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Abstract

A method is described for the formation of nearly 100% pseudohyphae populations of wild-type *Candida albicans* A72. The method employs fungal growth at 37 °C (ca. 5×10^6 cells/ml) in a glucose–proline–*N*-acetyl-glucosamine medium supplemented with up to 600 mM phosphate (KH_2PO_4/K_2HPO_4 1:1) at pH 6.5. Four other strains of *C. albicans* (MEN, 10261, SG5314 and CAI-4) also formed pseudohyphae under these conditions, although the phosphate response profiles differed in the concentration required for each strain to form pseudohyphae.

Keywords: *Candida albicans*, High phosphate, Pseudohyphae, Pseudomycelia

1. Introduction

The polymorphic fungus *Candida albicans* is one of the most commonly isolated fungal pathogens in humans. It is the fourth leading cause of nosocomial infections and *Candida* bloodstream infections

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are widespread due to chemotherapy, extensive use of antibiotics and indwelling intravenous catheters (Kullberg and Filler, 2002). The ability to switch morphologies is essential for pathogenesis. *C. albicans* undergoes reversible morphological transitions between at least three forms: (i) ovoid, unicellular, budding yeast cells; (ii) filamentous hyphae; and (iii) pseudohyphae. Pseudohyphae always display constrictions at their septa between individual cellular compartments, unlike true hyphae which show no such constrictions (Brown, 2002). The developmental relationship between yeast, pseudohyphal, and hyphal cells remains to be resolved. Do pseudohyphae represent developmental intermediates between budding and hyphal growth forms or are they formed via a separate developmental pathway (Brown, 2002)? Interestingly, in terms of cell cycle dynamics, recent evidence indicates that the pseudohyphae are more similar to yeasts than to true hyphae (Sudbery, 2001). For yeast and pseudohyphae, the septin filament ring forms at the incipient bud site and then the first mitosis and septation occur at this site. In contrast, in true hyphae, the first mitosis and septation take place entirely within the germ tube, usually 10–15 Am from the mother cell (Sudbery, 2001). Pseudohyphae are frequently observed at low rates in cultures of *C. albicans*, but their role in pathogenesis is poorly understood. In part, this ambiguity arises from the difficulty in obtaining pure preparations of pseudohyphal cells for study. The present paper describes a high phosphate method whereby pseudohyphae of wild-type *C. albicans* A72 can be readily obtained in large amounts at nearly 100% purity.

2. Materials and methods

2.1. Strain maintenance and media

C. albicans A72 and 10261 were obtained from Patrick Sullivan, University of Otago, Dunedin. *C. albicans* MEN was from Richard Cannon, University of Otago, and strains SG5314 and CAI-4 were from Burk Braun, University of California at San Francisco. All stock cultures were grown in a modified glucose–salts–biotin medium and inocula were prepared as previously described by washing cells in 50 mM potassium phosphate buffer, pH 6.5, and storing them in the same buffer (Hornby et al., 2001). Media for CAI-4 were supplemented with

50 Ag/ml uridine. Pseudohyphal induction was examined by increasing the phosphate concentration in a modified glucose–phosphate–proline (GPP) growth medium containing as final concentrations: 2.5 mM KH_2PO_4 (pH 6.5), 10.2 mM L-proline, 2.6 mM *N*-acetyl-D-glucosamine and 3 mM $MgSO_4 \cdot 7H_2O$. After autoclaving, 0.5 ml of a 20% (w/v) glucose stock solution was added to give a final volume of 5 ml (111 mM glucose).

2.2. Bioassay

Cells were grown for 4 h in 5 ml prewarmed (37 °C) modified GPP. The unsupplemented phosphate concentration in our GPP bioassay was 2.5 mM, with another 0.10 mM introduced with the inoculum. Phosphate was added as KH_2PO_4/K_2HPO_4 (1:1) adjusted to pH 6.5. Cells (5×10^6/ml) were incubated at 37 °C with shaking at 200 rpm as described by Hornby et al. (2001). After 4 h, cellular morphology was examined by phase contrast microscopy. At least 100 cells were counted for each sample. Initial characterization of yeast versus hyphae versus pseudohyphae was done microscopically using the criteria described by Odds (1988). Briefly, budding yeasts are the unicellular form of the fungus that exist as ovoid cells with a forming growth, or bud, which is smaller than the mother cell. Hyphae contain multiple cell units divided by septa. New growth arises from the mother cell in the form of a cylinder, or germ tube. Germ tubes have characteristic parallel walls. Pseudohyphae resemble hyphae but in the process of pseudohyphal development, each generation of buds remains attached to its parent leading to chains of progeny yeast, which are also called bud-on-bud. The distinction from hyphae is that pseudohyphae have constrictions at the septae, whereas hyphae have parallel walls that constitute the germ tube. Subsequent characterization of the pseudohyphae used the more quantitative measurements of Merson-Davies and Odds (1989).

3. Results

For *C. albicans* A72 cells growing in modified GPP, the percent pseudohyphae in the culture increased continuously at elevated phosphate concentrations (Fig. 1). There was a roughly linear increase in percent

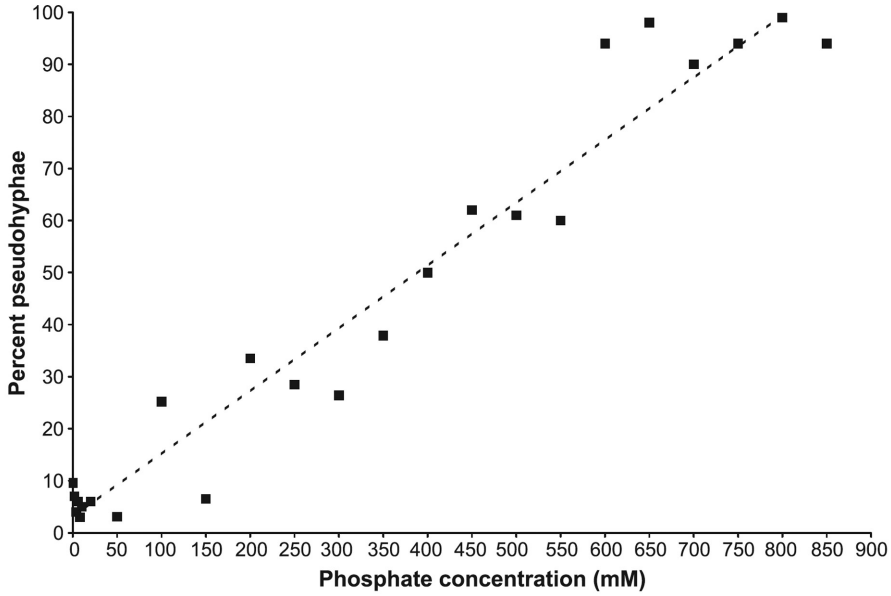

Fig. 1. Influence of high phosphate concentrations on the percentage of pseudohyphae for *C. albicans* A72. Cells were grown for 4 h in a modified GPP medium with an unsupplemented phosphate concentration of 2.5 mM. Values represent the average of four or more measurements made by two researchers over a 3-year time span. Dashed line represents linear regression of data for percent pseudohyphae.

pseudohyphae up to 600 mM added phosphate, at which time nearly 100% of the cells were pseudohyphae (Fig. 1). *C. albicans* A72 was still able to grow in GPP with up to 850 mM added phosphate, the highest concentration tested. The degree of elongation can vary considerably for pseudohyphal cells (Brown, 2002) and, in this regard, we observed a steady progression from the relatively elongated pseudohyphae present at 100 mM added phosphate (Fig. 2b) and 300 mM added phosphate (Fig. 2c) to the short, thick pseudohyphae present at 600 mM added phosphate (Fig. 2d). A more objective measure of cell morphology is the morphology index ls/d^2 developed by Merson-Davies and Odds (1989), where l and d are the length and maximum diameter of a compartment and s is the diameter at the septal junction. The morphology index values are usually 1.0–1.5 for yeast cells, 2.5–3.4 for pseudohyphae and greater than 3.4 for hyphae (Merson-Davies and Odds, 1989). Microscopic examination of the pseudohyphae formed by high phosphate-grown cells indicated that the ls/d^2 values were between 2.8 and 3.0 at all added phosphate concentrations tested from 50 mM up to 600 mM.

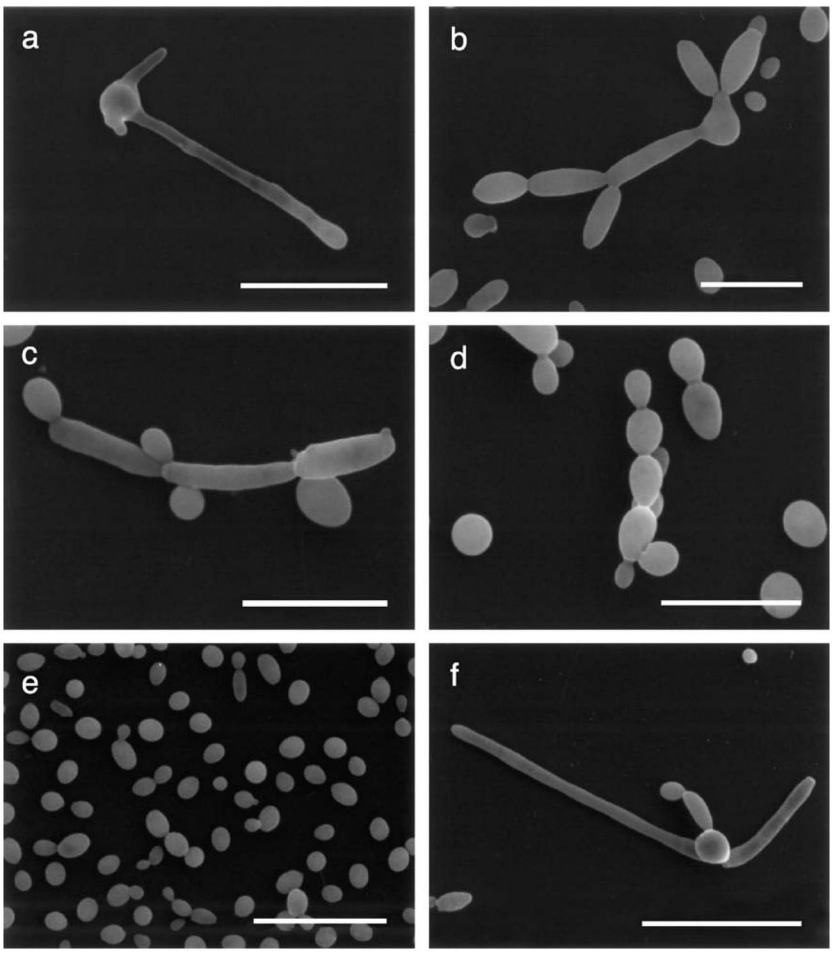

Fig. 2. (a– f) Pseudohyphae development in the presence of elevated potassium phosphate buffer, pH 6.5. Scanning electron micrographs with dimension bars = 5 μm. Cultures were grown for 18 h at 37 °C in GPP supplemented with: (a) no added phosphate, (b) 100 mM added phosphate, (c) 300 mM added phosphate and (d) 600 mM added phosphate. For comparison, (e) single celled budding yeasts (30 °C) and (f) germ tubes/hyphae as triggered by *N*-acetyl-glucosamine, are also shown.

With regard to chemical specificity, we routinely used KH_2PO_4/K_2HPO_4 (pH 6.5) for the data presented in Figs. 1 and 2. However, pseudohyphae were produced at equivalent levels with potassium, sodium and ammonium phosphate, all pH 6.5, while no pseudohyphae were produced with 0–600 mM potassium chloride, sodium sulfate or magnesium sulfate or with 15% sucrose, sorbitol or PEG. In each case, the growth was entirely as hyphae. Therefore, phosphate is the operative ion for pseudohyphal formation.

Table 1 High phosphate induction of pseudohyphae in four strains of *C. albicans*

Strain	Concentration of potassium phosphate added[a]			
	0 mM	100 mM	300 mM	600 mM
MEN	≥96% GT[b]	76% PH	30% PH	
		22% BY	70% BY	40% BY
		2% GT		60% Y
10261	≥96% GT	95% PH	95% PH	
		5% BY	5% BY	40% BY
60% Y				
SG5314	≥96% GT	50% PH	95% PH	
			5% BY	50% BY
		50% GT		50% Y
CAI-4	≥96% GT	95% PH		
		5% BY	5% BY	5% BY
			95% Y	95% Y

a. All experiments were conducted in the modified GPP growth medium described in Materials and methods. The medium for CAI- 4 was supplemented with uridine (50 Ag/ml).

b. PH = pseudohyphae, BY= budding yeasts, GT= germ tubes and Y= undifferentiated single cells.

With regard to strain specificity, the high phosphate method for pseudohyphae worked for *C. albicans* A72 (Fig. 1) and it also worked for each of the other four strains of *C. albicans* we tried, i.e. strains 10261, MEN, SG5314 and CAI-4 (Table 1). However, the phosphate levels needed to achieve 50% pseudohyphae varied greatly for the respective strains. Strain A72 required the highest phosphate levels (400 mM, Fig. 1), while the other strains required ≤100 mM (Table 1). For each strain, the morphological progression with increasing phosphate was germ tubes to pseudohyphae to budding yeasts to undifferentiated yeasts.

The questions of strain specificity and medium specificity are related. All five of the *C. albicans* strains tested (A72, 10261, MEN, SG5314 and CAI- 4) formed pseudohyphae in high phosphate, modified GPP (Fig. 1 and Table 1). However, only strain A72 responded in this fashion in the non-modified GPP growth medium (10) or in the *N*-acetyl-glucosamineimidazole differentiation medium (10). Significantly, the non-modified GPP growth medium does not contain *N*-acetyl-glucosamine and the differentiation medium does not contain proline, whereas the modified GPP contains both.

4. Discussion

Most fungi grow best in media with phosphate concentrations from 0.3 to 300 AM (Garraway and Evans, 1984) and thus the phosphate levels we are employing for 100% pseudohyphae are at least 2000 times higher than optimal. The ''normal'' operating range of the phosphate sensing component in a phosphate responsive signal transduction pathway of *Saccharomyces cerevisiae*, Pho81 provides a useful comparison (Lenburg and O'Shea, 1996). This system turns on extracellular phosphatases in low phosphate (200 AM) environments and then turns them off in high phosphate (11 mM) environments. Furthermore, phosphate transport rates in *S. cerevisiae* reach a maximum at an external phosphate concentration of 1 mM (Schmidt et al., 1949).

There are many possible mechanisms by which 100–600 mM added phosphate could promote pseudohyphae formation in *C. albicans*. These mechanisms could be either direct or indirect and they could act either as positive regulators for pseudohyphal growth or negative regulators for hyphal growth. The following ideas are neither inclusive nor mutually exclusive. (i) The Pho81 system for sensing external phosphate has been well characterized in *S. cerevisiae* (Lenburg and O'Shea, 1996). However, there could be another morphology related phosphate-sensing system in *C. albicans*. The genome of *C. albicans* contains ca. 2000 more genes than does that of *S. cerevisiae*. (ii) High external phosphate may elevate the cytoplasmic phosphate pool which is normally ca. 15 mM in *S. cerevisiae* (Rothstein, 1955). By mass action this elevated P^i could raise the energy charge (more ATP), which could, in turn, activate a protein kinase or the elevated P^i could inhibit a protein phosphatase. (iii) High phosphate could regulate production of a metabolite, which, in turn, regulates the hyphal/pseudohyphal ratio, e.g. high P^i turns off the production of ethylene by *Penicillium digitatum* (Chalutz et al., 1978). (iv) The effect could be polyphosphate-mediated. Inorganic polyphosphates (poly P) are linear polymers of hundreds of orthophosphate (P_i) residues linked by high-energy phosphoanhydride bonds (Kulaev, 1979). They are present in most living organisms, including fungi, and in yeast cells they may represent up to 10–20% of cellular dry weight, with 99% of the total poly P being present in the yeast vacuole (Kornberg, 1995; Urech

et al., 1978). However, the metabolic functions of poly P in yeasts are still poorly understood (Kornberg, 1995). In one scenario, the polyanionic vacuolar poly P would function as a strong chelator of metal ions such as Ca^{2+} or Mn^{2+}. Poly P acts as a Ca^{2+} sink within the vacuole, leading to the regulation of cytoplasmic Ca^{2+} by the yeast vacuoles (Dunn et al., 1994). This connection should be important in cell morphology because Muthukumar and Nickerson (1984) showed that a Ca^{2+}-calmodulin interaction was necessary for mycelial growth in *Ceratocystis ulmi*, the causative agent of Dutch elm disease. When the Ca^{2+}-calmodulin interaction was prevented, by drugs, chelating agents or Ca^{2+} starvation, the cells shifted to the yeast morphology (Muthukumar and Nickerson, 1984). Alternatively, poly P also binds Mn^{2+} and it has been suggested (Kornberg, 1995) that the Mn^{2+}-poly P complex acts as an inorganic catalyst for the removal of damaging superoxide radicals. Finally, in the yeast *Saccharomyces carlsbergensis*, there is a correlation between poly P accumulation and the synthesis and phosphorylation of cell wall mannoproteins (Kulaev et al., 1987). This link is attractive because it has long been known (Nickerson, 1963) that the cell wall mannoproteins play a major role in morphogenesis in *C. albicans*.

Regardless of its mode of action, our high phosphate protocol provides a reproducible means of obtaining large quantities of uniform 100% pseudohyphal cells from wild-type *C. albicans* A72. All previously reported methods for pseudohyphal production involve genetic manipulation and knock-out mutations. For instance, mutants of *C. albicans* lacking the forkhead transcription factor CaFkh2p formed constitutive pseudohyphae under all yeast and hyphal growth conditions tested (Bensen et al., 2002). One model suggested by these authors is that Fkh2p is a negative regulator of pseudohyphal growth and that this negative regulation is required for the formation of both yeast and true hyphal cells, i.e. that pseudohyphal growth may be a default pathway that must be repressed in order for yeast or true hyphal growth to proceed (Bensen et al., 2002).

Our protocol for pseudohyphal production will allow researchers to compare the biochemical composition and gene expression profiles of pseudohyphal cells versus those from yeasts and true hyphae. It also should help in establishing the role of pseudohyphae in biofilm formation and pathogenesis as well as the developmental relationships between pseudohyphae, yeasts and true hyphae.

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