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Calcium Dependence and Binding in Cultures of *Histoplasma capsulatum*

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***Histoplasma capsulatum* is a pathogenic fungus with two distinct morphologies and lifestyles. The saprophytic form of this organism, a mold, thrives in soil and is especially abundant in the Ohio and Mississippi River valleys. Its parasitic counterpart, a yeast, colonizes phagolysosomes of mammalian macrophages. We have observed a major difference in the calcium requirements of the two forms of *Histoplasma*, potentially implicating the phagolysosome as a calcium-limiting compartment. Deprivation of calcium by the addition of EGTA to culture media inhibited the growth of mycelial *H. capsulatum* but had no effect on yeast growth in vitro. In addition, yeasts released a calcium-binding protein (CBP) detectable by a $^{45}CaCl_2$ blotting technique. CBP was a major component of yeast culture supernatant and was also detectable by ruthenium red staining, another assay for calcium-binding activity. Conversely, mycelial *H. capsulatum* did not produce CBP, a finding that correlates with the dependence of mycelia on calcium for growth. We also describe here the purification of CBP from yeast culture supernatant by reversed-phase high-pressure liquid chromatography.**

The dimorphic fungus *Histoplasma capsulatum* establishes infection when hyphae and conidia are inhaled into the mammalian respiratory tract. This warm, moist environment triggers conversion of the saprophytic mycelial form to the parasitic yeast form, which is well suited to survival inside alveolar macrophages. *Histoplasma* yeasts proliferate within the phagolysosome (7), a particularly harsh compartment containing many enzymes that are typically effective at destroying microorganisms. However, Eissenberg et al. demonstrated that *H. capsulatum* yeasts maintain the phagolysosomal pH near neutral (6). Since acidic conditions are required for most lysosomal enzyme activities, modulating the phagolysosomal pH appears to be one of the mechanisms by which *H. capsulatum* survives intracellularly.

Histoplasma may also have to contend with surroundings which are low in calcium, based on observations for other intracellular pathogens. For example, *Toxoplasma gondii* appears to have a mechanism for survival within a potentially calcium-poor environment. Shortly after entry into host cell phagosomes, *T. gondii* secretes a protein-rich vesicular network which has been observed by electron microscopy. This network is also released in vitro when *T. gondii* is deprived of calcium, and one of the components in the network is a calcium-binding protein (16). In addition, García Véscovi et al. have shown that the PhoP-PhoQ two-component regulatory system in *Salmonella typhimurium* responds directly to low concentrations of magnesium or calcium (10). Transcriptional repression of PhoP-activated genes is mediated by either calcium or magnesium binding to distinct sites on the sensor protein PhoQ; therefore, activation of these genes demands an environment low in both cations (9). PhoP-PhoQ activates many genes that are expressed when *Salmonella* is inside macrophage phagolysosomes, implying that this intracellular compartment may be deficient in both calcium and magnesium.

We report here a dramatic difference in the levels of calcium required for yeast and mycelial *H. capsulatum* growth in vitro, as well as the identification and purification of a released *H. capsulatum* calcium-binding protein (CBP) which may be important for yeast intracellular survival.

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MATERIALS AND METHODS

Yeast strains. *H. capsulatum* G186A was obtained from the American Type Culture Collection. The parental strain is designated with an "R" suffix to denote its rough colony morphology. Variants which are avirulent in mouse and macrophage culture models were derived from the parental strains (8, 11). Spontaneously arising avirulent variants exhibit smooth colony morphology and are designated with an "S" suffix.

Mycelial strains. The *H. capsulatum* yeast strains mentioned above were grown at room temperature to induce the transition to the mycelial form. We verified that the cultures were fully mycelial by light microscopy.

Growth of yeast and mycelia in calcium-limiting conditions. *H. capsulatum* G186AR and G186AS were inoculated at 10^6 yeasts/ml into 24-well plates in HMM (17), either alone or supplemented with 150, 300, or 600 μM EGTA to chelate the 300 μM Ca^{2+} present in HMM. Since we are unable to count individual cells in mycelial cultures, a plug from a mycelial colony was placed into 1 ml of HMM in a microcentrifuge tube. After samples were vortexed and large clumps were allowed to settle, equal volumes of the suspension were inoculated into the wells. Growth was monitored visually and by assaying for metabolic activity (see below) of both types of organisms.

Preparation of culture supernatants for analysis of calcium-binding proteins. Five-milliliter cultures of *H. capsulatum* yeasts or mycelia were grown in HMM broth at 37 or 25°C, respectively. After centrifugation for 10 min at 1,200 × *g*, we collected the culture supernatants and sterilized them with a 0.2-μm-pore-size filter. Supernatants were concentrated in Centricon-3 filtration units (Amicon, Beverly, Mass.) and diafiltered with 2 volumes of 10 mM ammonium acetate, pH 7.0. Concentrated samples were lyophilized for subsequent analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

Quantitation of metabolic activity of *H. capsulatum*. To compare the quantities of released proteins in yeast and mycelial culture supernatants, we normalized the cultures based on their metabolic activities as measured by the reduction of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple crystals of formazan. We transferred 1-ml samples from the wells to microcentrifuge tubes and added 80 μl of MTT (12.5 mg/ml in water). After incubation for 2 h at 37°C (yeast) or room temperature (mycelia), the formazan was extracted with 2 ml of isopropanol containing 0.04 M HCl. Samples were sonicated for 10 min to facilitate formazan extraction and quantitated by measuring the absorbance at 570 nm (12).

$^{45}CaCl_2$ and ruthenium red blotting of calcium-binding protiens. $^{45}CaCl_2$ blotting was done by the method of Maruyama et al. (13). Briefly, proteins in concentrated culture supernatants (normalized as described above) or purified CBPs were separated by SDS-PAGE and transferred to 0.1-μm-pore-size nitrocellulose. After incubation in calcium-free hybridization buffer (10 mM Tris-HCl [pH 6.8], 60 mM KCl, and 5 mM $MgCl_2$), the filter was probed with 1 μCi of $^{45}CaCl_2$ per ml at 37°C, followed by washing for 5 min with water adjusted to pH 6.8. The filter was air dried and exposed for autoradiography. We also performed $^{45}CaCl_2$ blotting on proteins separated by nondenaturing gel electrophoresis, in

which SDS, β-mercaptoethanol, and dithiothreitol were omitted from the sample buffer and gel.

Ruthenium red staining was performed in a manner similar to that of $^{45}CaCl_2$ blotting after SDS-PAGE, with the exception that the nitrocellulose was stained with 25 mg of ruthenium red (Sigma, St. Louis, Mo.) per liter of calcium-free hybridization buffer (3). Silver staining after SDS-PAGE was performed by the method of Oakley et al. (14).

Reversed phase HPLC purification of CBP. A broth culture from a representative *H. capsulatum* yeast strain, G186AR, was centrifuged for 10 min at 1,200 × *g*, and the culture supernatant was collected and sterilized with a 0.2-μm-pore-size filter. The supernatant was concentrated in an Amicon filtration unit containing a 1,000-Da-cutoff ultrafiltration membrane and then diafiltered with 2 volumes of 10 mM ammonium acetate, pH 7.0. After lyophilization, the material was resuspended in 0.1% trifluoroacetic acid (TFA) and applied to a reversed-phase high-pressure liquid chromatography (HPLC) C_8 column (RP-300; Applied Biosystems, Foster City, Calif.). To separate CBP from the other supernatant components, we developed a linear gradient from 100% buffer A (0.1% TFA) to 100% buffer B (0.1% TFA plus 60% acetonitrile) over a period of 60 min. One-minute fractions were collected and dot blotted onto nitrocellulose with a MilliBlot-D System (Millipore, Bedford, Mass.). The filter was probed with $^{45}CaCl_2$, as described below, to determine which fraction contained CBP. Fractions that showed $^{45}CaCl_2$ binding on the dot blot were also analyzed by SDS-PAGE, as described above. A reversed-phase HPLC phenyl column (PH-300; Applied Biosystems) was used to purify further the CBP-containing fraction, with identical buffers, gradient conditions, and collection procedures. After collecting 1-min fractions over a 60-min gradient, we determined which fraction contained purified CBP by $^{45}CaCl_2$ dot blotting.

Electrospray ionization mass spectrometry. The mass of CBP was determined with a Platform II mass spectrometer (Micromass Instruments, Danvers, Mass.) and associated data system. The sample was dissolved in a 1:1 water-acetonitrile solution containing 0.1% formic acid. Samples were introduced into a 5-μl/min flow of the same buffer via a Rheodyne injector and loop and then flowed into the mass spectrometer ion source. Spectra were obtained and transformed (converted to the zero-charge spectrum) with the standard instrument software.

RESULTS

***H. capsulatum* dependence on calcium.** Yeast and mycelial growth in calcium-poor medium was examined by adding EGTA to chelate the Ca^{2+} in HMM broth. To generate growth curves, we measured the metabolic activities of the organisms at each time point by reduction of MTT. The yeast form of strain G186AS grew well under all conditions tested. However, mycelial growth was inhibited in a concentration-dependent fashion (Fig. 1). The growth curves of both forms of G186AR were similar to those of G186AS and are not shown. When 300 μM $CaCl_2$ was added to mycelial cultures containing 300 μM EGTA, mycelial growth was restored; in some cases, the growth was better than that of mycelia in HMM alone.

Identification of a released calcium-binding component. On the basis of the precedent set by *Toxoplasma* of calcium-binding protein production, we screened *H. capsulatum* yeast culture supernatants for the presence of calcium-binding proteins by $^{45}CaCl_2$ blotting. Autoradiography revealed a low-molecular-weight band with calcium-binding activity (designated CBP), as shown in Fig. 2A. When these results were compared with results from SDS-PAGE of culture supernatants, CBP migration was found to correspond to the region most densely stained with silver nitrate (Fig. 2B). In addition, we assayed yeast cell walls and extracts by $^{45}CaCl_2$ blotting and could detect no major calcium-binding molecules with this assay (data not shown).

Reversed-phase HPLC purification of CBP. The culture supernatant from a representative strain, G186AR, was processed as described in Materials and Methods and applied to a reversed-phase HPLC C_8 column. The chromatogram is shown in Fig. 3A. Fractions 59 and 60 showed calcium-binding activity by a dot blot assay, and the calcium-binding protein in these fractions comigrated with the 8- to 10-kDa calcium-binding protein in total culture supernatant (data not shown). These two fractions were further purified on a reversed-phase HPLC phenyl column followed by collection of 1-min fractions. A $^{45}CaCl_2$ dot blot of the samples revealed calcium-binding activity in a single fraction which corresponded to a single peak on the chromatogram, as shown in Fig. 3B and C. The positive fraction, no. 58, was also analyzed by nondenaturing PAGE followed by $^{45}CaCl_2$ blotting. Autoradiography revealed that this purified protein retained its calcium-binding activity as shown in Fig. 4A, and we have designated it CBP. Purified CBP appeared as a doublet after SDS-PAGE and silver nitrate staining, as shown in Fig. 4B.

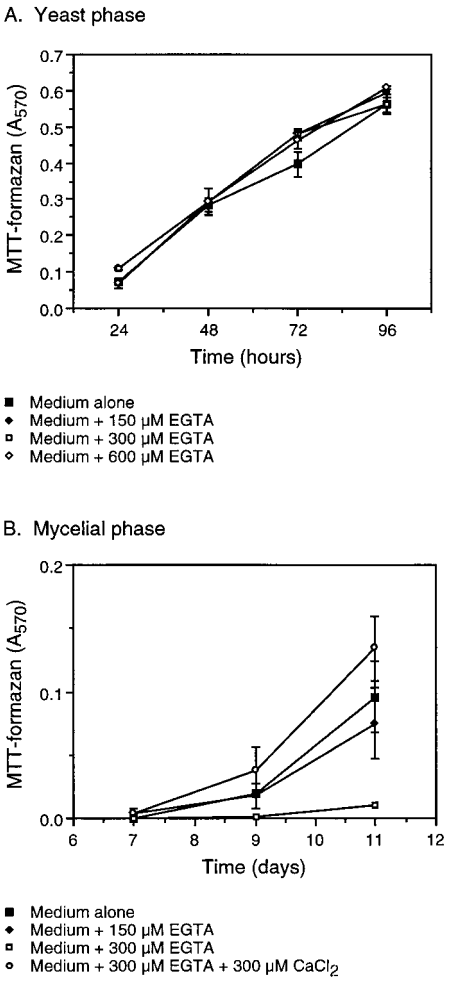

FIG. 1. Growth curves of *H. capsulatum* G186AS yeast and mycelia under calcium-limiting conditions. MTT assays were performed at the indicated time points, and the absorbance at 570 nm was plotted versus time. Each point indicates the mean for triplicate samples; error bars indicate standard deviations. (A) Yeasts were grown in HMM with different concentrations of EGTA. (B) Mycelia were grown in HMM with different concentrations of EGTA or with EGTA and equimolar $CaCl_2$ added exogenously.

From 1 liter of culture supernatant, we purified approximately 7 to 10 mg of CBP from a log-phase *H. capsulatum* yeast culture. The amount of CBP recovered from supernatant was at least 50% of the amount of CBP in total culture supernatant, as estimated by comparing the band intensities of culture supernatant and purified CBP after SDS-PAGE and silver nitrate staining.

Mass analysis of CBP. The molecular mass of purified CBP was determined by electrospray mass spectrometry. The spectrum showed a major peak at 7,858.0 Da and smaller peaks at 8,020.0 and 8,182.0 Da, each 162 mass units higher than the previous peak (data not shown). These data are consistent with

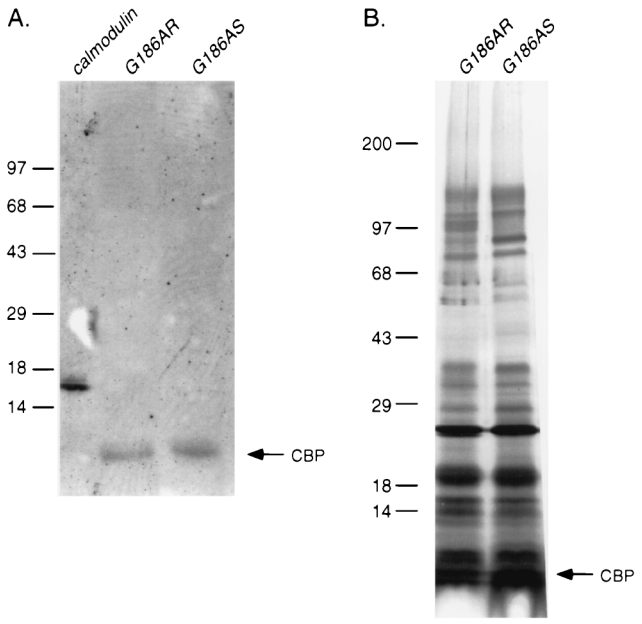

FIG. 2. *H. capsulatum* G186AR and G186AS yeast culture supernatants either blotted with $^{45}CaCl_2$ (A) or stained with silver nitrate after SDS-PAGE (B). Calmodulin was included as a positive control for $^{45}CaCl_2$ binding. The arrow designating CBP in panel B is positioned according to the migration of CBP detected in panel A. Numbers at left are molecular weights, in thousands.

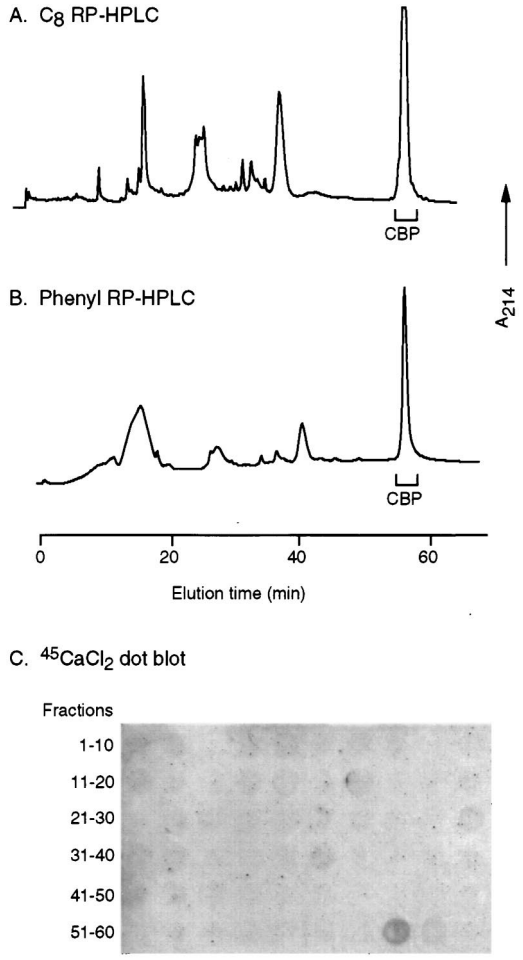

FIG. 3. Reversed-phase HPLC (RP-HPLC) purification of CBP. Peaks corresponding to CBP are indicated. (A) Chromatogram of total yeast culture supernatant separated on a C_8 column. (B) Chromatogram of the indicated peak from the C_8 column separated on a phenyl column. (C) $^{45}CaCl_2$ dot blot of fractions collected from the phenyl column to detect which fraction contained CBP (fraction 58).

posttranslational modification of CBP with one or two hexose residues.

Phase-specific release of CBP. Culture supernatants from the yeast and mycelial forms of 10 strains of *H. capsulatum* were assayed for the presence of CBP. Since we are unable to count individual cells in mycelial cultures, we chose an MTT assay for metabolic activity as a method of normalizing the samples; we also generated the same results when samples were normalized to the amounts of total protein released. Culture supernatants were analyzed by incubation with ruthenium red, a dye known to stain calcium-binding protein on nitrocellulose filters (3). The stained filter in Fig. 5 depicts the yeast and mycelial supernatants from a representative strain, G186AS; similar results have been observed with nine other *H. capsulatum* strains assayed by $^{45}CaCl_2$ blotting of culture supernatants (1). With either assay, we have never seen any evidence of CBP production by mycelial cultures of *H. capsulatum.*

DISCUSSION

H. capsulatum is well adapted to survive in several very different environments, due in large part to its dimorphic nature. The saprophytic form of *H. capsulatum*, a mold, lives in the soil until the mycelia are disturbed and inhaled by a mammalian host. After reaching the respiratory tract, which has a temperature of 37°C, conidia and hyphae convert to budding, unicellular yeasts. This parasitic form of *H. capsulatum* is adapted to survive and proliferate within several host cell types (5), including macrophages, within which yeasts reside in phagolysosomes. Inside the phagolysosomal compartment, the yeasts must contend with possible exposure to acid pH, lysosomal enzymes, toxic oxygen radicals, and a potentially calcium-poor milieu. In this paper, we have shown a major difference in the growth requirement for the yeast and mycelial forms of *Histoplasma*. We added EGTA, a calcium chelator, to the culture medium to create an environment with little free calcium. The yeasts grew well in medium containing EGTA, an observation that is consistent with the yeasts' ability to proliferate within phagolysosomes, which may contain little free calcium (10, 15, 16). Conversely, mycelial *Histoplasma* growth was inhibited by low free-calcium concentrations. The simultaneous addition of equimolar amounts of EGTA and $CaCl_2$ allowed normal growth of the mold form, implying that the inhibitory effect of EGTA was based solely on calcium limitation.

Another calcium-related difference between the mycelial and yeast phases of *H. capsulatum* is the production of CBP. Since mycelia do not make this protein and are also more dependent on calcium for growth, *Histoplasma* yeasts may use CBP as part of their adaptation to growth in a calcium-poor environment. We verified this protein's calcium-binding activity by two independent techniques: $^{45}CaCl_2$ blotting and ruthenium red staining. Both assays indicated that CBP was the only calcium-binding molecule released by *H. capsulatum* yeasts. In addition, neither assay detected CBP in yeast cell walls or extracts. The fact that CBP is a major component of the yeast

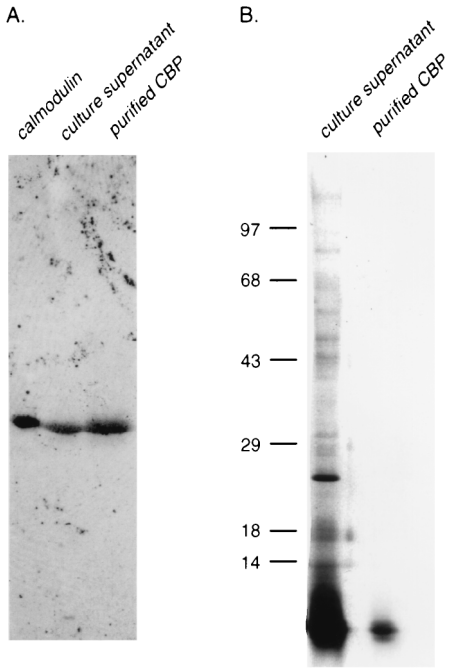

FIG. 4. Comparison of *H. capsulatum* G186AR culture supernatant and purified CBP either blotted with $^{45}CaCl_2$ after nondenaturing PAGE (A) or stained with silver nitrate after SDS-PAGE (B). In panel A, calmodulin was included as a positive control for $^{45}CaCl_2$ binding; purified CBP, like calmodulin, will also bind $^{45}CaCl_2$ following SDS-PAGE (data not shown). Numbers at left are molecular weights, in thousands.

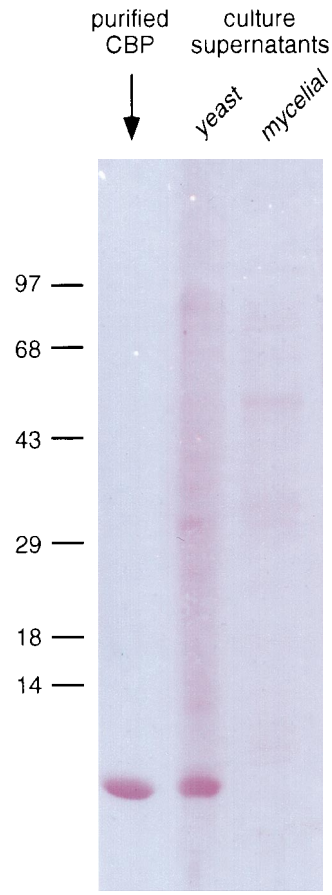

FIG. 5. Ruthenium red-stained blot of purified CBP and supernatants from yeast and mycelial cultures of *H. capsulatum* G186AS.

culture supernatant suggests an important function for this protein in modifying or responding to the environment.

In an earlier preliminary study (1), we showed CBP production by a variety of *H. capsulatum* yeast strains from diverse genetic classes and geographical origins. After these strains were induced to switch to the mold form by growing them at room temperature, none released CBP into their culture supernatants. This data, taken together with the differences between the yeast and mycelial forms in their dependence on calcium for growth, suggests that CBP has a significant and evolutionarily conserved role in the parasitic lifestyle of *Histoplasma*. Although we found that both virulent strains and their avirulent variants release CBP, it is not possible to conclude anything definitive regarding the linkage between virulence and CBP; it is likely that CBP is only one of many factors that act in concert to promote yeast survival and proliferation within host cells.

Once we had identified the presence of CBP in yeast culture supernatants, we took advantage of the $^{45}CaCl_2$ blotting method and modified it for use as an assay for determining the presence of CBP during fractionation and purification. After dot blotting an aliquot of each fraction collected from reversed-phase HPLC separation, we probed the filter with $^{45}CaCl_2$. The calcium-binding activities of samples that were positive in the dot blot assay were verified by $^{45}CaCl_2$ blotting of the samples after SDS-PAGE. The calcium-binding property of purified CBP was tested further by ruthenium red staining. In this assay, CBP appeared to bind calcium, since the dye stained CBP on nitrocellulose filters. In addition, purified CBP appeared as a doublet in SDS-PAGE, a finding consistent with the behavior of other calcium-binding proteins such as calmodulin, where the binding of calcium causes a mobility shift in electrophoresis (2, 4). In the mass analysis of purified CBP, the three peaks in the spectrum implicate glycosidase processing during or following CBP production. Alternatively, yeasts may glycosylate only a certain proportion of the CBP molecules as they are synthesized.

Although up to 50% of CBP is lost during the purification procedure, we routinely purified 7 to 10 mg from each liter of culture supernatant; this correlates with its appearance as the most abundant released protein detected by SDS-PAGE. We do not yet know the precise function of CBP, but its yeast-specific production and export imply a role in intracellular survival, potentially as a mechanism to sequester calcium during growth in the phagolysosomal compartment. Calcium binding by CBP may have a more indirect effect on *Histoplasma* growth in vivo, perhaps by altering the phagolysosomal environment or by changing host cell responses to the intracellular yeasts. Regardless, purified CBP has now become the primary tool in exploring the connections between calcium dependence and host cell parasitism by *H. capsulatum.*

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