

# Congenic Strains for Genetic Analysis of Virulence Traits in *Cryptococcus gattii*

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***Cryptococcus gattii* is responsible for a large outbreak of potentially fatal disease that started in the late 1990s on Vancouver Island, Canada. How this fungus and the outbreak isolates in particular cause disease in immunocompetent people is unknown, with differing hypotheses. To explore genetic contributions, a pair of congenic  $\alpha$  and  $\alpha$  mating type strains was generated by a series of 11 backcrosses to introgress the *MAT* locus from a nonoutbreak strain into the background of strain R265, isolated from a Vancouver Island patient. The congenic pair was used to investigate three traits: mitochondrial inheritance, the effect of the *MAT* alleles on virulence, and the impact of a predicted virulence factor on pathogenicity. The two congenic strains show the same virulence in different models of cryptococcosis and equivalent levels of competition in coinfection assays. These results rule out a role of the *MAT* locus and mitochondrial genotype as major virulence factors in the outbreak strains. Disruption of *Bwc2*, a light-dependent transcription factor, resulted in reduced virulence, consistent with a similar function in the related species *Cryptococcus neoformans*. The *C. gattii* congenic strains represent a new resource for exploring the evolution of virulence in the *C. neoformans*-*C. gattii* clade.**

The *Cryptococcus* species complex comprises distinct lineages that are currently assigned into two species, *C. neoformans* and *C. gattii*, with its members best recognized as the agents of cryptococcosis (1). These basidiomycete yeasts colonize the lungs of susceptible individuals and spread throughout the body to multiple organs, with dissemination to the central nervous system causing the most problematic symptoms. Cryptococcosis is considered an AIDS-defining illness, and most of the estimated 624,000 people who die from this disease are in sub-Saharan African countries where AIDS is most prevalent (2).

The majority of cryptococcosis cases are caused by *C. neoformans*. The species is distributed worldwide, often found in association with bird guano. Two serotypes (A and D) or varieties (*C. neoformans* var. *grubii* and var. *neoformans*) are recognized and have been extensively studied. In contrast, another species, *C. gattii* (serotypes B and C), is clinically rare at <1% of cryptococcosis cases and was historically considered to have a limited distribution within tropical and subtropical parts of the world (3, 4).

As a result of its rarity compared to *C. neoformans*, *C. gattii* had received less research attention. In the late 1990s, cryptococcal disease in healthy people, domestic pets, and wildlife caused by *C. gattii* appeared on Vancouver Island, and it subsequently spread to the mainland and into the northwest United States (5–8). This outbreak is one of the few examples of a newly endemic fungus capable of causing a life-threatening disease in a healthy human population. The origin of this outbreak is unknown, with several hypotheses to account for the emergence of this fungus beyond its traditional geographical range. One is a mating event, in this case between two *MAT* $\alpha$  strains, to generate a “super” pathogen (9). A second is that the outbreak strains have an altered mitochondrial metabolism (8, 10), presumably due to a unique genetic makeup of the strains. The third is that climate changes have promoted fungal reproduction in the region. Curiously, the NIH444 clinical strain of *C. gattii* isolated in Seattle in the 1970s is of the same molecular group as the outbreak strains isolated from the 1990s onwards (9). The NIH444 strain and the current presence of dis-

tinct lineages of *C. gattii* in the Pacific Northwest (11) would support a hypothesis of altered environment. Lastly, a combination of contributing factors is also a possibility.

One approach to understand the origin of an outbreak is to address virulence at the gene level through classical Mendelian genetics. Genetic analysis of *C. gattii* has been conducted on few occasions and often between different lineages (12–14). In the current classification scheme, four distinct lineages are recognized (15), named VGI to VGIV, with low fertility in crosses between them. Here we used crosses to generate a pair of congenic strains in the VGII background, using a sequenced strain from the Vancouver Island outbreak. The analysis of the virulence properties of the two congenic strains indicates no difference between them and the parental R265 strain, excluding a major contribution from the mitochondrial genotype and mating type in the virulence of the outbreak isolate and thereby implicating other factors. Furthermore, the strains add a new resource for future analysis of gene function and evolution in both *C. gattii* and *C. neoformans*, as discussed in the accompanying article by Zhai et al. (16).

## MATERIALS AND METHODS

**Strains and crossing.** Strains R265 (*MAT* $\alpha$ ) and CBS1930 (*MAT* $\alpha$ ) were used as the starting parents for crosses. R265 is a VGII strain isolated from

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a human case of cryptococcosis from Vancouver Island, and its genome has been sequenced (17). The available genome sequence and the origin of the strain provided a rationale for using it over strain NIH444, which has been used in other studies and has been recommended as a laboratory wild-type strain (18). CBS1930 was isolated from a veterinary sample from Aruba and is also a VGII strain (19). Strains were maintained on yeast extract-peptone-dextrose (YPD) medium. Crosses were set up by mixing yeast cells together on plates containing 5% V8 juice, 0.5 g/liter  $\text{KH}_2\text{PO}_4$ , and 4% agar. Plates were incubated in darkness at 19 to 21°C for 2 to 4 weeks. Basidiospores and parental yeasts were transferred with a pipette tip (the long and narrow type used to load polyacrylamide gels) onto YPD agar medium. Basidiospores were micromanipulated with a dissecting microscope.

**Molecular markers.** Most of the molecular markers were designed based on single nucleotide polymorphisms (SNPs) identified between CBS1930 and R265 in GenBank or comparing sequenced fragments of CBS1930 DNA to the R265 genome (K. Voelz, H. Ma, E. J. Byrnes, S. Phadke, P. Zhu, R. A. Farrer, D. A. Henk, Y. Lewit, Y.-P. Hseuh, M. C. Fisher, A. Idnurm, J. Heitman, and R. C. May, unpublished data). SNPs affecting restriction enzymes sites or with multiple differences between the strains were used for the design of oligonucleotide primers for PCRs: details are provided in Table S1 in the supplemental material. The PCR amplicons were either tested directly for their presence or absence or were digested with the appropriate restriction enzymes, and fragments were resolved on agarose gels. An SNP in the mitochondrial *COB2* gene was assessed by amplification with primers ALID1365 and ALI1373 and sequencing with primer ALID1373. The mating type was scored by crossing to the *C. neoformans* strains JEC20 (*MATa*) and JEC21 (*MAT $\alpha$* ) (20).

**Pulsed-field gel electrophoresis.** Chromosomal DNA was isolated from spheroplasts embedded in SeaPlaque low-melting-temperature agarose plugs (Lonza, Rockland ME), using previously described methods (21). The DNA was resolved in 1% agarose–0.5× Tris-borate-EDTA (TBE) gels using a CHEF-DR III system (Bio-Rad, Hercules, CA). The settings were as follows: block 1, 75 to 150 s of switching at 4 V/cm at 12°C for 24 h; block 2, 150 to 300 s of switching at 4 V/cm at 12°C for 24 h. The DNA was visualized by staining with ethidium bromide.

**Isolation of mutants.** Spontaneous mutants that were resistant to 5-fluorouracil (5-FU) or 5-fluoroorotic acid (5-FOA) were isolated by culturing independent colonies of strain R265 overnight in YPD medium and then plating the cells on media containing these chemicals: yeast nitrogen base (YNB) plus 20 mg/liter uracil plus 1 g/liter 5-FOA or YPD plus 250 mg/liter 5-FU. The stability of the mutation was tested by growing single-colony purified strains in nonselective YPD medium and plating onto YPD, YNB, or YPD–5-FU. The *URA5* and *FUR1* genes were amplified and sequenced from a subset of these strains, using primers shown in Table S2 in the supplemental material.

**Disruption of the *BWC2* gene and complementation.** The *BWC2* gene was targeted for gene replacement with the nourseothricin acetyltransferase (*NAT*) gene. The 5' flank was amplified with primers ALID1453 and ALID1454, the 3' flank was amplified with primers ALID1455 and ALID1456, and the *NAT* cassette was amplified from plasmid pAI3 with primers ai006 and ai290. Overlap PCR with primers ALID1453 and ALID1456 was used to join the three pieces together.

The DNA construct was precipitated onto gold beads (BioWorld, Dublin, OH) and transformed into strain R265 cells plated on YPD–1 M sorbitol, using a PDS/1000 particle delivery system (Bio-Rad) with standard parameters (22). Cells were recovered for 3 h at 30°C and then transferred to YPD, including 100  $\mu\text{g}/\text{ml}$  nourseothricin. Colonies appeared in 3 days at 30°C, and those with the correct gene replacement events were identified by PCR analysis.

To complement the *bwc2::NAT* strain, the wild-type copy of *BWC2* was amplified with primers ALID1602 and ALID1603 from R265 genomic DNA, the amplicon was digested with KpnI, and the fragment was ligated into the KpnI site of plasmid pPZP-NEO11. This plasmid was transformed into *Agrobacterium tumefaciens* strain EHA105 cells by electropo-

ration. The *BWC2-NEO* construct was introduced into the *bwc2::NAT* strain by transconjugation, using standard methods (23), with selection on YPD supplemented with 100  $\mu\text{g}/\text{ml}$  G418 and 200  $\mu\text{g}/\text{ml}$  cefotaxime.

**Northern blotting.** Overnight cultures of strain R265 and the *bwc2 $\Delta$*  mutant grown in YPD were diluted to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.5. Five hundred microliters was spread on 15-cm-diameter petri dishes containing YPD agar. Plates were wrapped in foil and incubated at room temperature in darkness for either 24 h or 22 h plus 2 h of exposure to white light. The cells were removed from the surface and washed in water prior to being pelleted in a centrifuge. The dark samples were harvested under dim red safe-light, and placed in black 50-ml tubes. RNA was extracted from lyophilized cells using Tri-Reagent (Sigma-Aldrich, St. Louis, MO). Twenty micrograms of each RNA sample was resolved on a 1.4% MOPS (morpholinepropanesulfonic acid)–formaldehyde gel, and blotted to Zeta-Probe membrane (Bio-Rad). The membrane was sequentially hybridized with probes for the *HEM15*, *UVE1*, and *ACT1* genes, amplified with the primers listed in Table S2 in the supplemental material. Autoradiographs were scanned, and the intensity of each band was measured with ImageJ software.

**Virulence assays of the congenic strains.** Mouse studies were performed basically as described previously (24, 25). For the inhalation models, groups (10 animals per group) of 6- to 10-week-old female A/J mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized by a mixture of ketamine and xylazine through intraperitoneal injection. Mice were then infected intranasally with  $1 \times 10^5$  fungal cells suspended in saline. For the intravenous models, mice were challenged with  $1 \times 10^4$  fungal cells by retro-orbital injection and monitored daily. Infected mice were sacrificed when they showed symptoms of being moribund, such as weight loss, rough fur, gait changes, or labored breathing. To test for organ dissemination in the animals infected with the congenic pair, the lungs, brains, and spleens of infected animals (5 animals per group) were dissected at the time of sacrifice. Each organ was homogenized in 2 ml sterile phosphate-buffered saline (PBS), and the suspension was then serially diluted and plated on YPD agar plates. The culture was incubated for 2 days to determine the CFU.

Infection of the wax moth (*Galleria mellonella*) followed previously described protocols (26). Larvae were purchased from Vanderhorst Wholesale, Saint Marys, OH. A total of  $1 \times 10^5$  cells suspended in PBS were inoculated into the rear proleg of each larva, with 11 larvae used for each strain or the PBS control. Larvae were incubated at 37°C and examined daily for survival.

***MATa* and *MAT $\alpha$*  coinfection *in vivo* and coculture *in vitro*.** For a and  $\alpha$  coinfection experiments, female A/J mice were challenged with a 1:1 ratio of a- $\alpha$  *C. gattii* cell mixture ( $1 \times 10^5$  fungal cells in inhalation models and  $1 \times 10^4$  fungal cells in intravenous models). The a and  $\alpha$  cell numbers of the initial mixed cell inocula were confirmed by measuring the CFU of serial dilutions. The organs were dissected from euthanized animals and processed as described above. To determine the ratio of the a and  $\alpha$  cells, 75 to 100 single colonies recovered from each organ of the dissected animals were examined for their mating type by crossing each with the reference strains JEC20 (*MATa*) and JEC21 (*MAT $\alpha$* ) separately. Hyphal formation and sporulation were scored as successful mating.

For the a and  $\alpha$  coculture experiments, the mixture of a and  $\alpha$  cells was inoculated into RPMI medium (pH 7.0) and incubated at 37°C under 5%  $\text{CO}_2$ . Aliquots taken at days 3 and 14 were plated on YPD agar with serial dilutions. Seventy-five to 100 single colonies were examined for their mating type through crossing with the reference strains JEC20 and JEC21.

**Statistical analysis.** Statistical significance of the survival data for comparisons between two groups was assessed by the log-rank (Mantel-Cox) test for the mouse and wax moth models. The comparison for groups of three or more was analyzed using one-way analysis of variance (ANOVA) tests. The one-way ANOVA tests were also used in the fungal burden studies. All statistical analyses were performed using the Graphpad Prism 5 program. *P* values lower than 0.05 were considered statistically significant.

**Animal ethics statement.** The mouse experiments were performed according to the guidelines of the National Institutes of Health and the Texas A&M University Institutional Animal Care and Use Committee (IACUC), protocol no. 2011-22.

## RESULTS

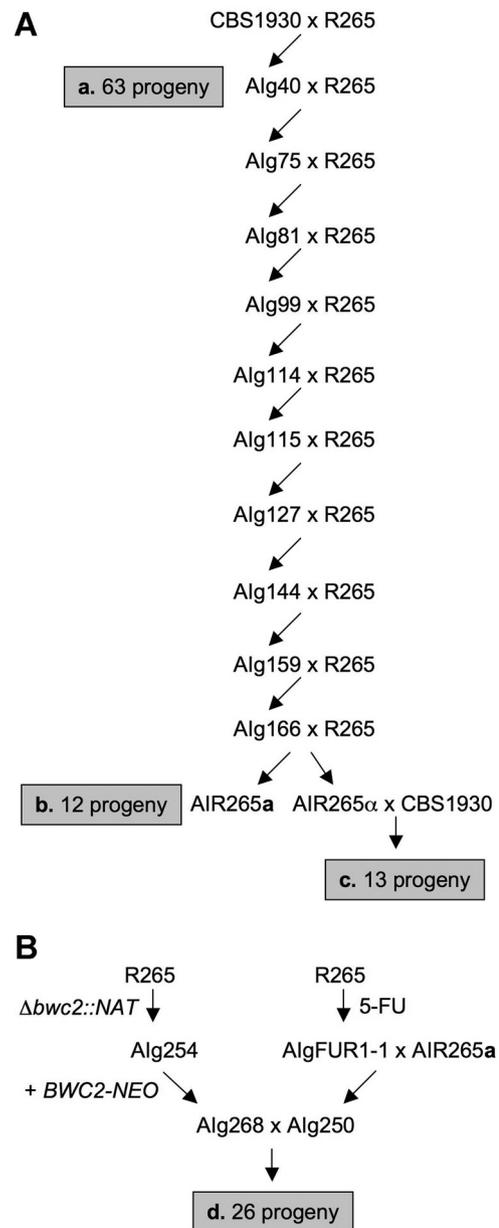
**Mendelian segregation of markers occurs in crosses between *C. gattii* strains R265 and CBS1930.** Genetic analysis of *C. gattii* has been rarely performed because many strains or strain combinations are infertile (27, 28). A series of crosses was used as part of generating a congenic pair, and four progeny sets were analyzed in detail to address different questions about the reliability of the strains for genetic analysis (Fig. 1). Each of these sets is described in subsequent sections.

Progeny set a in Fig. 1, comprising 63 progeny (basidiospore germination rate, 39%), was isolated from crosses between two VGII parents, R265 from a patient on Vancouver Island and CBS1930 isolated from a goat in Aruba. The distribution of alleles for 17 markers indicated that recombination has occurred in the progeny, as every strain differed in genotype from the two parents (Fig. 2A). Furthermore, comparison to the physical distances in the R265 genome indicated that the recombination frequencies between seven pairs of markers averaged 20.2 kb/cM (Fig. 2B). The range was from 5.6 to 42.1 kb/cM: note that the average includes two genetically unlinked regions (i.e., recombination of ~50%), which skews the calculation. For comparison, in a cross between two *C. neoformans* var. *neoformans* strains, the average was 13.2 kb/cM across the genome, ranging from 6.6 to 19.9 kb/cM on the two most extreme chromosomes (21).

**Generation of a congenic pair of *C. gattii* strains by backcrossing.** A *MATa* progeny from a series of crosses was backcrossed to strain R265 (Fig. 1; see Data Set S2 in the supplemental material). This was performed 11 times to yield strains AIR265a (*MATa*) and AIR265 $\alpha$  (*MAT* $\alpha$ ). In theory, both AIR265a and AIR265 $\alpha$  should be 99.95% identical to R265, with the exception of *MATa* in the AIR265a strain. Consistently, when the 17 markers used on the CBS1930  $\times$  R265 cross were tested on these two strains, all were derived from the R265 strain apart from the mating type allele in AIR265a.

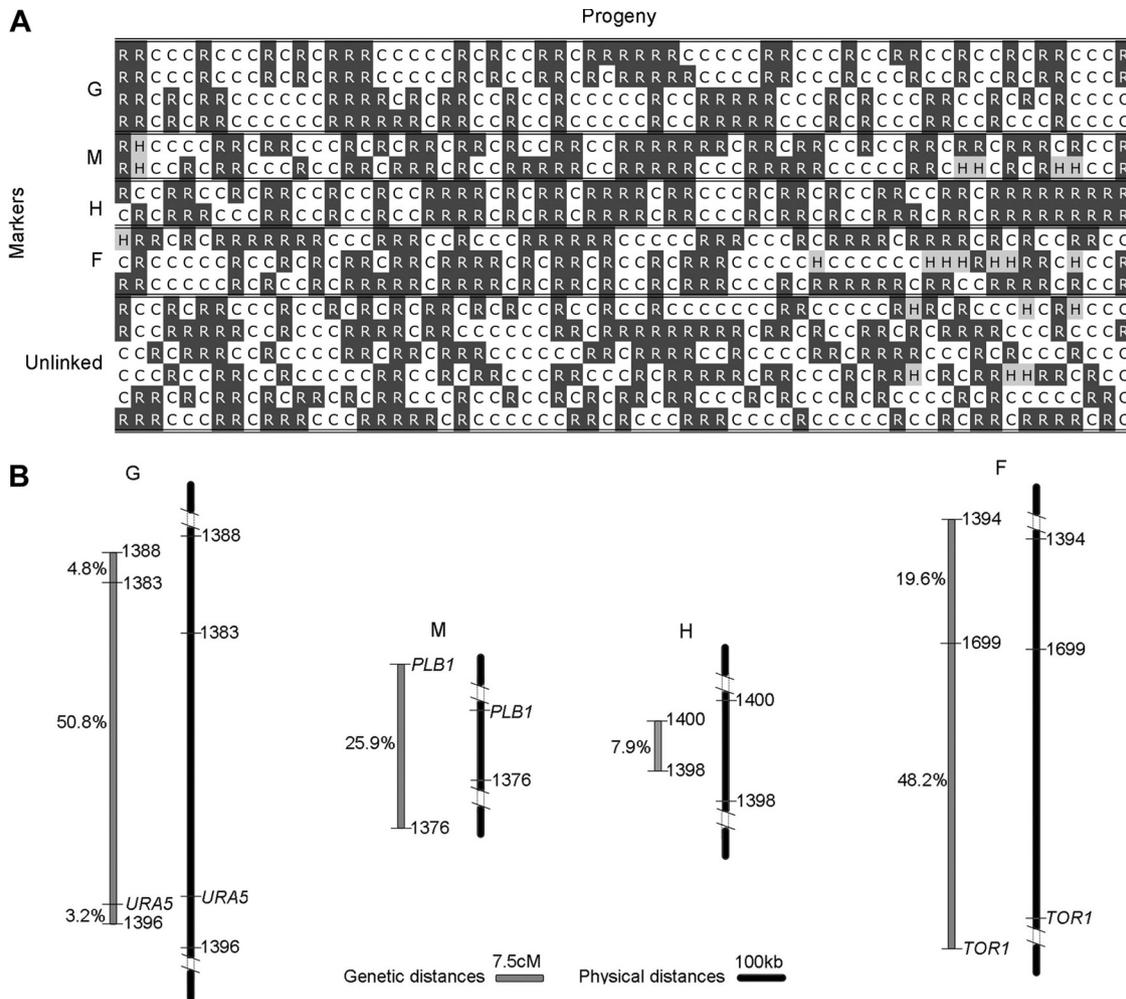
**Mitochondrial inheritance is predominantly uniparental from the *MATa* parent in crosses with congenic strains.** The mitochondrial genome in *C. neoformans* strains shows trends toward uniparental inheritance from the *MATa* parent (29–34). In studies on *C. gattii* with a subset of the CBS1930  $\times$  R265 progeny analyzed here and in the cross LA584  $\times$  R265, 19% and 31%, respectively, of the progeny had the mitochondrial genotype from the *MAT* $\alpha$  strain (Voelz et al., unpublished data). A fragment of the mitochondrial genome was amplified and sequenced from the 12 strains of backcross 11 (set b in Fig. 1). All 12 inherited the mitochondrial genome from the a parent, including strain AIR265 $\alpha$ , which is the *MAT* $\alpha$  strain characterized as the congenic pair. This suggests that the *MAT* locus is a key factor controlling inheritance of the mitochondrial genotype, while in noncongenic crosses, allelic differences of other non-*MAT* genes also play a role.

**Virulence of the *C. gattii* congenic strains in two murine models of cryptococcosis.** Several genetic factors may influence *Cryptococcus* virulence. One is the *MAT* locus, as demonstrated in *C. neoformans*. For instance, the a strain JEC20 is less virulent than the congenic  $\alpha$  strain JEC21 (20, 35, 36). Second, the outbreak strains are characterized by an unusual mitochondrial tubular



**FIG 1** Crossing strategies used to generate the strains in this study. (A) Production of the congenic pair AIR265a and AIR265 $\alpha$  from 11 backcrosses. (B) Generation of the *bwc2* $\Delta$  mutant and complemented + *BWC2-NEO* strain, spontaneous 5-fluorouracil-resistant (5-FU) strain, and derived strains. Four progeny sets (a to d) were characterized in further experiments. Set a provides information about recombination of genetic markers in crosses with the starting parents CBS1930 and R265. Set b tests for mitochondrial inheritance patterns. Set c compares recombination frequencies in the congenic  $\alpha$  strain with those of the original R265 parent. Set d establishes that recombination occurs between phenotypic genetic markers in the congenic pair. Additional information about the crosses is provided in the supplemental material.

morphology, which may reflect a specific factor within the mitochondrial genome. The *MATa* strain AIR265a and the *MAT* $\alpha$  strain AIR265 $\alpha$  have the same mitochondrial genomes derived from CBS1930, which does not have the tubular morphology (10), and their nuclear genomes differ at the mating type locus. Thus, these strains allowed us to test directly the impact of mating type and mitochondrial genomes on virulence.



**FIG 2** Recombination in R265 × CBS1930 crosses. (A) Diagram of allele segregation at 17 loci in 63 progeny. Four chromosomes (G, M, H, and F) have more than one locus, while the other six loci are on separate chromosomes (Unlinked). Black R, R265 allele; white C, CBS1930 allele; gray H, both alleles or ambiguous due to inefficient amplification or restriction digestion. The diagram is based on Data Set S1 in the supplemental material. (B) Comparison of physical and genetic distances between markers. The physical distances are based on the R265 genome sequence.

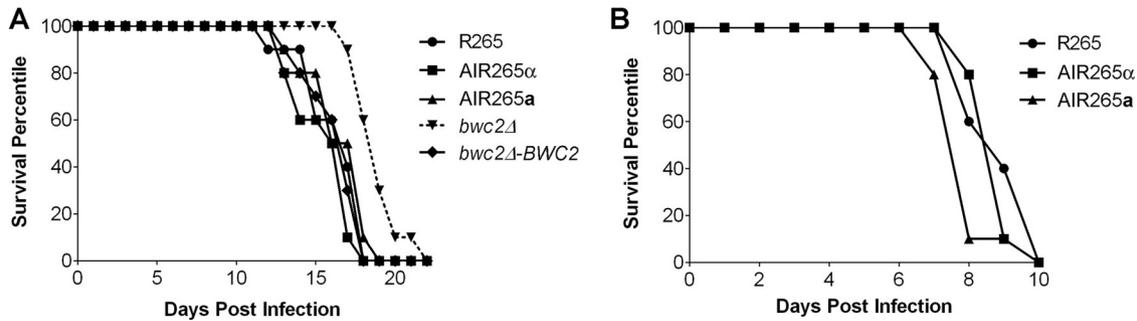
To compare the virulence levels of the congenetic strains of *C. gattii*, the well-established inhalation and intravenous infection models of murine cryptococcosis were used. The  $\alpha$  parental strain R265 was included in the assay to determine if the virulence potential of congenetic pair differs from that of the parental strain. There was no difference observed in survival rates among the animals infected by the parental strain R265 and the congenetic strains, either in the inhalation model (Fig. 3A) or in the intravenous model (Fig. 3B).

Furthermore, the fungal burden in the organs of mice infected by either the  $\alpha$  or the  $\alpha$  congenetic strain alone was examined after they were euthanized. When animals were challenged with the fungal cells intranasally, both the  $\alpha$  strain and the  $\alpha$  strain proliferate considerably in the lung. Surprisingly, animals infected by either the  $\alpha$  or  $\alpha$  *C. gattii* strain showed very low fungal loads in the brain (Fig. 4A). This is in contrast to what we and others observed in *C. neoformans* H99-infected animals, where the brain and the lung fungal burdens were both high at the time of sacrifice (37). The result suggests that the pulmonary infections, rather than the CSF infections, caused by the *C. gattii* strains are the likely cause of

fatality in this animal model. Interestingly, when the *C. gattii* fungal cells were inoculated directly to the bloodstream, high fungal burdens were observed in the brain (Fig. 4B), indicating a capability of both *C. gattii* strains to penetrate and proliferate in the brain.

**Virulence of the *C. gattii* strains in an insect model of cryptococcosis.** Alternative hosts can be used in place of mice to assay virulence, providing experimental advantages that include reduced cost, assessment of interactions with the innate immune system, and the ability to perform virulence assays at temperatures lower than mammalian 37°C (38, 39). The two congenetic strains, the two parental strains R265 and CBS1930, and the phosphate-buffered saline control were used as the inoculum for 11 larvae of the wax moth *G. mellonella* per strain. Survival was the same for the strains, with a slight delay for three larvae infected with strain CBS1930 that was not statistically significant (Fig. 5). The results from the insect model provide additional corroboration of the lack of difference in levels of virulence in the mouse models between the two congenetic strains and the outbreak strain R265.

**Coinfections with the congenetic strains.** One interesting phe-

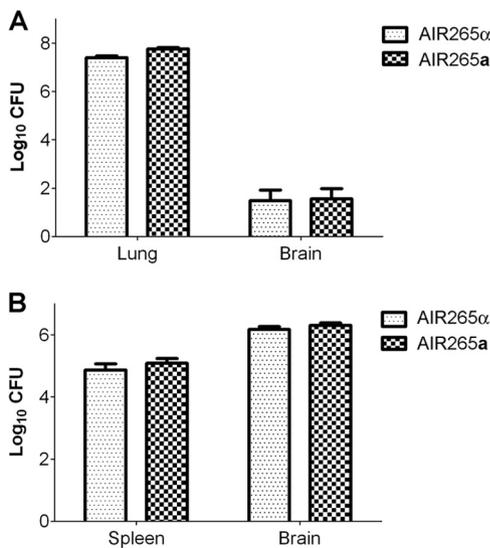


**FIG 3** The *C. gattii* congenic pair have equal virulence to each other and the original R265 strain. (A) Mice were inoculated with  $1 \times 10^5$  fungal cells intranasally, and survival was monitored. There was no difference observed in survival rates among the parental strain R265 and the congenic pairs. The *P* values were 0.361 (R265/AIR265 $\alpha$ ), 0.317 (R265/AIR265a), 0.949 (AIR265 $\alpha$ /AIR265a), 0.833 (AIR265 $\alpha$ /a +  $\alpha$ ), and 0.317 (AIR265a/a +  $\alpha$ ). The *bwc2* $\Delta$  mutant strain is attenuated in virulence compared to the wild-type R265 and the complemented strain (*P* = 0.02). (B) Mice were inoculated with  $1 \times 10^4$  fungal cells intravenously, and there was no difference among the strains. The *P* values were 1.0 (R265/AIR265 $\alpha$ ), 0.199 (R265/AIR265a), 0.362 (AIR265 $\alpha$ /AIR265a), 1.0 (AIR265 $\alpha$ /a +  $\alpha$ ), and 0.199 (AIR265a/a +  $\alpha$ ).

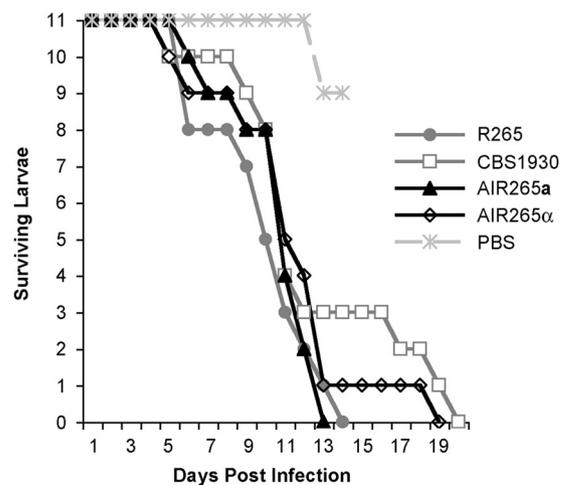
nomenon of the *C. neoformans* var. *grubii* congenic pair strains KN99a and KN99 $\alpha$  is that in infections with single strains both have similar virulence, whereas the  $\alpha$  strain tends to dominate in the brain when KN99a and KN99 $\alpha$  are used to coinfect the animals (40, 41). This neurotropism of the  $\alpha$  strain could contribute to the predominance of  $\alpha$  strains among clinical isolates, as cryptococcal meningitis is the most common clinical symptom (41). The impact of the mating type locus on the organ tropism during the *C. gattii* a and  $\alpha$  coinfection was tested. To avoid addition of

any potential variances by the introduction of markers to the strains, as was employed in previous studies (41), unmarked congenic strains were used.

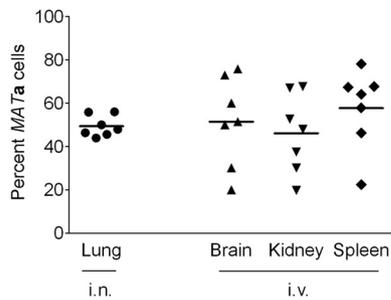
We first performed an *in vitro* experiment to determine if there is any proliferation advantage conferred by one mating type when the a and  $\alpha$  cells are cocultured under conditions that are relevant to the host physiology. A 1:1 ratio mixture of a and  $\alpha$  cells was inoculated into the mammalian cell culture medium RPMI at 37°C under 5% CO<sub>2</sub>. The 1:1 a/ $\alpha$  ratio of the initial inoculum was confirmed by measuring CFU of a and  $\alpha$  cells (see Table S3 in the supplemental material). The a/ $\alpha$  ratio in the coculture was measured after 3 and 14 days. The population was still increasing at 3 days postinoculation, while the population had long reached the stationary phase by 14 days postinoculation. The a/ $\alpha$  ratio maintained a 1:1 level in the coculture at both time points (see Table S3). Therefore, the a or the  $\alpha$  mating type locus allele does not



**FIG 4** Dissemination and growth of the two congenic strains are similar in the inhalation model and the intravenous infection model of cryptococcosis. (A) Animals were challenged with  $1 \times 10^5$  fungal cells intranasally. Animals were sacrificed in accordance with the virulence protocol, and the lungs and brains from five animals per group were harvested and homogenized. Serial dilutions of the homogenized tissue were plated to determine the organ fungal burden. There is no significant difference in the fungal burden at the time of euthanasia among three groups (AIR265 $\alpha$ , AIR265a, and  $\alpha$  + a) in the lungs (*P* = 0.078) or in the brains (*P* = 0.833). (B) Animals were challenged with  $1 \times 10^4$  fungal cells intravenously. When animals became moribund, spleens and brains from five terminated animals per group were harvested. Serial dilutions of the homogenized tissue were plated to determine the organ fungal burden. Neither the fungal burden in the brain (*P* = 0.204) nor that in the spleen (*P* = 0.620) showed any statistical difference among the groups in this model.



**FIG 5** The congenic strains have equal virulence in an insect model. Shown is survival of wax moth (*G. mellonella*) larvae inoculated with *C. gattii* strains or PBS. The PBS set was terminated at day 15, when the larvae had all formed cocoons. The *P* values were 0.639 (R265/CBS1930), 0.056 (R265/AIR265 $\alpha$ ), and 0.317 (R265/AIR265a). When these groups, excluding the PBS control, were analyzed by the one-way ANOVA test, there were no statistically significant differences among them (*P* = 0.7746).



**FIG 6** Mating type does not affect the competitiveness of the *MATa* and *MAT $\alpha$*  congenic strains in animals. Shown is *in vivo* competition between 1:1 starting ratios of the *MATa* and *MAT $\alpha$*  congenic strains, inoculated in mice by the intranasal (i.n.) or intravenous (i.v.) routes. The lungs from animals infected intranasally and the brains, kidneys, and spleens from animals infected intravenously were harvested and homogenized at the time of termination. Serial dilutions of the homogenized tissues were plated. Single colonies (75 to 100) from each organ were randomly picked, and their mating type was determined by crossing to reference strains. The overall proportions of *a* cells in all organs examined were close to 50%, indicating the absence of a competitive advantage of either mating type during infection in both murine models. Horizontal bars represent averages. Information for each mouse is provided in Table 1.

appear to confer any growth advantage in the coculture under physiological conditions.

We then coinfecting *a* and  $\alpha$  strains using both the inhalation and the intravenous models. In the inhalation infection model, only fungal cells recovered from lungs were further examined for their mating types. Cells recovered from the brain were not tested due to their low numbers in this organ. As shown in Fig. 6, the *a* and  $\alpha$  cells recovered from lungs from all seven animals examined (75 to 100 cells tested for each organ) displayed a similar 1:1 proportion in this inhalation model. There is no difference in terms of proliferation in the lungs between the *C. gattii a* and  $\alpha$  cells during coinfection.

In the intravenous infection model, mating type was assessed for fungal cells recovered from brains, spleens, and kidneys of seven animals (21 organs in total and 75 to 100 cells tested per organ). As shown in Fig. 6, the overall proportions of *a* and  $\alpha$  cells in all three organs were close to 1:1, although the variation of each organ is much larger than the tight distribution observed in the inhalation model. For a single mouse, the *a*/ $\alpha$  ratio could differ between organs, with no consistent pattern to suggest an advantage of a mating type in any organ (Table 1). For instance, for mouse 7, the *a*/ $\alpha$  cell ratios were 1:0.92 in the kidney, 1:2.1 in the spleen, and 1:0.43 in the brain. In contrast, for mouse 5, the *a*/ $\alpha$  cell ratios were 1:2.03 in the kidney, 1:0.29 in the spleen, and 1:3.13 in the brain. Therefore, unlike the modest predominance of brain infection observed of KN99 $\alpha$  during KN99 $\alpha$ -KN99*a* coinfection (41), our results suggest that the *C. gattii a* and  $\alpha$  strains are comparable in their organ tropism during coinfection.

**Characterization of *Bwc2* as a virulence factor common to *Cryptococcus* species.** In order to test the utility of these strains, a gene known to control virulence and mating efficiency was sought. The light-responsive *BWC2* homolog was selected while in the middle of the backcrossing process to isolate the congenic pair. The conserved *Bwc1-Bwc2* complex is responsible for UV/blue light perception in *C. neoformans* and influences mating, UV tolerance, and virulence (42–44). This pathway was tested to assess if the light-sensing machinery is involved in virulence more widely

than *C. neoformans* and to serve as a model to test the utility of the newly created congenic pair.

A *bwc2 $\Delta$*  mutant was isolated and tested for *in vitro* traits. The deletion strain was fertile and was crossed to the congenic AIR265*a* strain to isolate a *MATa* strain. However, due to the low level of *C. gattii* mating, it is difficult to obtain an accurate comparison of the mating efficiency in the light versus dark in the wild type or *bwc2 $\Delta$*  mutants. As expected, the *bwc2 $\Delta$*  mutants were more sensitive to UV light (Fig. 7A). Light induces expression of two genes, *HEM15* encoding ferrochelatase and *UVE1* encoding an endonuclease, in diverse fungi (45; S. Verma and A. Idnurm, unpublished data). Northern blot analysis indicated that the induction of the *HEM15* and *UVE1* genes by light is impaired in the *bwc2 $\Delta$*  mutant strain (Fig. 7B). Quantification of the levels of transcripts of these genes, normalized to the actin signal, indicates in the wild type 3- and 9-fold-higher levels in the light for *HEM15* and *UVE1*, respectively. This induction was absent in the *bwc2 $\Delta$*  mutant strain.

The virulence of the *bwc2 $\Delta$*  mutant was tested in the mouse inhalation model. As Fig. 3A shows, the deletion of *BWC2* in *C. gattii* leads to an attenuation in virulence in the inhalation animal model ( $P = 0.019$ ), and this defect was recovered in the strain in which the wild-type copy of the *BWC2* gene was introduced back to the *bwc2 $\Delta$*  mutant. Although the reduction in virulence in the *bwc2 $\Delta$*  mutant is not as dramatic in *C. gattii* as in the *C. neoformans* strains, these results are consistent with previous observations in *C. neoformans*.

#### Genetic analysis with the congenic pair and derived strains.

To be useful as a genetics resource, the congenic pair must be capable of normal chromosomal segregation during crosses. The segregation of genetic markers was examined in crosses using the congenic pair strains.

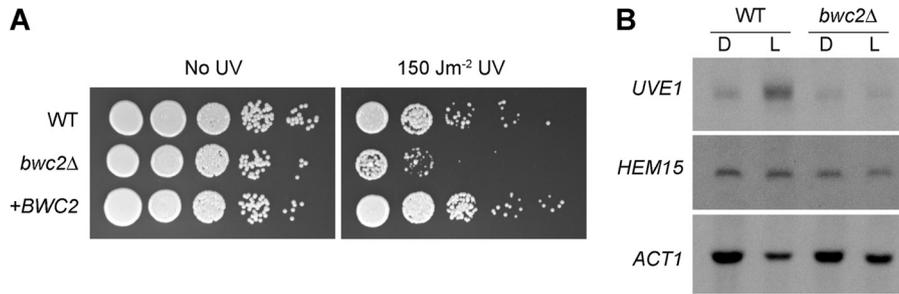
The *MAT $\alpha$*  strain AIR265 $\alpha$  was crossed to CBS1930, and 13 progeny were examined with 17 markers (Fig. 1, progeny set c; Fig. 8A). These progeny were all recombinant. Segregation of the markers, which includes evidence for recombination within a chromosome, was similar to that of the CBS1930  $\times$  R265 cross. Chromosomal DNA was isolated from cells and resolved by pulsed-field gel electrophoresis. No visible differences were observed between the congenic *a* and  $\alpha$  strains and the original R265 parent, whereas the second smallest chromosome of CBS1930 was of a different size (Fig. 8B). Thus, the  $\alpha$  strain AIR265 $\alpha$  of the congenic pair behaves similarly to R265 genetically and has an identical karyotype to strains R265 and AIR265*a*.

Spontaneous mutations affecting primary metabolism, for use

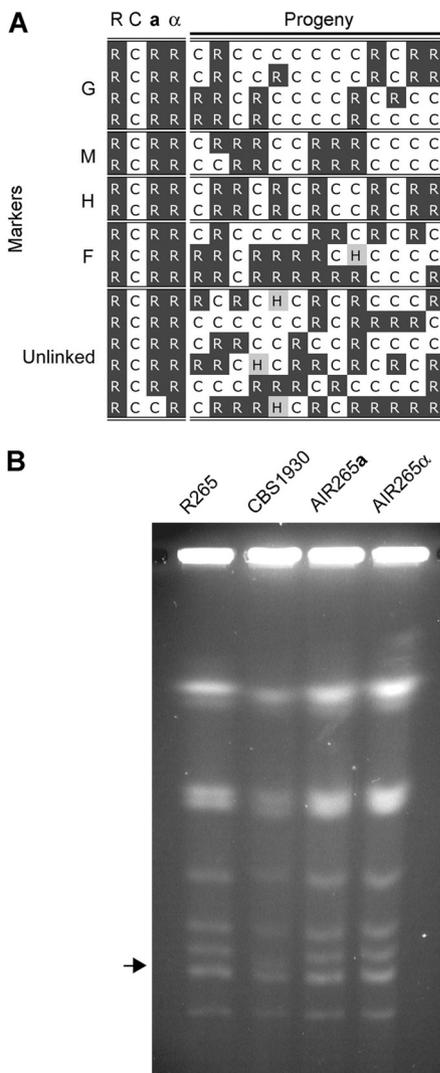
**TABLE 1** Results of *MATa*/*MAT $\alpha$*  competition in mice<sup>a</sup>

Mouse	No. (ratio) of <i>MATa</i> / <i>MAT<math>\alpha</math></i> cells in:		
	Kidney	Spleen	Brain
M1	77/19 (1:0.25)	21/75 (1:3.57)	65/69 (1:1.06)
M2	44/49 (1:1.113)	30/62 (1:2.07)	47/47 (1:1)
M3	31/65 (1:2.1)	33/59 (1:1.79)	76/19 (1:0.25)
M4	65/28 (1:0.43)	51/44 (1:0.86)	38/57 (1:1.5)
M5	31/63 (1:2.03)	69/20 (1:0.29)	23/72 (1:3.13)
M6	60/36 (1:0.6)	40/55 (1:1.38)	26/70 (1:2.7)
M7	50/46 (1:0.92)	31/65 (1:2.1)	67/29 (1:0.43)

<sup>a</sup> Organs were harvested, homogenized, and plated for colonies. The mating types of individuals were assessed by crossing them to the JEC20 *MATa* and JEC21 *MAT $\alpha$*  tester strains.



**FIG 7** Mutation of *BWC2* alters UV light sensitivity and transcript levels in response to light. (A) Ten-fold serial dilutions of wild-type strain R265, *bwc2Δ* strain Alg254, and the +*BWC2* complemented strain Alg268. One plate was exposed to 150 J m<sup>-2</sup> UV light. Plates were incubated for 2 days at 30°C and photographed. (B) Northern blots of total RNA isolated from cultures grown for 24 h in darkness (D) or with a 2-h light exposure (L). Blots were probed with fragments of the three genes. Normalized to actin levels, *UVE1* and *HEM15* are higher in the wild-type light sample, but not in the *bwc2Δ* mutant.



**FIG 8** (A) Recombination in 13 progeny from the AIR265 $\alpha$   $\times$  CBS1930 cross. Strain abbreviations: R, R265; C, CBS1930; a, AIR265a; and  $\alpha$ , AIR265 $\alpha$ . Four chromosomes (G, M, H, and F) have more than one locus, while the other six loci are unlinked on separate chromosomes. Black R, R265 allele; white C, CBS1930 allele; gray H, both alleles or ambiguous. The diagram is based on Data Set S1 in the supplemental material. (B) Resolution of chromosomal DNA from the parental and congenic pair by pulsed-field gel electrophoresis. The chromosome size polymorphism in strain CBS1930 is indicated by the arrow.

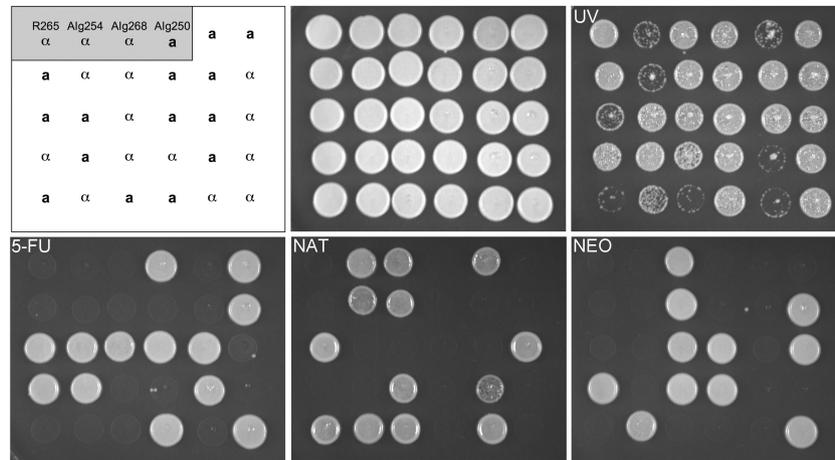
as genetic markers, were sought through positive selection. Strains resistant to 5-fluoroorotic acid or 5-fluorouracil were isolated. In other species, these arise due to mutation in the genes encoding orotidine-5'-phosphate decarboxylase or orotate phosphoribosyltransferase for 5-FOA, or uracil phosphoribosyltransferase for 5-FU. The *URA5* or *FUR1* genes were amplified and sequenced from five strains of each class of resistance. Mutations were identified in each strain (see Fig. S1 in the supplemental material). The uracil auxotrophs were less efficient at mating, including when the V8 juice medium was supplemented with uracil, so the *ura5* mutants were not pursued further. A 5-FU-resistant strain, AIgFUR1-1, mated as well as the wild type. The 19 progeny (germinated from 48 basidiospores) from the AIgFUR1-1  $\times$  AIR265a cross were as follows: 4 were 5-FU<sup>R</sup> MATa, 4 were 5-FU<sup>S</sup> MATa, 5 were 5-FU<sup>R</sup> MAT $\alpha$ , and 6 were 5-FU<sup>S</sup> MAT $\alpha$ .

A third example of marker segregation was in a cross between strain AIg268 (*bwc2::NAT BWC2-NEO*, MAT $\alpha$ ) and AIg250 (*fur1* MATa) (Fig. 1, progeny set d). Figure 9 shows the segregation of five traits, i.e., resistance to nourseothricin (*bwc2::NAT*), resistance to G-418 (*BWC2-NEO*; with the position of the T-DNA insertion in the genome unknown), mating type, UV tolerance (due to mutation of *BWC2*), and resistance to 5-FU (*fur1*). The results support the role of *BWC2* in conferring tolerance to UV light, since all NAT<sup>R</sup> NEO<sup>S</sup> strains are UV sensitive. Second, the markers segregate independently with one another, except for growth on 5-FU and nourseothricin (seven recombinants or 27 map units). The *BWC2* and *FUR1* genes are on the same chromosome. The *fur1* mutation is a T  $\rightarrow$  G change (see Fig. S1 in the supplemental material) at position 198527 on supercontig 9. The *bwc2* mutation replaces 1,174 bp with the *NAT* gene from positions 419309 to 420482. Thus, the genetic/physical distance ratio between *BWC2* and *FUR1* is 1 map unit per 8.2 kb. This experiment also demonstrates the utility of these strains for intrachromosomal segregation of genetic markers.

**Strain deposition.** The congenic pair and other *C. gattii* strains have been deposited at the Biodefense and Emerging Infections Resources repository ([www.beiresources.org](http://www.beiresources.org)) for free distribution.

**DISCUSSION**

*C. neoformans* and *C. gattii* diverged about 50 million years ago and are both complexes of distinct clades (46): all are capable of causing disease in people. The differences between these species at the level of gene function have been little explored. One useful tool



**FIG 9** Segregation of genetic markers in a cross between Alg268 (*bwc2::NAT BWC2-NEO*  $\alpha$ ) and Alg250 (*fur1 a*). The relationship between the transformation recipient or parental strains (indicated by the gray box) is provided in Fig. 1. Overnight cultures were spotted onto YPD medium or YPD supplemented with 250 mg/liter 5-FU, 100 mg/liter nourseothricin (NAT), or 100 mg/liter G-418 (NEO). One plate was irradiated with 150 J m<sup>-2</sup> UV. Plates were incubated for 2 days at 30°C. Mating type was determined by crossing to **a** and  $\alpha$  reference strains.

for research is strains that are identical in genetic background. For example, congenic pairs have been constructed for *C. neoformans* var. *neoformans* (20, 35) and var. *grubii* (40), and these strains have been widely used in biological and pathological studies. The strains reported here establish the first congenic pair for *C. gattii*. The congenic strains were characterized from two perspectives: a pathogenesis analysis to examine the ability of the strains to cause disease and dissemination patterns in animal models and a genetic analysis to ensure normal segregation of genes during crosses.

In contrast to the effects of *C. neoformans*, the *C. gattii* strains in the inhalation model of mice showed a severe pulmonary infection and a minor brain infection at the late stages of the disease. Similar patterns of pathogenesis are also described in a recent direct comparison between *C. neoformans* strain H99 and *C. gattii* strain R265 (37). Due to the high virulence potential of the parental R265 strain and its derived congenic strains observed in the inhalation model, we used a 10-fold-lower inoculum in the intravenous infection model. Despite the lower inoculum, the *C. gattii* strains caused rapid development of disease symptoms in the infected mice, and the fungal burdens of all three organs examined were high in the intravenous infection model. Our data indicate that the **a** and  $\alpha$  congenic strains behave similarly when they infect animals alone.

Furthermore, the **a** and  $\alpha$  congenic strains behave similarly in both mouse models when coinfecting; therefore, mating type does not impact the overall virulence or the tissue tropism of the *C. gattii* strains in the R265 genetic background. Although most of *C. gattii* Vancouver Island outbreak isolates are of the  $\alpha$  mating type, this is unlikely caused by any superiority of the  $\alpha$  mating type over the **a** mating type in terms of growth or organ tropism. Rather, predominance of the  $\alpha$  strains in the environment due to other factors is most likely the cause of the corresponding predominance of the  $\alpha$  strains in clinical settings. The strains used in this study, R265 and CBS1930, are classified in the VGII lineage. To conclude more broadly about a contribution of *MAT* to virulence in *C. gattii* would require similar studies in the VGI, VGIII, and VGIV lineages.

The analysis of genetic markers in the progeny of crosses pro-

vides evidence for recombination between the *C. gattii* strains and evidence that the congenic pair can be used to generate strains bearing multiple genetic changes via crossing. There have been few studies on the fertility of *C. gattii* strains, with most fertility being an estimate based on the formation of filaments and/or basidiospores. Compared to other congenic strains (e.g., JEC20/JEC21 or KN99a/KN99 $\alpha$ ), the *C. gattii* congenic pair filaments and sporulates poorly, which is a limitation to the use of these strains for studying genes that regulate mating. Indeed, it appears that the R265 background may be impaired in the production of spore chains because these are not observed in crosses between the congenic pair. Poor mating could simply reflect our lack of knowledge about the best conditions to induce mating in this strain background or actual low fertility.

Inheritance of mitochondria is often predominantly uniparental in *C. neoformans* crosses, and previous research has implicated the mating type locus as conferring this effect to drive inheritance from the *MATa* parent (34). In *C. gattii* VGII  $\times$  VGII crosses, the mitochondrial genome is inherited from either parent (Voelz et al., unpublished data). Thus, our results provide a different perspective on uniparental inheritance, in that this is controlled by more than one part of the genome. This hypothesis is further supported by evidence that factors like the transcription factor Mat2, which is located outside the *MAT* locus, control prezygotic aspects of mitochondrial inheritance (47). When the strains are identical other than the *MAT* alleles, the effects of the *MAT* locus predominate, leading to uniparental inheritance controlled by the *MAT* locus. Additional evidence for biparental inheritance in *Cryptococcus* species is shown by examples of mitochondrial recombination (48–50). The observation of uniparental transmission of mitochondria in *C. neoformans* has been made using crosses between congenic strains or across varieties. This inheritance pattern is worth revisiting in crosses between noncongenic strains.

The new *C. gattii* strains provide a way to examine the evolution of gene functions in *C. neoformans* varieties. *C. neoformans* is currently divided into two varieties, *neoformans* and *grubii*. The varieties have different biologies, and there are examples in which

genes have different functions in the two groups (for example, see references 51–55). Analysis of gene functions in *C. gattii*, such as has been done with *STE12α* (56), provides the outgroup taxon to explore which state is the original and which the derived in *C. neoformans*.

An advantage of a congeneric pair for *C. gattii* is their use in genetic analysis approaches. Crossing can be used to establish linkage between phenotype and genotype, cross out any inadvertent mutations arising from transformation or *in vitro* passaging, and compile complex genotypes. The congeneric pair also enables the effects of nuclear genes on mitochondrial inheritance to be tested. To facilitate future studies, the strains have been deposited in an international repository, BEI Resources, which is supported by the National Institutes of Health for no-cost distribution to researchers worldwide.

In summary, these results direct our understanding of the basis for pathogenesis of *C. gattii* strains responsible for the outbreak. First, we show that neither the mating type nor mitochondrial genotype impacts the virulence of these strains. Second, marker segregation indicates the ability to exchange genetic information between parents of VGII × VGII *C. gattii* crosses. Third, the poor mating exhibited by the congeneric pair, relative to *C. neoformans* strains, suggests that this process is less efficient in the outbreak strains, although the caveat to this observation is that the conditions that may best induce mating in the wild are unknown for *C. gattii*. Lastly, the new *C. gattii* strains represent a resource that will be essential in future molecular biology and genetic experiments directed toward understanding the origins of fungal outbreaks and virulence in *Cryptococcus* species.

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