2006

The Identification of RCY339 as a K.lactic cdc14 Mutant

Diana Zeilicovici
University of Redlands

The Identification of RCY339 as a *K. lactis cdc14* Mutant.

Diana Zeilicovici
Advisor: Linda Silveira
Honors Thesis
27 May 2006

Abstract

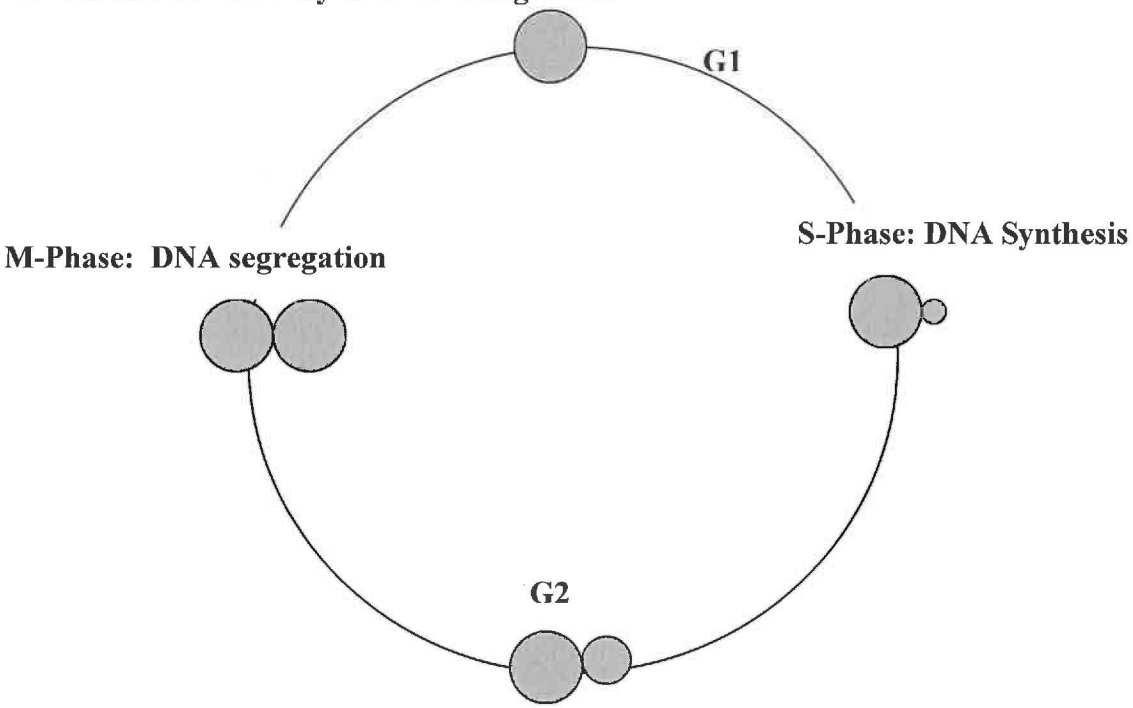
In budding yeast there exist regulators that control the steps in the process of forming the new daughter cell. Some of these regulators are called the mitotic exit network (MEN), which help the cell go from mitosis to G1.

We have been studying cell cycle control in the budding yeast *K. lactis*. Our *K. lactis* temperature sensitive mutant, RCY339, arrests after anaphase, which is suggested by its large bud, segregated DNA, and elongated spindle at the non-permissive temperature. A candidate gene altered in our mutant is *CDC14*, the last gene in the MEN pathway. A wild type copy of the *K. lactis CDC14* gene complements the temperature sensitive defect in RCY339. However, it remained to be determined whether suppression was occurring, or if *CDC14* was indeed mutated. In this study, classical genetic tests were used to confirm whether or not *CDC14* contains the mutation that has caused RCY339 to arrest after anaphase. Results from these tests verified that the *ts* mutation in RCY339 is linked to the *CDC14* locus. The *cdc14* allele of RCY339 was sequenced, to show that a mutation indeed existed, causing an amino acid change from a conserved proline to a serine in the encoded protein. Due to similarities observed in the *cdc14* mutant in *K. lactis* and *S. cerevisiae*, a *S. cerevisiae cdc14* mutant was transformed with a wild type copy of *CDC14* from *K. lactis*. Our transformant was no longer temperature sensitive, leading us to believe that Cdc14 proteins from *S. cerevisiae* and *K. lactis* are functionally equivalent.

Introduction

Cell division is essential for the life of all organisms. Without the ability for a cell to divide and duplicate, the organism could no longer grow. The process of cell division is called the cell division cycle. The organism we are studying is a budding yeast, meaning that it divides by producing a bud that continues to grow off of and become identical to the parent cell. The budded cell then breaks off and goes on to replicate and produce another bud and sister cell. Figure 1 shows the cell division cycle of budding yeast. (Alberts *et al.*, 2002)

Figure 1: The Cell Division Cycle of Budding Yeast



This cycle contains two main components which are the S and M phases. The S phase is where DNA synthesis/duplication occurs. M phase is mitosis, where DNA segregation takes place and the newly formed cell begins to separate as a daughter cell during cytokinesis. In between these two phases are the G1 and G2 phases which are gaps that occur to allow for repair of any mutations that may exist in the DNA due to its synthesis/duplication and allow for transition between the S and M phases (Alberts *et al.*, 2002). The steps are regulated by positive factors,

which promote cell cycle progression, and negative factors, which cause the cycle to stop in case of a disruption. A defect in the positive regulators would cause the cell cycle to halt, whereas a defect in the negative "checkpoint" regulators would cause progression to continue despite damage. In the first case, no new cells would form. In the latter case, new cells might be damaged and continue forming that way.

The replication of unhealthy/diseased cells has been found to be a major cause of cancer (Alberts *et al.*, 2002). For cancer, both positive and negative regulators are important. Inappropriate use of positive regulators can lead to increased cell cycle progression. Lack of negative regulators can lead to replication of damaged cells, some of which will have yet more defects in cycle regulation. Positive regulators can serve as drug targets for interfering with progression. Studies done to understand the cell division cycle and its mutations could potentially give a cure for cancer. By fully understanding the mechanisms of the complexes involved in the cycle, the malfunction in cancer cells could be identified and what we know about the malfunction could be used to fix the problem (i.e. cause the cells to stop replicating by using a drug to target a gene product).

Primary regulators driving progression in each of the stages of the cell division cycle are kinases called cyclin dependent kinases (CDKs), which rely on the presence of cyclin proteins to be activated. There are various types of cyclin, each of which corresponds to a stage of the cell cycle. There are different components in the cell that signal the production of cyclin which then activate the CDKs and allow them to go on and phosphorylate the appropriate targets. These targets then control and allow DNA synthesis and segregation to occur. Directly and indirectly, CDKs regulate the activity of DNA replication, spindle function, and even the activity of other

CDKs by affecting cyclin synthesis and degradation. Since each stage in the cycle is regulated by the presence or absence of cyclin, cyclin levels are key in the progression through the cell division cycle (Jaspersen *et al.*, 1998).

Another type of cell cycle regulators is ubiquitin ligases. One such ubiquitin ligase is the anaphase promoting complex (APC). The APC is activated by a previous cell cycle factor and goes on to ubiquitinate cyclin. The ubiquitinated cyclin is targeted for degradation by the proteosome allowing for mitotic CDKs to be inactivated and to allow cells to exit mitosis and enter into G1 (reviewed in McCollum and Gould, 2001).

Leland Hartwell won a Nobel Prize for his work on the genetic control of the cell division cycle in the budding yeast *Saccharomyces cerevisiae* beginning in the 1970's (Hartwell *et al.*, 1970). Hartwell wanted to see if the cell cycle is controlled by the same genes at each stage of the cycle as well as identifying these genes, and as a result started cell division cycle (cdc) studies in yeast. Hartwell looked for strains with mutations that halted cell cycle progression. These mutants would presumably be defective in factors required to drive the cycle forward, such as positive regulators or cell cycle machinery (Hartwell *et al.*, 1970).

How can one study mutants with defects in something required for growth of the cell? By creating temperature sensitive mutants, Hartwell was able to phenotypically characterize the strains he made. The reason these temperature sensitive mutants could be characterized was because the temperature sensitive allele allows for recovery of mutants with defects in essential functions. When a temperature sensitive mutant is exposed to a permissive temperature (24°C), the proteins produced function normally. When the temperature sensitive mutant is put at a restrictive temperature (i.e., 37°C), the proteins encoded by the mutated gene are unable to

function due to denaturing, and the mutant arrests. A wild type strain would still function at the restrictive temperature. Temperature sensitivity is a convenient form of testing for mutants, in that all one needs to do is put replicated plates at the permissive and restrictive temperatures and screen for results as opposed to using different mediums or solutions that cannot be interconverted as easily. At the restrictive temperature, the mutant should not be able to undergo a certain process.

Every protein in the cell division cycle works together in one way or another to initiate, terminate, or execute a function (i.e. exit from mitosis, cytokinesis, DNA replication, etc.). The point at which a job of a particular protein is done is known as the execution point. If a mutation exists in a gene that encodes for an essential Cdc protein, its job isn't done, and the mutant stops growing at what is known as its termination point. The termination point is often a checkpoint that screens to see that the job was done.

Hartwell separated strains with *ts* mutations in *cdc* and non-*cdc* functions using a morphological screen at non-permissive temperatures. Morphology of both *cdc* and non-*cdc* mutants can be viewed when exposing the cells to the restrictive temperature. Different *cdc* mutants are identified by the size and shape of the growing bud as well as the position of the DNA when arrest occurs. Each *cdc* strain has a characteristic cellular morphology at its termination point. *cdc* mutants can be distinguished from non-*cdc* mutants because bud size alters with cycle progression (Figure 1), so bud size is a quick indicator of cell cycle position. Non-*cdc* mutants are identified by non-uniform arrest morphology.

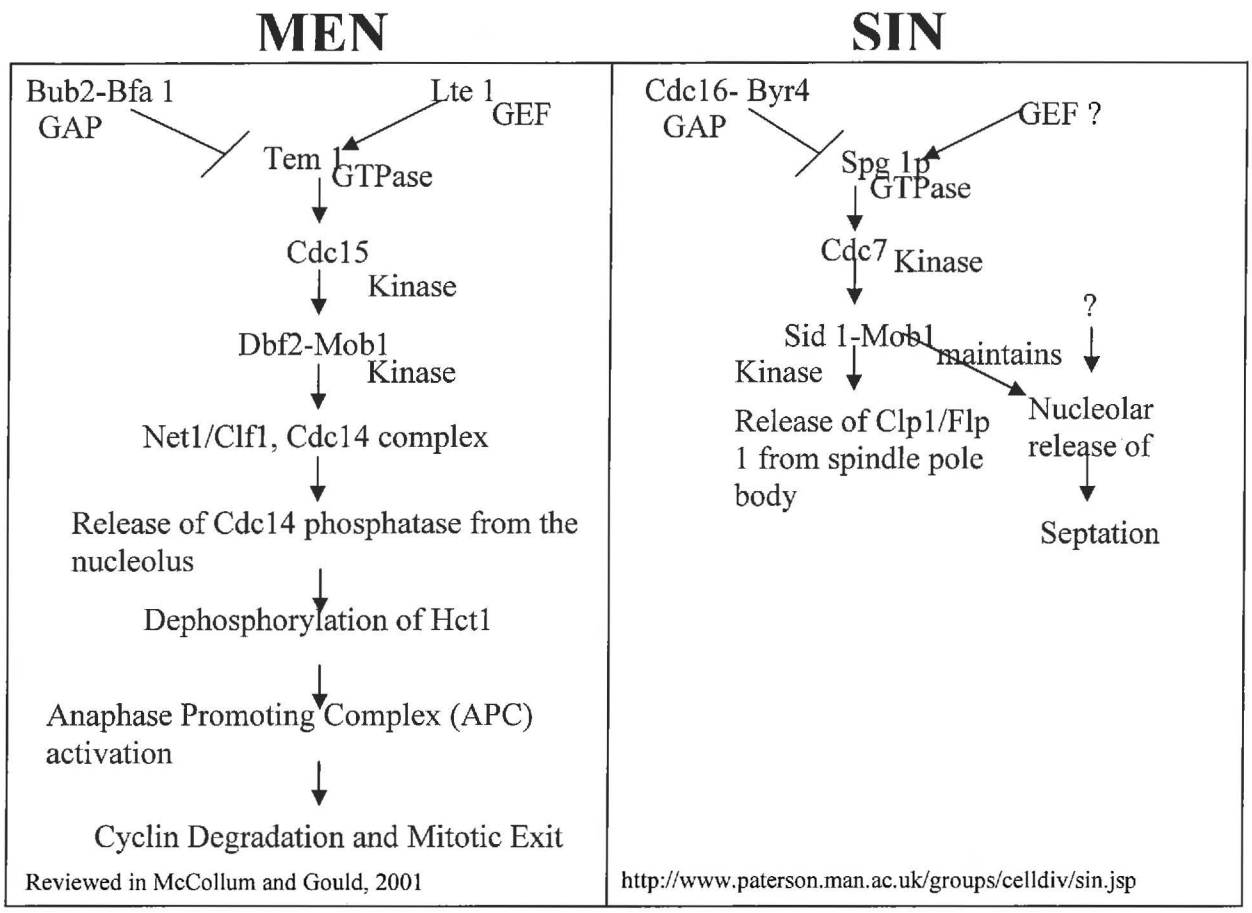
As shown in Figure 1, the cell division of a budding yeast has distinct stages, and each of the stages can be identified by the size of the bud and the position of the DNA in the dividing cell.

By looking at these characteristics, mutants can be classified by the phenotype they have when they arrest. For example, if a population of cells have arrested and the cells in the population all have a morphology of large buds, one can assume that the cells are stuck in mitosis, mitotic exit, or cytokinesis. Further analysis of the DNA's location in the cells would give further clues as to which point in the cell division cycle the mutant is arrested in.

Much research has been done not only on *S. cerevisiae,* but also on a fission yeast called *Schizosaccharomyces pombe* (*S. pombe*). Fission yeast forms by elongation of a rod-like cell that divides with formation of a septum (reviewed in McCollum and Gould, 2001). The experiments that have been done on these two types of yeast have led researchers to the conclusion that the two strains have two similar yet distinct mechanisms in mitosis.

One example of similar but non-identical regulation would be the MEN and SIN pathways illustrated in Figure 2.

Figure 2: Flowchart of the MEN pathway in *S. Cerevisiae* and the SIN pathway in *S. pombe*



In *S. cerevisiae*, the regulatory system shown to initiate the exit from mitosis is the mitotic exit network (MEN) (Jaspersen *et al.*, 1998). It is thought that the first protein in this network is Tem1, a GTPase. Tem 1 is located on the spindle pole body, and as it begins to move with the duplicated DNA into the newly formed bud, it comes into contact with a guanine exchange factor (GEF) called Lte1 which is located on the bud neck. It is thought that both proteins need to be in the same bud in order to be activated, giving the cell a way to detect whether DNA segregation has occurred and signal the initiation of mitotic exit. When the GEF binds to the GTPase it exchanges the GDP that is on the inactive Tem1 for a GTP which then activates Tem1 (Bardin *et al.*, 2000). Once activated, Tem1 goes on to activate the next protein in the MEN, and a cascade

effect takes place until a protein phosphatase, Cdc14, is released from the nucleolus. Once released, Cdc14 dephosphorylates Hct1 which then goes on to activate the anaphase promoting complex (APC). Once activated, the APC goes on to ubiquitinate mitotic cyclins. These cyclins then signal for their degradation by the proteosome and then allow for mitotic CDKs to be inactivated. This then allows for exit from mitosis and entrance into cytokinesis. (reviewed in McCollum and Gould, 2001)

The homologous network in *S. pombe* is known as the septation initiation network (SIN). The major difference that exists between the MEN and SIN is that instead of regulating mitotic exit like the MEN, the SIN regulates cytokinesis (Figure 2). Spg1p is a GTPase in *S. pombe* homologous to Tem1 in *S. cerevisiae.* Attached to the spindle pole body as well, Spg1p acts in the same way as Tem1 only instead of initiating mitotic exit, it initiates septation. At the moment it is not known which GEF is responsible for the activation of Spg1p, but once activated it goes on to activate the other proteins in the SIN which eventually cause the septum to form and allow cytokinesis to occur. (McCollum and Gould, 2001) Further research on *S. pombe* revealed a *CDC14* homolog called *clp*1 or *flp1* (Trautmann *et al.*, 2001; Cueille *et al.*, 2001). Though also not required for mitotic exit as with the MEN in *S. cerevisiae,* it is also located in the nucleolus and is released in order to carry out its functions. Though Clp 1 is not reliant on the SIN to be released from the nucleolus, the SIN does keep Clp1 from returning to the nucleolus until cytokinesis has taken place. It is thought that Clp1 inhibits CDK activity until the process finishes making sure that the current cell cycle is completed before the next begins. (Gould *et al.*, 2001). Though regulating different aspects of the cell division cycle, the components of the SIN are similar to those of the MEN.

Why do these cells use similar regulators in distinct ways? How does this relate to the biology of budding yeasts versus fission yeast? How can these questions be addressed?

In order to further understand the mechanisms cell cycle regulation in budding and fission yeasts, a different yeast may be studied. By looking at a different genus of yeast, we can see how it uses the MEN/SIN pathways, and see what biology it has in common or not in common with *S. cerevisiae* or *S. pombe.* Once the pathway of this different yeast is understood, it can lead to a clearer understanding of the functions of each component in one pathway or the other.

Our project looks at this third, different yeast. A series of experiments were performed to create mutants using mutagens such as ultraviolet light and ethylmethyl sulfonate on a budding yeast called *Kluyveromyces lactis* (*K. lactis*). Little information is presently known about the *K. lactis* cell cycle, a reason for which our research is important. Using a method similar to that of Hartwell's, mutants were created and screened for temperature sensitivity. Of one hundred and forty one temperature sensitive mutants examined using phase microscopy, eleven of them contained mutations in crucial cell division cycle genes. These eleven mutants underwent complementation testing to place them in complementation groups. Results showed that most of the mutants contained mutations in different cell division cycle genes, however, two pairs of mutants were in the same complementation groups. (Oldroyd, 2002)

Of these mutants, we focused on RCY339 which has large buds at arrest. Results from DAPI staining of RCY339 showed that DNA segregation has occurred (Oldroyd, 2002), and tubulin staining showed that the spindles have not yet been broken down (Silveira, personal communication).

RCY339's phenotype is more similar to budding yeast mitotic exit defects then to cytokinesis defects. *S. cerevisiae cdc* mutants 14 and 15 are MEN mutants, because these cells arrest with large single buds, segregated DNA, and spindles that have not yet broken down (Hartwell *et al.*, 1973). In contrast, study of Hartwell's mutants showed that *S. cerevisiae cdc* mutants 3, 10, 11, and 12 exhibited a phenotype of cells defective in cytokinesis (Hartwell, 1971). The DNA had fully segregated and the cells, despite not breaking apart, continue to form new cells/buds. These results lead us to conclude that our mutant is more MEN-like because our results show the same characteristics as Hartwell's MEN mutants. (Hartwell *et al.*, 1973)

RCY339 allows us to test the hypothesis that budding yeast monitor nucleus migration to the bud neck. The use of the MEN/SIN regulatory pathway in mitotic exit is thought to be critical to budding yeast because of their mode of cell division. Unlike *S. pombe, K. lactis* not only buds, but moves its nucleus to the bud neck and therefore it would make sense that it would require a mechanism similar to that of the MEN in order to monitor nuclear placement as opposed to using the SIN (as in *S. pombe*) for septation and cytokinesis. If we can understand the nature of the defect in RCY339, it will help us accept or reject the hypothesis that budding is the key reason why cells use the MEN in this way. RCY339 does appear to have a defect in the MEN/SIN because in previous work, the RCY339 mutant was complemented with a *K. lactis CDC14* gene on a plasmid (Wallace, 2002).

The major question of the current experiment was whether or not our mutant RCY339 was indeed a *cdc14* mutant, or if extra copies of *CDC14,* even one, was suppressing the actual mutation. Suppression would be caused by an overexpression of the Cdc14 protein. Because of the excess Cdc14 protein, a mutation in another gene could be suppressed, and allow the cell to exit mitosis. For example, both *cdc14* mutants and other MEN mutants have been shown to

function with extra copies of *CDC14* (Jaspersen *et al.*, 2001; Jimenez *et al.* 2001). Though Cdc14 is sequestered in the nucleolus by Net1/Cf1p, it can be released from this complex by the upstream MEN proteins such as Cdc15, Dbf2, and Tem1 (Shou *et al.*, 1999) (Figure 2). If a mutation exists in any of these, Jimenez *et al.* (2001) argued that an overexpression of Cdc14, even in a single copy, could "disturb the stoichiometric balance between Cdc14" and its Net1/Cfi1p complex, giving an active amount of Cdc14 released from the nucleolus, therefore not needing the function of Cdc15, Dbf2, or Tem1 to release it. Jaspersen *et al.* (1998) showed that even a single extra copy of *CDC14* was able to "restore growth" for *cdc15, dbf2,* or *tem1* mutants at the restrictive temperatures. When extra copies are present, Cdc14 could saturate nucleolar binding sites and therefore be released out of the nucleolus. Despite the fact that some Cdc14 cannot be released from the nucleolus, overexpression of it gives some amount of non-sequestered Cdc14 which then plays its role in the cells' exit from mitosis, suppressing the existing MEN mutant(s). This MEN-independent release could then lead to the activation of cyclin degradation and therefore the deactivatavation of CDKs, and consequently exit from mitosis. If suppression was to be occurring in our mutant, and another gene besides *CDC14* was mutated, a good copy of *CDC14* inserted from the plasmid would give an overexpression of Cdc14 and therefore suppress the mutation that did exist.

We can test whether RCY339 is a *cdc14* mutant by using a genetic approach. A previously constructed strain where the *CDC14* locus of RCY339 was marked by integration of a plasmid was used. This plasmid contained a wild type copy of *CDC14* and a *URA3* marker. Integration yielded two tandem copies of *CDC14* separated by the *URA3* marker. In this work, crosses were done in order to determine the linkage of the plasmid to the *ts* mutation. If the plasmid was indeed integrated into a chromosome containing a mutated copy of *cdc14,* the results from tetrad

analysis of a cross between an integrant and a wild type strain should ideally reveal that all the spores grow (Ts^+). Because a wild type *CDC14* gene is now at the *CDC14* locus, if this locus is mutated, the mutation and wild type copy will be tightly linked (Figure 3). Alternatively, if the *ts* mutation is not linked to the plasmid integrated at the *CDC14* locus, the results from tetrad analysis of the same cross would show a variety of combinations of Ts^+: Ts^- spores. Figure 4 shows a possible outcome of this experiment.

Figure 3: Results of Linkage Analysis if *cdc14* is Mutated

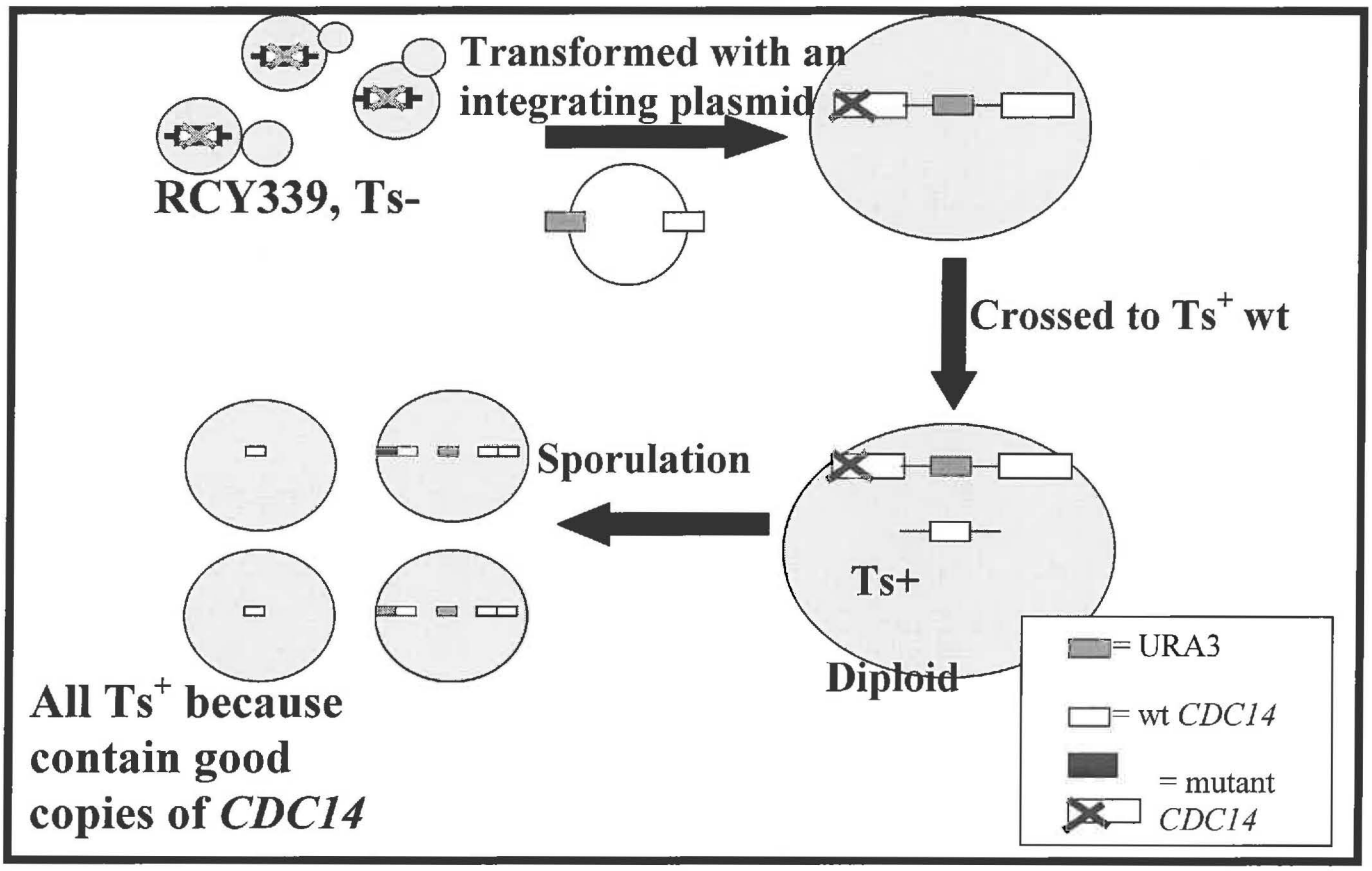
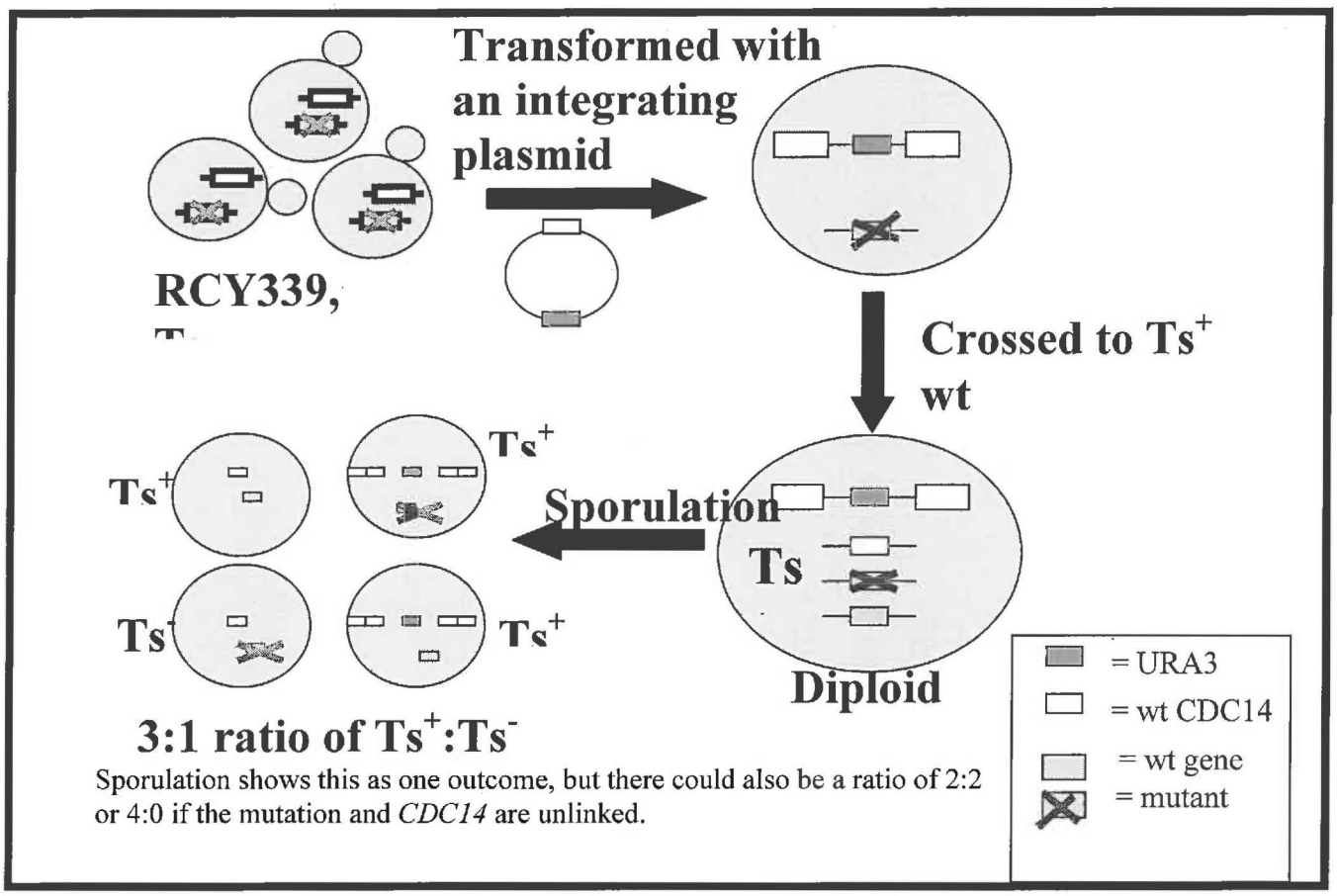


Figure 4: Linkage Analysis if Another Gene Besides *CDC14* is Defective



It would be likely that the *ts* mutation is far from the *CDC14* locus and therefore far from the integrated wild type gene. So, they will assort independently into spores, leaving some spores with the *ts* to not have a wild type *CDC14* to suppress it.

The experiment outlined in Figures 3 and 4 was performed in order to establish linkage data. Using the previously stated assumptions, the spores were scored for the temperature sensitive phenotype. The experiment showed linkage between the *ts* mutation and *CDC14* locus (Figure 3 results). However, the linkage was not complete.

To confirm the presence of a mutation in *CDC14,* DNA sequencing was performed in order to test whether the mutation was indeed in *CDC14.* Sequencing revealed that a single base change

mutation was found in an area homologous to other known sequences of *CDC14* in other species. Therefore, this sequence proved that a mutation does indeed exist in *CDC14* of RCY339.

In both the MEN and the SIN, the Cdc14 homolog acts as a protein phosphatase. A way to determine if the activity of the *K. lactis CDC14* is the same as in the MEN, is to determine its ability to make up for mutant copies in *S. cerevisiae*. In this work, a transformation of a *cdc14 S. cerevisiae* mutant with a wild type copy of *CDC14* from *K. lactis* was done in order to determine if this replacement could occur. The *S. cerevisiae* grew at the restrictive temperature, meaning that the *CDC14* from *K. lactis* could make up for the mutant copy in another species of yeast. It can therefore be concluded that *CDC14* from *K. lactis* has similar, if not the identical function as in the MEN in *S. cerevisiae.*

Future work will be done to further prove that the RCY339 mutant has arrested after anaphase and before mitotic exit. This will be done by doing further DAPI and tubulin stains on strains that have been further backcrossed to allow for a strain containing as few mutations as possible. Also, because Cdc14 is needed to initiate events culminating in cyclin degradation and mitotic exit, cyclin levels can be used as an indicator of whether mitotic exit is accomplished. Preliminary steps of these experiments were performed in this work.

Methods and Materials

Strain List-

Table 1 contains a list of the strains that were used and referenced in the experiment.

Table 1: Strains used for experiments

Strain	Phenotype/Genotype	Derivation
GG1888	MATα, Ura$^-$	Wild type (Cdc$^+$) from B. Zonneveld
LSY25	MATα, His$^-$, Ade$^-$	Wild type (Cdc$^+$) from B. Zonneveld
RCY290	MATα, Ura$^-$, Ts$^-$, Cdc$^-$	From GG1888 via mutagenesis
RCY339	MATα, Ura$^-$, Ts$^-$, Cdc$^-$	From GG1888 via mutagenesis
RCY424	MATα, Ura$^-$, Ts$^-$, Cdc$^-$	From GG1888 via mutagenesis
RCY435	MATα, Ura$^-$, Ts$^-$, Cdc$^-$	From GG1888 via mutagenesis
RCY770	His$^-$, Ade$^-$, Ts$^-$	Haploid progeny from RCY339 x LSY25
RCY773	MATα, Ts$^-$, Ade$^-$, Ura$^-$	Haploid progeny from RCY339 x LSY25
RCY784	His$^-$, Ts$^+$	Haploid progeny from RCY339 x LSY25
RCY818	*MAT a, URA3, CDC14* / *MATα, ura3, cdc14::CDC14/URA3*	Diploid from RCY784 x RCY821, contains *CDC14/URA3* integrating plasmid at *CDC14 locus*
RCY821	*MATα, ura3, cdc14::CDC14/URA3*	Transformant of RCY773 with *CDC14/URA3* integrating plasmid at *CDC14 locus*
RCY822	*MATα, ura3, cdc14::CDC14/URA3*	Transformant of RCY773 with *CDC14/URA3* integrating plasmid at *CDC14 locus*
RCY824	*MATα, ura3, cdc14::CDC14/URA3*	Transformant of RCY773 with *CDC14/URA3* integrating plasmid at *CDC14 locus*
RCY827	*MATα, ura3, cdc14::CDC14/URA3*	Transformant of RCY773 with *CDC14/URA3* integrating plasmid at *CDC14 locus*
RCY829	*MATa, URA3, CDC14, his7 ,ade2* / *MATα, ura3, cdc14, HIS7, ADE2*	Diploid from LSY25 x RCY424 backcross
RCY830	*MATa, URA3, CDC14, his7, ade2* / *MATα, ura3, cdc14, HIS7, ADE2*	Diploid from LSY25 x RCY435 backcross
RCY831	*MATa, URA3, CDC14, his7, ade2* / *MATα, ura3, cdc14, HIS7, ADE2*	Diploid from LSY25 x RCY290 backcross
RCY832	*MATa, ura3, cdc14* / *MATα, ura3, cdc14::CDC14/URA3*	Diploid from RCY339 x RCY824, transformant with integrating plasmid
RCY833	*MATa, ura3, cdc14* / *MATα, ura3, cdc14::CDC14/URA3*	Diploid from RCY339 x RCY822, transformant with *CDC14/URA3* integrating plasmid
RCY895	*MATa, ura3, cdc14* / *MATα, ura3, cdc14::CDC14/URA3*	Diploid from RCY339 x RCY821, transformant with *CDC14/URA3* integrating plasmid
RCY896	*MATa, ura3, cdc14* / *MATα, ura3, cdc14::CDC14/URA3*	Diploid from RCY339 x RCY827, transformant with *CDC14/URA3* integrating plasmid
RCY897	*MATa,URA3, CDC14* / *MATα ura3, cdc14::CDC14/URA3*	Diploid from RCY784 x RCY827, transformant with *CDC14/URA3* integrating plasmid
RJD1231	*MATα, ura3, his3, ade2,cdc14*	RJD1231, *S. cerevisiae* mutant that was transformed with *K. lactis CDC14* in this study

Media-

YPD and SD plates were used in order to grow colonies (Sherman, 1991). Liquid pre-sporulation and sporulation, and YPD media (Sherman, 1991) and malt plates (Zonneveld and Van Der Zanden, 1995) were made in order to mate and sporulate strains.

Use of Frozen Strains and Cell Freezing-

In order to have workable yeast strains, frozen strains were scraped and spread onto YPD media plates. The plates were placed into a 24° C incubator for approximately two days for enough growth to occur for the yeast to be used for other experiments. Frozen, in addition to spores dissected from tetrads and strains developed through backcrosses, were the diploids RCY895 (RCY821 x RCY339), RCY896 (RCY827 x RCY339), and RCY987 (RCY827 x RCY784) as well as the vector pCXJ22 *S. cerevisiae* RJD1231 transformant and pCXJ22-*CDC14* (Isha Wallace plasmid #9) transformant of *S. cerevisiae* RJD1231 transformant.

Cells that were chosen to be frozen were streaked onto YPD plates and placed into a 24˚C incubator for two days or until single colony growth could be observed. A single colony was then patched onto a YPD plates and placed into a 24˚C incubator for another two days. The entire patch of cells from the YPD plates were scraped up with a toothpick and resuspended into a cryo tube containing 800 µl of 15% glycerol. The cells were resuspended and the tubes were placed into a freezer at -70˚C.

Table 2 contains a list of strains that were frozen for future experiments.

Table 2: Strains Frozen

Strain Number (RCY#)	Original Name	Phenotype	Derivation
846	829 1A	Ts⁻, His⁻	829 tetrad haploid spores 829= LSY25 x RCY424
847	829 1B	Ts⁻, Ura⁻, His⁻	
848	829 2D	Ade⁻	
849	829 3D	Ade⁻, Ura⁻, His⁻	
850	829 4A	Ts⁻, Ura⁻	
851	829 4B	Ts⁻, His⁻	
852	829 5C	Ts⁻	
853	829 5D	Ts⁻	
854	829 7A	Ade⁻, Ura⁻, His⁻	
855	829 7C	Ade⁻	
856	830 1B	Ade⁻, Ts⁻, Ura⁻, His⁻	830 tetrad haploid spores 830= LSY25 x RCY435
857	830 1C	Ts⁻	
858	830 1D	Ade⁻, His⁻	
859	830 2A	Ade⁻, His⁻	
860	830 2C	Ade⁻, Ts⁻, Ura⁻	
861	830 3C	Ade⁻, Ts⁻, Ura⁻, His⁻	
862	830 3D	Ts⁻, His⁻	830 tetrad haploid spores 830=LSY25 x RCY435
863	830 4A	His⁻	
864	830 4B	Ade⁻, His⁻	
865	830 4D	Ts⁻, Ura⁻	
866	830 5A	Ts⁻, Ura⁻	
867	830 5B	Ade⁻, Ura⁻, His⁻	
868	830 5C	His⁻	
869	830 5D	Ade⁻	
870	830 6A	Ade⁻, Ts⁻	
871	830 6B	Ade⁻, His⁻	
872	830 6C	His⁻	
873	830 6D	Ade⁻, Ts⁻, Ura⁻	
874	831 1A	Ade⁻, Ts⁻, Ura⁻, His⁻	831 tetrad haploid spores 831= LSY25 x RCY290
875	831 1B	Ts⁻, Ura⁻	
876	831 1D	Ade⁻, His⁻	
877	831 2B	His⁻	
878	831 2C	Ade⁻, Ts⁻, His⁻	
879	831 2D	Ts⁻	
880	831 3C	Ade⁻, Ts⁻, Ura⁻, His⁻	
881	831 3D	Ade⁻, Ts⁻	831 tetrad haploid spores 831= LSY25 x RCY290
882	831 4C	Ade⁻, Ts⁻, Ura⁻	
883	831 4D	Ts⁻, Ura⁻, His⁻	
884	831 5B	Ts⁻	
885	831 5C	Ade⁻, Ts⁻, Ura⁻, His⁻	
886	831 7A	Ade⁻, His⁻	

887	831 8C	Ade$^-$, Ts$^-$, His$^-$	831 tetrad haploid spores 831= LSY25 x RCY290
888	831 8D	Wild type	
889	831 9C	Ade$^-$, Ts$^-$, Ura$^-$, His$^-$	
890	831 9D	Ade$^-$, Ts$^-$, His$^-$	
891	831 10B	Ura$^-$	
892	831 10C	Ade$^-$, Ts$^-$	
893	831 11B	Ts$^-$, Ura$^-$	
894	831 11D	Ts$^-$, Ura$^-$, His$^-$	
895	821 x 339	*MATa,ura3,cdc14 MATα,ura3,cdc14::CDC14 /URA3*	Diploid from RCY821 x RCY339 cross
896	827 x 339	*MATa,ura3,cdc14 MATα,ura3,cdc14::CDC14 /URA3*	Diploid from RCY827 x RCY339 cross
897	827 x 784	*MATa, URA3,CDC14 MATα, ura3, cdc14::CDC14/URA3*	Diploid from RCY827 x RCY784 cross
898	1B (GG1888 x 770)	Ade$^-$, Ts$^-$, His$^-$	Haploid tetrad spores from GG1888 x RCY770 second round backcross
899	1D (GG1888 x 770)	Ade$^-$, Ts$^-$, His$^-$	
900	2B (GG1888 x 770)	Ade$^-$, Ts$^-$, His$^-$	
901	3A (GG1888 x 770)	Ade$^-$, Ura$^-$	
902	4B (GG1888 x 770)	Ade$^-$, Ts$^-$, His$^-$	
903	6A (GG1888 x 770)	Ade$^-$, Ts$^-$, His$^-$	
904	7A (GG1888 x 770)	Ade$^-$, Ura$^-$	
905	8A (GG1888 x 770)	Ade$^-$, His$^-$	
906	1B (GG1888 x 902)	Ade$^-$, His$^-$	Haploid tetrad spores from GG1888 x 902 third round backcross
907	2D (GG1888 x 902)	Ade$^-$, Ts$^-$	
908	3C (GG1888 x 902)	Ade$^-$, His$^-$	
909	4B (GG1888 x 902)	His$^-$	
910	5A (GG1888 x 902)	Ts$^-$, His$^-$	

911	5B (GG1888 x 902)	Ade$^-$, His$^-$	Haploid tetrad spores from GG1888 x 902 third round backcross
912	6B (GG1888 x 902)	Ade$^-$, His$^-$	
913	6D (GG1888 x 902)	Ts$^-$	
S. cerevisiae (926)	Transformed strain	*MATα, ura3, his3, ade2, cdc14: MATα, URA3,*	Vector pCXJ22 *S. cerevisiae* RJD1231 transformant
S. cerevisiae (861)	Transformed strain	*MATα, ura3, his3, ade2, cdc14: MATα, URA3, CDC14*	Plasmid pCXJ22-*CDC14 S. cerevisiae* RJD1231 transformant

Mating Strains for Backcross Diploids-

Backcrosses were done by taking a Ts$^-$, Ura$^+$, and His$^-$ or Ade$^-$ derivative of RCY339 (*K. lactis*) and crossing it to GG1888, in order for diploid cells to be made. Diploid cells are essential in that they allow spores to form and for tetrad analysis to be done. Crosses were done by placing a 5 µl drop of YPD liquid media in a designated area on a malt plate and taking a toothpick with which a colony of interest was scraped off of the YPD plate and mixed into the drop on the malt plate. Another toothpick was then used to acquire a colony from the second strain in the cross and mixing it into the same drop. (Zonneveld, 1995) This was done for all the strains that fell under the genotypic requirements. The malt plates were left at 24°C for another 2 days to allow for the cells to grow, after which the now diploid cells were streaked onto SD plates. The SD plates are needed to select diploids and kill parent haploids. The entire patch was spread in sectors with 50 µl sterile water. After these cells grew at 24°C, they were placed into sporulation media.

Sporulation Tubes-

The cells were scraped from the SD plates with a toothpick and placed into separate sterile test tubes containing 2 ml of acetate sporulation media. The tubes were then spun on a test tube rotator at 24°C for 5 days (Sherman, 1991). This media and technique is done in order for the cells to sporulate (ascus formation). After this time period, the cells were looked at under phase contrast microscopy to find asci. Samples containing a high percentage of asci were dissected using a micromanipulator. If a sample was not dissected immediately, it was placed into a refrigerator.

Dissection of Tetrads-

Preparation of asci for tetrad dissection was based on protocol by Sherman and Hicks (1991). In order to analyze each individual spore of a tetrad from a cross, the tetrads need to be dissected using a micromanipulator. Asci (500 µl) were harvested from sporulation culture in a microcentrifuge. The asci pellet was resuspended in 1 ml of sterile water to wash the cells. The tube was spun at the highest setting on a microcentrifuge for about 1 min. The supernatant was removed and 1 ml of sterile water was added and the tube was spun again. These washes were done in order to remove the sporulation media. Once the supernatant was removed, 500 µl of sterile water was added to the tube, and the cells were resuspended using a vortex machine. An aliquot of the resuspended cells (100 µl) was placed into another tube. Sterile water (80 µl) and 20 µl of a 5.0% glusalase solution (Sigma-Aldrich Co., St. Louis MO) were also added to the tube. The glusalase is used to break the wall holding the tetrad together. The tube was left to sit for 10-15 min at room temperature, and then, 900 µl of water was added to the tube. An aliquot of the glusalase pre-treated cells (35 µl) were pipetted at the top of the YPD plate. The plate was then tilted in order to make a line down the center of the plate with the drop. Before dissecting

the plate, the strip was allowed to dry. The tetrads were dissected using a micromanipulator and a brightfield microscope. Once the tetrads were dissected, the plate containing the tetrads was placed into an incubator at 24° C. Colonies of cells appeared on the plate after approximately 4 days. The strains dissected are found in the "Scoring of Frogged, Dissected Tetrad Spores" section of the results and also in the appendix.

Frogging of Tetrads-

Spores used for the next backcross were those that appeared Ts^-, Ura^+, Ade^-, and His^- or any spores containing the Ts^-, Ura^+ phenotype and as many autotrophic markers as possible. In order to be able to score the phenotype of each spore in a tetrad, the colonies that grow on the dissected plate are replicated on plates containing various nutrients. To each well of a sterile metal plate with wells, 200 µl of sterile water was added. With a sterile toothpick, a bit of a yeast colony (amount varies with size of the actual colony) was scraped and stirred into the assigned well. This was repeated for each tetrad spore to be scored using a different toothpick and well for each colony. Next, the proper plates were prepared. For this experiment, three SD plates were topspread with the appropriate levels of nutrients. One SD plate topspread with 300 µl of a 0.002% Ura and 0.002% His solution. The second plate was topspread with 300 µl of a 0.003% Ade and 0.002% His solution. The third plate was topspread with 300 µl of a 0.003% Ade and 0.002% Ura solution. These SD plates were used to test the phenotypes of each spore, and two other plates, YPD plates, were used to test for temperature sensitivity. A "frogger" device with prongs that match the wells of the metal plate was placed into the "pond" wells and then placed onto the agar, making sure that each drop on the prongs of the frogger was transferred. The SD plates and one of the YPD plates were placed into the incubator set to 24° C and the remaining YPD plate was placed at 37° C. After about two days in the incubators, the

plates were scored. If a strain did not grow when a nutrient was absent, the strain was scored as auxotrophic for the nutrient. Strains that were frogged can be found in the results section ("Scoring of Frogged, Dissected Tetrad Spores") and appendix as well (same strains as those dissected).

Linkage Analysis-

RCY818 and RCY897 were used in order to test the linkage of RCY339's *ts* mutation to the *CDC14* locus. RCY818 is a diploid strain resulting from the cross between RCY821 and RCY784. RCY773 was derived from RCY339 via a backcross with wild type LSY25. RCY773 was then transformed with an integrating plasmid containing *K. lactis CDC14*, to create transformant L, which is RCY821 (Ts^+, Ura^+). RCY821 was then mated to a wild type strain, RCY784, to give RCY818. A similar regimen was used to derive RCY897. In order to test the linkage between the *ts* mutation and the wild type copy of *CDC14* integrated at the *CDC14* locus, the data for temperature sensitivity was scored, and the following equation was used to calculate the map distance (in centimorgans):

$$\frac{1/2\,(6NPD+TT)*100}{(PD + NPD + TT)}$$

(http://www.ergito.com/main-lcd.jsp?bcs=GNTC.2.7.10).

PD is the parental ditype, meaning that the spores have the same two combinations of phenotypes as the parental strains. TT is also known as the tetratype, or spores that have undergone recombination once, causing the spores to have a variation in their allele segregation, giving four spores with different combinations of genotypes.

NPD, is a non-parental ditype, meaning that the spores have undergone at least two recombination events, and therefore causing a greater variation in allele segregation and giving two different genotypes combinations, both dissimilar from the parental genotypes.
Also, the *URA3* and *ts* markers of RCY895 and RCY896 were scored, which acted as a control for RCY818 and RCY897, showing that the plasmid only integrated at one site.

DNA Preparation-

Protocol for *Purification of Genomic DNA from Yeast* from Qiagen DNeasy Tissue Handbook (Qiagen, Valencia, CA) was used to purify genomic DNA from RCY339 and GG1888. The samples used for purification were prepared by inoculating. This was done by placing a pinhead sized colony of RCY339 and GG1888 into separate test tubes containing 5 mL of YPD liquid media. The tubes were set to rotate at room temperature (~22° C) on a test tube rotator overnight. The DNA would be used to identify any change in base sequence of *CDC14* from wild type to mutant. The DNA samples were frozen for future use.

Amplification (Polymerase Chain Reaction)-

PCR is used to amplify/duplicate a portion of DNA in a DNA sample. *CDC14* from RCY339 and GG1888 was amplified using CDC14 upstr/CDC14r and Bam H1/EcoR1 primer sets. The sequences of primers used were Bam H1 primer: 5` TCCCGGGATCCTGCAACCAAAGCGTGTAATT 3`, EcoR1 primer: 5`CCCGAATTCCTTGAGCATGTATGCATCTC3`, CDC14 upstr primer: 5`TTATCCTACCTTTCCTTG 3`, and CDC14 r primer: 5` TGCAACCAAAGCGTGTAAT 3`. Pure Taq beads (Amersham Biosciences, Little Chalfont Buckinghamshire, England) and 5 µl of GG1888 or RCY339 DNA from the Qiagen prep and 0.4 µM of each primer were used for each

reaction tube. The PCR program titled CDC14 was used on the PCR instrument using the following temperature sequences:

94°C 2 min
94°C 15 sec
53°C 30 sec
72°C 1 min 20 sec
} 10 times
94°C 15 sec
53°C 30 sec
72°C 1 min 20 sec (+5 sec/cycle)
} 20 times
72°C 7 min
4°C Forever or until removed from instrument.

Agarose Gel Electrophoresis-

An agarose gel was run after each DNA purification experiment in order to make sure DNA was actually in each sample. After each PCR was completed, another gel was run to make sure that amplification products existed in each sample. After it was confirmed that amplification products existed, the entire sample was loaded onto the gel, in order to isolate the DNA for DNA extraction. After DNA extraction, a small amount of each sample along with a DNA ladder were run on the gel to again make sure that the sample contained DNA and to see how many base pairs existed in each sample of DNA. For each gel, 0.8% agarose in 1X TBE (Sambrook and Russell, 2001) and 0.005% ethidium bromide was used. About 2-3 µl of DNA and 2 µl of loading buffer (Sambrook and Russell, 2001) were mixed together and loaded into each well after which the gel was run.

DNA Extraction-

DNA was extracted from PCR gel bands using the *Qiagen QIAquick Gel Extraction Kit and Protocol* handbook (Qiagen, Valencia, CA). The DNA samples used were the PCR products described above from RCY339 and GG1888. The bands seen on the gel were cut out with a

razor blade under UV light and placed into separate labeled microcentrifuge tubes. The samples were eluted in 30 µl of the provided elution buffer. After extractions were completed, the tubes were labeled and placed in the freezer.

DNA Sequencing-

PCR products were sequenced with primers EcoR1/Bam H1, CDC14 upstr/CDC14r, and cdc midF at Cal State Northridge sequencing facility. The primers that were used for the amplification reaction were used to sequence that sample. The cdc midF sequence is 5`TTTGCAGCCGTTGGCTCA3`. This primer sequences an internal portion of the CDC14 gene in the forward direction and was used to sequence amplified DNA samples from RCY339 and GG1888. The sequencing data received from Cal State Northridge was analyzed using Editview (Applied Biosystems, Foster City CA) and Assemblylign (Oxford Molecular Group) to find the location of the mutation. MacVector and CLUSTALW were used in order to align the sequences and compare wild type and mutant sequences (Accelrys, San Diego CA).

***S. cerevisiae* Transformation –**

S. cerevisiae transformation was based on the procedure from Gietz *et al.* (1992).

S. cerevisiae RJD1231 cells, containing a *ts* allele of *cdc14*, were grown in 50 ml of YPD overnight with shaking at room temperature. The next day, the optical density (OD_{600}) of the solution was measured using a spectrophotometer, looking for a range between 0.2 and 0.5 OD_{600}/ml.

Once the appropriate density had been reached, (0.2939 OD_{600}/ml for the 50 ml solution) the culture was centrifuged in a 50 ml tube for 5 minutes at about 2500 rpm in a clinical centrifuge. The solution was poured off and the pellet was washed in 10 ml of sterile H_2O and centrifuged

again for 2.5 minutes at 2500 rpm. The pellet was then resuspended in 1 ml of sterile H_2O and transferred to microcentrifuge tube. The tube was then centrifuged for 15 seconds at maximum speed to pellet the cells. The cells were then resuspended in 1 ml of 1X TE/LiOAc freshly made from 10X TE (100mM Tris-HCl and 10 mM EDTA) and 10X LiOAC (1 M LiOAc). The solution was centrifuged again and the pellet resuspended in 125 µl 1X TE/LiOAc. Salmon sperm DNA (10 mg/ml) was put into a microcentrifuge tube and boiled for 5 minutes then put directly on ice. To one tube, 50 µl of cells, 990 ng pCXJ22 vector and 53 µg salmon sperm DNA were added. To another tube, 50 µl of cells, 10 µl of plasmid DNA pCXJ22w/*CDC14* (miniprep #9 by Isha Wallace) and 53 µg salmon sperm were added. To a third tube, the rest of the cells (~20 µl) and 53 µg salmon sperm DNA were added. To each of the three microcentrifuge tubes, 100 µl of 40% (w/v) PEG solution was added. The tubes were gently and thoroughly mixed and then incubated at 24° C for 30 minutes on a spinner. Heat shock was done at 37° C for 15 minutes. The microcentrifuge tubes were then centrifuged at 2300 rpm for 30 seconds and then the pellet was resuspended in 1 ml 1X TE (10 mM Tris-HCl, 1 mM EDTA). A fraction (200 µl) of each transformation was plated on an SD plate previously topspread with 300 µl of 0.003% w/v adenine and 0.002% histidine. The plates were incubated at 24° C and observed for growth several days later. Transformed colonies were tested for temperature sensitivity by streaking the colonies on a YPD plate and incubating at 37° C. One half of the YPD plate consisted of *S. cerevisiae* transformed with the *CDC14* plasmid and the other half were colonies that had received the pCXJ22 vector alone.

Arrest of GG1888 and RCY339 cells-

To observe Clb2 levels in GG1888 and RCY339 cells, the cells need to be arrested and observed at different cell cycle blocks. Also, we need to confirm that the antibody raised against *S. cerevisiae* Clb2 is recognizing *K. lactis* Clb2.

Initially, GG1888 cells were inoculated in order to produce stationary phase (saturated) cell cultures. This was done by taking a colony of cells and placing them in 5 ml of YPD in a test tube and allowing them to spin overnight. The tubes were then placed into the fridge until they were needed.

Using a spectrophotometer, the concentration of the stationary culture was identified. This concentration was used to determine the amount of the solution that was needed to spin overnight to obtain log phase asynchronous cultures. Using the following equation, the starting concentration was determined: $y = x_o(2^N)$. y is the final concentration which in this case is desired to be 2 OD/ml (midlog), N is the number of doubling times, which is the number of hours the culture will be spinning divided by 2.25 hr doubling time (hours which the culture will be spinning depends when the culture is started until the time that it can be checked), and x_o is the starting concentration. Using the concentration of the stationary culture found earlier, the amount of the culture that will be needed to add into the new culture can be calculated using the x_o that was calculated from the equation. Three different 50 ml Falcon tubes containing 30 ml of YPD media were set up using three different concentrations of the stationary cells. If the amount of stationary cells to be used was calculated to be 50 µl, two other concentrations, 125 µl and 250 µl, were used as well to make sure that the desired concentration of 2 OD/ml was reached. In most situations, the more concentrated solution had the concentration closest to the desired one after spinning. The tubes were taped onto a rotator and allowed to spin for the desired time.

After spinning, the concentrations of the cultures were identified using a spectrophotometer, and the culture closest to 2 OD/ml was used. The cells were then adjusted to 2 OD/ml with YPD. Two OD's were removed from the tube and put into a microcentrifuge tube. After 100% (w/v) TCA was added to 20% to the tube, the solution was vortexed and put on ice until the centrifuge was used. It was then spun for five minutes in a microcentrifuge that was placed in the cold room in order to keep the solution cold. The TCA was pipetted off and any excess acid was blotted off. The pellet was washed with cold acetone and quickly spun in the microcentrifuge for one minute and the solution was drained into the appropriate waste container. The microcentrifuge tube was then air dried and placed into -70° C. (protocol based on Azzam, personal communication)

Results

Mating Strains for Backcross Diploids and Sporulation Media Checks–

By backcrossing a temperature sensitive RCY339 derivative to the wild type strain GG1888, we are hoping to produce a strain containing only the mutation of interest. By crossing our mutant to the initial wild type strain, and choosing spores containing specific characteristics and producing further backcrosses, other mutations that are existent in our mutant will be weeded out. If other mutations exist in our strain, they may be causing some aspects of the phenotype. The data for these backcrosses can be found in the appendix; an example is provided below.

Scoring of Frogged, Dissected Tetrads Spores-

After tetrad dissection was completed, the haploid colonies were replica plated using the frogging method. Once the colonies on the replicated plates grew, they were scored for their

phenotypes. Table 3 shows the scoring results of the second round of backcrossing of the RCY339 derivative RCY770 to the wild type parent GG1888. Permissive temperature was 24° C. Restrictive temperature (used to test temperature sensitivity) was 37° C.

Table 3: Scoring of tetrads from RCY339 Second Round Backcross
This backcross was GG1888 x RCY770. GG1888 is the wild type parent of RCY339. RCY770 is a haploid derivative of RCY339 from the first round of backcrosses. Colonies arising from one tetrad are grouped between darker lines. Spores are given the number of the tetrad plus a letter, A-D. Spores used in another backcross are indicated with a ^. Frozen strains are indicated by a * and the strain number given to the spore is in parentheses. Also indicated are the phenotypes of Ura and Ts. Table 4 in the appendix contains the Ade and His phenotypes in addition to those of Ura and Ts.

Spore Number	Ura	Ts
1A	-	+
1B^*(898)	+	-
1C	-	+
1D^*(899)	+	-
2A	-	-
2B^*(900)	+	-
2C	+	+
2D	-	+
3A*(901)	-	+
3B	-	-
3C	+	+
3D	+	-
4A	-	+
4B^*(902)	+	-
4C	+	-
4D	-	+
5A	-	-
5B	-	-
5C	+	+
5D	+	+
6A^*(903)	-	-
6B	-	-
6C	+	+
6D	+	+
7A*(904)	-	+
7B	+	-
7C	+	+
7D	-	-
8A*(905)	+	+
8B	-	-
8C	-	-
8D	+	+

Table 4 shows the scoring results of progeny derived from RCY902 (listed as 4B in Table 3) crossed again to GG1888 for a third round of backcross.

Table 4: Scoring of RCY902 x GG1888 Dissected Tetrads
This backcross was RCY902 x GG1888. GG1888 is the wild type parent of RCY339. RCY902 is a haploid derivative of RCY339 from the second round of backcrosses. Colonies arising from one tetrad are grouped between darker lines. Spores are given the number of the tetrad plus a letter, A-D. Spores used in another backcross are indicated with a ^. Frozen strains are indicated by a * and the strain number given to the spore is in parentheses. Also indicated are the phenotypes of Ura and Ts. Table 7 in the appendix contains the Ade and His phenotypes in addition to those of Ura and Ts.

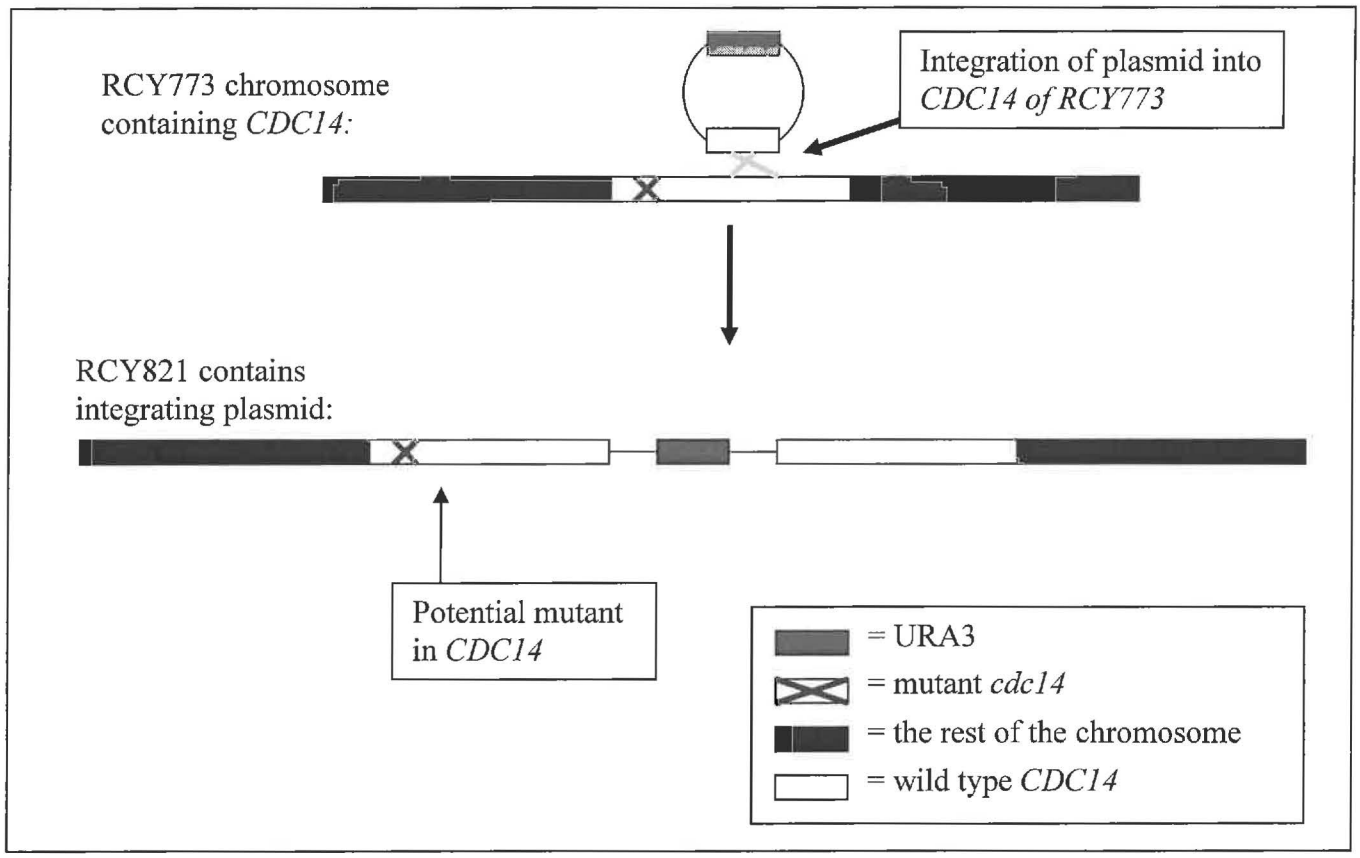
Spore Number	Ura	Ts
1A	-	-
1B*(906)	+	+
1C	+	+
1D	-	-
2A	-	-
2B	+	+
2C	-	+
2D^*(907)	+	-
3A	-	+
3B	-	-
3C*(908)	+	+
3D	+	-
4A	+	-
4B*(909)	+	+
4C	-	-
4D	-	+
5A^*(910)	+	-
5B*(911)	+	+
5C	-	+
5D	-	-
6A	-	-
6B*(912)	+	+
6C	-	+
6D^*(913)	+	-
7A	+	-
7B	-	-
7C	-	+
7D	+	+

The *ts* segregated 2:2 in both backcrosses as would be expected by a single *ts* allele. Many other tetrads were dissected throughout the course of the year to backcross other *cdc* mutants. These results are shown in the appendix.

Linkage Analysis-

It is possible that the *ts* mutation in RCY339 is in CDC*14,* but it could also be in another gene. A plasmid containing a wild type copy of *CDC14* and a *URA3* marker was integrated at the *CDC14* locus in an RCY339 derivative to create RCY821. This integration resulted in two copies of *CDC14* separated by the *URA3* gene. Correct integration was confirmed by PCR (Silveira, personal communication). In order to determine whether the inserted *CDC14* plasmid was linked to the *ts* mutation in RCY339 at the *CDC14* locus, linkage analysis was done. If the *ts* mutation is in *CDC14,* then it should be very tightly linked to the integrated wild type *CDC14.* In order to obtain the appropriate data to determine an accurate map distance, tetrad dissection of RCY818 was done. RCY818 is a diploid resulting from RCY821 (a *ts* mutant with a wildtype *CDC14* integrated at the chromosomal *CDC14* locus) crossed to a wild type strain (RCY784). Figure 5 illustrates what RCY773 looks like at the *CDC14* locus after plasmid integration to form RCY821.

Figure 5: RCY773 Plasmid Integration at the *CDC14* locus



If the *ts* allele is tightly linked to the *CDC14* locus, the Ts^- phenotype should no longer be observed in the progeny due to the presence of the wildtype *CDC14* integrated in the chromosome. Therefore, results should show a 4:0, Ts^+:Ts^- ratio. However, despite the fact that the summary of the results shown in table 5 mostly show the 4:0 ratio, approximately one in ten tetrads exhibit a 3:1 ratio of Ts^+:Ts^- phenotype. There were difficulties in scoring, because some spores grew better than the *ts* RCY339 but less well than the non-*ts* RCY773. When a spore developed a slight film of growth, the spores were labeled as +/- for ts, because they were not growing strong, yet some growth was observed. Figure 6 shows representative colony growth at 37° C.

Figure 6: Spore Growth for Replicated YPD Plate at 37° C
A suspension from each of the four colonies of a tetrad was spotted onto a YPD plate. Each row represents colonies from one tetrad. Plate was incubated at 37° C.

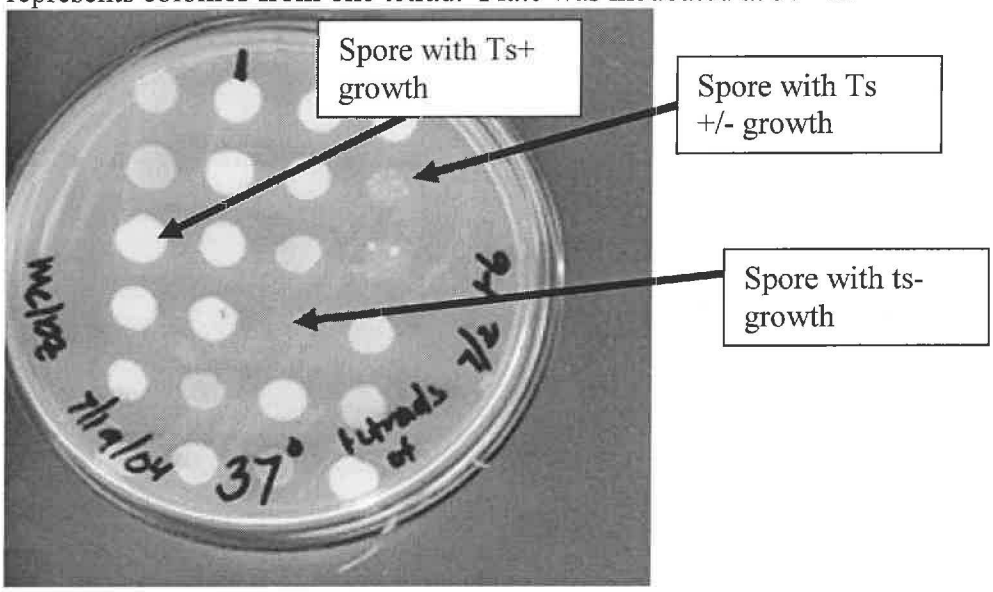


Table 5 consists of a summary of scoring of RCY818 dissected tetrad spores. The entire tables of results are in the appendix.

Table 5: Summary of Results from the Scoring of RCY818 (RCY784 x RCY821 cross) Dissected Tetrad Spores

Ts^+:Ts^- Segregation	Number of Tetrads
4:0 Ts^+:Ts^-	18
3:1 Ts^+:Ts^-	2
3:1 Ts^+:$Ts^{+/-}$	2
	Total : 22

RCY897 gave similar results as RCY818. RCY897 was formed by a cross between RCY827, a transformant of RCY773 containing the integrating plasmid, and the wild type RCY784. Table 6 contains a summary of the results given from the dissected tetrad spores of RCY897. The entire table of results is in the appendix. Because this is the exact same kind of experiment as that resulting in RCY818, results can be used to calculate more linkage data.

Table 6: Summary of Results from the Scoring of RCY897 (RCY784 x RCY827 cross)
Dissected Tetrad Spores

Ts^+:Ts^- Segregation	Number of Tetrads
4:0 Ts^+:Ts^-	10
3:1 Ts^+:Ts^-	7
2:2 Ts^+:Ts^-	1
	Total : 18

Because the data did not show the 4:0 ratio for all the tetrads tested, the following equation was used to determine the map distance and determine the linkage between the plasmid and the *ts* mutation:

$$\frac{1/2\ (6NPD+TT)*100}{(PD + NPD + TT)}$$

(http://www.ergito.com/main-lcd.jsp?bcs=GNTC.2.7.10).

-Map distance for RCY818 = $\frac{1/2\ (6(0) + 2)*100}{(18 + 0 + 2)}$ = 5 centimorgans ~ 5 kB

-Map distance for RCY897 = $\frac{1/2\ (6(1) + 7)*100}{(10 + 1 + 7)}$ = 38.2 centimorgans ~ 38.2 kB

The parental ditype (PD) is observed when the phenotypes of all the spores in the tetrad are Ts^+. This is due to the way segregation occurs between the cross of RCY784 and RCY821 for RCY818, and the cross of RCY784 and RCY827 for RCY897. The wildtype copy of *CDC14* from RCY784 and the plasmid wild type copy of *CDC14* integrated into the *CDC14* locus of RCY773 give the 4:0 Ts^+:Ts^- results that we see in the majority of the tetrads scored. The tetratype (TT), occurs, when one recombination event occurs, to give a 3:1 ratio of Ts^+:Ts^-. This recombination event could be due to a crossover between the integrated wild type *CDC14* and the ts mutation. Alternatively, the integrated plasmid could loop out (a crossover between the tandem *CDC14* copies) leaving only the *ts* genotype. If recombination occurs twice, a non-parental ditype (NPD) is seen, resulting in a 2:2 ratio of Ts^+:Ts^-, again because recombination causes the separation of the wild type copy of *CDC14* from the *ts* mutation on the chromosome.

spores exhibiting a Ts^- phenotype. If the integrated plasmid and the *ts* mutation were unlinked, a ratio of 1PD:4TT:1NPD would be expected. However, if they are linked, PD's would be primarily seen. In our case, we observe a 9:1:0 ratio of PD:TT:NPD for RCY818 and a ratio of 10:7:1 of PD:TT:NPD for RCY897. The map distance that was calculated for RCY818 shows that the plasmid and the *ts* mutation are linked, but not so tightly linked that the *ts* is inseparable from the *CDC14* locus as marked by the integrated plasmid. However, results for the map distance for RCY897 gives a greater distance. This is largely due to the trouble scoring the Ts phenotypes (a great deal of spores exhibit $Ts^{+/-}$ phenotypes), which gives an even bigger potential map distance between *ts* and *cdc14* locus. As a result, RCY339 could still have a *ts* mutation in a gene near *CDC14*.

Table 7 shows a summary of the results of the controls used for RCY818, RCY895 and RCY896, in order to show that the plasmid was only integrated at one site on the genome in RCY821 (Rose, 1991). This was done by observing the phenotypes that resulted from the *URA* marker. The cross that was used in order to get RCY895 was between RCY339 (Ura- and *ts*) and RCY821 (Ura-, RCY339 *ts* mutation, with an integrated plasmid containing a Ura+ marker and *CDC14*). The cross that was used in order to get RCY896 was between RCY339 (Ura- and *ts*) and RCY827 (Ura-, RCY339 *ts* mutation, with an integrated plasmid containing a Ura+ marker and *CDC14*). Therefore, we would expect to see a 2:2 ratio of Ura^+:Ura^-. This is because, according to the laws of segregation, all cells will contain mutant copies at the *URA3* locus (from both RCY339 and RCY773), however, wherever the plasmid segregates, it will contain a wild type copy of *URA3* making that cell Ura+. The results (in appendix) show a 2:2 ratio and therefore show that the plasmid integrated only once. This is important to know because if the plasmid had integrated multiple times into RCY773 to make RCY821, our linkage analysis of

RCY818 would be wrong because there would be multiple *CDC14's* integrated at various sites, increasing the chance that one would segregate to the spores that contain the *ts* mutation even though they were not linked.

Table 7: Summary of Results from the Scoring of RCY895 and RCY896Dissected Tetrad Spores

Number of Tetrads	Ura^+:Ura^-	Ts^+ co-segregating with Ura^+
RCY895		
16	2:2	16
RCY896		
14	2:2	11

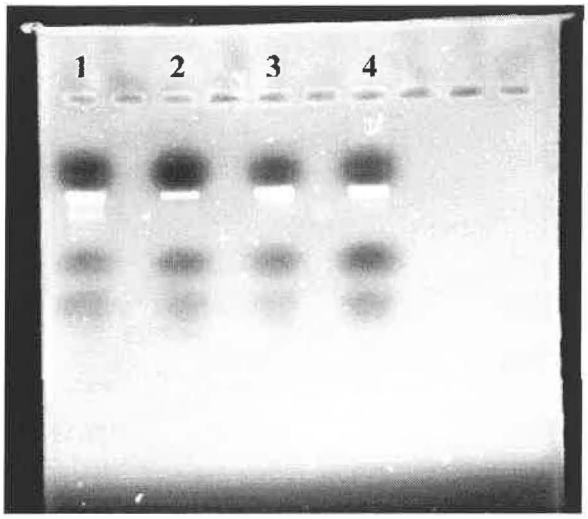
DNA Preparation-

Our linkage analysis proved to be ambiguous because though the *ts* mutation is near, it may not be in the same locus as *CDC14*. The way that we can see if the mutation is in the *CDC14* gene would be by sequencing. In order to sequence *CDC14* it needs to be amplified from RCY339 and its wild type parent, GG1888. A template DNA must be isolated RCY339 and GG1888 by DNA preparation. Once the experiment was completed, a small amount of the sample from the DNA preparation was run on a gel to verify that there was DNA in the sample so that it could be amplified.

Amplification and Product Purification-

The main purpose of the PCR is to amplify (multiply) a portion of desired DNA so that analysis, such as sequencing, can be done. Amplification of the *CDC14* locus was done on GG1888 and RCY339 genomic templates, on *CDC14* in hopes of identifying the location and characteristics of the mutation. For this experiment, a gel was run after each PCR to make sure that there were amplification products in the PCR tube. Figure 7 shows what a normal gel that was run with amplification products should look like when products are seen.

Figure 7: Amplification Products from RCY 339 and GG1888 a Gel
Agarose gel (0.8%) of *CDC14* PCR products with various primer pairs. Lanes 1&3 contain RCY339 *CDC14* amplification products. Lanes 2&4 contain GG1888 *CDC14* amplification products. Primers used in lanes 1&2 are CDC14 upstr and CDC14 r. Primers used in lanes 3&4 are Bam H1 and EcoR1



After the presence of the amplification products was confirmed, the entire sample was run on a preparative gel so as to fit the entire sample. The band was then cut out to be used for DNA extraction. The purified bands were sent out to be sequenced. Just as with DNA preparation, and amplification, a gel was run after DNA extraction was complete in order to determine if DNA was in the sample to be sent for sequencing. Also, a DNA marker was added onto the gel in order to determine the size of the DNA sequence (i.e. number of base pairs).

Sequencing-

Because results from linkage analysis were not 100% conclusive in RCY818 and even worse for RCY897, sequencing was done to show that the mutation is in *CDC14*. A portion (5` end) of the *CDC14* gene was sequenced on both strands. After receiving the sequence information from the *CDC14* loci amplified from GG1888 and RCY339, they were loaded onto a computer and the

analyzed for the presence of a mutation. Figure 8 shows the alignment of the wild type (GG1888) and mutant (RCY339) sequenced DNA. Analysis of the base peaks (as those shown in Figure 9) showed that the only mutation in the portion of the RCY339 sequence that was determined was that at the position not containing an asterisk underneath the alignment (Figure 8).

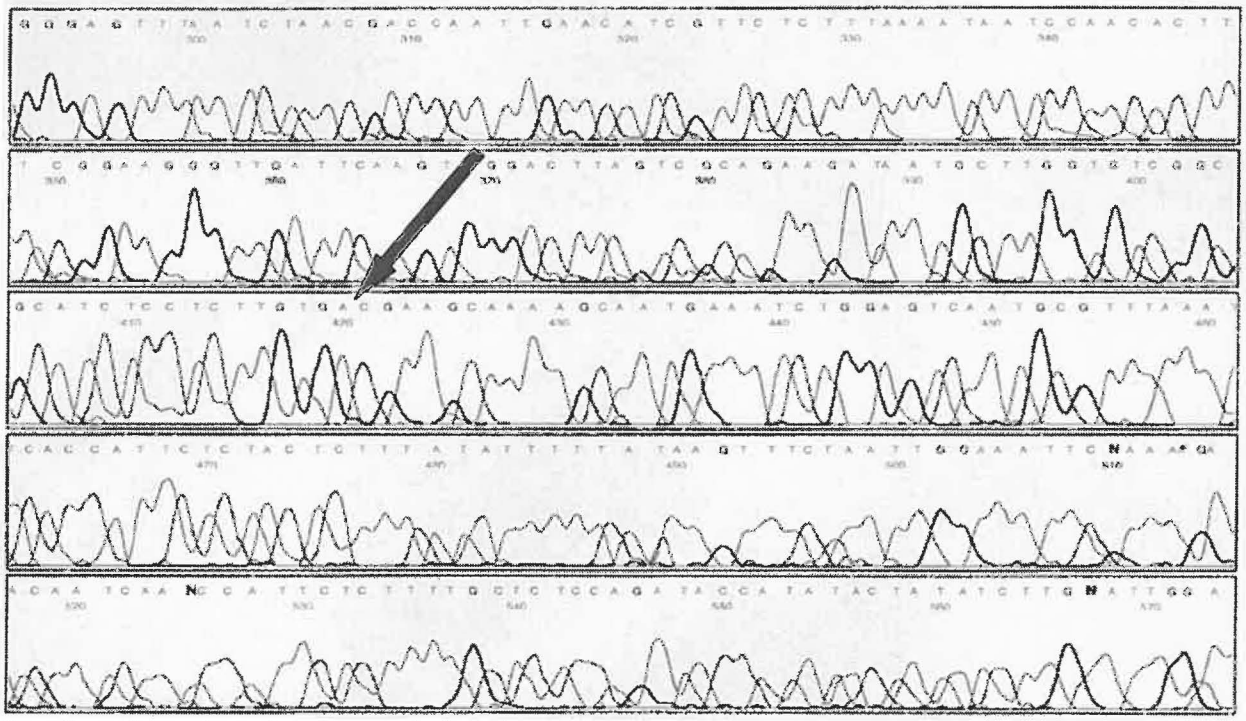
Figure 8: Alignment of Wildtype (GG1888) and mutant (RCY339) sequenced DNA
The base change in the sequence is in second line of the alignment. This is an alignment of partial *CDC14* sequences. * = matches between sequences

```
GG1888WildType      CGGAAGGGTTGATTCAAGTGGGACTTAGTCGCAGAAGATAATGCTTGGTGTCGGCGCATC 60
RCY339Mutant        CGGAAGGGTTGATTCAAGTGGGACTTAGTCGCAGAAGATAATGCTTGGTGTCGGCGCATC 60
                    ************************************************************

GG1888WildType      TCCTCTTGTGGCGAAGCAAAAGCAATGAAATCTGGAGTCAATGCGTTTAAATCACCATTC 120
RCY339Mutant        TCCTCTTGTGACGAAGCAAAAGCAATGAAATCTGGAGTCAATGCGTTTAAATCACCATTC 120
                    ********** ************************************************

GG1888WildType      TCTACTCTTT 130
RCY339Mutant        TCTACTCTTT 130
                    **********
```

The purpose of sequencing GG1888 was to make sure that the mutation that was found in RCY339 was truly the mutation we were looking for and was not already existent in the wild type parent. After the first round of RCY339 sequencing, the mutation was found to be a base change from a G to an A. The second round of RCY339 sequencing was puzzling in that the mutation had disappeared and was identical to the GG1888 wild type sequence. However, the third round of sequencing showed that the mutation was still there and that the error could have occurred due to mislabeling GG1888 DNA as RCY339. Each sequence analyzed represents a distinct PCR reaction. The identification of the mutation is therefore not a result of a PCR artifact because the base change appears in multiple PCR products. In addition to finding the mutation in the alignment shown in Figure 8, the mutation was ruled out as being a result of a PCR based error by looking at the second strand from a separate PCR reaction on which the

mutation was still seen. Figure 9 shows the base peaks on the electropherogram, with the arrow showing the location of the mutant on cdc14.

Figure 9: Sequence of RCY339 *cdc14* Mutant and the Location of the Mutation
Electropherogram of RCY339 *cdc14* locus sequenced with CDC14 Br (5` CCGCATGGTATATTTCCA3`). The arrow indicated the base changed from a G to an A.



Based on these data, it was concluded that RCY339 was indeed a *cdc14* mutant and the complementation by *CDC14* was not suppression. Also, because the majority of the tetrads scored for RCY818 exhibit a 4:0 ratio for Ts^+, it can be concluded that the plasmid and the *ts* mutation are linked because the mutation is in the *cdc14* gene. The times that 3:1 Ts^+:Ts^- is observed may be because of a loop out recombination event.

However, though there is an obvious mutation in *cdc14,* does it have an effect on the function of the encoded protein causing the arrest of the mutant cells? Figure 10 shows the protein sequence alignment of the protein sequences of GG1888 and RCY339. The unshaded aligned amino acids show the position where the mutation caused a change from a proline to a serine in the sequence (amino acid structures of proline and serine in Figure 11).

Figure 10: Alignment of Wild Type GG1888 and mutant RCY339 Protein Sequences
The third row contains the consensus sequence. Amino acid is given in single letter code for a region of Cdc14

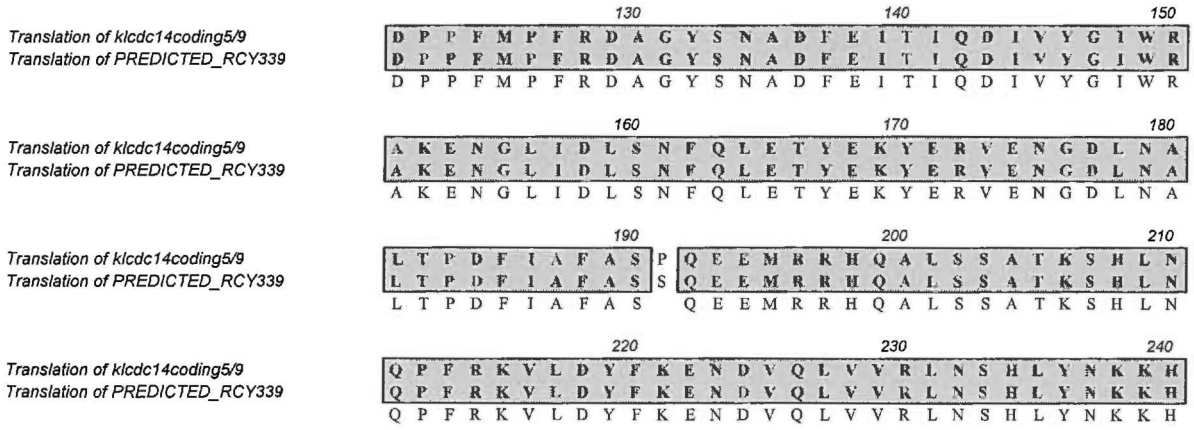


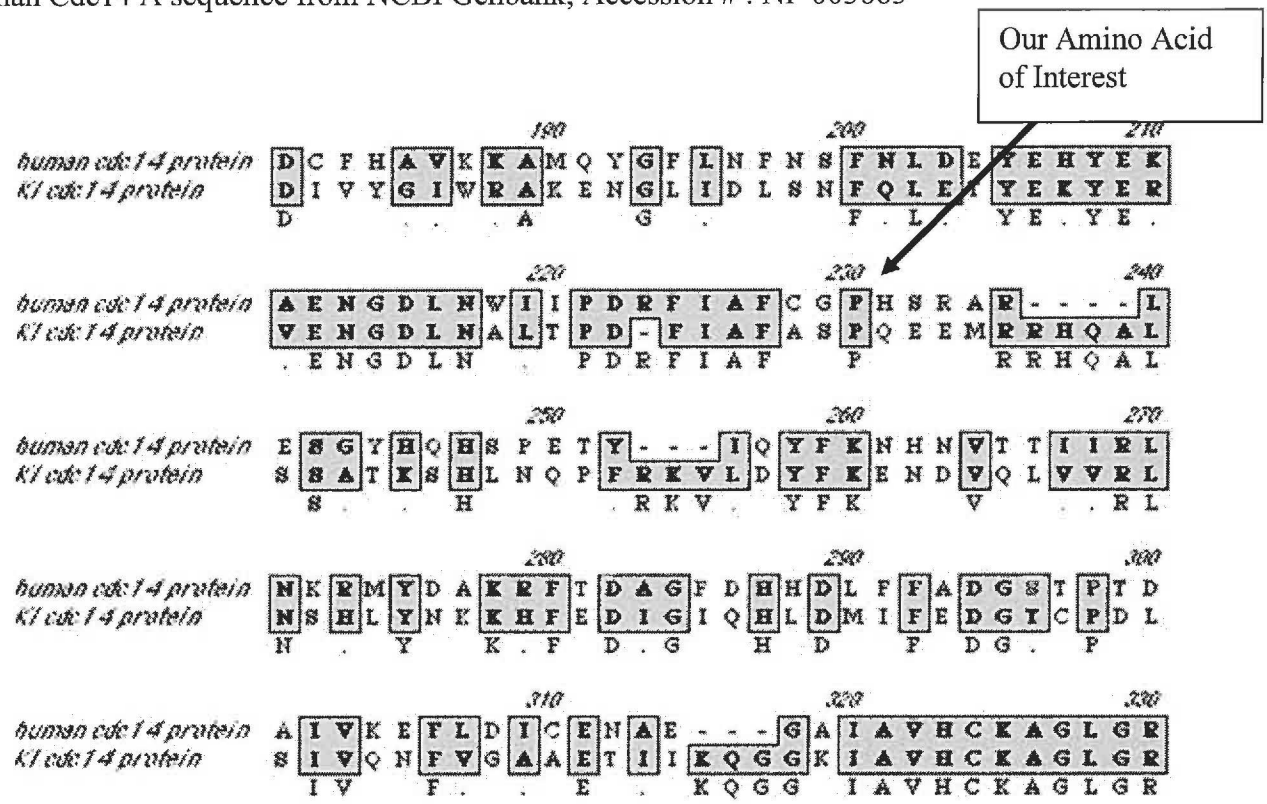
Figure 11: Proline and Serine Structures

http://www.cem.msu.edu/~cem252/sp97/ch24/ch24aa.html

Alignment of the human and *K. lactis CDC14* protein sequence alignment in Figure 12 show that there are portions of the gene that are conserved, one of which is the location where the mutation is found.

Figure 12: Alignment of Human and *K. lactis* Protein Sequences

Human Cdc14 A sequence from NCBI Genbank, Accession # : NP 003663



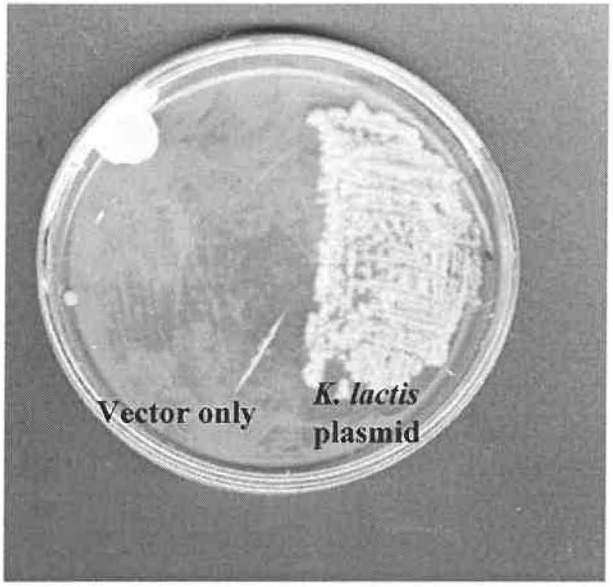
This information is important in that if the same amino acids exist in the same position in humans, it must mean that it is functionally conserved and most likely serves an important role in protein function.

S. cerevisiae Transformation-

A *S. cerevisiae cdc14* mutant was transformed with *K. lactis CDC14* wild type DNA in order to test whether the gene from *K. lactis* could complement a *S. cerevisiae* mutant. Figure 13 shows a plate consisting of the transformed cells. The half on the right consists of the transformant with the plasmid containing a wild type copy of *K. lactis CDC14.* The left half of the plate consists of the cells transformed with the vector and do not contain a wild type copy of *CDC14.* The plates were incubated at the restrictive temperature, 37° C, in order to test for temperature sensitive

growth. Our *K. lactis CDC14* gene is able to rescue the temperature sensitivity of a *S. cerevisiae cdc14* strain. These transformants have been frozen.

Figure 13: *S. cerevisiae* Transformation Plate
A *S. cerevisiae cdc14* mutant was transformed with a plasmid a wild type *K. lactis CDC14* insert. A control sample was transformed with the vector alone. Transformants were identified using the *URA3* marker on the vector. One transformant from each transformation was streaked on YPD medium and incubated at 37° C.



Discussion

Starting out this portion of the project, one of the most important things to figure out was whether our *K. lactis* RCY339 mutant was indeed a *cdc14* mutant, or if extra copies of Cdc14 were suppressing a mutation in another gene. It has been previously determined that both *cdc14* mutants and other MEN mutants have been able to function with extra copies of Cdc14 and grow at restrictive temperatures (Jaspersen *et al.*, 1998). Extra copies of Cdc14 presumably could cause it to be released out of the nucleolus and consequently lead to the exit from mitosis, because of the activation of cyclin degradation and deactivativation of CDK. Jimenez *et al.* (2001) showed that an overexpression of Cdc14 could "disturb the stoichiometric balance

between Cdc14" and its Net1/Cfi1p complex, therefore leaving an active amount of Cdc14 not sequestered/tethered to the complex and as a result released from the nucleolus. Over expression of Cdc14 gives some amount of non-sequestered Cdc14 which then plays it role in the cells exit from mitosis and suppressing the existing MEN mutant/s. Results gathered from our experiments showed that *CDC14* was mutated, and that suppression was not occurring.

Linkage analysis was done in order to determine if the location of the mutation was in *CDC14* or in another unlinked MEN gene. The plasmid that was inserted into the *K. lactis* mutant consisted of a wild type copy of *CDC14* and a *URA*3 tag. This plasmid was integrated into the mutant's *CDC14* locus. When this plasmid-containing strain was crossed to wildtype (resultant diploid = RCY818), the majority of the spores were Ts^+ (9 tetrads in 10), meaning that the *ts* mutation is tightly linked to the wild type *CDC14*. Had the mutation been in a different gene, results would have shown a variety of Ts^- spores, varying with the segregation of the genes. If unlinked, the expected pattern would have been 1:4:1 (2:2, 3:1, and 4:0 Ts^+:Ts^-) but this was not seen. However, how do we explain the fact that not all the spores in our analysis were Ts^+? An explanation for this is that recombination occurred within the chromosome and separated the mutant copy of *cdc14* from the wild type copy of the gene from the plasmid. A loop out occurs when this recombination happens between the tandem wild type and mutant copies, therefore the *CDC14* wild type copy of the gene is lost, leaving only the *ts* copy. Calculations showed that the linkage between the mutant and wild type copies of the genes were 5 centimorgans apart. To estimate the physical distance, we used information regarding *ARG1* and *ADE1*. Zonneveld (1995) showed that the genes *ARG1* and *ADE1* in *K. lactis* have a map distance of 20 centimorgans. Genbank's chromosome C from the *K. lactis* sequence (accession # CR382123) shows that this physical distance between these two genes in *K. lactis* is approximately 20 kb.

Therefore, if this relationship is generally true in *K. lactis,* then 1 centimorgan is about 1 kB physical distance. The problem is, we don't know if this relationship holds true to all parts of the *K. lactis* genome, but it provides a rough guide.

There are several issues that arose as to the accuracy of sequencing such as PCR based errors. Because amplification itself can cause mutations to occur in the DNA sample, it could be that our mutation was an artifact of the PCR. The error rate is estimated to be 2 x 10 -4 nucleotide/cycle (Ausubel *et al.*, 1993). However, because we sequenced several different samples from separate PCR reactions and the same mutation existed, it rules out the possibility that our mutation is a mere PCR error.

Despite the fact that we did find a mutation in *CDC14,* it has not been proven that it has any effect on protein function at all. It could be that the mutation that we have found has no effect on the function of the protein and our *K. lactis* mutant's inability to progress through the rest of the cell cycle is effected by another mutation in another gene. The amino acid change that results from the mutation is from a proline to a serine. Proline induces kinks in the protein because its side chain forms a ring with the backbone of the amino acid. It is usually found in tight turns of the protein. Serine on the other hand, does not have a side chain that forms a covalently bonded ring. However, its side chain can hydrogen bond with the backbone of the amino acid, and has been found to mimic proline. The hydrogen bond in serine is not as strong or stable as that of the covalent bond in proline, and will fall apart under stressful conditions, such as a rise in temperature. So, when place at a restrictive temperature, it affects the folded structure of the protein and its overall stability (http://www.russell.embl-heidelberg.de/aas/aas.html). Because our mutant is a temperature sensitive mutant, it makes sense that at the permissive temperature of

24° C it is able to function and when placed at the restrictive temperature of 37° C it is unable to because of the breaking of the hydrogen bond in serine. Though we have not proven that the function of the protein is altered, it is safe to assume that the amino acid change will have some effect on the protein and its function at the restrictive temperature, due to a change in protein shape of the serine.

Currently, backcrosses are being performed in order to eliminate any other existing mutations in RCY339. By backcrossing the strain, a purer form of the mutation can be observed. The strain may be healthier and mate better and we can be assured that any phenotypic changes from GG1888 behavior are due to our *ts* mutation.

Future work will be done in order to further support that the *K. lactis cdc14* mutant has indeed arrested after anaphase. The first step in doing so is checking cyclin levels in RCY339, in order to determine whether *CDC14* acts like a member of the MEN (involved in mitotic exit like in *S. cerevisiae*) or the SIN (involved in cytokinesis, like in *S. pombe*).

Mitotic exit is characterized by the cascade effect of the MEN in *S. cerevisiae*. Once activation of the first protein (Tem1) occurs, the other proteins in the network are sequentially activated until Cdc14 is released from its complex and therefore the nucleolus (reviewed in McCollum and Gould, 2001). One function of Cdc14 has been identified as dephosphorylating a protein (Hct1), which consequently goes on to activate the APC. This complex then ubiquitinates cyclin which signals for the degradation of cyclin to occur. This allows mitotic CDKs to be inactivated and allows exit from mitosis to occur. Thus a signature characteristic of a MEN mutant is high mitotic cyclin levels at non-permissive temperatures.

Cytokinesis in *S. pombe* is characterized by an analogous network of proteins, the SIN. This network allows cytokinesis (septum formation between the cells) to take place. In *S. pombe*, the homolog to Cdc14 is Clp1 and Flp1 (Trautmann *et al.*, 2001; Cueille *et al.*, 2001). Though in the SIN Clp1 is not needed for mitotic exit, the network does not allow Clp1 to return to the nucleolus until septation is complete. It is thought that Clp1 inhibits CDK activity until cytokinesis is completed, making sure that the current cell cycled is completed before a new one can begin.

The major question that will be attempted to be answered in a future experiment is whether Clb2 (a major type of mitotic cyclin) is stable or gone in the *K. lactis cdc14* mutant RCY339 at the restrictive temperature. If Clb2 levels are stable, our mutant is arrested before mitotic exit. If cyclin levels are low, the *K. lactis* mutant is not like a MEN mutant and Cdc14 may therefore play a role similar to that of Clp1 in the SIN during cytokinesis. In order to distinguish between these possibilities, the presence of Clb2 needs to be identified in *K. lactis*. To do this, an immunoblot using antibodies against *S. cerevisiae* Clb2 will be used to see if recognition of *K. lactis* Clb2 occurs. In this experiment, the samples were made, but due to time constraints, immunoblots were not run. If a band is observed, the next thing that needs to be done is to see if the identity of the band is truly Clb2. Because Clb2 is a mitotic cyclin, it should be seen in cells going through mitosis. Therefore, if the band is Clb2, it should not be seen in G1, but in mitosis. To verify the identity of the band, a microtubule inhibitor (nocodazole) will be added to two RCY339 cell cultures. Nocodazole will cause arrest in mitosis due to the lack of a spindle. One culture will be allowed to arrest at the permissive temperature (24°C) and the other culture at the restrictive temperature (37°C) (Azzam, personal communications). After arrest occurs, the cells will be observed at various time points after removal of the nocodazole. Cells observed at the

time points taken at the permissive temperature should show an initially high level of cyclin which then falls. The cells observed during mitosis would show high levels of Clb2, but as exit from mitosis progresses, the levels would decrease due to the degradation of the cyclin (this sample is therefore used as a control). Cells from the time points observed at the restrictive temperature, could give one of two results. A constant high level of Clb2 might be observed, meaning that the cells are stuck before mitotic exit because of their inability to exit mitosis and therefore would indicate a MEN-like mutant. Or, Clb2 levels might be observed to fall, meaning the cells are able to exit mitosis, and the mutant is stuck in cytokinesis, meaning that the *K. lactis CDC14* functions in a network homologous to the SIN.

According to the results already gathered, we would expect to see Clb2 levels stuck when tested at the restrictive temperature, because RCY339 appears to be arrested after anaphase in DAPI and tubulin staining, as it does not form chains of cells like *S. cerevisiae* cytokinesis mutants. The results to be gathered from the Clb2 project will just give us more insight into the functions of Cdc14.

The MEN and the SIN function have the similar processes but work to accomplish two different functions. The MEN is involved in the exit from mitosis and the SIN is involved in the completion of cytokinesis. Given the characteristics of our mutant, it would seem as though the *K. lactis CDC14* functions in the MEN as opposed to the SIN. The *S. cerevisiae* complementation experiment shows that the *K. lactis CDC14* is able to make up for a mutated *S. cerevisiae cdc14*, and shows that the activity of Cdc14 in both species is similar.
The identification of Cdc14's role in a cell can open doors to future discoveries. Cancer is characterized by the increase of cell production, and the inability of the body/cell, to turn off this

production. If the exact function of Cdc14 can be determined, there is future research that can be done to look into the potential of inhibiting a MEN gene product, such as Cdc14, in order to stop cell growth and allow the cancer to be cured.

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. 2002. The Cell Cycle and Programmed Cell Death. *Molecular Biology of the Cell.* 17: 983-997.

Ausubel, F. M., Brent, R. Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. 1993. Enzymatic Amplification of DNA by PCR. *Current Protocols in Molecular Biology.* 15:1.6.

Bardin, A., Visintin, R., and Amon, A. 2000. A Mechanism for Coupling Exit from Mitosis to Partitioning of the Nucleus. *Cell.* 102:21-31.

Cueille, N., Salimove, E., Esteban, V., Blanco, M., Moreno S., Vueno, A., and Simanis, V.. 2001. Flp1, a Fission Yeast Orthologue of the *S. cerevisiae CDC14* Gene, is Not Required for Cyclin Degradation or rum1p Stabilisation at the End of Mitosis. *Journal of Cell Science.* 114:2649-2664.

Gietz, D., St. Jean, A., Woods, R. A., and Schiest, R. H. 1992. Improved Method for High Efficiency Transformation of Intact Yeast Cells. *Nucleick Acids Research.* 20(6):1425.

Hartwell, L., Culotti, J., and Reid, B.. 1970. Genetic Control of the Cell-Division Cycle in Yeast, I. Detection of Mutants. *Proceedings of the National Academy of Sciences.* 66:352-359.

Hartwell, L. 1971. Genetic Control of the Cell Division Cycle in Yeast IV. Genes Controlling Bud Emergence and Cytokinesis. *Experimental Cell Research.* 69(2):265-276.

Hartwell, L., Mortimer, R., Culotti, J., and Culotti, M.. 1973. Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of *cdc* Mutants. *Genetics.* 74: 267-286.

Jaspersen, S. L., Charles, J. F., Tinker-Kulberg, R. L., and Morgan, D. O. 1998. A Late Mitotic Regulatory Network Controlling Cyclin Destruction in *Saccharomyces cerevisiae. Molecular Biology of the Cell*, vol. 9: 2803-17.

Jimenez, J., Cid, V. J., Nombela, C., and Sanchez, M. 2001 A Single-copy Suppressor of the *Saccharomyces cerevisiae* Late-Mitotic Mutants *cdc15* and *dbf2* is Encoded by the *Candida albicans CDC14* gene. *Yeast*, vol. 18, pg. 849-858

McCollum, D, Gould, K. 2001. Timing is Everything: Regulation of Mitotic Exit and Cytokinesis by the MEN and SIN. *TRENDS in Cell Biology.* 11:89-95.

Munson, A. 2003. Cell Division Cycle Control and Regulation Within the Budding Yeast *Kluyveromyces lactis: cdc14* Mutation or Suppression. University of Redlands. Honors Thesis.

Oldroyd, A. 2002. Identification and Characterization of Cell Division Cycle Mutants Followed by the Cloning of the Gene Responsible for a Cdc$^-$ Phenotype in the Budding Yeast *Kluyveromyces lactis*. University of Redlands. Honors Thesis.

Rose, M. D. and Broach, J. R. 1991. Cloning Genes by Complementation in Yeast. *Guide to Yeast Genetics and Molecular Biology.* 14:195-199.

Sambrook, J., and Russell, D. W. 2001. Electrophoresis Buffers. *Molecular Cloning: A Laboratory Manual.* 1:5.8

Sherman, F. 1991. Getting Started With Yeast. *Guide to Yeast Genetics and Molecular Biology.* 1:12-17,

Sherman, F. and Hick, James. 1991. Micromanipulation and Dissection of Asci. *Guide to Yeast Genetics and Molecular Biology.* 2:21-37.

Shou, W., Seol, J. H., Shevchenko, A., Baskerville, C., Moazed, D., Chem, Z. W. S., Jang, J., Shevchenko, A., Charbonneau, H., and Deshaies, R. J. 1999. Exit from Mitosis is Triggered by Tem1-dependent Release of the Protein Phosphatase Cdc14 from Nucleolar RENT Complex. *Cell.* 97(2):233-244.

Trautmann, S., Wolfe, B., Jorgensen, P., Tyers, M., Gould, K., and McCollum, D. 2001. Fission Yeast Clp1p Phosphatase Regulate G2/M Transition and Coordination of Cytokinesis with Cell Cycle Progression. *Current Biology.* 11:931-940.

Zonneveld, B.J.M., and Van Der Zanden, A.L. 1995. The Red *ade* Mutants of *Kluyveromyces lactis* and their Classification by Complementation with Cloned *ADE1* or *ADE2* Genes from *Saccharomyces cerevisiae. Yeast.* 11:823-27.

Appendix

Backcross Data-

The crosses done with GG1888 were done with RCY strains that are *ts* products of one round of RCY339 backcrosses.

The following crosses were done for the second round of backcrosses (Table 1 and Table 2):

Table 1: Backcrosses made with GG188 and RCY strains

GG1888 with:	
Cross Identification Name	**RCY#**
A1	754
B1	756
C1	760
D1	770
E1	776
F1	790
G1	781

Table 2: Backcrosses made with LSY25 and RCY strains

LSY25 with:	
Cross Identification Name	**RCY#**
A2	752
B2	759
C2	765
D2	774
E2	783
F2	785
G2	789

All strains crossed with GG1888 and LSY25 are from the RCY collection of strains, RCY339 derivatives.

The following crossed colonies developed diploids: A1, D1, E1, F1, G1, A2, B2, C2, D2, E2, F2, and G2. These diploids were then put in to sporulation media in order for tetrads to develop. Table 3 shows the results for the spore media check.

Table 3: Sporulation Check of Diploid Cells Put in Sporulation Media from 1st Backcross

Diploid Name	Appearance of Cells
A1	Dead, misshapen, dark, relatively small. No tetrads or triads.
D1	Many tetrads.
E1	Dead, dark, and misshapen.
F1	Some tetrads, some big clumps.
G1	Many tetrads.
A2	Many single cells.
B2	Several triads, many dead and misshapen.
C2	Many tetrads.
D2	Many tetrads.
E2	Several triads, not many cells.
F2	Dark, singles, misshapen.
G2	Some tetrads, many triads.

From the results in Table 3, D1 (RCY770 x GG1888) was randomly selected from the tubes that contained many tetrads to be dissected. Table 4 shows the scoring results for Ura, Ts, Ade, and His phenotypes.

Table 4: Scoring of Ura, Ts, Ade, and His phenotypes for RCY770 x GG1888 dissected spores

Growth observed is either + (healthy looking, strong growing colony), - (no growth).
^ = spores used in another backcross
* = indicates that the strain has been frozen
( ) = number in parenthesis signifies the strain number given to a frozen spore

Spore Number	Ura	Ts	Ade	His
1A	-	+	+	+
1B^ *(898)	+	-	-	-
1C	-	+	+	+
1D^ *(899)	+	-	-	-
2A	-	-	+	+
2B^ *(900)	+	-	-	-
2C	+	+	+	+
2D	-	+	-	-
3A	-	+	-	+
3B	-	-	+	-
3C	+	+	-	+
3D	+	-	+	-
4A	-	+	-	+
4B^ *(902)	+	-	-	-
4C	+	-	+	-
4D	-	+	+	+
5A	-	-	-	-
5B	-	-	-	+
5C	+	+	+	-
5D	+	+	+	+

6A^ *(903)	+	-	-	-
6B	-	-	-	-
6C	+	+	+	+
6D	+	+	+	+
7A*(904)	-	+	-	+
7B	+	-	+	+
7C	+	+	+	-
7D	-	-	-	-
8A*(905)	+	+	-	-
8B	-	-	+	-
8C	-	-	-	+
8D	+	+	+	+

From the dissected tetrads, spores bearing a Ts^-, Ura^+ phenotype were used for a second backcross with GG1888. Spores 1B, 1D, 2B, 4B, and 6A were used, and all the crosses produced diploids which were then put into sporulation media. Table 5 shows the results from the sporulation check.

Table 5: Sporulation Check of Diploid Cells Put in Sporulation Media from Second Backcross

Spore/Diploid Name	Appearance of Cells
1B	Dead and misshapen single cells and clumps.
1D	Dead and misshapen single cells and clumps.
2B	Dead and misshapen single cells and clumps.
4B	Several tetrads, but not many.
6A	One tetrad, the rest appear dead.

Because only the cross using RCY902 (spore 4B) yielded asci, another cross was done to get better results. Table 6 shows the results from the second attempt.

Table 6: Sporulation Check of Diploid Cells Put in Sporulation Media from Second Attempt of Second Backcross

Spore/Diploid Name	Appearance of Cells
1B	Some triads, singles, but a lot of misshapen cells.
1D	Singles, dark and dead.
2B	Several triads, dark and dead.
4B	Many tetrads, healthy looking.
6A	Singles.

Results were better from this cross, but RCY902 (4B) was the strongest looking of the set again. So the RCY902 x GG1888 asci were dissected. Table 7 shows the Ura, Ts, Ade, and His phenotypes of these dissected spores.

Table 7: Scoring of Ura, Ts, Ade, and Ura phenotypes for RCY902 x GG1888 dissected spores

Growth observed is either + (healthy looking, strong growing colony), - (no growth).
^ = spores used in another backcross
* = indicates that the strain has been frozen
( ) = number in parenthesis signifies the strain number given to a frozen spore

Spore Number	Ura	Ts	Ade	His
1A	-	-	-	+
1B*(906)	+	+	-	-
1C	+	+	+	+
1D	-	-	+	-
2A	-	-	-	-
2B	+	+	+	+
2C	-	+	+	-
2D^ *(907)	+	-	-	+
3A	-	+	-	-
3B	-	-	+	+
3C*(908)	+	+	-	-
3D	+	-	+	+
4A	+	-	+	+
4B*(909)	+	+	+	-
4C	-	-	-	-
4D	-	+	-	+
5A^*(910)	+	-	+	-
5B*(911)	+	+	-	-
5C	-	+	+	+
5D	-	-	-	+
6A	-	-	+	+
6B*(912)	+	+	-	-
6C	-	+	-	-
6D^*(913)	+	-	+	+
7A	+	-	-	+
7B	-	-	+	-
7C	-	+	-	+
7D	+	+	+	-

From the dissected tetrads, spores containing Ts^-, Ura^+ phenotype were used for a third backcross with GG1888. Spores 2A, 5A, 6D were used, and all the crosses produced diploids which were then put into sporulation media. Table 8 shows the results from the sporulation check.

Table 8: Sporulation Check of Diploid Cells Put in Sporulation Media from 3rd Backcross

Spore/Diploid Name	Appearance of Cells
2D	Some tetrads.
5A	Singles and clumps.
6D	Singles and clumps.

No further backcrosses were done but the spores that were used were frozen, and the tubes with the last diploids put into sporulation media (2A, 5A, and 6D) were placed into the cold room for future dissection and backcrosses.

Backcross of other *cdc* mutants

The following tables show the scoring information from the screening of the following strains backcrossed strains: RCY829, RCY830, RCY831, RCY832

The phenotypes that are recorded in each table are the uracil auxotropy and temperature sensitivity (*ts*) markers as well as the growth seen for the temperature sensitive spores.

Backcross of RCY424, a *K. lactis cdc, ura3* mutant

RCY424 was previously mated to the wild type strain LSY25 to create RCY829.

Table 9: Scoring of Ura, Ts, Ade, and His phenotypes of RCY829 dissected spores
Growth observed is either + (healthy looking, strong growing colony), - (no growth).
* = indicates that the strain has been frozen
( ) = number in parenthesis signifies the strain number given to a frozen spore

Spore Number	Ura	Ts	Ade	His
1A* (846)	+	-	+	-
1B* (847)	-	-	+	-
1C	-	+	-	+
1D	+	+	-	+
2A	-	+	-	+
2B	-	-	+	-
2C	+	-	+	-
2D* (848)	+	+	-	+
3A	-	+	-	+
3B	+	-	+	+
3C	+	-	+	-
3D* (849)	-	+	-	-
4A* (850)	-	-	+	+

4B* (851)	+	-	+	-
4C	+	+	-	+
4D	-	+	-	-
5A	-	+	-	-
5B	-	+	-	-
5C* (852)	+	-	+	+
5D* (853)	+	-	+	+
6A	-	-	+	-
6B	-	-	+	+
6C	+	+	-	+
6D	+	+	-	-
7A* (854)	-	+	-	-
7B	-	-	+	+
7C* (855)	+	+	-	+
7D	+	-	+	-
8A	-	-	+	-
8B	+	-	+	+
8C	-	+	-	+
8D	+	+	-	-
9A	+	+	-	+
9B	+	-	+	-
9C	-	-	+	+
9D	-	+	-	-

The *ts* and ade1 seem to be linked since they always segregate the same with respect to one another (if ts^-, then Ade^+). This suggests 424 might be mutated in *cdc15* since it is very close to ADE1 in *K. lactis* (genbank, accession number: CR382123).

Backcross of RCY435, a *K. lactis cdc, ura3* mutant

RCY435 was previously mated to the wild type strain LSY25 to create RCY830.

Table 10: Scoring of Ura, Ts, Ade, and His phenotypes of RCY830 dissected spores
Growth observed is either + (healthy looking, strong growing colony), - (no growth), weak growth (slight film observed), slight growth (more growth than just a film, but not strong enough to be positive growth), film, spotted (not an entire colony grown, but spotted growth around the frogged area).
* = indicates that the strain has been frozen
( ) = number in parenthesis signifies the strain number given to a frozen spore

Spore Number	Ura	Ts	Ade	His
1A	-	+	+	+
1B* (856)	-	-	-	-
1C* (857)	+/-	-	+	+/-
1D* (858)	+	+	-	-
2A* (859)	+	+	-	-
2B	+	-	+	+

2C* (860)	-	-	-	+
2D	-	+	+	-
3A	+	+	+	+
3B	-	+	-	+
3C* (861)	-	-	-	-
3D* (862)	+	-	+	-
4A* (863)	+	+	+	-
4B* (864)	+	+	-	-
4C	-	-	-	-
4D* (865)	-	-	+	+
5A* (866)	-	-	+	+
5B* (867)	-	+	-	-
5C* (868)	+	+	+	-
5D* (869)	+	+	-	+
6A* (870)	+/-	-	-	+/-
6B* (871)	-	+	+	-
6C* (872)	+	-	+	-
6D* (873)	-	-	-	+/-

Backcross of RCY290, a *K. lactis cdc, ura3* mutant

RCY290 was previously mated to the wild type strain LSY25 to create RCY831.

Table 11: Scoring of Ura, Ts, Ade, and His phenotypes of RCY831 dissected spores
Growth observed is either + (healthy looking, strong growing colony), - (no growth), weak growth (slight film observed), slight growth (more growth than just a film, but not strong enough to be positive growth), film, spotted (not an entire colony grown, but spotted growth around the frogged area).
* = indicates that the strain has been frozen
( ) = number in parenthesis signifies the strain number given to a frozen spore

Spore Number	Ura	Ts	Ade	His
1A* (874)	-	-	-	-
1B* (875)	-	-	+	+/-
1C	+	+/-	+	+/-
1D* (876)	+	+	-	-
2A	-	+/-	-	+/-
2B* (877)	+	+	+	-
2C* (878)	+	-	-	-
2D* (879)	+/-	-	+	+/-
3A	+	+/-	+	+
3B	+/-	+	+	+
3C* (880)	-	-	-	-
3D* (881)	+	-	-	+
4A	+	+/-	-	-
4B	+	+/-	+	+
4C* (882)	-	-	-	+
4D* (883)	-	-	+	-

5A	+	+/-	+	+
5B* (884)	+	-	+	+
5C* (885)	-	-	-	-
5D	-	+/-	+	+
6A	-	+	-	-
6B	+	+/-	+	+
6C	+	+	+	+
6D	+	-	+	+
7A* (886)	+	+	-	-
7B	+/-	+	+	+/-
7C	+	-	-	+/-
7D	+	-	+	+
8A	-	-	+	+/-
8B	-	-	-	-
8C* (887)	+	-	-	-
8D	+	+	+	+
9A	+	+	+	+
9B	+	-	+	+
9C* (889)	-	-	-	-
9D* (890)	+	-	-	-
10A	+/-	-	+	+/-
10B* (891)	-	+	+	+
10C* (892)	+	-	-	+
10D	-	+/-	-	-
11A	+	+/-	-	-
11B* (893)	-	-	+	+
11C	+	+	-	-
11D* (894)	-	-	+	-

First round backcrosses (such as RCY424, RCY435, and RCY290), the *ts* segregates 2:2, implying that these mutants have a single *ts* mutation.

Linkage Analysis of RCY339 Derivatives-

The following tables show the scoring information from the screening of RCY339 derivatives. These results were used in linkage analysis in order to determine a map distance of between the *ts* mutation and the integrated *CDC14* plasmid at the *CDC14* locus. The following strains were scored: RCY818, RCY832, RCY833, RCY895, RCY339 x RCY826, RCY339 x RCY827, and RCY784 x RCY827.

RCY818 created by a cross between wild type RCY784 and RCY821. RCY821 was formed when the *cdc14* mutant RCY773 was transformed with the *CDC14* integrating plasmid at the chromosomal *CDC14* locus. RCY784 is wild type for *URA3* and *CDC*.

Table 12: Scoring of Ura, Ts, Ade, and His phenotypes of RCY818 dissected spores
Growth observed is either + (healthy looking, strong growing colony), - (no growth).

Spore Number	Ura	Ts	Ade	His
1A	+	+	+	+
1B	+	+	+	-
1C	+	+	-	+
1D	-	+	-	-
2A	+	+	-	+
2B	+	+	+	-
2C	+	+	-	-
2D	-	+	+	+
3A	+	+	+	-
3B	-	+	-	+
3C	+	+	+	-
3D	-	+	-	+
4A	+	+	+	-
4B	-	+	+	+
4C	+	-	-	-
4D	+	+	-	+
5A	+	+	-	-
5B	+	+	+	+
5C	-	+	+	-
5D	+	+	-	+
6A	+	+	+	+
6B	+	+	-	-
6C	-	+	+	-
6D	+	+	-	+
7A	+	+	-	-
7B	+	+	+	+
7C	-	+	+	-
7D	+	+	-	+
8A	+	+	+	+
8B	+	+	-	+
8C	+	+	-	-
8D	+	+	+	-
9A	+	+	+	-
9B	+	+	-	-
9C	+	-	-	+
9D	-	+	+	+
10A	+	+	+	+
10B	+	+	-	-
10C	-	+	-	-
10D	-	+	+	+

Note: All spores are Ts^+ with the exception of 4C, 6D, and

9C
Ura does not segregate 2:2 because the *URA* locus is heterozygous and the *CDC14* locus also has a *URA3* marker.
Pictures of tetrads scanned in user file Diana Z. under:
818 tetrads 1-6 6/28/04 and
818 tetrads 7-10 6/28/04

A second round of spores from RCY818 were dissected in a separate experiment.

Table 13: Second set of scoring of Ura, Ts, Ade, and His phenotypes of RCY818 dissected spores
Growth observed is either + (healthy looking, strong growing colony), - (no growth), weak growth (slight film observed), slight growth (more growth than just a film, but not strong enough to be positive growth), film, spotted (not an entire colony grown, but spotted growth around the frogged area).

Spore Number	Ura	Ts	Ade	His
1A	+	+	+	+
1B	+	+	+	+
1C	+	+	+	+
1D	+	+	-	-
2A	+	+/-	-	+
2B	+	+	+	+
2C	+	+	+	-
2D	+	+	-	-
3A	+	+/-	-	-
3B	+	+	-	+
3C	+	+	+	+
3D	-	+	+	-
4A	+	+	-	+
4B	+	+/-	-	+
4C	+	+	+	-
4D	+	+	+	-
5A	-	+	-	+
5B	+	+	+	+
5C	-	+	-	-
5D	+	+	+	-
6A	+	+	+	+
6B	+	+	-	-
6C	-	+	+	+
6D	+	+	-	-
7A	+	+	+	-
7B	-	+	-	+
7C	+	+	-	-
7D	+	+	+	+
8A	+	+	+	+
8B	+	+	+	+
8C	+	+	-	-
8D	-	+	-	-

9A	+	+	+	-
9B	+	+	-	+
9C	+	+	+	-
9D	+	+	-	+
10A	+	+	+	+
10B	+	+	-	-
10C	+	+	+	-
10D	+	+	-	+
11A	+	+	+	-
11B	-	+	+	+
11C	+	+	-	-
11D	+	+	-	+
12A	+	+	-	+
12B	+	+	+	+
12C	+	+	+	-
12D	+	+	+	-

Note: Spores from Tetrads 1 and 12 are not tetrads because Ade marker did not segregate 2:2.
Pictures of tetrads scanned in user file Diana Z. under:
818 tetrads 1-6 7/2/04 and
818 tetrads 7-12 7/2/04

RCY897 is a diploid of a cross between wild type RCY784 and RCY827. RCY827 is a transformant of RCY773 (transformant XX) containing the integrating plasmid *CDC14/URA*. Because RCY897 is similar to that of RCY818, we ideally would expect to see the same results of 4:0 Ts^+:Ts^-. However, there are some 3:1 Ts^+:Ts^- seen, due to a potential loop out event.

Table 14: Scoring of Ura, Ts, Ade, and His phenotypes of RCY897 (RCY784 x RCY827) dissected spores

Growth observed is either + (healthy looking, strong growing colony), - (no growth), weak growth (slight film observed), slight growth (more growth than just a film, but not strong enough to be positive growth), film, spotted (not an entire colony grown, but spotted growth around the frogged area).

Spore Number	Ura	Ts	Ade	His
1A	-	+	-	-
1B	+	+/-	-	-
1C	+	+	+	+
1D	-	+	+	+
2A	-	+	-	-
2B	+	+	+	+
2C	+	+	+	-
2D	-	+	-	+
3A	+	+/-	-	-
3B	+	+	+	+
3C	+	+	-	+
3D	+	+	+	-
4A	+	+	-	-
4B	+	+	+	+

4C	+	+	+	+
4D	+	+	-	-
5A	+	-	-	-
5B	+	+	+	+
5C	+	+	+	-
5D	-	+/-	-	+
6A	+	+	+	+
6B	+	+	+	+
6C	-	+	-	-
6D	+	+/-	-	-
7A	+	-	-	-
7B	+	+	+	+
7C	+	+	-	-
7D	+	+	+	+
8A	+	+	-	-
8B	+	+	-	+
8C	+	+	+	-
8D	-	+	+	+
9A	+	+	+	-
9B	+	-	-	+
9C	+	+	+	+
9D	+	+	-	-
10A	+	+/-	-	-
10B	+	+	+	+
10C	-	+	-	-
10D	-	+	+	+
11A	+	+	+	+
11B	-	+	-	-
11C	+	-	-	-
11D	+	+	+	+
12A	+	+	+	-
12B	+	+/-	-	-
12C	-	+	+	+
12D	+	-	-	+
13A	+	+	+	-
13B	+	-	-	-
13C	-	-	-	+
13D	-	+	+	+
14A	+	+	-	-
14B	-	+	+	-
14C	-	+	+	+
14D	+	-	-	+
15A	+	+	+	-
15B	+	+	-	+
15C	-	+	-	-
15D	-	+	+	+
16A	+	+	+	-
16B	+	+	-	+

16C	+	+	-	+
16D	+	+	+	-
17A	-	+	-	-
17B	+	+	-	+
17C	+	+	+	+
17D	+	+	+	-
18A	-	+	+	-
18B	+	+	+	+
18C	+	+	-	-
18D	+	-	-	+

RCY895 was used as a control for RCY818 to show that the plasmid only integrated at one site on the genome in RCY821. The cross resulting in RCY895 was between RCY339 (*ts*, *ura3*) and RCY821. If only one integration event occurred, than Ura^+ and Ts+ will co-segregate 2:2. This is what is observed.

Table 15: Scoring of Ura, Ts, Ade, and His phenotypes of RCY895 (RCY339 x RCY821) dissected spores

Growth observed is either + (healthy looking, strong growing colony), - (no growth), weak growth (slight film observed), slight growth (more growth than just a film, but not strong enough to be positive growth), film, spotted (not an entire colony grown, but spotted growth around the frogged area).

Spore Number	Ura	Ts	Ade	His
1A	+	+	+	+
1B	-	-	+	+
1C	+	+	-	+
1D	-	-	-	+
2A	+	+	+	+
2B	-	-	-	+
2C	+	+	-	+
2D	-	-	+	+
3A	-	-	+	+
3B	+	+/-	-	+
3C	+	+	+	+
3D	-	-	-	+
4A	+	+	-	+
4B	+	+	+	+
4C	-	-	+	+
4D	-	-	-	+
5A	+	+	-	+
5B	+	+	+	+
5C	-	-	+	+
5D	-	-	-	+
6A	+	+	-	+
6B	+	+	+	+
6C	-	-	+	+
6D	-	-	-	+
7A	+	+	-	+

7B	+	+	+	+
7C	-	-	-	+
7D	-	-	+	+
8A	+	+	+	+
8B	-	-	-	+
8C	-	-	+	+
8D	+	+	-	+
9A	-	-	+	+
9B	+	+	-	+
9C	+	+	-	+
9D	-	-	+	+
10A	+	+	-	+
10B	-	-	-	+
10C	+	+	+	+
10D	-	-	+	+
11A	+	+	-	+
11B	-	-	+	+
11C	+	+	-	+
11D	-	-	+	+
12A	-	-	-	+
12B	+	+	-	+
12C	-	-	+	+
12D	+	+	+	+
13A	+	+/-	+	+
13B	+	+	-	+
13C	-	-	+	+
13D	-	-	-	+
14A	+	+/-	-	+
14B	-	-	+	+
14C	-	-	-	+
14D	+	+	+	+
15A	+	+	+	+
15B	-	-	-	+
15C	-	-	+	+
15D	+	+	-	+
16A	-	-	+	+
16B	+	+	-	+
16C	-	-	+	+
16D	+	+	-	+

A diploid of a cross between RCY339, a *K. lactis cdc, ura3* mutant, to RCY826. RCY826 is an RCY339 derivative containing an integrated *CDC14/URA3* plasmid. If only one integration event occurred, than Ura^+ and Ts^+ will co-segregate 2:2. This is what is observed.

Table 16: Scoring of Ura, Ts, Ade, and His phenotypes of RCY339 x RCY826 dissected spores

Growth observed is either + (healthy looking, strong growing colony), - (no growth).

Spore Number	Ura	Ts	Ade	His

1A	-	-	-	+
1B	-	-	+	+
1C	+	+	+	+
1D	+	+	-	+
2A	-	-	+	+
2B	-	-	-	+
2C	+	+	-	+
2D	+	+	+	+
3A	+	+	-	+
3B	-	-	+	+
3C	+	+	+	+
3D	-	-	-	+
4A	-	-	-	+
4B	-	-	-	+
4C	+	+	+	+
4D	+	+	+	+
5A	-	-	-	+
5B	+	+	-	+
5C	-	-	+	+
5D	+	+	+	+
6A	-	-	-	+
6B	+	+	+	+
6C	-	-	-	+
6D	+	+	+	+

RCY832 is a diploid of a cross between RCY339, a *K. lactis cdc, ura3* mutant, to RCY824. RCY824 is an RCY339 derivative containing an integrated *CDC14/URA3* plasmid. If only one integration event occurred, than Ura^+ and Ts^+ will co-segregate 2:2. However, though 2:2 segregation is observed, co-segregation is not. So, this does not seem to be the correct strain because the Ts^+ is not being co-inherited with the *URA3* from the same plasmid. This transformant should not be used in further studies because it does not follow the expected pattern of co-segregation.

Table 17: Scoring of Ura and Ts phenotypes of RCY832 dissected spores
Growth observed is either + (healthy looking, strong growing colony), - (no growth).

Spore Number	Ura	Ts	Ade	His
1A	-	-	+	-
1B	-	-	+	+
1C	+	-	-	-
1D	+	-	-	+
2A	+	-	-	+
2B	-	+	+	-
2C	-	-	+	+
2D	+	+	-	-
3A	-	-	+	+
3B	+	-	-	-
3C	-	-	+	+

3D	+	-	-	-
4A	+	-	+	-
4B	+	-	-	+
4C	-	-	-	-
4D	-	+	+	+
5A	+	+	+	-
5B	-	-	-	-
5C	-	-	+	+
5D	+	-	-	+
6A	-	-	+	-
6B	+	-	-	+
6C	-	-	+	+
6D	+	-	-	-
7A	+	-	-	+
7B	+	+	+	-
7C	-	+	-	-
7D	-	-	+	+
8A	-	-	+	+
8B	+	+	+	-
8C	+	-	-	+
8D	-	+	-	-
9A	-	+	-	-
9B	+	-	+	+
9C	-	-	+	+
9D	+	-	-	-
10A	+	-	-	+
10B	+	-	+	+
10C	+	+	+	+
10D	-	-	-	-
11A	-	-	+	-
11B	+	-	-	+
11C	-	+	+	+
11D	+	-	-	-
Conclusions for 832 are that it does not seem to have correct integration because Ura is not linked to Ts, which it should be				

RCY833 is a diploid of a cross between RCY339, a *K. lactis cdc, ura3* mutant, to RCY822. RCY822 is an RCY339 derivative containing an integrated *CDC14/URA3* plasmid. If only one integration event occurred, than Ura^+ and Ts+ will co-segregate 2:2. This is what is observed.

Table 18: Scoring of Ura, Ts, Ade, and His phenotypes of RCY833 dissected spores
Growth observed is either + (healthy looking, strong growing colony), - (no growth).

Spore Number	Ura	Ts	Ade	His
1A	-	-	+	+
1B	+	+	+	+
1C	-	-	-	+
1D	+	+	-	+

2A	-	-	+	+
2B	-	-	-	+
2C	+	+	+	+
2D	+	+	-	+
3A	+	+	-	+
3B	+	+	-	+
3C	-	-	+	+
3D	-	-	+	+
4A	-	-	+	+
4B	+	+	+	+
4C	+	+	-	+
4D	-	-	-	+
5A	+	+	-	+
5B	-	-	+	+
5C	-	-	-	+
5D	+	+	+	+
6A	+	+	+	+
6B	-	-	+	+
6C	-	-	-	+
6D	+	+	-	+
7A	-	-	-	+
7B	+	+	+	+
7C	+	+	-	+
7D	-	-	+	+
8A	-	-	+	+
8B	+	+	-	+
8C	+	+	-	+
8D	-	-	+	+
9A	-	-	+	+
9B	+	+	-	+
9C	+	-	-	+
9D	-	-	+	+
10A	-	-	+	+
10B	+	+	+	+
10C	-	-	-	+
10D	+	+	-	+

RCY896 is a diploid of a cross between RCY339, a *K. lactis cdc, ura3* mutant, to RCY827. RCY827 is an RCY339 derivative containing an integrated *CDC14/URA3* plasmid. If only one integration event occurred, than Ura^+ and Ts+ will co-segregate 2:2. This is what is observed, with some exceptions, some Ts^+ not seen.

Table 19: Scoring of Ura, Ts, Ade, and His phenotypes of RCY896 (RCY339 x RCY827) dissected spores

Growth observed is either + (healthy looking, strong growing colony), - (no growth), weak growth (slight film observed), slight growth (more growth than just a film, but not strong enough

to be positive growth), film, spotted (not an entire colony grown, but spotted growth around the frogged area).

Spore Number	Ura	Ts	Ade	His
1A	-	-	-	+
1B	+	+	+	+
1C	-	-	-	+
1D	+	+	+	+
2A	-	-	-	+
2B	+	+/-	+	+
2C	-	-	-	+
2D	+	+	+	+
3A	+	+/-	-	+
3B	-	-	-	+
3C	-	-	+	+
3D	+	+	+	+
4A	+	+	+	+
4B	+	-	-	+
4C	-	-	-	+
4D	-	-	+	+
5A	-	-	+	+
5B	+	-	-	+
5C	+	-	-	+
5D	-	-	+	+
6A	-	-	+	+
6B	-	-	+	+
6C	+	+	-	+
6D	+	+	-	+
7A	+	+	-	+
7B	-	-	-	+
7C	+	+	+	+
7D	-	-	+	+
8A	+	-	-	+
8B	-	-	+	+
8C	+	-	-	+
8D	-	-	+	+
9A	+	+	+	+
9B	-	-	-	+
9C	+	+	+	+
9D	-	-	-	+
10A	-	-	-	+
10B	-	-	+	+
10C	+	+	-	+
10D	+	+	+	+
11A	+	+	+	+
11B	-	-	-	+
11C	+	+	+	+
11D	-	-	-	+
12A	+	+	+	+

12B	-	-	+	+
12C	+	+	-	+
12D	-	-	-	+
13A	+	+	+	+
13B	-	-	-	+
13C	+	+	+	+
13D	-	-	-	+
14A	+	+	+	+
14B	+	+	-	+
14C	-	-	+	+
14D	-	-	-	+

Pictures of tetrads scanned in user file Diana Z. under:
339x827 1-8 7/27/04 and
333x827 9-14 7/27/04