

## Two Major Inositol Transporters and Their Role in Cryptococcal Virulence<sup>∇†</sup>

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***Cryptococcus neoformans* is an AIDS-associated human fungal pathogen and the most common cause of fungal meningitis, with a mortality rate over 40% in AIDS patients. Significant advances have been achieved in understanding its disease mechanisms. Yet the underlying mechanism of a high frequency of cryptococcal meningitis remains unclear. The existence of high inositol concentrations in brain and our earlier discovery of a large inositol transporter (*ITR*) gene family in *C. neoformans* led us to investigate the potential role of inositol in *Cryptococcus*-host interactions. In this study, we focus on functional analyses of two major *ITR* genes to understand their role in virulence of *C. neoformans*. Our results show that *ITR1A* and *ITR3C* are the only two *ITR* genes among 10 candidates that can complement the growth defect of a *Saccharomyces cerevisiae* strain lacking inositol transporters. Both *S. cerevisiae* strains heterologously expressing *ITR1A* or *ITR3C* showed high inositol uptake activity, an indication that they are major inositol transporters. Significantly, *itr1a itr3c* double mutants showed significant virulence attenuation in murine infection models. Mutating both *ITR1A* and *ITR3C* in an *ino1* mutant background activates the expression of several remaining *ITR* candidates and does not show more severe virulence attenuation, suggesting that both inositol uptake and biosynthetic pathways are important for inositol acquisition. Overall, our study provides evidence that host inositol and fungal inositol transporters are important for *Cryptococcus* pathogenicity.**

*Cryptococcus neoformans* is a major human fungal pathogen and the causative agent of fatal cryptococcal meningoencephalitis. It is the leading cause of fungal meningitis, with an estimated ~1 million cases worldwide in patients with AIDS and organ transplants and in those who are immunocompromised by cancer chemotherapy or by other treatments (1, 9, 11, 47, 49). Due to the medical significance and genetic tractability of *C. neoformans*, extensive studies have been conducted on the mechanism of its virulence. Several signal pathways important for *Cryptococcus* virulence have been identified (1, 5, 27, 33, 34, 63). However, fungal virulence is a complex trait, and new virulence-determining mechanisms remain to be explored.

Inositol is essential for cellular structure and intracellular signaling regulation of all eukaryotes. Inositol-derived products have been reported to be important for pathogenicity in fungi and parasites. Enzymes involved in sphingolipid biosynthesis and degradation pathways, such as inositol-phosphoryl ceramide synthase 1 (*Ipc1*) (35) and inositol phosphosphingolipid-phospholipase C1 (*Isc1*) (53), have been found to promote pathogenicity in *C. neoformans*. The importance of sphingolipids for pathogenicity has also been recognized in some parasitic pathogens, including *Leishmania* and *Trypano-*

*soma* species, and related enzymes have been proposed as potential drug targets (36, 37, 45, 60, 67). The diacylglycerol (DAG)-protein kinase C1 (*Pkc1*) signaling pathway is critical for virulence factor production and pathogenicity in *C. neoformans* (18, 19, 24, 25). The glycosylphosphatidylinositol (GPI)-anchored glycolipid is derived from inositol and is necessary for pathogenicity in *Candida* species (61).

There are nine inositol stereoisomers, and *myo*-inositol is the common one (here, referred to as inositol unless otherwise specified). There are two main sources by which fungal cells can acquire inositol. One is the internal synthesis of inositol. Intracellular glucose can be converted into inositol in a multiple-step biosynthetic pathway (14, 50). Inositol can also be imported from the extracellular environment via inositol transporters. The inositol transporter (*ITR*) gene family is part of the sugar transporter superfamily and plays an important role in inositol sensing in fungi, including *Saccharomyces cerevisiae* (30, 31, 42–44, 51), *Candida albicans* (12, 28, 50), *C. neoformans* (64), and *Schizosaccharomyces pombe* (40). Studying *ITR* genes in fungi has clinical significance as *ITR* genes identified in lower eukaryotes, such as fungi (28) and protozoa (15, 39, 52), are proton coupled and differ both kinetically and pharmacologically from the sodium-dependent inositol transporter system (*SMIT*) in humans. Therefore, they have the potential to be developed as potential targets for drug treatment.

Inositol plays a unique role in the development of cryptococci. It is known that *C. neoformans* is one of a few yeast organisms that can utilize inositol as a sole carbon source (6, 23). Recently, we identified inositol as one important compound for stimulating the sexual reproduction of this yeast (64, 65). Furthermore, an unusually large *ITR* gene family has been identified in *C. neoformans* based on the sequence homology

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with known *ITR* genes in other organisms. Functional characterization revealed that this *ITR* gene family is important for both sexual reproduction and fungal virulence (64, 65). Unlike most fungi that contain one or two inositol transporters, the H99 strain carries 10 genes that are considered *ITR* candidates, and most of them are located in the telomere regions of the chromosomes (64). The *ITR* homologues can be divided into two distinct groups (I and II). The seven members of group I (*Itr1*, *Itr1a*, *Itr2*, *Itr3*, *Itr3a*, *Itr3b*, and *Itr3c*) share strong sequence homology with well-characterized *ITR* genes in other fungi, such as *S. cerevisiae* and *C. albicans*. Group II has three members (*Itr4*, *Itr5*, and *Itr6*) that are more distantly related *ITR* genes that have been characterized in other yeasts but are closely related to *HGT19*, potential a second *ITR* in *C. albicans* (12). However, the role of *HGT19* in inositol function has not yet been confirmed.

Cryptococcal meningitis is the predominant form of cryptococcal infection and causes high mortality, but its disease mechanism is still poorly understood. It has been suggested that the high rate of cryptococcal meningitis may be related to the high inositol levels found in mammalian brains (38). The inositol concentration in human cerebrospinal fluid (CSF) is around 22 mg/liter, compared to an average 2.8 mg/liter in plasma, which makes the human brain the location with the most abundant free inositol (17, 55–58). High inositol levels are also found in animal brains (7, 17). Gene expression profiling during experimental cryptococcal meningitis in rabbits showed that the inositol 3-phosphate synthase gene (*INO1*) and inositol monophosphatase were highly expressed during brain infection, further suggesting a potential role of inositol in the development of cryptococcal meningitis (59). Because *Cryptococcus* contains an expanded *ITR* gene family and can utilize inositol as a sole carbon source, we hypothesize that sensing and utilizing host inositol play important roles during pathogen-host interactions. The high levels of inositol in the human brain could, then, be one reason why this pathogen so frequently causes meningitis. Our previous functional studies on the seven group I *ITR* genes revealed that individual single mutations had no clear impact on fungal virulence and that this may be due to functional redundancy, but the *ino1 itr1a* double mutant did show virulence attenuation. This result suggests that inositol acquisition is important for the pathogenicity of *C. neoformans* (64).

In this study, we continue a functional analysis of *ITR* homologues in *C. neoformans* to understand their role in virulence of *C. neoformans*. We expressed *ITR4*, *ITR5*, and *ITR6* in an *S. cerevisiae* heterologous expression system that had previously been used to express the other seven *ITR* genes in *C. neoformans* (64). Inositol uptake assays for all 10 *Saccharomyces* strains expressing *Cryptococcus ITR* genes revealed that *Itr1a* and *Itr3c* are two major inositol transporters. Significantly, *itr1a itr3c* double mutants and *ino1 itr1a itr3c* triple mutants showed significant virulence attenuation in murine infection models. Additional *ITR* genes were activated in the triple mutant background to partially compensate for the inositol defect. These results support the hypothesis that inositol acquisition in *C. neoformans* is important for the *Cryptococcus*-host interaction.

TABLE 1. Strains used in this study

<i>C. neoformans</i> var. <i>grubii</i> strain	Description	Reference or source
H99	<i>MATα</i> wild type	48
KN99a	<i>MATa</i> wild type	41
CDX99	<i>MATα itr1a::NAT</i>	64
CDX100	<i>MATa itr1a::NEO</i>	64
CDX166	<i>MATα itr3c::NAT</i>	64
CDX167	<i>MATa itr3c::NAT</i>	64
UBCINO1	<i>MATα ino1::NEO</i>	64
CUX8	<i>MATa ino1::NEO</i>	64
UBCINO11	<i>MATα ino1::NEO INO1</i>	64
CUX46	<i>MATα itr1a::NEO itr3c::NAT</i>	This study
CUX47	<i>MATa itr1a::NEO itr3c::NAT</i>	This study
CUX57	<i>MATα ino1::NEO itr1a::NEO itr3c::NAT</i>	This study
CUX58	<i>MATa ino1::NEO itr1a::NEO itr3c::NAT</i>	This study
CUX80	<i>MATα itr1a::NEO itr3c::NAT ura5</i>	This study
CUX74	<i>MATα itr1a::NEO itr3c::NAT ura5 ITR1A-URA5</i>	This study
CUX75	<i>MATα ino1::NEO itr1a::NEO itr3c::NAT ura5</i>	This study
CUX76	<i>MATa ino1::NEO itr1a::NEO itr3c::NAT ura5</i>	This study
CUX86	<i>MATα ino1::NEO itr1a::NEO itr3c::NAT ura5 INO1 ITR1A-URA5</i>	This study

MATERIALS AND METHODS

**Strains, media, and growth conditions.** *C. neoformans* strains used in this study are listed in Table 1. Strains were grown at 30°C on yeast extract-peptone-dextrose (YPD) agar medium and synthetic (SD) medium. V8 medium (pH 5.0) was used for mating assays. Modified MS medium (Murashige and Skoog medium) was used for mating and sporulation assays and was prepared as previously described (65). Yeast nitrogen base (YNB) medium without inositol was purchased from Sigma-Aldrich. Niger seed medium was used to test for melanin production. Dulbecco's modified Eagle's (DME) medium for assessing capsule production was prepared as previously described (3). All other media were prepared as described previously (1, 64).

**Database and sequence information.** All fungal *ITR* genes and other sequences were obtained from a variety of databases via the web link (<http://fungal.genome.duke.edu/>). All sequences for *C. neoformans* var. *grubii* were from the Broad Institute (<http://www.broadinstitute.org/>). Related *ITR* sequences in *S. cerevisiae* and *C. albicans* were obtained from the *Saccharomyces* genome database and *Candida* genome database. Phylogeny trees were generated using ClustalX, version 2.0 (32), and viewed via Treeview X (46).

**Detection of *ITR* gene expression using quantitative reverse transcription-PCR (qRT-PCR).** To test how the *ITR* genes respond to the presence of environmental *myo*-inositol, both *in vitro* and *in vivo*, we measured the mRNA levels for all 10 *ITR* genes under different conditions via quantitative real-time PCR (qPCR). Cultures of *C. neoformans* var. *grubii* wild-type strain H99 and its mutant strains were grown on YPD medium for 24 h at 30°C. Collected cells were washed with distilled H<sub>2</sub>O (dH<sub>2</sub>O), and pellets were used for total RNA extraction. Tissues infected by H99 or its mutants were also collected from infected animals right after they were terminated, and tissues were homogenized using a homogenizer and spun down. Pellets were washed with phosphate-buffered saline (PBS) and used for RNA preparation. Total RNAs were extracted using Trizol reagents (Invitrogen) and purified with a Qiagen RNeasy Cleanup Kit (Qiagen) 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) following the instructions provided by the manufacturer. Expression of *ITR* genes and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was analyzed with the comparative threshold cycle (*C<sub>t</sub>*) method using Brilliant SYBR green qPCR reagents (Stratagene) as described previously (64).

**Inositol uptake assay.** Full-length cDNAs of the *ITR4*, *ITR5*, and *ITR6* genes were amplified from *C. neoformans* H99 total cDNA and were cloned into the yeast expression vector pTH19 (22), under the control of the *ADHI* promoter. Each *ITR* expression plasmid was introduced into an *S. cerevisiae* strain lacking both inositol transporters, *Itr1* and *Itr2* (*Scitr1 Scitr2*) for heterologous expression as previously described (64). The expression of each *Cryptococcus ITR* in this yeast heterologous system was verified by RT-PCR using gene-specific primers (see Table S1 in the supplemental material). Yeast strains were tested for growth on YPD medium at 30°C and 37°C.

The inositol uptake assay protocol was adapted in part from protocols of Jin and Seyfang (28) and Chen and Reynolds (12). In brief, the *S. cerevisiae* control strain BY4742, the *S. cerevisiae* *itr1 itr2* mutant strain, and mutant strains expressing *ITR* genes from *C. neoformans* were grown in YPD liquid cultures overnight at 30°C. Cells were diluted in YPD medium to an optical density at 600 nm ( $OD_{600}$ ) of 1.0, grown at 30°C, and collected at an  $OD_{600}$  of 5.0 by centrifugation at  $2,600 \times g$  for 5 min. Cells were then washed twice with PBS at 4°C and resuspended in 2% glucose to a final concentration of  $2 \times 10^8$  cells/ml as determined by a hemacytometer. For the uptake assay, the reaction mixture (200  $\mu$ l) contained 2% glucose, 40 mM citric acid-KH<sub>2</sub>PO<sub>4</sub> (pH 5.5), 0.15  $\mu$ M *myo*-[2-<sup>3</sup>H]inositol (1  $\mu$ Ci/ $\mu$ l; MP BioMedicals). Additional 200  $\mu$ M unlabeled inositol (Sigma-Aldrich) was added to the reaction mixtures for competition assays. Equal volumes of the reaction and cell mixtures (60  $\mu$ l each) were warmed to 30°C and mixed for the uptake assay, which was performed for 10 min at 30°C. As negative controls, mixtures were kept at 0°C (on ice) during the 10-min incubation. Aliquots of 100  $\mu$ l were removed and transferred onto prewetted MetriCel filters (1.2  $\mu$ m pore size) on a vacuum manifold. The filters were washed four times each with 2 ml of ice-cold water. The washed filters were removed and added to liquid scintillation vials for measurements on a PerkinElmer Tri-Carb 2900TR scintillation counter.

**Generation of *itr1a itr3c* mutants and *ino1 itr1a itr3c* mutants.** In mating assays, *C. neoformans* cells of opposite mating types were mixed and cocultured on V8 or MS agar medium at 25°C in the dark for 10 days, and filamentation was examined by light microscopy. Spore production was visualized by microscopy and photographed. Basidiospores were dissected from matings performed on MS medium.

To generate *itr1a itr3c* double mutants, a mating between  $\alpha$  *itr1a::NEO* and *itr3c::NAT* mutants was conducted, and spores were visualized and isolated with an MSM microscope system (Singer Instrument, England). Genomic DNA was isolated from all progeny that grew on YPD medium with both nourseothricin and G418. PCR was used to screen for *ITR1A* gene deletion with primers JH16703/JH8994 and JH16705/JH16706 and for *ITR3C* gene deletion with primers JH19471/JH8994 and JH19469/JH19470 (see Table S1 in the supplemental material). To generate *ino1 itr1a itr3c* triple mutants, a mating between  $\alpha$  *itr1a::NEO itr3c::NAT* double mutant and a *ino1::NEO* mutant was conducted, and spores were isolated. Triple mutants were screened first by PCR on cultures initiated from single spores. All mutants confirmed by PCR were further confirmed by Southern blot analyses. The mating type of each confirmed mutant strain was determined by PCR using mating type-specific primers and by genetic crossing.

To generate complemented strains of the *itr1a itr3c* double mutant, *ura5* mutant strains were generated by selecting colonies grown on agar plates containing 0.1% 5-fluoroorotic acid (5-FOA). A genomic DNA fragment that contained a 1.5-kb upstream region of the *ITR1A* open reading frame (ORF), the *ITR1A* gene ORF, and its 500-bp downstream region was amplified by PCR. The *ITR1A* PCR fragment was fused with the *URA5* selective marker gene at its C terminus in an overlap PCR. The *ITR1A-URA5* overlap PCR product was biologically transformed in both *itr1a itr3c* mutant strains.

**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  $\mu$ l of each culture with  $10 \times$  series dilutions on Niger seed agar medium and L-3,4-dihydroxyphenylalanine (L-DOPA) medium. The agar plates were incubated at 30°C or 37°C for 2 days, and pigmentation of fungal colonies was assessed and photographed. To examine capsule production, 5  $\mu$ l of overnight cultures was inoculated on DME agar medium and incubated at 30°C for 3 days. Capsule was visualized with india ink negative staining and observed with an Olympus CX41 microscope equipped with an Infinity digital camera (Olympus).

To quantify the production of melanin by different strains, the activity of laccase enzyme was measured as previously described (26). Single colonies of each strain were inoculated in 5 ml of YPD liquid medium and incubated at 30°C overnight. A total of  $10^8$  cells from each overnight culture were inoculated in to 25 ml of L-DOPA medium in 125-ml flasks and incubated at 30°C for 16 h with shaking at 250 rpm. The cultures were further incubated at 25°C for 24 h with

shaking at 250 rpm. One milliliter of each culture was centrifuged, and the supernatants were spectrophotometrically read for optical density at a wavelength of 475 nm.

**Murine infection.** *Cryptococcus* strains were grown at 30°C overnight, and cultures were washed twice with  $1 \times$  phosphate-buffered saline (PBS) by centrifugation and resuspended at a final concentration of  $2 \times 10^6$  CFU/ml. Groups of 10 female A/Jcr mice (NCI-Frederick, MD) were used for each infection. For the intranasal inhalation model, mice were intranasally infected with  $10^5$  yeast cells of each strain in 50  $\mu$ l of PBS as previously described (13). For the intravenous injection model,  $5 \times 10^4$  yeast cells in a 100- $\mu$ l volume for each strain were inoculated via tail vein injection. For the intracerebral injection model, mice were sedated with a xylazine-ketamine combination, and the top of the head was sterilized using antiseptic until the hair was thoroughly wet. A total of 500 yeast cells in 50  $\mu$ l of PBS were directly injected into the cerebrum. Animals that appeared moribund or in pain were sacrificed by CO<sub>2</sub> inhalation. Survival data from the murine experiments were statistically analyzed between paired groups using a log rank test and the PRISM program, version 4.0 (GraphPad Software) (*P* values of  $<0.01$  were considered significant).

**Histopathology and organ fungal burden.** Infected animals were sacrificed at 3 and 7 days postinfection. Lungs and brains were isolated and fixed in 10% formalin solution and sent to the University of Medicine and Dentistry of New Jersey (UMDNJ) core facility for section preparation. Tissue slides were stained with hematoxylin and eosin (H&E) and examined by light microscopy. At each time point of the infection, infected lungs and brains were also isolated and homogenized using a homogenizer (Ultra-Trra T8; IKA) in  $1 \times$  PBS buffer. Resuspensions were diluted, and 50  $\mu$ l of each dilution was spread on YPD medium with antibiotics, and colonies were counted after 3 days of incubation at 30°C.

## RESULTS

***Cryptococcus Itr1a* and *Itr3c* are two major inositol transporters with high inositol uptake activity.** Two groups (group I and group II) of potential inositol transporters with a total of 10 members were identified in the H99 genome (Fig. 1A). In our previous studies, the group I seven *ITR* genes of *C. neoformans* were expressed in an *S. cerevisiae* mutant strain with deletions of both *ITR1* and *ITR2* (*Scitr1 Scitr2*), and we determined that *ITR1A* and *ITR3C* could rescue the growth defect of the *Scitr1 Scitr2* mutant (64). In this study, we also expressed *Cryptococcus* group II *ITR* genes (*ITR4*, *ITR5*, and *ITR6*) in the *Saccharomyces* tester mutant to further understand the role of each *ITR* in inositol sensing and uptake. Full-length cDNAs of *ITR4*, *ITR5*, and *ITR6* were cloned into the yeast expression vector pTH19 under the control of the *ADHI* promoter. The expression of these *ITR* genes was confirmed by RT-PCR (see Fig. S1 in the supplemental material). Strains expressing *ITR4*, *ITR5*, and *ITR6* could not complement the growth defect of *Scitr1 Scitr2* (see Fig. S1). The failure of expression of *ITR* genes other than *ITR1A* or *ITR3C* to complement the defect could reflect either the instability of the overexpressed *ITR* genes or their lack of functional inositol transporter activity.

To better understand the role of each *ITR* in inositol function, inositol uptake activity was measured for all 10 *Saccharomyces* strains expressing *C. neoformans ITR* genes by using <sup>3</sup>H isotope-labeled *myo*-inositol (*myo*-[2-<sup>3</sup>H]inositol). Uptake assays were performed at 30°C by incubating yeast cells with *myo*-[2-<sup>3</sup>H]inositol for 10 min. The results showed that yeast strains expressing *ITR1A* and *ITR3C* had very high uptake activity, which is consistent with their ability to complement the growth defect. None of the others showed significant inositol uptake activity (Fig. 1B). Our results indicated that *Itr1a* and *Itr3c* are major inositol transporters.

**Expression of *INO1* is not altered by inositol level or mutation of *ITR1A* and *ITR3C*.** There are two active pathways that

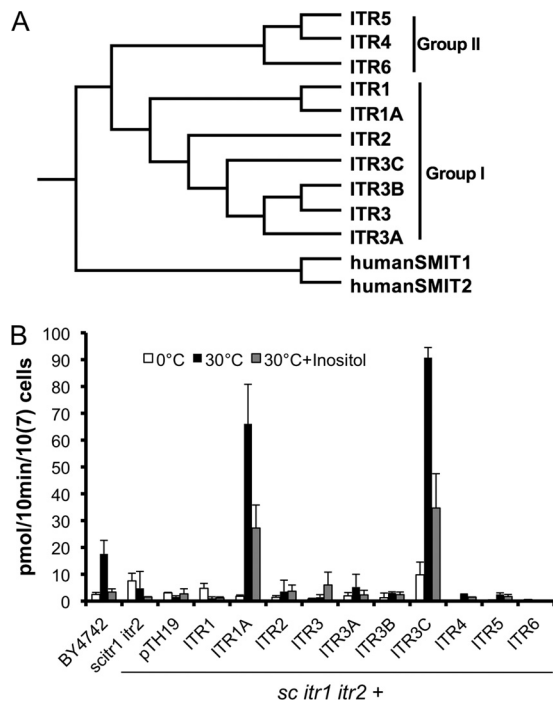


FIG. 1. *Saccharomyces* strains expressing *ITR1A* or *ITR3C* have high inositol uptake activity. (A) Phylogenetic tree of *ITR* gene family in *C. neoformans* (Itr1 to Itr6). The full-length protein sequences were used for alignment. The phylogram was generated using ClustalX, version 2.1, viewed by TreeView. (B) Inositol uptake analysis of *Cryptococcus ITR* genes expressed in a yeast heterologous system. Yeast cells were mixed with <sup>3</sup>H-labeled inositol and incubated at 0°C or 30°C for 10 min. Additional 200 μM unlabeled inositol was added to the reaction mixture for a competition assay as a control. Error bars indicate standard deviations of data from at least three independent experiments. *sc*, *S. cerevisiae*.

can supply inositol for fungal cellular function: importing inositol using *ITRs* or producing inositol via the Ino1-controlled biosynthetic pathway. A *C. neoformans ino1* mutant is inositol auxotrophic and cannot survive when inoculated on a medium without inositol (Fig. 2A). To further understand the role of the biosynthetic pathway, we measured the expression of the *INO1* gene under different inositol conditions. Overnight cultures of H99 were grown in YPD liquid medium, YNB medium lacking inositol, or YNB with 1% inositol. Total RNAs were prepared, and *INO1* expression was detected in a Northern blot assay. Our results showed that *INO1* is expressed under all tested conditions (Fig. 2B). The expression of *INO1* was much higher when *Cryptococcus* cultures were incubated on minimal medium than when cultured on rich medium (YPD), suggesting that *INO1* expression is regulated by nutrient availability.

To elucidate the potential effect of the inositol uptake on inositol biosynthesis, we generated the *itr1a itr3c* double mutants because of their importance in inositol uptake. The expression of the *INO1* gene in this double mutant background was analyzed by Northern blotting. Interestingly, the level of *INO1* expression observed in the *itr1a itr3c* double mutant background was similar to that of the wild type under all three culture conditions, suggesting that *INO1* expression was not altered by different expression of *ITR* genes. This result also

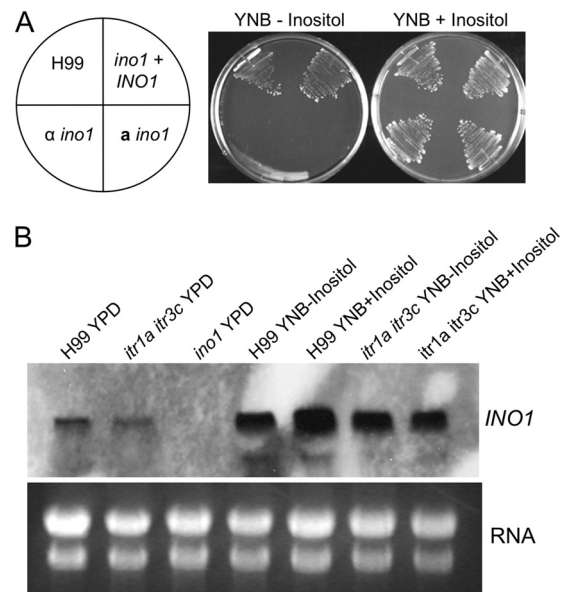


FIG. 2. *INO1* expression is not regulated by inositol. (A) *ino1* mutants are inositol auxotrophic. Yeast cells of H99, the *α ino1* mutant, the *a ino1* mutant, and its complemented strains were streaked on YNB medium with or without 1% inositol. Cultures were incubated at 30°C for 48 h. (B) Overnight cultures of H99, the *itr1a itr3c* mutant (CUX46), and the *ino1 itr1a itr3c* mutant (CUX57) grown on YPD or YNB medium without inositol or with 1% inositol were prepared and their total RNAs were purified. Total RNAs were separated on a denatured gel and transferred to a nylon membrane. The *INO1* gene fragment was used as the probe for detecting the expression of *INO1* transcripts. Total RNAs stained with ethidium bromide were used as loading controls.

indicated that the expression of *INO1* is not regulated by the availability of inositol in the medium since similar mRNA levels were detected in both wild-type and mutant cultures prepared from medium with or without supplemental inositol (Fig. 2B).

**Itr1a and Itr3c are required for sexual reproduction.** Although we previously found that inositol is important for stimulating fungal mating, each inositol transporter single mutant still produced normal dikaryotic mating hyphae and normal sporulation except the *itr1* mutant, which showed a mating defect in bilateral mating assays (64). Because of the importance of Itr1a and Itr3c in inositol uptake when they are expressed in *S. cerevisiae*, mating assays were performed in *itr1a itr3c* double mutants. Although no obvious mating defect was observed in unilateral mating assays between *itr1a itr3c* double mutants and the wild type, bilateral mating results (*itr1a itr3c* × *itr1a itr3c*) showed that such double mutants had a clear defect in the production of mating hyphae on both MS and V8 mating media, further suggestive of their functional importance (Fig. 3).

**Itr1a and Itr3c are important for fungal virulence.** *In vitro* analyses showed that the *itr1a itr3c* double mutant produced normal capsules and had normal growth at 37°C. Mutants produced normal melanin when incubated on Niger seed agar medium (data not shown). However, a modest melanin defect was observed when the mutants were incubated on L-DOPA agar medium (Fig. 4A). The difference in melanin production

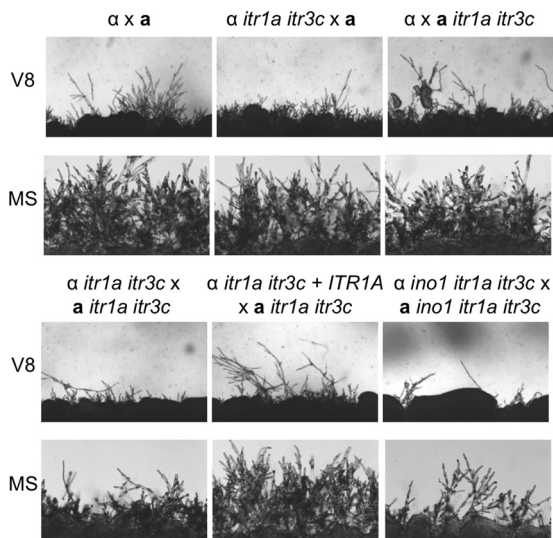


FIG. 3. *itr1a itr3c* mutants showed a mating defect. Unilateral and bilateral mating assays were performed in both MS and V8 media with *itr1a itr3c* double mutants and *ino1 itr1a itr3c* triple mutants. Mating cultures were incubated at room temperature in the dark for 5 days before photography. Significant reduction of mating hyphal production was observed in the bilateral mating assays of the double mutants and triple mutants.

between these two melanin detection media (Niger seed medium and L-DOPA medium) may be due to different substrate concentrations. Laccase activity analyses also revealed that the double mutants showed lower laccase activity (Fig. 4B). Such results indicate that *Itr1a* and *Itr3c* may also play a role in virulence factor development.

We examined the virulence of *itr1a itr3c* mutants in a murine inhalation model. Our results showed that mice infected by wild-type H99 or the *itr3c* mutant survived for less than 22 days postinoculation while mice infected by the *itr1a itr3c* double mutant had a prolonged survival rate between 27 and 30 days. Because *itr3c* single mutants did not show any visible defect in our studies, we generated functionally complemented strains of *itr1a itr3c* double mutants by reintroducing a copy of only the *ITR1A* gene. Mice infected by the complemented strain had a survival rate similar to that of the wild type, an indication of functional complementation. The *itr1a* mutant strain also showed modest virulence attenuation but was not statistically significant ( $P > 0.1$ ). These results indicate that the *itr1a itr3c* double mutant had significant virulence attenuation (Fig. 5A) and that both *Itr1a* and *Itr3c* are required for full virulence.

Because of the high inositol concentrations in human and animal brains, *ITRs* may be important during brain infection by affecting either fungal cell dissemination and blood brain barrier (BBB) crossing or fungal growth in the brain. Because we used an inhalation model, death of the mice in the experiments could have been caused by multiple organ failures even before the full progression of brain infection. To address this concern, we performed additional virulence assays by using other animal models. An intravenous injection model was used to specifically check virulence during dissemination and during brain infection. In this tail vein injection model, yeast cells are able to bypass the establishment of local infection in the lung and

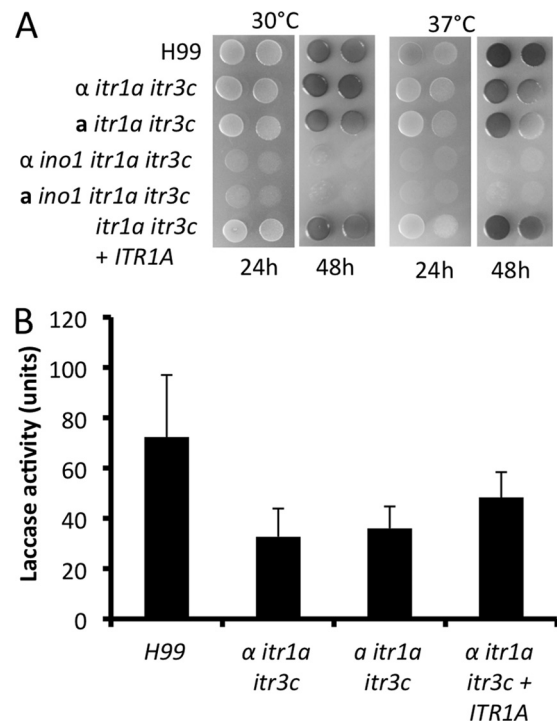


FIG. 4. *itr1a itr3c* mutants showed a melanin defect and reduced laccase activity. (A) *itr1a itr3c* double mutants showed modest melanin defect on L-DOPA medium. Overnight cultures of H99, *itr1a itr3c* mutants (CUX46 and CUX47), and the complemented strains (CUX74) as well as *ino1 itr1a itr3c* triple mutants (CUX57 and CUX58) were washed with dH<sub>2</sub>O and spotted on L-DOPA medium. Melanin production was photographed after plates were incubated at either 30°C or 37°C for 24 h. Triple mutants could not grow on L-DOPA medium due to lack of inositol in the medium. (B) Laccase activity assays were performed as described in Materials and Methods. Results presented were analyzed based on three independent replicates.

disseminate into the brain directly via the bloodstream. Ten female A/Jcr mice per group were used in our studies, and each mouse was injected with  $5 \times 10^4$  yeast cells; infected mice were carefully monitored, and their survival rates were recorded. Our results found that *itr1a itr3c* double mutants had significant virulence attenuation compared to wild-type H99 and the complemented strain (Fig. 5B).

We also performed the virulence assay using a murine intracerebral injection model to specifically investigate the role of *Itr1a* and *Itr3c* in brain infection. Five hundred yeast cells were injected into the cerebrum of each mouse directly. Mice infected by H99 died rapidly by 7 days postinjection, while animals infected by the *itr1a itr3c* mutant survived up to 12 days, indicating significant virulence attenuation of the mutant strain during brain infection (Fig. 5C).

To understand why *itr1a itr3c* double mutants have a virulence defect, the fungal burdens in infected lungs and brains at different time points of intravenous infection were measured by yeast CFU count. At 3 or 7 days postinfection, compared with mice infected with wild-type strain H99, mouse brains and lungs infected by the double mutant showed significant CFU reductions (Fig. 6A and B). Histopathology results of infected lungs also demonstrated less severe lesion development in

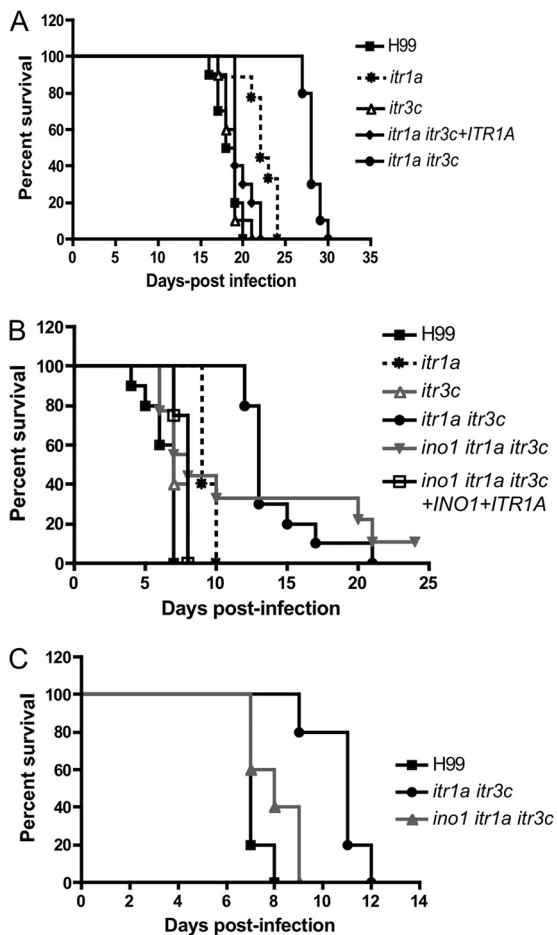


FIG. 5. *itr1a itr3c* mutants showed significant virulence attenuation in murine infection models. (A) Female A/Jcr mice were inoculated intranasally with the following strains: H99, *itr1a* mutant (CDX99), *itr3c* mutant (CUX42), and *itr1a itr3c* double mutant (CUX46) and its complement strain (CUX74). Groups of 10 mice were infected with  $1 \times 10^5$  yeast cells each strain. (B) Survival curves of infected mice in a murine intravenous injection model. Female A/Jcr mice were inoculated via tail veins with the following strains: H99, the *itr1a itr3c* mutant (CUX46), the *ino1 itr1a itr3c* mutant (CUX57), and the complemented strain (CUX74). Groups of 10 mice were infected with  $5 \times 10^4$  yeast cells each strain. (C) Groups of 10 mice were infected by H99, CUX46, or CUX57 via intracerebral injection with 500 yeast cells per mouse. Animals were monitored for clinical signs of cryptococcal infection and sacrificed at predetermined clinical endpoints that predict imminent mortality.

lungs and brains infected by the double mutant (Fig. 6C). These observations suggest that the *itr1a itr3c* mutant showed attenuated virulence during both lung and brain infection.

**The *ino1 itr1a itr3c* mutant strain showed attenuated virulence.** To evaluate the potential functional redundancy between the inositol uptake/sensing pathway and the inositol biosynthetic pathway, we generated the *ino1 itr1a itr3c* triple mutants in both mating types and analyzed their role in fungal virulence using murine models. The *ino1 itr1a itr3c* mutant showed normal growth both at 30°C and 37°C (data not shown) but showed a clear defect on mating filament production in bilateral mating assays (Fig. 3). In our virulence assay using the intravenous infection model with  $5 \times 10^4$  cells as an initial

inoculum, it was observed that 50% of mice infected by the triple mutant showed an increased survival rate, an indication that this mutant strain also showed attenuated virulence at a similar level as the *itr1a itr3c* double mutant (Fig. 5B). But the virulence attenuation phenotype was not as strong as that of the *itr1a itr3c* double mutants in the intracerebral infection model (Fig. 5C).

**The *itr1a itr3c* mutants showed defects in inositol uptake activity.** To understand whether the virulence attenuation and mating defect of the *itr1a itr3c* mutants were due to the defect in inositol uptake activity, we performed inositol uptake assays for H99, the *itr1a itr3c* double mutant, and the *ino1 itr1a itr3c* triple mutant following the method described in Materials and Methods. Our results showed that all three strains had similar uptake activities when a small amount of inositol was available. However, when the inositol concentrations in the reaction mixture were higher than 0.5  $\mu$ M, mutant cells could import much less free inositol in 10 min than the wild-type strain H99 (Fig. 7). Interestingly, the triple mutant showed a higher inositol uptake rate than the double mutant under the same conditions, suggesting that remaining *ITR* genes could be activated in the triple mutant to compensate for the mutation of *INO1* in the *itr1a itr3c* double mutant background.

**Additional *ITR* genes were induced in the double mutant and the triple mutant.** Because *ino1* mutants could not survive on medium without inositol (Fig. 3A), blocking both inositol internal biosynthetic pathway and inositol uptake activity would be lethal, a phenomenon called “inositol-less death.” The possible reason that the *ino1 itr1a itr3c* triple mutant did not show more severe virulence attenuation than the *itr1a itr3c* double mutant could be either that additional *ITR* genes play an increased role in inositol uptake or that the remaining *ITR* genes become active in the triple mutant background. Such a hypothesis is consistent with our inositol uptake results showing that the triple mutant had a higher uptake rate than the double mutant (Fig. 7). To test this hypothesis, we first measured the expression of *ITR* genes in the double mutant and the triple mutant and compared their expression with levels in the wild type when all strains were cultured in YPD rich medium. Our qRT-PCR results showed that the expression levels of most *ITR* genes were significantly increased in both the double mutant and the triple mutant (Fig. 8A). We also performed qRT-PCR to analyze the expression of all 10 *ITR* genes in lung and brain tissues that were infected by either H99, the double mutant, or the triple mutant strain in a intravenous injection model. Our qRT-PCR results showed that the expression of most *ITR* genes was highly induced in both lung and brain tissues infected by H99 compared to expression in H99 culture prepared on YPD rich medium (Fig. 8B). Compared to *ITR* expression in tissues infected by H99, *ITR5* expression was highly induced in tissues infected by the triple mutant in both lung and brain. Meanwhile, the expression levels of *ITR2*, *ITR3*, *ITR4*, and *ITR6* were induced in brain tissues infected by either the double mutant or the triple mutant (Fig. 8C and D). These results confirm that additional *ITR* genes are activated during infection with mutants lacking the two major *ITR* genes. The group II *ITRs* (Itr4, Itr5, and Itr6) may play an important role during *Cryptococcus*-host interactions, especially in the *itr1a itr3c* mutant backgrounds.

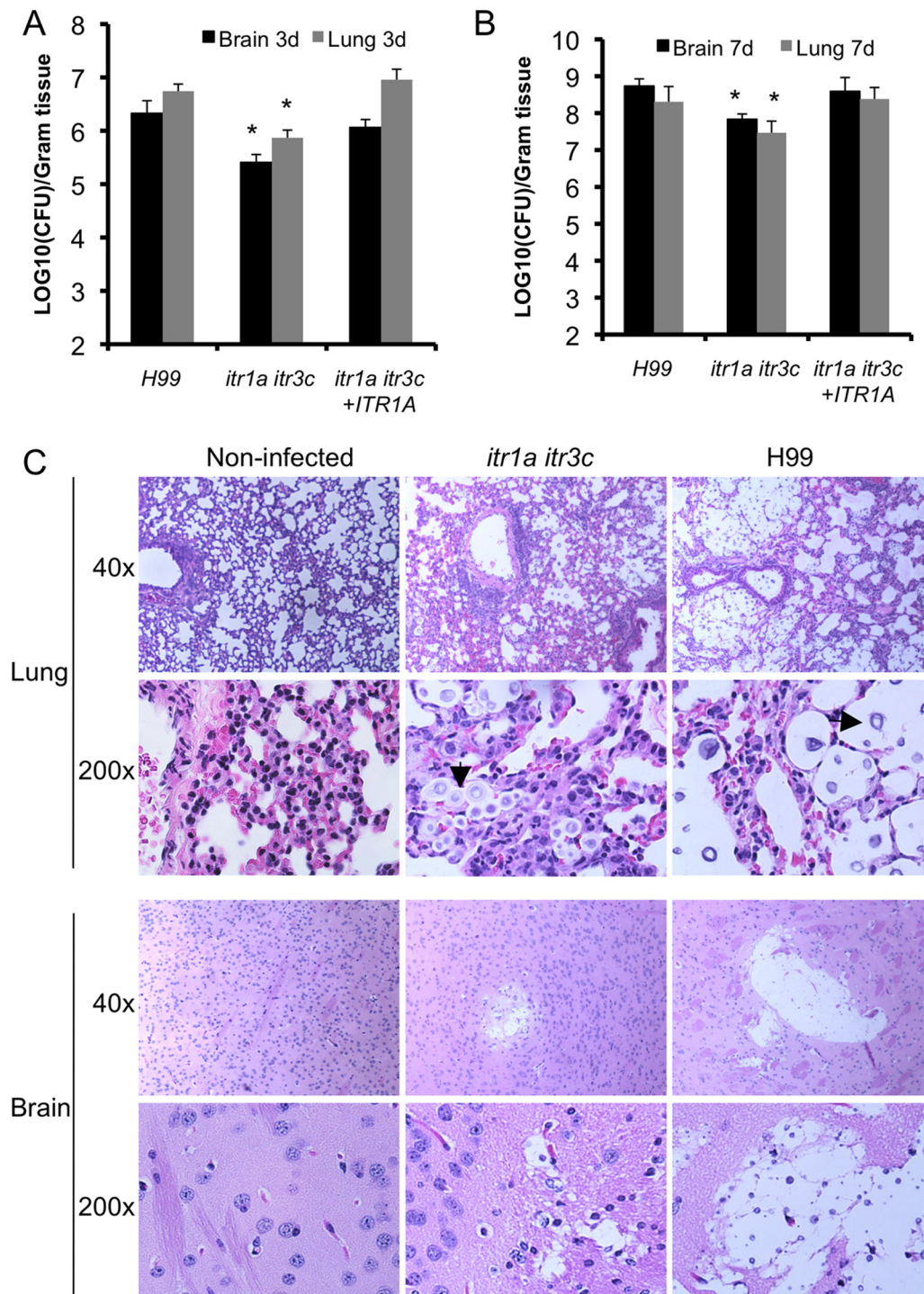


FIG. 6. H&E staining of lung and brain tissues infected by H99 or the *itr1a itr3c* mutant. (A) A/Jcr mice were infected with  $5 \times 10^4$  cells per mouse via tail vein injection, and infected lungs and brains were harvested at 3 days and 7 days postinoculation. Fungal burdens in organs infected by the *itr1a itr3c* mutant (CUX46) were compared with organs infected by H99. The number of yeast CFU per gram organ were measured in brain and lung homogenates. Each error bar indicates the standard error of the mean for values from three animals. The asterisk indicates statistical significance. (B) H&E-stained slides prepared as described in Materials and Methods. Arrows denote yeast cells in the infected tissue.

## DISCUSSION

Our previous studies revealed that inositol is important for the sexual reproduction of *C. neoformans* and that this fungus may utilize inositol from plants to complete its sexual cycle in

nature (65). We have also showed that *C. neoformans* contains a large inositol transporter (*ITR*) gene family, which is very unusual in fungi (64). In accord with the biological significance of inositol and inositol transporters (*ITRs*), it has been well

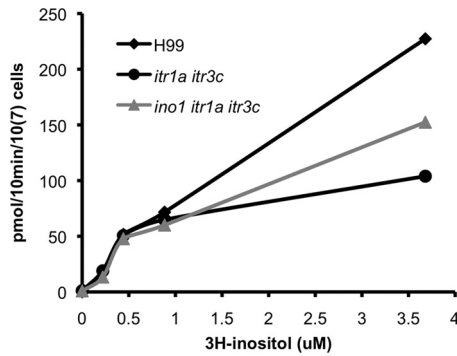


FIG. 7. The *itr1a itr3c* mutant had a lower inositol uptake rate than the wild-type H99. Inositol uptake assays were performed for *C. neoformans* strains (H99, CUX46, and CUX57) following the protocol described in Materials and Methods with several different concentrations of radioactively labeled inositol (0, 0.22  $\mu$ M, 0.44  $\mu$ M, 0.88  $\mu$ M, and 3.68  $\mu$ M). Representative results from three repeats were presented.

documented that human and animal brains contain high inositol levels and that abnormal brain inositol levels are related to a variety of important human diseases such as bipolar disorder (7, 54–56, 58). *C. neoformans* is the most common cause of fungal meningitis in AIDS patients. Hence, it is important to investigate whether high inositol levels in brains play a role in the development of cryptococcal meningitis. However, the functional redundancy of the inositol acquisition system in *C. neoformans* complicates the study of these transporters.

Our previous studies revealed that most single transporter deletion mutants do not have any obvious defective phenotype (64). Although the *ino1 itr1* and *ino1 itr1a* double mutants showed more clear defects in mating and virulence, it was still difficult to assess the function of each *ITR*, including its inositol uptake activity. In our previous study, we have utilized a yeast heterologous expression system that is based on a *Saccharomyces* mutant strain lacking inositol uptake activity caused by deletion of both inositol transporters *ITR1* and *ITR2* to assess the function of each *ITR* of *C. neoformans* (64). Here, we utilized these heterologous expression strains to assess inositol uptake activity of each *Cryptococcus* *ITR* and identified that *Itr1a* and *Itr3c* are major inositol transporters with high inositol uptake activity. Inositol uptake assays in *C. neoformans* strains also revealed that *Itr1a* and *Itr3c* are important for inositol uptake (Fig. 7). Because the *itr1a itr3c* double mutant had a much lower uptake rate than the wild-type strain only when a large amount of free inositol was available, *Itr1a* and/or *Itr3c* likely functions as a low-affinity transporter. Such an outcome is consistent with our early finding that both *ITR1A* and *ITR3C* are highly expressed during inositol induction based on our previous real-time PCR (qPCR) analyses (64). Among the rest of the *ITR* genes, high-affinity inositol transporters could exist. It is unclear why expressing the other eight *ITR* genes in this yeast heterologous system failed to complement the growth defect of the *Saccharomyces* mutant and did not show clear inositol uptake activity. Although all of them are expressed, based on RT-PCR analysis, it is possible that either overexpressed proteins are not stable or are not expressed properly in *S. cerevisiae*. Western blot analysis will be required to address this possibility, which is beyond the scope

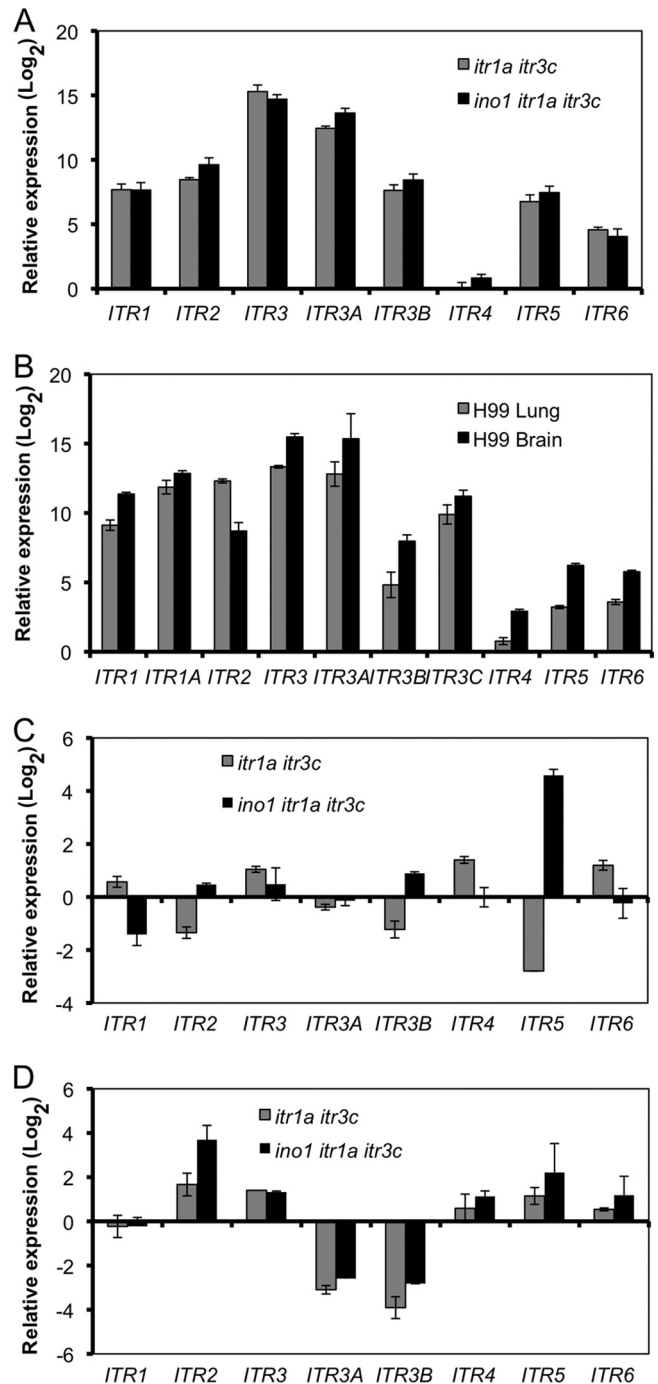


FIG. 8. Comparison of the expression of *Cryptococcus* *ITR* genes in *in vitro* cultures or in infected lungs and brains using qRT-PCR. (A) RNAs were purified from cultures of H99 or the *itr1a itr3c* (CUX46) or the *ino1 itr1a itr3c* (CUX57) mutant that were grown on YPD medium overnight. (B to D) RNAs were isolated from either infected mouse lungs (B and C) or infected mouse brains (B and D) at the endpoint of the infection experiment. 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 either H99 overnight liquid culture (A and B), lungs infected by H99 (C), or brains infected by H99 (D). cDNAs from noninfected lung was used as a negative control to make sure that only the fungal *GAPDH* gene was amplified. Error bars indicate the standard deviations from three independent PCR results.



of this study. It is also possible that some of them are not bona fide inositol transporters and, instead, function as either transporters for other sugars or as inositol sensors. Our previous study has demonstrated that *Itr1* may be a sensor based on its expression pattern and mutant phenotype (64). More study will be required to determine whether *Itr1* functions as a sensor.

In addition to acquiring inositol through uptake by *ITR* genes, fungal cells can also synthesize inositol by utilizing other sugars. The *INO1* gene encodes a rate-determining enzyme in the inositol biosynthetic pathway. Our results showed that *INO1* expression was not regulated by the availability of extracellular inositol; instead, it was upregulated by the nutrient limitation condition. Such an outcome is consistent with earlier studies in *C. neoformans*, which suggest that expression of *Cryptococcus* Ino1 and cellular phosphatidylinositol (PI) levels are not regulated by inositol (38, 62), but these results differ from what has been observed in *S. cerevisiae* (10) and *Candida glabrata* (8). In *S. cerevisiae*, Ino1 expression is highly regulated by inositol concentrations. A key inositol regulatory system has been identified that involves two positive regulators, Ino2 and Ino4, which form a heterodimer via their basic helix-loop-helix (bHLH) domains and bind to the promoter of *INO1* (2). The negative regulator Opi1 represses the expression and binding of the Ino2-Ino4 heterodimer (29). These inositol acquisition regulators in *S. cerevisiae* regulate not only phospholipid biosynthesis but also inositol uptake through *ITR1* expression (2, 21, 31). Interestingly, we could not find homologs of these three proteins in *C. neoformans* by BLAST search with H99 genome database. Thus, it is possible that the regulatory system in *C. neoformans* is very different from that in *S. cerevisiae*, and the role of Ino1 and its regulatory system in *C. neoformans* remains to be determined.

Identification of *Itr1a* and *Itr3c* as major transporters allowed us to focus on the functional significance of these two *ITR* genes. Mutagenesis analyses revealed that the *itr1a itr3c* double mutants showed significant defects in both sexual reproduction and virulence, further demonstrating the importance of inositol transporters on the fungal life cycle and disease development. Similar to finding in our earlier studies, *itr1a itr3c* double mutants demonstrated no obvious defects pertaining to two major virulence factors (production of capsule and growth at 37°C). However, the double mutant did exhibit reduced melanin production and laccase activity when assayed on L-DOPA medium, suggesting that *ITR* genes are involved in melanin production. It is unclear whether the modest melanin defect is part of the cause of virulence attenuation. Because no significant virulence defect was observed in either *itr1a* or *itr3c* single mutants, the virulence defect of *itr1a itr3c* double mutants suggested that *Itr1a* and *Itr3c* are both required for full virulence, and they likely play a redundant role in inositol function. It remains unclear how inositol and fungal inositol transporters affect fungal virulence and whether this fungus utilizes inositol as a carbon source, a signaling molecule, or both (see Fig. S2 in the supplemental material). Our findings of reduced yeast CFU in lungs and brains infected by the *itr1a itr3c* mutant at all time points postinfection suggest that this mutant may have decreased fitness *in vivo*, which could be due to a defect in host inositol utilization.

Although a significant virulence defect was observed, the mutant still caused infection resulting in morbidity and mor-

tality in the rodent models. While *Itr1a* and *Itr3c* appear to be the only major *ITR* genes, based on uptake assays in the yeast heterologous system, it is possible that other *ITR* genes also play a role in inositol sensing or uptake or that additional *ITR* candidates are activated in the double mutant background to partially compensate the defect of inositol acquisition. Also because the inositol internal biosynthetic pathway is still active, the potential functional redundancy between inositol biosynthesis and inositol uptake could compensate the defect of *itr1a itr3c* double mutants. We thus generated the *ino1 itr1a itr3c* triple mutants and evaluated the infectivity of such mutants. Interestingly, the triple mutant also showed virulence attenuation both in intravenous and intracerebral infection models but to a lesser extent than the double mutant in the intracerebral injection model. Because of the essentiality of Ino1 in inositol biosynthesis and the importance of *Itr1a* and *Itr3c* in inositol uptake, it is likely that other remaining *ITR* genes are activated for function in the triple mutant since completely blocking both pathways would trigger the so call inositol-less death and become lethal. Other remaining *ITR* genes must play a role in inositol acquisition in the triple mutant since this mutant is viable and has normal growth. Our inositol uptake results also agreed with this notion since the triple mutant still could acquire inositol at a rate even better than the *itr1a itr3c* double mutant.

Our qRT-PCR results on the expression of *ITR* genes shown in Fig. 8 revealed several interesting findings. First of all, compared to the expression of *ITR* genes in H99, the expression of the remaining *ITR* genes except *ITR4* was highly induced in either the double mutant or the triple mutant background when these strains were cultured on YPD rich medium. Such an outcome suggests that there is a functional redundancy within these *ITR* genes and that *Itr1a* and *Itr3c* are important for function, which is consistent with our previous study (64). Because the double mutant still showed inositol uptake activity but at a much lower uptake rate when abundant free inositol was available, the increased expression of remaining *ITR* genes did not fully rescue the defect of mutant uptake activity. Second, most *ITR* genes, including *ITR1A* and *ITR3C*, were highly induced in infected lung and brain during infection compared with the *in vitro* culture condition. Compared to the H99 infection, the expression of *ITR5* was highly induced in lung tissues infected by the triple mutant. The expression of *ITR2* and the group II *ITR* genes (*ITR4*, *ITR5*, and *ITR6*) was also induced in brain tissues infected by the triple mutant compared to H99 infection. These outcomes confirmed our hypothesis that remaining *ITR* genes are activated to overcome the defect of inositol acquisition in the triple mutants, which could potentially explain why the triple mutant did not show more severe virulence attenuation in our murine models. The role of group II *ITR* genes remains unknown both in *C. neoformans* and in other fungi, including *Candida* species. Our RT-PCR results suggest that they may have an important function during infection, and this lends itself to future studies.

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