A Mutant of *Candida albicans* Deficient in β -N-Acetylglucosaminidase (Chitobiase)

By H. F. JENKINSON* AND M. G. SHEPHERD

Department of Oral Biology and Oral Pathology, and Experimental Oral Biology Unit, University of Otago, Faculty of Dentistry, PO Box 647, Dunedin, New Zealand

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A mutant of *Candida albicans* ATCC 10261 was isolated that was defective in the production of β -N-acetylglucosaminidase (chitobiase). The mutant grew normally in minimal medium supplemented with either glucose or N-acetyl-D-glucosamine (GlcNAc) as carbon and energy source, and the cells formed germ-tubes at 37 °C when induced to do so with GlcNAc. However, unlike the wild-type parent strain, the mutant strain did not utilize N,N'-diacetylchitobiose for growth. The mutant and parent strains had similar growth rates on glucose or GlcNAc, similar rates of uptake of these sugars and similar rates of ¹⁴C-labelled amino acid incorporation. The chitobiase mutant did, however, contain 53–85% more chitin than the wild-type strain. No reversion of the mutant phenotype was observed following induction of mitotic recombination with UV light, suggesting that the mutant allele (*chi*) was carried homozygously in the chitobiase-deficient mutant. Although the chitobiase-deficient mutant was pathogenic, it was not as virulent as the wild-type strain.

INTRODUCTION

The addition of N-acetyl-D-glucosamine (GlcNAc) to starved cells of *Candida albicans* ATCC 10261 at 37 °C induces the formation of germ-tubes (Simonetti *et al.*, 1974; Shepherd & Sullivan, 1984). It also induces the synthesis of enzymes necessary for GlcNAc metabolism (Singh & Datta, 1979; Gopal *et al.*, 1982), and the synthesis of β -N-acetyl-D-glucosaminidase (Sullivan *et al.*, 1984). These enzymes are also induced in starved cells at 28 °C (Gopal *et al.*, 1982; Sullivan *et al.*, 1984) but at this temperature germ-tube formation does not occur. The β -N-acetyl-D-glucosaminidase (EC 3.2.1.30) acts on the synthetic substrate p-nitrophenyl-N-acetyl- β -D-glucosaminide, but not on p-nitrophenyl-N-acetyl- β -D-galactosaminide. Since the enzyme also readily hydrolyses N,N'-diacetylchitobiose, we refer to the enzyme as chitobiase in this paper. Chitobiase activity in yeast cells is mainly localized at the cell periphery (Pugh & Cawson, 1977), and during germ-tube formation at 37 °C as much as 70% of the total enzyme activity is in the extracellular medium (Sullivan *et al.*, 1984). Hydrolytic enzymes of *C. albicans*, particularly the extracellular acid protease, have been implicated as virulence factors (Macdonald & Odds, 1983; Negi *et al.*, 1984) and the secreted chitobiase may act on the GlcNAc residues of glycoproteins (Yamamoto *et al.*, 1985). This paper describes the isolation and characterization of a mutant of *C. albicans* deficient in chitobiase activity. The phenotypic properties of the mutant suggest that the enzyme is not normally essential for growth of *C. albicans*.

METHODS

Organisms. *Candida albicans* ATCC 10261 (prototroph) is referred to as the wild-type strain and was used throughout this work. Other strains of *C. albicans* used were: A72 and CA2 (A. Cassone, Istituto Superiore di Sanita, Rome); hOG301, a mycelial mutant derived from ATCC 10261; hOG1, an adenine-requiring auxotroph

Abbreviations: GlcNAc, N-acetyl-D-glucosamine; MUAG, 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide; YEP, yeast extract/peptone; NTG, N-methyl-N'-nitro-N-nitrosoguanidine.

(Poulter *et al.*, 1981) and hOG298, a mutant impaired in the utilization of GlcNAc (Corner *et al.*, 1986). Stocks of these strains were kept freeze-dried in horse serum (Difco), and cultured on malt extract agar (Difco).

Media. Yeast extract/peptone (YEP) medium contained (per litre): yeast extract (Difco), 5 g; Bacto-Peptone (Difco), 10 g; D-glucose, 20 g; NaCl, 1 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; pH 5.7. When required, agar was included to 3% (w/v) final concentration. When 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide (Sigma) (MUAG) was incorporated into agar medium, it was added to the autoclaved bulk medium as a filter-sterilized stock solution (7.5 mg ml⁻¹ in water) to a final concentration of 0.15 mg ml⁻¹. Salts/biotin minimal medium was as described by Shepherd *et al.* (1980), and for most experiments contained either GlcNAc or glucose (15 g l⁻¹) as carbon and energy source.

Mutagenesis. An exponentially growing culture (50 ml) of strain ATCC 10261 in YEP medium at 28 °C ($\text{OD}_{540} = 0.5$) was centrifuged (1000 g, 5 min) and the cells were suspended at a density of about 7×10^7 cells ml⁻¹ ($\text{OD}_{540} = 1.2$) in 0.2 M-sodium acetate, pH 7.0, containing N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Sigma; 0.1 mg ml⁻¹ final concn). The culture was incubated with gentle shaking at 28 °C for 1 h; the cells were then harvested by centrifugation as above, washed twice with YEP medium (20 ml), suspended in YEP medium at 28 °C (50 ml) and incubated for a further 2 h with vigorous shaking. Samples from the culture were taken before and immediately after treatment with NTG, appropriately diluted and spread onto YEP agar to determine viable counts. The mutagenesis treatment reduced the number of c.f.u. in the culture to about 3% of the initial number. The yeast cells were harvested by centrifugation, and for the isolation of a chitobiase-deficient mutant, the cells were suspended in salts/biotin/glucose medium (2 ml) in a 10 cm diameter glass Petri dish and irradiated for 40 s with a 30 W germicidal UV light ($1.3 \text{ J m}^{-2} \text{ s}^{-1}$) to induce mitotic segregation (Whelan *et al.*, 1980) and thus favour the formation of a mutant strain homozygous for the mutated allele. The UV treatment killed about 20% of the cells as estimated from plate counts of samples taken before and after irradiation. The UV-treated cells were then added to warm (28 °C) YEP medium (20 ml), incubated with shaking at 28 °C for a further 2 h, and portions (0.1 ml) were then diluted and plated onto YEP agar containing MUAG. Glycerol (final concn. 30%, v/v) was added to the remainder of the mutagenized culture, which was stored at -20 °C for up to 7 d, during which time further portions were used to screen for mutants. For the isolation of an adenine-requiring auxotroph, strain EOB4 (see Results) was mutagenized as described above and the yeast cell pellet obtained from the mutagenized culture (see above) was washed once with salts/biotin, suspended in salts/biotin/glucose and incubated with shaking at 28 °C for 3 h. Amphotericin B (2 $\mu\text{g ml}^{-1}$ final concn) was added and the culture was incubated for a further 2 h at 28 °C; amphotericin B kills only actively dividing cells, so this enriches for auxotrophs in the glucose minimal medium. The cells were harvested by centrifugation (1000 g, 5 min), washed twice with distilled water, and then diluted and plated onto YEP agar. Adenine-requiring auxotrophs grow as pink-red colonies on this medium and so are easily distinguished from the white prototrophs (Poulter & Rikkerink, 1983). An Ade⁻ derivative of strain EOB4 was obtained that formed pink colonies on YEP agar, and was denoted EOB5.

Induction of chitobiase and germ-tube formation. Cultures (200 ml) were grown for 20 h in salts/biotin medium containing glucose to a density of about 1.2×10^8 cells ml⁻¹ and induced to form germ-tubes as described by Shepherd *et al.* (1980).

Concentration of spent culture medium. Chitobiase activity (see below) was determined in extracellular culture fluid. The supernatant (200 ml) obtained after centrifuging the culture (3000 g, 10 min) was concentrated to a volume of about 20 ml by ultrafiltration using an Ulvac ultrafiltration membrane (exclusion limit 10 kDa). The protein concentration was determined by the Lowry method with bovine serum albumin as standard. Approximately 200 μg protein was obtained from 200 ml culture fluid.

Preparation of cell-free extracts. Cells from 200 ml culture (about 40 mg dry weight) were harvested, washed, broken with glass beads, and cell-free extracts were prepared as described by Gopal *et al.* (1982).

Determination of enzyme activities. β -N-Acetyl-D-glucosaminidase (chitobiase; EC 3.2.1.30) activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma) as described by Sullivan *et al.* (1984). β -Glucosidase (EC 3.2.1.21) activity was measured using p-nitrophenyl- β -D-glucoside (Sigma) as substrate (Ram *et al.*, 1983) and alkaline phosphatase (EC 3.1.3.1) activity was determined using p-nitrophenylphosphate (Sigma) as described by Ram *et al.* (1983). One unit of enzyme is defined as the amount catalysing the formation of 1 μmol p-nitrophenol min⁻¹ at 37 °C ($\epsilon = 16 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$). Enzyme specific activities are expressed as units (mg protein)⁻¹.

Chitin content of yeast cells. Yeast cells of strains ATCC 10261 and EOB4 were grown to late-exponential phase in either glucose/salts/biotin or GlcNAc/salts/biotin medium at 28 °C. The cells (approximately 5 g wet weight) were harvested by centrifuging (1000 g, 5 min) and washed three times in distilled water. The intact cells were sequentially extracted with alkali and acid essentially as described by Manners *et al.* (1973). Cells were incubated with 6 vols 3% (w/v) NaOH at 70 °C for 6 h, and the residue was collected and washed three times with water. The pellet was then extracted three times with 20 ml 0.5 M-acetic acid at 100 °C for 4 h. The insoluble residue, containing chitin and insoluble glucans, was collected by centrifugation (10000 g, 20 min), washed, suspended in 2 ml 0.1 M-sodium phosphate buffer (pH 6.9) containing 0.6 M-NaCl, 0.02% Na₂S₂O₃, chloramphenicol (50 $\mu\text{g ml}^{-1}$) and 2 mg α -amylase and incubated at 30 °C for 16 h. The suspension was centrifuged (10000 g, 20 min), and the

residue was washed twice with distilled water, suspended in 2 ml 0.2 M-sodium acetate buffer (pH 5.4) and incubated with a mixture of endo- and exo-(1,3)- β -glucanases (Zymolyase 100T, Miles Scientific, 100 units) for 18 h at 30 °C. The residue from the Zymolyase incubation was then analysed for chitin by two methods. In method I the residue was suspended in 2 ml 3 M-HCl. The tube was sealed and heated at 110 °C for 18 h after which the GlcNAc content was measured (Reissig *et al.*, 1955). In method II the residue was suspended in 0.1 M-phosphate buffer, pH 6.0, and treated with chitinase (Sigma C1525, 30 units) at 30 °C for 18 h. There was no residue after this treatment and the solution was analysed for GlcNAc content (Reissig *et al.*, 1955). Similar results were obtained by both methods.

Uptake assays. The uptake rates of glucose and GlcNAc were measured with yeast cells grown for 20 h in glucose/salts/biotin medium. The cells were harvested by centrifuging as before and washed three times in water. One half of the cells was suspended in 20 mM-imidazole/HCl buffer (pH 6.6) to a density of about 0.8×10^8 cells ml⁻¹ and the other half in the same buffer, but containing 5 mM-GlcNAc, to the same density. Each suspension was incubated at 30 °C for 3 h, before the cells were collected by centrifuging. For uptake assays, 8×10^6 cells were suspended in imidazole germination buffer (1.5 ml) and incubated at 37 °C for 2 min before addition of *N*-acetyl-D-[1-¹⁴C]glucosamine [0.05 μ Ci (1.85 kBq); 0.2 μ mol] or D-[U-¹⁴C]glucose [0.06 μ Ci (2.22 kBq); 0.16 μ mol]. Samples (200 μ l) were removed at intervals; the cells were collected by filtration (Whatman GF/C filter), washed three times with water and the radioactivity of the dried filters was determined by scintillation counting.

Measurement of protein synthesis. The incorporation of ¹⁴C-labelled Casamino acids into hot trichloroacetic acid precipitate was used as a measure of protein synthesis. Cells were suspended at 30 °C in 20 mM-imidazole/HCl buffer (pH 6.6) to a density of about 2×10^8 cells ml⁻¹ and incubated with shaking for 90 min. ¹⁴C-labelled Casamino acids [0.2 μ Ci (7.4 kBq); 20 μ g] were added and samples (1 ml) of each suspension were removed at intervals over 2 h and added to ice-cold 20% (w/v) trichloroacetic acid (1 ml) containing 100 μ g Casamino acids ml⁻¹. The mixtures were heated at 100 °C for 20 min, cooled to 0 °C, and the precipitates were collected by filtration (Whatman GF/C). Each precipitate was washed four times with 5 ml portions of 5% (w/v) ice-cold trichloroacetic acid containing 50 μ g Casamino acids ml⁻¹, and then washed twice with 5 ml 95% (v/v) ice-cold ethanol to remove the acid. The radioactivity of the filters was then determined by scintillation counting.

Pathogenicity studies. The pathogenicity of *C. albicans* ATCC 10261, the adenine auxotrophic mutant hOG1 and the chitinase-deficient mutants EOB4 and EOB5 was in each case tested by intravenous injection of yeast cells into the tail veins of mice. The male mice (6 weeks old) from a random colony were maintained in groups of 10 in a constant temperature (22 °C) animal room with an alternating 14 h light period and 10 h dark period. They were fed *ad libitum* on a diet of Standard-3/8 mouse and rat pellets (Reeve Stock Food Specialists, Dunedin, New Zealand). Approximately 30 mice were inoculated (0.25 ml cells) with each strain of *C. albicans* and mortalities were recorded each day. Kidneys were excised from dead animals, weighed and then homogenized in sterile YEP medium. The numbers of c.f.u. of *C. albicans* were determined by plating suitable dilutions on YEP agar. The histology of the induced candidosis was studied by staining 5 μ m sections of kidney with Gill's haematoxylin and phloxin.

Detection of secreted protease and phospholipase. The production of extracellular protease was measured on agar plates essentially as described by Germaine *et al.* (1978). The plates contained (per litre): yeast extract, 100 mg; glucose, 20 g; MgSO₄·7H₂O, 0.5 g; KH₂PO₄, 1 g; bovine serum albumin, 2 g; agar, 30 g; pH 4.0. For the preparation of these plates, the bovine serum albumin was autoclaved separately and then added as a 10 $\times$ concentrated solution. Plates were incubated for several days at 28 °C until colonies were apparent, then were flooded with amido black (1 g l⁻¹ in 3.5 M-acetic acid) (Ruchel *et al.*, 1982). After 10 min the amido black solution was removed and the agar destained by repeated washings with 1.2 M-acetic acid. Strains of *C. albicans* that produced extracellular protease showed zones of clearing around the colonies.

Agar plates used to show phospholipase production contained 1% (w/v) Neopeptone (Difco), 4% (w/v) glucose, 1 M-NaCl, 5 mM-CaCl₂, 8% (v/v) sterile egg yolk, 3% (w/v) agar. The sterile egg yolk was prepared by centrifuging fresh hen egg yolks at 500 g for 15 min and making the supernatant up to the original volume with sterile distilled water. Colonies of *C. albicans* that produce phospholipase have zones of precipitation around them (Price *et al.*, 1982).

RESULTS

Isolation and properties of a chitinase-deficient mutant

When cells of the wild-type *C. albicans* strain ATCC 10261 are plated onto YEP agar containing MUAG (0.15 mg ml⁻¹) and incubated at 28 °C for 48 h, chitinase activity is detected. The enzyme cleaves MUAG and under UV light the yeast colonies appear fluorescent with diffuse UV-fluorescent haloes. To isolate a mutant defective in chitinase, samples (0.1 ml) of mutagenized culture were diluted and plated onto YEP agar containing MUAG to give approximately 200 colonies per plate. From a single mutagenesis experiment approximately

Fig. 1. Hydrolysis of MUAG by *C. albicans* ATCC 10261 (a) and the Chi⁻ mutant EOB4 (b). Cultures were streaked on YEP agar containing MUAG, incubated for 36 h at 28 °C and photographed on a UV-transilluminator ($\lambda = 304$ nm).

4000 colonies were screened and one was found that did not fluoresce. Cells from this colony were streaked to single colonies on YEP agar containing MUAG and a single-colony isolate with the non-fluorescent phenotype (Chi⁻) was designated strain EOB4 (Fig. 1). A number of strains of *C. albicans* (ATCC 10261, A72, CA2, hOG301, hOG298 and hOG1) were tested for their ability to secrete chitinase; with the exception of the selected mutant EOB4, colonies of all strains produced haloes on agar plates containing MUAG.

Strain EOB4 was tested for its ability to secrete chitinase under conditions of germ-tube formation. Yeast-phase cells of strains EOB4 and ATCC 10261 were suspended in germination medium containing GlcNAc and shaken at 37 °C for 3 h, the cells from each culture were harvested by centrifugation (1000 *g*, 5 min) and the cell-free supernatants were concentrated by ultrafiltration and assayed for chitinase activity. The culture supernatant from the wild-type strain contained 0.41 units (mg protein)⁻¹ whereas no activity was detected in the supernatant from strain EOB4 (Table 1). In a separate experiment, even after 8 h incubation at 37 °C there was still no chitinase activity detectable in the culture supernatant from strain EOB4 (results not shown).

Since in the wild-type strain 80% of chitinase activity is periplasmic (Sullivan *et al.*, 1984) it was possible that in strain EOB4 chitinase activity was present but entirely intracellular. To test this possibility, cells of both EOB4 and ATCC 10261 were induced for chitinase as described above and cell-free extracts were assayed for chitinase activity; alkaline phosphatase and β -glucosidase activities were also determined as internal controls. No chitinase activity was detectable in extracts of strain EOB4, compared with 3.1×10^{-2} units (mg protein)⁻¹ in extracts of the wild-type strain (Table 1). The specific activities of alkaline phosphatase and β -glucosidase in strain EOB4 were similar to those in extracts prepared from the wild-type strain (Table 1).

The doubling time of strain EOB4 in glucose/salts/biotin (15 g l⁻¹) was 2 h at 28 °C, and 2.5 h at 37 °C; these doubling times are the same as those for strain ATCC 10261 grown under identical conditions. The growth of strain EOB4 in GlcNAc (15 g l⁻¹)/salts/biotin gave doubling times of 2.2 h at 28 °C and 2.8 h at 37 °C, which are similar to those obtained with the wild-type strain in the same medium.

Table 1. *Enzyme activities in culture supernatants and cell-free extracts from C. albicans strains ATCC 10261 (wild-type) and EOB4 (Chi⁻ mutant)*

Starved cells (8×10^7 cells ml⁻¹) of each strain were incubated at 37 °C with 2.5 mM-GlcNAc as described in Methods. Alkaline phosphatase and β -glucosidase activities were determined on cell-free extracts only. The values are the arithmetic means of duplicate estimations and the data represent typical examples of experiments which have been repeated.

Enzyme	10 ² × Specific activity [units (mg protein) ⁻¹]	
	ATCC 10261	EOB4
Chitobiase		
Culture supernatant	41.0	0
Cell-free extract	3.1	0
Alkaline phosphatase	3.0	3.2
β -Glucosidase	1.2	1.4

Table 2. *Glucose and GlcNAc uptake in C. albicans strains ATCC 10261 and EOB4*

Yeast cells of both strains were grown in glucose/salts/biotin medium, washed three times in water and suspended in 20 mM-imidazole/HCl buffer (pH 6.6) to a density of about 0.8×10^8 cells ml⁻¹. GlcNAc (5 mM) was added to one half of each suspension and the cells were incubated at 30 °C for 3 h. The uptakes of glucose and GlcNAc were then measured on each suspension as described in Methods.

Strain	Preincubation	Uptake [μ mol min ⁻¹ (mg dry wt) ⁻¹]	
		Glucose	GlcNAc
ATCC 10261	Buffer only	44	0.6
ATCC 10261	+ GlcNAc	36	26
EOB4	Buffer only	37	0.5
EOB4	+ GlcNAc	31	23

Table 3. *Protein synthesis in C. albicans strains ATCC 10261 and EOB4*

Washed cells were suspended in 20 ml 20 mM-imidazole/HCl buffer (pH 6.6) supplemented as indicated below. Cells were incubated with shaking at 30 °C for 90 min before adding the ¹⁴C-labelled Casamino acids and measuring uptake.

Medium	¹⁴ C-labelled amino acid incorporation [nmol h ⁻¹ (mg dry wt) ⁻¹]	
	ATCC 10261	EOB4
Buffer	27	32
Buffer + GlcNAc (2.5 mM)	38	44
Buffer + glucose (2.5 mM)	67	61

As shown in Table 2, induction of the GlcNAc uptake system was not impaired in the chitobiase mutant, and the constitutive transport of glucose was the same in the mutant and wild-type strains. The ability of the chitobiase-deficient mutant to synthesize protein was also compared with that of the parent strain (Table 3). The rates of amino acid incorporation were essentially the same in both strains and were maximal in the presence of glucose.

To determine if chitobiase was necessary for growth of *C. albicans* on the disaccharide *N,N'*-diacetylchitobiose, cultures of strains EOB4 and ATCC 10261 were first grown overnight at

Table 4. *Chitin contents of yeast cells of C. albicans strains ATCC 10261 and EOB4*

The values given are based on GlcNAc equivalents released by either acid hydrolysis or chitinase treatment. The results are the means of two different experiments (SEM < 10%).

Strain	Growth medium	Dry wt of cells (mg)	GlcNAc equivalents (nmol)	Chitin content (% w/w)
ATCC 10261	Glucose/salts/biotin	336	9.8	0.65
ATCC 10261	GlcNAc/salts/biotin	311	10.1	0.72
EOB4	Glucose/salts/biotin	274	14.8	1.2
EOB4	GlcNAc/salts/biotin	296	14.6	1.1

28 °C in GlcNAc/salts/biotin to induce enzymes necessary for GlcNAc metabolism. The cells were harvested by centrifugation (1000 g, 5 min), washed twice with salts/biotin and then resuspended at 28 °C to a density of approximately 5×10^6 cells ml⁻¹ in salts/biotin (1 ml) containing *N,N'*-diacetylchitobiose (5 mg). The cultures were incubated with shaking at 28 °C for 18 h, after which cell densities were calculated from a standard curve relating cell number and OD₅₄₀. The culture of strain ATCC 10261 contained approximately 3.2×10^7 cells ml⁻¹, while the culture of strain EOB4 had not increased in density, showing that it was unable to grow on *N,N'*-diacetylchitobiose.

Since GlcNAc induces germ-tube formation and chitinase synthesis in *C. albicans*, we determined whether chitinase activity was necessary for germ-tube formation. Accordingly, cells of strains EOB4 and ATCC 10261 were induced for germ-tube formation either with GlcNAc or with serum as described in Methods. After 3 h incubation at 37 °C, 70–75% of the cells in all cultures had formed germ-tubes, so this morphological transition was not impaired in strain EOB4.

The chitin contents of exponentially growing yeast cells of *C. albicans* strains ATCC 10261 and EOB4 are shown in Table 4. The chitin content of each strain was similar whether the cells were grown on glucose or GlcNAc. There was, however, a marked difference in the chitin content of the wild-type and mutant strains; the Chi⁻ mutant produced 53–85% more chitin depending on growth conditions.

The *C. albicans* Chi⁻ mutants EOB4 and EOB5 were not impaired in secretion of either phospholipase or protease as determined from growth on agar plates containing egg yolk or albumin respectively.

Reversion analysis

No spontaneous reversion of the non-fluorescent (Chi⁻) phenotype of strain EOB4 to Chi⁺ was observed on repeated subculture and we estimated the reversion frequency to be less than 10⁻⁴. However, if reversion to Chi⁺ could be induced with UV light, it might indicate that the Chi⁻ mutation was carried heterozygously (Whelan *et al.*, 1980). To induce mitotic recombination in strain EOB5 (Ade⁻ Chi⁻), cells from a single colony were suspended in sterile water, diluted and spread onto YEP agar to give, after incubation, approximately 200 colonies per plate. Some plates were spread and immediately exposed to UV light (1.3 J m⁻² s⁻¹) for 40 s, which reduced the colony count to about 40% of that obtained on the non-irradiated plates. After UV treatment and incubation of the plates for 4 d at 28 °C a foreground of small dark red colonies appeared at a frequency of 3.6% (38/1060) and these were easily distinguishable from the pink, larger colony type of the parent strain EOB5. No dark red colonies were obtained on plates that had not been exposed to UV light. The dark red colonies are characteristically adenine-requiring homozygous derivatives (Poulter & Rikkerink, 1983). We also obtained, interspersed with the pink colonies, larger white colonies at a frequency of about 0.5% (5/1060) and these were prototrophic. The fact that the dark red and white homozygous colonies were formed at different frequencies indicated that strain EOB5 carried more than one mutated *ade* allele. Nevertheless since the homozygotes were obtained at frequencies of >10⁴ above

Fig. 2. Relative pathogenicity of *C. albicans* ATCC 10261 (●) and the Chi⁻ mutant EOB4 (■). At each point shown, 15 mice were injected with the appropriate quantity of cells.

reversion frequency this showed that the UV treatment had induced mitotic instability. The colonies that formed on the plates after UV treatment were replica-plated onto YEP agar containing MUAG and after 2 d incubation at 30 °C the plates were examined for UV fluorescence. No fluorescent colonies were detected from about 10000 tested, suggesting that the Chi⁻ phenotype was not due to a mutated allele carried heterozygously.

Pathogenicity

The relative degrees of virulence of the prototrophic strain ATCC 10261, the mutant EOB4, and a dark red (homozygous Ade⁻) derivative of EOB5 (Chi⁻) were examined in mice. As shown in Fig. 2, the Chi⁻ mutant EOB4 was less virulent (LD₅₀ at 12 d 7.5×10^6 cells) than the parent strain ATCC 10261 (LD₅₀ at 12 d 5×10^5 cells). The numbers of *C. albicans* cells in the kidneys of mice that died as a result of inoculation with either EOB4 or ATCC 10261 cells were similar [$0.4\text{--}1.2 \times 10^8$ c.f.u. (g kidney)⁻¹].

Six of 24 mice injected with 10^6 EOB4 cells had survived after 53 d. The *C. albicans* count in two of these mice was $1.1\text{--}3.3 \times 10^2$ c.f.u. (g kidney)⁻¹; thus it appeared that these mice had overcome the infection. Microscopic examination of kidney tissue slices from mice that had died due to candidosis revealed extensive infection and a mixed population of yeast, pseudomycelial and mycelial forms. These kidneys were enlarged and had a discoloured, greyish appearance. The red adenine-requiring strain EOB5 was not pathogenic when up to 10^7 cells were injected and neither was another homozygous Ade⁻ auxotroph, hOG1 (Shepherd, 1985). In one experiment three mice from the group that had been injected with 10^7 cells of EOB5 were killed at 4, 16, and 37 d and *C. albicans* counts in their kidneys were 4.5×10^4 , 6×10^5 and 3×10^2 c.f.u. (g kidney)⁻¹, respectively. An inoculum of 10^8 cells of strains EOB4, EOB5, hOG1 or ATCC 10261 each killed the mice within 6 h; in these mice the numbers of *C. albicans* cells ranged from 1 to 7×10^6 c.f.u. (g kidney)⁻¹.

The *C. albicans* cells isolated from kidneys of mice injected with strains EOB4 and EOB5 were all Chi⁻ and did not produce haloes on YEP plates containing MUAG; the EOB5 cells grew as red colonies.

DISCUSSION

The metabolism of GlcNAc by *C. albicans* has attracted interest since this compound induces the conversion of yeast cells to hyphal cells (Simonetti *et al.*, 1974; Shepherd *et al.*, 1980; reviewed in Shepherd *et al.*, 1985). As well as triggering this change in morphology, GlcNAc induces the synthesis of enzymes for GlcNAc metabolism (Singh & Datta, 1979; Gopal *et al.*, 1982) and the synthesis of chitobiase (Sullivan *et al.*, 1984). Chitin metabolism has been implicated in the cell wall morphogenesis of *C. albicans* (Braun & Calderone, 1978; Shepherd *et*

al., 1980) and we therefore decided to examine in some detail a mutant deficient in chitinase. Secretion of chitinase was a property of all *C. albicans* strains tested, and with the exception of the selected mutant EOB4, all the strains fluoresced on agar plates containing MUAG. Mutant EOB4 could not hydrolyse MUAG, and did not secrete chitinase into the medium nor accumulate the enzyme intracellularly.

The Chi⁻ mutant was not affected in (a) growth rate on either glucose or GlcNAc, (b) germ-tube formation, (c) glucose or GlcNAc uptake and (d) amino acid incorporation. Collectively, these data indicate that chitinase is not associated with the growth and intermediary metabolism of *C. albicans*. However, chitinase activity is essential for the growth of *C. albicans* on *N,N'*-diacetylchitobiose since, unlike the wild-type strain, EOB4 was unable to grow on this disaccharide.

β -*N*-Acetyl-D-glucosaminidase activity has been detected in a wide variety of biological materials (e.g. Li & Li, 1970; Okada *et al.*, 1971; Mommsen, 1980) and this enzyme is generally assumed to be involved in the catabolism of hexosamine-containing compounds. The enzyme from *C. albicans* is specifically a chitinase (Sullivan *et al.*, 1984) and may act in concert with chitinase (EC 3.2.1.14), recently shown also to be present in *C. albicans* (Barrett-Bee & Hamilton, 1984), in the hydrolysis of chitin to form GlcNAc. The role of these enzymes in metabolism is not clear since chitin, supplied as sole carbon and energy source, does not support growth (Sullivan *et al.*, 1984); possibly exogenously supplied chitin is not accessible to the chitinase.

It was of some interest to find that replacing glucose with GlcNAc did not increase the chitin content of the yeast cells of strains ATCC 10261 and EOB4. GlcNAc stimulates the production of GlcNAc metabolic enzymes at both 28 °C and 37 °C (reviewed in Cassone *et al.*, 1985) and with the additional synthesis of GlcNAc-6-phosphate, a precursor of chitin, an increase in chitin synthesis may have been expected. It would therefore appear that the controls identified on *C. albicans* chitin synthase *in vitro* (Braun & Calderone, 1978; Chiew *et al.*, 1980) are also important *in vivo*. There was, however, an overproduction of chitin by the Chi⁻ mutant. These data suggest (a) that synthesis of chitin in *C. albicans* is tightly regulated and (b) that the absence of chitinase affects chitin turnover, leading to a net increase in chitin content. If the production of GlcNAc from chitobiose were in close proximity to the chitin synthase this would also assist in regulation of chitin synthesis since GlcNAc is a negative heterotropic allosteric effector of chitin synthase (Chiew *et al.*, 1980).

The overproduction of chitin has been observed previously with regenerating spheroplasts of *C. albicans* (Gopal *et al.*, 1984; Elorza *et al.*, 1983) and in cell cycle mutants of *Saccharomyces cerevisiae* (*cdc24*) (Sloat & Pringle, 1978). Roberts *et al.* (1983) have proposed that arresting the cell cycle at different stages results in generalized activation over the entire wall of the chitin synthase proenzyme. The role of chitin and GlcNAc metabolism in *C. albicans* morphogenesis and cell wall synthesis has recently been reviewed (Shepherd *et al.*, 1985).

A mutant of *C. albicans* (strain hOG298) has recently been described (Corner *et al.*, 1986) in which the first three steps in GlcNAc catabolism are not induced following addition of GlcNAc (Sullivan *et al.*, 1984). In addition, this mutant could not utilize GlcNAc for growth (Corner *et al.*, 1986); this distinguishes it phenotypically from the mutant strain EOB4 described in this paper which can utilize GlcNAc as a sole carbon and energy source.

The virulence studies indicated that the chitinase mutant was pathogenic to mice, although less so than the wild-type parent strain ATCC 10261. It has been proposed that the secreted hydrolytic enzymes of *C. albicans*, particularly the aspartic protease (Macdonald & Odds, 1983; Remold *et al.*, 1968) and phospholipase (Pugh & Cawson, 1977), are aggressins. Recently, a keratinolytic protease from culture filtrates of *C. albicans* grown on human stratum corneum has been purified and characterized (Negi *et al.*, 1984).

The sugar moieties of glycoproteins are essential in a number of biological processes such as cellular recognition (including tumour invasiveness, lymphocyte 'homing', trophoblast implantation and intercellular adhesion) (Ashwell & Harford, 1982). It is possible that secreted *C. albicans* chitinase acts on the GlcNAc residues of glycoproteins as has been found with the β -*N*-acetylhexosaminidase from *Penicillium oxalicum* (Yamamoto *et al.*, 1985). The inability of

the Chi⁻ mutant to carry out this function could affect its pathogenicity. It was of interest to find that strains EOB4 and EOB5 were not impaired in either protease or phospholipase secretion compared to strain ATCC 10261. The observation that the Ade⁻ auxotrophs EOB5 and hOG1 were not pathogenic at 10⁷ cells per mouse confirmed the earlier study of Shepherd (1985) and suggests that this auxotrophic requirement is critical for propagation of cells *in vivo*.

When the inoculum of either of the Ade⁻ derivatives was increased to 10⁸ cells, the mice died within 8 h. This may suggest either that a toxin was present or that possibly the massive inoculum caused blockage of the kidney tubules resulting in renal failure. A possible role for *Candida* toxins has been reviewed by Louria (1985). The numbers of *C. albicans* cells in the kidneys of mice infected with 10⁷ cells of ATCC 10261 increased over 25 d from an initial value of 10³–10⁵ c.f.u. (g kidney)⁻¹ to a fatal value of 0.4–1.2 × 10⁸ c.f.u. (g kidney)⁻¹. For a strain of *C. albicans* to cause death in this way it must be capable of propagating in soft tissues; the non-pathogenic strains EOB5 and hOG1 did not grow *in vivo* and reach a concentration in the kidneys that was fatal.

Classical genetic analysis of *C. albicans* has demonstrated that this organism is diploid (Whelan *et al.*, 1980; Poulter *et al.*, 1981). The experiments involving induction of mitotic instability suggest that the mutated *chi* allele is probably carried homozygously in strain EOB5. However, the possibility that the Chi⁻ phenotype is due to two or more mutations at distant genetic loci cannot be ruled out. We have attempted, using the same mutagenesis techniques as described above, to isolate further chitobiase-deficient mutants of strain ATCC 10261 and of some of its auxotrophic derivatives, so far without success. The isolation of more mutants for complementation analysis (Poulter & Rikkerink, 1983) should give some indication of the number of genetic loci involved in chitobiase synthesis.

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