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The [URE3] Prion in *Candida*

Herman K. Edskes, Reed B. Wickner

Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA

Ure2p, normally a regulator of nitrogen catabolism in *Saccharomyces cerevisiae*, can be a prion (infectious protein) by forming a folded in-register parallel amyloid called [URE3]. Using *S. cerevisiae* as a test bed, we previously showed that Ure2p of *Candida albicans* (CaUre2p) can also form a prion, but that Ure2p of *C. glabrata* (CgUre2p) cannot. Here, we constructed *C. glabrata* strains to test whether CgUre2p can form a prion in its native environment. We find that while CaUre2p can form a [URE3] in *C. glabrata*, CgUre2p cannot, although the latter has a prion domain sequence more similar to that of ScUre2p than that of CaUre2p. This supports the notion that prion formation is not a conserved property of Ure2p but is a pathology arising sporadically. We find that some [URE3*albicans*] variants are restricted in their transmissibility to certain recipient strains. In addition, we show that the *C. glabrata* HO can induce switching of the *C. glabrata* mating type locus.

Ure2p of the yeast *Saccharomyces cerevisiae* (ScUre2p) is involved in nitrogen catabolite repression, the ability to differentiate between the presence of good and poor nitrogen sources in the growth environment (1, 2). On rare occasions Ure2p, normally a soluble dimer, forms amyloid filaments, polymers of Ure2p. Once formed, Ure2p amyloid is maintained in the cell and is transferred from mother to daughter and between cells during incomplete matings (cytoductions) (3–6). Thus, Ure2p can misfold, resulting in the formation of a prion—an infectious protein—named [URE3]. Proteins with homology to *S. cerevisiae* Ure2p are found in many ascomycete yeasts, but few have been functionally characterized.

ScUre2p has an N-terminal Q/N-rich domain that is important for the stability of the protein *in vivo* (7), but it comprises the part that converts to amyloid in the prion form of the protein (5, 8). Because this N-terminal domain is sufficient for prion propagation or for infection in the complete absence of the remainder of the molecule (4, 6, 9), it is called the prion domain. The C-terminal domain is sufficient, if overexpressed, for the nitrogen regulation function and is homologous to glutathione-S-transferases. In the normal form, the Ure2p prion domain is unstructured (10), and in forming amyloid it becomes a folded in-register parallel β-sheet (5, 11).

Many of the Ure2p homologs in ascomycete yeasts have an asparagine/glutamine-rich N-terminal domain and a glutathione *S*-transferase-like C-terminal domain (12, 13). Recently, we showed that the Ure2p homologs of the human-pathogenic yeasts *Candida albicans* and *Candida glabrata* can functionally replace ScUre2p and in their native environment regulate a similar set of genes. In addition, we showed that CaUre2p can form a prion in *S. cerevisiae* with a similarly folded in-register parallel β-sheet architecture but that CgUre2p cannot (14, 15).

Because of the tools available for *S. cerevisiae*, few tests of non-*S. cerevisiae* prion candidates have been made in their native hosts. Sup35p is a subunit of the translation termination factor, and ScSup35p rarely can form a prion called [PSI+] (3). Sup35p of the ascomycete yeasts *Pichia methanolica*, *Candida albicans*, and *Kluyveromyces lactis* have been shown to be capable of prion formation, but in each case, *S. cerevisiae* was used as the host (16–19). Nakayashiki et al. have presented evidence that *K. lactis* Sup35p forms [PSI+] in *K. lactis* itself as well as in *S. cerevisiae* (19). In contrast, Ure2p of *K. lactis* does not form [URE3] in *K. lactis* (20) or in *S. cerevisiae* (13). *S. paradoxus* strains for the study of [URE3] have been developed (21), and in agreement with work in the *S. cerevisiae* host (12), *S. paradoxus* Ure2p can propagate [URE3] in its own environment (14). In the only three cases examined, results in *S. cerevisiae* are consistent with those in the native host, but the myriad of chaperones and other factors affecting prion propagation (reviewed in reference 22) suggest that there could be differences between the native host and *S. cerevisiae.*

Here, we constructed *C. glabrata* strains with the Ure2p-regulated *DUR3* promoter controlling *ADE2* and showed that CaUre2p can form a [URE3] prion in such cells, but that CgUre2p cannot, even though it is in its native environment. This supports our view that the prion-forming ability of Ure2p is not conserved but rather occurs sporadically among yeast species.

MATERIALS AND METHODS

***C. glabrata* strains for assaying Ure2p function.** We set up a Ure2p activity system in *C. glabrata* based on a similar system developed by Schlumpberger et al. (23). *C. glabrata* does not contain a *DAL5* gene, which is often used in *S. cerevisiae* to study nitrogen regulation and to monitor the presence of the [URE3] prion. However, *C. glabrata* Ure2p (CgUre2p) represses Cg*DUR3* transcription (14), so the Cg*DUR3* promoter was fused to Cg*ADE2* in *C. glabrata* to measure Ure2p activity. Yeasts were transformed by the Li acetate method (24), and media were as previously described (14).

The parental strain was BG88b [*MAT***a** *ura3*::*G418 his3*::*ura3*(5FOA)] (25). Cg*ADE2* was replaced with *FRT-HIS3-FRT*, producing strain HCg8, and then Cg*HIS3* was deleted by recombination, making strain HCg12 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *ade2*::*FRT*]. Specifically, the Cg*ADE2* 5′ untranslated region (UTR) (nucleotides [nt] −510 to +20 relative to the start ATG of *ADE2*) was amplified from BG88b using primers HE776 and HE782. The Cg*ADE2* 3′ UTR (nt 1714 to 2152 relative to the start ATG; the *ADE2* open reading frame [ORF] ends at nt 1713) was amplified from BG88b using primers HE780 and HE781. Cg*HIS3* flanked

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Address correspondence to Reed B. Wickner, wickner@helix.nih.gov.
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by FRT sites was amplified from strain 37A (26) using primers HE783 and HE779. Cg*HIS3* starts 514 bp upstream of the start codon and terminates at the stop codon. This HIS3 fragment is flanked by FRT sites (GAAGTT CCTATACTTTCTAGAGAATAGGAACTTC). Flanking this PCR product are nt 3 to 20 and nt 1714 to 1737 of *ADE2* (*ADE2* stops at 1713). The 3 PCR products were fused using oligonucleotides HE776 and HE781, and the resulting fusion product was transformed into BG88b, selecting for His$^+$ colonies. His$^+$ colonies unable to grow without adenine were identified.

Cg*HIS3* was deleted from the HCg8 *ADE2* locus by expression of the Flp recombinase using plasmid pRD16 (27), forming strain HCg12. HCg12 could not grow on medium without adenine and formed red colonies on medium containing 30 mg/liter adenine. Thus, the *ade2* mutant phenotype in *C. glabrata* is similar to that in *S. cerevisiae*, suggesting that Cg*ADE2* is useful as a marker in *C. glabrata* to assay for Ure2p activity.

Cg*DUR3* (CAGL0K03157g), the homologue of *S. cerevisiae YHL016c*, is a plasma membrane transporter for both urea and polyamines (28, 29). *DUR3* expression is derepressed more than 100-fold upon deletion of *URE2* in *C. glabrata*. Cg*GAP1* (CAGL0L03267g), the homologue of *S. cerevisiae* (Sc*YKR039w*) encoding the general amino acid permease, is the only other gene derepressed 100-fold, but Cg*GAP1* has a higher basal expression level than Cg*DUR3* (14).

HCg16. For the strain HCg16 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *dur3:ADE2-FRT-HIS3-FRT*] (the single colon refers to a promoter-ORF fusion) the Cg*DUR3* 5′ UTR (nt −685 to −1 of *DUR3*) was amplified from BG88b with a 5′ NotI site and a 3′ PstI site using oligonucleotides HE784 and HE788. The Cg*ADE2* ORF was amplified from BG88b with a 5′ PstI site and a 3′ HindIII site using oligonucleotides HE786 and HE787. Cg*HIS3* flanked by *FRT* sites and containing a 5′ HindIII site and a 3′ SalI site was amplified from strain 37A using oligonucleotides HE789 and HE790. The Cg*DUR3* 3′ UTR (nt 2153 to 2728 of *DUR3*; *DUR3* ends at nt 3175) was amplified from BG88b with a 5′ SalI site and a 3′ ApaI site using oligonucleotides HE791 and HE792. PCR products were cloned into pBC KS+ (Agilent Technologies) in the following order: *DUR3* 5′ UTR→*ADE2* ORF→*FRT-HIS3-FRT*→*DUR3* 3′ UTR, creating pH1104. All PCR products were checked by sequencing. The cassette was released from pH1104 by digestion with NotI and XhoI (which cleaves in the 3′ UTR of DUR3 at nt 2696) and transformed into BG88b, selecting for cells capable of growing without histidine, producing HCg16.

HCg18. For the HCg18 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *dur3: ADE2-FRT*] strain, the Cg*HIS3* marker was removed from HCg16 through Flp-mediated recombination using plasmid pRD16 (as described for HCg12).

HCg19 and HCg20. For the HCg19 and HCg20 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *dur3:ADE2-FRT ade2*::*FRT-HIS3-FRT*] strains, the Cg*ADE2* ORF was replaced with *FRT-HIS3-FRT* as described for HCg12. Transformants were selected on plates lacking histidine and containing either arginine (1 g/liter) or urea (10 mM) as a nitrogen source (arginine is converted to urea and ornithine). Although the *DUR3* urea permease is disrupted, HCg18 can still grow on plates containing urea as the sole nitrogen source, although very slowly. Growth on medium containing urea as the sole nitrogen source should activate the *DUR3* promoter and result in the production of adenine.

HCg23 and HCg25. For HCg23 and HCg25 [*MAT***a** *ura3*::*G418 his3*:: *ura3*(*5FOA*) *dur3:ADE2-FRT ade2*::*FRT*], the *HIS3* marker was removed from HCg19 (creating HCg23) and from HCg20 (creating HCg25) through Flp-mediated recombination using plasmid pRD16.

Deleting Cg*URE2* to create HCg27. For HCg27 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *dur3:ADE2-FRT ade2*::*FRT ure2*::*FRT-HIS3-FRT*], the Cg*URE2* 5′ UTR (nt −501 to −1 of *URE2*) was amplified from BG88b with a 5′ NotI site and a 3′ BamHI site using oligonucleotides HE815 and HE816. Cg*HIS3* flanked by *FRT* sites and containing a 5′ BamHI site and a 3′ PstI site was amplified from strain 37A using oligonucleotides HE819 and HE820. The Cg*URE2* 3′ UTR (nt 1074 to 1565 of *URE2*; the *URE2* ORF ends at nt 1068) was amplified from BG88b with a 5′ XhoI site and a 3′ ApaI site using oligonucleotides HE821 and HE822. The PCR products were cloned into pBC KS+ (Agilent Technologies) in the following order: *URE2* 5′ UTR→*FRT-HIS3-FRT*→*URE2* 3′ UTR, creating pH1111. All PCR products were checked by sequencing. The cassette was released from pH1111 by digestion with NotI and ApaI and transformed into HCg23, selecting for cells capable of growing without histidine.

HCg29. For HCg29 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *dur3:ADE2-FRT ade2*::*FRT ure2*::*FRT*], the *HIS3* marker was removed from HCg27 through Flp-mediated recombination using plasmid pRD16.

Replacing the Cg*URE2* ORF with the Ca*URE2* ORF. For HCg30 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *dur3:ADE2-FRT ade2*::*FRT* Cg*ure2:* Ca*URE2*–*FRT-HIS3-FRT*], the Ca*URE2* ORF from the Darlington strain (30) was amplified with a 5′ BamHI site and a 3′ PstI site using oligonucleotides HE817 and HE818. This Ca*URE2* PCR product was cloned between the Ca*URE2* 5′ UTR and *FRT-HIS3* in pH1111 (see HCg27), creating pH1113. The cassette was released by digestion with NotI and ApaI and transformed into HCg29, selecting for cells capable of growing without histidine.

HCg32. For HCg32 [*MAT***a** *ura3*::*G418 his3*::*ura3*(*5FOA*) *dur3*:*ADE2-FRT ade2*::*FRT ure2:URE2*(*C. albicans*)*-FRT*], the *HIS3* marker was removed from HCg30 through Flp-mediated recombination using plasmid pRD16.

***C. glabrata* expression plasmids.** The centromeric *S. cerevisiae* plasmid pRS316 (31) is capable of replicating in *C. glabrata* (32, 33) with a copy number of 10 to 30. The *ADH1* promoter cassette was amplified from pVT103 (34) (using oligonucleotides HE66 and HE67) and cloned in the PvuII window of pRS316, replacing the multiple cloning region. In the resulting plasmid, pH130, the *ADH1* and *URA3* promoters are facing each other. By site-directed mutagenesis using oligonucleotide HE127, a PstI site was removed from *URA3*, resulting in pH393.

No inducible promoter system is available in *C. glabrata*. The *TPI1* constitutive strong promoter (nt −959 to −1 of *TPI1*; CAGL0H08327g, triosephosphate isomerase) was amplified from *C. glabrata* CBS138 genomic DNA using oligonucleotides HE892 and HE893. The Sc*ADH1* promoter from pH393 was replaced with the Cg*TPI1* promoter using flanking NheI and BamHI sites, resulting in pH1232. In order to check if pH1232 could be used as an expression vector in *C. glabrata*, green fluorescent protein (GFP) was inserted behind the promoter as a BamHI-XhoI fragment from pH199 (35), resulting in plasmid pH1251. Transformation of this plasmid into *C. glabrata* BG88b resulted in very bright GFP fluorescence in all of the cells, while the vector pRS316 showed only very faint background fluorescence. Thus, pH1232 can be used as an expression plasmid in *C. glabrata*.

Cg*URE2* was amplified from BG88b with primers HE297 and HE299, introducing a BamHI site upstream of the start codon and an XhoI site downstream of the stop codon, and inserted as a BamHI/XhoI fragment into expression vector pH1232, resulting in pH1259. Ca*URE2* was transferred as a BamHI/XhoI fragment from pH563 (12) into pH1232, resulting in pH1258. Cg*URE2* lacking the 5′ prion domain (starting at nt 292; M98) was amplified with primers HE941 and HE299 and cloned as a BamHI/XhoI fragment into pH1232, resulting in pH1274. Ca*URE2* lacking the 5′ prion domain (starting at nt 262; Q88) was amplified with primers HE942 and HE188 and cloned as a BamHI/XhoI fragment into pH1232, resulting in pH1272.

[*URE3*] induction constructs. The centromeric *S. cerevisiae* plasmid pRS313 (31) containing *HIS3* was modified by site-directed mutagenesis to remove 2 BglII sites (oligonucleotides HE123 and HE124), 1 PstI site (oligonucleotide HE126), 1 NheI site (oligonucleotide HE125), and 2 HindIII sites (oligonucleotides HE122 and HE125), resulting in pH339. An *ADH1* promoter cassette was amplified from pVT103 (34) using oligonucleotides HE66 and HE67 and cloned in the PvuII window of pH339, replacing the multiple cloning region. In the resulting plasmid, pH403, the *ADH1* and *HIS3* promoters are facing each other.

The NheI/BamHI-bordered *ADH1* promoter in pH403 was replaced with the Cg*TPI1* promoter from pH1232, creating pH1286. The Cg*TPI1*

promoter and a fragment of Cg*URE2* containing the first 100 amino acids was transferred as an NheI/XhoI fragment into pH403, creating pH1287. The Cg*TPI1* promoter and a fragment of Ca*URE2* containing the first 89 amino acids was transferred as an NheI/XhoI fragment into pH403, creating pH1288.

Switching *C. glabrata* mating type loci. The *HO* ORF (CAGL0G05423) was amplified from *C. glabrata* BG88b using oligonucleotides HE894 and HE895 and cloned as a BamHI/XhoI fragment into pH1232, resulting in pH1254. pH1254 was transformed into HCg25, and transformants capable of growing without uracil were streaked as single colonies on YPAD (14). Nearly all of the YPAD colonies had lost plasmid pH1254. The *MAT* locus was scored using two sets of primers described by Butler et al. (36), as well as BG88b (*MAT**a***) and CBS138 (*MAT*α) as controls. Among 20 clones in which *HO* had been expressed using pH1254, four isolates were identified that had become *MAT*α. The same procedure, starting with strain HCg32, produced 7 *MAT*α clones of 20 examined. Thus, Cg*HO* has the ability to change the mating type locus. HCg25 *MAT*α was named HCg58.

The 5′ UTR of Cg*LEU2* (CAGL0H03795g; nt −399 to +17 of *LEU2*) was amplified from BG88b using oligonucleotides HE831 and HE833 with a 5′ BamHI site and a 3′ PstI site. Cg*HIS3*, flanked by FRT sites and containing a 5′ BamHI site and a 3′ PstI site, was amplified from strain 37A using oligonucleotides HE819 and HE820 and cloned into pBC KS+, creating pH1107. The 3′ UTR of Cg*LEU2* (nt 1099 to 1511 of *LEU2* with the *LEU2* ORF terminating at nt 1098) was amplified with oligonucleotides HE834 and HE832 with a 5′ XhoI site and a 3′ ApaI site. PCR products were cloned into pBS KS+ (Agilent Technologies) in the order LEU2 5′ UTR→FRT-HIS3-FRT→LEU2 3′ UTR, creating pH1285. The cassette was released from pH1285 by digestion with BamHI and ApaI and transformed into HCg58, selecting for cells capable of growing without histidine, producing the *leu2 C. glabrata* strain HCg62.

Transfection of [URE3albicans] from *C. glabrata* extracts into *S. cerevisiae* cells. Six Ade$^+$ *C. glabrata* isolates expressing CaUre2p (HCg32+pH1258+pH1288) were grown in SD medium to saturation, diluted into 50 ml YPAD, and grown for 2 to 3 doublings. As a positive control, *S. cerevisiae* YHE1161 (BY302 [URE3] (*MAT***a** *his3 leu2 trp1* Ca*URE2* P_{DAL5}:*ADE2* P_{DAL5}:*CAN1 kar1* [14]) was used, which propagates [URE3] based on CaUre2p (15). Cells were collected by centrifugation and washed 2 times with water, resuspended in 600 μl of water, and disrupted in a BioSpec Mini-Beadbeater-8. Lysates were cleared by 5 min of centrifugation at 4°C and sonicated with 3 cycles in a Branson sonifier 250 equipped with a microtip (45 s, 20% duty cycle, output 4).

S. cerevisiae BY302 (14) was used as the recipient for the protein extract. Cells were grown to early log phase (optical density at 600 nm [OD_{600}] of 0.8) in YPAD, washed once with water and 2 times with ST buffer (1 M sorbitol, 10 mM Tris-HCl, pH7.4), and resuspended in 5 ml ST buffer. Cells were protoplasted by adding 100 U lyticase (L2524; Sigma) and incubating them for 40 min at 30°C. Protoplasts were collected by centrifugation at 250 × *g*, washed twice in STC buffer (1 M sorbitol, 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$), and resuspended in 1 ml STC buffer. Protoplasts (100 μl) were mixed with 1 μl single-stranded DNA (ssDNA; 10 μg/μl; SPB1136; Open Biosystems), 4.6 μg *LEU2* plasmid pRS425 (37), and 9 μl sonicated protein extract. The mixture was incubated for 10 min at room temperature. PTC buffer (900 μl; 20% [wt/vol] polyethylene glycol 8000, 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$) was added, and incubation was continued at room temperature for 20 min. Protoplasts were collected by centrifugation at 400 × *g*, resuspended in 200 μl SOS buffer (1 M sorbitol, 7 mM $CaCl_2$, 1/3 yeast extract-peptone-dextrose [YPD]), allowed to recover for 30 min at 30°C, mixed with 10 ml CS+A5 medium (1 M sorbitol, leucine dropout medium containing 5 mg/liter adenine) at 50°C, and spread on plates containing 20 ml of the same medium. Plates were placed in a 30°C incubator for 6 days.

Cytoductions. A cytoduction recipient isogenic to BY302 was constructed with the opposite mating type and different markers. *TRP1* was amplified from *S. cerevisiae* S288c using oligonucleotides HE899 and HE900, and BY302 cells that had become Ade$^+$ after receiving protein extracts of HCg32 expressing Ca*URE2* and Ca*URE2N* were restored to Trp$^+$ by transformation with the PCR product. The mating type of BY302 was changed from *MAT***a** to *MAT*α by transient overexpression of *HO* from plasmid pJH298 (*CEN4 LEU2 GAL10*::*HO*; kindly provided by James Haber), resulting in YHE1355. *HIS3* was amplified from S288c using oligonucleotides HE950 and HE951 and used to make YHE1355 His$^+$, resulting in YHE1362. Subsequent growth on YPAD with ethidium bromide resulted in the [*rho*^{0}] strain YHE1364. *TRP1* amplified from *S. cerevisiae* as described above also was used to transform YHE1160, YHE1161, YHE1162, and YHE1170 to the corresponding Trp$^+$ strains YHE1345, YHE1346, YHE1347, and YHE1348, respectively.

RESULTS

Ure2p activity in *C. glabrata*. *Candida glabrata* strains HCg25 (Cg*URE2*), HCg32 (Ca*URE2*; genomic replacement), and HCg29 (*ure2*Δ) have the Ure2p-regulated *DUR3* promoter fused to *ADE2*, making growth on medium without adenine a measure of Ure2p activity (Table 1). We tested nitrogen sources for Ure2p activation (making cells Ade$^-$) (Fig. 1). The *ure2*Δ strain HCg29 grew equally well on all nitrogen sources, producing single colonies within 2 days, except on media containing urea, where the absence of the Dur3 urea permease slowed growth.

Ammonium, glutamine, asparagine, urea, or the combination of ammonium and glutamate activated *C. glabrata* Ure2p, but arginine, proline, or glutamate did not, a pattern similar to that observed in *S. cerevisiae*. CaUre2p showed the same pattern, except that urea did not activate CaUre2p and there was a bit more growth on ammonium and glutamine medium than for CgUre2p. When plates were incubated beyond the 2 days used to assess the effects of the various nitrogen sources described above, all strains formed single colonies on all media tested. This indicates that background expression of the *DUR3* promoter in *C. glabrata* is higher than that of the promoter routinely used in *S. cerevisiae*, *DAL5*. It is nevertheless remarkable that Ca*URE2* functions so well in both *S. cerevisiae* and *C. glabrata* given the limited homology between the Gln3 proteins of these three organisms and the fact that Ure2p largely acts through Gln3p in *S. cerevisiae* (1, 38). Homology is limited to the zinc finger domain in the middle of the protein and the last 9 amino acids (14). However, it is not known whether CgUre2p or CaUre2p acts largely through Gln3p in their native species.

Expressing CgUre2p or CaUre2p from plasmids in the *ure2*Δ strain HCg29 resulted in a tight Ade$^-$ phenotype on medium containing asparagine plus glutamine as nitrogen sources (1 g/liter each) for some transformants, but a substantial number of transformants were Ade$^+$ on the same medium.

[URE3] induction in *C. glabrata*. Because strains HCg25 (Cg*URE2*) and HCg32 (Ca*URE2*) with genomic *URE2* genes grew without adenine on all media tested (including the combination of asparagine and glutamine) when incubated for more than 2 days, we combined overexpression of each full-length Ure2p from plasmids with chromosomal expression, resulting in a tight Ade$^-$ phenotype and overexpressed N-terminal fragments (known to be the prion domain in the case of CaUre2p) in attempts to induce prion formation. Essentially all double transformants were Ade$^-$ in the presence of ammonium or asparagine plus glutamine even after 5 days of incubation. This was sufficient to allow selection of rare Ade$^+$ putative [URE3] clones (Table 2).

Confirming [URE3] generation in *C. glabrata*. (i) Mating in *C. glabrata*. If the *C. glabrata* Ade$^+$ colonies generated in strain

TABLE 1 Yeast strains and plasmids

Strain	Description	Source or reference
Saccharomyces cerevisiae		
BY302	*MAT***a** *his3 leu2 trp1* Ca*URE2* P_{DAL5}:*ADE2* P_{DAL5}:*CAN1 kar1*	14
YHE1160	BY302 [URE3*albicans*1160]	14
YHE1161	BY302 [URE3*albicans*1161]	14
YHE1162	BY302 [URE3*albicans*1162]	14
YHE1170	BY302 [URE3*albicans*1170]	14
YHE1181	*MAT*α *ura2 leu2 kar1* Ca*URE2* P_{DAL5}:*ADE2* P_{DAL5}:*CAN1* [rho^0]	This work
YHE1364	BY302 changed to *MAT*α *HIS3* [rho^0]	This work
Candida glabrata		
BG88b	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA)*	25
HCg8	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) ade2*::*FRT-HIS3-FRT*	This work
37A	Clinical isolate	26
HCg12	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) ade2*::*FRT*	This work
HCg16	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT-HIS3-FRT*	This work
HCg18	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT*	This work
HCg19, HCg20	*ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT ade2*::*FRT-HIS3-FRT*	This work
HCg23, HCg25	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT ade2*::*FRT*	This work
HCg27	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT ade2*::*FRT ure2*::*FRT-HIS3-FRT*	This work
HCg29	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT ade2*::*FRT ure2*::*FRT*	This work
HCg30	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT ade2*::*FRT ure2:*Ca*URE2–FRT-HIS3-FRT*	This work
HCg32	*MAT***a** *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT ade2*::*FRT ure2:*Ca*URE2-FRT*	This work
HCg62	*MAT*α *ura3*::*G418 his3*::*ura3(5FOA) dur3:ADE2-FRT ade2*::*FRT leu2*::*FRT-HIS3-FRT*	This work
Plasmids		
pRS316	*CEN ARS URA3* A^r	31
pVT103	*ADH1* promoter with multiple cloning site	34
pH1232	pRS316 with *ADH1* promoter, MCS from VT103 *CEN ARS URA3* A^r *ADH1* promoter	This work
pH1259	pH1232 with Cg*URE2*	This work
pH1258	pH1232 with Ca*URE2*	This work
pH1274	pH1232 with Cg*URE2N*Δ (lacks aa 1–97)	This work
pH1272	pH1232 with Ca*URE2N*Δ (lacks aa 1–87)	This work
pH1286	*CEN ARS HIS3* Cg*TPI1* promoter	This work
pH1287	*CEN ARS HIS3* Cg*TPI1* promoter Cg*URE2N* (aa 1–100)	This work
pH1288	*CEN ARS HIS3 TPI1* promoter Ca*URE2* (aa 1–89)	This work
pH1254	*CEN ARS URA3* A^r Cg*TPI1* promoter Cg*HO*	This work

HCg32 (Ca*URE2*) are indeed [URE3], the Ade$^+$ phenotype has to be transferable. However, no mating has been observed in *C. glabrata* (39). Three mating type-like loci, similar to those found in *S. cerevisiae*, are present in *C. glabrata* (40). Furthermore, *C. glabrata* has an *HO* endonuclease which is essential in *S. cerevisiae* for mating type switching (36). No mating type switching has been observed in *C. glabrata* grown in culture (41), but both *MAT***a** and *MAT*α cells are found in the same clade, suggesting the occurrence of mating type switching and mating in the wild (42).

Overexpression of Cg*HO* changed the mating type locus of HCg25 (or HCg32) from *MAT***a** to *MAT*α, indicating that Cg*HO* is active. HCg25 (*MAT***a** *his3*) was crossed with the switched strain, HCg62 (*MAT*α *leu2*), on plates containing various nitrogen sources or on plates containing acetate as a carbon source (*S. cerevisiae* sporulation medium). Plates were incubated at several temperatures. Single colonies emerged on plates containing glutamate as the sole nitrogen source that had been placed for 4 days at 37°C and then left at room temperature for 3 weeks. These colonies propagated when transferred to a fresh plate or to a plate containing ammonium as the nitrogen source, although the growth rate remained similarly low. However, when streaked on YPAD, all single colonies showed only the parental marker combinations. Thus, no mating was observed, indicating that *C. glabrata* cannot be used for prion infectivity studies.

(ii) Curing of the Ade$^+$ phenotype. *C. glabrata* grows well on YPAD with 120 mM guanidine HCl (GuHCl), but no curing of the Ade$^+$ phenotype was observed. A few colonies showed white to red sectoring, but these had to be purified several times on YPAD with 120 mM GuHCl in order to obtain Ade$^-$ colonies. Thus, guanidine curing does not seem to work in *C. glabrata*. It is unknown if CgHsp104p is sensitive to guanidine inhibition. CaHsp104 protein cannot be inhibited by guanidine (43).

(iii) Protein transformation into *S. cerevisiae*. When spheroplasts of *S. cerevisiae* are exposed to ScUre2p amyloid or to a protein extract from a [URE3] cell, the recipient cells can acquire the prion (6, 44). [URE3] based on CaUre2p but propagating in *S. cerevisiae* can be transferred to recipient *S. cerevisiae* cells by cytoduction or by exposure to a protein extract from infected cells (14, 15). If *C. glabrata* propagates [URE3], based on CaUre2p the prion should be transferable from a protein extract of these cells into recipient *S. cerevisiae* similarly expressing CaUre2p. Using the genetic tools available for *S. cerevisiae*, the prion then can be characterized.

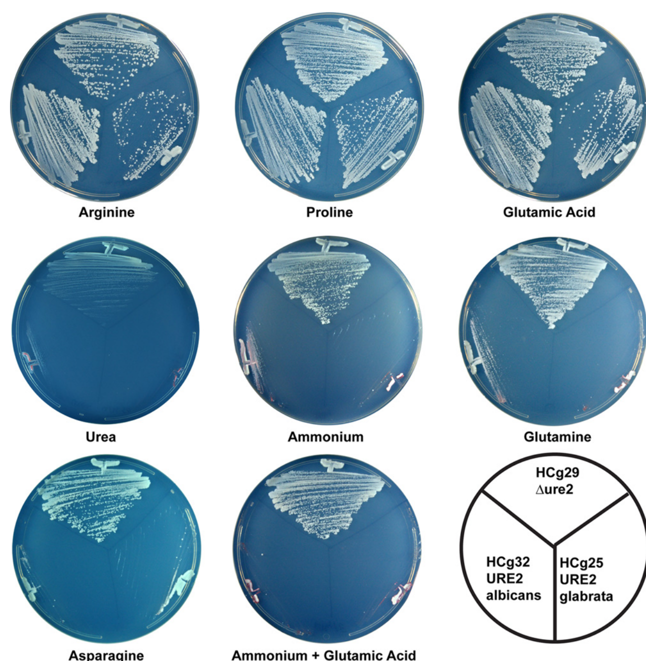

FIG 1 Nitrogen sources that activate Ure2p and repress *DUR3:ADE2*. Amino acids were tested at 1 g/liter, ammonium as present in yeast nitrogen base, and urea at 10 mM. Medium containing 1 g/liter glutamic acid was adjusted to pH 5.6. Growth was examined after 2 days at 30°C. At later times, all strains grew on all nitrogen sources.

TABLE 3 Infection of *S. cerevisiae* with [URE3*albicans*] from extracts of *C. glabrata* strains[a]

Protein extract source	Ade^+ ScBY302 of 48 tested	Ade^+ clone no.	No. Ade^+/color on YES after growth on:	
			YPAD	YPAD + 3 mM guanidine
YHE1161	39			
		19	12/white	0/red
		20	12/white	0/red
		21	12/white	0/red
		22	12/white	0/red
H_2O	0			
HCg32				
1	8			
		11	11/red	0/red
		12	12/red	0/red
		13	12/red	0/red
		14	12/red	0/red
		15	12/red	0/red
		16	12/red	0/red
		17	12/red	0/red
		18	12/red	0/red
2	4			
		8	10/red	0/red
		9	10/red	0/red
		10	7/white	7/white
3	0			
4	0			
5	1			
		7	12/white	12/white
6	6			
		1	12/red	0/red
		2	12/red	0/red
		4	12/red	0/red
		5	12/red	0/red
		6	12/red	0/red

[a] BY302 cells were transformed with the indicated extract and the *LEU2* plasmid pRS425 and plated on −Leu plates containing 5 mg/liter adenine. Forty-eight transformant colonies from each plate were picked and tested for growth on SD+H+W (−Ade −Leu). The number of colonies growing on the indicated plates is shown. The indicated Ade^+ clones were then streaked for single colonies on YPAD or YPAD with 3 mM guanidine, and 12 were tested from each plate for growth on −Ade medium (SD+H+W+L) and color on YES. The number of Ade^+ clones and their color on YES is shown.

Extracts of six Ade^+ *C. glabrata* clones (HCg32+pH1258+pH1288) expressing CaUre2p were transformed into spheroplasts of *S. cerevisiae* BY302 (Ca*URE2*), selecting first for clones which had been transformed with a *LEU2* plasmid included in the transformation mix and then checking Leu^+ clones for Ade^+, a sign that the same cell had been infected with the [URE3*albicans*] prion (Table 3). Since prion-infected cells should be guanidine curable, BY302 cells that received protein extracts from *C. glabrata* HCg32 colonies 1, 2, and 6 appear to contain [URE3*albicans*] (Table 3). This indicates that [URE3*albicans*] was present in these *C. glabrata* clones. All of the [URE3*albicans*] variants are weak, as colonies turn red on adenine-limiting (YES+W) medium or on minimal medium with no adenine (SD+H+W+L).

(iv) Cytoduction. A suitable cytoduction recipient was constructed from BY302 by changing its mating type and markers (see Materials and Methods). Each of several *S. cerevisiae* strains expressing CaUre2p and transformed to guanidine-curable Ade^+ by extracts of *C. glabrata* (Ca*URE2*) (Ade^+ clones 1, 2, and 6) could transmit [URE3*albicans*] by cytoplasmic mixing to the constructed recipient (Table 4).

Thus, transformation of a protein extract from *C. glabrata* cells selected to be Ade^+ into *S. cerevisiae* resulted in the recipient cells

TABLE 2 Induction of [URE3] in *Candida glabrata*[a]

Strain	*URA3* plasmid	*HIS3* plasmid	No. $Ade^+/10^6$ cells in expt:				
			1	2	3	4	5
HCg25 Cg*URE2*	Cg*URE2* (pH1259)	Vector (pH1286)	<1	<1	<1	<1	<1
	Cg*URE2* (pH1259)	Cg*URE2* aa 1–100 (pH1287)	<1	<1	<1	<1	<1
HCg32 *URE2albicans*	Ca*URE2* (pH1258)	Vector (pH1286)	<1	<1	12	1	1
	Ca*URE2* (pH1258)	Ca*URE2* aa 1–89 (pH1288)	36	15	30	24	95

[a] Cells were grown on medium without adenine, adjusted to pH 5.6, and contained both ammonium and glutamate as nitrogen sources. Colonies were counted after 5 days at 30°C. No histidine was added, as this seemed to increase the background.

TABLE 4 Cytoduction tests of *S. cerevisiae* transformed with extracts of *C. glabrata* Ade$^+$ clones[a]

Protein extract	*S. cerevisiae* BY302 Ade$^+$ clone no. and color on YES	No. of Ade$^+$ cytoductants/total
YHE1161	19; white	28/28
	20; white	30/30
HCg32		
1	11; red	26/28
	12; red	18/18
2	8; red	2/44
	9; red	27/43
6	1; red	41/42
	2; red	26/26

[a] The cytoduction recipient was YHE1364, isogenic with BY302, but with different markers. Cytoductants were tested for ability to grow without adenine. Numbers are expressed as no. of Ade$^+$ cytoductants out of the total number of cytoductants assayed.

becoming Ade$^+$. The Ade$^+$ phenotype was curable by growth on YPAD containing 3 mM guanidine HCl and was transferrable by cytoduction. This indicates that the Ade$^+$ phenotype in the recipient *S. cerevisiae* cells was due to the presence of [URE3*albicans*]. We conclude that the *C. glabrata* donor cells contain [URE3*albicans*].

Transmission variants of [URE3*albicans*]. Transient overexpression of CaUre2p in a *S. cerevisiae* strain in which Sc*URE2* has been replaced with Ca*URE2* results in an increase of cells containing [URE3*albicans*] (14). Each of four [URE3*albicans*]-containing isolates from strain BY302 (*MAT***a** Ca*URE2* P_{DAL5}:*ADE2 kar1*) could be cured of [URE3*albicans*] by growth on medium containing 3 mM guanidine hydrochloride (14), and, when crossed with strain YHE1181 (*MAT*α Ca*URE2* P_{DAL5}:*ADE2* [*rho*°]), diploids remained capable of propagating [URE3]. However, whereas [URE3*albicans*] was readily cytoduced from two of the [URE3] isolates, YHE1161 and YHE1162, into strain YHE1181, no [URE3*albicans*] cytoductants could be identified when the other two isolates, YHE1160 and YHE1170, were used as donors (Table 5).

Strains YHE1160 and YHE1170 were cured of [URE3*albicans*] by growth on guanidine, and [URE3*albicans*] originating in YHE1161 or YHE1162 was introduced into each by cytoduction (see Table S2 in the supplemental material). Those cytoductants could readily transfer [URE3*albicans*1161] or [URE3*albicans*1162] by cytoduction to YHE1181 (see Table S3, middle column). Thus, there is a difference in the [URE3*albicans*] variants between YHE1161 and YHE1162, which can transfer their prion to YHE1181, and YHE1160 and YHE1170, which cannot.

TABLE 5 Variants of [URE3*albicans*] differ[a]

[URE3*albicans*] donor	[ure-o] ρ^0 recipient	Cytoductants		Diploids		No. of Ade$^+$ mated cytoductants
		% Ade$^+$	*n*	% Ade$^+$	*n*	
YHE1160	YHE1181	0	86	35	48	0/86
YHE1161	YHE1181	100	88	72	47	86/88
YHE1162	YHE1181	100	89	54	48	89/89
YHE1170	YHE1181	0	91	38	38	0/91

[a] Cells were tested for the presence of [URE3*albicans*] by growth on adenine dropout media. When SD medium was used, lacking adenine and only containing the amino acids needed for growth, the same result was obtained. Cytoductants were mated to strain YHE1186 (BY302 [*rho*^{0}]), and the diploid phenotype was tested similarly.

TABLE 6 All [URE3*albicans*] variants are transmitted to a recipient isogenic to donors[a]

[URE3*alb*] donor	Recipient	No. of cytoductants growing on indicated medium/total	
		SD+U+L	−Ade
YHE1160 *TRP*$^+$	YHE1181	3/26	1/26
YHE1161 *TRP*$^+$	YHE1181	48/48	48/48
YHE1162 *TRP*$^+$	YHE1181	48/48	48/48
YHE1170 *TRP*$^+$	YHE1181	0/42	0/42
YHE1160 *TRP*$^+$	YHE1364	93/93	6/96
YHE1161 *TRP*$^+$	YHE1364	48/48	48/48
YHE1162 *TRP*$^+$	YHE1364	48/48	48/48
YHE1170 *TRP*$^+$	YHE1364	94/94	32/96

[a] YHE1364 is isogenic to BY302, the parent of the [URE3*albicans*] donors. Cytoductants are listed as Ade$^+$ colonies per number of cytoductants tested. Adenine prototrophy was assessed on minimal medium plus the other required amino acids (SD+U+L) or on complete defined medium lacking only adenine (−Ade).

A combination of the [URE3*albicans*] variant and the host background must block transfer of [URE3*albicans*] from YHE1160 and YHE1170 to YHE1181. Note that [URE3*albicans*1161] originating from YHE1161 changed during the transfers between the hosts BY302 and YHE1181, so that during its second time in the latter host it no longer supported growth on adenine dropout medium. The difference in results between the two adenine-lacking media was also displayed by the [URE3*albicans*] variants derived from *C. glabrata* when introduced into *S. cerevisiae* BY302.

The inability to transfer [URE3*albicans*] present in strain YHE1160 and YHE1170 to strain YHE1181 is due in part to the prion variant, as shown above. To determine if there is also a recipient host component to this phenomenon, isogenic donor and recipient strains were created. When using recipient strains with a background isogenic to the donor, the [URE3*albicans*] present in YHE1160 and YHE1170 is infectious (Table 6). It is thus a combination of the prion variant and the host background that prevents transfer. In meiotic crosses, the infection potential of these [URE3] prions segregated as a multigene trait in the spores, making definition of the genes involved impractical.

DISCUSSION

Here, we show that Ure2p of *C. glabrata* cannot form a prion at detectable frequency even in its own environment. As a control, we show that Ure2p of *C. albicans* can do so. Like previous results on *K. lactis* Sup35p and Ure2p (19, 20) and *S. paradoxus* Ure2p (14), this result supports the use of *S. cerevisiae* as a test bed for potential prion proteins (PrP). The one noted exception is the failure to find prion formation by PrP in *S. cerevisiae*. It is possible that the cell surface location of the normal form, PrP^C, and the formation of the prion form, PrP^{Sc}, either on the cell surface or in the endosomal system has not been properly set up in the attempts to make PrP a prion in yeast.

Our work confirms that prion-forming ability is not generally conserved. While the CgUre2p prion domain sequence is much closer to that of *S. cerevisiae* than is that of CaUre2p, only CaUre2p can form prions in *S. cerevisiae* or *C. glabrata*. Indeed, CgUre2p also does not form amyloid *in vitro*, while the *C. albicans* protein readily does so (15). Aigle's group has likewise found that the Ure2p of *Kluyveromyces lactis* is incapable of forming [URE3] in

K. lactis itself (20). The fact that prion formation by Ure2p or Sup35p is not restricted to *S. cerevisiae* has been used as an argument that it is a conserved trait, and that prion formation therefore must have a function for yeast (and those other species) (45, 46). Our results indicate that the ability of a protein from each of two species to form prions does not imply that that ability is conserved; it may well be a sporadically occurring trait. The same point is inferred from the finding that sequence is not important for prion-forming ability by the Ure2p and Sup35p prion domains (47–49). Our results parallel the fact, long known for mammalian prion proteins, that the ability of mammalian PrP to assume the prion form varies dramatically from one species to another and even within the same species (for examples, see reference 50).

In the course of preparing strains for this study, we have verified that, through Ure2p, *C. glabrata* selectively activates or represses transcription of genes in response to the availability of nitrogen sources in the growth environment. We showed previously that *C. glabrata* Ure2p regulates a set of genes similar to that of *S. cerevisiae*; however, we did not show that *glabrata* reacts to the nitrogen state of the environment, as does *S. cerevisiae*. As in *S. cerevisiae*, ammonia, asparagine, and glutamine activate Ure2p, whereas proline does not (1, 51, 52). Glutamate derepresses some Ure2p-regulated genes but not others in *S. cerevisiae* (53). Some genes, notably the general amino acid permease GAP1, are transcribed when glutamate is present as the sole nitrogen source but the protein is channeled to the vacuole for degradation (54, 55). Medium containing both ammonium and glutamate was used to isolate the first [URE3] prion (56). In *C. glabrata* the combination of ammonium and glutamate is among the strongest repressors of DUR3 promoter activity, and it was used here to select [URE3] candidates.

In preparing isogenic strains of opposite mating type to attempt cytoductions, we showed that the *S. cerevisiae* HO endonuclease homologue of *C. glabrata* can perform mating type locus switching, as is the case in *S. cerevisiae*. Although we did not observe mating, and no mating has been reported yet for *C. glabrata*, it is unclear why mating type switching has remained functional, if not for the purpose of creating mating partners.

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