# Functional significance of Hsp70 post-translational modification in prion propagation and cellular function


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## DECLARATION OF AUTHORSHIP

This thesis has not been submitted in whole or in part, to this or any other University, for any degree and is except otherwise stated the original work of the author.

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## ABSTRACT

The term prion (proteinaceous infectious particles) was first coined by Stanley Prusiner while naming the causative agent responsible for a group of invariably fatal neurodegenerative diseases collectively termed transmissible spongiform encephalopathies (TSE). A breakthrough in prion research came with the studies which revealed that yeast species *Saccharomyces cerevisiae* contains proteins that have the ability to form prions. Sup35 is a *S. cerevisiae* protein involved in termination of translation. In a prion state referred to as [*PSI*$^+$], a significant portion of the Sup35 protein in the cell coalesces into non-functional, self-propagating, amyloid-like polymers. Thus, yeast strains that are [*PSI*$^+$] show increased levels of nonsense suppression. Once present, [*PSI*$^+$] propagates by recruitment of the soluble form of Sup35 into the aggregate in a manner analogous to that of mammalian prions. A search for genetic factors affecting propagation and maintenance of [*PSI*$^+$] has identified an essential role for molecular chaperones, namely Hsp70 and Hsp104. The Hsp70 chaperone family and its associated co-chaperones are highly conserved from yeast to mammals. A major function of Hsp70 is to prevent the aggregation of denatured proteins by binding to exposed hydrophobic regions and preventing the accumulation of amorphous aggregates. In the model eukaryotic *S. cerevisiae*, to efficiently carry out such functions Hsp70 works in concert with a number of co-chaperones to regulate ATPase hydrolysis cycle of Hsp70, which in-turn dictates the peptide-binding status of Hsp70. While much data has accrued in relation to the ATPase and substrate binding cycles of Hsp70 there is a distinct lack of information regarding the regulation of this important chaperone at the post-translational level. Recent global proteomic studies have demonstrated that *in vivo* Hsp70 is phosphorylated. Using a simplified yeast system this study systematically assessed a variety of non-phosphorylateable and phosphomimetic Hsp70 mutants for phenotypic alterations in Hsp70 functions. It was found that alteration of Hsp70 phosphorylation status *in vivo* can impair prion propagation, alter both basal and acquired thermotolerance and in some cases render cells inviable. By looking at analogous mutants in closely related cytosolic Hsp70s this study identified functional similarities and differences between highly homologous Hsp70 species. This study shows a clear link between Hsp70 phosphorylation status and *in vivo* function. Given Hsp70s central role in a variety of important cellular metabolic pathways and the conservation of these phosphorylatable sites in higher eukaryotes, these findings have far reaching implications.

## ABBREVIATIONS

**CHAPTER1**

| | |
|---|---|
| TSE | Transmissible spongiform encephalopathies |
| CJD | Creutzfeldt-Jakob disease |
| GSS | Gerstmann-Straussler-Sheinker disease |
| BSE | Bovine spongiform encephalopathy |
| PrP | Prion protein |
| CNS | Central nervous system |
| kDa | Kilo Dalton |
| nm | Nano meter |
| UV | Ultraviolet |
| % | Percent |
| M/V | Methionine/Valine |
| vCJD | Variant Creutzfeldt-Jakob disease |
| HGH | Human growth hormone |
| SDS | Sodium dodecyl sulphate |
| RF | Release factors |
| GTP | Guanosine triphosphate |
| PrD | Prion domain |
| QNR | Glutamine Asparagine rich |
| OPR | Oligopeptide repeat region |
| mm | Milli meter |
| Q/N | Glutamine /Asparagine |
| Hsp | Heat shock protein |
| AIR | p-ribosylaminoimidazole |
| CAIR | p-ribosylaminoimidazolecarboxylate |
| ER | endoplasmic reticulum |
| ATP | Adenosine triphosphate |
| UPS | Ubiquitin−proteasome system |
| Ssa | Stress seventy subfamily A |
| NEF | Nucleotide exchange factors |
| RAC | Ribosome –associated complex |

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| °C | Degree Celcius |
| PBD | Peptide-binding domain |
| NBD | Nucleotide binding domain |
| SBD | Substarte binding domain |
| CTD | C-terminal domain |
| ADP | Adenosine triphosphate |
| Sti1 | Stress inducible protein 1 |
| Cns1 | Cyclophilin seven suppressor 1 |
| TPR | tetratricopeptide repeat |
| AAA | ATPase associated with a variety of cellular activities’ |
| WT | Wildtype |
| Gdn-HCl | Guanidine Hydrochloride |
| OR | Oligopeptide repeat |
| 3D | 3-dimensional |
| 2D | 2-dimensional |
| qPCR | Quantitative polymerase chain reaction |
| PRPP | P-ribosyl-PP |

**CHAPTER 2**

| | |
|---|---|
| SDM | Site directed mutagenesis |
| w/v | Weight per total volume |
| v/v | Volume per total volume |
| w/w | Weight per total weight |
| µM | Micromolar |
| RT | Room temperature |
| L | Litre |
| g | Gram |
| mM | Millimolar |
| ml | Millilitre |
| mg | Milligram |
| 5-FOA | 5-fluoro-orotic acid Medium |
| YPD | Yeast Peptone Dextrose |
| YPAD | Adenine-supplemented Yeast Peptone Dextrose |

| | |
|---|---|
| SC | Synthetic Complete |
| $H_2O_2$ | Hydrogen peroxide |
| µl | Microlitre |
| CW | Calcofluor white |
| LB | Luria Broth |
| rpm | Repetitions per minute |
| OD | Optical density |
| M | Mole |
| PEG | Polyethylene glycol |
| EDTA | Ethylenediaminetetraacetic acid |
| WT | wild-type |
| dNTP | Deoxynucleotide triphosphate |
| $dH_2O$ | Distilled water |
| pmol | Picomole |
| MGT | Mean generation time |
| BSA | Bovine serum albumin |
| SDS-PAGE | Sodium Dodecyl Sulphate-Poly acrylamide gel electrophoresis |
| TEMED | Tetramethylethylenediamine |
| V | Volt |
| PVDF | Polyvinylidene difluoride |
| mA | Milli ampere |
| TBS-t | Tris buffered saline-tween |
| NaCl | Sodium chloride |
| TBS | Tris buffered saline |
| MeOH | Methanol |
| 2D-GE | Two dimensional gel electrophoresis |
| IEF | Isoelectric focussing |
| DTT | Dithiothreitol |
| CHAPS | 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| APS | Ammonium persulfate |
| TCA | Trichloroacetic acid |
| µg | Microgram |
| cm | Centimetre |
| CIP | Calf intestinal phosphatases |

| | |
|---|---|
| RNA | Ribonucleic acid |
| RT | Reverse transcription |
| CT | Threshold cycle |
| RT-PCR | Real time polymerase chain reaction |
| LSD | Least Significance Difference |
| SPSS | Statistical Package for the Social Sciences |
| ANOVA | Analysis Of Variance |

**CHAPTER 3**

| | |
|---|---|
| SBD | Substrate binding domain |
| CWI | Cell wall integrity |
| MAPK | Mitogen-activated protein kinase |
| SBF | Swi4/6 cell cycle box binding factor |
| ROS | Reactive oxygen species |
| $O_2^-$ | Oxide anion |
| OH | Hydroxyl radical |
| NMR | Nuclear magnetic resonance |

**CHAPTER 5**

| | |
|---|---|
| SEM | Standard error of the mean |
| HOG | High osmolarity glycerol |
| UNC | Uncharacterised |
| cDNA | Complementary DNA |

**CHAPTER 6**

| | |
|---|---|
| S/T-P CDK | Serine/Threonine-Proline Cycline dependent kinase |
| SDD-AGE | Semi-Denaturating Detergent Agarose Gel Electrophoresis |

## PUBLICATIONS

### PAPERS

1. Truman, A.W., Kristjansdottir, K., **Hasin, N.**, Wolfgeher, D., Jones, G.W., Kron, S.J. Regulation of the cell cycle through phosphorylation of yeast Hsp70 (Ssa1) on Thr36. **Cell (In press).**

2. Linan X, **Hasin, N.**, Shen, M., Wang, He, J., Zhou, X, Jones, GW. , Song, Y. Predicting and assessing Hsp70 substrate binding domain mutants that alter prion propagation by steered molecular dynamic simulations. **Under review in PloS Computational Biology**

3. Fitzpatrick, D.A., O'Brien, J., Moran, C., **Hasin, N.**, Kenny, E., Cormican, P., Gates, A., Morris, D.W., Jones, G.W. 2011. Assessment of Inactivating Stop Codon Mutations in Forty *Saccharomyces cerevisiae* Strains: Implications for [$PSI^+$] Prion-Mediated Phenotypes**. PLoS ONE, 6(12), e28684.**

### PUBLISHED ABSTRACTS

1. **N. Hasin**, D.A. Fitzpatrick, J. O'Brien, C. Moran, E. Kenny, P. Cormican, A. Gates, D.W. Morris, G.W. Jones. "Assessment of Inactivating Stop Codon Mutations in Forty *Saccharomyces cerevisiae* Strains: Implications for [$PSI^+$] Prion-Mediated Phenotypes." SGM Spring Conference 2012, Convention Centre Dublin, 26th -29th March 2012 **(*Oral presentation*)**.

2. **N. Hasin**, G. W. Jones. "Functional significance of Hsp70 post-translational modification." British Yeast Group Meeting, University of Edinburgh, Scotland 21st - 23rd March, 2012 **(*Oral presentation*)**.

3. **N. Hasin**, S. A. Cusack, Li-Qong Xu, H. M. Loovers, S. Perrett, G. W. Jones. "Assessing the role of Hsp70 in prion propagation." British Yeast Group Meeting, University of Edinburgh, Scotland 21st -23rd March, 2012 **(*Poster presentation*)**.

4. **N. Hasin**, G. W. Jones. "Functional significance of Hsp70 post-translational modification." The Irish Area Section of the Biochemical Society, Glenroyal Hotel, Maynooth, Ireland 1st- 2nd December, 2011 **(*Oral presentation*)**.

5. **N. Hasin**, S. A. Cusack, Li-Qong Xu, H. M. Loovers, S. Perrett, G. W. Jones. "Assessing the role of Hsp70 in prion propagation." The Irish Area Section of the Biochemical Society, Glenroyal Hotel, Maynooth, Ireland 1st- 2nd December, 2011 **(*Poster presentation-Best poster prize*)**.

6. **N. Hasin**, S. A. Cusack, H. M. Loovers, G. W. Jones. "Assessing the role of Hsp70 in prion propagation." Irish Fungal Meeting, Trinity College, Dublin, Ireland 16th – 17th June, 2011 **(*Oral presentation*)**.

7. **N. Hasin,** S. A. Cusack, H. M. Loovers, G. W. Jones. "Role of Hsp70 in prion propagation: importance of peptide-binding domain and post-translational modification." SGM Irish Division Autumn Scientific Meeting, NUI Maynooth, Ireland 2nd – 3rd September, 2010 **(*Oral presentation*)**.

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# CHAPTER ONE

## GENERAL INTRODUCTION

## 1.1 Protein disaggregation and neurodegenerative diseases

One of the distinguishing characteristics of any living system is its ability to synthesise a new chain of amino acids and transform it into a fully functional protein with great precision and fidelity. Protein folding and unfolding are crucial ways of regulating biological activity and targeting proteins to different cellular locations. Protein misfolding and aggregation is a common feature of a wide range of highly devastating and increasingly prevalent diseases. In the past, more than a dozen unrelated human proteins have been identified that undergo aberrant assembly *in vivo* to form filaments or fibrils called amyloid (Sacchettini & Kelly, 2002). Amyloids can either be composed of an entire protein or a protein fragment and be present in tissues or extracellular matrix surrounding vital organs and are formed as a consequence of normal or abnormal endoproteolytic processing that can often result in debilitating and fatal consequences (Sacchettini & Kelly, 2002). To date 20, apparently unrelated, human proteins have been identified which can form amyloid and are responsible for human amyloid diseases. The structure of amyloid fibrils resembles an aircraft cable, in which 3–6 filaments wrap around one another to form the fibril (Blake & Serpell, 1996; Jiménez et al., 1999; Serpell et al., 2000; Sunde et al., 1997) (Figure 1.1). The individual filaments have a lamellar cross-β-sheet structure, which is composed of thousands of individual non-covalently associated protein or peptide subunits. Until recently it was believed that amyloid deposition in central nervous system results in neurodegenerative disorders like Alzheimer's, Hungtington's and Parkinson's disease in human. But amyloid-like disorders are far more widespread than previously thought, and include many common neurodegenerative and neuromuscular pathologies as well as prion diseases (Sacchettini & Kelly, 2002).

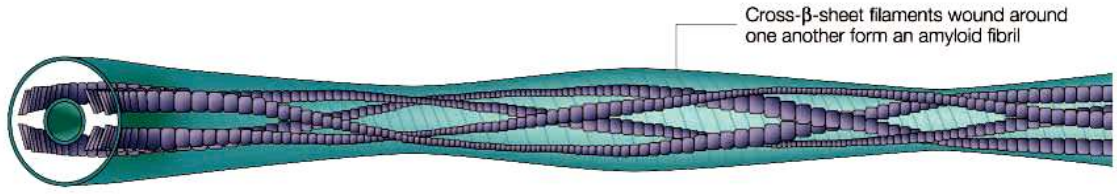



**Figure 1.1 Structure of an amyloid fibril.** Four individual cross β- sheet lamella are wound around one another, resembling the structure of aircraft cable. Figure adapted from Sacchettini & Kelly (2002).

### 1.2 Prion diseases in mammals

The term prion was first coined by Stanley Prusiner (1982) in describing the proteinaceous infectious particles identified as the causative agent of scrapie. Prions are infectious protein particles that cause a group of invariably fatal neurodegenerative diseases collectively termed as transmissible spongiform encephalopathies (TSE) and one of the earliest prion disease to be documented was scrapie in sheep (Detwiler, 1992). Other TSEs include Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Sheinker disease (GSS) and Kuru in humans and bovine spongiform encephalopathy (BSE) found in cattle (Figure 1.2) (Gajdusek et al., 1966; Holt & Phillips, 1988; Prusiner, 1998). One of the distinguishing features of prion diseases is aggregation of prion protein within the brain in the form of amyloid plaques leading to fatal neurodegeneration which currently cannot be treated (Prusiner, 1998; Trevitt & Collinge, 2006).

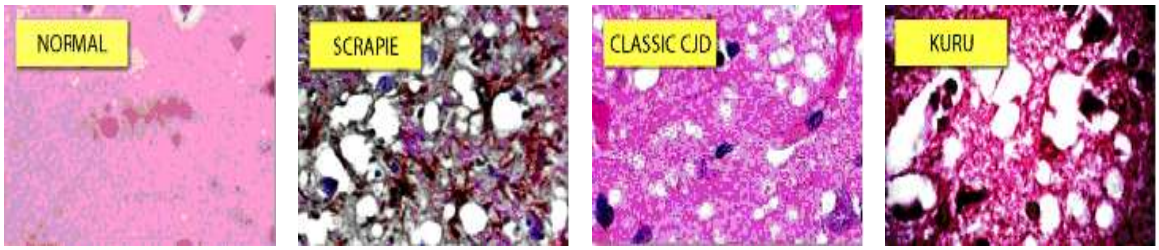



**Figure 1.2 Plaques form after the misfolded prion proteins kill neurons in the brain.** Image adapted from Genetic Science Learning Center, The University of Utah (http://learn.genetics.utah.edu).

#### 1.2.1 PrP protein

Prion diseases may occur sporadically, by infection or could be inherited, all of which involve modification of the prion protein (PrP), a constituent of the normal mammalian cell (Prusiner, 1991). The *PRNP* gene encodes the normal functional PrP protein ($PrP^C$ ) in mammals, which is expressed in the central nervous system (CNS). $PrP^C$ is a sialoglycoprotein expressed in brain and spinal cord and is thought to adhere to cell surface by its glycolipid (Bolton et al., 1985; Stahl et al., 1987). Although the normal function of this protein is not yet clear it has been suggested that the protein might be involved in protecting the cell against oxidative stress (Brown et al., 2001), cell signalling and cell adhesion (Brown et al., 2001; Brown et al., 1999; Gavín et al., 2005; Schmitt-Ulms et al., 2001).

Prions are devoid of nucleic acid and prion replication occurs exclusively by conversion of normal functional PrP protein ($PrP^{C}$) into non-functional, disease causing isoform ($PrP^{Sc}$) (Figure 1.3) (Prusiner, 1998). $PrP^{C}$ and $PrP^{Sc}$ are indistinguishable in their amino acid sequence but differ in conformation (Stahl et al., 1993). The generation of $PrP^{Sc}$ is characterised by a posttranslational event which involves refolding of a portion of its α-helical coiled structure into β-sheets accompanied by profound changes in the physiochemical properties of the $PrP^{C}$. $PrP^{Sc}$ differs from its normal isoform in that it is detergent insoluble (Meyer et al., 1986), resistant to proteinase K digestion (McKinley et al., 1991) and resistant to inactivation by radiation (Gibbs et al., 1978). The protease resistant core of $PrP^{Sc}$ has an apparent molecular weight of 27-30 kDa (Prusiner, 1998). The $PrP^{C}$ and $PrP^{Sc}$ also differ in their monomer conformation and state of aggregation (Riesner, 2003; Weissmann, 2004). There are about twenty *PRNP* mutations that have been identified to cause inherited human prion diseases, thus accounting for 10% of familial CJD cases (Prusiner, 1991; Prusiner, 1997).

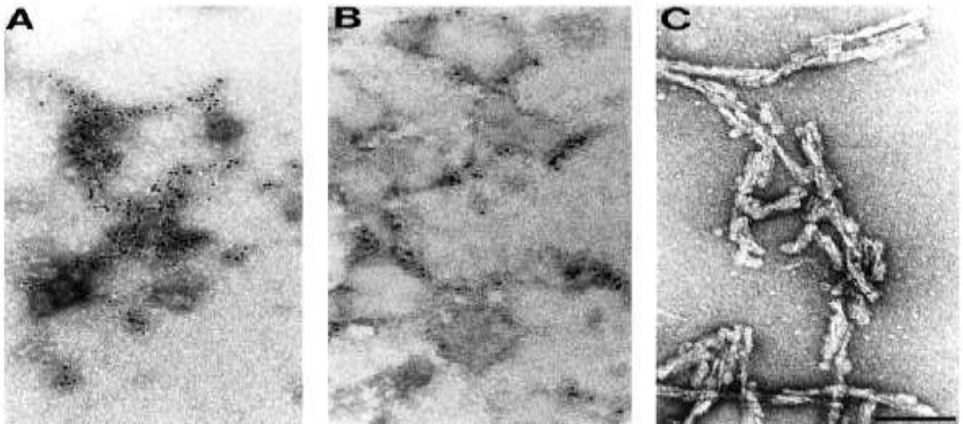



**Figure 1.3 Electron micrographs of negatively stained and immunogold- labelled prion proteins.** (A) $PrP^{C}$ (B) $PrP^{Sc}$ (C) $PrP^{Sc}$ amyloid showing presence of prion rods. Figure adapted from Prusiner (1998). Bar = 100 nm

### 1.2.2 Isolation and characterisation of the infectious agent

Prions have captured the attention of the scientific world for around two centuries since the discovery of scrapie, which had been a concern to scientists since the 19$^{th}$ century. In 1954, Bjorn Sigurdsson coined the term "slow virus" while he was working on scrapie and visna of sheep in Iceland. Five years later William Hadlow (1959) suggested that Kuru was similar to scrapie and thus believed that it was also caused by a slow virus. In a similar manner Igor Klatzo (1959) showed a connection

between kuru and CJD. It was not until 1966, when the radiobiological data demonstrated that the infectious agent that caused scrapie and CJD was extremely resistant to inactivation to UV and ionising radiation, thus suggesting that the infectious agent was too small to be viral particles and displayed protein like properties (Alper et al., 1966; Gibbs et al., 1978; Latarjet et al., 1970). These discoveries led to many unsolved questions until the early 1980s when Stanley Prusiner performed many chemical, physical and enzymatic studies on scrapie disease and hypothesised that this enigmatic disease may have a requirement for protein in order to cause infectivity as the infectious agent once believed to be viral particle was sensitive to protein damaging agents suggesting that it had a structure completely different from viruses. Prusiner (1982) assigned the term 'prion' to the disease and later defined prions as a 'proteinacious infectious agent that lacks nucleic acid' (Prusiner, 1997).

### 1.2.3 Molecular basis of prion disease transmission

Advances in molecular genetics and an in depth knowledge of molecular pathogenesis of neurodegenerative diseases enabled scientists to classify prion diseases based on aetiology into inherited, sporadic and acquired disorders.

***Inherited prion disease*** Approximately 15% cases of human prion disease has been identified as an inherited Mendelian disorder associated with mutations in the gene that encodes the PrP protein (*PRNP*) with over 30 autosomal dominant mutations identified in the *PRNP* gene (Collinge, 2005). The M/V polymorphism at codon 129 of PrP is associated with susceptibility to human prion disease (Collinge, 2005). All the cases of vCJD have been reported in individuals homozygous for methionine at position 129 in PrP, whereas humans heterozygous for this allele are protected from vCJD (Mead et al., 2009; Mead et al., 2003).

***Sporadic prion disease*** Sporadic forms of prion disease mainly constitute CJD, with a few cases of Gerstmann–Straussler–Scheinker disease (GSS) (Hsiao et al., 1989; Prusiner, 1998). Sporadic CJD accounts for 85% of the human prion diseases with 1-2 cases per million occurring annually around the world (Collinge, 2005). It has been hypothesised that sporadic forms of prion diseases occur in patients either by horizontal transmission of prions from humans or animals (Prusiner, 1998), somatic mutation of the PrP gene or spontaneous conversion of $PrP^{C}$ into $PrP^{Sc}$ (Hsiao et al., 1991; Prusiner, 1989).

***Acquired prion disease*** This forms of prion disease can best be illustrated by the prion disease called kuru of the Fore people in New Guinea, where prions were transmitted by ritualistic cannibalism (Prusiner, 1998). Acquired prion diseases are now rare but the 1990s saw a rise in iatrogenic CJD. Other sources causing acquired CJD include improperly sterilized depth electrodes, transplanted corneas, human growth hormone (HGH) and gonadotropin derived from cadaveric pituitaries, and dura mater grafts (Prusiner, 1998).

Though there is considerable evidence of relation between phenotypic diversity in human prion disease with the existence of distinct human prion strains encoded by abnormal PrP isoforms, the conformational array of pathological isoforms of wild-type (WT) human PrP and the various forms of mutant human PrP has not been fully defined (Wadsworth & Collinge, 2011). At the same time the appearance of a human prion disease, variant CJD and the evidence that it is caused by exposure to BSE has emphasised the need to understand the fundamental processes involved in prion propagation, seeded aggregation of misfolded host proteins and the barriers limiting inter- mammalian transmission (Collinge, 2005; Collinge, 2001).

The discovery of yeast prions and reports of several research finding indicating the structural and functional similarity between mammalian and yeast prion at the beginning of this century has opened a new area for studying mammalian prion using yeast as a model organism. There are a number of unifying features of mammalian and fungal prions, which supports why prion generation and propagation can be studied using the yeast model. The following review articles emphasize the structural and functional similarities between mammalian and yeast prions.

- Both mammalian and yeast prions assemble into self-propagating amyloid fibrils *in vitro* (Shorter & Lindquist, 2005).
- There are structurally related and distinct prion strains (Tessier & Lindquist, 2009).
- Both mammalian and yeast prions are composed of tightly packed β- sheets which are protease resistant and SDS soluble (Shorter & Lindquist, 2005).
- They both rely on cellular chaperones to propagate their abnormal conformation (Jones & Tuite, 2005).

The yeast *Saccharomyces cerevisiae* serves as an ideal model organism both to explore the molecular mechanisms underlying the generation of disease-associated protein misfolding and to map the cellular responses to potentially toxic misfolded

proteins. A number of assays at the cell and molecular level have been set up to report on specific protein misfolding events associated with endogenous or heterologous proteins. One major target is the mammalian prion protein PrP because we know little about what specific sequence and/or structural feature(s) of PrP are important for its conversion to the infectious prion form, $PrP^{Sc}$ .

The mammalian PrP and yeast Sup35 share several similar structural characteristics, including a well-folded C-terminal core and a natively unfolded N-terminus. The N-termini of both proteins contain oligopeptide repeats that influence their conformational conversion to the prion state (Goldmann et al., 1998; Liu and Lindquist, 1999; Chiesa et al., 1998; Flechsig et al., 2000; Bocharova et al., 2005). While the biophysical properties of the PrP repeats have been studied extensively, the role of the repeats in prion conformational conversion is not well understood, particularly because of the lack of knowledge on many details of PrP prion formation. One the other hand, the factors that guide prion conformational conversion have been best defined for Sup35. Several studies were aimed at exploring the role of the PrP octarepeats in the context of the yeast prion protein Sup35. One such study was carried out by Dong et. al. (2007) where they found that increasing the number of PrP repeats in the chimeric proteins increases the spontaneous appearance of the [*PSI*$^{+}$] phenotype *in vivo* and accelerates amyloid formation *in vitro*. Conformational conversion and amyloid formation by the chimeras are modulated by both pH and the presence of metal ions. Further, the manner in which these factors modulate conversion is highly sensitive to the number of PrP repeats. In another study carried out by Jossé et al. (2012) showed that by expressing fusion proteins in yeast that comprises the yeast prion protein Sup35 fused to various regions of mouse PrP protein, the PrP sequences can direct the formation of non-transmissible amyloids and thus focus in particular on the role of the mouse octarepeat region.

### 1.3 Fungal prions

Prions have also been identified in other eukaryotes such as fungi. Prion research was taken a step ahead into a new phase when the non-mendelian genetic elements [*PSI*$^{+}$] and [*URE3*] in *Saccharomyces cerevisiae* (Aigle & Lacroute, 1975; Cox, 1965; Lacroute, 1971) were proposed to be prion forms of the Ure2 and Sup35 proteins respectively (Wickner, 1994). Additionally the [*HET-s*] element in filamentous

fungi *Podospora anserina* has also been shown to function as a prion (Coustou et al., 1997).

### 1.3.1 Discovery of prions in yeast

A breakthrough in prion research came unknowingly many years ago when Brian Cox (1965) identified a non-mendelian genetic element in yeast which he termed as [$PSI^+$]. As is with many significant scientific discoveries, [$PSI^+$] was discovered by serendipity when Cox was monitoring the inheritance of a dominant nonsense suppressor called *SUQ5*, using *ade 2-1* mutant allele. *SUQ5* is a weak tRNA anticodon mutant that inserts serine residues at ochre stop codon sites. Cells carrying *ade 2-1* allele form red colonies and are adenine auxotrophs ($Ade^-$) but in *SUQ5* carrying strains this allele was suppressed by *SUQ5* giving $Ade^+$ cells and white colonies. Further analysis of this mutant led to the discovery of [*PSI*] with the non-suppressed cells designated as [$psi^-$] and the suppressed cells [$PSI^+$] (Cox, 1993; Cox, 1965). Another non-mendelian element [*URE3*] was discovered during genetic analysis to isolate nitrogen metabolism mutants (Aigle & Lacroute, 1975; Lacroute, 1971). Cox and Lacroute demonstrated that both [$PSI^+$] and [*URE3*] were dominant and demonstrated non-mendelian segregation (4:0) when crossed with another yeast strain lacking these elements. However, they were both unable to identify the exact mechanism of how these elements propagated.

### 1.3.2 Properties of yeast prions

Prions are infectious proteins that are found in yeast and filamentous fungi and prions ensure their transmission from cell to cell by cytoplasmic mixing, which occurs by mating parental strains. Yeast viruses are also passed from cell to cell by cytoplasmic mixing. Thus along with the transmission of prions, viruses can also be transferred by cytoplasmic mixing. In order to distinguish prions form viruses, three genetic properties were assigned to self-inactivating prions that are not found in nucleic acid replicons (Wickner, 1994).

***Reversible curing:*** If a yeast strain is cured of its prion, the prion should be able to arise again as the protein capable of forming that prion is still present in the yeast strain and should spontaneously convert into its prion form at some low frequency. In contrast, the reappearance of a virus is not possible when the yeast strain is cured of that virus unless introduced again.

***Overproduction of the normal protein increases the frequency of prion formation:*** Increasing the concentration of the protein capable of forming a prion should increase the frequency of that prion's appearance. In contrast there are no chromosomally encoded proteins whose overproduction will increase the frequency in which nucleic acid replicons arise *de novo*, in fact they will not arise *de novo* at all.

***Phenotypic relationship of prion and mutation of the gene for the protein:*** There should be similarities in phenotype exhibited by the prion form of a protein and the mutated protein, if the prion form of a protein simply inactivates the protein functions. For example, a null mutant for the protein should have the same phenotype as a strain that contains the prion form of the protein (assuming the prion is an inactive form of the protein).

### 1.3.3 Prions of yeast and filamentous fungi

The mammalian PrP prion can no longer be regarded as a stand-alone prion since several fungal non-mendelian genetic elements have been found to be prions. Since the proposal made by Wickner (1994) that the genetic behaviour of *S. cerevisiae* non-mendelian genetic elements [$PSI^+$] (Cox, 1965) and [*URE3*] (Aigle & Lacroute, 1975) could be explained if they were the prion of Sup35 and Ure2 proteins respectively, much evidence had accumulated to support this proposal. There are currently three confirmed prions in yeast [$PSI^+$], [*URE3*] and [$PIN^+$] (Derkatch et al., 2001). Several others might qualify as prions based on their genetic properties but still await molecular confirmation (see later section).

#### [*PSI*] – A prion of Sup35 protein

In eukaryotes, translation termination occurs when the mRNA carrying the stop codons (TAA, TGA or TAG) enters the ribosomal A-site. These stop codons in eukaryotes are recognised by a class I release factor (RF) called eRF1, encoded by the *SUP45* gene (in yeast) and recognizes all three stop codons (Kisselev et al., 2003) while the eRF3 encoded by *SUP35* gene (in yeast) is directly involved in translation termination and binds GTP which is required for polypeptide chain release from peptidyl-tRNA (Stansfield et al., 1995). The GTPase activity of eRF3 couples codon recognition and a peptidyl RNA hydrolysis mediated by eRF1 to ensure rapid and efficient peptide release (Salas-Marco & Bedwell, 2004).

In 1965, Cox discovered a non-mendelian genetic element, which he termed [*PSI*$^{+}$]. Around 30 years later Reed Wickner (1994) proposed that [*PSI*$^{+}$] was a prion form of the Sup35 protein. [*PSI* $^{+}$] is the prion form of Sup35 protein, and thus depends on the *SUP35* chromosomal gene (Ter-Avanesyan et al., 1994; Wickner, 1994). In [*PSI*$^{+}$] strains, the spontaneously altered prion protein form induces the conformation change of soluble, functional Sup35 protein to the prion form, resulting in protein aggregation (Patino et al., 1996). This aggregation and reduction in functional Sup35 protein, leads to suppression of nonsense mutations, characteristic of the [*PSI*$^{+}$] phenotype (Figure 1.4) (Cox et al., 1988; Tuite et al., 1987).

Sup35 protein is a multidomain protein and its structure can be divided into three regions the amino terminal N-domain, middle and C-terminus domains (Kushnirov et al., 1988), as illustrated in Figure 1.5 (a). The amino terminal N- domain is 123 amino acids long, typically high in glutamine and aspargine residues and retains prion activity and is thus referred to as the prion domain (PrD) (Derkatch et al., 1997; King et al., 1997; Ter-Avanesyan et al., 1994). The minimal PrD of Sup35 protein contains residues 1-97 (Tuite & Cox, 2003) and this region of 97 amino acids in length is again subdivided into the QN rich (QNR) region and the region containing five complete (R1–R5) and one part copy (R6) of an oligopeptide repeat (OPR, oligopeptide repeat region) (Figure 1.5 c). The highly charged middle-domain extends from amino acid 124-253 and assists in maintaining Sup35 protein solubility and enabling prion and non-prion state inter-conversion (Liu et al., 2002; Ter-Avanesyan et al., 1994). The C-terminal domain is responsible for the Sup35 protein translation termination function and is essential for cell viability (Ter-Avanesyan et al., 1994). This domain extends from amino acid 254-685 (Ter-Avanesyan et al., 1994). The N-terminus (PrD) of Sup35 contains five imperfect regions of an octapeptide sequence YQQYNPQGG resembling the octapeptide repeat PHGGGWGQ found in mammalian PrP (Figure 1.5 b). Deletion of these repeats in yeast interferes with [*PSI*$^{+}$] propagation (Ter-Avanesyan et al., 1994) and addition of repeats leads to an increase in frequency of [*PSI*$^{+}$] appearance (Liu & Lindquist, 1999).

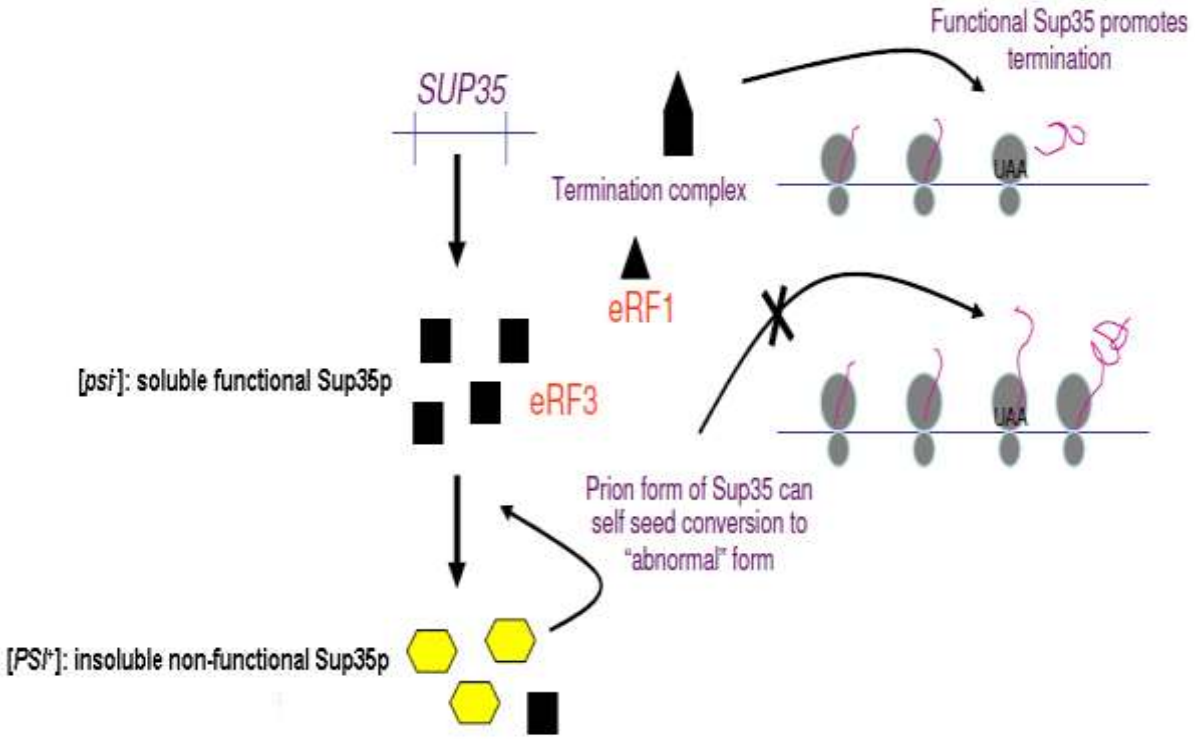



**Figure 1.4 Schematic representation of formation of [*PSI*$^+$].** In *S. cerevisiae* the *SUP35* gene encodes the Sup35 protein also known as eRF3 (eukaryotic release factor 3). Under normal circumstances the eRF3 binds to another release factor called eRF1 and forms the termination complex, which then causes the release of the nascent polypeptide chain from the ribosome. However the Sup35 protein can undergo spontaneous conversion to its prion form, which then recruits other native Sup35 protein into prion form and thus can no longer form the termination complex. This results in formation of insoluble, non-functional Sup35 protein no longer able to assist in the release of the nascent polypeptide chain from the ribosome. Figure adapted from Wickner et al. (2004).

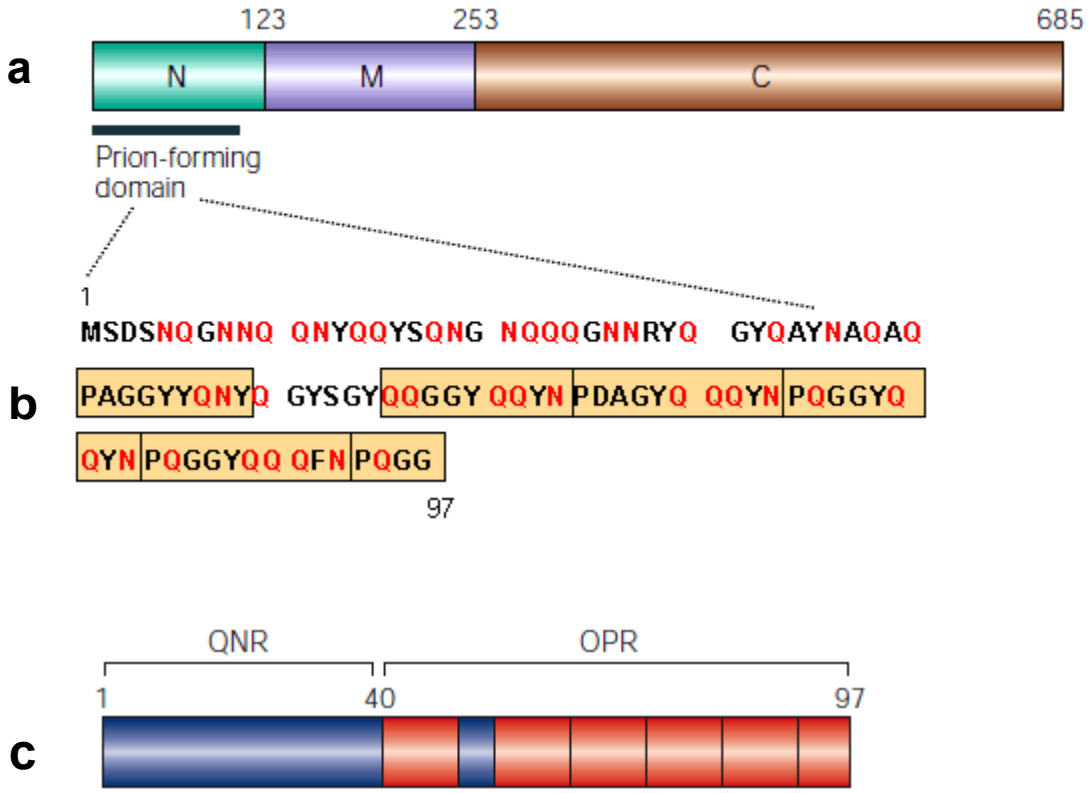



**Figure 1.5 Schematic diagram of the Sup35 protein.** (a) The Sup35 protein has been assigned with three domains - the N-terminus domain, the middle and the C-terminal domain. **(b)** The prion forming domain (PrD) extends from amino acid 1-97 in the N-terminal region and is rich in asparagine (N) and glutamine (Q) highlighted in red. (c) Within this minimal PrD region is the Q/N rich QNR and the OPR (oligopeptide repeat region) region. The OPR consist of 5 complete and 1 part copy of oligopeptide repeat that shows sequence similarity with the mammalian PrP octarepeat. Figure adapted from Tuite & Cox (2003).

**[*URE3*] – A non-mendelian genetic element affecting nitrogen catabolism**

The functional Ure2 protein is mainly involved in nitrogen catabolic repression. In yeast, the presence of good nitrogen source such as ammonia or glutamine, mediates the Ure2 to act as a catabolic repressor, repressing the genes involved in the synthesis of enzymes and transporters needed for assimilating poor nitrogen source (Cooper, 2002). The [*URE3*] is the non-Mendelian genetic element first reported by Lacroute (1971) and it fulfils all the three criteria expected of a prion of Ure2 protein (Wickner, 1994). The [*URE3*] prion disrupts the normal function of the Ure2 similar to a *ure2* mutants, resulting in inappropriate expression of genes and unnecessary uptake of nitrogen (Brachmann et al., 2005; Lacroute, 1971). Ure2 is a two-domain protein composed of a 65 amino acid N-terminal prion domain and a C-terminal domain extending from amino acid 66-354 necessary for nitrogen regulation. The overproduction of N-terminal

domain is sufficient to induce the expression of [*URE3*] at a rate 100-fold higher than the similar overproduction of full length protein (Masison & Wickner, 1995). In addition, the prion domain propagates the [*URE3*] prion in complete absence of the C-terminal domain (Masison et al., 1997).

**[*PIN* $^{+}$] – A prion that stimulates prion formation**

A third well documented yeast prion is [$PIN^{+}$], the amyloid form of Rnq1 protein (Derkatch et al., 2001). The first indication that different prions have the capability of influencing each other came with the discovery of [$PIN^{+}$] - the prion form of Rnq1 protein (for [$PSI^{+}$] inducibility). [$PIN^{+}$] was identified because it enabled transient overexpression of Sup35 protein and subsequent *de novo* appearance of [$PSI^{+}$] (Derkatch et al., 2001). It is now well known that [$PIN^{+}$] also enhances *de novo* appearance of [*URE3*] following overexpression of *URE2* prion domain (Bradley et al., 2002). [$PIN^{+}$] is now identified as the prion form of Rnq1 protein (Derkatch et al., 2001). Although no function has been assigned to the soluble form of Rnq1 protein, Rnq1 protein has now been identified as a candidate prion on the basis of sequence homology with the Sup35 prion domain and also based on the aggregation of Rnq1 protein which was inherited in a cytoplasmic manner (Sondheimer & Lindquist, 2000). Data so far may suggest that [$PIN^{+}$] prion aggregates act as an imperfect template on which Sup35 molecules misfold and assemble into infectious prion aggregates. This procedure, termed "cross-seeding", occurs only at the initial step of *de novo* formation of [$PSI^{+}$] and subsequently the two proteins stably co-aggregate (Bagriantsev & Liebman, 2004). There is also evidence for co-localization during initial cross-seeding (Derkatch et al., 2004; Salnikova et al., 2005). Furthermore, yeast two hybrid studies and co-immunoprecipitation analysis has detected interactions between soluble forms of Rnq1 and Sup35 *in vivo* (Tuite et al., 2008).

**[*Het-s*] of *Podospora anserina* – A prion in filamentous fungus**

[Het-s] is another well-documented prion, it is the infectious form of the protein HET-s found in filamentous fungal species *P. anserina.* This prion is involved in cell death phenomenon that is one of the common characteristics of all the filamentous fungi and is known as heterokaryon incompatibility (Coustou et al., 1997). The HET-s protein controls heterokaryon incompatibility- a somatic self/non-self recognition phenomenon common in filamentous fungi. The *het-s* locus exists as two distinct antagonistic alleles

termed as *het-s* and *het-S* (Rizet, 1952). Strains containing HET-s in its prion form are designated as [Het-s] whereas strains expressing HET-s in its soluble non-prion form are designated as [Het-s *]. Fusion of [Het-s] and a *het-S* strain leads to cell death of the mixed heterokaryotic cell. Since filamentous fungi spontaneously undergo vegetative cell fusion, the [Het-s] prion is efficiently and spontaneously transmitted horizontally from one strain to another. Because the mycelium is coenocytic, the [Het-s] prion can invade a [Het-s *] thallus at a high rate, up to 70 mm per day (Beisson-Schecroun, 1962).

Both [Het-s] and [*PIN*$^+$] differ from [*URE3*] and [*PSI*$^+$] because their transition into prion state results in no detectable loss of function of the corresponding protein. The transition to the prion state endows the corresponding proteins with new property, in case of HET-s, the ability to trigger cell death by incompatibility when interacting with HET-s (Benkemoun & Saupe, 2006).

### 1.3.4 Discovery of other yeast prions

Since the discovery of [*PSI*$^+$], [*URE3*] and [*PIN*$^+$] prion research was confined to this small group of proteins and its prion form but discoveries over the past few years have gone underway to hunt for novel prions in yeast. Recently a screen identified ten new prions whose over expression facilitated the *de novo* appearance of [*PSI*$^+$] (Derkatch et al., 2001). As this screen identified Ure2 as facilitating the de novo appearance of [*PSI*$^+$], so it was postulated that some of this proteins might act as prions as well, which included Cyc8, New1 and Swi1 (Table 1.1). Further, a bioinfomatic proteome – wide survey for prionogenic proteins in *S. cerevisiae* followed by experimental investigation identified twenty-four protein domains that satisfied prion behaviour. This includes the known prions Ure2, Sup35, Rnq1 and Swi1, the previously identified prion candidate New1 and a functionally diverse set of 19 new candidates (Alberti et al., 2009). However, [*MCA*] was not confirmed as a yeast prion by Alberti et al. (2009) but Erhardt et al. (2010) have suggested prion like properties of the Mca1 protein dependent upon N-terminus peptide stretch, an isoform not consistently expressed. [*MCA*] was identified as the first prion found by general molecular cloning (Nemecek et al., 2009). It is a prion form of the Mca1 protein which is a metacaspase (a member of a family of caspase related proteins) and is proposed to be involved in one of the yeast programmed cell death process (Mazzoni & Falcone, 2008). [*MCA*] satisfies two of the criteria for prion. Mca1 protein are aggregated in [*MCA*] cells and the N-

region that helps in propagation of [*MCA*] is Q/N rich. Unlike other amyloid based yeast prions, [*MCA*] is not cured by growth on millimolar guanidine but is cured by overexpression of Hsp104. But the paper which demonstrated the existence of [*MCA*] prion (Nemecek et al., 2009) was retracted as the results were not reproducable (Nemecek et al., 2011). Although Alberti et al. (2009) identified many potential prions with protein composition similar to known yeast prions and are Q/N rich but prion proteins may not be restricted to Q/N rich structure as both PrP and HET-s are not rich in glutamine and asparagine. There is still an urgency to carry out intriguing research in the prion field to account for how many more fungal prions exist (Tessier & Lindquist, 2009). Table 1.1 summarises the confirmed mammalian and fungal prions, their functions and prion manifestations.

### 1.3.5 Prion formation and polymerisation

Yeast prions are protein based elements of inheritance that propagate by a mechanism that resembles self-perpetuating alterations of protein conformation observed in prion based encephalopathies that are not responsible for diseases but accounts for epigenetically inherited phenotypes (Koo et al., 1999). As stated earlier the prion form of the Sup35 protein reduces its efficiency of translation termination leading to a heritable phenotypic change because protein with the altered conformation is passed from the cytoplasm of mother cells to their daughters, perpetuating the cycle of conformational change (DePace & Weissman, 2002; Glover et al., 1997; Scheibel & Lindquist, 2001; Serio et al., 2000). The *in vivo* conversion of Sup35 protein from the non-prion [$psi^-$] state to the prion [$PSI^+$] state is a complex mechanism and involves an array of cellular and environmental factors. Research carried out on the prion determining region of Sup35 (NM) by (Scheibel et al., 2004) showed that Sup35 (NM) converts to amyloid fibres by nucleated conformational conversion. It is referred to as NM, because it consists of the N-terminal asparagine and glutamine-rich region and the middle glutamate and lysine-rich region. Amyloid polymerisation is initiated by formation of a ‘nucleus’ or ‘seed’ of protein with different conformation to the normal soluble form. This nucleus promotes the conversion of the rest of the protein monomers to polymerise into prion conformation (Figure 1.6) (Jarrett et al., 1993; Scheibel et al., 2004; Wickner et al., 2000). The whole process of amyloid polymerisation occurs at a slower rate initially by addition of monomers to the fibre ends of Sup35NM and the process then accelerates by rapid and efficient addition of Sup35NM monomers

followed by fibre fragmentation (Collins et al., 2004). The partition of these prion 'propagons' onto the daughter cells occur by cytoplasmic distribution and approximately 500-1000 propagons can exist per cell (Byrne et al., 2009). In order for the prion to be maintained and passed to daughter cells, the prion protein must be able to propagate efficiently and chaperone proteins play a key role in this process. The chaperone Hsp104 (heat shock protein 104) is essential for prion maintenance and propagation of [$PSI^{+}$] within a yeast cell line (Chernoff et al., 1995) along with close co-operation of other chaperones, Hsp70 and Hsp40 (Glover & Lindquist, 1998). Details of the role of chaperones in prions propagation will be discussed in the sections to come.

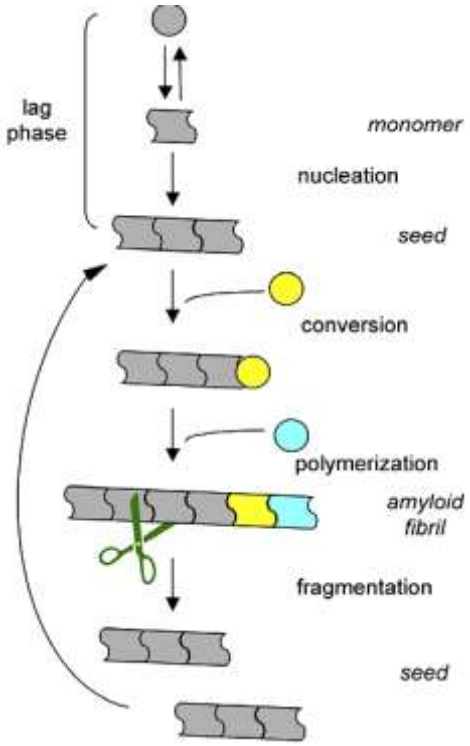



**Figure 1.6 Schematic representation of nucleation-polymerisation event.** during the nucleation process the 'seed' or nucleus formation takes place by association of small number of subunits corresponding to the lag phase of the process. During the second stage, amyloid fibrils rapidly elongate by monomer addition and conformational conversion at the end of the fibrils. When fibrils are long enough, they are fragmented into new 'seeds' making propagation possible. Figure adapted from Benkemoun & Saupe (2006).

**Table 1.1 Prions of mammals, yeast and the filamentous fungus *Podospora anserina.*** Table adapted from Wickner et al. (2009).

| Organism | Prion | Protein | Normal Function | Prion manifestation |
|---|---|---|---|---|
| Mammals | TSEs | PrP | Not known | Transmissible spongiform encephalopathy |
| *S. cerevisiae* | [*URE3*] | Ure2 | Nitrogen catabolite repression | Derepression of nitrogen catabolism enzymes and transporters |
| | [*PSI*$^+$] | Sup35 | Translation termination | Read-through of stop codons |
| | [*PIN*$^+$] | Rnq1 | Not known | Rare seeding of [*PSI*$^+$], other prions |
| | [*SWI*$^+$] | Swi1 | Chromatin remodeling | Poor growth on glycerol, raffinose, galactose |
| | [*OCT*$^+$] | Cyc8 | Repression of *CYC7* and other genes | Derepression of transcription |
| | [*MOT3*$^+$] | Mot3 | Transcription factor | Cell-wall changes |
| *P. anserina* | [Het-s] | HET-s | Heterokaryon incompatibility; meiotic drive (as a prion) | Heterokaryon incompatibility; meiotic drive (as a prion) |

### 1.3.6 A simplified method for monitoring yeast prions in laboratory

The discovery of prions in yeast allowed scientists to carry out prion research with great ease as yeast is a good model organism to be manipulated. The most well established method of assessing cellular [*PSI*$^+$] in yeast is the red/white screen based on a nonsense mutation on either *ADE1* or *ADE2* gene of the adenine biosynthesis pathway (Tuite & Cox, 2007) (Figure 1.7). In *S. cerevisiae* the *SUP35* and *SUP45* gene encodes the release factors called eRF3 and eRF1. In normal cells or [*psi*$^-$] cells the Sup35

protein or eRF3 is fully functional and forms a complex with eRF1 for translation termination (Stansfield et al., 1995). In [*PSI*$^{+}$] cells, the Sup35 protein becomes non-functional due to its aggregation resulting in defective or inefficient translation termination as there occurs no functional interaction between eRF3 and eRF1 (Paushkin et al., 1996; Tuite & Cox, 2007). Laboratory yeast strains can be generated with aberrant stop codons in *ADE1* or *ADE2* genes of adenine biosynthetic pathway. In [*psi* $^{-}$] cells Sup35 is fully functional leading to translation termination and accumulation of red pigment (AIR) due to oxidation of an accumulated precursor (Cox, 1965). In contrast, in [*PSI*$^{+}$] cells the non-functional Sup35 protein is in its aggregated insoluble state and thus leads to enhance readthrough of the nonsense mutation (Paushkin et al., 1996). Functional Ade1/Ade2 protein is produced and cells produce white colonies that grow on adenine lacking medium. This method is one of the most convenient and widely used assay in the field of [*PSI*$^{+}$] research (Shorter & Lindquist, 2005).

### 1.3.7 Adenine biosynthesis pathway

The adenine biosynthesis pathway in *S. cerevisiae* is a seven-step process by which P-ribosyl-PP (PRPP) is converted into adenine monophosphate. The *ADE* genes encode enzymes involved in the catalytic conversion of PRPP to adenine monophosphate. The *ADE2* gene encodes phosphoribosylaminoimidazole carboxylase that catalyses the sixth step in the biosynthesis of the purine nucleotide adenine. Mutation in *ADE2* gene results in accumulation of purine precursors in vacuoles leading to formation of red colonies. This pigmentation is one of the widely used markers in screening [*PSI*$^{+}$] prions in yeast (Jones & Fink, 1982). *ADE2* mutants that turn red are blocked in the step forming CAIR as the enzyme ADE2 responsible for catalyzing this step is no longer functional and AIR accumulates in cell. The cells that grow aerobically oxidises AIR to a red pigment (Figure 1.8).

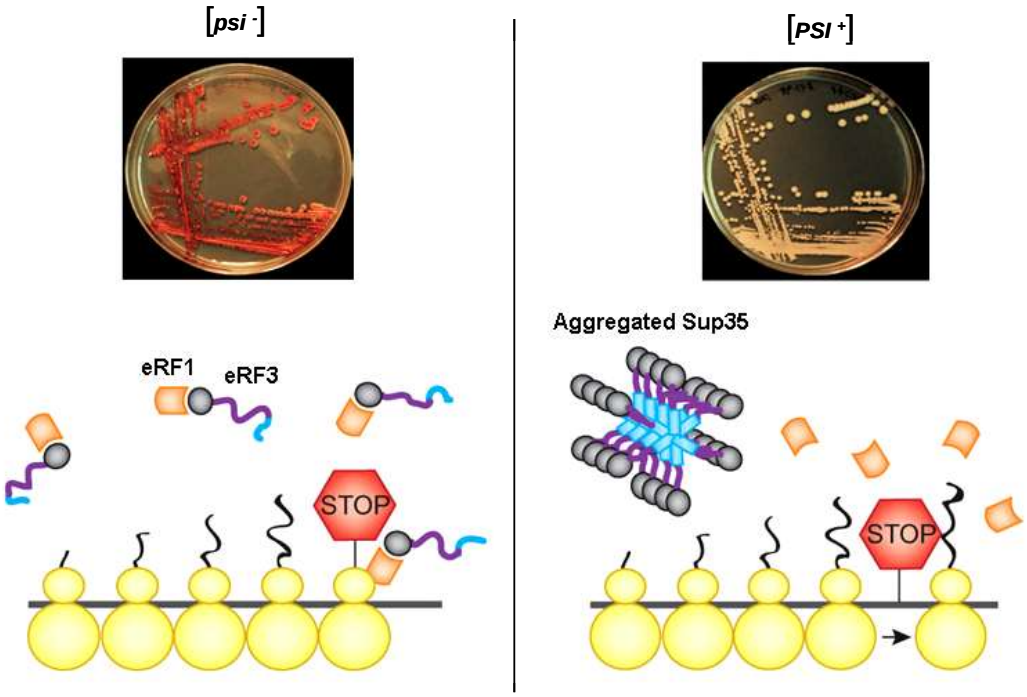



**Figure 1.7 Monitoring Sup35 aggregation in yeast using red/white assay.** Sup35, is soluble and complexed to Sup45 (orange) in the [*psi*⁻] state (left) or insoluble and inactive in the [*PSI*⁺] state (right). The inactivation of Sup35 causes read-through of premature stop codon resulting in the synthesis of full length Ade2 protein of the adenine biosynthesis pathway and thus the cells look white. Figure adapted from Tessier & Lindquist (2009).

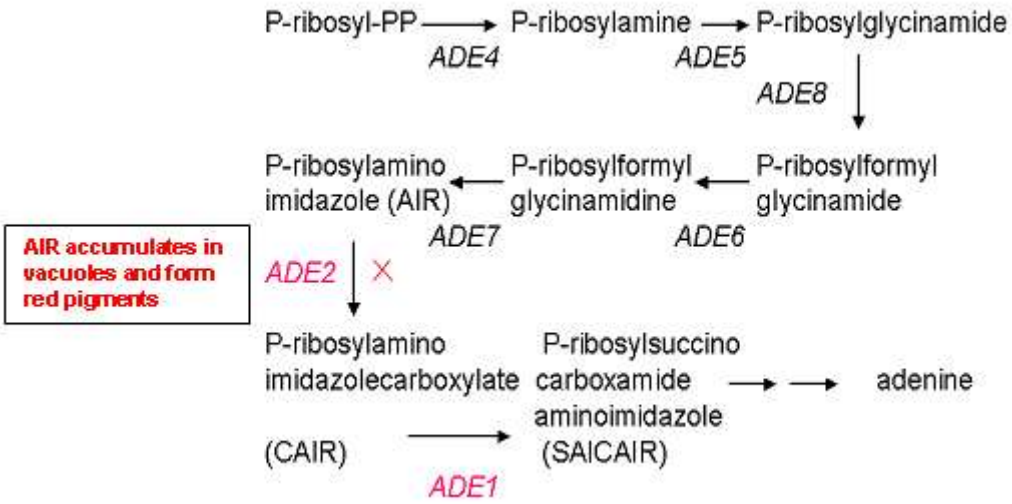



**Figure 1.8 The adenine biosynthesis pathway in *S. cerevisiae*.** Mutations in *ADE1* or *ADE2* cause accumulation of the intermediate AIR, which is converted to form the red pigment.

## 1.4 Protein folding and molecular chaperones

The mechanism involving the transformation of a newly synthesised chain of amino acids into its perfectly folded native three dimensional structure depends on the intrinsic properties of the primary amino acid sequence and also on numerous contributing factors from a crowded cellular environment (Dobson, 2003). Protein folding is a systematic, unbiased process whereby the proteins synthesised on the ribosome undergo a spectrum of transition states before arriving into their biologically active, pre-ordained structure. *In vivo* protein folding in some cases is 'co-translational' and is initiated before the completion of protein synthesis (Hardesty & Kramer, 2001). Other proteins, however, undergo the major part of their folding in the cytoplasm after release from the ribosome, whereas yet others fold in specific compartments, such as mitochondria or the endoplasmic reticulum (ER), after trafficking and translocation through membranes (Bukau & Horwich, 1998; Hartl & Hayer-Hartl, 2002). Thus, although the basic principles of protein folding are universal, each protein species has evolved a conserved and simplified folding pathway depending on the particular environment in which folding takes place (Walter & Buchner, 2002). The whole process of synthesis of a nascent polypeptide chain on the ribosome calls for investment of considerable amount of energy by the cell. Living systems have therefore evolved a range of strategies to promote productive folding of proteins into their active, functional state. Thus to ensure that the proteins fold into their native functional conformation and to prevent them from going 'off-pathway' and misfold, cells developed molecular chaperones - a set of proteins that associate with unfolded polypeptides thereby preventing aggregation and promoting productive folding in an ATP-dependent manner. (Buchner, 1996; Gething & Sambrook, 1992).

The main function of molecular chaperone is to bind to unfolded or partially folded polypeptide and prevent aggregation (Figure 1.9). Most of the protein species during the process of folding or misfolding have exposed hydrophobic residues that make the species susceptible to aggregation. Molecular chaperones associate with these hydrophobic residues and thus suppress aggregation (Walter & Buchner, 2002). Molecular chaperones can also induce conformational changes in the target protein such as controlled unfolding of the protein that help establish a link between the chaperones and cellular degradation system. Chaperone mediated unfolding can also provide the nascent polypeptide a chance to disrupt the non-productive interactions and thus reach its functional native structure (Walter & Buchner, 2002) .

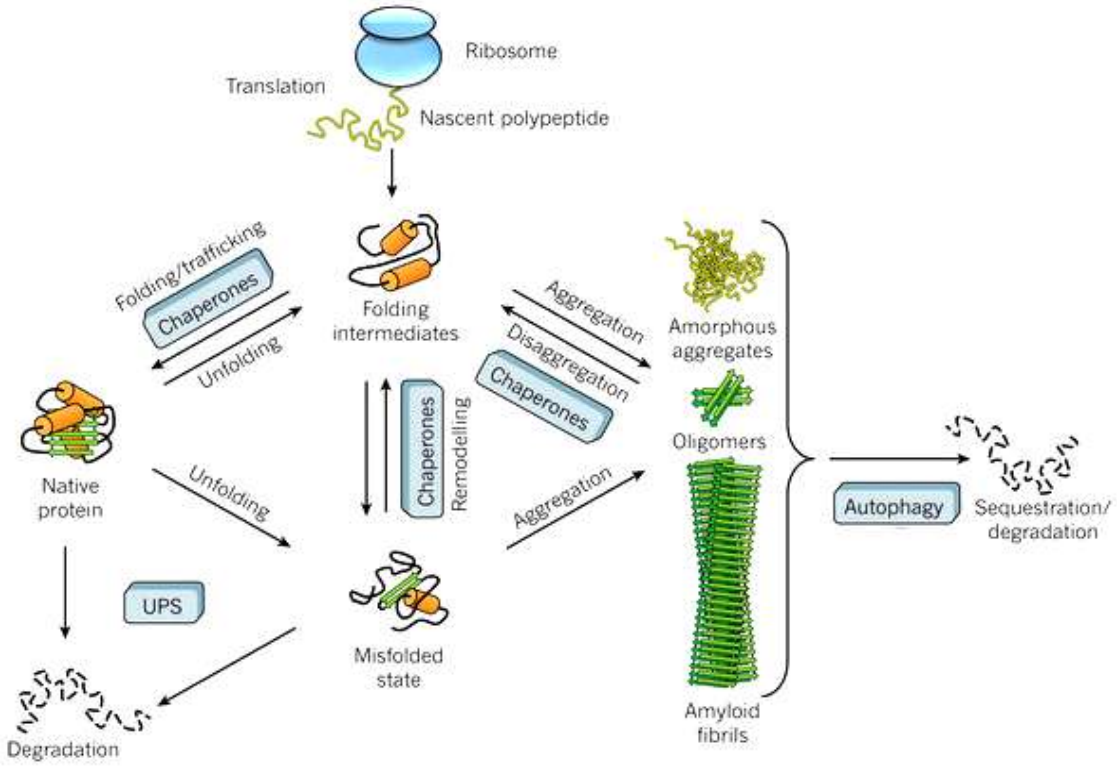



**Figure 1.9 Molecular chaperones in protein folding and protein homeostasis.** Proteins fold into their functional three-dimensional structure. But in a crowded cellular environment the proteins are susceptible to misfolding and aggregation. Thus the cell invests in a complex network of molecular chaperones to prevent aggregation and promote efficient folding. Because protein molecules are highly dynamic, constant supervision by chaperones is required to ensure protein homeostasis. This system incorporates the chaperone pathway for the folding of newly synthesized proteins, for the remodelling of misfolded states and for disaggregation with the protein degradation mediated by the UPS and the autophagy system. Figure adapted from Hartl et al. (2011).

### 1.5 Implication of molecular chaperones in prion diseases

Molecular chaperones are vital for cell survival and the significance of these proteins is clearly demonstrated by deletion of their genes that often results in lethal phenotypes and severe cellular defects such as reduced resistance to stress (Walter & Buchner, 2002). Although the primary role of chaperones in cells is to prevent protein misfolding and aggregation, prion proteins found in mammals and fungi appear to have evolved to exploit the molecular chaperone machinery to remodel their conformation into an aggregation-prone, infectious form (Prusiner, 1982). The lack of specific chaperone activity or reduction in their normal cellular levels often results in the deposition of fibrillous aggregates of the prion protein (PrP) in the brain- one of the

important characteristics of BSE or CJD (Prusiner, 1998). The presence of amyloid protein aggregates is often a characteristic of neurodegenerative diseases such as Hutington's and Alzheimer's disease (Ross & Poirier, 2004). While the involvement of chaperones in mammalian prion disease is still speculative, in yeast, prion formation and propagation was shown to be critically dependent on the well characterised molecular chaperone Hsp104 which has been shown to regulate the number and the size of prion seeds required for fibre polymerization and propagation (Figure 1.10). In this case, cells can be cured by the inactivation of Hsp104 (Chernoff et al., 1995; Eaglestone et al., 2000). In addition, chaperones are emerging as molecular targets for therapy in cancer, tissue transplantation, and septic shock syndrome, among others.

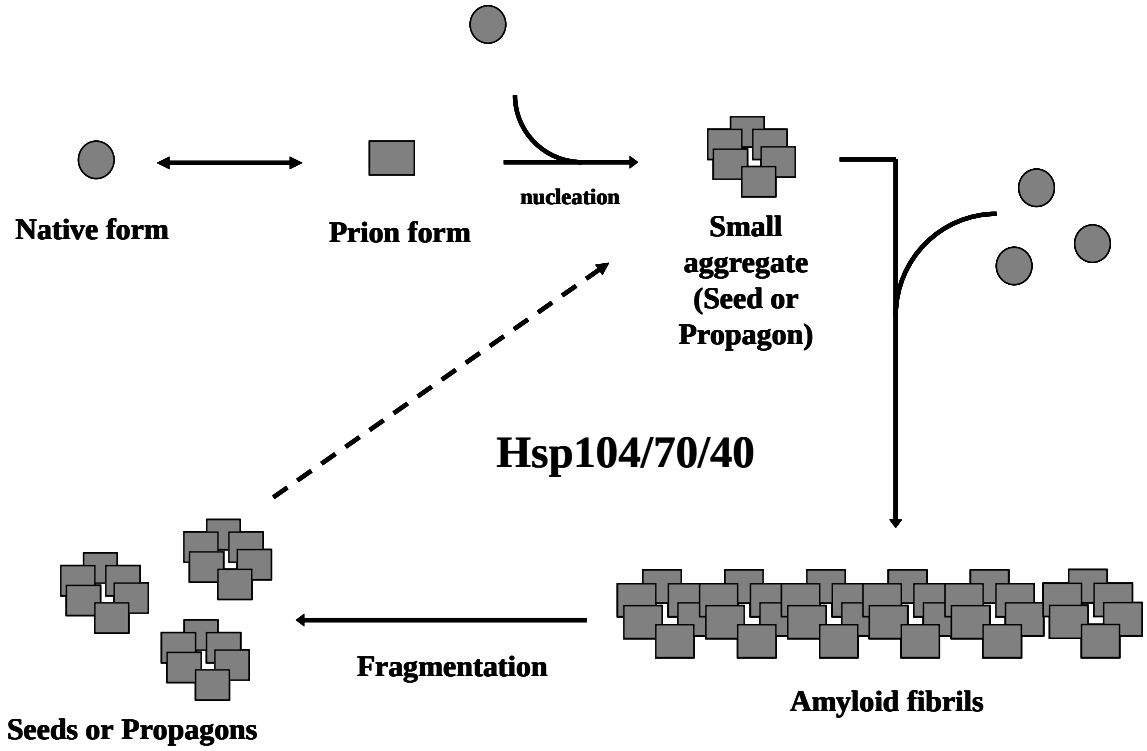



**Figure 1.10 Schematic representation of the essential role of molecular chaperones in yeast prion maintenance and propagation.** Once a prion is formed in *S. cerevisiae* , the chaperone machinery Hsp104-Hsp70-Hsp40 maintain the infectious protein with Hsp104 playing the key role in generating infectious prion seeds from the pre-existing amyloid fibrils.

### 1.6 Molecular chaperones and protein folding in yeast

The unicellular eukaryote *S. cerevisiae* is a budding yeast species that has been exploited at the molecular level by scientists for over four decades. Many of the genes in yeast are highly conserved and are now well characterised which has enabled scientists to study genetic and biochemical processes common to all eukaryotes (Baker

et al., 2008). One of such processes widely studied by scientists using *S. cerevisiae* as an invaluable model organism is the protein folding pathway within the cytosol of a eukaryotic cell. All the major classes of chaperones implicated in such pathways have been identified and extensively characterised in yeast (Table 1.2) with the majority of human orthologues being identified, except for Hsp104 chaperone for which no orthologue has been identified in mammals as yet although one is found in plants (Schirmer et al., 1994). The main classes of chaperones in eukaryotic cytosol is mainly classified as heat shock proteins (Hsps) as their expression is induced under heat shock conditions that elicit protein misfolding and aggregation.

**Table 1.2 Major chaperone and co-chaperone families in yeast cytosol.** Table adapted from Jones & Tuite (2005).

| Family | Members | General functions |
|---|---|---|
| Hsp100 | * **Hsp104** | Protein disaggregation, stress tolerance. |
| Hsp90 | Hsc82, Hsp82 | Protein folding and stress tolerance. Most substrates appear to be involved in signal transduction. |
| Hsp70 | * **Ssa1-4p** * **Ssb1-2p** Sse1-2p Ssz1p | Protein folding and stress tolerance. Bind to denatured proteins and prevent aggregation. Also involved in aspects of protein translocation and translation. |
| Hsp40 | * **Ydj1p,** * **Sis1p** | Deliver peptide substrates and stimulate ATPase activity of their relevant Hsp70 partner. Sis1p is involved in translation initiation. |
| Hsp70/Hsp90 co-factors | * **Sti1p,** Cpr6p, * **Cpr7p,** * **Cns1p** | Aid in the Hsp70-Hsp90 protein folding cycle. Sti1p bridges Hsp70 to Hsp90 and regulates ATPase activity of both proteins. |
| Small Hsps | Hsp26, Hsp42 | Form oligomeric complexes that bind to unfolded proteins and prevent aggregation. |

*Chaperones and co-chaperones in bold have been implicated in yeast prion propagation.

### 1.6.1 Hsp70

A major class of heat shock proteins (Hsps) belong to the Hsp70 family, a diverse collection of 70 kDa chaperones that exist in various compartments of the cell. This ubiquitous family of molecular chaperones perform essential housekeeping functions in protein folding, synthesis, assembly, translocation across membranes, presentation of substrates for degradation, assembly and disassembly of macromolecular

complexes or aggregates, gene induction and apoptosis (De Los Rios et al., 2006; Floer et al., 2008; Nollen & Morimoto, 2002; Sangster et al., 2004). They are also involved in quality control process such as protein refolding after stress and control the activity of regulatory proteins in signal transduction pathways (Mayer & Bukau, 2005). All of these cellular activities of Hsp70 depends on its ability to interact with hydrophobic stretches of proteins in an ATP-dependent manner preventing non-productive interactions that would lead to aggregation and to promote protein refolding (Sharma & Masison, 2009). Thus, Hsp70 has acquired a broad spectrum of cellular functions by amplification and diversification of *HSP70* gene across evolution, recruitment of co-chaperones for specialised functions and co-operation with other chaperone systems as well as their ability to bind non-specifically to hydrophobic peptides attributing Hsp70 to act on a broad spectrum of substrates. Hsp70 constitutes one of the most conserved protein families in evolution and is a predominant group of Hsps in all cell types ranging from bacteria to plants and humans (Hunt & Morimoto, 1985) . Comparison of amino acid sequences of human Hsp70 with *Drosophila* Hsp70 and *Escherichia coli* DnaK reveals that human Hsp70 is 73% identical to *Drosophila* Hsp70 and 47% identical to *E. coli* DnaK respectively (Hunt & Morimoto, 1985). Hsp70s share high structural homology and have conserved functional properties across species. For example, *Drosophila* Hsp70 can complement mammalian Hsp70 in protection from heat stress (Pelham et al., 1984).

#### 1.6.1.1 The Hsp70 system in yeast

The *S. cerevisiae* encodes five organelle specific and nine cytosolic Hsp70s (Table 1.3). The main Hsp70 component of yeast cytosol is the Ssa family (Stress seventy subfamily A) comprising of four closely related proteins Ssa1-4 which collectively provide essential cellular functions and are involved in various aspects of protein folding working in conjugation with other chaperone systems (Wegele et al., 2004). Other Hsp70s in yeast include the ribosome associated Ssb and Ssz and cytosolic Sse proteins.

**Table 1.3 *Saccharomyces cerevisiae* Hsp70 family constitutes five organelle specific and nine cytosolic Hsp70s.**

| Gene name | Function | Location |
|---|---|---|
| *SSA1*<br>*SSA2* | Constitutively expressed molecular chaperones that binds newly synthesized proteins to assist in protein folding and prevent aggregation/misfolding | Cytosol |
| *SSA3*<br>*SSA4* | Heat induced molecular chaperone that binds newly synthesized proteins to assist in protein folding and prevent aggregation/misfolding | |
| *SSE1*<br>*SSE2* | Nucleotide exchange factors (NEF) that assist Hsp70 during protein refolding | |
| *SSB1*<br>*SSB2*<br>*SSZ1* | The chaperone activity of Ssb and Ssz is localized to ribosome as a part of ribosome –associated complex RAC | |
| *KAR2* | Acts as a chaperone to mediate protein folding in the ER and may play a role in ER export of soluble proteins; regulates the unfolded protein response via interaction with Ire1p. | Lumen of ER |
| *LHS1* | Involved in polypeptide translocation and folding; nucleotide exchange factor for the ER lumenal Hsp70 chaperone Kar2p; regulated by the unfolded protein response pathway | |
| *SSC1* | *SSC1* encodes an essential chaperone that is the key component of the import motor sub-complex that mediates the transit of precursor proteins through the TIM23 complex. | Mitochondria |
| *SSQ1* | Required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis, and for maturation of Yfh1p, which is a homolog of human frataxin implicated in Friedreich's ataxia | |
| *ECM10* | Plays a role in protein translocation, interacts with Mge1p in an ATP-dependent manner; overexpression induces extensive mitochondrial DNA aggregations. | |

The highly homologous proteins exhibit functional redundancy and were originally thought to differ only by their spatio-temporal expression patterns (Werner-Washburne et al., 1987). The *SSA1* and *SSA2* are 96% identical to each other and are both expressed constitutively (Table 1.4). Although during normal growth the expression level of Ssa2 is several fold higher than Ssa1, following heat-shock the Ssa1 expression nearly equals Ssa2 (Werner-Washburne et al., 1987). Data from DNA sequence homology showed that the heat inducible genes *SSA3* and *SSA4* were closely related orthologues of *SSA1* and *SSA2* sharing approximately 80% identity with *SSA1* and *SSA2* and 88% identical with each other (Table 1.4) (Craig et al., 1985; Sharma & Masison, 2009). The heat inducible Ssa3 and Ssa4 are lowly expressed during normal vegetative growth but there is a dramatic increase in their expression levels upon stress; additionally there occurs an increased expression of Ssa3 on entry into stationary phase (Boorstein & Craig, 1990a; Boorstein & Craig, 1990b; Werner-Washburne et al., 1989; Werner-Washburne & Craig, 1989; Werner-Washburne et al., 1987). The simultaneous deletion of all the four genes is lethal but can be complemented by over-expression of either one of them suggesting functional redundancy (Werner-Washburne et al., 1987). Yeast strains harbouring deletion of either *SSA1* or *SSA2* genes are phenotypically indistinguishable from the WT but strains containing disruption of both the genes exhibit a dramatically altered phenotype (Werner-Washburne et al., 1987). However, a Δ*ssa1*Δ*ssa2* mutant strain is viable and thermotolerant due to the upregulation of *SSA3* and *SSA4* and other components of general stress response. The Δ*ssa1*Δ*ssa2* mutant strain forms small colonies at 23 °C and is thermosensitive at prolonged exposure to 37 °C suggesting that not all functions carried out by *SSA1* and/or *SSA2* can be complemented by the induction of *SSA3* and *SSA4* (Boorstein & Craig, 1990a; Boorstein & Craig, 1990b; Werner-Washburne et al., 1987).

**Table 1.4 Percent amino acid identity of Ssa proteins.** Table adapted from Sharma & Masison (2009).

| Proteins | Ssa1 | Ssa2 | Ssa3 |
|---|---|---|---|
| Ssa2 | 97% | - | - |
| Ssa3 | 79% | 79% | - |
| Ssa4 | 81% | 81% | 87% |

Both the Ssbs and Ssz1 forms a part of the ribosome associated complex (RAC) which also includes DnaJ homologue Zuo1 (Gautschi et al., 2001; Pfund et al., 1998). The three chaperones are genetically linked and forms a functional triad. Ssz1 and Zuo1 assemble into stable heterodimer complex termed RAC. It act as a co-chaperone for Ssb1/2 and stimulates its ATP hydrolysis. The function requires both RAC subunit.This complex ensures correct protein folding by binding with the ribosome and the emerging nascent polypeptide chain assisting translation fidelity (Gautschi et al., 2002; Rakwalska & Rospert, 2004). Unlike other heat shock genes in which expression is induced by heat, the expression of Ssb1/2 is repressed (Craig & Jacobsen, 1985), instead the expression of Ssb1/2 is coordinated with expression of ribosomal protein genes (Lopez et al., 1999). Both Ssb1/2 and Ssz1 are structurally similar to other Hsp70's, containing an ATPase domain and a peptide-binding domain (Pfund *et al*. 1998) but Ssz1 differs from other Hsp70's as its ATPase domain and PBD activities do not appear to be necessary to carry out its functions (Huang et al., 2005; Hundley et al., 2002). It has been postulated that the function of Ssz1 is to assist ATPase stimulation of Ssb1/2 by the DnaJ protein Zuo1 (Huang et al., 2005). The Sse family of cytosolic Hsp70's act as nucleotide exchange factors for Ssa1 and are discussed in Section 1.6.2.3.

### 1.6.1.2 Structure and mechanism of action of Hsp70

Hsp70s are a highly conserved family of proteins and are composed of an N-terminal 44 kDa ATPase domain (also called adenine nucleotide binding domain or NBD), an 18 kDa peptide-binding domain or substrate binding domain (PBD/SBD) and a C-terminal 10 kDa "lid" domain (CTD). The NBD is bilobular and each globular lobe (I and II) is further subdivided in two domains (A and B) (Flaherty et al., 1990). The interdomain linker connecting the NBD to the PBD is highly conserved and plays a critical role in allosteric regulation of Hsp70s (Swain et al., 2007; Vogel et al., 2006).

The Hsp70s cycle between two stable conformations with different affinities for substrates. The binding affinity and kinetics is dependent on the presence of ATP in the ATPase domain. In ATP bound state, Hsp70s display a fast rate of substrate binding in the PBD, whereas in the ADP-bound state PBD has higher affinity for its substrate resulting in tighter association of the substrate within the PBD and thus displays slower rate of exchange of the peptides (Figure 1.11) (McCarty et al., 1995; Schmid et al., 1994). The major structural difference between the ATP and ADP bound states of Hsp70 is the position of the CTD. In an ADP bound state the CTD lid is positioned over

the peptide binding cavity, therefore reduces the release of substrates (Han & Christen, 2003). Upon ATP hydrolysis, structural changes in the ATPase domain induce conformational changes in the PBD and CTD, which result in substrate trapping. Correspondingly, substrate binding to the PBD alters its structure, which results in a signal being transmitted to the ATPase domain that stimulates ATP hydrolysis, therefore increasing substrate-binding affinity. This allosteric regulation of Hsp70 is tightly regulated by specific co-chaperones that will be discussed in Section 1.6.2.

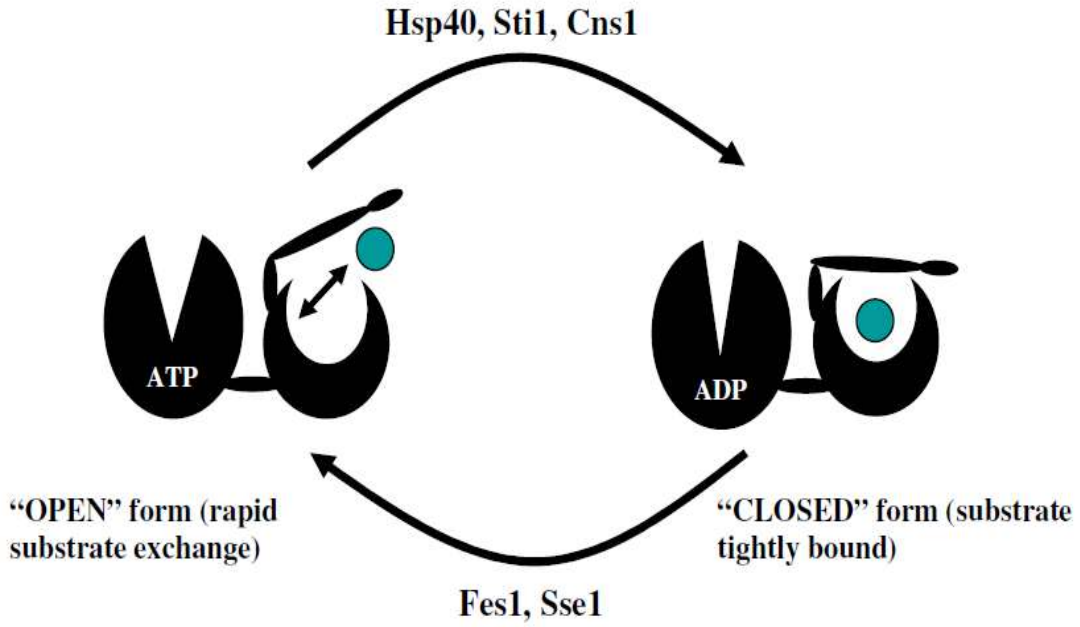



**Figure 1.11 Schematic representation of Hsp70 ATPase cycle.** In an ATP bound state Hsp70 is in an open conformation allowing rapid substrate exchange. ATP is hydrolyzed to ADP causing Hsp70 to become into a closed state in which the substrate is tightly bound. The ATPase cycle of Hsp70 is regulated by Hsp40s that stimulate ATP hydrolysis. The NEFs Fes1 and Sse1 assist in returning Hsp70 into its "open" conformation. Figure adapted from Jones & Tuite (2005) and Loovers et al. (2007).

### 1.6.2 Regulatory roles of Hsp70 co-factors

Hsp70 has a very slow intrinsic ATP hydrolysis and ADP release cycle, which provides key points for regulation of its reaction cycle. Accordingly, Hsp70 function is intimately associated with co-chaperones that regulate its function at these steps and recruit it to various sub-cellular locations or other chaperone machineries (Young et al., 2004)

#### 1.6.2.1 Hsp40 co-chaperones (J proteins)

The most abundant regulators of the ATPase cycle of Hsp70 are the Hsp40s also known as the "J proteins" which bind substrate with great specificity and stimulate Hsp70 ATP hydrolysis. This combination of activities allow Hsp40 to present substrates to Hsp70 and co-ordinate Hsp70 ATP hydrolysis with substrate binding (Greene et al., 1998; Wittung-Stafshede et al., 2003). Hsp40s are a ubiquitous family of co-chaperones and are represented by multiple isoforms, with twenty-two being expressed in *S. cerevisiae* with the major ones being Sis1 and Ydj1 (Higurashi et al., 2008). They are defined by a conserved stretch of 70 residues known as the J-protein (Craig et al., 2006; Peter Walsh et al., 2004). The co-chaperone Hsp40 assist Hsp70s function by binding to unfolded substrates and presenting it to Hsp70 (Laufen et al., 1999) and it physically interact with Hsp70 and stimulate ATP hydrolysis thus co-ordinating ATP hydrolysis in PBD (Cyr et al., 1992; Laufen et al., 1999; Liberek et al., 1991). There are some Hsp70s that show specificity to a single J-protein thus allowing Hsp70 to perform various cellular functions and localise to specific places in the cell. For example in *S. cerevisiae* while the Ssa1/2 have a general protein folding function in the cytosol, they interact with Hsp40 member Sis1 that assist Ssa1/2 on ribosomes in translation (Horton et al., 2001) and Ydj1 recruit Ssa1/2 to protein folding on ER membrane (Brodsky et al., 1998).

#### 1.6.2.2 Sti1 and Cns1

Both Sti1 (stress inducible protein 1) and Cns1 (cyclophilin seven suppressor) are also regulators of the ATPase cycle of Hsp70 member Ssa1. They both activate the ATPase activity of Ssa1 by accelerating the rate limiting step of ATP hydrolysis (Hainzl et al., 2004; Wegele et al., 2003). Sti1 consists mainly of three tetratricopeptide repeat (TPR) domains. TPRs are helical modules that mediate interactions between proteins (Honore et al., 1992). The N-terminal TPR domain of Sti1/Hop (Hop is the mammalian homologue of Sti1) interacts with Ssa1/Hsp70, and the TPR domains in the C-terminal half seems to be important for the interaction with Hsp90 thus providing a physical link between the Hsp70 and Hsp90 chaperone machinery (Chen & Smith, 1998; Dittmar et al., 1996; Scheufler et al., 2000). Wegele et al, (2003) have shown that binding of Sti1 to Ssa1 increases the ATPase activity of Ssa1 by a factor of 200. Cns1 from *S. cerevisiae* is a novel TPR containing modulator of yeast Hsp70 ATPase activity and is

able to activate the ATPase rate of Hsp70 (Ssa1) up to 30 fold. Cns1 binds via its TPR domain to the EEVD motif of yeast Hsp90 and Hsp70.

#### 1.6.2.3 Nucleotide Exchange Factors (NEFs) for Hsp70 – Sse1 and Fes1

Efficient cycling of Hsp70 requires nucleotide-exchange factors (NEFs) that accelerate the slow intrinsic release of ADP by Hsp70 thus shifting the equilibrium to the ATP-bound state, triggering the release of the substrate and the resumption of the chaperone cycle. Well known NEFs are GrpE in *E. coli*, Fes1 and Sse1 in *S. cerevisiae* (Dragovic et al., 2006a; Kabani et al., 2002a; Raviol et al., 2006) and the human Fes1 orthologue HspBP1 (Shomura et al., 2005). GrpE and Bag-1 were the first protein candidates identified as NEFs for *E. coli* DnaK and mammalian Hsc70 (Liberek et al., 1991; Zylicz et al., 1989). Like most other organisms *S. cerevisiae* also has multiple NEFs which include Snl1, Sls1, Sil1, Lhs1, Fes1 and Sse1 (Kabani et al., 2000; Sondermann et al., 2002; Steel et al., 2004; Tyson & Stirling, 2000). Although the significance of possessing multiple NEFs is not well documented, different NEFs might interact with different Hsp70s with different specificity. For example, Sse1 stimulates yeast and mammalian cytosolic Hsp70s (Shaner et al., 2006; Shaner et al., 2005). Some NEFs also exhibit functional redundancy. For example, overexpression of Fes1 confers viability to an otherwise lethal Δ*sse1*Δ*sse2* double deletion strain, but Sse1 is more potent with Ssa Hsp70s than Fes1(Raviol et al., 2006).

Sse1 is a member of the cytosolic Hsp70 family, comprised of an ATPase domain, a PBD containing an extended linker region separating the β and the α subdomains of the domain and an extended C-terminal region (Easton et al., 2000; Liu & Hendrickson, 2007). Binding of Sse1 to Ssa1 is a ATP-dependent process, whereby ATP hydrolyis results in a conformational change in Sse1 that promotes its association to Ssa1. It is thought that the ATPase domains of Ssa1 and Sse1 associate asymmetrically so that lobe I of Ssa1 contacts lobe II of Sse1 and the C-terminal α-helical domain of Sse1 plays a role in this interaction (Andréasson et al., 2008). Recent evidence suggests that Hsp110/Sse1 may be able to function as a protein disaggregase in mammalian systems and also to a lesser extent in yeast (Duennwald et al., 2012; Shorter, 2011).

Fes1 is also a NEF present in yeast (Kabani et al., 2002a), it is homologous to the mammalian HspBP1 which causes nucleotide dissociation in both Ssa1 and

mammalian Hsc70 (Kabani et al., 2002b). Fes1 facilitates translation, a process in which Ssa1 and Ydj1 have been implicated (Brodsky et al., 1998; Horton et al., 2001).

### 1.6.3 Hsp104

The yeast chaperone that appears to have no mammalian homologue, Hsp104, is a member of the AAA+ protein superfamily, which includes the 'ATPase Associated with a variety of cellular Activities' proteins and Clp/Hsp100 proteins (Neuwald et al., 1999) characterised by assembly of oligomeric ring complexes (usually hexamers) with a central pore, in presence of ATP (Parsell et al., 1994a). ATP binding results in ATP hydrolysis, leading to release of substrate from the chaperone (Bösl et al., 2005). Hsp104 plays a critical role in ensuring that cells subjected to prolonged exposure to physical (e.g high temperature) or chemical (e.g ethanol) stresses are able to survive by acting as 'protein disaggregase' leading to resolubilisation of protein aggregates. How Hsp104 resolves protein aggregates remained a mystery until Glover and Lindquist (1998) showed that it cooperated with the Hsp70 chaperone system *in vitro* to solubilize and refold aggregated proteins (Glover & Lindquist, 1998). Similar studies in other systems extended the analysis of this bi-chaperone network, and led to the proposal that Hsp104 and its orthologues might break large aggregates into smaller pieces that the Hsp70 system can then refold (Figure 1.12) (Glover & Tkach, 2001). Hsp104 have two highly conserved nucleotide binding domains (NBD1/NBD2) each displaying co-operative kinetics of ATP hydrolysis (Hattendorf & Lindquist, 2002; Parsell et al., 1994a). Mutational analysis has shown the importance of hexamer pore entrance in Hsp104 function (Lum et al., 2004). ATP binding to NBD1 of Hsp104 is essential for the chaperone to interact with substrate aggregate as mutations that prevent ATP binding to NBD1 impair Hsp104 substrate interaction. The NBD2 is also important for Hsp104 function as mutations to it severely impair Hsp104 hexamerisation (Schirmer et al., 1998). Tessarz et al, (2008) showed that substrate threading through the central channel of Hsp104 mediated substrate remodelling involves solubilization of heat aggregated proteins and prion-like conformers. While processes such as protein disaggregation and prion propagation rely on a threading activity of Hsp104, the relevance of this remodelling activity for the actual substrate (protein aggregate versus prion fibre) is rather different. In the case of heat-aggregated proteins, the extracted polypeptide itself represents the primary physiological target. In contrast, for extracted Sup35 molecules, the product of Hsp104-mediated threading of prion fibres, are

potentially not of physiological relevance, but the unthreaded fragmentized prion fibre that might serve as a new seed in prion propagation is of relevance.

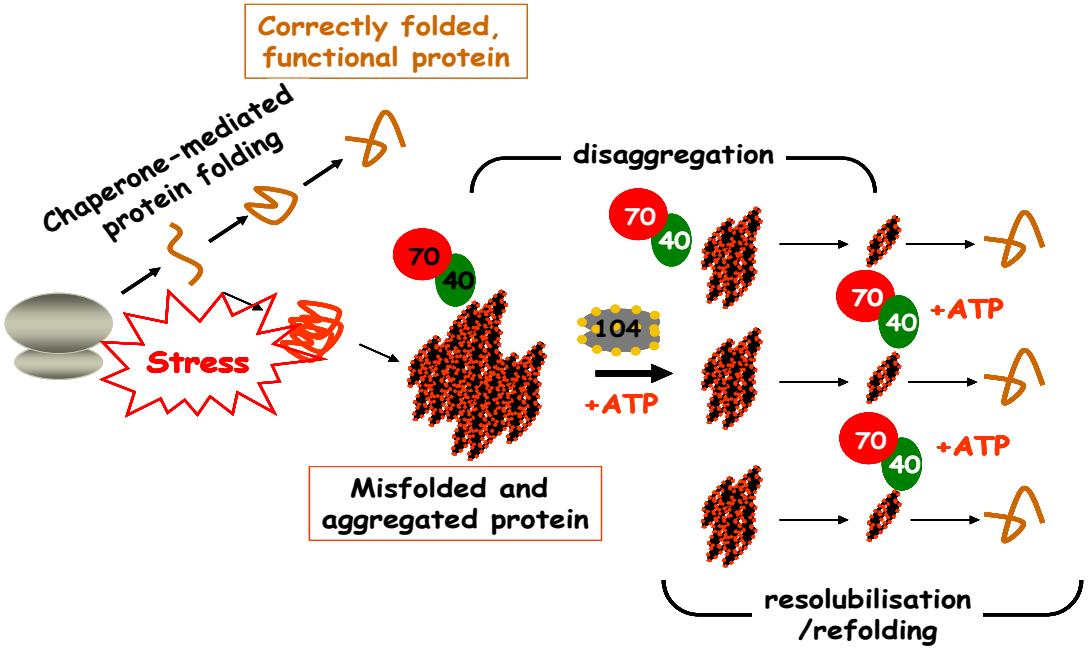



**Figure 1.12 Cooperative functions of Hsp104, Hsp70 and Hsp40.** When exposed to stress, such as heat-shock, proteins can become denatured or misfolded and form into amorphous aggregates. The action of Hsp104, aided by Hsp70 and Hsp40, results in a disaggregation of amorphous aggregates into substrates that Hsp70 and Hsp40 can act upon and aid in their correct refolding. Figure adapted from Jones & Tuite (2005).

### 1.6.4 Hsp90

Hsp90 is a highly conserved family of proteins that exist in all organisms and cell types prior to stress. In yeast *S. cerevisiae* there are two isoforms of Hsp90 reported *viz* Hsc82 and Hsp82, which share a sequence homology of 97%. Hsc82 is constitutively expressed at high levels and is only slightly induced by heat shock, whereas Hsp82 is strongly induced by both heat and stress (Borkovich et al., 1989).

Hsp90 is a flexible dimer, with each monomer consisting of three domains, each consisting of an N domain, connected by a long linker sequence to an M –domain that is followed by a C-terminal dimerization domain. The function of Hsp90 is dependent on ATP hydrolysis (Obermann et al., 1998; Panaretou et al., 1998).

The Hsp90 chaperone differs from Hsp70 in two ways: client proteins composed of kinase or transcription factors that rely on Hsp90 for correct signalling and unlike Hsp70, it binds partially folded proteins and induces structural changes and protein associations to gain full activity. The protein-folding pathway regulated by Hsp90 involves the requirement of Hsp70 and its co-chaperones. Initially, client proteins

interact with Hsp40 (Hernández et al., 2002), which targets them to Hsp70; proteins are transferred from Hsp70 to Hsp90 in order for folding to be completed. The co-chaperone Sti1 is thought to facilitate transfer by binding both Hsp70 and Hsp90 via TPR domains, forming a bridge on which proteins can be transferred (Chen et al., 1996; Chen & Smith, 1998; Smith et al., 1993). Sti1 may affect client folding through regulation of conformation and ATPase cycles of the two chaperones (Hernández et al., 2002; Johnson et al., 1998; Smith & Johnson, 2000).

### 1.7 "Prion maintenance and propagation" – The role of molecular chaperones

The discovery that proteins in yeast can behave as prions has greatly improved our understanding of prion biology (Wickner, 1994). Prion proteins appear to have evolved to exploit the molecular chaperone machinery in mammals and fungi and thus remodel their structure in a way that can be transmitted and propagated to the daughter cell. Genetic studies have identified a number of chaperones which interact with and modulate yeast prions, as reviewed by Jones & Tuite (2005). Yeast prions have provided a unique and a powerful genetic tool for identifying and characterizing alterations in protein chaperones in a way that considerably impairs ability of amyloid to propagate *in vivo* without having any affect on their normal cellular functions. Three classes of chaperone proteins (Hsp70, Hsp40 and Hsp104) along with a number of co-chaperones have been extensively analysed in their role in prion maintenance and propagation (Table 1.5).

#### 1.7.1 Hsp70 and its co-chaperones – a key modulator of prion propagation

A number of studies, predominantly genetics in nature, have identified Hsp70 as strongly influencing propagation of the two most well-studied prions in *S. cerevisiae* [*PSI*$^{+}$] and [*URE3*] which are the prion forms of Sup35 and Ure2 proteins respectively (Jones et al., 2004; Jones & Masison, 2003; Jung et al., 2000; Song et al., 2005; Tibor Roberts et al., 2004). In order to be maintained in a yeast population, the prions are dependent on the normal function of the chaperones and alterations in function or abundance of Hsp70 can greatly influence prion propagation and maintenance (Jones et al., 2004; Jones & Masison, 2003; Jung et al., 2000). The Hsp70s form a part of the large chaperone machinery consisting of Hsp104, Hsp40s and TPR-containing proteins and this chaperone machinery have a direct impact on prion propagation. Hsp70 might also exert its effect on prion propagation through direct or indirect alteration of its

cooperation with other chaperones (Song & Masison, 2005). For example in an *in vitro* study the Sup35 polymerisation was blocked by Ssa1 only in co-ordination with Ydj1 thus demonstrating the strong requirement of Ydj1 for Ssa1's effect on Sup35 polymerisation (Krzewska & Melki, 2006).

In the past decade, several biochemical studies carried out on prion proteins Sup35 and Rnq1 showed the involvement of the cytosolic Hsp70 members Ssa1 and Ssb1 and their co-chaperones Ydj1, Sis1 and Sse1 to actively bind the prion form of Sup35 in yeast (Allen et al., 2005; Bagriantsev et al., 2008; Sondheimer et al., 2001). Ssa1/Ssa2 are the most abundant proteins to co-purify with the Sup35 from [*PSI*$^+$] cells indicative of Hsp70s direct interaction with prion form of Sup35 *in vivo* and its co-chaperones may affect prion propagation in a Hsp70 mediated manner. In addition to the biochemical studies, there were several genetic studies carried out as well which has verified the involvement of Hsp70 in prion propagation. The first indication from genetic studies of active involvement of Hsp70 in prion propagation came from the study which showed that when Ssa1 is overexpressed it protects the [*PSI*$^+$] prion from the effects of overexpression of Hsp104 (Newnam et al., 1999). Ever since the discovery of the Ssa1 mutant Ssa1-21 (L483W) which impairs [*PSI*$^+$] propagation, several mutational studies were carried out on Ssa1 and Ssa2 that further implicated Hsp70 as playing a role in prion propagation (Jones & Masison, 2003; Jung et al., 2000; Loovers et al., 2007). In cells where Ssa1-21 replaces Ssa1, the number of [*PSI*$^+$] seeds were reduced while in cells where Ssa2 is also absent Ssa1-21 causes complete elimination of [*PSI*$^+$] prions (Jung et al., 2000). Most of the prion forming mutations were identified in the ATPase domain adjacent to PBD and has therefore suggested to impair [*PSI*$^+$] prions by affecting the interdomain communication resulting in altered ATPase regulation in opening and closing of the PBD. Furthermore, mutants of Ssa1 which affect prion propagation are different from those which affect cell growth suggesting that the effects are more specific and do not affect cellular functions of Hsp70 (Jones & Masison, 2003; Loovers et al., 2007).

Subsequent analysis confirmed that altering the ATPase cycle has an affect on prion propagation (Jones et al., 2004), for example, either increasing ATPase activity by overexpressing Sti1 or decreasing ADP release by deleting the NEF Fes1 weakens the [*PSI*$^+$] phenotype (Jones et al., 2004). This is indicative that shifting the ATPase cycle of Hsp70 to favour an ADP bound state appears to impair [*PSI*$^+$] propagation. The Ssa1 co-chaperones Ydj1 and Sis1 also play an important role in prion propagation. For

example over expression of Ydj1 eliminates [*URE3*] (Moriyama et al., 2000) and certain variants of [*PSI*$^+$] (Kushnirov et al., 2000), while over expression of Ydj1 or Sis1 eliminates artificial variants of [*PSI*$^+$] (Kryndushkin et al., 2002). Another member of the cytosolic Hsp70 family, Sse1 is also thought to play a role in prion propagation (Fan et al., 2007; Kryndushkin & Wickner, 2007; Sadlish et al., 2008). Studies show that overproduction of Sse1 dramatically enhances [*PSI*$^+$] formation when Sup35 is overexpressed, whereas deletion of Sse1 severely inhibits [*PSI*$^+$] formation (Fan et al., 2007; Sadlish et al., 2008; Shaner et al., 2008). The abundance of Sse1 also has an affect on the [*URE3*] prion; overproduction of Sse1 can efficiently cure [*URE3*] whereas deletion of Sse1 blocks [*URE3*] propagation (Kryndushkin & Wickner, 2007). The NEF Fes1 for Ssa1 in yeast accelerates release of ADP from substrate bound Ssa1 while Sti1 and Cns1 are activators of Ssa1 ATPase (Hainzl et al., 2004; Wegele et al., 2003). However, in *fes1* deleted *SSA1-21* cell, the [*PSI*$^+$] is no longer propagated (Jones et al., 2004) while over expression of *FES1* in *SSA1-21* cells produces a stronger [*PSI*$^+$] phenotype (Jones et al., 2004). Thus Hsp70 impair [*PSI*$^+$] propagation by enhancing substrate binding.

Thus, genetic studies have revealed that Hsp70 along with the assistance of its co-chaperones plays a central role in prion propagation. This involves the binding and release of prions or prion related substrate and Hsp70 interacts with these prion substrates in the same manner as with denatured or partially folded polypeptides. Thus the role of Hsp70 in prion maintenance and propagation relies upon the finely- tuned mechanism of Hsp70-prion substrate interaction and also links ATPase cycle of Hsp70 as the key influence on this process.

### 1.7.2 Effect of Hsp40 on prion propagation

The Hsp40 family of co-chaperones, which comprises Sis1, Ydj1 and Apj1 have been established as playing an important role in prion propagation of [*PSI*$^+$] cells and assembly of amyloid fibrils. Sis1 has been implicated in [*PSI*$^+$] propagation (Higurashi et al., 2008; Tipton et al., 2008) and both Sis1 and Ydj1 has been found to interact physically with large Sup35 aggregates (Bagriantsev et al., 2008). Apj1 is another member of the Hsp40 family in yeast, and overexpression of this co-chaperone cures certain variants of [*PSI*$^+$] (Kryndushkin et al., 2002).

The Hsp40 family of co-chaperones has also been implicated in prion propagation of [*PSI*$^+$], [*URE3*] and [*PIN*$^+$] cells. Sis1 is the only member of Hsp40 that

is important in propagation of all the three yeast prions [*PSI*$^+$], [*URE3*] and [*PIN*$^+$] (Aron et al., 2007; Higurashi et al., 2008). The interaction of Sis1 and Ssa1 with Rnq1 occurs only in [*PIN*$^+$] cells (Sondheimer et al., 2001), and inhibition of the ability of Sis1 to stimulate ATPase activity of Ssa1 also impairs [*PIN*$^+$] propagation, suggesting Sis1 acts as a Hsp70 co-chaperone with respect to [*PIN*$^+$] propagation (Aron et al., 2007; Higurashi et al., 2008; Lopez et al., 2003). There is an increase in Rnq1 polymer size and decrease in [*PIN*$^+$] seeds in cells depleted of Sis1 (Aron et al., 2007). This was due to the altered interaction of Sis1 with Hsp70 and Hsp104 underlying the importance of Sis1 in Rnq1 polymer fragmentation necessary for [*PIN*$^+$] propagation (Aron et al., 2007). A similar result was observed in the case of Sup35 polymers, where there was an increase in polymer size due to absence of Sis1 and inhibition of Hsp104 (Higurashi et al., 2008; Kryndushkin et al., 2003) thus suggesting that Sis1 may work in conjunction with Hsp70 to deliver prion substrates to Hsp104 (Tipton et al., 2008). Studies have also revealed that the J domain of Ydj1 is sufficient for the elimination of [*URE3*], the J domain of Ydj1 mediates interaction with Hsp70 and is responsible for stimulating ATPase activity, suggesting that the regulation of Ssa1 by Ydj1 is crucial for the [*URE3*] curing process (Masison et al., 2009; Sharma et al., 2009).

### 1.7.3 Role of Hsp104 in prion propagation

Among all the well characterised molecular chaperones, the Hsp104 chaperone protein is the first chaperone which showed evidence of involvement of molecular chaperones in yeast prion maintenance and propagation as deletion or overexpression of Hsp104 'cures' [*PSI*$^+$] (Figure 1.13) (Chernoff et al., 1995). The mechanism of [*PSI*$^+$] curing by overexpression of Hsp104 is still debatable although several studies have provided proposed models for explaining [*PSI*$^+$] curing by Hsp104 overexpression. The disaggregation based model provided by Wegrzyn et al. (2001) proposed that excess of Hsp104 cures [*PSI*$^+$] by dissolving large Sup35 polymers into monomers that can no longer be propagated to daughter cells that thus cures [*PSI*$^+$] but there lacks any direct evidence to support this. An alternative model proposes that excess of Hsp104 cures [*PSI*$^+$] by converting polymers into large structures that are no longer able to propagate. Electrophoretic analysis revealed that cells overexpressing Hsp104 resulted in large Sup35 polymers compared to its extracts from the same strain expressing normal levels of Hsp104 (Kryndushkin et al., 2003). Also adding Gdn-HCl to yeast cures the prion probably by Hsp104 inhibition (Ferreira et al., 2001).

The process of prion propagation is not solely dependent on Hsp104 but it also calls in the assistance of Hsp70 and Hsp40 where the Hsp70 co-factors along with Hsp40 acts as the components of this machinery and thus play a crucial role in prion propagation (Figure 1.10) (Guinan & Jones, 2009; Shorter & Lindquist, 2008) . Various studies have shown that shifting the cellular balance of various Hsp70 and Hsp40 chaperones have an affect on induction and propagation of [$PSI^+$] (Allen et al., 2005; Chernoff et al., 1999; Kryndushkin et al., 2002).

Just as prion propagation requires the active involvement of all the three molecular chaperones Hsp104, Hsp70 and Hsp40, in a similar manner the curing of [$PSI^+$] prions is not only dependent on overexpression of Hsp104 alone but also requires the assistance of Hsp70 and Hsp90 co-chaperones Sti1 and Cpr7 (Moosavi et al., 2010). The deletion of the region of Hsp104 that interacts with TPR containing co-chaperones is dispensable for curing , however cells expressing Sti1 defective in Hsp70 and Hsp90 interaction cured less efficiently. Recent studies have shown that deletion of Hsp70 and Hsp90 co-chaperones lead to significant reduction in generation of [$psi^-$] cells by over expression of Hsp104 and also presence of Hsp90 inhibitors like radicicol abolishes curing, implying the importance of Sti1 in curing through Hsp70 and Hsp90 interaction (Reidy & Masison, 2010).

It is still not clear about the part of the prion protein that Hsp104 targets but previous reports have shown that the 5.5 imperfect copies of oligopeptide repeat (OR) present within the prion domain (PrD) of Sup35 acts as a possible element of [$PSI^+$] transmission to daughter cells (Chernoff, 2004; Osherovich et al., 2004). However, studies have shown that Sup35 derivative with shuffled PrD (maintained amino acid but lacked repetitive order) are still able to propagate prions in a Hsp104 dependent manner (Ross et al., 2005). Moreover, not all prions contain OR regions. Also there were studies which have shown that the middle domain of Sup35 influence the effect of Hsp104 on prion (Liu et al., 2002).

Mutational analysis has revealed that the protein disaggregation machinery of Hsp104 for prion polymers is different from the disaggregation machinery for heat-denatured proteins. Mutations in Hsp104 revealed that it is the lateral channel of Hsp104 that threads off the amyloid fibres thus indicating that the lateral channel is involved in dissaggregation of prion polymers, while the central pore is used for disaggregating heat damaged proteins (Cashikar et al., 2002; Kurahashi & Nakamura, 2007; Lee et al., 2003).

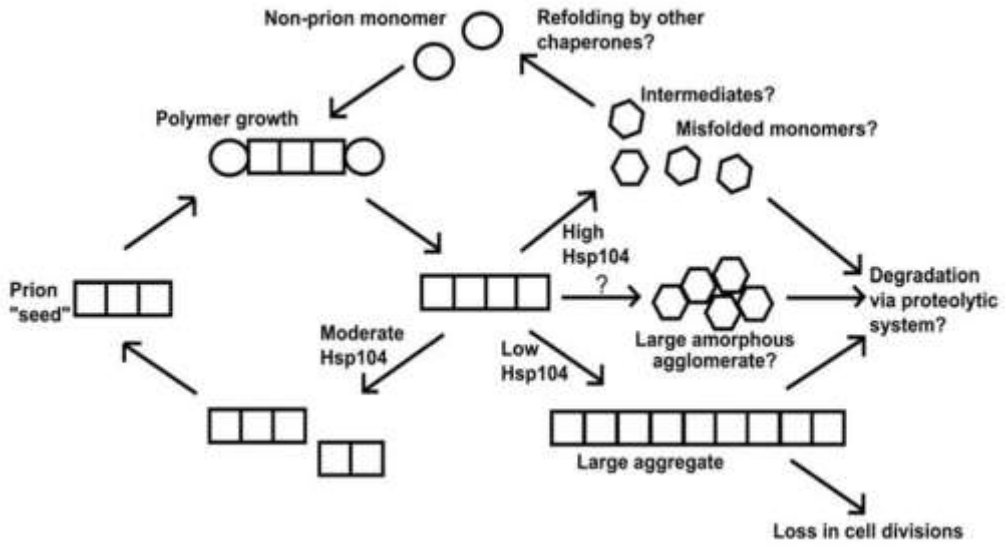



**Figure 1.13 Role of Hsp104 in Sup35 prion propagation.** At moderate levels of Hsp104 the initial polymer is broken down into seeds that initiate new rounds of prion propagation. At low levels of Hsp104 a large aggregate is formed that is eventually lost during cell division At high levels of Hsp104, amyloid polymers are hypothesized to be solubilized into presumably monomeric misfolded intermediates, whose fate remains unknown; they could be refolded into a non-prion conformation, probably with help of other chaperones, or degraded. Large aggregates (amorphous agglomerates) also appear. Figure adapted from Romanova & Chernoff (2009).

### 1.8 Chaperones and mammalian prions

Given the extreme conservation of Hsp70 family throughout evolution, it is possible that mammalian Hsp70 homologue may play a role in propagation of prion form of PrP although much research needs to be focussed on this area to finally elucidate the mechanism of propagation of mammalian prion. To date the only chaperone that appears to facilitate the *in vitro* conversion of $PrP^C$ to protease resistant form is bacterial chaperonin GroEL (DebBurman et al., 1997; Stöckel & Hartl, 2001) but it is not infectious. Other chaperones definitely play a role in correct folding of the $PrP^C$, for example inhibition of Hsp90 by geldenamycin leads to change in $PrP^C$ conformation and glycosylation (Ochel et al., 2003). It is still unknown how prions are generated in cells but research in the past decade on prion biology has opened an interesting area of molecular chaperone mediated process of aggregate dissociation and proves to be more fascinating due to these discoveries in yeast as it can prove to be a good model to further the investigation of involvement of molecular chaperones in prion research.

**Table 1.5 Role of molecular chaperones on yeast prion propagation.** Table adapted from Perrett & Jones (2008)

| Chaperones or co-chaperones | Cellular function | Effects on prion propagation | |
|---|---|---|---|
| | | **Deleted** | **Overexpressed** |
| Hsp104 | Protein disaggregation and stress tolerance | Cures all known naturally occurring yeast prions | Efficiently cures [*PSI*$^+$] but not [*URE3*] or [*PIN*$^+$]/[*RNQ*$^+$] |
| Hsp70 (Ssa1-4) | Protein folding and stress tolerance. Binds to denatured proteins and prevent aggregation.Also involved in aspects of protein translocation and translation | Deletion of all 4 Ssa members is lethal, constitutes an essential gene family | Can cure some variants of [*PSI*$^+$] when co-chaperones co-expressed. Can counteract the [*PSI*$^+$] curing effect of Hsp104 overexpression. Ssa1 can cure [*URE3*] while Ssa2 cannot. |
| Hsp70 (Ssb1/Ssb2) | Ribosome associated.Aid in folding of newly synthesised proteins. | Ten fold increase in spontaneous appearance of [*PSI*$^+$] in a [*PIN*$^+$] background. | Can cure some weak variants of [*PSI*$^+$] |
| Hsp40 (Ydj1, Sis1, Apj1) | Deliver peptide substrates and stimulate ATPase activity of their relevant Hsp70 partner. Sis1 is involved in translation initiation. | No effects of *YDJ1* or *APJ1* deletion. *SIS1* is essential. | Ydj1 efficiently cures [*URE3*] but not [*PSI*$^+$]. All three can cure artificial [*PSI*$^+$] variants. |
| Hsp110 (Sse1/2; related to Hsp70) | NEF of Hsp70 (Ssa and Ssb) | Reduced efficiency of propagation of some [*PSI*$^+$] variants. Cures [*URE3*]. | Efficiently cures [*URE3*] |
| Fes1 | NEF for Hsp70 (Ssa) | Reduced efficiency of propagation of some [*PSI*$^+$] variants. Cures [*URE3*]. | No reported effects |
| Sti1 | Aids in Hsp70-Hsp90 protein folding cycle.Sti1 bridges Hsp70 to Hsp90 and regulates ATPase activity of both proteins | Reduced stability of [*URE3*] | Cures artificial [*PSI*$^+$] variants and weakens wild type variant |

## 1.9 Significance of studying the post-translational modifications of Hsp70 on prion propagation and cellular functions

Cells are highly responsive to various environmental conditions and the phosphorylation status is affected by such environmental stimuli. Thus protein phosphorylation has evolved as an important post-translational modification that regulates the biological functions of the cell. The phosphorylation profile of a cell is highly complex and contains both high and low abundance proteins being phosphorylated and dephosphorylated at numerous sites as per cell requirement. Global phosphorylation analysis can provide a key insight into identifying the potential kinases that phosphorylate proteins in the cell and elucidate the signalling pathways. Recent global proteomics studies have demonstrated that *in vivo* yeast Hsp70 is phosphorylated (Albuquerque et al. 2008). Cytosolic Hsp70 is a ubiquitous molecular chaperone that is involved in responding to a variety of cellular stresses. A major function of Hsp70 is to prevent the aggregation of denatured proteins by binding to exposed hydrophobic regions and preventing the accumulation of amorphous aggregates. In the model eukaryotic *S. cerevisiae*, to efficiently carry out such functions Hsp70 works in concert with a number of co-chaperones such as the Hsp40 protein Ydj1 and nucleotide exchange factors (NEFs) such as Sse1 and Fes1. While much data has accrued in relation to the ATPase and substrate binding cycles of Hsp70 (primarily from assessment of the *E. coli* orthologue DnaK) there is a distinct lack of information regarding the regulation of this important chaperone at the post-translational level. Thus the primary objective of this study was to use a simplified yeast system to systematically assess a variety of non-phosphorylateable and phosphomimetic Hsp70 mutants for phenotypic alterations in Hsp70 functions and also to assess how Hsp70's phosphorylation status *in vivo* may affect prion propagation, to look into other affects on cellular functions of Hsp70 such as basal and acquired thermotolerance level and the effect of these mutations on cell viability. Given Hsp70s central role in a variety of important cellular metabolic pathways and the conservation of these phosphorylatable sites in higher eukaryotes, these findings have far reaching implications. Because of the extreme conservation of Hsp70s from yeast to humans the findings from this study can aid in the characterisation of Hsp70's cellular functions in mammalian systems.

## 1.10 Objectives of the study

The primary objective of this study was to assess the functional significance of post-translational modification on prion propagation and cellular functions. To initiate this primary goal of the study, the first step undertaken was to generate an array of non-phosphorylatable and phosphomimetic Hsp70 mutants. After the generation of these mutants the next step was to phenotypically characterise these mutants and investigate effects on prion maintenence and propagation and also to look into any affects of these mutations on other cellular functions of Hsp70.

Mutants can be modeled onto the 3D crystal structure of bacterial DnaK and bovine Hsc70 and thus decipher possible disruptions caused by the mutation to protein stability and function. This would further help to look into whether the mutations are directly having an effect on prion propagation or if the phenotypic differences observed in the mutant strains are due to structural defects on the protein brought about by these mutations. Using 2D-gel electrophoresis, this study looked into dephosphorylation of Hsp70 in response to heat stress, which may reflect a requirement for broadening chaperone/client interactions following stress.

Another objective of this project was to look into the functional specialization within the cytosolic Hsp70 Ssa family and also to analyse the gene expression patterns of individual Ssa's by carrying out microarray analysis and further confirmation of these results by performing qPCR. This project also aimed to carry out further analysis on previously isolated mutants of the Ssa family that has already been implicated in prion propagation and investigate the functional conservation of ATPase and PBD mutations across the cytosolic Ssa family and implicate a general role of the Hsp70 Ssa family in prion propagation.

# CHAPTER 2

## MATERIALS AND METHODS

## 2.1 *Saccharomyces cerevisiae* plasmids and strains used in the study

### 2.1.1 Plasmid vectors used in this study

Yeast expression vectors used in this study are listed in Table 2.1. Plasmids carrying mutated *SSA* genes generated by random mutagenesis (Loovers et al., 2007; Cusack, 2010) or site directed mutagenesis (SDM) are listed in Table 2.2. Plasmids carrying single and double phosphomutations of *SSA* genes generated by SDM are listed in Table 2.3. Multiple phosphomutations of *SSA1* gene were also generated by SDM and the mutant plasmids are listed in Table 2.4. All the plasmids used in this study were under the control of *SSA2* promoter.

### 2.1.2 *S. cerevisiae* strains used in this study

All the *S. cerevisiae* strains used in this study (Table 2.5) are [$PSI^{+}$] except for Ssa1-4 strains that are used in result chapter 5 are [$psi^{-}$]. The strains are all derivatives of G402. The working stocks of these strains were maintained on specific growth media containing 2% (w/v) agar. These strains were maintained at 4 ºC in the laboratory. Approximately every 15 days, these strains were sub-cultured onto fresh plates to avoid any kind of contamination. For long time storage, the strains were prepared by scraping a large number of cells from the plates and adding it into a solution that contains 15% glycerol (v/v), 85% liquid YPD (v/v) (Burke et al., 2000). These stocks were stored at -70ºC.

**Table 2.1 Yeast expression vectors used in this study.**

| Plasmid | Description | Source |
|---|---|---|
| pRS315 | Centromeric *Saccharomyces cerevisiae* shuttle vector - *LEU2* selection | Sikorski & Hieter, (1989) |
| pRS316 | Centromeric *Saccharomyces cerevisiae* shuttle vector - *URA3* selection | Sikorski & Hieter, (1989) |
| pDCM90 | *URA3*-based single-copy plasmid containing a gene for yeast expression of a thermolabile bacterial luciferase | Daniel C. Masison |
| pC210-Ssa1 | pRS315 carrying *SSA1* under *SSA2* promoter - *LEU2* selection | Yeast Genetics Laboratory |
| pC210-Ssa2 (pDCM64) | pRS313 carrying *SSA2* gene- *LEU2* selection | Yeast Genetics Laboratory |
| pC210-Ssa3 | pRS315 carrying *SSA3* under *SSA2* promoter - *LEU2* selection | Yeast Genetics Laboratory |
| pC210-Ssa4 (PA4) | pRS315 carrying *SSA4* under *SSA2* promoter - *LEU2* selection | Daniel C. Masison |
| pJ120 | pRS315 carrying *SSA1* with 500bp 5' and 3' flanking regions - *LEU2* selection | Jung et al.(2000) |

**Table 2.2 Mutant plasmids used in this study.**

| Plasmid | Description | Source |
|---|---|---|
| SA1-73 | G73D introduced by random mutagenesis into pJ120 | Loovers et al. (2007) |
| SA1-287 | G287D introduced by random mutagenesis into pJ120 | Loovers et al. (2007) |
| SA1-295 | T295I introduced by random mutagenesis into pJ120 | Loovers et al. (2007) |
| SA1-475 | F475S introduced by random mutagenesis into pJ120 | Loovers et al. (2007) |
| SA2-73 | G73D mutation introduced into pC210-Ssa2 by SDM | This study |
| SA2-287 | G287D mutation introduced into pC210-Ssa2 by SDM | Cusack (2010) |
| SA2-295 | T95I mutation introduced into pC210-Ssa2 by SDM | Cusack (2010) |
| SA2-475 | F475S mutation introduced into pC210-Ssa2 by SDM | This study |
| SA3-73 | G73D mutation introduced into pC210-Ssa3 by SDM | This study |
| SA3-287 | G287D mutation introduced into pC210-Ssa3 by SDM | Cusack (2010) |
| SA3-295 | T95I mutation introduced into pC210-Ssa3 by SDM | Cusack (2010) |
| SA3-475 | F475S mutation introduced into pC210-Ssa3 by SDM | Cusack (2010) |
| SA4-73 | G73D mutation introduced into pC210-Ssa4 by SDM | Cusack (2010) |
| SA4-287 | G287D mutation introduced into pC210-Ssa4 by SDM | Cusack (2010) |
| SA4-295 | T295I mutation introduced into pC210-Ssa4 by SDM | Cusack (2010) |
| SA4-475 | F475S mutation introduced into pC210-Ssa4 by SDM | Cusack (2010) |

**Table 2.3 Plasmids with single and double phosphomutations of *SSA* gene.**

| *Plasmid | Description |
|---|---|
| 1´2 | T36A mutation introduced into pC210-Ssa1 by SDM |
| 2´2 | T36E mutation introduced into pC210-Ssa1 by SDM |
| 1a | S62A mutation introduced into pC210-Ssa1 by SDM |
| 2d | S62E mutation introduced into pC210-Ssa1 by SDM |
| 3´1 | T113A mutation introduced into pC210-Ssa1 by SDM |
| 4’7 | T113E mutation introduced into pC210-Ssa1 by SDM |
| 3. D | S151A mutation introduced into pC210-Ssa1 by SDM |
| 4. A | S151E mutation introduced into pC210-Ssa1 by SDM |
| 3n | T492A mutation introduced into pC210-Ssa1 by SDM |
| 4d | T492E mutation introduced into pC210-Ssa1 by SDM |
| 5b | S535A mutation introduced into pC210-Ssa1 by SDM |
| 6h | S535E mutation introduced into pC210-Ssa1 by SDM |
| 7g | S551A mutation introduced into pC210-Ssa1 by SDM |
| 8v | S551E mutation introduced into pC210-Ssa1 by SDM |
| 1. B | S603A mutation introduced into pC210-Ssa1 by SDM |
| 2. B | S603E mutation introduced into pC210-Ssa1 by SDM |
| SA1-A | T378A mutation introduced into pC210-Ssa1 by SDM |
| SA1-E | T378E mutation introduced into pC210-Ssa1 by SDM |
| SA2-A | T378A mutation introduced into pC210-Ssa2 by SDM |
| SA2-E | T378E mutation introduced into pC210-Ssa2 by SDM |
| SA3-A | T379A mutation introduced into pC210-Ssa3 by SDM |
| SA3-E | T379E mutation introduced into pC210-Ssa3 by SDM |
| SA4-A | T379A mutation introduced into pC210-Ssa4 by SDM |

**Table 2.3 continued……**

| | |
|---|---|
| SA4-E | T379E mutation introduced into pC210-Ssa4 by SDM |
| 5´G | T113A mutation introduced into 1´2 by SDM |
| 6´P | T113E mutation introduced into 1´2 by SDM |
| 7´C | T113A mutation introduced into 2´2 by SDM |
| 8´C | T113E mutation introduced into 2´2 by SDM |
| 1´2 -1a | S62A mutation introduced into 1´2 by SDM |
| 1´2 -2d | S62E mutation introduced into 1´2 by SDM |
| 2´2 -1a | S62A mutation introduced into 2´2 by SDM |
| 2´2 -2d | S62E mutation introduced into 2´2 by SDM |
| 1a.5b | S535A mutation introduced into 1a by SDM |
| 5. F | S603A mutation introduced into 3. D by SDM |
| 6. C | S603A mutation introduced into 4. A by SDM |
| 7. E | S603E mutation introduced into 3. D by SDM |
| 8. J | S603E mutation introduced into 4. A by SDM |
| PDM1-a | S535A mutation introduced into 3n by SDM |

* All the mutated plasmids were generated in this study by SDM

**Table 2.4 Plasmids with multiple phosphomutations of *SSA1* gene.**

| *Plasmid | Description |
|---|---|
| 1-5´f | S62A mutation introduced into 5´Gby SDM |
| 8´C-1a-A | S62A mutation introduced into 8´C by SDM |
| 8´C-2d-A | S62E mutation introduced into 8´C by SDM |
| 1a.5b.7g-D | S551A mutation introduced into 1a.5b by SDM |
| PDM2-D | S551A mutation introduced into PDM1-a by SDM |
| ATP-c | S151A mutation introduced into 1-5´f by SDM |
| 1-5´f-5h | S535A mutation introduced into 1-5´f by SDM |
| 1-5´f-3h | T492A mutation introduced into 1-5´f by SDM |
| PDM3-A | S603A mutation introduced into PDM2-D by SDM |
| 1-5´f-5h-7c | S551A mutation introduced into 1-5´f-5h by SDM |
| 1-5´f-5h-7c-3D | S151A mutation introduced into 1-5´f-5h-7c by SDM |
| 1-5´f-5h-7c-3D-1B-b | S603A mutation introduced into 1-5´f-5h-7c-3D by SDM |

* All the mutated plasmids were generated in this study by SDM

**Table 2.5 Yeast strains used in this study.**

| *Strain | Genotype |
|---|---|
| G402 | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3*-1f/pRDW10 |
| Ssa1 | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-Ssa1 |
| Ssa2 | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-Ssa2 |
| Ssa3 | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210-Ssa3 |
| Ssa4 | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/pC210-Ssa4 |
| $Ssa1^{G73D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*,*ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{G73D}$ |
| $Ssa1^{G287D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{G287D}$ |
| $Ssa1^{T295I}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ $pJ120^{T295I}$ |

**Table 2.5 continued .....**

| | |
|---|---|
| Ssa1$^{F475S}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pJ120$^{F475S}$ |
| Ssa2 $^{G73D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA2* $^{G73D}$ |
| Ssa2 $^{G287D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA2* $^{G287D}$ |
| Ssa2 $^{T295I}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA2* $^{T295I}$ |
| Ssa2 $^{F475S}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA2* $^{F475S}$ |
| Ssa3 $^{G73D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA3*$^{G73D}$ |
| Ssa3 $^{G287D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA3*$^{G287D}$ |
| Ssa3 $^{T295I}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA3* $^{T295I}$ |
| Ssa3 $^{F475S}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA3*$^{F475S}$ |

**Table 2.5 continued.....**

| | |
|---|---|
| Ssa4 $^{G73D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA4*$^{G73D}$ |
| Ssa4 $^{G287D}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA4*$^{G287D}$ |
| Ssa4 $^{T295I}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA4*$^{T295I}$ |
| Ssa4 $^{F475S}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*, *ssa4*::*URA3-1f*/ pC210-*SSA4*$^{F475S}$ |
| Ssa1 $^{T36A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A}$ |
| Ssa1 $^{T36E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E}$ |
| Ssa1 $^{S62A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S62A}$ |
| Ssa1 $^{S62E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* |

**Table 2.5 continued…..**

| | |
|---|---|
| Ssa1 $^{T113A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S62E}$ |
| Ssa1 $^{T113E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T113E}$ |
| Ssa1 $^{S151A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S151A}$ |
| Ssa1 $^{S151E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S151E}$ |
| Ssa1 $^{T492A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T492A}$ |
| Ssa1 $^{T492E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T492E}$ |
| Ssa1 $^{S535A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S535A}$ |
| Ssa1 $^{S535E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S535E}$ |
| Ssa1 $^{S551A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S551A}$ |

**Table 2.5 continued.....**

| | |
|---|---|
| Ssa1 $^{S551E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S551E}$ |
| Ssa1 $^{S603A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S603A}$ |
| Ssa1 $^{S603E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S603E}$ |
| Ssa1 $^{T378A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T378A}$ |
| Ssa1 $^{T378E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T378E}$ |
| Ssa2 $^{T378A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA2* $^{T378A}$ |
| Ssa2 $^{T378E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA2* $^{T378E}$ |
| Ssa3 $^{T379A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA3* $^{T379A}$ |
| Ssa3 $^{T379E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA3* $^{T379E}$ |

**Table 2.5 continued.....**

| | |
|---|---|
| Ssa4 $^{T379A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA4* $^{T379A}$ |
| Ssa4 $^{T379E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA4* $^{T379E}$ |
| Ssa1 $^{T36A+T113A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+T113A}$ |
| Ssa1 $^{T36A+T113E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+T113E}$ |
| Ssa1 $^{T36E+T113A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E+T113A}$ |
| Ssa1 $^{T36E+T113E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E+T113E}$ |
| Ssa1 $^{T36A+S62A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E+T113E}$ |
| Ssa1 $^{T36A+S62E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+S62E}$ |
| Ssa1 $^{T36E+S62A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E+S62A}$ |

**Table 2.5 continued…..**

| | |
|---|---|
| Ssa1 $^{T36E+S62E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E+S62E}$ |
| Ssa1 $^{S151A+S603A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S151A+S603A}$ |
| Ssa1 $^{S151E+S603A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S151E+S603A}$ |
| Ssa1 $^{S151A+S603E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S151A+S603E}$ |
| Ssa1 $^{S151E+S603E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S151E+S603E}$ |
| Ssa1 $^{T492A+S535A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T492A+S535A}$ |
| Ssa1 $^{T36A+S62A+T113A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T492A+S535A}$ |
| Ssa1 $^{T36E+S62A+T113E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E+S62A+T113E}$ |
| Ssa1 $^{T36E+S62E+T113E}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36E+S62E+T113E}$ |

**Table 2.5 continued……..**

| | |
|---|---|
| Ssa1 $^{S62A+S535A+S551A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{S62A+S535A+S551A}$ |
| Ssa1 $^{T492A+S535A+S551A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T492A+S535A+S551A}$ |
| Ssa1 $^{T36A+S62A+T113A+S151A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+S62A+T113A+S151A}$ |
| Ssa1 $^{T36A+S62A +T113A+S535A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+S62A +T113A+S535A}$ |
| Ssa1 $^{T36A+S62A +T113A+T492A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+S62A +T113A+T492A}$ |
| Ssa1 $^{T492A+S535A+S551A+S603A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T492A+S535A+S551A+S603A}$ |
| Ssa1 $^{T36A+S62A+T113A+S535A+S551A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+S62A+T113A+S535A+S551A}$ |
| Ssa1 $^{T36A+S62A+T113A+S151A+S535A+S551A+ S603A}$ | *MAT*a *ade2-1 SUQ5 kar1-1 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX*, *ssa2*::*HIS3*, *ssa3*::*TRP1*,*ssa4*::*URA3-1f*/pC210-*SSA1* $^{T36A+S62A+T113A+S151A+S535A+S551A+ S603A}$ |

***** All the strains stated in Table 2.5 were generated in this study except for G402 strain that was generated by Jones & Masison (2003)

## 2.2 Bacterial strain used in the study

*Escherichia coli* strain DH5α was used in this study. Competent DH5α was prepared according to the protocol described by Maniatis et al. (1982) with minor modifications and maintained at -70 °C.

## 2.3 Sterilisation technique

The materials and growth media required for all the aseptic techniques were subjected to autoclaving at 121 °C for 15 min. Any solutions susceptible to disintegration during autoclaving were filter sterilised using 0.22 µm Millipore membrane filters. The workplace was wiped with 70% (v/v) ethanol prior to carrying out any experiments.

## 2.4 Yeast and bacterial growth media

All the growth media used in this research are as mentioned by Burke et al. (2000). The chemicals required for this study was purchased from Sigma-Aldrich Chemical Co. Ltd. U.K., unless otherwise stated and all the required growth media are sourced from Becton, Dickinson and Company, Le Point de Claix, France (BD). For preparation of all the growth media, the final volume was adjusted with $dH_2O$, autoclaved and stored at room temperature (RT). If solidification of the growth medium was required, bacto-agar was added to the growth media (1.5%, w/v), autoclaved and allowed to cool at 60 $^{o}$C. Medium was then poured into sterile petri dishes under sterile conditions and allowed to set. Agar plates were stored at 4 $^{o}$C.

### 2.4.1 Media composition for culturing yeast

**Yeast Peptone Dextrose (YPD), 1L**

10 g Bacto- yeast extract

20 g Bacto-peptone

20 g D-glucose

20 g Bacto-agar (for solid media)

**YPD-guanidine hydrochloride (Gdn-HCl), 1L**

10 g Bacto- yeast extract

20 g Bacto-peptone

20 g D-glucose

20 g Bacto-agar (for solid media)

The media was cooled to approximately 60 °C prior to addition of Gdn-HCl. Gdn-HCl stock solution (300 mM) was prepared by dissolving 2.9 g of Gdn-HCl in 100 ml of $dH_2O$ followed by filter sterilisation. Then 10 ml of the Gdn-HCl stock solution was added to 990 ml of the growth media to get a final concentration of 3 mM.

**YPD-Sorbitol, 1L**

10 g Bacto- yeast extract

20 g Bacto-peptone

20 g D-glucose

20 g Bacto-agar (for solid media)

To get a final concentration of 1 M sorbitol 182 g of sorbitol was added to 500 ml of $dH_2O$ and stirred until the sorbitol was completely dissolved. The components of the YPD media listed above were then added to 500 ml of the prepared sorbitol solution and the mixture was then adjusted with $dH_2O$ to get a final volume of 1000 ml.

**YPAD (Slant Medium), 1L**

10 g Bacto- yeast extract

20 g Bacto-peptone

20 g D-glucose

40 mg Adenine sulphate

20 g Bacto-agar (for solid media)

**Synthetic Complete (SC), 1L**

6.7 g Bacto-yeast nitrogen base without amino acid and ammonium sulphate

20 g D-glucose

2 g Dropout mix (Table 2.6)

20 g Bacto-agar (for solid media)

The above media can then be supplemented depending on the growth requirements of the strain with the required amino acid listed in Table 2.7.

**Table 2.6 Composition of dropout mixture .**

| Amino acid | Quantity (g)* |
|---|---|
| Alanine | 2 |
| Arginine | 2 |
| Aspartic acid | 2 |
| Asparagine | 2 |
| Aminobenzoic acid | 2 |
| Cysteine | 2 |
| Glutamine | 2 |
| Glutamic acid | 2 |
| Glycine | 2 |
| Isoleucine | 2 |
| Inositol | 2 |
| Lysine | 2 |
| Methionine | 2 |
| Proline | 2 |
| Phenylalanine | 2 |
| Serine | 2 |
| Threonine | 2 |
| Tyrosine | 2 |
| Valine | 2 |

*The components were added together, ground using a pestle and mortar and stored at RT.

**Table 2.7 Concentrations of stock solutions and final concentration of amino acids supplemented into SC media.**

| Amino acid | Quantity added to 100 ml $dH_2O$ to make stock solutions (g) | Volume of stock added to 1 L SC media (ml) | Final concentration in the media (µg/ml) |
|---|---|---|---|
| Leucine | 1 | 10 | 100 |
| Adenine | 0.2 | 10 | 20 |
| Histidine | 1 | 2 | 20 |
| Tryptophan | 1 | 2 | 20 |
| Uracil | 0.2 | 10 | 20 |

**5-fluoro-orotic acid Medium (5-FOA), 1L**

6.7 g Bacto-yeast nitrogen base without amino acid and ammonium sulphate

20 g D-glucose

2 g Dropout mix (Table 2.6)

50 mg Uracil

Amino acids stock solution (Table 2.7) leucine (10 ml), adenine (10 ml), histidine (2 ml), tryptophan (2 ml), uracil (10 ml) was added to the above components along with 1 g 5-FOA and the mixture was brought to a final volume of 500 ml with $dH_2O$ in a Schott Duran bottle and filter sterilised. After that 20 g of Bacto-agar was added to 500 ml $dH_2O$ in a separate Schott Duran bottle and autoclaved. The agar was then allowed to cool to approximately 60 °C and then the components of both the bottles were mixed before pouring into sterile Petri dishes. The final concentration of 5-FOA was 1 mg/ml.

**SC-$H_2O_2$**

SC media was made as described above. After autoclaving, the media was allowed to cool to approximately 60 ºC. Then $H_2O_2$ [30% (w/w) in $H_2O$] was added at the required concentration (Table 2.8).

**Table 2.8 Volume of 30% $H_2O_2$ added to 250 ml SC.**

| Concentration required (mM) | Volume of 1 mM stock added (µl) |
|---|---|
| 1 | 21 |
| 2 | 42 |
| 3 | 63 |
| 4 | 84 |
| 5 | 105 |

**Additional Stress Medium**

YPD was made as described above. After autoclaving, the media was allowed to cool to approximately 60 ºC. The cell wall damaging agent was then added as required.

- **Caffeine**: A 100 mM stock solution of caffeine was prepared by adding 9.6 g caffeine to 500 ml $dH_2O$. This was followed by preparation of 10 mM working solution of caffeine and the solution was filter sterilised prior to adding in the media. The final concentration of caffeine required was 8 µM. This was prepared by adding 160 µl of 10 mM stock solution to 199.84 ml of cooled media.

- **SDS**: A 1% SDS stock solution was prepared by adding 1 g SDS in a final volume of 100 ml $dH_2O$. This stock solution was then filter sterilised prior to adding in the media. The final concentration of SDS required was 0.005% and 0.01%. This was prepared by adding 1 ml and 2 ml of 1% SDS stock solution to 199 ml and 198 ml of cooled media to get a final concentration of 0.005% and 0.01% SDS respectively.

- **Calcofluor white (CW)**: 1000X calcofluor white stock solution was prepared by adding 1 g CW to 100 ml of $dH_2O$. 1000X stock was filter sterilised prior to use and then diluted 1/10 in sterile $dH_2O$ to make 100X stock solution. The final concentration of CW required was 10 µg/ml and this was prepared by adding 2 ml of the 100X stock solution to 298 ml of cooled media.

### 2.4.2 Media for culturing *E. coli*

**Luria Broth (LB) Media, 1L**

25 g Luria bertani broth

20 g Bacto agar (for solid media)

LB media was supplemented with ampicillin at a concentration of 100 µg/ml by adding 2 ml of stock solution per L of media. The ampicillin stock solution was prepared by dissolving 5 g ampicillin to 100 ml of sterile $dH_2O$ to get a final concentration of 50 mg/ml. This solution was then filter sterilised, aliquot into 1 ml volumes and stored at -20 °C.

## 2.5 Yeast and bacterial culture conditions

### 2.5.1: Yeast culture conditions

Yeast strains were grown on YPD plates or the required selective media at 30 ºC static (BD series Binder incubator, Mason Technology) for 48 h and kept at 4 °C for short term storage.

For liquid culturing, yeast strains were inoculated in 5 ml appropriate media in 14 ml round bottom falcon tubes (BD). Cultures were then incubated overnight in an orbital shaker (Innova 4000 Orbital Shaker, New Brunswick Scientific, UK) at 30 °C and 200 rpm.

### 2.5.2 Bacterial culture conditions

DH5α bacterial strain was cultured overnight on LB-ampicillin plates at 37 °C (B series, Binder incubator, Mason Technology) prior to liquid culturing.

For liquid culturing, a single DH5α colony was selected from the overnight cultured LB-ampicillin plate and inoculated in 5 ml LB-ampicillin media. These cultures were incubated overnight at 37 ºC, 200 rpm (Innova 44 Orbital Shaker, New Brunswick Scientific, UK).

### 2.5.3 Harvesting yeast and bacterial liquid cultures

Yeast and bacterial liquid cultures were harvested by centrifugation for 5 min at 2500 rpm and 4000 rpm respectively (Centrifuge 5810R, Eppendorf).

## 2.6 Determination of yeast cell density

The yeast overnight culture was first diluted 1/100 with sterile $dH_2O$. An 8 µl of the diluted culture was loaded onto a haemocytometer (Bright Line, Hausser Scientific) and the cells were counted using a light microscope (INGENIUS, Bio imaging Syngene) at a magnification of 32 X (Burke et al., 2000). To calculate the number of cells/ml in a particular culture, the following formula was used:

cells/square x $10^4$ x dilution factor = cells/ml in the culture

For some experiments indirect assessment of cell density were measured based on the absorbance of yeast cultures. Absorbance was determined using a spectrophotometer (Eppendorf) at $OD_{600nm}$ (Burke et al., 2000).

## 2.7 Yeast transformation

The yeast transformation carried out in this study was as mentioned by Burke et al. (2000) with minor modifications. All the chemicals used in this method were stored at RT unless otherwise mentioned.

### 2.7.1 Materials required

- **1 M Lithium acetate (Sigma):** 10.2 g of lithium acetate was dissolved in 100 ml of $dH_2O$, followed by autoclaving.
- **100 mM Lithium acetate:** 1 ml of 1 M lithium acetate was dissolved in 9 ml of sterile $dH_2O$ to get a final concentration of 100 mM.
- **50 % Polyethylene glycol (PEG) (Sigma):** 50 g PEG was dissolved in 50 ml sterile $dH_2O$ and adjusted to a final volume of 100 ml followed by autoclaving.
- **Carrier DNA:** 200 mg of single stranded DNA (Sigma) was dissolved in 100 ml of 10 mM Tris/1 mM EDTA solution giving a final concentration of 2 mg/ml. This was aliquoted into 1 ml volumes in 1.5 ml microfuge tubes (Eppendorf) and boiled at 100 °C for 5 min on an Accublock Digital Dry Bath (Labnet International.Inc). The aliquots were subsequently cooled on ice and stored at -20 °C.
- **10 mM Tris/1 mM EDTA [pH=7.5] (Sigma):** 100X stock concentrations was prepared by adding 12.14 g Tris and 2.92 g EDTA to sterile $dH_2O$ and the final volume was adjusted to 100 ml, followed by autoclaving. 1 ml of the stock solution was pipetted out and dissolved in 99 ml sterile $dH_2O$ to give a final concentration of 10 mM Tris/1 mM EDTA.

- **Plasmid DNA:** The desired plasmid DNA to be transformed into the yeast cell is prepared by method described in Section 2.10. The DNA concentration was determined on a Nanodrop Spectrophotometer (Mason Technology) and diluted to a volume of 50 µl containing 750 ng of total DNA.

### 2.7.2 Preparation of competent yeast

Yeast strains were inoculated and harvested as previously described in Section 2.5.2 and 2.5.3. The cell density was then determined as described in Section 2.6 and then cells were resuspended in 50 ml of fresh media to get a cell density of 5 x $10^6$ cells/ml. Yeast cultures were incubated in an orbital shaker (Innova 4000 Orbital Shaker, New Brunswick Scientific, UK) at 30 °C and 200 rpm for 3-5 h until the cell concentrations were reached 1-2 x $10^7$ cells/ml. The cells were harvested by centrifugation at 2500 rpm, washed in 25 ml of sterile $dH_2O$ and centrifuged again. The supernatants were disposed of and pellets were resuspended in 1 ml of 100 mM lithium acetate. The cells were transferred to microfuge tubes and pelleted in a table top centrifuge (Centrifuge 5415D, Eppendorf AG, Hamburg) at 13200 rpm for 5 s and resuspended in 500 µl of 100 mM Lithium acetate. The competent yeast cells were then aliquoted to 50 µl volume and could be stored at 4 °C for 1-2 weeks or used immediately for transformation.

### 2.7.3 Transformation of competent yeast cells

The competent cells were centrifuged at 13200 rpm for 15 s and the residual lithium acetate was removed. The following components were then added to the cells in the order listed below:

240 µl 50% (w/v) PEG

36 µl 1 M Lithium acetate

25 µl single stranded carrier DNA (taken from 2 mg/ml stock)

50 µl mix of molecular $H_2O$ and desired DNA

The cell suspensions were then vortexed and incubated at 30 °C for 30 min. The mixtures were then heat-shocked for 20-25 min at 42 °C in an Accublock Digital Dry Bath (Labnet International.Inc). The microfuge tubes were centrifuged at 13000 rpm for 15 s and the pellets were resuspended in 200 µl of sterile $dH_2O$ and plated onto selective agar plates. These plates were incubated at 30 °C for 2-3 days.

## 2.8 Site-directed mutagenesis of plasmid DNA

Site directed mutagenesis (SDM) is an effective molecular biology technique that can be used to incorporate nucleotide changes in a fragment of the DNA molecule thus generating mutants of interest at specific amino acid residues. The technique was first described by Hutchison et al. (1978). Complementary primer pairs were designed to anneal to *SSA1, SSA2, SSA3* or *SSA4* DNA with one or more of the base pairs being mismatched to the wild-type (WT) sequence resulting in a single amino acid change in the protein sequence. The primer pairs were designed to replace the threonines and serines at the positions of interest with either alanine which is immune to phosphorylation or glutamic acid that is phosphomimetic. Plasmid DNA was prepared as described in Section 2.10 and PCR reaction was carried out using PTC-200 Peltier Thermal Cycler (MJ Research) as described below using primers listed in Table 2.9. The PCR products were treated with 1 µl *Dpn1* (New England Biolabs) and 2.5 µl NEB Buffer 4 (New England Biolabs) and incubated for 2 h at 37 °C to get rid of any methylated parental plasmid DNA. Approximately 8 µl of PCR product was transformed into competent *E. coli* strain DH5α using transformation method as described in Section 2.9.2. Four colonies were picked randomly from each transformation and the plasmid DNA extracted as described in Section 2.10. The plasmid DNA was then quantified in Nanodrop Spectrophotometer (Mason Technology) and sent for sequencing to LGC genomics as described in Section 2.11 using primers listed in Table 2.12. After confirmation of the presence of desired mutation by DNA sequence analysis, the plasmid was then transformed into yeast for phenotypic characterisation. The plasmids were also fully sequenced to confirm that there is no other mutations in the gene except for the desired one introduced by SDM.

| **PCR Mixture (25 µl)** | **PCR Cycle (Time in min)** |
|---|---|
| PFU Ultra Buffer (Stratagene) = 1 X | Step1 = 95 °C 1:00 |
| dNTP mix (Promega) = 0.2 mM each | Step2 = 95 °C 0:30 |
| Forward primer (Sigma) = 100 ng | Step3 = 55 °C 1:00 |
| Reverse primer (Sigma) = 100 ng | Step4 = 68 °C 2:00 per kb |
| Template DNA = 50 ng | Step5 = Go to Step2, 12 times |
| HF PFU ULTRA (Stratagene) = 2.5 U | Step6 = 68 °C 7:00 |
| Molecular grade $H_2O$ adjusted to 25 µl | Step7 = 4 °C forever |
| | Step8 = End |

**Table 2.9 PCR primers used for site-directed mutagenesis .**

| PRIMER | OLIGONUCLEOTIDE |
|---|---|
| T36A-F | CAAGGTAACAGAACCGCTCCATCTTTTGTCGCT |
| T36A-R | AGCGACAAAAGATGGAGCGGTTCTGTTACCTTG |
| T36E-F | CAAGGTAACAGAACCGAGCCATCTTTTGTCGCT |
| T36E-R | AGCGACAAAAGATGGCTCGGTTCTGTTACCTTG |
| S62A-F | GCTGCTATGAATCCTGCGAATACCGTTTTCGACGC |
| S62A-R | GCGTCGAAAACGGTATTCGCAGGATTCATAGCAGC |
| S62E-F | GCTGCTATGAATCCTGAGAATACCGTTTTCGACGC |
| S62E-R | GCGTCGAAAACGGTATTCTCAGGATTCATAGCAGC |
| T113A-F | GAAACCAAGAACTTTGCCCCAGAACAAATCTCC |
| T113A-R | GGAGATTTGTTCTGGGGCAAAGTTCTTGGTTTC |
| T113E-F | GAAACCAAGAACTTTGAGCCAGAACAAATCTCC |
| T113E-R | GGAGATTTGTTCTGGCTCAAAGTTCTTGGTTTC |
| S151A-F | CAGCTTACTTCAACGATGCTCAAAGACAAGCTACC |
| S151A-R | GGTAGCTTGTCTTTGAGCATCGTTGAAGTAAGCTG |
| S151E-F | CAGCTTACTTCAACGATGAGCAAAGACAAGCTACC |
| S151E-R | GGTAGCTTGTCTTTGCTCATCGTTGAAGTAAGCTG |
| T378A-F | GCTGCTATTTTGGCTGGTGACGAATCTTC |
| T378A-R | GAAGATTCGTCACCAGCCAAAATAGCAGC |
| T378E-F | GCTGCTATTTTGGAGGGTGACGAATCTTC |
| T378E-R | GAAGATTCGTCACCCTCCAAAATAGCAGC |

**Table 2.9 continued…….**

| | |
|---|---|
| T379A-3F | CCGTTCAAGCTGCCATTTTAGCCGGCGATCAATC |
| T379A-3R | GATTGATCGCCGGCTAAAATGGCAGCTTGAACGG |
| T379E-3F | CCGTTCAAGCTGCCATTTTAGAGGGCGATCAATC |
| T379E-3R | GATTGATCGCCCTCTAAAATGGCAGCTTGAACGG |
| T379A-4F | GTACAGGCTGCCATCTTAGCGGGTGACCAGTCGTC |
| T379A-4R | GACGACTGGTCACCCGCTAAGATGGCAGCCTGTAC |
| T379E-4F | GTACAGGCTGCCATCTTAGAGGGTGACCAGTCGTC |
| T379E-4R | GACGACTGGTCACCCTCTAAGATGGCAGCCTGTAC |
| T492A-F | GCCGTCGAAAAGGGTGCTGGTAAGTCTAACAAGATC |
| T492A-R | GATCTTGTTAGACTTACCAGCACCCTTTTCGACGGC |
| T492E-F | GCCGTCGAAAAGGGTGAGGGTAAGTCTAACAAGATC |
| T492E-R | GATCTTGTTAGACTTACCCTCACCCTTTTCGACGGC |
| S535A-F | GAATCTCAAAGAATTGCTGCCAAGAACCAATTGGAATC |
| S535A-R | GATTCCAATTGGTTCTTGGCAGCAATTCTTTGAGATTC |
| S535E-F | GAATCTCAAAGAATTGCTGAGAAGAACCAATTGGAATC |
| S535E-R | GATTCCAATTGGTTCTTCTCAGCAATTCTTTGAGATTC |
| S551A-F | CTTTGAAGAACACCATTGCTGAAGCTGGTGACAAATTG |
| S551A-R | CAATTTGTCACCAGCTTCAGCAATGGTGTTCTTCAAAG |
| S551E-F | CTTTGAAGAACACCATTGAGGAAGCTGGTGACAAATTG |
| S551E-R | CAATTTGTCACCAGCTTCCTCAATGGTGTTCTTCAAAG |

**Table 2.9 continued…..**

| | |
|---|---|
| S603A-F | GCCAACCCAATCATGGCTAAGTTGTACCAAGCTGG |
| S603A-R | CCAGCTTGGTACAACTTAGCCATGATTGGGTTGGC |
| S603E-F | GCCAACCCAATCATGGAGAAGTTGTACCAAGCTGG |
| S603E-R | CCAGCTTGGTACAACTTCTCCATGATTGGGTTGGC |
| Ssa2-G73D-F | CGACGCTAAGCGTTTGATCGATAGAAACTTCAATGACCC AGAAGTC |
| Ssa2-G73D-R | GACTTCTGGGTCATTGAAGTTTCTATCGATCAAACGCTTA GCGTCG |
| Ssa2-F475S-F | CAAATTGAAGTCACTTCCGATGTCGACTC |
| Ssa2-F475S-R | GAGTCGACATCGGAAGTGACTTCAATTTG |
| Ssa3-G73D-F | AAGCGGTTAATTGATCGTAAATTTGATG |
| Ssa3-G73D-R | CATCAAATTTACGATCAATTAACCGCTT |

## 2.9 Preparation and transformation of *E. coli*

### 2.9.1 Preparation of competent *E. coli* DH5α

Competent DH5α was prepared as described by Maniatis et al. (1982) with minor modifications in the method. A single colony of DH5α was inoculated in 10 ml of LB media and incubated overnight at 37 °C shaking incubator at 200 rpm. The overnight culture was then added to 1 L of fresh LB media and incubated at 37 °C shaking incubator at 200 rpm for approximately 2 h. The culture was then split equally into 4 x 250 ml centrifugation tubes and kept in ice for 10 min. The chilled culture was then harvested by centrifugation at 5000 rpm for 10 min at 4 °C in a GSA rotor. The pellets were resuspended in 10 ml RF1 Buffer (Table 2.10) and chilled on ice for 30 min followed by centrifugation at 5000 rpm for 10 min at 4 °C. The pellets were resuspended in 3.2 ml RF2 Buffer (Table 2.11) and left on ice for 15 min. Finally, the competent DH5α were aliquoted into 100 µl volumes and were transferred into pre-cooled 1.5 ml microfuge tubes and stored at -70 °C.

**Table 2.10 Components of buffer RF1 used in preparation of competent DH5α.**

| Components | Amount added | Final concentration/volume |
|---|---|---|
| K-Acetate | 1.47 g | 30 mM |
| $CaCl_2.2H_2O$ (dehydrate) | 5 ml of 1 M stock | 10 mM |
| Glycerol | 7.5 ml | 15% |
| $dH_2O$ | - | Adjusted to 450 ml* |
| RbCl | 6 g | 100 mM |
| $MnCl_2.4H_2O$(tetrahydrate) | 4.95 g | 50 mM |
| $dH_2O$ | | Adjusted to 500 ml |

* The solution was adjusted to pH 5.92 with 0.2 M acetic acid (0.2 M = 3 g in 250 ml $dH_2O$).

**Table 2.11 Components of buffer RF2 used in preparation of competent DH5α.**

| Components | Amount added | Final concentration/volume |
|---|---|---|
| RbCl | 5 ml of a 1 M stock | 10 mM |
| $CaCl_2.2H_2O$ | 5.5 g | 75 mM |
| $dH_2O$ | - | Adjusted to 500 ml |

All chemicals and solutions were added in consecutive order from top to bottom, as illustrated in the Tables. Both RF1 and RF2 buffers was filter sterilised and stored at 4 °C. All chemicals were purchased from Sigma.

### 2.9.2 Transformation of plasmid DNA into *E. coli*

Transformation of plasmid DNA was carried out as mentioned by Maniatis et al. (1982) with minor modifications in the method. Prior to doing the transformation of DH5α, LB-ampicillin plates were pre-warmed at 37 °C for approximately 1 h and the competent DH5α cells were thawed on ice for 30 min to ensure efficient transformation. 1-20 µl (approximately 1 µg) of plasmid DNA was added to 100 µl of defrosted competent DH5α and incubated on ice for 30 min. The cells were then exposed to a heat shock at 42 °C for 1 minute on a digital dry bath and then placed on ice for further 2 min. For the recovery of the heat shocked cells, approximately 400 µl of fresh LB media was added followed by incubation at 37 °C shaking for 1 h. The cells were then spread onto LB-ampicillin plates and incubated at 37 °C overnight.

### 2.9.3 High efficiency 5 minutes transformation of *E. coli*

High efficiency transformation of DH5α was carried out as essentially described by Pope & Kent (1996). This method was followed for high yield of plasmid DNA after the desired mutants were generated. In brief, LB-ampicillin plates were pre-warmed at 37 °C for approximately 1 h prior to transformation to ensure optimum conditions for efficient transformation. 50 µl of competent DH5α were thawed on ice and 1 µl of plasmid DNA was added to 50 µl of competent DH5α immediately after defrosting. The cells were then left on ice for 5 min and finally spread onto warm LB-ampicillin plates and incubated overnight at 37 °C.

## 2.10 Isolation of plasmid DNA from *E. coli*

Isolation of plasmid DNA from *E.coli* was done using plasmid miniprep kit from Qiagen according to the manufacturer's recommendation. Following transformation of plasmid DNA into *E. coli*, individual bacterial colonies were isolated from the LB-ampicillin plates. To isolate DNA, a single colony was inoculated in 5 ml of LB-ampicillin liquid media and grown overnight at 37 °C at 200 rpm. The next day, cells were harvested by centrifugation for 5 min at 4000 rpm. The pellets were resuspended in 250 µl resuspension buffer. This was followed by addition of 250 µl of lysis buffer and 350 µl of neutralising buffer, mixed well and centrifuged for 10 min at 13000 rpm. The supernatants were then removed and transferred to DNA binding columns where they were centrifuged for 1 min at 13000 rpm. Flow-through in catchment tubes was discarded and 750 µl of wash buffer was added to each column followed by centrifugation for 1 min at 13000 rpm. Flow-through was discarded again and columns were centrifuged for an additional 1 minute to remove any residual buffer from the binding column. DNA was eluted by adding 50 µl of elution buffer directly into the binding columns and allowing it sit for 1-2 min. The binding columns were transferred to fresh 1.5 ml microfuge tubes and plasmid DNA was isolated by a 1 min centrifugation at 13000 rpm. Plasmid DNA was stored at -20 °C.

### 2.11 DNA sequence analysis

Plasmid DNA was prepared as described in Section 2.10. Approximately 1 µg of DNA was mixed with 20 pmol of an appropriate primer and the final volume was adjusted to 15 µl with sterile $dH_2O$ in a 1.5 ml microfuge tube. The microfuge tubes were then labelled with prepaid barcode purchased from LGC Genomics and the samples were sent to Sanger sequencing (LGC Genomics Germany). DNA was sequenced by dye-terminator sequencing and results were available for downloading after 3-5 days from LGC Genomics website. Primers were designed to cover complete sequences of *SSA1*, *SSA2*, *SSA3* and *SSA4*. A list of sequencing primers used to screen mutants in *SSA* family is listed in Table 2.12.

**Table 2.12 DNA sequencing primers**

| Primer Name | Oligonucleotide |
|---|---|
| Sp1-R | CTCATTATACCCAGATCA |
| Sp2-R | CTTTTCGACGGCGGAAAC |
| Sp3-R | CATCGACTTGAGATTTG |
| Sp4-R | CAGCGTCATTGACCTTGGC |
| Sp5-F | CGAGAAGGGATTGAGTTG |
| Sp6-F | GGTCTTGGGTAAGATG |
| Sp7-F | CAGATCTACTTTGGACCC |
| Sp8-F | CACAAATTGAAGTCAC |
| SSA2-1-F | CCTGTGTTGCTCAC |
| SSA2-2-F | CACTTCATCCAAGAATTC |
| SSA2-3-F | CTACTTATGCTGACAAC |
| SSA3-1F | GATGATCCTGAAGTGACG |
| SSA3-2F | GATTCGAAGCTGGACAAGTC |
| SSA3-3F | GTAGGCTCTCGAAGGATG |
| SSA3-7F | CTTGTATGTCAATGTTTG |

**Table 2.12 continued……**

| | |
|---|---|
| SSA3-8F | GACACTCATTTAGGTG |
| SSA3-9R | GGTAGAGAAGGTTTCCG |
| SSA3-8R | CACCTAAATGAGTGTC |
| SSA4-1F | GAACGATGCTAAGCATTACC |
| SSA4-2F | GATCTACATTGGAGCCAG |
| SSA4-3F | GAACGTATCTGCCGTTGA |
| SSA4-4R | GCGAAGCATCTAACCAAT |
| SSA4-5R | GTTAAGATGGCAGCCTGTAC |
| SSA4-6R | GATACGAAGAACGTTCAAGC |

## 2.12 Isolating mutants by plasmid shuffle technique

To express mutants of Ssa1, Ssa2, Ssa3 or Ssa4 as the sole source of the Ssa in *S.cerevisiae*, the plasmid shuffle technique was employed as essentially described by Loovers et al. (2007). The G402 strain contains the plasmid pRDW10, which contains a uracil (*URA3*) marker and is the sole source of Ssa in the strain. Strain G402 was transformed with the Ssa1, Ssa2, Ssa3 or Ssa4 mutants. Transformants were then selected on medium lacking leucine as described in Section 2.7.3. Transformation plates were then replica plated onto medium containing limiting adenine and also 5-fluoroorotic acid (5-FOA), a chemical that selects against *URA3* cells and hence against the presence of the pRDW10 plasmid. After 2 days at 30 °C incubation, colonies were screened for appearance of red or dark pink phenotype at this stage, and were scored as potentially harbouring a mutant *SSA* allele that cannot maintain [$PSI^{+}$]. The colour phenotype of colonies was scored subjectively from 0 to 9, with 0 being white and 9 being red with the help of Image J- java based image processing program (Abràmoff et al., 2004).

## 2.13 Monitoring the presence of the prion phenotype [*PSI*$^+$] in *S. cerevisiae*

Monitoring of [*PSI*$^+$] was carried out as described by Jones & Masison (2003). The presence or absence of the [*PSI*$^+$] prion can be monitored in yeast strains by a simple colour assay (Figure 2.1 A).

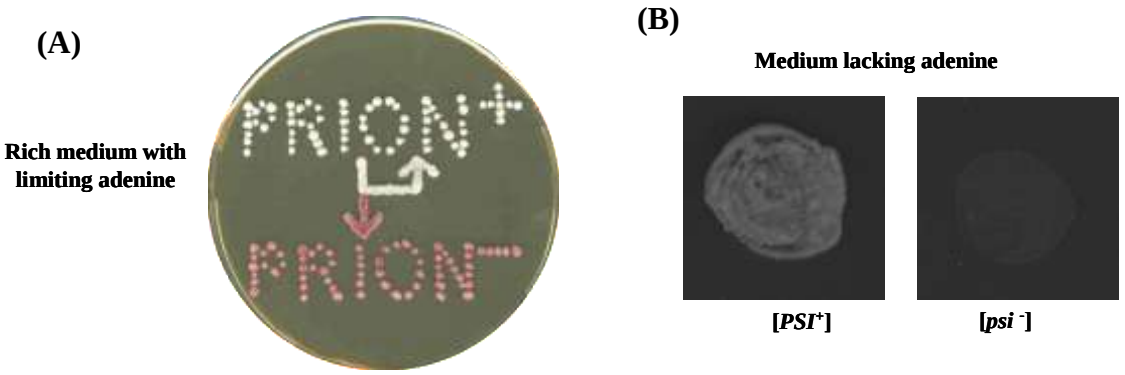



**Figure 2.1 Monitoring the presence/absence of [*PSI*$^+$] using a simple colour and growth assay.**(A) [*PSI*$^+$] cells grow as white colonies on media containing limited adenine, while [*psi*$^-$] cells grow as red colonies (Loovers et al., 2007). (B) [*PSI*$^+$] cells grow on medium lacking adenine, while [*psi*$^-$] cells do not grow on medium lacking adenine

The strains that were used to carry out experiments involving the monitoring of [*PSI*$^+$] contain a pre-mature ochre mutation (*ade2.1*) in a gene responsible for adenine biosynthesis. Sup35 is a *S. cerevisiae* protein involved in translation termination and when in its fully functional state, halts translation at this premature stop codon, leading to the build-up of a red pigment in the cells. [*psi*$^-$] cells are red when grown with limiting amounts of adenine (*i.e.*, on YPD medium) because of accumulation of pigmented form of the substrate of Ade2 protein, aminoamidizole ribotide (Silver & Eaton, 1969). [*PSI*$^+$] cells are self-replicating aggregated form of the translation termination factor Sup35. The presence of [*PSI*$^+$] and the weak suppressor tRNA SUQ5 in our strains causes partial suppression of the *ade 2.1* mutation and read through of the of the *ochre* mutation resulting in the synthesis of the full length Ade2 protein and the cells appear white on limiting adenine media (*i.e.*, on YPD medium) (Cox, 1965; Liebman et al., 1975).

A second way of monitoring the presence of [*PSI*$^+$] is by growth of yeast cells on medium lacking adenine (Figure 2.2). [*PSI*$^+$] cells grow on medium lacking adenine (Cox, 1965) whereas [*psi*$^-$] cells do not (Figure 2.1 B). This is because [*PSI*$^+$] cells

produce functional Ade2 protein which is involved in the adenine biosynthesis pathway. In [*psi*$^-$] cells the adenine biosynthesis pathway is disrupted and cells require supplemented adenine for normal growth.

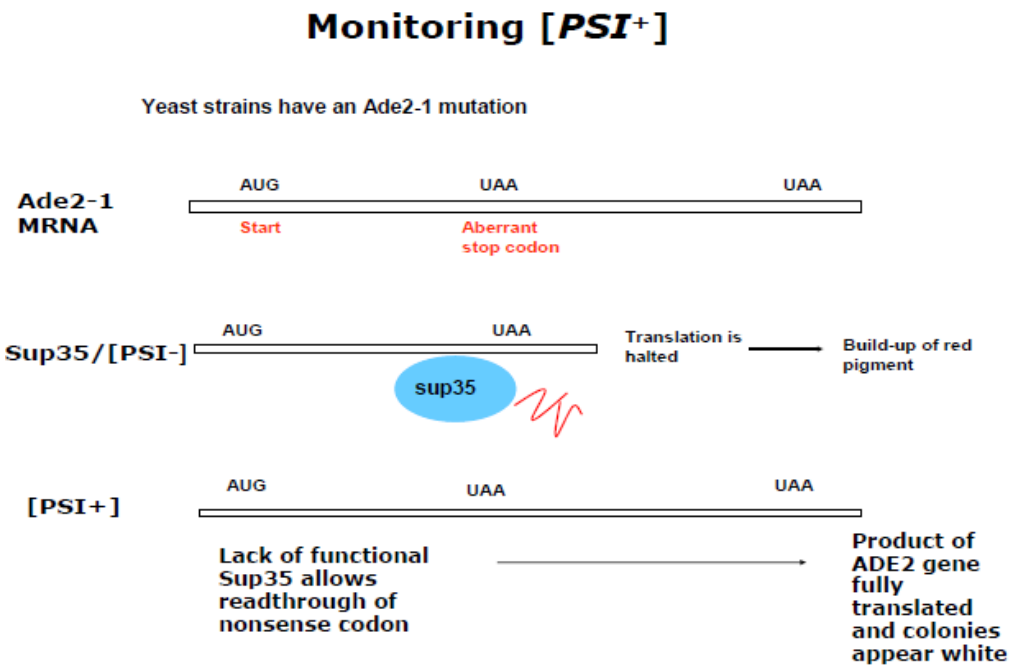



**Figure 2.2 Consequences of [*PSI*$^+$].** Non-functional, aggregated Sup35p [*PSI*$^+$] allows read through of the *ade2-1* mutation.

### 2.14 Yeast growth curve

The mean generation time (MGT) or the growth curve of the yeast strains were determined as essentially described by Amberg et al. (2005). In brief, the yeast strains were inoculated in 5 ml YPAD media at 30 °C overnight in shaking incubator. The next day, $OD_{600nm}$ was determined for each of the overnight cultures and diluted back to $OD_{600nm}$ 0.1 (t = 0 min) in 30 ml of fresh YPAD. These cultures were then placed at 30 °C shaking incubator. Cell density was measured at $OD_{600nm}$ at intervals of every 2 h (t = 2 h, 4 h, 6 h and 8 h). Three technical replicates were taken for all the yeast strains. The data was then entered into excel worksheet and compiled into linear graphs. This was achieved by plotting log values of $OD_{600nm}$ determined against time. The time points which gave the most linear points on the graph were used in the following formula to calculate the MGT for each yeast strain.

$$\frac{\textbf{Time (minutes)}}{\textbf{(Log } t_1 \textbf{ – Log } t_0\textbf{) / Log2}}$$

## 2.15 Comparative growth analysis

Spot growth assays were carried out as essentially described by Fitzpatrick et al. (2011). Briefly, yeast strains were cultured in 5 ml of YPD or selective medium overnight at 30 °C and 200 rpm in a shaking incubator. Next day, the overnight culture was diluted back to an $OD_{600nm}$ = 0.1 in 5 ml fresh medium and cells were grown to exponential phase to a density of 3 x $10^6$ cells/ml by incubating at 30 °C in a shaking incubator. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a 96 well microtiter plate (Starstedt). 200 µl of the culture was placed in A1 in the 96 well microtiter plate and 160 µl media was added to wells A2-A6. A 1 in 5 dilution of the culture was done by dispensing 40 µl cultures from well A1 through to A6 with the help of multi-pronged replicator (Sigma). Following a 1 in 5 serial dilution, the cells were transferred to appropriate agar plates and left for drying under the laminar flow. The plates were then incubated at 30 °C and in case of temperature sensitive assays plates were incubated at 30 °C, 37 °C and 39 °C for 3 days and growth were monitored over the period of time.

## 2.16 Acquired thermotolerance assay

Thermotolerance assays were performed as described by Jung & Masison (2001) with minor modifications. Yeast strains were prepared as described in Section 2.15 to a cell density of 3 x $10^6$ cells/ml and were resuspended in fresh media to a final cell density of 5 x $10^6$ cells/ml in 14 ml round bottom tubes (BD Falcon). Then 1 ml of the cultures were aliquoted and transferred to ice before exposing it to a temperature of 39 °C for 1 h to induce Hsp104 that is important for conferring acquired thermotolerance to the cell and thus preventing protein denaturation while the cells are experiencing heat shock. After 1 h at 39 °C another 1 ml aliquot was immediately transferred to ice. Subsequently, 1 ml volumes of cell aliquots were maintained at 47 ºC shaking incubator at 200 rpm and the aliquots were then transferred to ice at an interval of every 10 min for duration of 40 min. As described in Section 2.15 a 1 in 5 serial dilution of the aliquots were carried out and the cells were spotted onto appropriate agar plates and incubated for 2 days at 30 °C and left 1 day on bench and growth were monitored over the period of time.

### 2.17 Luciferase assay

Luciferase refolding was assayed as essentially described by Parsell et al. (1994b). Prior to the assay, the strains were transformed with pDCM90 plasmid as described in Section 2.7. To perform the luciferase assay, yeast strains containing pDCM90 was grown in 5 ml of SC media lacking uracil at 30 °C shaking incubator overnight at 200 rpm. Next day, the cultures were diluted to an $OD_{600nm}$ = 0.2 into the same medium and three biological replicates for each strain was prepared. Diluted cultures were incubated at 37 °C shaking for 30 min to induce expression of heat shock proteins. After 30 min at 37 °C, cellular luciferase activity of each strain was measured in triplicate for each of the three biological replicates of each strain by immediately adding 10 µl of decanal (Sigma) to 200 µl of culture in 5 ml rohren tubes (Starstedt) in a FB 12 Luminometer (Berthold Detection Systems). The cells were then transferred to 45 °C and incubated with shaking for 1 h. Cyclohexamide (Sigma) was added after 50 min at 45 °C at a concentration of 10 µg/ml to prevent synthesis. Cultures were then incubated at 45 ºC for a further 10 min. After 1 h heatshock, cellular luciferase activity of each strain was measured as mentioned above. Cultures were shifted to 25 °C for the recovery of cells from heatshock. After 15 min at 25 °C, luciferase refolding activity was measured as described earlier in the section. This was done at an interval of every 15 min to check for the recovery over time for duration of 1 h.

### 2.18 Guanidine curing

Routine curing of [*PSI*$^+$] strains was done by streaking the yeast strains on YPD plates containing 3 mM Gdn-HCl and incubating the plates at 30 °C for 3 days as essentially described by (Jung et al., 2000) with minor modifications. Cells were then restreaked onto YPD and red colonies isolated. Both the [*PSI*$^+$] and [*psi*$^-$] versions of the strains were maintained at 4 °C and also stock was stored at -70 °C as described in Section 2.1.2.

### 2.19 Western blot analysis

#### 2.19.1 Preparation of cell lysates

Overnight YPD cultures were diluted in 25 ml fresh YPD to an $OD_{600nm}$ = 0.1 - 0.2 and grown to $OD_{600nm}$ = 0.5 – 0.8 at 30 °C and cell lysates were prepared as described by DePace et al. (1998) and Newnam et al. (1999) with minor modifications. Cells were collected by centrifugation at 4 °C for 5 min at 2500 rpm and were

immediately placed on ice. Prior to centrifugation, 2 ml screw capped tubes (Starstedt) were filled ¾ with 0.5 mm soda lime glass beads and left to be sitted on ice for 10 – 15 min. Pellets were resuspended in 1 ml of cell lysis buffer (Sigma) and transferred to chilled 2 ml screw capped tubes. The tubes were then inverted to remove air bubbles. Cells were then broken using mini – bead beater (Biospec products) by agitating with glass beads for 20 s at maximum speed and incubating on ice for 30 s between the agitations. This was repeated three times to ensure that the cells were completely disrupted. The cell debris was then removed by centrifugation at 7000 rpm for 5 min and the supernatant containing the total lysate was collected and transferred to pre-chilled 1.5 ml microfuge tubes and stored at –20 °C.

### 2.19.2 Protein quantification

Protein concentration of the cell lysates was carried out using Bradford assay (Bradford, 1976). In brief, protein standard curve was constructed on a Nanodrop Spectrophotometer (Nanodrop 1000, Mason Technology) at $OD_{595nm}$ by using a range of BSA (New England Biolabs) dilutions (125 – 2000 µg/ml). Bradford reagent (Quickstart Bradford dry reagent 1X, Biorad) was allowed to come to RT for 15 min. The cell lysates were thawed on ice for 15 min and then 1 in 2 dilutions was carried out for the cell lysates with sterile $dH_2O$. Then 5 µl of the diluted lysates and 5 µl cell lysis reagent (to provide blank) were added to 500 µl Bradford reagent (Quickstart Bradford dry reagent 1X, Biorad) and incubated at RT for approximately 20 – 30 min. Protein concentrations were then determined following manufacturer's recommendations.

### 2.19.3 Preparation of Sodium Dodecyl Sulphate-Poly acrylamide (SDS-PAGE) gels

12.5 % SDS-PAGE gels were prepared using 1 mm Bio-Rad glass plates with 15 well combs having 20 µl sample capacity. The components of running and stacking gel are listed in Tables 2.13 and 2.14. The glass plates were sealed in a Biorad Gel Cassette and a casting frame. The running gel was prepared first by adding the chemical in the order described in Table 2.13 in a 50 ml tube and the mixture was gently pipetted between two clean glass plates to approximately 70% capacity of the plates. 1-2 ml of 100% isopropanol was layered on top of the running gel to ensure that gel solidification occurred with a smooth levelled surface. Stacking gel was prepared subsequently as described in Table 2.14 and allowed to set between the two glass plates on top of the solidified running gel after discarding 100% isopropanol and washing off with $dH_2O$. Combs were placed between plates directly after stacking gel was added to create wells

into which protein samples would be placed. This arrangement was left to set for 15-20 min.

**Table 2.13 Composition of running gel**

| Reagents | Volume |
|---|---|
| 4X running buffer (34.75 g Tris base and 4 g SDS dissolved in 500 ml $dH_2O$, pH adjusted to 8) | 2.5 ml |
| $dH_2O$ | 3.3 ml |
| Protogel | 4.15 ml |
| 10% ammonium persulphate* | 100 µl |
| TEMED (added just before casting the gel) | 10 µl |

**Table 2.14 Composition of stacking gel**

| Reagents | Volume |
|---|---|
| 2X stacking buffer (15 g Tris base and 0.2 g SDS dissolved in 500 ml $dH_2O$, pH adjusted to 8) | 1.25 ml |
| $dH_2O$ | 0.825 ml |
| Protogel | 400 µl |
| 10% ammonium persulfate* | 25 µl |
| TEMED (added just before casting the gel) | 2.5 µl |

*10% ammonium persulfate is prepared by dissolving 10 g in a final volume of 100 ml sterile $dH_2O$. This was aliquoted to 250 µl volumes and stored at – 4 °C.
** All the reagents for running and stacking gel were purchased from Sigma except for protogel that was purchased from National Diagonostics, England. The reagents were stored at RT except for 10% ammonium persulfate.

### 2.19.4 Sample preparation and SDS-PAGE

The 4X protein sample buffer is prepared as described in Table 2.15. The samples were prepared by mixing the lysate at a concentration of 10 µg to required amount of 4X sample buffer and boiled at 100 °C for 5 min in a digital dry bath. The prepared gels were then assembled in the casting frames with the short plate facing inwards and the clamping frames were then sealed. This setup was then placed into the electrophoresis tank (Biorad) that was filled to appropriate levels with 1X protein running buffer (Table 2.16) and protein samples were then loaded along with the protein ladder (Page ruler plus prestained protein ladder 10-250 kDa, Fermentas, Life Sciences).

The gels were then resolved in 1X protein running buffer at 100 V per gel for 1.5 h in a Biorad Powerpac Basics.

**Table 2.15 Preparation of 4X protein sample buffer**

| Reagents | Concentration | Volume |
|---|---|---|
| Tris pH 6.8 | 10 mM | 625 µl |
| EDTA | 200 mM | 500 µl |
| SDS | 20 % ($wv^{-1}$) | 15 ml |
| glycerol | 100 % ($vv^{-1}$) | 10 ml |

The above reagents were added and final volume was adjusted to 50 ml with sterile $dH_2O$. A pinch of bromophenol blue was added finally and the buffer was stored at RT. All the reagents were purchased from Sigma.

**Table 2.16 Preparation of 10X protein running buffer**

| Reagents | Concentration | Amount |
|---|---|---|
| Tris | 25 mM | 15 g |
| Glycine | 192 mM | 72 g |
| SDS | 0.1 % ($wv^{-1}$) | 5 g |

The above reagents were added and final volume was adjusted to 500 ml with sterile $dH_2O$ and the buffer was stored at RT. All the reagents were purchased from Sigma.

### 2.19.5 Protein transfer to polyvinylidene difluoride (PVDF) membrane

Proteins separated in the gels were then electrophoretically transferred to PVDF membranes (Perkin Elmer) and processed for blotting as per recommendations for use with Bio-Rad gel transfer system. In brief, after the proteins were separated the stacking gel was removed and the gel was equilibriated in transfer buffer for 15 min. In the mean time, the Whatmann filter paper was cut into 4 pieces (Schleicher and Schuell) and was made slightly smaller then the size of the fiber pads. The Whatmann filter paper and the fiber pads were kept soaked in chilled transfer buffer (Table 2.17). The PVDF transfer membrane was cut approximately the size of the gel (8 cm x 5 cm) and soaked in 100% Methanol (Sigma) for 5 s. The membrane was then washed with $dH_2O$ for 1 minute and

then equilibrated for 5 min in chilled transfer buffer. For assembly of the gel and membrane sandwich, a fiber pad was first placed on the black side of the cassette followed by two layers of Whatmann filter paper. The pre-equilibriated gel was then placed gently on top of the Whatmann filter paper and a wet gloved finger was gently run across the gel to avoid formation of any air bubbles that may be trapped underneath the gel. Subsequently, the pre-equilibriated membrane was gently placed on top of the gel and care was taken to avoid formation of any air bubble between the membrane and the gel. Finally, another two layers of Whatmann filter paper was placed on top of the membrane and the second fiber pad that was soaked in transfer buffer was gently placed on top of the Whatmann filter paper and the cassette was closed. Once the cassette was closed and locked it was inserted into the tank with the latch side up and the black side of the cassette facing the black electrode plate. The transfer buffer was then poured to a level that reached the fill line, the Bio-Ice™ cooling unit was then placed inside the tank and a magnetic stirrer bar was dropped into the transfer buffer. The lid was then closed making sure that the colour-coded cables on the lid are attached to the electrode cards of the same colour. The cables were then connected to the power supply and an electric current was applied (100 V/ 350 mA) for precisely 1 h while the buffer was stirred (Stuart Scientific magnetic Stirrer).

**Table 2.17 Preparation of transfer buffer.**

| Reagents | Concentration | Volume |
|---|---|---|
| Tris | 25 mM | 3.03 g |
| Glycine | 192 mM | 14.4 g |
| Methanol | 20 % | 200 ml |
| Deionised water | - | Adjusted to 1 L |

The transfer buffer was prepared and left at – 20 °C for 1 h prior to use. All the chemicals used in the preparation of transfer buffer were purchased from Sigma.

### 2.19.6 Immunoblotting

After the proteins were transferred to PVDF membranes, protein detection using primary and secondary antibodies listed in Table 2.19 and 2.20 were carried out according to manufacturer's recommendation. Briefly, after the proteins were transferred to the PVDF membrane, the membrane was washed 4 times in 30 ml TBS-t (Table 2.18) and in each wash the membrane was left rocking with the TBS-t solution

on it for 5 min before the next wash. Subsequently, blocking was done for 2 h at RT in 40 ml blocking solution (5% Marvel milk powder in TBS-t). After blocking for 2 h, the membrane was incubated overnight with primary antibody and left rocking (Rocker 35A, Labnet) overnight. The following day, the membrane was washed 4 times with TBS-t as described above to remove excess antibody that might cause non-specific binding. This was followed by incubation with alkaline phosphatase labelled secondary antibody precisely for 1 h at RT and left rocking. After 1 h incubation with secondary antibody, the membrane was again washed 4 times with TBS-t as described earlier in this section.

**Table 2.18 Components of 10X TBS buffer and 1X TBS-t**

| **Buffer components** | **Concentrations** | **Volume** |
|---|---|---|
| **10X TBS stock:** | | |
| Tris pH 7.5 | 100 mM | 12.14 g |
| NaCl | 1 M | 58.44 g |
| Deionised water | - | Adjusted to 1 litre |
| **TBS-t:** | | |
| 10X TBS buffer | 1X | 100 ml |
| Tween | 0.1 % | 1 ml |
| Deionised water | - | 899 ml |

* All the reagents were purchased from Sigma and buffer was stored at room temperature

**Table 2.19 Primary antibodies used in this study.**

| **Name of antibody** | **Animal of origin** | **Source** | **Dilution** |
|---|---|---|---|
| Hsp70 SPA-822 | Mouse | Stressgen (Victoria, BC, Canada) | 1/2,000 |
| Hsp104 | Rabbit | John Glover (University of Toronto) | 1/150,000 |

**Table 2.20 Secondary antibodies used in this study.**

| Name of antibody | Animal of origin | Source | Dilution |
|---|---|---|---|
| Anti-Mouse IgG (Fab specific) – Alkaline Phosphatase antibody | Goat | Sigma-Aldrich Chemical Co. Ltd. U.K. | 1/10,000 |
| Anti-Rabbit IgG (whole molecule) – Alkaline Phosphatase antibody | Goat | Sigma-Aldrich Chemical Co. Ltd. U.K. | 1/10,000 |

### 2.19.7 Chemiluminescence and developing

Chemiluminescence using substrate CDP – star (Perkin Elmer) and exposure to X-ray film (Kodak Biomax Light Film, U.S.A) used for detection was carried out as per manufacturer's recommendation. In brief, 5 ml of CDP-star was used to cover the membrane and kept rocking precisely for 5 min at RT. The membrane was then placed between two acetate sheets and care was taken to avoid air bubbles that might be trapped between the acetate sheets and the membrane. Subsequently, the membrane was placed in an autoradiography cassette (FBAC 810 Fisher Biotech) in darkness and an X-ray film (Kodak Biomax Light Film, U.S.A) was gently placed on top of the membrane and left for varying periods of time ranging from 20 - 40 s. The film was immediately transferred to developer (25 ml Kodak developer and 100 ml of $dH_2O$) for approximately 10 s or until image began to appear. The film was then washed in $dH_2O$ and transferred to fixer (25 ml Kodak fixing solution and 100 ml of $dH_2O$) for approximately 40 s. Finally, the film was washed in $dH_2O$ and allowed to dry.

### 2.19.8 Stripping membrane

In order to probe a membrane with a second primary antibody, it is necessary to "strip" the membrane to remove traces of the first antibodies used. The membrane was first washed twice for 5 min with 10 ml of $dH_2O$, followed by 10 ml of TBS-t. This was followed by membrane incubation with 40 ml 0.2 M sodium hydroxide for 5 min, rocking at RT. Sodium hydroxide was removed by washing with 5 ml of $dH_2O$ for 5

min and then the membrane was blocked as described in Section 2.19.6 and then the membrane was ready for incubation with another primary antibody.

### 2.19.9 Coomassie staining of protein gels

Coomassie staining was carried out as essentially described by Burnette (1981) . In brief, following SDS-PAGE, the gel was transferred to coomassie brilliant blue stain (Serva) and left rocking at RT overnight. The next day, gel was destained with destaining solution that consisted of 10% Ethanol, 10% Acetic acid and 80% $dH_2O$. The destaining was carried out for approximately 2 h until proteins in the gel became clearly visible.

### 2.19.10 Amido black staining of membrane

Amido black staining was carried out as described by Towbin et al. (1979) with minor modifications. PVDF membranes were stained in 10 ml of amido black stain (0.1% Naphthol blue black dissolved in 2% Acetic acid and 45% Methanol) for 1 minute. The membranes were then washed in $dH_2O$ and incubated with destain (2% Acetic acid and 45% Methanol) until a clear banding pattern appeared.

## 2.20 Two-dimensional gel electrophoresis (2D-GE)

Two dimensional gel electrophoresis was carried out as essentially described by Agrawal & Rakwal (2006) and Hajduch et al. (2001) with minor modifications. The buffers and reagents required for the process are listed as below (Table 2.21 – 2.25). All the chemicals required were purchased from Sigma.

### 2.20.1 Preparation of buffers and reagents for 2D-GE.

**Table 2.21 IEF Buffer constituents.**

| Component | Concentration | Quantity in 50 ml |
|---|---|---|
| Urea | 8 M | 24.024 g |
| Thiourea | 2 M | 7.612 g |
| CHAPS | 4% | 2 g |
| Triton X-100 | 1% | 500 µl |
| Tris Base | 10 mM | 0.0606 g |
| DTT* | 65 mM | 0.01 g in 1 ml IEF buffer |
| Ampholyte pH 3-10* | 0.8% | 8 µl in 1 ml IEF buffer |

After preparation of 50 ml of the buffer it was aliquoted into 1 ml volumes and kept at – 20 °C.
* DTT and ampholyte was added to the 1 ml aliquot prior to the experiment

**Table 2.22 IPG strip Equilibration Buffer constituents.**

| Component | Concentration | Quantity in 500 ml |
|---|---|---|
| Glycerol | 30% | 150 ml |
| SDS | 2% | 10 g |
| Urea | 6 M | 180 g |
| Tris | 50 mM | 3.03 g |

IPG strip equilibration buffer was adjusted to pH 6.8, aliquoted into 20 ml volumes and stored at -20ºC.

**Table 2.23 10X SDS Buffer constituents.**

| Component | Concentration | Quantity in 2 L |
|---|---|---|
| Tris | 25 mM | 60.57 g |
| Glycine | 192 mM | 288.82 g |
| SDS | 0.1% | 20 g |

10X SDS buffer was stored at RT. 1X SDS buffer was prepared from 10X prior to the experiment by adding 100 ml of 10X SDS buffer to 900 ml of sterile $dH_2O$

**Table 2.24 Agarose sealing solution.**.

| Components | Volume |
|---|---|
| Trizma base | 1.5 g |
| Glycine | 7.2 g |
| SDS | 0.5 g |
| Agarose | 0.5 g |
| Deionised $H_2O$ | Adjusted to 100 ml |

100 ml of agarose sealing solution was prepared, a pinch of bromophenol blue was added and stored at RT

**Table 2.25 Volume of reagents required to prepare two 12% gels.**

| Reagents | Volume |
|---|---|
| Protogel | 12.17 ml |
| Resolving buffer | 7.91 ml |
| Deionised $H_2O$ | 10.01 ml |
| 10% APS | 121.7 µl |
| TEMED(added just before casting the gel) | 12.17 µl |

1.5 mm glass plates were used for casting of the 12% gel. The casting procedure is similar to that described in Section 2.19.3, except that no isopropanol was used to level the gel. Once the gel solidified it was topped with sterile $dH_2O$ to prevent the gel from drying.

### 2.20.2 Trichloroacetic acid (TCA) protein precipitation

TCA protein precipitation was carried out as essentially described by Granier (1988). Briefly, cell lysates were prepared as described in Section 2.19.1 except that the samples were done in duplicates and the $OD_{600nm}$ was adjusted to 0.1 for both the samples and after the $OD_{600nm}$ reached to 0.5-1.0 one of the duplicates were given a heat shock at 39 °C prior to protein extraction. TCA was added to the thawed cell lysates in the ratio of 1:4 *i.e* 250 µl of TCA (Sigma) was added to 1 ml of the lysate in a 1.5 ml microfuge tube and incubated for 10 min at 4 °C. Lysates were then harvested by centrifugation at 13200 rpm for 7 min. The pellets were washed in 300 µl of chilled acetone followed by centrifugation a 13200 rpm for 6 min. The washing with chilled acetone was repeated another three times to ensure that there is no more TCA left in the protein sample. The pellets were then dried to get rid of the left over acetone by keeping the lids of the 1.5 ml microfuge opened under laminar flow for 5-10 min. DTT and

ampholyte was added to appropriate volume of thawed IEF buffer and the pellets were then resuspended in the 300 µl IEF buffer.

Precipitated proteins were then quantified (Section 2.19.2) and 10 µg of proteins were run under SDS-PAGE (Section 2.19.3 and 2.19.4), followed by coomassie staining (Section 2.19.9) to check for equal loading. It was ensured that the proteins had been quantified accurately and that equal loading was obtained before proceeding.

### 2.20.3 Isoelectric focusing (IEF), first dimension

IEF was carried out as described by Agrawal & Rakwal (2006) and Hajduch et al. (2001) with minor modifications. In brief, 125 µg of the precipitated proteins were resuspended in 125 µl of thawed IEF buffer (DTT and ampholyte added prior to use) to get a concentration of 1 µg/µl. It was ensured that all the protein samples that were to be run had the same protein concentration. A pinch of bromophenol blue was then added to the protein samples that were then pippetted out into the 7 cm coffins (GE Healthcare). The thin plastic strip was then removed gently from the IPG strips (Immobiline Drystrip, pH 3-10, 7 cm, GE Healthcare) which were then placed into the coffin containing the protein sample. It was ensured that the gel side of the IPG strip was facing down into the coffin containing the protein samples and care was taken to avoid any air bubbles between the gel side of the IPG strip and the protein sample. The IPG strip codes were recorded to later identify the protein samples. 1 ml cover fluid (GE Healthcare) was added into each of the coffin until the strips were covered fully, to prevent strip desiccation and the cover slide was placed gently to cover the coffin. Following this, the strips immediately underwent isoelectric focusing.

IEF was carried out on protein samples using Ettan IPG phor II IEF system (GE Healthcare) according to manufacturer's recommendations. Briefly, the coffins with the protein samples were placed on the gold coated surface of the Ettan IPG phor II IEF system ensuring that the positive side of the strip in the coffin is aligned towards the positive end of IEF machine. The lid was then closed and the programme was run under conditions given in Table 2.26.

**Table 2.26 IEF conditions**

| Step/Gradient | Volts (V) | Time (h) |
|---|---|---|
| Step 1 | 50 | 12 |
| Step2 | 250 | 0.15 |
| Gradient3 | 5000 | 2 |
| Step4 | 5000 | 5 |
| Gradient5 | 8000 | 2 |
| Step6 | 8000 | 1 |
| Step7 | 250 | 1 |

### 2.20.4 Gel electrophoresis, second dimension

Gel electrophoresis was carried out as described by Agrawal & Rakwal (2006) and Hajduch et al. (2001) with minor modifications. In brief, in preparation of the second dimension protein separation by electrophoresis, the focussed 7 cm IPG strips were equilibriated in appropriate volumes of IPG strip equilibration buffer. Prior to starting the experiment, a 20 ml aliquot of IPG strip equilibration (Table 2.22) buffer was thawed and divided into two separate 10 ml aliquots for preparation of equilibration buffer A and B. Equilibration buffer A was made by adding 2% DTT while equilibration buffer B was prepared by adding 2.5% Idoacetamide to each of the 10 ml aliquots of IPG strip equilibration buffer. A small amount of bromophenol blue was added to both the buffers to act as a tracking dye. The strips were first equilibriated in equilibration buffer A by keeping it rocking for 15 min followed by 15 min rocking in equilibration buffer B. Each equilibrated strip was placed horizontally on top of a solidified 12% gel with gel-side facing forward and positive side to the left. The agarose sealing solution was boiled and pipetted to cover the strip completely, sealing it to the gel. The gels were then resolved in 1X gel running buffer at 100 V per gel for 1.5 h in a Biorad Powerpac Basics.

### 2.20.5 Protein transfer to PVDF membrane

Protein transfer to PVDF membrane was done as described in Section 2.19.5

### 2.20.6 Immunoblotting

Immunoblotting of the separated proteins was done as described in Section 2.19.6. The primary and the secondary antibody used for immunoblotting was Hsp70 SPA-822 and Anti-Mouse IgG (Fab specific) – Alkaline Phosphatase antibody. The dilution for the primary and the secondary antibody are as described in Table 2.19 and 2.20.

### 2.20.7 Chemiluminescence and developing

Chemiluminescence using substrate CDP – star (Perkin Elmer) and exposure to X-ray film (Kodak Biomax Light Film, U.S.A) used for detection of protein was carried out as described in Section 2.19.7.

### 2.20.8 Alkaline phosphatase treatment

Alkaline phosphatase treatment was done for some of the protein samples prior to IEF focussing to check for any shift in the pH range of the protein isoforms in presence of alkaline phosphatase. In brief, the method involved treatment of 125 µg of the protein with 10 U (10 U = 1 µl) of CIP (calf intestinal phosphatases) (New England Biolabs) and 1X concentration of NEB Buffer 3. The mixture was then incubated for 1 h at 37 °C prior to sample preparation for IEF. After 1 h incubation at 37 °C the final volume was adjusted to 125 µl with IEF buffer and then focussing was carried out as described in Section 2.20.3.

## 2.21 SSA1 3D crystal structure analysis

The SSA1 phosphomutants were modelled onto the 3D crystal structure using the programme PyMOL molecular graphic system (DeLano, 2002). This work has been done in collaboration with Youtao Song (Liaoning University, China). The models created are based on the 3D crystal structure of bovine Hsc70 ATPase domain (Accession no.: 2BUP) and *E.coli* Hsp70 DnaK (Accession no.: 2KHO) obtained from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do).

## 2.22 Differential gene expression analysis of the Ssa family

Microarray analysis was carried out by Toray Industries, Japan and qPCR was done in collaboration with UCD.

### 2.22.1 RNA extraction

Prior to RNA extraction the [*psi*$^-$] yeast strains Ssa1, Ssa2, Ssa3 and Ssa4 were inoculated overnight in 5 ml YPD culture. The following day RNA was extracted using Qiagen RNAesy kit as per manufacturer's recommendation. In brief, the overnight culture was harvested at 2500 rpm for 5 min at 4 °C. Pellets were then resuspended in 600 µl of Buffer RLT and transferred to 1.5 ml microfuge tubes and left on ice. Equal volume of 0.5 mm soda lime glass beads were added to the 1.5 ml microfuge tubes and vortexed for 30 s and left on ice for 30 s. This was repeated 5 times to ensure that the pellets were completely disrupted. The samples were then centrifuged at 4 °C for 3 min at 3000 rpm and the supernatants were then transferred to 1.5 ml microfuge tubes. One volume of 70% molecular ethanol (Merck) was added to the lysate, mixed and transferred to spin columns and centrifuged at 4 °C for 15 s at 10000 rpm. This was followed by addition of 700 µl of Buffer RW1 and samples were centrifuged again at 4 °C for 15 s at 10000 rpm. Then 500 µl of Buffer RPE was added to the spin columns and centrifuged as above. Another 500 µl of Buffer RPE was added to the columns and centrifuged at 4 °C for 2 min at 10000 rpm. The RNA was then eluted in 40 µl RNase free $H_2O$ by centrifugation at 10000 rpm for 2 min at 4 °C. For each strain, three replicate flasks were used and each experiment was conducted twice.

RNA was DNased-treated using TURBO DNA-free kit (Ambion, USA), according to the manufacturers' recommendations. In brief, 0.1 volumes of 10X TURBO DNase Buffer and 1 µl TURBO DNase was added to the RNA, mixed gently and incubated at 37 °C for 25 min. Then 0.1 volume of DNase inactivation reagent was added and incubated at RT for 5 min followed by centrifugation at 10000 rpm for 2 min at 4 °C. RNA solution was then transferred to fresh tubes. RNA concentrations were measured using NanoDrop 1000 Spectrophotometer (Mason Technologies).

### 2.22.2 Microarray analysis

For microarray analysis equal amount of RNA from all three replications from two separate experiments were combined and sent to Toray Industries, Japan. Microarray analysis was carried out as per the *3D-Gene*™ analysis protocol. In brief the method involved synthesizing of aRNA from 1 µg total RNA with Amino Allyl MessageAmp TM II aRNA Amplification kit (Ambion). This was followed by labeling 10 µg of aRNA with Cy5 Mono-ReactiveDyePack (GE Health Care Bioscience) and hybridization of labelled RNA to DNA chip at 37 °C for 16 h at 250 rpm. After

hybridization the DNA chips were washed with wash buffers and scanned by ScanArray Lite (Perkin Elmer). The scanned images of all the DNA chips were quantified using GenePix Pro6.0. The normalized data was then summarised and sent back to us in separate excel files.

### 2.22.3 qPCR reaction

Reverse transcription (RT) of total RNA was conducted as described by Ansari et al. (2007). In brief, 1 µg of total RNA (DNase-treated) and 2.4 µM of oligo $dT_{12-18}$ (Invitrogen, UK) was incubated at 72 °C for 10 min in a 10 µl reaction and then chilled on ice. Then 15 µl reaction mix containing 100 U of MMLV reverse transcriptase, 1X first strand buffer (Promega, USA), 100 µM each of dATP, dGTP, dCTP and dTTP (Gibco BRL, UK), 1 mM dithiothreitol and 20 U of RNaseOut (Invitrogen, UK) was added to the first reaction and incubated at 37 ºC for 2 h. Reactions were stopped by incubation at 95 ºC for 5 min. Solutions were then chilled on ice. 1 µl RNase H was added to the solutions, followed by incubation at 37 ºC for 20 min. cDNA was then stored at -80 ºC until used.

The qPCR analysis was carried out in collaboration with University College Dublin. In brief, RT products (25 µl) were diluted to 200 µl and 2.5µl was PCR-amplified in a 25µl volume reaction containing 12.5µl Premix Ex Taq™ (Perfect Real Time) (Takara, Japan) and 100 nM each of forward and reverse transcript-specific primers (Table 2.27). Primer specificity was confirmed by melting curve analyses of real-time RT-PCR products (81 cycles of 55 °C for 30 s). Real-time RT-PCR reactions were conducted in a Stratagene Mx3000$^{TM}$ real-time PCR machine (Stratagene, USA) and the programme consisted of 1 cycle of 95 °C for 10 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 1 cycle of 95 °C for 60 s. Data were analysed using Stratagene Mx3000$^{TM}$ software (Stratagene, USA). The housekeeping gene used for normalisation of real-time RT-PCR data was *ACT1* (YFL039C); real-time quantification of target gene and of the housekeeping gene was performed in separate reactions. The threshold cycle (CT) values obtained by real-time RT-PCR were used to calculate the accumulation of target gene (relative mRNA accumulation), relative to *ACT1* transcript, by $2^{\wedge-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct$ = (Ct, Target gene - Ct, β-tubulin) (Livak & Schmittgen, 2001). Results were based on the average obtained for two replica RT-PCR reactions per sample.

**Table 2.27 Primers for qPCR**

| Primers | Oligonucleotide |
|---|---|
| ACT1-F | GGGTTTGGAATCTGCC |
| ACT1-R | CCACCAATCCAGACGGAG |
| CBP6-F | TAATTAACAGAACTTCCGGTCCA |
| CBP6-R | TACCGTCAACGGCATTTATTAAC |
| YBR197C-F | TTCCACCAACAGAAGGAAATCTA |
| YBR197C-R | GAGTGCATTGTTATACGGCTACC |
| TOS6-F | CTCTACTCTCTCCACCGTTGCT |
| TOS6-R | GTGGTGTTAGCTTGGCTACTGAT |
| YOX1-F | ATCACCAAGCTTCACTAATCCAA |
| YOX1-R | TCGTGTGATTCATCAACTGTTTC |
| CLN1-F | CTAATCAAGATCAAGGCCCTTTT |
| CLN1-R | CTATTGTAGAGGCCAGTTGCAGT |
| CLN2-F | TATGTGGTAGCACCGTTAGTGTG |
| CLN2-R | TCCATGGTTAATGAAGCTTGTCT |
| MRPL6-F | TAGGTGTTACTGAGGGGCATCT |
| MRPL6-R | CTCTGGTGGATGGAATTTTCTC |
| ATG8-F | CCGTAGGGCAATTTGTTTATGT |
| ATG8-R | ACAAAAACCCGTCCTTATCCTT |
| HSP31-F | CAAGACCGGTGTTTTTGTTGTA |
| HSP31-R | AATTTGGTAATCATCGGCATTC |
| VMA13-F | CCGTGATACCAGAGTATCGTGA |
| VMA13-R | AAACTGGGTTAAAGGTCAGCAA |
| MSS116-F | ATTAGGTTTGAGCCGTAGTCCA |
| MSS116-R | ACGGTTCGTCATCGTAATCTCT |
| OYE3-F | TTTAGCCATCCATGATTGTCAG |
| OYE3-R | GATAGAATTCTTAGCCGCATGG |
| HXT1-F | CCAGCCATGTTTCAACGTACTA |
| HXT1-R | GGTCAACGGTGTACAGAGAACA |
| YNR014W-F | TCACTACTGCCCTCTTCTAGGC |
| YNR014W-R | GTACGAGACGGACCTTTTCATC |

**Table 2.27 continued……**

| | |
|---|---|
| HUF1-F | ACCGGTATTGATAACGATGTCC |
| HUF1-R | CTCCTACACCACGATCGACATA |
| ILV6-F | AACACCGAGGTCAAAGACCTAA |
| ILV6-R | CTGGCCATCACTAGCTCTCTTT |
| MIF2-F | GTAGAAGAGGAGGCTGAGGTGA |
| MIF2-R | ATGTCAAGAACGGGTTTATTGG |
| GPI8-F | AGGCTGTTAACGGATAGATGGA |
| GPI8-R | TTTTCGTACATTTGCTGGAATG |
| UBP10-F | AAACTTGGGTTCTCCATTAGCA |
| UBP10-R | CAGAAATTTTGGAGCTGGAATC |
| SCJ1-F | TGATCAAGGTCCAGGAAAAACT |
| SCJ1-R | ACCACACATCTGTTGAATCTGC |
| ARO9-F | ACAGAGAGTCCCAGGGTATTGA |
| ARO9-R | TCAACAATGAGGAAATCGTGTC |
| YOR387C-F | ATTGGTGATGCTATGGTTCCTT |
| YOR387C-R | GGGGTGCTCAAAATAACAGAAG |
| ADH2-F | CATTGTTAGCGCAGTCGTTAAG |
| ADH2-R | ACAACGTGGTTGAAGACATCAG |
| LYS20-F | ACTGCTGCTAAACCAAATCCAT |
| LYS20-R | ATGTAGTCCACACCGAAATCGT |
| HXT7-F | GGGCTGTTTGGTCTTCATGT |
| HXT7-R | ACCTCTTCTGGATGGTGGAA |

### 2.23 Statistical analysis

Normal distributon of data set were determined using the Ryan Joiner test (Ryan & Joiner, 1976) within Minitab (Minitab release 13.32©, 2000 Minitab Inc.). Non-normally distributed data were transformed to fit a normal distribution using the Johnson transformation (Johnson, 1978) within Minitab (Minitab release 13.32©, 2000 Minitab Inc.). The homogeneity of data sets across replicate experiments was confirmed by one-tailed correlation analysis conducted using mean data values (non-normal data: Spearman Rank; normal data: Pearson product moment) conducted within the Statistical Package for the Social Sciences (SPSS 11.0, SPSS Inc.) ($r \geq 0.610$; $P < 0.050$)

(Snedecor & Cochran, 1980) .Therefore, data sets from the replicate experiments were pooled for the purposes of further statistical analysis. The significance of treatment effects was analysed within SPSS by either (i) normally distributed data - one-way ANOVA with Post Hoc pair wise Least Significance Difference (LSD) comparisons ($P = 0.050$), or (ii) non-normally-distributed data - the Kruskal-Wallis H test. Correlations between mean values from different normally-distributed data sets were calculated using Pearson product moment analysis.

# CHAPTER 3

## MOLECULAR AND PHENOTYPIC CHARACTERISATION OF NON-PHOSPHORYLATABLE AND PHOSPHOMIMETIC MUTANT RESIDUES OF HSP70

## 3.1 Introduction

Protein misfolding is an important criterion attributed to many age related diseases and is also an important factor in aging. In order to assist protein folding, many proteins require the assistance of other cellular proteins called "molecular chaperones" (Walter & Buchner, 2002). Molecular chaperones are also required for degradation or refolding of a misfolded protein into their native or biologically functional form. Molecular chaperones exist in various compartments of the cell. Most of the cytosolic molecular chaperones are often referred to as "heat shock proteins" (*hsps*) as they are mainly up-regulated when cell experiences stress, such as exposure to high temperature (Guinan & Jones, 2009).

The Hsp70s are a highly conserved family of molecular chaperones found in all organisms and exist in various compartments of the cell. This class of chaperones are in the centre of the ubiquitous protein folding system (Wegele et al., 2004) and they also assist protein translocation across membranes, translation and regulation of heat shock response (Bukau & Horwich, 1998; Wegele et al., 2004; Young et al., 2004). In yeast, there are fourteen distinct Hsp70-family proteins that exist in various compartments of the cell. The main component of Hsp70 in the yeast cytosol is the Ssa family (Stress seventy subfamily A) comprising of four closely related proteins Ssa1- 4 which collectively provide Hsp70s essential cellular functions. The presence of at least one Ssa is required for vegetative growth. Among the four Ssas, *SSA1* and *SSA2* are constitutively expressed and under normal growth conditions 80% of Ssa in the cell are Ssa2 whereas *SSA3* and *SSA4* are stress inducible (Werner-Washburne et al., 1989). The function of Hsp70 is regulated by its ATPase cycle. Structurally, Hsp70 consist of a conserved, N-terminal 44 kDa bi-lobal ATPase domain (also known as nucleotide binding domain or NBD), an 18 kDa peptide/substrate binding domain (PBD or SBD) and a C-terminal 10 kDa lid-domain (CTD). The inter-domain linker between NBD and SBD is highly conserved and is critical for allosteric regulation of Hsp70 ATPase cycle (Swain et al., 2007; Vogel et al., 2006). The ATPase cycle of Hsp70 alters between the ATP state with low affinity and rapid exchange of substrates, and the ADP state with high affinity and slow exchange of substrates (McCarty et al., 1995; Schmid et al., 1994). This ADP-ATP exchange is essential for substrate release and recycling of Hsp70. The ATPase cycle of Hsp70 is tightly controlled by some co-chaperones.

The cellular function of Hsp70 relies on many co-chaperones and other factors involved in regulation of the ATPase cycle. The co-chaperone Hsp40 recruits Hsp70 to

specific sites in the cell and therefore helps Hsp70 perform a wide range of cellular functions. Hsp40 binds unfolded protein and presents it to Hsp70, which then refolds them back into its functional conformation (Hennessy et al., 2005; Qiu et al., 2006). Another molecular chaperone Hsp110 represented by Sse1 and Sse2 are divergent members of the Hsp70 superfamily. The Sse1 acts as a "holdase" by binding denatured proteins (Oh et al., 1999) and it has been shown that Sse1 also binds aggregated polypeptides and presents them to Hsp70.The Hsp110 (Sse1) plays a major role in ATPase cycle. It acts as a nucleotide exchange factor (NEF) helping liberate bound ADP and thus triggering peptide release and rebinding of ATP (Dragovic et al., 2006a). Another chaperone called Hsp104 is a member of the $AAA^+$ superfamily of ATPase and its also acts in conjugation with Hsp70 and Hsp40, acting as "protein disaggregation machinery" that breaks down the high molecular weight aggregated proteins into smaller forms that are then more effectively dealt with by Hsp70 and Hsp40 (Glover & Lindquist, 1998).

The non-mendelian genetic element of *S. cerevisiae* [*PSI*$^+$] is the prion form of the translation termination protein Sup35. Recent studies have implicated the essential role of molecular chaperones in [*PSI*$^+$] propagation (Jones & Tuite, 2005; Masison et al., 2009). The first evidence of involvement of Hsp70 in prion propagation came from studies which showed that over expression of the Ssa1 protein can counteract the [*PSI*$^+$]- curing effect of over-expression of Hsp104 (Newnam et al., 1999). This was followed by isolation of the Ssa1-21 (L483W) mutation in the Ssa1 protein which impairs [*PSI*$^+$] but exhibits no effect on stress tolerance or cell growth (Jung et al., 2000). Since then many mutations were generated on Ssa1 and Ssa2 which have a dominant effect on [*PSI*$^+$] propagation. The majority of *SSA1* mutants that impair [*PSI*$^+$] propagation are located on the ATPase domain of Ssa1 protein (Jones & Masison, 2003; Loovers et al., 2007). In addition, the Ssa1 mutants that had an effect on growth at high temperature did not correlate with [*PSI*$^+$] – curing ability, suggesting a difference in function of Hsp70 for growth and prion propagation (Jones & Masison, 2003; Loovers et al., 2007). Also, recent studies have found that Hsp110 (Sse1) is required for efficient prion propagation (Fan et al., 2007; Kryndushkin & Wickner, 2007).

Cells respond to a wide variety of environmental stimuli and the protein phosphorylation status of the cell is highly affected by such environmental stimuli (Albuquerque et al., 2008). One of the important post-translational modifications of proteins in cells is protein phosphorylation, which can control and influence many

biological functions within a cell. Cells possess a complex phosphorylation profile with both high and low abundance protein being phophorylated and dephosphorylataed at numerous sites as per requirement of the cell (Albuquerque et al., 2008). Previous work has shown that global phosphorylation analysis can provide a key towards gaining in depth knowledge about the protein kinases that phosphorylate various proteins in the cell and thus elucidate the signalling pathways. Global proteomic analysis has revealed that the molecular chaperone Hsp70 appears to be hyperphosphorylated in yeast (*S. cerevisiae*) (Albuquerque et al., 2008). Currently, very little information is available the scientific community about Hsp70 phosphorylation in yeast and the biological significance attributed to it. Thus, the main aim of this chapter was to assess the *in vivo* functional effects of phosphorylation of a yeast cytosolic Hsp70 chaperone protein on its various cellular functions and to determine its effect on yeast prion propagation and maintenance. To carry out this work, several non-phosphorylatable and phosphomimetic mutations were generated at the known phosphorylation sites of yeast Hsp70 (Ssa1), based on the global mass spectrometry data and then these phosphorylation mutants were assessed for their affect on Hsp70 cellular functions and prion propagation.

In summary, the main objectives of this chapter were:

- Construction of phosphorylation mutants of Ssa1.
- Phenotypic characterization of the phosphomutants with respect to growth and stress.
- Investigate the effects of these mutations on [*PSI*$^{+}$] prion propagation.

## 3.2 Generation of Ssa1 phosphorylation mutants that affect [*PSI* $^{+}$] propagation and chaperone activity

Phosphorylation is an important post-translational modification that is responsible for various cellular functions. Global proteomic analysis of the *S. cerevisiae* identified 18 phosphorylation sites in Ssa1 (Albuquerque et al., 2008). These phosphorylation sites have not been assessed by any group of researchers, and as such no biological significance is assigned to these sites as yet. Of the 18 phosphorylation sites present in Ssa1, the 9 phosphorylation sites shown in Figure 3.1 were selected from phosphopep database (http://www.uniprot.org/uniprot/P10591) and site directed mutagenesis (SDM) was carried out as described in Section 2.8 to generate non-phosphorylatable and phosphomimetic mutants as shown in Tables 3.1, 3.2, 3.3 and 3.4

by replacing the serine's and threonine's at those sites with either alanine (non-phosphorylatable) or glutamic acid (phosphomimetic).

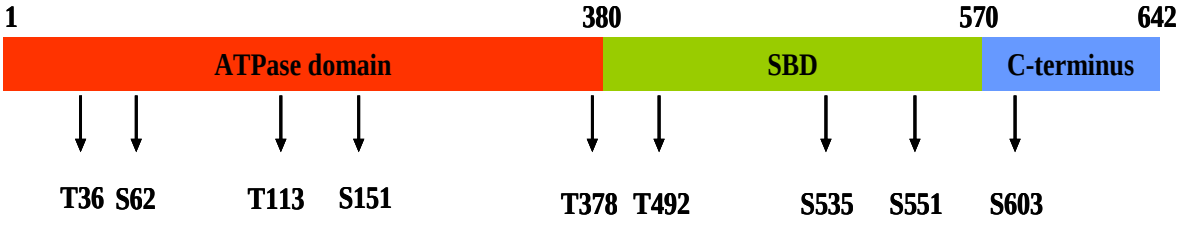



**Figure 3.1 Schematic representation of Hsp70 (Ssa1) protein showing the three domains and the 9 phosphorylation sites selected for the study.** The serines or the threonines at the phosphosites are either replaced with alanine (non-phosphorylatable) or glutamic acid (phosphomimetic) by SDM.

**Table 3.1 Ssa1 single phosphomutants on ATPase domain.** SDM was carried out to generate the phospho-mutations on the ATPase domain of Ssa1. The table shows the nucleotides that were changed by SDM to get either alanine or glutamic acid at the required position.

| Mutation | Nucleotide Position Changed | Nucleotide Changed | Codon Changed | Amino Acid Changed |
|---|---|---|---|---|
| T36A | 106 | A ⇨ T | 36 | T ⇨ A |
| T36E | 106,107,108 | ACT ⇨ GAG | 36 | T ⇨ E |
| S62A | 184 | T ⇨ G | 62 | S ⇨ A |
| S62E | 184, 185 | TC ⇨ GA | 62 | S ⇨ E |
| T113A | 337 | A ⇨ G | 113 | T ⇨ A |
| T113E | 337, 338, 339 | ACC ⇨ GAG | 113 | T ⇨ E |
| S151A | 451 | T ⇨ G | 151 | S ⇨ A |
| S151E | 451,452,453 | TCT ⇨ GAG | 151 | S ⇨ E |

**Table 3.2 Ssa1 single phosphomutants on the substrate binding domain (SBD).** SDM was carried out to generate the phospho-mutations on the SBD of Ssa1. The table shows the nucleotides that were changed by SDM to get either alanine or glutamic acid at the required position.

| Mutation | Nucleotide Position Changed | Nucleotide Changed | Codon Changed | Amino Acid Changed |
|---|---|---|---|---|
| T492A | 1474 | A ⇨ G | 492 | T ⇨ A |
| T492E | 1474,1475,1476 | ACT ⇨ GAG | 492 | T ⇨ E |
| S535A | 1603 | T ⇨ G | 535 | S ⇨ A |
| S535E | 1603,1604,1605 | TCC ⇨ GAG | 535 | S ⇨ E |
| S551A | 1651 | T ⇨ G | 551 | S ⇨ A |
| S551E | 1651,1652,1653 | TCT ⇨ GAG | 551 | S ⇨ E |

### 3.3 Isolation of Ssa1 phosphorylation mutants that effect [*PSI*$^{+}$] propagation and chaperone activity.

The plasmid shuffle technique was carried out as described in Section 2.12 to investigate the phenotypic effect of Ssa1 phosphorylation mutations on prion propagation and other cellular functions of Ssa1. For this experiment, the yeast strain G402 was used which consisted of a *URA3* based plasmid with exogenous expression of Ssa1 protein as the sole source of Ssa in the cell required for cell viability. The method involved transforming this strain with plasmid bearing a *LEU2* selectable marker expressing WT [Ssa1] or the phosphorylation mutants as the sole source of Ssa protein in the cell. The transformants were then replica plated onto 5-FOA (Figure 3.2) and the prion phenotype was assessed on YPD plates at 30 °C and −ade plates at room temperature (RT) and at 30 °C.

**Table 3.3 Ssa1 single and double phosphomutants on the C-terminus and ATPase domain.** SDM was carried out to generate the phospho-mutations on the C-terminus domain of Ssa1. The table shows the nucleotides that were changed by SDM to get either alanine or glutamic acid at the required position.

| Mutation | Nucleotide Position Changed | Nucleotide Changed | Codon Changed | Amino Acid Changed |
|---|---|---|---|---|
| S603A | 1807 | T ⇨ G | 603 | S ⇨ A |
| S603E | 1807,1808,1809 | TCT ⇨ GAG | 603 | S ⇨ E |
| S151A+S603A | 451 + 1807 | (T ⇨ G) + ( T ⇨ G) | 151+ 603 | (S ⇨ A) + ( S ⇨ A) |
| S151E+S603A | (451,452,453) + (1807) | (TCT ⇨ GAG)+ ( T ⇨ G) | 151+ 603 | (S ⇨ E) + (S ⇨ A) |
| S151A+S603E | (451)+(1807,1808,1809) | (T ⇨ G) + (TCT ⇨ GAG) | 151+ 603 | (S ⇨ A) + (S ⇨ E) |
| S151E+S603E | (451,452,453)+(1807,1808,1809) | (TCT ⇨ GAG) + (TCT ⇨ GAG) | 151+ 603 | (S ⇨ E) + (S ⇨ E) |

**Table 3.4 Ssa1 double phosphomutants on the ATPase domain.** SDM was carried out to generate double phospho-mutations on the ATPase domain of Ssa1. The table shows the nucleotides that were changed by SDM to get either alanine or glutamic acid at the required position.

| Mutation | Nucleotide Position Changed | Nucleotide Changed | Codon Changed | Amino Acid Changed |
|---|---|---|---|---|
| T36A+T113A | 106 + 337 | (A ⇨ T) + (A ⇨ G) | 36 + 113 | (T ⇨ A) + (T ⇨ A) |
| T36A+T113E | (106) + (337, 338, 339) | (A ⇨ T) + (ACC ⇨ GAG ) | 36 + 113 | (T ⇨ A) + (T ⇨ E) |
| T36E+T113A | (106, 107,108) + (337) | ( ACT ⇨ GAG) + (A ⇨ G) | 36 + 113 | (T ⇨ E) + (T ⇨ A) |
| T36E+T113E | (106,107,108) + (337, 338, 339) | ( ACT ⇨ GAG) + (ACC ⇨ GAG ) | 36 + 113 | (T ⇨ E) + (T ⇨ E) |
| T36A+S62A | 106 + 184 | (A ⇨ T) + (T ⇨ G) | 36 + 62 | (T ⇨ A) + (S ⇨ A) |
| T36A+S62E | (106) + (184, 185) | (A ⇨ T) + ( TC ⇨ GA) | 36 + 62 | (T ⇨ A) + (S ⇨ E) |
| T36E+S62A | (106,107,108) + (184) | ( ACT ⇨ GAG) + (T ⇨ G) | 36 + 62 | (T ⇨ E) + (S ⇨ A) |
| T36E+S62E | (106,107,108) + (184, 185) | ( ACT ⇨ GAG) + ( TC ⇨ GA) | 36 + 62 | (T ⇨ E) + (S ⇨ E) |

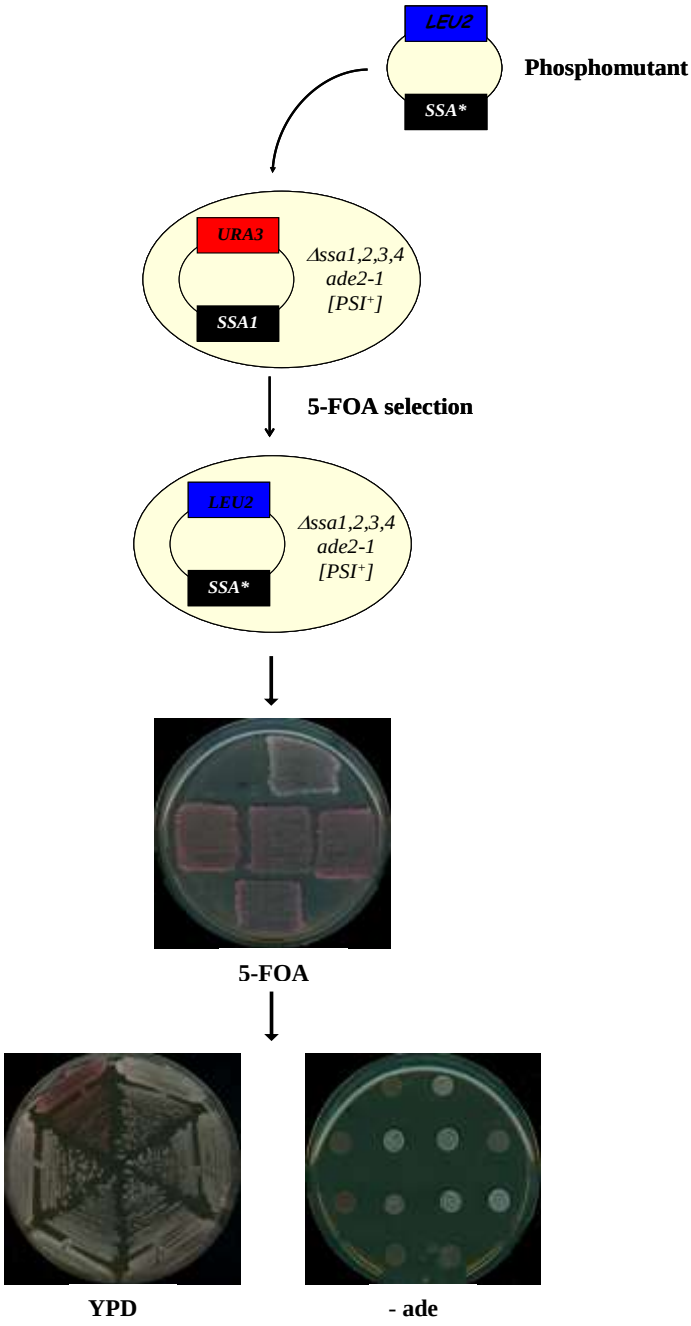



**Figure 3.2 Plasmid shuffle technique for isolation of phosphorylation mutants of Ssa1 that may impair prion propagation and chaperone activity.** Phosphomutants were generated in the plasmid pC210-Ssa1 by SDM and then transformed into G402. Medium containing 5-FOA selected plasmids with *LEU2* marker and plasmids containing *URA3* marker were non-viable thus allowing selection of only the phosphorylation mutants of Ssa1 or the WT [Ssa1]. The phosphomutants were then assessed for their prion phenotype on YPD plates at 30 °C and on − ade plates at 30 °C and RT.

### 3.4 Phenotypic analysis of Ssa1 phosphorylation mutants and its effect on [*PSI*$^+$] prion propagation and cell growth

[*PSI*$^+$] is a self-replicating amorphous aggregate of the Sup35 protein. Sup35 protein (eRF3) under normal conditions in the cell encode a translation release factor (Stansfield et al., 1995; Zhouravleva et al., 1995). Aggregation of Sup35 in [*PSI*$^+$] cells causes nonsense suppression because of the depletion of the Sup35 protein into an insoluble prion aggregate that is no longer able to function in translation termination. The strength of the prion phenotype is thus directly proportional to amount of Sup35 protein that has been depleted to insoluble prion aggregates (Jung et al., 2000), which is easily depicted in the extent of pigment accumulation on YPD plates and growth on − ade plates. Weaker prion propagation is seen as intermediate (pink) colony colour on YPD plate and/or slower growth without adenine. [*PSI*$^+$] thus provides a simplified system for studying prion propagation *in vivo*.

[*PSI*$^+$] phenotype can be assessed by its ability to suppress *ade 2-1* premature *ochre* mutation in a gene responsible for adenine biosynthesis. On YPD and − ade plates [*psi*$^-$] cells are red whereas [*PSI*$^+$] cells are white and Ade$^+$ (as described in Section 2.13). When [*PSI*$^+$] is in a "weak" state, cells will grow pink and display intermediate growth in − ade plates. All the strains used in this work had this *ade 2-1* mutation and were [*PSI*$^+$]. Using plasmid shuffle technique 8 single phosphomutants were isolated from ATPase domain, 6 from SBD and 2 from C-terminus domain (Table 3.1, 3.2 and 3.3). Several double phosphomutants were also generated in Ssa1 (Table 3.3 and 3.4). These phosphomutants were assessed for their ability to impair prion propagation by growing them on YPD or − ade plates.

#### 3.4.1 Impairment of [*PSI*$^+$] propagation by ATPase domain single phosphorylation mutants of Ssa1

To assess the effect of [*PSI*$^+$] mediated nonsense suppression, the growth and pigmentation of [*PSI*$^+$] cells were compared on YPD plates and – ade plates. The WT Ssa1[*PSI*$^+$] cells were white on YPD and showed good growth without adenine at 30 °C and RT and the Ssa1[*psi*$^-$] cells were red on YPD and showed weak growth without adenine at 30 °C and RT (Figure 3.3). [*PSI*$^+$] was weak in cells expressing T36A mutation as seen by the pink colour in YPD and weaker growth on – ade plates (Figure 3.3 A, B, C). Apart from T36A mutation, all other mutations were similar to WT in their ability to propagate [*PSI*$^+$].

### 3.4.2 Impairment of [*PSI*$^{+}$] propagation by SBD single phosphorylation mutants of Ssa1

[*PSI*$^{+}$] was normally propagated in cells expressing SBD phosphomutants of Ssa1 as seen by white colour on YPD and good growth on media without adenine (Figure 3.4). The Ssa1$^{T492E}$ mutation was lethal, perhaps suggesting some kind of major structural or functional defect in the protein due to this mutation.

### 3.4.3 Impairment of [*PSI*$^{+}$] propagation by C-terminus domain phosphorylation mutants of Ssa1

Several single and multiple phosphorylation mutations were generated at the C-terminus and ATPase domain to investigate the effects of phosphorylation on prion propagation. As shown in Figure 3.5 the WT Ssa1[*PSI*$^{+}$] was white on YPD and grew well on media without adenine. On the other hand, Ssa1[*psi* $^{-}$] was red on YPD and growth was very weak on media without adenine. Subsequently, [*PSI*$^{+}$] was normally propagated in all the phosphomutants except for Ssa1$^{S603A}$ where [*PSI*$^{+}$] was slightly weaker compared to the WT.

### 3.4.4 Impairment of [*PSI*$^{+}$] propagation by ATPase domain double phosphorylation mutants of Ssa1

As previous studies have shown the importance of ATPase domain of Hsp70 in impairment of prion propagation (Loovers et al., 2007), apart from generating single phosphomutants several double phosphomutants were also generated in the ATPase domain and assessed for their prion phenotype. While most of the mutants greatly impair [*PSI*$^{+}$] propagation when they are the sole source of Ssa in the cell as seen by the pigmentation on YPD plates and growth on adenine lacking media, only one mutant *viz.* Ssa1$^{T36E+T113E}$ was lethal suggesting some kind of structural or functional defect in the Ssa1 protein due to this mutation (Figure 3.6). Interestingly, all the double mutants except for Ssa1$^{T36E+T113E}$ having the T36A or T36E show impairment in prion propagation, which might implicate T36 to be an important site of Ssa1 in context of prion propagation. Also it can be speculated from the results from Figure 3.6 that there might be complex interactions going on between the phosphorylation sites at T36, T113 and S62 that have a dominant affect on prion propagation.

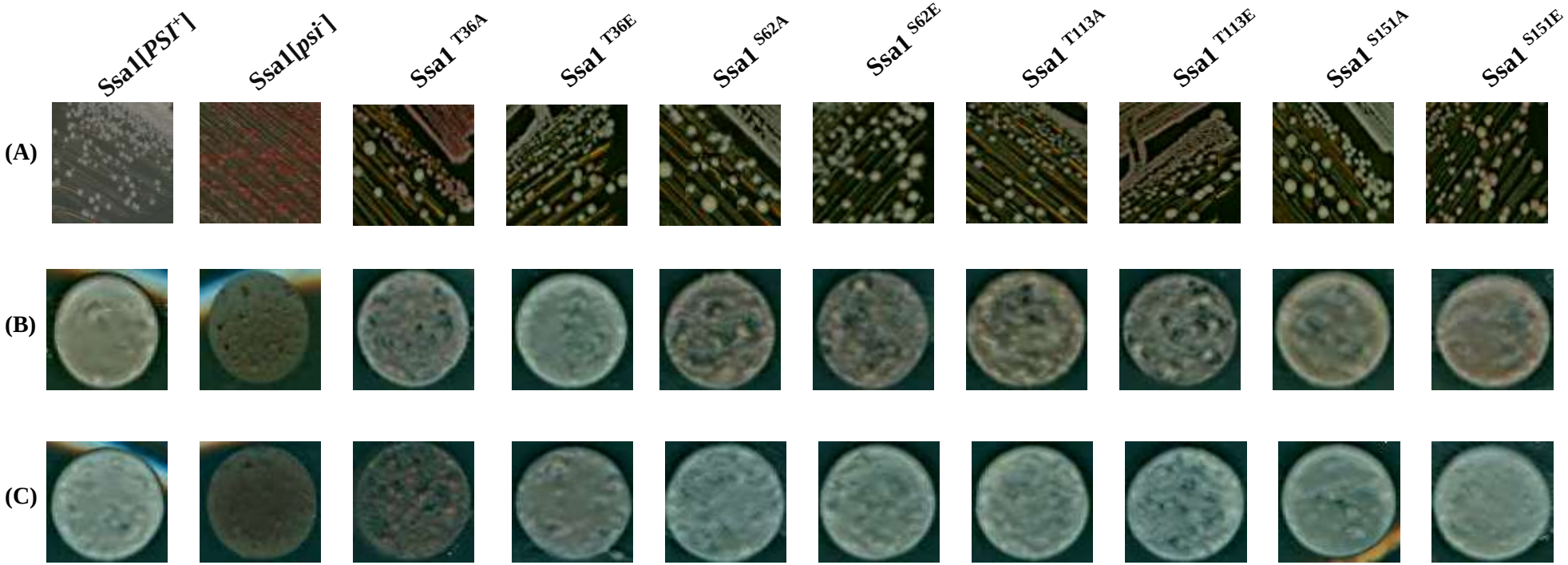



**Figure 3.3 Effect of ATPase domain single phosphomutants on [*PSI*$^+$] phenotype.** (A) Cells were streaked on YPD and colonies came up after 2 days of incubation at 30 °C followed by 2 days at 4 °C. (B) 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and (C) at RT for 3 days. Ssa1[*PSI*$^+$] is the WT; Ssa1[*PSI*$^+$] and Ssa1[*psi*$^-$] are taken as controls; Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text) . The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates.

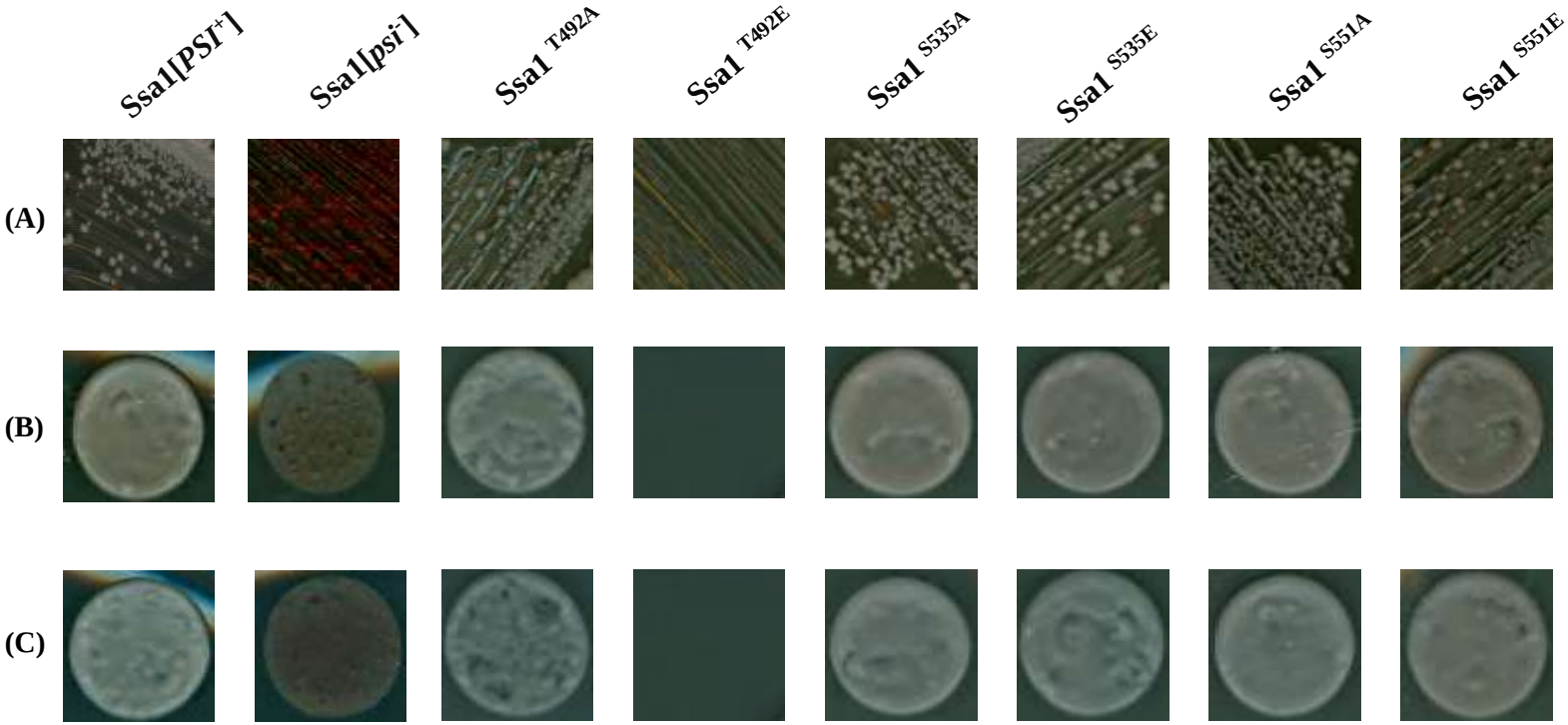



**Figure 3.4 Effect of SBD single phosphomutants on [*PSI*$^+$] phenotype.** (A) Cells were streaked on YPD and colonies appeared up after 2 days of incubation at 30 °C followed by 2 days at 4 °C. (B) 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and (C) at RT for 3 days. Ssa1[*PSI*$^+$] is the WT; Ssa1[*PSI*$^+$] and Ssa1[*psi*$^-$] are taken as controls; Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text) . The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates.

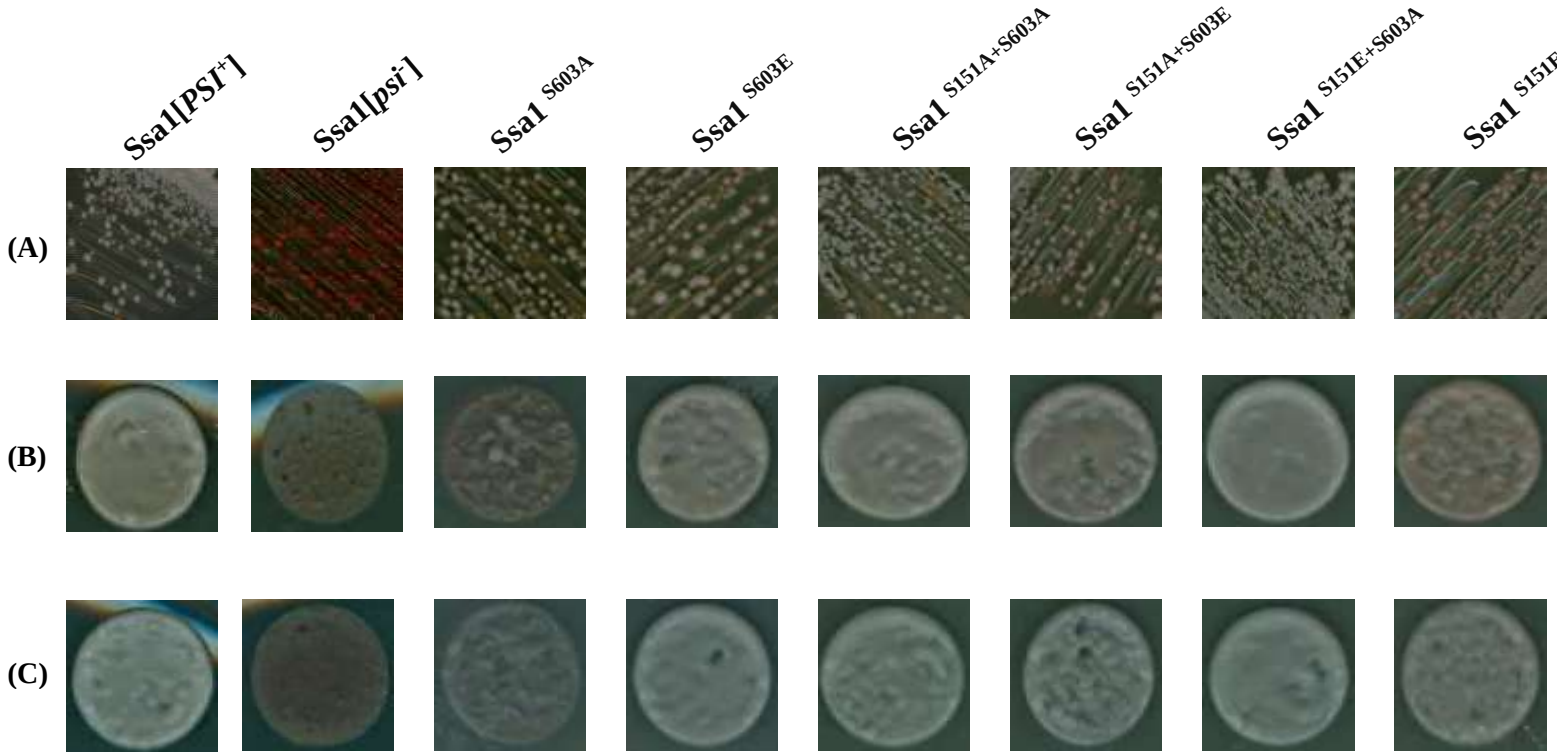



**Figure 3.5 Effect of C-terminus domain and ATPase domain phosphomutants on [*PSI*$^+$] phenotype.** (A) Cells were streaked on YPD and colonies came up after 2 days of incubation at 30 °C followed by 2 days at 4 °C. (B) 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and (C) at RT for 3 days. Ssa1[*PSI*$^+$] is the WT; Ssa1[*PSI*$^+$] and Ssa1[*psi*$^-$] are taken as controls; Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text) . The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates.

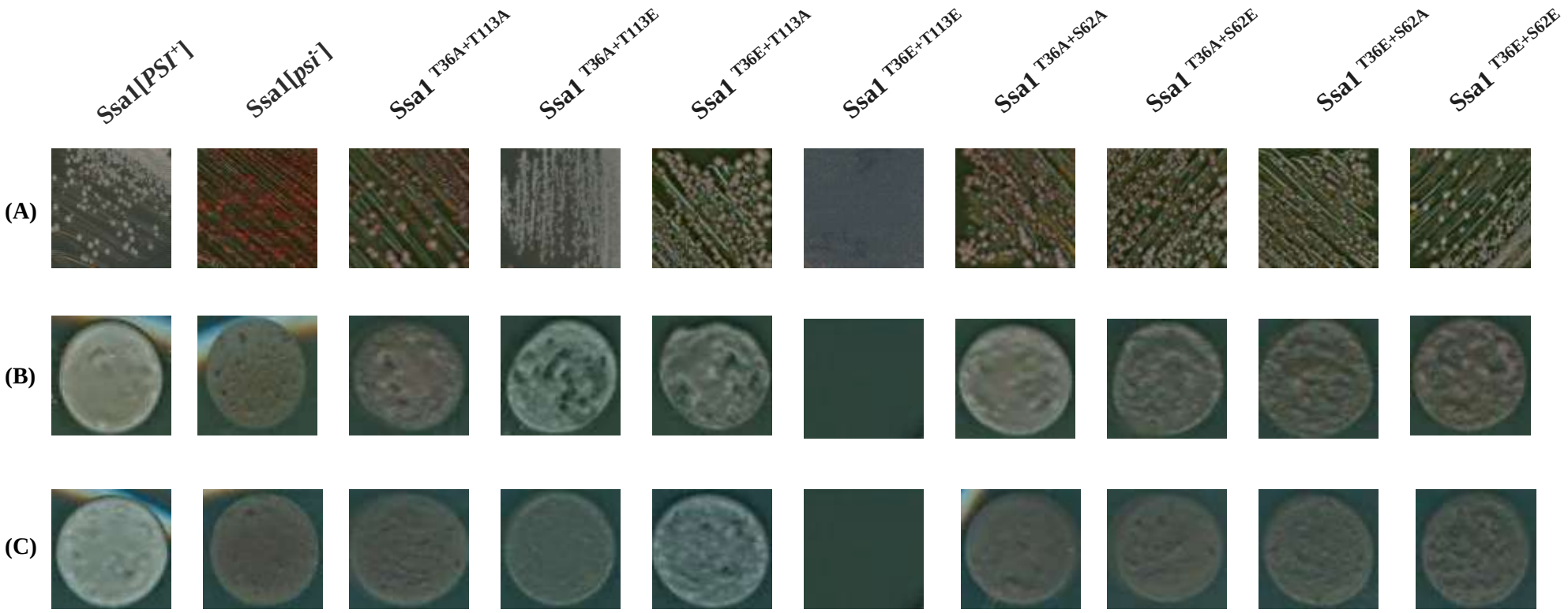



**Figure 3.6 Effect ATPase domain double phosphomutants on [*PSI*$^+$] phenotype.** (A) Cells were streaked on YPD and colonies appeared after 2 days of incubation at 30 °C followed by 2 days at 4 °C. (B) 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and (C) at RT for 3 days. Ssa1[*PSI*$^+$] is the WT; Ssa1[*PSI*$^+$] and Ssa1[*psi*$^-$] are taken as controls; Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text) . The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates.

### 3.4.5 Comparative analysis of the effect of Ssa1 phosphomutants on cell growth and [*PSI*$^{+}$] propagation

Apart from looking into the prion phenotype, the Ssa1 phosphorylation mutants were also characterised based on the effect of these mutants on growth rate. A substantial difference in growth rates may be indicative that phosphomutants not only impair prion propagation but also have an effect on other cellular functions of Hsp70.

Most of the phosphorylation mutants of Ssa1 showed a significant difference in growth rate of yeast cell ($p<0.05$) compared to WT, but for some strains *viz*. Ssa1 $^{T36E}$, Ssa1 $^{T36A+T113E}$, Ssa1 $^{T36E+S62E}$ and Ssa1 $^{T492A}$ the difference in growth rate was much more pronounced compared to WT. From the Table 3.5 it can be deduced that most of the SBD and the C-terminus domain mutations did not have a major effect on prion propagation when Ssa is the sole source of Hsp70 in the cell. The only mutant with the biggest difference in growth rate compared to WT on the SBD is the Ssa1 $^{T492A}$ which is approximately 19% slower compared to WT. Also [*PSI*$^{+}$] was weak in the phospmutants strain Ssa1$^{603A}$.

On the other hand, three of the ATPase domain mutant *viz.* Ssa1 $^{T36E}$, Ssa1 $^{T36A+T113E}$ and Ssa1 $^{T36E+S62E}$, was found to have a prominent effect on growth rate (Table 3.5). The Ssa1 $^{T36E}$, Ssa1 $^{T36A+T113E}$ and Ssa1 $^{T36E+S62E}$ were approximately 31%, 131% and 29% slower compared to WT respectively. Also most of the ATPase domain phosphomutants particularly Ssa1 $^{T36A}$, Ssa1 $^{T36A+T113A}$ and Ssa1 $^{T36A+S62A}$ were seen to have a prominent effect on [*PSI*$^{+}$] propagation as analysed with the Image J- java based image processing program. Thus the ATPase domain phosphomutants not only impair prion propagation but also have a major effect on growth rate. This result also highlights the importance of ATPase domain compared to SBD and C-terminus domain with respect to prion propagation and cell growth.

**Table 3.5 Relative effects of Ssa1 phosphorylation mutations on prion propagation and cell growth**

| Domain | Phosphomutants | ± MGT (mins) | Colour in G402 |
|---|---|---|---|
| | WT [Ssa1] | 108.63 ± 0.2 | 0 |
| ATPase domain | *Ssa1 $^{T36A}$ | 114.5 ± 1.4 | 8 |
| | *Ssa1 $^{T36E}$ | 142.1 ± 3.5 | 2 |
| | Ssa1 $^{S62A}$ | 105.3 ± 1.3 | 4 |
| | *Ssa1 $^{S62E}$ | 100.4 ± 0.7 | 4 |
| | *Ssa1 $^{T113A}$ | 100.9 ± 0.3 | 6 |
| | Ssa1 $^{T113E}$ | 106.3 ± 0.3 | 6 |
| | Ssa1 $^{S151A}$ | 103.5 ± 0.8 | 3 |
| | Ssa1 $^{S151E}$ | 105.2 ± 0.3 | 5 |
| | *Ssa1 $^{T36A+T113A}$ | 115.1±0.7 | 9 |
| | *Ssa1 $^{T36A+T113E}$ | 251.6±8.5 | 6 |
| | *Ssa1 $^{T36E+T113A}$ | 99.3±0.1 | 6 |
| | *Ssa1 $^{T36A+S62A}$ | 111.9±1.7 | 8 |
| | *Ssa1 $^{T36A+S62E}$ | 99.4±1.7 | 7 |
| | *Ssa1 $^{T36E+S62A}$ | 118.2±0.4 | 7 |
| | *Ssa1 $^{T36E+S62E}$ | 141.8±2.3 | 7 |
| Substrate binding domain | *Ssa1 $^{T492A}$ | 129.07 ± 1.7 | 4 |
| | *Ssa1 $^{S535A}$ | 99.02 ± 2.2 | 3 |
| | *Ssa1 $^{S535E}$ | 86.58 ± 1.1 | 1 |
| | Ssa1 $^{S551A}$ | 106.2 ± 0.8 | 1 |
| | *Ssa1 $^{S551E}$ | 95.7 ± 1.5 | 5 |
| C-terminus domain | Ssa1 $^{S603A}$ | 102 ± 0.3 | 7 |
| | Ssa1 $^{S603E}$ | 100 ± 0.2 | 3 |
| C-terminus+ATPase domain | Ssa1 $^{S151A+S603A}$ | 103 ± 1.1 | 3 |
| | *Ssa1 $^{S151E+S603A}$ | 99 ± 0.8 | 2 |
| | Ssa1 $^{S151A+S603E}$ | 102 ± 0.2 | 3 |
| | *Ssa1 $^{S151E+S603E}$ | 98 ± 1.3 | 4 |

± The mean generation time of the yeast strains were determined by diluting the overnight 30 °C culture in 30 ml fresh media to an $OD_{600nm}$ of 0.1(t = 0 min). Cultures were transferred to 30 °C shaking incubator and cell density was measured at an interval of every 2 h as described in Section 2.14. The table also numerically categorizes the phophomutants according to their colour in G402, zero representing white Ssa1 [$PSI^{+}$] and ten representing red Ssa1 [$psi^{-}$] based on Image J-java based image processing program. (* indicate significant difference between WT [Ssa1] and its phosphomutants).

## 3.5 Investigation of temperature sensitivity of the Ssa1 mutants by comparative growth analysis

Hsp70 is an essential, highly conserved chaperone protein that helps in protein folding and also rescues cells from stress by preventing aggregation of stress-denatured proteins. Therefore, defective cellular thermostability of the cells expressing Ssa1 phosphomutants growing at higher temperature is indicative of altered Hsp70 function due to the mutation. As Hsp70 is an important heat shock cellular chaperone one objective of this chapter was to assess the effect of the phosphorylation mutants on cellular thermostability. Therefore, comparative growth analysis was carried out on all the Ssa1 phosphorylation mutants as described in Section 2.15 and the plates were incubated at 30 °C, 37 °C and 39 °C and growth was monitored after 3 days.

### 3.5.1 The ATPase domain phosphomutants showed increased temperature sensitivity

Analysing the ATPase domain phosphomutants for temperature sensitivity revealed a varying degree of growth rate at 37 °C and 39 °C (Figure 3.7 and 3.8). Some of the mutants were temperature sensitive at 39 °C whereas some of the mutants were similar to WT [Ssa1]. The Ssa1 $^{T36A}$, Ssa1 $^{T36E}$, Ssa1 $^{T113A}$ and Ssa1 $^{T113E}$ were temperature sensitive at 39 °C whereas the other single phosphomutants on the ATPase domain behaved similarly to the WT (Figure 3.7). The Ssa1 $^{S151E}$ displayed partial growth at 39 °C but was still inhibited slightly in comparison to WT [Ssa1]. All the double phosphomutants on the ATPase domain were temperature sensitive at 39 °C (Figure 3.8).

The results indicate that Hsp70 is defective in preventing aggregation of stress-denatured proteins at elevated temperature due to the mutations and normal functioning of Hsp70 is required for protection of the cell at extreme environmental stress temperatures. These mutations could affect stability and folding of Hsp70 under extreme heat shock conditions also.

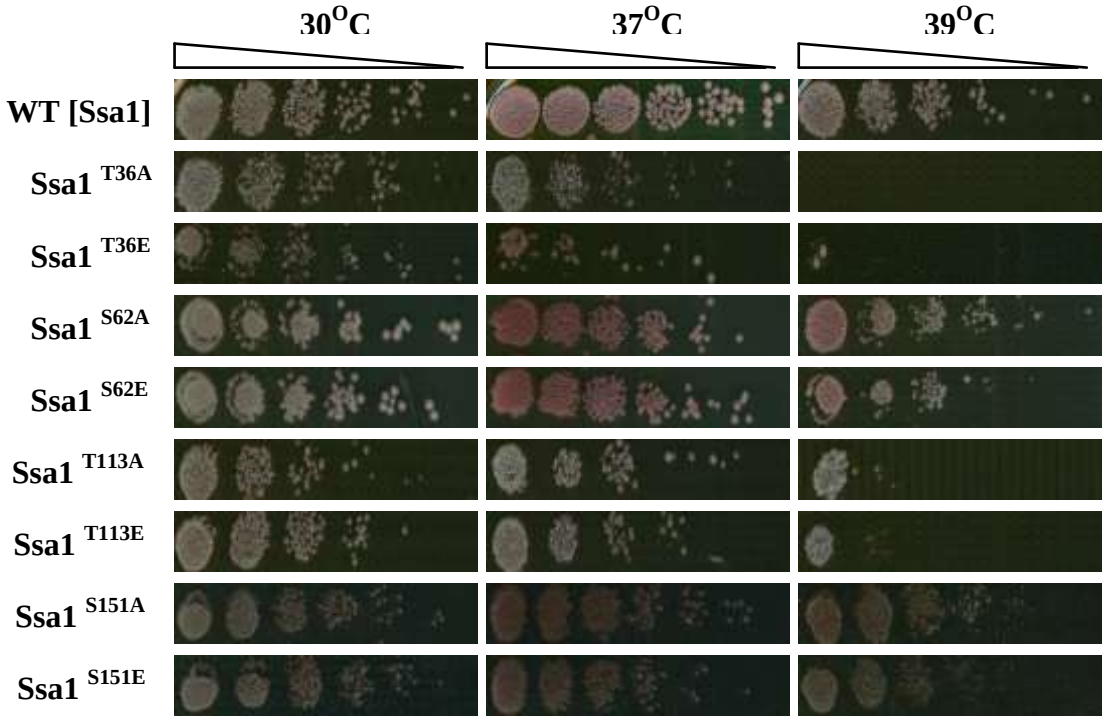



**Figure 3.7 Comparative growth analysis of the Ssa1 single phosphorylation mutants of the ATPase domain on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

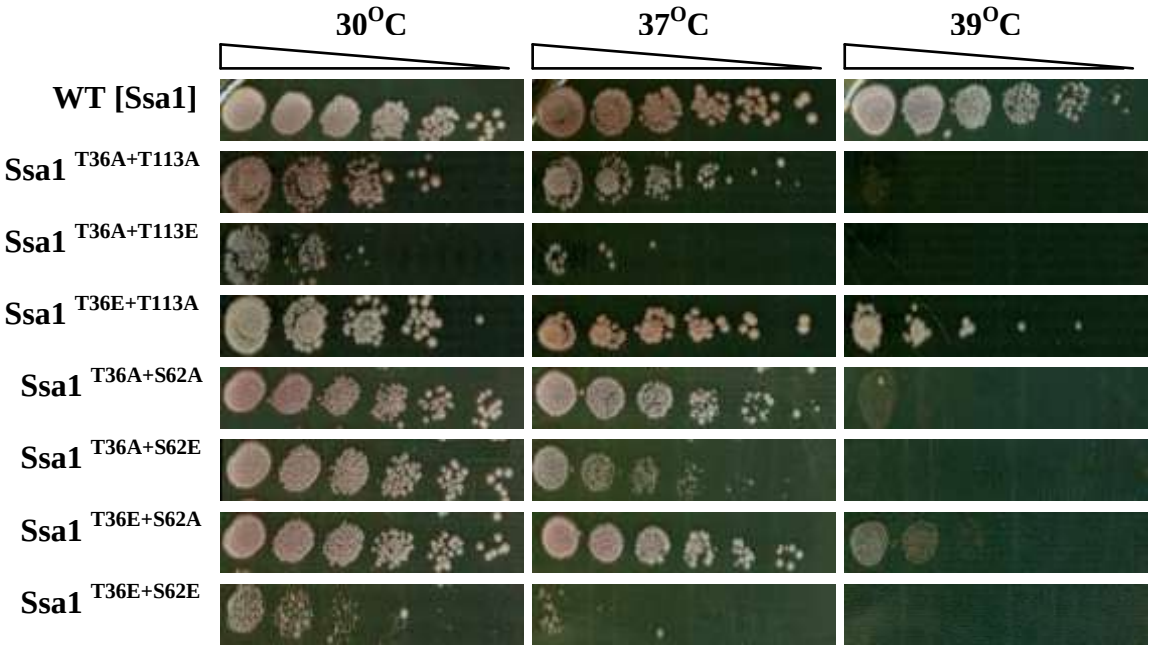



**Figure 3.8 Comparative growth analysis of the Ssa1 double phosphorylation mutants of the ATPase domain on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

### 3.5.2 Temperature sensitivity of the SBD and the C-terminus phosphomutants

The comparative growth analysis of the SBD (Figure 3.9) and C-terminus domain (Figure 3.10) phosphomutants showed that these phosphomutants were not temperature sensitive and all the mutants behaved similarly to WT [Ssa1] except for Ssa1 $^{T492A}$ that displayed some degree of temperature sensitivity at 37 °C and 39 °C. This mutant also did not grow properly at 30 °C in comparison to the WT [Ssa1]. This might be indicative of some kind of structural or functional defect in the protein due to the Ssa1 $^{T492A}$ mutation. Thus, the mutations on the SBD and C-terminus domain did not have a prominent effect on the protein disaggregation machinery of Hsp70 and thus Hsp70 is fully functional under extreme heat shock conditions despite of the mutations on the SBD and C-terminus domain.

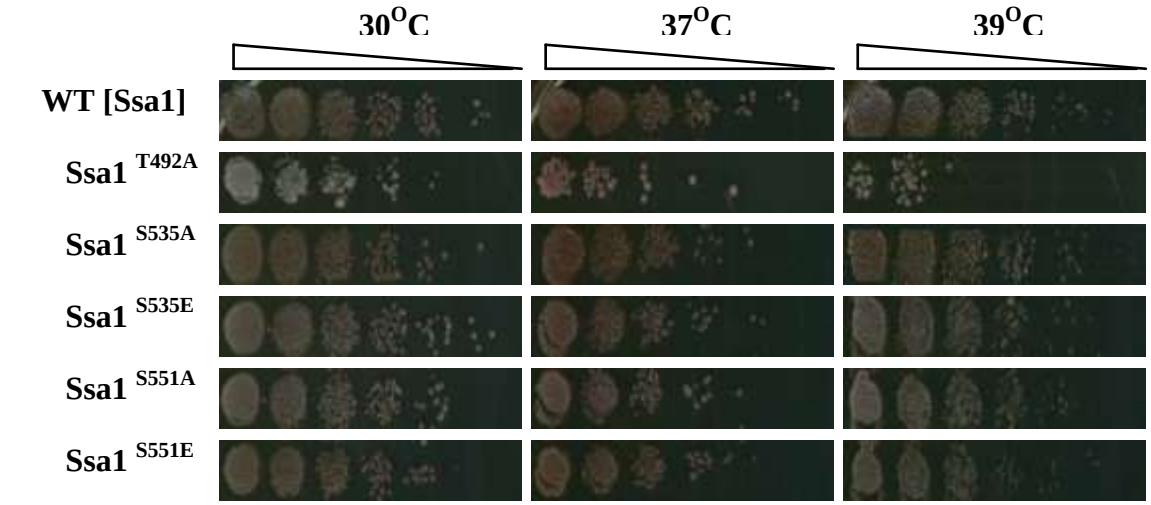



**Figure 3.9 Comparative growth analysis of the Ssa1 single phosphorylation mutants of the SBD on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

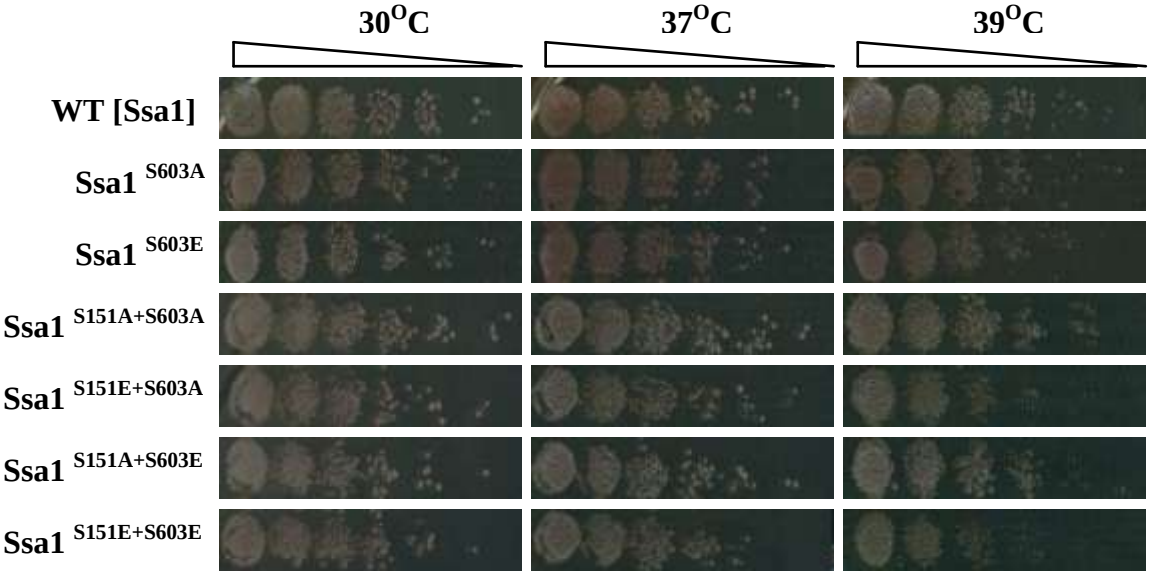



**Figure 3.10 Comparative growth analysis of the Ssa1 single and double phosphorylation mutants of C-terminus and ATPase domain on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

### 3.6 High osmotic medium recovers the temperature tolerance of the phosphorylation mutants

Molecular chaperones ensure refolding and support signal transduction responses to cytotoxic stress. Mutations that destabilize the cell wall at higher temperatures are often found in the genes involved in cell wall maintenance (Paravicini et al., 1992). Shaner et al. (2008) and Paravicini et al. (1992) examined some temperature sensitive mutants for cell wall permeability and observed that temperature sensitive mutants show substantial levels of leakage of the cellular components into the external environment at higher temperature which is indicative of defect in cell wall integrity.

Yeast can be subjected to a diverse array of environmental conditions that include pH changes, osmotic changes, thermal changes, oxide radicals, nutrient deprivation and exposure to chemicals. The cell wall integrity (CWI) pathway provides a means to survive such extreme environmental conditions by repairing cell wall damage as well as supporting the cell wall to withstand such stressful environments (Fuchs & Mylonakis, 2009). When yeast grows at high temperatures on hypo-osmotic solutes such as YPD, the CWI pathway is activated and mutants which hamper this pathway lyse in absence of osmotic stabilisers such as 1M sorbitol (Lee & Levin, 1992; Davenport et al., 1995).

As can be seen from Figure 3.11 most of the temperature sensitive mutants recovered in presence of 1M sorbitol and thus it might be predicted that in case of temperature sensitive phosphomutants the cell wall might become permeable at higher temperatures due to alteration of the CWI pathway or some other signalling pathway, making growth on hypotonic medium such as YPD lethal compared to the cell wall stability provided in presence of osmotic stabilisers such as 1M sorbitol.

The Ssa1 $^{S151E}$ mutant that displayed partial growth at 39 °C also did recover well on 1M sorbitol (Figure 3.11 A) which is indicative of cell wall integrity defect due to the mutation. But the phosphomutants Ssa1 $^{T492A}$, Ssa1 $^{T36E}$, Ssa1 $^{T36A+T113E}$ and Ssa1 $^{T36E+S62E}$ (Figure 3.11 and 3.12) could not recover on 1M sorbitol suggesting that the mutation have brought some functional defect in the protein that renders the cell to become lethal at higher temperature.

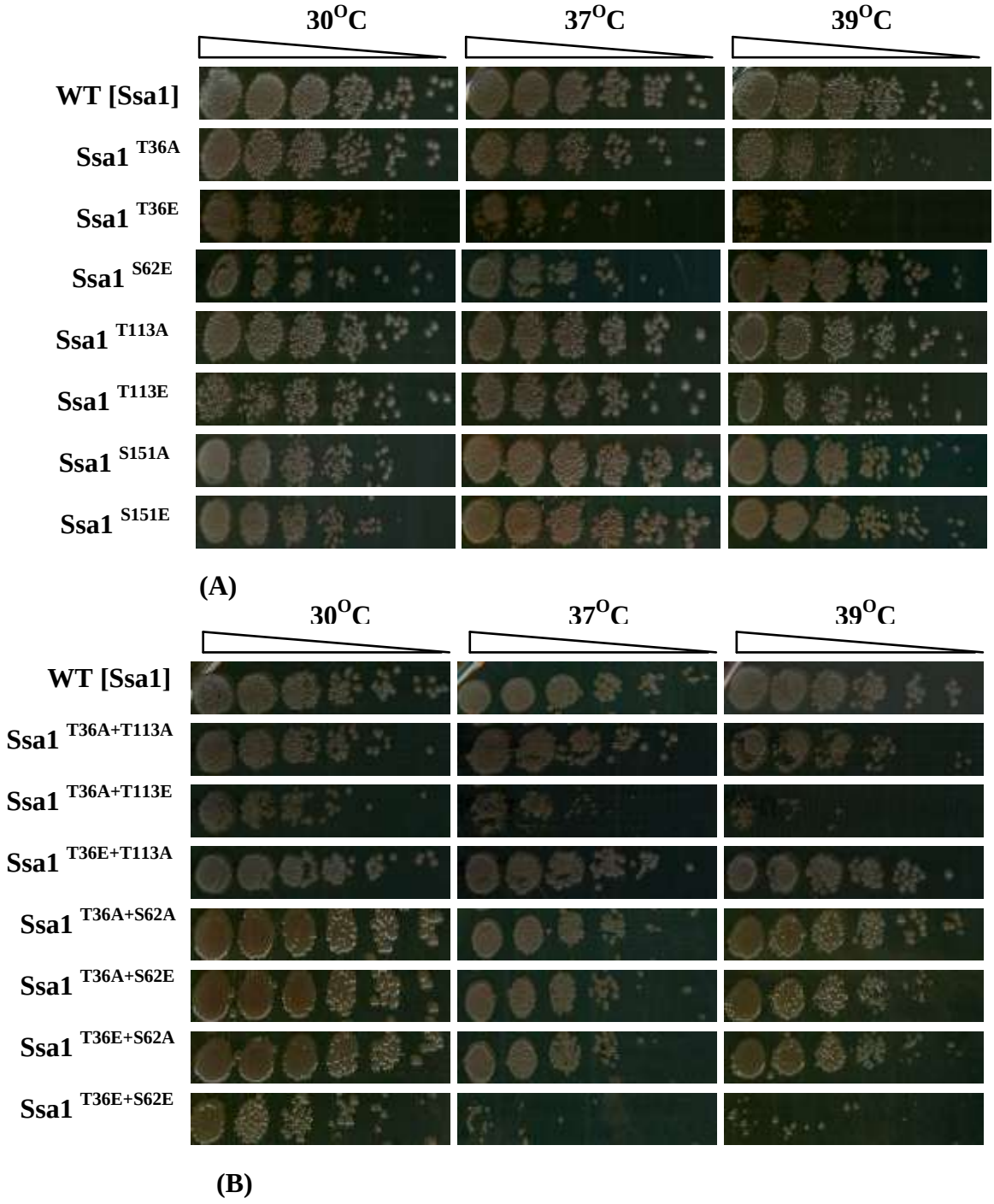



**Figure 3.11 Comparative growth analysis of the Ssa1 single (A) and double (B) phosphorylation mutants of ATPase domain on 1M sorbitol plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for recovery over time on 1M sorbitol plate

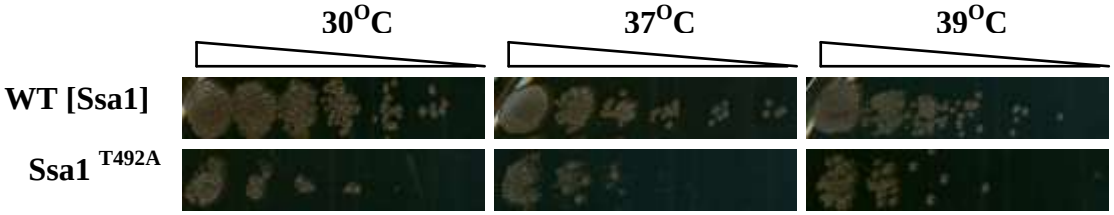



**Figure 3.12 Comparative growth analysis of the Ssa1 phosphorylation mutant of SBD on 1M sorbitol plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for recovery over time on 1M sorbitol plate.

### 3.7 Assessment of acquired thermotolerance activity in the Ssa1 phosphorylation mutants

The tolerance that yeast cells exhibit at elevated temperature is triggered by a small group of proteins called heat shock proteins (hsps) (Lindquist & Craig, 1988; Morimoto et al., 1990). One of such important hsps found in yeast is the Hsp104 which ensures cell survival on prolonged exposure to high temperature (Jones & Tuite, 2005). At elevated temperature, Hsp104 activity allows cells to survive by resolubilising heat-denatured proteins (Parsell et al., 1994b). Apart from Hsp104, refolding of misfolded protein requires the activity of additional chaperone proteins Hsp70 and Hsp40. Thus, Hsp104 in conjugation with Hsp70 and the co-chaperone Hsp40 act as a protein disaggregation machinery leading to resolubilization of protein aggregates (Glover & Lindquist, 1998). The well-established thermotolerance assay (described in Section 2.16) was used to assess the activity of Hsp104 whereby cell survival predominantly represents functional Hsp104 activity.

Figure 3.13 shows the ability of the WT [Ssa1] and the Ssa1 single phosphorylation mutants in the ATPase domain to withstand growth at an elevated temperature of 47 °C. Cell viability is decreased with longer period of exposure to 47 °C on YPD plate. In presence of 3mM Gdn-HCl which inhibits Hsp104 a significant decrease in growth rate can be observed after a short period of exposure to 47 °C compared to cell viability on YPD plate at elevated temperature due to lack of ability to refold heat-damaged proteins. The phosphomutants Ssa1 $^{T36A}$, Ssa1 $^{S62A}$, Ssa1 $^{S62E}$ behaved similar to the WT [Ssa1] suggesting that the thermotolerance activity of Hsp104 is not inhibited by these phosphomutations. Another phosphomutant Ssa1 $^{T113A}$

behaved slightly better than WT [Ssa1] which might suggest that Hsp104 activity is slightly enhanced due to the presence of this mutation. But two of the phosphomutants Ssa1 $^{T36E}$ and Ssa1 $^{T113E}$ behaved better than the WT [Ssa1] on YPD indicating increased Hsp104 activity due to the mutation. These two mutants also showed viability for a longer period of time at an exposure to 47 °C heat shock compared to WT [Ssa1] on 3mM Gdn-HCl which may indicate that Hsp70 might have a greater role to play in acquired thermotolerance than is currently thought.

When the Ssa1 double phosphorylation mutants on the ATPase domain were assessed for acquired thermotolerance activity of Hsp104 a varying degree of thermotolerance was shown by different phosphomutants (Figure 3.14). The double phosphomutants Ssa1 $^{T36E+T113A}$ and Ssa1 $^{T36E+S62E}$ showed similar levels of acquired thermotolerance compared to WT [Ssa1] on YPD and 3mM Gdn-HCl indicating that the Hsp104 is not defective in its activity to show acquired thermotolerance at elevated temperature due to these mutations. The phosphomutants Ssa1 $^{T36A+T113A}$ and Ssa1 $^{T36A+T113E}$ could not survive for more than 20 min at 47 °C on YPD and were not viable after 10 min heat shock at 47 °C on 3 mM Gdn-HCl plate. This suggests that Hsp104 is unable to compensate for lack of Hsp70 function in these strains. The fact that the cells lose viability on 3 mM Gdn-HCl after 10 min heat shock at 47 °C further validates this suggestion. The phosphomutants Ssa1 $^{T36A+S62A}$, Ssa1 $^{T36A+S62E}$ and Ssa1 $^{T36E+S62A}$ although defective in showing acquired thermotolerance compared to WT behaved slightly better than Ssa1 $^{T36A+T113A}$ and Ssa1 $^{T36A+T113E}$ on YPD suggesting that Hsp104 activity can be impaired to a variable extent due to the phosphomutations within the ATPase domain.

Figure 3.15 shows the acquired thermotolerance activity of the phosphomutants within the SBD. The phosphorylation muations Ssa1 $^{S535A}$, Ssa1 $^{S535E}$ and Ssa1 $^{S551A}$ on the SBD showed a similar level of Hsp104 tolerance compared to WT [Ssa1] on YPD and 3 mM Gdn-HCl on exposure to elevated temperature of 47 °C suggesting that Hsp104 was not impaired in its function due to the mutation. However, the phosphomutant Ssa1 $^{S551E}$ showed slightly decreased thermotolerance activity compared to WT [Ssa1] suggesting reduced thermotolerance activity. A similar level of acquired thermotolerance activity was observed for the phosphomutants *viz.* Ssa1 $^{S603A}$ and Ssa1 $^{S603E}$ in the C-terminus domain compared to WT [Ssa1] (Figure 3.16).

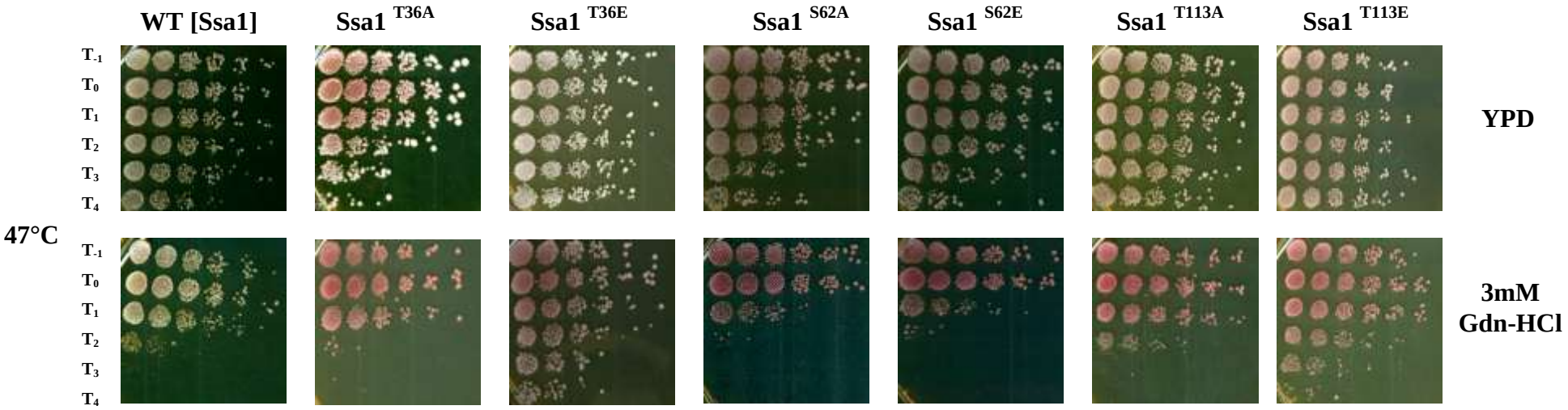



**Figure 3.13 Thermotolerance assay of Ssa1 single phosphorylation mutations on the ATPase domain.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39 ºC to induce Hsp104 expression to protect against heat shock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD and 3mM Gdn-HCl as indicated above for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated at 30 ºC for 2 days followed by 1 day at RT and were monitored for cellular thermotolerance.

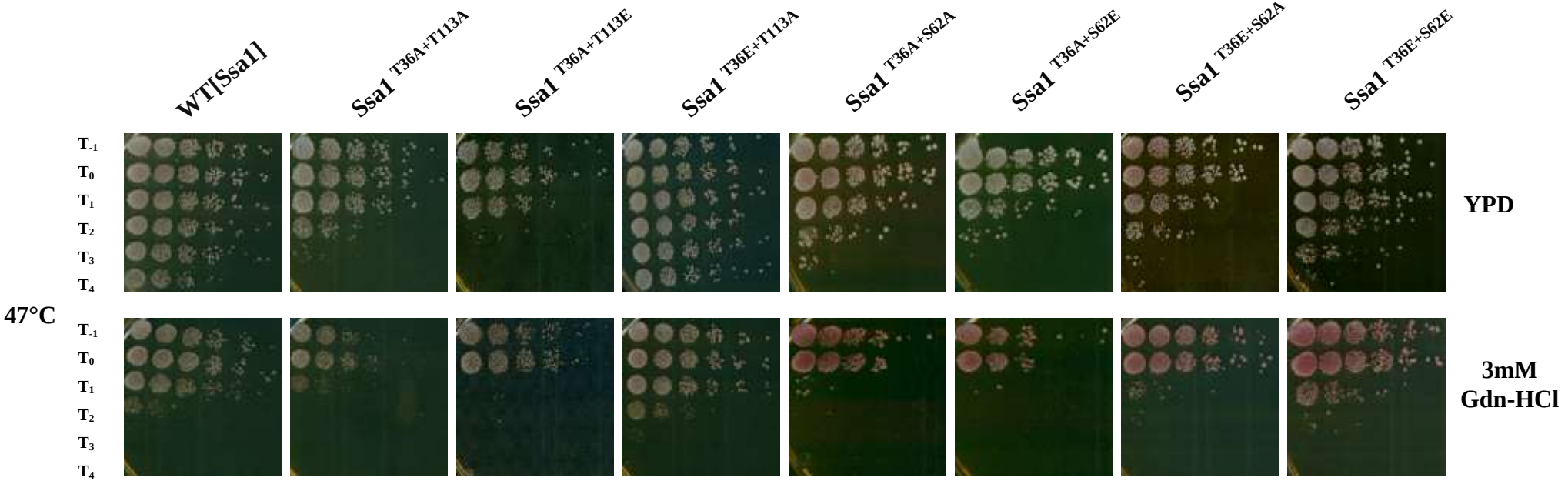



**Figure 3.14 Thermotolerance assay of Ssa1 double phosphorylation mutations on the ATPase domain.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39 ºC to induce Hsp104 expression to protect against heat shock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD and 3mM Gdn-HCl as indicated above for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series.The plates were then incubated at 30 ºC for 2 days followed by 1 day at RT and were monitored for cellular thermotolerance.

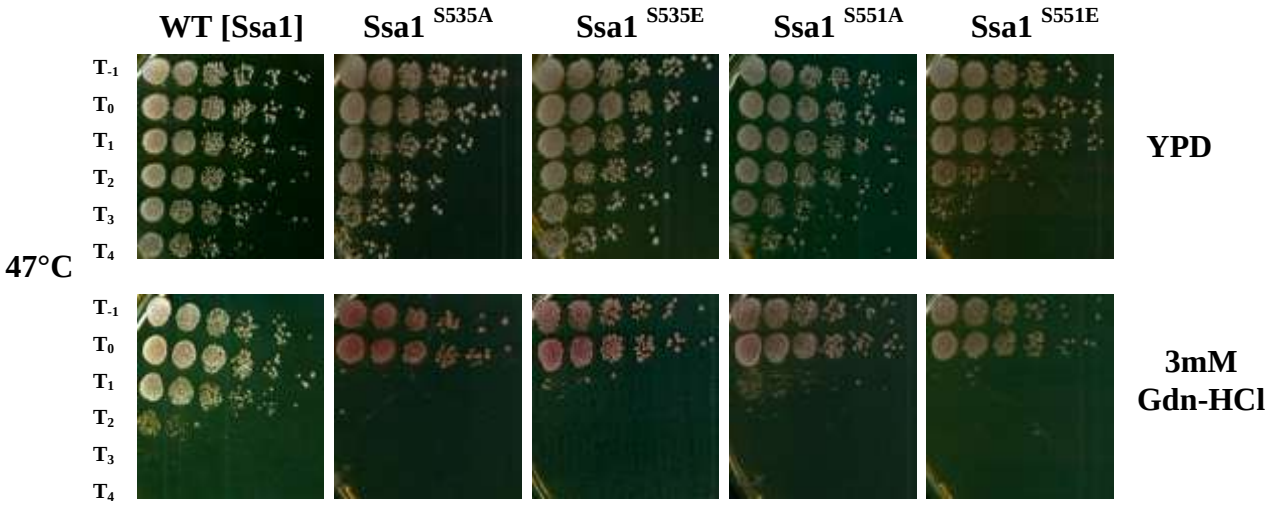



**Figure 3.15 Thermotolerance assay of Ssa1 single phosphorylation mutations on the SBD.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39 ºC to induce Hsp104 expression to protect against heat shock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD and 3mM Gdn-HCl as indicated above for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated at 30ºC for 2 days followed by 1 day at RT and were monitored for cellular thermotolerance.

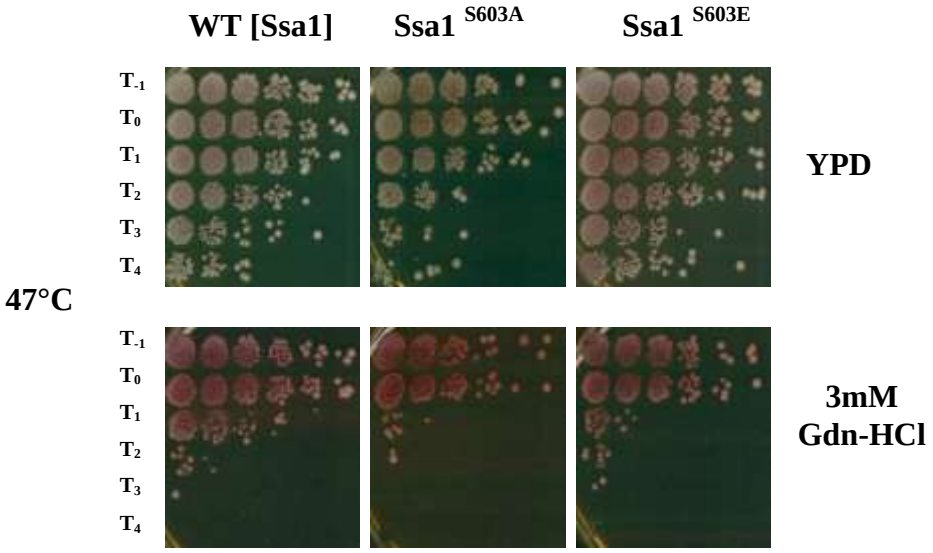



**Figure 3.16 Thermotolerance assay of Ssa1 single phosphorylation mutations on the C-terminus domain.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39 ºC to induce Hsp104 expression to protect against heat shock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD and 3mM Gdn-HCl as indicated above for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated at 30 ºC for 2 days followed by 1 day at RT and were monitored for cellular thermotolerance.

### 3.8 Assessing the effects of phosphorylation mutations of Hsp70 on *in vivo* refolding of bacterial luciferase model substrate

Hsp104 is a crucial chaperone that is required for thermotolerance in *S. cerevisiae* enhancing survival at elevated temperature (Sanchez & Lindquist, 1990). In cells expressing bacterial luciferase, the enzyme activity is lost in the presence or absence of Hsp104 (Parsell et al., 1994b). However recovery of luciferase takes place only in cells expressing Hsp104. Thus the ability of chaperones to refold misfolded proteins can be measured by performing a luciferase assay. The luciferase enzyme is unstable at high temperatures and omits light in presence of its substrate decanal, which can be measured on a luminometer. Hence, luciferase assays were performed to investigate how the phosphorylation mutants may effect yeast chaperone function with respect to influencing refolding activity.

For the assay, the pDCM90 plasmid was used which is a *URA3*-based plasmid containing a gene for expression of a thermolabile bacterial luciferase and was used as a reporter vector allowing analysis of luciferase protein function after heat shock. Decanal is a substrate for bacterial luciferase and upon reaction light is emitted, which can be measured using a luminometer. The plasmid pDCM90 was transformed into strains used in this assay, which in culture were subsequently incubated at 37 ºC to induce heat shock protein expression. Luciferase activity was measured at this point and taken to represent full protein activity. Cultures were then exposed to 45 ºC to subject the cells to heat shock conditions, after which luciferase activity was measured again. Cells were incubated at 25 ºC to allow recovery and luciferase activity measurements were taken at regular intervals.

Figure 3.17 A and B shows a differential rate of luciferase refolding activity over time between the WT and the phosphomutants ($p < 0.05$) after heat shock, reflecting the ability of chaperone proteins to refold luciferase into its functional state. As seen from the Figure 3.17 A, all the mutants behaved similar to WT in refolding luciferase after a recovery period of 45 min. Although the Ssa1 $^{T113E}$ mutant performed better than WT at an initial recovery period after 15 min, but at the later time point the Ssa1 $^{T113E}$ mutant behaved similar to WT in luciferase activity.

The luciferase refolding activity was also analysed for the double phosphomutants on the ATPase domain as shown in Figure 3.17 B. All the double phosphomutants on the ATPase domain showed similar ability to recover following heat shock compared to WT as represented by their luciferase refolding activity

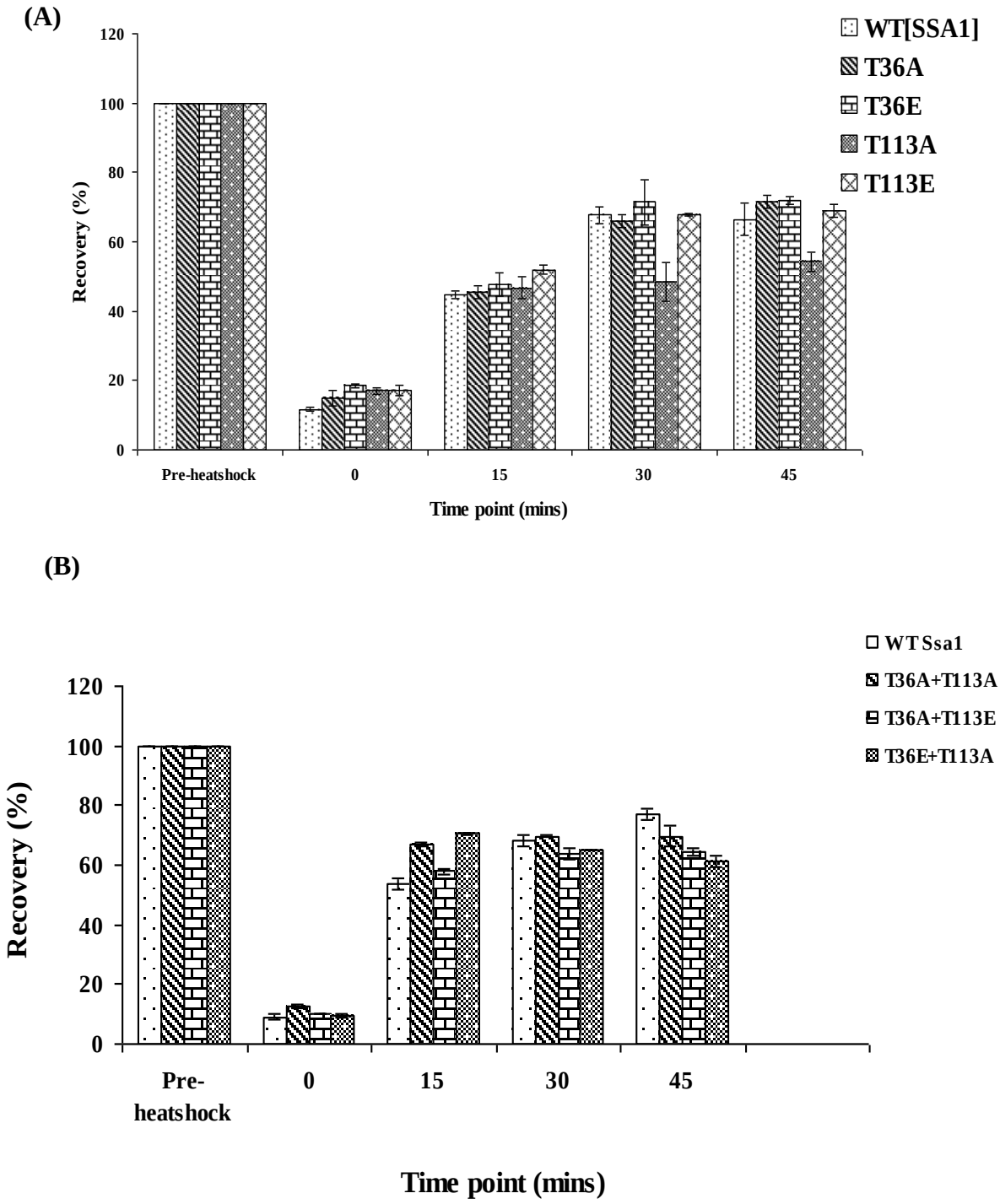



**Figure 3.17 Luciferase activity of the Ssa1 single (A) and double (B) phosphorylation mutations on the ATPase domain.** Overnight cultures were diluted in fresh SC medium lacking uracil to an $OD_{600nm}$ = 0.1. The cultures were then shifted to 37 °C for 30 min to induce the expression of Hsp104. After 30 min at 37 °C, the cultures were shifted to 45 °C for one hour. Cyclohexamide was added to the cultures after 50 min at 45 °C to prevent any further synthesis of luciferase during the recovery period. Luciferase activity, which is expressed as a percentage of pre-heat shock activity was measured at regular intervals during the recovery period of 45 min at 25 °C. [LSD at 0.05 for (A) = 8.52 and (B) = 2.41].

### 3.9 Basal levels of Hsp70 and Hsp104 in yeast cells expressing Ssa1 phosphorylation mutations.

The assessment of basal levels of Hsp70 and Hsp104 proteins in cells expressing phosphorylation mutations of Ssa1 that affect [$PSI^+$] propagation is critical as the levels of these molecular chaperones may have an effect on prion propagation. Previous studies have shown that the expression levels of molecular chaperones has an impact on [$PSI^+$] propagation (Chernoff et al., 1995; Kushnirov et al., 2000). Thus, western blot analysis was performed to assess the levels of these molecular chaperones in yeast cells that harboured the mutations with each of the phosphomutants representing the sole source of Ssa1 protein in the cell. The western blot analysis was carried out by loading 10 µg total protein onto a polyacrylamide gel as described in Section 2.19. The phosphomutants were then probed with Hsp70 and Hsp104 primary antibody to look for their expression levels.

Previous studies have shown that the basal expression levels of Hsp70 and Hsp104 were not affected by mutations within the Hsp70 ATPase domain (Jones & Masison, 2003; Loovers et al., 2007). As consistent with the previous results carried out by Loovers et al. (2007), the expression levels of Hsp70 and Hsp104 in yeast cells expressing ATPase domain single and double phosphorylation mutants were not considerably different in comparison to the protein levels in WT [Ssa1], except for the Ssa1 $^{T36E}$ mutant which showed an increased level of expression of Hsp70 (Figure 3.18 and 3.19). These results suggest that impairment of [$PSI^+$] propagation is not a result of altered amounts of Hsp70 and Hsp104.

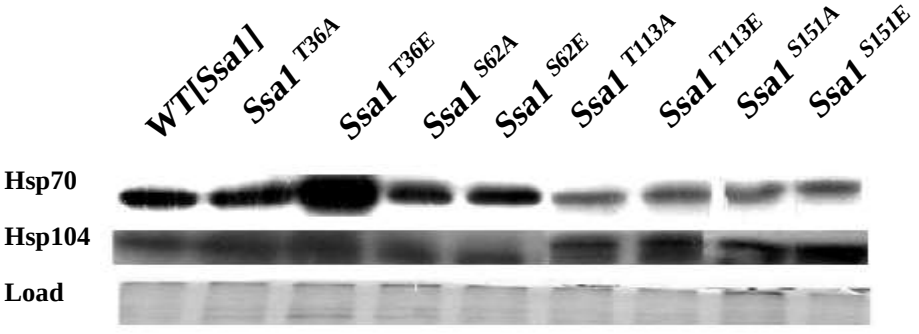



**Figure 3.18 Relative abundance of Hsp70 and Hsp104 in yeast cells expressing the single phosphorylation mutations on the ATPase domain.** Western blot analysis was performed to examine the abundance of Hsp70 and Hsp104. Blots probed with anti-Hsp70 antibody were stripped and reprobed with anti-Hsp104 antibody. Membrane was then stained by amido black as a loading and transfer control, are shown (Load). The phosphomutants along with the WT are indicated on the top.

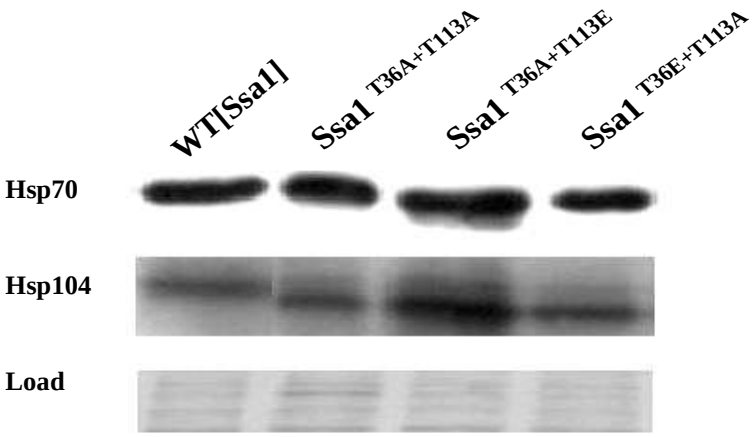



**Figure 3.19 Relative abundance of Hsp70 and Hsp104 in yeast cells expressing the double phosphorylation mutations on the ATPase domain.** Western blot analysis was performed to examine the abundance of Hsp70 and Hsp104. Blots probed with anti-Hsp70 antibody were stripped and reprobed with anti-Hsp104 antibody. Membrane was then stained by amido black as a loading and transfer control, are shown (Load). The phosphomutants along with the WT are indicated on the top.

While looking into the expression levels of Hsp70 and Hsp104 in the mutants within the SBD and C-terminus domains (Figure 3.20 and 3.21), a similar result was observed as the ATPase domain phosphorylation mutants suggesting any phenotypic effects are not due to the alterations of cellular chaperone levels.

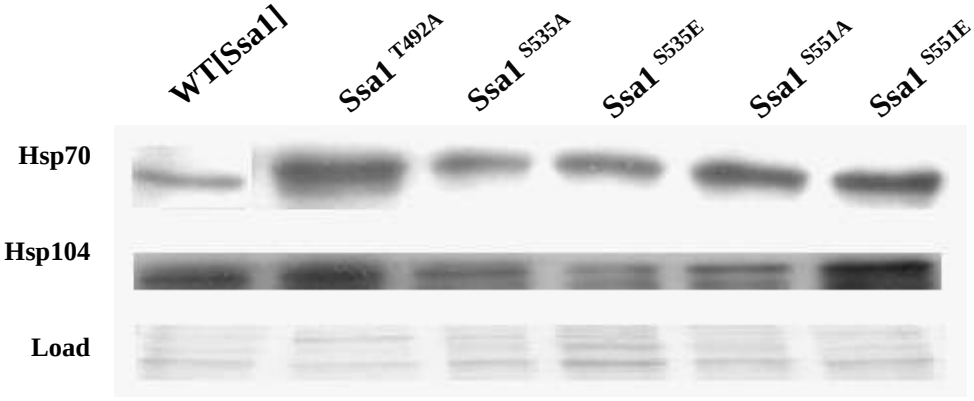



**Figure 3.20 Relative abundance of Hsp70 and Hsp104 in yeast cells expressing the phosphorylation mutations on the SBD.** Western blot analysis was performed to examine the abundance of Hsp70 and Hsp104. Blots probed with anti-Hsp70 antibody were stripped and reprobed with anti-Hsp104 antibody. Membrane was then stained by amido black as a loading and transfer control, are shown (Load). The phosphomutants along with the WT are indicated on the top.

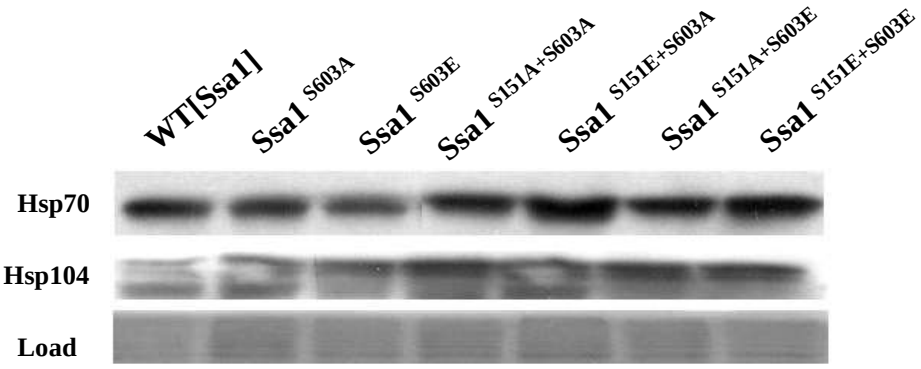



**Figure 3.21 Relative abundance of Hsp70 and Hsp104 in yeast cells expressing the phosphorylation mutations on the C-terminus and ATPase domain.**Western blot analysis was performed to examine the abundance of Hsp70 and Hsp104. Blots probed with anti-Hsp70 antibody were stripped and reprobed with anti-Hsp104 antibody. Membrane was then stained by amido black as a loading and transfer control, are shown (Load). The phosphomutants along with the WT are indicated on the top.

### 3.10 Role of phosphorylation at the linker region of yeast Hsp70 in chaperone function and prion maintenance and propagation

The *S. cerevisiae* genome encodes nine cytosolic proteins of the Hsp70 family – Ssa1-4, Sse1/2, Ssb1/2 and Ssz1 (Kabani & Martineau, 2008).Viability of the yeast cells is dependent on constitutive expression of at least one of the Ssa protein. The Ssa1 and Ssa2 are constitutively expressed and cells lacking the constitutive expression of these two isoforms causes the upregulation of the stress-inducible isoforms Ssa3 and Ssa4 as a compensatory mechanism (Sharma & Masison, 2008). The linker region between the ATPase domain and SBD in the Ssa family would be an ideal location for adapting a phosphorylation control mechanism for influencing inter-domain communication. There is one phosphorylatable residue in this region that is conserved between all four members of the Ssa family. Thus, site directed mutagenesis (SDM) was carried out as described in Section 2.8 to generate non-phosphorylatable and phosphomimetic mutants at the linker region of these Ssa proteins as shown in Table 3.6 by replacing the serine's and threonine's at those sites with either alanine (non-phophorylatable) or glutamic acid (phosphomimetic).

**Table 3.6 Single phosphomutants generated on the linker region of the Ssa family.** SDM was carried out to generate the phospho-mutations. The table shows the nucleotides that were changed by SDM to get either alanine or glutamic acid at the required position.

| Mutation | Ssa | Nucleotide Position Changed | Nucleotide Changed | Codon Changed | Amino Acid Changed |
|---|---|---|---|---|---|
| T378A | Ssa1 | 1132 | A ⇨ G | 378 | T ⇨ A |
| T378E | Ssa1 | 1132,1133,1134 | ACT ⇨ GAG | 378 | T ⇨ E |
| T378A | Ssa2 | 1132 | A ⇨ G | 378 | T ⇨ A |
| T378E | Ssa2 | 1132,1133,1134 | ACT ⇨ GAG | 378 | T ⇨ E |
| T379A | Ssa3 | 1135 | A ⇨ G | 379 | T ⇨ A |
| T379E | Ssa3 | 1135,1136,1137 | ACC ⇨ GAG | 379 | T ⇨ E |
| T379A | Ssa4 | 1135 | A ⇨ G | 379 | T ⇨ A |
| T379E | Ssa4 | 1135,1136,1137 | ACG⇨ GAG | 379 | T ⇨ E |

### 3.10.1 Phenotypic characterization of the phosphomutants at the linker region of the Ssa family

The plasmid shuffle technique was employed as described in Section 2.12 to isolate the phosphomutants generated at the linker region of the Ssa proteins and the prion phenotype of these mutants were then assessed on YPD and − ade plates (Figure 3.22 and 3.23).The [*PSI*$^+$] mediated nonsense suppression was assessed by comparing the growth and pigmentation of [*PSI*$^+$] cells on YPD plates and – ade plates. When Ssa1 [*PSI*$^+$] and Ssa2 [*PSI*$^+$] were used as WT strains, the [*PSI*$^+$] status was maintained in Ssa1 but the [*PSI*$^+$] was not strong in Ssa2 compared to Ssa1 on YPD. Also on − ade plates, Ssa1 [*PSI*$^+$] showed good growth without adenine but the Ssa2 [*PSI*$^+$] showed weak growth without adenine at 30 °C and RT. This might suggest that members of the Ssa family are distinct from one another with respect to prion maintenance and propagation. [*PSI*$^+$] was efficiently propagated in both the Ssa1 and Ssa2 mutant strains compared to WT, as seen by the pigmentation on YPD and growth on − ade plate (Figure 3.22).

As depicted from Figure 3.23, [*PSI*$^+$] was maintained in cells expressing Ssa3 as the sole source of Ssa in the cell but [*PSI*$^+$], although present in cells expressing Ssa4, was very weak . When these two strains were taken as WT and the phosphomutants of

Ssa3 and Ssa4 was assessed for prion propagation, it was observed that the [*PSI*$^{+}$] status was strongly maintained in Ssa3 $^{T379A}$ and Ssa3 $^{T379E}$ mutant strains as seen by the colour on YPD and growth on −ade medium. But the weak [*PSI*$^{+}$] status of the WT [Ssa4] could not be altered by the Ssa4 $^{T379A}$ and Ssa4 $^{T379E}$ mutation and the cells were strongly [*psi*$^{-}$] as seen by the red colour on YPD and very weak growth on − ade plates (Figure 3.23). It has been shown previously that [*PSI*$^{+}$] is weak in cells expressing Ssa4 as the sole source of Ssa in the cell, and there occurs functional similarities between Ssa1 and Ssa3 and between Ssa2 and Ssa4 in regard to prion propagation (Sharma et al., 2009; Sharma & Masison, 2008)

### 3.11 A comparison of the growth rate and strength of [*PSI*$^{+}$] status among the phosphomutants of the Ssa family.

All the *SSA* genes support yeast growth although to a variable extent suggesting that the different isoforms of the Ssa subfamily of Hsp70 are significant in their function (Sharma et al., 2009). To assess whether the phosphomutants in the linker region of the Ssa proteins affected growth rate of *S. cerevisiae* differently, we analyzed the growth rate of the cells expressing the phosphorylation mutants on the Ssa proteins as sole source of Ssa in the cell. Mutations in the linker region of both the constitutive and heat inducible isoforms of Hsp70 affected growth rate of yeast cells significantly ($p < 0.05$) but the mutations generated on the heat inducible isoforms of Hsp70 had a more pronounced affect on growth rate of the yeast cell compared to its constitutive counterpart when considered from a biological point view (Table 3.7). The phosphomutants Ssa3 $^{T379E}$ generated on heat inducible Ssa3 showed 9% slower growth compared to its WT [Ssa3] (Table 3.7). The Ssa4 mutants had a significant effect on growth rate, with the growth rate being reduced by 25% for Ssa4 $^{T379A}$ and 16% for Ssa4 $^{T379E}$ mutant (Table 3.7). The colour of the phosphomutants in G402 was scored with Image J- java based image processing program, zero being [*PSI*$^{+}$] (white) and 10 being [*psi*$^{-}$] (red). The Ssa4 phosphorylation mutants on the linker region appeared to have the strongest [*psi*$^{-}$] phenotype as shown in Table 3.7.

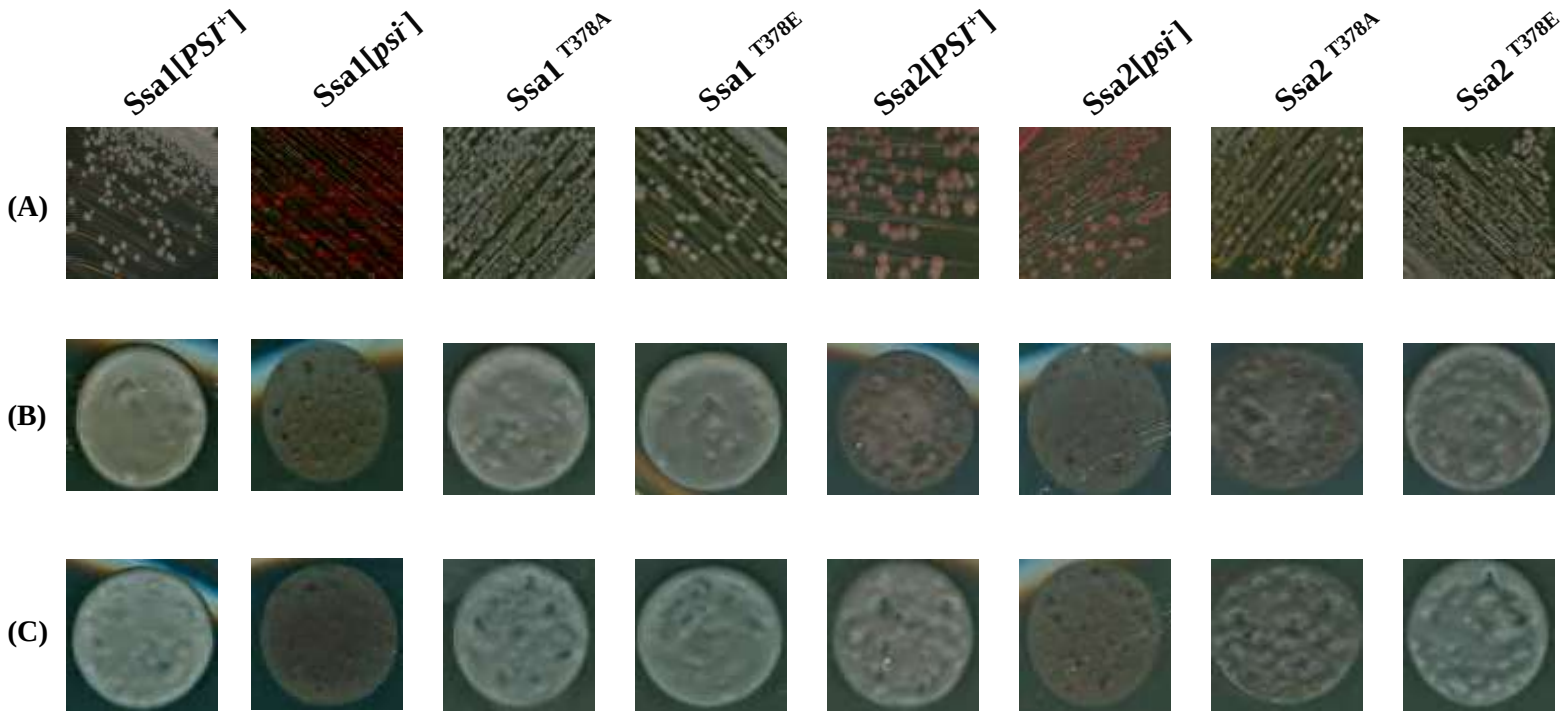



**Figure 3.22 Effect of Ssa1 and Ssa2 phosphomutants on [*PSI*$^+$] phenotype.** (A) Cells were streaked on YPD and colonies appeared after 2 days of incubation at 30 °C followed by 2 days at 4 °C. (B) 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and (C) at RT for 3 days. Ssa1[*PSI*$^+$] is the WT and Ssa1[*PSI*$^+$] and Ssa1[*psi* $^-$] are taken as controls for Ssa1 mutants; Ssa2[*PSI*$^+$] is the WT and Ssa2[*PSI*$^+$] and Ssa2[*psi* $^-$] are taken as controls for Ssa2 mutants. Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text). The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates.

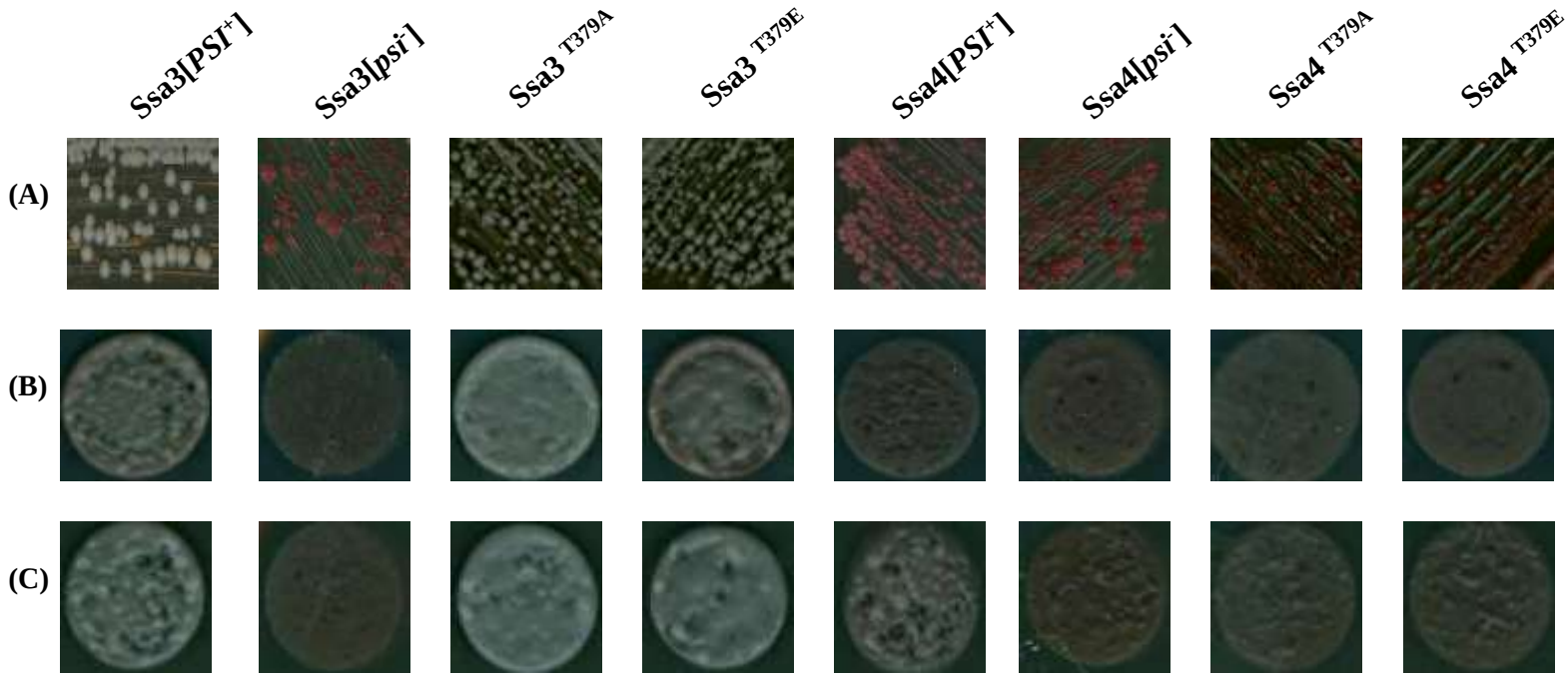



**Figure 3.23 Effect of Ssa3 and Ssa4 phosphomutants on [*PSI*$^+$] phenotype.** (A) Cells were streaked on YPD and colonies appeared after 2 days of incubation at 30 °C followed by 2 days at 4 °C. (B) 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and (C) at RT for 3 days. Ssa3[*PSI*$^+$] is the WT and Ssa3[*PSI*$^+$] and Ssa3[*psi*$^-$] are taken as controls for Ssa3 mutants; Ssa4[*PSI*$^+$] is the WT and Ssa4[*PSI*$^+$] and Ssa4[*psi*$^-$] are taken as controls for Ssa4 mutants. Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text). The extent of *ade 2-1* suppression is also reflected as density of growth on −ade plates.

**Table 3.7 Relative effects of Ssa1-4 phosphorylation mutations on prion propagation and cell growth**

| Phosphomutants | ±MGT (min) | Colour in G402 |
|---|---|---|
| WT [Ssa1] | 108.63 ± 0.2 | 0 |
| Ssa1 $^{T378A}$ | 103.67 ± 0.5 | 4 |
| *Ssa1 $^{T378E}$ | 101.76 ± 1.4 | 4 |
| WT [Ssa2] | 107.96 ± 0.5 | 0 |
| *Ssa2 $^{T378A}$ | 100.61 ± 0.5 | 2 |
| Ssa2 $^{T378E}$ | 101.82 ± 0.2 | 4 |
| WT [Ssa3] | 199.71 ± 1.1 | 0 |
| Ssa3 $^{T379A}$ | 190.07 ± 1.9 | 3 |
| *Ssa3$^{T379E}$ | 218.12 ± 5.7 | 3 |
| WT [Ssa4] | 114.00 ± 0.9 | 0 |
| *Ssa4 $^{T379A}$ | 143.28 ± 0.6 | 8 |
| *Ssa4 $^{T379E}$ | 132.99 ± 0.6 | 9 |

±The mean generation time of the yeast strains were determined by diluting the overnight 30 °C culture in 30 ml fresh media to an $OD_{600nm}$ of 0.1(t = 0 min). Cultures were transferred to 30 °C shaking incubator and cell density was measured at an interval of every 2 h as described in Section 2.14. The table also numerically categorizes the phophomutants according to their colour in G402, zero representing white [$PSI^+$] and ten representing red [$psi^-$] based on Image J-java based image processing program. (* indicate significant difference between the respective WT and its phosphomutants at $p < 0.05$)

### 3.12 Comparative growth analysis of the phosphomutants of the Ssa family

The phosphomutants generated at the linker region of Ssa1 and Ssa2 did not show any temperature sensitive phenotype at 39 °C (Figure 3.24). Thus, the mutations did not have a prominent effect on constitutively expressed Hsp70 function under heat shock conditions.

Differences in temperature sensitivity were more pronounced for the Ssa3 and Ssa4 phosphomutants as shown in Figure 3.25 (A). The Ssa3 mutant T379E displayed mild temperature sensitivity at 37 °C. Also the mutants Ssa4 $^{T379A}$ and Ssa4 $^{T379E}$ were

slightly temperature sensitive at 37 °C and its temperature sensitive phenotype became more prominent at 39 °C. All the three mutants *i.e* Ssa3 $^{T379E}$, Ssa4 $^{T379A}$ and Ssa4 $^{T379E}$ recovered from temperature sensitivity on subsequent growth at 37 °C and 39 °C on 1M sorbitol (Figure 3.25 B). The recovery of these mutants on 1M sorbitol might be indicative of the importance of the T379 site of Ssa3 and Ssa4 protein in cell wall integrity signalling.

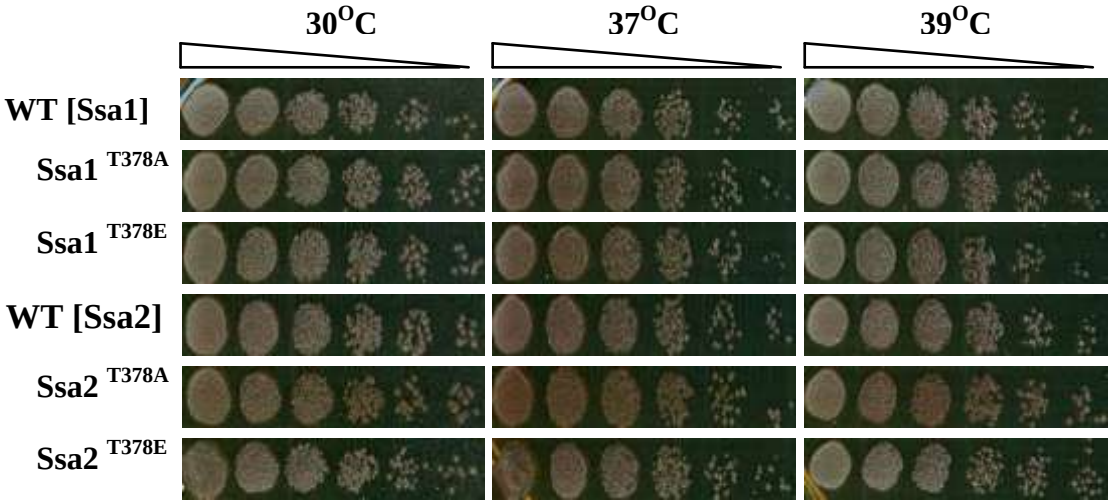
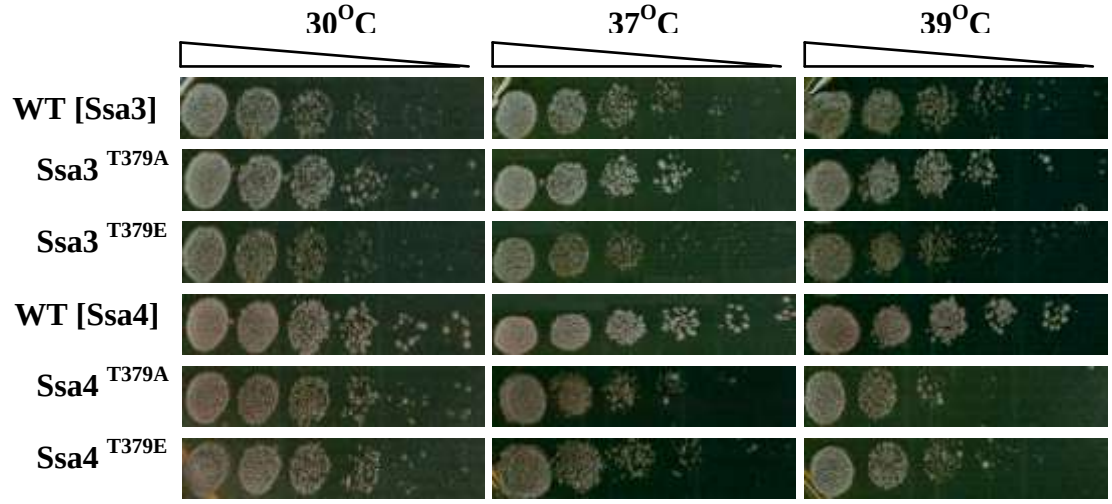
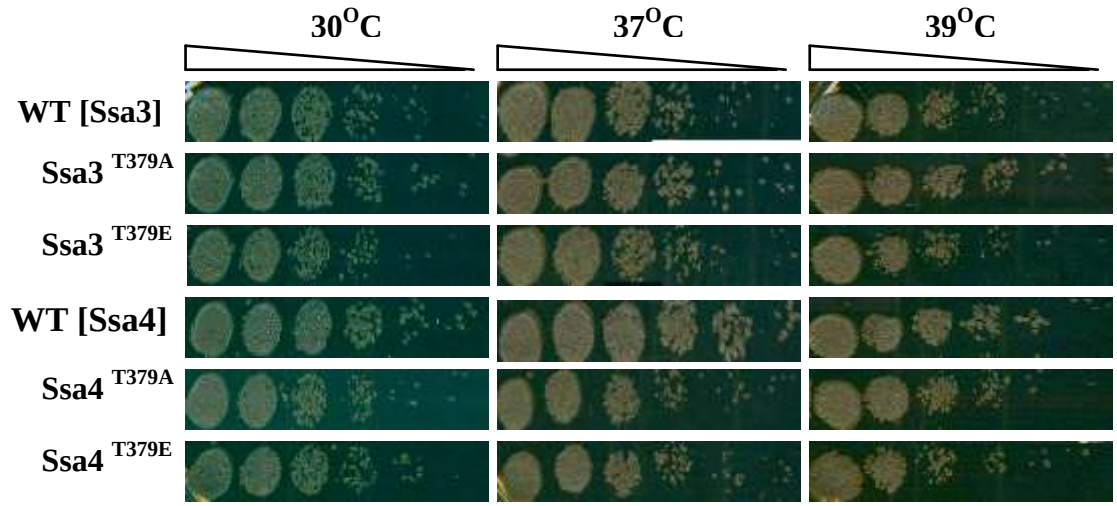



**Figure 3.24 Comparative growth analysis of the phosphorylation mutants of Ssa1 and Ssa2 on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.




**(A)**




**(B)**

**Figure 3.25 Comparative growth analysis of the phosphorylation mutants of Ssa3 and Ssa4 on (A) YPD plate and (B) 1M sorbitol plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

### 3.13 Investigation of the influence of phosphorylation mutants of the Ssa family in cell wall integrity signalling

The cell wall integrity (CWI) signalling is activated in response to a variety of environmental stimuli that cause cell wall stress (Levin, 2005). In *S. cerevisiae*, there are a total five separate mitogen-activated protein kinase (MAPK) cascades that are activated in response to various environmental stimuli such as heat shock and oxidative stress (Chen & Thorner, 2007), and CWI signalling utilizes one of these five cascades for cell wall maintenance and biosynthesis (Levin, 2005). This cascade in yeast is triggered by several cell wall stress sensors such as Mid2 and Wsc1 (Verna et al., 1997; Ketela et al., 1999). As a result, kinase Pkc1 (Nonaka et al., 1995) is activated and in turn activates a cascade of phosphorylations through MAP kinases resulting in activation of two transcription factors Rlm1 and SBF (Swi4/Swi6). These transcription factors then trigger gene expression involved in cell wall biogenesis (Madden et al., 1997; Jung et al., 2002) (Figure 3.26)

Defects in the kinase or the transcription factors involved in this cascade can lead to temperature-sensitive cell lysis that can be recovered by osmotic support (Lee & Levin, 1992). Agents that cause cell wall stress like caffeine, calcofluor white, congo

red can also activate the CWI signalling (Levin, 2005; Martín et al., 2000; Errede et al., 1995). Protein chaperones Hsp90, Hsp70 nucleotide exchange factor (NEF) Sse1 and Hsp40 have been implicated in the CWI signalling (Millson et al., 2005; Piper et al., 2006; Wright et al., 2007). Hsp90 interacts with Slt2 to activate Rlm1 (Millson et al., 2005). The NEF Sse1 of the cytosolic Hsp70 has been shown to work in conjugation with Hsp90 and Slt2 in the CWI signalling cascade (Shaner et al., 2008). A number of cell wall sensitivity tests with different cell wall stress agents were performed on the phosphomutants of the Ssa family to look for their effects on the CWI signalling cascade.

### 3.13.1 Analysis of the sensitivity of the phosphorylation mutants of Ssa family to cell wall damaging agents

When *S. cerevisiae* are exposed to cell wall damaging agents like SDS, calcofluor white, caffeine and congo red, the cell wall is damaged leading to subsequent activation of MAP kinase pathway (Ketela et al., 1999; de Nobel et al., 2000). Thus, if the phosphomutants of the Ssa family are defective in CWI signalling pathway then they should show sensitivity to cell wall damaging agents. A comparative growth analysis was performed as described in Section 2.15 and the cells were then selected on medium containing different concentrations of SDS (0.005-0.01%), caffeine (8 µm) and calcofluor white (10 µg/ml) with YPD used as a control plate (Figure 3.27 and 3.28). The plates were then left at 30 °C for 3 days and the phenotype assessed after 3 days. The phosphomutants on the constitutively expressed Ssa proteins i.e Ssa1 $^{T378A}$, Ssa1 $^{T378E}$ and Ssa2 $^{T378A}$ did not show a marked difference in sensitivity to the cell wall damaging agents compared to their WT except for the Ssa2 $^{T378E}$ that showed a slight difference in sensitivity on SDS (0.005%). However, a difference in sensitivity in response to the chemical stress could be observed for the phosphomutants on the heat inducible Ssa4 compared to the respective WT on SDS (0.01%) as shown in Figure 3.28. The Ssa3 phosphomutants did not show any sensitivity to cell wall damaging agents suggesting that Ssa3 phosphomutants do not cause any defect to the CWI signalling pathway in response to chemical stress.

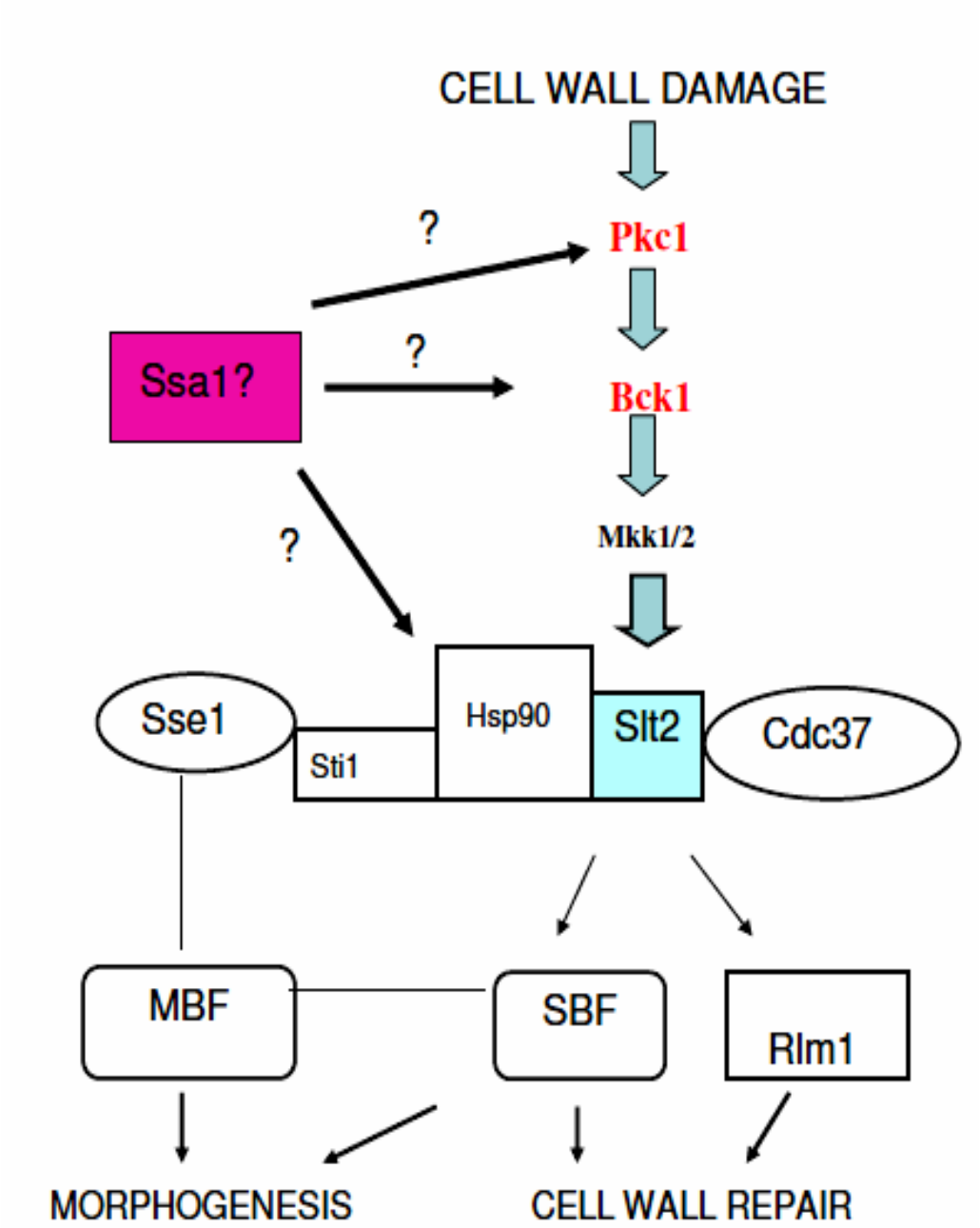



**Figure 3.26 Cell wall integrity signalling pathway.** A linear MAP kinase signalling pathway is involved in amplification of signals due to cell wall damage that is elicited by several environmental stimuli. It has been shown that Pkc1 activates Bck1which in turn activates Mkk1/2 leading to the phosphorylation of Slt2. This phosphorylation event activates transcription factors Rlm1 and SBF. These transcription factors then trigger expression of genes involved in cell wall biogenesis. This CWI signalling pathway is supported by a multi-chaperone complex that includes Hsp90, Hsp70 (Ssa), Hsp110 (Sse1) and Sti1 that interacts with Slt2 and thus leads to activation of downstream transcription factors. This figure is adapted from Shaner et al. (2008).

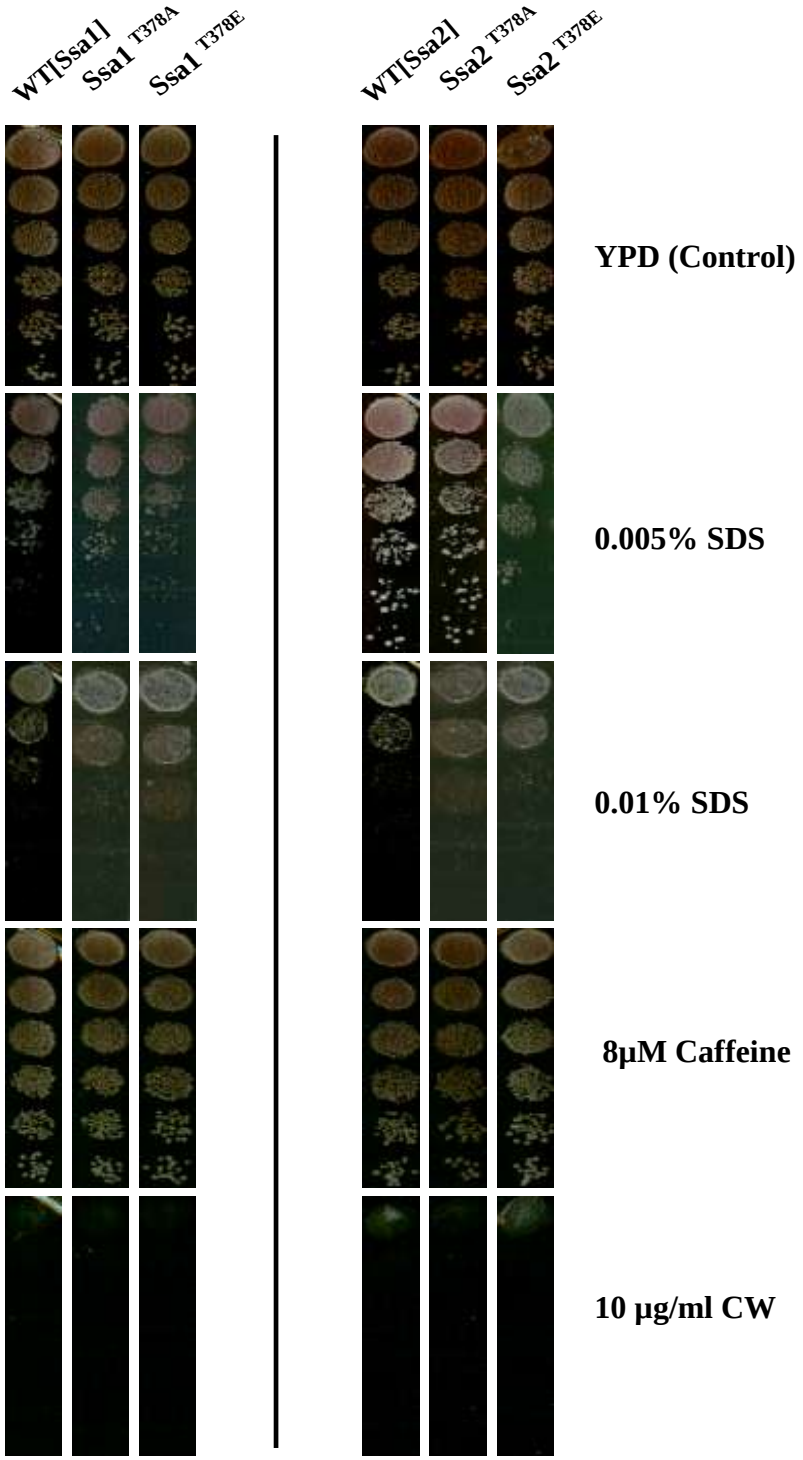



**Figure 3.27 Comparative growth analysis of the phosphorylation mutants of Ssa1 and Ssa2 on cell wall damaging agents.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C and were monitored for sensitivity.

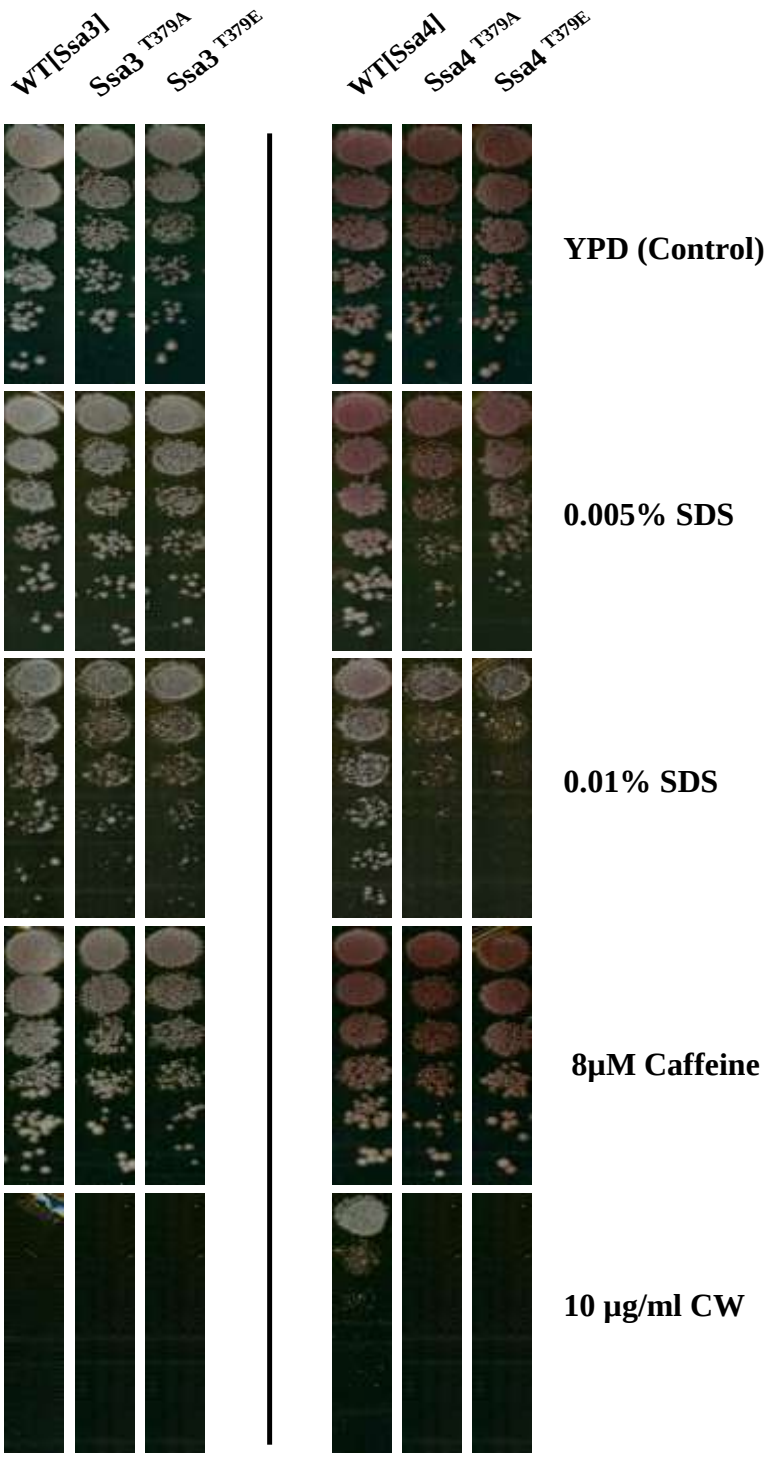



**Figure 3.28 Comparative growth analysis of the phosphorylation mutants of Ssa3 and Ssa4 on cell wall damaging agents.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C and were monitored for sensitivity.

### 3.14 Reactive oxygen species, oxidative stress and oxidative stress response

Oxygen is a highly reactive molecule and all the aerobically growing organisms are continuously exposed to chemically reactive agents known as reactive oxygen species (ROS) such as super oxide anion ($O_2^-$), hydroxyl radical (OH) and hydrogen peroxide ($H_2O_2$) that is formed when molecular oxygen is reduced during oxidative phosphorylation (Lushchak, 2011). The ROS is highly damaging to various cellular components like DNA, proteins and lipids (Storz et al., 1987; Wolff et al., 2005). The generation of ROS is inevitable to all aerobic forms of life and is generated in cells as a consequence of many metabolic processes (Lushchak, 2011). The generation and degradation of ROS is under refined cellular control, maintaining a steady state ROS in the cell (Halliwell & Gutteridge, 1989). However under some unknown circumstances, when the balance between production and elimination of ROS is disturbed leading to enhanced steady-state level is called "oxidative stress". Thus all the aerobic forms of life have evolved to develop various defense mechanisms to degrade ROS through production of detoxifying enzymes and molecular scavengers (Moradas-Ferreira & Costa, 2000). These molecules serve as the first line of defense against oxidative stress, and the cellular mechanisms that induce production of these molecules in response to oxidative stress are termed "oxidative stress response".

One of the highly conserved signalling pathways found in eukaryotes is the mitogen-activated protein kinase (MAPK) cascades that can be utilized in response to various environmental stimuli (Schaeffer & Weber, 1999). In *S. cerevisiae*, there are in total five separate mitogen-activated protein kinase (MAPK) cascades that are activated in response to various environmental stimuli such as heat shock and oxidative stress (Chen & Thorner, 2007) and CWI signalling utilizes one of these five cascades for cell wall maintenance and biosynthesis (Levin, 2005). As MAPK cascades are conserved throughout evolution, yeast as a model organism provides an excellent tool for gaining in depth perspective towards understanding how eukaryotes utilize this cascade to regulate gene expression in response to oxidative stress.

#### 3.14.1 Investigation of the effect of phosphorylation mutants of Ssa family on growth of yeast cells in response to hydrogen peroxide.

Comparative growth analysis was performed for the phosphomutants generated on the Ssa genes and the yeast cells were then tested for oxidative stress response to hydrogen peroxide. The cells were selected on SC medium having varying

concentrations of $H_2O_2$ (0-5 mM) and grown at 30 °C for 3 days and the cells were then monitored for sensitivity towards $H_2O_2$ (Figure 3.29 & 3.30). The Ssa1 mutants, exhibited similar response to WT [Ssa1] at 4 mM and 5 mM concentrations of $H_2O_2$ (Figure 3.29). However, for the Ssa2 mutants, sensitivity to $H_2O_2$ was observed at 3mM concentration for both WT [Ssa2] and Ssa2 $^{T378A}$ but the mutant Ssa2 $^{T378E}$, which was resistant to a concentration of 3 mM $H_2O_2$ and started showing sensitivity only at a higher concentration (Figure 3.29).

On the other hand, the phosphomutants on the inducible isoform of Hsp70 did not show a marked difference in sensitivity and their response towards $H_2O_2$ was comparable to the WT. But the mutant Ssa3 $^{T379A}$ showed a difference in sensitivity compared to WT [Ssa3] (Figure 3.30). While 3-5 mM concentration of $H_2O_2$ inhibits WT growth, the mutant Ssa3 $^{T379A}$ showed better growth compared to WT at these concentrations.

### 3.15 Investigation of acquired thermotolerance activity of the phosphomutants of the Ssa family.

A major role played by Hsp104 molecular chaperone is to ensure cell survival after prolonged exposure to elevated temperature or chemical stress (Jones & Tuite, 2005). Unlike Hsp70 and other chaperones, Hsp104 function is not directly related to protein disaggregation of misfolded protein. In fact it works in concert, with Hsp70 and Hsp40 and forms a protein disaggregation machinery which then reactivates misfolded proteins (Glover & Lindquist, 1998). The survival rate of yeast cells to lethal heat shock can be greatly improved by pre-treatment of the cells to mild non-lethal heat shock that induces the expression of heat shock proteins involved in stress response pathways (Lindquist & Craig, 1988; Landry et al., 1982). Since Hsp104 expression level is crucial for growth at elevated temperature, a well-established thermotolerance assay (described in Section 2.16) was used to assess the activity of Hsp104 whereby cell survival represented functional Hsp104 activity.

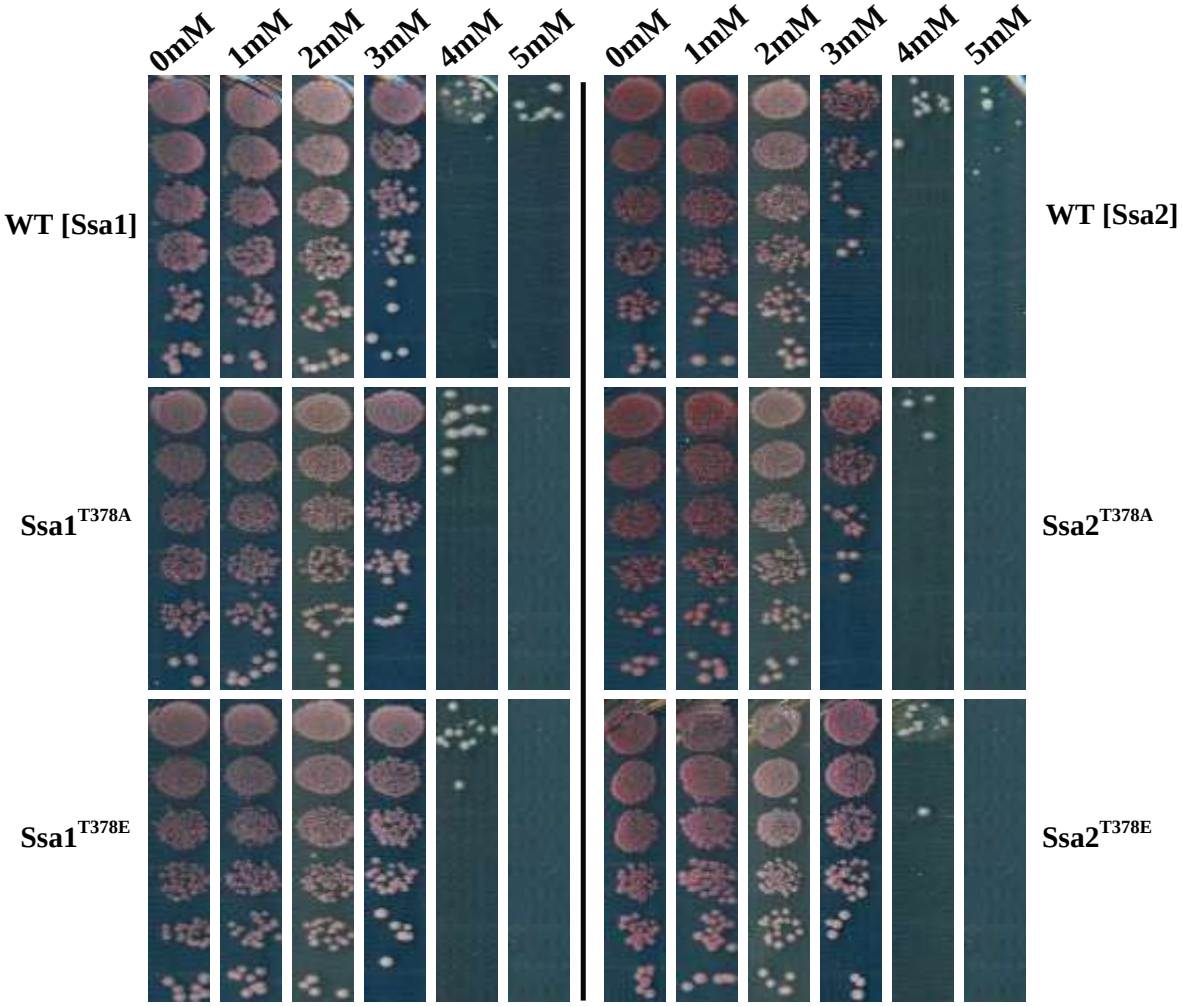



**Figure 3.29 Comparative growth analysis of the phosphorylation mutants of Ssa1 and Ssa2 on varying concentrations of $H_2O_2$ (0-5 mM).** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C and were monitored for oxidative stress response.

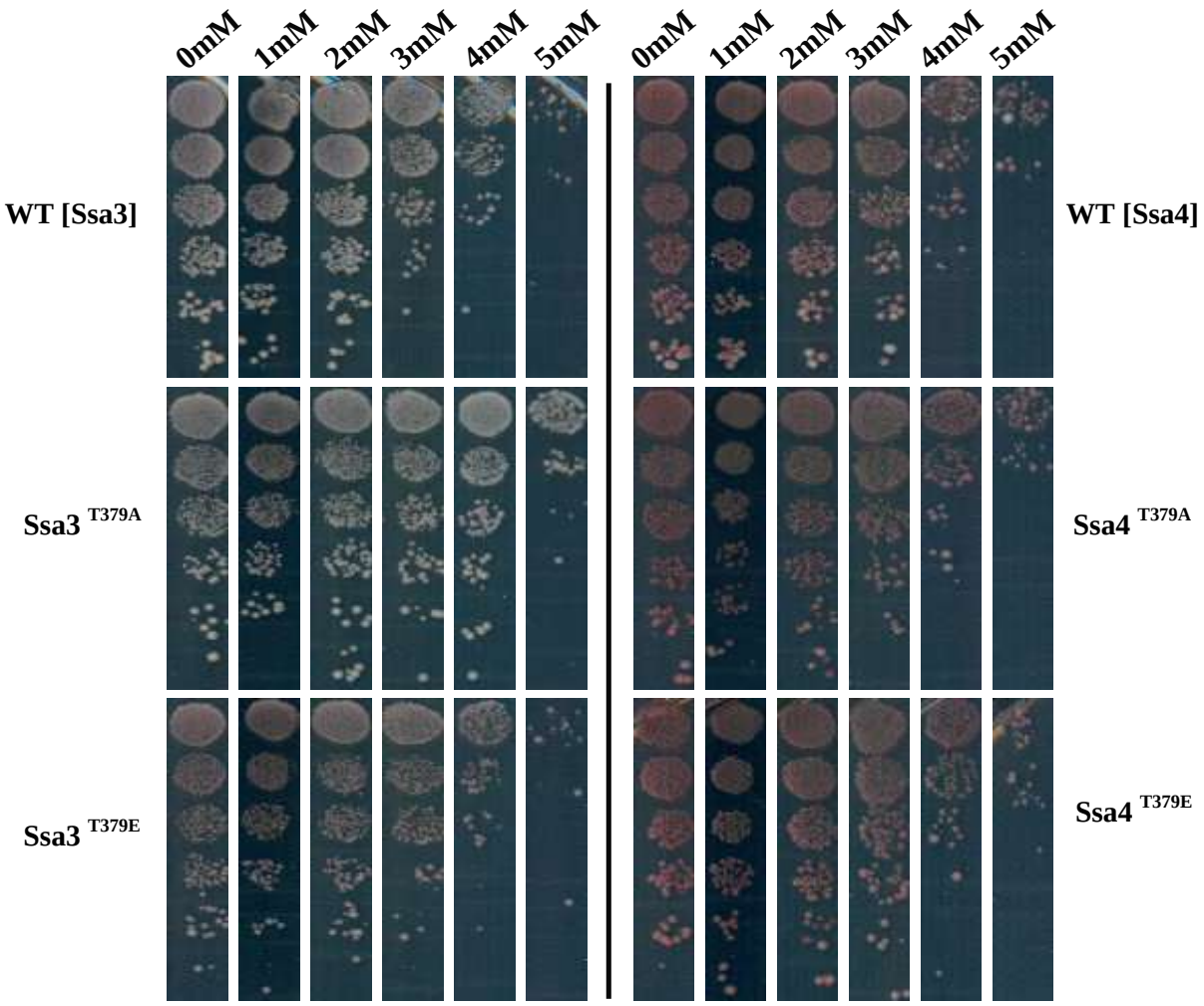



**Figure 3.30 Comparative growth analysis of the phosphorylation mutants of Ssa3 and Ssa4 on varying concentrations of $H_2O_2$ (0-5 mM).** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C and were monitored for oxidative stress response.

### 3.15.1 The phosphomutant T378A in the Ssa2 protein alters acquired thermotolerance

The thermotolerance of the phosphomutant strains on constitutively expressed isoforms of Hsp70 revealed that the Ssa2 $^{T378A}$ phosphomutant showed a marked sensitivity in acquired thermotolerance compared to WT. Cell viability of this mutant strain was decreased after 10 min of exposure to heat shock at 47 °C on YPD plate. In presence of 3mM Gdn-HCl, which inhibits Hsp104 a significant decrease in growth rate can be observed after a short period of exposure to 47 °C (Figure 3.31). This result might indicate that Hsp104 is impaired in its function due to this Ssa2 mutation. As shown in the Figure 3.31 the thermotolerance of all other Ssa1 and Ssa2 mutants were comparable to the WT except for the Ssa2 $^{T378A}$.

### 3.15.2 Phosphomutants in inducible isoforms of Hsp70s enhance acquired thermotolerance

The basal thermotolerance level of the phophomutants on the inducible isoforms of Hsp70 showed that the thermotolerance level of the Ssa3 and Ssa4 phosphomutants did not show any significant difference compared to their respective WT except for the Ssa3 $^{T379A}$ mutants, which showed increased thermo-resistance (Figure 3.32). Also the mutant strain showed increased viability on 3 mM Gdn-HCl. These results indicate that phosphomutants in inducible Hsp70 may be more efficient in cooperating with Hsp104 during heat stress and have more affinity towards heat denatured substrates.

## 3.16 Phosphomutations on the inducible and constitutive isoforms of Hsp70 vary in their ability to assist *in vivo* protein refolding

Hsp104 is one of the crucial molecular chaperones in yeast *S. cerevisiae* that becomes critical for survival at elevated temperature, which causes widespread protein disaggregation (Sanchez & Lindquist, 1990) .This function of Hsp104 is dependent on Hsp70 and Hsp40 (Lindquist & Craig, 1988). The ability of the chaperones to help refold misfolded proteins can be assessed *in vivo* by monitoring reactivation of a thermolabile bacterial luciferase after exposing cells to elevated temperature. The luciferase activity was monitored *in vivo* in the various phophomutant strains of the Ssa family (Figure 3.33 & 3.34) by transferring exponentially growing cells from 30 °C to 37 °C for 30 min to induce the expression of the heat shock proteins, particularly

Hsp104 and then shifted to 45 °C to cause extensive aggregation of luciferase. After this treatment, the cells were allowed to recover from the heat shock at 25 °C for 45 min.

There was significant difference in protein refolding between ($p < 0.05$) the WT [Ssa1] and WT [Ssa2] compared to the mutant proteins (Figure 3.33 A and B). As seen from the Figure 3.33 A, in WT [Ssa1] over 60% of the luciferase activity was recovered and the Ssa1 $^{T378A}$ showed more than 50% recovery while the phosphomimetic mutant Ssa1 $^{T378E}$ showed less than 50% recovery. The results showed marked difference in luciferase activity for the WT [Ssa2] and its phosphomutants (Figure 3.33 B). In WT [Ssa2] cells, 40% luciferase activity was restored, while in cells with Ssa2 $^{T378A}$ mutation only around 15 % was reactivated. But the Ssa2 $^{T378E}$ behaved better than the WT in luciferase refolding showing over 50% recovery.

Luciferase reactivation of the phosphomutants on the inducible isoforms of Hsp70 also showed significant variations ($p < 0.005$). The Ssa4 phosphomutants Ssa4 $^{T379A}$ and Ssa4 $^{T379E}$ showed a similar level of luciferase refolding activity compared to WT [Ssa4], thus suggesting no defect in Hsp104 activity due to the mutations (Figure 3.34 B). Although the luciferase refolding activity of Ssa3 $^{T379E}$ showed a significant difference in refolding compared to WT [Ssa3] but after a full recovery period of 45 min it can be suggested that luciferase refolding of the Ssa3 $^{T379E}$ was similar to the WT [Ssa3] whereas the difference in refolding between WT [Ssa3] and Ssa3 $^{T379A}$ mutant was not statistically significant (Figure 3.34 A) thus indicating that Ssa3 was not defective in its Hsp104 activity due to the mutations.

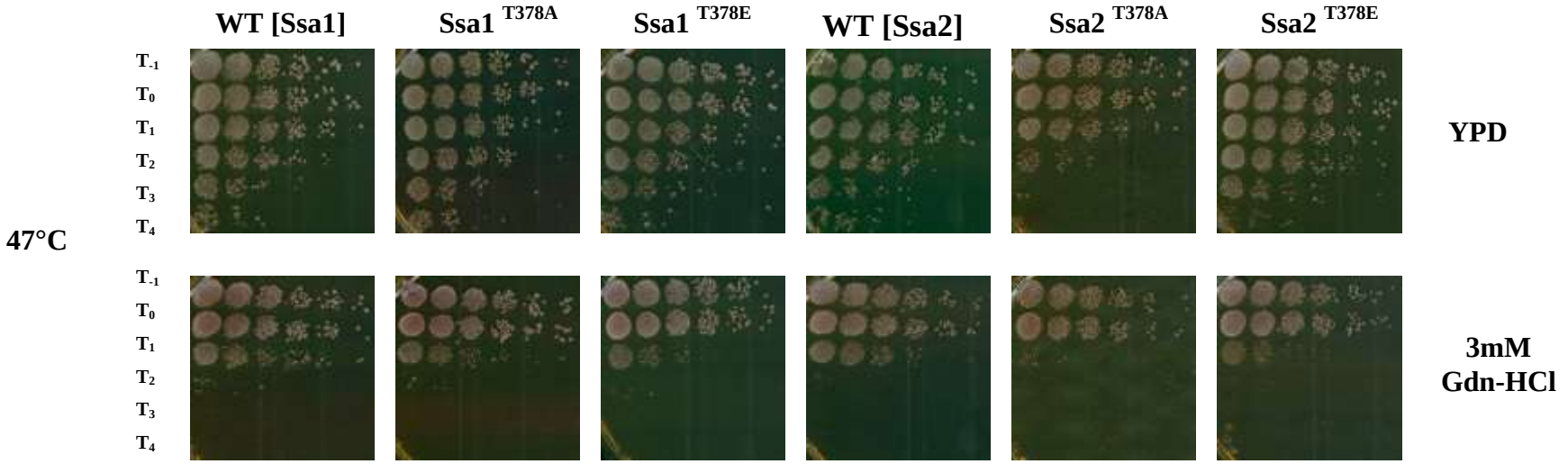



**Figure 3.31 Thermotolerance assay for phosphorylation mutations on Ssa1 and Ssa2 isoforms of Hsp70.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39ºC to induce Hsp104 expression to protect against heat shock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD and 3mM Gdn-HCl as indicated above for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated at 30 ºC for 2 days followed by 1 day at RT and were assessed for cellular thermotolerance.

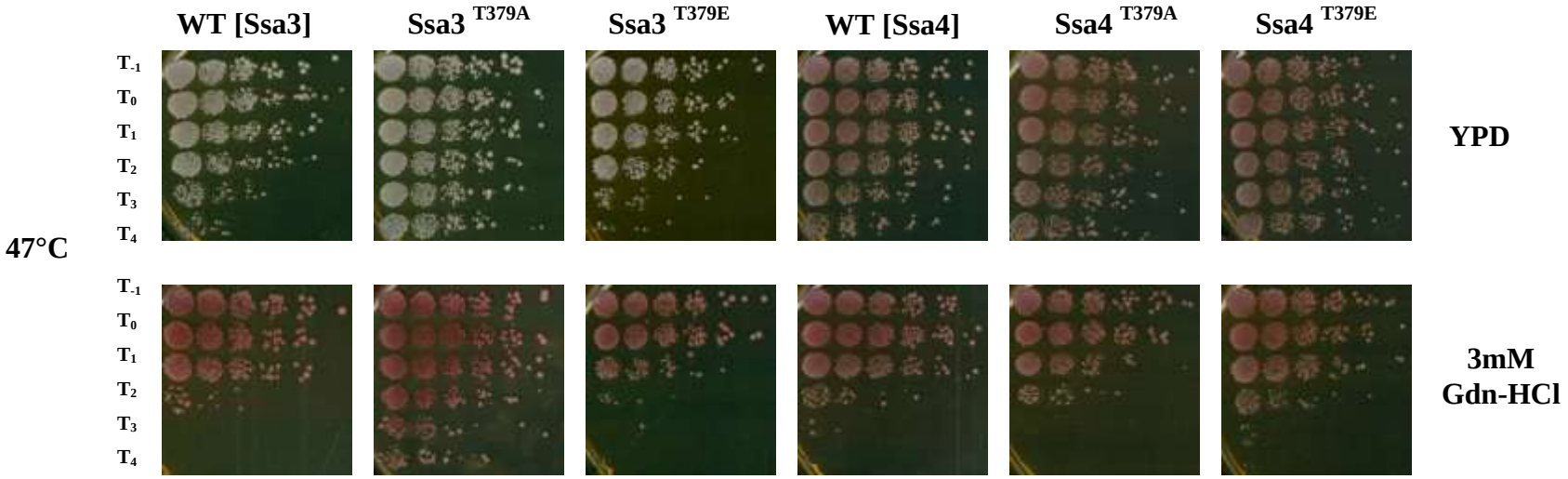



**Figure 3.32 Thermotolerance assay for phosphorylation mutations on Ssa3 and Ssa4 isoforms of Hsp70.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39 ºC to induce Hsp104 expression to protect against heat shock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD and 3mM Gdn-HCl as indicated above for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated at 30 ºC for 2 days followed by 1 day at RT and were assessed for cellular thermotolerance.

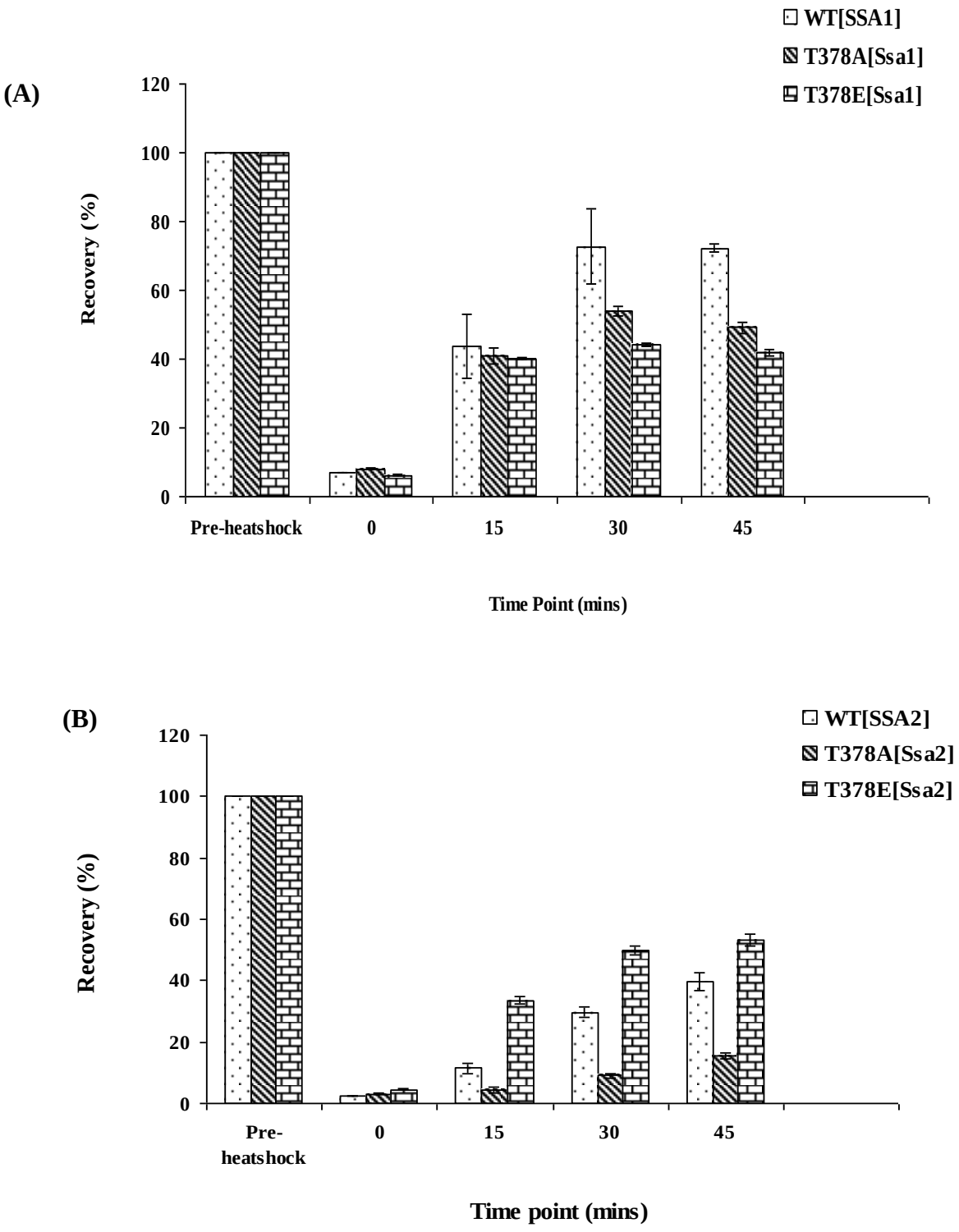
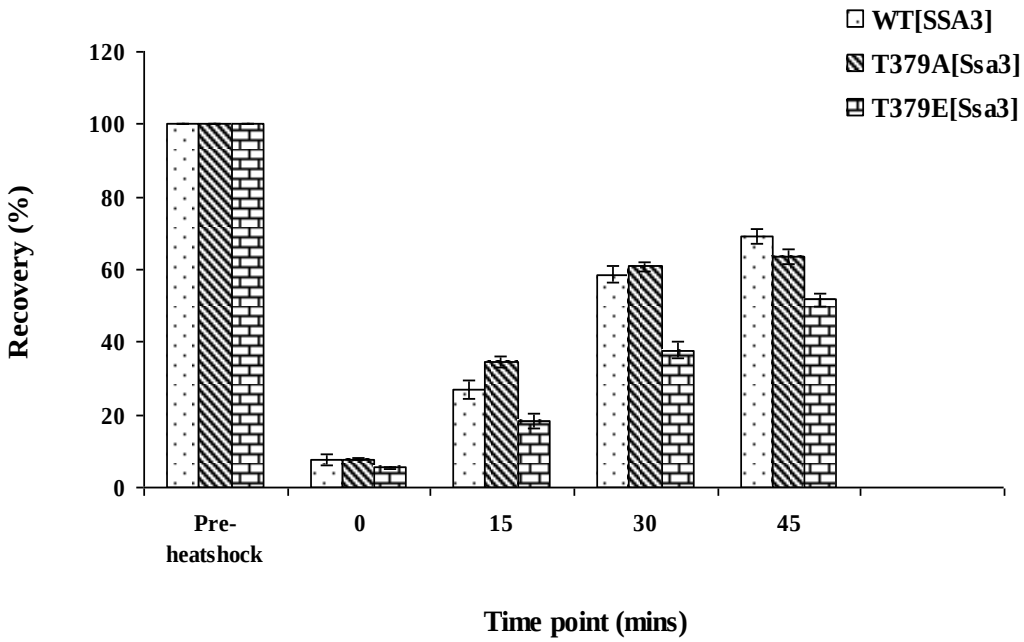



**Figure 3.33 Luciferase activity of the Ssa1 (A) and Ssa2 (B) phosphorylation mutations of Hsp70.** Overnight cultures were diluted in fresh SC medium lacking uracil to an $OD_{600nm}$ = 0.1. The cultures were then shifted to 37 °C for 30 min to induce the expression of Hsp104. After 30 min at 37 °C, the cultures were shifted to 45 °C for one hour. Cyclohexamide was added to the cultures after 50 min at 45 °C to prevent any further synthesis of luciferase during the recovery period. Luciferase activity, which is expressed as a percentage of pre-heat shock activity was measured at regular intervals during the recovery period of 45 min at 25 °C. [(A) LSD = 9.76 and (B) LSD = 7.13]

**(A)**




**(B)**

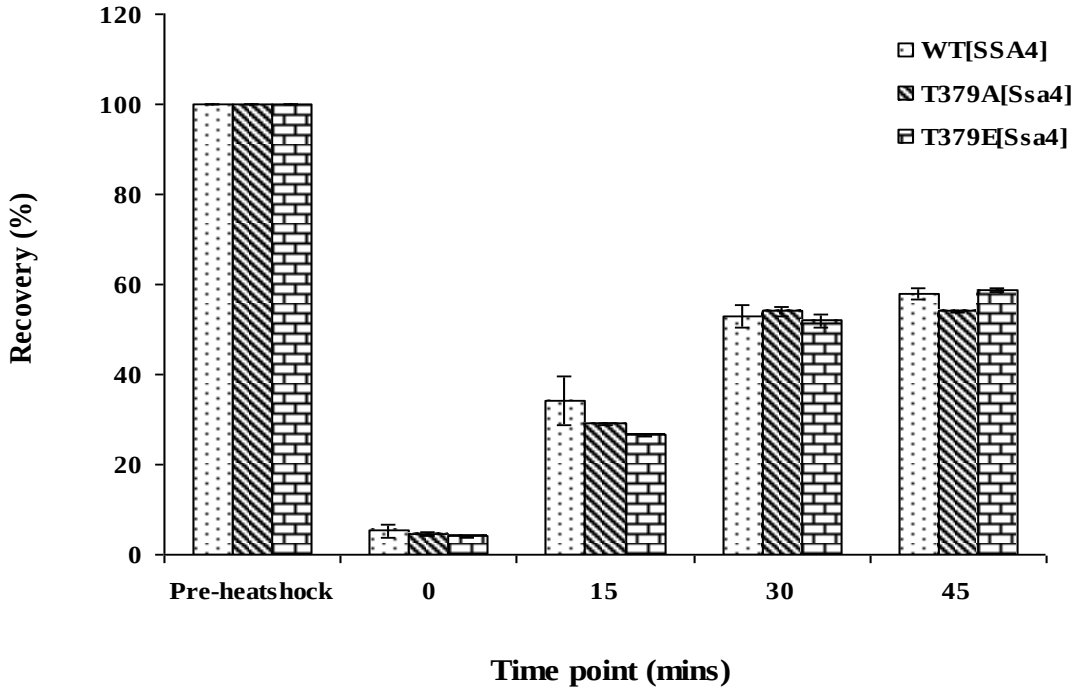



**Figure 3.34 Luciferase activity of the Ssa3 (A) and Ssa4 (B) phosphorylation mutations of Hsp70.** Overnight cultures were diluted in fresh SC medium lacking uracil to an $OD_{600nm}$ = 0.1. The cultures were then shifted to 37 °C for 30 min to induce the expression of Hsp104. After 30 min at 37 °C, the cultures were shifted to 45 °C for one hour. Cyclohexamide was added to the cultures after 50 min at 45 °C to prevent any further synthesis of luciferase during the recovery period. Luciferase activity, which is expressed as a percentage of pre-heat shock activity was measured at regular intervals during the recovery period of 45 min at 25 °C. [(A) LSD = 8.78 and (B) LSD = non-significant]

### 3.17 Chapter discussion

In this chapter, there were a total of thirty six phosphorylation mutants generated within the ATPase domain, SBD and C-terminus domain of the major cytosolic Hsp70-Ssa chaperone protein of *S. cerevisiae* and these were phenotypically assessed for their effect on [*PSI*$^{+}$] propagation, growth, temperature sensitivity, basal thermotolerance level and *in vivo* protein refolding activity. This chapter mainly focussed on a domain-targeted approach and site directed mutagenesis with the main aim to isolate an array of Hsp70 mutants and assess whether they altered the ability to propagate yeast prions. This approach shows that post-translational modifications of Hsp70 do influence Hsp70's role in [*PSI*$^{+}$] prion propagation. Phenotypic characterisation of these mutants highlights both overlapping and distinct Hsp70 functions in relation to prion propagation and other cellular activities. In addition, a new cellular role for Hsp70 in cell wall integrity signalling has been implicated.

Previous Hsp70 genetic screens have mainly isolated mutant residues that affect [*PSI*$^{+}$] propagation as being located in the ATPase domain, highlighting the importance of this domain in prion propagation (Jones & Masison, 2003; Jung et al., 2000; Loovers et al., 2007). Consistent with the previous results, this study identified several phosphomutants on the ATPase domain of cytosolic Hsp70-Ssa1 of *S. cerevisiae* that impair [*PSI*$^{+}$] propagation. One of the important ATPase domain mutants identified in this study was the T36A mutant which was seen to impair [*PSI*$^{+}$] propagation. Also the T36 residue was implicated to be an important site in context of prion propagation because most of the double mutants with T36 mutation present were seen to impair prion propagation. As most of the double mutants on the ATPase domain had an affect on prion propagation, thus it can be speculated that there might occur complex interactions between the phosphorylation sites 36, 113 and 62. Also three of the ATPase domain mutants *viz*. Ssa1 $^{T36E}$, Ssa1 $^{T36A+T113E}$, Ssa1 $^{T36E+S62E}$ and one SBD mutant Ssa1 $^{T492A}$ had a major affect on growth rate. Thus this results suggest that phosphomutants on Hsp70 that impair prion propagation may also affect other cellular functions of Hsp70 like growth rate thus highlighting overlapping and distinct functions of Hsp70 in context of prion propagation and other cellular activities. The PBD and C-terminus domain mutants did not have a major effect on [*PSI*$^{+}$] propagation and cell growth, except for alteration of the T492 residue, which affected both viability (T/E) and also cell growth and prion propagation (T/A). Thus this study reveals several new conserved phosphorylation sites of Hsp70 and highlights the significance of these sites in prion

propagation and also supports previous studies, which show the importance of ATPase domain in prion propagation. Also these results suggest that the ATPase domain mutations that impair prion propagation may directly alter the intrinsic ATPase activity or nucleotide exchange or may also alter the transition between open and closed state conformation of the ATPase cycle as impairment of this cycle has also been shown previously to impair [*PSI*$^{+}$] propagation (Needham & Masison, 2008).

Yeast can be subjected to a diverse array of environmental stimuli that include pH changes, osmotic changes, thermal changes, oxide radicals, nutrient deprivation and exposure to chemicals and yeast can survive such environmental stress by activating a variety of stress response primarily through MAP kinase cascades (Chen & Thorner, 2007). When yeast grows on hypo-osmotic solutes such as YPD, the MAP kinase CWI pathway is activated and mutants which hamper this pathway lyse in absence of osmotic stabilisers such as 1M sorbitol (Lee & Levin, 1992; Davenport et al., 1995). A number of single and double mutations within the ATPase domain were osmoremedial at 39 °C on YPD with 1M sorbitol which is indicative of the involvement of Ssa1 protein of Hsp70 in CWI pathway. Chaperones and co-chaperones that function in concert with Hsp70 are involved in the CWI signalling pathway. Protein chaperones Hsp90, Hsp70 nucleotide exchange factor (NEF) Sse1 and Hsp40 have been implicated in CWI signalling (Millson et al., 2005; Piper et al., 2006; Wright et al., 2007). Hsp90 interacts with Slt2 to activate one of the downstream transcription factor Rlm1(Millson et al., 2005). The NEF Sse1 of the cytosolic Hsp70 has been shown to work in conjugation with Hsp90 and Slt2 in the CWI signalling cascade (Shaner et al., 2008). Cells containing *ydj1* deletion also exhibit phenotypes consistent with cell wall defects and overexpression of Pkc1 enhances cell growth (Wright et al., 2007). Ssa1-Hsp70 may be indirectly involved in the CWI pathway and thus affect any of the components of the pathway or alternatively it might directly affect the pathway by regulating some of the essential components of the pathway. Previous studies have shown that Ssa1 mutants that impair [*PSI*$^{+}$] propagation do not have a significant effect on cell growth and thus the essential cellular functions of Ssa1 can be separated from its prion related functions (Jones & Masison, 2003; Jung et al., 2000; Loovers et al., 2007). However, the findings of this study did not fully correlate with the previous studies as it showed that phosphomutants on the ATPase domain that impaired prion propagation also had an impact on temperature sensitivity indicating that in some cases (mutants) Ssa's essential

cellular functions cannot be separated from its function in prion maintenance and propagation.

Further to these results, the phosphorylation mutants were also assessed for their acquired thermotolerance. Hsp104 is a key regulator in enabling cells to withstand heat shock. Under environmental stress, Hsp104-Hsp70-Hsp40 act as a protein disaggregation machinery and thus rescue the heat denatured, aggregated proteins (Glover & Lindquist, 1998). Upon induction of Hsp104 at 39 °C followed by heat shock at 47 °C, a clear growth gradient was observed for WT [Ssa1] cells on YPD. Prolonged exposure resulted in increased cell death. On subsequent exposure of the cells to 3 mM Gdn-HCl inhibited cell growth after a short period of exposure to heat shock temperature, illustrating inhibition of Hsp104 activity by GdnHCl, thus hindering thermotolerance. The ATPase mutants showed a varying degree of acquired thermotolerance in response to the heat shock. The phosphomutants Ssa1 $^{T36A}$, Ssa1 $^{S62A}$, Ssa1 $^{S62E}$, Ssa1 $^{T36E+T113A}$, Ssa1 $^{T36E+S62E}$ were similar in its cellular thermotolerance compared to WT [Ssa1], demonstrating that these mutants did not have an influence on the activity of Hsp104 in response to heat shock. However, the phophomutants Ssa1 $^{T36A+T113A}$ and Ssa1 $^{T36A+T113E}$ were reduced in their activity of acquired thermotolerance which reflects impairment in Hsp104 activity in these mutations, perhaps reflecting an inability to cooperate in the disaggregation process. Although the mutants Ssa1 $^{T36A+S62A}$, Ssa1 $^{T36A+S62E}$ and Ssa1 $^{T36E+S62A}$ showed enhanced thermotolerance compared to Ssa1 $^{T36A+T113A}$ and Ssa1 $^{T36A+T113E}$ these mutants were still defective in cellular thermotolerance compared to WT. Interestingly, the Ssa1 $^{T36E}$ and Ssa1 $^{T113E}$ mutants showed increased levels of acquired thermotolerance on YPD as well as on 3 mM Gdn-HCl. These results could reflect a more efficient Hsp70 system that is functioning with Hsp104 for enhanced thermotolerance or it can be speculated that yeast Ssa1-Hsp70 might have a greater role to play in acquired thermotolerance than has previously been thought. The involvement of Hsp70 in acquired thermotolerance has also been shown by Sanchez et al. (1993). Also Hsp70 has been shown to be required for thermotolerance in many different organisms (Atkinson & Walden, 1985; Hahn & Li, 1982; Li et al., 1991; Lindquist & Craig, 1988; Riabowol et al., 1988; Solomon et al., 1991). The phosphomutants within the SBD and the C-terminus domain did not have a prominent effect on basal thermotolerance level suggesting that Hsp104 was not impaired in its activity due to the mutations. These findings further highlight the importance of the ATPase domain of Hsp70 in regard to its various cellular functions.

We also assessed Hsp104 *in vivo* activity by measuring refolding of a model denatured protein substrate, bacterial luciferase. In theory a demonstrated reduction in acquired thermotolerance by plate assay should be reflected in a reduced ability to refold denatured luciferase. This was the case for some mutants, but not for others. All the mutants showed similar luciferase refolding *in vivo* compared to WT [Ssa1]. But results from the luciferase assay did not necessarily correlate with thermotolerance plate assay. For example Ssa1 $^{T36E}$ and Ssa1 $^{T113E}$ were behaving better than the WT [Ssa1] in showing acquired thermotolerance both on YPD and 3 mM Gdn-HCl implicating the increased thermoresistance of the cell is independent of Hsp104 regulation as 3 mM Gdn-HCl inhibits Hsp104. Also most of the phosphomutants on the ATPase domain could not tolerate heat stress for longer duration compared to WT [Ssa1] suggesting a defect in Hsp104 activity due to the mutation. But similar results were not reflected in luciferase refolding assay. Thus the results implicate that phosphorylation of Ssa1-Hsp70 at specific residues might have an effect on acquired thermotolerance, either directly or through influencing the functionality of Hsp104 during heat shock. Also previous studies have shown that Hsp104 and Hsp70 are implicated in facilitating cells to survive heat stress (Glover & Lindquist, 1998; Sanchez & Lindquist, 1990), thus these results are indicative that Hsp70 in conjunction with Hsp104 might have more important role to play then expected to help in recovery of the proteins in the cells following a thermo-stress. Further to this, the results from thermotolerance plate assay also implies that these may be a complex interaction going on between the various phosphorylation sites of Hsp70 in response to heat stress in cells that further enhances Hsp70s activity.

While investigating the effects of Hsp70 phosphorylation mutations on chaperone activity and prion propagation, it was important to look for the abundance of Hsp104 and Hsp70 in the cell to ensure that the protein expression levels are not having an impact on prion propagation and other observed chaperone activity. Thus western blot analysis was performed and the results indicated that the effects of the phosphomutants on [*PSI*$^{+}$] propagation and other chaperone activity are not influenced by changes in chaperone abundance. These results correlate with previous studies which showed that the abundance of Hsp70 or Hsp104 was not affected by mutations within the ATPase domain (Jones & Masison, 2003; Loovers et al., 2007). However, an increase in expression level was observed for one of the ATPase domain mutant, Ssa1 $^{T36E}$. Given that all expressed proteins are under the same promoter, this apparent

increase in abundance may reflect an increased inherent stability for this protein. This result may also explain the findings derived from the luciferase and thermotolerance assay which showed that the Ssa1 $^{T36E}$ mutant strain was showing improved acquired thermotolerance both on YPD and 3 mM Gdn-HCl and also showed a better heat shock recovery compared to WT. Also there was no difference in basal expression levels for Hsp70 and Hsp104 compared to WT for PBD and C-terminus domain phosphomutants. Overall, western analysis suggests that the observed phenotypes for the Ssa1 phosphomutants are not due to alteration in expression levels of Hsp70 or Hsp104.

Previous mutational, NMR and biophysical studies in *Escherichia coli* Hsp70 (DnaK) have shown the importance of the linker region between the ATPase domain and PBD in the allosteric regulation of the ATPase cycle of Hsp70 and also in inter-domain communication (Swain et al., 2007; Vogel et al., 2006). Assessment of Hsp70-Ssa family linker region identifies one potentially phosphorylatable residue, which was speculated could potentially influence inter-domain communication, depending on phosphorylation status. Thus, in this study several phosphomimetic and non-phosphorylatable mutants were generated at the linker region of yeast Hsp70 Ssa family and assessed for its effect on prion propagation and other cellular functions of Hsp70. It was observed that the [*PSI*$^{+}$] was efficiently propagated in both the Ssa1 and Ssa2 mutants *viz* Ssa1 $^{T378A}$, Ssa1 $^{T378E}$ and Ssa2 $^{T378A}$, Ssa2 $^{T378E}$ compared to their WT. For the heat inducible Ssa3 and Ssa4 mutants, it was observed that Ssa3 $^{T379A}$ and Ssa3 $^{T379E}$ could not impair the [*PSI*$^{+}$] propagation whereas the weak [*PSI*$^{+}$] status of Ssa4 could not be altered by either Ssa4 $^{T379A}$ or Ssa4 $^{T379E}$ mutants. The findings from this study imply that the phosphorylation of T378/T379 residue in linker region of the Hsp70 may be important for regulating inter-domain communication. For example the mutants generated on constitutive isoforms of Hsp70 exhibited more sensitivity at higher concentrations of $H_2O_2$ compared to their WT then their inducible counterparts.

As the stress inducible Hsp70s are distinct from their constitutively expressed counterparts in terms of their function (Sharma & Masison, 2008), there was a difference observed in the growth rate between the phosphomutants generated at the linker region between the stress inducible and constitutively expressed isoforms of Hsp70. The Ssa4 mutants had a maximum effect on growth rate, with the growth rate being reduced by 25% and 16% for the Ssa4 $^{T379A}$ and Ssa4 $^{T379E}$ mutants. Thus, these results suggest that mutations on the heat inducible isoforms of Hsp70 have a much

more significant effect on growth rate compared to its constitutively expressed isoforms.

Introducing mutations to components of the cell wall integrity (CWI) signalling pathway results in common phenotypes such as temperature sensitivity at 39 °C, sensitivity to drugs that damage the cell wall and oxidative stress response. These phenotypes can be rescued in the presence of an osmotic stabilizer such as sorbitol in growth media. The Ssa3 $^{T379E}$, Ssa4 $^{T379A}$ and Ssa4 $^{T379E}$ were temperature sensitive at 39 °C but these mutants showed recovery in presence 1M sorbitol thus it was speculated that Hsp70 might have a role to play in CWI signalling pathway. To test this hypothesis, the phosphomutants were tested for response to cell wall damaging agents and oxidative stress induced by varying concentrations of $H_2O_2$. Additionally, a difference in sensitivity was observed for the Ssa4 phosphomutants Ssa4 $^{T379A}$ and Ssa4 $^{T379E}$ in response to cell wall damaging agent SDS (0.01%) and this might implicate that the heat inducible Hsp70 might have a more significant role to play in the CWI pathway then its constitutive counterpart. However, in contrast, on 3-5 mM concentration of $H_2O_2$ the Ssa3 $^{T379A}$ mutant showed better growth compared to WT [Ssa3] whose growth was inhibited or slowed down after 3 mM concentration of $H_2O_2$. Also, the Ssa2 $^{T378E}$ mutant grew normally at 3 mM $H_2O_2$ compared to WT and started showing sensitivity only at higher concentrations of $H_2O_2$. Thus, these results are not conclusive and it can be predicted that Hsp70 may be indirectly involved in the CWI pathway and thus affect any of the components of the pathway or alternatively it might directly affect the pathway by regulating some of the essential components of the pathway.

Hsp104 is a crucial thermotolerance related protein of *S. cerevisiae*, enhancing survival at higher temperature (Sanchez & Lindquist, 1990) and this chaperone protein works in concert with Hsp70 and Hsp40 and forms a protein disaggregation machinery that rescues heat denatured proteins and thus ensures survival (Glover & Lindquist, 1998). Thus, to assess the Hsp104 activity, thermotolerance assay was performed that revealed a difference in Hsp104 protein refolding activity between the heat inducible and constitutively expressed isoforms of Hsp70. The Ssa2 $^{T378A}$ phosphomutant showed a marked sensitivity in showing acquired thermotolerance whereas Ssa3 $^{T379A}$ showed an increased thermoresistance. These results are indicative that heat inducible Hsp70 are more efficient in cooperating with Hsp104 during heat shock and have increased affinity towards heat denatured substrates than its constitutive counterparts.

The ability of chaperones to refold misfolded proteins can be measured by performing a luciferase assay. Luciferase reactivation of the phosphomutants on the constitutive isoforms of Hsp70 showed significant variations. Despite the apparent confliction of results from thermotolerance and luciferase assays, the results cannot be compared in some cases. Thermotolerance assays involve the assessment of the whole cellular response, leading to cell survival. Thus, it must be considered that a range of proteins contribute to the observed result. Conversely, luciferase assays examine the refolding activity of one protein, thus, the two assays do not equate.

Using a simplified yeast system a variety of non-phosphorylatable and phosphomimetic Hsp70 mutants were assessed systematically for phenotypic alterations in Hsp70 functions and it was found that alteration of Hsp70 phosphorylation status *in vivo* can impair prion propagation, alter both basal and acquired thermotolerance and in some cases render cells inviable. These mutants also identified a new potential role for Hsp70 in cell wall integrity signalling. Previous genetic screens have demonstrated the importance of tightly regulating the Hsp70 ATPase cycle in efficiently propagating prions. This approach identified ATPase domain and post-translational modification of Hsp70 as playing pivotal roles in prion propagation. Phenotypic characterisation of these mutants highlights both overlapping and distinct Hsp70 functions in relation to prion propagation and other cellular activities.

**Table 3.8 Result summary of the chapter**

| Mutations | Phenotypic and molecular characterisation of the phosphomutants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Prion Phenotype** | **MGT** | **Colour scale for prion phenotype** | **Ts phenotype** | **Sorbitol recovery** | **Acquired thermotolerance (ATT)** | **Luciferase refolding** | **Protein expression level** | **SDS sensitivity** | **Oxidative stress response (OSR)** |
| **ATPase domain single phosphomutants** | | | | | | | | | | |
| $Ssa1^{T36A}$ | Impaired [*PSI*$^+$] propagation | 114.5 ± 1.4 | 8 | Ts at 39 °C | Recovered on sorbitol | Similar to WT in showing ATT | Similar to WT in refolding luciferase | Equal expression | - | - |
| $Ssa1^{T36E}$ | [*PSI*$^+$] propagation similar to WT | 142.1 ± 3.5 | 2 | Ts at 39 °C | Did not recover on sorbitol | Better than WT in showing ATT | Similar to WT in refolding luciferase | Expression slightly higher than WT | - | - |
| $Ssa1^{S62A}$ | [*PSI*$^+$] propagation similar to WT | 105.3 ± 1.3 | 4 | Did not display Ts phenotype | - | Similar to WT in showing ATT | - | Equal expression | - | - |
| $Ssa1^{S62E}$ | [*PSI*$^+$] propagation similar to WT | 100.4 ± 0.7 | 4 | Did not display Ts phenotype | - | Similar to WT in showing ATT | - | Equal expression | - | - |
| $Ssa1^{T113A}$ | [*PSI*$^+$] propagation similar to WT | 100.9 ± 0.3 | 6 | Ts at 39 °C | Recovered on sorbitol | Slightly better than WT in showing ATT | Similar to WT in refolding luciferase | Equal expression | - | - |

**Table 3.8 continued……**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Ssa1$^{T113E}$ | [*PSI*$^{+}$] propagation similar to WT | 106.3 ± 0.3 | 6 | Ts at 39 °C | Recovered on sorbitol | Better than WT in showing ATT | Similar to WT in refolding luciferase | Equal expression | - | - |
| Ssa1$^{S151A}$ | [*PSI*$^{+}$] propagation similar to WT | 103.5 ± 0.8 | 3 | Did not display Ts phenotype | - | - | - | Equal expression | - | - |
| Ssa1$^{S151E}$ | [*PSI*$^{+}$] propagation similar to WT | 105.2 ± 0.3 | 5 | Slightly Ts at 39 °C | Recovered on sorbitol | - | - | Equal expression | - | - |
| **SBD single phosphomutants** | | | | | | | | | | |
| Ssa1$^{T492A}$ | [*PSI*$^{+}$] propagation similar to WT | 129.07 ± 1.7 | 4 | Ts at 37 °C and 39 °C | Did not recover on sorbitol | - | - | Equal expression | - | - |
| Ssa1$^{T492E}$ | Lethal | - | - | - | - | - | - | - | - | - |
| Ssa1$^{S535A}$ | [*PSI*$^{+}$] propagation similar to WT | 99.02 ± 2.2 | 3 | Did not display Ts phenotype | - | Similar to WT in showing ATT | - | Equal expression | - | - |
| Ssa1$^{S535E}$ | [*PSI*$^{+}$] propagation similar to WT | 86.58 ± 1.1 | 1 | Did not display Ts phenotype | - | Similar to WT in showing ATT | - | Equal expression | - | - |

**Table 3.8 continued……**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $Ssa1^{S551A}$ | [*PSI*$^+$] propagation similar to WT | 106.2 ± 0.8 | 1 | Did not display Ts phenotype | - | Similar to WT in showing ATT | - | Equal expression | - | - |
| $Ssa1^{S551E}$ | [*PSI*$^+$] propagation similar to WT | 95.7 ± 1.5 | 5 | Did not display Ts phenotype | - | Slightly defective in showing ATT | - | Equal expression | - | - |
| **C-terminus domain single phosphomutants** | | | | | | | | | | |
| $Ssa1^{S603A}$ | Impaired [*PSI*$^+$] propagation | 102 ± 0.3 | 7 | Did not display Ts phenotype | - | Similar to WT in showing ATT | - | Equal expression | - | - |
| $Ssa1^{S603E}$ | [*PSI*$^+$] propagation similar to WT | 100 ± 0.2 | 3 | Did not display Ts phenotype | - | Similar to WT in showing ATT | - | Equal expression | - | - |
| **ATPase and C-terminus domain double phosphomutants** | | | | | | | | | | |
| $Ssa1^{S151A+S603A}$ | [*PSI*$^+$] propagation similar to WT | 103 ± 1.1 | 3 | Did not display Ts phenotype | - | - | - | Equal expression | - | - |

**Table 3.8 continued……**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $Ssa1^{S151E+S603A}$ | [*PSI*$^+$] propagation similar to WT | 99 ± 0.8 | 2 | Did not display Ts phenotype | - | - | - | Equal expression | - | - |
| $Ssa1^{S151A+S603E}$ | [*PSI*$^+$] propagation similar to WT | 102±0.2 | 3 | Did not display Ts phenotype | - | - | - | Equal expression | - | - |
| $Ssa1^{S151E+S603E}$ | [*PSI*$^+$] propagation similar to WT | 98 ± 1.3 | 4 | Did not display Ts phenotype | - | - | | Equal expression | - | - |
| **ATPase domain double phosphomutants** | | | | | | | | | | |
| $Ssa1^{T36A+T113A}$ | Impaired [*PSI*$^+$] propagation | 115.1±0.7 | 9 | Ts at 39 °C | Recovered on sorbitol | Defective in showing ATT | Similar to WT in refolding luciferase | Equal expression | - | - |
| $Ssa1^{T36A+T113E}$ | Impaired [*PSI*$^+$] propagation | 251.6±8.5 | 6 | Ts at 39 °C | Did not recover on sorbitol | Defective in showing ATT | Similar to WT in refolding luciferase | Equal expression | - | - |
| $Ssa1^{T36E+T113A}$ | Impaired [*PSI*$^+$] propagation | 99.3±0.1 | 6 | Ts at 39 °C | Recovered on sorbitol | Similar to WT in showing ATT | Similar to WT in refolding luciferase | Equal expression | - | - |
| $Ssa1^{T36E+T113E}$ | Lethal | - | - | - | - | - | - | - | - | - |
| $Ssa1^{T36A+S62A}$ | Impaired [*PSI*$^+$] propagation | 111.9±1.7 | 8 | Ts at 39 °C | Recovered on sorbitol | Defective showing ATT | - | Equal expression | - | - |

**Table 3.8 continued……**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Ssa1$^{T36A+S62E}$ | Impaired [*PSI*$^{+}$] propagation | 99.4±1.7 | 7 | Ts at 39 °C | Recovered on sorbitol | Defective in showing ATT | - | Equal expression | - | - |
| Ssa1$^{T36E+S62A}$ | Impaired [*PSI*$^{+}$] propagation | 118.2±0.4 | 7 | Ts at 39 °C | Recovered on sorbitol | Defective in showing ATT | - | Equal expression | - | - |
| Ssa1$^{T36E+S62E}$ | Impaired [*PSI*$^{+}$] propagation | 141.8±2.3 | 7 | Ts at 39 °C | Did not recover on sorbitol | Similar to WT in showing ATT | - | Equal expression | - | - |

**Single phosphomutants generated on the linker region of the Ssa family**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Ssa1$^{T378A}$ | [*PSI*$^{+}$] propagation similar to WT | 103.67 ± 0.5 | 4 | Did not display Ts phenotype | - | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - | Displayed sensitivity similar to WT | Exhibited OSR similar to WT |
| Ssa1$^{T378E}$ | [*PSI*$^{+}$] propagation similar to WT | 101.76 ± 1.4 | 4 | Did not display Ts phenotype | - | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - | Displayed sensitivity similar to WT | Exhibited OSR similar to WT |
| Ssa2$^{T378A}$ | [*PSI*$^{+}$] propagation similar to WT | 100.61 ± 0.5 | 2 | Did not display Ts phenotype | - | Defective in showing ATT | Defective in refolding luciferase | - | Displayed sensitivity similar to WT | Exhibited OSR similar to WT |

**Table 3.8 continued……**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $Ssa2^{T378E}$ | [*PSI*$^+$] propagation similar to WT | 101.82 ± 0.2 | 4 | Did not display Ts phenotype | - | Similar to WT in showing ATT | Better than WT in refolding luciferase | - | Started showing sensitivity at 0.005% | Displayed sensitivity at higher concentration compared to WT |
| $Ssa3^{T379A}$ | [*PSI*$^+$] propagation similar to WT | 190.07 ± 1.9 | 3 | Did not display Ts phenotype | - | Better than WT in showing ATT | Similar to WT in refolding luciferase | - | Displayed sensitivity similar to WT | Displayed sensitivity at higher concentration compared to WT |
| $Ssa3^{T379E}$ | [*PSI*$^+$] propagation similar to WT | 218.12 ± 5.7 | 3 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - | Displayed sensitivity similar to WT | Exhibited OSR similar to WT |
| $Ssa4^{T379A}$ | Weak [*PSI*$^+$] of WT could not be altered | 143.28 ± 0.6 | 8 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - | Sensitive to 0.01% SDS | Exhibited OSR similar to WT |
| $Ssa4^{T379E}$ | Weak [*PSI*$^+$] of WT could not be altered | 132.99 ± 0.6 | 9 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - | Sensitive to 0.01% SDS | Exhibited OSR similar to WT |

# CHAPTER 4

## MOLECULAR AND PHENOTYPIC CHARACTERISATION OF SSA1 CONTAINING MULTIPLE NON-PHOSPHORYLATABLE MUTANT RESIDUES

## 4.1 Introduction

In most eukaryotic organisms, Hsp70 protein is a member of a multigene family and is expressed under a variety of physiological circumstances (Lindquist & Craig, 1988). The functional pleiotropy of Hsp70s is achieved through the evolutionary amplification and diversification of *HSP70* genes, generating Hsp70 chaperones and co-chaperones that are required for different cellular functions and co-operation of Hsp70 with other cellular systems constituting complex network of folding machinery (Mayer & Bukau, 2005). The evolutionary history of this multigene family is not well understood but the similarities are greater among genes from different species than among genes of same organism reflecting gene duplication and conservation throughout evolution (Boorstein et al., 1994) . There are 14 different kinds of Hsp70s that exist in various compartments of the yeast cell. Table 4.1 shows the conservation of the Ssa1 phosphorylation sites selected for this study across the 14 different Hsp70s in yeast. Given Hsp70s central role in a variety of important cellular metabolic pathways and the conservation of the phosphorylatable sites from yeast to humans (Figure 4.1), findings regarding post-translational control of yeast Hsp70, will aid in the characterisation of Hsp70's cellular functions in mammalian systems.

Over expression of Ssa1 under stress conditions protects [*PSI*$^{+}$] prions from the effects of over expression of Hsp104, thus ensuring prion maintenance under stress conditions (Newnam et al., 1999). This is the first published data indicating the involvement of Hsp70 in prion propagation. Shortly after this finding, the mutant Ssa1-21 (L483W) of Ssa1 was discovered which impaired [*PSI*$^{+}$] propagation (Jung et al., 2000). Since then a number of genetic screening were carried out, that identified Ssa1 that could impair [*PSI*$^{+}$] propagation (Loovers et al., 2007). Most of these prion impairing mutations were located in the regions of ATPase domain that are important for inter-domain communication (Loovers et al., 2007).

Previous studies carried out random mutagenesis screening to identify mutations in Hsp70 that impair prion propagation (Jones et al., 2004; Jones & Masison, 2003; Jung et al., 2000; Loovers et al., 2007). While much data has accrued in relation to the ATPase and substrate binding cycles of Hsp70 (primarily from assessment of the *Escherichia coli* orthologue DnaK) there is a distinct lack of information regarding the regulation of this important chaperone at the post-translational level. Recent global proteomics studies have demonstrated that *in vivo* Hsp70 is phosphorylated (Albuquerque et al., 2008). In the previous chapter, using a simplified yeast system,

systematic assessment was carried out for a variety of non-phosphorylateable and phosphomimetic Hsp70 mutants for phenotypic alterations in Hsp70 functions. It was observed that alteration of Hsp70 phosphorylation status *in vivo* can impair prion propagation, alter both basal and acquired thermotolerance and in some cases render cells inviable. Through domain targeted approach and site directed mutagenesis an array of Hsp70 mutants that are impaired in the ability to propagate prions were isolated identifying specific regions on two domains of Hsp70 and post-translational modification as playing a central role in prion propagation.

The aim of this chapter is to generate multiple non-phosphorylatable and phosphomimetic mutants on the two domains of Hsp70 and finally generate a mutant with all the phosphorylation sites made non-phosphorylatable by carrying out site directed mutagenesis. This chapter assesses the phenotypic alterations in Hsp70 brought about by these mutations and their affect on prion propagation. Another aim of this chapter was to carry out 2D-GE to look into the phosphorylation status of Hsp70 in response to heat stress.

Thus to understand the precise role of post-translational modification of Hsp70 in amyloid prevention, formation and propagation and also other cellular functions of Hsp70, it is essential to carry out genetic as well as biochemical approach to investigate the complex interactions going on between the various phosphorylation sites on the two domains of Hsp70 which has an important role to play on prion propagation as well as on the cellular functions of Hsp70.

The main objectives of this chapter were to:

- Analyse the effect of ATPase domain and SBD mutations on prion propagation and cellular functions of Hsp70.
- To investigate the complex interactions between the various phosphorylation sites of Hsp70 by generating phosphomutants with the phosphorylation sites made non-phosphorylatable and assessing its effect on prion propagation and other cellular functions of Hsp70.
- To carry out 2D-GE to check the phosphorylation status of Hsp70 and its phosphomutants in response to heat shock.

**Table 4.1 The conservation of the phosphorylation sites of Ssa1 protein across the 14 different Hsp70s in yeast.** * Indicates conserved site.

| Cellular Compartment | Hsp70s | Phosphorylation sites on Ssa1 protein | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **T36** | **S62** | **T113** | **S151** | **T492** | **S535** | **S551** | **S603** |
| Cytosolic | **SSA1** | * | * | * | * | * | * | * | * |
| Cytosolic | **SSA2** | * | - | * | * | * | * | * | * |
| Cytosolic | **SSA3** | * | - | * | * | * | - | - | - |
| Cytosolic | **SSA4** | * | - | * | * | * | - | * | * |
| Cytosolic | **SSE1** | * | - | - | - | * | - | - | - |
| Cytosolic | **SSE2** | * | - | - | - | * | - | - | - |
| Ribosomal | **SSB1** | * | - | - | - | * | - | - | - |
| Ribosomal | **SSB2** | * | - | - | - | * | - | - | - |
| Ribosomal | **SSZ1** | - | - | * | - | * | - | - | - |
| Mitochondrial | **SSC1** | * | - | - | * | * | - | - | - |
| Mitochondrial | **ECM10** | * | - | - | - | - | - | - | - |
| Mitochondrial | **SSQ1** | * | - | - | * | - | - | - | - |
| ER-associated | **KAR2** | * | - | * | - | * | * | - | * |
| ER-associated | **LSH1** | - | - | - | - | - | - | - | - |

```
Human Hsp70: 6   AIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVALNPQN 65
                 A+GIDLGTTYSCV  F + +V+IIANDQGNRTTPS+VAFTDTERLIGDAAKNQ A+NP N
Yeast Hsp70: 4   AVGIDLGTTYSCVAHFANDRVDIIANDQGNRTIPSFVAFTDTERLIGDAAKNQAAMNPSN 63

Human Hsp70: 66  TVFDAKRLIGRKFGDPVVQSDMKHWPFQVINDGDKPKVQVSYKGETKAFYPEEISSMVLT 125
                 TVFDAKRLIGR F DP VQ+DMKH+PF++I+   KP++QV +KGETK F PE+ISSMVL
Yeast Hsp70: 64  TVFDAKRLIGRNFNDPEVQADMKHFPFKLIDVDGKPQIQVEFKGETKNFTPEQISSMVLG 123

Human Hsp70: 126 KMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGL 185
                 KMKE AE+YLG  V +AV+TVPAYFNDSQRQATKDAG IAGLNVLRIINEPTAAAIAYGL
Yeast Hsp70: 124 KMKETAESYLGAKVNDAVVTVPAYFNDSQRQATKDAGTIAGLNVLRIINEPTAAAIAYGL 183

Human Hsp70: 186 DRTGKGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNRLVNHFVEEF 245
                 D+ GK E +VLIFDLGGGTFDVS+L+I+DGIFEVKATAGDTHLGGEDFDNRLVNHF++EF
Yeast Hsp70: 184 DKKGK-EEHVLIFDLGGGTFDVSLLSIEDGIFEVKATAGDTHLGGEDFDNRLVNHFIQEF 242

Human Hsp70: 246 KRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSITRARFEEL 305
                 KRK+KKD+S N+RA+RRLRTACERAKRTLSSS Q S+EIDSLFEGIDFYTSITRARFEEL
Yeast Hsp70: 243 KRKNKKDLSTNQRALRRLRTACERAKRTLSSSAQTSVEIDSLFEGIDFYTSITRARFEEL 302

Human Hsp70: 306 CSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPKVQKLLQDFFNGRDLNKSINP 365
                 C+DLFRSTL+PVEK LRDAKLDK+Q+ ++VLVGGSTRIPKVQKL+ D+FNG++ N+SINP
Yeast Hsp70: 303 CADLFRSTLDPVEKVLRDAKLDKSQVDEIVLVGGSTRIPKVQKLVTDYFNGKEPNRSINP 362

Human Hsp70: 366 DEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLETAGGVMTALIKRNSTIPTKQT 425
                 DEAVAYGAAVQAAIL GD+S   QDLLLLDVAPLSLG+ETAGGVMT LI RNSTIPTK++
Yeast Hsp70: 363 DEAVAYGAAVQAAILTGDESSKTQDLLLLDVAPLSLGIETAGGVMTKLIPRNSTIPTKKS 422

Human Hsp70: 426 QIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGRFELSGIPPAPRGVPQIEVTFDIDANGI 485
                 +IF+TY+DNQPGVLIQV+EGERA TKDNNLLG+FELSGIPPAPRGVPQIEVTFD+D+NGI
Yeast Hsp70: 423 EIFSTYADNQPGVLIQVFEGERAKTKDNNLLGKFELSGIPPAPRGVPQIEVTFDVDSNGI 482

Human Hsp70: 486 LNVTATDKSTGKANKITITNDKGRLSKEEIERMVQEAEKYKAEDEVQRERVSAKNALESY 545
                 LNV+A +K TGK+NKITITNDKGRLSKE+IE+MV EAEK+K EDE + +R+++KN LES
Yeast Hsp70: 483 LNVSAVEKGTGKSNKITITNDKGRLSKEDIEKMVAEAEKFKEEDEKESQRIASKNQLESI 542

Human Hsp70: 546 AFNMKSAVEDEGLKGKISEADKKKVLDKCQEVISWLDANTLAEKDEFEHKRKELEQVCNP 605
                 A+++K+ + + G   K+ +ADK  V  K +E ISWLD+NT A K+EF+ K KEL+ + NP
Yeast Hsp70: 543 AYSLKNTISEAG--DKLEQADKDTVTKKAEETISWLDSNTTASKEEFDDKLKELQDIANP 600

Human Hsp70: 606 IISGLYQGAGGPG------PGGFGAQGPKGGSGSGPTIEEV 640
                 I+S LYQ  G PG      PGGF    P      GPT+EEV
Yeast Hsp70: 601 IMSKLYQAGGAPGGAAGGAPGGFPGGAPPAPEAEGPTVEEV 641
```

**Figure 4.1 The alignment of yeast Hsp70 (Ssa1) and human Hsp70 1A/1B (NP_005337.2) shows their remarkable evolutionary conservation.** The sights highlighted in red are the phosphorylations sites of Hsp70 taken in this study that are conserved both in humans and in yeast. (This figure was made using *Saccharomyces* Genome Database)

### 4.2 Hsp70 structural analysis

Hsp70s mediate protein folding, translocation and macromolecular complex remodeling reactions. These biochemical activities of Hsp70 are regulated by exchange of ADP for ATP from the nucleotide binding domain (NBD). Thus, the crystal structures of the two domains of Hsp70 provide a detailed biochemical understanding of chaperone activity of Hsp70 (Figure 4.2). One of the first crystal structure of NBD of Hsp70 was from bovine Hsc70 (bHsc70) provided by Flaherty et al. (1990). It consists of four subdomains (IA, IB, IIA, and IIB) made up of two lobes, which are structurally similar. The deep cleft between the lobes provide binding sites for adenosine nucleotides that makes contacts with all the four subdomains (Flaherty et al., 1990). The *E. coli* Hsp70 called DnaK provided one of the early prototype SBD crystal structure (Zhu et al., 1996). It has a compact β sandwich subdomain (SBDβ) followed by an extended α-helical subdomain (SBDα). A substrate peptide is bound to DnaK in an extended conformation through a channel defined by loops from the β subdomain and covered by the α -helical subdomain.

All the phosphomutants were modelled onto the 3D crystal structure using the programme PyMOL molecular graphic system (DeLano, 2002). This work has been done in collaboration with Professor Youtao Song (Liaoning University, Shenyang, China). The models created are based on the 3D crystal structure of bovine Hsc70 ATPase domain (Accession no.: 2BUP) and *E. coli* Hsp70 DnaK (Accession no.: 2KHO) obtained from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The Ssa1 phosphomutants are well dispersed across the structure of the bovine Hsc70 ATPase domain (Figure 4.3 A) and *E. coli* Hsp70 DnaK (Figure 4.3 B) and are not clustered together. This analysis revealed that the phosphomutations may be located at important sites of Hsp70 interaction points and also at sites for interdomain communication. The movement of protein or "protein dynamics" aids protein functionality. Phosphorylation can have an impact on protein dynamics (Wales & Engen, 2006). The multiple mutations generated on the two domains of Hsp70 may cause alterations to protein by disrupting intramolecular interactions. These mutations can also cause loss of intramolecular bonds and interactions formed normally in a wild type Hsp70. Mutations can also disrupt amino acid polarity, charge and hydrophobicity, which may ultimately lead alteration of protein function. Phosphorylation sites within Hsp70 are not localised but are well distributed throughout the protein.

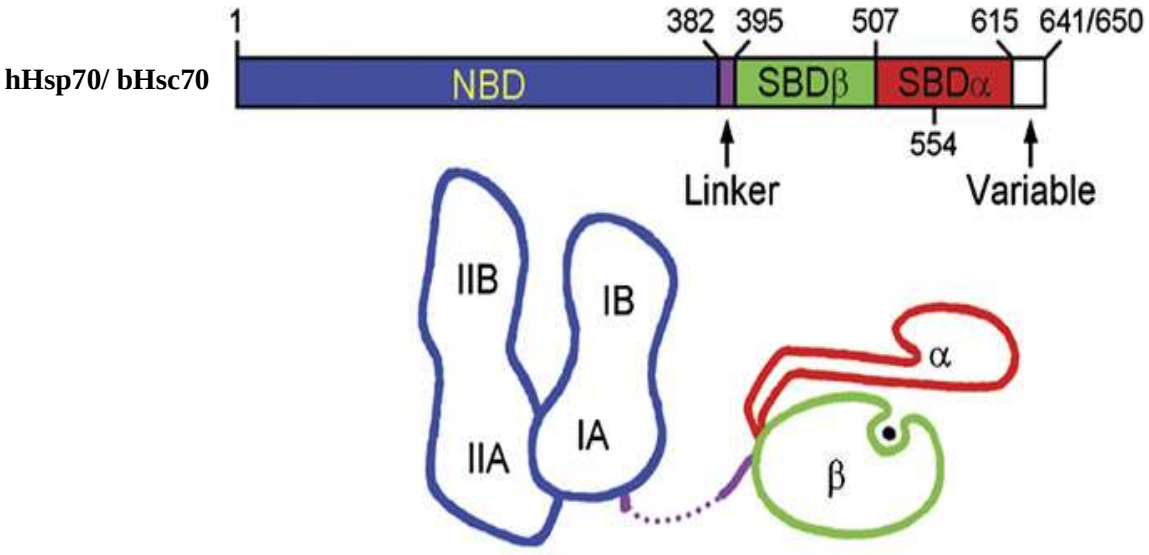
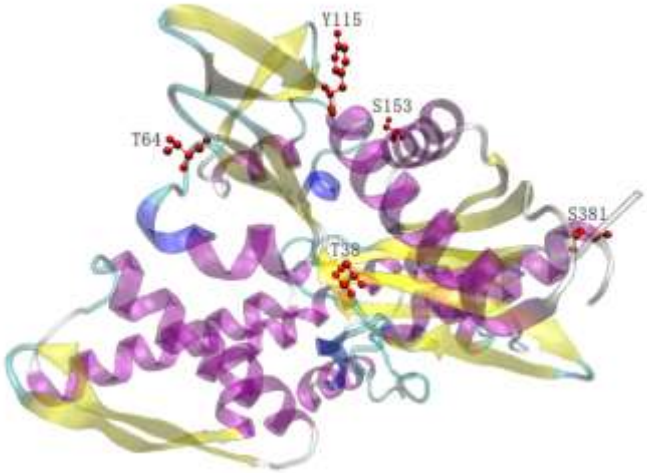


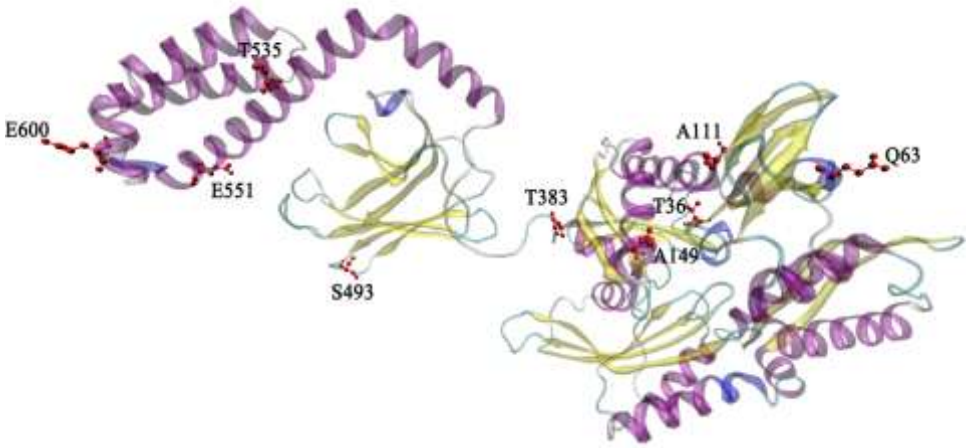

**Figure 4.2 Schematic representation of Hsp70 sequence and prototypic domain structure.** Figure adapted from Liu & Hendrickson (2007). The colour code is NBD (blue), interdomain linker (purple), SBDβ (green), SBDα (red).

## 4.3 Generation of domain specific multiple non-phosphorylatable mutants that effect prion propagation and Hsp70 function

The previous chapter mainly focussed on the generation of an array of phosphomimetic and non-phosphorylatable mutants within the three domain of Hsp70 and these mutants were assessed for their effects on prion propagation and other cellular functions of Hsp70. To further gain an in depth understanding of effect of Hsp70 phosphorylation on prion propagation and other cellular functions a domain targeted approach was carried out to investigate the effect of making all the phosphorylation sites of the ATPase domain and SBD non-phosphorylatable (Table 4.2) by replacing the serines and threonines at specific sites with alanine (non-phosphorylatable) .




**(A)**




**(B)**

**Figure 4.3 Yeast Hsp70 phosphorylation mutations model developed on the crystal structure of the two prototypes (A) bovine Hsc70 NBD and (B) *E. coli* DnaK.** The phosphomutants are modelled onto the 3D crystal structure using the programme PyMOL molecular graphic system. The models created are based on the 3D crystal structure of bovine Hsc70 ATPase domain (A) and *E. coli* Hsp70 DnaK (B) obtained from Protein Data Bank.

**Table 4.2 Phosphomutations generated within the two domains of Hsp70.** SDM was carried out to generate the phospho-mutations within the ATPase and SBD domain of Ssa1 protein. The table shows the nucleotides that were changed by SDM to get alanine (non-phosphorylatable) at the required position.

| Domain | Mutation | Nucleotide Position Changed | Nucleotide Changed | Codon Changed | Amino Acid Changed |
|---|---|---|---|---|---|
| ATPase domain mutant | T36A+S62A+T113A+S151A | 106 + 184 + 337 + 451 | (A ⇨ T) + (T ⇨ G) + (A ⇨ G) + (T ⇨ G) | 36 + 62 + 113 + 535 +151 | (T ⇨ A) + (S ⇨ A) + (T ⇨ A) + (S ⇨ A) |
| SBD mutants | T492A+S535A | 1474+ 1603 | (A ⇨ G) + ( T ⇨ G) | 492 + 535 | (T ⇨ A) + (S ⇨ A) |
| | T492A+S535A+S551A | 1474 + 1603 + 1651 | (A ⇨ G) + ( T ⇨ G) + (T ⇨ G) | 492 + 535 + 551 | (T ⇨ A) + (S ⇨ A) + (S ⇨ A) |
| | T492A+S535A+S551A+S603A | 1474 + 1603 +1651 + 1807 | (T ⇨ G) + (T ⇨ G) + (T ⇨ G) + ( T ⇨ G) | 492 + 535 551 + 603 | (T ⇨ A) + (S ⇨ A) + (S ⇨ A) + (S ⇨ A) |

## 4.4 Phenotypic assessment of mutated ATPase domain and SBD on prion propagation

Previous genetic studies had identified the ATPase domain of Hsp70 as playing an important role in prion propagation (Jones & Masison, 2003). The results from the previous chapter also showed that phosphorylation mutations on the ATPase domain of Ssa1 have an effect on prion propagation. To further investigate the effect of mutating the phosphorylation sites on the ATPase domain and SBD into non-phosphorylatable sites, SDM (as mentioned in Section 2.8) was carried out followed by plasmid shuffle technique (Section 2.12) and then the mutated domains of Hsp70 was phenotypically characterised based on their ability to propagate and maintain [*PSI*$^{+}$]. When the ATPase domain mutant Ssa1 $^{T36A+S62A+T113A+S151A}$ was expressed as the sole source of Ssa1 in yeast cell, the [*PSI*$^{+}$] propagation was impaired as seen by the colour on YPD plates and growth on medium lacking adenine (Figure 4.4). Contrary to this, the SBD phosphomutants did not have an effect on [*PSI*$^{+}$] propagation as seen by growth and pigmentation on -ade plates and YPD plates (Figure 4.4).

## 4.5 Investigation of the ATPase domain and SBD phosphomutations on yeast cell growth and prion propagation

The time required for a yeast cell to double is the mean generation time. The phosphomutants within the two domains of Hsp70 were characterised based on the growth rate. A substantial difference in growth rate of yeast cell due to the mutations is suggestive of an effect of these mutations on important cellular functions of Hsp70.

Although the ATPase and SBD mutants showed significant difference in growth rate of yeast cell ($p<0.05$) compared to WT, this difference may be less relevant when considered from biological point of view. The difference in growth rate was much more pronounced for Ssa1 $^{T492A+S535A+S551A}$ that showed a 27% slower growth compared to WT (Table 4.3).The prion phenotype of the phosphomutants on both the domains of Hsp70 was also assessed based on the Image J-java based imaging program (as described in Section 2.12) (Table 4.3). The ATPase domain phosphomutant had a major effect on [*PSI*$^{+}$] propagation compared to WT and the SBD phosphomutants (Table 4.3).

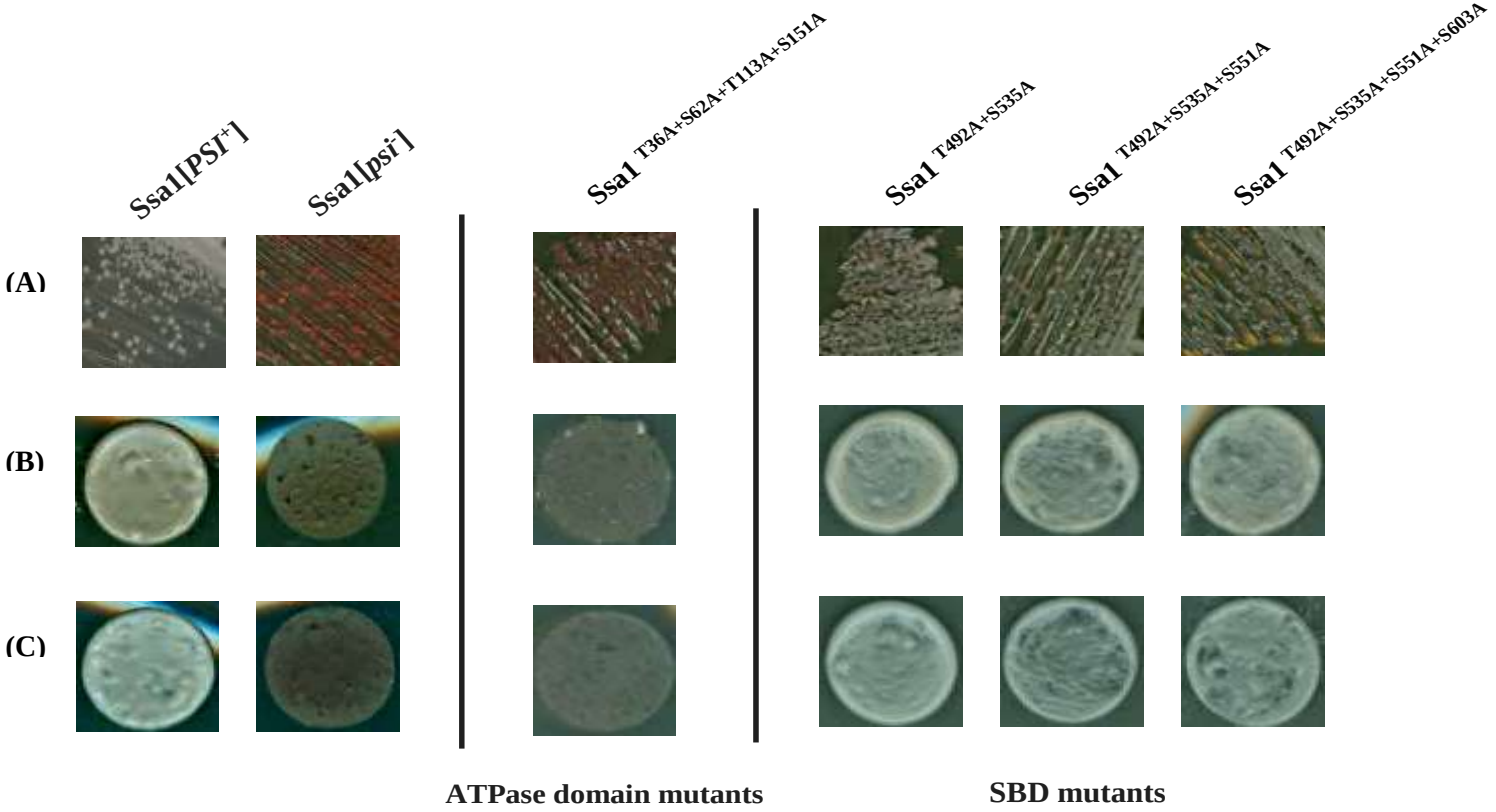



**Figure 4.4 Effect of ATPase domain and SBD multiple phosphomutants on [*PSI* $^{+}$] phenotype.** Cells were streaked on YPD and colonies appeared after 2 days of incubation at 30 °C followed by 2 days at 4 °C (A). 20 µl spots were placed on −ade plates and incubated at 30 °C for 3 days (B) and at RT for 3 days (C). Ssa1 [*PSI*$^{+}$] is the WT; Ssa1 [*PSI*$^{+}$] and Ssa1 [*psi*$^{-}$] are taken as controls; Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text) . The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates.

**Table 4.3 Relative effects of the ATPase domain and SBD phosphomutations on prion propagation and cell growth**

| Domain | Phosphomutants | ±MGT (min) | Colour in G402 |
|---|---|---|---|
| | WT [Ssa1] | *105.63 ± 0.3 | 0 |
| ATPase domain mutant | Ssa1 $^{T36A+S62A+T113A+S151A}$ | *113.69 ± 1.2 | 9 |
| SBD mutants | Ssa1 $^{T492A+S535A}$ | *108.68 ± 1.1 | 4 |
| | Ssa1 $^{T492A+S535A+S551A}$ | *134.86 ± 0.6 | 4 |
| | Ssa1 $^{T492A+S535A+S551A+S603A}$ | *117.40 ± 0.5 | 4 |

±The mean generation time of the yeast strains was determined by diluting the overnight 30 °C culture in 30 ml fresh media to an $OD_{600nm}$ of 0.1(t = 0 min). Cultures were transferred to 30 °C shaking incubator and cell density was measured at an interval of every 2 h as described in Section 2.14. The table also numerically categorizes the phosphomutants according to their colour in G402, zero representing white Ssa1 [$PSI^+$] and ten representing red *SSA1* [$psi^-$] based on Image J-java based image processing program. (* indicate significant difference between WT [Ssa1] and its phosphomutants).

## 4.6 Temperature sensitivity testing of the ATPase domain and SBD mutants by comparative growth analysis

The findings from Chapter 3 revealed the importance of some ATPase domain single and double phosphomutants on cellular thermostability as well as on prion propagation. As Ssa1 is a heat shock protein and an important cellular chaperone, one of the objectives of this chapter was to characterise the multiple phosphomutants in terms of their cellular thermostability. Comparative growth analysis was carried out on the phosphomutants as described in Section 2.15 and the plates were then incubated at 30 °C, 37 °C and 39 °C for 3 days. The growth of the phosphomuatnts was then monitored over time at the three different temperatures. After 3 days it was evident that the ATPase domain mutant was temperature sensitive at 39 °C (Figure 4.5). For the SBD mutants, except for the Ssa1 $^{T492A+S535A}$ mutant, showed temperature sensitivity at 37 °C and 39 °C (Figure 4.5).

The results from this experiment are indicative of the importance of the ATPase domain and SBD of the Ssa1 protein in protecting the cell against extreme environmental temperatures. The protein stability and folding under heat shock

conditions can be affected by these mutations on the two domains. Given the structural analysis data (Figure 4.3) it might be speculated that these mutations may have an affect on protein dynamics of Hsp70 thus hampering the Hsp70 protein function.

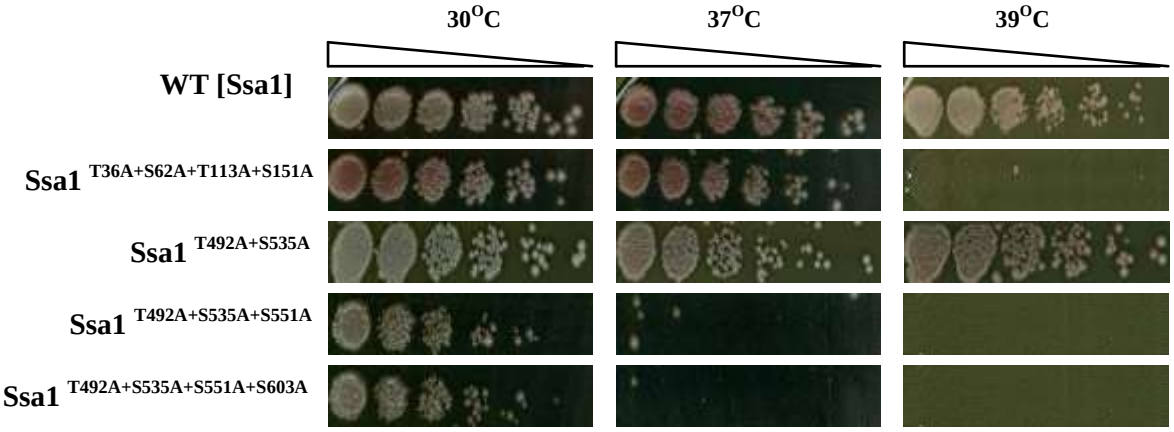



**Figure 4.5 Comparative growth analysis of phosphorylation mutants of the ATPase domain and SBD on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then re-suspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

## 4.7 High osmotic medium could rescue the temperature sensitive phenotype of ATPase domain phosphomutants

In Chapter 3 it was established that some of the temperature sensitive mutants could be recovered on 1M sorbitol thus implicating that the cell wall becomes permeable at higher temperature due to alteration of CWI pathway or some other signalling pathway resulting in a lethal phenotype on hypotonic medium such as YPD. But this lethal phenotype could be recovered in presence of osmotic stabilisers such as 1M sorbitol. Therefore it was tested whether this was also the case for multiple phosphomutants. As described in previous sections, a comparative growth analysis was performed by making lysates from yeast cultures expressing ATPase domain and SBD phosphomutants that were grown at 30 °C, 37 °C and 39 °C for 3 days in YPD supplemented with 1M sorbitol. The ATPase domain mutant Ssa1 $^{T36A+S62A+T113A+S151A}$ that was temperature sensitive at 39 °C (Figure 4.5) did recover on 1M sorbitol (Figure 4.6). But the SBD mutants did not show recovery on 1M sorbitol (Figure 4.6).

The recovery of the ATPase domain mutant on 1M sorbitol is indicative of defect in CWI pathway due to the multiple mutations on the ATPase domain. But as the

SBD mutants did not recover on 1M sorbitol, these results are suggestive of possible structural or functional defects in the protein due the multiple mutations generated within the SBD.

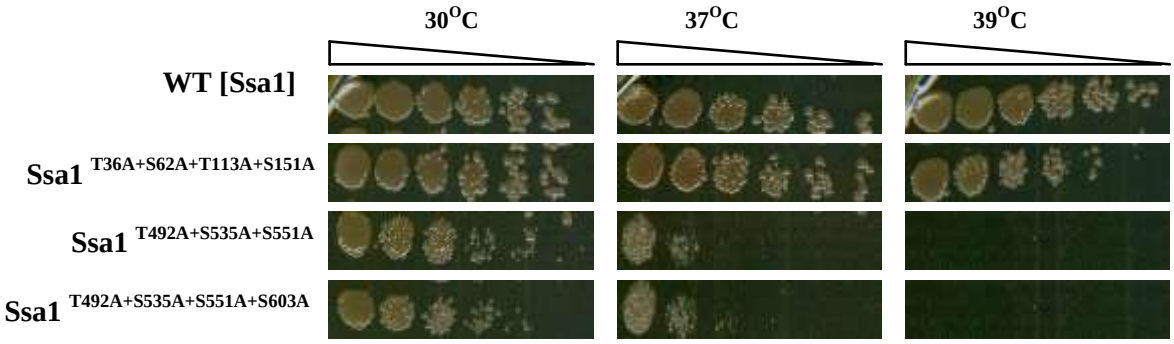



**Figure 4.6 Comparative growth analysis of phosphorylation mutants of the ATPase domain and SBD on 1M sorbitol plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then re-suspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for recovery.

## 4.8 Basal levels of Hsp70 and Hsp104 in yeast cells expressing multiple phosphomutants within the ATPase domain and SBD

Mutations within Ssa1 may affect the basal expression levels of Ssa1 protein or influence the action of other chaperone proteins like Hsp104. [$PSI^+$] propagation can also be affected by the expression levels of the molecular chaperones (Chernoff et al., 1995; Kushnirov et al., 2000).

Western blot analysis was performed to assess the levels of Hsp70 and Hsp104 in yeast that harboured phosphomutations within the ATPase domain and SBD of Ssa1. As described in Section 2.19, 10 µg of total protein was loaded onto polyacrylamide gels and the mutants were probed with antibodies for Hsp70 and Hsp104. There were no obvious differences observed in the abundance of Hsp70 and Hsp104 (Figure 4.7). This suggests that the effect of Ssa1 mutants on [$PSI^+$] propagation is not due to alterations in cellular chaperone levels. The results from western blot analysis are consistent with previous results which showed that expression levels of Hsp70 and Hsp104 are not affected by mutations on the ATPase domain of Hsp70 (Jones & Masison, 2003; Loovers et al., 2007).

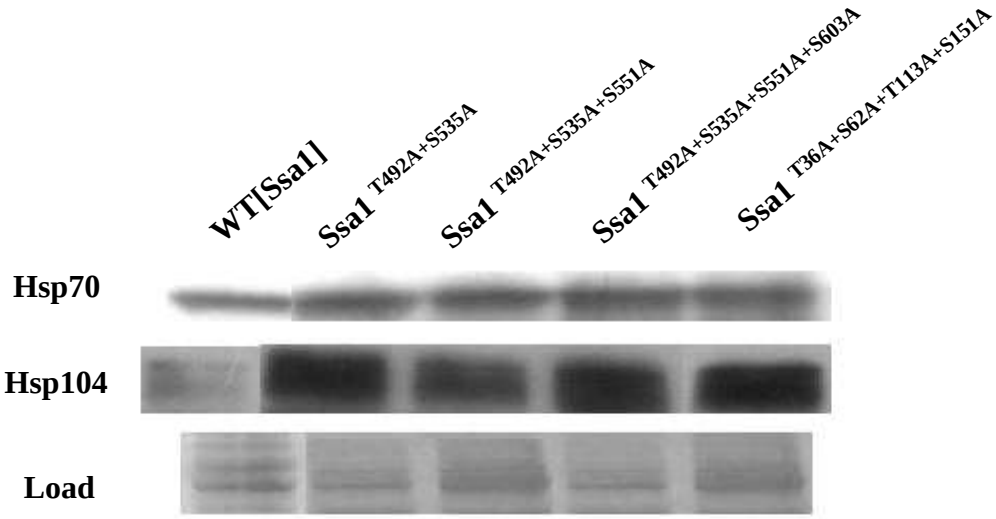



**Figure 4.7 Relative abundance of Hsp70 and Hsp104 in yeast cells expressing the multiple phosphorylation mutations on the ATPase domain and SBD.** Western blot analysis was performed to examine the abundance of Hsp70 and Hsp104. Blots probed with anti-Hsp70 antibodies were stripped and re-probed with anti-Hsp104 antibodies. Membrane was then stained by amido black as a loading and transfer control (Load). The phosphomutants along with the WT are indicated on the top.

### 4.9 Creating multiple phosphorylation mutations for assessing effect on yeast prion propagation and cellular activity of Hsp70

Based on the global proteomic analysis of *S. cerevisiae* (Albuquerque et al., 2008), the previous chapter focussed on nine phosphorylation sites of the Ssa1 protein of Hsp70 and mutants were generated at these sites and assessed for their effect on prion propagation and other cellular functions of Hsp70. This approach highlighted the importance of ATPase domain and post-translational modifications of Hsp70 as playing an important role in prion propagation and other cellular activities of Hsp70. Thus, one of the objectives of this chapter was to generate multiple phosphorylation mutants and finally to get a mutant with maximum phosphorylation sites made non-phosphorylatable within Ssa1 and then assess the effect on prion propagation and chaperone activity of Hsp70. The multiple phosphorylation mutations were generated by introducing subsequent mutations on the Ssa1 protein by carrying out site directed mutagenesis described in Section 2.8 (Table 4.4).

## 4.10 Analysis of the effects of multiple phosphomutants of Ssa1 on [*PSI*$^+$] propagation and yeast cell growth

[*PSI*$^+$] phenotypes were assessed in the multiple phosphomutants of Ssa1 by their colour on adenine limiting YPD medium and their ability to grow on adenine lacking medium (as described in Section 2.13). The varying degree of colour intensity of the Ssa1 multiple phosphomutants revealed the strength of prion propagation in the mutant yeast strain (Figure 4.8) after 2 days of incubation at 30 °C followed by 2 days at 4 °C. All the mutants that did survive, had weaker [*PSI*$^+$] phenotypes compared to WT as they were either pink or dark pink in colour. This indicates that multiple mutations generated at several phosphorylation sites did have an effect on the ability of [*PSI*$^+$] propagation.

The WT grew well on medium lacking adenine both at 30 °C and at RT when monitored after 3 days of incubation. The Ssa1 multiple phosphomutants showed varying degrees of growth in medium lacking adenine both at 30 °C and RT compared to WT (Figure 4.8 B and C) indicating impairment of prion propagation due to the mutations. The most pronounced affect on prion propagation was observed for the multiple phosphomutant with the maximum sites made non – phosphorylatable as seen by the weak growth on medium lacking adenine both at 30 °C and RT (Figure 4.8 B and C). These multiple phosphorylation mutations also had an effect on growth as some of the mutations viz. Ssa1 $^{T36A+S62A+T113A+T492A}$, Ssa1 $^{T36E+S62A+T113E}$, Ssa1 $^{T36E+S62E+T113E}$ were lethal and the yeast strain bearing the mutation was not able to survive (Figure 4.8).

In order to further characterise the Ssa1 multiple phosphomutants they were quantitatively scored based on the Image-J Java based image processing program by their red or white colour (Table 4.5). This involves assigning each mutant a colour score based on their weak or strong [*PSI*$^+$] phenotype. This helped to confirm whether any of the mutants had a dominant effect over WT. The [*PSI*$^+$] cells that were white were scored as zero and the [*psi*$^-$] cells that were dark red was scored as ten. Any colour between white and red was scored quantitatively between 0-10 scale. The Table 4.5 summarises the results of these assessments.

**Table 4.4 Multiple Phosphomutations generated on Hsp70.** SDM was carried out to generate the multiple phosphomutations on Ssa1 protein of Hsp70. The table shows the nucleotides that were changed by SDM to get either alanine (non-phosphorylatable) or glutamic acid (phosphomimetic) at the required position.

| Mutation | Nucleotide Position Changed | Nucleotide Changed | Codon Changed | Amino Acid Changed |
|---|---|---|---|---|
| T36A+S62A+ T113A | 106 + 184 + 337 | (A ⇨ T) + (T ⇨ G) + (A ⇨ G) | 36 + 62 + 113 | (T ⇨ A) + (S ⇨ A) +( T ⇨ A) |
| T36A+S62A +T113A+S535A | 106 + 184 + 337 + 1603 | (A ⇨ T) + (T ⇨ G) + (A ⇨ G) + ( T ⇨ G) | 36 + 62 + 113 + 535 | (T ⇨ A) + (S ⇨ A) + (T ⇨ A) + (S ⇨ A) |
| T36A+S62A+T113A+S535A+S551A | 106 + 184 + 337 + 1603+ 1651 | (A ⇨ T) + (T ⇨ G) +(A ⇨ G) + ( T ⇨ G) + (T ⇨ G) | 36 + 62 + 113 + 535 +551 | (T ⇨ A) + (S ⇨ A) + (T ⇨ A) + (S ⇨ A) + (S ⇨ A) |
| T36A+S62A+T113A+S151A+S535A+S551A+ S603A | 106 + 184 + 337 + 451+ 1603+ 1651 + 1807 | (A ⇨ T) + (T ⇨ G) +(A ⇨ G) +(T ⇨ G) + ( T ⇨ G) + (T ⇨ G) + ( T ⇨G) | 36 + 62 + 113 + 151 + 535 +551 + 603 | (T ⇨ A) + (S ⇨ A) + (T ⇨ A) + (S ⇨ A) + (S ⇨ A) + (S ⇨ A) + (S ⇨A) |
| T36A+S62A +T113A+T492A | 106 +184 + 337 + 1474 | (A ⇨ T) + (T ⇨ G) + (A ⇨ G) + (A ⇨ G) | 36 + 62 113 + 492 | (T ⇨ A) + (S ⇨ A) + (T ⇨ A) + (T ⇨ A) |
| S62A+S535A+S551A | 184 + 1603 + 1651 | (T ⇨ G) + (T ⇨ G) + (T ⇨ G) | 62 + 535 + 551 | (S ⇨ A) + (S ⇨ A) + (S ⇨ A) |
| T36E+ S62A+T113E | (106,107,108) + (184) +( 337, 338, 339) | ( ACT ⇨ GAG) + (T ⇨ G) + ( ACC ⇨ GAG) | 36 + 62 + 113 | (T ⇨ E) + (S ⇨ A) + (T ⇨ E) |
| T36E+S62E+T113E | (106,107,108) + (184, 185) + (337, 338, 339) | ( ACT ⇨ GAG) + ( TC ⇨ GA) + ( ACC ⇨ GAG) | 36 + 62 + 113 | (T ⇨ E) +(S ⇨ E) +(T ⇨ E ) |

The Ssa1 phosphomutants showed varying degree of prion impairment as most of the mutants scored 8 indicating that they have a strong ability to weaken [*PSI*$^{+}$] propagation. The mutant Ssa1 $^{S62A+S535A+S551A}$ scored 7 suggesting that in comparison to the other Ssa1 multiple phosphomutants, this mutant had a slightly strong [*PSI*$^{+}$] phenotype.

It was observed that the multiple phosphorylation mutations of Ssa1 protein of Hsp70 chaperone had a significant affect on growth rate of yeast ($p<0.05$) but these differences in growth rate was not of particular importance and is less relevant in terms of biological impact (Table 4.5).

**Table 4.5 Relative effects of Ssa1 multiple phosphorylation mutations on prion propagation and cell growth.**

| **Phosphomutants** | **±MGT (min)** | **Colour in G402** |
|---|---|---|
| WT [Ssa1] | *105.63 ± 0.3 | 0 |
| Ssa1 $^{T36A+S62A+T113A}$ | *117.26 ± 1.8 | 8 |
| Ssa1 $^{T36A+S62A+T113A+S535A}$ | *117.84 ± 0.4 | 8 |
| Ssa1 $^{T36A+S62A+T113A+S535+S551A}$ | *119.33 ± 1.6 | 8 |
| Ssa1 $^{T36A+S62A+T113A+S535+S551A+S151A+S603A}$ | *110.81 ± 0.6 | 9 |
| Ssa1 $^{S62A+S535A+S551A}$ | 97.16 ± 1.2 | 7 |

±The mean generation time of the yeast strains were determined by diluting the overnight 30 °C culture in 30 ml fresh media to an $OD_{600nm}$ of 0.1(t = 0 min). Cultures were transferred to 30 °C shaking incubator and cell density was measured at an interval of every 2 h as described in Section 2.14. The table also numerically categorizes the phophomutants according to their colour in G402, zero representing white Ssa1 [*PSI*$^{+}$] and ten representing red Ssa1[*psi*$^{-}$] based on Image J-java based image processing program. (* indicate significant difference between WT [Ssa1] and its phosphomutants).

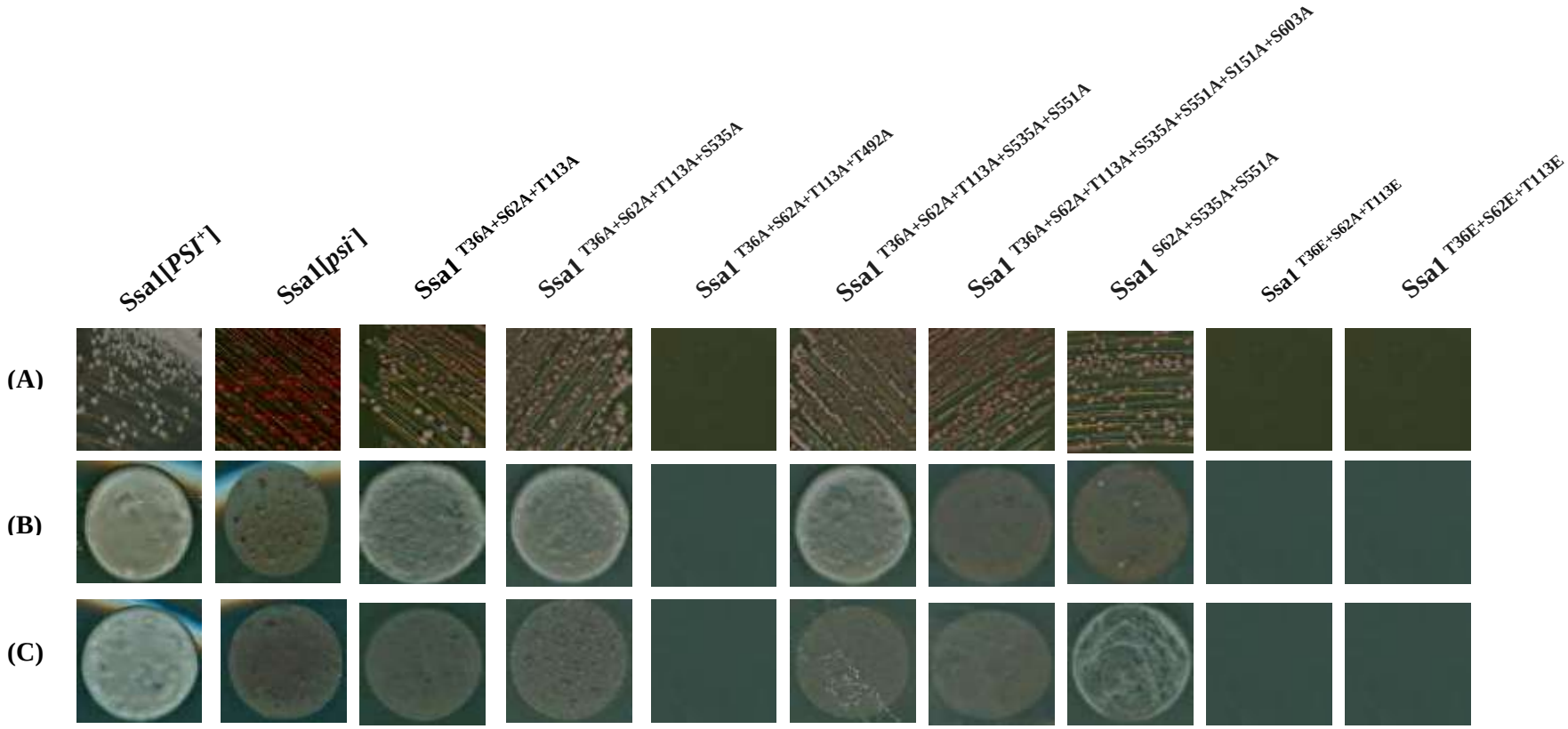



**Figure 4.8 Effect of multiple phosphomutants of Ssa1 on [*PSI*$^+$] phenotype.** (A) Cells were streaked on YPD and colonies appeared after 2 days of incubation at 30 °C followed by 2 days at 4 °C. (B) 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and (C) at RT for 3 days. Ssa1[*PSI*$^+$] is the WT; Ssa1[*PSI*$^+$] and Ssa1[*psi*$^-$] are taken as controls; Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text) . The extent of *ade 2-1* suppression is also reflected as density of growth on ade plates.

## 4.11 The multiple phosphorylation mutations of Ssa1 exhibited increased temperature sensitivity

In Chapter 3, it was established that phosphorylation mutations that impaired [*PSI*$^{+}$] propagation also had an effect on the cellular thermostability at higher temperature. Hsp70 is a heat shock protein, refolding misfolded or aggregated proteins at higher temperature. Therefore, measuring the ability of the cells expressing multiple phosphomutations on Hsp70 (Ssa1) growing at higher temperature (37 °C and 39 °C) is indicative of altered Hsp70 function. Comparative growth analysis was carried out on each of the mutant strain. The plates were then incubated at 30 °C, 37 °C and 39 °C for 3 days. The growth of the phosphomuatnts was then monitored over time at the three different temperatures. After 3 days it was evident that except for the Ssa1 $^{S62A+S535A+S551A}$ mutant, all other multiple phosphorylation mutations were temperature sensitive at 39 °C (Figure 4.9). This is suggestive of the instability of the Ssa1 protein due to the multiple phosphomutations. These results might also indicate that the mutations at multiple sites on the Ssa1 protein may have an effect on the protein dynamics hampering the ability of the Hsp70 protein to rescue aggregated, heat denatured proteins at elevated temperature.

## 4.12 Recovery of the temperature sensitive phenotypes in presence of high osmotic medium

Yeast is subjected to various kinds of environmental stimuli and cell wall damage occurs when yeast encounters chemical or heat stress (Levin, 2005). This results in activation of a MAP kinase pathway which is comprised of a linear array of components terminating with the phosphorylation of transcription factors Rlm1 and SBF which in turn activates the expression of genes involved in cell wall repair and synthesis (Figure 3.26) (Jung et al., 2002; Madden et al., 1997). Introducing mutations to cell wall integrity (CWI) signalling pathway results in common phenotypes such as temperature sensitivity that can then be recovered by osmotic support like 1M sorbitol in growth medium (Lee & Levin, 1992). The multiple phosphomutations may implicate Ssa1 as playing a role in CWI signalling pathway as the phosphomutations exhibited temperature sensitivity at 39 °C. To test this hypothesis comparative growth analysis was performed on the temperature sensitive phosphomutants and the mutants were then plated on growth media containing 1M sorbitol and incubated at 30 °C, 37 °C and 39 °C

for 3 days (Figure 4.10). Recovery of the 39 °C temperature sensitive mutants on 1 M sorbitol is indicative that Ssa1 is a component of CWI signalling pathway.

### 4.13 Basal levels of expression of Hsp104 and Hsp70 in yeast expressing multiple phosphorylation mutations of Ssa1

Analysis of the basal expression levels of Hsp70 and Hsp104 in cells expressing phosphomutations that affect [*PSI*$^+$] propagation is essential as their levels may effect prion propagation. Western blot analysis was performed to assess the levels of Hsp70 and Hsp104 in yeast that harboured phosphomutations on Ssa1 protein. As described in Section 2.19, 10 µg of total protein was loaded onto polyacrylamide gels and the mutants were probed with antibodies for Hsp70 and Hsp104. Previous studies indicated that abundance of Hsp70 or Hsp104 was not affected by mutations in the ATPase domain (Jones & Masison, 2003; Loovers et al., 2007). As previously observed, levels of Hsp70 or Hsp104 in yeast cells expressing Ssa1 phosphomutations were not considerably different in mutants compared to WT (Figure 4.11). This indicates that impairment of [*PSI*$^+$] propagation is not result of altered amounts of Hsp70.

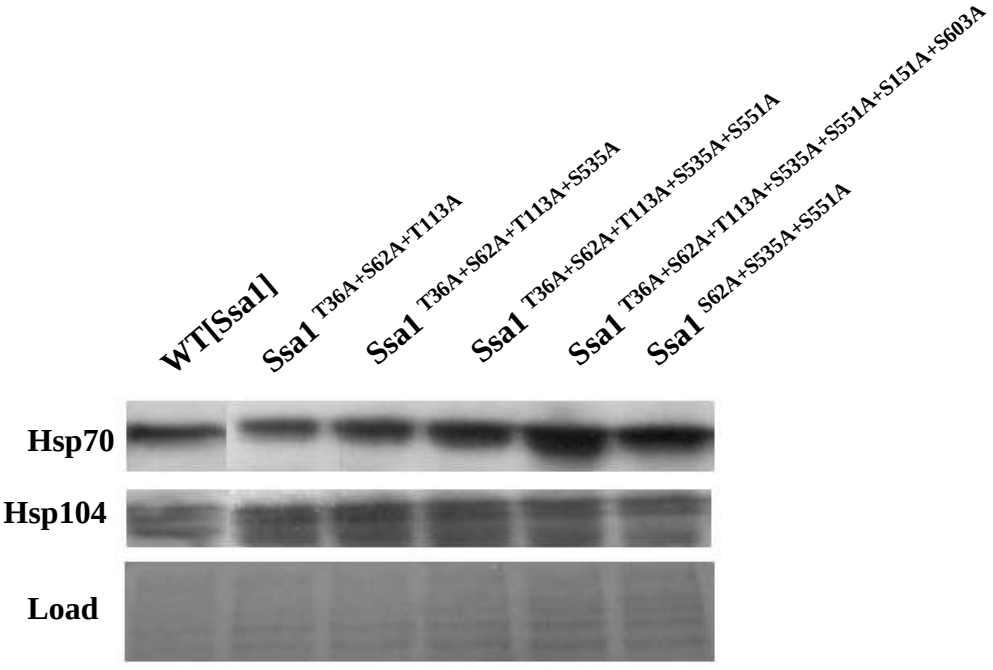



**Figure 4.11 Relative abundance of Hsp70 and Hsp104 in yeast cells expressing the multiple phosphorylation mutations on the Ssa1 protein.** Western blot analysis was performed to examine the abundance of Hsp70 and Hsp104. Blots probed with anti-Hsp70 antibodies were stripped and reprobed with anti-Hsp104 antibodies. Membrane was then stained by amido black as a loading and transfer control, are shown (Load). The phosphomutants along with the WT are indicated on the top.

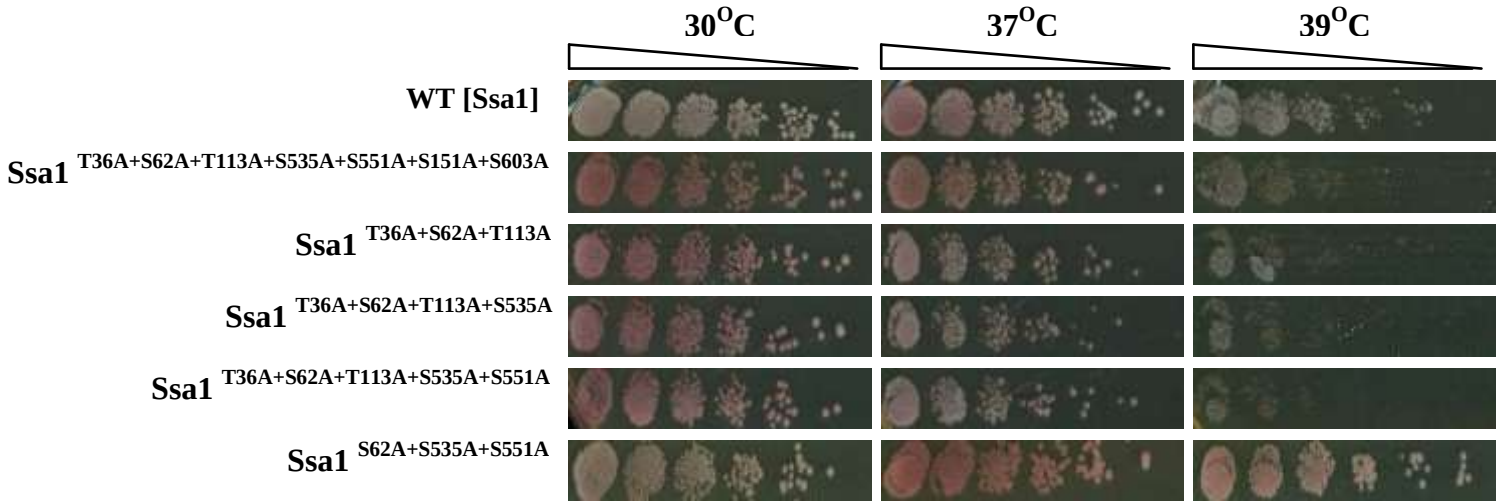



**Figure 4.9 Comparative growth analysis of the Ssa1 multiple phosphorylation mutants on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then re-suspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

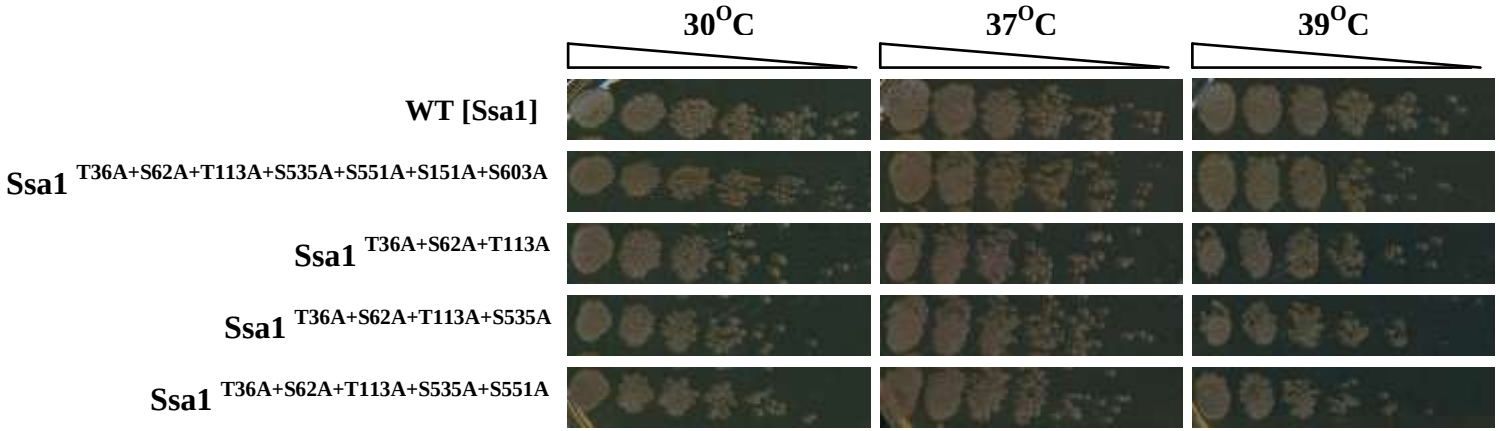



**Figure 4.10 Comparative growth analysis of the Ssa1 multiple phosphorylation mutants on 1M sorbitol plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then re-suspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for recovery over time on 1M sorbitol plate.

## 4.14 Investigation of the effect of heat shock on Hsp70 phosphorylation by 2D-gel electrophoresis

Global proteomic analysis has revealed that the Ssa1 protein is phosphorylated (Albuquerque et al., 2008). Currently, very little data is available on the post-translational modifications of Hsp70. Phosphorylation of *E. coli* DnaK was studied before but no functional significance was attributed (Sherman & Goldberg, 1993; Zylicz et al., 1983). In vertebrates, it has been shown that Hsp70 is phosphorylated in response to exercise (Melling et al., 2009) but the sites of phosphorylation and its biological significance has not yet been resolved. Not much is known about the phosphorylation of yeast Hsp70 in response to heat shock and thus one of the objectives of this chapter was to perform 2D-GE to investigate the phosphorylation status of Hsp70 (Ssa1) in response to heat shock.

The 2D-GE carried out involved separation of the proteins on the basis of pH in first dimension and then on the basis of molecular mass in second dimension (Section 2.20). The protein lysates were prepared from the cultures at 30°C and 1 h heat shock temperature at 39 °C. Approximately 125 µg protein was re-suspended in 125 µl of IEF to get a concentration of 1 µg/ µl and then was focussed on 7 cm Immobiline Drystrips pH = 3-10 overnight. This was followed by separation of the proteins on the 2$^{nd}$ dimension on the basis of molecular weight. The proteins were transferred to PVDF membrane and probed with anti-Hsp70 antibody.

The 2D-GE of WT showed that Hsp70 (Ssa1) exists in predominantly only one isoform under normal conditions at 30 °C but several other isoforms appear following a heat shock for 1 h at 39 °C (Figure 4.12 A). When the phosphorylation sites on the ATPase domain and SBD were made non-phosphorylatable, the protein had a tendency to aggregate and destabilise under heat shock conditions (Figure 4.12 B and C), although a number of potential isoforms were observed. When the mutations on the ATPase domain and SBD were brought together to generate a phosphomutant with maximum phosphorylation sites made non-phosphorylatable, the protein seemed to stabilise and the potential different isoforms disappeared (Figure 4.12 D). These findings suggest that a complex array of interactions may occur between the phosphorylation sites on the ATPase domain and SBD that can directly impact upon protein stability and perhaps in the generation of isoforms in response to heat shock.

When 2D-GE was carried out on some of the representative single phosphomutants of Ssa1 on the ATPase domain and SBD (Figure 4.13) several potential

isoforms appeared at 30 °C and 39 °C. For the Ssa1$^{T36A}$ mutant (Figure 4.13 B) the protein tended to aggregate in the wells and also became destabilised at 39 °C although several potential isoforms did appear under heat shock at 39 °C . For the Ssa1$^{T36E}$ mutant (Figure 4.13 C) the protein disaggregation and destabilization was more pronounced compared to the Ssa1$^{T36A}$ mutant suggesting that this mutation has a more severe effect on protein structure and function although several potential isoform appeared under heat shock conditions. Also for the Ssa1$^{S551A}$ and Ssa1$^{S551E}$ a similar protein aggregation and destabilization was observed (Figure 4.13 D and E). But on comparison of the single mutations of ATPase and SBD it is clear that the ATPase domain mutants (Ssa1$^{T36A}$ and Ssa1$^{T36E}$ ) have a greater tendency to aggregate and destabilise the protein than the mutations on SBD (Ssa1$^{S551A}$ and Ssa1$^{S551E}$). This highlights the importance of ATPase domain in Hsp70 function. Again these findings suggest that the process of phosphorylation and the consequences of such is complex and there occurs potential multiple interactions between the various phosphorylation sites of Hsp70 in response to heat shock.

The differential phosphorylation status of the observed potential isoforms seems to be confirmed as treatment with alkaline phosphatase alters the isoform pattern observed for WT (Figure 4.14).

However, a caveat exists in the interpretation of these results in terms of phosphorylation status. The pI for Ssa1 is 4.82 and the different isoforms observed results in major changes in pI values. The possibility exists that there is a consistent problem in focussing and aggregation for mutated Ssa1 proteins, which produces such obscure 2D-GE results. Having said this, a number of these gels were produced multiple times with reproducible results.

Equal loading was tested for all the phosphomutants prior to 2D-GE to ensure that equal amounts of protein was loaded onto the gel. The gel was first run in one dimension where 10 µg protein was loaded in each well and then after one dimensional gel electrophoresis the gel was transferred to membrane and stained by 10 ml amido black (Section 2.19.10) to check for loading and transfer control (Figure 4.15**)**

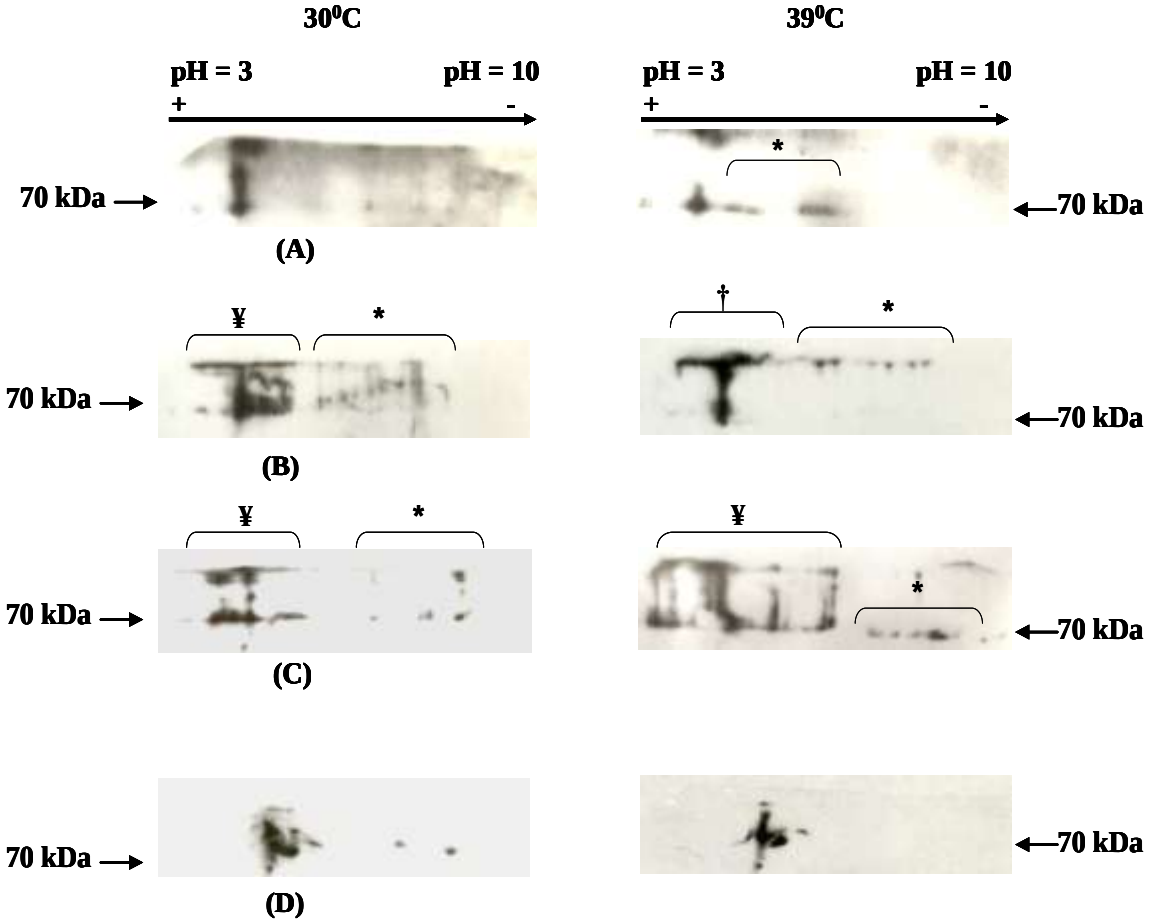



**Figure 4.12 Comparison of phosphorylation and dephosphorylation isoforms of some multiphosphomutants of Ssa1.** (A) WT [Ssa1] (B) Ssa1 $^{T36A+S62A+T113A+S151A}$ (C) Ssa1 $^{T492A+S535A+S551A+S603A}$ (D) Ssa1 $^{T36A+S62A+T113A+S151A+S535A+S551A+S603A}$. A 2D-GE was carried out to analyse the phosphorylation status of Hsp70 and its multiple phosphomutants. The proteins were first separated on the basis of pH and then on the basis of their molecular mass followed by transfer to PVDF membrane. The membrane was immunoblotted with anti Hsp70 antibody. Here * indicates potential isoforms, ¥ indicates protein destabilization and † indicates protein aggregation

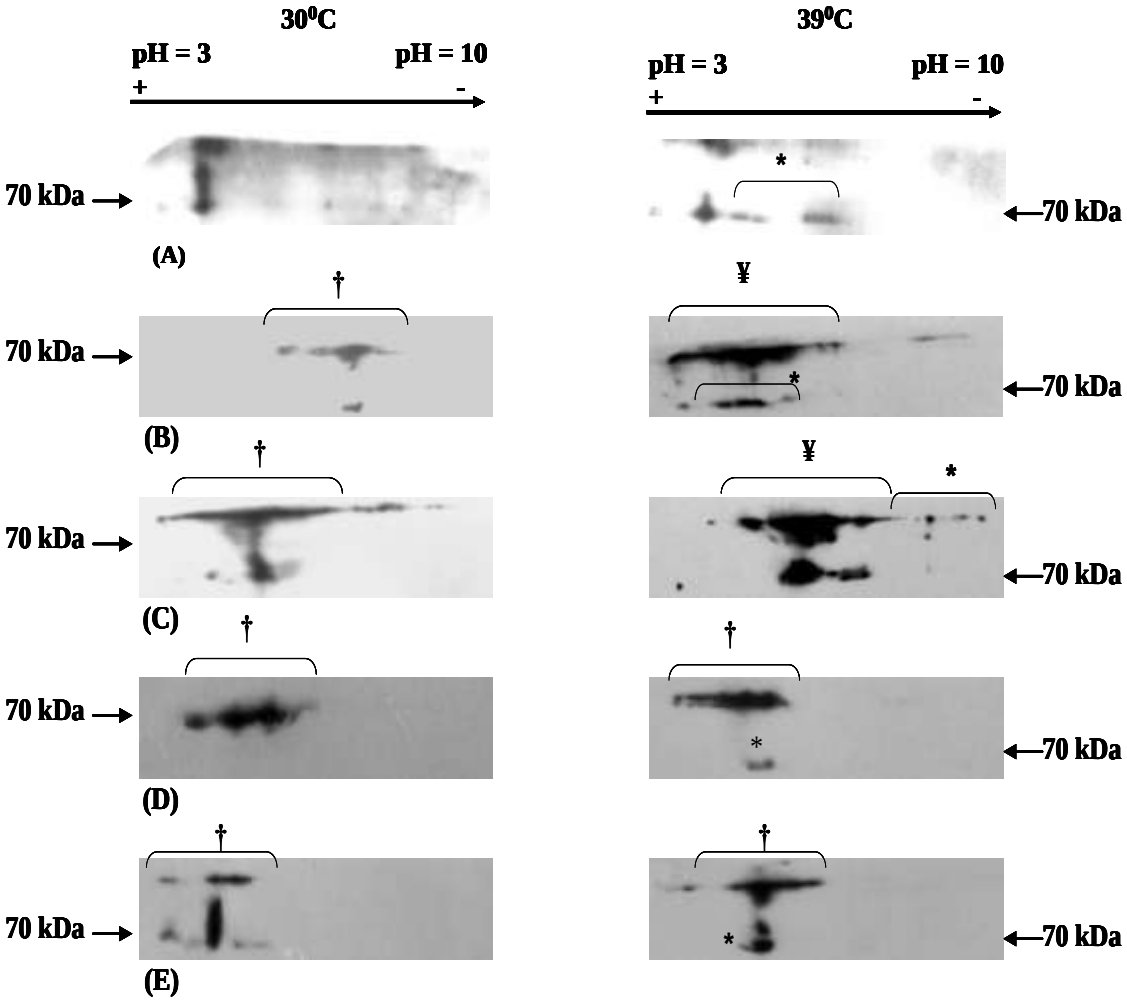



**Figure 4.13 Comparison of phosphorylation and dephosphorylation isoforms of some single phosphomutants of Ssa1.** (A) WT [Ssa1] (B) Ssa1 $^{T36A}$ (C) Ssa1 $^{T36E}$ (D) Ssa1 $^{S551A}$ (E) Ssa1 $^{S551E}$. A 2D – GE was carried out to analyse the phosphorylation status of Hsp70 and some of the representative phosphomutants on the ATPase domain and SBD. The proteins were first separated on the basis of pH and then on the basis of their molecular mass followed by transfer to PVDF membrane. The membrane was immunoblotted with anti Hsp70 antibody. Here * indicates potential isoforms, ¥ indicates protein destabilization and † indicates protein aggregation

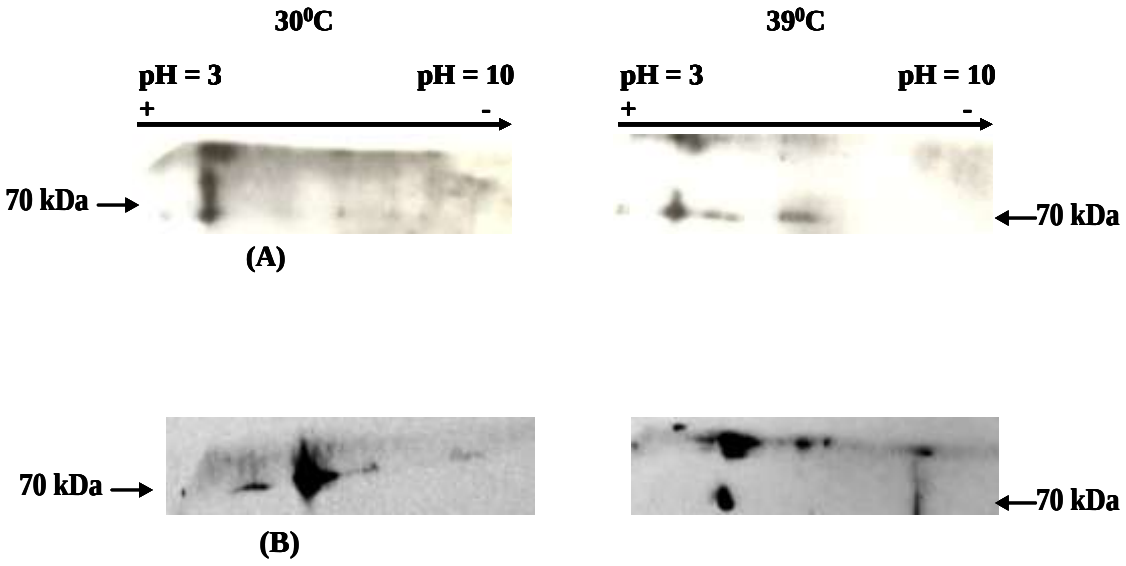
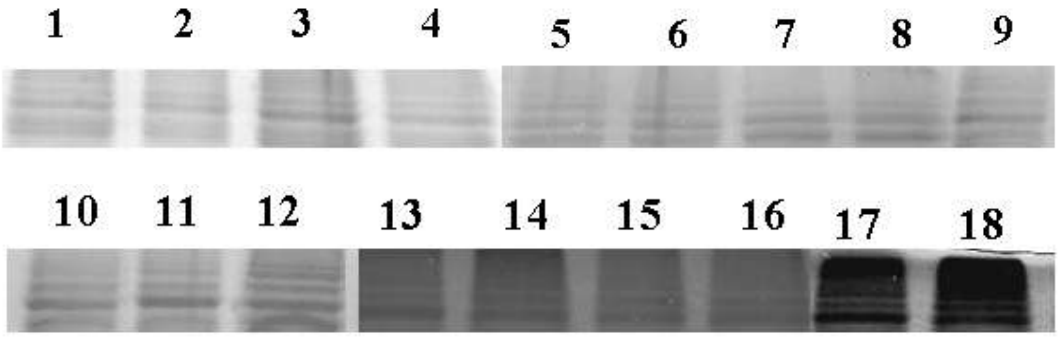



**Figure 4.14 Comparison of phosphorylation and dephosphorylation isoforms of WT [Ssa1] with and without alkaline phosphatase treatment.** (A) WT [Ssa1] (B) WT[Ssa1] after alkaline phosphatase treatment. The WT [Ssa1] was treated with alkaline phosphatase (B) as described in Section 2.20.9. A 2D-GE was carried out to analyse the phosphorylation status of WT [Ssa1] (A), alkaline phosphatase treated WT [Ssa1] (B). The proteins were first separated on the basis of pH and then on the basis of their molecular mass followed by transfer to PVDF membrane. The membrane was immunoblotted with anti Hsp70 antibody.




1. **WT Ssa1-30°C**
2. **WT Ssa1-39°C**
3. **T36A+S62A+T113A+S151A+S535A+S551A+S603A-30°C**
4. **T36A+S62A+T113A+S151A+S535A+S551A+S603A-39°C**
5. **T36A+S62A+T113A+S151A-30°C**
6. **T36A+S62A+T113A+S151A-39°C**
7. **T492A+S535A+S551A+S603A-30°C**
8. **T492A+S535A+S551A+S603A-39°C**
9. **T36A-30°C**
10. **T36A-39°C**
11. **T36E-30°C**
12. **T36E-39°C**
13. **S551A-30°C**
14. **S551A-39°C**
15. **S551E-30°C**
16. **S551E-39°C**
17. **WT Ssa1-30°C (alk.phosphatse treatment)**
18. **WT Ssa1-39°C (alk.phosphatse treatment)**

**Figure 4.15: Equal loading to check for equal amount of protein loaded to gel.** Equal loading gel was run in one dimension to ensure equal loading. The proteins from the gel were then transferred to membrane. Membrane was then stained by amido black as loading and transfer control. The phosphomutants along with the WT are numbered

## 4.15 Chapter Discussion

In this chapter, several multiple phosphorylation mutations were generated within the ATPase domain and SBD of Hsp70 by carrying out site directed mutagenesis. The approach was domain specific and multiple mutations were at first generated individually within the ATPase and SBD and were phenotypically assessed for their effect on [*PSI*$^{+}$] propagation, growth, temperature sensitivity and involvement in cellular signalling process. The second aim of this chapter, was to create an array of multiple phosphomutants throughout the two main domains of Ssa1 protein and finally a phosphomutant with all nine phosphorylation sites analysed in this study replaced with non-phosphorylatable by alanines. These multiple phosphorylation mutants were then characterised based on their effect on [*PSI*$^{+}$] propagation, growth, temperature sensitivity and involvement in cellular signalling process. Using 2D-GE, this chapter demonstrated the potential existence of Hsp70 isoforms and the possible change in isoform distribution in response to heat shock. Additionally, 2D analysis suggests the possibility of a complex interplay and communication network of Ssa1 phosphorylation sites.

"*In vivo* protein folding requires chaperone assistance"- this statement has been a topic of debate ever since the role of chaperones in mediating cellular folding were uncovered. It has been proposed that chaperones are needed to assist the folding of aggregation-prone proteins (Lorimer, 1996). Genetic and biochemical studies in *S. cerevisiae* has shown that the yeast Hsp70 homologs Ssa1–4 assist the *in vivo* folding of model proteins (Crombie et al., 1994; Kim et al., 1998). Thus realising the importance of yeast Hsp70 in protein folding, the phosphomutants generated in this study were modelled onto the 3D crystal structures of the highly homologous bovine Hsc70 ATPase domain (Accession number: 2BUP) and *E. coli* Hsp70 DnaK (Accession number: 2KHO) to assess the location of these mutations on the 3D crystal structure. The movement of proteins or "protein dynamics" makes the protein functional. Phosphorylation can have an effect on protein dynamics (Wales & Engen, 2006). As shown by the 3D crystal structures, the Ssa1 phosphomutants were well dispersed across the structure of bovine Hsc70 and *E. coli* Hsp70 DnaK and are not clustered together. However, the multiple mutations generated on the two domains of Hsp70 could possibly cause protein alterations by disrupting intramolecular interactions and the interactions formed normally in a WT Hsp70 protein. For example, Ssa1 $^{T492E}$ mutation made the cell inviable. Also the mutations Ssa1$^{T492A+S535A+S551A}$ and Ssa1

$^{T492A+S535A+S551A+S603A}$ which were temperature sensitive at 37 °C and 39 °C could not be recovered on 1M sorbitol suggesting some kind of structural or functional defect in the protein due to the multiple mutations. Thus there is a possibility that in various combinations these phosphomutations might disrupt amino acid polarity, charge and hydrophobicity which may ultimately lead to loss of protein function.

Previous genetic screens have identified an array of mutations within the constitutively expressed isoforms (Ssa1 and Ssa2) of Hsp70, which impair [$PSI^+$] propagation in *S. cerevisiae*. Some of these mutations are located within the ATPase domain of Hsp70 highlighting the importance of regulation of Hsp70-Ssa ATP hydrolysis in prion propagation (Loovers et al., 2007). Also, other studies carried out by Jones & Masison (2003) have revealed the importance of the ATPase domain in prion propagation and showed that the mutations most likely weaken Hsp70's substrate – trapping ability, implying that the impairment of [$PSI^+$] due to these mutations are mainly due to altered ability of the ATPase domain to regulate substrate binding. These studies demonstrated the importance of ATPase regulation of Hsp70 for [$PSI^+$] propagation. Also results from the previous chapter showed that the phosphorylation mutations within the ATPase domain can impact on prion propagation. The multiple ATPase domain mutants were found to impair [$PSI^+$] propagation as seen by the colour and growth on YPD and −ade plates. These results are consistent with the previous studies highlighting the importance of the ATPase domain in [$PSI^+$] propagation. It was observed that the multiple phosphorylation mutations within both main domains could alter both prion propagation and also cell growth. The impact on prion propagation again highlights the importance of the ATPase domain and how this can override phenotypes generated through mutations in the SBD. Considering data from this and the previous chapter, the residue T36 is clearly an important site for influencing Ssa1 activity. Some of the multiple mutants generated a lethal phenotype when these mutants were the sole source of Ssa in the cell. Thus this is indicative of the effect of these multiple phosphomutants both on growth and prion phenotype of Hsp70 suggesting that phosphorylation not only has an impact on prion propagation but also on cell viability. These results also indicate that complex interaction occurs between various phosphorylation sites of Hsp70 as per cell requirement.

The mutants were also assessed for their growth rates as a difference in growth rate of the yeast cell due to the mutations is indicative of the effect of these mutations on the important cellular functions of Hsp70. There was a significant effect on growth

observed for the ATPase and SBD mutants ($p < 0.05$) but this difference may not be as relevant in terms of biological impact in the experiment. The most prominent effect on growth rate was exhibited by the SBD mutant Ssa1 $^{T492A+S535A+S551A}$ that was 27% slower in growth compared to WT. These suggest that the SBD mutants had an affect on growth rate of the yeast cell although it could not influence prion propagation. These results are consistent with a previous study carried out by Jones & Masison (2003) where they showed that Hsp70 functions required for [*PSI*$^{+}$] propagation and cell growth are different implicating that Hsp70 has a different role to play with [*PSI*$^{+}$] propagation than with its other cellular functions. The prion phenotype of the ATPase domain and SBD mutant was also assessed based on the Image J-java based imaging program, which revealed that the ATPase domain mutant had a prominent effect on [*PSI*$^{+}$] propagation compared to SBD mutants. The array of multiple phosphorylation mutants generated across the Ssa1 gene also exhibited a significant effect on growth rate ($p<0.05$) but this difference in growth rate may not have much impact biologically. The Image-J java based image-processing program scored the multiple phosphomutants on red or white coloured phenotype. All the multiple phosphomutations showed a prominent effect on [*PSI*$^{+}$] propagation compared to WT except for the Ssa1 $^{S62A+S535A+S551A}$ that only had a slight effect on [*PSI* $^{+}$] propagation .

It was important to look at the abundance of Hsp104 and Hsp70 in the cell to ensure that the protein expression levels are not having an impact on prion propagation and observed chaperone activity. Thus, western blot analysis was carried out which suggested that effect of the Ssa1 multiple phosphomutants and the ATPase domain and SBD mutants on [*PSI*$^{+}$] propagation are not influenced by changes in chaperone abundance. All the phosphomutants had comparable protein chaperone levels irrespective of their effect on [*PSI*$^{+}$] propagation.

Previous studies have shown that Ssa1 mutants that impair [*PSI*$^{+}$] propagation did not have a significant effect on cell growth, leading to the conclusion that Ssa function in essential cellular pathways can be separated from Ssa function in prion propagation (Jones & Masison, 2003; Jung et al., 2000; Loovers et al., 2007). However, the multiple mutations generated in this chapter appears to be unique as it demonstrates temperature sensitivity at elevated temperature, highlighting both overlapping and distinct Hsp70 functions in relation to prion propagation and other cellular activities. When the ATPase domain and SBD mutants were assessed for temperature sensitivity, it was observed that the ATPase domain mutant was temperature sensitive at 39 °C and

this temperature sensitive phenotype was recovered in presence of the osmotic stabiliser sorbitol (1M). The SBD mutants Ssa1 $^{T492A+S535A+S551A}$ and Ssa1 $^{T492A+S535A+S551A+S603A}$ that exhibited temperature sensitivity at 37 °C and 39 °C could not be recovered by 1M sorbitol. Remediation of the temperature sensitive phenotype by osmotic support lead us to believe that the ATPase domain mutant may implicate Ssa1 as having role in cell wall integrity (CWI) signalling pathway. From these results it can also be concluded that the multiple phosphomutations on the SBD might affect the protein stability and folding under heat shock conditions. It can also be speculated that mutations within the SBD may have more of an effect on the protein dynamics of Hsp70 compared to ATPase domain mutants, thus hampering the Hsp70 protein function. When the multiple phosphomutants that were generated in Ssa1 were assessed for temperature sensitivity, it was observed that except for the Ssa1 $^{S62A+S535A+S551A}$ mutant, all the four multiple phosphomutants exhibited temperature sensitivity at 39 °C. At this point, it is interesting to note that the multiple phosphomutants that had T36A and T113A mutation did exhibit temperature sensitivity but when the multiple phosphomutant Ssa1 $^{S62A+S535A+S551A}$ was generated without these two mutations at position 36 and 113, there was no temperature sensitivity phenotype observed. This suggests that 36 and 113 might be important phosphorylation sites of the Ssa1 protein in regard to the cellular functions of Hsp70 and there is a complex communication between sites in the ATPase domain and SBD. The recovery of the temperature sensitive phenotype of these multiple phosphomutants in the presence of osmotic stabiliser further supports the hypothesis of involvement of Ssa1 in CWI signalling pathway. Chaperones and co-chaperones that function in concert with Hsp70 are involved in CWI signalling pathway. A linear MAP kinase signalling pathway is activated in response to various environmental stimuli like heat shock response, response to various cell wall damaging agents. Ssa1-Hsp70 may be indirectly involved in this pathway and thus affect any of the components of the pathway or alternatively it might directly affect the pathway by regulating some of the essential components of the pathway.

For further investigation into the phosphorylation status of Hsp70, 2D-GE was carried out for both the WT Hsp70 and some of its single and multiple phosphomutants. This analysis suggested that WT Hsp70 is hyperphosphorylated at 30 °C and perhaps several potential dephosphorylation isoforms appear in response to heat shock at 39 °C. The hyperphosphorylation of Hsp70 was confirmed by alkaline phosphatase treatment prior to 2D gel electrophoresis. This result might suggest that under heat shock

conditions “something might be happening” to the phosphorylation sites of Hsp70 and perhaps reflects that Hsp70 recognises a wider variety of substrates under heat shock conditions due to the presence of different isoforms. The non-phosphorylatable mutants within the ATPase domain and SBD generated a whole new array of isoforms under normal and heat shock conditions and also indicated a greater tendency of these proteins to become aggregated and destabilised. When the mutations on the ATPase domain and SBD were brought together to generate a phosphomutant with “maximum” phosphorylation sites made non-phosphorylatable, all the different isoforms disappeared. Thus it can be speculated that there is a complex array of cross-talk going on between the various phosphorylation sites on the two domains of Hsp70. When several representative single phosphomutants were selected from the ATPase domain and SBD and assessed for its phosphorylation status, revealed that the process of phosphorylation in Hsp70 is complex and there occurs multiple interactions between various phosphorylations sites of Hsp70 in response to heat shock.

A caveat to the interpretation of the 2D data is the fact that the range of isoforms produced covers such a wide pH range. The addition of individual phosphate groups to a protein causes a change of 0.1 pH units. Our data suggests that in exponentially growing unsynchronised yeast populations, Ssa1 is hyperphosphorylated. The spread of isoforms produced in our results cannot solely be explained by dephosphorylation, the pH shifts are just too large. Therefore, the results obtained could possibly be artefacts due to problems in focussing. However, the results were reproducible and focussing problems would need to have been confined to and representative of specific Ssa1 phosphomutants, which seems unlikely. To my knowledge there is as yet no single type of post-translational modification that could produce the pH shifts we observe. Currently work is ongoing to use mass spectrometry analysis to assess the post-translational changes that occur to Ssa1 following heat shock.

In summary, this chapter used a domain-targeted approach to create multiple phosphomutants within solely the ATPase domain or SBD, and then to combine these mutants. Phenotypic analysis of these mutants revealed a complex relationship between the possible post-translational control of Hsp70 by phosphorylation and resulting biological function. While some sites seem particularly important in this regard (eg, T36) it seems that there is a complex interaction and cross-talk between various phosphorylated residues in Ssa1.

**Table 4.6 Result summary of the chapter**

| **Mutations** | **Phenotypic and molecular characterisation of the phosphomutants** | | | | | |
|---|---|---|---|---|---|---|
| | **Prion Phenotype** | **MGT** | **Colour scale for prion phenotype** | **Ts phenotype** | **Sorbitol recovery** | **Protein expression level** |
| **ATPase domain mutant** | | | | | | |
| T36A+S62A+T113A+S151A | Impaired [*PSI*$^+$] propagation | 113.69 ± 1.2 | 9 | Ts at 39 °C | Recovered on sorbitol | Equal expression |
| **SBD mutants** | | | | | | |
| T492A+S535A | [*PSI*$^+$] propagation similar to WT | 108.68 ± 1.1 | 4 | Did not display Ts phenotype | - | Equal expression |
| T492A+S535A+S551A | [*PSI*$^+$] propagation similar to WT | 134.86 ± 0.6 | 4 | Ts at 37 °C and 39 °C | Did not recover on sorbitol | Equal expression |
| T492A+S535A+S551A+S603A | [*PSI*$^+$] propagation similar to WT | 117.40 ± 0.5 | 4 | Ts at 37 °C and 39 °C | Did not recover on sorbitol | Equal expression |
| **Multiple phosphomutants** | | | | | | |
| T36A+S62A+ T113A | Impaired [*PSI*$^+$] propagation | 117.26 ± 1.8 | 8 | Ts at 39 °C | Recovered on sorbitol | Equal expression |
| T36A+S62A +T113A+S535A | Impaired [*PSI*$^+$] propagation | 117.84 ± 0.4 | 8 | Ts at 39 °C | Recovered on sorbitol | Equal expression |

**Table 4.6 continued……**

| | | | | | | |
|---|---|---|---|---|---|---|
| T36A+S62A+T113A+S535A+ S551A | Impaired [*PSI*$^+$] propagation | 119.33 ± 1.6 | 8 | Ts at 39 °C | Recovered on sorbitol | Equal expression |
| T36A+S62A+T113A+S151A+ S535A+S551A+ S603A | Impaired [*PSI*$^+$] propagation | 110.81 ± 0.6 | 9 | Ts at 39 °C | Recovered on sorbitol | Equal expression |
| T36A+S62A +T113A+T492A | Lethal | - | - | - | - | - |
| S62A+S535A+S551A | Impaired [*PSI*$^+$] propagation | 97.16 ± 1.2 | 7 | Did not display Ts phenotype | - | Equal expression |
| T36E+ S62A+T113E | Lethal | - | - | - | - | - |
| T36E+S62E+T113E | Lethal | - | - | - | - | - |

**Table 4.7 Summary of the 2D data of the phosphomutants and WT**

| **Mutations** | **Effect of temperature on phosphorylation status** | |
|---|---|---|
| | **At 30 °C** | **At 39 °C** |
| WT [Ssa1] | Only one isoform | Several potential isoforms appeared |
| T36A+S62A+T113A+S151A | Protein got destabilised; Appearance of several potential isoforms | Aggregated proteins; Appearance of several potential isoforms |
| T492A+S535A+S551A+S603A | Protein got destabilised; Appearance of several potential isoforms | Protein got destabilised; Appearance of several potential isoforms |
| T36A+S62A+T113A+S151A+ S535A+S551A+ S603A | Similar to WT | Disappearence of all potential isoforms |
| T36A | Aggregated proteins | Aggregated proteins; Appearance of several potential isoforms |
| T36E | Aggregated proteins | Protein got destabilised; Appearance of several potential isoforms |
| S551A | Aggregated proteins | Aggregated proteins; Appearance of several potential isoforms |
| S551E | Aggregated proteins | Aggregated proteins; Appearance of several potential isoforms |

# CHAPTER 5

## A PHENOTYPIC AND MOLECULAR ANALYSIS OF HIGHLY HOMOLOGOUS HSP70 SSA FAMILY

## 5.1 Introduction

Hsp70 constitutes one of the most highly conserved and well studied Hsps across species ranging from archaebacteria to plants and humans with the prokaryotic Hsp70 protein DnaK sharing approximately 50% amino acid homology with eukaryotic Hsp70 proteins (Daugaard et al., 2007; Gupta & Singh, 1994; Hunt & Morimoto, 1985; Lindquist & Craig, 1988). Hsp70 is conserved in its functional properties across species. For example, *Drosophila* Hsp70 expressed in mammalian cells efficiently protects against heat stress (Pelham, 1984), and rodent Hsp70 can be functionally complemented by human Hsp70 to grant cellular protection against various stresses both *in vitro* (Jäättelä et al., 1992; Li et al., 1992; Li et al., 1991) and in transgenic animals. Also plant Hsc70 and eukaryotic stress inducible (iHsp70) isoform behave similarly implicating evolutionary conservation. (Angelidis et al., 1996; Plumier et al., 1995; Radford et al., 1996; Tutar et al., 2006).

Ever since the discovery of *hsp70* gene family in *Drosophila melanogaster*, several homologous genes have been identified in other organisms as well (Craig & Schlesinger, 1985; Lindquist, 1986). In yeast *Saccharomyces cerevisiae*, the *hsp70* gene family comprises of fourteen genes, which share a sequence homology of approximately 50-96%. Of these fourteen Hsp70 homologues, nine are cytosolic (Ssa1, Ssa2, Ssa3, Ssa4, Ssb1, Ssb2, Sse1, Sse2 and Ssz1) and five are compartmental specific. In yeast, the cytosolic Hsp70 family have both overlapping and diverse functions. For example, the cytosolic Ssb proteins cannot substitute for the survival function of Ssa proteins but the four Ssa proteins can compensate for each other (Boorstein et al., 1994; James et al., 1997).

The Ssa sub-family, of cytosolic Hsp70, consists of four members Ssa1-4, which are functionally redundant and abundance of at least one protein is essential for growth (Werner-Washburne et al., 1987). Constitutively expressed Ssa1 and Ssa2 are 98% identical to each other and under optimal conditions Ssa2 is roughly fourfold more abundant than Ssa1 and depletion of Ssa2 induces expression of Ssa1, maintaining overall Hsp70 abundance. The heat-inducible Ssa3 and Ssa4 are 88% identical to each other and share an identity of 80% with Ssa1/2. The heat inducible isoforms are expressed under non-optimal growth conditions and help protect cells from the adverse effects of stress (Sharma & Masison, 2008).

It had been suggested for a long time that the Hsp70 isoforms are functionally redundant and differ only by their spatio-temporal expression pattern. However, this

was challenged by several findings in yeast and higher eukaryotes demonstrating functional specificity among Hsp70 isoforms (Kabani & Martineau, 2008). Early evidence of functional specificity among the Hsp70 isoforms was given by Eisenberg and his collaborators by their work on yeast cytoplasmic Hsp70s to dissociate clathrin from bovine brain coated vesicle (Gao et al., 1991). They showed that *in vitro* clathrin uncoating activity is associated with Ssa but not with Ssb proteins. Further they showed that Ssa2 has higher uncoating ability compared to Ssa1 and Ssa1 can inhibit the uncoating activity of Ssa2 (Gao et al., 1991). Another functional distinction between Ssa1 and Ssa2 stems from their role in prion propagation. Overexpression of Ssa1 appears not to weaken [$PSI^+$] prion but cures [*URE3*] prion and conversely depleting Ssa1 weakens [$PSI^+$] but not [*URE3*] (Schwimmer & Masison, 2002; Roberts et al., 2004). Hsp70 has also been implicated in biofilm production in yeast which is another good example of functional specificity among the Hsp70 orthologues (Martineau et al., 2007). Ssa1 deletion had a more adverse effect on biofilm formation in yeast compared to Ssa2 deletion. Additionally, Ssa3 and Ssa4 deletion enhanced the defects brought about by Ssa1/Ssa2 deletion suggesting cooperation between constitutive and inducible isoforms of Hsp70 (Martineau et al., 2007).

Previous studies have shown the importance of the ATPase domain of Hsp70 in prion propagation by isolation of numerous mutants from the ATPase domain that have an effect on prion propagation (Jones & Masison, 2003; Loovers et al., 2007). Some of these mutants are located in the region that was shown to be important for inter-domain communication in the *Escherichia coli* Hsp70, DnaK (Revington et al., 2005). Still others are found in the IIB region of the ATPase domain that has been implicated as interacting with the Fes1 homologue, HspBP1, in mammals (Shomura et al., 2005). By specifically targeting the Hsp70 peptide-binding domain (PBD) for mutagenesis, previous work carried out in the laboratory has shown the importance of a localized region in allowing efficient prion propagation. When mapped onto the Hsp70 PBD crystal structure the location of this region suggests it has the ability to influence Hsp70 substrate specificity (Cusack, 2010). Thus, looking into the functional conservation of these mutations that impair prion propagation located on the two domains of Hsp70 across the Ssa family may be useful targets for therapeutics or designing treatments for prion diseases.

Thus the main objectives of this chapter were to:

- To investigate the similarities and differences between yeast strains expressing individual members of the Ssa family.
- To investigate the functional conservation of ATPase and PBD mutations across the cytosolic Ssa family and implicate a general role of the Hsp70 Ssa family in prion propagation.
- To analyse the differential gene expression patterns in yeast strains expressing individual Ssa's.

## 5.2 Strategy for Ssa1-4 comparison

Comparative analysis of Ssa1-4 proteins required plasmids expressing each gene under the control of a constitutively expressed promoter. To achieve this each member of the Ssa family was cloned into the plasmid under the control of the constitutively expressed promoter Ssa2. The Ssa1 and Ssa3 gene was successfully cloned into the pRS315 plasmid under the control of Ssa2 promoter (pC210) by former members of our laboratory. The plasmids expressing Ssa2 (pDCM64) and Ssa4 (PA4) gene was obtained as gift from Dr. Daniel Masison (NIDDK/NIH, Bethesda, MD, USA).

### 5.2.1 Phenotypic characterisation of the Ssa family required expression of each member of the family in the yeast strain G402

To assess the functions of individual members of the Ssa family, each member must be expressed as a sole source of Ssa in a strain. To achieve this, each plasmid carrying the individual Ssa gene and bearing *LEU2* selectable marker was transformed into the [$PSI^+$] version of a yeast strain G402 by carrying out plasmid shuffle technique (Section 2.12). The strain G402 consisted of a *URA3* based pRDW10 plasmid with all the four major cytosolic *SSA* genes deleted from the chromosomal DNA. The potential transformants were selected on SD agar lacking leucine. Colonies from SD agar plates lacking leucine was replica plated on 5-FOA to eliminate pRDW10 plasmid and were restreaked onto YPD and SD agar lacking adenine to assess growth and prion propagation supported by individually members of the Ssa family (Figure 5.1). All the members of the Ssa family individually supported growth of G402. [$PSI^+$] was well maintained in cells expressing Ssa3 as the sole source of Ssa as seen by the pigmentation on YPD and growth on adenine lacking media (Figure 5.1 upper panel). The prion phenotype was also maintained in cells expressing Ssa1 and Ssa2 but not to

the same extent as Ssa3 whereas among all the four Ssa's, cells expressing Ssa4 was seen to impair the propagation of [*PSI*$^{+}$] (Figure 5.1 upper panel). All the strains expressing individual Ssa's were curable by 3 mM Gdn-HCl as observed by the pigmentation and growth on YPD and SD agar plates lacking adenine (Figure 5.1 lower panel). These results are indicative of the fact that prion propagation and maintenance is affected to a variable degree by different members of the cytosolic Ssa family of Hsp70 which is one of the distinguishing characteristics of the members Ssa family.

### 5.2.2 Investigation of the individual members of Ssa family on growth rate of yeast cell

The *SSA* subfamily of Hsp70 is essential for the growth of *S. cerevisiae* (Werner-Washburne et al., 1987). It has been shown in the previous section that each member of the Ssa family individually support growth of *S.cerevisiae* strain G402 that lacks all the four chromosomal *SSA* genes (Figure 5.1). For further phenotypic characterisation of the Ssa family, the growth rate of yeast was assessed in the presence of individual members Ssa family as the sole source of Ssa in the cell. Cells expressing constitutively expressed isoforms of Hsp70 *i.e* Ssa1 and Ssa2 grow quicker than its heat inducible counterpart's i.e Ssa3 and Ssa4 (Figure 5.2). Also the [*psi*$^{-}$] versions of Ssa1 and Ssa2 requires less generation time than the [*psi*$^{-}$] versions of Ssa3 and Ssa4 (Figure 5.2). Among the [*PSI*$^{+}$] version, the Ssa4 appears to have the greatest effect on growth, with the growth rate being reduced by approximately 63% compared to Ssa1 (Figure 5.2). This result is consistent with the previous studies which showed that Ssa4 had the greatest affect on yeast cell growth (Sharma et al., 2009). But when the [*psi*$^{-}$] versions of the Ssa family was assessed for it growth rate, it was observed that Ssa3 exerted greatest effect on growth, with the growth rate being reduced by 59% compared to Ssa1 (Figure 5.2). Overall, the [*psi*$^{-}$] versions of the Ssa family have a greater effect on growth of yeast compared to the [*PSI*$^{+}$] version. But interestingly, this was not the case for Ssa4 where Sss4 [*PSI*$^{+}$] exhibited slower growth compared to its [*psi*$^{-}$] versions (Figure 5.2). However from these results, it is clear that the heat inducible isoforms of the Ssa family do not support growth of *S. cerevisiae* as efficiently as its constitutively expressed isoforms.

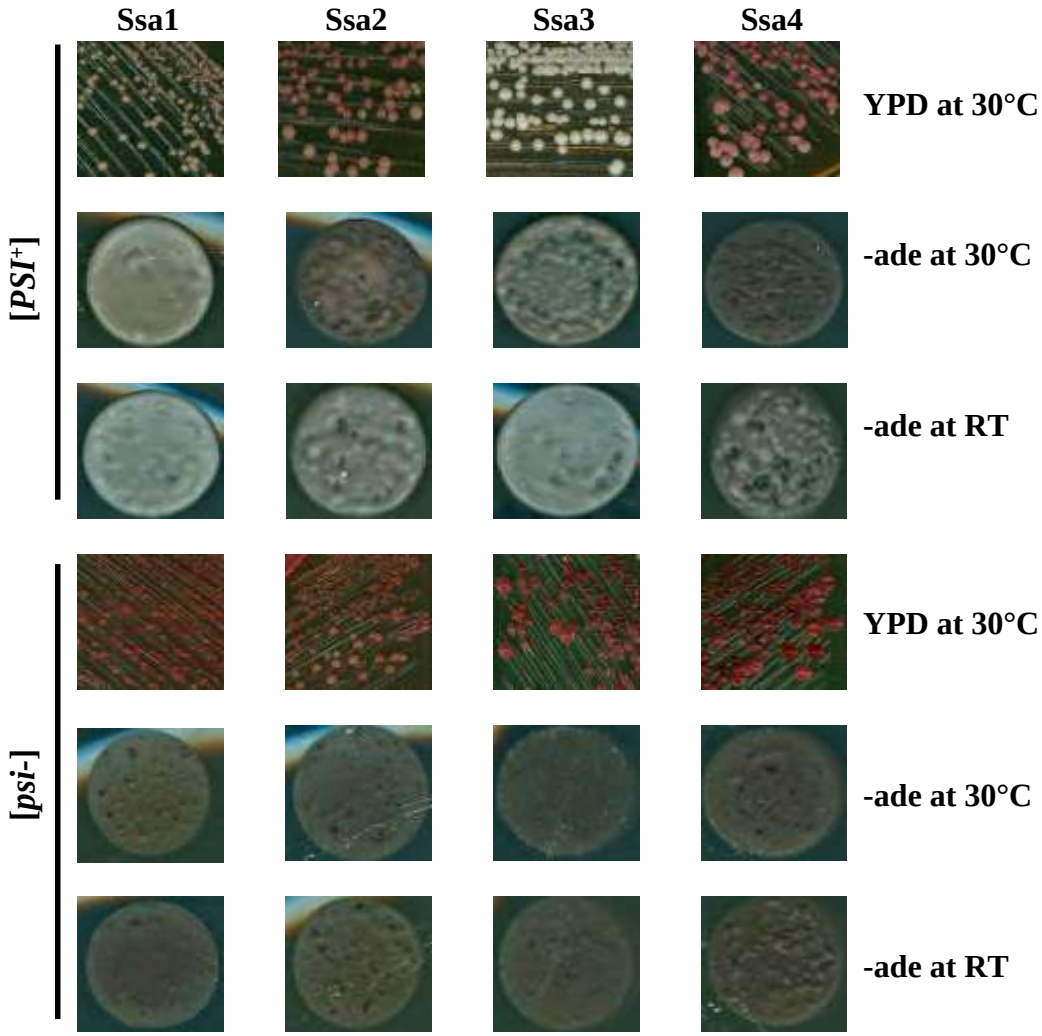



**Figure 5.1 Effect of individual members of the Ssa family on [*PSI*$^+$] phenotype.** Each member of the Ssa family was expressed as the sole source of Ssa in the yeast strain G402 by plasmid shuffle technique. Cells were streaked from 5-FOA onto YPD and colonies came up after 2 days of incubation at 30 °C followed by 2 days at 4 °C. 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and at RT for 3 days. Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text). The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates. Figures on the upper panel shows the [*PSI*$^+$] version of the Ssa family. Strains were cured by incubating the cells on 3 mM Gdn-HCl plates for 2 days at 30 °C and then the cured strains were streaked onto YPD and colonies came up after 2 days of incubation at 30 °C followed by 2 days at 4 °C. 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and at RT for 3 days. Figures on the lower panel shows the [*psi*$^-$] version of the Ssa family.

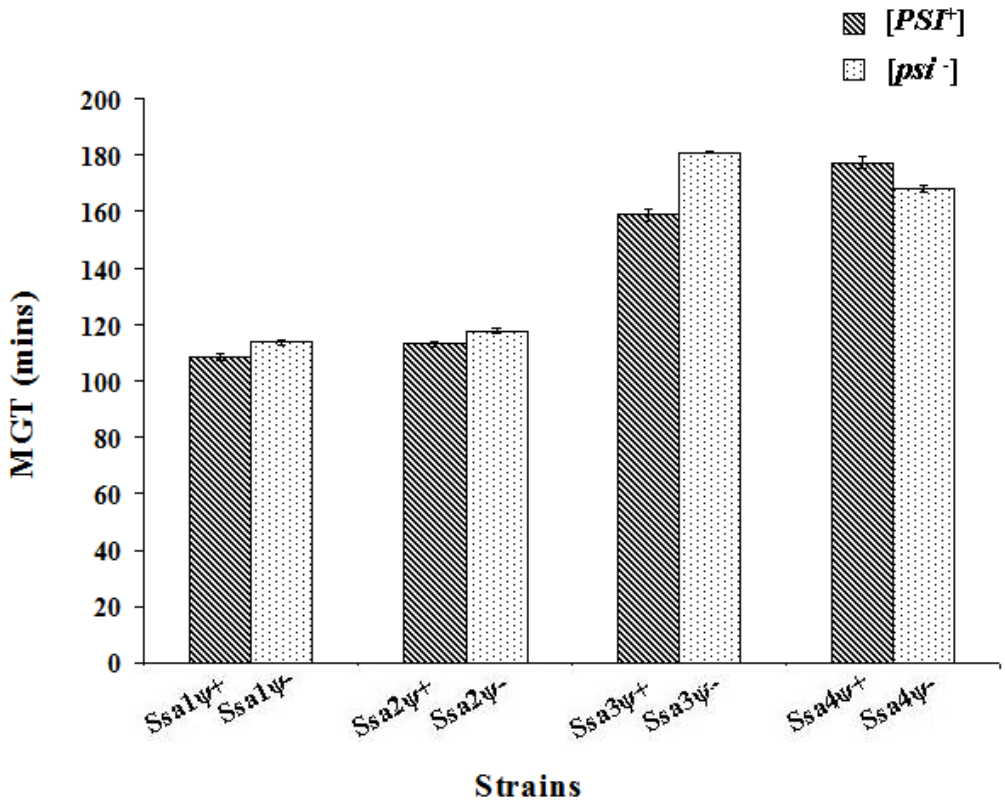



**Figure 5.2 Relative effects of cells expressing individual members of Hsp70 Ssa subfamily on growth of *S.cerevisiae*.** The mean generation time of the yeast strains were determined by diluting the overnight 30 °C culture in 30 ml fresh media to an $OD_{600nm}$ of 0.1(t = 0 min). Cultures were transferred to 30 °C shaking incubator and cell density was measured at an interval of every 2 h as described in Section 2.14. Bars indicate SEM. [LSD at 0.05 = 4.7]

### 5.2.3 Investigation of temperature sensitivity of the Ssa family by comparative growth analysis

Previous studies from different research groups have shown that yeast strains lacking all the four chromosomal *SSA* genes and expressing individual Ssa proteins from plasmids under the control of Ssa2 promoter confirms that Ssa1-Ssa4 each support cell growth (Sharma & Masison, 2008; Werner-Washburne et al., 1987). These findings were also confirmed in the present study as discussed in the previous section (Section 5.1.1). The molecular chaperone Hsp70 functions to protect cells from stress injuries by preventing aggregation of partially unfolded proteins (Hartl & Hayer-Hartl, 2002; Mayer et al., 2001). In order to gain insight into whether individual Ssa's can protect cells at elevated temperatures, cells expressing individual Ssa's were tested at various temperatures. Comparative growth analysis was carried out as described in Section 2.15 on the yeast strains expressing individual Ssa's and the plates were incubated at 30 °C,

37 °C and 39 °C and growth was monitored after 3 days. Importantly, cells expressing inducible Hsp70s (Ssa3 and Ssa4) grew more slowly then their constitutive counterparts (Ssa1 and Ssa2). These results suggest that stress inducible isoforms of Hsp70 lacks important housekeeping functions that require more efficient activities possessed by the constitutive isoforms. These results are also indicative that Ssa3 and Ssa4 are not efficient in protecting yeast cell against stress injuries when each protein is expressed as the sole source of Ssa in the cell. Also when the [*PSI*$^{+}$] and the [*psi*$^{-}$] version of the Ssa family was compared, it was observed that [*psi*$^{-}$] versions of constitutive isoforms of Hsp70 did not show any difference in growth even at elevated temperatures but a difference in growth was observed for the inducible isoforms (Figure 5.3).

### 5.2.4 Recovery of the temperature sensitive phenotype in presence of osmotic stabiliser

Yeast can be subjected to a diverse array of environmental stimuli and to survive such extreme environmental conditions, their exists a signalling pathway called the cell wall integrity (CWI) pathway that provides a means of survival under stressful environments by repairing cell wall damages as well as providing support to the cell wall to withstand such extreme environmental conditions (Fuchs & Mylonakis, 2009). Loss of function of any of the components in this pathway can lead to temperature sensitive cell lysis that can be remediated by osmotic support (Lee & Levin, 1992). As described in the previous chapters, phosphomutants that cause the temperature sensitive phenotype can be recovered in presence of osmotic stabilisers like 1 M sorbitol. This may implicate the role of Ssa1 in CWI signalling pathway, as mutations which hamper this pathway lyse in absence of osmotic stabilisers like 1 M sorbitol. As the heat inducible isoforms of Hsp70 *i.e* Ssa3 and Ssa4 exhibited a temperature sensitive phenotype thus these two isoforms were tested for recovery on 1 M sorbitol (Figure 5.4 A and B). The recovery of both the [*PSI*$^{+}$] and [*psi*$^{-}$] versions of Ssa3 and Ssa4 is indicative of the role of Ssa family of Hsp70 in CWI signalling pathway. It may also suggest that the Ssa3 and Ssa4 proteins become unstable at elevated temperature and sorbitol being an osmotic stabiliser might somehow make the protein stable.

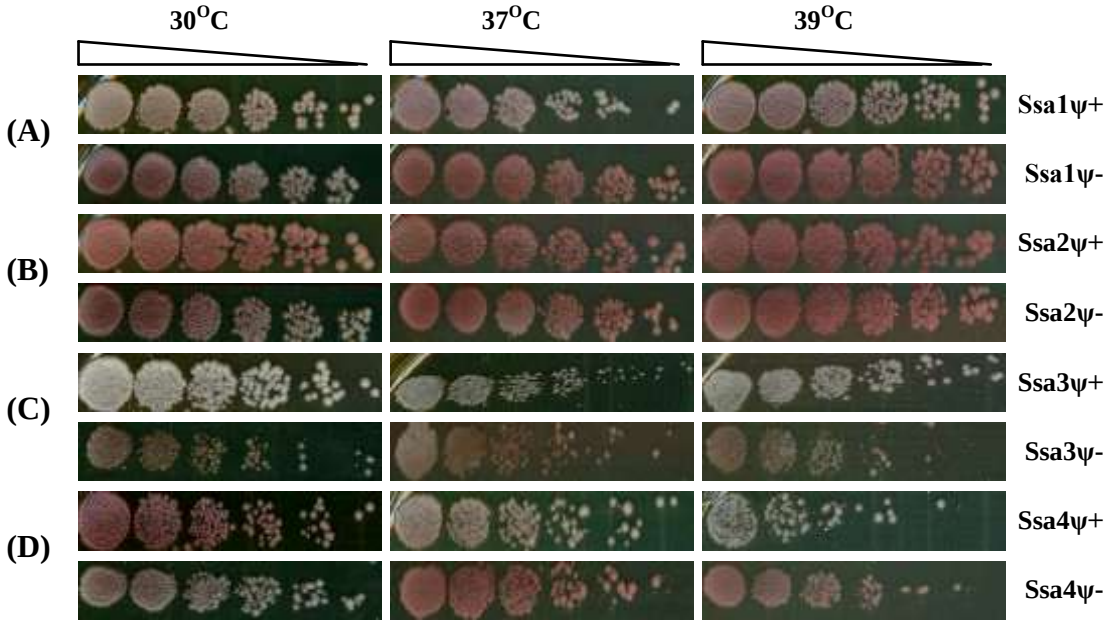



**Figure 5.3 Comparative growth analysis of the Ssa family on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then re-suspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity. Ssa1-4 expressed in G402 as sole source of Ssa in the yeast strain.

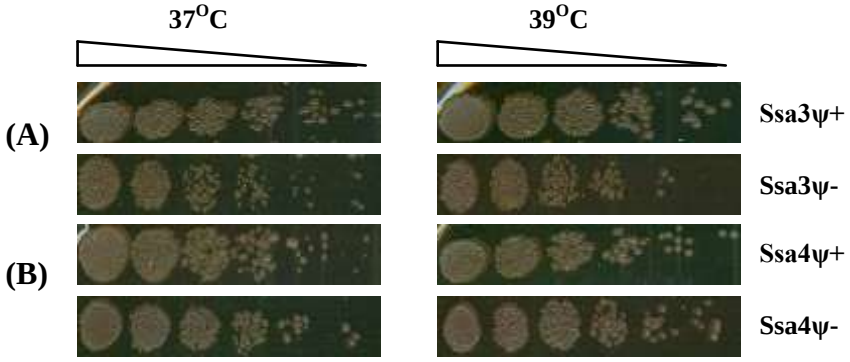



**Figure 5.4 Recovery of temperature sensitive phenotype of Ssa3 and Ssa4 on 1 M sorbitol plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity. Ssa1-4 expressed in G402 as sole source of Ssa in the yeast strain.

### 5.2.5 Sensitivity to cell wall damaging agents for G402 cells expressing individual members of the Ssa family

Many organisms have developed defensive mechanisms against fungal invasion and most of this defence mechanisms target the cell wall. In the same way, yeast have developed a response mechanism to compensate these attacks (Smits et al., 2001). Several environmental factors such as heat stress, mating factors, cell wall damaging agents or inactivation of genes involved in cell wall biogenesis contribute to impairment or damage to cell wall (Popolo et al., 2001). Cell wall maintenance is essential for preserving the osmotic integrity of the cell and thus several responses are triggered in response to cell wall damage (Popolo et al., 2001). One such response found in yeast is the cell wall integrity (CWI) pathway that can be activated in various ways (Smits et al., 2001).

Exposure of *S. cerevisiae* to environmental stress results in activation of five separate mitogen-activated protein kinase (MAPK) cascades (Chen & Thorner, 2007). The CWI signalling pathway uses one of these cascades to mediate cell wall biosynthesis and maintenance (Levin, 2005). Loss of function of any of the components of this cascade can lead to temperature sensitive phenotype that can be remediated by osmotic support (Lee & Levin, 1992). Other mutant phenotypes of CWI signalling pathway also includes sensitivity to cell wall damaging agents such as caffeine, calcofluor white and congo red (Errede et al., 1995; Martín et al., 2000). The use of sodium dodecyl sulphate (SDS), an anionic detergent also has been shown to destabilise the cell wall at very low concentrations (de Nobel et al., 2000; Delley & Hall, 1999).

Previous studies have shown the involvement of molecular chaperones in CWI signalling (Millson et al., 2005; Piper et al., 2006) Thus the aim of this section was to perform an array of cell wall sensitivity tests in presence of several cell wall damaging agents to determine whether the temperature sensitive phenotypes of the Ssa family can have any impact on signalling cascade.

If the Ssa family of Hsp70 is involved in CWI signalling pathway they should show sensitivity to cell wall damaging agents as damage to cell wall elicits this pathway. Comparative growth analysis was performed as described in Section 2.15 and cells were selected on medium with varying concentrations of SDS, caffeine, calcofluor white with YPD as a control plate and monitored after 3 days of incubation at 30 °C (Figure 5.5). All the members of the Ssa family were sensitive to calcoflour white at a concentration of 10 µg/ml but did not exhibit any sensitivity to caffeine. This was due to

the strain background used and thus the assay for sensitivity with calcofluor white and caffeine needs to be tested with a different strain background like BY4741. However, there was a varying degree of sensitivity observed on two different concentrations of SDS. Both the [*PSI*$^{+}$] and the [*psi*$^{-}$] versions of the constitutive isoforms of Hsp70 exhibited sensitivity to 0.01% SDS (Figure 5.5 A and B). The Ssa3 [*psi*$^{-}$] was sensitive to SDS at a concentration of 0.005% but Ssa4 [*psi*$^{-}$] exhibited sensitivity at higher concentrations of SDS (0.01%). However, due to varying degree of sensitivity seen for the members of Ssa family on SDS, an accurate comparison could not be made. These results are suggestive but not conclusive of the involvement of the Ssa family of Hsp70 in CWI signalling pathway.

### 5.2.6 Investigation of oxidative stress response in G402 cells expressing different members of the Ssa family as sole cytosolic Hsp70

Almost every living cell in nature is exposed to oxidative stress with no exception to fungal cells. Exposure to aerobic conditions, UV light, production of reactive oxygen species, hydrogen peroxide and hydroxyl radicals by host cells are the major cause of oxidative stress for fungi. Such stresses are also the consequence of normal cell metabolism. The fungal cell wall is the first line of defence against cell damage from exogenous oxide radicals. The cell wall senses the oxidative stress and reactive measures are taken by signal transduction pathways to protect fungi from the adverse environment. Though the key defence against oxidative stress is the HOG pathway (Alonso-Monge et al., 2003; Arana et al., 2005; Singh, 2000), the CWI signalling pathway provides additional oxide defence through cross talk with the HOG pathway (Fuchs & Mylonakis, 2009). Transcription factors such as Skn7, Yap1, Msn2, and Msn4 control the expression of numerous genes in response to peroxide stress and thus play an important role in oxidative stress resistance. As orthologous sequences of these transcription factors are shared between the *S. cerevisiae*, the *S. pombe*, and some other pathogenic fungi like *C. albicans, Candida glabrata*, and *Ustilago maydis* (Cuéllar-Cruz et al., 2008), their role in oxidative stress resistance become more established. Chemicals such as hydrogen peroxide and diamide which causes lipid peroxidation, protein oxidation and DNA damage in living cells are often used to deduce the CWI pathway response to oxide stress in *S. cerevisiae.* Both of these two chemicals are capable of activating the dual phosphorylation of Slt2/Mpk1, although with differing kinetic activity (Fuchs & Mylonakis, 2009).

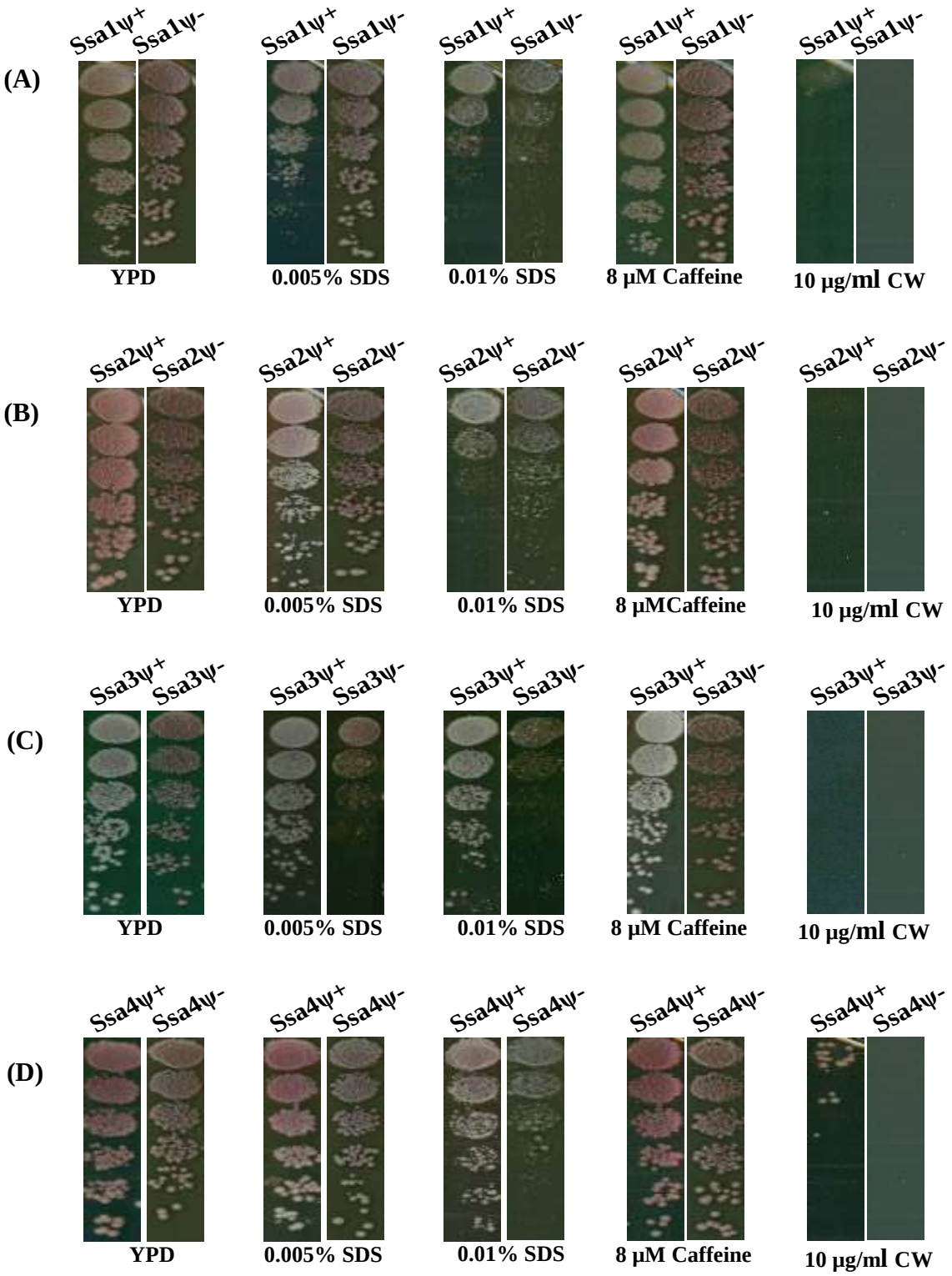



**Figure 5.5 Comparative growth analysis of of the Ssa1-4 in response to cell wall damaging agents.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C. Ssa1-4 expressed in G402 as sole source of Ssa in the yeast strain.

Hydrogen peroxide does not require one of the known CWI stress sensors of *S. cerevisiae* to provoke the phosphorylation of Slt2/Mpk1 and is suspected to elicit an intracellular means of activation (Vilella et al., 2005). Cell surface sensors activate Rom2 (a guanine exchange factor protein) of the GTP-binding protein Rho1 which in turn activate Pkc1 protein kinase leading to the activation of MAPK signalling cascade (Vilella et al., 2005). Exposure of a *rom2 m*utant strain, to either diamide or hydrogen peroxide results in reduced phosphorylation of Slt2/Mpk1 thus affecting the downstream MAPK pathway. Pkc1 is also required, but the MAPK module downstream of Pkc1 is not vital to the cellular response in defense of oxidative stress (Vilella et al., 2005). Thus, the activation of the CWI pathway by oxidative stress by either inducer requires the upper part of the CWI pathway for cell survival (Vilella et al., 2005).

Comparative growth analysis was performed on yeast strain G402 expressing individual members of the Ssa family as the sole source of Ssa and the yeast strain was then tested for oxidative stress response to hydrogen peroxide. The strains were grown on SC medium having varying concentrations of $H_2O_2$ (0-5 mM) and incubated at 30 °C for 3 days (Figure 5.6). Amongst the two constitutively expressed members of the Ssa family, there was a clear difference in sensitivity observed between Ssa1 and Ssa2 at 3 mM concentration of $H_2O_2$ with a pronounced sensitivity observed in Ssa2 at this concentration compared to Ssa1. Both [$PSI^+$] and [$psi^-$] versions of Ssa1 and Ssa2 displayed sensitivity at 3 mM concentration of $H_2O_2$ with Ssa2 showing more sensitivity compared to Ssa1 (Figure 5.6 A and B).

When the heat inducible isoforms of Hsp70 were assessed for oxidative stress in response to $H_2O_2$ a varying degree of sensitivity was exhibited by the two isoforms. The Ssa3 [$psi^-$] started showing sensitivity at 3 mM concentration of $H_2O_2$ but the Ssa3 [$PSI^+$] was resistance up till 4mM concentration of $H_2O_2$ (Figure 5.6 C). Also when Ssa4 was assessed for oxidative stress response to $H_2O_2$ a marked difference in sensitivity was observed only at higher concentration of $H_2O_2$ (4mM) for both the [$PSI^+$] and [$psi^-$] (Figure 5.6 D). Since a varying degree of sensitivity persisted among the different members of Ssa family to $H_2O_2$, these results are suggestive but not conclusive of the involvement of the Ssa family of Hsp70 in CWI signalling pathway. On the other-hand, oxidative stress causes protein denaturation and interferes with protein folding. Thus the proteins are denatured at higher concentrations of $H_2O_2$ and as observed from the Figure 5.6 C and D, heat inducible isoforms of Hsp70 are more

efficient in dealing with stress response compared to its constitutively expressed isoforms.

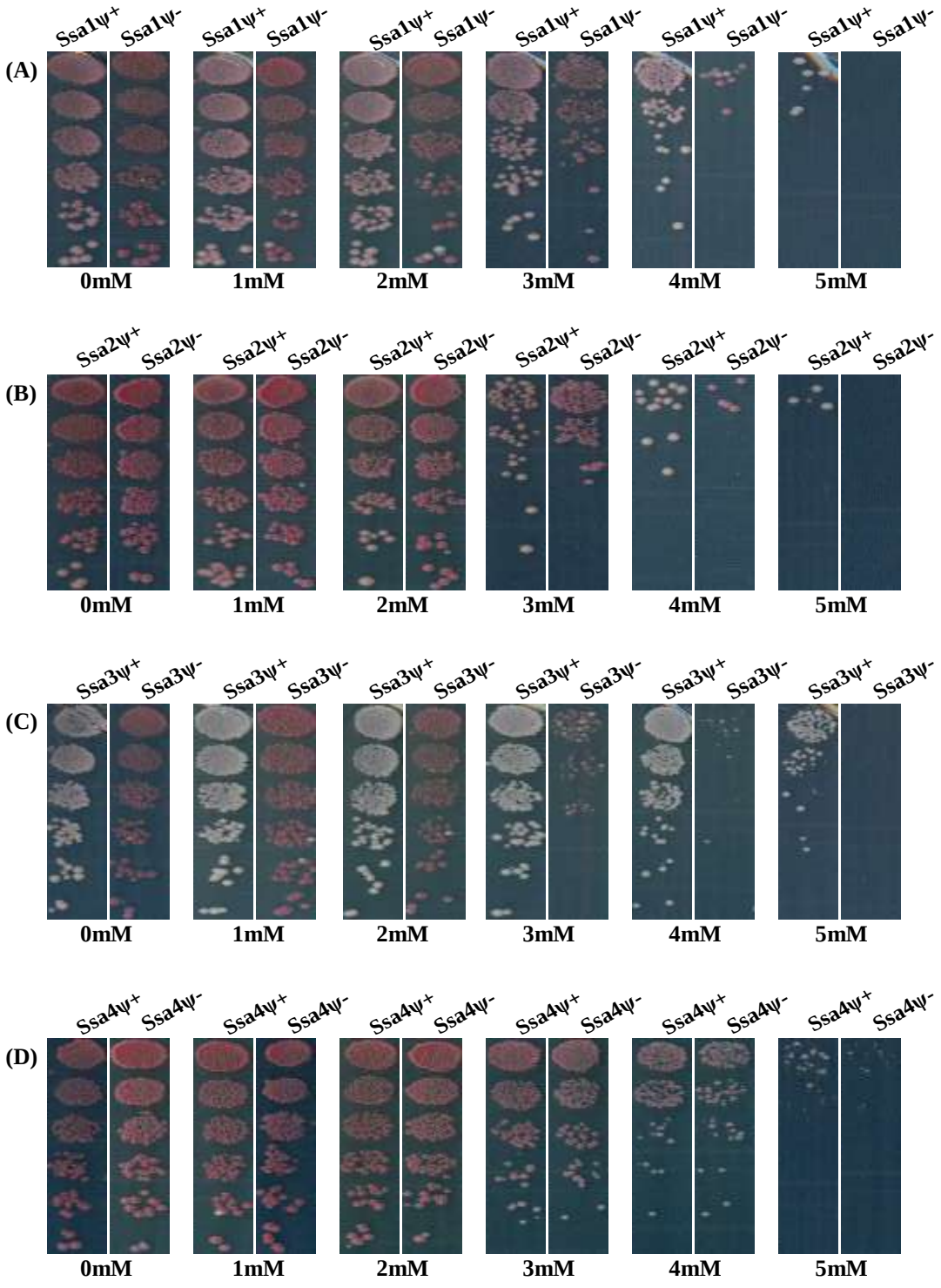



**Figure 5.6 Comparative growth analysis of the Ssa1-4 in response to oxidative stress.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then re-suspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C. Ssa1-4 expressed in G402 as sole source of Ssa in the yeast strain.

### 5.2.7 Assessment of acquired thermotolerance activity of the Ssa family

The molecular chaperone Hsp104 is an important chaperone involved in acquired thermotolerance of *S. cerevisiae* ensuring survival at extreme temperature (Sanchez & Lindquist, 1990). This chaperone is highly conserved in its ability to protect cells from environmental stress under wide range of physiological conditions and can be found in organisms ranging from yeast to *Arabidopsis* (Parsell et al., 1994b). Additionally, Hsp104 forms a protein refolding machinery with Hsp70 and Hsp40 that acts in resolubilisation of aggregated proteins (Glover & Lindquist, 1998). The survival rate of yeast post lethal heat shock can be greatly improved by pre-treating cells with mild non-lethal heat shock stimulating elevation of heat shock proteins including Hsp70 and Hsp104 (Landry et al., 1982; Lindquist & Craig, 1988). To assess the activity of Hsp104, a well established acquired thermotolerance assay (described in Section 2.16) was carried out whereby cell survival represented *in vivo* functional Hsp104 activity.

As shown in (Figure 5.7 A), the [*psi*$^-$] strains expressing inducible Hsp70 isoforms were more resistant to heat shock at 47 °C than those expressing constitutive Hsp70s. After different periods of exposure to 47 °C, more heat resistant cells were recovered for inducible isoforms than constitutive isoforms of the [*psi*$^-$] version. Also on 3 mM Gdn-HCl that inhibits Hsp104, the heat inducible isoforms show more survival rate compared to its constitutive counterparts (Figure 5.7 B). The [*PSI*$^+$] version of Ssa family was efficient in Hsp104 activity and no distinction could be made between heat inducible and constitutive isoforms of [*PSI*$^+$] in showing acquired thermotolerance except for Ssa3 [*PSI*$^+$] on 3 mM Gdn-HCl which showed more recovery of heat denatured proteins.

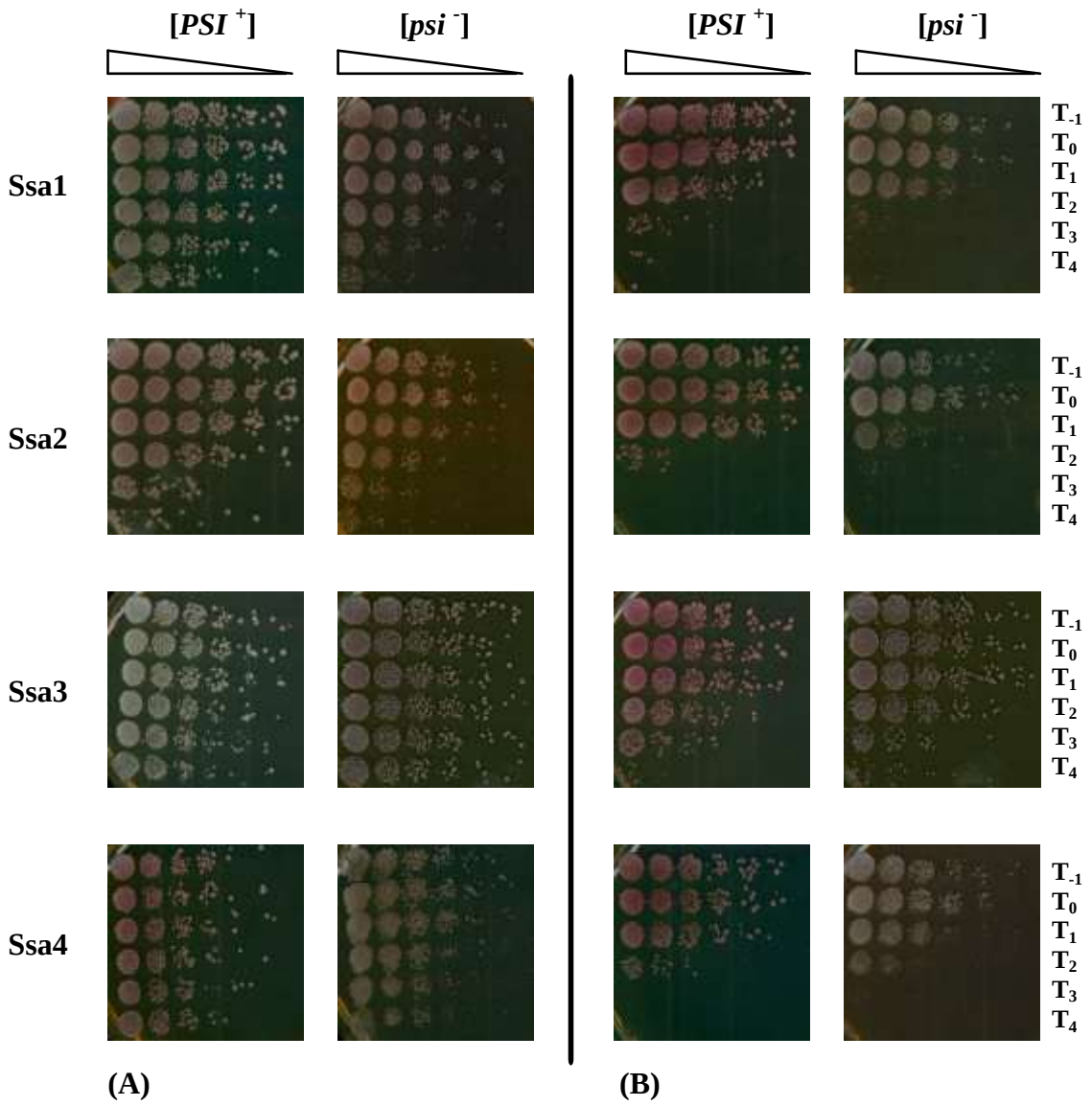



**Figure 5.7 Thermotolerance assay of Ssa1-4.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39ºC to induce Hsp104 expression to protect against heatshock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD (A) and 3mM Gdn-HCl (B) for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated at 30 ºC for 2 days followed by 1 day at RT and were monitored for cellular thermotolerance.

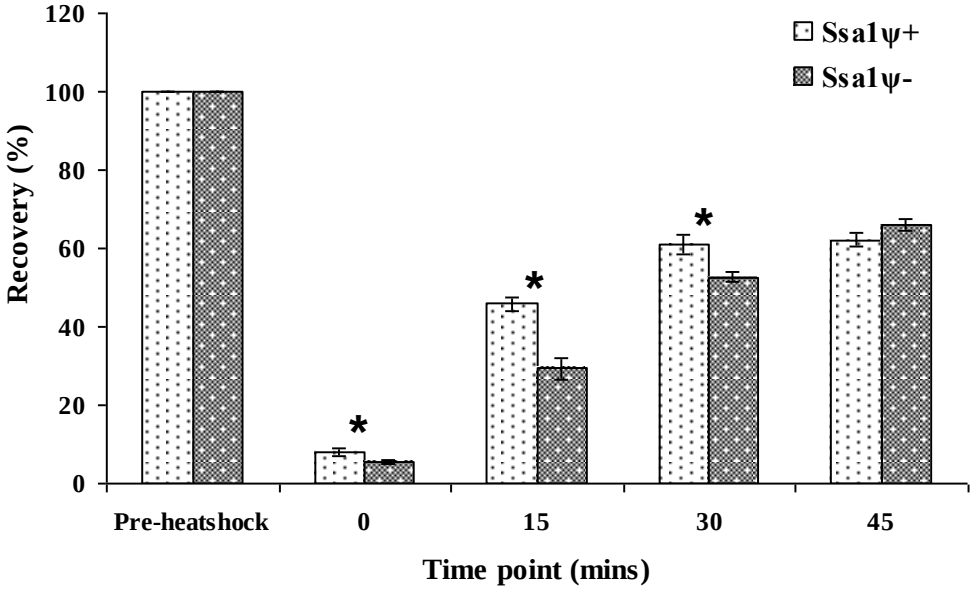
### 5.2.8 Individual Ssa's vary in their protein refolding ability *in vivo*

The protein refolding ability of the chaperones can be assessed by monitoring reactivation of luciferase protein which is a thermolabile bacterial protein at elevated temperature. Hsp104 along with the assistance of Hsp70 and Hsp40 becomes critical for survival at elevated temperature that causes wide spread protein aggregation. The Hsp104-Hsp70-Hsp40 protein refolding machinery causes protein disaggregation and thus assist in protein refolding (Glover & Lindquist, 1998). The luciferase reactivation was monitored in Ssa1-4 by shifting exponentially growing cells from 30 °C to 37 °C for 30 min to induce the expression of heat shock proteins and then to 45 °C to cause extensive aggregation of luciferase. This was followed by recovery of the strains at 25 °C for 45 min and the recovery was assessed at intervals of every 15 min.

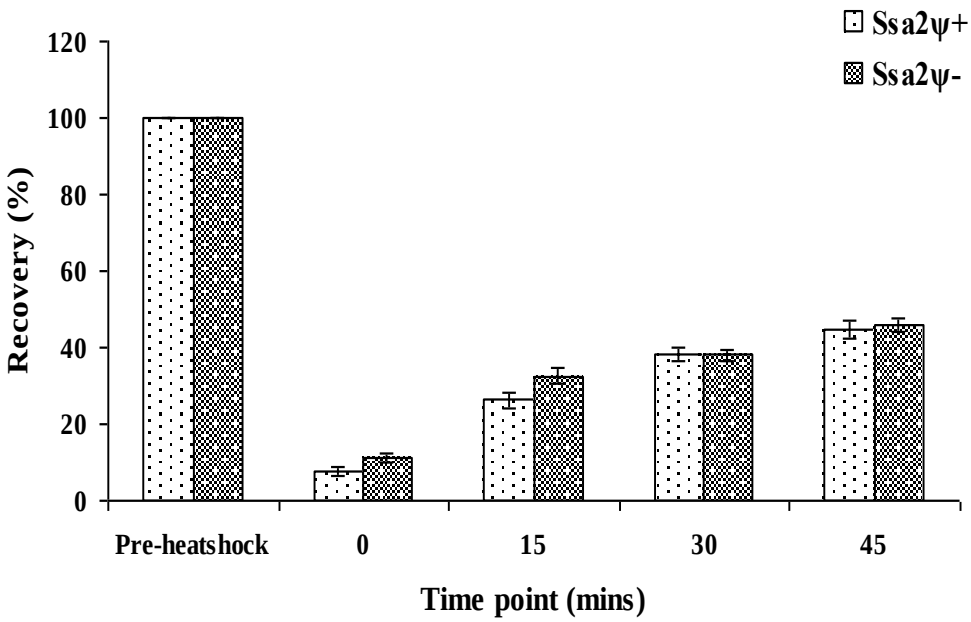
As shown in Figure 5.8 A and B there was no difference observed between the [$PSI^+$] and [$psi^-$] versions of Ssa1 and Ssa2 in their protein refolding activity whereas a difference was observed for the [$PSI^+$] and [$psi^-$] versions of Ssa3 and Ssa4 in refolding luciferase during the recovery period (Figure 5.8 C and D).

Also among the [$PSI^+$] version of the Ssa family, Ssa1 showed the highest recovery of 73% whereas in Ssa3 which showed the second highest recovery, 67% of the luciferase activity was restored. Ssa2 and Ssa4 had a similar luciferase recovery rate of 48% and 50% respectively (Figure 5.9). A similar trend of recovery was observed among the [$psi^-$] strains as well, with Ssa1 showing the highest recovery rate of 65% and Ssa3 being second highest (46%) in recovering luciferase (Figure 5.9). Thus from these results it can be speculated that Ssa1 and Ssa3 are more efficient in luciferase reactivation compared to Ssa2 and Ssa4.

**(A)**


120
100
80
60
40
20
0
Recovery (%)
Ssa1ψ+
Ssa1ψ-
*
*
*
Pre-heatshock
0
15
30
45
Time point (mins)

**(B)**


120
100
80
60
40
20
0
Recovery (%)
Ssa2ψ+
Ssa2ψ-
Pre-heatshock
0
15
30
45
Time point (mins)

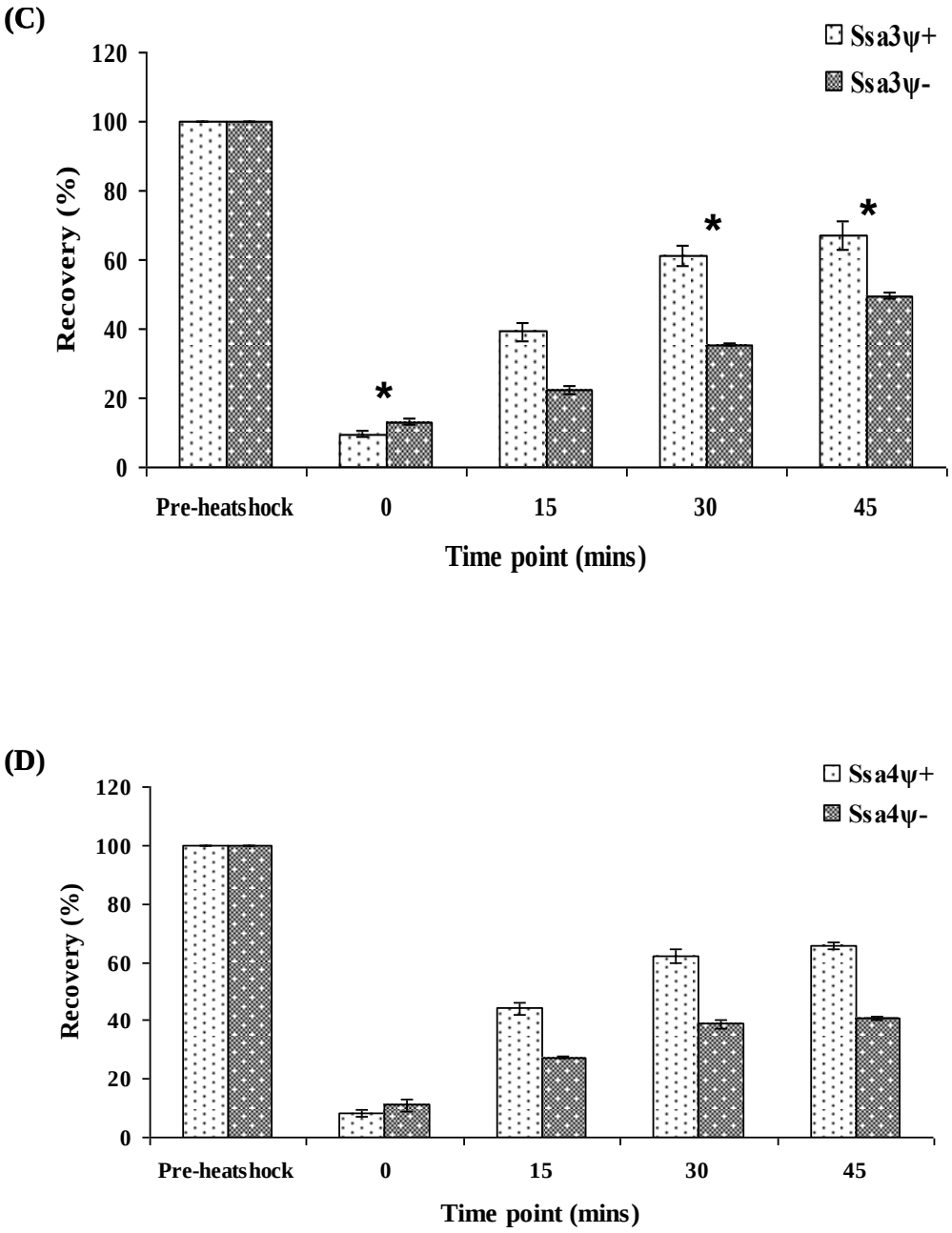



**Figure 5.8 Comparison of luciferase activity of [*PSI*$^{+}$] and [*psi*$^{-}$] version of the Ssa family.** Overnight cultures were diluted in fresh SC medium lacking uracil to an $OD_{600nm}$ = 0.1. The cultures were then shifted to 37 °C for 30 min to induce the expression of Hsp104. After 30 min at 37 °C, the cultures were shifted to 45 °C for one hour. Cyclohexamide was added to the cultures after 50 min at 45°C to prevent any further synthesis of luciferase during the recovery period. Luciferase activity, which is expressed as a percentage of pre-heat shock activity was measured at regular intervals during the recovery period of 45 min at 25 °C. (Bar indicates SEM. Value followed by * are significantly different at $p \leq 0.05$)

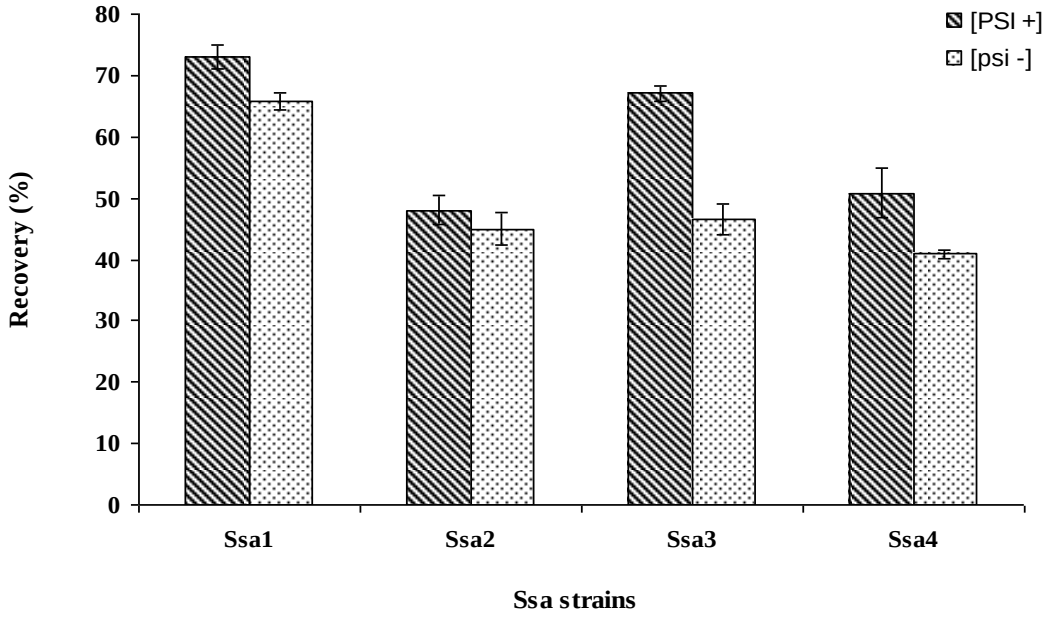



**Figure 5.9 Luciferase refolding activity of Ssa family at final recovery period of 45 min.** Overnight cultures were diluted in fresh SC medium lacking uracil to an $OD_{600nm}$ = 0.1. The cultures were then shifted to 37 °C for 30 min to induce the expression of Hsp104. After 30 min at 37 °C, the cultures were shifted to 45 °C for one hour. Cyclohexamide was added to the cultures after 50 min at 45 °C to prevent any further synthesis of luciferase during the recovery period. Luciferase activity, which is expressed as a percentage of pre-heat shock activity was measured after allowing the cells to recover for 45 min at 25 °C. [LSD at 0.05 = 21.59]

### 5.2.9 Basal levels of expression of Hsp104 and Hsp70 in yeast cells expressing individual Ssa's

The basal levels of expression of Hsp104 and Hsp70 in yeast cells expressing individual Ssa's are critical in contribution of these molecular chaperones for impairment of [*PSI*$^+$] propagation. Thus western blot analysis was performed to assess the levels of Hsp104 and Hsp70. In addition to analysing abundance of Hsp104 and Hsp70 in cell, western blot should also confirm that each strain is expressed to similar level in both [*PSI*$^+$] and [*psi*$^-$] variants. Abundance of [*psi*$^-$] version of Ssa's appears to be slightly lower than the [*PSI*$^+$] versions when probed with general Hsp70 antibody (Figure 5.10). The reason for this difference is unknown but may provide an answer to some phenotypic stress differences between these variants. Also there appears to be no difference in the amount of Hsp104 being expressed between the strains.

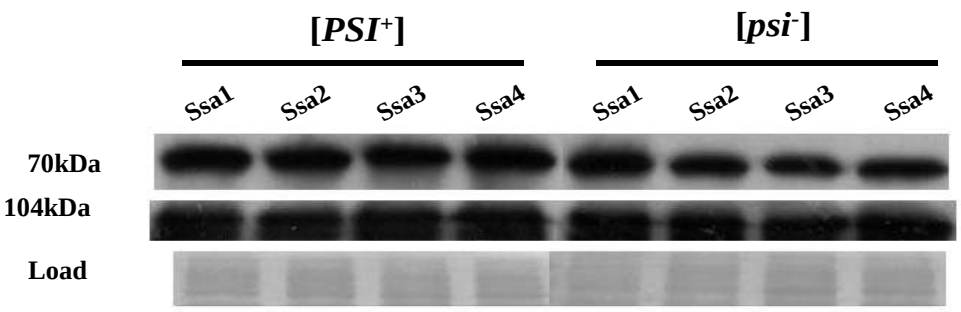



**Figure 5.10 Relative abundance of Hsp70 and Hsp104 in yeast cells expressing individual Ssa's.** Western blot analysis was performed to examine the abundance of Hsp70 and Hsp104. Blots probed with anti-Hsp70 antibodies were stripped and reprobed with anti-Hsp104 antibodies. Membrane was then stained by amido black as a loading and transfer control, are shown (Load). The Ssa strains are indicated on the top.

## 5.3 Strategy for isolating Hsp70 ATPase domain mutations that impair [*PSI*$^+$] propagation

Previous work carried out by Jones & Masison (2003) showned that the ATPase domain of Hsp70-Ssa plays a significant role in yeast prion propagation. Further insight into the influence of ATPase domain of Hsp70-Ssa on prion propagation comes from the extensive genetic screen carried out to identify around 25 mutants in both the major cytosolic Ssa proteins, Ssa1 and Ssa2, that impair prion propagation (Loovers et al., 2007). Some of the mutations isolated both in *SSA1* and *SSA2* had a similar affect on [*PSI*$^+$] propagation thus supporting the suggestion that the *SSA* gene family are redundant in [*PSI*$^+$] propagation (Allen et al., 2005). A subset of these mutants (A53, G73, R74, A146 and T223) are located in a region, which has recently been shown to be important for inter-domain communication in the *E. coli* Hsp70, DnaK (Revington et al., 2005). Another subset of the mutant residues (R259, G287, T295 and A297) is located within the IIB region of the ATPase domain. The IIB region of the ATPase domain has been implicated as interacting with the Fes1 homologue, HspBP1, in mammals (Shomura et al., 2005). Therefore, mutants may be altering the IIB region in a way that Hsp70 cannot interact with the NEF Fes1 in yeast, which then alters [*PSI*$^+$] propagation. To further assess the role of Hsp70 ATPase mutants on [*PSI*$^+$] propagation, this study proposed to further characterise three mutations *viz.* G73D, G287D and T295I which are representative of both subsets. Also as G287D and T295I were isolated from Ssa2 as well this might reveal a similar function of Ssa1 and Ssa2 in regard to prion propagation.

This study also set to investigate into the functional conservations of the ATPase mutations across the cytosolic Ssa family by generating mutations by doing site directed

mutagenesis in plasmids expressing each individual Ssa protein. All the mutations (G73D, G287D, T295I) in the Ssa1-4 were generated by previous members of the laboratory except for the G73D mutation in Ssa2 and Ssa3 that was generated in this study due to failed attempts of generating these mutations by previous laboratory members.

### 5.3.1 Conservation of ATPase mutations across Ssa family

A high degree of conservation of Hsp70 family is observed in all forms of life ranging from Archaea, eubacteria, eukaryotes and organelles (mitochondria, chloroplast) (Karlin & Brocchieri, 1998). One of the subfamily of cytosolic Hsp70 implicated in yeast prion propagation is the Hsp70-Ssa family. Therefore, conservation of mutations of Hsp70, that impair prion propagation, may be useful targets for therapeutics or designing treatments for prion diseases. The results from this study supported the work carried out by Loovers et al. (2007) which showed that the mutations G287D and T295I impair prion propagation in Ssa1 as well as in Ssa2 (Figure 5.11). Also investigation into the functional conservation of these two mutations in Ssa3 and Ssa4 revealed impairment of [*PSI*$^+$] propagation thus supporting the fact that the two mutations G287D and T295I are functionally conserved in Ssa3 and Ssa4 (Figure 5.11). When the mean generation time was assessed for the mutants of the Ssa family it was observed that the T295I exhibited a slower growth rate compared to its WT [Ssa3] but the growth rate of the same mutant in Ssa4 was faster compared to the WT [Ssa4]. No difference in growth rate was observed for the other mutants of the Ssa family compared to its appropriate WT when considered from biological point of view although these differences in growth rate was statistically significant ($p < 0.05$). The generation time of the yeast strains expressing *SSA3* or *SSA4* mutant alleles as sole source of Ssa protein appeared to grow at a much slower rate compared to the mutant alleles of its constitutive counterparts (Table 5.1). The G73D mutation appeared to have different effect on both the constitutive and heat inducible isoforms of Hsp70. The G73D mutation impaired [*PSI*$^+$] propagation in Ssa1 and Ssa2 but appeared to make Ssa3 and Ssa4 protein non-functional as this mutation generated a lethal phenotype (Figure 5.11). The impairment [*PSI*$^+$] propagation by this mutation on the Ssa family was assessed by the colour on YPD at 30 °C and growth on medium lacking adenine at 30 °C and RT.

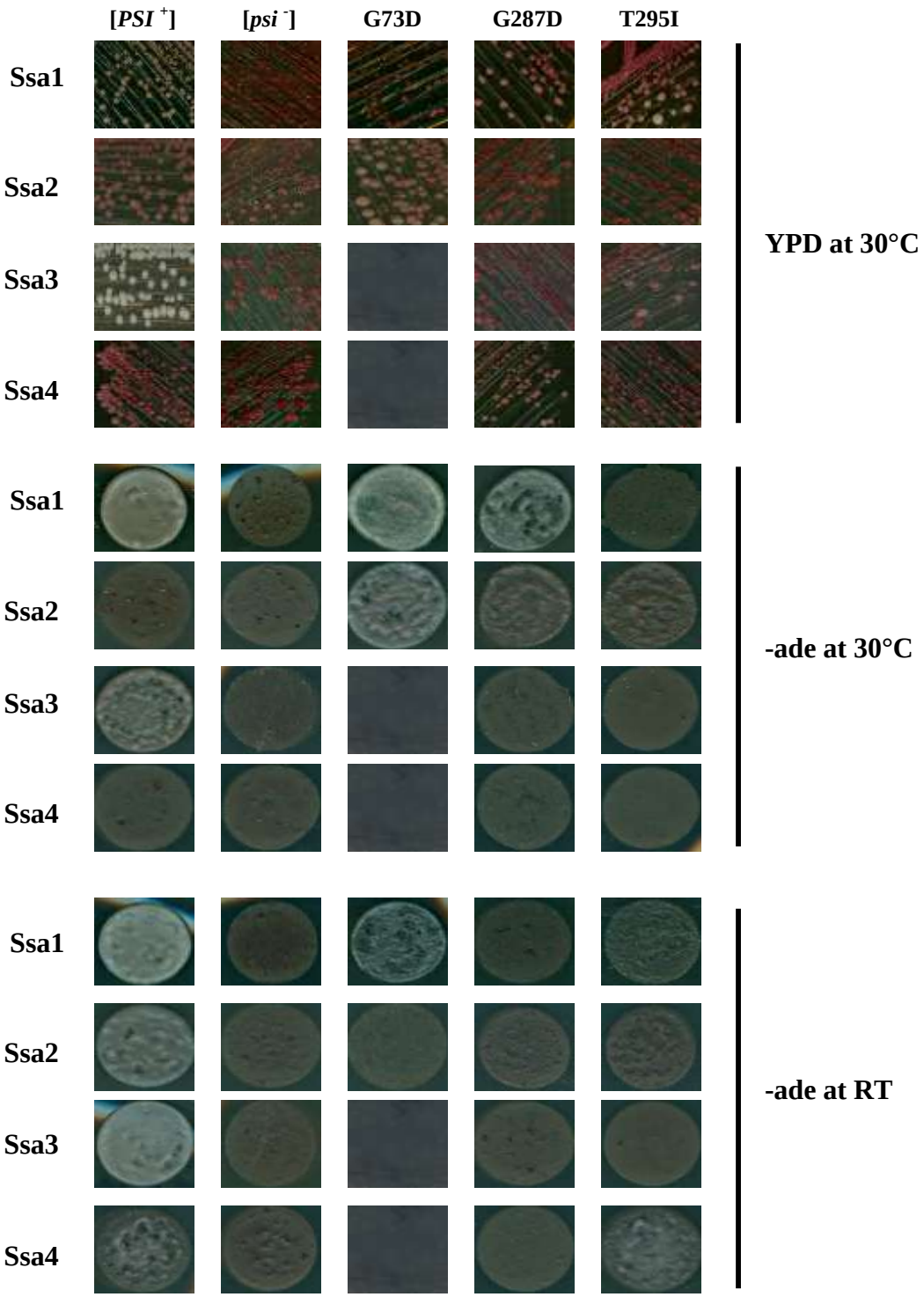



**Figure 5.11 Prion phenotype of yeast cells expressing *SSA1-SSA4* mutant alleles as a sole source of Ssa protein.** Cells were streaked from 5-FOA onto YPD and colonies came up after 2 days of incubation at 30 °C followed by 2 days at 4 °C. 20 µl spots were placed on − ade plates and incubated at 30 °C for 3 days and at RT for 3 days. Colour of the mutant strains ranged from white to pink reflecting the varying degree of *ade 2-1* suppression due to the mutation (as described in text). The extent of *ade 2-1* suppression is also reflected as density of growth on − ade plates

**Table 5.1 Relative effects of *SSA1-SSA4* mutations on cell growth.**

| **Mutants** | ±**MGT (min)** |
|---|---|
| WT [Ssa1] | 108.18 ± 1.1 |
| Ssa1 $^{G73D}$ | *115.12 ± 1.7 |
| Ssa1 $^{G287D}$ | *117.11 ± 0.2 |
| Ssa1 $^{T295I}$ | *116.26 ± 1.0 |
| WT [Ssa2] | 113.25 ± 0.8 |
| Ssa2 $^{G73D}$ | *109.19 ± 0.8 |
| Ssa2 $^{G287D}$ | *112.17 ± 0.3 |
| Ssa2 $^{T295I}$ | *103.44 ± 0.6 |
| WT [Ssa3] | 158.67 ± 2.0 |
| Ssa3 $^{G287D}$ | *167.64 ± 2.4 |
| Ssa3 $^{T295I}$ | *178.30 ± 1.8 |
| WT [Ssa4] | 177.52 ± 1.9 |
| Ssa4 $^{G287D}$ | 180.29 ± 1.1 |
| Ssa4 $^{T295I}$ | *151.38 ± 3.1 |

±The mean generation time of the yeast strains were determined by diluting the overnight 30 °C culture in 30 ml fresh media to an $OD_{600nm}$ of 0.1(t = 0 min). Cultures were transferred to 30 °C shaking incubator and cell density was measured at an interval of every 2 h as described in Section 2.14. (* indicate significant difference between WT and the mutants at $p < 0.05$)

### 5.3.2 Heat shock response of the ATPase mutants of Ssa family

As highly conserved Hsp70 assist protein folding and also protect cells from stress by preventing aggregation of stress denatured proteins, it is important to look into the heat shock functions of the ATPase mutants of Hsp70. The mutations on Ssa1 and Ssa2 appear not have any affect on Hsp70 general functions, as the cells expressing each mutant as the sole source of Ssa were viable at 37 °C and 39 °C. However, the mutations G287D and T295I on Ssa3 and the mutation G287D on Ssa4 produced a temperature sensitive phenotype at 37 °C and 39 °C (Figure 5.12). But, the recovery of these temperature sensitive mutations on 1M sorbitol (Figure 5.13) suggest that these mutations may affect the function of Hsp70 at elevated temperature and also implicates Hsp70-Ssa's involvement in CWI signalling pathway.

### 5.3.3 Response of the *SSA1-SSA4* mutant to cell wall damaging agents

To further support the hypothesis of involvement of Hsp70-Ssa's in CWI signalling pathway, the *SSA1-SSA4* mutant alleles were tested in presence of different cell wall damaging agents like SDS (0.005-0.01%), caffeine (8 µm), calcofluor white ( 10 µg/ml) when these mutant alleles were expressed as the sole source of Ssa in the cell. If the Ssa family of Hsp70 is involved in CWI signalling pathway they should show sensitivity to cell wall damaging agents as damage to the cell wall elicits this pathway. Comparative growth analysis was performed as described in Section 2.15 and cells were selected on medium with varying concentrations of SDS, caffeine, calcofluor white with YPD as a control plate and monitored after 3 days of incubation at 30 °C (Figure 5.14). All the *SSA1-SSA4* mutant alleles were sensitive to calcoflour white at a concentration of 10 µg/ml but did not exhibit any sensitivity to caffeine (data not shown). This sensitivity displayed to calcofluor white and caffeine may be due to strain background and need to be tested in another strain background. However, there was a varying degree of sensitivity observed on two different concentrations of SDS (Figure 5.14). All the mutant alleles on *SSA1-SSA4* showed sensitivity to SDS at a concentration of 0.01%. The G73D mutation on Ssa1 showed marked sensitivity to SDS at a concentration of 0.005% compared to its wild type. For the G287D mutation, sensitivity was observed on SDS at a concentration of 0.05% for all the Ssa's. In case of T295I mutation, sensitivity to 0.05% SDS was more pronounced on the heat inducible isoforms than its constitutive counterparts (Figure 5.14). However, due to varying degree of sensitivity seen for *SSA1-SSA4* mutant alleles on SDS, an accurate comparison could not be made. These results

are suggestive but not conclusive of the involvement of the Ssa family of Hsp70 in CWI signalling pathway.

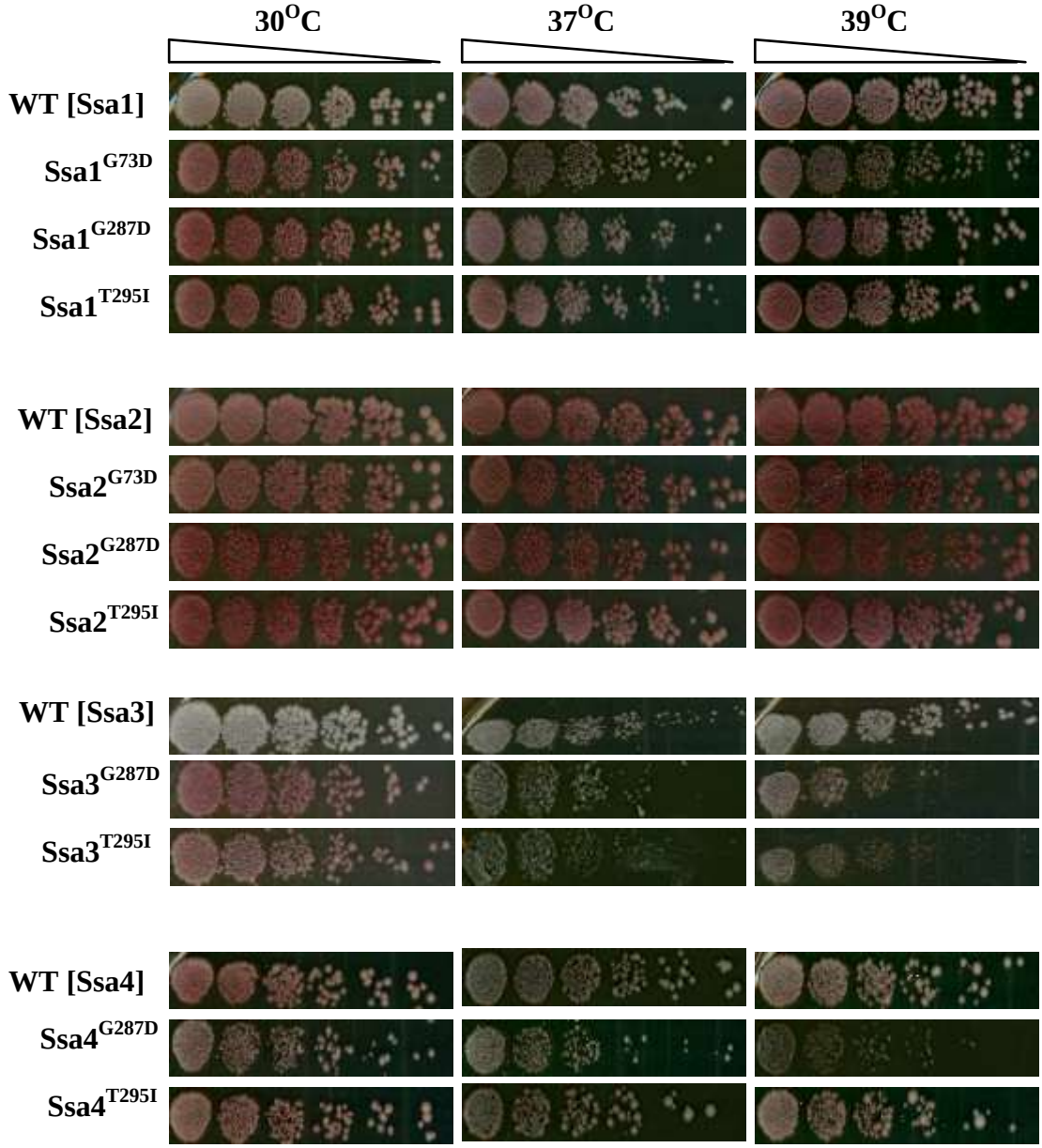



**Figure 5.12 Temperature sensitive phenotype of yeast cells expressing *SSA1-SSA4* mutant alleles as a sole source of Ssa protein on YPD plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

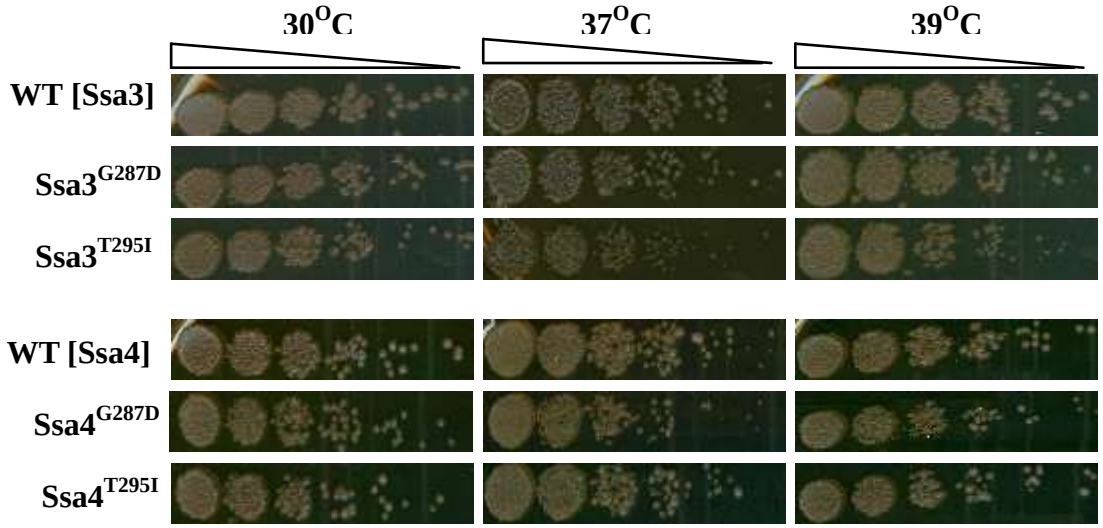



**Figure 5.13 Recovery of temperature sensitive phenotype of yeast cells expressing *SSA3* and *SSA4* mutant alleles as a sole source of Ssa protein on 1 M sorbitol plate.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C, 37 °C and 39 °C and were monitored for temperature sensitivity.

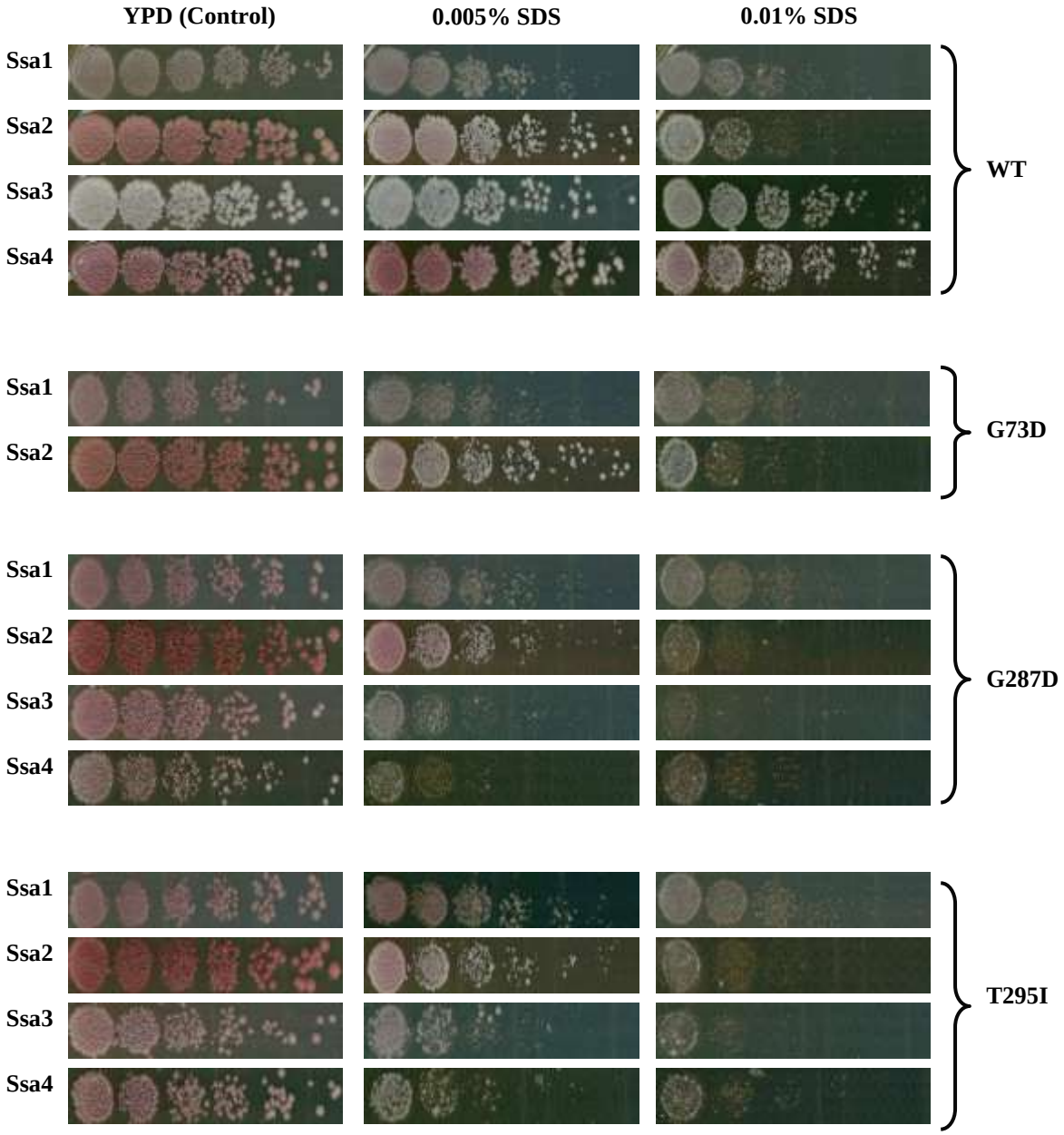



**Figure 5.14 Comparative growth analysis of the *SSA1-SSA4* mutant alleles in response to cell wall damaging agents.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C. *SSA1-SSA4* mutant alleles expressed in G402 as sole source of Ssa protein in the yeast strain.

### 5.3.4 Analysis of Ssa1-Ssa4 mutant in response to oxidative stress

Oxygen which is vital for life can also induce oxidative stress response and thus cause cellular damage (Ames et al., 1993). It is a highly reactive molecule and all aerobically growing organisms are continuously exposed to chemically reactive agents known as reactive oxygen species (ROS) such as super oxide anion ($O_2^-$), hydroxyl radical (OH) and $H_2O_2$ and all these forms of ROS is inadvertently formed when molecular oxygen is reduced during oxidative phosphorylation (Lushchak, 2011). The generation and degradation of ROS is under refined cellular control and when the balance between the production and elimination of ROS is disturbed it causes oxidative stress. In *S. cerevisiae*, the PKC1-MAPK cell integrity pathway is involved in responses to a wide variety of stresses (Davenport et al., 1995; Kamada et al., 1995; Torres et al., 2002). Previous studies have shown that Pkc1 and the upper elements of the cell wall integrity pathway are required for survival and adaptation to the oxidative stress provoked by oxidizing agents like hydrogen peroxide and diamide (Vilella et al., 2005).

Comparative growth analysis was performed and the yeast strains were tested for oxidative stress in response to varying concentrations of $H_2O_2$ when the *SSA1-SSA4* mutant alleles were expressed as a sole source of Ssa in the cell (Figure 5.15). Growth of the mutants G73D, G287D and T295I of Ssa family were assessed in the presence of 1-4 mM concentrations of $H_2O_2$. At a concentration of 2 mM $H_2O_2$ exposure there was a clear difference observed in growth capacities of the strains expressing G73D, G287D and T295I mutations in Ssa1 and Ssa2 (Figure 5.15) while the appropriate WT was virtually unaffected at this concentration. However, the G287D and T295I mutation in Ssa3 and Ssa4 does not appear to be greatly inhibited by 2 mM $H_2O_2$ exposure. But the G287D mutation in Ssa3 started showing sensitivity to $H_2O_2$ at a concentration of 3 mM while the WT was still unaffected at this concentration. The maximum resistance among the *SSA1-SSA4* mutant alleles to varying concentrations of $H_2O_2$ was exhibited by the mutations on Ssa4 which did not show sensitivity to $H_2O_2$ until a concentration of 4 mM was reached.

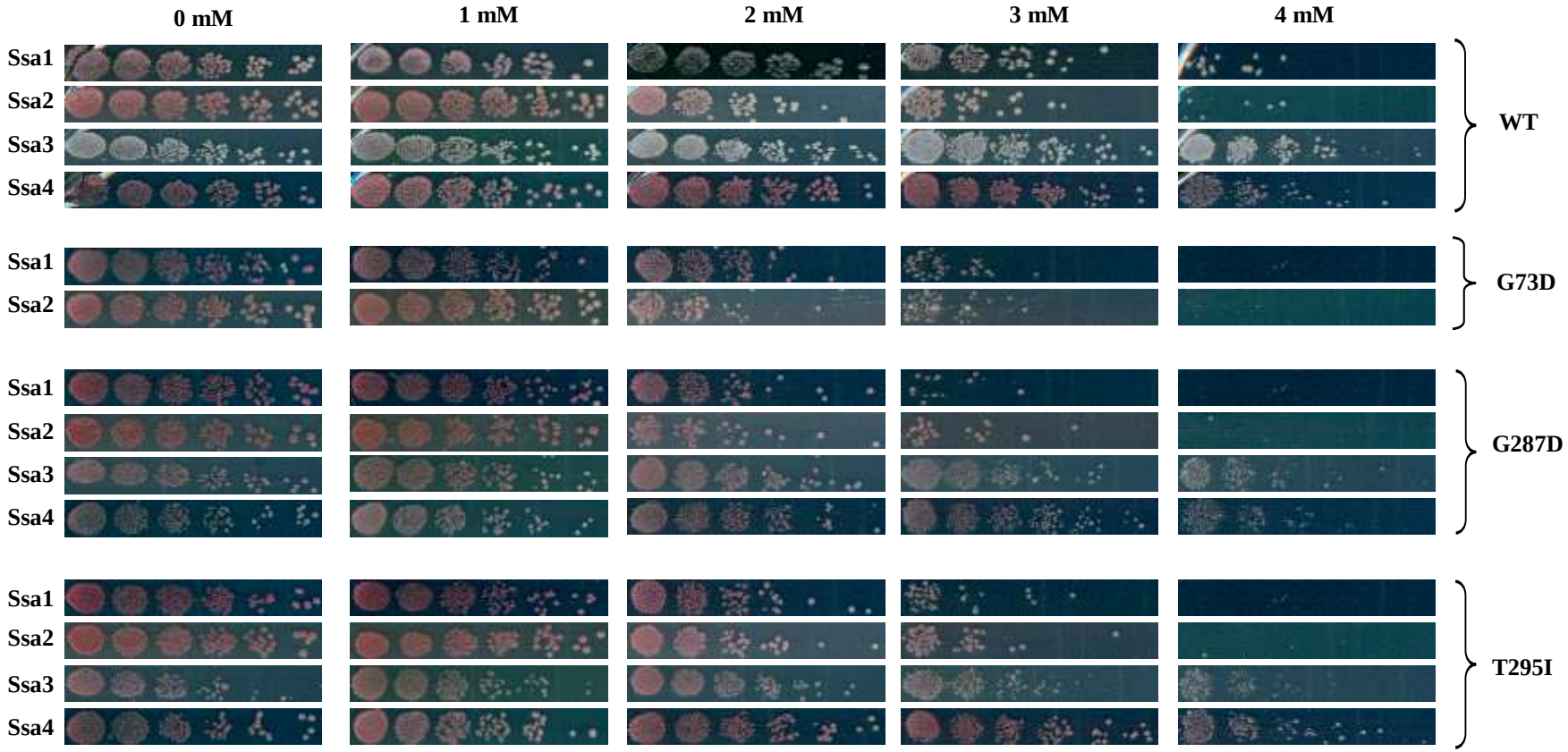



**Figure 5.15 Comparative growth analysis of of the *SSA1-SSA4* mutant alleles in response to oxidative stress.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml and transferred to a microtitre plate. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated for 3 days at 30 °C. *SSA1-SSA4* mutant alleles expressed in G402 as sole source of Ssa protein in the yeast strain.

### 5.3.5 Assessment of acquired thermotolerance ability of *SSA1-SSA4* mutants

As some of the mutant alleles of the Ssa family exhibited temperature a sensitive phenotype at 37 °C and 39 °C, thus the effect of short lethal heat shock (47 °C) on cells individually expressing *SSA1-SSA4* mutant alleles were analysed by performing a well established thermotolerance assay. The Ssa1 mutant strain G73D cannot survive at 47ºC for more than 10 min due to lack of ability to refold heat-damaged proteins. In presence of 3 mM Gdn-HCl that inhibits Hsp104, cell death occurred at initial 10 min exposure to 47ºC indicating that Hsp104 is non-functional in cell due to the G73D mutation in Ssa1(Figure 5.16 A and B). As depicted in Figure 5.16, the T295I mutant strain of Ssa3 exhibited reduced inhibition of growth on YPD as well as 3 mM Gdn-HCl due to the lack of ability of Hsp104 in this mutant strain to refold heat denatured protein. The G287D mutation on Ssa4 also could not survive for more than 20 min at 47 ºC on YPD and were not viable after 10 min heat shock at 47 ºC on 3mM Gdn-HCl plate (Figure 5.16). These results are indicative of reduced Hsp104 activity due to these Hsp70 mutations, perhaps through reduced capacity for the different chaperones to work together.

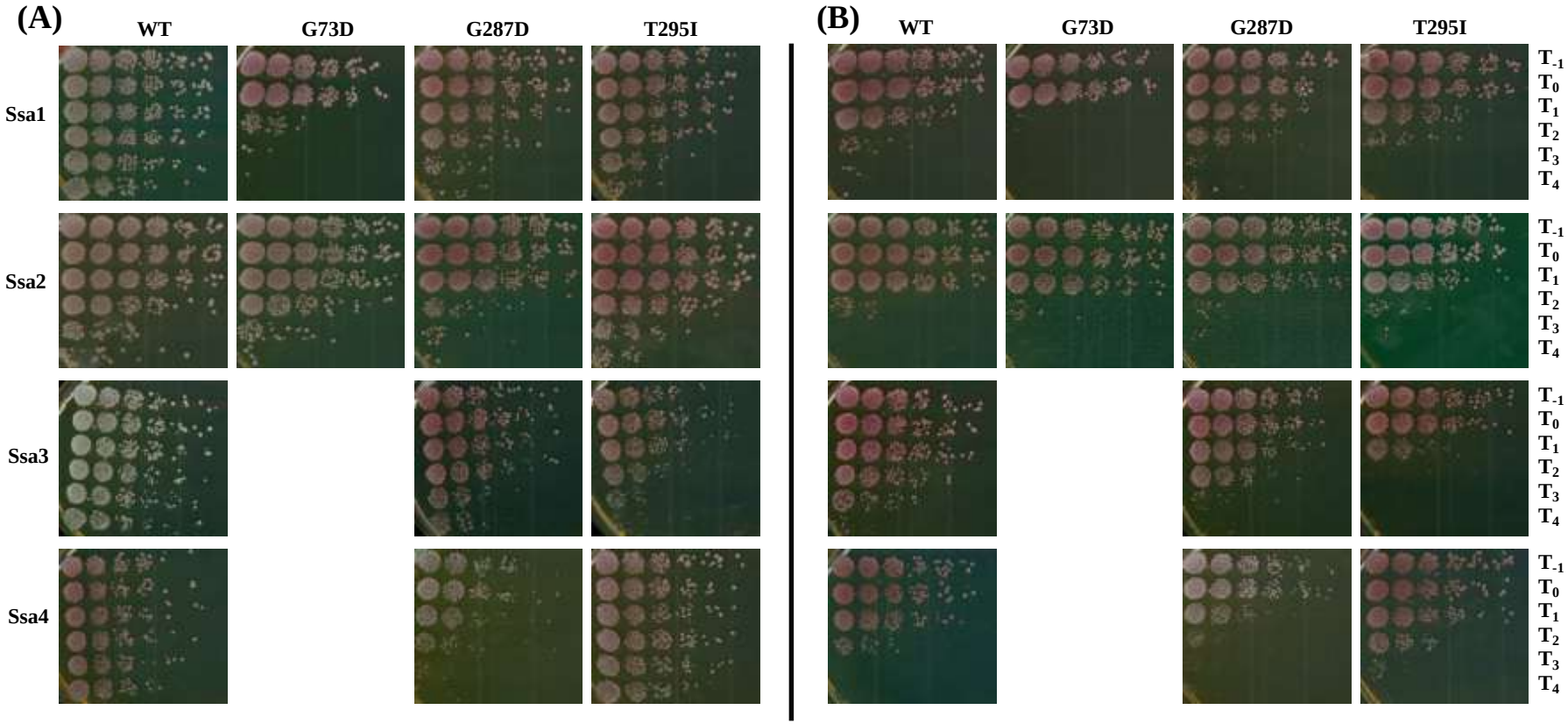
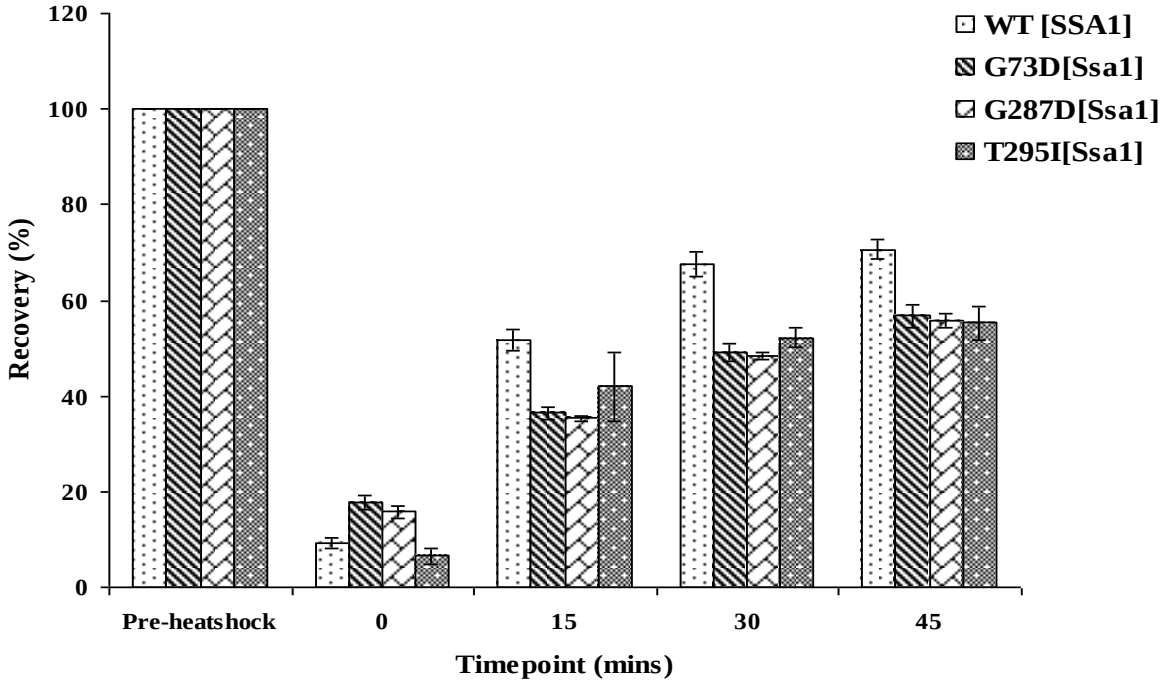



**Figure 5.16 Thermotolerance assay of *SSA1* - *SSA4* mutant alleles of Hsp70.** Overnight culture was diluted in fresh YPD medium to an $OD_{600nm}$ = 0.1 and then the cells were grown to an exponential phase to a density of 3 x $10^6$ cells/ml. Cells were then resuspended in fresh medium to a density of 5 x $10^6$ cells/ml. An aliquot ($T_{-1}$) was then shifted to ice. The cultures were then incubated at 39ºC to induce Hsp104 expression to protect against heatshock. Cells were then incubated at 47 ºC for 0, 10, 20, 30 and 40 min ($T_0$-$T_4$) and plated on YPD (A) and 3mM Gdn-HCl (B) for comparative growth analysis. Representative spots shown in the figure are a neat concentration from a 1 in 5 serial dilution series. The plates were then incubated at 30ºC for 2 days followed by 1 day at RT and were monitored for cellular thermotolerance.

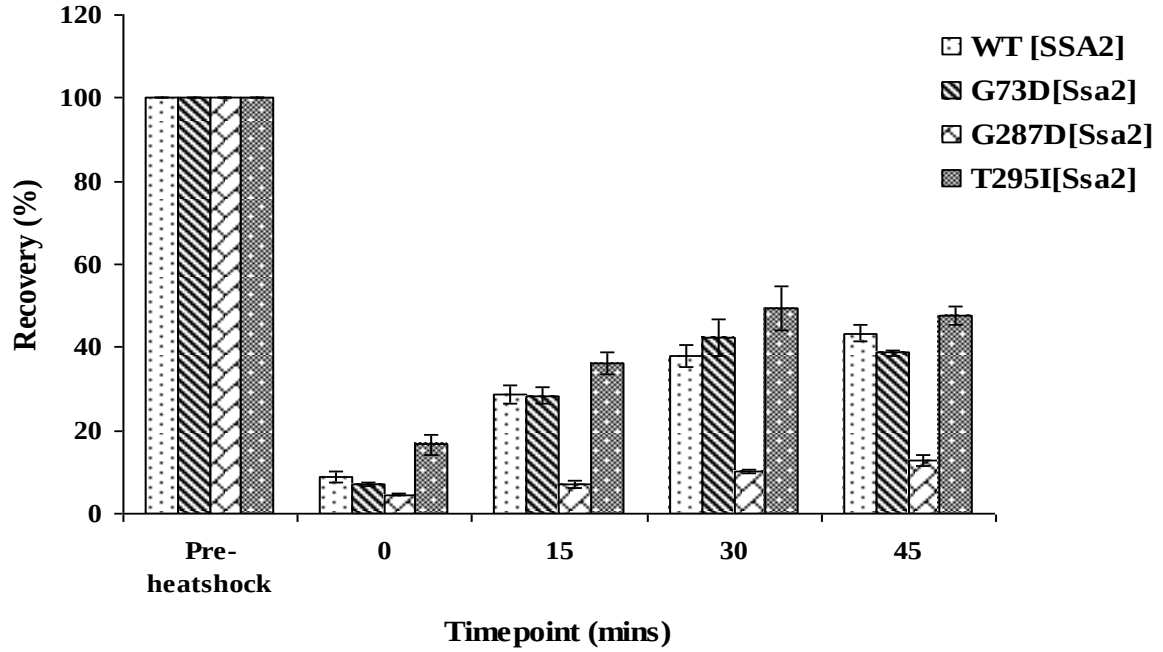
### 5.3.6 Analysis of the effect of Ssa1-Ssa4 mutants in their protein refolding ability *in vivo*

To further support the results obtained from the acquired thermotolerance assay, the luciferase assay was performed that enabled further investigation into the effects of the mutation of the Ssa family on heat shock recovery. As both the assays assessed the Hsp104 protein refolding activity after a heat shock, thus it was expected that this assay would produce results that would more or less correlate with the results obtained from acquired thermotolerance. As chaperone activity, particularly that of Hsp104, is essential in facilitating cellular recovery after heatshock, this particular aspect of chaperone function was examined. The Figure 5.17 A-D shows the rate at which luciferase activity recovers over time after heatshock, reflecting the ability of chaperone proteins to refold luciferase into its functional state. There was a significant difference ($p<0.05$) in the rate of luciferase folding between the WT [Ssa1] and its mutants (Figure 5.17 A). All the mutants of Ssa1 showed a similar luciferase refolding activity compared to WT [Ssa1]. For the Ssa2 mutants the maximum significant recovery was obtained for the T295I mutant after 30 min whereas the mutant G287D behaved like a Δ *hsp104* strain (Figure 5.17 B). The G287D mutation is located within the IIB region of the ATPase domain. The IIB region of the ATPase domain has been implicated as interacting with the Fes1 homologue, HspBP1, in mammals (Shomura et al. 2005). Therefore, the mutant $Ssa1^{G287D}$ may be altering the IIB region in a way that Hsp70 cannot interact with the NEF Fes1 in yeast and this alteration is more pronounced in Ssa2 compared to the other Ssa's thus making Ssa2 less efficient in interacting with Hsp104. This result also reflects some difference between Ssa2 and other members of Ssa family in interacting with NEFs thus providing acquired thermotolerance. In case of the Ssa3 mutants, the maximum recovery was exhibited by the G287D mutant that was significantly more efficient in retaining chaperone activity compared to WT [Ssa3] (Figure 5.17 C). Further to this for the Ssa4 mutants, the T295I mutant showed a significant recovery of 69% over time compared to the WT [Ssa4] whereas the G287D mutant was not as efficient as the WT [Ssa4] in restoring chaperone activity (Figure 5.17 D). Despite the apparent confliction of results from thermotolerance and luciferase assays, the results cannot be compared. But these results from luciferase can be considered because thermotolerance assays involve the assessment of the whole cellular response, leading to cell survival. Thus, it must be considered that a range of proteins contribute to the observed result. Conversely, luciferase assays examine the refolding activity of one protein, thus, the two assays do not equate.

**(A)**


120
100
80
60
40
20
0
Recovery (%)
WT [SSA1]
G73D[Ssa1]
G287D[Ssa1]
T295I[Ssa1]
Pre-heatshock
0
15
30
45
Timepoint (mins)

**(B)**


120
100
80
60
40
20
0
Recovery (%)
WT [SSA2]
G73D[Ssa2]
G287D[Ssa2]
T295I[Ssa2]
Pre-heatshock
0
15
30
45
Timepoint (mins)

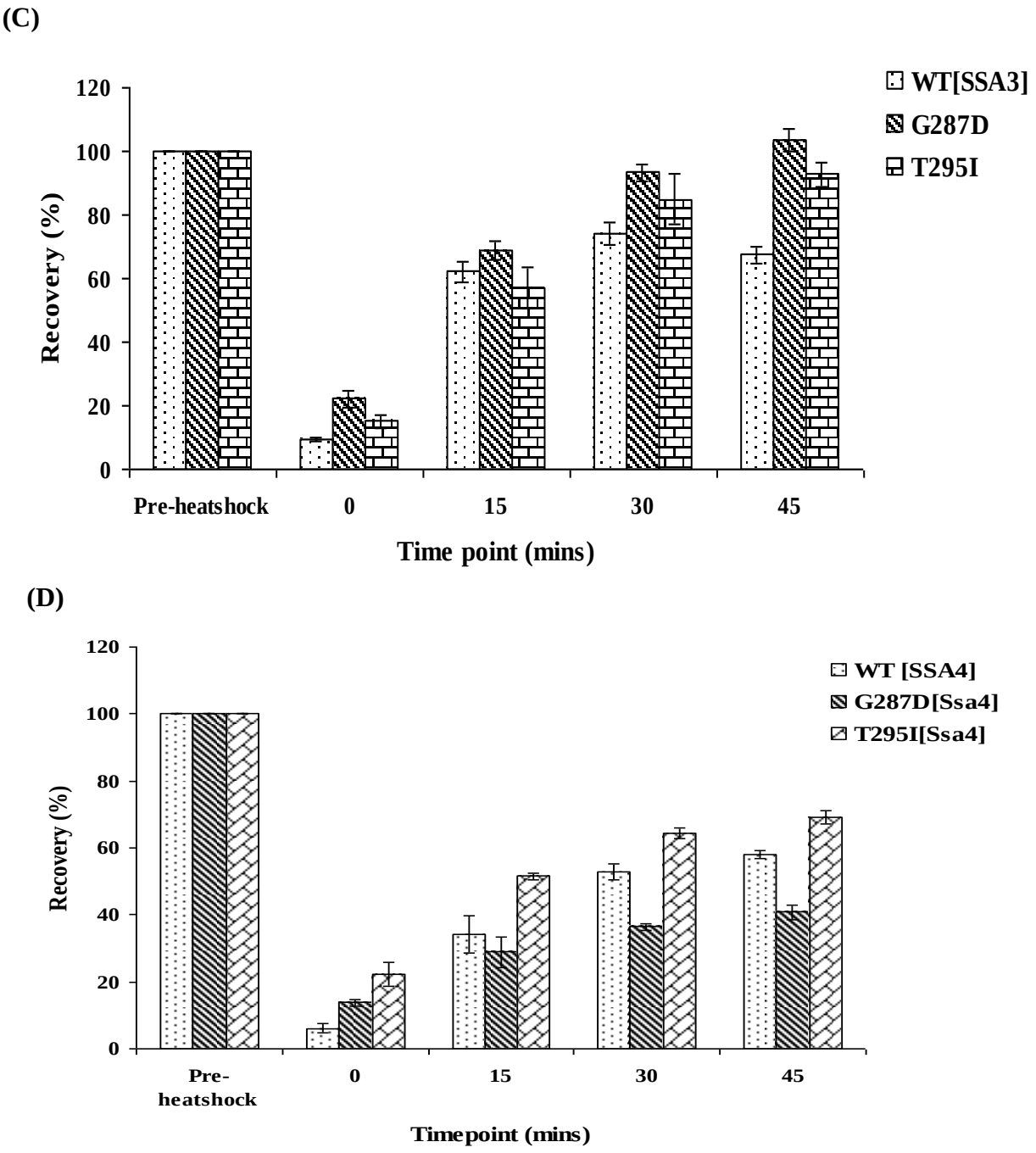



**Figure 5.17 Comparison of luciferase activity of *SSA1-SSA4* mutant alleles.** Overnight cultures were diluted in fresh SC medium lacking uracil to an $OD_{600nm}$ = 0.1. The cultures were then shifted to 37 °C for 30 min to induce the expression of Hsp104. After 30 min at 37 °C, the cultures were shifted to 45 °C for one hour. Cyclohexamide was added to the cultures after 50 min at 45 °C to prevent any further synthesis of luciferase during the recovery period. Luciferase activity, which is expressed as a percentage of pre-heat shock activity was measured at regular intervals during the recovery period of 45 min at 25 °C. [Bar indicates SEM. LSD at 0.05 for (A) = 13.78, (B) = 11.30, (C) = 7.11 and (D) = 16.11]

### 5.4 Strategy for isolating Hsp70 PBD mutations that impair [*PSI* $^{+}$] propagation.

Random mutagenesis screen of Ssa1 identified mutants predominantly on the ATPase domain of Ssa1 that has been shown to affect [*PSI*$^{+}$] propagation (Jones & Masison, 2003; Loovers et al., 2007). Thus, previous laboratory members employed a modified version of the plasmid shuffle technique to locate mutants in PBD and C-terminal domain that effect [*PSI*$^{+}$] propagation (Cusack, 2010). This Ssa1 mutagenesis screen identified four novel mutants (V439I, F475S, M515I, S545F) and two previously characterised mutants (G481D, L483W) localised to the PBD to impair prion propagation (Cusack, 2010). The mutant F475S (Cusack, 2010) and previously characterised mutant L483W (Jung et al., 2000) appeared to have strongest [*psi*$^{-}$] phenotypes. Also attempts were made to generate the F475S mutation by SDM on Ssa2, Ssa3 and Ssa4 to check for the functional conservation of the PBD mutant across Ssa family of Hsp70 to implicate its general role in [*PSI*$^{+}$] propagation. Previous work carried out in the lab were able to generate the F475S mutation only in Ssa3 (Cusack, 2010) which gave a lethal phenotype. Thus, in the present study the F475S mutations were generated in Ssa2 and Ssa4 and analysed for its effect on [*PSI*$^{+}$] propagation and other cellular functions of Hsp70. This F475S mutation was selected from the PBD as previous work has shown that it impairs [*PSI* $^{+}$] propagation in Ssa1 (Cusack, 2010) and thus this mutant was taken as a representative of the PBD to analyse the functional conservation of PBD across the cytosolic Ssa family of Hsp70 and to implicate a general role of Hsp70 in prion propagation.

#### 5.4.1 Phenotypic characterisation of the F475S mutant across Ssa family of Hsp70 implicate a role in prion propagation and cellular function of Hsp70

Previous work carried out in the laboretary on the PBD Ssa1 $^{F475S}$ highlighted that this is the only PBD mutant to date that impair prion propagation and also at the same time exhibit a temperature sensitive phenotype at 37 °C reflecting the significance of this mutant not only in prion propagation but also on the other cellular functions of Hsp70 (Cusack, 2010) . Thus after successfully generating this mutant in Ssa2, Ssa3 and Ssa4 by SDM, these mutants were phenotypically characterised to assess it importance in prion propagation and other cellular functions of Hsp70.

The plasmid shuffle technique was carried out to express the F475S mutant in Ssa1-4 as the sole source of Ssa in the cell and then the mutant strains were assessed for

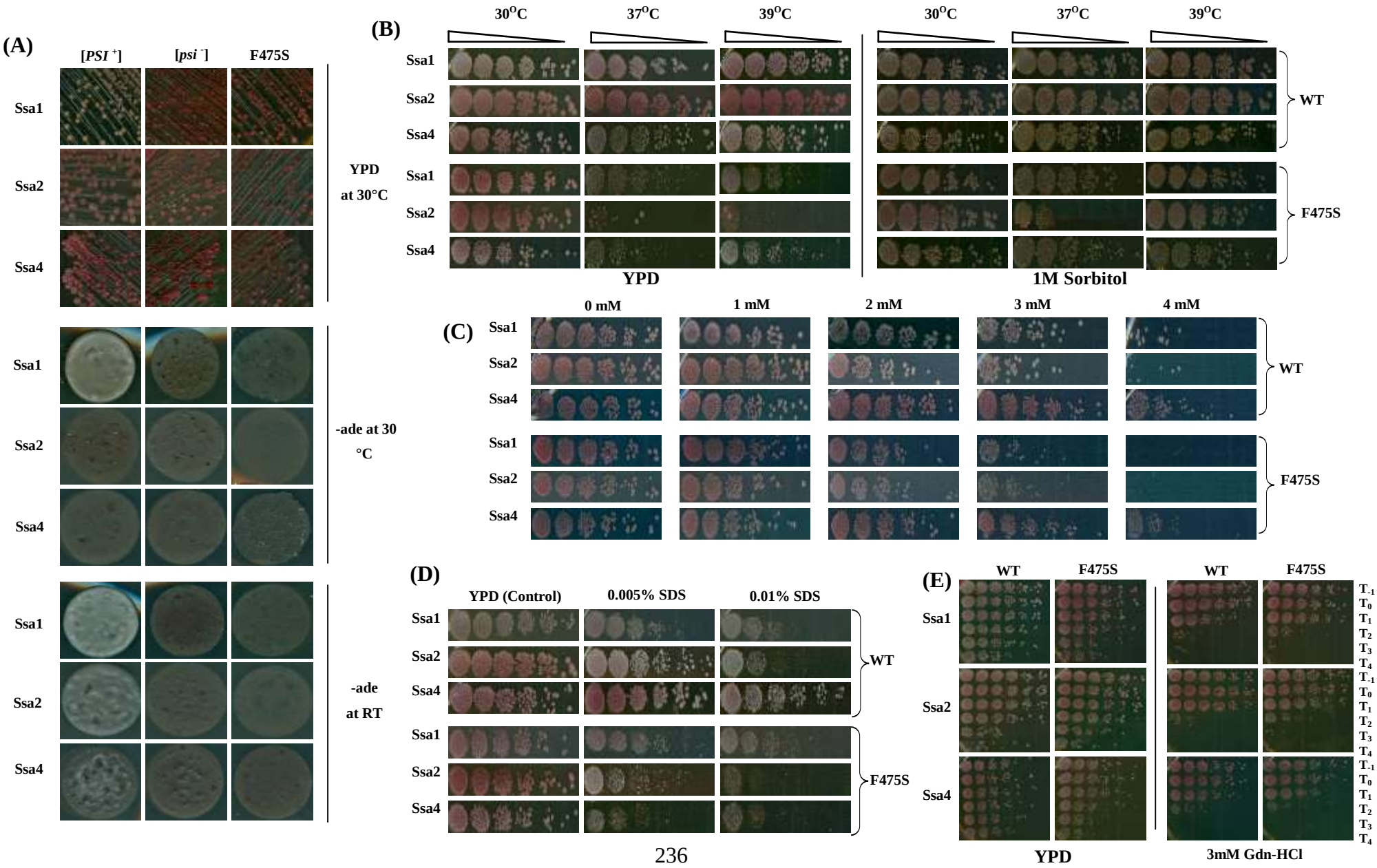
prion phenotype based on the pigmentation on YPD plate and growth on adenine lacking medium. The [*PSI*$^{+}$] propagation was severely impaired by this mutant in all the members of Ssa family as seen by the colour on YPD and growth on adenine lacking medium (Figure 5.18 A). Testing for temperature sensitivity revealed that all the mutant strain were temperature sensitive at 37 °C and 39 °C. These results suggest that the F475S mutant renders all the Ssa's unstable at elevated temperature (Figure 5.18 B). Also it can be speculated that the ability of Hsp70 to rescue aggregated proteins at elevated temperature is greatly hampered by this mutation on PBD. When these temperature sensitive mutants were tested on 1M sorbitol plate, the Ssa1 $^{F475S}$, Ssa4 $^{F475S}$ did recover on 1 M sorbitol, thus indicating the possible involvement of Hsp70 in CWI signalling pathway (Figure 5.18 B). Also the Ssa2 $^{F475S}$ mutant showed recovery on 1 M sorbitol at 39 °C but there was no recovery observed at 37 °C. This might indicate some kind of structural or functional defect in the protein due to the mutation that could be restored by 1 M sorbitol only at an elevated temperature of 39 °C (Figure 5.18 B). When the mutant alleles were tested for their oxidative stress response to varying concentrations of $H_2O_2$, it was observed that the Ssa1 $^{F475S}$ and Ssa2 $^{F475S}$ mutants started showing sensitivity to $H_2O_2$ at a concentration of 2 mM compared to their WT that did grow well at this concentration. But the Ssa4 $^{F475S}$ mutant was resistant to 3 mM $H_2O_2$ and started showing sensitivity only at 4 mM concentration of $H_2O_2$ (Figure 5.18 C). When the F475S mutants were tested in presence of cell wall damaging agents like SDS (0.005-0.01%), calcofluor white (10 µg/ml), caffeine (8 µm) all the mutants exhibited lethal phenotype in presence of calcoflour white but they showed resistance to caffeine (data not shown). The mutants showed varying degree of sensitivity to SDS at different concentrations. The Ssa4 $^{F475S}$ and Ssa2 $^{F475S}$ mutant showed highest sensitivity at a 0.05% concentration of SDS compared to their appropriate WT whereas there was no significant difference in sensitivity observed for Ssa1 $^{F475S}$ at 0.005-0.01% SDS compared to its WT (Figure 5.18 D). When these mutants were assessed for their acquired thermotolerance ability, there appeared to be no significant difference observed between the F475S mutants and its WT with regard to thermotolerance, indicating that the mutants did not have any effect on the Hsp104 function and thus can cope with lethal heat shock in presence of elevated levels of chaperones (Figure 5.18 E). Also the luciferase assay did not reveal any significant difference between the WT and F475S mutants in refolding luciferase during the recovery except for the Ssa2 $^{F475S}$ mutant that was significantly more efficient in refolding luciferase compared to its WT

(Figure 5.18 F). From the Table 5.2 it can be deduced that the F475S mutants have a major effect on the growth rate of *S.cerevisiae* with the biggest difference in growth rate observed for the Ssa2 $^{F475S}$ mutant which is approximately 28% slower in comparison to its WT.

**Table 5.2 Relative effects of Ssa1-Ssa4 mutations of F475S on cell growth.**

| **Mutants** | ±**MGT (min)** |
|---|---|
| WT [Ssa1] | 108.18 ± 1.1 |
| Ssa1 $^{F475S}$ | *121.04 ± 0.2 |
| WT [Ssa2] | 113.25 ± 0.8 |
| Ssa2 $^{F475S}$ | *145.82 ± 3.9 |
| WT [Ssa4] | 177.52 ± 1.9 |
| Ssa4 $^{F475S}$ | *183.31 ± 1.41 |

±The mean generation time of the yeast strains were determined by diluting the overnight 30 °C culture in 30 ml fresh media to an $OD_{600nm}$ of 0.1(t = 0 min). Cultures were transferred to 30 °C shaking incubator and cell density was measured at an interval of every 2 h as described in Section 2.14. (* indicate significant difference between WT and the mutants at $p < 0.05$)


(A)
[PSI +]
[psi -]
F475S
Ssa1
Ssa2
Ssa4
YPD at 30°C
-ade at 30 °C
-ade at RT
(B)
30°C
37°C
39°C
WT
F475S
YPD
1M Sorbitol
(C)
0 mM
1 mM
2 mM
3 mM
4 mM
(D)
YPD (Control)
0.005% SDS
0.01% SDS
(E)
T-1
T0
T1
T2
T3
T4
3mM Gdn-HCl

**(F)**

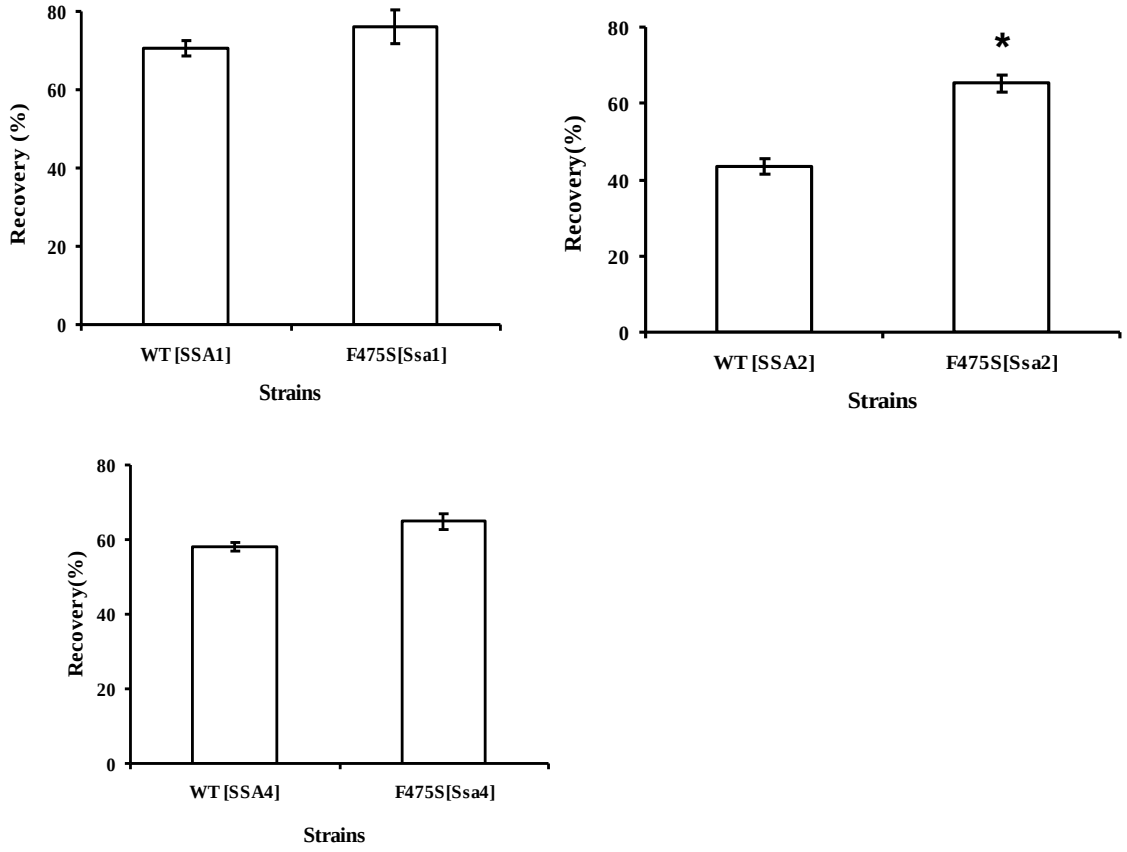



**Figure 5.18 Phenotypic characterisation of the F475S mutant across Ssa family of Hsp70.** (A) Prion phenotype of the mutant alleles on YPD at 30 °C and – ade plates at 30 °C and RT. (B) Temperature sensitive phenotype of the mutant alleles on YPD and 1 M sorbitol for 3 days. (C) Oxidative stress response of the mutant alleles to varying concentrations of $H_2O_2$ (0-4 mM). (D) Effect of cell wall damaging agents on the F475S mutants of Ssa1, Ssa2 and Ssa4. (E) Acquired thermotolerance activity of the mutant alleles. (F) Luciferase refolding activity exhibited by the mutant alleles at a final recovery period of 45 min at 25 °C (Bar indicates SEM and * indicate significant difference between WT [Ssa2] and Ssa2 $^{F475S}$ at $p < 0.05$).

### 5.5 Transcriptional profiling of Hsp70 Ssa family in *Saccharomyces cerevisiae*

As stated earlier in this thesis, Hsp70 is a ubiquitous family of molecular chaperones that perform essential housekeeping functions in protein folding, synthesis, assembly, translocation across membranes, presentation of substrates for degradation, assembly and disassembly of macromolecular complexes or aggregates, gene induction and apoptosis (De Los Rios et al., 2006; Floer et al., 2008; Nollen & Morimoto, 2002; Sangster et al., 2004). They are also involved in quality control process such as protein refolding after stress injury and control the activity of regulatory proteins in signal transduction pathways (Mayer & Bukau, 2005). The broad spectrum of cellular functions attributed to Hsp70 is the outcome of amplification and diversification of *HSP70* gene across evolution. They constitute one of the most conserved protein families in evolution and is a predominant group of Hsps in all cell types ranging from bacteria and plants to humans (Hunt & Morimoto, 1985). One of the main Hsp70 subfamily of yeast cytosol discussed earlier in this chapter is the Ssa family. It has already being stated earlier that these highly homologous orthologs of Hsp70-the Ssa proteins exhibit functional redundancy and differ only by their spatio-temporal expression patterns (Werner-Washburne et al., 1987). The simultaneous deletion of all the four genes is lethal but can be complemented by over-expression of either one of them suggesting functional redundancy (Werner-Washburne et al., 1987). However, this finding was challenged by several other findings in yeast and higher eukaryotes demonstrating functional specificity among Hsp70 isoforms (Kabani & Martineau, 2008). The phenotypic characterisation of the Ssa family discussed in the earlier sections of this chapter also supports this finding of existence of functional specificity among Hsp70 isoforms. For example all the Ssa's exhibited a different phenotype with regard to prion propagation, temperature sensitivity, response to oxidative stress *etc*. Therefore, the current evidence depicts a scenario in which the cytosolic Ssa family controls a large number of cellular functions in yeast cell, probably through a complex interplay of functions that in some cases could be rather unique but in some other cases may be overlapping. Thus in this study a broader and a systematic overview was obtained by carrying out a comparative analysis of the transcription profile from yeast cells expressing Ssa1-4 each as a sole source of Ssa in a [*psi*$^-$] background. One of the hypotheses set in this study was that each of the Ssa may affect downstream regulation of other genes involved in various cellular processes either by its own or in conjugation with other Ssa's. Thus to further gain insight into the functional relationship between

Ssa1-4, the differential gene expression patterns were compared in [*psi*$^{-}$] strains expressing either Ssa2,Ssa3 or Ssa4 as the sole source of Ssa. Strains expressing Ssa1 was taken as a control in this study to compare gene expression levels. RNA was extracted from each of the four strains expressing individual Ssa's and sent to Toray industries, Japan for microarray analysis (as a part of 3D Microarray award to Dr. Gary Jones – custom analysis carried out).

### 5.5.1 Expression profile analysis of yeast strains expressing individual Ssa's

To identify overlapping and specific functions of the members of the Ssa family, this study analysed the alterations generated in the expression patterns by the presence of individual Ssa in the yeast strain. The expression profile of an Ssa deletion strain expressing either Ssa2, Ssa3 or Ssa4 (mutant strain) was compared with the "wild type" (Ssa1 expressed as sole source of Ssa).

The total number of genes induced or repressed in each of the Ssa deletion mutant strains is shown in Figure 5.19. There were a total of 78, 134 and 298 genes induced (>2- fold induction) when Ssa2, Ssa3 or Ssa4 was expressed as a sole source of Ssa in the cell respectively. On the other hand, a total of 147, 120 and 426 genes were repressed (>2-fold repression) when Ssa2, Ssa3 or Ssa4 was expressed as a sole source of Ssa in the cell respectively (Figure 5.19). The Δ*ssa1*Δ*ssa2*Δ*ssa3* mutant strain yielded a large response with 339 genes repressed and 209 genes induced when Ssa4 was the sole source of Ssa in the cell. In contrast, Δ*ssa1*Δ*ssa3*Δ*ssa4* mutant strain and Δ*ssa1*Δ*ssa2*Δ*ssa4* mutant strain resulted in a relatively small number of induced (16 and 47 respectively) and repressed (86 and 79 respectively) genes which in most cases may be due to close structural similarity between Ssa1 and Ssa2 and between Ssa3 and Ssa4 revealed by sequence homology. When the expression profile was analysed for the co-regulated set of genes in mutant strains compared to wildtype, there was 38 genes induced and 13 genes repressed in Δ*ssa1*. Also the differential gene expression profile analysis revealed that there were 38 genes induced and 27 genes repressed in Δ*ssa1*Δ*ssa2* whereas there were 13 genes induced and 47 genes repressed in Δ*ssa1*Δ*ssa3*. It was worth noting that only one gene (*FAR7*) was down regulated and 11 genes were up regulated in Δ*ssa1*Δ*ssa4*.

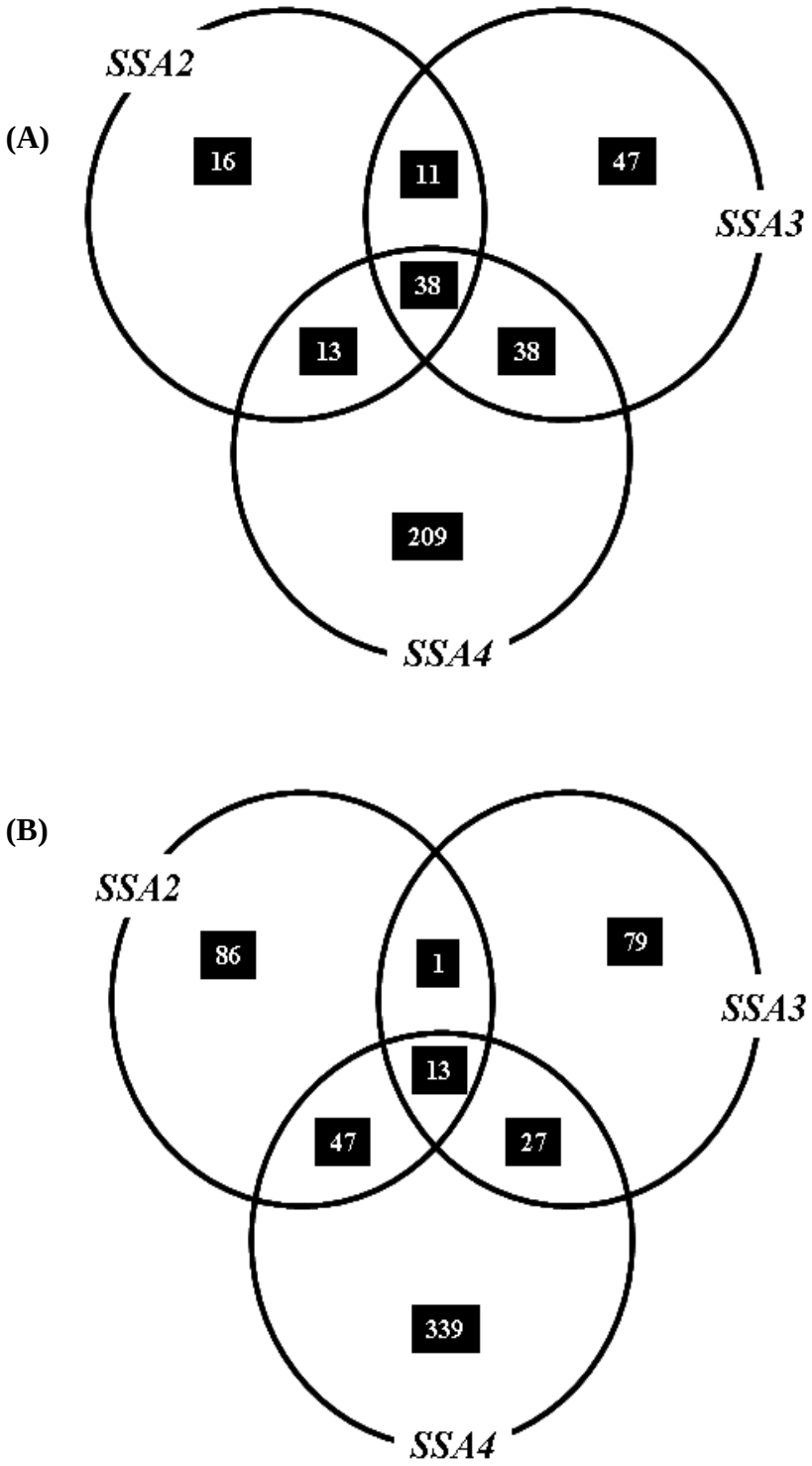



**Figure 5.19 Comparative transcriptome profiling of the Ssa family** A Venn diagram representation of genes up-regulated (A) or down-regulated (B) in different Δ*ssa* strains

### 5.5.2 Analysis of the top ten genes differentially expressed in Δ*ssa* strains

The top 10 differentially expressed genes were analysed in all the three mutant strain *viz.,* Δ*ssa1*Δ*ssa3*Δ*ssa4,* Δ*ssa1*Δ*ssa2*Δ*ssa4* and Δ*ssa1*Δ*ssa2*Δ*ssa3* as stated in Table 5.3 and represented by Venn diagram in Figure 5.20. The transcriptional profile analysis of the top ten genes in the deletion strains showed differential gene expression for a number of genes including genes involved in oxidative stress response and chemical stimulus, genes involved in cell cycle and cell wall and cytoskeleton organisation, various genes involved in metabolic processes in the cell, cellular homeostatsis, DNA and RNA metabolic processes and processing, enzyme regulator activity, structural molecule activity, oxidoreductase activity, transmembrane transporter activity *etc.*

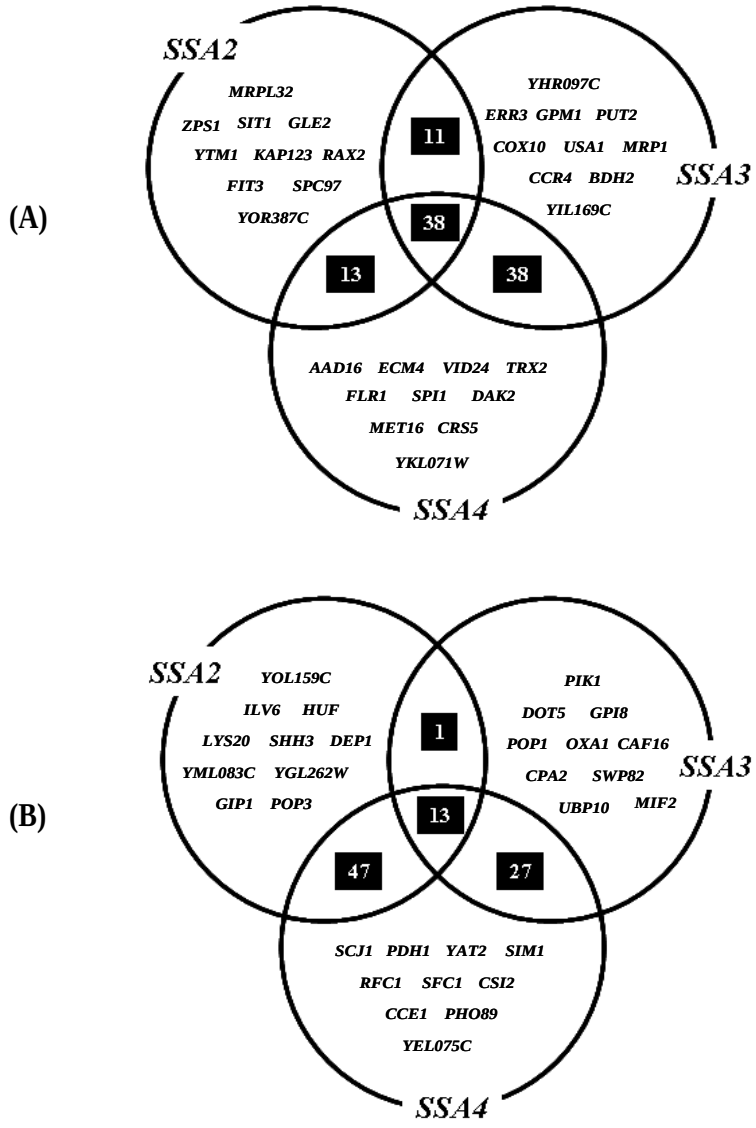



**Figure 5.20 Comparative transcriptome profiling of the top 10 differentially expressed genes of Ssa family** A Venn diagram representation of top ten genes up-regulated (A) or down-regulated (B) in different Δ*ssa* strains.

The plasma membrane is involved in many cellular processes ensuring adaptation to changed environmental conditions. These include maintenance of intracellular ion homeostasis, regulation of signaling pathways, morphogenesis and cell wall maintainance. An important aspect associated with the survival of environmental stress situations, like osmotic or heat stress is the adjustment of ion fluxes across the plasma membrane (Hohmann, 2002). Transporters, exchangers and channels, embedded in a highly flexible membrane system, allow an accurate adaptation of the cellular ion homeostasis to changing environmental conditions (Levin, 2005).

In Ssa2 the upregulation of genes like *FIT3* (3.02), *SIT1* (2.66) which are involved in maintainance of cellular ion homeostasis and *GLE2* (2.57) involved in response to heat stress might account for Ssa2's enhanced survival under heat shock conditions and also better survival compared to other Ssa's at higher concentration of SDS. Also among one of the top 10 downregulated genes in Ssa2 were *GIP1* (0.31) which is a gene involved in cell wall organization and its downregulation might also explain some of the phenotypes associated with Ssa2.

It is worth noting that the microarray results showed that the *DOT5* (0.16) gene which responds to chemical stimuli and oxidative stress response was among the top 10 genes that were down regulated in Ssa3 and this might contribute to the sensitivity of Ssa3 [*psi*$^-$] at 3 mM concentration of $H_2O_2$.

Among a few top 10 upregulated genes of Ssa4 which is of special importance in relation to the stress resistant phenotypes observed for Ssa4 like resistance to increased concentration of $H_2O_2$ (4 mM) and SDS (0.01%) are *ECM4* (4.79) involved in cell wall organisation, *TRX2* (4.34) involved in cell wall protection against oxidative stress, *SPI1* (4.17) whose expression is induced under stress conditions and *DAK2* (3.91) is a stress adaptor. Upregulation of all this genes might provide an explanation for some of the stress resistant phenotypes of Ssa4. The gene *RFC1* (0.16) was among one of the top 10 downregulated genes of Ssa4. *RFC1* is involved in mitotic cell cycle. Thus its downregulation in a strain with Ssa4 as a sole source of Ssa might explain why Ssa4 containing strains takes longer time to grow compared to other Ssa's.

**Table 5.3 Differential gene expression pattern of top ten genes unique to Ssa2, Ssa3 or Ssa4 compared to Ssa1 as wildtype**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| **Differentially expressed genes of Ssa2** | | | |
| YOR383C | *FIT3* | 3.02 | Mannoprotein that is incorporated into the cell wall via a GPI anchor, involved in the retention of siderophore-iron in the cell wall, maintains cellular ion homeostasis and ion transport |
| YOR387C | *UNC* | 2.96 | Putative protein of unknown function; regulated by the metal-responsive Aft1p transcription factor; highly inducible in zinc-depleted conditions |
| YEL065W | *SIT1* | 2.66 | Involved in transmembrane transporter activity and ion transport, maintains cellular ion homeostasis. It is a Ferrioxamine B transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; transcription is induced during iron deprivation and diauxic shift; potentially phosphorylated by Cdc28p. |
| YER107C | *GLE2* | 2.57 | Component of the Nup82 subcomplex of the nuclear pore complex; required for polyadenylated RNA export; important in nuclear transport, organisation and regulation; response to heat stress |
| YOR272W | *YTM1* | 2.45 | Constituent of 66S pre-ribosomal particles forms a complex with Nop7p and Erb1p that is required for maturation of the large ribosomal subunit. |
| YER110C | *KAP123* | 2.40 | Karyopherin beta, mediates nuclear import of ribosomal proteins prior to assembly into ribosomes and import of histones H3 and H4; exhibits genetic interactions with RAI1; have protein transporter and protein activity; also involved in nuclear transport and pseudohyphal growth. |

**Table 5.3 continued……**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YCR003W | *MRPL32* | 2.16 | Mitochondrial ribosomal protein of the large subunit involved in mitochondrial translation and organisation |
| YLR084C | *RAX2* | 2.15 | N-glycosylated protein involved in cytokinesis and maintenance of bud site selection during bipolar budding |
| YHR172W | *SPC97* | 2.14 | Involved in cytoskeleton organisation; Component of the microtubule-nucleating Tub4p (gamma-tubulin) complex; interacts with Spc110p at the spindle pole body (SPB) inner plaque and with Spc72p at the SPB outer plaque |
| YOL154W | *ZPS1* | 2.12 | Putative GPI-anchored protein; transcription is induced under low-zinc conditions, as mediated by the Zap1p transcription factor, and at alkaline pH |
| YNL282W | *POP3* | 0.18 | Involved in processing of mRNA, tRNA, rRNA and snoRNA processing; Subunit of both RNase MRP and nuclear RNase P; RNase MRP cleaves pre-rRNA, while nuclear RNase P cleaves tRNA precursors to generate mature 5' ends and facilitates turnover of nuclear RNAs |
| YNL108C | *HUF* | 0.18 | Putative protein of unknown function with similarity to Tfc7p and prokaryotic phosphotransfer enzymes; null mutant shows alterations in glucose metabolism |

**Table 5.3 continued……**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YOL159C | - | 0.20 | Soluble protein of unknown function; deletion mutants are viable and have elevated levels of Ty1 retrotransposition and Ty1 cDNA |
| YCL009C | *ILV6* | 0.22 | Involved in cellular amino acid metabolic process and regulates enzyme activity; regulatory subunit of acetolactate synthase, which catalyzes the first step of branched-chain amino acid biosynthesis; enhances activity of the Ilv2p catalytic subunit |
| YML083C | *UNC* | 0.25 | Putative protein of unknown function; transcriptionally regulated by Upc2p via an upstream sterol response element; strong increase in transcript abundance during anaerobic growth compared to aerobic growth; cells deleted for YML083C do not exhibit growth defects in anerobic or anaerobic conditions |
| YDL182W | *LYS20* | 0.26 | Involved in cellular amino acid metabolic process and transfer of acyl groups; Homocitrate synthase isozyme, catalyzes the condensation of acetyl-CoA and alpha-ketoglutarate to form homocitrate, which is the first step in the lysine biosynthesis pathway |
| YMR118C | *SHH3* | 0.26 | Putative mitochondrial inner membrane protein of unknown function |

**Table 5.3 continued……**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YAL013W | *DEP1* | 0.26 | Transcriptional modulator involved in regulation of structural phospholipid biosynthesis genes and metabolically unrelated genes, as well as maintenance of telomeres, mating efficiency, and sporulation; also involved in DNA replication, carbohydrate and lipid metabolic process, chromatin organisation and histone modification, regulation of DNA metabolic process; response to heat stress |
| YBR045C | *GIP1* | 0.31 | Enzyme binding and regulates enzyme activity, Meiosis-specific regulatory subunit of the Glc7p protein phosphatase required for expression of some late meiotic genes and for normal localization of Glc7p; cell wall organisation or biogenesis and sporulation, |
| YGL262W | *UNC* | 0.33 | Putative protein of unknown function; null mutant displays elevated sensitivity to expression of a mutant huntingtin fragment or of alpha-synuclein |
| **Differentially expressed genes of Ssa3** | | | |
| YHR097C | *UNC* | 5.26 | Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and the nucleus. |
| YMR323W | *ERR3* | 2.92 | Protein of unknown function, has similarity to enolases; lyase activity |
| YKL152C | *GPM1* | 2.91 | Involved in carbohydrate metabolic process and generation of precursor metabolites and energy; Tetrameric phosphoglycerate mutase, mediates the conversion of 3-phosphoglycerate to 2-phosphoglycerate during glycolysis and the reverse reaction during gluconeogenesis |

**Table 5.3 continued……..**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YHR037W | *PUT2* | 2.86 | Delta-1-pyrroline-5-carboxylate dehydrogenase, nuclear-encoded mitochondrial protein involved in utilization of proline as sole nitrogen source; deficiency of the human homolog causes HPII, an autosomal recessive inborn error of metabolism; involved in cellular amino acid metabolic processes. |
| YPL172C | *COX10* | 2.81 | Heme A:farnesyltransferase, catalyzes the first step in the conversion of protoheme to the heme A prosthetic group required for cytochrome c oxidase activity; human ortholog is associated with mitochondrial disorders; have transferase activity like transfer of alkyl or aryl groups; involved in cofactor metabolic processes. |
| YML029W | *USA1* | 2.76 | Involved in RNA splicing and mRNA processing, protein complex biogenesis, proteolysis involved in cellular protein catabolic process; scaffold subunit of the Hrd1p ubiquitin ligase that also promotes ligase oligomerization; involved in ER-associated protein degradation (ERAD); interacts with the U1 snRNP-specific protein, Snp1p |
| YDR347W | *MRP1* | 2.75 | Structural constituent of ribosome and involved in mitochondrial translation and organisation; mitochondrial ribosomal protein of the small subunit; MRP1 exhibits genetic interactions with PET122, encoding a COX3-specific translational activator, and with PET123, encoding a small subunit mitochondrial ribosomal protein |

**Table 5.3 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YAL021C | *CCR4* | 2.71 | Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening; also involved in regulation of mitotic cell cycle. |
| YAL061W | *BDH2* | 2.69 | Putative medium-chain alcohol dehydrogenase with similarity to BDH1; transcription induced by constitutively active PDR1 and PDR3; have oxidoreductase activity |
| YIL169C | *UNC* | 2.55 | Putative protein of unknown function; serine/threonine rich and highly similar to YOL155C, a putative glucan alpha-1,4-glucosidase; transcript is induced in both high and low pH environments |
| YKL089W | *MIF2* | 0.08 | Kinetochore protein with homology to human CENP-C, required for structural integrity of the spindle during anaphase spindle elongation, interacts with histones H2A, H2B, and H4, phosphorylated by Ipl1p; involved in chromosome segregation, cytoskeleton organisation and mitotic cell cycle. |
| YNL267W | *PIK1* | 0.12 | Phosphatidylinositol 4-kinase; catalyzes first step in the biosynthesis of phosphatidylinositol-4,5-biphosphate; may control cytokinesis through the actin cytoskeleton |
| YIL010W | *DOT5* | 0.16 | Nuclear thiol peroxidase which functions as an alkyl-hydroperoxide reductase during post-diauxic growth; have oxidoreductase activity and is responsive to chemical stimulus and oxidative stress. |

**Table 5.3 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YDR331W | *GPI8* | 0.16 | Involved in lipid metabolic processes and protein lipidation; ER membrane glycoprotein subunit of the glycosylphosphatidylinositol transamidase complex that adds glycosylphosphatidylinositol (GPI) anchors to newly synthesized proteins; human PIG-K protein is a functional homolog. |
| YNL221C | *POP1* | 0.18 | Have nuclease activity and involved in tRNA, mRNA, rRNA and snoRNA processing; Subunit of both RNase MRP and nuclear RNase P; RNase MRP cleaves pre-rRNA, while nuclear RNase P cleaves tRNA precursors to generate mature 5' ends and facilitates turnover of nuclear RNAs; binds to the RPR1 RNA subunit in RNase P. |
| YER154W | *OXA1* | 0.20 | Have protein transporter activity and involved in protein complex biogenesis and mitochondrial organisation; mitochondrial inner membrane insertase, mediates the insertion of both mitochondrial- and nuclear-encoded proteins from the matrix into the inner membrane, interacts with mitochondrial ribosomes; conserved from bacteria to animals |
| YFL028C | *CAF16* | 0.23 | Part of evolutionarily-conserved CCR4-NOT regulatory complex; contains single ABC-type ATPase domain but no transmembrane domain; interacts with several subunits of Mediator; have ATPase activity and involved in transcription from RNAse II promoter. |
| YJR109C | *CPA2* | 0.24 | Have ligase activity and involved in cellular amino acid metabolic processes; large subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline, an arginine precursor |

**Table 5.3 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YFL049W | *SWP82* | 0.27 | Involved in transcription from RNAse II promoter; member of the SWI/SNF chromatin remodeling complex in which it plays an as yet unidentified role; has identifiable counterparts in closely related yeast species; abundantly expressed in many growth conditions; paralog of Npl6p |
| YNL186W | *UBP10* | 0.28 | Have peptidase activity and involved in protein modification by small protein conjugation and removal; ubiquitin-specific protease that deubiquitinates ubiquitin-protein moieties; may regulate silencing by acting on Sir4p; involved in posttranscriptionally regulating Gap1p and possibly other transporter |
| **Differentially expressed genes of Ssa4** | | | |
| YFL057C | *AAD16* | 5.06 | Putative aryl-alcohol dehydrogenase; similar to P. chrysosporium aryl-alcohol dehydrogenase; mutational analysis has not yet revealed a physiological role; have oxidoreductase activity. |
| YKR076W | *ECM4* | 4.79 | Involved in cell wall organisation or biogenesis and cellular amino acid metabolic processes; have transferase activity transferring aryl or alkyl groups; omega class glutathione transferase; similar to Ygr154cp |

**Table 5.3 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YBR105C | *VID24* | 4.66 | Involved in carbohydrate metabolic process, membrane and organelle fusion,protein targeting and in proteolysis involving cellular protein catabolic processes; vacuole and vesicle organisation; Peripheral membrane protein located at Vid (vacuole import and degradation) vesicles; regulates fructose-1,6-bisphosphatase (FBPase) targeting to the vacuole; promotes proteasome-dependent catabolite degradation of FBPase |
| YGR209C | *TRX2* | 4.34 | Have oxidoreductase activity; membrane fusion; involved in DNA replication and regulation of DNA metabolic processes; respond to chemical stimulus and oxidative stress; cytoplasmic thioredoxin isoenzyme of the thioredoxin system which protects cells against oxidative and reductive stress, forms LMA1 complex with Pbi2p, acts as a cofactor for Tsa1p, required for ER-Golgi transport and vacuole inheritance |
| YBR008C | *FLR1* | 4.27 | Have transmembrane transporter activity and respond to chemical stimulus; plasma membrane multidrug transporter of the major facilitator superfamily, involved in efflux of fluconazole, diazaborine, benomyl, methotrexate, and other drugs; expression induced in cells treated with the mycotoxin patulin |
| YER150W | *SPI1* | 4.17 | Shows response to chemical stimulus; GPI-anchored cell wall protein involved in weak acid resistance; basal expression requires Msn2p/Msn4p; expression is induced under conditions of stress and during the diauxic shift; similar to Sed1p |

**Table 5.3 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YKL071W | *UNC* | 4.00 | Putative protein of unknown function; expression induced in cells treated with the mycotoxin patulin, and also the quinone methide triterpene celastrol |
| YFL053W | *DAK2* | 3.91 | Have kinase activity and involved in carbohydrate metabolic process; dihydroxyacetone kinase, required for detoxification of dihydroxyacetone (DHA); involved in stress adaptation |
| YPR167C | *MET16* | 3.84 | Have oxidoreductase activity and involved in cellular amino acid metabolic processes; 3'-phosphoadenylsulfate reductase, reduces 3'-phosphoadenylyl sulfate to adenosine-3',5'-bisphosphate and free sulfite using reduced thioredoxin as cosubstrate, involved in sulfate assimilation and methionine metabolism |
| YOR031W | *CRS5* | 3.66 | Promotes ion binding and respond to chemical stimulus; copper-binding metallothionein, required for wild-type copper resistance |
| YMR214W | *SCJ1* | 0.08 | Promotes protein folding and proteolysis involved in cellular protein catabolic process; respond to chemical stimuli; one of several homologs of bacterial chaperone DnaJ, located in the ER lumen where it cooperates with Kar2p to mediate maturation of proteins |
| YPR002W | *PDH1* | 0.15 | Mitochondrial protein that participates in respiration, induced by diauxic shift; homologous to E. coli PrpD, may take part in the conversion of 2-methylcitrate to 2-methylisocitrate |

**Table 5.3 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YER024W | *YAT2* | 0.15 | Have transferase activity and involved in cellular amino acid metabolic processes; it's a carnitine acetyltransferase; has similarity to Yat1p, which is a carnitine acetyltransferase associated with the mitochondrial outer membrane |
| YIL123W | *SIM1* | 0.15 | Involved in cell wall organisation or biogenesis; protein of the SUN family (Sim1p, Uth1p, Nca3p, Sun4p) that may participate in DNA replication, promoter contains SCB regulation box at -300 bp indicating that expression may be cell cycle-regulated |
| YOR217W | *RFC1* | 0.16 | Have ATPase activity; involved in DNA binding, repair and replication; respond to DNA damage stimulus; involved in mitotic cell cycle; subunit of heteropentameric Replication factor C (RF-C), which is a DNA binding protein and ATPase that acts as a clamp loader of the proliferating cell nuclear antigen (PCNA) processivity factor for DNA polymerases delta and epsilon |
| YBR296C | *PHO89* | 0.18 | Have transmembrane transporter activity and promotes ion transport; Na+/Pi cotransporter, active in early growth phase; similar to phosphate transporters of Neurospora crassa; transcription regulated by inorganic phosphate concentrations and Pho4p |
| YOL007C | *CSI2* | 0.19 | Protein of unknown function |

**Table 5.3 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | Biological Functions |
|---|---|---|---|
| YEL075C | *UNC* | 0.19 | Putative protein of unknown function |
| YKL011C | *CCE1* | 0.20 | Have nuclease activity and involved in mitochondrial organisation; mitochondrial cruciform cutting endonuclease, cleaves Holliday junctions formed during recombination of mitochondrial DNA |
| YJR095W | *SFC1* | 0.20 | Have transmembrane transporter activity and promotes ion transport; Mitochondrial succinate-fumarate transporter, transports succinate into and fumarate out of the mitochondrion; required for ethanol and acetate utilization |

### 5.5.3 Analysis of differentially expressed genes in Δ*ssa1 strains.*

Trancriptiomic analysis revealed that there were 38 genes that were induced and 13 genes that were repressed in a Δ*ssa1* deletion (Table 5.4). Among the differentially expressed genes that were either up-regulated or down-regulated included genes involved in cell wall organisation, morphogenesis and cell cycle regulation, enzyme regulatory activity, chemical stimuli, starvation and DNA damage responsive gene, genes involved in RNA catabolic process, modification and splicing, oxidoreductase activity, transmembrane transporter activity, ion transport,amino acid and co-factor metabolic process *etc*.

## 5.6 Confirmation of transcriptional profiling by quantitative PCR (qPCR)

To assess the efficacy of microarray results, qPCR was performed (Figure 5.21). Total RNA was extracted from yeast cells expressing Ssa1, Ssa2, Ssa3 or Ssa4 as a sole source of Ssa in the cell in a [*psi*$^-$] background. cDNA was synthesised as described in Section 2.23.1 and was used as a reaction template for carrying out qPCR. The expression levels of 25 genes were analysed. These 25 genes were selected based on up-regulation or down regulation recorded in transcriptomic data (Table 5.5). The housekeeping gene used was *ACT1*. qPCR was carried out using primers for the 25 genes as described in Section 2.23.2 of materials and methods. A comparative overview of the qPCR and microarray results is in Table 5.5 presenting the efficacy of the two experiments. There was an excellent correlation between qPCR and microarray results for approximately 14 genes which further supports the microarray results.

Comparative analysis of the fold change between microarray and qPCR showed excellent correlation for genes involved in cell cycle regulation (*YOX1, CLN1, CLN2, YBRO14W*), structural integrity (*MIF2*), cell wall protein (*TOS6*), oxidative stress response and cell death (*OYE3*). There was one gene (*SCJ1*) that encoded the homolog of bacterial chaperone DnaJ that was seen to be uniformly downregulated across the Ssa family from both qPCR and microarray analysis.

The comparative data from qPCR and microarray (Table 5.5) showed that most of the genes involved in cell cycle regulation (*CLN1, CLN2, YOX1*) were down regulated which might account for most of the phenotypes associated with the Ssa family discussed earlier in this chapter.

One of the interesting cell cycle regulated gene was the *YNRO14W*. It is a putative protein of unknown function and whose expression is cell cycle regulated. It is

also a heat inducible protein. Among all the Ssa's this gene was highly upregulated in Ssa4 as given by the microarray analysis (10.37) and qPCR (7.24). Upregulation of this gene in Ssa4 might explain some of the stress phenotypes observed for Ssa4.

Another important gene that was shown to be upregulated in both qPCR and microarray analysis (Table 5.5) was *OYE3*. The highest upregulation was found in Ssa4. *OYE3* is a conserved NADPH oxidoreductase containing flavin mononucleotide (FMN) that has potential roles in oxidative stress response and programmed cell death. This might provide an explanation to the resistance displayed by Ssa4 to higher concentrations of $H_2O_2$.

**Table 5.4 Differential gene expression pattern of Δ*ssa1* strains compared to Ssa1 as wild type**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YAL039C | *CYC3* | 2.06 | 2.06 | 3.09 | Cytochrome c heme lyase (holocytochrome c synthase), attaches heme to apo-cytochrome c (Cyc1p or Cyc7p) in the mitochondrial intermembrane space. |
| YBL078C | *ATG8* | 6.69 | 5.76 | 10.23 | Involved in Golgi vesicle transport, organelle assembly, protein targeting, vacuole organisation; respond to starvation; Component of autophagosomes and Cvt vesicles; undergoes conjugation to phosphatidylethanolamine (PE); Atg8p-PE is anchored to membranes, is involved in phagophore expansion, and may mediate membrane fusion during autophagosome formation |
| YBR204C | *LDH1* | 2.88 | 2.70 | 3.97 | Serine hydrolase; exhibits active esterase plus weak triacylglycerol lipase activities; proposed role in lipid homeostasis, regulating phospholipid and non-polar lipid levels and required for mobilization of LD-stored lipids; localizes to the lipid droplet (LD) surface; contains a classical serine containing catalytic triad (GxSxG motif) |
| YCL064C | *CHA1* | 2.40 | 3.12 | 2.76 | Catabolic L-serine (L-threonine) deaminase, catalyzes the degradation of both L-serine and L-threonine; required to use serine or threonine as the sole nitrogen source, transcriptionally induced by serine and threonine |

**Table 5.4 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YDL243C | *AAD4* | 2.02 | 2.21 | 5.65 | Have oxidoreductase activity; putative aryl-alcohol dehydrogenase; involved in oxidative stress response; similar to P. chrysosporium aryl-alcohol dehydrogenase; expression induced in cells treated with the mycotoxin patulin |
| YDR101C | *ARX1* | 2.82 | 2.25 | 2.44 | Shuttling pre-60S factor; involved in the biogenesis of ribosomal large subunit biogenesis; interacts directly with Alb1; responsible for Tif6 recycling defects in absence of Rei1; associated with the ribosomal export complex |
| YDR194C | *MSS116* | 5.70 | 3.09 | 4.83 | DEAD-box protein required for efficient splicing of mitochondrial Group I and II introns; non-polar RNA helicase that also facilities strand annealing |
| YDR253C | *MET32* | 2.08 | 2.98 | 3.94 | Zinc-finger DNA-binding protein, involved in transcriptional regulation of the methionine biosynthetic genes, similar to Met31p; mitotic cell cycle regulation; cellular amino acid metabolic process; |
| YDR261C-D | | 2.15 | 2.01 | 3.06 | Retrotransposon TYA Gag and TYB Pol genes; in YDRCTY1-3 TYB is mutant and probably non-functional. |
| YDR471W | *RPL27B* | 3.01 | 3.05 | 6.53 | Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl27Ap and has similarity to rat L27 ribosomal protein; involved in cytoplasmic translation. |

**Table 5.4 continued……..**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YDR533C | *HSP31* | 6.64 | 5.55 | 14.08 | Possible chaperone and cysteine protease with similarity to E. coli Hsp31; member of the DJ-1/ThiJ/PfpI superfamily, which includes human DJ-1 involved in Parkinson's disease; exists as a dimer and contains a putative metal-binding site; involved in unfolded protein binding and exhibits peptidase activity |
| YEL039C | *CYC7* | 3.04 | 2.84 | 8.18 | Involved in cellular respiration and generation of precursor metabolites and energy; Cytochrome c isoform 2, expressed under hypoxic conditions; electron carrier of the mitochondrial intermembrane space that transfers electrons from ubiquinone-cytochrome c oxidoreductase to cytochrome c oxidase during cellular respiration |
| YGR147C | *NAT2* | 3.07 | 2.60 | 2.76 | Involved in peptidyl-amino acid modification; protein acylation; it’s a protein with an apparent role in acetylation of N-terminal methionine residues. |
| YGR169C | *PUS6* | 3.16 | 3.62 | 2.19 | Have isomerise activity and involved in RNA modification and tRNA processing; catalyzes the conversion of uridine to pseudouridine at position 31 in cytoplasmic and mitochondrial tRNAs; mutation of Asp168 to Ala abolishes enzyme activity |
| YHL036W | *MUP3* | 2.07 | 2.06 | 5.66 | Involved in amino acid transport and exhibits transmembrane transporter activity |

**Table 5.4 continued……**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YHL040C | *ARN1* | 2.40 | 2.44 | 2.86 | Involved in ion transport and maintains cellular ion homeostasis; Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to ferrirubin, ferrirhodin, and related siderophores |
| YHR094C | *HXT1* | 2.69 | 3.05 | 16.90 | Exhibits transmembrane transporter activity; low-affinity glucose transporter of the major facilitator superfamily, expression is induced by Hxk2p in the presence of glucose and repressed by Rgt1p when glucose is limiting. |
| YHR147C | *MRPL6* | 9.81 | 13.31 | 11.98 | Involved in mitochondrial translation and organisation; it's a mitochondrial ribosomal protein of the large subunit |
| YIR021W | *MRS1* | 3.85 | 4.40 | 3.22 | Protein required for the splicing of two mitochondrial group I introns (BI3 in COB and AI5beta in COX1); forms a splicing complex, containing four subunits of Mrs1p and two subunits of the BI3-encoded maturase, that binds to the BI3 RNA. |
| YJL209W | *CBP1* | 3.30 | 3.94 | 2.71 | Involved in RNA catabolic process; promotes RNA and mRNA binding; also involved in generation of precursor metabolites and energy; mitochondrial protein that interacts with the 5'-untranslated region of the COB mRNA and has a role in its stability and translation; found in a complex at the inner membrane along with Pet309p |

**Table 5.4 continued…**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YJL212C | *OPT1* | 2.35 | 2.85 | 5.40 | Proton-coupled oligopeptide transporter of the plasma membrane; also transports glutathione and phytochelatin; member of the OPT family |
| YJR132W | *NMD5* | 3.02 | 2.65 | 2.89 | Have protein transporter activity; also involved in nuclear transport and protein targeting; it's a karyopherin, a carrier protein involved in nuclear import of proteins. |
| YKL140W | *TGL1* | 2.21 | 2.09 | 2.25 | Steryl ester hydrolase, one of three gene products (Yeh1p, Yeh2p, Tgl1p) responsible for steryl ester hydrolase activity and involved in sterol homeostasis |
| YKL224C | *PAU16* | 3.30 | 3.22 | 4.58 | Protein of unknown function, member of the seripauperin multigene family encoded mainly in subtelomeric regions. |
| YLL009C | *COX17* | 2.67 | 2.85 | 3.31 | Involved in ion binding and transport; mitochondrial organisation and protein complex biogenesis; copper metallochaperone that transfers copper to Sco1p and Cox11p for eventual delivery to cytochrome c oxidase |
| YLR092W | *SUL2* | 2.93 | 3.22 | 7.63 | Have transmembrane transporter activity and ion transport; high affinity sulfate permease; sulfate uptake is mediated by specific sulfate transporters Sul1p and Sul2p, which control the concentration of endogenous activated sulfate intermediates |
| YLR108C | *UNC* | 2.58 | 3.46 | 6.83 | Protein of unknown function |

**Table 5.4 continued…….**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YML077W | *BET5* | 4.14 | 4.35 | 3.85 | Have enzyme regulator activity; component of the TRAPP (transport protein particle) complex, which plays an essential role in the vesicular transport from endoplasmic reticulum to Golgi |
| YNL134C | *UNC* | 2.62 | 2.20 | 5.92 | Have oxidoreductase activity; putative protein of unknown function with similarity to dehydrogenases from other model organisms and is induced by the DNA-damaging agent MMS. |
| YNL277W | *MET2* | 2.52 | 3.38 | 5.29 | Involved in cellular amino acid metabolic process, have transferase activity transferring acyl groups; L-homoserine-O-acetyltransferase, catalyzes the conversion of homoserine to O-acetyl homoserine which is the first step of the methionine biosynthetic pathway |
| YNR014W | *UNC* | 3.38 | 2.44 | 10.37 | Putative protein of unknown function; expression is cell-cycle regulated, Azf1p-dependent, and heat-inducible |
| YOL155C | *HPF1* | 2.70 | 2.73 | 3.11 | Have hydrolase activity and acts on glycosyl bonds; involved in cell wall organisation or biogenesis; haze-protective mannoprotein that reduces the particle size of aggregated proteins in white wines |

**Table 5.4 continued……..**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YOR226C | *ISU2* | 2.19 | 2.05 | 5.45 | Maintains cellular ion homeostasis and involved in tRNA processing; conserved protein of the mitochondrial matrix, required for synthesis of mitochondrial and cytosolic iron-sulfur proteins, performs a scaffolding function in mitochondria during Fe/S cluster assembly |
| YOR346W | *REV1* | 4.83 | 4.49 | 5.18 | Have nucleotidyltransferase activity involved in DNA repair and response to DNA damage stimulus; deoxycytidyl transferase; involved in repair of abasic sites and adducted guanines in damaged DNA by translesion synthesis (TLS); forms a complex with the subunits of DNA polymerase zeta, Rev3p and Rev7p |
| YPL123C | *RNY1* | 2.67 | 3.53 | 3.67 | Have nuclease activity; involved in RNA catabolic process and cellular morphogenesis; vacuolar RNase of the T(2) family, relocalizes to the cytosol where it cleaves tRNAs upon oxidative or stationary phase stress; promotes apoptosis under stress conditions and this function is independent of its catalytic activity |
| YPL171C | *OYE3* | 3.31 | 3.31 | 12.45 | Conserved NADPH oxidoreductase containing flavin mononucleotide (FMN), homologous to Oye2p with different ligand binding and catalytic properties; has potential roles in oxidative stress response and programmed cell death. |
| YPR011C | *UNC* | 2.32 | 2.50 | 2.12 | Putative transporter, member of the mitochondrial carrier family; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies |

**Table 5.4 continued……..**

| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YPR036W | *VMA13* | 5.93 | 8.02 | 5.94 | Have ATPase activity and involved in transmembrane transporter activity; maintains cellular ion homeostasis; Subunit H of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; serves as an activator or a structural stabilizer of the V-ATPase |
| YDR384C | *ATO3* | 0.45 | 0.44 | 0.12 | Have transmembrane transporter activity and involved in ion transport; plasma membrane protein, regulation pattern suggests a possible role in export of ammonia from the cell; phosphorylated in mitochondria; member of the TC 9.B.33 YaaH family of putative transporters |
| YER039C | *HVG1* | 0.27 | 0.35 | 0.30 | Protein of unknown function, has homology to Vrg4p |
| YER039C-A | *UNC* | 0.17 | 0.44 | 0.15 | Putative protein of unknown function |
| YER180C | *ISC10* | 0.42 | 0.41 | 0.60 | Protein required for sporulation, transcript is induced 7.5 h after induction of meiosis, expected to play significant role in the formation of reproductive cells |
| YIL134C-A | *UNC* | 0.31 | 0.40 | 0.47 | Putative protein of unknown function |

**Table 5.4 continued…..**

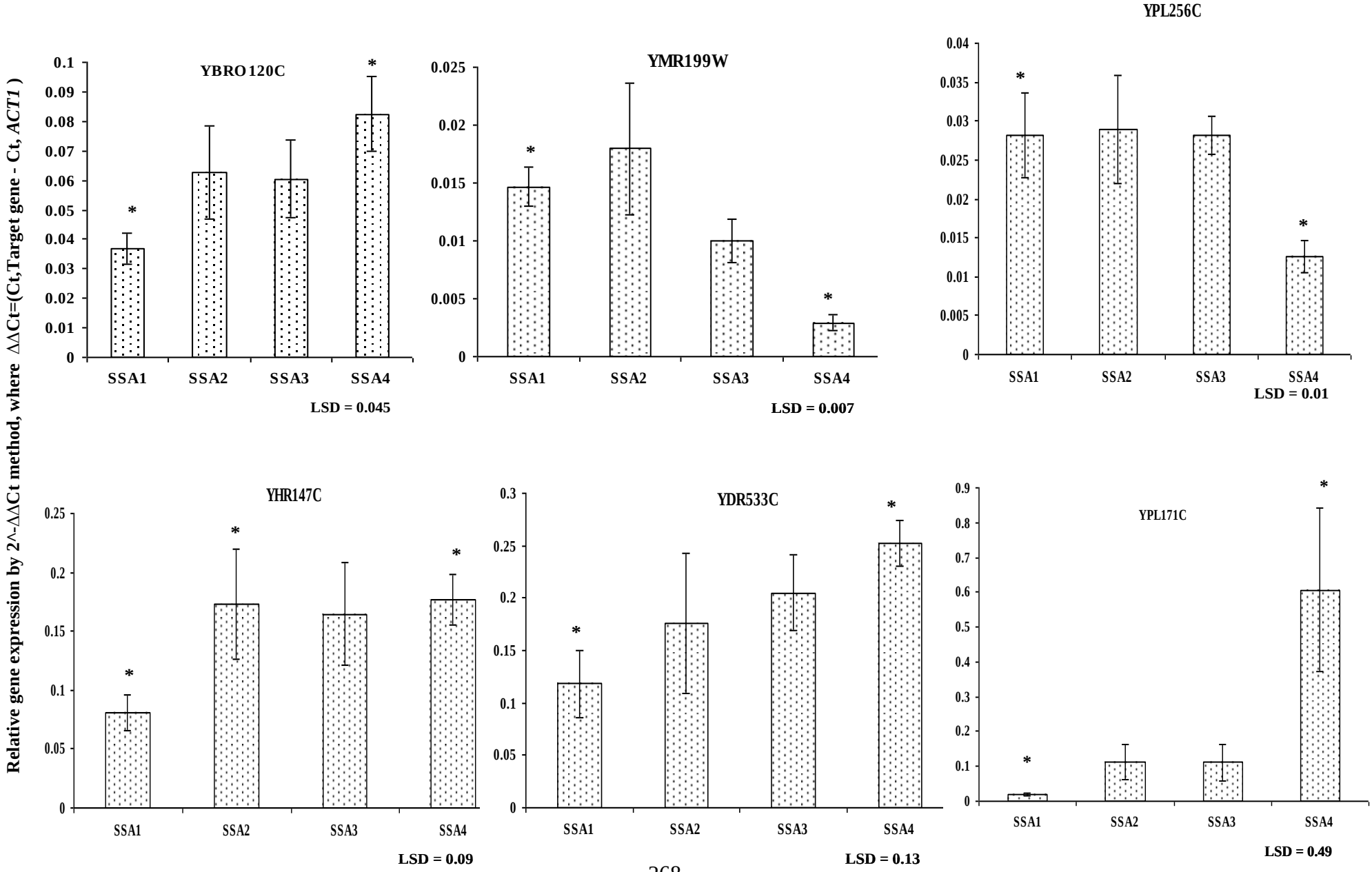
| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YIR031C | *DAL7* | 0.34 | 0.36 | 0.41 | Have transferase activity, transfer acyl groups; malate synthase, role in allantoin degradation unknown; expression sensitive to nitrogen catabolite repression and induced by allophanate, an intermediate in allantoin degradation |
| YLR125W | *UNC* | 0.38 | 0.50 | 0.31 | Putative protein of unknown function |
| YLR308W | *CDA2* | 0.41 | 0.26 | 0.13 | Have hydrolase activity acting on carbon-nitrogen bonds; involved in cell wall organisation or biogenesis and sporulation; chitin deacetylase, together with Cda1p involved in the biosynthesis ascospore wall component, chitosan; required for proper rigidity of the ascospore wall |
| YMR065W | *KAR5* | 0.38 | 0.47 | 0.48 | Protein required for nuclear membrane fusion during karyogamy, localizes to the membrane with a soluble portion in the endoplasmic reticulum lumen, may form a complex with Jem1p and Kar2p; expression of the gene is regulated by pheromone; involved in organelle fusion and nuclear organisation. |

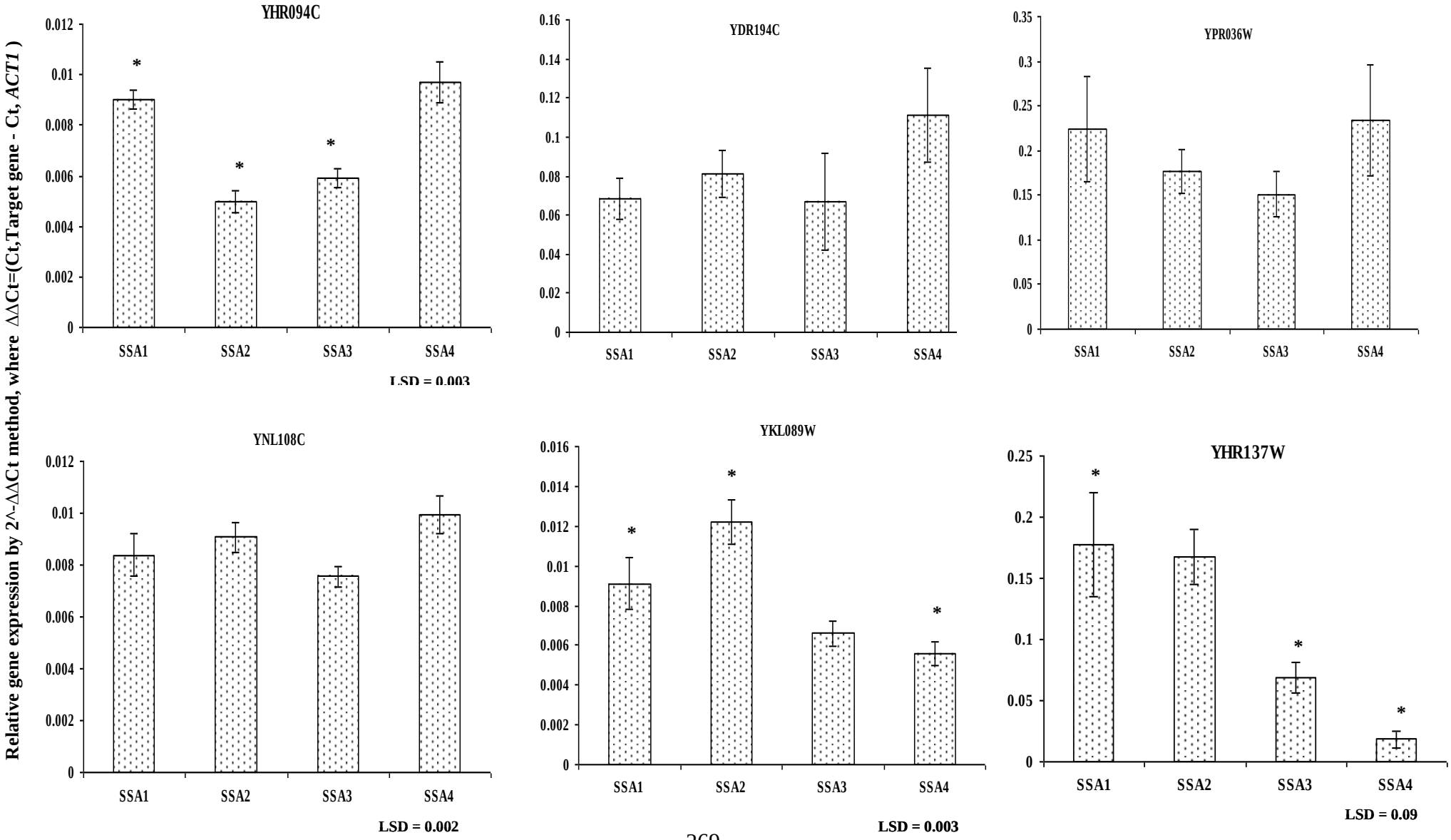
**Table 5.4 continued……**

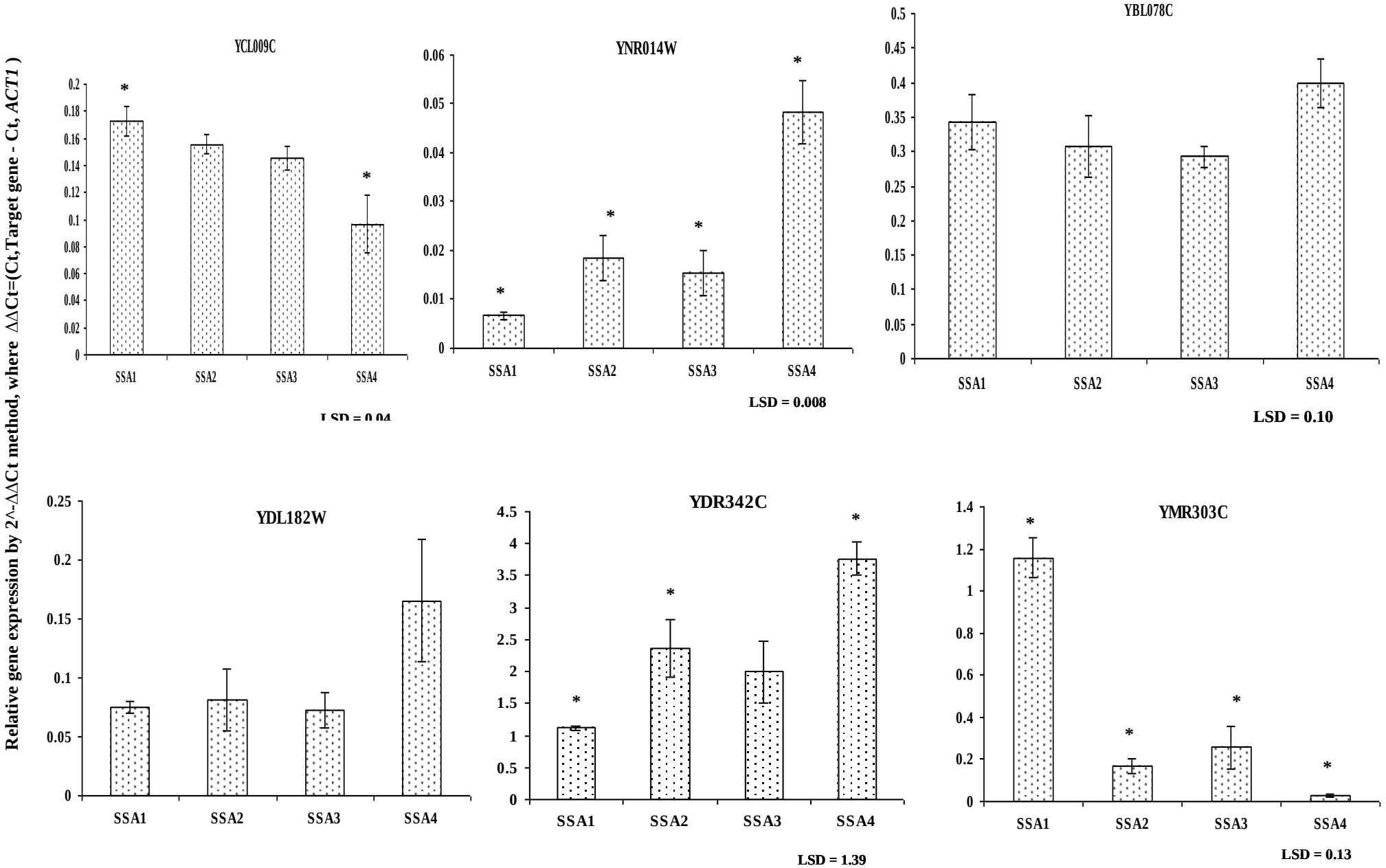
| Systemic name | Gene name | Fold change compared to Ssa1 | | | Biological functions |
|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | |
| YMR303C | *ADH2* | 0.21 | 0.38 | 0.04 | Involved in cellular amino acid and co-factor metabolic process; generates precursor metabolites and energy; involved in nucleobase-containing small molecule metabolic process; Glucose-repressible alcohol dehydrogenase II, catalyzes the conversion of ethanol to acetaldehyde; involved in the production of certain carboxylate esters |
| YOR348C | *PUT4* | 0.36 | 0.50 | 0.12 | Have transmembrane transporter activity and involved in amino acid transport; proline permease, required for high-affinity transport of proline; also transports the toxic proline analog azetidine-2-carboxylate (AzC); PUT4 transcription is repressed in ammonia-grown cells |
| YOR388C | *FDH1* | 0.29 | 0.13 | 0.42 | Have oxidoreductase activity; involved in co-factor metabolic process ; also involved nucleobase-containing small molecule metabolic process; NAD(+)-dependent formate dehydrogenase, may protect cells from exogenous formate |
| YPL272C | *UNC* | 0.49 | 0.33 | 0.37 | Putative protein of unknown function; gene expression induced in response to ketoconazole |

**Table 5.5 Comparative overview of fold change for microarray and qPCR of 25 differentially expressed genes in Ssa2, Ssa3 and Ssa4.**

| Gene name | Systemic name | Microarray | | | qPCR | | |
|---|---|---|---|---|---|---|---|
| | | Ssa2 | Ssa3 | Ssa4 | Ssa2 | Ssa3 | Ssa4 |
| *CBP6* | YBR120C | 1.39 | 1.94 | 2.14 | 1.71 | 1.65 | 2.24 |
| - | YBR197C | 0.44 | 0.62 | 0.60 | 0.75 | 0.86 | 0.88 |
| *ILV6* | YCL009C | 0.22 | 1.10 | 1.00 | 0.90 | 0.84 | 0.56 |
| *LYS20* | YDL182W | 0.26 | 1.64 | 2.46 | 1.09 | 0.97 | 2.21 |
| *GPI8* | YDR331W | 1.12 | 0.16 | 0.97 | 1.21 | 0.81 | 0.94 |
| *HXT7* | YDR342C | 2.76 | 6.15 | 1.51 | 2.12 | 1.79 | 3.37 |
| *ARO9* | YHR137W | 0.83 | 0.50 | 0.11 | 0.94 | 0.39 | 0.10 |
| *MIF2* | YKL089W | 1.12 | 0.08 | 1.13 | 1.34 | 0.72 | 0.61 |
| *YOX1* | YML027W | 1.30 | 0.56 | 0.23 | 0.50 | 0.41 | 0.39 |
| *CLN1* | YMR199W | 2.48 | 2.24 | 0.81 | 1.22 | 0.68 | 0.20 |
| *SCJ1* | YMR214W | 1.29 | 0.60 | 0.08 | 1.16 | 0.50 | 0.45 |
| *ADH2* | YMR303C | 0.21 | 0.38 | 0.04 | 0.14 | 0.22 | 0.02 |
| *HUF1* | YNL108C | 0.18 | 1.18 | 1.17 | 1.08 | 0.90 | 1.19 |
| *UBP10* | YNL186W | 1.07 | 0.28 | 1.03 | 0.97 | 0.78 | 0.78 |
| *TOS6* | YNL300W | 1.39 | 1.11 | 0.21 | 1.57 | 0.71 | 0.17 |
| - | YNR014W | 3.38 | 2.44 | 10.37 | 2.77 | 2.33 | 7.24 |
| - | YOR387C | 2.96 | 0.54 | 0.26 | 2.21 | 0.55 | 0.42 |
| *OYE3* | YPL171C | 3.31 | 3.31 | 12.45 | 6.11 | 6.00 | 32.87 |
| *CLN2* | YPL256C | 0.90 | 1.48 | 0.47 | 1.03 | 1.00 | 0.45 |
| *HXT1* | YHR094C | 2.69 | 3.05 | 16.90 | 0.55 | 0.65 | 1.07 |
| *MRPL6* | YHR147C | 9.81 | 13.31 | 11.98 | 2.13 | 2.03 | 2.18 |
| *ATG8* | YBL078C | 6.69 | 5.76 | 10.23 | 0.89 | 0.85 | 1.16 |
| *HSP31* | YDR533C | 6.64 | 5.55 | 14.08 | 1.48 | 1.73 | 2.13 |
| *VMA13* | YPR036W | 0.97 | 1.00 | 1.02 | 0.78 | 0.67 | 1.04 |
| *MSS116* | YDR194C | 5.70 | 3.09 | 4.83 | 1.18 | 0.98 | 1.62 |


Relative gene expression by 2^-ΔΔCt method, where ΔΔCt=(Ct,Target gene - Ct, ACT1 )
YBRO120C
YMR199W
YPL256C
YHR147C
YDR533C
YPL171C
SSA1
SSA2
SSA3
SSA4
LSD = 0.045
LSD = 0.007
LSD = 0.01
LSD = 0.09
LSD = 0.13
LSD = 0.49


YHR094C
YDR194C
YPR036W
YNL108C
YKL089W
YHR137W
Relative gene expression by 2^-ΔΔCt method, where ΔΔCt=(Ct,Target gene - Ct, ACT1 )
SSA1
SSA2
SSA3
SSA4
LSD = 0.003
LSD = 0.002
LSD = 0.003
LSD = 0.09


Relative gene expression by 2^-ΔΔCt method, where ΔΔCt=(Ct,Target gene - Ct, ACT1 )
YCL009C
YNR014W
YBL078C
YDL182W
YDR342C
YMR303C
SSA1
SSA2
SSA3
SSA4
LSD = 0.008
LSD = 0.10
LSD = 1.39
LSD = 0.13

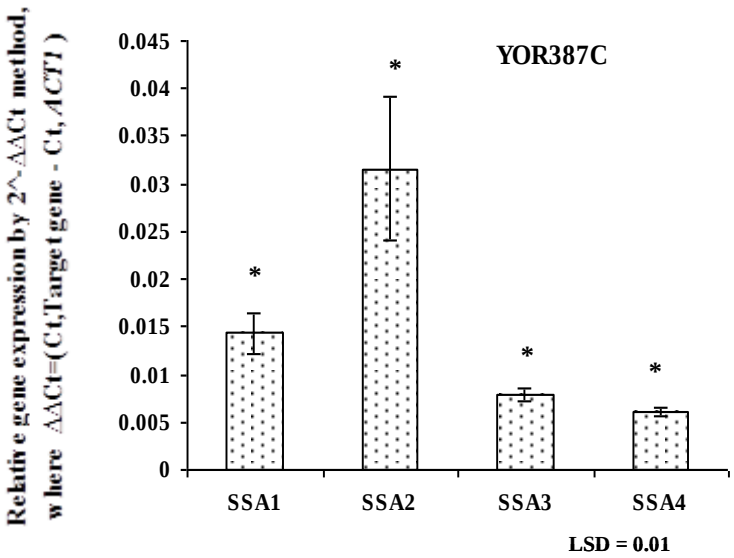



**Figure 5.21 Quantitative real-time RT-PCR analysis of 25 differentially expressed genes in Ssa2, Ssa3 and Ssa4** The 25 genes were identified by microarray technique as being primed by *S. cerevisiae* strain Δ*ssa1*Δ*ssa3*Δ*ssa4*, Δ*ssa1*Δ*ssa2*Δ*ssa4* and Δ*ssa1*Δ*ssa2*Δ*ssa3*; both qPCR and microarray analysis was conducted using RNA from samples harvested overnight. Relative gene expression pattern was quantified relative to that of the housekeeping gene *ACT1*.Results are based on two experiments, each with three replicates. Bars indicate SEM. Values followed by '*' are statistically significant as compared to Δ*ssa2*Δ*ssa3*Δ*ssa4* as wild-type at $p \leq 0.05$

## 5.7 Chapter discussion

It was believed for a long time that canonical Hsp70s display functional redundancy, with the main difference between the isoforms lying in their spatio-temporal expression pattern. However, several studies in yeast and higher eukaryotic organisms challenged this widely accepted idea by demonstrating functional specificity among Hsp70 isoforms (Kabani & Martineau, 2008). One of the earliest reports addressing the functional specialization among the cytosolic Hsp70 isoforms came from the research carried out by Sharma et al. (2009). They showed that despite of high degree of homology and overlapping functions displayed by intra- and inter-species Hsp70, the different Hsp70 orthologs have evolved to possess distinct activities required to cope with different substrates as well as with diversified stress conditions. As stated earlier by Sharma et al. (2009), yeast prions provide an excellent model to uncover this

functional specificity of Hsp70 and also to investigate the complex system of underlying networks for chaperone/co-chaperone/substrates interaction. Thus, in this study, the yeast prion [*PSI*$^{+}$] was used as a model to provide insight into the functional specificity of Hsp70 isoforms of Ssa family and look into several distinct and overlapping functions of Hsp70-Ssa family.

Consistent with previous studies, this study also supported the concept of existence of functional specialisation among the cytosolic members of the Ssa family of Hsp70 (Derkatch et al., 2001; Gao et al., 1991; Sharma et al., 2009). The Ssa family differs by expression pattern; both Ssa1 and Ssa2 are constitutively expressed but Ssa1 is expressed at a lower level than Ssa2. The inducible isoforms- Ssa3 and Ssa4 are lowly expressed during normal vegetative growth but their expression increases several fold upon encounter with stress conditions (Boorstein & Craig, 1990a; Boorstein & Craig, 1990b). All the four Ssa's in *S. cerevisiae* supported growth as the sole source of Ssa, although the inducible isoforms Ssa3 and Ssa4 was not as efficient as Ssa1 and Ssa2. Also the strains expressing Ssa3 and Ssa4 displayed higher degree of temperature sensitivity suggesting that functions carried out by Ssa1 and Ssa2 cannot be fully be complemented by Ssa3 and Ssa4. But both Ssa3 and Ssa4 could be recovered in presence of osmotic stabiliser (1 M sorbitol) implicating that Hsp70-Ssa might have a role to play in CWI signalling pathway.

Propagation of [*PSI*$^{+}$] was also affected differently by members of the Ssa family, which uncovered additional distinctions. Ssa3 was the most effective in maintenance and propagation of [*PSI*$^{+}$] compared to other Ssa's. This result is consistent with the previous studies carried out by Sharma et al. (2009). Ssa4 was weak in maintaining [*PSI*$^{+}$]. Research carried out by Schwimmer & Masison (2002) and Roberts et al. (2004) also found different effects of the Ssa family on [*URE3*] propagation. Overexpression of Ssa1 but not Ssa2 cures [*URE3*], whereas a mutation in Ssa2 but not Ssa1 impairs [*URE3*] propagation. These differences could reflect the differences in specific intrinsic activities of Hsp70 or in ways Hsp70 interact with prion proteins as substrate or with other components of the chaperone machinery. Also the differences that exist among the four Ssa's with regard to prion propagation can be attributed to the sequence specificity. For example a screen undertaken to find components that bind to the prion form of Sup35 found that Ssa1/2 efficiently bind to the prion domain of Sup35 in [*PSI*$^{+}$] cells but had a weak interaction with Sup35 in [*psi*$^{-}$] cells (Bagriantsev et al., 2008). Ssa3/Ssa4 was not isolated in the screen as binding to

Sup35, indicating difference in binding to prions between Ssa members. In *S. cerevisiae*, the Ssa proteins are recruited and activated by J-proteins (Sahi & Craig, 2007) and regulated by NEFs such as Fes1 and Sse1/2 (Dragovic et al., 2006a; Dragovic et al., 2006b; Kabani et al., 2002a; Raviol et al., 2006; Shaner et al., 2005; Yam et al., 2005). It has not been systematically tested as yet whether the different Ssa's have different affinities for J-proteins and NEFs but remains an attractive possibility that could partly explain different prion phenotypes when members of the Ssa family are individually expressed as a sole source of Ssa in the cell.

Both the constitutive and heat-inducible isoforms of Hsp70 functioned well with Hsp104 in refolding thermally denatured luciferase but preferentially, Ssa1 and Ssa3 were more active in protein refolding compared to Ssa2 and Ssa4. Also the Hsp104 functions differently with regard to prion propagation and thermotolerance suggesting that the disaggregation machinery interacts differently with amorphous aggregates than with highly ordered prion aggregate and such differences may be reflected by specific Hsp70 component of the protein disaggregation machinery. The thermotolerance exhibited by all the Ssa strains were comparable except for the Ssa3 strain that was significantly more resistant than the other three Ssa strains. These results might indicate that heat inducible isoforms of Hsp70 may be more efficient in cooperating with Hsp104 during the process of protein disaggregation and have more affinity for heat denatured substrates. These results are consistent with previous studies carried out by Sharma et al. (2009). The western blot analysis on the four Ssa's also revealed that there was no difference in the expression levels of Hsp70 and Hsp104 in the Ssa strains suggesting that the difference in functions observed among the individual Ssa's are not attributed by difference in protein expression levels.

Most molecular chaperones are induced in response to a wide variety of stress conditions and are important for protection of cells from these adverse conditions reviewed in Craig et al. (1993) and Mager & Ferreira (1993). They assist abnormal proteins accumulating under stress conditions to regain their proper folding or help their proteolytic degradation. Most of the chaperones and proteases have essential roles under non-stress conditions by assisting protein biogenesis, oligomer assembly, traffic between cellular organelles, and selective protein degradation (Ciechanover et al., 1995; Hilt & Wolf, 1996). Hence, in addition to their protective functions, they may help to re-orchestrate cellular metabolism to the needs of the stress response. The oxidative stress response of the Ssa family to varying concentrations of $H_2O_2$ revealed that Ssa2

and Ssa3 [*psi*$^-$] cells showed sensitivity to 3 mM concentration of $H_2O_2$ whereas both Ssa1 and Ssa4 started showing sensitivity to $H_2O_2$ only at higher concentrations. These results can be supported from previous studies carried out by Godon et al. (1998). They showed that Ssa1 and Ssa3 are upregulated (2.7 and 4.2) but Ssa2 (0.33) is downregulated in response to $H_2O_2$. Although this finding do not correlate with the sensitivity observed at 3 mM concentration for Ssa3 [*psi*$^-$] cell, it is worth noting that the microarray results showed that the *DOT5* gene which responds to chemical stimuli and oxidative stress response was among the top 10 genes that were down regulated in Ssa3 and this might contribute to the sensitivity of Ssa3 [*psi*$^-$] at 3 mM concentration of $H_2O_2$. The transcriptional profiling of the Ssa family also revealed that the *TRX2* gene which respond to chemical stimuli and oxidative stress response were also among the top 10 induced gene in Ssa4 strain which might provide an explanation of resistance displayed by Ssa4 to $H_2O_2$. Taken together the response of all the Ssa isoforms to $H_2O_2$, it can be speculated that $H_2O_2$ might be important in switching cellular activities of molecular chaperones from biosynthetic toward protective functions.

Chemicals such as hydrogen peroxide and diamide which causes lipid peroxidation, protein oxidation, and DNA damage in living cells are often used to deduce the CWI pathway response to oxide stress in *S. cerevisiae.* Both of these two chemicals are capable of activating the dual phosphorylation of Slt2/Mpk1, although with differing kinetic activity (Fuchs & Mylonakis, 2009). Hydrogen peroxide does not require one of the known CWI stress sensors of *S. cerevisiae* to provoke the phosphorylation of Slt2/Mpk1 and is suspected to elicit an intracellular means of activation (Vilella et al., 2005). Thus these results are also suggestive but not conclusive of the involvement of the Ssa family of Hsp70 in CWI signalling pathway.

The involvement of Ssa family in CWI signalling pathway can be further supported by the sensitivity displayed towards cell wall damaging agents (SDS) as damage to the cell wall elicits this pathway. Both the [*PSI*$^+$] and the [*psi*$^-$] versions of the constitutive isoforms of Hsp70 exhibited sensitivity to 0.01% SDS. The Ssa3 [*psi*$^-$] was sensitive to SDS at a concentration of 0.005% but Ssa4 [*psi*$^-$] exhibited sensitivity at higher concentrations of SDS (0.01%). Again the sensitivity observed for Ssa3 [*psi*$^-$] at a concentration of 0.005% and the sensitivity displayed by Ssa4 [*psi*$^-$] at higher concentrations of SDS can be attributed to the down regulation of the gene *DOT5* in Ssa3 strain and upregulation of *TRX2* in Ssa4 obtained from microarray analysis. However, due to varying degree of sensitivity seen for the members of Ssa family on

SDS, an accurate comparison could not be made, but these results are suggestive of the involvement of the Ssa family of Hsp70 in CWI signalling pathway.

Previous genetic screens carried by Loovers et al. (2007) identified an array of mutations within the two major cytosolic Hsp70 chaperones Ssa1 and Ssa2 which impair [*PSI*$^+$] propagation. There were five mutations identified in identical residues of both Ssa1 and Ssa2 (A53V, T64I, R74K, G287D, T295I). These findings further support the functional redundancy of the Hsp70-Ssa family with respect to [*PSI*$^+$] propagation. One of the mutants (G73D) that was unique to Ssa1 was located within the ATPase domain highlighting the importance of ATPase regulation of Hsp70-Ssa in prion propagation. Thus this study investigated into the functional redundancy of the ATPase domain of Hsp70-Ssa family by taking a subset of mutations viz. G73D, G287D, T295I generated in Ssa1-4.

The results from this study supported the work carried out by Loovers et al. (2007) which showed that the mutations G287D and T295I impair prion propagation in Ssa1 as well as in Ssa2. Also investigation into the functional conservation of these two mutations in Ssa3 and Ssa4 revealed impairment of [*PSI*$^+$] propagation thus supporting the fact that the two mutations G287D and T295I are functionally conserved in Ssa3 and Ssa4. Although the G73D mutation impaired [*PSI*$^+$] propagation of the constitutive isoforms of Hsp70 it generated a lethal phenotype for the heat inducible isoforms. The mutations on the heat inducible isoforms had a more prominent effect on growth rate than those on its constitutive counterpart supporting the suggestion that the function of Ssa family in essential cellular pathways can be separated from its function in prion propagation (Jones & Masison, 2003; Jung et al., 2000). The mutations G287D and T295I on Ssa3 and the mutation G287D on Ssa4 produced a temperature sensitive phenotype at 37 °C and 39 °C which did recover on 1M sorbitol suggesting that these mutations may affect the function of Hsp70 at elevated temperature and also implicates Hsp70-Ssa's involvement in CWI signalling pathway. The involvement of Hsp70-Ssa's in CWI signalling pathway was further supported by varying degree of sensitivity represented by the *SSA1-SSA4* mutant alleles compared to their wild type in presence of SDS (0.005 and 0.01%) which is a cell wall damaging agent. Although no accurate comparisons can be made based on this results and requires further investigation, but the findings from this study implicate a role of Hsp70-Ssa in CWI signalling pathway.

To further support the hypothesis of involvement of Hsp70-Ssa in CWI signalling pathway, the *SSA1-SSA4* mutant alleles were tested in presence of varying

concentrations of $H_2O_2$ which induces oxidative stress response. In *S. cerevisiae*, the PKC1-MAPK cell integrity pathway is involved in responses to a wide variety of stresses (Davenport et al., 1995; Kamada et al., 1995; Torres et al., 2002). Previous studies have shown that Pkc1 and the upper elements of the cell integrity pathway are required for survival and adaptation to the oxidative stress provoked by oxidizing agents like hydrogen peroxide and diamide (Vilella et al., 2005). Thus when the mutant strains were tested for oxidative stress response to $H_2O_2$ a varying degree of sensitivity was observed among all the mutant alleles compared to their wild type. The mutations on the constitutive isoforms of Hsp70 exhibited sensitivity at lower concentrations of $H_2O_2$ compared to their wild type. But the G287D mutation in Ssa3 started showing sensitivity to $H_2O_2$ at a concentration of 3 mM while the WT was still unaffected at this concentration. The maximum resistance among the *SSA1-SSA4* mutant alleles to varying concentrations of $H_2O_2$ was exhibited by the mutations on Ssa4 which did not show sensitivity to $H_2O_2$ until a concentration of 4 mM was reached. These findings might implicate that the heat inducible isoforms of Hsp70 have a much greater role to play in CWI signalling pathway either by direct interaction with the essential components of the pathway or indirectly affecting the pathway. On the other hand, oxidative stress causes protein denaturation and interferes with the protein folding. Thus the proteins are denatured at higher concentrations of $H_2O_2$ and heat inducible isoforms of Hsp70 are more efficient in dealing with stress response compared to its constitutively expressed isoforms.

Further to this, the mutant strains were also assessed for their ability to show acquired thermotolerance under heat shock conditions. Under environmental stress Hsp104 in conjugation with Hsp70 and Hsp40 acts as a key regulator and helps the cell to withstand heat shock conditions and thus rescue heat denatured, aggregated proteins (Glover & Lindquist, 1998). Upon induction of Hsp104 at 39 °C followed by heat shock at 47 °C, a clear growth gradient was observed for WT cells on YPD. Prolonged exposure resulted in increased cell death. On subsequent exposure of the cells to 3 mM Gdn-HCl inhibited cell growth after a short period of exposure to heat shock temperature, illustrating inhibition of Hsp104 activity by GdnHCl, thus hindering thermotolerance. The mutants $Ssa1^{G73D}$, $Ssa3^{T295I}$ and $Ssa4^{G287D}$ were reduced in their activity of acquired thermotolerance compared to their WT which reflects impairment in Hsp104 activity in these mutations and inability to cooperate in the disaggregation process. On assessment of Hsp104 *in vivo* activity for measuring the protein refolding

of bacterial luciferase did not correlate with the results obtained from acquired thermotolerance, whereas in theory a demonstrated reduction in acquired thermotolerance by plate assay should be reflected in a reduced ability to refold denatured luciferase. For example the luciferase refolding activity of Ssa2$^{G287D}$ was similar to a Δ*hsp104* strain and thus was completely defective in protein refolding. But the acquired thermotolerance for Ssa2$^{G287D}$ gave a phenotype that was similar to WT [Ssa2]. Also the mutants, Ssa1$^{G73D}$, Ssa3$^{T295I}$ and Ssa4$^{G287D}$ could not tolerate heat stress for a longer duration of time but the same was not reflected in their ability to refold luciferase. Thus this results are indicative of Hsp70 having a role to play in acquired thermotolerance either directly or through complex interactions with Hsp104 during heat shock. Also previous studies have shown that Hsp104 and Hsp70 are implicated in facilitating cells to survive heat stress (Glover & Lindquist, 1998; Sanchez & Lindquist, 1990), thus these results are indicative that Hsp70 in conjunction with Hsp104 might have more important role to play then expected to help in recovery of the proteins in the cells following a thermo-stress.

Previous studies on Hsp70 genetic screens have mainly identified mutations on the ATPase domain highlighting the importance of this domain on prion propagation (Jones & Masison, 2003; Jung et al., 2000; Loovers et al., 2007). Previous work carried out in the laboratory on the PBD Ssa1 $^{F475S}$ highlighted that this is the only PBD mutant to date that impair prion propagation and also at the same time exhibit a temperature sensitive phenotype at 37 °C reflecting the significance of this mutant not only in prion propagation but also on the other cellular functions of Hsp70 (Cusack, 2010) . Thus to further assess the overlapping and distinct functions of this PBD mutant, this mutation was generated in all the members of the Ssa family phenotypically characterised for its role in prion propagation and other cellular functions of Hsp70. The heat inducible member of Hsp70 Ssa3 was rendered non-functional due to this mutation and thus gave a lethal phenotype. Apart from Ssa3, this mutation severely impaired [*PSI*$^{+}$] propagation of all the members of Hsp70-Ssa family. The F475S mutation was also unique as it demonstrated temperature sensitivity at elevated temperature suggesting overlapping functions of the Ssa family in regard to cellular functions and prion propagation. Recovery of the temperature sensitive phenotype of the F475S mutant in presence of osmotic stabiliser is also indicative of a role of Hsp70-Ssa in CWI signalling pathway. Analysis of the effect of this mutation on oxidative stress response and damage induced by cell wall stress agents like SDS futher supports the hypothesis of involvement of

Hsp70-Ssa in CWI signalling pathway. The acquired thermotolerance and luciferase refolding activity of this mutant was similar to the WT in all the Ssa strains indicating that Hsp104 was not defective in its ability of protein refolding ability. The growth rate of yeast was also affected by the mutation. Thus these results are indicative of overlapping functions of Hsp70-Ssa due to the F475S mutation suggesting that the cellular pathways are not separate to Ssa function in prion propagation.

Microarray analysis was carried out to identify possible differential expression profiles caused by the Ssa members when expressed as a sole source of Ssa in the cell. This analysis revealed that highest number of genes were induced or repressed in Ssa4 compared to the WT Ssa1 thus suggesting that Ssa4 is more distantly related to Ssa1 then the other two members in terms of transcriptional profiling. The transcription profiling data shows distinct and overlapping functions of the Ssa family. It was worth noting that among the top ten genes that were upregulated in Ssa4, there were genes involved in response to chemical stimuli and oxidative stress response (*TRX2*) which might explain the increased resistance of Ssa4 to oxidative stress response and SDS. Also in Ssa3, *DOT5* was among the top ten down regulated genes which also explains the sensitivity displayed by Ssa3 in response to oxidative stress and chemical stimuli like SDS. Groups of genes involved in cellular ion homeostasis were uniquely upregulated in Ssa2 thus indicating functional specialisation. Also there were groups of genes involved in heat stress, cell cycle regulation, cell wall organisation that were uniquely upregulated or downregulated in Ssa2, Ssa3 or Ssa4. This further supports the hypothesis of distinct functions of the Ssa family. The microarray analysis was further supported by qPCR analysis of 25 genes. The results from both microarray and qPCR were 60-70% similar.

Thus investigation of expression of individual Ssa's provided us with a new insight to several overlapping and distinct functions of the Ssa family of Hsp70. This was further supported by the ATPase domain and the PBD mutant which showed that Hsp70 functions can be separate in cellular pathways and prion propagation. Also the microarray data highlighted specific genes that are differentially regulated when members of the Ssa family are expressed individually further highlighting functional specialisation.

**Table 5.6 Result summary of the chapter**

| **Mutations** | **Phenotypic and molecular characterisation of the Ssa family and its mutants** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Prion Phenotype** | **MGT** | **Ts phenotype** | **Sorbitol recovery** | **SDS sensitivity** | **Oxidative stress response (OSR)** | **Acquired thermotolerance (ATT)** | **Luciferase refolding** | **Protein expression level** |
| **Phenotypic and molecular characterisation of the Ssa family** | | | | | | | | | |
| Ssa1ψ+ | Intermediate in its efficiency to propagate [*PSI*$^+$] | 108.1±1.1 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 5 mM | Displayed functional Hsp104 activity | Showed 73% recovery of denatured luciferase | Equal expression when probed with Hsp70 ad Hsp104 antibody |
| Ssa1ψ- | Impaired [*PSI*$^+$] propagation when cured on 3 mM Gdn-HCl | 113.7±0.9 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.01% | Displayed sensitivity to $H_2O_2$ at 4 mM | Was not as efficient as its inducible counterparts in displaying functional Hsp104 activity | Showed 65% recovery of denatured luciferase | Expression level slightly lower compared to [*PSI*$^+$] version when probed with Hsp70 |

**Table 5.6 continued……**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ssa2ψ+ | Intermediate in its efficiency to propagate [*PSI*$^+$] | 92.2±0.8 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.01% | Displayed sensitivity to $H_2O_2$ at 4 mM | Displayed functional Hsp104 activity | Showed 48% recovery of denatured luciferase | Equal expression when probed with Hsp70 ad Hsp104 antibody |
| Ssa2ψ- | Impaired [*PSI*$^+$] propagation when cured on 3 mM Gdn-HCl | 117.7±0.9 | Did not display Ts phenotype | - | Compared to Ssa2ψ+ showed better growth on 0.01% SDS | Displayed sensitivity to $H_2O_2$ at 3 mM | Was not as efficient as its inducible counterparts in displaying functional Hsp104 activity | Showed 45% recovery of denatured luciferase | Expression level slightly lower compared to [*PSI*$^+$] version when probed with Hsp70 |
| Ssa3ψ+ | Most efficient in [*PSI*$^+$] propagation | 181±0.5 | Was Ts compared to its constitutive counterparts | Recovered on sorbitol | Did not display sensitivity to SDS | Displayed sensitivity to $H_2O_2$ at 5 mM | Displayed functional Hsp104 activity | Showed 67% recovery of denatured luciferase | Equal expression when probed with Hsp70 ad Hsp104 antibody |

**Table 5.6 continued……**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ssa3ψ- | Impaired [*PSI*$^+$] propagation when cured on 3 mM Gdn-HCl | 158.6±2 | Was Ts compared to its constitutive counterparts | Recovered on sorbitol | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 3 mM | Displayed better functional Hsp104 activity compared to its constitutive counterparts | Showed 46% recovery of denatured luciferase | Expression level slightly lower compared to [*PSI*$^+$] version when probed with Hsp70 |
| Ssa4ψ+ | Was very poor in [*PSI*$^+$] propagation | 177.5±1.9 | Was Ts compared to its constitutive counterparts | Recovered on sorbitol | Did not display sensitivity to SDS | Displayed sensitivity to $H_2O_2$ at 5 mM | Displayed functional Hsp104 activity | Showed 50% recovery of denatured luciferase | Equal expression when probed with Hsp70 ad Hsp104 antibody |
| Ssa4ψ- | Impaired [*PSI*$^+$] propagation when cured on 3 mM Gdn-HCl | 141±1.2 | Was Ts compared to its constitutive counterparts | Recovered on sorbitol | Displayed sensitivity to SDS at 0.01% | Displayed sensitivity to $H_2O_2$ at 5 mM | Displayed better functional Hsp104 activity compared to its constitutive counterparts | Showed 41% recovery of denatured luciferase | Expression level slightly lower compared to [*PSI*$^+$] version when probed with Hsp70 |

**Table 5.6 continued……**

**Phenotypic and molecular characterisation of the ATPase domain mutations of the Ssa family**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $Ssa1^{G73D}$ | Impaired [*PSI*$^+$] propagation | 115.12 ± 1.7 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 2 mM | Defective in showing ATT | Similar to WT in refolding luciferase | - |
| $Ssa1^{G287D}$ | Impaired [*PSI*$^+$] propagation | 117.11 ± 0.2 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.01% | Displayed sensitivity to $H_2O_2$ at 2 mM | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - |
| $Ssa1^{T295I}$ | Impaired [*PSI*$^+$] propagation | 116.26 ± 1.0 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.01% | Displayed sensitivity to $H_2O_2$ at 3 mM | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - |
| $Ssa2^{G73D}$ | Impaired [*PSI*$^+$] propagation | 109.19 ± 0.8 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.01% | Displayed sensitivity to $H_2O_2$ at 2 mM | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - |
| $Ssa2^{G287D}$ | Impaired [*PSI*$^+$] propagation | 112.17 ± 0.3 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 3 mM | Similar to WT in showing ATT | Behaved like a Δ*hsp104* strain | - |
| $Ssa2^{T295I}$ | Impaired [*PSI*$^+$] propagation | 103.44 ± 0.6 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 3 mM | Similar to WT in showing ATT | Better than WT in refolding luciferase | - |
| $Ssa3^{G73D}$ | Lethal | - | - | - | - | - | - | - | - |

**Table 5.6 continued……**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $Ssa3^{G287D}$ | Impaired [*PSI*$^+$] propagation | 167.64 ± 2.4 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 3 mM | Similar to WT in showing ATT | Better than WT in refolding luciferase | - |
| $Ssa3^{T295I}$ | Impaired [*PSI*$^+$] propagation | 178.30 ± 1.8 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 3 mM | Defective in showing ATT | Better than WT in refolding luciferase | - |
| $Ssa4^{G73D}$ | Lethal | - | - | - | - | - | - | - | - |
| $Ssa4^{G287D}$ | Impaired [*PSI*$^+$] propagation | 180.29 ± 1.1 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 4 mM | Defective in showing ATT | Defective in refolding luciferase | - |
| $Ssa4^{T295I}$ | Impaired [*PSI*$^+$] propagation | 151.38 ± 3.1 | Did not display Ts phenotype | - | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 4 mM | Similar to WT in showing ATT | Better than WT in refolding luciferase | - |

**Phenotypic and molecular characterisation of the SBD mutant (F475S) across the Ssa family**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $Ssa1^{F475S}$ | Impaired [*PSI*$^+$] propagation | 121.04 ± 0.2 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Displayed sensitivity to SDS at 0.01% similar to WT | Displayed sensitivity to $H_2O_2$ at 2 mM | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - |

**Table 5.6 continued……**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $Ssa2^{F475S}$ | Impaired [*PSI*$^+$] propagation | 145.82 ± 3.9 | Ts at 37 °C and 39 °C | Recovered on sorbitol | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 3 mM | Similar to WT in showing ATT | Better than WT in refolding luciferase | - |
| $Ssa3^{F475S}$ | Lethal | - | - | - | - | - | - | - | - |
| $Ssa4^{F475S}$ | Impaired [*PSI*$^+$] propagation | 183.31 ± 1.41 | Ts at 37 °C but better at 39 °C | Recovered on sorbitol | Displayed sensitivity to SDS at 0.005% | Displayed sensitivity to $H_2O_2$ at 4 mM | Similar to WT in showing ATT | Similar to WT in refolding luciferase | - |

# CHAPTER 6

## GENERAL DISCUSSION AND FUTURE DIRECTIONS

## 6.1 General discussion and future work

The Hsp70 family of molecular chaperones constitute the central components of complex cellular networks of molecular chaperones and they also act as catalysts for protein folding processes in the cell. By binding and releasing hydrophobic surfaces on partially folded proteins, Hsp70 plays a general role in helping proteins adopt and maintain native conformations. It also contributes to various cellular processes such as translation and transport across membranes, protects cells during stress injury by preventing protein disaggregation. Thus the Hsp70 family of molecular chaperones have housekeeping functions as well as involved in protein quality control functions. It is well established that all these functions of Hsp70 are regulated via altered expressions of Hsp70 in the cell, differential expression of Hsp70 isoforms and the cooperation of Hsp70 with other chaperone systems and co-chaperones which are selectively recruited by Hsp70 chaperones to fulfil specific cellular functions and provide Hsp70 with a broad spectrum of activity. While much data has accrued in relation to the ATPase and substrate binding cycles of Hsp70 (primarily from assessment of the *E. coli* orthologue DnaK) there is a distinct lack of information regarding the regulation of this important chaperone at the post-translational level. In both yeast and mammalian cells, cell cycle dependent phosphorylation of Hsp90 has been shown to alter Hsp90-client interactions (Mollapour et al., 2010; Shock, 2010). Also in vertebrates it has been shown that Hsp70 is phosphorylated in response to exercise (Melling et al., 2009), but the site of activation, the kinase(s) responsible and biological significance of these modifications has not been determined. In this study using prions in yeast as model for studying post-translational modification of Hsp70 it is shown that Hsp70 cellular functions can be regulated by direct phosphorylation and phosphorylation of Hsp70 also have a significant role to play in prion propagation in yeast.

A recent global phophoproteomics study carried out by Albuquerque et al. (2008) demonstrated that *in vivo* Hsp70 is phosphorylated. Using a simplified yeast system, this study have systematically assessed a variety of non-phosphorylateable and phosphomimetic Hsp70 mutants for phenotypic alteration of functions. It has been shown in this study that alteration of Hsp70 phosphorylation status *in vivo* can impair prion propagation, alter both basal and acquired thermotolerance and in some cases render cells inviable. Among the various phospho-sites analysed in this study the T36 phospho-site appeared to be an important site as it is located within a consensus S/T-P CDK phosphorylation motif and is highly conserved among Hsp70 family proteins.

This site is speculated to have a potential regulatory role as it is proximal to ATP binding and co-chaperone binding region (Truman et al., accepted). Also this study has shown that the T36A mutation caused the cells to become pink as revealed by the colour on YPD and growth in adenine lacking medium suggesting impairment in prion propagation due to this mutation. This might suggest that the efficient propagation of [*PSI*$^+$] is critically dependent on the regulation of substrate interaction by the ATPase domain of Hsp70 and T36 may be altered in its ATPase activity. Determination of ATPase activity of this mutant could further provide insight into the role of this mutation in prion propagation.

Previous genetic screens carried out by Jones & Masison (2003) and Loovers et al. (2007) have shown the importance of regulation of the ATPase domain in prion propagation. They had shown that the mutations in the ATPase domain weaken the Hsp70's substrate – trapping ability, implying that the impairment of [*PSI*$^+$] due to these mutations are mainly due to altered ability of the ATPase domain to regulate substrate binding. The multiple phosphomutants generated on the ATPase domain had a major effect on the [*PSI*$^+$] propagation indicating that post–translational modifications like phosphorylation of Hsp70 also targets the ATPase domain of Hsp70 in prion propagation. Also it can be speculated from this study that there might occur complex interactions between the various phospho-sites on the ATPase domain that might contribute to impairment in [*PSI*$^+$] propagation. Thus the need for assessing the ATPase activity of these mutants is of prime importance as this could provide an important insight into the role of phosphorylation of Hsp70 in prion propagation. The PBD mutants did not have a major effect on [*PSI*$^+$] propagation and cell growth, except for alteration of the T492 residue, which affected both viability and as well as cell growth.

The phosphomutant with maximum sites made non-phosphorylatable also had a major impact on prion propagation. Some of the mutations also generated lethal phenotypes which is indicative of the effect of these multiple phosphomutants both on growth and prion phenotype of Hsp70 suggesting that phosphorylation not only have an impact on prion propagation but also on cell viability. This result also further supports the hypothesis of complex interaction between various phosphorylation sites of Hsp70 as per cell requirement. Also the survival of non-phosphorylatable mutant of Hsp70 is indicative of the fact that phosphorylation of Hsp70 is regulated by complex interactions between the various phosphosites within, Hsp70 is tolerant to not being phosphorylated at any one time at these multiple phosphosites.

As previously discussed, there were a number of phosphomutants that were temperature sensitive at elevated temperature and were recovered in presence of osmotic stabilisers like 1 M sorbitol indicating that Ssa might have a role to play in CWI signalling pathway. To further verify that the temperature sensitive phenotypes of the mutants are due to phosphorylation and not due to strain background, these mutants should be tested in a different strain background like the commonly used yeast strain BY4741. In order to achieve this, the Ssa family must be deleted in this background so that the mutants may be expressed as the sole source in the strain. Phenotypes of strains expressing Ssa mutants can then be tested at elevated temperatures and compared to phenotypes observed in the G402 background. Sensitivity of the phosphomutants may also be tested by growth on cell wall damaging drugs such as caffeine, SDS and calcofluor white.

Using 2D-GE this study tried to demonstrate that dephosphorylation of Hsp70 in response to heat stress and the production of a variety of different iso-forms, which may reflect a requirement for broadening chaperone/client interactions following stress. But due to possible experimental error more likely at the focussing point, these results were not conclusive and require further investigation.

The acquired thermotolerance results for some of the phosphomutants could reflect a more efficient Hsp70 system that is functioning with Hsp104 for enhanced thermotolerance or it can be speculated that yeast Ssa1-Hsp70 might have a greater role to play in acquired thermotolerance than has previously been thought. It might directly affect Hsp104 function by avidly binding to denatured substrates and thus enhancing Hsp104 function by preventing aggregate formation so that at high expression levels of Hsp104 under heat shock conditions, it has a less number of substrates to refold back into their functional conformation. The involvement of Hsp70 in acquired thermotolerance has also been shown by Sanchez et al. (1993). Also Hsp70 has been shown to be required for thermotolerance in many different organisms (Atkinson & Walden, 1985; Hahn & Li, 1982; Li et al., 1991; Lindquist & Craig, 1988; Riabowol et al., 1988; Solomon et al., 1991).

While investigating the effects of Hsp70 phosphorylation mutations on chaperone activity and prion propagation, it was important to look for the abundance of Hsp104 and Hsp70 in the cell to ensure that the protein expression levels are not having an impact on prion propagation and other observed chaperone activity. Thus western blot analysis was performed and the results indicated that the effects of the

phosphomutants on [*PSI*$^{+}$] propagation and other chaperone activity are not influenced by changes in chaperone abundance.

Previous mutational, NMR and biophysical studies in *E. coli* Hsp70 (DnaK) have shown the importance of the linker region between the ATPase domain and PBD in the allosteric regulation of the ATPase cycle of Hsp70 and also in inter-domain communication (Swain et al., 2007; Vogel et al., 2006). One of the hypotheses thus set was that the potential phosphorylatable residue identified on the linker region of Hsp70-Ssa family could potentially influence inter-domain communication, depending on phosphorylation status. The findings from this study imply that the phosphorylation of T378/T379 residue in linker region of the Hsp70 may be important for regulating inter-domain communication. Introducing mutations to components of the cell wall integrity (CWI) signalling pathway results in common phenotypes such as temperature sensitivity at 39 °C, sensitivity to drugs that damage the cell wall and oxidative stress response. These phenotypes can be rescued in presence of an osmotic stabilizer such as sorbitol in growth media. The mutations in the linker region of Hsp70-Ssa family also showed that Hsp70 may be indirectly involved in the CWI pathway and thus affect any of the components of the pathway or alternatively it might directly affect the pathway by regulating some of the essential components of the pathway. Phenotypes of the strains expressing mutations in the linker region of Ssa could be tested in presence of components of the CWI signalling pathway at elevated temperature to further investigate into the role of Hsp70 in this pathway.

Recently work carried out by Truman et al., (submitted) has shown that phosphorylation of conserved CDK consensus site, Ssa1 T36, is cell cycle regulated and has a specific role to play in regulating G1/S progression. Also proteomic analysis carried out revealed that T36 phosphorylation alters global interaction of partner and client proteins. Thus, it would be convenient to carry out any further investigation on T36 phospho-site by first synchronising the cell in G1 or G2 phase prior to carrying out phenotypic analysis.

Many studies carried out in the past decade strongly supported structural homology and functional redundancy among the members of the cytosolic Hsp70 Ssa-family as single isoforms were more generalised to explain the functions of other isoforms. However, the idea of specialised role of Hsp70 isoforms are now being well appreciated in humans, primates and *E. coli* (Brocchieri et al., 2008; Daugaard et al., 2007; Genevaux et al., 2007; Tutar et al., 2006). The question of functional

specialisation among the yeast cytosolic Hsp70 isoforms of the Ssa-subfamily has never been systematically tested, although several reports indicated such possibility (Kabani & Martineau, 2008). Previous work carried out by Sharma et al. (2009) and Sharma & Masison (2008) provided a new insight into redundant yet clearly distinct functions of yeast cytosolic Ssa Hsp70's with regard to propagation of yeast prions. The results from this study also support the functional specialisation among the members of cytosolic Ssa Hsp70. By expressing each member individually as the sole source of Ssa, it is clear that each member exhibit functional specialization with respect to growth and prion propagation. This functional specialization between members of the Ssa family may be due to differences between members which may determine how they interact with Sup35. Alternatively, it is possible that individual members of the Ssa family are regulated differently. Ssa proteins are recruited and activated by J proteins (Hsp40/DnaJ family) and regulated by nucleotide exchange factors such as Fes1 and Sse1. It is conceivable that Ssa isoforms have different preferences or affinities for particular J proteins and NEF's, which in turn may explain different prion phenotypes when members of the Ssa family are individually expressed.

Although the stress inducible Hsp70's functions differently than their constitutively expressed counterparts, there was a similar distinction with regard to the interaction with [*PSI*$^{+}$] among the constitutive and inducible isoforms of Hsp70 which uncovered functional differences among the constitutive and inducible isoforms of Hsp70 as well as similarity in functional differences between the isoforms expressed under different conditions. These are suggestive of the fact that the functions of Hsp70 isoforms for cell survival under stress are different from the functions of constitutive isoforms expressed under optimal conditions. Also these observations are indicative of the subtle variations in Hsp70 function that might exist to improve fitness under optimal conditions, which are also important during stress.

The *SSA* subfamily of Hsp70 is essential for the growth of *S. cerevisiae* (Werner-Washburne & Craig, 1989). Cells expressing constitutively expressed isoforms of Hsp70 grow quicker than its heat inducible counterparts. The constitutive isoforms refold protein under optimal growth conditions but the heat inducible isoforms are not as good in protein folding under normal conditions. These reflect differences in protein folding ability under normal and stressful conditions, perhaps reflecting a different distribution of client protein for these chaperones.

Apart from sequence homology that determines the interaction of chaperones with prion proteins, it is possible that the structural composition of the prion aggregate could contribute to determining the importance of a particular isoform of Hsp70-Ssa for prion propagation. For example, if [*PSI*$^{+}$] prions are organised in a way that makes them less accessible to certain isoforms, they might be more sensitive to the changes in abundance of that isoform. Difference in phenotype could then reflect differences in efficiency with which different Hsp70s interact functionally with other components of the disaggregation machinery. Thus further investigation can be carried out in determining the structural organisation of prion aggregates by SDD-AGE analysis of Sup35p polymers in G402 [*PSI*$^{+}$] cells expressing individual Ssa's as the sole source of Hsp70.

Furthermore, by carrying out transcriptomic profiling, it appears that numerous genes are regulated differently in the presence of individual members of the Ssa family. Groups of genes involved in regulation of the yeast cell cycle, chemical stimuli and oxidative stress response appear to be differentially regulated in cells individually expressing Ssa3 and Ssa4. Genes involved in cellular ion homeostasis were uniquely upregulated in Ssa2 thus indicating functional specialisation. Also there were groups of genes that were involved in heat stress, cell cycle regulation, cell wall organisation that was uniquely upregulated or downregulated in Ssa2, Ssa3 or Ssa4. This further supports the hypothesis of distinct functions of the Ssa family. The microarray analysis was further supported by qPCR analysis of 25 genes.

Previous studies have shown the importance of ATPase domain of Hsp70 in prion propagation by isolation of numerous mutants from the ATPase domain that has an effect on prion propagation (Jones & Masison, 2003; Loovers et al., 2007). As most of the mutants that impair prion propagation, isolated by random mutagenesis were located on the ATPase domain, thus this study carried out further analysis on a subset of mutants of Ssa family (G73D, G287D and T295I) of the ATPase domain. By specifically targeting the Hsp70 peptide-binding domain (PBD) for mutagenesis, previous work carried out in the laboratory have shown the importance of a localized region in allowing efficient prion propagation. When mapped onto the Hsp70 PBD crystal structure the location of this region suggests it has the ability to influence Hsp70 substrate specificity (Cusack, 2010). Among the previously isolated PBD mutants was the Ssa1$^{F475S}$ which is among the first Ssa1 mutation to affect both prion propagation and cellular function (Cusack, 2010). The F475S mutation was also generated in Ssa2-

Ssa4 to look into its affect on prion propagation and cellular functions. Thus this study also tried to investigate the functional conservation of ATPase and PBD mutations across the cytosolic Ssa family and implicate a general role of the Hsp70 Ssa family in prion propagation. It was observed that all the mutations on the ATPase domain and PBD could impair prion propagation and also did affect the cellular functions of Hsp70. These results are indicative of overlapping and distinct functions of Hsp70 with regard to prion propagation and other cellular functions. By constructing analogous mutants in closely related cytosolic Hsp70s this study identified functional similarities and differences between highly homologous Hsp70 species. Further genetic and biochemical analysis of these Ssa mutants will thus aid in understanding the precise role of Hsp-70 Ssa in amyloid prevention, formation and propagation within yeast cell.

The ability to separate Hsp70 essential and prion- related functions suggests that the Hsp70 chaperone machinery may be a good target for possible therapeutics for prion and other amyloid diseases. Also this is the first study that showed a clear link between Hsp70 phosphorylation status and *in vivo* function. Given Hsp70s central role in a variety of important cellular metabolic pathways and the conservation of these phosphorylatable sites in higher eukaryotes, these findings have far reaching implications and will aid in the characterisation of Hsp70's cellular functions in mammalian systems.

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