THE ISOLATION AND CHARACTERIZATION OF MUTANTS DEFECTIVE IN NITRATE ASSIMILATION IN *NEUROSPORA CRASSA*

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ABSTRACT

The isolation and characterization of mutants altered for nitrate assimilation in *Neurospora crassa* is described. The mutants isolated can be subdivided into five classes on the basis of growth tests that correspond to the growth patterns of existing mutants at six distinct loci. Mutants with growth characteristics like those of *nit-2*, *nit-3* and *nit-6* are assigned to those loci on the basis of noncomplementation and lack of recombination. Mutants that, from their growth patterns, appear to lack the molybdenum-containing co-factor for both nitrate reductase and xanthine dehydrogenase subdivide into three loci (*nit-7*, *nit-8* and *nit-9*), all of which are genetically distinct from *nit-1*. *nit-9* is a complex locus consisting of three complementation groups and thus appears similar to the *cnxABC* locus of *Asperillus nidulans*. Extensive complementational and recombinational analyses reveal that *nit-4* and *nit-5* are alleles of the same locus, and two new alleles of that locus have been isolated. The results indicate that, as in *A. nidulans*, nitrate assimilation in *N. crassa* requires at least four loci (*nit-1*, *7*, *8* and *9*) to produce the molybdenum co-factor for nitrate reductase (and xanthine dehydrogenase), one locus (*nit-3*) to code for the nitrate reductase apoprotein, one locus (*nit-6*) to code for the nitrite reductase approtein and only one locus (*nit-4/5*) for the regulation of induction of the pathway by nitrate and nitrite.

THE pathway of nitrate assimilation in *Neurospora crassa* involves the complete reduction of nitrate to ammonium, *via* nitrite. This process occurs in two steps: the reduction of nitrate to nitrite by nitrate reductase (NADPH: nitrate oxidoreductase, E.C.1.6.6.3.), a reaction involving a two-electron transfer; and the reduction of nitrite to ammonium by nitrite reductase (NADPH: nitrite oxidoreductase, E.C.1.6.6.4.) a six-electron transfer. The ammonium thus formed is then assimilated into glutamate and glutamine by NADP-linked glutamate dehydrogenase and glutamine synthetase.

Nitrate reductase is a dimer of molecular weight 228,000 and has a sedimentation coefficient of 8.0S (GARRETT and NASON 1969). Sodium dodecyl sulfate and 8 M urea polyacrylamide gel electrophoresis of the pure enzyme revealed two protein bands with molecular weights of 130,000 and 115,000, respectively (PAN and NASON 1976). Nevertheless, since they appear to have identical N-terminals and tryptic peptide maps, the working hypothesis is that the enzyme is a dimer of identical polypeptides (PAN and NASON 1978). The possibility remains that there is some heterogeneity in the size of the subunits.

In addition to the two apparently identical polypeptides, nitrate reductase possesses a molybdenum-containing co-factor (NICHOLAS and NASON 1954; NASON *et al.* 1970). This co-factor is found in a wide variety of enzymes from diverse phylogenetic sources (KETCHUM *et al.* 1970; NASON *et al.* 1971). Although little is known of the structure of this co-factor, it is very similar in many enzymes in which it is found since *in vitro* complementation can be used to reactivate *N. crassa* co-factor-deficient nitrate reductase with co-factor from other sources.

Nitrite reductase has a molecular weight 290,000 and a sedimentation coefficient of 9.4S (LAFFERTY and GARRETT 1974). It also appears to be a homodimer of identical 140,000 molecular weight protomers (PRODOUZ and GARRETT 1979). The enzyme has a novel prosthetic group, siroheme (VEGA, GARRETT and SIEGEL 1975).

The synthesis of these two enzymes is subject to two control mechanisms: nitrogen metabolite repression and induction by nitrate and/or nitrite. Nitrogen metabolite repression prevents the expression of the pathway in the presence of ammonium or its assimilated forms, glutamate and glutamine (DUNN-COLEMAN, TOMSETT and GARRETT 1979; PREMAKUMAR, SORGER and GOODEN 1979). For many years, the co-repressor of the pathway was believed to be ammonium itself, which was thought to inactivate a positive regulator required for the synthesis of the nitrate assimilation enzymes and a number of other enzymes and permeases involved with nitrogen metabolism (CODDINGTON 1976; FACKLAM and MARZLUF 1978). Recent data, however, indicate that glutamine is the important metabolic signal acting *via* glutamine synthetase to repress or inactivate the positive gene regulation system (DUNN-COLEMAN, TOMSETT and GARRETT 1979). Ammonium is thus effective in repression by its conversion to glutamine. In the presence of ammonium or glutamine, it is obviously energetically advantageous to repress nitrate assimilation and thus conserve four equivalents of reduced nicotinamide adenine dinucleotide consumed for each nitrate-nitrogen reduced.

In the absence of ammonium and glutamine, the pathway of nitrate assimilation is expressed only in the presence of nitrate and/or nitrite. Nitrate/nitrite induction is also believed to be mediated by a positive gene regulator that is stimulated in the presence of nitrate or nitrite. The mechanism of induction remains obscure although, through analogy to the mechanism of nitrate induction in *Aspergillus nidulans*, nitrate reductase is believed to be its own co-repressor in the absence of nitrate/nitrite (COVE 1970; CODDINGTON 1976).

The enzymes and the regulation of their synthesis appear to be somewhat similar in both *N. crassa* and *A. nidulans* (GARRETT and AMY 1978; COVE 1979). Until now, however, an apparent great difference existed in the number of genes known to be involved in these processes. A summary of these loci is given in Table 1. Two major differences directly affecting nitrate assimilation are: (1) five genes (seven complementation groups) are implicated in the formation of the molybdenum-containing co-factor in *A. nidulans* whereas only one gene is known in *N. crassa*; and (2) only one gene is responsible for the nitrate induction regulator in *A. nidulans*, whereas two appear necessary in *N. crassa*.

TABLE 1

Genes known to encode nitrate assimilation in N. crassa *and* A. nidulans*

N. crassa locus	A. nidulans locus	Function
nit-1	*cnx ABC,E,F,G,H*	Formation of the molybdenum-containing co-factor required by both nitrate reductase and xanthine dehydrogenase.
nit-2	*areA, tamA*	Regulator gene(s) responsible for nitrogen metabolite repression of nitrate assimilation and other pathways of nitrogen acquisition.
nit-3	*niaD*	Nitrate reductase structural gene encoding the apoprotein.
nit-4, nit-5	*nirA*	Regulator gene(s) necessary for nitrate induction of nitrate assimilation.
nit-6	*niiA*	Nitrite reductase structural gene encoding the apoprotein.

* See GARRETT and AMY (1978) and COVE (1979) for full discussion and literature.

a foundation for the investigation of the genetic regulation of nitrate assimilation in Neurospora.

The major method employed exploits the finding that cells lacking nitrate reductase are resistant to the toxic analog, chlorate (ABERG 1947). The exact mechanism of chlorate toxicity is unknown. The simplest hypothesis is that nitrate reductase catalyzes the conversion of chlorate to chlorite, a highly reactive substance (ABERG 1947). This mechanism is almost certainly correct, but is insufficient to explain all the data (COVE 1976a,b). However, resistance to chlorate has been used successfully to select mutants defective in nitrate assimilation in a variety of organisms (OOSTINDIER-BRAAKSMA and FEENSTRA 1973; COVE 1976a; MÜLLER and GRAFE 1978). Chlorate has been successfully employed here to select mutants defective in nitrate assimilation in *Neurospora crassa*. This report presents the initial findings of genetic studies with these mutants. A preliminary report of this data has appeared (TOMSETT and GARRETT 1979). The present study was undertaken to examine these differences and to provide

MATERIALS AND METHODS

Media and routine procedures: The media and routine manipulative procedures used have been described in DAVIS and DE SERRES (1970).

Strains: Standard wild types were Oak Ridge strains 74-OR8-1a and 74-OR23-1A. The following mutant alleles were used: *nit-1* (34547), *nit-2* (nr37, I7, K31), *nit-3* (14789), *nit-4* (2, 5), *nit-5* (nr15), *nit-6* (OP4), *inl hetCde, fl*^{P}. These strains were obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA.

Mutagenesis: Conidial suspensions were prepared from 10-day-old wild-type cultures on solid media containing nitrate as sole nitrogen source. Twenty-five ml of filtered conidial susepension containing 10^{7} to 10^{8} conidial/ml were irradiated with UV at 25° in a 14 cm petri dish. UVSL-58 lamp (Ultra-Violet Products Inc., California) was used on short wavelength to yield 20 to 50% survivors.

Isolation of mutants: (1) Resistance to chlorate. Irradiated conidia were spread on growth test medium that contained uric acid as sole nitrogen source (2mg/ml) and 100 mM potassium chlorate, at a density of 2 to 3 $\times$ 10^3 conidia/petri dish. After 2 days incubation at 25°, colonies growing and conidiating normally were selected, subcultured and purified from a single conidium. (2) Inability to utilize nitrate. Irradiated conidia were spread at a density of 50 to 100 conidia/petri dish on growth test medium containing ammonium (10 mM) as sole nitrogen source. The petri dishes were incubated for 1 day at 25°, at which time a sterile filter paper (cut to fit the petri dish exactly) was laid upon the surface of the medium. The papers adhere tightly to the medium by surface tension. The petri dishes were then incubated for a further 1 to 2 days at 25° until colonies could be seen growing through the filter paper. The filter paper was then removed and placed on the surface of a fresh petri dish containing growth medium with nitrate (10 mM) as sole nitrogen source. Both plates were then incubated for 1 to 2 days more. The ammonium plate was stored at 4° once colonies appeared on the plates. The filter paper was then removed from the nitrate plate and discarded. The nitrate plate was incubated for another 1 to 2 days until colonies were visible. The plates were thus replicates. Careful examination of the nitrate plate revealed colonies that had the characteristic "nitrogen starved" morphology. These colonies were either cut directly from the medium or the equivalent colony on the ammonium medium was subcultured. Isolated colonies were purified by subsequent reisolation from a single conidium.

Growth tests: The medium used for growth tests contained Fries' salts (modified to exclude sucrose and nitrogen sources), 0.05% fructose, 0.05% glucose, 2% sorbose, 2% agar and the required nitrogen source. For inoculation, conidial suspensions were prepared for each strain by the addition of 5 ml of sterile water to a 7- to 10-day-old slant. The conidial concentration was not critical, provided the strains conidiated well. Petri dishes were inoculated by allowing a small drop of conidial suspension to be withdrawn by surface tension from the tip of a sterile pasteur pipette. After 2 to 4 days of incubation at 25°, colonies 0.5 to 1 cm in diameter appeared on the surface of the agar. Strains unable to grow did not form colonies and careful examination showed the opaque mark of the conidial inoculum. Strains that were leaky on a given medium were identified by a thin, spidery growth or slower appearance of the colony. When nitrite was the nitrogen source, the medium was adjusted to pH 7.3 with potassium phosphate (at lower pH, nitrite becomes nitrous acid, which is toxic).

Complementation: Tests of complementation were made in pairwise combinations in tubes containing solid minimal medium with nitrate as sole nitrogen source. Drops of suspension containing 10^6 to 10^7 fresh conidia/ml were used as inocula. The *nit* mutants, when placed on solid media containing *no* nitrogen source, showed a characteristic, thin spidery morphology. The basis of this growth is unknown, but is easily distinguished from the normal condition. Negative results from complementation tests showed this characteristic growth.

Mapping: Newly isolated mutants were crossed to known *nit* mutants to determine linkage and locus assignments. Ascospores from these crosses were plated directly onto growth test medium containing nitrate (10 mM) as sole nitrogen source. Recombination frequency was calculated from the number of prototrophs by assuming reciprocal recombination.

Plate test for nitrate reductase activity: Nitrate reductase activity may be assayed *in vitro* by measurement of the amount of nitrite produced when the enzyme is incubated at 25° with the assay mixture. Addition of sulfanilic acid and N-1-naphthylethylenediamine dihydrochloride (NED) to the assay mixture produces a pink coloration, which can be measured by absorption at 540 nm. The plate test employs this pink coloration as an indication of nitrate reductase activity. Plates of nitrate growth test medium that have been incubated for 2 to 3 days at 25° are treated with 3 to 5 ml of sulfanilic acid, followed by 3 to 5 ml of NED. Under these conditions, wild-type colonies show a slight pink coloration. Mutants lacking nitrite reductase, however, are stained in deep pink color and have a pink halo round the colony due to excreted nitrite. Colonies utilizing other nitrogen sources may also be stained for nitrate reductase activity by initially adding 1 M $NaNO_3$, and 10 mM Na_2SO_3 solution for 20 min prior to staining. [The sulfite inhibits nitrite reductase activity in strains possessing this enzyme (VEGA, GREENBAUM and GARRETT 1975; GARRETT and COVE 1976).]

RESULTS

The selection of mutants: As a result of selection of colonies resistant to 100 mM chlorate on uric acid, 76 putative mutant strains were isolated from approximately 3–5 × 10^5 conidia plated. Of the 76 isolated, 65 were shown by subsequent analysis to be defective in nitrate assimilation. Of six colonies selected as nitrate nonutilizers, two were subsequently shown to be defective in nitrate assimilation. All new isolates were growth-tested, purified by reisolation from a single conidium and retested.

Growth tests: The existing *nit* mutants each gave an unequivocal pattern of growth when analyzed on solid medium. For these tests, nitrogen sources were added to the described growth test medium at 10 mM. The results are shown in Table 2.

nit-1 is unable to utilize either nitrate or hypoxanthine, since it lacks the co-factor for both nitrate reductase and xanthine dehydrogenase (SORGER 1966; NASON *et al.* 1970, 1971; KETCHUM *et al.* 1970). It is resistant to chlorate since it lacks nitrate reductase. *nit-2* is able to effectively utilize only ammonium, glutamate or glutamine as nitrogen sources, since it is thought to lack a positive regulator necessary for the expression of many genes necessary for the utilization of alternative nitrogen sources (CODDINGTON 1976; FACKLAM and MARZLUF 1978). *nit-3* is able to utilize all nitrogen sources except nitrate, since it lacks only nitrate reductase (SORGER 1966; NASON *et al.* 1970, 1971; CODDINGTON 1976). This defect also confers chlorate resistance. Both *nit-4* and *nit-5* are thought to be involved with the induction of nitrate reductase and nitrite reductase. These mutants cannot utilize nitrate or nitrite (CODDINGTON 1976). Presumably these strains express a low level of nitrate reductase, which is sufficient to render them sensitive to chlorate but insufficient to support growth on nitrate. *nit-4* and *nit-5* are, however, more resistant to chlorate than is wild type. *nit-6*

TABLE 2

Results of growth tests

Existing mutant type	Growth media*†						Plate test for nitrate reductase§	No. of new mutants isolated	New mutant type
	NH_4^+	NO_3^-	NO_2^-	Hypoxanthine	Uric acid	Uric acid + 100 mM ClO_3^-‡			
Wild-type	+	+	+	+	+	VS	+		
nit-1	+	—	+	—	+	R	—	25	I
nit-2	+	—	—	—	—	—	—	4	II
nit-3	+	—	+	+	+	R	—	35	III
nit-4/5	+	—	—	+	+	S	—	2	IV
nit-6	+	—	—	+	+	VS	++	1	V

* + indicates growth on that medium, — indicates no growth (nitrogen-starved morphology).
‡ R indicates resistance to chlorate; S, sensitivity; VS extreme sensitivity; — indicates that the strain cannot grow.
† All nitrogen sources were added at 10 mM.
§ + indicates colony stained pink; ++ colony had a pink halo from excreted nitrite; — colonies did not stain.

is also unable to grow on nitrate or nitrite as sole nitrogen source because it lacks nitrite reductase (CHANG *et al.* 1975). Such mutants, however, do retain nitrate reductase; thus, they excrete nitrite when growing on nitrate and remain as sensitive to chlorate as wild type.

Growth tests of the new mutants revealed that they all fell into these five patterns of growth, although there was some variation in their resistance to chlorate. The groups were numbered I through V in Table 2.

Complementation of the new mutants: The new mutants were tested for complementation by mixing conidial suspensions in pairwise combinations. The mutants that by growth tests were shown to be type II, III, IV, V proved in complementation tests to be homogenous groups, exhibiting complementation between groups, but not within groups. It would seem likely therefore that these groups represent four genetic loci.

The 25 type I mutants did not form a single complementation group. These mutants formed five complementation groups (IA through IE). Furthermore, one of these complementation groups (IB) could not complement two of the other groups (IA and IC).

The results are summarized in Table 3.

Backcrosses: Representatives of each complementation group of the new mutants were taken and backcrossed to wild type. All were shown to be single mutants. These representatives were used throughout the rest of the study as markers for that complementation group.

The existing *nit* loci (*nit-1* through *nit-6*) were also backcrossed to wild type (74-OR23-1A) in order to obtain isogenic strains so that the new complementation groups could be assigned to existing loci.

Assignment of new mutants to existing loci: Initial assignment of the new complementation groups to existing loci was by complementation. Complementation tests unequivocally assigned mutants of type II, III and V to the *nit-2*, *nit-3* and *nit-6* loci, respectfully. Type IV mutants failed to complement either

TABLE 3

Complementation of the new mutants

	V	IV	III	II	IE	ID	IC	IB	IA
IA	+	+	+	+	+	+	+	—	—
IB	+	+	+	+	+	+	—	—	
IC	+	+	+	+	+	+	—		
ID	+	+	+	+	+	—			
IE	+	+	+	+	—				
II	+	+	+	—					
III	+	+	—						
IV	+	—							
V	—								

+ indicates complementation, *i.e.*, normal growth on nitrate.
— indicates noncomplementation, *i.e.*, nitrogen-starved growth on nitrate.

TABLE 4

Complementation of new mutants to existing loci*

		nit-1	*nit-2*	*nit-3*	*nit-4*	*nit-5*	*nit-6*
IA	(V1M5)	+	+	+	+	+	+
IB	(V1M32)	+	+	+	+	+	+
IC	(V1M50)	+	+	+	+	+	+
ID	(V1M17)	+	+	+	+	+	+
IE	(V1M44)	+	+	+	+	+	+
II	(V1M7)	+	—	+	+	+	+
III	(V1M4; V1M31)	+	+	—	+	+	+
IV	(V1M41)	+	+	+	—	—	+
V	(V1M82)	+	+	+	+	+	—

+ indicates complementation.
— indicates noncomplementation.
* Allele numbers of representative new mutants is given in parentheses.

nit-4 or *nit-5* mutant strains. Mutants in groups IA through IE could all complement the *nit-1* mutant. These results are summarized in Table 4.

Representatives of the new mutants were also crossed to existing *nit* mutations to confirm the locus assignments made by complementation. The results are summarized in Table 5. Mutants of types II, III and V showed no recombination with *nit-2*, *nit-3* and *nit-6*, respectively, and therefore can be unequivocally assigned to these loci. Mutants of type IV showed no recombination with *nit-4*

TABLE 5

Assignment of new mutants to existing loci

Mutant complementation group		Linkage group assignment	Mapping to existing loci			Locus assignment
			Locus	Recombinant frequency	No. progeny tested	
IA	(V1M5)	IVR	*nit-3*	38%	810	*nit-9A*
			nit-4	11%	305	
IB	(V1M32)	IVR	—	—	—	*nit-9B**
IC	(V1M50)	IVR	*nit-3*	35%	618	*nit-9C*
			nit-4	8%	880	
			nit-9A	0%	134	
ID	(V1M17)	?†	*nit-1*	50%	793	*nit-7*
IE	(V1M44)	IR‡	*nit-1*	32%	295	*nit-8*
II	(V1M7)	IL	*nit-2*	0%	145	*nit-2*
III	(V1M4)	IVR	*nit-3*	0%	639	*nit-3*
	(V1M31)	IVR	*nit-3*	0%	>1000	*nit-3*
IV	(V1M41)	IVR	*nit-4*	0%	1878	*nit-4/5*
			nit-5	0%	399	
V	(V1M82)	VIL§	*nit-6*	0%	321	*nit-6*

* On the basis of complementation only.
† Not yet assigned to a linkage group.
‡ On the basis of linkage to *nit-1* only.
§ D. D. PERKINS, personal communication.

TABLE 6

Mapping of nit-4 *and* nit-5† *alleles*

Cross	No. colonies screened	No. prototrophs	Recombination frequency (%)
nit-4 (5) × *nit-4* (2)	104	0	0
nit-4 (5) × *nit-4* (V1M2)	440	0	0
nit-4 (5) × *nit-4* (V1M41)	1878	0	0
nit-4 (5) × *nit-5* (nr15)	102	0	0
nit-4 (V1M41) × *nit-5* (nr15)	399	0	0
nit-4 (V1M41) × *nit-5* (nr15*)	1098	0	0
nit-4 (2) × *nit-5* (nr15*)	481	0	0
nit-5 (nr15) × *nit-5* (nr15*)	439	0	0
nit-4 (2) × *nit-3* (14789)	916	152	33
nit-4 (2) × *nit-3* (V1M31)	593	118	39
nit-4 (2) × *nit-3* (V1M4)	290	57	39
nit-4 (V1M2) × *nit-3* (14789)	817	144	35

† *nit-5* (nr15*) was obtained from D. D. PERKINS as a duplicate of the original *nit-5* (nr15), which had been stored on silica gel since 1963.

and *nit-5* and appeared to be mutant in both of these loci. As expected from the complementation tests, none of the type I mutants mapped to the *nit-1* locus. Therefore, these have been assigned to three new loci: *nit-7*, *nit-8* and *nit-9*. These new loci have not been definitely located in the seven linkage groups, although *nit-9* is clearly on IVR by linkage to *nit-3* and *nit-4*. The location of *nit-7* is unknown and *nit-8* may be on IR since it shows slight linkage to *nit-1*.

Complementation and mapping of nit-4 *and* nit-5: None of the complementation tests between *nit-4* and *nit-5* mutants showed complementation, suggesting that these mutants were in fact alleles of the same locus. Mapping of *nit-5* (nr15) to existing and newly isolated *nit-4* alleles also showed no recombination. The data are summarized in Table 6.

Table 6 also shows data from crosses between various *nit-4* and *nit-3* alleles. The results indicate that *nit-3* and *nit-4* are approximately 36 cM apart on the right arm of linkage group IV.

DISCUSSION

We have isolated 67 mutant strains of *Neurospora crassa* that are altered in nitrate assimilation. Two of these were isolated as being unable to grow on nitrate as the sole nitrogen source. The remaining 65 were selected as being resistant to chlorate on uric acid. The exact cause of their resistance is uncertain, but it certainly reflects the fact that all are unable to utilize nitrate.

These mutants could be divided into five classes by their patterns of growth on solid media containing various nitrogen sources and these classes appeared equivalent to known mutant loci. Under the conditions of the growth test, *nit* mutants unable to utilize a given nitrogen source show a characteristic thin, spidery morphology. This characteristic nitrogen-starved morphology has also

been described in *Aspergillus nidulans* (COVE 1976a). The reason for this apparent leaky growth is uncertain, but it presumably reflects low levels of either contaminating nitrogen sources in the medium or the conidial suspension, endoproteolytic digestion, or a combination of the two. This morphology, however, varies little, is highly characteristic and can be distinguished unmistakably in routine testing. The growth tests revealed 25 mutants like *nit-1*, four like *nit-2*, 35 like *nit-3*, two like *nit-4* and *nit-5* and one like *nit-6*.

Complementation of these five classes of mutants showed that those distinguished by growth tests to be like *nit-2*, *nit-3* and *nit-6* did not complement with existing mutants at those loci, but did complement mutants at other loci. No recombination could be detected between the new mutants and existing mutants at the locus to which they were assigned by complementation. All mutants that by growth tests appeared to be like *nit-1* complemented *nit-1*. Further, they could be subdivided into five complementation groups, one of which overlapped two of the others. These mutants have been assigned to new loci termed *nit-7*, *nit-8* and *nit-9*. The three complementation groups, IA, IB and IC, have been assigned to the *nit9ABC* locus, since it is uncertain at this time whether this represents one, two or three genes. The map positions of these three loci have not yet been precisely determined, although *nit-9* appears to be on linkage group IVR.

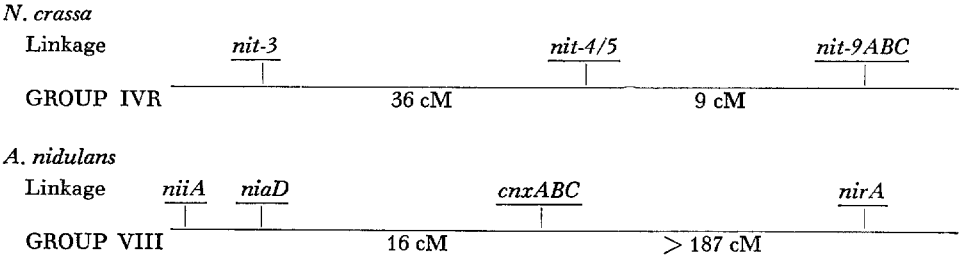
Mutants with growth characteristics like *nit-4* and *nit-5* did not complement each other or known mutants at either of these loci. Extensive complementation tests and genetic mapping were conducted to distinguish between the existing *nit-4* alleles, the existing *nit-5* alleles and the new *nit-4/5* alleles. In all combinations, the mutant alleles failed to complement. Furthermore, no recombination was found in crosses between a number of these mutant alleles. There are only two possible explanations of this anomaly: (1) *nit-4* and *nit-5* are actually the same locus and the initial identification was mistaken, or (2) the *nit-5* culture at some time during the long period since its isolation has been contaminated or confused with a *nit-4* culture (such that the existing *nit-5* is all *nit-4*). In an attempt to distinguish these, a *nit-5* (nr15) culture that had been stored on silica gel since 1963 (D. D. PERKINS, personal communication) was tested to distinguish it from *nit-4*. This culture also failed either to complement or to recombine with *nit-4*. Therefore, we favor the explanation of initial mistaken identification, especially since no cross between *nit-4* and *nit-5* was reported (SORGER and GILES 1965). We cannot exclude the second hypothesis, however, since SORGER and GILES (1965) stated that *nit-5* (nr15) was able to grow on nitrite as sole nitrogen source, whereas all of our tests and those of CODDINGTON (1976) fail to show such growth.

At this point, it is interesting to note the similarity between the genetics of nitrate assimilation in *N. crassa* and *A. nidulans*. This pathway has undergone extensive genetic analysis in *A. nidulans*, and many of the genes have been well characterized (see Table 1). The present study has resolved a number of differences between the two systems. First, the *nit-4/5* locus appears to be a single gene necessary for nitrate induction of this pathway and is thus equivalent to

the *nirA* gene of *A. nidulans*. Second, several genes are necessary for the formation of the molybdenum-containing co-factor required by both nitrate reductase and xanthine dehydrogenase. In *A. nidulans*, five genes (seven complementation groups) are required to encode co-factor formation, while in *N. crassa* at least four genes (six complementation groups) can be implicated. Perhaps a fifth gene is also present in *N. crassa*, but thus far no mutants in it have been isolated.

It seems probable that these two ascomycete fungi would have fundamentally similar genetic systems to encode and control nitrate assimilation. Similarities and differences between the two systems are both striking. Although the two organisms differ in chromosome number (*N. crassa* has seven, while *A. nidulans* has eight), some of the genes responsible for nitrate assimilation display similar linkage relationships in both organisms (Figure 1). For example, although in a different arrangement, the genes responsible for the nitrate reductase apoprotein, the regulatory gene for nitrate/nitrite induction and the complex locus required for the formation of the molybdenum co-factor (*cnxABC* or *nit-9ABC*) are all on a single chromosome arm in each of the organisms. This organization could reflect a relatively recent evolutionary divergence of the two organisms or, perhaps, that it is selectively advantageous to maintain these genes on the same chromosome arm. A more significant difference, however, may be that while the structural genes for nitrate reductase and nitrite reductase (*niiA* and *niaD*) are tightly linked in *A. nidulans* (Cove 1970; Tomsett and Cove 1979), in *N. crassa* they are on separate chromosomes (this report; Sorger and Giles 1965; D. D. Perkins, personal communication). In both organisms, these two structural genes are subject to the similar control mechanisms of nitrate induction and nitrogen metabolite repression. While it is possible that the *Aspergillus* genes may share some control sites, it is clear that the Neurospora genes must be regulated independently (or by post-transcriptional control). It is also possible, however, that the *niiA* and *niaD* genes of *A. nidulans*, though clustered, are regulated through independent control sites. In such an instance, it would have been fortuitous that they had not been separated through evolution. Recent data on a mutant strain (*nis*-5) of *A. nidulans*, which resulted from an insertional translocation between the *niiA* and *niaD* genes, have shown that, although both gene products are normal and *niaD* remains normally regulated, *niiA* gene

Figure 1.—Comparison of linkage relationships in *N. crassa* and *A. nidulans*.



Linkage relationships for *N. crassa* were determined in the present study. Linkage relationships for *A. nidulans* were taken from Clutterbuck (1974).

regulation is altered. The basis of this abnormal regulation has yet to be identified (RAND and ARST 1977; ARST, RAND and BAILEY 1979; TOMSETT and COVE, unpublished observations). Whether fundamental differences in the regulation of this pathway in these organisms really exist must await further analysis.

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LITERATURE CITED

ABERG, B., 1947 On the mechanism of the toxic action of chlorates and some related substances upon young wheat plants. Kungliga Lantbrukshögskolans Annaler. **15**: 37–107.

ARST, H. N., K. N. RAND and C. R. BAILEY, 1979 Do the tightly linked structural genes for nitrate and nitrite reductases in *Aspergillus nidulans* form an operon? Evidence from an insertional translocation which separates them. Molec. Gen. Genet. **174**: 89–100.

CHANG, H. C. P., G. J. MULKINS, J. C. DYER and G. J. SORGER, 1975 Enzymatic and non-enzymatic reduction of nitrite by extracts of *Neurospora crassa*. J. Bacteriol. **123**: 755–758.

CLUTTERBUCK, A. J., 1974 *Aspergillus nidulans*. pp. 447–510. In: *Handbook of Genetics*, Vol. 1. Edited by R. C. KING. Plenum Press, New York and London.

CODDINGTON, A., 1976 Biochemical studies on the *nit* mutants of *Neurospora crassa*. Molec. Gen. Genet. **145**: 195–206.

COVE, D. J., 1970 Control of gene action in *Aspergillus nidulans*. Proc. Roy. Soc. Lond. [Biol.] **176**: 267–275. ——, 1976a Chlorate toxicity in *Aspergillus nidulans*: the selection and characterization of chlorate resistant mutants. Heredity **36**: 191–203. ——, 1976b Chlorate toxicity in *Aspergillus nidulans*. Studies of mutants altered in nitrate assimilation. Molec. Gen. Genet. **146**: 147–159. ——, 1979 Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol. Rev. **54**: 291–327.

DAVIS, R. M. and F. J. DE SERRES, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17A**: 79–143.

DUNN-COLEMAN, N. S., A. B. TOMSETT and R. H. GARRETT, 1979 Nitrogen metabolite repression of nitrate reductase in *Neurospora crassa*: Effect of the *gln*-la locus. J. Bacteriol. **139**: 697–700.

FACKLAM, T. J. and G. A. MARZLUF, 1978 Nitrogen regulation of amino acid catabolism in *Neurospora crassa*. Biochem. Genet. **16**: 343–355.

GARRETT, R. H. and N. K. AMY, 1978 Nitrate assimilation in fungi. Adv. Microb. Physiol. **18**: 1–65.

GARRETT, R. H. and D. J. COVE, 1976 Formation of NADPH-nitrate reductase activity *in vitro* from *Aspergillus nidulans niaD* and *cnx* mutants. Molec. Gen. Genet. **149**: 189–196.

GARRETT, R. H. and A. NASON, 1969 Further purification and properties of *Neurospora* nitrate reductase. J. Biol. Chem. **244**: 2870–2882.

KETCHUM, P. A., H. Y. CAMBIER, W. A. FRAZIER, C. H. MADANSKY and A. NASON, 1970 *In vitro* assembly of *Neurospora* assimilatory nitrate reductase from protein subunits of a *Neurospora* mutant and the xanthine oxidizing or aldehyde oxidase systems of higher animals. Proc. Natl. Acad. Sci. U.S. **66**: 1016–1023.

LAFFERTY, M. A. and R. H. GARRETT, 1974 Purification and properties of the *Neurospora crassa* assimilatory nitrite reductase. J. Biol. Chem. **249**: 7555–7567.

MÜLLER, A. J. and R. GRAFE, 1978 Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Molec. Gen. Genet. **161**: 67–76.

NASON, A., A. D. ANTOINE, P. A. KETCHUM, W. A. FRAZIER and D. K. LEE, 1970 Formation of assimilatory nitrate reductase by *in vitro* inter-cistronic complementation in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **65**: 137–144.

NASON, A., K-Y. LEE, S-S. PAN, P. A. KETCHUM, A. LAMBERTI and J. DEVRIES, 1971 *In vitro* formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate reductase from a *Neurospora* mutant and a component of molybdenum-enzymes. Proc. Natl. Acad. Sci. U.S. **68**: 3242–3246.

NICOLAS, D. J. D. and A. NASON, 1954 Mechanism of action of nitrate reductase from *Neurospora*. *J. Biol. Chem.* **211**: 183–197.

OOSTINDIER-BRAAKSMA, F. J. and W. J. FEENSTRA, 1973 Isolation and characterization of chlorate-resistant mutants of *Arabidopsis thaliana*. Mutation Res. **19**: 175–185.

PAN, S-S. and N. NASON, 1976 Further characterization of purified assimilatory NADPH: nitrate reductase from *Neurospora crassa*. Fed. Proc. **35**: 1530. ——, 1978 Purification and characterization of homogeneous assimilatory reduced nicotinamide adenine dinucleotide phosphate-nitrate reductase from *Neurospora crassa*. Biochem. Biophys. Acta **523**: 297–313.

PREMAKUMAR, R., G. J. SORGER and D. GOODEN, 1979 Nitrogen metabolite repression of nitrate reductase in *Neurospora crassa*. J. Bacteriol. **137**: 1119–1126.

PRODOUZ, K. N. and R. H. GARRETT, 1970 *Neurospora crassa* NADPH-nitrate reductase: a unique multi-electron transferring homodimeric enzyme. Abstr. XIth Int. Cong., Biochem. p. 261.

RAND, K. N. and H. N. ARST, 1977 A mutation of *Aspergillus nidulans* which affects the regulation of nitrite reductase and is tightly linked to its structural gene. Molec. Gen. Genet. **155**: 67–75.

SORGER, G. J., 1966 Nitrate reductase electron transport system in mutant and in wild-type strains of *Neurospora*. Biochim Biophys. Acta. **118**: 484–494.

SORGER, G. J. and N. H. GILES, 1965 Genetic control of nitrate reductase in *Neurospora crassa*. Genetics **52**: 777–788.

TOMSETT, A. B. and D. J. COVE, 1979 Deletion mapping of the *niiA niaD* gene region of *Aspergillus nidulans*. Genet. Res., Camb. **34**: 19–32.

TOMSETT, A. B. and R. H. GARRETT, 1979 Mutants defective in nitrate assimilation in *Neurospora crassa*. Amer. Soc. Microbiol. Abstracts. **79**: 129.

VEGA, J. M., R. H. GARRETT and L. M. SIEGEL, 1975 Siroheme: a prosthetic group of the *Neurospora crassa* assimilatory nitrite reductase. J. Biol. Chem. **250**: 7980–7989.

VEGA, J. M., P. GREENBAUM and R. H. GARRETT, 1975 Studies on the *in vitro* inactivation of the *Neurospora crassa* assimilatory nitrite reductase in the presence of reduced pyridine nucleotides plus flavin. Biochim. Biophys. Acta. **377**: 251–257.

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