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Evidence for a nonsense mutation at the *niaD* locus of *Aspergillus nidulans*

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SUMMARY

Two mutations at the *niaD* locus (structural gene for nitrate reductase apo-protein) are genotypically suppressible. Both mutations result in loss of nitrate reductase enzyme activity and cross reacting material and are non complementing, nonleaky and highly revertible. They have the properties of nonsense mutations. This implies that some of the allele specific suppressors, which act on these and alleles at several other loci, are nonsense suppressors.

INTRODUCTION

Roberts, Martinelli & Scazzocchio (1979) isolated genetically co-suppressible alleles in three genes: *alX4*, *sB43* and *alcA125* (renamed *alcR125* by Pateman *et al.* 1983). These three suppressible alleles were thought to be of chain termination or nonsense type since they are revertible, nonleaky mutations in three unrelated genes. *alX* codes for allantoinase (Scazzocchio & Darlington, 1968), *sB* for sulphate permease (Arst, 1968) and *alcR* for a *trans*-acting regulatory protein necessary for transcription of alcohol dehydrogenase (Pateman *et al.* 1983). By the nature of their products, these genes are unsuitable or inconvenient for biochemical analysis. Consequently we isolated suppressible alleles in *niaD* (encoding the apo-protein for nitrate reductase) since the enzyme can be assayed and antibodies were already available for immunological work. Also, the *niaD* gene has been the subject of detailed study (Tomsett & Cove, 1979; Cove, 1979).

It is desirable to establish the nature of the suppressible mutations for two reasons. Firstly it would aid in the characterization of our allele specific suppressors (Roberts *et al.* 1979) which are thought to fall into both tRNA and ribosomal categories on genetical grounds. Secondly, nonsense mutations in one gene can be used to isolate the same kind of mutation in another gene using genetic co-suppression techniques (Roberts *et al.* 1979). This would be particularly useful for studying genes whose protein products are unknown, e.g. those whose products are involved in development or regulation.

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MATERIALS AND METHODS

Genetical techniques and general cultural conditions were those of Pontecorvo *et al.* (1953).

(i) *Strains*

Numbers refer to Birkbeck stock numbers. The basic strain was 77 *fwA1*, *pabaA1*, and *alX4*. *fwA1* results in fawn conidia, *pabaA1* leads to requirement for *p*-aminobenzoic acid. Succeeding mutations were obtained in single steps (as described in Roberts *et al.* 1979) to give strain 390 with *sB43* then strain 391 with *alcR125*. *niaD17* (strain 17), *niaD52* (strain 279) and *niaD* deletion mutant strains with various backgrounds were obtained from Cambridge. Details of the informational suppressors used, strains containing suppressors and the method of isolation are given in Roberts *et al.* (1979).

(ii) *Medium*

Media are described in Roberts *et al.* (1979). The abbreviation SC refers to minimal medium supplemented with all the nutrients necessary to compensate for the background nutritional markers. Unless specified, the carbon source is glucose and the nitrogen source is ammonium tartrate. Sodium thiosulphate supplements the *sB* strains.

(iii) *Mutagenesis, selection and coreversion of* niaD *mutants*

Two mutagens were used, diethyl sulphate (DES) and 8-methoxypsoralen with a Woods u.v. light source (photodynamic mutagenesis) (Roberts *et al.* 1979). Conidia were mutagenized, added to soft, chlorate containing SC and poured onto chlorate SC plates to give 10^6 viable conidia per plate. This medium, which selects for nitrate non-utilising mutants (Cove, 1979), contains 2·5 mM glutamic acid plus 2·5 mM arginine as nitrogen sources and 50 mM-$KClO_3$, a toxic analogue of nitrate. About 30 resistant colonies appeared per plate.

The mutants were characterized by testing on SC with 10 mM-$NaNO_3$ or 5 mM-$NaNO_2$ or 10 mM nicotinate as nitrogen sources. Colonies unable to grow on nitrate had *niaD*, *cnx*, *nir* mutations or *niaD niiA* deletions. The last two were screened out by their lack of growth on nitrite, and *cnx* mutants by their inability to grow on nicotinate whilst *niaD* mutants can utilize both. The *alX4* mutation precluded testing for *cnx* mutations on hypoxanthine as nitrogen source.

niaD mutations were obtained in the following strains: the D9 series by diethyl mutagenesis of strain 390 (allele numbers: 530–535), the 541 series by photodynamic mutagenesis of strain 77 (allele numbers: 540, 542), and the P9 series by PD of strain 391 (allele numbers: 500–526).

Nonleaky *niaD* mutants (i.e. with a phenotype on nitrate equivalent to that of a *niaD niiA* deletion) were mutagenized to 10% survival: 10^7 viable conidia were added as an overlay to each plate of SC nitrate or SC allantoin to obtain reversion rates for *niaD* and *alX4* respectively. Colonies were transferred from SC allantoin to SC nitrate by velvet replication to detect simultaneous co-reversion.

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(iv) *Complementation tests*

In preliminary work, aimed at finding complementing *niaD* mutations, Roberts (unpublished work) used the following technique. Seventeen inocula of 1 mutant were stabbed onto 1 plate of SC nitrate. These were over stabbed with inocula from a corresponding 17 position master plate containing 17 different *niaD* mutants. With a large sample of mutants some positive complementation was detected. Complementary mutants were kept for the second series of tests. In this case, the two mutants under test were stabbled 3 mm apart. Up to 8 were performed per plate. Positive complementation was clearly visible after 4–5 days at 37 °C along the join of the two colonies. The strength of complemented growth was less than that found between two complementary genes.

(v) *Preparation of cell free extracts*

Mycelia were grown in minimal medium containing 5 mM urea as nitrogen source. After 16 h at 25 °C, 10 mM sodium nitrate was added for a further 5 h incubation. At 37 °C, nitrate was added 9 h after inoculation and the cultures harvested after 2 h more. Non induced mycelia were grown for 21 h at 25 °C or 11 h at 37 °C on urea. The preparation of cell free extracts has been described previously (Scazzocchio, Holl & Foguelman, 1973).

(vi) *Assay of nitrate reductase*

NADPH-nitrate reductase (EC. 1.6.6.3) was assayed as described in Cove (1966). The specific activity is defined as the production of 1 nmole of nitrite per minute per mg protein. Protein was measured by the microbiuret method (Goa, 1953).

(vii) *Cross-Immunoelectrophoresis*

This was performed according to Weeke (1973). A tris maleate buffer pH 8·6 was used in the first and second dimensions. Antibodies against nitrate reductase were provided by N. J. Lewis (1976) and were incorporated at a concentration of 1 % in the second dimension.

(viii) *Phenotypic suppression*

This was performed exactly as described in Martinelli & Roberts (1983). Most antibiotics were supplied by Parke–Davis, Eli–Lilly or J. Davies of Biogen. Others were purchased from Sigma.

RESULTS

(i) *Isolation of* niaD *mutants*

A total of 135 chlorate-resistant mutants were induced by photodynamic mutagenesis; 33 had mutations in *niaD*, 68 in various *cnx* genes; the rest were altered in morphology or nitrite non-utilizing mutants, i.e. *nirA*, *niiA niaD* deletion mutants or *niiA*, *niaD* double mutants. Of the chlorate-resistant mutants, 102, induced by DES mutagenesis, were characterized. These came from two separate experiments. Overall, there were 49 *niaD*, 25 *cnx*, 1 *nir* and 27 prototrophs.

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(ii) *Genetic characterization of* niaD *mutations by reversion, recombination and complementation*

Of the above *niaD* mutants, 24 induced by photodynamic mutagenesis were selected for further work by their nonleaky phenotype on SC nitrate. They were induced to revert by photodynamic mutagenesis and plated on SC allantoin and SC nitrate to compare reversion frequencies. Coreversion of *alX4* and *niaD* were

Table 1. *Revertibility of selected** niaD *mutants by photodynamic mutagenesis*

niaD allele	Number of revertant colonies from 5×10^7 viable treated conidia plated on: SC allantoin†	SC nitrate†	Corevertants‡
500§	85	19	34
505	114	1	0
506	120	19	0
507	111	1	0
508	103	6	0
509	98	0	0
510	228	0	0
511	86	24	0
501	91	50	42

* Only those used in complementation tests (Table 2) or phenotypic suppression tests have been detailed.

† Selects for revertants of *alX4* and *niaD* respectively.

‡ Corevertants were identified by replica plating from SC allantoin to SC nitrate.

§ These mutants are all from the P9 series of *niaD* mutants induced in strain 391, i.e. *fwA1 pabaA1 alX4 sB43 alcR125*.

assessed by replica plating from SC allantoin to SC nitrate. Results are given in Table 1 for a selection of mutants, i.e. those used in complementation (ibid) and phenotypic suppression tests (Martinelli & Roberts, 1983). Two mutants, designated *niaD500* and *niaD501* were shown to be corevertible with *alx4*. Amongst other mutants tested, (and which do not appear in Table 1), 5 did not revert, 9 gave reversion rates for *niaD* similar to those given in Table 1 and *niaD526* reverted at higher frequency than *niaD501* although it gave no corevertants.

Coreversion of *niaD* mutations with *sB43* and *alcR125* was not tested directly. However, several colonies were isolated from SC nitrate and SC allantoin plates after mutagenesis of *niaD500* and *niaD501*, and tested on SC ethanol and SC minus thiosulphate. All 39 $niaD^+$ revertants were mutant for the other 3 suppressible alleles. In contrast, 7 alX^+ colonies were revertant at all 4 loci and amongst these strains, 3 were cold sensitive. They were all assumed to contain allele-specific suppressors.

A series of complementation tests were carried out with 17 new *niaD* mutants and 2 control mutants: (*a*) *niiA niaDΔ516* as a noncomplementing mutant, and (*b*) *niaD17* which showed interallelic complementation in extensive preliminary tests (see methods): 9 mutants complemented with at least 1 other strain. *niaD500*, *niaD501* and 6 other mutants showed no inter-allelic complementation (Table 2).

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In a wider series of complementation tests with 46 *niaD* mutants, 30 were noncomplementing and 16 showed interallelic complementation.

In crosses between *niaD500* or *niaD501* and the deletion mutation *niiA niaD516*, no wild type progeny were seen in 70000 ascospores, indicating that these

Table 2. *Complementation of* niaD *mutants on SC nitrate*

Mutant allele

17	530	531	532	533	540	534	535	542	500	501	505	506	507	508	509	510	511	
	0*	0	+	+++	++	+	0	0	0	0	0	0	0	—	—	0	0	17†
		+	+	+++	++	0	0	0	0	0	—	—	—	—	—	—	—	530
			0	+++	+++	0	0	0	0	0	—	—	—	0	0	—	—	531
				+++	+++	0	0	0	0	0	0	0	0	0	0	0	0	532
					+	+++	++	0	0	0	+++	0	+++	0	0	0	0	533
						++	+	0	0	0	+	0	++	0	0	0	0	540
							+	0	0	0	0	0	0	0	0	0	0	534
								0	0	0	0	0	0	0	0	0	0	535
									0	0	0	0	0	0	0	0	0	542
										0	0	0	0	0	0	0	0	500
											—	—	—	—	—	—	—	501

* Indicates strength of complementation 0, none; +, weak; ++, medium; +++, strong complementation. –, not tested. Strong complementation between *niaD* mutations is weaker than that given between mutants complementing for different defects in nitrate utilisation when grown on SC nitrate. Results for the non-complementing deletion mutation *niiA niaDΔ516* are not given, but were all negative.

† Alleles 530 to 535 inclusive are part of the D9 series (Diethylsulphate mutagenesis of strain 390). Alleles 540 and 542 are part of the 541 series (Diethylsulphate mutagenesis of strain 77). Alleles 500 to 511 inclusive are part of the P9 series (Photodynamic mutagenesis of strain 391). Alleles 530 and 533 cause nitrate reductase activity to be temperature sensitive *in vitro*. Allele 17 was isolated by D. J. Cove (Pateman *et al.* 1967) and is the standard allele used in this work.

mutations map at this locus. Complementing and non-complementing mutants were crossed to known point mutants *niaD52* and *niaD17*. The frequency of $niaD^{+}$ progeny was between 0·01 and 0·04 %.

niaD500 was recessive to the wild type allele in a heterozygous diploid.

(iii) *Suppression of* niaD *mutations*

Strains containing *niaD500* or *D501* were crossed to a series of strains each containing a characterized allele specific suppressor. Suppressors *suaA105*, *suaD103*, *suaD108* and *suaB111* were isolated by coreversion of *alX4* and *sB43* and suppress only these mutations. *suaC109* and *sua-115* were isolated in the same background but later shown to suppress *alcR125* also. *suaA101* was isolated by coreversion of *alcR125* with *alX4* and *sB43*. *sua-115* is very similar to *suaC109* but its genetic location is unknown. For further details of suppressors, see Roberts *et al.* (1979). The crosses were homozygous for *alX4* and *sB43*. Thus it was possible to score independently for the presence of a suppressor by growth of progeny on SC allantoin and SC minus thiosulphate. *A priori*, the occurrence of progeny containing suppressors which have a mutant phenotype on SC nitrate establishes that a particular *niaD* allele is not suppressed. *niaD500* and *D501* were not

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suppressed by *suaD103*, *suaD108*, *suaB111* or *suaA105*. Colonies containing suppressors *suaA101* and *suaC109* are recognizable on all media by their slow growth rate resulting in smaller colonies than normal. On SC nitrate it was possible to distinguish 4 classes of progeny; wild type *niaD*$^+$ *sua*$^+$, mutant *niaD sua*$^+$, *niaD*$^+$ *sua* which was similar to wild type but smaller, and *niaD sua* which was small and had a hyphal density intermediate between wild type and mutant. A sample of *niaD sua* progeny were outcrossed to wild type to detect the presence of the *niaD* mutation and confirm suppression. Similarly, *sua-115* suppressed *niaD500*. Crosses between *niaD501* and *sua-115* strains have not been successful. *niaD52* and *niaD17* were not suppressed by *suaA101* or *suaC109* therefore the suppression of *niaD500* and *D501* is allele specific.

(iv) *Activity of nitrate reductase and cross immuno electrophoresis*

Neither strains containing *niaD500* nor *niaD501* showed any nitrate reductase activity when grown at 25 and 37 °C. *niaD500*, *suaA101* and *niaD501 suaA101* combinations gave detectable activity at both temperatures, between 2 and 13% of wild type activity. *niaD501 suaC109* gave 7% of wild type activity at 37 °C. The latter could not be grown at 25 °C because *suaC109* confers cold sensitivity for growth. No other combinations of suppressors with *niaD* mutants were assayed. These results indicate that both *suaA101* and *suaC109* restore nitrate reductase activity.

Both *niaD500* and *D501* mutants lacked cross reacting material for nitrate reductase. Plate 1 shows a cross-immuno electrophoresis experiment involving the *niaD500* strain and wild type. There was no deflection of the reference line by cross reacting material for nitrate reductase. It should be noted that this method does not detect cross reacting material at levels below 10% of the fully induced wild type.

(v) *Independence of* niaD500 *and* D501

niaD$^+$ colonies were obtained from both homo-allelic and hetero-allelic crosses with *niaD500* and *D501* but no marked difference was observed since all crosses gave approximately 1 in $3{\cdot}5 \times 10^4$ *niaD*$^+$ progeny.

Homoallelic *niaD500/niaD500* and *niaD501/niaD501* diploids and the hetero-allelic diploid *niaD500/niaD501* were constructed. The heteroallelic diploids were constructed in two different genetic backgrounds. Conidia from these 4 diploids were plated at high concentrations on SC nitrate to select *niaD*$^+$ colonies. It is interesting that up to 80-fold more *niaD*$^+$ colonies came from the two heteroallelic diploids than from the 2 homoallelic ones. The frequency of *niaD*$^+$ production from the latter was 7×10^{-8} viable conidia. The increased number of *niaD*$^+$ colonies could have arisen by mitotic crossing over or forward mutation at suppressor loci but probably not by spontaneous mutation. Although the origin of these *niaD*$^+$ colonies is uncertain, they suggest that *niaD500* and *D501* map at different sites within the gene.

(vi) *Phenotypic suppression of* niaD501

Discs containing antibiotics were placed on top of SC nitrate plates which had been spread with conidial suspensions of a strain containing *niaD501*. After

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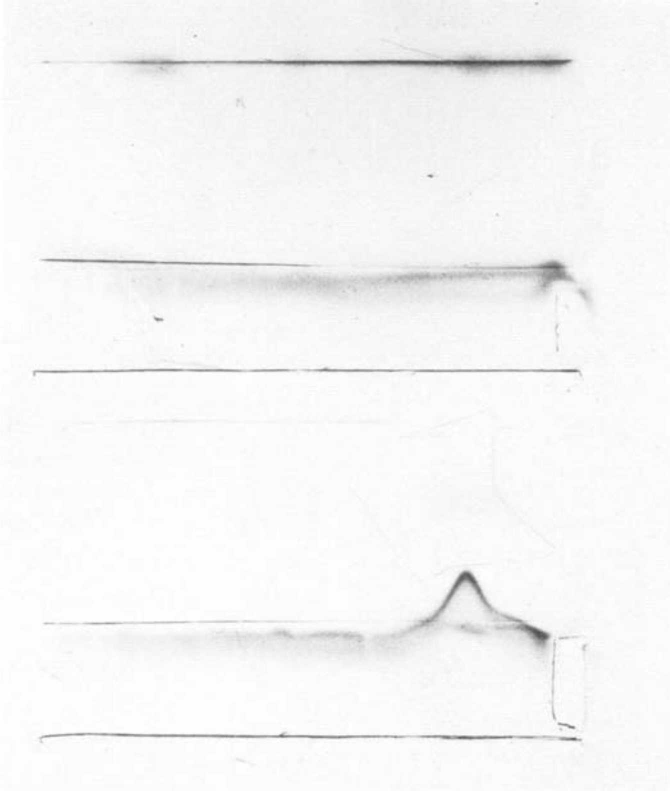

Cross immunoelectrophoresis of *niaD500* and wild type extracts. The strains were grown at 25 °C on urea as nitrogen source, then induced with 10 mM nitrate. Each sample well contained 25 μl of a crude extract containing 50 μg protein. Top: *fwA1 pabaA1 alX4 sB43 alcR125 niaD500*, Bottom: *pabaA1* = wild type. The reference trough contained 100 μl of wild type extract (= 100 μg protein). The plate was stained for NADPH/FAD tetrazolium activity (Lewis, 1976).

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incubation for 3–5 days rings of growth appeared around discs impregnated with paromomycin, gentamiacin, tobramycin, neomycin and sisomycin, but not with neamine, hygromycin, apramycin, kanamycin or G418. Experiments with *niaD500* gave the same results. Results of tests on *niaD500*, *niaD52* and the P9 mutants listed in Table 2 are reported elsewhere (Martinelli & Roberts, 1983). It was not possible to distinguish between suppression of *niaD501* and *niaD500* qualitatively or quantitatively.

DISCUSSION

The two mutant colonies representing *niaD500* and *niaD501* were isolated from different plates after mutagenesis and were therefore presumed to be of independent origin. However in reversion, coreversion, complementation, recombination, genetic and phenotypic suppression studies, no difference between them is apparent. They could be very closely linked mutations or mutations at the same site. The heterogeneity of these two alleles has only been shown by presumed mitotic recombination in a hetero allelic diploid.

The evidence presented here suggests that *niaD500* and *niaD501* are nonsense mutations. None of the following criteria alone indicate a nonsense mutant with certainty: i.e. nonleakiness, noncomplementation, revertibility, lack of enzyme activity, lack of cross reacting material and suppression by allele specific suppressors. However, altogether, these facts point to nonsense or frameshift mutations rather than missense.

In *Saccharomyces cerevisiae*, there is a strong correlation between phenotypic suppression and nonsense suppression (Singh, Ursic & Davies, 1979; Chattoo *et al.* 1979). Suppression of *niaD500* and *niaD*501 but not eight other *niaD* mutants by aminoglycoside antibiotics (ibid; Martinelli & Roberts, 1983) lends support to the hypothesis that *niaD500* and *D501* are chain termination mutants.

In an extensive search for complementing *niaD* mutants intracistronic complementation was observed between a few newly isolated *niaD* mutants and more surprisingly between some mutants and *niaD17* which is the standard allele used in genetical and biochemical work on this gene. No intra-cistronic complementation amongst $niaD^{-}$ alleles has previously been reported.

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