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Identification of the dialysable serum inducer of germ-tube formation in *Candida albicans*

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Yeast cells of *Candida albicans* are induced by serum at 37 °C to produce germ tubes, the first step in a transition from yeast to hyphal growth. Previously, it has been shown that the active component is not serum albumin but is present in the dialysable fraction of serum. In this study, serum induction of germ-tube formation is shown to occur even in the presence of added exogenous nitrogen sources and is therefore not signalled by nitrogen derepression. The active component in serum was purified by ion-exchange, reverse-phase and size-exclusion chromatography from the dialysable fraction of serum and was identified by NMR to be D-glucose. Enzymic destruction of glucose, using glucose oxidase, demonstrated that D-glucose was the only active component in these fractions. Induction of germ-tube formation by D-glucose required a temperature of 37 °C and the pH optimum was between pH 7·0 and 8·0. D-Glucose induced germ-tube formation in a panel of clinical isolates of *C. albicans*. Although D-glucose is the major inducer in serum, a second non-dialysable, trichloroacetic acid precipitable inducer is also present. However, whereas either 1·4 % (v/v) serum or an equivalent concentration of D-glucose induced 50 % germ-tube formation, the non-dialysable component required a 10-fold higher concentration to induce 50 % germ-tube formation. Serum is, therefore, the most effective induction medium for germ-tube formation because it is buffered at about pH 8·5 and contains two distinct inducers (glucose and a non-dialysable component), both active at this pH.

INTRODUCTION

The yeast *Candida albicans* is a ubiquitous commensal member of the human microflora but also an opportunistic pathogen that is able to cause both superficial and, in immunocompromised patients, systemic infections. It is the most common cause of fungal infections in humans (Navarro-Garcia *et al.*, 2001). *C. albicans* is capable of growing as budding cells, as pseudohyphae and as hyphal cells and it can undergo reversible transitions between these different forms. The ability to make the transition from budding to hyphal growth is essential for virulence (Lo *et al.*, 1997; Bahn & Sundstrom, 2001; Rocha *et al.*, 2001) and the first stage in this transition is the formation of a germ tube.

The formation of germ tubes can be triggered by a variety of inducers, including serum (Odds, 1988). The induction of germ-tube formation by *C. albicans* in serum was first described by Reynolds & Braude (1956) and was applied to the diagnosis of *C. albicans* infections soon after (Taschdjian *et al.*, 1960). Serum has been described as the 'magic potion' for induction of germ-tube formation by *C. albicans* (Ernst, 2000) because it is the most effective induction medium known (Gow, 1997), but the active components have never been identified. It has been suggested that the lack of readily available nitrogen may be the trigger for germ-tube formation in serum (Gow, 1997), but other reports have suggested that peptides (Barlow *et al.*, 1974), haemin (Casanova *et al.*, 1997), β-endorphin (Witkin & Kalo-Klein, 1991) or hormones (White & Larsen, 1997) may be the active component. Albumin is not the inducer because serum from a mutant rat lacking albumin can induce germ-tube formation while recombinant human albumin expressed and purified from *Pichia pastoris* can not (Feng *et al.*, 1999). Serum also triggers germ-tube formation in the yeast *Yarrowia lipolytica* (Perez-Campo & Dominguez, 2001) and the emerging fungal pathogen *Trichosporon beigelii* (Walsh *et al.*, 1994).

In *C. albicans*, the major route for assimilation of ammonium is via the glutamine synthetase/glutamate synthase pathway (Holmes *et al.*, 1991). Glutamine, which signals nitrogen repression (Marzluf, 1997), can also be utilized as

Abbreviations: ACCORD-HMBC, ACCORDIAN heteronuclear multiple bond correlation; COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; DQF-COSY, double-quantum filtered COSY; HMQC, heteronuclear multiple quantum coherence; J_{HH}, scalar proton–proton coupling constant.

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a sole nitrogen source (Limjindaporn *et al.*, 2003). Several lines of evidence suggest that germ-tube formation is not induced by nitrogen derepression. Not only do cells take up glutamine during glucose/glutamine-induced germ-tube formation (Sullivan *et al.*, 1983), but nitrogen derepression reportedly inhibits germ-tube formation (Holmes & Shepherd, 1987). The MAP kinase signal transduction pathway, which has been implicated in signalling nitrogen limitation on solid medium, is not required for germ-tube formation on serum agar medium (Csank *et al.*, 1998). The transcription factor *GAT1*, which activates gene expression in response to nitrogen derepression, is not required for germ-tube formation (Limjindaporn *et al.*, 2003) and in particular, the *gat1*Δ mutant is unimpaired in its ability to form germ tubes in serum.

Germ-tube formation in *C. albicans* is triggered by several independent signal transduction pathways that respond to different environmental cues but the receptors that act upstream of these pathways are not known (Liu, 2001; Ernst, 2000; Whiteway, 2000). The environmental cues include pH, temperature and specific chemical inducers (Odds, 1988). Serum induction of germ-tube formation may, therefore, result from signalling via more than one pathway. It has been possible, however, to characterize individual chemical inducers of germ-tube formation, for example *N*-acetylglucosamine (Cassone *et al.*, 1985), and therefore a reductionist approach was adopted in this present study to identify the chemical inducer present in serum.

METHODS

Organism and growth conditions. Strains of *C. albicans* used during this study were: A72 (Niimi *et al.*, 1998), ATCC 10261, ATCC 10231, IR40, SA310, 3153A (Olaiya *et al.*, 1980), five strains (CH3, OTG10, HUN61, HUN68 and Gay-mc) that do not belong to cluster A, as defined by Schmid *et al.* (1999), and eight strains (CLB42, Ysu568, Sc5314, OD8807, HUN96, FJ23, FJ26, RIHO10) that belong to cluster A. Strain A72 was used for the assay of serum fractions during purification of the serum inducer of germ-tube formation. All strains were maintained as glycerol stocks stored at −70 °C. For assessment of the germ-tube-inducing activity of different serum fractions, cells were grown overnight at 30 °C in liquid YPD medium (Ross *et al.*, 1990) that had been inoculated with a single colony. The culture was then stored at 4 °C for up to 3 days and aliquots of cells removed for use in the bioassay as required. These aliquots were centrifuged to remove spent medium, washed twice with sterile distilled water and then the cells were resuspended in sterile distilled water. An aliquot of cells from each new culture was tested for germ-tube formation with and without added serum (20 %, v/v). Occasionally a significant proportion of cells (typically 10–20 %) produced germ tubes in the absence of added serum and these cultures were discarded.

Purification of the serum inducer of germ-tube formation. Bovine serum (400 ml) was diluted to 20 % (v/v) with distilled water and fractionated on a Vivaflow 200 tangential-flow module (10 kDa cut-off membrane) set up for diafiltration. The retentate was recovered and stored at −20 °C. The permeant (5 l) (called F1) was applied, at a flow rate of 2 ml min^{-1}, to a DEAE-Sepharose column (5 × 53 cm, Pharmacia), that had been pre-equilibrated with 10 mM Tris/HCl buffer pH 9·0. The unbound fraction was collected, treated with Dowex SAX anion-exchange resin (40 g l^{-1}), filtered to remove the resin and lyophilized. The dry material was resuspended in one-hundredth the original volume of distilled water and filtered (0·2 μm). Trifluoroacetic acid (0·1 % w/v) was added and the sample was applied, in 1 ml aliquots at a flow rate of 1 ml min^{-1}, to a Jupiter C18 reverse-phase column (1 × 25 cm, Phenomenex) that had been pre-equilibrated with 0·1 % (w/v) trifluoroacetic acid. The absorbance of the column eluate was monitored at 230 nm and the leading unbound fraction from each run was collected and lyophilized. The dry material was dissolved in distilled water at a concentration of 50 mg ml^{-1}, treated sequentially with Dowex SCX cation-exchange resin (100 mg ml^{-1}) and Dowex SAX anion-exchange resin (100 mg ml^{-1}) and lyophilized. After each treatment with the ion-exchange resin the sample was filtered and centrifuged to remove the resin. The resin was washed with distilled water to recover entrapped inducer and the washes were added to the unbound fraction. The dry material was dissolved in one-tenth the original volume of 100 mM ammonium formate buffer pH 5·5 (nominally 500 mg ml^{-1}) and applied, in 0·1 ml aliquots at a flow rate of 0·5 ml min^{-1}, to a TSK G-Oligo PW column (0·78 × 30 cm, Phenomenex) that had been pre-equilibrated in the same buffer. The refractive index of the eluate was monitored and the leading peak was collected and lyophilized. The dry material was dissolved in the same buffer and reapplied, in 10-fold smaller aliquots, to the TSK G-Oligo PW column. The active peak (elution time 18 min) was collected, lyophilized, dissolved in buffer and reapplied to the column a third time to completely resolve the peak of active material (called F5) from the side fractions. Each time an eluate from the TSK G-Oligo PW column was lyophilized, the sample was dissolved in distilled water and lyophilized again, at least three times, to remove residual ammonium formate before further processing or assaying for activity.

Bioassay. The bioassay was performed in microtitre plates (Brightman & Dumbreck, 1989). The assay contained: yeast cells (about 10^6 cells ml^{-1}), $MgSO_4$ (1·20 mM), $CaCl_2$ (0·04 mM), $MnSO_4$ (0·014 mM) and the sample to be tested in a total volume of 103 μl. Assays in which glucose was the inducer also contained either 50 mM Bicine/NaOH buffer pH 8·0 or 50 mM potassium phosphate buffer pH 8·0 unless stated otherwise. The plates were covered with a plate sealer and incubated with shaking (250 r.p.m.) at 37 °C for 2 h. Germ-tube formation was assessed using an inverted microscope as described previously (Shepherd *et al.*, 1980). All assays were performed in duplicate. Immobilized *β*-D-glucose (Sigma) used in some experiments was linked to 4 % agarose beads via a 12 atom spacer. The pH of the serum used in this study was 8·5 and the concentration of glucose in this serum was 4·90 ± 0·35 mM (n = 22).

Glucose estimation and destruction. Glucose was specifically removed from selected samples using glucose oxidase. Samples to be treated were first adjusted to about pH 7·0 and then incubated in 2·5 mM phosphate buffer pH 7·0 with 2 mg recombinant glucose oxidase (Roche) ml^{-1} and 0·2 mg crystalline catalase (Roche) ml^{-1} for 4 h at room temperature. Glucose concentrations were determined before and after this treatment by the hexokinase/dehydrogenase method essentially as described by Chaplin (1986).

NMR analysis. All NMR experiments were performed at 25 °C with a Bruker Avance spectrometer operating at 400·13 MHz. The sample (approx. 0·4 mg F5) was dissolved in 0·5 ml D_2O. Data were also collected for an equilibrium mixture of approximately 30 mg D-glucose in D_2O.

Other methods. Ammonium ion concentrations were determined using the indophenol reagent as described previously (Farley &

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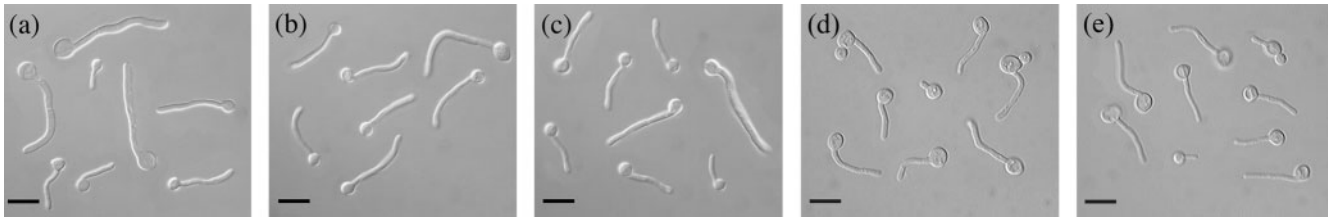

Fig. 1. Comparison of germ tubes induced by serum, serum containing an easily utilizable nitrogen source, buffered glucose and dialysed serum. Assays were performed as described in Methods with (a) 10 % (v/v) serum, (b) 10 % (v/v) serum containing 50 mM ammonium chloride, (c) 10 % (v/v) serum containing 50 mM aspartate, (d) 0·5 mM glucose in 50 mM Bicine buffer pH 8·0 and (e) 10 % (v/v) dialysed serum. Bar, 10 μm.

Santosa, 2002). Ultra-clean water was prepared by distillation over charcoal. Statistical significance was determined using Student's *t* test (http://www.graphpad.com/quickcalcs/index.cfm).

RESULTS

Germ-tube formation in serum supplemented with readily available nitrogen sources

Addition of either ammonium chloride or aspartate to the germ-tube assay (final concentrations between 5 and 50 mM) did not inhibit induction of germ tubes by 10 % (v/v) serum (100 % germ-tube formation with or without addition of the nitrogen source) (Fig. 1). In the case of ammonium chloride, the concentration of ammonium ions remaining at the end of the incubation was determined and found to be at least 90 % of that added to the assay.

Identification of the dialysable serum inducer

A 10 kDa cut-off membrane did not retain the serum component that induced germ-tube formation (Fig. 2).

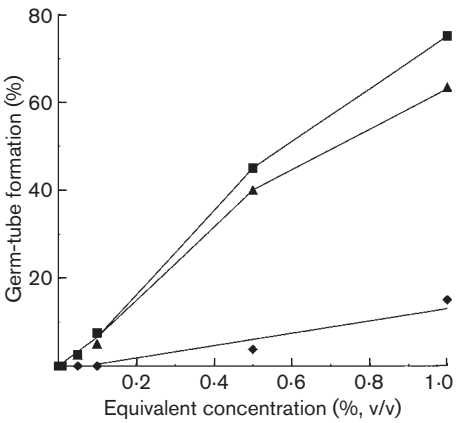

Fig. 2. Comparison of germ-tube induction by serum and serum fractions obtained by filtration. Assays were performed as described in Methods with serum (■) and either the filtrate (F1) (▲) or the fraction retained by a 10 kDa cut-off membrane (◆).

The permeant (F1) still contained a number of proteins, most of which could be removed, without significant loss of activity, by passage through a DEAE ion-exchange column (data not shown). Treatment of this fraction with a strong anion exchanger was essential for successful fractionation on the C18 reverse-phase column. This was discovered by trial and error, but possibly the glucose interacts with several components of serum, one of which was removed during this treatment, making the complex more labile and facilitating subsequent fractionation on the C18 column. All the activity applied to the C18 column was recovered in the unbound fraction. This fraction had no significant absorbance at either 260 nm or 280 nm and could be treated with a strong cation exchanger without loss of activity. The SCX treatment did, however, drop the pH of the solution from pH 7 to about pH 2 and the solution had to be neutralized before activity could be detected. This was accomplished by a subsequent treatment with the SAX anion exchanger. Fractionation of the deionized material on a TSK gel-filtration column gave a single active fraction that eluted at 18 min. To facilitate the purification, the column was deliberately overloaded with sample and the active fraction was rechromatographed twice to obtain a single symmetrical peak of active material (F5). The elution time for this material was coincident with that for glucose. The material in the active peak from the TSK column was heat stable (complete retention of activity after either 55 °C for 18 h or 2 h at 100 °C) and tested positive for reducing sugar. It was subjected to NMR analysis. One-dimensional ^{1}H, ^{13}C and ^{13}C-DEPT spectra displayed a set of major peaks consistent with a 5 : 3 mixture of monosaccharide epimers. Subsequent measurement of selective COSY, DQF-COSY, HMQC and ACCORD-HMBC spectra revealed a distribution of ^{1}H and ^{13}C chemical shifts and J_{HH} couplings consistent with D-glucose (Bock & Thøgersen, 1982). A comparison of the ^{1}H spectrum with that of a reference equilibrium mixture of D-glucose in D_2O is shown in Fig. 3. The ^{1}H spectra contained a number of low-intensity peaks distributed between 1 and 5 p.p.m. Additional correlated peaks at 8·32 p.p.m. and 172·6 p.p.m. in ^{1}H and ^{13}C spectra were consistent with a small amount of formic acid, most likely retained from the HPLC purification.

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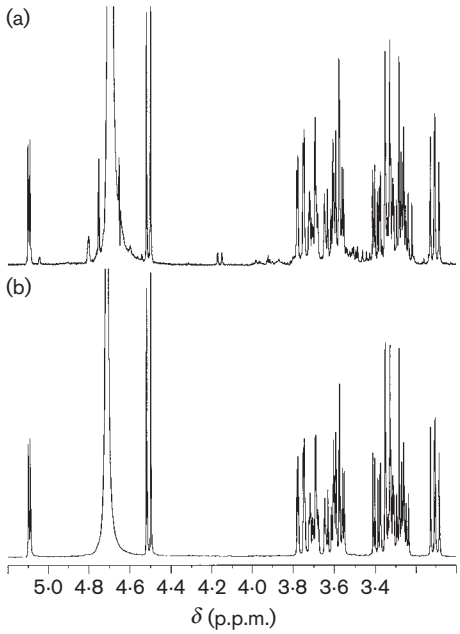

Fig. 3. Comparison of the ^{1}H NMR spectra of (a) the purified inducer and (b) a reference spectrum of D-glucose, both in D_2O.

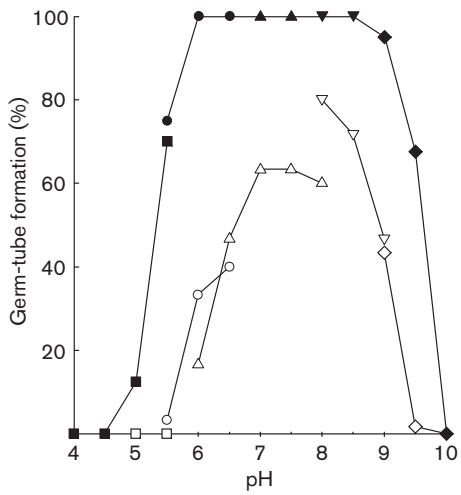

Fig. 4. Germ-tube formation induced by serum or glucose as a function of pH. Assays were performed as described in Methods with either 10 % (v/v) serum (filled symbols) or 0·5 mM glucose (open symbols) in the following buffers: potassium acetate, pH 4·0–5·5 (■, □); MES, pH 5·5–6·5 (●, ○); potassium phosphate, pH 6·0–8·0 (▲, △); Bicine, pH 8·0–9·0 (▼, ▽) and CAPSO, pH 9·0–10·0 (◆, ◇). All buffers were 90 mM.

Glucose oxidase treatment of serum fractions

Glucose oxidase treatment of fraction F5 destroyed both the glucose (5·65 mM before and not more than 0·015 mM after treatment) and the germ-tube-inducing ability of this fraction (80 % germ-tube formation pre-treatment compared to 0 % post-treatment). The glucose oxidase and catalase enzymes used to destroy the glucose did not themselves inhibit germ-tube formation. The extent of germ-tube formation induced by *N*-acetylglucosamine (70 % with 0·45 mM *N*-acetylglucosamine) was unaffected by pre-incubation of the *N*-acetylglucosamine with these two enzymes (75 % germ-tube formation). Treatment of glucose with catalase alone did not alter its effectiveness as an inducer of germ-tube formation. In addition, a reduction in glucose-induced germ-tube formation could be demonstrated in the presence of both glucose oxidase and catalase when a mixture of the enzymes was added directly to the assay (30 % compared to 70 % in the control). That some germ-tube formation was able to occur is presumably because the glucose was only being destroyed during the assay. Gluconolactone, produced during the destruction of glucose, neither induced germ-tube formation nor inhibited induction of germ-tube formation by either serum [100 % germ-tube formation in 10 % (v/v) serum containing 0·56 mM gluconolactone (the concentration of gluconolactone present in the assay following glucose oxidase treatment) compared to 100 % germ-tube formation in 10 % (v/v) serum alone] or glucose (60 % germ-tube formation in 0·5 mM glucose containing 0·5 mM gluconolactone and 50 mM Bicine pH 8 compared to 60 % germ-tube formation in buffered glucose alone).

More than 80 % of the serum germ-tube-inducing activity was recovered in F1 (Fig. 2). Glucose oxidase treatment of F1 destroyed both the glucose (4·13 ± 0·48 mM before and 0 mM after treatment) and the germ-tube-inducing ability of this fraction (80 % germ-tube formation pre-treatment compared to 0 % post-treatment, for the equivalent volume of F1). Glucose oxidase treatment of serum destroyed the glucose (0·00 ± 0·01 mM after treatment, $n=8$) and made a statistically significant difference ($P<0·05$) to the percentage of germ tubes formed in the treated serum (68 ± 16 %, $n=3$) compared to untreated serum (100 ± 0 %, $n=3$).

Glucose-induced germ-tube formation

Germ-tube formation was induced by glucose over a slightly narrower range of pH than for serum (Fig. 4). At pH 8·0 the concentration of Bicine buffer had no significant effect in the range 5–50 mM (data not shown) but the level of germ-tube formation induced by glucose buffered with Bicine at pH 8·0 (69 ± 7, $n=11$) was statistically significantly different ($P<0·001$) from that observed with glucose alone (7 ± 10, $n=11$). Germ-tube formation was dependent on glucose concentration (Fig. 5). The concentration of serum that gave 50 % germ-tube formation was 1·4 % (v/v); this corresponds to a glucose concentration of about 0·07 mM, which is similar to that required for 50 % germ-tube formation by buffered glucose (0·12 mM).

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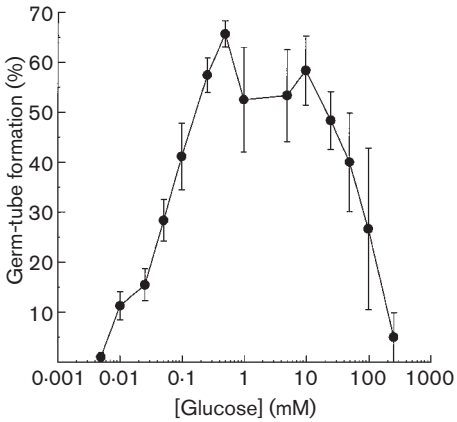

Fig. 5. Germ-tube formation as a function of glucose concentration. Assays were performed as described in Methods.

C. albicans cells can be induced to form germ tubes in 0·5 % serum (33 ± 26 %, $n=15$), which for bovine serum corresponds to a glucose concentration in the range 0·013–0·025 mM (Knowles *et al.*, 2000; http://amvetlab.com/bovine.htm#chem; this study). This concentration of glucose is sufficient to trigger germ-tube formation (Fig. 5). There was no statistically significant utilization of glucose during the time-course of the assay (5·19 ± 0·14 mM compared with 5·67 ± 0·39 mM in the control; $P>0·05$, $n=4$). No germ-tube formation was observed at 25 °C with either serum or buffered glucose over the time-course of the assay (2 h). Intriguingly, although high concentrations of glucose inhibited germ-tube formation in the buffered glucose assay, this was not observed when 10 % (v/v) serum was supplemented with additional glucose (data not shown). In 10 % (v/v) serum supplemented with additional glucose, no statistically significant utilization of glucose was observed (5·87 ± 0·25 mM compared with 5·97 ± 0·32 mM in the control; $P>0·05$, $n=4$). The length of germ tubes formed in buffered glucose (15·5 ± 5·5 μm, $n=40$) was statistically significantly different ($P<0·001$) from those formed in serum (34·8 ± 16·2 μm, $n=40$).

No germ tubes were formed when β-D-glucose immobilized on beaded agarose was added to cells in 50 mM Bicine pH 8·0, whereas a comparable concentration of free glucose induced 70 % germ-tube formation. Using cells grown in YPG medium (YPD medium in which the glucose was replaced by glycerol), no germ-tube formation was obtained with immobilized β-D-glucose in 50 mM phosphate buffer pH 8·0 nor with immobilized β-D-glucose to which 0·1 mM glycerol had been added. With these cells, 0·1 mM glycerol neither inhibited induction of germ-tube formation by free glucose (71 % germ-tube formation compared to 65 % in the control) nor induced significant germ-tube formation (1 %). Addition of free glucose, instead of glycerol, to the assay containing immobilized β-D-glucose induced germ-tube formation (50 % compared to 65 % for free glucose alone).

Neither 0·5 mM 3-*O*-methyl glucopyranoside, 0·5 mM methyl-α-glucopyranoside, nor 0·5 mM methyl-β-glucopyranoside induced germ-tube formation under the conditions used, nor did these methylated sugars inhibit germ-tube formation induced by glucose (data not shown). Addition of 0·5 mM 6-deoxyglucose to the assay did not inhibit germ-tube induction by glucose nor did 6-deoxyglucose itself induce germ-tube formation (data not shown).

Induction of germ tubes by dialysed serum

Glucose accounts for most (>80 %), but not all, of the germ-tube-inducing activity in serum. Fractionation of serum by dialysis or filtration on a 10 kDa cut-off membrane removed the free glucose but produced a fraction (the dialysed serum or retentate) that contained a low level of an inducer of germ-tube formation (Fig. 2). Increasing the concentration of dialysed serum in the assay increased the level of germ-tube formation (Fig. 6). However, whereas 50 % germ-tube formation was obtained with 1·4 % (v/v) serum (and a comparable concentration of glucose), this level of germ-tube formation required 14 % (v/v) dialysed serum. Induction of germ-tube formation by dialysed serum is due to a component of serum that is precipitable with trichloroacetic acid. After trichloroacetic acid precipitation of dialysed serum the supernatant (neutralized with NaOH) had lost its ability to induce germ tubes whereas the supernatant obtained after the same treatment of serum retained the ability to induce germ-tube formation. This was not due to the presence of an inhibitor in the supernatant from the dialysed serum because when 0·5 mM glucose was added to this supernatant, germ tubes were formed (60 % compared to 60 % for glucose alone and 0 % for the neutralized trichloroacetic acid supernatant alone). In addition, a solution of glucose treated with trichloroacetic acid in the same way as dialysed serum retained its ability to induce germ-tube

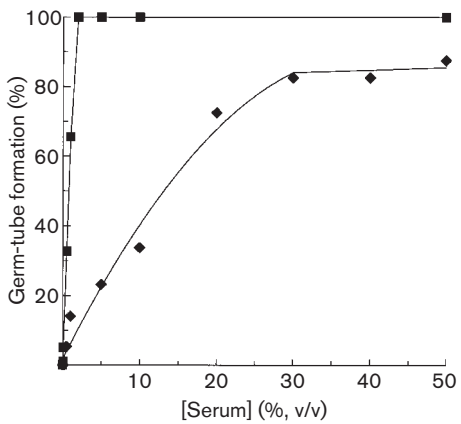

Fig. 6. Comparison of germ-tube induction by serum and dialysed serum. Assays were performed as described in Methods with serum (■) or dialysed serum (◆).

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Table 1. Induction of germ-tube formation in YPD and YP medium

Assays were performed as described in Methods. The final concentration of serum and dialysed serum was 10 % (v/v). The results are means ± SD for the number of experiments shown in parentheses.

Medium	Germ-tube formation (%)
Water	0 ± 0 (7)
Dialysed serum	45 ± 22 (4)
Buffered glucose (0·5 mM)	71 ± 13 (4)
Serum	100 ± 0 (7)*
YPD	40 ± 20 (7)
YPD + glucose (0·5 mM)	49 ± 23 (4)
YPD + dialysed serum	90 ± 9 (4)†
YPD + serum	90 ± 12 (7)†
YP	61 ± 14 (4)
YP + glucose (0·5 mM)	71 ± 14 (4)
YP + dialysed serum	94 ± 3 (4)‡
YP + serum	96 ± 5 (4)‡

*Statistically significantly different from the percentage germ-tube formation in dialysed serum (Student's *t* test, $P<0{\cdot}001$).
†Statistically significantly different from the percentage germ-tube formation in YPD (Student's *t* test, $P<0{\cdot}005$).
‡Statistically significantly different from the percentage germ-tube formation in YP (Student's *t* test, $P<0{\cdot}005$).

formation (75 % germ-tube formation before and 70 % after treatment). It should be noted that the supernatant obtained by trichloroacetic acid precipitation of serum would contain free glucose. The pH optimum for germ-tube induction by dialysed serum was pH 8·5 (data not shown).

Induction of germ-tube formation in YPD medium was examined (Table 1). The induction of germ tubes in either YPD medium containing serum or YPD medium containing dialysed serum was statistically significantly different from that obtained in YPD medium alone (Student's *t* test, $P<0{\cdot}005$). Whereas the percentages of germ-tube formation in serum and dialysed serum were statistically significantly different ($P<0{\cdot}001$), the percentages of germ-tube formation in YPD medium containing serum and YPD medium containing dialysed serum were not statistically significantly different ($P>0{\cdot}05$). As expected, because the additional glucose was less than 1 % of the total glucose in the medium, germ-tube formation in YPD medium containing additional glucose (equivalent to the amount in the serum added to YPD medium) was not statistically significantly different from germ-tube formation in YPD medium alone ($P>0{\cdot}05$). Similarly, addition of F1 to YPD medium did not promote germ-tube formation (30 % compared to 35 % for YPD alone and 100 % for F1 alone). The experiments with glucose, serum and dialysed serum were also performed in YP medium (YPD medium without the glucose) with identical results. The background level of germ-tube formation in YP medium was not statistically different from that obtained in YPD medium ($P>0{\cdot}05$). No germ-tube formation was observed at 25 °C in any of these media over the time-course of the assay (2 h).

Glucose-induced germ-tube formation in clinical isolates

A panel of clinical isolates of *C. albicans* and some additional laboratory strains were examined (Table 2). All strains formed germ tubes in serum, although the extent of germ-tube formation varied (from 4 % to 100 %) and all but one strain formed germ tubes in buffered glucose (the exception, strain CLB42, produced the least number of germ tubes in serum). In only one of the 19 strains tested (strain OTG10) was the level of germ-tube formation in F1 statistically significantly different from the level obtained with serum ($P<0{\cdot}01$). In all 19 strains tested there was no statistically significant difference between germ-tube formation in F1 and in buffered glucose ($P>0{\cdot}01$) and in 17 of the strains (A72 and Sc5314 were the exceptions) there was no statistically significant difference between germ-tube formation in buffered glucose and serum ($P>0{\cdot}01$). In eight strains, for which the percentage germ-tube formation in dialysed serum was statistically significantly different from that in serum ($P<0{\cdot}01$), addition of 0·5 mM glucose to the dialysed serum restored the percentage germ-tube formation to a level that was not significantly different ($P>0{\cdot}01$) from that observed in serum (Table 2). The effect on germ-tube formation of adding other carbon sources to dialysed serum was examined with strain A72. The percentage of cells that formed a germ tube in dialysed serum supplemented with either 1 mM glycerol or 0·5 mM sorbitol was no different from that observed for dialysed serum alone (data not shown).

DISCUSSION

Many media that induce *C. albicans* to form germ tubes, including serum, are poor in available nitrogen (Gow, 1997). However, supplementation of serum with a readily available nitrogen source (ammonium chloride) did not inhibit germ-tube formation. Nitrogen derepression, the absence of a readily available nitrogen source, is therefore either a redundant signal in serum-induced germ-tube formation or not a signal at all. This observation is consistent with previously reported genetic analysis of the MAP kinase signal transduction pathway implicated in signalling nitrogen limitation on solid medium (Csank *et al.*, 1998) and the transcription factor *GAT1*, which activates gene expression in response to nitrogen derepression (Limjindaporn *et al.*, 2003). Alternatively, any inhibitory signal from an easily utilizable nitrogen source (Ernst, 2000) is overridden by the inducer present in serum.

Fractionation of serum by filtration or dialysis demonstrated that most of the germ-tube-inducing ability of serum could be attributed to a dialysable component(s). The inducer passed through a 1 kDa cut-off membrane

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Table 2. Germ-tube induction in various *C. albicans* strains

Germ-tube formation was assayed as described in Methods. Induction with 0·5 mM glucose was performed in 50 mM Bicine buffer pH 8·0 except for two strains, where the buffer was 10 mM imidazole buffer pH 6·6 (indicated by an asterisk). The final concentration of serum and dialysed serum was 10 % (v/v). The concentration of F1 was equivalent to 10 % (v/v) serum. The concentration of glucose added to dialysed serum was 0·5 mM. The results are means ± SD for the number of experiments shown in parentheses.

Strain	Germ-tube formation (%)				
	Serum	F1	Dialysed serum	Glucose	Dialysed serum + glucose
Sc5314	92 ± 4 (3)	65 ± 28 (3)	22 ± 10 (2)†	51 ± 1 (3)†	93 ± 1 (2)
FJ26	82 ± 5 (3)	80 ± 6 (3)	28 ± 13 (2)†	45 ± 16 (3)	67 ± 4 (2)
FJ23	83 ± 2 (2)	73 ± 9 (2)	ND	69 ± 19 (2)	ND
HUN96	82 ± 14 (3)	79 ± 15 (3)	21 ± 29 (2)†	43 ± 17 (3)	29 ± 41 (2)
RIHO10	62 ± 36 (4)	37 ± 24 (4)	1 ± 1 (4)†	24 ± 24 (4)	21 ± 2 (4)
Ysu568	50 ± 43 (2)	72 ± 23 (2)	ND	68 ± 2 (2)	ND
OD8807	37 ± 35 (3)	22 ± 22 (3)	1 ± 0 (1)	18 ± 30 (3)	ND
CLB42	4 ± 1 (2)	2 ± 1 (2)	0 ± 0 (2)†	0 ± 0 (2)	ND
Gay-mc	85 ± 5 (4)	79 ± 10 (4)	5 ± 3 (3)†	70 ± 11 (4)	55 ± 29 (3)
HUN61	85 ± 4 (3)	82 ± 10 (3)	81 ± 0 (1)	79 ± 17 (3)	ND
CH3	73 ± 5 (2)	78 ± 9 (2)	ND	71 ± 1 (2)	ND
HUN68	79 ± 13 (2)	56 ± 20 (2)	ND	59 ± 6 (2)	ND
OTG10	71 ± 5 (3)	55 ± 1 (3)†	5 ± 6 (2)†	36 ± 20 (3)	31 ± 28 (2)
ATCC 10261	100 ± 0 (2)	92 ± 10 (3)	ND	63 ± 25 (2)*	ND
ATCC 10231	100 ± 0 (2)	97 ± 6 (3)	ND	63 ± 25 (2)*	ND
A72	95 ± 5 (8)	93 ± 4 (8)	23 ± 20 (5)†	86 ± 6 (8)†	82 ± 21 (5)
SA310	75 ± 23 (3)	81 ± 19 (3)	6 ± 3 (3)†	47 ± 20 (3)	22 ± 12 (3)
IR40	51 ± 37 (2)	22 ± 31 (2)	0 ± 0 (2)	25 ± 31 (2)	20 ± 5 (2)
3153A	25 ± 30 (4)	25 ± 20 (4)	1 ± 2 (4)	23 ± 20 (4)	8 ± 7 (4)

ND, Not determined.

†Statistically significantly different from the percentage germ-tube formation in serum (Student's *t* test, $P < 0{\cdot}01$).

(data not shown), as reported also by Feng *et al.* (1999), but a 10 kDa cut-off membrane was used to facilitate the processing of large volumes of serum. Subsequent fractionation of the filtrate by ion-exchange, reverse-phase and gel-filtration chromatography demonstrated that it contained a single active component that was identified as glucose. That the only active component in this fraction was glucose was then confirmed by the complete loss, in both the highly purified fraction (F5) and the crude filtrate (F1), of germ-tube-inducing activity following specific enzymic destruction of the glucose and by the demonstration that addition of glucose alone to dialysed serum was sufficient to restore germ-tube formation to a level indistinguishable from that obtained with serum.

Glucose has previously been reported to induce germ-tube formation in *C. albicans* (Hrmova & Drobnica, 1981; Vidotto *et al.*, 1996) and there have been reports of a link between candidiasis and both hyperglycaemia (Goswami *et al.*, 2000) and insulin-dependent diabetes mellitus (Guggenheimer *et al.*, 2000). Germ-tube formation in response to exogenous buffered glucose was observed in clinical isolates of *C. albicans*. Schmid *et al.* (1999) identified a geographically widespread genotype that predominated amongst isolates from patients with candidiasis. Of the strains with this genotype tested, all but one formed germ tubes in response to glucose. The exception, strain CLB42, produced only a few (< 5 %) germ tubes in serum. Of the other strains tested, all formed germ tubes in response to glucose. Although expression of a putative glucose transporter (orf19.3668) is up-regulated during germ-tube formation in serum (Nantel *et al.*, 2002), no significant glucose uptake was detected in this study and deletion of both copies of orf19.3668 did not prevent germ-tube formation in serum or buffered glucose (Q. L. Sciascia & P. C. Farley, unpublished).

Feng *et al.* (1999) implicated Ras1p-mediated signal transduction, via an adenylate cyclase pathway, as acting downstream of the dialysable serum inducer. The upstream receptor has not yet been identified but a glucose receptor is one possible candidate. In *Saccharomyces cerevisiae*, a glucose receptor, Gpr1p, located in the plasma membrane, is required for pseudohyphal growth (Palecek *et al.*, 2002). Activation of the downstream adenylate cyclase signal transduction pathway requires both a Gpr1p-mediated signal and an intracellular phosphorylated glucose signal (Rolland *et al.*, 2000). Two other glucose receptors are known in *S. cerevisiae*, Snf3p and Rgt2p, which have high and low affinity for glucose, respectively (Rolland *et al.*,

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2002). Signalling by these receptors induces expression of different members of the glucose transporter gene family. A putative *GPR1* orthologue (orf19.1944) and a large family of putative glucose transporters/receptors are present in the *C. albicans* genome (Fan *et al.*, 2002) but as yet only one member, a glucose transporter, has been functionally characterized (Varma *et al.*, 2000). Although a glucose sensor that is essential for germ-tube formation has yet to be identified, it seems unlikely that glucose is acting simply as a source of energy because (i) germ-tube formation can occur without significant uptake of the exogenous glucose, (ii) the expression of genes encoding glycolytic enzymes does not correlate with germ-tube formation (Swoboda *et al.*, 1994) and (iii) although *C. albicans* can grow on gluconolactone, glycerol or sorbitol, they can not be used in place of glucose to induce germ-tube formation.

A common protocol for induction of germ-tube formation by *C. albicans* is incubation of cells in YPD medium supplemented with 10 % serum (Lo *et al.*, 1997). The serum contribution to the total glucose present in this medium (2 %, w/v) is insignificant and has no effect on germ-tube formation. Rather, it is a non-dialysable component of serum that induces germ-tube formation under these conditions. Serum is, therefore, the 'magic potion' for the induction of germ-tube formation by *C. albicans*, not because of a lack of readily available nitrogen but because it is buffered at a slightly alkaline pH and contains two inducers that are both active at this pH, glucose and a non-dialysable component, the identity of which remains to be established.

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REFERENCES

Bahn, Y. S. & Sundstrom, P. (2001). *CAP1*, an adenylate cyclase-associated protein gene, regulates bud-hypha transitions, filamentous growth and cyclic AMP levels and is required for virulence of *Candida albicans*. *J Bacteriol* **183**, 3211–3223.

Barlow, A. J. E., Aldersley, T. & Chattaway, F. W. (1974). Factors present in serum and seminal plasma which promote germ tube formation and mycelial growth of *Candida albicans*. *J Gen Microbiol* **82**, 261–272.

Bock, K. & Thøgersen, H. (1982). Nuclear magnetic resonance spectroscopy in the study of mono- and oligosaccharides. *Annu Rep NMR Spectrosc* **13**, 1–57.

Brightman, C. A. J. & Dumbreck, L. A. (1989). The use of microtitre plates to observe germ tube formation in *Candida albicans*. *Med Lab Sci* **46**, 270–271.

Casanova, M., Cervera, A. M., Gozalbo, D. & Martínez, J. P. (1997). Hemin induces germ tube formation in *Candida albicans*. *Infect Immun* **65**, 4360–4364.

Cassone, A., Sullivan, P. A. & Shepherd, M. G. (1985). *N*-Acetyl-D-glucosamine-induced morphogenesis in *Candida albicans*. *Microbiologica* **8**, 85–99.

Chaplin, M. F. (1986). Monosaccharides. In *Carbohydrate Analysis: a Practical Approach*, pp. 1–36. Edited by M. F. Chaplin & J. F. Kennedy. Oxford: IRL Press.

Csank, C., Schroppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S., Thomas, D. Y. & Whiteway, M. (1998). Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect Immun* **66**, 2713–2721.

Ernst, J. F. (2000). Transcription factors in *Candida albicans* – environmental control of morphogenesis. *Microbiology* **146**, 1763–1774.

Fan, J., Chaturvedi, V. & Shen, S. H. (2002). Identification and phylogenetic analysis of a glucose transporter gene family from the human pathogenic yeast *Candida albicans*. *J Mol Evol* **55**, 336–346.

Farley, P. C. & Santosa, S. (2002). Regulation of expression of the *Rhizopus oryzae* uricase and urease enzymes. *Can J Microbiol* **48**, 1104–1108.

Feng, Q., Summers, E., Guo, B. & Fink, G. (1999). Ras signalling is required for serum-induced hyphal differentiation in *Candida albicans*. *J Bacteriol* **181**, 6339–6346.

Goswami, R., Dadhwai, V., Tejaswi, S., Datta, K., Paul, A., Haricharan, R. N., Banerjee, U. & Kochupillai, N. P. (2000). Species-specific prevalence of vaginal candidasis among patients with diabetes mellitus and its relation to their glycaemic status. *J Infect* **41**, 162–166.

Gow, N. A. (1997). Germ tube growth of *Candida albicans*. *Curr Top Med Mycol* **8**, 43–55.

Guggenheimer, J., Moore, P. A., Rossie, K., Myers, D., Mongelluzzo, M. B., Block, H. M., Weyant, R. & Orchard, T. (2000). Insulin-dependent diabetes mellitus and oral soft tissue pathologies: II. Prevalence and characteristics of *Candida* and candidal lesions. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **89**, 570–576.

Holmes, A. R. & Shepherd, M. G. (1987). Proline-induced germ tube formation in *Candida albicans*: role of proline uptake and nitrogen metabolism. *J Gen Microbiol* **133**, 3219–3228.

Holmes, A. R., McNaughton, G. S., More, R. D. & Shepherd, M. G. (1991). Ammonium assimilation by *Candida albicans* and other yeasts: a ^{13}N isotope study. *Can J Microbiol* **37**, 226–232.

Hrmova, M. & Drobnica, L. (1981). Induction of mycelial type of development in *Candida albicans* by low glucose concentration. *Mycopathologia* **76**, 83–96.

Knowles, T. G., Edwards, J. E., Bazeley, K. J., Brown, S. N., Butterworth, A. & Warriss, P. D. (2000). Changes in the blood biochemical and haematological profile of neonatal calves with age. *Vet Rec* **147**, 593–598.

Limjindaporn, T., Khalaf, R. A. & Fonzi, W. A. (2003). Nitrogen metabolism and virulence of *Candida albicans* require the GATA-type transcriptional activator encoded by *GAT1*. *Mol Microbiol* **50**, 993–1004.

Liu, H. (2001). Transcriptional control of dimorphism in *Candida albicans*. *Curr Opin Microbiol* **4**, 728–735.

Lo, H.-J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939–949.

Marzluf, G. A. (1997). Genetic regulation of nitrogen metabolism in the fungi. *Microbiol Mol Biol Rev* **61**, 17–32.

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Nantel, A., Dignard, D., Bachewich, C. & 12 other authors (2002). Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol Biol Cell* **13**, 3452–3465.

Navarro-Garcia, F., Sanchez, M., Nombela, C. & Pla, J. (2001). Virulence genes in the pathogenic yeast *Candida albicans*. *FEMS Microbiol Rev* **25**, 245–268.

Niimi, K., Shepherd, M. G. & Cannon, R. D. (1998). *Candida albicans HEX1* gene, a reporter of gene expression in *Saccharomyces cerevisiae*. *Arch Microbiol* **170**, 113–119.

Odds, F. C. (1988). *Candida and Candidosis*, 2nd edn. London: Baillière Tindall.

Olaiya, A. F., Steed, J. R. & Sogin, S. J. (1980). Deoxyribonucleic acid-deficient strains of *Candida albicans*. *J Bacteriol* **141**, 1284–1290.

Palecek, S. P., Parikh, A. S. & Kron, S. J. (2002). Sensing, signalling and integrating physical processes during *Saccharomyces cerevisiae* invasive and filamentous growth. *Microbiology* **148**, 893–907.

Perez-Campo, F. M. & Dominguez, A. (2001). Factors affecting the morphogenetic switch in *Yarrowia lipolytica*. *Curr Microbiol* **43**, 429–433.

Reynolds, R. & Braude, A. I. (1956). The filament inducing property of blood for *Candida albicans*; its nature and significance. *Clin Res Proc* **4**, 40.

Rocha, C. R. C., Schroppel, K., Harcus, D., Marcil, A., Dignard, D., Taylor, B. N., Thomas, D. Y., Whiteway, M. & Leberer, E. (2001). Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol Biol Cell* **12**, 3631–3643.

Rolland, F., de Winde, J. H., Lemaire, K., Boles, E., Thevelein, J. M. & Winderickx, J. (2000). Glucose-induced camp signaling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separate hexose kinase-dependent sensing process. *Mol Microbiol* **38**, 348–358.

Rolland, F., Winderickx, J. & Thevelein, J. M. (2002). Glucose-sensing and -signaling mechanisms in yeast. *FEMS Yeast Res* **2**, 183–201.

Ross, I. K., De Bernardis, F., Emerson, G. W., Cassone, A. & Sullivan, P. A. (1990). The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant. *J Gen Microbiol* **136**, 687–694.

Schmid, J., Herd, S., Hunter, P. R. & 13 other authors (1999). Evidence for a general-purpose genotype in *Candida albicans*, highly prevalent in multiple geographical regions, patient types and types of infection. *Microbiology* **145**, 2405–2413.

Shepherd, M. G., Chiew, Y. Y., Ram, S. P. & Sullivan, P. A. (1980). Germ tube induction in *Candida albicans*. *Can J Microbiol* **26**, 21–26.

Sullivan, P. A., Yin, C. Y., Molloy, C., Templeton, M. D. & Shepherd, M. G. (1983). An analysis of the metabolism and cell wall composition of *Candida albicans* during germ tube formation. *Can J Microbiol* **29**, 1514–1525.

Swoboda, R. K., Bertram, G., Delbruck, S., Ernst, J. F., Gow, N. A., Gooday, G. W. & Brown, A. J. (1994). Fluctuations in glycolytic mRNA levels during morphogenesis in *Candida albicans* reflect underlying changes in growth and are not a response to cellular dimorphism. *Mol Microbiol* **13**, 663–672.

Taschdjian, C. L., Burchall, J. J. & Kozinn, P. J. (1960). Rapid identification of *Candida albicans* by filamentation on serum and serum substitutes. *Am J Dis Child* **99**, 212–215.

Varma, A., Singh, B. B., Karnani, N., Lichtenberg-Frate, H., Hofer, M., Magee, B. B. & Prasad, R. (2000). Molecular cloning and functional characterization of a glucose transporter, *CaHGT1*, of *Candida albicans*. *FEMS Microbiol Lett* **182**, 15–21.

Vidotto, V., Accattatis, G., Zhang, Q., Campanini, G. & Aoki, S. (1996). Glucose influence on germ tube production in *Candida albicans*. *Mycopathologia* **133**, 143–147.

Walsh, T. J., Kelly, P., Peebles, R., Lee, J., Lecciones, J. & Pizzo, P. A. (1994). Biochemical and pharmacological factors causing induction and suppression of germination of *Trichosporon beigelii*. *J Med Vet Mycol* **32**, 123–132.

White, S. & Larsen, B. (1997). *Candida albicans* morphogenesis is influenced by estrogen. *Cell Mol Life Sci* **53**, 744–749.

Whiteway, M. (2000). Transcriptional control of cell type and morphogenesis in *Candida albicans*. *Curr Opin Microbiol* **3**, 582–588.

Witkin, S. S. & Kalo-Klein, A. (1991). Enhancement of germ tube formation in *Candida albicans* by β-endorphin. *Am J Obstet Gynecol* **164**, 917–920.

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