EUKARYOTIC CELL, June 2007, p. 949–959 Vol. 6, No. 6
1535-9778/07/$08.00+0 doi:10.1128/EC.00097-07

Cryptococcus neoformans Mates on Pigeon Guano: Implications for the Realized Ecological Niche and Globalization▽

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Received 27 March 2007/Accepted 6 April 2007

The ecological niche that a species can occupy is determined by its resource requirements and the physical conditions necessary for survival. The niche to which an organism is most highly adapted is the realized niche, whereas the complete range of habitats that an organism can occupy represents the fundamental niche. The growth and development of *Cryptococcus neoformans* and *Cryptococcus gattii* on pigeon guano were examined to determine whether these two species occupy the same or different ecological niches. *C. neoformans* is a cosmopolitan pathogenic yeast that infects predominantly immunocompromised individuals, exists in two varieties (*grubii* [serotype A] and *neoformans* [serotype D]), and is commonly isolated from pigeon guano worldwide. By contrast, *C. gattii* often infects immunocompetent individuals and is associated with geographically restricted environments, most notably, eucalyptus trees. Pigeon guano supported the growth of both species, and a brown pigment related to melanin, a key virulence factor, was produced. *C. neoformans* exhibited prolific mating on pigeon guano, whereas *C. gattii* did not. The observations that *C. neoformans* completes the life cycle on pigeon guano but that *C. gattii* does not indicates that pigeon guano could represent the realized ecological niche for *C. neoformans*. Because *C. gattii* grows on pigeon guano but cannot sexually reproduce, pigeon guano represents a fundamental but not a realized niche for *C. gattii*. Based on these studies, we hypothesize that an ancestral *Cryptococcus* strain gained the ability to sexually reproduce in pigeon guano and then swept the globe.

Emerging infectious diseases are those that have newly appeared in a population or geographic range (60). One route to understand and ultimately prevent these diseases is to define the specific factors precipitating their emergence. Responsible factors can include ecological changes, human demographic alterations, travel and commerce, technology, microbial adaptation, and breakdown of public health measures (35, 37, 81). Expansion of the pathogen's geographic range can also result in disease outbreaks, such as the ongoing outbreak of the fungal pathogen *Cryptococcus gattii* on Vancouver Island (VI) in British Columbia, Canada (28).

Most infections are caused by pathogens already present in the environment that gain a selective advantage by changing conditions or have an opportunity to infect new hosts (61). For example, Legionnaires' disease is caused by the intracellular bacterium *Legionella pneumophila*, which colonizes amoebae and, when present in cooling towers, is exposed to and infects humans (23, 77). Cooling towers thus provide a "man-made" reservoir for *L. pneumophila* growth that simulates the ecological niche of *L. pneumophila*—ponds where amoebae are readily available to sustain the organism's reproduction and survival (2, 3). Thus, when studying human infections acquired from environmental sources, knowledge of not only the infecting reservoir but also the natural ecology and life cycle of the microorganism is important.

Certain combinations of environmental conditions are necessary for species to tolerate the physical environment, obtain energy and nutrients, and evade predators. The total requirements of a species for resources and physical conditions determine its abundance and distribution in nature. In ecology, these requirements govern the niche for a species or population in an ecosystem. More formally, the niche includes how the population responds to available resources and competitors and establishes the organism's life history, habitat, and place in the food chain. However, according to the competitive exclusion principle, no two species can occupy the same niche in the same environment for a prolonged period, which has resulted in a distinction between fundamental and realized niches (32). The full range of environmental conditions (biological and physical) under which an organism can exist defines its fundamental niche. However, as a result of pressure from interactions with other organisms, as well as changes in the environment, species are usually forced to occupy a niche that is narrower than the fundamental niche. This is termed the realized niche and represents the environment to which a species becomes most highly adapted.

Cryptococcus neoformans and the closely related species *Cryptococcus gattii* are human fungal pathogens. Humans are thought to be exposed by inhalation of basidiospores, which are small enough to lodge in the alveoli of the lung (78). The organism can then spread from the lungs to the central nervous system to cause meningoencephalitis (11, 33, 47). *C. neoformans* occurs in two varieties—*grubii* (serotype A) and *neoformans* (serotype D)—and diverged from *C. gattii* ~40 million years ago (11, 90). The *grubii* and *neoformans* varieties have different disease epidemiologies, with var. *grubii* causing the vast majority of cryptococcosis worldwide (11, 83). While the *C. neoformans* varieties are cosmopolitan and cause disease

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▽ Published ahead of print on 20 April 2007.

predominantly in immunocompromised individuals, *C. gattii* is found predominantly in tropical regions and frequently causes disease in individuals with no known immune deficiency (33). An outbreak of *C. gattii* is currently ongoing in British Columbia (28).

A complete life cycle, including a sexual cycle, has been described for both *Cryptococcus* species (21, 38, 43, 44, 50). The sexual cycle was first described for var. *neoformans*, and early studies examining genetic virulence determinants were conducted with this variety (43, 45). The sexual cycle for the more commonly pathogenic variety, *grubii*, has recently been characterized and applied to define virulence characteristics (38, 57, 62, 63, 65). While *C. gattii* mating had been identified 30 years ago, evidence of recombination has only recently been shown in the Australian Northern Territory, which may have played a role in the cryptococcosis outbreak on VI (9, 10, 20, 21, 44).

Over the past 2 decades, *C. neoformans* infections have increased in prevalence as the population of immunocompromised individuals expanded due to the AIDS pandemic, aggressive cancer therapy, and organ transplantation. The sporadic nature of human cryptococcosis and rarity of documented human-to-human transmission indicate that infection is acquired from the environment (11). Pigeon guano is a common source for infectious propagules of *C. neoformans* and is postulated to play a central role in transmission from the environment to humans (11, 15, 22, 27, 29, 39, 40, 52, 70, 72, 76, 79, 80, 91, 92). *C. neoformans* can readily be isolated from pigeon guano and has been shown to grow and mate on medium containing pigeon guano (31, 73–75, 85). The closely related species *C. gattii* is not isolated from pigeon guano and is instead associated with various tree species (13, 14, 71). The different environmental sources of these two species has led to the hypothesis that *C. neoformans* is ubiquitous in the environment due to dissemination by pigeons following migratory and trade routes and that *C. gattii* is restricted to tropical/subtropical regions because it is not associated with pigeons. If this is the case, then how has the outbreak of *C. gattii* developed in the Pacific Northwest? Two highly related strains have been identified on VI, both of which are associated with soil and various tree species (41). Furthermore, the major genotype has been found only in the Pacific Northwest, leading to the hypothesis that this new strain has gained the ability to proliferate in a temperate environment and/or is highly virulent.

This study characterizes the growth of *C. neoformans* and *C. gattii* strains on pigeon guano so that we may understand factors influencing species survival. We show that both species are capable of growth on pigeon guano. Moreover, *C. neoformans* undergoes robust sexual reproduction on pigeon guano, whereas *C. gattii* does not. These results provide evidence that pigeon guano could be the realized niche for *C. neoformans* and highlight why it is not a preferred ecological niche for *C. gattii*. These results also illuminate a possible explanation for why *C. neoformans* is cosmopolitan and *C. gattii* is geographically restricted. The implications of these studies for the emergence of global infectious diseases are considered.

MATERIALS AND METHODS

Strains and media. Strains used in this study are listed in Table 1. Strains were grown on yeast peptone dextrose (YPD) prior to transfer to other medium types.

TABLE 1. Strains used in this study

Species	Strain	Genotype	Reference
C. neoformans var. *grubii*	H99	*MAT*α	84
	KN99**a**	*MAT***a**	63
	KN99α	*MAT*α	63
	YSB119	*MAT*α *NAT*	4
	YSB121	*MAT***a** *NEO*	4
	F99	*MAT*α *ura5*	87
	MO49	*MAT*α *ade2*	84
	ST303B12	*MAT*α *arg3*	34
	ST225B9	*MAT*α *pro2*	34
	ST232C4	*MAT*α *arg8*	34
	MDC16	*MAT*α *lac1*	68
	RCP26	*MAT*α *lac2*	68
	RCP29	*MAT*α *lac1 lac2*	68
	KN119/21-1	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN119/21-2	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN119/21-3	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN119/21-4	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN119/21-5	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN119/21-6	*MAT*α/*MAT***a** *NAT NEO*	This paper
C. neoformans var. *neoformans*	JEC21	*MAT*α	45
	JEC20	*MAT***a**	45
	XL405	*MAT*α *hxk2*::NEO	This paper
	XL465	*MAT***a** *gsy1*::*NAT*	46
	JEC31	*MAT*α *lys1*	59
	JEC33	*MAT*α *lys2*	59
	JEC38	*MAT*α *met1*	59
	JEC43	*MAT*α *ura5*	59
	JEC47	*MAT*α *arg1*	59
	JEC50	*MAT*α *ade2*	59
	KN405/465-1	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN405/465-2	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN405/465-3	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN405/465-4	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN405/465-5	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN405/465-6	*MAT*α/*MAT***a** *NAT NEO*	This paper
C. gattii	NIH312	*MAT*α	21
	B4546	*MAT***a**	21
	R265	*MAT*α	21
	JF65	*MAT*α *NAT*	21
	JF66	*MAT***a** *NEO ura5*	21
	KN65/66-1	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN65/66-2	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN65/66-3	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN65/66-4	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN65/66-5	*MAT*α/*MAT***a** *NAT NEO*	This paper
	KN65/66-6	*MAT*α/*MAT***a** *NAT NEO*	This paper
Candida albicans	SC5314	*MTL*α/*MTL***a**	19

Yeast nitrogen base (YNB) minimal medium, V8 (pH 5.0 and pH 7.0) medium, and Murashige and Skoog (MS) medium were as described previously (63).

Guano media. Pigeon guano was collected in Durham, NC, at the intersection of state road NC147 and interstate highway 40 (35°53′51.37″N, 78°52′34.34″W), where pigeons were observed continuously roosting for 3 years. "Contaminated" soil was taken from the same area as the pure pigeon guano after the guano had been removed. "Uncontaminated" soil was collected 10 m from the pure pigeon guano samples, where no guano was observed. Desert bat, dry-bar cave bat, fossilized seabird, and original seabird guanos were purchased from Guano Co. International, Inc. Guano or soil was ground to a fine powder using a coffee grinder. Guano (2.5%, 12%, 25% [wt/vol]) or soil was added to boiling distilled water, incubated for 5 min with occasional stirring, and then filtered through a coffee press (style 1028; Bonjour, Inc.). Agar (4% [wt/vol]) was added to the mixture and autoclaved for 50 min. For combination media, 12.5% (wt/vol) of each guano was combined to give a final guano concentration of 25%. For UV irradiation, plates were exposed to 240 mJ of UV light in a Stratalinker apparatus (Stratagene, La Jolla, CA).

Medium with and without L-DOPA. A minimal medium containing 15.0 mM glucose, 10.0 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13.0 mM glycine, 3.0 μM thiamine, and 2% (wt/vol) agar with a pH of 5.5 was prepared. For positive 3,4-dihydroxy-L-phenylalanine (L-DOPA) plates, 1 mM L-DOPA (Sigma Chemical Co., St. Louis, MO) was also added.

Environmental-isolation medium. Minimal medium for environmental isolation contained 50 mM glucose, 100 mM glycine, 200 mM KH_2PO_4, 20 mM

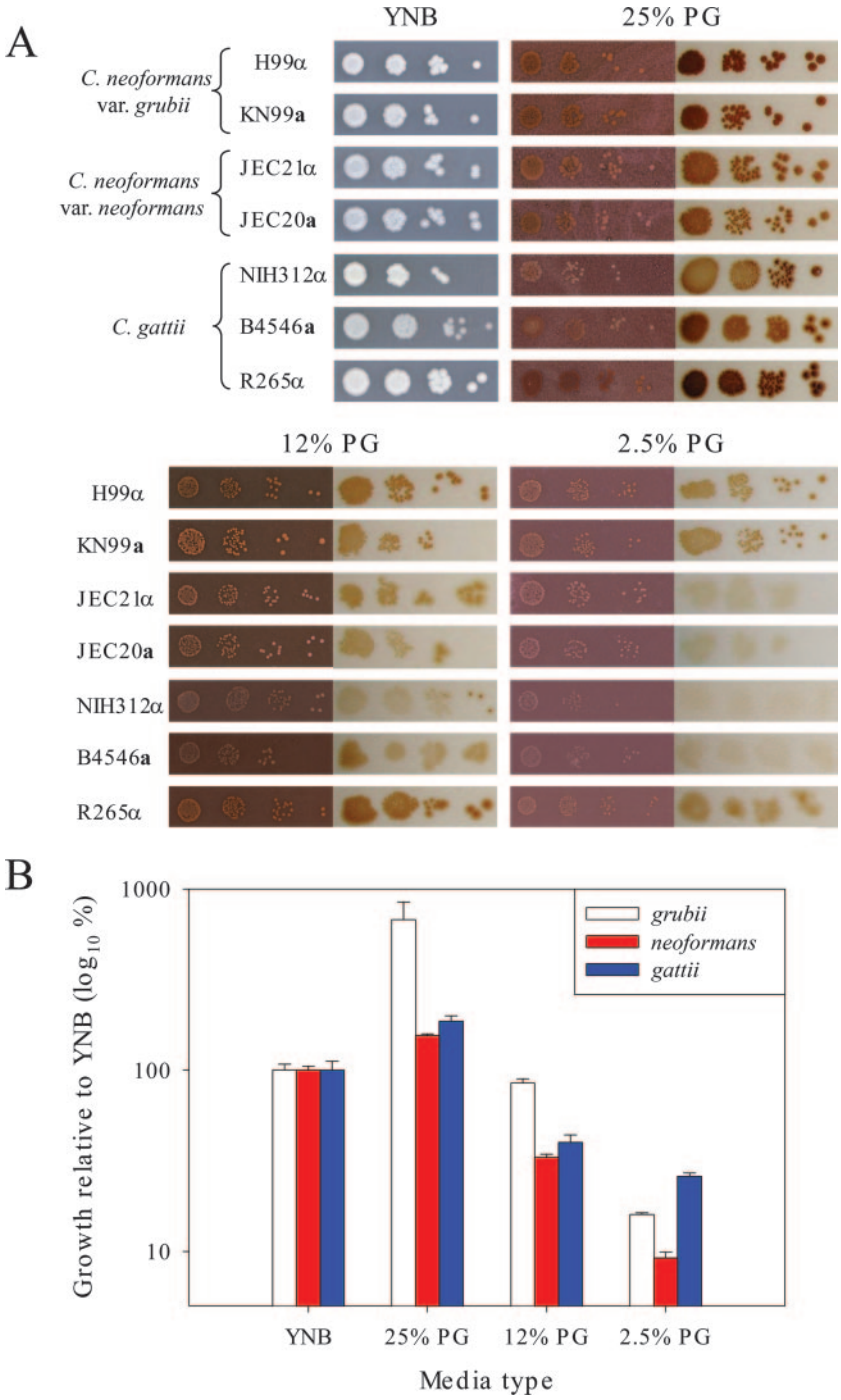

FIG. 1. Growth and pigmentation of *Cryptococcus* species on medium containing pigeon guano. *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii s*trains were grown overnight at 30°C in YPD medium, washed with PBS, and 10-fold serially diluted (10^3 to 10^6 dilutions). (A) Two microliters of each diluted cell suspension was spotted directly onto YNB or pigeon guano medium containing 25%, 12%, or 2.5% pigeon guano and incubated at 25°C for 7 days. To examine pigmentation, a sterilized 14-kDa-cutoff dialysis membrane was placed on the medium surface and then 0.5 μl of each diluted cell suspension was spotted onto the membrane. After 7 days of incubation at 25°C, membranes were removed from the medium and placed on moist filter paper to examine colony pigmentation. (B) To quantify growth, the 10^3 dilutions of JEC21α (*neoformans*), KN99α (*grubii*), and B4546α (*gattii*) grown on membranes were removed after 7 days and placed in PBS, and CFU were counted by serial dilution on YPD. Numbers of CFU resulting from growth on the various media are expressed as percentages of the number for the strain grown on YNB.

$MgSO_4 \cdot 7H_2O$, 5 μM $CuSO_4$, 2 mg/ml L-DOPA, 10 μg/ml thiamine-HCl, 0.5 μg/ml biotin, 0.5 mg/ml ampicillin, 40 μg/ml rose bengal, and 2% (wt/vol) agar (pH 5.6).

Growth and pigmentation comparisons. YPD broth overnight cultures were inoculated with the desired yeast strain and incubated with shaking at 30°C overnight. The overnight culture was centrifuged at 4,000 rpm for 5 min to pellet cells and then resuspended in phosphate-buffered saline (PBS). Serial dilutions from 1:1 to $1:10^6$ were prepared in PBS. Two microliters of each dilution was plated, allowed to dry completely, and then incubated in the dark with Parafilm and/or Ziploc bags as protection against contamination. For studies including

membranes, a sheet of sterilized 3.5- or 14-kDa-cutoff dialysis membrane (Spectrum Laboratories) was placed on the plate prior to inoculation with 0.5 μl of culture. Membranes were placed on moist filter paper for analysis. To quantify growth, colonies grown on membranes were suspended in 1 ml PBS and then CFU were enumerated from serial dilutions onto YPD medium.

Mating and monokaryotic fruiting assays. Strains were suspended in PBS, and 5-μl droplets were plated onto all medium types, allowed to dry completely, and then incubated at 25°C in the dark. Alternatively, the strains were mated as described previously (63). In crosses with genetically marked strains on 25% pigeon guano medium, spores were microdissected and progeny analyzed for genetic-recombination events.

Fusion and filamentation assays. To assess the level of cell fusion on various medium types, cell fusion assays were performed as described previously (4). Variety *grubii* parental strains were YSB119α (nourseothricin resistant [NAT^r]) and YSB121**a** (neomycin resistant [NEO^r]); var. *neoformans* parental strains were XL465α (NEO^r) and XL405**a** (NAT^r); and *C. gattii* parental strains were JF65 (NAT^r) and JF66 (NEO^r). Briefly, 10^8 cells of each parental mating type were mixed in equal volumes, and 5 μl was spotted onto the various medium types. For *C. gattii*, the V8 and PG media were supplemented with uracil to allow growth of JF66. After incubation for 24 h in the dark, the cells were scraped from the plate and resuspended in 1 ml water. Serial dilutions were prepared and spread onto YPD plates containing both NAT and NEO. Plates were incubated at 37°C for 72 h to induce diploid formation. Strains that were thermally dimorphic (budding at 37°C and filamentous at 30°C) and contained both sex-determining genes, *SXI1*α and *SXI2***a**, were isolated. Six strains each were isolated for var. *grubii* (named KN119/21-1 to KN119/21-6), var. *neoformans* (KN405/465-1 to KN405/465-6), and *C. gattii* (KN65/66-1 to KN65/66-6) to verify that measurements were representative for multiple strains. To examine filament length, 10^8 cells of the diploid strains for each variety/species were spotted as 5-μl drops onto the various medium types. Filament length was measured after incubation for 7 days in the dark at 30°C.

Environmental isolation. One gram of guano or soil was suspended in 5 ml PBS, and then 50 μl was spread onto culture medium containing L-DOPA and rose bengal. Pigmented colonies were isolated and DNA was obtained as described previously (49). PCR with *STE20* primers was used to identify *C. neoformans* strains as well as their serotypes and mating types (5).

Elemental analysis. Guanos were analyzed for percent carbon, hydrogen, nitrogen, oxygen, and sulfur (Galbraith Laboratories, Knoxville, TN). For glucose concentration determination, powdered guano was added to boiling distilled water, incubated for 5 min, filtered through a French press, and autoclaved for 50 min. The medium was allowed to cool and solids to settle out of the solution. The glucose concentration in the resulting supernatant was determined using the QuantiChrom glucose assay kit (BioAssay Systems, Hayward, CA).

RESULTS

Pigeon guano supports the growth of *C. neoformans* and *C. gattii*. *C. neoformans* is readily isolated from pigeon guano, and var. *neoformans* strains have been cultured and mated on medium containing pigeon guano extracts (31, 73–75, 85), but *C. gattii* is not typically isolated from avian excreta (13, 14, 71), suggesting that *C. gattii* might not grow well in pigeon guano (11). This hypothesis was tested by examining the growth of *C. neoformans* (var. *grubii* and *neoformans*) and *C. gattii* on pigeon guano medium in which sterilized pigeon guano serves as the sole nutrient source. As shown in Fig. 1, both varieties of *C. neoformans* as well as *C. gattii* exhibited robust growth on pigeon guano medium. All strains tested had higher growth on medium containing 25% (wt/vol) pigeon guano than on YNB minimal medium (six times higher for var. *grubii*). The growth was reduced when pigeon guano in the medium was reduced (25% to 12% or 2.5%) (Fig. 1). No significant difference in growth was observed for the VI outbreak major strain, R265. Additional global *C. neoformans* and *C. gattii* strains were screened on pigeon guano medium, and no significant differences in growth were observed (data not shown).

We next tested commercially available excreta for their ability to support *Cryptococcus* growth (data not shown). Medium containing desert bat guano supported the growth of all strains, but at a lower level than YNB. The growth on seabird guano, fossilized seabird guano, and dry-bar cave bat media was extremely poor. Two possibilities could explain the inability of *Cryptococcus* to grow on these media: the media could contain compounds that inhibit growth, or the medium types could lack required nutrients. To test this, growth was examined on media containing 12.5% seabird guano and 12.5% pigeon guano. Supplementation of seabird medium with pigeon guano restored growth (data not shown). Similar results were seen with other medium types tested, suggesting that other guanos lack an essential nutrient. Supplementation of seabird medium with components of YNB, including amino acids, nitrogen, and glucose, revealed growth only with glucose addition (data not shown). Elemental analysis revealed similar levels of total carbon in pigeon, bat, and seabird guanos; however, an analysis of glucose concentration revealed decreased levels in bat and seabird guanos compared to that in pigeon guano (Table 2). These data suggest that seabird medium lacks a sufficient utilizable carbon source.

TABLE 2. Elemental analysis of guanos[a]

Element	% in:		
	Pigeon guano	Bat guano	Seabird guano
Total carbon	14.38	13.73	16.70
Hydrogen	2.12	2.09	3.92
Nitrogen	1.51	3.89	14.41
Oxygen	14.69	8.93	29.40
Sulfur	0.21	0.46	1.92

[a] The glucose concentrations for pigeon, bat, and seabird guanos were 1,000, 24, and 12 ppm, respectively.

The pigeon guano used for these studies was obtained from the environment. Because guano was collected from the ground, we sought to verify that growth on pigeon guano medium was due to pigeon guano and not contamination with other nutrients from the collection site. Soil isolated from the same area resulted in very poor growth of *Cryptococcus* (data not shown). As with results with other guanos, growth on soil medium could be achieved by supplementation with pigeon guano, suggesting that soil medium is nutrient limited (data not shown). There was no increase in the growth of *Cryptococcus* on soil contaminated with pigeon guano compared to growth on an uncontaminated sample, suggesting that growth factors in pigeon guano do not readily diffuse into the surrounding soil or that these factors are metabolized by resident soil microbes. *Cryptococcus* was isolated from pigeon guano but not from soil at the collection site, confirming pigeon guano as the source of *Cryptococcus* from this environment (data not shown).

***Cryptococcus* produces pigment during growth on pigeon guano medium.** *C. neoformans* and *C. gattii* grown on pigeon guano medium produced brown pigmentation, which we hypothesized could be melanin. However, we found that this pigmentation was only partially generated via the well-characterized melanin biosynthesis pathway and that black particles resulting from another pigment production pathway could also be isolated from cells grown on pigeon guano.

Pigmentation consistently increased as the concentration of

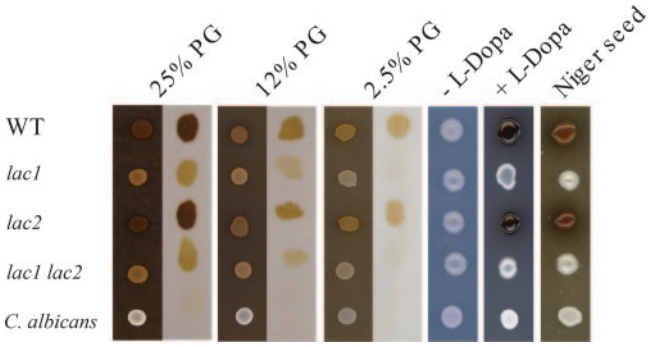

FIG. 2. Laccase mutant strains retain partial pigmentation on medium containing pigeon guano but not on medium containing L-DOPA. *C. neoformans* var. *grubii* H99 (wild type [WT]), *lac1* and *lac2* single mutants, a *lac1 lac2* double mutant, and a *Candida albicans* strain were washed with PBS, and 2 μl of each cell suspension was spotted directly onto minimal medium (medium lacking L-DOPA [−L-DOPA]), Niger seed medium, medium containing L-DOPA to induce melanin production, or onto medium containing 25%, 12%, or 2.5% pigeon guano (PG). To examine the pigmentation of the strains grown on the pigeon guano medium, a sterilized 14-kDa-cutoff dialysis membrane was placed on the medium surface and then 0.5 μl of each diluted cell suspension was spotted onto the membrane. After 7 days of incubation at 25°C, membranes were removed from the medium, placed on moist filter paper, and photographed.

pigeon guano in the medium increased (Fig. 1). Treatment of the guano with activated charcoal significantly decreased pigmentation, suggesting that the components in pigeon guano stimulating pigmentation can be absorbed to a carbonaceous surface. When a dialysis membrane (cutoff, either 3.5 kDa or 14 kDa) was used to separate cryptococcal cells from the medium, pigmentation was still observed, suggesting that the component(s) involved in pigment formation is less than 3.5 kDa in size and can readily diffuse through the membrane (Fig. 1).

While the formation of a brownish pigment when *Cryptococcus* is grown on medium containing pigeon guano has been observed previously (74), pigment formation has not been characterized. The well-defined laccase pathway produces the brown/black pigment melanin (33). Recent studies have identified two laccase genes (*LAC1* and *LAC2*) in *Cryptococcus* (56, 68, 93). Mutation of the *LAC1* gene blocks melanin production, while mutation of the *LAC2* gene has no discernible effect on melanin production on medium containing L-DOPA (68, 86). Previous studies have also identified melanized cells present in pigeon guano (66). Thus, we hypothesized that the brown pigment produced on pigeon guano medium is melanin.

The wild-type strain, laccase mutant strains, and *Candida albicans*, which does not produce melanin, were tested for pigment production on pigeon guano medium (Fig. 2). The *lac1* and *lac1 lac2* mutant strains, while less pigmented than the

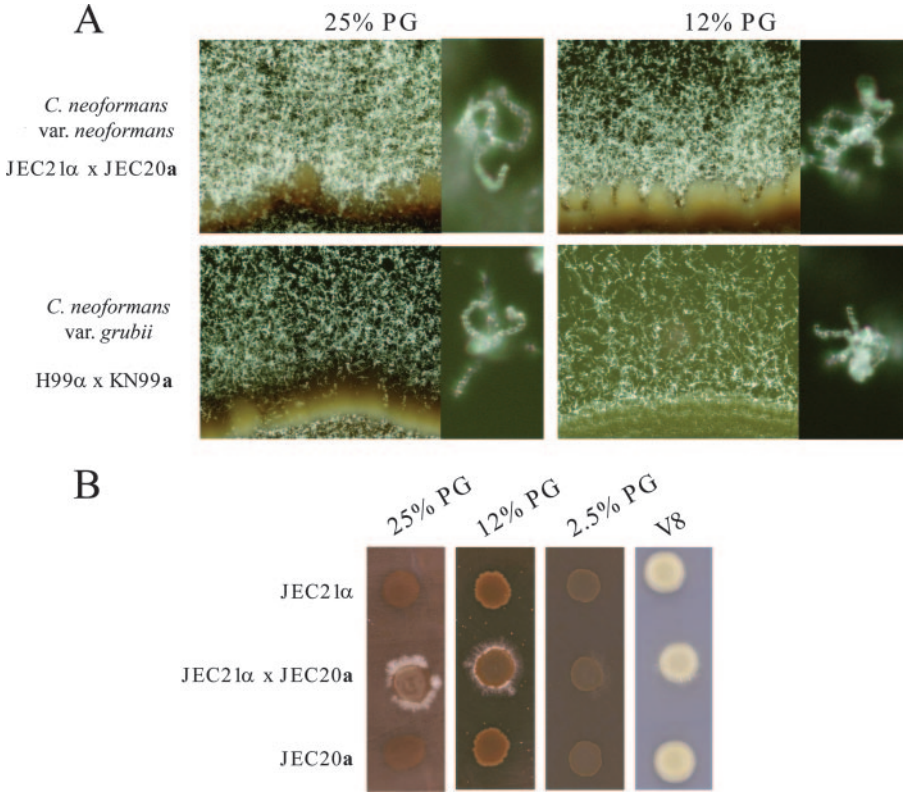

FIG. 3. *Cryptococcus neoformans* var. *grubii* and var. *neoformans* mate robustly on pigeon guano. Opposite-mating-type strains of *C. neoformans* var. *grubii* and var. *neoformans* were washed with PBS, and equal volumes of each mating type were combined. The mixture was placed as a 10-μl drop onto V8 medium (pH 5 for var. *grubii* and pH 7 for var. *neoformans*) or medium containing 25%, 12%, or 2.5% pigeon guano (PG). Plates were incubated in the dark at 25°C for 7 days. (A) Filamentation (×20) and sporulation (×400) of var. *neoformans* and var. *grubii* strains on media containing 25% and 12% pigeon guano. (B) Comparison of *C. neoformans* var. *neoformans* mating colonies on V8 (pH 7) medium or pigeon guano medium containing increasing levels of pigeon guano.

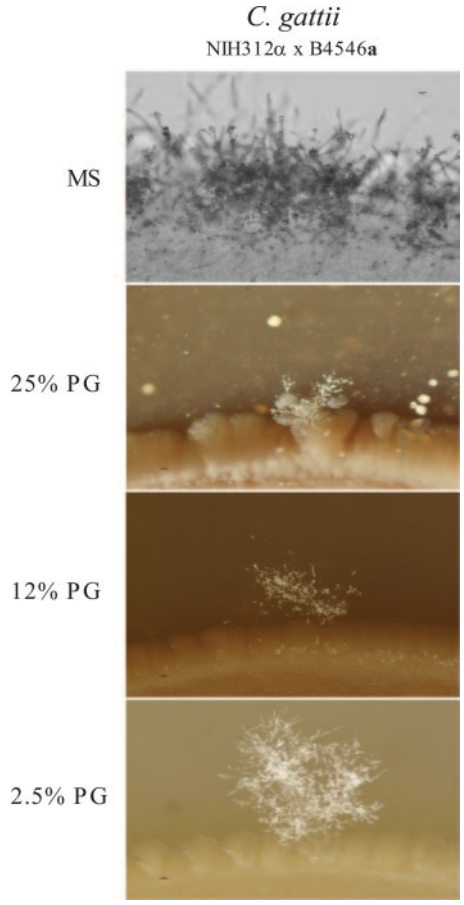

FIG. 4. *Cryptococcus gattii* mating is inhibited on pigeon guano medium (PG). *C. gattii* strains of opposite mating types were washed with PBS, and equal volumes of each mating type were combined. The mixture was placed as a 10-μl drop onto MS medium or medium containing 25%, 12%, or 2.5% pigeon guano. Plates were incubated in the dark at 25°C for 3 days and then photographed at a ×20 magnification. The top panel shows a random patch at the edge of the mating colony on MS medium. The lower three panels show patches of the mating colonies where filamentation was observed on media containing 2.5%, 12%, and 25% pigeon guano, respectively.

isogenic wild-type strain, still produced pigment on pigeon guano medium but were unpigmented on minimal medium containing L-DOPA. In contrast, pigmentation of the *lac2* mutant was unaffected on either pigeon guano medium or L-DOPA medium. The absolute amount of pigmentation observed with the laccase mutants was somewhat varied, with some batches of pigeon guano medium producing a slighter difference in pigmentation between the wild-type and laccase mutant strains (data not shown). Melanin ghosts were recovered from wild-type and *lac2* strains grown on pigeon guano medium but not from the *lac1* or *lac1 lac2* mutants. Small black particles were observed in these mutants instead of cell-sized melanin ghosts (J. D. Nosanchuk, K. Nielsen, and J. Heitman, unpublished data). These results suggest that only some of the brown pigment observed on pigeon guano medium is produced via the classical laccase-dependent melanin pathway and that another, as-yet-uncharacterized pigment is also generated on pigeon guano medium.

***C. neoformans* but not *C. gattii* strains mate on pigeon guano medium.** No difference in the ability of *C. neoformans* and *C. gattii* to grow on pigeon guano medium was observed even though *C. gattii* is not typically isolated from pigeon guano. We next determined whether both *Cryptococcus* species can complete their life cycle by undergoing sexual reproduction on pigeon guano, a hallmark of realized ecological niches, and found that only *C. neoformans* strains were able to robustly mate on pigeon guano medium; *C. gattii* strains did not.

Crosses were performed between **a** and α strains of both *C. neoformans* (var. *grubii* and *neoformans*) (Fig. 3) and *C. gattii* (Fig. 4). The *C. neoformans* var. *neoformans* and var. *grubii* strains exhibited prolific mating on pigeon guano medium. The robustness of mating increased with the concentration of pigeon guano in the medium and exceeded that on V8 mating medium (Fig. 3). Spores produced from matings with genetically marked strains were microdissected and germinated, and based on marker analysis, they exhibited classical Mendelian segregation consistent with sexual reproduction (Table 3). *C. neoformans* strains were also able to mate at higher temperatures on pigeon guano medium than on V8 medium (data not shown). Mating was observed at 37°C on pigeon guano medium but not on V8 medium. In contrast, *C. gattii* mating was significantly reduced on pigeon guano medium compared to that on V8 or MS mating medium, and the inhibition of mating increased as the concentration of pigeon guano in the medium increased (Fig. 4). The mating of the *C. gattii* VI outbreak strain R265 was also significantly reduced (data not shown).

Mating is generally thought to occur in response to nutrient limitation. The two cell types produce peptide pheromones that trigger conjugation tube formation in α cells and uniform cell expansion of **a** cells, leading to cell fusion. Nuclear fusion is delayed, and the resulting heterokaryon adopts a filamentous state. The filaments ultimately produce basidia, where nuclear fusion and meiosis occur, and long chains of recombinant basidiospores are produced (33, 55). Thus, enhanced mating of *C. neoformans* on pigeon guano medium could occur at two stages of mating: cell fusion and filamentation (Fig. 5A).

To examine cell fusion of the *C. neoformans* varieties on

TABLE 3. Recombinational analysis of mating of *C. neoformans* KN99α NAT with KN99**a** NEO on medium containing pigeon guano[a]

Marker combination	No. of strains (%)
α NAT	5 (11) (parental)
a NEO	7 (16) (parental)
α	5 (11)
a	3 (7)
α NEO	7 (16)
a NAT	6 (13)
α NAT NEO	9 (20)
a NAT NEO	3 (7)
Total	45 (100)

[a] Opposite-mating-type strains of *C. neoformans* var. *grubii* genetically marked with a NAT or NEO resistance gene were washed with PBS, and equal volumes of each mating type were combined. The mixture was placed as a 10-μl drop onto medium containing 25% pigeon guano. Plates were incubated in the dark at 25°C for 7 days. Spores were microdissected, and the resulting colonies were screened for mating type and the presence of each marker.

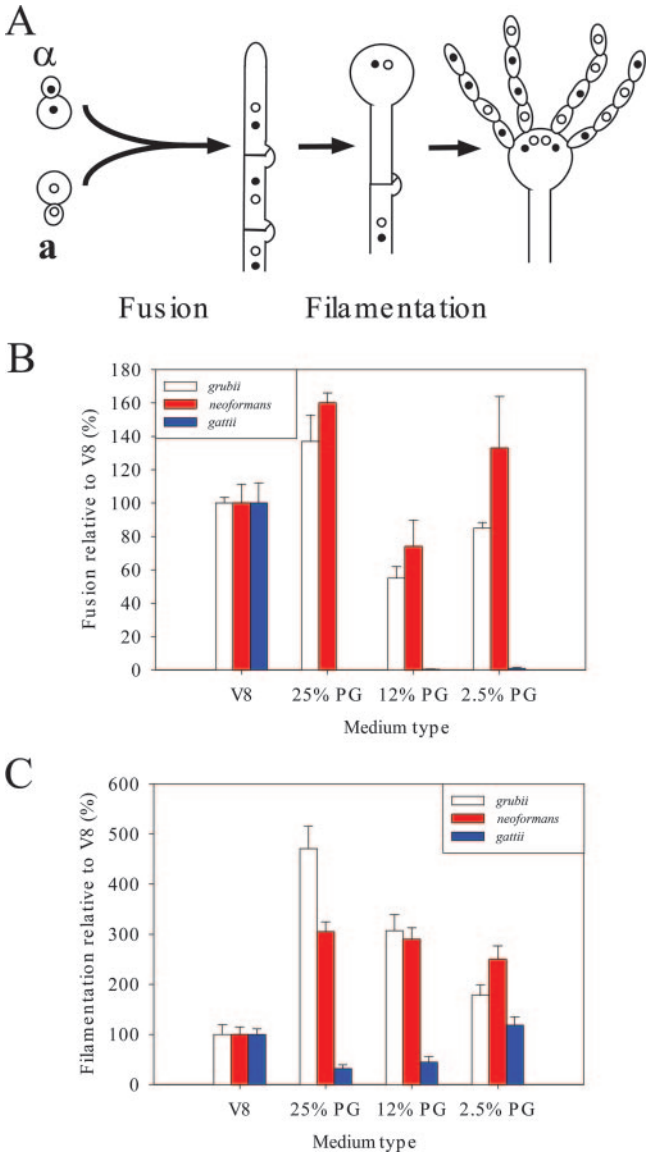

FIG. 5. Regulation of *C. neoformans* and *C. gattii* mating on V8 medium compared to that on medium containing pigeon guano (PG). (A) Schematic diagram of *Cryptococcus* mating. (B) Cells (10^8) of opposite mating types, genetically marked with a NAT or NEO resistance marker, were mixed, and 5-μl spots were placed onto V8 medium (pH 5 for *C. neoformans* var. *grubii* and pH 7 for *C. neoformans* var. *neoformans* and *C. gattii*) or medium containing 25%, 12%, or 2.5% pigeon guano. After 24 h of incubation, the resulting cells were screened for fusion products containing both markers. The numbers of fusion events on the various media are expressed as percentages of the number of fusion events occurring on V8 medium for each mating pair. (C) Six diploid strains for each variety/species were washed with PBS, inoculated onto V8 medium (pH 5 for *C. neoformans* var. *grubii* and pH 7 for *C. neoformans* var. *neoformans* and *C. gattii*) or pigeon guano medium contain differing levels of pigeon guano, and incubated in the dark for 7 days. Filament length is based on the average distance from the edge of the colony to the outer edge of filamentation and is expressed as a percentage of the average filament length on V8 medium.

pigeon guano medium, strains were neutrally marked with dominant NAT and NEO markers and cultured on pigeon guano or V8 medium and the levels of cell-cell fusion were compared. An increase in cell fusion was observed on 25% pigeon guano medium compared to cell fusion on V8 medium for both *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* strains (Fig. 5B). Thus, components in pigeon guano stimulate *C. neoformans* **a**-α cell fusion during mating.

Next, the impact of pigeon guano medium on filamentation was analyzed using diploid **a**/α strains to allow an examination of filament length irrespective of fusion. Both var. *neoformans* and var. *grubii* diploid strains exhibited significantly increased filament lengths on pigeon guano medium (3-fold and 4.5-fold, respectively) compared to lengths on V8 medium. Even 2.5% pigeon guano medium increased filament length twofold in both *C. neoformans* varieties, indicating that pigeon guano stimulates filamentation, ultimately leading to the production of basidia and infectious basidiospores (Fig. 5C).

In contrast to *C. neoformans* strains, *C. gattii* strains showed significantly reduced mating on pigeon guano medium. Instead of the prolific mating observed on V8 or MS medium surrounding the entire colony periphery (Fig. 4A), only a few regions of mating were observed on pigeon guano medium, and the size and number of these regions decreased as the concentration of pigeon guano increased (Fig. 4). To determine whether *C. gattii* cell fusion is reduced on pigeon guano medium, the levels of fusion of NAT^r and NEO^r strains were compared on pigeon guano and V8 media and found to be dramatically reduced (20-fold) on pigeon guano medium (Fig. 5B). Furthermore, the filament length of *C. gattii* **a**/α diploid strains was decreased by 80% on pigeon guano medium compared to that on V8 medium (Fig. 5C). Interestingly, as the concentration of pigeon guano in the medium decreased, filament length increased and was the same on 2.5% pigeon guano and V8 media. These results indicate that pigeon guano inhibits both the cell-cell fusion and the filamentation of *C. gattii* during mating.

DISCUSSION

Our results indicate that pigeon guano has the properties expected of the realized ecological niche for *C. neoformans*. The fact that *C. neoformans* var. *grubii* and *neoformans* produced higher numbers of CFU on pigeon guano than on YNB suggests that the nutritional composition of pigeon guano provides a highly favorable environment for the growth of *Cryptococcus*.

Increased growth with increasing pigeon guano concentration correlates with glucose levels in the medium. The fact that seabird guano did not support the growth of *Cryptococcus* unless it was supplemented with glucose suggests that guanos differ in the amounts of utilizable carbon sources that they contain. Bat guano also supported the growth of *Cryptococcus*, but to a lesser degree than pigeon guano, and *Cryptococcus* has occasionally been isolated from bat guano and caves (25, 58). The growth of *Cryptococcus* on chicken guano was previously found to be inhibited due to high alkalinity and the presence of a low-molecular-weight substance (85). The guanos tested here did not differ in alkalinity (data not shown). The fact that *Cryptococcus* was able to grow on all of the media tested when supplemented with pigeon guano or glucose indicates that nutrients are limiting in these guanos rather than that an inhibitory agent is present.

Many studies have examined the presence of *Cryptococcus* in

association with birds and their guanos, including in aviaries where multiple species are housed in close proximity (6, 7, 12, 24, 26, 42, 51, 54, 67, 75). These studies have identified specific avian species as carriers for *Cryptococcus*, but no reason for this specificity has been determined. Our data comparing the levels of growth of *Cryptococcus* on multiple guanos suggests that the preference of *Cryptococcus* for certain avian species is likely due to the nutrient composition of the corresponding guano. This finding may help to more clearly define the role of birds and bird excreta, particularly pigeons, in the transmission of *C. neoformans*. The growth of these organisms on pigeon guano provides a potential mechanism for explaining how pigeons might play a role in harboring *C. neoformans*, either internally or on external parts of their anatomy that come into contact with guano, such as their feathers or feet (1, 8, 15, 30, 36, 39, 48, 54, 67, 80).

Pigmentation is observed when *Cryptococcus* is grown on pigeon guano and increases as the concentration of guano in the medium increases. Pigmentation was also observed in other media with carbon source supplementation. The pigment melanin is a virulence factor of *Cryptococcus*. Interestingly, melanin production is suppressed in standard laboratory media by carbon supplementation (88), yet a utilizable carbon source does not appear to be limiting in pigeon guano medium. The pigment observed on pigeon guano is in part produced by the classical laccase pathway involved in melanin production because laccase mutants exhibit a reduction in pigmentation. However, the laccase pathway is not solely responsible for the pigmentation, and *lac1* and *lac1 lac2* mutants still produce pigment on pigeon guano. It is unclear whether the remaining pigment is actively produced by cryptococcal cells or results from the transport of pigmented compounds from the medium into the cells. If the latter, it is specific to *Cryptococcus*, as a *C. albicans* strain did not accumulate pigment and produced white colonies on pigeon guano medium.

That *C. neoformans* var. *grubii* and var. *neoformans* strains grow and mate on pigeon guano medium and therefore complete their entire life cycle supports the hypothesis that pigeon guano is a realized ecological niche for *C. neoformans*. Finding the true realized ecological niche for an organism is challenging. While all evidence to date suggests that pigeon guano is an ecological niche for *C. neoformans*, we cannot exclude the possibility that there is another, as-yet-uncharacterized niche to which this organism is even better adapted.

While *C. neoformans* is well adapted to survive and sexually reproduce on pigeon guano, *C. gattii* is not well suited for long-term survival in this environment. *C. gattii* growth on pigeon guano is equivalent to that of *C. neoformans*, showing that pigeon guano is a fundamental niche for *C. gattii* and can sustain its growth. However, *C. gattii* mates poorly on pigeon guano. The inability to reproduce efficiently on pigeon guano shows that pigeon guano is not a suitable substrate overall for the species survival of *C. gattii* and therefore is not a realized ecological niche for this organism. These findings and our conclusions about the differing realized ecological niches for *C. neoformans* and *C. gattii* correlate well with environmental-isolation studies that show that *C. neoformans* is readily isolated from pigeon guano but that *C. gattii* is not. While the mating results highlight the importance of mating in pathogenic fungi, they also raise a paradox. In both *C. neoformans* and *C. gattii*, sexual reproduction is limited by a nearly unisexual population in which sexual reproduction might be uncommon (reviewed in reference 64). If sexual reproduction is a significant component of species survival, as these findings suggest, why is the population largely unisexual?

A monokaryotic fruiting cycle that produces spores has been identified in *C. neoformans* var. *neoformans* (89) and has recently been shown to produce sexual recombinant progeny (47). While this cycle has not been characterized yet in the laboratory for *C. neoformans* var. *grubii* or *C. gattii*, recent evidence suggests that monokaryotic fruiting may occur in nature (20, 69). *C. neoformans* var. *neoformans* strains were able to undergo filamentation on pigeon guano medium, but no spore production was observed (K. Nielsen, X. Lin, and J. Heitman, unpublished results). That filamentation could be induced suggests that monokaryotic fruiting might occur on pigeon guano under appropriate environmental conditions. If so, then both same-sex and **a**-α sexual reproduction may contribute to species survival.

Pigeon guano as a realized ecological niche for *C. neoformans* provides a plausible explanation for the cosmopolitan nature of this organism. Because of the intimate interaction between *C. neoformans* and pigeons (and possibly other avian species), the organism can disseminate worldwide along bird migratory routes and, due to the domestication of the pigeon, along trade routes. In contrast, *C. gattii* is associated with sedentary trees and thus has a more restricted global movement thought to be associated with tree export and planting. These observations also suggest that mating and sexual reproduction are required for the long-term survival of *C. gattii*, and thus the spread of the organism is limited. The *C. gattii* VI major outbreak strain exhibited no increase in mating on pigeon guano, suggesting that its introduction into the Pacific Northwest was not due to mating on pigeon guano. Instead recent studies suggest that the emergence of *C. gattii* in temperate environments is likely due to the expansion or alteration of the ecological niche by a subset of the population that allows for environmental proliferation predominantly in soil instead of in association with tree species (41, 53). Based on these studies, we hypothesize that at least two distinct events significantly altered the *Cryptococcus* ecology. First, an ancestral *Cryptococcus* strain gained the ability to sexually reproduce in pigeon guano and then swept the globe, likely as a result of the seafaring migration of humans and associated birds. Second, and perhaps more recently, another ecological-niche change has resulted in the survival of *C. gattii* in a temperate environment to allow further spread of a subset of this species.

The distribution of most primary fungal pathogens, including the dimorphic species *Coccidioides immitis* and *Coccidioides posadasii*, *Histoplasma capsulatum*, *Penicillium marneffei*, *Paracoccidioides brasiliensis*, and *Blastomyces dermatitidis*, is geographically restricted, likely due to an inability to reproduce or survive outside of their realized environmental niches. In many of these organisms, sexual reproduction and the requirements for reproduction are not clearly defined. However, with *Cryptococcus neoformans* and now *C. gattii* as examples, expansion of the environmental niches for the dimorphic primary pathogens could result in pandemic disease. The spread of *C. immitis* from North America to South America concomitant with Amerindian colonization exemplifies the ability of

pathogenic fungi to adapt to environmental change (16). *C. immitis* outbreaks in regions of endemicity occur due to climactic changes rather than due to the emergence of pathogenic strains (17). However, the population exhibits high levels of genetic exchange, and thus the emergence of a new strain with the expansion of an ecological niche is conceivable and may have contributed to the migration of the population from North to South America (18). By studying differences between *C. neoformans* and *C. gattii*, we may be able to identify key events punctuating environmental-niche expansion that might apply to the emergence of or increased risk for other environmental fungal pathogens.

The findings presented here on emerging fungal pathogens are also applicable to other microbial pathogens. Both ecological changes and microbial evolution are significant determinants for bacterial- and viral-disease emergence. For example, all pandemic and epidemic influenza A virus outbreaks arise by genetic drift or reassortment to generate new viruses with differing pathogeneses (81, 82). This illustrates the pressing need to understand not only the driving force behind genetic alterations but also how these genetic changes affect the ecology of the organism and thereby impact disease prevalence.

ACKNOWLEDGMENTS

We thank Josh Nosanchuk and Arturo Casadevall for melanin ghost analysis; Andrew Alspaugh, Yong-Sun Bahn, James A. Fraser, Alexander Idnurm, and Xiaorong Lin for strains; Diane Inglis for discussions and suggestions; and Ana Litvintseva and Xiaorong Lin for helpful comments on the manuscript.

This work was supported by NIAID R01 grant AI50113 to Joseph Heitman. Kirsten Nielsen was supported by NIAID grant T32 and Ruth L. Kirschstein NRSA in Molecular Mycology and Pathogenesis grant AI052080.

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