RESEARCH ARTICLE

Quorum Sensing-Mediated, Cell Density-Dependent Regulation of Growth and Virulence in *Cryptococcus neoformans*

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ABSTRACT Quorum sensing (QS) is a cell density-dependent mechanism of communication between microorganisms, characterized by the release of signaling molecules that affect microbial metabolism and gene expression in a synchronized way. In this study, we investigated cell density-dependent behaviors mediated by conditioned medium (CM) in the pathogenic encapsulated fungus *Cryptococcus neoformans*. CM produced dose-dependent increases in the growth of planktonic and biofilm cells, glucuronoxylomannan release, and melanin synthesis, important virulence attributes of this organism. Mass spectrometry revealed the presence of pantothenic acid (PA) in our samples, and commercial PA was able to increase growth and melanization, although not to the same extent as CM. Additionally, we found four mutants that were either unable to produce active CM or failed to respond with increased growth in the presence of wild-type CM, providing genetic evidence for the existence of intercellular communication in *C. neoformans*. *C. neoformans* CM also increased the growth of *Cryptococcus albidus*, *Candida albicans*, and *Saccharomyces cerevisiae*. Conversely, CM from *Cryptococcus albidus*, *C. albicans*, *S.* cerevisiae, and *Sporothrix schenckii* increased *C. neoformans* growth. In summary, we report the existence of a new QS system regulating the growth and virulence factor expression of *C. neoformans in vitro* and, possibly, also able to regulate growth in other fungi.

IMPORTANCE Quorum sensing is a strategy of communication used by pathogenic microorganisms to coordinate the expression of attributes necessary to cause disease. In this work, we describe a quorum sensing system in *Cryptococcus neoformans*, a yeast that can cause severe central nervous system infections. Adding conditioned medium—culture medium in which *C. neoformans* has previously grown—to fresh cultures resulted in faster growth of *C. neoformans* both as isolated cells and in microbial communities called biofilms. The addition of conditioned medium also increased the secretion of capsule carbohydrates and the formation of melanin pigment, two tools used by this microorganism to thrive in the host. This remarkable example of microbial communication shows that *C. neoformans* cells can act in unison when expressing attributes necessary to survive in the host, a finding that could point the way to improvements in the treatment of cryptococcosis.

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Quorum sensing (QS) is a communication system used by microbes to coordinate the expression of selected genes in response to population density and/or the presence of other species of microbes. QS is mediated by exogenous signaling molecules, called autoinducers or quorum sensing molecules (QSMs). These small, autostimulatory molecules accumulate during cell growth and, after reaching threshold concentrations, induce changes in microbial gene expression that trigger population cooperation (1, 2). QS has been shown to regulate the expression of bacterial virulence factors, among several other important processes (2).

Eukaryotic regulation by QS was unknown until the reports that farnesol and tyrosol functioned as QSMs for *Candida albicans* (3, 4). These molecules regulate cell morphology, growth, biofilm formation, resistance to oxidative stress, and other processes in the life of *C. albicans* (3–6). So far, only a few other eukaryotic QSMs have been described, even though phenomena consistent with QS are known in several other fungi (7–10).

Cryptococcus neoformans is the etiologic agent of cryptococcosis, a life-threatening systemic mycosis that predominantly affects immunocompromised people (11). A QS-like effect was observed after the deletion of the general regulator *TUP1* in *C. neoformans* serotype D (12). The mutant showed a cell density dependency for growth mediated by an oligopeptide; however, this phenotype was found to be very specific, since it was not observed in wild-type serotype D cells or even in *tup1* mutants from *C. neoformans* serotype A. We also investigated the presence of cell density regula-

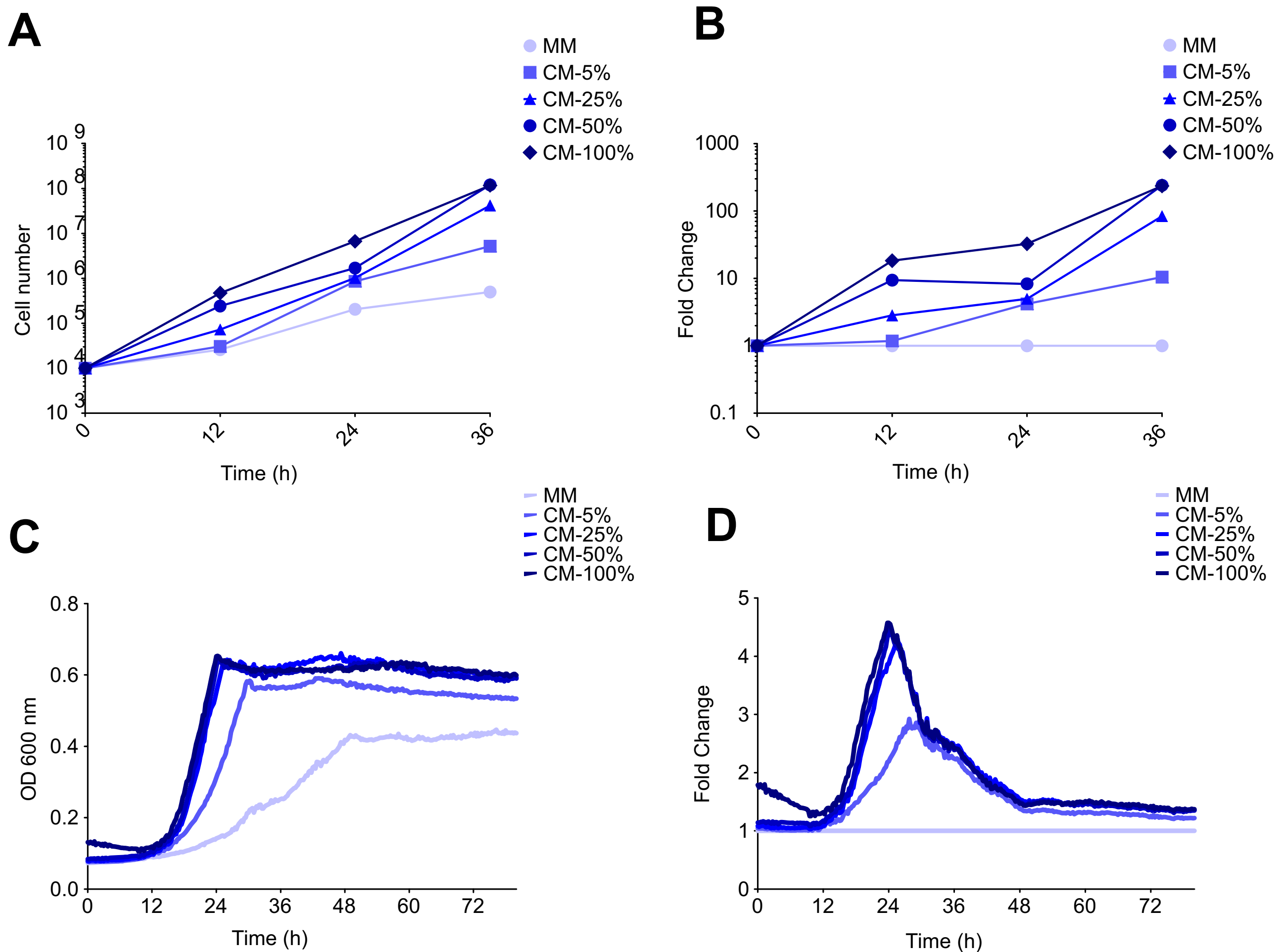

FIG 1 CM accelerates the growth of *C. neoformans* strain 24067 and decreases the time for growth resumption after culture dilution. *C. neoformans* 24067 cells were grown overnight and diluted to new cultures at 10^4 cells/ml. The cells were grown in MM or MM complemented with increasing concentrations of CM (5 to 100%) at 30°C. The results shown are the averages of three measurements. (A) To determine cell number, the cultures were followed for 36 h and aliquots collected every 12 h for cell counting in a hemocytometer. (B) To determine fold change in growth, the number of cells in each system containing CM was normalized by the number of cells in the control system (MM not supplemented with CM). (C) To evaluate cell density, the cultures were incubated in an automated microbiology growth curve analysis system, and their absorbance was read every 30 min for 84 h. OD, optical density. (D) To determine fold change in growth, the absorbance of each system containing CM was normalized to the absorbance of the cells in the control system (MM not supplemented with CM).

tion in this fungus, and in this study, we report the existence of quorum sensing in *C. neoformans*, regulating the cell growth of planktonic and biofilm cells, glucuronoxylomannan (GXM) release, and melanin synthesis, important virulence attributes of this organism.

(Data in this study were submitted by P. Albuquerque in partial fulfillment of the requirements for the degree of doctor of philosophy in the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY, 2011.)

RESULTS

CM accelerates *C. neoformans* growth. To evaluate the presence of QS regulation in *C. neoformans*, we prepared conditioned medium (CM) from cell-free supernatants of stationary-phase cultures of this fungus grown in minimal medium (MM). CM was then added to low-density fresh cultures of *C. neoformans* to evaluate possible effects on its growth or expression of virulence factors. The addition of CM produced a significant and dose-dependent increase in growth compared to the growth of cultures in MM without added CM (Fig. 1; see also Fig. S1 in the supplemental material). At 12 h after the addition of the CM, the numbers of cells in CM-supplemented medium were 2- to 18-fold greater than in the control medium without CM. The increase in growth was easily visualized at 24 h, when the cultures in CM-supplemented medium were turbid while the ones in MM alone remained clear. At this time, the fold increase in growth varied from 4 to 32 times the growth of the control, and the growth reached its maximum at 36 to 48 h, with fold increases up to 200 times relative to the growth observed in control cultures without CM supplementation. After 36 to 48 h, the cultures in CM-supplemented medium slowed their growth rates, while the cultures in control medium without CM reached exponential growth, resulting in a gradual diminishing of the fold change differences between them. Similar growth changes were observed when the cultures were monitored by spectrophotometry, al-

TABLE 1 Cross-reactivity of CM for growth among the four serotypes of *Cryptococcus neoformans* and *C. gattii*

Serotype of cells	Growth in CM from cryptococci of serotype[a]:			
	A	B	C	D
A	+++	+++	+++	+++
B	−	+	+	+
C	−	−	−	−
D	+++	+++	+++	+++

[a] +++, strong activity; ++, intermediate activity; +, little activity; −, no activity.

TABLE 2 Effects of CM produced by other fungal strains on *C. neoformans* growth

Source of CM	Activity[a]
Cryptococcus neoformans	+++
Cryptococcus albidus	++
Saccharomyces cerevisiae	+
Candida albicans	+
Candida glabrata	+/−
Blastomyces dermatitidis	+
Sporothrix schenckii	++
Histoplasma capsulatum	+/−

[a] +++, strong activity; ++, intermediate activity; +, little activity; +/−, inhibitory and/or stimulatory effects depending on CM concentration; −, no activity.

though the fold increases were not as large as the ones obtained by cell counting (Fig. 1B and D; Fig. S1 and S2). When comparing the growth curves from strain 24067 and H99 cells in the presence or absence of CM using an automated cell counter, it was apparent that cultures grown in the presence of CM had decreased lag phase and higher growth rates than the ones grown in MM alone (Fig. 1B; Fig. S1).

We tested the growth of *C. neoformans* in MM supplemented with up to 10 times the normal concentration of glucose or glycine but found no significant increase in the growth rate; this implies that the observed growth rate acceleration was not solely due to the increased presence of these nutrients. We also prepared CM from different days of culture growth, starting at low cell density, and we were able to observe a gradual increase in the growth-inducing activity. As the culture from which the CM was obtained aged, a plateau was reached in the early stationary phase (see Fig. S3 in the supplemental material).

We also tested the influence of the initial number of cells on growth. Lower initial cell densities resulted in greater induction of growth in cultures in the presence of CM than was measured in cultures in MM without CM supplementation. Cultures started with 10^6 cells/ml or a higher inoculum had lower fold growth increases, and the effect was virtually absent when cultures were begun at an initial cell density of 10^7 cells/ml (data not shown).

We did not observe any significant difference in the growth-inducing activities of CM derived from cultures grown at 30°C or 37°C. Similarly, faster growth was induced equally in CM-supplemented cultures grown at both temperatures (data not shown).

***C. neoformans* CM modulates the growth of other cryptococcal strains.** To evaluate possible differences in the CM produced by different *Cryptococcus* strains, we produced CM from several strains of the four cryptococcal serotypes and tested for their stimulatory effects in growth assays (Table 1; see also Fig. S4 in the supplemental material). The growth of serotype A or D *C. neoformans* cells was induced by the addition of CM from any of the other 4 serotypes. Cells from serotype D strain 24067 were tested with CM from 10 other strains, and their growth was affected by all of them. In contrast, cells from serotypes B or C, corresponding to *Cryptococcus gattii*, showed little or no response to the addition of their own CM or CM from all other strains tested. Additionally, we produced CM from the serotype D congenic strains JEC21 (MATα) and JEC20 (MATa), and we did not observe any significant difference due to their different mating types in the production of active CM or in response to CM produced by another strain. The acapsular mutant Cap67, derived from the B3501 strain, only responded to CM from its parental strain.

CM cross-stimulatory activity among different fungal species. We produced CM from different fungal species from cultures grown in MM or synthetic defined (SD) medium and tested their effects on *C. neoformans* growth. We observed that CM from *Cryptococcus albidus*, *C. albicans*, *Saccharomyces cerevisiae*, *Sporothrix schenckii*, and *Blastomyces dermatitidis* were able to elicit higher growth rates of *C. neoformans* cells, although not to the same extent as the induction with *C. neoformans* endogenous CM (Table 2). In contrast, CM from *Candida glabrata* and *Histoplasma capsulatum* manifested no increase or even slightly inhibitory effects on *C. neoformans* growth. Conversely, we tested the effects of CM from *C. neoformans* cultures on the growth of these other fungi. *C. neoformans* CM produced similar dose-dependent effects on the growth of *Cryptococcus albidus*. Moreover, supplementation with *C. neoformans* CM enabled the growth of *C. albicans* and *S. cerevisiae* cultures in MM, starting at low cell densities (10^4 cells/ml), whereas cultures of those fungi in MM alone grew very slowly or did not grow at all even after a week postinoculation.

Effects of CM on GXM release, capsule size, and melanization. Given the importance of the capsule, GXM release, and melanin synthesis for cryptococcal pathogenesis, we measured the effects of CM on each of these virulence attributes. An ultrafiltration step after CM production was used to remove all the secreted GXM in the CM, since the molecular weight of GXM is higher than 1 million (13). Using the same time intervals as previously established and that showed major increases in cell density in the growth assay, we collected the supernatant of *C. neoformans* cultures grown in MM with or without CM supplementation and measured their GXM concentration by capture enzyme-linked immunosorbent assay (ELISA). The addition of CM resulted in a dose-dependent increase in extracellular GXM for all cultures containing CM at the three time intervals analyzed (Fig. 2). Since cultures grown in the presence of CM had a higher cell density, we calculated under all conditions the rate of GXM released per cell by dividing the measured concentration of GXM by the number of cells. The rate of GXM secretion per cell was previously shown not to be linear during growth, but instead, it increases drastically several days after the onset of stationary phase (14). As shown by the results in Fig. 2, this dramatic surge in the GXM release rate occurred in the first 24 h of growth in cultures containing CM. Thus, the effect of CM on GXM release was not simply to promote an increase in the total amount of exopolysaccharide but, rather, a decrease in a dose-dependent manner during the time it took for the surge in GXM release rate to occur. We did not observe any significant differences in capsule diameter during this experiment (data not shown), implying that the effect on the release rate of

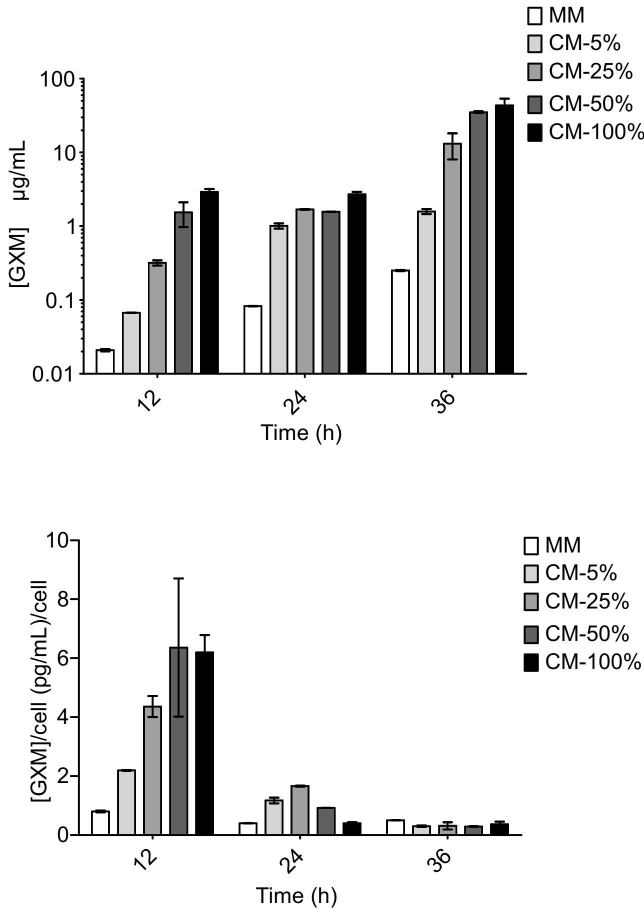

FIG 2 CM affects GXM release in culture. *C. neoformans* strain 24067 cells were grown overnight and diluted to new cultures at 10^4 cells/ml in minimal medium alone or in the presence of increasing concentrations of CM (5 to 100%). (A) The supernatants of the cultures were collected at three different time points and used in a capture ELISA assay to measure GXM released to the medium during cell growth. (B) The concentration of GXM (μg/ml) at each time point was divided by the cell number of each culture at each time interval. Error bars show standard deviations.

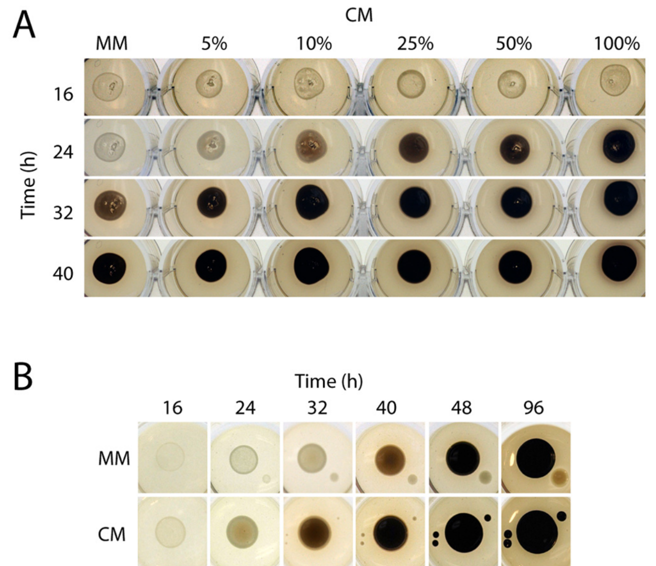

FIG 3 CM effects on *C. neoformans* melanization. (A) *C. neoformans* strain H99 cells were grown overnight and washed 3 times, and 10^5 cells were spotted on solid minimal medium supplemented with L-DOPA and with increasing concentrations of CM. The colonies were followed visually for melanin production for 40 h. We can observe that cultures in the presence of CM already showed melanization on a dose-response basis at 24 h, while the control in MM started to show the pigment only at 32 h. (B) We followed colonies growing in MM or in the presence of 10% CM and observed that small satellite colonies in the wells containing CM melanized much earlier than the satellite colonies in the wells containing MM alone, even though the main colony was already melanized 48 h earlier. Similar results were obtained with *C. neoformans* strain 24067 cells. This experiment is representative of several replicates done over 5 years.

soluble GXM was probably a result of *de novo* synthesis of exopolysaccharide.

To analyze the effects of CM on melanin synthesis, we spotted 10^5 *C. neoformans* cells in 1 ml of agar MM supplemented with 3,4-dihydroxy-L-phenylalanine (L-DOPA) and six different concentrations of CM. We observed that at 24 h, the colonies in wells containing CM melanized faster than those grown in MM alone (Fig. 3). Importantly, the effect of CM on the cells was dose dependent, just as we observed before in respect to cell growth. Similar results were observed regardless of whether the CM was obtained from serotype A or D cultures and whether the cells plated were serotype A or D (data not shown).

Since glucose suppresses melanin synthesis in *C. neoformans* (15), it is important to take into account the additional glucose introduced with CM. Reducing sugars corresponded to 6% of the CM dry weight (phenol sulfuric acid quantification), and the presence of glucose in CM was confirmed by proton and carbon nuclear magnetic resonance (NMR). However, the conditions under which glucose was increased upon the addition of CM were found only when melanization occurred faster. Therefore, the results we observed were not due to an increased concentration of glucose. An alternative explanation for the results observed is that the CM-induced growth leads to faster depletion of glucose in the medium, leading to faster melanization simply by removing a suppressor instead of acting directly on melanization. In addition, to control for the effects of glucose regulation, we tested the CM effects using minimal medium in which glucose and glycine were replaced with acetate and asparagine as the sole carbon and nitrogen sources (see Fig. S5 in the supplemental material). This medium has previously been shown not to repress melanization (16). Even in this medium, the addition of CM resulted in the same dose-dependent increase in melanization as observed in regular minimal medium containing glucose and glycine. A second alternative explanation is that the faster melanization observed in response to CM was due solely to a faster replication rate. The first indication that this was not the case was serendipitously observed during the experiments for which results are shown in Fig. 3B. In this experiment, we observed that smaller satellite *C. neoformans* colonies, present in the wells containing CM at 48 h, were more melanized than the larger satellite colony present in the well containing MM at 96 h. In summary, the observation that smaller colonies in CM melanize much faster than larger colonies in MM suggests that QS regulates melanization directly. In addition to this evidence, we repeated the melanization tests in medium containing glucose and creatinine as the sole carbon and nitrogen sources (Fig. S5), which is known to allow melanization in a setting of poor growth (16). Even with the slower growth of *C. neoformans* in this medium, we

still observed the same dose-dependent effect of CM. Thus, CM directly induced melanization of *C. neoformans* cells.

Effects of CM on growth of *C. neoformans* biofilms. Having observed that CM stimulated the growth of planktonic cultures, we evaluated the effects of CM on the growth of *C. neoformans* biofilm cells. To test this, suspensions of *C. neoformans* at different initial cell densities were added to polystyrene plates and coincubated with CM added at different time intervals (0, 4, and 24 h), allowing us to follow its effects at different phases of biofilm development. We found that CM induced a dose-dependent increase in the growth of *C. neoformans* biofilm cells when added at any of the chosen time points and in biofilms that were started at low cell densities (Fig. 4A). We observed an inverse correlation between the CM effect on biofilm growth and the initial fungal cell density. In biofilms started with 10^7 cells/ml, we only observed changes in biofilm growth when we added CM to mature biofilms.

These experiments were performed using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay to evaluate biofilm growth. To rule out the possibility that the results observed were due to CM-induced metabolic changes and not biofilm growth, we repeated the experiments by growing biofilms in glass coverslips followed by confocal microscopy using probes for *C. neoformans* capsule and cell wall. This experiment confirmed that the presence of CM increases both the number of individual colonies and the number of *C. neoformans* cells in each colony (Fig. 4B).

Stability of CM activity We evaluated the stability of the CM activity under a variety of conditions. With regard to thermal stability, CM was kept at 4°C, −20°C, and room temperature for at least a year and submitted to multiple freeze-thaw cycles without a significant decline in activity. Also, the activity was not affected by treatment with low or high temperatures or even by two sequential rounds of exposure to high temperature (121°C) and pressure (104 kPa) in an autoclave (data not shown). To evaluate the stability of the activity when exposed to enzymatic degradation, we treated *C. neoformans* CM with proteinase K, trypsin, pronase, DNase I, RNase T1, α-glucosidase, and β-glucosidase. None of these enzyme treatments significantly altered the CM activity. Also, no significant decline in CM activity was observed after acid or alkali treatment: CM was tested for activity after treatment with 1 M HCl or 1 M NaOH for 1 h and neutralization of the acid or base.

Farnesol, tyrosol, and QSP1 do not reproduce CM activity. We tested the two previously described *Candida albicans* QSMs, farnesol and tyrosol, for their ability to function as QSMs for *C. neoformans*. Farnesol and tyrosol were tested in concentrations up to 250 μM and 200 μM, respectively, but neither reproduced any of the effects of the *C. neoformans* CM (data not shown). We also tested the QSP1 peptide (quorum sensing-like peptide 1) described by Lee et al. in a *C. neoformans* TUP1 mutant (12), but this peptide also did not replicate the activity that we observed with *C. neoformans* CM (data not shown).

CM solubility. One of our first approaches to determine the chemical characteristics of the QSM activity consisted of a series of liquid-liquid or liquid-solid extractions of the CM using organic solvents. The activity was not present in the lipid extract obtained after CM fractioning by the Bligh and Dyer method, suggesting that the QSM is not derived from a lipid molecule. We also observed no extraction of the activity by nonpolar solvents, such as hexane, chloroform, and ethyl acetate, whereas extracts with a series of alcohols of increasing polarity were also increasingly active (butanol < propanol < ethanol < methanol), indicating that the QS molecule is hydrophilic. However, even the most polar alcohol, methanol, was not able to extract the activity completely or as well as water.

Purification of the QSM. To purify the active molecule(s) in CM, we tested several chromatographic methods of separation. Due to its hydrophilic nature and poor solubility in organic solvents, the activity showed poor binding and/or separation in numerous combinations of reverse-phase, normal-phase, and ion exchange matrices and using different solvents. Despite these difficulties, we were able to design a method for partial purification of the QSM. The sample obtained by this method was submitted to mass spectrometry (MS) and NMR analyses, suggesting several candidates for the active molecule. Among the candidates, MS analysis of an ion at m/z 238 produced several product ions that matched the product ions of commercial pantothenic acid (PA) (data not shown). When tested at high concentrations, commercially available pantothenic acid increased the growth of *C. neoformans* cells similarly to CM (Fig. 5A and B) and also reduced the time to melanization (data not shown). Despite reproducing the effect of the QS activity found in CM, the effect observed with pure pantothenic acid was quantitatively smaller than that observed with CM. Additionally, we used high-performance liquid chromatography (HPLC) to evaluate the presence of PA in CM derived from cultures of *C. neoformans*, *C. gattii*, and other fungal species. In these samples, we observed a peak with a retention time similar (but not identical) to that of commercial pantothenic acid (Fig. 5C; also see Fig. S6A and B in the supplemental material). This analysis showed that the pantothenic acid concentrations in CM were in the range of 4 to 6 μM (data not shown), while the range of pantothenic acid concentrations that produced increased *C. neoformans* growth was from 16 μM to 1 mM (Fig. 5).

Screening for mutants that do not generate the active QSM. To confirm the results obtained with CM in wild-type cells that are susceptible to genetic defects, we screened a library of *C. neoformans* mutants (17) to isolate mutants that were either unable to produce active CM or failed to increase their growth in response to wild-type CM. The first screening was done with wild-type cells grown in medium supplemented with CM derived from each mutant to search for those that did not produce active CM. The second screening was made with wild-type CM and cells from each mutant to detect those that did not respond to wild-type CM. After 3 rounds of screening, only one mutant had significantly lower activity in its CM. This mutant has a deletion in the *C. neoformans* open reading frame (ORF) CNI27702, which is annotated as a stomatin-like protein because its hypothetical sequence contains a prohibitin domain (18).

Supplementation of wild-type cultures with *CNI27702*Δ CM did not result in the expected increase in growth observed with the wild-type CM at 24 h, even though the mutant cells themselves responded to the wild-type CM (Fig. 6). However, at 48 h, as the fold change in the growth of cells grown in wild-type CM started to decrease, the cells grown in *CNI27702*Δ CM began to show a greater fold change in growth (Fig. 6). These results suggest that the CM produced by the mutant probably still has some QS activity but that either the kinetics of QSM secretion is slower, its degradation is faster, or a modification in the secreted molecule renders it less active. We also observed a peak of retention time

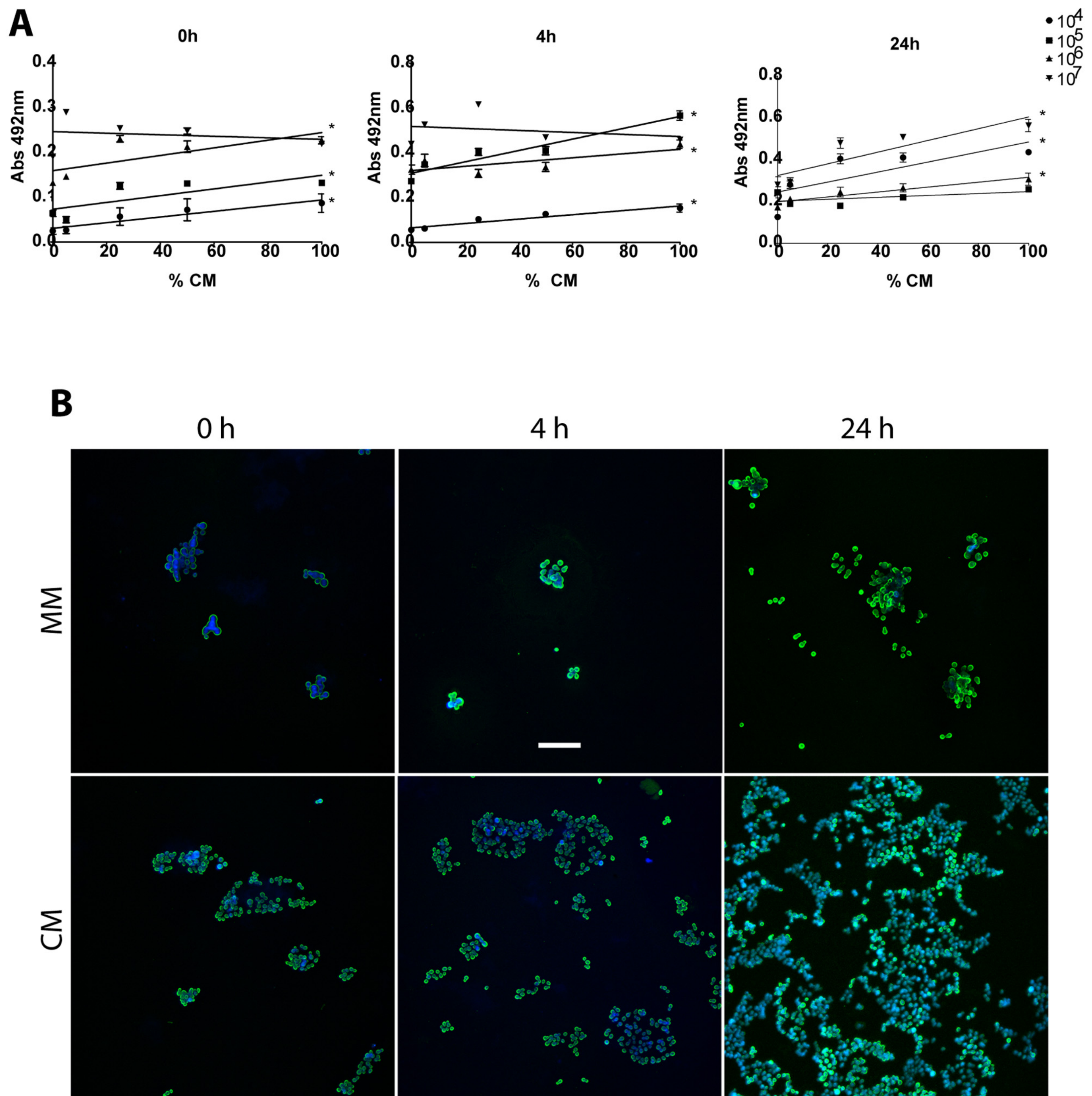

FIG 4 CM affects the growth of *C. neoformans* cells in biofilms. Cells of *C. neoformans* strain B3501 were grown overnight, washed 3 times with PBS, and inoculated at different initial cell densities (10^4, 10^5, 10^6, or 10^7 cells/ml) in 96-well polystyrene plates with increasing concentrations of CM that was added to the plates at different time points. (A) 0h, *C. neoformans* cells and CM were added at the same time; 4h, *C. neoformans* cells were added to the plate 4 h before the addition of CM; 24h, *C. neoformans* cells were added to the plate 24 h before the addition of CM. After CM addition, the plates were incubated for 24 h at 37°C and the biofilm formation was evaluated with the XTT reduction assay. Error bars represent standard deviations. The results were analyzed with linear regression to generate trend lines and evaluate whether the slopes of these lines were significantly different from zero (*, $P < 0.05$). (B) B3501 biofilms were prepared with initial cell density of 10^4 cells/ml in coverslips placed in 6-well culture plates. As described above, after 0, 4, and 24 h, CM (100% final concentration) or MM was added and the coverslips incubated for another 24 h. After vigorous washing, attached cells were stained with the cell wall stain Calcofluor white (blue) and the GXM antibody 18B7 (green) and imaged by confocal microscopy. The scale bar measures 50 μm.

similar to that of pantothenic acid in the *CNI27702*Δ CM (see Fig. S6C in the supplemental material), and its concentration was similar to the concentration of pantothenic acid in CM derived from wild-type cells (4.37 μM). The effect of the *CNI27702*Δ CM in the melanization of wild-type cells was even more pronounced, since as early as 24 h, there was already a noticeable dose-dependent increase in the melanization of cells incubated with wild-type CM, whereas at 32 and 40 h, the cells growing in the presence of the mutant CM still showed melanization levels similar to the melanization in control cells, and the melanization was

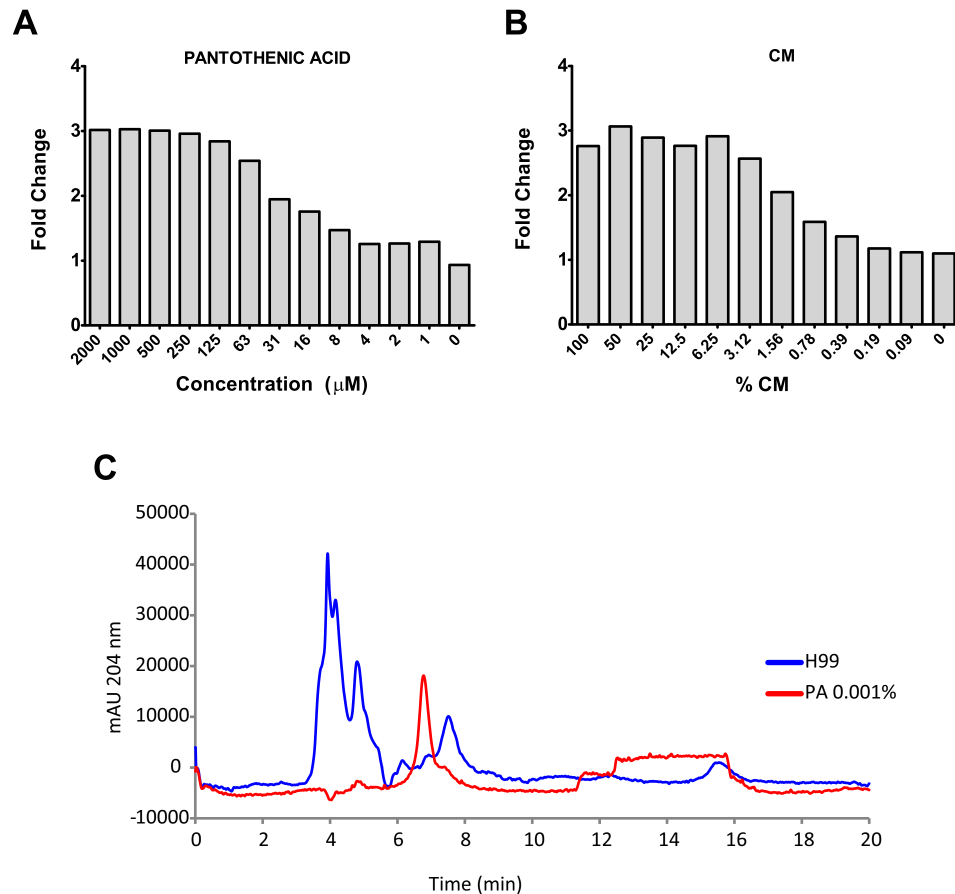

FIG 5 Comparison of the activities of pantothenic acid and CM on *C. neoformans* cells. Cells of the H99 strain of *C. neoformans* were grown overnight, washed three times, and inoculated at a density of 10^4 cells/ml in different concentrations of pantothenic acid (A) or CM (B). The cultures were incubated at 30°C for 24 h, and their growth was measured by their absorbance at 600 nm. The fold increase represents the ratio between the growth in the cultures containing PA or CM and the growth of the control culture in MM. (C) HPLC analysis to evaluate the presence of PA in H99 CM. CM from H99 cells was analyzed in a C_{18} column. Commercial PA was used as a standard, and its elution profile was compared to the elution profile of CM derived from H99 cells. mAU, milli-absorbance unit.

lower at higher concentrations of CM (Fig. 7), possibly due to a higher glucose concentration in those wells. This screening had the main objective of finding genes that are involved in the QSM biosynthetic pathway. As the CNI27702 ORF product has not been characterized before, we tried to obtain as much information as possible from its sequence. Searches in several databases did not identify any known catalytic domain, reducing the likelihood that this protein acts directly in the synthesis of the active molecule. On the other hand, the available evidence does not exclude the possibility of a regulatory role in the biosynthetic pathway. The sequence did not show possible mitochondrial target sequences, as many members of the prohibitin family do, nor did it show transmembrane domains or secretory sequences, meaning that the protein possibly acts in the cytoplasm or nucleus.

Screening for mutants that do not respond to the wild-type QS activity. To search for proteins that participate in the response to the wild-type QSM, we selected mutants whose growth rates in MM with or without wild-type CM supplementation were not significantly different and whose fold change in growth in the presence of CM was at least two standard deviations lower than the fold change presented by wild-type cells. After 3 rounds of screening, 3 mutants with a phenotype of low response to QS activity were recovered. Two of the mutants were deficient for methyltransferases (OPI3 and CHO2) involved in the synthesis of phosphatidylcholine from phosphatidylethanolamine, and the third had lost the gene that coded for the cyclic AMP (cAMP)-dependent protein kinase catalytic subunit (Pka1). Although it was very interesting to find two enzymes from the same pathway in the screening, we could not characterize the *cho2*Δ mutant cells further because of their very slow growth in MM.

Both the *opi3*Δ and the *pka1*Δ mutant exhibited a weaker response to wild-type CM, albeit in different ways. Cells of the *opi3*Δ mutant had a very low growth rate in MM, and their response to supplementation with wild-type CM was delayed and less intense in comparison to the response of wild-type cells (Fig. 8). The mutant cells also manifested a delayed response to wild-type CM in our melanization assay (Fig. 9). In MM, the mutant colonies took a much longer time to darken than wild-type cells. When comparing the mutant cells grown in MM or CM, the rates of melanization were not as different as those observed with wild-type cells. The phenotype presented by the *pka1*Δ cells was interesting since the fold change was reduced not because the mutant cells failed to grow faster in response to CM but because their growth rate in MM was much higher than that of wild-type cells (Fig. 8). This phenotype suggests that the mutant could have a constitutive activation of the signaling pathway that responds to the QSM and

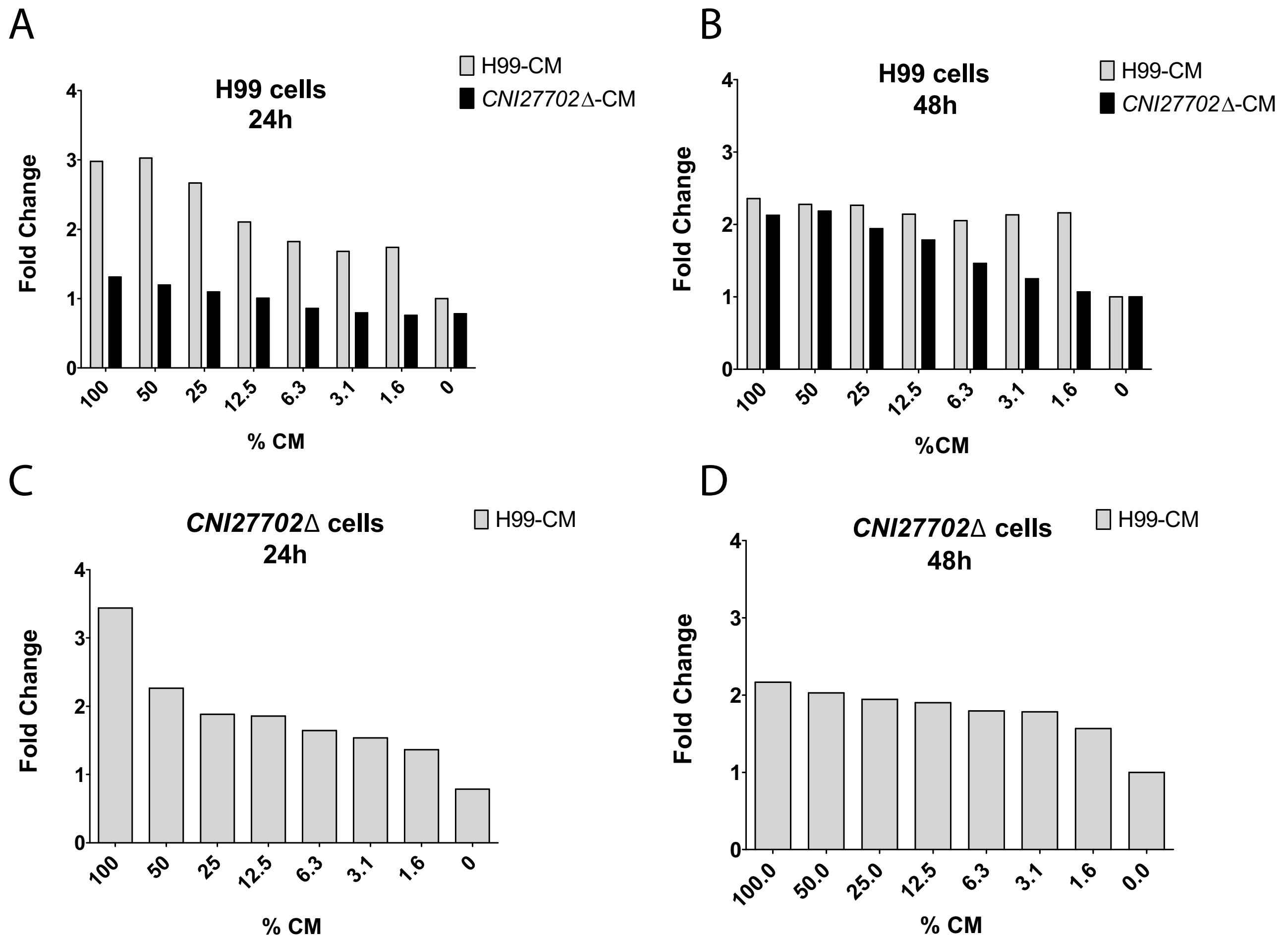

FIG 6 Effects of CNI27702 deletion on CM growth effects. *C. neoformans* H99 cells were grown for 24 h and diluted to begin new cultures at 10^4 cells/ml. The cells were grown in MM or MM complemented with increasing concentrations of H99 CM or *CNI27702*Δ CM at 30°C. (A and B) Fold change values (growth in CM/growth in MM) for H99 wild-type cells grown in the presence of their own CM (gray) or with *CNI27702*Δ CM (black) at 24 h or 48 h. (C and D) Fold change values of *CNI27702*Δ cells incubated with H99 CM after 24 h or 48 h of incubation. At 24 h, we observed a dose-dependent increase in growth of the H99 cells in the presence of H99 CM, while the cells growing in *CNI27702*Δ CM were not different from the MM control. At 48 h, the effects of the H99 CM began to decrease and approached the control levels, while the cells grown in the presence of *CNI27702*Δ CM finally began to show some increase in the fold change. The results shown in panels C and D demonstrate that *CNI27702*Δ cells respond similarly to H99 cells with faster growth when exposed to wild-type CM.

that the cAMP pathway would consequently be a repressor of QS activity. With regard to melanization, the *pka1*Δ cells did not melanize at all, either in the presence or absence of CM (Fig. 9), as described previously (19).

In contrast to the defects of the mutants in response to wild-type CM, the CM produced by the *opi3*Δ and *pka1*Δ cells was not significantly different from that produced by wild-type cells, as it was able to induce faster growth in wild-type cultures (data not shown).

***lhc1*Δ mutant.** In addition to the mutants discovered through library screening, we tested a lactonohydrolase mutant (*lhc1*Δ) that has recently been shown to have defects in capsule architecture (unpublished data). The rationale for testing this mutant was the possibility that lactone rings could be involved in the QS mechanism. The *lhc1*Δ cells produced CM that, when added to wild-type cells, was as active as the wild-type CM (data not shown). However, the *lhc1*Δ cells themselves had a delayed growth response to CM derived from wild-type cells (Fig. 10) or their own CM (data not shown). The mutant cells also had delayed melanization in response to their own CM or CM derived from wild-type cells (Fig. 11). Comparing the growth of the mutant with that of wild-type cells in the absence of CM suggested the possibilities that the mutant had a constitutively activated QS pathway that did not require the QS signaling for growth or that this enzyme could act in the inactivation of the QSM. However, the opposite was observed when we analyzed effects on melanin formation. One possible explanation is that since this mutant has alterations in the capsule, the altered capsule binds small molecules like the QSM or melanin polymers differently. However, further experiments will be required to answer this question.

DISCUSSION

QS is a well-known mechanism of regulation of virulence in bacteria but has only recently been discovered in eukaryotes (20). The knowledge that this phenomenon is more widespread than previously thought prompted us to look for QS behaviors in *C. neoformans*. A central feature of the pathogenesis of cryptococcal infections is the occurrence of large tissue burdens with the formation

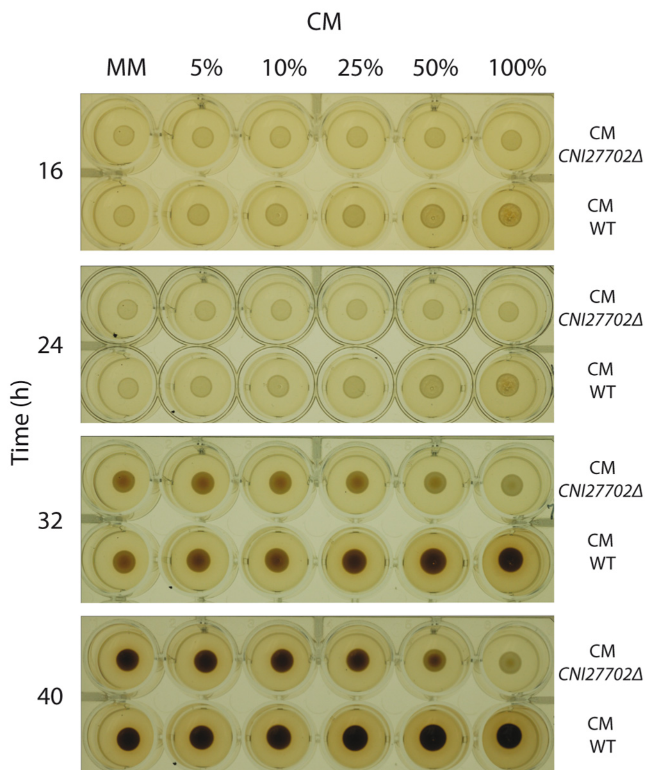

FIG 7 Comparison of the effects of *CNI27702*Δ CM and wild-type (WT) CM on melanization. *C. neoformans* H99 cells were grown for 24 h and washed 3 times, and 10^5 cells were spotted in solid MM supplemented with L-DOPA and with increasing concentrations of H99 CM or *CNI27702*Δ CM. The colonies were visually followed for melanin production for 40 h. At 24 and 32 h, it was possible to visualize the dose-dependent melanization of cells at higher concentrations of wild-type CM. Only at 32 h was the melanization of cells grown in *CNI27702*Δ CM noticeable; however, the melanization levels are similar to those of the control cells grown in MM alone.

of masses of organisms associated with large amounts of GXM (21). The large number of organisms in these fungal conglomerates suggests the possibility that some kind of cell-cell communication could exist in *C. neoformans* to coordinate features that improve their survival in the host. To search for the presence of microbial communication in *C. neoformans*, we studied the effects of CM. We found that the CM from early-stationary-phase *C. neoformans* cultures increased the growth of planktonic and biofilm cells and regulated multiple virulence factors of this fungus, such as GXM release and melanization. Moreover, we found four mutants that appeared to be defective in cellular communication, a result that if confirmed by future rigorous genetic studies and functional characterization of the encoded proteins should clarify QS mechanisms in *C. neoformans*.

The addition of CM to fungal medium reduced the time required to resume logarithmic growth by *C. neoformans* cultures in MM, decreased the doubling time during logarithmic growth, and increased the final density of the stationary culture. The inverse relationship between the initial cell density and the duration of the lag phase was shown previously in *Mycobacterium tuberculosis*, *C. albicans*, and other organisms (4, 22, 23). The addition of CM to cryptococcal cultures reduced this delay, suggesting that secretion of an autostimulatory molecule potentiated *C. neoformans* growth. We produced CM from cryptococci of different serotypes, comprising *C. neoformans* varieties *grubii* (serotype A) and *neoformans* (serotype D) and *C. gattii* (serotypes B and C). We observed that the two varieties of *C. neoformans* could have their growth stimulated by CM from all four serotypes, whereas the addition of CM to the two tested strains of *C. gattii* cells produced very little to no response to this stimulatory factor. Consequently, we conclude that the QS activity could have evolved differently between the two *Cryptococcus* species. However, more systematic screening of a large number of *Cryptococcus* strains is necessary to confirm such a hypothesis, since cryptococcal isolates can be quite variable. Moreover, CM from other fungal species, including *S. cerevisiae*, *C. albidus*, *C. albicans*, and *S. schenckii*, induced a growth response in *C. neoformans*, and the addition of *C. neoformans* CM stimulated the growth of *C. albicans* and *S. cerevisiae* in MM. These results suggest the presence of similar signaling systems in other fungal species.

None of the growth-related effects were observed when the cells were grown on complex media, such as yeast peptone dextrose (YPD) or Sabouraud's dextrose broth (SDB), both of which have yeast extract in their composition. As we observed that *S. cerevisiae* CM was able to induce faster growth in *C. neoformans*, we believe that yeast extract could contain the same or a similar active molecule, explaining our inability to observe an effect with these media. The fold increase in growth was also present but less intense when *C. neoformans* cells were grown on a richer minimal medium (SD medium).

A similar QS-like effect on growth was described in cultures of *C. neoformans tup1* mutants (12, 24). However, in contrast to the QSP1 peptide, the effects we observed were not restricted to growth and were observed in wild-type cells in several different strains and backgrounds. We tested the QSP1 peptide in our assay, and it was not able to induce activity, confirming that we had observed different phenomena. Similarly, none of the QS molecules produced by *C. albicans* was able to induce the effects we observed (data not shown) or to inhibit *C. neoformans* growth as has been shown with *Aspergillus nidulans* (25).

Recently, we proposed a set of necessary conditions for classifying a molecule as a fungal QSM (20), based on criteria previously established for bacterial QS molecules (26, 27). In short, the molecule should accumulate during fungal growth in a density-dependent manner and, after reaching a threshold concentration, it should trigger a coordinated behavior in the entire population. The response to the molecule should be restricted to a specific growth phase and should not solely reflect detoxification or metabolic adaptation to the molecule itself. Additionally, the exogenous addition of the molecule should reproduce the QS behavior and the molecule should not be a simple catabolism by-product. Our activity meets most of these criteria, although the lack of a definitive QSM identification does not allow us to meet all the conditions. The *C. neoformans* CM activity accumulated during growth, reaching high concentrations at stationary phase. At this phase, the effect of CM stopped increasing, suggesting an autoregulatory mechanism for secretion of the QSM. We also observed that the response to CM was highly correlated with cell density and that cells in late-logarithmic- or stationary-phase growth apparently became unresponsive to the CM stimulatory effects. It is noteworthy that the cell densities at which a response was observed in our experiments are similar to the ones found in cerebrospinal fluid (CSF) from patients with cryptococcosis (28, 29). This raises the possibility that similar growth-related phenomena

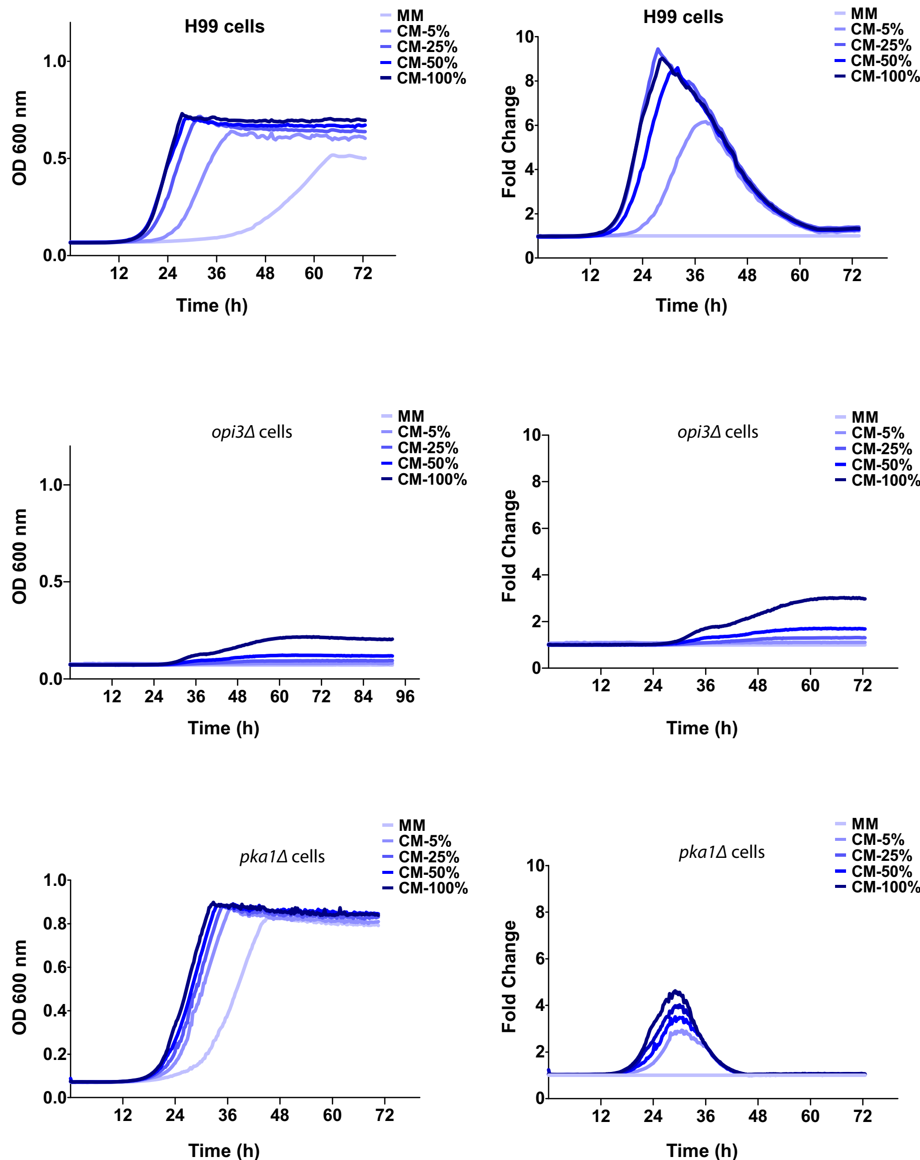

FIG 8 Growth effects of wild-type CM on wild-type, *opi3*Δ, and *pka1*Δ cells. Each strain was grown for 24 h and diluted to new cultures at 10^4 cells/ml. The cells were then grown in MM or MM supplemented with increasing concentrations of H99 CM at 30°C in an automated microbiology growth curve analysis system where their absorbance was read every 30 min for 72 h. Left side plots show the absorbance of each culture at 600 nm, while the right plots show fold change (growth in CM/growth in MM). The values shown represent the means of three wells for each condition.

could occur during infection and suggests the need to test fluids like cerebrospinal fluid from infected hosts for their ability to mediate comparable effects. In addition, we found a mutant with defective secretion of the CM activity, and this mutant responds normally to the addition of wild-type CM. The effects observed regarding biofilm formation also support our hypothesis. When we started the experiment using low cell densities, we observed a significant dose-dependent increase in biofilm growth that was not observed when the experiment was initiated at high cell densities.

We began the characterization of the QSM by defining some basic features of the molecule, such as its solubility and stability. The activity showed high hydrophilicity and poor solubility in organic solvents, characteristics that contrast with those of other fungal QSMs. The activity was resistant to heating, acid, alkali, and the action of proteases, nucleases, and glucosidases. The development of a purification strategy for the CM activity proved extremely challenging because of the hydrophilicity of the QSM substance(s). We identified pantothenic acid in *C. neoformans* CM and showed that this molecule can mediate the cell density effects attributed to the QSM. One possible explanation for the inability of exogenous pantothenic acid to fully reproduce the effects with CM is that *C. neoformans* also produces other pantothenic acid derivatives modified with other functional groups and/or that the activity observed with CM is produced by more than one molecule, of which pantothenic acid is only one component. The presence of a pantothenic acid derivative stimulating growth in *C. neoformans* is supported by the work of Amachi and colleagues, who

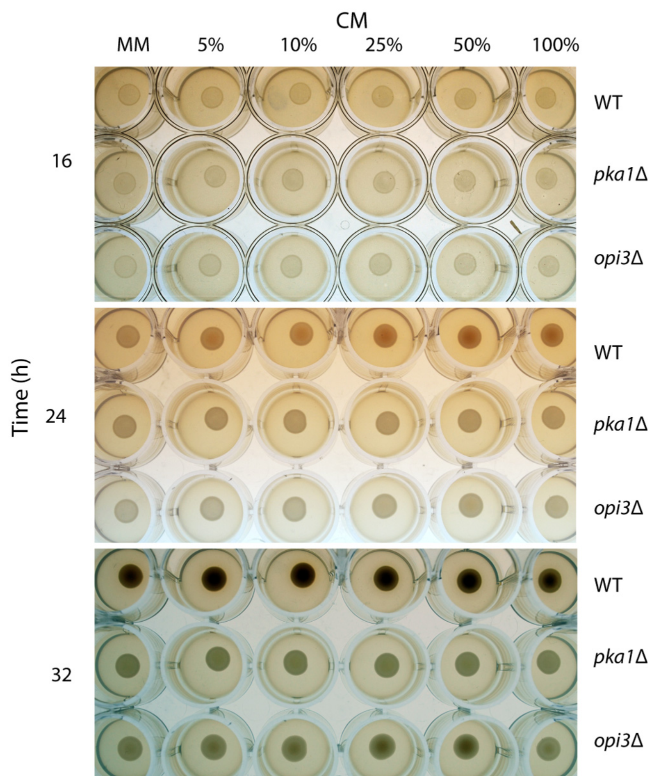

FIG 9 Comparison of the effects of wild-type CM on melanization of wild-type, *pka1*Δ, and *opi3*Δ cells. Each strain was grown for 24 h and washed 3 times, and 10^5 cells were spotted in agar MM supplemented with L-DOPA and with increasing concentrations of CM from wild-type cells (H99). The colonies were followed visually for melanin production for 32 h. At 24 h, it was possible to discriminate visually the dose-dependent melanization of wild-type cells. The *opi3*Δ colonies only started to melanize at 32 h, whereas the *pka1*Δ colonies did not melanize at all.

reported that a β-glucoside derivative of pantothenic acid had strong effects on the growth of lactic acid bacteria (30). Despite considerable efforts spanning several years, we were not able to definitively identify the additional molecule(s) responsible for the *C. neoformans* QS effects. At this time, our best interpretation of our findings suggests that at least one of the *C. neoformans* bioactive molecules is pantothenic acid or one of its derivatives. This would be in agreement with the fact that we replicated QS effects with purified pantothenic acid, albeit at lower activities than were observed with CM. Furthermore, it is possible that CM contains other metabolites which work synergistically with pantothenic acid and/or its derivatives. In this regard, we note that pantothenic acid is an important metabolite present in biological fluids like CSF (31). The definitive identification of the full complement of QSMs remains an experimental challenge given the hydrophilic nature of these molecules, but this knowledge is necessary for understanding QS-related phenomena in *C. neoformans*.

In summary, microbial communication can involve much more than the simple evaluation of population density. Our findings about QS transform our former view of microbes as single and isolated organisms. Further research on microbial social interactions is likely to reveal a good deal of complexity and could provide us with new tools to better manage the detrimental effects that microbes can present to human beings. This would be particularly important for pathogenic fungal species, considering the toxicity, high cost, and low efficiency generally presented by available antifungal drugs.

MATERIALS AND METHODS

Fungal strains. *C. neoformans* strains 24067 and B3501 (serotype D) were obtained from ATCC (Manassas, VA), and *C. neoformans* strain Cap67, an acapsular mutant derived from the B3501 strain, and the congenic serotype D strains JEC21 (*MAT*α) and JEC20 (*MATa*) were provided by J. Kwon-Chung. *Cryptococcus neoformans* var. *grubii* strain H99 (serotype A) was provided by John Perfect (Durham, NC). *Cryptococcus gattii* serotype B strain NIH198 and serotype C strain 1343 were obtained from Thomas Mitchell (Durham, NC). *Cryptococcus albidus* was obtained from Stuart Chaskes (Farmingdale, NY). *Candida albicans* (SC 5314), *Sporothrix schenckii* (ATCC 14285), *Blastomyces dermatitidis*, and *Histoplasma capsulatum* (G217B) were provided by Joshua D. Nosanchuk (Bronx, NY). *Saccharomyces cerevisiae* strain S288C was obtained from Viviane C. B. Reis (Brasilia-DF, Brazil). The *C. neoformans* deletion mutant library (CNKO-PS mutants) was purchased from ATCC (Manassas, VA). The library spans about 1,200 target genes of *C. neoformans* strain H99, and each mutant carries the nourseothricin selection marker (natR) and a signature bar code of 40 bp (17). Mutants with mutations in different signaling pathways of *C. neoformans* were kindly provided by Joseph Heitman (Duke University) and Andrew Alspaugh (Duke University).

Media. *C. neoformans* minimal medium (MM) contained 15 mM glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, and 3.0 μM thiamine. The melanization assays were done in 2% minimal medium agar supplemented with 1 mM 3,4-dihydroxy-L-phenylalanine (L-DOPA; Sigma, St. Louis, MO). Minimal medium with different carbon and nitrogen sources had glucose and glycine replaced with (i) 15 mM glucose and 7.6 mM asparagine (GA), (ii) 15 mM glucose and 8.8 mM creatinine (GC), or (iii) 122 mM sodium acetate and 7.6 mM asparagine (AA). *Candida* SD medium contained 0.7% (wt/vol) yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI) and 2% (wt/vol) glucose. Yeast peptone dextrose (YPD) medium and Sabouraud's dextrose broth (SBD) were purchased from Becton Dickinson (Franklin Lakes, NJ).

Conditioned medium from wild-type cells. The conditioned medium (CM) was generated by growing cultures initiated with 10^5 cells/ml of *C. neoformans* in MM for 5 days (stationary phase) at 30°C, removing the cells by centrifugation, and then filtering the supernatant through 0.2-μm filters. For the GXM release assays, the CM was filtered through a 1-kDa membrane to remove previously shed GXM and other high-molecular-mass molecules. The cell-free supernatant was concentrated 10 times by lyophilization and kept at 4°C. We also obtained CM from different culture times to ascertain CM activity throughout the *C. neoformans* growth phases.

Growth assay in the presence of CM. *C. neoformans* cells were grown overnight in MM at 30°C. Cells were collected by centrifugation, washed three times with fresh MM, and inoculated at the cell densities indicated in the figures into MM alone or MM complemented with different concentrations of 10×-concentrated, 1-kDa-filtered CM. The cultures were incubated at 30°C, and the cell density was evaluated every 12 h by cell counting in a hemocytometer or, in most situations, by measurement of the optical density at 600 nm in a microplate spectrophotometer or measurements every 30 min in an automated microbiology growth curve analysis system (Bioscreen C; Growth Curves USA).

GXM release in the presence of CM. Samples from the cultures used in the growth curve assay were simultaneously collected for GXM concentration measurement by ELISA capture assay, as modified from Casadevall et al. (14), and for capsule measurement by India ink preparation. For the ELISA capture assay, 96-well polystyrene plates were coated with 10 μg/ml of goat anti-mouse IgM (Fisher Scientific) for 1 h and then blocked with 2% bovine serum albumin, followed by an overnight incubation at 4°C with the anti-GXM monoclonal antibody (MAb) 2D10 (10 μg/ml). The plates were washed 3 times with a solution of Tris-

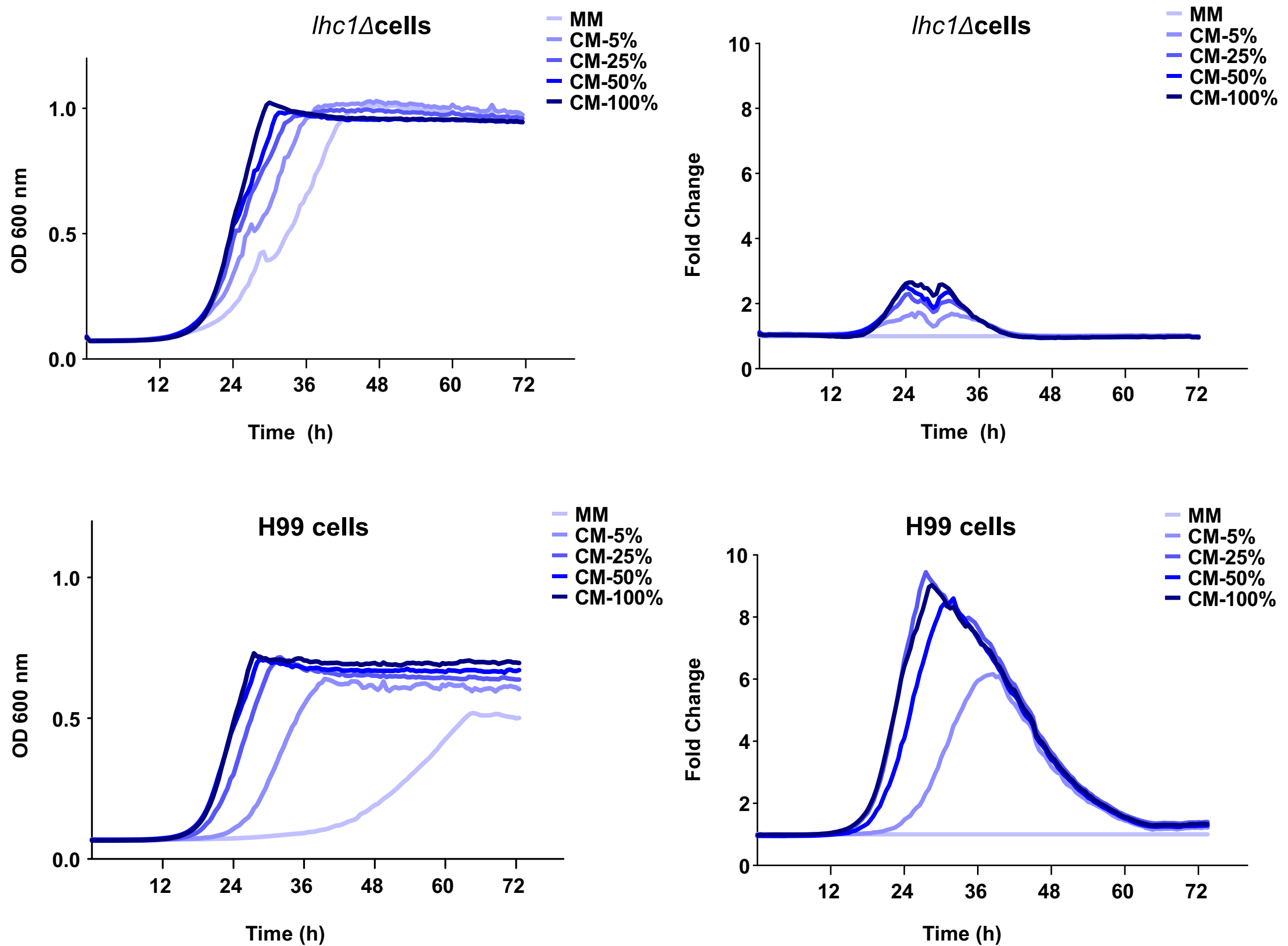

FIG 10 Comparison of the growth effects of wild-type CM on wild-type and *lhc1*Δ cells. Both strains were grown for 24 h and diluted to seed new cultures starting at 10^4 cells/ml. The cells were grown in either MM or MM supplemented with increasing quantities of H99 CM at 30°C. The plots on the left show the absorbance at 600 nm, whereas the plots on the right show the fold change values (growth in CM/growth in MM) during the growth curve. The values represent the means of three wells.

buffered saline–0.1% Tween 20 (TBST), and after that, appropriate dilutions of each sample of *C. neoformans* cultures were loaded into the plates and incubated for 1 h at 37°C. The plates were washed again 3 times with TBST and incubated for 1 h at 37°C with the GXM-binding MAb 18B7. The plates were then washed 3 times with TBST, and MAb 18B7 binding was detected with an alkaline phosphatase-conjugated goat anti-mouse IgG1 antibody for 1 h. The ELISA was developed with 1 mg/ml of *p*-nitrophenyl phosphate (Sigma), and the absorbance was measured at 405 nm with a Multiscan MS.

Melanization assay. *C. neoformans* cells were grown overnight in MM at 30°C. Cells were then collected by centrifugation, washed three times with fresh MM, and inoculated at the cell densities indicated in the figures in MM agar supplemented with 1 mM L-DOPA alone or in the presence of different concentrations of 10×-concentrated CM. The cultures were incubated in the dark at 30°C, and photographs were taken every 8 or 12 h using a Nikon D70 or D90 to visually follow the melanization of the cultures. Image processing consisted of white balance correction of the whole figure using Photoshop CS5 (Adobe Systems Inc., San Jose, CA). The melanization assays were done using different strains of *C. neoformans*, with similar results. *C. neoformans* H99 cells were usually chosen for these experiments because they melanize faster than other tested strains.

Biofilm growth. To evaluate the effects of CM on biofilm growth, *C. neoformans* cells from strain B3501 were grown overnight at 37°C in SDB medium, washed three times with phosphate-buffered saline (PBS), resuspended at different final cell concentrations (10^4, 10^5, 10^6, and 10^7 cells/ml) and added to the wells of a 96-well plate. As controls, we had wells containing medium without cells. We added CM to the wells in different concentrations and at different time points to follow its effects on different phases of the biofilm development. The following conditions were used: (i) cells and CM were added to a polystyrene plate at the same time and incubated for 24 h at 37°C; (ii) *C. neoformans* cells were preincubated in the plate for 4 h, unbound cells were removed by washing the plates 3 times using MM, CM was added, and the plate was incubated for 24 h at 37°C; and (iii) *C. neoformans* cells were incubated in the plate for 24 h, unbound cells were removed, CM was added, and the plate was incubated for another 24 h. Under all conditions, after the final 24-h incubation, the amount of biofilm cells was evaluated with the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay (32). In addition to XTT reduction assays, we used immunofluorescence and confocal microscopy to evaluate biofilms. Cells of the *C. neoformans* strain B3501 were inoculated at an initial cell density of 10^4 cells/ml in MM on 6-well plates containing sterile glass coverslips. The same three conditions described above (addition of CM 0, 4, or 24 h after addition of cells, followed by 24 h of incubation) were used. After the final incubation period, the wells were washed twice with PBS to remove nonadherent cells and incubated over-

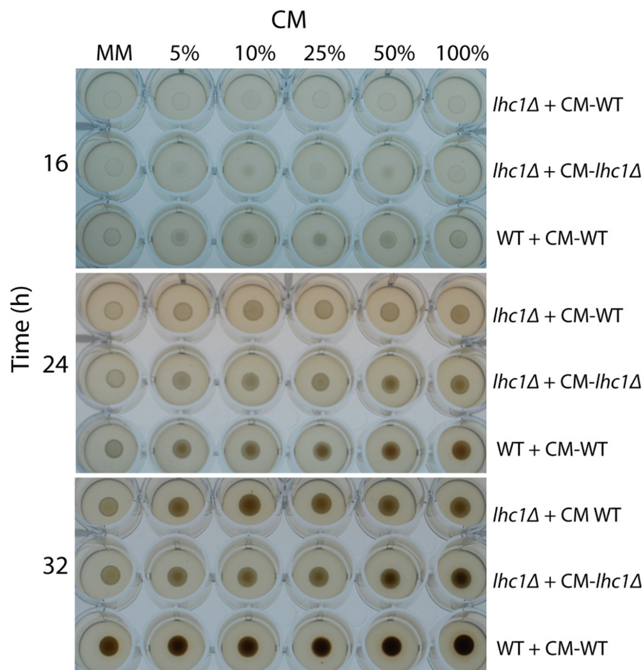

FIG 11 Comparison of the effects of CM on melanization of wild-type and *lhc1*Δ cells. Cells of the two strains were grown for 24 h and washed 3 times, and 10^5 cells were spotted in solid MM supplemented with L-DOPA and with increasing concentrations of H99 CM or *lhc1*Δ CM. The colonies were visually followed for melanin production for 32 h. At 24 h, it was possible to visualize the dose-dependent melanization of H99 cells but not yet of *lhc1*Δ cells, which began to show melanization only by 32 h of incubation.

night at 4°C with 10 μg/ml MAb 18B7 in PBS. Afterward, the coverslips were washed twice with PBS and incubated overnight at 4°C with 20 μg/ml of the cell wall chitin stain Calcofluor white and a 1:1,000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody. After two washes in PBS, the coverslips were mounted in ProLong Gold antifade medium (Invitrogen) and imaged in a Leica SP5 laser-scanning confocal microscope using a 20× oil immersion objective with 0.7 numeric aperture (NA). The images collected were processed with ImageJ and Adobe Photoshop software, with no nonlinear alterations.

Cross-stimulatory effects among different serotypes. CM from different strains of *C. neoformans* and *C. gattii*, including the four different serotype groups based on capsular agglutination reactions, were obtained following the same procedures described above.

Treatment with enzymes. To evaluate the possible biological class of the molecule responsible for the activity, we treated the CM with proteinase K (100 μg/ml for 2 h), trypsin (100 μg/ml for 2 h), RNase T_1 (2,000 U/ml/ for 12 h), DNase I (100 U/ml for 12 h), pronase (250 μg/ml for 12 h), α-glucosidase (10 U/ml for 12 h), or β-glucosidase (10 U/ml for 12 h). After the treatments, the CM was tested for activity.

Treatment with acid or alkali. To evaluate the effects of acid or alkali treatment upon the CM activity, the CM was treated with 1 M HCl or 1 M NaOH for 1 h at room temperature, neutralized with the same molar concentration of base or acid, and tested for activity.

Temperature stability. To evaluate the temperature stability of *C. neoformans* CM, aliquots were submitted to extremes of temperature and pressure in one or two rounds of autoclaving (121°C/20 min). Additionally, we also tested the activity of CM that was stored at 4°C, −20°C, or room temperature for several weeks or subjected to multiple freeze-thaw cycles.

Purification of CM activity. Cells from *C. neoformans* strain H99 were grown in MM for 5 days, and the cell-free supernatant was ultrafiltered by passage through a 1-kDa membrane for removal of exopolysaccharides and other high-molecular-mass molecules. The CM was then lyophilized for concentration. The dried sample was resuspended in ultrapure water to a final concentration 100× greater than the initial volume of CM. The CM was loaded in a Superdex peptide 10/300-Gl column (GE Healthcare, Uppsala, Sweden). The active fractions were collected, dried, tested for activity, and subsequently loaded into a Hypercarb column (Thermo Fisher Scientific, Waltham, MA). The active samples were dried and resuspended in the appropriate solvent for mass spectrometry and NMR analysis.

Mass spectrometry. Samples were diluted 1/10 or 1/100 in 50% MeOH-H_2O or 50% acetonitrile– 0.1% formic acid. Electrospray MS and tandem mass spectrometry were performed on a linear trap quadrupole (LTQ) linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The diluted samples were either infused at flow rates of 5 μl/min or injected (10 or 20 μl) for flow injection analysis (FIA) with 50% methanol-H_2O at a flow rate of 50 μl/min. Tandem mass spectrometry was performed using an isolation width of 1.5 *m/z* and normalized collision energy of ~35%. electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) was performed on a Varian 12.0 T quadrupole Fourier transform (QFT) mass spectrometer. Samples were infused at a flow rate of 5 μl/min into the ESI FT-ICR MS for high-resolution measurements providing mass measurements below 5 ppm (parts per million).

NMR. One-dimensional (1-D) proton spectra were collected at 25°C on a Bruker DRX 300-MHz spectrometer using 8 scans, a sweep width of 14 ppm sampled with 16 K points, and a recycle delay of 1.5 s. Spectra were processed with a 0.3-Hz exponential window function. Two-dimensional (2-D) ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra were collected at 25°C on a Bruker DRX 300-MHz spectrometer using 1 K and 256 complex points in t2 (^{1}H) and t1 (^{13}C), respectively, with 8 scans per t1 point and a recycle delay of 1.3 s. All experiments used a proton sweep width of 8 ppm and a ^{13}C sweep width of 200 ppm with the ^{1}H and ^{13}C carriers set to 4 ppm and 75 ppm, respectively. The proton dimension was zero filled to 2 K points, and the ^{13}C dimension was linearly predicted to 512 points before processing with a cosine bell window function in each dimension. The 2-D ^{1}H-^{15}N heteronuclear multiple bond correlation (HMBC) spectrum was collected at 25°C on a Bruker DRX 600-MHz spectrometer using 2 K and 256 points in t2 (^{1}H) and t1 (^{15}N), respectively, with 160 scans per t1 point and a recycle delay of 1.5 s. All experiments used a proton sweep width of 12 ppm and a ^{15}N sweep width of 200 ppm with the ^{1}H and ^{15}N carriers set to 4.7 ppm and 100 ppm, respectively. A sine bell window function was used to process the data in each dimension. The 2-D ^{1}H-^{13}C HMBC spectrum was collected at 25°C on a Bruker DRX 600-MHz spectrometer using 2 K and 512 points in t2 (^{1}H) and t1 (^{13}C), respectively, with 96 scans per t1 point and a recycle delay of 1.5 s. All experiments used a proton sweep width of 12 ppm and a ^{13}C sweep width of 225 ppm with the ^{1}H and ^{13}C carriers set to 4.7 ppm and 100 ppm, respectively. A sine bell window function was used to process the data in each dimension.

Pantothenic acid identification and quantification by HPLC. The pantothenic acid concentration in CM was evaluated by HPLC in an Alltech Alltima HP C_{18}-AQ HPLC column (250 by 4.6 mm and with 5-μm particle size) with 0.1% mol/liter KH_2PO_4 (pH 7.0)-methanol (90:10) as the mobile phase (0.7 ml/min) in isocratic mode using a Shimadzu (Kyoto, Japan) HPLC system. The detection was made using a UV-visible diode array at 205 nm as described previously (33). Shimadzu software was used to calculate the peak areas and concentration of pantothenic acid in CM based on a calibration plot with different concentrations of commercial pantothenic acid as the standard.

Treatment with farnesol and tyrosol. Concentrations of up to 250 μM farnesol and 100 μM tyrosol were tested for their ability to produce effects on the growth, capsule size, GXM release, or melanization of *C. neoformans*.

Activity test with QSP1 peptide. The QSP1 peptide NFGAPGGAYPW (12) was synthesized by the Proteomics Resource Center of the Rockefeller University (New York, NY), dissolved in minimal medium, and tested for activity in several concentrations up to 500 μM.

Capsule measurement. The capsule from *C. neoformans* cells was measured by light microscopy using India ink exclusion. Cells were suspended in India ink and photographed in an Axiovert 200 M inverted microscope using a 40× objective (Carl Zeiss MicroImaging, Thornwood, NY) and a Hamamatsu ORCA ERJ camera (Hamamatsu Photonics, Hamamatsu City, Japan). The volume of the *C. neoformans* capsule was measured using AxioVision software (Carl Zeiss MicroImaging, Thornwood, NY). The size of the whole cell and the cell body were each measured, and the capsule width was considered to be the difference between the whole-cell measurement and the cell body measurement. The volume of the capsule was calculated using the equation for the volume of a sphere, $4/3 \cdot \pi \cdot (D/2)^3$, and subtracting the volume of the cell body from the whole-cell volume. For each condition tested, we averaged the capsule volumes of 50 *C. neoformans* cells.

Conditioned medium from CNKO-PS mutants. The 1,200 CNKO-PS mutants were distributed in 14 96-well microtiter plates so that there was one mutant strain per well. To obtain CM from each mutant, we grew the 14 plates of mutant cells in YPDA (yeast peptone dextrose adenine) medium for 3 days for a preinoculum. Then, 2 μl of each mutant culture was added as an inoculum into 1 ml of minimal medium in 96-deep-well plates. These cultures were grown for 5 days at 30°C; the cell-free supernatant was obtained by plate centrifugation and filtration through a multiscreen 96-well plate from Millipore. The resulting CM samples were kept at 4°C.

Screening for mutants that do not produce CM activity. To screen the CNKO-PS mutant collection, *C. neoformans* cells were grown for 24 h in MM at 30°C, washed three times with fresh MM, and inoculated at 10^4 cells/ml in 96-well plates containing MM alone or MM supplemented with 25% CM prepared from each mutant strain as described above. The cultures were incubated at 30°C, and the cell density was evaluated after 24 h by measuring absorbance at 600 nm in a microplate spectrophotometer. Each mutant was assayed at least three times on different days. We calculated the fold change in growth for H99 cells in the presence of CM from each mutant relative to their growth in MM alone and compared this value to the fold change induced by native H99 CM. We selected candidates whose CM activity was significantly different from the activity of the CM produced by wild-type cells based on statistical parameters as described below. Positive hits (as described in "Statistical analysis" below) were submitted to a second, more stringent round of screening which yielded candidates for a final round. In this last step, mutant cells were grown in flasks instead of deep-well plates because we observed that, under this condition, their CM resulted in much stronger induction of growth. The CM obtained was also concentrated 10× before being added to the new cultures to minimize the dilution of nutrients that the addition of CM produces. Mutants that were unable to grow in minimal medium were excluded from the analysis.

Screening for mutants that do not respond to wild-type CM activity. The CNKO-PS mutant strains from the 96-well plates were grown for 2 days in YPD at 30°C, and then 2 μl of each culture was diluted to 1 ml with fresh MM in 96-well plates to yield a suspension with approximately 10^3 to 10^4 cells/ml. We transferred 100 μl of the dilutions from each of these plates to 6 new microtiter plates. Three of those plates received another 100 μl of MM, and the other three received MM supplemented with wild-type CM to a final concentration of 25%. The cultures were incubated at 30°C, and the cell density was evaluated after 16 to 18 h by measuring absorbance at 600 nm in a microplate spectrophotometer. We calculated the fold change in the growth of each mutant grown in the presence of wild-type CM relative to their growth in MM alone and then compared this value to the fold change induced by wild-type CM in wild-type cells. After statistical analysis, clones identified as potentially positive (as described in "Statistical analysis" below) were selected and submitted to a second and a third round of selection. In the third round, the activity test was done in tubes instead of 96-well plates. Mutants that presented major growth problems were excluded from the analysis.

Statistical analysis. Statistical analysis was done using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA) or Excel (Microsoft, Redmond, WA) data analysis tools. Data were evaluated for normal distribution using the Shapiro-Wilk test. Normally distributed data were analyzed for significance using one-way analysis of variance, and nonnormally distributed data were submitted to the Kruskal-Wallis test. P values lower than 0.05 were considered significant. For the mutant screening, statistical analyses were done with the Student t test. All experiments were done in triplicate. The fold change in growth represents the ratio between the growth of a specific strain in CM and its growth in MM alone. In screening for mutants that did not produce the QS-like activity, we calculated the means and standard deviations for the fold changes obtained from the control wells, which contained wild-type cells grown in MM or in CM from wild-type culture. Positive clones were selected based on the following two criteria: (i) their CM produced a smaller mean fold change (2 standard deviations below the mean fold change in the control well containing wild-type CM), and (ii) this difference between the two sets of values was statistically significant (P value of <0.05). Positive hits were submitted to two additional rounds of screening. To screen for mutants that presented problems in their response to wild-type CM activity, we produced wild-type CM and grew each of the mutants in triplicate in either MM or MM supplemented with wild-type CM. We calculated the means and standard deviations for the fold changes in growth obtained from the control wells, which contained wild-type cells grown in MM or in wild-type CM. Potential candidates were selected based on the following two conditions: (i) the cells did not grow more in the presence of CM than they did in MM, and (ii) the fold change in growth for the mutant cells in the presence of CM was statistically lower than the fold change in growth displayed by wild-type cells ($P < 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00986-13/-/DCSupplemental.

Figure S1, EPS file, 0.9 MB.
Figure S2, EPS file, 1.7 MB.
Figure S3, EPS file, 0.7 MB.
Figure S4, EPS file, 1.6 MB.
Figure S5, TIF file, 6.5 MB.
Figure S6, EPS file, 1.6 MB.

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