Depolarized cell growth precedes filamentation during the process of ethanol-induced pseudohyphal formation in the yeast *Candida tropicalis*

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Ethanol has been reported to cause mycelial growth in *Candida tropicalis* Pk233, and mycelial growth has also been shown to be abolished by concomitant addition of myo-inositol. In this study, the process of ethanol-induced mycelial growth in this organism was examined in combination with cytological characterization of actin localization. Cultivation with ethanol gave biphasic growth curves. During the first growth phase (doubling time 2.4 h), there was an accumulation of swollen spherical yeast cells, instead of the oblong ones observed in the control culture, followed by the appearance of spherical daughter cells in chains. Randomly distributed actin patches were observed on these swollen yeast cells and the bud initiation sites of these cells appeared random. These observations suggested that ethanol caused depolarization of cell growth during the first phase. During the second growth phase (doubling time 7.4 h), pseudohyphal cells appeared, projecting from the swollen yeast cells. Activity of chitinase in the control culture rose during the exponential phase. In the ethanol culture the activity stayed at a low level throughout the growth phases. When pseudohyphal cells were transferred to fresh ethanol medium, yeast cells appeared from pseudohyphal filaments and changed their shape to spherical, and filamentation appeared to be inhibited during the first phase. From these observations, an initial effect of ethanol on *C. tropicalis* cells appeared to be depolarization of cell growth, and the resulting swollen cells grew as polar pseudohyphal cells. In the culture supplemented with both ethanol and inositol, or with both ethanol and sorbitol, the accumulation of swollen cells was not observed and single yeast cells with normal oblong shape were seen throughout the growth phases.

Keywords: *Candida tropicalis*, hyphal growth, ethanol, chitinase, actin localization

INTRODUCTION

Dimorphic transitions in fungi describe the ability of fungal cells to alternate between different growth patterns, especially to grow either as a yeast form or as a mycelial one. There has been great interest in fungal

dimorphism as a simple model of cellular morphogenesis (Harold, 1995).

The yeast-to-pseudohyphal transition seems to be coupled to the control of polarity in cell growth. When a bud has grown to two-thirds of its circumference, the apical growth zone shuts down and general growth completes bud expansion during yeast growth. In marked contrast, during pseudohyphal growth, the apical growth zone does not shut down as long as the daughter cell grows in the pseudohyphal form (Gimeno *et al.*, 1992). Bud site assembly and yeast growth are

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Abbreviations: DAPI, 4',6'-diamidino-2-phenylindole; Rh-Ph, rhodamine-phalloidin.

coupled to reorganization of the actin cytoskeleton; and the distribution of actin cytoskeleton changes through the cell cycle in *Saccharomyces cerevisiae* (Novick & Botstein, 1985; Drubin, 1991; Evangelista *et al.*, 1997), as well as in *Candida albicans* (Anderson & Soll, 1986). Actin localization has been suggested to play a role in cell wall deposition and growth in both organisms. Especially, there is a strong correlation between occurrence of active growth at the bud tip and clustering of cortical actin patches in *S. cerevisiae* (Qadota *et al.*, 1996). In terms of function, the actin cytoskeleton has been implicated in maintenance of cell polarity, especially with respect to membrane growth and protein secretion, in changes in cell shape, in resistance to osmotic forces, and in response to environmental signals (Botstein *et al.*, 1997).

The dimorphic transition from yeast to pseudohyphae in the petroleum-assimilating yeast *Candida tropicalis* occurs following the addition of ethanol (Tani *et al.*, 1979; Yamada *et al.*, 1982) but the addition of inositol prevents the ethanol-induced pseudomycelial formation (Tani *et al.*, 1979; Omi & Kamihara, 1989). This ethanol-induced dimorphic transition can be controlled in liquid cultures and seems to be a suitable system for physiological and cytological studies on individual cells during the process of dimorphic transition. In this study, actin distribution on individual cells during the process of ethanol-induced pseudohyphal development was examined, and chitinase activity in cell wall fractions of the cultures was monitored.

METHODS

Strains and culture conditions. The strain used in this study was *Candida tropicalis* Pk233. Yeast cells were precultured as colonies on YPD solid medium as described previously (Suzuki *et al.*, 1991). Inocula were prepared by seeding a loopful of cells into 100 ml synthetic medium (pH 6.0) containing (g l⁻¹): KH₂PO₄, 3.4; Na₂HPO₄, 3.9; glucose, 20; vitamin-free Cas-amino acids (Difco), 5; (NH₄)₂SO₄, 5; MgSO₄·7H₂O, 0.125; FeCl₃·6H₂O, 0.025; KCl, 0.425; CaCl₂·2H₂O, 0.125; and biotin, 20 × 10⁻⁶. Inoculated cultures were incubated at 30 °C with shaking (90 r.p.m.) for approximately 30 h. They were then diluted 200-fold into a fresh synthetic medium (control medium) and incubated at the same temperature (control culture). The ethanol medium was prepared by adding ethanol (25 ml l⁻¹) to the control medium, and the ethanol-inositol medium by adding *myo*-inositol (5 mg l⁻¹) to the ethanol medium. To examine the transition from hyphae to yeast, an ethanol culture which had been incubated for 48 h was diluted 100-fold into fresh medium (control medium or ethanol medium). At various times during incubation, 5–10 ml portions of each culture were taken for measuring chitinase activity and for fixation by adding an equal volume of 6% (w/v) formaldehyde solution in 0.2 M potassium phosphate buffer (pH 6.5). The fixed sample was used for monitoring growth by measuring turbidity at 660 nm and for the observation of cell shape by phase-contrast microscopy.

Measurement of chitinase activity. Chitinase activity was measured in cell wall fractions of the cultures, according to the method described by Kuranda & Robbins (1991). Samples of culture fluid containing approximately 2 × 10⁸ cells were

centrifuged at 3000 r.p.m. for 5 min at 4 °C. Harvested cells were suspended in 200 µl solution A, consisting of 0.8% NaCl, 0.02% KCl, 0.12% Na₂HPO₄, 0.02% KH₂PO₄ and 0.1% Triton X-100. Acid-washed quartz sand was added to a level approximately 2 mm below the meniscus. The suspension was vortexed at maximum speed for 30 s at 4 °C. The tube was placed on ice for 1–2 min. This procedure was repeated four times. The supernatant was recovered with a pipette and the residue washed twice with 0.5 ml solution A. The supernatants were pooled and pelleted by centrifugation at 16000 r.p.m. for 1 min. The cell wall pellet was then washed twice with 1 ml solution A by centrifugation, and finally suspended in 200 µl 0.1 M sodium citrate buffer, pH 3.0. Portions (20 µl) of cell wall suspensions were mixed with 80 µl 250 µM 4-methylumbelliferyl β-D-N,N',N''-triacylchitotrioxide in 0.1 M sodium citrate buffer (pH 3.0), and incubated at 30 °C for 1 h. The reaction was stopped by adding 2.9 ml 0.5 M glycine/NaOH buffer (pH 10.4). Debris was removed by centrifugation at 15000 r.p.m. for 5 min. The liberated 4-methylumbelliferone (4MU) was measured with a Hitachi F-2000 spectrophotofluorometer (excitation at 350 nm, emission at 440 nm). The calibration curve was calculated by fluorescence of standard concentrations of 4MU and chitinase activity was defined as nmol 4MU released min⁻¹.

Fluorescence microscopy. To obtain well-fixed specimens for observing actin filaments, the microwave fixation method (Mizuhira *et al.*, 1994) was used. Culture media were transferred into a test tube, and mixed with an equal volume of cold 0.2 M potassium buffer (pH 6.5) containing 6% (w/v) formaldehyde and 6% (w/v) DMSO. The test tube was immediately floated on ice-water, exposed to microwaves for 30 s using a Hitachi electronic oven (500 W), and then incubated for 20 min at 37 °C. Fixed specimens were washed three times with PBS (10 mM phosphate buffer, pH 6.9, containing 137 mM NaCl and 2.7 mM KCl) by centrifugation (3000 g for 5 s). The cells were stained with rhodamine-phalloidin (Rh-Ph; Molecular Probes) which had been diluted 1:20 in PBS, at 25 °C for 3–4 h. For double-staining, the cells were stained for 3–4 h with Rh-Ph solution (1:20 in PBS) containing either Calcofluor White at 0.5 mg l⁻¹ or 4',6'-diamidino-2-phenylindole (DAPI) at 0.5 mg l⁻¹. Stained samples were examined with an epifluorescence microscope equipped with a phase-contrast apparatus (BH-2-RFL; Olympus).

RESULTS

Biphasic growth of *C. tropicalis* Pk233 in the ethanol culture

To clarify the events leading up to the formation of pseudohyphae, cell morphology was examined at various times in ethanol cultures and control cultures. At zero time, when 2.5% ethanol was added and the total count of inoculated cells was 2 × 10⁶ ml⁻¹, unbudded cells accounted for 90% of the cell population, since the inoculum was from a stationary-phase culture. After 2 h incubation, the control culture entered the exponential phase. However, in the case of the ethanol culture, the lag phase lasted 3–4 h (Fig. 1a). Whereas the control culture showed apparently monophasic growth, the ethanol culture showed biphasic growth, changing when the OD₆₀₀ of the culture reached around 1.0, at 12–15 h after inoculation (Fig. 1a). The doubling time in the mid-exponential phase was 1.8 h in the control culture. In the

Fig. 1. Growth curve (a) and chitinase activity (b) of *C. tropicalis* Pk233 grown in control medium (○), ethanol medium (△) or ethanol-inositol medium (●).

ethanol culture, doubling time was 2.4 h during the first phase and 7.4 h during the second phase.

The control culture did not show any significant presence of pseudohyphal cells throughout its growth cycle (Fig. 2a, b). Pseudohyphal cells appeared in the ethanol culture at 16–20 h, when the culture entered into the second phase (Fig. 2e). As Tani *et al.* (1979) had described, the production of pseudohyphal cells depended on the continuous presence of the ethanol. During the first growth phase, individual yeast cells appeared to alter their shape from ellipsoidal to spherical (Fig. 2b, c). New-born daughter buds from spherical mother cells swelled isodiametrically. Until a late stage of the first phase of growth in the ethanol culture (12–15 h), several spherical yeast cells formed a chain, and small filamentous daughter buds appeared from the cells (Fig. 2d). Each cell of the chain increased in volume until the end of the first growth phase. These features were quite different from those of ellipsoidal yeast cells in the control culture, which were separated from each other (Fig. 2a, b). Until the inflection period of the ethanol culture, three to four spherical cells were in chains. After the culture entered the second phase of growth curve, polar elongated daughter cells grew from chained cells, and became pseudohyphae (Fig. 2d, e). Some of the chained cells changed their shape from spherical to spatulate or pyriform and produced a

pseudohyphal cell at their tips (Fig. 2e). Until 30 h of incubation in the ethanol culture, many of the swollen cells produced more than two daughter pseudohyphal cells and each of the pseudohyphal ones had two or three septa (Fig. 2e).

Cytological characterization of ethanol-induced pseudohyphal formation

To clarify the morphological events occurring prior to the production of pseudohyphal cells, especially on the chained spherical cells, individual cells were examined for their distribution pattern of actin patches, because the pattern should reflect polarity of cell growth, as has been shown in a related species, *C. albicans* (Anderson & Soll, 1986; Yokoyama *et al.*, 1990; Akashi *et al.*, 1994). The cells of the control culture were classified into six types according to their pattern of actin localization: type A was characterized by edge granules in a pre-elongated cell (Fig. 3a), type B by clustered granules in a pre-elongated cell (Fig. 3b), type C by generally distributed granules in a small bud (Fig. 3c), type D by edge granules in bud (Fig. 3d), type E by an apical cluster in an elongated bud (Fig. 3e), and type F by the presence of large neck granules on a septum (Fig. 3f). Budding sites of cells of the control culture were limited to the cell poles. At 15 h after ethanol addition, just before growth shifted to the second phase, formation of chains consisting of three to five yeast spheres occurred in the ethanol culture (Fig. 2c, Fig. 3g, h). Rh-Ph-stained cells at this period of the culture showed spherical swollen cells with edge actin granules (Fig. 3g–i). Small buds of the swollen cells had clustered granules. These swollen cells in a chain were counted as one chain and classified into three types according to the number of cells. If the size of a bud in a chain was at least two-thirds the size of the mother cell of type F in the control culture, the bud was regarded as mature and counted as one cell, since DAPI staining of such a bud showed a nucleus. Actin patches of such a mature bud were generally distributed. No elongated bud was detected until the culture entered the second growth phase. Locations of daughter-budding sites on these swollen cells appeared to be random (Fig. 3g–i), compared to the sites of those of the control culture, which were limited to a cell pole (Fig. 3c–f). These observations supported the notion that the spherical yeast cells which appeared during the first phase of growth in the ethanol culture displayed depolarized cell growth. After entering the second phase of growth in the ethanol culture, the location of new-born daughter cells was limited to the tips of pyriform mother cells. Some of the spherical mother cells produced several pseudohyphal cells (Fig. 2e), indicating that they remained depolarized.

To examine cell development in the first growth phase, the percentage of each type cell was examined at a given time in the cell population. Experiments were repeated three times; results from a representative one are shown in Table 1. At the start of the culture, the cell population consisted mainly of type A cells. The frequency of type

Fig. 2. Phase-contrast micrographs of *C. tropicalis* Pk233 cells grown in control culture at zero time (a) and at mid-exponential phase (b); or in the ethanol culture at the middle (c) and the late stage (d) of the first phase and the early stage (e) of the second phase. Bar, 30 μ m.

B–F cells increased up to the mid-exponential phase. On the other hand, the population of the ethanol culture at the early stage of the first growth phase (7 h) showed about 60% type C and D cells, each having a daughter bud without elongation. At the middle stage of the first growth phase (11 h), 80% of the population were swollen yeast cells, suggesting that individual cells of ethanol culture transformed their shape to a swollen one. At the later stage of the first growth phase (15 h), chains of three swollen cells occupied 40% of the population.

These observations suggested that the depolarized yeast cells in a chain, which were produced in the ethanol culture during the first growth phase, recovered their polarization during the second growth phase and produced daughter buds with polar growth. It should be noted that the degree of polarization of new daughter cell-growth at this period was rather higher than that of the control culture.

Budding of yeast cells was observed when filamentous cells of the ethanol culture were transferred to fresh control medium by 100-fold dilution of a 48 h ethanol culture (Fig. 4a, b). On the other hand, after the ethanol-induced filamentous cells were similarly transferred to a fresh ethanol medium, the filamentous cells produced spherical daughter cells with depolarized growth (Fig. 4c). This agrees with the idea that the first event in the ethanol culture was depolarization of cell growth rather than filamentous polar growth. Prolonged incubation of

each of the transferred cultures gave mostly yeast cells in the control medium and chains of spherical yeast cells in the ethanol medium (Fig. 4d, e).

Prevention of ethanol-induced pseudohyphal formation by addition of *myo*-inositol or sorbitol

Addition of *myo*-inositol (0.03 mM) to the ethanol culture medium (ethanol-inositol culture) prevented pseudohyphal formation, as observed by Tani *et al.* (1979). This culture showed monophasic growth with a doubling time at mid-exponential phase of 1.8 h (Fig. 1a). In this culture, the transient appearance of yeast cells in chains was observed at mid-exponential phase; the majority of these cells were ovate to oblong. The pattern of actin localization of these ovate to oblong cells was apparently similar to that in the control culture (not shown). Further incubation of the ethanol-inositol culture to late-exponential phase showed single yeast cells with an ovate to oblong shape occupying more than 98% of the cell population, and no significant formation of pseudohyphae was observed. Addition of 1 M sorbitol to the ethanol culture also prevented pseudomycelial formation. The addition of 1 M sorbitol to the yeast cultures gave a prolonged lag phase: 3 h in the control culture or 4 h in the ethanol culture. However, the doubling time in the mid-exponential phase was 1.8 h for the control culture and 2.2 h for the ethanol culture. The addition of 1 M sorbitol to the ethanol culture prevented both growth inhibition and pseudo-

Fig. 3. Fluorescence and phase-contrast micrographs of *C. tropicalis* Pk233 cells stained with Rh-Ph showing the distribution of actin in the control culture (a–f), or in the ethanol culture at the middle stage (g–i) of the first phase and the early stage (j) of the second phase. Bar, 30 μ m.

mycelial formation by ethanol. The transient appearance of yeast cells in chains was also observed at the mid-exponential phase in the ethanol culture containing 1 M sorbitol, but further incubation of the culture produced single yeast cells. No spherical cells appeared during growth in the presence of 1 M sorbitol.

Dimorphic transition and chitinase activity

In the ethanol culture, daughter-cell separation appeared to be blocked and cells were in chains. Chitinase activity was examined in cell wall fractions of cells in the three cultures. From the control culture, chitinase activity

Table 1. Percentage of cell types, classified according to the distribution pattern of actin patches, in various cultures

Culture (total cells)	Cell type:								
	A	B	C	D	E	F	Swollen*		
							1 cell	2 cells	3 cells
Start [0 h] (<i>n</i> = 520)	88	1.7	4.2	2.1	3.7	0	0	0	0
Control [15 h] (<i>n</i> = 491)	32	9	17	14	19	9	0	0	0
Ethanol [7 h] (<i>n</i> = 229)	14	23	24	33	1	4	1	0	0
Ethanol [11 h] (<i>n</i> = 154)	0	1	1	8	0	0	28	53	9
Ethanol [15 h] (<i>n</i> = 189)	0	0	1	0	0	0	23	34	42
Et-Ino* [8 h] (<i>n</i> = 128)	5	9	32	16	10	28	0	0	0
Et-Ino* [12 h] (<i>n</i> = 257)	23	16	23	11	18	9	0	0	0

*Ethanol-inositol culture.

decreased during the early exponential phase and then increased until it reached the original level (Fig. 1b). This seemed to parallel the alteration of growth phase, from late-exponential to stationary (Fig. 1a). However, in the ethanol culture, chitinase activity was at a low level during the first phase of growth, in parallel with the chain formation of cells (Fig. 1b, Fig. 3g–i). Addition of *myo*-inositol to the ethanol culture resulted in an increase in chitinase activity, but the increase occurred a few hours later than in the control culture.

DISCUSSION

Inhibition of daughter-cell separation followed the depolarization in the ethanol culture. The appearance of the chains of yeast cells paralleled low levels of chitinase activity. The pseudohyphal cells that grew from these spherical yeasts did not separate from each other. Chitinase activity has been shown to be required for daughter cell separation in the normal cell cycle of *S. cerevisiae* (Cabib *et al.*, 1992; Kuranda & Robbins, 1991). The process of yeast-to-pseudomycelial transition in *C. tropicalis* seems to require low levels of chitinase activity.

In the presence of ethanol, addition of 0.03 mM *myo*-inositol or 1 M sorbitol restored the chitinase activity to the control level and prevented the formation of both depolarized spherical cells in chains and polarized filamentous growth. The addition of ethanol is associated with a decrease in the molar ratio of the saturated acid content of phospholipids, especially of palmitic acid in phosphatidylinositol (Uejima *et al.*, 1987; K.

Kono & T. Kamihara, unpublished). However, in the presence of ethanol, addition of 0.03 mM inositol prevented the decrease in the molar ratio of saturated acid content (K. Kono & T. Kamihara, unpublished). Indirect evidence for inositol-phospholipid-linked signal transduction during filamentation has been obtained for this organism (Kamihara & Omi, 1989), as well as for *Fusarium graminearum* (Prior *et al.*, 1993). During the dimorphic transition from yeast cells to germ tubes in *C. albicans*, a sustained increase in cellular inositol trisphosphate levels was also reported to occur (Gadd & Foster, 1997). The role in the dimorphic transition of phosphatidylinositol metabolism and a signal transduction pathway linked to it requires further investigation.

The morphogenetic pathway for development of pseudohyphae in *C. tropicalis* may consist of two steps: an inhibition of daughter-cell separation and formation of filamentous cells. These two steps also apply in the case of filamentous growth of *S. cerevisiae* on solid medium (Gimeno *et al.*, 1992), but differ from the case of germ tube formation in *C. albicans* (Anderson & Soll, 1986; Gow & Gooday, 1987). Germ tubes represent the initiation of hyphal growth directly from yeast cells, have parallel walls at their point of origin, and are not constricted at septa (McGinnis, 1980). However, filamentous growth in nitrogen-limited *S. cerevisiae* and that in ethanol-induced *C. tropicalis* consists of a series of elongated yeast cells remaining attached, and their walls typically contain marked constrictions at the septa. In *S. cerevisiae*, some diploid strains switch from budding cells to pseudohyphae under conditions of nitrogen starvation on solid medium (Gimeno *et al.*, 1992;

Fig. 4. Phase-contrast micrographs of *C. tropicalis* Pk233 cells grown in ethanol medium for 48 h (a) and then transferred to control conditions for 9 h (b) and 12 h (c), or to ethanol medium for 9 h (d) and 12 h (e). Newly formed yeast cells are shown by arrows. Bars, 30 μ m.

Gimeno & Fink, 1994). Elements of the yeast mating signal transduction pathway including the mitogen-activated protein (MAP) kinase cascade, are required for pseudohyphal growth (Liu *et al.*, 1993; Madhani & Fink, 1997). The same signalling kinase cascade that activates Ste12p in *S. cerevisiae* may also work in the filamentous growth of *C. albicans* (Liu *et al.*, 1993; Gavrias *et al.*, 1996). In the case of the dimorphic transition in *C. tropicalis*, elements of signal transduction pathways may work under ethanol induction. Identifications of specific genes for the process of ethanol-induced filamentation are in progress.

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