Importance of calcium to the regulation of polymorphism in *Wangiella (Exophiala) dermatitidis*

S. M. KARUPPAYIL & P. J. SZANISZLO
Department of Microbiology, University of Texas at Austin, Austin, TX, USA

Critical steps implicated in the polymorphism of *Wangiella dermatitidis* were found to be sensitive to calcium ion availability. When grown in a defined, synthetic medium under various pH and temperature conditions, two thresholds of calcium ion concentrations were identified: a lower concentration favouring non-polarized growth leading to multicellular form development and a higher concentration promoting polarized growth characterized by yeast budding or pseudo/true hyphal growth. The phenotypic transition of yeasts to multicellular forms or to hyphae was induced at both 25 and 37 °C in the wild-type strain by the addition of calcium to the synthetic medium adjusted to pH 2-5, which was otherwise not conducive to the production of either growth form. However, the calcium additions did not allow maintenance of polarized growth of yeasts or hyphae in a temperature-sensitive, cell-division-cycle mutant (*wdcdc2*) derived from the same strain and grown at 37 °C in the same medium adjusted to either pH 2.5 or 6.5. Instead these conditions allowed only the non-polarized, multicellular form development associated with this conditional mutant cultured in rich media at the 37 °C restrictive temperature for yeast bud formation. Results from experiments using the calcium chelator EGTA added to the synthetic medium supported these conclusions at neutral pH with both the wild type and the *wdcdc2* mutant cultured at 37 °C. The results suggested that during infection different concentrations of calcium may be encountered by *W. dermatitidis* in different tissues, which might directly regulate its growth and polymorphism and indirectly its virulence depending on host conditions.

**Keywords** calcium, cell cycle, polymorphism, *Wangiella dermatitidis*

### Introduction

*Wangiella dermatitidis* (Kano) McGinnis (*Exophiala dermatitidis* (Kano) de Hoog) is a vegetatively polymorphic fungal pathogen of humans, which traditionally has been most associated with dermotrophic forms of phaeohyphomycosis [1]. However, this melanized fungus has become better known recently as a paradigm for this emerging mycosis because of its increasing detection as a systemic pathogen with a marked neurotropic tendency [2-4]. Predisposing factors for systemic infections with *W. dermatitidis* include cystic fibrosis, lymphocytic leukaemia, diabetes mellitus, bronchiectasis, rheumatoid arthritis and catheterization [4-6]. Initiation of both the subcutaneous and systemic forms of phaeohyphomycosis usually is attributed to the traumatic implantation of fungal propagules into tissue, although in some cases the route of infection is unknown and pulmonary entry has not been ruled out completely [3,5]. *In vivo* *W. dermatitidis* exhibits a wide range of dark-walled, vegetative morphologies including ovoid yeasts, sepatate, branched or unbranched hyphal filaments, catenulate cells, toruloid hyphae and isotropically enlarged spherical cells [1,3-5]. The latter often resemble sclerotic body (SB) precursors similar to those of chromoblastomycosis fungi but are reported to have thinner walls and only infrequent septation in one plane [3,4]. In granulomatous lesions, *W. dermatitidis* usually exhibits its more spherical...
morphology but on some occasions the fungus develops mycelium [4,7].

Because of its ease of laboratory manipulation, and a number of other attributes, W. dermatitidis has been established as a dermatiaceous fungal model for studies of phenotypic switching between polarized yeast or hyphal forms and non-polarized isotropic forms that may become multicellular/sclerotic [8,9]. By altering growth conditions, W. dermatitidis can be induced to produce in vitro near homogenous populations of each of its predominant vegetative phenotypes [10,11]. Yeast (Y) phase to multicellular (Mc) form (Y→Mc) transition is achieved by subculturing logarithmically growing yeasts achieves multicellular/sclerotic [8,9]. By altering growth phase to multicellular (Mc) form (Y→Mc) transition is predominant vegetative phenotypes [10,11]. Yeast (Y) phase to multicellular (Mc) form (Y→Mc) transition is achieved by subculturing logarithmically growing yeasts rich in rich media acidified to pH 2.5 or by the incubation of certain temperature-sensitive (ts), cell-division-cycle (wdc2) mutants in rich medium at the restrictive temperature of 37 °C [12–14]. Because conversion to the Mc forms appears to result from the cessation of bud emergence, without the inhibition of cell wall growth, nuclear division or cytokinesis, it was hypothesized that the acidic condition with the wild-type and the elevated temperature with the wdc2 mutants were inhibiting cell cycle events normally responsible for bud emergence [13,15]. It was also noted that Y→Mc form conversion proceeds through a two-stage process regardless of the induction conditions. Stage I is characterized by the formation of non-septate, swollen, unbudded cells having one or more nuclei and thickened cell walls [16,17]. Wall thickness increases from about 0.04 µm to 0.3 µm during yeast to stage I conversion and constitutes greater than 24% of the cell volume [18]. In stage II, growth continues and the isotropically enlarged cells form one or more transverse septa resulting in the Mc morphology [14,17,19]. Recently, additional ts mutants of W. dermatitidis have been derived which produce hyphae (H) from yeasts (Y→H) at the restrictive temperature (R. Rennard, N. D. P. McIntosh and P. J. Szaniszlo, unpublished results). Of significance is the fact that Y→H transition by these mutants, like the wild type, seems to require the formation of stage I-type cells prior to hyphae production by polarized apical extension mechanism (ref. 12; N. D. P. McIntosh and P. J. Szaniszlo, unpublished results).

Our previous studies indicate that a calcium/proton exchange mechanism may play an important role in the pH-induced dimorphism of W. dermatitidis cultured at 25 °C and also in the H→SB dimorphism of three classical agents of chromoblastomycosis cultured at either 25 or 37 °C [8,9,20,21]. At 25 °C and pH 2.5, critical but low calcium concentrations in a defined, synthetic medium favour non-polarized, Mc-form development in W. dermatitidis whereas higher concentrations allow polarized yeast/hyphal growth [9]. Additional support for this important role of calcium in the regulation of polymorphism in W. dermatitidis and in the dimorphism of the chromoblastomycosis fungi was derived by growing wild-type cells in a similar defined medium containing the calcium chelator EGTA and buffered at pH 6.0 [8,9,21]. High EGTA concentrations caused yeast-phase cells of W. dermatitidis cultured at 25 °C to arrest in a terminal phenotype that is normal in size and shape but characterized by the presence of a bud initial that tends not to enlarge [9]. In contrast, EGTA at lower concentrations induces Y→Mc form conversion in W. dermatitidis [9] or H→SB conversion in the chromoblastomycosis fungi [8,21].

This communication extends our previous studies with W. dermatitidis and provides evidence that Y→Mc conversion at 37 °C, a temperature commensurate with in vivo host environments, is also under the direct influence of calcium. These new studies were made possible by our finding that the completely defined synthetic medium described previously [9,17,21], when supplemented with calcium concentrations higher than those required at 25 °C, allowed for the first time significant levels of Y→Mc conversion at 37 °C in both the wild-type strain cultured at pH 2.5 and the wdc2 mutant strain cultured identically, but at pH 6.0. In all previous studies with these two strains, significant levels of Y→Mc conversion only had been observed at 37 °C in rich, non-defined media [12,14,17,20,21]. Other results are presented that suggest that nuclear division and septation in these strains are also influenced by calcium availability. Finally, the results provide additional clues about the mechanisms that might be regulating cellular morphogenesis in other dermatiaceous fungi during infections, and particularly the production of the SB morphology of the chromoblastomycosis fungi [1,7,22].

Materials and methods

Strains, media and culture conditions

A wild-type strain of Wangiella dermatitidis (Kano) McGinnis (strain 8656/ATCC 43100), and a ts cell-division-cycle mutant (wdc2) derived previously [13] from it by NTG mutagenesis (strain Mc3/ATCC 38716), were used in this study. The basal, synthetic growth medium for most experiments was prepared according to Cooper et al. [17]. This medium contained the following components added to deionised distilled water (g L⁻¹): 30 g glucose; 3 g NaNO₃; 1 g K₂HPO₄; 0.5 g MgSO₄·7H₂O; 0.01 g FeSO₄·7H₂O; 0.265 g NH₄Cl and 0.003 g thiamine (added as a filter sterilized solution after autoclaving). The pH of the medium was initially adjusted with HCl or NaOH to 6.0 or 2.5, prior to autoclaving, depending on the experiment. Calcium was added as a CaCl₂ solution and the pH 6.0 medium was

© 1997 ISHAM, Journal of Medical & Veterinary Mycology 35, 379–388
Calcium and polymorphism in Wangella dermatitidis U 8.5

Effect of Ca$^{2+}$ concentration on the total growth of $W$. dermatitidis wild type at pH 2.5 and at 25 °C (A) or 37 °C (B). Ca$^{2+}$ concentrations used in mM were 0.0 (■), 0.1 (□), 0.5 (♦), 1.0 (▼), 5.0 (○), 10.0 (△). The controls (●) at both temperatures represent growth in the same medium without added Ca$^{2+}$, but having an initial pH of 6.0.

Measurement of cellular parameters

Cells were fixed at selected times by adding 37% formaldehyde to a final concentration of 3.75% (w:v). Cell numbers were determined using a Neubauer Hemacytometer (Spencer Bright Line, 1/10 mm deep). Viable cell counts were performed when required using standard plate count techniques [13]. The data presented represent the averages of a minimum of two independent experiments each with duplicate cultures. To disrupt cell clumps, samples were sonicated on ice for 30 s with 15 s intervals in between sonications using a Biosonic III Sonicator (Bronswill Scientific, Rochester, NY). The pH of the culture media was determined using a Corning pH meter (Corning, Model 125).

Nuclei were visualized using the fluorescent dye mithramycin following Slater [23]. Cells were fixed by treatment for 10 min in absolute alcohol and acetone (1:1 vol:vol). Fixed cells were stored at 4 °C until used. After removing ethanol by washing with water, cells were stained overnight at 4 °C. For Calcofluor staining to visualize chitin, cells were collected by centrifugation and fixed in formaldehyde for 24 h at room temperature. Cells were then rinsed in water and resuspended in a minimal volume of 10-fold diluted Calcofluor stock solution (1 mg ml$^{-1}$ water) and incubated for 30 min at room temperature in the dark. After washing with water (5 ×), cells were observed with UV excitatory illumination using a Zeiss ICM 405, Inverted Photomicroscope (Carl Zeiss, Oberkochen, Germany) equipped with differential-interference-contrast (DIC) or phase-contrast optics and tungsten light source [24]. For mithramycin and Calcofluor stained preparations, the fluorescence filter pack contained excitor filters BG3, LP397, BP 390/440 and dichroic reflector LP460, and barrier filter LP470. The light source was an HBO 50 Mercury vapour bulb. Cells were photographed with the 35 mm microscope camera attachment (M35) using Ilford HP5 black and white film.

Chemicals

Inorganic salts, EGTA and Calcofluor White M2R (Fluorescent brightener 28) were purchased from Sigma Chemical Company (St Louis, MO). Media components were from Difco (Detroit). Mithramycin was a generous gift from Pfizer, Groton, CN, USA.

Results

Comparison of the effects of Ca$^{2+}$ additions on the total growth of the wild type at pH 2.5 and at 25 or 37 °C

The basal synthetic medium without added Ca$^{2+}$ and adjusted to pH 2.5 did not support normal yeast reproductive growth of the wild-type strain of $W$. dermatitidis at 37 °C (Fig. 1), as reported previously for this strain cultured at 25 °C [9]. In contrast, calcium additions enhanced yeast reproductive growth in the same medium at both temperatures as determined by cell counts (Fig. 1)
Table 1  Effect of temperature and calcium on morphology, cell number, biomass and final culture pH of *Wangiella dermatitidis* wild type grown in medium with initial 2-5 pH for 5 days

<table>
<thead>
<tr>
<th>Growth characteristics</th>
<th>Calcium ion concentration (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td><strong>25 °C</strong></td>
<td></td>
</tr>
<tr>
<td>Yeast*</td>
<td>19</td>
</tr>
<tr>
<td>Stage I*</td>
<td>79</td>
</tr>
<tr>
<td>Stage II*</td>
<td>2</td>
</tr>
<tr>
<td>Pseudohyphal or isotropic forms regaining polarity*</td>
<td>0</td>
</tr>
<tr>
<td>Cell number (log)t</td>
<td>5.94</td>
</tr>
<tr>
<td>Biomass (mg)$</td>
<td>30</td>
</tr>
<tr>
<td>pH§</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>37 °C</strong></td>
<td></td>
</tr>
<tr>
<td>Yeast*</td>
<td>0</td>
</tr>
<tr>
<td>Stage I*</td>
<td>97</td>
</tr>
<tr>
<td>Stage II*</td>
<td>3</td>
</tr>
<tr>
<td>Cell number (log)t</td>
<td>6.04</td>
</tr>
<tr>
<td>Biomass (mg)$</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>pH§</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*Morphological types are presented as percent of total number of cells.
*Cell number denotes log of final cell concentration.
*Biomass represents mg dry weight 100 ml~1 culture.
§pH denotes change from the initial 2-5 pH.

and biomass measurements (Table 1). The increased reproductive growth was most apparent initially at 37 °C (Fig. 1B) and was dependent on the Ca{}^{2+} concentration in the medium, with more Ca{}^{2+} generally causing more rapid initial rates of growth. However, significantly more biomass by weight was associated with the Ca{}^{2+} additions at 25 °C (Table 1), which possibly was more reflective of wall thickening than with reproductive growth per se. Because the culture pH decreased with increasing calcium concentrations, the increased growth in the pH 2-5 media was not due to a rise in pH during culture (Table 1). In fact, it was more likely that the increasing external Ca{}^{2+} concentrations were causing a proton efflux as more Ca{}^{2+} was internalized by cells.

Comparisons of the effects of Ca{}^{2+} additions on the morphogenesis of the wild type at pH 2-5 and at 25 or 37 °C

Unlike the rich non-synthetic media investigated previously [12], the synthetic pH 2-5 medium without added Ca{}^{2+} used initially in this study and previously [9] did not favour Y→Mc conversion of the wild-type strain, although it did cause considerable inhibition in budding and induced the development of isotropic (stage I) forms (Table 1). However, with the addition of increasing amounts of Ca{}^{2+} to this same medium, dramatic changes in growth habit resulted (Table 1). For example, with the addition of as little as 0-1 mM Ca{}^{2+}, 19% of the initial inoculum converted to Mc (stage II) forms during culture at 25 °C (Table 1). At higher Ca{}^{2+} ion concentrations at this temperature, the yeast cells or isotropic forms regained considerable polarity and tended to resume growth as pseudohyphae or budding yeasts. When this experiment was repeated at 37 °C, similar trends, but also some important differences, were observed. As at 25 °C, the synthetic pH 2-5 medium without added Ca{}^{2+} did not favour Y→Mc conversion at 37 °C but did induce isotropism (Table 1; Fig. 2A). With increased Ca{}^{2+} concentrations there was an enhancement of isotropic growth and an increase in Y→Mc conversion to a high of 21% (Table 1; Fig. 2B), but this required 10 mM calcium instead of the 0-1 mM concentration noted for maximum Mc formation at 25 °C. In general, between 50 and 100% of all inoculum cells became isotropic at 37 °C at Ca{}^{2+} concentrations up to 5 mM. However, at 10 mM concentrations of Ca{}^{2+} Y cells maintained their ability to grow reproductively by budding at pH 2-5 (Figs 1 and 2). At this Ca{}^{2+} concentration, more than 70% of the cells were in the pseudohyphal phase after 4 days of incubation.
in the pH 2.5 medium at 25 °C whereas at 37 °C the predominant growth form was a reproductive budding yeast (Fig. 2C; Table 1), which suggests that the Ca\(^{2+}\) additions were differentially affecting Y cell separation kinetics at the two temperatures. In contrast, Ca\(^{2+}\) additions to the pH 6.0 control medium at either 25 or 37 °C did not markedly influence the growth rate or growth habit of *W. dermatitidis* (data not shown).

**Effects of EGTA, a Ca\(^{2+}\) chelator, on growth, morphogenesis and nuclear division of the wild type at pH 6.0 and at 37 °C**

Because Ca\(^{2+}\) must have been present as a medium contaminant or as inoculum intracellular stores at quantities sufficient to allow near normal growth at pH 6.0 and 25 and 37 °C in the basal synthetic medium, the Ca\(^{2+}\) chelator EGTA was used to limit Ca\(^{2+}\) availability for subsequent Ca\(^{2+}\) studies with the wild type at near neutral pH. As reported previously for cells cultured at 25 °C [9], EGTA concentrations of 1 mM or higher almost completely inhibited the yeast reproductive growth of *W. dermatitidis* at pH 6.0 during incubation at 37 °C (data not shown). The predominant terminal phenotype produced at 37 °C at these high EGTA concentrations was again a yeast-phase cell arrested with a tiny bud (bud initial) (Fig. 3A; see also fig. 3 of ref. 9). More than 80% of the yeast-phase cells were arrested in this phenotype by the 30 mM concentration of EGTA. In contrast, lower EGTA concentrations, for example 5.0 to 10.0 mM, which inhibited isotropic growth, also reduced viability somewhat as determined by viable cell counting (data not shown).

As expected, the addition of EGTA to the synthetic medium having a pH of 6.0 also influenced nuclear division in *W. dermatitidis*. Concentrations that allowed some isotropism and planate cell formation at 37 °C also sometimes allowed one or more mitotic nuclear divisions. This was particularly evident at the 5.0 and 10.0 mM EGTA concentrations. However, higher EGTA concentrations inhibited nuclear division causing most of the cells to arrest with a single nucleus (Fig. 3B; see also fig. 3 of ref. 9). Thus, most wild-type Y cells cultured at the highest EGTA concentrations arrested at 37 °C with a bud initial and contained only one nucleus in the mother cell (Fig. 3).

**Effects of Ca\(^{2+}\) additions and pH 6.0 on Y→Mc conversion and nuclear division in *wdcdc2*, a ts mutant, at 37 °C**

In prior studies, Y→Mc conversion at 37 °C in the ts mutant *wdcdc2* was only observed to occur to any extent in rich media, although isotropic form development was common in some synthetic media devoid of added Ca\(^{2+}\) (refs 13, 15, 24; R. L. Roberts, C. W. Jacobs and P. J. Szaniszlo, unpublished results). Temperature induced Y→Mc conversion in *wdcdc2* was also poor at 37 °C in the synthetic medium devoid of added calcium and having a pH of 6.0 (Figs 4A and 5A). Only 5 to 10% of the inoculum yeasts converted to Mc forms, as confirmed by the general lack of septa in isotropic forms after Calcofluor staining (Fig. 5D), whereas the rest were arrested in stage I after 48 h of incubation (Fig. 4A). Addition of Ca\(^{2+}\) again enhanced Y→Mc conversion, with as little as 0.1 mM Ca\(^{2+}\) concentrations promoting conversion to about 40% in 48 h (Fig. 4A). This increased to 60–80% Mc forms with additions of 0.5–10 mM calcium (Figs 4A and 5B and E). Extended incubation at the same temperature improved the Y→Mc form induction in all treatments, except in those with 5.0 and 10.0 mM Ca\(^{2+}\) additions, where it peaked at the 80% Mc form level (data not shown).

The Ca\(^{2+}\) additions also enhanced nuclear division in *wdcdc2* cultured at 37 °C (Fig. 4B). Although almost 70% of the stage I and II phenotypes that developed from yeasts of *wdcdc2* cultured at the restrictive temperature for
yeast budding in the absence of added Ca\(^{2+}\) were multinucleate, only about 40% had more than two nuclei after 48 h (Figs 4B and 5G). However, that percentage of isotropic forms with more than two nuclei increased to 60–70% of the cell population on addition of 0.1–0.5 mM calcium (Figs 4B and 5H) and to 90–95% on addition of 1.0–10 mM calcium (Fig. 4B).

**Effects of EGTA additions and pH 6.0 on Y→Mc conversion, and nuclear division in wdcdc2 at 37 °C**

As expected, the addition of EGTA to the synthetic medium without added Ca\(^{2+}\) caused considerable inhibition of Y→Mc form conversion of wdcdc2 at 37 °C. With the addition of as little as 0.1 mM EGTA, a 50% inhibition was observed in the induction of the few stage II forms that developed, compared with those of control cultures (Fig. 6A). At 5.0 mM concentrations and above, the EGTA additions totally inhibited Y→Mc conversion, as confirmed by Calcofluor staining (Fig. 5C and F). In addition, 25% of the yeast inoculum was inhibited in isotropic growth by these higher EGTA concentrations (Fig. 5C), as indicated by the presence of many normalized yeasts without tiny buds in the populations, even after 48 h culture at 37 °C. Also the EGTA additions again notably affected nuclear division, with higher concentrations of EGTA being progressively inhibitory up to concentrations of 10 mM (Fig. 6B). Fully 80–90% of the inoculum cells were arrested as single cells with a single nucleus after culture for 48 h at 37 °C at EGTA concentrations of 10.0 mM or higher (Figs 5I and 6B).

**Discussion**

Earlier studies suggested that perturbations in the yeast cell cycle of *W. dermatitidis* can result in transitions from
enlarged, unbudded cells with multiple nuclei as was a terminal phenotype characterized only by isotropically found in nuclear divisions are often followed by cytokinesis and

However, at 37 °C, inhibition of bud emergence in the wdcdc mutants incubated at the non-permissive temperature [25,26].

mutants is similar to that associated with the cell-division-cycle proteins are involved in budding in the polymorphic fungus W. dermatitidis. Our experiments with EGTA at pH 6-0 and the non-synchronized wild-type yeast cells cultured at 37 °C showed that the highest concentrations tested affected polarized yeast-phase reproductive growth by arresting cells with a single nucleus and a bud initial, which failed to expand. A calcium-dependent mutant (call-l) of S. cerevisiae, which requires a high concentration of calcium for growth, arrests similarly in its cell cycle with a single nucleus and a tiny bud [29]. When used in combination, the calcium ionophore A23187 and EGTA also affected yeast cell cycle progression in S. cerevisiae and caused a rapid decrease in intracellular calcium and a transient G1 arrest, which was followed by a block at the G2/M boundary. This suggested that

The inhibition of bud emergence and its consequences in W. dermatitidis cultured under acidic conditions for the wdcde2 (Mc3) cultured for 48 h at pH 6-0 at 37 °C. Bar in C = approximately 10 μm and applies to A-I.

Calcium and polymorphism in W. dermatitidis

polarized to non-polarized developmental pathways (for comprehensive reviews see references 8 and 9). For example, polarized yeast and hyphal growth forms were induced to form non-polarized, isotropically enlarged cells that might become multicellular [15,24]. The initial event signalling that a phenotypic transition might be about to occur in actively growing yeasts was usually identified as the inhibition of yeast bud emergence. Initially this was achieved by growing wild-type yeast cells of W. dermatitidis at pH 2-5 in rich media [12] and subsequently by culturing certain ts wdcde bud emergence mutants at 37 °C in rich media at more neutral pH [13,14]. The inhibition of bud emergence and its consequences in W. dermatitidis cultured under acidic conditions for the wild-type or elevated temperatures for the wdcde mutants is similar to that associated with the cell-division-cycle mutants cdc24, 42 or 43 of Saccharomyces cerevisiae incubated at the non-permissive temperature [25,26]. However, at 37 °C, inhibition of bud emergence in the wdcde mutants of W. dermatitidis does not cause arrest in a terminal phenotype characterized only by isotropically enlarged, unbudded cells with multiple nuclei as was found in S. cerevisiae. Instead the isotropic growth and nuclear divisions are often followed by cytokinesis and considerable additional cell wall synthesis in wall and septal regions, which results in the Mc morphology [10,12,13,16].

The central role for calcium in the many steps involved in the phenotypic transition between the polarized and non-polarized growth forms leading to Y→Mc development in W. dermatitidis has been clarified. This dimorphism was induced in the wild type by the simple addition of Ca2+ to a synthetic medium adjusted to pH 2-5, which otherwise was not ideal for the induction of Mc forms. Most likely in the absence of added exogenous Ca2+, the acidic culture conditions caused inoculum cells to lose or not accumulate the critical amounts of intracellular Ca2+ required to maintain polarized growth mechanisms. In general, our low pH experiments showed that at either 25 or 37 °C critical but low concentrations of Ca2+ favoured Y→Mc form development. In contrast, lower concentrations allowed only isotropic growth, no septation and few or no nuclear divisions, whereas higher concentrations of Ca2+ allowed maintenance of polarized cell growth characterized either by yeast budding or hyphal development. These results confirmed that at 37 °C isotropic growth, mitosis, septation and polarized yeast budding and hyphal apical extension at pH 2-5 are all Ca2+-dependent processes in W. dermatitidis, as had been suggested by earlier preliminary experiments carried out exclusively at 25 °C [9,20]. Our new results derived from complimentary studies with the Ca2+ chelator, EGTA, and the wild-type strain and with the Ca2+ and EGTA additions to the media used to culture wdcde2 at 37 °C and at pH 6-0 also strongly supported this conclusion.

The bud emergence gene, CDC24, of S. cerevisiae encodes a putative calcium-binding protein [27]. Mutants of this fungus having a lesion in CLS4 (CDC24) are unable to bud, but nevertheless undergo spindle-pole duplication and DNA synthesis [27,28]. By analogy with what is known about the importance of Ca2+ in S. cerevisiae, we speculate that similar calcium-responsive proteins are involved in budding in the polymorphic fungus W. dermatitidis. Our experiments with EGTA at pH 6-0 and the non-synchronized wild-type yeast cells cultured at 37 °C showed that the highest concentrations tested affected polarized yeast-phase reproductive growth by arresting cells with a single nucleus and a bud initial, which failed to expand. A calcium-dependent mutant (call-l) of S. cerevisiae and which requires a high concentration of calcium for growth, arrests similarly in its cell cycle with a single nucleus and a tiny bud [29]. When used in combination, the calcium ionophore A23187 and EGTA also affected yeast cell cycle progression in S. cerevisiae and caused a rapid decrease in intracellular calcium and a transient G1 arrest, which was followed by a block at the G2/M boundary. This suggested that

© 1997 ISHAM, Journal of Medical & Veterinary Mycology 35, 379-388

Fig. 5 Effects of calcium at 0 (A, D, G) and at 0.5 mM (B, E, H) added concentrations, or of EGTA at 15 mM (C, F, I) on morphology (A, B, C), calcofluor staining pattern to reveal septa (D, E, F) and mithramycin staining to reveal nuclear conditions (GHI) of wdcde2 (Mc3) cultured for 48 h at pH 6-0 at 37 °C. Bar in C = approximately 10 μm and applies to A-I.
Calcium ions are required at all stages of the *S. cerevisiae* cell cycle, with the possible exception of the initiation of DNA synthesis [30]. In spite of these observations, the kinetics of nuclear division in wild-type cells of *W. dermatitidis* at different EGTA concentrations had not been investigated prior to the studies in this report, even though nuclear division is probably a prerequisite for the cytokinesis required for the production of Mc forms. The finding of a window of EGTA concentrations (5.0 and 10.0 mM at 37 °C) that allowed some amount of isotropism and also allowed a few rounds of nuclear division and septation in *W. dermatitidis* implies that calcium deprivation influences both nuclear division and septation. Whether septation can occur under any scenario of culture leading to Y-Mc development in this species in the absence of mitosis is unknown.

The dissection of some cell cycle events associated with polymorphism in *W. dermatitidis* was previously facilitated by study of the cell-division-cycle mutant *wdcde2* (Mc3), which undergoes nearly uniform Y-Mc form conversion at 37 °C in rich media at near neutral pH [13,15,16,31]. We also exploited this strain in the present investigation to confirm the involvement of Ca\(^{2+}\) in the regulation of Y-Mc morphogenesis at 37 °C. At this temperature in rich non-acidic medium, this mutant is uniformly inhibited in bud emergence, but is still capable of continuing the nuclear division, and the cytokinesis by intracellular septation that are required for the Y-Mc form-conversions [13,15,16,31]. However, in the present study, the same Y-Mc form conversion in *wdcde2* was found to be very poor at 37 °C and at near neutral pH in the synthetic medium devoid of added Ca\(^{2+}\). However, by the simple addition of Ca\(^{2+}\) to this same medium adjusted to either pH 2.5 or 6.0, a dramatic increase in Y-Mc form induction at 37 °C occurred. In both cases, it appeared that critical but different levels of Ca\(^{2+}\) were again required before mitosis and before septation were carried out by the mutant and allowed Y-Mc conversion. In addition, in the presence of EGTA adequate Ca\(^{2+}\) appeared to be required for isotropic forms to become multinucleate, and then most likely even more Ca\(^{2+}\) had to be available for these forms to become multicellular. Even with the *wdcde2* mutant, extreme limitations of Ca\(^{2+}\) availability (for example by 5–30 mM EGTA concentrations; see Fig. 6) arrested some cells in growth in ways that inhibited even isotropic development, but unlike with the wild type without tiny buds. Possibly these cells represented a subpopulation of inoculum cells devoid of the adequate intracellular stores of Ca\(^{2+}\) necessary to initiate even the isotropic growth required to be classified as a stage 1 type isotropic form. However, the arrest of these cells without tiny buds was not unexpected because the ts block is known to inhibit yeast bud emergence in *wdcde2*, whereas the EGTA block apparently allows progression through bud emergence, but subsequently does not allow the new bud to enlarge (ref. 9; this study). The fact that most cells of *wdcde2* cultured at 37 °C in the presence of the high EGTA concentrations have only a single nucleus further suggested that they are extremely starved for Ca\(^{2+}\).

The calcium ion requirements of *W. dermatitidis* for each of the different morphological events studied seems to be different, probably because each event requires a unique calcium concentration. Evidence from other higher and lower eukaryotes supports this hypothesis [32,33]. No information, however, is currently available on the Ca\(^{2+}\) stoichiometry requirements leading to any of these events. Nevertheless, Ca\(^{2+}\) is a well known participant in relevant biomolecular systems. For example, both spindle pole body duplication and nuclear division have been demonstrated to be calcium-requiring events in fungi.
The gene CDC31, which encodes a calcium-binding protein, is recognized as a component of the half-bridge of the spindle body, and in S. cerevisiae is known to be involved in spindle pole body duplication [36,37]. A mutant with the lesion in this gene, although unable to duplicate its spindle pole body, can still bud and replicate its DNA [25,36]. The calcium-binding protein calmodulin also has been implicated in nuclear division in S. cerevisiae and has been shown to accumulate at sites of budding [38,39]. Temperature-sensitive calmodulin mutants of this fungus have been shown to lose viability during mitosis at the inhibitory temperature [40]. Also, such mutants containing the calmodulin gene under the control of GAL1 promoter, when placed under calmodulin-depriving conditions, arrest with a single nucleus and a short mitotic spindle, which suggests a role for calmodulin in nuclear division [34]. In addition, an interaction between actin and calmodulin has been documented in S. cerevisiae, where calmodulin and actin sometimes were found concentrated in overlapping regions during cell cycle progression [39]. Calmodulin was also found to localize to shmoo tips in cells treated with alpha factor, implicating calmodulin in polarized cell growth [39]. The polarized distribution of actin and calmodulin are apparently interdependent in S. cerevisiae, with a mutation in the actin gene causing dispersal of calmodulin. Conversely, disruption of calmodulin function disorganizes actin cytoskeletons in most of the cells [38]. Our results suggest that W. dermatitidis also has calcium-binding proteins functionally similar to these encoded by CDC24, CDC31 and the calmodulin gene of S. cerevisiae and searches for such genes are in progress.

Although transitions between polar and non-polar growth forms may not be very pronounced during infections with W. dermatitidis, such transitions are very common during infections with other related fungi [1,7,8,21]. Recently, calcium ion concentrations were found to be important in the regulation in vitro of SB morphogenesis in agents of chromoblastomycosis in humans, in the same general manner that these factors affected Y→Mc forms conversion in W. dermatitidis [9,21]. Because the SB is the predominant phenotype generally expressed by these fungi in vivo [1], it appears that the chromoblastomycosis fungi may be more sensitive than W. dermatitidis to calcium concentrations during infections. In the chronic granulomatous lesions of chromoblastomycosis, calcium withholding conditions possibly are more restrictive than those found in the cystic lesions or CNS invasions associated with phaeohyphomycosis caused by W. dermatitidis, thus inducing only sclerotic bodies in the former at the expense of the polarized hyphal phenotype. In contrast, in phaeohyphomycotic sites of infection, critical calcium ion concentrations in host microenvironments may seldom be so limited that all the events in Y→Mc transition occur with W. dermatitidis. Instead, the Ca²⁺ stoichiometry in tissues infected by this species is either generally adequate for yeast growth or so inadequate that mainly isotropic forms without septa develop. The occasional report of so-called planate forms (two-celled isotropic forms) observed in some W. dermatitidis infections suggests that infrequently an isotropically arrested cell has enough intracellular Ca²⁺ available to allow at least one mitosis followed by the formation of one septum [1]. The absence of reports of Mc forms of W. dermatitidis in tissue, which consist of more than two cells, strongly suggests that the Ca²⁺ concentrations conducive to Y→Mc conversion are rare in humans, particularly in view of their relative ease of production at 37 °C in vitro, as reported in this communication. Nevertheless, the inherent ability of W. dermatitidis to react in a sensitive manner to different Ca²⁺ concentrations may offer a survival advantage during infections by allowing transient or prolonged pauses at certain developmental stages. Thus, whenever the Ca²⁺ concentrations become limiting in vivo, the predominant polarized yeast form of W. dermatitidis may become transiently arrested developmentally and then initiate new growth patterns having greater survival potential. It is assumed that one of these produces the more spore-like, isotropically enlarged, thicker walled forms that are often observed in infected cutaneous and subcutaneous tissues together with yeasts and hyphae. Whenever the Ca²⁺ concentrations are restored or are not limiting, for example in blood or brain tissue during systemic infections, the fungus grows rapidly as a yeast or alternatively the isotropic forms essentially germinate and produce the hyphal phase of this very versatile polymorphic pathogen.

Acknowledgements

We thank N. D. Philip McIntosh for critically reading the manuscript and helping with its preparation. This research was supported by a grant from National Institute of Allergy and Infectious Diseases (AI 33049).

References

14 Kester SA, Garrett DC. Morphometry and stereology of the conversion of thin-walled yeasts to phase I yeast cells of Wangiella dermatisidis. Mycologia 1995; 87: 153-60.

© 1997 ISHAM. Journal of Medical & Veterinary Mycology 35, 379–388