EUKARYOTIC CELL, Mar. 2009, p. 315–326 Vol. 8, No. 3
1535-9778/09/$08.00+0 doi:10.1128/EC.00375-08

Identification of *ENA1* as a Virulence Gene of the Human Pathogenic Fungus *Cryptococcus neoformans* through Signature-Tagged Insertional Mutagenesis▽

Alexander Idnurm,[1,2]* Felicia J. Walton,[1]† Anna Floyd,[1] Jennifer L. Reedy,[1] and Joseph Heitman[1]*

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina 27710,[1] and Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri—Kansas City, Kansas City, Missouri 64110[2]

Received 26 November 2008/Accepted 8 January 2009

A library of more than 4,500 signature-tagged insertion mutants of the human pathogenic fungus *Cryptococcus neoformans* was generated, and a subset was screened in a murine inhalation model to identify genes required for virulence. New genes that regulate aspects of *C. neoformans* virulence were also identified by screening the entire library for in vitro phenotypes related to the ability to cause disease, including melanin production, growth at high temperature, and growth under conditions of nutrient limitation. A screen of 10% of the strain collection in mice identified an avirulent mutant strain with an insertion in the *ENA1* gene, which is predicted to encode a fungus-specific sodium or potassium P-type ATPase. The results of the deletion of the gene and complementation experiments confirmed its key role in mammalian virulence. *ena1* mutant strains exhibited no change in sensitivity to high salt concentrations but were sensitive to alkaline pH conditions, providing evidence that the fungus may have to survive at elevated pH during infection of the mammalian host. The mutation of the well-characterized virulence factor calcineurin (*CNA1*) also rendered *C. neoformans* strains sensitive to elevated pH. *ENA1* transcripts in wild-type and *cna1* mutant strains were upregulated in response to high pH, and *cna1 ena1* double mutant strains exhibited increased sensitivity to elevated pH, indicating that at least two pathways in the fungus mediate survival under alkaline conditions. Signature-tagged mutagenesis is an effective strategy for the discovery of new virulence genes in fungal pathogens of animals.

One of the challenges in combating human pathogens is the identification of genes required for virulence through forward genetic approaches. First, humans can rarely be used as hosts, so a suitable animal model must be found and the caveats associated with a divergent host and different immunological responses must be taken into consideration. Second, animal experiments become prohibitively expensive for testing large numbers of mutant strains. Third, there is an ethical consideration in using large numbers of animals with a low probability of identifying new avenues for therapy. In contrast, there have been many successful forward genetic screens of plant pathogens for changes in the ability to cause disease, including a recent saturation screen for pathogenicity genes by T-DNA insertional mutagenesis in the rice blast fungus *Magnaporthe grisea* (26).

For bacterial pathogens, signature-tagged mutagenesis (STM) has circumvented the issue of using large numbers of animals (21). Pools of strains, each containing a unique DNA code, are inoculated into animals, the organisms are recovered at the end of the experiment, and changes in tag abundance are used to measure virulence properties for each strain. This approach has been underutilized for human pathogenic fungi. In a classic experiment, STM in the pathogenic yeast *Candida glabrata* was used to screen for mutants unable to adhere to epithelial cells, resulting in the identification of the Epa1 adhesin (12). STM has also been applied in mouse models for two other human pathogenic fungi. For the ascomycete *Aspergillus fumigatus*, 4,648 strains were screened, but to date only one gene, encoding PABA synthetase, has been identified (7), and it had been discovered previously as a virulence gene through other approaches (56). For the basidiomycete *Cryptococcus neoformans*, 672 tagged strains were screened (44). Although no new virulence genes were identified, this study provided technological insight for subsequent experiments. For instance, it revealed potential bottleneck events during dissemination from the lung to the brain and demonstrated that plasmids are often targeted by homologous recombination to the native promoter region they carry for the expression of the drug resistance marker (44). For *C. neoformans*, tags have also been incorporated into the generation of targeted gene deletion strains and used to assess pools of strains in mouse models (32, 39). Liu et al. generated 1,201 specific gene deletion strains and tested their virulence levels in a murine inhalation model (32), highlighting the power of using signature tags to identify strains with reduced or increased growth in mammalian models.

C. neoformans and the closely related species *Cryptococcus gattii* are basidiomycete yeasts with worldwide distribution and are characterized by their abilities to cause disease in both

* Corresponding author. Mailing address for Alexander Idnurm: Room 513, Spencer Chemistry Building, 5100 Rockhill Rd., School of Biological Sciences, Division of Cell Biology and Biophysics, University of Missouri—Kansas City, Kansas City, MO 64110. Phone: (816) 235-2265. Fax: (816) 235-1503. E-mail: idnurma@umkc.edu. Mailing address for Joseph Heitman: Room 322, CARL Building, Box 3546, Research Dr., Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710. Phone: (919) 684-2428. Fax: (919) 684-5458. E-mail: heitm001@duke.edu.

† Present address: The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, United Kingdom.

▽ Published ahead of print on 16 January 2009.

healthy and immunocompetent individuals (3, 9). A number of research tools for *C. neoformans*, including five completed genome sequences, that enable facile investigation of traits of interest are available (22, 34). However, with the exception of the recent partial systematic mutagenesis screen of *C. neoformans* (32), almost all investigations of genes required for virulence in this fungus have focused on candidate genes or used in vitro screening approaches for known virulence traits like melanin and capsule production and the ability to grow at mammalian body temperature. Screens of insertion mutants generated by biolistic transformation in nonmammalian alternative hosts, the nematode *Caenorhabditis elegans* and the amoeba *Acanthamoeba castellani*, have led to the identification of three genes: *KIN1* and *ROM2*, which are required for killing of the host, fertility, and viability (41, 57), and *ECA1*, whose role in virulence depends on the host used (16). Importantly, both *kin1* and *rom2* deletion mutants are attenuated for virulence in murine inhalation models while *eca1* mutants are temperature sensitive and, as such, are expected to be unable to survive in the mammalian host. However, not all genes that are required for virulence in mouse models are required for virulence in these heterologous host systems (40), and thus, screens in murine systems have the potential to reveal novel virulence attributes.

Here, we report the generation and screening of an insertion mutant library comprising 4,608 strains generated by *Agrobacterium* T-DNA insertion events. The strains were assessed for in vitro phenotypes associated with virulence. By incorporating a signature tag into the strains, pools of a subset of the collection were screened in a murine inhalation model of cryptococcosis. This screening led to the identification of the *ENA1* gene, encoding a P-type ATPase, whose deletion results in increased sensitivity to high pH and the loss of virulence in mice. This type of gene could not have been identified by in vitro phenotypic screens, thereby demonstrating the utility of genetic screens of STM strain pools in animal models for elucidating novel determinants of virulence.

MATERIALS AND METHODS

***C. neoformans* strains and growth.** Serotype A strains KN99α and KN99**a**, a congenic pair differing in mating type, were used as the wild-type strains (46). Gene disruption strains and other strains are listed in Table 1. Strains were maintained on yeast extract-peptone-dextrose (YPD) agar and stored frozen in 15% glycerol at −80°C for the long term. Yeast extract-nitrogen base (YNB) with 2% dextrose was used as a minimal medium. For melanin production, strains were grown in the presence of 3,4-dihydroxyphenylalanine (L-DOPA; 100 mg/liter) in melanin-inducing medium. Capsule production was induced by growing the cells in low-iron medium containing the iron chelator EDDHA (ethylenediamine dihydroxyphenylacetate; 20 mg/liter) and was visualized microscopically by the exclusion of India ink particles from the cell wall (58). For assessing UV tolerance, cells were plated onto YPD agar medium, exposed to a UV light dose (120 J/m^2 in a Stratalinker apparatus with 280-nm maximum emission), and then grown for 2 days at 30°C.

Signature tag plasmid and *C. neoformans* library construction. Plasmids containing previously generated signature tags (44) were obtained from Jennifer Lodge (St. Louis University School of Medicine). New signature tags were created by PCR from the oligonucleotide 5′-CTTACCTACAACCTCCAACCC TTTAAA-(NM)$_{20}$-TTTAAAGGTGTAGAATGGGTATGGAGT-3′, where N is any nucleotide and M is either an A or C nucleotide, according to previous methods (21). Previously generated tags were designated by the Lodge laboratory numbering system, while new tags were numbered 301 to 314. The tags were amplified by PCR, cut with KpnI, and cloned into plasmid pPZP-NATcc (60). The plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen) by electroporation. *C. neoformans* wild-type strain KN99α was transformed with T-DNA from these *Agrobacterium* strains by using previously described methods (24), and for each signature tag, 96 strains with apparent wild-type growth were streaked to obtain single colonies, grown in YPD liquid medium in 96-well plates, and stored at −80°C after the addition of glycerol (final concentration, 15%).

TABLE 1. Strains used in this study[a]

Name	Genotype	Parent(s)	Reference
KN99α	*MAT*α	Wild type	46
KN99**a**	*MAT***a**	Wild type	46
JLCN488	*cku80*Δ::*NEO*	KN99α	19
KK1	*cna1*Δ::*NAT*	H99	28
KK5	*cna1*Δ::*NAT CNA1-NEO*	KK1	28
KK8	*cna1*Δ::*NEO*	KN99**a**	28
AI146	*plr1*Δ::*NAT*	KN99α	This study
FJW18	*ena1*Δ::*NAT cku80*Δ::*NEO*	JLCN488	This study
AI167	*ena1*Δ::*NAT MAT*α	FJW18 and KN99**a**	This study
AI168	*ena1*Δ::*NAT MAT***a**	FJW18 and KN99**a**	This study
AI173	*ena1*Δ::*NAT ENA1-HYG*	AI167	This study
AI180	*ena1*Δ::*NAT ENA1-HYG*	AI167	This study
AI205	*cna1*Δ::*NEO ena1*Δ::*NAT MAT***a**	KK8 and AI167	This study
AI206	*cna1*Δ::*NEO ena1*Δ::*NAT MAT*α	KK8 and AI167	This study

[a] The 4,608 T-DNA insertion strains were created in the KN99α background and are not listed.

Murine infections and virulence studies. A/Jcr mice were infected with *C. neoformans* yeast cells (10^5) via intranasal inoculation and monitored daily (13). The murine protocol was approved by the Duke University Animal Use Committee. Log rank tests were used to compare the survival rates of animals infected with individual strains. When pools of strains were used, mice were sacrificed 21 days postinoculation. For signature tag studies, a sample of the inoculum pool was used to isolate genomic DNA as the input control. Brains, spleens, and lungs from the infected animals were dissected. For the output samples, genomic DNA isolated directly from homogenized tissues or from cultures of cells isolated from mouse tissue and grown overnight in YPD liquid was also obtained by using a cetyltrimethylammonium bromide extraction buffer protocol (50). Tags were amplified from genomic DNA by PCR (21 cycles) using primers JOHE8958 and JOHE8959 to incorporate [^{32}P]dCTP. The unlabeled ends were removed by digestion with a DraI restriction enzyme. Tags were hybridized to nylon membranes (Pall Biodyne B) onto which each tag was spotted twice (once at a 1/10 dilution). Signals were detected on X-ray film, and those for input and output DNA hybridization were visually compared.

Identification of mutated genes and construction of gene deletion strains. Inverse PCR was used to identify regions flanking the T-DNA insertions in strains of interest with primers JOHE8956 and JOHE8957 (24). In situations in which the restriction enzyme cut within the T-DNA molecule, primer combinations were JOHE8956 with the universal M13F primer and JOHE8957 with the universal M13R primer. Briefly, 2 μg of genomic DNA was digested with restriction enzymes (ClaI, EcoRI, NcoI, and NdeI being the most commonly used) and self-ligated with T4 DNA ligase, and the ligation reaction products were used as templates in PCR. The PCR products were precipitated or gel purified and directly sequenced, and the sequences were used for BLAST comparisons with sequences in the Broad Institute and GenBank databases. Gene replacement alleles were generated by overlap PCR and introduced into strain KN99α or JLCN488 (*cku80*::*NEO*) (19) by transformation with the Bio-Rad biolistic apparatus. The 5′ and 3′ fragments of the gene *PLR1* were amplified with primers JOHE16914-JOHE16915 and JOHE16916-JOHE16917, and those of *ENA1* were amplified with primers JOHE18299-JOHE18301 and JOHE18303-JOHE18304. The nourseothricin acetyltransferase cassette was amplified with primers JOHE8677 and JOHE11866. Overlaps of the three PCR products for each disruption cassette were amplified with JOHE16914 and JOHE16917 (*PLR1*) or JOHE18299 and JOHE18304 (*ENA1*). Candidate gene disruption strains were screened by PCR and confirmed by Southern blot analysis. For the *ena1* mutant, the deletion was made initially in the *cku80*::*NEO* background; the deletion strain was then crossed with strain KN99**a,** and Nat^r Neo^s strains of both mating types containing only the *ena1*::*NAT* mutation were isolated. The comple-

TABLE 2. Oligonucleotide primers used in this study

Name	Sequence[a] (5′–3′)	Purpose or description (reference)
JOHE8956	AACAGTTGCGCAGCCTGAATG	Inverse PCR analysis of T-DNA
JOHE8957	AGAGGCGGTTTGCGTATTGG	Inverse PCR analysis of T-DNA
JOHE8962	CTTACCTACAACCTCCAACCCTTTAAA-$(NM)_{20}$-TTTAAAGGTGTAGAATGGGTATGGAGT	Generation of new signature tags
JOHE8958	CTTACCTACAACCTCCAACC	STM5′ (44)
JOHE8959	ACTCCATACCCATTCTACACC	STM3′ (44)
JOHE16914	TCCGCCTGTGGCACATCAGC	Disruption construct for *PLR1*, 5′ flank
JOHE16915	GCTTATGTGAGTCCTCCCCGGTGACAGTAGAAACGG	Disruption construct for *PLR1*, 5′ flank
JOHE16916	CTCGTTTCTACATCTCTTCTCAAGCAATGGCCCACC	Disruption construct for *PLR1*, 3′ flank
JOHE16917	TGGATCAATCCACCTTTGGG	Disruption construct for *PLR1*, 3′ flank
JOHE18299	GAGGAAAGAATGAGAGGG	Disruption construct for *ENA1*, 5′ flank
JOHE18301	GCTTATGTGAGTCCTCCCAGAAGACATCGTTCATCC	Disruption construct for *ENA1*, 5′ flank
JOHE18303	TCGTTTCTACATCTCTTCTGACACTGCAGTCTTGCC	Disruption construct for *ENA1*, 3′ flank
JOHE18304	TCCTCGAGATGGTGTGTG	Disruption construct for *ENA1*, 3′ flank
JOHE8677	GAAGAGATGTAGAAACGAG	*NAT* cassette amplification for overlap PCR
JOHE11866	GGGAGGACTCACATAAGC	*NAT* cassette amplification for overlap PCR
JOHE16918	GGCAGCAAGCTCACATCATG	Amplification of *ENA1* for complementation
JOHE16921	TCAATAATGACAGGATCATC	Amplification of *ENA1* for complementation
ALID0325	CTCGAGCCTGATAGACG	Amplification of *ENA1* for Northern blot analysis
ALID0343	TGACAGGCGATCATCCTG	Amplification of *ENA1* for Northern blot analysis
JOHE8713	ATGGAAGAAGAAGGTACG	Amplification of *ACT1* for Northern blot analysis
JOHE8714	TTAGAAACACTTTCGGTG	Amplification of *ACT1* for Northern blot analysis

[a] N, any nucleotide; M, A or C.

mentation of the *ena1* mutation was achieved by amplifying the wild-type gene from genomic DNA of strain KN99α with primers JOHE16918 and JOHE16921, cloning the gene into TOPO plasmid pCR2.1 (Invitrogen), and then subcloning the HindIII-XbaI fragment containing *ENA1* into the HindIII-SpeI site of plasmid pPZP-HYG2 (60). These constructs were introduced into *A. tumefaciens* strain EHA105 by electroporation and used to transform the *ena1* mutant strain AI167 to confer hygromycin resistance. The sequences of the primers used as described above are provided in Table 2.

Northern blot analysis. Strains were grown overnight in 50 ml of liquid YPD. The next day, 10 ml of the culture was added to 40 ml of fresh YPD or 40 ml of YPD adjusted to pH 8.3 with either NaOH or Tris base, depending on the experimental replicate. The cells were harvested either 1 or 2 h later and lyophilized, and the RNA was extracted with Trizol reagent according to the instructions of the manufacturer (Invitrogen). Ten micrograms of total RNA was resolved on formaldehyde-agarose gels, blotted onto Zeta-Probe membranes (Bio-Rad), and probed with fragments of *ENA1* and secondarily, as the loading control, the actin gene, all radiolabeled with [^{32}P]dCTP from the RediPrime II kit (Amersham, United Kingdom).

Genetic analysis. To generate progeny for the establishment of linkage between phenotypes and the T-DNA insertion events, strains with mutant phenotypes (all MATα) were mixed with congenic strain KN99**a** (MAT**a**) on 5% V8 juice agar medium. Plates were incubated in the dark for approximately 2 weeks or until chains of basidiospores developed. Basidiospores were micromanipulated onto YPD agar plates, and the resultant colonies were scored for their phenotypes and resistance to nourseothricin as the marker for T-DNA insertion. For the majority of crosses, the progeny were backcrossed to representative MATα and MAT**a** strains to assess the mating type as a control to demonstrate the independent assortment of markers. To generate *ena1 cna1* double mutants, strains AI167 and KK8 (Table 1) were crossed and basidiospore-derived progeny were analyzed for resistance to nourseothricin and neomycin. The deletions of the *ENA1* and *CNA1* genes in these strains were confirmed by PCR analysis.

RESULTS

Generation of a library of 4,608 T-DNA insertion mutants. A set of 48 plasmids, each containing a unique 40-bp signature tag within a sequence that confers nourseothricin resistance when introduced into *C. neoformans*, was created. The plasmids also contain all the components to function in T-DNA transfer from *Agrobacterium* into fungal cells. This insertional mutagenesis method creates random insertion events and alleviates the targeting to the *ACT1* locus previously observed in STM strains created by biolistic transformation (44). *Agrobacterium* transconjugate strains of *C. neoformans*, including 96 from each signature tag plasmid, were isolated for a total of 4,608 strains. All strains were subjected to single-colony purification and grew at rates similar to that of the wild type on YPD medium. Strains with unusual colony morphology were generally excluded from the library and analyzed independently, as reported previously (59). Single-colony isolates were placed into a 96-well plate format for subsequent analysis and storage at −80°C.

In vitro screens reveal strains with mutant phenotypes in the library. The library was screened for the following in vitro phenotypes: UV sensitivity, growth on minimal YNB medium, melanin production on the L-DOPA substrate, and growth at 37°C. A subset was analyzed for genetic linkage, and the T-DNA flanks in this subset were amplified by inverse PCR and sequenced to identify the mutated gene by BLAST analysis of the sequences against the Broad and GenBank databases (Fig. 1 and Table 3).

Seven temperature-sensitive strains with reduced growth at mammalian body temperature (37°C) were isolated. In two of the three mutants that were tested, the *NAT* marker insertions were linked to the mutant phenotype (all nourseothricin-resistant segregants were temperature sensitive and all nourseothricin-sensitive segregants were not temperature sensitive). Analyses of genomic sequences flanking the T-DNA insertions revealed insertions in the ortholog of the gene for Dph1 (33) and a gene for a putative bZIP class transcription factor with no previously characterized orthologs. Dph1 contributes to the production of diphthamide, a posttranslationally modified histidine found only in translation elongation factor 2 of eukaryotes and archaea and best known as the specific residue targeted for ADP-ribosylation by diphtheria toxin. While the mutation of *DPH1* in *Saccharomyces cerevisiae* causes no dra-

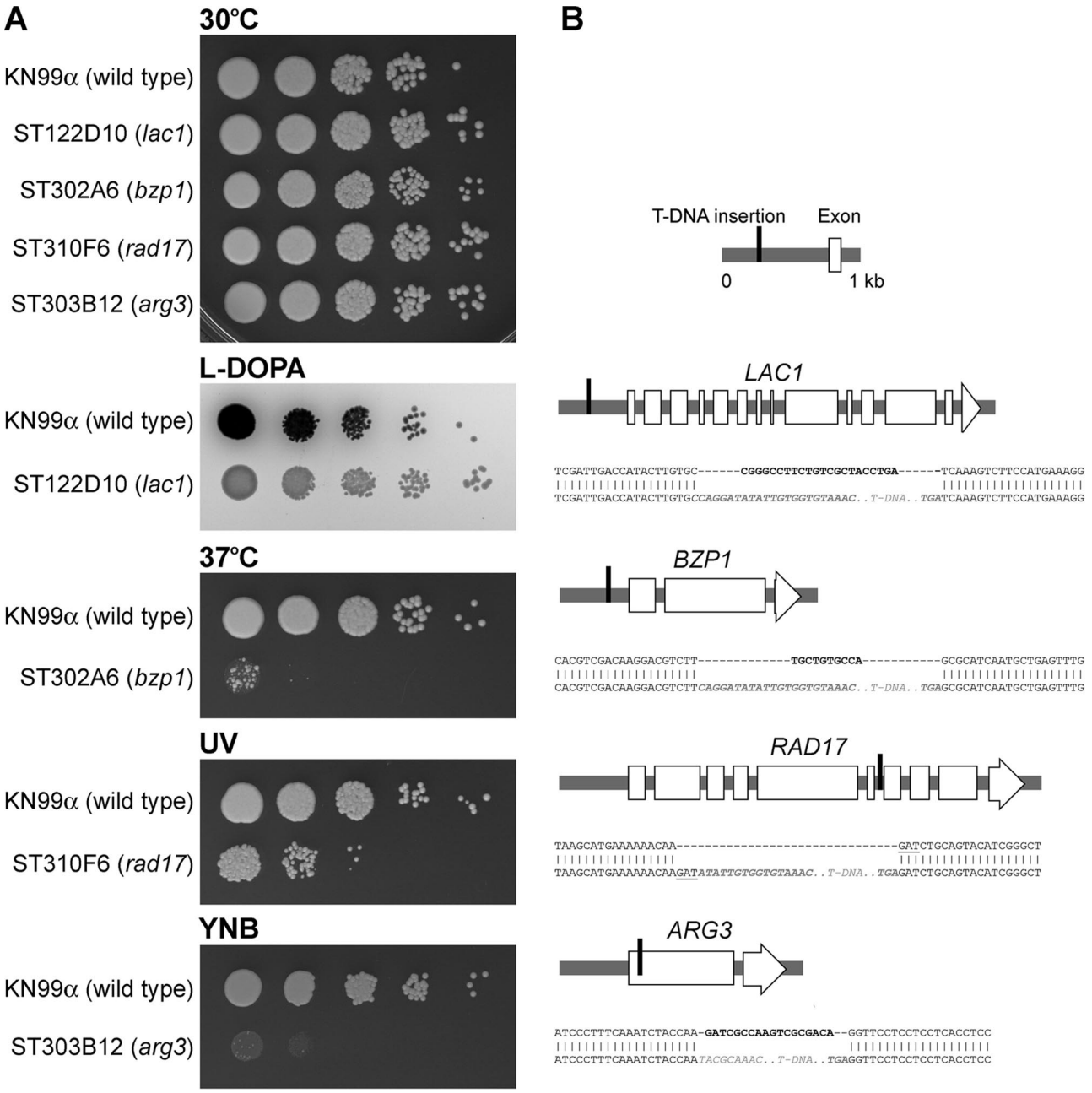

FIG. 1. *C. neoformans* T-DNA insertional mutants with in vitro phenotypes and associated mutated genes. (A) Cells were 10-fold serially diluted and spotted onto YPD, YNB, or L-DOPA agar medium. One YPD plate was exposed to UV irradiation (180 J/m^2). Plates were incubated at 30 or 37°C for 2 days or at 22°C for 4 days with L-DOPA. (B) Regions flanking the T-DNA in each strain were identified by inverse PCR and used to determine the disrupted gene by comparison against the *C. neoformans* genome database. For DNA alignments, the top sequence is from the wild-type strain, with bold letters denoting those nucleotides replaced by the T-DNA insertion event, except in the case of strain ST310F6, which has a duplication of GAT (underlined). Gray font indicates the T-DNA sequence, with italics indicating right- or left-border nucleotides.

matic phenotype (33), altering the conserved histidine residue of *S. cerevisiae* elongation factor 2 is reported to increase temperature sensitivity (27), providing a link to the temperature sensitivity of the *C. neoformans DPH1* mutant strain. In contrast to this characterized gene, the other gene identified from the 37°C screen has not been analyzed previously (the GenBank accession number for the genome sequence of the corresponding strain is CNM01380). The gene product contains a bZIP domain and has homologs in other basidiomycete fungi, but in BLAST analyses, only the bZIP domain shows similarity to sequences from other organisms beyond the basidiomycete phylum. The role of this novel gene in growth at high temperature remains to be further elucidated.

Five UV-sensitive strains were isolated by their increased sensitivity to UV treatment, and four exhibited linkage between UV sensitivity and nourseothricin resistance. UV sensi-

TABLE 3. Insertion mutants characterized by in vitro phenotypes associated with virulence

Strain[a]	Phenotype	Phenotype linked to virulence (no. of progeny analyzed)[b]	Affected gene[c]
ST104E11	Temp sensitive	Yes (23)	*DPH1*
ST302A6	Temp sensitive	Yes (21)	*BZP1*
ST311A1	Temp sensitive	No (11)	NA
ST128E8	Auxotrophic	No (21)	NA
ST219C10	Auxotrophic	Not determined	*BNA5* or *BNA6*
ST225B9	Auxotrophic	Not determined	*PRO2*
ST232C4	Auxotrophic	Not determined	*ARG8*
ST303B12	Auxotrophic	Yes (13)	*ARG3*
ST305D6	Auxotrophic	Yes (23)	Not determined
ST27E1	UV sensitive	No (5)	NA
ST38F5	UV sensitive	Yes (22)	Not determined
ST146E3	UV sensitive	No (14)	NA
ST239E6	UV sensitive	Yes (15)	*UVE1*
ST310F6	UV sensitive	Yes (11)	*RAD17*
ST122D10	Defective in melanin production	Yes (29)	*LAC1*

[a] ST refers to a signature tag, the number refers to the specific tag, and the final letter and number refer to the well position within a 96-well plate.

[b] Numbers in parentheses indicate the number of analyzed progeny from crosses of the strain with congenic strain KN99**a**.

[c] NA, not applicable.

tivity is not considered a standard virulence trait of *C. neoformans*. It was included as a screen because of the correlation between reduced virulence and increased UV sensitivity in the *bwc1* and *bwc2* mutant strains (23) and because UV sensitivity is in some cases indicative of increased sensitivity to other stresses. The flanks of two T-DNA insertions linked to UV sensitivity were obtained, revealing insertions in homologs of the *RAD17* and *UVE1* genes, both associated with resistance to UV irradiation in other organisms. Rad17 homologs function in a clamp-like complex with two other proteins as a checkpoint control system for DNA damage (35, 54). Uve1 is an endonuclease that functions in a base excision repair pathway for damage caused by UV irradiation (55, 63).

Nine auxotrophic mutants that exhibited reduced growth on YNB medium were isolated. Genetic linkage was not established for every strain because many auxotrophic mutants do not grow well on V8 juice agar medium that is used for crossing *C. neoformans* strains and is limiting for some nutrients. Nevertheless, T-DNA insertions were identified as present in the genes *BNA5* or *BNA6* (adjacent in the *C. neoformans* genome), *PRO2*, *ARG3*, and *ARG8*. *BNA5* encodes kynureninase, *BNA6* encodes quinolinate phosphoribosyl transferase, and both enzymes are required for the biosynthesis of nicotinic acid. The T-DNA inserted into the shared promoter region of these two genes; thus, either one or both may be affected, impairing growth on YNB medium that does not contain nicotinic acid. *PRO2* encodes γ-glutamyl phosphate reductase, the second step in proline biosynthesis. *ARG3* encodes ornithine carbamoyltransferase and *ARG8* encodes acetylornithine aminotransferase, corresponding to steps six and four, respectively, in the arginine biosynthetic pathway. Neither proline nor arginine is present in YNB medium. While genetic linkage was demonstrated only for the T-DNA insertion into *ARG3*, the mutation of the homologs of these five genes in other fungi produces auxotrophic defects and the results suggest that these phenotypes seen in the *C. neoformans* strains are attributable to the insertion of the T-DNA molecules, leading to the loss of function.

One melanin mutant that exhibited cosegregation of the loss of pigmentation with nourseothricin resistance (and wild-type pigmentation with nourseothricin sensitivity) was characterized. The T-DNA inserted in the promoter of the laccase (*LAC1*) gene that has been extensively studied since being cloned over a decade ago (62). This gene has also been identified previously by T-DNA insertions, with four other T-DNA strains containing insertions into the promoter region while no insertions within the exons of the laccase gene have yet been found (24, 60).

The results of in vitro screening demonstrate that mutants with interesting phenotypes are present in the T-DNA library of 4,608 strains and that many of the phenotypes are due to the insertion of the T-DNA molecules. However, genetic segregation analysis indicates that not all mutant phenotypes are caused by the T-DNA insertion event, highlighting the importance of genetic linkage analysis for subsequent characterization of insertional mutagenic screens for *C. neoformans* and other fungal species for which classical Mendelian genetic analysis is feasible.

Insertion of *Agrobacterium* T-DNA molecules. From the first reports on the transformation of plants and fungi by *Agrobacterium*, it was observed that this alphaproteobacterium introduces T-DNA molecules into the host genome in a mostly random fashion. The regions flanking the T-DNA insertions in 41 of the *C. neoformans* strains were identified by inverse PCR using restriction enzymes that recognize 6-bp sites. A summary of these events is as follows. Twenty-seven strains displayed "perfect" integration, wherein the T-DNA replaces a small number of nucleotides and only the T-DNA region is integrated. For three strains, only one side of the insertion was obtained due to the enzyme used, with only one side of the T-DNA amplifying when specific left- or right-border primers were used in the inverse PCR. Five strains had extra DNA from the T plasmid integrated beyond the left border, hindering the identification of this side of the insertion. Three strains showed evidence of genomic rearrangements, as suggested by sequences from the T-DNA flanks that matched different contigs from the genome sequencing project, with two additional strains having potential intrachromosomal rearrangements. In Southern blot analysis with randomly selected *Agrobacterium*-derived transformants, we had previously observed double insertion events at a frequency of approximately 15% (A. Idnurm, F. J. Walton, and J. Heitman, unpublished data); one strain produced two different PCR products, and sequencing results from inverse PCR indicated an example of multiple T-DNA insertion events at independent places in the genome. The T-DNA insertions were mapped to the genome sequence of strain H99 at the Broad Institute, revealing insertions into all 14 chromosomes (Fig. 2).

Strains with reduced survival in a murine model are present in pooled inocula as detected with signature tags. Mice were inoculated with 10 pools of 48 strains, with each strain containing a unique signature tag. Animals were monitored daily and were sacrificed 21 days postinoculation, a time point when the disease was well established and the symptoms of labored

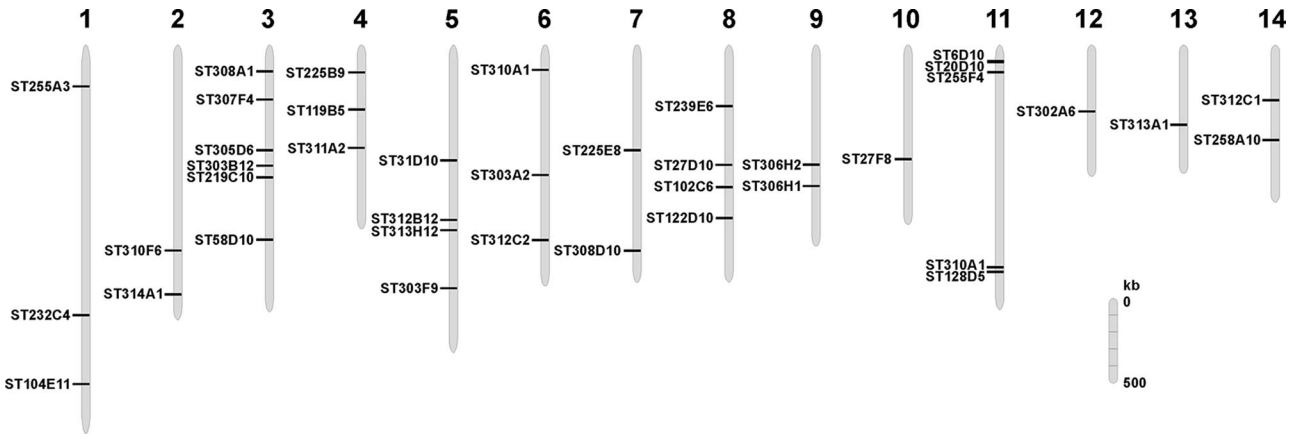

FIG. 2. Random T-DNA insertion points in the *C. neoformans* genome. The regions flanking the T-DNAs were compared to the serotype A genome sequence (Broad Institute chromosome designation, 8 December 2008) and mapped onto all 14 chromosomes.

breathing and daily weight loss were visible. The brains, lungs, and spleens were harvested and homogenized. Genomic DNA was extracted from this material and used as the template in PCR to amplify the signature tags incorporated into the genomes of the *C. neoformans* strains present in those organs. The spleen provides an indicator of the dissemination of the fungus from the lung. DNA extraction from spleens and PCR amplification were unreliable, and furthermore, CFU counts indicated low levels of fungal cells in the spleens; thus, spleen tissue was excluded from subsequent analyses. The amplified mixture of sequence tags was radiolabeled and hybridized to nylon membranes onto which each of the tags had been spotted individually. DNA from brain samples yielded only a small number of hybridizing spots, suggesting that only a few strains predominated in the brain, consistent with the findings of previous studies indicative of a bottleneck in crossing the blood-brain barrier (44, 45). The lung material provided the most robust tissue for the detection of fungal strains. From this analysis, 11 strains appeared to have reduced levels in the lung compared to those of the other 469 strains inoculated into the animals. These strains were reisolated from the *C. neoformans* library, DNA was extracted, and inverse PCR was applied to identify the mutated genes.

In an ideal genetic situation, the phenotype can be correlated to the genotype by segregation analysis. However, testing the numerous progeny from crosses in the animal model was contradictory to the aim of reducing the number of animals required for virulence studies. From those genes identified in the analysis, two were chosen for further study, and their functions in *C. neoformans* virulence were reexamined in the murine model. One of the corresponding strains had a T-DNA insertion in an uncharacterized gene, and the other strain had a T-DNA insertion in the homolog of a gene well characterized in other fungi. The first strain, ST258A10, had an insertion in the hypothetical conserved gene *PLR1*, encoding a protein with a predicted aldo-keto reductase or pyridoxal reductase domain. The only characterized homolog of this gene, in *Schizosaccharomyces pombe*, suggests that it may play a role in vitamin B_6 metabolism (38). The second strain, ST310D10, had an insertion in the putative *ENA1* gene, whose homologs are required for maintaining sodium, potassium, and lithium ion homeostasis in ascomycete fungal species (2, 20, 52).

Results from the analysis of in vitro phenotypes and other studies show that T-DNA insertions are not always the cause of the mutant phenotype. Therefore, independent deletion alleles of *PLR1* and *ENA1* were created by overlap PCR, and the DNA fragments were introduced by biolistic transformation. *plr1*::*NAT* and *ena1*::*NAT* gene replacements were confirmed by PCR analysis and Southern hybridization (data not shown), as well as by Northern blot analysis in the case of the *ena1* deletion strain (see Fig. 5B). In an initial experiment, the *plr1* and *ena1* mutant strains, along with the wild-type strain, were inoculated individually into five mice and mouse survival was monitored over time. There was no difference in virulence between the *plr1*Δ deletion mutant and the wild type, suggesting that some mutation other than the T-DNA insertion was responsible for the reduced in vivo survival of strain ST258A10 or that it was less fit than other strains in the lung competition assay. In contrast, the results of this animal pilot study suggested that *ENA1* was essential for virulence, as none of the mice infected with the *ena1* mutant strain developed symptoms of cryptococcal infection. A complemented strain for use as a control was constructed as follows. The *ENA1* gene from wild-type *C. neoformans* was amplified by PCR, cloned into the pCR2.1 TOPO vector, and subcloned into a vector that confers resistance to hygromycin. The *ena1* deletion strain was transformed with the construct to complement the *ena1* mutation. Because *ENA1* is a large gene, two independent PCR amplicons of the 6,099-bp fragment including the *ENA1* open reading frame, promoter, and terminator were used in complementation experiments to minimize the chances of PCR-induced errors causing mutations that would impair function.

The wild-type strain, two *ena1* deletion strains (one of each mating type), and the two independent *ena1 ENA1* complemented strains were inoculated into 9 or 10 mice each, and mouse survival was monitored daily. All mice inoculated with wild-type or complemented strains succumbed to cryptococcosis within 24 days. In contrast, no symptoms in the mice infected with the *ena1* deletion strains were observed, even up to 139 days postinoculation, when the experiment was ended (Fig.

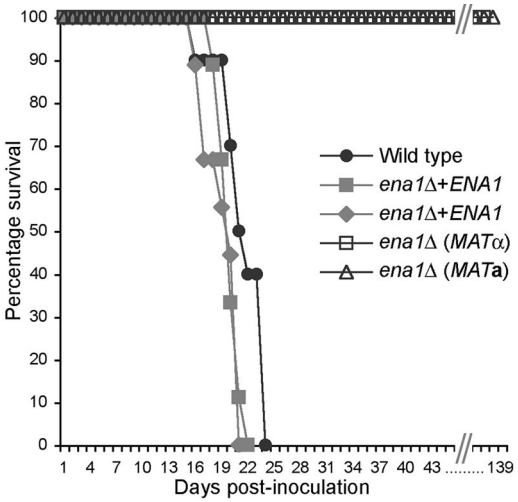

FIG. 3. *ENA1* is required for virulence. Mice were inoculated with wild-type *C. neoformans* strain KN99α ($n = 10$), *ena1* deletion mutants of both mating types ($n = 9$ for both groups), and two independent *ena1 ENA1* (*ena1*Δ+*ENA1*) complemented strains ($n = 9$ for both groups).

3). This difference in survival between mice infected with wild-type or complemented strains and those infected with the *ena1* mutant strains is significant as assessed by log rank tests ($P < 0.0001$). Thus, *ENA1* is required for the virulence of *C. neoformans* in a murine model.

***ENA1* is required for growth at high pH but not at high salt concentrations.** The dramatic virulence phenotype of the *ena1* mutants raised the question as to the reason behind this reduction in virulence. The initial insertion mutant, ST310D10, did not emerge from other in vitro screens for traits associated with virulence. A set of traits of the *ena1* deletion and complementation strains was assessed. There were no changes in properties like melanin and capsule production, growth at 37°C, and growth on minimal medium (YNB medium).

The mutation of *ENA1* in other fungal species results in changes in sensitivity to monovalent ions, i.e., lithium, sodium, or potassium, and an increased sensitivity to high pH. The *C. neoformans ena1* mutants were examined for similar phenotypes. Surprisingly, no increased sensitivity of *ena1* mutant strains to monovalent ions (Li^+, Na^+, or K^+) or calcium ions or to another osmotic stress (1 M sorbitol) was observed. There was no change in sensitivity to high concentrations of H^+ ions (acidity; pH ~4). In contrast, the *ena1* mutants were highly sensitive to low H^+ concentrations, i.e., medium with high pH (Fig. 4A), and the wild-type phenotype was fully restored in the *ena1 ENA1* complemented strain. To ensure further that these findings were not an artifact of increased Na^+ or K^+ ion concentrations in the medium with KOH or NaOH used to adjust the pH, the medium was also altered to pH ~9 with Tris base. The same inhibition of growth was observed as when the pH was adjusted with KOH or NaOH, suggesting that Ena1 plays a role in maintaining the homeostasis of H^+ ions, rather than monovalent ions.

For the experiments described above, pH indicator strips were used to assess the pH of the molten agar before it was poured onto plates; however, the strips are not sensitive

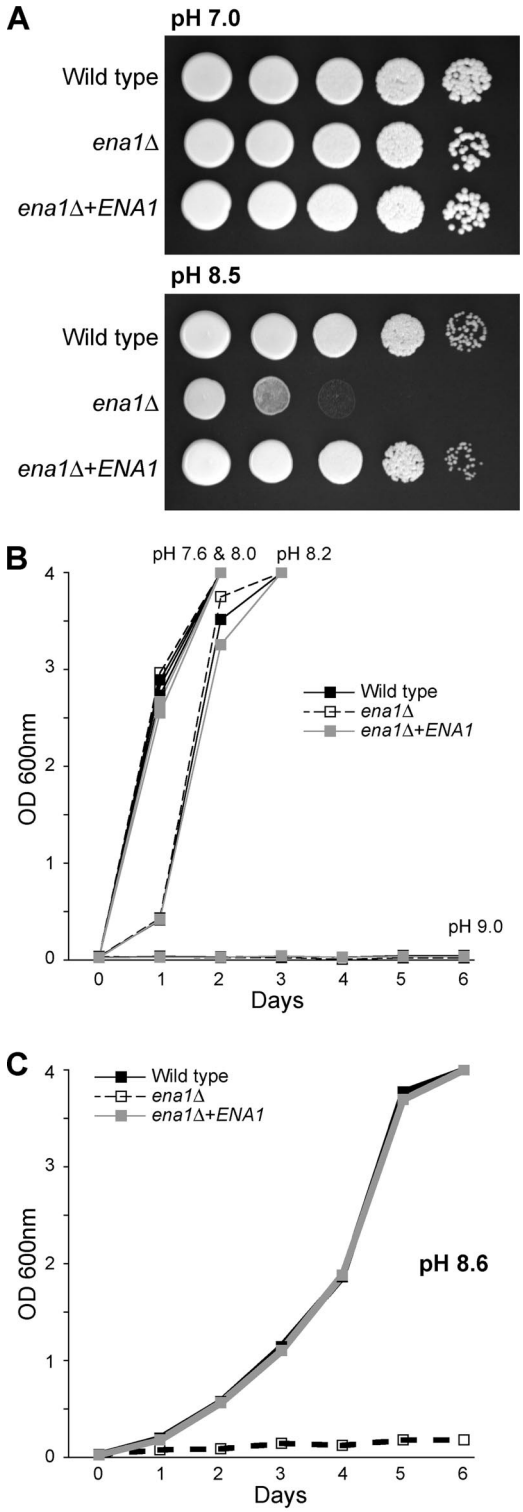

FIG. 4. The mutation of *ENA1* causes increased sensitivity to alkaline pH. (A) Cells were 10-fold serially diluted and spotted onto YPD at a pH of 7 or at a pH adjusted to ~8.5 with KOH, and the cultures were incubated at 30°C for 2 or 3 days, respectively. (B and C) Strains were grown in liquid YPD adjusted to different pHs with Tris base at 22°C, and the optical density at 600 nm (OD 600nm) was measured daily. An optical density of 4 was the maximum reading. (B) Comparative growth rates for all three strains under four pH conditions. (C) Reduced growth of the *ena1* mutant at pH 8.6 compared to that of the wild-type and complemented strains.

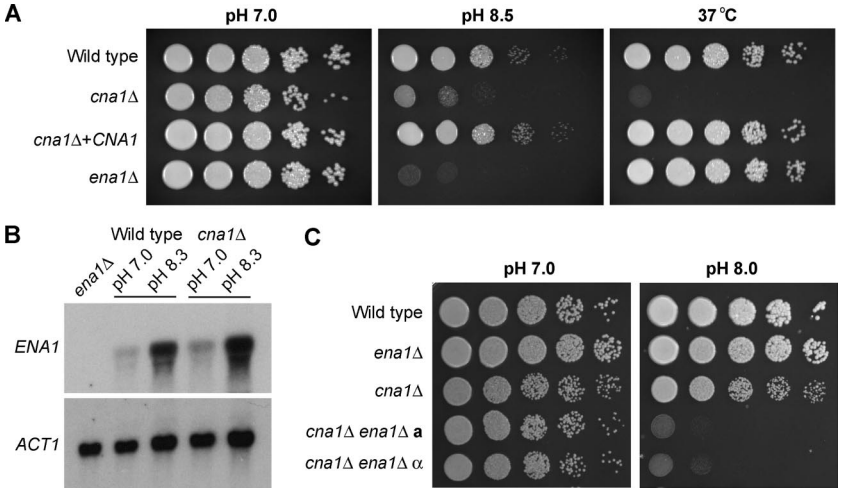

FIG. 5. Calcineurin and Ena1 jointly contribute to growth under alkaline conditions. (A) The mutation of *CNA1* causes increased sensitivity to alkaline pH. Cells were 10-fold serially diluted and spotted onto YPD at a pH of 7 or at a pH adjusted to ~8.5 with NaOH, and the cultures were incubated at 30°C for 2 days (pH 7) or 3 days (pH 8.5) or at 37°C for 2 days. (B) *ENA1* transcript levels increase with high pH. Strains were grown in regular YPD liquid or YPD liquid adjusted to pH 8.3 with NaOH for a 2-h incubation. Total RNA was extracted from the cells and was probed for *ENA1* and actin (*ACT1*) mRNA levels. (C) The combined mutation of *CNA1* and *ENA1* causes an additional increase in sensitivity to alkaline pH. Cells were 10-fold serially diluted and spotted onto YPD at a pH of 7 or at a pH adjusted to ~8.0 with NaOH, and the cultures were incubated at 30°C for 2 days (pH 7) or 3 days (pH 8).

enough for pH increments of less than 1. A liquid growth assay was used to assess more accurately the range of pH sensitivities of wild-type and mutant *ena1 C. neoformans* strains (Fig. 4B). In the assay with liquid YPD medium, the pH was adjusted with Tris base and measured with a pH meter equipped with a Tris-compatible electrode. *C. neoformans* exhibits reduced growth in YPD at pH 8.2, and the *ena1* mutant strain was no more sensitive at this pH than the wild type. At pH 8.6, there was greater inhibition of growth than at pH 8.2 and the growth of the *ena1* mutant strain was minimal compared to that of the congenic wild-type and *ena1 ENA1* complemented strains. At pH 9.0, no growth of any of the strains was observed. Thus, we conclude that the pH sensitivity of the *ena1* mutant strain, in contrast to that of the wild type, starts above pH 8.2 but below pH 8.6 in YPD medium and that by pH 9.0, all strains are unable to grow.

Calcineurin and Ena1 function in separate pathways to enable growth under alkaline conditions. At present, few genes from *C. neoformans* or fungal species outside the ascomycete phylum that regulate growth at elevated pH are known. However, in the ascomycete fungi, a suite of genes that enable growth at alkaline pH and in some cases transcriptionally regulating the expression of *ENA1* orthologs, has been characterized previously (52). Two corresponding pathways in *C. neoformans* have been characterized and involve the calcium-dependent phosphatase calcineurin and the high-osmolarity glycerol mitogen-activated protein kinase pathway (1, 28). Calcineurin was of particular interest because its mutation results in reduced growth at alkaline pH (48). Calcineurin A subunit and Hog1 mitogen-activated protein kinase deletion strains (*cna1*Δ and *hog1*Δ) were tested for growth under alkaline conditions. The *hog1* mutant strain was unaffected, while the *cna1* mutant strain exhibited sensitivity to high pH (Fig. 5A), and both phenotypes are consistent with those of the equivalent *S. cerevisiae* mutants.

The hypothesis that calcineurin is required for Ena1 regulation or function was tested by two approaches. First, *ENA1* mRNA levels in wild-type and *cna1* mutant strains in normal YPD medium or YPD at pH 8.3 were assessed. Increased pH induced accumulated levels of *ENA1* transcripts in both the wild-type and calcineurin mutant strains, showing that *ENA1* is induced under conditions in which it is required to function and that this induction is not mediated by calcineurin (Fig. 5B). Second, strains bearing both *ena1* and *cna1* mutations were generated by genetic crosses. In contrast to other meiotic progeny (Fig. 5C), all seven such double mutants identified showed increased sensitivity to pH at a lower range than that to which single mutants showed sensitivity. Thus, Ena1 and calcineurin function independently in regulating growth under alkaline conditions.

DISCUSSION

Screens of large numbers of mutagenized strains are a powerful approach to identify new gene functions. Not surprisingly, this strategy has been applied to human pathogenic fungi, including *C. neoformans*, to identify genes required for the ability to cause disease. Genetic research on the basidiomycete yeast *C. neoformans* started with the isolation of spontaneous or induced mutations and the cloning of the genes by complementation with plasmid libraries (10). More recently, insertional mutagenesis with DNA delivery by electroporation or biolistic transformation, combined with the ability to identify the affected gene by using the known inserted DNA sequence and the complete genome sequences of *C. neoformans* strains, has been a more efficient method (15, 24, 65). *Agrobacterium*-mediated transformation has been implemented for *C. neoformans*, with the advantage of yielding stable (integrated) insertions, and has been used previously to identify new gene functions such as those required for melanin production, col-

ony morphology, growth under hypoxia-mimicking conditions, and responses to light (23–25, 36, 59, 60, 64). Here, we developed a large library of T-DNA insertion strains of *C. neoformans* that incorporate signature tags enabling the screening of pools of strains in animal models of cryptococcosis.

A number of large-scale T-DNA insertion libraries for fungi are under development or have been described recently. These studies have examined the patterns of T-DNA integration into the genome, suggesting a bias in insertion into promoter regions of genes but an otherwise even distribution throughout fungal genomes in species like *S. cerevisiae* (8) and the plant pathogens *Leptosphaeria maculans* (6) and *M. grisea* (5, 11, 31, 37). We previously noticed a possible bias in the promoters of genes or hot spots like the laccase (*LAC1*) promoter: in this study, the insertion into *LAC1* in the characterized ST122D10 strain is the fifth example of an insertion into this region, whereas no T-DNA insertions into the *LAC1* open reading frame have yet been identified. We have also found evidence of chromosomal rearrangements, as suggested by highly reduced basidiospore germination after crosses and by inverse PCR results yielding sequences from two different contigs in the genome sequencing assembly. Furthermore, in one example, these suspected changes were associated with changes in chromosomal DNA migration after resolution on contour-clamped homogeneous electric field gels (data not shown). Chromosomal rearrangements in plant T-DNA transformants have been observed previously (43), and in three independent studies of the fungus *M. grisea*, chromosomal rearrangements occurred with a collective frequency equivalent to 5 of 193 transformants (2.6%) (11, 31, 37). Thus, the *Agrobacterium* T-DNA insertion method is perhaps accurately described as a "silver" bullet rather than the "gold" bullet it was proposed to be when first developed for filamentous fungal species more than a decade ago (14): nevertheless, it should be remembered that whenever tested, all methods for mutation have some level of bias as to their effects across the genome of an organism.

While mutant screens have produced the majority of our initial knowledge on gene function, a recent paper on a systematic deletion strategy for *C. neoformans* (32) places the approaches of forward versus reverse genetic screens into a new perspective. The study generated more than 1,000 specific gene disruption strains that were then screened in the mouse inhalation model. In species in which recombination frequencies are high, it is likely a better use of time and resources to perform large-scale reverse genetic screens rather than forward genetic screens since the nature of the mutation is known prior to the observation of the phenotype associated with it. However, mutant screens remain an invaluable resource in gaining insights into genetic functions in specific strain backgrounds or in the majority of fungi in which the efficiency of homologous recombination is low.

A subset of the insertional mutant library was inoculated into the lungs of mice. In approximately half of the 480 unique strains used, it is estimated that the T-DNA inserted into the open reading frame or the promoter of a gene that would impair function, and in the other half, the T-DNA inserted into an intergenic region. For one gene, *PLR1*, found in this screen, the deletion strain showed virulence equal to that of the wild type. This result may reflect that the T-DNA insertion event was unrelated to the reduction in virulence in the original strain or that the original strain was less fit than other strains in the signature tag assay that measures competitive fitness levels among a collection of strains. In contrast, the *ENA1* gene identified in this screen was further characterized as a gene required for virulence. At present, the only in vitro phenotype associated with mutating *ENA1* is increased sensitivity to elevated pH. In vitro in a rich medium (YPD), this phenotype is manifested once the pH rises above 8.2, and this finding raises the question of where in the mammalian host the pH is as high, other than the small intestine, which is not part of the normal route of dissemination for *C. neoformans*. The more stressful environment within a mammalian host compared to in vitro may mean that modest increases in pH, relative to those tested in vitro, coupled with *ena1* mutation may influence the ability of the fungus to cause disease. Alternatively, *C. neoformans* may cause localized alkaline microenvironments, mediated by the virulence factor urease (13), within the host in which the fungus has to be able to survive. Whatever the reason behind the loss of virulence of the *ena1* mutant strains, Ena1 orthologs are conserved in many fungal species and are not present in animals (or higher plants), making them attractive for further investigation as fungus-specific proteins representing novel targets for therapeutic intervention.

Alternative host models for investigating the virulence of *C. neoformans* have been developed previously (17, 40). Both the *plr1* and *ena1* gene replacement strains were tested in the *Galleria mellonella* wax moth model of cryptococcal disease (42). No differences in the virulence of either strain compared to that of the wild type in the moth larvae were observed (data not shown). This finding may not be surprising in the case of the *PLR1* gene, since the deletion strain had wild-type virulence in mice. However, for *ena1*, this information suggests that there is a significant difference in physiology between the wax moth and murine models and that the larval environment does not mimic that in which *C. neoformans* requires Ena1. Furthermore, while insect models may enable a new level of understanding of how fungi evade the innate immune responses of the host, murine models will still be required for an understanding of the interaction of fungi with mammalian hosts.

The *ENA1* gene was cloned in *S. cerevisiae* originally because of the ability of the gene to suppress lithium sensitivity in a subset of strains of this yeast, and subsequently, *ENA1* was shown to control the efflux of Na^+, Li^+, and probably K^+ ions from the cell (20). Its homolog in *S. pombe*, *cta3*, was identified by the ability of the *cta3* product to transport Ca^{2+}, although the protein has also been shown more recently to transport K^+ ions (4, 18). *ENA1* of *S. cerevisiae* is regulated by many environmental conditions, and the signaling pathways identified include homologs of genes known to be involved in virulence in pathogenic fungi, such as calcineurin, Rim101, and Nrg1 genes (29, 30, 51–53). *S. cerevisiae* strains contain tandem copies of *ENA1*, up to five, that exhibit functional redundancy (61). However, a search of the *C. neoformans* genome reveals only a single copy of the *ENA1* gene, with the next best BLAST match to the *S. cerevisiae* Ena1 gene being the *C. neoformans* Eca1 gene. Eca1 is a putative sarcoplasmic-endoplasmic reticulum calcium ATPase-like Ca^{2+} P-type ATPase that was identified in an amoeba virulence screen, and the mutant strains are

predicted to be attenuated for virulence in mammalian models due to temperature sensitivity (16).

The mechanism of action of Ena1 in regulating *C. neoformans* virulence is a mystery, even more so since the mutation of the gene does not confer increased ion sensitivity like the mutation of genes for Ena1 homologs. It has been proposed that these P-type ATPases originally functioned as K^+ ion transporters and have gained additional ion specificities (4). For example, in the yeast *Schwanniomyces occidentalis*, one Ena1-like ATPase mediates Na^+ extrusion while the other mediates K^+ extrusion and is required for growth at high pH (2). One possible function for *C. neoformans* Ena1 may be in proton transport, thus explaining the increased sensitivity to elevated pH. While H^+ ion transport has been demonstrated in only a few cases, it was recently proposed that all P-type ATPases may require antiport activity to be functional (47). Thus, *C. neoformans* Ena1 may transport monovalent ions in a mechanism coupled to a functional role in the active transport of H^+ into the cell. Furthermore, given that the mutation of *ENA1* homologs in other fungi causes phenotypes that include alkaline sensitivity and that the transcripts of these *ENA1* homologs are induced by alkalinity, this H^+ transport activity may have been the original primary function of this class of P-type ATPases that could act to balance the activity of the H^+ ATPase Pma1 class of proton pumps found in fungi and plants. An alternative hypothesis explaining the loss of virulence in *C. neoformans ena1* mutants is that high pH may change the solubility and, thereby, the availability of some other ion that is the bona fide substrate for Ena1 activity.

A suite of regulators control the transcription of *ENA1* in *S. cerevisiae* and other fungi (52). One such regulatory factor is the protein phosphatase calcineurin, which has also been extensively studied in *C. neoformans*. In light of the regulation of *ENA1* by calcineurin in *S. cerevisiae*, we hypothesized that one of the reasons that the calcineurin mutant strains of *C. neoformans* are avirulent in the mouse model (in addition to the role of calcineurin in growth at high temperatures) is the misregulation of *ENA1*. We confirmed the *C. neoformans* calcineurin functions in growth at alkaline pH, illustrated by the increased sensitivity of a *cna1* calcineurin mutant strain to high pH, as originally reported (48). However, strains containing double *cna1-ena1* mutations were even more sensitive to high pH than the single mutant strains, and *ENA1* was still induced by alkaline pH in *cna1* mutant strains, demonstrating that at least two pathways promote *C. neoformans* growth under alkaline conditions. How Ena1 is regulated is currently unknown.

While it is unclear why a fungus would need to grow at high pH in the mammalian host, there are a number of links between pH sensing and virulence, with one clear link being a need to grow in the slightly alkaline environment found in the blood (49). pH sensing in the fungi has been studied for decades, with a key component being the Rim101/PacC transcription factor. The mutation of the corresponding gene impairs the virulence of both plant and animal pathogenic fungi (49). During the preparation of this paper, Liu et al. reported a *C. neoformans* mutagenesis approach using specific gene deletion constructs that include signature tags (32). That study revealed more than 60 genes, including *ENA1* (annotated as *ENA2*), required for growth in the murine lung, further corroborating our results by independently showing that an *ena1* mutant strain is less fit than the wild-type strain in a competition assay in the lung. Additional genes of relevance to pH sensing that were identified in that global study include *RIM20* and *RIM101*, which are components of the pH-signaling pathway in other fungi (49). However, both the *rim20* and *rim101* mutant strains of *C. neoformans* exhibit a melanin defect and only minor changes or even increased survival in the mouse lung. To date, mutations in *C. neoformans* homologs of proteins characterized as regulators of *ENA1* in *S. cerevisiae* (e.g., calcineurin, Hog1, and Rim101) have produced phenotypes that suggest that these regulators do not play a similar role in *C. neoformans*. All research thus far on pH sensing and *ENA1* homologs has been performed with species from a single phylum of the fungal kingdom (the ascomycetes), and it is anticipated that future studies on the basidiomycete *C. neoformans* will reveal conserved and divergent components of both pH sensing and Ena1 function.

In summary, the application of signature-tagged insertional mutagenesis to *C. neoformans* has resulted in (i) the identification of genes required for in vitro phenotypes associated with virulence and (ii) the identification of Ena1 as a novel virulence factor in *C. neoformans*, suggesting that high pH should be examined in further detail for its impact on pathogenesis for this and other fungal species.

ACKNOWLEDGMENTS

We thank Gray Kimbrough for technical assistance, John Perfect for maintaining animal protocols, Xiaorong Lin, Kirsten Nielsen, Rajesh Velagapudi, and Marianela Rodriguez-Carres for assistance with animal experiments, and Jennifer Lodge and Kim Gerik for providing signature tag plasmids and the *cku80* mutant strain. Gene identification was greatly facilitated by access to the *C. neoformans* strain H99 genome sequence at Duke University and the Broad Institute.

This research was supported by NIH grants AI073917 (A.I.), AI42159 (J.H.), and AI063443 (J.H.).

REFERENCES

1. **Bahn, Y.-S., K. Kojima, G. M. Cox, and J. Heitman.** 2005. Specialization of the HOG pathway and its impact on differentiation and virulence of *Cryptococcus neoformans*. Mol. Biol. Cell **16:**2285–2300.
2. **Bañuelos, M. A., and A. Rodríguez-Navarro.** 1998. P-type ATPases mediate sodium and potassium effluxes in *Schwanniomyces occidentalis*. J. Biol. Chem. **273:**1640–1646.
3. **Bartlett, K. H., S. E. Kidd, and J. W. Kronstad.** 2007. The emergence of *Cryptococcus gattii* in British Columbia and the Pacific Northwest. Curr. Fungal Infect. Rep. **1:**108–115.
4. **Benito, B., B. Garciadeblás, and A. Rodríguez-Navarro.** 2002. Potassium- or sodium-efflux ATPase, a key enzyme in the evolution of fungi. Microbiology **148:**933–941.
5. **Betts, M. F., S. L. Tucker, N. Galadima, Y. Meng, G. Patel, L. Li, N. Donofrio, A. Floyd, S. Nolin, D. Brown, M. A. Mandel, T. K. Mitchell, J.-R. Xu, R. A. Dean, M. L. Farman, and M. J. Orbach.** 2007. Development of a high throughput transformation system for insertional mutagenesis in *Magnaporthe oryzae*. Fungal Genet. Biol. **44:**1035–1049.
6. **Blaise, F., E. Rémy, M. Meyer, L. Zhou, J.-P. Narcy, J. Roux, M.-H. Balesdent, and T. Rouxel.** 2007. A critical assessment of *Agrobacterium tumefaciens*-mediated transformation as a tool for pathogenicity gene discovery in the phytopathogenic fungus *Leptosphaeria maculans*. Fungal Genet. Biol. **44:**123–138.
7. **Brown, J. S., A. Aufauvre-Brown, J. Brown, J. M. Jennings, H. Arst, Jr., and D. W. Holden.** 2000. Signature-tagged and directed mutagenesis identify PABA synthetase as essential for *Aspergillus fumigatus* pathogenicity. Mol. Microbiol. **36:**1371–1380.
8. **Bundock, P., H. van Attikum, A. den Dulk-Ras, and P. J. J. Hooykaas.** 2002. Insertional mutagenesis in yeasts using T-DNA from *Agrobacterium tumefaciens*. Yeast **19:**529–536.
9. **Casadevall, A., and J. Perfect.** 1998. *Cryptococcus neoformans.* American Society for Microbiology Press, Washington, DC.
10. **Chang, Y. C., and K. J. Kwon-Chung.** 1994. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. Mol. Cell. Biol. **14:**4912–4919.

11. **Choi, J., J. Park, J. Jeon, M.-H. Chi, J. Goh, S.-Y. Yoo, J. Park, K. Jung, H. Kim, S.-Y. Park, H.-S. Rho, S. Kim, B. R. Kim, S.-S. Han, S. Kang, and Y.-H. Lee.** 2007. Genome-wide analysis of T-DNA integration into the chromosomes of *Magnaporthe oryzae*. Mol. Microbiol. **66:**371–382.
12. **Cormack, B. P., N. Ghori, and S. Falkow.** 1999. An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. Science **285:**578–582.
13. **Cox, G. M., J. Mukherjee, G. T. Cole, A. Casadevall, and J. R. Perfect.** 2000. Urease as a virulence factor in experimental cryptococcosis. Infect. Immun. **68:**443–448.
14. **Dunn-Coleman, N., and H. Wang.** 1998. *Agrobacterium* T-DNA: a silver bullet for filamentous fungi? Nat. Biotechnol. **16:**817–818.
15. **Erickson, T., L. Liu, A. Gueyikian, X. Zhu, J. Gibbons, and P. R. Williamson.** 2001. Multiple virulence factors of *Cryptococcus neoformans* are dependent on *VPH1*. Mol. Microbiol. **42:**1121–1131.
16. **Fan, W., A. Idnurm, J. Breger, E. Mylonakis, and J. Heitman.** 2007. Eca1, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, is involved in stress tolerance and virulence in *Cryptococcus neoformans*. Infect. Immun. **75:**3394–3405.
17. **Fuchs, B. B., and E. Mylonakis.** 2006. Using non-mammalian hosts to study fungal virulence and host defense. Curr. Opin. Microbiol. **9:**346–351.
18. **Ghislain, M., A. Goffeau, D. Halachmi, and Y. Eilam.** 1990. Calcium homeostasis and transport are affected by disruption of *cta3*, a novel gene encoding Ca^{2+}-ATPase in *Schizosaccharomyces pombe*. J. Biol. Chem. **265:** 18400–18407.
19. **Goins, C. L., K. J. Gerik, and J. K. Lodge.** 2006. Improvements to gene deletion in the fungal pathogen *Cryptococcus neoformans*: absence of Ku proteins increases homologous recombination, and co-transformation of independent DNA molecules allows rapid complementation of deletion phenotypes. Fungal Genet. Biol. **43:**531–544.
20. **Haro, R., B. Garciadeblas, and A. Rodríguez-Navarro.** 1991. A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. **291:**189–191.
21. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400–403.
22. **Idnurm, A., Y.-S. Bahn, K. Nielsen, X. Lin, J. A. Fraser, and J. Heitman.** 2005. Deciphering the model pathogenic fungus *Cryptococcus neoformans*. Nat. Rev. Microbiol. **3:**753–764.
23. **Idnurm, A., and J. Heitman.** 2005. Light controls growth and development via a conserved pathway in the fungal kingdom. PLoS Biol. **3:**615–626.
24. **Idnurm, A., J. L. Reedy, J. C. Nussbaum, and J. Heitman.** 2004. *Cryptococcus neoformans* virulence gene discovery through insertional mutagenesis. Eukaryot. Cell **3:**420–429.
25. **Ingavale, S. S., Y. C. Chang, H. Lee, C. M. McClelland, M. L. Leong, and K. J. Kwon-Chung.** 2008. Importance of mitochondria in survival of *Cryptococcus neoformans* under low oxygen conditions and tolerance to cobalt chloride. PLoS Pathog. **4:**e1000155.
26. **Jeon, J., S.-Y. Park, M.-H. Chi, J. Choi, J. Park, H.-S. Rho, S. Kim, J. Goh, S. Yoo, J. Choi, J.-Y. Park, M. Yi, S. Yang, M.-J. Kwon, S.-S. Han, B. R. Kim, C. H. Khang, B. Park, S.-E. Lim, K. Jung, S. Kong, M. Karunakaran, H.-S. Oh, H. Kim, S. Kim, J. Park, S. Kang, W.-B. Choi, S. Kang, and Y.-H. Lee.** 2007. Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. Nat. Genet. **39:**561–565.
27. **Kimata, Y., and K. Kohno.** 1994. Elongation factor 2 mutants deficient in diphthamide formation show temperature-sensitive cell growth. J. Biol. Chem. **269:**13497–13501.
28. **Kojima, K., Y.-S. Bahn, and J. Heitman.** 2006. Calcineurin, Mpk1, and Hog1 MAPK pathways independently control fludioxonil antifungal sensitivity in *Cryptococcus neoformans*. Microbiology **152:**591–604.
29. **Kullas, A. L., S. J. Martin, and D. Davis.** 2007. Adaptation to environmental pH: integrating the Rim101 and calcineurin signal transduction pathways. Mol. Microbiol. **66:**858–871.
30. **Lamb, T. M., and A. P. Mitchell.** 2003. The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes *NRG1* and *SMP1* in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **23:**677–686.
31. **Li, G., Z. Zhou, G. Liu, F. Zheng, and C. He.** 2007. Characterization of T-DNA insertion patterns in the genome of rice blast fungus *Magnaporthe oryzae*. Curr. Genet. **51:**233–243.
32. **Liu, O. W., C. D. Chun, E. D. Chow, C. Chen, H. D. Madhani, and S. M. Noble.** 2008. Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. Cell **135:**174–188.
33. **Liu, S., G. T. Milne, J. G. Kuremsky, G. R. Fink, and S. H. Leppla.** 2004. Identification of the proteins required for biosynthesis of diphthamide, the target of bacterial ADP-ribosylating toxins on translation elongation factor 2. Mol. Cell. Biol. **24:**9487–9497.
34. **Loftus, B. J., E. Fung, P. Roncaglia, D. Rowley, P. Amedeo, D. Bruno, J. Vamathevan, M. Miranda, I. J. Anderson, J. A. Fraser, J. E. Allen, I. E. Bosdet, M. R. Brent, R. Chiu, T. L. Doering, M. J. Donlin, C. A. D'Souza, D. S. Fox, V. Grinberg, J. Fu, M. Fukushima, B. J. Haas, J. C. Huang, G. Janbon, S. J. M. Jones, H. L. Koo, M. I. Krzywinski, J. K. Kwon-Chung, K. B. Lengeler, R. Maiti, M. A. Marra, R. E. Marra, C. A. Mathewson, T. G. Mitchell, M. Pertea, F. R. Riggs, S. L. Salzberg, J. E. Schein, A. Shvartsbeyn, H. Shin, M. Shumway, C. A. Specht, B. B. Suh, A. Tenney, T. R. Utterback, B. L. Wickes, J. R. Wortman, N. H. Wye, J. W. Kronstad, J. K. Lodge, J. Heitman, R. W. Davis, C. M. Fraser, and R. W. Hyman.** 2005. The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. Science **307:**1321–1324.
35. **Majka, J., and P. M. J. Burgers.** 2003. Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint. Proc. Natl. Acad. Sci. USA **100:** 2249–2254.
36. **McClelland, C. M., Y. C. Chang, and K. J. Kwon-Chung.** 2005. High frequency transformation of *Cryptococcus neoformans* and *Cryptococcus gattii* by *Agrobacterium tumefaciens*. Fungal Genet. Biol. **42:**904–913.
37. **Meng, Y., G. Patel, M. Heist, M. F. Betts, S. L. Tucker, N. Galadima, N. M. Donofrio, D. Brown, T. K. Mitchell, L. Li, J.-R. Xu, M. Orbach, M. Thon, R. A. Dean, and M. L. Farman.** 2007. A systematic analysis of T-DNA insertion events in *Magnaporthe oryzae*. Fungal Genet. Biol. **44:**1050–1064.
38. **Morita, T., K. Takegawa, and T. Yagi.** 2004. Disruption of the *plr1*$^+$ gene encoding pyridoxal reductase of *Schizosaccharomyces pombe*. J. Biochem. **135:**225–230.
39. **Moyrand, F., T. Fontaine, and G. Janbon.** 2007. Systematic capsule gene disruption reveals the central role of galactose metabolism on *Cryptococcus neoformans* virulence. Mol. Microbiol. **64:**771–781.
40. **Mylonakis, E., A. Casadevall, and F. M. Ausubel.** 2007. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog. **3:**e101.
41. **Mylonakis, E., A. Idnurm, R. Moreno, J. El Khoury, J. B. Rottman, F. M. Ausubel, J. Heitman, and S. B. Calderwood.** 2004. *Cryptococcus neoformans* Kin1 protein kinase homologue, identified through a *Caenorhabditis elegans* screen, promotes virulence in mammals. Mol. Microbiol. **54:**407–419.
42. **Mylonakis, E., R. Moreno, J. B. El Khoury, A. Idnurm, J. Heitman, S. B. Calderwood, F. M. Ausubel, and A. Diener.** 2005. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. Infect. Immun. **73:**3842–3850.
43. **Nacry, P., C. Camilleri, B. Courtial, M. Caboche, and D. Bouchez.** 1998. Major chromosomal rearrangements induced by T-DNA transformation in Arabidopsis. Genetics **149:**641–650.
44. **Nelson, R. T., J. Hua, B. Pryor, and J. K. Lodge.** 2001. Identification of virulence mutants of the fungal pathogen *Cryptococcus neoformans* using signature-tagged mutagenesis. Genetics **157:**935–947.
45. **Nielsen, K., G. M. Cox, A. P. Litvintseva, E. Mylonakis, S. D. Malliaris, D. K. Benjamin, Jr., S. S. Giles, T. G. Mitchell, A. Casadevall, J. R. Perfect, and J. Heitman.** 2005. *Cryptococcus neoformans* α strains preferentially disseminate to the central nervous system during coinfection. Infect. Immun. **73:** 4922–4933.
46. **Nielsen, K., G. M. Cox, P. Wang, D. L. Toffaletti, J. R. Perfect, and J. Heitman.** 2003. Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic **a** and α isolates. Infect. Immun. **71:**4831–4841.
47. **Niggli, V., and E. Sigel.** 2008. Anticipating antiport in P-type ATPases. Trends Biochem. Sci. **33:**156–160.
48. **Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman.** 1997. Calcineurin is required for virulence of *Cryptococcus neoformans*. EMBO J. **16:**2576–2589.
49. **Peñalva, M. A., J. Tilburn, E. Bignell, and H. N. Arst, Jr.** 2008. Ambient pH gene regulation in fungi: making connections. Trends Microbiol. **16:**291–300.
50. **Pitkin, J. W., D. G. Panaccione, and J. D. Walton.** 1996. A putative cyclic peptide efflux pump encoded by the *TOXA* gene of the plant-pathogenic fungus *Cochliobolus carbonum*. Microbiology **142:**1557–1565.
51. **Platara, M., A. Ruiz, R. Serrano, A. Palomino, F. Moreno, and J. Ariño.** 2006. The transcriptional response of the yeast Na^+-ATPase *ENA1* gene to alkaline stress involves three main signaling pathways. J. Biol. Chem. **281:** 36632–36642.
52. **Ruiz, A., and J. Ariño.** 2007. Function and regulation of the *Saccharomyces cerevisiae ENA* sodium ATPase system. Eukaryot. Cell **6:**2175–2183.
53. **Serrano, R., A. Ruiz, D. Bernal, J. R. Chambers, and J. Ariño.** 2002. The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signalling. Mol. Microbiol. **46:**1319–1333.
54. **Shinohara, M., K. Sakai, T. Ogawa, and A. Shinohara.** 2003. The mitotic DNA damage checkpoint proteins Rad17 and Rad24 are required for repair of double-strand breaks during meiosis in yeast. Genetics **164:**855–856.
55. **Takao, M., R. Yonemasu, K. Yamamoto, and A. Yasui.** 1996. Characterization of a UV endonuclease gene from the fission yeast *Schizosaccharomyces pombe* and its bacterial homolog. Nucleic Acids Res. **24:**1267–1271.
56. **Tang, C. M., J. M. Smith, H. N. Arst, Jr., and D. W. Holden.** 1994. Virulence studies of *Aspergillus nidulans* mutants requiring lysine or *p*-aminobenzoic acid in invasive pulmonary aspergillosis. Infect. Immun. **62:**5255–5260.
57. **Tang, R. J., J. Breger, A. Idnurm, K. J. Gerik, J. K. Lodge, J. Heitman, S. B. Calderwood, and E. Mylonakis.** 2005. *Cryptococcus neoformans* gene involved in mammalian pathogenesis identified by a *Caenorhabditis elegans* progeny-based approach. Infect. Immun. **73:**8219–8225.
58. **Vartivarian, S. E., E. J. Anaissie, R. E. Cowart, H. A. Sprigg, M. J. Tingler, and E. S. Jacobson.** 1993. Regulation of cryptococcal capsular polysaccharide by iron. J. Infect. Dis. **167:**186–190.

59. **Walton, F. J., J. Heitman, and A. Idnurm.** 2006. Conserved elements of the RAM signaling pathway establish cell polarity in the basidiomycete *Cryptococcus neoformans* in a divergent fashion from other fungi. Mol. Biol. Cell **17:**3768–3780.
60. **Walton, F. J., A. Idnurm, and J. Heitman.** 2005. Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. Mol. Microbiol. **57:**1381–1396.
61. **Wieland, J., A. M. Nitsche, J. Strayle, H. Steiner, and H. K. Rudolph.** 1995. The *PMR2* gene cluster encodes functionally distinct isoforms of a putative Na^+ pump in the yeast plasma membrane. EMBO J. **14:**3870–3882.
62. **Williamson, P. R.** 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. J. Bacteriol. **176:**656–664.
63. **Yajima, H., M. Takao, S. Yasuhira, J. H. Zhao, C. Ishii, H. Inoue, and A. Yasui.** 1995. A eukaryotic gene encoding an endonuclease that specifically repairs DNA damaged by ultraviolet light. EMBO J. **14:**2393–2399.
64. **Yeh, Y.-L., Y.-S. Lin, B.-J. Su, and W.-C. Shen.** 2009. A screening for suppressor mutants reveals components involved in the blue light-inhibited sexual filamentation in *Cryptococcus neoformans*. Fungal Genet. Biol. **46:**42–54.
65. **Zhu, X., and P. R. Williamson.** 2003. A CLC-type chloride channel gene is required for laccase activity and virulence in *Cryptococcus neoformans*. Mol. Microbiol. **50:**1271–1282.