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Genetics of nitrate reductase in *Ustilago maydis*

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SUMMARY

Mutants of *Ustilago maydis* have been isolated both deficient and derepressed for nitrate reduction. Those deficient in enzyme fall into six groups, one of which is the structural gene. Enzyme which has proved to be more labile than that of wild-type has been isolated from a temperature-sensitive mutant at this locus. All the mutants in the structural gene have xanthine dehydrogenase activity and the situation closely parallels that of *Aspergillus nidulans*.

The derepressed mutants fall into four complementation groups and all are partially derepressed in that they are further inducible by nitrate. Full derepression can be conferred by induction of a second mutation. In one analysed case the second reinforcing mutation proved to be phenotypically similar to the first one when separated from it.

1. INTRODUCTION

The utilization of nitrate is an important metabolic process and the enzymes concerned with its assimilation have been identified in a diversity of systems from micro-organisms through to higher plants (Hewitt & Afridi, 1959; Morris & Syrett, 1963; Nason & Evans, 1953; Nicholas & Nason, 1955*a*; Nicholas & Nason, 1955*b*; Pateman *et al.* 1964; Rijven, 1958; Sorger, 1966). Nitrate reductase is the first enzyme involved in this process and in all those cases which have been studied it has proved to be induced by nitrate. In view of the relative importance of the enzyme one might expect it to be under rather stringent control.

The genetic control of the enzyme has been most thoroughly examined in *Aspergillus nidulans* where Pateman *et al.* (1964) have established that at least six loci and eight complementation groups are involved. The activity of xanthine dehydrogenase is closely connected with that of nitrate reductase since mutations at all except one locus result in the loss of both activities. It was concluded that mutants able to grow on hypoxanthine, those with xanthine dehydrogenase activity, were mutant in the structural gene for nitrate reductase and the other mutants were postulated to be somehow involved in the synthesis of a hypothetical co-factor, essential for the activity of both enzymes. Later, Pateman, Rever & Cove (1965); Cove (1967); Cove & Pateman (1969) have suggested that the enzyme

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is intimately concerned with its own synthesis, postulating that when the enzyme is not bound to nitrate it acts as its own repressor or co-repressor.

The presence of a functional nitrate reductase is not the only factor concerned with the enzyme's control: constitutive production of enzyme can also be evoked by a mutation which maps very close to another locus whose mutation results in the loss of all the enzymes required for the reduction of nitrate to ammonium (Pateman & Cove, 1967*a*).

The control of nitrate reductase in *Neurospora crassa* evidently differs, for genetic and biochemical studies carried out by Sorger (1963, 1966) and Sorger & Giles (1965) suggest that only four loci are involved.

It would seem that control mechanisms in the Eucaryotes may be somewhat different from the classical systems of negative and positive control that have been identified in bacteria, and the following paper is an account of an attempt to determine the regulatory mechanisms in the basidiomycete *Ustilago maydis* using mutants deficient and derepressed in nitrate reductase activity.

2. METHODS

The genetical methods employed were essentially the same as those described by Holliday (1961*a*). Wild-type cells and strains of various types were kindly supplied by Dr Holliday.

Media. The medium was the same as that described by Holliday; ammonium minimal medium contained 3 g/l. ammonium sulphate, nitrate minimal medium contained 3 g/l. potassium nitrate and ammonium nitrate minimal contained 1·5 g/l. of ammonium nitrate.

Preparation of crude extracts. Cells grown in shaken culture in liquid medium at 30 °C. They were harvested by centrifuging the suspension for 5 min at 3000 rev/min in a M.S.E. High Speed centrifuge at 4 °C. The packed cells were resuspended in distilled water and recentrifuged, this operation being carried out twice in order to wash the cells. Extracts were made by grinding the cells with an equal volume of buffer, 0·1 M potassium in orthophosphate pH 7, and a similar amount of Ballotini (Jencons grade II). This was done in a Mickle shaker for a period of 5 min. Centrifuging at 12000 rev/min for 30 min separated the debris from the supernatant crude extract.

Enzyme assays. Nitrate reductase was assayed using essentially the same method as Nason & Evans (1953), where the nitrite produced during the reaction was measured colorimetrically. The quantities used were the same as those used by Cove (1966). The specific activity is 10^{-10}-moles of nitrite formed/mg of protein/min.

Protein determinations. Protein concentrations were estimated by a modification of the Folin method described by Lowry *et al.* (1951) using casein as a standard.

Xanthine dehydrogenase activity of the cell-free extracts was determined by the method used by Pateman *et al.* (1964), following the production of the coloured formazan derivative of neotetrazolium. The neotetrazolium reduced in the reaction acts as a terminal electron acceptor during the oxidation of the purine. The assay

system contained 1·6 mg hypoxanthine; 1·25 mg neotetrazolium and 0·5 ml crude extract in a total volume of 3 ml buffered with 0·1 M phosphate at pH 8·5. One unit of xanthine dehydrogenase activity is the amount of enzyme which produces an increase in optical density of 0·1 units in 1 min at 510 mμ. Assays were carried out using a Beckman DB recording spectrophotometer.

Isocitrate lyase activity was measured by following the change in optical density at 324 mμ on the Beckman DB spectrophotometer. The assay system contained a final concentration of phenylhydrazine HCl, 10 μM; 0·1 ml of extract; 5 μM trisodium isocitrate and 0·1 M pH 7 phosphate buffer with 1 % cysteine and 0·1 % magnesium chloride, to a total volume of 3 ml.

Isolation of mutants. Ultraviolet light and 1-methyl-3-nitro-1-nitrosoguanidine were used as mutagenic agents. The source of u.v. light was a low pressure mercury lamp emitting 1300 ergs/cm^2/sec at a target distance of 16 cm. When 10 ml of a suspension of wild-type sporidia (10^4–10^5/ml) were agitated for 60 sec under this irradiation there was a survival of about 20 %.

Nitrosoguanidine was added to an exponentially dividing culture of cells so that the final concentration was 10 μg/ml. Under these conditions exposure to the chemical for a period of 20 min gave an approximately 50 % kill.

Total isolation. Mutants lacking nitrate reductase are unable to grow on nitrate minimal medium yet can grow on ammonium minimal medium. In view of the ease with which subculturing can be effected, total isolation of mutants does not present the same difficulties as are inherent in the use of mycelial fungi for such a method. The method was essentially the same as that used by Holliday (1961*a*).

Inositol starvation. In spite of the ease with which mutants can be collected using total isolation, other methods were also tried. *Ustilago* lends itself to a number of selective techniques. Inositol starvation (Lester & Gross, 1959) has been widely used in many fungi to isolate mutants, and the availability of an inositol-requiring mutant in *Ustilago* enabled the method to be used here.

Cells of an *in-2aB* strain were incubated overnight on plates of complete medium, the freshly grown cells being harvested on the end of a spatula and spread thinly on nitrate minimal medium. The plates were then incubated for 2–3 days, after which they were supplemented with ammonium minimal medium containing inositol. Despite the specific exclusion of other mutants by this method, not all the surviving colonies proved to be nitrate or nitrite mutants, and it may well be that a longer period of incubation in the absence of inositol would enhance selection.

Selection with nystatin. Selection of mutants in bacteria has proved possible with the aid of antibiotics which actively kill dividing cells whilst not killing mutants unable to grow. Snow (1966) utilized the antibiotic Nystatin to enrich the yield of auxotrophic mutants in yeast and since this antibiotic is effective against *Ustilago* the method was tried here.

Preliminary experiments using the antibiotic at a concentration of 10 μg/ml showed that a nitrate mutant was much more resistant to the drug than wild-type when grown on nitrate minimal, and experiments were carried out in the same

manner as those of Snow. The ratio of nitrate mutants to other mutants was considerably lower than that obtained by total isolation. This is perhaps a reflexion of the rather leaky nature of the nitrate mutants which may thus be more sensitive to nystatin than other, more clear-cut auxotrophs. There was, however, an overall increase in the proportion of auxotrophs.

Isolation of depressed mutants. Prototrophic cells of *Ustilago* excrete nitrite when grown on minimal medium containing nitrate and this can be readily detected with *N*(1-naphthyl)ethylenediamine and sulphanilamide as in the assay procedure. Colonies which are derepressed for the enzyme might be expected to produce more nitrite or to produce nitrite under conditions when the enzyme would be normally repressed. This was found to be the case.

Cells which had been treated with a mutagen were plated on ammonium minimal medium (whose ammonium sulphate contains 0·005 % nitrate), and after colonies had developed the plates were overlaid with 1 % molten agar containing 0·05 % sulphanilamide in N-HCl and 0·1 % NED. (Chilling the plates prior to NED and sulphanilamide treatment caused the overlay to set more rapidly and the distinction between the colours of the various colonies was considerably improved.)

It was also found possible to isolate nitrate mutants by this method, since when grown on ammonium nitrate medium *nar*$^-$ mutant colonies remained white after the overlay treatment.

3. RESULTS

Classification of the nitrate mutants

In *A. nidulans* Pateman *et al.* (1964) has established that mutants in one gene, taken to be the structural gene for nitrate reductase, are able to grow on medium containing hypoxanthine as a nitrogen source whilst other nitrate mutants cannot. Those nitrate mutants of *Ustilago* able to use hypoxanthine are shown in Fig. 2; nitrate minimal medium was supplemented with hypoxanthine at a concentration of 1 mg/ml. The following evidence suggests that these mutants are indeed mutant in the structural gene for nitrate reductase.

Temperature-sensitive mutant

A temperature-sensitive mutant, unable to grow on nitrate and able to grow on hypoxanthine at 30° C but prototrophic at 25 °C was isolated and kindly donated by Dr R. Holliday. It seemed probable, should this locus represent the structural gene, that an altered enzyme was responsible for the temperature-sensitivity of the mutant: an enzyme which, in fact, was sufficiently stable at 25 °C *in vivo* to render growth on nitrate minimal possible but which at 30 °C was too labile to be useful.

Crude extracts were prepared from both wild-type and mutants cells which had been induced by nitrate at room temperature, and the extracts were compared for lability of enzyme at 25, 30 and 35 °C. Fig. 1 shows the results and provides almost conclusive evidence that this locus in *Ustilago* is that of the structural gene for nitrate reductase.

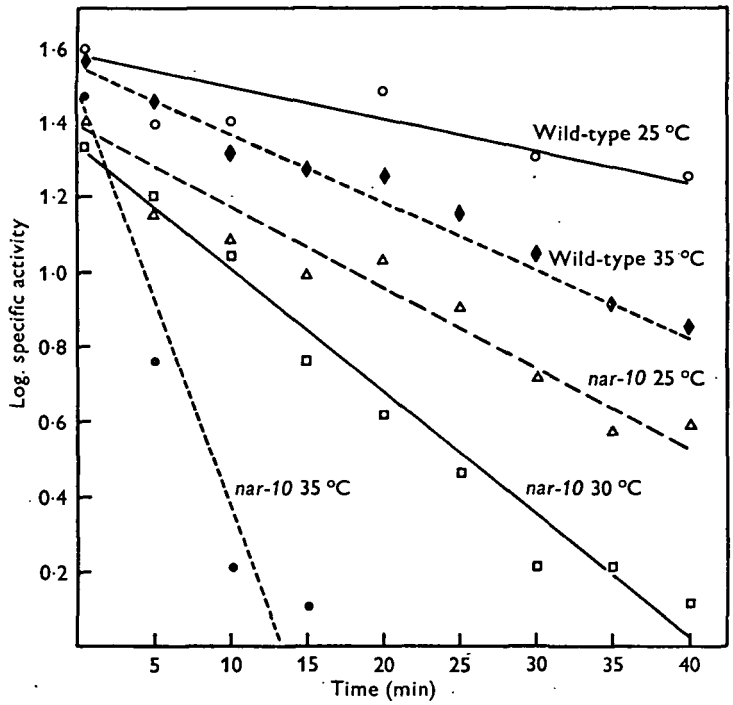

Fig. 1. Lability of nitrate reductase enzyme in crude extracts of wild-type and *nar*-10 at different temperatures. The preparation of crude extracts and measurements of enzyme activity were done as described under Methods. Protein concentrations were adjusted to 5 mg/ml. prior to assay.

Complementation tests

Fig. 2 shows the complementation patterns of the nitrate mutants. Complementation was effected by forcing diploids. Although the table is incomplete, the available data allow the establishment of five further groups, a situation which appears closely to parallel that in *A. nidulans* where six loci are also concerned with nitrate reduction.

Response to nitrite

When routine tests were being carried out for the maintenance of stocks, a number of nitrate mutants were found to be unable to grow on minimal medium supplemented with 0·1 % sodium nitrite. These were, in fact, mutants which had been collected using the colour method where the characteristics of no growth on nitrate minimal and the absence of excreted nitrite were taken as criteria of *nar*$^-$ mutants. In other methods for the selection of the nitrate mutants, care had been taken to exclude all mutants which did not grow on nitrite, since many of these possess functional nitrate reductase and excrete nitrite in large quantities.

Moreover, these apparently doubly deficient mutants were the sole occupants of one complementation group, C, and are apparently similar to the recently reported *niiB* mutants of Pateman, Rever & Cove (1967*b*). Cove (1969) has more

recently termed these *nir*⁻ mutants. Perhaps, as in *Aspergillus*, this locus of *Ustilago* is responsible for some component of the regulatory system of the nitrate reductase pathway.

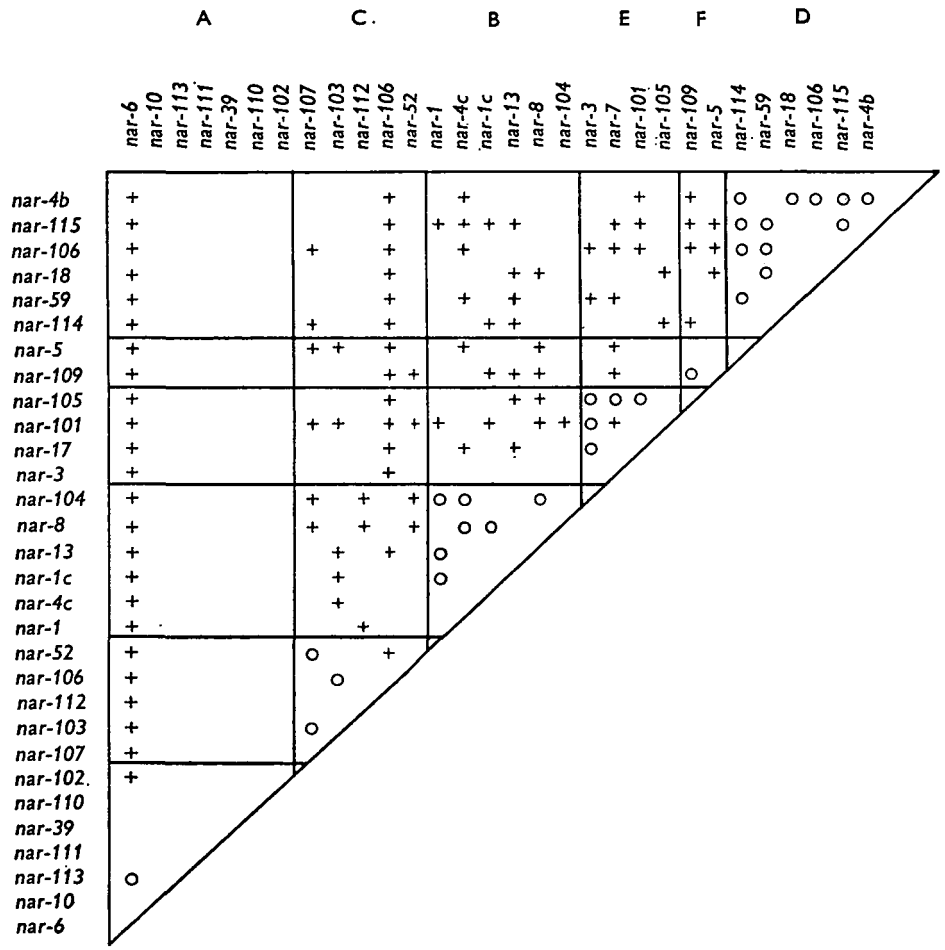

Fig. 2. Complementation of nitrate mutants. + = complementation; ○ = no complementation.

Chlorate resistance

Azoulay, Puig & Pichinoty (1967) selected mutants in *Escherichia coli* which were resistant to chlorate and which showed alterations in respiratory particles. The mutants showed deficiency for a number of enzyme activities, nitrate reductase being one. Pichinoty & Metenier (1966) had previously shown that nitrate reductase of the assimilatory type was capable of reducing chlorate to chlorite which, since this was more toxic than the chlorate, inhibited the growth of wild-type. In view of this it seemed reasonable to suppose that nitrate mutants might be resistant to chlorate, since they would lack the ability

to reduce the compound to the more toxic chlorite. On minimal medium containing 0·1 M potassium chlorate and 0·25 % hydrolysed casein, wild-type growth was inhibited but all the nitrate mutants without exception divided actively. This would provide an easy method of selecting mutants deficient in nitrate reductase.

Isolation of mutants derepressed for nitrate reductase

The colour method was used for the isolation of these mutants, initially by growing the colonies on medium containing ammonium nitrate. Despite screening large numbers of colonies, no derepression was at first detected, those colonies which appeared red after overlayering with the NED and sulphanilamide in agar were simply nitrite-excreting colonies, and did not have higher basal levels of nitrate reductase when grown on medium containing ammonium sulphate as sole source of nitrogen. However, some of the isolated strains did have slightly different properties, one being inducible by nitrate to a level higher than that of wild-type whilst another appeared to have a more readily inducible nitrate reductase.

Further scanning of the colonies was continued, and this led to the isolation of a mutant, *c-1*, which had a level of nitrate reductase appreciably higher than that of wild-type when grown on ammonium minimal medium. A closer examination of the properties of this mutant enabled further derepressed mutants to be isolated more easily. Thus, it was found that the mutant appeared pink when overlaid following growth on ammonium minimal, due, presumably, to the reduction of trace amounts of nitrate in the ammonium sulphate (0·005 %). This provided a clearer differentiation from wild-type and led to the collection of further derepressed mutants on ammonium minimal medium.

Classification of derepressed mutants

Fig. 3 shows the complementation patterns of the derepressed mutants, the complementation being effected by forcing diploids. Diploids were tested for derepression by overlaying on ammonium minimal medium.

All the derepressed mutants proved to be recessive in that combination of each with a non-allelic mutant resulted in the loss of the derepressed character.

Levels of activity of the derepressed mutants

Table 1 shows the levels of enzyme activities of the derepressed mutants on nitrate minimal, ammonium sulphate minimal and ammonium chloride minimal, and these vary considerably within, as well as between, groups so that a particular level of activity cannot be ascribed to any one group.

It was noted whilst these tests were being carried out that the mutant *c-8* did not grow on nitrate minimal yet nitrate reductase could be fully induced by the nitrate. In this respect the mutant differed from all the others.

Resistance to methylamine

Arst & Cove (1969) isolated mutants in *Aspergillus* by selecting for mutants which were resistant to methylamine. The mutants showed derepressed nitrate

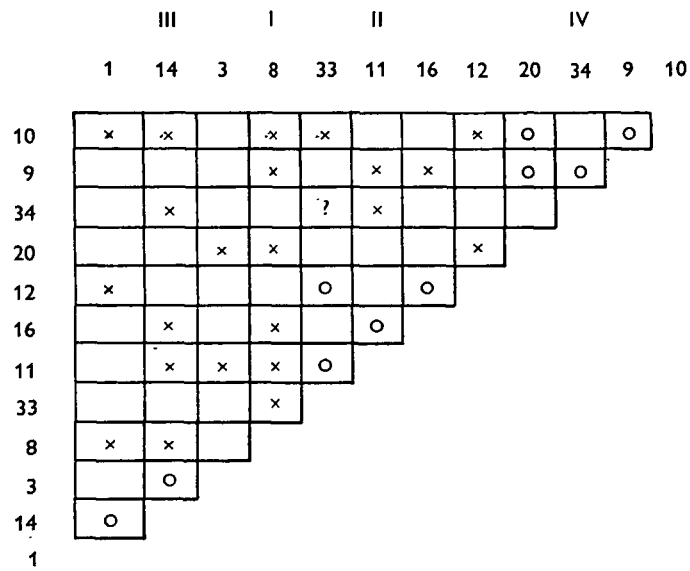

Fig. 3. Table showing the groups of derepressed mutants classed according to their complementation. Diploids were forced as described by Holliday. When colonies grown on ammonium minimal medium were overlaid with NED and sulphanilamide, non-complementary mutants turned pink, complementary mutants remained white. × = complementation of mutants; ○ = non-complementation.

Table 1. *Levels of various enzyme activities in crude extracts of cells of derepressed mutants grown on various nitrogen sources*

(The levels of activity of enzyme unrelated to nitrate reductase are also given. The units are defined under Methods)

	Xanthine dehydrogenase activity, uninduced	Isocitrate lyase activity, uninduced	Nitrate reductase activity on			Growth on nitrate minimal medium with 10^{-2} M Methylamine
			29 mM-NO_3	23 mM $(NH_4)_2SO_4$	56 mM-NH_4Cl	
c-1	0	2	54	22	25	+
c-14	0	4	49	24	21	+
c-3	0	6	52	19	21	+
c-8	0	9	43	9	10	+
c-33	0	1	44	20	19	—
c-11	0	2	50	18	14	—
c-16	0	1	45	7	7	—
c-12	0	2	53	28	21	—
c-20	0	6	55	11	12	—
c-34	0	1	51	14	16	—
c-9	0	1	59	18	14	—
c-10	0	2	46	23	9	—
	Induced					
Wild-type	8	32	56	0	0	—

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reductase synthesis in the presence of ammonium and they also proved to be derepressed for a number of other enzymes, nitrite and hydroxylamine reductases, xanthine dehydrogenase, and three independent systems concerned with the uptake of hypoxanthine, uric acid and allantoin. The derepressed mutants of *Ustilago* were therefore tested for their resistance to methylamine; the results are shown in Table 1. Some were indeed resistant to the compound but this was not a universal characteristic.

Yudkin (1966) had reported that a longer-lived messenger was concerned with constitutive production of penicillinase and it seemed possible that any effect on messenger stability might be a general one, derepression being conferred on a number of inducible enzymes by some alteration which would result in a longer life for all short-lived messengers. As Table 1 shows, the levels of other inducible enzymes, isocitrate lyase and xanthine dehydrogenase, were not in any way elevated and it would seem that the derepression in this case was confined to the nitrate reduction pathway.

Doubly derepressed mutants

All these mutants, listed in Fig. 3, had a significant but low level of nitrate reductase activity on ammonium minimal medium which could be increased in nitrate minimal to a level approaching that of wild-type. It seemed possible that

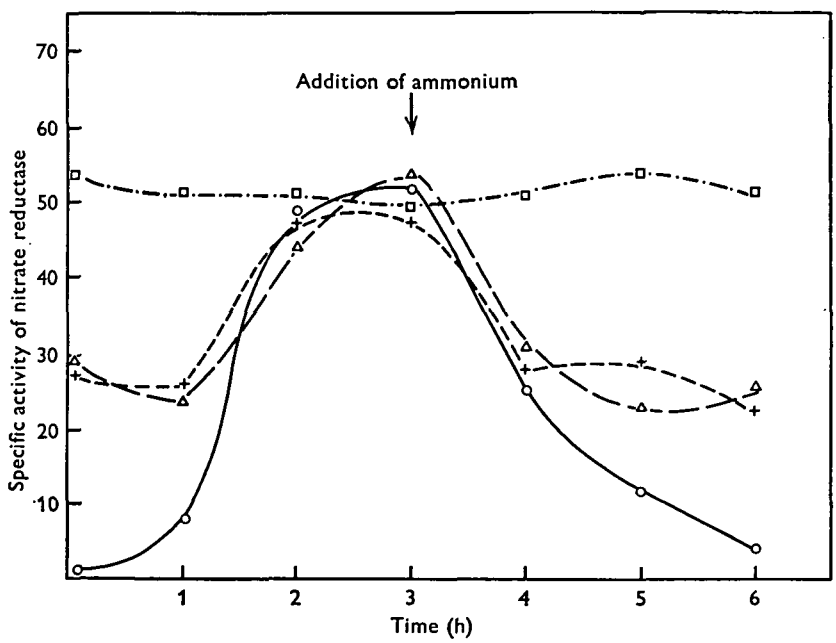

Fig. 4. Induction by nitrate and repression by ammonium of nitrate reductase in four different strains isolated from a tetratype tetrad of cross $+ + \times C_1 C_{22}$.

Cells were initially grown on ammonium minimal medium and after washing transferred to nitrate minimal medium. 3 g/l. of solid ammonium sulphate was added to the induced culture after 3 hr.

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further mutation of one such strain might produce a strain which was more fully derepressed. This proved to be the case. Cells of the *c-1* strain, initially isolated following u.v.-treatment, were subjected to further mutagenesis with nitrosoguanidine, plated and overlaid as described in the Methods. Colonies which had a more dense red colour than the original mutant were picked off. Many of these proved to have a very high level of nitrate reductase when grown on ammonium minimal.

In order to establish whether the fully derepressed nature of these mutants was the result of a further mutation at the same locus or a second mutation at a different locus, one, apparently derepressed, was crossed with wild-type. The types of tetrad could be distinguished easily, non-parental ditype, all pink colonies; parental ditype with red and white colonies and tetratype tetrads with red, pink and white colonies. Ten Parental ditype, nine non-parental ditype and 26 tetratype tetrads were isolated indicating that there is no linkage between two apparently identical partially derepressed mutants which have cumulative effects. The induction and repression patterns of the four strains isolated from a tetratype tetrad are shown in Fig. 4 and there is apparently no difference between the two single mutant strains.

4. DISCUSSION

Mutants of *Ustilago* lacking nitrate reductase fall into six groups, one of which probably represents the structural gene *nar-A*. Nitrate reductase extracted from a temperature-sensitive mutant at this locus was more temperature-sensitive in crude extracts than the wild-type enzyme. Again, mutants at this locus are unable to utilize hypoxanthine and are therefore similar to those mutants of *Aspergillus* which lack both inducible nitrate reductase and inducible cytochrome-*c* reductase. The weight of evidence from both *Aspergillus* and *Neurospora* now indicates that these two activities are functions of the same nitrate reductase protein.

Another group of mutants of *Ustilago*, *nar-C*, resemble those termed *niiB* by Pateman *et al.* (1967*b*) in that they are unable to grow on either nitrite or nitrate. In *Aspergillus* these mutants lack nitrate, nitrite and hydroxylamine reductases, and a number of mechanisms were proposed whereby a single mutation could result in the loss of three activities. The most likely explanation, however, was that the gene product was a regulator protein whose alteration affected the levels of both enzymes simultaneously.

Another idea which they considered was the postulate that the reduction of nitrate to ammonium was effected on a single molecule consisting of an aggregate of polypeptides. This postulate may be the key to the whole question in *Ustilago*. Bussey (1966) has suggested that the reduction of nitrate to ammonium was effected by a 'nitrosome' and, more recently, Showe & De Moss (1968) have established that the nitrate reductase of *E. coli* was localized to a particulate fraction of the cell, less than 1·5% of the total nitrate reductase occurring in a soluble form. It is possible that a similar situation exists in *Ustilago*, the *nar* gene loci being directly concerned not with nitrate reductase but with the stability of an aggregate or nitrosome.

The isolation of the derepressed mutants and the examination of their properties has shed very little light on the mechanisms of regulation of nitrate reductase in *Ustilago*. Biochemical evidence (Lewis & Fincham, 1970) suggests that ammonium ions either alter the confirmation of, or result in the induction of a protein which both represses and inactivates nitrate reductase. At the present time it seems impossible to correlate the biochemical evidence with the four, and possibly more, genetic loci which are concerned with the repression of the enzyme.

The following are suggested reasons for the derepression which was observed with these mutants. One mutant, *c-8*, was unable to grow on nitrate minimal although having an appreciable level of derepressed nitrate reductase activity. Complementation tests on nitrate minimal show that *c-8* falls in the *nar-E* group. It is possible, although by no means proven, that this locus is responsible for the synthesis of a repressor protein. *Nar-E* mutants could be the result of a mutant form of this protein which was unresponsive to nitrate and which would constantly repress the formation of nitrate reductase. On the other hand, a mutation in the same region could result in the synthesis of an altered repressor protein with a different sensitivity to nitrate and ammonium and which would allow some synthesis in the absence of nitrate. The *c-8* mutant may be one such example, although the fact that this *c-8* mutant will not grow on nitrate still needs explaining. The following is a possible explanation. The regulation of nitrate reductase may not be the only function of the *nar-E* locus. The protein product may play some role in the biosynthesis of amino acids or other molecules from ammonium: a not unlikely function. If this were so then all the aspects of mutants at this locus are explicable. The level of the *c-8* protein on ammonium minimal medium might be expected to be at its highest (if it is induced by ammonium) and if the molecule were only slightly altered then even a low level of enzyme activity might be sufficient to support growth. When the mutant is grown on nitrate minimal the level of the mutant protein might be lower and just insufficient to support growth.

The other three groups of derepressed mutants are obviously somewhat different. Those of group III may owe their derepression simply to an altered assimilation of ammonium, since these are resistant to methylamine. This possibility could be tested. The reasons for the derepression of the other two groups remain obscure. It is possible that they are mutations which affect the rate of protein turnover and hence the steady-state levels of nitrate reductase or some regulator protein.

On the other hand, it is possible that the various *c*-mutants all contribute in some way to the level of repressor. Histidinyl-tRNA has been implicated as co-repressor in the histidine operon in *Salmonella* (Fink & Roth, 1968), there being two genes which normally contribute to its level in the wild-type. It is possible that a similar situation exists in *Ustilago*, the *c*-genes being in reality glutaminyl-tRNA genes or some other amino acyl tRNA whose amino acid moiety is closely related to ammonium. There could be many copies of the gene for this tRNA and it might be necessary to mutate at least two of them to reduce the level to the point where nitrate reductase was fully derepressed.

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