INFECTION AND IMMUNITY, Nov. 2003, p. 6155–6164 Vol. 71, No. 11
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.11.6155–6164.2003

Induction of Capsule Growth in *Cryptococcus neoformans* by Mammalian Serum and CO_2

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Received 28 May 2003/Returned for modification 25 June 2003/Accepted 25 July 2003

The pathogenic fungus *Cryptococcus neoformans* has a polysaccharide capsule that is essential for virulence in vivo. Capsule size is known to increase during animal infection, and this phenomenon was recently associated with virulence. Although various conditions have been implicated in promoting capsule growth, including CO_2 concentration, osmolarity, and phenotypic switching, it is difficult to reproduce the capsule enlargement effect in the laboratory. In this study, we report that serum can induce capsule growth, and we describe the conditions that induce this effect, not only by serum but also by CO_2. Capsule enlargement was dependent on the medium used, and this determined whether the strain responded to serum or CO_2 efficiently. Serum was most effective in inducing capsule growth under nutrient-limited conditions. There was considerable variability between strains in their response to either serum or CO_2, with some strains requiring both stimuli. Sera from several animal sources were each highly efficient in inducing capsule growth. The cyclic AMP (cAMP) pathway and Ras1 were both necessary for serum-induced capsule growth. The lack of induction in the *ras1* mutant was not complemented by exogenous cAMP, indicating that these pathways act in parallel. However, both cAMP and Ras1 were dispensable for inducing a partial capsule growth by CO_2, suggesting that multiple pathways participate in this process. The ability of serum to induce capsule growth suggests a mechanism for the capsular enlargement observed during animal infection.

The yeast *Cryptococcus neoformans* is a human pathogen that is ubiquitous in certain environments, such as soil contaminated with pigeon excreta. Human infection is believed to result from inhalation of infectious particles and, in some areas, 50 to 70% of individuals have antibodies against *C. neoformans*. However, cryptococcal disease in normal hosts is rare (22). Although most cases of human infection with *C. neoformans* are not recognized clinically, the infection can become latent and/or disseminate in the setting of immune impairment. The most common clinical manifestation of cryptococcosis is meningitis, a condition that is lethal unless treated.

C. neoformans has several virulence factors (for review, see reference 12), including a capsule, which is mainly composed of the polysaccharide glucuronoxylomannan (GXM). The size of the capsule of *C. neoformans* is variable, ranging from 5 to 30 μm (19, 43) and varying between strains (38). In soils and under laboratory conditions, the capsule size of most *C. neoformans* strains is relatively small but can increase during mammalian infection (5, 14, 33). Littman showed that the size of the capsule was highly variable and dependent on the environmental conditions (32). During in vivo infection, the size of the capsule varies depending on the organ studied. For instance, the lung environment is a powerful inducer of capsule growth (43). Another compartment that induces capsule growth, although not as efficiently as the lung, is the brain (18, 43). There are several reports indicating that the induction observed in vivo contributes to the virulence of the pathogen. Strains unable to induce capsule growth showed reduced virulence (3, 16, 23). In this regard, increase in capsule size has been associated with resistance to phagocytosis (8, 30, 37). But paradoxically, there is no correlation between capsule size at the moment of infection and virulence (3, 16, 24, 28). Several conditions induce capsule growth in vitro (for review, see reference 36). The most commonly used are high CO_2 concentration (23) and iron deprivation (25, 46). Other factors have also been described, such as availability of vitamins, the amino acids present, the type of carbon source (32) and osmolarity (17, 27). Unfortunately, it is difficult to achieve capsule growth under laboratory conditions, and not all the strains respond to these stimuli. Consequently, this phenomenon has not been extensively studied despite its relevance to cryptococcal infection.

The phenomenon of capsular growth is believed to be relevant because it increases the size of the cell and thus poses a problem for phagocytosis (29, 51). Furthermore, the capsule and the capsular polysaccharide interfere with a large number of processes involved in the immune response (15, 29, 34, 42, 47). The capsule is required for virulence, since acapsular mutants are avirulent (13, 21), and it is also necessary for survival inside phagocytic cells (45). However, several reports suggest that it is not necessary for protection in killing assays (9, 31).

Prior studies have shown that serum can have an inhibitory effect on yeast growth (41). However, studies about the effect of serum on capsule size are scarce (4), and it is not clear whether it can be used as a modulator of capsule growth. Here we demonstrate that sera from multiple sources can be potent inducers of capsule growth. We have also analyzed the requirement of CO_2 and gained insight into the putative pathways involved in this process. We conclude that both serum and CO_2 induce capsule growth and that this induction is also controlled by additional environmental factors.

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TABLE 1. Induction of capsule growth by serum and CO_2

Strain	Serotype	Induction[a]				Reference or source
		CO_2		No CO_2		
		Serum	PBS	Serum	PBS	
H99	A	+	+/−	+	−	20
J4	A	+	−	+	−	35
J8	A	+	−	+	−	35
J11	A	+	−	−	−	35
J20	A	+/−	−	+	−	35
J47	A	−	−	+/−	−	35
I6	B	+	−	+	+/−	U. Banerjee (Dehli, India)
I23	B	+	+	−	−	U. Banerjee
I24	B	+	+	+	−	U. Banerjee
102.97	B	+	+	−	−	T. Mitchell (Durham, N.C.)
103.97	B	+	+	+	+	T. Mitchell
105.97	B	+	+	+/−	−	T. Mitchell
106.97	C	+	+	−	−	T. Mitchell
107.97	C	+	+	−	−	T. Mitchell
1343	C	+	+	−	−	Ira Salkin (New York, N.Y.)
24067	D	+	+	+	−	26
3501	D	+/−	−	+/−	−	26
11	D	+/−	+/−	−	−	35
13	D	+	+	+	−	35
14	D	−	−	−	−	35
16	D	+/−	+/−	−	−	35

[a] Yeast were grown in Sabouraud medium and transferred to PBS in the presence or absence of 10% FCS and in the presence or absence of 10% CO_2. Capsule size was determined after examining the cells in an India ink suspension. −, no induction; +/−, induction of 1 to 2 times, +, induction of >2 times.

MATERIALS AND METHODS

Strains and growth conditions. *C. neoformans* strains are listed in Table 1. Additionally, to study the role of cyclic AMP (cAMP) and Ras1 pathways, the following strains, kindly provided by J. Heitman, were used: RPC3 (*cac1*::*URA5* [3]), RPC7 (*cac1*::*URA5 CAC1* [3]), CDC1 (*pka1*::*URA5* [16]), CDC16 (*pka1*::*URA5 PKA1* [16]), LCC1 (*ras1*::*ADE2* [1]), and LCC2 (*ras1*::*ADE2 RAS1* [1]). For each strain, the cells were grown overnight in Sabouraud dextrose broth medium (Difco, Sparks, Md.) at 30°C in a rotating shaker with moderate agitation (150 to 180 rpm). The cells were collected, washed three times with phosphate-buffered saline solution (PBS), and counted with a hemocytometer.

Capsule growth induction. To study capsule growth, the yeast cells were incubated at 37°C for the time interval indicated in one of the following media: PBS, Sabouraud dextrose broth, or Dulbecco's modified Eagle medium (DME; Life Technologies, Rockville, Md.). As serum source, 10% heat-inactivated fetal calf serum (FCS) was used in all experiments unless otherwise indicated. In some experiments, 10% human, rat, mouse, or guinea pig serum was used. Inactivation of sera was performed by incubation at 56°C for 30 min. The yeast cells (around 2×10^6 to 4×10^6) were placed in six-well plates containing 2 ml of medium and incubated at 37°C in the absence or presence of 10% CO_2. To supplement the media with iron, EDTA-ferric sodium salt (Sigma, St. Louis, Mo.) was added at the concentration indicated in each case. For some experiments, the pH of the medium was adjusted with HCl or NaOH. Since incubation of the yeast did not significantly change the pH of the medium (most probably due to the low density of the cells used), no additional buffer was required. In some experiments, cAMP (Sigma Aldrich) was added at a final concentration of 10 mM.

India ink staining and microscopy. To visualize the size of the capsule, a drop of India ink was added to the cell suspension on the slide. The samples were observed in an Olympus AX70 microscope. Pictures were taken with a QImaging Retiga 1300 digital camera using the QCapture Suite V2.46 software (QImaging, Burnaby, British Columbia, Canada) and processed with Adobe Photoshop 7.0 for Windows (San Jose, Calif.).

Measurement of capsule volume. To calculate the capsule volume, the diameters of the whole cell (D_{wc}) and the cell body (D_{cb}) were each measured with Adobe Photoshop 7.0, and capsule volume was defined as the difference between the volume of the whole cell (yeast cell plus capsule) and the volume of the cell body (no capsule). Volumes were determined using the equation for volume of a sphere as $4/3 \times \pi \times (D/2)^3$. In parallel, a relative measurement was calculated by representing the percentage of capsule in the whole cell as follows: $[(D_{wc} - D_{cb}) \times 100]/D_{wc}$. Between 15 and 40 cells were measured for each determination.

Immunofluorescence. To detect the capsule of *C. neoformans*, a monoclonal antibody to GXM 18B7 (11) and a goat anti-mouse (GAM) immunoglobulin G conjugated to tetramethylrhodamine isothiocyanate (TRITC) were used. Briefly, cells were incubated in 1% bovine serum albumin–0.5% horse serum for 1 h at 37°C, washed, incubated with primary antibody 18B7 (10 μg/ml [11]) for 30 min at 37°C, washed, and incubated with GAM-immunoglobulin G-TRITC (5 μg/ml; Southern Biotechnology Associates, Inc., Birmingham, Ala.). After incubation at 37°C for 1 h and a final wash, the cells were suspended in mounting medium (50 mM *n*-propylgallate, 50% glycerol in PBS) and observed with an Olympus AX70 microscope. To detect complement bound to the *C. neoformans* capsule, the yeast cells were grown in Sabouraud and washed with PBS, and 2×10^6 cells were suspended in 80 μl of mouse serum. After 1 h of incubation at 37°C, the cells were washed and incubated for 24 h in PBS plus 10% FCS at 37°C. To detect complement fluorescein-conjugated antibody (C), the yeast was incubated in 1% bovine serum albumin–0.5% horse serum for 1 h at 37°C, washed, incubated with conjugated GAM-complement (5 μg/ml; Cappel, ICN, Aurora, Ohio) for 30 min at 37°C, and visualized under the microscope after suspension in the mounting medium described above.

Statistics. The data were assessed for normal distribution by using the Shapiro-Wilk test. For measurements where the data were normally distributed, statistical analysis was done with an analysis of variance and *t* test. For measurements where the data were not normally distributed, statistical analysis was done by using the Kruskal-Wallis statistic. *P* values of <0.05 were considered significant. All the statistics were performed with the Unistat 5.5 (Unistat Ltd., London, England) and Analyze-it (Analyze-it Ltd., Leeds, England) software for Excel.

RESULTS

Effect of serum, CO_2, and growth medium on capsule growth induction. Although serum is known to induce morphological changes in other fungi, such as the phenomenon of germ tube formation in *Candida albicans*, exposure to serum is not generally believed to affect *C. neoformans*. While carrying out other studies in our laboratory, we noted that under certain conditions media containing 10% heat-inactivated FCS induced a significant increase in capsule size. To characterize this

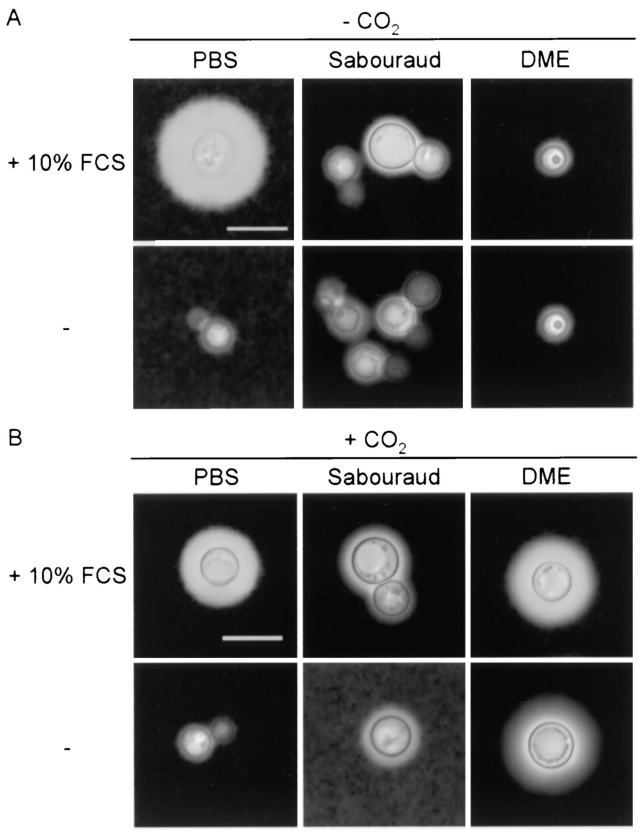

FIG. 1. Effect of the medium in the induction of the *C. neoformans* capsule by serum and/or CO_2. Strain H99 was grown in Sabouraud overnight, washed with PBS, and suspended in different media (PBS, Sabouraud, or DME) containing 10% heat-inactivated FCS as indicated in Materials and Methods. Two equivalent sets of media were prepared: one was placed at 37°C (A), and the other one was placed at 37°C in an atmosphere containing 10% CO_2 (B). The panels show *C. neoformans* cells in a suspension of India ink particles after 24 h of incubation. In both panels A and B, the bar in the first panel denotes 10 μm and is applicable to the other panels.

phenomenon systematically, we studied the effect of both serum and CO_2 in the induction of capsule growth. We examined capsule growth of strain H99, because this strain was used in prior capsule studies (19, 23, 40) and most of the auxotrophic mutants are derived from this strain (39, 48). Under laboratory growth conditions, the capsule of H99 has a relatively small size, which is in marked contrast to the large capsule variants observed during murine infection (19). We first studied the capsule induction in the presence and/or absence of CO_2 and/or 10% heat-inactivated FCS. Capsule induction was studied in three different media: PBS, Sabouraud, and DME. As shown in Fig. 1A, in the absence of CO_2 serum induced a prominent capsule in strain H99 when the cells were incubated in PBS, indicating that serum alone is a potent inducing factor for capsule growth. However, when H99 was cultured in Sabouraud or DME, serum did not induce capsule growth. We repeated this experiment, but in an atmosphere containing 10% CO_2. In DME, capsule growth occurred in response to CO_2, even in the absence of serum. This result indicated that the growth medium affected the ability to induce capsule growth and suggested the need for additional stimuli. Most of the experiments described in this paper were carried out in PBS because its composition is defined, and in this solution the effect of serum on capsule growth was prominent. Serum induced capsule growth at concentrations of 5% or higher, whereas below 5% the proportion of cells exhibiting increased capsule size was very small. Hence, we selected a serum concentration of 10% as our standard, because at this concentration strain H99 consistently demonstrated induced capsule growth. The induction of capsule growth was noticeable after only 6 h of incubation in inducing medium. Full induction of capsule growth, however, required 24 h (results not shown). The kinetics of capsule growth were not affected by incubation in CO_2.

Sera from fetal calf, mouse, human, rat, and guinea pig induced capsule growth (Fig. 2). There were no significant differences in the capsule volume of cells incubated in sera from different animals; however, there were small differences between mammalian sera, with rat and human sera being the most and less effective, respectively. Heat inactivation of serum had no effect on capsule induction (results not shown), suggesting no role of complement in the induction process.

Effect of pH and temperature on the serum-induced capsule growth. Several reports have indicated that capsule induction is a pH-dependent phenomenon (18, 23, 44). We observed that in the media in which the size of capsule increased, the pH was higher than 7. This suggested that a pH higher than 7 was necessary to induce capsule growth. However, pH was not a sufficient stimulus to induce capsule growth, since in a medium such as DME (pH around 7.5), serum did not induce capsule growth in the absence of CO_2. To investigate the effect of pH, we adjusted the pH to 5.6, from the slightly alkaline value of 7.3 found under our induction conditions (PBS plus serum). However, at a pH of 5.6, the serum did not efficiently increase capsule size, with only 15% of the cells having a large capsule, which was defined as a diameter of more than 45% of the total volume of the cell, capsule included (Fig. 3A).

Given the inefficiency of capsule induction by serum at pH 5.6, we investigated whether the lack of capsule growth in serum-supplemented Sabouraud medium was due to the acidic pH of this medium (range, 5.5 to 6). Consequently, we adjusted the pH of Sabouraud medium to 7.5 and studied serum induction. After incubation with the yeast, the pH of the suspension dropped to 7, but only a small proportion of cells (around 15%) demonstrated an increase in capsule size. However, statistical analysis indicated that the proportion of capsule in the cells after overnight incubation in Sabouraud without agitation in the presence or absence of serum decreased slightly (Fig. 3B). This indicates that in Sabouraud medium the lack of capsule induction was not due to the low pH of the medium.

The effect of temperature on capsule growth was studied. As shown in Fig. 4A, serum induced capsule growth at 24, 30, and 37°C, but the volume of the capsule was significantly larger at 37°C. However, the differences between the absolute volumes did not correlate with differences in the relative amount of capsule compared to the size of the cell. As shown in Fig. 4B, the percentage of the capsule after induction was very similar at all temperatures. To investigate the discrepancy between the data referred in volume or in percentage, we correlated the volume and the diameter with the size of the cell and found

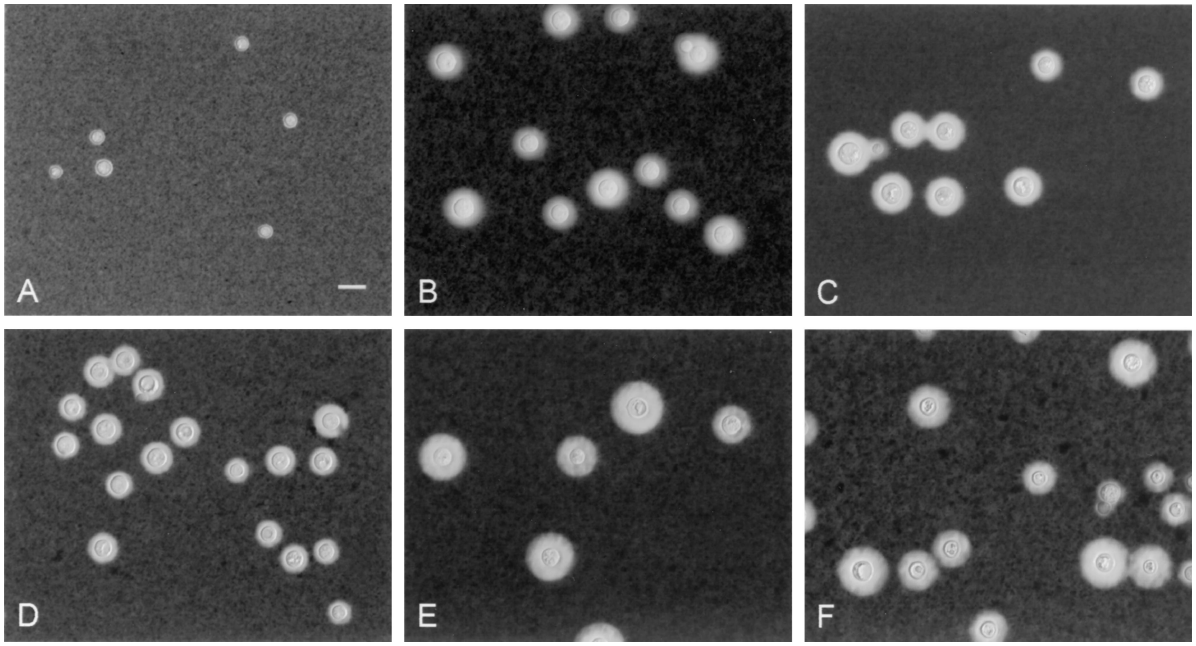

FIG. 2. Efficiency of different sources of serum in the induction of capsule growth. H99 was placed in PBS (A) or PBS containing 10% of the following sources of serum: FCS (B), mouse (C), human (D), rat (E), and guinea pig (F). Pictures of the cells suspended in India ink particles were taken after overnight incubation at 37°C.

positive correlations between both parameters (Fig. 4D). The apparent discrepancy arises because the size of the cell body is smaller at lower temperatures (Fig. 4C). So, we conclude that temperature does not have any effect on the proportion of capsule produced by the cell, but it does affect the size of the cell body.

Capsule growth in the presence of serum and CO_2 is highly strain dependent. Prior studies have noted differences in capsule growth among strains (19, 23, 32). To investigate this possibility, we compared capsule induction among various strains, including 24067 (serotype D) and H99 (serotype A). Strain 24067 exhibited two major differences compared to H99.

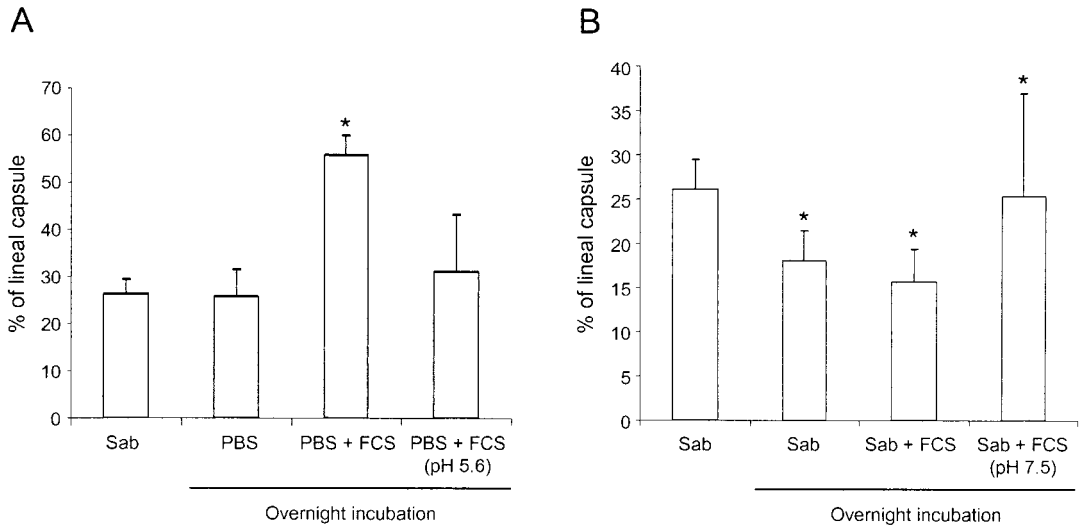

FIG. 3. Effect of pH on capsule growth. (A) Strain H99 was grown in Sabouraud and in the logarithmic phase cells were transferred to PBS, PBS plus 10% heat-inactivated FCS (pH 7.4), or the previous medium with the pH adjusted to 5.6 with HCl. After incubating overnight, the proportion of the size occupied by the capsule in the whole cell was calculated as described in Materials and Methods, measuring at least 100 cells in each case; the average and standard deviation are plotted in the left panel. Asterisks denote statistical differences between the size of the capsule after growth in Sabouraud medium and after incubation under the rest of the conditions, using the *t* test or Kruskall-Wallis test, dependent on the normality of the distribution of the samples. (B) H99 was grown as described for panel A but transferred to Sabouraud, Sabouraud plus 10% heat-inactivated FCS, or Sabouraud plus 10% FCS with pH adjusted to 7.5 with NaOH. The size of the capsule was calculated and is represented as described for panel A. Statistical differences are noted with an asterisk and were calculated as described for panel A.

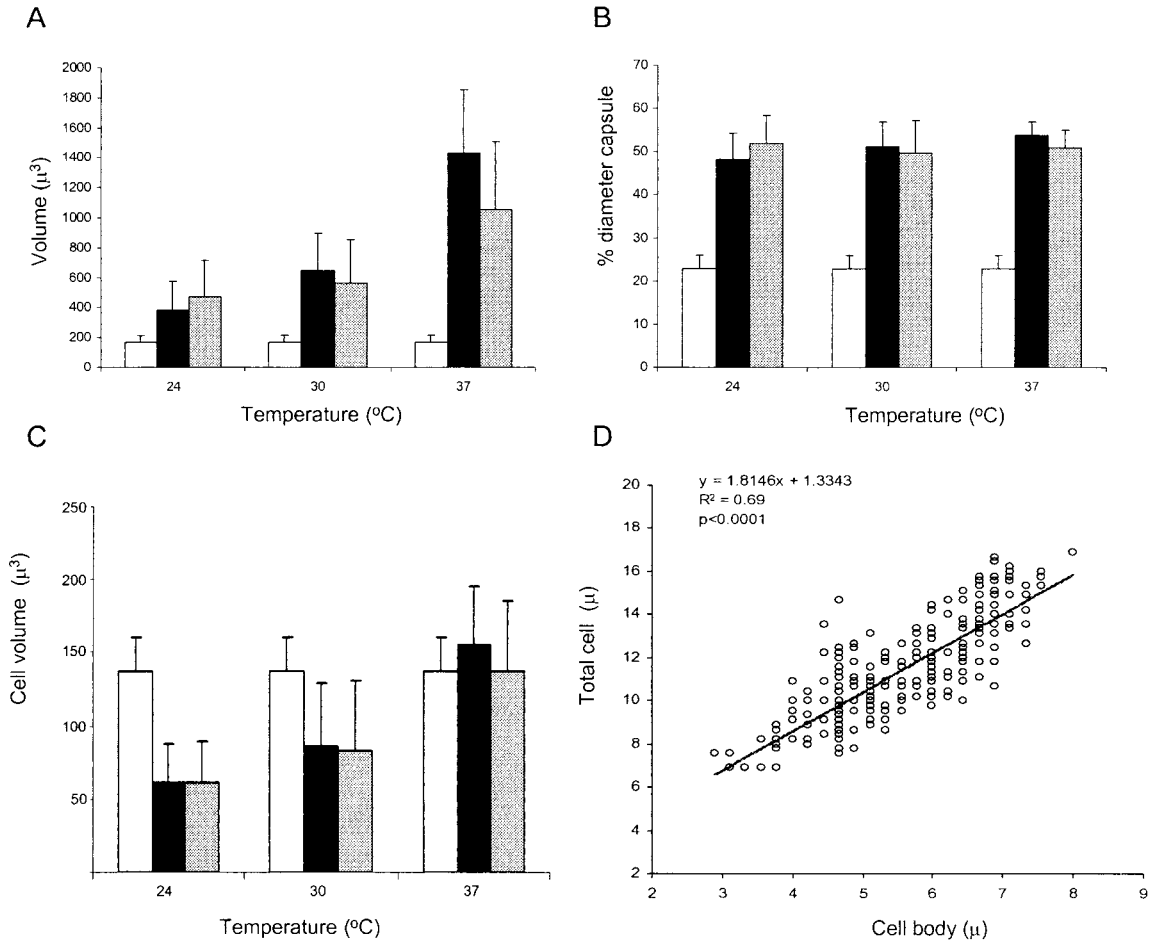

FIG. 4. Effect of temperature on capsule induction. Strain H99 was grown in Sabouraud (open bars), and capsule was induced by transferring the cells to PBS containing 10% heat-inactivated FCS and incubating at 24, 30, or 37°C for 24 h (black bars) or 48 h (grey bars). After induction, capsule volume (A), relative size of the capsule (B), and cell volume (C) were calculated as described in Materials and Methods. The mean and standard deviation in each case of at least 25 cells are indicated in panels A to C. (D) Correlation between cell body and total cell diameter (cell body plus capsule). The equation and the R^2 and P values for each regression are also indicated.

First, it manifested capsule growth in PBS in the presence of CO_2, without serum and additional nutrients (Table 1). Second, induction of capsule growth in strain 24067 was accompanied by a great heterogeneity in cell size and shape, such that cells with big capsules were mixed with other cells with a very small size of both cell body and capsule. In contrast, capsule induction in H99 produced a more homogenous population, with more than 95% of the cells demonstrating large capsules. We found that the cells that were placed in the induction medium had increased capsule size, and the small cells with small capsules were buds originated during the overnight incubation. We confirmed this finding by labeling the cells at the beginning of the incubation with C, which binds covalently to the capsule and can be easily visualized by immunofluorescence using an anti-C fluorescein isothiocyanate-conjugated antibody and does not segregate to the daughter cells. After overnight incubation in serum, none of the cells with a small capsule had C labeling, whereas more than 90% of the cells with a large capsule had C bound to the capsule (results not shown).

Capsule induction by serum and CO_2 was studied in other serotype A, B, C, and D *C. neoformans* strains. We found great variability in the response of different strains to the stimuli for capsule induction (Table 1). Serum strongly induced capsule growth in most serotype A strains, whereas for strains of this serotype CO_2 had little or no effect. In contrast, most of the serotype B strains responded very efficiently to either CO_2 or serum, and the combination of both stimuli induced a strong increase in capsule size for five of six strains. However, the serotype C strains studied did not respond to serum but did manifest increased capsule growth when exposed to CO_2. Serotype D strains demonstrated considerable variability in response to both serum and CO_2. For two strains (24067 and 13), both serum and CO_2 efficiently induced the capsule, whereas this induction was absent in other serotype D strains. We considered the possibility that the inability of serum to induce capsule growth for some strains was due to a limiting concentration of serum. Hence, we repeated the experiments using 100% FCS with seven different strains that did not respond to serum, but we did not observe induction of capsule growth

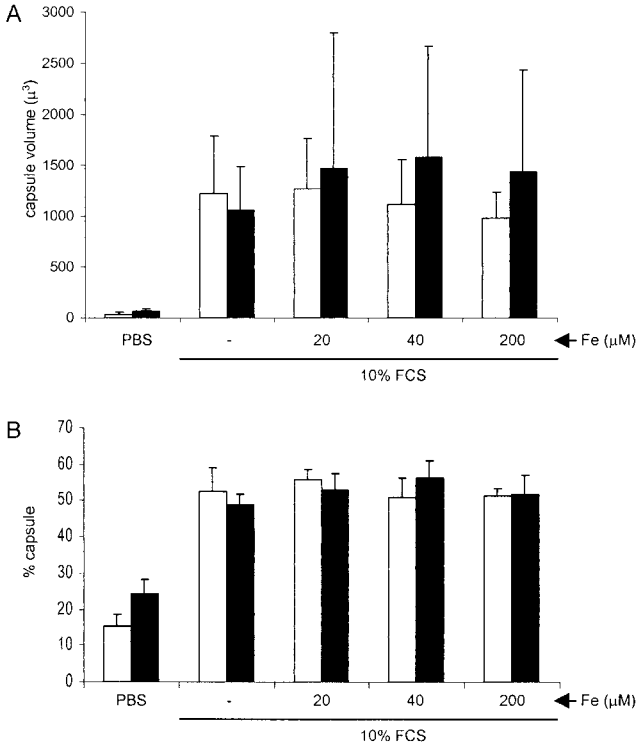

FIG. 5. Effect of iron on the induction of capsule by serum. Cells from strain H99 were placed on 10% heat-inactivated FCS containing different amounts of ferric-EDTA in the absence or presence of 10% CO_2. Controls without iron and without serum were also studied. The average and standard deviation of capsule volume (A) and relative size of the capsule (B) under various conditions where FCS was supplemented with Fe are represented. Open bars denote the data of cells incubated in the absence of CO_2, and closed bars denote that in cells incubated in 10% CO_2.

(results not shown). This result suggested that the inability of some strains to respond to serum was due to the genetic background of the strains and not to the experimental conditions used.

Serum-induced capsule growth is not due to iron limitation. Iron limitation can stimulate capsule growth (46). Since serum contains iron-binding proteins that sequester iron, we explored whether the phenomenon of serum-induced capsule growth was due to iron limitation. Supplementation of serum-containing medium with different concentrations of iron did not inhibit the induction of capsule growth regardless of the presence or absence of CO_2 (Fig. 5).

The cAMP pathway and *RAS1* are involved differently in the serum- and CO_2-induced capsule growth. cAMP is necessary to increase capsule size under conditions of iron limitation (3, 16). Hence, we investigated whether this pathway was involved in induction of capsule growth by studying the responsiveness of mutant strains lacking the adenylate cyclase (*CAC1*) and the cAMP-dependent protein kinase (*PKA1*) to serum and CO_2. When cells from these mutant strains were incubated in 10% FCS, no capsule growth was observed (Fig. 6). Since induction of capsule growth by serum was not observed in these mutant strains, we investigated whether capsular polysaccharide was present by indirect immunofluorescence using a monoclonal antibody to GXM. All the mutants were positive for the staining, indicating the presence of a capsule. Hence, absence of capsule induction was not a consequence of a lack of an encapsulated phenotype. Measurement of capsule volume and relative size of mutant strains under conditions of capsule growth induction revealed no significant differences relative to cells in the control media (Fig. 7A and B). In contrast, when CO_2 was used as a stimulus for capsule growth, both *cac1* and *pka1* cAMP mutants manifested capsule growth compared to control condition, although the induction was significantly lower than that observed in the reconstituted or wild-type strains (Fig. 6 and 7C and D).

We also studied the role of Ras1 in the serum- and CO_2-induced capsule growth. *RAS1* encodes a G-protein which has been mainly involved in *C. neoformans* in the control of the mitogen-activated protein kinase pathway (49). In the presence of serum, *ras1* mutant cells failed to induce capsule growth (Fig. 6 and 7A and B). These experiments were performed at 37°C, a temperature where *ras1* mutants show impaired growth (1). So, we repeated this experiment at room temperature and 30°C, but *ras1* mutant cells did not manifest capsule growth at the lower temperatures. When CO_2 was used as inducing factor, *ras1* mutant cells induced significant capsule growth (Fig. 6), although the size was smaller than the size of the complemented strain (Fig. 7C and D).

We considered that the lack of serum-induced capsule growth for the *ras1* mutants could reflect a defective cAMP pathway, which would involve an activation of the adenylate cyclase by Ras1. Hence, we studied the capsule induction by serum of *ras1* mutant cells in the presence of exogenous cAMP. Addition of cAMP increased capsule size in the wild-type strain compared to the control without cAMP, but it did not have any effect on the behavior of the *ras1* mutant (results not shown), suggesting that a defective cAMP pathway was not the cause of the lack of capsule induction by serum in the *ras1* mutant.

DISCUSSION

Capsule growth is a morphological response of *C. neoformans* to a variety of stimuli, including infection of mammalian hosts. Here we report that incubation of *C. neoformans* in serum induces an increase in the capsule volume. Furthermore, we have studied the relationship between this phenomenon and other stimuli that are known to induce capsule growth, such as iron and CO_2 levels. To our knowledge, the serum induction phenomenon was unknown in the cryptococcal field. Previous studies have used a serum-containing medium to induce the capsule size (6, 7), but neither demonstrated that the effect was due to serum. In fact, both studies employed conditions that included other factors that can increase capsule size, such as a 5% CO_2 atmosphere. Other studies (4, 32) reported no induction of capsule growth when using human pooled serum, although one of these reports (4) described capsule induction by lyophilized rabbit coagulase plasma.

One striking finding of the serum inducing effect was its dependence on the composition of the medium. When cells were incubated in PBS with serum, capsule growth was induced, whereas in Sabouraud medium no increase in capsule

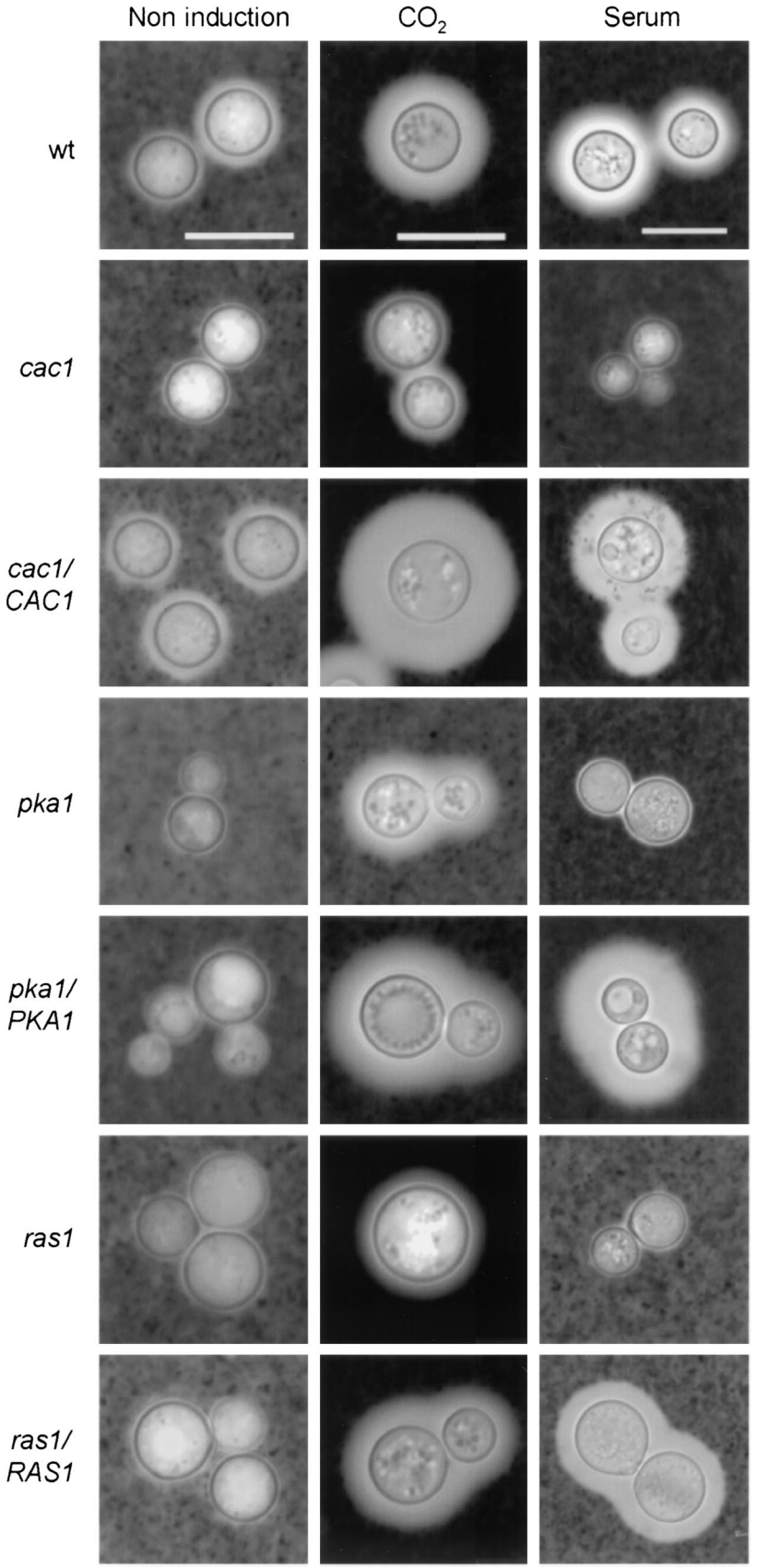

FIG. 6. Role of the cAMP pathway and Ras1 on serum- and CO_2-induced capsule growth. (A) The strains H99, RPC3 (*cac1*), RPC7 (*cac1 CAC1*), CDC1 (*pka1*), CDC16 (*pka1 PKA1*), LCC1 (*ras1*), and LCC2 (*ras1 RAS1*) were incubated in the presence of 10% CO_2 in DME medium or in PBS containing 10% heat-inactivated FCS in the absence of CO_2. The pictures show cells in a suspension of India ink after incubation for 24 h, and the scale bar in the first picture (10 μm) applies for the rest of the pictures in the rest of the column.

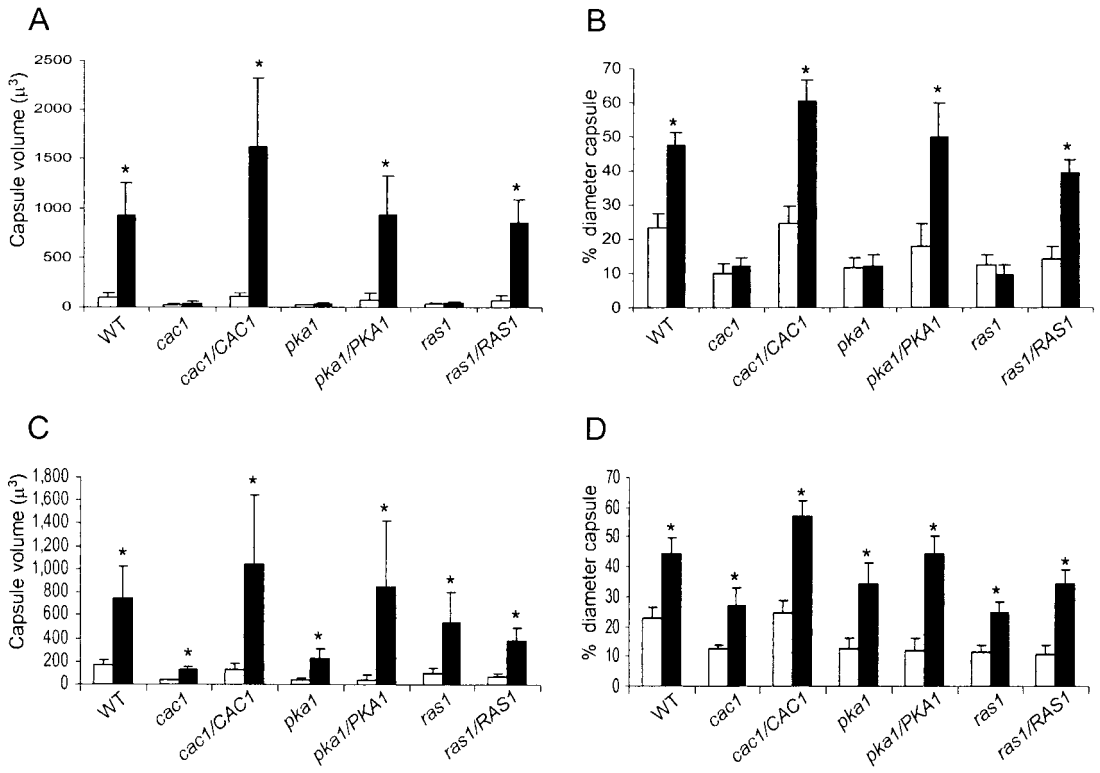

FIG. 7. Capsule size after serum- and CO_2-induced capsule growth in mutant strains of the cAMP and Ras1 pathways. Histograms in panels show capsule sizes of the mutant cells described in the legend for Fig. 6. The bars denote the mean and standard deviation of at least 20 cells after growth in Sabouraud (open bars), after 24 h of incubation (closed bars) in PBS plus 10% heat-inactivated FCS (A and B), or in DME and 10% CO_2 (C and D). Determined as described in Materials and Methods, results in panels A and C represent the absolute capsule volume and results in panels B and D represent the relative size of the capsule. Asterisks denote statistical differences ($P < 0.05$) in capsule size between time zero and 24 h of induction.

size was observed. Dykstra et al. (17) reported that a high concentration of glucose (16%) repressed capsule induction, and they correlated this phenomenon to changes in osmolarity. However, Littman observed that the capsule induction by thiamine was not prevented by the addition of 10% glucose (32). We do not think that this explanation is relevant to our observations, since the glucose concentration under our conditions was lower (2%). With regards to induction by CO_2, Granger et al. (23) demonstrated that capsule induction by CO_2 only occurred in DME with 22 mM $NaHCO_3$. Although we did not find that the addition of $NaHCO_3$ to the DME was required for capsule growth, this discrepancy could be due to the different CO_2 concentrations used in each study. Furthermore, it is conceivable that the requirement for $NaHCO_3$ is not applicable to all *C. neoformans* strains. The dependence of the phenomenon on the composition of inducing medium may explain why the serum-induced capsule growth has not been reported before, despite the fact that *C. neoformans* is commonly incubated in solutions containing serum during immunological studies.

Another factor that influences capsule growth is the pH of the medium. Solutions with a pH lower than 7 inhibited the induction of capsule growth. This is consistent with the fact that all the media reportedly used to induce capsule growth (such as low-iron medium) have a pH around 7.3. The importance of the pH has been previously noted (18, 23, 44), and it is known that a basic pH can enhance capsule growth (17) and affect the morphology of the colonies on plates. Although a pH higher than 7 is required to induce capsule growth by serum, it is not a sufficient condition, since increasing the pH in media that do not allow capsule growth in the presence of serum, such as Sabouraud, did not result in larger capsules. The mechanism by which pH regulates capsule growth is not known, but in other organisms, such as *C. albicans*, morphological transitions are pH dependent (10). Sera from each of the four different mammalian species tested induced capsule growth. This result suggests the existence of a common inducing factor in mammalian sera. We noted some differences in the efficiency of the capsule growth induction, which could be related to differences in the concentration of the inducing compound. Littman studied the assimilation of most of the cerebrospinal fluid components by *C. neoformans* and their effect on capsule size (32). He reported that the most common lipids found in nervous tissue did not affect capsule size in vitro, whereas glutamic acid did induce capsule size. One of the most abundant proteins in serum is albumin, and we studied its role on capsule growth but did not find any effect of this protein (results not shown). Iron deprivation is one of the main factors that induces capsule growth (46). In our study, iron deprivation did not explain the capsule induction, since addition of saturating concentrations of iron did not prevent the induction. The identification of the

inducing compound present in the serum is an important future goal that is outside the scope of this study.

While evaluating the role of temperature in the capsule growth process, we noticed a strong correlation between capsule size and cell size. Although at 37°C there was a larger capsule volume, the percentage of volume corresponding to the capsule was the same in all the cases. This result is in agreement with previous work that indicated that a shift from 24 to 37°C did not affect the proportion of capsule present in the cells (25). This suggests that in some strains the cell regulates the size of the capsule after induction. This is potentially a very interesting finding, because it implies the existence of mechanisms to control capsule size and thus avoid unlimited growth of the capsule. With regards to cell size, a potential control mechanism is cell cycle. In other yeast (50), the size of the cell determines the moment of cell division, and it is conceivable that in *C. neoformans* the process of capsule growth induction by serum is regulated not only by the cell size but also by the capsule size.

We observed considerable interstrain variability with regards to the response to serum. In general, serotype A and B strains manifested fewer interstrain differences. However, serotype D strains were highly variable in their response to serum. For some serotype D strains, such as 24067, we observed the simultaneous presence of macro- and microforms in the inducing medium. Similar microforms and heterogeneity have been described in vivo (19). Under our conditions, this heterogeneity was probably caused by the absence of capsule induction by the daughter cells arising during the incubation in serum. We do not have an explanation for this phenomenon, but it could represent phenotypic switching or changes in the medium during the incubation of the yeast, which would make this medium no longer efficient in inducing capsule size for the new cells produced. For other strains, such as H99, the presence of serum induced a homogenous response, although it has been reported that H99 can undergo a great variability after long incubations under conditions of capsule growth (23). The interstrain differences indicate the importance of the genotype for the capsule growth response. Our results establish great interstrain variation in capsule growth in response to serum, CO_2, and the inducing medium components. Consequently, the optimal conditions for each strain must be determined empirically.

cAMP is involved in *C. neoformans* capsule growth under low-iron conditions (2, 3, 16). Hence, we studied whether this pathway was required for induction of capsule growth by serum or CO_2. The cAMP pathway and Ras1 were each essential for induction of capsule growth by serum. Since Ras1 seems to act mainly through a cAMP-independent pathway (mitogen-activated protein kinase pathway [1]), we interpret this result as implying that serum induction is required for the interplay of several independent pathways. However, although reduced, some degree of capsule growth was found in the presence of CO_2 in the cAMP and *ras1* mutants. Hence, *RAS1* seemed to play a role under these conditions, even though *ras1* mutants have impaired growth at 37°C (1). It is possible that the putative role of Ras1 is performed under these conditions by the homolog Ras2 (48). On the basis of the observations with the various mutants, we conclude that CO_2-induced capsule growth can occur through different pathways. At this point, we cannot distinguish whether both cAMP and Ras1 pathways cooperate as different but overlapping pathways or if there is another different pathway involved in the induction. Interestingly, induction of capsule growth by a low iron concentration seems to involve only the activation of the cAMP pathway, without any involvement of Ras1 (3, 16). This indicates that the growth of the capsule responds to different stimuli that are integrated by different pathways of the cell which, according to the conditions, will act in an overlapping manner or, in some other way, cooperate to increase capsule size efficiently.

Our results establish that capsule growth is induced in *C. neoformans* strains by mammalian sera. This effect may contribute to virulence by promoting the induction of large capsule variants after animal infection. Depending on the *C. neoformans* strain, this effect is independent of, or can be enhanced by, CO_2. Serum-mediated growth of the *C. neoformans* capsule provides a potential explanation for the observation that cells in tissue often manifest large capsules that are not evident when grown in fungal media in vitro.

ACKNOWLEDGMENTS

We thank J. Heitman (Duke University, Durham, N.C.) for the gift of strains and A. Telzak for careful reading of the manuscript.

This work was supported by the following National Institutes of Health grants: HL59842, AI33142, and AI33774.

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Editor: T. R. Kozel