INTERACTIONS OF THE CHAPERONES AND COMPONENTS OF UB SYSTEM IN THE FORMATION AND PROPAGATION OF THE YEAST PRION [PSI^+]

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INTERACTIONS OF THE CHAPERONES AND COMPONENTS OF UB SYSTEM IN THE FORMATION AND PROPAGATION OF THE YEAST PRION [PSI^{+}]

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I dedicate this thesis to my parents, Mr. Arthur E. Tennant and Ms. Patricia F. Tennant, and to my children John Tennant, Christopher Clegg and Jeremy Clegg.

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Chapter One
Introduction

What Are Prions?

Over 30 years ago, it was proposed that a protein could be an agent of infection if it could exists in a self-propagating infectious form (Griffen, 1967). A prion (proteinaceous infectious particles) is a protein isoform that has undergone changes that diminish its ability to perform its normal function, but enables it to convert the normal form of the protein into the prion isoform (Wickner and Chernoff, 1999). The term, prion, was originally proposed by S. Prusiner to explain the unusual transmission pattern of neurodegenative diseases (Prusiner, 1982).

The prion concept reveals a new method of inheritance independent of nucleic acid or amino acid sequences, but instead, will efficiently transfer from cell to cell by cytoplasmic mixing. Neither the genetic sequence nor the amino acid sequence is changed, but the prion is passed to progeny cells in a non-Mendelian pattern of segregation in meiosis in yeast cells. A single copy of a genetic element will result in a 2+:2- segregation. The mating of a cell containing the prion element with one that lacks the prion element produces mietotic offspring which all contains the prion element. This is a 4+: 0 segregation (Cox et al., 1988).

Prions form highly ordered insoluble aggregates, called amyloids, that seem to reproduce themselves by dissociating into smaller oligomers that serve as ‘seeds’ to new rounds of prion propagation. The formation of aggregates is associated with a conformational change in the secondary structure resulting in increase of ß-sheet structures in the effected protein. This conformational shift can be induced by the over production of the normal cellular protein.

An additional characteristic of a prion is the curability by exposure to non-mutagenic substances, such as guanine hydrochloride, and re-infection independent of exposure to another cell. The cured cell should be capable of producing prion-containing progeny in a manner dependent only upon the production of the normal cellular protein.

Prion diseases

The prion concept was first used to explain pathogen transmission consistent with bovine spongiform encephalopathy (BSE), sheep scapie disease, and human Creutzfeldt-Jacob disease. The cellular protein, PrP^{C}, normally located on the cell surface, is converted to an abnormal isoform PrP^{Sc}. Both isoforms have the same amino acid sequence and can be distinguished from each other by structure alone. The non-infectious form of PrP contains mostly alpha helical structures. This is in contrast to the infectious form with in rich in B-structures (Pan 1993). PrP^{C} is monomeric and remain soluble in non-denaturing detergents, and is degradable by proteases. PrP^{Sc} is an insoluble aggregate that is resistant to proteaolysis (Prusiner, 1994).

The accumulation of insoluble protein aggregates is associated with amyloidosis such as Alzheimer's disease, neuro inclusion disorders such as Amyotrophic Lateral Sclersosis, and polyglutamine diseases such as Huntington's disease. Although these diseases are non-infectious, the autocatalyzed misfolding, perpetuation of incorrect conformation and aggregation of proteins is consistent with the prion concept observed in transmission of BSE. The presence of aggregates is a prominent cytopathological

feature of many neurodegenerative diseases, and has been associated with the inhibition of certain cellular functions (Bence et al., 2001).

Prions in Yeast

Three of the best-characterized prions of Saccharomyces cerevisiae are [PSI^+], [URE3], and [PIN^+]. [URA3] is a prion form of the protein Ure2 that is a posttranslational regulator in the nitrogen metabolism pathway. Because of the partial loss of function of Ure2, in the presence of [URA3], cells are able to uptake ureidosuccinic acid from the media. These cells are then able to use ureidosuccinic acid as a source of uracil (Lacroute, 1971).

The prion [PIN^+] is the isoform of the protein Rnq1, and this protein's function is unknown. The presence of the prion [PIN^+] is necessary for the de novo formation of the prion [PSI^+] (Derkatch et al., 1997). [PSI^+] is the prion isoforms of the protein Sup35 that functions as a eRF3 translational termination factor. The presence of [PSI^+] is detected by the partial loss of function of Sup35.

All three of these protein elements are inherited in a non-Mendelian manner and require the nuclear elements for maintenance, *URA2* gene in the case of [URA3], (Wickner, 1994) the N terminus of the *SUP35* gene in the case of [PSI^+] (Doel et al., 1994), and *RNQ1* in the case of [PIN^+]. All three are reversibly curable using agents such as guanidine hydrochloride, and the prion form can be induced by the over expression of the related genetic element. The majority of the related protein is found in an insoluble, protease resistant, aggregated form in cells that are phenotypically

consistent with the presence of the prions. Most of the related protein is found in the insoluble form in cells that are not suspect for the presence of the prions.

The Yeast Prion [PSI$^+$]

The structure of an eRF1 protein mimics that of a tRNA molecule. It recognizes the stop codon and catalyzes the hydrolysis of the bond between the nascent polypeptide and tRNA. eRF3 is a GTPase that binds to eRF1 facilitating its release activity.(Zhouravleva et al., 1995) In yeast eRF3 is encoded by the *SUP35* gene. (Zhouravleva et al.,1995; Stansfield et al.,1995) Mutations in either *SUP35* or *SUP45*, which codes for eRF1, results in suppression of nonsense codons (Inge-Vechtomov and Andrianova, 1970).

Sup35 protein consists of three regions. The C-proximal region is the most conserved and is required for viability and termination. The N-proximal portion is not required for viability or termination, but in necessary for the propagation, induction and maintenance of [PSI$^+$] (Derkatch et al., 1996). The M region, middle region, is not directly required for proper function of the protein nor is it responsible for [PSI$^+$] propagation, maintenance or induction. The minimal region of the Sup35 protein required for the induction of [PSI$^+$] is the first 61 amino acids of the N-region, and the first 97 amino acids are required for [PSI$^+$] maintenance.

The self-propagation of the prion isomers may be responsible for the partial loss of function of Sup35. As new Sup35 is created, it is converted to the prion conformation, and then incorporated into aggregates, which interferes with its ability to

perform as a translational release factor (Paushkin et al.,1997). This loss of function is also self perpetuating and becomes a heritable change (Patino et al., 1996).

The Yeast Prion [PIN$^+$]

The over production of Sup35 or Sup35N induces the de novo formation of [PSI$^+$] in [psi$^-$] strains. In most cases, this induction is dependent upon the presence of an additional prion [PIN$^+$] (Derkatch et al., 1997). De novo formation of [PSI$^+$] is dependent upon the prion [PIN$^+$], but the maintenance of [PSI$^+$] is not dependent upon the presence of [PIN$^+$].

[PIN$^+$] is the prion isoforms of the protein, Rnq1. Deletion of gene that codes for Rnq1 eliminates [PIN$^+$] phenotype, but other prions, such as [URA3] can substitute of [PIN$^+$] in *rnq1Δ* strains (Derkatch et al., 2001). The deletion of the *URA3* gene, in these circumstances, eliminates the [PIN$^+$] phenotype. This interaction among these prions suggests that self-propagating protein structures may perform functions yet unknown throughout many eukaryotic cells.

Prions and Chaperones

The concentration of proteins in the cellular plasma is extremely dense, and nascent proteins, in order to function properly, need to obtain their native structures while in this highly competitive environment. Chaperones are a group of proteins that facilitate the proper folding of nascent proteins, to prevent the aggregation of denatured and damaged proteins, to aide in the correction of misfolded proteins, and to assist in the elimination of proteins that cannot be properly refolded (Gottsman et al. 1997). Protein aggregation is a common cellular event, and occurs at a magnified level during

thermal and oxidative stress (Fink, 1998). The chaperone system is instrumental in preventing the accumulation of aggregates within the cell.

How can prions form under the action of the many chaperones used to control protein aggregation? In the case of induction by over expression of the genetic element related to prion development, the major facilitator could be the over whelming number of nascent proteins is too great for the system. The lack of action of the chaperone system upon nascent proteins may allow for the development of the prion isoforms. The self-propagating property of this isoforms further facilitates the development of the aggregate by its contact with nascent, unfolded proteins or proteins that have already taken on the native conformation.

In the case of the extremely rare spontaneous occurrence of prions, where over production of proteins is not a factor; the protein control system may have an occasional error that allows the creation of the self-propagating isoform of a protein. This allows for the exponential creation of proteins that are in the prion isoform and that readily aggregate. The correction of excessive misfolded proteins in conjunction with the normal function may again over whelm the chaperone system.

Hsp104 and [PSI^{+}]

The chaperone, Hsp104, belongs to an evolutionary conserved Hsp100 family of proteins that participate in a various number of cellular processes (Schirmer et al., 1996). Hsp104, in particular, is responsible for the cells adaptation to heat shock, it controls spore viability and the long-term viability of starving vegetative cells. (Sanchez

and Linquist, 1990; Sanchez et al.,1992) It is an ATPase that has been shown to promote solubilization of aggregated protein (Parsel et al., 1991).

A unique relationship exists between Hsp104 levels within the cell and the maintenance of the prion [PSI^+]. The over production of Hsp104 eliminates [PSI^+] (Chernoff 1995). This seems logical considering Hsp104 is a disaggregase, and it is reasonable to assume that the over production provides sufficient resources to break the aggregates into portions that are accessible to either other chaperones which would facilitate the proper folding or perhaps the system responsible for the elimination of unusable proteins, such as the ubiquitin-proteasome system.

What is both unique and counter-intuitive is the elimination of [PSI^+] by the inhibition of elimination of Hsp104. The disaggregase activity of Hsp104 may allow the propagation of [PSI^+] by facilitating the seeding of daughter cells. When the action of Hsp104 is inhibited or eliminated, the strong tendency for prion isoforms to aggregate may decrease the available proteins to the extent that none are passed to the daughter cells but remain in the aggregated form in the mother cell and are lost in cell division (Paushkin et al., 1996).

It is necessary to note that the conditions that induce Hsp104, such as mild heat shock sporulation, or incubation in the stationary phase do not cure cells of [PSI^+]. This suggests that other proteins or changes associated with these conditions could influence the Hsp104 effects on [PSI^+].

Hsp70 Proteins and [PSI$^+$]

The major cytosolic subfamily of the Hsp10 family in yeast is Ssa. It includes four closely related homologous proteins, Ssa1,2,3, and 4 Like Hsp104, the level of Ssa proteins is increased with temperature, stationary phase and sporulation. Ssa1 protein participates in disaggregating and refolding of heat damaged protein agglomerates. However, the over production of Ssa prevents efficient [PSI$^+$] curing by the over production of Hsp104.The high ordered structure of prion aggregates may cause Hsp70-Ssa protein to recognize them as legitimate sub cellular structures rather than the disorganized aggregates formed by general misfolded proteins. It may actually help to convert Sup35 back to the prion state promoting prion propagation (Chernoff, 2000).

Ssb1 and Ssb2 are also members of the Hsp-70 family. They are constitutively expressed and are not essential for viability. Genetic and biochemical data suggest that Ssb proteins are involved in cotranslational folding of nascent polypeptides and protein turnover (Nelson 1992; Pfund et al., 1998; Obha 1997). The over production of Ssb proteins enhances the curing ability of Hsp104 while the deletion inhibits curing. Both spontaneous [PSI$^+$] formation and induction by over production of Sup35 in increased in *ssb1,2*Δ strains (Chernoff et al., 1999). Figure 1.1 illustrates the possible role of refolding in curing by Hsp104.

The two major subfamilies of the Hsp70 family, Ssa and Ssb, have functionally diverged from each other in regard to their roles in prion formation and propagation. Ssb protein is involved in a proofreading system that is aimed at the prevention of the formation of prion aggregates (Chernoff et al., 1999).

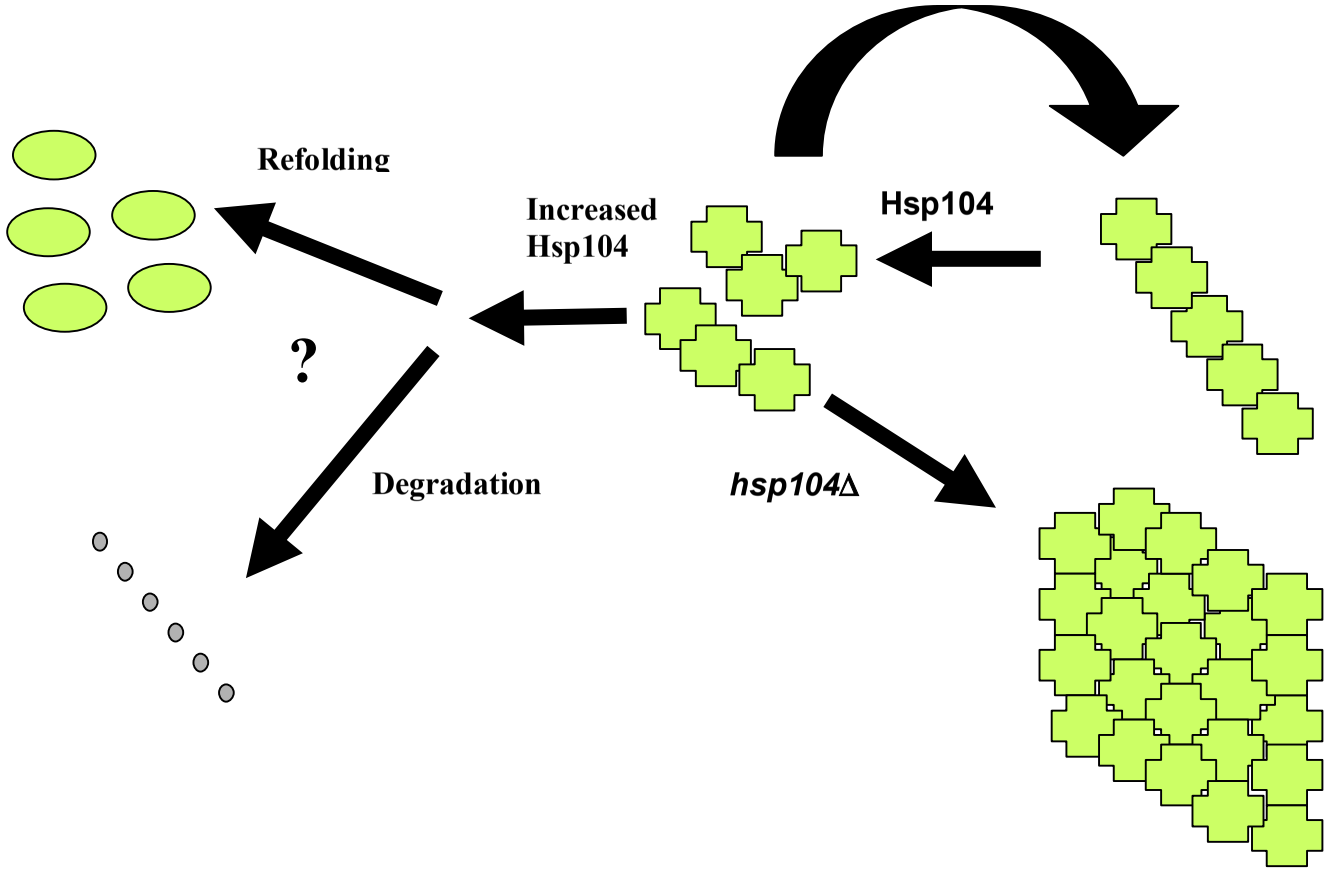


Figure 1.1 Model of Hsp104 Curing. The action of intermediate levels of Hsp104 provides a mechanism that provides the seeds necessary for prion propagation. When Hsp104 is over produced, the disaggregase activity breaks the aggregates into small enough sizes to allow the action of either the ubiquitin-proteasome pathway which leads to the degradation of misfolded proteins or the action of Ssb proteins to refold the Sup35 to its native conformation. The prion seeds are not passed to daughter cell when Hsp104 activity is either elimination or inhibited.

Ubiquitin-Proteasome Pathway

The ubiquitin system is a highly conserved, multi-enzyme system that is found in all eukaryotes. It's function if primarily to target proteins for proteolytic degradation (Varshavsky et al., 2000; Hochstrasser, 1996). This system plays a major role in many biological processes such as cell differentiation, the cell cycle, apoptosis, signal transduction, DNA repair, transmembrane and vesicular transport, stress responses as well as degradation of ubiquitin-conjugated proteins by the 26S proteasome (Varshavsky 1997). Figure 1.1 demonstrates the proposed role of degradation in curing by Hsp104. Figure 1.2 illustrates the recycling of ubiquitin within the degradation pathway. It can be seen that by the potential for inhibition of this pathway exists either in the suppression of available ubiquitin or in the interference of ubiquitin conjugation.

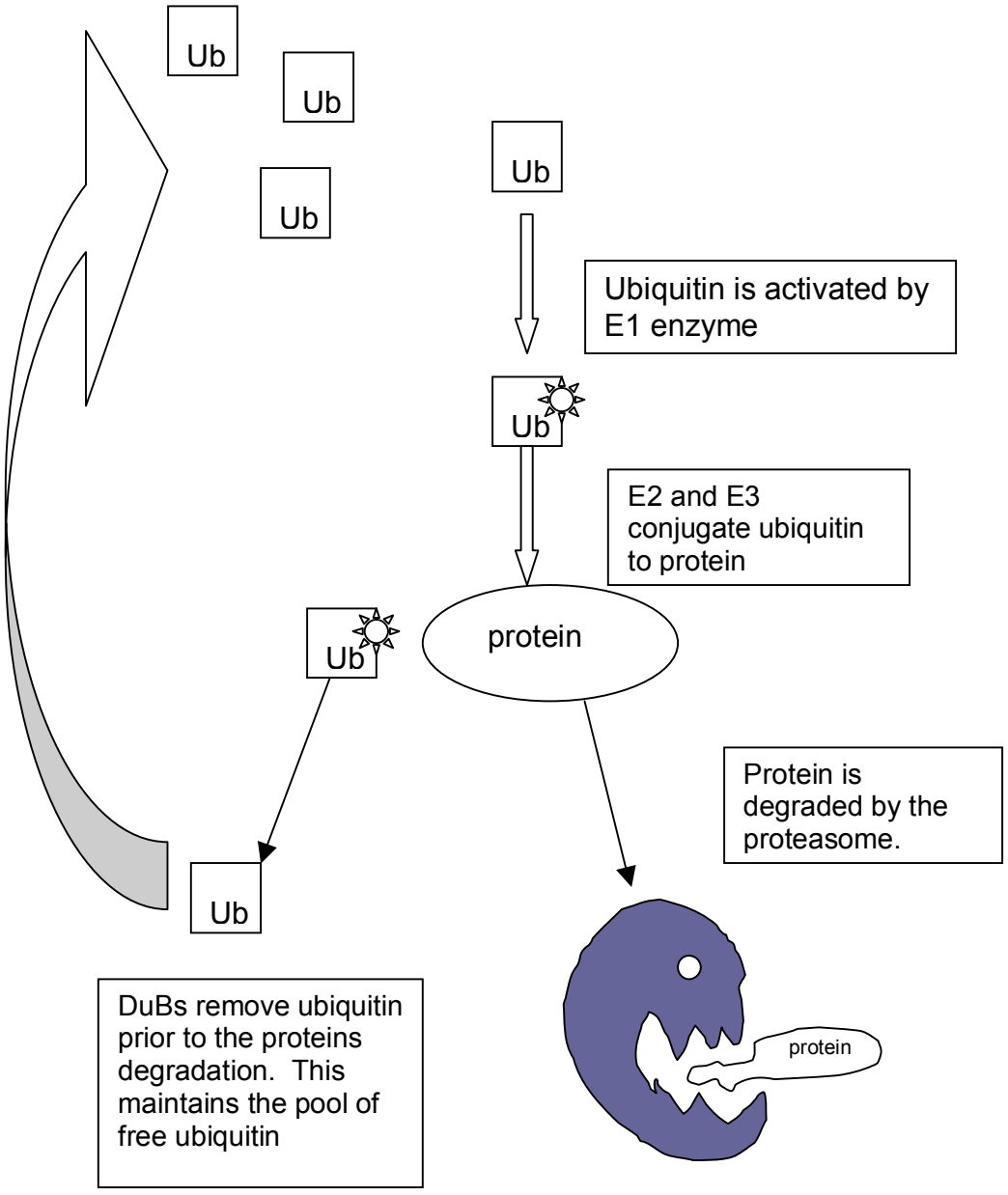


Figure 1.2. The Ubiquitin System. The ubiquitin molecule is recycled. First it is activated by E1, conjugated to the targeted protein by E2, and is then removed prior or during degradation by DUBs. Ubiquitin is then again available to re-enter the

The primary element of the system is a 76-residue protein that exists in cells either free or covalently linked to other proteins. Ubiquitin is activated for conjugation to other proteins by a ubiquitin-activating enzyme (E1) and then moved to an ubiquitin cunjugatin enzyme (E2). The E2 enzyme, along with an E3 enzyme involved in recognition, attaches the ubiquitin to a Lysine residue of an acceptor protein, yielding an ubiquitin-protein conjugate (Varshavsky 1997).

Ubiquitin is a long-lived, recycled protein. It must be removed from the ubiquitin-protein conjugates before or during degradation. This is accomplished by and number of deubiquitinating enzymes (Dubs). The best characterized of the 17 Dubs of Saccharomyces cerevisiae, is Doa4. A significant fraction of the enzyme has been found associated with the 26S proteasome. In exponentially growing cultures of *doa4* mutants, small ubiquitinated species accumulate; these species were suggested to be the proteolytic remnants of ubiquitinated proteins (Papa and Hochstrasser, 1993; Papa et al.,1999). Also ubiquitin pools in the mutant become depleted, particularly in stationary-phase cultures as a result of the proteolysis of ubiquitin. Partial suppression of the ubiquitin depletion by mutations in components of the 26S proteasome suggests that this protease is partly responsible for the degradation of ubiquitin (Amerik et al., 2000).

Chapter Two
Material and Methods

Materials

Yeast Strains

All yeast strains used in this study are listed in table 2.1. Single deletions listed were accomplished using a PCR-mediated gene deletion protocol (Longtine et al.1998). A more detailed protocol is provided in the methods section. Double and triple deletion strains were obtained through mating appropriate knock-out strains and dissecting the resulting diploids. (see Methods for more detailed protocol)

Table 2.1
Yeast Strains

Strain	Genotype	References
GT81-1C	MAT a *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52* [PSI$^+$][PIN$^+$]	Chernoff et al., 2000
GT159	MAT a *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52* [psi$^-$] [PIN$^+$]	Chernoff et al, 1999
GT409	MAT a *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52* [psi$^-$] [pin$^-$]	Chernoff et al, 1999
GT349	MAT a ade1-14 *his3-Δ200 or 11.15 leu2-3,112 lys2 trp1-Δ ura3-52 ubc4::HIS3* [psi$^-$] [PIN$^+$]	This study. See Appendix
GT386	MAT a *ade1-14 his3-Δ200 or 11.15 leu2-3,112 lys2 trp1-Δ ura3-52 ubc4::HIS3* [PSI$^+$] [PIN$^+$]	This study. See Appendix
GT387	MAT a *ade1-14 his3-Δ200 or 11.15 leu2-3,112 lys2 trp1-Δ ura3-52 ubc4::HIS3* [psi$^-$] [pin$^-$]	This study. See Appendix
GT532-9C	MAT α ade1-14 *his3-Δ200 or 11.15 leu2-3,112 lys2 trp1-Δ ura3-52 ubc4::HIS3* [PSI$^+$] [PIN$^+$]	This study. See Appendix
OT116	MAT a *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 ubp6::HIS3* [PSI$^+$] [PIN$^+$]	Chernova et al., 2003
OT117	MAT a *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 ubp6::HIS3* [psi$^-$] [PIN$^+$]	Chernova et al., 2003
GT685	MAT a *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 ubp6::HIS3* [psi$^-$] [pin$^-$]	This study. See Appendix
OT144	MAT α *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 ubp4 :: HIS5Sp* [PSI$^+$] [PIN$^+$]	Chernova et al., 2003
GT147	MAT a ade1-14 *his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 MAT a ssb1::HIS33 ssb2::URA3* [PSI$^+$] [PIN$^+$]	Chernoff et al, 1999

Table 2.1 Continued

GT823	MAT a *ade1-14 leu2-3, 112 his3-Δ200(or 11,15) lys2 trp1-Δ ura3-52 ubc4::HIS3 ssb1::HIS3 ssb2::URA3* [psi$^-$] [PIN$^+$]	This study. See Appendix
GT858	MAT a *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 ubc4::HIS3 ssb1::HIS3 ssb2::URA3* [psi$^-$] [pin$^-$]	This study. See Appendix
GT563	MAT *a ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 rnq::HIS3* [PSI$^+$]	This study. See Appendix
GT564	MAT a ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 14 *rnq1:: HIS3* [psi$^-$]	This study. See Appendix
GT832-7B	MAT a *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 ubp6::HIS3 ubc4::HIS3* [PSI$^+$] [PIN$^+$]	This study. See Appendix
GT949	MATa *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 ubp6::HIS3 ubc4::HIS3* Ψ-[PIN$^+$]	This study. See Appendix
GT950	MATa *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 ubp6::HIS3 ubc4::HIS3* [psi$^-$] [pin$^-$]	This study. See Appendix
GT784-8B	MAT a *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 ubc4::HIS3 rnq1::HIS3* [PSI$^+$] [PIN$^+$]	This study. See Appendix
GT389-14A	MAT a *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 ubc4::HIS3 ssb1::HIS3 ssb2::URA3* [PSI$^+$] [PIN$^+$]	This study. See Appendix
GT784-8C	MATα ade1-14 his3Δ (or 11,15) leu2-3,112 lys2 trp1-Δ ura3-52 *ubc4::HIS3 rnq1::HIS3* [PSI$^+$]	This study. See Appendix
GT820	MAT a *ade1-14 his3Δ (or 11,15) lys2 ura3-52 leu2-3,112 trp1 ubc4::HIS3 rnq1::HIS3* [psi$^-$]	This study. See Appendix
GT684-5B	MAT a *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 ubp6::HIS3 ubp4::HIS3* [PSI$^+$] [PIN$^+$]	This study. See Appendix
OT37	MATα *his4 lys2*	S. Liebman
OT38	MAT a *his4 lys2*	S. Liebman

Plasmids

Table 2.2
Plasmids

Plasmid	Protein	Type	Marker	Promoter	Source
pFL39	Vector	CEN	*TRP*		Bonneaud, et al., 1991
YEp13	Vector	2 micron	*LEU2*		Ma et al., 1987
pRS316GAL	Vector	CEN	*URA3*		Liu et al., 1992
pGAL104-URA3	*HSp104*	CEN	*URA3*	GAL	Sanchez et al., 1993
pLH105	*HSp104*	2 micron	*LEU2*	GPD	Chernoff et al., 1999
PFL39-HSP104GAL	*HSP104*	CEN	*TRP*	GAL	This study. See Appendix

Transformation of Yeast Cells by Lithium-Acetate Treatment

Strains are pre-cultured overnight for two days, depending upon growth rate, in 5-10 mls of complete media. The pre-cultures are diluted with fresh media, by 3 to 10 fold, and then cultured for an additional 3-5 hours until the cells have re-entered exponential growth phase verified by an OD_{600} of 1.0-5.0. Cells are collected by centrifugation at 3000 rpm (6000g). The cells are washed with TE (10mM Tris-HCl, pH7.5, 1mM EDTA) and re-suspended in 5-10mls of 100mM LiAc-TE, and then cultured for an hour at 30^{o}C while shaken at 200 rpm. The cells are again collected by centrifugation at 3000 rpm for 5 minutes. The cells are then transferred to microfuge tubes (50-100 µl cells per tube) containing 20ul of carrier DNA and 1-10 µg of transforming DNA. Microfuge tubes are incubated for 45 minutes at 30oC. Following incubation, 350ul of LiAc-PEG (40%PEG 3350, 100mM LiAc, 10mM Tris-HCl, pH7.5, 1mM EDTA) is added per 50 µl of cells. After one hour of incubation and agitation by shaking at 30o and 200rpm, the cells are heat shocked at 42^{o}C for 5 minutes. The cells are left, at a minimum, for an hour, or overnight at 4^{o}C. At this point cells can be plated on complete media and cultured at 30^{o}C overnight, and then velveteened onto media selective for the inserted DNA, or they can be plated directly upon the selective media.

PCR-Mediated Gene Transplacement

Gene deletion strains, used in this study, were created using the PCR-based gene deletion protocol described by Longtine et al. (1998) Primers are designed that have 5' ends that contain 40 base pair sequences that are homologous to the target gene and 3' ends that anneal to and allow PCR amplification of the selectable marker gene from a template plasmid cassette. The amplified disruption fragment is then transformed directly into yeast cells by using the previously described transformation procedure with the exception of the transforming DNA concentration. All of the fragment generated by PCR is used in the transformation procedure, or the fragments from several PCR reactions may be pooled and precipitated by ethanol. The transformation product is plated on YPD, and after incubation, is velveteened to media selective of the marker used to displace the gene. The selective media plates are incubated at standard conditions for 3-5 days, and then duplicated once again on the selective media.

If homologous recombination takes place, the target gene will be transplaced by the amplified cassette containing the marker gene. Two passes on the selective media eliminates cells that have not truly incorporated the maker gene. The transplacement is then verified through PCR and/or by restriction analysis.

Mating of Yeast Strains

Haploid yeast strains are one of two switchable mating types, MAT a and MATα. Diploid strains are heterozygous, MATa/MATα. Mating of haploid strains was carried out according to convention as described in Kaiser et al, 194. Haploid parental strains were streaked together on YPD media and cultured at 30^{o}C overnight. If each haploid contains convenient markers, the mating plate can be duplicated on media selective for diploids. In some cases, the genotypes of the haploids do not provide such convenient markers. In this situation, cells are again mated to 'tester' strains that contain makers different from the haploids. Cells that fail to mate with the tester strains of either mating type are selected as diploids resulting from the original mating. This procedure is illustrated in figure 2.1 below.

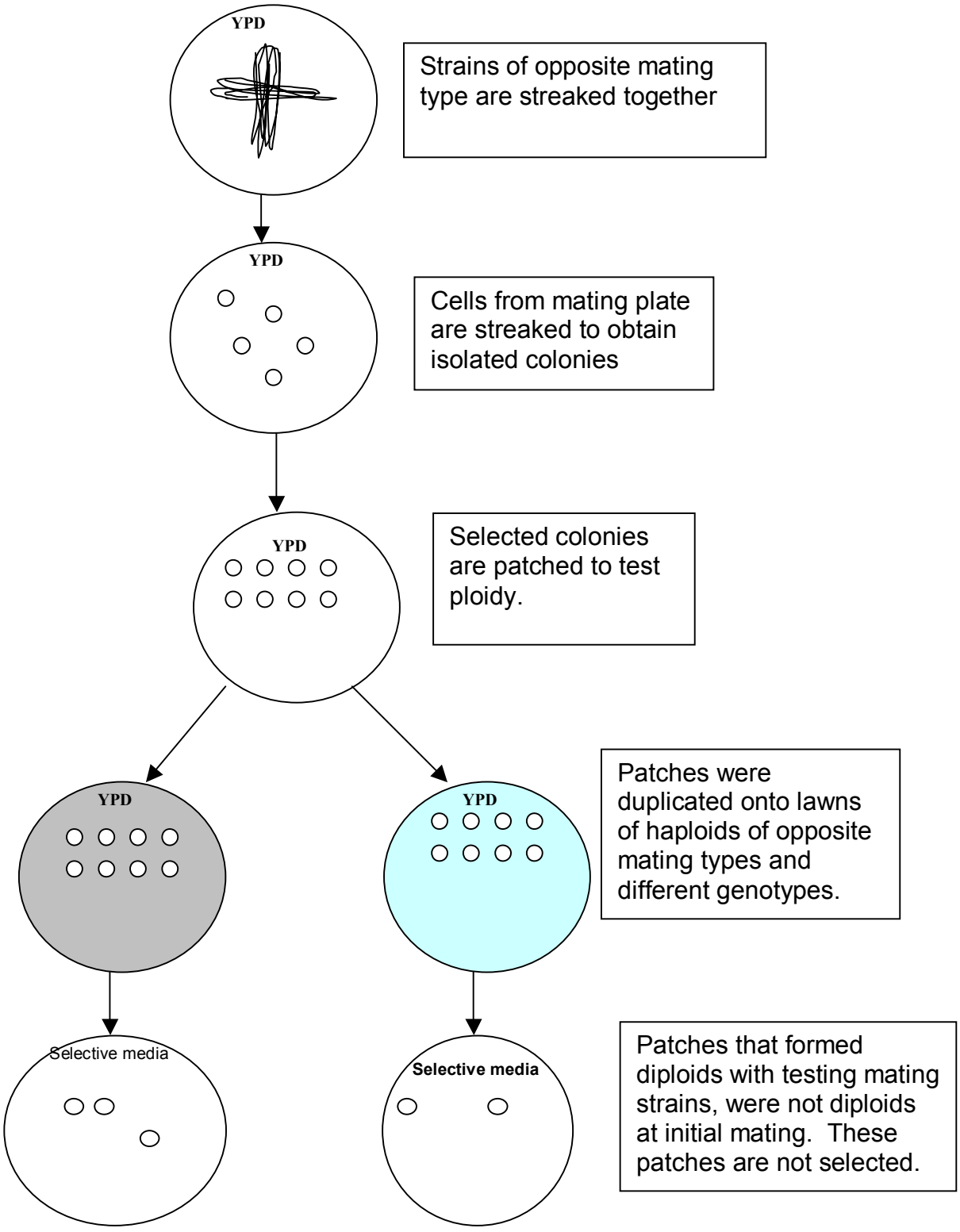


Figure 2.1. Selection of diploids resulting from the mating of haploids whose genotypes lack markers that can be used for diploid selection. Patches that fail to grow in the last step of this procedure are diploids that were formed on the initial mating plate.

Tetrad Dissection and Analysis

1. Diploids are placed upon a rich pre-sporulation media for 1 day and incubated at 30oC, then velveteened onto sporulation media which provides limited nutritional resources and incubated for 3-5 days until a sufficient number of cells have sporulated. (a minimum of 40%).
2. Cells are then subjected to lyticase treatment (1mg/ml) for 3-5 minutes at room temperature to remove cell wall and weaken the ascus.
3. Lyticase digestion is halted by collection of cells at 300 rpm for 2 minutes, and then by resuspension in sterile water.
4. A small portion of the cells are placed upon a small slab of YPD and dissected into their individual spores using a micromanipulator.
5. The spores are incubated at 30^{o}C until colonies are formed.

Construction of Double Knockout Strains

The double knockout strains for this study were generated by crossing two isogenic strains with deletions of different alleles resulting in a diploid heterozygous for each deletion. The diploids were sporulated and dissected. In these cases, the single deletion strains were constructed using the same *HIS3* marker cassette, so identification of segragants containing both deletions was accomplished by selecting tetrads that exhibited a 2:2 segregation of the *HIS*$^{+}$ phenotype.

Qualitative Curing of [PSI$^+$] by *HSP104* expressed under the GPD Promoter

1. Transform strains with a YEp13 and pLH105 plasmids using the lithium acetate procedure. Select for transformants on media lacking leucine.
2. Patch selected colonies of each plasmid/strain combination onto –Leu media and culture using standard conditions for 3-5 days.
3. Duplicate –Leu plate on YPD, YPG, -Leu –Ade, and –Ade media and culture selective media plates for 3-5 days at standard conditions. Culture the YPD plate at 25^{o}C for 3-5 days then move to 4^{o}C and wait for color to develop.
4. Compare growth on –Ade media and color development on YPD to evaluate curing.
5. Use YPG to eliminated petites from experimental evaluation.

Qualitative Curing of [PSI$^+$] by *HSP104* expressed under GAL Promoter

1. Transform strains with a pRS316GAL and pGAL104 plasmids using the lithium acetate procedure. Select for transformants on media lacking leucine.
2. Patch selected colonies of each plasmid/strain combination onto –Ura media and culture using standard conditions for 3-5 days.
3. Duplicate –Ura plate on YPD, YPG, -URA GAL, –Ade-Ura, and –Ade media and culture the –Ura GAL plates for 5 days at standard conditions, and the other selective media is cultured 3-5 days at standard conditions. Culture the YPD plate at 25^{o}C for 3-5 days then move to 4^{o}C and wait for color to develop.
4. Compare growth on –Ade and color on YPD to check for curing. Use growth on YPG to eliminate petites.
5. Duplicate the –Ura GAL plate to YPD and –Ade. Culture the –Ade at standard conditions for 3-5 days, and culture the YPD plate at 25^{o}C for 3-5 days then move to 4^{o}C and wait for color to develop.
6. Compare growth on –Ade and color on YPD to check for curing.

Quantitative Curing of [PSI$^+$] by HSP104 expressed under the GAL Promoter

1. Transform strains with a pRS316GAL and pGAL104 plasmids using the lithium acetate procedure. Select for transformants on media lacking uracil.
2. Patch selected colonies of each plasmid/strain combination onto –Ura media and culture using standard conditions for 3-5 days.
3. Duplicate –Ura plate onto –Ura, YPD, YPG, and –Ade media. Culture selective media for 3-5 days at standard conditions. Culture the YPD plate at 25^{o}C for 3-5 days then move to 4^{o}C and wait for color to develop
4. Select three PET+ colonies will be selected using YPG. These colonies should show no evidence of curing.
5. Use cells from each selected colony to inoculate 10 mls of liquid –Ura media and culture 24 hours at standard conditions.
6. Centrifuge cultures at 3000 rpm for 5 minutes and remove supernatant.
7. Resuspend cells in 5 ml of sterile water, and centrifuge again.
8. Resuspend cells in 3 ml of sterile water. Transfer 1 ml of cells to a microfuge tube.
9. Create serial dilutions of each cells from each colony, and perform cell counts using a hemocytometer.
10. Use cell counts to create time zero plates containing approximately 300 cells per plate, and to inoculate 150 mls of liquid –Ura, 2% GAL, 2% RAF media to the concentration of 1.0 x 10^5 cells per ml.

11. Culture the flasks of liquid cultures at standard conditions, then remove 1 ml from each to create serial dilutions. These will be used for cell counts to plate approximately 300 cells per plate onto – Ura media. This will be repeated for each time point
12. Culture the –Ura plates for 3-5 days at 30^{o}C.
13. Duplicate these plates by velveteen onto –Ade and YPD.
14. Incubate –Ade plates at 30^{o}C for 3-5 days. Culture the YPD plate at 25^{o}C for 3-5 days then move to 4^{o}C and wait for color to develop.

Assay for presence of [PSI$^+$]

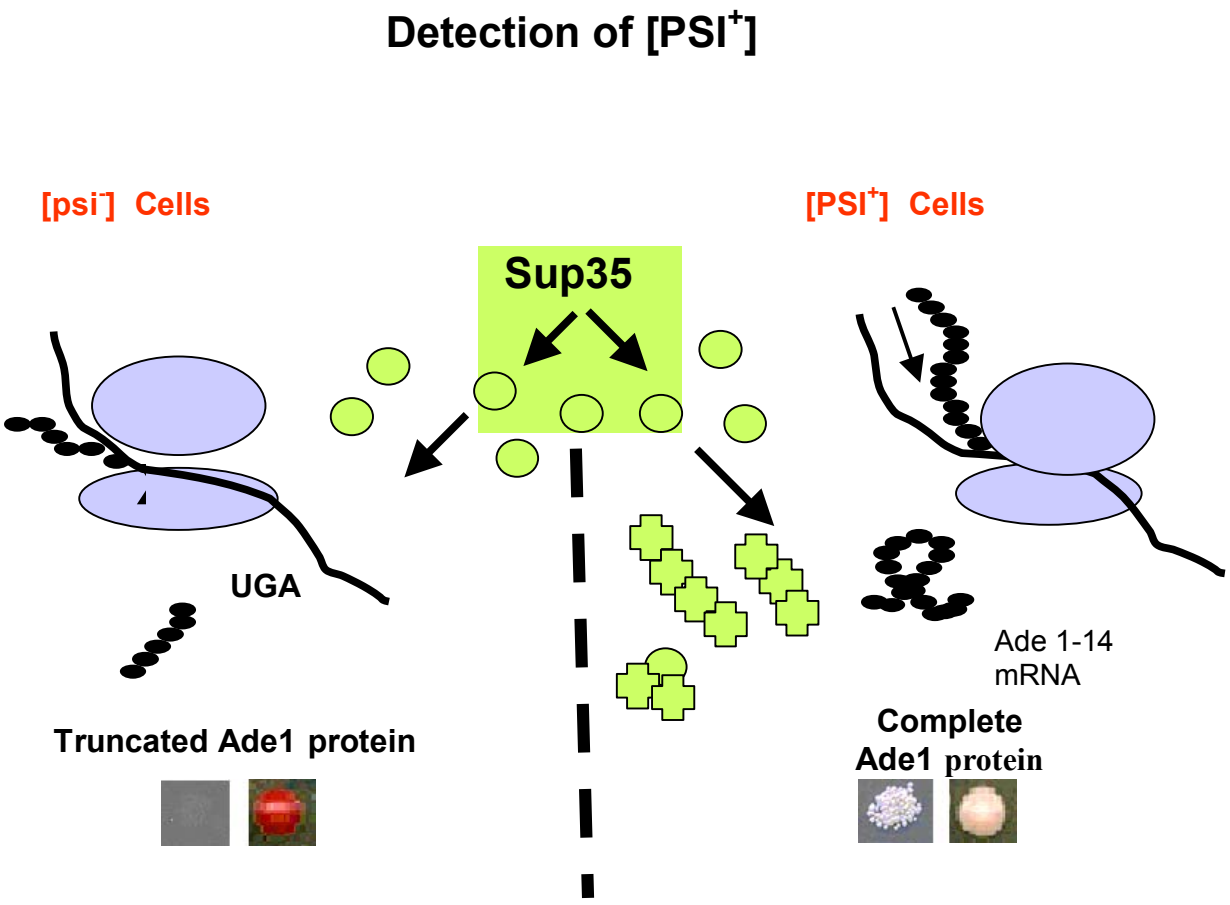


Figure 2.2. Assay to detect [PSI$^+$]. Functional Sup35 stops translation at a nonsense mutation in the *ADE1* gene. This causes a development of red pigment on YPD and prohibits growth on –Ade media. When Sup35 is not functional, there is a read through of the nonsense mutation allowing production of functional Ade1. This eliminates pigment development and permits growth on –Ade.

The structure of eRF1 protein mimics that of a tRNA molecule. It recognizes the stop codon and catalyzes the hydrolysis of the bond between the nascent polypeptide and tRNA. eRF3 is a GTPase that binds to eRF1 facilitating its release activity.(Zhouravleva, 1995) In yeast eRF3 is encoded by the *SUP35* gene. (Zhouravleva 1995; Stansfield 1995) Mutations in either *SUP35* or *SUP45*, which codes for eRF1, results in suppression of nonsense codons. (Inge-Vechtomov 1970)

The self-propagation of the prion isomers may be responsible for the partial loss of function of Sup35. As new Sup35 is created, it is converted to the prion conformation, and then incorporated into aggregates which interferes with its ability to perform as a translational release factor.(Paushkin 1997) This loss of function is also self perpetuating and becomes a heritable change. (Patino, 1996).

A nonsense mutation in the *ADE1* gene provides an assay using this loss of function to detect the presence of [PSI^+]. When Sup35 is functional, a truncated version of the Ade1 protein is produced. An accumulation of the precurser in this pathway creates a red pigment , and the elimination of this functional protein prohibits growth on media lacking adenine. When Sup35 is not functional, the nonsense mutation is read through creating a fully functional Ade1 protein. This permits growth on media lacking adenine, and eliminates the pigment development on YPD.

Viability Study of *doa4Δ* Strain

1. [PSI$^+$] and [psi$^-$] versions of isogenic *DOA4*$^+$ strains and the *doa4Δ* strains were streaked from –70^{o}C stock collection onto YPD and cultured at 30^{o}C for 3 days.
2. Isolated colonies were patched on YPD, cultured for 3 days, then velveteened onto YPG to select for PET$^+$ cells.
3. The PET$^+$ cells are used to inoculate 10 mls of liquid SD+13 (synthetic media containing all necessary elements per the genotype of the strain).
4. A 500 ul aliquot of each culture is used to create serial dilutions. Cell counts were performed using a hemocytometer, and cells were plated on SD+13 agar in a concentration to allow 300 cells per plate.
5. At indicated time points, aliquots were taken and cell counts were performed. Sufficient cells were plated on SD+13 to allow approximately 300 cells per plate of the *DOA4*$^+$ strain, and 300, 600,1500, 3000 and 6000 of the *doa4Δ* strains.
6. Percentage of viable cells was calculated by dividing the number of anticipated colonies by the number of colonies that developed.

Spontaneous Occurrence of [PSI$^+$]

1. Cells of each strain to be tested are obtained from –70^{o}C stock collection and cultured on YPD for 3 days.
2. Isolated colonies of each strain is used to create patches of equal size on YPD and the plates are cultured for 2 days.
3. The YPD plates are duplicated by velveteen onto media lacking adenine.
4. The –Ade plates are cultured for 20 days at 30^{o}C.
5. Small pink papillae are counted as occurrences of [PSI$^+$].

Chapter Three
Results and Conclusions

Curing of *ubc4, rnq1*Δ strain by *HSP104* expressed under the GPD

Results

The [PSI$^+$][PIN$^+$] versions of a *UBC4*+, *RNQ1*+ strain, a *ubc4*Δ strain, a *rnq1*Δ strain, and the *ubc4, rnq1*Δ strain were transformed with a plasmid containing *HSP104* expressed under the GPD promoter and a *LEU* marker, pLH105, and also a control plasmid that contained only the *LEU* marker, YEp13. Several colonies were selected from the transformation plates and were patched onto media lacking leucine. These plates were cultured at standard conditions for 5 days, and were then duplicated by velveteen onto YPG, media lacking adenine, media lacking leucine, media lacking both adenine and leucine, and YPD. The curing was assessed by examining the color of the patches on YPD and growth on media lacking adenine. (For a detailed protocol, see Materials and Methods) As seen in figure 3.1, *ubc4*Δ strains were partially cured and *UBC4*+ strains were completely cured by *HSP104*.

Conclusion

The absence of *RNQ1* had no detectable effect on efficiency of curing by *HSP104* expressed under the GPD promoter. As expected the *ubc4*Δ strains were cured at a lower efficiency compared to wild type, and this remained true

when *rnq1* was also deleted. This suggest that the function of Rnq1 protein is not one involved in curing of [PSI^{+}] by Hsp104.. This result may also indicate that the effect, if any, is not strong enough to be detected when *HSP104* is expressed at this level.

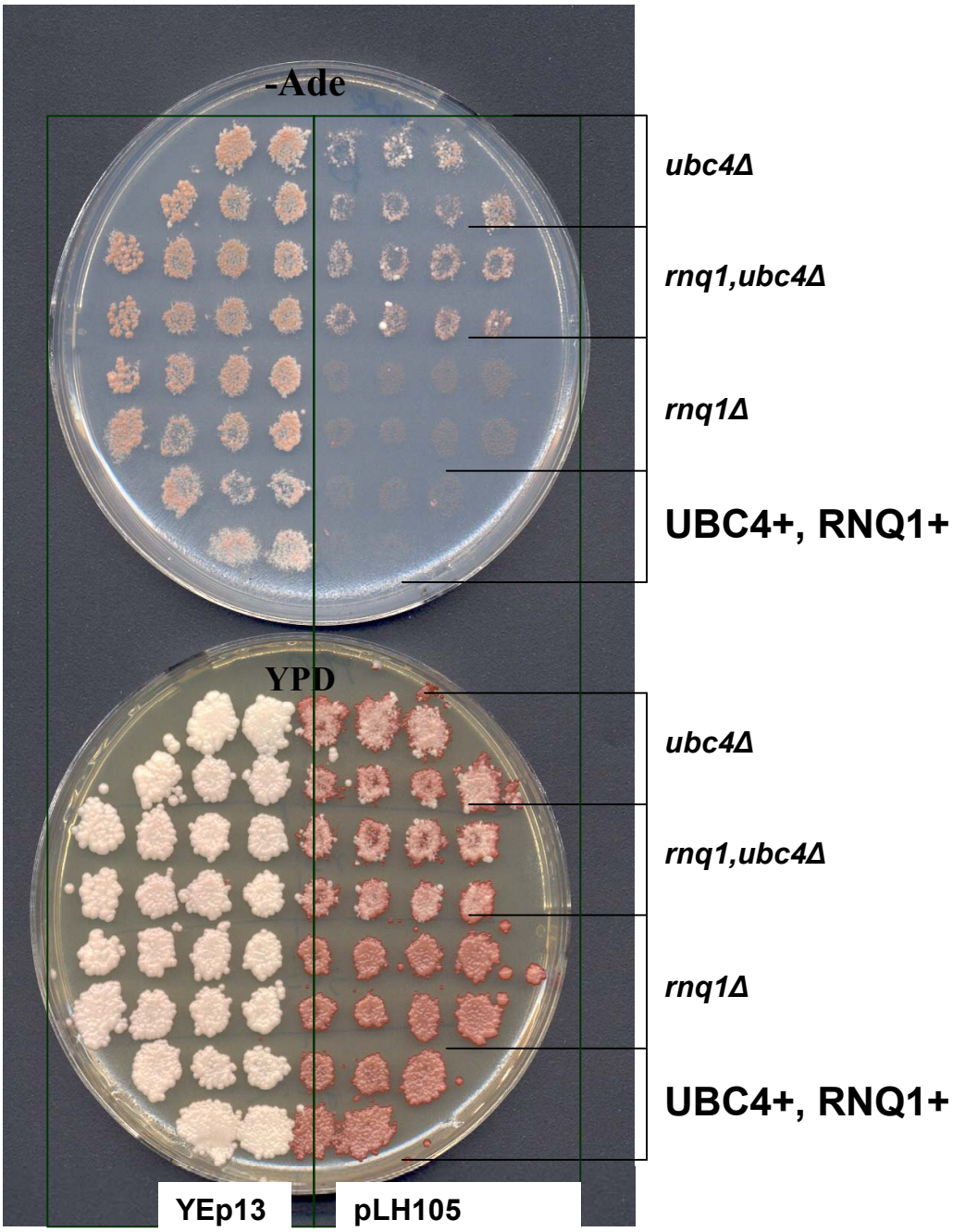


Figure 3.1. **Curing of *ubc4, rnq1*Δ strain by *HSP104* expressed under the GPD** Strains GT81-1C, *UBC4*$^{+}$*, RNQ1*$^{+}$*,* GT349, *ubc4Δ*, GT563, *rnq1Δ*, and GT784-8B, *ubc4,rnq1Δ* were transformed with Yep13,an empty vector plasmid and pLH105, the plasmid expressing *HSP104* under the GPD promoter. Transformants were cultured on media lacking leucine and then duplicated by velveteen onto media lacking adenine and YPD. All patches of *UBC4*$^{+}$ cells appear completely cured. They are red on YPD, and fail to grow on –Ade media. The patches of *ubc4*Δ cells are partially cured. The deletion of *rnq1* does not appear to effect curing efficiency.

Curing of *ubc4, rnq1*Δ strain by *HSP104* expressed under the Galactose Promoter

Results

The [PSI$^+$][PIN$^+$] versions of a *UBC4*+, *RNQ1*+ strain, a *ubc4*Δ strain, a *rnq1*Δ strain, and the *ubc4, rnq1*Δ strain were transformed with a plasmid containing *HSP104* expressed under the Galactose promoter and a *URA* marker,pGAL104, and also a control plasmid that contained only the *URA* marker,pRS316GAL. Several colonies were selected from the transformation plates and were patched onto media lacking uracil. These plates were cultured at standard conditions for 5 days, and were then duplicated by velveteen onto media lacking uracil with galactose as sole carbon source. These plates are cultured for 5 days and then duplicated onto YPD and media lacking adenine. The curing was assessed by examining the color of the patches on YPD and growth on media lacking adenine. (For a detailed protocol, see Materials and Methods) As seen in figure 3.2, *ubc4*Δ strains appear to have less red cells on YPD and slightly more growth on media lacking adenine than the *UBC4*+ strains.

Conclusion

Curing by *HSP104* expressed under the galactose promoter is not effected by the deletion of *rnq1*. Curing is decreased in both *ubc4*Δ strains independent of the presence of the Rnq1 protein. Curing is similar in *rnq1*Δ and *RNQ1*+ strains. The function of Rnq1 is not involved in the curing of [PSI$^+$] by the over expression of *HSP104*.

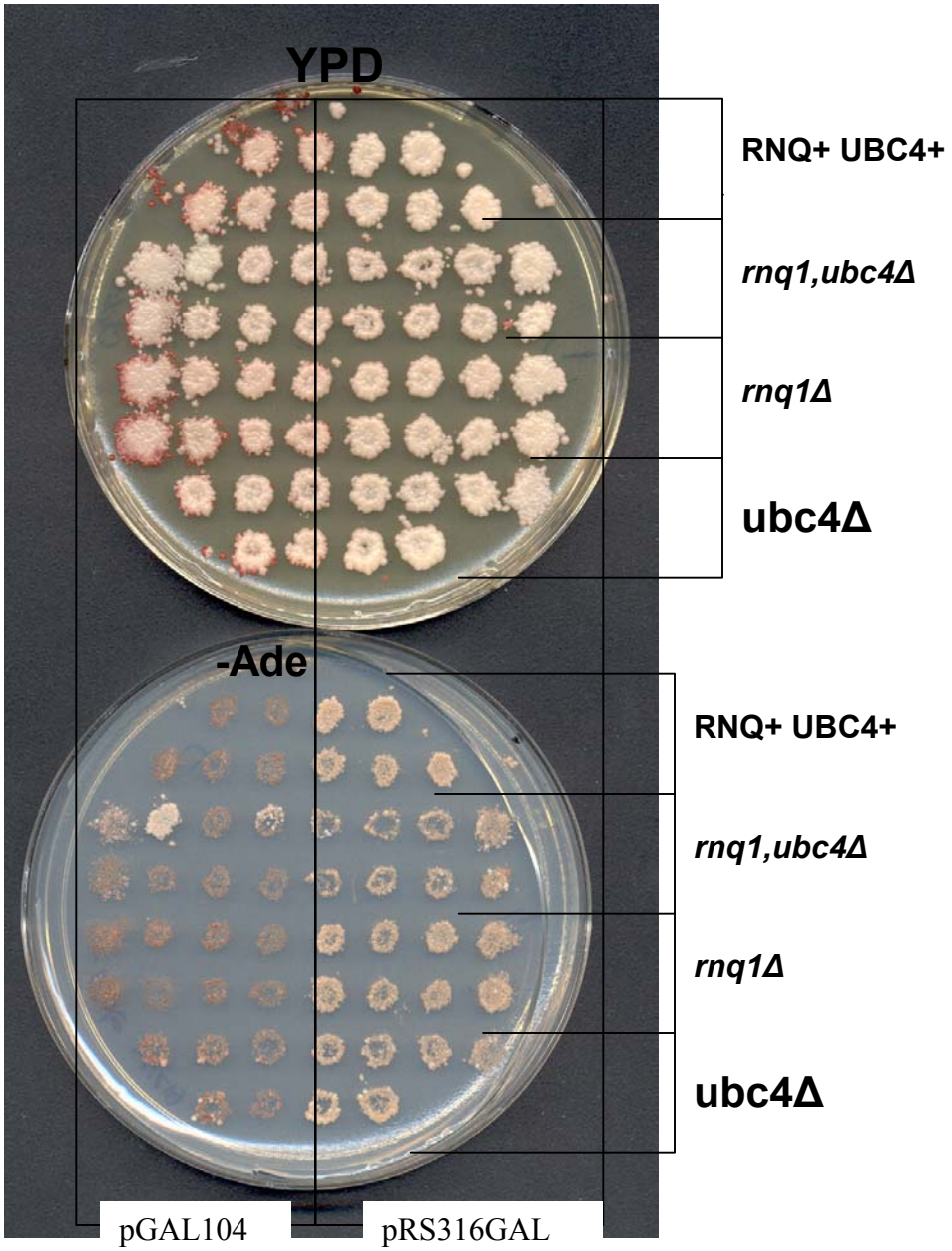


Figure 3.2. **Curing of *ubc4, rnq1*Δ strain by *HSP104* expressed under the Galactose Promoter** Strains GT81-1C, *UBC4*$^{+}$, *RNQ1*$^{+}$, GT349, *ubc4Δ*, GT563, *rnq1Δ*, and GT784-8B, *ubc4,rnq1Δ* were transformed with pRS316GAL,an empty vector plasmid and pGAL104, the plasmid expressing *HSP104* under the galactose promoter for 5 days. Transformants were cultured –Ura media and then duplicated by velveteen onto –Ura media with galactose as the sole carbon source and cultured for 5 days. This plate was duplicated one –Ade media and YPD. All patches of *UbC4*$^{+}$ cells appear only partially cured by color on YPD after 1 week. The patches of *ubc4*Δ appear less cured. On –Ade media after 5 days, a greater portion of curing seems to be apparent, but there is still less curing in the *ubc4*Δ strains. The deletion of *rnq1* does not appear to effect curing efficiency.

Spontaneous Occurrence of [PSI$^+$] in the *ubc4, rnq1*Δ strain

Results

Isolated colonies of a *RNQ1*+, *UBC4*+ strain, *an ubc4* Δ strain, a *rnq1* Δ strain, and an *ubc4, rnq1* Δ strain were used to create patches of approximately the same size on YPD. These plates were cultured using standard conditions for 3 days, and then duplicated by velveteen upon the selective media lacking adenine. The –Ade plates were cultured using standard conditions for 20 days.

There are two types of papillae that develop. The larger white papillae are not curable by GuHCl, and are therefore, assumed to be tRNA suppressor mutations. The smaller pink papillea are curable by GuHCl, so are considered to be indicative of the presence of the prion, [PSI$^+$].

As predicted from previous experimentation, spontaneous [PSI$^+$] was a relatively rare event in the *RNQ*+, *UBC4*+ strains. Papillae appear at a much lower frequency when the prion [PIN$^+$] is absent. Previous repetitive experimentation has shown that the papillation occurs at a much higher rate in both the [PIN$^+$] and the [pin$^-$] strains in the absence of *ubc4*. The patches in figure 3.3 show that these results are consistent with the papillation predicted for that of the *UBC4*+ and the *ubc4*Δ strains.

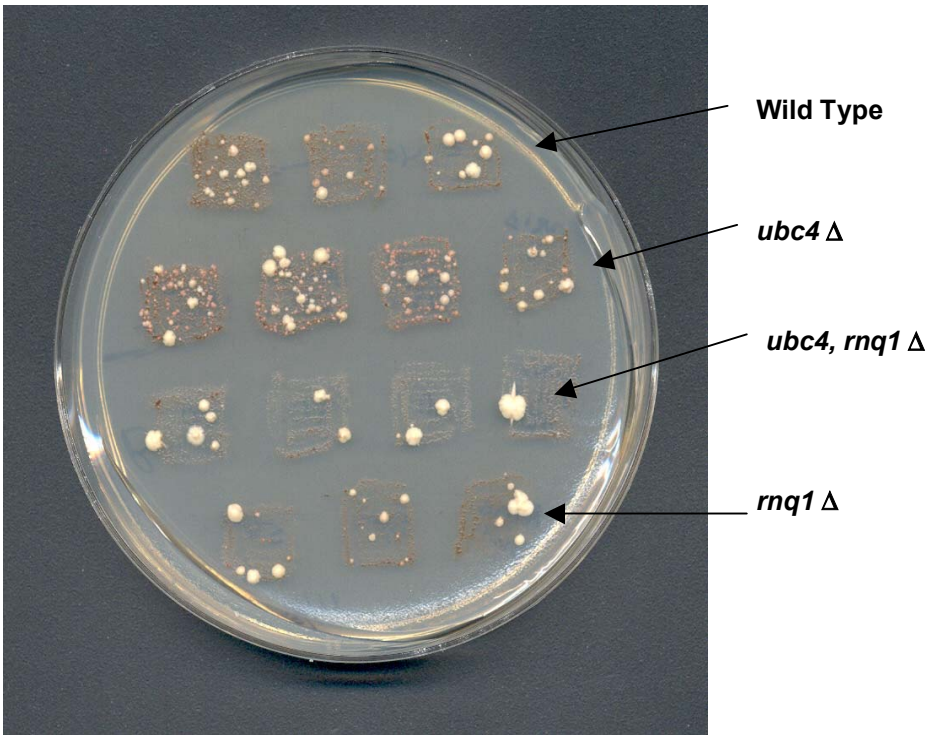


Figure 3.3. **Spontaneous Occurrence of [PSI$^+$] in the *ubc4, rnq1*Δ strain**
The [psi$^-$],[pin$^-$] strains GT409, *UBC4*$^+$, *RNQ1*$^+$, GT387, *ubc4Δ*, GT564, *rnq1Δ*, and GT830, *ubc4,rnq1Δ* were patched on YPD and allowed to grow for 3 days, then duplicated on –Ade media. After 20 days, it became evident that the double deletion strain had relatively less papillae than that of WT or either single deletion strains. This illustrates the requirement for the Rnq1 protein for the formation of spontaneous [PSI$^+$].

There was absence of any papillae in the *ubc4*, *rnq1* double deletion strain, and very little papillation was evident in the *rnq1* Δ strain. This is best illustrated in figure 4. The only growth evident in the *ubc4,rnq1* Δ strain patch, is those consistant with tRNA suppressor mutations that are not curable by GuHCl. The spontaneous occurrence of [PSI$^+$] in independent of the presence of [PIN$^+$] in the absence of *ubc4*, but this pathway of prion formation is dependent upon some action of the Rnq1 protein.

Table 3.1: Spontaneous Occurrence of [PSI$^+$] in the *ubc4* Δ, *rnq1*Δ Strain The number of papillae counted on patches –Ade media				
Genotype	*RNQ1+*, *UBC4+*	*rnq1*Δ	*ubc4*Δ	*ubc4*Δ, *rnq1*Δ
Number of Papillae	5	2	1	3
	1	6	53	0
	0	15	150	0
	7	30	76	0
	4	2	75	1
	0	3	4	0
		0		0
Average	2.83	8.29	59.83	0.57

Conclusion

The function of the Rnq1 protein has yet to be determined. It is known that the prion form of Rnq1, [PIN$^+$], is required for the de novo formation of [PSI$^+$] induced by the over production of Sup35. Also, in most strains, the spontaneous occurrence of [PSI$^+$] is extremely rare in [pin$^-$] strains when compared to their [PIN$^+$] versions. The *ubc4Δ* strain is an exception in which the substantial increase of spontaneous [PSI$^+$] appearance is independent of the presence of [PIN$^+$]. This research shows that the *rnq1*Δ strain is comparable to wild type such that the occurrence of spontaneous [PSI$^+$] is rare. However, when both *ubc4* and *rnq1* are absent, the development of [PSI$^+$] cells is totally eliminated.

Efficiency of curing by Hsp104 is not effected in the *rnq1*Δ strain unlike the *ubc4* deletion strain in which curing is decreased. Toxicity caused by the over expression of *SUP35* in [PSI$^+$] strains is also unaffected by the deletion of either *ubc4* or *rnq1*. This data suggests that the function of Rnq1 is related specifically to the pathway responsible for the spontaneous occurrence of [PSI$^+$].

Curing of *ubc4, ssb1,2*Δ strain by *HSP104* expressed under the GPD promoter

Results

The [PSI$^+$][PIN$^+$] versions of a *UBC4*+, *SSB1,2*+ strain, a *ubc4*Δ strain, a *ssb1,2*Δ strain, and the *ubc4, ssb1,2*Δ strain were transformed with a plasmid containing *HSP104* expressed under the GPD promoter and a *LEU* marker, pLH105, and also a control plasmid that contained only the *LEU* marker,Yep13. Several colonies were selected from the transformation plates and were patched onto media lacking leucine. These plates were cultured at standard conditions for 5 days, and were then duplicated by velveteen onto YPG, media lacking adenine, media lacking leucine, media lacking both adenine and leucine, and YPD. The curing was assessed by examining the color of the patches on YPD and growth on media lacking adenine. (For a detailed protocol, see Materials and Methods) As seen on the –Ade plate in figure 3.4, both the deletion of *ubc4* and ssb1,2 decrease curing by HSP04 over expression. This effect is much stronger in the triple deletion strain and is seen well on both the YPD and the - Ade plate.

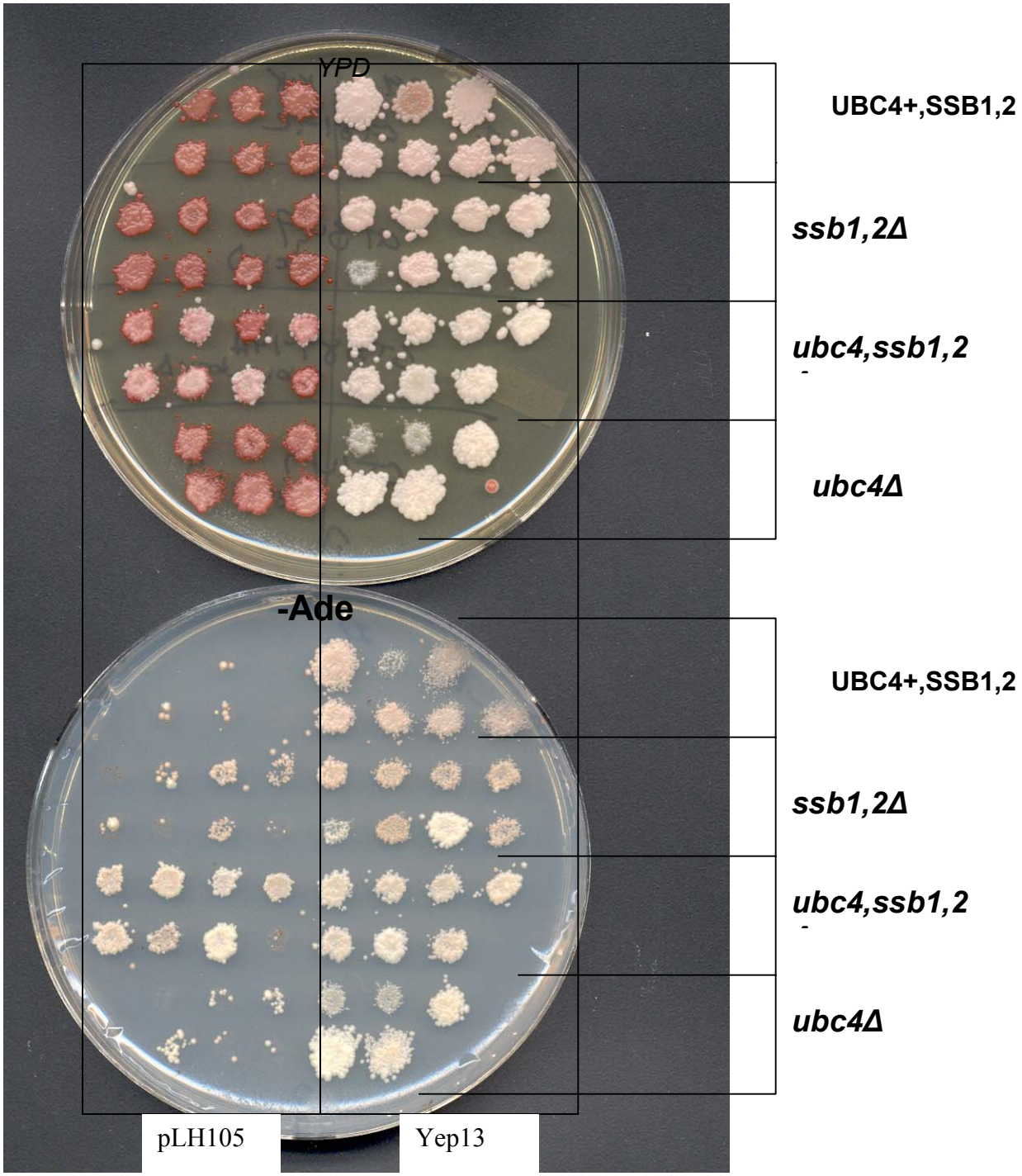


Figure 3.4. **Curing of *ubc4, ssb1,2*Δ strain by *HSP104* expressed under the GPD promoter.** Strains GT81-1C, *UBC4*$^{+}$*, SSB1,2*$^{+}$*,* GT349, *ubc4Δ*, GT147, *ssb1,2Δ*, and GT7389-14A, *ubc4,ssb1,2Δ* were transformed with YEp13,an empty vector plasmid and pLH105, the plasmid expressing *HSP104* under the GPD promoter for 5 days. Transformants were cultured on –Leu media and then duplicated by velveteen onto –Ade media and YPD. The –Ade was cultured for 7 days, and the YPD was cultured at standard conditions for 3 days and then placed at 4^{o}C for 1 week for color development. The *ssb1,2Δ* and wild type cells appear completely cured. The *ubc4Δ* cells are only partially cured. Curing is almost eliminated in the triple deletion strains.

Conclusion

The deletion of *ubc4* may have decreased curing by inhibiting the pathway that leads to protein degradation. The deletion of *ssb1* and *ssb2* decreased curing by Hsp104 by disabling the pathway in which proteins are refolded. The absence of SSB*1,2* and *UBC4* significantly reduced curing. The functions of two pathways involved in curing by Hsp104 are disabled in the triple deletion strain. This effect is sufficiently strong to be easily detected even when *HSP104* is expressed at a relatively much greater level as in P_{GPD}-HSP104.

Curing of *ssb1,2,ubc4Δ* strain by *HSP104* expressed under the Galactose Promoter

Results

The [PSI$^+$][PIN$^+$] versions of a *UBC4*+, *SSB1,2*+ strain, a *ubc4*Δ strain, a *ssb1,2*Δ strain, and the *ubc4, ssb1,2*Δ strain were transformed with a plasmid containing *HSP104* expressed under the Galactose promoter and a *TRP* marker, pFL39-HSP104GAL and also a control plasmid that contained only the *TRP* marker., pFL39 Three colonies that were PET+ and TRP+ were selected and cultured in liquid media containing galactose as the sole carbon source and lacking tryptophan. One-milliliter aliquots were taken at zero time point, at 24 hours, and then again at 54 hours. Cells were plated onto media selective for the plasmid from serial dilutions and allowed to culture at standard conditions for 5 days. These plates were duplicated by velveteen onto YPD and media lacking adenine. The colonies judged as cured of [PSI$^+$] failed to grow on media lacking adenine and also developed a red color on YPD. (For a detailed protocol, see Materials and Methods)

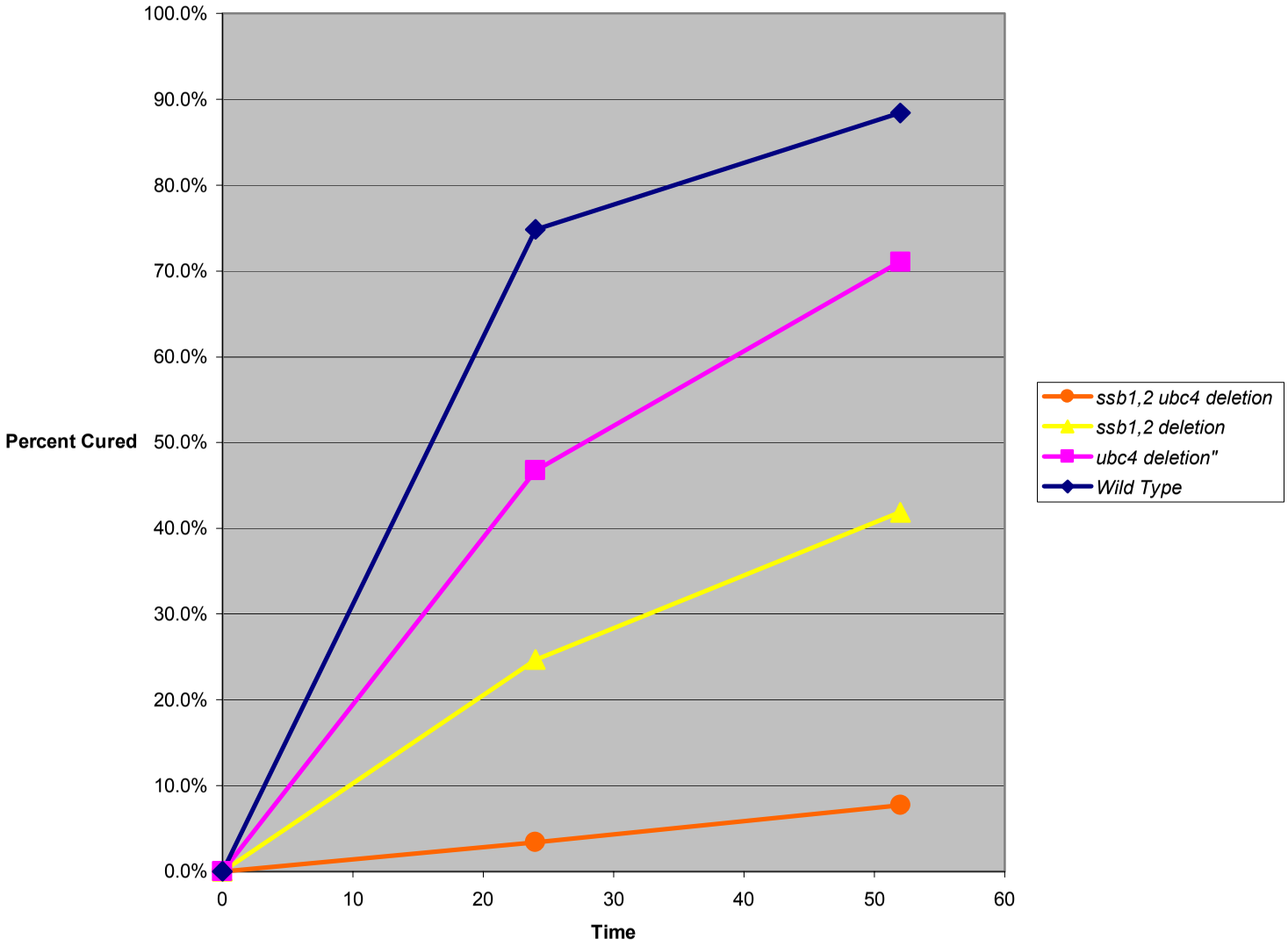


Figure 3.5. **Curing of *ssb1,2,ubc4Δ* strain by *HSP104* expressed under the Galactose Promoter**
Strains GT81-1C, Wild Type, GT349, *ubc4Δ*, GT147, *ssb1,2Δ*, and GT7389-14A, *ubc4,ssb1,2Δ* were transformed with pFL39,an empty vector plasmid and pFL39-HSP104GAL, the plasmid expressing *HSP104* under the galactose promoter. Transformants were cultured –Trp media and then grown in 150mls of liquid –Ttp media with galactose as the sole carbon source. Aliquots were removed and serial dilutions performed at 22 and 54 hours. Cells were plated on –Trp agar, and then duplicated upon –Ade and YPD. Curing was assessed using color on YPD, and further verified by growth on –Ade media. Curing was less efficient in the *ssb1,2Δ* strain relative to the *ubc4Δ* strain, and was further decreased in the strain which lacked all three genes.

Repetitive previous experimentation, throughout this project, has shown that curing by the over expression of *HSP104* is decreased in the *ubc4Δ* strain when ralative to the *UBC4*+ strain. Similarly, curing is also decreased in the *ssb1,2* Δ strain. At all time points examined, curing was further decreased in the strain in which all three genes, *ubc4*, *ssb1*,and *ssb2* were deleted. There was no evidence of curing in any of the colonies that contained the empty vector plasmid. (Data not shown) These results suggest that curing is being decreased by influencing two separate pathways.

Conclusion

Ubc4 is one of the enzymes responsible for the conjugation of the ubiquitin tag to proteins marked for degradation by the proteasome. The decreased efficiency of curing by Hsp104 suggests the utilization of the degradation pathway during Hsp104 curing.

The Ssb proteins are involved in cotranslational folding of nascent polypeptides (Neilson et al, 1992, Cell). It has been previously shown that curing efficiency is decreased in the *ssb1,2 Δ* background (Newnan et al, 1999, MCB). The decrease in efficiency of curing in the *ssb1,2 Δ* strain suggests that curing by Hsp104 simultaneously utilizes this folding pathway in addition to the degradation pathway.

The model in figure 3.6 illustrates the alternate destinations of Sup35 protein after its release from the aggregate facilitated by the action of Hsp104. The deletion of *ubc4* inhibits the degradation path, and the deletions of the

ssb1,2 genes inhibit the refolding path. The triple deletion strain creates a compounded inhibitory effect because both pathways are negatively modified leading to greater loss of curing efficiency by Hsp104 relative to each single deletion.

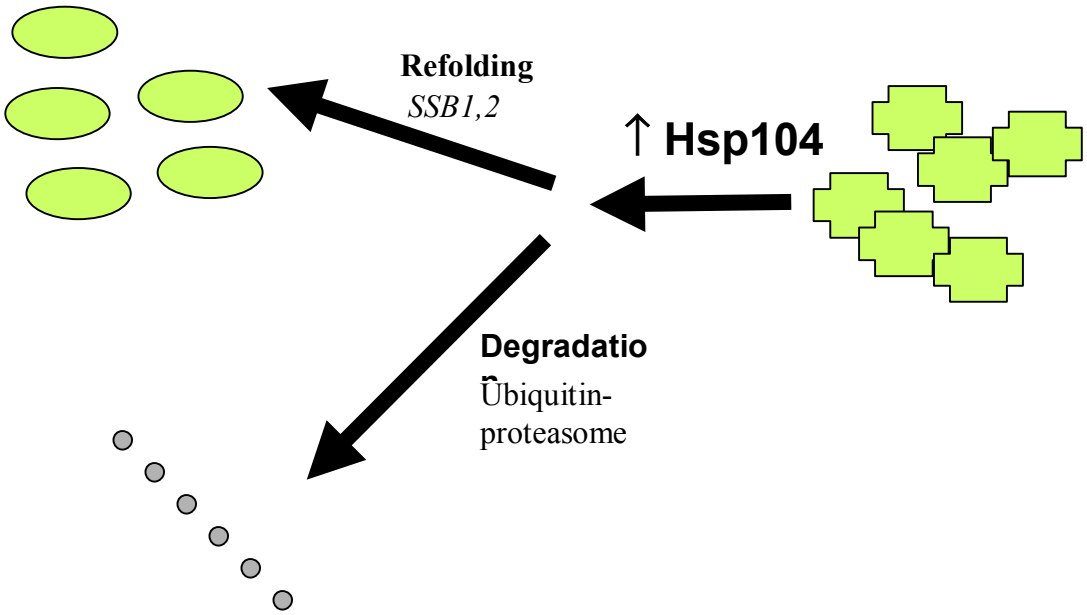


Figure 3.6. Model proposing two pathways involved in curing by Hsp104. This model proposes how curing can occur by two pathways. Both pathways are initiated by the disaggregase action of Hsp104. In one pathway the misfolded Sup35 is degraded by the action of the ubiquitin-proteasome pathway. The other is the correction in the folding of Sup35 facilitated by Ssb1,2 chaperone.

Spontaneous Occurrence of [PSI$^+$] in the *ubc4, ssb1,2* deletion strains

Results

Isolated colonies of both [PIN$^+$] and [pin$^-$] versions of [psi$^-$] *SSB1,2*+, *UBC4*+ strains, *a ubc4*Δ strain, *ssb1,2*Δ strains, and *ubc4, ssb1,2*Δ strains were used to create patches of approximately the same size on YPD. These plates were cultured using standard conditions for 3 days, and then duplicated by velveteen upon the selective media lacking adenine. The –Ade plates were cultured using standard conditions for 20 days. The smaller pink papillea were counted as spontaneous occurrences of [PSI$^+$].

As predicted from previous experimentation, spontaneous [PSI$^+$] was a relatively rare event in the *SSB1,2*+, *UBC4*+ strains on either plates containing [PIN$^+$] or [pin$^-$] versions of the strain. The *ubc4*Δ strains had a significantly greater number of papillae independent of the presence of [PIN$^+$]. The *ssb1,2Δ* strain had occurrences of [PSI$^+$] similar to that of the wild type strains All *ubc4*Δ strains had increased papillation independent of both [PIN$^+$] and the presence of SSB1 and SSB2. The deletion of *ssb1* and *ssb2* seem to have no effect upon the spontaneous occurrence of [PSI$^+$].

Table 3.2. **Spontaneous Occurrence of [PSI$^+$] in the [PIN$^+$] *ubc4, ssb1,2* Δ strains** GT159, *SSB1,2*$^+$, *UBC4*$^+$, GT408, *ssb1,2*Δ, GT386, *ubc4Δ* and GT823, *ubc4, ssb1,2*Δ strains were used to create patches of equal size on YPD and cultured at standard conditions for 2 days. The YPD plates were duplicated by velveteen on –Ade media and cultured for 20 days. The small pink papillae were counted and recorded for each patch. The wild type and the *ssb1,2*Δ strains show a similar rate of [PSI$^+$] occurrence. Likewise both strains with the deletion of *ubc4* show an increase occurrence relative to wild type. The *ubc4, ssb1,2*Δ deletion strain showed a slight increase of papillae when compared to the *ubc4Δ strain*, but this difference does not even represent a 2 fold increase, so it may not be significant.

The number of papillae counted on patches of [PIN$^+$] strains on –Ade media				
Genotype	*SSB1,2+*, *UBC4+*	*ssb1,2*Δ	*ubc4Δ*	*ubc4, ssb1,2*Δ
Number of Papillae	4	8	50	>300
	8	14	>300	40
	9	5	57	53
	10	10	54	>300
	7	6	56	150
	4	5	51	65
		7		250
Average	5.67	7.86	94.67	165.43

Table 3.3. **Spontaneous Occurrence of [PSI$^+$] in the [pin$^-$] *ubc4, ssb1,2* Δ strains.** GT409, *SSB1,2*$^+$, *UBC4*$^+$, GT175, *ssb1,2*Δ, GT387, *ubc4Δ* and GT858, *ubc4, ssb1,2*Δ strains were used to create patches of equal size on YPD and cultured at standard conditions for 2 days. The YPD plates were duplicated by velveteen on –Ade media and cultured for 20 days. The small pink papillae were counted and recorded for each patch. The wild type and the *ssb1,2*Δ strains show a similar rate of [PSI$^+$] occurrence. Likewise both *ubc4*Δ strains show an increase occurrence relative to wild type independent of the presence of *SSB1* and *SSB2*.

The number of papillae counted on patches of [pin$^-$] strains on –Ade media				
Genotype	*SSB1,2+*, *UBC4+*	*ssb1,2*Δ	*ubc4*Δ	*ubc4, ssb1,2*Δ
Number of Papillae	16	29	68	58
	30	24	46	62
	17	17	>300	89
	15	19	50	70
	33	9	63	150
	24	22	51	51
		16		37
Average	22.5	19.43	96.33	73.86

Conclusion

The spontaneous occurrence of [PSI$^+$] is greater in the *ubc4*Δ strain, and this increase in independent of the presence of the prion [PIN$^+$]. There is no effect when *SSB1* or *SSB2* are deleted. The mechanisms responsible for spontaneous [PSI$^+$] are not dependent upon the action of the Ssb proteins.

Curing of *ubc4, ubp6*Δ strain by *HSP104* expressed under the GPD Promoter

Results

The [PSI$^+$][PIN$^+$] versions of a *UBC4*+, *UBP6*+ strain, a *ubc4*Δ strain, a *ubp6*Δ strain, and the *ubc4, ubp6*Δ strain were transformed with a plasmid containing *HSP104* expressed under the GPD promoter and a *LEU* marker, pHS105, and also a control plasmid that contained only the *LEU* marker, YEp13. Several colonies were selected from the transformation plates and were patched onto media lacking leucine. These plates were cultured at standard conditions for 5 days, and were then duplicated by velveteen onto YPG, media lacking adenine, media lacking leucine, media lacking both adenine and leucine, and YPD. The curing was assessed by examining the color of the patches on YPD and growth on media lacking adenine. (For a detailed protocol, see Materials and Methods)

There was no obvious difference between any strains when cured by the high level of Hsp104 from plamsid P_{GPD}-Hsp04. In figure 3.7, all strains appear to be completely cured when looking at color on YPD. There is some evidence of [PSI$^+$] cells when looking at the cells transformed with pLH105 on the –Ade plate, particularly in the *ubc4Δ* background.

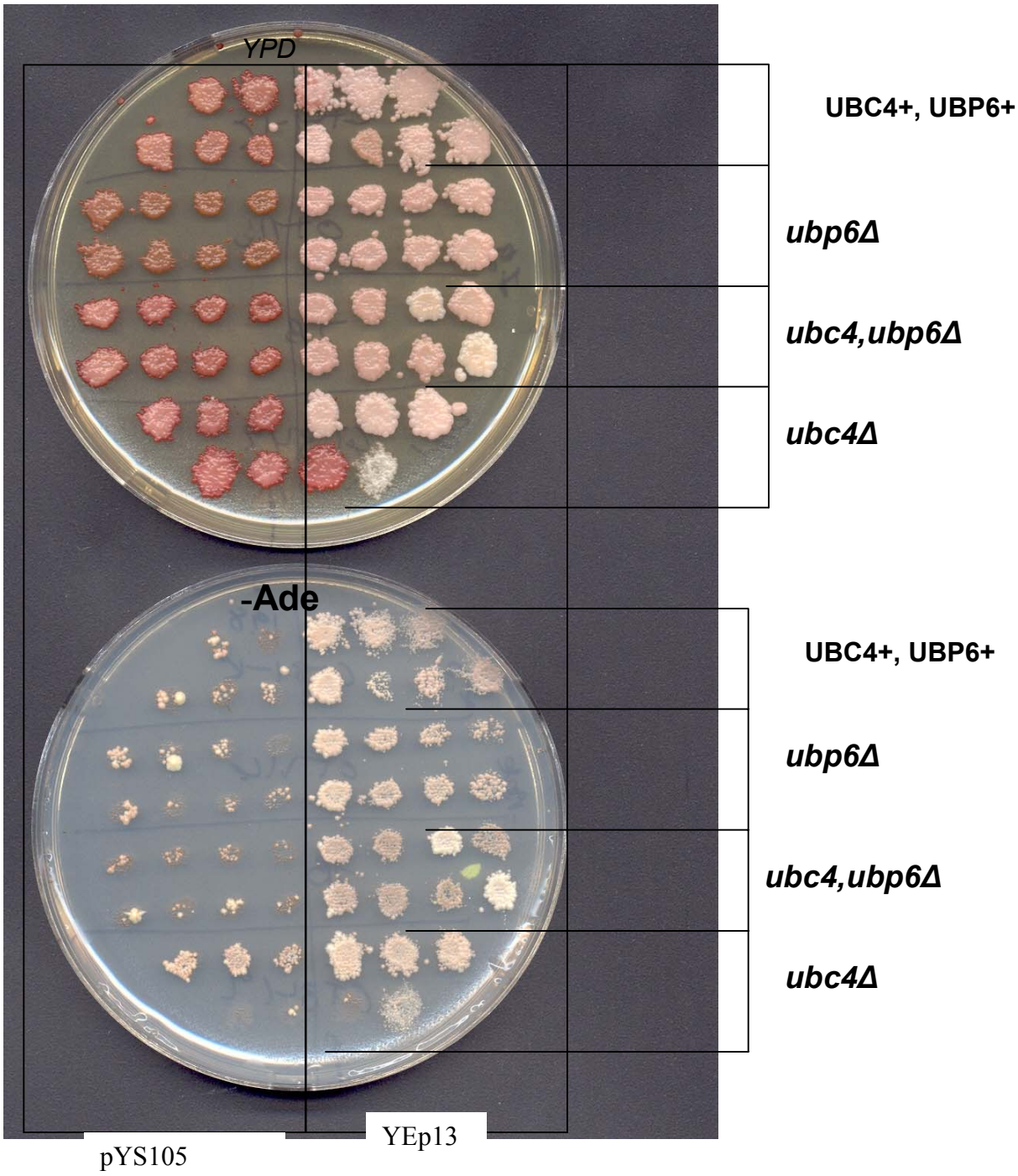


Figure 3.7. Curing of *ubc4, ubp6*Δ strain by *HSP104* expressed under the GPD Promoter. Strains GT81-1C, Wild Type, GT349, *ubc4Δ*, OT116, *ubp6Δ*, and GT684-8B,*ubc4,ubp6Δ* were transformed with YEp13, an empty vector plasmid and pLH105, the plasmid expressing *HSP104* under the GPD promoter for 5 days. Transformants were cultured on –Leu media and then duplicated by velveteen onto –Ade media and YPD. The *ubp6Δ* and wild type cells appear completely cured. The *ubc4Δ* cells are only partially cured. All strains appear to cure at similar rates on the YPD plate, but the *ubc4Δ*,strain has more growth on –Ade media. YPD was cultured at standard conditions for 2 days and then placed at 4oC for 1 week for color development. –Ade plates were cultured for 6 days.

Conclusion

Curing by HSP104 under the GPD promoter does not seem to be significantly reduced in any of the test strains. The high expression level allowed by the P_{GPD}-HSP104 promoter may mask effects that are not of sufficient strength to overcome the strong curing effect. This does not eliminate the possible role of the Ubp6 protein in Hsp104 curing, the results only show that the effect of the deletion of *ubp6* is not strong enough to be detected when using the GPD promoter.

Curing of *ubc4, ubp6*Δ strain by *HSP104* expressed under the Galactose Promoter

Results

The [PSI^+][PIN^+] versions of a *UBC4*+, *UBP6*+ strain, a *ubc4Δ* strain, a *ubp6*Δ strain, and the *ubc4,ubp6*Δ strain were transformed with a plasmid containing *HSP104* expressed under the Galactose promoter and a *URA3* marker,pGAL104, and also a control plasmid that contained only the *URA3* marker, pRS316GAL. Three colonies that were rho+ and URA+ were selected and cultured in liquid media containing 2% galactose as the sole carbon source but lacking uracil. One-milliliter aliquots were taken at zero time point, at 24 hours, 48 hours and then again at 80 hours. Serial dilutions were made and cells were plated onto media selective for the plasmid and allowed to culture at standard conditions for 5 days. These plates were duplicated by velveteen onto YPD and media lacking adenine. The colonies judged as cured of [PSI^+] failed to

grow on media lacking adenine and also developed a red color on YPD. (For a detailed protocol, see Materials and Methods)

Curing by the over expression of *HSP104* is decreased in the *ubc4*Δ strain when compared to the *UBC4*+ strain. These results show that curing is also decreased in the *ubp6*Δ strain. At 22 hours and also at 48 hours curing is decreased further in the double deletion strain relative to wild type and both single deletion strains. At the 80 hour time point, curing is decreased in all three mutant strains relative to wild type. There was no evidence of curing in any of the colonies that contained the empty vector plasmid or at the zero time point.

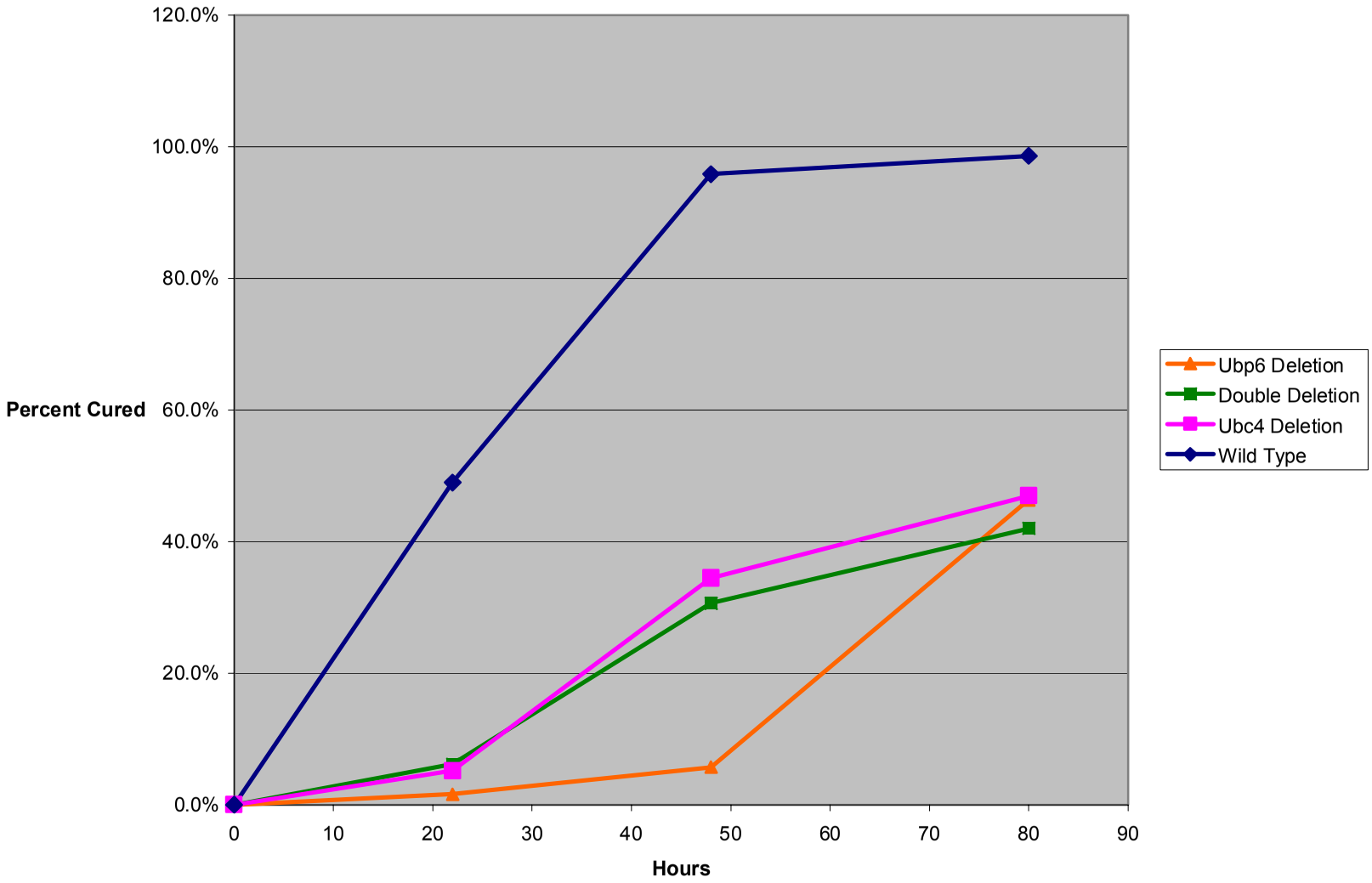


Figure 3.8. Curing of *ubc4, ubp6*Δ strain by *HSP104* expressed under the Galactose Promoter. Strains GT81-1C, *UBC4*$^{+}$, *UBP6*$^{+}$, GT349, *ubc4Δ*, OT116, *ubp6Δ*, and GT684-8B,*ubc4,ubp6Δ* were transformed with pGAL104, a plasmid that contains *HSP104* expressed under the galactose promoter, and pRS316GAL, and empty vector plasmid. Transformants were cultured –Ura media and then cultured in 150mls of liquid –Ura media with glactose as the sole carbon source. Aliquots were removed and serial dilutions performed at 22 and 54 hours. Cells were plated on –Ura, and then duplicated upon –Ade and YPD. Curing was assessed using color on YPD, and further verified by growth on –Adea. Curing was decreased in all three mutant strain relative to wild type. These single mutant strains have reduced curing efficiencies relative to wild type. The double deletion strain is most similar to the *ubc4Δ* strain.

Conclusion

The enzyme Ubc4 is one of the enzymes responsible for the conjugation of ubiquitin to the protein targeted for degradation by the proteasome. Ubp6 removes the ubiquitin tag prior to degradation allowing the ubiquitin to re-enter the cycle. In *ubp6Δ* strains, pools of free ubiquitin are reduced as a result of the degradation of the ubiquitin by the proteasome. When conjugation of ubiquitin is decreased in a background of decreased ubiquitin concentration, fewer proteins are able to enter the degradation pathway leading to degradation by the proteasome.

The efficiency of curing is similarly decreased in both the *ubc4*Δ strain and the *ubp6*Δ strain, as could be predicted by the known function of each of the proteins. The interaction between *ubc4*Δ and *ubp6*Δ is epistatic: Curing in the double deletion strain occurs at a efficiency similar to that of the single deletion of *ubc4.* This is also predictable when the function of the proteins is considered, as they work in the same pathway of ubiquitination-degradation.

Spontaneous Occurrence of [PSI$^+$] in the *ubc4, ubp6Δ* strains

Results

Isolated colonies of both [PIN$^+$] and [pin$^-$] versions of [psi$^-$] *UBP6*+, *UBC4*+ strains, *a ubc4*Δ strain, *ubp6*Δ strains, and *ubc4, ubp6*Δ strains were used to create patches of approximately the same size on YPD. These plates were cultured using standard conditions for 3 days, and then duplicated by velveteen upon the selective

media lacking adenine. The –Ade plates were cultured using standard conditions for 20 days. The smaller pink papillae were counted as spontaneous occurrences of [PSI^+].

As predicted from previous experimentation, spontaneous [PSI^+] was a relatively rare event in the *UBP6*+, *UBC4*+ strains on both the plates containing [PIN^+] an [pin^-] versions. The *ubc4Δ* strains had a significantly greater number of occurrences of [PSI^+] independent of the presence of [PIN^+]. The *ubp6Δ* strain had occurrences of [PSI^+] similar to that of the wild type strains. All *ubc4*Δ strains had increased papillation independent of both [PIN^+] and the presence of *UBP6* and *SSB2*. The deletion of *ssb1* and *ssb2* seem to have no effect upon the spontaneous occurrence of [PSI^+].

Table 3.4. **Spontaneous Occurrence of [PSI$^+$] in the [PIN$^+$] *ubc4, ubp6*Δ strains** GT159, *SSB1,2*$^+$, *UBC4*$^+$, OT117, *ubp6*Δ, GT386, *ubc4Δ* and GT949, *ubc4, ubp6*Δ strains were used to create patches of equal size on YPD and cultured at standard conditions for 2 days. The YPD plates were duplicated by velveteen on –Ade and cultured for 20 days. The small pink papillae were counted and recorded for each patch. The wild type and the *ubp6*Δ strains show a similar rate of [PSI$^+$] occurrence. Likewise both strains with the deletion of *ubc4* show an increase occurrence relative to wild type.

The number of papillae counted on patches of [PIN$^+$] strains on –Ade media				
Genotype	*UBP6+*, *UBC4+*	*ubp6*Δ	*ubc4Δ*	*ubc4, ubp6 Δ*
Number of Papillae	2	5	36	32
	7	15	>300	40
	9	5	62	42
	5	8	47	56
	12	4	>300	64
	9	11	26	76
		6		22
Average	7.33	7.71	128.5	98.86

Table 3.5. Spontaneous Occurrence of [PSI$^+$] in the [pin$^-$] *ubc4, ubp6*Δ strains. GT409, *UBP6*$^+$, *UBC4*$^+$, GT685, *ubp6*Δ, GT387, *ubc4Δ* and GT950, *ubc4, ubp6*Δ strains were used to create patches of equal size on YPD and cultured at standard conditions for 2 days. The YPD plates were duplicated by velveteen on –Ade and cultured for 20 days. The small pink papillae were counted and recorded for each patch. The wild type and the *ubp6Δ* strains show a similar rate of [PSI$^+$] occurrence. Likewise both *ubc4*Δ strains show an increase occurrence relative to wild type independent of the presence of *UBP6*.

The number of papillae counted on patches of [pin$^-$] strains on –Ade media				
Genotype	*UBP6+*, *UBC4+*	*ubp6*Δ	*ubc4Δ*	*ubc4, ubp6 Δ*
Number of Papillae	18	9	54	56
	20	12	43	72
	22	5	21	62
	2	7	19	22
	0	3	70	76
	6	0	87	92
		4		42
Average	11.33	11.42	49.0	60.28

Conclusion

The spontaneous occurrence of [PSI$^+$] is greater in the *ubc4*Δ strain, and this increase is independent of the presence of the prion [PIN$^+$]. There is no effect when *ubp6* is deleted. It is possible that the deletion of this gene does not produce a decrease in the free ubiquitin pool sufficient to see an effect on spontaneous [PSI$^+$]. The phenotypes associated with the deletion of genes coding for DUBs, de-ubiquitinating enzymes are much stronger in *doa4Δ* strains, but because of the defect of suppression in this strain, it is not possible to examine the spontaneous occurrence of [PSI$^+$].

Curing of *doa4Δ* and *ubp6Δ* strains by *HSP104* expressed under the GPD promoter and comparison of suppression

Results

The [PSI$^+$][PIN$^+$] versions of a *UBP6*+, *DOA4*+ strain, a *ubp6*Δ strain, and *dao4*Δ strain, were transformed with a plasmid containing *HSP104* expressed under the GPD promoter and a *LEU* marker and also a control plasmid that contained only the *LEU* marker. Several colonies were selected from the transformation plates and were patched onto media lacking leucine. These plates were cultured at standard conditions for 5 days, and were then duplicated by velveteen onto YPG, media lacking adenine, media lacking leucine, media lacking both adenine and leucine, and YPD. The curing was assessed by examination of the color of the patches on YPD and growth on the media lacking adenine.

The strain containing the wild type genes and the *ubp6Δ* strain were completely cured by Hsp104. This is evident by both color on YPD and by lack of growth on media lacking adenine. Color development on YPD shows a slight decreased in curing in the *doa4Δ* strain when compared to the *ubp6Δ* strain and the strain containing with wild type genes. This difference is not seen on media lacking adenine because growth of the *doa4Δ* strain is severely decreased on media lacking adenine due to the strong defect in nonsense suppression consistent with this strain.

Conclusion

The lack of expected growth on media lacking adenine is characteristic of a suppression defect. The *Ade1-14* nonsense mutation is not suppressed sufficiently in

the [PSI$^+$] version, so translation of the *Ade1* gene does not permit production of the full-length protein at a level equivalent to that of either the wild type strain or the *ubp6Δ* strain. Comparison of the [psi$^-$] *doa4Δ* with the [PSI$^+$] patches shows that curing can be assessed efficiently by color on YPD, but not efficiently on media lacking adenine.

Both *UBP6* and *DOA4* are de-ubiquitinating enzymes. The *doa4Δ* strain shows stronger phenotypes than that of the *ubp6Δ* strains. Temperature sensitivity, growth on media with galactose as the only carbon source and loss of viability at stationary phase are all phenotypes that are strongly evident in the *doa4Δ* strain (data shown in separate section of results). This suggests that the deletion of *doa4* decreases the pool of free ubiquitin to a greater extent than that of the deletion of *ubp6*.

A decreased pool of ubiquitin available for conjugation should have a similar effect as the deletion of a ubiquitin conjugating enzyme. Both mutations result in an inhibition of the conjugation of ubiquitin to the protein substrate. The YPD plate in figure shows that, as expected, curing is decreased in the *doa4Δ* deletion strain, but not in the *ubp6Δ* strain where the ubiquitin pools may not be sufficiently decreased to inhibit curing by *HSP104* by P_{GPD}-HSP04.

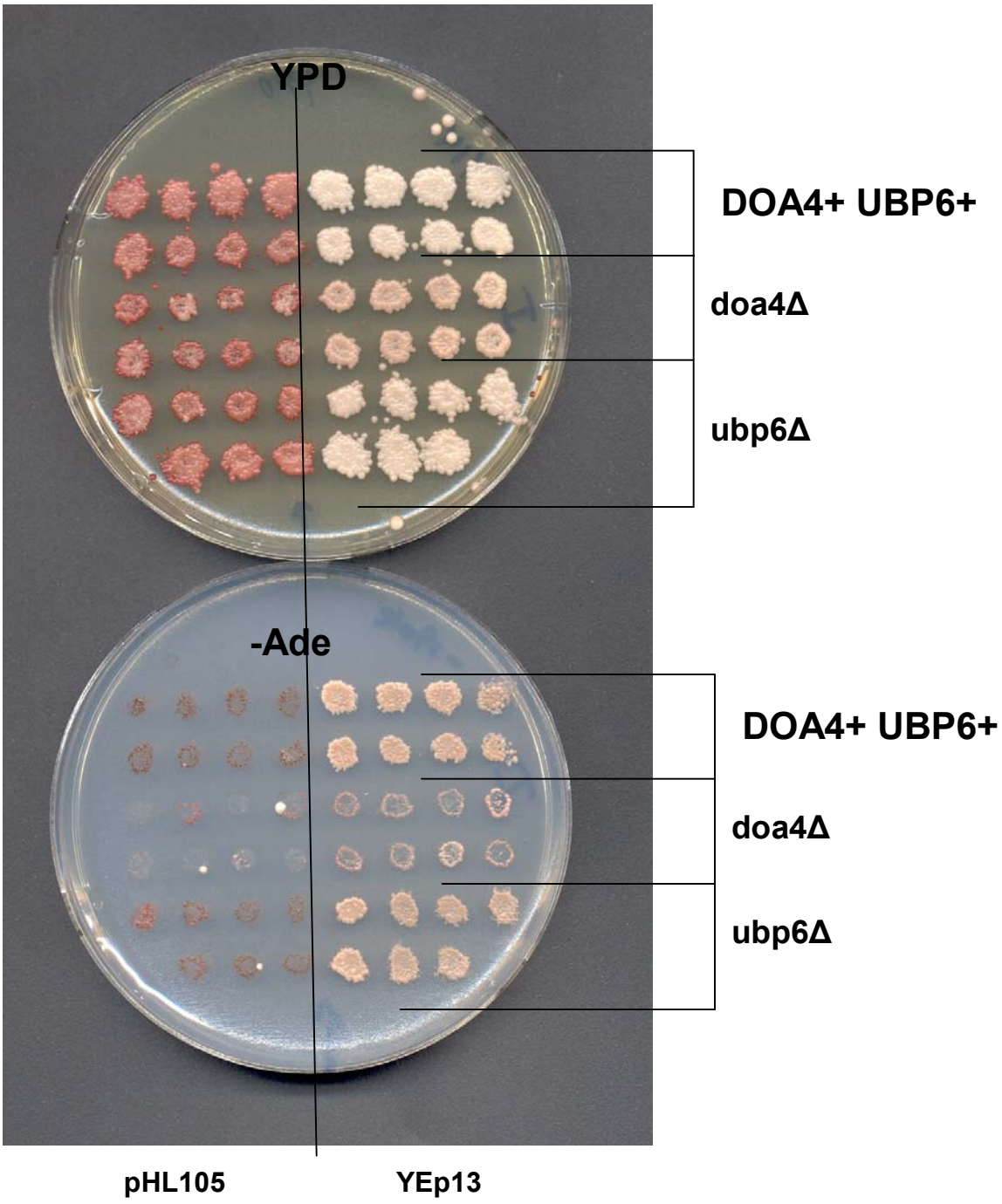


Figure 3.9. Curing of doa4Δ and ubp6Δ strains by HSP104 expressed under the GPD promoter and comparison of suppression. Strains GT81-1C, Wild type, OT116, *ubp6Δ*, and OT144, *doa4Δ* were transformed with YEp13, an empty vector plasmid and pLH105, the plasmid expressing *HSP104* under the GPD promoter. Transformants were cultured on –Leu media and then duplicated by velveteen onto –Ade and YPD. The *ubp6Δ* and wild type cells appear completely cured. The *doa4Δ* cells appear partially cured on YPD. The suppression defect prevents adequate assessment of curing of this strain. Color on YPD can be used to determine the status of [PSI^+]. YPD was cultured at standard conditions for 2 days, and placed on 4^oC for 1 week to develop color. –Ade was cultured for 7 days.

Curing of *ubc4, doa4*Δ strain by *HSP104* expressed under the GPD Promoter

Results

The [PSI$^+$][PIN$^+$] versions of a *UBC4*+, *DOA4*+ strain, a *ubc4*Δ strain, a *dao4*Δ strain, and the *ubc4, doa4*Δ strain were transformed with a plasmid containing *HSP104* expressed under the GPD promoter and a *LEU* marker and also a control plasmid that contained only the *LEU* marker. Several colonies were selected from the transformation plates and were patched onto media lacking leucine. These plates were cultured at standard conditions for 5 days, and were then duplicated by velveteen onto YPG, media lacking adenine, media lacking leucine, media lacking both adenine and leucine, and YPD. The curing was assessed by examination of the color of the patches on YPD. Lack of growth on adenine is not an efficient method to determine curing in the *doa4*Δ strain because of the severe nonsense suppression that is characteristic of this strain. Growth on media lacking adenine is only useful to double check controls. (For a detailed protocol, see Materials and Methods)

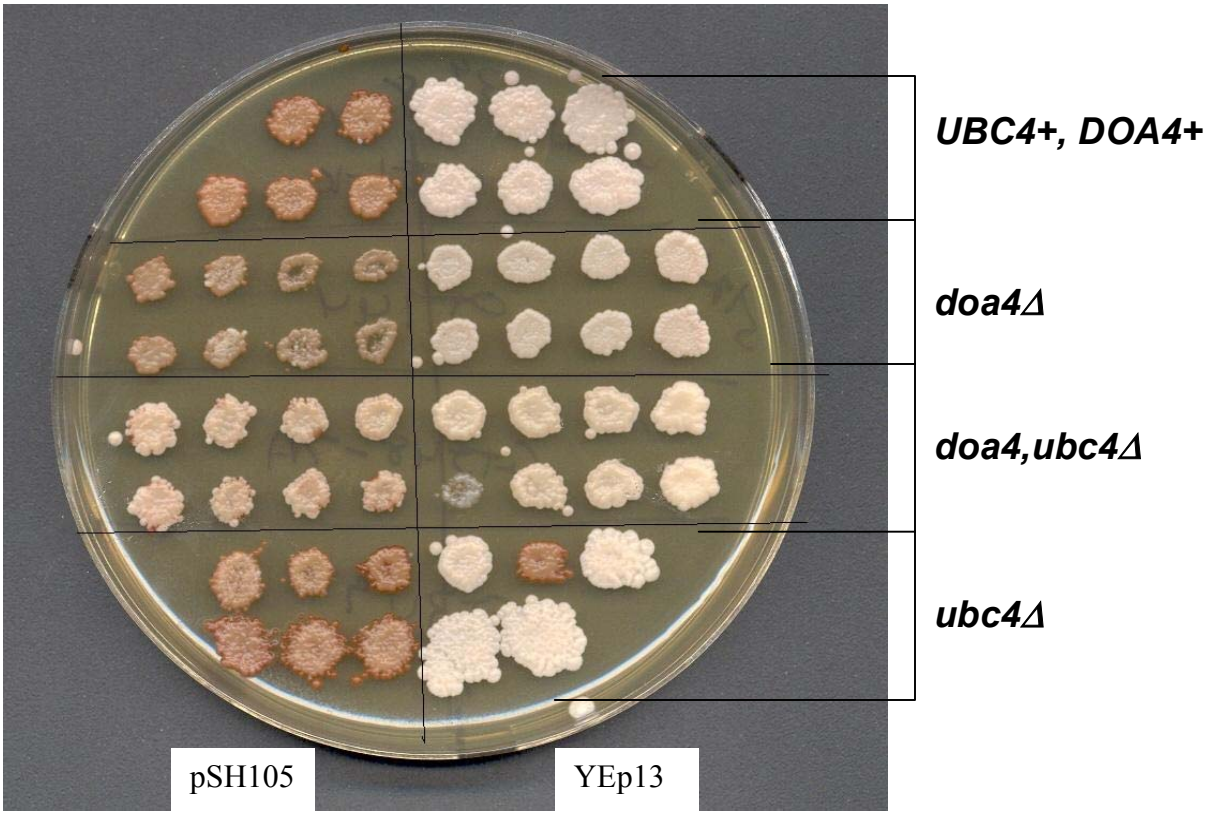


Figure 3.10. **Curing of *ubc4, doa4Δ* strain by *HSP104* expressed under the GPD Promoter.** Strains GT81-1C, Wild Type, GT349, *ubc4Δ*, OT144, *doa4Δ*, and GT538-7A, *ubc4,doa4Δ* were transformed with YEp13, an empty vector plasmid and pLH105, the plasmid expressing *HSP104* under the GPD promoter Transformants were cultured on –Leu and then duplicated by velveteen onto –Ade and YPD. The YPD seen above was cultured at standard conditions for 2 days and placed at 4^{o}C for 1 week for color development. Curing is almost totally eliminated in the double deletion strain. A slight decrease in curing efficiency is seen in *doa4Δ* strain, but not in the *ubc4Δ* deletion strain. The red colony on the right most likely represents a spontaneous loss of [PSI^{+}] that may occur as a result of the transformation procedure.

Conclusion:

Doa4 is one of the proteins responsible for the removal of ubiquitin from proteins prior to degradation by the proteasome. The deletion of *doa4* or *ubp6* results in a defect in the suppression of the nonsense mutation in the *ADE1* gene. (Chernova et al., 2003,

JBC 278:52). This phenotype, and others, are much more prevalent in the *doa4*Δ strain. This strain, unlike the *ubp6*Δ strain, fails to grow on media containing galactose as the sole carbon source. Therefore, it was necessary to use an alternative promoter to control the expression of *HSP104*.

Since the phenotypes are much stronger in cells lacking the Doa4, deubiquitinating enzyme, relative to the *ubp6*Δ strain, it is predictable that the efficiency of curing would be affected to a greater extent than that of *ubp6*Δ strain. The results support this prediction. Curing was almost eliminated in the double deletion strain, and slightly decreased in the *doa4Δ* strain.

Since the deletion of *ubp4* inhibits the removal of ubiquitin from protein prior to degradation, the level of free ubiquitin in the cells in substantially decreased. This decreases the amount of ubiquitin available for conjugating enzymes. The deletion of *ubc4* inhibits the conjugation of ubiquitin to proteins marked for degradation. The decreased ubiquitin pool and the further inhibition of conjugation by elimination of the enzyme responsible for conjugation should result in a magnified effect on curing efficiency. This is what is seen by the near elimination of curing by *HSP104* over expression in the double deletion strain. The results further support the role of the ubiquitin-proteasome system in curing by the over expression of *HSP104*.

Additional Phenotypes of the *doa4Δ* strain

Temperature Sensitivity

Serial dilutions of the *doa4Δ* strain, the *ubc4Δ* strain and the wild type strain, all [PSI^+], were created. These dilutions were used to create spots on YPD media, and then the plates were cultured at 25^{o}C, 30^{o}C, and 37^{o}C for three days.

Growth of all three strains was similar at both 25^{o}C and 30^{o}C. Growth was eliminated in the *doa4Δ* strain at 37^{o}C, but even more interesting is that the growth was partially restored in the *ubc4, doa4* double deletion strain. This supports the conclusion that these two genes code for proteins that act in the same pathway. When ubiquitin conjugation is inhibited, the free pool of ubiquitin is increased. This decreases phenotypes caused by severe depletion of the ubiquitin pool.

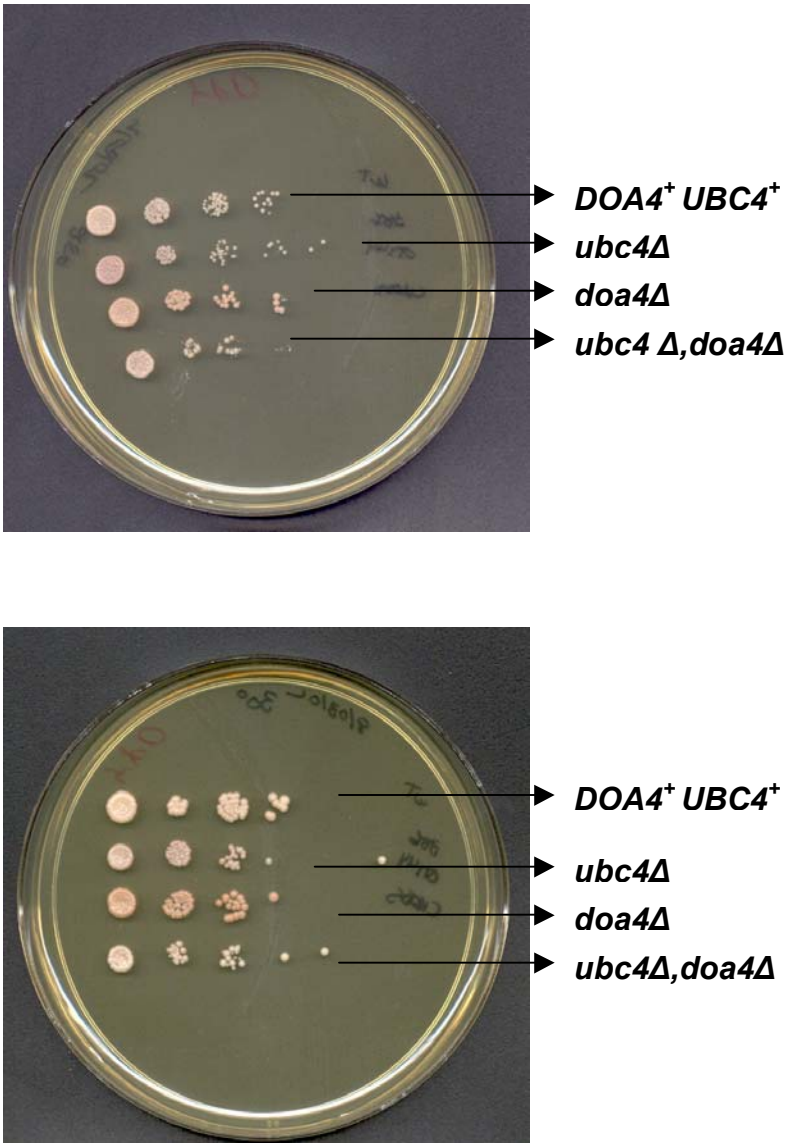
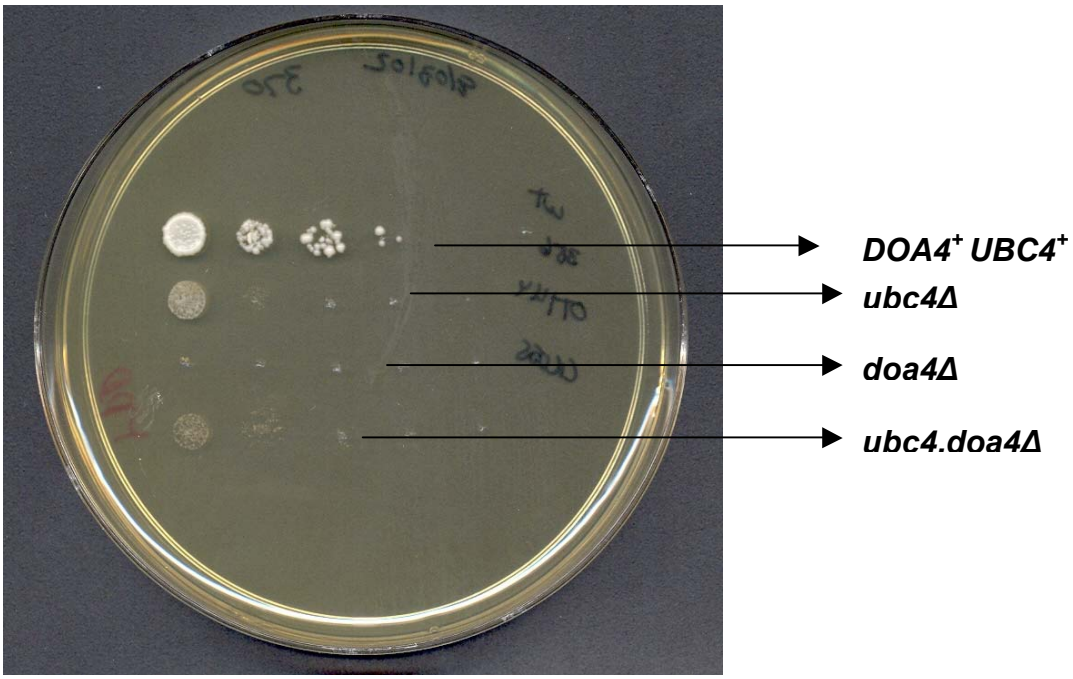


Figure 3.11. Growth of the *doa4Δ* and *ubc4Δ* strains. Dilution spots of OT144,*doa4Δ,* GT349*, ubc4Δ, and GT81-1C,* Wild Type, strains were cultured at 25°C and 30°C and 37°C for 4 days. There does not appear to be any difference between the four strains at either 25°C or 30°C temperature. Growth was eliminated in the *doa4Δ* strain at 37°C, but shows some restoration in the double deletion strain. The levels of free ubiquitin may be elevated in the *ubc4,doa4Δ* strain compared to that of the *doa4Δ* strain allowing for some reduction in phenotypes related to the decreased ubiquitin pools.

Figure 3.11 Continued



Growth of *doa4Δ* on media with galactose as sole carbon source

Strains GT81-1C, Wild Type GT349, *ubc4Δ*, OT144, *doa4Δ, and* OT116, *ubp6 Δ* strains were transformed with pGAL104, a plasmid that contains *HSP104* expressed under the galactose promoter, and pRS316GAL, and empty vector plasmid in an attempt to asses curing by Hsp104. The transformation was plated on –Ura and cultured at standard conditions for 5 days Colonies were selected and patched on –Ura, cultured, and then duplicated by veleveteen onto –Ura with 2% galactose as the sole carbon source. The *doa4Δ* strain failed to grow after 7 days. An –Ura plate was then again duplicated on –Ura media that contained 2% galactose and 2% raffinose as the carbon sources. The *doa4Δ* strain failed to grow after 7 days. This eliminated the use of the galactose inducible promoter for experimentation with the *doa4Δ* strain. (For detailed protocol for curing with *HSP104* under the galactose promoter, see Material and Methods section)

APPENDIX

Curing of *ssb1,2 Δ, ubc4 Δ* by *HSP104* Expressed Under the Galactose Promoter

			Time 24 Hours				Time 52 Hours			
Strain	Plasmid	Isolate	Total Colonies Examined	Percent Cured	Avg of Cured Isolates	Generations by CFU	Total Colonies Examined	Percent Cured	Avg of Cured Isolate	Generations by CFU
GT81-1C	123	A	65	0.00%	0.0%	3.55	93	0.0%	0.0%	8.27
			112	0.00%			207	0.0%		
							169	0.0%		
	123	B	51	0.00%	0.0%	3.99	123	0.0%	0.0%	7.16
			59	0.00%			270	0.0%		
			95	0.00%			408	0.0%		
	123	C	82	0.00%	0.0%	4.35	88	0.0%	0.0%	6.53
			74	0.00%			208	0.0%		
			170	0.00%			214	0.0%		
	474	A	135	76.30%	75.5%	4.78	30	90.0%	88.8%	7.51
			177	76.27%			36	91.7%		
			366	74.04%			210	88.1%		
	474	B	114	74.56%	75.2%	4.76	13	92.3%	88.1%	7.09
			202	73.27%			62	93.5%		
			166	77.71%			70	87.1%		
							91	84.6%		
	474	C	72	73.61%	74.3%	5.05	99	88.9%	88.4%	6.96
			153	74.51%			159	88.1%		
			191	74.35%						
GT349	123	A	85	0.00%	0.0%	2.64	236	0.0%	0.0%	5.33
			184	0.00%			476	0.0%		
			409	0.00%			612	0.0%		
	123	B	116	0.00%	0.0%	2.42	125	0.0%	0.0%	5.85

Curing of *ssb1,2 Δ, ubc4 Δ* by *HSP104* Expressed Under the Galactose Promoter Continued

Strain	Plasmid	Isolate	Total Colonies Examined	Percent Cured	Avg of Cured Isolates	Generations by CFU	Total Colonies Examined	Percent Cured	Avg of Cured Isolate	Generations by CFU
			147	0.00%			157	0.0%		
			182	0.00%			273	0.0%		
	123	C	61	0.00%	0.0%	2.77	84	0.0%	0.0%	5.09
			95	0.00%			259	0.0%		
			159	0.00%			332	0.0%		
	474	A	77	45.45%	43.7%	1.78	212	72.2%	68.7%	4.18
			207	43.00%			428	70.6%		
							497	65.6%		
	474	B	102	35.29%	47.4%	1.07	10	60.0%	73.5%	2.37
			157	47.13%			48	72.9%		
			178	54.49%			40	77.5%		
	474	C	132	43.18%	49.2%	1.22	99	76.8%	81.6%	5.8
			328	47.26%			195	84.6%		
			318	53.77%			250	81.2%		
GT147	123	A	87	0.00%	0.0%	3.15	158	0.0%	0.0%	6.86
			261	0.00%			199	0.0%		
			415	0.00%			314	0.0%		
		B	79	0.00%	0.0%	2.05	183	0.0%	0.0%	5.89
			128	0.00%			253	0.0%		
			140	0.00%						
		C	89	0.00%	0.0%	3.66	98	0.0%	0.0%	7.65
			158	0.00%			197	0.0%		
			206	0.00%						
	474	A	81	17.28%	24.0%	3.27	136	32.4%	38.0%	6.45
			244	23.77%			244	37.7%		
			205	26.83%			406	40.1%		
		B	232	19.40%	22.5%	3.43	144	38.9%	41.3%	4.92

Curing of *ssb1,2 Δ, ubc4 Δ* by *HSP104* Expressed Under the Galactose Promoter Continue

Strain	Plasmid	Isolate	Total Colonies Examined	Percent Cured	Avg of Cured Isolates	Generations by CFU	Total Colonies Examined	Percent Cured	Avg of Cured Isolate	Generations by CFU
			298	24.50%			171	43.3%		
			442	22.85%						
		C	118	27.97%	27.5%	3.34	111	51.4%	46.3%	6.48
			190	27.89%			131	48.1%		
			262	27.10%			246	43.1%		
GT389-14A	123	A	49	0.00%	0.0%	1.53	71	0.0%	0.0%	6.01
			167	0.00%			159	0.0%		
			340	0.00%			299	0.0%		
		B	82	0.00%	0.0%	3.01	70	0.0%	0.0%	6.59
			154	0.00%			170	0.0%		
			270	0.00%			288	0.0%		
		C	94	0.00%	0.0%	5.6	74	0.0%	0.0%	9.96
			180	0.00%			247	0.0%		
			234	0.00%			200	0.0%		
	474	A	106	8.49%	4.4%	3.78	123	5.7%	7.21%	6.95
			246	2.85%			213	9.9%		
			310	4.19%			235	11.1%		
		B	147	2.72%	2.3%	3.45	61	6.6%	5.8%	6.51
			403	0.99%			112	5.4%		
			589	3.06%						
		C	153	3.27%	3.6%	4.42	32	9.4%	10.1%	6.24
			240	5.00%			100	9.0%		
			307	2.61%			125	11.2%		

Curing of *ubp6 Δ*, *ubc4 Δ* Strain by *HSP104* Expressed Under the Galactose Promoter

Strain	Isolate	Total Colonies Examined	Percent Cured	Avg of Cured Isolate	Generations by CFU	Total Colonies Examined	Percent Cured	Avg of Cured Isolate	Generations by CFU	Total Colonies Examined	Percent Cured	Avg of Cured Isolate	Generations by CFU
GT81-1C	A	236	59.3%	48.9%	1.85	217	94.1%	95.8%	6.55	322	98.6%	97.8%	7.84
	B	404	42.6%		3.58	281	96.1%		6.87	420	98.1%		8.26
	C	485	44.9%		5.17	292	97.3%		7.94	478	96.6%		8.82
GT349	A	252	9.1%	5.2%	1.03	205	36.9%	34.4%	4	711	46.9%	37.9%	6.45
	B	708	3.8%		4.16	631	29.9%		5.81	990	29.7%		7.84
	C	333	2.6%		1.66	208	36.5%		3.81	208	37.1%		5.6
OT116	A	261	0	1.6%	0	427	9.5%	5.7%	4.35	850	46.9%	46.3%	7.11
	B	414	4.8%		1.82	288	3.5%		4.06	202	53.8%		4.92
	C	109	0		0	331	4.1%		1.08	730	38.3%		6.16
GT832-7B	A	690	3.5%	6.2%	1.82	783	31.7%	30.6%	5.44	517	41.9%	42.0%	8.23
	B	392	13.8%		2.15	417	34.5%		5.24	222	44.2%		6.21
	C	548	1.3%		0.7	594	25.7%		5.47	299	39.9%		7.77

Mosaics are counted as [PSI^{+}]. Growth on –Ade was used to verify curing, and a colony that is mosaic will grow on –Ade.

Construction of Strains for This Study

Strain	Construction
GT349	PCR-mediated gene transplacement was accomplished using plasmid pFA6A-HIS3MX6 to displace *UBC4* with *HIS3* in the strain GT81-1C
GT386	GT349 cured by *HSP104* over expression
GT387	GT349 cured by GuHCl
GT685	OT116 cured by GuHCl
GT823	GT398-14A cured by *HSP104* over expression
GT563	PCR-mediated gene transplacement was accomplished using plasmid pFA6A-HIS3MX6 to displace *RNQ1* with *HIS3* in the strain GT81-1C
GT564	PCR-mediated gene transplacement was accomplished using plasmid pFA6A-HIS3MX6 to displace *RNQ1* with *HIS3* in the strain GT159
GT832-7B	Cross and dissection of OT116 and GT532-9C
GT532-9C	Created by a cross between GT580 (*ubc4::HIS3*) and GT385-13A (*ubc4::HIS3*) for previous research.
GT949	GT832-7B cured by *HSP104* over expression
GT950	GT832-7B cured by GuHCl
GT784-8B	Cross and dissection between GT563 and GT532-9C
GT389-14A	Cross GT127 (*ssb1::HIS3, ssb2::URA3*) and GT385-13A (*ubc4::HIS3*)
GT820	GT784-8B cured by *HSP104* over expression

Plasmid Constructions for This Study

PFL39-HSP104GAL	PGAL104 was cut with HXO1 and SAC1. The entire *HSP104* under the *GAL1* promoter was inserted into pFL39 with was cut with SAL1 and SAC1

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