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Interaction Between Genetic Background and the Mating-Type Locus in *Cryptococcus neoformans* Virulence Potential

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ABSTRACT

The study of quantitative traits provides a window on the interactions between multiple unlinked genetic loci. The interaction between hosts and pathogenic microbes, such as fungi, involves aspects of quantitative genetics for both partners in this dynamic equilibrium. One important pathogenic fungus is *Cryptococcus neoformans*, a basidiomycete yeast that can infect the human brain and whose mating system has two mating type alleles, **a** and α. The α mating-type allele has previously been linked to increased virulence potential. Here congenic *C. neoformans* strains were generated in the two well-characterized genetic backgrounds B3501α and NIH433**a** to examine the potential influence of genes outside of the mating-type locus on the virulence potential of mating type. The congenic nature of these new strain pairs was established by karyotyping, amplified fragment length polymorphism genotyping, and whole-genome molecular allele mapping (congenicity mapping). Virulence studies revealed that virulence was equivalent between the B3501 **a** and α congenic strains but the α strain was more virulent than its **a** counterpart in the NIH433 genetic background. These results demonstrate that genomic regions outside the mating type locus contribute to differences in virulence between **a** and α cells. The congenic strains described here provide a foundation upon which to elucidate at genetic and molecular levels how mating-type and other unlinked loci interact to enable microbial pathogenesis.

HUMAN pathogenic fungi are increasing in prevalence as the population of immunocompromised individuals escalates due to human immunodeficiency virus (HIV)/AIDS and to immunosuppression associated with cancer and its therapy. Like their human hosts, pathogenic fungi are eukaryotic cells and therefore current antifungal treatments are limited and often either stimulate the emergence of drug-resistant isolates or are quite toxic to humans. Thus, an understanding of the mechanisms by which fungal pathogens have adapted to survive and cause disease in their hosts is of paramount importance. For pathogenic fungi, which like most fungi can reproduce both sexually and asexually, recent studies have begun to forge a link between mating and virulence.

The role of mating in pathogenicity differs among human pathogenic fungi. For example, mating is thought to be an integral part of the Pneumocystis infection cycle on the basis of morphological analysis of infected lung tissue (reviewed in Cushion 2004; Thomas and Limper 2004). In other pathogenic fungi, such as *Coccidioides immitis* or *Aspergillus fumigatus*, a sexual cycle has not yet been described in the laboratory but population genetics studies provide evidence of actively recombining populations (Burt *et al.* 1996; Koufopanou *et al.* 1997; Varga and Toth 2003; Paoletti *et al.* 2005). The propagation of some organisms, such as *Candida albicans* and *Cryptococcus neoformans*, is largely clonal although there is evidence of recombination and both organisms have retained mating-type loci, mating machinery, and either a complete sexual cycle or at least a parasexual one (Kwon-Chung 1975; Pujol *et al.* 1993; Gräser *et al.* 1996; Franzot and Casadevall 1997; Hull and Johnson 1999; Xu *et al.* 1999; Hull *et al.* 2000; Magee and Magee 2000; Lengeler *et al.* 2002; Litvintseva *et al.* 2003). Mating of *C. neoformans* has thus far been observed only in the laboratory, although isolation of intervarietal hybrid strains reflects mating events that can occur in nature (Lengeler *et al.* 2001; Hull and Heitman 2002). There is also substantial evidence that several components involved in Cryptococcus mating are associated with virulence, including the transcription factor Ste12 and the PAK kinase Ste20 (Yue *et al.* 1999; Chang *et al.* 2000; Wang *et al.* 2002; Davidson *et al.*

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2003). Here we address the role of mating type in the virulence of *C. neoformans*.

C. neoformans occurs in two varieties—*grubii* (serotype A) and *neoformans* (serotype D)—and diverged from the sibling species *C. gattii* ~40 million years ago (Xu *et al.* 2000). The *grubii* and *neoformans* varieties have different disease epidemiologies with var. *grubii* causing the vast majority of cryptococcosis worldwide and >99% of infections in AIDS patients (Casadevall and Perfect 1998). However, in Europe var. *neoformans* can account for up to 20% of cryptococcosis cases, many in the context of an AD hybrid background.

Cryptococcus has two mating types—**a** and α. Yet, the vast majority of human cryptococcosis is caused by strains of the α mating type. Mating-type alleles in Cryptococcus are determined by a *MAT* locus that is >100 kb and contains >20 genes (Lengeler *et al.* 2002; Fraser *et al.* 2004). Analysis of markers within and flanking the *MAT* locus has shown that recombination is suppressed in the *MAT* locus (Lengeler *et al.* 2002). Due to the large size and complexity of the *MAT* locus, simple gene exchange experiments may not be sufficient to elucidate the role of this large genomic region in virulence. Here, **a** and α congenic strains were generated by a series of 10 backcrosses, yielding strains that are identical except at the *MAT* locus. In a similar previous study, the var. *neoformans* α mating-type strain JEC21α was found to be more virulent than the congenic **a** mating-type strain JEC20**a**, suggesting that α strains are more virulent in mice than **a** strains (Kwon-Chung *et al.* 1992). In contrast, the var. *grubii* congenic strains KN99**a** and KN99α showed no difference in murine virulence (Nielsen *et al.* 2003). However, following co-infection with the var. *grubii* congenic strains, the α strain more efficiently colonized the central nervous system than the **a** mating-type strain and provided evidence for a contribution of the *MAT* locus at least during co-infection (Nielsen *et al.* 2005).

These observations lead to a number of questions. Are there innate differences in the pathogenicity of varieties *grubii* and *neoformans*? Does the genetic background of the strain affect the role of mating type in virulence? Are 10 backcrosses sufficient to produce congenic strains? Can minor differences between the genomes of the congenic strains account for their virulence differences? To address these questions we generated two additional congenic strain pairs in var. *neoformans* (serotype D) and examined their levels of congenicity and their virulence. Our findings reveal that the virulence of the congenic strains differs from that of the parental strains and that genetic background can determine whether a virulence difference is observed between **a** and α cells in a murine model of cryptococcosis. The congenic strains described here can be used in future studies to identify other unlinked loci that interact with the mating-type locus to quantitatively affect virulence of Cryptococcus.

MATERIALS AND METHODS

Strains and media: Parental var. *neoformans* strains used in this study were NIH433**a**, B3501α, JEC20**a**, and JEC21α (Kwon-Chung *et al.* 1992). B3501Aα denotes the freezer isolate of strain B3501α that was sequenced at Stanford, and it is identical to strain B3501α with the exception of one minor chromosome length polymorphism noted by pulsed-field gel electrophoresis (PFGE) (Loftus *et al.* 2005). Strains were grown on yeast extract-peptone-dextrose (YPD) medium. Matings were on V8 medium [5% (v/v) V8 juice, 3 mm KH_2PO_4, 4% (w/v) bactoagar] (Kwon-Chung *et al.* 1982).

Congenic strain construction: To generate the congenic strain pair KN3501**a** and KN3501α, the parental strain JEC20**a** was crossed with strain B3501α and single basidiospores were isolated (Figure 1B). One of the F_1 **a** progeny from this mating was backcrossed to B3501α and single basidiospores were isolated. The process of isolating **a** single-basidiospore cultures and backcrossing to B3501α was repeated an additional eight times. After the last backcross sibling **a** and α progeny were selected and designated KN3501**a** and KN3501α. The process was repeated to generate the congenic KN433**a** and KN433α strain pair from the parental strains NIH433**a** and JEC21α (Figure 1C). The α progeny strains were backcrossed to NIH433**a** and sibling strains were isolated from the tenth backcross.

PFGE: PFGE was as described previously (Marra *et al.* 2004). Cells were grown in YPD at 30° with shaking at 250 rpm to an optical density at 600 nm of 0.5. Spheroplasts were prepared according to Lengeler *et al.* (2000), with modifications based on Wickes *et al.* (1994). Plugs containing ~1 μg DNA were electrophoresced in a 13 × 14-cm 1% PFGE-grade agarose gel (Bio-Rad, Hercules, CA) in 0.5× Tris-borate-EDTA (TBE). Chromosomes were separated in 0.5× TBE using contour-clamped homogeneous electric field (CHEF) in a Bio-Rad DRII apparatus with the following settings: initial A time, 75 sec; final A time, 150 sec; start ratio, 1.0; run time, 40 hr; mode, 10; initial B time, 200 sec; final B time, 400 sec; start ratio, 1.0; run time, 56 hr; mode, 11. The voltage was set to 4 V/cm and the buffer temperature was set to 12°. The gel was stained in 0.5 μg/ml ethidium bromide for 0.5–1.0 hr, visualized, and photographed using ultraviolet light on a Gel-Doc imager (Bio-Rad).

AFLP: Amplified fragment length polymorphisms (AFLPs) were carried out as previously described (Boekhout *et al.* 2001). Briefly, the restriction and ligation reactions were performed simultaneously on 10 ng DNA using *Mse*I, *Eco*RI, and T4 DNA ligase, combined with *Eco*RI and *Mse*I adaptors from PE Biosystems AFLP microbial fingerprinting kit. The first PCR was performed using *Eco*RI and *Mse*I core sequence preselective primers. The second PCR used a more selective *Eco*RI primer labeled at the 5′ end with 6-carboxyfluorescein (FAM) combined with a more selective *Mse*I primer. The AFLP products were electrophoresced on a 5% polyacrylamide gel on an ABI310 sequencer (PE Biosystems).

Congenicity mapping: Genomic DNA was prepared using the Camgen yeast genomic DNA extraction kit (Whatman Bioscience) with the addition of 425- to 600-μm glass beads and vortexing for 1 min as a preparatory step. DNA concentration was adjusted to 10 ng/μl and diluted 10-fold for PCRs in 10 mm Tris-Cl, pH 8.0. A subset of microsatellites and restriction fragment length polymorphisms (RFLPs) identified as polymorphic in the B3501/B3502 linkage map (Marra *et al.* 2004) were selected to span each linkage group.

Microsatellite polymorphisms were characterized as described by Marra *et al.* (2004). Briefly, 100–300 bp of sequence flanking the microsatellite locus was amplified by PCR. The PCR amplicons were evaluated for size polymorphisms by

electrophoresis in 6.7% polyacrylamide in 1× TBE in a 30 × 38-cm Bio-Rad SequiGen GT vertical gel apparatus. After electrophoresis the gels were silver stained and then exposed for 1–2 sec with white light onto X-ray duplication film (Kane X-Ray) and developed according to standard procedures.

RFLPs were characterized as described by Marra *et al.* (2004). Briefly, ~800 bp of sequence flanking the RFLP was amplified by PCR. The PCR amplicons were then digested with the appropriate restriction enzyme and electrophoretically separated in 1% agarose gels in 1× TBE. Gels were visualized with UV light and photographed in a MultiImage light cabinet (Alpha Innotech).

Virulence studies: Virulence studies were performed using the murine tail-vein injection model. Four- to 6-week-old female DBA mice (10 per strain) were injected directly in the lateral tail vein with 5×10^6 or 1×10^6 cells. The concentration of cells in the inoculum was confirmed by plating serial dilutions and enumerating colony-forming units (CFUs). Mice were monitored twice daily and those that showed signs of severe morbidity (weight loss, abnormal gait, extension of the cerebral portion of the cranium) were killed by CO_2 inhalation. The animal protocol was approved by the Duke University Animal Use Committee. Survival data from the mouse experiments were analyzed by the Kruskal-Wallis test, and for animals that survived to the termination of the experiment the last day was considered the date of death for these analyses. Statistical results did not change if we assumed that the surviving animals at the termination of the experiment survived an additional 1000 days.

RESULTS

Congenic strain development: Additional congenic strains were developed to determine whether genetic background affects the virulence of the **a** and α mating types in *C. neoformans*. The original var. *neoformans* congenic strains JEC20**a** and JEC21α were generated by crossing the environmental strain NIH433**a**, which has relatively low virulence in mice, with the clinical strain NIH12α (high virulence) to generate B3502**a** (low virulence) and B3501α (high virulence). Mating-type α progeny from a cross of B3501α to B3502**a** were backcrossed nine times to generate JEC21α and JEC20**a**, which is analogous to B3502**a** (Figure 1A). JEC21α was originally found to be more virulent than JEC20**a** but both strains had relatively low virulence (Kwon-Chung *et al.* 1992). The low virulence observed in the JEC20/21 congenic strains has at least two possible explanations. First, the low virulence could be due to the B3502**a** genetic background. Alternatively, the low virulence could be due to the multiple passages involved in generating the congenic strains. Previous studies have shown that continuous *in vitro* culture can reduce the virulence of *C. neoformans* strains (Franzot *et al.* 1998).

To differentiate between these two possibilities, and to determine the role of genetic background in mating-type virulence potential, we isolated congenic strains in two related strain backgrounds, B3501α and NIH433**a** (Figure 1A). The B3501α strain background has high virulence whereas the environmental isolate NIH433**a** has low virulence. Congenic strains were not isolated in the clinical NIH12α genetic background due to abundant monokaryotic fruiting by this strain. Because recombination is suppressed in the *MAT* locus (Lengeler *et al.* 2002), the sequenced JEC20**a** *MAT* locus should be identical to the NIH433**a** *MAT* locus. Similarly, the sequenced JEC21 *MAT*α allele is identical to the *MAT*α allele of B3501Aα (Loftus *et al.* 2005). Therefore, JEC20**a** and JEC21α were used as the parental strains to generate the congenic strains. Figure 1B illustrates how the JEC20**a** *MAT* locus was backcrossed into the B3501α genetic background to generate the sibling strains KN3501**a** and KN3501α. The KN3501 congenic strains, like their B3501α parental strain, are derived from a 50% clinical and 50% environmental genetic background. The JEC21α *MAT* locus was backcrossed into the NIH433**a** genetic background to generate the sibling strains KN433**a** and KN433α (Figure 1C), which are in an environmental genetic background.

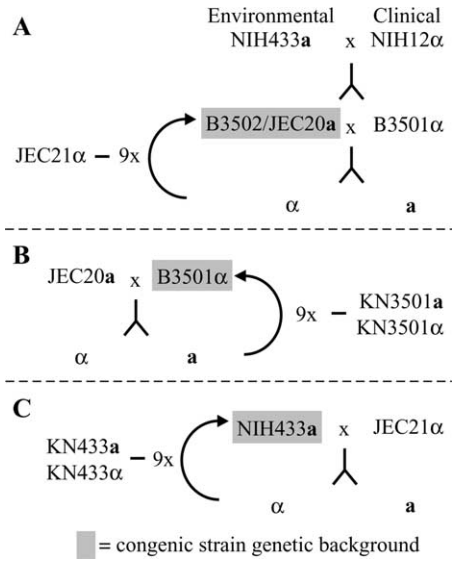

Figure 1.—The mating schemes used to produce *C. neoformans* congenic strain pairs. (A) The congenic pair JEC20**a** and JEC21α were produced by Jeff Edman and June Kwon-Chung through an initial cross between the environmental isolate NIH433**a** and the clinical isolate NIH12α to generate the F_1 strains B3501α and B3502**a**. Mating-type α progeny from a B3501α and B3502**a** cross were backcrossed nine times to produce JEC21α, which is congenic with B3502/JEC20**a** (Kwon-Chung *et al.* 1992; Marra *et al.* 2004). (B) Here the congenic pair KN3501**a** and KN3501α were produced through an initial cross of JEC20**a** to B3501α followed by additional backcrosses to B3501α. KN3501**a** and KN3501α are sibling progeny from the final backcross. (C) The congenic pair KN433**a** and KN433α were produced through an initial cross of NIH433**a** to JEC21α followed by additional backcrosses to NIH433**a**. KN433**a** and KN433α are sibling progeny from the final backcross.

Congenicity of strain pairs: The KN3501 and KN433 congenic strain pairs were compared to each other and to the parent strains to identify any differences between the strains. First, the karyotype of the strains was analyzed using PFGE. Figure 2 shows that the KN3501

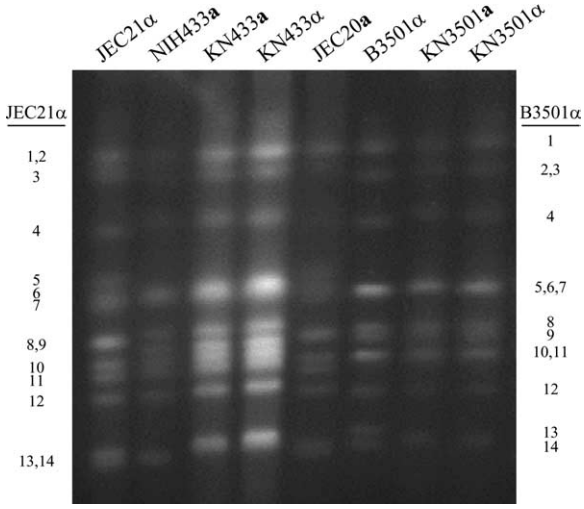

FIGURE 2.—Karyotype analysis of *C. neoformans* congenic and parental strains. Chromosomes were separated by PFGE and the gel was strained with ethidium bromide. JEC21α and B3501α chromosome numbers are based on MARRA *et al.* (2004) and LOFTUS *et al.* (2005).

parental strains JEC20**a** and B3501α have different karyotypes. The most striking differences are the sizes of chromosomes 2, 5, 6, 8, 11, and 13. The sibling congenic strains KN3501**a** and KN3501α have identical karyotypes and their karyotype is most similar to B3501α. However, chromosomes 13 and 14 in the KN3501 congenic strains comigrate with those present in JEC20**a**.

The KN433 parental strains NIH433**a** and JEC21α also have different karyotypes. Chromosome 4 is larger in NIH433**a** than in JEC21α but chromosomes 5, 6, and 9 are all smaller than those of JEC21α. The sibling congenic strains KN433**a** and KN433α have identical karyotypes and show all the characteristics of the NIH433**a** karyotype. Thus, the KN3501 and KN433 congenic strain pairs are identical at the chromosome level, although the KN3501 strains differ from either parental strain.

AFLPs were used to genotype each strain to analyze the congenicity of the genomes. Comparison of the parental strains JEC20**a** with B3501α (Figure 3A) and of NIH433**a** with JEC21α (Figure 3B) reveals polymorphic fragments between the parental strains (Figure 3, A and B, arrows). The KN3501 congenic strain pair has AFLP genotypes that differ at only one location (Figure 3A, *) and their genotypes most resemble B3501α. The KN433 congenic strains have identical AFLP genotypes. Interestingly, one band present in NIH433**a** is absent in the congenic strains (Figure 3B, star), indicating that the KN433 strains might not be exactly identical to NIH433**a**. These data suggest that at a genomic level there is at least one difference between KN3501**a** and KN3501α but no difference could be detected between KN433**a** and KN433α.

One advantage of constructing congenic strains in the NIH433**a** and B3501α genetic backgrounds is the availability of the B3501/B3502 linkage map (MARRA *et al.* 2004). This linkage map is based on microsatellites, RFLPs, and insertions/deletions (INDELs) between the B3501α and B3502/JEC20**a** genomes. To identify any detectable differences between the KN3501**a** and KN3501α congenic strains, microsatellite and RFLP markers that span the linkage groups and chromosomes were chosen (Figure 4). Chromosome 10 could not be analyzed because it is monomorphic (and presumably congenic) between B3501α and B3502**a**. All markers found to be polymorphic between B3502**a** and B3501α on the linkage map were also monomorphic between JEC20**a** and B3502**a,** indicating that these strains are isogenic. After 10 backcrosses into the B3501α genetic background, both KN3501**a** and KN3501α retained three regions (six markers in KN3501**a** but five markers in KN3501α) with JEC20**a** identity (Figure 4) that constitute ~626 kb or ~3% of the genome. Thus, KN3501**a** and -α are 97% congenic with B3501α.

To determine whether the remaining 3% of the genome could be rendered congenic we analyzed strains from an additional cross of KN3501**a** to B3501α. We sought to identify an **a** progeny strain that was congenic to B3501α for the two largest regions of dissimilarity on chromosomes 6 and 11. One **a** strain was found to contain the B3501 allele at both regions but this strain was sterile when crossed to B3501α. These regions of the genome do not appear to contain genes that might be implicated in self/nonself recognition or other mating processes so it is unclear what role, if any, these polymorphisms might play in fertility.

Nonetheless, comparison of the sibling strains KN3501**a** and KN3501α showed that the two strains differ from each other only at the *MAT* locus and in

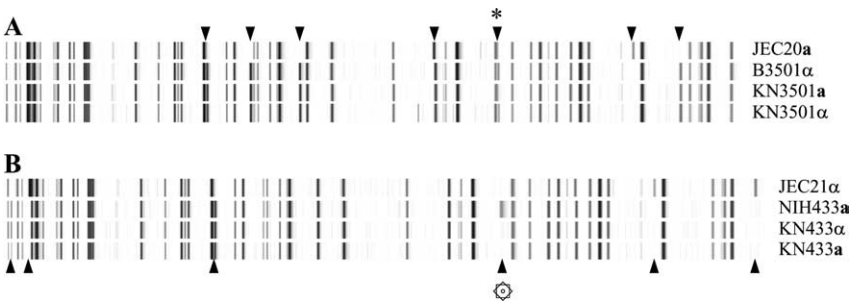

FIGURE 3.—AFLPs from *C. neoformans* parental and congenic strains. Polymorphic products are indicated by arrowheads. (A) AFLP products for the congenic strains KN3501**a** and KN3501α and their parental strains JEC20**a** and B3501α. The polymorphism between the congenic pair is indicated by an *. (B) AFLP products for the congenic strains KN433**a** and KN433α and their parental strains NIH433**a** and JEC21α. The polymorphism between the parental strain NIH433**a** and the congenic pair is indicated by a star.

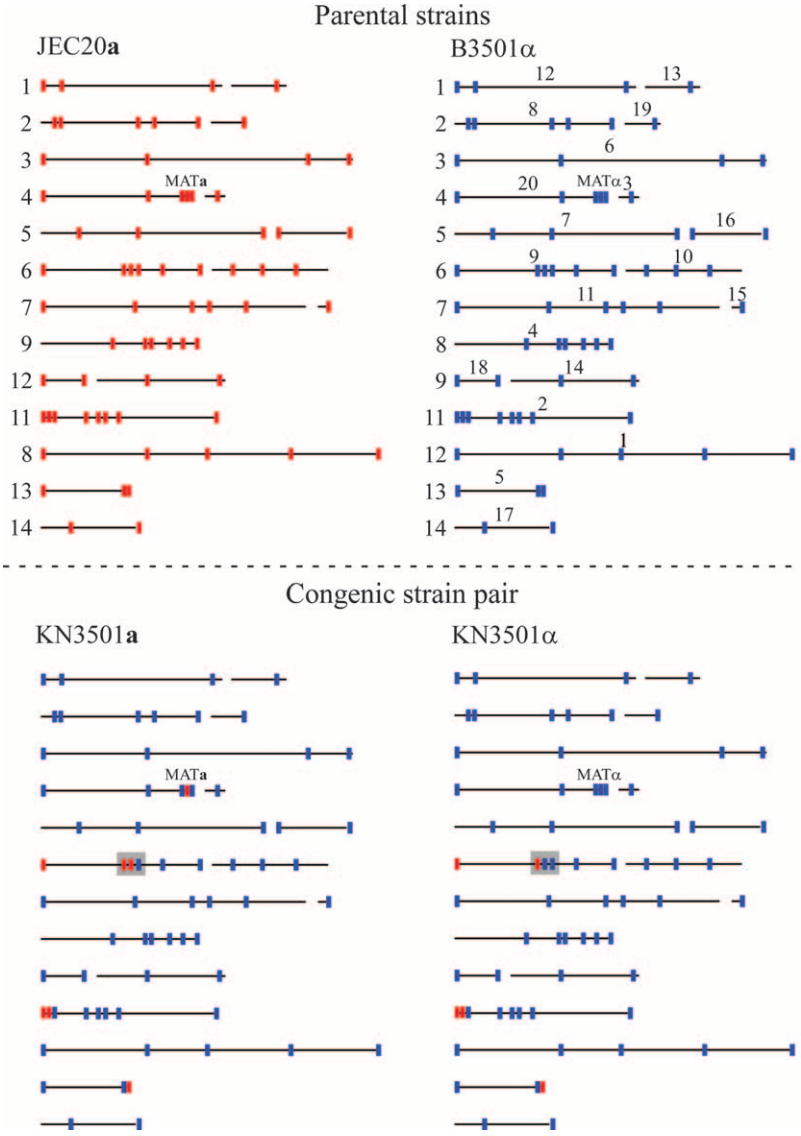

FIGURE 4.—Congenicity maps for the KN3501**a** and KN3501α congenic strain pair. This analysis was based on 28 microsatellite markers, 34 RFLP markers, and 1 *MAT* locus marker. Black lines designate the B3501α linkage groups (numbered as in the B3501α map by MARRA *et al.* 2004). The chromosome containing each linkage group for JEC20**a** and B3501α is designated at the left of each line. Red bars indicate the JEC20**a** allele and blue bars designate the B3501α allele at each locus tested. The area of dissimilarity between KN3501**a** and KN3501α is indicated by a shaded box.

an ~15-kb region on chromosome 9 and thus are 99.93% identical. These data indicate that KN3501**a** and KN3501α are congenic with each other, even though they differ by ~3% from the B3501α parental background. Because the genomes of both B3501α and JEC20/21 are known, we can infer the complete genome sequence of the KN3501 congenic strains.

Since B3501α and B3502**a** are siblings from a cross of NIH12α to NIH433**a**, the polymorphisms observed between B3501α and B3502**a** are due to the presence of the NIH12α or NIH433**a** allele at each locus. Therefore, B3502**a** received 50% of its genome from NIH12α and the other 50% from NIH433**a**. Because B3502**a** and JEC20/21 are isogenic (Figure 1 and MARRA *et al.* 2004), when we compared NIH433**a** and JEC21α we anticipated that 50% of the markers would be monomorphic (Figure 5, black bars) and 50% would be polymorphic (Figure 5, blue bars *vs.* red bars), in accord with our experimental observations. After 10 backcrosses into the NIH433**a** genetic background, both KN433**a** and KN433α were identical to each other and to NIH433**a** at all markers examined except those in the *MAT* locus.

In summary, the KN3501 and KN433 congenic strains were mapped and both strain pairs were estimated to be >99.9% identical. Furthermore, no differences were observed between the KN433 congenic strains and their parental strain NIH433**a** but the KN3501 congenic strains differed from the B3501α parental strain.

Congenic strain virulence: We next compared the genomic profile of the strains with their virulence in a murine tail-vein model of cryptococcosis. Figure 6A shows that the congenic KN3501**a** and KN3501α strains produced very similar survival curves with no significant difference ($P = 1.00$). Similar results were obtained when the experiment was repeated with an inoculum of 5×10^6 cells (data not shown). Interestingly, the congenic strains were significantly less virulent than the genetic background strain B3501α ($P \leq 0.009$),

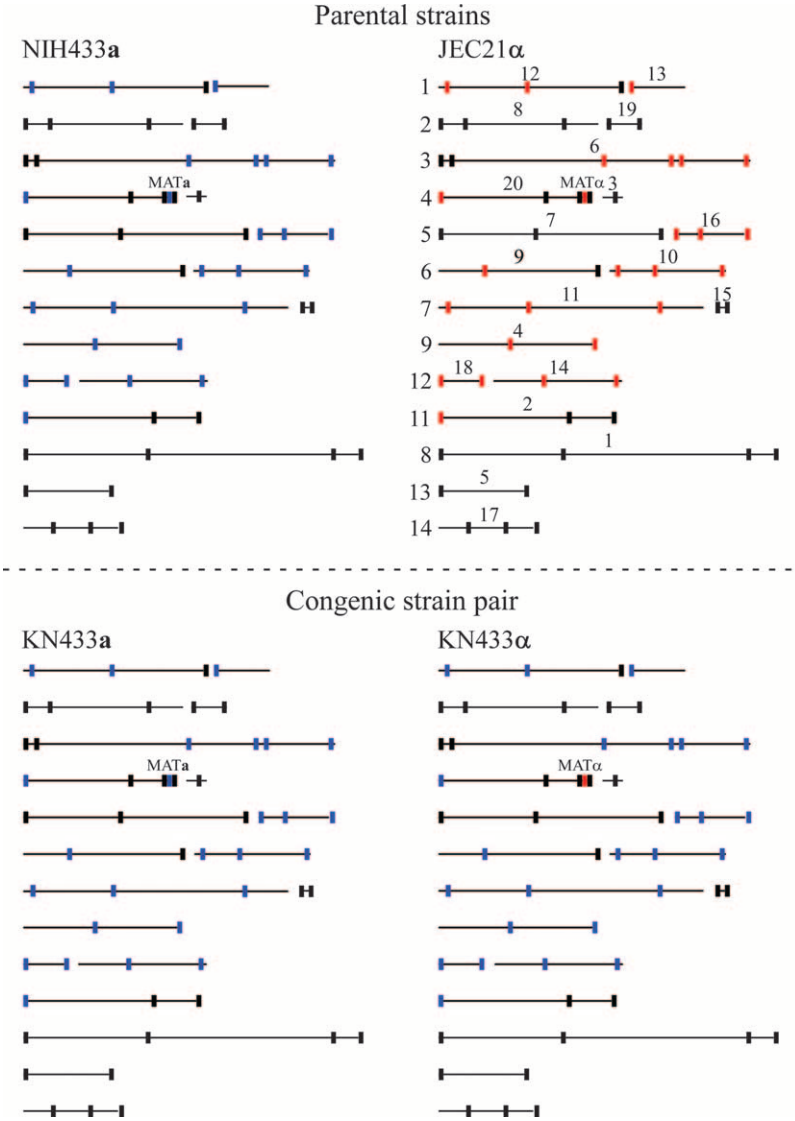

FIGURE 5.—Congenicity maps for the KN433**a** and KN433α congenic strain pair. This analysis was based on 54 RFLP markers and 1 *MAT* locus marker. Black lines designate the linkage groups based on the B3501/B3502 linkage map by MARRA *et al.* (2004) (numbered as in the JEC21α map). The chromosome containing each linkage group for JEC21α is designated at the left of each line. Black bars indicate loci that contained the same allele in NIH433**a** and JEC21α; blue bars indicate the NIH433**a** allele; and red bars designate the JEC21α allele at each locus tested.

supporting the conclusion that their reduced virulence was attributable to either multiple passages or the 626-kb region persisting from JEC20**a**. The parental strains JEC20**a** and B3501α differed slightly in their survival curves with the α strain appearing to cause lethal infections of all animals faster than the **a** strain. However, this apparent difference in virulence between JEC20**a** and B3501α did not meet statistical significance ($P = 0.427$). Thus, the congenic KN3501**a** and KN3501α strains showed no difference in virulence in two independent experiments.

The survival curves in Figure 6B demonstrate that KN433α is significantly more virulent than the congenic strains NIH433**a** or KN433**a** ($P = 0.001$ and 0.009, respectively). These data support the conclusion that the NIH433 genetic background somehow influences the observed differences in virulence between **a** and α cells. No difference in growth at high temperature, auxotrophy, capsule size, or melanin production was observed *in vitro* that could account for the difference in virulence between KN433α and KN433**a** or NIH433**a** (data not shown). There was no statistically significant difference in virulence between the parental strains NIH433**a** and JEC21α ($P = 0.473$). We also note that the KN433 congenic strains were as or more virulent than both parental strains, suggesting that passage has contributed to enhanced virulence of the NIH433 environmental background.

DISCUSSION

The model yeast *Saccharomyces cerevisiae* and the human pathogenic fungus *C. albicans* have relatively small *MAT* loci consisting of just a few genes, which facilitates the generation of isogenic strains by allele exchange or single-gene disruptions. In contrast, the Cryptococcus *MAT* locus spans >100 kb and contains >20 genes, which makes the generation of isogenic strains considerably more challenging. To overcome this, we generated

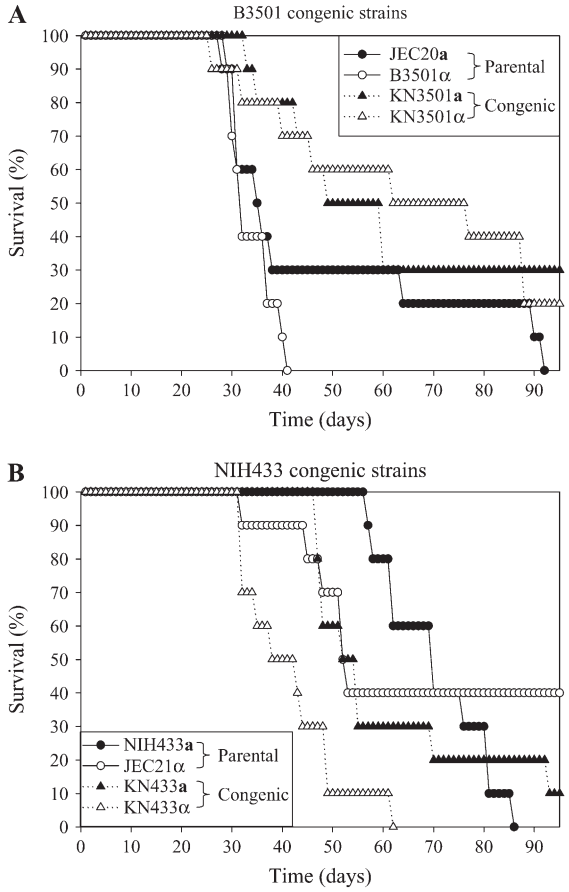

FIGURE 6.—Virulence of *C. neoformans* parental and congenic strains. Groups of 10 DBA mice were infected with 10^6 cells by lateral tail-vein injection and progression to severe morbidity was monitored. (A) Comparison of the survival of mice infected with the KN3501**a** and KN3501α congenic pair compared to that of the parental strains. *P*-values were 1.000 (KN3501**a**/KN3501α), 0.002 (KN3501**a**/B3501α), 0.081 (KN3501**a**/JEC20**a**), 0.009 (KN3501α/B3501α), 0.162 (KN3501α/JEC20**a**), and 0.427 (B3501α/JEC20**a**). (B) Comparison of the survival of mice infected with the KN433**a** and KN433α congenic pair compared to that of the parental strains. *P*-values were 0.009 (KN433**a**/KN433α), 0.064 (KN433**a**/NIH433**a**), 0.734 (KN433**a**/JEC21α), 0.001 (KN433α/NIH433**a**), 0.016 (KN433α/JEC21α), and 0.473 (NIH433**a**/JEC21α).

C. neoformans var. *neoformans* congenic strains in different, but related, genetic backgrounds by a backcrossing approach in which the *MAT* locus alleles were introgressed onto different genetic backgrounds. This approach yields strains in which the *MAT* locus **a** or α allele is present on otherwise identical genetic backgrounds.

The completely sequenced genomes of the *C. neoformans* strains B3501Aα and JEC21α (and by inference its congenic partner JEC20**a**) represent unique and invaluable tools for conducting genetic and molecular studies (LOFTUS *et al.* 2005). These genomic sequences were used to generate a highly saturated *C. neoformans* var. *neoformans* linkage map for B3501α and B3502/JEC20**a** (MARRA *et al.* 2004). The congenic strains developed in this article are all closely related to the sequenced and mapped genomes. Genetic manipulation of the JEC21α genome was previously possible using the JEC20/21 congenic strain pair. The KN3501 congenic strain pair now enables similar approaches for the other sequenced strain, B3501Aα. Production of the congenic strains in sequenced and mapped genetic backgrounds has allowed us to scrutinize the congenicity of the strains and also to determine the precise differences between the congenic strains and their relationship to the parental strains.

Given that the meiotic progeny of a genetic cross inherit, on average, half of their genome from each parent, 10 backcrosses should result in congenic strains with 99.9% identity. Our data confirm this for NIH433 and the congenic strains KN433**a** and KN433α. However, the KN3501 congenic strains contain remnants from the opposite parental genome that persisted after 10 backcrosses and account for ~3% of the genome. It is unclear why these portions of the JEC20**a** genome have persisted in the KN3501 congenic pair, but when additional backcrossing was conducted to obtain isolates in which this genomic region corresponded to the B3501α allele, the isolates were sterile. Despite this small difference from the B3501α parental strain, KN3501**a** and KN3501α are 99.9% identical. These data clearly demonstrate that the KN433 and KN3501 strain pairs are highly similar except at the *MAT* locus and therefore can be used to examine the role of mating type in virulence without significant confounding genetic variables.

In earlier studies, the α strain was more virulent than the **a** strain in the var. *neoformans* congenic strains JEC20**a** and JEC21α but both mating types displayed equivalent virulence in the var. *grubii* congenic strains KN99**a** and KN99α (KWON-CHUNG *et al.* 1992; NIELSEN *et al.* 2003). A number of hypotheses have been proposed to explain these differences (NIELSEN *et al.* 2003; MCCLELLAND *et al.* 2004). First, the JEC20**a** and JEC21α strains might not be identical and differences in the genetic background of the strains could account for their virulence differences. This possibility seems unlikely since α progeny from a cross of JEC20**a** and JEC21α are more virulent than **a** progeny, providing evidence that the observed virulence difference is linked to mating type in these strains (KWON-CHUNG *et al.* 1992).

Alternatively, there could be innate differences between variety *grubii* and *neoformans* strains, which have diverged for ~20 million years. Mating type might play a role in virulence of var. *neoformans* strains but not of var. *grubii* strains. In single infections, the var. *grubii* strain KN99α was equivalent to KN99**a** in virulence. However, KN99α cells more readily penetrate the blood-brain barrier than KN99**a** cells during co-infection (NIELSEN *et al.* 2005). Thus, differences in virulence potential between **a** and α strains in var. *grubii* are apparent during co-infection.

Finally, the genetic background of the congenic strains could affect the impact of the mating-type allele on virulence. This phenomenon could apply to either var. *neoformans* or var. *grubii*. In this model, different congenic strains could have different mating-type virulence characteristics. Our data support this hypothesis. Similar to the var. *neoformans* JEC20/21 results, the KN433 congenic strains showed that the α strain is more virulent than the **a** strain. Likewise, the KN3501 congenic strains, as well as an analogous pair of independently derived congenic strains in the B3501Aα genetic background (K. J. Kwon-Chung, personal communication), resemble the var. *grubii* KN99 congenic strains where no difference was observed in the virulence of **a** and α strains.

A correlation was observed between the overall virulence of the genetic background and differences in virulence associated with mating type. For example, both the JEC20/21 and KN433 genetic backgrounds had an overall low virulence, and in both cases the difference in virulence between congenic **a** and α strains was significant (Kwon-Chung *et al.* 1992 and this article). By contrast, the KN3501 and KN99 genetic backgrounds had high overall virulence, with no significant difference in virulence between the congenic **a** and α strains (Nielsen *et al.* 2003 and this article). Thus, the contribution of the α allele of the *MAT* locus may be more apparent in strains with lower virulence. In this case, var. *grubii* α congenic strains generated in genetic backgrounds with decreased virulence potential may exhibit enhanced virulence compared to their congenic **a** partner strains.

In contrast, no link was observed between the virulence of the congenic strains compared to the parental strains and mating-type virulence potential. The KN433 and KN99 congenic strains are as virulent or more virulent than their parental strains NIH433**a** and H99α but the KN433 pair shows a difference in mating-type virulence potential whereas the KN99 pair does not. Likewise, the KN3501 and JEC20/21 congenic strains are less virulent than their parental strains but the KN3501 pair shows no difference in virulence between mating types whereas the α strain is more virulent in the JEC20/21 pair.

Taken together, these data support the conclusion that genetic background plays a significant role in determining the potential effect of mating type on virulence in *C. neoformans*. Interestingly, the α strain was more virulent in all cases where a difference in virulence between mating types was observed. This observation suggests that there is an overall virulence advantage to being mating-type α that is more pronounced in certain genetic contexts. These studies provide a foundation from which to identify the genetic determinants that influence the virulence impact of the *MAT* locus α allele, and it will be of considerable interest to elucidate how the α allele collaborates with other unlinked genetic determinants to enhance the virulence composite, which on the basis of these findings represents a quantitative trait.

Mating type also contributes to virulence of other human pathogenic fungi. Both mating types of *Histoplasma capsulatum* (+ and −) are found in environmental soil samples in an equal ratio yet the vast majority of clinical isolates possess the − mating type (Kwon-Chung 1973; Kwon-Chung *et al.* 1974). Similar to some of the *C. neoformans* congenic strains, no difference in virulence was observed between *H. capsulatum* + and − mating types in murine infection experiments, but this issue has not yet been examined rigorously with congenic strains (Kwon-Chung 1981). In the diploid pathogenic fungus *C. albicans*, most clinical isolates are **a**/α and thus heterozygous at the mating-type locus (Lockhart *et al.* 2002). Recent studies have revealed that homozygous **a**/**a** and α/α strains are less virulent than **a**/α heterozygous strains and this difference could explain how the **a**/α mating type is conserved in the natural population (Lockhart *et al.* 2005). These data clearly implicate the mating-type locus in the virulence of divergent human pathogenic fungi. The next challenge will be to unravel how the *MAT* locus collaborates with other unlinked loci and to define the molecular differences that alter the impact of the **a** and α alleles on this process. With this understanding, we can better predict the genetic virulence composite of strains, which will aid in the identification of antifungal and vaccine targets for human pathogenic fungi.

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