A METHOD FOR IDENTIFYING PROTEINS INVOLVED IN HEAT SHOCK PROTEIN SECRETION IN Saccharomyces cerevisiae

by

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CHAPTER I

INTRODUCTION

Saccharomyces cerevisiae: A Model Organism

Yeasts have made a massive contribution to our lives in a variety of ways. They have been used for brewing, baking, pharmaceuticals and other industrial processes for a number of reasons.

They possess relatively simple growth requirements and can be cultured easily. The conservation of most biochemical activities throughout a wide range of organisms has allowed for the use of *S. cerevisiae* as a model eukaryote which has contributed immensely to our understanding of cell biology. *S. cerevisiae* exists in a haploid or diploid state and can reproduce both asexually and sexually, having both the a and α mating types. It has a genome that has sixteen linear chromosomes and has been classified in the ascomycetes group of fungi. It is generally considered nonpathogenic except in a very small number of susceptible individuals. The entire genome of *S. cerevisiae* has been sequenced and is available on various public databases. The *Saccharomyces* proteome is currently being mapped, which covers the entire range of proteins and thier function. All of these factors allow us to use *S. cerevisiae* as a model for studying many processes of cellular function including secretion through temperature sensitive mutants, cellular and nuclear organization as well as protein function. For example, one model has utilized the *Saccharomyces* invertase protein as a marker for the external localization of a gene fused to a hybrid transcript (51). Being a nonpathogenic

model organism allows us to utilize the qualities of S. *cerevisiae* to learn about other pathogenic organism including *Candida albicans*, *C. dublienensis*, *Cryptococcoccus neoformans* and other eukaryotic pathogenic fungi.

Cell Wall of *S. cerevisiae*

The cell walls of a variety of fungi have been studied vigorously for a number of years. After disposing of the myth that the cell wall is an innocent bystander in the life of the organism, scientist now believe that the cell wall plays an important role in almost all areas of cellular function and processes. The structure (Figure 1.1) and function of the cell wall of the yeast *S. cerevisiae* has been of particular importance to a number of researchers. As with all fungi, this cell wall provides the structural support for the cell, protects against osmotic stress, maintains the physical shape characteristic and interacts with the environment surrounding the cell.

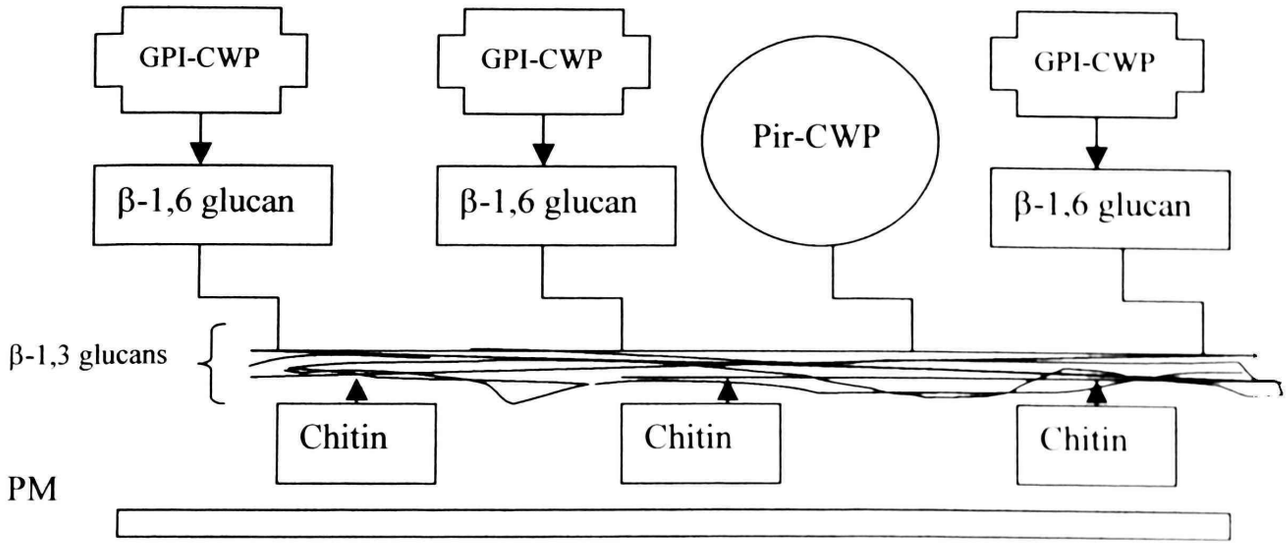

Figure 1.1. Molecular Model of the Yeast Cell Wall. The GPI-anchored cell wall proteins are linked to the β-1,3 glucans by a β-1,6 glucan molecule whereas the Pir cell wall proteins are linked directly to the β-1,3 glucans. The β-1,3 glucan layer is held together by hydrogen bonding between individual chains and this is then linked to the innermost chitin layer.

Previous research has focused on *S. cerevisiae* and the structural components of the cell wall, and has allowed for the development of our current molecular model. The cell wall is made up of a network of β-1,3 glucan molecules that provides much of the structural integrity of the cell wall. Since β-1.3 glucan molecules have multiple nonreducing ends, it has been postulated that this may be the site of attachment for other cell wall molecules (44). Chitin, which is a β-1,4 polymer of N-acetyl-D-glucosamine and makes up a small amount of the cell wall by mass, is closely associated with the plasma membrane and adds to the rigidity of the cell wall (35). Chitin can also be linked to side chains of β-1,6 glucans (33). These β-1,6 glucan molecules are mainly found at the very outside of the structural components of the cell wall and are closely associated with glycosyl phosphatidylinositol (GPI) anchored proteins (35). These GPI anchored cell wall proteins (CWP) are covalently linked to the cell wall by a β-1,6 glucan, which then is covalently attached to a β-1,3 glucan molecule. Fifty-one GPI anchored proteins have been postulated by genome in *silico analysis (7).* These GPI-CWP differ from another class of cell wall associated proteins called the proteins with internal repeats (PIR). PIR proteins are covalently attached to the β-1,3 glucans directly (47).

Proteins of the Cell Wall

Even after the elucidation that there are fifty-one genes encoding GPI anchored proteins in the genome of *S. cerevisiae*, the function of these has remained largely unsolved. Other cell wall proteins are involved in wall biosynthesis and breakdown and examples of these are glycosidases, glucanses, and chitinase. However, most of the cell

wall proteins have an effect on the porosity of the cell wall. These mainly influence the passing of high-molecular-weight molecules into and out of the cell. Zlotnik *et al.* showed that *S. cerevisiae* is resistant to spheroplasting by β-1-3 glucanases (69). The cells are only susceptible to the action of glucanase if they have been pretreated with a protease. This means that the proteins in the external leaflet of the cell wall exclude the glucanase from reaching the glucan which is in the internal leaflet of the cell wall. This porosity changes with the stage of growth of the cell and correlates to the different rates of incorporation of the mannoproteins onto the glucan (69).

Another major function of the cell wall is flocculation. This involves the formation of large clumps of cells when grown in liquid media by utilizing the interaction of lectin-like proteins on the flocculating cell and mannan side chains of glycoproteins on the other cells (69). This process is dependent on the flocculation genes *FLO1-5* (49). *FLO1* codes for a large protein that is GPI-anchored and may either act as the lectin itself or provide the framework for the lectin (34).

Some of these cell wall proteins are also involved in cell to cell interaction and in adhesion, as is the case for the sexual agglutinins (Aga1, Aga2 and Sag1)(41, 65). When *S. cerevisiae* mates, it need to recognize the other cell and change its conformation. It does this through shmoo formation (39). This distinct cell shape is necessary for mating to occur and requires massive cell wall restructuring. The protein Fig2 allows for successful recognition and subsequent cell wall changes. Another GPI-anchored protein, Egt2, is required for cell separation after mating has occurred (19, 38).

Secretion

In order for the GPI anchored and PIR proteins to reach the periplasmic space and beyond they may proceed through the classical secretion pathway: An overview this pathway is shown in Figure 1.2 (53). The GPI anchored proteins can only be released by treating the cell with glucanase and the PIR proteins are released by treating the cells with base (47). Other proteins can be released solely by reducing agents and may reach beyond the cell plasma membrane by an alternative mechanism; this subject has recently been reviewed by Karl Kuchler (40).

Classical Secretion

Classical secretion proceeds through a number of steps in *S. cerevisiae* and is the same as was originally described by Palade in mammalian cells (50). Classically secreted proteins are first transported to the lumen of the endoplasmic reticulum (ER). This can be achieved while the protein is being translated or it can be done posttranslationally. The first step in the export of a protein to the cell wall requires a signal recognition particle to recognize a signal sequence in the protein that is to be exported. The signal recognition particle is a cytoplasmic protein that selectively binds to ribosomes that are translating proteins destined to be secreted. The entire complex then migrates to the endoplasmic reticulum where it interacts with the signal sequence

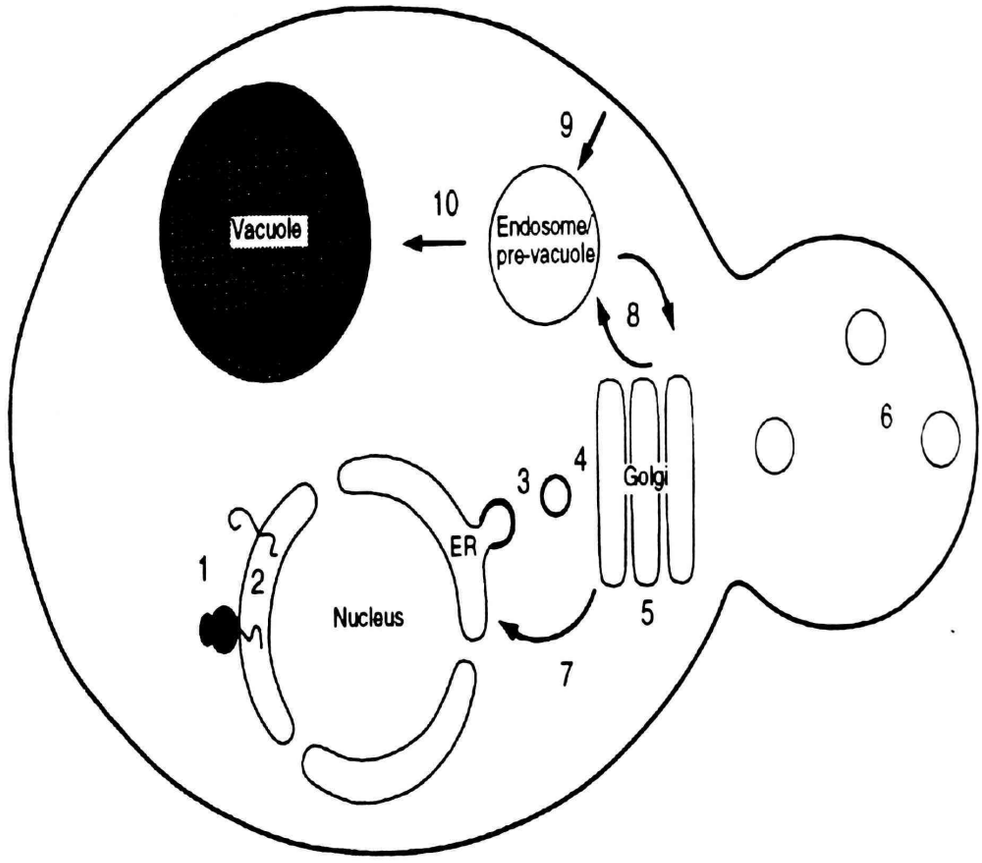

Figure 1.2. Overview of the stages of secretion. (1). The cotranslational extrusion of protein to be secreted into ER. (2). Posttranslational extrusion into the ER. (3). Vesicle budding off from ER destined to the cis-golgi network. (4). Vesicle before it is docking with the Golgi system. (5). Transition through the Golgi network where glycosylation and phosphorylation modifications occur. (6). Secretory vesicles bound to the newly forming bud. (7). Retrograde transport from the Golgi stacks back to the ER. (8). Cycling between the Golgi network and endosomes/ prevacuoles. (9). Endocytosis of plasma membrane components. (10). Maturation of prevacuoles into vacuoles.

receptor and the translocation machinery. The newly forming protein is then extruded through the membrane into the lumen of the endoplasmic reticulum. This signal sequence is located on the N-terminus in the first 20-25 amino acid residues. This is composed of 4-5 positively charged residues on the amino terminus followed by a hydrophobic region of 5-15 residues and then a final 5-7 polar amino acids (56). This entire signal sequence will be cleaved off the protein by a signal peptidase directly after the signal sequence reaches the lumen of the ER. This signal peptidase cleavage site is located in the final polar residues of the signal sequence (66). Once the signal sequence has been proteolitically cleaved, the protein can continue through the secretory process. While in the ER, proteins undergo a variety of changes including covalent modifications by N and O-linked glycosylation with oligosaccharides, the formation of disulfide bonds, phosphorylation and the addition of the GPI anchors (17). It is also in the ER that the protein assumes its natural conformation.

The proteins are then packaged into secretory vesicles to be transported to the Golgi apparatus. The secretory vesicles bud off from the ER to fuse with the cis-Golgi network and are coated by soluble cytosolic proteins. The coating proteins mediate the proteins being transported and the docking of the vesicles between compartments within the cell. Once inside the Golgi network, the protein undergoes further glycosylation while being transported from the cis-Golgi system to the trans Golgi system (50). There are three possible fates for proteins that reach the trans-Golgi network. They can be transported to vacuoles, be sent to the plasma membrane or become a resident protein of the Golgi system (36, 63, 11). Proteins to be secreted arrive at the plasma membrane via

coated vesicles that will fuse with the membrane and release their contents to the extracellular space. The lipid components and the proteins embedded in the lipid component of the vesicles become fixtures in the plasma membrane.

Alternative Secretion

Another class of rapidly growing proteins reaches beyond the plasma membrane but do not possess the consensus sequence for the N-terminal secretion signal sequence or the GPI- anchoring sequence. It has been postulated that these peptides get to the extracellular space by an alternative mechanism or non-classical secretion pathway. These types of proteins have been shown to exist in all types of bacteria, yeast and mammalian species. One of the most well defined pathways for alternative secretion proceeds through a family of ATP-dependent transport proteins, also called ATP binding cassette-transporters (ABC transporters) (29).

ABC-Transporter Proteins

The ABC-transporter proteins are very well conserved among all the different organisms that possess them. Most are comprised of two ATP-binding motifs and two membrane spanning regions containing six α-helices (40). These ABC-transporters appear to be very specific for the protein or other type of molecule that they transfer and most only transport one substrate (27). There are also a family of ABC-transporter proteins that only possess one ATP-binding region and one membrane-spanning region (40). The ABC-transporter family has been shown to transport a variety of compounds

across the membrane including proteins, sugars, ions, amino acids and peptides of all sizes.

Alternative Secretion in Bacteria

There are many different kinds of substrates exported from bacteria including toxins, proteases, cytolysins and hemolysins (24). It has been shown that the ABC-transporters can secrete certain toxins and several different compounds in the same family, even ones from different species of bacteria (25). Hemolysin A is the most characterized substrate for non-classical secretion in bacteria. This compound is secreted by a ABC-transporting system and requires the five genes *hylA-D* (37). The HlyA protein is synthesized as a pro-protein and is converted to the active form by a fatty acyl transfer by the HlyC enzyme. The HlyB protein is the ABC-transporter and may form a dimer to allow the active HlyA to cross the bacterial membranes (32). The function of the HlyD protein has not yet been characterized, but may involve interaction with TolC pump (67). The polypeptide product of the *hlyA* gene does produce a region that directs the completed protein to the transport system and can be considered a signal sequence. This sequence however, is not cleaved and is located in the terminal 48 residues of the mature peptide. However, it is not considered a typical secretion signal sequence in bacteria (37).

Non-Classical Export in Mammals

The evidence supporting the existence of an alternative secretion pathway is greater than the actual cloning and identification of the proteins responsible for such a pathway in mammals. The majority of substrates that are candidates for alternative secretion in mammals have been identified in humans. It seems that the immune system possesses most of the proteins that may be secreted by a non-classical mechanism. Examples of these include interluekins 1α and 1β, complement factor XIIIa, two different fibroblast growth factors, among several other proteins (45, 1, 28). These proteins are thought to be secreted by this route for several reasons: they lack a traditional secretion signal, they are secreted in the presence of brefeldin A (which blocks classical secretion), none are glycosylated even though they possess the correct signals (meaning that they did not pass through the golgi). They do however still undergo some post-translational modification (55). ABC-transport systems have been proposed in the case of the TAP1 and TAP2 proteins for the secretion of viral antigens attached to the MHC, which of course lack a hydrophobic signal sequence (55).

Export of a-Factor in Yeast

The most thoroughly studied example of a non-classically exported compound in yeast is the mating a-factor that is required for mating in the budding species. The a-factor was shown to be secreted in temperature sensitive mutants of the classical secretion pathway after imposing the restrictive block by McGrath *et al.* (46). Active a-factor is a 12-amino acid oligopeptide that is covalently modified at a number of residues

This is directed by a C-terminal sequence of 4 amino acids. The mature form of a-factor is farnesylated and carboxymethylated on the C-terminal cysteine residue after the final three residues have been cleaved off (26). An N-terminal extension of several hydrophilic residues is added to the pro-product, and it is only after all these modifications that the active form can be secreted. Once these modifications have taken place, the ABC-transporter Ste6p can then transport a-factor across the membrane. This is dependent on the lysis of ATP by Ste6p (5). Other members of the Ste family of proteins interact with a-factor, but none have been postulated to make up the membrane pore except Ste6p. Ste14p have been shown to possess farnesyl transferase activity and is probably involved in the maturation of a-factor (30).

Candidate Proteins for Alternative Secretion in Yeast

The secretion of a-factor is not the only candidate for a protein being secreted by a non-classical mechanism in yeast. There are several other proteins that meet the requirements for being considered as alternatively secreted proteins, but the mechanisms have not yet been elucidated. These candidate proteins lack the signal sequence for exportation through the classical pathway, yet are still proven to be found outside the cell plasma membrane. In most cases, the idea of cell lysis or cell wall damage has been ruled out. Some of the first evidence for an alternative pathway utilized a temperature-sensitive mutant defective in the *sec18* gene of the classical pathway. In this study, they found that some proteins were still exported even though the block had been imposed (10).

There have been several proteins that have been found in the cell wall of a variety of different fungi. Enolase is an enzyme that catalyses the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. Enolase was proven to be in the inner leaflet of the cell wall of *C. albicans* associated with glucan, and later was discovered in the cell wall of *S. cerevisiae* (4, 18). The presence of glycolytic enzymes in the cell wall of fungi is not without precedence. Phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase, and alcohol dehydrogenase have all been identified in the cell wall of *C. albicans,* and have all been experimentally verified through a variety of techniques (3, 23, 52). *Kluyveromyces lactis* also expresses glyceraldehyde phosphate dehydrogenase, and its protein product has been identified in the cell wall. Pardo *et al.* also discovered the Hsp150 and Bgl2 proteins in the cell wall of *S. cerevisiae* (15). The function of these proteins in the cell wall still remains a mystery. Some have postulated that they may not be functional; however, it has now been shown that glyceraldehyde phosphate dehydrogenase retains its enzymatic function while it is in the cell wall. This may also be the case for the other proteins of the glycolytic pathway in the cell wall. A 70 kDa heat shock protein (Hsp70), which also lacks the secretory signal sequence, has been reported to be in the cell walls of both *S. cerevisiae* and *C. albicans* (42, 43).

The Hsp70 Family in *Saccharomyces cerevisiae*

The ability to respond rapidly to variation in environmental temperatures is one of the most fundamental responses to change possessed by any organism. To a large part, these are done by a group of heat shock proteins, which were first identified as having a

heat shock response element in the promoter region of the gene. Now many families of Hsps have been identified, and it has been found that they are encoded by some of the most highly conserved genes throughout all forms of life (31). These genes are present from the most primitive bacteria all the way up to the complex human body. In the Hsp superfamily of genes, our laboratory is concerned with the Hsp70 family.

There have now been more than 14 genes in *S.cerevisiae* classified as Hsp70s, which have been grouped into five different families from *SSA* through *SSE* (48, 60). The synthesis of the mRNA transcript from all of these genes is induced during a variety of stressful situations, however most also display relatively high levels of transcription throughout the stages of growth. The *SSA* family is a group of four genes ranging in similarity from 98% to 83% and is labeled *SSA1, SSA2, SSA3* and *SSA4* (31). In this family, *SSA1* and *SSA2* are 98% alike; and if one is deleted, the remaining gene serves the necessary cellular function and there is no apparent phenotype (68). However, if both of these genes are disrupted, the organism has trouble growing at higher temperatures (13). It is thought that this is possible due to the overproduction of *SSA4*. If all the genes of this family are missing, the organism cannot survive. A single mutation of *SSA3* and *SSA4,* or a double mutant of both genes, has no obvious phenotype (68).

It was once believed that the Hsp70 group of proteins was confined to the intracellular space, but it has recently been found in the cell wall of both *S. cerevisiae* and *C. albicans*. The only family of Hsp70s that has been localized to the cell wall is that of the *SSA* genes and then only the Ssa1 and Ssa2 proteins (43). Several genes coding for cell surface proteins in *C. albicans* were isolated from a cDNA expression library. These

were then shown to be members of the *SSA* family of Hsp70 genes. This was confirmed by the use of an indirect immunofluorescence assay using affinity-purified antibody specific to the fusion protein produced by the cDNA clone. Intact cells were also biotinylated with a non-membrane-permeable derivative so cytosolic proteins can be distinguished from cell surface proteins. The cellular extracts were then affinity purified on a strepavidin column and Hsp70 detected by immunoblotting in the biotinylated fraction. Lopez-Ribot *et al.* also utilized immunoelectron microscopy to show that Hsp70 is a cell wall protein. The biotinylation and indirect immunofluorescence experiments were repeated with *S. cerevisiae* and the *SSA1* and *SSA2* genes of the Hsp70 family were proven to be in the cell wall of this organism. These experiments confirm that SSA1 and SSA2 are true cell wall proteins, and their extracellular location is not due to cell lysis. With a single *SSA1* or *SSA2* mutant, Hsp70 is still detected in the cell wall suggesting that one deletion can replace the other.

Functions of Hsp70s

The main function of most Hsp70s is to bind polypeptides in their unfolded state. In this instance they are acting like a molecular chaperone and can also participate in the folding of a polypeptide in a stepwise fashion (22). Therefore, Hsp70s are required in order for proteins to assume their natural, functional conformation inside the cell. The polypeptide-binding domain is located close to the C-terminus in the final 27 kDa of the mature Hsp70, and the N-terminus possesses an ATP-binding domain with very weak ATPase activity (20). This activity is so weak that the Hsp70 itself cannot be responsible

for hydrolyzing ATP. and must therefore stay in the ATP-bound form in which it cannot bind polypeptides. A DnaJ protein is needed to carry the polypeptide and associate with the Hsp70 before it can proceed. The DnaJ then causes the hydrolysis of ATP and the peptide can now move and bind to the Hsp70 (21). Another major function of Hsp70 proteins is their ability to help proteins translocate across cellular membranes. This may be due to the fact that proteins that have already reached a defined three-dimensional structure cannot cross membranes. Hsp70 proteins hold other proteins in the linear state. This is why they have been shown to be involved in a large 250 kDa complex that is required to help certain proteins cross membranes (59).

Hsp70s and the Heat Shock Response

As their name suggests, the Hsp70s are intimately involved in the heat shock response. They play an active role in protecting naturally folded proteins from undergoing denaturization while in the stressful situation of increased temperature. The Hsp70s and their homologs have been shown to protect some of the most sensitive and crucial elements of the cell from heat inactivation (12). They may accomplish this by simply not allowing the denatured proteins to form large aggregates. The Hsp70s are also involved in the regulation of the heat shock response. When the gene for the Ssa1 protein is overexpressed, there is a reduction in expression from both the *SSA1* and *SSA4* loci (16). This suggests that the role of Ssa1 is one of negative control. Also supporting this is the fact that when the *SSA1* gene is knocked out, one sees a dramatic up regulation from the *SSA4* locus (64).

Multiple Functions of the Hsp70s

The super family of Hsp70 proteins are involved in numerous cellular activities. They are needed to survive an increase in temperature, and are needed inside the cell in order to carry out normal cellular functions. Along with aiding in the regulation of the heat shock response and acting as a molecular chaperone, they also are involved in uncoating clathrin coated vesicles and preventing proteolysis (9). The Hsp70s have also been shown to be associated with steroid hormone receptors and the tumor suppressor protein p53 (14). Because Hsp70s display so many functions within the cell and seem to interact with such a variety of proteins, it has long been the goal of researches to elucidate how the protein accomplishes this. It is now believed that the partner protein that interacts with Hsp70 causes it to customize its conformation and perform so many different functions inside and outside the cell (54). Different Hsp70s are found in almost every cellular compartment, and these Hsp70s are not functionally interchangeable even though the similarity of the proteins may be very dramatic (98%). This phenomenon, along with the multiple actions of Hsp70s, could be due to the localization of certain partner proteins and their binding to the Hsp70 protein at highly specific regions (54). Since Hsp70 has been localized to the cell wall, its function in the cell wall is still under investigation and may have more to do with its cytosolic functions than with any function it has in the cell wall.

Aim of this Research

While the existence of alternative secretory pathways has now been hypothesized for a number of different proteins, over a wide range of organisms, and only relatively few pathways have been completed. It is the goal of this research to identify pathways that are involved in the secretion of Hsp70 proteins to the cell wall in *S. cerevisiae*. This can be accomplished by several different mechanisms. Hsp70 can interact with proteins involved in an alternative secretory pathway, or it may self-tranlocate across the plasma membrane. If it is a substrate for an alternative secretory pathway, it would have to interact with other proteins to accomplish the secretion. If self-translocation accounts for the appearance of Hsp70 in the cell wall, the requirements for secretion would lie within Hsp70 itself. The goal of this project it to devise a mechanism to identify proteins that interact and translocate Hsp70 to the cell surface.

While trying to accomplish the task of identifying proteins involved in the secretion of the Ssa1 protein, we first decided to use methods described by Lopez-Ribot *et al.* (43). The observation that Hsp70 can be located at the cell surface by immunofluorescence was used to try and select for mutants. In this case, fluorescence-activated cell sorting may be used to separate cells expressing surface Hsp70 from non-expressing cells in a mutagenizing population. However, the antibody utilized in the original study was different from that used in this study and may have not recognized surface exposed epitopes of intact cells. A selection approach was then devised to use a physiological marker for Hsp70 secretion. This approach relied on the secretion of an Hsp70-invertase fusion protein in a *suc*$^-$ (invertase negative) strain such that cells that

secrete the fusion would grow on media where sucrose is the only available carbon source and those that fail to secrete the fusion would not grow. This could then be used to identify secreting and non-secreting cells in a mutagenized population.

CHAPTER II

MATERIALS AND METHODS

Microbial Strains and Vectors

Saccharomyces cerevisiae strains T211, a mating type, GAL2, *his*3-11,15, *leu*2-3,112, *lys*1, *lys*2, *trp*1, *ura*3-52, and T212, a mating type, *GAL2, his* 3-11,15 *leu*2-3,112, *lys1, lys2, trp*1-Δ1, *ura* 3-52, *ssa1*::*HIS3, ssa2*:: *LUE2* were obtained from Dr. Elizabeth Craig at the University of Wisconsin in Madison, WI. *S. cerevisiae* strain BY4742, α mating type, *his*$^-$, *leu*$^-$, *lys*$^-$, and *ura*$^-$ was used in the mating experiments. A derivative of BY4742 was also used in the mating experiments and is *his*$^-$, *leu*$^-$, *lys*$^-$, *ura*$^-$ *and SUC2*::G418. The BY4742 strains are from Research Genetics and the Yeast Deletion

Table 2.1. Yeast Strains Used in this Study.

Strain Name	Genotype	Reference
T211	Mat a, *his3-11,15, leu2-3,112, lys1, lys2, trp1, ura3-52*	Mol.& Cell. Biol. 7:2568-2577 (1987)
T212	Mat a, *his3-11,15, leu2-3,112, lys1, lys2, trp1, ura3-52*$^-$, *ssa1::HIS3, ssa2:: LUE2*	Mol.& Cell. Biol. 7:2568-2577 (1987)
BY4742	Mat alpha, *his3D1, leu2D0, lys2D0, ura3D0*	Research Genetics
BY4742*suc2*$^-$	Mat alpha, *his3D1, leu2D0, lys2D0, ura3D0, SUC2::G418*	Research Genetics Ref. # 12321
T211*suc2*$^-$	Mat a, *his3-11,15, leu2-3,112, lys1, lys2, trp1, ura3-52, suc2::URA3*	This Study
211U*suc2*$^-$	Mat a, *his3-11,15, leu2-3,112, lys1, lys2, trp1, ura3-52, suc2*	This Srudy

Project. *Escherichia coli* strain TOP10F′ was purchased from Invitrogen (Carlsbad, CA) with the genotype F′{*lac*I^{q}Tn*10*(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS*mcr*BC) ϕ80ZΔM15 Δ*lac*X74*deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rsp*L *end*A1 *nup*G and was used for simplified production of the vectors used in this study. All the yeast strains used in this study are shown in Table 2.1.

Several different vectors were created for use in this study utilizing the yeast integrative vector pRS306 (Figure 2.1), first described by Sidorski *et al.* (62). The presence of the replication machinery for propagation in both *E. coli* and *S. cerevisiae* allowed this plasmid to be used as a shuttle vector; the ampicillin resistance and uracil genes permitted for selection in *E. coli* and *S. cerevisiae*, respectively. The pMPY-ZAP plasmid was utilized in the deletion of the *SUC2* gene in the *S. cerevisiae* strain T211 and was provided by Dr. Brandt Schneidar (TTUHSC, Lubbock, TX).

Media and Growth Conditions

E. coli strains were grown in Luria Bertani (LB) medium containing 1% tryptone (Difco Laboratories, Detroit, MI), 0.5% yeast extract (Difco Laboratories), and 1% NaCl (Fischer Scientific, Fair Lawn, NJ). Ampicillin (Sigma Chemical Corporation, St. Louis, MO) was added to a final concentration of 50 ug/ml when selective pressure in *E. coli* was needed. These cultures were grown in 50 ml flasks containing 10 ml LB in an orbital shaker at 220 rpm at 37° C. Luria Bertani agar plates were made by supplementing the LB media with 20% granulated agar (Fisher Scientific).

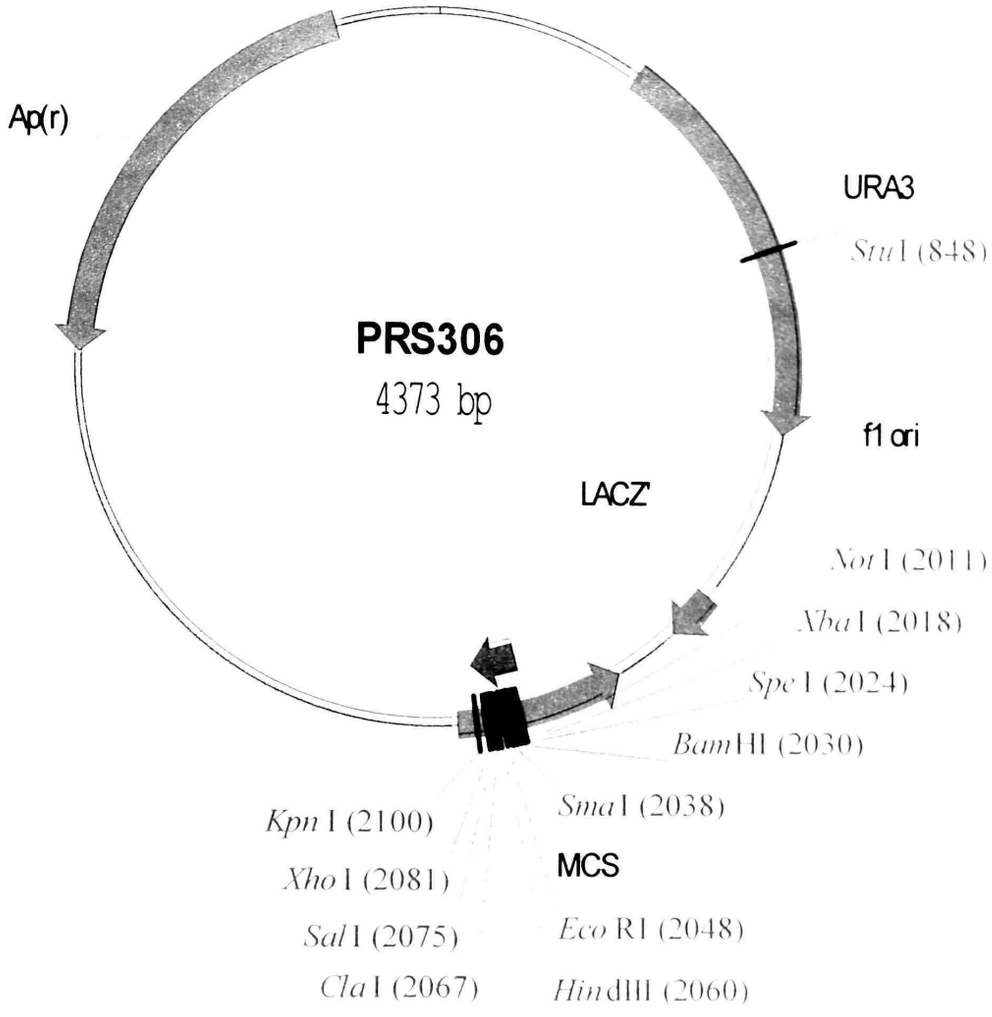

Figure 2.1. The Yeast Vector pRS306. This vector was used to construct several different plasmids. It contains ampicillin resistance and uracil genes to allow for selection in *E. coli* and *S. cerevisiae*, respectively.

S. cerevisiae cultures were grown at 30° C on the orbital shaker at 180 rpm in Yeast Extract Peptone and Dextrose medium (YEPD). YEPD is composed of 10% yeast extract (Difco Laboratories), 20% peptone (Difco Laboratories) and 20% Dextrose (Fischer Scientific). Yeast Nitrogen Base medium (YNB, 0.67% [Fischer Scientific]) supplemented with 20% Dextrose, 20% granulated agar and the appropriate concentration of individual amino acids without uracil was used for selection of plasmid retention in *S. cerevisiae* as described in Methods in Yeast Genetics by (2). Sucrose plates contained 2% sucrose (Fisher), 0.67% YNB (Fisher), 0.2% amino acid mix lacking uracil, and 2% agar (Fisher).

Preparing Cell Wall Extracts

In this study, we were interested in a soluble protein component of the cell wall. These proteins can be extracted from the cell wall under mild reducing conditions. These proteins were removed from the cell wall as describe by Cassnova *et al.* (8). A culture of the appropriate strain was inoculated and allowed to grow overnight to the midlogorithmic phase of growth. The cells were harvested by centrifugation at 3000 rpm and then washed twice in sterile water. The cell wall proteins were then isolated by resuspending the cells in ammonium carbonate (1.6g/L [Sigma]) and adding 2-mercaptoethanol to 2% and then incubating at 37°C for 30 min. The suspension was centrifuged at 3000 rpm and then the supernatant filtered through a 0.45 µm (Fisher) to remove any cells that may have remained. The filtrate was dialyzed for 48 hrs against water to remove the 2-mercaptoethanol and then lyophilized.

Preparation and Purification of a Polyclonal Antibody to Hsp70

The 17 C-terminal amino acids of the Ssa1 protein (APPAPEAEGPTVEEVD) were synthesized and conjugated to the hemaglutinin molecule by the Louisiana State University core protein laboratory. A solution was prepared by the addition of 0.5 ml this conjugated peptide to 1.0 ml of the Ribi Adjuvant System (RIBI ImmunoChem Research Inc. Hamilton, MT). This mixture was then injected intradermally into the back of a rabbit. Three weeks later the injection was repeated. The rabbit was then bled 10 days after the second boosting and the antibody (PAb-Ssa1) purified by ammonium sulfate (Fisher) precipitation. The whole blood obtained from the rabbit was incubated at 37°C for one hour then transferred to 4°C overnight. This was then centrifuged at 3000g for 30 minutes. The supernatant was transferred to a clean beaker. While the serum was being stirred, saturated ammonium sulfate was added until the final mixture was 50% saturated ammonium sulfate. This solution was again centrifuged at 3000g for 30 minutes. The supernatant was removed and discarded. The pellet was suspended in 0.5 volumes of the starting volume in PBS. This was dialyzed against PBS overnight and the concentration determined by the Bradford method.

Western Blotting

Western blotting was done by standard techniques (57). Protein extracts were resuspended in water and the concentration determined by the Bradford method (6). Ten micrograms of the protein extracts were loaded onto a 12.5% sodium dodecyl sulfate-

polyacrylamide gel and run at 100 volts. The proteins were transferred using a semi-dry electric blotter (Hoefer Scientific Instruments, San Francisco) to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was then incubated at room temperature for 1 hr in a 10% Bovine Serum Albumin (BSA [Sigma]) solution to effectively block any nonspecific binding. The membrane was rinsed with Tris-Buffered-Saline (TBS) and a 1 to 100 dilution of the antibody, anti-Hsp70, was applied in BSA and incubated for 1 hr at 37°C. The primary antibody was rinsed and the membrane washed 3 times in TBS. A 1/100 solution of the secondary antibody, goat anti-rabbit conjugated to peroxidase (Sigma), in TBS was then incubated with the membrane for 1 hour at 37°C. Three final washes were done in TBS and the peroxidase was developed according to the supplier with 4-chloro-1-napthol (Sigma) and hydrogen peroxide (Sigma).

Indirect Immunofluorescence

The indirect immunofluorescence assays were done on the T211 and T212 strains of *S.cerevisiae*. The polyclonal anti-Hsp70 antibody, that was described earlier, was used to detect the cell surface location of Hsp70 using a fluorescein isothiocyanate (FITC) conjugated secondary antibody. The cells were grown overnight in 10 mls of YPD in a 50 ml flask. The cell density of the culture was determined microscopically on a hemacytometer, and a new 50 ml culture was inoculated to a density of $5x10^6$ cells/ml culture. This culture was incubated on an orbital shaker at 180 rpm and 30°C for 3 to 5 hours, to a final density of $2x10^7$ cells/ml. The cells were then harvested in a sterile 50 ml Falcon polypropelene centrifuge tube (Fisher Scientific) at 3000 x g for 5 minutes.

The supernatant was decanted and the cells washed three times with 25 mls of PBS. The cell were then resuspended in 50 mls of PBS keeping the concentration $2x10^7$ cells/ml. Two hundred microliters were then taken and the anti-Hsp70 polyclonal antibody was added to a concentration of 1 to 100. This was then incubated at 37°C for 1 hr. After the incubation the cells were again wash three times in PBS and resuspended in 200 µl PBS. The anti-IgG antibody conjugated to FITC (Sigma) was then added to a concentration of 1 to 100 and incubated at 37°C for 1 hr. The cells were again wash three times in PBS and resuspended in 200 µl PBS. The cells were then examined with a Nikon Labophot microscope equipped for epifluoresence.

Construction of Plasmid

The *SUC*2 gene was utilized as a genetic marker to select for cells that either can or cannot secrete the *SSA*1 gene. In order to do this, an in-frame fusion between the two genes (the entire *SSA*1 gene fused to the N-terminus of the *SUC*2 gene lacking its own secretion signal) was created and then cloned into pRS306 with the inducible *MET3* promotor just upstream of the fusion as described below.

Splice Overlap Extension (SOE) Polymerase Chain Reaction (PCR)

SOE PCR was used to splice two genes together in the correct orientation. The primary PCR reactions (Figure 2.2) were carried out in an Ericomp thermocycler using

250 µl thin walled microfuge tubes (Phenix Research, Hayward, CA). The 50 µl reactions consisted of 1 ng of *S. cerevisiae* genomic DNA Research Genetics, Huntsville,

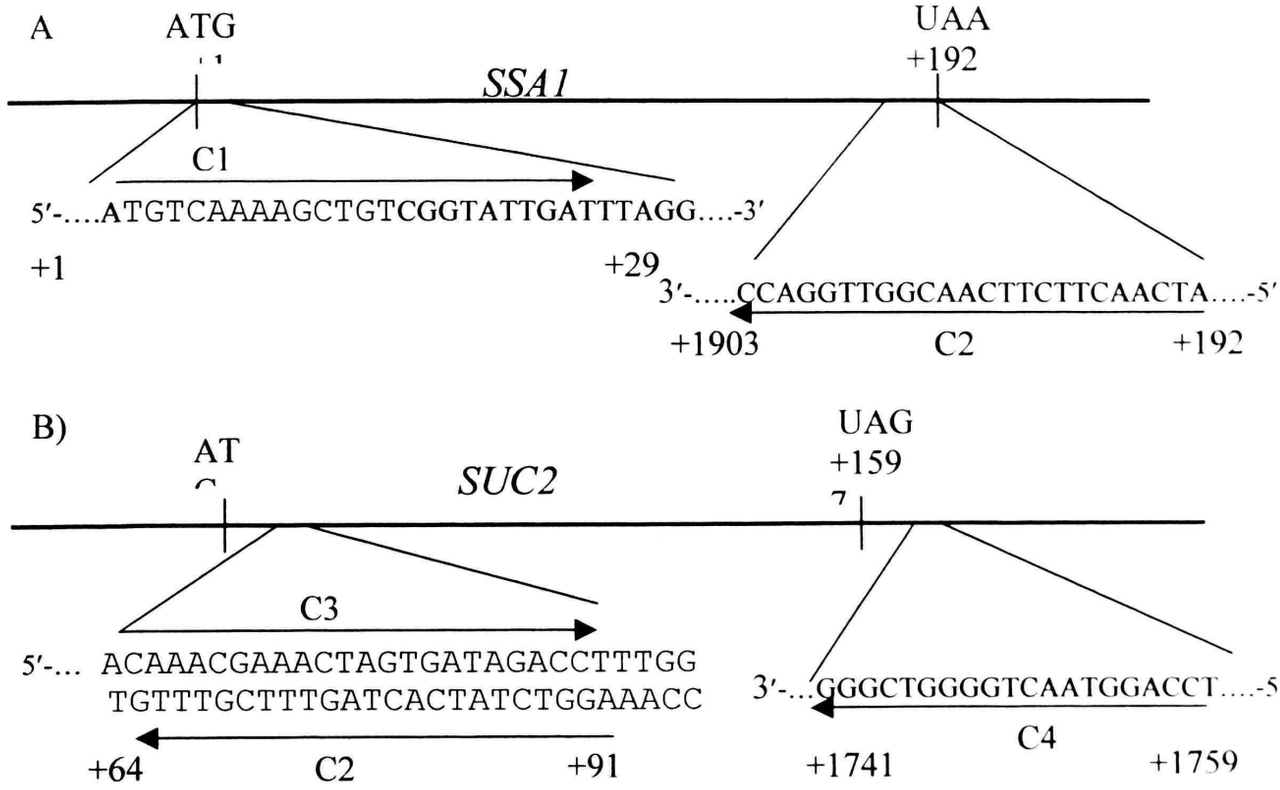

Figure 2.2. Primers Designed for SOE PCR. (A) The PCR primer design for the *SSA1* gene uses primers C1 and C2. C1 was designed starting at the start codon and has 29 bases of homology to the *SSA1* gene with a *HindIII* restriction enzyme site incorporated to the 5′ side of the *SSA1* homology as detailed in Table 2.2. Primer C2 was designed at the end of the *SSA1* gene not including the stop codon with another 28 bases complement to the C3 primer and *SUC2*. (B). Primer C3 was designed to amplify the *SUC2* gene lacking its own signal sequence (+1-+63). Primer C4 was designed 200 bases downstream of the stop codon of *SUC2*. It has 20 bases of homology to *SUC2* and has a *NotI* restriction enzyme site (Table 2.2) to clone the fusion into pRS306.

Table 2.2. Oligonucleotides Used in PCR Reactions. Restriction sites: *HindIII* sites are in red. XhoI sites are in blue. KpnI sites are in green. NotI sites are pink.

Primer	Sequence
C1	5′-GCGATCGTCAAGCTTGAGCTCAT-GTCAAAAGCTGTCGGTATTGATTT-AGG-3′
C2*	5′-GCCCTTGTTGGGTGTGAAGTGGA-CCAAAGGTCTATCACTAGTTTCGTT-TGTATCAACTTCTTCAACGGTTGGA-CC-3′
C3	5′-ACAAACGAAACTAGTGATAGACC-TTTGG-3′
C4	5′-GCTATCTGCCTCGAGGCGGCCGC-TCCAGGTAACTGGGGTCGGG-3′
S1	5′-ACGATCGTCAAGCTTGAGCTCAT-GACAAACGAAACTAGTGAGACCTT-TGG-3′
ZAP1	5′-TTTTCTCTCAGAGAAACAAGCAA-AACAAAAAGCTTTTCTTTTCACTAA-CGAAAGGGAACAAAAGCTGG-3′
ZAP2	5′-CTATTTTACTTCCCTTACTTGGAA-CTTGTCAATGTAGAACAAATTATCG-ACCACTATAGGGCGAATTGG-3′
U1	5′-ACGACTTTTTTTTTTTGG-3′
U2	5′-ACATAAAGAACAAATTCC-3′
Z2	5′- GCATATTTGAGAAGATGCGGCC-3′
M1	5′- ACGATCGTCGGTACCCCGCGGC-GTTTAATTTAGTACTAACAGAGAC-3′
M2	5′-GCTATCTGCAAGCTTATCGATGTT-AATTATACTTTATTCTTGTTATTATT-ATAC-3′

AL), (200 μM of dCTP, 200 μM of dTTP, 200 μM of dATP, 200 μM of dGTP, 1μM of each appropriate primer (C1 with C2 or C3 with C4 [Table 2.2 and Figure 2.2]), 20 units of High Fidelity Polymerase (Boerinhger-Mannheim, Pleasanton, CA) and 1X concentration of reaction buffer (20mM Tris-HCl, 100mM KCl, 1mM dithiothreitol, 0.1 mM EDTA).

The primers were designed by using sequence information from the Saccharomyces Genome Database. A *HindIII* site was incorporated into the 5′ primer for *SSA1* PCR product and a *NotI* site was incorporated into the 3′ side of the *SUC2* product. A list of the primers used is outlined in Table 2.1, and depending on which reaction was run, a different set of primers may be utilized. The PCR was run with a denaturization step at 94 °C for two minutes, followed by 30 cycles of 95°C for 30 seconds, 60° for 30 seconds and 68°C for 3 minutes. Then a final extension was done at 72°C for 10 minutes. Each PCR reaction was run at room temperature for 1 hour at 80 volts on a 0.8% SeaKem GTG agarose (BioWhittaker, Vallensbaek Strad, Denmark) TBE gel (0.045 M Tris-Borate, 0.001 M EDTA pH 8.0), and then gel extracted using the Qiagen Qiaex II gel extraction kit (Qiagen, Valencia, CA) to remove primers and excess dNTPs. The gel was stained with 15 μl 10 mg/ml ethidium bromide. The 50μl product of the gel extraction was then checked for purity by loading 5 μl on another 0.8% TBE gel. The concentration was determined by UV spectroscopy.

In the second PCR reaction, the overlapping sequences that are present between the first two PCR products annealed and extension occurred at the free 3′ phosphates. This resulted in the entire fusion product. The reaction was carried out under

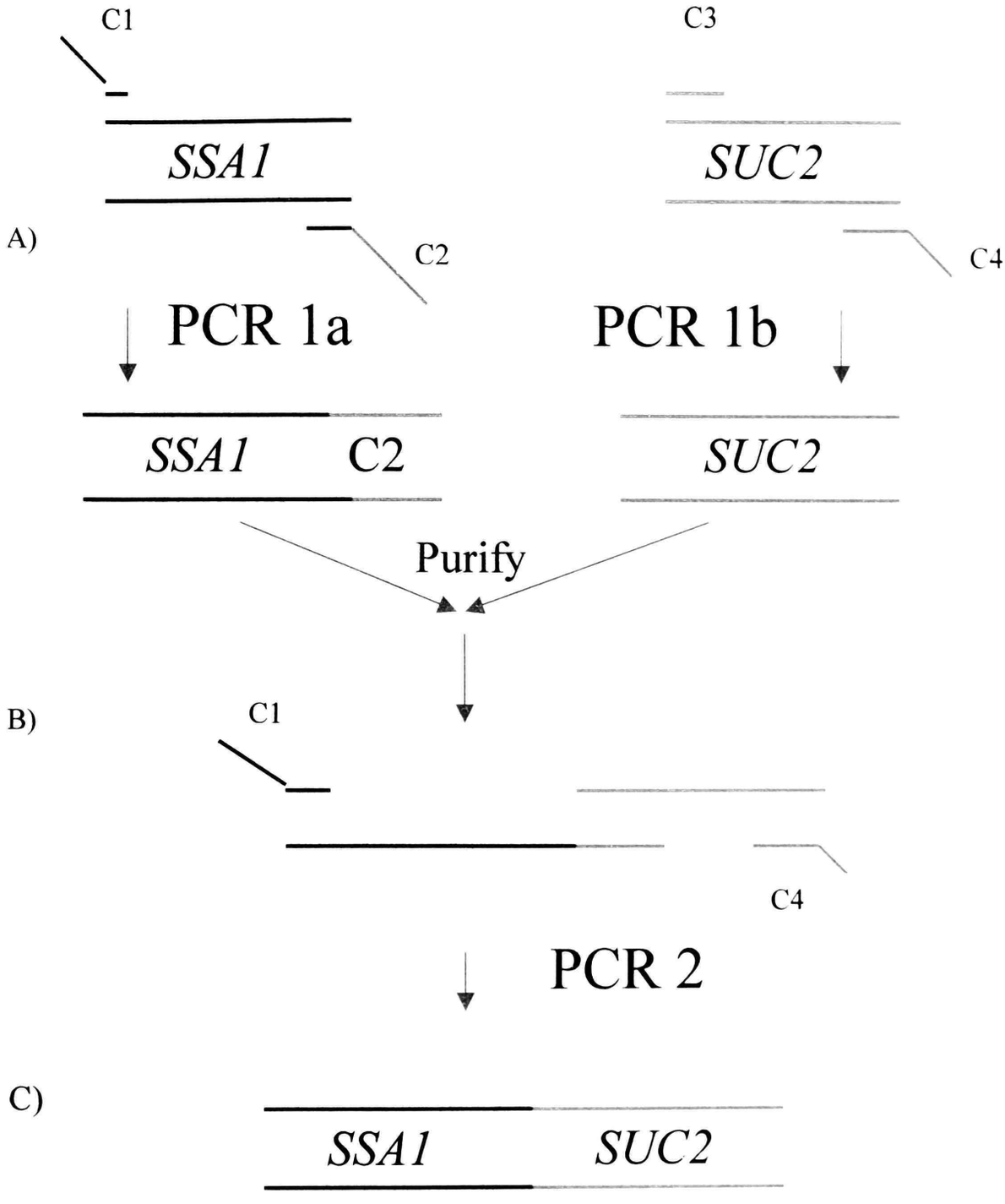

Figure 2.3. SOE PCR. (A). In the first PCR reactions, two separate fragments of DNA are amplified using primers C1, C2, C3, C4. Primer C3 has 50 basepairs that are complementary to the terminal portion of the C4 primer and the *SUC2* gene. (B). The two separate fragments are then gel extracted to rid the mixture of primers C2 and C3. After this is done they are combined together in another PCR reaction using only C1 and C4 as the amplification primers. (C). Since there is homology between the two pieces of DNA, the entire in-frame fusion will be produced.

conditions similar to the first PCR except that the primers and template used changed. Only primer C1 and C4 were required in this reaction and the templates used were the primary PCR products generated earlier. The entire SOE PCR is shown in Figure 2.3.

Cloning into pRS306

All the genetic techniques were performed as previously described (Maniatis, CSHL (57)). Before the fusion could be cloned into the pRS306 (Figure 2.5), the *MET3* promoter had to be cloned into the plasmid. The *MET3* promoter was amplified from *S. cerevisiae* genomic DNA using the same technique as described for the amplification of *SSA1* and *SUC2*, except primers M1 and M2 were added to the reaction. M1 and M2 (Figure 2.4) ampified the 500 basepairs of the region immediately upstream of the *MET3* gene. The MET3 promoter PCR product was digested with 20 units *Hind*III (New England Biolabs,

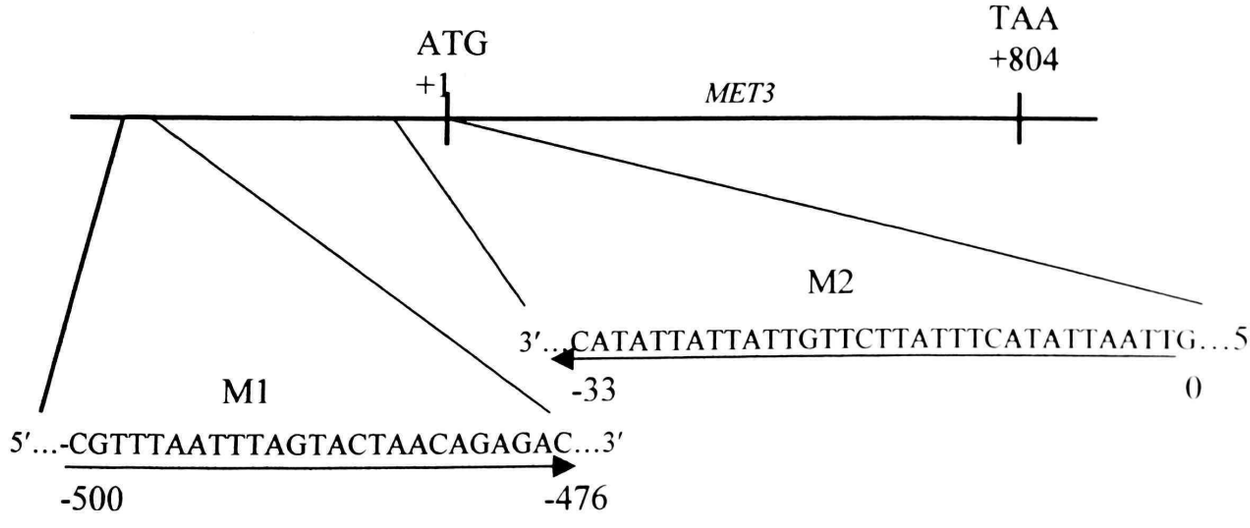

Figure 2.4. Primers Used in Cloning the *MET3* Promoter. The primers were designed as shown above with a *KpnI* site incorporated into the 5' side of the M1 primer and a *HindIII* site incorporated into the M2 primer.

Beverly, MA [NEB]) in NEB buffer 2 (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, pH 7.0) for three hours at 37°C, followed by addition of 20 units *Kpn*I (NEB) and subsequent incubation at 37°C for 3 hours. This digested product was then run on a 0.8% agarose (BioWhittaker) TBE gel and extracted as described earlier in this manuscript. The same procedure was used to prepare the plasmid, pRS306, for ligation. All ligations were carried out at 15°C overnight using 10 units of T4 DNA ligase (NEB) in 10µl volumes according to the manufacturers protocol. The next day, one microliter of the ligation mixture was used to transform *E. coli* cells as described by Maniatis *et al.*

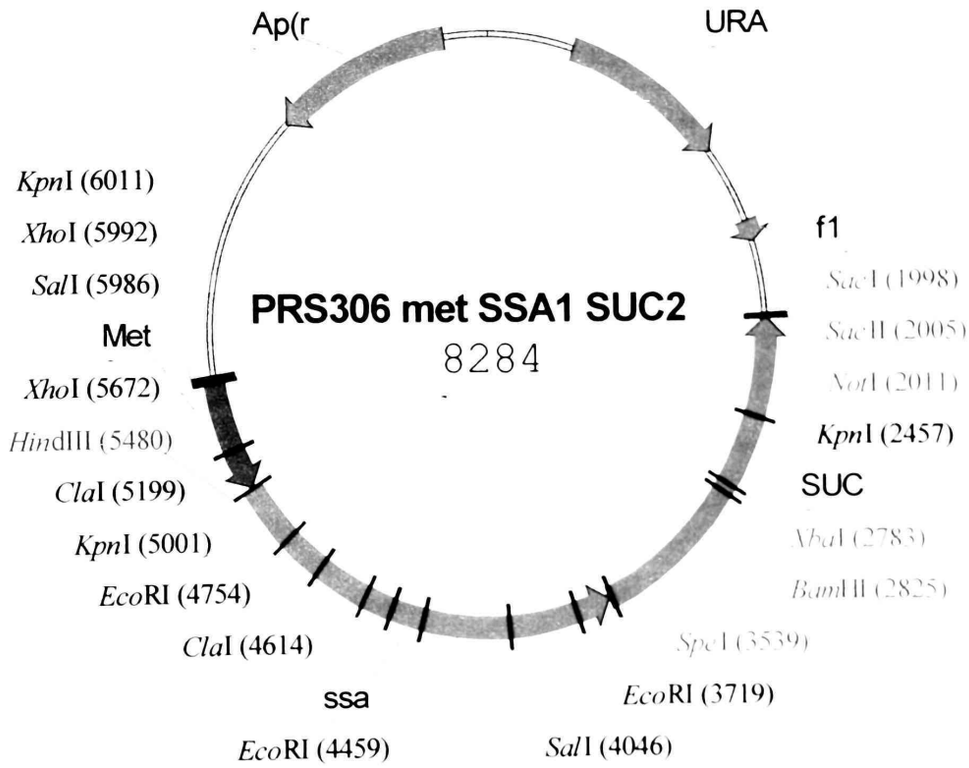

Figure 2.5. The SSA::SUC2 Fusion Cloned into pRS306met3. The *SSA1::SUC2* gene fusion was cloned into the *HindIII* and *NotI* sites in front of the *MET3* promoter, which was cloned into the *KpnI* and *HindIII* sites.

Briefly, the TOP10F′ cells purchased from Invitrogen were incubated on ice for 30 minutes with 1 µl of the ligation mixture. The cells were then heat shocked for 90 seconds at 42°C. One milliliter of sterile LB was then added and the cells incubated at 37°C for 1 hr. The *E. coli* cells were then plated on LB-ampicillin (50mg ml), and were grown up at 37°C overnight. A single colony was chosen from the transformation plate and grown to saturation LB-ampicillin media, and the new plasmid pRS306-MET3P was isolated using the QIAprep Spin Miniprep kit (Qiagen). This DNA was then used to clone the *SSA1::SUC2* fusion. The fusion and plasmid pRS306-MET3P were digested with *HindIII* followed by digestion with *XhoI* as describe above. After gel purification of the two digested products, they were ligated together and transformed into *E. coli*. The entire construct containing the *MET3* promoter and fusion, shown in Figure 2.5, was purified using the QIAprep Spin Miniprep kit (Qiagen) from *E. coli* and transformed into *S. cerevisiae* as described later in this manuscript.

In order to determine if the fusion was made in-frame, primers were designed to sequence across the junction. The plasmid was isolated as previously described using the Qiagen Midi Prep kit and sent to the Texas Tech Biotechnology Core Facitlity and sequenced using the Big-Dye Terminator Cycle Sequencing Ready kit (Perkin-Elmer, Shelton, CT) on an automated ABI PRISM 377 XL DNA Sequencer (Perkin-Elmer) using the manufacturer's protocol. The primer used was 100 basepairs immediately upstream of the junction inside the *SSA1* gene and its sequence was

5′-CATGTCTAAGTTGTACCAAGCTGGTGG-3′

Before the experiments could proceed, a negative control plasmid had to be constructed. Using the same techniques described above, the *SUC2* gene lacking its own signal sequence was cloned into the pRS306-MET3P. After the *SUC2* gene was amplified out with primers S1 and C4, the product was cloned into the plasmid using *HindIII* and *NotI*. This plasmid was given the name pRS306*SUC2*

Deletion of the *SUC2* gene in *S. cerevisiae*

In order to detect the Ssa1 protein at the cell surface using the enzymatic activity of invertase, the *SUC2* gene must first be deleted in the *S. cerevisiae* strain (Figure 2.6). The gene deletion was carried out using pMPY-ZAP, a reusable PCR-directed disruption cassette.

Amplification of the pMPY-Zap Cassette

The pMPY-Zap cassette contains the URA3 gene flanked by 500 basepairs of *his*G repeats on a plasmid, which is stable and can replicate in *E. coli*. About 50 basepairs of homology was needed to insure the recombination event would occur and replace the *SUC2* gene with the uracil auxotrophic marker. These 50 basepairs were designed into a 5′ extension of the primer, which also has 18-20 basepairs of homology to the region immediately upstream of the *hisG* repeats of the pMPY-Zap plasmid. Table

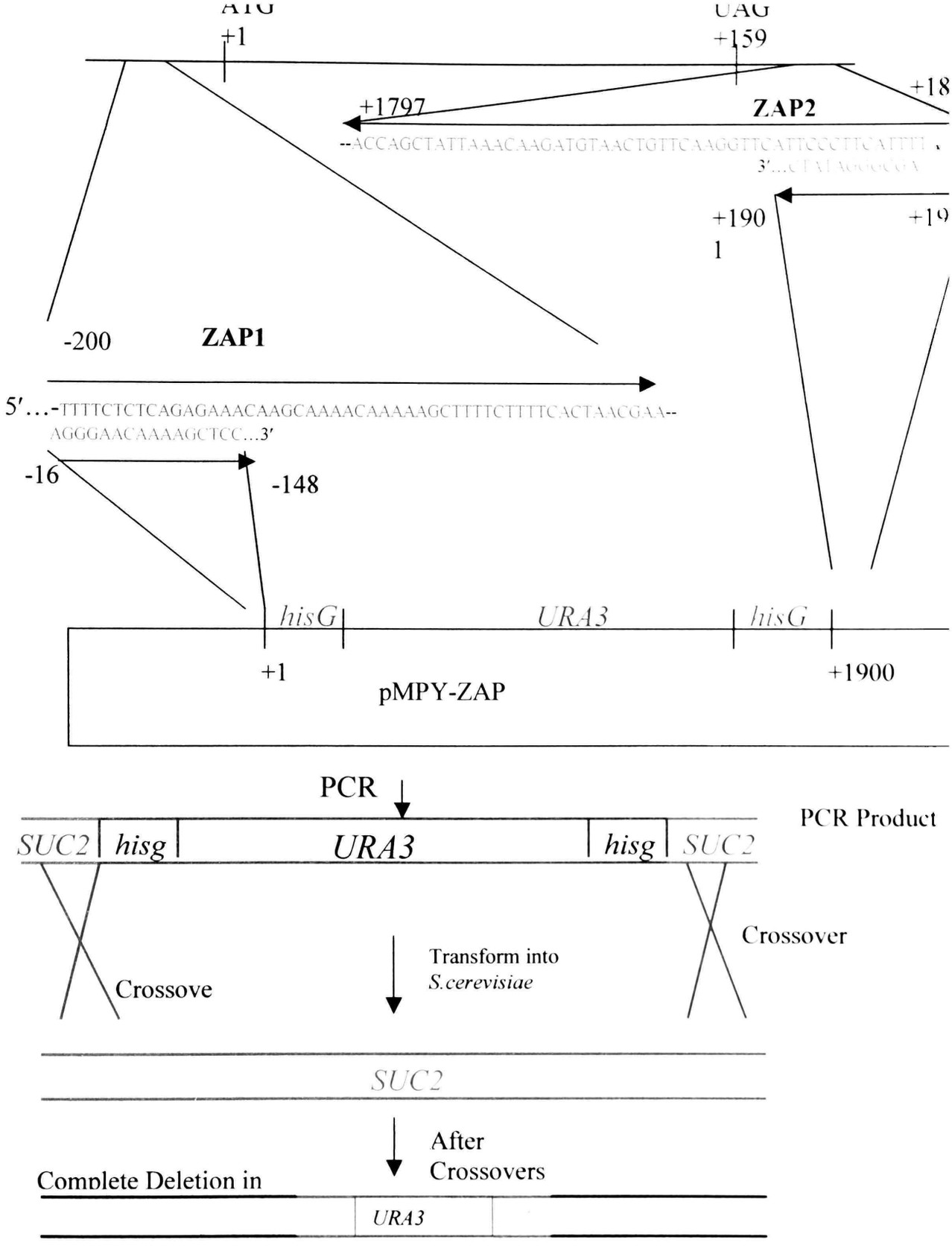

Figure 2.6. Deletion of the *SUC2* Gene with the pMPY-ZAP Gene Deletion Cassette. Using primers ZAP1 and ZAP2, with homology to the *SUC2* gene (red) and the pMPY-ZAP (blue) plasmid, the PCR reaction was run and the product shown. The product has homology to the *SUC2* gene and contains the *URA3* gene for selection of the crossover event. The crossover event occurs after the PCR product is transformed into *S. cerevisiae* and the complete deletion is shown. This experiment effectively replaces the *SUC2* gene with the gene deletion cassette.

2.1 shows the ZAP1 and ZAP2 primers that were used for this experiment. The PCR reaction was carried out as described by Schneider *et al.* and was a 50 µl reaction, which contained 20 ng of pMPY-ZAP, 100 pmol of each primer with the appropriate buffer and $MgCl_2$ concentrations (58). Five units of the High Fidelity polymerase (Boehringer Mannheim) were added and the following reaction was run: one cycle for 5 minutes at 94° C, one minute at 50° C, and three minutes at 68° C followed by 20 cycles at one minute at 94° C, one minute at 50°C, and three minutes at 68°C.

Transformation of *S. cerevisiae*

The PCR product pMPY-ZAP is a linear piece of DNA with 50 bp of homology on both sides that are homologous to the regions immediately upstream and downstream of the *SUC*2 gene, which was used directly in the transformation of *S. cerevisiae* strain T211. A 5ml culture of was grown overnight to saturation in YEPD. The cell density of the culture was determined microscopically on a hemacytometer, and a new 50 ml culture was inoculated to a density of $5x10^6$ cells/ml culture. This culture was incubated on an orbital shaker at 180 rpm and 30°C for 3 to 5 hours, to a final density of $2x10^7$ cells/ml. The cells were then harvested in a sterile 50 ml Falcon polypropelene centrifuge tube (Fisher Scientific) at 3000 x g for 5 minutes. The supernatant was decanted and the cells washed with 25 mls of sterile water. The cells were suspended and centrifuged again. After decanting the supernatant, 1 ml of 1.0M LiAc (Sigma) was used to resuspend the cells, which were then transferred to a 1.5 ml microfuge tube (Phenix). The cells were

pelleted at 15,000 rpm on a bench top microfuge, and the LiAc was removed with a pippette. The cells were again resuspended in LiAc to a final volume of 500µl.

The cells were then transferred to separate 1.5 ml microfuge tubes in 50 µl aliquots. The basic transformation mix was added in the following order: 240 µl polyethylene glycol (Sigma) 50% w/v, 36 µl of 1.0 M LiAc, 25 µl ss-DNA (Sigma) 2.0 mg/ml, and 50 µl of water containing the SOE-PCR product. There was 10 µg of the PCR product added. The entire mixture was then vortexed and incubated at 30° C for 30 min. The microfuge tubes were then transferred to a 42° C water bath for 20 minutes to heat shock the cells. Following a centrigugation at 6000 rpm for 15 seconds, the transformation mix was removed. The pellet was resuspended very carefully in 1 ml of sterile water to wash the cells. After this, 200 µl of the mixture was spread on YNB plates that lack uracil and grown for two to four days to select for the recombination event between the PCR product and the *SUC2* gene.

In order to transform the now *suc2*$^-$ cells with a plasmid that also utilizes the URA selectable marker, the *URA* gene used in the pMPY-ZAP cassette needed to be excised. This recombination event occurred between the flanking *his*G repeats and leaves the *his*G in the place of the *SUC*2 gene. Five-fluoroorotic acid (5-FOA [USBiological, Swampcott, MA]) is converted into a lethal by-product in cells that possess a functional *URA*3 gene, and therefore the recombination event between the *his*G repeats is selected for when the cells are grown in the presence of 5-FOA. Briefly, the cells were grown to the mid-logarithmic phase in YEPD and then transferred to a YNB agar plate containting 1g/L 5-FOA. Ten *ura*$^-$ colonies were selected and tested for the lack of growth on sucrose

media, and these then be transformed using a functional *URA* gene as the selectable marker. This strain was named 211U*suc2*$^-$

Confirmation of the *SUC2* Deletion

At this point, the yeast have been transformed with the pMPY-ZAP PCR product, and transformants have been selected for by growth on YNB agar plates that lack uracil. The next step is to confirm that the cassette was integrated in the correct location within the *SUC*2 locus. This was tested in a variety of ways.

Confirmation by Growth on Sucrose

Since a functional *SUC2* gene is required for the cells to grow when sucrose is the only available carbon source, the strains in which the pMPY-ZAP cassette is correctly integrated will not be able to grow on sucrose plates. Individual colonies were plated onto –URA YNB glucose plates lacking uracil, and then replica plated onto YNB sucrose plates lacking uracil and incubated for 3-5 days at 30° C.

Confirmation by Mating

Mating experiments can be useful for determining which gene has been deleted if a known deletion is available. Dr. Brandt Schneider (Lubbock, TX) provided a suitable *S. cerevisiae SUC*2 deletion strain (reference number 12321), a derivative of BY4742 from Research Genetics (Table 2.1), and this used in crosses to the *SUC2* deletion (T211*suc2*$^-$) that was produced in this study. Mating experiments are a form of nutritional

complementation; a functional *SUC2* gene in one strain will be able to allow for growth of the diploid on sucrose media. One small colony of T211*suc2*$^-$, mating type a, was picked with a sterile toothpick from a YPD plate and innoculated onto another YPD plate in a spot roughly 5mm in diameter. The BY4742 strain, mating type α, was then inoculated and spread in exactly the same spot. This was allowed to grow overnight at 30° C. The colonies were then transferred to a YNB plate which lack tryptophan and uracil to select for cells that have mated, and are now diploid in nature. This was again incubated at 30° C overnight, and then colonies were transferred to a sucrose plate that lack uracil and tryptophan to determine if the correct gene has been disrupted.

Confirmation by PCR

PCR can also be used to determine if the disruption cassette has integrated in the correct location. Colonies from T211*suc2*$^-$ were used in this step. These cells have the *SUC2* gene deleted and the entire disruption cassette integrated into the genome, and is therefore URA$^+$. Primers Z1 and Z2, along with U1 and U2 (Table 2.1), were used in the orientation shown in Figure 2.7, to produce several distinct bands when the reaction were run. A toothpick was used to pick up a small portion of the T211*suc2*$^-$ strain, and was placed in 5 µl solution of water in a 250 µl microfuge tube and boiled for 5 minutes. This solution was used to run the appropriate PCR reaction. There was one cycle of 5 minutes at 94°C, 1 minute at 50°C, and 3 minutes at 72°C, followed by 20 cycles at 1 minute at 94°C, 1 minute at 50°C, and 3 minutes at 72°C using *Taq* polymerase (Sigma) in the appropriate buffer and magnesium chloride concentrations as recommended by the

supplier. In order to further characterize the legitimacy of using PCR to show that the cassette has been integrated properly, one of the PCR products was cloned using a TOPO TA Cloning Kit purchased from Invitrogen. After the product was cloned, it was sent for sequencing from the T7 and M13 reverse primers at the Texas Tech Biotechnology Core Facility.

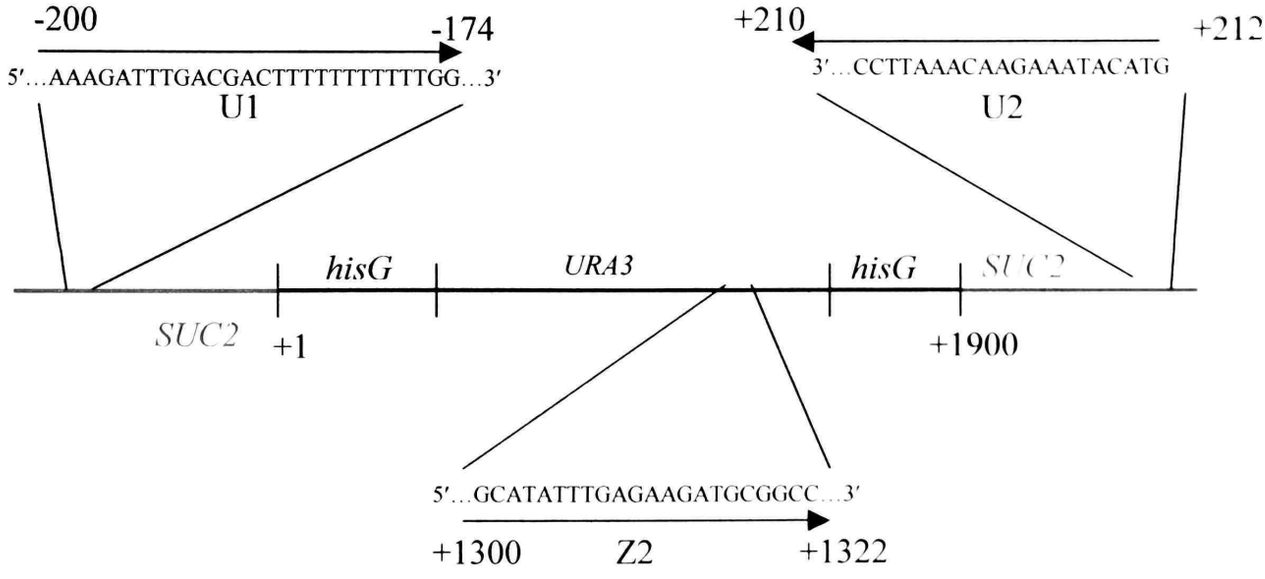

Figure 2.7. The Orientation of the Primers Used in the PCR Confirmation of the *suc2* Deletion. U1 is in the immediately upstream of the *SUC2* gene and U2 is immediately downstream where as *Z2* is inside the functional *URA3* gene. If the cassette is in the correct location there will be distinct bands produce by the PCR reactions.

Transformation of 211U:*suc2*⁻ with pRS306-*SSA1*:*SUC2*

The same transformation procedure for the introduction of pRS306-*SSA1*:*SUC2* into T211:*suc2*⁻ was used for the transformation of T211 with the PCR product from pMPY-ZAP. Rather than the 20 ng used previously, 100 ng of plasmid DNA was added to the transformation mix. Again, the transformants were selected for on YNB glucose plates that lack uracil and allowed to grow for 3-5 days at 30° C. After the transformation of T211 *suc2*⁻ with pRS306-*SSA1*:*SUC2*, the transformants were tested to see if the secretion of *SSA1* could be detected using the enzymatic activity of invertase. The transformants were grown on YNB glucose plates that lack methionine and uracil and then serial dilutions were transferred to YNB sucrose plates that lack methionine and uracil. If the *SSA1* portion of the fusion is able to direct the construct to the cell surface, the cell will be able to grow on the sucrose plates. A negative control plasmid, pRS306*SUC2*, was also transformed into the T211 *suc2*⁻ that had the SUC2 gene without its own leader sequence under the control of the MET3 promoter. This will be used as a negative control in order to make sure that it is the *SSA1* portion and not the *SUC2* portion that targets the fusion to the cell surface.

CHAPTER III

RESULTS

Confirmation of Hsp70 in the Cell Wall

In order to study the secretion of *SSA1* and *SSA2* to the cell wall, Hsp70 had to be confirmed to be in the cell wall. Cell wall extracts were prepared as described from T211 and T212 and run on a 12.5% SDS-PAGE gel. The cell wall protein extracts were then transferred to a nitrocellulose membrane and visualized using Western blotting with the polyclonal Hsp70 antibody as the primary antibody and anti-IgG conjugated to horse radish peroxidase (HRP) as the secondary antibody. The results of this experiment are shown in Figure 3.1. A single sharp band was produced in lane 1 (the T211 strain) and had an electrophoretic mobility around 70 KDa MW. As expected, there was no reactivity in the *ssa1* and *ssa2* mutant strain T212 (lane 2).

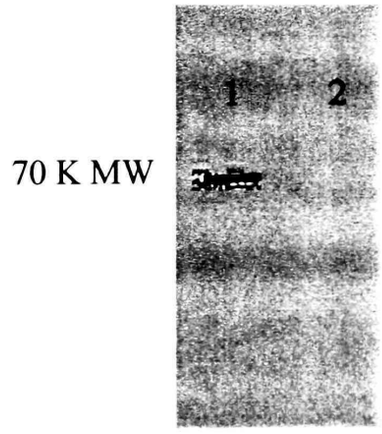

Figure 3.1. Western Blot of Hsp70 in the Cell Wall of *S. cerevisiae*. Lane 1: Cell wall extract from S. cerevisiae strain T211. Lane 2: Cell wall extract from T212. The reactivity shown in lane 1 is at 70 KDa MW and corresponds to the Hsp70 protein.

Indirect Immunofluorescence Assays

Since the polyclonal anti-Hsp70 antibody exhibited specific reactivity to one protein in the cell wall extract of T211 and none in strain T2112, it was postulated that immunofluorescence assays could be performed that would allow the detection of Hsp70 at the cell surface. This would allow for the selection of mutants that do not secrete Hsp70. Therefore, strain T211 was used as a positive control and strain T212 as a negative control in testing if Hsp70 could be detected using immunofluorescence. After repeated experiments testing the fluorescent activity of both strains after incubation with the primary (anti-Hsp70) and secondary antibodies (anti-IgG conjugated to FITC), no significant distinction could be made between the two types of cells. Some experiments produced too high background, and some produced no detectable fluorescence. Due to the irreproducibility of the immunofluorescence assays, this method was abandoned for a physiological assay.

Construction of Gene Fusion

In order to study the secretion and identify other proteins that interact with Hsp70 while it is being secreted, a selection method had to be designed. The *SUC2* encodes the invertase enzyme that allows cells to grow on sucrose. Therefore, a gene fusion between an *SSA* gene and the *SUC2* gene was designed that would hopefully allow for the selection of secretion of Hsp70. The Ssa1 portion should direct the fusion to the cell wall where the Suc2 portion will have enzymatic activity. If this is placed in a *suc2*⁻ strain, we will be able to select for growth on sucrose. The *SSA1* and *SUC2* gene fusion was

constructed using SOE PCR, then cloned into a vector that would allow for the expression of the fusion clone. The first PCR products were amplified as detailed in the materials and methods section and can be seen in Figure 3.2. The band at approximately 1.9 Kb product in Lane 2 of Figure 3.1a (created using primers C1 and C2) represents the amplified product of the *SSA*1 gene. The product in Lane 2 of Figure 3.1b represents the *SUC*2 and is 1.6 Kb in size.

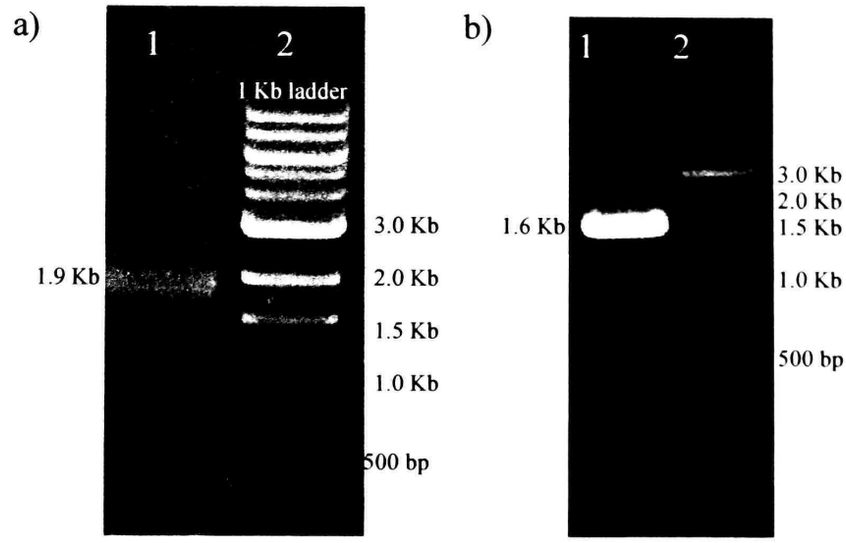

Figure 3.2. The Primary PCR Products of the *SSA*1 Gene and the *SUC*2 Gene. Lane 1a: 1 Kb ladder. Lane 2a: *SSA*1 primary PCR product. Lane 1b: *SUC*2 primary PCR product. Lane. 2b: 1 Kb ladder

After the first PCR products were obtained and purified by gel extraction, the second PCR reaction was done. This reaction utilized the C1 and C4 oligonucleotides, as shown in Figure 2.2, for primers and the primary PCR products as the template DNA. The 3.4 Kb fragment in Figure 3.3 represents the final SOE PCR product, and some major side products can be seen at about 1.6 Kb.

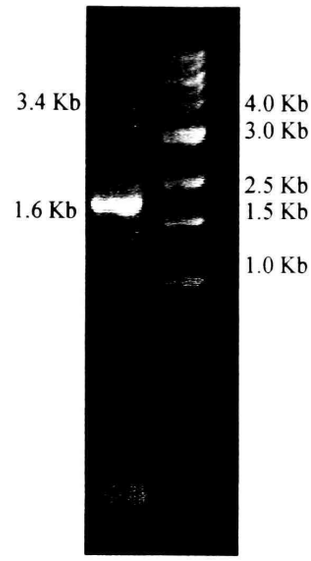

Figure 3.3. The SOE PCR Product. The 3.4 Kb product is the gene fusion produced by SOE PCR between the *SSA*1 gene and the *SUC*2 gene. The 1 Kb ladder is shown in the far right lane.

Cloning Gene Fusion Into pRS306

After the SOE PCR fusion product was generated, it was incorporated into the pRS306 plasmid. This plasmid allows for selection and replication in both *E.coli* and *S. cerevisiae* and has the *MET3* promoter for expression in *S. cerevisiae*. The inducible *MET3* promoter had been cloned into the *Kpn*I and *Hind*III sites of the multiple cloning site in pRS306 as shown in Figure 2.5. A *Hind*III restriction enzyme site was incorporated on the 5′ side of the N-terminus of the *SSA*1 PCR product and a *Not* I restriction site was incorporated into the 3′ side of the C-terminus of the *SUC*2 product (Figure 2.2 and Table 2.2). Therefore, these sites were used to clone the entire fusion, under the control of the *MET3* promoter, into pRS306. After the fusion was ligated into pRS306, the mixture was transformed into *E. coli* and isolated by a plasmid prep. A restriction digest was performed on the resulting plasmid, which is shown in Figure 3.4.

This gel was run after the plasmid had been digested with *Hind*III and *Not*I. The 3.4 Kb band represents the digested fusion and the 4.9 Kb band represents the linearized pRS306 plasmid containing the *MET3* promoter.

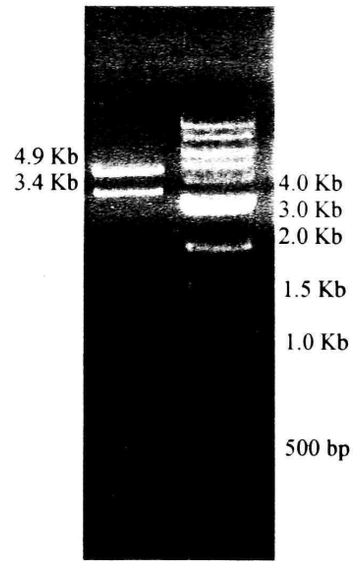

Figure 3.4. Restriction Digest of pRS306metSSA1SUC2. The 3.4 Kb band is the result of a *Hind*III and *Not*I double digest, dropping the fusion out of the pRS306 plasmid (4.9 Kb band) that still contains the *MET3* promoter. The far right lane contains the 1 Kb ladder.

Verification of the Fusion Gene

After plasmid pRS306metSSA1SUC2 was constructed, and the size of the insert confirmed by restriction digest, the junction between the two genes needed to be verified. This was to be done in order to prove that the fusion was constructed in-frame. A primer was created to sequence across the junction as detailed in the Materials and Methods section and the sequence determined. After analysis, the sequence of the junction between the two genes was shown correct. This is shown in Figures 3.5. and A.3.

5'-...CCGTTGAACAAGTTCATACAAACGAAACTAGTGATTA...3'

Figure 3.5. Sequence from the *SSA1* and *SUC2* Fusion Junction. The red sequence is from the C-terminus of the *SSA1* gene and the blue sequence is from the N-terminus of the SUC2 gene.

Deletion of the SUC2 Gene

In order to use invertase activity to select cells that transport the fusion, the cells, which will express the fusion construct (211U*suc2*), must not have any invertase activity. Therefore, the *SUC2* gene must be deleted from this strain to provide the strain for transformation. Utilizing PCR with primers ZAP1 and ZAP2 to amplify a portion of the pMPY-ZAP cassette that contains the *URA3* gene flanked by *hisG* repeats on either side (Figure 2.6), and transforming this product into *S. cerevisiae* strain T211, carried out the site-specific deletion of the *SUC2* gene. This method allowed the *URA3* gene to first replace the gene of interest, and then select for cells with the deletion by plating on media without uracil. The PCR product of the cassette is shown in Figure 3.6 as a band of approxamately 2.1 Kb in length. A smaller band around 600 bp in length is also shown, and probably represents amplification of the *hisG* repeats.

Confirmation of the *SUC2* Deletion

After the transformation with the PCR disruption cassette, the integration of the cassette needs to be verified. Fifty colonies were selected from the transformant cells that grew on –URA YNB agar plates. These URA$^+$, strain T211*suc2*$^-$, colonies have the pMPY-ZAP cassette PCR product integrated somewhere in the genome. The 50

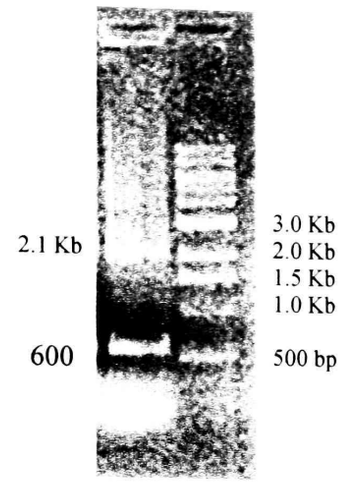

Figure 3.6. pMPY-ZAP PCR Product. In the right lane is the 1 Kb ladder. In the left lane is the result of the PCR reaction with primers that have 18-20 bp complementary to the cassette and 50 bp of homology to the *SUC*2 gene. The major product at 2.0 Kb represents the entire PCR cassette, while the band at 600 bp represents the amplification of the *his*G repeats alone.

transformants selected were grown up on –URA plates with glucose as the carbon source, and then replica plated to –URA sucrose plates. Out of the 50 colonies, 48 of them still grew when only sucrose was present as the carbon source; therefore, the cassette is not integrated into the *SUC2* gene. The two that did not grow represent the desired strain and were further characterized. Before other experiments could proceed, the uracil auxotrophic marker had to be recovered in the T211*suc2*$^-$ strain. This was accomplished by selecting for recombination between hisG repeats by growing the cells in the presence of 5-FOA. 5-FOA is toxic to cells that have a functional *URA3* gene and therefore selects for this recombination event. The cells that were recovered after plating on 5-FOA plates were given the name 211U*suc2*$^-$.

The next confirmation experiment involved mating the 211U*suc2*$^-$ strain to several different strains of *S. cerevisiae*. T211*suc2*$^-$ was mated to a *SUC2*$^+$ strain and the daughters should be *SUC2*$^+$. T211*suc2*$^-$ was also mated to a *suc2*$^-$ where the daughters

should be *suc2*$^-$. However, the mating of the two *suc2*$^-$ strain produced a *SUC2*$^-$ strain. After this conflicting result, the control plates were done, shown in figure 3.8, to try and discern why this occurred. In region 1 of Figure 3.7, the 211U*suc2*$^-$ strain was mated to a *SUC2*$^+$ strain, BY4742. In region 2, the same 211U*suc2*$^-$ strain was mated to a mutant of BY4742*suc2*. Both of these mating experiments produced offspring that can metabolize sucrose and grow when sucrose is the only available carbon source. Region 3 is 211U*suc2*$^-$, and regions 4 and 5 are BY4742 strains that cannot grow without tryptophan in the media, all three showed no growth in this experiment. The last two regions, 6 and 7, are BY4742*suc2*$^-$ strains that are *trp*$^-$ and did not grow. It should be noted that growth in quadrant 2 of Figure 3.8 could possibly be due to a mix up in strains from Research Genetics and may not be due to the incorrect deletion done in this study. In order to prove that the correct deletion was made the PCR confirmation was done.

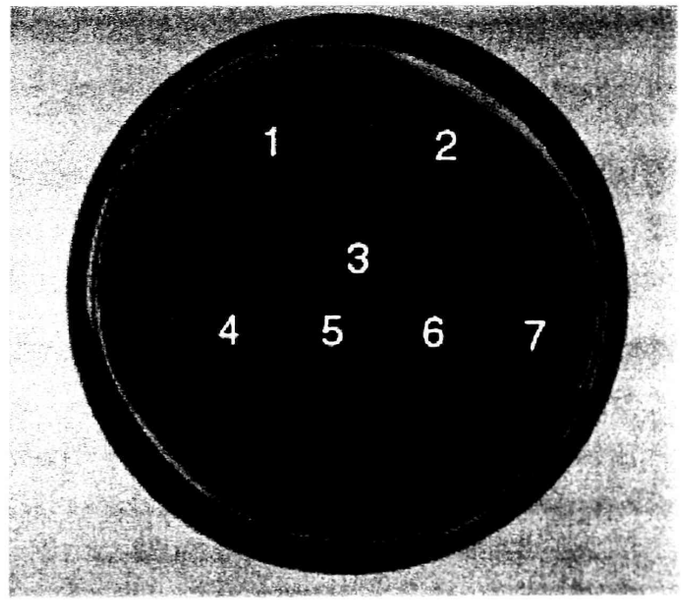

Figure 3.7. Mating Experiments Plated on a –URA, -TRP YNB Sucrose Plate. Region 1: Mate between 211U*suc2*$^-$ and BY4742. Region 2: Mate between 211U*suc2*$^-$ and BY4742*suc2*$^-$ strain. Region 3: 211U*suc2*$^-$. Region 4 and Region 5: BY4742. Region 6 and Region 7: BY4742*suc2*$^-$.

Other controls were done to determine the ability of these strains to grow on sucrose plates. These plates are shown in Figure 3.8. Plate a is a –URA YNB sucrose and plate b is a –TRP YNB sucrose plate. Strain 1 is a cross between 211U*suc2*$^-$ and BY4742. This diploid organism grows on sucrose without either uracil or tryptophan. Strain 2 is 211U*suc2*$^-$ and does not grow under either condition. Strain 3 is the BY4742 *suc2*$^-$ mutant and showed only growth on the – TRP plate. The last strain, strain 4, is BY4742 and was able to grow on the –TRP plate but not on the –URA plate.

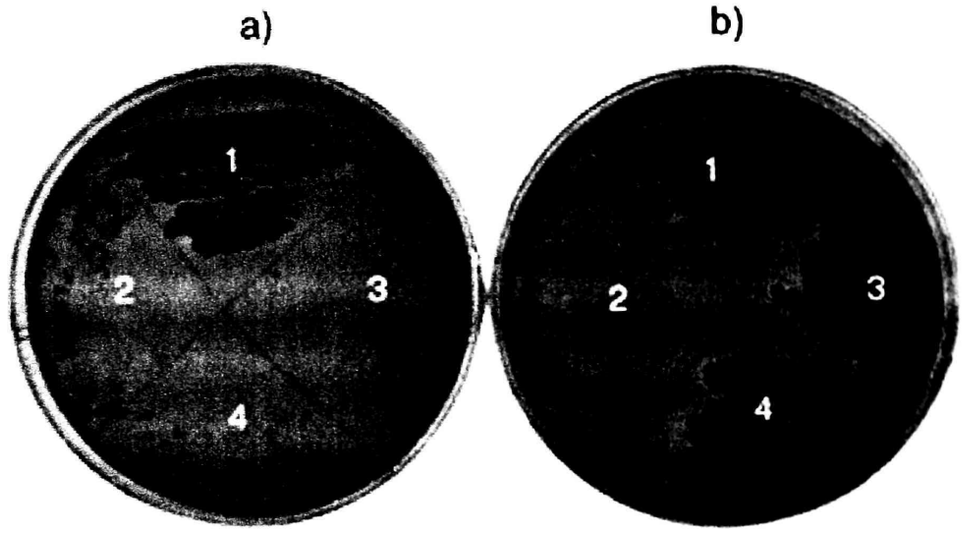

Figure 3.8. Mating Experiments. Cells were plated on (a) – URA or (b) –TRP sucrose YNB Plates. Section 1: Mate between 211U*suc2*$^-$ and BY4742 *suc2*$^-$ strain. Section 2: 211U*suc2*$^-$. Section 3: BY4742 *suc2*$^-$. Section 4: BY4742.

Another method employed to determine if the pMPY-ZAP PCR product integrated into the correct site in *S. cerevisiae* strain T211*suc2*$^-$ utilized the specificity of PCR. PCR was run using primers primer pairs U1 with U2 and U2 with Z2 (Figure 2.7).

The latter primer pair will only amplify a specific region of DNA if the cassette has been integrated properly into strain T211*suc2*$^-$ (lane 8, Figure 3.9). When primers U1 and U1 are used, a 1.9 Kb fragment should be observed when compared to the 2.3 Kb fragment of the deletion to the parental strain. Lanes 1 and 2 contain DNA from a wild type *S. cerevisiae* strain. Lane 1 used primers U1 and Z1 (Table 2.1) and showed no amplification. Lane 2 had the U1 and U2 primers and produced a band approximately 1.7 Kb in length. Lane 3 was left empty as a negative control. Lanes 4-6 utilized template DNA from the original T211*suc2*$^-$ strain. Lane 4 contains primers U1 and Z1. Lane 5 had no template DNA and was a negative control. Lane 6 again used the U1 and U2 primers, and a band of approximately 1.7 Kb in size was produced. Lane 7 and 11 contain the Kb ladder. Lanes 8-10 contain template DNA from the T211 *suc2*$^-$ strain. Inlane 8 the primers U1 and Z1 were added and the product is about 900 bp in length. There was no template DNA added to the lane 9 reaction. The band of approximately 2.3 Kb shown in lane 10 was produced by the U1 and U2 primers. Therefore, I am confident that the correct deletion was made and the results of the mating of the two *SUC2* minus strains can be ignored.

To further show that the deletion cassette was integrated into the correct location of the chromosome, the PCR product in lane 10 of Figure 3.9 was cloned into a PCR cloning vector and sequenced. The partial sequence is shown in Figure 3.10 and noted in the appendix in Figures A.5 and A.6, the sequence analysis shows 200 basepairs of *SUC2* interrupted by the deletion cassette containing the hisG repeats.

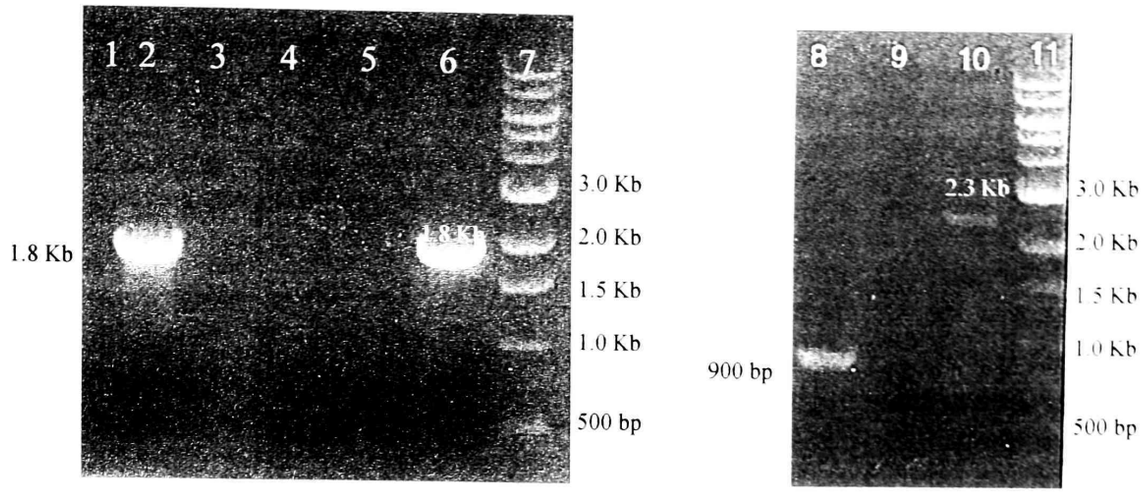

Figure 3.9. PCR Deletion Confirmation. Lane 1 used primers U1 and Z1 and contained wild type *S. cerevisiae*. The same DNA was used in lane 2 but primers U1 and U2 were used. Lanes 4-6 use DNA from the T211 strain. Lane 4 utilized primers U1 and Z1 whereas lane 5 contained no template DNA. The 1.7 Kb band in lane 6 was produced by the U1 and U2 primers. Lane 8 is the product of the U1 and Z1 primers when DNA from T211 *suc2*⁻ was used. Lane 9 contained no template DNA. The 2.2 Kb band in lane 10 was produced by the U1 and U2 primers with T211 *suc2*⁻ DNA. Lanes 7 and 11 contained the 1 Kb ladder.

A) 5′...GCATTCGCCCTTGCCTAAGGGCTCT...AAAGCTTTTCTTTTCACTAACGAAAGGGAA
CAAAAGCTGGTACCGGGCCCCCCCTCGAGACT...3′

B) 5′...TGGTAATTCGCCCTTAACAAATTCCAGG...AACAAATTATCGACCACTATAGGGC
GAATTGTAGCTCCACTCAGCGCCTGATTGCGAT...3′

Figure 3.10. Sequence Analysis of the PCR Confirmation. (A) The upstream sequence from primer U1 (Figure 2.7). (B) The downstream sequence from primer U2 (Figure 2.7). The sequence in blue is a portion of the TA vector used to clone the PCR product. The portion in red is the region of the *SUC2* gene. The green segment is the start of the deletion cassette and the black is the *hisG* repeats.

Growth of 211U*suc2*⁻ cells with the Fusion on Sucrose Media

In order to test our hypothesis that the *SSA1* portion of the fusion will transport the fusion to the cell wall and the *SUC2* portion will allow the cells to grow on sucrose plates, the plasmid needs to be transformed into 211U*suc2*⁻. The transformed cells were

selected for retention of the plasmid on –URA YNB glucose plates and then transferred to –URA YNB sucrose plates to test the hypothesis. In Figure 3.11, Fus denotes the 211U*suc2*$^-$ carrying pRS306metSSA1SUC2 strain. The Suc lanes are the same 211U*suc2*$^-$ strain except that it carries only the *SUC2* gene without its leader sequence. The wt stands for a URA$^+$ strain that is able to grow without supplemented uracil or methionine. The I strain is a strain that is unable to grow without uracil supplemented in the media. Methionine was not added to the media because transcription from the *MET3* promoter is induced in its absence. Plate a is –URA, +MET glucose, whereas plate b is –URA, -MET glucose. The wt, Fus, and Suc all show growth under these conditions. The I strain, however, does not show growth. Plate c is a –URA, +MET sucrose plate and plate d is –URA, -MET sucrose. Only the wild-type strain showed growth on plates c and d. From these observations, it is obvious that the *SSA1-SUC2* fusion did not restore growth on sucrose in a *suc2*$^-$ strain.

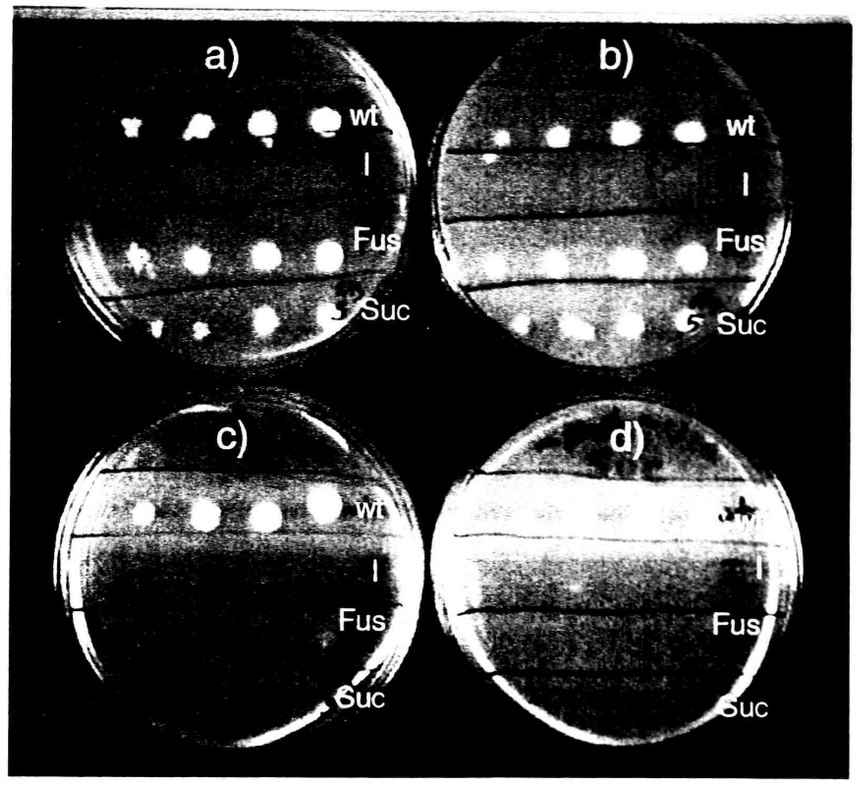

Figure 3.11. Growth of Strains on Glucose and Sucrose Plates. Plate a: -URA, +MET YNB glucose. Plate b: -URA, -MET YNB glucose. Plate c: -URA, +MET YNB sucrose. Plate d: -URA, -MET YNB sucrose. The wild type strain grew on all the plates. The Fus and Suc strains only grew only on the glucose containing plates.

CHAPTER IV

DISCUSSION

The main goal of this project was to enable the identification of proteins present in *S. cerevisiae* that may be involved in the secretion of Hsp70 to the cell wall. In order to achieve this goal, a selection method to screen for the presence of Hsp70 in the cell wall needed to be developed.

Immunofluorescence

Hsp70 was detected in the cell wall of *S. cerevisiae* by Lopez-Ribot (43) through a variety of methods. This was confirmed through the use of Western blot analysis using a polyclonal antibody to the C-terminus of the Hsp70 protein. Therefore, immunofluorescence assays on intact cells using the same antibody were attempted. This would allow a Fluorescence-Activated-Cell-Sorter (FACS) to screen for mutants that are not able to secrete Hsp70. However, repeated indirect immunofluorescence experiments, using a parental *S. cerevisiae* strain (T211) that has *SSA1* and *SSA2* and a mutant strain (T212) that does not possess *SSA1* and *SSA2,* did not allow significant distinction between the degree of fluorescence emitted by the positive and negative controls. Potential reasons for this include either the lack of specific antibody binding or the problem of high background intensity.

There are several reasons why there would have been an absence of fluorescence in the cells. The polyclonal antibody produced in this study was created using the last 17

C-terminal amino acids of the Hsp70 protein. Therefore, the localization of the protein and that of the C-terminal amino acids becomes important once Hsp70 reaches the cell wall. The epitopes recognized by the antibody may not have been accessible to the antibody on intact cells. This could be due to fact that the epitope on the mature protein was not in the correct conformation and therefore did not allow sufficient binding of the antibody to the epitope. This epitope could have been masked by the three-dimensional conformation of the protein itself. Another possible explanation is that the epitope was in the correct conformation and on the exterior of the protein, but that it was hidden by some of the other components of the cell wall. All of these would have led to the failure of the Hsp70 specific antibody to bind to Hsp70.

Another possibility for the inability to distinguish between the positive and negative control would be the presence of high background fluorescence on both samples. This would mask any lower levels of fluorescence emitted by the antibody when Hsp70 is present in the cell wall. The secondary antibody, anti-IgG conjugated to FITC, could have exhibited non-specific binding to the cell wall, or the cells themselves could also have exhibited a high level of autofluorescence. Both of these options could account for the high levels of background fluorescence. Because there were difficulties detecting the presence of Hsp70 in the cell wall through immunofluorescence, an alternate approach was taken.

Invertase as a Reporter Enzyme

Since the immunofluorescence assays did not offer a viable option to screen for cells that secreted Hsp70, another experiment was designed that allowed for the detection of Hsp70 when it is present in the cell wall. The *SUC2* gene encodes for two differently regulated gene products that cleave sucrose into monosaccharides. The larger RNA encodes a protein that is secreted into the periplasmic space while the smaller form of invertase is located within the cell. In order for a cell to metabolize sucrose, the enzymatic activity of the protein from the larger gene product is required to be localized outside the cell membrane. Therefore, cells that do not have a functional Suc2 protein beyond the cell membrane cannot grow on media where sucrose is the only available carbon source. Since the *SSA1* protein is secreted to the cell wall, a protein fusion to the *SUC2* protein should allow *suc2⁻ S. cerevisiae* cells to then grow on sucrose.

Gene Fusion

The difference between the two gene products that are produced from the *SUC2* locus involves the secretion signal. The larger construct is transcribed with the secretion signal sequence; whereas, the shorter product does not have this sequence. The gene fusion produced in this study attaches the N-terminus of the *SUC2* gene without its signal sequence to the C-terminus of the *SSA1* gene. Therefore, the Ssa1 protein portion of the gene fusion should direct the entire construct to the cell wall and the Suc2 portion should display its enzymatic activity and allow the cell to metabolize sucrose. The gene fusion was constructed as shown in Figures 2.2 and 2.7. The *SSA1* gene was amplified without

the stop codon and with 50 bp of homology to the N-terminus of the *SUC2* gene (Figure 3.1 and 2.2); whereas, the *SUC2* gene was amplified without its signal sequence and a stop codon (Figure 2.2 and 3.1). These two PCR products were then used in the SOE PCR reaction shown in Figure 2.7. This construct was designed so that the two genes were fused together in-frame. This is important because the Suc2 protein has to retain enzymatic activity and an out-of-frame fusion would not preserve this activity. This fusion was then cloned into the *HindIII* and *NotI* sites of the pRS306 plasmid (Figures 2.5 and 3.3).

SUC2 Deletion

Before the experiments could proceed, a *SUC2* deletion had to be constructed. The *SUC2* gene was deleted using a reusable PCR mediated disruption cassette (Figure 2.6). The cassette replaced the *SUC2* gene in the chromosome with a functional *URA3* gene flanked on either side by hisG repeats. This allows for the cells to grow on plates that lack uracil.

Confirmation of the *SUC2* Deletion

After the *SUC2* gene was deleted, the correct integration of the cassette had to be confirmed. This was done through several different methods. The first experiment simply tested the *SUC2* deletion's ability to grow on a plate where sucrose is the only carbon source. As expected, the *SUC2* deletion exhibited no growth on the sucrose plates, showing that integration was indeed in the correct gene.

Another method to determine if the cassette integrated into the correct location in the chromosome utilized PCR. As shown in Figure 2.7, primers were designed that would produce distinctively sized PCR products when the reaction was run. The *SUC2* gene is 1.6 Kb in length and the primers U1 and U2 were designed 200 basepair on either side of the open reading frame (Figure 2.7). The *URA3* gene and the *higG* repeats are a total of 1.9 Kb in length. Therefore, the mutant T211*suc2*⁻ produced a 2.3 kilobase band and the parental T211 produced a 1.9 Kb band as shown in Figure 3.7. The U2 primer was also used in conjunction with the Z2 primer. This combination will only produce a band when the *URA3* gene is in the correct orientation and located next to the downstream region of *SUC2* (Figure 2.7). This situation is only present in the deletion strain and not in the parental strain, as seen as the 900 basepair band shown in Figure 3.7 The 900 basepair band is comprised of the 400 bp of *hisG* repeats, a short section of the *URA3* gene, and the 200 basepairs of the region immediately downstream of the *SUC2* gene. These PCR results confirm that the deletion cassette was replaced with the *SUC2* gene.

Complementation experiments were also utilized to determine if the correct gene was disrupted. This was accomplished through the use of mating experiments. When *S. cerevisiae* strains mate, a diploid organism is produced. Therefore, when a strain with a functional *SUC2* gene is crossed to a strain without a functional *SUC2* gene, the daughter cells are able to metabolize sucrose. Likewise, when a *suc2*⁻ strain is crossed to another *suc2*⁻ strain the daughter cells will not be able to metabolize sucrose. This experiment is shown in Figure 3.5. The crosses were done with the 211U*suc2*⁻ strain produced in this

study to strain BY4742*suc2*⁻ from the Yeast Deletion Project. The results of this cross-produced cells which were able to grow on sucrose plates. As controls, the strains used in the mating experiment were plated individually on sucrose containing media and the BY4742*suc2*⁻ strain showed growth on the sucrose media (Figure 3.6). Therefore, it was concluded that either the strain received from Research Genetics (BY4742*suc2*⁻) must have been mislabeled, or that the *SUC2* gene was not correctly deleted.

Sequence analysis was also used to determine if the pMPY-ZAP cassette integrated into the correct location within the chromosome. Using primers U1 and U2 in Figure 2.7, a 800 bp band was amplified from 211U*suc2*⁻ and then cloned into a PCR cloning vector. The insert was then sequenced using the T7 and M13 reverse primers. This sequence matched the expected sequence containing 200 basepairs of homology to the *SUC2* gene followed by a portion of the *hisG* repeats. This proves that the PCR product used to delete the *SUC2* gene has been inserted into the correct location.

Before the pRS306met3SSASUC plasmid could be transformed into T211*suc2*⁻, this strain, which has the pMPY-ZAP cassette integrated into the genome, needs to undergo one last genetic manipulation. The cells were grown in the presence of 5-FOA, which is lethal to cells with a functional *URA3* gene. This selects for the recombination between the two *hisG* repeats flanking the *URA3* gene and therefore restoring the auxotrophic marker. Then plasmid pRS306met3SSASUC, that utilizes uracil for the auxotrophic marker, could be transformed into these cells.

Yeast Transformation and Growth

Once the plasmids containing the gene fusion constructs were created, and the *suc2*$^-$ cells restored to the *ura*$^-$ phenotype, the transformation and subsequent growth on sucrose experiments could be done. *S. cerevisiae* strain T211*suc2*$^-$ was transformed with pRS306metSSASUC and selected for by growth on media without available uracil. A single colony was isolated and transferred to a plate where methionine was absent and sucrose was the only carbon source. The cells were also transferred to plates without histidine but had glucose as the only available carbon source. The methionine was absent in order to activate transcription from the met3 promoter on the pRS306met3SSASUC plasmid. The cells were able to grow on the glucose plates, but were unable to grow on the sucrose plates. Therefore, the fusion was not able to complement the cells' *SUC2* deletion.

Transcription, Translation and Activity of the Gene Fusion: What went wrong?

After the plasmid (pRS306met3SSASUC) was constructed and then transformed into the *SUC2* deletion, the cells still showed no growth on media when sucrose was the only available carbon source. This could be due to a number of problems associated with the nature of constructing a gene fusion or expressing this gene from a plasmid.

Expression of the Gene Fusion

The first logical explanation for the failure of the gene fusion to restore growth on sucrose media has to do with the induction of transcription from the *MET3* promoter. The inducible *MET3* promoter was originally chosen over a constitutive promoter to guard against the fusion protein being toxic to the cell. Transcription from the *MET3* promoter has been characterized and is induced when methionine is not supplemented in the growth media. There could have been a small amount of methionine contaminating the media and this could have caused the inhibition of transcription from the promoter. There could have been problems with the transcription machinery binding and subsequently producing the mRNA. The entire fusion is 3.4 Kb and the length of the construct as well as its foreign nature could have halted transcription. Running Northern blot analysis could test this outcome. In this experiment, the total mRNA would have to be isolated and a radioactive probe specific to the fusion would have to be used. Since the *SUC2* gene has been deleted from T211*suc2*$^-$, a probe specific to this gene could have been used. This would confirm if the fusion was being transcribed.

Translation of the Gene Fusion Product

Once the gene fusion has been shown to be transcribed, the next step is translation of the mRNA. The fusion will produce a long transcript that is not normally present in the cell, and this may form a secondary structure that inhibits the translation machinery from recognizing or producing the amino acid sequence associated with the fusion. The amino acid chain itself may also form irregular structure and interfere with the elongation

of the sequence. The most plausible explanation for the cell not producing an translated product is if a premature stop codon was inserted. Running Western blots on cytosolic extracts with an antibody to the Suc2 protein could test this theory. This would be done by comparing the signal in a cell culture that has the gene fusion and one that has not been transformed with the plasmid.

Transportation of the Fusion Protein

The inability to transcribe or translate the gene fusion and product are not as likely as a problem associated with transporting the fusion protein to the exterior of the cell. The Ssa1 portion is meant to direct the entire fusion to the cell wall, and this may not be able to happen with another large protein hanging off the C-terminus of the protein. Since alternative secretory pathways have not been characterized, it is not known how other proteins, if any, interact with a substrate for non-classical export. The transportation apparatus may have to interact intimately with the amino acid sequence of Ssa1 at any stage during its development. The Suc2 portion of the fusion may interfere with this interaction and delay or halt secretion beyond the cell membrane. The fusion may also not be capable of crossing the membrane in general. Suc2 may not be able to pass through an alternative secretory pathway due to an amino acid sequence or final conformation that does not interact correctly with the proteins involved in secretion by an alternative mechanism. In this case, the Hsp70 protein may reach the external environment, but the Suc2 protein would be held in the membrane or in the cytosol. Both of these problems could be addressed by again using an antibody specific to the Suc2

protein. However, in this case the Western blot signal in the cell wall extract could be compared to the signal in the cytosolic extract. If the fusion was not transported, the cytosol would exhibit a reaction with the antibody but the cell wall would not.

Enzymatic Activity of Invertase

Another likely possibility that would not allow the fusion protein to complement the cell's inability to grow on sucrose media is if the fusion is transcribed, translated, and then transported, but the invertase is not functional. In this case, the Ssa1 protein could be interfering with the activity of the Suc2 protein. Invertase has to recognize the sucrose molecule and bind efficiently so that it can hydrolyze the compound into glucose and fructose. If being involved in the fusion compromises either the binding efficiency or the hydrolyzing ability of invertase, the cell will not be able to grow on sucrose media. In order to address this problem, assays for invertase activity could be preformed. If there is no activity inside the cell, but the fusion protein has been shown to be inside and outside of the cell, then the inactivity of the invertase is probably the cause of the inability of the fusion to complement the *SUC2* deletion in the cell. In the case of any cloning and amplification steps, the most sound reason of why an expected observation did not occur is that a mutation occurred which did not allow the correct protein sequence to be created.

Future Directions

The first step in continuing the research into the secretion of Hsp70 should involve determining what caused the inability of the cells carrying pRS306metSSA4SUC

to metabolize sucrose. This could be done by performing the experiments listed above for each of the possible explanations.

There are also a number of design changes that one could do that may solve some of the problems discussed. The gene fusion was constructed so that the C-terminus of the *SSA1* gene was directly fused to the N-terminus of the *SUC2* gene. The fusion could be designed so that the C-terminus of the *SUC2* gene is linked to the N-terminus of the *SSA1* gene. This may alleviate some of the conformational issues associated with the transportation or activity of the fusion protein. Since the fusion was constructed leading from the *SSA1* gene directly into the *SUC2* gene, a linker polypeptide may add to the flexibility of the construct and allow the correct molecular interactions to occur so that the fusion will be transported and have activity. This linker should be constructed using ten to fifteen amino acids that are relatively nonreactive and uncharged, such as glycine. This linker would have to allow for the separation of the two independent proteins without causing any transcriptional, translational or conformational problems.

The use of a reporter enzyme has been shown to be a valid and sound method to detect when a protein arrives at the cell exterior. Invertase has been used by a variety of researchers to show that a fusion construct was secreted (51). Due to the reasons discussed above, it should be noted that the most probable reason that the fusion plasmid did not complement the *SUC2* deletion is that there was a mutation in the fusion plasmid. This possible problem could be solved by creating primers that could be used to sequence the entire fusion construct and search for an inserted premature stop codon or another

major frameshift mutation. It is important to note that this method is still a valuable way to screen for mutants, however the quirks in using it to study the secretion of Hsp70.

Invertase is not the only candidate for a reporter enzyme. Acid phosphatase was used in the study of the transport of α-factor in *S. cerevisiae* (61). In this case, the *SSA1* gene could be fused to the *PHO5* gene and colonies screened by staining with diazocoupling dye or using a colormetric assay by determining the amount of *p*-nitrophenol from *p*-nitrophenyl-phosphate.

In order to study alternative secretory pathways and identify any proteins involved in this process, a candidate for non-classical export must be used. In this study, Hsp70 was used to try and learn more about these mechanisms. However, there are other proteins that may be used to study this phenomenom, and this may allow the elucidation of an alternative secretory pathway.

In light of the inability of the fusion construct to restore growth of the strain on sucrose, this approach has sound scientific basis and the work has yet to be finished on this project. The fusion should be sequenced, and the reason for the observed results determined and dealt with appropriately.

As our knowledge of the cell wall continues to expand, it is important to continue studying the export of proteins through a non-classical mechanism. These proteins have been shown to exist in a number of species and may one day provide an important piece of information to solve a bigger problem.

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APPENDIX

SEQUENCE ANALYSIS RESULTS

CAGNANTTTGGTACCCTGCCGCGTTTATTTAGTACAAACAGAGAATTTTGTC

ACAACTACATCTAAGTGTACAAATATAGTACAGATATGACACACTTGTAGC

GGCCAACGCGCATCCTACGGATTGCTGACAGAAAAAAAGGTCACGTGACCA

GAAAAGTCACGTGTAATTTTGTAACTCACCGCATTCTAGCGGTCCCTGTCGT

GCACACTGCACTCAACACCATAAACCTTAGCAACCTCCAAAGGAAAATCACC

GTATAACAAAGCCACAGTTTTACAACTTAGTCTCTTATGAGGTTACTTACCA

ATGAGAAATAG

Figure A.1. Sequence Analysis of pRSMETSSA1SUC2 from the T3 Promoter. The sequence in black is from the plasmid and the sequence in red is from the *MET3* promoter.

GGNNANCCTGGTGTACTGTGCGTCGTTGGCAATAATGTCAACACNATCATTA

GAGAAGTGAGCAACACAGGAGTAGGTAGTACCTAAATCAATACCGACAGCT

TTTGACATGAGCTCAAGCTTATCGATGTTAATTATACTTTATTCTTGTTATTA

TTATACTTTCTTAGTTCCTTTTCAATTGTTAAGAAACGATATCACAACTGTTA

CGACAGAGAGAGACCCAAGCTAG

Figure A.2. Sequence Analysis of pRSMET*SSA1SUC2* Across the MET3 and SSA1 Junction. Primers were designed that would sequence across the junction between the MET3 promoter and SSA1. The MET3 portion is in black, whereas the SSA1 portion is in red.

GGNCCATNCCCGNANGAAAGGCGCAGCTCCAGGTGGTTTCCAGNTGGTGCT

CCTCCAGCTCCAGAAGCTGAAGGTCCAACCGTTGAAGAAGTTGATACAAAC

GAAACTAGTGATAGACCTTTGGTCCACTTCACACCCAACAAGGGCTGGATG

AATGACCCAAATGGGTTGTGGTACGATGAAAAAGATGCCAAATGGCATCTG

TACTTTCAATACAACCCAAATGACACC

Figure A.3. Sequence Analysis of pRSMET*SSA1SUC2* across the SSA1 and SUC2 Junction. The black sequence is SSA1 and the red sequence in SUC2.

CNATTTNGNAGTTCCNCCTGCCCGTGCGCGGCCNGCTCCAGAGTAACTTGGGG

TCGGGAGAAAATAAAAATAAAAATACATTTTCAATGGTTTGGAGGTATTCTTT

GAAATCATAAAGTTTTACATTCGTCACTCGTTAGCTAAAGCCCTTTAGAATGG

CTTTTGAAAAAAATAAAAAAGACAATAAGTTTTATAACCTCTATTTTACTTTC

CCTTACTTGGAACTTGTCAATGTAGAACAAATTATCGACACCAGTGGT

Figure A.4. Sequence Analysis of pRSMETSSA1SUC2 from the T3 Promoter. The black sequence is from the plasmid and the red sequence is the terminal portion of *SUC2*.

TANCNGGCATGCTCGNAGCGGCCGCCAGANNTGATGGATATCTGCAGCATTC

GCCCTTGCCTAAGGGCTCTATAGTAAACCATTTGGAAGAAAGATTGACGACTT

TTTTTTTTGGATTTCGATCCTATAATCCTTCCTCCTGAAAAGAAACATATAAAT

AGATATGTATTATTCTTCAAAACTATCTCTTGTTCTTGTGCNTTTTTTTTACCAT

ATATCTTACTTTTTTTTTTTCTCTCAGAGAAACAAGCAAAACAAAAAGCTTTTC

TTTTCACTAACGAAAGGGAACAAAAGCTGGTACCGGGCCCCCCCTCGAGACT

AGTTACCAAATCGCAG

Figure A.5. Sequence Analysis from the T7 Promoter of the Deletion Confirmation PCR Product. The black portion is the TA vector used to clone the PCR product, and the red portion is the inserted PCR product.

NGNTTCGCCGCTCGGNATCACTTAGNTAACGGCAACCAGTGNTGCTGGAATTC

GCCCTTGNAACAAATTCCAGGTAACTGGGGTCGGGAGAAAATAAAAAATAAAA

ATACATTTTCAATGGTTTGGAGGTATTCTTTGAAGTCATAAAGTTTTACATTCG

TCACTCGTTAGCTAAAGCCCTTTAGAATGGCTTTTGAAAAAAATAAAAAAGAC

AATAAGGTTTAATAACACTCTATTTTACTTCCCTTACTTGGAACTTGTCAATGT

AGAACAAATTATCGACCACTATAGGGCGAATGGTAGCTCCACTCAGCGCCTG

ATTGCGATGGCGGAAAACAT

Figure A.6. Sequence Analysis from the M13 Reverse Promoter of the Deletion Confirmation PCR Product. The black portion is the TA vector used to clone the PCR product, and the red portion is the inserted PCR product.

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Student Signature Date