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ENVIRONMENTAL MICROBIOLOGY

Divalent Metal Cations Potentiate the Predatory Capacity of Amoeba for *Cryptococcus neoformans*

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ABSTRACT Among the best-studied interactions between soil phagocytic predators and a human-pathogenic fungus is that of *Acanthamoeba castellanii* and *Cryptococcus neoformans*. The experimental conditions used in amoeba-fungus confrontation assays can have major effects on whether the fungus or the protozoan is ascendant in the interaction. In the presence of Mg^{2+} and Ca^{2+} in phosphate-buffered saline (PBS), *C. neoformans* was consistently killed when incubated with *A. castellanii*. *A. castellanii* survived better in the presence of Mg^{2+} and Ca^{2+}, even when incubated with *C. neoformans*. In the absence of Mg^{2+} and Ca^{2+}, *C. neoformans* survived when incubated with *A. castellanii*, and the percentage of dead amoebae was higher than when incubated without yeast cells. These results show that the presence of Mg^{2+} and Ca^{2+} can make a decisive contribution toward tilting the outcome of the interaction in favor of the amoeba. Of the two metals, Mg^{2+} had a stronger effect than Ca^{2+}. The cations enhanced *A. castellanii* activity against *C. neoformans* via enhanced phagocytosis, which is the major mechanism by which amoebae kill fungal cells. We found no evidence that amoebae use extracellular killing mechanisms in their interactions with *C. neoformans*. In summary, the presence of Mg^{2+} and Ca^{2+} enhanced the cell adhesion on the surfaces and the motility of the amoeba, thus increasing the chance for contact with *C. neoformans* and the frequency of phagocytosis. Our findings imply that the divalent cation concentration in soils could be an important variable for whether amoebae can control *C. neoformans* in the environment.

IMPORTANCE The grazing of soil organisms by phagocytic predators such as amoebae is thought to select for traits that enable some of them to acquire the capacity for virulence in animals. Consequently, knowledge about the interactions between amoebae and soil microbes, such as pathogenic fungi, is important for understanding how virulence can emerge. We show that the interaction between an amoeba and the pathogenic fungus *C. neoformans* is influenced by the presence in the assay of magnesium and calcium, which potentiate amoebae. The results may also have practical applications, since enriching soils with divalent cations may reduce *C. neoformans* numbers in contaminated soils.

KEYWORDS amoeba, fungi, *Cryptococcus neoformans*, predation, cations

Cryptococcus neoformans is a soil-dwelling fungus that is a frequent cause of life-threatening meningoencephalitis in individuals with impaired immunity (1). One of the fascinating aspects of *C. neoformans* biology is that it has the capacity for virulence in very different animal and plant species, and yet this organism has no need for pathogenicity, as it can survive in soils without hosts. To explain this phenomenon, the concept of accidental virulence was proposed, which posits that environmental pressures select for traits that confer upon the microbe the capacity for pathogenicity independent of the final host (2, 3). In the case of *C. neoformans*, the environmental

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pressure was proposed to include amoeboid predators such as amoebae (4, 5). Supporting this hypothesis is the fact that many virulence factors function in the same manner during the interaction between macrophages and amoebae (5, 6). Furthermore, the passage of an avirulent strain of *C. neoformans* in social amoebae restored virulence (7). Recently, the interaction of *C. neoformans* with *Acanthamoeba castellanii* was shown to result in increased fungal virulence for *Galleria mellonella* as a result of amoeba-induced changes in susceptibility to microbicidal oxidants and the cell wall (8). Studies of amoeba interactions with other pathogenic fungi such as *Histoplasma capsulatum* (9), *Sporothrix schenckii* (9), *Blastomyces dermatitidis* (9), *Aspergillus fumigatus* (10, 11), and entomopathogenic fungi (12) suggest that fungus-amoeba interactions could be important in their virulence for animal hosts.

In recent years, amoebae have emerged as a major system for studying bacterial and fungal host-microbe interactions (13, 14), including *C. neoformans* biology and virulence (15–19). The realization that amoebae served as reservoirs and training hosts for animal virulence together with the ease that these protozoa can be maintained in the laboratory have popularized the study of their interactions with various microbes. Insights gained with amoebae on mechanisms of intracellular pathogenesis were shown to apply to microbe-macrophage interactions and vice versa. For example, the discovery that phospholipids are a trigger for capsular enlargement in *C. neoformans* followed the observation that cryptococcal cells enlarged their capsules in the presence of amoebae, and then the same phenomenon was shown in macrophages (20). On the other hand, a *Mycobacterium avium* pathogenicity island important for intracellular infection was first identified in a macrophage screen and then shown to also be important for infecting amoebae (21). Clearly, macrophages and amoebae provide complementary systems for the study of virulence determinants and the evolution of pathogenicity.

Prior studies have shown that the outcome of the *C. neoformans-Acanthamoeba castellanii* interaction is highly dependent on the conditions of the experiment. For example, confrontation experiments involving cryptococci and amoebae under nutrient-poor conditions such as phosphate-buffered saline resulted in fungal growth and the death of amoebae (4, 22). However, when cryptococci and amoebae were suspended on amoeba growth medium, the protozoa were ascendant, with a reduction of fungal cells by predation and killing (23). Those results were interpreted as suggesting that the nutritional state of amoebae was an important variable in their predatory capacity (23). However, while carrying out *C. neoformans*-amoeba experiments, we made the serendipitous observation that the presence of Ca^{2+} and Mg^{2+} was all that was required to potentiate amoeba predation for fungal cells. These results identify the concentration of divalent cations as an important variable in studies of microbe-amoeba interactions.

RESULTS

A serendipitous observation. While studying *C. neoformans* interactions with *A. castellanii* in phosphate-buffered saline, we noted that the amoebae killed a substantial proportion of the fungal cells. This result was unexpected given that prior work has shown that under these conditions, *C. neoformans* tended to kill *A. castellanii* and replicate (4, 23). A review of the conditions of the experiment revealed that the solution used was not the standard laboratory formulation but instead a commercial product manufactured by Corning (Corning, NY) known as Dulbecco's phosphate-buffered saline (DPBS), which differed from the phosphate-buffered saline (PBS) used in prior experiments by supplementation with magnesium and calcium. This suggested that the difference in the findings was the presence of these two divalent cations. We then repeated the experiments with DPBS supplemented with or without Ca^{2+} and Mg^{2+}, jointly and singly, and verified that the addition of the metal salts was the ingredient potentiating the activity of *A. castellanii* against *C. neoformans*. In the presence of magnesium and calcium in DPBS, *C. neoformans* CFU decreased when incubated with *A. castellanii* but increased without *A. castellanii* (Fig. 1A and B). *A. castellanii* survived

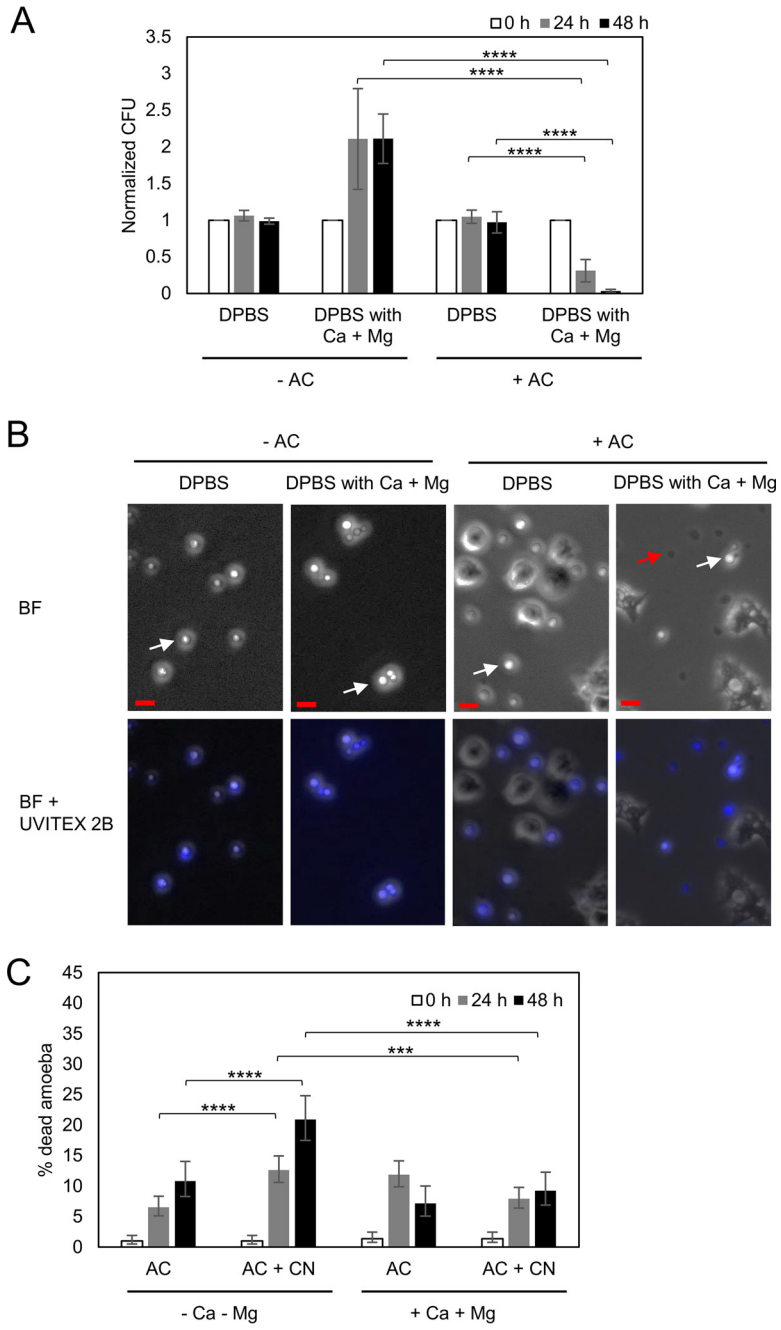

FIG 1 Presence of magnesium and calcium affects the outcome of the *A. castellanii-C. neoformans* interaction. (A) The presence of magnesium and calcium decreases the survival of *C. neoformans* during the incubation with *A. castellanii* (AC). The survival of *C. neoformans* was determined by CFU after incubation with *A. castellanii* for 0, 24, and 48 h. The CFU counts at 24 h and 48 h were normalized to the initial CFU at time zero. Data represent the means from four biological samples. Error bars are SDs. ****, $P < 0.0001$ by Student's *t* test. (B) Phase-contrast and fluorescence images of Uvitex 2B-stained *C. neoformans* cells coincubated with or without *A. castellanii* in DPBS with or without magnesium and calcium. In the phase-contrast images, disrupted *C. neoformans* cells are dark (red arrow), while intact cells are refractile (white arrows). More disrupted *C. neoformans* cells appeared under the condition with *A. castellanii* incubation in DPBS containing magnesium and calcium. Scale bars are 10 μm. (C) *A. castellanii* cells survived better in the presence of Mg^{2+} and Ca^{2+} during the incubation with *C. neoformans* (CN). The viability of *A. castellanii* cells was determined by a trypan blue exclusion assay. The percentages of dead *A. castellanii* cells were determined by counting the numbers of blue stained cells per the total cell number counted. Four independent biological experiments were performed. Error bars represent 95% confidence intervals of the means. ***, $P = 0.0008$; ****, $P < 0.0001$ by Fisher's exact tests.

better in the presence than in the absence of Ca^{2+} and Mg^{2+} during the incubation with *C. neoformans* (Fig. 1C). The percentage of dead *A. castellanii* after incubation with *C. neoformans* was higher than that without yeast cells in the absence of divalent metal cations (Fig. 1C). The results show that magnesium and calcium are key determinants of the outcome of the *C. neoformans* and amoeba interaction.

The commercial DPBS is supplemented with $CaCl_2$ and $MgCl_2$. DPBS (without Ca^{2+} and Mg^{2+}) also contains KCl and NaCl, ruling out chloride ions as the component responsible for the enhanced predation of amoebae. However, to further confirm that the divalent metals were responsible for the enhanced protozoa fungicidal effects, we performed the experiment with DPBS supplemented with different forms of Ca^{2+} and Mg^{2+} (calcium nitrate and magnesium sulfate). Under these conditions, we observed an enhanced killing ability of amoebae, establishing that magnesium and calcium ions are the ones responsible for this effect (see Fig. S1 in the supplemental material).

Mg^{2+} is more effective than Ca^{2+} in potentiating *A. castellanii*. To ascertain which of the divalent cations was responsible for potentiating amoebae, we evaluated the outcome of the *C. neoformans-A. castellanii* interaction under conditions where DPBS was supplemented with Ca^{2+}, Mg^{2+}, or both. The addition of either Ca^{2+} or Mg^{2+} had a significant effect in reducing the *C. neoformans* CFU relative to that under conditions with no divalent cations, but the effect was much greater with Mg^{2+} and was maximal when both cations were present simultaneously (Fig. 2A to D). The presence of Mg^{2+} also increased the viability of *A. castellanii* in the presence and absence of *C. neoformans* (Fig. 2G).

Divalent cations enhanced amoeba surface area, phagocytosis, and mobility. To investigate the mechanism by which divalent cations potentiated *A. castellanii* against *C. neoformans*, we evaluated three parameters with a high likelihood to impact the fungal-protozoal interaction: amoeba adhesion, phagocytosis, and mobility. Both Ca^{2+} and Mg^{2+} are known to be important for cellular adhesion, with Mg^{2+} having a stronger effect than Ca^{2+} (24, 25). We measured cellular areas as a measure of adhesion and noted that amoebae in the presence of Ca^{2+} and Mg^{2+} manifested significantly greater areas when placed on a glass surface (Fig. 3A and B). Amoebae moved more and made more contacts with *C. neoformans* in the presence of Ca^{2+} and Mg^{2+} (Fig. 3C and D), which translated into a significantly greater phagocytosis of yeast cells (Fig. 3E).

Amoeba fungicidal activity requires protozoa-fungus cell contact. Next, we considered whether contact was necessary for an amoeba to kill *C. neoformans* by separating fungal and amoeboid cells in wells where the fluid was connected through a semipermeable membrane. We observed no reduction in fungal CFU under conditions where the fungal and protozoal cells were separated. However, when *C. neoformans* and amoeba were placed in the same chamber, there was a reduction in fungal colonies (Fig. 4A and B). The incubation of *C. neoformans* in amoeba-conditioned medium, which was collected after the coincubation of *A. castellanii* and *C. neoformans*, had no effect on fungal colonies, arguing against the release of fungicidal products from amoebae (Fig. 4C and D). These experiments indicated a necessity for contact between the amoeba and *C. neoformans* for protozoal fungicidal activity. Cinematographic analysis of the *C. neoformans-A. castellanii* interaction revealed the phagocytosis followed by the regurgitation of fungal carcasses, with the latter being easily distinguishable from live fungal cells, as these were shriveled structures that had lost their characteristic translucent appearance by light microscopy (Fig. 4E; see also Movie S1). The average time from phagocytosis to regurgitation of cryptococcal cellular remnants was 2.04 ± 0.53 h ($n = 5$).

Interaction of *C. neoformans* and amoeba. To better understand how divalent cations potentiated an amoeba against *C. neoformans*, we carried out several experiments to probe variables that might contribute to their interaction. Given that the capsule of *C. neoformans* has negatively charged glucuronic acid residues that can interact with cations and that capsule enlargement can be triggered by Ca^{2+} (26), we

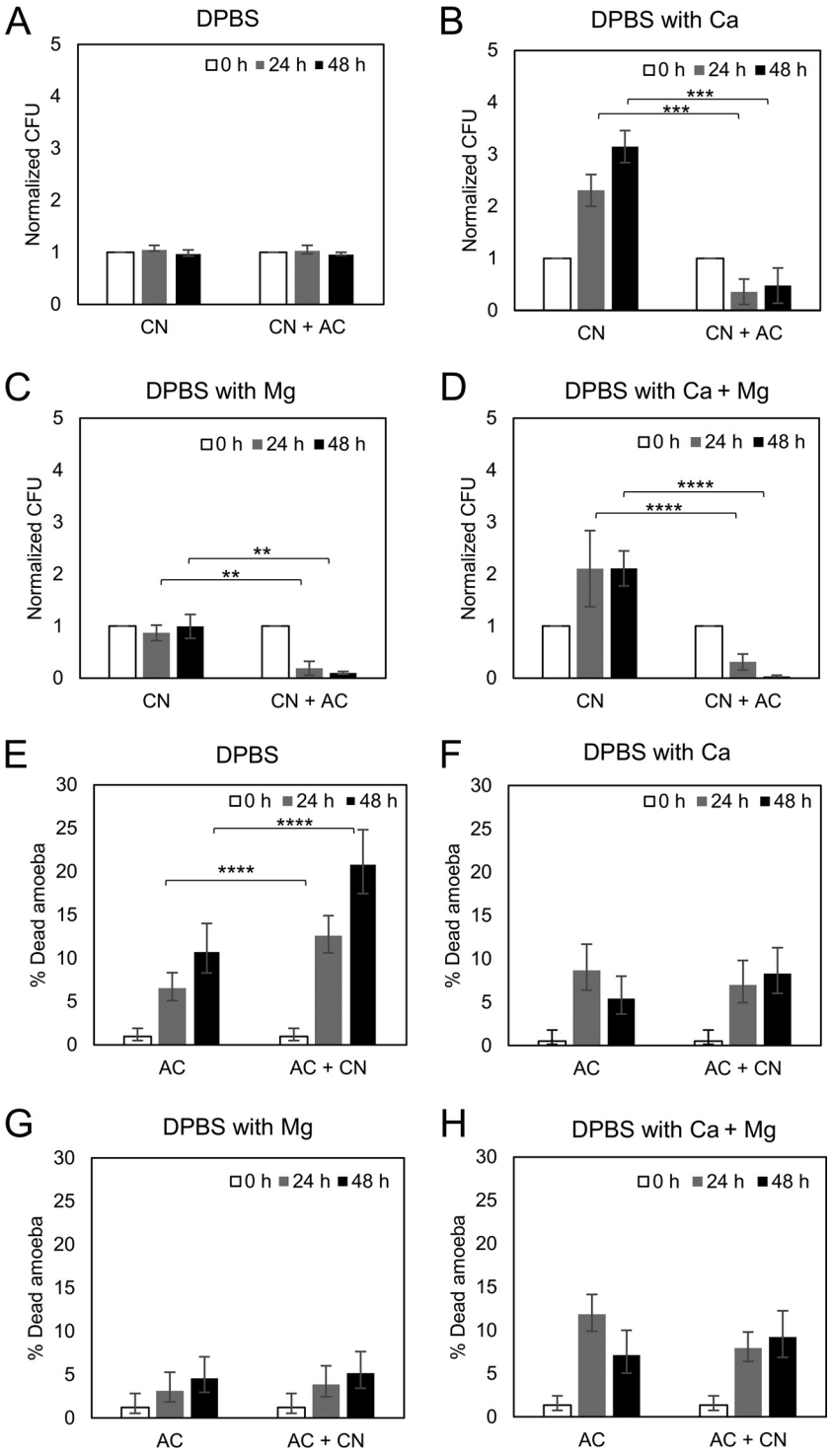

FIG 2 Magnesium had a stronger effect than calcium on reducing the survival of *C. neoformans* when incubated with *A. castellanii*. The survival of *C. neoformans* (CN) was determined by CFU after incubation with *A. castellanii* (AC) for 0, 24, and 48 h in DBPS (A), DPBS with calcium (B), DPBS with magnesium (C), and DPBS with calcium and magnesium (D). The CFU counts were normalized to the initial CFU at time zero. Data represent the means from three biological samples. Error bars are SDs. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by Student's *t* tests. The viabilities of *A. castellanii* cells were also determined by trypan blue exclusion assays after incubation with *C. neoformans* in DBPS (E), DPBS with calcium (F), DPBS with magnesium (G), and DPBS with calcium and magnesium (H). The percentages of dead *A. castellanii* cells were determined by counting the numbers of blue stained cells per total cell number counted. Three independent biological experiments were performed. Error bars represent 95% confidence intervals of the mean. ****, $P < 0.0001$ by Fisher's exact tests.

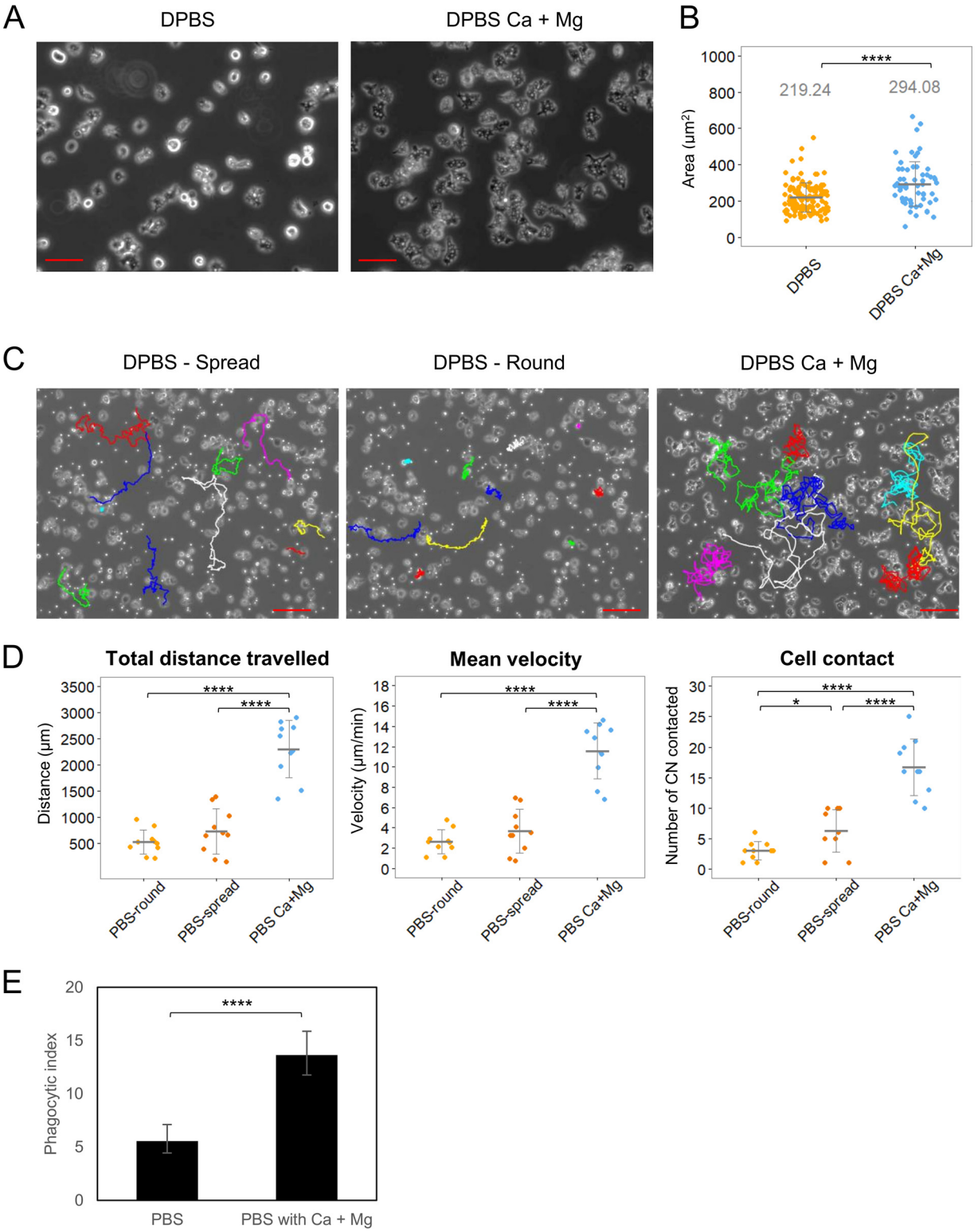

FIG 3 Presence of calcium and magnesium enhances the adherence of *A. castellanii* to the surface and its motility. (A) Phase-contrast images of *A. castellanii* cells on glass surfaces in DPBS with or without Ca^{2+} and Mg^{2+}. Scale bars are 50 μm. (B) To quantify cell spreading, the areas of *A. castellanii* cells in DPBS with or without Ca^{2+} and Mg^{2+} were measured. At least 100 cells were analyzed for each condition. Error bars indicate SDs. *, $P = 0.01572$; ****, $P < 0.0001$ by Student's *t* test. (C) Images of *A. castellanii* cell trajectories on glass surfaces in DPBS with or without Ca^{2+} and Mg^{2+}. Ten *A. castellanii* cells were randomly selected for manually centroid tracked for a total duration of 2 h under each condition. The interval of each track is 30 s. Color lines indicate manually generated tracks. Two distinct morphologies of amoeboid cells, round and spread, in DPBS were tracked separately. Scale bars are 100 μm. (D) The total distance, the mean velocity, and the frequency of contact with *C. neoformans* cells were quantified. The total distance was defined as the sum of the distances the amoeboid cells travelled from the starting point to the

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first incubated yeast cells with Mg^{2+} and Ca^{2+}, and then washed and added the fungal cells to the amoebae (see Fig. S2A and B). We measured no effect, suggesting that the increased susceptibility of *C. neoformans* to an amoeba in the presence of cations was not due to effects on the yeast cell and/or capsule. The pretreatment of the amoeba or both the amoeba and *C. neoformans* with Mg^{2+} and Ca^{2+} (Fig. S2C to F) followed by washing and incubation with *C. neoformans* also had no effect on fungicidal activity. These results indicate that the divalent cations have to be in the solution containing amoeboid and fungal cells for their potentiating effect on amoeba fungicidal activity.

DISCUSSION

Amoebae are an increasingly popular alternative host system for studying interactions between microbes and environmental phagocytic cells, but relatively little work has been done on the effect of experimental conditions on the outcome of their interaction with fungi. In earlier studies, we had noted that carrying out confrontation experiments between *A. castellanii* and *C. neoformans* in ATCC medium 712 with peptone-yeast extract-glucose (PYG) potentiated the fungicidal capacity of the protozoa and inferred that it reflected an improved nutritional status for the amoeboid cells (23). Here, we show that the addition of the divalent cations Ca^{2+} and Mg^{2+} is sufficient to potentiate *A. castellanii* activity against *C. neoformans*, making it very likely that they are the active ingredients in ATCC medium 712, which has 0.05 M $CaCl_2$ and 0.4 M $MgSO_4$. Our findings establish that the ionic composition of the solution can have an important effect on the outcome of the experiment with *C. neoformans* and suggest that the same may apply in other amoeba-microbe confrontation systems.

The ability of amoebae to prey on *C. neoformans* was first described in the 1970s (27, 28). The evidence for the importance of this interaction in the environment comes from the observation that the prevalence of *C. neoformans* in soils is reduced proportionally to the presence of amoebae (29). Consequently, amoebae probably play a major role in reducing the fungal burden in contaminated soils and, as such, may reduce the amount of human and animal exposure. Our results suggest that the efficacy of amoebae in soils may depend on environmental divalent cation concentrations. In this regard, soils can vary greatly on free Ca and Mg depending on the presence of vegetable matter detritus and the pH, which in turn can affect worm concentrations (30). In the United States, soil Ca and Mg concentrations average 0.375 M (range, 0.02 to >4 M) and 0.6 M (range, 0.0025 to 8 M), respectively (31). Such ranges in concentrations imply that the availability of these cations to soil amoebae would vary greatly depending on the soils, given that that these concentrations do not necessarily imply that the element would be available in a soluble cationic form.

The potentiation of amoeba fungicidal activity by divalent cation ions was associated with increases in surface area, mobility, contact with fungal cells, and phagocytosis. Hence, divalent cations appear to have global effects on amoeboid cell biological function that may work synergistically to enhance their predatory capacity. An increased surface area together with greater mobility and enhanced contacts with *C. neoformans* cells are likely to increase the probability of phagocytosis, which was indeed measured. Amoebae were visualized ingesting cryptococcal cells that were later regurgitated as opaque and shriveled forms that were easily distinguishable from live translucent cells. The requirement for contact and phagocytosis in fungal killing was shown by the absence of fungicidal activity through diffusible substances. In contrast, facilitating the contact between amoebae and yeast cells reduced the fungal CFU. The treatment of fungal cells with divalent cations had no discernible impact on the

FIG 3 Legend (Continued)
endpoint of the cell trajectory. The mean velocity was calculated as the mean from all velocity measurements from amoeboid cells moving in each 30-s interval. Error bars indicate SDs. ****, $P < 0.0001$ by Student's *t* tests. (E) The presence of magnesium and calcium induces the phagocytosis of *C. neoformans* by *A. castellanii*. *A. castellanii* and Uvitex 2B-labeled *C. neoformans* were incubated at a 1:1 ratio in DPBS with or without magnesium and calcium for 2 h to allow phagocytosis. The phagocytic index was determined by the number of internalized *C. neoformans* cells per 100 *A. castellanii* cells. Data were obtained from four biologically independent experiments. Error bars represent 95% confidence intervals of the means. ****, $P < 0.0001$ by Fisher's exact test.

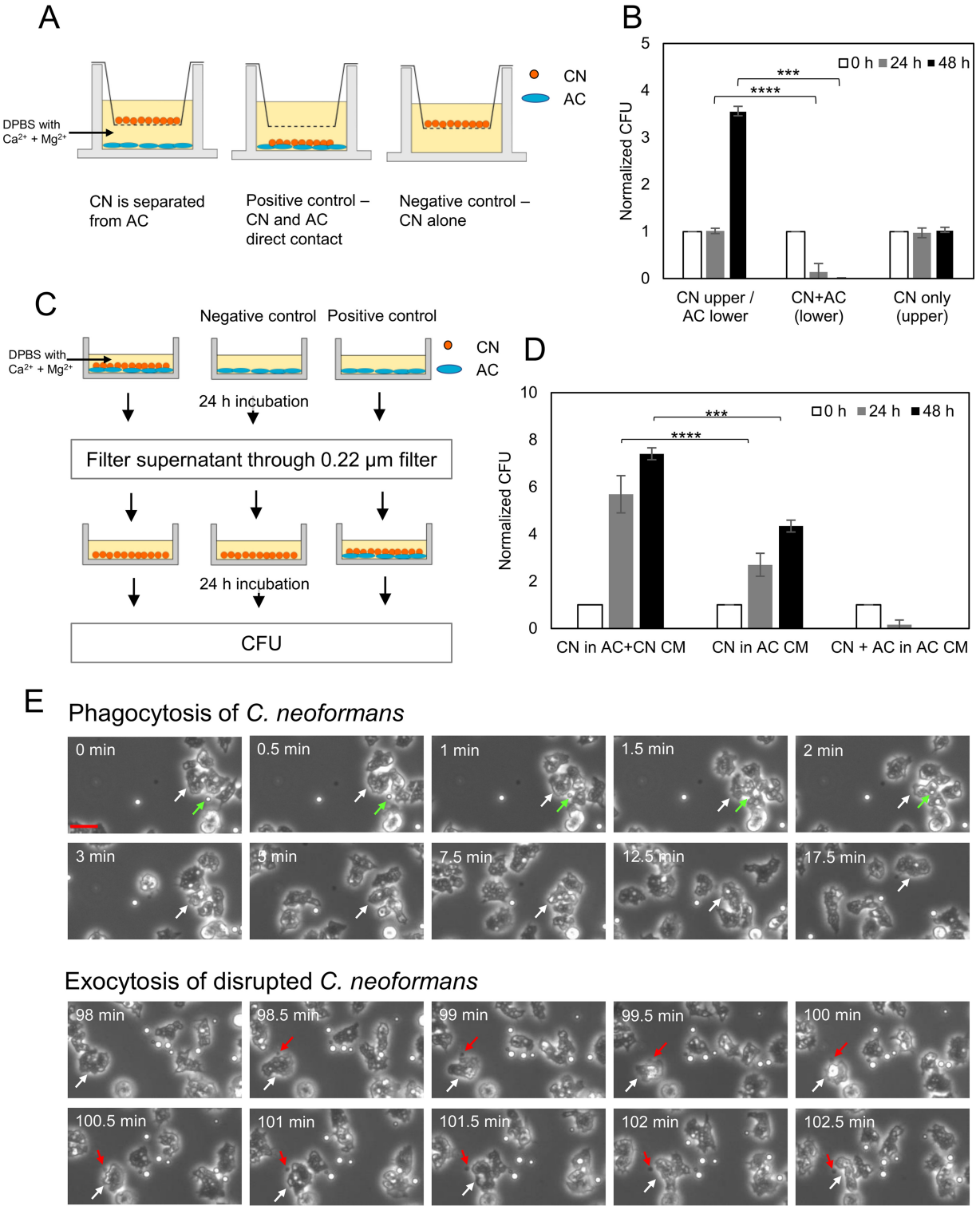

FIG 4 Phagocytosis is the major mechanism for *A. castellanii* to kill *C. neoformans*. (A) Scheme of the Transwell assay. *A. castellanii* and *C. neoformans* were separated in DPBS with Ca^{2+} and Mg^{2+} by porous membrane (pore size, 0.4 μm). The upper compartment contained *C. neoformans* while the lower compartment contained *A. castellanii*. As a positive control, *C. neoformans* was incubated with *A. castellanii* in the lower compartment. As a negative control, *C. neoformans* was incubated without *A. castellanii* in the upper compartment. (B) The survival of *C. neoformans* was determined by CFU after the Transwell assay for 0, 24, and 48 h. The CFU counts were normalized to the initial CFU at time zero. Data represent the means from four biological samples. Error bars are SDs. ***, $P = 0.0004$; ****, $P < 0.0001$ by Student's *t* tests. (C) Schematic for the production of conditioned media from coincubation of *C. neoformans* (CN) and *A. castellanii* (AC) and exposure of the fungal cells. After exposure of *A. castellanii* to *C. neoformans* in DPBS with Ca^{2+} and Mg^{2+}, conditioned media were collected, filtered, and added to fresh cultures of *C. neoformans*. As a negative control, *A. castellanii*-conditioned medium was collected and added to *C. neoformans*. As a positive control, *A. castellanii*-conditioned medium was collected and added to the coincubation of *C. neoformans* and *A. castellanii*. (D) The survival of *C. neoformans*

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interaction, suggesting that the effect of Ca^{2+} and Mg^{2+} in augmenting amoeba fungicidal activity was primarily, if not exclusively, due to the effects on the protozoan cells.

Although the mechanism by which Ca^{2+} and Mg^{2+} affect *A. castellanii* has not been investigated, there is evidence from related systems that these cations can have powerful effects on amoebae. Pinocytosis and locomotion of amoebae can be influenced by monovalent and divalent cations (32). *A. castellanii* is known to accumulate Ca^{2+} in the presence of excess cations, with cells forming electron-dense deposits (33). The displacement of cell surface-associated Ca^{2+} with drugs was shown to inhibit phagocytosis (34). Both Ca^{2+} and Mg^{2+} trigger signaling pathways that promote chemotaxis in the social amoeba *Dictyostelium discoideum* (35). For *Entamoeba invadens*, the signaling program that triggers encystation is dependent on Ca^{2+} (36). Ca^{2+} has been shown to increase the adhesion of *Acanthamoeba polyphaga* to extracellular matrix proteins (37) in a manner that recapitulates the increased adhesion effects we describe in this study. However, not all interactions of amoebae with other cells require divalent cations. In this regard, *Entamoeba histolytica* adhered and killed Chinese hamster ovary cells in the presence of EDTA, suggesting that the divalent cations were not needed for the interaction (38). These observations illustrate the protean effects of divalent cations on protozoa and, in aggregate, support the view for major differences in the physiological states of amoeboid cells under conditions with and without Ca^{2+} and Mg^{2+}, which translate into large differences in their ability to kill *C. neoformans*. However, it is noteworthy that *C. neoformans* also responds to divalent cations with capsular enlargement (26), which protects against amoebae (39). Hence, the fungal-protozoal balance of power could reflect not only cation concentrations but also the time each type of organism had to adapt to the prevailing conditions.

Several publications describing amoeba interactions with various pathogens report the use of different experimental conditions that could vary in divalent cations. For example, *A. castellanii* interactions with *Pseudomonas aeruginosa* (41), *Corynebacterium* spp. (41), and *Campylobacter jejuni* (43) were studied in peptone-yeast extract-glucose (PYG) medium, while interactions with *Vibrio cholerae* (40) and *Aspergillus fumigatus* (11) were done separately in defined artificial seawater and Dulbecco's modified Eagle medium that included Ca^{2+} and Mg^{2+} cations and interactions with *Bacillus anthracis* (44) was studied in autoclaved creek water supplemented with divalent cations. Given the powerful effects of divalent cations on amoeba function, comparisons across studies need to take into account the possibility that differences in Ca^{2+} and Mg^{2+} could affect experimental outcomes.

C. neoformans is commonly found in soils enriched in bird feces (reviewed in reference 1). In urban centers, *C. neoformans* is often found in high densities in soils near sites where pigeons roost, where guano provides a rich source of nutrients (45). Human and animal infection is believed to originate from the inhalation of desiccated yeast cells or basidiospores that become aerosolized from *C. neoformans*-contaminated soils (1, 46). Consistent with an environmental source of infection, isolates recovered from environmental sites were indistinguishable from those recovered from patients by the use of several genetic markers (47, 48). The contaminated soils near a hospital have been suggested as sites for hospital-acquired infection of *C. neoformans* (49). The remediation of *C. neoformans*-contaminated soils is a formidable challenge, and current methods involve the application of noxious chemicals such as formalin (50) and quaternary ammonium salts (51). Given that amoeba predation can reduce fungal

FIG 4 Legend (Continued)
(CN) was determined by CFU after exposure to conditioned medium (CM) for 0, 24, and 48 h. The CFU counts were normalized to the initial CFU at time zero. Data represent the means from three biological samples. Error bars are SDs. ***, $P = 0.00223$; ****, $P < 0.0001$ by Student's *t* tests. (E) Time-lapse phase-contrast images of coincubation of *C. neoformans* and *A. castellanii* in DPBS with Ca^{2+} and Mg^{2+}. Top images show the phagocytosis of *C. neoformans* (green arrows) by *A. castellanii* (white arrows). Bottom images show the exocytosis of disrupted *C. neoformans* (appears dark; red arrows) from *A. castellanii* (white arrows). Scale bar is 25 μm.

burden in contaminated soils (29), our results suggest that increasing the soil concentration of divalent cations with calcium and magnesium salts could potentiate the fungicidal capacity of amoebae and provide a more environmentally friendly biological alternative to chemical decontamination.

In summary, we establish the divalent cation concentration as a major variable in the outcome of *A. castellanii* interactions with *C. neoformans*. Given the dramatic effects that divalent cations have on amoeba physiology, we suspect that this effect will apply to other microbe-amoeba interactions and is likely to be an important experimental variable in other systems. This effect may also have applications for the environmental control of *C. neoformans*, since increasing Ca and Mg concentrations in soils could potentiate amoeba predation and reduce the fungal burden in contaminated sites.

MATERIALS AND METHODS

Cell culture. *Acanthamoeba castellanii* strain 30234 was obtained from the American Type Culture Collection (ATCC). Cultures were maintained in PYG broth (ATCC medium 712) at 25°C according to instructions from ATCC. *C. neoformans* var. *grubii* serotype A strain H99 was used in all experiments, and this strain was originally obtained from John Perfect (Durham, NC). Cryptococcal cells were cultivated in Sabouraud dextrose broth with shaking (120 rpm) at 30°C overnight (16 h) prior to use in amoeba assays.

Assay of *A. castellanii* and *C. neoformans* interaction. The survival of *C. neoformans* in amoeba culture was performed as described previously (22). Briefly, *A. castellanii* cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS; Corning, Corning, NY) and diluted in DPBS to an appropriate density. This buffer is available with and without calcium and magnesium supplementation. *A. castellanii* cells (1×10^4 cells/well) were added to 96-well plates and allowed to adhere for 1 h at 25°C. *C. neoformans* cells were washed twice with DPBS and diluted in DPBS to an appropriate density. Fungal cells (1×10^4) were added to wells containing amoebae or control wells containing DPBS alone, and the plates were incubated at 25°C. At 0, 24, and 48 h, the amoebae were lysed by pulling the culture through 27-gauge syringe needles five to seven times. The extent of cell lysis was examined using a light microscope, and approximately 98% of amoeboid cells were lysed. The lysates were serially diluted, plated on Sabouraud agar, and incubated at 30°C for 48 h for CFU determination. To study the effect of calcium and/or magnesium on the survival of *C. neoformans* during the interaction with amoebae, DPBS was replaced with DPBS containing 0.9 mM calcium and/or 0.5 mM magnesium. To study if the cations affect the adhesion between *C. neoformans* and *A. castellanii*, either or both *C. neoformans* and *A. castellanii* cells were pretreated with DPBS containing 0.9 mM calcium and 0.5 mM magnesium for 1 h. Cells were then washed with DPBS. The viability of *A. castellanii* was also determined under the same conditions and time intervals by adding a 1:80 dilution of trypan blue stain. The percentage of dead amoebae was determined by counting the number of trypan blue-stained cells per total cell number counted. A minimum of 100 cells were counted. Control wells contained *A. castellanii* without *C. neoformans*.

Microscopy and time-lapse imaging. For measuring the spread area of *A. castellanii* in the presence or absence of calcium and magnesium, *A. castellanii* cells (5×10^5 cells) were allowed to adhere for 2 h on a glass surface of poly-D-lysine-coated coverslip-bottom MatTek petri dishes with 14-mm microwells (MatTek Corporation, Ashland, MA) in DPBS with or without magnesium and calcium. Images were then taken using a Zeiss Axiovert 200M inverted microscope with a 20× phase objective. The areas of amoeboid cells (minimum 100 cells) were measured using ImageJ software.

For fluorescence imaging, *A. castellanii* cells were washed twice with DPBS, and 2×10^4 amoeboid cells were seeded on an 8-well-chambered cover glass (Nunc, Roskilde, Denmark) in DPBS with or without magnesium and calcium at 25°C for 2 h. *C. neoformans* cells were stained with 0.01% Uvitex 2B (Polysciences, Warminster, PA) for 10 min and washed twice with DPBS. Uvitex 2B-stained *C. neoformans* cells (2×10^4 cells) were added to an *A. castellanii* culture and incubated at 25°C. After 2 h and 24 h of incubation, images were taken using a DAPI (4′,6-diamidino-2-phenylindole) filter-equipped Zeiss Axiovert 200M inverted microscope with 20× phase objective. Images were also used for measuring the phagocytosis index, which was determined by the number of *C. neoformans* cells per 100 amoeboid cells.

For time-lapse imaging, *A. castellanii* cells were seeded (5×10^5 cells) on MatTek petri dishes in DPBS with or without magnesium and calcium. Cells were then incubated at 25°C for 2 h. Cryptococcal cells (5×10^5 cells/well) were added to an amoeba culture. After a 15-min incubation to allow fungal cells to settle down, images were taken every 30 s for 24 h using a Zeiss Axiovert 200M inverted microscope with a 10× phase objective in an enclosed chamber under conditions of 25°C.

The movements of *A. castellanii* cells were tracked manually using ImageJ manual tracking. Ten amoeboid cells were randomly selected and tracked after the first 2 h of incubation with *C. neoformans* under each tested condition. The total distance, mean velocity, and the number of contacts between *A. castellanii* and *C. neoformans* were measured. The total distance was defined as the sum of the distances the amoeboid cells travelled from the starting point to the endpoint of the cell trajectory. The mean velocity was calculated as the mean from all velocity measurements from amoeboid cells moving in each 30-s interval.

Supernatant toxicity assay. To examine the effect of the secretion of *A. castellanii* on *C. neoformans*, Transwell plates (6-well, polycarbonate membrane, 0.4-μm-pore size; Corning) were used to separate *C. neoformans* and *A. castellanii* into two compartments with a continuity of saline. *A. castellanii* cells were

washed twice and suspended with DPBS containing magnesium and calcium, and 2×10^5 cells were added to the lower compartments. The plates were then incubated at 25°C for 2 h. *C. neoformans* cells were washed and suspended with DPBS containing magnesium and calcium, and 2×10^5 cryptococcal cells were added to the inserts. After 0 h and 24 h of incubation, *C. neoformans* cells in the inserts were serially diluted, plated on Sabouraud agar, and incubated at 30°C for 48 h for CFU determination.

To examine whether the secretion of *A. castellanii* products required direct cell contact between amoebae and fungi, *C. neoformans* cells were treated with amoeba-conditioned medium collected after the incubation of *A. castellanii* and *C. neoformans*. *A. castellanii* cells (1×10^4 cells) in DPBS containing magnesium and calcium were added to 96-well plates and allowed to adhere for 2 h at 25°C. *C. neoformans* cells were washed twice with DPBS containing magnesium and calcium, and then 1×10^4 cells were added to wells containing amoebae and the plates were incubated at 25°C for 24 h. The conditioned medium was collected and filtered through 0.22-μm syringe filters. Fresh cultures of *C. neoformans* cells were washed twice with DPBS containing magnesium and calcium and suspended under the conditioned medium. Fungal cells (1×10^4) were added to wells and incubated at 25°C. Control wells contained a coincubation of *A. castellanii* and *C. neoformans* as well as *C. neoformans* alone with *A. castellanii*-conditioned medium. After 0 h and 24 h of incubation, the amoebae were lysed by pulling the cultures through 27-gauge syringe needles five to seven times. The lysates were serially diluted, plated on Sabouraud agar, and incubated at 30°C for 48 h for CFU determination.

Statistical analysis. All comparisons were analyzed by either unpaired two-tailed Student's *t* tests or two-tailed Fisher's exact tests.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01717-17.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, AVI file, 7.1 MB.

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REFERENCES

1. Casadevall A, Perfect JR. 1998. *Cryptococcus neoformans*. American Society for Microbiology, Washington, DC.
2. Casadevall A, Pirofski LA. 2007. Accidental virulence, cryptic pathogenesis, martians, lost hosts, and the pathogenicity of environmental microbes. Eukaryot Cell 6:2169–2174. https://doi.org/10.1128/EC.00308-07.
3. Guimaraes AJ, Gomes KX, Cortines JR, Peralta JM, Peralta RH. 2016. *Acanthamoeba* spp. as a universal host for pathogenic microorganisms: one bridge from environment to host virulence. Microbiol Res 193:30–38. https://doi.org/10.1016/j.micres.2016.08.001.
4. Steenbergen JN, Shuman HA, Casadevall A. 2001. *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. Proc Natl Acad Sci U S A 18:15245–15250. https://doi.org/10.1073/pnas.261418798.
5. Casadevall A, Nosanchuk JD, Steenbergen JN. 2003. "Ready-made" virulence and "dual-use" virulence factors in pathogenic environmental fungi–the *Cryptococcus neoformans* paradigm. Curr Opin Microbiol 112:1164–1175.
6. Adiba S, Nizak C, van Baalen M, Denamur E, Depaulis F. 2010. From grazing resistance to pathogenesis: the coincidental evolution of virulence factors. PLoS One 5:e11882. https://doi.org/10.1371/journal.pone.0011882.
7. Steenbergen JN, Nosanchuk JD, Malliaris SD, Casadevall A. 2003. *Cryptococcus neoformans* virulence is enhanced after intracellular growth in the genetically malleable host *Dictyostelium discoideum*. Infect Immun 71:4862–4872. https://doi.org/10.1128/IAI.71.9.4862-4872.2003.
8. Rizzo J, Albuquerque PC, Wolf JM, Nascimento R, Pereira MD, Nosanchuk JD, Rodrigues ML. 2017. Analysis of multiple components involved in the interaction between *Cryptococcus neoformans* and *Acanthamoeba castellanii*. Fungal Biol 121:602–614. https://doi.org/10.1016/j.funbio.2017.04.002.
9. Steenbergen JN, Nosanchuk JD, Malliaris SD, Casadevall A. 2004. Interaction of *Blastomyces dermatitidis*, *Sporothrix schenckii*, and *Histoplasma capsulatum* with *Acanthamoeba castellanii*. Infect Immun 72:3478–3488. https://doi.org/10.1128/IAI.72.6.3478-3488.2004.
10. Hillmann F, Novohradska S, Mattern DJ, Forberger T, Heinekamp T, Westermann M, Winckler T, Brakhage AA. 2015. Virulence determinants of the human pathogenic fungus *Aspergillus fumigatus* protect against soil amoeba predation. Environ Microbiol 17:2858–2869. https://doi.org/10.1111/1462-2920.12808.
11. Van Waeyenberghe L, Bare J, Pasmans F, Claeys M, Bert W, Haesebrouck F, Houf K, Martel A. 2013. Interaction of *Aspergillus fumigatus* conidia with *Acanthamoeba castellanii* parallels macrophage-fungus interactions. Environ Microbiol Rep 5:819–824. https://doi.org/10.1111/1758-2229.12082.
12. Bidochka MJ, Clark DC, Lewis MW, Keyhani NO. 2010. Could insect phagocytic avoidance by entomogenous fungi have evolved via selection against soil amoeboid predators? Microbiology 156:2164–2171. https://doi.org/10.1099/mic.0.038216-0.
13. Mylonakis E, Casadevall A, Ausubel FM. 2007. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog 3:e101. https://doi.org/10.1371/journal.ppat.0030101.
14. Hilbi H, Weber SS, Ragaz C, Nyfeler Y, Urwyler S. 2007. Environmental predators as models for bacterial pathogenesis. Environ Microbiol 9:563–575. https://doi.org/10.1111/j.1462-2920.2007.01238.x.
15. Lin J, Idnurm A, Lin X. 2015. Morphology and its underlying genetic regulation impact the interaction between *Cryptococcus neoformans* and its hosts. Med Mycol 53:493–504. https://doi.org/10.1093/mmy/myv012.
16. Madu UL, Ogundeji AO, Mochochoko BM, Pohl CH, Albertyn J, Swart CW, Allwood JW, Southam AD, Dunn WB, May RC, Sebolai OM. 2015. Cryptococcal 3-hydroxy fatty acids protect cells against amoebal phagocytosis. Front Microbiol 6:1351. https://doi.org/10.3389/fmicb.2015.01351.
17. Magditch DA, Liu TB, Xue C, Idnurm A. 2012. DNA mutations mediate microevolution between host-adapted forms of the pathogenic fungus *Cryptococcus neoformans*. PLoS Pathog 8:e1002936. https://doi.org/10.1371/journal.ppat.1002936.
18. Derengowski LS, Paes HC, Albuquerque P, Tavares AH, Fernandes L, Silva-Pereira I, Casadevall A. 2013. The transcriptional response of *Cryptococcus neoformans* to ingestion by *Acanthamoeba castellanii* and macrophages provides insights into the evolutionary adaptation to the mammalian host. Eukaryot Cell 12:761–774. https://doi.org/10.1128/EC.00073-13.

19. Araujo Gde S, Fonseca FL, Pontes B, Torres A, Cordero RJ, Zancope-Oliveira RM, Casadevall A, Viana NB, Nimrichter L, Rodrigues ML, Garcia ES, Souza W, Frases S. 2012. Capsules from pathogenic and non-pathogenic *Cryptococcus* spp. manifest significant differences in structure and ability to protect against phagocytic cells. PLoS One 7:e29561.
20. Chrisman CJ, Albuquerque P, Guimaraes AJ, Nieves E, Casadevall A. 2011. Phospholipids trigger *Cryptococcus neoformans* capsule enlargement during interactions with amoebae and macrophages. PLoS Pathog 7:e1002047. https://doi.org/10.1371/journal.ppat.1002047.
21. Danelishvili L, Wu M, Stang B, Harriff M, Cirillo SL, Cirillo JD, Bildfell R, Arbogast B, Bermudez LE. 2007. Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci U S A 104:11038–11043. https://doi.org/10.1073/pnas.0610746104.
22. Malliaris SD, Steenbergen JN, Casadevall A. 2004. *Cryptococcus neoformans* var. *gattii* can exploit *Acanthamoeba castellanii* for growth. Med Mycol 42:149–158. https://doi.org/10.1080/13693780310001616500.
23. Garcia-Solache MA, Izquierdo-Garcia D, Smith C, Bergman A, Casadevall A. 2013. Fungal virulence in a lepidopteran model is an emergent property with deterministic features. mBio 4:e00100-13. https://doi.org/10.1128/mBio.00100-13.
24. Takeichi M, Okada TS. 1972. Roles of magnesium and calcium ions in cell-to-substrate adhesion. Exp Cell Res 74:51–60. https://doi.org/10.1016/0014-4827(72)90480-6.
25. Hornby JE. 1973. Measurements of cell adhesion. II. Quantitative study of the effect of divalent ions on cell adhesion. J Embryol Exp Morphol 30:511–518.
26. Nimrichter L, Frases S, Cinelli LP, Viana NB, Nakouzi A, Travassos LR, Casadevall A, Rodrigues ML. 2007. Self-aggregation of *Cryptococcus neoformans* capsular glucuronoxylomannan is dependent on divalent cations. Eukaryot Cell 6:1400–1410. https://doi.org/10.1128/EC.00122-07.
27. Bunting LA, Neilson JB, Bulmer GS. 1979. *Cryptococcus neoformans*: gastronomic delight of a soil ameba. Sabouraudia 17:225–232. https://doi.org/10.1080/00362177985380341.
28. Neilson JB, Ivey MH, Bulmer GS. 1978. *Cryptococcus neoformans*: pseudohyphal forms surviving culture with *Acanthamoeba polyphaga*. Infect Immun 20:262–266.
29. Ruiz A, Neilson JB, Bulmer GS. 1982. Control of *Cryptococcus neoformans* in nature by biotic factors. Sabouraudia 20:21–29. https://doi.org/10.1080/00362178285380051.
30. Reich PB, Oleksyn J, Modrzynski J, Mrozinski P, Hobbie SE, Eissenstat DM, Chorover J, Chadwick OA, Hale CM, Tjoelker MG. 2005. Linking liter calcium, earthworms and soil properties: a common garden test with 14 tree species. Ecol Lett 8:811–818. https://doi.org/10.1111/j.1461-0248.2005.00779.x.
31. Shacklette HB, JG. 1984. Element concentration in soils and superficial materials of the conterminous United States, p 1–10. U.S. Geological Survey professional paper 1270. United States Government Printing Office, Washington, DC.
32. Stockem W, Klein HP. 1988. Pinocytosis and locomotion of amoebae: XVII. Influence of different cations on induced pinocytosis in *Amoeba proteus*. Eur J Protistol 23:317–326. https://doi.org/10.1016/S0932-4739(88)80021-X.
33. Sobota A, Przelecka A, Janossy AG. 1978. X-ray microanalysis of calcium-dependent deposits at the plasma membrane of *Acanthamoeba castellanii*. Cytobiologie 17:464–469.
34. Głowacka SK, Sobota A, Przelecka A. 1985. Displacement of cell-surface associated calcium inhibits phagocytosis and Ca-ATPase activity in amoeba. Cell Biol Int Rep 9:183–191. https://doi.org/10.1016/0309-1651(85)90093-1.
35. Korohoda W, Madeja Z, Sroka J. 2002. Diverse chemotactic responses of *Dictyostelium discoideum* amoebae in the developing (temporal) and stationary (spatial) concentration gradients of folic acid, cAMP, Ca(2+) and Mg(2+). Cell Motil Cytoskeleton 53:1–25. https://doi.org/10.1002/cm.10052.
36. Makioka A, Kumagai M, Kobayashi S, Takeuchi T. 2002. Possible role of calcium ions, calcium channels and calmodulin in excystation and metacystic development of *Entamoeba invadens*. Parasitol Res 88:837–843. https://doi.org/10.1007/s00436-002-0676-6.
37. Wang L, Asem EK, McLaughlin GL. 1994. Calcium enhances *Acanthamoeba polyphaga* binding to extracellular matrix proteins. Invest Ophthalmol Vis Sci 35:2421–2426.
38. Ravdin JI, Guerrant RL. 1981. Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*. Study with mammalian tissue culture cells and human erythrocytes. J Clin Invest 68:1305–1313.
39. Zaragoza O, Chrisman CJ, Castelli MV, Frases S, Cuenca-Estrella M, Rodriguez-Tudela JL, Casadevall A. 2008. Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. Cell Microbiol 10:2043–2057. https://doi.org/10.1111/j.1462-5822.2008.01186.x.
40. Van der Henst C, Scrignari T, Maclachlan C, Blokesch M. 2016. An intracellular replication niche for Vibrio cholerae in the amoeba *Acanthamoeba castellanii*. ISME J 10:897–910. https://doi.org/10.1038/ismej.2015.165.
41. Siddiqui R, Lakhundi S, Khan NA. 2015. Interactions of *Pseudomonas aeruginosa* and *Corynebacterium* spp. with non-phagocytic brain microvascular endothelial cells and phagocytic *Acanthamoeba castellanii*. Parasitol Res 114:2349–2356. https://doi.org/10.1007/s00436-015-4432-0.
42. Reference deleted.
43. Bui XT, Winding A, Qvortrup K, Wolff A, Bang DD, Creuzenet C. 2012. Survival of *Campylobacter jejuni* in co-culture with *Acanthamoeba castellanii*: role of amoeba-mediated depletion of dissolved oxygen. Environ Microbiol 14:2034–2047. https://doi.org/10.1111/j.1462-2920.2011.02655.x.
44. Dey R, Hoffman PS, Glomski IJ. 2012. Germination and amplification of anthrax spores by soil-dwelling amoebas. Appl Environ Microbiol 78:8075–8081. https://doi.org/10.1128/AEM.02034-12.
45. Larsen RA, Pappas PG, Perfect JR, Aberg JA, Casadevall A, Cloud GA, James R, Filler S, Dismukes WE. 2005. A phase I evaluation of the safety and pharmacodynamic activity of a murine-derived monoclonal antibody 18B7 in subjects with treated cryptococcal meningitis. Antimicrob Agents Chemother 49:952–958. https://doi.org/10.1128/AAC.49.3.952-958.2005.
46. Botts MR, Hull CM. 2010. Dueling in the lung: how *Cryptococcus* spores race the host for survival. Curr Opin Microbiol 13:437–442. https://doi.org/10.1016/j.mib.2010.05.003.
47. Currie BP, Freundlich LF, Casadevall A. 1994. Restriction fragment length polymorphism analysis of *Cryptococcus neoformans* isolates from environmental (pigeon excreta) and clinical isolates in New York City. J Clin Microbiol 32:1188–1192.
48. Nosanchuk JD, Shoham S, Fries BC, Shapiro DS, Levitz SM, Casadevall A. 2000. Evidence for zoonotic transmission of *Cryptococcus neoformans* from a pet cockatoo to an immunocompromised patient. Ann Intern Med 132:205–208. https://doi.org/10.7326/0003-4819-132-3-200002010-00032.
49. Rustan ME, Rubinstein HR, Siciliano C, Masih DT. 1992. Possibility of in-hospital infection by *Cryptococcus neoformans* in patients with AIDS. Rev Inst Med Trop Sao Paulo 34:383–387. https://doi.org/10.1590/S0036-46651992000500002.
50. Pal M, Dave P. 2016. Cryptococcosis: an emerging airborne mycosis of global concern. Air Water Borne Dis 5:127. https://doi.org/10.4172/2167-7719.1000127.
51. Krangvichain P, Niyomtham W, Prapasarakul N. 2016. Occurrence and susceptibilities to disinfectants of *Cryptococcus neoformans* in fecal droppings from pigeons in Bangkok, Thailand. J Vet Med Sci 78:391–396. https://doi.org/10.1292/jvms.15-0594.