Chlorate Resistance and Nitrate Assimilation in Industrial Strains of *Penicillium chrysogenum*

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Several chlorate-resistant mutants of *Penicillium chrysogenum* were isolated and analysed; all were affected in nitrate assimilation. Nine loci were recognized by complementation analysis and these appear to be equivalent to the *niaD* (nitrate reductase structural gene), *nirA* (control locus) and seven *cnx* loci (responsible for the biosynthesis of a cofactor for nitrate reductase) of *Aspergillus nidulans*. The organization of the nitrate assimilation genes appears to be similar in both organisms, even to the extent of having contiguous genes coding for the nitrate and nitrite reductase enzymes.

INTRODUCTION

Chlorate toxicity and mutations conferring resistance have been studied in fungi (Lewis & Fincham, 1970; Cove, 1976*a*, *b*), algae (Nichols & Syrett, 1978; Huskey *et al.*, 1979) and bacteria (Stouthamer, 1967; Piéchaud *et al.*, 1967). In all these studies a loss of nitrate reductase activity was identified as one cause of chlorate resistance.

The most extensive genetical studies have been carried out with *Aspergillus nidulans* (Cove, 1976*a*, *b*). These studies suggested that the toxic effects of chlorate probably resulted from its activity as a non-metabolizable analogue of nitrate. Mutations in at least ten genes (twelve loci) in *A. nidulans* can produce a chlorate-resistant phenotype; seven of these genes (nine loci) are involved in the production of an active nitrate reductase (Cove, 1976*b*). One is the structural gene for nitrate reductase (*niaD*), one (*nirA*) is believed to code for a regulatory element needed for the synthesis of nitrate and nitrite reductase, and the remaining five (*cnxA*, *B*, *C*, *E*, *F*, *G*, *H*) are involved in the synthesis of a cofactor shared with xanthine dehydrogenase (Pateman *et al.*, 1964, 1967; Pateman & Cove, 1967; Cove, 1970, 1976*b*; Scazzocchio, 1974).

Aspergillus nidulans has often been used to study problems that have industrial significance especially in relation to penicillin production (Holt & Macdonald, 1968*a*, *b*; Edwards *et al.*, 1974; Merrick, 1975*a*, *b*; Merrick & Caten, 1975*a*, *b*; Ditchburn *et al.*, 1976; Simpson & Caten, 1979). The present paper reports a study of chlorate resistance in high-yielding strains of *Penicillium chrysogenum* which has been carried out to determine if there are any similarities in the control of nitrate assimilation in the two organisms. Such comparison could provide a useful insight into how applicable are the studies with *A. nidulans*.

METHODS

Strains. The strains of *P. chrysogenum* used in these studies were derived from Q176 (Stauffer & Backus, 1954) by serial mutation and selection for increased penicillin production.

Media and genetical techniques. The media and methods used were similar to those of Macdonald *et al.* (1963*a*, *b*, *c*) and Ball (1971) but with the following exceptions. The basal medium used to study the assimilation of different nitrogen sources was a modified Czapek–Dox medium, similar to that used by Macdonald *et al.* (1963*a*, *b*, *c*) but with NaCl replacing the $NaNO_3$. The mutants were replicated on to the test media using the methods of

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Roberts (1959). Nitrogen sources were added to the basal medium to give the following concentrations: $NaNO_3$, 12 mM; $NaNO_2$, 1·5 mM; $(NH_4)_2SO_4$, 0·8 mM; adenine hydrochloride, 0·6 mM; L-arginine, 6 mM; uric acid, 0·6 mM.

Isolation of mutants. Mutants were isolated spontaneously, or after ultraviolet light induction, by plating spores on to basal medium with 94 mM-$NaClO_3$ and a suitable nitrogen source. The strains were irradiated with ultraviolet light to give approximately 10% survival.

Classification of mutants. The mutants were grouped into four classes using two criteria – their growth responses on media containing nitrate, nitrite or adenine as sole nitrogen source, and their degree of resistance to chlorate. Adenine was used in preference to hypoxanthine (Cove, 1976*a*) because the former is more soluble and the differing growth responses were easier to distinguish.

RESULTS

Growth responses of the mutants. The four mutant classes (Cove, 1976*a*, *b*) were as follows. (1) *nia* mutants: these showed very sparse growth on nitrate, similar to that on medium without added nitrogen sources. The degree of chlorate resistance varied from mutant to mutant, some being weaker than others. (2) *cnx* mutants: these showed very sparse growth on nitrate and much reduced growth on adenine. There was some variation in chlorate resistance, as with *nia* mutants. (3) *nir* mutants: these showed more growth on nitrate than *nia* and *cnx* mutants, but less than the wild-type; growth on nitrite was reduced, but better than on nitrate. These mutants were weakly resistant to chlorate, responding like the weaker *nia* and *cnx* mutants. (4) *niinia* mutants: these were relatively rare. All gave very sparse growth on nitrate and nitrite, and a strong response on chlorate.

Molybdate repair. The *cnx* mutants were tested on medium containing 33 mM-molybdate with nitrate as sole nitrogen source. The growth of one mutant (*cnx-7*) was restored to the wild-type level on this medium.

Nitrite toxicity. The growth of the *nir* mutants was inhibited by 10 mM-nitrite even when 5 mM-urea or 10 mM-ammonium was present as an alternative nitrogen source. The *niinia* mutants responded similarly on 10 mM-nitrite and 5 mM-urea but grew more strongly on 10 mM-nitrite and 10 mM-ammonium although there was still some inhibition of growth. The *cnx-6* mutant gave an unexpected response on these two media; its growth was inhibited in the presence of ammonium but not of urea. The possibility that this strain could be a double mutant is under investigation.

Specificity effects. For these analyses, *niinia*-like mutants were grouped with *nia* mutants. There was no significant difference (χ^2 test, 95% level) in the distribution of mutant types isolated either on L-arginine or on uric acid as sole nitrogen source (Table 1). There did appear to be a difference in the distribution of types isolated before and after far ultraviolet mutagenesis (Table 2). The results in Table 2 are from several experiments independently conducted in an effort to remove any clonal distortions from the spontaneous data.

Complementation studies. The following mutants were used for complementation studies: *nia-1* to *4*, *nir-1* to *4*, *cnx-1* to *14*, and one *niinia*. 'Pair-wise' combinations of the mutants were randomly selected and tested for their complementation reaction. The results

Table 1. *Distribution of mutant types isolated on different nitrogen sources*

Mutant type	Distribution on L-arginine (%)	Distribution on uric acid (%)
nia	46	43
cnx	40	31
nir	14	26
CRUN	0	0
Total no. tested	74	63

Table 2. *Comparison of mutant types isolated before and after mutagenesis*

Mutant type	Spontaneous mutants		Induced mutants	
	No. isolated	Distribution (%)	No. isolated	Distribution (%)
nia	13	28	235	41
cnx	27	57	221	39
nir	7	15	116	20

from these tests were used to draw up a short list of possible loci. Tester strains representing the recognized loci were then used in specific combinations to yield further information (Armitt *et al.*, 1976). The results were interpreted thus:

(1) *nia* mutants. The four *nia* mutants tested did not complement with each other or with the *niinia* mutant but they did complement with the *nir* and *cnx* mutants. This suggests that there is only one locus (*niaA*) and that the *niinia* mutant carries a lesion in this locus.

(2) *nir* mutants. Four *nir* mutants were tested. They did not complement with each other but did complement with the *nia* and *cnx* mutants. This suggests that there is only one locus (*nirA*).

(3) *cnx* mutants. Some *cnx* mutants complemented with each other but others did not. On this basis they could be split into seven complementation groups: *cnxA2*, *4*; *cnxB1*, *6*, *11*; *cnxC3*; *cnxD10*; *cnxE7*; *cnxF8*; *cnxG13*. One group gave the non-standard complementation pattern observed with the *cnxA*, *cnxB*, *cnxC* loci of *A. nidulans* (Cove & Pateman, 1963; Rever, 1965). Within this group the *cnxB* mutants did not complement with the *cnxA* and *cnxC* mutants, but the last two did complement with each other.

(4) *niinia* mutants. The one *niinia* mutant failed to complement with the *nia* mutants but did complement with the *nir* and *cnx* mutants. It carried one lesion in the *niaA* gene and, presumably, another in the *nii* gene (Cove, 1976*a*).

Map location. Of the nine loci, two have been allocated to linkage groups – *niaA* on linkage group III and *cnxG* on group I of Ball's (1971) map. The *niinia* double mutant has been demonstrated to segregate as a single mitotic unit (unpublished data), supporting the hypothesis that it is similar to the deletion mutants of *A. nidulans* (Cove, 1976*a*).

DISCUSSION

Cove (1976*a*) demonstrated that the nitrogen source in the medium had a profound effect upon the spectra of chlorate-resistant mutants isolated from *A. nidulans*. Using two contrasting nitrogen sources (L-arginine and uric acid), no difference could be detected in the distribution of mutant types from *P. chrysogenum*. Cove also reported that the spectra of types differed before and after mutagenesis. This appeared to be generally true of *P. chrysogenum* but, unlike *A. nidulans*, the relative frequency of its *nia* mutants increased after mutagenesis.

The general growth responses of the *P. chrysogenum* mutants were similar to those of mutants isolated from *A. nidulans*. Four types of mutant were recognized, corresponding to the *nia*, *cnx*, *nir* and *niinia* classes (Cove 1976*a*). The main difference was the complete lack of *P. chrysogenum* mutants that were resistant to chlorate and able to utilize nitrate as sole nitrogen source (CRUN mutants; Cove, 1976*a*). The *cnxE* mutants of *A. nidulans* are believed to have lost the ability to incorporate molybdenum into the nitrate reductase/xanthine dehydrogenase cofactor. Such mutants can be repaired by incorporating high levels of molybdate in the medium (Arst *et al.*, 1970). One mutant of *P. chrysogenum*, designated *cnxE7*, behaved in an identical manner.

It has been reported that the *nirA* and *niiA* (similar to *niiAniaD*) mutants of *A. nidulans* could be distinguished by their growth responses on medium containing 20 mM-nitrite with 20 mM-ammonium or 5 mM-urea (Rand & Arst, 1977; Rand, 1978). Growth of the *nirA* mutants is inhibited in the presence of ammonium or urea but that of the *niiA* mutants only in the presence of urea. A similar response was found with the *P. chrysogenum* mutants, the only differences being that slightly different levels of nitrite, ammonium and urea were needed and that the growth of the *niinia* mutants was not fully restored by ammonium.

The results from complementation analysis were identical to those reported for *A. nidulans*: a single *nia* locus, a group of mutants carrying a lesion in the *nia* gene and probably in the *nii* gene (the behaviour of these mutants was identical to that of the mutants of *A. nidulans* which carry a deletion extending from the *niaD* gene into the adjacent *niiA* gene), a single *nir* locus and seven *cnx* loci with a group of three showing a complex complementation pattern.

The similarities in the genetical architecture of nitrate assimilation by *P. chrysogenum* and *A. nidulans* strengthen the link between these two groups of filamentous fungi. It would be interesting to see if the similarities in organization extend to other, more distantly related fungi.

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