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UGE1 and *UGE2* Regulate the UDP-Glucose/UDP-Galactose Equilibrium in *Cryptococcus neoformans*▽†

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The genome of the basidiomycete pathogenic yeast *Cryptococcus neoformans* carries two UDP-glucose epimerase genes (*UGE1* and *UGE2*). *UGE2* maps within a galactose cluster composed of a galactokinase homologue gene and a galactose-1-phosphate uridylyltransferase. This clustered organization of the *GAL* genes is similar to that in most of the hemiascomycete yeast genomes and in *Schizosaccharomyces pombe* but is otherwise not generally conserved in the fungal kingdom. *UGE1* has been identified as necessary for galactoxylomannan biosynthesis and virulence. Here, we show that *UGE2* is necessary for *C. neoformans* cells to utilize galactose as a carbon source at 30°C but is not required for virulence. In contrast, deletion of *UGE1* does not affect cell growth on galactose at this temperature. At 37°C, a *uge2*Δ mutant grows on galactose in a *UGE1*-dependent manner. This compensation by *UGE1* of *UGE2* mutation for growth on galactose at 37°C was not associated with upregulation of *UGE1* transcription or with an increase of the affinity of the enzyme for UDP-galactose at this temperature. We studied the subcellular localization of the two enzymes. Whereas at 30°C, Uge1p is at least partially associated with intracellular vesicles and Uge2p is on the plasma membrane, in cells growing on galactose at 37°C, Uge1p colocalizes with Uge2p to the plasma membrane, suggesting that its activity is regulated through subcellular localization.

Cryptococcus neoformans is an environmental microbe responsible for severe diseases in immunocompromised individuals (4). It is found in soils, decaying vegetation, and bird droppings. *C. neoformans* is thought to be acquired early in life and to stay in dormancy until an immune defect occurs (11). Then, *C. neoformans* multiplies and disseminates to various organs, including the central nervous system, where it causes meningoencephalitis that is fatal if untreated. Its main virulence factor is the polysaccharide capsule, which is essential for virulence. This capsule is composed mainly of two polysaccharides, a large molecule called glucuronoxylomann (made of an α-mannose chain with glucuronic acid, xylose, and *O*-acetyl residues) and a smaller molecule called galactoxylomannan (GalXM) (made of a galactose chain with mannose and xylose residues) (for reviews, see references 21 and 3).

We have been conducting a program of systematic deletion of all genes potentially involved in capsule polysaccharide biosynthesis. Analysis of virulence and organ dissemination profiles of the resulting mutant strains led to the identification of a gene named *UGE1* as necessary for GalXM biosynthesis and virulence (28). *UGE1* encodes a putative UDP-glucose epimerase, an enzyme involved in galactose metabolism. Confirming the central role of galactose metabolism in *C. neoformans* virulence, we have also described a second gene, named *UGT1*, encoding a putative UDP-galactose transporter and similarly necessary for GalXM biosynthesis and virulence (28).

In species ranging from *Escherichia coli* to mammals, galactose is metabolized via a series of reactions collectively known as the Leloir pathway (reviewed in reference 17). A set of four enzymes (galactose mutarotase, galactokinase, galactose-1-phosphate uridylyltransferase, and UDP-galactose epimerase) converts β-D-galactose into glucose-1-phosphate. In *Saccharomyces cerevisiae*, *GAL1* and *GAL7* encode the galactokinase and galactose-1-phosphate uridylyltransferase, respectively; the mutarotase and UDP-galactose epimerase activities are catalyzed by a single protein encoded by the gene *GAL10* (7). Mutation of the epimerase-mutarotase gene or the galactose-1-phosphate uridyl transferase gene results in galactose sensitivity: these mutant strains stop growing in response to even trace quantities of galactose even in the presence of an alternative carbon source (glycerol/ethanol) (33). Similarly, in humans, mutation in one these genes results the inherited metabolic disorder named galactosemia (31). Chinese hamster ovary cells lacking UDP-galactose epimerase activities exhibit impaired growth when exposed to galactose at concentrations of above 0.125 mM, with this phenotype being bypassed by the addition of uridine to the culture medium (36).

Surprisingly, in *C. neoformans*, the deletion of *UGE1* is not associated with galactose sensitivity. Thus, a *uge1*Δ mutant can grow on galactose at 30°C, and the addition of galactose to the medium bypasses the thermosensitive phenotype associated with this gene deletion (28). Analysis of the *C. neoformans* genome sequence identified a *UGE1* gene paralogue which we named *UGE2*. Here, we report an investigation of how *UGE1* and *UGE2* contribute to galactose metabolism, GalXM biosynthesis, and *C. neoformans* virulence by regulating the equilibrium between UDP-galactose and UDP-glucose.

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MATERIALS AND METHODS

Strains and culture conditions. The *C. neoformans* strains used in this study all originated from the serotype A strain KN99α (30) and are listed in Table SA in the supplemental material. The strains were routinely cultured on yeast extract-peptone-dextrose (YPD) medium at 30°C (39). Synthetic dextrose was prepared as described previously (39). The capsule sizes were estimated after 24 h of growth in capsule-inducing medium at 30°C as previously described (22). Production of melanin and urease was assessed after spotting 10^5 cells of each strain on L-Dopa or Christensen agar medium, respectively (32, 44); the plates were read after 48 h of incubation at 30°C. The bacterial strain *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) was used for the propagation of all plasmids.

RNA extraction and Northern blot analysis. Cells were grown in YPD liquid culture to a density of 5×10^7 cells/ml. RNA was extracted with Trizol reagent (Invitrogen) following the manufacturer's instructions. Total RNA (10 μg) was separated by denaturing agarose gel electrophoresis, transferred onto a Hybond-N+ membrane (Amersham), and probed with [^{32}P]dCTP-radiolabeled DNA fragments. The banding pattern was quantified with a Typhoon 9200 imager and Image Quantifier 5.2 software (Molecular Dynamics).

Strain construction. The genes described in this report were deleted by transforming the KN99α strain with a disruption cassette constructed by overlapping PCR as previously described (27). The primer sequences used are given in Table SB in the supplemental material. The transformants were then screened for homologous integration, first by PCR and then by Southern blotting, as previously described (29). The tagged plasmids, pNATSTM and pHYGSMT, used to amplify the selective marker were kindly provided by Jennifer Lodge (St. Louis University School of Medicine). The *uge2*Δ strain was reconstituted by inserting a 3.9-kbp PCR-amplified fragment (using the primers UGE2-5′5 and UGE2-3′3) between the KspI and SpeI sites of a plasmid containing a nourseothricin resistance cassette (18). The resulting plasmid, pNE394, was digested with SpeI and used to transform strain NE369 (*MAT*α *uge2*Δ) by biolistic DNA delivery. Transformants were selected on YPD medium containing 100 μg/ml of nourseothricin (Werner BioAgents). Three nourseothricin-resistant strains were obtained; all grew on galactose. Two were stored at −80°C for further studies.

Recombinant protein production. *UGE1* and *UGE2* cDNAs were amplified by PCR and inserted into the pQ-30 *E. coli* expression vector (Qiagen). The *E. coli* BL21 transformant strains were grown in 50 ml of yeast extract tryptone medium containing ampicillin (50 μg/ml) and kanamycin (30 μg/ml) to an optical density at 600 nm of 0.5; gene expression was induced by addition of 50 μM of IPTG (isopropyl-β-D-thiogalactopyranoside) and incubation overnight at room temperature. The cells were then disrupted by sonication and centrifuged at 3,000 × *g*. The supernatant was recovered, and the recombinant proteins were purified by affinity chromatography on an Ni-nitrilotriacetic acid column (Qiagen) following the manufacturer's procedures. The protein solution was adjusted to 20% (wt/vol) glycerol (final concentration, 700 μg/ml) and stored in aliquots at −80°C.

UDP-galactose epimerase assay. Epimerase activity was assayed using an NADH-coupled assay developed by Wilson and Hogness (46) with some minor modifications. The 1-ml assay mixture consisted of 100 mM glycine buffer (pH 8.7), 1 mM β-NAD^+ (Sigma), and 0.8 mM UDP-galactose (Sigma). The reaction was started by adding 10 μl of epimerase (140 μg/ml) in 50 mM Tris HCl (pH 7.6)–1% bovine serum albumin–1 mM dithiothreitol–1 mM EDTA–1 mM β-NAD^+ and stopped by incubation for 10 min at 100°C. The UDP-glucose produced was determined by addition of 0.04 unit of bovine UDP-glucose dehydrogenase (Sigma) and incubation for 10 min at 30°C; the increase in absorbance due to the formation of NADH was then measured at 340 nm. K_m values were determined by varying the UDP-galactose concentration between 0.4 mM and 3.2 mM. The experiment was conducted in triplicate.

Subcellular localization with fluorescent protein fusions. To localize the Uge proteins, the *UGE1* and *UGE2* genes under the control of their own promoters were joined in frame to the sequences encoding the green fluorescent protein or the DsRed protein at their C-terminal ends. Primers used for amplification are listed in Table SB in the supplemental material. The *uge1*Δ and *uge2*Δ strains and the *uge1*Δ *uge2*Δ double mutant were transformed with plasmids containing the Hyg or the Neo selectable marker and the Uge-fluorescent protein fusion by biolistic delivery (42). Tagged fluorescent *UGE1* versions were integrated in the genome using the Hyg marker. It should be noted that the *UGE1* deletion is associated with hypersensitivity to hygromycin such that the *uge1*Δ::*NAT1* *uge2*Δ::*HYG1* double mutants were sensitive to hygromycin (data not shown), and this allowed the reutilization of this marker for the reintroduction of this gene. Tagged fluorescent *UGE2* versions were integrated in the genome using the Neo selectable marker. Transformants were analyzed for fluorescence under various growth conditions.

Expression of *UGE1* and *UGE2* in *S. cerevisiae*. *UGE1* and *UGE2* cDNAs were amplified by PCR and inserted into the vector pCM190 (12). The Euroscarf *S. cerevisiae* strain BY4742 (*Mat*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 gal10*::*kanMX4*) was transformed using a lithium acetate procedure (13) and tested on 1% galactose medium.

Analysis of the sugar composition. Cells were grown at 30°C or 37°C for 3 days in capsule induction medium. After elimination of the cells by centrifugation and filtration, the total polysaccharide in the culture supernatant was precipitated with ethanol, resuspended in distilled water, filtered, and lyophilized. The polysaccharide compositions were analyzed by gas-liquid chromatography after acid hydrolysis with trifluoroacetic acid as previously described (35).

Virulence assays. Six-week-old male BALB/c mice (Charles River Laboratories, France) were used for all in vivo experiments. Strains were grown on YPD medium at 30°C overnight. For the dissemination assay, 10^5 *C. neoformans* cells were injected into the tail vein of each mouse. On day 1, the mice were killed and the brain, spleen, and lungs were recovered. Each organ was homogenized, and adapted dilutions were plated on Sabouraud-chloramphenicol plates. The experiments were repeated independently three times.

The relative virulence of the strains was evaluated by intravenously inoculating groups of seven mice with 10^5 cells of each cryptococcal strain. Animals were observed daily, and any deaths were recorded.

Gene identification. The *Cryptococcus neoformans* var. *grubii* Uge2p sequence we used as a query in BLASTP searches against the proteomes of 36 fungi (*Ashbya gossypii*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus*, *Batrachochytrium dendrobatidis*, *Botrytis cinerea*, *Candida albicans*, *Candida glabrata*, *Candida guillermondii*, *Candida lusitaniae*, *Candida tropicalis*, *Chaetomium globosum*, *Coccidioides immitis*, *Coprinus cinereus*, *Cryptococcus neoformans* var. *neoformans*, *Debaryomyces hansenii*, *Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Histoplasma capsulatum*, *Kluyveromyces lactis*, *Lodderomyces elongisporus*, *Magnaporthe grisea*, *Neosartorya fischeri*, *Neurospora crassa*, *Phanerochaete chrysosporium*, *Pichia stipitis*, *Puccinia graminis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Sclerotinia sclerotiorum*, *Stagonospora nodorum*, *Uncinocarpus reesei*, *Ustilago maydis*, and *Yarrowia lipolytica*). For each query, the top hits were retained provided that their E values were less than 1e−20. Proteins sharing at least 25% sequence identity with *Cryptococcus neoformans* var. *grubii* Uge2p as analyzed with ClustalW were selected (see Table SC in the supplemental material). Phylogenetic analysis was performed via the server http://www.phylogeny.fr/, using MUSCLE (9) for the multiple alignment, Gblocks (6) to remove the poorly aligned positions, and PHYML (14) for tree reconstruction. Branch support was calculated using the approximate likelihood ratio test described previously (1). The substitution model used was WAG plus gamma distribution with an estimated alpha parameter and an estimated proportion of invariable sites.

RESULTS

***C. neoformans* possesses two putative UDP-glucose epimerase genes in its genome.** The gene *UGE1* is necessary for GalXM biosynthesis and virulence in *C. neoformans* (28). BLAST analysis revealed the presence of a paralogue of *UGE1*, called *UGE2*, in the *C. neoformans* var. *grubii* genome. Uge1p and Uge2p share 65% amino acid sequence identity (Fig. 1A). Hydropathy analysis indicates that both proteins have a potential N-terminal transmembrane domain (Fig. 1B); both sequences include an epimerase motif, but neither has a mutaronase domain similar to that in the Gal10 protein of *S. cerevisiae* (25). In fact, we were unable to identify any putative maturonase gene in the *C. neoformans* genome (24). The presence of two UDP-glucose epimerases has not previously been described for organisms in which this enzyme has been mostly studied (*S. cerevisiae*, *E. coli*, and human), where only one UDP-glucose epimerase equilibrates the UDP-glucose/UDP-galactose ratio (17). We looked for putative UDP-glucose epimerase genes in the large number of fungal genomes now sequenced and annotated (Table 1). We identified at least one *GAL10* orthologue in most fungi but not in *Candida glabrata* and *Ashbya gossypii* (8, 16). The list of fungi covered most of the evolutionary tree of fungi, and although not statistically

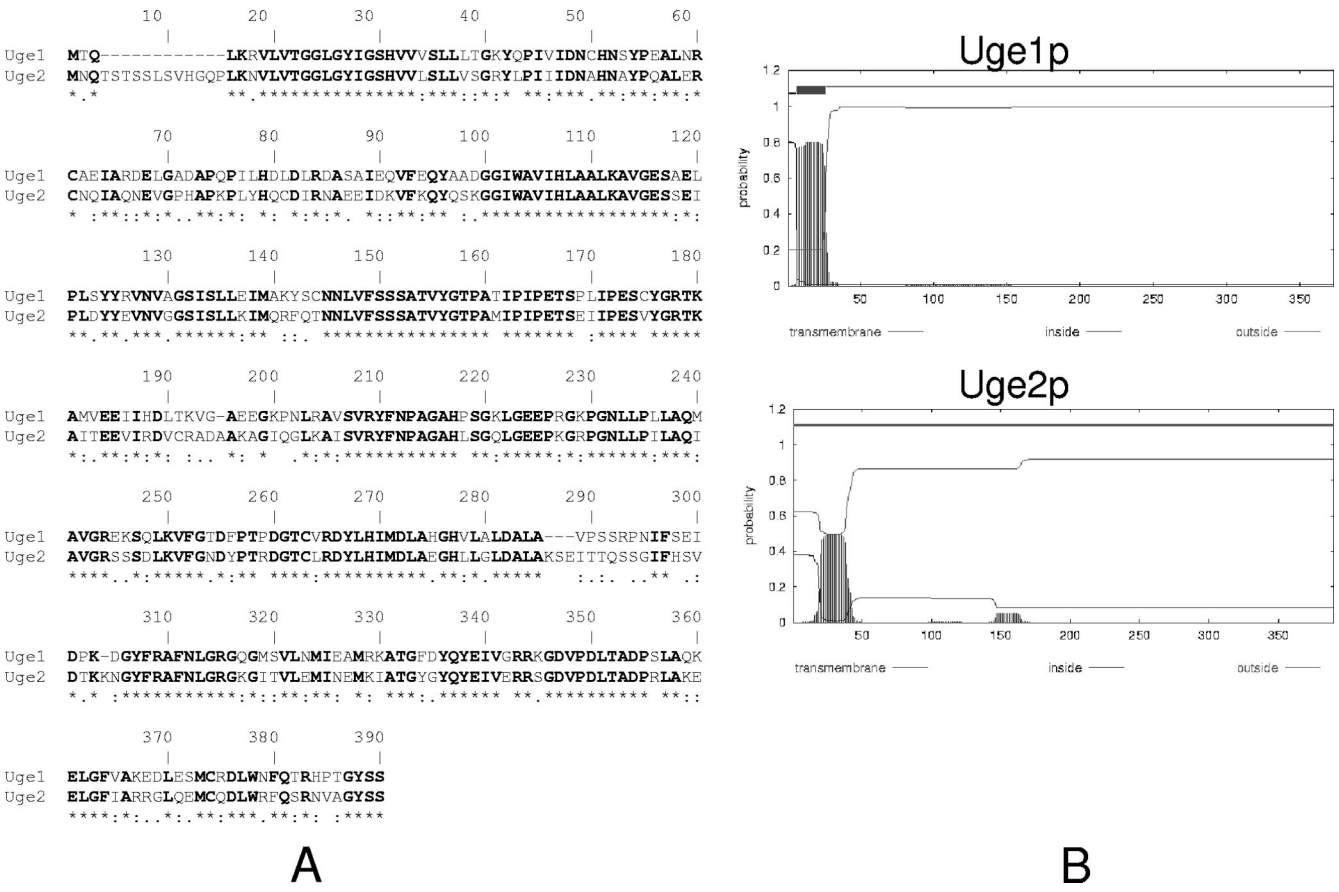

FIG. 1. (A) Amino acid alignment of the two *C. neoformans* Uge sequences. (B) Hydropathy profiles of the Uge proteins determined by TMHMM1.0 analysis.

representative because some genera are clearly overrepresented, it is clear that the presence of more than one UDP-glucose epimerase is not unusual.

UGE2 belongs to a cluster of genes involved in galactose assimilation, whereas *UGE1* is not clustered with any other *GAL* genes. *UGE2* is localized immediately upstream from a close homologue of *S. cerevisiae GAL1* in the opposite orientation, and a close homologue of *S. cerevisiae GAL7* is downstream from *GAL1* on the same strand (Fig. 2). This galactose gene cluster organization is very similar to those in *S. cerevisiae* and *Kluyveromyces lactis* (45) (Fig. 2) except that the *GAL7* gene in *C. neoformans* is on the other side of the *GAL10-GAL1* cluster. We manually reannotated all the regions surrounding the *GAL10* genes in the different fungal genomes. We found that *GAL10* and *GAL1* orthologues are generally not clustered. Indeed, this particular clustered organization of the galactose genes is restricted to the hemisacomycete genomes, with *Yarrowia lipolytica* being the only member of this class of fungi to not have any *GAL* cluster. As shown in Fig. 2, the *GAL* cluster can be restricted to the three *GAL* genes, like in *S. cerevisiae* and *K. lactis*. For all other members, the *GAL* cluster includes two additional putative open reading frames (ORFs). An ORF encoding a protein sharing similarities with dTDP glucose-6-dehydratases is present upstream from the *GAL7* gene on the opposite strand (26); this ORF is also present in *S. pombe*. Another small ORF, encoding a putative sybindin-like protein, is present downstream (10). This ORF is not present in *C. lusitaniae* and in *S. pombe* but is duplicated in *D. hansenii*. It overlaps the dTDP glucose-6-dehydratase gene in *C. albicans* and *C. tropicalis*.

Outside the hemiascomycetes, the clustered organization of the *GAL* genes is conserved only in *S. pombe* and *C. neoformans*; *C. neoformans* is the only organism outside the ascomycete phylum to have a *GAL* gene cluster.

Both *UGE1* and *UGE2* can complement a *gal10* mutation in *S. cerevisiae*. The cDNAs of *UGE1* and *UGE2* were amplified by PCR and inserted into an *S. cerevisiae* expression vector under the control of a Tet-off promoter. The resulting plasmids were used to transform an *S. cerevisiae gal10* mutant strain. In the absence of doxycycline, the transformants were able to grow on 1% galactose, whereas the strain transformed with the control plasmid was not (Fig. 3). These results indicate that both *UGE* genes encode UDP-glucose epimerases and that both enzymes are able to catalyze the transformation of UDP-galactose to UDP-glucose.

***UGE2* is not necessary for *C. neoformans* dissemination and virulence.** We deleted the *UGE2* gene using the hygromycin marker (see Materials and Methods). The *uge2*Δ strains displayed a normal colonial morphology and a wild-type growth rate on glucose, and production of capsule, melanin, and urease was not affected (data not shown). The polysaccharide secreted by these mutants at 30°C and 37°C was the same as that secreted by the wild type; thus, GalXM production was not

TABLE 1. Putative *GAL10* paralogues in fungi

Fungus	UDP-glucose epimerase-homologous gene(s)	Clustering of *GAL1* with one *GAL10* paralogues
Ashbya gossypii	None	
Aspergillus fumigatus	Afu3g07910, Afu4g14090, Afu5g10780	No
Aspergillus nidulans	AN2951, AN4727	No
Aspergillus terreus	ATEG 07631, ATEG 01678	No
Batrachochytrium dendrobatidis	BDEG07987	No
Botrytis cinerea	BC1G 0445, BC1G11467	No
Candida albicans	*GAL10*	Yes
Candida glabrata	None	
Candida guillermondii	PCGUG 05863	Yes
Candida lusitaniae	CLUG02291	Yes
Candida tropicalis	CTRG04618	Yes
Chaetomium globosum	CHGG 04003	No
Coccidioides immitis	CIMG 00721	No
Coprinus cinereus	CC1G_00553	No
Cryptococcus neoformans var. *grubii*	*UGE1*, *UGE2*	Yes
Cryptococcus neoformans var. *neoformans*	*UGE1*, *UGE2*	Yes
Debaryomyces hansenii	DEHA0C02838g	Yes
Fusarium graminearum	FG05689, FG03048	No
Fusarium oxysporum	FOXG09188, FOXG02479	No
Fusarium verticillioides	FVEG06791, FVEG05667	No
Histoplasma capsulatum	HCAG00955	No
Kluyveromyces lactis	*GAL10*	Yes
Lodderomyces elongisporus	LELG_01648	Yes
Magnaporthe grisea	MGG8012	No
Neosartorya fischeri	NFIA076450, NFIA069260, NFIA102220	No
Neurospora crassa	NCU4442 and NCU5133	No
Phanerochaete chrysosporium	PC160.1	No
Pichia stipitis	*GALK*	Yes
Puccinia graminis	PGTG06253, PGTG14524	No
Rhizopus oryzae	RO3G12257, RO3G01911, RO3G05148	No
Saccharomyces cerevisiae	*GAL10*	Yes
Schizosaccharomyces pombe	SPBPB2BE-12c, SPB23G5-14c	Yes
Sclerotinia sclerotiorum	SS1G08326, SS1G04477	No
Stagonospora nodorum	SNOG14014, SNOG 14228	No
Uncinocarpus reesei	UREG 00714	No
Ustilago maydis	UM06057	No
Yarrowia lipolytica	YALI0E26829g	No

affected by deletion of this gene (data not shown).We studied the dissemination of *uge2*Δ and wild-type strains to various target organs (Fig. 4). In contrast to the case for the *uge1*Δ strain (28), the dissemination of the *uge2*Δ strain was indistinguishable from that of the wild type. Moreover, the virulence of the *uge2*Δ and reconstructed strains did not differ significantly from that of the wild-type. Thus, unlike *UGE1*, *UGE2* is clearly inessential for *C. neoformans* virulence.

***UGE2* is necessary for *C. neoformans* growth on galactose at 30°C but not at 37°C.** Like *S. cerevisiae gal10* mutants, the *uge2*Δ strains were not able to grow on galactose (1%) at 30°C (Fig. 5) and displayed galactose sensitivity (defective growth on ethanol-galactose and glycerol-galactose media at 30°C [data not shown]). This galactose sensitivity was not relieved by the addition of uracil or uridine to the medium (data not shown). Reintroduction of the *UGE2* gene into the mutant strains restored growth on galactose. In contrast, *uge1*Δ strains grew on galactose at 30°C, suggesting that *uge1* is not necessary for galactose assimilation/detoxification at this temperature.

Surprisingly, at 37°C, the *uge2*Δ strains were able to grow on galactose (Fig. 5) suggesting that another enzyme assimilated/detoxified galactose at this temperature. To test whether the enzyme was Uge1p, we deleted the *UGE1* gene from a *uge2*Δ background using a nourseothricin marker. The double mutant strain cumulated all the phenotypes associated with each single deletion: it did not grow on glucose at 37°C, displayed a larger-than-wild-type capsule, did not produce GalXM, had a growth defect on glucose at 30°C, and did not grow on galactose at 30°C. However, the double mutant did not grow on galactose at 37°C, whereas both single mutants did, suggesting that at this temperature and on this medium, *UGE1* compensates for the absence of *UGE2*.

***UGE2* expression, but not *UGE1* expression, is induced by the presence of galactose.** The fact that *UGE1* is able to compensate for the absence of *UGE2* on galactose at 37°C but not at 30°C (see above) suggests that Uge1p is regulated in a temperature-dependent manner on galactose. The easiest hypothesis was that *UGE1* transcription would be upregulated at 37°C on galactose in the absence of *UGE2* and thus compensate for the absence of its closest paralogue. We thus studied the expression of the two *UGE* genes in strains grown on various carbon sources by Northern blotting. As shown in Fig. 6, transcription of *UGE2* was undetectable in cells grown on glucose and was very strongly induced in the presence of galactose at 30°C and 37°C. This regulation is similar to that of the *S. cerevisiae GAL10* gene. In contrast, *UGE1* expression was not affected by the presence of galactose in the medium; the expression was similar under all condition tested in the absence or the presence of *UGE2*.

The enzymatic properties of Uge1p are not affected by the temperature. Our second hypothesis was that Uge1p has different affinities for UDP-galactose or UDP-glucose at different temperatures. We made various constructions to produce both Uge1 and Uge2 recombinant enzymes in *E. coli*. For each, we made two constructs including or excluding the putative transmembrane domain described above. For Uge2p, although denaturing gel electrophoresis unambiguously showed production of a recombinant protein, the protein obtained was always in the insoluble cell extract at all temperatures (4°C, 25°C, and 37°C) and IPTG concentrations (1 mM and 50 μM) tested (data not shown). For Uge1p, the truncated gene similarly gave an insoluble protein, but the vector with the complete coding sequence produced a soluble protein of the correct size, which was purified on an affinity column (Fig. 7A). We tested the activity of the recombinant enzyme at 30°C and 37°C on UDP-galactose (Fig. 7B). The recombinant Uge1p converted UDP-galactose to UDP-glucose, but the affinity of the enzyme was largely unaffected by a change in the temperature; the affinity of the enzyme for UDP-galactose was, if anything, lower at 37°C (apparent $K_m = 610 \pm 75$ μM) than at 30°C (apparent $K_m = 282 \pm 34$ μM).

Uge1p and Uge2p subcellular localization. Our third hypothesis was that Uge1p has different subcellular localizations at 30 and 37°C: at one location Uge1p may be involved exclusively in GalXM biosynthesis, transforming UDP-glucose to

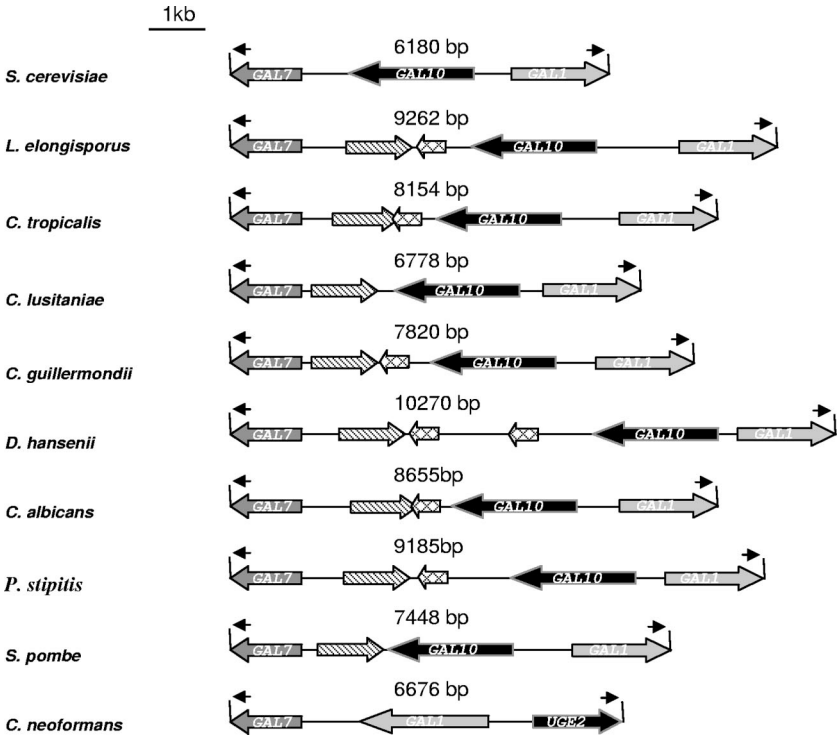

FIG. 2. Organization of the galactose gene cluster in fungi. A galactose cluster is found in only some hemiascomycete yeasts and in *S. pombe* and *C. neoformans*. In ascomycetes, *GAL10* encodes a double enzyme (UDP-glucose epimerase-mutaronase), whereas *UGE2* and *UGE1* in *C. neoformans* and the paralogue of *GAL10* in *S. pombe* (SBPB23G5-14c), which is located outside of the galactose cluster, are shorter and encode only putative UDP-glucose epimerase activity. ⇒, encodes a putative dTDP glucose-6-dehydratase. ⇨, encodes a sybindin-like protein.

UDP-galactose, whereas at the other it may participate in galactose assimilation, transforming UDP-galactose to UDP-glucose. We constructed fluorescently tagged versions of Uge1p and Uge2p. These protein fusions were completely functional (data not shown). Strains producing these proteins were cultivated at 30°C on galactose and searched for the fluorescent tags: Uge1p fluorescence appeared at least partly in a punctate, dotted pattern, indicating that the protein was partly associated with vesicles, while Uge2p mainly had a plasma membrane-like localization, although there was some cytoplasmic labeling (Fig. 8). We checked that the fluorescence observed was indeed specific for the Uge proteins (Fig. 8). We also checked that the localization of Uge1 was not affected by the deletion of *UGE2* (data not shown). We then constructed a strain expressing the fluorescent versions of both Uge1p and Uge2p. When the cells were cultivated at 30°C on galactose, the localizations of the proteins were similar to those observed using the strains expressing only one fluorescent protein (Fig. 9). Overlaying the pictures on each other showed that the two proteins did not colocalize to any great extent at this temperature (Fig. 9). When the temperature was shifted to 37°C and the cells grown on galactose, the subcellular localization of Uge1p changed such that it was also found at the plasma membrane, like Uge2p. Overlaying the pictures on each other showed at least partial colocalization of the two proteins (Fig. 9). Thus, the carbon source and the temperature regulate the subcellular localization of Uge1p. These results strongly suggest that the subcellular localization of Uge1p determines, at least in part, its function in *C. neoformans*.

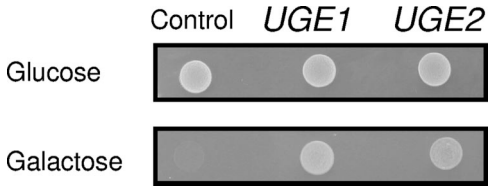

FIG. 3. Both *UGE1* and *UGE2* can complement an *S. cerevisiae gal10* mutation. An *S. cerevisiae gal10* mutant strain was transformed with plasmids containing *UGE1* or *UGE2* cDNAs under the control of a Tet-off promoter, and 10^5 cells were spotted onto each glucose and galactose medium. Cells were grown at 30°C for 3 days and photographed. As a control, the *S. cerevisiae gal10* mutant strain was transformed with the same plasmid containing no cDNA.

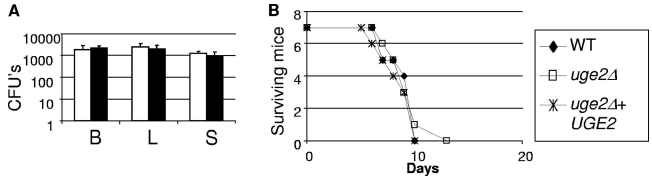

FIG. 4. Virulence of the uge2Δ strains. (A) Dissemination of *uge2*Δ (black bars) and wild-type (white bars) cells after 1 day of infection. The various organs (brain [B], lungs [L], and spleen [S]) were homogenized and plated, and the numbers of CFU per organ were recorded after 3 to 5 days of incubation at 30°C. Three mice were used for each strain at each time point, and the results reported are mean values and standard deviations. (B) Survival of mice after infection with strains KN99α (wild type [WT]), NE369 (*uge2*Δ), and NE447 (*uge2*Δ + *UGE2*).

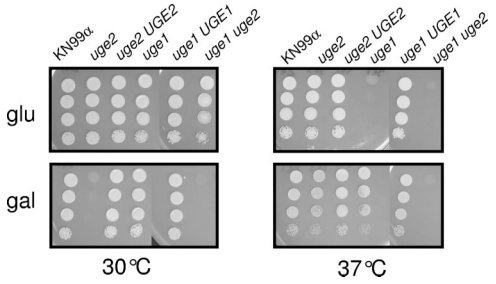

FIG. 5. Growth defects associated with *UGE1* and *UGE2* disruptions. *C. neoformans* cells were grown in liquid YPD medium overnight and washed with sterile water, and serial dilutions (5×10^5, 5×10^4, 5×10^3, and 5×10^2 cells) of each strain were spotted onto various media and observed after incubation for 3 days.

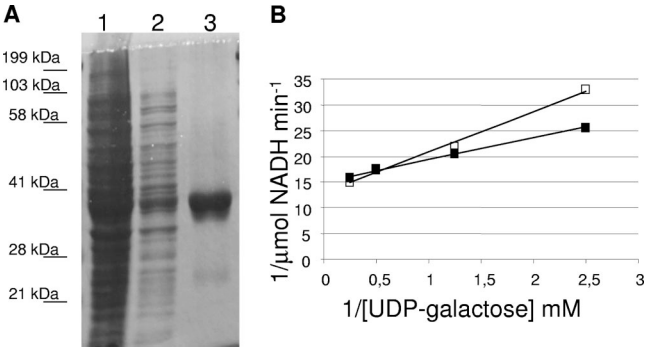

FIG. 7. Uge1p can catalyze the epimerization of UDP-galactose in UDP-glucose at 30°C and 37°C. (A) Purification of recombinant Uge1p in *E. coli*, showing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining of total *E. coli* lysate (lane 1), soluble supernatant (lane 2), and the purified protein after affinity column purification (lane 3). (B) Lineweaver-Burk plots of purified recombinant Uge1p UDP-galactose epimerase activity at 30°C (■) and at 37°C (□).

DISCUSSION

Origin of the *GAL* gene cluster. *UGE2* is located within a *GAL* cluster very similar in organization to those in most of the hemiascomycete yeasts and in the archeascomycete yeast *S. pombe*. The presence of this similar genomic organization in these evolutionarily very distant organisms is surprising. These clusters may have originated from horizontal transfer from a bacterium. Indeed, the *E. coli gal* operon, containing the four Leloir pathway genes (*galM*, *galK*, *galT*, and *galE*), has a similar organization. Were this the case, there must have been either multiple independent gene transfers or one transfer to an ancestral organism followed by the loss of this gene organization in most fungi. Constructions of phylogenetic trees provide no evidence for specific clustering of any bacterial protein sequences with those of any fungal phylum containing the *GAL* cluster (see Fig. SA and Table SC in the supplemental material), as would be expected if there had been multiple horizontal gene transfers. Alternatively, the *GAL* clusters may have been formed by genomic rearrangements leading to a similar organization in the three different classes representing two phyla. Indeed, there is now evidence that the gene order in eukaryotic genomes is not random (19). According to current models of the dynamics of gene orders in eukaryotes, clustering of genes is selected to keep particular combinations of alleles in linkage disequilibrium (19). In the present case, the galactose sensitivity associated with the loss of the *GAL10* or *GAL7* gene can be compensated for by the loss of *GAL1*. In other words, it is better to lose the complete pathway than only part of it. Analysis of the galactose assimilation pathway in hemiascomycete yeast has recently illustrated this phenomenon. When one of the four Leloir genes is lost or mutated, the three others are also lost or mutated (16). Thus, in this case, linkage disequilibrium might be the driving force for natural selection to cluster these genes. The same has recently been suggested for the *DAL* gene cluster, which is the only other cluster of at least three genes in the *S. cerevisiae* genome whose products catalyze successive enzymatic steps (47). Also, a study has reported evidence for the independent origin of the maltase gene cluster in two *Drosophila* species, *D. viridis* and *D. melanogaster* (43).

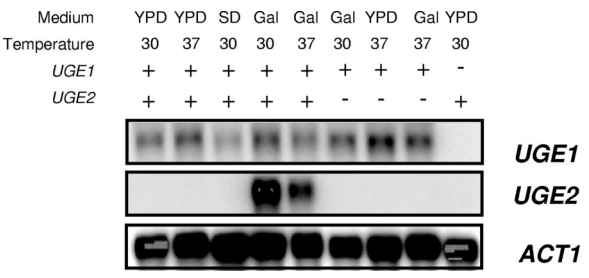

FIG. 6. Expression of *UGE* genes under various growth conditions. Cells were cultured on YPD overnight and subcultured on various media and at various temperatures up to a cell density of 5×10^7 cells/ml. Total RNA was extracted and separated under denaturing condition and probed with *UGE1*, *UGE2*, and *ACT1* gene-specific probes.

The absence of the *GAL* gene cluster from some filamentous ascomycetes is associated with the absence of a *GAL4* orthologue and a different type of *GAL* gene regulation (15, 37), suggesting that this class uses another metabolic and gene regulation strategy to assimilate this alternative carbon source. The absence of coregulation of the *GAL* genes might also explain why the *GAL* clusters, whether formed or acquired, have not been maintained in these fungi. Indeed, natural selection favors conservation of clusters of coregulated genes (20). The presence of the galactose gene cluster in diverse fungi may thus be a unique example of evolutionary convergence leading to the formation or maintenance of gene clustering.

Uge1p delocalization and Uge2p transcription regulate their functions. In the absence of galactose, *UGE2* is not transcribed and thus does not participate in the anabolism of GalXM. This result by itself explains why a *uge1*Δ mutant strain is GalXM negative even in the presence of a functional *UGE2* gene. However, at 37°C, the *uge2*Δ mutant grows on galactose but the *uge1*Δ *uge2*Δ double mutant does not: therefore, Uge1p participates in the assimilation of galactose at 37°C. Neither *UGE1* transcription nor the activity of the protein in vitro toward UDP-galactose is higher at this temperature than at 30°C. The only pertinent difference we observed was the subcellular localization of the protein. Uge1p was clearly associated, at least partially, with Golgi-like vesicles at 30°C on

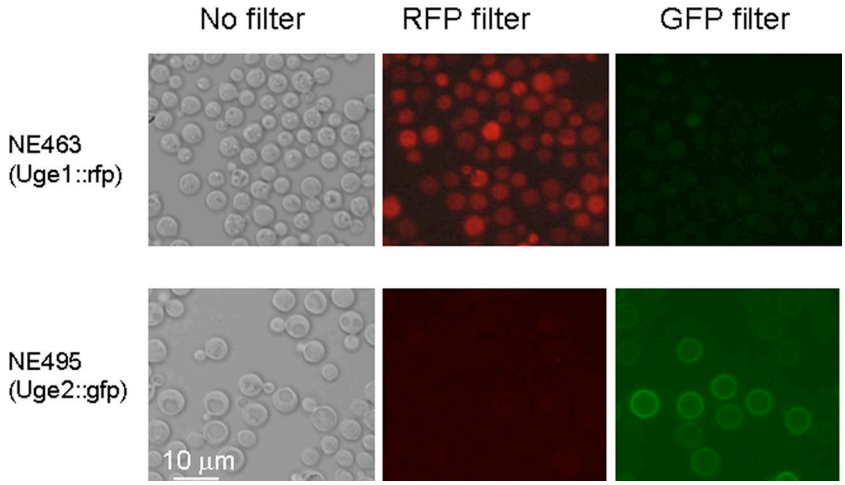

FIG. 8. Subcellular localization of Uge proteins. Cells expressing the fluorescently tagged versions of the Uge proteins were grown on galactose at 30°C and examined by epifluorescence or by bright-field microscopy. It should be noted that the level of autofluorescence is negligible when cells are grown under such conditions. RFP, red fluorescent protein; GFP, green fluorescent protein.

glucose whereas at 37°C on galactose it localized at least partly to the plasma membrane. We have no definitive proof of a causal relationship between the differential localization of the protein and its metabolic function. However, the colocalization of Uge1p and Uge2p at 37°C on galactose at the plasma membrane suggests strongly that under these conditions, Uge1p delocalizes from the Golgi-like vesicles to the plasma membrane to back up Uge2p to transform UDP-galactose to UDP-glucose. Neither the galactose nor the temperature is by itself sufficient to modify the function of Uge1p. Indeed, the *uge2*Δ mutant strain cannot grow on galactose at 30°C; thus, the presence of galactose is not sufficient to modify the function of Uge1. Similarly, the *uge2*Δ mutant strain grown at 37°C on glucose does not display any GalXM defect, as demonstrated by the composition of the secreted polysaccharide. This suggests that at this temperature, Uge1 still fully participates in the transformation of UDP-glucose to UDP-galactose. Thus, both a temperature of 37°C and the presence of galactose seem to be necessary to signal a modification of Uge1p function.

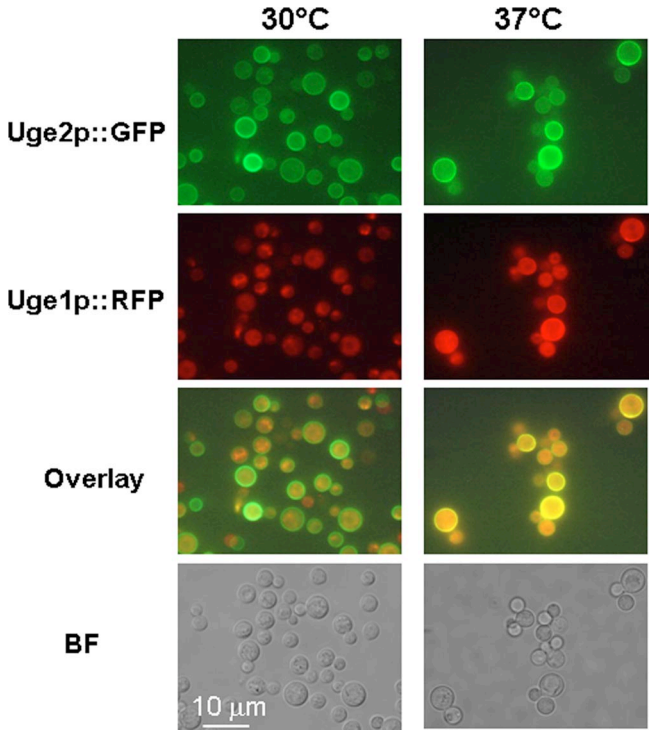

FIG. 9. Temperature-dependent colocalization of Uge proteins. Cells producing the fluorescence-tagged Uge proteins (strain NE537) were grown on galactose at 30°C and 37°C and examined by epifluorescence or by bright-field (BF) microscopy.

The presence of two UDP-glucose epimerases in *C. neoformans* is reminiscent of the situation in plants, in which five paralogous proteins are present (2). However, unlike in *C. neoformans*, all these proteins have particular functions in various carbohydrate biosynthesis pathways, and galactose tolerance parallels the total UDP-galactose-epimerase activity rather than being determined by the presence or absence of a particular *UGE* isoform (34). On the other hand, recent cytological data and transcriptional pattern analysis suggest that, like for the Uge proteins in *C. neoformans*, the function of each of the proteins might be dependent on the subcellular localization of the protein and on its transcriptional regulation during plant development (2, 34, 38).

Is this metabolic mechanism an adaptation for *C. neoformans* to live in the environment or in the host? In the host, *C. neoformans* may be subjected to the two signals sufficient to drive the translocation of Uge1p (i.e., a temperature of 37°C and the presence of galactose at the surface of the host cells), and therefore this metabolic regulation may be an adaptation to the host. Some recent observations seem to contradict this view. First, *Candida glabrata*, which is not present in the environment, has completely lost its galactose assimilation pathway (8) but can nevertheless survive and multiply in the host. Second, the deletion of the *Candida albicans GAL10* homologue renders the cells galactose sensitive but does not significantly reduce virulence as assessed by mouse studies (40). These

findings suggest that other carbon sources are available in the host and that the concentration of free galactose is very low.

In the environment, plant polysaccharides can be galactose rich and *C. neoformans* probably uses this source of carbon, but the temperature is rarely close to 37°C. However, 37°C is not the optimal growth temperature for *C. neoformans*. Indeed, this yeast grows much better at lower temperatures (unlike fungal pathogens not acquired from the environment, such as *Candida* species), and the pattern of gene regulation at 37°C seems to indicate that this temperature is stressful for *C. neoformans* (23). Thus, the signal that changes the function of Uge1p in the presence of galactose might not be specifically temperature related but might be associated with extreme environmental conditions. We looked at the effects of various other stresses (heavy metals, high osmolarity, and presence of oxidants) on the capacity of a *uge2*Δ strain to grow on galactose but could not identify any in vitro growth conditions that mimic the effect of temperature (data not shown). However, environmental conditions are most certainly much more complex than those in a petri dish in the laboratory, and *C. neoformans* has to cope with potential predators such as amoebae and worms and also with fluctuations of the temperature, humidity, and pH. It also has to find nutrients and adapt its metabolism to be able to use them (5). Thus, the environment should be considered to be a very stressful situation in which *C. neoformans* has to maintain both functional virulence factors (i.e., capsule and melanin) (41) and also the plasticity of its metabolism to be able to exploit as many carbon and nitrogen sources as possible.

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REFERENCES

1. **Anisimova, M., and O. Gascuel.** 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. **55:**539–552.
2. **Barber, C., J. Rösti, A. Rawat, K. Findlay, K. Roberts, and G. J. Seifert.** 2006. Distinct properties of the five UDP-D-glucose/UDP-D-galactose 4-epimerase isoforms of *Arabidopsis thaliana*. J. Biol. Chem. **281:**17276–17285.
3. **Bose, I., A. J. Reese, J. J. Ory, G. Janbon, and T. L. Doering.** 2003. A yeast under cover: the capsule of *Cryptococcus neoformans*. Eukaryot. Cell **2:**655–663.
4. **Casadevall, A., and J. R. Perfect.** 1998. *Cryptococcus neoformans.* ASM Press, Washington, DC.
5. **Casadevall, A., and L. A. Pirofski.** 2007. Accidental virulence, cryptic pathogenesis, Martians, lost hosts, and the pathogenicity of environmental microbes. Eukaryot. Cell **6:**2119–2174.
6. **Castresana, J., R. Guigó, and M. M. Albà.** 2004. Clustering of genes coding for DNA binding proteins in a region of atypical evolution of the human genome. J. Mol. Evol. **59:**72–79.
7. **Douglas, H. C., and D. C. Hawthorne.** 1964. Enzymatic expression and genetic linkage of genes controlling galactose utilization in *Saccharomyces*. Genetics **49:**837–844.
8. **Dujon, B., D. Sherman, G. Fisher, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. B. Barbe, S., S. Blanchin, J. M. Beckerich, Beyne, E., C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J. L. Souciet.** 2004. Genome evolution in yeasts. Nature **430:**35–44.
9. **Edgar, R. C.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **19:**1792–1797.
10. **Ethell, I. M., K. Hagihara, Y. Miura, F. Irie, and Y. Yamaguchi.** 2000. Synbindin, a novel syndecan-2-binding protein in neuronal dendritic spines. J. Cell Biol. **151:**53–68.
11. **Garcia-Hermoso, D., G. Janbon, and F. Dromer.** 1999. Epidemiological evidence for dormant *Cryptococcus neoformans* infection. J. Clin. Microbiol. **37:**3204–3209.
12. **Gari, E., L. Piedrafita, M. Aldea, and E. Herrero.** 1997. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. Yeast **13:**837–848.
13. **Gietz, R. D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **220:**1425.
14. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
15. **Hartl, L., C. P. Kubicek, and B. Seiboth.** 2007. Induction of the *gal* pathway and cellulase gene involves no transcriptional inducer function of the galactokinase in *Hypocrea jecorina*. J. Biol. Chem. **282:**18654–18659.
16. **Hittinger, C. T., A. Rokas, and S. B. Carroll.** 2004. Parallel inactivation of multiple *GAL* pathway genes and ecological diversification in yeasts. Proc. Natl. Acad. Sci. USA **101:**14144–14149.
17. **Holden, H. M., I. Rayment, and J. B. Thoden.** 2003. Structure and function of enzymes of the Leloir pathway for galactose metabolism. J. Biol. Chem. **278:**43885–43888.
18. **Hua, J. H., J. D. Meyer, and J. K. Lodge.** 2000. Development of positive markers for the fungal pathogen, *Cryptococcus neoformans*. Clin. Diagn. Lab Immunol. **7:**125–128.
19. **Hurst, L. D., C. Pal, and M. J. Lercher.** 2004. The evolutionary dynamics of eukaryotic gene order. Nat. Rev. Genet. **5:**299–310.
20. **Hurst, L. D., E. J. B. Williams, and C. Pal.** 2002. Natural selection promotes the conservation of linkage of co-expressed genes. Trends Genet. **18:**604–606.
21. **Janbon, G.** 2004. *Cryptococcus neoformans* capsule biosynthesis and regulation. FEMS Yeast Res. **4:**765–771.
22. **Janbon, G., U. Himmelreich, F. Moyrand, L. Improvisi, and F. Dromer.** 2001. Cas1p is a membrane protein necessary for the *O*-acetylation of the *Cryptococcus neoformans* capsular polysaccharide. Mol. Microbiol. **42:**453–469.
23. **Kraus, P. R., M. J. Boily, S. S. G. Giles, J. E. Stajich, A. Allen, G. M. Cox, F. S. Dietrich, J. R. Perfect, and J. Heitman.** 2004. Identification of *Cryptococcus neoformans* temperature-regulated genes with a genomic microarray. Eukaryot. Cell **3:**1249–1260.
24. **Loftus, B., E. Fung, P. Roncaglia, D. Rowley, P. Amedeo, D. Bruno, J. Vamathevan, M. Miranda, I. Anderson, J. A. Fraser, J. Allen, I. 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. Haas, J. C. Huang, G. Janbon, S. Jones, M. I. Krzywinski, K. J. Kwon-Chung, K. B. Lengeler, R. Maiti, M. Marra, R. E. Marra, C. Mathewson, T. G. Mitchell, M. Pertea, F. Riggs, S. L. Salzberg, J. Schein, A. Shvartsbeyn, H. Shin, C. Specht, B. Suh, A. Tenney, T. Utterback, B. L. Wickes, N. Wye, J. W. Kronstad, J. K. Lodge, J. Heitman, R. W. Davis, C. M. Fraser, and R. W. Hyman.** 2005. The genome and transcriptome of *Cryptococcus neoformans*, a basidiomycetous fungal pathogen of humans. Science **307:**1321–1324.
25. **Majumdar, S., J. Ghatak, S. Mukkerji, H. Bhattacharjee, and A. Bhaduri.** 2004. UDPgalactose 4-epimerase from *Saccharomyces cerevisiae*. Eur. J. Biochem. **271:**753–759.
26. **Marolda, C. L., and M. A. Valvano.** 1995. Genetic analysis of the dTDP-rhamnose biosynthesis region of the *Escherichia coli* VW187 (O7:K1) *rfb* gene cluster: identification of functional homologs of *rfbB* and *rfbA* in the *rff* cluster and correct location of the *rffE* gene. J. Bacteriol. **177:**5539–5546.
27. **Moyrand, F., Y. C. Chang, U. Himmelreich, K. J. Kwon-Chung, and G. Janbon.** 2004. Cas3p belongs to a seven member family of capsule structure designer proteins. Eukaryot. Cell **3:**1513–1524.
28. **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.
29. **Moyrand, F., and G. Janbon.** 2004. *UGD1* encoding the *Cryptococcus neoformans* UDP-glucose dehydrogenase is essential for growth at 37°C and for capsule biosynthesis. Eukaryot. Cell **3:**1601–1608.
30. **Nielsen, K., G. Cox, P. Wang, D. Toffaletti, J. Perfect, and J. Heitman.** 2003. Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic **a** and alpha isolates. Infect. Immun. **71:**4831–4841.
31. **Novelli, G., and J. K. V. Reichardt.** 2000. Molecular basis of disorders of human galactose metabolism: past, present, and future. Mol. Genet. Metab. **71:**62–65.

32. **Roberts, G. D., C. D. Horstmeier, G. A. Land, and J. H. Foxworth.** 1978. Rapid urea broth test for yeasts. J. Clin. Microbiol. **7:**584–588.
33. **Ross, K. L., C. N. Davis, and J. L. Fridovich-Keil.** 2004. Differential roles of the Leloir pathway enzymes and metabolites in defining galactose sensitivity in yeast. Mol. Genet. Metab. **83:**103–116.
34. **Rösti, J., C. J. Barton, S. Albrecht, P. Dupree, M. Pauly, K. Findlay, K. Roberts, and G. J. Seifert.** 2007. UDP-glucose 4-epimerase isoforms *UGE2* and *UGE4* cooperate in providing UDP-galactose for cell wall biosynthesis and growth of *Arabidopsis thaliana*. Plant Cell **19:**1565–1579.
35. **Sawardeker, J. S., J. H. Sloneker, and A. Jeanes.** 1965. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Biochem. **37:**1602–1604.
36. **Schulz, J. M., K. L. Ross, K. Malmstrom, M. Krieger, and J. L. Fridovich-Keil.** 2005. Mediators of galactose sensitivity in UDP-galactose 4′-epimerase-impaired mammalian cells. J. Biol. Chem. **280:**13493–13502.
37. **Seiboth, B., L. Karaffa, E. Sandor, and C. P. Kubicek.** 2002. The *Hypocrea jeronica gal10* (uridine 5′-diphosphate-glucose 4-epimerase-encoding) gene differs from yeast homologues in structure, genomic organization and expression. Gene **295:**143–149.
38. **Seifert, G. J., C. Barber, B. Wells, L. Dolan, and K. Roberts.** 2002. Galactose biosynthesis in Arabidopsis: genetic evidence for substrate channelling from UDP-D-galactose into cell wall polymers. Curr. Biol. **12:**1840–1845.
39. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **194:**3–21.
40. **Singh, V., S. V. Satheesh, M. L. Raghavendra, and P. P. Sadhale.** 2007. The key enzyme in galactose metabolism, UDP-galactose-4-epimerase, affects cell-wall integrity and morphology in *Candida albicans* even in the absence of galactose. Fungal Genet. Biol. **44:**563–574.
41. **Steenbergen, J. N., H. A. Shuman, and A. Casadevall.** 2001. *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. Proc. Natl. Acad. Sci. USA **98:**15245–15250.
42. **Toffaletti, D. L., T. H. Rude, S. A. Johnston, D. T. Durack, and J. R. Perfect.** 1993. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. J. Bacteriol. **175:**1405–1411.
43. **Vieira, C. P., J. Viera, and D. L. Hartl.** 1997. The evolution of small gene clusters: evidence for an independent origin of the maltase gene cluster in *Drosophila viridis* and *Drosophila melanogaste*r. Mol. Biol. Evol. **14:**985–993.
44. **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.
45. **Webster, T. D., and R. C. Dickson.** 1988. The organization and transcription of the galactose gene cluster of *Kluyveromyces lactis*. Nucleic Acids Res. **25:**8011–8028.
46. **Wilson, D. B., and D. S. Hogness.** 1964. The enzymes of the galactose operon in *Escherichia coli*. I. Purification and characterisation of the uridine diphosphogaclactose 4-epimerase. J. Biol. Chem. **239:**2469–2481.
47. **Wong, S., and K. H. Wolfe.** 2005. Birth of a metabolic gene cluster in yeast by adaptive gene relocation. Nat. Genet. **37:**777–782.