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Ca(II)-Calmodulin Regulation of Fungal Dimorphism in *Ceratocystis ulmi*

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We have shown that Ca(II) ions, ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid, $LaCl_3$, and six known calmodulin inhibitors shift the yeast-mycelium dimorphic potential of *Ceratocystis ulmi*. Our data are consistent with the conclusions that Ca(II)-calmodulin interaction is necessary for mycelial growth in *C. ulmi* and that the absence of this interaction leads to the yeast phase.

Fungal dimorphism is defined (20) as an environmentally controlled reversible interconversion of the yeast and mycelium morphologies. We have studied dimorphism in the ascomycete *Ceratocystis ulmi* (Buism.) C. Moreau, the causative agent of Dutch elm disease. The nutritional conditions sufficient for the production of yeasts (10), mycelia (10), and chlamydospores (13) have been described previously. In a defined glucose-salts liquid medium, the presence of proline as a nitrogen source induced the yeast phase, whereas arginine, ammonium, or asparagine induced the mycelial phase (10). Both the yeast and the mycelial phase cells differentiated to form chlamydospores at pH ≤3 (13).

Numerous environmental parameters have been found to regulate yeast-mycelial dimorphism in other fungi. Among these are temperature (12), the presence of transition metal ions (26) and respiratory inhibitors (22), and the composition of the gaseous atmosphere (2). The diversity of these environmental factors has made it difficult to identify a unifying explanation for fungal dimorphism (1, 16, 20, 21). Any such explanation must ultimately be based on the site of cell wall synthesis (1, 15, 16). The Ca(II)-binding protein calmodulin has recently been detected in the filamentous fungi *Neurospora crassa* (17), *Agaricus bisporus* (3), and *Blastocladiella emersonii* (8), but not in the yeast *Saccharomyces cerevisiae* (5, 9). Because none of the higher fungi have an absolute requirement for calcium for growth (7), this apparent discrepancy prompted us to examine the roles of Ca(II) and calmodulin in fungal development (dimorphism). This paper reports the influence of Ca(II), the Ca(II) chelator ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA), the Ca(II) antagonist $LaCl_3$, and six known calmodulin inhibitors (25) on the morphology of *C. ulmi*.

C. ulmi NRRL 6404 was used throughout this study. Blastospores and conidiospores of *C. ulmi* were prepared as described previously (10). The cell cultures (5 ml) were grown at room temperature (22 to 23°C) in 25-ml glass Erlenmeyer flasks and aerated by rotary agitation on a New Brunswick Scientific G-2 shaker at 200 rpm. The defined glucose-salts media GPP and GPR contained L-proline and L-arginine, respectively, as the nitrogen source (10, 13). EGTA, $LaCl_3$, the calmodulin inhibitors, and the anesthetics (Table 3) were purchased from Sigma Chemical Co., St. Louis, Mo. Each culture was prepared with an inoculum of 2×10^7 spores per ml and examined microscopically after 24 h of growth. Data on the percentage of cells in each morphological state were obtained by counting ≥200 cells.

Ca(II) regulation. Glucose-salts defined media, in which *C. ulmi* developed in the yeast phase with proline as the nitrogen source (GPP medium) and in the mycelial phase with arginine as the nitrogen source (GPR medium), have been described previously (10). These media did not contain added calcium; induction of the yeast phase in GPP depended on this absence (Table 1). Progressively higher germination percentages for conidiospores of *C. ulmi* were obtained in GPP supplemented with $CaCl_2$. In contrast, added $CaCl_2$ did not influence the expected germination percentages (98 to 100%) in GPR medium.

EGTA and $LaCl_3$. The ability of Ca(II) to promote germination in GPP led us to ask whether its absence could promote budding in GPR. The trace amounts of contaminating Ca(II) present in our media (ca. 0.8×10^{-7} M) could still be sufficient for Ca(II)-calmodulin to control the morphological state because the cytoplasmic Ca(II) concentration in cells is generally very low, frequently $\leq 10^{-7}$ M (7). We therefore sought to prevent any morphogenetic action by residual Ca(II) by adding either a Ca(II) chelator or a Ca(II) antagonist. EGTA is known to sequester Ca(II) ions (25). When 10 mM EGTA was added to GPR, only 10% of the conidiospores germinated, whereas 98 to 100% germinated in GPR without EGTA (Table 2). Similarly, when 200 μM of the Ca(II) competitor $LaCl_3$ was added to GPR, only 70% of the conidiospores germinated, whereas 98 to 100% germinated in GPR without $LaCl_3$. Concentrations of $LaCl_3 \geq 300$ μM were toxic to *C. ulmi*.

Calmodulin inhibitors. Most Ca(II)-dependent regulatory phenomena are mediated by the Ca(II)-binding protein calmodulin (4, 14). Because calmodulin is so highly conserved (4), we sought to determine the effect of six known inhibitors of mammalian and plant calmodulin (23–25) on *C. ulmi* morphology in GPR. The quintessential calmodulin inhibitor

TABLE 1. Effect of Ca(II) on the morphology of *C. ulmi* conidiospores[a] in GPP

$CaCl_2$ concn (mM)	% of cells showing:		
	Germination	Intermediate form[b]	Budding
0	11	1	88
1.0	9	18	73
2.0	12	36	52
3.0	48	1	51
4.0	53	4	43
5.0	60	5	35
20.0	85	9	6

[a] Similar results were obtained with *C. ulmi* blastospores.
[b] Transient mycelial stage (10) in which the spores germinate but do not branch; instead, the hyphae bud from the tips to produce yeasts.

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TABLE 2. Effect of EGTA on the morphology of *C. ulmi* conidiospores in GPR[a]

EGTA concn (mM)	% of cells showing:		
	Germination	Intermediate form	Budding
0	94	3	3
0.10	72	7	21
0.25	73	6	21
0.50	71	5	24
1.00	56	9	35
2.50	52	6	42
5.00	40	6	54
10.00	10	5	85

[a] See Table 1, footnotes *a* and *b*.

is trifluoperazine (11). Adding trifluoperazine to GPR caused blastospores to shift from 94 to 96% germination with no trifluoperazine to 50 and 10% germination with 20 and 40 μM trifluoperazine, respectively, and there was 0% germination (100% budding) with 50 μM trifluoperazine (Fig. 1). At somewhat higher concentrations, five other recognized calmodulin inhibitors (chlorpromazine, quinacrine, dibucaine, propranolol, and tetracaine) also caused shifts leading to budding in GPR (Table 3). Significantly: (i) the effect of each of the six calmodulin inhibitors could be completely reversed by the simultaneous addition of 5 mM $CaCl_2$; (ii) two anesthetics not reported to be calmodulin inhibitors, procaine and lidocaine, did not affect the expected mycelial morphology (Table 3); and (iii) none of the compounds listed in Table 3 altered the expected morphology (90 to 95% budding) in GPP.

We have shown that Ca(II) ions (Table 1), a Ca(II) sequestering agent (Table 2), a Ca(II) antagonist, and six calmodulin inhibitors (Table 3) shift the dimorphic potential of *C. ulmi*. The effective concentrations and the order of effectiveness of the six calmodulin inhibitors used (trifluoperazine > chlorpromazine > quinacrine > dibucaine > propranolol > tetracaine) closely approximated those found to inhibit calmodulin stimulation of cyclic nucleotide phosphodiesterase (24) and betacyanin synthesis (6). Furthermore, the effect of each of these calmodulin inhibitors could be reversed by adding 5 mM $CaCl_2$, and the presence of two local anesthetics not reported to be calmodulin inhibitors, lidocaine and procaine, did not alter the *C. ulmi* morphology

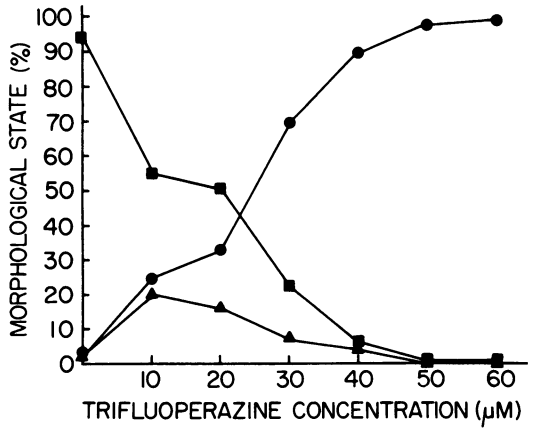

FIG. 1. Effect of trifluoperazine on the morphology of *C. ulmi* blastospores in GPR. Morphological state was determined 24 h after inoculation with 2×10^7 spores per ml. Symbols: ■, percent germination; ●, percent budding; ▲, percent intermediates.

TABLE 3. Effect of calmodulin inhibitors and anesthetics on the morphology of *C. ulmi* blastospores in GPR[a]

Compound	Concn (μM) required for:	
	50% Germination	50% Budding
Trifluoperazine	20	25
Chlorpromazine	35	55
Quinacrine	80	160
Dibucaine	300	450
Propranolol	520	580
Tetracaine	2,500	3,500
Lidocaine[b]	>50,000	
Procaine[b]	>50,000	

[a] Differences between the concentrations necessary to achieve 50% germination and 50% budding reflect the presence of intermediates (Fig. 1).
[b] These compounds did not affect the expected mycelial morphology.

(Table 3). Thus, two of the criteria established (4) for determining whether a biological phenomenon is regulated by calmodulin have been fulfilled by the findings for its effects on dimorphism in *C. ulmi*.

Our data indicate that Ca(II)-calmodulin interaction is necessary for mycelial growth in *C. ulmi* and that its absence leads to the yeast phase. Others (5, 9) have failed to detect calmodulin in the yeast *S. cerevisiae*. Our data agree with the observation that Ca(II) acts as a branching signal in *N. crassa* (19) and are consistent with the speculation that a Ca(II) gradient is necessary for tubular growth in both plants and *Achyla* (7, 18). We are presently attempting to: (i) measure the internal and external concentrations of Ca(II), calmodulin, and other Ca(II)-binding proteins for each morphological state of *C. ulmi*; (ii) identify the Ca(II)-calmodulin-activated enzymes ultimately responsible for this dimorphism; and (iii) identify the gene products responsible for the differences observed between growth in GPR and growth in GPP.

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