A *Candida albicans* Mutant Impaired in the Utilization of *N*-Acetylglucosamine

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Indicator plates containing eosin, methylene blue, glucosamine and proline were used to select mutants of *Candida albicans* impaired in the utilization of glucosamine. One such mutant, strain hOG298, grew on glucosamine at a slower rate than the parent and was severely impaired in growth on *N*-acetylglucosamine. The mutant was unable to express the first three steps in the *N*-acetylglucosamine pathway: *viz* the permease, *N*-acetylglucosamine kinase and *N*-acetylglucosamine-6-phosphate deacetylase. Glucosamine-6-phosphate deaminase was, however, induced by *N*-acetylglucosamine. The mutant still possessed a constitutive uptake system and kinase activity for glucosamine but glucosamine neither increased the glucosamine kinase activity nor induced *N*-acetylglucosamine kinase. These findings accounted for the decreased growth rate on glucosamine. The parent strain formed germ-tubes in *N*-acetylglucosamine or 4% (v/v) serum but the mutant formed germ-tubes only in serum.

INTRODUCTION

Germ-tube formation, the initial stage of the transition from yeast to mycelium in *Candida albicans*, may be induced at 37 °C by many nutrients including *N*-acetylglucosamine (GlcNAc) (Simonetti *et al.*, 1974; Shepherd *et al.*, 1980*a*). In the presence of GlcNAc the specific activities of the GlcNAc uptake system, GlcNAc kinase, GlcNAc-6-phosphate deacetylase and glucosamine (GlcN)-6-phosphate deaminase increase 25–40-fold (Singh & Datta, 1979; Shepherd *et al.*, 1980*b*; Sullivan & Shepherd, 1982; Gopal *et al.*, 1982; Shepherd & Sullivan, 1984) but these increases are abolished by inhibitors of RNA and protein synthesis. However, induction of this pathway occurs at either 28 °C or 37 °C whereas the dimorphic transition is limited to incubations above 33 °C (Mattia & Cassone, 1979). Recent studies have shown that analogues and derivatives of GlcNAc such as *N*-acetylmannosamine, colloidal chitin and GlcNAc covalently attached to agarose beads (Sullivan & Shepherd, 1982; Shepherd & Sullivan, 1983, 1984) induce germ-tube formation and the uptake system for GlcNAc in a gratuitous manner. These data suggest that the metabolism of GlcNAc *per se* is not necessary for GlcNAc-induced germ-tube formation but that this formation is mediated via the GlcNAc permease. To investigate further the relationship between GlcNAc and germ-tube formation we describe in this paper the isolation and characterization of a mutant impaired in GlcNAc utilization.

METHODS

Strains. All mutant strains were derived from *C. albicans* ATCC 10261. *C. albicans* ATCC 10261 hOG1 (hereafter known as strain hOG1) is a red adenine auxotroph derived from ATCC 10261 (Poulter *et al.*, 1981). Amino sugar utilization mutants were derived from strain hOG1.

Abbreviations: EMB, eosin/methylene blue; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine.

Media and culture conditions. For long term maintenance all strains were stored in sterile 50% (v/v) glycerol at -20°C . Strains were maintained for short periods on yeast extract/peptone agar slopes or plates at 4°C . Plates and slopes were solidified with 2% (w/v) agar. All growth media were prepared in a salts/biotin medium (Shepherd & Sullivan, 1976) containing 3.0 mM-adenine. Unless otherwise stated the carbon source was 83 mM-glucose (Glc). The growth conditions for plates (Poulter *et al.*, 1981) and liquid cultures (Shepherd & Sullivan, 1976) have been described previously. Yeast cultures were grown at 28°C . For germ-tube formation starved yeast cells (8×10^7 cells ml^{-1}) were incubated for 3 or 4 h at 37°C in 10 mM-imidazole/HCl buffer, pH 6.6 containing 0.1 mM-MnCl₂ and either 2.5 mM-GlcNAc or 4% (v/v) serum (Shepherd *et al.*, 1980a). Adenine supplementation was not necessary for germ-tube formation. The percentage germ-tube formation was assessed by light microscopy.

Isolation of amino sugar utilization mutants. Preliminary experiments indicated that in the pH range 4.6 to 7, GlcN was utilized as a carbon source by *C. albicans*. On GlcN (83 mM)/eosin (200 mg ml^{-1})/methylene blue (32 mg ml^{-1}) (EMB) plates strain hOG1 gave rise to pink colonies after 5 d growth because the utilization of GlcN was producing acid. The replacement of GlcN with proline resulted in deep blue alkaline colonies. The following indicator medium was therefore selected: GlcN (55 mM)/proline (33 mM)/EMB (pH 6.7). On this medium strain hOG1 gave pink to pale blue colonies after 7–8 d growth, indicating that the colonies were acid or neutral. Mutants impaired in GlcN metabolism grew as more intensely coloured blue colonies (see below).

Yeast cells of strain hOG1 were harvested in late exponential phase, centrifuged and suspended in 0.2 M-sodium acetate (pH 7.0) at approximately 10^8 cells ml^{-1} . *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was added to give a final concentration of 0.15 mg ml^{-1} . The culture was incubated for 90 min at 30°C , centrifuged and the pellet suspended in distilled water. The viability following this procedure was approximately 10%. The cells were plated on the GlcN/proline/EMB medium and screened for abnormal colour development. Colonies showing a different colouration from strain hOG1 were purified by replating on GlcN/proline/EMB medium, and then tested for growth on GlcN, GlcNAc and proline.

Induction of sugar uptake and enzymes for GlcNAc metabolism. The standard procedure for induction of GlcNAc permease and enzymes of the GlcNAc metabolic pathway involved incubating cells (8×10^7 ml^{-1}) for 3 h at 28°C in 10 mM-imidazole/HCl, pH 6.6 containing 3.0 mM-adenine and 0.1 mM-MnCl₂ (Gopal *et al.*, 1982). Sugars (2.5 mM) were added as indicated in the text.

Enzyme assays. Cell-free extracts were prepared and GlcNAc-6-phosphate deacetylase (EC 3.5.1.25) and GlcN-6-phosphate deaminase were assayed as described previously (Gopal *et al.*, 1982). GlcNAc kinase (EC 2.7.1.59) was assayed by the method of Shepherd *et al.* (1980b). GlcN kinase activity was measured by the same method except that the substrate was 40 mM-D-[1-¹⁴C]glucosamine hydrochloride (110000 c.p.m. per assay). For each enzyme, a unit of enzyme activity transforms 1 μmol reactant min^{-1} . Specific activity is given as units (mg protein)⁻¹. Protein was estimated by a modified Lowry method (Eggstein & Kreutz, 1967) with crystalline bovine serum albumin as a standard.

Uptake assays. The method used has been described previously (Sullivan & Shepherd, 1982). The substrates used were: *N*-acetyl-D-[1-¹⁴C]glucosamine (0.05 μCi ; 0.2 μmol), D-[1-³H]glucosamine hydrochloride (0.19 μCi ; 0.2 μmol) and D-[U-¹⁴C]glucose (0.05 μCi ; 0.2 μmol).

Materials. The following chemicals were obtained from Sigma: adenine, *N*-acetyl-D-glucosamine, glucosamine hydrochloride, methylene blue, eosin, proline and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The radiochemicals *N*-acetyl-D-[1-¹⁴C]glucosamine (58 mCi mmol^{-1}), D-[1-³H]glucosamine hydrochloride (2.9 Ci mmol^{-1}), D-[1-¹⁴C]glucosamine hydrochloride (54 mCi mmol^{-1}) and D-[U-¹⁴C]glucose (230 mCi mmol^{-1}) were purchased from Amersham (1 Ci = 3.7×10^{10} Bq). Bactopeptone was from Difco and human serum was provided by the Dunedin Public Hospital.

RESULTS AND DISCUSSION

Approximately 12000 mutagenized colonies of strain hOG1 were screened on the GlcN/proline/EMB indicator medium. Of these, 60 colonies showing abnormal colour development were purified and tested for growth on GlcNAc, GlcN and proline. On plates one mutant strain was impaired in its growth on GlcN but grew normally on proline. This strain, designated hOG298, was also markedly impaired in its growth on GlcNAc plates when compared with hOG1. Strain hOG298 showed a low spontaneous reversion frequency (10^{-5}) to the GlcNAc-utilizing state.

The growth rates and growth yields on glucose/salts/biotin were similar for hOG298 and the parent strain hOG1 (doubling time approximately 2.5 h) (Fig. 1). Both strains grew to the same extent on proline. The growth rate of strain hOG1 on GlcNAc was the same as on Glc but the growth of hOG298 on GlcNAc was slow (doubling time 14 h). Both of these strains showed GlcN-dependent growth but strain hOG298 was impaired (doubling time 14 h) when compared to strain hOG1 (doubling time 9 h; Fig. 1).

Fig. 1. Growth of strains of *C. albicans* on amino sugars. Strains hOG1 and hOG298 were grown on liquid cultures at 28 °C on salts/biotin/adenine (3 mM) and either Glc or GlcNAc or GlcN (all at 83 mM) as indicated below. The growth of hOG1 (closed symbols) and hOG298 (open symbols) was determined from the OD₅₄₀ of samples taken at the times indicated. The OD₅₄₀ values were converted to dry weight with reference to a standard curve. ●, ○, Growth on Glc; ▲, △, growth on GlcNAc; ■, □, growth on GlcN.

Table 1. Uptake of sugars by strains of *C. albicans*

Yeast cells of strains hOG1 and hOG298 were incubated for the induction of sugar uptake systems as described in Methods with the additions listed below. After 3 h incubation the cells were washed and used in uptake assays. Low rates of uptake [0–0.5 nmol min⁻¹ (mg dry wt)⁻¹] are given as <0.5. The values shown are the means of at least duplicate determinations.

Addition	Strain	Uptake [nmol min ⁻¹ (mg dry wt) ⁻¹]		
		Glc	GlcNAc	GlcN
Glc	hOG1	69	<0.5	2.6
	hOG298	42	<0.5	2.2
GlcNAc	hOG1	47	20	1.0
	hOG298	30	<0.5	1.4
GlcN	hOG1	43	9.9	4.3
	hOG298	25	<0.5	2.0

Table 1 summarizes the measurements of sugar uptake by strains hOG1 and hOG298 after preincubation of the cells under various conditions. The rate of glucose uptake varied from 43 to 69 nmol min⁻¹ (mg dry wt)⁻¹ in strain hOG1 and from 25 to 42 nmol min⁻¹ (mg dry wt)⁻¹ in strain hOG298. Induction of GlcNAc uptake was observed with strain hOG1 after preincubation with either GlcNAc or GlcN; strain hOG298 showed no induced GlcNAc uptake. Consistent with the slow growth rates on GlcN, the uptake rates for this sugar were lower than those for Glc and GlcNAc. Uptake of GlcN was, however, constitutive in both strains hOG1 and hOG298. The GlcN uptake rate was increased in strain hOG1 after preincubation in GlcN, but a similar incubation had no effect on strain hOG298.

Cell-free extracts of strains ATCC 10261, hOG1 and hOG298 were prepared from cells which had been incubated with GlcNAc to induce the enzymes of the GlcNAc pathway. The enzymes GlcNAc kinase, GlcNAc-6-phosphate deacetylase and GlcN-6-phosphate deaminase were induced in ATCC 10261 and strain hOG1. Only the deaminase was induced in strain hOG298 (Table 2). Cell-free extracts were also prepared from cells that had been incubated with GlcN. Constitutive GlcN kinase activity was present in both strains hOG1 and hOG298. However,

Fig. 2. Pathways for the catabolism of GlcNAc and GlcN. The enzymes involved in the steps shown are: (1) GlcNAc permease, (2) GlcNAc kinase, (3) GlcNAc-6-phosphate deacetylase, (4) GlcN-6-phosphate deaminase, (5) general sugar permease and (6) GlcN kinase.

Table 2. *Enzyme activities in strains of C. albicans*

Cell-free extracts were prepared from yeast cells of strains ATCC 10261, hOG1 and hOG298 which had been incubated in (a) 2.5 mM-GlcNAc or (b) 5 mM-GlcN for 3 h. The values in parentheses are for cell-free extracts from cells incubated in buffer plus 0.1 mM-MnCl₂ and 3 mM-adenine only; ND, not determined.

Enzyme	10 ⁻² × Specific activity [units (mg protein) ⁻¹]		
	Strain . . . ATCC 10261	hOG1	hOG298
(a) <i>Extracts from cells incubated with GlcNAc</i>			
GlcNAc kinase	7.1 (0.7)	ND	0.47 (0.4)
GlcNAc-6-phosphate deacetylase	7.1 (0.05)	5.8 (0.04)	0.05 (0.1)
GlcN-6-phosphate deaminase	6.3 (0.2)	7.1 (0.3)	5.3 (0.2)
(b) <i>Extracts from cells incubated with GlcN</i>			
GlcN kinase	ND	5.3 (1.9)	3.5 (3.6)
GlcNAc kinase	ND	1.5 (ND)	0 (ND)

incubation with GlcN increased the GlcN kinase activity in strain hOG1 but not in strain hOG298 (Table 2). GlcN also partially induced GlcNAc kinase in strain hOG1.

The pathway for the uptake and the metabolism of GlcNAc and GlcN is summarized in Fig. 2. Previous studies with wild-type strains showed that the rates of induction for the GlcNAc uptake system (Sullivan & Shepherd, 1982), the GlcNAc kinase (Shepherd *et al.*, 1980*b*), the deacetylase and the deaminase (Gopal *et al.*, 1982) were essentially the same. Although the uptake system and the first two enzymes of the pathway were not induced in strain hOG298 the mutant grew, albeit at a much slower rate, on GlcNAc. The slow growth could have been due to a leaky mutation or *C. albicans* could possess another pathway for the catabolism of GlcNAc. The deaminase was however induced in strain hOG298 by GlcNAc and the synthesis of this enzyme could be regulated independently from the other enzymes because this step is required for the catabolism of both GlcN and GlcNAc (Fig. 2).

Contrary to a previous report (Odds, 1979), GlcN was utilized by *C. albicans* but the growth rate was much slower than that attained with Glc and GlcNAc. GlcN is transported via a constitutive uptake system (T. M. Kirkpatrick, M. G. Shepherd & P. A. Sullivan, unpublished results) and cell-free extracts contained constitutive kinase activity for GlcN. These permease and kinase activities may be involved in the metabolism of hexoses such as Glc. GlcN induced GlcNAc permease and kinase activities in strain hOG1 but not in strain hOG298. It is possible GlcN may be a poor substrate for both the constitutive kinase and the inducible GlcNAc kinase. Low levels of the latter would then account for the altered GlcN phenotype of strain hOG298.

Consistent with this suggestion it was reported previously (Shepherd *et al.*, 1980*b*) that the induction of GlcNAc kinase activity also increased GlcN kinase activity.

Germ-tubes (>80% yields) were formed when strains hOG1 and hOG298 were incubated in 4% (v/v) serum at 37°C. Similar results were obtained when strain hOG1 was incubated in GlcNAc for 3–4 h but strain hOG298 did not form germ-tubes under these conditions.

It has been suggested (Simonetti *et al.*, 1974; Mattia *et al.*, 1982; Dabrowa & Howard, 1983) that the metabolism of GlcNAc may provide intermediary metabolites required for germ-tube formation. However, the gratuitous induction of germ-tube formation by *N*-acetylmannosamine (Sullivan & Shepherd, 1982) and GlcNAc covalently bound to agarose (Shepherd & Sullivan, 1983) showed that GlcNAc metabolism was not essential and that the GlcNAc signal was mediated via a surface receptor. The present study with strain hOG298 suggests that there is a common signal for GlcNAc metabolism and morphogenesis.

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