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Genetics and Physiology of *Neurospora crassa* Glutamine Auxotrophs

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This work reports on the isolation and characterization of two glutamine auxotrophs in *Neurospora crassa*. The mutations responsible for the glutamine-requiring phenotype were very closely linked, and one of them proved to be recessive to wild type. The mutations impaired the conversion of glutamic acid to glutamine and resulted in changes of both the activity and oligomeric structure of the enzyme glutamine synthetase.

Recently, the role of glutamine synthetase (EC 6.3.1.2) in the regulation of nitrogen metabolism as well as in its own regulation has been established in procaryotes by the detailed studies of Magasanik and co-workers (6, 9, 11, 14). The isolation and characterization of glutamine auxotrophs in eucaryotes should help to elucidate whether the enzyme plays a similar role in these organisms. Glutamine auxotrophs have been isolated from two eucaryotes, namely, *Saccharomyces cerevisiae* (2, 3) and *Neurospora crassa* (E. Reich and S. Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49-50, 1963). However, characterization of the enzyme products of these mutations is lacking.

Studies from our laboratory have established that *N. crassa* accumulates glutamine and arginine as carbon and nitrogen reservoirs and that a metabolic link exists between the synthesis of glutamine and the catabolism of arginine (15; Y. Mora, G. Espín, K. Willms, and J. Mora. J. Gen. Microbiol., in press). In addition, we have purified and partially characterized glutamine synthetase from the wild-type 74-A strain of *N. crassa* (10) and studied the regulation of enzyme concentration (12; I. Vichido, Y. Mora, C. Quinto, R. Palacios, and J. Mora, J. Gen. Microbiol., in press). In this paper we report the isolation and partial characterization of two glutamine auxotrophs from *N. crassa*.

MATERIALS AND METHODS

Stocks. All stocks came from the Fungal Genetics Stock Center at Humboldt State University Foundation, Arcata, Calif. or from the collection of J. Mora. The basic stocks were wild-type strains 74-A and 73-a, glutamine auxotroph *gln-1a* (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49-50, 1963), arginine auxotroph *arg-5*, tryptophan auxotroph *tryp-2*, and proline auxotroph *prol-3*. All double mutants were obtained from appropriate crosses of the mentioned stocks.

Growth conditions. *N. crassa* was grown on liquid minimal medium (N medium) of Vogel (16) supplemented with 1.5% sucrose. Conidia were harvested from slants of N medium supplemented with 1.5% sucrose that had been incubated in the dark for 3 days at 29°C followed by 2 days under incandescent light at 25°C. Cultures were started by inoculating conidia into 2-liter Florence flasks containing 1 liter of N medium. The initial optical density of the culture at 540 nm was 0.05. Incubation was for 12 to 36 h at 25°C with continuous bubbling with hydrated air. Other nitrogen sources in place of or in addition to NH_4NO_3 were employed as indicated in the text.

Fed-batch cultures of *Neurospora* were incubated at 25°C in 6-liter Florence flasks containing 5 liters of N medium without nitrogen source. After inoculation with conidia, the cultures were fed with 9.2 mM NH_4Cl at a dilution rate of 3.2×10^{-3} h^{-1} (7).

Protein determination. Samples of mycelium were collected on membrane filters (Millipore Corp., Bedford, Mass; type HA, 0.45 μm) and washed with 2 volumes of distilled water. The suspended samples were then precipitated in 2 ml of 5% trichloroacetic acid and centrifuged for 5 min at 2,000 rpm, and the pellets were suspended in 1.0 N NaOH. Protein was determined by the method of Lowry et al. (8), using bovine serum albumin as a standard.

Mutagenesis and mutant selection. A conidial suspension (2×10^7) in twice-distilled water was incubated with 1.36 mM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) for 1 h in the dark at 25°C with shaking. Conidia were then concentrated by filtration on a membrane filter (Millipore Corp.; type RAWP, 0.47 μm) and washed with cold water. After mutagenesis, conidia were incubated in a 500-ml Erlenmeyer flask with 250 ml of N medium supplemented with 1.5% sucrose and 5 mM glutamate as the sole nitrogen source. The flask was incubated with shaking at 37°C. Every 6 h the entire culture was filtered through cheesecloth into an Erlenmeyer flask containing fresh medium, the process being repeated eight times. The enriched spore population was concentrated by filtration, washed, resuspended in twice-distilled water, and plated on N agar medium supplemented with glucose and fructose (0.05% each) in place

of sucrose and containing 1% sorbose and 1.35 mM glutamine as the sole nitrogen source. Plates were incubated at 29°C, and the colonies that appeared after 2 to 5 days of incubation were transferred to slants of N agar medium supplemented with 1.5% sucrose and 1.36 mM glutamine.

Spot testing, crosses, and progeny analysis were carried out as previously reported (1). Complementation tests were performed in tubes with N medium by incubating conidia of both mutant strains at a final concentration of 5×10^4 each per ml for 5 days at 29°C. Mutants were purified by crosses with the wild-type strain.

Glutamate and glutamine pools. Glutamate and glutamine were extracted as described by Vaca and Mora (15) and separated as reported by Ferguson and Simms (4). Both amino acids were measured by an isotopic dilution method after addition of [^{14}C]glutamine and [^{14}C]glutamic acid (New England Nuclear Corp., Boston, Mass.) to the crude extract.

Determination of glutamine synthetase activity. Cell-free extracts were prepared as previously described (10). Glutamine synthetase was measured by its transferase and synthetase activities by the methods of Ferguson and Simms (5). Units of activity were expressed as micromoles of γ-glutamyl hydroxamate produced per minute at 30°C.

Sucrose gradient sedimentation. Samples of 0.5 ml of the cell-free extracts were layered over a 5 to 20% continuous sucrose gradient in either buffer A (5 mM phosphate–0.5 mM ethylenediaminetetraacetic acid–50 mM K_2SO_4 [pH 7.2]) or buffer B (50 mM imidazole–0.5 mM ethylenediaminetetraacetic acid–50 mM K_2SO_4–5 mM 2-mercaptoethanol–80 mM MgCl–50 mM glutamate [pH 7.1]) as indicated and centrifuged for 12 h at 4°C in a Spinco L5-75 ultracentrifuge at 40,000 rpm with a SW40 rotor. After centrifugation, 0.3-ml fractions were collected from the top of the tubes, and glutamine synthetase activity was determined in each fraction. Globular proteins were used as markers of sedimentation as previously described (10).

Purification of in vivo-labeled glutamine synthetase. Batch cultures of the wild-type strain grown on N medium with 5 mM glutamate as the sole nitrogen source received a 60-min pulse of [^{3}H]methionine (New England Nuclear Corp.; 1 μCi/ml). From these cultures, glutamine synthetase was purified as previously described (10).

RESULTS

Spot testing, complementation, and allelism of glutamine auxotrophs. Table 1 shows the growth patterns on plates of the two different glutamine auxotrophs. Mutant *gln-1a* was not able to grow on ammonia and/or glutamic acid at 29°C. It grew at 37°C with glutamate as the sole nitrogen source, but not when both glutamate and ammonia were present in the medium; only glutamine was used as a nitrogen source at both temperatures.

The selection procedure used to obtain mutant *gln-1a* is unknown (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49–50, 1963). The mutant strain *gln-1b*, which was selected because of its inability to grow on glutamate at 37°C, also did not utilize ammonia as a nitrogen source at either 29 or 37°C, and only glutamine supported growth.

TABLE 1. *Growth response of N. crassa glutamine auxotrophs to different nitrogen sources and temperatures*

Strain	Temp (°C)	Growth[a]			
		NH_4NO_3	Glu	NH_4NO_3 + Glu	Gln
74-A	29	+	+	+	+
	37	+	+	+	+
gln-1a	29	−	−	−	+
	37	−	+	−	+
gln-1b	29	−	−	−	+
	37	−	−	−	+

[a] Plates were incubated for 36 h before growth was scored as + (good growth) or − (no growth); Glu and Gln were added at a final concentration of 200 μg/ml, and NH_4NO_3 was added at a final concentration of 25 mM.

The recessiveness of the *gln-1b* mutation was established by complementation analysis. Heterocaryons were produced when conidia of the double mutant *gln-1b tryp-2* plus conidia of the mutant strain *arg-5* were cultured together in N medium, in which neither strain alone was able to grow. We were not able to perform the complementation analysis of the *gln-1a* strain because of its leakiness after prolonged incubation in N medium.

The mutant *gln-1b* segregated as a monogenic mutation. When this mutant was crossed with the 73-a wild-type strain, dissection of two asci demonstrated that half of the ascospores were glutamine auxotrophs, whereas the remainder were prototrophs. The cross between *gln-1a* and *gln-1b* only gave one prototroph out of 5,000 viable ascospores. Thus, the two mutations map very close to one another and are probably allelic.

Physiological and biochemical characterization of the mutants. As shown in Fig. 1, the mutant strains *gln-1a* and *gln-1b* started growing slowly only after 18 h of incubation in N medium.

The conidia used as the inoculum in these experiments were obtained from slants of N medium in which the NH_4NO_3 was substituted by 10 mM glutamine. A different situation held when conidia were harvested from slants of N medium plus 200 μg of glutamine per ml. In this case, growth started after 6 to 8 h of incubation and at a rate higher than that above (Fig. 1A).

In fed-batch ammonium-limited cultures, the

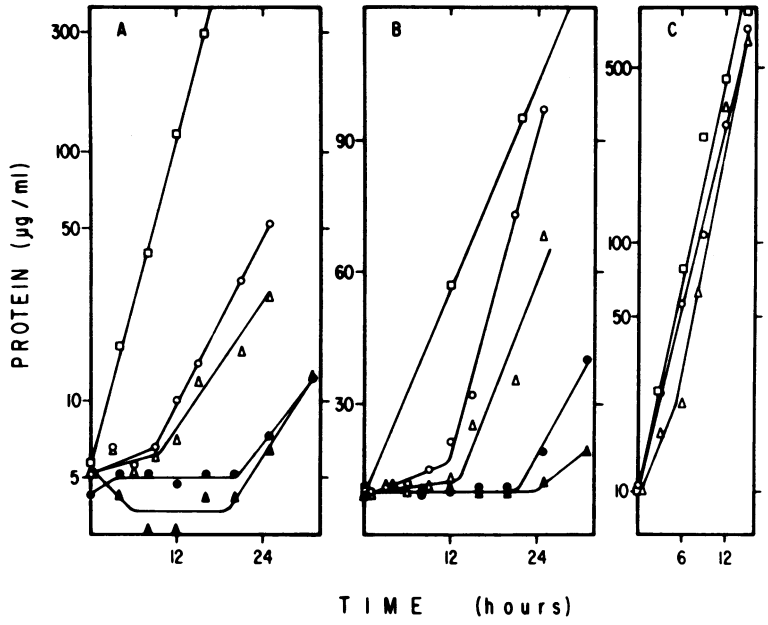

FIG. 1. *Growth curves of wild type* (□) *and mutants gln-1a* (○, ●) *and gln-1b* (△, ▲) *on 25 mM* NH_4NO_3 *(A), on fed-batch ammonium-limited cultures (B), and on 5 mM glutamine (C). Open symbols indicate conidia harvested from slants containing 25 mM* NH_4NO_3 *plus 1.36 mM glutamine; closed symbols indicate conidia harvested from slants containing 10 mM glutamine as the sole nitrogen source.*

mutants grew linearly after a lag similar to that observed in an excess of ammonium (Fig. 1B). Optimal growth of both mutants was observed when 5 mM glutamine was present as the sole nitrogen source (Fig. 1C).

In the presence of either low or high concentrations of ammonium, as the sole nitrogen source, i.e., under nongrowing conditions, the mutants accumulated a high glutamate pool, indicating a block in the synthesis of glutamine. Accordingly, the glutamine pool was found to be very low under this condition (Table 2). A lowering of the accumulated glutamate was observed after prolonged incubation when the mutants started to grow on ammonium (data not shown). The *prol-3* mutant was included as a control in these experiments to compare the pool size of glutamic acid in a different amino acid auxotroph under nongrowing conditions.

Glutamine synthetase activity as judged by the synthetase assay (see above) was 20- to 30-fold lower than the wild-type activity when the mutants were grown in the presence of glutamine as the sole nitrogen source (Table 3). Enzyme activities, when measured by the transferase assay, were about 10-fold lower in the mutants than in the wild type. The ratios of synthetase to transferase activities were, therefore, lower in the mutants than in the wild type.

Previous studies in our laboratory have established that *N. crassa* glutamine synthetase can exist in two different oligomeric states. When the organism is grown exponentially in the presence of glutamate, glutamine, or ammonia as the sole nitrogen source, the enzyme is found as an octamer (Vichido et al., J. Gen. Microbiol., in press). On the other hand, when limiting ammonia is present as the nitrogen source, the enzyme is found as a tetramer (7).

Instead of the octameric structure of the enzyme present in the wild-type mycelium grown on ammonium excess, the mutants had lower oligomeric forms under similar conditions (Fig.

TABLE 2. *Glutamate and glutamine pools of glutamine auxotrophs under different growth conditions*

Strain	Ammonium condition	Glutamate[a]	Glutamine[a]
74-A	Limiting	0.094	0.036
	Excess	0.170	0.265
gln-1a	Limiting	0.595	0.042
	Excess	0.702	0.029
gln-1b	Limiting	0.424	0.043
	Excess	0.565	0.061
prol-3	Excess	0.170	0.330

[a] Amino acid pools were determined at 12 h after inoculation of conidia in liquid medium and are expressed as micromoles per milligram of extracted protein.

2A). Figure 2B shows the sedimentation profiles of glutamine synthetase from wild-type and mutant strains *gln-1a* and *gln-1b* grown with limited ammonia. Both showed the presence of glutamine synthetase as a tetramer. In addition, the *gln-1b* mutant also showed an oligomeric form corresponding to a dimer. An abnormal oligomeric pattern was also observed in the mutant strains when they were grown on glutamine at 37°C, and extracts were prepared in the presence of glutamate, magnesium, and 2-mercaptoethanol (Fig. 2C). This experiment was carried out at 37°C since growth is optimal and glutamine synthetase activities are higher than those found in cultures grown in glutamine at 25°C. In this case, wild-type glutamine synthetase was present as an octamer, whereas in the mutants the enzyme was found in oligomeric structures of lower molecular weight.

TABLE 3. *Glutamine synthetase activity in glutamine auxotrophs*

Strain	Glutamine synthetase activity[a]		
	S	T	S/T ratio
74-A	0.0200	0.060	0.33
gln-1a	0.0010	0.005	0.20
gln-1b	0.0006	0.006	0.10

[a] Glutamine synthetase specific activity was determined after 12 h of growth at 25°C on glutamine as the sole nitrogen source. Specific activity is expressed as micromoles of γ-glutamyl hydroxamate produced per minute per milligram of protein at 30°C. S, Synthetase activity; T, transferase activity.

The extracts of mutant strains did not seem to alter significantly the purified octameric form of the wild type as evidenced by the presence of both the mutant tetramer and the wild-type octamer when they were centrifuged together in the same gradient (Fig. 3).

DISCUSSION

Of the two glutamine auxotrophs partially characterized, one of them, *gln-1b*, was selected because of its inability to synthesize glutamine at 37°C in the presence of glutamic acid as the sole nitrogen source. The rationale for this was to maintain conditions whereby mutant strains would show a negative phenotype while the wild-type strain has a fully induced glutamine synthetase.

The other mutant strain included in this study, *gln-1a*, isolated some years ago (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 40–50, 1963), showed somewhat different phenotypic characteristics. In particular, it was able to grow at 37°C with glutamate as the sole nitrogen source, whereas mutant *gln-1b* was not.

The low frequency of prototrophs obtained after crossing the two mutant strains indicates

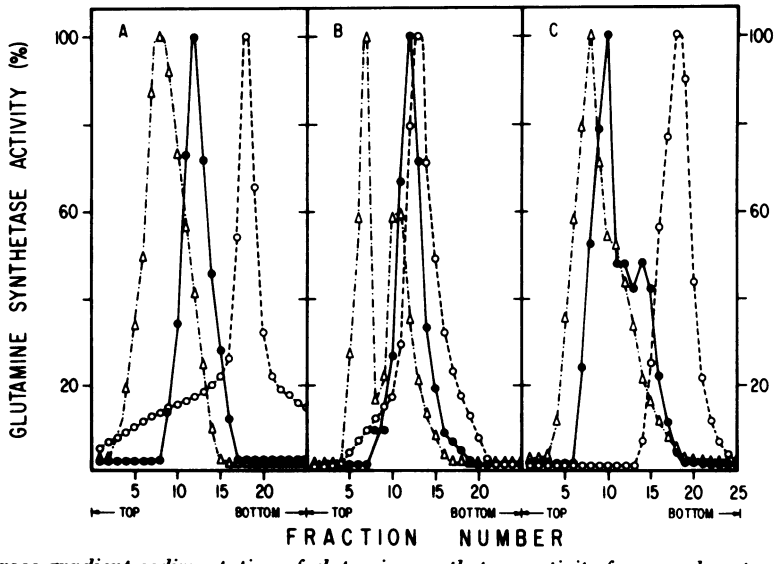

FIG. 2. *Sucrose gradient sedimentation of glutamine synthetase activity from crude extracts of wild type (○) and gln-1a (●) and gln-1b (△) mutants. (A) Wild-type strain grown for 12 h at 25°C and mutant strains grown for 24 h at 25°C on 25 mM NH_4NO_3; extracts and sucrose gradients prepared in buffer A (see text). (B) Three strains grown for 24 h at 25°C on fed-batch ammonium-limited cultures; extracts and sucrose gradients prepared in buffer A. (C) Three strains grown for 12 h at 37°C on 5 mM glutamine; extracts and sucrose gradients prepared in buffer B. Glutamine synthetase activity was normalized to the peak fraction in each gradient.*

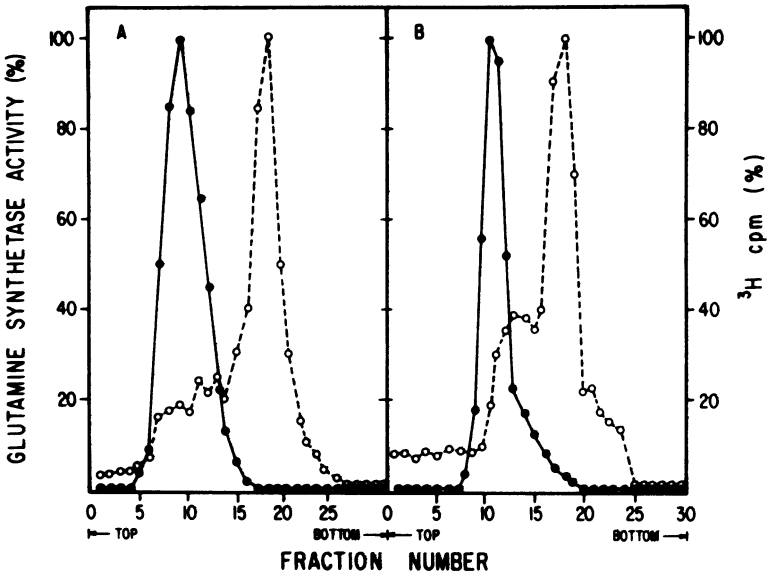

FIG. 3. *Sucrose gradient sedimentation of crude extracts from mutant strains (●) mixed with ^{3}H-labeled purified glutamine synthetase (○) from the wild-type strain. Mutant strains were grown for 12 h at 37°C on 5 mM glutamine as the nitrogen source. Extracts were prepared in buffer A and mixed with purified in vivo-labeled glutamine synthetase. (A) gln-1a strain; (B) gln-1b strain. Glutamine synthetase activity and radioactivity were normalized to the peak fraction in each gradient.*

that both mutations are very closely linked in chromosome VR, where mutant *gln-1a* has already been mapped (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49–50, 1963). The complementation analysis, possible only in mutant *gln-1b*, showed that this mutation is recessive to the wild type.

The fact that glutamate was accumulated by the mutant strains in either excess or limiting ammonium during the lag period and the decrease of this amino acid pool after growth resumed indicate that these mutations impair the synthesis of glutamine. The adaptation of the mutants to grow on ammonium after a lag, as well as the relationship between the nitrogen source present in the inoculum slants and the duration of this lag phase, is being studied.

The ability of the mutant strains to grow on ammonium after a prolonged lag phase, together with the presence of the tetramer in mutant *gln-1a* and of the tetramer and dimer in the mutant *gln-1b*, suggests a physiological role for these oligomeric structures of glutamine synthetase in the fixation of ammonium. These data reinforce the contention that the prevalence of the tetrameric over the octameric forms of the enzyme in wild-type *N. crassa* is a regulatory response to the presence in the medium of a limiting concentration of ammonium, and vice versa with ammonium excess (Fig. 2) (7).

The differences in glutamine synthetase activity of the mutants as compared with those found in the wild type growing under a variety of nitrogen conditions (Table 3) could be due to either structural or regulatory alterations in the mutant strains. The differences in behavior of the mutants in regard to the oligomeric structure of glutamine synthetase, namely, (i) the absence of the octameric form, (ii) the impairment of the in vitro conversion of the mutant tetramer to the octameric form as occurs in the wild-type strain (unpublished data), and (iii) the presence of dimeric forms, indicate alterations in the structure of the enzyme. This view is further supported by an immunochemical study of enzyme activity (Sánchez, Dávila, Mora, and Palacios, submitted for publication). The possibility that these mutant enzymes could alter the regulation of the enzyme during its biosynthesis is now being studied. The autogenous regulation of glutamine synthetase has been well documented in *Escherichia coli* (13, 17) and *Klebsiella aerogenes* (6, 9, 14).

The isolation of mutants unable to grow on limited amounts of ammonia and deficient of glutamine synthetase activity will help to correlate the structure and function of eucaryotic glutamine synthetase as well as to better understand the genetics of the stucture and regulation of this enzyme.

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