Title
Genetic characterization of the Saccharomyces cerevisiae cyclin-dependent kinase encoded by PHO85

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Genetic characterization of the *Saccharomyces cerevisiae* cyclin-dependent kinase encoded by *PHO85*

by

Marc Elliott Lenburg

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

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To my parents and Laura Grabel

Acknowledgments

I thank my parents for all of their support during my many years in school.

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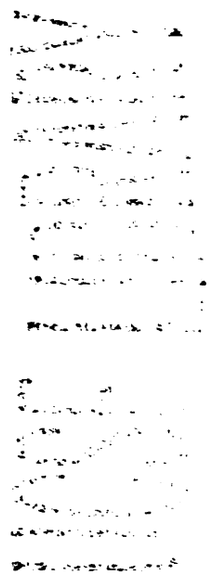

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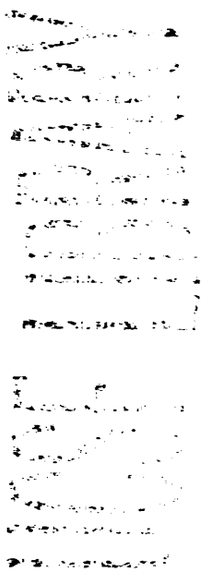

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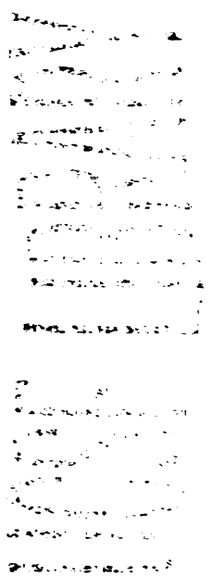

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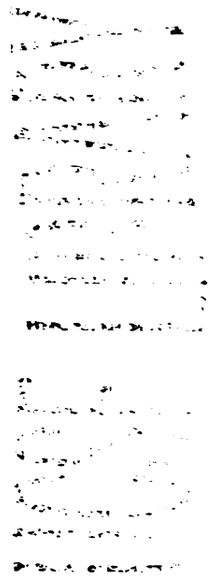
Abstract

Genetic characterization of the *Saccharomyces cerevisiae* cyclin-dependent kinase encoded by *PHO85*

Marc Elliott Lenburg

Cyclin-dependent kinases are heterodimeric kinases that regulate diverse processes in all eukaryotes. Most catalytic subunits associate with several different activating cyclin subunits. A longstanding goal has been to understand the functional consequences of a single catalytic subunit associating with different activating subunits. The *S. cerevisiae PHO85* gene encodes a nonessential cyclin-dependent kinase that associates with ten cyclins. The functions Pho85 performs in association with most of these cyclins is unknown. Several functions are involved in regulating responses to nutrient availability, while another function is regulated by the cell-division cycle. I am interested in understanding the functions of Pho85 in association with its cyclins in order to understand the functional consequences of Pho85 associating with so many different cyclins. To survey the functions provided by Pho85, I identified mutants that require *PHO85* for viability. I identified mutations that define seven Pho **E**ighty-**F**ive **R**equiring or Efr loci, six of which are previously-identified genes -- *BEM2* (*YER155C*), *SPT7* (*YBR081C*), *GCR1* (*YPL075W*), *SRB5* (*YGR104C*), *HFI1* (*YPL254W*) and *BCK1* (*YJL095W*) -- with one novel gene (*YMR212C*). I found that mutations in the EFR genes involved in morphogenesis are specifically inviable when the Pho85-associated G1 cyclins encoded by *PCL1* and *PCL2* are absent. *pcl1Δ bem2*, *pcl1Δ pcl2Δ cla4Δ* and

pcl1Δ pcl2Δ cdc42-1 strains are inviable. *pcl1Δ pcl2Δ mpk1Δ*, *pcl1Δ pcl2Δ bck1* and *pcl1Δ pcl2Δ cln1Δ cln2Δ* strains are also inviable, but are rescued by osmotic stabilization with 1M sorbitol. I propose that the G1 cyclins encoded by *PCL1* and *PCL2* positively regulate *CDC42* or another morphogenesis promoting function.



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CHAPTER 1

The role of *PHO85* in phosphate metabolism

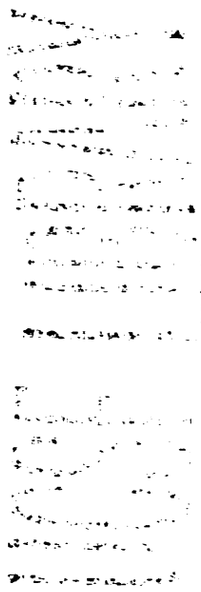
Phosphate is an essential constituent of all living things. It is a major component of membranes and nucleic acids and is also used by the cell for energy storage and information transfer. Almost 5% of an *S. cerevisiae* cell's dry weight is phosphate (National Research Council, 1971). Assimilation of phosphate from the environment is therefore an important task to which yeast must devote considerable resources. Many of these processes are induced when the concentration of free inorganic phosphate in the growth medium is low. It is the regulation of these processes by phosphate availability and our understanding of the ways in which this regulation contributes to a yeast cell's ability to adapt to low phosphate environments that is the subject of this chapter.

There are three phosphate assimilation mechanisms that are induced transcriptionally in response to phosphate starvation: high-affinity phosphate transport (Rautanen and Miikkulainen, 1951), phosphate scavenging (Suomalainen et al., 1960), and phosphate storage (Harold, 1966). These three processes are regulated by a common phosphate-responsive signal transduction pathway called the PHO pathway. Yeast have high and low affinity phosphate transporters for delivering inorganic phosphate from the environment into the cell. In response to phosphate starvation, expression of the high-affinity phosphate transporter, as well as several other genes with roles in phosphate transport, is induced (Bun-Ya et al., 1991; Yompakdee et al., 1996; Martinez and Persson, 1998). As yeast grow in the wild primarily on rotting fruit, most of the phosphate in a yeast cell's environment is covalently attached to organic molecules. To make this organic phosphate available for the yeast's own metabolic needs, yeast secrete

extracellular phosphatases to scavenge phosphate (Suomalainen et al., 1960). Production of these secreted phosphatases is induced by the PHO pathway when levels of free inorganic phosphate are low (Toh-e et al., 1973). Yeast have a vacuolar phosphate reserve which is stored in the form of long- and short-chained polyphosphates (for review see Kornberg et al., 1999). The enzymes responsible for the production and utilization of polyphosphate in yeast have only recently been discovered, and their expression is also regulated by the PHO pathway (N. Ogawa, personal communication). This chapter focuses specifically on how the activity of a cyclin-dependent kinase (CDK) regulates a critical PHO pathway transcription factor. As the non-PHO pathway functions of this cyclin-dependent kinase (CDK) are the focus of the remainder of this thesis, understanding the role of this CDK in the PHO pathway provides an introduction to this kinase.

Much of the work on understanding how fungi respond to phosphate availability started with attempts to understand the genetic pathways which regulate the secretion of the scavenging phosphatases in response to phosphate limitation (for review see Oshima et al., 1996). It was only through subsequent work that it became apparent that these pathways played a more general role in modulating a variety of processes in response to phosphate availability. One strategy for identifying mutants with altered phosphatase expression, used by A. Toh-e in the early 1970s, was to identify *N. crassa* mutants that are unable to grow on plates which contain RNA as the sole source of phosphate (Toh-e and Ishikawa, 1971). Screens for *S. cerevisiae* mutants more specifically defective in secreted phosphatase regulation were performed by Toh-e, Ueda, and Oshima in the mid

1970s (Toh-e et al., 1973; Ueda et al., 1975). They took advantage of a colorimetric phosphatase assay which can be performed directly on yeast colonies. Earlier work had shown that yeast's major phosphate-regulated secreted phosphatase had an acidic pH optimum (Boer et al., 1975). The acidic pH optimum of the secreted phosphatase may be a significant advantage for *S. cerevisiae* as a common habitat for this yeast is the acidic environment of rotting fruit. Most fruit only contains 5 - 20 mg of phosphate per liter (Sherman, 1952); and this concentration of phosphate strongly induces the expression of the phosphatase (Springer and O'Shea, unpublished observations). The acidic pH optimum of the phosphatase suggests that it is adapted for its role in this environment. By adjusting the pH of the plate overlay solution, Toh-e and Oshima were able to make their screens specific for mutants with altered regulation of this particular phosphatase. By screening directly for phosphatase activity rather than using conditions that require phosphatase expression for growth, like the *N. crassa* screen, Toh-e and Oshima were able to do two screens: one screen for mutants unable to induce acid phosphatase activity in response to phosphate limitation (which would uncover a subset of the *N. crassa* mutants), and another screen for mutants unable to repress this activity when phosphate is abundant.



These screens identified seven complementation groups. Recessive mutations at four loci result in an inability to induce acid phosphatase activity (Toh-e et al., 1973). These mutations are in the genes *PHO81*, *PHO4*, *PHO2* and *PHO5*. Recessive mutations at three other loci result in a failure to repress acid phosphatase activity when phosphate is abundant (Ueda et al., 1975). These genes are *PHO84*, *PHO85* and *PHO80*. Epistasis

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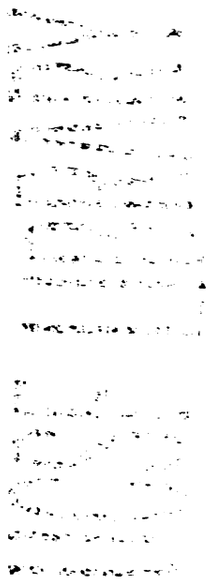
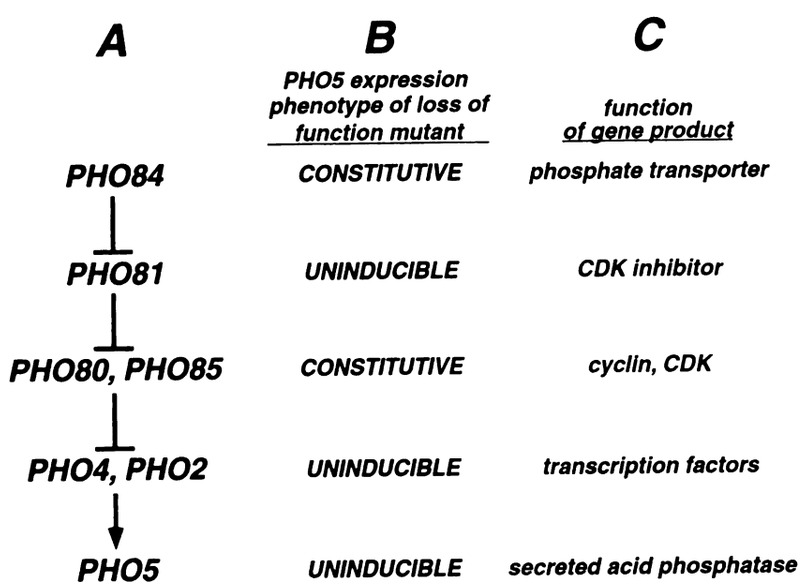
studies using both dominant and recessive alleles of these genes allowed them to be ordered in a genetic pathway (Figure 1).

As predicted from the epistasis studies, subsequent work demonstrated that *PHO5* is the structural gene for the regulated secreted acid phosphatase (Rogers et al., 1982) and further demonstrated that transcription of *PHO5* is induced in response to phosphate limitation (Bostian et al., 1980). The genes immediately upstream of *PHO5* in the genetic pathway encode the transcription factors that are directly responsible for this induction. *PHO4* encodes a basic helix-loop-helix protein that is a transcriptional activator (Koren et al., 1986; Ogawa and Oshima, 1990) and *PHO2* encodes a homeobox protein (Sengstag and Hinnen, 1987; Burglin, 1988) that forms a complex with Pho4 (Shao et al., 1996; Barbaric et al., 1996). In addition to binding to Pho4, Pho2 binds other transcription factors such as Bas1 (Zhang et al., 1997) and Swi5 (Brazas and Stillman, 1993). There are no known Pho2-dependent genes that are not also dependent on one of these Pho2-binding partners suggesting that Pho2 is a coactivator unable to activate transcription on its own. Consistent with this idea, while high-affinity sequence-specific DNA-binding sites have been determined for Pho4 (Fisher et al., 1991), a similar high-affinity binding site has not been determined for Pho2. The identification of high-affinity binding sites for Pho4 in the *PHO5* promoter has aided in the search for other genes regulated by the PHO pathway (Martinez and Persson, 1998). The genes that are regulated by the various Pho2-containing complexes are not regulated by the same environmental signals, indicating that any pathway-specific regulation is likely to occur on -- or be targeted by -- the Pho2-binding partner rather than Pho2 itself. The *PHO5*

FIGURE 1. – Genetic analysis of PHO-pathway mutants. A. Epistasis relationships between PHO pathway mutants. B. Phenotypes of PHO pathway mutants. C. Gene function of PHO pathway mutants.

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pho4 and *pho2* are epistatic to loss-of-function mutations in two genes named *PHO80* and *PHO85* (Oshima, 1982). Loss of function mutations in either *PHO80* or *PHO85* result in the constitutive expression of *PHO5* even when phosphate is abundant (Ueda et al., 1975), suggesting that Pho80 and Pho85 somehow function to antagonize the activity of Pho4 and or Pho2. *PHO85* encodes a cyclin-dependent kinase which when partnered with the cyclin Pho80 phosphorylates Pho4 (Kaffman et al., 1994). *In vivo*, when phosphate is abundant, Pho4 is phosphorylated in a *PHO80*- and *PHO85*-dependent manner (Kaffman et al., 1994). In addition to associating with Pho80, Pho85 associates with nine other cyclin subunits (Measday et al., 1997). The functions performed by most of these cyclins are not yet known, but only Pho80 seems to be involved in regulating the PHO pathway. Two Pho85-associated cyclins, Pcl8 and Pcl10, repress the synthesis of glycogen when glucose is abundant by phosphorylating the glycogen synthase Gsy2 (Huang et al., 1998; Wilson et al., 1999). It is interesting that both the Pho4-regulation and Gsy2-regulation functions of Pho85 kinases involve the repression of starvation-specific behaviors. Three of the remaining Pho85 cyclins are cell-cycle regulated (Measday et al., 1997). Rather than repressing a starvation-specific behavior, at least two of these cyclins appear to be involved in promoting cellular morphogenesis during the G1 phase of the cell cycle.

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The activity of the Pho80-Pho85 kinase is inhibited in response to phosphate starvation: Pho80-Pho85 kinase isolated from cells grown in low phosphate is less active than kinase purified from cells grown in high phosphate (Schneider et al., 1994). Phosphate-dependent regulation of Pho80-Pho85 kinase activity requires *PHO81* (Schneider et al., 1994), a positive regulator of *PHO5* expression that functions upstream of *PHO80* and *PHO85* (Oshima, 1982). *In vitro*, Pho81 functions as a dose-dependent inhibitor of Pho80-Pho85. At physiological concentrations, Pho81 forms a complex with the Pho80-Pho85 kinase in conditions of both high and low phosphate (Schneider et al., 1994). As measured by co-immunoprecipitation and yeast two-hybrid assay, the amount of Pho81 that associates with Pho80-Pho85 does not change upon phosphate starvation (Schneider et al., 1994; Hirst et al., 1994). These data suggest that Pho81 is always associated with Pho80-Pho85, but only inhibits the kinase when cells are starved for phosphate.

Some mutations that cause constitutive *PHO5* expression interfere with phosphate transport into the cell. Loss of the *PHO84* gene, which encodes a high-affinity phosphate transporter, results in constitutive expression of *PHO5* (Ueda and Oshima, 1975). Epistasis experiments suggest that *PHO84* functions upstream of *PHO81*. It is possible that in addition to being a transporter, Pho84 senses extracellular phosphate levels and directly regulates the signal transduction pathway. Alternatively, defects in phosphate transport may result in a decreased level of intracellular phosphate or some phosphate-dependent metabolite, which in turn affects the signal transduction pathway. Both types of nutrient-sensing mechanisms, in which the starvation signal is either the extracellular

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concentration of a nutrient or the intracellular concentration of a metabolite, have precedent in bacteria (Rao and Torriani, 1990; Keener et al., 1987).

What makes the *S. cerevisiae* PHO pathway so important for understanding how *S. cerevisiae* respond to environmental phosphate availability is, that in addition to regulating the secreted phosphatase that comprises the phosphate-scavenging pathway, the PHO pathway also transcriptionally regulates many critical components in other phosphate-assimilation pathways. Consistent with this idea, while wild type and *pho5* mutant strains are able to grow in laboratory media with limiting amounts of inorganic phosphate, mutations in the positive regulators of *PHO5* such as *PHO4* or *PHO81* result in an inability to grow in these media. Therefore, whereas the scavenging phosphatase is dispensible for growth in at least some low phosphate environments, the PHO pathway itself is an essential component of a yeast cell's ability to adapt to low phosphate environments. Regulation of Pho5 activity is just one among several of this pathway's outputs. The remainder of this chapter will focus on the mechanics of how the PHO pathway responds to phosphate availability since this is a critical determinant of how yeast adapt to changing phosphate levels. Our description will focus on *PHO5* regulation since this output of the PHO pathway is the most studied due to its experimental tractability.

A critical observation that frames most of our understanding of how the PHO pathway is regulated is that transcription of *PHO5* can be induced in response to phosphate starvation as well as repressed in response to phosphate abundance in the

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absence of new protein synthesis (Lemire et al., 1985). The induction and repression of the PHO pathway in the absence of new protein synthesis suggests that cells growing in any concentration of phosphate have at least the core machinery required to sense phosphate availability and modulate expression of *PHO5*. These data also indicate that the PHO pathway is regulated post-translationally.

One form of post-translational regulation in the PHO pathway is the regulation of Pho4 activity by phosphorylation. Pho4 contains six serine-proline potential CDK phosphorylation sites, and Pho80/Pho85 phosphorylates five of them (O'Neill et al., 1996) (Figure 2). Of these five, four are phosphorylated both *in vivo* and *in vitro* while the fifth site does not seem to be phosphorylated efficiently. Interestingly, these five sites conform to a consensus which is different from other CDKs. While CDKs such as Cdc28 phosphorylate S/T-P-X-K/R the Pho80/Pho85 phosphorylation sites are S/T-P-X-I/L. The S-P site in Pho4 which is not used is S-P-A-T; the S-P site which is not phosphorylated efficiently is S-P-V-T.

Upon phosphate starvation, Pho4 becomes dephosphorylated. The simplest interpretation of this pattern of phosphorylation is that phosphorylation of Pho4 by Pho80/Pho85 in high phosphate inhibits its ability to activate *PHO5* transcription. Consistent with this model, a Pho4 mutant lacking the five Pho80/Pho85 phosphorylation sites constitutively expresses *PHO5* at a level similar to mutants lacking Pho80 or cells grown in low phosphate (Komeili and O'Shea, 1999). These data further argue that relieving the inhibition of Pho4 by Pho80 and Pho85 in high phosphate is sufficient to

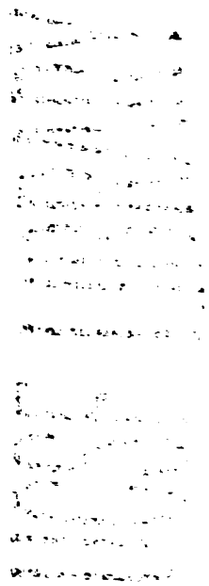
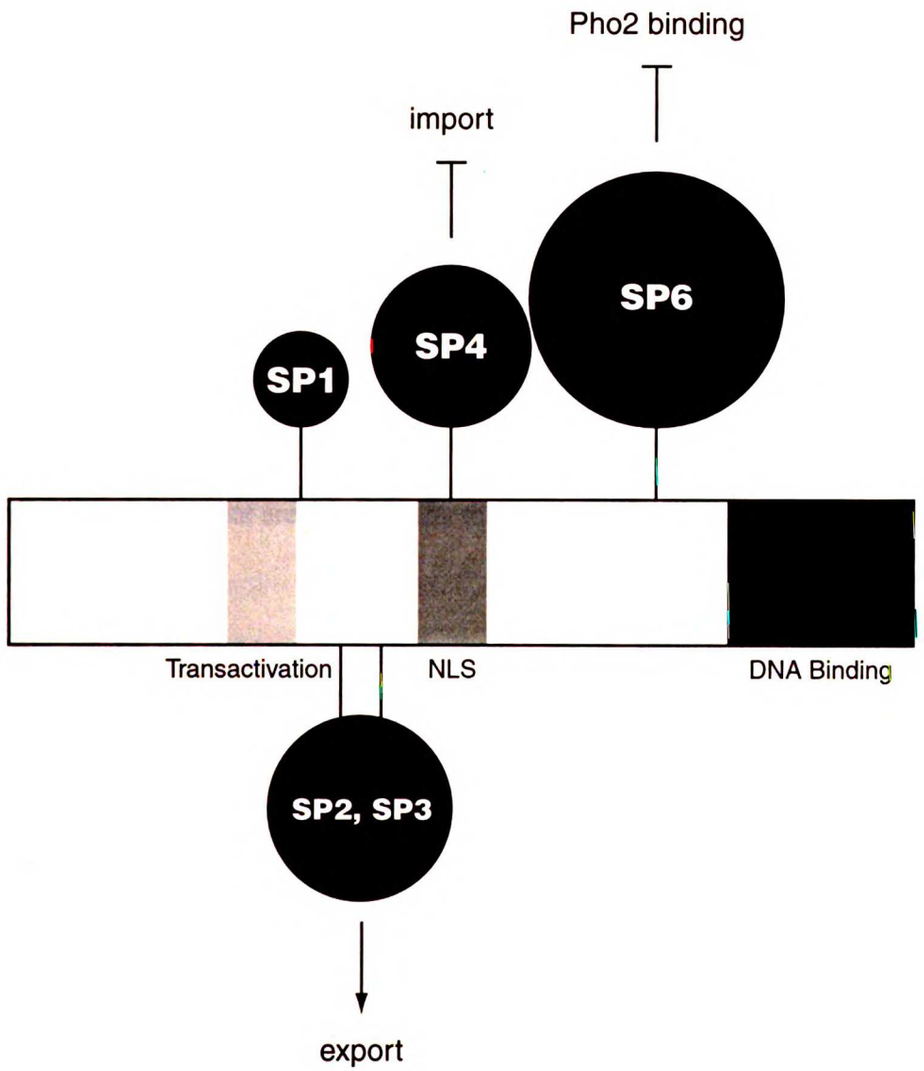


FIGURE 2. – Sites of Pho4 phosphorylation. The five sites on Pho4 which are phosphorylated both in vitro and in vivo by Pho80-Pho85 are labelled SP1, SP2, SP3, SP4, SP6. The area of each circle is proportional to the relative probability of that site being phosphorylated. The effect of each phosphorylation is indicated next to each circle.



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cause a physiological induction of *PHO5* activity. These data indicate that regulation of Pho4 by phosphorylation is sufficient to regulate the transcriptional response to decreasing availability of phosphate. There is some evidence, however, that hints at the presence of a non-Pho80/Pho85-dependent pathway which contributes to Pho5 regulation. This idea comes from the finding that while a *pho80* mutant expresses physiologically induced levels of Pho5 in phosphate-rich conditions that normally repress *PHO5*, the level of *PHO5* expression increases slightly when *pho80* mutants are transferred to low phosphate (Gregory et al., 1998). If this induction does indeed reflect another layer of *PHO5* regulation, it will be of great interest to determine if it also influences the expression of other PHO pathway outputs and if there are other responses to phosphate starvation that are regulated by this additional pathway alone.

Even if there is another phosphate-responsive pathway that contributes to phosphate adaptation, it is clear that the predominant regulation of the low-phosphate survival mechanisms involving phosphate transport, phosphate scavenging, and phosphate storage works through the regulation of Pho4 phosphorylation. Critical to the upregulation of these processes upon phosphate starvation is dephosphorylation of Pho4. Pho4 becomes dephosphorylated in response to phosphate starvation by two mechanisms. The inhibition of Pho80-Pho85 kinase activity by Pho81 blocks or at least greatly reduces the rate at which additional Pho4 is phosphorylated. The fact that the PHO pathway can be induced in the absence of protein synthesis indicates that there is a Pho4-phosphatase

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One of the more recent advances in understanding how the yeast PHO pathway is regulated in response to phosphate availability has been understanding in some detail how phosphorylation of Pho4 leads to the repression of Pho4's transcriptional activity. One of the questions framing this investigation has been the desire to understand the consequences of Pho4 phosphorylation on five different sites. One possibility is that phosphorylation of each of the different sites affects just one property of Pho4. Another possibility is that phosphorylation of the different sites affects multiple distinct properties of Pho4. Either of these biochemical consequences of Pho4 phosphorylation could result in different physiological effects. Multiple phosphorylation sites could tune the level of Pho4 activity from fully active (fully unphosphorylated) to fully inactive (fully phosphorylated). Another possibility is that partially phosphorylated Pho4 behaves the same as Pho4 which is either fully phosphorylated or fully dephosphorylated and that multiple phosphorylation sites make the transition from active to inactive highly cooperative. The different sites of phosphorylation on Pho4 affect different properties of Pho4 and this appears to allow partially phosphorylated Pho4 to have intermediate activity.

One of the fundamental mechanisms of Pho4 regulation is the regulation of its subcellular localization by phosphorylation (Figure 3). In high phosphate, when Pho4 is

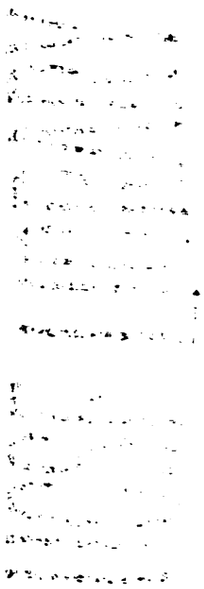

FIGURE 3. – Regulation of nuclear localization by phosphorylation. In low phosphate environments, Pho81 inhibits Pho80-Pho85 and Pho4 is unphosphorylated. Unphosphorylated Pho4 accumulates in the nucleus as a result of increased nuclear import and decreased nuclear export. Nuclear unphosphorylated Pho4 is also able to bind Pho2 and activate transcription. In high phosphate environments, Pho80-Pho85 is active and Pho4 is phosphorylated. Phosphorylated Pho4 is unable to bind Pho2. Phosphorylated Pho4 is exported from the nucleus and is imported more slowly than unphosphorylated Pho4.

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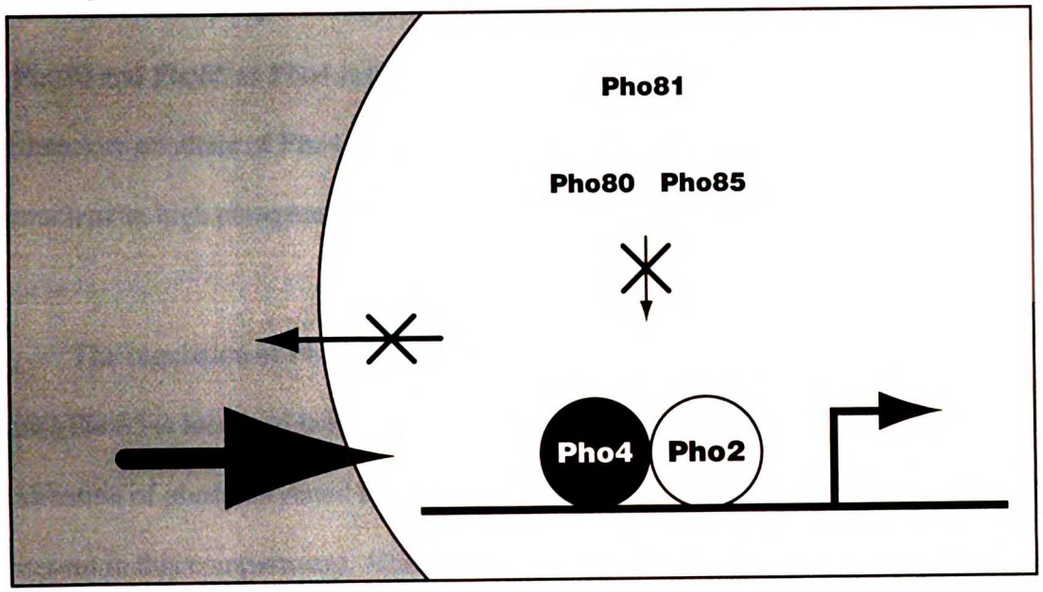

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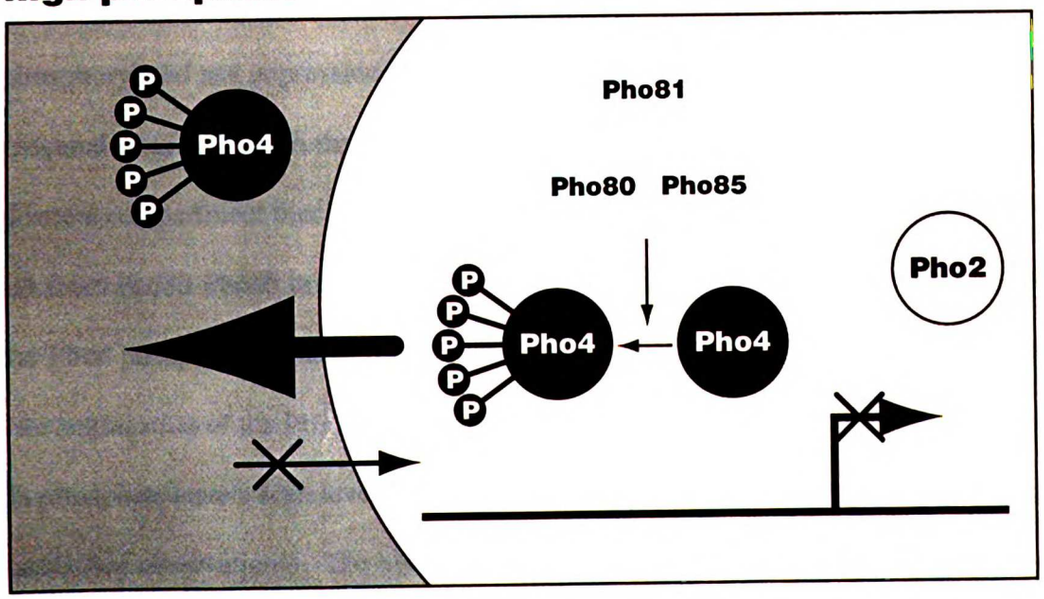

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phosphorylated and its transcriptional activity is repressed, Pho4 is in the cytoplasm. In low phosphate, when Pho4 is unphosphorylated and active, it is in the nucleus (O'Neill et al., 1996). These changes in subcellular localization are dependent on phosphorylation by Pho80 and Pho85 as Pho4 is constitutively nuclear in strains lacking Pho80. Furthermore an allele of Pho4 which lacks all phosphorylation sites is also localized to the nucleus in high phosphate (O'Neill et al., 1996).

The regulation of Pho4 nuclear localization is particularly interesting because Pho80-Pho85 is localized predominantly to the nucleus (O'Neill et al., 1996). The localization of phosphorylated Pho4 to the cytoplasm implies that the Pho4 phosphatase is present in this compartment. The localization of Pho80-Pho85 to the nucleus is clearly advantageous. When yeast encounter abundant phosphate conditions, the kinase is in the same compartment as Pho4, and Pho4 can be rapidly phosphorylated and inactivated. The nuclear localization of Pho80-Pho85 also means that in high phosphate, when Pho4 is phosphorylated and expression of Pho4-dependent genes is repressed, Pho4 is segregated away from both the kinase that phosphorylates it, as well as its site of action, and into a compartment that contains the activating Pho4 phosphatase. The segregation of Pho4 from Pho80-Pho85 in high phosphate would be a particularly efficient arrangement if the Pho4 phosphatase was inactive when phosphate is abundant as it would allow for a stable segregation of the PHO pathway components. But it appears that cells grown in high phosphate have a high level of Pho4 phosphatase activity (A. Carroll and E. O'Shea unpublished observations). The high level of Pho4 phosphatase activity in high phosphate implies that in high phosphate Pho4 is constantly moving between the

cytoplasm and the nucleus, with the balance of these processes resulting in a steady-state cytoplasmic localization.

The change in Pho4's subcellular localization by phosphorylation could be the result of several mechanisms. One possibility is that phosphorylated Pho4 is prevented from entering the nucleus as the result of being tethered to a cytoplasmic anchor. The localization of NF-kB is regulated by such a mechanism (for review see Karin and Ben-Neriah, 2000). Another possibility is that unphosphorylated Pho4 is prevented from exiting the nucleus due to tethering by a nuclear anchor. If there is a nuclear anchor, it is clear that it is not DNA, as Pho4 lacking its DNA-binding domain is still able to localize to the nucleus. It is also clear that binding to Pho2 does not result in the nuclear retention of Pho4 as Pho4's localization is appropriately regulated in strains lacking *PHO2* (O'Neill et al., 1996). Two mechanisms which regulate the nuclear localization of Pho4 are the regulation of Pho4 import into the nucleus as well as the regulation of its export (Kaffman et al., 1998; Kaffman et al., 1998).

The ferrying of proteins in and out of the nucleus is accomplished by a family of related Ran GTPase-dependent cargo receptors which shuttle back and forth between the nucleus and cytoplasm. Much progress has been made recently in understanding the details of how these proteins transport cargoes in and out of the nucleus and how in some cases the transport of cargos is regulated. Several valuable reviews of this progress have recently been written (Komeili and O'Shea, 2000; Nakielny and Dreyfuss, 1999; Kaffman and O'Shea, 1999).

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What is interesting about Pho4 nuclear transport is that transport in both directions is regulated and that these distinct processes are regulated by phosphorylation of distinct sites on Pho4. We have numbered all of the potential sites of Pho80/Pho85 phosphorylation sites SP1 through SP6, as they appear in the primary sequence of Pho4 from the N to C terminus (Figure 2). The fifth SP does not appear to be phosphorylated, and the first site is phosphorylated poorly.

The export of phosphorylated Pho4 from the nucleus in high phosphate is mediated by the Msn5 cargo receptor, and occurs only when SP2 and SP3 are phosphorylated (Kaffman et al., 1998). Interestingly, Far1 and Mig1 are Msn5 cargoes, and they are also subject to regulated nuclear localization. In both cases, this change in localization, like Pho4, is regulated by phosphorylation (Blondel et al., 1999; DeVit and Johnston, 1999). In an *msn5* mutant, Pho4 is localized predominantly to the nucleus independent of Pho4's phosphorylation state (Kaffman et al., 1998). Phosphorylated Pho4 binds to Msn5 *in vitro* in a Ran-GTP dependent manner while unphosphorylated Pho4 is unable to bind. This phosphorylation-induced binding is due to phosphorylation at sites SP2 and SP3, as phosphorylated Pho4 lacking either of these phosphorylation sites is unable to bind Msn5, whereas Pho4 lacking all three of the other Pho80/Pho85 phosphorylation sites binds Msn5 in a phosphorylation-dependent manner. Consistent with these biochemical data, *in vivo*, a mutant Pho4 which cannot be phosphorylated on sites SP2 or SP3 is localized predominantly to the nucleus regardless of phosphate levels (Kaffman et al., 1998). One unresolved question related to the phosphorylation-

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dependent interaction between Pho4 and Msn5 is how phosphorylation of Pho4 leads to Msn5 binding. Two possibilities are that phosphorylation of Pho4 changes its conformation to expose an Msn5 binding site or that the phosphoserine residues at sites 2 and 3 are themeselves part of the Msn5 binding domain.

The import of Pho4 into the nucleus is mediated by the cargo receptor Pse1 (Kaffman et al., 1998). Pho4 binds to Pse1 *in vitro* in a Ran-GTP-dependent manner. The interaction between Pho4 and Pse1 is reduced about three-fold by phosphorylation of site SP4, while phosphorylation at none of the other Pho4 SP sites affects this interaction. A twenty-seven amino acid peptide containing the SP4 phosphorylation site is sufficient to localize a heterologous protein to the nucleus in a *PSE1*-dependent manner. *PSE1* is an essential gene, but strains carrying a temperature-sensitive allele of *PSE1* are blocked for Pho4 import into the nucleus even at a temperature permissive for growth (Kaffman et al., 1998). A *PHO4* mutant with a negatively charged aspartic acid residue in place of the serine at SP4 has a reduced rate of nuclear import suggesting that the negative charge which results from phosphorylation of SP4 slows down Pho4 import *in vivo* (Komeili and O'Shea, 1999). Interestingly though, unlike a *PHO4* mutant that cannot be phosphorylated at site SP2 or SP3, a *PHO4* mutant which cannot be phosphorylated at SP4 is still predominantly cytoplasmic in high phosphate (Kaffman et al., 1998). The cytoplasmic localization of this constitutively imported allele of *PHO4* suggests that the rate of Pho4 export mediated by phosphorylation of sites SP2 and SP3 is sufficient to localize the bulk of Pho4 to the cytoplasm even when the SP4-dependent block to Pho4 import is absent.

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If subcellular localization was the only way in which Pho4 activity is regulated, one would expect that a Pho4 mutant that could not be phosphorylated at sites SP2, SP3, and SP4 would constitutively express Pho4-dependent genes at the same level as these genes are expressed upon phosphate starvation. However, strains carrying an allele of *PHO4* that encodes a Pho4 molecule that cannot be phosphorylated at sites SP2, SP3, and SP4 is not fully repressed for *PHO5* expression in high phosphate, but only expresses *PHO5* at about 20% of low-phosphate levels (Komeili and O'Shea, 1999). There is a third level of Pho4 regulation that is independent of subcellular localization and is dependent on phosphorylation of site SP6. SP6 resides in the region of Pho4 which is important for Pho2 interaction (Hirst et al., 1994). Pho4 that is phosphorylated at site SP6 has a greatly reduced affinity for Pho2 (Komeili and O'Shea, 1999), which greatly reduces its ability to activate expression of *PHO5,* as seen from the subtle *PHO5* expression phenotype of *PHO4* mutants lacking the other sites of Pho80-Pho85 phosphorylation. Interestingly, blocking phosphorylation of site SP6 alone also results in an only a modest increase in high-phosphate *PHO5* expression, while blocking phosphorylation of sites SP2 SP3 SP4 and SP6 together causes *PHO5* to be expressed at levels nearly identical to those seen in low phosphate (Komeili and O'Shea, 1999). These data indicate that either enhancing Pho4 export or blocking Pho4's interaction with Pho2 is largely sufficient to repress expression of *PHO5*. These data also indicate that neither mechanism alone leads to complete repression of *PHO5* expression, as mutants defective for cytoplasmic localization, but competent to interact with Pho2 express some *PHO5* in

high phosphate. The same is true for mutants that are defective for binding to Pho2 but can be localized to the cytoplasm.

The presence of these overlapping Pho4 regulatory mechanisms indicates that phosphorylation of different sites on Pho4 does indeed affect different biochemical properties of Pho4. The non-additive effect of these overlapping regulatory mechanisms on Pho4 activity further indicates that these mechanisms act cooperatively to repress expression of *PHO5*. At the same time, the analysis of Pho4-phosphorylation mutants indicates that partially phosphorylated Pho4 can activate low levels of *PHO5* expression, raising the possibility that there could be physiologically significant intermediate states of Pho4 activity that result from partially phosphorylated Pho4. This possibility becomes even more likely when one considers the targets of Pho4 beyond *PHO5*. *PHO8* is a Pho4-dependent phosphate-regulated gene that unlike *PHO5* is not Pho2 dependent (Magbanua et al., 1997; Munsterkotter et al., 2000). Since the effects of phosphorylation at SP6 on Pho2 interaction seem to be independent from the effects of phosphorylation at sites SP2, SP3 and SP4 on cytoplasmic localization, a partially phosphorylated pool of Pho4 containing Pho4 molecules phosphorylated at site SP6 but not phosphorylated at site SP2 and SP3 would have different properties than completely phosphorylated or completely unphosphorylated Pho4. If such a Pho4 species existed, it would be largely blocked for activating *PHO5* expression due to reduced interaction with Pho2, but would be competent for activating expression of Pho2-independent genes such as *PHO8*.

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This discussion of how Pho4 is regulated by Pho80-Pho85 in response to phosphate availability highlights how much we have learned about how yeast respond to phosphate starvation in the last several years. It is a powerful example of how understanding the regulation of a kinase's activity, the identity of a it's substrates, and understanding the consequences of a substrate phosphorylation can result in a richer understanding of an organism's survival plan. As such, the story of Pho80-Pho85's phosphorylation of Pho4 provides an especially good introduction to the remainder of this thesis which sets out to understand the functions of the other nine Pho85-associated kinases.

CHAPTER 2

Functions of *PHO85* outside of phosphate metabolism and the identification of more such functions.

Cyclin-dependent kinases are heterodimeric protein kinases that are present in all eukaryotes. The phosphotransfer reaction is catalyzed by the cyclin-dependent kinase (CDK) subunit only when activated by association with a cyclin. Most catalytic subunits are able to associate with several different cyclins. The first CDKs identified play important roles in regulating key transitions in the cell cycle. The name of this kinase family reflects the periodic activity of those kinases (for review see Hunt, 1991). However, the discovery of other CDKs has made it clear that other members of this kinase family participate in events that are not directly related to cell cycle regulation and that the activity of many CDKs is more constant than periodic (for review see Morgan, 1997).

The prevalence and diversity of CDKs among eukaryotes has led us to speculate about the evolutionary advantages of their heterodimeric nature. In many metazoans, it appears that although any particular CDK is capable of associating with multiple cyclin subunits, these different cyclin-CDK complexes have the same biological activity. For example, CDK4 associates with four different D-type cyclins, but there are no clear differences in the functions performed by these different CDK4-containing complexes in promoting cell-cycle progression through the restriction point. Rather, there is evidence that the different D-type cyclins are subject to transcriptional regulation that results in cell-type specific differences in Cyclin D levels (for review see Sherr, 1995). These observations suggest that one consequence of a single CDK associating with multiple cyclins is that it can provide a mechanism for adjusting kinase activity through

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transcriptional regulation of the different cyclins. However, there are other examples in which a single CDK performs different functions in association with different cyclins. Perhaps the best example of this phenomenon is the *S. cerevisiae* CDK encoded by *CDC28*: in association with the Cln-type cyclins, Cdc28 promotes G1 progression; in association with Clb5 and Clb6, it promotes DNA synthesis; and in association with Clb1-4, it promotes mitosis (for review see Nasmyth, 1996).

The ability of Cdc28 to perform different functions in association with different cyclins could be the consequence of a number of mechanisms. For example, it is possible that the different cyclins are capable of phosphorylating the same substrates but that their activity is subject to differential regulation (transcriptional and/or post-transcriptional). Another possibility is that the different cyclins localize an equivalent Cdc28-dependent kinase activity to different subcellular locations. An oft-invoked possibility is that a single CDK subunit acquires the ability to recognize and phosphorylate different substrates as a result of association with different cyclins. Evidence that cyclins can modulate the properties of the CDK subunits with which they interact by each of these mechanisms follows.

The expression of each of the different Cdc28-associated cyclins is restricted to the phase of the cell cycle in which their function is required. *CLN* genes are expressed only in G1, the *CLB5* and *CLB6* genes are expressed only in S phase, and the *CLB1* - *CLB4* genes are expressed only in mitosis. Moreover, while a strain lacking all the Cln-type cyclins is inviable, removing the G1-phase Clb inhibitor enoded by the *SIC1* gene

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restores viability (Schneider et al., 1996). The viability of a *cln1Δ cln2Δ cln3Δ sic1Δ* strain suggests that Clb-associated Cdc28 kinase activity can at least partially substitute for Cln-associated Cdc28 kinase activity and that Clb5- and Clb6-associated Cdc28 kinase activity is regulated by transcriptional and post-transcriptional mechanisms.

Cyclin association can also serve to localize CDK activity to different subcelluar compartments. For example, Cyclin E/Cdk2 complexes, but not Cyclin A/Cdk2 complexes, localize to Cajal bodies in the nucleolus (Liu et al., 2000). The differential localization of cyclins may be important for directing kinase activity to substrates that are present in these different locations. For example, Cln2 localizes predominantly to the cytoplasm, while Cln3 localizes predominantly to the nucleus (Miller and Cross, 2000). Redirecting Cln3 to the cytoplasm partially suppresses some phenotypes associated with the loss of *CLN2,* suggesting that a critical difference between Cln2- and Cln3-containing Cdc28-associated complexes is their localization to different subcellular compartments.

Cyclin binding can also confer substrate specificity on the CDK subunit, as seen by the phosphorylation of p107 by CyclinA/CDK2 or CyclinA/CDC2 and not by CyclinB/CDK2 or CyclinB/Cdc2 (Peeper et al., 1993). These findings are results from *in vitro* experiments in which equivalent amounts of kinase are used -- as determined by their equivalent ability to phosphorylate other substrates. Therefore, the differential activity of these kinases toward specific substrates indicates that cyclin binding can confer some degree of substrate specificity on the CDK subunit. Taken together, these data indicate that in addition to activating the CDK subunit, cyclin association can also

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alter other properties of the kinase and suggests that there can be a variety of physiological consequences that arise from a single CDK associating with multiple cyclin subunits. Although cyclin binding can alter a CDK's properties it seems that the functions performed by a single CDK in association with different cyclin subunits are related to each other. Cdc28 is a particularly striking example -- the different Cdc28-associated kinases are involved in regulating diverse processes, but all of them concern determining the correct order of events during the cell-division cycle.

It was with these ideas about the functional consequences of cyclin binding in mind that I became interested in the *S. cerevisiae* cyclin-dependent kinase encoded by *PHO85*. The budding yeast *S. cerevisiae* has five CDKs. Perhaps the best understood of these is Cdc28, which we have already discussed in some detail. Three of the other *S. cerevisiae* CDKs (Srb10, Kin28, and Ctk1) associate with only one or two cyclins and all of these kinases seem to be involved in regulating transcription by RNA Pol II (Liao et al., 1995; Sterner et al., 1995; Valay et al., 1995). The remaining *S. cerevisiae* CDK is Pho85.

Pho85 is a nonessential CDK which associates with ten different cyclins, suggesting that it may be involved in regulating a variety of cellular processes, none of which are required for cell viability. The Pho85-associated cyclins can be divided into two broad families based on sequence similarity. The Pho80 family contains five members: Pho80, Pcl6, Pcl7, Pcl8 and Pcl10. The Pcl1,2 family also contains five members: Pcl1, Pcl2, Clg1, Pcl5, and Pcl9 (for review see Andrews and Measday, 1998).

PHO85 was first discovered for its role, along with the cyclin Pho80, in inhibiting the expression of the phosphate starvation-induced gene *PHO5* when phosphate is abundant, as described in Chapter 1 (see also Lenburg and O'Shea, 1996). Pho80 is the only Pho85-associated cyclin which appears to be involved in this process. Two other cyclins, Pcl8 and Pcl10, are involved in repressing glycogen synthase activity during fermentation (Timblin et al., 1996; Wilson et al., 1999). None of the functions of the remaining seven cyclins is clear. Transcription of three of these Pho85-associated cyclins is periodic across the cell cycle. Pcl1 and Pcl2 are expressed in mid to late G1 as a result of the effects of the same transcription factors that are responsible for Cln1 and Cln2 expression (Measday et al., 1997). Pcl9 is expressed in late mitosis and early G1 by the same activity that is responsible for the periodic expression of Cln3 (Tennyson et al., 1998; Aerne et al., 1998). The cell-cycle dependent transcription of these cyclins suggests that Pho85 could participate in a G1-regulated process. This notion is further bolstered by the observation that deletion of two of the Pho85 G1 cyclins (*PCL1* and *PCL2*) causes synthetic lethality with deletion of the Cdc28 G1 cyclins *CLN1* and *CLN2* (Espinoza et al., 1994; Measday et al., 1994). The exact role performed by these G1-specific Pho85 kinases is unclear. There is evidence that Pho85 in general and the cyclins in the Pcl1,2 family in particular play a role in a process which effects cell polarity and morphogenesis – processes which are regulated by progression from G1 to S. Strains which lack these Pho85-associated kinases display a variety of phenotypes such as salt sensitivity, reduced endocytosis, and a random budding pattern – phenotypes which are commonly observed in mutants defective in cell polarity and morphogenesis. Pcl2 can phosphorylate Rvs167, a protein involved in actin polymerization, *in vitro*, providing a possible mechanism by

which Pho85 participates in establishment of cell polarity during G1 (Lee et al., 1998). However, cells must lack all members of the Pcl1,2 family before these cell polarity-related phenotypes are apparent (Lee et al., 1998; Tennyson et al., 1998), so it is possible that these phenotypes could be a secondary consequence of lesions in several distinct Pho85-regulated processes.

One factor that makes the analysis of the multiple cyclins that associate with either Pho85 or Cdc28 complicated is a relatively recent genome duplication event in the *S. cervisiae* evolutionary lineage and the retention of about 16% of the genome in a duplicated state (Wolfe and Shields, 1997; Seoighe and Wolfe, 1999). Many genes encoding Pho85- and Cdc28-associated cyclins fall into this category. The pairs of duplicated Pho85-associated cyclins are *PCL2/PCL9*, *PCL6/PCL7,* and *PCL8/PCL10.* The pairs of duplicated Cdc28-associated cyclins are *CLN1/CLN2*, *CLB5/CLB6, CLB1/CLB2*, and *CLB3/CLB4.* A variety of factors could explain why both copies of a particular gene have been although most genes have reverted to a single copy. One large class of duplicated genes encodes enzymes involved in glycolysis (Wolfe and Shields, 1997). Since *S. cerevisiae* grow most rapidly when fermenting glucose, one possibility is that the increased gene dosage of these glycolytic genes confers a growth advantage. A benefit derived from increased gene dosage could explain the persistence of any number of the other duplicated genes as well. For most of the duplicated cyclins, that there is little evidence for any functional difference between members of a duplicated pair. There are several exceptions which point to the possibility that the functions of some duplicated cyclins have acquired divergent functions. While either *CLN1* or *CLN2* is sufficient to

provide the G1-promoting function in vegetatively growing cells, *CLN1* may play a more important role than *CLN2* in G1 progression when yeast are undergoing pseudohyphal growth (Madhani et al., 1999). Furthermore, *CLN1* is expressed more strongly than *CLN2* specifically in cells growing pseudohyphally, providing at least one possible explanation for the more dramatic phenotype of *cln1* mutants in pseudohyphal growth conditions. A similar example is the induction of *CLB1* but not *CLB2* during sporulation (Chu and Herskowitz, 1998). The differences in the expression patterns of *CLN1* and *CLN2* as well as *CLB1* and *CLB2* indicates that the promoters of these cyclin pairs have diverged to regulate the appearance of Cln- and Clb-Cdc28 activity during different aspects of the *S. cerevisiae* life cycle. The acquisition of divergent transcriptional regulation is also seen among closely related mammalian cyclins. For example the Cyclin D promoters seem to have diverged to regulate the appearance of Cyclin D-Cdk4 activity in different cell types. An even more dramatic example of the divergence of duplicated cyclins is the apparent divergence in function between the Pho85-associated cyclins Pcl2 and Pcl9. The transcriptional regulation of these genes is distinct, and there is no evidence for any overlap in their functions. What is interesting about the diverging functions of these duplicated genes is that the genome duplication occurred after the last known speciation event in the *S. cerevisiae* lineage. Thus any new functionality acquired through the divergence of duplicated genes is likely to be functionality that is specific to *S. cerevisiae*.

I am interested in understanding the processes regulated by Pho85-associated kinases for a variety of reasons. I would like to determine how the various functions of

Pho85 are related to each other. Are they unrelated, or is there a theme which unites the functions of the different Pho85-associated kinases? If so, what is that theme? I am particularly intrigued by the fact that the two best understood functions of Pho85-associated kinases – the repression of phosphate starvation-specific gene expression and the repression of glycogen synthase activity – share the common feature of inhibiting nutrient stress-induced behaviors when nutrients are abundant. Although the cyclins responsible for these activities belong to the Pho80 subfamily of Pho85-associated cyclins, it remains possible that a theme of Pho85 acting to repress starvation-specific behaviors might also be shared by the Pho85 kinases containing the Pcl1/Pcl2 subfamily of cyclins.

I sought to identify the processes in which the different Pho85-containing kinases participate. I reasoned that the phenotypes that result from removing a particular *PHO85* function would reflect something about the processes in which that function of *PHO85* participates. The challenge of such a strategy is devising a method for determining the phenotypes which result from defects in a particular Pho85 function. The number of different phenotypes that have been described for various yeast mutants is quite large. It would be impossible to systematically determine if any particular mutation had each of these phenotypes. I turned to a genetic strategy for uncovering phenotypes that arise as a result of defects in Pho85-mediated processes -- I looked for mutations that result in inviability in combination with such *PHO85* defects. This type of genetic interaction is known as synthetic lethality because either mutation is viable on its own, but the combination of mutations results in lethality. Though there are many possible causes of

synthetic lethality, a common interpretation is that the mutations which are synthetically lethal affect genes that perform overlapping functions and thus share a degree of redundancy. In this interpretation, lethality is a result of the more profound defect in the shared process when both contributions are removed than results when either contribution is removed individually. This type of analysis has been used to identify components of processes such as RNA splicing (Frank et al., 1992) and nuclear import (Koepp et al., 1996). A screen for mutations that are synthetically lethal with the loss of the Cdc28-associated cyclins Cln1 and Cln2 identified a number of genes involved in budding and morphogenesis (Cvrckova and Nasmyth, 1993; Cvrckova et al., 1995; Benton et al., 1997). These data serve as the foundation for the hypothesis that Cln1 and Cln2 are involved in these processes. The advantage of looking for synthetic-lethal mutants is that unlike the directed approach of searching for phenotypes, this strategy explores the phenotype space of a given mutation in a relatively unbiased manner.

As the function of different Cdc28-associated kinases seem to be directed by cyclin binding, I reasoned that removing the functions that Pho85 acquires as a result of association with a specific cyclin might result in the loss of a definable subset of the functions Pho85 performs. One strategy would be to remove a specific Pho85-associated cyclin and determine the spectrum of synthetic-lethal interactions that results from this mutation. The problem with this approach is that there is apparent functional redundancy among the cyclins that associate with any particular CDK. Thus mutations in a single cyclin might not abrogate a particular Pho85-mediated process and would not give rise to synthetic-lethal interactions. To avoid these and other complications, I performed a

screen for phenotypes that result from removing all the functions of PHO85 simultaneously, by doing a screen for Pho Eighty-Five Requiring (Efr) mutants – mutants that require *PHO85* for viability.

This collection of mutants would contain *efr* mutants requiring different functions of PHO85. I predicted I could phenocopy the inviability of different *efr⁻ pho85⁻* strains by creating *efr* strains lacking the Pho85-associated cyclins that perform the specific Pho85 function required for viability in those mutants. Thus, if I could classify the various Efr mutants by identifying which Pho85-associated cyclins direct Pho85 to perform the necessary function in the different *efr* mutants, it might be possible to determine the functional similarities between the different Pho85-associated cyclins. By then looking for similarities in the wild-type functions of the different *efr* mutants that require a specific subset of Pho85-associated cyclins, I might be able to infer something about the function of those particular Pho85-containing heterodimers.

My screen uncovered mutations in eight genes which are inviable in the absence of *PHO85* function. Characterization of these mutants as well as directed tests for synthetic lethality between *PHO85* and a variety of other genes has provided clues to the functions provided by the G1-specific Pho85-associated kinases and a function of *PHO85* that is related to its role in regulating responses to phosphate starvation.

RESULTS

Identification of mutants that require *PHO85* for viability. I conducted two screens for Efr mutants using two different strategies. Both strategies had been used successfully to look for synthetic-lethal interactions with other genes (Cvrckova and Nasmyth, 1993; Benton et al., 1997). One strategy involves the identification of strains that are inviable in the absence of a plasmid containing the gene of interest; the other strategy involves the identification of strains that are inviable when a regulated promoter driving the gene of interest is repressed. The first strategy looked for mutants in a *pho85Δ* background that could not grow in the absence of a *PHO85*-containing plasmid. The inability to grow in the absence of the *PHO85* plasmid became a readily-scorable phenotype by placing the *URA3* and *ADE3* genes onto this plasmid and mutagenizing a strain that contains this plasmid and lacks the chromosomal copies *PHO85*, *URA3*, *ADE3* and *ADE2*. An *ade2 ade3* strain harboring an *ADE3*-containing plasmid gives rise to red colonies. If cells that lose the *ADE3*-containing plasmid are able to proliferate, they will give rise to white progeny that form white sectors in the otherwise red colony. The first screen for Efr mutants involved looking for derivatives of a *pho85Δ ura3 ade2 ade3* strain carrying a *PHO85 ADE3 URA3* plasmid that unlike the unmutagenized strain gave rise to colonies with no white sectors and then determining that the inability of these strains to give rise to white sectors is the result of an inability to proliferate in the absence of *PHO85*. I mutagenized the starting strain by exposure to UV irradiation, scored the sectoring phenotype of about 21,000 colonies, and identified eighteen mutants that can only grow in the presence of *PHO85*. These eighteen recessive mutants are in seventeen complementation groups suggesting that the number of loci that can be mutated to give rise to an Efr phenotype is quite large.

I identified the mutated locus in two of these Efr mutants. I identified the mutated loci by first identifying plasmids from a yeast genomic library (Nasmyth and Tatchell, 1980) which allowed the *efr pho85Δ* (*PHO85*; *ADE3*; *URA3*) strains to become 5-FOA^r. I recovered a mixture of 5-FOA^r colonies that were either Pho^c or Pho$^+$ and rescued plasmids from the colonies that were Pho^c, as this indicated that the complementing plasmid did not contain *PHO85*. One mutant was complemented by plasmids containing *TFC3* and *BRF1*. *TFC3* and *BRF1* are both essential genes involved in transcription of RNA Pol III genes (Lefebvre et al., 1992). *TFC3* is tightly linked to *CDC15*, and the mutation responsible for the Efr phenotype of this strain is also tightly linked to *CDC15*, as determined by the preponderance of parental ditype tetrads resulting from an *efr pho85Δ* strain crossed to a *cdc15-2 pho85Δ* strain (data not shown). This indicates that *TFC3* is the mutated locus responsible for the Efr phenotype of this strain. The other mutant was complemented by plasmids containing a previously uncharacterized ORF YMR212C which I named *EFR3*. An *efr3Δ pho85Δ* strain is inviable. I have not pursued the lethality of *pho85 tfc3* strains further, while the lethality of *pho85 efr3* strains is discussed in more detail in Appendix A.

The plasmid-dependent phenotype used to identify *efr* mutants in this screen was time consuming. Scoring the plasmid dependence of these mutants required streaking each of the red colonies several times to ensure that each was incapable of giving rise to colonies with white sectors. Many of the isolates that repeatedly yielded uniformly red colonies began to produce colonies with white sectors upon being transformed with an

irrelevant plasmid – suggesting that many of the mutants had an increased plasmid-stability phenotype rather than an Efr phenotype. Identification of the mutated locus required identifying the minimal complementing fragment on the genomic clones recovered from the library and then showing that the relevant locus was indeed linked to the mutation which causes the Efr phenotype, in the case of the *tfc3* mutant; or that mutations in the locus contained on the minimal complementing fragment have an Efr phenotype. To circumvent each of these problems I modified my screen: 1) I used a screening strategy that involved identifying Efr mutants that could not grow in conditions in which *PHO85* expression is repressed; 2) I used a mutagenesis scheme which tagged the mutation with both genetic and molecular markers to aid in cloning and linkage determination; 3) I developed a preliminary characterization scheme that would allow the mutants to be categorized without any knowledge of the mutated locus.

In a second screen for Efr mutants that cannot grow in the absence of *PHO85*, I constructed a strain in which expression of *PHO85* is galactose dependent and identified strains that can only grow in the presence of galactose. I mutagenized the *GAL1-10::PHO85* strain with transposon-containing fragments from a yeast genomic DNA library (Burns et al., 1994). These transformants were plated on galactose-containing yeast nitrogen base (YNB) plates that lacked leucine to select for the presence of transposon-containing sequences which had presumably integrated into the genome by homologous recombination directed by flanking genomic DNA. These transformants were then replica plated to dextose-containing YNB plates that also lacked leucine. Those colonies that could not grow on the dextrose plates were retained for further

characterization. Among 25,000 transposon transformants, twenty-six displayed a galactose-dependent phenotype.

Using the transposon-insertion library presented a challenge for identifying Efr mutants. Unlike the twenty-six mutants that were characterized further, approximately 700 primary isolates displayed a galactose-dependent phenotype on plates lacking leucine but became galactose-independent and Leu$^-$ on plates containing leucine. The reversion of these strains to a Transposon$^-$ Efr$^+$ phenotype after removing selection for the transposon suggests that the great majority of the primary *efr* mutant isolates maintained the transposon episomally. The Efr$^-$ phenotype that results from these episomal transposons suggests that at least a subset of the fragments in the library that can be maintained episomally cause an Efr$^-$ phenotype.

The inviability of *pho85* mutants in the presence of episomal DNA fragments may be related to the increased sensitivity of *pho85* mutants to DNA damage. I first observed this phenotype when I noticed that *pho85* strains are much more sensitive than wild-type strains to hydroxyurea, a competitive inhibitor of ribonucleotide reductase that slows down the rate of DNA synthesis (Elford, 1968). This increased sensitivity to hydroxyurea likely reflects a sensitivity to DNA damage rather than a defect in DNA synthesis, as I have also observed that *pho85* mutants are also supersensitive to the DNA-alkylating agent methyl-methane sulfonate (MMS). There are at least two responses to DNA damage that depend on a common damage-sensing mechanism: the arrest of the cell-cycle until the damage is repaired and the induction of enzymes required to repair the

damage (for review see Weinert, 1998). To determine if *pho85* mutants are unable to sense DNA damage or are unable to inhibit the cell cycle in response to DNA damage, I compared the ability of wild type, *pho85,* and *mec1* cells to arrest in response to MMS treatment. *MEC1* encodes a kinase that is involved in the DNA-damage sensing signal-transduction pathway (Sanchez et al., 1996). As a result, *mec1* mutants are unable to either arrest the cell cycle or induce expression of repair enzymes. To assay growth arrest in these strains, I used a halo assay in which I spotted MMS onto plates that contained a lawn of each strain. The size of the zone of growth inhibition around the spot of MMS reflects the sensitivity of each strain to MMS treatment, cells in the growth-inhibited zone were examined to determine if the inhibited cells had arrested properly or had undergone lethal cell divisions as a result of dividing in the presence of unrepaired DNA damage. I found that while both *pho85* and *mec1* strains were much more sensitive to MMS than wild-type strains, the *mec1* strain continued to divide in the presence of MMS while the *pho85* strain arrested properly. The ability of *pho85* mutants to arrest cell division in the presence of DNA damage suggests that *pho85* mutants may have a defect in inducing the expression of DNA-damage repair enzymes rather than a defect in DNA-damage sensing. Interestingly, the Pho85-associated cyclin encoded by the *PCL5* gene is induced in response to DNA damage (Jelinsky and Samson, 1999). Adam Carroll in our lab has been interested in pursuing this *pho85* phenotype further.

After eliminating the Efr strains that carried episomal transposon fragments, I had twenty-six strains which exhibited a stable Efr phenotype. In addition to mutants requiring *PHO85*, my primary screen would also identify mutants unable to grow on

dextrose even in the presence of *PHO85* (which we name **D**ead **O**n **D**extrose, or Dod mutants). To differentiate between these two classes of mutants, I crossed candidate Efr mutants to strains with a temperature sensitive-allele of *PHO85*. Eleven of the twenty-six mutants had a transposon-linked Efr phenotype: spores containing the transposon were dead or very sick in the presence of the *GAL1-10::PHO85* allele on dextrose but grew in the presence of the *pho85-9* allele at room temperature. These eleven mutants defined seven complementation groups.

A temperature-sensitive *PHO85* mutation is suppressed by overexpression of Pho85-associated cyclins: To investigate which cyclins are required to perform the various functions of *PHO85*, I tested if high-copy plasmids containing the different Pho85-associated cyclins could suppress the different phenotypes of a temperature-sensitive *PHO85* allele. This idea is based on the identification of *CLN1* and *CLN2* as high copy suppressors of the *cdc28-4* temperature-sensitive G1 arrest (Reed et al., 1989). I first determined whether high-copy plasmids containing *PHO80*, *PCL1* or *PCL2* suppress the temperature-sensitive inviability phenotype of a *cln1Δ cln2Δ pho85-9* strain (Figure 4A) or the ts-Pho^c phenotype of a *pho85-9* strain (Figure 4B). Since both *pho85Δ cln1Δ cln2Δ* and *pcl1Δ pcl2Δ cln1Δ cln2Δ* mutants are inviable, the function of *PHO85* that allows *cln1Δ cln2Δ* strains to grow requires Pcl1- and Pcl2-containing Pho85 kinases (Espinoza et al., 1994, Measday et al., 1994). High-copy plasmids containing *PCL1* suppress the ts-growth phenotype of a *cln1Δ cln2Δ pho85-9* strain. In contrast, high-copy plasmids containing *PHO80* suppress the ts-Pho^c phenotype of a *pho85-9* strain. Given the ability of plasmids containing *PCL1* to suppress the ts-growth phenotype of a *cln1Δ*

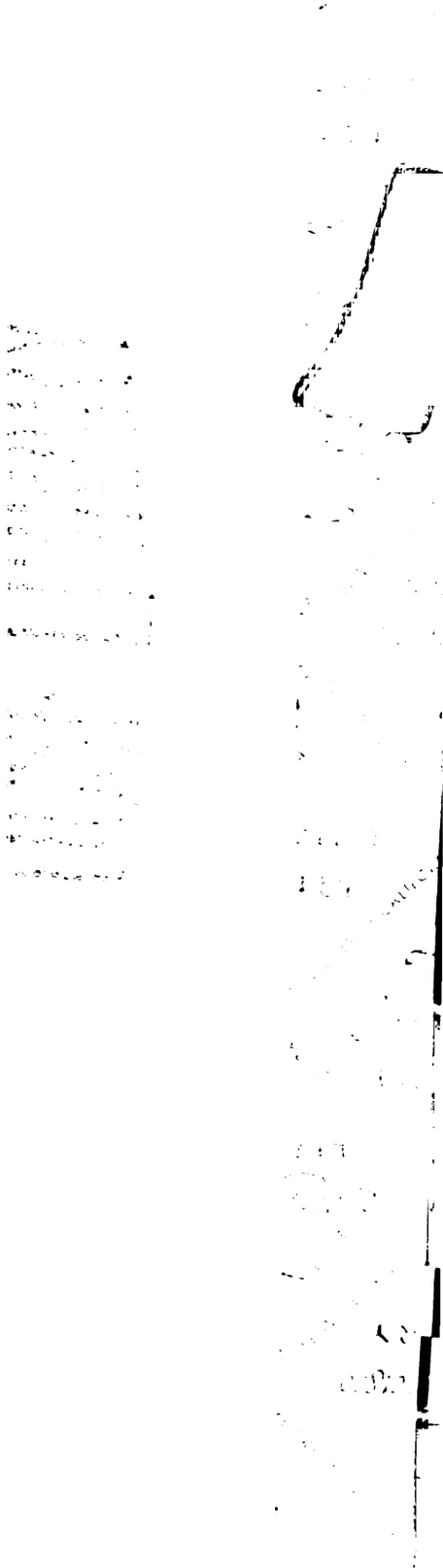
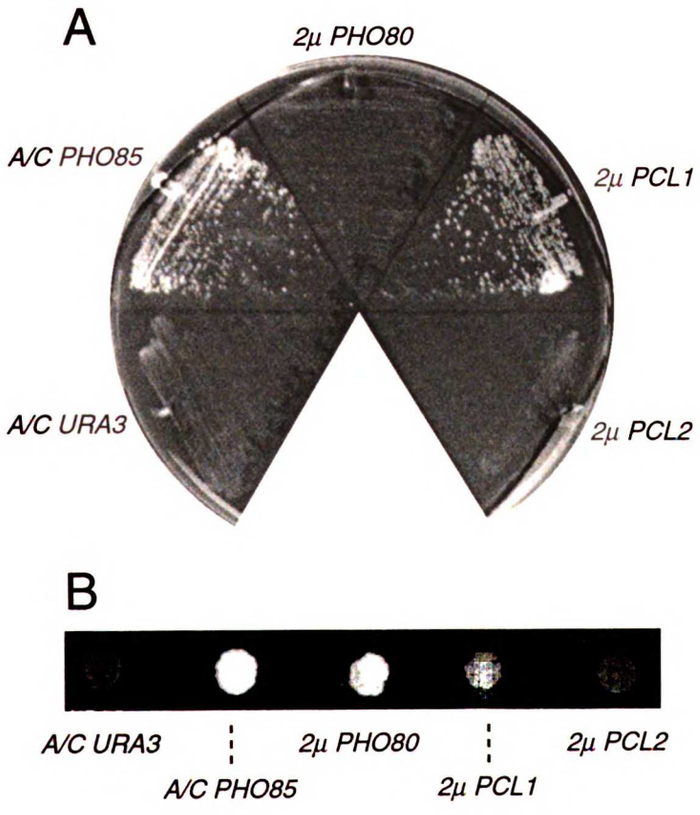


FIGURE 4. – Suppression of *pho85-9* temperature sensitive phenotypes by high copy cyclin plasmids. A. Suppression of the synthetic temperature sensitive lethality of the *pho85-9 cln1Δ cln2Δ* mutant. Strain MY0205 (*bf264-15D pho85-9 cln1Δ cln2Δ*) transformed with *ARS/CEN URA3* (EB0009), *ARS/CEN PHO85* (EB0327), *2μ PHO80* (MP0115), *2μ PCL1* (MP0120), or *2μ PCL2* (MP0121) was streaked on SD-Ura plates and placed at 30° for three days. B. Suppression of the temperature sensitive constitutive acid phosphatase expression phenotype of the *pho85-9* mutant. Strain MY0158 (*K699 pho85-9*) (transformed with the same plasmids) was placed on SD-Ura and placed at 37° overnight and then stained for acid-phosphatase activity.


A
2μ PHO80
A/C PHO85
2μ PCL1
A/C URA3
2μ PCL2
B
A/C URA3
2μ PHO80
2μ PCL2
A/C PHO85
2μ PCL1

cln2Δ pho85-9 strain, the inability of high-copy plasmids containing *PCL2* to suppress the same phenotype is surprising. This *PCL2* plasmid is functional, as it suppresses the temperature-sensitive lethality of an *mpk1Δ* strain (Madden et al., 1997, data not shown). High-copy plasmids containing each of the other seven Pho85-associated cyclins do not suppress either of these temperature-sensitive *pho85-9* phenotypes (data not shown).

Characterization of *efr pho85-9* strains: I next tested whether high-copy plasmids containing the Pho85-associated cyclins suppress the temperature-sensitive growth phenotypes of different *efr pho85-9* strains (Figure 5). High-copy plasmids containing *PCL1* suppress the ts-growth phenotype of *efr1 pho85-9* and *efr8 pho85-9* strains, whereas plasmids containing *PHO80* suppress the *efr3 pho85-9* strain. None of the Pho85-cyclin containing plasmids suppress the ts phenotype of *efr2 pho85-9, efr6 pho85-9,* or *efr7 pho85-9* strains. The ts phenotype of these strains is, however, *pho85-9* dependent as it is complemented by *PHO85* (Figure 5 and data not shown). I could not perform this analysis on the *efr5 pho85-9* strain as it is very slow growing.

The positions of the mutations responsible for the Efr phenotype are indicated in Table 1. The class of mutants that is not suppressed by cyclin overexpression contains mutations in a variety of genes involved in transcriptional regulation: *SPT7*, *HFI1* (*ADA1*), *SRB5,* and *GCR1*. With the exception of *GCR1,* which plays a specific role in regulating expression of genes involved in carbohydrate metabolism, the other genes encode general regulators of transcription by RNA polymerase II. The *PCL1*-suppressible class of Efr mutants is due to mutations in *BEM2* and *BCK1* -- genes

FIGURE 5. – Suppression of the synthetic temperature sensitive lethality of various *efr pho85-9* double mutants by high copy cyclin plasmids. Strains MY0276 (*efr1 pho85-9*), MY0282 (*efr2 pho85-9*), MY0176 (*efr3Δ pho85-9*) and MY0321 (*efr8 pho85-9*) transformed with high-copy plasmids containing each of the Pho85-associated cyclins or low-copy plasmids containing *PHO85* (EB0327) or *URA3* (EB0009) were diluted from an OD_{600}=0.3 by three-fold serial dilutions and plated on SD-Ura plates. Plates were incubated at the indicated temperature for three days.

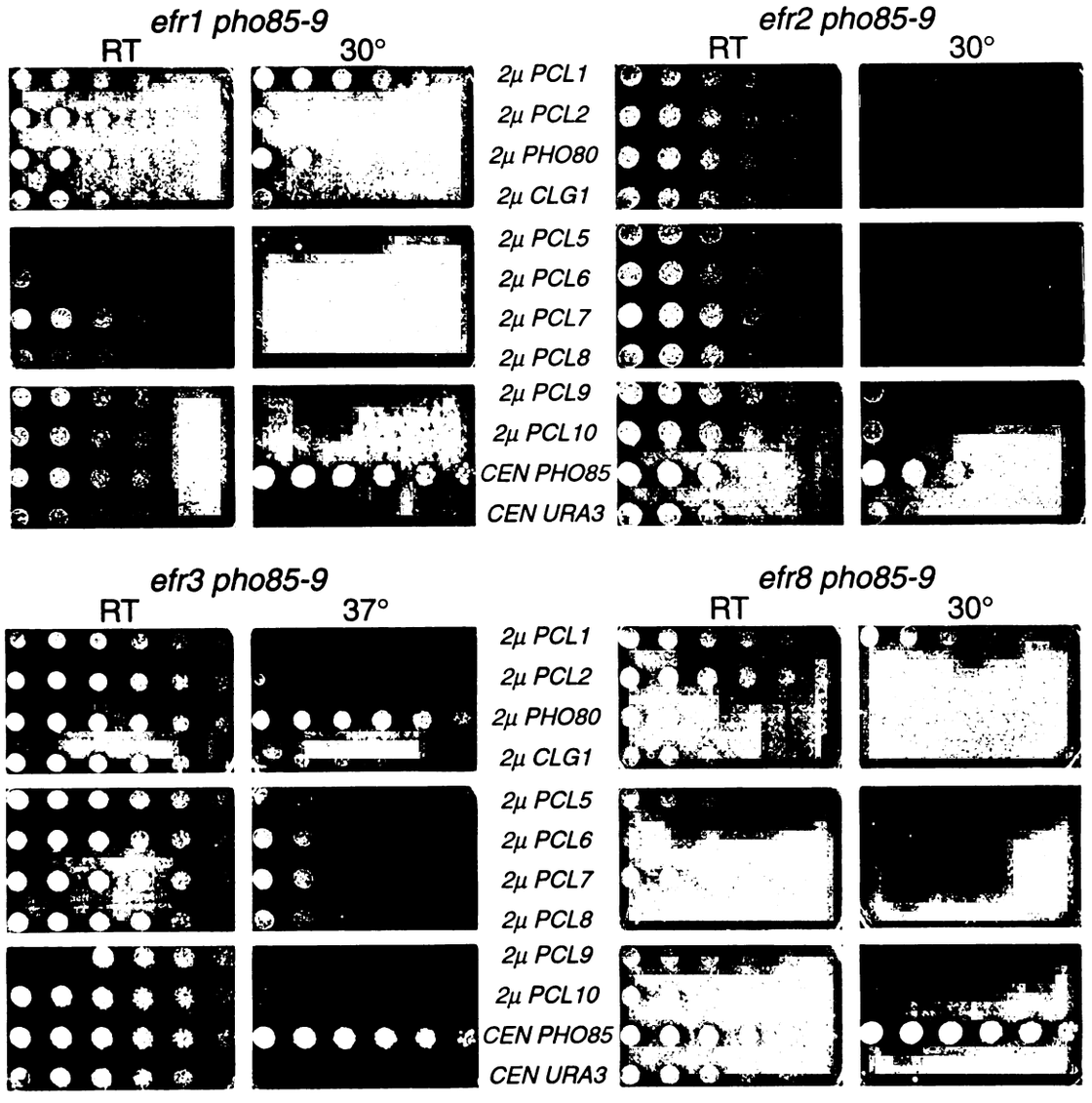

efr1 pho85-9
RT
30°
efr2 pho85-9
RT
30°
2μ PCL1
2μ PCL2
2μ PHO80
2μ CLG1
2μ PCL5
2μ PCL6
2μ PCL7
2μ PCL8
2μ PCL9
2μ PCL10
CEN PHO85
CEN URA3
efr3 pho85-9
RT
37°
efr8 pho85-9
RT
30°

TABLE 1.

Identification of Efr Loci

complementation group	# of alleles	gene disrupted	Position of transposon relative to start codon[a]
EFR1	4	*BEM2*	3,033 / 6,503
EFR2	2	*SPT7*	1,750 / 3,998
EFR3	1	*YMR212c*	see note[b]
EFR5	1	*GCR1*	3,013 / 3,108
EFR6	1	*SRB5*	-15 / 923
EFR7	1	*HFI1*	358 / 1,466
EFR8	1	*BCK1*	1,789 / 4,436

[a] When more than one allele was identified, the transposon position is reported for the allele used in subsequent experiments.

[b] We had identified UV-induced alleles of *EFR3* in an earlier screen and generated an *efr3Δ* strain during the course of those experiments. The transposon-insertion allele of *EFR3* was identified by noncomplementation of this strain and was not characterized further. The deletion allele is used in subsequent experiments.

involved in morphogenesis and cell-wall biosynthesis. Mutations in a previously uncharacterized ORF, YMR212C, which I have named *EFR3,* account for the *PHO80*-suppressible mutant.

DISCUSSION

Our strategy for identifying the cyclins responsible for different functions of Pho85 involved determining if high-copy plasmids carrying the genes for the Pho85-associated cyclins could suppress various phenotypes of a temperature-sensitive allele of *PHO85*. This strategy was of limited utility as only *PHO80*- and *PCL1*-containing plasmids had activity in this assay, and some *efr pho85-9* mutants were not suppressed by either plasmid. Mutations in activators of transcription account for the largest class of non-cyclin suppressible mutants. It is striking that our screen identified mutations in two different components of the SAGA histone acetylase complex (*SPT7* and *HFI1/ADA1*) (Eberharter et al., 1998). We do not know if these synthetic lethal interactions indicate a role for *PHO85* as a general regulator of transcription -- like the yeast CDKs Srb10, Kin28, and Ctk1 (Liao et al., 1995; Valay et al., 1995; Sterner et al., 1995) or if they cause a defect in expression of a particular gene with which *PHO85* is also synthetically lethal.

Our screen for mutants that require *PHO85* for viability was designed to survey the functions that Pho85 performs in association with its ten cyclin subunits. I identified mutations in seven genes. Because I found only one allele of many of these genes and

found additional synthetic-lethal interactions involving *PHO85* by directed tests (as described in Chapter 3), it is apparent that the screen was not saturating. A similar screen for Efr mutants using ultraviolet light as a mutagen uncovered eighteen recessive mutations in seventeen complementation groups, suggesting that the number of loci which can be mutated to give rise to an Efr phenotype is large. Two of these UV-induced mutants contained mutations in *EFR3*. Rather than attempting to saturate my screen, I decided to determine if I could use the synthetic lethality of *pho85* with *efr3* and *bem2* or *bck1* as tools to understand more about the functions of Pho80 (which we already know a great deal about) and Pcl1 (about which we know very little). If analysis of these synthetic-lethal interactions would result in a better understanding of the functions of these Pho85-containing kinases it would provide incentive to identify a more complete set of Efr mutants.

CHAPTER 3

A role for Pcl1- and Pcl2-Pho85 in morphogenesis

S. cerevisiae has two CDKs that associate with multiple cyclins: Pho85 and Cdc28. One interesting difference between these two CDKs is that whereas the transcription of each of the Cdc28-associated cyclins is regulated by the cell cycle (Nasmyth, 1996; McInerny et al., 1997), transcription of some of Pho85 cyclins is not cell-cycle regulated (Measday et al., 1997). The cell-cycle regulation of the Cdc28-associated cyclins results in the levels of different cyclin peaking at different points in the cell cycle where Cdc28 activity is required (Nasmyth, 1996). In contrast, expression of all the cell cycle-regulated Pho85 cyclins (*PCL1, PCL2 and PCL9*) peaks in the G1 phase of the cell cycle (Measday et al., 1997). The maximal expression of Cdc28-associated cyclins at different points across the cell cycle is an indication of Cdc28's function as a general cell-cycle regulator. In contrast, the cell cycle-regulated cyclins of Pho85 are expressed exclusively in G1, suggesting that the cell-cycle regulated functions of Pho85 are restricted to a G1. One of the central questions about Pho85's G1-regulated cyclins has been understanding the process to which these cyclins contribute.

A critical observation which has framed most of the thinking about the function of the G1-regulated cyclins of Pho85 is that a *cln1Δ cln2Δ pcl1Δ pcl2Δ* mutant is inviable and arrests in G1 (Espinoza et al., 1994; Measday et al., 1994). *CLN1* and *CLN2* encode Cdc28-associated cyclins that peak in G1 (Wittenberg et al., 1990). *PCL1* and *PCL2* encode two of the three G1-regulated cyclins that associate with Pho85 (Measday et al., 1997). One interpretation of the inviability of *cln1Δ cln2Δ pcl1Δ pcl2Δ* mutants is that

Pho85 and Cdc28 have overlapping functions in G1 and promote a process which is essential for G1 exit. Understanding the causes and consquences of the functional overlap between Pho85 and Cdc28 requires first understanding in detail the functions of Pho85 and Cdc28 that result in the inviability of *cln1Δ cln2Δ pcl1Δ pcl2Δ* mutants.

The screen for Efr mutants identified two mutants -- *bem2* and *bck1* -- that share phenotypes with a *cln1Δ cln2Δ* mutant: like the *cln1Δ cln2Δ pho85*-9 mutant, the temperature-sensitive lethality of *bem2 pho85-9* and *bck1 pho85-9* mutants is suppressed by high-copy plasmids containing *PCL1*. Further analysis of the phenotypes of *cln1Δ cln2Δ, bem2,* and *bck1* mutants might provide additional clues about the functions of the G1-specific Pho85-associated cyclins and the defects that result in the G1 arrest of *cln1Δ cln2Δ pho85Δ* strains.

RESULTS

Morphogenesis-related Efr mutants: Since Bck1 is the MAPKKK responsible for activation of Mpk1 (Kamada et al., 1995), and *bck1* mutants have an Efr phenotype, I examined synthetic interactions between *mpk1* and *pho85*. *mpk1Δ pho85Δ* strains are inviable (this result was subsequently reported in HUANG *et al.* 1999; *mpk1Δ pho85-9* strains exhibit a synthetic temperature-sensitive growth phenotype (data not shown).

Since high-copy *PCL1* plasmids suppress the ts-lethality of *bem2 pho85-9* and *bck1 pho85-9* strains, I determined whether Pcl1-Pho85 complexes provide the *PHO85*

function required for viability in these mutant backgrounds. *bem2 pcl1Δ* (Table 2) and *bem2Δ pcl1Δ* strains are inviable (data not shown). *mpk1Δ pcl1Δ pcl2Δ* and *bck1 pcl1Δ pcl2Δ* strains are inviable when dissected onto standard medium, but are viable on medium containing 1M sorbitol (Figure 6). The inviability of *mpk1Δ pcl1Δ pcl2Δ* strains is different from the *bem2 pcl1Δ* lethality because *mpk1Δ pcl1Δ* and *bck1 pcl1Δ* strains have growth rates similar to *mpk1Δ* or *bck*1 strains. Furthermore, sorbitol does not rescue the inviability of *bem2 pcl1Δ* strains (data not shown). Removing *PCL9,* a cyclin highly homologous to *PCL2*, does not exacerbate the sorbitol-requiring phenotype of *mpk1Δ pcl1Δ pcl2Δ* or *bck1 pcl1Δ pcl2Δ* strains nor do *bck1 pcl1Δ pcl9Δ* or *bck1 pcl2Δ pcl9Δ* strains exhibit any growth defects (data not shown).

The screen identified several Efr mutants that in the presence of *pho85-9* exhibited a temperature-sensitive lethality phenotype that is not suppressed by high-copy plasmids containing any of the genes that encode Pho85-associated cyclins. I also tested for genetic interactions between these Efr mutants (*spt7*, *gcr1*, *srb5* or *hfi1*) and *pcl1Δ pcl2Δ pcl9Δ.* All of these *efr pcl1Δ pcl2Δ pcl9Δ* strains grow similarily to the corresponding *efr* strain (data not shown), suggesting that the cause of the synthetic lethality in these Efr mutants is distinct from that of the *PCL1*-suppressible Efr mutants.

Table 2.

Inviability of *bem2 pcl1Δ* strains

BEM2	PCL1	PCL2	Viable Spores
-	+	+	9 / 9
-	-	+	**0 / 10**
-	-	-	**0 / 7**
-	+	-	8 / 8
+	+	+	7 / 7
+	-	+	8 / 8
+	-	-	9 / 9
+	+	-	10 / 10

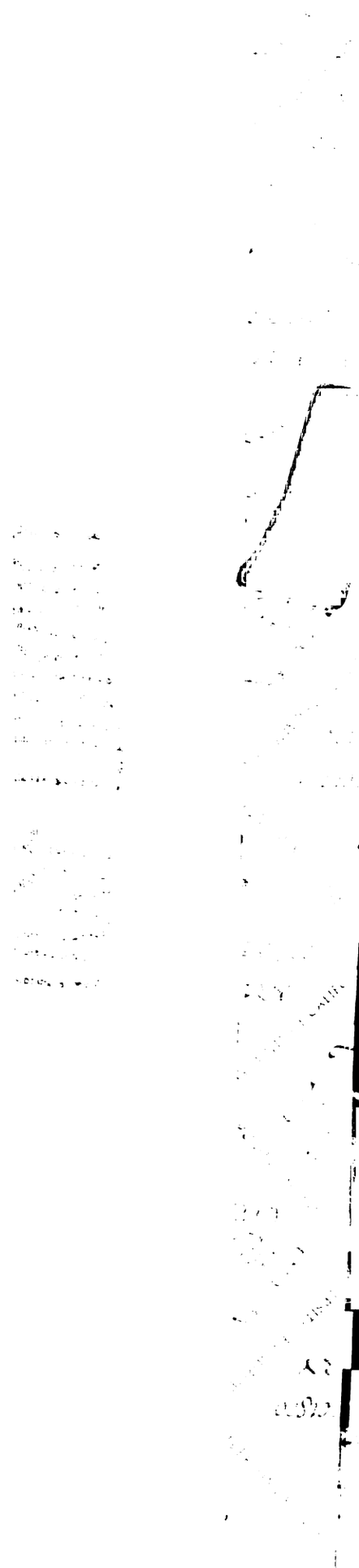
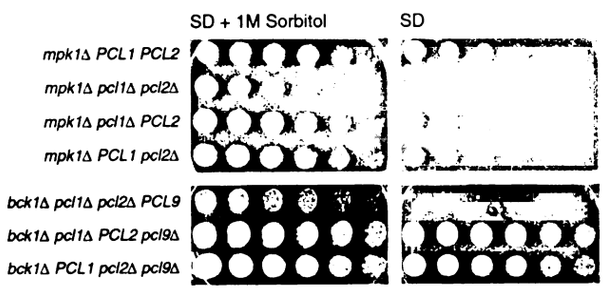


FIGURE 6. -- Sorbitol suppressible synthetic lethal phenotype of the *pcl1Δ pcl2Δ mpk1Δ* mutant. Progeny of a cross between MY0263 (*mpk1Δ*) and EY0535 (*pcl1Δ pcl2Δ*) or of a cross between MY0296 (*efr8*) and EY0552(*pcl1Δ pcl2Δ pcl9Δ*) were diluted from an OD_{600}=0.3 by three-fold serial dilutions and plated on SD-complete plates or SD-complete plates that had been supplemented with 1M sorbitol. Plates were placed at 30° for two days.

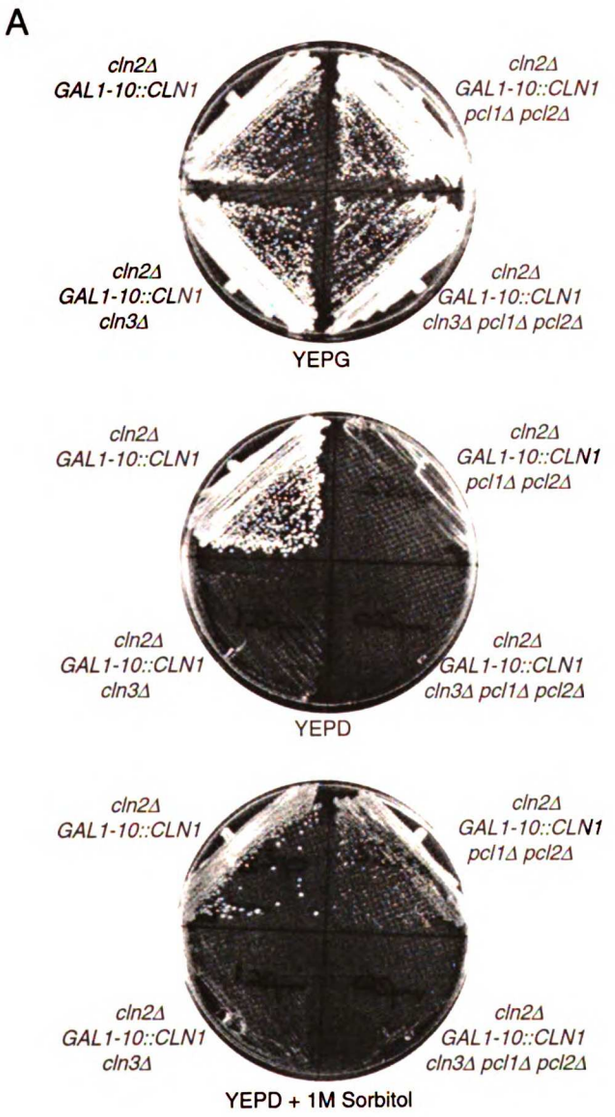
A


SD + 1M Sorbitol
SD
mpk1Δ PCL1 PCL2
mpk1Δ pcl1Δ pcl2Δ
mpk1Δ pcl1Δ PCL2
mpk1Δ PCL1 pcl2Δ
bck1Δ pcl1Δ pcl2Δ PCL9
bck1Δ pcl1Δ PCL2 pcl9Δ
bck1Δ PCL1 pcl2Δ pcl9Δ

Suppression of *cln1Δ cln2Δ pcl1Δ pcl2Δ* lethality by sorbitol-containing medium:

The screen identified two types of *efr pho85-9* mutants that are suppressed by high-copy *PCL1* plasmids. *bem2 pcl1Δ* mutants are inviable while *bck1 pcl1Δ pcl2Δ* exhibit high osmolarity-suppressibe inviability. Since high-copy *PCL1* also suppresses the *cln1Δ cln2Δ pho85-9* strain (Figure 4), the lethality of a *cln1Δ cln2Δ pcl1Δ pcl2Δ* strain (ESPINOZA *et al.* 1994, MEASDAY *et al.* 1994) might also be suppressed by high osmolarity. I compared growth of a *GAL1-10::CLN1 cln2Δ* strain with *GAL1-10::CLN1 cln2Δ cln3Δ*, *GAL1-10::CLN1 cln2Δ pcl1Δ pcl2Δ*, and *GAL1-10::CLN1 cln2Δ cln3Δ pcl1Δ pcl2Δ* strains on YEPG, YEPD, and YEPD + 1M sorbitol. Whereas *GAL1-10::CLN1 cln2Δ pcl1Δ pcl2Δ* strains are unable to grow on YEPD plates, they grow on YEPD plates containing sorbitol (Figure 7A). In contrast, the *GAL1-10::CLN1 cln2Δ cln3Δ* strain is unable to grow on YEPD with or without sorbitol. Like the *GAL1-10::CLN1 cln2Δ* strain, my *WT* strain grows more slowly on YEPD + 1M sorbitol than on YEPD (data not shown). The dominant *PKC1 R398P* gain-of-function allele suppresses the temperature-sensitive lethality of *mpk1Δ* and *swi4Δ* strains (data not shown). The *PKC1 R398P* allele also suppresses the temperature-sensitive lethality of a *cln1Δ cln2Δ pho85-9* strain (Figure 7B). These data indicate that the synthetic lethality of the *cln1Δ cln2Δ pcl1Δ pcl2Δ* strain has many similarities with the synthetic lethality of *mpk1Δ pcl1Δ pcl2Δ* and *bck1 pcl1Δ pcl2Δ* strains.

FIGURE 7. Comparison of the phenotypes of the *pcl1Δ pcl2Δ cln1Δ cln2Δ* and the *pcl1Δ pcl2Δ mpk1Δ* mutants. A. The lethality of *pcl1Δ pcl2Δ cln1Δ cln2Δ* is suppressed by 1M sorbitol. The following strains were streaked on the indicated plates and placed at 37° for two days: *cln2Δ GAL1-10::CLN1* (EY0233); *cln2Δ GAL1-10::CLN1 pcl1Δ pcl2Δ* (EY0409); *cln2Δ GAL1-10::CLN1 cln3Δ* (MY0261); *cln2Δ GAL1-10::CLN1 cln3Δ pcl1Δ pcl2Δ* (MY0260). B. The temperature-sensitive lethality of the *pho85-9 cln1Δ cln2Δ* mutant is suppressed by a PKC1 gain of function allele. Strain MY0205 (*cln1Δ cln2Δ pho85-9*) transformed with plasmids containing *PKC-R398P* (MP0143), *2μ PCL1* (MP0120), *2μ CLN2* (EB0459), *ARS/CEN PHO85* (EB0327), or *ARS/CEN URA3* (EB0009) was streaked on SD-Ura plates and placed at 30° for three days.


A
cln2Δ GAL1-10::CLN1
cln2Δ GAL1-10::CLN1 pcl1Δ pcl2Δ
cln2Δ GAL1-10::CLN1 cln3Δ
cln2Δ GAL1-10::CLN1 cln3Δ pcl1Δ pcl2Δ
YEPG
cln2Δ GAL1-10::CLN1
cln2Δ GAL1-10::CLN1 pcl1Δ pcl2Δ
cln2Δ GAL1-10::CLN1 cln3Δ
cln2Δ GAL1-10::CLN1 cln3Δ pcl1Δ pcl2Δ
YEPD
cln2Δ GAL1-10::CLN1
cln2Δ GAL1-10::CLN1 pcl1Δ pcl2Δ
cln2Δ GAL1-10::CLN1 cln3Δ
cln2Δ GAL1-10::CLN1 cln3Δ pcl1Δ pcl2Δ
YEPD + 1M Sorbitol
B
2μ PKC-R398P
CEN URA3
2μ PCL1
CEN PHO85
2μ CLN2

Other morphogenesis-related phenotypes of *pho85* mutants: Because *pcl1Δ* and *pcl1Δ pcl2Δ* are synthetically lethal with mutations in genes involved in morphogenesis, and some of these phenotypes can be reversed by osmotic stabilization, mutations in *PCL1* and *PCL2* might show interactions with mutations in other morphogenesis-promoting genes. Interactions between *PHO85* and the p21-activated (PAK) kinases Ste20, Cla4, or Skm1 would be particularly interesting because these kinases are involved in morphogenesis-related processes, and mammalian CDK5, which is involved in regulating PAK kinase activity in neurons (NIKOLIC *et al.* 1998), can complement a *pho85Δ* strain (HUANG *et al.* 1999; NISHIZAWA *et al.* 1999). *pho85Δ ste20Δ* strains are temperature sensitive (Figure 8). *cla4Δ pcl1Δ pcl2Δ* strains are inviable (Table 3) and not suppressed by osmotic stabilization with sorbitol (data not shown). The *cla4Δ pcl1Δ pcl2Δ* inviability is therefore different from the sorbitol-requiring phenotype of strains carrying the *mpk1Δ*, *bck1,* or *cln1Δ cln2Δ* mutations in combination with *pcl1Δ pcl2Δ.*

As sorbitol suppresses the inviability of *cln1Δ cln2Δ pcl1Δ pcl2Δ*, *mpk1Δ pcl1Δ pcl2Δ,* and *bck1 pcl1Δ pcl2Δ* strains, *cln1Δ cln2Δ, mpk1Δ,* and *bck1* might cause similar defects. Since *CLA4* is required for viability in the absence of *CLN1* and *CLN2* (CVRCKOVA *et al.* 1995), other *PCL1*- and *PCL2*-requiring mutants might also require *CLA4*. Both *cla4Δ mpk1Δ* and *cla4Δ bem2* strains are inviable (data not shown). These data demonstrate another similarity between the phenotypes of *mpk1Δ* and *cln1Δ cln2Δ* mutants and suggest that despite the differences in synthetic lethality with *pcl1Δ pcl2Δ*, the defects of a *bem2* mutant are related to those of *mpk1Δ* and *cln1Δ cln2Δ* mutants.

FIGURE 8. The *ste20Δ pho85Δ* mutant is synthetically temperature sensitive. Progeny of a cross between MY0363 (*ste20Δ*) and MY0147 (*pho85Δ*) were diluted from an OD_{600}=0.3 by three fold serial dilutions and plated on YEPD plates that were then placed

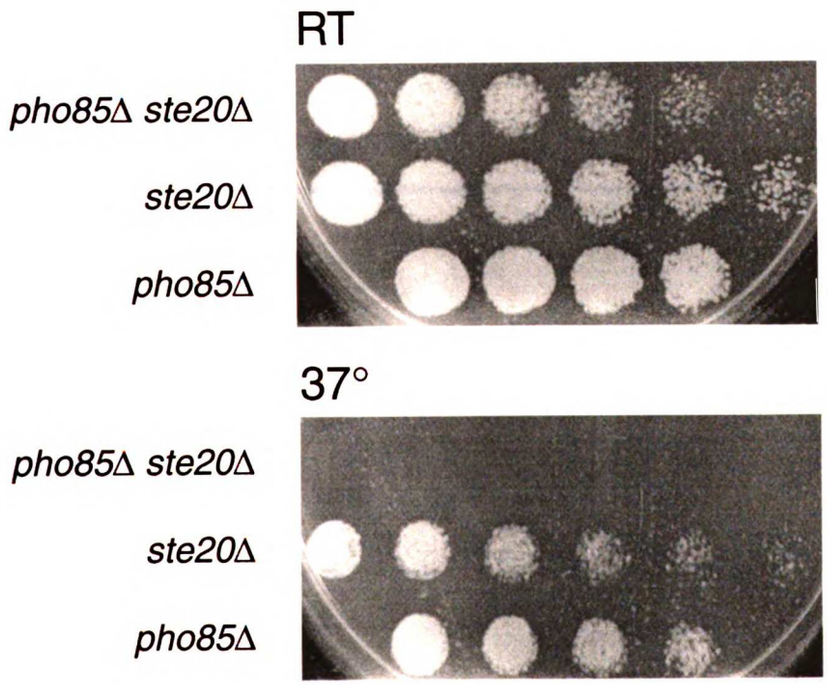

RT
pho85Δ ste20Δ
ste20Δ
pho85Δ
37°
pho85Δ ste20Δ
ste20Δ
pho85Δ

Table 3.

Inviability of *cla4Δ pcl1Δ pcl2Δ* strains

CLA4	PCL1	PCL2	Viable Spores
-	+	+	50 / 52
-	-	+	7 /8
-	-	-	**0 / 8**
-	+	-	31 / 35
+	+	+	3 / 3
+	-	+	39 / 39
+	-	-	50 / 52
+	+	-	13 / 13

Since *CDC42* is a GTPase that performs an essential function involved in actin polarization and is also required for the activation of Ste20 and Cla4 (ADAMS *et al.* 1990; BENTON *et al.* 1997; PETER *et al.* 1996), mutations in *PCL1* or *PCL2* might alter the permissive temperature of strains carrying a temperature-sensitive allele of *CDC42*. *cdc42*-1 *pcl1Δ pcl2Δ* strains are inviable at all temperatures (Table 4) and are not rescued by sorbitol (data not shown).

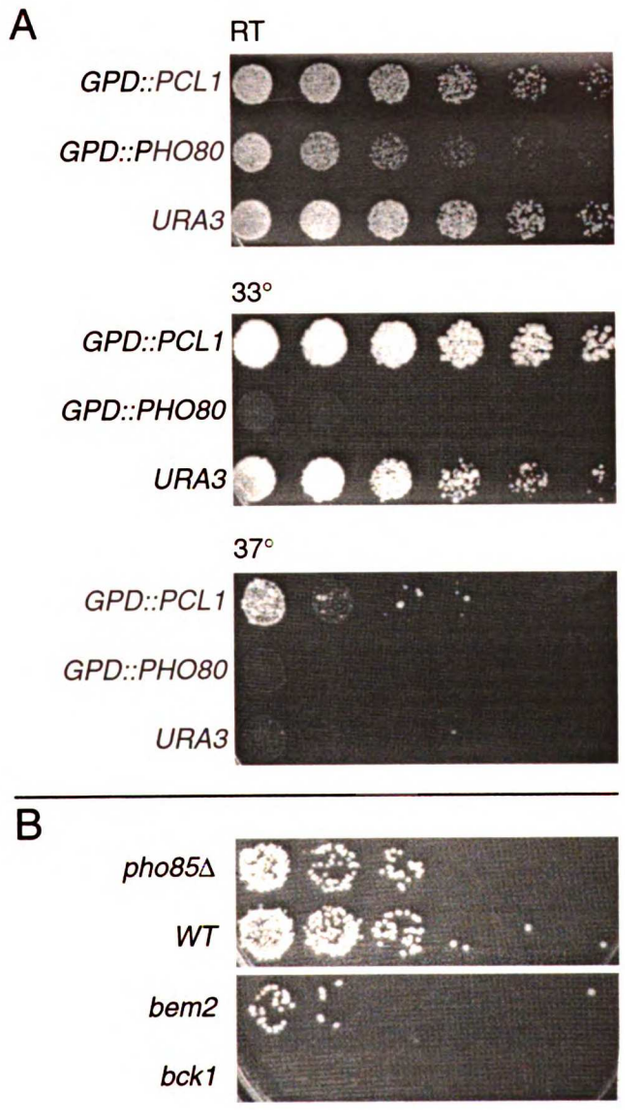
The synthetic lethality between *cdc42-1* and *pcl1Δ pcl2Δ* could have many causes. Expression of *PCL1* from the *GPD1* promoter raises the permissive temperature of the *cdc42-1* strain (Figure 9A), suggesting that overexpression of *PCL1* either promotes a process that substitutes for *CDC42* function or promotes Cdc42 activity. In contrast, overexpression of *PHO80* lowers the permissive temperature of a *cdc42-1* strain, suggesting that *PHO80* is not able to perform the function *PCL1* provides. The lower permissive temperature of *cdc42-1* strains overexpressing *PHO80* also suggests that increasing Pho80 activity interferes with the Pcl1-mediated functions of Pho85, perhaps by decreasing the amount of Pho85 available to associate with Pcl1.

Table 4.

Inviability of *cdc42-1 pcl1Δ pcl2Δ* strains

CDC42	PCL1	PCL2	Viable Spores
-	+	+	4 / 5
-	-	+	8 / 9
-	-	-	**1 / 6**
-	+	-	11 / 11
+	+	+	6 / 6
+	-	+	10 / 11
+	-	-	8 / 8
+	+	-	7 / 7

FIGURE 9. Other phenotypic consequences of altering Pho85 activity. A. The effect of Pho85-associated cyclin overexpression of the permissive temperature of *cdc42-1* strains. Strain MY361 (*MAT a cdc42-1*) transformed with plasmids containing *GPD1::PCL1* (EB0128), *GPD1::PHO80* (EB0049) or *URA3* (EB0009) were diluted from an OD_{600}=0.3 by three-fold serial dilutions and plated on YEPD plates and placed at the indicated temperature for three days. B. *pho85Δ* strains mate as efficiently as *WT* strains. Strains EY0140 (*MAT a pho85Δ*), EY057 (*MAT a WT*), MY0277 (*MAT a bem2*), and MY0296 (*MAT a bck1*) were diluted from an OD_{600}=0.3 by three-fold serial dilutions and plated on a lawn of 2.0 OD_{600} of EY0478 (*MAT α far1-c* Lys^-) that had been spread on YEPD plates and placed at 30° for six hours. These plates were then replica-plated to SD-Min plates (lacking amino acid supplements) and placed at 30° for two days.


A
RT
GPD::PCL1
GPD::PHO80
URA3
33°
GPD::PCL1
GPD::PHO80
URA3
37°
GPD::PCL1
GPD::PHO80
URA3
B
pho85Δ
WT
bem2
bck1

Since many of the processes required for morphogenesis during the cell cycle are also required for the specialized morphogenetic processes that occur during mating, many mutants with defects in morphogenesis and cell-wall biosynthesis also have mating defects. Subtle mating defects can be detected by looking for mating to a *far1-c* strain (VALTZ *et al.* 1995). WT and *pho85Δ* strains mate with similar efficiency to a *far1-c* strain whereas *bem2* and *bck1* mutants show reduced mating ability (Figure 9B). The wild-type mating ability of *pho85Δ* strains suggests that *pho85Δ* mutants are unlikely to have a defect in the physical processes of morphogenesis or cell-wall biosynthesis.

DISCUSSION

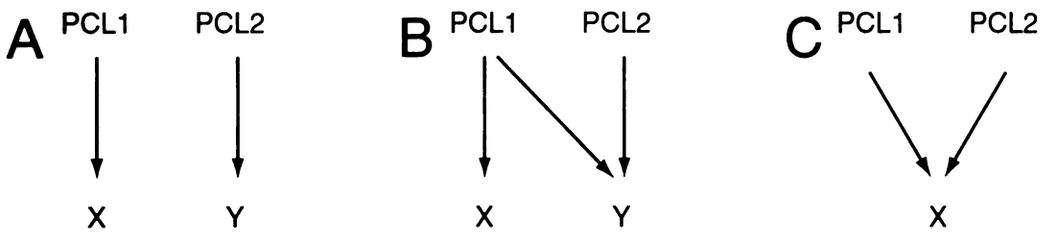
There are several different classes of mutations that require *PCL2* and/or *PCL1* for viability. *bem2Δ* and *pcl1Δ* are synthetic lethal, and this interaction is not suppressed by growth in the presence of 1M sorbitol. *bck1*, *mpk1Δ*, and *cln1Δ cln2Δ* are synthetically lethal with *pcl1Δ pcl2Δ*, and this interaction is suppressed by sorbitol. *cla4Δ* and *cdc42-1* are synthetic lethal with *pcl1Δ pcl2Δ*, and this interaction is not suppressed by sorbitol.

The inviability of *bem2Δ pcl1Δ* strains is the first example of a *pcl1Δ* phenotype that does not also depend on the removal of *PCL2* function. There are several models which can account for the requirements for *PCL1* versus the requirement for *PCL1* or *PCL2* (Figure 10). One possibility is that *PCL1* and *PCL2* perform distinct functions and that mutants

such as *bem2Δ* require the functions provided by *PCL1* for viability and that mutants like *mpk1Δ* are inviable when the functions provided by *PCL1* and the distinct functions provided by *PCL2* are both removed. Another model to explain why some mutants require *PCL1* while others require *PCL1* or *PCL2* is that *PCL1* has distinct functions, some of which can only be performed by *PCL1* and others which can be performed by *PCL1* or *PCL2*. The overlapping function model is similar to the first model in that it posits that the cause of a *bem2Δ pcl1Δ* strain's inviability and the inviability of *mpk1Δ pcl1Δ pcl2Δ* strains is caused by fundamentally different defects in the *pcl1Δ* as compared to the *pcl1Δ pcl2Δ* strain. A third possibility is that *PCL1* and *PCL2* both perform similar functions and that *bem2Δ* mutants require different thresholds of the shared functions performed by *PCL1* and *PCL2* for viability than *mpk1Δ* mutants.

The possibility that *PCL1* and *PCL2* perform distinct functions is intriguing. In most cases in which yeast cyclins perform highly overlapping functions, these cyclins were duplicated during the most recent gene duplication event. Two examples are the functions of the Cdc28-associated cyclins encoded by *CLN1* and *CLN2* and the Pho85-associated cyclins encoded by *PCL8* and *PCL10*. In both cases, there are only subtle, if any, differences in the functions performed by the members of the pair. *PCL1* and *PCL2* encode proteins that are clearly related to each other in primary amino acid sequence but are not contained in duplicated regions of the genome. *PCL2* and *PCL9* (which encode proteins with even more primary sequence similarity than Pcl1 and Pcl2) are in duplicated regions of the genome. The inviability of *cln1Δ cln2Δ pcl1Δ pcl2Δ* and *mpk1Δ pcl1Δ pcl2Δ* mutants suggests functional overlap between *PCL1* and *PCL2*, but

FIGURE 10. Models to explain the functional relationships between Pcl1- and Pcl2-associated Pho85 kinase activities. A. *PCL1* and *PCL2* perform non-overlapping functions. B. *PCL1* participates in multiple processes. Some of these are independent of *PCL2* (labeled X) and some are shared with *PCL2* (labeled Y). C. The functions of *PCL1* and *PCL2* overlap.


A
PCL1
PCL2
X
Y
B
PCL1
PCL2
X
Y
C
PCL1
PCL2
X

there is no evidence of functional overlap between *PCL2* and *PCL9*. In fact since *PCL9* transcription peaks at the M/G1 boundary and is dependent on *SWI5* while *PCL2* transcription peaks later in G1 and is *SWI4* dependent, there is evidence that the functions of *PCL2* and *PCL9* are at least somewhat divergent.

Determining if the *PCL2/PCL9* ancestral gene performed a more *PCL2*- or *PCL9*-like function could result in a better understanding of the functional distinction between *PCL1* and *PCL2*. Such a determination should be possible by looking at the functions of the *PCL1* and *PCL2/PCL9* homologs in a modern yeast species that diverged from the *S. cerevisiae* lineage before the genome duplication event. The model that *PCL1* and *PCL2* perform similar functions is attactive as it allows the various Efr mutants to be arranged along a phenotypic spectrum and from this information make a prediction about the site of action of the *PCL1/PCL2* function.

If *PCL1* and *PCL2* perform the same function, the requirement of *bem2* mutants for the Pcl1- and Pcl2- mediated function of Pho85 is the most because *bem2* mutants are inviable when just the Pcl1 fraction of the Pcl1- and Pcl2- associated Pho85 is removed. This is in contrast to other mutants that are inviable only when both *PCL1* and *PCL2* are both absent. Similarly, the inviability of *mpk1Δ pcl1Δ pcl2Δ* strains can be rescued by sorbitol, while the inviability of the *cla4Δ pcl1Δ pcl2Δ* strain cannot. The differential ability of sorbitol to suppress these synthetic-lethal interactions could indicate that *cla4Δ* strains are more dependent on Pho85 function than *mpk1Δ* strains. If these suppositions

are correct, these Efr mutants can be arranged along a phenotypic spectrum: *bem2* > *cla4Δ, cdc42-1* > *mpk1Δ, bck1, cln1Δ cln2Δ.*

A model to explain the defect that accounts for the phenotypic spectrum is that *PCL1* and *PCL2* function together with the *PCL1*- and *PCL2*-requiring *EFR* genes to positively regulate Cdc42 activity (Figure 11). As the novel elements of the model are based on circumstantial evidence, I have summarized my principal findings in Table 5. One aspect of the model proposes that *PKC1* positively regulates *CDC42* activity. A functional connection between *BEM2* and *CDC42* is supported by the observation that *GIC1* and *GIC2* are *CDC42* effectors and are also high copy suppressors of the *bem2Δ* mutation (CHEN *et al.* 1997). *PKC1* may function in between *BEM2* and *CDC42* as *in vitro* studies show Bem2 displays strong GAP activity toward Rho1 (KIM *et al.* 1994; PETERSON *et al.* 1994), a GTPase that activates *PKC1* (DRGONOVA *et al.* 1996; KAMADA *et al.* 1996, NONAKA *et al.* 1995). There is a distinction among the functions of the Swi4-dependent genes *CLN1, CLN2, PCL1,* and *PCL2* because, of these, only *PCL1* and *PCL2* high-copy suppress the temperature-sensitive phenotype of *mpk1Δ* strains (MADDEN *et al.* 1997). *PCL1* and *PCL2* high-copy suppress an *mpk1Δ* mutation by increasing Cdc42 activity. *CLN1* and *CLN2* are inferred to be positive regulators of *PKC1* function because gain-of-function alleles of *PKC1* suppress the temperature-sensitive phenotypes of *swi4Δ* and *mpk1Δ* strains (foreshadowed by GRAY *et al.* 1997) and also suppress the synthetic temperature sensitivity of a *cln1Δ cln2Δ pho85-9* strain, suggesting that *PKC1* functions downstream of each of these genes. As *PKC1* is an activator of the *MPK1* MAP kinase pathway at the level of either *BCK1* or *MKK1 MKK2*

FIGURE 11. A model to explain the functional relationships between Pcl1- and Pcl2-associated Pho85 kinase activities and some of the Efr mutants identified in our screen. This model hypothesizes that the underlying cause of many of the synthetic-lethal interactions is a defect in *CDC42* activity.

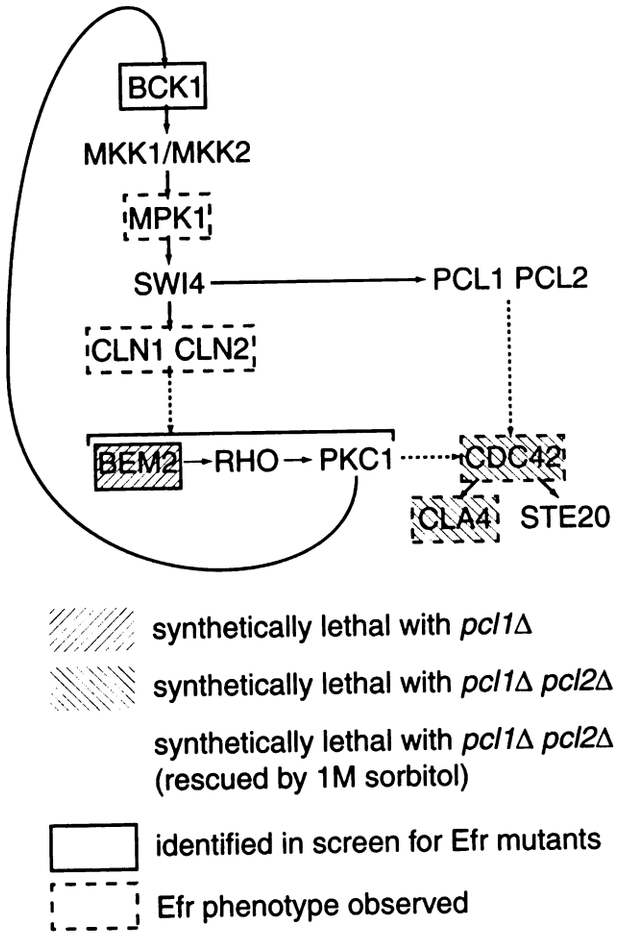

BCK1
MKK1/MKK2
MPK1
SWI4
PCL1 PCL2
CLN1 CLN2
BEM2
RHO
PKC1
CDC42
CLA4
STE20
synthetically lethal with pcl1Δ
synthetically lethal with pcl1Δ pcl2Δ
synthetically lethal with pcl1Δ pcl2Δ
(rescued by 1M sorbitol)
identified in screen for Efr mutants
Efr phenotype observed

Table 5.

Summary of Synthetic Lethal Interactions

Synthetic lethal with *pho85Δ*	Recovered in Efr Screen	Synthetic ts phenotype with *pho85-9*	ts phenotype suppressed by 2μ *PCL1*	ts phenotype suppressed by 2 μ *PHO80*	ts phenotype suppressed by other 2 μ *PCL* plasmids	Synthetic lethal phenotype with *pcl1Δ pcl2Δ pcl9Δ*	Synthetic lethal phenotype with *pcl1Δ pcl2Δ*	Synthetic lethal phenotype with *pcl1Δ*	Synthetic lethal phenotype with *pcl2Δ pcl9Δ*	Synthetic lethal with *cla4Δ*	Other Results
cln1Δ cln2Δ	no	yes	yes	no	no		yes[a]	no		yes[b]	• *cln1Δ cln2Δ* *pho85-9* ts phenotype suppressed by *PKC-R398P*
bck1	yes	yes	yes	no	no	yes[a]	yes[a]	no	no		• Reduced mating to *far1-c*
mpk1Δ	no	yes	no	no	no	yes[a]	yes[a]	no	no	yes	• ts phenotype of *mpk1Δ* mutant suppressed by *PKC-R398P*.

bem2	yes	yes	yes	no	no	yes	yes	yes	no	yes	• Reduced mating to *far1-c*
cdc42-1	no						yes	no			• Mutant phenotype partially suppressed by *GPD1::PCL1* and exacerbated by *GPD1::PHO80*
cla4Δ	no	yes					yes	no			
efr3Δ	yes	yes	no	yes	no	no	no	no	no		• No growth defect of *efr3Δ pho80Δ* strain
spt7	yes	yes	no	no	no	no	no	no	no		
gcr1	yes	yes	no	no	no	no	no	no	no		
srb5	yes	yes	no	no	no	no	no	no	no		
hfi1	yes	yes	no	no	no	no	no	no	no		

[a] Synthetic lethal phenotype with pcl1D pcl2D is rescued by growth in 1M Sorbitol

[b] CVRCKOVA *et al.* 1995

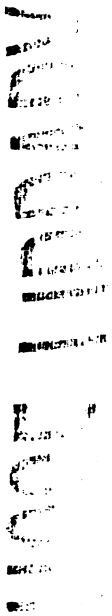
(HUANG and SYMINGTON 1995), overexpression of *CLN1* and *CLN2* fails to suppress the *mpk1Δ* phenotype because, in the *mpk1Δ* mutant, increased Pkc1 function is unable to increase the Swi4-dependent transcription of *PCL1* and *PCL2* necessary for full activation of Cdc42.

The hypothesis that Pcl1 and Pcl2 are positive regulators of Cdc42 is strengthened by the observation that *cdc42-1 pcl1Δ pcl2Δ* strains are inviable, and perhaps even more strongly by the ability of Pcl1 overexpression to partially suppress the temperature-sensitive lethality of *cdc42-1* strains. The ability of Pcl1 overexpression to partially suppress the temperature-sensitive growth phenotype of *cdc42-1* strains suggests that Pcl1 is able to promote a process which either substitutes for Cdc42 or acts to increase Cdc42 function.

As a variety of synthetic lethal interactions between mutants with defects in morphogenesis and cell wall biogenesis are suppressed by osmotic stabilzation with sorbitol (eg. *rho3Δ rho4Δ* [Matsui and Toh-e, 1992], *mid2Δ slg1Δ* [Ketela et al., 1999] and *chs5Δ spa2Δ* [Santos and Snyder, 2000]), the suppression of the *cln1Δ cln2Δ pcl1Δ pcl2Δ* G1 arrest by sorbitol suggests that these strains do not have an absolute defect in G1 progression but rather have a defect in a morphogenesis or cell-wall biogenesis related function that results in a G1 arrest. The suppression of the *cln1Δ cln2Δ pcl1Δ pcl2Δ* G1 arrest by sorbitol is distinct from the non-sorbitol suppressible inviability of *cln1Δ cln2Δ cln3Δ* strains and the unbudded G2-phase terminal phenotype of *cln1Δ cln2Δ bud2Δ* strains (CVRCKOVA and NASMYTH 1993). Perhaps the morphogenesis defect

in *pcl1Δ pcl2Δ cln1Δ cln2Δ* strains occurs upstream of a checkpoint that inhibits passage through START. Such a checkpoint might operate through stabilization of Sic1, an S-phase inhibitor, as *pho85Δ sic1Δ* cells are temperature sensitive for growth (AERNE *et al.* 1998), and Sic1 is more stable in *pho85Δ* strains (NISHIZAWA *et al.* 1998). The increased stability of Sic1 suggests that entry into S phase is delayed in the *pho85Δ* mutant and that removing this delay has deleterious consequences. Consistent with the hypothesis that *pho85Δ* strains have a defect that activates a Sic1-dependent checkpoint, whereas the synthetic lethality of *cln1Δ cln2Δ cln3Δ* is suppressed by deletion of *SIC1* (SCHNEIDER *et al.* 1996), the lethality of *pcl1Δ pcl2Δ cln1Δ cln2Δ* is not suppressed by deletion of *SIC1* (M. TYERS and B. ANDREWS, personal communication).

In contrast to mutants with defects in morphogenesis and cell-wall biogenesis, *pho85Δ* strains mate efficiently. Furthermore, I have been unable to detect any defects in morphogenesis-related processes such as actin polarization or cell-wall deposition in either *pcl1Δ pcl2Δ* or *pho85Δ* mutants (data not shown). Perhaps Pcl1- and Pcl2-associated Pho85 kinases regulate morphogenetic events specifically during a specialized G1. Pho85 might, for example, play a more important role in promoting morphogenesis during pseudohyphal growth, as pseudohyphal differentation involves morphogenetic changes that are regulated by both nutrient availability and the cell cycle. However, homozygous *pho85Δ* mutants in are able to undergo the transition to the pseudohyphal growth pattern when starved for nitrogen (data not shown).



These data help to clarify the cause of inviability in *cln1 cln2 pcl1 pcl2* strains, as they suggest that *PCL1* and *PCL2* promote Cdc42 activity or promote a process which can substitute for Cdc42 rather than participating in the START step of G1 itself. I report a *pcl1Δ* phenotype that does not also depend on removing *PCL2*. It will be interesting to determine if the inviability of *bem2Δ pcl1Δ* and *mpk1Δ pcl1Δ pcl2Δ* strains reflects the ability of Pcl1 and Pcl2 to phosphorylate different substrates or if instead it reflects differential requirements for phosphorylation of a common substrate. The challenge of understanding how cyclin binding confers functional specificity on Pho85 requires the identification the *in vivo* substrates of these different Pho85-containing kinases.

CHAPTER 5

Perspectives and Future Directions

The goal of understanding the different functions of Pho85 presents two rather unusual challenges. The first is to determine the process or processes in which a particular protein, in this case Pho85, fuctions. As more genes with interesting homology to known genes are uncovered in genome sequencing efforts, this is likely to become a common situation. I approached the challenge of understanding the various functions of Pho85 with a genetic strategy to uncover phenotypes associated with the loss of *PHO85*, by looking for mutations that are inviable in the absence of *PHO85*. These phenotypes have provided clues to some of the specific processes in which different Pho85-containing kinases participate. The approach of understanding the functions of Pho85 through the identification of *PHO85*-requiring mutants has a variety of limitations. One is that the mutations with which *pho85Δ* is synthetically lethal can be in genes whose functions are not known. Such is the case with *EFR3*. While I have been able to determine that *efr3Δ pho85Δ* strains are inviable in part because of Pho85's function in repressing the expression of Pho4- and Pho2-dependent genes (see Appendix A), this function was not suggested simply by the identification of *EFR3*, but rather required the identification of mutations which suppress the inviability of *efr3Δ pho85Δ* strains.

Perhaps a more serious limitation is that the synthetic-lethal phenotype is much more abstract than the more common kinds of phenotypes that are often looked for in genetic screens. Although the interpretation of synthetic lethal interactions can be challenging, such interactions provide a powerful tool for further explorations that can

lead to the identification of downstream components. For example, *pho4Δ* suppresses the inviability of an *efr3Δ pho85Δ*, strain and Pho4 is a Pho85 substrate. Similarily, *sic1Δ* suppresses the synthetic lethality of a *cln1Δ cln2Δ cln3Δ* strain (Schneider et al., 1996), and Sic1 needs to be targeted for degradation by Cln1- and Cln2-mediated phosphorylation for entry into S phase (Verma et al., 1997; Verma et al., 1997).

It is worth considering how the strategy of uncovering *PHO85* functions through the identification of Efr mutants compares with other approaches that are being used to identify Pho85 functions. In addition to the Pho4-repression function, two other functions of *PHO85* have been revealed in undirected screens for mutants with a particular phenotype. The role of *PHO85* in repressing glycogen synthesis was identified as a result of identifying *pho85* alleles in a screen for mutants with constitutive glycogen accumulation (Huang et al., 1996). The notion that *PHO85* participates in a G1-regulated process is a consequence of identifying high-copy *PCL1*-containing plasmids in a screen for suppressors of a temperature-sensitive-lethal allele of the G1 transcription factor encoded by *SWI4* (Ogas et al., 1991).

The other main strategy for identifying *PHO85* function has been to look for proteins that interact with Pho85 or the Pho85-associated cyclins by two-hybrid analysis. The identification of Rvs167 as a Pcl9-interacting protein is the foundation of the hypothesis that Rvs167 is a substrate of Pho85-containing kinases. However, the *in vivo* evidence to support the hypothesis that Rvs167 is a substrate of Pho85-containing kinases is not compelling. One limitation of identifying functions of Pho85 by looking for

Pho85-interacting proteins is that kinases and their substrates may not have high-affinity interactions. It may therefore be difficult to detect physiologically relevant kinase substrates on the basis of their ability to bind to the kinase in the two-hybrid assay or by biochemical techniques. For example, the interaction between Pcl8 and the glycogen synthase encoded by GSY2 as detected by two-hybrid analysis is barely above background (Wilson et al., 1999).

Our lab is currently taking an approach that circumvents the interaction issue by identifying proteins *in vitro* that can be phosphorylated specifically by one Pcl-containing kinase and not by others. It remains to be determined if the *in vitro* specificity of the different Pcl-containing kinases will correspond with the functional distinctions of these different kinases *in vivo*. For example, although Rvs167 interacts specifically with Pcl9 in the two-hybrid assay, it appears that a number of other Pcl-associated kinases can phosphorylate Rvs167 *in vivo* (Lee et al., 1998). Genetic interactions between *efr* mutants with defined Pho85-cyclin requirements and the genes that encode the substrates identified in the *in vitro* phosphorylation assays using those cyclin-containing kinase may prove to be a useful tool for determining the *in vivo* relevance of the phosphorylation.

For such a strategy to be generally applicable to all of the functions of the various Pho85-containing kinases, it would be necessary to obtain a larger collection of Efr mutants that contained members that are synthetically lethal with *pho85Δ* as a result of defects in each of the processes in which *PHO85* participates. As the present strategy for identifying Efr mutants had a similar goal and failed to identify every Efr locus, it is

important to consider ways to make any additional screens for such mutants more efficient. One problem with the current screen involves the use of the galactose-regulated allele of *PHO85*, as the primary screen would only identify *efr* mutants that are able to grow in galactose-containing media. Since galactose is a poorly utilized carbon source for *S. cerevisiae*, and *pho85Δ* mutants themselves grow poorly in galactose, there may be *efr* mutants that are unable to grow in galactose. Another pitfall of the galactose-regulated allele of *PHO85* is that in the presence of galactose, Pho85 is induced to very high levels. I found that it took between eight and twenty-four hours for a strain carrying the *gal1-10::pho85* allele to become Pho85$^-$ after being transfered from galactose-containing media to dextrose-containing media (data not shown). A powerful way to circumvent the long lag before *gal1-10::pho85* strains become Pho85$^-$, but still regulate *PHO85* function is to use an allele of *PHO85* generated by Adam Carroll in our lab which replaces the phenylalanine at aminoacid position 82 in the ATP-binding pocket with glycine. The *pho85-F82G* allele of *PHO85* is specifically and rapidly inhibited by the synthetic compound 1-Na-PP1. Isolation of mutants in a *pho85-F82G* background that cannot grow in the presence of 1-Na-PP1 could be a more powerful screen for *efr* mutants than the screens that I performed.

Before any future screen for additional Efr mutants is performed, the mutagen should be considered carefully. Although the use of the transposon library aids in the molecular identification of the mutated locus, the transposon library may lack fragments with insertions into a large proportion of yeast genes. Furthermore, the apparent inviability of *pho85Δ* mutants in the presence of episomal transposon-containing DNA

fragments makes using the transposon library for generating Efr mutants especially problematic. One strategy for circumventing the episomal DNA-induced lethality of *pho85Δ* strains while maintaining the ability to identify the mutated locus rapidly would be to perform a screen for Efr mutants using a library of yeast strains that are deleted for each non-essential gene. A useful feature of this approach of massive directed tests for synthetic lethality is that it could be done to completion much more readily than any screen could be performed to saturation. The central challenge of such an approach would be finding a way to inactivate *PHO85* in each of the deletion strains. One approach would be to introduce plasmid-borne dominant-negative alleles of *PHO85* under the control of an inducible promoter.

The second challenge for understanding the functions provided by the Pho85-containing kinases is that the functions of Pho85 are carried out by a variety of Pho85 activities formed by the association of Pho85 with different cyclin subunits. The goal of understanding the functions of Pho85 really involves understanding the combined functions of each of these different kinases. The challenge of understanding the functions of each of the different Pho85-containing kinase is particularly daunting when using a strategy which begins with the identification of different Pho85 functions and then requires the assignment of these functions to different Pho85-containing kinases. The strategy I used for assigning Pho85 functions to particular Pcl-containing kinases has only been partially successful, and any attempts to use *PHO85*-requiring mutants to understand additional functions of Pho85 will need to find ways to make the process of assigning Pho85 functions to particular Pho85-containing kinases more efficient.

My strategy for identifying the cyclins that associate with Pho85 to mediate different functions involved determining if high-copy plasmids containing any of the genes which encode Pho85-associated cyclins could suppress a temperature-sensitive allele of *PHO85, pho85-9*. In its present form, the *pho85-9* suppression strategy was only partially successful. The temperature-sensitive lethality of of *bem2Δ pho85-9* mutants is suppressed by high-copy plasmids containing *PCL1*. The temperature sensitivity of *bck1 pho85-9* is also suppressed only by high-copy plasmids containing *PCL1*, but unlike the *bem2Δ pcl1Δ* mutant, the *bck1 pcl1Δ* mutant is viable. Since a *cln1Δ cln2Δ pho85-9* mutant is also only suppressed by high-copy plasmids containing *PCL1,* and a *cln1Δ cln2Δ pcl1Δ pcl2Δ* mutant is inviable, it was straightforward to determine that like *cln1Δ cln2Δ* mutants, *bck1* mutants are synthetically lethal with *pcl1Δ pcl2Δ*. Similar predictions about combinations of cyclin mutations that would result in the inviability of other *efr* mutants was not possible where there were no precedents for such synthetic-lethal interactions, as was the case with *cln1Δ cln2Δ*. For example, the temperature-sensitive lethality of an *efr3Δ pho85-9* mutant is suppressed only by high-copy plasmids containing *PHO80*, and *efr3Δ pho80Δ* mutants are viable. There is no obvious choice of which *pcl*-deletion strains to test for synthetic-lethal interactions with *efr3Δ*. The remainder of the *efr pho85-9* mutants are not suppressed by any of the high-copy plasmids containing the different *PCL* genes. These data indicate that in most cases only a subset of the relevant Pho85-associated cyclins can suppress the different temperature-sensitive defects of the *pho85-9* strain.

One possible explanation of the failure of relevant cyclins to suppress the *pho85-9* defects is that the increase in activity that results from the cyclin genes being on high-copy plasmids is not great enough to overcome some of the *pho85-9* temperature-sensitive defects. The lack of cyclin activity resulting from high-copy plasmids might be solved by expressing the cyclins at high levels by replacing the different cyclin-promoters with a promoter from a regulated and highly induced gene. As *cdc42-1* mutants are killed by overexpression of *PHO80* from the *GPD1* promoter, it is possible that high-level expression of cyclins that are not relevant to a required *PHO85* function could generally decrease the fitness of different *efr* mutants. The sickness of strains overexpressing different Pho85-associated cyclins could be even more dramatic in a *pho85-9* mutant. Therefore, the phenotypes of *efr pho85-9* strains harboring highly-expressed Pho85-associated cyclin plasmids could be informative both for those cyclins that suppress the temperature-sensitive lethality and those that lower the permissive temperature for growth.

Another potential problem with utilizing the *pho85-9* allele as the cornerstone of the strategy for determining the cyclins that mediate the different functions of Pho85 is that some of the transposon-induced *efr* mutations displayed growth defects at room temperature when combined with the *pho85-9* allele, compared to WT *PHO85*. The growth defects exhibited by some of the *efr pho85-9* strains were very pronounced. The sickness of some *efr pho85-9* strains raises the possibility that there may be *efr* mutants that are inviable in combination with the *pho85-9* allele. Other strategies for identifying

which Pho85-associated cyclins contribute to the Pho85 functions required by the different *efr* mutants may be useful.

One strategy for identifying the Pho85-associated cyclins required for viability in *efr* mutant backgrounds would be to construct a strain which lacked the genes encoding all ten of the Pho85-associated cyclins and cross it to the various *efr* mutants. These deletion alleles could be created with unique molecular tags to identify which mutations were harbored by the progeny that result from such a cross. The advantage of the "ten-cyclin-delete" approach is its ability to be done to completion and its applicability to any *efr* mutant. The disadvantage of the cyclin-deletion approach is the considerable labor required to generate a strain lacking the genes for all ten Pho85-associated cyclins and the labor required to genotype the progeny from the cross.

Other strategies for obtaining information about the cyclins required for the various functions of the different Pho85-containing kinases is to perform screens for mutations that are specifically inviable in the absence of certain cyclins or certain combinations of cyclins. To follow such a strategy, one would have to decide which combinations of cyclin mutations to use. With our current level of understanding about the functional relationships among the different Pho85-associated cyclins, choosing which cyclin mutations to combine is still a daunting task. One possibility would be to use single-cyclin mutants and hope that removing a single cyclin can result in inviability in different *efr* backgrounds, as removing *PCL1* causes inviability in a *bem2Δ* mutant background.

If it is possible to obtain more Efr mutants that can be sorted as to their requirements for specific Pho85-associated cyclins, they could serve as valuable tools for determining the *in vivo* relevance of Pho85's phosphorylation of substrates identified through *in vitro* biochemical analysis and also serve as useful reagents for less directed genetic approaches to uncovering new cyclin-specific Pho85 substrates. The identification of substrates of the different Pho85-containing kinases and understanding the physiological consequences of their phosphorylation will help us to understand the spectrum of functions performed by Pho85, as well as any consistency among these different functions. The similarities and differences between the functions performed by the different Pho85-associated kinases will allow us to eventually understand the sources of both functional differences and commonality among different the cyclin-CDK complexes that share a common catalytic subunit. A theme from the study of the development of multicellular organisms is that the evolution of new traits is often the result of subtle changes in regulatory molecules. Because of the regulatory nature of CDKs, a deep understanding of CDK functions may not only provide a richer understanding of how cells regulate their responses to an extra- and intra-cellular environment that changes on a timescale of seconds to hours, but also how organisms respond to evolutionary pressures which occur on a timescale of millenia.

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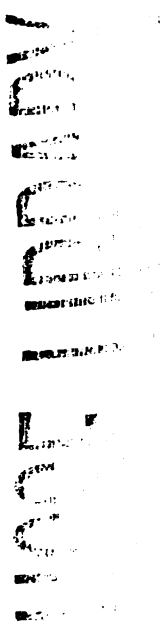

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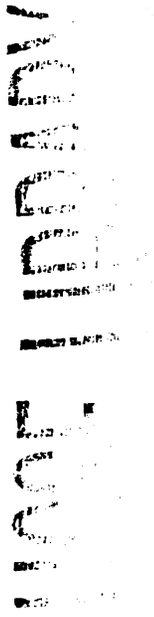

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Appendix A

Analysis of the inviability of *efr3Δ pho85Δ* strains

Understanding the underlying cause of the inviability of *pho85 efr3* strains is interesting because the temperature-sensitive lethality of *pho85-9 efr3Δ* strains is suppressed by overexpressing *PHO80*, and *PHO80* is involved in repressing the expression of phosphate-starvation specific genes when phosphate is abundant. The inviability of *pho85 efr3* strains could therefore be caused by the constitutive expression of phosphate starvation-specific genes, by a defect in an additional function of the Pho80-Pho85 kinase, or by a defect in a function of a Pho80-related Pho85-containing kinase. I learned a number of things about the inviability of *efr3Δ pho85Δ* strains and about *EFR3* that may be useful to others interested in pursuing the functions of *EFR3* or the functions of *PHO85* that are required for viability when *EFR3* is absent.

Despite the wild-type growth rate of *efr3Δ* strains in either SD or YEPD media, I found that *efr3Δ* strains grow much more poorly than WT strains in S- or YEP-glycerol or S- or YEP-ethanol medium. I also found that homozygous *efr3Δ* diploids are unable to sporulate. The sporulation defect of *efr3Δ* strains might be a consequence of the inability of *efr3Δ* strains to grow on non-fermentable carbon sources as sporulation is triggered by transfering yeast to media with low nitrogen and a non-fermentable carbon source. However, other mutants that are completely unable to grow on non-fermentable carbon sources, such as pho85Δ mutants, are able to sporulate. Therefore, the sporulation defect

of *efr3Δ* strains might be unrelated to the non-fermentable carbon source growth defect. My finding that *pho85Δ* strains sporulate efficiently is at odds with reports that other pho85Δ strains are defective for sporulation (Tennyson et al., 1998). It may be interesting to determine if this difference is the result of a strain background difference or different sporulation conditions.

If *efr3Δ* mutants are inviable in the presence of the constitutive expression of phosphate-starvation specific genes or if there are additional defects that result from removing the Pho80-mediated functions of Pho85 that are required for growth in an *efr3Δ* mutant then an *efr3Δ pho80Δ* mutant would similarily be inviable and *efr3Δ* mutants would not be able to grow in low-phosphate medium as this results in the inhibition of the Pho80-Pho85 kinase. Rather, *efr3Δ* mutants grow as well as WT in low phosphate medium or in the absence of *PHO80*. Both of these observations indicate that the constitutive expression of phosphate-starvation specific genes cannot be the only defect of *pho85Δ* strains that results in the inviability of *efr3Δ pho85Δ* strains. These observations further indicate that there is at least one other cyclin beyond Pho80 which is able to perform the function of Pho85 required for viability in an *efr3Δ* strain. The ability of *efr3Δ* strains to grow in low phosphate medium indicates that this other Pho85-associated kinase is unlikely to be repressed by phosphate availability.

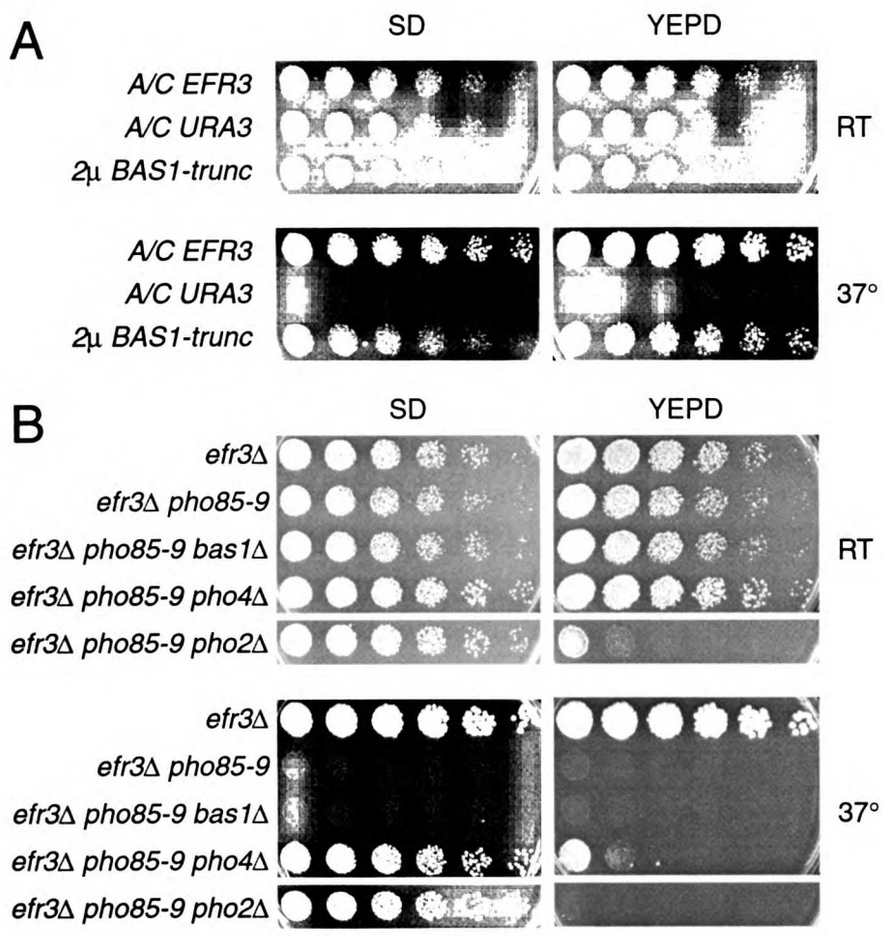
To understand the cause of inviability in *efr3 pho85* strains I performed a selection for high-copy plasmids that suppress the temperature-sensitive lethality of an *efr3Δ pho85-9* strain. To do this selection I used a YEp24-based genomic library

(Carlson et al., 1984). From this selection I recovered multiple plasmids with inserts containing PHO85, *EFR3*, *PHO80* as well as a plasmid containing the N-terminal regions of the *BAS1* and *UBP11* genes. What was interesting about this plasmid is that I recovered plasmids containing the identical insert at least a dozen times, while I expected that the library should contain plasmids with overlapping inserts that contained these genes. This led me to consider the possiblity that the truncation of the ORFs contained on this plasmid was required for their ability to suppress the temperature-sensitive lethality of an *efr3Δ pho85-9* strain.

I found that a plasmid containing the truncated *BAS1* allele but lacking the *UBP11* allele was also able to suppress the temperature-sensitive lethality of an *efr3Δ pho85-9* strain, indicating that the *BAS1* fragment is responsible for the suppression I observed with the original plasmid (Figure 12A). This genetic interaction between *BAS1*, *EFR3* and *PHO85* is particularly intriguing because *BAS1* encodes a transcription factor that in association with Pho2 is responsible for activating transcription of a number of genes required for the synthesis of adenine and histidine, and the expression of these genes is induced in response to phosphate starvation (Daignan-Fornier and Fink, 1992).

Since the activity of the Pho2-binding partner Pho4 is repressed by a Pho85-containing kinase, Bas1 might also be phosphorylated and inactivated by a kinase that contains Pho85. The hypothesis that Bas1 is regulated by Pho85 in a manner analogous to the regulation of Pho4 by Pho80-Pho85 suggests that in a *pho85* strain, Bas1 would be constitutively active, and that an *efr3Δ* strain might be inviable in the presence of

FIGURE 12. – Suppression of *efr3Δ pho85-9* temperature-sensitive inviability by genetic manipulation of Pho2-associated transcription factors. A. Suppression of *efr3Δ pho85-9* temperature-sensitive inviability by overexpression of *BAS1*. Strain MY0176 transformed with low copy plasmids containing *EFR3* (MP0019), *URA3* (pRS316), or a high-copy plasmid containing a truncation allele of *BAS1* (MP0086) were diluted from an OD_{600}=0.3 by three-fold serial dilutions and plated on different plates as indicated, and then incubated at the indicated temperature for two days. B. Suppression of *efr3Δ pho85-9* temperature-sensitive inviability by deletion of *PHO2* or *PHO4* but not *BAS1*. Strains of the indicated genotype were diluted from an OD_{600}=0.3 by three-fold serial dilutions and plated on different plates as indicated, and then incubated at the indicated temperature for two days.


A
SD
YEPD
A/C EFR3
A/C URA3
2μ BAS1-trunc
RT
A/C EFR3
A/C URA3
2μ BAS1-trunc
37°
B
SD
YEPD
efr3Δ
efr3Δ pho85-9
efr3Δ pho85-9 bas1Δ
efr3Δ pho85-9 pho4Δ
efr3Δ pho85-9 pho2Δ
RT
efr3Δ
efr3Δ pho85-9
efr3Δ pho85-9 bas1Δ
efr3Δ pho85-9 pho4Δ
efr3Δ pho85-9 pho2Δ
37°

constitutive Bas1 activity. The hypothesis further predicts that the truncated *BAS1* allele is a dominant negative allele and that deletion of *BAS1* would also suppress the temperature-sensitive lethality of an *efr3Δ pho85-9* strain. An alternative hypothesis is that Bas1 might be positively regulated by a Pho85-containing kinase, the suppressing *BAS1* allele is a gain-of-function allele, and that *efr3Δ* strains are inviable in the presence of reduced Bas1 activity. If this hypothesis is true, a deletion of *BAS1* should be synthetically lethal with *EFR3*.

The truncated allele of *BAS1* rescues the histidine auxotrophy of a *gcn4Δ bas1Δ* strain as well as wild-type *BAS1*, suggesting that the Bas1-truncation allele does not have a dominant-negative phenotype. Consistent with this finding, removing *BAS1* does not raise the permissive temperature of an *efr3Δ pho85-9* strain. *bas1Δ efr3Δ* strains grow as well as either parental strain (Figure 12B). These data indicate that neither of the simple hypotheses to explain the suppression of the temperature-sensitive lethality of an *efr3Δ pho85-9* strain by high-copy plasmids containing the truncated-*BAS1* are correct.

However, removing the Bas1-binding partnter encoded by *PHO2* suppresses the the temperature-sensitive lethality of an *efr3Δ pho85-9* strain (Figure 12B). This suggests that the temperature-sensitive lethality of *efr3Δ pho85-9* strains is the result of the expression of Pho2-dependent but not Bas1-dependent genes. The suppression of the temperature-sensitive lethality of an *efr3Δ pho85-9* strain by high-copy *BAS1*-containing plasmids might result from increased levels of Bas1 titrating Pho2 away from another Pho2-binding partner. The analysis of interactions between *bas1Δ*, *pho2Δ*, *efr3Δ* and

pho85-9 was performed on synthetic-complete media due to the increased adenine and histidine requirements of strains lacking *BAS1* or *PHO2*. As a result *bas1Δ* and *pho2Δ* strains grow more rapidly on synthetic-complete media than YEP-based media.

If the expression of Pho2-dependent but not Bas1-dependent genes is responsible for the inviability of *efr3Δ pho85Δ* strains, removing the functions of a Pho2-binding partner other than that encoded by *BAS1* would also suppress the temperature-sensitive lethality of *efr3Δ pho85-9* strains. I crossed strains which lacked the Pho2-binding partners encoded by *PHO4* and *SWI5* to an *efr3Δ pho85-9* strain. Since neither *pho4Δ* or *swi5Δ* strains have a growth defect on YEPD, I examined the growth of *efr3Δ pho85-9 pho4Δ* and *efr3Δ pho85-9 swi5Δ* strains at elevated temperatures on YEPD and found that these strains grow similarily to an *efr3Δ pho85-9* strain.

To see if another Pho2-bidning partner might suppress the temperature-sensitive lethality of an *efr3Δ pho85-9* strain, I performed a selection for mutations that suppress the synthetic-temperature sensitivity of an *efr3-1 pho85-9* strain using the transposon-insertion library as a mutagen. A UV-induced *efr3* allele was used for the suppressor selection because of an inability to make deletion alleles of *EFR3* marked with anything other than the *LEU2* gene which is also contained in the transposon. I provisionally named these *efr3-1 pho85-9* suppressors TSL mutants for EFR-**T**hree **S**uppressors of **L**ethality.

I isolated several hundred *tsl* mutants which allowed an *efr3-1 pho85-9* strain to grow at 37°C from a pool of approximately 2 x 10^5 transposon mutagenized colonies. I determined the genomic positions of sixty different transposon insertions. Most of the transposon insertions ocurred at loci that were only recovered once, two were insertions into the *PHO4* locus.

An interesting property of the transposon-insertion *pho4* alleles I recovered is that they suppress the temperature-sensitive lethality of *efr3-1 pho85-9* strains on synthetic media but do not suppress on YEP-based media. I next constructed an *efr3Δ pho85-9 pho4Δ* strain and found that unlike an *efr3Δ pho85-9* strain which is temperature sensitive for growth on both synthetic media and YEP-based media (Figure 12B), the *efr3Δ pho85-9 pho4Δ* strain is able to grow at 37°C on synthetic media but is temperature sensitive for growth on YEP-based media. These data suggest that expression of Pho4- and Pho2-dependent genes results in the inviability of *efr3 pho85* strains grown on synthetic media. Consistent with this hypothesis, I found that high-copy plasmids containing *BAS1* suppress the temperature-sensitive lethality of an *efr3Δ pho85-9* strain only when grown on synthetic media and not when grown on YEP-based media.

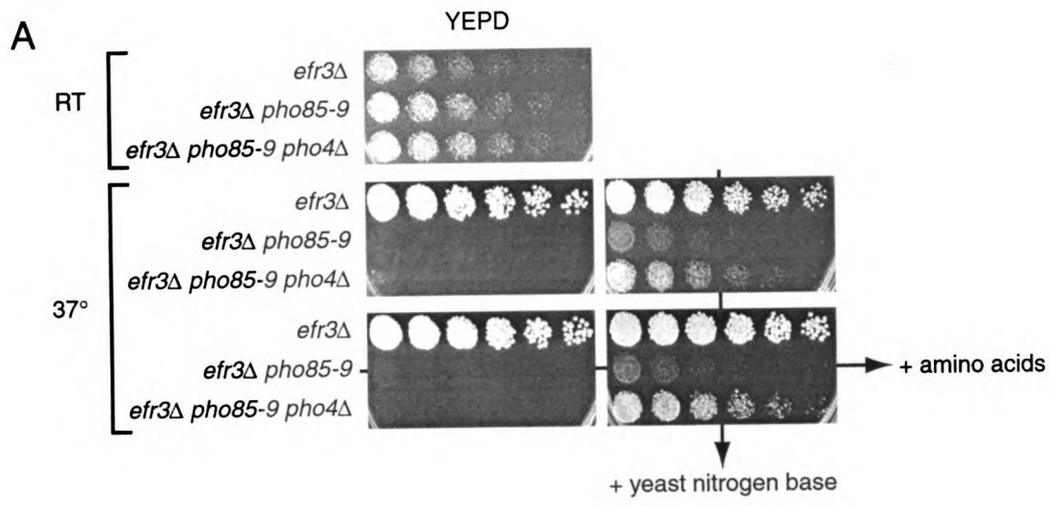
The growth rate of neither *efr3Δ* nor *pho85Δ* strains is effected by removing *PHO4* function. *efr3Δ* strains grow similarily on synthetic and YEP-based media when cultured at a variety of temperatures, as do *pho85Δ*, and *pho85-9* strains. These data indicate that there is a synthetic defect in *efr3 pho85-9* strains that is rescued by removing *PHO4*, and that removing *PHO4* is only able to rescue this defect when the strains are

grown on synthetic media. Media mixing experiments were next performed to determine if either YEP or synthetic media acts dominantly to effect the growth of *efr3Δ pho85-9 pho4Δ* strains, and found that at the non-permissive temperature, *efr3Δ pho85-9 pho4Δ* strain grow in media containing both YEP and the YNB components of synthetic media, but not in YEP and the amino acid components (Figure 13). indicates that SD is dominant to YEP and suggests that YEP might lack a

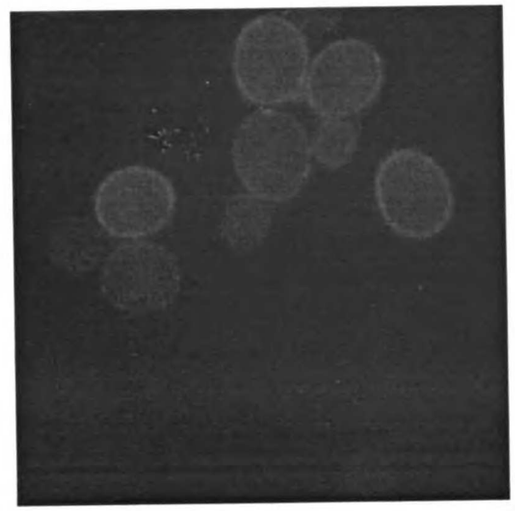
The ability of *efr3Δ pho95-9 pho4Δ* strains to grow in media containing both YEP and the YNB suggests that YNB contains a component that is necessary for the growth of an *efr3Δ pho85-9 pho4Δ* strain that is not present in sufficient quantities in YEP-based media. *efr3Δ pho85-9 pho4Δ* strains were then tested for their ability to grow at 37°C in different YEPD media that contained one of each of the individual components of synthetic media. Supplementing YEPD with the amount of potassium phosphate contained in synthetic media allowed an *efr3Δ pho85-9 pho4Δ* strain to grow at 37°C, while supplementing YEPD with an equimolar amount of potassium chloride had no effect. YEPD supplemented with potassium phosphate supports growth of an *efr3Δ pho85-9 pho4Δ* strain but not an *efr3Δ pho85-9* strain at 37°C. *efr3Δ pho85-9 pho4Δ* strains therefore require a level of inorganic phosphate higher than that found in YEPD in order to be able to grow at 37°C.

One model which explains these observations is that Pho85 contributes to two processes: one is the Pho80-dependent phosphate-specific repression of Pho4 activity, and the other is a Pho80-independent non-phosphate regulated function that might act

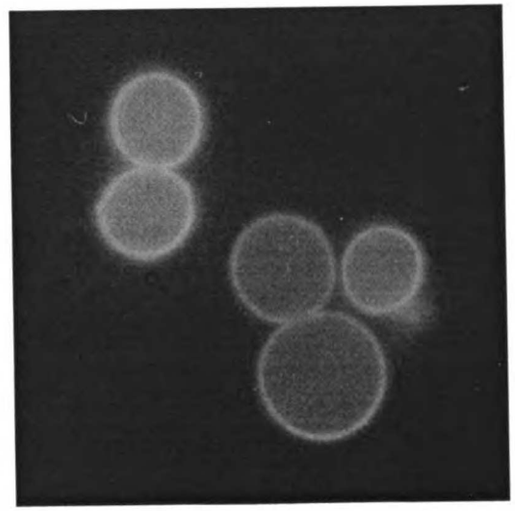
FIGURE 13. -- Yeast nitrogen base is required for suppression of *efr3Δ pho85-9* by *pho4Δ*. Strains of the indicated genotype were were diluted from an OD_{600}=0.3 by three-fold serial dilutions and plated on YEPD plates, or YEPD plates which contained YNB, amino acids, or both of these SD components and placed at the indicated temperature for two days.


A
YEPD
RT
efr3Δ
efr3Δ pho85-9
efr3Δ pho85-9 pho4Δ
37°
efr3Δ
efr3Δ pho85-9
efr3Δ pho85-9 pho4Δ
efr3Δ
efr3Δ pho85-9
efr3Δ pho85-9 pho4Δ
+ amino acids
+ yeast nitrogen base

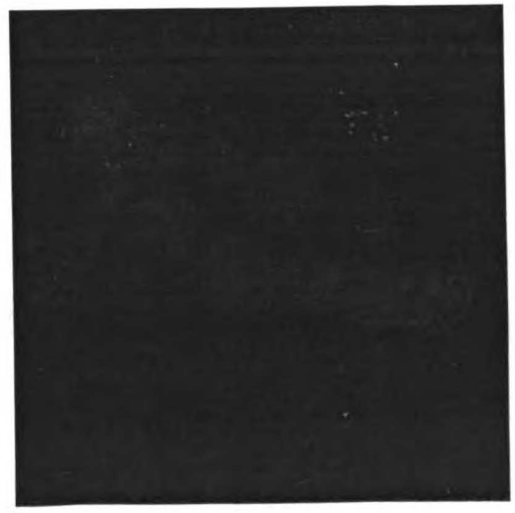
ARS/CEN EFR3::GFP



2μ EFR3::GFP



ARS/CEN EFR3



upon Pho4-dependent gene products. One example of this type of function is if Pho85 in association with a cyclin other than Pho80 was required for the maturation of some Pho4-dependent gene product such as the phosphate transporters. In this model, the removal of *PHO85* function results in two related defects: one is the constitutive expression of Pho4-dependent genes and the other is a defect in the function of these Pho4-dependent gene products. The physiological state caused by the removal of *PHO85* is therefore distinct from that caused by removal of *PHO80* or that caused by low phosphate because in these latter cases, the functions of the Pho4-dependent gene products are intact. This model predicts that it is specifically the presence of these non-functional Pho4-dependent gene products that causes an *efr3Δ* strain to be inviable. This model explains the suppression of the inviability of *efr3 pho85* strains by removing *PHO4* as resulting from a lack of non-functional Pho4-dependent gene products.

The model has a more difficult time explaining the increased phosphate dependence of an *efr3 pho85 pho4* strain. One possibility is that *pho85 pho4* strains have an increased dependence on inorganic phosphate as a result of the combined defect in the expression of Pho4-dependent genes caused by the removal of *PHO4*, and a defect in the function of these gene-products as a result of a defect in Pho85's independent effect on their activity. In order for this to be true, however, there would have to be non-Pho4 dependent expression of these Pho4-regulated genes. Consistent with this model, a *pho85Δ pho4Δ* strain grows more slowly than either a *pho85Δ* or *pho4Δ* strain (data not shown). Another possibile explanation for the increased phosphate dependence of an

[redacted]

efr3 pho85 pho4 strain is that along with Pho85, Efr3 might also play a role in promoting the function of Pho4-dependent genes.

This model predicts that mutations in components of the pathway that promotes the function of Pho4-dependent gene products would be inviable when both the expression of Pho4-dependent genes is induced and *EFR3* is absent. If such mutants exist, they could be in components that are either upstream or downstream of *PHO85* in this pathway, and might help identify the pathway to which *PHO85* contributes. One way to screen for such mutants would be to transform a *PHO80*-containing plasmid into a *pho80Δ gal1-10::efr3* strain and look for mutants that are unable to grow in the absence of the *PHO80* plasmid when grown in dextrose (when expression of *EFR3* is repressed), but are able to lose the plasmid when grown in galactose.

A perhaps less pointed screen would be to look for other Efr mutants that share the same secondary phenotypes as Efr3 -- namely suppression by *pho4Δ* on synthetic media. This screen could identify other components of the pathway in which Efr3 functions, and could therefore further elucidate the nature of the defect which results in the inviability of *pho85Δ* mutants. Such mutants could be found by mutagenizing a *gal1-10::pho85 pho4Δ* strain and looking for mutants that could grow on SD but not YEPD. This strains could then be checked for their ability to grow on both SG and YEPG to determine if the synthetic-media dependence phenotype of these strains is dependent on the loss of *PHO85* function.

Despite our incomplete understanding of the defect that results in the inviability of *efr3 pho85* strains, the discovery of this synthetic interaction among the collection of Efr mutants is encouraging for several reasons. The suppression of *efr3 pho85* strains by *pho4Δ* indicates that the screen was successful in identifying a synthetic lethal interaction which is at least in part the result of a known defect of *pho85Δ* mutants -- the expression of Pho4-dependent genes when phosphate is abundant. This suggests that understanding the defects that lead to the inviability of other *efr pho85* strains could lead to the discovery of *bona fide* functions of Pho85. It is also encouraging that it was possible to identify a substrate of Pho85 by looking for mutations which suppress the *efr3 pho85* synthetic lethality. This suggests that genetic analysis of other synthetic-lethal interactions could lead to the identification of other Pho85 targets in other Pho85-dependent pathways.

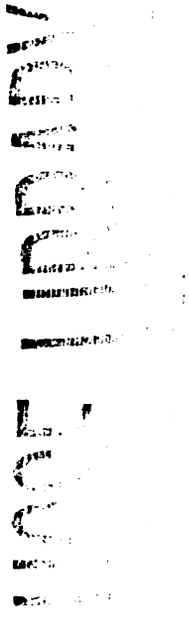
Appendix B

Biocemical characterization of Efr3

As *EFR3* is a novel gene with no significant homology to any previously studied gene, I conducted a preliminary characterization of the Efr3 protein in the hopes that information about Efr3 could serve to help constrain hypotheses about the functions of this gene.

To begin this characterization, I first sought to determine the subcellular localization and the expression pattern of Efr3. I constructed an allele of *EFR3* that contains the *EFR3* promoter sequence and an in-frame fusion between the 3' end of the *EFR3* coding sequence and a highly fluorescent mutant of the gene which encodes the green fluorescent protein (GFP) from the jellyfish *A. victoria*. Both high- and low-copy plasmids containing this allele of *EFR3* complement the inviability of an *efr3Δ pho85Δ* strain. WT, *efr3Δ* and *pho85Δ* strains harboring this plasmid exhibit a plasmid-dependent green fluorescence that localizes to the periphery of the cell (Figure 14). This pattern of GFP localization is in contrast to strains harboring a GFP plasmid that lacks the *EFR3* coding sequence -- in these strains, GFP appears to localize throughout the cell (data not shown). Strains harboring the high-copy plasmid exhibit a much brighter GFP signal indicating that expression of the *EFR3* promoter is not tightly regulated in vegetatively growing cells. The Efr3-dependent localization of GFP to the very periphery of the cell is apparent in cells at all stages of the cell-cycle, is not effected by the presence of

FIGURE 14. Localization of Efr3-GFP. Strain MY0114 transformed with *ARS/CEN EFR3::GFP*$^+$ (MP 0015), *2μ EFR3::GFP*$^+$ (MP0016), or *ARS/CEN EFR3* (MP0017) were grown in SD medium that lacked histidine to an OD_{600} = 0.5. GFP was visualized by fluorescence microscopy using a 100X objective. Photographs with identical exposure times were taken on Kodachrome 400 film and processed identically.



PHO85, and is not effected by phosphate availability (data not shown). The distribution of Efr3-GFP is different from Pho80-Pho85 as Pho80-GFP localizes to the nucleus.

I next purified the Efr3 protein from yeast to determine if it associated with any other proteins and to determine if anything about its mobility or interactions was affected by the presence of *PHO85*. I constructed an allele of *EFR3* which contained the *EFR3* promoter and coding sequence fused in frame at the 3' end to two tandem copies of an epitope recognized by the αPy monoclonal antibody. Either low- or high-copy plasmids containing this allele of EFR3 complement the inviability of an *efr3Δ pho85Δ* strain. Whole cell extracts from yeast strains harboring the *EFR3::Py²* allele have a single plasmid-dependent αPy cross-reactive band that migrates at approximately 92 kDa on SDS-PAGE. *EFR3* is predicted to encode a protein of 90 kDa, and its apparent moelcular weight of 92 kDa is equal to the mass of the predicted Efr3 protein and the mass of the Py^2 tag. Consistent with the observed difference in the fluorescent signal of cells harboring either the low- or high-copy *EFR3::GFP* plasmid, cells harboring the high-copy *EFR3::Py²* plasmid have a stronger αPy cross-reactive band. I purified the Efr3-Py^2 protein from whole-cell extracts obtained from *efr3Δ* strains harboring the *EFR3::Py²* allele by binding it to αPy mAb that was cross-linked to Protein G beads (Figure 15). I estimate that I was able to purify either 25 ng or 5 ng of Efr3-Py^2 from 3 mg of whole-cell extract using 30 μl of αPy cross-linked beads, depending on whether the yeast strain from which this extract was prepared harbored the high- or low-copy *EFR3::Py²* plasmid, respectively. Qualitatively all of the Efr3-Py^2 was purified from the whole-cell extract under these conditions.

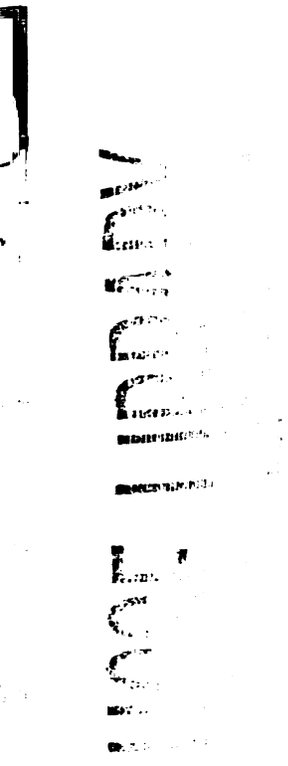
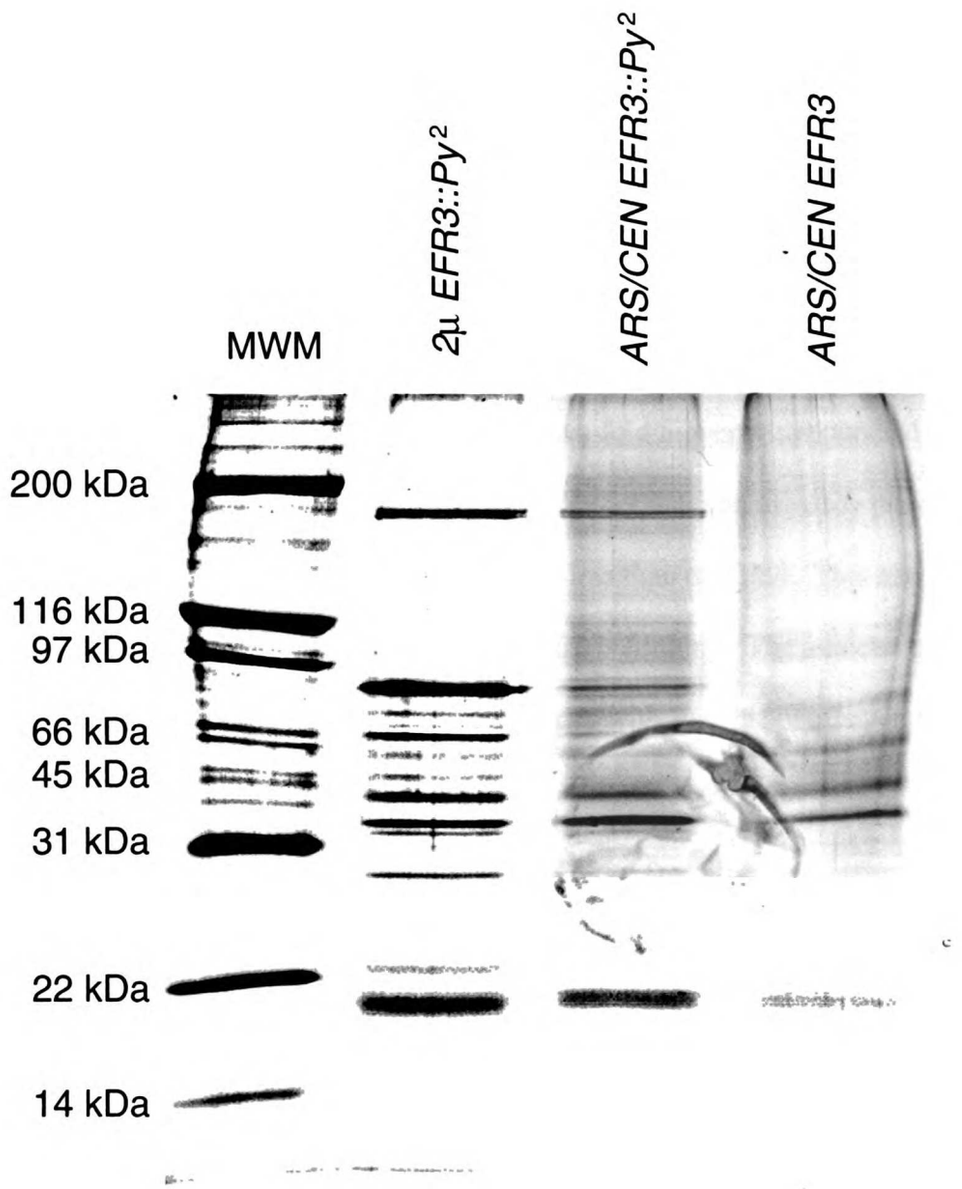
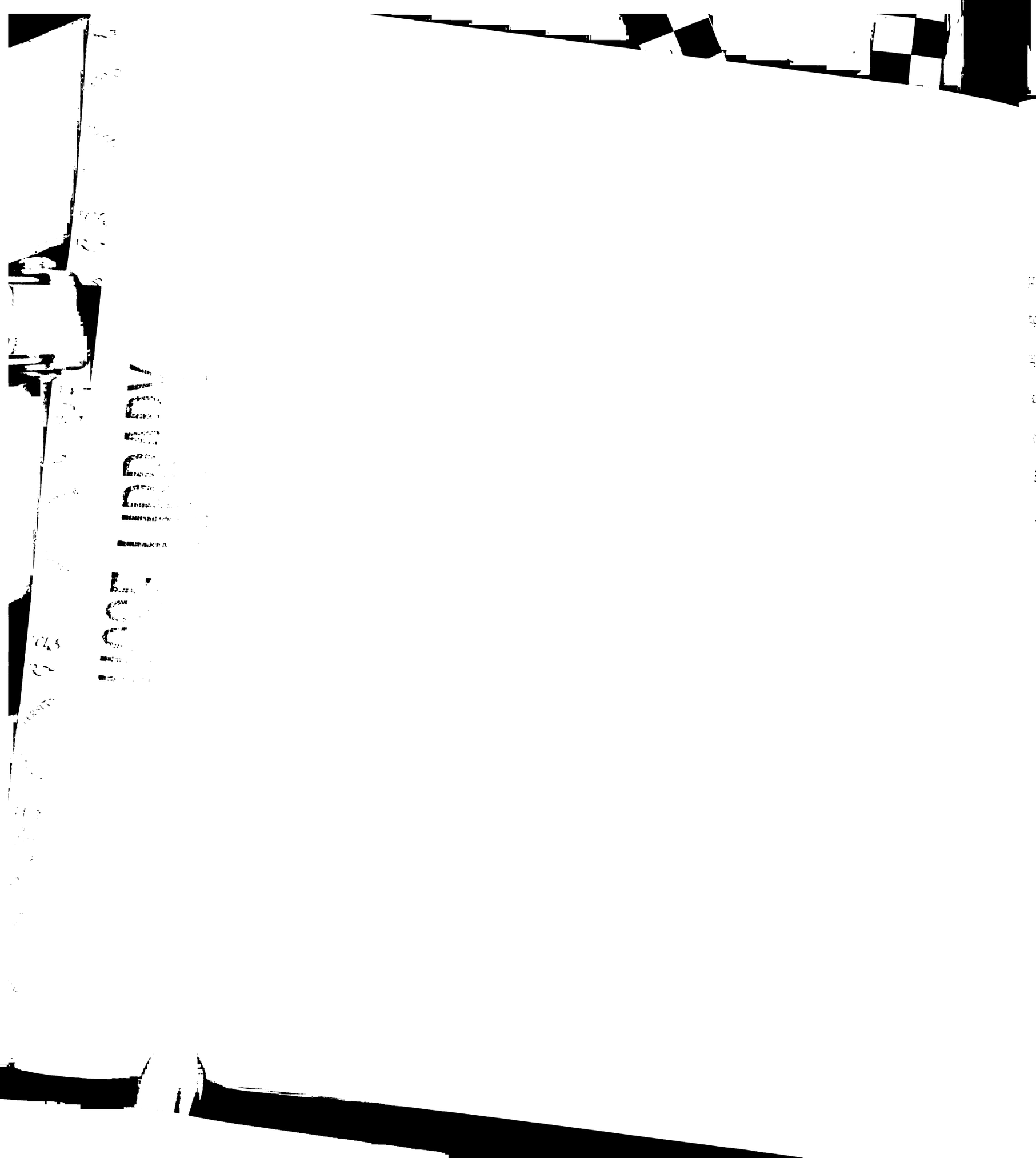


FIGURE 15. Immunoprecipitation of Efr3-Py^2. Strain MY0114 transformed with *ARS/CEN EFR3::Py*$^+$ (MP 0013), *2μ EFR3::Py*$^+$ (MP0014), or *ARS/CEN EFR3* (MP0017) was grown in SD medium that lacked histidine to an OD_{600} = 0.8 and whole-cell extracts were prepared and Efr3 immunopurified using the αPy antibody as described in Appendix B. The purified Efr3 material was electrophoresed on a 3 - 20% SDS-PAGE gradient gel and silver stained.


MWM
2μ EFR3::Py2
ARS/CEN EFR3::Py2
ARS/CEN EFR3
200 kDa
116 kDa
97 kDa
66 kDa
45 kDa
31 kDa
22 kDa
14 kDa



I found that there were several non αPy cross-reactive bands that specifically co-purified from whole-cell extracts of *EFR3::Py*^{2} strains. Many of these bands are faster migrating than the Efr3 band, and might be Efr3 break down products that lack the Py^2 tag. However, several of the Efr3-copurifying proteins migrate more slowly than Efr3, and these are likely to be Efr3-associated proteins. A roughly equimolar amount of one of these proteins, which migrates at approximately 170 kDA, co-purifies with Efr3 when Efr3-Py^2 is expressed from a low-copy plasmid. I provisionally named this protein Eap170, for **E**fr3 **A**ssociated **P**rotein with an approximate molecular weight of 170 kDa. While the amount of Efr3-Py^2 was approximately five-fold higher when purified from strains harboring the high-copy *EFR3::Py*^{2} plasmid rather than thelow-copy plasmid, the amount of Eap170 that purified with Efr3 increased less than two fold. This suggests that the amount of Eap170 that is available to bind to Efr3 is limiting. The association of Efr3 and Eap170, as well as the other bands which copurify with Efr3 is not effected by the presence of *PHO85*. Neither is the mobility of any of these bands. On some gels Eap170 appears as a doublet.

I attempted to purify larger quantities of Efr3 in the hopes that this would allow me to purify enough Eap170 to subject it to microsequencing. This effort was motivated by a hope that mutations in the gene which encodes Eap170 would show interactions with *pho85Δ* or *efr3Δ pho85Δ* strains. It is also possible that Eap170 could be a known protein, and that its function would help elucidate the function of Efr3. I first tried to scale up the immunopurification stratgey by passing 200 mg of whole-cell extract from

an *efr3Δ* yeast strain harboring a low copy *EFR3::Py²* plasmid over a 1 ml column of αPy cross-linked beads, and eluting the Efr3-containing complexes with a Py peptide. The immunopurification strategy did not adapt well to being conducted on this scale. Unlike the small-scale purification, it was difficult to deplete all of the Efr3-Py² from the whole-cell extract, and it was also difficult to completely elute the Efr3-containing complexes from the column.

I next, constructed a variant of the *EFR3::Py²* allele that contained ten histidine-encoding codons downstream of the Py² encoding sequence. I constructed this allele to be able to be able to follow the purification of Efr3 by virtue of its interaction with the αPy antibody. This allele of *EFR3* also complements the inviability of an *efr3Δ pho85Δ* strain. I used the affinity of poly-histidine tracts for divalent cations to purify Efr3-Py²-10His on an iminodiacetic acid-coupled agarose resin that had been charged with either Ni^{++} or Co^{++}. Unlike the immunopurification protocol, there were many bands that bound to this resin that were not-dependent on the whole-cell extract being prepared from a *EFR3::Py²::10His* harboring strain. One common solution for dealing with these sorts of non-specific interactions is to increase the ionic strength of the extract and the wash solutions. However, the interaction between Efr3-Py² and Eap170 was detected in the presence of 1% TX-100 and 100 mM sodium chloride. In the presence of 1% TX-100 and 200 mM sodium chloride, I was unable to detect any Eap170 copurifying with Efr3. I was therefore unable to make the Efr3-Py²-10His purification any more stringent. Also as a result of the salt sensitivity of the interaction between Efr3 and Eap170, I was unable to use ion-exchange chromatography to purify Efr3-containing complexes.

Appendix C

MATERIALS & METHODS

Yeast Manipulation: Yeast were cultured and manipulated according to standard laboratory procedures which have been described previously (GUTHRIE and FINK 1991).

The *GAL1-10::PHO85* allele was generated by transforming MY0192 with plasmid MP136 linearized with *Bgl* II. The vast majority of transformants displayed a Pho^+ phenotype on galactose-containing media and a constitutive *PHO5* expression, or Pho^c, phenotype on dextrose-containing media. One of these transformants was plated on 5-FOA containing plates. A Ura^- colony that displayed a galactose-dependent $Pho85^+$ phenotype was identified and the presence of the *GAL1-10* promoter replacing the *PHO85* promoter was confirmed by PCR. This strain was named MY0246.

The transposon-insertion containing DNA fragments we used as a mutagen in the screen for Efr mutants were derived from eight different pools of a yeast genomic library that had been mutagenized with a Tn3 transposon Burns et al., 1994. Four alleles of *EFR1* were obtained from yeast transformed with fragments from two separate pools. Two alleles of *EFR2* were obtained from yeast transformed with fragments from one pool of the library.

To generate new alleles of *PHO85*, *PHO85* was amplified by error prone PCR using

Taq DNA polymerase at standard conditions except the concentration of $MgCl_2$ was lowered from 1.5 mM to 1.34 mM and $MnCl_2$ was added to 160 μM. We screened for temperature-sensitive alleles of *PHO85* by cotransforming a *cln1Δ cln2Δ GAL1-10::CLN1 pho85Δ* strain (MY0126) with these PCR products and MP056 digested with *Eco*RI. Transformations were plated on SD at room temperature. Temperature-sensitive alleles of *PHO85* were identified by replica plating the transformation plates and screening for colonies that were unable to grow at 37°C. Positives from this screen were then tested for temperature-sensitive constitutive Pho5 activity (a ts-Pho^c phenotype) by an acid-phosphatase activity plate assay performed on strains growing in SG medium at room temperature or 37°C. We recovered the *PHO85* plasmid from three strains with a strong temperature-dependent Pho85 phenotype, and the *PHO85* allele from each was used to replace the wildtype *PHO85* locus. The allele that we named *pho85-9* displays the most dramatic temperature sensitive phenotype and was used in all experiments. *pho85-9* strains display a variety of Pho85 phenotypes at elevated temperature such as an inability to grow in glycerol medium (GILLIQUET and BERBEN 1993) and supersensitivity to hydroxyurea (data not shown) though I found that the non-permissive temperature for different Pho85 phenotypes varied from 30°C to 37°C. I could not test whether the *pho85-9* allele results in a glycogen hyper-accumulation phenotype (HUANG *et al.* 1996, TIMBLIN *et al.* 1996) because we cannot detect this phenotype in our *pho85Δ* strains.

High copy plasmids carrying each of the Pho85-associated cyclins were made as follows. Genomic clones of *PCL7*, *PCL8*, *PCL9* and *PCL10* were identified in a YEp13 genomic library (NASMYTH and TATCHELL 1980) by colony hybridization using fragments isolated

from pBA949, pBA946a, pBA950 and pBA945a, respectively. Fragments containing the cyclin locus from these plasmids were then subcloned into pRS426 using naturally occurring restriction enzyme sites. Plasmids containing other Pho85-associated cyclins were generated by subcloning fragments containing *PCL1* (from pFHE27), *PCL2* (from pBA619), *PHO80* (from pAC800), *PCL5* (from pBA906), and *PCL6* (from pRS425-*PCL6*, a generous gift of ANITA SIL) into pRS426. pRS426-*CLG1* (pBA904) and the other pBA plasmids were the generous gift of BRENDA ANDREWS.

Strains containing deletions of various Pho85-associated cyclins were constructed as follows. Deletion of *PCL1* was performed by transformation of strain EY057 with *Sph* I - *Sal* I digested EB0149 and selecting for integrants on medium lacking histidine. Integration of *HIS3* at *PCL1* was confirmed by PCR. Deletion of *PCL2* was performed by transformation with *Kpn* I - *Xba* I digested EB0226 and selecting for integrants on medium lacking uracil. Integration of *URA3* at *PCL2* was confirmed by PCR. One such strain was named EY0535. *PCL9* was deleted by transforming EY0535 with EB0727 that had been digested with *Not* I and *Xho* I, and selecting for integrants on medium lacking tryptophan. Integration of *TRP1* at *PCL9* was confirmed by PCR. One such strain was named EY0552.

A deletion of the *EFR3* locus marked with the *LEU2* gene was generated by transforming EY0099 with MP0030 cut with *Sal* I and *Bam*H I and selecting for integrants on medium lacking leucine. Integration at the *EFR3* locus was confirmed by PCR.

Cloning of transposon insertions: The genomic position of transposon insertions which give rise to an Efr phenotype was determined by first using T4 DNA ligase to circularize fragments obtained by digesting genomic DNA from the Efr strains with *Csp*6 I. Primers that hybridize to the sense strand of the transposon just downstream of the 5' end or to the antisense strand just upstream of the transposon's first *Csp*6 I site were used to perform PCR with the circularized DNA fragments, and the resulting PCR products were sequenced directly. We identified the genomic sequence adjacent to the transposon end by searching the yeast genome sequence database (CHERRY *et al.* 2000).

Efr3-GFP localization: The microscopic examination of Efr3-GFP localization was performed as described (O'Neill et al., 1996)

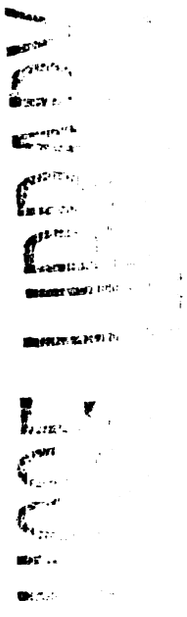
Immunopurification of Efr3-Py^2: The immunopurification of Efr3-Py^2 was performed as described (Schneider et al., 1994).

APPENDIX D

Yeast Strains

Strain	Genotype
MY025	W303 a *pho85Δ::LEU2*
MY033	RD270-4C a *CDC28::HA::TRP1*
MY034	MTY263 *CLN2::HA::LEU2*
MY035	W303 diploid (maybe heterozygous for ade3 or ade2)
MY036	W303 a *ade2 ade3 pho85Δ::LEU2* with plasmid *CD160-PHO85* (strain used for sectoring synthetic lethal screen)
MY037	W303 a *ade2 ade3 pho85Δ::LEU2*
MY038	W303 *a ade2 ade3*
MY039	W303 *α ade2 ade3*
MY040	K699 a *ade2 trp1-1 can1-100 leu2-3,112, his3-11,15 ura3 GAL^{+} PSI^{+}* (original lab K699 isolate from 'This Peter)
MY080	YPH274 diploid *ura3-52 lys2-801^{amb} ade2-101^{och} leu2Δ-1 his3Δ-200 trp1Δ-1 cir^{+} gal2$^{+/-}$*
MY081	K699 *nankΔ::HIS3* #1 (checked by PCR)
MY082	K699 *nankΔ::HIS3* #2 (checked by PCR)
MY087	NY180 *α ura3 leu2* (from Allan Myers, WT for MY088)
MY088	NY180 a *ura3 leu2 pps1* (from Allan Myers)
MY089	W303-1A a *ura3 leu2 trp1 his3 ade2* (from Allan Myers, WT for MY090)

Strain	Genotype
MY090	W303-1A a *pps1Δ::URA3 ura3 leu2 trp1 his3 ade2* (from Allan Myers)
MY091	W303 α *pho85Δ::TRP1* (made from MY039)
MY099	K699 diplod (made by crossing MY100 with MY040)
MY100	K699 α (made from MY040 by *GAL-HO* 1/2/95 -- lab original MAT α K699 strain)
MY101	W303 diplod *pho85Δ::TRP1* (there may be red sectors as one parent had CB160-*PHO85*, made from MY091)
MY108	W303 α *pho85Δ::LEU2 ade2* (MY025 x MY091)
MY109	W303 a *pho85Δ::LEU2 ade2* (MY025 x MY091)
MY110	K699 α *sl12 ade3?* (allele of *TFC3*, 3rd backcross after mutagenesis)
MY111	K699 a *sl12 ade3?* (allele of *TFC3*, 3rd backcross after mutagenesis)
MY112	K699 α *sl28 ade3* (allele of *EFR3*, 3rd backcross after mutagenesis)
MY123	K699 a *efr3Δ::LEU2 PHO81^{c}-38* (my114 crossed to a strain from Doug Jeffery)
MY124	K699 α *efr3Δ::LEU2 PHO81^{c}-38* (my114 crossed to a strain from Doug Jeffery)
MY125	K699 a *pho85Δ::TRP1 his3::PHO5p::HIS3*
MY126	BF264-15D a *cln1Δ cln2Δ pho85Δn::TRP1 leu2::GAL1-10p::CLN2::LEU2 lys2*? (made by crossing EY0233 to EY0340, disrupting *PHO85* with MP054 and dissecting onto galactose. Strains are galactose dependent, Pho^{c}, Trp^{+})



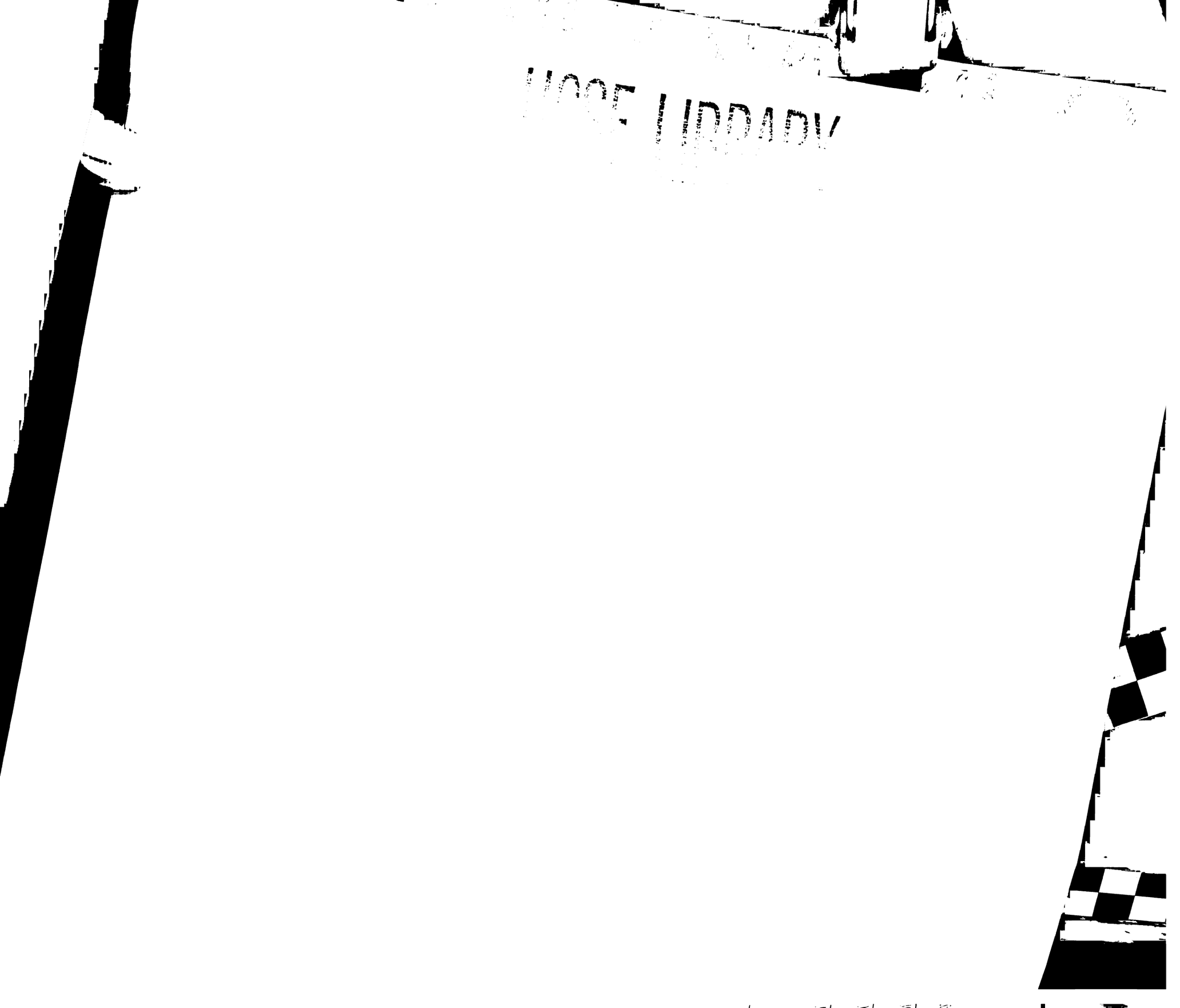
Strain	Genotype
MY127	BF264-15D α *cln1Δ cln2Δ pho85Δn::TRP1 leu2::GAL1-10p::CLN2::LEU2 lys2?* (made by crossing EY0233 to EY0340, disrupting *PHO85* with MP054 and dissecting onto galactose. Strains are galactose dependent, Pho^c, Trp^+)
MY128	K699 a pRS313
MY129	K699 a pRS314
MY130	K699 a pRS315
MY131	K699 α pRS313
MY132	K699 α pRS314
MY133	K699 α pRS315
MY134	IH2703 Ura^- *met6 mat?*
MY135	W303 a *ade2 ade3 pho85Δn::TRP1*
MY136	K699 a *pho85Δn::HIS3*
MY137	K699 α *pho85Δn::HIS3*
MY138	BF264-15D *cln1Δ/cln1Δ cln2Δ/cln2Δ lys2/+ leu2::GAL1-10p::CLN2::LEU2/+* (parent of MY126 and MY127)
MY150	K699 α *PHO85::*Py^2 #2 (Ura^- Leu^- $Pho85^+$ loop outs of EY0141 transformed with MP0048. The presence of the Py^2 Tag has not been verified)
MY151	K699 α *PHO85::*Py^2 #3 (Ura^- Leu^- $Pho85^+$ loop outs of EY0141 transformed with MP0048. The presence of the Py^2 Tag has not been

Strain	Genotype
	verified)
MY152	K699 α *PHO85::Py*^{2} #4 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with MP0048. The presence of the Py^2 Tag has not been verified)
MY153	K699 α *PHO85::Py*^{2} #5 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with MP0048. The presence of the Py^2 Tag has not been verified)
MY154	K699 α *PHO85::Py*^{2} #6 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with MP0048. The presence of the Py^2 Tag has not been verified)
MY155	K699 α *pho85-6::Py*^{2} #1 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY156	K699 α *pho85-6::Py*^{2} #2 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY157	K699 α *pho85-6::Py*^{2} #3 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY158	K699 α *pho85-9::Py*^{2} #1 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has

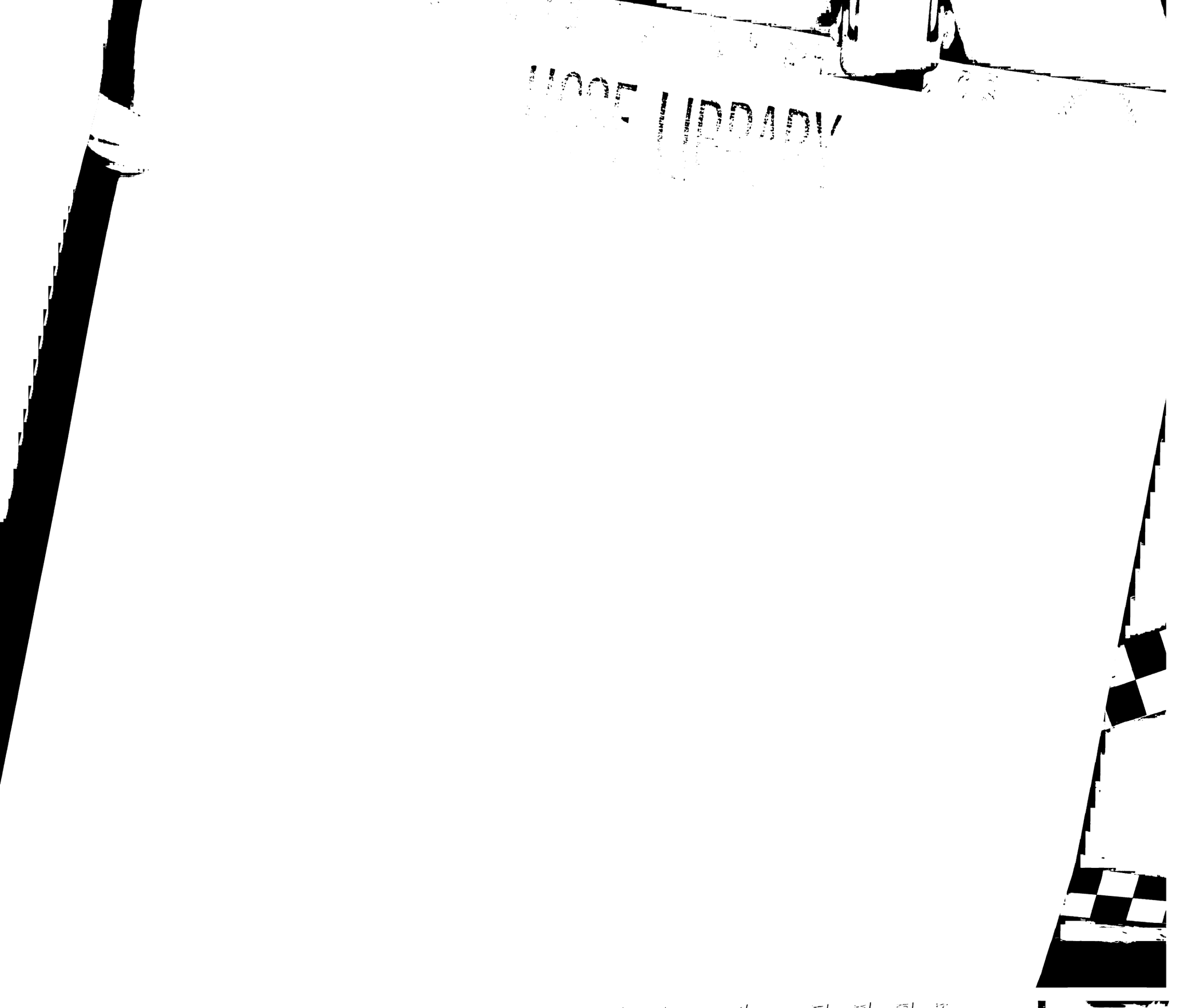
Strain	Genotype
	not been verified)
MY159	K699 α *pho85-9::Py*^{2} #2 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY160	K699 α *pho85-9::Py*^{2} #3 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY161	K699 α *pho85-10::Py*^{2} #1 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY162	K699 α *pho85-10::Py*^{2} #2 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY163	K699 α *pho85-10::Py*^{2} #3 (Ura$^-$ Leu$^-$ Pho85$^+$ loop outs of EY0141 transformed with *pho85-ts* allele plasmids. The presence of the Py^2 Tag has not been verified)
MY164	W303 a *mec1-1 bar1 CLB2::LACZ ura3 leu2 his3 trp1-1 ade2 can1 ADE3* (from Lena Hwang LW113)
MY165	BF264-15D α *pho85Δo::LEU2* #1 (derived from EY0230 with plasmid pYU19)
MY166	BF264-15D α *pho85Δo::LEU2* #2 (derived from EY0230 with plasmid

Strain	Genotype
	pYU19)
MY167	K699 a *pho85-6* (MY155 x EY0150)
MY168	K699 a *pho85-6 pho81Δ::TRP1* (MY155 x EY0150)
MY169	K699 α *pho85-6 pho81Δ::TRP1* (MY155 x EY0150)
MY170	K699 a *pho85-10* (MY160 x EY0150)
MY171	K699 a *pho85-10 pho81Δ::TRP1* (MY160 x EY0150)
MY172	K699 α *pho85-10 pho81Δ::TRP1* (MY160 x EY0150)
MY173	K699 a *pho85-9* (MY158 x EY0150)
MY174	K699 a *pho85-9 pho81Δ::TRP1* (MY158 x EY0150)
MY175	K699 α *pho85-9 pho81Δ::TRP1* (MY158 x EY0150)
MY176	K699 α *efr3Δ::LEU2 pho85-9* (MY158 x MY115)
MY177	K699 a *tfc3 pho85-9 ADE3*
MY178	K699 α *sl13 pho85-9 ade3?*
MY179	K699 α *sl15 pho85-9 ADE3*
MY180	K699 a *sl17 pho85-9 ade3?*
MY181	K699 α *sl22 pho85-9 ade3?*
MY182	K699 a *sl38 pho85-9 ADE3*
MY183	K699 α *efr3-2 pho85-9 ade3?*
MY184	K699 a *sl43 pho85-9 ADE3*
MY185	K699 α *efr3Δ::LEU2 pho85-9 pho4Δ::TRP1* (10a)
MY186	K699 diploid cir^0 (made by Heather Fitzgerald)

Strain	Genotype
MY187	K699 a *cir*^{0} (made by Heather Fitzgerald)
MY188	K699 α *cir*^{0} (made by Heather Fitzgerald)
MY189	K699 a *cir*^{0} *TRP1* #1 (DO NOT USE, not stably Trp$^+$, made by Heather Fitzgerald by transforming M*Y1*87 with TRP1 *frag*ment)
MY190	K699 a cir0 TRP1 #2 (made by H*eathe*r Fitzgerald by transforming M*Y1*87 with TRP1 fragment)
MY191	K699 a cir0 TRP1 #3 (made by Heather Fitzgerald by transforming MY187 with TRP1 fragment)
MY192	K699 α *cir*^{0} *HIS3* #1 (made by Heather Fitzgerald by transforming MY188 with *HIS3* fragment)
MY193	K699 α *cir*^{0} *HIS3* #2 (made by Heather Fitzgerald by transforming MY188 with *HIS3* fragment)
MY194	K699 α *cir*^{0} *HIS3* #3 (made by Heather Fitzgerald by transforming MY188 with *HIS3* fragment)
MY195	BF264-15D α *pho85-9* (made by Heather Fitzgerald by scoring loop-outs of MY165 TX'ed with *pho85-9::Py*^{2} loop-in vector. Integrant may or may not have *Py*^{2} tag)
MY196	K699 a *efr3Δ::LEU2 pho2Δ::LEU2*
MY197	K699 α *efr3Δ::LEU2 pho2Δ::LEU2*
MY198	K699 *MAT? efr3Δ::LEU2 pho2Δ::LEU2 pho85-9* (#7B)
MY199	K699 a *ADE2*$^+$ (do not use, not stably Ade$^+$)



Strain	Genotype
MY200	K699 α *ADE2*$^{+}$ (do not use, not stably Ade^{+})
MY201	Y13/Y16 *pho85Δn::LEU2/pho85Δn::LEU2* pRS316 (prototroph)
MY202	Y13/Y16 *pho85Δn::LEU2/pho85Δn::LEU2* pRS316-*PHO85::PY*[2] (EB0327) (prototroph)
MY203	BF264-15D a *cln1Δ cln2Δ pho85-9* (MY195 x EY173?) 5A, Made by Heather Fitzgerald
MY204	BF264-15D α *cln1Δ cln2Δ pho85-9* (MY195 x EY173?) 24B, Made by Heather Fitzgerald
MY205	BF264-15D a *cln1Δ cln2Δ pho85-9*
MY206	K699 α *efr2 pho85-9 ade3?*
MY207	K699 diploid *pho85Δn::TRP1/+ efr3Δ::LEU2/+*
MY208	SBY132 *cir*^{0} diplod from Mike Snyder via Sue Biggins (control strain)
MY209	K699 a *pho85Δn::TRP1 pho2Δ::LEU2*
MY210	K699 α *pho85Δn::TRP1 pho2Δ::LEU2*
MY211	K699 a *pho85Δn::HIS3 pho4Δ::TRP1*
MY212	Fink pseudohyphal strain (MY141) a *efr3Δ::LEU2* (checked by PCR and EFR3 plasmid complementation of slow growth on glycerol)
MY213	PLY122 a *ura3-52 lys2-201 leu2-3,112*. From Fink Lab (Daignan-Fornier PNAS paper strain)
MY214	K699 a *ADE2+* (made by TX of EY0057 with Hind III cut mp0102 to replace strain MY199)

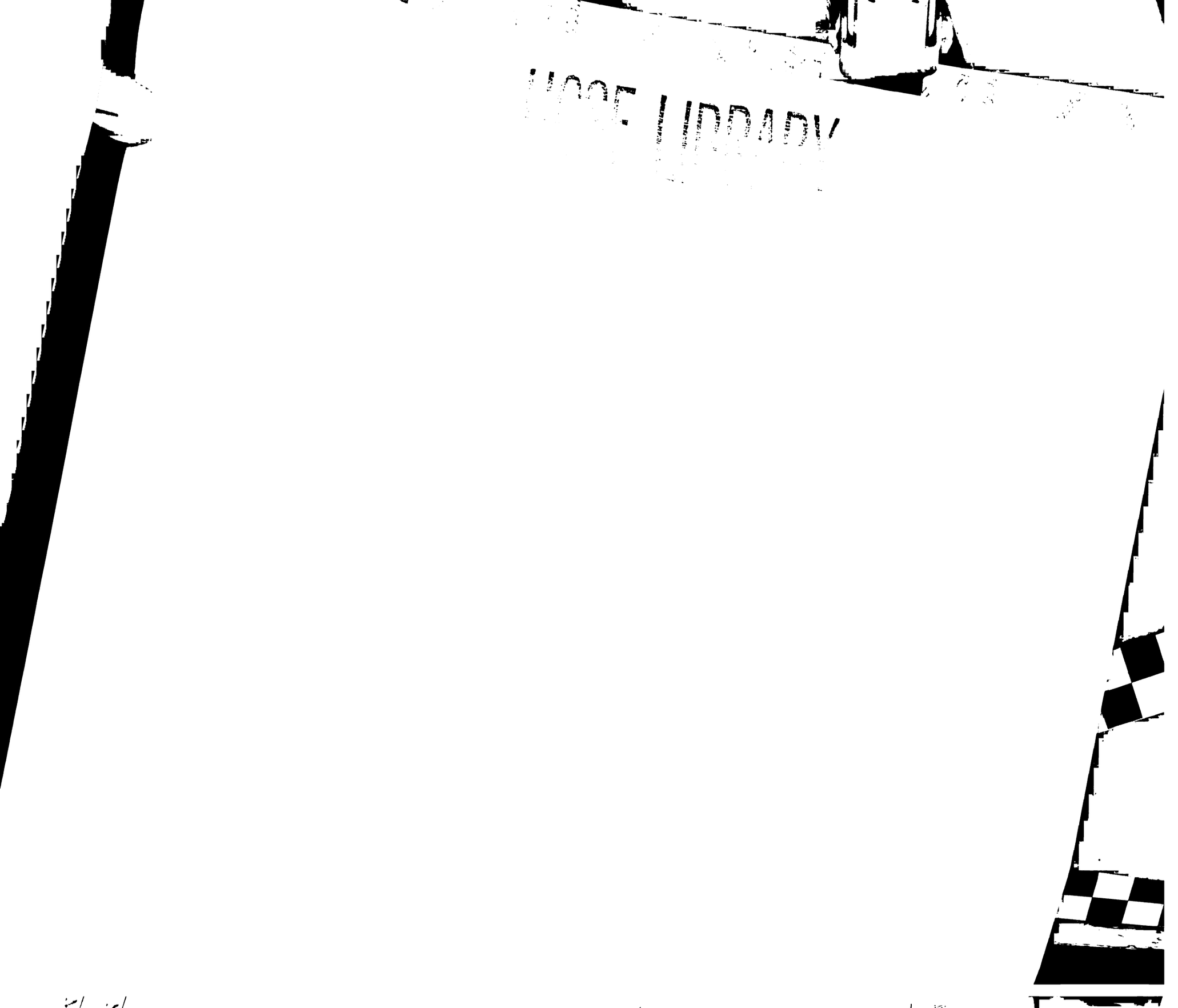


Strain	Genotype
MY215	K699 a *bas1Δ::HIS3* (made from MY207, checked by PCR)
MY216	K699 α *bas1Δ::HIS3* (made from MY207, checked by PCR)
MY217	K699 a *bas1Δ::HIS3 pho85Δn::TRP1* (made from MY207, checked by PCR)
MY218	K699 α *bas1Δ::HIS3 pho85Δn::TRP1* (made from MY207, checked by PCR)
MY219	K699 a *bas1Δ::HIS3 efr3Δ::LEU2* (made from MY207, checked by PCR)
MY220	K699 α *bas1Δ::HIS3 efr3Δ::LEU2* (made from MY207, checked by PCR)
MY221	K699 a *bas1Δ::HIS3 efr3Δ::LEU2 pho85-9* (made from MY207, checked by PCR)
MY222	K699 α *bas1Δ::HIS3 efr3Δ::LEU2 pho85-9* (made from MY207, checked by PCR)
MY223	K699 a *bas1Δ::HIS3 pho85-9* (made from MY207, checked by PCR)
MY224	K699 α *bas1Δ::HIS3 pho85-9* (made from MY207, checked by PCR)
MY225	K699 α *ADE2*$^+$
MY226	K699 a *ADE2*$^+$ *bas1Δ::HIS3*
MY227	K699 α *ADE2*$^+$ *bas1Δ::HIS3*
MY228	K699 α *ADE2*$^+$ *pho85Δn::TRP1*
MY229	K699 α *ADE2*$^+$ *pho85Δn::TRP1 bas1Δ::HIS3*
MY230	W303 α *swi5Δ::hisG::URA3* (from Mary Maxon YM93. Grow on -Uracil

Strain	Genotype
	to keep marker)
MY231	K699 a *efr3Δ2::TRP1* (disrupted with MP0125, checked by PCR)
MY232	K699 a *pho85Δn::HIS3 pho2Δ::LEU2*
MY233	K699 α *pho85Δn::HIS3 pho2Δ::LEU2*
MY234	K699 a *swi5Δ::hisG::URA3* (MY230 x MY221, #7B)
MY235	K699 a *swi5Δ::hisG::URA3 pho85-9* (MY230 x MY221, #9C)
MY236	K699 a *swi5Δ::hisG::URA3 pho85-9 efr3Δ::LEU2* (MY230 x MY221, #1A)
MY237	K699 a *swi5Δ::hisG::URA3 pho85-9 efr3Δ::LEU2 bas1Δ::HIS3* (MY230 x MY221, #3A)
MY238	K699 α *swi5Δ::hisG::URA3 pho85-9 bas1Δ::HIS3* (MY230 x MY221, #2A)
MY239	K699 α *swi5Δ::hisG::URA3 bas1Δ::HIS3* (MY230 x MY221, #6C)
MY240	K699 a *cir^0 pho85Δn::TRP1* #5D (made by disrupting *PHO85* in MY187 and MY188 followed by mating and dissecting tetrads. A lot of heterogeneity in colony size. These spores are the fastest growing)
MY241	K699 α *cir^0 pho85Δn::TRP1* #1B (made by disrupting *PHO85* in MY187 and MY188 followed by mating and dissecting tetrads. A lot of heterogeneity in colony size. These spores are the fastest growing)
MY242	K699 a *cir^0 pho85Δn::HIS3* #10C (made by disrupting *PHO85* in MY187 and MY188 followed by mating and dissecting tetrads. A lot of

Strain	Genotype
	heterogeneity in colony size. These spores are the fastest growing)
MY243	K699 α *cir^0 pho85Δn::HIS3* #2A (made by disrupting *PHO85* in MY187 and MY188 followed by mating and dissecting tetrads. A lot of heterogeneity in colony size. These spores are the fastest growing)
MY244	K699 a *HIS3 pho85-9* #1C (MY192 x MY173)
MY245	K699 α *HIS3 pho85-9* #2D (MY192 x MY173)
MY246	K699 α *HIS3 cir^0 gal1-10p::pho85* (loop-in-out of MY 192 (FOA^r, Galactose-dependent Pho$^+$)
MY247	K699 a *HIS3 cir^0 gal1-10p::pho85* #3B (MY246 x MY190)
MY248	K699 a *TRP1 cir^0 gal1-10p::pho85* #4B (MY246 x MY190)
MY249	K699 α *TRP1 cir^0 gal1-10p::pho85* #3D (MY246 x MY190)
MY250	K699 a *HIS3 cir^0* #3A (MY246 x MY190)
MY251	K699 α *TRP1 cir^0* #10D (MY246 x MY190)
MY252	K699 a *TRP1 pho85-9* #4B (MY245 x MY190)
MY253	K699 α *TRP1 pho85-9* #3A (MY245 x MY190)
MY254	K699 a *efr3Δ::LEU2 gal1-10p::pho85* #7C (MY255 x MY115)
MY255	K699 a *cir^0 gal1-10p::pho85*
MY256	S288C *TRP1* (IH2534)
MY257	S288C *TRP1 swi4Δ::HIS3* (JO22)
MY258	K699 a *bas1Δ::HIS3 gcn4Δ::LEU2* #19, by TX of MY215 to Leu$^+$ and His$^-$
MY259	K699 a *bas1Δ::HIS3 gcn4Δ::LEU2* #20, by TX of MY215 to Leu$^+$ and His$^-$

Strain	Genotype
MY260	BF264-15D *cln1Δ cln2Δ cln3Δ pcl1Δ::HI3 pcl2Δ::URA3 leu2::LEU2::GAL1::CLN1 ade1 his3 ura3 trp1* (from Fred Cross #1591-10D)
MY261	BF264-15D *bar1 cln1Δ cln2Δ cln3Δ pcl1Δ::HI3 pcl2Δ::URA3 leu2::LEU2::GAL1::CLN1 ade1 his3 ura3 trp1* (from Fred Cross #1254-14D)
MY262	K699 α *mpk1Δ::TRP1* #1D, "big segregant" (From AFS151 x MY137, Trp^+ ts-lethality rescued by sorbitol 5/28/97)
MY263	K699 a *mpk1Δ::TRP1* #6B, "big segregant" (From AFS151 x MY137, Trp^+ ts-lethality rescued by sorbitol 5/28/97)
MY264	K699 α *mpk1Δ::TRP1* #2C, "small segregant" (From AFS151 x MY137, Trp+ ts-lethality rescued by sorbitol 5/28/97) MY265 K699 _ mpk1_::TRP1 pho85-9 his3 #2D (MY245 x MY263) MY266 K699 a CAN1 (made from MY099 by loop-in-out with MP0148, 2:2 segregation of Cans/Canr) MY267 K699 _ CAN1 (made from MY099 by loop-in-out with MP0148, 2:2 segregation of Cans/Canr) MY268 K699 a CAN1 KAR1::kar1_::URA3 #1B (made from heterozygous can1/+ parent of MY266 and MY267 by loop-in of EB0416. These strains are Kar+. Grow on -Ura to keep them that way, or put on FOA to select for Kar-. Correct loop-in verified by Kar- loop-outs) MY269 K699 _ CAN1 KAR1::kar1_::URA3 #4D (made from heterozygous can1/+ parent of



Strain	Genotype
	MY266 and MY267 by loop-in of EB0416. These strains are Kar+. Grow on -Ura to keep them that way, or put on FOA to select for Kar-. Correct loop-in verified by Kar- loop-outs) MY270 K699 a can1-1 KAR1::kar1_::URA3 #3B (made from heterozygous can1/+ parent of MY266 and MY267 by loop-in of EB0416. These strains are Kar+. Grow on -Ura to keep them that way, or put on FOA to select for Kar-. Correct loop-in verified by Kar- loop-outs)
MY271	K699 _ can1-1 KAR1::kar1_::URA3 #1B (made from heterozygous can1/+ parent of MY266 and MY267 by loop-in of EB0416. These strains are Kar+. Grow on -Ura to keep them that way, or put on FOA to select for Kar-. Correct loop-in verified by Kar- loop-outs)
MY272	K699 _ CAN1 kar1_-13 (loop out of MY271)
MY273	K699 a/_ 96-4/+ gal1-10::pho85/+ his3/+ trp1/+ This mutant is either dead on dextrose or synthetically lethal with pho85-9 at room-temperature. Phenotype is LEU2 linked.
MY274	K699 a 76-9 pho85-9 his3 TRP1 leu2 (this mutant is dead at 37°C but phenotype is unliked to LEU2 transposon, may be spontaneous efr mutant
MY275	K699 _ 96-5::LEU2 pho85-9 his3 TRP1 (96-5 is an insertion into BEM2)
MY276	K699 a 96-3::LEU2 pho85-9 HIS3 trp1 (96-3 is an insertion into BEM2 -- independent of 96-5)
MY277	K699 a 96-3::LEU2 PHO85 his3 trp1
MY278	K699 _ 77-4::LEU2 pho85-9 his3 trp1 (77-4 is an insertion into SRB5

Strain	Genotype
	5'UTR)
MY279	K699 a 77-4::LEU2 pho85-9 HIS3 trp1
MY280	K699 _ 77-4::LEU2 PHO85 his3 trp1
MY281	K699 a 77-4::LEU2 PHO85 his3 trp1
MY282	K699 a 76-5::LEU2 pho85-9 his3 TRP1 (76-5 is an insertion into SPT7)
MY283	K699 _ 76-5::LEU2 pho85-9 HIS3 trp1
MY284	K699 _ 76-5::LEU2 PHO85 his3 trp1
MY285	K699 a 76-5::LEU2 PHO85 his3 trp1
MY286	K699 _ 97-6::LEU2 pho85-9 his3 trp1 (97-6 is an insertion into ADA2/HFI1)
MY287	K699 a 97-6::LEU2 pho85-9 his3 trp1
MY288	K699 _ 97-6::LEU2 PHO85 HIS3 trp1
MY289	K699 a 97-6::LEU2PHO85 his3 trp1
MY290	K699 _ 97-3::LEU2 pho85-9 his3 TRP1 (97-3 is an insertion into YLR114C)
MY291	K699 a 97-3::LEU2 pho85-9 his3 TRP1
MY292	K699 a 78-1::LEU2 pho85-9 his3 trp1 (78-1 is an insertion into the very 3' end of GCR1)
MY293	K699 a *78-1::LEU2 PHO85 his3 trp1*
MY294	K699 α *78-1::LEU2 PHO85 his3 trp1*
MY295	K699 α *33-31::LEU2 PHO85 his3 trp1 ade3* (*33-31* is an insertion into

Strain	Genotype
	BCK1)
MY296	K699 a *33-31::LEU2 PHO85 his3 trp1 ade3*
MY297	K699 a *pho85-6 HIS3 TRP1*
MY298	K699 α *pho85-6 HIS3 TRP1*
MY299	K699 a pho85-6 his3 TRP1
MY300	K699 _ pho85-6 his3 TRP1
MY301	K699 a pho85-6 HIS3 trp1
MY302	K699 _ pho85-6 HIS3 trp1
MY303	K699 _ pho85-10 HIS3 TRP1
MY304	K699 a pho85-10 his3 TRP1
MY305	K699 _ pho85-10 his3 TRP1
MY306	K699 a pho85-10 HIS3 trp1
MY307	K699 _ pho85-10 HIS3 trp1
MY308	K699 a SSD1-V::URA3::ssd1-d pho85-9
MY309	K699 _ SSD1-V::URA3::ssd1-d pho85-9
MY310	K699 a 77-4::LEU2 pho85-10
MY311	K699 a 77-4::LEU2 pho85-6
MY312	K699 a 76-2::LEU2 HIS3 TRP1 pho85-6
MY313	K699 a 76-2::LEU2 HIS3 trp1 pho85-10
MY314	K699 _ 76-5::LEU2 pho85-6
MY315	K699 a 76-5::LEU2 pho85-10

Strain	Genotype
MY316	K699 a 78-1::LEU2 pho85-6
MY317	K699 a 78-1::LEU2 pho85-10
MY318	K699 a 97-6::LEU2 pho85-6
MY319	K699 a 97-6::LEU2 pho85-10
MY320	K699 _ 33-31::LEU2 pho85-6
MY321	K699 _ 33-31::LEU2 pho85-9
MY322	K699 a 33-31::LEU2 pho85-10
MY323	K699 a gal1-10::sit4::HIS3 SSD1-V::URA3 pho85-9 (3b)
MY324	K699 α *gal1-10::sit4::HIS3 SSD1-V::URA3 pho85-9* (6c)
MY325	K699 a *gal1-10::sit4::HIS3 SSD1-V pho85-9* (FOA^r of MY323)
MY326	K699 α *gal1-10::sit4::HIS3 SSD1-V pho85-9* (FOA^r of MY324)
MY327	K699 α *96-5::LEU2 SSD1-V::URA3* (3c)
MY328	K699 α *96-5::LEU2 SSD1-V::URA3 pho85-9* (4b)
MY329	K699 α *mpk1Δ::TRP1 SSD1-V::URA3* (3c)
MY330	K699 a *mpk1Δ::TRP1 SSD1-V::URA3 pho85-9* (4c)
MY331	K699 α *mpk1Δ::TRP1 SSD1-V::URA3 pho85-9* (5b)
MY332	K699 a *cln1Δ cln2Δ SSD1-V::LEU2 pho85-9 ade3?* (11c)
MY333	K699 α *cln1Δ cln2Δ SSD1-V::URA3 pho85-9 ade3?* (1c)
MY334	K699 a *cln1Δ cln2Δ SSD1-V::URA3 pho85-9 ADE3?* (12b)
MY335	K699 α *76-2::LEU2 CDC28-VF::URA3* (1a)
MY336	K699 α *76-2::LEU2 CDC28-VF::URA3 pho85-10* (5b)

Strain	Genotype
MY337	K699 a *CDC28-VF::URA3 pho85Δ::HIS3*
MY338	K699 a *cdc55Δ::HIS3 pho85-9* (2d)
MY339	K699 α *cdc55Δ::HIS3 pho85-9* (5c)
MY340	K699 a *SSD1-V::URA3::ssd1-d pho85-9* (S1-1c)
MY341	K699 α *SSD1-V::URA3::ssd1-d pho85-9* (S1-6c)
MY342	K699 a *SSD1-V::LEU2 pho85-9* (6c)
MY343	K699 α *SSD1-V::LEU2 pho85-9* (1c)
MY344	K699 α *SSD1-V::LEU2 pho85-9 CDC28-VF::URA3*(20a)
MY345	K699 a *swe1Δ::TRP1 pho85-9* (R1-2d)
MY346	K699 α *swe1Δ::TRP1 pho85-9* (R2-3b)
MY347	K699 a *mih1Δ::LEU2 pho85-9* (S2-3a)
MY348	K699 α *mih1Δ::LEU2 pho85-9 TRP1*(S2-2b)
MY349	K699 α *cln1Δ cln2Δ SSD1-V::LEU2 pho85-9 CDC28-VF::URA3* (11b)
MY350	K699 a *cln1Δ cln2Δ SSD1-V::LEU2 pho85-9? CDC28-VF::URA3* (14d)
MY351	K699 a *76-2::LEU2 swe1Δ::TRP1 CDC28-VF::URA3* (T2-3c)
MY352	K699 α *76-2::LEU2 swe1Δ::TRP1 CDC28-VF::URA3* (T2-9b)
MY353	K699 α *mpk1Δ::TRP1 pcl1Δ::HIS3 pcl2D::URA3* (A1-3b, this strain requires sorbitol for growth)
MY354	K699 a *76-5::LEU2 pcl1Δ::HIS3 pcl2D::URA3 pcl9Δ::TRP1* (I1-3b)
MY355	K699 α *76-5::LEU2 pcl1Δ::HIS3 pcl2D::URA3 pcl9Δ::TRP1* (I1-3a)

Strain	Genotype
MY356	K699 a *pcl1Δ::HIS3 CDC28-VF::URA3* (J3a)
MY357	K699 α *pcl1Δ::HIS3 CDC28-VF::URA3* (J2b)
MY358	K699 a/α *pho85Δ::HIS3/ pho85Δ::TRP1*
MY359	K699 a/α *pho85Δ::HIS3/+ CDC28-VF::URA3/+*
MY360	??? *hog1*⁻ (stolen from Sean's bench as a positive control)
MY361	W303 a *cdc42-1* (from Sean, SO686)
MY362	W303 a *cla4Δ::LEU2 ste20Δ::LEU2 (pCLA4-ts; TRP1)*

APPENDIX E

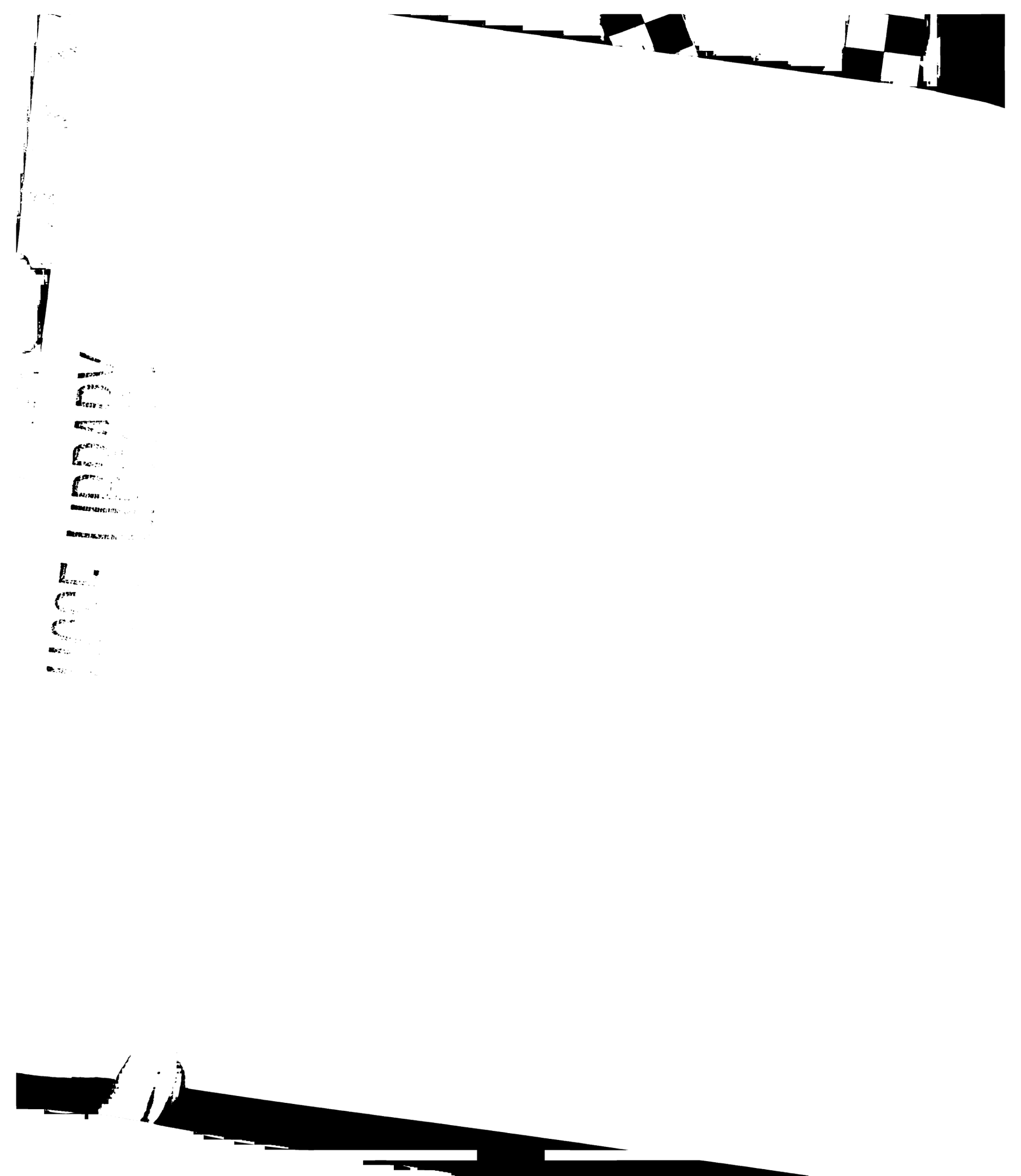
Plasmids

Strain	Contents	Description	Date	Host
MP0001	pKS+Nde	Pst-Nde linker inserted into Pst site of pKS+	5/10/96	TG2
MP0002	pRS315.Nde	Pst-Nde linker inserted into Pst site of pRS315	5/10/96	TG2
MP0003	pRS314.Nde	Pst-Nde linker inserted into Pst site of pRS314	5/10/96	TG2
MP0004	pKS+.*EFR3*	Nde-PvuII fragment containing *EFR3* ORF, ~300 bp of promoter sequence and ~30bp of 3'UTR cloned from YEp13 plasmid isolated from Nasmyth library which complements SL28. Cloned into Nde-SmaI sites of MP0001	5/10/96	TG2
MP0005	pRS315.*EFR3*	Sal.Bam fragment containing entire insert from MP0004 cloned into Sal/Bam of pRS315. This construct complements *efr3*.	5/10/96	TG2
MP0006	pRS425.*EFR3*	Sal.Bam fragment from MP0004 cloned into same sites of pRS425. This vector complements *efr3*.	5/10/96	TG2
MP0007	pKS.*EFR3*.Nco	generated by site directed mutagenesis of pKS.*EFR3* Puts NcoI site at stop codon, destroying stop codon.	5/10/96	TG2
MP0008	pKS.*EFR3*.Myc	Myc tag inserted as Nco.Bam into same sites in MP0007	5/10/96	TG2
MP0009	pKS.*EFR3*.Py^2	Py-Py tag inserted as Nco.Bam from EB0327 into same sites of MP0007	5/10/96	TG2
MP0010	pKS.*EFR3*.GFP+	GFP cloned as Bam.Nco partial into same	5/10/96	TG2

Strain	Contents	Description	Date	Host
		sites of MP0007		
MP0011	pRS313.*EFR3*.Myc	Sal.Bam from MP0008	5/10/96	TG2
MP0012	pRS423.*EFR3*.Myc	Sal.Bam from MP0008	5/10/96	TG2
MP0013	pRS313.*EFR3*.Py^2	Sal.Bam from MP0009	5/10/96	TG2
MP0014	pRS423.*EFR3*.Py^2	Sal.Bam from MP0009	5/10/96	TG2
MP0015	pRS313.*EFR3*.GFP+	Sal.Bam from MP0010	5/10/96	TG2
MP0016	pRS423.*EFR3*.GFP+	Sal.Bam from MP0010	5/10/96	TG2
MP0017	pRS313.*EFR3*	Sal.Bam from MP0004	5/10/96	TG2
MP0018	pRS423.*EFR3*	Sal.Bam from MP0004	5/10/96	TG2
MP0019	pRS316.*EFR3*.Py^2	Sal.Bam fragment from pKS+.*EFR3*.Py^2 cloned into same sites of pRS316 complementation of *efr3 pho85* phenotype confirmed by TX into *efr3 pho85 GALpPHO85* and recovering Ura+ colonies on SD -Ura (but not with empty pRS316)	5/13/96	TG2
MP0020	pKS+.*EFR3*.Py^2.Sph	SphI site introduced at -2 to ATG (to replace *EFR3p* with *GAL1p*) by site directed mutagenesis. SphI site confirmed by digest. This freeze corresponds to miniprep A. Plasmids B-E not frozen down as bacterial stocks.	5/14/96	TG2
MP0021	*GALpPHO81-38*	GAL1 promoter inserted as a PCR product into Eag-Spe sites of pDJ4 which is a gap repaired version of EB0262. pDJ4 contains the $PHO81^c$ mutation found in strain wk38. This	5/19/96	TG2

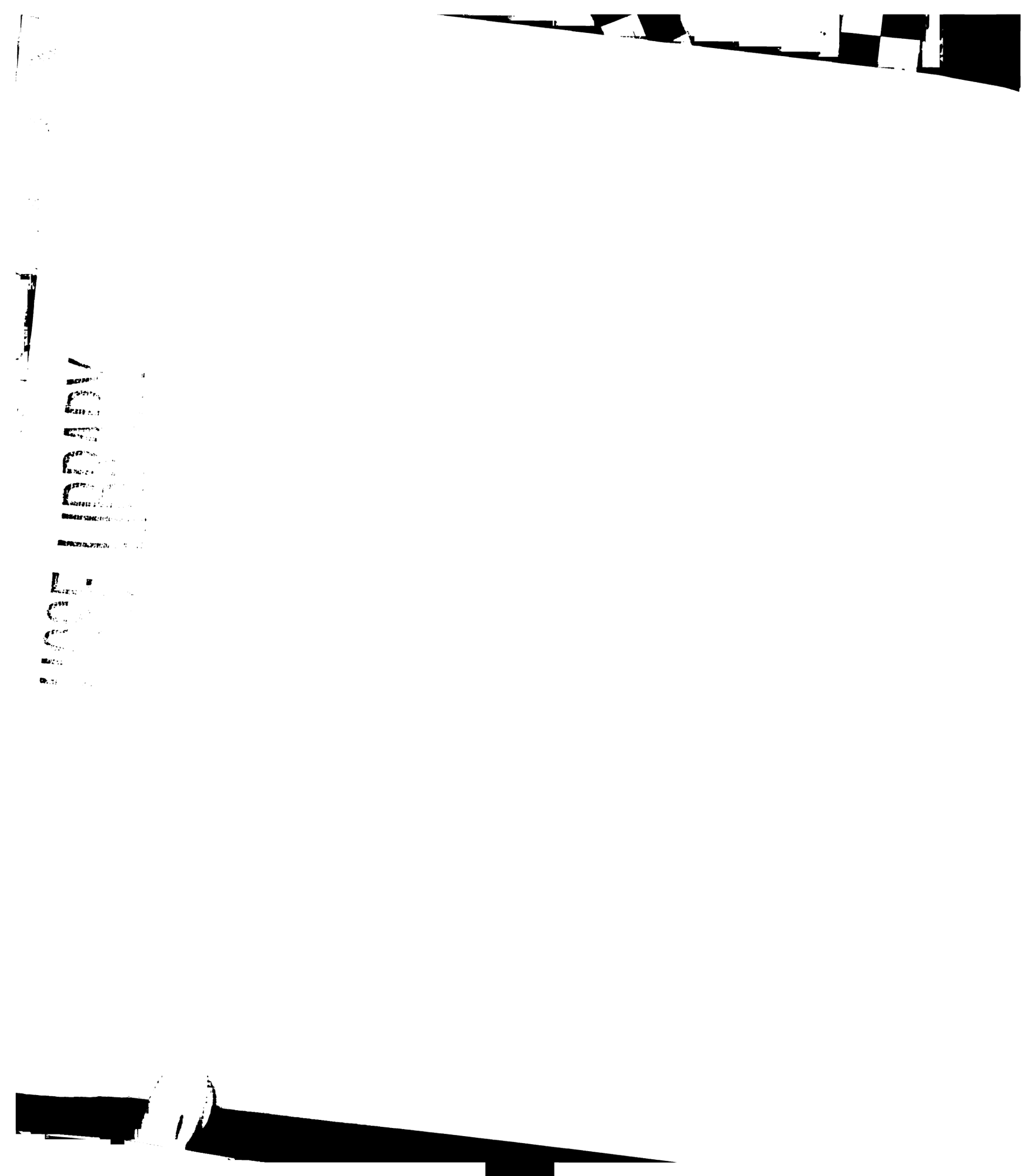
Strain	Contents	Description	Date	Host
		construct has to date only been checked by restriction digest confirming replacement of the *PHO81p* with the *GAL1p*.		
MP0022	pKS+.*GALpEFR3*.Py2	made from MP0020 by inserting *GAL1* promoter as Pst-Sph fragment just upstream of the *EFR3* ATG	5/19/96	TG2
MP0023	pET16-B with Nde linker	synthetic linker inserted at Nco site of pET16-B to create an in frame Nde site, and kill the 5' Nco site. The HIS tag in this construct goes Nde.Nco.HIS.Nde The HIS tag can be purified as an Nde fragment and inserted at the 3'Nde site in the Py2 tag present in EFR3 and PHO85. The orientation and frame of the linker has been confirmed by sequencing.	5/22/96	TG2
MP0024	pRS313.*GAL1pEFR3*.Py^2	Sal.Bam from mp022 into same sites of pRS313	5/26/96	TG2
MP0025	pRS316.*EFR3*.Py^2 (hydroxylamine mutant library)	hydroxylamine mutagenesis of mp0019(45 minutes, ~3% Ura-) This and mp0026 constitute the primary freeze of the library library contains between 1 and 2x106 members	5/28/96	TG2
MP0026	pRS316.*EFR3*.Py^2 (hydroxylamine mutant library)	see mp0025	5/28/96	TG2

Strain	Contents	Description	Date	Host
MP0027	pRS316.*PHO85*.Py[2] (hydroxylamine mutant library)	hydroxylamine mutagenesis of eb0327_(45 minutes, ~3% Ura-) This and mp0028 constitute the primary freeze of the library library contains 1 - $2x10^6$ members	5/28/96	TG2
MP0028	pRS316.*PHO85*.Py[2] (hydroxylamine mutant library)	see mp0027	5/28/96	TG2
MP0029	pRS313.*EFR3*(-Xba)	This is a cloning intermediate in which the C-terminal ~ third of the *EFR3* ORF extending into the polylinker (Xba/Xba) is removed. Now, C-terminal tags can be swapped into this vector as Xba/Xba fragments and oriented with RI/Bam double digests. This will allow for more reliable detection of recombinants versus plasmids arising from the input vector alone (as the Xba/Xba frag will often be similar in length). This vector was derived from mp013	5/29/96	TG2
MP0030	pUC18-*efr3Δ::LEU2*	Cut with Sal & Bam, disruption fragment is 3.5kb in notebook refered to as *SL28::LEU2* miniprep 6/30/95 cloning of *SL28* into pUC18 6/28 cloning of *SL28* PCR product into pKS 6/22	5/29/96	TG2

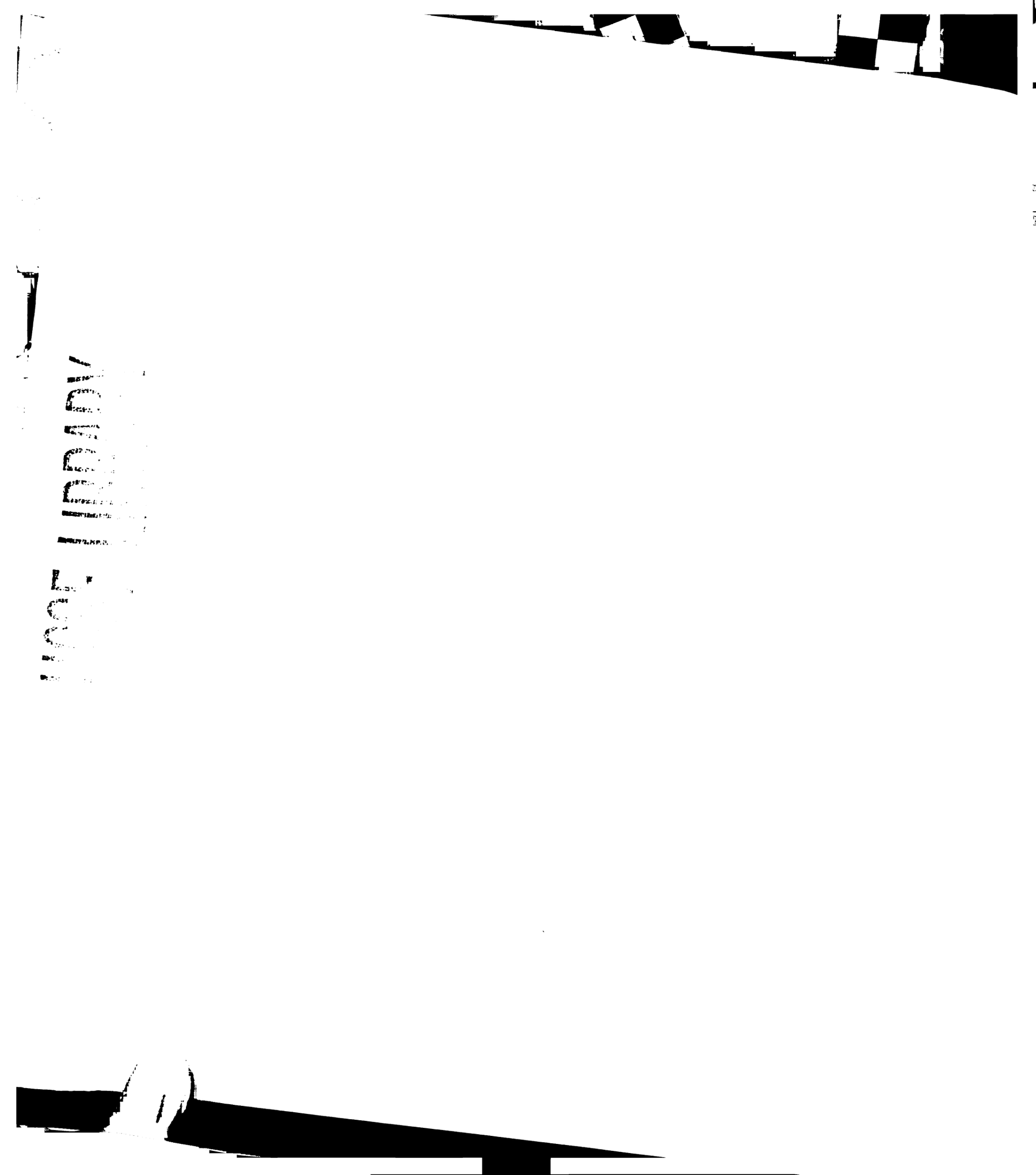


Strain	Contents	Description	Date	Host
		(see explanation of why this cloning step was necessary (not originally planned)) primer design 6/15/95		
MP0031	pKS+.*GAL1pEFR3*.Py^2.His (A)	10xHis tag from pET-16b(+Nde) mp023 cloned as Nde fragment into the Nde site at the 3' end of the Py2 tag on mp022. 10xHis tag oriented by Nco digest. Will sequence this and candidate B (mp0032) from polylinker into *EFR3_*	6/2/96	TG2
MP0032	pKS+.*GAL1pEFR3*.Py^2.His (B)	Different candidate for the same construct as mp0031	6/2/96	TG2
MP0033	pKS+.*GAL1pEFR3*.10xHis	Py^2 tag in MP022 replaced with 10xHis tag from pET-16b (EB0042) as Nco.Nde fragment. Checked by sequencing.	6/5/96	TG2
MP0034	pRS313.*EFR3*.Py^2.10xHIS	~ 1 kb Xba fragment from mp0032 containing 3' end of *EFR3* including Py^2 and 10xHIS tag cloned into Xba site of mp0029. Orientation of Xba fragment confirmed by Sal/Bam digest (2.8 kb vs 1.8 kb)	6/5/96	TG2
MP0035	pRS313.*EFR3*.Py^2.10xHIS (candidate #4)	same as mp0034	6/5/96	TG2
MP0036	pRS313-*PHO85*.Py^2	Sal.Bam from pRS315-*PHO85*.Py^2 cloned into same sites of pRS313. I'm not sure if the pRS315 version is in any strain collection.	7/7/96	TG2
MP0037	pRS313-*pho85-9*.Py^2	Sal.Bam from pRS315- *pho85-9*.Py^2 cloned	7/7/96	TG2

Strain	Contents	Description	Date	Host
		into same sites of pRS313. This allele of *PHO85* contains a mutation analogous to the temperature sensitive mutation in *cdc28-9*. This mutation seems to be cold sensitive in *PHO85*. I'm not sure if the pRS315 version is in any strain collection.		
MP0038	pRS313-*pho85-13*.Py[2]	Sal.Bam from pRS315- *pho85-13*.Py[2] cloned into same sites of pRS313. This allele of *PHO85* contains a mutation analogous to the temperature sensitive mutation in *cdc28-13*. This mutation seems to be cold sensitive in *PHO85*. I'm not sure if the pRS315 version is in any strain collection.	7/7/96	TG2
MP0039	pBS(URA3)(-Xho)	pBS(URA3) EB0431 cut with Xho, blunted and religated. Removal of Xho checked by double digest with ApaLI and Xho. Integrity of polylinker sites flanking Xho not yet checked.	7/9/96	TG2
MP0040	pRS313(-Xho)	pRS313 EB0248 cut with Xho, blunted and religated. Removal of Xho checked by double digest with ApaLI and Xho. Integrity of polylinker sites flanking Xho not yet checked. DNA made from original glycerol would not cut. Retransformed plasmid (DNA now cuts) and replaced glycerol stock (7/29/96)	7/9/96	TG2
MP0041	pKS+.*PHO85*.3'	does not contain entire ORF!!!	7/18/96	TG2

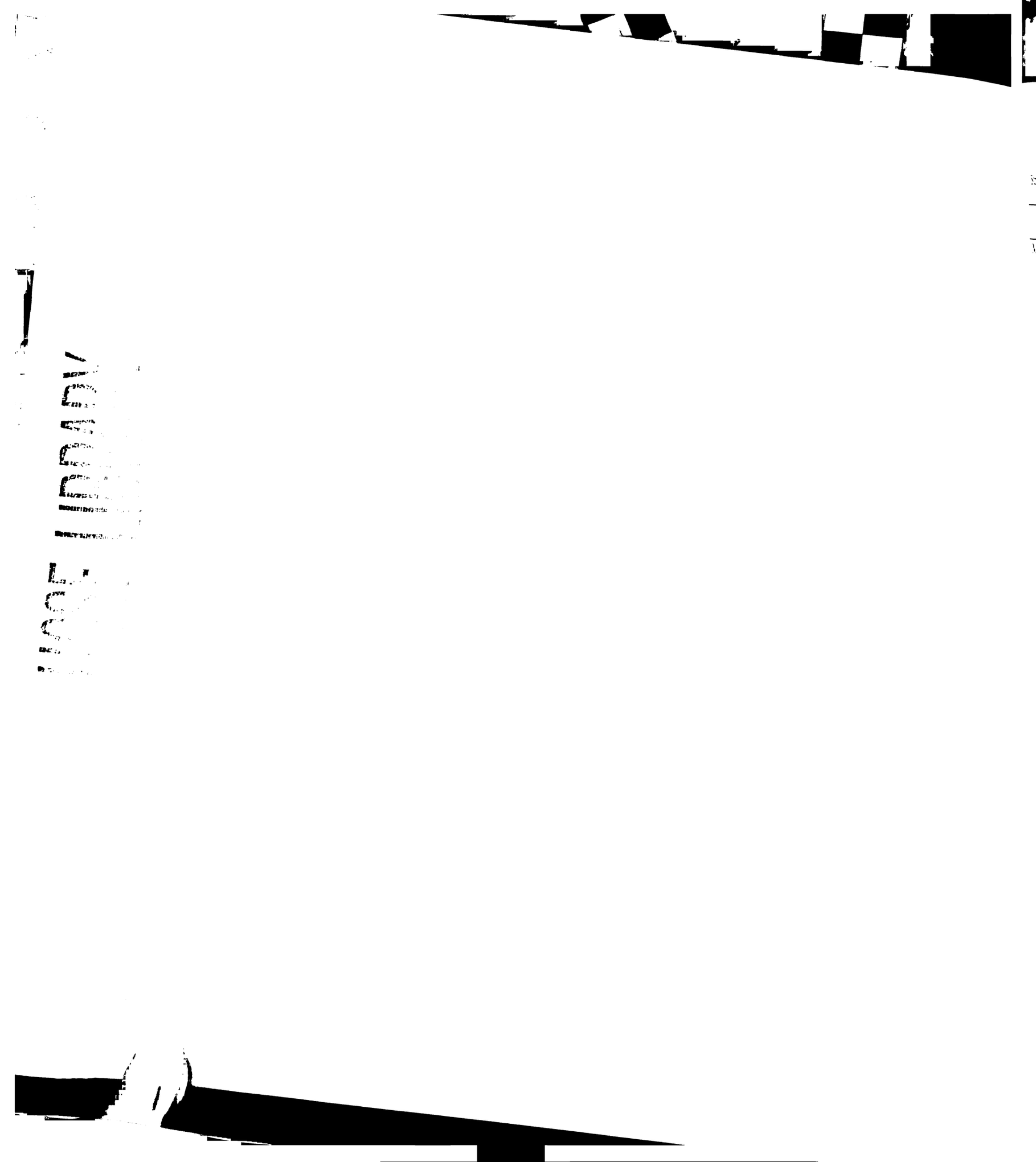


Strain	Contents	Description	Date	Host
		contains a 4.2kb Kpn.Spe fragment from EB0323 (PHO85 genomic chunk isolated from LEU2; A/C library) cloned into same sites of bluescript. The Kpn site is towards the end of the PHO85 ORF. The Spe site is ~3.8 Kb downstream of the stop codon. The genome sequence from this region indicates an Xba site @ ~1.4 Kb downstream of the stop codon which is not found in this fragment. I need to do other digests, or use this plasmid as a PCR template to confirm that it is in fact contains the right insert.		
MP0042	YEp24 Carlson-Botstein library	this glycerol stock contained approx $1.5x10^5$ cfu in one ml when frozen down. These are SURE bacteria from stratagene which were electroporated with 0.002ul of Amy Kistler's amplification of Hiten Madhani's transformation of the DNA obtained directly from Botstein	7/25/96	TG2
MP0043	YEp24 Carlson-Botstein library	see MP0042	7/25/96	TG2
MP0044	YEp24 Carlson-Botstein library	see MP0042 for description of glycerol stock this plasmid position contains 1ml of Amy Kistler's DNA diluted 1:500 in TE. 2ul of this dilution gave $2.9x10^5$ cfu when electroporated into SURE cells	7/25/96	TG2



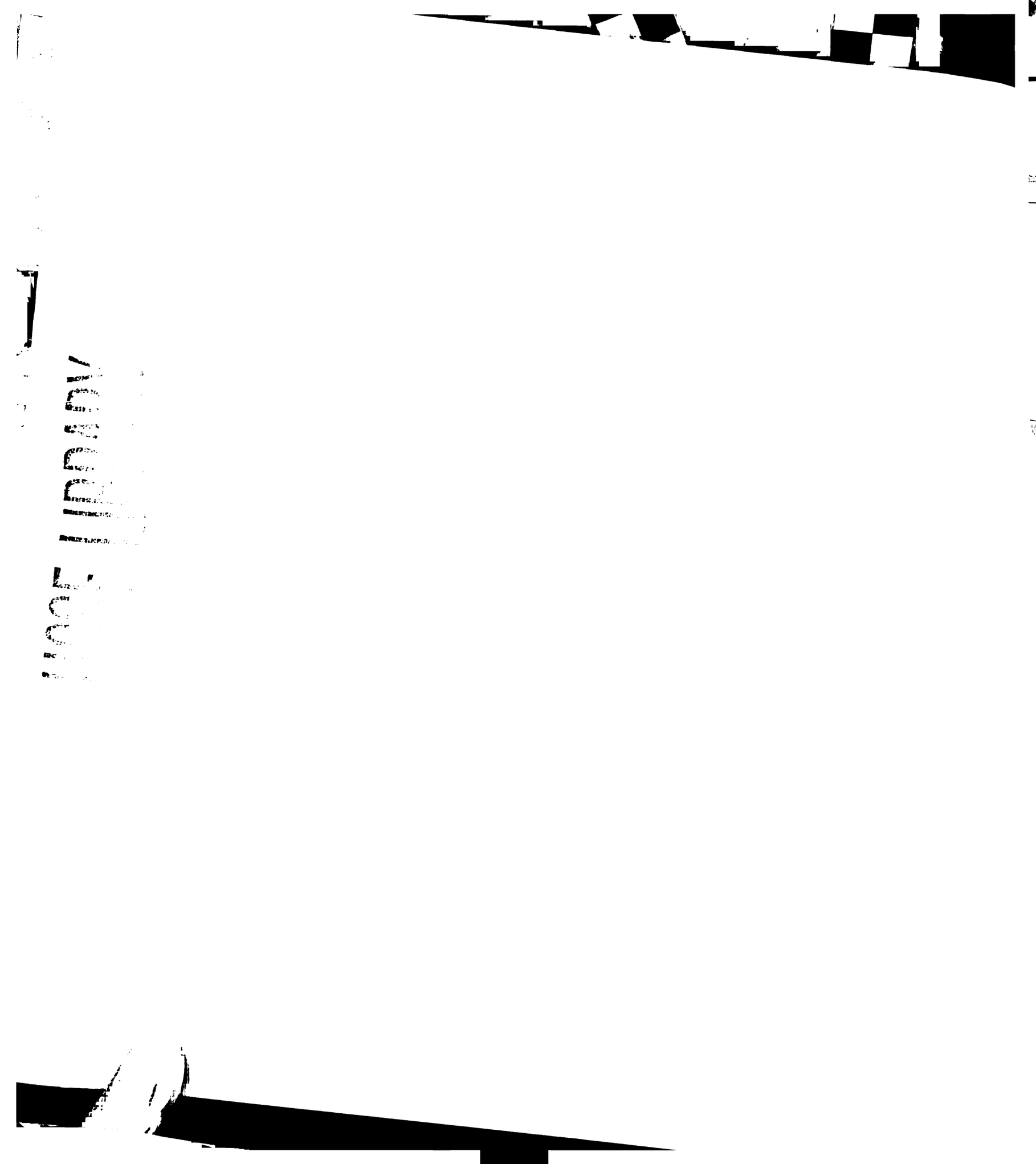
Strain	Contents	Description	Date	Host
MP0045	pKS.*PHO85*.3'	This plasmid contains an ~2kb fragment of genome immediately downstream of *PHO85*. It was generated by PCR with Vent. The primer closest to *PHO85* contains a BamHI site. The distal primer contains a Bgl II site. This product has been blunted into the SmaI site of pKS and is oriented such that the BamHI site in the PCR product is immediately adjacent to the BamHI site in the pKS poly linker. This is a cloning intermediate	8/1/96	TG2
MP0046	pRS316.*PHO85*myc.*ADE3*	contains Nhe/Sal fragment from eb0153 cloned into Spe/Sal sites of pRS316. this vector is an alternative to eb0153 (YEp24.*PHO85*myc.*ADE3*) for doing *pho85* synthetic lethal screens	8/2/96	TG2
MP0047	pBS(URA3)(-Xho).*PHO85*.3'	Bam.BglII PCR product cloned from pKS(mp045) into Bam site of mp039. Orientation of insert confirmed by EcoRI digest (1.5 vs 0.6kb). Bam site is immediately downstream of ORF at beginning of PCR product.	8/6/96	TG2
MP0048	pKS(-Xho)*PHO85*.Py^2.*PHO85*-3'.*URA3* (*PHO85* loop-in)	*PHO85*.Py^2 as Sal.Bam from EB0327 cloned into same sites of MP0047. Linearize with Xho in intron to loop-in	8/11/96	TG2
MP0049	pRS313(-Xho).*PHO85*.Py^2	*PHO85*.Py^2 cloned as Sal.Bam from EB0327 into same sites of MP0040	8/11/96	TG2

Strain	Contents	Description	Date	Host
MP0050	pKS(-Xho)*PHO85*.Py^2.*PHO85*-3'.*URA3* (*PHO85* loop-in) HA mutagenized	mp0048 mutagenized with hydroxylamine_for 45', and about 500,000 bacterial colonies amplified on plates	8/14/96	TG2
MP0051	pKS+.*PHO85*.Promoter	contains ~500bp PCR product. 5' EcoRI site. 3' BamHI site. Bam site is immediately upstream of ATG. PCRed with Taq from eb0327 as template (30 cycles). Cloned into EcoRI/BamHI sites of pKS+ (eb0077). To be used for making *pho85Δ* vector.	8/14/96	TG2
MP0052	pKS+.*PHO85*.5'.3'	2.1 kb PCR product containing sequence immediately downstream of stop codon cloned as Bam/Bgl2 from mp045 into Bam site of mp051. Insert oriented with EcoRI	8/16/96	TG2
MP0053	pKS-*pho85Δ::HIS3*	1.8 Kb BamHI frag containing *HIS3* from pJJ215 (eb0098) ligated into BamHI site of mp052. Cut with EcoRI to generate two ~3.7Kb fragments for disrupting *PHO85*	8/20/96	TG2
MP0054	pKS-*pho85Δ::TRP1*	0.9 Kb BamHI BglII frag containing *TRP1* from pJJ248 (eb0103) ligated into BamHI site of mp052. Cut with Xho and Not (in buf #3) to generate ~3.4Kb fragment for disrupting *PHO85*	8/20/96	TG2
MP0055	pKS-*pho85Δ::LEU2*	2.9 Kb BglII frag containing *LEU2* from YEp13 (eb0257) ligated into BamHI site of mp052. Cut with HindIII to generate ~4.3Kb	8/20/96	TG2

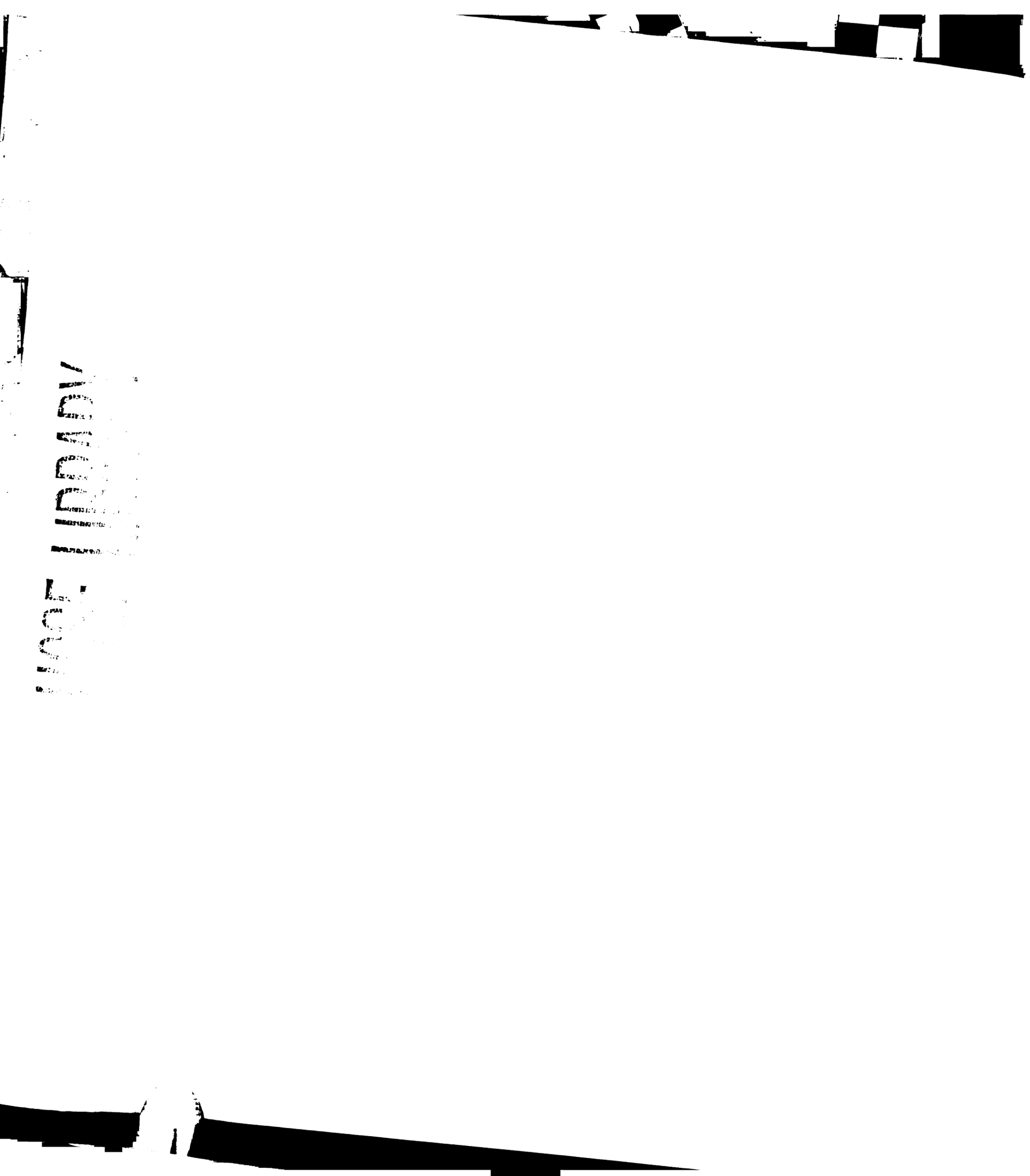


Strain	Contents	Description	Date	Host
		fragment for disrupting *PHO85*		
MP0056	pRS313.*PHO85*.Py.gapped	This plasmid is designed for gap-repairing mutagenized PCR products to regenerate *PHO85* ORF. Xho-Nco removed from mp049 and replaced with 40bp polylinker from LITMUS-29. EcoRI in this inserted polylinker is unique (as are Xho and Nco). This removes almost entire ORF. Xho is in intron, Nco is artificial at C-Term._	8/23/96	TG2
MP0057	pRS313.*pho85*.Py^2.ts-6-2	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0058	pRS313. *pho85*.Py^2.ts-6-3	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A	9/17/96	TG2

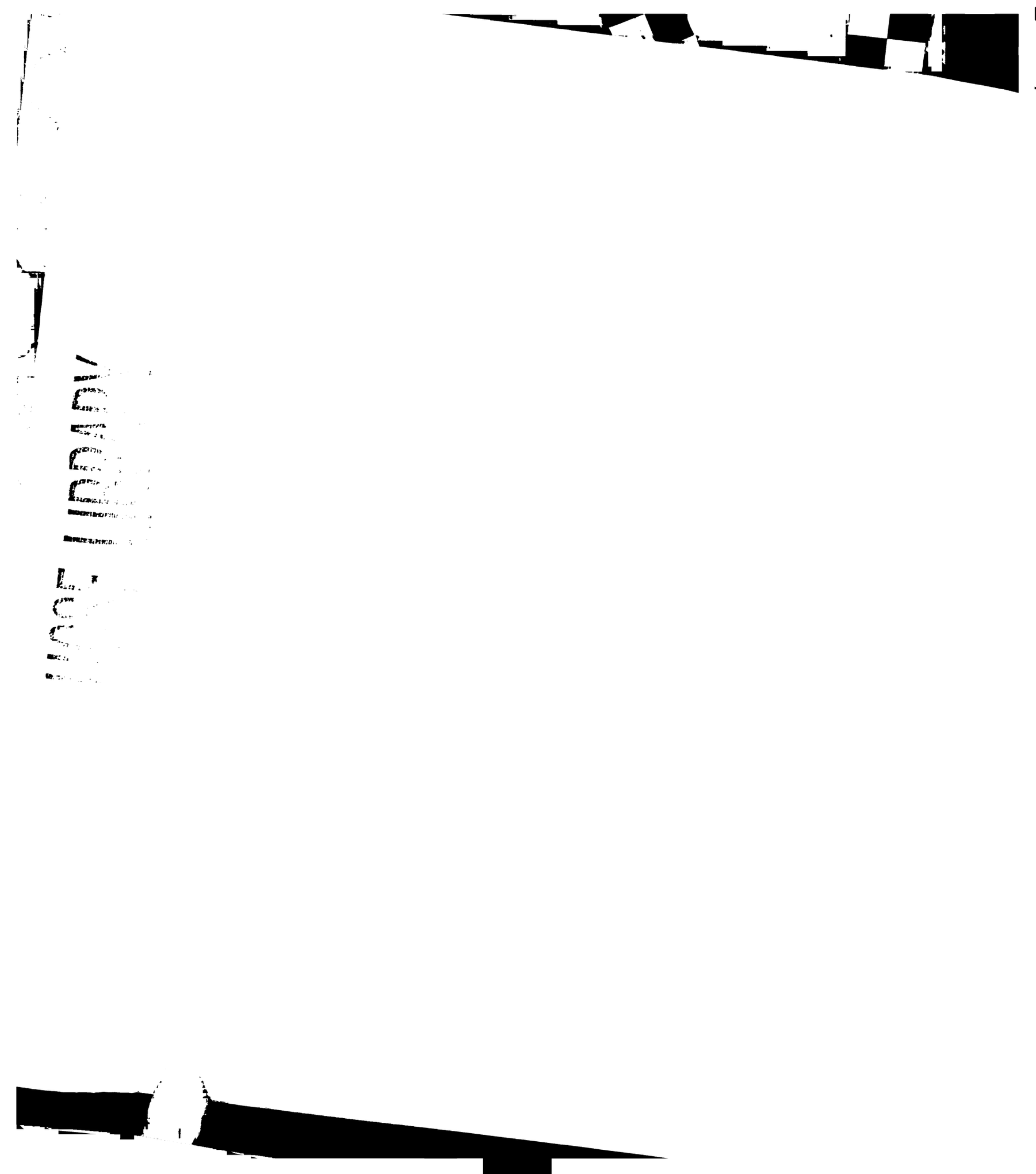
Strain	Contents	Description	Date	Host
		library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details		
MP0059	pRS313. *pho85*.Py^2.ts-8-1	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0060	pRS313. *pho85*.Py^2.ts-8-3	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a	9/17/96	TG2



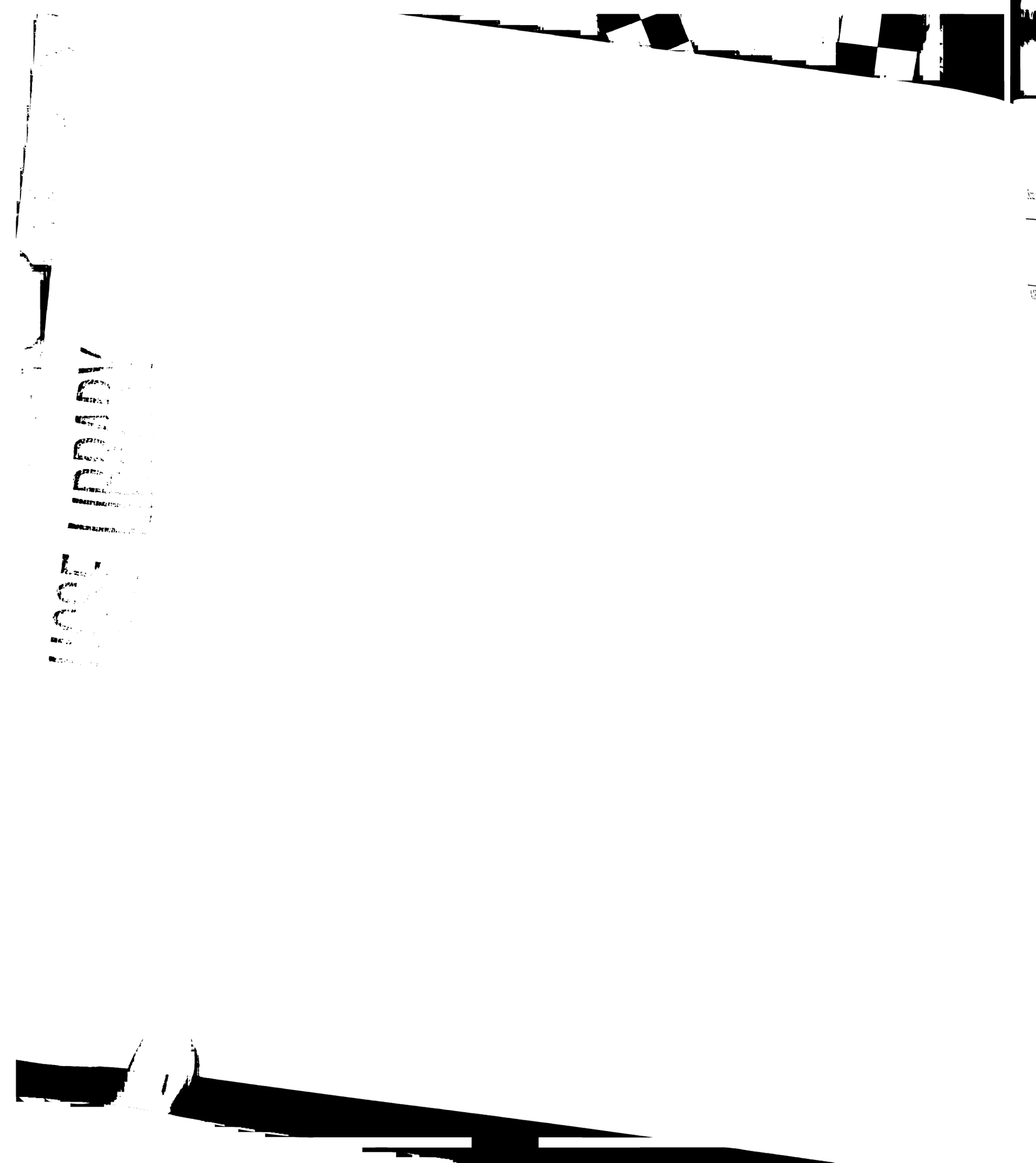
Strain	Contents	Description	Date	Host
		cln1Δ cln2Δ pho85Δ GAL1pCLN2 strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details		
MP0061	pRS313. *pho85*.Py^2.ts-8-4	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0062	pRS313. *pho85*.Py^2.ts-9-1	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and	9/17/96	TG2



Strain	Contents	Description	Date	Host
		this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details		
MP0063	pRS313. *pho85*.Py^2.ts-9-2	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0064	pRS313. *pho85*.Py^2.ts-9-3	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony	9/17/96	TG2



Strain	Contents	Description	Date	Host
		was also characterized for ts Pho^{c} phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details		
MP0065	pRS313. *pho85*.Py^{2}.ts-9-4	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^{c} phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0066	pRS313. *pho85*.Py^{2}.ts-10-1	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^{c} phenotype	9/17/96	TG2



Strain	Contents	Description	Date	Host
		and ts glycogen hyper- accumulation on galactose. See notebook for further details		
MP0067	pRS313. *pho85*.Py^2.ts-10-2	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0068	pRS313. *pho85*.Py^2.ts-10-3	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2

Strain	Contents	Description	Date	Host
MP0069	pRS313. *pho85*.Py^2.ts-10-4	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0070	pRS313. *pho85*.Py^2.ts-10-5	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0071	pRS313. *pho85*.Py^2.ts-10-6	This plasmid generated by in vivo recombination between mutagenic PCR	9/17/96	TG2

Strain	Contents	Description	Date	Host
		product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details. KpnI site in ORF is no longer present		
MP0072	pRS313. *pho85*.Py^2.ts-6-1	This plasmid generated by in vivo recombination between mutagenic PCR product and mp0056 cut with Xho and Nco. Part of the promoter, the entire ORF, and the Py tag were included in the PCR product. A library of recombinants was generated in a *cln1Δ cln2Δ pho85Δ GAL1pCLN2* strain and this plasmid was isolated from a colony which was ts for growth on dextrose. This colony was also characterized for ts Pho^c phenotype and ts glycogen hyper- accumulation on galactose. See notebook for further details	9/17/96	TG2
MP0073	*pho85-6-1*.Py^2.loop-in	Sal.Bam from mp073 cloned into same sites of mp047. Cut with Xho to loop-in	9/29/96	TG2

Strain	Contents	Description	Date	Host
MP0074	*pho85-8-3*.Py^2.loop-in	Sal.Bam from mp060 cloned into same sites of mp047. Cut with Xho to loop-in	9/29/96	TG2
MP0075	*pho85-9-3*.Py^2.loop-in	Sal.Bam from mp064 cloned into same sites of mp047. Cut with Xho to loop-in	9/29/96	TG2
MP0076	*pho85-10-6*.Py^2.loop-in	Sal.Bam from mp071cloned into same sites of mp047. Cut with Xho to loop-in	9/29/96	TG2
MP0077	pRS316.*pho85-8-3*.Py^2	Sal.Bam from mp060 cloned into same sites of pRS316 (eb009)	9/29/96	TG2
MP0078	pRS316.*pho85-6-1*.Py^2	Sal.Bam from mp072 cloned into same sites of pRS316 (eb009)	10/18/96	TG2
MP0079	pRS316.*pho85-9-3*.Py^2	Sal.Bam from mp064 cloned into same sites of pRS316 (eb009)	10/18/96	TG2
MP0080	pRS316.*pho85-10-6*.Py^2	Sal.Bam from mp071 cloned into same sites of pRS316 (eb009) KpnI site in *PHO85* ORF is dead in this mutant	10/18/96	TG2
MP0081	pLITMUS29	subcloning vector from NEB (got the poly-linker to join the Xho and Nco sites in mp056 from this vector)	10/18/96	TG2
MP0082	pBS.*MET3p.PHO4*	1.3 kB HindIII/Blunt/EcoRI fragment from pRS316.*MET3p.PHO4*-GFP+ containing MET3p.Nco.Bgl II.PHO4 from eb0421 cloned into HindIII/Blunt/EcoRI sites of bluescript. This is a cloning intermediate. Next step is to clone N-terminal Nco/EcoRI fragment of *PHO85* in frame with ATG of *MET3p*	10/24/96	TG2

Strain	Contents	Description	Date	Host
MP0083	pBS.*MET3p.PHO85*.5'	PHO4 in mp082 replaced with ~250 bp Nco-EcoRI fragment from eb082. This vector contains part of a *PHO85* cDNA with the NcoI site covering the ATG, and extending to the EcoRI site (the intron is removed). This vector contains all poly-linker sites between KpnI and Hind III (which is dead), and EcoRI and Sac	10/26/96	TG2
MP0084	pKS.*MET3p.PHO85*.5'.*URA3*	this vector contains KpnI/EcoRI fragment from mp083 (containing dead Hind III site and MET3p in frame with ATG-EcoRI of *PHO85* cDNA) cloned into same sites of pBS.*URA3* (eb0431). With the addition of 5' sequence genomic sequence upstream of the *MET3p* this will become a loop-in vector to replace the genomic *PHO85p* with the *MET3p* (and to remove the intron)	10/28/96	TG2
MP0085	pKS.*PHO85* upstream genomic region	contains PCR product which contains region upstream of *PHO85* (from ~200 upstream of ATG to 2,200 upstream of ATG) blunt cloned into EcoRV site of bluescript. The PCR product has Xho site at end nearest the ATG and Sal site at the far upstream end. This isolate is oriented such that Xho site is near Xho site in the polylinker.	11/13/96	TG2
MP0086	YEp24.*BAS1* (C-terminal	This plasmid isolated from Carlson-Botstein	11/29/96	TG2

Strain	Contents	Description	Date	Host
	deletion)	library as a high copy suppressor of *efr3Δ* *pho85-9* ts-lethal phenotype. This plasmid contains from 633942-637563 from chromosome XI which contains all but last ~400 bp (~1/6) of *BAS1* and only the first ~1/2 of *UBP11*		
MP0087	YEp13.*ADE2*	3.6 Kb Bgl II partial containing *ADE2* region ligated to BamHI linkers and cloned into BamHI site of YEp13. This plasmid from Joachim Li (BJL952) who says it is originally from Jeff Lamont. My digest with BamHI dropped out appropriate size fragment. SacchDB confirms that 3.7Kb partial contains *ADE2* ORF.	12/3/96	TG2
MP0088	pUC18-*efr3Δ::HIS3*	THIS PLASMID SUCKS! INTEGRATION AT EFR3 CAUSES DEATH!!!!! 1.4 Kb Xho/Xba fragment containing *HIS3* from pJJ217 cloned into same sites of pUC18-SL28 (see 6/95). This disruption vector is very similar to pUC18-*efr3Δ::LEU2* (mp030) except that *HIS3* fragment contains a BamHI site Cut with Sal and RI to release ~2.8 Kb disruption fragment (which is almost the same size as the backbone)	12/20/96	TG2
MP0089	pFN8 x-n *HIS4-LacZ*	*HIS4* promoter driving *LacZ* in YCp50	12/27/96	TG2

Strain	Contents	Description	Date	Host
		from fink lab: bacterial stock B929		
MP0090	AB243 *BAS1*	~12 Kb genomic clone containing bas1-complementing insert in YCp50 from fink lab: bacterial stock B1570	12/27/96	TG2
MP0091	pCB159 *GALpBAS1*	*BAS1* under the control of the *GAL* promoter from fink lab: bacterial stock B1990	12/27/96	TG2
MP0092	p63 *ADE3-LacZ*	*ADE3* promoter driving *LacZ* from fink lab: bacterial stock B2394	12/27/96	TG2
MP0093	p107 *ADE2-LacZ*	*ADE2* promoter driving *LacZ* from fink lab: bacterial stock B2403	12/27/96	TG2
MP0094	p115 *ADE1-LacZ*	*ADE1* promoter driving *LacZ* from fink lab: bacterial stock B2404	12/27/96	TG2
MP0095	p121 *ADE8-LacZ*	*ADE8* promoter driving *LacZ* from fink lab: bacterial stock B2406	12/27/96	TG2
MP0096	pCB114 lPL-*BAS1*	bacterial expression vector for *BAS1* from fink lab: bacterial stock B2411	12/27/96	TG2
MP0097	pKS-*MET3pPHO85-URA3* loop-in vector	2.2 Kb of *PHO85* upstream genomic sequence starting ~200bp upstream of ATG cloned as Sal/Xho from mp085 into Sal site of mp084. Contains ATG(Nco)-EcoRI of cDNA for *PHO85* ORF frag. Cut with HindIII to linearize within *PHO85* ORF	12/27/96	TG2
MP0098	pKS.*BAS1*.flank	fusion PCR product containing 500bp upstream of ATG and 500bp downstream of *BAS1* stop codon cloned into HindIII/Xba sites	1/3/97	TG2

Strain	Contents	Description	Date	Host
		of pKS (eb077). The 1kb PCR product contains a BamHI site in the middle for cloning in markers		
MP0099	pKS-*bas1Δ::HIS3*	*HIS3* from pJJ215 (eb098) cloned as 1.8Kb Bam frag. into Bam site of mp098. Cut with Sal I / Xba to release 2.8 Kb disruption fragment Originally I thought HindIII/Xba would work, this is quite wrong	1/8/97	TG2
MP0100	pKS-*bas1Δ::TRP1*	*HIS3* from pJJ248 (eb103) cloned as 1.8Kb Bam/Bgl II frag. into Bam site of mp098. Cut with Xho / Not to release 1.9 Kb disruption fragment	1/8/97	TG2
MP0101	pKS-*ADE2*-C-term	contains 1.2 Kb HindIII fragment from YEp13-*ADE2* (mp087) cloned into HindIII of pKS+ (eb077) the fragment does not contain complete ORF and does not extend to the very end of the ORF, to be used to try and repair *ade2*$^{-}$ allele of k699 (by transforming with HindIII digested plasmid)	1/30/97	TG2
MP0102	pKS-*ADE2*-N-term	contains 1.0 Kb HindIII fragment from YEp13-*ADE2* (mp087) cloned into HindIII of pKS+ (eb077) the fragment does not contain complete ORF it begins ~500 bp upstream of ATG, to be used	1/30/97	TG2

Strain	Contents	Description	Date	Host
		to try and repair *ade2*$^-$ allele of k699 (by transforming with HindIII digested plasmid)		
MP0103	pBA686, pET-HMK-*PCL2*		1/30/97	TG2
MP0104	pBA949, pET-HMK-*PCL5*		1/30/97	TG2
MP0105	pBA885, pET-*CLG1*		1/30/97	TG2
MP0106	pBA914b, pRSETB-*PCL1*		1/30/97	TG2
MP0107	pBA945a, pRSETB-*PCL10*		1/30/97	TG2
MP0108	pBA946a, pRSETC-*PCL8*		1/30/97	TG2
MP0109	pBA948, pRSETA-*PCL6*		1/30/97	TG2
MP0110	pBA949, pRSETB-*PCL7*		1/30/97	TG2
MP0111	pBA950, pRSETB-*PCL9*		1/30/97	TG2
MP0112	pBA619, 2μ *PCL2, LEU2*	HindIII/Sal fragment containing *PCL2* and upstream sequence cloned into *LEU2* 2μ "skorski type" vector	1/30/97	TG2
MP0113	pBA904, 2μ *CLG1, URA3*	*CLG1* with its own promoter cloned into *URA3* 2μ "skorski type" vector	1/30/97	TG2
MP0114	pBA906, 2μ *PCL5, TRP1*	*PCL5* with its own promoter cloned into *TRP1* 2μ "skorski type" vector	1/30/97	TG2
MP0115	pRS426-*PHO80*	contains EcoRI/HindIII from pAC800 (eb053) into same sites of pRS426. This is the same fragment of *PHO80* contained in pACD80 (YEp13-PHO80)	2/3/97	TG2
MP0116	pRS316-*MET3pPHO85*.Py^2	Sal/HindIII fragment from pKS-*PHO85.5'-MET3p-PHO85*.RI-*URA3* (mp097) cloned into same sites of pRS316-*PHO85*.Py^2 . This	2/3/97	TG2

Strain	Contents	Description	Date	Host
		contains full length *PHO85* as well as 2Kb of *PHO85* upstream sequence. The HindIII junction is downstream of the intron so the intron is not included in this construct. BamHI releases an ~3Kb frag from within the upstream region to just after the Py tag.		
MP0117	pRS425-*PCL6*	made by Anita Sil by subcloning fragment from a Nasmyth Library YEp13 plasmid into pRS425. I haven't got the map from her yet. This plasmid confers a "Ash1 mislocalization" phenotype. Plasmid contains 2.86 Kb NheI/Xho fragment cloned into SpeI/XhoI sites of pRS425	2/5/97	TG2
MP0118	YCp50-*PHO85*upstream-*MET3p-PHO85*.Py	Bam fragment from mp0116 cloned into Bam site of YCp50. This plasmid contains *PHO85* cDNA and considerable sequence upstream (starting @ ~200bp upstream of ATG)	2/7/97	TG2
MP0119	pKS+.*efr3*.5'.3'	This vector is a cloning intermediate for making a new *efr3Δ* vector. It contains a fusion PCR product cloned into EcoRI/Xba that contains ~500bp upstream of ATG and ~500bp downstream of stop codon with a BamHI site in the middle. It appears that either the EcoRI or the Xba site did not survive the subcloning	2/19/97	TG2

Strain	Contents	Description	Date	Host
MP0120	PRS426-*PCL1*	1.7 Kb EcoRI/Xho fragment from pFHE27 (eb0373) containing *PCL1* promoter and ORF and 3'UTR cloned into same sites of pRS426	2/19/97	TG2
MP0121	pRS426-*PCL2*	Xho/HindIII fragment from mp112 cloned into same sites of pRS426	2/21/97	TG2
MP0122	pKS+.*efr3Δ2::HIS3*.1	contains 1.8Kb BamHI *HIS3* fragment from pJJ_ cloned into same sites of mp0119. *HIS3* gene runs in the same direction as *EFR3* ORF. Cut with EcoRV/NotI to release ~2.9Kb disruption fragment	2/23/97	TG2
MP0123	pKS+. *efr3Δ2::HIS3*.2	contains 1.8Kb BamHI *HIS3* fragment from pJJ_ cloned into same sites of mp0119. *HIS3* gene runs in the opposite direction as *EFR3* ORF. Cut with EcoRV/NotI to release ~2.9Kb disruption fragment	2/23/97	TG2
MP0124	pKS+.*efr3Δ2::TRP1*.1	contains 0.9Kb BamHI/BglII *TRP1* fragment from pJJ_ cloned into same sites of mp0119. *TRP1* gene runs in the opposite direction as the *TRP1* contained in mp0125. Cut with Xho/NotI to release ~1.9Kb disruption fragment	2/23/97	TG2
MP0125	pKS+.*efr3Δ2::TRP1*.2	contains 0.9Kb BamHI/BglII TRP1 fragment from pJJ_ cloned into same sites of mp0119. *TRP1* gene runs in the opposite direction as the *TRP1* contained in mp0124.	2/23/97	TG2

Strain	Contents	Description	Date	Host
		Cut with Xho/NotI to release ~1.9Kb disruption fragment		
MP0126	pRS426-*PCL5*	Bam HI fragment from mp114 (re-tx#4) cloned into Bam site of pRS426 Saving three different clones	3/3/97	TG2
MP0127	pRS426-*PCL6*	Xho-Not fragment from mp117 cloned into same sites of pRS426	3/3/97	TG2
MP0128	pM12-*gcn4::LEU2*	from hinnebusch lab digest with Pst I /Pvu II to disrupt (Pst I is in the *URA3* gene upstream of the *gcn4* cassette)_I'm guessing about the fragment to be ~4.5 Kb it seems that the bacbone is YCp50? reference MCB 15:1220-1233 (1995)	3/16/97	TG2
MP0129	pKS.*GALpPHO85*.5'ORF	GALp cloned from pRS316.*GALpPHO4*.GFP+ (eb417) as Xho/Nco into same sites pKS.*MET3pPHO85*.5' (mp083) thereby replacing *MET3p* with *GALp* and restoring the dead HindIII piece. See mp083 for info about *PHO85* chunk This is a cloning intermediate for making loop-in *GALpPHO85*	3/25/97	TG2
MP0130	pKS+*GALpPHO85*.5'.URA 3	Xho/EcoRI from pKS+*GALpPHO85*.5' (mp129) cloned into same sites of pKS+*URA3* (eb0431)	3/27/97	TG2

Strain	Contents	Description	Date	Host
MP0131	pTAC in INVa	from Jonathon Weisman	4/1/97	TG2
MP0132	pKS+.*PHO85* upstream.*URA3*	2.2 Kb of *PHO85* upstream sequence cloned as Sal / Hind III fragment from mp085 into Xho / Hind III sites of pKS+.*URA3* (eb0431) This kills Xho site in bluescript backbone but leaves the Xho site contained at the 3' end of the *PHO85* upstream fragment. This vector can now receive *GALpPHO85* as Xho / R I fragment from mp129	4/2/97	TG2
MP0133	YEp13-*PCL7*	Plasmid obtained from Nasmyth YEp13 genomic library by colony hyb with *PCL7* probe from ... Contains sequence from Ch IX 251700-260445 with low end being near the origin *PCL7* (YIL050W) is from 258912-259769 (watson)	4/2/97	TG2
MP0134	YEp13-*PCL9*#1	Plasmid obtained from Nasmyth YEp13 genomic library by colony hyb with *PCL9* probe from ... Contains sequence from Ch IV 136311-142469 with high end being near the origin *PCL9* (YDL179W) is from 138292-139206 (watson)	4/2/97	TG2
MP0135	YEp13-*PCL9*#2	Plasmid obtained from Nasmyth YEp13 genomic library by colony hyb with *PCL9* probe from ...	4/2/97	TG2

Strain	Contents	Description	Date	Host
		Contains sequence from Ch IV 136080-142186with high end being near the origin *PCL9* (YDL179W) is from 138292-139206 (watson)		
MP0136	pKS+.*PHO85*.5'.*GALp*.*PHO85*.*URA3* (aka *GALpPHO85* loop-in vector)	*GALpPHO85* cloned as Xho/RI fragment from mp129 into same sites of mp132 (pKS.*PHO85*.5'.*URA3*) The piece of *PHO85* ORF downstream of the *GALp* is a partial cDNA (from start codon to EcoRI) linearize with Bgl II to target integration to within the ORF. The correct loop-out replaces the *PHO85p* with the *GALp* and removes the intron	4/4/97	TG2
MP0137	YEp13.*PCL8*.partial	Nasmyth YEp13 library plasmid which hybridizes to *PCL8* probe 4/6/97: This is not a useful plasmid Plasmid contains 130946-137232 of Ch XVI_*PCL8* = YPL219W = 136749-138227 Ch XVI	4/4/97	TG2
MP0138	pRS426-*PCL7*.A	contains ~ 4.2 Kb Mfe I fragment from mp133 cloned into EcoRI site of pRS426 (eb0252). Insert is oriented such that 3' UTR of *PCL7* is located closer to BamHI site in polylinker This vector contains several Kb of upstream sequence	4/11/97	TG2

Strain	Contents	Description	Date	Host
MP0139	pRS426-*PCL7*.B	This plasmid is no good. Saved the wrong miniprep_contains ~ 4.2 Kb Mfe I fragment from mp133 cloned into EcoRI site of pRS426 (eb0252). Insert is oriented such that 3' UTR of *PCL7* is located closer to Hind III site in polylinker This vector contains several Kb of upstream sequence	4/11/97	TG2
MP0140	YEp13.*PCL10*	Isolated from Nasmyth YEp13 Library using fragment from mp0107 as probe	4/14/97	TG2
MP0141	pRS315-*PHO85*.Py[2]	construct made ... summer of '95? but never frozen down until now construction is very similar to eb0327	4/17/97	TG2
MP0142	YEp351-HA-*MPK1*	from Herskowitz lab CY559, pBC95 No other information	4/28/97	TG2
MP0143	YCp50-*PKC-R398P*	*PKC1* gain of function allele Plasmid from Herskowitz lab (CY676) plasmid made in Takai lab	4/28/97	TG2
MP0144	pRS314-*BCK1-20*	*BCK1* gain of function allele_From Herskowitz Lab (CY547)_Made in David Levin's lab. (pDL636) Ref: MCB (12)172	4/28/97	TG2
MP0145	pRS426.*PCL7*.2	This construct has a trimmed version of the insert in mp139 (removing 2.4Kb of extraneous upstream sequence) 1.8Kb BamHI fragment from mp139 cloned	5/15/97	TG2

Strain	Contents	Description	Date	Host
		into BamHI site of pRS426 (eb0252). BamHI site in genomic fragment is ~500bp upstream of *PCL7* ATG (& ~130bp upstream of next ORFs ATG)		
MP0146	pRS426-*PCL9*	contains 2.1 Kb EcoRI/Xho fragment from YEp13 genomic clone (mp134) cloned into same sites of pRS426. EcoRI site is 971 bp upstream of ATG (includes ~150 bp of next ORF which is quite big) Xho site is 66bp downstream of stop codon	5/15/97	TG2
MP0147	*mpk1Δ::TRP1* plasmid	mysterious plasmid from Aaron Straight in the Murray Lab. Cut with EcoRI and Sal and TX Aaron says it works very well	5/15/97	TG2
MP0148	pKS-*CAN1-URA3*	most of *CAN1* ORF cloned as 1.6 Kb Xho/MfeI fragment from eb0472 into Xho/EcoRI sites of pKS-*URA3* (eb0431). This fragment does not contain promoter or the last ~5% of the ORF. The construct only contains one Sal site. Will try Afl II, EcoRI and Sal to loop-in. This construct is very toxic to bacteria, and strains harboring the plasmid grow very slowly.	5/18/97	TG2
MP0149	YEp13.*PTP2*	8.8 Kb genomic chunk with unknown	5/19/97	TG2

Strain	Contents	Description	Date	Host
		boundaries "containing *PTP2* -- complements sln1D" in YEp13. Plasmid from Sean O'Rourke in Herskowitz lab who got it from some woman named Irene. Sean says we should check with her before publishing anything using this plasmid		
MP0150	pKS-*PCL10*	*PCL10* from mp140 cut out as BamHI/SnaBI. BamHI is at the junction between between YEp13 and the insert (in the *PCL10* promoter region -- BamHI site made by Sau3A). SnaBI is just downstream of the *PCL10* stop codon. This 2.6Kb fragment cloned into BamHI/EcoRV sites of pKS+ (3B077)	5/20/97	TG2
MP0151	pRS425-*PTP2*	~3Kb Taq PCR product with HindIII (5') and Xba (3') ends amplified from mp0149 cloned into HindIII/Spe sites of pRS425. Identity of PCR product confirmed by EcoRI digest. Not yet checked for functionality	5/23/97	TG2
MP0152	pRS426-*PCL10*	Insert from pKS-*PCL10* cloned into pRS426 as ... Use mp159 for complementing yeast! This plasmid does not contain the full *PCL10* promoter	5/23/97	TG2
MP0153	pRS425-*YNL074c*	2.6 Kb Sal/Bam fragment from eb0567 cloned into same sites of pRS425. This vector contains all of *YNL074c* and upstream	5/23/97	TG2

Strain	Contents	Description	Date	Host
		intergenic region. It also contains C-terminal 2/3 of *YNL075w*, and only the first little bit of *MSK1* (with promoter)		
MP0154	YEp13.*PCL8*	Plasmid isolated from Nasmyth library by colony hyb using probe from Brenda Andrew's plasmid Ends not sequenced, but appears to contain considerable upstream and downstream sequence (by Kpn I digest)	5/26/97	TG2
MP0155	YEp13.*PCL10*.2	Plasmid isolated from Nasmyth YEp13 library by colony hyb with probe from mp0150. By AseI digest, this insert is likely to contain the upstream intergenic region. By HindIII digest the insert probably contains considerable downstream sequence with the promoter region of *PCL10* distal to the origin of YEp13	6/3/97	TG2
MP0156	YEp13.*PCL10*.3	Plasmid isolated from Nasmyth YEp13 library by colony hyb with probe from mp0150. By AseI digest, this insert is unlikely to contain the complete upstream intergenic region. It may be identical to mp140. By HindIII digest the insert probably contains considerable downstream sequence with the promoter region of *PCL10* distal to the origin of YEp13	6/3/97	TG2

Strain	Contents	Description	Date	Host
MP0157	YEp13.*npt1Δ*	This plasmid is a derivative of pcl1-ts highcopy suppressor =4.8 eb563 in which most of the *NPT1* ORF has been removed leaving only c-terminal stub and the n-terminally truncated allele of *PTP2* eb563 cleaved with SphI and religated. 0.9 Kb dropout (not including YEp13 sequences deleted) confirmed by Pvu II digest.	6.8.97	TG2
MP0158	pRS426.*PCL8*	2.6 Kb Hind III Bgl II fragment from mp154 (YEp13.*PCL8*) cloned into HindIII Bam HI sites of pRS426	6.8.97	TG2
MP0159	pRS426.*PCL10*.full promoter	1.X Kb Bgl II fragment from YEp13.*PCL10*.2 (mp155) cloned into Bam/Bgl II digest of pRS426.*PCL10* (mp152). Insert oriented with Mfe I/ Cel II (Esp I) digest. Use this plasmid for complementing yeast	6.15.97	TG2
MP0160	pRS426.*PCL10D*	This plasmid contains the Bgl II fragment in mp0159 in the opposite orientation such that the promoter and first ~500 bp of the ORF are in the antisense orientation. It is unlikely to complement *pcl10Δ* and thus could serve as a control (saved only because it was a by-product of mp0159) 1.X Kb Bgl II fragment from YEp13.PCL10.2 (mp155) cloned into Bam/Bgl II digest of pRS426.PCL10 (mp152). Insert confirmed by	6.15.97	TG2

Strain	Contents	Description	Date	Host
		Sac II / Esp I digest and oriented with Mfe I/ Cel II (Esp I) digest (couldn't see the ~150 bp band)._		
MP0161	pKS.*ADE3.URA3*	2.8 Kb Xho/Nhe I fragment containing almost the full length *ADE3* gene (missing promoter and first ~350 bp of ORF) cloned from eb0151 into Xho/Xba sites of pKS-*URA3* (eb0431) First step in making a loop-in-out *ade3Δ* vector	6/15/97	TG2
MP0162	pKS-*ade3Δ-URA3*	1.5 Kb Hind III fragment from mp0161 removed This vector is a loop-in loop-out vector for removing a large chunk of the *ADE3* ORF. Cut with Bgl II to direct loop-in. Both correct loop-in and loop-out should be Ade3$^-$	6/17/97	TG2
MP0163	pKS-*ptp2Δ*	fusion PCR product containing ~500bp upstream of *PTP2* ATG, BamHI site, and ~360bp downstream of stop codon cloned into Hind III / Xba sites of pKS (eb077) cloning intermediate for making *ptp2Δ* vector	6/19/97	TG2
MP0164	pRS426-*ptp2Δ*	fusion PCR product containing ~500bp upstream of *PTP2* ATG, BamHI site, and ~360bp downstream of stop codon cloned into Hind III / Spe sites of pRS426 (eb252) could be used to do PCR mutagenesis of *PTP2*	6/19/97	TG2

Strain	Contents	Description	Date	Host
MP0165	pRS316-*ptp2Δ*	fusion PCR product containing ~500bp upstream of *PTP2* ATG, BamHI site, and ~360bp downstream of stop codon cloned into Hind III / Xba sites of pRS316 (eb009) could be used to gap repair genomic *PTP2* ORF	6/19/97	TG2
MP0166	pKS-*ptp2Δ::LEU2*	*LEU2* inserted into BamH I site of mp0163 as ~2.9 Kb Bgl II fragment from YEp13. The insert is oriented with the *LEU2* Sal I site being closer to Sal I site in pKS poly linker indicating that *LEU2* transcription runs opposite to that of the deleted *PTP2* gene. Cut with Hind III and Xba to release ~3.7 kb disruption fragment	6/22/97	TG2
MP0167	pSWM29 (2-micron HA-*HOG1-HIS3*)	plasmid from Haruo Saito's lab. Reference is MCB (1997) 17:1289-1297	6/30/97	TG2
MP0168	pDBT2 (*ADH1pPTP2*, 2-micron, *LEU2*)	plasmid from Haruo Saito's lab. Reference is MCB (1997) 17:1289-1297. Backbone is pDBL2 (G&D 7:1755-1765) "2.65 Kb AvrII (blunt) Xba (blunt) from pPTP21 inserted at HindIII (blunt) of pDBL2. Xba site is regenerated"	6/30/97	TG2
MP0169	pDBT2 (*ADH1pPTP2-C/S*, 2-micron, *LEU2*)	plasmid from Haruo Saito's lab. Reference is MCB (1997) 17:1289-1297. Dominant negative Cys666Ser mutation Backbone is pDBL2 (G&D 7:1755-1765)	6/30/97	TG2

Strain	Contents	Description	Date	Host
		"0.2 Kb Nco/Pst from pPDS21 replaces corresponding fragment from pDBT2"		
MP0170	2 micron-*GPD-PCL1*-Py^2-*URA3*	HA3 tag of eb0128 replaced with a Not fragment containing Py^2 stop. This Not fragment generated by PCR using eb0324 as a template. PCR product also contains an Nco site just 3' of 5' Not site, and an Nde site just 5' of stop codon. Stop codon is followed by a Bam site and then the 3' Not site (see eb0324 and my notebook ~7/14 for details). Orientation checked by Pst / Nco.	7/21/97	TG2