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Isolation and Characterization of *Neurospora crassa* Mutants Impaired in Feedback Control of Ornithine Synthesis

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Thirty-two independent mutants were isolated which overcame the proline requirement of *pro-3* mutations in *Neurospora crassa*. The mutations were not revertants, appeared to be allelic, were closely linked or allelic to *arg-6*, and in strains unable to degrade ornithine no longer suppressed the proline requirement. The suppressor mutations did not alter the levels of biosynthetic or catabolic enzymes, yet allowed accumulation of ornithine. Suppressed strains unable to degrade arginine still produced ornithine (as detected by growth) in arginine-supplemented medium. The results suggest that the suppressor mutants were impaired in the feedback inhibition of ornithine synthesis by arginine. The activity of the appropriate biosynthetic enzyme was less sensitive to inhibition by arginine. The potential usefulness of such mutations is discussed.

The regulation of arginine metabolism in *Neurospora crassa* involves the complex interaction of a number of regulatory and organizational features. Regulation involves (i) modulation of enzyme activity, (ii) variations in the levels of biosynthetic and catabolic enzymes (induction and repression), and (iii) controlling the availability of substrate for potentially competing metabolic reactions (reviewed in reference 9). Although many regulatory processes have been described, their relative importance, exact function and mechanisms, and interaction are not fully understood.

Arginine is synthesized from glutamate via ornithine. Arginine degradation to glutamate occurs via ornithine and glutamic-γ-semialdehyde. The latter is a normal biosynthetic intermediate in the pathway of proline production. These metabolic relationships are shown in Fig. 1. Both ornithine and arginine are substrates for potentially competing anabolic and catabolic enzymes. *N. crassa* is known to prevent the simultaneous operation of both pathways (24). The exact mechanisms responsible for the exclusive operation of either the biosynthetic or catabolic pathway are not fully understood.

The synthesis of arginine from glutamate ceases in the presence of exogenous arginine (12; I. Goodman and R. L. Weiss, manuscript in preparation). An early enzyme of the biosynthetic pathway, acetylglutamate kinase, is inhibited by arginine (5; E. Wolf and R. L. Weiss, manuscript in preparation). There is no evidence for additional sites of feedback inhibition (8). Only one enzyme of arginine biosynthesis, carbamylphosphate synthetase-A (arginine-specific), is repressed (6, 8). The biosynthetic enzymes are derepressed when *N. crassa* is starved for arginine, histidine, lysine, or tryptophan (general control) (3, 4). Similar observations have been made in yeast (26). The catabolic enzymes arginase and ornithine aminotransferase are inducible approximately threefold (a significant basal level of both enzymes is observed in cells growing in minimal medium). Induction is influenced by the availability of alternative nitrogen sources (18). The synthesis of ornithine and its use for arginine biosynthesis are separated by the mitochondrial membrane from the site of ornithine degradation, which occurs in the cytoplasm (23). Arginine synthesis, its use in protein synthesis, and its degradation are catalyzed by cytosolic enzymes (23). Only a low substrate concentration is available to the cytosolic catabolic enzymes during growth in minimal medium. The majority of the ornithine and arginine is sequestered in the vacuole (16, 22). Similar organizational features are found in yeast (9, 14, 25).

Regulatory mutants affecting arginine metabolism have been isolated in yeast by a modification of the analog resistance method (2) or by selection procedures requiring highly specific regulatory alterations (17). Such methods have not been successful in *N. crassa*. An alternative approach involves the isolation of phenotypic revertants (suppressors) of proline auxotrophs (e.g., *pro-3*) unable to synthesize glutamic-γ-semialdehyde (Fig. 1). Regulatory or organizational mutants might suppress *pro-3* mutations by accelerating ornithine degradation to glutamic-γ-semialdehyde. This selection procedure

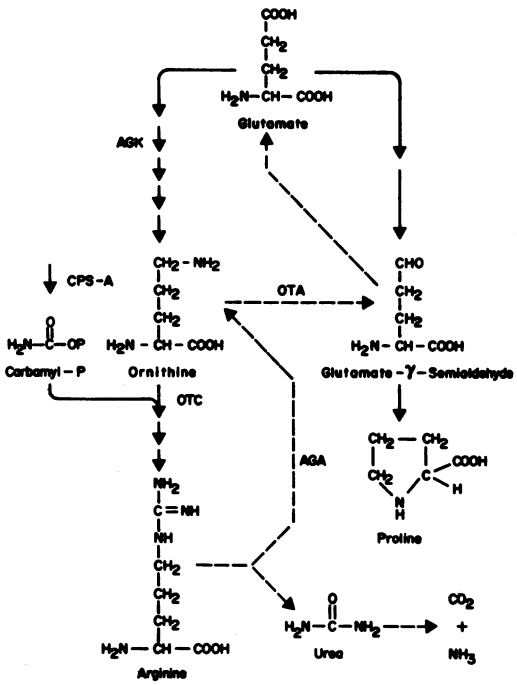

FIG. 1. *Metabolic relationships between arginine, ornithine, and proline metabolism and identification of relevant enzymes in Neurospora: AGK, acetylglutamate kinase; CPS-A, carbamylphosphate synthetase-A (arginine-specific); OTC, ornithine transcarbamylase; OTA, ornithine aminotransferase; AGA, arginase. The pro-3 mutation represents an unknown defect between glutamate and glutamate-γ-semialdehyde and is allelic to arg-8 (9).*

has produced a variety of apparent regulatory mutants in *Aspergillus nidulans* (20).

This paper reports the isolation of suppressors of the *pro-3* mutation of *N. crassa*. Suppression is shown to involve the catabolism of ornithine to glutamic-γ-semialdehyde. Genetic and biochemical evidence indicates that suppression is due to an alteration in the feedback sensitivity of acetylglutamate kinase. The usefulness of such mutants for isolating mutants in processes controlling the expression of biosynthetic and catabolic enzymes is discussed.

MATERIALS AND METHODS

Strains. The strains used and their origins are shown in Table 1. All operations including stock maintenance were performed with Vogel medium N (19). Supplementation of medium was 10 μg/ml for vitamins or 200 μg/ml for amino acids. Genetic techniques were those described by Davis and de Serres (11). Mutagenesis was with UV light to approximately 20% survival. Purification was by single conidial isolation. Strains chosen for further analysis were back-crossed twice to strain LA31. Crosses were analyzed by plating activated ascospores on appropriate plating medium (11) and transferring colonies to test tubes after 48 h of growth. Spot tests were then made on appropriately supplemented medium (11). Forced heterokaryons were produced by mixing conidia of appropriate strains (same mating type) in liquid medium. Growth rates were determined as previously described (1).

Enzyme activities. Enzyme activities were determined as previously described: acetylglutamate kinase (5; Wolf and Weiss, manuscript in preparation), arginase (1), ornithine transcarbamylase (1), and ornithine aminotransferase (1). One unit of activity is defined as the formation of 1 μmol of product per min. Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

Arginine and ornithine pools. The soluble pools of arginine and ornithine were determined as previously described (1).

RESULTS

Isolation of suppressors of proline auxotrophs. The *pro-3* mutant strain (LA30) was unable to synthesize glutamic-γ-semialdehyde from glutamate (Fig. 1). The alternative source of this compound was ornithine (Fig. 1). This catabolic pathway normally operates only in the presence of exogenous ornithine or arginine. Thirty-two independent isolates (CAL1 to CAL32) were obtained from UV-mutagenized LA30 which were capable of growth on unsupplemented medium. The isolates were characterized as described below.

Reversion and recombination analysis. Two types of genetic crosses were performed to identify suppressor-containing strains and to detect possible allelism among the suppressors: (i) *pro-3 su-x* × *pro-3*$^+$ *su*$^+$ and (ii) *pro-3 su-x* × *pro-3 su-y*. A sample of the data obtained in

TABLE 1. *Origin and genotype of Neurospora strains*

Strain	Genotype[a]	Allele	Source
LA1	Wild-type (74A)		R. H. Davis
LA30[b]	*pro-3* A	*44207*	R. H. Davis
LA31	*pro-3* a	*44207*	This work
LA6	*aga* A	*UM906*	R. H. Davis
LA22	*ota* A	*UM728*	R. H. Davis
LA45	*al-2* A	*15300*	R. H. Davis
LA82	*ota pro-3* A	*UM728 44207*	This work
LA80	*pro-3 aga* A	*44207 UM906*	This work
LA86	*al-2 pro-3* A	*15300 44207*	This work
LA107	*arg-6 aga* A	*CD-25 UM906*	R. H. Davis
LA24	*pan-1* A	*5531*	FGSC[c]
LA26	*pdx-1* A	*35405*	FGSC[c]
LA92	*pan-1 pro-3* A	*5531 44207*	This work
LA143	*pdx-1 pro-3* A	*35405 44207*	This work

[a] Requirement or enzymatic deficiency; *pro-3*, proline; *aga*, arginase; *ota*, ornithine aminotransferase; *al-2*, albino; *arg-6*, acetylglutamate phosphate reductase; *pan-1*, pantothenate; *pdx-1*, pyridoxine. A and a, mating types.

[b] A particularly nonleaky isolate (R20) of this genotype (8).

[c] Fungal Genetics Stock Center, Arcata, Calif.

crosses of the first type is shown in Table 2. Proline-requiring progeny were obtained from crosses of all mutant strains with the wild type, indicating the presence of a suppressor mutation. All strains yielded such proline auxotrophs in frequencies sufficiently high (about 25%) to indicate no linkage between suppressors and the *pro-3* mutation.

Crosses of the second type were performed to detect allelism between the suppressor mutations. All strains were crossed with CAL3. Nonallelic suppressor mutations should yield proline-requiring progeny. Twenty-four progeny from each cross were tested for a possible proline requirement. No proline-requiring progeny were detected from any of the crosses. It was concluded that all isolates were closely linked or allelic. This conclusion was confirmed by the results of genetic mapping (see below). Seven isolates were back-crossed to LA31 before further analysis.

Origin of suppression. Catabolism of ornithine to glutamic-γ-semialdehyde was the most likely mechanism of suppression (Fig. 1). If this were the case, introduction of a mutant structural gene for ornithine aminotransferase should restore the Pro$^-$ phenotype (Fig. 1). Such strains were obtained from the cross *pro-3 su* × *pro-3 ota*. Progeny containing mutations in ornithine aminotransferase were identified by their inability to use ornithine as a sole nitrogen source. Thirty-four progeny from crosses to various CAL strains were obtained which failed to use ornithine as a sole nitrogen source. None of these 34 progeny could grow on minimal medium. Seventeen of the thirty-four strains yielded Pro$^+$ progeny from crosses with LA30. Only strains carrying a *su(pro-3)* mutation should yield progeny capable of growth on minimal medium. The results indicate that suppression occurred via ornithine degradation and that the suppressor mutation was unlinked to *ota*.

Enzyme activities in mutant strains. Suppression of *pro-3* might involve changes in the levels or catalytic activities of enzymes of arginine metabolism. The growth rates and activities of the biosynthetic enzyme ornithine transcarbamylase and the catabolic enzymes arginase and ornithine aminotransferase were determined (Table 3). The low growth rates of the mutant strains were reversed by proline. This suggested that the growth of such cells was proline limited in minimal medium. The activity of ornithine transcarbamylase was slightly elevated in the mutants grown in minimal medium. This elevation disappeared in the presence of proline. This change in activity was probably related to the proline limitation of growth since it was also found with proline-starved LA30. This result is inconsistent with a mutation in ornithine transcarbamylase of the type previously observed to suppress *pro-3* mutations (7).

TABLE 2. *Detection of suppressor mutations*[a]

Strain	Genotype	Colonies tested	Pro$^-$ (%)
CAL3	*pro-3 su(pro-3)*	48	10 (21)
CAL4	*pro-3 su(pro-3)*	144	36 (25)
CAL8	*pro-3 su(pro-3)*	96	22 (23)
CAL9	*pro-3 su(pro-3)*	52	9 (17)
CAL14	*pro-3 su(pro-3)*	41	7 (17)
CAL18	*pro-3 su(pro-3)*	72	27 (38)
CAL21	*pro-3 su(pro-3)*	96	26 (27)

[a] Mutants were crossed to the wild type, and progeny were isolated on proline-supplemented medium and scored for proline requirement on minimal medium as described in the text.

The catabolic enzymes appeared to be at normal levels in mutant cells grown in proline-supplemented medium. Ornithine aminotransferase was slightly elevated in cells grown in minimal medium. This may be the result of ornithine accumulation (see below) in the mutant strains. The levels of both enzymes were considerably below those observed in cells grown in arginine-supplemented medium (data not shown). The results are inconsistent with derepression of the biosynthetic enzymes or constitutive production of the catabolic enzymes.

Arginine and ornithine pools in selected *pro-3 su(pro-3)* strains. The pools of ornithine and arginine in several suppressed strains were compared with those of wild-type (LA1) and parental (LA30) strains (Table 4). The arginine pool was unchanged or slightly elevated in the suppressed strains grown in minimal medium. Proline limitation of the parental strain resulted in a large elevation of the arginine pool. The ornithine pool was elevated in suppressed strains grown in minimal medium. In contrast, proline limitation of the parental strain resulted in a decrease in the ornithine pool. Both the arginine and ornithine pools were very similar in all strains grown in proline-supplemented medium. The results suggest an inability to modulate ornithine production upon arginine accumulation during proline-limited growth. A mutation resulting in the loss of feedback sensitivity of acetylglutamate kinase would be consistent with this result. At normal growth rates (upon proline supplementation), the activity of the enzyme would be rate-limiting and no excess ornithine production would occur. Acetylglutamate kinase has been shown to be the rate-limiting enzyme for ornithine production in *Saccharomyces cerevisiae* (13).

TABLE 3. *Generation times and enzyme levels in selected pro-3 su strains*[a]

Strain	Genotype	Generation time (h)		Enzyme activity (U/mg of protein)					
				Ornithine transcarbamylase		Arginase		Ornithine aminotransferase	
		M	M + Proline	M	M + proline	M	M + proline	M	M + proline
LA1	Wild type (74A)	2.0	1.9	0.54	0.48	1.28	1.65	0.021	0.020
LA30	*pro-3*	>20	1.9	1.00[b]	0.52	0.79[b]	1.49	N.D.[c]	0.018
CAL3	*pro-3 su(pro-3)*	3.0	2.1	0.74	0.54	1.35	1.64	0.027	0.020
CAL4	*pro-3 su(pro-3)*	3.0	1.9	0.95	0.51	1.52	1.72	0.027	0.020
CAL8	*pro-3 su(pro-3)*	2.6	2.1	1.04	0.54	1.58	1.66	0.021	0.018
CAL18	*pro-3 su(pro-3)*	2.7	2.1	1.01	0.49	1.43	1.54	0.025	0.022
CAL21	*pro-3 su(pro-3)*	2.6	2.1	0.99	0.59	1.42	1.80	0.031	0.022

[a] Cells were grown in minimal (M) or proline-supplemented medium as described in the text. Enzyme assays were performed as described in the text.

[b] Cells were grown in proline-supplemented medium and then transferred to minimal medium for 8 h before determination of enzyme activities.

[c] Not done.

TABLE 4. *Arginine and ornithine pools of selected pro-3 su(pro-3) strains*[a]

Strain	Genotype	Arginine (nmol/mg of protein)		Ornithine (nmol/mg of protein)	
		M	M + proline	M	M + proline
LA1	Wild type (74A)	133	169	186	209
LA30	*pro 3*	952[b]	211	59[b]	223
CAL3	*pro-3 su(pro-3)*	271	190	388	233
CAL4	*pro-3 su(pro-3)*	198	187	411	210
CAL8	*pro-3 su(pro-3)*	205	216	369	213
CAL18	*pro-3 su(pro-3)*	301	206	494	215
CAL21	*pro-3 su(pro-3)*	262	196	460	207

[a] Cells were grown in minimal (M) or proline-supplemented medium as described in the text.

[b] Cells were grown in proline-supplemented medium and then transferred to minimal medium for 8 h before determination of amino acid pools.

Sensitivity of ornithine synthesis to exogenous arginine. Ornithine is the sole source of polyamines in *N. crassa* (12). The latter are required for normal germination and growth. Strains lacking the enzyme arginase (Fig. 1) germinate poorly and grow slowly in the presence of exogenous arginine. To test for sensitivity to arginine, strains with the genotype *pro-3 su(pro-3) aga* were constructed from crosses of *pro-3 su(pro-3)* with *pro-3 aga*. The *aga* mutation was detected in progeny by the inability of such strains to use arginine as a sole nitrogen source. The *su(pro-3)* mutation was detected by the ability of the strains to grow on minimal medium. Strains with the genotype *pro-3 su(pro-3) aga* germinated and grew on arginine-supplemented solid medium.

Dominance of the suppressor phenotype. One expectation for a mutation resulting in the loss of feedback inhibition of ornithine synthesis is that such a mutation would be dominant or semidominant with respect to the wild-type allele in heterokaryons (the mutant enzyme would continue to overproduce ornithine). Appropriate forced heterokaryons were constructed. Their growth in minimal medium is shown in Table 5. The results indicated that the suppressor mutation was dominant.

Genetic mapping of suppressor mutation. The previous results suggested that the suppressor phenotype might be a consequence of the loss of feedback inhibition of the arginine biosynthetic pathway. The feedback-sensitive enzyme is thought to be acetylglutamate kinase (Fig. 1, reference 5). The structural gene for this enzyme has recently been identified as the previously mapped *arg-6* locus (E. Wolf, R. H. Davis, and R. L. Weiss, unpublished data). Possible linkage to *arg-6* on the right arm of linkage group one was examined with a cross between CAL3 and LA86: *pro-3 su(pro-3)* × *pro-3 al-2*. The *al-2* (albino) mutation was closely linked (less than 2 map units) to *arg-6*. The 130 progeny tested yielded the genotypes shown in Table 6. Only two recombinants were obtained. The linkage to *al-2* was confirmed in subsequent crosses of the recombinant, *pro-3 su(pro-3) al* with LA80 and LA82. Of 30 unselected albino progeny, 29 carried the suppressor mutation. Of 52 progeny which did not carry the suppressor mutation, only one was albino. The linkage of the suppressor and *al-2* suggested that the former might be allelic with *arg-6*.

Possible allelism between the suppressor mutation and *arg-6* was examined by the cross: *pro-*

3 su(pro-3) aga × *arg-6 aga*. Recombination between *arg-6* and *su(pro-3)* would yield a strain of the genotype *pro-3 aga*. Among 154 arginine-independent progeny, no proline-requiring strains were detected. Twenty-five percent such progeny would be expected for unlinked genes. The results suggest that *arg-6* and the suppressor mutation are either very closely linked or allelic.

Feedback sensitivity of acetylglutamate kinase. The sensitivity of acetylglutamate kinase (Fig. 1) to inhibition by arginine in the wild type and in several suppressor-containing strains is shown in Fig. 2. The sensitivity of the enzyme from the suppressed mutant strains was 100× less than the enzyme from the wild-type strain.

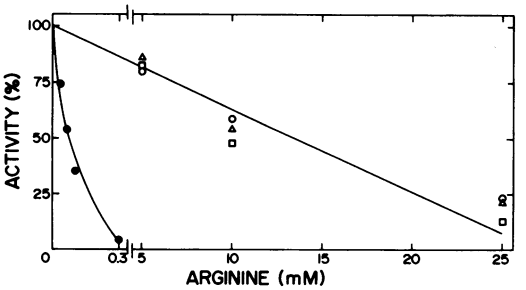

FIG. 2. *Inhibition by arginine of acetylglutamate kinase from various strains: pro-3* (●); *CAL3* (○); *CAL8* (□); *and CAL21* (△). *The enzyme from a crude organellar fraction was assayed as described in the text.*

DISCUSSION

All 32 independently isolated phenotypic revertants of the *pro-3* mutation appeared to be linked, possibly allelic to one another. Those examined further appeared to have an acetylglutamate kinase with altered sensitivity to feedback inhibition by arginine. The mutations were all closely linked or allelic to *arg-6*, which was previously reported to be the structural gene for acetylglutamate phosphate reductase (9). This locus has recently been shown to be complex and to include both the kinase and reductase (Wolf et al., unpublished data).

TABLE 5. *Growth of heterokaryons carrying suppressor mutations*[a]

Heterokaryon	Dry wt (mg)
pan-1 pro-3 + pro-3 pdx-1	<2
pan-1 pro-3 + pdx-1 pro-3 su(pro-3) (3)	21.1
pan-1 pro-3 + pdx-1 pro-3 su(pro-3) (4)	20.7
pan-1 pro-3 + pdx-1 pro-3 su(pro-3) (8)	20.7
pan-1 pro-3 + pdx-1 pro-3 su(pro-3) (18)	19.3
pan-1 pro-3 + pdx-1 pro-3 su(pro-3) (21)	16.8

[a] Flasks containing 25 ml of minimal medium were inoculated with conidia of the indicated strains (A mating type). After 48 h at 30°C, the cells were collected by filtration, dried overnight at 110°C, and weighed.

TABLE 6. *Progeny of cross between al-2 su(pro-3)*$^+$ *pro-3 and al-2*$^+$ *su(pro-3) pro-3*[a]

Genotype	No. of progeny
al-2$^+$ *su(pro-3) pro-3*	64
al-2 su(pro-3)$^+$ *pro-3*	64
al-2 su(pro-3) pro-3	1
al-2$^+$ *su(pro-3)*$^+$ *pro-3*	1

[a] The suppressor mutation was detected by the ability of the cells to grow on minimal medium. Germination percentage was 84%.

The ability of *pro-3 su(pro-3) aga* triple mutants to grow in arginine-supplemented medium suggests that feedback inhibition alone was responsible for preventing wasteful ornithine production in the presence of exogenous arginine. This is consistent with the previously reported absence of repression of ornithine biosynthetic enzymes (6). It also suggests that control of acetylglutamate synthase (9) had little influence on ornithine production.

The suppressor mutations led to an accumulation of ornithine in cells growing in minimal medium (Table 4). This accumulation disappeared when the cells were grown in proline-supplemented medium (Table 4). The most likely explanation is that catalytic ability of acetylglutamate kinase (in the absence of feedback inhibition) was unable to produce ornithine at a rate sufficient for its accumulation at the more rapid growth rates characteristic of cells growing in proline-supplemented medium. This suggests that the enzyme normally acted at maximum capacity (presumably uninhibited by arginine) in wild-type cells growing in minimal medium or that the suppressor mutations might have also affected the catalytic properties of the enzyme in addition to its sensitivity to inhibition by arginine. The properties of acetylglutamate kinase are currently under investigation.

The isolation of proline suppressors has also been performed with *A. nidulans* (20). Weglenski identified three unlinked loci. He observed that one class of mutants had barely detectable levels of ornithine transcarbamylase, presumably a leaky mutant analogous to the *arg-12*^{s} mutation of *N. crassa* (7). Mutations at the other loci resulted in constitutive production of ornithine aminotransferase or both arginase and ornithine aminotransferase. No mutations of the type described here were obtained (21). The results suggest that in *N. crassa* ornithine ac-

cumulation may be a more significant determinant of ornithine catabolism than the level of the catabolic enzymes. This has been shown to be the case for arginine metabolism in *N. crassa* (R. L. Weiss and J. M. Lipeles, manuscript in preparation).

The absence of regulatory mutations with derepressed levels of either biosynthetic or catabolic enzymes suggests that such mutations may not be sufficient to suppress the *pro-3* mutation in *N. crassa*. This would be the case if such mutations failed to accumulate sufficient cytosolic ornithine. The "leaky" phenotype of most *pro-3* mutations (8) suggests that small increases in the level of the catabolic enzymes or in the production of ornithine ought to result in suppression of the proline requirement. The nonleaky nature of the R20 isolate (8) used here may be responsible for the failure to isolate mutants with derepressed levels of ornithine aminotransferase. In addition, increased accumulation of ornithine might not occur in mutants with derepressed levels of the biosynthetic enzymes if the feedback-inhibitable enzyme is extremely sensitive to low concentrations of cytosolic arginine. Acetylglutamate kinase appears to exhibit such properties (Wolf and Weiss, manuscript in preparation). These properties might also account for the inability to isolate regulatory mutants with increased resistance to the arginine analog canavanine. The required overproduction of cytosolic arginine would be compensated by efficient feedback inhibition, and little arginine accumulation would occur.

The availability of mutants with impaired sensitivity to feedback inhibition provides a means of overcoming some of the inherent difficulties in isolating mutants impaired in other regulatory process. Similar mutants have been used in yeast to isolate mutants affecting the general amino acid regulatory system (26). A similar system appears to be involved in controlling the expression of genes for arginine metabolism in *N. crassa* (3, 4). Hopefully, the mutants described here will be valuable in characterization of the mechanisms responsible for the control of arginine metabolism in *N. crassa*.

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