

Role of an Expanded Inositol Transporter Repertoire in *Cryptococcus neoformans* Sexual Reproduction and Virulence

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ABSTRACT *Cryptococcus neoformans* and *Cryptococcus gattii* are globally distributed human fungal pathogens and the leading causes of fungal meningitis. Recent studies reveal that *myo*-inositol is an important factor for fungal sexual reproduction. That *C. neoformans* can utilize *myo*-inositol as a sole carbon source and the existence of abundant inositol in the human central nervous system suggest that inositol is important for *Cryptococcus* development and virulence. In accord with this central importance of inositol, an expanded *myo*-inositol transporter (*ITR*) gene family has been identified in *Cryptococcus*. This gene family contains two phylogenetically distinct groups, with a total of 10 or more members in *C. neoformans* and at least six members in the sibling species *C. gattii*. These inositol transporter genes are differentially expressed under inositol-inducing conditions based on quantitative real-time PCR analyses. Expression of *ITR* genes in a *Saccharomyces cerevisiae* *itr1 itr2* mutant lacking inositol transport can complement the slow-growth phenotype of this strain, confirming that *ITR* genes are *bona fide* inositol transporters. Gene mutagenesis studies reveal that the *Itr1* and *Itr1A* transporters are important for *myo*-inositol stimulation of mating and that functional redundancies among the *myo*-inositol transporters likely exist. Deletion of the inositol 1-phosphate synthase gene *INO1* in an *itr1* or *itr1a* mutant background compromised virulence in a murine inhalation model, indicating the importance of inositol sensing and acquisition for fungal infectivity. Our study provides a platform for further understanding the roles of inositol in fungal physiology and virulence.

IMPORTANCE *Cryptococcus neoformans* is an AIDS-associated human fungal pathogen that causes over 1 million cases of meningitis annually and is the leading cause of fungal meningitis in immunosuppressed patients. The initial cryptococcal infection is caused predominantly via inhalation of sexual spores or desiccated yeast cells from the environment. How this fungus completes its sexual cycle and produces infectious spores in nature and why it frequently infects the central nervous system to cause fatal meningitis are critical questions that remain to be understood. In this study, we demonstrate that inositol acquisition is important not only for fungal sexual reproduction but also for fungal virulence. We identified an expanded inositol transporter gene family that contains over 10 members, important for both fungal sexual reproduction and virulence. Our work contributes to our understanding of how fungi respond to the environmental inositol availability and its impact on sexual reproduction and virulence.

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Inositol is essential for all eukaryotes, including model and pathogenic fungi. *myo*-Inositol is the precursor of phosphatidylinositol (PI), which plays key roles in both cellular structure and intracellular signal regulation. The intracellular inositol level is precisely regulated by specific PI kinases, phosphatases, and phospholipases. Inositol polyphosphates derived from PI orchestrate myriad cell functions, including nuclear export, telomere length, chromatin remodeling, and transcription (1–5). Inositol-derived products have been reported to be important for fungal pathogenicity. Some enzymes involved in sphingolipid biosynthesis and degradation pathways have also been found to promote pathogenicity in *Cryptococcus neoformans*, such as inositol-phosphoryl ceramide synthase 1 (*Ipc1*) (6) and inositol phosphosphingolipid-

phospholipase C1 (*Isc1*) (7). The diacylglycerol (DAG)-protein kinase C1 (*Pkc1*) branch signaling pathway is also critical for virulence factor production and pathogenicity in *C. neoformans* (8–11). Inositol also functions as the precursor for phospholipomannan, a glycosphosphatidylinositol (GPI)-anchored glycolipid on the cell surface of *Candida albicans* that binds to human macrophages and is necessary for pathogenicity (12).

Inositol can serve as a carbon source for fungi. There are two main sources by which fungal cells can acquire inositol. One route involves the conversion of intracellular glucose into *myo*-inositol by a multiple-step inositol biosynthesis pathway (13). Inositol 1-phosphate synthase (*Ino1*) is the key enzyme for this synthetic route and converts glucose 6-phosphate to inositol 3-phosphate in

the rate-determining step (14). Inositol can also be imported from the extracellular environment via inositol transporters. The *myo*-inositol transporter gene family is part of the sugar transporter superfamily and plays an important role in inositol acquisition in fungi, including *Saccharomyces cerevisiae* (15–20), *C. albicans* (13, 21, 22), and *Schizosaccharomyces pombe* (23).

There are two *myo*-inositol transporters (Itrs) in *S. cerevisiae*, which were first isolated by complementation of a yeast mutant defective in *myo*-inositol uptake. Itr1 is the major transporter, and its abundant mRNA is transcriptionally and posttranslationally repressed by inositol and choline. Itr2 is a minor transporter that is constitutively expressed at a low level (18, 19). Depletion of inositol from the growth medium stimulates *ITR1* expression, while addition of inositol to the medium triggers repression of *ITR1* expression and inhibits uptake activity (15). The *C. albicans* *myo*-inositol transporter CaItr1 exhibits high substrate specificity for inositol, and interactions between the C-2, C-3, and C-4 hydroxyl groups of *myo*-inositol and the transporter are critical for substrate recognition and binding (22). A recent study showed that, similar to *S. cerevisiae*, *C. albicans* can generate inositol *de novo* through Ino1 and also import it from the environment through CaItr1. *C. albicans* may utilize these two complementary mechanisms to obtain inositol during host infection (21). There is another phylogenetically distinct transporter (orf19.5447) that may represent a second *ITR* gene, but it is expressed at a low level and less well studied (21). The fission yeast *S. pombe* is a natural auxotroph for inositol due to the absence of Ino1 and therefore cannot grow in the absence of inositol. Two transporters, Itr1 and Itr2, are involved in inositol uptake in *S. pombe*. High concentrations of inositol in the culture medium stimulate mating and sporulation, while a low concentration supports only vegetative growth (23). It was reported that *myo*-inositol regulates the production of pheromone P and the response of cells to pheromones, but production of pheromone M is inositol independent. It is likely that inositol or one of its metabolites is involved in pheromone P secretion and pheromone signaling and thereby influences sexual reproduction (24).

Cryptococcus neoformans is a major AIDS-associated human fungal pathogen that often infects immunocompromised individuals to cause fatal meningoencephalitis. Inositol metabolism is important for the growth and development of *C. neoformans* and may be involved in its survival both in environmental niches, including plants and soil, and in humans and other hosts (25, 26). The development of cryptococcosis is thought to be initiated by inhalation of spores and/or desiccated yeasts from the environment, as no human-to-human transmission (other than iatrogenic) has been reported, and spores are small enough to lodge in the alveoli of the lung (27). Recent studies also demonstrate that spores are fully virulent (28, 29). Our recent study revealed that high concentrations of inositol on plants ($\sim 6.5 \mu\text{g}/\text{cm}^2$ on the *Eucalyptus camaldulensis* leaf surface) and in media stimulate *Cryptococcus* to complete the sexual cycle and produce spores, which could provide an explanation as to how spores are produced in nature (26). Understanding how inositol triggers fungal mating will provide insights into the interactions between *C. neoformans* and its environmental niches.

The high rate of cryptococcal meningitis may be related to the elevated inositol levels found in the mammalian brain and the preference of the fungus for proliferation in this tissue (30–32). Inositol concentrations in human cerebrospinal fluid (CSF) are

$\sim 25 \text{ mg/liter}$ compared to an average concentration of 4.3 mg/liter in plasma (33). Serial analysis of gene expression (SAGE) has been applied to characterize gene expression profiles during experimental cryptococcal meningitis in a rabbit model (34). The *INO1* gene and inositol monophosphatase were found to be abundant in a SAGE library generated from RNAs isolated during brain infection, suggesting that the *myo*-inositol internal synthesis pathway is functional and that inositol could be important for the development of meningitis (34). Inositol metabolism has also been linked to PKA signaling, which is critical for the virulence of *C. neoformans* (35). Another unique feature of *C. neoformans* is that it can utilize inositol as a sole carbon source, which was first described in 1976 (36, 37). We recently identified an undefined *myo*-inositol transporter gene family that contains seven members with high similarity based on the *ITR* sequences in *S. cerevisiae* (26). It is unusual to find this expanded family of inositol transporters in *C. neoformans*, considering that most other fungi have only two or three related inositol transporters; this finding also supports the importance of inositol for *Cryptococcus* physiology and virulence.

In this study, we describe the full suite of expanded *ITR* genes found in *Cryptococcus*. Our results with real-time PCR showed that these *ITR* genes are differentially expressed in response to *myo*-inositol induction. Phenotypic complementation of an *S. cerevisiae* *itr1 itr2* double mutant strain by some *ITR* genes from *C. neoformans* supports the idea that these *ITR* homologs are *bona fide* inositol transporters. Phenotypic analyses of *itr* mutants revealed that inositol acquisition is important for fungal mating and that virulence and functional redundancy exist among *ITR* genes and between the *ITR* gene family and the inositol internal biosynthesis pathway. Our results further support the hypothesis that inositol is important for *C. neoformans* sexual reproduction and virulence.

RESULTS

***myo*-Inositol is a key compound for triggering mating and sporulation.** In previous studies, we discovered a defined medium, modified MS (Murashige and Skoog) medium, which supports the efficient mating of both *C. neoformans* and *Cryptococcus gattii* (26). By omission and readdition of components, *myo*-inositol was shown to be the active component stimulating mating. Standard MS medium contains 100 mg/liter *myo*-inositol. *myo*-Inositol was added at different concentrations to MS inositol dropout medium, and a concentration as low as 15 mg/liter *myo*-inositol was found to stimulate mating. A medium made from V8 juice is classically used for mating assays in *C. neoformans*. In related studies, the *myo*-inositol concentration in V8 juice has been measured (38). Based on this information, standard V8 mating medium contains 16.675 mg/liter inositol, and this level is sufficient to support mating in MS medium. The addition of more *myo*-inositol to V8 medium triggers increased mating filament and spore production (26). These observations suggest that *myo*-inositol is an active compound in V8 medium that stimulates mating. Compared with mating on V8 medium, mating on MS medium shows significantly increased sporulation efficiency.

Because *myo*-inositol is the main carbon source in MS medium, we replaced *myo*-inositol with other carbon sources, including glucose, sucrose, galactose, and glycerol. None of those sugars stimulated mating at the same concentration, indicating that *myo*-inositol elicits mating in addition to its known ability to serve as a carbon source (Fig. 1A).

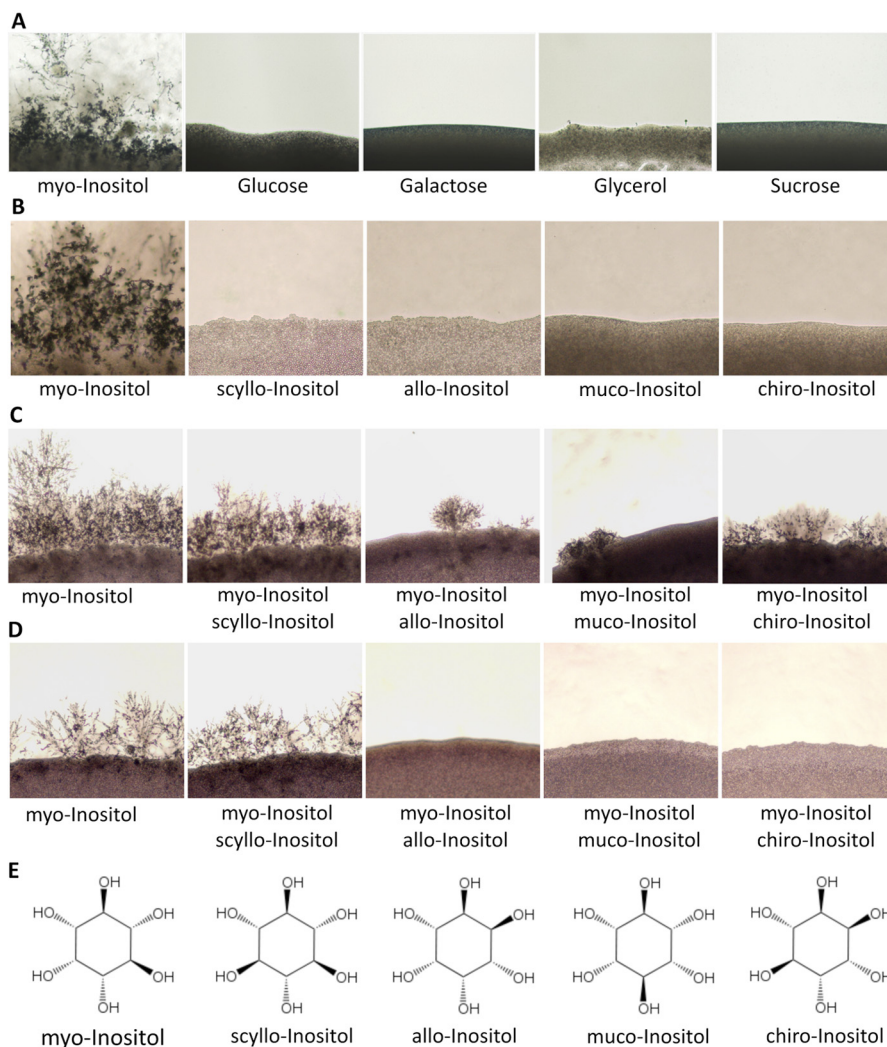


FIG 1 Mating stimulation is *myo*-inositol specific. (A) Mating assays between strains H99 and KN99a were performed in MS medium containing different carbon sources at a concentration of 100 mg/liter, including *myo*-inositol, glucose, galactose, glycerol, or sucrose. No other tested carbon source except *myo*-inositol stimulated fungal mating. (B) Mating assays were performed in MS medium containing different inositol isomers, including *myo*-, *scyllo*-, *allo*-, *muco*-, and *chiro*-inositol. No other isomers except *myo*-inositol stimulated mating at a concentration of 100 mg/liter. (C and D) Mating assays were performed in MS medium containing 50 mg/liter (C) or 20 mg/liter (D) *myo*-inositol and 100 mg/liter of each other form, such as *scyllo*-, *allo*-, *muco*-, or *chiro*-inositol. The presence of *allo*-, *muco*-, or *chiro*-inositol competed with *myo*-inositol and inhibited mating. No obvious inhibition by *scyllo*-inositol was observed. (E) Structures of the inositol isomers studied.

We also tested several other inositol isomers, including *scyllo*-inositol, *allo*-inositol, *muco*-inositol, and *chiro*-inositol. None stimulated mating, indicating that mating stimulation is specific to *myo*-inositol (Fig. 1B). We have also investigated whether any of these inositol isoforms can compete to inhibit *myo*-inositol-stimulated mating. By adding 100 mg/liter of each isomer to MS medium containing 50 mg/liter *myo*-inositol, respectively (yielding a 2:1 ratio), a clear inhibition of mating by *allo*-, *muco*-, or *chiro*-inositol was observed (Fig. 1C). Lowering the *myo*-inositol concentration to 20 mg/liter in these experiments to yield a 5:1 ratio (at a constant concentration of 100 mg/liter of the inositol isomers) completely blocked mating filament production (Fig. 1D). These results suggest that *allo*-, *muco*-, and *chiro*-inositol antagonize mating by competing with *myo*-inositol. However, no antagonistic effect was observed by adding *scyllo*-inositol to MS medium with 20 mg/liter or 50 mg/liter *myo*-

inositol (Fig. 1C and D). Because *scyllo*-inositol is an inactive form of inositol, these results may reflect an inability of *scyllo*-inositol to compete with *myo*-inositol for binding.

Identification of an expanded ITR gene family. Fungal cells can acquire inositol from the following two main sources: internal inositol synthesis from glucose or transport from the environment via inositol transporters. We have identified a large *myo*-inositol transporter (*ITR*) gene family in *Cryptococcus* as encoding potential inositol permeases or sensors based on sequence identity shared with inositol transporter genes identified in *S. cerevisiae* and *S. pombe* (26). Because the *ITR* genes belong to the sugar transporter superfamily, here we identified all sugar transporter homologs from the genomes of the *C. neoformans* strain H99, *S. cerevisiae*, and *C. albicans*, and the phylogenetic relationships among them were compared (see Fig. S1 in the supplemental material). Homologs of *Itrs* in *S. cerevisiae* and *C. albicans* form a

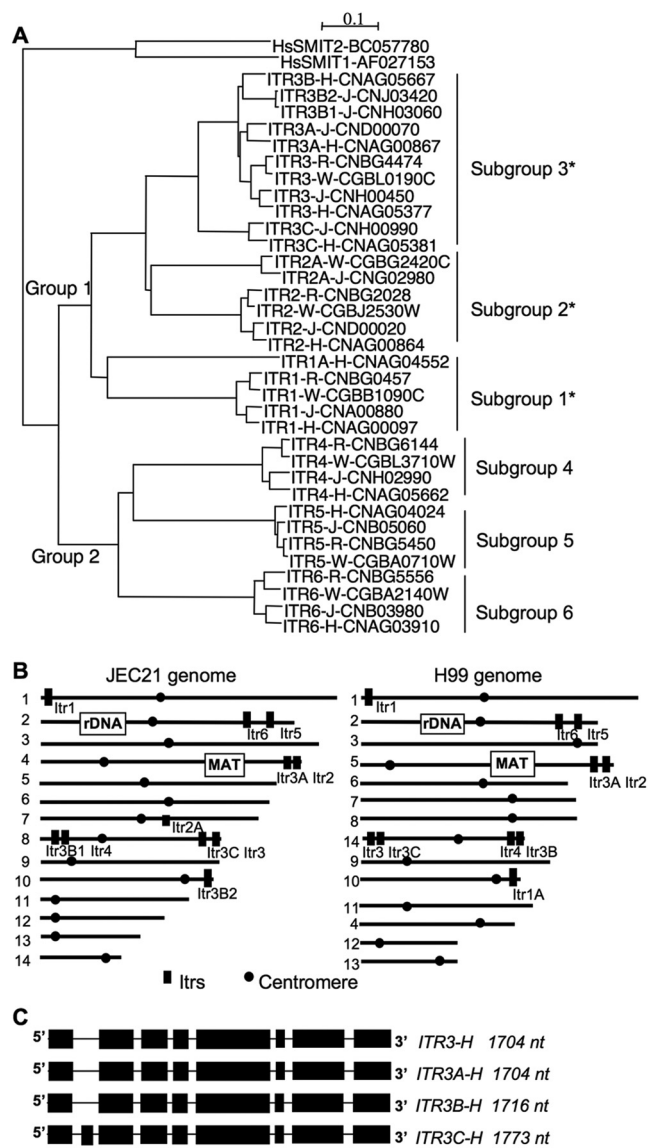


FIG 2 Phylogenetic analysis of *myo*-inositol transporter (*Itr*) proteins in *C. neoformans* and *C. gattii*. (A) Potential *myo*-inositol transporters from H99, JEC21, WM276, and R265 were identified using BLASTn and BLASTp analyses. The phylogram was generated using ClustalX 2.0.1 and viewed with the TreeView program. Two human sodium-dependent *myo*-inositol transporters (HsSMIT1 and HsSMIT2) served as outgroup controls. *, genes with closely related duplicated paralogs. (B) *ITR* gene locations in the genomes of JEC21 and H99. All *ITR* homologs in JEC21 and H99 were labeled at the appropriate locations on the 14 chromosomes of the genomes. (C) Subgroup 3 *ITR* genes of H99 have similar intron locations. Transcripts of *ITR3*, *ITR3A*, *ITR3B*, and *ITR3C* are shown in black, and lines indicate introns in each gene. nt, nucleotides.

cluster in the phylogenetic tree generated. A total of 10 proteins in H99 were considered *ITR* candidates and included in this cluster. We then compared all of the *Itr* homologs identified from the following four complete *Cryptococcus* genome sequences: *C. neoformans* var. *grubii* H99 (serotype A, VNI), *C. neoformans* var. *neoformans* JEC21 (serotype D, VNIV), *C. gattii* WM276 (serotype B, VGI), and *C. gattii* R265 (serotype B, VGII) (Table 1). The *Itr* homologs can be divided into two distinct groups (Fig. 2A). The

TABLE 1 Potential *myo*-inositol transporters in *Cryptococcus* species

Strain	Gene name	Locus tag	Chromosome
H99	<i>ITR1-H</i>	CNAG_00097	1
H99	<i>ITR1A-H</i>	CNAG_04552	10
H99	<i>ITR2-H</i>	CNAG_00864	5
H99	<i>ITR3-H</i>	CNAG_05377	14
H99	<i>ITR3A-H</i>	CNAG_00867	5
H99	<i>ITR3B-H</i>	CNAG_05667	14
H99	<i>ITR3C-H</i>	CNAG_05381	14
H99	<i>ITR4-H</i>	CNAG_05662	14
H99	<i>ITR5-H</i>	CNAG_04024	2
H99	<i>ITR6-H</i>	CNAG_03910	2
JEC21	<i>ITR1-J</i>	CNA00880	1
JEC21	<i>ITR2-J</i>	CND00020	4
JEC21	<i>ITR2A-J</i>	CNG02980	7
JEC21	<i>ITR3-J</i>	CNH00450	8
JEC21	<i>ITR3A-J</i>	CND00070	4
JEC21	<i>ITR3B1-J</i>	CNH03060	8
JEC21	<i>ITR3B2-J</i>	CNJ03420	10
JEC21	<i>ITR3C-J</i>	CNH00990	8
JEC21	<i>ITR4-J</i>	CNH02990	8
JEC21	<i>ITR5-J</i>	CNB05060	2
JEC21	<i>ITR6-J</i>	CNB03980	2
WM276	<i>ITR1-W</i>	CGB_B1090C	2
WM276	<i>ITR2-W</i>	CGB_J2530W	10
WM276	<i>ITR2A-W</i>	CGB_G2420C	7
WM276	<i>ITR3-W</i>	CGB_L0190C	12
WM276	<i>ITR4-W</i>	CGB_L3710W	12
WM276	<i>ITR5-W</i>	CGB_A0710W	1
WM276	<i>ITR6-W</i>	CGB_A2140W	1
R265	<i>ITR1-R</i>	CNBG_0457	Supercontig 1
R265	<i>ITR2-R</i>	CNBG_2028	Supercontig 4
R265	<i>ITR3-R</i>	CNBG_4474	Supercontig 11
R265	<i>ITR4-R</i>	CNBG_6144	Supercontig 23
R265	<i>ITR5-R</i>	CNBG_5450	Supercontig 16
R265	<i>ITR6-R</i>	CNBG_5556	Supercontig 16

group 1 genes are homologs of *Itr* genes identified from *S. cerevisiae* and *S. pombe* and of the *Itr1* gene from *C. albicans*. The group 2 genes are homologs of the *HGT19* (orf19.5447) gene from *C. albicans*, which also exhibits *myo*-inositol transporter activity based on computational analysis presented in the *Candida* Genome Database (<http://www.candidagenome.org/>) but has not been extensively studied (21). The first group may have undergone recent duplication because the numbers of *Itr* paralogs differ among *Cryptococcus* strains, while the second group is well conserved among all of the strains that were compared. Based on the similarity among these two groups of *Itrs* in *Cryptococcus*, they can be further divided into six different subgroups (*Itr1* to -6). Among these, group 1 *Itrs* can be further divided into subgroups 1, 2, and 3, while group 2 *Itrs* can be divided into subgroups 4, 5, and 6. Subgroups 4, 5, and 6 are well conserved, and each subgroup has one *Itr* (*Itr4*, *Itr5*, and *Itr6*) from each *Cryptococcus* strain. Interestingly, subgroup 3 has expanded in *C. neoformans* to include four homologs in H99 (*ITR3*, *ITR3A*, *ITR3B*, and *ITR3C*) and five homologs in JEC21 (*ITR3*, *ITR3A*, *ITR3B1*, *ITR3B2*, and *ITR3C*) but only one each for R265 and WM276 (Fig. 2A; Table 1).

When the group 1 and group 2 *Itrs* from *Cryptococcus* were compared with related *Itr* homologs from other fungi, group 1 was found to share a high level of identity with the *Itrs* in other yeasts, such as *S. cerevisiae* and *S. pombe*. There are two *Itrs* in *S. cerevisiae* and *S. pombe*, and both cluster within group 1. In most other ascomycetes with completed genome sequences, only one

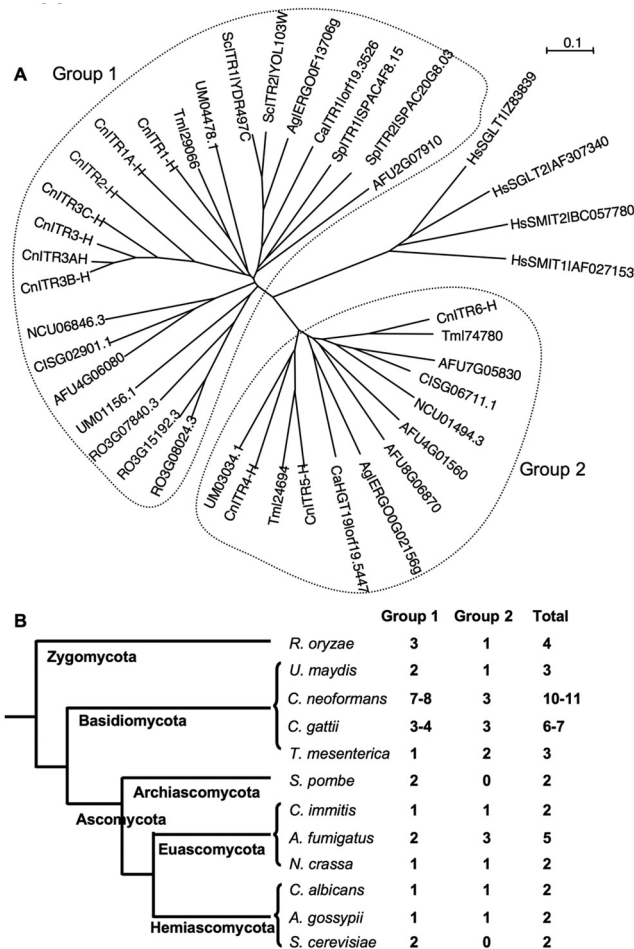


FIG 3 Phylogenetic analysis of *myo*-inositol transporter (*Itr*) proteins among different fungi. (A) *myo*-Inositol transporter homologs from the Hemiascomycota (*S. cerevisiae*, *A. gossypii*, and *C. albicans*), Euascomycota (*N. crassa*, *Aspergillus fumigatus*, and *C. immitis*), Archiascomycota (*S. pombe*), Basidiomycota (*U. maydis* and *C. neoformans* var. *grubii*), and Zygomycota (*R. oryzae*) were identified based on BLAST searches. The human sodium-dependent inositol transporters served as outgroup controls. (B) Numbers of *Itr* homologs in different fungi.

ITR gene was found in this group, including in hemiascomycetes (*C. albicans* and *Ashbya gossypii*) and euascomycetes (*Coccidioides immitis* and *Neurospora crassa*) (Fig. 3). Two basidiomycete genomes were used for comparison. The closely related species *Tremella mesenterica* has only one *ITR* homolog, while the corn smut fungus *Ustilago maydis* has two (Fig. 3). One exception in this group is the zygomycete *Rhizopus oryzae*, which has three *Itr* paralogs in group 1.

There are three *Itrs* in the four *Cryptococcus* strains that belong to group 2. Most fungi analyzed have only one *Itr* that belongs to group 2, including *C. albicans*, *A. gossypii*, *C. immitis*, *N. crassa*, *U. maydis*, and *R. oryzae*. *T. mesenterica* has two *Itrs* that belong to group 2, and interestingly, *A. fumigatus* has three group 2 representatives.

Based on the comparative genomic analysis, it is clear that the *ITR* gene family in *Cryptococcus* has expanded (Fig. 3). Because *C. neoformans* is an environmental microorganism that associates with a variety of niches, the expansion of the *ITR* gene family may

reflect adaptation to nutrient-limited niches, such as survival on plants or in soil containing plant debris, where free inositol or phytic acid (IP6) is abundant. *C. neoformans* can utilize inositol as a sole carbon source, which may require this fungus to have more efficient inositol transport systems both to survive in natural habitats and to successfully infect the host and cause infection.

Most of the *ITR* genes in *C. neoformans* are located in telomeric regions of the chromosomes, which may have contributed to the expansion of this gene family (Fig. 2B) via recombination-mediated exchange of telomeric and subtelomeric regions between different chromosomes. Interestingly, in JEC21 chromosome 8 and its corresponding chromosome, 14, in H99, four *ITR* genes are located in telomeric regions, and three of these belong to subgroup 3, the least conserved transporter cluster. In addition, all four genes in subgroup 3 of H99 have very similar intron locations in the genes except *ITR3C*, which contains an extra exon (Fig. 2C). Comparison of 20-kb flanking sequence regions of the *ITR* genes in JEC21 and H99 revealed synteny between *ITR3A* (chromosome 4) and *ITR3B2* (chromosome 10) in JEC21, an indication that this genomic region underwent duplication (see Fig. S2 in the supplemental material). These observations suggest that the subgroup 3 *ITR* genes on these chromosomes may have undergone recent duplications. In this study, we focused further effort on functional analyses of the seven group 1 *Itr* homologs in H99 to understand their roles in mating and virulence of *C. neoformans*.

Inositol transporters are differentially expressed under different culture conditions. To understand how the *Itrs* are involved in inositol sensing and transport, quantitative real-time PCR (qRT-PCR) was performed to detect the expression of *ITR* genes during growth under high-inositol conditions and during mating on MS medium containing inositol. In this study, we designed specific primers for seven *ITR* genes of H99 that belong to group 1 and that are closely related to well-studied *ITR* genes in *S. cerevisiae* and *S. pombe*. Cells of strain H99 and mating coculture of H99 and KN99a were inoculated on MS medium containing 100 mg/liter inositol. Cells were recovered from the medium surface at 24 h, 48 h, and 72 h postinoculation. Total RNAs were purified, and first-strand cDNAs were synthesized. Each cDNA sample was used as a template to amplify all seven *ITR* genes using gene-specific primers in a qRT-PCR. Our qRT-PCR results showed that these *ITR* genes are differentially expressed both during mating and during growth as a single strain in the presence of inositol (Fig. 4). Within the first 24 h of incubation, the mating mixture undergoes cell fusion, and all seven *ITR* genes were found to be expressed. Increased expression was observed when incubation was continued for 48 h or 72 h. The expression of *ITR1* at all time points tested was significantly repressed in the single-strain culture but was not repressed during mating. Meanwhile, *ITR1A* and *ITR2* were constitutively highly induced under both culture conditions, especially *ITR1A*, which represented the highest expression at all conditions; this suggests that these two may play important roles in inositol uptake. *ITR3*, *ITR3B*, and *ITR3C* are highly induced only during mating but not in the single-strain culture, possibly also consistent with a role in mating. *ITR3A* was expressed at a low level under both culture conditions, and thus, it may not play a significant role in inositol transport.

Overall, our qRT-PCR results suggest that there is a mating effect on the expression of *ITR* genes, since several *ITR* genes are expressed at a much higher level in mating culture than in single-strain culture. Our expression profiles of the inositol transporter

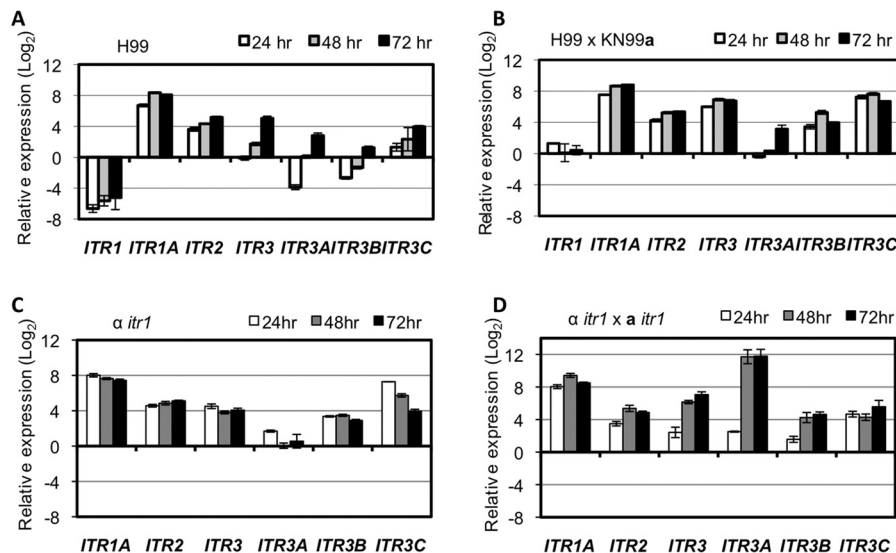


FIG 4 Comparison of the expression of seven *ITR* genes in *C. neoformans* at both mating and growth stages in the presence of inositol. H99 cultures (A), H99 × KN99a mating mixtures (B), the α *itr1* cultures (C), and *itr1* × *itr1* mating cultures (D) were spotted on agar plates and collected at 24, 48, and 72 h after plating. Gene expression was measured by qRT-PCR in triplicate, and the comparative C_T method was used for the relative quantification. Values are expressed as relative expression (\log_2) (means \pm SD) of *ITR* genes, normalized to the *GAPDH* gene endogenous reference and relative to the 0-h time point (H99 overnight liquid culture was considered the 0-h time point). Error bars show standard deviations of three repeats.

gene family also provide an important indication that this gene family may play redundant but distinguishable roles in response to inositol, during both mating coculture and growth in monoculture. It will be of interest to investigate the expression profiles of these *ITR* genes at different sexual development stages or in other strain backgrounds, such as in *ino1* and *itr* mutant strains.

Inositol transporters (*Itrs*) from *C. neoformans* complement the growth defect of *S. cerevisiae* *itr1 itr2* double mutants. To understand the potential role of the *C. neoformans* *ITR* gene family in inositol uptake, we generated *S. cerevisiae* *itr1 itr2* double mutants lacking both inositol transporters. Such double mutants showed a growth defect in yeast extract-peptone-dextrose (YPD) at 37°C. Full-length cDNAs for all seven *ITR* genes from the *C. neoformans* H99 strain were cloned into the yeast expression vector pTH19 under the control of the *ADH1* promoter and expressed in an *S. cerevisiae* *itr1 itr2* double mutant strain. The heterologous expression of all seven *ITR* genes of H99 was confirmed by RT-PCR analyses (data not shown). Growth assays were performed for these *S. cerevisiae* strains expressing *C. neoformans* *ITR* genes, and the potential complementation of the *S. cerevisiae* *itr1 itr2* growth defect phenotype was investigated. Expression of *ITR1A* and *ITR3C* fully complemented the growth defect of the *S. cerevisiae* *itr1 itr2* mutant strain, while *ITR3A* and *ITR3B* partially rescued the slow growth on YPD medium (Fig. 5). Yeast strains expressing *ITR1*, *ITR2*, or *ITR3*, on the other hand, showed growth rates similar to that of the original *S. cerevisiae* *itr1 itr2* mutant strain, suggesting no obvious complementation. These results suggest that *Itr1A* and *Itr3C* are important for inositol uptake, which is consistent with our qRT-PCR results suggesting that both *ITR1A* and *ITR3C* are highly expressed in response to the availability of environmental *myo*-inositol. The *ITR* genes that did not complement the growth defect of *S. cerevisiae* *itr1 itr2* might be caused by either insufficient inositol uptake or expressed proteins that are unstable or not functional.

***Itr1* and *Itr1A* are important for fungal mating.** Gene deletion mutations were generated by biolistic transformation and homologous recombination for all seven *ITR* genes in *C. neoformans* var. *grubii* in both the α and **a** mating-type backgrounds. Each individual mutation was not sufficient to block sexual reproduction based on mating assays with both MS and V8 mating media. Normal mating hyphae and spores were observed in all bilateral mutants by mutant mating assays. The exception was that bilateral mating between *itr1* mutants exhibited reduced mating hypha

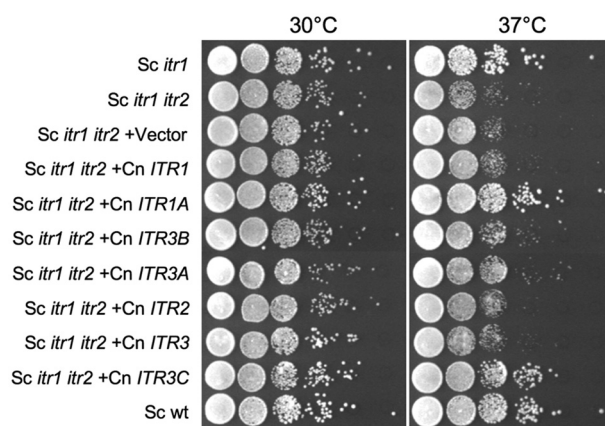


FIG 5 *ITR* genes from *C. neoformans* complement the growth defect of an *S. cerevisiae* *itr1 itr2* mutant strain. *S. cerevisiae* *itr1*, *itr1 itr2*, and *itr1 itr2* expressing vector pTH19 or one of the seven *C. neoformans* *ITR* genes were cultured in YPD or SD-Ura medium. Concentrations of overnight cultures were determined by measuring the optical density at 600 nm (OD_{600}) and adjusted to the same cell density with YPD. Serial 10-fold dilutions were prepared, and 5 μ l of each dilution was spotted on YPD plates and incubated at 30°C or 37°C for 48 h before photography. This assay was repeated multiple times, with similar results. Sc, *S. cerevisiae*; Cn, *C. neoformans*; wt, wild type.

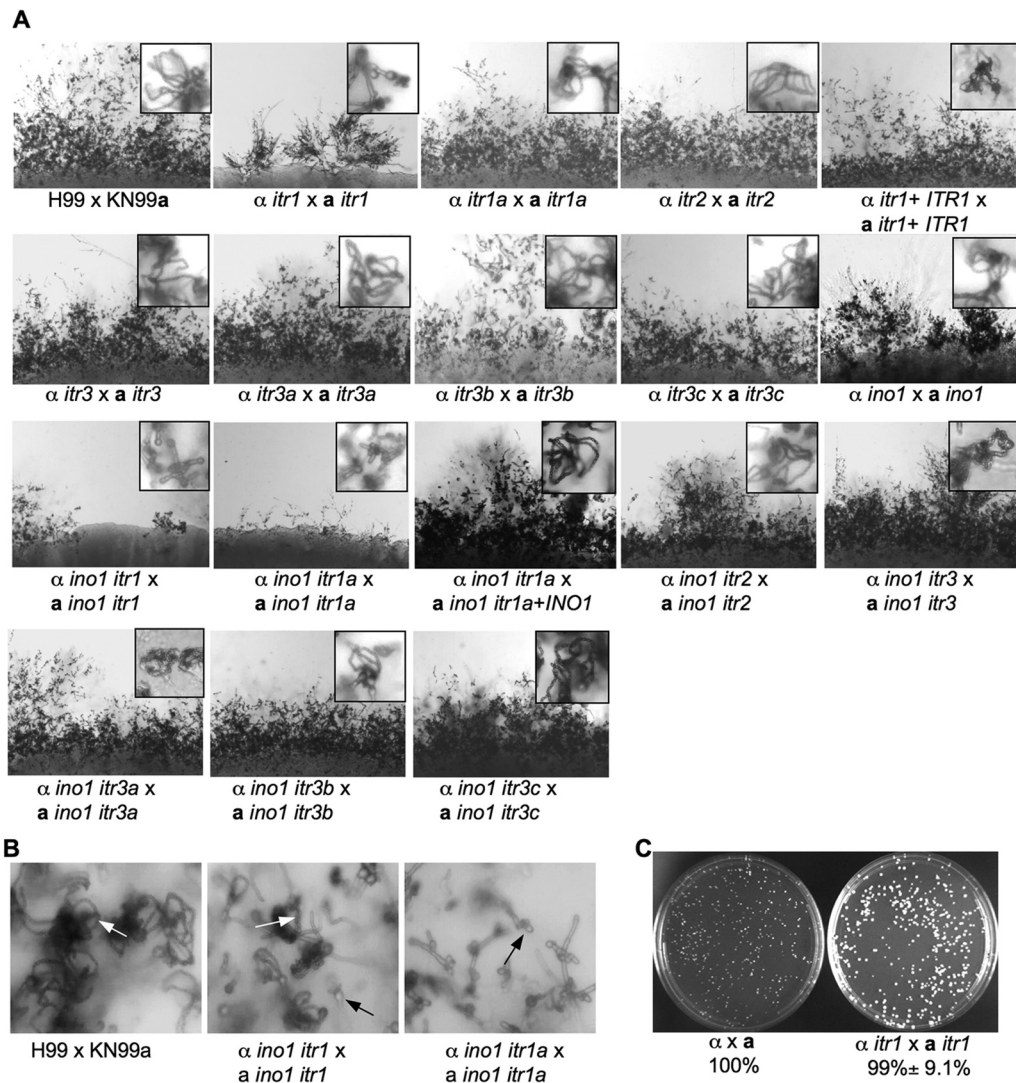


FIG 6 *Itr1* and *Itr1A* promote mating in response to inositol. (A) Bilateral mating assays were performed in MS medium with all seven inositol transporter mutants and with double mutants lacking *ino1* and each *itr* gene. Mating cultures were incubated at room temperature in the dark for 7 days before being examined by microscopy and photographed. Both mating filaments (40 \times magnification) and spores (insets, 400 \times magnification) were observed for all mating cultures. Mating of *itr1*, *ino1 itr1*, and *ino1 itr1a* mutants showed a defect in mating filament production and sporulation, suggesting an important role in inositol sensing. (B) Bilateral mating cultures of wild-type strains, *ino1 itr1* mutants, and *ino1 itr1a* mutants were incubated under the same conditions as described in the legend to panel A for 14 days, and sporulation results were photographed under 200 \times magnification. White arrows indicate basidiospore chains; black arrows indicate basidia. (C) Cell fusion assays were performed for wild-type strains (YSB119 and YSB121) and *itr1* mutants (CDX175 and CUX50). Cell fusion results were quantified from repeat assays, and representative images are shown.

production (Fig. 6A). Cell fusion assays showed that *itr1* mutants have cell fusion efficiency similar to that of wild-type strains, suggesting that the mating defect could be caused by post-cell fusion events, such as insufficient dikaryotic hypha production or a meiosis defect (Fig. 6C). Because expression of *ITR1* in an *S. cerevisiae* *itr1 itr2* mutant strain failed to rescue the growth defect of that yeast strain, *Itr1* may not be important for inositol uptake but may play other roles, such as functioning as an inositol sensor. The fact that *ITR1* expression is repressed by high concentrations of inositol also suggests that *Itr1* may be functional as an inositol sensor. To further understand the potential role of *Itr1* in the regulation of other *Itrs*, qRT-PCR analysis was performed to test the expression of the other six *ITR* genes in the *itr1* mutant background, both as

single cultures and during mating, using the same approach as that used for wild-type strains (Fig. 4C and D). Our results revealed that *ITR3*, *ITR3B*, and *ITR3C* are expressed at a much higher level in *itr1* single cultures than in the H99 wild-type strain. During mating, most *ITR* genes were highly expressed, with similar expression patterns in both *itr1* mating cultures and wild-type strains, except that *ITR3A* was highly expressed only in *itr1* mating cultures (Fig. 4). These results indicate that the *ITR3* subgroup genes may be regulated by *Itr1*, which further suggests that *Itr1* could be an inositol sensor that regulates other *Itrs*.

We have also tested the development of virulence factors and found that all mutants produced normal amounts of capsule and melanin and had normal growth at 37 $^{\circ}$ C (data not shown). The

absence of notable phenotypes may indicate that these transporters could be functionally redundant. Another possibility is that although one *Itr* could play a major role in inositol uptake, the inositol internal biosynthesis pathway might produce sufficient inositol to compensate for reduced inositol uptake from the environment in *itr* mutants.

The key enzyme for the *myo*-inositol biosynthetic pathway, inositol 1-phosphate synthase (*Ino1*), has been identified, and *ino1* mutants have been generated, thereby blocking the inositol internal biosynthesis pathway. Importantly, *ino1* mutants failed to grow on medium without *myo*-inositol (data not shown). No obvious mating defect was observed in an *ino1* × *ino1* bilateral mating assay using MS medium (Fig. 6A). To study the function of *Itrs* without the potential interference of the inositol biosynthesis pathway, *ino1* with *itr1*, *itr1a*, *itr2*, *itr3*, *itr3a*, *itr3b*, or *itr3c* double mutants were generated in both of the mating-type backgrounds by genetic crossing. While none of the three major virulence factors were significantly altered in these double mutants, mating filament production and sporulation were clearly reduced in bilateral matings of *ino1 itr1* × *ino1 itr1* and *ino1 itr1a* × *ino1 itr1a* double mutants, suggesting that *Itr1* and *Itr1A* are important for inositol transport or sensing (Fig. 6A). Compared with wild-type strains, the *ino1 itr1* double mutant produced sporadic mating hyphae and reduced spore production, while the *ino1 itr1a* double mutant produced evenly distributed mating filaments but at much lower hyphal density and produced very few spores in MS medium. The sporulation defect in the *ino1 itr1a* double mutant is much more severe than in the *ino1 itr1* double mutant (Fig. 6B). Bilateral mating of other double mutants produced normal mating structures, including dikaryotic hyphae and basidiospores. The identification of the *ITR* gene family and generation of *itr* and *ino1* single and double mutants provide a valuable system to further study how *C. neoformans* senses and acquires inositol.

Virulence study of inositol transporters in a murine systemic infection model. Although none of the *itr* mutants generated showed significant effects on several key virulence factors, such as capsule and melanin production and growth at 37°C (data not shown), their potential involvement in fungal virulence was assessed because virulence is a complex trait. All seven *itr* mutants (*itr1*, *itr1a*, *itr2*, *itr3*, *itr3a*, *itr3b*, and *itr3c*) were examined in a murine inhalation model of systemic *C. neoformans* infection. Groups of 10 female A/JCr mice were intranasally inoculated with 10⁵ yeast cells from each strain, and animals were monitored daily. As previously demonstrated, all mice infected with the wild-type strain H99α survived between 16 and 22 days (39, 40). Mice infected with the mutants exhibited mortality rates similar to those of mice infected with the wild-type H99 strain, indicating that none of these seven *itr* mutants are attenuated for virulence of *C. neoformans* under these conditions (Fig. 7). It is possible that the functional redundancy of this gene family or the existence of the inositol biosynthesis pathway is sufficient for the virulence of each single mutant.

Virulence studies were also conducted in the murine model for an *ino1* mutant, an *ino1 itr1* double mutant, and an *ino1 itr1a* double mutant. While the *ino1* mutant was fully virulent, the *ino1 itr1* double mutant was moderately attenuated for virulence, with statistical significance ($P = 0.001$) (Fig. 7). The median survival time was delayed from 18 days in H99 to 21.5 days in the *ino1 itr1* double mutant. Interestingly, the *ino1 itr1a* double mutant exhibited an even more severe virulence attenuation ($P < 0.0001$), and

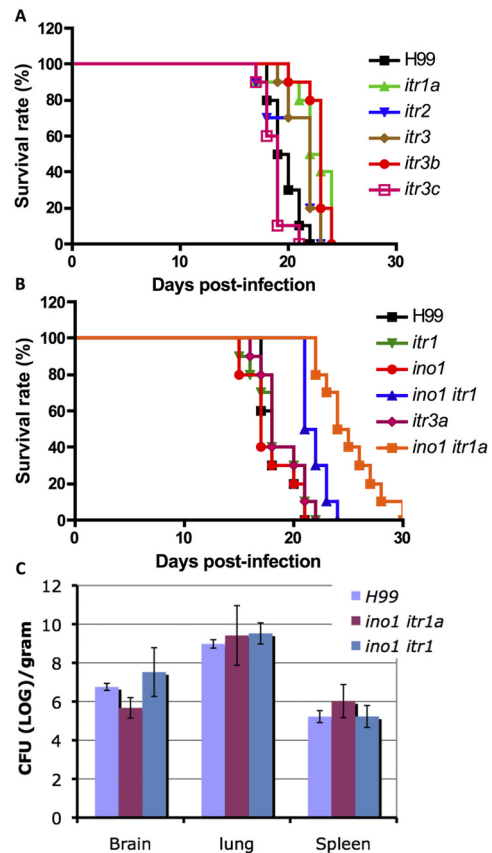


FIG 7 *itr* mutant strains are still virulent in mice. (A and B) Female A/JCr mice were inoculated intranasally with the following strains: H99, *itr1* mutant (CUX7), *itr1a* mutant (CDX99), *itr2* mutant (CDX103), *itr3* mutant (CDX105), *itr3a* mutant (CDX102), *itr3b* mutant (CDX101), *itr3c* mutant (CUX42), *ino1* mutant (UBCINO1), *ino1 itr1* double mutant (CUX17), and *ino1 itr1a* double mutant (CUX23). Two experiments were performed to finish the virulence test for all above-mentioned strains. Groups of 10 mice were infected with each strain. Animals were monitored for clinical signs of cryptococcal infection and sacrificed at predetermined clinical end points that predict imminent mortality. (C) Fungal burdens in organs infected by *ino1 itr1* and *ino1 itr1a* mutants were compared with those in organs infected by H99 at the end point of the infection. The numbers of colony-forming units (CFU) per gram of organ tissue were measured in brain, lung, and spleen homogenates. Each error bar indicates the standard error of the mean for values obtained from testing three animals.

the median survival time was prolonged to 24.5 days (Fig. 7). Evaluation of fungal burdens at the end point of the infection revealed that brains infected by the *ino1 itr1a* mutant contained 10-fold fewer yeast cells than those with H99 infection, which may indicate that the *ino1 itr1a* mutant either has a defect in central nervous system (CNS) entry or proliferates at a lower rate in the brain (Fig. 7C). These results indicate that both *Itr1* and *Itr1A* are important for inositol acquisition in *C. neoformans* and that inositol acquisition is important for fungal virulence.

Genes involved in inositol metabolic pathways are highly up-regulated by inositol. To investigate how inositol affects *Cryptococcus* mating, the effect of inositol on the global transcriptional profile was evaluated by conducting whole-genome microarray experiments. Global gene expression profiles during mating under inositol induction conditions were evaluated in both wild-type strains (H99 and KN99a) and *ino1* mutants (α *ino1* and a

TABLE 2 Genes upregulated by inositol during mating

Gene and function	Product/description	Average fold change in gene upregulation	
		α <i>ino1</i> \times a <i>ino1</i>	H99 \times KN99a
Metabolism			
180.m00186	<i>myo</i> -Inositol oxygenase	38.05	11.81
177.m03138	Inositol oxygenase	14.82	17.79
184.m04662	Isocitrate lyase	4.93	1.78
184.m04739	Phosphatidylinositol 3-kinase	3.41	0.86
162.m02888	Phosphoenolpyruvate carboxylase	3.14	4.59
181.m07830	Fructose-1,6-bisphosphatase	2.85	2.23
162.m02888	Phosphoenolpyruvate carboxylase	3.14	4.59
185.m02442	Pyruvate decarboxylase- related protein	2.32	2.74
179.m00355	Cytochrome <i>c</i> oxidase	1.04	3.05
181.m08517	Acetate-coenzyme A ligase	1.55	2.88
177.m03189	Malate dehydrogenase	1.12	2.44
180.m00160	Formate dehydrogenase	1.57	2.75
Transporter/receptor			
180.m00244	α -Glucoside transporter	11.51	5.16
180.m00262	α -Glucoside transporter	3.01	0.97
162.m02883	α -Glucoside transporter	3.44	1.01
177.m03407	Itr2	3.02	0.70
185.m02698	Itr3B2	2.78	1.17
181.m07866	Itr1	2.34	1.4
163.m03771	Itr3A	1.99	1.75
179.m00178	Putative sugar transporter	3.38	0.71
186.m03862	Ste3	2.05	2.07
164.m01642	Amino acid transporter, putative	2.53	0.76
177.m03009	GDP-mannose transporter	2.59	1.03
183.m01606	Hxt1, putative	3.45	0.85
185.m02415	Uracil permease	2.59	1.31

ino1). Mating assays were performed in MS medium without inositol and in MS medium with 100 mg/liter inositol for 48 h before cells were collected and RNA was purified. C-3- and C-5-labeled cDNAs were hybridized to JEC21 70-mer whole-genome arrays. When the transcriptomes of wild-type mating mixtures in MS medium were compared with that in MS medium without inositol, only a small set of genes (less than 50 genes) were upregulated over 2-fold by inositol. A large proportion of these genes encode putative proteins (see Fig. S3 in the supplemental material). Among these, two inositol oxygenase genes (180.m00186 and 177.m03138) were upregulated over 10-fold. An α -glucoside transporter gene was also significantly upregulated (Table 2). When the transcriptomes of *ino1* mutant mating mixtures in MS medium with and without inositol were compared, over 300 genes were found to be upregulated over 2-fold by inositol. Inositol oxygenase and glucoside transporter genes were also among the genes with the highest induction levels. The pheromone receptor Ste3 was found to be upregulated in both of the experiments. In the *ino1* mutant results, more genes involved in inositol sensing and metabolism were found to be upregulated, including a phosphatidylinositol 3-kinase gene and several inositol transporter

genes, including *ITR1*, *ITR2*, *ITR3A*, and *ITR3B2*, indicating that *ino1* mutant cells are more responsive to environmental inositol than wild-type cells. Results obtained from such systemic analyses provide rich information for further understanding of inositol acquisition and cellular function in *C. neoformans*, especially with respect to sexual reproduction and fungal virulence.

DISCUSSION

Inositol metabolism and catabolism have been found to play important roles in both cellular structure and intracellular signaling and, thus, are critical for cell development. Our previous study identified *myo*-inositol as an important elicitor for *Cryptococcus* mating, both in media and on living plants (26). The importance of *myo*-inositol for fungal mating and the facts that *Cryptococcus* can utilize *myo*-inositol as a sole carbon source and that the human brain contains a high concentration of *myo*-inositol all indicate that *myo*-inositol is important for this fungus. In accord, we found that *Cryptococcus* has an unusually large, expanded inositol transporter gene family. These findings raise the following questions. How do fungal cells sense and transport inositol, and how does inositol affect fungal mating? What is the role of each inositol transporter? Does inositol sensing/transport play a role in fungal virulence? Is there a correlation or causative role between inositol sensing and the high rate of cryptococcal meningitis?

It is likely that the presence of a large *ITR* gene family in *Cryptococcus* is the result of coevolution between this yeast and its environmental niches and host conditions. As one major environmental niche, soil often contains abundant decayed plant materials that are rich in phytic acid (IP6) and other inositol-derived compounds. Although the addition of phytic acid to media did not yield a clear effect on fungal development or mating (data not shown), this may be because IP6 is charged and cannot enter fungal cells directly; IP6 may be converted into other inositol forms that can be utilized by *Cryptococcus*. *Cryptococcus* species, especially *C. gattii*, are commonly isolated from living plants, including *Eucalyptus* species, as environmental niches. We found that free *myo*-inositol exists on several living plant surfaces, and the concentration on *Eucalyptus* is much higher than on *Arabidopsis*, which could have significant implications for how this human pathogen completes its sexual cycle in nature, since *Eucalyptus* is the major environmental niche for *C. gattii* (26).

Sexual reproduction in *Cryptococcus* is important for both genetic manipulation and the production of the suspected primary infectious particles (basidiospores) and has been extensively studied (28, 29, 41–44). In *Cryptococcus* species that infect humans and animals, *C. neoformans* strains are mostly fertile. *C. neoformans* var. *neoformans* (serotype D) is even more fertile than *C. neoformans* var. *grubii* (serotype A), while most isolates of the sibling species *C. gattii* strains (serotype B and serotype C) have lower levels of mating efficiency or are even sterile. We found that the serotype D strain JEC21 has 11 potential *ITR* genes, more *ITR* homologs than any other *Cryptococcus* strain, while the serotype A strain H99 contains 10 *ITR* genes, the serotype B VGI strain WM276 has 7, and the serotype B VGII strain R265 has only 6 *ITR* homologs. Thus, there is an interesting potential correlation between the number of *ITR* genes in each serotype strain and fertility. Further functional studies are required to verify whether all of the candidate *ITR* genes indeed encode functional inositol transporters. The comparison of *ITR* genes among different *Cryptococcus* strains clearly indicates that subgroup 3 has undergone a major

expansion in *C. neoformans* compared to those in *C. gattii*. The facts that most of the *ITR* genes in this subgroup are localized in the telomeric regions of a single chromosome and that all of them share similar intron locations provide further insight into this expansion. Besides environmental niches, human and animal brains have been widely reported to have high concentrations of *myo*-inositol (30–32, 45, 46). Because the major lethal infection caused by *Cryptococcus* is in the CNS and results in cryptococcal meningoencephalitis, there may be a potential correlation between inositol sensing and acquisition and cryptococcal pathogenesis. It is possible that the organism adapted to inositol-rich environments by expanding the *ITR* gene family to promote survival under harsh host conditions.

The subtelomeric location of the *ITR* gene family is interesting and may explain the rapid expansion of *ITR* genes in *C. neoformans*, since genes in such a chromosomal location are commonly rapidly evolving. Our functional genomic analysis also revealed gene duplication events within the genomes of H99 and JEC21 (see Fig. S2 in the supplemental material). Other large gene families have also been observed to have telomeric or subtelomeric locations. The *EPA* (epithelial adhesin) gene family in *Candida glabrata* is important for cell adhesion and contains at least 17 members in the CBS138 strain and 23 in the BG2 strain, and most of them are located in telomeric or subtelomeric regions (47). Interestingly, many are subject to chromatin-based transcriptional silencing that involves telomere-associated proteins, including the Rap1, Hdf1, and Sir proteins (Sir2, Sir3, and Sir4) (47–49). In contrast, we did not observe such transcriptional silencing for the *ITR* genes we studied, because all seven *ITR* genes in H99 were expressed based on our RT-PCR results (Fig. 4).

We identified two groups of *ITR* homologs based on the known inositol transporter sequences in other yeasts; functional studies are important to verify whether they are indeed involved in inositol transport or sensing. In this study, we focused on the seven *ITR* homologs in group 1 of H99. Our quantitative RT-PCR results showed that all seven *ITR* genes are expressed with different expression patterns in response to inositol induction, an indication that this gene family might have both functional redundancy and specificity. Overall, most *ITR* genes were expressed at higher levels at the later time points, suggesting that at later incubation stages, fungal cells require more inositol from the environment as internal inositol and other stored carbon sources are depleted, triggering the induction of *ITR* expression. Also, most *ITR* genes were induced at a higher level in strains during mating than when individual strains were grown alone, suggesting that mating may require more inositol, either as an energy source, a signaling compound, or both. *ITR1A* and *ITR3C* were highly induced by inositol, and both of them complemented the growth defect of the *S. cerevisiae itr1 itr2* double mutant in YPD at 37°C, suggesting that these two are *bona fide* *ITR* genes and play important roles in inositol uptake in H99. Inositol uptake assays in this yeast heterologous expression system will be important to understand the inositol affinity and specificity for each *Itr* from *C. neoformans*.

Interestingly, single-gene deletion mutants of the first seven *ITR* genes in group 1 still undergo cell fusion, mating filament production, and sporulation, indicating that none of these *Itrs* is essential for fungal mating. However, the mating dikaryotic hyphal production in the bilateral *itr1* × *itr1* mutant mating was reduced, especially at early time points, indicating that *Itr1* may be important for fungal inositol acquisition. In our qRT-PCR study,

ITR1 is the only *ITR* gene whose expression was consistently repressed when grown in MS medium as a single strain. Because the expression of the major inositol transporter in *S. cerevisiae*, *ITR1*, is also repressed when additional inositol is available (18), *Itr1* could also be important for inositol transport or sensing in *C. neoformans*. The fact that expression of *ITR1* in the *S. cerevisiae itr1 itr2* double mutant strain failed to rescue the YPD growth mutant phenotype suggests that *Itr1* may be a sensor rather than a transporter. In *S. cerevisiae*, a large glucose transporter gene family has been identified to differentially control glucose uptake, and two permease homologs, *Snf3* and *Rgt2*, function as sensors rather than transporters; these two proteins play important roles in regulating the expression of the other hexose transporters (50, 51). A similar paradigm may exist in *Cryptococcus* to sense inositol using one or more members of the expanded *ITR* gene family. The significant changes in other expression patterns of *ITR* genes in the *itr1* mutant background provide further evidence that *Itr1* could be an inositol sensor that regulates the function of other *Itrs*. It is also possible that the expression of *ITR1* from *C. neoformans* in *S. cerevisiae* may not be stable or functional. On the other hand, *Itr1A* is clearly an important inositol transporter in *C. neoformans* because *ITR1A* is constitutively highly expressed under inositol induction conditions and it also complemented the growth defect of the *S. cerevisiae itr1 itr2* double mutant.

There are two possible explanations as to why none of the *itr* mutants was completely blocked in mating or significantly altered for virulence factor production. First, potential functional redundancy of these *ITR* genes could compensate for a defect conferred by any *ITR* single mutation. Second, it is also possible that since the internal inositol biosynthesis pathway is functional in *C. neoformans* and converts glucose-6-phosphate into *myo*-inositol, this may compensate for any defect in inositol uptake caused by an *itr* mutation. Although none of the seven *itr* single mutants tested showed an obvious effect on fungal virulence in a murine systemic infection model, the mating and virulence defects observed in the *ino1 itr1* and *ino1 itr1a* double mutants provide evidence for a coordinated inositol acquisition mechanism that includes inositol internal biosynthesis and environmental inositol sensing/transport pathways. The virulence attenuation in these two double mutants supports our hypothesis that inositol acquisition is important not only for fungal sexual reproduction but also for fungal disease development. Interesting, although *Itr3C* could complement the growth defect of the *S. cerevisiae itr1 itr2* mutant strain, *ino1 itr3c* double mutants did not show any obvious phenotype in either mating or virulence factor development. It will still be of interest to test this double mutant in the murine model in the future to determine whether *Itr3c* is also involved in virulence. It will also be important and necessary in future studies to generate multiple *itr* mutations in the *ino1* mutant strain background (such as *ino1 itr1 itr1a*) to understand the function of each *Itr* in *C. neoformans*.

We found that *ino1* mutants, similar to *itr* single mutants, *C. neoformans* are still fully virulent and have normal *in vitro* growth, which is consistent with what has been reported in *C. albicans* (21) but different from what has been reported in the bacterial pathogen *Mycobacterium tuberculosis* (52) and the parasite *Trypanosoma brucei* (53). Similar to *C. neoformans*, both *M. tuberculosis* and *T. brucei* maintain functional inositol biosynthetic pathways and active inositol transporters, but the imported inositol is not sufficient for full cell activity, and the *Ino1* protein is

required for cell growth and full virulence. However, despite the fact that the *C. albicans itr1* and *ino1* homozygous single mutants have normal growth and full virulence, similar to what we found in *C. neoformans*, inositol is essential for the viability of *C. albicans*, and the *itr1/itr1 ino1/ino1* double mutant is inviable. The lethality of such double mutations suggests that *C. albicans* also utilizes both the internal biosynthetic pathway and environmental uptake mechanism to acquire inositol, and these two pathways can complement each other (13, 21). Such inositol acquisition machinery could be conserved in other pathogens as well. Because *itr1 ino1* double mutants of *C. albicans* are inviable, it is a challenge to score an effect on virulence. The conditional double mutant (*ino1/ino1 itr1/P_{MET3}::ITR1*) was found to be avirulent, suggesting that inositol is also important for the development of candidiasis (21). In *C. neoformans*, *ino1 itr1* and *ino1 itr1a* double mutants have normal *in vitro* growth and show virulence attenuation, which indicates that *Cryptococcus* has a more well-developed system for acquiring inositol than *Candida albicans* or other pathogens and thus provides a valuable system to evaluate the effect of inositol sensing/transporting on fungal disease development.

It is still unclear how these mutations affect virulence. Although *ino1 itr1* and *ino1 itr1a* double mutants show virulence attenuation, they still produce normal virulence factors *in vitro* and cause lethal infection. One possibility is that the high concentrations of inositol in the human central nervous system (CNS) play a role in the development of cryptococcal meningitis. *C. neoformans* cells may sense inositol via a mechanism similar to chemotaxis in other microorganisms. Such a chemotactic response could attract yeast cells to cross the blood-brain barrier (BBB) and to reach the CNS, where large amounts of inositol are present. Once inside the brain, yeast cells could utilize inositol in the brain, either as a carbon source, signaling molecule, or both; thus, inositol may facilitate fungal proliferation and accelerate the development of meningitis. *Itr1* and *Itr1A* may be required for yeast cells to cross the BBB and/or proliferate in the CNS. In fact, our preliminary animal study results have shown that the *ino1 itr1a* double mutant produced ~10 times fewer CFU in the brain (average, 1.7×10^5 cells/g organ) than the wild-type strain (average, 7.1×10^6 cells/g organ) at the end point of the infection, while in the lung and spleen, both the mutant and wild-type strains reached equivalent levels of fungal burden (Fig. 7C). This result suggests that the *ino1 itr1a* mutant strain may be slower to disseminate and cross the BBB and/or proliferate in the CNS, and thus, mice infected with the *ino1 itr1a* mutant survived longer in our experiments. It would be very interesting to further investigate this hypothesis, which may ultimately contribute to our understanding of why *C. neoformans* so frequently causes lethal cryptococcal meningitis.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *C. neoformans* strains used in this study are listed in Table 3. Strains were grown at 30°C in yeast extract-peptone-dextrose (YPD) agar medium and synthetic defined (SD) medium. V8 medium (pH 5.0) was used for mating assays. Modified MS medium was used for mating and sporulation assays and prepared as previously described, with modification (26). Niger seed medium was used to test for melanin production. Dulbecco modified Eagle's (DME) medium for assessing capsule production was prepared as previously described (54). All other media were prepared as described previously (55–57).

TABLE 3 Strains used in this study

<i>C. neoformans</i> var. <i>grubii</i> strain	Description	Reference
H99	<i>MATα</i> wild type	(69)
KN99a	<i>MATa</i> wild type	(70)
CDX175	<i>MATα itr1::NAT</i>	This study
CUX7	<i>MATa itr1::NAT</i>	This study
CUX50	<i>MATa itr1::NEO</i>	This study
CUX53	<i>MATα itr1::nat ITR1-NEO</i>	This study
CUX54	<i>MATa itr1::nat ITR1-NEO</i>	This study
CDX99	<i>MATα itr1a::NAT</i>	This study
CDX100	<i>MATa itr1a::NEO</i>	This study
CDX103	<i>MATα itr2::NAT</i>	This study
CDX104	<i>MATa itr2::NEO</i>	This study
CDX105	<i>MATα itr3::NAT</i>	This study
CDX106	<i>MATa itr3::NEO</i>	This study
CDX196	<i>MATα itr3a::NEO</i>	This study
CDX197	<i>MATa itr3a::NEO</i>	This study
CDX101	<i>MATα itr3b::NAT</i>	This study
CDX102	<i>MATa itr3b::NEO</i>	This study
CDX166	<i>MATα itr3c::NAT</i>	This study
CDX167	<i>MATa itr3c::NAT</i>	This study
UBCINO1	<i>MATα ino1::NEO</i>	This study
CUX8	<i>MATa ino1::NEO</i>	This study
UBCINO11	<i>MATα ino1::NEO INO1</i>	This study
CUX17	<i>MATα ino1::NEO itr1::NAT</i>	This study
CUX18	<i>MATa ino1::NEO itr1::NAT</i>	This study
CUX23	<i>MATα ino1::NEO itr1a::NAT</i>	This study
CUX24	<i>MATa ino1::NEO itr1a::NAT</i>	This study
CUX29	<i>MATα ino1::NEO itr2::NAT</i>	This study
CUX30	<i>MATa ino1::NEO itr2::NAT</i>	This study
CUX15	<i>MATα ino1::NEO itr3::NAT</i>	This study
CUX16	<i>MATa ino1::NEO itr3::NAT</i>	This study
CUX27	<i>MATα ino1::NEO itr3a::NAT</i>	This study
CUX28	<i>MATa ino1::NEO itr3a::NAT</i>	This study
CUX25	<i>MATα ino1::NEO itr3b::NAT</i>	This study
CUX26	<i>MATa ino1::NEO itr3b::NAT</i>	This study
CUX33	<i>MATα ino1::NEO itr3c::NAT</i>	This study
CUX34	<i>MATa ino1::NEO itr3c::NAT</i>	This study
YSB119	<i>MATα aca1::NAT ura5 ACA1-URA5</i>	(54)
YSB121	<i>MATa aca1::NEO ura5 ACA1-URA5</i>	(54)

Database and sequence information. All fungal ITR gene sequences and other sequences were obtained from a variety of databases. All sequences for *C. neoformans* var. *grubii* and *C. gattii* were obtained from the Broad Institute (<http://www.broadinstitute.org/>), and sequences for *C. neoformans* var. *neoformans* were obtained from the TIGR database (<http://www.tigr.org/>). Related ITR sequences in *S. cerevisiae* and *C. albicans* were obtained from the *Saccharomyces* genome database and *Candida* genome database. The well-defined ITR genes in *S. cerevisiae*, *S. pombe*, and *C. albicans* were used as queries to identify the initial ITR homologs in the H99 genome via the BLAST algorithm with the default setting, and the output was sorted with top hits ranked by BLASTp scores (58). Phylogeny trees were generated using ClustalX 2.0 (59) and viewed via TreeView X (60).

To identify ITR gene homologs/paralogs in different *Cryptococcus* species, the identified ITR gene homologs in H99 were used as queries to conduct BLASTn and BLASTx searches in the genome database for *C. neoformans* strains H99 and JEC21 and *C. gattii* strains R265 and WM276. BLAST results were parsed using an in-house Perl script. Alignment of the deduced amino acid sequences of all ITR genes was performed with ClustalW (61). The aligned amino acid sequences were imported to PhyML to construct their phylogenetic organization using the maximum likelihood method (62). The generated phylogenetic tree was viewed and edited with FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>).

Detection of *ITR* gene expression using quantitative RT-PCR. To test how the *ITR* genes respond to the presence of environmental *myo*-inositol, we measured the mRNA levels for all seven *ITR* genes under inositol induction conditions via quantitative real-time PCR (qRT-PCR). Mating assays were performed by mixing H99 and KN99a and coculturing on MS medium containing 100 mg/liter *myo*-inositol. A single-strain H99 culture was also grown in MS medium. Plates were incubated at room temperature in the dark. Mating mixtures and H99 cultures were collected from agar surfaces at 24 h, 48 h, or 72 h after plating using cell scrapers. Collected cells were washed with distilled water (dH₂O), and pellets were used for total RNA extraction. Total RNAs were extracted using Trizol reagents (Invitrogen) and purified with the Qiagen RNeasy cleanup kit (Qiagen) by following the manufacturer's instructions. Purified RNAs were quantified using a NanoDrop instrument (Thermo Scientific). The same approach was used to prepare RNAs from *itr1* mutant strains.

First-strand cDNAs were synthesized using a SuperScript III cDNA synthesis kit (Invitrogen) by following the instructions provided by the manufacturer. Expression of the *ITR* genes and the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was analyzed using Brilliant SYBR green quantitative PCR reagents (Stratagene). The gene expression level was normalized using the endogenous control *GAPDH* gene, and the relative levels were determined using the comparative threshold cycle (C_T) method. Real-time PCRs were performed using a Mx4000 quantitative PCR system (Stratagene), with an initial denaturation step at 95°C for 10 min, followed by 40 PCR cycles in which each cycle consisted of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. SYBR green fluorescence emissions were monitored after each cycle. The primers for each gene are listed in Table S1 in the supplemental material. Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 60°C and heating it slowly to 95°C, with measurement of fluorescence) at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis and sequencing.

Generation of *S. cerevisiae itr1 itr2* double mutants and heterologous expression of *C. neoformans ITR* genes. *S. cerevisiae itr1* and *itr2* single mutants were obtained from Open Biosystems. A genetic cross between *S. cerevisiae* α *itr1* and *S. cerevisiae* **a** *itr2* was conducted in YPD medium, and the mating mixture was colony purified in yeast nitrogen base (YNB) supplemented with histidine, leucine, and uracil and incubated at 30°C for 2 days. Single colonies were inoculated in sporulation medium and incubated for 5 days, and meiotic products were then isolated by tetrad dissection. Five tetrads were analyzed, and two double mutants (*S. cerevisiae itr1 itr2*) were confirmed by PCR using gene-specific primers (see Table S1 in the supplemental material) and selected for further study. The growth rate of these double mutants was compared with those of wild-type yeast strain BY4742 and *S. cerevisiae itr1* and *itr2* single mutants at both 30°C and 37°C.

Seven *ITR* genes from *C. neoformans* were cloned into the yeast expression vector pTH19 (63), under the control of the *ADH1* promoter. Each of the seven *ITR* expression plasmids was introduced into an *S. cerevisiae itr1 itr2* double mutant background, and colonies were selected on SD-Ura medium. The expression of all of these *ITR* genes in this yeast heterologous system was verified with RT-PCR using gene-specific primers (see Table S1 in the supplemental material). Yeast strains were tested for growth in YPD at 30°C and 37°C.

Generation of inositol transporter mutants and *ino1* mutants. Seven inositol transporters have been identified in the genome of H99 that share significant identity with the *Itrs* of *S. cerevisiae* (*Itr1* and *Itr2*), *S. pombe* (*Itr1* and *Itr2*), and *C. albicans* (*Itr1*). *itr* mutants were generated in the congeneric *C. neoformans* serotype A α (H99) and **a** (KN99a) strains by overlap PCR as previously described (64). The 5' and 3' regions of each *ITR* gene were amplified with primers F1 and R1 and primers F2 and R2, respectively (see Table S1 in the supplemental material for primer sequences), from H99 or KN99a genomic DNA. The dominant selectable markers (Nat^r or Neo^r) were amplified with the M13 primers (M13F/

M13R) from plasmid pNATSTM#122 or pJAF1 (65), respectively. Each target gene replacement cassette was generated by overlap PCR with primers F1 and R2 (see Table S1 in the supplemental material). Purified overlap PCR products were precipitated onto 600- μ g gold microcarrier beads (0.8 μ m [Bioworld Inc.] or 0.6 μ m [Bio-Rad]), and strains H99 and KN99a were biologically transformed as described previously (66). Stable transformants were selected in YPD medium containing nourseothricin (100 mg/liter) or G418 (200 mg/liter). To screen for mutants of each *ITR* gene, diagnostic PCR was performed by analyzing the 5' junction of the disrupted mutant alleles with primers F4 and JH8994 (see Table S1 in the supplemental material). Positive transformants identified by PCR screening were further confirmed by Southern blot analysis. The *ino1* deletion mutants in the H99 background were generated using the same strategy used to generate *itr* mutants, as described above. Because the *ino1* mutant is *myo*-inositol autotrophic, all mutants were tested for growth in YNB medium without *myo*-inositol to verify the deletion of the *INO1* gene.

To generate complemented strains of *itr1* mutants, a genomic DNA fragment that contains a 1.5-kb upstream region of the *ITR1* open reading frame (ORF) and its 500 bp downstream region was amplified with a PCR using primers JH19630 and JH19631. This PCR fragment was fused with the Neo^r selective marker gene at its C-terminal end with an overlap PCR using primers JH19630 and M13R. The overlap PCR product was biologically transformed in both α *itr1* and **a** *itr1* mutant strains. Mating assays were performed to identify the transformants that complemented the *itr1* phenotype.

Generation of *ino1 itr* double mutants via genetic crosses. In a mating assay, *C. neoformans* cells of opposite mating types were mixed and cocultured in V8 or MS agar medium at 25°C in the dark for 10 days, and filamentation was examined by light microscopy. Spore production was also visualized by microscopy and photographed. Basidiospores were also dissected from mating performed in MS medium.

To generate *ino1 itr1* double mutants, a mating assay between α *ino1::NEO* and **a** *itr1::NAT* mutants was conducted, and spores were visualized and isolated with an MSM system (Singer Instruments, England). All progeny that grew in YPD with both nourseothricin and G418 were tested in YNB without inositol, and those that failed to grow were subjected to genomic DNA extraction. PCR was used to screen for *ITR* gene deletion with primers F4/JH8994 and F3/R3 (see Table S1 in the supplemental material). *INO1* gene mutations for all potential double mutants were also tested with primers JH18517/JH18518 and confirmed by Southern blotting. The same approach was used to generate double mutants between *ino1* and other *itr* mutants.

Microarray analyses for genes regulated by inositol. To understand the effect of inositol on gene expression profiles during cell development, microarray experiments were performed to monitor the genes regulated during mating by inositol. Overnight cultures of H99 and KN99a were mixed in equal cell amounts and washed with dH₂O once, and cell mixtures were inoculated on MS medium with or without inositol. Cells were collected from MS plates 48 h postinoculation and washed with dH₂O, and total RNA was purified. Total RNAs were extracted using Trizol reagents (Invitrogen) and purified using the Qiagen RNeasy cleanup kit (Qiagen). Cy3- and Cy5-labeled cDNA were generated by incorporating amino-allyl-dUTP during reverse transcription of 5 μ g of total RNA as described previously (67) and competitively hybridized to a JEC21 whole-genome array generated previously at Washington University in Saint Louis, MO. After hybridization, arrays were scanned with a GenePix 4000B scanner (Axon Instruments) and analyzed by using GenePix Pro version 4.0 and BRB array tools (developed by Richard Simon and Amy Peng Lam at the National Cancer Institute [http://linus.nci.nih.gov/BRB-ArrayTools.html]) as described previously (68).

Assays for melanin and capsule production. Melanin production was assayed by inoculating *C. neoformans* strains into 2 ml of YPD liquid medium, incubating the culture overnight at 30°C, and spotting 5 μ l of each culture on Niger seed agar medium. The agar plates were incubated at 30°C or 37°C for 2 days, and pigmentation of fungal colonies was as-

sessed and photographed. To examine capsule production, 5 μ l of overnight cultures was inoculated on DME agar medium and incubated at 30°C for 3 days. The capsule was visualized with India Ink negative staining and observed with a 100 \times Olympus CX41 equipped with an Infinity digital camera (Olympus).

Virulence study. Yeast strains were grown at 30°C overnight, and cultures were washed twice with 1 \times phosphate-buffered saline (PBS) and resuspended at a final concentration of 2×10^6 CFU/ml. Groups of 10 female A/JCr mice (NCI-Frederick, MD) were intranasally infected with 10^5 yeast cells of each strain as previously described (39). Animals that appeared to be moribund or in pain were sacrificed by CO₂ inhalation. Survival data obtained from the murine experiments were statistically analyzed between paired groups using the log rank test and PRISM program 4.0 (GraphPad Software) (P values of <0.01 were considered significant). At the end point of the infection, infected lungs, brains, and spleens were also isolated and homogenized using a homogenizer in 1 \times PBS buffer. Resuspensions were diluted, 50 μ l of each dilution was spread on YPD medium with antibiotics, and colonies were counted after 3 days of incubation at 30°C.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/content/1/1/e00084-10.full#SUPPLEMENTAL>.

Table S1, DOC file, 0.18 MB.

Fig. S1, TIF file, 2.14 MB.

Fig. S2, TIF file, 2.51 MB.

Fig. S3, TIF file, 0.29 MB.

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