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Physiological Characterization of a *Neurospora crassa* Mutant with Impaired Regulation of Nitrate Reductase

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This report describes the isolation and characterization of a *Neurospora crassa* mutant with an impaired regulation of nitrate reductase. Glutamine, which prevents the induction of nitrate reductase in *N. crassa*, did so relatively ineffectively in this mutant. The mutation did not affect the regulation of all enzymes regulated by "nitrogen metabolite regulation"; it did affect the regulation of nitrate reductase, nitrite reductase, histidase, and acetamidase, as well as that of thiourea sensitivity. The mutation was not allelic with *nit-2*, the gene controlling a general positive effector of nitrogen metabolite-regulated enzyme formation.

Nitrate reductase from *Neurospora crassa*, the first enzyme in the assimilation of nitrate, is induced by nitrate (15, 18, 24) and repressed by ammonium (15, 18, 31), by what seems to be a form of nitrogen metabolite regulation (1, 22, 23, 33). There is evidence that the nitrogen metabolite preventing the induction of nitrate reductase is glutamine (8, 20). The isolation and characterization of mutants insensitive to the above effect of glutamine (ammonium) in these systems should contribute to our understanding of the mechanism of repression. In this report, we describe the isolation and physiological characterization of one such mutant.

MATERIALS AND METHODS

***N. crassa* strains.** The strains of *N. crassa* came either from the Fungal Genetics stock center at Humboldt State University, Arcata, Calif., or from our own collection. Strain 3.1a (FGSC no. 935) was used as the wild-type strain. Strain *gln-1b*, a glutamine-requiring mutant, was obtained from R. Garrett (Department of Biology, University of Virginia, Charlottesville). The other mutant strains used were *nit-3A* (FGSC no. 3009), *nit-4A* (FGSC no. 2993), *nit-5A* (FGSC no. 985), and NR37.6A (FGSC no. 983), which is allelic to *nit-2* (28). There is no induced formation of nitrate or nitrite reductase in mutants *nit-4A*, *nit-5A*, and NR37.6A; hence, these are postulated to be regulatory mutants. Mutant *nit-3A*, which is thought to be the structural gene for nitrate reductase, produces an altered nitrate reductase possessing only a partial enzymatic activity, namely, reduced benzyl viologen-nitrate reductase.

Culture conditions. The basic medium without a nitrogen source has been described (28) and contained sucrose (20 g/liter) and macro- and micro- elements. One of the following nitrogen sources was included in the medium: ammonium tartrate, 4 g/liter (ammonia medium); glutamine, 2 g/liter (glutamine medium); sodium glutamate, 2 g/liter (glutamate medium); sodium nitrate, 20 mM (nitrate induction medium); or sodium nitrite, 3 mM (nitrite induction medium); the last was neutralized to pH 6.5 before use. The general culture conditions were as described previously (35). When the induction of nitrate reductase was studied, the mycelial pads were grown from a conidial inoculum for 39 h at 27°C in standing culture in ammonia medium and then washed. They were subsequently transferred to induction medium (three pads per 30 ml of medium), supplemented or not with the test compound of interest at the required concentration, and incubated with shaking for the desired time at 27°C. The mycelia were then collected, washed with distilled water, blotted dry between paper towels, and either extracted immediately or stored frozen in liquid nitrogen. When the decay of nitrate reductase was studied, the mycelia were grown for 39 h in stationary culture at 27°C, subsequently transferred, and incubated in induction medium, with shaking, for 3.5 h, to permit the formation of nitrate reductase; the pads were then washed and transferred to basic medium containing 6 mM sodium tungstate (decay medium), supplemented or not with the test compound (i.e., either nitrate, 20 mM, or glutamine, 20 mM), and were shaken for 3.5 h, at 27°C, to permit the decay of active nitrate reductase in the absence of any new synthesis of active enzyme (27). The mycelial pads were then harvested, washed with distilled water, blotted dry between paper towels, and extracted immediately, or frozen and stored in liquid nitrogen until required.

Mutagenesis and mutant selection. A conidial suspension (approximately 6×10^8 conidia) was incubated in 100 ml of basal medium containing 3 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 1 h at 27°C with shaking in the dark. The survivors were diluted into soft agar (induction medium containing 10 mM glutamine, 17.5 g of sorbose per liter, 0.5 g each of fructose and dextrose per liter, and 0.75% (wt/vol) agar, i.e., glutamine-nitrate solid medium) and overlaid on plates containing the above glutamine-nitrate solid medium and 1.5% (wt/vol) agar. The colonies appearing after 2 days of incubation at 27°C were flushed with nitrite color reagent [1% sulfanilamide in 1 M HCl, 0.02% (wt/vol) α-(*N*-1)-naphthylethylenediamine dihydrochloride in distilled water, and distilled water at a ratio of 1:1:3)]. After 5 min, some colonies turned pink; these were isolated into and grown to conidiation in liquid ammonia medium. Conidia of these putative

mutants were then plated, and resulting individual colonies were picked. Mycelia arising from these colonies were then screened for inhibition of nitrate reductase induction by glutamine. Two such putative mutants appeared to be non-inhibitable, one of these (MS5) was chosen for further characterization and is described herein.

Extractions. Mycelial pads were extracted by grinding them with an equal weight of silica in a buffer appropriate for the enzyme to be studied (2 ml of buffer per three pads), in an ice-cold mortar. The resulting brei was centrifuged at 13,000 × *g* for 15 min in a Sorvall refrigerated centrifuge. The supernatant was used for enzyme assays.

Determination of the intracellular pool size of glutamine. Glutamine was extracted from the mycelia as described by Vaca and Mora (34) and separated from acidic amino acids as reported by Ferguson and Simms (9). Glutamine thus separated was converted to glutamic acid by incubating it overnight at 27°C with 0.2 U of commercial glutaminase prepared from *Escherichia coli*; the resulting glutamic acid was isolated (9) and measured by the method of Yemm and Cocking (36) as modified by Ferguson and Simms (9).

Enzyme assays. NADPH-nitrate reductase was assayed as described previously (20), and reduced benzyl viologen-nitrate reductase activity was determined as reported by Subramanian and Sorger (32). One unit of activity in both cases is defined as the production of 1 nmol of nitrite per min at 30°C.

Glutamine synthetase was extracted and subsequently measured by its transferase activity as described by Ferguson and Simms (9). One unit of activity is defined as the production of 1 μmol of γ-glutamyl hydroxamate per min in the assay.

Nitrite reductase activity was extracted and measured as described by Chang and Sorger (2). One unit of nitrite reductase is defined as the production of 1 nmol of ammonia per min at 30°C. For each assay there was a control with no nitrite in the assay mixture.

Histidase (13) and acetamidase (12) were extracted and measured as described by Hynes. One unit of histidase is defined as the production of 1 nmol of urocanate per min and one unit of acetamidase, as the production of 1 μmol of ammonia per min, in their respective assays.

NADP-glutamate dehydrogenase was assayed by the method of Kapoor and Grover (14), by following the decrease in absorbance at 340 nm with an SP 1801 Unicam recording spectrophotometer. One unit of activity is defined as 1 nmol of NADPH oxidized per min at ambient temperature.

For the assay of protease, mycelia were extracted in 0.1 M citrate buffer (pH 5) plus 10^{-3} M disodium EDTA as described above under Extractions. The crude extract was heated at 45°C for 20 min, as reported by us previously (29); the treated extract was then clarified by centrifugation, and the supernatant was used as crude protease. The reaction mixture contained crude protease, 0.05 ml of 1 M phosphate, pH 7.0, 5 mg of Azocoll, and water, in a final volume of 0.5 ml. The reaction mixture was shaken at 37°C for 30 min. The reaction was terminated by the addition of 1.5 ml of 1 N HCl. The suspension was then clarified by centrifugation at top speed in a bench-top centrifuge. The absorbance of the supernatant was read at 520 nm against water. One unit of activity is defined as a change of 1 absorbance unit in 30 min at 37°C. Specific activity in all cases is given in units of activity per milligram of protein.

Determination of protein. Protein concentrations were determined by using the biuret reagent (11) and crystalline bovine serum albumin as standard.

Materials. NADPH, flavin adenine dinucleotide, bovine serum albumin, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and glutaminase A were from Sigma Chemical Co. Azocoll was purchased from Calbiochem. Benzyl viologen was from BDH, and Nessler reagent was from Paragon Co. All other chemicals were of reagent grade and were purchased locally.

RESULTS

Effect of L-glutamine on the induction of nitrate reductase in the wild-type strain and MS5. Ammonium ions, L-glutamate, L-glutamate plus ammonium ions, and L-glutamine all inhibited nitrate reductase formation in the wild type but not in strain MS5 (Table 1). The inhibition of nitrate reductase induction was virtually complete at a concentration of 3 mM L-glutamine in the induction medium (Fig. 1; 20). This concentration of L-glutamine had no noticeable effect on the induction of the reductase in strain MS5 (Fig. 1).

Is the lack of inhibition of induction of nitrate reductase by glutamine due to cellular exclusion or elimination of glutamine? Strain MS5 grew more rapidly on glutamine as sole nitrogen source than on ammonia,

TABLE 1. *Effect of different nitrogen compounds on the induction of nitrate reductase in the wild-type and MS5 strains of N. crassa*[a]

Additions	Nitrate reductase specific activity	
	Wild type	MS5
To nitrate induction medium		
None	77	58
Ammonium ions	20	62
Glutamine	4	71
Glutamate	39	59
Ammonium ions + glutamate	2	57
To nitrite induction medium		
None	60	85
Ammonium ions	20	66
Glutamine	4	70
Glutamate	40	62
Ammonium ions + glutamate	2	69

[a] The wild-type and the mutant strains were grown on ammonia medium for 39 h at 27°C, as described in Materials and Methods. The mycelial felts were washed, transferred to induction media, supplemented or not with the test compound at 20 mM final concentration, and incubated for 3.5 h at 27°C, with shaking. The mycelial pads were then harvested, and extracts were made and assayed as described in the text. The pattern of results in this experiment is representative of more than four repetitions of each combination of additions.

nitrate, or glutamate as nitrogen sources (Table 2). The concentrations of glutamine in extracts of MS5 and wild-type mycelia, when exposed to nitrate induction medium containing 20 mM

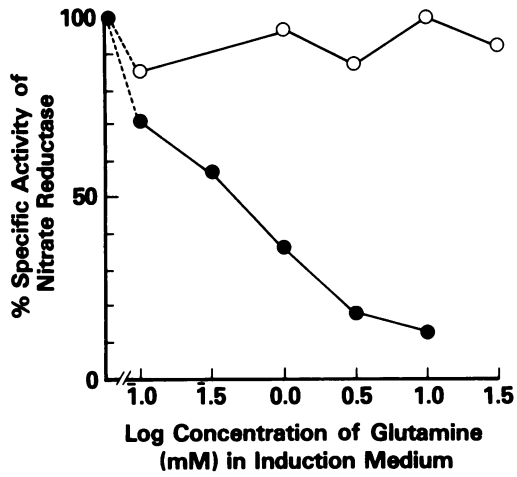

FIG. 1. *Effect of glutamine on the induction of nitrate reductase in strain MS5 and in the wild-type strain. Mycelia of the wild-type strain (●) and of the mutant (○) were grown in standing culture for 39 h at 27°C in ammonia medium; they were then transferred, after washing, to nitrate induction medium containing the indicated concentrations of L-glutamine and were shaken for 3.5 h at 27°C. The mycelia were then harvested and extracted with 0.1 M phosphate, pH 7.0, containing 10^{-3} M disodium EDTA, and the extracts were assayed for nitrate reductase activity and protein as described in Materials and Methods. The specific activities of nitrate reductase in MS5 and wild-type extracts of mycelia induced in the absence of glutamine in this experiment were 158 and 21 U per mg of protein, respectively.*

TABLE 2. *Growth response of wild type and mutant strain MS5 to different nitrogen sources*[a]

N source in growth medium	Concn (mM)	Dry wt of mycelium (mg/10^6 colony-forming units)	
		Wild type	MS5
Ammonium tartrate	20	91	95
Glutamine	10	68	99
Glutamate	20	59	71
Ammonium tartrate + glutamate	10	83	86
Nitrate	20	66	40

[a] Roughly equal numbers of spores (approximately 4×10^7 conidia) from each strain were inoculated into three flasks containing basal medium supplemented with the additions indicated in the table and grown in standing culture at 27°C as described in the text. The resulting pads, three in each case, were washed with distilled water, collected on a membrane filter, blotted dry with paper towels, dried overnight at 200°F (92°C) in an oven, and weighed. The efficiency of plating of MS5 was approximately 0.4 times that of the wild-type strain.

glutamine, were 0.066 and 0.069 μmol of glutamine per mg of protein, respectively. A serine protease, which is repressed by rich sources of nitrogen including glutamine (29) responded to glutamine in strain MS5 mycelia as in the wild-type strain (Table 6). These three observations indicate that glutamine was able to enter the cells of the mutant strain.

Strain MS5 grew more slowly than the wild-type strain on all nitrogen sources, presumably because the number of colony-forming units per conidium in the mutant was lower than in the wild-type strain (Table 2).

Strain MS5 could, perhaps, be resistant to the regulatory effects of glutamine because the amino acid was metabolized or destroyed very rapidly in the mutant cells, not accumulating in sufficient quantity, therefore, to shut off nitrate reductase synthesis. If this were true, one would predict that: (i) culture filtrates of MS5 mycelia incubated in glutamine-containing nitrate induction medium, but which are fully induced for nitrate reductase, should contain much less glutamine than comparable culture filtrates of wild-type mycelia incubated in the same type of medium and which have no nitrate reductase; and (ii) extracts of the above MS5 mycelia should contain much less glutamine than extracts of the above wild-type mycelia. To test the first prediction, we incubated 39-h-old wild-type and MS5 mycelial pads for 3.5 h with shaking at 27°C in nitrate induction medium containing 100 or 20 mM L-glutamine. Under these conditions the wild-type mycelia contained no nitrate reductase, whereas the MS5 mycelia contained plenty of reductase activity (see Table 3). The spent culture filtrates were then mixed in various proportions with fresh nitrate induction medium and incubated, with shaking, for 3.5 h at 27°C with 39-h old *gln-1b* mycelia. The induction of nitrate reductase in these *gln-1b* mycelia (*gln-1b* cannot convert ammonium or amino acids to glutamine) will respond to the concentration of glutamine in the environment by being inhibited.

A culture filtrate of MS5 mycelia that had been incubated for 3.5 h in 100 mM glutamine-containing nitrate induction medium had about 10 times as much inhibitor of nitrate reductase induction as did a comparable culture filtrate of wild-type mycelia that had been incubated for 3.5 h in 20 mM glutamine-containing nitrate induction medium (Table 3A). Culture filtrates of wild-type mycelia incubated for 3.5 h in 100 mM glutamine-containing nitrate induction medium had roughly as much inhibitor of nitrate reductase induction as did comparable filtrates of MS5 mycelia treated in the same fashion

TABLE 3. *Effect of the presence during induction of spent glutamine-containing nitrate induction medium and of extract from mycelia exposed to glutamine-containing induction medium on the induction of nitrate reductase in strain gln-lb*

Medium component	Expt no.	Strain	Glutamine concn (mM)	Proportions (%) in induction medium		Sp act of nitrate reductase in extracts of *gln-lb*
				Spent medium or extract	Fresh medium	
A. Spent medium from culture of strain indicated, initially containing the indicated concentration of glutamine[a]	1	Wild type (sp act of mycelial extract, 1.1)	20	1	99	47.5
				3	97	46.3
				10	90	45.0
				30	70	7.3
				100	0	4.2
	1	MS5 (sp act, 684)	100	1	99	40.9
				3	97	7.3
				10	90	5.4
				30	70	4.3
				100	0	3.1
	1	Control		0	100	43.2
	2	Wild type (sp act, 39.1)	0	100	0	37.0
	2	Control		0	100	44.3
B. Concentrated extract from mycelia of strain indicated, incubated in nitrate induction medium containing the indicated concentration of glutamine[b]	1	Wild type (sp act of mycelial extract, 1.1)	20	10	90	2.2
				50	50	1.3
	1	MS5 (sp act, 684)	100	10	90	2.2
				50	50	1.6
	2	Wild type (sp act, 39.1)	0	10	90	10.5
				50	50	4.5
		Control		0	100	42.2

[a] Wild-type or MS5 mycelia were grown for 39 h in standing culture at 27°C in ammonia medium and were subsequently induced in nitrate induction medium containing the concentration of glutamine shown in the table, with shaking at 27°C for 3.5 h. The spent medium was separated from the mycelia by filtration through a Nalgene 0.2-μm filter (Nalge Co., Rochester, N.Y.). The filtered medium was then mixed with fresh nitrate induction medium in the proportions shown above. Mycelia of strain *gln-lb*, which had been previously grown for 39 h at 27°C in standing culture in glutamine medium, were then induced in the above mixture of fresh and spent medium, with shaking at 27°C for 2.5 h. The *gln-lb* mycelia were then extracted, and the specific activity of nitrate reductase in the extracts was measured as described in Materials and Methods.

[b] The wild-type and MS5 mycelia from part A were extracted and assayed for nitrate reductase activity and protein. The specific activity of nitrate reductase in these extracts is given in parentheses after the strain name. The remainder of the extracts were then lyophilized, suspended in 0.10 the original volume of water, boiled for 5 min, and clarified by centrifugation at 12,000 × *g* in a Sorvall refrigerated centrifuge. The resulting clear extract was mixed with nitrate induction medium in the proportions shown in the table and were incubated for 2.5 h with 39-h-old *gln-lb* mycelia in the same manner as described in footnote *a*. The *gln-lb* mycelia were then extracted and assayed for nitrate reductase activity and protein as described in Materials and Methods.

(results not shown). Evidently, the reason that MS5 mycelia induced in the presence of glutamine contain copious amounts of nitrate reductase activity is not that the MS5 mycelia destroy or use up glutamine from the surrounding medium at a much faster pace than their wild-type counterparts.

Tenfold concentrated extracts of wild-type mycelia that had been incubated in the presence of 20 mM glutamine-containing nitrate induction medium had a very low level of nitrate reductase and also some material (presumably glutamine) that inhibited the induction of nitrate reductase in strain *gln-1b*. Tenfold concentrated extracts of MS5 mycelia incubated in 100 mM glutamine-containing nitrate induction medium had high levels of nitrate reductase activity and approximately the same concentration of nitrate reductase induction inhibitor as the above wild-type extracts incubated in 20 mM

glutamine-containing nitrate induction medium (Table 3B). Tenfold concentrated extracts of fully induced wild-type mycelia also contained an inhibitor(s) of nitrate reductase induction (Table 3B). The latter inhibitor either was weaker or, if it was the same material as the inhibitor present in extracts of mycelia incubated with glutamine-containing induction medium, its concentration was lower in extracts of fully induced wild-type mycelia than in extracts of mycelia exposed to glutamine. If one assumes a typical concentration of 0.07 μmol of glutamine per mg of protein (see above) in extracts of mycelia exposed to 20 mM glutamine, then the wild-type concentrated extracts used in Table 3B contained 3.5 mM glutamine (5 mg of protein per ml, concentrated 10-fold) and the MS5 mycelia contained at least 2.2 mM glutamine (3.2 mg of protein per ml, concentrated 10-fold). On the basis of previous observations (see Fig. 2 of reference 20), one-half the concentration of either of the above levels of glutamine in the induction medium would be sufficient to cause more than 95% inhibition of the induction of nitrate reductase. This was, in fact, observed (Table 3B). One-tenth the concentration of the above levels of glutamine in the induction medium would cause an inhibition of nitrate reductase induction between 40 and 90% (see Fig. 2 of reference 20). The concentrated extracts of mycelia exposed to 20 and 100 mM glutamine-containing induction medium had a somewhat greater inhibitor potency than expected on the basis of this reasoning (Table 3B); however, if these extracts were present in the induction medium at a concentration of 3% or lower, there was virtually no effect on nitrate reductase induction (results not shown), as expected. The level of nitrate reductase induction inhibitor in these extracts was, by the above reasoning, in the expected concentration range if the inhibitor is glutamine. The potency of nitrate reductase induction inhibition of fully induced wild-type mycelial extracts was less than one-half that of cell-free preparations of wild-type or MS5 mycelia exposed to 20 or 100 mM glutamine (Table 3B). Ferguson et al. (9) observed that the intracellular concentration of glutamine in *Candida* growing on glutamine was approximately four times that of the yeast growing on nitrate. It is difficult to extrapolate meaningfully from one system to another when dealing with quantitative data of this kind; however, it is reassuring that our data are not grossly out of line with the findings of Ferguson et al. (9), assuming that the inhibitor of nitrate reductase induction is glutamine.

The data in Table 3B show that the level of nitrate reductase induction inhibitor present in extracts of MS5 mycelia induced in glutamine-containing induction medium was higher than that in extracts of wild-type mycelia induced on nitrate induction medium and comparable to that in extracts of wild-type mycelia exposed to glutamine-containing nitrate induction medium. Evidently, the presence of relatively large amounts of nitrate reductase in MS5 mycelia induced on glutamine-containing nitrate induction medium was not due to the absence of effective intracellular quantities of nitrate reductase induction inhibitor (presumably glutamine) in this strain.

Effect of the MS5 mutation on the decay of nitrate reductase in vivo. The lack of repression of nitrate reductase by glutamine observed in the mutant could conceivably be due to a much lowered rate of decay of nitrate reductase in the mutant. This was tested by inducing the mycelia as described in Materials and Methods and subsequently transferring them to decay medium containing sodium tungstate, supplemented with glutamine as nitrogen source, so that any nitrate reductase synthesized de novo during incubation in the decay medium would be inactive (27). Any remaining nitrate reductase activity would be due to the active enzyme synthesized before the mycelia were transferred to the decay medium. There appeared to be a fivefold difference in the rates of decay of the reductase in vivo in the wild type and in the mutant strain in the presence of glutamine (Fig. 2). The fivefold difference in the rate of decay of nitrate reductase in strain MS5 and the wild type in the presence of glutamine was quantitatively insufficient to account for the near-qualitative difference in the effect of glutamine on the rate of induction (Fig. 2) of the reductase in the two strains. The difference in decay in vivo just described was not due to a difference in heat lability between the nitrate reductase from wild-type and MS5 strains. The half-life of the enzyme activity in crude extracts of both strains at 45°C was 1.4 min (results not shown).

Is nitrate reductase induction by nitrate resistant to glutamine in strain MS5? A study of the effect of the concentration of nitrate in the induction medium on the synthesis of nitrate reductase in the wild-type and mutant strains revealed that nitrate was required for the formation of nitrate reductase in both strains (Fig. 3).

The possibility that the transcription of the nitrate reductase gene was resistant to the effect of glutamine was tested indirectly, as follows. Wild-type and MS5 mycelia were grown at 27°C

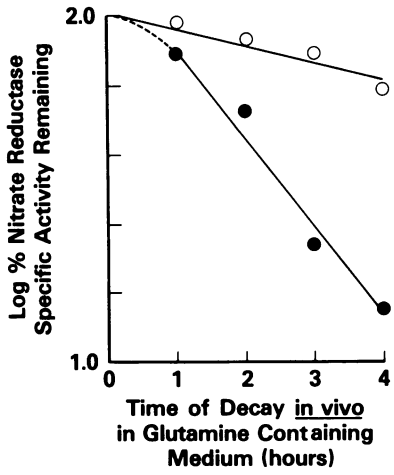

FIG. 2. *Decay of nitrate reductase in vivo in MS5 and in the wild-type strain. The experiment is described in the text. Specific activity of nitrate reductase in the wild-type extract was 164 and in the MS5 extract was 210 at time zero of decay. The experiment was repeated three times, and although the specific activities varied from one experiment to the next, the patterns of decay were very similar. Symbols: ○, MS5; ●, wild type.*

in standing culture in ammonia medium for 39 h, washed, and transferred to nitrate induction medium containing 0.5 mM nitrate and 6 mM sodium tungstate with or without 5 mM glutamine, with shaking, at 27°C for various lengths of time (transcription stage). Under these conditions nitrate reductase mRNA should accumulate but will not be translated into active enzyme because the tungstate-containing analog of nitrate reductase is inactive (27). After this incubation, the mycelia were washed with distilled water and transferred for 20 min at 27°C, with shaking, to nitrate induction medium containing 20 mM nitrate, 20 mM glutamine, and 6 mM sodium molybdate. During this stage of incubation (translation stage), whatever mRNA has accumulated should be translated. Controls containing cycloheximide during the translation-stage incubation showed that there was very little activation of the tungstate-containing enzyme (27; Fig. 4). The results of this experiment (Fig. 4) show that the accumulation of the capacity to synthesize nitrate reductase (presumably this was nitrate reductase mRNA) was resistant to glutamine in strain MS5, unlike the situation in the wild-type strain.

Is the MS5 phenotype due to the lack of glutamine synthetase or glutamate dehydrogenase? Both glutamine synthetase and glutamate dehydrogenase have been implicated in the regulation of nitrogen-metabolizing enzymes (6, 10, 16, 19, 21, 30). The level of NADP-glutamate dehydrogenase was at least as high in extracts of strain MS5 as in extracts of the wild-type strain, no consistent differences being found in this respect between the two strains when subjected to a variety of nitrogen nutritional conditions (results not shown). The specific activity of glutamine synthetase was consistently higher in extracts of strain MS5 than in those of the wild-type strain; interestingly, glutamine did not repress glutamine synthetase in strain MS5 as it does in the wild-type strain (Table 4). These results suggest that the phenotype of strain MS5 is not accounted for by a lack of glutamine synthetase or NADP-glutamate dehydrogenase and that the regulation of glutamine synthetase is affected by the MS5 mutation.

Does the MS5 mutation affect other nitrogen metabolite-regulated functions? Thiourea is a basic analog of urea, which presumably gains entry to *N. crassa* cells by the urea transport system (7). The urea transport system is not formed when cells are growing in rich sources of nitrogen (7). The growth of the wild-type strain of *N. crassa* was sensitive to thiourea when grown on nitrate, but much less so when grown on glutamine (Table 5). Strain MS5, on the other hand, was highly sensitive to thiourea when growing on nitrate or on glutamine (Table 5). This observation suggests that the regulation

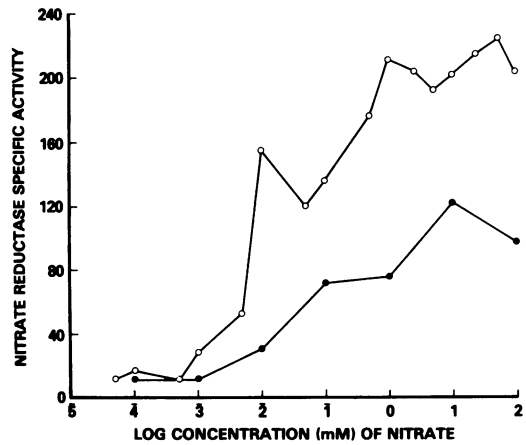

FIG. 3. *Effect of the concentration of nitrate on the induction of nitrate reductase in the wild-type and MS5 strains of N. crassa. The two strains were grown on ammonia medium for 39 h at 27°C in stationary culture. The mycelial pads of the wild type (●) and the mutant (○) were then transferred to basal medium containing the indicated final concentrations of nitrate and were incubated for 90 min at 27°C with shaking. The pads were harvested and extracted, and the nitrate reductase activity of the extracts was determined, as described in Materials and Methods.*

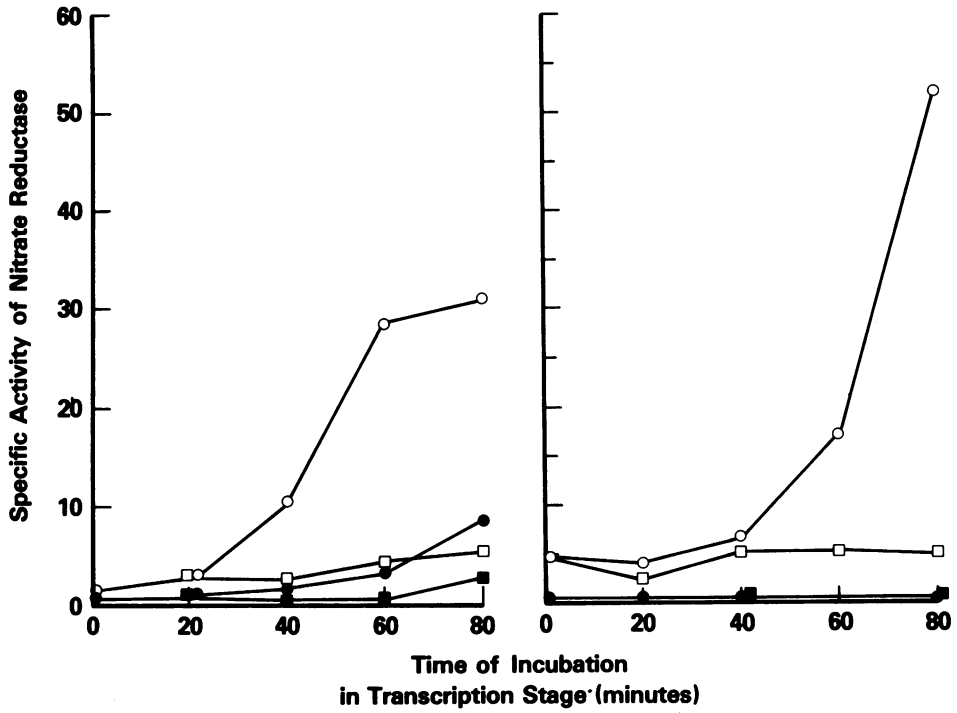

FIG. 4. *Effect of glutamine on the accumulation of presumptive nitrate reductase mRNA in wild-type and MS5 strains. Mycelia were grown, and presumptive mRNA then accumulated in the absence of translation into active nitrate reductase, for the times indicated in the presence (right panel) and absence (left panel) of glutamine. The accumulated presumptive mRNA was then translated for 20 min in each case into active nitrate reductase (circles) or mock treated in the same fashion in the presence of 1 μg of cycloheximide per ml (squares), all as described in the text. Filled symbols indicate the wild-type strain; empty symbols, strain MS5.*

TABLE 4. *Glutamine synthetase activity in the wild-type and MS5 strains of N. crassa*[a]

Addition to induction medium	Sp act[b] of glutamine synthetase	
	Wild type	MS5
None	.24	.38
Glutamate	.27	.32
Ammonium tartrate	.23	.26
Glutamine	.16	.30
Ammonium tartrate + glutamate	.15	.27

[a] The two strains were grown on ammonia medium for 39 h at 27°C in standing culture and were subsequently transferred to induction medium with or without the compounds indicated in the table at a final concentration of 20 mM. The incubation was continued with shaking for 3.5 h at 27°C. The harvested pads were extracted in 0.1 M phosphate buffer, pH 7.1, containing disodium EDTA (1 mM) and mercaptoethanol (1 mM). Glutamine synthetase activity was determined as described in Materials and Methods.

[b] Specific activity of glutamine synthetase is expressed as micromoles of γ-glutamyl hydroxamate produced per minute per milligram of protein at 30°C.

of the urea uptake system was less sensitive to glutamine in strain MS5 than in the wild-type strain.

The formation of nitrite reductase, histidase, and acetamidase, was induced by their substrates, and rich sources of nitrogen inhibited the induced formation of these enzymes (Table 6). Unlike the wild-type strain, mycelia of MS5 were relatively insensitive to the inhibitory effect of glutamine and other rich sources of nitrogen on the induced formation of these enzymes (Table 6). This is in sharp contrast to the situation with the nitrogen metabolite-regulated intracellular neutral serine protease, which was repressed by rich sources of nitrogen in both strains (Table 6) and to the same extent. The nitrate reductase inactivase that is associated with the above nitrogen metabolite-regulated intracellular serine protease activity (29) was repressed in the same manner in mutant MS5 and the wild-type strain (results not shown).

Is MS5 allelic to *nit-2* or *nit-3*? It is believed that *nit-2* is the gene controlling a positive effector involved in the induced (or derepressed, or both) formation of a number of nitrogen metabolite-regulated enzymes (1, 17). Mutants at this locus fail to synthesize a number of the above enzymes (1, 17, 28, 29).

Unfortunately, crosses between MS5 and all

TABLE 5. *Effect of thiourea on the growth of the wild type and mutant strain MS5 of N. crassa*[a]

N source in growth medium	Wild type		Mutant MS5	
	Wt of mycelium (g)	Inhibition of growth (%)	Wt of mycelium[b] (g)	Inhibition of growth (%)
Glutamine (5 mM)	.0198	0	.0097	0
Glutamine (5 mM) + thiourea (5 mM)	.0149	25	.0023	75
Nitrate (20 mM)	.0098	0	.0052	0
Nitrate (20 mM) + thiourea (5 mM)	.0005	95	.0003	94
Thiourea (5 mM)	.0005		.0005	

[a] Wild-type and mutant strains were grown from a conidial inoculum on the medium indicated for 39 h at 27°C in stationary culture, as described in the text. The resulting pads (two in each case) were washed, pressed between folds of filter paper, placed in aluminum foil, dried overnight at 200°F (ca. 92°C), and then weighed.

[b] The values given are the average weights of one mycelial pad and are typical of three repeats.

TABLE 6. *Effect of glutamine on the induction of nitrogen metabolite-regulated enzymes in the wild-type and mutant MS5 strains of N. crassa*[a]

Strain	Additions to induction medium	Specific activity			
		Nitrite reductase	Histidase	Acetamidase	Neutral serine protease
Wild type	None	6.6	1.6	0.30	0.61
	Ammonium ions	2.8	—	0.08	0.28
	Glutamine	1.1	0.3	0.12	0.19
	Glutamate	1.9	—	—	0.40
	Ammonium ions plus glutamate	0.6	—	0.05	0.25
MS5	None	16.8	2.7	3.3	0.60
	Ammonium ions	7.3	—	2.6	0.22
	Glutamine	6.8	2.9	4.2	0.16
	Glutamate	7.1	—	—	0.22
	Glutamate plus ammonium	3.7	—	3.3	0.33

[a] Wild-type and mutant mycelia were grown on ammonia medium as described in Materials and Methods. For nitrite reductase determinations mycelia were transferred to nitrate induction medium with or without the additions shown above, at a concentration of 20 mM, and shaken at 27°C for 4 h before mycelial extracts were harvested and assayed as described by Chang and Sorger (2). One unit of nitrite reductase is defined as the production of 1 nmol of ammonia per min in the assay. For histidase determinations mycelia were transferred to medium without nitrogen containing histidine at a concentration of 5 mM and the additions shown in the table at 20 mM. The mycelia were shaken at 27°C for 4 h, harvested, extracted, and assayed as described by Hynes (13). One unit of activity is defined as the production of 1 nmol of urocanate per min in the assay. For acetamidase determinations the mycelia were transferred to medium without nitrogen containing acetamide at a concentration of 5 mM and were shaken at 27°C for 4 h. The mycelia were then extracted and assayed by the procedure of Hynes (12), with formamide as substrate. One unit of activity is defined as the production of 1 μmol of ammonia per min in the assay. For protease determinations the mycelia were induced on medium without nitrogen containing the additions shown in the table at a concentration of 20 mM and shaken for 4 h at 27°C. The mycelia were then harvested and extracted, and protease was assayed with Azocoll as substrate, as described in Materials and Methods. One unit of activity is defined as the production of soluble material absorbing 1 A_{520} unit per min in the assay. Boiled extract inhibited the protease activity as described previously (29). Specific activity is defined in terms of units of activity per milligram of protein. Each experiment was repeated at least once with very similar patterns of results being observed.

nit genes were highly infertile, even after the first backcross; however, 109 ascospores were isolated from two of the crosses with *nit-2* and were heat shocked. Only 17 ascospores germinated, and of these 2 were wild type. It would appear, consequently, that MS5 and *nit-2* are not allelic.

It is thought that *nit-3* is the structural locus for nitrate reductase (3, 17, 27) because mutations at this gene alter the structure of the reductase (3, 25). Nitrate reductase has been postulated to be implicated in its own regulation (4) because mutants at the *nit-3* and *nit-1* locus of *Neurospora* (3, 25) are altered in the regulation of this enzyme. The same situation applies to *Aspergillus* (4). Forty ascospores from a cross between *nit-3* and MS5 were isolated and heat shocked; 18 of these germinated and 2 were wild-

type progeny. The MS5 mutation was obviously not allelic with *nit-3*. In support of this observation, the heat sensitivity of the active nitrate reductase of MS5 in vitro was not detectably different from that of the wild type (see section on stability), and the electrophoretic mobility in tube gels (6.5 cm by 0.5 cm in diameter) in 5% polyacrylamide (the buffer in the positive electrode reservoir was 0.05 M Tris-hydrochloride, pH 8.1, and in the negative electrode reservoir was 0.05 M Tris-glycine, pH 8.9; the current was 2.5 mA per gel, applied for 3 h) appeared to be indistinguishable from that of the wild-type strain (the relative mobility of both was 0.45 times that of the dye front; the band of enzyme was visualized in the gel by assaying its nitrate-dependent oxidation of reduced benzyl viologen). The above observations suggest that the enzyme nitrate reductase in strain MS5 and the wild-type strain is identical. This, in turn, means that the relatively high specific activity of nitrate reductase in induced mycelia of strain MS5, when compared with that of the wild-type strain, is due to a relatively higher number of enzyme molecules per cell in the former strain than in the latter.

DISCUSSION

Our results show that glutamine did not prevent the induction of nitrate reductase effectively in mutant strain MS5. At levels of glutamine (2.5 mM) that inhibited nitrate reductase formation almost completely in the wild-type strain, there was virtually no inhibition in the mutant. The lack of effect observed was not due to cellular impermeability to glutamine or due to its greater utilization in the mutant (Tables 2 and 3), nor can it be explained by a lowered rate of decay of nitrate reductase in the presence of glutamine (Fig. 2).

The specific activities of various nitrogen metabolite-regulated enzymes in the mutant were consistently higher than in the wild-type strain (Table 6). This was possibly due to the fact that ammonia and metabolites of ammonia, which are end products of inorganic nitrogen assimilation, amino acid degradation, and general scavenging of metabolic nitrogen, do not regulate these enzymes efficiently in this mutant.

N. crassa can utilize a variety of compounds as nitrogen sources. It appears that the normal product of gene *nit-2* is required for the synthesis of a number of enzymes that are all regulated by metabolites of ammonia, such as glutamine. Mutants altered in the *nit-2* gene display a pleiotropic loss of numerous enzymes involved in nitrogen metabolism and the inability to utilize some nitrogen sources such as nitrate. As the *nit-2* gene regulates the synthesis of enzymes whose structural genes are scattered throughout the *N. crassa* genome, it has been postulated that the *nit-2* gene codes for an intracellular inducer of enzymes regulated by nitrogen metabolite repression. This postulated inducer has been hypothesized to bind to control regions adjacent to structural genes and "turn on" their expression (23). In *Aspergillus* spp., there is a locus analogous to the *N. crassa nit-2* gene—*areA* (1). Nitrate reductase in one revertant of *areA* or *Aspergillus* spp. is not repressible by ammonia, as is the case in the wild-type strain (1). A simple interpretation of this observation is that ammonium, or a nitrogen metabolite derived from it, interacts with the *areA* (or *nit-2*) gene product and converts it into an inactive intracellular inducer or a repressor, or both. There is increasing evidence to suggest that it is not ammonium that is responsible for the lack of gene expression (2, 5) but that glutamine is, in fact, an effector or interacts with the effector, at least in so far as nitrate reductase is concerned (8, 20). Thus, according to the above hypothesis, interaction of the *nit-2* gene product with glutamine would be expected to render it inactive as an inducer of nitrogen metabolite-regulated enzymes or active as repressor of the same, or both, leading to nonexpression of the corresponding genes.

In view of this postulate, it is interesting to note that in mutant MS5 glutamine did not prevent the induction of nitrate reductase, nitrite reductase, histidase, and acetamidase, but it did so to an intracellular protease. All of these enzymes are under the control of *nit-2* locus; i.e., they are nitrogen metabolite-controlled enzymes (29; our unpublished data). It is also noteworthy that the MS5 locus is not allelic with *nit-2*, the gene controlling the presumptive intracellular inducer of numerous nitrogen metabolite-regulated enzymes. It seems the MS5 mutation has probably not altered the postulated *nit-2* gene product's recognition of glutamine directly, if such a recognition exists. It seems difficult, in fact, to reconcile the simple model described above with our results. An alternative interpretation is that there is a negative effector(s) controlling nitrogen metabolite-regulated genes as well as positive effectors. The negative effector for the genes controlling nitrate reductase, nitrite reductase, histidase, acetamidase, and the urea transport system would, by this interpretation, be different from the negative effector controlling the intracellular neutral serine protease. The former would be under the control of the MS5 locus; the latter would be under the control of a different locus.

Mutants such as MS5 should be most useful in finding out how nitrogen metabolite regulation works.

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