INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Role of the v-SNARE Bet1p in Cell Wall Biosynthesis

by

Pearl Kipnis

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2002

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/12/02
Date

Chair of Examining Committee
Dr. Peter Lipke, Hunter College

4/13/02
Date

Executive Officer
Dr. Richard L. Chappell

Dr. David Foster, Hunter College

Dr. Shirley Raps, Hunter College

Dr. Anne Draginis, St. John's University

Dr. Jeanne Hirsch, Mount Sinai Medical Center

Supervising Committee

The City University of New York

Abstract

Role of the v-SNARE Bet1p in Cell Wall Biosynthesis

by

Pearl Kipnis

Advisor: Professor Peter N. Lipke

The cell wall of *Saccharomyces cerevisiae* is a complex structure of cross-linked glucans, chitin and mannoproteins. The biosynthesis of the cell wall depends on the secretory pathway for components and required enzymes. To elucidate mechanisms involved in secretion and biosynthesis of the cell wall, I used W303-1B mutants previously selected for temperature sensitivity for growth and inability to cross-link the mannoprotein alpha-agglutinin to the cell wall. Twenty mutants which did not bind to a-cells were further screened for excretion of alpha-agglutinin, sensitivity to lysis by the enzyme Zymolyase, and for growth rate. Ten of these were tested for sensitivity to calcofluor white. Various strains exhibited abnormal levels of excretion of alpha-agglutinin, and all strains exhibited some degree of cell wall aberration.

To identify the genes responsible for these aberrant cell wall phenotypes I transformed the mutants with a YCp50 yeast genomic library. A mutant strain that is hyper-sensitive to Zymolyase and calcofluor white, AC59, was restored to viable growth at the restrictive temperature of 37°C by a particular plasmid from the library. The complementing gene on the plasmid proved to be *BET1*, which codes for a v-SNARE, an

integral membrane protein on vesicles involved in ER to Golgi transport.

In AC59, incubated at the restrictive temperature, levels of invertase were diminished at the cell surface, consistent with a *bet1* block in secretion, but alpha-agglutinin continued to be excreted. This apparent paradox could be explained by the existence of a post-ER pool of alpha-agglutinin.

In parent strain W303-1B the existence of a pool of alpha-agglutinin was shown by continued secretion of alpha-agglutinin to the cell wall after a cycloheximide block. In AC59 at 37°C the results were similar, except alpha-agglutinin was excreted into the media instead of being cross-linked to the cell wall.

Such a cell wall anchorage deficit could result from the absence of beta-1,6 glucan and/or enzyme activities required for anchorage. The levels of beta-1,6 glucan were assayed and found to be significantly diminished at the restrictive temperature. This work thus demonstrates a linkage between the yeast v-SNARE Bet1p, secretion and cell wall biosynthesis.

Acknowledgments

I would like to thank many people who have made this work possible.

First and foremost, I want to thank my advisor Dr. Peter Lipke for his scientific guidance, support, and encouragement during the years I spent in his laboratory.

To the members of my thesis committee: Dr. Ann M. Dranginis, Dr. Jeanne P. Hirsch, and Dr. Shirley Raps for their advice and encouragement.

To my colleague Dr. Rafael Ovalle for providing invaluable suggestions.

To Dr. Rivka Rudner and Dr. Ann Henderson for their encouragement.

To my parents

Table of Contents

List of Tables

List of Figures

INTRODUCTION

I. The life cycle and mating type control of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a single cell organism that exists in three yeast forms. Two haploid mating types, designated as **a** and α can mate and form the third cell type, the diploid a/α. Both haploid and diploid cells can replicate asexually by budding. The diploid form under adverse environmental conditions can undergo meiosis, form an ascus, and produce **a** and α haploid spores. Haploid cells can switch mating type after they have produced a daughter cell. The mating type is determined by the mating-type (*MAT*) locus. *MATα* codes for two regulatory proteins α1 and α2 and *MAT**a*** codes for the regulatory protein **a**1 and for **a**2. The **a** and α proteins control transcription of genes involved in mating. They function independently in haploids and function conjointly in diploids. In **a** cells the expression of **a**-type mating genes is constitutive. In α cells, α2 represses **a**-specific genes and α1 activates α-specific genes. In diploid cells the **a**1/α2 heterodimer represses haploid-specific genes functions (see for review, Sprague *et al.*, 1983; Nasmyth and Shore, 1987).

A. Sexual agglutination and mating

The mating of **a**- and α-cells is regulated by the pheromones, **a**- and α-factor. Each pheromone binds to a receptor on the cell membrane of the opposite mating type and initiates a mating response. This consists of the arrest of replication in the G1 phase, formation of a mating projection, and secretion to the cell wall of **a**- or α-agglutinin, primarily to the tip of the mating projection (Smits *et al.*, 2001). The adhesion of the agglutinins mediates the

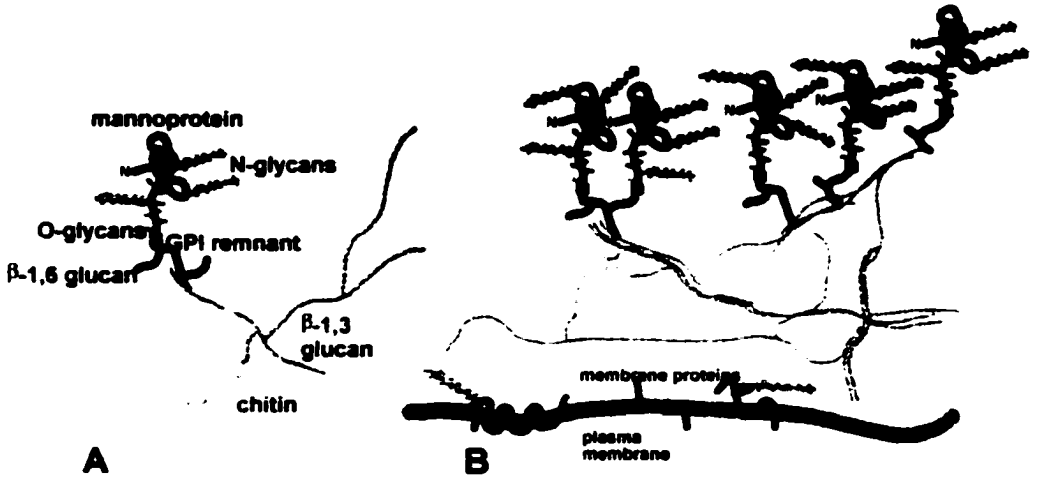
fusion of the a- and α-cells in the process of mating.

II. The cell wall

The cell wall of *S. cerevisiae* protects the membrane-bounded cell from osmotic stress and controls the passage of molecules (Brul *et al.*, 1997). It is dynamically restructured during mating in the formation of the mating projection and during replication in bud formation.

The cell wall is composed of an outer layer of mannoproteins and an inner layer of β-1,3 glucans, β-1,6 glucans and a small amount of chitin (Kapteyn *et al.*, 1997; Lipke and Ovalle, 1998). β-1,3 glucans account for the major part of the wall. β-1,3 glucans together with chitin maintain the shape and strength of the cell wall (Kapteyn *et al.*, 1999). The four components of the cell wall, β-1,3 glucan, β-1,6 glucan, chitin, and mannoproteins are all linked together via the highly branched polymers of β-1,6 glucans (Kapteyn *et al.*, 1996; Kapteyn *et al.*, 1997). β-1,6 glucan has β-1,3 glucan branches, linked by the reducing end of β-1,6 glucans to the terminal glucose non-reducing end of β-1,3 glucan (Kollar *et al.*, 1997; Kapteyn *et al.*, 1998). The four-unit complex has been described by Lipke and Ovalle (1998) as a lattice, composed of unit modules built around β-1,3 glucan, to which one or two β-1,6 glucan and mannoprotein moieties are attached, with chitin linked to β-1,3 or β-1,6 glucan in a small number of instances (Figure 1). The modules in the glucan-chitin layer are associated by non-covalent interactions and in the mannoprotein layer by covalent cross-links, including disulfide bonds.

Figure 1. Relationships among components of *S.cerevisae* cell walls



A) Prototypical module with components individually labeled. The mannoprotein polypeptide and oligosaccharides are labeled as N or O linked. Chitin can also be linked to β-1,6 glucan. (B) Association of modules to form a wall lattice. The β-1,3 glucan chains are intertwined to designate triple helices and chitin is shown as a crystalline microdomain. Cross-linking of mannoproteins through disulfide and other bonds is not shown.

(Lipke and Ovalle, 1998)

III. Cell wall mannoproteins and GPI anchors

Some mannoproteins are covalently bound to β-1,6 glucan in the cell wall via a remnant of a glycosylphosphatidylinositol (GPI) anchor (Kapteyn *et al.*, 1996). The GPI anchor is synthesized in the endoplasmic reticulum and transferred to newly formed proteins (Leidich and Orlean, 1996). The precursor of the GPI anchor consists of a lipid group, phosphatidyl-inositol which is the membrane anchor, glucosamine, a carbohydrate head group consisting of four α-linked mannoses, designated M1-M4, and a phosphoethanolamine group on M3, the point of protein attachment (Gaynor *et al.* 1999; Sipos *et al.*, 1994). The addition of a fifth mannose occurs in the early and late Golgi compartments (Sipos *et al.*, 1995) (Figure 2A).

The existence of five α-linked mannose residues attached to the nonreducing end of β-1,6 glucans was demonstrated by Kollar (Kollar *et al.*, 1997). The connection between cell wall mannoproteins and β-1,6 glucan through a portion of the GPI anchor was shown by the simultaneous binding of mannoproteins and β-1,6 glucan to ConA-Sepharose column. Kollar subsequently determined the nature of this bond between β-1,6 glucans and mannoproteins. To isolate the linkage region between the protein and β-1,6 glucan, the ConA fraction was digested with β-1,6 glucanase, and then hydrolyzed with cold hydrofluoric acid to cleave the bond between the phosphate and mannose in the GPI remnant. The resulting oligosaccharides consisted of five α-linked mannose residues which were attached to the nonreducing end of either β-1,6 -linked di- or trisaccharides. This result thus identified the part of the GPI anchor that remained attached to the protein as ethanolamine-phosphate-

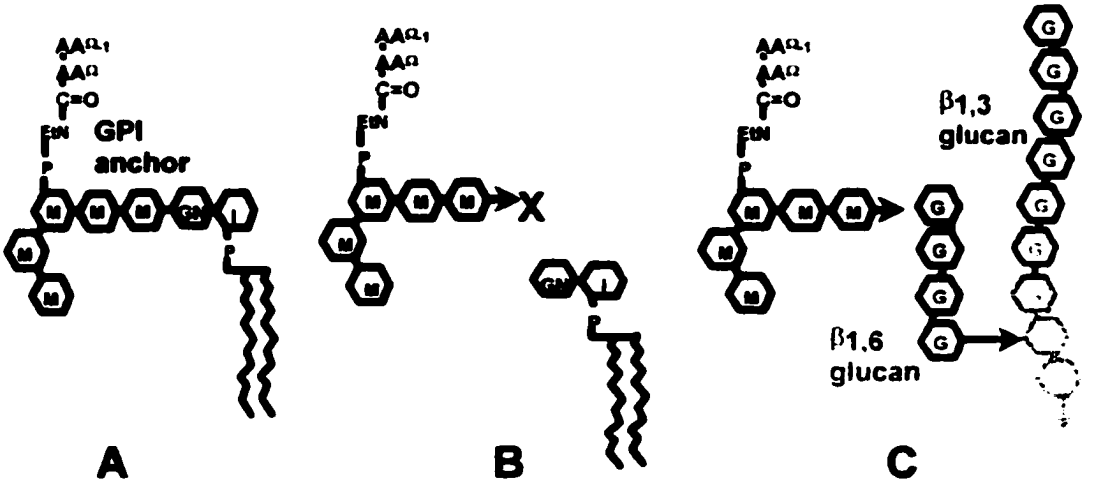


Figure 2: Structure of a yeast GPI anchor and model for assembly of a mannoprotein into a module: (A) GPI anchor: AA, Amino acid residue; EtN, ethanolamine; P, phosphate; M, mannose; GN, glcosamine; I, myoinositol. (B) Proposed cleavage of the glucan of the GPI anchor. (C) Formation of a glycosidic linkage between the GPI-remnant and glucans. G, glucose.

(Lipke and Ovalle, 1998)

Man_5. The rest of the anchor, the glucosamine residue and the phosphatidylinositol group, was eliminated in what was thought to be the transglycosylation reaction which initiated the protein-glucan reaction (Kollar *et al.*, 1997).

Based on the above results, the transition of α-agglutinin from a GPI plasma membrane anchored form to a cell wall bound form is thus thought to occur by a cleavage between the glucosamine and first mannose. In this model the remnant of the GPI anchor, containing five α-linked mannosyl residues, is then covalently bound to β-1,6 glucan in the cell wall (Lipke and Ovalle, 1998).

IV. The secretory pathway, from the endoplasmic reticulum (ER) to the cell wall

A. Modification of proteins can be demonstrated with secretory (*sec*) mutants

In the synthesis of secretory proteins the nascent protein precursors emerge from the ribosomes and enter the endoplasmic reticulum (ER) (Lyman and Schekman, 1996), then proceed on a pathway of modification before developing into a mature form at the cell surface.

One way of exploring the manner in which cell wall proteins are modified during transport to the cell surface is by the use of secretory (*sec*) mutants, in which the biosynthesis of these proteins can be studied by an analysis of their form and stage of development in *sec* blocked organelles (Hibbs and Meyer, 1988). Lu (Lu *et al.*, 1994) described the sequential forms of α-agglutinin from the ER to the cell surface, using *sec* blocked mutants.

1. How *sec* mutants block the secretory pathway

In the ER *sec53* mutants (at the restrictive temperature) do not synthesize guanosine diphosphate (GDP)- mannose nor add the GPI anchor (Fankhauser and Conzelmann,1991; Conzelmann *et al.*,1990). In *sec53* mutants GPI anchored proteins such as α-agglutinin would remain attached to the ER. Solubility studies suggested that adherence to the ER membrane is the result of protein-protein interaction (Ruohola and Ferro-Novick, 1987). Sec53p has been implicated in protein translocation across the ER membrane and mutants block the transport of all cell surface proteins at the restrictive temperature (Ruohola and Ferro-Novick,1987).

Sec18p is required for ER to Golgi transport (Eakle *et al.*, 1988) as well as for subsequent inter-organelle transport (Graham and Emr, 1991; Rothman, 1994). Sec18p is homologous to NSF (N-ethylmaleimide-sensitive fusion protein) in mammals. Sec 18p and NSF, both ATPases, play a role in disassembly and priming of SNAREs (soluble NSF attachment protein receptors). In Sec18p defective mutants, unprimed SNARE complexes accumulate (Steel *et al.*, 2000).

In the Golgi, Sec7p is thought to coordinate membrane transitions in Golgi biogenesis (Deitz *et al.*, 2000). In *sec7* mutants, protein transport through the Golgi was deficient and at 37°C an increased array of Golgi-like cisternae was formed (Franzusoff *et al.*, 1991).

In the late secretory pathway, Sec1p is required for vesicular transport between the Golgi and the cell surface (Pevsner, 1996). *sec1* mutants exhibit an accumulation of post-Golgi secretory vesicles (Egerton *et al.*, 1993). Sec1p is a cytosolic protein and is associated with membranes through interaction with the t-SNARE syntaxin. Sec1p is thought to be a

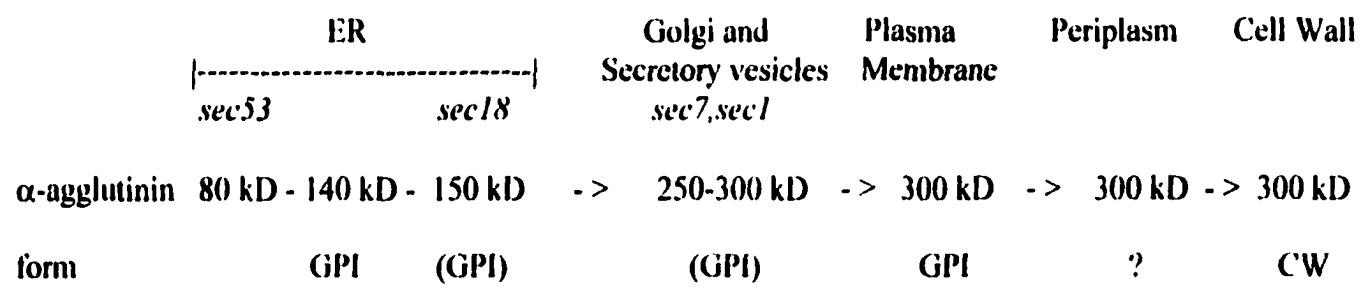
chaperone that puts syntaxin into the proper conformation required for SNARE-SNARE interaction (Bock *et al.*, 2001).

B. The secretory pathway of α-agglutinin

The progression of α-agglutinin from its pro-α-agglutinin form in the ER to its mature form in the cell wall (Figure 3) was described by Lu (Lu *et al.*, 1994). The earliest form of α-agglutinin observed was 80 kDa, in a *sec53* mutant. This form was thought to represent the unmodified peptide in the ER. In wild-type cells and *sec* mutants, at the permissive temperature, a 140 kDa form was observed. In *sec18* mutants, a 150 kDa membrane bound form was observed at the restrictive temperature. Pulse-chase experiments showed that the 140 kDa form preceded the 150-kDa form. Since *sec18* blocks transport of proteins from the ER, a 140 kDa GPI anchored form of α-agglutinin would be in the ER and a 150 kDa form would indicate a modification of the 140 kDa form. The addition of tunicamycin, which blocks N-glycosylation, decreased the 140 and 150 kDa forms in *sec18* to 125 and 135 kDa, respectively, which indicated that N-glycosylation occurs in the ER. The increase in size from 80-kDa was thought to be due to O-glycosylation in the ER (Lu *et al.*, 1994).

In *sec7* mutants, blocked in transport through the Golgi, a 150 kDa form of α-agglutinin was observed. In *sec1* mutants, blocked in vesicle transport from the Golgi to the cell surface, a 300 kDa form of α-agglutinin was observed. With the addition of tunicamycin this form was reduced to 200 kDa, indicating that elongation of N-glycosylation occurs in the Golgi.

Figure 3. Model for processing of α-agglutinin



The *sec* mutants in which particular forms were detected at the restrictive temperature are indicated. The positions of all forms within the secretory pathway are inferred from the known positions of the blocks in the *sec* mutants. The >300kD form includes membrane bound, soluble periplasmic, and cell wall-anchored forms. The 140 and >300kDa membrane linked forms have been demonstrated to contain GPI anchors. The 150 and 250- to 300-kDa forms are proposed to be intermediate between the two demonstrated GPI-anchored forms. It was proposed that they also contain GPI anchors. The periplasmic >300kD form was not labeled by inositol or fatty acid but might retain a portion of the GPI anchor (Lu *et al.*, 1994).

A membrane linked, > 300 kDa GPI-anchored form of α-agglutinin was released by phosphatidylinositol phospholipase C (PI-PLC) and labeled by [^{3}H]inositol or [^{3}H]fatty acid. Since this form was susceptible to exogenous protease treatment it was thought to be exposed to the outside surface of the plasma membrane. A soluble > 300 kDa form of α-agglutinin which was not labeled by [^{3}H]inositol or [^{3}H]fatty acid, and which was also susceptible to exogenous protease treatment was observed, indicating that it did not have an intact GPI anchor. This form, suggesting a modified GPI anchor, had the properties of a periplasmic protein that was consistent with the cell wall form of α-agglutinin which was > 300 kDa and also not labeled with [^{3}H]palmitate or [^{3}H]inositol. β-1,6 glucan antibodies reacted with cell wall α-agglutinin but not with plasma membrane or periplasmic forms, indicating that cell wall α-agglutinin was bound to β-1,6 glucan. In addition, α-agglutinin was released from the cell wall by β-1,3 glucanase but not by SDS, indicating that the binding was covalent (Lu *et al.*, 1994).

C. Cleavage of GPI anchored α-agglutinin at the plasma membrane

Proteins such as α-agglutinin, destined to be attached to a GPI anchor, contain an N-terminal signal sequence which is cleaved after translocation to the ER and a C-terminal region which anchors the protein to the ER and contains a GPI-signal sequence (Gaynor *et al.*, 1999). The GPI-signal sequence contains a GPI attachment region comprising an omega site (the site of cleavage), an omega+1, and omega+2 sites, a spacer domain of approximately 8-12 amino acids and a terminal hydrophobic domain of at least 11 amino acids (Hamada *et al.*, 1997). In the ER the GPI-signal is cleaved at the omega site and the resulting carboxy

terminal becomes covalently bound to the GPI moiety. The GPI-attached proteins are then transported to the cell surface.

Some of the GPI-anchored proteins, such as Yap3, remain at the plasma membrane, and others, such as α-agglutinin, are further processed and are incorporated into the cell wall (Hamada *et al.*, 1998, Hamada *et al.*, 1999). Only the amino acid sequences in the short omega-minus region were found to be significant in determining the final location of GPI-anchored proteins (Table 1, Figure 2). The amino acids Val or Ile at the omega - 4/5 site and Val, Ile, Asn, or Tyr at the omega - 2 site are thought necessary for GPI-attached proteins to be efficiently incorporated into the cell wall. This is known as the omega - 4/5 and omega - 2 rule. This rule was demonstrated in Yap3p, when the omega-minus region was mutated to sequences containing Val or Ile at the omega - 4 or - 5 site and Val or Tyr at the omega - 2 site, the fusion protein became localized in the cell wall. It is thought possible that the dibasic residues, Lys and Arg at the omega - 2 and omega -1 sites may act negatively resulting in a protein not being incorporated into the cell wall. A GPI-anchored protein containing the dibasic motif will then remain on the plasma membrane (Hamada *et al.*, 1998). By default, other GPI-proteins are cleaved at the plasma membrane and are incorporated into the cell wall by covalently binding to β-1,6 glucan.

Table 1. *S. cerevisiae* GPI-proteins. The expected omega (Ω) site, Ω-2 and Ω-5 sites are indicated by bold letters

Protein	location	GPI-signal
		Ω-5 Ω-2 Ω ↓ ↓ ↓
Yap3	cell membrane	TASATSTSS**K**R**N**VGDHIVPSLPLTLISLLFAFI
Agα1	cell wall	TSTSLMIST**Y**EGKASIFFSAELGSIIFLLLSYLLF

(Caro *et al.*, 1997)

V. The synthesis of β-1,6 glucan and the role of *KRE* genes

KRE genes are involved in the synthesis of β-1.6 glucans. These genes have been identified through resistance of *kre* mutants to the K1 killer toxin which binds to cell wall β-1,6 glucan (Brown *et al.*, 1993; Shahinian and Bussey, 2000). Mutants with defects in the cell wall toxin receptor are toxin-resistant. Based on their killer resistant (*KRE*) phenotype, a number of yeast genes involved in glucan synthesis have been identified (Boone *et al.*, 1990; Meaden *et al.*, 1990). A functional analysis of the killer resistant genes thus defined a hypothetical pathway for the assembly of β-1.6 glucans (Bussey, 1991). The *KRE* gene products have been localized to the endoplasmic reticulum, the Golgi, the cytoplasm , and the cell surface. These results have suggested that β-1,6 glucan synthesis occurs via a series of intracellular events along the secretory pathway, and is completed at the cell surface (Shahinian and Bussey, 2000).

KRE5 codes for an ER protein involved in the synthesis of β-1,6 glucan (Brown *et al.*, 1993; Shahinian and Bussey, 2000). *kre5* mutants demonstrated a severe deficiency in the synthesis of β-1,6 glucan and secreted most of their α-agglutinin into the growth medium (Lu *et al.*, 1995).

KRE6 codes for a protein located in the Golgi and is required for the synthesis of β-1,6 glucans in the cell wall. *kre6* disruptants were defective in β-1,6 glucan, had an aberrant cell morphology (Roemer and Bussey, 1991) and were lacking in a dense mannoprotein layer (Roemer *et al.*, 1994). It is thought that Kre6p might be responsible for an intermediate step in the synthesis of β-1,6 glucan (Shahinian and Bussey, 2000).

KRE11 codes for a cytoplasmic protein (Shahinian and Bussey, 2000) and disruption in the *KRE11* locus resulted in a 50% reduction in the level of β-1.6 glucan in the cell wall (Brown *et al.*, 1993). Kre11p has been identified as a component of TRAPP, a cis-Golgi complex required for the docking of ER-derived transport vesicles. This suggested that *KRE11* might play a role in transport of β-1,6 glucan through the secretory pathway (Sacher *et al.*, 2000).

KRE1 codes for a cell surface protein required for the production of β-1.6 glucan (Roemer and Bussey, 1995). A genomic analysis predicted that Kre1p was a plasma membrane GPI-anchored protein (Caro *et al.*, 1997). This was in agreement with the results of immunofluorescence which localized Kre1p to the cell surface (Roemer and Bussey, 1995).

kre9 disruptants are defective in β-1,6 glucan (Brown *et al.*, 1993) and α-agglutinin cell wall anchorage (Lu *et al.*, 1995). *kre9* disruptants are multiply budded and unable to form a mating projection (Brown *et al.*, 1993). The defects in growth in β-1.6 glucan and anchorage of cell wall proteins together with a cell surface location imply that Kre9p

functions late in the secretory pathway of β-1,6 glucan synthesis (Shahinian and Bussey, 2000).

β-1,6 glucan is a component of the cell wall, and its synthesis is dependent on the secretory pathway. Newly formed cell walls require the synthesis and cross-linking of glucan polymers, mannoproteins and chitin. The biosynthesis of β-1,3 glucan and chitin which occur at the cell surface require secreted enzymes. Mannoproteins and possibly β-1,6 glucan are dependent on the secretory pathway to reach the cell surface and become integrated into the cell wall. In order for these events to occur the cell has developed a complex secretory system.

VI. Transport from the endoplasmic reticulum to the cell wall

Secretion of proteins from the ER to the exterior of the cell involves the budding of vesicles carrying the protein cargo from a donor organelle and delivery of cargo to an acceptor or target organelle. Interaction of the transport vesicle with its target organelle is initiated by recognition, followed by tethering of the vesicle to the target. Subsequent fusion of the donor and acceptor membrane bilayers allows the translocation of the protein cargo from the vesicle to the target organelle (Grote *et al.*, 2000; Schekman and Orci, 1996).

A. COPI and COPII vesicles in ER to Golgi transport

The formation of vesicles is initiated by cytoplasmic (coat) proteins, recruited to the membrane by small GTP-binding proteins (Gaynor and Emr, 1997). Transmembrane proteins act as receptors for the cytosolic coat proteins and are thought to play a role in linking coat

proteins with cargo (Wieland and Harter, 1999). There are three classes of coat proteins that are well characterized: clathrin, coatomer or COPI, and COPII (Gaynor and Emr, 1997). Clathrin coated vesicles are involved in endocytosis and vesicular interaction between endosomes and the trans-Golgi network.

The vesicles involved in anterograde transport from the ER to the Golgi are coated with COPII (coat protein complex II) proteins. The COPII coat is composed of the small GTPase Sar1p and the heterodimeric protein complexes Sec23/Sec24p and Sec13/Sec31p. These five proteins are required for the formation of COPII vesicles from ER microsomes (Barlowe *et al.*, 1994; Springer *et al.*, 1999).

COPI coated vesicles play an important role in retrograde traffic, from the Golgi to the ER (Klumperman, 2000). The formation of COPI coated vesicles involves interaction with a membrane bound GTPase, ARF1 (ADP-ribosylation factor1)-GTP and with a complex of seven pre-assembled cytosolic subunits, (α, β, β', γ, δ, ε, ζ) called coatomer. ARF1 activates the binding of coatomer to Golgi enriched membranes in the formation of coatomer coated COPI vesicles. (Gaynor *et al.*, 1998).

B. SNAREs: Function in protein transport

Fusion between transport vesicle and receptor organelle membranes requires SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). SNAREs are integral membrane proteins located on transport vesicles (v-SNAREs) and on target membranes (t-SNAREs). The association between SNAREs forms a bridge between the two membrane bilayers leading to a very stable cis-SNARE complex (complex formed when the

vesicle SNARE [v-SNARE] and target membrane SNARE [t-SNARE] combine in the same membrane) (Weber *et al.*, 2000). After membrane fusion, the binding of v-SNARE and t-SNARE is disrupted by the ATPase NSF/Sec18p (N-ethylmeleimide sensitive factor) and its cofactor α-SNAP/Sec17p (soluble NSF attachment protein), to allow the recycling of SNAREs for another round of transport (Weber *et al.*, 2000; Katz and Brennwald, 2000).

1. Specificity in intracellular transport

The initial SNARE hypothesis was based on the belief that specificity in intracellular transport depended on the interaction of v-SNAREs and cognate t-SNAREs (Rothman and Warren, 1994). This concept was later modified to one in which specificity is conferred at the targeting and tethering stage (Chen and Scheller, 2001). Vesicles are thought to be captured and tethered by proteins emanating from the target membrane (Pfeffer, 1999). Small GTPases, Rab/Ypt1, found on distinct cellular compartments are believed to play a role in the determination of transport specificity. SNAREs might then be involved in membrane fusion after the initial vesicle docking mediated by Rab GTPases.

2. The four-helix model for SNARE complexes

The concept of the helical structure of SNAREs, suggested by Gerst (1997) was further developed into a model of a four-helix bundle consisting of three transport vesicle membrane anchored v-SNAREs combining with their cognate t-SNARE on the target membrane. The SNAREs comprising the four-helix bundle fall into four classes (Table 2): Syntaxins, Bet1p, Bos1p, and R-SNAREs. The SNARE complexes appear to

require one helix from each group. The designation of R-SNARE is based on the crystal structure of the SNARE complex which revealed a unique ionic layer in the hydrophobic core of the complex in which one SNARE contributed a conserved arginine, and other SNAREs contributed a glutamine. An exception to this was Bet1p which contributed a serine. (Fasshauer *et al.*, 1998; Katz and Brennwald, 2000; Sutton *et al.*, 1998). The term R-SNARE is used here (Table 2) to designate that class which contributes an arginine to the helix bundle.

Some v-SNAREs appear to be associated with only one syntaxin, whereas others can interact with more than one syntaxin (Pelham, 2001). In a SNARE complex, the t-SNARE Sed5p together with v-SNAREs Bos1p and Sec22p were shown to interact only with Bet1p, and not with any others from the same class such as Sft1p and Tig1p. Sed5p, however, interacts with both Sft1p and Tig1p in a later compartment. This would indicate that Sed5p interacts with multiple specificity and that other proteins are required for specificity in vesicle docking.

3. SNARE-mediated membrane fusion

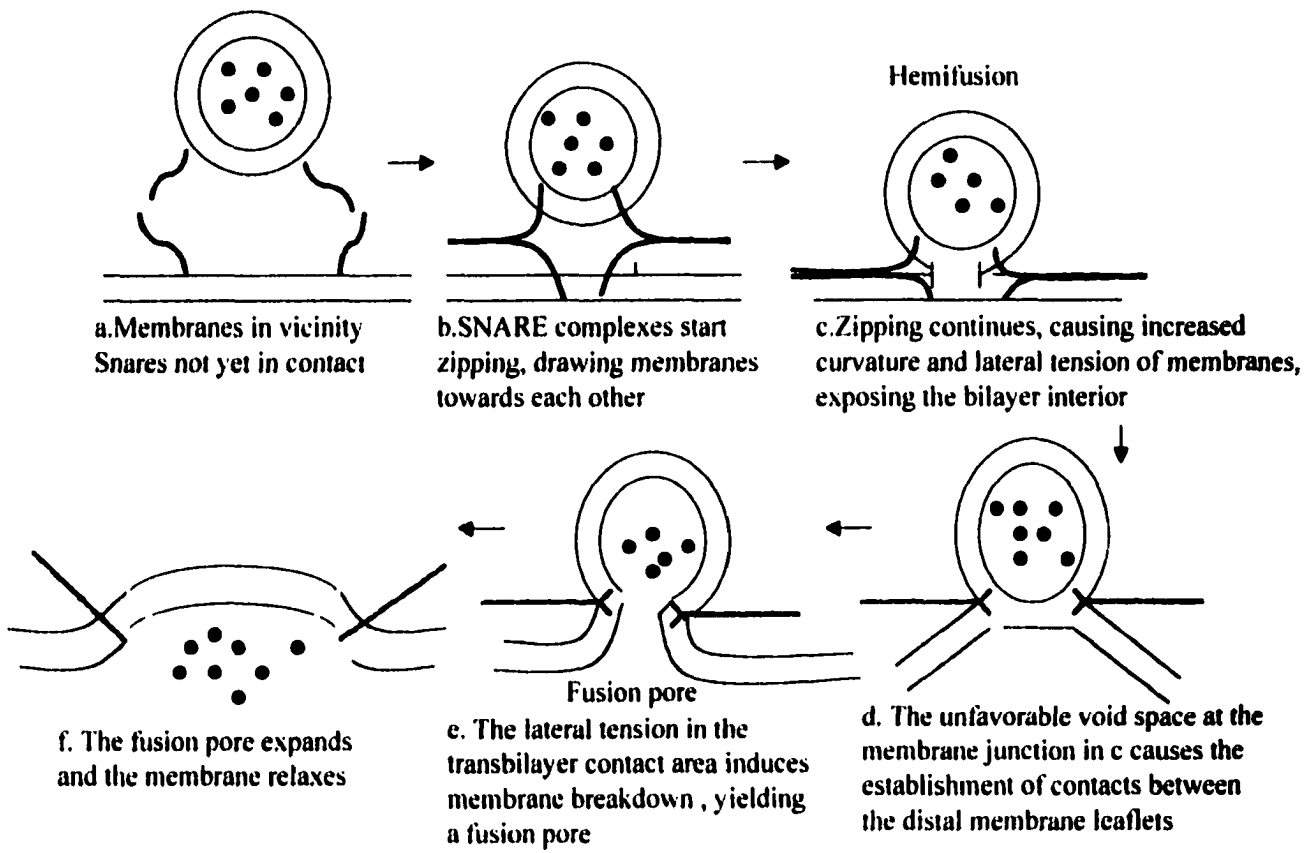
In order to achieve fusion between the vesicle and target membrane lipid bilayers, repulsive ionic and hydration forces between the two bilayers must be overcome. A 'zipper' model of bilayer fusion postulates that SNAREs form a stable complex by zipping from the membrane-distal amino terminal of the vesicle to the membrane-proximal carboxyl terminal of the target membrane (Figure 4 a,b) (Chen, 2001). The 'zipper' model would then overcome the energy barrier and drive the fusion of the lipid bilayers. In this model fusion occurs (Figure 4 c,d) when the distance between the two bilayers is sufficiently reduced.

Table 2. SNARE classes comprising four-helix model

t-SNAREs	v-SNAREs		
1.Syntaxins (location)	2. Bet1p	3. Bos1p	4. R-SNAREs
Sed5p (early golgi)	**Bet1p**	Bos1p	Sec22p
Tig2p (late golgi/early endosome)	Sft1p	Gos1p	Ykt6p
Pep12p (late endosome)	Tig1p	Sec20p	

(Pelham, 2001)

Figure 4. Model of SNARE-mediated lipid fusion



(Chen *et al.*, 2001)

This step is followed by the formation of a fusion pore (Figure 4 e) which then expands releasing the vesicle contents (Figure 4 f).

VII. *BET1* codes for a v-SNARE involved in ER to Golgi Transport

A. Bet1p, a v-SNARE, defined

Bet1p is a v-SNARE located on vesicles involved in transport between the ER and the Golgi in *S. cerevisiae* (Stone *et al.*, 1997). Bet1p interacts with v-SNAREs Bos1p and Sec22p on ER-derived vesicles, transporting protein cargo to the Golgi, where the v-SNAREs bind to Sed5p, a t-SNARE, on the Golgi (Newman *et al.*, 1990; Lian and Ferro-Novick, 1993; Tsui and Banfield, 2000). The four helix bundle formed by these SNAREs mediates the fusion of the ER derived transport vesicle to the Golgi target membrane (Parlati *et al.*, 2000).

B. *bet1* mutant phenotypes

The *BET1* gene is essential for the viability of the cell. This was shown in a tetrad analysis of a *BET1*::*LEU2* disruption in a *leu2/leu2* diploid strain. The transformed diploid was then sporulated and dissected. The only viable spores were leu$^-$, which demonstrated that disruption of *BET1* was lethal. Additionally, a *bet1* conditional mutant is temperature sensitive for growth and protein secretion (Newman *et al.*, 1990).

C. *bet1* was initially identified in 1987

In order to identify proteins that are involved in export of proteins from the ER Newman and Ferro-Novick (1987) used a technique that involved mutants temperature

sensitive for secretion and cell surface growth which become dense during incubation at 37°C. Sedimentation of the dense cells then allows for selection of secretory mutants (Novick *et al.*, 1980). This technique required that mutants survive at the restrictive temperature for a 3 hour period required for the cells to become dense. Those mutants blocked early in the secretory pathway that did not survive during this time period could thus not be selected. In order to isolate those mutants blocked early in the secretory pathway, Newman and Ferro-Novick (1987) used a [^{3}H] mannose suicide selection (Huffaker and Robbins, 1982; Huffaker and Robbins, 1983). Since secretory proteins are mostly glycosylated in the Golgi, by using [^{3}H] mannose, those mutants would survive that were blocked at the ER and therefore would incorporate less [^{3}H] mannose into their glycoproteins. In that way they isolated the mutant *bet1* (blocked in early transport). Cells surviving the suicide selection were then selected for temperature sensitivity for growth at 37°C and for blocking secretion of the periplasmic mannoprotein invertase. *bet1* mutants were also characterized as blocked in the secretion of α-factor, the mating pheromone and for defective processing of carboxypeptidase Y, the vacuolar protein. At the restrictive temperature, *bet1* cells became dense, as had occurred in previously identified ER mutants (Novick *et al.*, 1980; Ferro-Novick *et al.*, 1984).

D. *BET1* and *BOS1* complement *bet1* mutation, 1990

In screening a yeast library for genes that complement *bet1*, Newman (Newman *et al.*, 1990) isolated two genes, BET1 and BOS1 (Bet One Suppressor). Transformation with

plasmids containing the *BET1* gene resulted in wild-type growth of the *bet1-1* mutant at 37°C. Transformation with *BOS1* resulted in full growth at 36°C but only partial growth at 37°C. It was determined that *BOS1* was not functionally equivalent to *BET1* since overproduction of Bos1p did not compensate for the lethality of disruption of *BET1*.

E. Sec22p interacts with Bos1p and Bet1p, 1990

SEC22 encodes a v-SNARE. Genetic interaction between *BOS1* and *BET1* with *sec22-3* was shown when overproduction of either Bos1p or Bet1p suppressed the growth and secretory defects of *sec22-3*. Thus *sec22-3* was identified as another temperature-sensitive mutant blocked early in the secretory pathway. Additional evidence of genetic interaction was shown by the inviability of a *bet1 sec22* double mutant (Newman *et al.*, 1990).

F. Bos1p, Sec22p, Bet1p (v-SNAREs) interact with Sed5p, 1994

Sed5p was found to be required for docking/fusion of ER-derived vesicles with Golgi in vivo, and physically localized to the cis side of the Golgi (Hardwick and Pelham, 1992; Banfield *et al.*,1995). When spheroplasts of *sec18* mutant cells were incubated at the restrictive temperature of 37°C, Bos1p, Sec22p, Bet1p, and Sec17p coimmunoprecipitated with antibodies to Sed5p. Thus, Bet1p, Sec22p and Bos1p were likely v-SNAREs specifying attachment of ER-derived vesicles with Golgi, forming a match with a cognate t-SNARE Sed5p (Sogaard *et al.*, 1994).

G. Bet1p may function in both retrograde and anterograde traffic, 2000

In yeast the t-SNARE Sed5p is known to bind to at least 7 SNAREs: Sft1p, Ykt6p. Vti1p, Gos1p, Sec22p, Bos1p, and Bet1p, involved in ER-Golgi and intra-Golgi transport. In a binding assay Tsui and Banfield (2000) showed that SNARE-SNARE combinations could be functionally redundant. Specifically, the over-expression of Bet1p can bypass the requirement for Sft1p, a SNARE required for intra-Golgi retrograde traffic. In this study, Bet1p was shown to share many of the same binding partners as Sft1p such as Gos1p and Ykt6p. Thus it was thought that Bet1p might function either in a parallel pathway to Sft1p or be incorporated into SNARE complexes in place of Sft1p. These results suggested that Bet1p can function both in anterograde traffic between the ER and the Golgi and in intra-Golgi retrograde traffic (Tsui and Banfield, 2000). In a previous study the requirement for Bet1p in both ER-Golgi and retrograde from the Golgi to the ER was shown (Spang and Schekman, 1998). The various combinations SNAREs formed with Sed5p and the presence of SNAREs, such as Bet1p, in more than one trafficking location suggested that specificity in vesicle fusion could not be explained only on the basis of SNARE-SNARE interaction (Tsui and Banfield, 2000).

VIII. Specificity of target membrane

A. *BET3*, a new gene, interacts with *BET1*

The finding that the interaction of SNAREs was not adequate to determine target specificity led to a search for other components involved in ER to Golgi transport. In an attempt to find genes whose products might interact with *BET1*, Rossi (Rossi *et al.*, 1995)

identified *BET3* in a synthetic lethal screen with *bet1-1*. *bet1-1* cells that harbored *BET1* on a *CEN* plasmid (pAN102) were subject to ethylmethanesulfonate mutagenesis. Mutants were selected for inability to grow at 37°C and for defective secretion of invertase. These mutants were mated to wild-type cells and the plasmid pAN102 containing *BET1* was displaced. The diploids were sporulated and during tetrad analysis the *bet1-1* mutation was separated from the newly identified temperature sensitive mutation. Only one mutant was found to be defective in a gene that is lethal in combination with *bet1-1*. To determine if the defect in this mutant strain was due to one mutation, the strain was crossed to the wild-type, sporulated and subject to tetrad analysis. 50 tetrads were examined and the growth defect segregated 2:2 in all cases indicating that it was due to a single mutation. To demonstrate that the new mutation was lethal in combination with *bet1-1*, colonies were crossed to *bet1-1* and tetrad analysis was performed. 12 tetrads were examined and in each case the double mutants were inviable. The new mutant was called *bet3-1* (Rossi *et al.*, 1995).

As in *bet1*, at 37°C *bet3-1* failed to transport invertase, α-factor, and carboxypeptidase Y (CPY) from the ER to the Golgi (Rossi *et al.*, 1995). *bet3-1* was also found to genetically interact with *BOS1*, *SEC21*, *SEC22*, and *YPT1* since over-production of any of these proteins resulted in *bet3-1* growing at the previously restrictive temperature of 30°C. Despite this interaction, Bet3p was not physically associated with the SNARE complex. This was shown when a detergent extract of *sec18* mutant cells (in which SNAREs accumulate) was precipitated with antibody to Bos1p. Sec22p and Sed5p components of the SNARE complex, were co-precipitated but Bet3p was not co-precipitated (Rossi *et al.*, 1995).

B. TRAPP (transport protein particle)

1. Bet3p is part of a large complex: TRAPP

Although Bet3p was shown to interact with the v-SNARE Bet1p, and was not part of the SNARE complex, it was subsequently shown to be part of a larger complex called TRAPP. In order to determine the function of Bet3p, Sacher (Sacher *et al.*, 1998) added three c-myc tags to the C-terminus of Bet3p and precipitated the associated proteins from a radiolabeled lysate with anti-c-myc antibodies. In addition to Bet3p, nine radiolabeled bands were specifically precipitated. There were five low molecular weight polypeptides (18, 20, 23, 31 and 33 kDa) and five high molecular weight bands (65, 85, 105, 120 and 130 kDa). Bet3p was present with the radiolabeled bands in an approximately 800 kDa complex that was given the term TRAPP (transport protein particle) (Sacher *et al.*, 1998). The 18 kDa band was identified as *BET5*, a suppressor of *bet3-1* (Jiang *et al.*,1998). The 65 kDa band was identified as *KRE11*. *KRE11* codes for a protein which when absent results in defects in cell wall β-1,6 glucan (Brown *et al.*, 1993) and defective anchorage of α-agglutinin (Lu *et al.*, 1995). As part of the TRAPP complex, it is thought that Kre11p may play a role in transport of β-1,6 glucan biosynthetic components through the secretory pathway (Shahinian and Bussey, 2000). The TRAPP complex was found to be exclusively localized on the Golgi (Barrowman *et al.*, 2000).

2. TRAPP plays a role in vesicle tethering

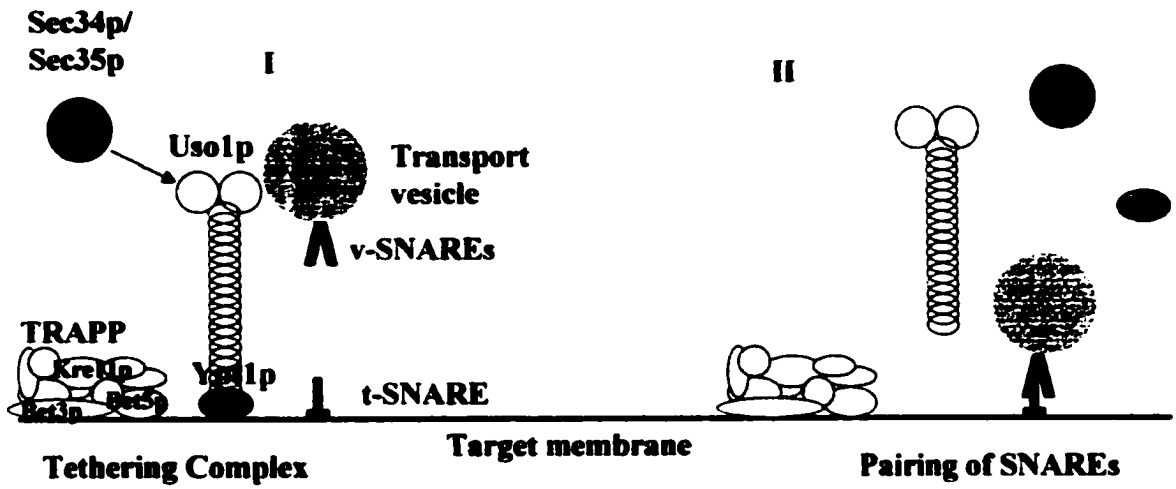
The TRAPP multi-subunit complex plays a role in tethering, the initial attachment of ER-derived transport vesicle to Golgi membranes prior to pairing of SNAREs (Lowe, 2000).

In addition to TRAPP, the other factors that play a role in the tethering process include Ypt1p, Uso1p, and the Sec34p-Sec35p complex. Ypt1p is a member of the Rab family of small GTPases required in vesicle docking. Uso1p interacts with Ypt1p and is largely cytosolic, in contrast to TRAPP which is located on the Golgi. The depletion of either Sec34p or Sec35p prevented the tethering of ER vesicles in an in vitro assay (VanRheenen *et al.*, 1998; VanRheenen *et al.*, 1999).

The idea that TRAPP might determine the site of delivery of ER-derived transport vesicles resulted when the depletion of TRAPP component Bet3p prevented the attachment and fusion with Golgi membranes of ER-derived vesicles (Lowe, 2000). In a model of ER to Golgi transport proposed by Lowe (Figure 5), tethering was thought of as establishing the site of vesicle delivery, followed by the recruitment of components required for vesicle capture. A bridge would then be formed between the transport vesicle and the target membrane by linkage between Uso1p and Ypt1p. A conformational change might then occur which would bring the v-SNAREs on the transport vesicle into proximity with the t-SNARE on the target membrane. Three v-SNAREs and one t-SNARE would form a four helix coiled-coil bundle, mediating the fusion of the respective vesicle and target membrane bilayers and the subsequent translocation of protein cargo (Lowe, 2000).

An indication that the COP II coat had to be removed for TRAPP to bind to COP II vesicles was shown in an experiment by Barlowe (Barlowe *et al.*, 1994). GTP is known to

Figure 5. Model for transport vesicle tethering complex and pairing of SNAREs



Model shows: I. Assembly and binding of tethering complex, followed by II. The pairing of v- and t-SNARES

Tethering complex	Function
Sec34p/Sec35	Unclear function. Depletion prevents tethering
Uso1p	Cytosolic coiled-coil tethering protein recruited to membrane in a Rab dependent manner. Acts in conjunction with other tethering complexes
TRAPP	Peripheral membrane protein complex associated with Golgi. Thought to determine site of delivery of ER-derived transport vesicles. Depletion of Bet3p prevents attachment of transport vesicle
Ypt1p	GTPase of Rab family. Thought to be important in determining transport specificity.
Transport vesicle	Contains cargo to be transported from the ER to the Golgi
v-SNARE	Peripheral membrane protein on transport vesicle. Binds to cognate t-SNARE on target membrane, mediating fusion of membranes
t-SNARE	Peripheral membrane protein on target membrane. Binds to v-SNAREs thus mediating membrane fusionthe fusion of transport vesicle and target membranes.

stabilize the COP II coat. It is thought that the uncoating of COP II vesicles is stimulated by the GTPase Sar1p. In the presence of GTPγS, a nonhydrolyzable analog of GTP, the binding of COPII to TRAPP was greatly reduced, suggesting the uncoating COP II was required for binding TRAPP.

A model of ER to Golgi transport involving COP II and TRAPP is shown in Figure 6. Recognition of the transport vesicle with its target membrane is followed by the binding of the uncoated COP II vesicle to TRAPP as the tether thought to confer specificity. TRAPP binding then activates Ypt1p, converting it from its GDP-bound form to its GTP-bound form, which is the signal that the vesicle has reached its correct acceptor compartment. This would lead to the recruitment of other tethers since the binding of COPII vesicles to TRAPP might otherwise be reversible (Sacher *et al.*, 2001). The later tethering events, the binding of Uso1p, dependent on Ypt1p (Cao *et al.*,1998) and Sec34p/Sec35p (VanRheenen *et al.*, 1998; VanRheenen *et al.*, 1999) might be required for the subsequent pairing of SNAREs which is the last step in COPII vesicle-Golgi docking.

IX. Parallel pathways

In yeast there are two types of vesicle populations leaving the ER, those proteins with a GPI anchor, such as α-agglutinin, and all other secretory proteins. Proteins with a GPI anchor attachment have at least two specific requirements (Sutterlin *et al.*, 1997). First, when there is a mutation in the α-subunit of coatomer (COPI), the transport of GPI-anchored proteins is blocked while no other secretory proteins are affected. Second, ER-to-Golgi transport of the GPI-anchored Gas1p and Yps1p requires ongoing synthesis of sphingoid

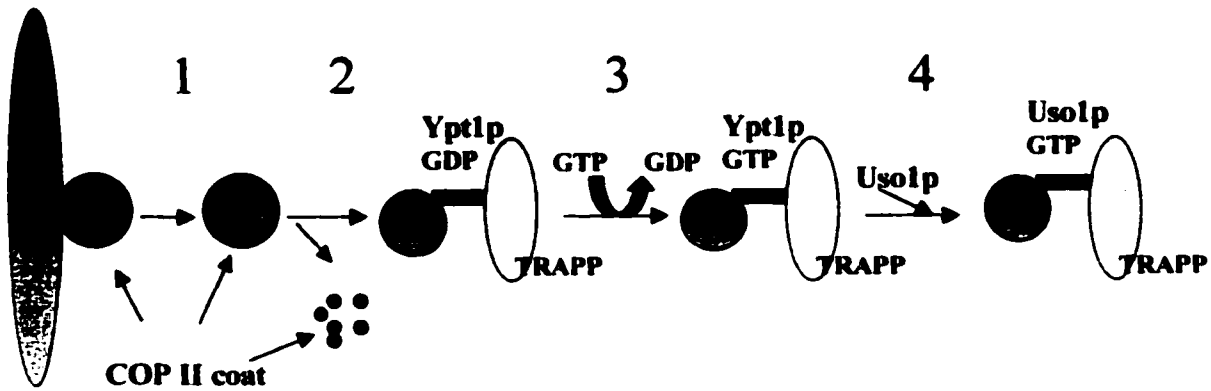


Figure 6. Model of TRAPP I in tethering ER to Golgi vesicles.

1. Cargo contained in COP II coated vesicles buds from the ER; 2. Cop II coat is shed then binds to TRAPP; 3. TRAPP binding activates Ypt1p (bar) converting it from its GDP-bound form to its GTP-bound form thus signaling target specificity; 4. Uso1p and other possible effectors are recruited to aid in vesicle tethering.

From Susan Ferro-Novick (www.med.yale.edu/cellbio/SFN.html)

bases and/or ceramide. Inhibition of ceramide synthesis by the fungal metabolite, myriosin resulted in defective transport of these two GPI anchored proteins while other proteins such as invertase and CPY were not affected (Horvath *et al.*, 1994).

Having specific requirements for transport of GPI anchored proteins suggested the possibility of separate sorting and selection and the existence of two distinct ER-derived vesicles (Muniz *et al.*, 2001; Muniz *et al.*, 2000). In order to test whether GPI-anchored proteins and other secretory proteins were transported in the same or different ER-derived vesicles, the incorporation of cargo proteins into ER-derived vesicles was measured. Since GPI-anchored proteins are lumenal and do not have a cytosolic domain, vesicles containing GPI-anchored proteins could not be directly immunoisolated. Therefore, vesicles carrying transmembrane plasma membrane proteins were immunoisolated and their contents were examined for the co-isolation of GPI-anchored proteins. Gap1p, which does not have a GPI anchor, was tagged with a hemaglutinin (HA) epitope tag on its cytosolic tail and was subjected to immunoisolation using HA antibody. The results were that 61% of Gap1pHA was found in the vesicle pellet after immunoisolation. In contrast, only 6% of the GPI-anchored Gas1p, was detected in the vesicle pellet. This result suggested that Gas1p and Gap1 are not incorporated into the same vesicles. In another experiment, in vitro generated primary ER-derived vesicles were separated by sucrose density-gradient floatation. The different fractions were collected and analyzed by immunoprecipitation. The GPI-anchored proteins Gas1p and Yps1p were found mainly in the top fractions of the gradient. In contrast, the non-GPI-anchored proteins were found in the two bottom fractions of the gradient. This showed that was possible to separate ER-derived vesicle populations based on their densities.

The results were confirmation that GPI-anchored proteins are packaged into ER-derived vesicles that are distinct from and have different physical properties than the vesicle used to package other secretory proteins.

X. Genetic analysis of cell wall mutants

A. Selection of cell wall mutants

1. Conzelmann and Benghezal mutants

The objective of Conzelmann and Benghezal (Benghezal *et al.*, 1995) was to investigate the biosynthesis of the GPI anchor. They mutagenized W303-1B, *MATα* cells with ethylmethanesulfonate and selected those cells in which the cell wall mannoprotein, α-agglutinin was absent. Since α-agglutinin was a GPI anchored protein, its absence from the cell wall was a way to select potential mutants in GPI biosynthesis. The selection of mutants involved several successive screens. The mating pheromone a-factor was used to induce the secretion of α-agglutinin. The cells were then incubated at 37°C for 3 hours followed by incubation with anti-α-agglutinin antibody coated magnetic beads. Non-adherent cells, assumed to be deficient in α-agglutinin expression, were plated and screened for growth at 24°C and no growth at 37°C. To select for those cells in which the secretory process had not been mutagenized the cells were screened for the secretion of acid phosphatase. The screening for acid phosphatase secretion was done by replica plating clones grown at 24°C onto phosphate-free plates which induces the secretion of acid phosphatase by the cells, and incubating the replicas at 37°C for 1-2 days. To select for those cells in which the secretory

process had not been mutagenized, the replica plates were processed for the detection of secreted phosphatase at 37°C and only those that secreted phosphatase were chosen. To isolate those cells deficient in GPI biosynthesis cells were selected for non-incorporation of [^{3}H] into proteins or for accumulated abnormal GPI lipids. Those cells that had not adhered to α-agglutinin antibodies, did not grow at 37°C, had secreted acid phosphatase at 37°C, but did not exhibit deficiency in GPI biosynthesis were kindly donated to the Lipke Lab by Drs. Conzelmann and Benghezal.

Non-adherence of cells to α-agglutinin antibodies is selective for the diminution or absence of α-agglutinin on the cell surface. This suggested a deficiency in the pathway of secretory proteins to the cell surface, or a deficiency in cell wall anchorage of α-agglutinin. Transport to the cell wall requires a number of steps during which a mutation at some point might block α-agglutinin from reaching its destination. Since anchorage to the cell wall requires the availability of cell wall glucans and enzymes required for linkage, a deficiency in anchorage might reveal mutations involving cell wall biosynthesis.

2. Methods of selection of cell wall mutant phenotypes

Elucidation of the genes involved in cell wall synthesis has been approached using gene disruption, then screening for selected phenotypes (Lussier *et al.*, 1997). Another method is using random mutagenesis, screening for selected phenotypes, then determining the gene responsible. One way of detecting and classifying cell wall mutants is based on the calcofluor white effect on cell wall structure. Cells with weakened walls are killed by calcofluor white at a lower concentration than the parental wild-type (Klis, 1994). Based on this initial

identification, it was found that calcofluor white hypersensitivity was correlated with underglucosylated and undermannosylated cell walls (Ram *et al.*,1994). Hypersensitivity to calcofluor white was also associated with an increase in chitin in the cell wall (Roncero and Duran, 1985), the increase found to be a compensatory mechanism to cell wall damage (Valdivieso *et al.*, 2000; Popolo *et al.*, 1997; Kapteyn *et al.*, 1997). Another method used to determine mutations in the structure and composition of the cell wall was to measure cell wall sensitivity to Zymolyase, a β-1,3 glucanase and protease (Mehta and Gregory, 1981). The phenotypes of hypersensitivity to calcofluor white and to Zymolyase have been found to coexist in many mutants (Ram *et al.*, 1994).

3. Zymolyase: a protease and β-1,3 glucanase

In an attempt to find enzymes that would lyse the yeast cell wall, an enzyme mixture able to lyse the cell wall of *S. cerevisiae*, was isolated from the bacterium *Arthrobacter luteus*. (Kitamura *et al.*, 1971; Kitamura *et al.*, 1972). Zymolyase (ICN Biochemical, Inc.), a commercial preparation from the culture of *Arthrobacter luteus*, which lyses viable yeast cells, is a complex of a β-1,3 glucan laminaripentaohydrolase and an alkaline protease. The primary enzyme responsible for lysing yeast cells is the β-1,3 glucan laminaripentaohydrolase which hydrolyzes glucose polymers at the β-1,3 glucan linkages releasing laminaripentaose. In order to determine the effect that glucanase contained in Zymolyase had on intact cells Zlotnik (Zlotnik *et al.*, 1984) separated β-1,3 glucanase (z-glucanase) from the protease (z-protease) by affinity chromatography. The protease-free glucanase had very little effect on lysis of *S. cerevisiae* untreated cells, but after cells were pre-treated with z-protease, the

pentaohydrolase lysed cells rapidly. This result led to the conclusion that mannoproteins had shielded the glucan layer from attack by z-glucanase since protease treatment showed specificity for mannoproteins as substrates. When cells were treated by z-protease there were significant changes in the cell wall. The z-protease removed most of the mannoprotein layer from the cell wall resulting in a loss of the outer electron dense dark layer of the wall (Zlotnik *et al.*, 1984). The protease induced increase in cell wall porosity allowed the accessibility of horseradish peroxidase to the plasma membrane, resulting in cell lysis. These results indicate the role played by the mannoprotein layer in cell wall protection from glucanase penetration.

a. Cell wall sensitivity or resistance to lysis by Zymolyase

The relative sensitivity of the cell wall to lysis by Zymolyase is related to its reaction to both protease and β-1,3 glucanase. A diminished mannoprotein outer layer will be degraded more quickly by protease treatment, allowing faster access by β-1,3 glucanase to the inner glucan layer, and a shorter "lag time" (Ovalle *et al.*, 1999) before the beginning of rapid cell wall lysis. Thus the "lag time" might be indicative of the condition of the outer cell wall layer. If the inner layer is diminished in β-1,3 glucan the cell wall might be lysed more rapidly, resulting in an increased "maximal lysis rate" (MLR) (Ovalle *et al.*, 1999). It has been shown that in *gas1*Δ and *fks1*Δ cells, in which the synthesis and assembly of β-1,3 glucan is defective, there is an increase in chitin and in cell wall proteins cross-linked to chitin via a β-1,6 glucosyl moiety. The consequence of chitin anchorage was an increase in β1,3 glucanase-resistant proteins (Kapteyn *et al.*, 1997; Popolo *et al.*, 1997). In a spheroplast

lysis assay with Zymolyase, resistance to β-1,3 glucanase would be shown by a low "maximal lysis rate." Thus the "maximal lysis rate" would be an indication of the condition and composition of the inner cell wall layer.

b. Correlation of cell wall integrity and growth rate in cell wall mutants

A slow growth rate might be a consequence of a defect in cell wall synthesis and structure affecting synthesis of new walls (Vossen *et al.*, 1997). A slow rate of growth might also be the result of a mutation in the budding process, either of synthesizing new buds or in the septation or separation of new buds. The correlation of a slow growth rate and other manifestations of a mutant phenotype has been shown in several instances. In *Saccharomyces cerevisiae*, *kre6* mutations where cells possess half the level of wild-type cell wall β-1,6 glucan also manifest a slow rate of growth (Roemer *et al.*, 1993). *kre5* mutants, which in *Saccharomyces cerevisiae* has significant homology to *Schizosacchromyces pombe* UDP-glucose glycoprotein glucosyltransferase (GT), mutants lack cell wall β-1,6 glucan and grow very slowly. It has been shown in *Neurospora crassa*, a filamentous fungus, inactivation of the chitin synthase gene, *chs-1* by point mutations resulted in progeny that grew slowly and formed hyphae with morphologic abnormalities (Yarden and Yanofsky, 1991). In the case of *Saccharomyces cerevisiae GNS1*, required for the synthesis of β-1,3-glucan, mutations resulted an 80 to 90% reduction of β-1,3 glucan synthase specific activity. *gns1* mutant strains displayed a pleiotrophic phenotype including slow growth, and mating and sporulation defects (el-Sherbeini and Clemas, 1995). Deletion of *SEC3*, a late-acting secretory gene, required in the fusion of post-Golgi secretory vesicles to the plasma membrane in

Saccharomyces cerevisiae results in slow growth, temperature sensitivity, and defects in bud site selection in homozygous diploids (Finger and Novick, 1997).

XI. Cell wall biogenesis in response to stress

A great many organisms have developed mechanisms to protect themselves against fungal invasion. These mechanisms often target the cell wall of the fungus since it is essential to the cell and sufficiently different from the plant or animal host. As a response, yeast have developed compensatory mechanisms to insure cell wall integrity (Smits *et al.*, 2001).

In response to mutations that weaken the cell wall, or under hypo-osmotic stress several cell wall components are increased. This was shown with *FKS1*, which codes for a subunit of β1,3 glucan synthase (Mazur *et al.*, 1995) and Gas1p, which is involved in remodeling of β-1,3 glucan (Popolo and Vai, 1999). Both fks1Δ and gas1Δ displayed an increase in chitin content. Such changes are thought to be compensatory in order to ensure cell wall integrity.

Under cell wall weakening or stress the cell can switch to alternative mechanisms. When there was a decrease in cell wall β-1,3 glucan, GPI anchored cell wall proteins were linked through β-1,6 glucan binding directly to chitin in place of β-1,3 glucan (Kapteyn *et al.*, 1999a). When β-1,6 glucan was diminished, there was an increase in the number of cell wall proteins that were bound to β-1,3 glucan and to chitin (Kapteyn *et al.*, 1999b).

Defects in the cell wall are sensed in the plasma membrane, possibly in a way that involves membrane stretch (Banuett, 1998). The probable sensors are a set of single-span

integral membrane proteins, Hcs77p, Wsc2p, Wsc3p, Wsc4p and Mid2p (Gray *et al.*, 1997; Ketela *et al.*, 1999). The Wsc proteins are thought to be upstream activators of the stress induced PKC1-MAP kinase cascade and essential for the maintenance of cell wall integrity (Verna and Ballester, 1999). Hcs77p is a putative cell surface sensor involved in signaling cell wall stress during vegetative growth. Mid2p is required for cell integrity signaling in response to pheromone and its primary role is signaling cell wall stress during pheromone induced morphogenesis (Rajavel *et al.*, 1999).

XII. Conclusions

The interdependence of the secretory system and the biosynthesis of the cell wall has been explored and the mechanisms of this interaction have been elucidated. The quest for such knowledge has had significance in the practical realm of yeast pathology and in yeast as a model for the processes of nature. In order to continue in pursuit of such knowledge I have undertaken this research.

My approach was to use mutant cells in which α-agglutinin was absent or diminished on the cell wall. This selection provided cells in which there might be a mutation in the transport and /or anchoring of a cell wall mannoprotein. By complementation of the mutant gene or genes involved in this phenotype mechanisms of how the secretory pathway contributes to cell wall biosynthesis might be revealed. The work presented here shows the results of such a quest.

RESULTS

I. Cell wall mutants

We obtained 37 mutant *MATα* strains from Dr. Conzelmann (Benghezal *et al.*, 1995) which had been selected for the absence of or diminished α-agglutinin on the cell wall. These strains were wild-type for lipid synthesis and secretion of acid phosphates but the defect in cell wall α-agglutinin was of value in investigating secretion and cell wall biosynthesis. Of these strains, I selected 20 which did not agglutinate to a-cells and further tested these strains for the secretory and cell wall related functions. I assayed the strains for level of α-agglutinin secreted into the growth medium. Henceforth I will use the term excreted or excretion to indicate secreted or secretion of α-agglutinin into the growth medium. I also assayed these strains for sensitivity to cell lysis with Zymolyase, relative to the wild-type and for growth rate.

The results, summarized in Table 3, show that there was great variability in phenotypes. Relative to the wild-type, excretion of α-agglutinin varied from greater than, similar to, or less than the wild-type. In all but one strain the doubling time was greater than the wild-type. Ten of the mutant strains were selected for further testing and were initially classified on the basis of level of excretion of α-agglutinin: class I (excretion greater than the wild-type), class II (excretion similar to the wild-type), and class III (excretion less than the wild-type). Most of these strains were more sensitive to Zymolyase than the wild-type. These results demonstrated the manifold phenotypes that can be associated with diminished α-agglutinin on the cell wall.

Table 3. Summary of Mutant Phenotypes

Cell strain	Agglutination to a-cells(a)	Excretion of α-agglutinin(b)	Cell wall lysis by Zymolyase(c)	Doubling time Hours	Doubling time Fold-WT
Wild Type					
W303-1B	+	+	+	2.2	1
Mutants					
Class I (Excretion > WT)					
17	-	++	++	3.6	1.6
48	-	++	++	4.6	2.1
59	-	++	++	2.4	1.1
Class II (Excretion ≈ WT)					
55	-	+	-	2.6	1.2
56	-	+	+	2.4	1.1
70	-	+	++	3.4	1.5
76	-	+	++	4.9	2.2
Class III (Excretion < WT)					
31	-	-	++	4.6	2.1
60	-	-	+	3.8	1.7
75	-	-	++	4.8	2.2
Not classified					
53	-	++	n.d.	4.4	2.0
50	-	+	n.d.	3.0	1.4
64	-	+	n.d	3.7	1.7
81	-	+	n.d	2.0	0.9
83	-	+	n.d	5.4	2.5
54	-	-	n.d	3.3	1.5
62	-	-	n.d.	2.1	1.0
73	-	-	n.d.	3.4	1.5
74	-	-	n.d.	3.5	1.6
78	-	-	n.d.	n.d.	n.d.

(a) Agglutination assays were performed as previously described by Lipke (Lipke *et al.*,1989). Strains were incubated overnight with tester strains, W303-1A. Agglutination was indicated by a lacy pellet and non-agglutination by a compact pellet.
(b) Cells were incubated at 30°C to late exponential phase. The respective supernatants were quantitated for excreted α-agglutinin by ELISA.
(c) Cells were grown at 25°C to mid-exponential phase and the sensitivity or resistance to cell wall lysis was assayed in a spheroplast lysis assay with Zymolyase.

A. Phenotype analysis of strains

There were 10 strains which were further analyzed.

1. Excretion of α-agglutinin (Figures 7a, 7b, 7c 1,2,3)

Because α-agglutinin would be absent from the cell wall if it were excreted into the growth medium, I tested these mutants for excreted α-agglutinin. Growth media were collected and concentrated and α-agglutinin activity was determined, as described in Materials and Methods.

The results of ELISA for excretion of α-agglutinin are summarized in Table 4. These results show that the variability in amount of excreted α-agglutinin ranged from 74 percent to 155 percent of the wild-type. Since all of these strains had the same phenotype of non-agglutination to **a**-cells, the variation in excretion suggests that there are several different mutations represented. Based on the phenotypes shown, mutations might exist in these mutants in the pathways of mating (defect in cell wall α-agglutinin) and of cell wall integrity (inability to anchor α-agglutinin).

2. Sensitivity to cell wall lysis by Zymolyase

The absence of α-agglutinin from the cell wall due to excretion might result if there were an aberrant cell wall structure which prevented anchorage. I therefore tested the mutant strains for cell wall integrity in spheroplast lysis assays with Zymolyase, a mixture of protease and β-1,3 glucanase. Zymolyase lyses yeast in a two step process: first protease degrades the outer mannoprotein layer, then the glucanase lyses the inner

Strain	Slope	R-value	Ratio to W303-1B
AC17	0.0419	0.9868	1.406
AC48	0.0465	0.9954	1.560
AC59	0.0397	0.9894	1.332
W303-1B	0.0298	0.9969	1.0

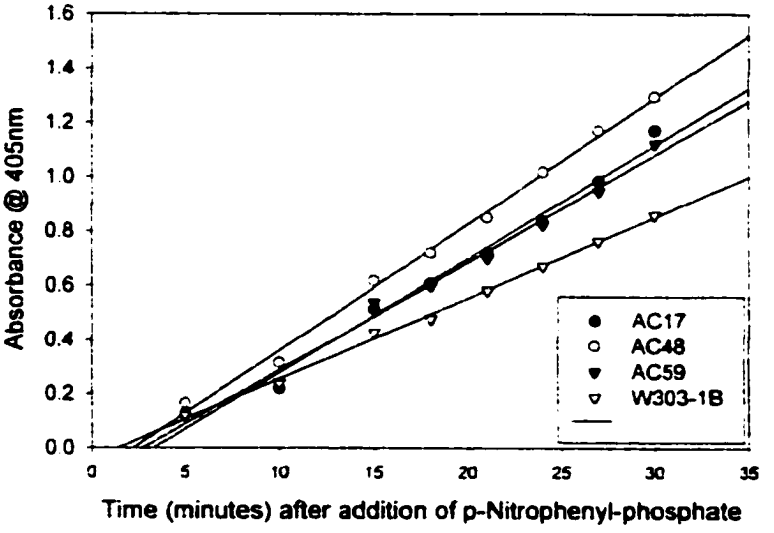


Figure 7a. Results of ELISA for excretion of α-agglutinin by Class I strains: AC17, AC48, AC59, and wild-type W303-1B

Strain	Slope	R-value	Ratio to W303-1B
AC55	0.0356	0.9862	0.9544
AC56	0.0363	0.9891	1.0831
AC70	0.0307	0.9634	0.9732
AC76	0.0402	0.9849	1.0777
W303-1B	0.0373	0.9826	1.0

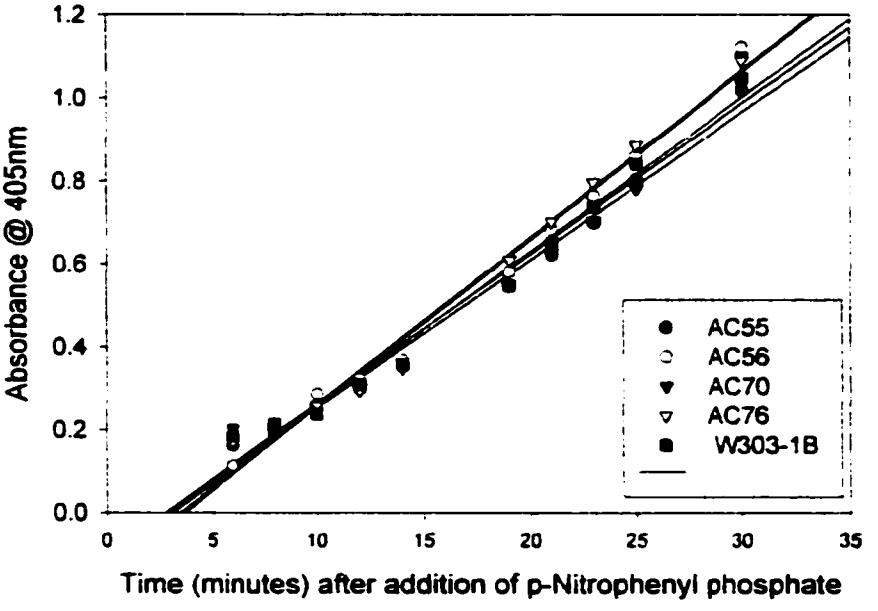


Figure 7b. Results of ELISA for excretion of α-agglutinin by Class II strains: AC55, AC45, AC70, AC76, and wild-type W303-1B.

Strain	Slope	R-value	Ratio to W303-1B
AC31	0.0229	0.9851	0.8500
W303-1B	0.0269	0.9896	1.0

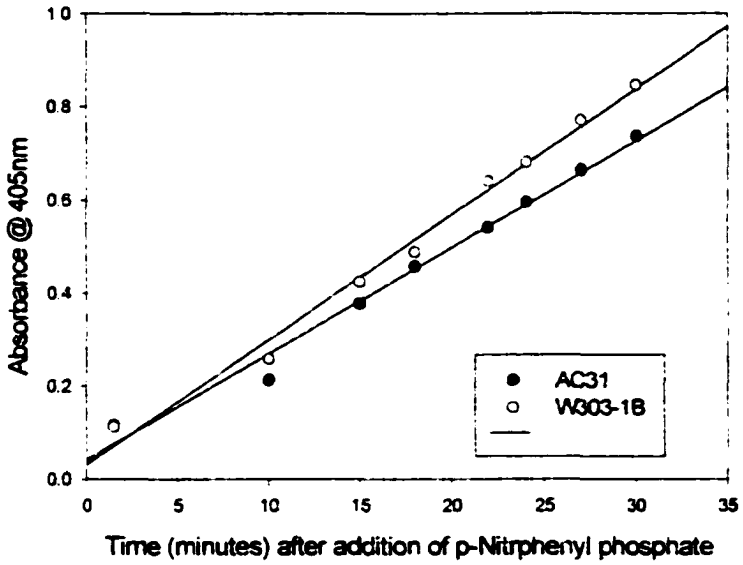


Figure 7c (1). Results of ELISA for excretion of α-agglutinin by Class III strain AC31and wild-type W303-1B.

Strain	Slope	R-value	Ratio to W303-1B
AC60	0.0199	0.9538	0.7463
W303-1B	0.0267	0.9885	1.0

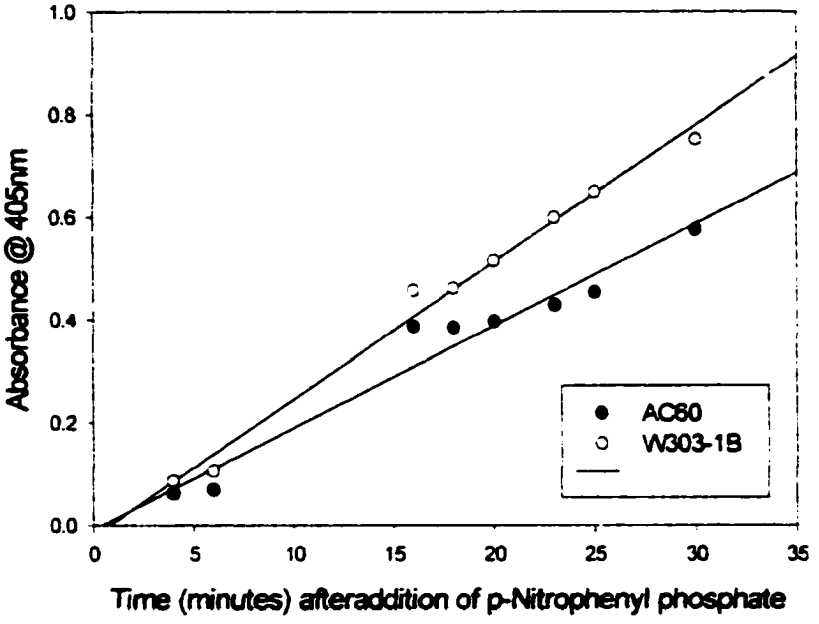


Figure 7c (2). Results of ELISA for excretion of α-agglutinin by Class III strain AC60 and wild-type W303-1B.

Strain	Slope	R-value	Ratio to W303-1B
AC75	0.0307	0.9799	0.8231
W303-1B	0.0373	0.9826	1.0

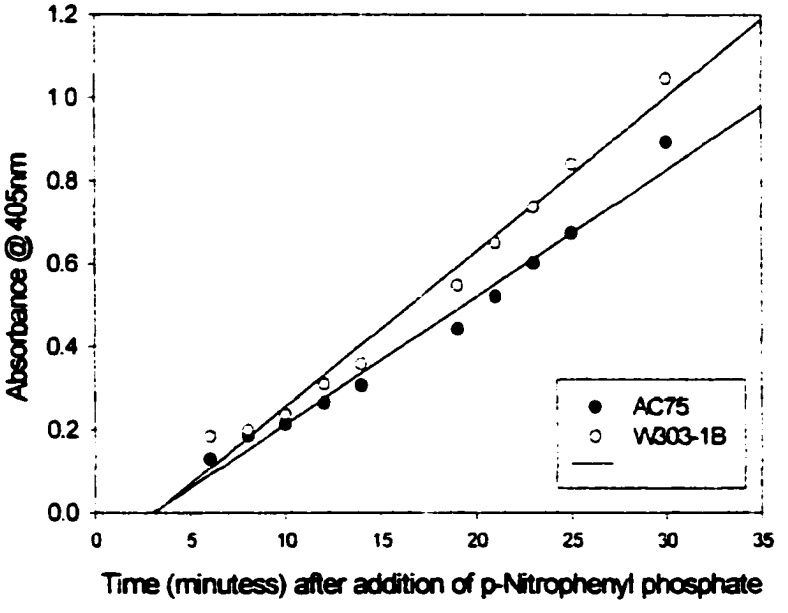


Figure 7c (3). Results of ELISA for excretion of α-agglutinin by Class III strain AC75 and wild-type W303-1B.

Table 4.

Excretion of α-agglutinin
Regression analysis of ELISA
Absorbance @ 405 nm

Strain	Number of Experiments	Rate of Increase in Absorbance/min @ 405 nm	Standard Deviation	Ratio of Excretion to W303-1B
W303-1B (wild-type)	5	0.031	0.004	
Class I (Excretion > WT)				
AC17	3	0.045	0.000	1.45
AC48	3	0.047	0.003	1.55
AC59	1	0.038	n.a.	1.29
Class II (Excretion = WT)				
AC55	1	0.036	n.a.	0.95
AC56	1	0.040	n.a.	1.08
AC70	1	0.036	n.a.	1.09
AC76	1	0.040	n.a.	1.08
Class III (Excretion < WT)				
AC31	1	0.023	n.a.	0.85
AC60	1	0.020	n.a.	0.74
AC75	1	0.031	n.a.	0.82

glucan layer (Zlotnik, 1984; Ovalle *et al*, 1998). In these assays the "lag time" is the time before the start of the most rapid lysis. The lag time was an indicator of the condition and thickness of the outer layer. Subsequent to the lag time, the rapidity with which β-1,3 glucanase lysed the inner glucan layer was called the "maximal lysis rate" (MLR) and was an indicator of density and/or composition of the inner layer.

a. Class I (Excretion > WT) AC17, AC48, AC59 (Figures 8a 1,2,3)

For all strains in class I the maximal lysis rate was more than double that of the wild-type, and there was also a shorter lag time. The shorter lag time suggested an outer mannoprotein layer showing reduced resistance to degradation by the protease in Zymolyase. The greater maximal lysis rate suggested that the inner glucan layer was more quickly lysed by the β-1,3 glucanase in Zymolyase.

b. Class II (Excretion ≈ WT) AC55, AC56, AC70, AC76 (Figures 8b 1,2,3,4)

The results of the spheroplast lysis assay further subdivided this class into categories based on either sensitivity or resistance to cell lysis. Both strains AC55 and AC56 were lysed more slowly than the wild-type. In AC55 the lag time was almost four-fold that of the wild-type, and in AC56 the lag time was almost twice that of the wild-type, showing. These lag times showed resistance of the outer mannoprotein layer of the cell wall to protease digestion. The relatively slow lysis rate showed resistance to lysis of the inner glucan layer by β-1,3 glucanase.

Strains AC70 and AC76 were lysed more rapidly and had a shorter lag time than

W303-1B. The shorter lag time and relatively more rapid lysis rate were indicative of sensitivity of the mannoprotein layer to protease and the glucan layer to β-1,3 glucanase.

c. Class III (Excretion < WT) AC31, AC60, AC75 (Figures 8c 1,2,3)

In class III each of the strains was unique. In AC31 there was a long lag time, which was four-fold that of the wild-type, and rapid cell lysis which was twice the rate of the wild-type. These results show resistance to protease of the outer layer of the cell wall and sensitivity to lysis of the inner glucan layer. In AC60 there was a short lag time, which was half that of the wild-type and maximal lysis rate which was relatively slower than the wild-type. This type of cell wall indicates sensitivity of the outer mannoprotein layer to protease digestion and resistance of the inner layer to lysis by β-1,3 glucanase. Strain AC75 had a shorter lag time and more rapid rate of cell lysis than the wild-type. This was indicative of the sensitivity to protease digestion of the mannoprotein layer and sensitivity of the inner glucan layer to β-1,3 glucanase lysis.

d. Cell lysis with β-1,3 glucanase only (Figure 8d)

In order to determine the condition of the outer mannoprotein layer in the class I strains, AC17, AC48, and AC59, I assayed the sensitivity of these strains to cell lysis with β-1,3 glucanase only, in the absence of protease. As can be seen in Table 5 most of the cells in strains AC48, AC59, and wild-type W303-1B remained intact after 11 hours of incubation. In AC17, only 44.6 percent of the cells remained intact after 11 hours of

Spheroplast Lysis Assay for AC17

	Maximal Lysis Rate (MLR)	Lag time (minutes)
AC17	0.0295	6.21
W303-1B	0.0126	10.89

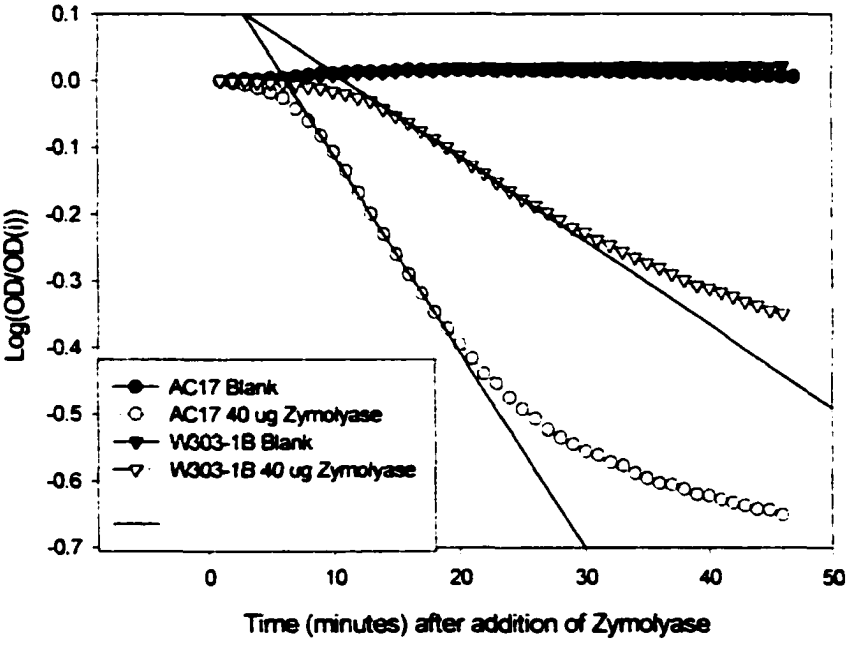


Figure 8a (1). Zymolyase-induced spheroplast lysis of class I strain AC17 and wild-type W303-1B

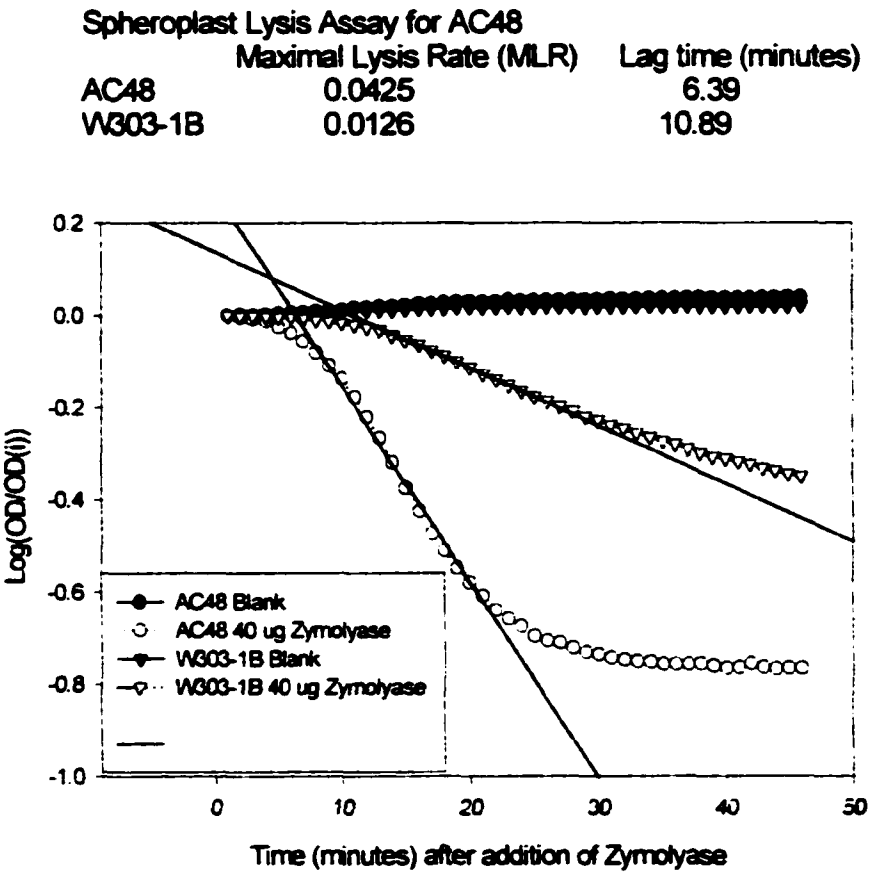


Figure 8a (2). Zymolyase-induced spheroplast lysis of class I strain AC48 and wild-type W303-1B

Spheroplast Lysis Assay for AC59

	Maximal Lysis Rate (MLR)	Lag Time (minutes)
AC59	0.0293	4.83
W303-1B	0.0131	9.07

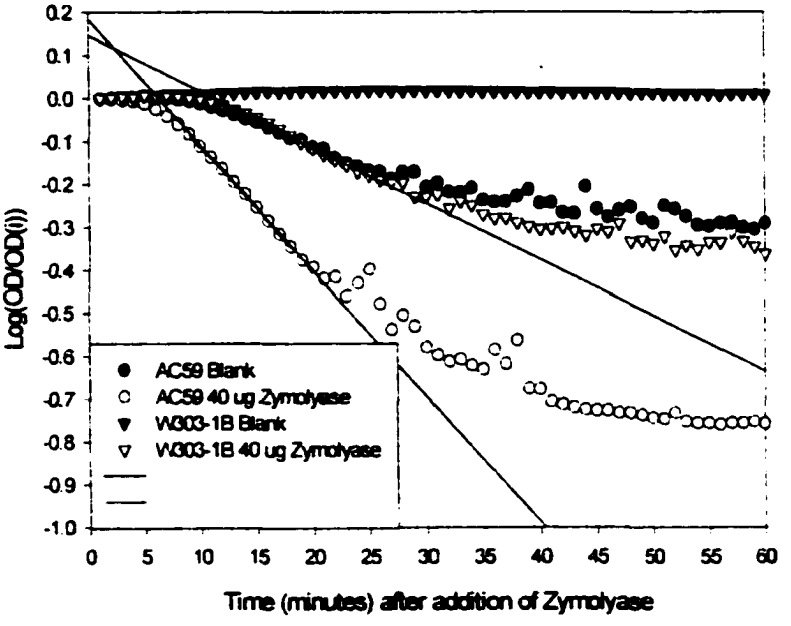


Figure 8a (3). Zymolyase-induced spheroplast lysis of class I strain AC59 and wild-type W303-1B

Spheroplast Lysis Assay for AC55

	Maximal Lysis Rate (MLR)	Lag time (minutes)
AC55	0.0033	31.54
W303-1B	0.0052	8.24

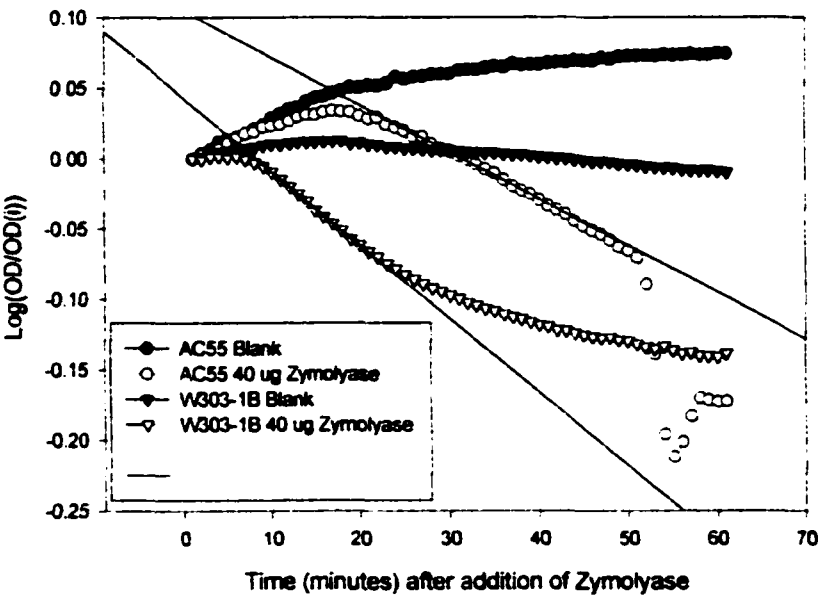


Figure 8b (1). Zymolyase-induced spheroplast lysis of class II strain AC55 and wild-type W303-1B.

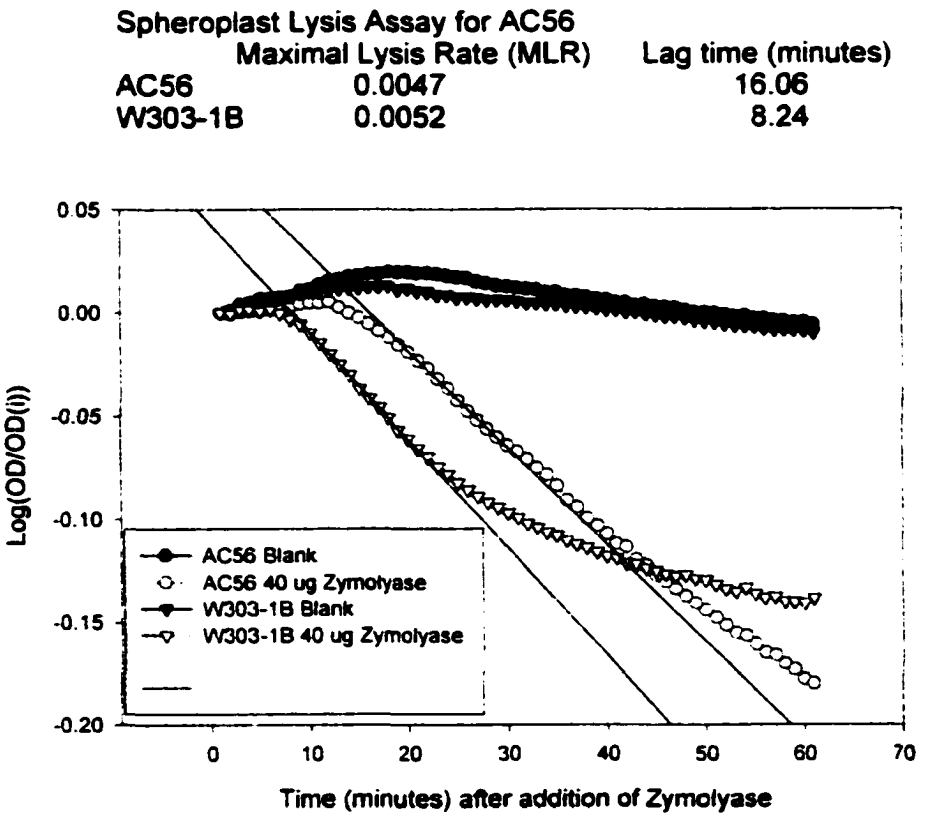


Figure 8 b (2). Zymolyase-induced spheroplast lysis of class II strain AC56 and wild-type W303-1B.

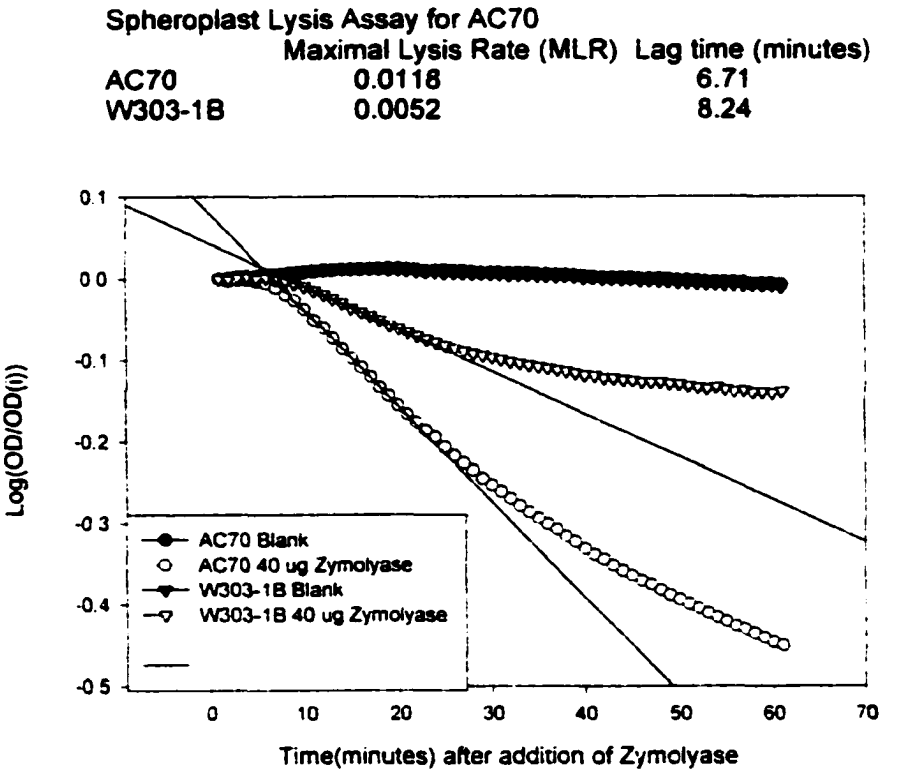


Figure 8b (3). Zymolyase-induced spheroplast lysis of class II strain AC70 and wild-type W303-1B.

Spheroplast Lysis Assay for AC76

	Maximal Lysis Rate (MLR)	Lag time (minutes)
AC76	0.0202	6.40
W303-1B	0.0052	8.13

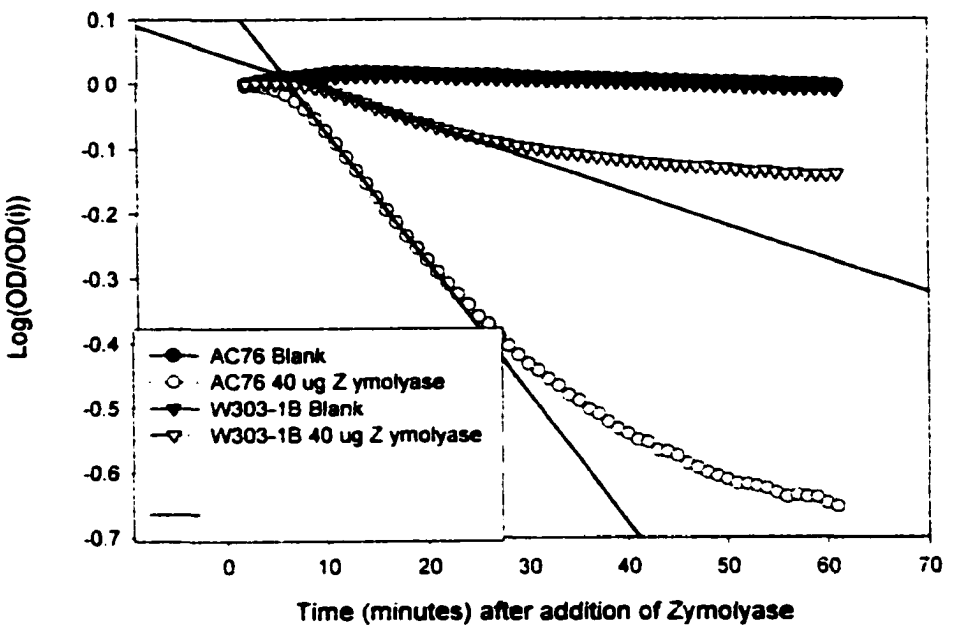


Figure 8b (4). Zymolyase-induced spheroplast lysis of class II strain AC76 and wild-type W303-1B.

Spheroplast Lysis Assay for AC31

	Maximal Lysis Rate (MLR)	Lag Time (minutes)
AC31	0.0121	33.83
W303-1B	0.0052	8.20

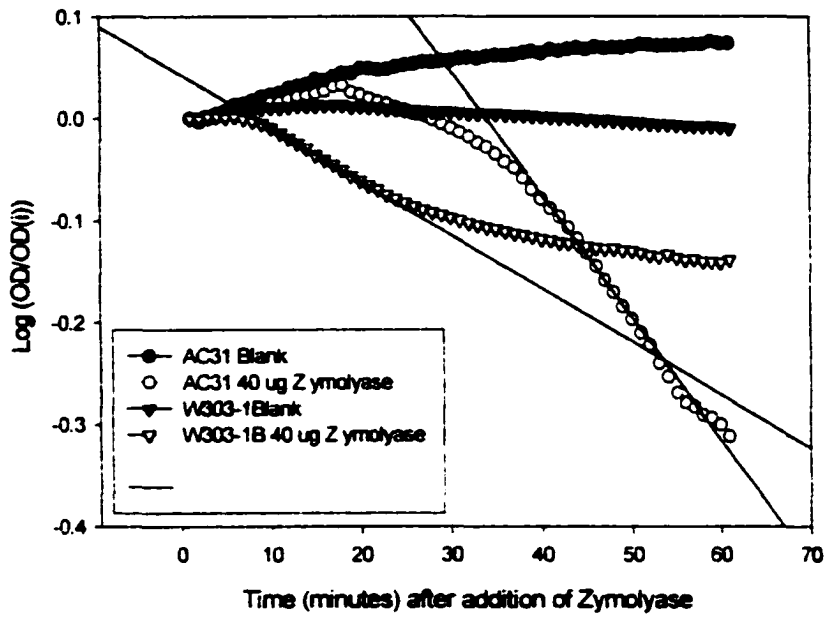


Figure 8c (1). Zymolyase-induced spheroplast lysis of class III strain AC31 and wild-type W303-1B

Spheroplast Lysis Assay for AC60

	Maximal Lysis Rate (MLR)	Lag time (minutes)
AC60	0.0118	5.64
W303-1B	0.0134	11.48

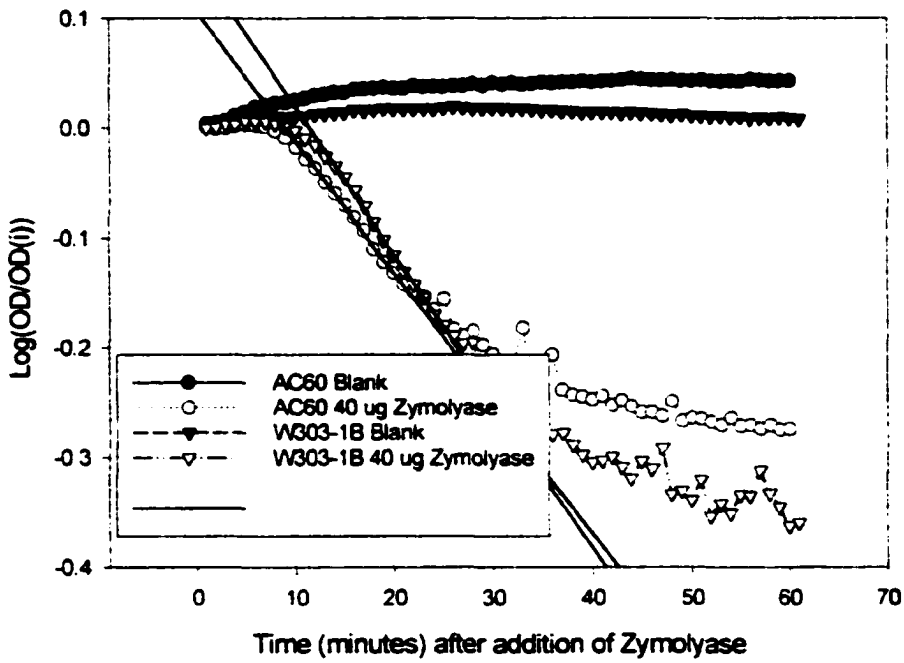


Figure 8c (2). Zymolyase-induced spheroplast lysis of class III strain AC60 and wild-type W303-1B

Spheroplast Lysis Assay for AC75

	Maximal Lysis Rate (MLR)	Lag time (minutes)
AC75	0.0096	5.71
W303-1B	0.0052	8.20

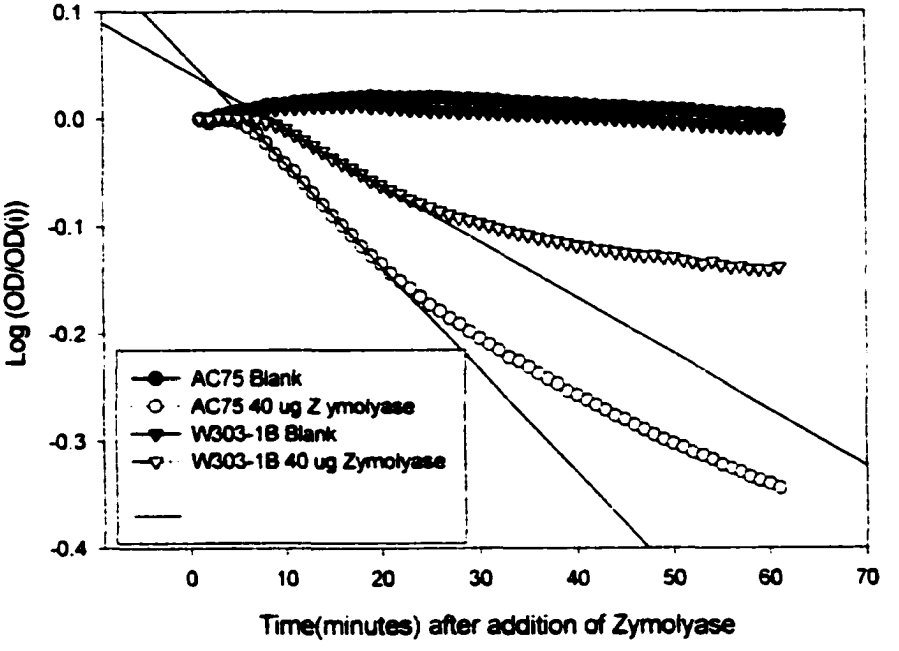


Figure 8c (3). Zymolyase-induced spheroplast lysis of class III strain AC75 and wild-type W303-1B

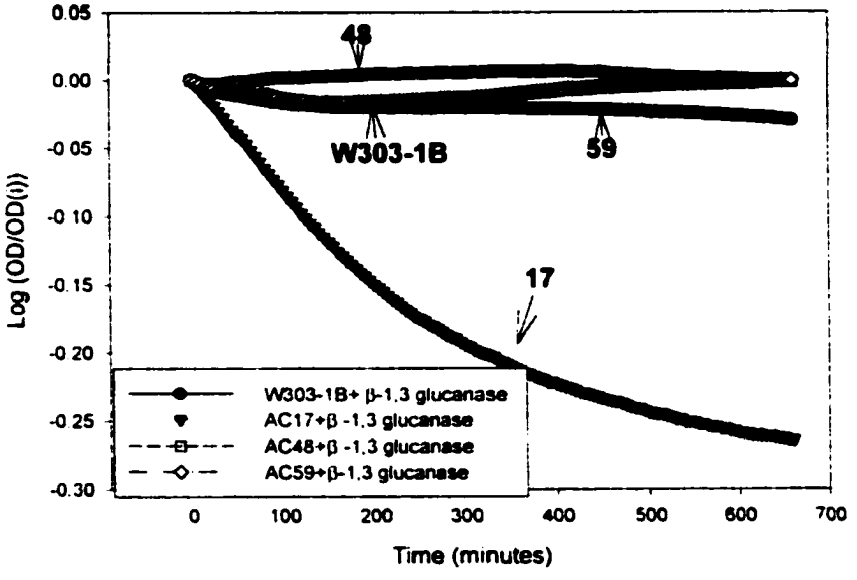


Figure 8d. Spheroplast lysis assay of strains AC17, AC48, AC59, and W303-1B, using β-1,3 glucanase.

incubation with β-1,3 glucanase, compared to at least 98 percent of the wild-type. These results suggest that the outer mannoprotein layer of strain AC17 was greatly diminished which allowed access to the inner glucan layer by β-1,3 glucanase without protease digestion of an outer mannoprotein layer. A graphic representation of the results is shown in Figure 8d.

Table 5. Cell wall lysis with β-1,3 glucanase

Strain	Percent of cells remaining intact after 11 hours
AC17	44.6
AC48	99.6
AC59	98.5
W303-1B	93.3

3. Sensitivity to calcofluor white.

In many mutants defective in the synthesis of cell wall glucans there is a compensatory increase in cell wall chitin (Ketala *et al.*, 1999). Calcofluor white is a fluorescent dye that by intercalation into newly formed chitin chains prevents the assembly of microfibrils. At sufficiently high concentrations calcofluor white can cause cell death by blocking the assembly of the cell wall (Lussier *et al.*,1997; Ketala *et al.*, 1999). In order to evaluate the degree of cell wall damage in these strains I tested them for sensitivity to calcofluor white, as described in Materials and Methods.

As can be seen in Figure 9, the strains in class I (hyper-excretors) all exhibited sensitivity to calcofluor white but varied in degree of sensitivity. At a concentration of 5 ug/ml, AC48 did not grow at all. Although AC17 and AC59 grew at 5 ug/ml, at a higher calcofluor white concentration of 10 ug/ml growth of these two strains was greatly impaired. The wild-type, W303-1B, was able to grow at 10 ug/ml calcofluor white at all dilutions. These results suggest that, relative to the wild-type, there was significantly more damage in the cell walls of AC17, AC48, and AC59, with the greatest amount in AC48.

The strains in class II (WT-excretors) exhibited the least sensitivity to calcofluor white of all classes. Even at the highest calcofluor white concentration, of 10 ug/ml the growth of all class II strains was at most moderate.

The strains in class III (non-excretors), showed the greatest sensitivity to calcofluor white. Compared to W303-1B which grew at a calcofluor white concentration of 10 ug/ml for all dilutions, AC31 did not grow at 10 ug/ml at any dilution. AC75 exhibited little growth at 10^6 cells/ml and AC60 did not grow below 10^6 cells/ml. These results suggest that, in addition to strain AC48 in class I, the strains in class III have the greatest amount of cell wall damage.

II. Transformation of mutants with a *Saccharomyces cerevisiae* genomic library

The foregoing phenotype analysis of the AC mutants supported the existence of mutations related to secretion and/or cell wall biosynthesis. Since my objective was to identify a mutant gene or genes involved, I commenced transformations of respective strains with a YCp50 yeast genomic library, kindly given to us by Dr. Jeanne Hirsch (Hirsch and Cross, 1993).

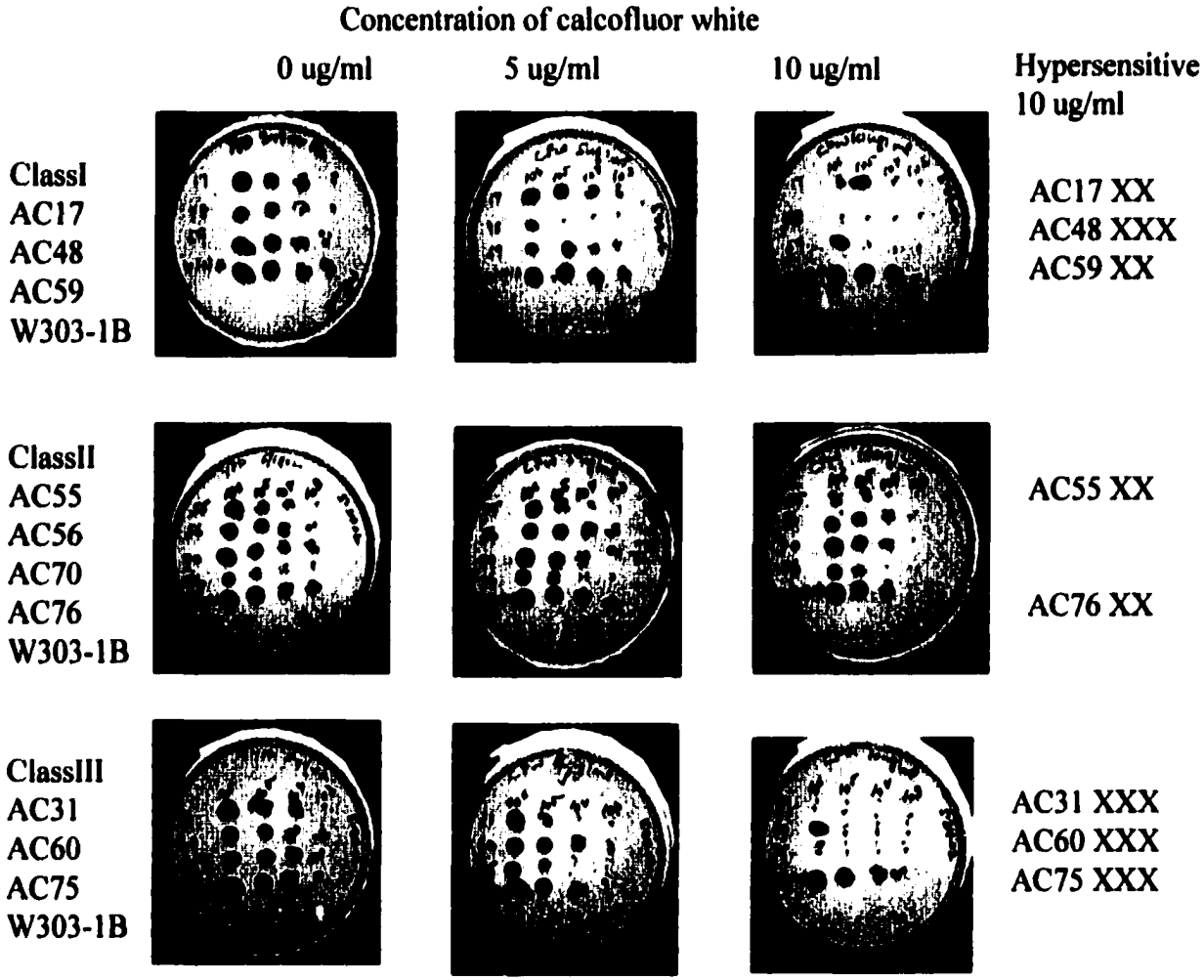


Figure 9. Sensitivity of strains to calcofluor white. Cells were spotted at an initial dilution of 10^6 cells/ml and in decrements of 0.1. XXX, no growth or severely impaired growth; XX, moderate growth.

A. Transformation of mutants with a yeast genomic library

1. Growth at 37°C

A plasmid in the yeast genomic library complemented strain AC59 for growth at 37°C. The result after transformation was that after 2 days of incubation at 37°C there was a total of 4 colonies on 3 plates. Each colony was restreaked and again grew at 37°C, confirming the presence in the plasmid insert of a complementing gene for growth at the restrictive temperature.

2. Extraction of and re-transformation with complementing plasmid, PK1

I extracted the DNA from one of the AC59 colonies that had grown at 37°C. The extracted DNA which included the complementing plasmid (PK1) DNA and chromosomal DNA was used to transform *E. coli* (Stratagene XL1 Blue) in order to isolate and proliferate the complementing plasmid, PK1. AC59 was then re-transformed with PK1 and after 1 day at room temperature and 2 days at 37°C there were approximately 1,000 AC59 colonies per plate (Figure 10).

3. Restriction enzyme digest of *S. cerevisiae* genomic insert in PK1

In order to determine the length of the DNA insert in PK1, I did a restriction enzyme digest of the DNA with four of the re-transformed AC59 colonies. The result is shown in an agarose gel (Figure 11). The five bands in this gel are due to the presence of four EcoRI sites within the insert. The 7 Kb band represents the YCp50 vector and the 3

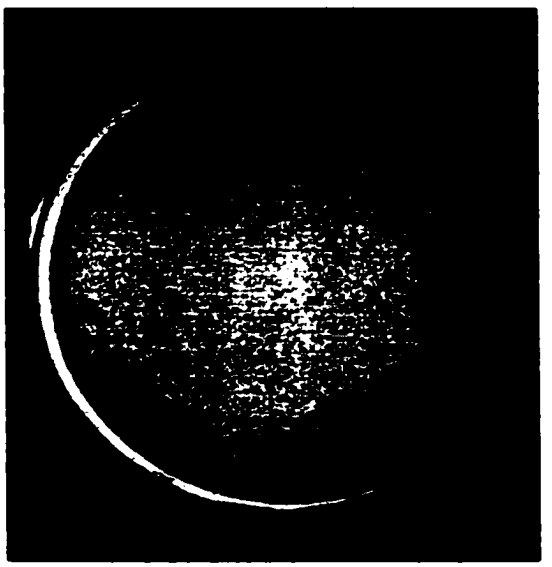


Figure 10. Growth at 37°C of AC59 transformed with PK1.

AC59 was transformed with PK1. Plates were incubated at room temperature for 20 hours then transferred to 37°C.

Figure 11. Restriction enzyme digest of plasmid PK1. The DNA of colonies 1,2,4 and 9 was cut with *EcoRI* and *SalI*, restriction enzymes encompassing the BamHI insert site in YCp50.

Lane	Polyacrylamide gel		ug DNA
1	10 ul 1Kb ladder		
2	Colony 1	cut with EcoRI and SalI	1.40
3	Colony 2	"	0.66
4	Colony 4	"	1.24
5	Colony 9	"	0.26
6	Colony 1	"	2.80
7	Colony 9	uncut	0.26

Band at 7.0 kb represents the YCp50 vector

Insert Bands

3.0 Kb

1.6

0.8 *

0.7 *

6.1 Kb

*Bands at 0.8 and 0.7 seen on negative

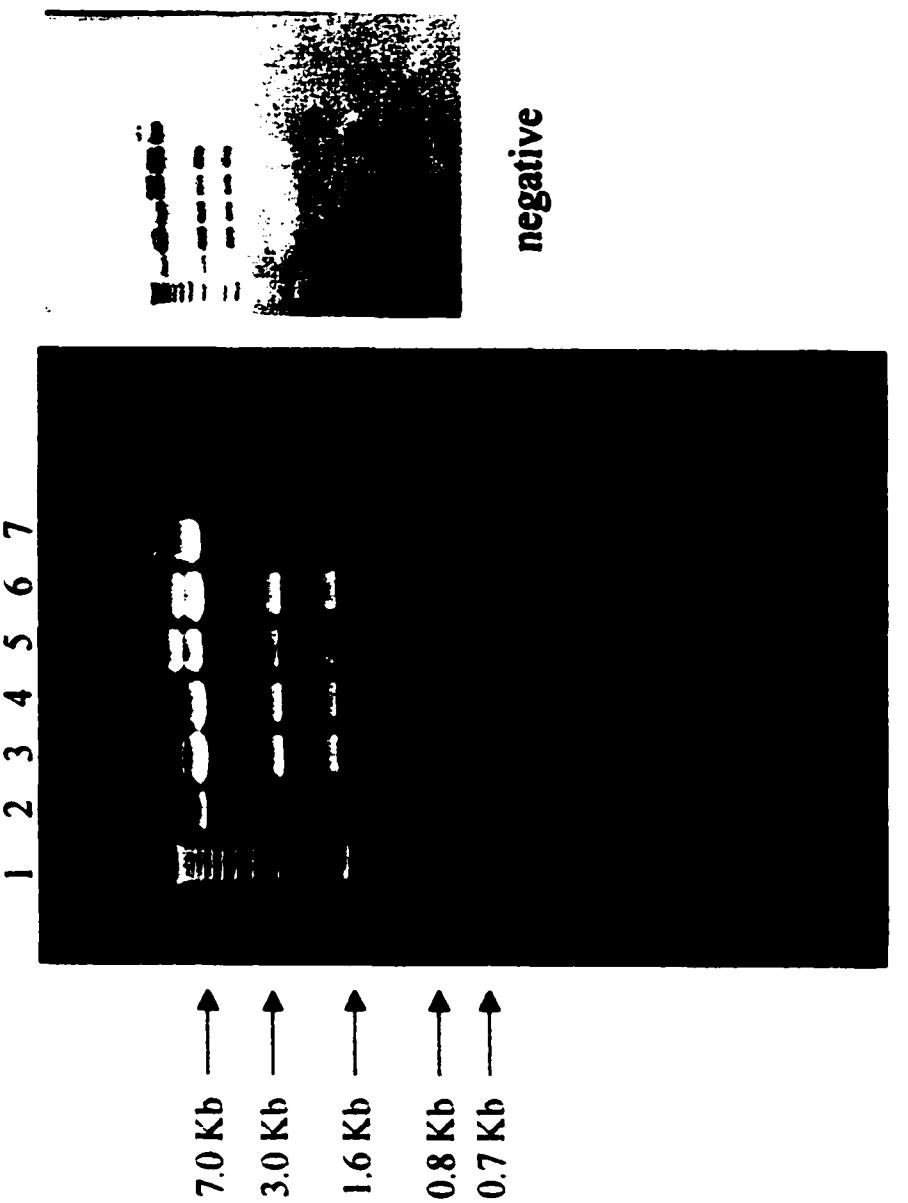


Figure 11

Kb, 1.7 Kb, 0.8 Kb, and 0.7 Kb bands represent the insert. The sum of the bands, exclusive of the vector was 6.1 Kb.

4. The insert contained three open reading frames including *BET1*

The left end of the DNA insert in PK1 was sequenced by the Hunter College Sequencing Facility, using primers generated on each side of the BamHI insert site. The result returned was a 900 nucleotide sequence. A BLAST search of this sequence identified 805 homologous residues on chromosome IX of *S. cerevisiae*, beginning with pair 343605. Extended for 6.1 Kb, the size of the insert, the sequence included three open reading frames (ORFs) (Table 6). Two of the ORFs, YIL006W and YIL005W had no known function. The third ORF, YIL004C from base pair 348502 to 347943, on the reverse strand was identified as *BET1*. Subsequent to this search YIL005W was identified as *EPS1*, thought to be a chaperone, functioning in protein folding in the ER (*Saccharomyces* database, genome-www.stanford.edu/Saccharomyces/).

B. Transformation of AC59 with *BET1*

1. Growth at 37°C

A plasmid containing the *BET1* gene (pAN101) kindly sent to us by Dr. Ferro-Novick (Newman *et al.*, 1990) was used to transform AC59. After 16 hours at room temperature small colonies began to appear. After 2 days at 37°C the colonies had increased in size and there were approximately 1500 colonies per plate (Figure 12). *BET1* therefore permitted the growth of AC59 at 37°C.

Table 6. Open reading frames on insert in PK1

ORF	Gene	Basepair Coordinates	Function
YIL006W	-	344059 - 345180	Carrier Protein ?
YIL005W	*EPS1*	345689 - 347794	Isomerase/Chaperone
YIL004W	*BET1*	348502 - 347943	Protein Transport

The insert in PK1, homologous to chromosome IX of *S. cerevisiae*, extending from nucleotide 343605 to 349805, includes three open reading frames.

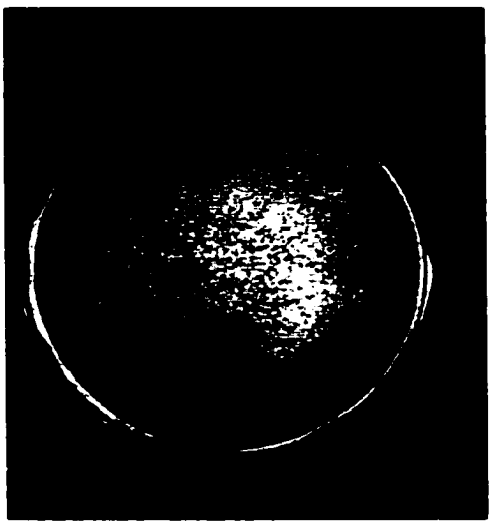


Figure 12. Growth at 37°C of AC59 transformed with pAN101. Strain AC59 was transformed with the *BET1* gene in YCp50 (pAN101α *BET1 amp tet ARS1 CEN4 URA3*) kindly given to us by Dr. Susan Ferro-Novick. The plates were incubated at room temperature for 22 hours then transferred to 37°C.

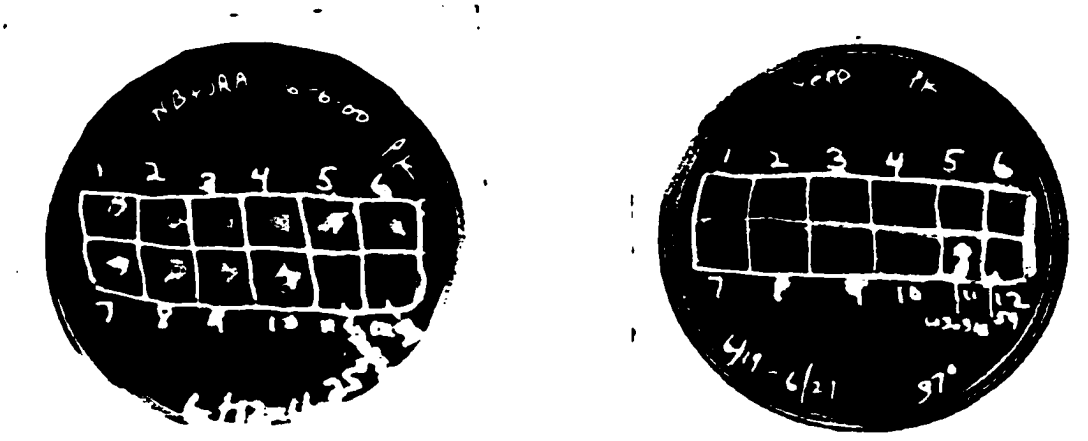
2. Evidence that *bet1* was the mutant gene in AC59

The possibility existed that over-expression of *BET1* rescued a mutant gene in AC59 other than *bet1*. If *BET1* were actually wild-type in AC59, then in a cross of AC59 with an authentic *bet1-1* mutant the resulting diploid would grow at 37°C. To test this possibility I crossed AC59 with the *bet1-1* mutant RSY944, kindly given to us by Dr. Randy Schekman (Wuestehube *et al.*, 1996). I selected 10 of the resulting diploid colonies, spotted them on a YEPD plate and incubated them at 37°C. I incubated corresponding colonies at 25°C. None of the diploids grew at 37°C and all grew at 25°C (Figure 13). This result was evidence that the cross of AC59 and RSY944 was homozygous for *bet1* and therefore *bet1* was mutant in AC59.

However, it was possible that if the *bet1* mutation in RSY944 were dominant and *BET1* in AC59 were wild-type, a cross of AC59 with RSY944 would also not have grown at 37°C. I therefore crossed RSY944 with the Wild-type *MATα*, W303-1B. The resulting diploids grew at 37°C showing that *bet1-1* in RSY944 was recessive (data not shown). Similarly AC59 was crossed with the wild-type *MATa* strain N4351. The resulting diploids grew at 37°C demonstrating that the *bet1* mutation in AC59 was also recessive (data not shown).

III. Phenotype of AC59, as a *bet1* mutant

AC59 was a class I mutant characterized by excretion of α-agglutinin, sensitivity to cell lysis by Zymolyase, and reduced growth rate was now identified as having a mutation



(a) (b)

Figure 13. Temperature sensitivity of the diploid colonies AC59 x RSY944, W303-1B, and AC59. (a) After 4 days growth at 25°C; (b) After 4 days growth at 37°C: (1-10) diploids; (11) W303-1B; (12) AC59

in the *BET1* gene. I therefore added experiments related to the manifestation in AC59 of a *bet1* phenotype.

A. Excretion of α-agglutinin into the growth medium at 25°C and 37°C

Since *bet1-1* was temperature sensitive for growth (Newman and Ferro-Novick, 1987; Newman *et al.*, 1990), I quantitated the amount of α-agglutinin excreted by AC59 and W303-1B cells at both the restrictive temperature of 37°C and the permissive temperature of 25°C. The results shown in Table 7, indicate that at 37°C the excretion of α-agglutinin by AC59 was significantly greater than at 25°C and greater than W303-1B at both temperatures.

B. Sensitivity of AC59 to lysis by Zymolyase

AC59 was shown to be sensitive to cell lysis by Zymolyase (Figure 8a). The maximal lysis rate of AC59 was 2.24 fold greater than the wild-type W303-1B and the lag time was only 54% that of W303-1B. These results showed the greater fragility of the cell wall of AC59 relative to that of W303-1B even at the permissive temperature.

C. Growth Rate

I also measured the growth rate of AC59 compared to W303-1B, at 25°C (Figure 14a) and at 36°C (Figure 14b). At 25°C strain AC59 grew at 78% of the rate of W303-1B. This indicated that the growth of AC59 was inhibited at the permissive temperature. At 36°C AC59 grew at 58% the rate of W303-1B during the first 2 hours, then the rate of

Table 7. Excretion of α-agglutinin by strains AC59 and W303-1B

Strain	Incubation Temperature	α-Agglutinin Excreted Relative to W303-1B at 37°C (units/cells x10^7)	Standard Error (units/cellx10^7)
AC59	25°C	0.073	+/- 0.145
	37°C	6.017	+/- 0.427
W303-1B	25°C	0.360	+/- 0.006
	37°C	0.000(a)	+/- 0.008

AC59 and W303-1B were grown at 25°C to early exponential phase then continued at 25°C or 37°C for three more hours. The growth medium of the respective cultures was collected, lyophilized, resuspended to 1/10 of the original volume, and the α-agglutinin quantitated in an aggltination assay.

(a) The activity of W303-1B at 37°C was used as the base-level of excretion.

Figure 14(a). Growth rate at 25°C of strains AC59 (circles) and W303-1B (triangles). Cells were grown in YEPD with shaking at 120 rpm, to mid-exponential phase and the absorbance at 660nm was measured.

Figure 14(b). Growth rate at 36°C of strains AC59 (circles) and W303-1B (triangles). Cells were grown overnight at room temperature, then pelleted, resuspended in media and incubated at 36°C with shaking at120 rpm for 3 more hours. The initial ODs were: AC59: 0.349; W303-1B: 0.358.

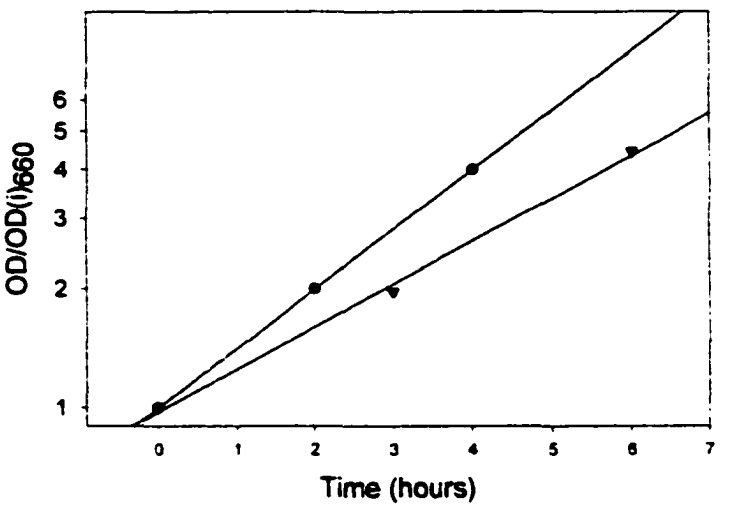


Figure 14(a)

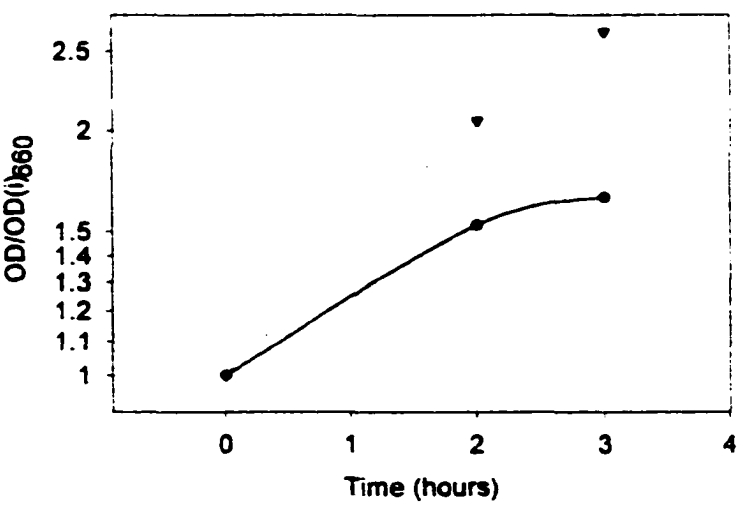


Figure 14(b)

growth tapered off. This indicated that there was a significant inhibition of growth in AC59 at a restrictive temperature of 36°C.

D. Secretion of invertase in AC59

bet1-1 was originally identified as temperature sensitive for growth and protein secretion (Newman and Ferro-Novick, 1987). In the *bet1-1* mutant, at the restrictive temperature of 37°C invertase was blocked in secretion from the ER to the Golgi and failed to be transported to the cell surface. In order to determine whether in AC59 the secretion of invertase to the cell surface was similarly blocked, I assayed for invertase on the cell wall and in the supernatant. Invertase expression was derepressed by glucose deprivation both at 25°C and 37°C for 2 ½ hours, then invertase activity was determined. As seen in Table 8, secretion of invertase both to the cell wall and into the growth medium was significantly less at 37°C than at 25°C. These results demonstrated in AC59 a block in secretion of invertase at the restrictive temperature which was consistent with the *bet1-1* phenotype (Newman and Ferro-Novick, 1990).

IV. *BET1* rescue of AC59 mutant phenotypes

It remained to be shown that *BET1* would complement AC59 for excretion of α-agglutinin. I transformed AC59 with pAN101, containing *BET1*, and with YCp50 without an insert, and tested for excretion of α-agglutinin. The results in Table 9 show that when AC59 was transformed with pAN101 the amount of α-agglutinin excreted was greatly reduced at 37°C and reduced to a lesser extend at 25°C. Therefore, *BET1* complemented

Table 8. Secretion of invertase to the cell surface and into the supernatant of strain AC59

Fraction assayed	Growth temperature	Number of cells	Reducing sugar produced (ug)	Invertase units of activity (μMol/min·cell/10^8)
Cell Surface	25°C	1.04×10^7	36	1.25
	37°C	0.87×10^7	11	0.45
Supernatant	25°C	1.04×10^7	8	0.28
	37°C	0.87×10^7	3	0.13

Table 9. α-Agglutinin excreted by strain AC59

Strain	Plasmid	Incubation temperature	α-Agglutinin excreted (units/cell x10^7)	Standard error (units/cell x10^7)
AC59	pAN101 (*BET1*)	25°C	0.126	+/-0.020
		37°C	0.028	+/-0.002
AC59	YCp50	25°C	0.934	+/-0.007
		37°C	1.940	+/-0.010

Cultures AC59 transformed by pAN101 or by YCp50 were incubated to mid-exponential phase. The growth medium was collected, lyophilized and resuspended to 1/10 of the original volume. The excreted α-agglutinin was quantitated in an agglutination assay.

the excretion phenotype of AC59 for excretion in increased amounts of α-agglutinin at both the restrictive and permissive temperatures.

To determine whether *BET1* would complement the growth defect in AC59 I streaked plates with AC59, AC59 transformed with pAN101, and W303-1B. As can be seen in Figure 15, AC59 grew at 37° only when transformed with pAN101. This result showed that *BET1* complemented AC59 for temperature sensitivity at 37°C.

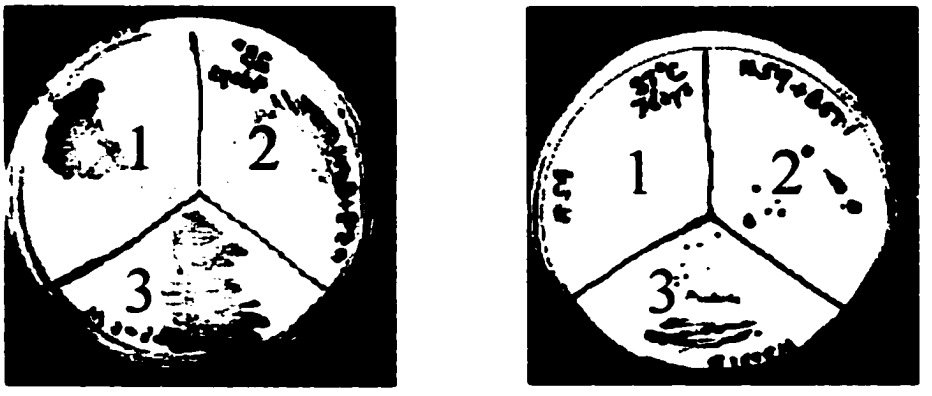
I also tested the ability of *BET1* to complement AC59 for inability to agglutinate to the opposite mating type, **a**-cells. I transformed AC59 with pAN101 and performed a qualitative agglutination assay, as described in Materials and Methods. As indicated in Table 10, after transformation with *BET1*, the increased formation of a lacy circumference was evidence that the agglutinability of AC59 to the opposite mating type had increased.

V. Paradox of excretion of α-agglutinin

The excretion of α-agglutinin by AC59 at 37°C appeared to be a contradiction. The *bet1-1* mutation blocks secretion from the ER to the Golgi, as demonstrated with invertase (Newman and Ferro-Novick, 1990). My results for AC59 were similar (Table 8). The apparent paradox of excretion of α-agglutinin in a pathway blocked at the ER had two possible explanations. One possibility was the existence of a post-ER pool of α-agglutinin, that was present in the cell prior to being transferred to 37°C, as the source of excreted α-agglutinin. Another possibility was that α-agglutinin was secreted to the cell wall via an alternative, *BET-1* independent secretory pathway. In the following experiments I tested these two hypotheses.

Table 10. Agglutination Assay: AC59 +/- transformation with *BET1*

Strain	Transfomed by *BET1*	Pellet formation	Agglutination to a-cells
AC59	-	Compact pellet, few lacy edges	-/+
AC59	+	Pellet with increased lacy edges	++
W303-1B		Pellet in center, lacy around edges	+++
W303-1A		Compact pellet, no lacy edges	-



(a) (b)

Figure 15. Temperature sensitivity of strains AC59 (1), AC59 + *BET1* (2), W303-1B (3). (a) After 4 days of incubation at 25°C; (b) After 7 days of incubation at 37°C.

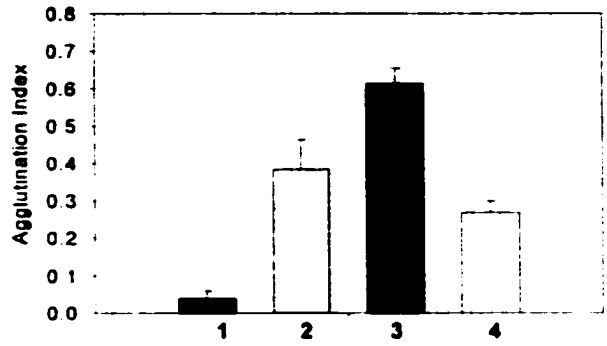
A. Existence of a post-ER pool of α-agglutinin

1. Cycloheximide insensitivity of expression of α-agglutinin in W303-1B

In order to determine if a pool of α-agglutinin existed in the wild-type W303-1B, I blocked synthesis of α-agglutinin with cycloheximide. If α-agglutinin continued to be secreted to the cell wall in the presence of this block, that might imply the pre-existence of a pool as the source of α-agglutinin. I incubated W303-1B cells in the presence and absence of cylcoheximide, with or without the added pheromone **a**-factor and assayed the amount of α-agglutinin secreted to the cell wall. As can be seen in Figure 16, column 2, when cycloheximide and **a**-factor were added to the culture at the same time, α-agglutinin continued to be secreted to the cell wall, though in lesser amounts than in cells induced without a cycloheximide block (Figure 16, column 3). This amount (column 2) was significantly greater than that of the control where α-agglutinin was blocked by cycloheximide ½ hour before induction by **a**-factor (Figure 16, column 1). These results suggested that a pool of α-agglutinin existed in the cells prior to the addition of cycloheximide. The results also show there was a significant amount of α-agglutinin cross-linked to the cell wall in controls not treated with pheromone (Figure 16, column 4), suggesting that the source of the pool was constitutively synthesized α-agglutinin.

2. Cycloheximide insensitivity of α-agglutinin expression in AC59

The existence of a pool in the mutant AC59 remained to be shown. I did an experiment similar to that of W303-1B, incubating cultures with or without cycloheximide,



Time of addition (minutes)

a-factor	30	30	30	-
cycloheximide	0	30	-	-

Figure 16. Effect of cycloheximide and a-factor on secretion of α-agglutinin to the cell wall of W303-1B. W303-1B cells were grown in YNB-C to OD_{660} = 0.3, pelleted and resuspended in one-half volume of fresh YNB-C to OD = 0.6. The cells were then divided into four tubes of 5 mls each. To tube 1, cycloheximide (10 ug/ml) was added ½ hour before adding the pheromone, a-factor (supernatant of induced X2180-1A cells grown to stationary phase) (5 mls). To tube 2, a-factor (5 mls) and cycloheximide (10 ug/ml) were added at the same time. To tube 3, only a-factor (5 mls) was added. To tube 4 neither a-factor nor cycloheximide were added, but YNB-C (5 mls) was added. BSA (0.2 ug/ml) was added to each tube to facilitate solubility of the pheromone (Terrance and Lipke, 1981). All tubes were incubated at 30°C, shaking at 150 rpm, for a total of 1 ½ hours. The cells were then pelleted, resuspended in 0.1M sodium acetate, pH 5.5, to 1/10 of the original volume and used in a quantitative agglutination assay as described in Materials and Methods.

with or without induction by **a**-factor. Since in AC59 α-agglutinin was not cross-linked to the cell wall but was excreted, I assayed the amount of α-agglutinin excreted into the supernatant. If after a cycloheximide block, excretion of α-agglutinin were to continue, that would suggest the existence of a pool in the cell as a source of the excreted α-agglutinin.

As Figure 17 shows the amount of α-agglutinin excreted was the same whether or not cells were induced by **a**-factor and whether or not cycloheximide had been added. This result indicated that in the presence of a cyloheximide block of α-agglutinin synthesis (Figure 17, columns 1 and 2) or by a block at 37°C in secretion of *bet1* from the ER (Figure 17, columns 3 and 4), α-agglutinin was similarly excreted.

If in the absence of cycloheximide the amount of α-agglutinin excreted was not greater than but similar to the amount excreted in the presence of cycloheximide, that would suggest that there was a *bet1* block in the secretory pathway at the restrictive temperature. These results support the existence of a post-ER pool, as the source of the excreted α-agglutinin, which was present in the cell prior to transfer to the restrictive temperature.

3. Depletion of α-agglutinin pool in AC59 after incubation at 37°C

In order to determine whether a post-ER pool of α-agglutinin in AC59 was finite, I assayed AC59 for excretion of α-agglutinin during 2 successive rounds of incubation. As shown in Figure 18, the excretion of α-agglutinin at 37°C during the first round (Figure 18, column 1) was significantly greater than during the second round (Figure 18, column 2),

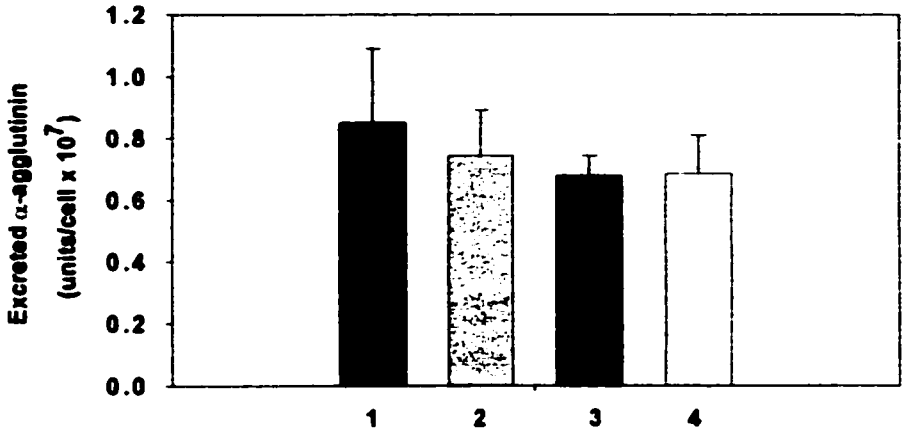


Figure 17. Effect of cycloheximide and a-factor on excretion of α-agglutinin into the growth medium by AC59. AC59 cells were grown in YEPD to OD_{660} = 0.3. The cells were pelleted and resuspended in one-half volume YNB-C to OD = 0.6 and divided into 4 tubes at 5 mls/tube. a-Factor (supernatant of induced X2180-1A cells grown to stationary phase) (5 mls) was added to 2 tubes. 10 ug/ml of cycloheximide was added to one of these tubes. α-factor (supernatant of X2180-1B cells grown to stationary phase) (5 mls) was added to to 2 tubes, and 10 ug/ml of cycloheximide was added to one of these tubes. The tubes were incubated at 37°C for 1 hour, shaking at 150 rpm. The cells were pelleted and the supernatants were saved, lyophylized and resuspended to 1/10 of the original volume. A quantitative agglutination assay for excretion of α-agglutinin was done, as described in Materials and Methods. (1) + cycloheximide, + a-factor; (2) + cycloheximide, - a-factor; (3) - cycloheximide, + a- factor; (4) - cycloheximide, - a-factor. Cells were incubated at 37°C for 1 hour.

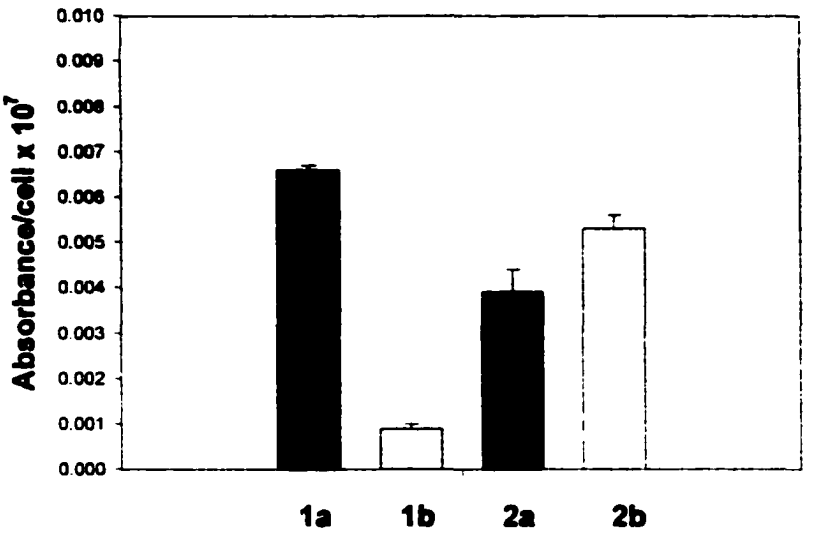


Figure 18. Excretion of α-agglutinin by AC59, at 25°C and 37°C, during 2 rounds of incubation. AC59 cells were grown in 40 mls of YNB-C, at 25°C to mid-exponential phase, pelleted, resuspended in 40 mls of fresh medium and divided into 2 flasks. The respective cultures were incubated at 25°C or 37°C for 2 hours, pelleted and the supernatants saved (Round 1). The pellets were washed 3 times with media warmed to 37°C or at room temperature, relative to the original culture. The pellets were then resuspended in media pre-warmed to 37°C or at room temperature and incubated for a second 2 hour period at 25°C or 37°C. The cells were pelleted and the supernatants saved (Round 2). The respective supernatants were lyophilized and resuspended in 1.5 mls of water. The amount of α-agglutinin excreted into the supernatant was determined in an ELISA assay as described in Materials and Methods. (1) Incubation at 37°C: (a) Round 1 (b) Round 2; (2) Incubation at 25°C: (a) Round 1, (b) Round 2.

and was greater than incubation at 25°C during either round (Figure 18, columns 3 and 4). These results are consistent with the existence of a post-ER pool of α-agglutinin. That the pool is finite as shown by depletion of α-agglutinin in the supernatant during the second round, is supportive of a *bet1-1* block in secretion at the restrictive temperature.

VI. Mutation in *bet1* correlated with reduced β-1,6 glucan on cell wall

Since α-agglutinin is covalently bonded to β-1.6 glucan in the cell wall (Lu *et al.*, 1995; Kapteyn *et al.*, 1996; Shahinian *et al.*, 1998), the excretion by AC59 of α-agglutinin would occur if there were a diminution of β-1.6 glucan in the cell wall. I therefore assayed for cell wall β-1,6 glucan at 37°C and at 25°C. As shown in Figure 19, when AC59 was incubated at 37°C the amount of β-1.6 glucan in the cell wall was reduced ten-fold, relative to incubation at 25°C. These results suggest that the *bet1* block in secretion from the ER inhibited the expression of cell wall β-1,6 glucan.

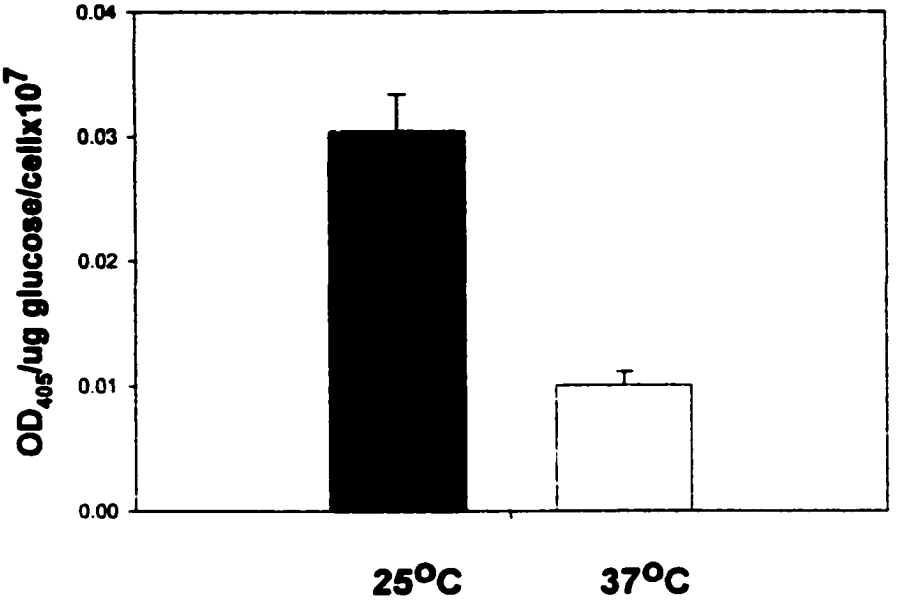


Figure 19. β-1,6 glucan in a laminarinase extract of cell walls of AC59. Cells were grown to mid-exponential phase, pelleted, and β-1,6 glucan was extracted, as described in Materials and Methods, and assayed by ELISA.

DISCUSSION

I. Introduction

This work involves an investigation of cellular processes with a focus on the secretory pathway and fungal cell wall biosynthesis, using *Saccharomyces cerevisiae* as a model. Although much progress has been made in understanding the biosynthesis of the yeast cell wall and the significance of the part played by the secretory pathway there is still much to be determined. The cell wall consists of β-1, 3 glucan, β-1, 6 glucan, a small amount of chitin, and mannoproteins. The role of the secretory pathway in the synthesis of cell wall β-1,6 glucan, and the process of glucosylation of cell wall proteins are among the factors involved in cell wall biosynthesis. In pursuit of such issues shown here are specific consequences to wall structure and biogenesis after disruption of the secretory pathway.

II. Classification of mutant phenotypes

Conzelmann (Benghezal *et al.*,1995) selected temperature sensitive mutants that did not express α-agglutinin on the cell surface, a condition that should include mutations in functions related to secretion and cell wall synthesis. To further refine our selection of putative cell wall mutants we used only those strains or mutants that at the restrictive temperature, were unable to bind to cells of the opposite mating type. These strains were divided into three classes based on level of excretion of α-agglutinin. Within each class we determined the sensitivity of strains to Zymolyase and calcofluor white, and

Table 11 **Summary**

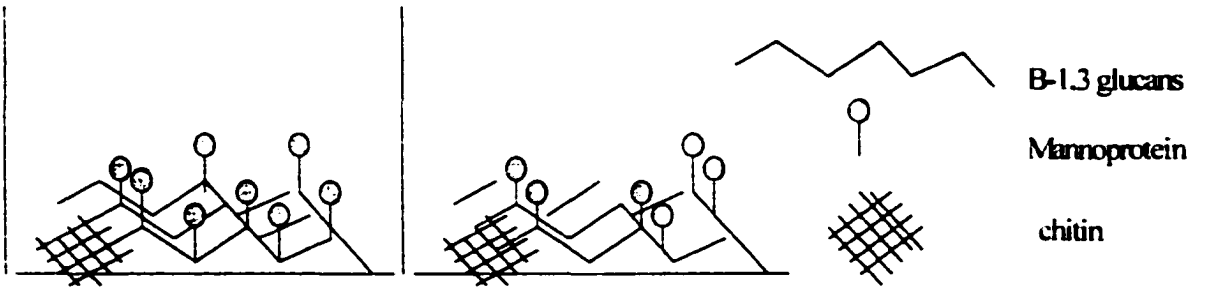
Excretion, MLR, Lag Time, and sensitivity to calcofluor white

Strain	Ratio to Wild-Type, W303-1B: Secretion into Growth Media (Excretion)	Lysis with Zymolyase: Maximal Lysis Rate (MLR)	Lysis with Zymolyase: Lag Time	Growth on calcofluor white agar: 5 ug/ml	Growth on calcofluor white agar: 10 ug/ml
Wild-type,W303-1B	1.00	1.00	1.00	+	+
Class I (Excretion > WT)					
AC17	1.45	2.34	0.57	X	XX
AC48	1.55	3.37	0.59	XXX	XXX
AC59	1.26	2.24	0.53	X	XX
Class II (Excretion ≈ WT)					
AC55	0.95	0.67	3.89	X	XX
AC56	1.08	0.90	1.96	+	X
AC70	0.97	2.27	0.94	+	X
AC76	1.07	3.88	0.78	X	XX
Class III (Excretion < WT)					
AC31	0.85	2.33	4.13	XX	XXX
AC60	0.74	0.88	0.49	X	XXX
AC75	0.82	1.85	0.70	XX	XXX

XXX, no growth or severely impaired growth; XX, moderate growth; X slightly inhibited growth; + normal growth

correlated these phenotypes with strain excretion type (Table 11).

A. Class I. Excretion greater than wild-type: Strains AC17, AC48, and AC59



Wild-type cell wall Mutant cell wall

Figure 20. Model of wild-type and class I mutant cell wall. Mutant cell wall has a diminished mannoprotein layer and a thin inner glucan layer.

Class I strains showed similarities in several phenotypes. These strains, which excreted greater levels of α-agglutinin than the wild-type, were also sensitive to lysis with Zymolyase, and to calcofluor white. During spheroplast lysis assays, cells were lysed at more than twice the rate of the wild-type and had a shorter lag time than the wild-type (Figures 8a 1,2,3). A short lag time is indicative of rapid degradation of the cell wall outer layer of mannoproteins by the protease in Zymolyase. This suggests a thin mannoprotein layer. The rapid lysis rate in these strains suggests, relative to the wild-type, a diminished inner glucan layer which was therefore more rapidly lysed by the β-1,3 glucanase in Zymolyase. Indeed, AC17 is lysed by β-1,3 glucanase alone (Figure 8d). Figure 20

illustrates the type of cell depicted here, compared to the wild-type.

All the strains in class I were impaired in growth on calcofluor white agar. Since calcofluor white blocks the synthesis of chitin, sensitivity to calcofluor white can be indicative of an increase in cell wall chitin, a compensatory reaction to a weakened cell wall (Valdivieso *et al.*, 2000). However, the cell walls in strains AC17, AC48, and AC59 were all sensitive to lysis with Zymolyase and an increase in cell wall chitin would result in resistance to Zymolyase (Kapteyn *et al.*, 1997; Hong *et al.*, 1994). This contradiction suggests that sensitivity to calcofluor white in class I strains is not the result of increased cell wall chitin. Another possible explanation is based on the ability of calcofluor white to form hydrogen bonds with β-1,3 linked polysaccharides (Albani *et al.*, 2000). By hydrogen bonding to β-1,3 glucan polymers in the cell wall calcofluor white might sterically block the addition of β-1,3 glucan and β-1,6 glucan branches to cell wall β-1,3 glucan polymers or inhibit assembly of β-1,3 glucan into fibrils or helices and thus inhibit cell wall biosynthesis. The thinner the inner β-1,3 glucan layer, the less calcofluor white is required to inhibit cell growth (Figure 20). These results, based on a decreased inner β-1,3 glucan layer were shown most emphatically in strain AC48, which had the shortest lag time, the most rapid maximal lysis rate, and the most severely impaired growth on calcolfluor white agar.

B. Class II. Excretion similar to wild-type (AC55, AC56, AC70 and AC76)

Class II strains did not agglutinate to a-cells, but the excretion of α-agglutinin was similar to that of the wild-type. In all class II strains there were aberrations in the structure

of the cell wall. These were manifested in two types of strains in this class: either there was a long lag time and then a slow MLR or a short lag time and a rapid MLR, relative to the wild-type (Table 11, Figures 8b 1,2,3,4).

Class II A. Wild-type excretion and long lag time in strains AC55 and AC56

Strains AC55 and AC56 (Figures 8b 1,2) had a long lag time before the start of a slow cell lysis. This result suggests a thick outer mannoprotein layer that would slow the rate of degradation by the protease in Zymolyase delaying access to the inner glucan layer by β-1,3 glucanase. The slow maximal lysis rate suggests a more dense inner glucan layer, resistant to β-1,3 glucanase in Zymolyase.

Class II B. Wild-type excretion and rapid lysis rate in AC70 and AC76

The rapid MLR of these strains (Figures 8b 3,4) indicates a thin glucan layer, lysed more rapidly by the β-1,3 glucanase in Zymolyase. The lag time was less than the wild type, suggesting a diminished outer mannoprotein layer.

Sensitivity to calcofluor white and conclusion

The sensitivity to calcofluor white in class II strains ranged from slight to moderate and was not correlated with sensitivity to Zymolyase. The variations in phenotypes in this class did not suggest any general conclusions relating to class II mutants.

C. Class III. Excretion less than wild-type and hyper-sensitivity to calcofluor white

The strains in class III were all hyper-sensitive to calcofluor white. This phenotype was correlated with excretion of α-agglutinin less than the wild-type (Table 11, Figures 8c 1,2,3). Otherwise, the strains in class III were all unique.

Class III A. Excretion less than wild-type and four-fold greater lag time in AC31

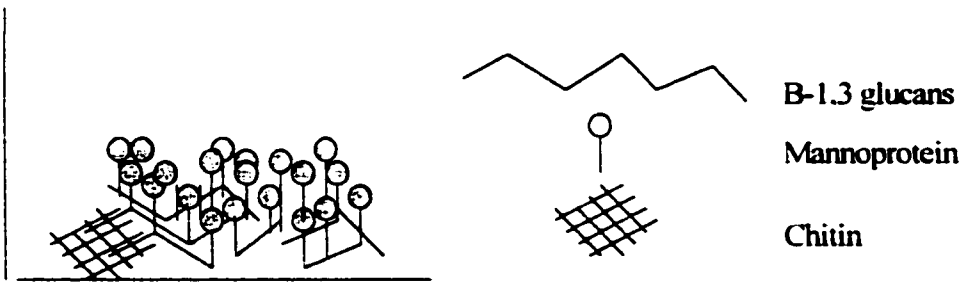


Figure 21. Class IIIA. Model of cell wall of strain AC31. Cell wall has a thick mannoprotein layer which can be associated with a long lag time.

AC31 (Figure 8c 1) had a long lag time, possibly indicating an outer layer dense in mannoproteins which might block excretion of α-agglutinin and result in an excretion level lower than the wild-type. In AC31 there was also a two-fold greater maximal lysis rate, indicating a diminished glucan layer, more easily lysed by β-1,3 glucanase. These results make it unlikely that the defect is compensated for by increased chitin. A model of this mutant is depicted in Figure 21. AC31 was the only mutant in which there was

both a long lag time and a high MLR.

Class III B. Excretion less than wild-type with uncleaved GPI anchor bound to plasma membrane in AC60 and AC75.

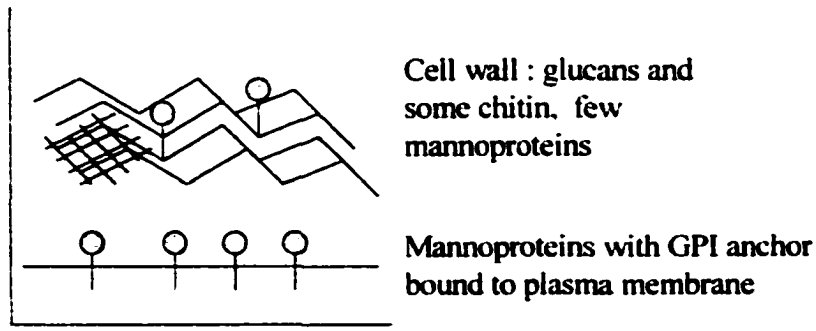


Figure 22. Class III B. Cell wall of strains showing mannoproteins with uncleaved GPI anchor bound to plasma membrane: Class III (AC60 and AC75)

In AC60 and AC75 (Figures 8c 2,3), the low level of excreted α-agglutinin might occur if secretion of the mannoprotein were blocked before reaching the cell wall. This might result if α-agglutinin were secreted to the cell membrane but was not cleaved from its GPI anchorage to the membrane. The short lag time in both AC60 and AC75, indicating a thin mannoprotein layer, easily degraded by protease, is consistent with other mannoproteins in addition to α-agglutinin being blocked in reaching the cell wall. This type of cell wall is depicted in Figure 22.

Both AC60 and AC75 were severely inhibited in growth on calcofluor white agar. In AC75, sensitivity to Zymolyase in association with sensitivity to calcofluor white was

similar to this association in the strains of class I and of AC31 in class III. As stated above, a thin β-1,3 glucan layer in these mutants, would facilitate the binding of calcofluor white to β-1,3 glucan polymers, thereby blocking further growth.

D. Summary

In all classes of strains the absence of cell wall α-agglutinin was associated with attained sensitivity to some aspect of lysis by Zymolyase. Our results showed that co-incidence of phenotypes occurred in class I (excretion of α-agglutinin greater than wild-type), where hypersensitivity to cell lysis by Zymolyase, and sensitivity to calcofluor white were present in strains AC17, AC48, and AC59.

Also in class III, in which excretion of α-agglutinin was less than the wild-type, strains AC31, AC60, and AC76 were hypersensitive to cell lysis by Zymolyase and to calcofluor white.

The sensitivity of all class I and class III strains to calcofluor white, strains that were also sensitive to Zymolyase suggests that calcofluor white can inhibit growth in cells with a diminished inner glucan layer, as discussed above (Table 11). The greater the reduction in β-1,3 glucans the less calcofluor white was required for inhibition of growth. This was shown particularly in strain AC48, which, compared to other strains, had the most rapid maximal lysis rate (Table 11) and was the most severely growth impaired at the lowest concentration of calcofluor white (Figure 9).

These results suggest that sensitvity to calcoflour white might be used as one criterion for the selection of cells with reduced glucan content in the cell walls. The

association of calcofluor white and Zymolyase sensitive phenotypes was shown in a study by Lussier (Lussier *et al.*, 1997). In this study, encompassing the identification of genes involved in cell surface biosynthesis, transposon-mutagenized cells were screened for altered sensitivity to calcofluor white. The results showed that three of four transposon-disrupted genes directly involved in cell surface assembly, *KRE6*, *KRE2*, and *CWP2*, had phenotypes that were hypersensitive to calcofluor white and also hypersensitive to Zymolyase.

III. AC59, class I mutant: complementation by *BET1*

A. Complementation by *BET1*

AC59 is a class I mutant, which by transformation with a YCp50 *Saccharomyces cerevisiae* genomic library, I was able to complement for growth at 37°C. The genomic insert in the complementing plasmid contained three open reading frames, one of which was the *BET1* gene. When I transformed AC59 with pAN101 containing *BET1* it grew at 37°C suggesting that AC59 was a *bet1* mutant.

B. *BET1* and the mutant phenotype of AC59

BET1 encodes a v-SNARE involved in transport of cargo from the ER to the Golgi. The initial selection of AC59 for temperature sensitive absence of cell wall α-agglutinin and for growth at 37°C, and a class I phenotype, can now be associated with a *bet1* mutation. Therefore, an effect of the *bet1* mutation was that at the restrictive temperature AC59 excreted increased levels of α-agglutinin relative to the wild-type.

Another effect of the *bet1* mutation was an aberrant structure of the cell wall. Zymolyase, a protease and β-1,3 glucanase lysed AC59 at a relatively rapid rate after a relatively short lag time compared to the wild-type. When AC59 cells were incubated at 37°C, there was significantly less β-1,6 glucan in the cell walls than at 25°C. The phenotype of the *bet1* mutant AC59 was the first indication in this research that a cell wall aberration as seen in a class I mutant can be caused by a mutation in the secretory pathway.

C. Evidence that *BET1* was the mutant gene in AC59

In order to show that *bet1* was the actual mutant gene in AC59 and did not by over-expression suppress another mutation, I crossed AS59 with the *bet1-1* mutant RSY944. The resulting diploid did not grow at 37°C which was evidence that it was homozygous for *bet1*. I also showed that both AC59 and RSY944 were recessive for temperature sensitivity in a cross with their respective wild-type opposite mating type in which both diploids grew at 37°C.

D. Complementation by *BET1* of mutant phenotype in AC59

Transformation of AC59 with pAN101, containing the *BET1* gene, complemented mutant phenotypes of AC59 for (1) excretion of α-agglutinin: when I transformed AC59 with pAN101 and incubated the cells at 37°C, the amount of α-agglutinin that AC59 excreted was only 1.5% of the amount excreted when transformed by the YCp50 plasmid without an insert (Table 9), (2) growth at the restrictive temperature: when I streaked agar plates with AC59, transformed with *BET1*, and AC59,

untransformed, and incubated the cells at 37°C, only those cells which had been transformed by the *BET1* gene grew at the restrictive temperature (Figure 15), (3) agglutination to cells of the opposite mating type: AC59 was unable to agglutinate to **a**-cells, the opposite mating type and after transformation with *BET1*, AC59 then agglutinated to **a**-cells (Table 10).

These results supported the existence of a *bet1* mutation in AC59 and complementation by the *BET1* gene of the mutant phenotype.

IV. The *BET1* Gene

A. *BET1* identified

BET1 was first identified by Drs. Newman and Ferro-Novick, (1987) while looking for genes in yeast which function in the early part of the secretory pathway. Strains containing *bet1-1* are conditional mutants, temperature sensitive for growth and protein secretion (Newman *et al.*, 1990). At the restrictive temperature, *bet1-1* blocks the transport of the secretory proteins invertase, acid phosphatase, and α-factor to the cell surface, and the processing of the vacuolar protease carboxypeptidase Y is incomplete. In these mutants precursors of the hexose-repressible form of invertase are retained in the ER (Newman and Ferro-Novick, 1987).

B. *BET1* is a v-SNARE

The role of *BET1* in secretion has been well documented. *BET1* encodes a v-SNARE (Whiteheart *et al.*, 1994), an integral membrane protein located on vesicles

involved in transport between the endoplasmic reticulum (ER) and the Golgi (Newman and Ferro-Novick, 1987) Bet1p interacts with v-SNAREs Bos1p and Sec22p on ER-derived vesicles, transporting protein cargo to the Golgi, where the v-SNAREs bind to Sed5p, a t-SNARE on the Golgi. Parlati (Parlati *et al.*, 2000) proposed that in yeast the four integral membrane proteins, Sed5p, Bos1p, Sec22p, and Bet1p each contribute a single helix to form a SNARE complex. The resulting four-helix bundle would function in bringing the vesicle and Golgi membranes into the correct proximity to allow for restructuring of the membranes and the passage of proteins from the vesicle to the target organelle.

C. Bet1p: Size and Location

Bet1p is a relatively small protein, composed of 142 amino acids, lacks a signal sequence, is mostly hydrophilic and has a hydrophobic carboxyl terminal of 19 amino acids. In fractionation studies, Bos1p was found to co-localize with Bet1p and the ER membranes. These data were suggestive that Bos1p and Bet1p were membrane bound on the cytoplasmic side of the ER (Newman *et al.*, 1992).

D. Neuronal homolgues and helical conformation in SNAREs (Table 12)

1. Neuronal homologues of v-SNAREs Bet1p, Bos1p, Sec22p, and t-SNARE Sed5p

Bet1p has similarities to neuronal v-SNAREs, including synaptobrevins, a family of proteins which are found on synaptic vesicles. The synaptobrevins are composed of three domains: an initial region rich in prolines or asparagines, a hydrophilic "core"

middle segment, and a hydrophobic C-terminal transmembrane anchor. The hydrophilic core of synaptobrevins has a high α-helical potential. Similarly the secondary structure of the Bet1p middle region (amino acids 50-115) predicted significant α-helical potential. This indicated that *BET1* coded for a putative membrane protein similar in structure to a protein found on synaptic vesicles (Newman *et al.*, 1992).

Further evidence of homology to neuronal synaptic vesicle release existed in ER to Golgi transport. The v-SNAREs Bos1p and Sec22p. also have regions homologous to synaptobrevins (Sacher *et al.*, 1997). Sed5p, a receptor on the Golgi, classified as a t-SNARE has regions of homology to syntaxin.

In a database search for the optical alignment of the best segment of similarity between two segments, Stone (Stone *et al.*, 1997) found that Bet1p amino acids 61-106, found to be 21% homologous with 5 gaps to amino acids 50-102 of *Drosophila melanogaster* synaptobrevin, was more significantly similar to *D. melanogaster* SNAP-25 (Figure 23). A BLAST program (Altschul *et al.*, 1990) revealed that the same approximate region of Bet1p, amino acids 55-93 was 36% identical and 61% similar to amino acids 151 of *D. melanogaster* SNAP-25 (synaptosomal-associated protein of 25 kDa). There are no gaps in this region of the alignment (Gonzalo and Linder, 1998). The structure of SNAP-25 suggested a functional homology with *BET1*. SNAP-25 forms a ternary complex with two other exocytotic proteins: syntaxin and the synaptic vesicle synaptobrevin (Hodel, 1998). In neurons SNAP-25 facilitates the binding of synaptobrevin to syntaxin (Pevsner *et al.*, 1994) If there were a similar binding

```
SNAP-25 143 ENEMDENLEQV S GI IGN LRHMALDM GNEIDTQNRQ IDRI 181
Human       | . :  : |. : : . : : . | .| : : . |. | | :| | . |. | | . :
Bet1p    55 ESQ S EEQMGAMGQRIKALKS L S LKMGDEIRGS NQTIDQL 93
            | .:  | | . | | . : .  .|  .| : . : . | . | | .| : . |. | | . :
SNAP-25 151 EDEMEENMGQVNTMIGN LRNM ALDM GSELENQNRQIDRI 189
D. melanogaster
```

Region	Bet1p percent similarity	Bet1p percent identity
SNAP-25		
Human	**82% (no gaps)**	**31%**
D. melanogaster	**85% (no gaps)**	**36%**

Figure 23. Bet1p domain similar to region of SNAP-25. Amino acid sequence of Bet1p from residues 55-93 compared with residues 151-189 of *D. melanogaster* SNAP-25 and residues 143-181 of human SNAP25. Identity is indicated by a line and conserved changes are indicated by two dots (two corresponding bases in their codons) or one dot (one corresponding base in their codons) between the seqences.

(Stone *et al.*, 1997)

mechanism in yeast, then the SNAP-25-like Bet1p would potentiate the binding of the synaptobrevin-like Bos1p to the syntaxin-like Sed5p.

Table 12. Yeast SNAREs structurally related to neuronal components

Yeast SNAREs	Neuronal Counterpart	Neuronal Function	Yeast Function
Bos1p	Synaptobrevin	v-SNARE in fusion of synaptic vesicle and terminal membranes during exocitosis	v-SNARE in fusion of ER derived transport vesicle and Golgi membranes
Sec22p	Synaptobrevin	Same	Same
Bet1p	SNAP-25	Potentiates binding of synaptobrevins and syntaxins	v-SNARE. Potentiates binding of Bos1p to Sec22p and Sed5p
Sed5p	Syntaxin	Neuronal t-SNARE. Binds to synaptobrevins and SNAP-25, forming four-helix bundle	Yeast t-SNARE. Binds to Bet1p, Bos1p, and Sec22p, forming four-helix bundle

(Ferro-Novick and Jahn, 1994; Hodel, 1998)

2. X-Ray crystal structure showed the SNARE complex to be a four-helix bundle

The X-ray crystal structure of a core synaptic complex containing syntaxin-1A, synaptobrevin-II, and the N and C terminals of SNAP-25-B revealed a four helix bundle (Sutton *et al.*, 1998). This result was supported by the crystal structure of a post-Golgi SNARE complex in yeast showing that the synaptic SNARE complex is a four helix coiled-coil with a hydrophobic core. The helix was composed of a syntaxin homologue,

Sso1, a synaptobrevin homolologue, Snc2, each of which contributes one helical element and a SNAP 25 homologue, Sec9 which contributes two helices (Katz *et al.*,1998; Samuel Lunenfeld Research Institute, Internet). Although the X-ray crystal structure of the ER to Golgi SNARE complex has not been done, the existence of yeast homologues of neuronal SNAREs supports the existence of an ER to Golgi four helix SNARE model, as shown for the post-Golgi SNARE complex.

E. Loss of α-helical conformation and binding capacity in a mutant Bet1p

Since EMS mutagenesis was used in creating the strains used in this research this method of mutagenesis might then explain the source and consequences of the *bet1* mutation in AC59.

1. Amino acid substitution results in a loss of α-helical conformation

A mutation in Bet1p resulting in a change in one amino acid demonstrated the loss of α-helical conformation and inability to interact with other SNAREs (Stone *et al.*,1997). A mutated form of Bet1p in which leucine at amino acid 72 was substituted by phenylalanine (L72F) failed to bind to Bos1p as well as to Sed5p, suggesting that Bet1p (L72F) lacked the ability to enable v-SNARE-t-SNARE interaction. CD spectroscopy of Bos1p showed minima at 208 and 222 nm, indicative of classic α-helix formation. Bet1p had a minimum only at 222nm indicative of an α-helix, but otherwise random coil conformation. The α-helical form of Bet1p and Bos1p is consistent with the protein-protein binding of SNAREs in a coiled-coil formation. The mutant Bet1p (L72F) no longer displayed a minimum at 222 nm, indicating it had lost the α-helix

structure. The inability of Bet1p (L72F) to interact with Bos1p or Sed5p was a consequence of a change in the α-helical structure of mutant Bet1p (L72F) which resulted in the loss of binding capacity (Stone, 1997). The specific mutation in *bet1* in AC59 has not been determined, however based on the above results EMS mutagenesis in AC59 might have similar consequences.

V. The *bet1* Paradox: Excretion of α-agglutinin into the medium in a secretion blocked pathway

A. Introduction

Here we have seen in strain AC59 that a mutation in the v-SNARE *BET1* resulted in a cell wall with a greatly reduced ability to resist lysis and a cell wall in which α-agglutinin and possibly other mannoproteins were not anchored and were excreted. The mutation also resulted in a diminution of β-1,6 glucan in the cell wall, demonstrating the dependence of cell wall biosynthesis on the secretory system. The remaining question was how a *bet1* block in the secretory pathway resulted in the AC59 phenotype described.

B. The *bet1* paradox

The paradox of excretion of α-agglutinin into the medium in a secretion-blocked pathway was resolved by the discovery of a post-ER pool of α-aggltinin. In parent strain W303-1B α-agglutinin continued to be secreted to the cell wall in the presence of cycloheximide, an inhibitor of translation (Figure 16). In the mutant strain AC59 the

results were similar, in the presence of cycloheximide or at the restrictive temperature, except that α-agglutinin was excreted into the medium instead of being cross-linked to the cell wall (Figure 17). Consistent with the existence of a pool of α-agglutinin I showed that the pool was finite when after two hours of incubation at the restrictive temperature the pool was depleted and was not regenerated during a second two hours (Figure 18).

C. Origin of a post-ER pool of α-agglutinin

The existence of a pool leads to the question of the origin of such a pool. It is possible that the accumulation of a pool of α-agglutinin might be the result of the rate limiting process of secretion of α-agglutinin from the Golgi to the cell wall. A pulse-chase analysis of the secretory pathway of α-agglutinin (Lu *et al.*, 1994) showed some evidence for this possibility. A 150-kDa form of α-agglutinin first appeared in the Golgi in 5 minutes. In the post-Golgi sequence, the membrane bound > 300-kDa form peaked at 5 to 10 minutes and a soluble > 300-kDa form peaked at 10 to 20 minutes, with both forms subsequently disappearing. The cell wall form of α-agglutinin was initially detected at 20 minutes and reached a maximal level at about 45 minutes (Lu et al. 1994). The difference between the maximal cell wall form appearing at 45 minutes and a Golgi form first appearing in 5 minutes would represent a 40 minute time period during which α-agglutinin, after a block in secretion, could continue to be transported to the cell wall in W303-1B, wild-type cells and excreted in AC59, a *bet1* mutant.

A rate limiting pathway for the secretion of α-agglutinin was suggested in a time- course assay done by Terrance and Lipke (1981). When α-cells were induced to synthesize α-agglutinin by a-factor, it took 60 to 90 minutes for full induction to occur. A comparison with a-cells showed that maximal induction was reached in only 18 minutes. These results suggest that in α-cells the length of time required for full induction of α-agglutinin creates an opportunity for a reserve to accumulate.

D. Existence of another intracellular pool and its functional value in *S. cerevisiae*

An example in *S. cerevisiae* in which an intracellular pool is used to provide an enzyme within a specific time period is that of Chs3p. Chs3p is the catalytic subunit of chitin synthase III which synthesizes a ring of chitin at the onset of bud emergence.Chs3 is synthesized continuously and remains metabolically stable. It has been shown that Chs3 is temporarily sequestered in chitosomes, endosomal structures distinct from vacuoles and mature secretory vesicles (Lagorce *et al.*, 2002; Ziman *et al.*, 1996). It was proposed by Ziman that the function of chitosomes is to act as a pool of chitin synthase enzymes, ready to be mobilized, for chitin ring formation, as regulated by the cell cycle (Ziman *et al.*, 1996; Ziman *et al.*, 1998). Transport of Ch3p to the site of formation of the chitin ring might then be mediated by a secretory pathway involving vesicular transport from the endosome to the plasma membrane (Chuang and Schekman, 1996; Ziman *et al.*, 1996).

E. Functional significance of a pool of α-agglutinin in *S. cerevisiae*

The existence of an internal pool of α-agglutinin might be an advantage in mating. Upon the detection of pheromones of the opposite mating type, cell division is arrested in G1 phase. A mating projection is then formed after polarization of the actin cytoskeleton and subsequent polarized cell wall deposition. This type of polarized cell is called a "shmoo." The formation of the mating projection requires the synthesis and integration of new cell wall components. During the formation of the mating projection the cell wall changes and α-agglutinin becomes highly expressed and is deposited mostly in the shmoo tip (Smits *et al.*, 2000). As described previously, in the time course assay of Terrance and Lipke (1981), after the addition of the pheromone a-factor, full induction of α-agglutinin took 60 to 90 minutes. Subsequent to induction, the mating projection (shmoo) was visible within 1 hour (Lipke, Ph.D. thesis, 1979). In view of the length of time required for α-agglutinin to reach the cell wall after pheromone induction, the existence of a pool of α-agglutinin might be an advantage or a requirement in achieving coordination between the formation of a shmoo and secretion of α-agglutinin to the shmoo tip.

VI. Evidence contradicting a *BET1*-independent secretion pathway

The existence of a pool of α-agglutinin does not preclude a hypothesis that an alternate *BET1*-independent pathway is responsible for secretion of α-agglutinin to the

cell wall and excretion from the cell in AC59 at the restrictive temperature. Consistent with this possibility, Muniz (Muniz *et al.*, 2001) showed that GPI-anchored proteins exit the endoplasmic reticulum in vesicles distinct from those of other secretory proteins.

However, there is experimental evidence against a *BET1*-independent secretion of α-agglutinin. If the excretion of α-agglutinin were *BET1*-independent, then at the restrictive temperature, excretion of α-agglutinin by AC59 would be greater in the absence of cycloheximide than in the presence of cycloheximide. Our results showed that, at 37°C, in the presence or absence of cycloheximide, excretion by AC59 of α-agglutinin was the same (Figure 17). That result shows that secretion to the cell surface and subsequent excretion was blocked by a source other than cycloheximide. Therefore, if an alternate secretion pathway does exist for α-agglutinin it is *BET1*-dependent.

VII. Secretion pathway and synthesis of the cell wall components

A. β-1,6 glucan expression is *BET1*-dependent

The excretion of α-agglutinin would occur if the *bet1* mutation blocked it from anchorage to the cell wall. Our results suggested this possibility. We showed that when AC59 was incubated at the restrictive temperature, the amount of β-1,6 glucan on the cell wall was greatly diminished relative to incubation at the permissive temperature. Since α-agglutinin binds to β-1,6 glucan, the result of diminution of β-1,6 glucan at the restrictive temperature is consistent with excretion of α-agglutinin from the cell. These results would be explained if β-1,6 glucan synthesis were dependent on the secretory

pathway and α-agglutinin anchorage were dependent on β-1,6 glucan synthesis.

B. Location of β-1,6 glucan synthesis

There are two current views regarding the method of synthesis of β-1,6 glucan. One view is that the synthesis of β-1,6 glucan occurs intracellularly, beginning in the ER with the formation of protein bound primer structures, and then is extended in the Golgi, with some Kre proteins acting as glucosyl transferases. This view is based on the fact that both β-1,3 glucan synthase and chitin synthase are located at the plasma membrane, but a similar synthase for β-1,6 glucan has not been found (Shahinian and Bussey, 2000). In support of this view, is the result with *Schizosaccharomyces pombe*, where gold labeled β-1,6 glucan was found on the Golgi and on small vesicles (Humbel *et al.*, 2001). Another view is that synthesis of β-1,6 glucan occurs at the plasma membrane. This view is based on the inability of β-1,6 glucan antibodies to detect β-1,6 glucan, except at the cell surface (Montijn *et al.*, 1999). The inability of antibodies to detect intracellular β-1,6 glucan might occur if the synthesis of β-1,6 glucan were not completed until reaching the cell wall. Since a *bet1* block in secretion from the ER to the Golgi resulted in a diminution of β-1,6 glucan in the cell wall, this suggests the possibility that the synthesis of β-1,6 glucan is initiated in the ER and continues in intracellular biosynthetic steps to the cell wall. It is also possible that β-1,6 glucan is synthesized at the cell wall but that enzymes required for synthesis are transported via the secretory pathway and would be blocked by *bet1* at the ER.

C. *KRE* genes and cell wall β-1,6 glucan

KRE genes were first identified as involved with β-1,6 glucan synthesis and/or assembly, in screening for the resistance to K1 killer toxin. The K1 killer toxin binds to β-1,6 glucan and those cells resistant to killer toxin called *kre* (Killer Resistant) mutants, were deficient in β-1,6 glucan. Based on the location of Kre proteins which correspond to complemented *kre* mutants, Shahinian (Shahinian and Bussey, 2000) proposed that the synthesis of β-1,6 glucan occurs in intracellular biosynthetic steps, following the secretory pathway from the ER to the cell wall. The *KRE5* gene encodes an ER glycoprotein containing an ER retention signal. Deletion of *KRE5* led to the complete loss of β-1,6 glucan (Meaden *et al.*, 1990). The function of Kre5p is not known (Shahinian and Bussey, 2000). It has been proposed that *KRE5* gene products are involved in the core synthesis of β-1,6 glucan in the ER. This concept was based on the likelihood that UDP-glucose is a substrate for β-1,6 glucan synthesis since it is a substrate for β-1,3 glucan synthesis (Meaden *et al.*, 1990). There is evidence that uridine diphosphate (UDP)-glucose can be transported into the lumen of the ER (Castro and Abeijon, 1999). Since a glucosyl transferase has not been found in *Saccharomyces cerevisiae*, it has also been suggested the Kre5p, located in the ER, might perform this function (Castro and Abeijon, 1999).

A hypothetical model of β-1,6 glucan synthesis, based on the above sequence of *kre* mutants' defects in β-1,6 glucan synthesis, is shown in Figure 24. In support of core

synthesis of β-1,6 glucan in the ER was the precedent of ER synthesis of glycoconjugates of proteins (Kornfeld R., and S. Kornfeld, 1985). Kre5p, using UDP-glucose as substrate might then function as a glucosyltransferase (Shahinian *et al.*, 1998).

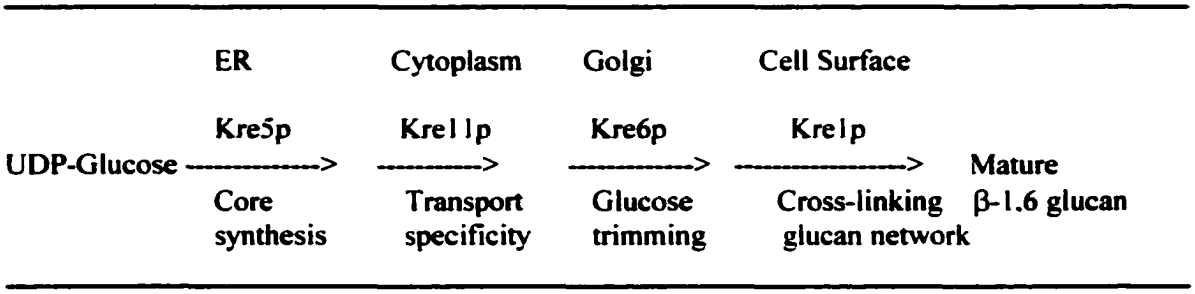


Figure 24. Model for β-1,6 glucan biosynthesis.
Kre5p in the ER is involved in core synthesis of β-1,6 glucan. In the Golgi, Kre6p acts as a glucanase. Kre11p, a TRAPP subunit, plays a role in trafficking of β-1,6 glucan components from the ER to the Golgi. At the cell surface, Kre1p functions in cross-linking β-1,6 glucan in the cell wall (Meaden *et al.*, 1990; Shahinian and Bussey, 2000).

D. Kre5p function and the *bet1* block

The *kre5* mutation and *bet1* have similar effects on the diminution of cell wall β-1,6 glucan, *bet1* in blocking ER to Golgi secretion, and Kre5p with a still unknown function. It might be possible that Kre5p is involved in core β-1,6 glucan synthesis in the ER and that *bet1* blocks transport to the Golgi. It might also be possible that the product of Kre5p activity is transported via the secretory pathway from the ER and is required for the synthesis of β-1,6 glucan at the cell surface but can be blocked by *bet1*. This product may be β-1,6 glucan itself, or an enzyme required in the process of β-1,6 glucan synthesis.

E. Consequences of *bet1* in AC59

The *bet1* mutation in AC59 was manifested in several observed phenotypes. AC59 was hypersensitive to cell lysis with Zymolyase and was growth impaired on calcofluor white. At the restrictive temperature, AC59 excreted increased levels of α-agglutinin. As suggested by previous sections, a *bet1* block in ER to Golgi transport of a β-1,6 glucan precursor and/or biosynthetic enzymes might explain these observed cell wall phenotypes. A model of the consequences of *bet1* in AC59 is shown in Figure 25.

VIII. The significance of the secretory system and biosynthesis of the cell wall in the life of the cell.

The functions of growth and reproduction, basic to continuity of a species, are carried out within a single yeast cell. In order to carry out the chemical and molecular activities needed for cell survival yeast cells require distinct cellular compartments or organelles and the protection of a cell membrane and a wall. The organelles and cell wall in turn require a continuous supply of newly synthesized proteins, polysaccharides, and lipids. Essential in the organization of the cell is the ability to transport molecular cargoes within the cell in a time-dependent fashion to the appropriate target. The biosynthesis of the cell wall depends on secretion of mannoproteins destined for the outer wall, enzymes required in the structure of the glucan inner wall and enzymes required for cross-linking the constituent subunits of the entire wall. The absence of α-agglutinin from the cell wall was used as a marker for both a compromised secretion

system and compromised cell wall integrity. We were thus able to show that when the secretion system was blocked in its function with a v-SNARE mutation (in *BET1*) the result was an aberrant cell wall and excretion of α-agglutinin. I thus demonstrated how the integrity of the secretory system and the cell wall are interdependent.

Figure 25. Model in AC59 of Transport from ER to Golgi at 37°C.

Figure 25 (a) Transport from ER to cell wall in *bet1* cells at permissive temperature. In this figure α-agglutinin and enzymes and/or core factors required for β-1,6 glucan synthesis are being transported from the ER to the Golgi. Based on a two-vesicle hypothesis in which GPI-anchored proteins were shown to be transported in vesicles separate from other proteins (Muniz *et al.*, 2001), α-agglutinin and β-1,6 glucan factors are represented in separate vesicles. Bet1p is depicted as an intact v-SNARE and α-agglutinin is depicted attached to β-1,6 glucan on the cell wall. Transport of β-1,6 glucan factors to the cell wall is shown to precede α-agglutinin as symbolic of the relatively faster rate of β-1,6 glucan synthesis.

Figure 25 (b) *bet1* cells 5 minutes after transfer to 37°C. The *bet1* mutation manifested at 37°C has resulted in the inability of Bet1p to form an α-helix conformation required for binding to Bos1p. Consequently the four-helix bundle consisting of the three v-SNAREs, Bet1p, Bos1p, and Sec22p and one t-SNARE Sed5p cannot be formed. This failure prevents the fusion of the transport vesicle and Golgi membranes, blocking transport of proteins from the ER to the Golgi. At 5 minutes β-1,6 glucan that was in the Golgi or further along in the secretory pathway would have reached the cell wall and been secreted. Similarly some post-ER α-agglutinin would have reached the cell wall

but due to the relatively slower kinetics of α-agglutinin synthesis (Lu *et al.*, 1995), there is still a pool of α-agglutinin in the process of being transported to the cell wall.

Figure 25 (c) *bet1* cells 30 minutes after transfer to 37°C. After 30 minutes at 37°C, the *bet1* block prevents more α-agglutinin and β-1,6 glucan precursors or glucan synthase from reaching the Golgi or other late compartments. Thus there is no β-1.6 glucan to serve as anchor for α-agglutinin, which continues to be secreted from its post-ER pool The unanchored α-agglutinin is excreted into the growth medium.

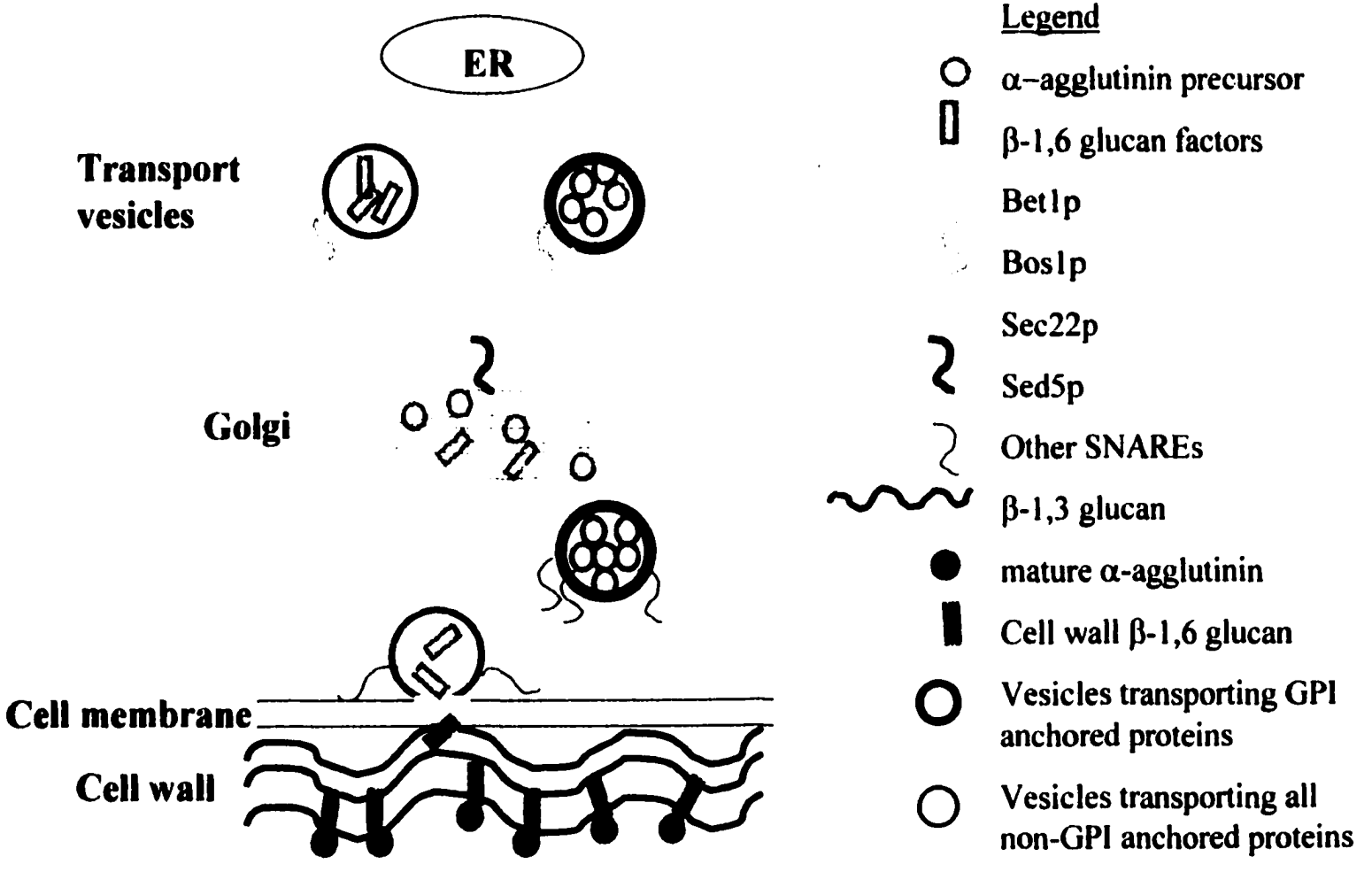


Figure 25(a). Transport from ER to cell wall in *bet1* cells at permissive temperature

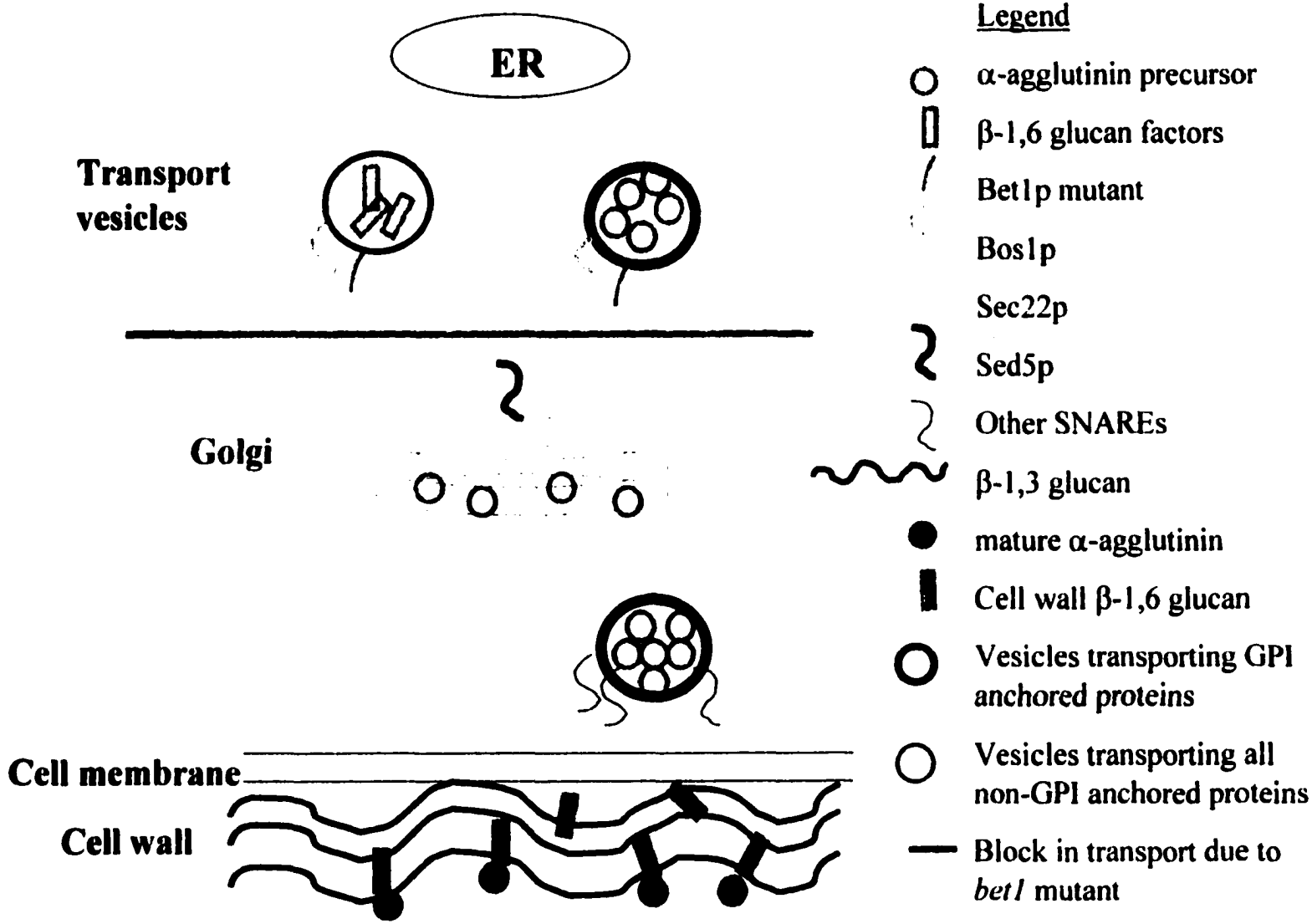


Figure 25 (b) *bet1* cells 5 minutes after transfer to 37°C

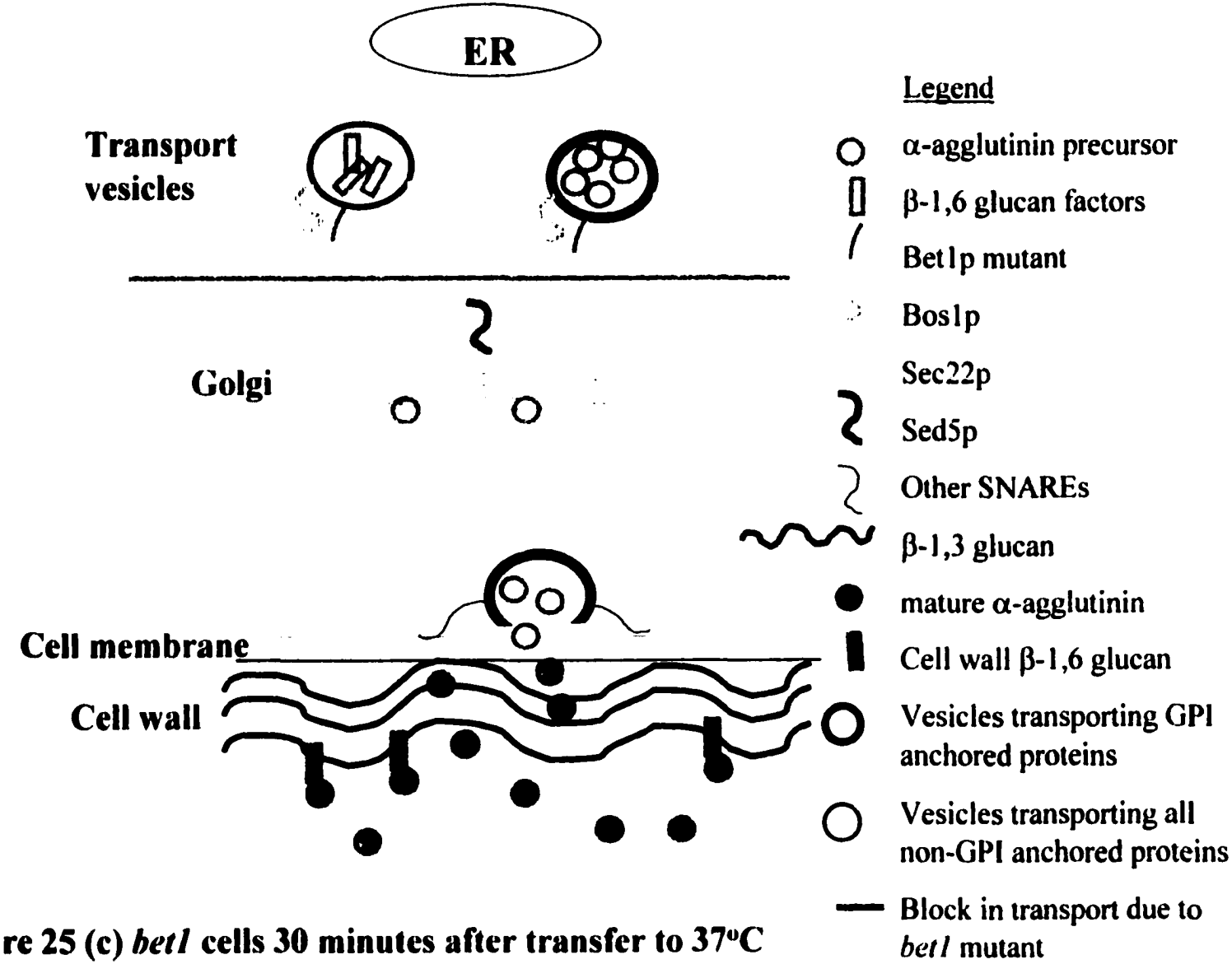


Figure 25 (c) *bet1* cells 30 minutes after transfer to 37°C

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids are listed in Table 13.

Table 13

Strain	Genotype or derivation	Source
S. cerevisiae		
W303-1A	*MATa ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11,15*	R. Rothstein
W303-1B	*MATa ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11,15*	R. Rothstein
X2180-1A	*MATa SUC2 mal mel gal2 CUP1*	Yeast Stock Center
X2180-1B	*MATα SUC2 mal mel fal2 CUP1*	Yeast Stock Center
RSY 944	*MATa bet1-1 lys2-801 ura3-52*	R. Schekman
N4351A	*MATa his7(ts) met6 arg1 gal4 MAL25 SUC*	Yeast Stock Center
Escherichia coli		
XL1Blue		Stratagene (LaJolla, CA)
Plasmids		
YCp50	*Amp Tet ARS1 CEN4 URA3*	J. Hirsch
pAN101	*MATα BET1 amp tet ARS1 CEN4 URA3*	S. Ferro-Novick

Media and Growth Conditions

Yeast and *E. coli* Transformation

The YCp50-based yeast genomic library 3JDAF2 (kindly provided by Dr. Jeanne Hirsch, Mt. Sinai School of Medicine) was used for yeast transformation. The library was created by insertion of *Sau*3A fragments of yeast genomic DNA into the *Bam*HI site in YCp50. Transformation of yeast cells was done by the method of Ito *et al.* (1983)

Plasmid Isolation

Plasmids from yeast transformants were isolated using the method of Hoffman and Winston (1987). A Qiagen Plasmid Purification Kit (Qiagen, Inc., USA) was used to purify plasmid DNA for yeast transformation and sequencing.

Media

Minimal medium (YNB): contains (in grams per liter) yeast nitrogen base (1.7) (Bio 101), ammonium sulfate, 4.5; glucose 20.

Complete medium (YNB-C): YNB supplemented with amino acids as appropriate

Assay buffer: 0.1M sodium acetate, pH 5.5.

YEPD: contains (in grams per liter) yeast extract, 10; Bacto-peptone, 20; glucose, 20.

Growth Rate

Cells were grown in 5 mls of YEPD or YNB-C, shaking at 120 rpm. The optical density (OD) was measured beginning at early logarithmic phase and continued at 1 or 2 hour intervals for at least 3 readings. Since the rate of growth in *S. cerevisiae* is exponential, the results are shown on a logarithmic graph. The OD is represented on the graph as the OD divided by the initial optical density, OD(i) for each strain. The number of cells per ml is based on the following formula: OD_{660} 0.3 = 10^7 cells/ml.

Quantitative Agglutination Assay for α-agglutinin on the cell surface

Agglutination assays were performed as described previously Terrance and Lipke (1981). W303-1A (tester **a**-cells) and W303-1B cells, to be tested, were grown in YNB-C to OD 0.1 to 0.5 (660 nm). The cells were washed and resuspended in Assay buffer to OD 3.0. The **a**-cells had been induced with 50 ng/ml α-factor for 45 minutes. Samples (1 ml) of each cell type were added to 13-by 100-mm test tubes. Control tubes received 2 ml of a single cell type. The volume of each tube was brought to 3 ml with assay buffer The tubes were vortexed and centrifuged at 200 x g (900 rpm) for 5 minutes at 20° C. The cells were resuspended with a 7- by 90-mm paddle at 1.000 rpm for 6 sec. A stop maintained the paddle at 6 mm above the bottom of the tube. A constant speed control unit (Cole-Parmer Instrument Co., model 4420) assured reproducible results. The suspensions were left undisturbed for 20 min to allow settling of the aggregates and, using the test tubes as cuvettes, the optical density was read at 660 nm in a Bausch & Lomb Spectronic 21 DV. The samples were run in duplicate or triplicate.

The agglutination index (AI) was defined as

$$AI = 1 - \frac{[2 \cdot A(a+\alpha)]}{(Aa + A\alpha)}$$

$A(a+\alpha)$ = Average OD of incubation of a + α cells
Aa = Average OD of incubation of a cells only
$A\alpha$ = Average OD of incubation of α cells only

The AI varies from 0 to 1, with higher indices denoting a greater degree of agglutination.

Quantitative Agglutination Assay for α-Agglutinin Excreted into the Media

X2180-1B (tester α-cells) and X2180-1A (tester **a**-cells) were grown separately in YNB media to OD 0.1 - 0.3. The **a**-cells were then induced with α-factor (50 ng/ml) for 45 minutes. The cells were harvested and washed in assay buffer and resuspended in assay buffer to OD 3.0. Samples (0.2 mls) of **a**-cells and samples of supernatant containing putative secreted α-agglutinin of strain AC59 or of W303-1B were added to 13 by 100-mm test tubes. Assay buffer was added to total 2.8 mls. Control samples (0.4 mls) of **a**-cells only were added to 3 test tubes containing 2.6 mls assay buffer. All cells were incubated for 90 minutes at room temperature, shaking at 120 rpm. After incubation, samples (0.2 mls) of X2180-1B α-cells were added to the tubes containing the tester **a**-cells and the supernatant of strain AC59 or of W303-1B cells. As a control samples (0.2 mls) of X2180-1B α-cells were also added to 3 tubes containing 0.2 mls of a-cells. Control samples (0.4 mls) of α-cells only were added to 3 tubes containing 2.6 mls of assay buffer. All tubes were vortexed and centrifuged at 200 x g (900 rpm) for 5 minutes at 20°C. The subsequent procedure followed was as described above under "Quantitative Agglutination Assay for α-agglutinin on Cell Surface." The units of activity

of α-agglutinin in the supernatant was defined as

$$\text{Units of Activity} = \frac{10 \cdot [OD\ (sample)] - [OD\ (a + \alpha)]}{\frac{[OD(a)) + (OD(\alpha)]}{2}}$$

The greater the amount of α-agglutinin in the supernatant sample the more the a-cell receptors will be blocked by α-agglutinin, the less α-cells added later will be able agglutinate to a-cells. and the greater will be the absorption or optical density (OD) of the sample.

Cell Lysis by Zymolyase

Enzyme preparation and cell wall lysis assays were done based on the previously decribed method (Ovalle. *et al.*, 1999) which was modified. Cells were grown in 5 mls of 1% yeast extract, 2% peptone, 2% glucose (YEPD) to OD 0.4 (660nm), harvested. and washed 3 x with deionized water. The pellets were resuspended to OD 0.6 in 3 mls of TE buffer pH 7.5 (50 mM Tris-HCl + 5 mM EDTA) + 5% PEG 8000 which was added just before use. The cells were then incubated at 25°C for 30 minutes. 200 ul of each sample was added to microtiter plate wells in sets of 3 wells per sample. 40 µg/ml of Zymolyase (100 T; ICN) was added to each set of 3 wells. As a control, water only was added to one set of wells. The Zymolyase and substrate were mixed in the wells with a multipipetter and the readings of the results were immediately started using an ELISA reader, read at one minute intervals, and was continued for 1 hour. For each type of sample the optical density (OD) for each well is divided by the initial optical density for that well (ODi). The statistical results of a multiple regression curve is used to determine the maximum lysis rate and lag time. The lag time (LT) is the period of time before the beginning of rapid

cell lysis. The maximum lysis rate (MLR) refers to the slope of the least-squares fit line for the region of the curve with the steepest decline (Ovalle *et al.*, 1998).

Invertase Assays

The amount of invertase on the cell surface and secreted into the medium was determined by measuring the glucose present after hydrolysis by invertase of sucrose into glucose and fructose (Kwon-Chung *et al.*, 1990; Jue and Lipke, 1985). AC59 was grown to OD 0.225, pelleted at 4000 rpm for 6 minutes and resuspended in 1% yeast extract/1% peptone (YP) containing 0.1% glucose, shaking at 120 rpm, at room temperature, for 35 minutes to derepress the expression of invertase. The culture was divided into 2 flasks, and incubated at 37°C and 28°C, respectively, for 2 hours. The cells were harvested and the pellets and supernatants saved. The supernatants were dialyzed in 0.01M sodium acetate, pH 5.5, lyophilized, and resuspended in H_2O to 1/4 of the original volume. The pellets were resuspended in 1 ml of 0.1 M sodium acetate, pH 5.5 and 10 mM sucrose. Tubes were incubated at 30° C for 30 minutes to allow the hydrolysis by invertase of sucrose into glucose and fructose. 40 ul of each sample was added to13x100 mm tubes in sets of 3. To determine the amount of glucose in the mixture, 4 mls of tetrazolium blue reagent was added to each tube, the tubes were heated in a boiling water bath for 3 minutes, cooled, a spectrophotometer reading was taken at 660 nm and the µg of glucose was calculated based on a standard curve (Jue and Lipke, 1985). The units of activity of invertase were defined as

µMol reducing sugar/(min · cell)/2.

Calcofluor White Plate Assays

Calcofluor white plates were prepared according to Ram (Ram *et al.*, 1994). Cells were grown overnight in YEPD and dilutions of 10^6,10^5, 10^4, and 10^3 cells/ml were made. Three microliters of each dilution series were then spotted onto a series of petri dishes containing calcofluor white in concentrations of 0, 5, and 10 μg/ml. The growth of respective strains was determined after 4 days at room temperature.

Experimental procedure using cycloheximide and a-factor to determine existence of pools of α-agglutinin.

I. W303-1B, wild type cells

W303-1B cells were grown in YNB-C to OD 0.3. Cells were pelleted and resuspended in one-half volume of fresh YNB-C to OD 0.6. The cells were then divided into four tubes of 5 mls each. Cycloheximide (10 μg/ml) was added to tube 1 only. All four tubes were incubated at 30 C , 150 rpm. After 30 min. 5 mls of **a**-factor pheromone (supernatant of **a**-cells grown to stationary phase) was added to tubes 1,2 and 3. At that time cycloheximide (10 μg/ml) was added to tube 2. Tube 4 contained neither cycloheximide nor **a**-factor. BSA (0.2 μg/ml) was added to each tube. The content of tubes, in addition to BSA, was as follows:

Tube 1:	cycloheximide added 30 min. before **a**-factor
Tube 2	cycloheximide added at same time as **a**-factor
Tube 3	no cycloheximide, only **a**-factor
Tube 4	no cycloheximide, no **a**-factor

The tubes continued to incubate at 30° C, at 150 rpm for 1 hour. The cells were then pelleted, resuspended in 0.1M sodium acetate, pH 5.5, to be used in a quantitative agglutination assay as described in Materials and Methods.

II. AC59, mutant cells.

Strain AC59 cells were grown in YEPD to OD 0.3 (660 nm). The cells were pelleted and resuspended in ½ volume YNB-C to OD 0.6 and divided into 4 tubes at 5 mls/tube. a-Factor (supernatant of induced X2180-1A cells grown to stationary phase) was added to 2 tubes.10 μg/ml of cycloheximide was added to one of these tubes. As a control. α-factor (supernatant of X2180-1B cells grown to stationary phase) was added to 2 tubes. and 10 μg/ml of cycloheximide was added to one of these tubes. The tubes were incubated at 37°C for 1 hour. shaking at 150 rpm. The cells were harvested and the supernatants were saved, lyophilized and resuspended to 1/10 of the original volume. A quantitative agglutination assay was done for secretion of α-agglutinin, as described in Materials and Methods.

Assay for β-1,6 glucan in cell walls of AC59 *bet1* mutants, grown at 37°C vs 25° C

AC59 cells were grown in YEPD to OD 0.1, divided into 2 flasks and incubated at 25° C and 37° C resp., shaking at 120 rpm, for 3 hours. The cells were pelleted, washed in wash buffer, and lysed, as described previously (Lu *et al.*, 1994). with modifications. Cells were broken with 3 g of glass beads in 1.5 mls of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 ul protease inhibitor cocktail/ml (Sigma)]. The cells

were centrifuged at 5000 rpm (1000g) for 10 min. A hot SDS extraction of the pellet was done twice with 10% SDS in lysis buffer heated for 5 min at 95° C., in order to remove non-covalently bound cell wall proteins. The extracted pellet was washed three times with cold 1 M NaCl containing protease inhibitor cocktail, and three times with sterile cold distilled water containing protease inhibitor cocktail. The cells were treated with 25 U of Laminarinase (Sigma cat # 5272) in 120 µl of 50 mM sodium acetate (pH 5.5) containing 2 mM EDTA and protease inhibitor cocktail. After 4 hours incubation at 35°C, the insoluble residue was pelleted at 13000 rpm for 5 min at 4° C. The supernatant containing covalently bound cell wall glucans and proteins was used in an ELISA assay for β-1,6 glucan content.

ELISA for β-1,6 glucan

ELISA Assays were carried out by the method of Reen (1994). Microtiter plate wells were coated with 2 µl of laminarinase extract in 50 µl of 0.1M carbonate/bicarbonate antigen coating buffer, pH 9.6: 0.1M Na_2CO_3 (29.3 ml) and 0.1M $NaHCO_3$ (70.7 ml) in sets of 3 wells per sample. The plates were incubated at 37° C. for 2 h, washed 3x with PBS, and blocked with 3% BSA for 1 h at room temperature. The plates were washed as before and 50 µl of β-1,6 glucan antibody (#8296, P.N. Lipke Lab) was used at a concentration of 1:1000 in PBS was added to each well. The β-1,6 glucan antibodies used were provided by Dr. Fred W. Quimby, College of Veterinary Medicine. Cornell University, and purified in the P.N. Lipke lab. The microtiter plates were incubated at 37 °C for 1 h, washed, and 50 ul of the secondary antibody goat anti-rabbit alkaline

phosphatase (Sigma) was added at a concentration of 1:100. The plates were incubated at 37° C. for 1 h, washed and the developed with the substrate pNitrophenyl phosphate in diethanolamine. After 8 minutes the plates were read at 405 nm.

Phenol Sulfuric Acid determination of hexose content of laminarinase extract of β-1,6 glucans

A. Preparation of a standard curve for glucose

A standard curve for glucose was created as follows: Glucose was prepared at 1mg/ml and aliquoted in duplicate into tubes at 0,10, 20 and in increments of 20 thereafter to 100 µl per tube. To each tube 0.5 ml 4% phenol (diluted in water) was added. Then to each tube 2.5 mls of sulfuric acid was added, the tube was vortexed and allowed to stand at room temperature for 10 minutes. The absorbance was read at 405 nm for each tube and a standard curve for µg glucose vs optical density was thus established.

B. Determination of glucose in laminarinase extract and pellet in cell wall

Samples of laminarinase extract of supernatant and pellet of isolated cell walls of AC59 were aliquoted into tubes, in duplicate, at 1 µl, 3 µl, and 10 µl per tube. One tube contained 25 µg glucose, as a control. To each tube 0.5 mls of 4% phenol was added and 2.5 mls of sulfuric acid was then added. The tubes were vortexed, allowed to cool and the optical density was read ad 405 nm. Using the standard curve for µg of glucose vs absorbance, the µg of glucose per sample was determined, and the glucose/cell then established.

Assay for α-Agglutinin excreted into the media at 30°C

Cells were grown in 5 mls of YNB-C to mid-exponential phase (OD_{660} 0.3 - 0.6). The respective strains were separated into 1ml samples and incubated for 3 more hours at 30°C, centrifuged at 5000 rpm, and the supernatants saved to be used in an ELISA assay.

ELISA for excreted α-agglutinin

Mictrotiter plate wells were coated with Concanavalin A (Con A) (10 μg/ml in PBS, pH 7.5, + 20 μM $CaCl_2$ + 20 μM $MnSo_4$), 50μl/well, overnight at 4°C. The wells were blocked by overlaying ConA with 1% BSA, 100 μl/well, incubated at room temperature for 1 hour. The plates were then washed with PBS-Tween 20, the samples of supernatant added in multiples of 3 wells per sample, and incubated for 2 hrs at room temperature, or overnight at 4°C. After washing, the wells were blocked with 1% Invertase in PBS + 20 μM $CaCl_2$ + 20 μM $MnSO_4$, 100 μl/well, for 2 hours at room temperature. After washing, α-agglutinin antibody was added at a concentration of 1:100 in PBS, 50 μl/well, and incubated for 2 hrs at room temperature, or overnight at 4°C. After washing, the secondary antibody, goat anti-rabbit alkaline phosphatase (Sigma) was added at a concentration of 1:100 and incubated for 2 hours at room temperature. The cells were washed and developed with the substrate p-nitrohenyl phosphate in diethanolamine. The plates were read at 5 minute intervals and the optical density graphed with the results recorded as a regression analysis of the slope of the readings.

BIBLIOGRAPHY

Albani, J. R., Sillen, A., Plancke, Y. D., Coddeville, B., and Engelborghs, Y. (2000). Interaction between carbohydrate residues of alpha1-acid glycoprotein (orosomucoid) and saturating concentrations of Calcofluor White. A fluorescence study. Carbohydr Res 327, 333-340.

Alberts, Bruce, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson eds. Molecular Biology of the Cell. New York: Garland Publishing, Inc., 1994. 441-442.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. J Mol Biol 215, 403-410.

Banfield, D. K., Lewis, M. J., and Pelham, H. R. (1995). A SNARE-like protein required for traffic through the Golgi complex. Nature 375, 806-809.

Banuett, F. (1998). Signalling in the yeasts: an informational cascade with links to the filamentous fungi. Microbiol Mol Biol Rev 62, 249-274.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 77, 895-907.

Barrowman, J., Sacher, M., and Ferro-Novick, S. (2000). TRAPP stably associates with the Golgi and is required for vesicle docking. Embo J 19, 862-869.

Benghezal, M., Lipke, P. N., and Conzelmann, A. (1995). Identification of six complementation classes involved in the biosynthesis of glycosylphosphatidylinositol anchors in Saccharomyces cerevisiae. J Cell Biol 130, 1333-1344.

Bock, J. B., Matern, H. T., Peden, A. A., and Scheller, R. H. (2001). A genomic perspective on membrane compartment organization. Nature 409, 839-841.

Boone, C., Sommer, S. S., Hensel, A., and Bussey, H. (1990). Yeast KRE genes provide evidence for a pathway of cell wall beta-glucan assembly. J Cell Biol 110, 1833-1843.

Brown, J. L., Kossaczka, Z., Jiang, B., and Bussey, H. (1993). A mutational analysis of killer toxin resistance in Saccharomyces cerevisiae identifies new genes involved in cell wall (1-->6)-beta- glucan synthesis. Genetics 133, 837-849.

Brul, S., King, A., van der Vaart, J. M., Chapman, J., Klis, F., and Verrips, C. T. (1997). The incorporation of mannoproteins in the cell wall of S. cerevisiae and filamentous Ascomycetes. Antonie Van Leeuwenhoek 72, 229-237.

Bussey, H. (1991). K1 killer toxin, a pore-forming protein from yeast. Mol Microbiol 5, 2339-2343.

Cao, X., Ballew, N., and Barlowe, C. (1998). Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. Embo J 17, 2156-2165.

Caro, L. H., Tettelin, H., Vossen, J. H., Ram, A. F., van den Ende, H., and Klis, F. M. (1997). In silicio identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of Saccharomyces cerevisiae. Yeast 13, 1477-1489.

Castro, O., Chen, L. Y., Parodi, A. J., and Abeijon, C. (1999). Uridine diphosphate-glucose transport into the endoplasmic reticulum of Saccharomyces cerevisiae: in vivo and in vitro evidence. Mol Biol Cell 10, 1019-1030.

Chen, Y. A., and Scheller, R. H. (2001). SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol 2, 98-106.

Chuang, J. S., and Schekman, R. W. (1996). Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. J Cell Biol 135, 597-610.

Conzelmann, A., Fankhauser, C., and Desponds, C. (1990). Myoinositol gets incorporated into numerous membrane glycoproteins of Saccharomyces cerevisiae: incorporation is dependent on phosphomannomutase (sec53). Embo J 9, 653-661.

Deitz, S. B., Rambourg, A., Kepes, F., and Franzusoff, A. (2000). Sec7p directs the transitions required for yeast Golgi biogenesis. Traffic 1, 172-183.

Eakle, K. A., Bernstein, M., and Emr, S. D. (1988). Characterization of a component of the yeast secretion machinery: identification of the SEC18 gene product. Mol Cell Biol 8, 4098-4109.

Egerton, M., Zueco, J., and Boyd, A. (1993). Molecular characterization of the SEC1 gene of Saccharomyces cerevisiae: subcellular distribution of a protein required for yeast protein secretion. Yeast 9, 703-713.

el-Sherbeini, M., and Clemas, J. A. (1995). Cloning and characterization of GNS1: a Saccharomyces cerevisiae gene involved in synthesis of 1.3-beta-glucan in vitro. J Bacteriol 177, 3227-3234.

Fankhauser, C., and Conzelmann, A. (1991). Purification, biosynthesis and cellular localization of a major 125-kDa glycophosphatidylinositol-anchored membrane glycoprotein of Saccharomyces cerevisiae. Eur J Biochem 195, 439-448.

Fasshauer, D., Sutton, R. B., Brunger, A. T., and Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci U S A 95, 15781-15786.

Ferro-Novick, S., Novick, P., Field, C., and Schekman, R. (1984). Yeast secretory mutants that block the formation of active cell surface enzymes. J Cell Biol 98, 35-43.

Ferro-Novick, S., and Jahn, R. (1994). Vesicle fusion from yeast to man. Nature 370, 191-193.

Finger, F. P., and Novick, P. (1997). Sec3p is involved in secretion and morphogenesis in Saccharomyces cerevisiae. Mol Biol Cell 8, 647-662.

Franzusoff, A., Redding, K., Crosby, J., Fuller, R. S., and Schekman, R. (1991). Localization of components involved in protein transport and processing through the yeast Golgi apparatus. J Cell Biol 112, 27-37.

Gaynor, E. C., and Emr, S. D. (1997). COPI-independent anterograde transport: cargo-selective ER to Golgi protein transport in yeast COPI mutants. J Cell Biol 136, 789-802.

Gaynor, E. C., Graham, T. R., and Emr, S. D. (1998). COPI in ER/Golgi and intra-Golgi transport: do yeast COPI mutants point the way? Biochim Biophys Acta 1404, 33-51.

Gaynor, E. C., Mondesert, G., Grimme, S. J., Reed, S. I., Orlean, P., and Emr, S. D. (1999). MCD4 encodes a conserved endoplasmic reticulum membrane protein essential for glycosylphosphatidylinositol anchor synthesis in yeast. Mol Biol Cell 10, 627-648.

Gerst, J. E. (1997). Conserved alpha-helical segments on yeast homologs of the synaptobrevin/VAMP family of v-SNAREs mediate exocytic function. J Biol Chem 272, 16591-16598.

Gonzalo, S., and Linder, M. E. (1998). SNAP-25 palmitoylation and plasma membrane targeting require a functional secretory pathway. Mol Biol Cell 9, 585-597.

Graham, T. R., and Emr, S. D. (1991). Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast sec18 (NSF) mutant. J Cell Biol 114, 207-218.

Gray, J. V., Ogas, J. P., Kamada, Y., Stone, M., Levin, D. E., and Herskowitz, I. (1997). A role for the Pkc1 MAP kinase pathway of Saccharomyces cerevisiae in bud emergence and identification of a putative upstream regulator. Embo J 16, 4924-4937.

Grote, E., Carr, C. M., and Novick, P. J. (2000). Ordering the final events in yeast exocytosis. J Cell Biol 151, 439-452.

Hamada, K., Terashima, H., Arisawa, M., and Kitada, K. (1998). Amino acid sequence requirement for efficient incorporation of glycosylphosphatidylinositol-associated proteins into the cell wall of Saccharomyces cerevisiae. J Biol Chem 273, 26946-26953.

Hamada, K., Terashima, H., Arisawa, M., Yabuki, N., and Kitada, K. (1999). Amino acid residues in the omega-minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. J Bacteriol 181, 3886-3889.

Hardwick, K. G., and Pelham, H. R. (1992). SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. J Cell Biol 119, 513-521.

Hibbs, A. R., and Meyer, D. I. (1988). Secretion in yeast: in vitro analysis of the sec53 mutant. Embo J 7, 2229-2232.

Hirsch, J. P., and Cross, F. R. (1993). The pheromone receptors inhibit the pheromone response pathway in Saccharomyces cerevisiae by a process that is independent of their associated G alpha protein. Genetics 135, 943-953.

Hodel, A. (1998). Snap-25. Int J Biochem Cell Biol 30, 1069-1073.

Hoffman, C. S., and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. Gene 57, 267-272.

Hong, Z., Mann, P., Shaw, K. J., and Didomenico, B. (1994). Analysis of beta-glucans and chitin in a Saccharomyces cerevisiae cell wall mutant using high-performance liquid chromatography. Yeast 10, 1083-1092.

Horvath, A., Sutterlin, C., Manning-Krieg, U., Movva, N. R., and Riezman, H. (1994). Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. Embo J 13, 3687-3695.

Huffaker, T. C., and Robbins, P. W. (1982). Temperature-sensitive yeast mutants deficient in asparagine-linked glycosylation. J Biol Chem 257, 3203-3210.

Huffaker, T. C., and Robbins, P. W. (1983). Yeast mutants deficient in protein glycosylation. Proc Natl Acad Sci U S A 80, 7466-7470.

Humbel, B. M., Konomi, M., Takagi, T., Kamasawa, N., Ishijima, S. A., and Osumi, M. (2001). In situ localization of beta-glucans in the cell wall of Schizosaccharomyces pombe. Yeast 18, 433-444.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153, 163-168.

Jiang, Y., Scarpa, A., Zhang, L., Stone, S., Feliciano, E., and Ferro-Novick, S. (1998). A high copy suppressor screen reveals genetic interactions between BET3 and a new gene. Evidence for a novel complex in ER-to-Golgi transport. Genetics 149, 833-841.

Jue, C. K., and Lipke, P. N. (1985). Determination of reducing sugars in the nanomole range with tetrazolium blue. J Biochem Biophys Methods 11, 109-115.

Kapteyn, J. C., Montijn, R. C., Vink, E., de la Cruz, J., Llobell, A., Douwes, J. E., Shimoi, H., Lipke, P. N., and Klis, F. M. (1996). Retention of Saccharomyces cerevisiae cell wall proteins through a phosphodiester-linked beta-1,3-/beta-1,6-glucan heteropolymer. Glycobiology 6, 337-345.

Kapteyn, J. C., Ram, A. F., Groos, E. M., Kollar, R., Montijn, R. C., Van Den Ende, H., Llobell, A., Cabib, E., and Klis, F. M. (1997). Altered extent of cross-linking of beta1,6-glucosylated mannoproteins to chitin in Saccharomyces cerevisiae mutants with reduced cell wall beta1,3-glucan content. J Bacteriol 179, 6279-6284.

Kapteyn, J. C., Van Den Ende, H., and Klis, F. M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall. Biochim Biophys Acta 1426, 373-383.

Katz, L., Hanson, P. I., Heuser, J. E., and Brennwald, P. (1998). Genetic and morphological analyses reveal a critical interaction between the C-termini of two SNARE proteins and a parallel four helical arrangement for the exocytic SNARE complex. Embo J 17, 6200-6209.

Katz, L., and Brennwald, P. (2000). Testing the 3Q:1R "rule": mutational analysis of the ionic "zero" layer in the yeast exocytic SNARE complex reveals no requirement for arginine. Mol Biol Cell 11, 3849-3858.

Ketela, T., Green, R., and Bussey, H. (1999). Saccharomyces cerevisiae mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. J Bacteriol 181, 3330-3340.

Kitamura, K., Kaneko, T., and Yamamoto, Y. (1971). Lysis of viable yeast cells by enzymes of Arthrobacter luteus. Arch Biochem Biophys 145, 402-404.

Kitamura, K., and Yamamoto, Y. (1972). Purification and properties of an enzyme, zymolyase, which lyses viable yeast cells. Arch Biochem Biophys 153, 403-406.

Klis, F. M. (1994). Review: cell wall assembly in yeast. Yeast 10, 851-869.
Klumperman, J. (2000). Transport between ER and Golgi. Curr Opin Cell Biol 12, 445-449.

Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J., Ashwell, G., Drgonova, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. (1997). Architecture of the yeast cell wall. Beta(1-->6)-glucan interconnects mannoprotein, beta(1-->)3-glucan, and chitin. J Biol Chem 272, 17762-17775.

Kornfeld, R., and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 54, 631-664.

Kwon-Chung, K. J., Hicks, J. B., and Lipke, P. N. (1990). Evidence that Candida stellatoidea type II is a mutant of Candida albicans that does not express sucrose-inhibitable alpha-glucosidase. Infect Immun 58, 2804-2808.

Lagorce, A., Le Berre-Anton, V., Aguilar-Uscanga, B., Martin-Yken, H., Dagkessamanskaia, A., and Francois, J. (2002). Involvement of GFA1, which encodes glutamine-fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in Saccharomyces cerevisiae. Eur J Biochem 269, 1697-1707.

Leidich, S. D., and Orlean, P. (1996). Gpi1, a Saccharomyces cerevisiae protein that participates in the first step in glycosylphosphatidylinositol anchor synthesis. J Biol Chem 271, 27829-27837.

Lian, J. P., and Ferro-Novick, S. (1993). Bos1p, an integral membrane protein of the endoplasmic reticulum to Golgi transport vesicles, is required for their fusion competence. Cell 73, 735-745.

Lipke, P. N., and Ovalle, R. (1998). Cell wall architecture in yeast: new structure and new challenges. J Bacteriol 180, 3735-3740.

Lowe, M. (2000). Membrane transport: tethers and TRAPPs. Curr Biol 10, R407-409.
Lu, C. F., Kurjan, J., and Lipke, P. N. (1994). A pathway for cell wall anchorage of Saccharomyces cerevisiae alpha- agglutinin. Mol Cell Biol 14, 4825-4833.

Lussier, M., White, A. M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S. B., Horenstein, C. I., Chen-Weiner, J., Ram, A. F., Kapteyn, J. C., et al. (1997). Large scale identification of genes involved in cell surface biosynthesis and architecture in Saccharomyces cerevisiae. Genetics 147, 435-450.

Lyman, S. K., and Schekman, R. (1996). Polypeptide translocation machinery of the yeast endoplasmic reticulum. Experientia 52, 1042-1049.

Mazur, P., Morin, N., Baginsky, W., el-Sherbeini, M., Clemas, J. A., Nielsen, J. B., and Foor, F. (1995). Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. Mol Cell Biol 15, 5671-5681.

Meaden, P., Hill, K., Wagner, J., Slipetz, D., Sommer, S. S., and Bussey, H. (1990). The yeast KRE5 gene encodes a probable endoplasmic reticulum protein required for (1----6)-beta-D-glucan synthesis and normal cell growth. Mol Cell Biol 10, 3013-3019.

Mehta, R. D., and Gregory, K. F. (1981). Mutants of Saccharomyces cerevisiae and Candida utilis with increased susceptibility to digestive enzymes. Appl Environ Microbiol 41, 992-999.

Montijn, R. C., Vink, E., Muller, W. H., Verkleij, A. J., Van Den Ende, H., Henrissat, B., and Klis, F. M. (1999). Localization of synthesis of beta1,6-glucan in Saccharomyces cerevisiae. J Bacteriol 181, 7414-7420.

Muniz, M., and Riezman, H. (2000). Intracellular transport of GPI-anchored proteins. Embo J 19, 10-15.

Muniz, M., Morsomme, P., and Riezman, H. (2001). Protein sorting upon exit from the endoplasmic reticulum. Cell 104, 313-320.

Newman, A. P., and Ferro-Novick, S. (1987). Characterization of new mutants in the early part of the yeast secretory pathway isolated by a [3H]mannose suicide selection. J Cell Biol 105, 1587-1594.

Newman, A. P., Shim, J., and Ferro-Novick, S. (1990). BET1, BOS1, and SEC22 are members of a group of interacting yeast genes required for transport from the endoplasmic reticulum to the Golgi complex. Mol Cell Biol 10, 3405-3414.

Newman, A. P., Groesch, M. E., and Ferro-Novick, S. (1992). Bos1p, a membrane protein required for ER to Golgi transport in yeast, co-purifies with the carrier vesicles and with Bet1p and the ER membrane. Embo J 11, 3609-3617.

Nasmyth, K., and Shore, D. (1987). Transcriptional regulation in the yeast life cycle. Science *237*, 1162-1170.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post- translational events in the yeast secretory pathway. Cell 21, 205-215.

Ovalle, R., Lim, S. T., Holder, B., Jue, C. K., Moore, C. W., and Lipke, P. N. (1998). A spheroplast rate assay for determination of cell wall integrity in yeast. Yeast 14, 1159-1166.

Ovalle, R., Spencer, M., Thiwanont, M., and Lipke, P. N. (1999). The spheroplast lysis assay for yeast in microtiter plate format. Appl Environ Microbiol 65, 3325-3327.
Parlati, F., McNew, J. A., Fukuda, R., Miller, R., Sollner, T. H., and Rothman, J. E. (2000). Topological restriction of SNARE-dependent membrane fusion. Nature 407, 194-198.

Pelham, H. R. (2001). SNAREs and the specificity of membrane fusion. Trends Cell Biol 11, 99-101.

Pevsner, J., Hsu, S. C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K., and Scheller, R. H. (1994). Specificity and regulation of a synaptic vesicle docking complex. Neuron 13, 353-361.

Pevsner, J. (1996). The role of Sec1p-related proteins in vesicle trafficking in the nerve terminal. J Neurosci Res 45, 89-95.

Pfeffer, S. R. (1999). Transport-vesicle targeting: tethers before SNAREs. Nat Cell Biol 1, E17-22.

Popolo, L., Gilardelli, D., Bonfante, P., and Vai, M. (1997). Increase in chitin as an essential response to defects in assembly of cell wall polymers in the gpg1delta mutant of Saccharomyces cerevisiae. J Bacteriol 179, 463-469.

Popolo, L., and Vai, M. (1999). The Gas1 glycoprotein, a putative wall polymer cross-linker. Biochim Biophys Acta 1426, 385-400.

Rajavel, M., Philip, B., Buehrer, B. M., Errede, B., and Levin, D. E. (1999). Mid2 is a putative sensor for cell integrity signaling in Saccharomyces cerevisiae. Mol Cell Biol 19, 3969-3976.

Ram, A. F., Wolters, A., Ten Hoopen, R., and Klis, F. M. (1994). A new approach for isolating cell wall mutants in Saccharomyces cerevisiae by screening for hypersensitivity to calcofluor white. Yeast 10, 1019-1030.

Reen, D. J. (1994). Enzyme-linked immunosorbent assay (ELISA). Methods Mol Biol 32, 461-466.

Roemer, T., and Bussey, H. (1991). Yeast beta-glucan synthesis: KRE6 encodes a predicted type II membrane protein required for glucan synthesis in vivo and for glucan synthase activity in vitro. Proc Natl Acad Sci U S A 88, 11295-11299.

Roemer, T., Delaney, S., and Bussey, H. (1993). SKN1 and KRE6 define a pair of functional homologs encoding putative membrane proteins involved in beta-glucan synthesis. Mol Cell Biol 13, 4039-4048.

Roemer, T., Paravicini, G., Payton, M. A., and Bussey, H. (1994). Characterization of the yeast (1-->6)-beta-glucan biosynthetic components, Kre6p and Skn1p, and genetic interactions between the PKC1 pathway and extracellular matrix assembly. J Cell Biol 127, 567-579.

Roemer, T., and Bussey, H. (1995). Yeast Kre1p is a cell surface O-glycoprotein. Mol Gen Genet 249, 209-216.

Roncero, C., and Duran, A. (1985). Effect of Calcofluor white and Congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. J Bacteriol 163, 1180-1185.

Rossi, G., Kolstad, K., Stone, S., Palluault, F., and Ferro-Novick, S. (1995). BET3 encodes a novel hydrophilic protein that acts in conjunction with yeast SNAREs. Mol Biol Cell 6, 1769-1780.

Rothman, J. E. (1994). Intracellular membrane fusion. Adv Second Messenger Phosphoprotein Res 29, 81-96.

Ruohola, H., and Ferro-Novick, S. (1987). Sec53, a protein required for an early step in secretory protein processing and transport in yeast, interacts with the cytoplasmic surface of the endoplasmic reticulum. Proc Natl Acad Sci U S A 84, 8468-8472.

Sacher, M., Stone, S., and Ferro-Novick, S. (1997). The synaptobrevin-related domains of Bos1p and Sec22p bind to the syntaxin-like region of Sed5p. J Biol Chem 272, 17134-17138.

Saccharomyces Genome Database. http://genome-www4.stanford.edu/Saccharomyces/ (accessed July 14, 2002).

Sacher, M., Jiang, Y., Barrowman, J., Scarpa, A., Burston, J., Zhang, L., Schieltz, D., Yates, J. R., 3rd, Abeliovich, H., and Ferro-Novick, S. (1998). TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion. Embo J 17, 2494-2503.

Sacher, M., Barrowman, J., Schieltz, D., Yates, J. R., 3rd, and Ferro-Novick, S. (2000). Identification and characterization of five new subunits of TRAPP. Eur J Cell Biol 79, 71-80.

Sacher, M., Barrowman, J., Wang, W., Horecka, J., Zhang, Y., Pypaert, M., and Ferro-Novick, S. (2001). TRAPP I implicated in the specificity of tethering in ER-to-Golgi transport. Mol Cell 7, 433-442.

Samuel Lunenfeld Research Institute Site. [illegible] (accessed July 14, 2002)

Schekman, R., and Orci, L. (1996). Coat proteins and vesicle budding. Science 271, 1526-1533.

Schmitt, M. J., and Compain, P. (1995). Killer-toxin-resistant kre12 mutants of Saccharomyces cerevisiae: genetic and biochemical evidence for a secondary K1 membrane receptor. Arch Microbiol 164, 435-443.

Shahinian, S., Dijkgraaf, G. J., Sdicu, A. M., Thomas, D. Y., Jakob, C. A., Aebi, M., and Bussey, H. (1998). Involvement of protein N-glycosyl chain glucosylation and processing in the biosynthesis of cell wall beta-1,6-glucan of Saccharomyces cerevisiae. Genetics 149, 843-856.

Shahinian, S., and Bussey, H. (2000). beta-1,6-Glucan synthesis in Saccharomyces cerevisiae. Mol Microbiol 35, 477-489.

Sipos, G., Puoti, A., and Conzelmann, A. (1994). Glycosylphosphatidylinositol membrane anchors in Saccharomyces cerevisiae: absence of ceramides from complete precursor glycolipids. Embo J 13, 2789-2796.

Sipos, G., Puoti, A., and Conzelmann, A. (1995). Biosynthesis of the side chain of yeast glycosylphosphatidylinositol anchors is operated by novel mannosyltransferases located in the endoplasmic reticulum and the Golgi apparatus. J Biol Chem 270, 19709-19715.

Smits, G. J., van den Ende, H., and Klis, F. M. (2001). Differential regulation of cell wall biogenesis during growth and development in yeast. Microbiology 147, 781-794.

Sogaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E., and Sollner, T. (1994). A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell 78, 937-948.

Spang, A., and Schekman, R. (1998). Reconstitution of retrograde transport from the Golgi to the ER in vitro. J Cell Biol 143, 589-599.

Sprague, G. F., Jr., Blair, L. C., and Thorner, J. (1983). Cell interactions and regulation of cell type in the yeast Saccharomyces cerevisiae. Annu Rev Microbiol *37*, 623-660.

Springer, S., Spang, A., and Schekman, R. (1999). A primer on vesicle budding. Cell 97, 145-148.

Steel, G. J., Harley, C., Boyd, A., and Morgan, A. (2000). A screen for dominant negative mutants of SEC18 reveals a role for the AAA protein consensus sequence in ATP hydrolysis. Mol Biol Cell 11, 1345-1356.

Stone, S., Sacher, M., Mao, Y., Carr, C., Lyons, P., Quinn, A. M., and Ferro-Novick, S. (1997). Bet1p activates the v-SNARE Bos1p. Mol Biol Cell 8, 1175-1181.

Terrance, K., and Lipke, P. N. (1981). Sexual agglutination in Saccharomyces cerevisiae. J Bacteriol 148, 889-896.

Trilla, J. A., Duran, A., and Roncero, C. (1999). Chs7p, a new protein involved in the control of protein export from the endoplasmic reticulum that is specifically engaged in the regulation of chitin synthesis in Saccharomyces cerevisiae. J Cell Biol 145, 1153-1163.

Tsui, M. M., and Banfield, D. K. (2000). Yeast Golgi SNARE interactions are promiscuous. J Cell Sci 113, 145-152.

Valdivieso, M. H., Ferrario, L., Vai, M., Duran, A., and Popolo, L. (2000). Chitin synthesis in a gas1 mutant of Saccharomyces cerevisiae. J Bacteriol 182, 4752-4757.

VanRheenen, S. M., Cao, X., Lupashin, V. V., Barlowe, C., and Waters, M. G. (1998). Sec35p, a novel peripheral membrane protein, is required for ER to Golgi vesicle docking. J Cell Biol 141, 1107-1119.

VanRheenen, S. M., Cao, X., Sapperstein, S. K., Chiang, E. C., Lupashin, V. V., Barlowe, C., and Waters, M. G. (1999). Sec34p, a protein required for vesicle tethering to the yeast Golgi apparatus, is in a complex with Sec35p. J Cell Biol 147, 729-742.

Verna, J., and Ballester, R. (1999). A novel role for the mating type (MAT) locus in the maintenance of cell wall integrity in Saccharomyces cerevisiae. Mol Gen Genet 261, 681-689.

Vossen, J. H., Muller, W. H., Lipke, P. N., and Klis, F. M. (1997). Restrictive glycosylphosphatidylinositol anchor synthesis in cwh6/gpi3 yeast cells causes aberrant biogenesis of cell wall proteins. J Bacteriol 179, 2202-2209.

Weber, T., Parlati, F., McNew, J. A., Johnston, R. J., Westermann, B., Sollner, T. H., and Rothman, J. E. (2000). SNAREpins are functionally resistant to disruption by NSF and alphaSNAP. J Cell Biol 149, 1063-1072.

Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R., and Rothman, J. E. (1994). N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. J Cell Biol 126, 945-954.

Wieland, F., and Harter, C. (1999). Mechanisms of vesicle formation: insights from the COP system. Curr Opin Cell Biol 11, 440-446.

Yarden, O., and Yanofsky, C. (1991). Chitin synthase 1 plays a major role in cell wall biogenesis in Neurospora crassa. Genes Dev 5, 2420-2430.

Ziman, M., Chuang, J. S., and Schekman, R. W. (1996). Chs1p and Chs3p, two proteins involved in chitin synthesis, populate a compartment of the Saccharomyces cerevisiae endocytic pathway. Mol Biol Cell 7, 1909-1919.

Ziman, M., Chuang, J. S., Tsung, M., Hamamoto, S., and Schekman, R. (1998). Chs6p-dependent anterograde transport of Chs3p from the chitosome to the plasma membrane in Saccharomyces cerevisiae. Mol Biol Cell 9, 1565-1576.

Zlotnik, H., Fernandez, M. P., Bowers, B., and Cabib, E. (1984). Saccharomyces cerevisiae mannoproteins form an external cell wall layer that determines wall porosity. J Bacteriol 159, 1018-1026.