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Nitrogen Regulation of Amino Acid Utilization by *Neurospora crassa*

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The production of an extracellular deaminase activity involved with the utilization of amino acids as sole sources of nitrogen is under the control of the *nit-2* locus of *Neurospora crassa*. This locus is the sole major nitrogen regulatory locus described for *N. crassa* and is believed to encode a positive effector required for induction of activities involved with the utilization of alternate nitrogen sources. Production of deaminase activity requires the lifting of nitrogen metabolite repression, the presence of a functional *nit-2* gene product, and specific induction by amino acids. Additional parameters of enzyme production are described.

Nitrogen is an essential component of all living systems. Accordingly, a significant proportion of the metabolic activities of an organism is concerned with procuring and assimilating nitrogen. Studies with microbial systems are providing insight into the processes by which nitrogen is obtained and converted into a utilizable form, the regulation of these processes, and the various structural and regulatory genes involved (17, 18). The amino acid glutamine is a major nitrogen donor for intermediary metabolism, and when glutamine or its precusors glutamate and ammonium are available to an organism, they serve as the preferred nitrogen sources. When these primary nitrogen sources are limiting, however, an organism can selectively express genes whose products enable it to use secondary nitrogen sources. Expression of these genes is prevented through nitrogen metabolite repression when preferred nitrogen sources are available (2, 11, 12, 17, 18, 20, 22). Several microbial systems have been developed to study the processes involved with the utilization of secondary nitrogen sources, to determine how these processes are regulated, and to identify the roles of various structural and regulatory genes in nitrogen metabolism.

The filamentous fungus *Neurospora crassa* can utilize nitrate, nitrite, purines, amino acids, nucleic acids, and proteins as secondary nitrogen sources (14, 15, 25, 26, 29). The utilization of nitrate and nitrite has been extensively characterized (1, 3, 21–23, 29, 30, 33). Two reductases work sequentially to reduce nitrate first to nitrite and then to ammonium. These enzymes are the products of unlinked genes that are kept silent by nitrogen metabolite repression. Through the isolation of nitrate-nonutilizing strains, a major control locus, *nit-2*, has been identified (5, 14, 25). This locus is not specific to the nitrate assimilatory pathway but instead is believed to code for a positive effector required for expression of structural genes involved with the utilization of secondary nitrogen sources. Nitrogen metabolite repression is thought to interfere with *nit-2* activation of the structural genes.

Additional systems need to be developed as comprehensively as the nitrate system. Such systems would allow us to define the major nitrogen regulatory circuit in terms of the broad range of activities controlled by the *nit-2* locus and to develop other approaches for the identification of both major and minor control loci through the isolation of mutant strains defective in one or more of these activities. In this paper we describe an amino acid utilization system that is under control of the *nit-2* locus. The production of a nitrogen-regulated extracellular deaminase in response to amino acids as sole sources of nitrogen provides a ready assay for gene expression and an alternate approach for the isolation of mutant strains defective in nitrogen regulation.

MATERIALS AND METHODS

Strains. The following strains were obtained from the Fungal Genetics Stock Center, Humboldt State University, Arcata, Calif.: *nit-2* (FGSC 2698, allele I7), *nit-2* (FGSC 33, allele K31), and *nit-2* (FGSC 982, allele nr37). Wild-type strain 74a and the *pmn*; *pmb*; *pmg* strain, which is defective for the three constitutive amino acid permeases, were isolated by tetrad analysis following four backcrosses of the *pmn*; *pmb*; *pmg* strain to strain 74a (= FGSC 988) and are available from us. The *pmn*; *pmb*; *pmg* strain contains three unlinked single-gene mutations, *pmn*, *pmb*, and *pmg*, that inactivate the neutral amino acid-specific permease, the basic amino acid-specific permease, and the general permease, respectively (16, 24, 27). We constructed the *pmn*; *pmb*; *pmg*; *nit-2* (allele I7), *pmn*; *pmb*; *pmg*; *nit-2* (allele K31), and *pmn*; *pmb*; *pmg*; *nit-2* (allele nr37) strains by crossing the *pmn*; *pmb*; *pmg* strain with the appropriate *nit-2* strain. Identification of the *pmn*; *pmb*; *pmg*; *nit-2* strains took advantage of the amino acid analog resistance conferred by the permease mutations, as well as the inability to utilize potassium nitrate as a nitrogen source as a result of the *nit-2* mutation. The presence of all three transport mutations in the putative *pmn*; *pmb*; *pmg*; *nit-2* isolates was confirmed by transport assays with radiolabeled amino acids. The transport methodology used has been described previously by DeBusk and DeBusk (8) and Ogilvie-Villa et al. (19).

Growth of cultures. Cultures were maintained on Vogel medium N (32) containing 2% sucrose and 1.5% agar as previously described (8).

Growth assays. Growth assays were performed in 125-ml Erlenmeyer flasks containing 25 ml of medium as previously described (7, 9). The basal medium used (NNV) was Vogel medium N in which alternate nitrogen sources were substituted for the NH_4NO_3. Cultures were incubated at 35°C for 72 h unless otherwise noted. The mycelial growth was subsequently harvested, dried, and weighed. All weights

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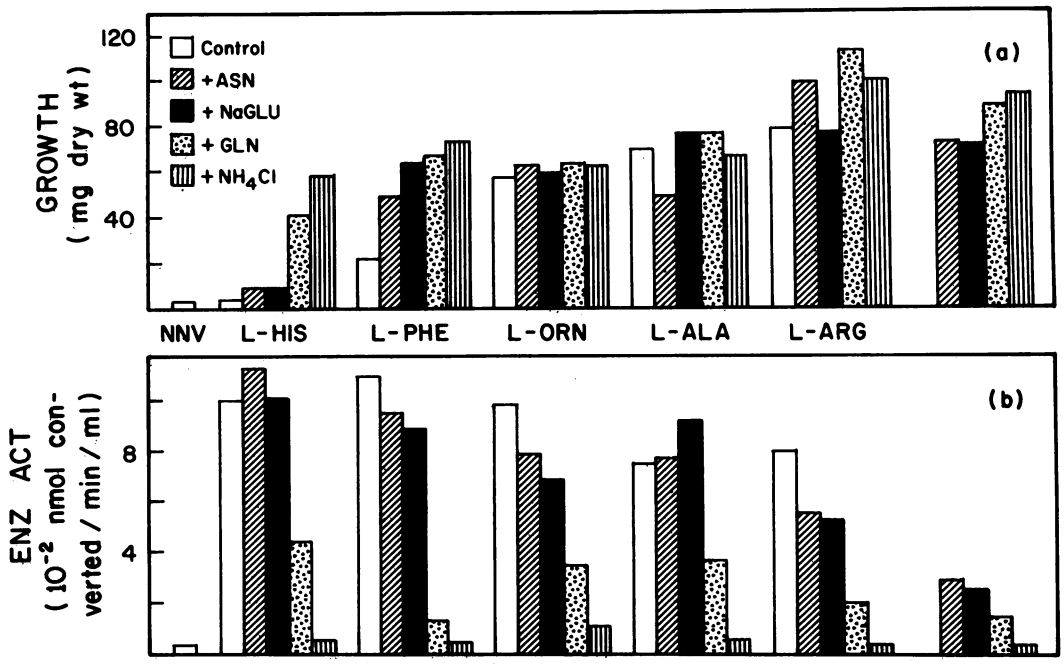

FIG. 1. Growth (a) and production of an extracellular deaminase activity (b) by the *pmn*; *pmb*; *pmg* strain in the presence of putative nitrogen metabolite repressors. The amino acids L-histidine, L-phenylalanine, L-ornithine, L-alanine, and L-arginine were presented as sole nitrogen sources in the absence of repressors (control) or in the presence of L-asparagine (ASN), sodium L-glutamate (NaGLU), L-glutamine (GLN), or ammonium chloride (NH_4Cl). In addition, the ability of each repressor in itself to support growth and elicit deaminase production was examined. Growth assays were performed at 35°C for 72 h. Deaminase assays were performed at 37°C for 2 h. All nitrogen sources and repressors were present at concentrations of 15 mM. All media contained NNV salts as the basal component. Enz Act, Enzyme activity.

reported are averages of duplicate flasks and in many instances represent averages of three to five separate assays, each in duplicate.

To determine the minimum concentration of amino acid capable of triggering extracellular deaminase production, standard 72-h growth assays were performed. Various amino acids were presented as the sole nitrogen sources over a concentration range of 1 nM to 15 mM.

For determination of the time course of extracellular deaminase production in response to an amino acid, standard growth assays were performed with 15 mM L-arginine as the nitrogen source. Cultures were sampled at intervals as indicated below. Mass was determined by obtaining the dry weights of duplicate cultures. At the time that the dry weight of a culture was determined, the culture filtrate was assayed for deaminase activity. The preparation of samples for the deaminase assay and the assay itself are described below.

Preparation of culture media for deaminase assay. Growth assays were performed in Erlenmeyer flasks as described above. The media from duplicate flasks were combined, filtered through glass fiber filters (Whatman GF/A), and dialyzed extensively at 4°C against distilled water.

Deaminase assay. The assay used to detect deaminase activity is a modification of one developed by Woodward and Cirillo (34). Our assay is a microassay that detects the ability of dialyzed culture medium to generate radiolabeled keto acid from the radiolabeled amino acid L-phenylalanine. The details of the method have been described previously (7).

Glutamine transport assays. Conidial cultures were harvested aseptically into cold sterile glass distilled water, filtered, and stored at 4°C in an ice bath as previously described (8, 19).

Transport assays were performed with conidial suspensions that had been developed to the postconidial stage. Conidia were incubated at a final concentration of 0.1 mg/ml for 3 h at 35°C in 1× Vogel medium N containing 1% D-glucose. Assays were initiated by adding uniformly labeled L-[^{14}C]glutamine at a final concentration of 3.5×10^{-5} M (0.57 mCi/mmol). Samples were removed at 12-min intervals and counted in a Beckman gas flow proportional counter as previously described (8).

Chemicals. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., and were of the highest purity available. Radiolabeled L-[^{14}C]phenylalanine was uniformly labeled and was purchased from Schwarz/Mann, Cambridge, Mass.

RESULTS

Control of deaminase production by nitrogen catabolite repression. Ammonia, glutamate, and glutamine are each believed to mediate nitrogen metabolite repression in the fungi, with glutamine being the end metabolite and true effector and ammonia and glutamate acting through conversion to glutamine. We examined the effect of each of these repressors on extracellular deaminase production by the *pmn*; *pmb*; *pmg* strain in response to amino acids as sole sources of nitrogen (Fig. 1). This strain is defective for each of the three constitutive amino acid permeases, the neutral amino acid-specific permease, the basic amino acid-specific permease, and the general permease. When presented with an amino acid as the sole source of nitrogen, this strain

TABLE 1. Growth and deaminase production by the *pmn*; *pmb*; *pmg* strain in response to arginine in the presence of various nitrogen sources

Nitrogen Source	Concn (mM)	− Arginine		+ Arginine	
		Growth[a]	Activity[b]	Growth[a]	Activity[b]
None		0.5	0.4	68	8.6
Ammonium chloride	0.50	5	1.1	52	3.8
Potassium nitrate	0.50	5	1.2	48	5.1
Uric acid	0.50	16	1.1	64	3.8
Xanthine	0.25	3	1.6	48	8.3
Allantoin	0.25	5	0.7	62	4.7
Urea	0.50	10	1.1	62	5.6

[a] Growth is expressed in milligrams (dry weight) after 72 h of incubation at 35°C. All media contained NNV basal salts. L-Arginine was added at a final concentration of 15mM.

[b] Extracellular deaminase activity is expressed as $\times 10^{-2}$ nmol of L-phenylalanine converted per min per ml of culture filtrate. Assays were performed at 37°C.

adapts by producing an extracellular deaminase that cleaves the alpha amino nitrogen from the amino acid (9, 10). The wild-type strain cannot produce deaminase in response to the amino acids that we tested (9).

Each amino acid substrate examined was previously shown to elicit production of an extracellular deaminase by this strain but to differ considerably in the ability to support growth (9, 10). Both ammonium chloride and L-glutamine greatly reduced deaminase production, whereas sodium glutamate and L-asparagine had little effect (Fig. 1).

Role of nitrogen limitation in production of the extracellular deaminase. To determine whether enzyme production resulted from derepression in response to nitrogen limitation or from specific induction by an amino acid, we tested the ability of several secondary nitrogen sources to elicit deaminase production in the absence and presence of the amino acid arginine (Table 1). The nitrogen sources used are those known to cause nitrogen limitation when they are present at the concentrations that we used (25). Similar results were obtained when arginine was replaced by either the neutral amino acid alanine or the basic amino acid ornithine (data not shown). The inability of the secondary nitrogen source to elicit deaminase production in the absence of amino acid suggests that production requires substrate induction in addition to nitrogen limitation.

TABLE 2. Growth and deaminase production by the *pmn*; *pmb*; *pmg* strain in response to various amino acids in the presence of allantoin as the nitrogen source

Amino acid addition	Concn (mM)	− Allantoin		+ Allantoin	
		Growth[a]	Activity[b]	Growth[a]	Activity[b]
None		0.5	0.4	14	0.7
α-Aminoisobutyrate	15	Tr	0.5	10	2.9
L-Lysine	15	1	3.0	15	2.9
L-Tryptophan	15	6	1.6	16	2.6
D-Methionine	15	4	2.0	26	5.2
D-Phenylalanine	15	1	1.2	10	8.8
L-Histidine	15	3	7.2	3	6.9

[a] Growth is expressed in milligrams (dry weight) after 72 h of incubation at 35°C. All media contained no-nitrogen Vogel basal salts. Allantoin was present at a concentration of 0.5 mM. In the absence of allantoin, the amino acid was the sole nitrogen source.

[b] Extracellular deaminase activity is expressed as $\times 10^{-2}$ nmol of L-phenylalanine converted per min per ml of culture filtrate. Assays were performed at 37°C.

The discovery that various secondary nitrogen sources could support growth of the *pmn*; *pmb*; *pmg* strain and allowed deaminase production in response to amino acids enabled us to separate the ability of an amino acid to elicit deaminase from its ability to support growth. Allantoin was chosen as the nitrogen source because it supported growth adequately and allowed deaminase production. Several amino acids previously found to be unable to support growth were examined for their ability to elicit deaminase production in the presence of allantoin as the nitrogen source. Each of these amino acids was capable of inducing the deaminase (Table 2).

Minimum concentration of amino acid required for deaminase induction. We then used allantoin as the nitrogen source and examined a variety of amino acids for the minimum concentration required to induce the deaminase. All amino acids were tested at several concentrations, spanning the nanomolar to millimolar range. No detectable induction occurred in the nanomolar range, induction could be seen when the concentration of amino acid reached 20 μM, and each amino acid was a competent inducer at millimolar concentrations (Table 3).

Time course of deaminase production. Culture filtrates of the *pmn*; *pmb*; *pmg* strain incubated with arginine as the sole nitrogen source were sampled at intervals over the standard 72-h growth period to determine the timing of extracellular deaminase production. Activity was first detected at 26 h and steadily increased over the ensuing 48 h (Fig. 2).

Control of deaminase production by the *nit-2* locus. Since deaminase production in response to nitrogen limitation plus the presence of substrate was subject to nitrogen metabolite repression, we examined whether production was under control of the *nit-2* locus, which regulates expression of activities subject to such repression in *N. crassa*. This locus is the sole major nitrogen regulatory locus that has been identified in this organism. By virtue of its ability to regulate expression of a number of permeases and metabolic activities that enable the organism to utilize secondary nitrogen sources, this locus has defined the activities that constitute the nitrogen regulatory circuit.

We constructed *pmn*; *pmb*; *pmg*; *nit-2* strains by using three alleles of the *nit-2* locus and tested the ability of each strain to utilize amino acids as sole sources of nitrogen. The effect of the I7 allele on amino acid utilization and deaminase production is shown in Fig. 3a. Similar results were obtained with the K31 and nr37 alleles (data not shown).

Since in most instances the *pmn*; *pmb*; *pmg*; *nit-2* strains gave no measurable growth when an amino acid was the sole

TABLE 3. Induction of extracellular deaminase by the *pmn*; *pmb*; *pmg* strain in response to different concentrations of amino acids

Amino acid inducer	Deaminase activity at a concn of:[a]				
	1 nM	1 μM	10 μM	20 μM	15 mM
L-Histidine	0.7	1.0	1.7	2.1	11.2
L-Phenylalanine	0.5	0.2	2.3	2.3	9.3
L-Alanine	0.5	0.5	1.7	2.0	4.3
L-Ornithine	1.0	0.7	0.6	2.5	11.8
L-Arginine	1.0	1.0	1.7	2.0	10.5

[a] Extracellular deminase activity is expressed as $\times 10^{-2}$ nmol of L-phenylalanine converted per min per ml of culture filtrate. All media contained no-nitrogen Vogel basal salts and 0.5 mM allantoin as the nitrogen source. The extracellular deaminase activity produced in response to allantoin in the absence of amino acid was 0.9×10^{-2} nmol converted per min per ml.

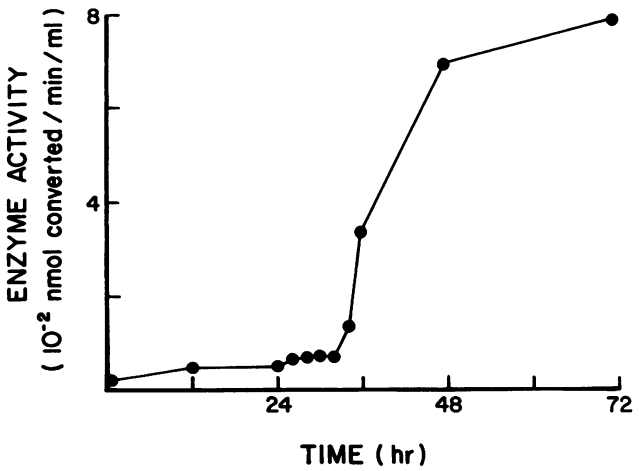

FIG. 2. Time course of production of the extracellular deaminase by the *pmn*; *pmb*; *pmg* strain in response to 15 mM L-arginine as the sole nitrogen source. Growth and deaminase assays were conducted as described in the legend to Fig. 1.

nitrogen source, we retested the *pmn*; *pmb*; *pmg* and *pmn*; *pmb*; *pmg*; *nit-2* strains by using nitrogen sources that established conditions of nitrogen limitation, supported growth of the *pmn*; *pmb*; *pmg*; *nit-2* strains, and yet did not interfere with the ability of the *pmn*; *pmb*; *pmg* strain to produce deaminase. Such conditions allowed us to distinguish between failure of the *pmn*; *pmb*; *pmg*; *nit-2* strains to produce detectable enzyme as a consequence of not attaining a critical growth mass and failure to express the enzyme as a direct consequence of the absence of the *nit-2* gene product. Allantoin was initially used since it is one of the few alternate nitrogen sources that permits growth of *nit-2* strains. However, insufficient growth of the *pmn*; *pmb*; *pmg*; *nit-2* strains was obtained at allantoin concentrations that allowed deaminase production in the *pmn*; *pmb*; *pmg* strain. We then used limiting concentrations of ammonium chloride (1.5 mM) as the nitrogen source. The data obtained with the *pmn*; *pmb*; *pmg*; *nit-2* (I7 allele) strain suggest that the *pmn*; *pmb*; *pmg* strain is unable to produce the extracellular deaminase under induction conditions in the absence of a functional *nit-2* gene product (Fig. 3). Similar results were obtained with the K31 and nr37 alleles (data not shown).

Glutamine transport by the *pmn*; *pmb*; *pmg*; *nit-2* strain. The ability of the *pmn*; *pmb*; *pmg*: *nit-2* strain to utilize glutamine as a nitrogen source in the absence of measurable deaminase production suggests that an alternate mechanism for glutamine utilization exists and that this mechanism is not controlled by the *nit-2* locus. As an initial approach to identifying this mechanism, we tested the possibility that glutamine, as a major nitrogen source, may be transported by a system distinct from the constitutive amino acid permeases. We attempted a preliminary examination by choosing a developmental stage, the postconidial stage, which is known to be very active for the transport of amino acids. No transport of L-glutamine by the *pmn*; *pmb*; *pmg*; *nit-2* strain could be detected in this developmental stage (Fig. 4).

DISCUSSION

When the amino acid transport-defective *pmn*; *pmb*; *pmg* strain of *N. crassa* is presented with amino acids as sole nitrogen sources, it produces an extracellular deaminase that can generate usable nitrogen, in the form of ammonium, from the amino-acid molecules. Nitrogen metabolite repression prevents production of deaminase activity when primary nitrogen sources are plentiful. Production requires the lifting of nitrogen metabolite repression, the presence of amino acid inducer, and a functional gene product of the major nitrogen regulatory locus, *nit-2*. Activity is elicted by

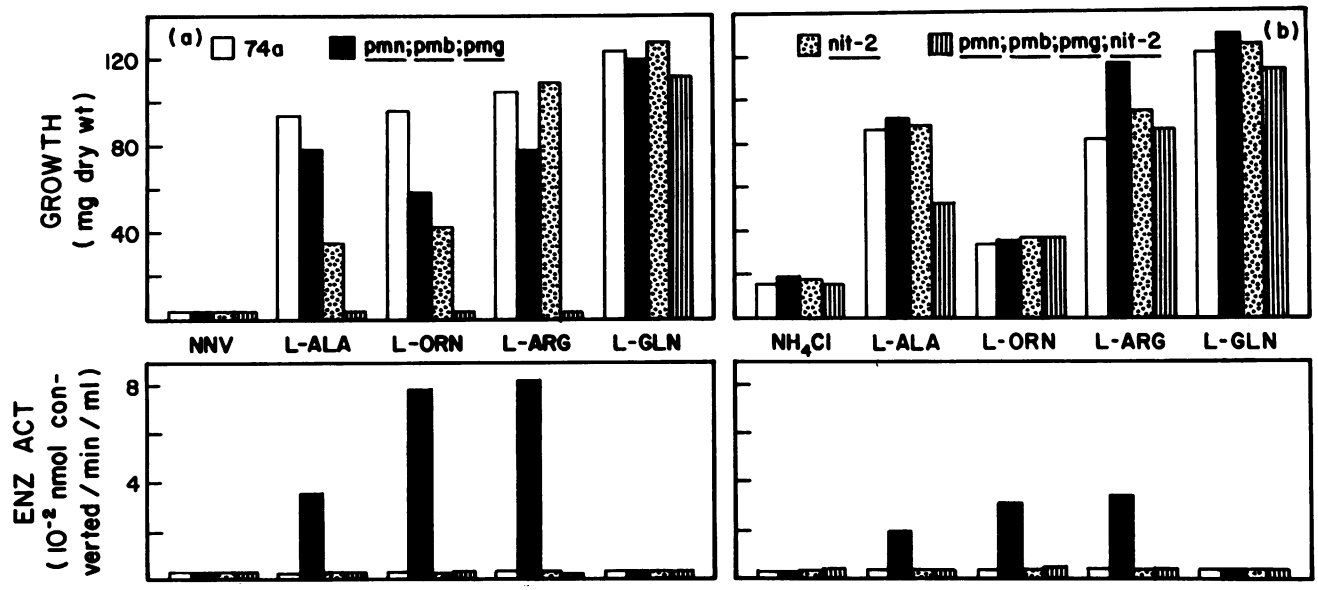

FIG. 3. Effect of the *nit-2* locus (allele I7) on growth and deaminase production by the *pmn*; *pmb*; *pmg* strain. (a) Response to amino acids as sole nitrogen sources. The amino acids L-alanine, L-ornithine, L-arginine, L-glutamine, and L-asparagine were present at concentrations of 15 mM. The media contained no-nitrogen Vogel (NNV) salts as the basal component. (b) Response to amino acids when 1.5 mM ammonium chloride (NH_4Cl) was the nitrogen source. Growth and deaminase assays were conducted as described in the legend to Fig. 1. Enz Act, Enzyme activity.

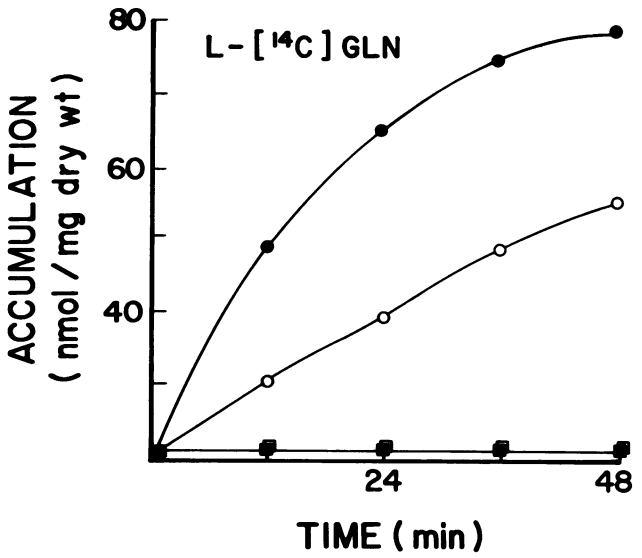

FIG. 4. Transport of L-glutamine during the postcondidial developmental stage. Strains were incubated at 35° for 3 h in Vogel medium N containing 1% D-glucose and subsequently incubated with L-[^{14}C]glutamine for 48 min. Symbols: ●, wild type; ○, *nit-2*; ■, *pmn*; *pmb*; *pmg*; □, *pmn*; *pmb*; *pmg*; *nit-2*.

inducer concentrations as low as 20 μM. When the time course of the appearance of activity in response to inducer is monitored, activity is detectable at 26 h and rises steadily for the remainder of a 72-h growth period.

The most effective nitrogen metabolite repressors with respect to deaminase production appear to be L-glutamine and ammonium, with sodium glutamate being moderately effective and L-asparagine virtually ineffective. L-Asparagine was tested because of its similarity in structure to L-glutamine but proved to be completely ineffective in repressing deaminase production. As a direct precursor to glutamine, glutamate might be expected to mimic glutamine. However, since the *pmn*; *pmb*; *pmg* strain is defective for amino acid transport, the amino acid repressors must first enter cells by a mechanism other than the normal transport route, presumably via the deaminase route, and then be reassembled internally. Glutamine, but not glutamate, appears to have yet another route of entry (see below) which may result in its being readily available as a repressor, in contrast to glutamate.

The requirement of a functional *nit-2* gene product for deaminase production is clear, and the role of this gene product is presumably as a positive effector for gene expression, as has been postulated for other structural genes on the nitrogen regulatory circuit. Although no transport activity has been detected in numerous transport assays similar to those displayed in Fig. 4, we cannot conclusively rule out the possibility that the *pmn*; *pmb*; *pmg* strain retains residual amino acid transport activity and that loss of a functional *nit-2* gene results in loss of this residual activity so that the inducer is excluded and cannot affect deaminase production. We are currently examining transport activity in the *pmn*; *pmb*; *pmg* strain with respect to very high concentrations of amino acids in an effort to detect any residual activity that might be present.

Despite the *nit-2* effect on deaminase production, this mutation does not prevent glutamine from being used as a sole nitrogen source. The fact that the *pmn*; *pmb*; *pmg*; *nit-2* strain can neither produce deaminase nor transport glutamine under the transport conditions which we tested suggests that this amino acid serves as a nitrogen source by an independent, as-yet-undetermined mechanism. We feel that such a mechanism is likely an external degradative mechanism, such as that described by Mora and co-workers for *N. crassa* (13). In a series of reactions, glutamine is converted to ammonium plus alpha-ketoglutarate and ultimately is resynthesized internally as glutamine, which is then available as both a nitrogen donor and a repressor. We are pursuing studies of this mechanism, along with glutamine transport studies at millimolar concentrations that will allow detection of an additional permease or residual activity of the known permeases, as a possible explanation for the ability of the *pmn*; *pmb*; *pmg*; *nit-2* strain to utilize glutamine in the absence of deaminase activity.

Several amino acids have previously been shown to be unable to support growth (9). With the discovery that using allantoin as the alternate nitrogen source provides a means for uncoupling amino acid as inducer from amino acid as nitrogen source, we were able to test these amino acids for their ability to induce deaminase activity. All were inducers. The failure of glutamate and glutamine to fully repress deaminase production likely reflects a balance between their role as repressors and their role as inducers. Ammonium, which is not an amino acid and therefore not an inducer, should be capable of full repression of deaminase production, and this was observed. Second, since L-tryptophan and L-lysine are capable of inducing the deaminase, the failure of the *pmn*; *pmb*; *pmg* strain to utilize these amino acids as sole nitrogen sources is not due to their inability to induce the deaminase. Possibly these amino acids can serve as inducers but not substrates for the enzyme. However, since they are competitive inhibitors of phenylalanine conversion, we suspect that they are substrates for the deaminase (10). Alternatively, these amino acids or their metabolites may be inhibitory to growth. Amino acid inhibition has been reported by several investigators, and explanations for this phenomenon include metabolite starvation, interference with cell division, and alteration of macromolecular synthesis (4, 6, 31). In addition, it may be that the keto acids generated by the deaminase from lysine and tryptophan are growth inhibitory. We will be able to test this possibility through the use of strains defective for keto acid transport.

The finding that D-methionine, when not required to serve as a sole nitrogen source, could elicit deaminase production suggests that the extracellular deaminase described here may be the same activity as the nitrogen-regulated L-amino acid oxidase reported by Sikora and Marzluf (28). D-methionine is a gratuitous inducer of the oxidase. We previously observed no induction by this amino acid when it was present as the sole nitrogen source, presumably because it could not support sufficient growth to allow detection of deaminase activity rather than because it was unable to induce deaminase (9). Preliminary biochemical studies in our laboratory suggest that the deaminase is an L-amino acid oxidase. Induction of both enzymes requires the lifting of nitrogen metabolite repression and a functional *nit-2* gene product. L-histidine is a strong inducer of both enzymes. The main difference between the data presented for the two enzymes to date concerns the ability of the wild-type strain to produce activity in response to particular amino acids. Marzluf and co-workers have detected oxidase production by the wild type in response to all amino acids tested. We did not detect deaminase production by the wild type in response to L-alanine, L-ornithine, or L-arginine; only strains

defective for amino acid transport activity produced deaminase activity in response to these particular amino acids. Since data for oxidase production in response to these three amino acids are not available and we would expect to detect the activity in our assay if it were present in wild-type cultures, we feel that the discrepancies in the available data are attributable to different test conditions rather than to different enzymatic activities. The two activities are likely the same, and a comparative study of the biochemical properties of these two activities is currently in progress.

The deaminase-amino acid utilization system provides an additional approach for examining the role of nitrogen metabolite repression and of the *nit-2* gene in the regulation of gene expression. Additionally, since the *pmn*; *pmb*; *pmg*; *nit-2* strain cannot utilize most amino acids as sole nitrogen sources, we should be able to select for mutant strains capable of utilizing amino acids as nitrogen sources and thereby localize the region of the deaminase structural gene with which the *nit-2* gene product interacts.

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