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Deletion mapping of the *nii*A *nia*D gene region of *Aspergillus nidulans*

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SUMMARY

The genetic fine-structure of the *nii*A *nia*D gene region of *Aspergillus nidulans* has been studied using deletion mapping. Deletions were identified as *nii*A *nia*D double mutants and comprised 1% of spontaneous chlorate-resistant mutants. All such double mutants were shown to involve deletions and their frequency was not increased by mutagenic treatment with either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or with ultraviolet light. Deletion maps of the *nia*D and *nii*A genes have been constructed. A further class of mutation was also mapped using the deletions. These *crn* mutations, which affect a gene whose function is as yet unknown, map on the centromere proximal side of *nii*A. This analysis of a eukaryote gene cluster will provide a framework upon which to base studies of the regulation of the nitrate assimilation pathway.

1. INTRODUCTION

In *Aspergillus nidulans* the core-polypeptide of nitrate reductase (NADPH-nitrate oxidoreductase, E.C. 1.6.6.3) is specified by the *nia*D gene (Pateman, Rever & Cove, 1967; Macdonald & Cove, 1974; Cove, 1979) and nitrite reductase (NADPH-nitrite oxidoreductase, E.C. 1.6.6.4) is specified by the *nii*A gene (Pateman *et al.* 1967; Rand & Arst, 1977; Cove, 1979). The *nii*A and *nia*D genes are closely linked on chromosome VIII, but are thought not to comprise an operon (Cove, 1970, 1979). However, both are subject, through similar mechanisms, to nitrate induction (Cove, 1967; Pateman & Cove, 1967; Cove & Pateman, 1969; Cove, 1979), and to ammonium repression (Arst & Cove, 1973; Pateman *et al.* 1973; Kinghorn & Pateman, 1975; Pateman & Kinghorn, 1975; Cove, 1979), and the structural organisation of these two genes is consequently of considerable interest. This study was undertaken to obtain a genetic fine-structure map of the *nii*A *nia*D gene region, such a map being essential for a complete understanding of the regulation of nitrate assimilation, in particular enabling the accurate location of

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cis-acting mutations which may occur in control sites for the induction or repression systems. The mapping of the *nii*A *nia*D gene region has been carried out using deletion mapping in an essentially similar way to that employed by Benzer (1961) to map the rII region of phage T_4. In earlier work, Benzer (1959) had concentrated on the topology of the genetic fine-structure, and considered how the parts of the structure were connected to each other, instead of measuring distances between them. We have adopted a similar approach in this study.

2. MATERIALS AND METHODS

(i) *Strains and general genetic techniques*

The genetic techniques used were modified after Pontecorvo *et al.* (1953). Most of the markers carried by the strains used in this study are in general use (see Clutterbuck, 1974).

(ii) *Media and supplements*

The nitrogen-less minimal medium (containing 1% D-glucose) described by Cove (1966) was used. Unless specified otherwise, nitrogen sources were added to give a final concentration of 10 mg-atoms of nitrogen per litre, with the exceptions of hypoxanthine and uric acid which were used at 100 mg/ml. Ammonium was added as ammonium(+)-tartrate. Nitrate and nitrite were added as sodium salts. The toxic analogues chlorate and bromate were added as the potassium salts. Sodium desoxycholate was added to selective media at 800 mg/ml.

(iii) *Mutagenesis and the selection of mutant strains*

Mutant strains were either isolated as being spontaneously resistant to the toxic analogue chlorate or were induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) or ultraviolet light (UV). For NTG mutagenesis, conidiospores were treated according to the method of Adelberg, Mandel & Chen (1965) as modified by Alderson & Hartley (1969), so that survival was between 5 and 15%. For UV mutagenesis, a suspension containing 10^9 conidia per ml was placed in a 14 cm sterile petri dish so that it just covered the bottom. The dish was placed 6 cm distant from an UV lamp and exposed for 20 minutes to obtain a survival between 1 and 30%.

Mutant strains were selected in one of three ways:

(*a*) *Selection of chlorate-resistant mutants.*

The method was based on that used by Cove (1976*a*). A conidial suspension was diluted to give 5×10^6 conidia per ml in appropriate selective molten medium at 45 °C. Twenty ml of this mixture was then poured into each petri dish to give plates containing 10^8 spores. The medium contained sodium desoxycholate to induce the formation of microcolonies (Mackintosh & Pritchard, 1963). After 3–4 days' indubation at 37 °C about 100 resistant microcolonies developed on each plate. These were subcultured and subsequently reisolated from single conidiospores. The sample of resistant mutants was picked to ensure that there was no

bias in favour of a particular morphology of resistant colony, except that only colonies which conidiated were chosen.

When deletions were to be selected by this method, plates having *c.* 100 resistant microcolonies were replicated using velvet (Mackintosh & Pritchard, 1963) onto medium containing sodium desoxycholate and nitrite as sole nitrogen source. These replica plates were then incubated for 1 day at 37 °C. Colonies which failed to grow on the nitrite medium were picked from the selective plate, subcultured and reisolated as above.

(*b*) *Selection of nitrate non-utilizing mutants from putrescine-requiring auxotrophs by the use of putrescine starvation*

The method was that used by Cove (1976*a*).

(*c*) *Selection of temperature-sensitive nitrate non-utilizing mutants*

The conidiospores after mutagenic treatment were diluted to give a density of 2×10^4 conidia per ml. This suspension was further diluted 100-fold into molten minimal medium, cooled to 45 °C, and containing nitrate as nitrogen source, and 5 ml of this mixture was then pipetted on to the surface of a similar solid medium in a petri dish. These media contained sodium desoxycholate to induce the formation of microcolonies. After incubation for 4–5 days at 25 °C the plates were replicated with velvet onto identical media and incubated for one day at 37 °C. Colonies which failed to grow at 37 °C were then picked from the 25 °C plate, subcultured and reisolated as above.

(iv) *Classification of mutant strains*

The classification of mutant strains was based on the method of Cove (1976*a*). Growth tests were used initially to classify mutants according to the criteria shown in Table 1. Confirmation of these assignments was made by complementation tests to representative stock cultures carrying mutations in the loci concerned.

(v) *Method of deletion mapping.*

Crosses between two strains carrying different auxotrophic markers and having different conidial colours were set up using modifications of the technique described by Pontecorvo *et al.* (1953).

One strain carried a putative deletion of part of the *nii*A *nia*D gene region while the other carried a single gene mutation in the *nii*A or *nia*D gene. Four cleistothecia from each cross were cleaned by rolling them on the surface of 3% agar. The back of a petri dish containing medium with all appropriate biochemical supplements, sodium desoxycholate and nitrate as sole nitrogen source was marked into quarters. Each cleistothecium was squashed on the surface of the medium to release the ascospores which were then spread over one quarter of the plate with a loopful of sterile distilled water. The ascospores remaining on the loop after spreading were streaked on the surface of a plate containing complete medium. Both plates were incubated at 37 °C for 3 days. Hybrid cleistothecia were identified

Table 1. *Criteria used for the classification of selected strains*

	Nitrogen source utilization				Resistance to chlorate on			Resistance to bromate on glutamate
Mutation	NH_4^+	NO_3^-	NO_2^-	Hypoxanthine	Uric acid	Arginine	Glutamate	
*nia*D$^-$	+	−	+	+	Varies	Varies	Varies	S
cnx$^-$	+	−	+	−	Varies	Varies	Varies	S
*nii*A$^-$	+	−	−	+	S	S	S	I
*nii*A *nia*D$^\Delta$	+	−	−	+	R	R	R	S or R
*nir*A$^-$	+	−	−	+	R	I	S	R
crn	+	+	+	+	R	S	S	R
wild-type	+	+	+	+	S	S	S	I

For nitrogen source, + indicates growth on that medium and − indicates no growth. For resistance to toxic analogues, R indicates resistance, S sensitivity and I intermediate resistance. Chlorate resistance was scored on media containing 100 mM-$KClO_3$ and either 100 mg/l uric acid, 10 mM L-arginine HCl or 10 mM L-glutamate. Bromate resistance was scored on media containing 1 mM-$KBrO_3$ and 10 mM L-glutamate. The resistance to bromate of *nii*A *nia*D deletion strains varies depending on whether the *crn* locus is also deleted (see text).

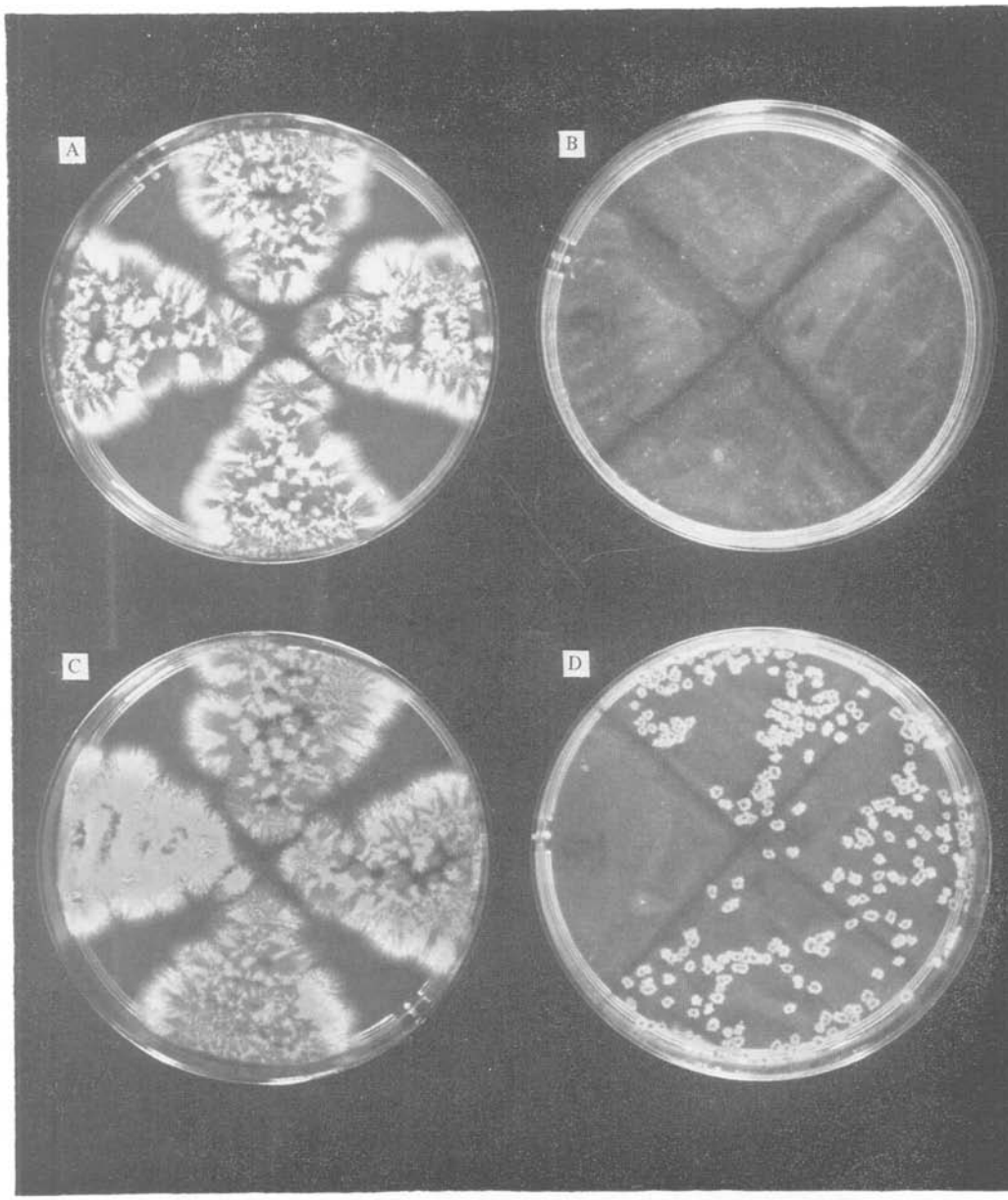

Fig. 1. Results of crosses used for deletion mapping. Petri dishes A and C contain complete media to test which cleistothecia are of hybrid origin. Petri dishes B and D contain media with nitrate as sole nitrogen source, upon which only 'wild-type' recombinants can grow. The cross analysed in dishes A and B shows no recombination has occurred and hence the single gene mutation is located within the deletion. Dishes C and D show a cross in which recombination has occurred and hence the single gene mutation is outside the deletion. The selfed cleistothecium in dish C does not contain any recombinants as shown by the corresponding sector in dish D.

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by the segregation of conidiospore colour among progeny plated on the complete medium, and the corresponding quarters could then be scored for the presence or absence of progeny able to grow on nitrate. Four cleistothecia were usually sufficient to give a clear indication of whether recombination occurred between the single gene mutant and the deletion, but in cases where the result was in doubt, four or more further cleistothecia were tested. Typical results are shown in Plate I, fig. 1.

3. RESULTS

(i) *The selection of mutant strains*

In the selection of mutant strains attempts were made to use several different methods to isolate mutants at as many different sites as possible.

(*a*) *The selection of deletions*

The selection and characterization of chlorate-resistant mutants in *Aspergillus nidulans* has been studied extensively by Cove (1976*a*). Chlorate appears to be a toxic analogue of nitrate, although the mechanism of its toxicity is unknown (Åberg, 1947; Cove, 1976*b*). Mutations which result in a chlorate-resistant phenotype are generally altered in nitrate assimilation. Cove (1976*a*) showed that among *nia*D mutants which arose spontaneously, 1–5% also carried a *nii*A mutation. He concluded that these strains were unlikely to carry two independent point mutations since they did not arise with the same relative frequency in mutagenized samples. Since some preliminary studies showed these strains to be non-revertible (Cove, unpublished data) and the *nii*A and *nia*D genes map near to one another (Cove, 1970), these double mutants might involve deletions. In the studies described here, these double mutants have been shown to be deletions involving loss of part or the whole of both genes.

Since all other chlorate-resistant mutants except *nir*A$^-$ could be screened out in the selection of *nii*A *nia*D deletions, it would be useful to select against *nir*A$^-$ strains. Cove (1976*a*) showed that the spectrum of chlorate-resistant mutants obtained was dependent on the nitrogen source used for selection. The effect on the proportion of *nir*A$^-$ mutants was very marked. For example, whereas when urea served as nitrogen source, 22% of chlorate-resistant strains were *nir*A$^-$, when L-glutamate was the nitrogen source, no *nir*A$^-$ mutants were recovered in a sample of 607 chlorate-resistant strains. By the use of L-glutamate as nitrogen source, we were able to ensure that almost all the chlorate-resistant nitrite non-utilizers selected were *nii*A *nia*D deletions.

(*b*) *The selection of single gene mutations.*

Strains carrying *nii*A and *nia*D single gene mutations were isolated after *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine or ultraviolet mutagenesis and either selection using putrescine starvation or by replica plating. Spontaneous *nia*D mutants were selected as chlorate-resistant colonies.

(ii) *Principles of deletion mapping*

(*a*) *Theory of the method*

The theory of the method was exactly the same as that used by Benzer (1961) and by others. Deletions are defined as those mutations which fail to recombine with two other mutations which recombine with one another. The position of mutant sites relative to a given deletion can be determined by simply scoring the crosses for the presence or absence of progeny growing on nitrate.

(*b*) *Sample size*

Although the method does not rely upon recombination frequencies, large numbers of progeny must be tested in order to detect the lowest recombination rates. Despite the fact that *Aspergillus nidulans* is homothallic, crosses vary in their ability to produce hybrid cleistothecia and this appears to be related partly to the genetic backgrounds of the two parental strains. Certain strains cross easily while others produce only a few small cleistothecia. Whether this reflects the quality of the mixing of nuclei within the heterokaryon or some other variation in the genetic backgrounds of the component strains is unknown. In these studies, it was found that the best crosses were between strains of genetic background *y*A1 *pu*A2 and *bi*A1 since they produced consistently large numbers of very large cleistothecia. Other strains which also gave good results were those carrying the *panto*B100 or the *paba*A1 mutation with or without the conidial colour markers *y*A1 or *fw*A1.

The cleistothecia used for deletion mapping usually had diameters of 0·4 mm or larger, but most were in the range of 0·4–0·5 mm diameter. Tests of cleistothecia of this size indicated the number of viable ascospores they contained varied from 10000 to 150000 per cleistothecium and averaged 60000 per cleistothecium. Furthermore, the number of viable ascospores increased with storage for one month. After 2 weeks' storage at 4 °C the average number of viable ascospores was 40000 per cleistothecium, while after 1 month's storage the average was 120000 per cleistothecium. Clutterbuck (1975) had similar findings and concluded that ascospores go through a definite maturation process which cannot be completed once they have been plated. These values indicate that the number of progeny tested was probably in the range of 10^5–10^6 ascospores per cross.

(*c*) *Limits of resolution*

Before the order of point mutations could be assigned, the limits of resolution for deletion mapping had to be defined. Since recombinants and revertants were indistinguishable, recombination could only be measured if it were significantly higher than the reversion rate. Reversion of each mutation was checked by ensuring that it produced no recombinants in at least one cross. Thus any strains which reverted with a fairly high frequency could be detected. To ensure the recombination rate was higher than the reversion rate, recombination was only scored if the average number of wild-type recombinant progeny was equal to or greater than

1 per cleistothecium. In practice, therefore, the lowest rate of recombination detected by this system will be about 2×10^5.

In an attempt to avoid confusion resulting from reversion or occasional airborne contaminants, crosses the results of which were in doubt, i.e. with less than three recombinants per cleistothecium, were tested further to ensure the consistency of the results. Furthermore, the test streak on complete medium gave an indication of the number of spores tested. Cleistothecia containing low numbers of viable ascospores were detected by the reduced density of colonies comprising the test streak.

(iii) *Deletion mapping of the* nia*D gene*

The *nii*A *nia*D double mutants, which arose as spontaneous chlorate-resistant colonies, could plausibly carry deletion mutations. However, many of the single gene mutants that arose spontaneously could also carry deletions restricted to the *nia*D gene. These would be phenotypically indistinguishable from point mutants, but they should be non-revertible. A study of mutation in *Aspergillus nidulans* (Hartley, 1969) investigated the reversion of *nia*D alleles. This study revealed that *nia*D10, 14, 26, 32, 35 and 40 were non-revertible. Deletion mapping was started therefore using the six non-revertible *nia*D mutations, a selection of *nii*A *nia*D spontaneous double mutants and a number of *nia*D single gene mutant strains.

Results of these early crosses showed that *nia*D10, 14, 26, 32 and 40 were all deletions; however, this has not been shown for *nia*D35. Subsequent analysis showed that all 140 *nii*A *nia*D double mutants isolated as spontaneous chlorate-resistant double mutants were deletions. These strains are referred to as *nii*A *nia*D$^{\Delta}$ or Δ501–Δ640.

The deletion map of the *nia*D gene is shown in Fig. 2. In this map, each interval, defined by the difference between two deletions, is drawn as if it were the same length irrespective of the number of single gene mutants that map within it.

(iv) *Deletion mapping of the* nii*A gene*

The mapping of the *nii*A gene is basically similar to that of *nia*D except that no deletions involving only the *nii*A gene were available. Here the deletion mapping is less extensive. The deletion map is shown in Fig. 3.

(v) *Deletion mapping of* crn *mutations*

crn mutants were obtained as one type of a new class of mutation (CRUN), members of which were chlorate-resistant but still able to grow up on nitrate, nitrite and hypoxanthine (Cove, 1976*a*). Studies by Cove (unpublished data) showed that *crn* mutations mapped close to the *nii*A *nia*D region. Mapping by recombination was impossible since the *crn* mutant phenotype does not allow for the selection of recombinants in crosses with *nii*A and *nia*D strains. Use can be made, however, of the finding that *crn* mutations are recessive. A heterozygous diploid between wild type and *crn* (*crn*/+) was almost as sensitive to 100 mM chlorate as was the wild-type homozygous diploid (+/+). The *crn* homozygous

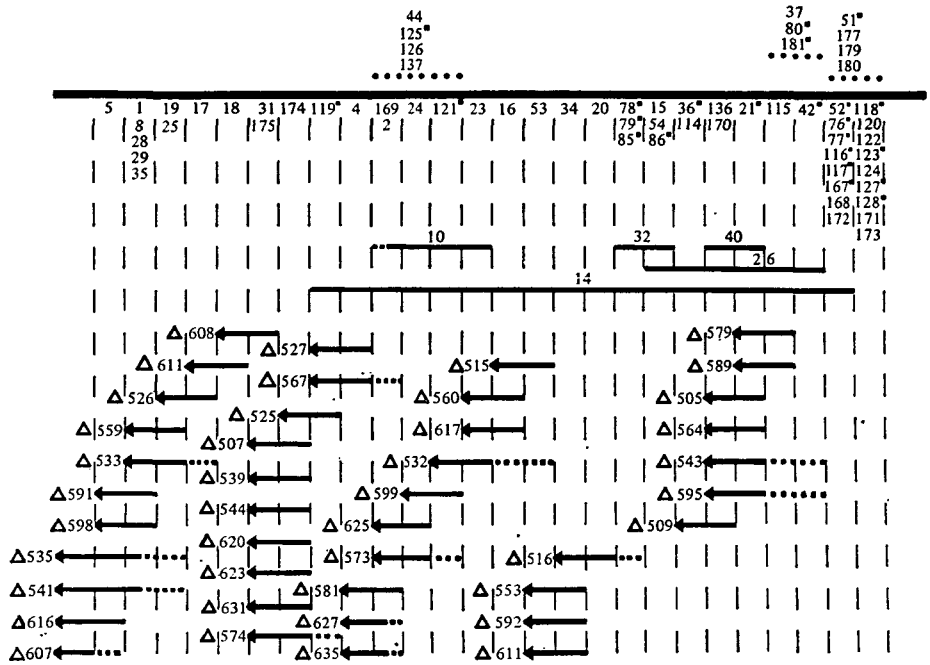

Fig. 2. A deletion map of the *nia*D gene in the conventional orientation, so that the *nii*A gene and the centromere are to the left of the *nia*D gene. In this map, deletion intervals are assumed to be of equal size irrespective of the number of single gene mutants that map within them. The lines depicting deletions 10, 14, 26, 32 and 40 represent the full extent of the deleted region. Deletions Δ501–Δ635 all extend in from the *nii*A gene and hence only their right-hand limit is shown. Dotted extensions to deletions represent the region within which a deletion end-point is known to occur if it has not been fully defined. The dotted lines above the main map represent single gene mutants which have not been fully defined. The dotted line covers the region within which those mutants map. The mutations, marked with a black spot, lead to a temperature-sensitive phenotype.

diploid (*crn*/*crn*) was resistant to 100 mM chlorate when either uric acid or asparagine but not when arginine served as nitrogen source. It was possible therefore that a deletion which also involved the whole or part of the *crn* locus would, when combined with a *crn* point mutation in a diploid, have the same phenotype as a *crn*/*crn* homozygote.

Diploids were constructed between three different *crn* mutant strains and a series of representative strains including four deletions. The homozygous wild-type diploid was sensitive to 100 mM chlorate when either uric acid, asparagine or arginine were used as nitrogen source. Diploids which were homozygous for chlorate-resistant *nia*D alleles showed complete resistance to chlorate on all three nitrogen sources and were unable to grow on nitrate. All diploids between strains carrying a *crn* mutation and strains carrying a *nii*A *nia*D deletion were sensitive to chlorate when arginine was the nitrogen source, but their resistance when either uric acid of asparagine was the nitrogen source depended on which deletion was

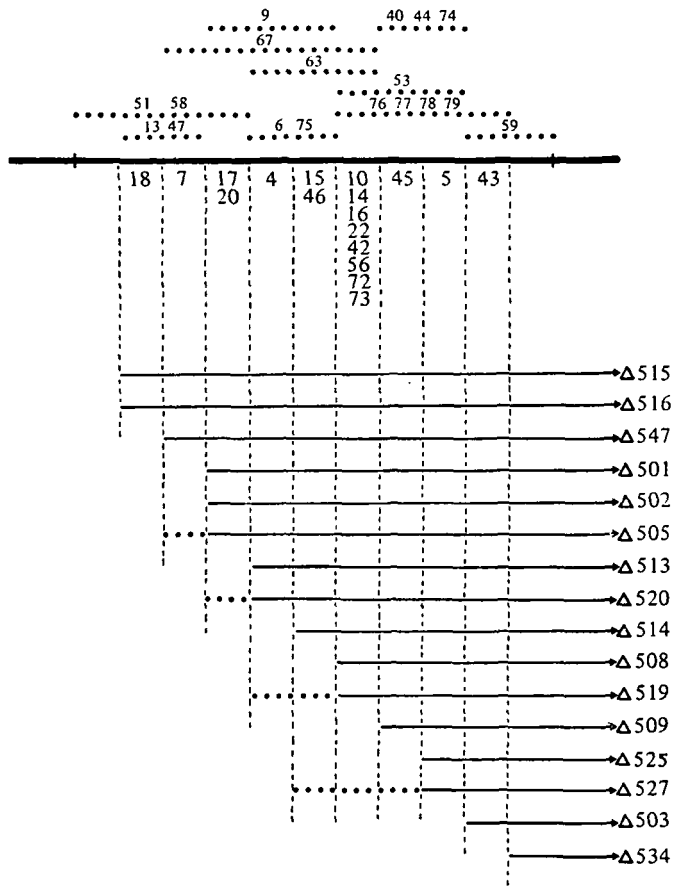

Fig. 3. A deletion map of the *nii*A gene in the conventional orientation so that the centromere is to the left and the *nia*D gene is to the right of the *nii*A gene. This map was constructed using the same principles as those used in Fig. 2. All deletions in this map extend into the *nia*D gene and hence only their left-hand limit is shown.

involved. Results obtained for diploids involving the *crn*-1 allele are given in table 2. Similar results were obtained for diploids involving the *crn*-2 and *crn*-3 alleles. The four deletions which were used allow the location of *crn* in relation to the *nii*A and *nia*D genes to be determined. Δ506 and Δ507 are examples of deletions which fail to recombine with all *nii*A point mutants tested and are therefore likely to extend at least to the left-hand (centromere proximal) end of the *nii*A gene. The chlorate resistance phenotype of *crn* heterozygous diploids involving these deletions is the same as *crn*/*crn* homozygous diploids, and so Δ506 and Δ507 are likely to have the *crn* locus deleted also. On the other hand diploids between *crn* strains and deletions Δ501 and Δ505, the left-hand end-points of which are within the *nii*A gene, resemble *crn*/+ diploids, and are therefore likely to retain the *crn* locus. There is however no similar correlation between the phenotype of a *crn*/deletion heterozygous diploid and the extent of the deletion rightwards into

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Table 2. *Summary of growth tests of diploid strains involving* crn-1 *mutation*

Partial Genotype	Nitrogen source utilization		Resistance to 100 mM chlorate on		
	NH_4^+	NO_3^-	Uric acid	Asparagine	Arginine
+/+	+	+	S	S	S
crn-1/+	+	+	SR	SR	S
crn-1/*nia*D17	+	+	SR	SR	S
*nia*D17/+	+	+	SR	SR	S
*nia*D17/*nia*D17	+	−	R	R	R
crn-1/*crn*-1	+	+	R	R	S
crn-1/*nii*A4	+	+	SR	SR	S
crn-1/Δ501	+	+	SR	SR	S
crn-1/Δ505	+	+	SR	SR	S
crn-1/Δ506	+	+	R	R	S
crn-1/Δ507	+	+	R	R	S
Δ501/+	+	+	SR	SR	S
Δ505/+	+	+	SR	SR	S
Δ506/+	+	+	SR	SR	S
Δ507/+	+	+	SR	SR	S

For nitrogen source utilization, + indicates growth on that medium and − indicates no growth. For resistance to the toxic analogue chlorate, S indicates the diploid was sensitive, R indicates it was resistant, while SR indicates it was only slightly resistant.

the *nia*D gene. Thus of the two *nii*A *nia*D deletions which retain the *crn* locus, Δ501 is likely to extend at least to the right-hand border of the *nia*D gene, whereas Δ505 terminates within it. A similar situation holds for Δ506 and Δ507 which are likely to have the *crn* locus deleted. The *crn* locus is therefore likely to be located to the left-hand (centromere proximal) side of *nii*A (see Fig. 4). Growth tests of diploids between a *crn* allele and further deletions were also consistent with this location. Heterozygous diploids involving one of the following deletions: Δ502, 503, 508, 509, 513, 514, 519, 520, 524, 525 or 527, all of which deletions have their left-hand end point within the *nii*A gene, were undistinguishable from *crn*/+ diploids with respect to chlorate sensitivity, as were diploids involving Δ516 and Δ517, which fail to recombine with any *nii*A point mutations so far investigated. Diploids were also made between a *crn* mutant and 16 deletions whose left-hand end-points had not been determined. Of these, the ones involving Δ504, 510, 511 and 512 were phenotypically similar to *crn*/*crn* diploids, and it is therefore to be predicted that these should fail to recombine with all *nii*A point mutations.

Preliminary studies of bromate toxicity show that *crn* mutant strains are more resistant than the wild-type to 1 mM bromate when uric acid serves as nitrogen source. Point mutations involving either the *nii*A gene or the *nia*D gene do not lead to similar bromate resistance. *nii*A *nia*D deletions could however be divided into two groups according to their resistance to bromate. Those deletions which, on the basis of the chlorate-resistance phenotype of the *crn* heterozygous diploid were thought to delete the *crn* locus, were all bromate resistant, and those which, on similar grounds, were thought not to have the *crn* locus deleted, were bromate

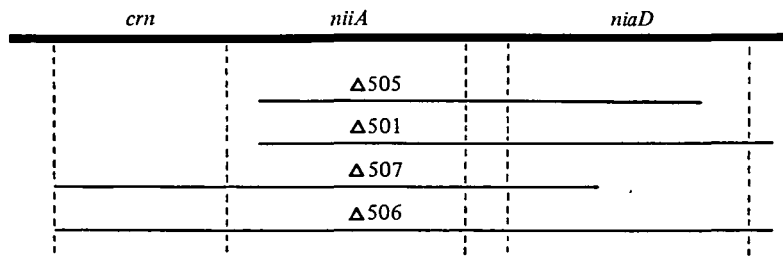

Fig. 4. A deletion map to determine the location of *crn* mutations in relation to the *nii*A and *nia*D genes. Deletions Δ506 and Δ507 delete the *crn* locus, but it is not known how much further they extend to the left. Deletions Δ501 and Δ506 fail to recombine with those *nia*D point mutations located furthest to the right in the *nia*D gene, and are likely therefore to be deleted for the whole *nia*D gene, although it is not known at present if they extend leftwards beyond it.

sensitive. This growth test should provide a quicker method than the construction of *crn* heterozygous diploids to decide whether deletions extend into the *crn* region.

4. DISCUSSION

In this study, a simple procedure has been devised to allow the analysis of the genetic fine-structure of the *nii*A *nia*D gene region of *A. nidulans*. The procedure involves deletion mapping and has been used to consider the organization of the *nii*A and *nia*D genes. A new class of mutation, *crn*, has also been mapped using the deletions. The deletions are easily recognized among spontaneous chlorate-resistant colonies and arise at a rate of about 1 %. The single gene mutations were derived from the use of a number of different mutagens and several different selection procedures. All mutations used in this study were obtained from a large number of sources in an attempt to avoid 'hot-spots' and have alterations at as many different sites as possible.

The deletion map of the *nia*D gene (Fig. 2) shows a non-random distribution of point mutations. Since the map was drawn on the assumption that all intervals between deletion endpoints were equal, the clusters of point mutations may represent large regions of the gene although they may also represent 'hot-spots'. The large number of point mutations at the right-hand end of the map most probably represents a large deletion interval. Unfortunately, *nia*D52 was the most rightward point mutation mapped through most of the course of these studies. It was late in this work that the *nia*D118 group was added to the map. It is possible therefore that the interval between *nia*D14 and the end of the *nia*D gene is quite large. However, the possibility that this represents a 'hot-spot' cannot be ruled out.

It is possible that the distribution of deletion end-points is non-random in the *nia*D gene. Seven deletions terminate between *nia*D174 and *nia*D119. This may represent a deletion endpoint 'hot-spot'. Such clustering of deletion endpoints has been observed in the *gal* region of *E. coli* and has been shown to occur as a

result both of heat induction of λ (Pfeifer *et al.* 1974) and of *IS*1-mediated deletion formation (Reif & Saedler, 1975). Three clusters of endpoints also occur in the *lac*I gene (Schmeissner, Ganem & Miller, 1977) although the cause in this case is unknown.

Mapping of the *nii*A gene has not yet proceeded far enough to make detailed comments. All clusters of point mutations and deletion termini almost certainly represent large deletion intervals.

Mutations at the *crn* locus were known to map in the *nii*A *nia*D gene region and hence the most likely explanation for their phenotype was that they were alleles of the *nia*D gene which were not altered sufficiently to lose catalytic function of nitrate reductase but which led to some resistance to chlorate. The mapping of *crn* mutations using diploids, however, reveals that they map on the centromere-proximal side of the *nii*A gene (i.e. distal to *nia*D). It is unlikely that they map within the *nii*A gene, since no link between chlorate toxicity and nitrite reductase has been found. They could perhaps define a control site for the *nii*A *nia*D genes, however, but this is also extremely unlikely since evidence suggests that the control sites for the *nii*A gene are on its centromere distal side (Rand & Arst, 1977; Tomsett & Cove, unpublished data). A further possibility is that they define a new gene. Since 30 % of all *nii*A *nia*D deletions are likely to delete the *crn* locus also, *crn* is probably very close and perhaps contiguous to the *nii*A gene.

The fine-structure mapping of the *nii*A *nia*D gene region, for which the studies described in this paper lay the foundation, should be of considerable use in the understanding of eukaryote gene function and, in particular, of the significance of gene clusters. The deletions characterised here have already been used to locate a *cis*-acting regulatory mutation affecting the expression of the *nii*A gene, into the region between the *nii*A and the *nia*D genes (Rand & Arst, 1977; Tomsett & Cove, unpublished data). It will also be possible to study the relationship of *nia*D gene structure to nitrate reductase structure and function. To illustrate one aspect of these studies, those *nia*D point mutations which result in a temperature-sensitive phenotype are indicated in Fig. 2. *In vitro* studies can determine whether the observed phenotype is caused by alterations in the nitrate reductase protein and hence whether the mutations occur within a region of the *nia*D gene which codes directly for the structure of the gene product, rather than being in a region coding for, for example, a leader or an internal non-coding sequence. It will be seen from Fig. 2 that point mutations resulting in a temperature-sensitive phenotype appear to be distributed non-randomly and this could be a reflexion of the distribution of sequences coding for protein structure within the *nia*D gene. However, it is not implausible that alterations in certain regions of the protein are more likely to result in a temperature-sensitive phenotype than in others, and thus at this stage it is not profitable to comment at length on the possible significance of this non-random distribution of temperature-sensitive mutations.

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