

Fungal Virulence in a Lepidopteran Model Is an Emergent Property with Deterministic Features

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ABSTRACT Virulence has been proposed to be an emergent property, which by definition implies that it is not reducible to its components, but this application of a philosophical concept to the host-microbe interaction has not been experimentally tested. The goals of our study were to analyze the correlation of the phenotype with the ability to cause disease and to determine the dynamics of an experimental cryptococcal infection in *Galleria mellonella* and *Acanthamoeba castellanii*. By studying the outcome of infection as host death, we showed that the dynamics of virulence in the *G. mellonella*/*Cryptococcus neoformans* interaction follow a predictable pattern. We also found that the experimental temperature and not the presence of virulence factors was a critical parameter defining the pathogenic potential of cryptococcal species. Our results established that cryptococcal species not considered pathogenic could be pathogens given suitable conditions. Our results support the idea that virulence is an emergent property that cannot be easily predicted by a reductionist approach and yet it behaves as a deterministic system in a lepidopteran cryptococcal infection. These findings provide a road map for evaluating whether host-microbe interactions in other systems are chaotic, deterministic, or stochastic, including those with public health importance.

IMPORTANCE Virulence is a complex phenotype that cannot be easily studied by analyzing its individual components in isolation. By studying the outcome of infection as the death of the host, we found that a given microbial phenotype does not necessarily correlate with its ability to cause disease and that the presence of so-called virulence factors does not predict pathogenicity, consistent with the notion that virulence is an emergent property. This paper reports that the dynamics of virulence in *Galleria mellonella* larvae infected with the fungus *Cryptococcus neoformans* follows a predictable pattern. Establishing that virulence is an emergent property is important because it implies that it is not reducible to its components, and consequently, this phenomenon needs to be studied by a holistic approach.

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Virulence is one outcome of the interaction of a microbe and a host and is a complex phenotype that depends on factors displayed both by the microbe and the host. Virulence is a dependent variable that is “contingent on the availability of a susceptible host and the context and nature of the host-microbe interaction” (54). In this regard, virulent organisms can be defined as such only in the presence of susceptible hosts, and the phenomenon of virulence has been proposed to be an emergent property (1, 2). Emergent properties are properties of a system that cannot be fully explained by the addition of the individual system components (3).

In evolutionary terms, virulence has been mostly regarded as the outcome of the coevolution of a pathogen and its host, and such has been the acquisition of virulence factors (4); however, in accidental pathogens acquired from the environment, the major-

ity of encounters with a host are dead-end events. The presence of virulence traits and the ability to survive within the host might be seen as the side products of the survival adaptations to their natural environment (5). Plasticity and nonspecificity with regard to susceptible hosts are common features of human environmental fungal pathogens such that most can infect phylogenetically distant hosts.

Among the accidental pathogens are the pathogenic cryptococci (PC), which are defined as such because they do not have a known need for a host and are environmentally acquired (6). The PC comprise the *Cryptococcus neoformans*-*Cryptococcus grubii* group and their sister species *Cryptococcus gattii* (7, 8). The closest relatives to the PC are *Cryptococcus amyloletus*-*Tsuchiyaea wingfieldii* and *Filobasidiella depauperata* (9, 10). Outside the PC group, the other members of the *Filobasidiella* clade are saprobes

mostly associated with insect frass. Even without the need for a mammalian host to complete their life cycle, the PC are responsible for a considerable burden of mortality and morbidity in humans, with an estimated 1 million cases of cryptococcal meningitis/year, leading to about 600,000 deaths (11). The PC have a battery of well-described virulence factors, including heat tolerance, the presence of a polysaccharide capsule, melanin production via a phenoloxidase enzyme (laccase), and extracellular secretion of urease, type B phospholipase (12–15), etc. However, the known virulence factors other than heat tolerance contribute less than half of the total virulent phenotype of the PC (16), and other attributes might be important in host colonization and disease progression.

In this study, we investigated the dynamics of cryptococcal infection with the goal of determining whether the virulence of an environmental microbe followed a predictable pattern. Unpredictability of microbial virulence may be due to stochastic (non-deterministic) or deterministic chaotic systems. Even though chaos is intrinsically deterministic, it is difficult to make predictions in chaotic systems due to their nonlinear dynamics and the high sensitivity to initial conditions (17–19). We have developed two methods to study virulence. First, we analyzed the pattern of deaths in a time series in a given host-microbe interaction to determine the dynamics of our system. Second, we formulated a quantitative measure of virulence using death of the host as the observable outcome. Our results suggest that virulence in our experimental system is intrinsically deterministic and most of the variations observed are due to random factors present in the system rather than to deterministic chaotic components. We also evaluated the pathogenic potential of traditionally nonpathogenic cryptococcal species to determine whether phenotypic characters shared with the pathogenic cryptococci can be useful predictors of virulence. Our findings indicate that isolated fungal traits do not predict virulence, thus supporting the notion that virulence is an emergent property.

RESULTS

Virulence in three cryptococcal species. To determine whether the phenotypic characteristics correlated with the pathogenic potential as a measurement of predictability for virulence, we compared two cryptococcal species, *Cryptococcus amylo lentus* and *Cryptococcus podzolicus*, to the well-described pathogen *C. neoformans*. We compared the presence of canonical virulence factors like those found in *C. neoformans*, such as the capsule, extracellular urease and phospholipase activity, and melanin production, with the capacity to cause death in *G. mellonella* larvae and the protozoan *Acanthamoeba castellanii*.

C. amylo lentus is the closest known relative to the PC (9), whereas *C. podzolicus* (20) is more distantly related (21) but was reported to be able to melanize and possess a capsule (7, 22).

We observed positive staining by immunofluorescence assays (IFA) on the surface of *C. amylo lentus* cells using the monoclonal antibody (MAb) 18B7 to glucuronoxylomannan (GXM), which is consistent with the presence of a capsule. The polysaccharide in *C. amylo lentus* was in close proximity to the cell body and formed a very thin capsule (Fig. 1A and C). The India ink exclusion assay revealed a clear area surrounding some of the yeasts (Fig. 1B and D), and transmission electron microscopy (TEM) also suggested the presence of a small capsule (see Fig. S3 in the supplemental material). Capsular growth was observed primarily in aging cul-

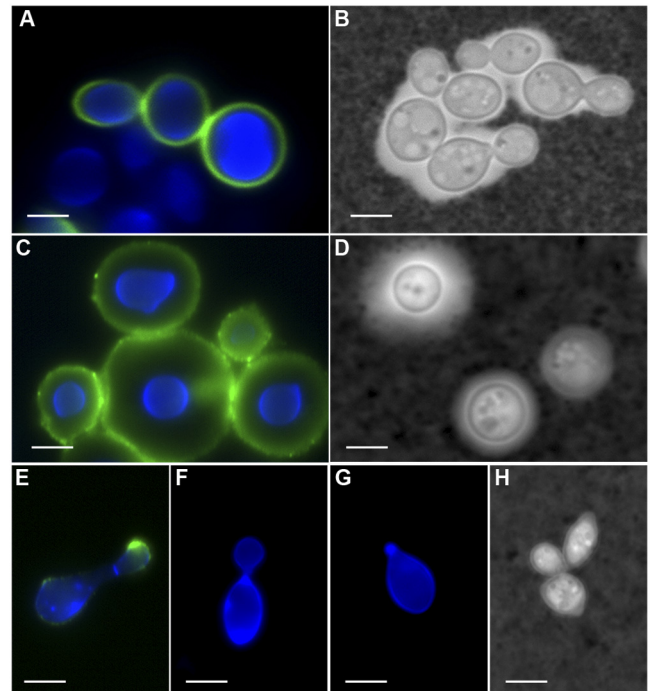


FIG 1 Capsular detection in different cryptococcal species. (A, C, and E to G) IFA for GXM with MAb 18B7 (green); the cell wall is stained with UviTex2B (blue). (B, D, and H) India ink. Cells were grown for 6 days in MM at 25°C. (A and B) *C. amylo lentus*; (C and D) *C. neoformans* var. *grubii*; (E to H) *C. podzolicus*. (A) Some cells showed positive staining for GXM, closely surrounding the cell wall. (B) The India ink exclusion test revealed the presence of a clear halo that is not homogeneous for all cells. (C) Characteristic capsular pattern for GXM stain. (D) Clear halo corresponding to the capsule. (E) Only a small number of cells in strain CBS 6490 stained positive for MAb 18B7, showing a punctate pattern around the cell body and stronger intensity in the budding daughter cell. (F and G) Strains CBS 9357 and CBS 9358 did not show any positive staining for MAb 18B7. (H) *C. podzolicus* did not exhibit positive India ink exclusion. The deposition of GXM in *C. amylo lentus* and *C. podzolicus* did not suggest the presence of a capsule similar to that of *C. neoformans*. Scale bar, 10 μ m.

tures and was not induced by the conditions that favored capsular growth in *C. neoformans*. The capsule never reached the dimensions of *C. neoformans* capsules regardless of the conditions tested (Fig. 1A and B). We did not observe a capsule for any strain of *C. podzolicus* under the experimental conditions used in this study (Fig. 1E to G and H; also, see Fig. S3A in the supplemental material).

In the presence of phenolic substrates like L-3,4-dihydroxyphenylalanine (L-DOPA) and bird seed agar, *C. podzolicus* melanized as previously reported (20, 22), although melanization was slower than for *C. neoformans* var. *grubii*. There were strain differences in the time of melanization and intensity of the pigmentation (see Fig. S1A and Table S1 in the supplemental material). No melanization was observed for *C. amylo lentus* at any temperature.

Both *C. podzolicus* and *C. amylo lentus* manifested urease activity similar to that of *C. neoformans* var. *grubii* at 25°C and 28°C, only weak activity at 31°C, and no growth at higher temperatures (see Fig. S1B in the supplemental material).

The extracellular phospholipase B of *C. neoformans* (CNPLB1) contributes to the virulence of this fungus (14). This enzyme has

TABLE 1 PP and PI for cryptococci^a

Fungal strain	2 h			6 h		
	Mean no. of fungi/cell	PP	PI	Mean no. of fungi/cell	PP	PI
<i>C. neoformans</i> H99	0.96	16.99	16.34	1.07	32.37	34.53
<i>C. amyloletus</i> CBS 6039	1.97	46.53	91.67	1.59	31.40	50.00
<i>C. podzolicus</i> CBS 6490	1.85	52.15	96.32	1.57	44.77	70.35
<i>C. podzolicus</i> CBS 9357	1.57	33.65	52.88	1.85	38.40	71.20

^a *A. castellanii* ATCC 30234 was incubated 2 and 6 h with the different cryptococci at a 1:1 ratio. The non-heat-tolerant cryptococci have a higher PP (phagocytic percentage), PI (phagocytic index), and mean number of yeast per amoebae than *C. neoformans* var. *grubii* (H99).

preferential activity for DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), a major mammalian membrane phospholipid and a component of the lung surfactant, and for Lyso-PC (1-palmitoyl-sn-glycero-phosphocholine) (23), a short-lived derivative of phosphatidylcholine that stimulates neutrophils and is a chemoattractant for macrophages (24–26). To test for phospholipid utilization in the non-heat-tolerant cryptococci, they were plated on media supplemented with either a simple triglyceride, DPPC, or Lyso-PC as the carbon source. All fungi manifested lipase activity as measured by growth on tributyrates (see Fig. S2A in the supplemental material). All fungi grew and consumed Lyso-PC at 25°C and 28°C, but only *C. neoformans* var. *grubii* and *C. podzolicus* strain CBS 9358 grew at 31°C (see Fig. S2B). In addition to *C. neoformans*, only *C. podzolicus* strain CBS 9358 grew on DPPC-containing medium and consumed the lipid at 25°C but weakly at 28°C (see Fig. S2C).

***C. neoformans* and *C. amyloletus* are pathogenic in amoebae.** The PC can cause disease in distantly related phyla, such as plants, amoebae, and metazoans (27–29). To ascertain whether this pathogenic capacity extended to other non-heat-tolerant cryptococci, we tested the virulence of *C. amyloletus* and *C. podzolicus* in two model hosts, the free-living amoeba *Acanthamoeba castellanii* and the larvae of the lepidopteran *Galleria mellonella*.

A. castellanii ingested the non-heat-tolerant cryptococcal species more efficiently than *C. neoformans*. There were more amoebae with ingested fungi (phagocytic percentage [PP]) and more intracellular fungi per amoeba (phagocytic index [PI]) 2 h after coincubation. The greater phagocytic activity against *C. amyloletus* and *C. podzolicus* could reflect the absence of a developed capsule, given that the capsule protects against protozoan ingestion (28). However, at 6 h, the PP and the PI diminished and were comparable to those observed with *C. neoformans* var. *grubii* (Table 1).

Next we evaluated the outcome of the interaction of the three cryptococcal species with amoebae after 24 h of coincubation. The amoeba survival was highest in the *C. podzolicus* CBS 9357 group and lowest for *C. neoformans* var. *grubii* (see Table S2 in the supplemental material). Our PP, PI, and amoeba killing results for *C. neoformans* var. *grubii* were similar to what was previously reported (28). For the interaction between the non-heat-tolerant cryptococci with *A. castellanii*, we found that under nutrient-rich conditions, not only was *C. amyloletus* able to survive in the presence of amoebae, but it also grew better than in rich medium only. However, the outcome of this interaction was very different when the experiment was carried out in starvation. If *C. amyloletus* was coincubated with *A. castellanii* in phosphate-buffered saline (PBS), no CFU were recovered after 24 h (Fig. 2). In other words, *C. amyloletus* was able to survive as well as *C. neoformans*

in the presence of amoebae if there were nutrients in the medium. However, unlike *C. neoformans*, *C. amyloletus* was unable to grow or even survive the amoeba interaction under stress conditions and could not efficiently kill the amoebae or use them as a nutrient source.

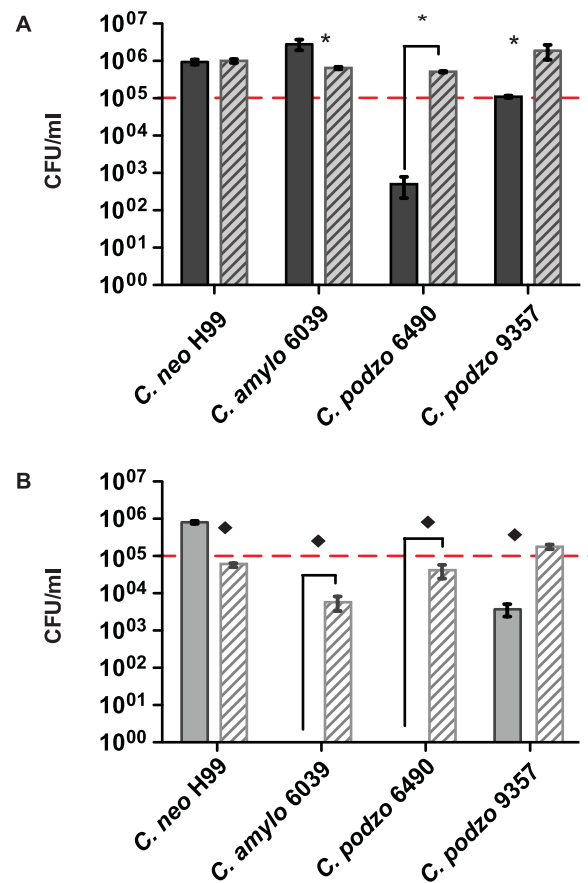


FIG 2 Fungal survival after the interaction with *A. castellanii*. Solid bars correspond to fungi coincubated with amoebae. Striped bars correspond to fungi alone. Dotted lines indicate the initial number of fungal CFUs used to inoculate each well. (A) Fungi and amoebae incubated in rich amoeba medium (PYG). In the presence of amoebae but without other stress conditions in the system, *C. neoformans* var. *grubii* (H99) and *C. amyloletus* (CBS 6039) were able to grow; *C. podzolicus*, on the other hand, was not able to grow and in the case of strain CBS 6490 was efficiently killed by the amoebae. (B) Fungi and amoebae incubated in PBS. *C. neoformans* var. *grubii* was able to grow feeding on amoebae. *C. amyloletus* was highly susceptible to nutrient deprivation and in the presence of amoebae was completely cleared. *C. podzolicus* CBS 6490 was cleared by amoebae, whereas strain CBS 9357 diminished but survived. * and ♦, $P < 0.05$.

TABLE 2 Mean virulence rate as death outcome

Temp (°C)	Mean (SD) ViRaDO at indicated inoculum ^a					
	5 × 10 ⁴	5 × 10 ⁵	5 × 10 ⁶	5 × 10 ⁴	5 × 10 ⁵	5 × 10 ⁶
	<i>C. neoformans</i> H99			<i>C. amyloletus</i> CBS 6039		
25	17.65 (4.24)	39.71 (15.32)	78.68 (6.02)	2.33 (1.53)	30.76 (7.45)	84.51 (10.04)
28	34.81 (3.86)	40.43 (11.52)	71.57 (8.09)	2.47 (2.16)	10.99 (4.07)	77.06 (8.38)
30	68.28 (6.29)	73.90 (4.09)	83.21 (1.39)	5.51 (4.78)	6.86 (5.86)	41.93 (19.29)
32	63.11 (7.47)	70.47 (3.59)	82.05 (2.05)	0.20 (0.28)	14.37 (0.99)	25.55 (8.06)
35	74.62 (5.90)	82.72 (6.18)	86.07 (7.23)	21.25 (10.11)	28.72 (13.58)	38.06 (13.76)
	<i>C. podzolicus</i> CBS 6490			<i>C. podzolicus</i> CBS 9357		
25	5.34 (3.66)	1.72 (1.49)	5.64 (2.45)	1.72 (1.49)	1.10 (1.33)	7.72 (2.21)
28	3.05 (2.77)	1.70 (2.94)	27.75 (7.13)	1.11 (1.92)	11.36 (2.73)	36.95 (4.70)
30	10.05 (14.24)	4.44 (6.39)	34.30 (19.01)	16.05 (1.56)	5.08 (2.06)	22.79 (12.28)
32	6.89 (1.94)	8.97 (4.47)	6.41 (1.82)	15.44 (13.52)	7.24 (1.92)	14.72 (2.58)
35	35.82 (7.99)	33.06 (4.61)	45.97 (4.81)	38.06 (20.09)	38.33 (8.55)	31.81 (7.57)
	<i>C. podzolicus</i> CBS 9358			NI	PBS	
25	0.49 (0.42)	5.15 (2.94)	9.80 (11.54)	6.50 (6.43)	6.50 (8.14)	
28	11.19 (9.76)	8.11 (5.99)	33.93 (9.90)	1.43 (1.43)	1.60 (2.37)	
30	10.05 (7.71)	6.13 (6.46)	17.72 (6.98)	3.98 (1.05)	6.37 (2.09)	
32	6.86 (4.94)	18.19 (0.24)	24.92 (5.09)	3.14 (2.84)	2.52 (2.20)	
35	20.42 (25.32)	20.69 (2.65)	19.31 (10.34)	21.45 (13.56)	26.94 (12.51)	

^a The mean virulence rate as death outcome (ViRaDO) and SD were obtained from three independent experiments for each condition (inoculum and temperature).

In summary, *C. amyloletus* was readily phagocytized by amoebae, but the drop in the PI and PP after 6 h, which was accompanied with an increase in CFU counting at 24 h, indicated that this fungus was able to survive the interaction either by avoiding intracellular killing or by rapid replication in the presence of amoebae. In contrast, *C. podzolicus* CBS 6490 was highly susceptible to amoeba killing under both nutrient-rich and under nutrient-depleted conditions, and *C. podzolicus* strain CBS 9357 persisted in the presence of amoebae, suggesting that this strain had significant resistance to amoeba predation (Fig. 2).

Pathogenicity for *G. mellonella* larvae. To ascertain the pathogenic potential of *C. amyloletus* and *C. podzolicus*, we used the *G. mellonella* larva model (30). We studied the virulence of the three different cryptococcal species as a function of inoculum size and temperature. Since *G. mellonella* is an ectothermic organism, we controlled its body temperature by controlling the ambient experimental temperature and compared the effect of temperature changes in the survival of infected larvae. In this series of experiments, we tested three different inocula and five different temperatures. The lowest was the optimal growing temperature for non-heat-tolerant cryptococci, and the maximum was not permissive for growth of non-heat-tolerant fungi (data not shown).

To measure the relative virulence of the different fungi studied and to better analyze the large amount of data generated, we developed a parameter named “virulence rate as death outcome” (ViRaDO). Our function measures the interaction of a host-microorganism pair resulting in death of the host, extracting the data from the survival proportions in survival curves. To develop the ViRaDO concept, we used the population biology definition of virulence, namely, increased host mortality due to pathogen infection (31). The ViRaDO allowed us to evaluate the pathogenic capability of the different cryptococci against *G. mellonella* larvae and to compile the data obtained in different *G. mellonella* survival assays to analyze the outcome of a given interaction under different conditions.

We observed that for *C. neoformans* var. *grubii*, the virulence increased as a function of the temperature. For *C. neoformans* var. *grubii*, the effect of inoculum size on the outcome of infection was less dramatic than that of temperature (Table 2; Fig. 3A). Faster replication could account for greater virulence of *C. neoformans* var. *grubii* at higher temperatures, and this could explain the higher virulence at 32°C than at 25 to 30°C but not at 35°C and above, since replication slows above 32°C. The increased virulence of *C. neoformans* var. *grubii* at temperatures higher than the optimal growing temperature could be a consequence of an increased stress response in the fungus, making it more fitted to survive in hostile conditions (32).

Virulence in the non-heat-tolerant cryptococci was dependent of the number of inoculated organisms and the experimental temperature. *C. amyloletus* was the most virulent for *G. mellonella* larvae, manifesting a bimodal pattern. At 25°C and 28°C, *C. amyloletus* was highly pathogenic for *G. mellonella* larvae, but only at the highest inoculum, 5 × 10⁶ CFU/larva (Fig. 3B; Table 2). At 30°C and 32°C, the virulence decreased. At 35°C, there was an increase in larval mortality in the three inoculation groups (Fig. 3B; Table 2). At 25°C, the killing curves of *C. neoformans* var. *grubii* and *C. amyloletus* were not statistically different for 5 × 10⁶ CFU/larva (Fig. 4).

In the case of *C. podzolicus*, we observed that its capability to kill larvae was strain dependent, showing peaks of virulence at 28°C and 35°C (Fig. 3C and D; Table 2). The overall virulence of *C. podzolicus* was low compared to that of the other cryptococci. At 25°C larval mortality was no higher than in the controls. The slight increase in larval mortality infected with *C. podzolicus* at 35°C can be partially attributed to a basal increase in mortality in untreated larvae and PBS-injected larvae (Fig. 3E). The results suggested that heat stress contributed to increased host susceptibility for infection. To rule out the possibility that larval deaths were caused by the large amount of yeast inoculated into the hemocoel, we tested the effect of injecting heat-killed yeast on larva survival for all strains and found no evidence of reduced

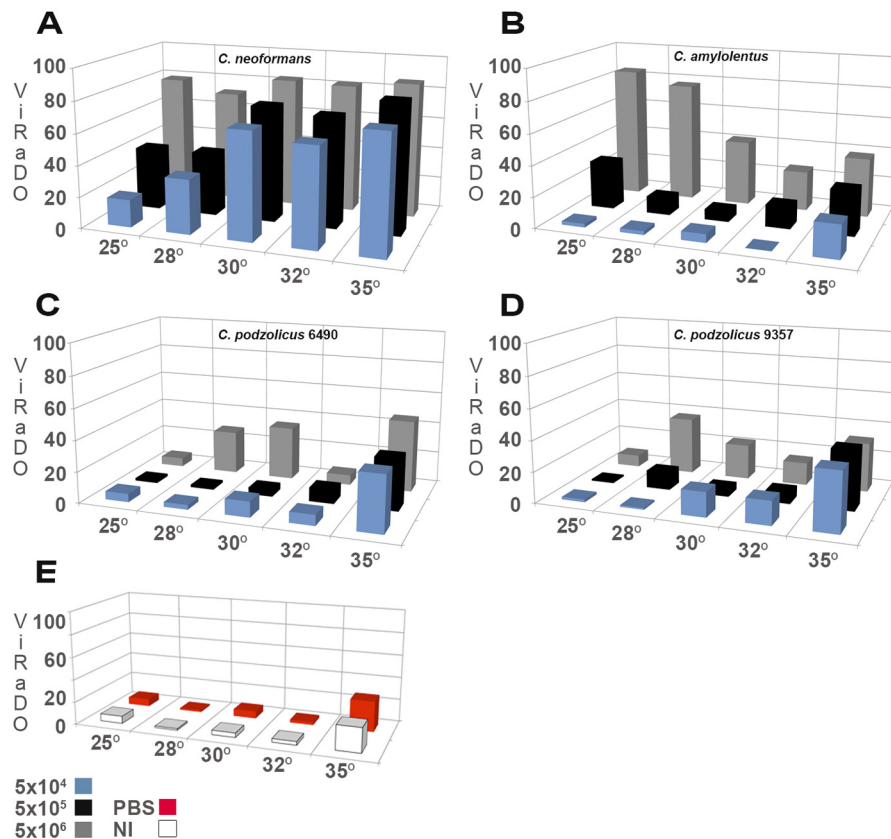


FIG 3 Virulence as death outcome. 3D graphic comparison of three inocula and 5 temperatures for the fungi used. (A) The *C. neoformans* var. *grubii* curve shows that the ViRaDO increased as a function of inoculum and temperature. (B) *C. amyloletus* had a bimodal pattern. At 25°C and 28°C, *C. amyloletus* was highly pathogenic for *G. mellonella* larvae but only at the highest inoculum, 5×10^6 CFU/larva. At 30°C and 32°C, the virulence decreased for the three different tested inocula. At 35°C there is an increase in larval mortality in the three inoculation groups. (C) *C. podzolicus* CBS 6490 showed an increased virulence as a function of the temperature up to 30°C, and at 32°C, the fungus virulence decreased, followed by an increase at 35°C. (D) *C. podzolicus* CBS 9357 manifested its greatest virulence at 28°C. (E) Noninjected (NI) and PBS-injected larva controls.

survival even when 5×10^6 yeast cells/larva were given (data not shown).

Virulence displays a deterministic behavior. *G. mellonella* larvae are a well-validated metazoan host model to study *C. neoformans* infection (30). In the present study, we used this model to analyze whether virulence followed a predictable or a nonpredictable pattern. For this analysis, we infected the larvae as described above and followed the survival of 240 individuals at 12-h intervals, in a time series over ca. 100 concatenated days. To determine the dynamic temporal behavior of our virulence model, we calculated the autocorrelation, $R_{MM}(t)$, and the rate of accumulated events (deaths), $Ac(t)$, of the concatenated time series (18, 33). We found that under the tested conditions, virulence followed a predictable pattern, suggesting that virulence may be a deterministic process explained by the periodic pattern of both the autocorrelation function, $R_{MM}(t)$, and the accumulated events, $Ac(t)$ (Fig. 5). Our analysis showed no indication of chaos.

DISCUSSION

In this study, we tested the idea of virulence as an emergent property by evaluating the virulence of related species that differed in virulence factors. Consistent with the notion that virulence is an emergent property (1), measures of virulence such as mortality were not predictable, since microbes that do not possess any ob-

vious attributes of virulence were highly virulent in a suitable host, while those that express known virulence factors were avirulent. In contrast to unpredictability of a virulent phenotype, our results showed that virulence in an infected population follows a deterministic pattern. These results showed no evidence of the chaotic behavior found in other biological systems, such as prey-predator interactions (34), and the transmission of human diseases like measles (35), suggesting that the outcome of the host-pathogen interaction in our system was not very sensitive to the initial conditions. Our results implied that most of the variation could be attributed to noise present in the system due to host genetic heterogeneity, small variations in inoculum preparation, and differences in the general health of the larvae but that this variation does not affect the determinism of the system. To our knowledge, this is the first study that specifically attempts to distinguish between noisy periodicity and chaos in the outcome of a host-pathogen interaction, to gain a better understanding of virulence dynamics.

The mechanism by which environmental fungi evolve the necessary traits to become facultative human pathogens is a fundamental question of microbial pathogenesis. The traits involved in mammalian virulence have been proposed to have emerged from complex selective pressures encountered by the PC in their natural habitat (36). Due to the similar interactions between phagocytic

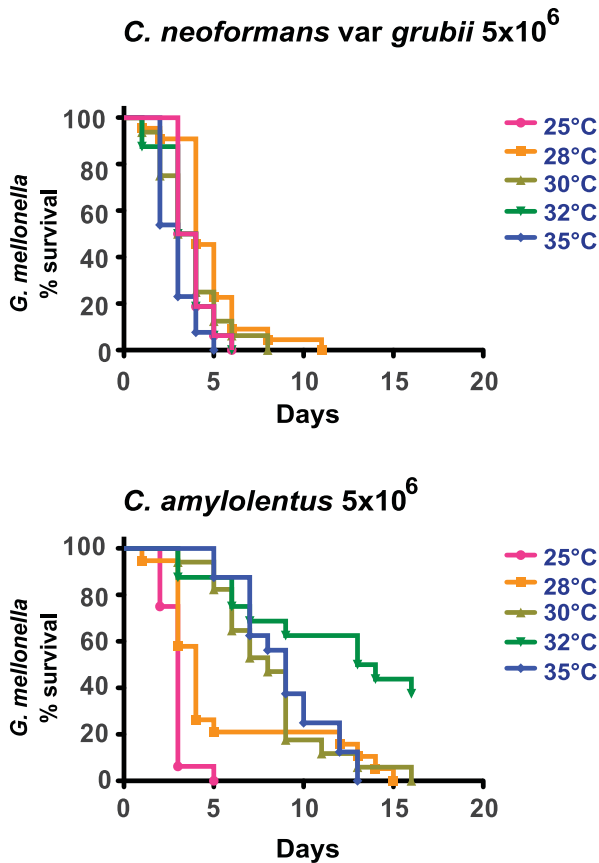


FIG 4 *G. mellonella* survival after infection with cryptococcal spp. Larvae were infected with 5×10^6 CFU of *C. neoformans* var. *grubii* (H99) (top) or *C. amyloletus* (bottom). Survival experiments were performed at a range of temperatures from 25 to 35°C. The killing curve at 25°C showed no statistical difference ($P = 0.8507$) for larvae infected with *C. neoformans* var. *grubii* or *C. amyloletus*.

soil amoebae and mammalian macrophages, it was proposed that one of the selective forces that have shaped the pathogenic phenotype of these organisms is the predation pressure exerted by soil dwellers such as amoebae or small metazoans like nematodes (28, 29, 36, 37).

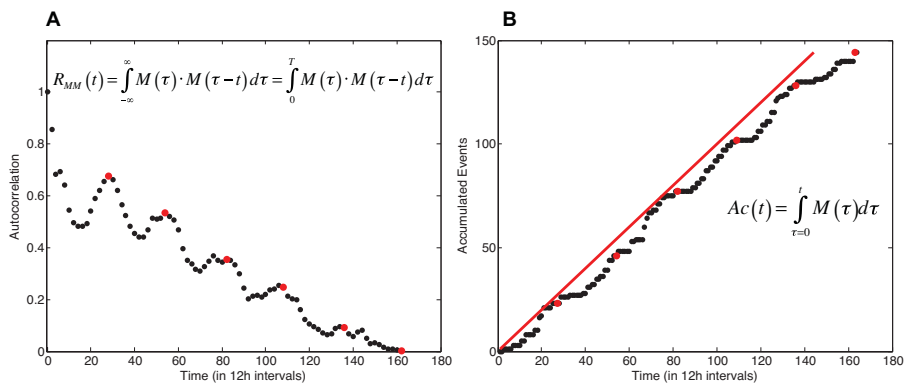


FIG 5 Virulence has a predictable periodicity. (A) The autocorrelation function plot, $R_{MM}(t)$, shows a periodic pattern over time (red dots). (B) The accumulated-events function, $Ac(t)$, also shows a periodic shape (red dots) and runs parallel to the identity equation ($y = x$; red line), demonstrating its predictability. These results suggest the deterministic and predictable nature of virulence in our host-pathogen model.

This hypothesis has found support in other systems. For example, for the bacterium *Escherichia coli* O175:H7, the Shiga toxin VF improves its survival in the presence of grazing *Tetrahymena* (38), demonstrating that a trait that may have been selected for bacterial survival in its natural ecological niche can function as a virulence factor in mammals. Both *C. neoformans* and *C. gattii* can also be pathogenic to plants (27), and considering that fungus-plant interactions for the PC clade seem to be ancestral, the adaptation for plant colonization might also have played a role in the acquisition of the virulence composite.

Our results with *C. amyloletus* suggest that certain members of the *Filobasidiella* clade already had the potential for metazoan pathogenicity before the split of the PC species complex about 24.5 million years ago (9, 39). In fact, the similarities in pathogenicity between *C. amyloletus* and *C. neoformans* at ambient temperatures suggest considerable virulence potential for the former, such that this species may be avirulent for mammals by virtue of its thermal intolerance (40). Consequently, one might infer that the key event in the mammalian pathogenicity of the PC species is its ability for heat tolerance, a trait that is not present in the other members of the *Filobasidiella* clade. Although it is not clear how heat tolerance has evolved, it has been proposed that extended exposure to high environmental temperature is important for the evolution and maintenance of this capacity in *C. neoformans* (41). Heat adaptation occurs as a trade-off between good performance of an organism across a range of different temperatures and fitness loss (42). If the ecological niche of the PC involved an environment with sustained high mean yearly temperatures then one can imagine a scenario whereby a gain in heat resistance compared with related species inhabiting cooler niches could have driven the early evolution of the PC clade. Recent studies suggesting that *C. neoformans* var. *grubii* originated from southern Africa (43, 44) in tropical/subtropical regions are consistent with this notion.

Our work demonstrated that the experimental temperature was a critical parameter for defining the pathogenicity of two members of the *Filobasidiella* clade, *C. amyloletus* and the human pathogen *C. neoformans* var. *grubii*. Previously, it was reported that the virulence of *C. neoformans* in the *G. mellonella* larva model was increased at 37°C relative to 30°C (30). *C. amyloletus* was highly pathogenic for *G. mellonella* larvae despite the absence of clearly identified virulence factors. At its optimal growing temper-

ature, its pathogenicity was comparable to that of *C. neoformans*. The pathogenic potential of *C. amyloletus* was manifested in a narrow window between optimal growing conditions versus thermal stress. In a previous study using an intermediate inoculum, it was reported that at 24°C *C. amyloletus* had intermediate virulence against *G. mellonella*, but this observation was inconsistent between experiments (9). In addition to the findings obtained with the *G. mellonella* infection model, challenging *C. amyloletus* with the soil amoeba *A. castellanii* showed that this fungus was highly susceptible to nutrient deprivation and stress. Under nutrient-rich conditions, *C. amyloletus* was able to grow regardless of the presence of the amoebae. However, under nutrient deprivation, it was not possible to recover the fungus, suggesting that the amoebae consumed the ingested fungi. These results indicate that even if *C. amyloletus* and *C. neoformans* share similar virulent capacities for the moth system, the former is more susceptible to environmental conditions and to stress, perhaps indicating the different lifestyle choices of the two fungi.

For *C. podzolicus* the presence of factors associated with virulence shared with *C. neoformans*, like melanization and phospholipid utilization, did not correlate with virulence. The PC capsule is known to function in avoiding phagocytosis by organisms of distantly related phyla, such as amoebae, invertebrates, and mammals (28, 45). However, capsule-independent mechanisms to avoid phagocytosis have recently been described (46). The presence of other strategies to avoid phagocytosis and the finding that clinical isolates of virulent *C. neoformans* can be capsule deficient (47) suggest that the capsule is important, even if not absolutely required, for pathogenesis. We found that *C. amyloletus* bears a small capsule but that this fungus does not respond to the capsule-inducing conditions that are effective for the PC.

In this study, we developed a new parameter to quantify virulence as a function of host death, ViRaDO, which allowed easier comparison of large amounts of data from survival studies. This parameter could be useful in studies where comparison of several survival experiments is necessary. Virulence is a complex phenotype, and for the purposes of our study, we used host death as the quantifiable outcome while also considering time of infection. Given that many systems, from weather to prey-predator interactions, exhibit chaotic behavior, we considered whether the same quality was present for virulence in our studied system. The study of the dynamics of host-microbe interactions poses significant challenges because each host-microbe interaction is unique, and it is difficult if not impossible to determine that a system exhibits chaotic dynamics by designing experiments where initial conditions are systematically varied. Here, we took a simple yet powerful approach to search for chaotic signatures in our experimental host-pathogen system. We found no evidence of chaotic behavior in the virulence outcome of the *C. neoformans*-*G. mellonella* interaction. Instead, we found that the system was deterministic and the observed variation can be attributed to random events such as differences in experimental infection and/or genetic heterogeneity of the insect host. The finding that in certain systems virulence has deterministic features that lack chaotic signatures, i.e., that the system is not highly sensitive to small variations in the initial conditions, could help explain humanity's success in controlling various infectious diseases through vaccines, sanitation, and therapeutic drugs. Whether determinism is a general feature of virulence in host-pathogen interactions is a question whose answer must await the completion of other studies addressing simi-

lar questions with other host-microbe systems. The method used here to study the dynamics of virulence should be applicable to other host-microbe interactions.

In conclusion, our results show that species that are not traditionally considered pathogenic can be pathogens given suitable conditions and suitable hosts. Furthermore, our findings are consistent with the notion that human activities, such as climate disruption, in particular global warming (48), and its consequent selection for greater thermal tolerance (49) in conjunction with fungal adaptability can favor the emergence of new fungal pathogens. Our work shows that the presence of so-called "canonical" virulence factors such as a capsule, melanization, urease activity, and phospholipid consumption is important to explain virulence in some hosts but not predictive of pathogenicity of an organism. These results are consistent with the notion that virulence is an emergent property that cannot be easily predicted or fully explained by studying its component parts (1). The results suggest the existence of epistemological limits on our ability to explain and predict the emergence of virulence and new pathogenic microbes.

MATERIALS AND METHODS

Species, strains, and media used in this work. *C. neoformans* var. *grubii* strain H99 was obtained from John Perfect (Durham, NC); *C. neoformans* var. *neoformans* Cap67 (acapsular strain) ATCC 52817 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA), *C. podzolicus* strain CBS 6490 was obtained from K. J. Kwon-Chung (Bethesda, MD); *C. amyloletus* CBS 6039 and *C. podzolicus* strains CBS 9357 and CBS 9358 were obtained from the CBS-KNAW Fungal Biodiversity Centre (Utrecht, Netherlands). Fungi were grown on YPD broth or agar (1% yeast extract, 2% peptone, 2% dextrose; Becton Dickinson) at 25°C, 28°C, or 31°C depending on the experiment. *A. castellanii* strain ATCC 30234 was acquired from the ATCC. The amoebae were cultured at 28°C in peptone-yeast extract-glucose broth (PYG ATCC medium 712) as previously described (50).

Phenotypic analysis of known virulence traits in *C. neoformans*. The optimal growth temperature for each of the used strains was determined using a BioScreen analyzer (Growth Curves USA) at 25°C, 28°C, 30°C, 32°C, and 35°C; absorbance was measured every 30 min at 600 nm.

To search for the presence of known cryptococcal virulence traits in *C. podzolicus* and *C. amyloletus* we evaluated melanization on L-DOPA minimal medium (MM) and bird seed plates (Becton Dickinson), urease activity on Christensen's medium (Becton Dickinson), extracellular lipase activity using simple triglycerides (tributyrin) or phospholipids (Lyso-PC [1-palmitoyl-sn-glycero-phosphocholine] [200 μM] and DPPC [1,2-dipalmitoyl-sn-glycero-3-phosphocholine] [800 μM; Sigma-Aldrich]) on spirit blue agar (Becton Dickinson), and capsule production by growing the cells in capsule induction medium (9), MM, or YPD, followed by India ink staining, electron microscopy, and immunofluorescence assays.

Immunofluorescence assays were performed on 4-day-old cells grown at either 25°C or 31°C using the monoclonal antibody (MAb) 18B7, which binds to *C. neoformans* glucuronoxylomannan (GXM), following published protocols (51, 52).

Electron microscopy. Samples were fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 2% uranyl acetate, dehydrated through a graded series of ethanol, and embedded in LX112 resin (Ladd Research Industries, Burlington, VT). Ultrathin sections were cut on a Reichert Ultracut UCT microtome, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

***Acanthamoeba castellanii* phagocytosis assay.** *A. castellanii* cells were plated in 96-well plates at 1×10^4 cells/well and incubated for 24 h at 28°C. After 24 h, inocula were prepared from fresh fungal cultures washed

three times in phosphate-buffered saline (PBS), resuspended in PBS, and added to the amoeba culture at a multiplicity of infection (MOI) of 10. Fungal cells were then incubated with *A. castellanii* at 28°C for various times. Following incubation, the plates were stained with Giemsa, and 100 cells per condition were counted to determine the amount of internalized fungi. Phagocytic index and phagocytic percentage were calculated for each condition. Phagocytic percentage (PP) is the percentage of cells containing fungi; phagocytic index (PI) is the percentage of cells containing at least one yeast multiplied by the mean number of yeasts per positive cell.

Fungal survival assay after *A. castellanii*-fungus coincubation.

Amoebae and fungi were prepared as described for the phagocytosis assay, with the following differences. Fungi were plated at a MOI of 1. The amoebae and fungi were cocultured in either PYG or PBS for 24 h. After 24 h, the entire contents of each well was passed 6 times through a 27-gauge needle to disrupt the amoebae, and the viability of extracellular and internalized fungi was evaluated by plating serial dilutions in duplicate YPD plates which were incubated at 28°C for 48 h and counting the CFU in each plate. Each experimental group was tested in triplicate.

Amoeba survival. The percentage of viable *A. castellanii* cells from the plate used in the fungal survival assay was determined after 24 h interaction with the different fungi at a MOI of 1. Amoeba viability was determined by an exclusion assay using 1:10 trypan blue.

***Galleria mellonella* survival assays.** To analyze the virulence of the different fungi in *G. mellonella*, we followed a previously described protocol (30, 53). Briefly, fungal inocula were prepared by growing the different fungi at 25°C, 28°C, or 30°C overnight for *C. neoformans* H99 or for 48 h for the other species (growing above 28°C); for the 32°C and 35°C experiments, fungi were grown at 30°C. Yeasts were collected by centrifugation, washed twice, and resuspended with PBS. Last-instar *G. mellonella* larvae (Vanderhorst Wholesale, OH) were injected with a 10- μ l Hamilton syringe in one of the prolegs of the fifth abdominal segment with either 5×10^4 , 5×10^5 , or 5×10^6 CFU/larva or PBS. Each group contained 16 randomly allocated larvae weighing between 200 and 400 mg. Larvae were kept at either 25, 28, 30, 32, or 35°C. Larval survival was monitored and recorded daily. Survival curves were plotted using Prism software to create Kaplan-Meier survival curves (version 5.0d; Prism Computational Sciences, Inc., Madison, WI). For curve comparison, we used the Mantel-Cox test; a *P* value of ≤ 0.05 was considered significant. Each experimental condition was tested at least three independent times.

Virulence quantification. For a quantitative value of the virulence suitable for comparisons between experiments, we developed a normalized method to quantify the data obtained from survival curves. The ViRaDO (virulence rate as death outcome) is measured as the rate of death as the observed outcome. It is a weighted function of the daily death rate of the experiment:

$$\text{ViRaDO} = \frac{1}{D} \cdot \sum_{i=1}^D w_i \cdot \tau^i$$

where $w_i = (D - i + 1)$ is the weight at day i , defined as the number of days remaining from day i until the end of experiment, $D =$ length of the experiment, and $\tau^i =$ number of deaths on day i /total number of larvae, the daily death rate. The ViRaDO varies between 0 and 1, where 0 corresponds to no deaths during the length of the experiment and 1 corresponds to 100% mortality by day 1. Therefore, the ViRaDO is inversely proportional to the mean survival.

Determination of virulence pattern. Larvae were infected as described above using 5×10^4 CFU/larva and 40 larvae per group, with 6 repetitions. Larvae were kept at 25°C for 17 days, and mortality was measured every 12 h. To determine the correlation pattern of our virulence model throughout the time series, we performed an autocorrelation analysis on $M(t)$, where $M(t)$ is the concatenated time series of the six survival experiments, defined as death events per period of time (t). Let $R_{MM}(t)$ be the autocorrelation of $M(t)$:

$$R_{MM}(t) = \int_{-\infty}^{\infty} M(\tau) \cdot M(\tau - t) d\tau = \int_0^T M(\tau) \cdot M(\tau - t) d\tau$$

Let $Ac(t)$ be the total accumulated number of deaths at time t :

$$Ac(t) = \int_{\tau=0}^t M(\tau) d\tau$$

Both $R_{MM}(t)$ and $Ac(t)$ characterized the behavior of the distribution pattern of the survival series over time. An in-house program in Matlab (MathWorks, Natick, MA) was used to calculate both $R_{MM}(t)$ and $Ac(t)$ functions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00100-13/-/DCSupplemental>.

Figure S1, TIFF file, 2.6 MB.

Figure S2, TIFF file, 2.6 MB.

Figure S3, TIFF file, 2.6 MB.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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