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Yeast prions: Could they be exaptations? The *URE2*/[*URE3*] system in *Kluyveromyces lactis*.

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## Abstract

We examined aspects of the *URE2*/[*URE3*] prion system in *Kluyveromyces lactis*, which lies on a different evolutionary branch from *Saccharomyces*. We first analysed polymorphism of the prion-forming domain in 38 strains. Considerable differences were found between these two genera, with little variation within *K. lactis*. We then analysed the regulatory function of Ure2p, using a deletion of *URE2*. We assessed the deregulation of two reporter genes,

*DAL5* and *GDH2*. Both were derepressed in the mutant strain, as in *Saccharomyces*. Finally, we tried to obtain the [URE3] prion from *K. lactis*. Despite the use of many different experimental conditions, we were unable to obtain a prion from Ure2p. This finding calls into question the extent to which the prion form of Ure2p may be considered an evolutionary adaptation, instead suggesting that an exaptation phenomenon may be more likely than a continuous selection history.

Many proteins with the potential to become prions have been identified in *Saccharomyces cerevisiae* (Halfmann *et al.*, 2009). These proteins have a wide range of physiological functions. The conversion of these proteins to the prion form abolishes some or all of their functions and results in new cellular phenotypes (Halfmann *et al.*, 2009). A key unanswered question concerns the possible selection of this switch during evolution. In other words, is the ability to form a prion subject to selection, or is it just a side effect of the structure of the protein? One way to address this question is to determine whether the coding sequence of a prion is always associated with the folded prion structure. Sequences orthologous to prion forming domains (PFDs) are readily detected in other hemiascomycetous yeasts, but their mere existence does not provide proof that they actually switch to the prion form (Talarek *et al.* 2005). We analysed the *URE2*/[URE3] prion system in *Kluyveromyces lactis*. This yeast is on a different

evolutionary branch from *Saccharomyces* and displays respiratory metabolism, rather than the fermentative metabolism of *Saccharomyces*. Previous attempts to generate a prion from the *K. lactis* Ure2p through heterologous expression in *Saccharomyces* were unsuccessful (Baudin-Bailleu *et al.* 2003, Talarek *et al.* 2005).

We began by using YGOB (Byrne & Wolfe, 2005) to identify the principal members of the *URE2* network. The *URE2*, *GLN3*, *DAL5* and *GDH2* genes of *S. cerevisiae* were found to have *bona fide* orthologous genes in *K. lactis*. As in all hemiascomycetous yeasts, Ure2p was found to have an N-terminal prion-forming domain and a C-terminal, GST-like domain. The GST-like domain is well conserved between the two species, whereas the PFD is longer, with a higher proportion of glutamine residues and a very different sequence in *K. lactis* (Baudin-Baillieu *et al.* 2003). We investigated whether this difference between species was also found within the *K. lactis* species, by sequencing PFD and an equivalent length of the GST-like domain (800 nucleotides in total) in 36 *K. lactis var. lactis* strains from CIRM-Levures. Comparison with the *K. lactis* reference sequence (Baudin-Baillieu *et al.* 2003) resulted in the identification of only six polymorphisms in the PFD and two in the GST-like domain. We repeated this analysis with two more distantly related *K. lactis var. drosophilarum* strains (CBS2105 and CBS2103, Naumova *et al.*, 2004). In these two strains, we detected 17 polymorphisms in the PFD and 15 in the GST-

like domain (corresponding to mutation frequencies per nucleotide of  $22 \times 10^{-3}$  and  $17 \times 10^{-3}$ , respectively). An external control gene (*URA3*) was found to have a polymorphism frequency of  $11 \times 10^{-3}$ . Thus, within the *K. lactis* species, the PFD has evolved no more than the GST-like domain. This result may simply reflect isolation times, which are clearly likely to be considerably longer between species than between clones within a given species. However, isolation times alone cannot account for the striking difference in the rate of evolution between the two parts of the protein in the two species.

In *S. cerevisiae*, Ure2p downregulates the expression of numerous genes, including *GDH2* and *DAL5*. We investigated the possible conservation of Ure2p function in *K. lactis*, by deleting the entire *URE2* open reading frame and replacing it with the KanMX marker (Talarek *et al.*, 2005) by homologous recombination, resulting in the (*ure2::KanR*) genotype. We then evaluated the regulation of *GDH2* and *DAL5* expression in wild-type and (*ure2::KanR*) *K. lactis* strains.

*GDH2* regulation. The oxidoreduction pathways used by the two species differ considerably. *S. cerevisiae* uses essentially the NAD/NADH<sup>+</sup> shuttle, whereas *K. lactis* uses principally the NADP/NADPH<sup>+</sup> shuttle (Tarrío *et al.*, 2006). *GDH2* encodes a glutamate dehydrogenase that functions with NADH<sup>+</sup>. In *S. cerevisiae*, its activity is derepressed by a factor of 20 in *ure2*- mutant strains (Drillien *et al.* 1973), potentially creating a trough in the concentration of this

metabolite. This situation is easily resolved in *S. cerevisiae*, by its fermentative NADPHH<sup>+</sup>-producing metabolism. It was therefore important to determine whether this activity was also derepressed in *K. lactis*, which produces more NADPHH<sup>+</sup>. We quantified the specific activity of Gdh2p in wild-type and (*ure2::KanR*) strains, during culture in complete medium. The ratio between the activities of the two strains was 1/23, in the same range as for *S. cerevisiae*. Thus, the fundamental difference in oxidative metabolism between these two species has no effect on this part of the Ure2p network.

*DAL5* regulation. In *S. cerevisiae*, *pDAL5* is strongly repressed by Ure2p and expressed in the absence of Ure2p. We therefore constructed a reporter system based on the *ADE2* gene in *K. lactis* (*ade2-*) strains. The open reading frame of the *ADE2* gene from *S. cerevisiae* was inserted between the promoter (*pDAL5*, 500 bp) and the terminator (*tDAL5*, 500 bp) of the *K. lactis DAL5* gene. This construct was integrated into the genome of *K. lactis*, at an unknown site. In complete or minimal medium, recombinants (*ade2-*, *pDAL5::ADE2::tDAL5*) were red and auxotrophic for adenine. The double-mutant strain (*ure2::KanR*, *ade2-*, *pDAL5::ADE2::tDAL5*) was white and prototrophic for adenine, demonstrating similar regulation in *K. lactis* and in *S. cerevisiae*.

We then tried to obtain evidence for the existence of [URE3], the prion form of Ure2p, in *K. lactis*. For this purpose, we used an (*ade2-*, *pDAL5::ADE2::tDAL5*) background in all selection experiments and further crosses. In normal

conditions, this strain is red and auxotrophic for adenine. If Ure2p becomes [URE3], the strain should display an ure2- phenotype: white and prototrophic for adenine. We therefore used adenine-free medium to select for ADE+ clones among the original ade- strains. We used several different sets of conditions. First, we used haploid or diploid homozygous strains. As the (*ure2*-) mutation is essentially recessive, the use of diploid strains should make it possible to avoid selecting this mutation. Second, as *URE2* overexpression is known to increase the frequency of prion generation (Wickner, 1994), selection was carried out with strains overexpressing the *K. lactis* *URE2* gene from a multicopy pCXJ3 plasmid (Chen, 1996). Third, we also used stress conditions (strong limitation of growth by LiCl) previously reported to increase greatly prion formation in *S. cerevisiae* (Tyedmers *et al.* 2008). As PSI+ prion has been obtained in *K.lactis* (Nakayashiki *et al.*), we considered that all conditions to obtain prions in this species are present (e.g. the PIN+ status). We first differentiated between *ure2* mutations and [URE3] prions, by exposing ADE+ clones to guanidium chloride (GuCl), to which prions are specifically sensitive (Talarek *et al.* 2005). GuCl acts through the Hsp104p, which has a true orthologous gene in *K.lactis*. Moreover, PSI+ has been shown to be sensitive to GuCl in *K.lactis*. We plated about 300 clones of each ADE+ candidate on complete medium with or without 3 mM GuCl. Control experiments showed that (1) the (*ure2::KanR*) ADE+ prototrophic, white phenotype was not cured on GuCl medium. (2) single-mutant (*ade2*-) clones from *K. lactis* grew well and were red on GuCl medium

and (3) *S. cerevisiae* was totally cured of the [URE3] prion in this assay. However, none of the 40 ADE+ *K. lactis* candidate clones obtained in the various selection procedures was sensitive to GuCl treatment.

More detailed genetic analysis was carried out for four candidates. All harboured Mendelian mutations, segregating 2/2 on crossing with a tester strain. Two of these candidates were analysed further by crossing with the (*ure2::KanR*) strain. The 4/0 segregations obtained demonstrated that the original ADE+ phenotype of the candidates resulted from chromosomal mutations in *URE2*.

In conclusion, (1) the *ure2* regulation network in *K. lactis* is similar to that in *S. cerevisiae* and (2) the frequency of [URE3] prion occurrence is very low in *K. lactis*, well below that of (*ure2-*) mutation, such that it may even be impossible to obtain this prion. This has also been shown to be the case in *S. paradoxus* (Talarek *et al.* 2005). This low frequency, if indeed the prion occurs at all, provides strong evidence against selective pressure currently operating on the prion property of Ure2p throughout hemiascomycetes. We therefore suggest that the prion-forming domain was first selected in the ancestor of hemiascomycetous yeast for another purpose, such as the stabilization of Ure2p (Shewmaker *et al.*, 2007). Prion formation occurs by chance and may resemble mild illness (Saupe & Wickner, 2010). Shifting a protein to its prion state generates numerous strong phenotypes, due to loss of function (Halfmann *et al.*,

2009). These phenotypes can be beneficial in laboratory conditions, but no better fitness has been demonstrated in natural conditions in consequences of these new phenotypes. We therefore suggest that the phenotypes generated by prions of hemiascomycetous yeasts may constitute a classical case of exaptation, as defined by Gould and Vrba (1982) — “features that now enhance fitness, but were not built by natural selection for their current role” — as opposed to adaptation — “features built by selection for their current role”. One essential property of exaptations, that we can apply to yeast prions is that “adaptations have functions, exaptations have effects”.

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