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Isolation and characterization of *Aphanocladium album* chitinase-overproducing mutants

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A wild-type strain of the fungus *Aphanocladium album* was mutagenized by UV irradiation in order to obtain chitinase-overproducing mutants. Mutants were screened on agar medium containing colloidal chitin and selected for their ability to produce large clearing zones around the colonies. Two mutant strains, designated E3 and E12, showed respectively a 26- and a 2·5-fold increase in maximal extracellular chitinase activity, determined in liquid medium with crystalline chitin as sole carbon source, compared to the wild-type strain. This is believed to be the first report on the induction of stable chitinase-overproducing mutants in a filamentous fungus. Regulation of enzyme activity was investigated in mutant E3 and the wild-type strain.

Introduction

Chitin, an unbranched polysaccharide composed primarily of β-1→4-linked *N*-acetyl-D-glucosamine residues, is hydrolysed to *N*-acetyl-D-glucosamine (GlcNAc) by two separate hydrolases: an endochitinase (EC 3.2.1.14) which produces soluble low-molecular-mass multimers of GlcNAc, the dimer *N,N'*-diacetylchitobiose being predominant, and chitobiase (EC 3.2.1.30), which hydrolyses the intermediates to GlcNAc. In the literature both enzymes are often grouped under the name chitinase (Jeuniaux, 1963; Monreal & Reese, 1969; Reid & Ogrydziak, 1981). Chitinases have been detected in a variety of organisms: bacteria, fungi, plants, insects and vertebrates (for reviews see Boller, 1986; Gooday, 1986). These enzymes are known to perform several biological functions: together with chitin synthases (EC 2.4.1.16) they are required for the morphogenesis of fungal and arthropod cell walls. They may play a nutritional role in the case of soil saprophytes such as *Aspergillus* (Monreal & Reese, 1969) and *Trichoderma*. In the case of pathogens of crustacea, insects and fungi, chitinase production allows them to penetrate their hosts. Moreover, these enzymes are produced in large quantities by actinomycetes and streptomycetes and presumably contribute to soil antibiosis (Sneh, 1981). Plant chitinase is involved in defence reactions against fungal pathogens, either through a direct inhibitory effect (cell wall lysis) (Schlumbaum *et al.*, 1986; Roberts & Selitrennikoff, 1988), or via an indirect effect (release of fungal cell wall elicitors) (Keen & Yoshikawa, 1983; Hadwiger *et al.*, 1986). During the last decade, chitinases have received increasing attention due to their potentially wide range of applications (Cosio *et al.*, 1982; Jones *et al.*, 1986). One use of chitinase is the digestion of the chitin component of many agronomic pests, including insects, fungi and nematodes. The enzyme could be used directly in biological control via a micro-organism (Ordentlich *et al.*, 1987) or indirectly, using the purified protein or the gene encoding the chitinase (introduction of chitinase gene into plants or bacteria) (Lund *et al.*, 1989; Shapira *et al.*, 1989).

A survey of micro-organisms able to degrade crystalline chitin (native form) showed that the bacteria *Serratia marcescens* and *Enterobacter liquefaciens* produced almost 10-fold more chitinase than the second-best producer, the fungus *Aspergillus fumigatus* (Monreal & Reese, 1969). *S. marcescens* and other bacteria such as *Vibrio vulnificus* and *V. harveyi* appeared to be very promising tools for the production of large amounts of chitinase (Soto-Gil & Zyskind, 1984; Fuchs *et al.*, 1986; Wortman *et al.*, 1986). Different studies have since been initiated on *Serratia marcescens* with subsequent genetic and biochemical investigations carried out in order to

Abbreviation: GlcNAc, *N*-acetyl-D-glucosamine.

better characterize chitinase and to optimize the level of enzyme production (Tom & Carroad, 1981; Roberts & Cabib, 1982; Young *et al.*, 1985*a*, *b*). Reid & Ogrydziak (1981) reported the isolation of chitinase-overproducing mutants of *S. marcescens*, but the mutants were unstable, presumably because the mutation was a tandem gene duplication.

Fungi have so far received little attention in this field. A study by Srivastava *et al.* (1985) revealed that the deuteromycete fungus *Aphanocladium album* produced high levels of extracellular chitinase when grown in minimal medium with crystalline chitin as sole carbon source. A preliminary study in our laboratory (unpublished data) showed that *A. album* grew better on chitin agar medium than did several entomopathogenic fungi such as *Beauveria bassiana*, *B. tenella*, *Metarhizium anisopliae* and *Verticillium lecanii*, which are known to be chitinase producers (Smith & Grula, 1983; Coudron *et al.*, 1984; St Leger *et al.*, 1986).

Here we report the isolation of stable chitinase-overproducing mutants of *Aphanocladium album* after one-step UV mutagenesis, showing extensive clearing of chitin on agar plates. Two mutants, E3 and E12, were compared to the wild-type strain for their chitinase production in submerged culture. The mechanisms of regulation of chitinase activity were investigated in mutant E3 and the wild-type strain. The possible mechanism(s) of chitinase overproduction by mutant E3 are discussed.

Methods

Strain and culture maintenance. The wild-type strain of *Aphanocladium album*, ETH M483 (referred to as E1 in this study), was provided by the Institute of Phytomedicine, ETH-Zürich, and maintained at 25 °C on cristomalt medium, consisting of 1% (w/v) cristomalt (Difal, France) and 1·7% (w/v) Bacto Agar (Difco).

Preparation of colloidal chitin. A modification of the method described by Skujins *et al.* (1965) was used. Thirty grams of crystalline chitin (Fluka) were mixed with 250 ml 10 M-HCl and left for 4 h at room temperature, then 5 litres of distilled water were added. After chitin precipitation, the water was removed and 5 litres of fresh distilled water were added. The precipitate was removed by centrifugation and rinsed several times with water until the pH was neutral. The colloidal chitin was then lyophilized.

Mutagenesis. Mycelia-free spore suspensions were subjected to UV irradiation for various time intervals corresponding to different survival rates. Aliquots of 0·1 ml were then plated on colloidal chitin agar, consisting of 1% (w/v) colloidal chitin and 2% (w/v) agar, to give approximately 50 viable colonies per plate, as previously established from dose–response curves.

Plate screening methodology. After mutagenesis, plates were incubated at 24 °C and observed periodically for colony growth and for visible clearing of chitin around the colonies. Isolated colonies showing either a larger clearing zone or a better growth rate than the wild-type strain were subcultured on cristomalt medium and tested again on the screening medium after being cloned.

Culture conditions. Cultures were grown in 250 ml Erlenmeyer flasks containing a 1% (w/v) carbon source, which was either D-glucose (Prolabo, France), L-malic acid (Sigma), crystalline chitin or *N*-acetyl-D-glucosamine (GlcNAc) (Sigma) in 50 ml of modified medium of Srivastava *et al.* (1985), containing: 8·25 g $(NH_4)_2SO_4$; 0·5 g $MgSO_4.7H_2O$; 2·5 g KH_2PO_4; 0·06 g $FeCl_3.6H_2O$; 0·01 g $ZnSO_4.7H_2O$; 1·6 g $MnSO_4.H_2O$ per litre distilled water. The pH of the medium was adjusted to 5·0 prior to autoclaving. Media containing L-malic acid and D-glucose were buffered with 0·2 M-2-(*N*-morpholino)ethanesulphonic acid (Sigma). The chitin was ground in a cylinder mill (Schleicher & Schüll) and then sifted in order to obtain a particle fraction with a diameter of 44–125 μm. Flasks were shaken for different time intervals on a rotary incubator (80 r.p.m.) at 24 °C.

Diffusion capsules. Diffusion capsules consisted of cylindrical containers (2·5 ml), filled with a solution of GlcNAc. Rates of monomer diffusion were controlled by altering the concentration of solute inside the capsules and the number of membranes through which diffusion occurred. Capsules containing a solution of 5% (w/v) GlcNAc and sealed with one semi-permeable membrane, i.e. a cellulose dialysis tube with a molecular mass cut-off of 10000 Da, gave linear diffusion rates of about 30 μg ml^{-1} h^{-1} over 17 h. Capsules were replaced twice a day to ensure linear release.

Sterilization. Culture media were sterilized by autoclaving at 121 °C for 30 min. D-Glucose and GlcNAc were sterilized by membrane filtration (Millex-GS 0·22 μm, Millipore) under reduced pressure and incorporated in autoclaved media. Diffusion capsules were autoclaved for 30 min at 121 °C and filled with the sterile monomer solution. Dialysis membrane and empty capsules were washed with ethanol, rinsed with sterile water and immersed in the medium.

Inoculum. Chitin medium was inoculated with conidia taken from a 5-d-old cristomalt culture (2×10^6 conidia per 50 ml medium). In the other experiments, a standardized inoculum was prepared from 60 h shake cultures containing 1% (w/v) D-glucose. Mycelium was removed by sterile filtration, washed with sterile water and transferred to the following media: GlcNAc capsule, 1% (w/v) GlcNAc, 1% (w/v) D-glucose or 1% (w/v) L-malic acid. The biomass of fungus grown on soluble carbon sources was estimated by measurement of the dry weight. The mycelium was dried for 24 h at 70 °C and then weighed. When the fungus was grown on chitin (insoluble polymer), the dry weight could not be estimated because of the strong adsorption of chitin to the mycelium.

Enzyme production. After several hours, cultures were filtered through Whatman no. 1 filter paper and rinsed with 0·2 M-sodium acetate buffer (pH 5·0). The culture filtrates were filtered successively through 8, 3 and 0·2 μm cellulose nitrate filters (Sartorius). Filtrates were analysed for GlcNAc, D-glucose or L-malic acid content. They were assayed for chitinase activity after dialysis (molecular mass cut-off of cellulose dialysis tube: 10000 Da) against stirred distilled water with the final bath against 0·2 M-sodium acetate buffer (pH 5·0).

Concentration of crude extracts. When cultures were grown on D-glucose or L-malic acid, the crude extracts were concentrated with polyethylene glycol (20000 Da exclusion limit) and then dialysed against 0·2 M-sodium acetate buffer (pH 5·0).

Titration of L-malic acid. L-Malic acid was measured enzymically with a test kit (Boehringer Mannheim).

Titration of D-glucose and GlcNAc. D-Glucose was measured by the modified method of Nelson (1944). Somogyi's reagent (0·2 ml) was added to 0·2 ml of the sample. The tubes were heated at 100 °C for 45 min. After cooling in ice, 0·2 ml Nelson's reagent and 4·4 ml distilled water were added successively. Absorbance was read at 500 nm.

GlcNAc was measured by the method of Reissig *et al.* (1955).

Chitinase activity. Dialysed filtrate (1 ml) was incubated for 30 min at 37 °C with 0·5 ml of a 2% (w/v) reacetylated crystalline chitin (Molano *et al.*, 1977) suspended in 0·2 M-sodium acetate buffer (pH 5·0). After centrifugation, GlcNAc was measured in the supernatant by the method of Reissig *et al.* (1955). One unit (U) of chitinase activity was defined as the amount of enzyme that catalysed the release of 1 μmol GlcNAc ml^{-1} in 30 min.

Results

Isolation and characterization of the mutants

When spores of the wild-type strain, E1, were plated on colloidal chitin agar medium, they developed a relatively dense mycelium without any visible clearing zone around the colony. Approximately 10000 colonies were examined after mutagenesis on this screening medium. Two of them showed an extensive clearing zone around the colony between days 4 and 8 of incubation and were designated E3 and E12 respectively. Both were recovered at a UV dose corresponding to 10% survival. When grown on chitin agar medium, the two mutants exhibited a similar radial growth rate but with considerably narrower hyphae than the wild-type strain. This particular morphology was not due to auxotrophy, because all the strains displayed a dense mycelial morphology on the same medium when glucose was used as the sole carbon source. Several colonies with altered growth patterns were also recovered. Those with an increased radial growth on colloidal chitin were selected but when cultivated in liquid medium they did not show higher chitinase activity than the wild-type strain.

Stability of the mutants

The stability of E3 and E12 was tested by successive monoconidial subcultures on cristomalt medium. After five subcultures in the absence of chitin, the mutants were still able to develop a large clearing zone on colloidal chitin. The two mutants were cloned each month and always displayed the ability to develop a clearing zone on colloidal chitin.

Chitinase production in shake flasks

Mutants E3 and E12 showed markedly higher chitinase activity than the wild-type strain E1 cultured under similar conditions (Table 1). Maximum activities of E3 and E12 were observed after 20 d of culture, and were 26- and 2·5-fold higher, respectively, than the maximum reached by strain E1 on day 10. Mutant E3 showed the highest chitinase activity throughout the culture.

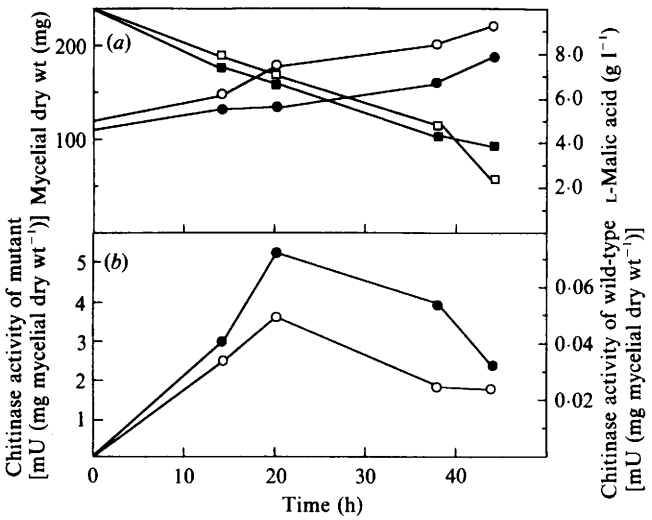

Fig. 1. (*a*) Mycelial growth (○, ●) and L-malic acid content (□, ■), and (*b*) chitinase activity on 1% (w/v) L-malic acid medium. Open and filled symbols represent, respectively, data for the wild-type strain E1 and for the mutant E3. Values are means of three independent assays.

Table 1. *Production of chitinase by A. album grown in shake flasks on 1% chitin medium*

Incubation time (d)	Chitinase activity (mU ml^{-1})*		
	Wild-type E1	Mutant E3	Mutant E12
5	10·7	72·0	52·5
10	108·0	600·0	178·8
15	51·8	1992·0	182·4
20	33·6	2868·0	268·8
30	12·6	145·6	30·4

* One unit (U) of chitinase activity was defined as the amount of enzyme that catalysed the release of 1 μmol GlcNAc ml^{-1} in 30 min.

Constitutive level of chitinase activity

We attempted to find a carbon source which induces no catabolic repression: Krebs cycle intermediates are generally used for this purpose because they are less rapidly utilized than glucose and should therefore cause less repression. The effect of L-malic acid on growth and chitinase activity was tested. In 1% (w/v) L-malic acid medium, the increase in biomass after 44 h of culture was 107 mg dry weight for E1 and 60 mg dry weight for E3 (Fig. 1*a*). The concentration of L-malic acid in the medium remained very high for both strains up to 20 h of culture (7·14 g l^{-1} for E1 and 6·98 g l^{-1} for E3); in the medium after 44 h of culture, however, there remained 23% of the initial concentration with E1 and 40% with E3 (Fig. 1*a*). The two strains displayed a constitutive

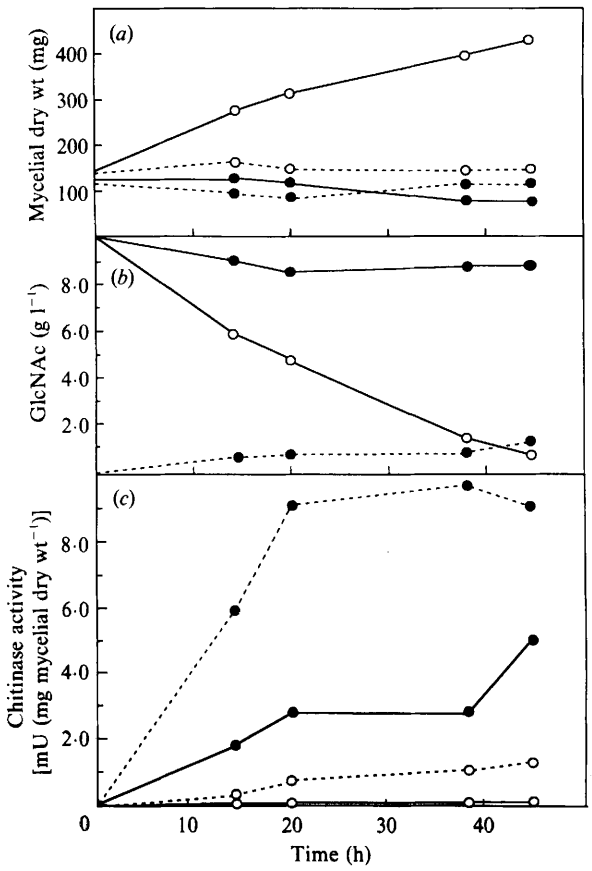

Fig. 2. (*a*) Mycelial growth, (*b*) GlcNAc content and (*c*) chitinase activity on 1% (w/v) GlcNAc medium (solid lines) and on GlcNAc capsule medium (broken lines). Open and filled symbols represent, respectively, data for the wild-type strain E1 and for the mutant E3. Values are means of three independent assays.

level of chitinase activity which followed the same accumulation pattern (Fig. 1*b*), increasing up to 20 h, then decreasing to about 50% of the maximum activity. Different hypotheses may be put forward to explain this phenomenon, e.g. destruction or inactivation of chitinase by proteases secreted by this fungus (see Manning & Wood, 1983). Chitinase activity in the mutant strain E3 was on average 100-fold higher than that measured in the wild-type strain E1.

Induction and repression of chitinase activity by GlcNAc

When diffusion capsules were used, growth of the wild-type strain E1 was considerably lower than in the 1% (w/v) GlcNAc medium (Fig. 2*a*). Growth was limited by the restricted rate of diffusion of the monomer through the capsule (feed limitation condition). No GlcNAc accumulation was observed throughout the culture of E1 in diffusion capsule conditions. In the 1% (w/v) GlcNAc medium, the concentration of monomer decreased throughout the culture period and fell to about 4·6% of the initial concentration after 44 h (Fig. 2*b*). Poor growth of the mutant strain E3 and accumulation of GlcNAc were observed on media containing the monomer independent of conditions: GlcNAc capsule or 1% (w/v) GlcNAc (Fig. 2*a*, *b*). In the GlcNAc capsule medium, 0·811 g GlcNAc l^{-1} accumulated in the medium after 44 h of culture. Theoretically, the final concentration expected if no GlcNAc was taken up by the fungus would be 1·3 g l^{-1}. Thus nearly 38% of the GlcNAc accumulated was taken up by mutant E3. In the 1% (w/v) GlcNAc medium the concentration of GlcNAc remained very high – on average 8·9 g l^{-1} – throughout all the culture; only 11% of the initial concentration had disappeared after 44 h.

In GlcNAc capsule and in 1% (w/v) GlcNAc conditions, the viability of both strains was checked by testing the ability of a small mycelial sample to grow on cristomalt agar. After 4–5 d, both strains displayed good growth comparable to untreated mycelium.

Chitinase activity of both strains was consistently higher in the GlcNAc capsule than in the 1% (w/v) GlcNAc medium. Chitinase activity of the mutant was always higher (on average 10-fold higher) than that of the wild-type strain, independent of conditions (Fig. 2*c*).

The results showed that GlcNAc at a concentration of 1% could repress chitinase activity in both strains: in the wild-type strain, there was a strong correlation between the concentration of GlcNAc in the medium and the repressor effect of the monomer on chitinase activity. The mutant strain had a high level of chitinase activity although GlcNAc concentration remained high in the medium (8·9 g l^{-1}). An estimation was made of a 'repression ratio', the ratio between chitinase activity detected in GlcNAc capsule medium and chitinase activity measured in 1% (w/v) GlcNAc medium. At 14 h, this ratio was 24 in the wild-type strain and 3·5 in the mutant. The repressor effect of GlcNAc is thus more significant in the wild-type than in the mutant strain.

In both strains, chitinase activity was higher in GlcNAc capsule than in 1% L-malic acid medium (Figs 1*b* and 2*c*). A low concentration of monomer in the medium could also increase the basal level of chitinase activity. At 14 h, the ratio between chitinase activity in GlcNAc capsule medium and that in 1% (w/v) L-malic acid medium was 10 in strain E1 and 2 in mutant E3.

'Glucose effect' on chitinase activity

As shown in Fig. 3(*a*), the growth of E1 and E3 in glucose medium increased rapidly up to 38 h and reached maximum values at 44 h (393 mg dry weight for E1 and 400 mg for E3): at this time, the glucose was almost exhausted. Glucose is a carbon source which is easily and

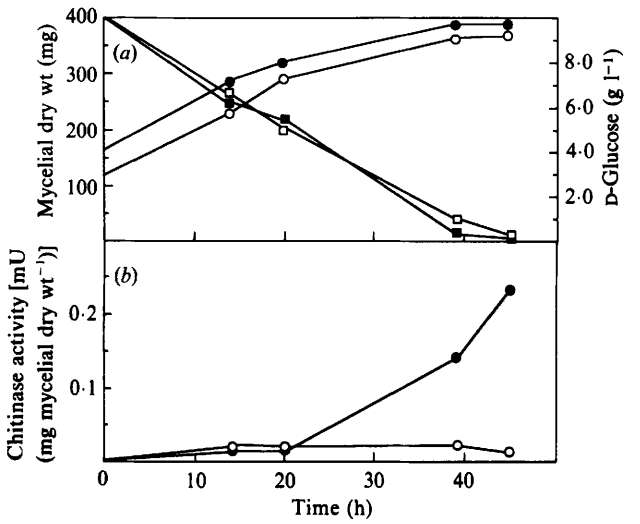

Fig. 3. (*a*) Mycelial growth (○, ●) and D-glucose content (□, ■), and (*b*) chitinase activity on 1% (w/v) D-glucose medium. Open and filled symbols represent, respectively, data for the wild-type strain E1 and for the mutant strain E3. Values are means of three independent assays.

rapidly assimilated by both strains compared to L-malic acid (Figs 1*a* and 3*a*).

As shown in Fig. 3(*b*), the two strains displayed the same low level of chitinase activity during the first 20 h of culture; at the same time, the concentration of glucose in the medium was high (5 g l^{-1}). Chitinase activity of strain E1 remained very low throughout the culture. In contrast, chitinase activity of mutant E3 increased after 20 h of culture.

These results demonstrate that chitinase activity in both strains is repressed by glucose during early stages of growth, i.e., when glucose concentration in the medium is high. It is a well-known phenomenon in micro-organisms that glucose is an easily assimilable carbon source which enters the glycolytic pathway directly. This is a self-regulating process whereby cells avoid wastage of resources and energy in synthesizing unnecessary enzymes. In our case, chitinase activity is not necessary in the presence of another rapidly utilizable carbon source. In the mutant strain, chitinase activity was repressed during 20 h of culture, and was derepressed after glucose had reached a low concentration in the medium. The constitutive level of chitinase activity was then detected.

Discussion

To our knowledge, this is the first report of chitinase-overproducing mutants in a filamentous fungus. The selection of colonies forming clearing zones on colloidal chitin agar constitutes a qualitative screening method, which allows rapid examination of a large number of colonies after mutagenesis. We suggest that this method could be employed in other fungi known as chitinase producers to induce chitinase-overproducing mutants.

Chitinase production of mutants E3 and E12 and the wild-type strain was compared quantitatively in liquid culture. E3 and E12 exhibited greater chitinase activities than the wild-type strain. Chitinase activity was increased 26-fold in mutant E3: the increase was very high compared with that in overproducing mutants of *Serratia marcescens* (Reid & Ogrydziak, 1981), which produced about two- to threefold as much chitinase activity as the wild-type strain.

The possible mechanism(s) responsible for the overproduction of chitinase in the E3 mutant was investigated.

The morphological aspect and growth rate of strains E3 and E1 were compared in various liquid media. In 1% (w/v) D-glucose and 1% (w/v) L-malic acid media, they were nearly identical. On 1% (w/v) chitin medium, the strains displayed apparently the same growth, but it was impossible to estimate dry weight accurately because of the strong adsorption of chitin to the mycelium. Moreover, an increase in chitinase activity in E3 was not correlated with an increase in biomass. These results indicate that E3 is not a morphological mutant.

The regulation of chitinase activity in strains E3 and E1 was also compared. There are few reports on the regulation of chitinase activity in filamentous fungi. Smith & Grula (1984) showed that the chitinase activity of *Beauveria bassiana* was inducible by GlcNAc, glucosamine or chitobiose. St Leger *et al.* (1986) demonstrated that chitinase activity in the entomopathogenic fungus *Metarhizium anisopliae* was regulated by products of chitin degradation through an inducer-repressor mechanism. The latter authors used, as we have done, a capsule diffusion test (Pirt, 1971) to demonstrate the induction of chitinase activity by GlcNAc. The use of these capsules prevents catabolite repression; micro-organisms use the solute at the rate of diffusion, which can be modulated and controlled according to the concentration of the substrate in the capsule and the number of membranes through which diffusion occurred. If, as in the present study, the capsules are changed regularly, before diffusion ceases to be linear, this system simulates a continuous culture. It is a very convenient method which has seldom been used in physiological studies on filamentous fungi.

Chitinase activity in both strains we studied (E1 and E3) is constitutive, inducible by GlcNAc, and sensitive to feedback repression and to the 'glucose effect'. The mutant strain E3 differs from E1 in displaying a high level of constitutive chitinase activity (100-fold higher

than in E1) and being more resistant to the repressor effect of GlcNAc. The sequence of events involved in induction of chitinase activity by insoluble chitin is not clearly understood. There is strong evidence supporting the role of constitutive chitinase activity in the induction phenomenon. Chitin is a large insoluble polymer and cannot enter the cell. The constitutive level of chitinase activity may be sufficient to initiate chitin degradation. The soluble products liberated could induce either the biosynthesis or activation of enzyme(s). The product of chitin degradation could act as either inducer or repressor according to its concentration in the medium. As a consequence, the production of enzyme depends on a delicate balance between induction and repression. In our case, the existence of a constitutive level of chitinase activity was demonstrated in the wild-type and the mutant strains. Moreover, mutant E3 had a high level of constitutive chitinase activity which allowed an early degradation of chitin and an early induction of chitinase activity, compared to the wild-type strain. Chitinase activity of the mutant strain was also less repressed by GlcNAc than that of the wild-type strain. Low transport of GlcNAc or poor metabolization of this molecule in the mutant could perhaps explain this result. We are currently investigating this. The high level of constitutive chitinase activity and high resistance to repressor effect of GlcNAc could explain the overproduction of chitinase activity by mutant E3.

In conclusion, we have isolated a mutant, E3, of *A. album* which is genetically stable and secretes high amounts of extracellular chitinase that is active on native chitin. This mutant is resistant to feedback repression and displays a high level of constitutive chitinase activity. *A. album*, which is a *Cephalosporium*-like fungus, can be easily grown in fermenters (G. Défago, personal communication). We consider this mutant to be a good candidate for testing the potential applications of chitinase.

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