

## Ammonium Repression of Extracellular Protease in *Aspergillus nidulans*

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### SUMMARY

Extracellular protease of *Aspergillus nidulans* is repressible by ammonium. A mutant (*xprDI*) selected for derepression of protease in the presence of ammonium is also derepressed for nitrate reductase, xanthine dehydrogenase and glutamate uptake; it retains the ability to use ammonium as sole nitrogen source. The mutant is partially dominant in heterozygous diploids. The mutation does not confer methylamine resistance, nor are methylamine-resistant (*meaA*) strains derepressed for protease.

### INTRODUCTION

The formation of extracellular proteases by fungi has been reported frequently, and in many cases the enzymes have been purified and characterized, often being of some significance in an industrial process (Matsubara & Feder, 1971; Nakagawa, 1971). In the course of such investigations it has often been observed that enzyme levels are reduced when cultures are grown on rich media or those containing nitrate or ammonium (e.g. Matile, 1965), whereas nutrient depletion and stationary-phase conditions increase enzyme yields. In *Aspergillus niger*, Tomonaga, Ohama & Yanagita (1964) have shown that depletion of sulphur compounds specifically derepresses synthesis of extracellular acid proteases. Interpreted in terms of the repression of enzyme synthesis (or release) by nitrogenous and sulphur-containing metabolites, these observations are immediately recognizable as reflecting what may be an ecologically valuable adaptation for a free-living fungus; hydrolysis of exogenous proteins can supply both nitrogen and sulphur when low molecular weight nutrients are scarce. The biochemical and genetic basis of the regulation of extracellular protease has not, however, been investigated in any detail.

The release of some extracellular enzymes has been studied in *Saccharomyces cerevisiae* and its protoplasts, but this organism does not produce protease (Lampen, 1968). In *Neurospora crassa* (Matile, 1965; Matile, Jost & Moor, 1965), however, it has been shown that extracellular protease is located in lysosome-like cytoplasmic particles which are liberated to the exterior by a process of reverse pinocytosis.

Despite the wide interest of these systems and their possible bearing on the regulation of enzyme synthesis, the control of cell secretion processes and the relations between intra- and extracellular enzymes, the present study appears to be the first in which protease release is studied in relation to its regulation by specific metabolites and genes. In this paper it is shown that, in common with other nitrogen-assimilatory enzymes, the extracellular protease of *Aspergillus nidulans* is repressible by ammonium. A mutant (*xprDI*) insensitive to ammonium repression, but still able to utilize ammonium, has been isolated and is shown to be partially dominant. In view of this and of the fact that strains carrying this mutation are also derepressed for a wide variety of other ammonium-repressible functions, it seems likely that

this mutation affects a control mechanism. It has previously been shown that ammonium repression of many functions in *A. nidulans* can be abolished by mutations at the *meaA* locus which simultaneously confer methylamine-resistance (Arst & Cove, 1969). The *xprD* mutation is not allelic with *meaA* and does not confer methylamine-resistance, nor do *meaA* mutations derepress extracellular protease. The results do not permit any definite conclusion as to the role of the *xprD* gene product but suggest strongly that it is involved (probably very centrally) in ammonium repression.

It should be noted that the results described are based simply upon observation of extracellular proteolysis. It is not known, therefore, whether the control process revealed operates on synthesis, activation or release of protease, or on some combination of these functions.

#### METHODS

*General techniques, media and strains.* General techniques for *Aspergillus nidulans* are those described by Pontecorvo, Roper, Hemmons, MacDonald & Bufton (1953). Minimal medium (MM) was as described by Pontecorvo *et al.* (1953). Minus-nitrate medium (-N) is MM without sodium nitrate and solidified with Difco Bacto Agar. The modified -N medium (JAP-N) used for methylamine-resistance tests is described by Cove (1966).

Strains were from the Glasgow and Cambridge stock cultures; Cambridge strains were supplied by courtesy of Professor J. A. Pateman and Dr H. N. Arst.

*Plate tests for protease.* Medium containing skimmed milk can conveniently be used for the detection of proteolytic enzymes (Gorini, 1934; cited in Gorini & Fromageot, 1949) and was found to be suitable for *Aspergillus* by Bufton in 1950 (G. Pontecorvo, personal communication). Oxoid skimmed milk, stock solution 10% (w/v) is added to -N medium to give a final concentration approximately 0.1% (w/v). Distinct zones of milk clearing develop under and around colonies during the course of growth, and phenotypic classification is usually made after 48 h at 37 °C.

That the zones of milk clearing are indeed the result of proteolytic activity is demonstrated by the failure of medium within the zones to bind protein stains such as Naphthalene Black 10B in acetic-methanol solution.

Ammonium repression of protease release is demonstrated by growth on -N milk medium to which ammonium tartrate has been added to a final concentration of 5 to 10 mM-ammonium.

*Plate tests for nitrate reductase and xanthine dehydrogenase derepression.* Following Arst & Cove (1969), failure of growth on medium containing 5 to 10 mM-ammonium in the presence of 100 mM-KClO<sub>3</sub> is taken as evidence for derepression of nitrate reductase.

Yellowing of the conidiospores of a green-spored strain when grown in the presence of 5 to 10 mM-ammonium and 0.5 mM-2-thioxanthine is taken as evidence for derepression of xanthine dehydrogenase.

*Plate test for methylamine-resistance.* Wild-type strains will not grow on JAP-N medium + 100 mM-methylammonium chloride on which *meaA* strains grow well (Arst & Cove, 1969).

*Isolation of protease-derepressed mutants.* Mutations were induced by ultraviolet light irradiation of translocation-free *pabaA1*, *biA1*; *tryC801*, *hisC38* conidia to give approximately 10% survival. Treated conidia were held in the dark for 1 h at 6 °C and then spread on -N milk medium supplemented with the nutrients required by the strain and with the addition of 10 mM-ammonium. Sodium deoxycholate was also added to the medium (0.09%, w/v) to induce microcolony formation (Mackintosh & Pritchard, 1963), enabling about 300 viable spores to be spread on each 90 mm Petri dish.

Colonies with a distinct zone of milk clearing were picked after 36 to 40 h of growth at 37 °C. Although the procedure was cumbersome, three mutants were recovered from  $3 \times 10^5$  colonies tested but only one (*xpr807(10)-4*) was sufficiently vigorous in habit to be retained for further study.

*Genetic analysis.* The mutant strain *xpr807(10)-4* was crossed with *pabaA1*, *yA2*; *pyroA4* (translocation-free) and *his*<sup>+</sup>, *try*<sup>+</sup> recombinants were isolated. The mutant phenotype was recovered in approximately one half of the progeny and all further work made use of such recombinant strains.

Haploidization of the diploid between *pabaA1*, *biA1*; *xpr807(10)-4* and master strain E by the method of McCully & Forbes (1965) yielded segregants which showed free assortment between *xpr* and all markers except *galA1* in linkage group III. This mutation therefore defines a locus *xprD*, allele *xprD1* in this linkage group.

Analysis of a single perithecium from the cross: *yA2*, *wA3*; *methH3*, *argB2*, *galA1* × *pabaA1*, *biA1*; *xprD1* located *xprD1*  $17 \pm 2$  map units to the left of *methH* (45 recombinants amongst 264 progeny tested).

A heterozygous diploid strain (*xprD1*/+) to test the mutant for dominance was selected from the heterokaryon *pabaA1*, *biA1*; *xprD1* + *yA2*; *riboA2*; *nicB8*. Diploidy was confirmed by measurement of conidial diameter.

## RESULTS

### *Extracellular protease of wild-type strains*

The liberation of protease by wild-type strains occurs under conditions of nitrogen-deprivation, e.g. on -N milk medium (Fig. 1). Protease is repressed when small molecular weight nitrogen sources are added to -N milk medium. When ammonium, nitrate or methylamine are added the repression is detectable as a reduction in the sharpness and extent of the milk-clearing halo at initial concentrations greater than 2 mM, and is complete at 5 to 10 mM (Fig. 2). When urea is added repression is evident at 5 to 10 mM but is not complete below about 20 mM.

Wells cut in milk agar plates with or without added ammonium, nitrate, urea or methylamine (10 mM) were filled with culture filtrates of wild-type and *xprD1* strains and incubated at 37 °C. Milk clearing of equal sharpness and extent was observed in all plates, from which it was concluded that the reduction in milk clearing that follows growth in the presence of these nitrogen sources is due to repression rather than inhibition of enzyme activity.

### *Extracellular protease of xprD1 haploid and diploid strains*

The recombinant *xprD1* strains produce a very strong zone of milk clearing on -N milk medium. This halo is clearly visible after 20 to 24 h of incubation, and is very pronounced after 48 h (Fig. 1).

Utilization of ammonium, nitrate, nitrite, methylamine and urea is normal, although sporulation is weak on nitrate. Protease is not repressed when 100 mM-ammonium, -nitrate or -urea or 50 mM-methylamine are present in -N milk medium during growth.

The milk clearing zone produced by the heterozygous diploid *xprD1*/+ on -N milk medium is less strong than that produced by haploid *xprD1* strains (Fig. 2). Protease of the diploid is not repressed by 100 mM-ammonium or -nitrate nor by 50 mM-methylamine, but it is repressed by 100 mM-urea. The mutation is therefore partially dominant.

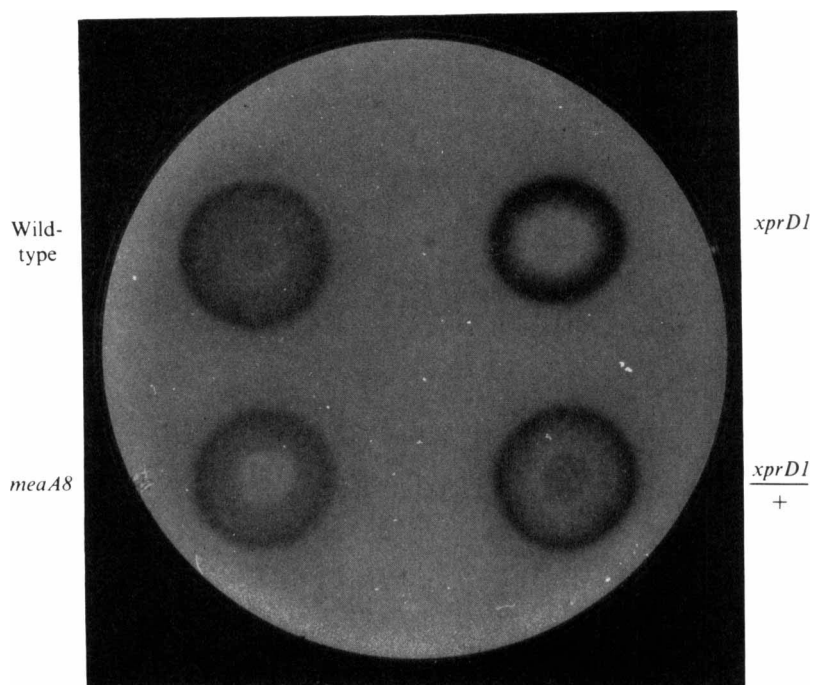


Fig. 1. Protease production by wild-type and mutant strains of *Aspergillus nidulans* grown on  $-N$  milk medium. All strains show derepression of protease.

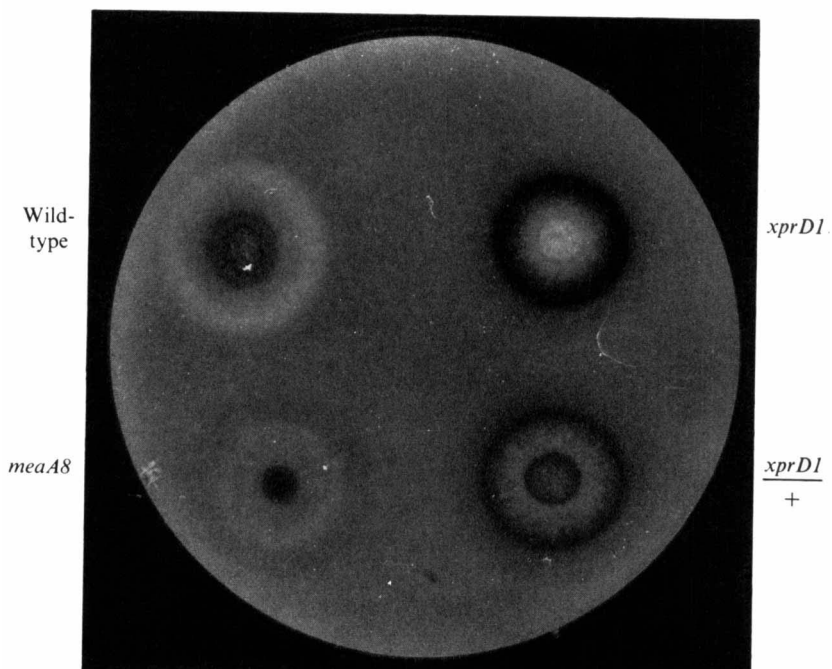


Fig. 2. Protease production by wild-type and mutant strains of *Aspergillus nidulans* grown on 10 mM-ammonium milk medium. *xprD1* and diploid *xprD1/+* strains do not show ammonium repression of protease.

*Extracellular protease of mea<sup>R</sup> strains*

Like wild-type, the methylamine-resistant strains *meaA1*, *meaA2*, *meaA6* and *meaA8* are derepressed for protease on -N milk medium and repressed by ammonium, nitrate, urea and methylamine (Fig. 1, 2). Within the limits of accuracy of the plate tests used, *mea-1*, -2 and -8 are as sensitive as wild-type in this respect, but *mea-6* is somewhat less sensitive to ammonium and nitrate repression and also confers less resistance to methylamine toxicity than do the other alleles. Arst & Cove (1969) also noted that *mea-6* was atypical\*, being least effective in derepressing synthesis of nitrite reductase and xanthine dehydrogenase.

*Derepression of synthesis of other enzymes in xprDI strains*

Many of the enzymes simultaneously repressed by ammonium in *Aspergillus nidulans* participate either in the nitrate reduction or purine oxidation pathways. To test for derepression of these enzymes one in each pathway was selected on the grounds that a plate test was available.

(1) *Nitrate reductase*. In the wild-type, this enzyme is induced by nitrate and repressed by ammonium; it can catalyse the reduction of chlorate to chlorite which is toxic. On 5 to 10 mM-ammonium synthesis of nitrate reductase is repressed to very low levels, so that in the presence of 10 mM-ammonium and 100 mM-chlorate wild-type strains grow normally (Arst & Cove, 1969).

Under the same conditions, both *xprDI* and *mea<sup>R</sup>* haploids are unable to grow, indicating derepressed synthesis of nitrate reductase. The diploid *xprDI/+* can grow on ammonium plus chlorate, though not as well as a diploid homozygous for the wild-type allele at this locus. Thus *xprDI* is partially dominant for the derepression of nitrate reductase.

(2) *Xanthine dehydrogenase*. Growth in the presence of 2-thioxanthine leads to yellowing of the conidiospore pigment of genotypically green-spored strains if the enzyme xanthine dehydrogenase is also present at derepressed levels. In the presence of 10 mM-ammonium, synthesis of this enzyme is repressed in wild-type strains. Thus when grown in the presence of 10 mM-ammonium and 0.05 mM-2-thioxanthine, genotypically green-spored wild-type strains remain green, whereas genotypically green-spored *mea<sup>R</sup>* strains (which have derepressed xanthine dehydrogenase) develop yellow spore pigment (Arst & Cove, 1969).

Under the same conditions, *xprDI* strains develop yellow conidiospores, despite ammonium concentrations up to 100 mM, whereas with the *xprDI/+* diploid the spores are yellow at 10 mM but green at higher concentrations. Thus the *xprDI* mutation causes derepression of xanthine dehydrogenase and derepression is partially dominant.

*Derepression of glutamate uptake*

Recent studies (J. A. Pateman, personal communication) have established that the uptake of glutamate is ammonium-repressible in *Aspergillus nidulans*. Under conditions where wild-type strains are fully repressed, *xprDI* and *xprDI/+* are both fully derepressed for glutamate uptake. Thus *xprDI* is dominant for this function.

*Methylamine sensitivity of xprDI strains*

Wild-type strains are able to grow well on methylamine as sole nitrogen source at concentrations below about 10 mM, but growth is strongly inhibited at 100 mM. At this inhibitory concentration *mea<sup>R</sup>* strains grow well but *xprDI* haploids and the heterozygous diploid (*xprDI/+*) do not grow.

\* Note added in proof: *meaA6* has been found to recombine freely with *meaA8* and is now designated *meaB6* (H. N. Arst, personal communication).

Thus the ammonium-derepression brought about by the *xpr* mutation is not accompanied by resistance to methylamine toxicity.

#### DISCUSSION

In *Aspergillus nidulans*, ammonium has been found to cause the simultaneous repression of many intracellular enzymes concerned with the metabolism of nitrogenous substances (Cove, 1966; Pateman & Cove, 1967; Pateman, Rever & Cove, 1967; Scazzocchio & Darlington, 1968; Cove & Pateman, 1969; Cove, 1970). A feature common to all these examples is that ammonium repression is relieved by mutations that confer resistance to the toxic effects of the ammonium analogue methylamine. All such mutations so far known occur at a single locus, designated *meaA*, which has been interpreted as specifying a macromolecular control element of a general ammonium-repression system (Arst & Cove, 1969).

From the results described here it may be concluded that the ammonium repression mechanism affected by *meaA* mutations, whilst having widespread effects on uptake and intracellular enzyme syntheses, does not control extracellular protease. The ammonium repression mechanism affected by the *xprD* mutation, however, controls both extracellular protease, glutamate uptake and the intracellular enzymes (at least the two so far tested) repressed by the *meaA*-dependent system.

Further interpretation of the results must await investigation into the possibility that the apparent derepression of protease is not due to increased enzyme synthesis. With this reservation in mind the results can be interpreted as indicating that general ammonium repression may be dependent on the concentration of the *xprD* gene product, acting alone in the case of extracellular protease, but after interaction with (or sequential action by) the *meaA* gene or its product in the cases of uptake and intracellular enzyme syntheses.

Ammonium repression can also be relieved by mutations affecting ammonium transport (J. A. Pateman, personal communication) but the alternative hypothesis that *xprDI* causes extreme reduction of the intracellular ammonium pool can probably be rejected since mutant strains grow and sporulate on limiting ammonium almost as well as wild-type strains.

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