1986

The lysosomal trehalase of Saccharomyces cerevisiae.

Steven David. Harris
University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000ext. 3208.
NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SCR 1970, c. C-30.

LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RECEUE
THE LYSOSONAL TREHALASE OF SACCHAROMYCES CEREVISIAE

by

Steven David Harris

A thesis
submitted to the Faculty of Graduate Studies
through the Department of
Biology in Partial Fulfillment
of the Requirements for the Degree
of Master of Science at
The University of Windsor

Windsor, Ontario, Canada
1988
Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilm cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ABSTRACT

The yeast, *Saccharomyces cerevisiae* possesses two distinct trehalases. One trehalase is located in the cytoplasm and the other is found in the vacuole. The cytoplasmic trehalase is regulated by cAMP-dependent phosphorylation.

Vacuolar trehalase activity is induced by starvation conditions. It is initially synthesized as an inactive proenzyme which traverses the secretory pathway through the endoplasmic reticulum to the Golgi body. Vacuolar trehalase activity is *PEP4*-dependent, indicating that protease A activates the proenzyme to its active form in the vacuole.

Since they are each regulated and processed in a different manner, it is unlikely that a precursor relationship exists between the cytoplasmic and vacuolar trehalases. It is proposed that each enzyme is coded for by a separate transcript, but from a common gene.

Preliminary attempts to isolate trehalase deficient mutants yielded two mutants with phenotypes similar to petite mutants. The evidence that vacuolar trehalase is processed by the same mechanism(s) that operate on other vacuolar enzymes should allow isolation of the trehalase structural gene by employing a vacuolar enzyme-specific cloning procedure.
ACKNOWLEDGEMENTS

I would like to thank all the members of Dr. Cotter's lab, as well as the faculty and staff of the Department of Biological Sciences, for their support and assistance during the course of my studies. Appreciation is also expressed to J. DeAngelo, R. Needleman, J. Rothman, T. Stevens and C. Low for the use of their facilities, their gifts of strains and their technical advice.

I would also like to thank the members of my committee, Dr. A. Warner and Dr. K. Taylor, for their advice. Finally, greatest appreciation is expressed to Dr. D. Cotter, my advisor, for financial support and technical and theoretical advice during this project.
TABLE OF CONTENTS

I. Abstract ............................................................... iv
II. Acknowledgement ................................................... v
III. List of Tables ...................................................... viii
IV. List of Illustrations ............................................... ix
V. Introduction ........................................................ 1

A. Life cycle of *Saccharomyces cerevisiae*
B. Reserve carbohydrate metabolism in *S. cerevisiae*
C. The *S. cerevisiae* lysosome-like vacuole
D. The secretory pathway in *S. cerevisiae*
E. Hypothesis

VI. Materials and Methods .......................................... 21

A. Strains and Media
B. Culture conditions
C. Preparation of crude enzyme extracts
D. Trehalase assays
E. Determination of trehalase specific activity
F. Protein determination
G. Isoelectric focusing gel electrophoresis
H. Procedures for mutagenesis
I. Tunicamycin experiments
J. Detection of extracellular trehalase
K. Yeast genetic manipulations

VII. Results ........................................................... 34

A. Trehalase activities during a growth cycle
B. Trehalase activities in trehalose-grown cells
C. Trehalase activities in cells growing on other carbon sources
D. Trehalase activities in pep4 mutants

E. Trehalase activities in secretory mutants
   1. sec18 mutant
   2. sec7 mutant
   3. sec5 mutant

F. Trehalase levels in tunicamycin treated cells

G. Isoelectric point of vacuolar trehalase

H. Isolation of mutants unable to grow on trehalose

I. Detection of extracellular trehalase activity

VIII. Discussion ------------------------------- 72

A. Starvation induced derepression

B. Processing of vacuolar trehalase

C. Isolation of trehalase mutants

IX. References -------------------------------------- 88

X. Vita Auctoris ------------------------------------- 97
LIST OF TABLES

Tables

1. *Saccharomyces cerevisiae* strains used in this study --------------------------------- 22
2. The ratio of trehalase activities in extracts of cells grown in YEP-2% trehalose medium compared to cells grown in YEPD medium ------------------------------- 41
3. Growth of DBY747 on various carbon sources ------- 42
4. Growth of a pep4 mutant on a trehalose containing medium ------------------------------------ 46
5. Trehalase activities in a pep4 mutant --------- 48
6. Trehalase activities in a pep4 deletion mutant --- 50
7. Effect of a temperature shift on trehalase activities ------------------------------------------ 52
8. Trehalase activities in a sec18 mutant ------- 54
9. Trehalase activities in a sec7 mutant ------- 58
10. Trehalase activities in a sec5 mutant ------- 59
11. Effects of tunicamycin on trehalase activities in strain X2180-1A ------------------------ 59
12. Trehalase activities in mutants ---------------------------------- 64
13. Summary of mutant phenotypes ---------------------------------- 66
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The life cycle of <em>Saccharomyces cerevisiae</em></td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Secretory and vacuolar protein transport pathways in <em>S. cerevisiae</em></td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Growth of strain DBY747 in YEPD or YEP-2% trehalose-0.3% glucose</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>Trehalase activities in strain DBY747 grown in YEPD or YEP-2% trehalose-0.3% glucose</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>Trehalase activities in strain DBY747 grown on various carbon sources</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>Representation of isoelectric focusing gel electrophoresis results</td>
<td>61</td>
</tr>
<tr>
<td>7</td>
<td>Growth of strain SH3 in YEPD or YEP-2% trehalose-0.3% glucose</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>Trehalase activities in strain SH3 grown in YEPD or YEP-2% trehalose-0.3% glucose</td>
<td>69</td>
</tr>
</tbody>
</table>
INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is a unicellular lower eukaryote with a simple developmental program (Figure 1). Laboratory strains of *S. cerevisiae* are primarily heterothallic. In the haploid state, the cells can be either of two mating types, a or alpha. Each cell secretes a mating pheromone that triggers a series of reactions in cells of the opposite mating type. This leads to conjugation followed shortly by karyogamy. The resulting diploids can continue mitotic growth until nutrients, particularly nitrogen, are depleted. This triggers the sporulation program, which consists of meiosis and ascospore development. The spores will germinate upon incubation in glucose, and the four haploid products of meiosis can be readily recovered (Klar et al., 1984; Byers, 1981; Herskowitz and Oshima, 1981).

Wild type strains of *S. cerevisiae* are homothallic. Haploid cells can switch mating type approximately once per generation in culture (Klar et al., 1984). This leads to a mixed population with cells of each mating type undergoing mating and diploid formation.

The ease with which biochemical and genetic approaches can be combined, utilizing the yeast *S. cerevisiae*, has made this organism ideally suited for the study of eukaryotic cell biology.

RESERVE CARBOHYDRATE METABOLISM IN *S. CEREVISIAE*
FIGURE 1

The life cycle of *Saccharomyces cerevisiae*.

The top panel depicts the heterothallic life cycle and the bottom panel depicts the homothallic life cycle. Taken from Klar et al., 1994.
Saccharomyces cerevisiae can synthesize and degrade internally both the polysaccharide glycogen and the disaccharide trehalose. These carbohydrates can represent less than 1% or greater than 23% of the dry weight of the cells, depending on the environmental conditions and the stage of the life cycle (Lillie and Pringle, 1980). This suggests that each carbohydrate plays a critical role in the yeast life cycle. Physiological studies relating the levels of these carbohydrates to the developmental cycle have suggested that they have roles as reserves in starving cells (Lillie and Pringle, 1980; Panék, 1963), in cells undergoing respiratory adaptation (Lillie and Pringle, 1980; Panek and Mattoon, 1979, in sporulating cells (Panek and Bernardes, 1983; Colonna and Magee, 1978; Kane and Roth, 1974), in germinating spores (Panek and Bernardes, 1983; Kane and Roth, 1974; Thevelein, 1984a), in vegetative cells initiating new rounds of growth in fresh media (Panek, 1963; van der Plaat, 1974), and in cells traversing the mitotic cycle under conditions of carbon and energy limitation (Lillie and Pringle, 1980; Kuenzi and Feichter, 1969; Kuenzi and Feichter, 1972).

Glycogen is accumulated by cells in rich media when there is still considerable glucose remaining (Lillie and Pringle, 1980). The mechanism which initiates glycogen accumulation is still unclear. This glycogen may be utilized for respiratory adaptation, but there is no evidence to indicate this. Glycogen is accumulated by
sporulating cells prior to the appearance of mature ascis, however it is degraded late in sporulation (Kane and Roth, 1974). A glycogenolytic activity appears at this time, and likely consists of a glucoamylase, degrading α-1,4 bonds, and a debranching enzyme, degrading α-1,6 bonds (Colonna and Magee, 1978). A presumably sporulation-specific glucoamylase gene has been cloned, but its expression does not increase during meiosis and sporulation. Also, cells with no glucoamylase activity fail to degrade accumulated glycogen during sporulation, yet still produce four viable spores (Yamashita and Fukui, 1985). The glcI mutant fails to accumulate glycogen since glycogen synthetase is maintained in its low activity form (Rothman-Denes and Cabib, 1970). This mutant sporulates poorly, leading to speculation that glycogen is required for sporulation (Panek and Bernardes, 1983). However, the glcI mutation is allelic to the ppdI mutation, which results in the deficiency of a phosphoprotein phosphatase (Matsumoto et al., 1985b). Since a number of proteins are controlled by phosphorylation in S. cerevisiae (Matsumoto et al., 1985a), it is unclear if the failure of glcI mutants to sporulate is due to an inactive glycogen synthetase.

Trehalose appears to serve primarily as a storage carbohydrate during periods of non-proliferation (Thevelein, 1984b). Cells growing in rich media begin to accumulate trehalose as they approach stationary phase and
glucose is depleted (Lillie and Pringle, 1980). This trehalose is slowly degraded if the cells are maintained under prolonged starvation conditions (Lillie and Pringle, 1980). When starved cells initiate a new round of growth in fresh media, stored trehalose is rapidly mobilized (Panek, 1963; van der Platt, 1974; Thevelein, 1984b). Only glucose stimulates this degradation, and it is quickly followed by resynthesis of trehalose, resulting in a net increase. Nitrogen sources enhance degradation, but prevent resynthesis (van der Platt, 1974; Thevelein, 1984b). It has also been shown that trehalose is mobilized during the mitotic cycle at the G1/S phase interface. During the remainder of the cycle, trehalose is accumulated (Kuenzi and Feichter, 1969). Thus, it is evident that trehalose is utilized as a storage carbohydrate for survival by starved cells, and as an energy source for cells initiating new rounds of growth or about to enter mitosis.

Diploid cells undergoing sporulation also accumulate trehalose (Kane and Roth, 1974). A mutant unable to accumulate trehalose due to a specific lesion in the putative trehalose 6-phosphate synthetase gene sporulated well, but failed to germinate (Panek and Bernardes, 1983). They proposed that trehalase is required for spore germination, as in many other fungi (Thevelein, 1984b). The trehalose reserve may serve as a source of energy in dormant spores, with its metabolism regulated by an ATP
feedback mechanism (Barton et al., 1982; Thevelein et al., 1982; Thevelein et al., 1984). It may also serve as an energy source during spore germination (Thevelein, 1984b; Thevelein et al., 1982; Thevelein and Jones, 1983).

Many strains of *Saccharomyces cerevisiae* grow very poorly, if at all, on exogenous trehalose (Barnett et al., 1984). Trehalose can reportedly be transported into the cell through the maltose transport system (Kotyk and Michaljancova, 1979). Internally, trehalose is accumulated through the action of trehalose 6-phosphate synthetase, which uses UDP-glucose and glucose 6-phosphate as substrates. Associated with this enzyme is a phosphatase which removes the phosphate from trehalose 6-phosphate as soon as it is synthesized (Cabib and Leloir, 1958). The *sst1* mutant reportedly lacks this synthetase activity (Panek and Bernardes, 1983). An alternative pathway of trehalose accumulation, linked to maltose fermentation has been proposed. This pathway is dependent upon one of the genes regulating maltose fermentation (*MAL2, 4 or 6*) being constitutive. This pathway can apparently substitute for the UDPG-dependent pathway when that pathway is inactivated by mutation (Panek and Bernardes, 1983). They suggested that the glucose derived from maltose degradation is directly converted into trehalose.

The only enzyme known to hydrolyze trehalose in *S. cerevisiae*, as in all fungi, is trehalase (α,α-trehalose 1-
D-glucohydrolase, EC 3.2.1.28 (Elbein, 1974). Two classes of trehalases exist in the fungi. "Non-regulatory" trehalases are purely hydrolytic and are regulated by compartmentalization. Regulatory trehalases are regulated by a cAMP-dependent phosphorylation cascade. It has been proposed that each type exists in S. cerevisiae (Thevelein, 1984b).

Early purifications of trehalase from S. cerevisiae yielded essentially similar results in different laboratories (Panek and Souza, 1964; Panek, 1969; Kelly and Catley, 1976). Final specific activities were roughly similar, as were the pH optima for activity (5.5-5.7) and the $K_m$ for trehalose ($4\times10^{-4}$). At this time it was also observed that trehalase could be stimulated by cAMP during the lag phase preceding growth (van der Plaat, 1974). Subsequent evidence has shown that trehalase is a substrate for a cAMP-dependent protein kinase (Ortiz et al., 1983; Uno et al., 1983; Thevelein, 1984b, c). It was shown that addition of glucose to ascospores elicited a ten-fold increase in trehalase activity, preceded by a two- to seven-fold increase in intracellular cAMP levels. The trehalase was then inactivated, but this could be reversed by the addition of a nitrogen source. Adding glucose along with the nitrogen source caused a two-fold increase in cAMP levels, concomitant with trehalase reactivation in spores, but not in vegetative cells (Thevelein, 1984c). It was later suggested that there may be an alternative second
messenger other than cAMP or cGMP which stimulates the protein kinase activating trehalase in vegetative cells (Thevelein and Buellens, 1985). Accompanying the phosphorylation of a partially purified 320 kD trehalase, an 80 kD protein was phosphorylated. This could possibly be a subunit of trehalase, which when phosphorylated caused the activation of the enzyme. Mutants with a thermolabile adenylate cyclase showed low levels of phosphorylation of the 80 kD protein and no trehalase activation. Another mutant with a cAMP-independent protein kinase exhibits constitutive trehalase activation (Uno et al., 1983). The protein kinase which activates trehalase by phosphorylation may be the same one which maintains glycogen synthetase in its low activity form (Ortiz et al., 1983), but there is now evidence which contradicts this proposal (Matsumoto et al., 1985b).

It has been proposed that the glucose stimulated activation of trehalase occurred via membrane depolarization caused by glucose transport (Thevelein, 1984b). Various membrane depolarizing agents mimicked the glucose stimulated activation of trehalase (Thevelein, 1984d). Membrane depolarization apparently stimulated adenylate cyclase, which caused a rise in internal cAMP levels and activation of trehalase (Thevelein, 1984b). However, recent evidence indicates that the glucose stimulated rise in cAMP levels and the activation of trehalase are each the result of internal acidification
(Caspani et al., 1985; Valle et al., 1986).

It was initially observed that trehalase was located in the soluble fraction of an S. cerevisiae protoplast lysate (Souza and Panek, 1968). Subsequent studies indicated that active trehalase was found in the vacuole (Keller et al., 1982), while a trehalase zymogen was located in the cytoplasm (Wiemken and Schellenberg, 1982). These workers proposed that cAMP-dependent phosphorylation converted the trehalase zymogen to active trehalase and initiated its transfer from the cytoplasm into the vacuole following its degradation of trehalose. In the vacuole the enzyme would be proteolytically degraded. However, recent work on the characterization of two trehalases in S. cerevisiae contradicts this proposal (Londesborough and Varimo, 1984). They characterized a vacuolar trehalase with an acidic pH optimum and a cytoplasmic trehalase with a neutral pH optimum. The vacuolar activity bound strongly to Concanavalin A, had a molecular weight near 215 kD, was EDTA insensitive and acetate sensitive. It had a \( K_m \) for trehalose of 1.4 mM. The cytoplasmic activity did not bind to Concanavalin A, had an estimated molecular weight of 170 kD and was EDTA sensitive. The cytoplasmic activity could be activated by incubation with ATP and cAMP, but not to as great an extent as earlier reported. This activation was absolutely dependent on Ca\(^{2+}\) or Mn\(^{2+}\) ions. Since the vacuolar trehalase is glycosylated and the cytoplasmic trehalase is not, the authors concluded that
phosphorylation of cytoplasmic trehalase could not lead to its translocation into the vacuole (Londesborough and Varimo, 1984). Such a precursor relationship between the two trehalases would require a novel glycosylation mechanism. Each trehalase also possessed distinct catalytic properties, leading to the proposal that the two trehalases are synthesized from different transcripts and possibly different genes (Londesborough and Varimo, 1984). A similar relationship between two trehalase activities has been proposed in the yeast *Candida utilis* (Arguellos and Gacto, 1985).

**THE S. CEREVISIAE LYSOSOME-LIKE VACUOLE**

The *S. cerevisiae* vacuole is functionally analogous to the eukaryotic lysosome (Matile and Weimken, 1967; Weimken *et al.*, 1979). The vacuole contains a number of hydrolytic enzymes, primarily proteases, and also serves as a storage pool for amino acids, poly-phosphate and other compounds (Weimken *et al.*, 1979; Jones, 1984). During vegetative growth on rich media, a single cell possesses a small fragmented vacuole, but during starvation and sporulation, the vacuole becomes very large. It appears phase dark as opposed to the phase bright cell using phase contrast microscopy (Byers, 1981). At these times, intracellular protein degradation reaches a peak (Weimken *et al.*, 1979; Jones, 1984; Achstetter and Wolf, 1985). The proteases in the vacuole are generally non-specific and are involved in nitrogen starvation-induced protein degradation during
stationary phase and sporulation (Jones, 1984; Achstetter and Wolf, 1985). It seems evident that there is a correlation between the dominance of the vacuole in the starved cell and the general derepression of the vacuolar enzymes.

In order to study the synthesis and maturation of the vacuolar enzymes on a biochemical and genetic basis, the vacuolar proteases carboxypeptidase Y, protease A and protease B have been used as models. These three proteases are all mannanproteins of the N-linked type (Hasilik and Tanner, 1976b; Hasilik and Tanner, 1978; Jones, 1984). However, vacuolar proteins are less extensively glycosylated than secreted proteins. They primarily contain elaborations of the core carbohydrate, with no outer chain addition (Jones, 1984). Protease A and carboxypeptidase Y are synthesized, but are inactive, in the presence of the N-linked glycosylation inhibitor tunicamycin (Hasilik and Tanner, 1976a; Jones, 1984). Endoglycosidase H, which cleaves N-linked glycoproteins such that only a sole N-acetylglucosamine residue remains (Trimble and Maley, 1977), removes the carbohydrate from protease A and carboxypeptidase Y, but does not affect protease B (Mechler et al., 1982; Jones, 1984). Even with the carbohydrate removed, carboxypeptidase Y is still transported into the vacuole (Schwaiger et al., 1982; Stevens et al., 1982).

Carboxypeptidase Y is synthesized on membrane bound
ribosomes (Muller and Muller, 1981). It is initially synthesized as a 67 kD precursor which is converted to a 69 kD form by the addition of carbohydrate (Stevens et al., 1982; Distel et al., 1983). Active carboxypeptidase Y in the vacuole is a 60 kD glycoprotein (Hasilik and Tanner, 1978). The carboxypeptidase Y transcript level increases ten-fold during the end of logarithmic growth on glucose, and decreases during stationary phase (Distel et al., 1983).

In a search for mutants affecting vacuolar protease levels, the pleiotropic pep4-1 mutant was isolated (Jones, 1977). Activity of protease A, protease B and carboxypeptidase Y were all only a few percent of wild-type in this mutant. An additional pleiotropic mutant causing deficiencies in vacuolar enzyme levels, called pep4-3, was later isolated (Jones et al., 1981; Hemmings et al., 1981). Besides the proteases, the levels of vacuolar ribonuclease and alkaline phosphatase are diminished in the pep4-3 mutant (Jones et al., 1981). The pep4-3 mutant accumulates the 69 kD precursor of carboxypeptidase Y in the vacuole (Hemmings et al., 1981). pep4-3 homozygous diploids fail to sporulate, and protein degradation during sporulation is greatly diminished (Hemmings et al., 1981). The pep4-3 gene product was shown to be a vacuolar protease which cleaves an 8-10 kD amino-terminal fragment from vacuolar proenzymes, thus activating them (Hemmings et al., 1981). The pep4-3 gene product has recently been identified as
protease A (Ammerer et al., 1986).

Carboxypeptidase Y is co-translationally translocated into the endoplasmic reticulum, where it undergoes core glycosylation. It is then transported to the Golgi body, where limited modification of the core carbohydrate occurs. From the Golgi body, carboxypeptidase Y is transported into the vacuole in a process not requiring secretory vesicles (Stevens et al., 1982). The compartment of the Golgi body from which carboxypeptidase Y is sorted from other secretory proteins is unknown, but is likely to be an early compartment (cis-compartment) due to the lack of outer chain carbohydrate addition (Dunphy and Rothman, 1985). Mutants defective in the sorting of carboxypeptidase Y to the vacuole have been isolated (Emr et al., 1985; Rothman et al., 1985). These mutants mislocalize carboxypeptidase Y to the cell surface where it can be detected immunologically (Rothman et al., 1985). In addition, the sorting reaction for carboxypeptidase Y is saturable, as indicated by gene dosage experiments with its structural gene PRC1 (Stevens et al., 1986). The carbohydrate moiety of carboxypeptidase Y plays no role in targeting it to the vacuole (Stevens et al., 1982), as opposed to the case for mammalian lysosomal enzymes (Sly and Fisher, 1982).

Based on the above evidence, the following pathway which vacuolar enzymes follow to the vacuole has been proposed (Stevens et al., 1982). They are co-translationally translocated into the lumen of the
endoplasmic reticulum as inactive proenzymes. Core glycosylation occurs there, and the proenzymes are then transported to the Golgi body. Modifications to the core carbohydrate are made in the Golgi body, and the inactive proenzymes are sorted out from other secretory proteins and transported to the vacuole. This sorting may occur via a peptide-recognizing receptor mediated process (J.H.Rothman, pers. commun.). In the vacuole, the proenzymes are activated through the action of the pep4 \textsuperscript{-}3 gene product, protease A.

THE SECRETORY PATHWAY IN S. CEREVISIAE

The pathway which secretory proteins travel in S. cerevisiae is structurally analogous to that in other eukaryotes (Schekman and Novick, 1982; Kelly, 1985; Schekman, 1985). Secreted proteins are translated on membrane-bound ribosomes, and are translocated into the endoplasmic reticulum in a process mediated by an amino-terminal signal sequence (Walter et al., 1984). The proteins then travel from the endoplasmic reticulum to the Golgi body, with core glycosylation occurring in the endoplasmic reticulum and outer chain carbohydrate addition in the Golgi body. While proteins destined for the vacuole are sorted out in the Golgi body, the true secretory proteins travel via secretory vesicles to the cell surface (Figure 2). In S. cerevisiae, some secreted proteins, such as invertase, remain in the periplasmic space. Others, such as the mating pheromone \alpha-factor are released into the
FIGURE 2

Secretory and vacuolar protein transport pathways in *S. cerevisiae*.

N: nucleus. NM: nuclear membrane.
ER: endoplasmic reticulum.
SEC: wild-type gene product.
VAC: vacuole. V: vesicle.
PM: plasma membrane. CW: cell wall.
CPY: 61 kd mature carboxypeptidase Y.
p1 CPY: 67 kd proCPY. p2 CPY: 69 kd proCPY.
Taken from Stevens *et al.*, 1982.
media (Schekman and Novick, 1982; Schekman, 1985). The S. cerevisiae secretory pathway has been defined genetically at all steps by a series of reversible temperature-sensitive or growth mutations (Schekman and Novick, 1982; Schekman, 1985). Recently, various components of the S. cerevisiae secretory pathway have been reconstituted in vitro (Hansen et al., 1986; Haselback and Schekman, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). Also, late stages of the pathway are required for endocytosis (Riezman, 1985).

The first step of the secretory pathway, translocation into the endoplasmic reticulum, is defective in the mutants sec53 and sec59. Each mutant accumulates inactive forms of invertase at the restrictive temperature of 37°C. The mutation is reversible when the cells are returned to the permissive temperature (Ferro-Novick et al., 1984a; Ferro-Novick et al., 1984b; Ferro-Novick, 1985). In each mutant, the inactive invertase is partially glycosylated and firmly embedded in the membrane of the endoplasmic reticulum (Ferro-Novick et al., 1984a; Ferro-Novick, 1985). Even though the sec53 mutant synthesizes invertase containing little or no carbohydrate, it is not defective in oligosaccharide synthesis (Ferro-Novick et al., 1984b; Ferro-Novick, 1985). The sec59 mutant synthesizes invertase with 0-3 oligosaccharide units added (Ferro-Novick et al., 1984b). Recently, the SEC53 gene product was found to be located in the cytoplasm, and it was
suggested that it acts indirectly to facilitate protein assembly in the endoplasmic reticulum (Bernstein et al., 1985). These mutants are useful for demonstrating that a protein is translocated into the endoplasmic reticulum.

The remaining 23 complementation groups of secretory mutants (sec mutants) accumulate active invertase at the restrictive temperature. The blocks can be reversed in the absence of protein synthesis, indicating that each mutation likely defines a thermolabile intermediate which facilitates the transport of proteins through the pathway (Schekman and Novick, 1982; Schekman, 1985). These mutants can be divided into three classes: 1. Those defective in transit from the endoplasmic reticulum to the Golgi body (sec18 group) 2. Those defective in the formation of secretory vesicles from the Golgi body (sec7 group) 3. Those defective in the transport of the secretory vesicles to the cell surface (sec1 and sec5 group). Vacuolar carboxypeptidase Y accumulates as an inactive precursor in the first two classes, but is active and located in the vacuole in the third class (Stevens et al., 1982). True secretory proteins accumulate as active enzymes, except in the case of prepro-α-factor, in all three mutant classes (Schekman and Novick, 1982; Schekman, 1985).

HYPOTHESIS

It has been postulated that the cytoplasmic trehalase was a zymogen that upon phosphorylation became active. After trehalose degradation, the trehalase was translocated
into the vacuole for proteolytic degradation (Weimken and Schellenberg, 1982). Contradicting this hypothesis are the observations that dephosphorylated trehalase can be reactivated (Thevelein, 1984c; Thevelein and Buellens, 1985). In light of the recent findings that the vacuolar trehalase was glycosylated while the cytoplasmic enzyme was not, and that they possessed distinct catalytic properties, it was suggested that the two trehalases were separate translation products (Londesborough and Varimo, 1984). If the vacuolar trehalase from yeast is a true vacuolar (lysosomal) enzyme, then it should show increased activity during stationary phase as do most other vacuolar proteins. Also, in a manner similar to vacuolar carboxypeptidase Y, the enzyme should have diminished activity in pep4 mutants. The sec18 and sec7 mutations should affect vacuolar trehalase activity, while the sec1 and sec5 mutations should not. The precedent for a true vacuolar trehalase in S. cerevisiae is the trehalase from the cellular slime mold Dictyostelium discoideum which is known to be a lysosomal enzyme (Seshadri et al., 1986).
MATERIALS AND METHODS

A. STRAINS AND MEDIA

All strains used are listed in Table 1, along with their genotype and source. Stock cultures were maintained in rich media (YEPD) at room temperature.

All media used were standard yeast media (Sherman et al., 1970). Rich media was YEPD, consisting of 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. Media with alternative carbon sources were the same, except that glucose was substituted with 2% maltose, 2% trehalose, 2% sucrose or 2% glycerol. In each of these media, 0.3% glucose was also added. For derepressing media, YEPD containing only 0.1% glucose was used. Synthetic minimal media was SD, consisting of 0.67% Difco yeast nitrogen base without amino acids and 2% glucose with the appropriate nutritional supplements (Sherman et al., 1970). Synthetic complete media, SC, was SD with all nutritional supplements added. Trehalose minimal media was the same as SD or SC, except that 2% trehalose was the carbon source. Presporulation media was YEPA, consisting of YEP with 1% potassium acetate. Sporulation media was 1% potassium acetate, pH 7.

For liquid media, 50 ml culture volumes were used in 250 ml baffled Bellco flasks. For solid media, 2% agar was added to the liquid media following autoclaving and 15-20 mls was poured per plate.

B. CULTURE CONDITIONS
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>X2190-1A</td>
<td>α SUC2 mal mel gal2 CUP1</td>
<td>YGSC</td>
</tr>
<tr>
<td>DBY747</td>
<td>a his3-Δ1 leu2-3,112 ura3-52 trp1-286</td>
<td>YGSC</td>
</tr>
<tr>
<td>DBY746</td>
<td>α his3-Δ1 leu2-3,112 ura3-52 trp1-286</td>
<td>YGSC</td>
</tr>
<tr>
<td>YNN282</td>
<td>α trp1-Δ his3-Δ200 ura3-52 lys2-801 ade2-1o</td>
<td>YGSC</td>
</tr>
<tr>
<td>YNN281</td>
<td>a trp1-Δ his3-Δ200 ura3-52 lys2-801 ade2-1o</td>
<td>YGSC</td>
</tr>
<tr>
<td>HMSF176</td>
<td>a SUC2 mal mel gal2 CUP1 sec18-1</td>
<td>YGSC</td>
</tr>
<tr>
<td>SF294-2B</td>
<td>a SUC2 mal mel gal2 CUP1 sec7-1</td>
<td>YGSC</td>
</tr>
<tr>
<td>HMSF174</td>
<td>a SUC2 mal mel gal2 CUP1 sec5-24</td>
<td>C. Low</td>
</tr>
<tr>
<td>208-12</td>
<td>α pep4-3 trp1</td>
<td>YGSC</td>
</tr>
<tr>
<td>JHRY20-2C</td>
<td>a his3-Δ200 leu2-3,112 ura3-52 PEP4</td>
<td>J. Rothman</td>
</tr>
<tr>
<td>JHRY20-2Ca</td>
<td>a his3-Δ200 leu2-3,112 pep4::URA3</td>
<td>J. Rothman</td>
</tr>
</tbody>
</table>

* Yeast Genetic Stock Center, Univ. of California, Berkeley, CA.
* Dept. of Microbiology, North Carolina State University, Raleigh, N. C.
* Institute of Molecular Biology, Univ. of Oregon, Eugene, OR.
For routine growth in liquid media, cultures were grown on a gyratory shaker (New Brunswick, USA) at 200 rpm at room temperature.

The experiments to examine trehalase levels during the growth cycle were initiated with 2 day old stationary phase cultures that were synchronized in G1 using nutrient depletion (Pringle and Hartwell, 1981). These cells were harvested and transferred into YEPD. They were grown for 30 hours at room temperature, with samples taken every 10 hours starting at time zero.

The experiments comparing trehalase levels on different carbon sources were also initiated with stationary phase cells. These cells were harvested and transferred into the appropriate media. They were grown at room temperature, with samples taken at time zero and after 20 hours growth, when the 0.3% glucose had been depleted and the cells had shifted to growth on the alternative carbon source.

The first set of experiments comparing trehalase levels in the pepl4 mutant to its wild-type parent were performed as described above. Cells were grown in either YEPD or YEP-2% trehalose and samples were taken at time zero and after 20 hours growth. The second set of these experiments, using the pepl4 deletion mutant and its parent, were performed under different growth conditions. This was done to conform to standard conditions for the derepression of vacuolar enzymes (J.H. Rothman, pers. commun.).
Overnight cultures of mid-exponential phase cells grown in YEPD at room temperature were used as starter cultures. These are standard repressing conditions (Jones, 1984). These cultures were harvested and split in half. One-half of the cells were transferred back into repressing media, while the second half was transferred into derepressing media. These cultures were grown overnight (8–12 hours) and samples were then taken.

Mid-exponential phase cultures growing in YEPD at room temperature were also used as starter cultures for the experiments comparing trehalase levels in the sec mutants and the wild-type parent. Each culture was split in half, and each half was transferred into derepressing media. One culture was then incubated at room temperature, and the second culture was incubated in a water bath with the temperature slowly raised to 37°C. Following three hours of incubation, samples were taken from each culture. The cultures incubated at the restrictive temperature (37°C) were then shifted back to room temperature. At this time 0.2 mg/ml cycloheximide was added. Samples were taken from these cultures following two hours of incubation.

Larger scale cultures were used to obtain samples for gel electrophoresis. Synchronized stationary phase cells in YEPD were inoculated into 800 ml of fresh YEPD in a 2500 ml flask. These cultures were grown to stationary phase, as monitored by the appearance of phase bright cells (Pringle and Hartwell, 1981). At this time the cells were
harvested.

For all of the above experiments, the starter cultures were harvested at room temperature. The cells were transferred to sterilized 30 ml Corex tubes (Corning, USA), and centrifuged at setting 6 in an IEC clinical table top centrifuge. The cells were washed once in sterilized double-distilled water, and were then resuspended in 5 mls of the sterilized water. This "cell concentrate" was then used to inoculate the various media. Each flask received an equal volume. That each flask received an equal number of cells was determined by reading a sample from each one at OD₆₅₀ in an MSE Spectro-Plus spectrophotometer (MSE Scientific Instruments, UK) or by counting in a hemocytometer.

C. PREPARATION OF CRUDE ENZYME EXTRACTS

In all experiments, cells were harvested in sterilized 30 ml Corex tubes (Corning, USA). Care was taken to ensure that each sample contained an equal number of cells. In cases where trehalase activities in cells growing on YEPD were being compared to cells growing on poorer carbon sources (such as trehalose, ethanol or 0.1% glucose), the sample volumes were adjusted such that the total cell number in each sample was equal.

The samples were harvested by centrifugation for 5 minutes at setting 6 in an IEC clinical table top centrifuge. Each sample was washed and resuspended in 5 mls of sterile double distilled water. The cells were
completely disrupted by three passages through a French Press chamber (Aminco, USA) at 20,000 psi per passage. The samples were centrifuged at 10,000 g for 20 minutes in a Beckman J21-C refrigerated centrifuge using a Beckman JA-20 rotor. The resulting supernatant was used as a crude enzyme extract. In all cases, particularly with the sec mutants, the pellet was resuspended in 5 ml of sterile double distilled water and also examined for activity.

D. TREHALASE ASSAYS

The assay employed was a discontinuous trehalase assay (Chan and Cotter, 1980). However, the incubation buffers were changed (Londesborough and Varimo, 1984). For measuring the vacuolar trehalase levels, 200 µl of crude extract was incubated with 400 µl 50 mM MES buffer with 0.1 mM EDTA at pH 5 and 200 µl of 12.5 mM trehalose suspended in the same buffer. The cytoplasmic activity is sensitive to EDTA (Londesborough and Varimo, 1984). Cytoplasmic trehalase activity was measured the same way, except that the incubation buffer was 50 mM PIPES buffer with 2.5 mM CaCl₂ at pH 7 and the trehalose was also suspended in this buffer. Ca²⁺ stimulates the protein kinase which activates cytoplasmic trehalase (Uno et al., 1983; Londesborough and Varimo, 1984). Even though these assay conditions tend to eliminate "cross-contamination" of the activities in each assay, it has been noted that when the cytoplasmic activity is fully activated, it can retain 10-20% of its activity at pH 5 (Londesborough and Varimo, 1984).
The samples were incubated under the above conditions for one hour at 23°C. It was verified that during this incubation, the amount of trehalase present in the extract was the limiting factor, and that the reaction was linear. The reactions were then terminated by incubating the samples at 90°C for 10 minutes. This incubation was found to be just as effective as boiling for 10 minutes at terminating the reaction, and it was much easier to process large numbers of samples. The tubes were allowed to sit in ice for 10-20 minutes, and if necessary, precipitated proteins were removed by centrifuging the samples in a Fisher Micro-centrifuge (model 23SB) for 5 minutes.

Liberated glucose was then measured by incubating 100 ul of each sample with 300 ul of 0.1 M acetate buffer, pH 5.5, in the presence of 400 ul of trehalase assay reagent. The reagent consists of 45 U/ml of glucose oxidase (EC 1.1.3.4), 7.5 U/ml horseradish peroxidase (EC 1.11.1.7) and 2.3 mM ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate]) all suspended in 0.1 M acetate buffer, pH 5.5 (Chan and Cotter, 1980). The incubation time was one hour at 23°C or room temperature. The absorbance of each sample was measured in a spectrophotometer at 414 nm.

For each sample tested, an enzyme control was used to measure background glucose in the sample. This consisted of 200 ul of sample plus 600 ul of the appropriate buffer. A substrate control was also included in each assay to measure the amount of free glucose in the trehalose. This
consisted of 200 ul of 12.5 mM trehalose suspended in the appropriate buffer, plus 600 ul of that buffer. These tubes were then treated in the same manner as the sample tubes.

Finally, for each assay, a series of glucose standard controls were used. These consisted of 20 nmoles, 40 nmoles and 80 nmoles of glucose, made by the appropriate dilutions of a 100 uM stock solution of glucose in the appropriate buffer. These tubes were also treated in the same manner as the sample tubes.

E. DETERMINATION OF TREHALASE SPECIFIC ACTIVITY

The value for nmoles glucose released per minute was determined for a particular sample by subtracting the OD values for the substrate and enzyme controls from the sample OD value. This value was then converted to OD per minute, and the value for nmoles glucose released per minute was read from a standard curve. Specific activity was expressed as nmoles of glucose released per minute per mg protein.

F. PROTEIN DETERMINATION

Total protein was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, USA) (Bradford, 1976). Gamma-globulin was used as the standard.

G. ISOELECTRIC FOCUSING GEL ELECTROPHORESIS

Precast LKB IEF gels were used. The cells were stored at 4°C until use. The 5% polyacrylamide gels contained ampholines in the range 3.5 to 9.5 at a concentration of
2.4%. The dimensions of the gel were 245 x 110 x 1 mm. The electrode solutions were 1 M NaOH, applied to the cathode, and 1 M H₃PO₄, applied to the anode. The gel was pre-run at 50 mA for 30 minutes to allow the gradient to establish. The samples were applied using filter paper wicks. As a control, 10 ul of mouse hemoglobin was applied at the acidic end, less than 1 cm from the acidic wick. As an additional control 10 ul of partially purified lysosomal trehalase from D. discoideum was applied at the basic end. The pI of this enzyme is less than 2.5 (Seshadri et al., 1986). 10 ul of the test sample was also applied at the basic end.

The test sample was prepared in the following manner. A crude extract, prepared as described above, was obtained from a large-scale culture of the particular strain. This crude extract was concentrated for approximately 2 hours in an Amicon ultrafiltration unit at 4°C. A PM-30 membrane (Amicon, USA) was used. The concentrate was collected and centrifuged for 3 minutes in a microcentrifuge. The resulting supernatant was applied to the gel.

The gel was run for 3 hours at room temperature with cooling. During this time, the hemoglobin control moved towards the basic end and focused.

At the completion of the run, the gel was removed and immersed in 100 ml of 50 mM MES buffer, pH 5. To this, 50 mls of 12.5 mM trehalose dissolved in the same buffer was added. Finally, 50 mls of trehalase assay reagent,
suspended in 50mM MES buffer at pH 5, was added. The gel was stained for 4 hours at room temperature, and then photographed.

H. PROCEDURES FOR MUTAGENESIS

The strains DBY747 and YNN282 were used in attempts to isolate trehalase deficient mutants. The mutagen EMS (ethyl methanesulfonate) was used. This chemical alkylates guanine residues, and is used for standard yeast mutagenesis experiments (Sherman et al., 1970).

A modification of a previously described procedure for EMS mutagenesis in S. cerevisiae was used (Kaback et al., 1984). A 10 ml culture of late exponential phase cells growing in YEPD at room temperature were harvested by centrifugation. The cells were washed and resuspended in 10 mls of sodium phosphate-glucose buffer (0.2 M sodium phosphate, 0.11 M glucose, pH 8), then treated with 3% (v/v) EMS (Sigma, USA) for 1 hour at 30°C. Mutagenic activity was terminated by diluting the cells 50-fold into 6% sodium thiosulfate. An aliquot of these cells was then centrifuged and resuspended in sterile double distilled water. Appropriate dilutions were then plated on YEPD plates at 23°C for three days. The colonies arising were then replicated onto YEPD or SC-2% trehalose plates using sterile replica filter pads (FMC, USA). These plates were incubated at either 23°C or 37°C for 2 days. Colonies able to grow on glucose, but not on trehalose, were picked. Also, all colonies temperature sensitive for growth were
picked. The colonies were then grown in liquid cultures and their trehalase activities examined.

I. TUNICAMYCIN EXPERIMENTS

Stationary phase cultures of strain X2180-1A grown on YEPD were used to initiate these experiments. These cells were transferred to derepressing media as described in Section B, such that four 50 ml cultures were established. One was a control and received no additions. The second received 500 ul of 95% methanol, and was the methanol control. The third received 500 ul of a 1 mg/ml stock solution of tunicamycin (Sigma, USA) suspended in 95% methanol. The fourth culture received 500 ul of a 2 mg/ml stock solution of tunicamycin, also suspended in 95% methanol. The tunicamycin stock solutions were stored at -20°C. All of these cultures were incubated at room temperature for 5 hours, and samples were then taken.

J. DETECTION OF EXTRACELLULAR TREHALASE

Extracellular trehalase activity was detected by a modification of a procedure designed to detect extracellular invertase activity (Schauer et al., 1985). Single colonies of various strains were grown on YEPD at 30°C for one day. These colonies were then replica plated onto YEP-0.1% glucose and incubated at 23°C for 8-12 hours. The colonies were then replica plated onto a sheet of Whatman No. 1 filter paper. The filter paper had previously been immersed for two minutes in a trehalase assay mixture, which contained 10 mls of 50 mM MES buffer
at pH 5, 10 mls of 12.5 mM trehalose suspended in the same buffer and 20 mls of trehalase assay reagent also suspended in the same buffer. Any extracellular trehalase activity cleaved the trehalose, and the liberated glucose produced a green colour around the patched colony. This reaction occurred slowly during the incubation time. Residual glucose reacted immediately to give a green colour. The filter paper was generally examined after 30 minutes. As a positive control, a similar YEP-0.1% glucose plate was inverted over paper towels pre-soaked with chloroform for 10 minutes. Microscopic examination verified that this treatment completely permeabilized the cells. The permeabilized colonies were then replica plated on to a sheet of Whatman No. 1 filter paper and assayed.

This procedure was also used to screen for trehalase deficient colonies amongst mutagenized cells. The mutagenized cells were treated as described in Section H, and were plated on to YEPD. Following three days incubation, any colonies arising were replica plated on to YEP-0.1% glucose. These plates were incubated for 8-12 hours at 23°C, and were then assayed as described above.

K. YEAST GENETIC MANIPULATIONS

Standard techniques were used for the construction of diploid strains (Sherman et al., 1970). Individual haploids of similar mating type were streaked in a line across a YEPD plate, which was incubated overnight at 30°C. A plate of strains of one mating type was then patched on
to a replica pad. The pad was turned 90° and patched on the plate containing the strains of opposite mating type. The replica pad was then stamped on to a fresh YEPD plate, creating a grid pattern where mating occurred at the intersections. This plate was incubated overnight at 30°C. Non-isogenic strains carrying complementary auxotrophic markers were mated. This allowed for the selection of diploids on SD minimal media with no supplements. The appropriate supplement was added if the strains each carried a common auxotrophic marker. Hence, the mating grids were replica plated on to SD plates (with the appropriate supplement if required) and incubated at 30°C overnight. Diploids grew in small patches at the grid intersections, while haploids failed to grow.
RESULTS

A. TREHALASE ACTIVITIES DURING A GROWTH CYCLE

Trehalase activities were determined in cells at various points during a growth cycle. The strain DBY747 was grown on YEPD and samples were taken over a 30 hour period. The growth cycle was monitored by measuring cell growth, determining the budding index and measuring the external glucose concentration. Cell growth was monitored by reading the OD600 of an aliquot from the culture. The cells grew exponentially for the first 15 hours, and then reached stationary phase by 20 hours (Figure 3). The onset of stationary phase correlated with the depletion of glucose from the media (Figure 3). The budding index is used to express the proportion of actively growing cells in the population. It is calculated by counting the number of cells with observable buds during a random screen of 200 cells (Lillie and Pringle, 1980). As is characteristic for exponentially growing cells, the budding index is high (Figure 3). As the cells approached stationary phase and fewer were dividing, the budding index decreased gradually.

Vacuolar trehalase activity was initially very high as stationary phase arrested cells were inoculated into fresh YEPD (Figure 4). However, prior to inoculation into YEPD, the arrested cells had these high levels. The vacuolar activity then decreased considerably during exponential growth. The activity increased slowly as the cells approached stationary phase. Stationary phase cells (30
FIGURE 3

Growth of strain DBY747 on YEPD or YEP-2% trehalose-0.3% glucose.

Samples were taken at the indicated times and growth was monitored by measuring the absorbance of the sample at OD$_{600}$. The budding index was determined by counting the number of budded cells in a random population of 200 cells using phase contrast microscopy. The amount of glucose in the spent media was determined using the continuous assay for detecting glucose (Chan and Cotter, 1980). Each sample tube received 200 ul of spent media, 200 ul of 0.1 M acetate buffer, pH 5.5, and 400 ul of assay reagent. A series of glucose standards were included, and, the tubes were incubated for 1 hour. The absorbance of each sample was measured at OD$_{600}$ and the amount of glucose present was determined by comparing these readings to those for the glucose standards. The results are from one of 5 independent experiments.

(□) Amount of glucose in spent YEPD
(●) Amount of glucose in spent YEPT
(▲) Budding index in YEPD
(▲) Budding index in YEPT
(○) Growth in YEPD
(●) Growth in YEPT
FIGURE 4

Trehalase activities in strain DBY747 grown in YEPD or YEP-2% trehalose-0.3% glucose.

Samples were taken at the indicated times and assayed for trehalase activities. The amount of glucose in the spent media was determined using the continuous assay for detecting glucose (Chan and Cotter, 1980). The results are from one of 5 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein.

(○) Amount of glucose in spent YEPD
(●) Amount of glucose in spent YEPT
(☐) V-Tre activity in YEPD
(●) V-Tre activity in YEPT
(☐) C-Tre activity in YEPD
(■) C-Tre activity in YEPT
hour sample) had 8-fold higher levels of vacuolar trehalase activity compared to exponential phase cells (10 hour sample).

Cytoplasmic trehalase activity was also very high as the cells were inoculated into YEPD (Figure 4). This too appears to be a due to carry-over from the stationary phase, since the cells had these high levels prior to inoculation. Cytoplasmic activity then settled to approximately 35 units during the exponential growth phase. Cells in the stationary phase showed a 3-fold increase in activity compared to cells in the exponential growth phase (Figure 4).

B. Trehalase Activities in Trehalose-Grown Cells

Trehalase activities were examined in cells of strain DBY747 incubated in YEP-2% trehalose-0.3% glucose over a 30 hour period. Growth parameters were monitored as described above. The cells grew exponentially for the first 5-10 hours on the 0.3% glucose (Figure 3). Following glucose depletion, the cells grew poorly on the trehalose, as they failed to undergo complete doubling. As evidenced by the low budding index, the cells were not dividing during exposure to trehalose, and had likely entered stationary phase.

Vacuolar trehalase activity was high at time zero as the cells were inoculated into trehalose containing media (Figure 4). Activity then decreased, but not to as great an extent as it did in cells grown in YEPD. After 10
hours, vacuolar trehalase activity was about 10-fold higher in cells in trehalose containing media than in cells growing in YEPD for 10 hours (Figure 4, Table 2). After 20 hours, activity had risen slightly, but was still 3-fold higher than that in cells grown for 20 hours in YEPD. After 30 hours, or 20 hours exposure to trehalose, vacuolar trehalase activity was almost 75 units. It was still higher than the activity in cells incubated in YEPD for 30 hours (Figure 4, Table 2).

Cytoplasmic trehalase activity was also higher after 10 hours in YEP-2% trehalose-0.3% glucose when compared to cells grown for 10 hours in YEPD (Figure 4, Table 2). As the cells were exposed to the trehalose, cytoplasmic activity increased 2-fold. However, by 30 hours the activity had decreased slightly (Figure 4).

C. Trehalase Activities in Cells Growing on Other Carbon Sources

Trehalase activities were examined in cells growing on YEP with either 2% maltose, 2% sucrose or 2% glycerol, with each also containing 0.3% glucose. Strain DBY747 was grown on each of these media, as well as YEPD, and trehalase activities were checked after 20 hours growth. At this time, the glucose had been depleted and the cells were growing on the alternative carbon source.

Cell growth, as monitored by the OD600 of an aliquot from each culture, was as efficient on sucrose as it was on YEPD (Table 3). However, growth was poorer on maltose, and
TABLE 2  The ratio of trehalase activities in extracts of cells grown in YEP-2% trehalose medium compared to cells grown in YEPD medium

<table>
<thead>
<tr>
<th>TIME (hours)</th>
<th>V-TRE(^1)</th>
<th>C-TRE(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>10</td>
<td>10.3</td>
<td>2.1</td>
</tr>
<tr>
<td>20</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>30</td>
<td>1.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

\(^1\) Vacuolar trehalase activity ratios
\(^2\) C, tonplasmic trehalase activity ratios
<table>
<thead>
<tr>
<th>MEDIA</th>
<th>CELL NUMBER ($\times 10^7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 HOUR</td>
</tr>
<tr>
<td>YEPD</td>
<td>0.4</td>
</tr>
<tr>
<td>YEP-2% SUCROSE</td>
<td>0.4</td>
</tr>
<tr>
<td>YEP-2% MALTOSE</td>
<td>0.4</td>
</tr>
<tr>
<td>YEP-2% GLYCEROL</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Synchronized stationary phase cells in YEPD were harvested. The cells were transferred to four separate cultures with the appropriate medium. Following a 20 hour incubation, the cell number in each culture was determined using a hemocytometer. The results presented are from one of 5 independent experiments.
was very slow on glycerol (Table 3).

Cells grown on sucrose showed essentially the same profile of vacuolar and cytoplasmic trehalase activities as cells grown on glucose (Figure 5). Sucrose is hydrolyzed externally by the periplasmic enzyme invertase, and the glucose generated is transported into the cell (Schekman and Novick, 1982). Thus the cells are essentially growing on glucose.

Cells grown on maltose or on glycerol exhibit the same level of cytoplasmic trehalase activity as do cells grown on YEPD (Figure 5). However, vacuolar trehalase activity was approximately 3-fold higher in these cells than in those grown in YEPD (Figure 5).

D. TREHALASE ACTIVITIES IN pep4 MUTANTS

The pep4 mutant has diminished levels of all vacuolar enzymes (Hemmings et al., 1981; Jones, 1984). Such mutants should possess diminished levels of vacuolar trehalase activity if that enzyme is a true vacuolar enzyme. Cytoplasmic trehalase activity should be relatively unaffected.

The pep4 mutant strain 20B-12 and its otherwise isogenic parent X2190-1A were examined for trehalase activities after 20 hours growth in either YEPD or YEP-2% trehalose-0.3% glucose. Both strains grew similarly on each carbon source (Table 4).

On YEPD, vacuolar trehalase activity was almost 10-fold lower in the pep4 mutant compared to its parent.
FIGURE 5
Trehalase activities in strain DBY747 growing on various carbon sources.

Cells were grown in the indicated media and samples were taken after 20 hours growth and assayed for trehalase activities. The results are from one of 5 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein.

C: cytoplasmic trehalase activity.
V: vacuolar trehalase activity.
YEPD: YEP-2% glucose. YEPS: YEP-2% sucrose.
YEPM: YEP-2% MALTOS. YEPG: YEP-2% glycerol.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>MEDIA</th>
<th>CELL NUMBER ($\times 10^7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hour</td>
<td>20 hours</td>
</tr>
<tr>
<td>X2180-1A</td>
<td>YEPD</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>YEP-2% TRE</td>
<td>1.9</td>
</tr>
<tr>
<td>208-12</td>
<td>YEPD</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>YEP-2% TRE</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Mid-exponential growth phase cells growing on YEPD were harvested. One-half of the cells were inoculated into YEPD and the remaining half were inoculated into YEP-2% trehalose-0.7% glucose. Following 20 hours incubation, the cell number in each culture was determined using a hemocytometer. The results are from one of 10 independent experiments.
Cytoplasmic activity was similar in each strain (Table 5). On trehalose, 20B-12 exhibited almost 5-fold lower vacuolar trehalase activity than did X2180-1A. However, there was a similar decrease in cytoplasmic activity in 20B-12 (Table 5).

Trehalase activities were examined in a second pep4 mutant. However, this mutant, JHRY20-2Ca, is a null mutant generated by the insertion of the S. cerevisiae UP43 gene into the PEP4 coding region using standard yeast genetic procedures (Ammerer et al., 1986; Rothstein, 1983).

The pep4 mutant JHRY20-2Ca and its otherwise isogenic parent, JHRY20-2C, grew equally poorly on trehalose containing plates. Trehalase activities in these strains were compared on repressing media (YEPD) and on derepressing media (YEP-0.1% glucose). This was done to conform to the conditions in which other vacuolar enzymes had been studied in this mutant (J.H.Rothman, pers. commun.). It was also done to remove the possible effects on the trehalase levels of having an alternative carbon source present following glucose depletion. Finally, these two strains are known to be isogenic since the mutation was specifically created in the PEP4 gene. There could potentially be background mutations in 20B-12 that are not present in the strain from which it was derived, X2180-1A, due to the mutagenic treatment (Hemmings et al., 1981). These background mutations may affect the trehalase levels in 20B-12.
TABLE 5  Trehalase activities in a pep4 mutant

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>V-TRE ACTIVITY</th>
<th>C-TRE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>X2190-1A (wild-type)</td>
<td>9</td>
<td>56</td>
</tr>
<tr>
<td>208-12 (pep4-3)</td>
<td>1</td>
<td>12</td>
</tr>
</tbody>
</table>

Synchronized stationary phase cells in YEPD were harvested. One-half of the cells were inoculated into YEPD and the remaining half were inoculated into YEP-2% trehalose-0.3% glucose. After a 20 hour incubation the cultures were harvested and crude extracts were prepared. The results presented are from one of 10 independent experiments. Specific activity is defined as the number of mmols of glucose released per minute per mg of protein.
There was no vacuolar trehalase activity present in JHRY20-2Co when it was grown under repressing conditions. The parent strain had a low level of vacuolar activity (Table 6). Under derepressing conditions, there was a 5-fold reduction in vacuolar trehalase activity in the pep4 mutant compared to its parent (Table 6).

Each strain exhibited similar levels of cytoplasmic trehalase activity under repressing and derepressing conditions (Table 6). The levels of cytoplasmic activity on YEPD were comparable in strains JHRY20-2C and X2180-1A, but they were higher than the activity in DBY747.

E. TREHALASE ACTIVITIES IN SECRETORY MUTANTS

True vacuolar enzymes traverse the early part of the secretory pathway in S. cerevisiae (Stevens et al., 1982). If "vacuolar trehalase" is a true vacuolar enzyme, then it should also pass through these stages of the secretory pathway. Since mutants defective at each stage of the pathway are available (Schekman and Novick, 1982; Schekman, 1985), trehalase activities were examined in them and their parent strain X2180-1A.

Cells were grown in derepressing media at either 27°C or 37°C. The sec mutants are temperature sensitive for growth at 37°C. After 3 hours incubation to allow for derepression, samples were taken from each culture. Incubation of the mutants at the restrictive temperature for longer periods of time results in lysis of the cells. The block to secretion in the mutants are reversible, thus
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>V-TRE ACTIVITY</th>
<th>C-TRE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REP</td>
<td>DREP</td>
</tr>
<tr>
<td>JHRY20-2C (PEP4 ura3-52)</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>JHRY20-2Ca (pep4::URA3)</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

Mid-exponential growth phase cells growing on YEPD were harvested. One-half of the cells were inoculated into YEPD (repressing conditions) and the remaining half were inoculated into YEP-0.1% glucose (derepressing conditions). The cultures were incubated for 8-12 hours after which they were harvested and crude extracts were prepared. The results presented are from one of 10 independent experiments. Specific activity is defined as the number of moles of glucose released per minute per mg of protein.
the culture incubated at 37°C was shifted back to 23°C. Since the reversibility is not dependent on new protein synthesis, 0.2 mg/ml cycloheximide was added at the time of the temperature shift. Samples were taken following 2 hours incubation.

Initially, the effects of a temperature shift from 23°C to 37°C on trehalase levels under repressing conditions was examined in the wild-type strain X2180-1A. After 3 hours incubation in YEPD at 23°C, there was a 3-fold increase in vacuolar trehalase activity and a 10-fold increase in cytoplasmic activity (Table 7). At 37°C, the vacuolar trehalase level was 2-fold higher than it was at 23°C, while there was a 50% increase in cytoplasmic activity.

Thus the shift from 23°C to 37°C seems to cause an approximate 2-fold increase in vacuolar trehalase activity under repressing conditions, while there is a smaller increase in cytoplasmic activity.

1. sec18 Mutant

The sec18 mutant is blocked in transport from the endoplasmic reticulum to the Golgi body (Schekman and Novick, 1982; Schekman, 1985). In this mutant, vacuolar carboxypeptidase Y accumulates as an inactive precursor (Stevens, et al., 1982). The effects of a temperature shift on trehalase activities under repressing conditions were examined in the sec18 mutant strain HMSF176. After 3 hours at 23°C, there was a 2-fold increase in vacuolar
TABLE 7  Effect of a temperature shift on trehalase activities

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>V-TRE ACTIVITY</th>
<th>C-TRE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr.</td>
<td>3 hr.</td>
</tr>
<tr>
<td>X2190-1A (wild-type)</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>HMSF176 (sec19-I)</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

Mid-exponential growth phase cells growing on YEPD were harvested, a sample was taken, and the remainder were used to inoculate two separate cultures in YEPD. One culture was maintained at 23°C while the other culture was transferred to a water bath where the temperature was slowly raised to 37°C. Following 3 hours incubation, the cultures were harvested and crude extracts were prepared. The results presented are from one of 4 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein.
trehalase activity and a 3-fold increase in cytoplasmic activity (Table 7). At 37°C, there was no vacuolar trehalase activity detected, while cytoplasmic activity was similar to that from X2180-1A.

Trehalase levels were then examined in HMSF176 and X2180-1A under derepressing conditions. Each strain had similar low levels of vacuolar trehalase activity after 13 hours incubation in YEP-0.1% glucose at 23°C (Table 8). At 37°C, X2180-1A exhibited a fold higher levels of vacuolar activity than was found at 23°C. However, HMSF176 showed a 3-fold reduction in vacuolar trehalase activity at 37°C (Table 8). Vacuolar activity recovered in HMSF176 when the cells were shifted back to 23°C.

While the absolute levels of cytoplasmic trehalase activity were about 4-fold lower in HMSF176 than in X2180-1A at 23°C and 37°C, there were similar increases in activity in cells incubated at 37°C (Table 8).

Thus the secl8 mutation had a drastic effect on vacuolar trehalase activity, while cytoplasmic trehalase activity was unaffected.

2. sec7 Mutant

The sec7 mutant is blocked in the assembly of secretory vesicles in the Golgi body (Schekman and Novick, 1982; Schekman, 1985). Vacuolar carboxypeptidase Y accumulates as an inactive precursor in this mutant (Stevens et al., 1982). At 37°C, the sec7 mutant SF294-2B forms irreversible Golgi-like structures called Berkeley
TABLE 2  Trehalase activities in a sec18 mutant

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>V-TRE ACTIVITY</th>
<th>C-TRE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REP</td>
<td>DREP</td>
</tr>
<tr>
<td>X2180-1A (wild-type)</td>
<td>0</td>
<td>5 19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMSF176 (sec18)</td>
<td>0</td>
<td>4 1</td>
</tr>
</tbody>
</table>

Mid-exponential growth phase cells growing on YEPD were harvested, a sample was taken, and the remainder were used to inoculate two separate cultures in YEP-0.1% glucose (derepressing conditions). One culture was maintained at 27°C while the other was transferred to a water bath where the temperature was slowly raised to 37°C. Following 3 hours incubation, the cultures were harvested and crude extracts were prepared. The results presented are from one of 4 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein. Activity in cells shifted back to 27°C from 37°C and incubated for 2 hours in the presence of 0.2 mg/ml cycloheximide.
bodies in 2% glucose. In 0.1% glucose, such structures are not formed and the mutation is reversible (Schekman, 1985).

Trehalase levels were examined in SF294-2B and its parent strain X2180-1A under derepressing conditions.

After 3 hours incubation in YEP-0.1% glucose at 23°C, X2180-1A and SF294-2B had similar low levels of vacuolar trehalase activity (Table 9). At 37°C, there was a 3-fold increase in vacuolar activity in X2180-1A. However, there was no increase in vacuolar trehalase activity in SF294-2B, instead there was a slight decrease in activity (Table 9). Vacuolar trehalase levels recovered when SF294-2B was returned to 23°C.

SF294-2B and X2180-1A showed similar levels of cytoplasmic trehalase activity after 3 hours at 23°C. Activity increased in each strain at 37°C, 2.5-fold in SF294-2B and 5-fold in X2180-1A (Table 9).

Thus the sec7 mutation affected vacuolar trehalase activity while the cytoplasmic activity was not affected.

3. sec5 Mutant

The sec5 mutant HMSN134 is blocked in the transport of secretory vesicles to the cell surface (Schekman and Novick, 1982; Schekman 1985). In this mutant, vacuolar carboxypeptidase Y is active and located in the vacuole (Stevens et al., 1982). Trehalase activities were examined in HMSN134 and its parent X2180-1A under derepressing conditions.

Following 3 hours incubation in YEP-0.1% glucose at
TABLE 9  Trehalase activities in a sec7 mutant

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>V-TRE ACTIVITY</th>
<th>C-TRE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REP DREP</td>
<td>REP DREP</td>
</tr>
<tr>
<td>23 37</td>
<td>59 286</td>
<td>(22) (282)</td>
</tr>
<tr>
<td>X2180-1A (wild-type)</td>
<td>0 18 (22)</td>
<td>6 5 45</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>52 127 (175)</td>
</tr>
<tr>
<td>SF294-2B (sec7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The results are from one of 4 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein.
2 Activity in cells shifted back to 23°C from 37°C and incubated for 2 hours in the presence of 0.2 mg/ml cycloheximide.
23°C, X2180-1A had 2-fold more vacuolar trehalase activity than did HMSF134 (Table 10). After 3 hours at 37°C, X2180-1A showed a 4-fold increase in vacuolar trehalase activity, while HMSF134 had a 5-fold increase in activity (Table 10). Each strain exhibited further increases in vacuolar trehalase activity when shifted back to 23°C from 37°C.

X2180-1A also had 2-fold more cytoplasmic trehalase activity than did HMSF134 after 3 hours at 23°C. Each strain showed a similar increase in activity at 37°C, 12-fold in HMSF134 and 8-fold in X2180-1A (Table 10).

Thus the sec5 mutation did not affect vacuolar or cytoplasmic trehalase activity.

F. TREHALASE LEVELS IN TUNICAMYCIN TREATED CELLS

The vacuolar enzymes carboxypeptidase Y and protease A are properly localized to the vacuole in the presence of the N-linked glycosylation inhibitor tunicamycin, but they are inactive (Jones, 1984). Thus the effects of tunicamycin on trehalase levels in the strain X2180-1A were determined.

Mid-exponential phase cells growing in YEPD at 37°C were transferred to four flasks of fresh YEP-0.1% glucose. Two flasks were treated with tunicamycin at 10 μg/ml and 20 μg/ml, and the remaining two served as controls. Since the tunicamycin was suspended in methanol, one control received an equal volume of methanol. The cultures were incubated for 5 hours to allow for derepression of the vacuolar trehalase.
TABLE 10  Trehalase activities in a sec5 mutant

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>V-TRE ACTIVITY</th>
<th>C-TRE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>REP DREP REP DREP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23 37</td>
<td>23 37</td>
</tr>
<tr>
<td>X2180-1A (wild-type)</td>
<td>0 5 20 (26)</td>
<td>38 44 312 (246)</td>
</tr>
<tr>
<td>HMSF134 (sec5)</td>
<td>0 3 12 (17)</td>
<td>42 20 246 (205)</td>
</tr>
</tbody>
</table>

1 The results are from one of 4 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein.
2 Activity in cells shifted back to 33°C from 37°C and incubated for 2 hours in the presence of 0.2 mg/ml cycloheximide.

TABLE 11  Effects of tunicamycin on trehalase activities in strain X2180-1A

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>V-TRE ACTIVITY</th>
<th>V-TRE FOLD-INCREMENT</th>
<th>C-TRE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hour1</td>
<td>5</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>5 hour control</td>
<td>27</td>
<td>5.4</td>
<td>165</td>
</tr>
<tr>
<td>5 hour MeOH control2</td>
<td>44</td>
<td>4.8</td>
<td>138</td>
</tr>
<tr>
<td>5 hour 10 ug/ml TM3</td>
<td>10</td>
<td>2.0</td>
<td>112</td>
</tr>
<tr>
<td>5 hour 20 ug/ml TM4</td>
<td>8</td>
<td>1.6</td>
<td>34</td>
</tr>
</tbody>
</table>

1 Mid-exponential growth phase cells growing on YEPD were harvested and a sample was taken. The remaining cells were then transferred to YEP-0.1% glucose in four separate cultures.
2 Received 500 ul of 95% methanol.
3 Received 500 ul from a 1 mg/ml stock solution of tunicamycin suspended in 95% methanol.
4 Received 500 ul from a 2 mg/ml stock solution of tunicamycin suspended in 95% methanol.
5 The results presented are from one of 5 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein.
After 5 hours incubation, there was a 5.4-fold increase in vacuolar trehalase activity in the non-treated control (Table 11). There was also a large increase in the cytoplasmic activity. The methanol treated control had slightly lower levels of each activity, but still showed a 4.8-fold derepression of vacuolar activity (Table 11). The culture treated with 10 ug/ml tunicamycin showed only a 2-fold increase in vacuolar trehalase activity, with 40% of the level of activity seen in the untreated control. There was also a sharp decrease in cytoplasmic trehalase activity, but not to as great an extent as for the vacuolar activity (Table 11). The culture treated with 20 ug/ml tunicamycin exhibited a small 1.6-fold derepression of vacuolar trehalase activity, but the cytoplasmic activity was also drastically diminished.

G. ISOELECTRIC POINT OF VACUOLAR TREATRASE

Trehalases from D. discoideum and Neurospora crassa are compartmentalized enzymes (Seshadri et al., 1986; White et al., 1985). They each possess an acidic isoelectric point. The vacuolar trehalase of S. cerevisiae should also possess an acidic isoelectric point if it is a compartmentalized enzyme. To examine this, a concentrated crude extract derived from an 800 ml stationary phase culture of strain X2180-1A in YEPD was run on an IEF polyacrylamide gel.

The gel was run as described in Materials and Methods, section G. It was run until the mouse hemoglobin had
focused as a number of discrete bands. Hemoglobin has known isolectric points in the range from 6.8 to 7.1. The lysosomal trehalase of *D. discoideum* has an isolectric point of less than 4.0 (Seshadri et al., 1986). It can be seen as a wide band at the acidic end of the gel (Figure 6). The broadness of the band indicates that the protein has not yet focused.

The yeast samples each show two bands staining faintly (Figure 6). In each lane, one band is at the acidic end of the gel and is above the region where the *D. discoideum* trehalase is found. These bands have not yet focused. Two lanes possess a second band at the basic end of the gel (Figure 6). However, cytoplasmic trehalase should not be detected under the staining conditions used. These bands are more likely the result of a portion of the sample not entering the gel. This is verified in the third lane, where the sample was applied at the middle of the gel. The second band in this lane appears at this point, not at the basic end of the gel (Figure 6).

Thus, the vacuolar trehalase has an acidic isolectric point; and by analogy with the compartmentalized trehalases in *D. discoideum* and *N. crassa*, is also likely to be compartmentalized.

**H. ISOLATION OF MUTANTS UNABLE TO GROW ON TREHALOSE**

In each of four separate mutagenesis experiments utilizing EMS as a mutagen, approximately $5 \times 10^6$ cells were mutagenized and plated on YEPD at 50 cells per plate.
FIGURE 6

Representation of isoelectric gel electrophoresis results:

Crude extracts from strain X2180-1A were prepared and run on an IEF gel as described in the Materials and Methods. The gel was stained in a trehalase assay mixture (see Materials and Methods) and the banding patterns were depicted diagramatically.

Lanes 1-3: Crude extracts from S. cerevisiae strain X2180-1A. Lanes 2 and 3 were loaded at the basic end of the gel, while lane 1 was loaded near the midpoint of the gel.

Lane 4: Mouse hemoglobin
Lane 5: Partially purified D. discoideum lysosomal trehalase.
Three of the experiments were based on selection on YEP-2% trehalose, and these yielded 2 mutants unable to grow on trehalose which appeared not to be petite mutants. The extracellular trehalase plate assay was used for selection in the fourth experiment, and one mutant with no detectable extracellular activity was isolated.

The 2 colonies picked on the basis of their inability to grow on YEP-2% trehalose grew adequately on glucose, but not as well as the parent strains DBY747 and YNN282. The size of the colonies on YEPD was similar to the size of petite mutants which lack functional mitochondria, and can only grow fermentatively. However, each of the 2 putative mutants had a smaller cell size compared to petites. They also had prominent vacuoles during exponential growth, which neither the parent strain nor petites have.

Each of the putative mutants was tested for trehalase activities. Synchronized cells (see Materials and Methods, Section B) were inoculated into YEP-2% trehalose-0.1% glucose and were grown for 20 hours at 23°C. Each of these isolates, SH1 (derived from DBY747) and SH19 (derived from YNN282), had vacuolar trehalase activities less than 25% of that in a wild-type parent (Table 12). There was also a deficiency in the cytoplasmic trehalase activity of each isolate, but not as great as that for the vacuolar activity (Table 12). It was observed that there was a large degree of variation in the percentage of wild-type vacuolar activity in each mutant, but it was generally less than
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GROWTH ON TREHALOSE</th>
<th>TREHALASE ACTIVITIES</th>
<th>YEPD</th>
<th>YEPT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-TRE</td>
<td>C-TRE</td>
<td>V-TRE</td>
<td>C-TRE</td>
</tr>
<tr>
<td>DBY746</td>
<td>13</td>
<td>86</td>
<td>56</td>
<td>207</td>
</tr>
<tr>
<td>YNN282</td>
<td>19</td>
<td>118</td>
<td>44</td>
<td>204</td>
</tr>
<tr>
<td>SH3</td>
<td>-</td>
<td>28</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>SH18</td>
<td>-</td>
<td>19</td>
<td>13</td>
<td>38</td>
</tr>
<tr>
<td>SH200</td>
<td>-</td>
<td>123</td>
<td>18</td>
<td>67</td>
</tr>
</tbody>
</table>

Synchronized stationary phase cells in YEPD were harvested. One-half of the cells were transferred to YEPD, and the remaining half were transferred to YEP-2% trehalose-0.7% glucose. Following a 20 hour incubation, the cells were harvested and crude extracts were prepared. The results are from one of five independent experiments. Specific activity is defined as the number of nanomoles of glucose released per minute per mg of protein.
The third mutant, SH200 (derived from YNN282), possessed no extracellular trehalase activity as determined by the plate assay. However, it had internal vacuolar trehalase levels similar to those in YNN282 (Table 12). Using the plate assay, no extracellular trehalase activity was detected from SD3 or SD18.

SH3 is mating type a, while SH18 is mating type alpha. They were mated and the complementation behaviour of their phenotypes was examined. Diploid YSH1 (SH3 x SH18) failed to grow on trehalose and exhibited no extracellular trehalase activity (Table 13). Diploids YSH2 (SH3 x YNN282) and YSH1 (SH18 x DBY747) each exhibited some growth on trehalose (wild-type strains grow poorly on trehalose), and showed weak staining on the trehalase plate assay (Table 13). Thus the mutations are non-complementing and are recessive.

The mutant SH3 was studied in further detail. Trehalase activities were examined during a growth cycle as they were for strain DBY747. On YEPD, SH3 ceased growth once glucose was depleted (Figure 7). DBY747 continued to grow past this point as the cells switch to respiratory growth (Figure 7). On YEP-2% trehalose-0.2% glucose, SH3 again failed to grow once the glucose was depleted, even though the trehalose was available (Figure 7). Following glucose depletion, DBY747 continued to grow slowly on the trehalose (Figure 7). SH3 exhibited virtually no vacuolar trehalase activity on either YEPD or YEP-2% trehalose-0.2%.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GROWTH ON TREHALOSE</th>
<th>EXTRACELLULAR ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH3</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>SH18</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>SH200</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>YSH-1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>YSH-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>YSH-3</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Single colony isolates of each strain were streaked onto YEP-2% trehalose and SC-2% trehalose plates and were incubated at room temperature for 3 days. SC-2% trehalose media was supplemented with uracil, histidine, and tryptophan, or adenine and lysine at 20 mg/l when necessary. Leucine was added at 20 mg/l when required. *As determined by the trehalase plate assay.*
FIGURE 7

Growth of strain SH3 on YEPD or YEP-2% trehalose-0.3% glucose.

Samples were taken at the indicated times and growth was monitored by measuring the absorbance of the sample at OD_600. The budding index was determined by counting the number of budded cells in a random population of 200 cells using phase contrast microscopy. The amount of glucose in the spent media was determined using the continuous assay for detecting glucose (Chan and Cotter, 1980). The results presented are from one of 5 independent experiments.

(○) Amount of glucose in spent YEPD
(●) Amount of glucose in spent YEP
(△) Budding index in YEPD
(▲) Budding index in YEP
(○) Growth in YEPD
(●) Growth in YEP
FIGURE 8

Trehalase activities in strain SH7 grown in YEPD or YEP-2% trehalose-0.3% glucose.

Samples were taken at the indicated times and assayed for trehalase activities. The amount of glucose in the spent media was determined using the continuous assay for detecting glucose (Chan and Cotter, 1980). The results presented are from one of 5 independent experiments. Specific activity is defined as the number of nmoles of glucose released per minute per mg of protein.

(○) Amount of glucose in spent YEPD.
(●) Amount of glucose in spent YEPT.
(○) V-Tre activity in YEPD.
(●) V-Tre activity in YEPT.
(□) C-Tre activity in YEPD.
(■) C-Tre activity in YEPT.
glucose (Figure 8). The levels of cytoplasmic trehalase activity in SH3 were low throughout the growth cycle (Figure 8).

I. DETECTION OF EXTRACELLULAR TREHALASE ACTIVITY

The strains X2180-1A, DBY746, DBY747, YNN281 and YNN282 all exhibited varying degrees of extracellular trehalase activity. This was only observed if the colonies were incubated 8-12 hours on YEP-0.1% glucose to derepress the vacuolar enzymes. Microscopic examination of cells from the colonies staining positive revealed that no cell lysis had occurred. When all of the colonies were permeabilized, they all demonstrated an immediate positive stain. However, the non-permeabilized colonies that stained positive developed the stain slowly. Attempts were made to recover extracellular trehalase activity from 50 ml YEPD culture supernatants, but only negligible amounts of activity were detected.
DISCUSSION

The yeast S. cerevisiae possesses two distinct trehalase activities. The cytoplasmic activity is regulated by cAMP-dependent phosphorylation. It has a neutral pH optimum and is not glycosylated. The vacuolar activity is not regulated by phosphorylation. It has an acidic pH optimum and is glycosylated. The $K_m$ for trehalose for the cytoplasmic and vacuolar activities are 5.7 mM and 1.6 mM respectively (Londesborough and Varimo, 1984).

An earlier proposal suggested that a precursor relationship existed between the two trehalase activities. Upon activation by phosphorylation and subsequent degradation of trehalose, it was postulated that the cytoplasmic enzyme was translocated into the vacuole for degradation (Weimken and Schellenberg, 1982). However, since the vacuolar enzyme is glycosylated and the cytoplasmic enzyme is not, such a translocation would require a novel method of glycosylation.

Instead, it is possible that each trehalase is a distinct enzyme, and the two are subjected to different types of regulation. The cytoplasmic enzyme is regulated by phosphorylation. The vacuolar enzyme could possibly be under general vacuolar enzyme control or carbon catabolite repression. The existence of two distinct trehalases in S. cerevisiae has been previously suggested (Thevelein, 1984b).
For the vacuolar trehalase to be a true vacuolar enzyme, it should meet a number of criteria. It should show starvation induced derepression. The protein should have an acidic isoelectric point, and be synthesized as a proenzyme that is processed through the early stages of the secretory pathway followed by subsequent conversion to the active enzyme in the vacuole. The evidence presented here demonstrates that the vacuolar trehalase does satisfy these criteria.

STARVATION INDUCED DEREPRESSION

The three vacuolar proteases, carboxypeptidase Y, protease A and protease B, are all derepressed just prior to the onset of stationary phase in rich media (Jones, 1984). This coincides with the time of glucose depletion from the media. Vacuolar trehalase exhibits a similar derepression. Stationary phase activity is generally 4-8 fold higher than exponential phase activity. This increase in activity coincides with glucose depletion as the cells enter stationary phase.

The same strain, DBY747, was grown on sucrose, trehalose, maltose and glycerol and vacuolar trehalase activity was examined. Each carbon source was present at 2%, with 0.7% glucose present to allow standardization of growth. After 20 hours incubation on sucrose, the vacuolar activity was repressed. This was similar to the repressed activity in glucose grown cells. Growth on sucrose is essentially equivalent to growth on glucose, since sucrose
is rapidly hydrolyzed by a periplasmic enzyme, and the glucose generated is internalized by the cells. Thus, no glucose depletion occurs. On the maltose, trehalose and glycerol based media, glucose depletion occurs within ten hours of inoculation. In each case, vacuolar trehalase activity showed a 3-fold derepression. This increase in activity is not due to the presence of an alternative carbon source following glucose depletion, since it still occurs in the presence of only 0.1% glucose. However, the alternative carbon sources may minimize the extent of derepression.

The derepression of vacuolar trehalase upon glucose depletion could be due to starvation induced general vacuolar enzyme derepression. The release of carbon catabolite repression most likely plays a significant role in this. Recently, vacuolar protease B repression has been shown to be under carbon catabolite control. Mutants defective in glucose repression show significantly higher protease B activity under normally repressing conditions (Moehle and Jones, 1986). It would be interesting to examine vacuolar trehalase activity in these mutants.

It should be noted that although the absolute values for vacuolar trehalase activity varied between experiments, the differences in the level of activity between cells grown on glucose and on the other carbon sources were consistent. This also applies to the experiments comparing vacuolar trehalase activity in wild-type cells and the pep
and sec mutants. The variance in the absolute levels of activity is due in large part to the extent of vacuolar enzyme derepression at the time the cultures were harvested (Jones et al., 1981).

Cytoplasmic trehalase activity was consistently higher than the vacuolar activity. There was generally an increase in cytoplasmic activity following glucose depletion, but this was strain dependent. Whereas all strains showed derepression of vacuolar trehalase, the increase in cytoplasmic activity varied. Strain X2180-1A exhibited a greater increase than did DBY747, while JHY20-2C showed no increase. The extent of the increase for each strain varied between experiments as well. Since the cytoplasmic activity is regulated by a cAMP-dependent phosphorylation cascade, it is largely inactive when that cascade is not functioning. In the crude enzyme extract, which was obtained by rather harsh means (French pressing at 20,000 psi), the cascade had most likely been disrupted. The cAMP-dependent protein kinase which utilizes cytoplasmic trehalase as a substrate is also calcium dependent (Uno et al., 1983). Thus, 2.5 mM calcium was included in the assay to allow partial activation of the cytoplasmic enzyme. This amount of calcium is unphysiologically high, which results in higher cytoplasmic trehalase activity than might actually be present in the cell (Londesborough and Varimo, 1984).

The cytoplasmic trehalase shows no increase in
activity on sucrose, maltose or glycerol compared to glucose. Only on trehalose is there an increase in activity, with levels 2-3 fold higher than at comparable incubation times on glucose. This could be due to trehalose being the poorest growth substrate of those tested, causing the cells to enter stationary phase. *S. cerevisiae* grows poorly on trehalose with a generation time in excess of 20 hours (Barnett et al., 1984).

A possible scenario for the regulation of the two trehalases is described below. In a situation analogous to that for *S. cerevisiae* invertase, the protein could be translated from separate transcripts, which are coded for by a common gene (Carlson and Botstein, 1982). The transcript for the vacuolar enzyme would be longer due to the presence of the signal sequence and pro-sequence. The estimated molecular weights of the vacuolar and cytoplasmic enzymes are 215,000 and 170,000 daltons respectively (Londesborough and Varino, 1984). The difference could be accounted for solely by the presence of carbohydrate on the vacuolar enzyme.

Transcription of the gene may be induced by the depletion of glucose and the relief of glucose repression. This occurs prior to the onset of stationary phase. At this time, a number of unidentified new proteins appear as detected by two-dimensional gel electrophoresis (Bouche, 1985). The vacuolar enzyme would be activated in the vacuole. Its role may be to degrade limited amounts of trehalose during starvation, in order to generate glucose.
for maintenance metabolism in the resting cells (Pringle and Hartwell, 1981). The cytoplasmic activity would be relatively inactive in starved cells due to the low intracellular cAMP concentration in such cells (Eraso and Gancedo, 1984; Matsumoto et al., 1985b). Upon introduction into new growth media, cAMP levels increase and the cytoplasmic trehalase is activated (Thevelein, 1984c). The location of trehalose in cells of S. cerevisiae has not yet been determined. If it is located in the cytoplasm, the cAMP induced activation of cytoplasmic trehalase is a way in which the cell could prevent premature degradation of stored trehalose.

It is possible that the trehalase gene in S. cerevisiae is duplicated. A significant number of protein-coding genes are present in two or three functional copies per haploid genome, and in some cases it appears that only one copy is needed to allow for normal growth of a haploid cell (Kaback et al., 1984). There are five copies each of the genes encoding maltase and invertase (Mortimer and Schild, 1985). Since trehalose degradation may be a critical aspect of growth control, the gene coding for the two trehalases could be duplicated. The strain differences in trehalase activities may be due to differences in the number of functional copies of the genes in each strain.

PROCESSING OF VACUOLAR TREHALASE

In S. cerevisiae, all vacuolar enzymes are synthesized as inactive proenzymes. They are synthesized on membrane-
bound ribosomes and then enter the endoplasmic reticulum. They are transported to the Golgi body, from which they are diverted to the vacuole. In the vacuole, the pro-sequence is cleaved by protease A (the PEP4 gene product) to generate the active enzyme (Jones, 1984).

The vacuolar trehalase traverses the secretory pathway up to the Golgi body. This was determined by examining trehalase levels in a number of temperature-sensitive secretory mutants. Mutants defective in transport from the endoplasmic reticulum (sec18) to the Golgi body and in the formation of secretory vesicles in the Golgi body (sec7) show deficiencies in vacuolar trehalase activity at the restrictive temperature. A mutant defective in the transport of the secretory vesicles (sec5) showed no deficiency in activity at 37°C. Thus, this step is not required for the transport of vacuolar enzymes to the vacuole. Finally, a mutant defective in the conversion of vacuolar proenzymes to their active form in the vacuole (pep4) has diminished levels of vacuolar trehalase activity.

The basis for determining that vacuolar trehalase activity was diminished in the sec7 and sec18 mutants was the observation that there was a large increase in both trehalase activities in cells incubated at 37°C compared to those incubated at 27°C. When mid-exponential cells were transferred into fresh YEPD, those cells incubated at 37°C had 2-fold higher vacuolar trehalase activity compared to
cells, incubated at 23°C. There was also an increase in cytoplasmic trehalase activity. Thus, the higher temperature played some role in the increase in activity. However, wild-type cells showed a 4-fold increase in vacuolar trehalase activity at 37°C compared to 23°C when transferred from repressing conditions to derepressing conditions. Since 37°C is closer to the optimal growth temperature for wild-type S. cerevisiae than 23°C, it is possible that cells incubated at this temperature deplete the small amount of glucose (0.1%) present in derepressing media much quicker than do those cells incubated at 23°C. This would result in an earlier derepression of vacuolar trehalase activity in those cells incubated at 37°C, such that at the time of harvest they had higher levels of activity than in cells incubated at 23°C. It is also conceivable that derepression is slower at 23°C than at 37°C.

The se18 and se17 mutants show a decrease, rather than an increase, in vacuolar trehalase activity at 37°C compared to 23°C. The cytoplasmic activity increases similarly to that in the wild-type strain. Thus each mutation is likely resulting in the accumulation of inactive proenzyme at the particular stage of the secretory pathway where transit is blocked. The presence of a small amount of active vacuolar trehalase in these mutants at the restrictive temperature could be accounted for by the proteolytic activation of some of the accumulated proenzyme.
in the crude extract. The lack of any effect of these mutations on the cytoplasmic activity indicates that the cytoplasmic enzyme does not enter the secretory pathway.

The block to secretion in the mutants can be reversed by shifting the cells back to the permissive temperature in the presence of cycloheximide to prevent the synthesis of new enzyme. The accumulated proenzymes can then complete their transit to the vacuole where they are activated. A recovery of the diminished vacuolar trehalase activity in the sec18 and sec7 mutants was seen upon such a shift.

The sec5 mutant exhibits the same increase in vacuolar trehalase activity as is seen in the wild-type strain. This indicates that the proenzyme does not traverse the post-Golgi stage of the secretory pathway, but is active in the vacuole. The secretory vesicles are not used to transport the proenzymes into the vacuole (Stevens et al., 1982).

The PEP1 gene product, protease A, is required to cleave the prosequence off the proenzyme once it arrives in the vacuole, thus activating it (Jones, 1984; Ammerer et al., 1996). In the absence of this activity, all vacuolar enzymes tested to date show diminished activity. The pep1 mutant 20B-12 exhibits less than 10% of the vacuolar trehalase activity seen in its wild-type parent on YEPD. On trehalose, it shows about 25% of the wild-type activity. However, the cytoplasmic trehalase from 20B-12 is also 4-fold less active on trehalose. In order to determine if
the pep4 mutation had an effect on the cytoplasmic trehalase, another mutant from a different genetic background was examined. JHRY20-2Ca carries a complete pep4 deletion, generated by the insertion of the URA3 gene into the pep4 coding region such that any residual activity is eliminated (Ammerer et al., 1986). Trehalase levels were examined on derepressing media compared to repressing media because these are the conditions that were used to study the effects of the pep4 mutation on other vacuolar enzymes. It also removed any possible effect that trehalase might have on the trehalase activities. In JHRY20-2Ca, there was no vacuolar trehalase activity under repressing conditions. Under derepressing conditions, about 20% of the wild-type level of activity was present. The cytoplasmic trehalase activity was unaffected. These results clearly demonstrate that the vacuolar trehalase is dependent on the protease A for activation, while the cytoplasmic activity is not. The presence of some active vacuolar trehalase in the pep4 mutant under derepressing conditions may be due to the partial activation of some accumulated proenzyme in the crude extract, or due to the uncovering of a pep4 independent trehalase activity in the vacuole.

The vacuolar enzymes protease A and carboxypeptidase Y are properly targeted to the vacuole, but are inactive in the presence of the N-linked glycosylation inhibitor tunicamycin (Jones, 1984). Vacuolar trehalase activity
could not be as effectively derepressed in the presence of 10 ug/ml of tunicamycin as it was in its absence. A 2-fold derepression occurred in the presence of tunicamycin, compared to a 5-fold derepression in the control. Tunicamycin also affected the cytoplasmic trehalase, but not to as great an extent as the vacuolar trehalase. This probably reflects the effect of the general metabolic perturbation caused by tunicamycin on the cytoplasmic activity, since this enzyme is not N-glycoylated (Londesborough and Varimo, 1984). These results demonstrate, that like the vacuolar protease A and carboxypeptidase Y, vacuolar trehalase is inactive, if synthesized, in the presence of tunicamycin.

Soluble vacuolar enzymes in *S. cerevisiae* have isoelectric points in the range of 4-5 (T. Stevens, pers. commun.). This is indicative of enzymes located in acidic cellular compartments. Vacuolar trehalase has a pI in this range. The cytoplasmic trehalase could not be visualized by this staining procedure, thus its pI could not be determined. The compartmentalized trehalases of *D. discoideum* and *N. crassa* have acidic isoelectric points (Seshadri et al., 1986; White et al., 1985). This suggests that the vacuolar trehalase of *S. cerevisiae* is compartmentalized as are these other trehalases.

The results obtained clearly refute the hypothesis that the cytoplasmic trehalase is translocated into the vacuole for proteolytic degradation (Weinken and
Schellenberg, 1982). Vacuolar trehalase is an N-linked glycoprotein with an acidic isoelectric point, which are features common to all vacuolar enzymes in S. cerevisiae. Like those enzymes, vacuolar trehalase traverses the secretory pathway through the endoplasmic reticulum to the Golgi body. In the vacuole, vacuolar trehalase is activated by protease A, as are all other vacuolar enzymes. The mechanism by which vacuolar enzymes are transported to the vacuole from the Golgi body is unknown. It is clear that the sorting determinants do not reside in the carbohydrate portions of the enzymes (Stevens et al., 1982). Vacuolar carboxypeptidase Y contains putative sorting information in its pro-sequence (Valls et al., 1986). The sorting machinery can be saturated when carboxypeptidase Y is expressed from a high copy number plasmid, and mutants defective in sorting have been isolated (Stevens et al., 1986; Rothman et al., 1985). In each of these cases, vacuolar enzymes are mislocalized to the cell surface. Vacuolar trehalase has not yet been extensively studied in these mutants.

When following a protein through an intracellular pathway, it is preferable to work with the immunoprecipitated protein. Since no antibody towards trehalase is yet available, enzyme activity in various processing mutants was used to follow the enzyme. The results demonstrate that vacuolar trehalase is processed through the same pathway as all other vacuolar enzymes in
S. cerevisiae.

ISOLATION OF TREHALASE MUTANTS

Attempts were made to isolate trehalase deficient mutants using a standard yeast mutagenesis protocol. The mutants isolated appear to show the characteristics of petite mutants which have non-functional mitochondria. The mutants failed to grow following glucose depletion, and could not grow on carbon sources other than glucose or sucrose. These are typical characteristics of petite mutants. However, the cellular morphology of the mutant was unlike that of petite mutants. The cells were smaller than wild-type cells and possessed dark vacuoles throughout the growth cycle. The percentage of wild-type trehalase activities observed for each mutant was always less than 25%, but there was considerable variance in this value. This indicates that a tight trehalase mutation was not present. The mutations were recessive and defined one complementation group. However, a homozygous diploid showed very poor sporulation, preventing an analysis of the segregation of the mutation. Although it is possible that these mutants are petites, they could also be mutants defective in the regulation of one or both of the trehalases. The small size of the mutants when they are in stationary phase indicates that maintenance metabolism is almost shutdown. Normal cells swell in size when they are in an arrested growth situation (Pringle and Hartwell, 1991). Since it is possible that the vacuolar trehalase
may be required during such a time, it is conceivable that the mutant phenotype may be due to an inefficient or poorly regulated vacuolar trehalase.

Screens for trehalase deficient mutants may be facilitated by the use of the trehalase plate assay. A number of strains possessed a small amount of extracellular trehalase activity as detected by this assay. Control experiments showed that the activity was not due to cell lysis. Since the activity was measured under conditions used to assay vacuolar trehalase, the extracellular activity may be derived from the vacuolar compartment. It is possible that during derepression, the vacuolar sorting apparatus may become saturated, such that some vacuolar trehalase escapes to the cell surface where it is activated. There is no evidence for this, and it was not possible to quantitate the extracellular activity. An effective screen for trehalase deficient mutants could be based on the absence of any extracellular trehalase activity in a strain which normally possesses such activity. An ade2 mutant such as YMN282 should be used, since these mutants accumulate a red pigment. Petite mutants lose this red pigment, thus they could be selected against by picking only red colonies without extracellular trehalase activity (Sherman, et al., 1970).

The advanced state of molecular genetic techniques employed to study the yeast *S. cerevisiae* should enable the isolation of the trehalase gene(s) without requiring a
trehalase mutant. The isolated gene(s) could then be mutagenized in vitro and used to replace the wild-type gene(s) using standard techniques (Rothstein, 1983). The phenotypes of such mutants could then reveal the roles that each trehalase plays in the growth and life cycle of S. cerevisiae.

The λgt11 expression library could be used to isolate the gene(s). This vector contains random inserts of genomic DNA expressed as α-galactosidase fusion proteins. These fusion proteins can then be screened using an antibody to the protein of interest, in this case trehalase (Young and Davis, 1983a,b). When a positive signal is obtained, the corresponding cloned insert should contain at least a partial copy of the structural gene for the particular protein. All that is required for this screen is an antibody to the protein.

An alternative procedure for isolating the trehalase gene(s) is one used to isolate the structural genes for vacuolar enzymes (Rothman et al., 1986). This procedure is based on the mislocalization to the cell surface of vacuolar enzymes when their structural gene is present on a high-copy number plasmid (Stevens et al., 1986). The enzymes are activated at the cell surface in a pep4-independent manner. A recipient strain such as YKM282 or DBY747 could be transformed with a S. cerevisiae gene library on the high-copy plasmid YEp24. The presence of the trehalase gene in high-copy number should result in
mislocalization of vacuolar trehalase to the cell surface, where it could be detected using the trehalase plate assay.

Using these techniques, it should be possible to clone the trehalase gene(s) from \textit{S. cerevisiae} and to create trehalase mutants. Such mutants could then be used to determine the role of trehalose degradation during the growth and life cycles of this yeast.
REFERENCES


Mechler, B., M. Muller, H. Muller, F. Muessdoerffer and D.


Yamashita, Y. and S. Fukui. 1985. Transcriptional control of the sporulation-specific glucoamylase gene in the


VITA AUCTORIS

Steven David Harris

Born: August 17, 1961; Scarborough, Ontario

Parents: Elizabeth and David Harris

Education: David and Mary Thomson Collegiate Institute, Scarborough, Ont.
Diploma awarded in 1979

University of Windsor
Bachelor of Science (Hons.) Degree
Degree awarded in 1983