Investigation of Complemented Transformants of "Kluyveromyces lactis" Temperature Sensitive Cell Division Cycle Mutants RCY426 and RCY435

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Investigation of Complemented Transformants of
*Kluyveromyces lactis* Temperature Sensitive Cell Division Cycle
Mutants RCY246 and RCY435

Elysia Carnagey

Honors Thesis

13 April 2009

Dr. Linda Silveira
Abstract:

The cell division cycle is a complex process necessary for the survival of all organisms. This highly controlled series of phases requires the proper functioning of all proteins involved to yield accurate cell cycle progression. Dr. Leland H. Hartwell is well known for his cell cycle research in the budding yeast *Saccharomyces cerevisiae* in which he identified and classified cell division cycle mutants (*cdc*) in order to better understand cell cycle controls. Hartwell used conditional mutants that were viable at the restrictive temperature to accurately study the mutant phenotype of cells with a cell cycle gene deficiency. While this research looks uses similar methods, it is useful to study another strain of budding yeast, *Kluyveromyces lactis*, in order to discover new *CDC* genes or other possible roles for *CDC* genes previously discovered.

This study involves the previously identified temperature sensitive (*ts*) *cdc* *K. lactis* mutants RCY246 and RCY435. In order to determine its defective gene, RCY246 was transformed with a *K. lactis* genomic DNA library and screened for transformants that grew at the restrictive temperature. A complementing transformant for RCY246 was found and designated RCY946. To amplify the complementing plasmid, the plasmid DNA from RCY946 was isolated and transformed into *E. coli*. Retransformation of RCY246 with the proposed complementary plasmid indicated partial complementation of RCY246; however, the plasmid was unstable. In an attempt to amplify the insert region of the plasmid, a polymerase chain reaction was used and the major products were sequenced. To determine the genes of interest, the sequence was input into a BLAST program that revealed the presence of *E. coli* genomic DNA. Information about the complementing plasmid has yet to be discovered. In addition, RCY435 has a previously
identified complemented transformant, RCY947. The complementing plasmid was isolated from RCY947 and amplified in *E. coli*. To verify complementation, the plasmid DNA could be retransformed into RCY435 and transformants could grow at the restrictive temperature.

**Introduction:**

The cell division cycle is the process necessary for all cell survival and thus the survival of all organisms. If cells are unable to reproduce, replicate and transmit their DNA, they will cease to exist. The cell cycle involves highly controlled mechanisms that yield the accurate replication and preservation of genetic information. Disruptions within cell cycle genes could lead to mutations of the cell and may negatively affect the entire organism. The study of the cell cycle and its controls may help to provide insight on why some cells have uncontrollable abnormal growth, such as in malignant tumors (Alberts *et al.*, 2002; Botstein *et al.* 1997).

Four distinct phases define the cell cycle; G1, S, G2, and M phase. The cell’s DNA is replicated during S phase and packed tightly into chromosomes. M phase includes chromosomal segregation and DNA distribution to two daughter cells, followed by the physical separation of cells, cytokinesis. The two gap phases, G1 and G2, are essential for cell growth and monitoring of the environment to ensure optimal conditions for the cell cycle to proceed (Alberts *et al.*, 2002). Each of these phases includes a series of transitions and checkpoints to guarantee the cycle has the components necessary to progress to the next phase. Transition regulators are promoters of phase changes, when the biochemistry changes from one state to the next. Checkpoint regulators act as the
surveillance mechanisms to check the integrity of the cell; they stop transition regulators until the monitored cell components are ready to progress (Morgan, 2007).

Many of these transitions are highly regulated by cyclin dependent kinases (Cdk). Cdk are enzymes that activate or inactivate protein substrates by phosphorylating them, thus changing the enzymatic activity of the substrates. These changes in the cell cycle components initiate cell cycle events or transitions (Morgan, 2007).

The activation and inactivation of Cdk may be controlled by the binding of the protein cyclin and cyclin’s degradation through ubiquitin-mediated proteolysis respectively. While Cdk levels remain constant throughout the cell cycle, the level of cyclin fluctuates, thus controlling cell cycle events (Alberts et al., 2002). While cyclin proteins regulate Cdk activity, cyclin protein levels are controlled as well. Two methods to regulate cyclin are through ubiquitination of cyclin protein and transcription of cyclin genes. Two ubiquitin ligases are present in the cell cycle, SCF (Skp1/Cullin/F-box) and APC (anaphase promoting complex), each of which has a different control (Morgan, 2007). SCF activity is regulated by the phosphorylation of its target protein. For example, the cyclins bound to activate Cdk can be phosphorylated and are then recognized by SCF as targets for ubiquitination. Proteosomes degrade the ubiquitinated subunits to render the Cdk inactive (Alberts et al., 2002). APC, on the other hand, is activated by phosphorylation of the enzyme itself. For instance, before the transition from anaphase to telophase, a subunit of APC, Cdh1 (Hct1), is dephosphorylated by the CDC14 gene product, a gene that functions during this transition. Due to this dephosphorylation, the
APC is activated causing the degradation of M-cyclins, thus inactivating M-Cdk and enabling the cell to exit mitosis (Morgan, 1999).

CdkS themselves can also be regulated by cyclin kinase inhibitors (CKIs) as well as inhibitory phosphorylation. For example, the yeast CKI, Sic1, inhibits S-Cdks by altering the structure of its active site before cells enter S phase. Sic1 must be ubiquitinated by SCF and degraded before the S-Cdk can become active and allow entry into S phase (Barberis et al., 2005). An example of inhibition through phosphorylation involves the M-Cdk and mitotic entry seen mostly in the fission yeast Schizosaccharomyces pombe. The protein kinase, Wee1, phosphorylates the active site of the M-cyclin-Cdk complex making it inactive. To activate M-Cdk, the protein phosphatase cdc25 must dephosphorylate the active site allowing the cell to proceed into mitosis (Harvey et al., 2003, Dunphy et al., 1991).

The cell cycle begins in G1; however cells are not committed to the cell cycle until they have progressed through the Start transition in late G1. Important protein regulators of Start are G1/S-cyclins. Other important transitions in the cell cycle include G1 to S phase, G2 to M phase, metaphase to anaphase, and mitotic exit.

Entry into mitosis requires the accumulation of M-cyclins and the dephosphorylation of M-Cdks, to activate the M cyclin-Cdk complex. This complex is involved in essential roles including mitotic spindle formation and chromosome condensation (Morgan, 2007).

The APC controls both the transition from metaphase to anaphase and mitotic exit. The metaphase to anaphase transition requires sister chromatid separation. Sister chromatids are held together by the protein complex cohesin. To initiate this transition,
the anaphase promoting complex (APC) must mark the protein securin with ubiquitin for degradation. Securin degradation releases a protease, separase, that cleaves the cohesin complexes and allows the mitotic spindle to separate the sister chromatids leading to mitotic exit. Mitotic exit is triggered by the inactivation of M-Cdk5. The APC targets the M-cyclins on activated M-Cdk5 for degradation by ubiquitination. After the M-cyclins are degraded, their M-Cdk5 are inactivated, allowing the cell to exit mitosis (Alberts et al., 2002).

It is important to recall that each of these transitions is governed by checkpoints that allow the cell to confirm if all conditions have been met before proceeding to the next phase. Checkpoints for damage to replicated DNA halt the cell cycle in G2 until repairs have been made before entering mitosis (Longhese et al., 1998). In most eukaryotes, including fission yeast, damaged DNA prevents mitotic entry. When incomplete or damaged chromosomes are present, protein kinases of the DNA damage response system are activated. This system acts on the cell-cycle control system to prevent mitotic Cdk activation and thus blocks the cell from entering mitosis. However, in budding yeast, cells with damaged DNA enter mitosis and arrest in metaphase. Although the mechanism isn't entirely clear, one likely theory is the damage response kinases prevent the ubiquitination of securin by the APC and thus block sister chromatid separation (Morgan, 2007).

Spindle assembly is another important checkpoint in the cell. Before chromosome separation, the cell must ensure that there are no defects in the mitotic spindle and that all chromosomes are attached to the spindle properly. In the event that the spindle is
damaged or defective, the checkpoint mechanism halts the cell pre-anaphase allowing time for accurate attachment (Lew et al., 1997).

In order to study the controls of the cell cycle, many past and recent studies have used yeast mutants to genetically analyze the control system (Alberts et al., 2002). Yeast is widely used due to its quick reproduction and its ability to grow either diploid or haploid; genetic disruptions can be easily studied because, in many cases, mutations cannot be masked by the existence of a second active gene when cells are in the haploid state. In addition, yeast is advantageous for its genes homologous to those of humans and other mammals (Lee et al., 1987; Botstein et al. 1997). This evolutionary conservation of genes between eukaryotic organisms can lead to more in depth understanding of the overall cell cycle in organisms where it can be studied less easily.

One of the most recognized researchers of cell cycle controls is Leland H. Hartwell. He is well known for his identification and classification of cell division cycle mutants (cdc) in the budding yeast Saccharomyces cerevisiae (see for example, Hartwell et al., 1970; Hartwell et al., 1973). S. cerevisiae is a budding yeast whose morphology correlates to the cell cycle progression; as the cell cycle proceeds a small bud begins to form off the parent bud and continues to grow until pinched off at the end of mitosis. If stopped at an unknown point in the cell cycle, the morphology of the yeast at this arrest enables researchers to determine which phase the yeast is in.

Mutations in cdc genes led to a halt in the cell cycle and couldn’t be studied unless the mutant phenotypes were conditional. Conditional mutations in yeast allow growth, or wild type phenotype, under one set of conditions and no growth, or mutant phenotype, under another. These are identified as temperature sensitive (ts) mutants.
Temperature sensitive genetic mutations are not severe enough to affect growth at low (permissive) temperatures and thus allow normal progression through the cell cycle. However, mutated proteins at a higher (nonpermissive or restrictive) temperature have more difficulty folding and thus fail to allow continued growth of yeast (Morgan, 2007).

Hartwell used this information to study \( ts \) mutant strains in order to find \( cdc \) mutants (Hartwell et al., 1970). Hartwell predicted that \( cdc \ ts \) mutations would yield a uniform morphology in \( S. \ cervisiae \) at the nonpermissive temperature; he used 36°C. These results would indicate that the cells are able to complete the cell cycle up to a specific point and then arrest leading to a similar morphology, which includes bud size and the amount of DNA segregation, among cells. However, \( ts \) mutations that affect essential processes unrelated to the cell cycle yield bud morphologies in all different points of the cell cycle. This is because mutations unrelated to the cell cycle may affect genes necessary for overall cell growth. Therefore, when shifted to the restrictive temperature, they cannot continue to cycle, so they arrest with varying morphologies. This difference allows for an easy screen to identify \( ts \) mutants that are \( cdc \). Hartwell found that while \( cdc \ ts \) mutations yield uniform morphology among one strain, arrested morphologies may differ between various \( cdc \) mutant strains. These differing morphological arrest points implied that mutations in different genes have distinct effects on the cell cycle. In addition, different gene products may have been used at different times.

In order to determine at which point in the cell cycle the mutant gene was needed, Hartwell used time-lapse photomicroscopy to look at cells blocked at specific stages in the cell cycle through a shift to the restrictive temperature. His experimental results
allowed him to define two important points: the execution and termination points. The execution point defines the point in the cell cycle when the defective gene product is normally needed, while the termination point defines the point at which the cells arrest. Hartwell noticed that these two points may not be at the same point in the cell cycle (Hartwell et al., 1970). One explanation is that some event earlier in the cell cycle may produce products that may not be needed for regulation until a later stage. Thus, the later stage is dependent on the completion of the earlier event. Also, the role of a defective gene product may be carried out earlier in the cell cycle, but is not screened until a later checkpoint. Most, if not all, cell cycle events are linked through these “dependent pathways” and thus checkpoints are vital to the integrity of the cell (Lew et al., 1997).

After this earlier discovery of cdc mutant identification, Hartwell went on to classify cdc mutants by their prototype phenotype and complementation groups (Hartwell et al., 1973). He completed many complementation crosses that yielded diploid mutants, some possessing the wild type phenotype, while others did not. Mutants that continued to display a mutant phenotype after crossing were placed in the same complementation group. Hartwell found 32 complementation groups overall in S. cerevisiae. In addition, he classified the 32 groups into their prototype phenotype, or the arrested phenotype displayed by most mutants within a group. Researchers can use his results to examine their cdc mutants’ termination phenotypes and determine which cdc genes may be defective (Figure 1, Hartwell et al., 1973). Since the genome of S. cerevisiae has been sequenced and the roles of many of its genes discovered, the Saccharomyces Genome Database (SGD) can be used to search for the specific role of CDC genes within the cell cycle (www.yeastgenome.org). Thus far, these subsequent studies of Hartwell’s mutants
have helped identify the important roles of many cdc genes including Cds (CDC28), anaphase promoting complex (APC) genes (CDC16, CDC 20, CDC23, CDC26, CDC27), and mitotic exit network (MEN) genes (CDC14, CDC15) among others which can be obtained via the SGD. Therefore, a continuation of these studies may be valuable in identifying and classifying additional important players in the cell cycle of yeast and often in other eukaryotes.

While there have been numerous studies on S. cerevisiae, it is still worthwhile to study a second budding yeast, Kluyveromyces lactis. Evidence indicates that after the divergence of S. cerevisiae from K. lactis, the entire S. cerevisiae genome duplicated. However, most of the duplications were deleted over time and reciprocal translocations led to a rearrangement in gene order (Wolfe et al., 1997). Thus, while S. cerevisiae can still be haploid, it contains more redundant genes due to blocks within homologous DNA that appear to be left from the whole-genome duplication. Although many cdc genes have been discovered in S. cerevisiae in the haploid state, there is a great probability that more can be identified in K. lactis since it contains fewer redundancies (Dujon et al., 2004). Duplications of genes may differ between these two types of yeast. It should be noted that due to redundant copies of some genes in S. cerevisiae, mutations in multiple genes may be required to cause a cell cycle arrest. This makes it difficult to identify redundant genes in a cdc screen. One example is the B-Type CLB genes found in S. cerevisiae (Fitch et al., 1992). Fitch et al. found that at least three CLB genes must be mutated in order to create inviable cells. Therefore, three or more mutations would be needed to identify cells in a ts cdc screen.
Additionally, *Schizosaccharomyces pombe*, a fission yeast, is another popular species of study. In the wild, *S. pombe* grows as a haploid and *S. cerevisiae* lives as a diploid. Another contrasting factor between these two types of yeast is that *S. cerevisiae* spends more time in G1, while *S. pombe* spends most of its time in G2. One theory explaining this difference is that if there were a double stranded break in the DNA, diploids would have the ability to repair the damage due to their second copy of DNA to act as a template for repair. Haploids, however, lack this second copy to repair damage until they replicate their DNA; they move through G1 rapidly in order to replicate their DNA quickly and then spend more time in G2 determining if the cell is ready to proceed to mitosis (Murray *et al.*, 1993). *K. lactis* can be used to test the theory of how ploidy and budding are involved in cell cycle regulation, since it is predominantly haploid (Dujon *et al.*, 2004).

Another reason to study a variety of yeast species was concluded from studies by Kaback *et al.* (1984) who looked at lethal *ts* mutations on chromosome I in yeast. While they found 32 different mutations, they determined that these mutations only represented three genes. Because there are many essential genes on chromosome I (thirteen, according to SGD), this implies that some genes may be more difficult to mutate to a *ts* phenotype than others. Also, Hartwell *et al.* helped conclude that per screening method, there is a limited number of *cdc* genes that can be found given that the screens have biases that yield non-random mutant recovery (Figure 3, Hartwell *et al.*, 1973). Therefore, *cdc* *ts* mutations require a strict set of conditions that could be easier to meet for some *cdc* genes in *K. lactis* over *S. cerevisiae*. 
Furthermore, *K. lactis* studies could deepen our understanding of possible regulator roles. It should be noted that while regulators exist for each of these different species of yeast, the roles or importance of the regulators may differ between species. For example, *MIHI* in *S. cerevisiae* is the homolog to *cdc25* in *S. pombe* (Russell et al., 1989). *Cdc25* is the phosphatase needed to dephosphorylated M-Cdks and allow mitotic entry. However, mutations in *MIHI* only cause G2 delay, while mutations in *cdc25* are known to cause G2 arrest. These results helped lead to the conclusion that *S. cerevisiae* doesn’t require much *MIHI* because very few of its M-Cdks are phosphorylated. This *cdc25* regulator was discovered due to its observable mutant phenotype in *S. pombe*, yet it is difficult to identify the role of *MIHI* in *S. cerevisiae* because the cells don’t arrest indicating that *cdc* screens can only identify regulators that play essential roles within a species (Booher et al., 1993). Since this important regulator was not identified in *cdc* screens in *S. cerevisiae*, it may have gone unnoticed if not for the study of another yeast species. Therefore, studying *K. lactis* may reveal additional regulators.

Also, it was hypothesized in a previous study that similar mutations of the target site for Cdc25/Wee1, in tyrosine phosphorylation of the Cdk homologs, Cdc2 in *S. pombe* and Cdc28 in *S. cerevisiae* would lead to similar results (Sorger et al., 1992). These proteins, Wee1/Cdc25, are involved in the inhibitory phosphorylation and activating dephosphorylation of the active site of Cdks. It was evident from previous research that mutations preventing tyrosine phosphorylation of Cdc2 could lead to premature initiation of mitosis and disruption in S-phase feedback controls triggered by unreplicated DNA. Since this phosphorylation site was known to be conserved among many species, the authors hypothesized that a disruption of this site in the *S. cerevisiae* homolog, Cdc28,
would yield similar results. Their conclusions indicate, however, that while Cdc28 is phosphorylated on the homologous tyrosine, mutations of this phosphorylation site do not lead to premature acceleration into mitosis or disruption in feedback controls (Sorger et al., 1992). Thus while some regulatory genes may appear similar, it is still worthwhile to study their function because they may yield different results and could lead to an understanding behind those differences.

This research involves the study of two different mutant strains of *K. lactis*: RCY435 and RCY246. RCY435 has been previously characterized at the University of Redlands as a *ts* Ura^- cdc mutant. Earlier studies have backcrossed this *ts* cdc mutant to a wild type strain and determined that the mutant phenotype is due to a single recessive mutation (Zeilicovici, 2006). The mutant phenotype of RCY435 at its termination point reveals a single large bud with a mass of unsegregated DNA (Figure 1). Since the DNA appears unsegregated, the cells must not have completed anaphase, categorizing RCY435 as a pre-anaphase arresting mutant strain. In addition, RCY246 has also been characterized as a pre-anaphase arrest mutant (Figure 2).

There are numerous types of mutations that could result in this phenotypic arrest, which may include disruptions to the APC, or defects in DNA replication, spindle formation, or other genes that could trigger a pre-anaphase checkpoint if defective. Hartwell found mutants involved in DNA replication such as *cdc6, cdc7, cdc8, cdc9, cdc13* and *cdc21* that demonstrated a pre-anaphase arrest (Hartwell et al., 1973, SGD). As discussed earlier, DNA damage in budding yeast prevents the separation of sister chromatids most likely through blocking the degradation of securin, (Morgan, 2007) and thus can also cause a pre-anaphase arrest.
Along with DNA replication mutations, this phenotype can also be seen in strains that have mutations in their APC. Some examples of genes that encode for an APC protein subunit are *CDC16, CDC23, CDC26* and *CDC27* (SGD). In addition, *CDC20* is another gene involved in the activation of the APC, among other roles (Lim *et al.*, 1998). Research has shown that *CDC20* is a substrate specific activator of the APC and may be required for APC degradation of substrates, such as Pds1, also known as securin, in the metaphase-anaphase transition (Visintin *et al.*, 1997). Thus if there is a mutation in this gene, the APC will not be activated and thus securin will not be degraded. Due to this, sister chromatids will remain bound together by cohesin and the cell will not progress through this transition, leading to pre-anaphase arrest. Also, APC is involved in mitotic exit, regulated by *CDC14*, but this is less important since a mutation affecting only this aspect of its function would not lead to pre-anaphase arrest.

Alternatively, this phenotype may reflect a problem with the mitotic spindle. Another study by Hwang *et al.* (1998) proved that Cdc20 is also the target for the spindle checkpoint in budding yeast. Thus, additionally, if genes encoding the mitotic spindle were mutated, Cdc20 would be the target to inhibit the APC and prevent the cell from progressing through anaphase (Hwang *et al.*, 1998). Thus if mitotic spindle genes are mutated, they would cause a similar arrest phenotype. This idea is supported by studies using nocodazole as a microtubule inhibitor to cause cell cycle arrest in *K. lactis* and *S. cerevisiae*, which show the pre-anaphase arrest phenotype expected from altering spindle formation (Visintin *et al.*, 1997; Personal communication with Linda Silveira).

Furthermore, a previously studied *K. lactis* strain with a probable mutation in tubulin, the structural protein component of microtubules, also led to a pre-anaphase arrest (Fonseca,
2008; Personal Communication with Linda Silveira). Therefore, RCY246 and RCY435 could have defects in DNA replication, mitotic spindle formation, or the APC.

This research is designed to study the two mutant strains, RCY246 and RCY435, and their complementing genes. To find the complemented transformant, each mutant strain was grown at 24°C and a *K. lactis* library was transformed into the cells. The inserted plasmid contained a marker, *URA3*, which allowed only the transformants to grow when plated on minimal media, SD, plates. The conditional mutant that survived at both the permissive temperature of 24°C and the restrictive temperature of 36°C was expected to contain the complementary gene to the mutated copy. Both RCY435 and RCY246 have proposed complemented transformants, RCY947 and RCY946, respectively, thus far.

In order to verify if the complementing transformant is plasmid-dependent and not rescued by a second mutation suppressing the defect, each of these *ts cdc* mutant strains, RCY435 and RCY246, have previously been analyzed using a 5-FOA test (Molinos, 2008; Louie, 2008). For this test, the complementing transformant for each strain was streaked onto plates containing 5-FOA (5-fluoroorotic acid) and grown at both the permissive and restrictive temperatures. In the presence of *URA3*, the 5-FOA is converted to a toxin and kills all of the Ura+ cells (Leeuwen and Gottschling, 2002). Therefore, *cdc* mutants that were successfully transformed and contain the *URA3* from the plasmid will die. Yet, when the cells divide after transformation, not all of the cells will receive a plasmid and thus some cells are expected to live. However, they would only live at the permissive temperature because even though the *URA3* is absent, the cells are now *ts* because they have lost the complementing gene and therefore should die at the
restrictive temperature. If these results were found, the complementing transformant would be considered plasmid-dependent. After conducting this test, RCY435 was determined to be plasmid dependent (Molinos, 2008). However, previous experiments have shown that RCY246 is only temperature sensitive on rich media, YPD, and the 5-FOA test failed to work on this type of media (Louie, 2008). Thus, RCY246’s rescue has not yet been determined to be plasmid-dependent.

Due to its sensitivity to growth on specific media, research shows that RCY246 may be an osmotic remedial mutant (Louie, 2008). This implies that a mutant, if provided with higher osmotic support, loses its mutant phenotype, in this case, temperature sensitivity. Past research shows that SD medium can suppress the ts phenotype of mutations in genes required for chitin synthesis. This suppression is believed to be due to the greater osmotic support on SD media. Chitin is involved in the essential role of creating the septum that separates daughter cells (Bulawa, 1992). These results indicate that the mutant gene in RCY246 may be involved in resistance to low osmolarity since it shows preferences for higher osmotic support found in minimal media (Louie, 2008). However, RCY246’s loss of temperature sensitivity may also suggest that higher osmotic support aids in stabilizing the folding of the mutant protein such that it cannot be destabilized when put at higher temperatures (Hawthorne et al., 1964). If that were the case, the mutated gene in RCY246 may function only in the cell cycle rather than normally functioning in osmoregulation or protection against osmotic stress.

The studies of this experiment are dedicated to recovering the rescuing complementing plasmid, amplifying in E. coli, and transforming it back into the original mutant to see if it still rescues the mutant phenotype. If the cells still display the mutant
phenotype, cell death at the restrictive temperature, this may indicate that the transformants gained another mutation or that they have multiple plasmids and the correct one must be studied. A 5-FOA test, previously mentioned, should be done to rule out multiple mutations. However, if the transformants are able to grow at the restrictive temperature, then a correct plasmid has been isolated. Each plasmid insert is about 8-10 kilobases (kb) in size and studies of the *K. lactis* genome indicate that this correlates to approximately 4 genes per insert (Weslowski-Louvel; Dujon *et al.*, 2004). Therefore, the gene responsible for rescuing the mutation must be identified from this group. This can be done by sequencing the insert and then inputting the results into BLAST to search for possible homologs in SGD or Genbank, to the identified gene sequence in order to better understand its role/s in the cell. If that gene is previously known to yield a mutant phenotype similar to that found in RCY246 or RCY435, then it is likely to be the complementary gene to the specific mutation. For example, genes of interest would include those known to encode DNA replication factors, spindle proteins or APC subunits. Yet, if no genes functioning in these processes are found on the insert, genes of unknown function can be investigated by complementation testing to determine if they contain a mutation in the genomic copy of genes in RCY246 or RCY435.
Figure 1: RCY435 Arrest Phenotype
Photograph of cell division cycle mutant RCY435 after inducing cell arrest by incubation at 36°C for nine hrs. Phase microscopy was used at a magnification of 1,000X (Molinos, 2008)

Figure 2: RCY246 Arrest Phenotype
Arrested RCY246 cells visualized by phase microscopy at 1000X after a 7 hour shift to 37°C (Andrew Oldroyd, 2002).

Materials and Methods:

Media and Strains:

YPD, SD, and LB + ampicillin (LB + amp) plates and liquid media were prepared as stated in the protocols of Ausubel et al. (1993). On SD + uracil plates, 0.3ml of 100X uracil (50μg/ml 100X stock) was topspread on SD media plates.

Strains used include LSY17, RCY246, RCY435, RCY946 and RCY947. The table below summarizes their sources and genotypes.

Table 1: Strains used in methods

<table>
<thead>
<tr>
<th>Strain</th>
<th>Original Name</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSY17</td>
<td>GG1888</td>
<td>Zonneveld and Van der Zanden (1995)</td>
<td>MAT a, TS, ura3</td>
</tr>
<tr>
<td>RCY246</td>
<td>-----</td>
<td>Derived from LSY17 by EMS Mutagenesis</td>
<td>MAT a, cdc, ts, ura3</td>
</tr>
<tr>
<td>RCY435</td>
<td>-----</td>
<td>Derived from LSY17 by EMS Mutagenesis</td>
<td>MAT a, cdc, ts, ura3</td>
</tr>
<tr>
<td>RCY946</td>
<td>-----</td>
<td>Hypothesized RCY246</td>
<td>MAT a, cdc, ts, ura3</td>
</tr>
<tr>
<td>RCY947</td>
<td>Hypothesized RCY435 complemented transformant</td>
<td>[TS, URA3 on KCp491 derived plasmid]</td>
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**K. lactis genomic DNA library:**

The *K. lactis* genomic library was a gift from M. Wesolowski-Louvel. Made in a *CEN2 E.coli/K. lactis* shuttle vector, KCp491, this library contains a common yeast *URA3* marker in order to select for transformants. Genomic DNA inserts for this library underwent a partial Sau3A digest that created fragmented inserts with an average size of 9 kb.

**Electroporation transformation of RCY435 with a *K. lactis* genomic library**

In order to find a plasmid that rescues the mutant phenotype in RCY435, the mutant strain was transformed with the Wesolowski-Louvel *K. lactis* genomic DNA library. The complementing plasmid should rescue the *ts cdc* mutation in RCY435 at the restrictive temperature. For successful transformation, after overnight growth, a single colony of RCY246 was grown in 5ml of liquid YPD. The overnight cell culture was diluted 1/50 in H2O and the sample’s O.D.600 was read using a Jasco V-530 UV/Vis Spectrophotometer. Once obtained, the O.D.600 reading is multiplied by 50 to account for the dilution factor. This stationary phase culture was used to inoculate a log phase culture for transformation. At the time of transformation, cells should yield an O.D.600 reading between 0.8-1.4 indicating approximately 2x10^7 cells/ml. To determine the correct amount to inoculate to obtain this level of growth, the starting O.D.600 is determined by the formula:
Final O.D./ml = (2^N) x (Starting O.D./ml)

where N is the amount of doubling times, assuming 2.25 hours per doubling. The OD reading of the first culture was divided by the calculated starting O.D.₆₀₀ to determine the dilution factor needed to obtain the desired starting O.D.₆₀₀ reading. Using twice the calculated amount most often led to the best results.

The next day the log phase culture was centrifuged at 3000rpm for five minutes, and the supernatant was poured off. The cells were washed with sterile water and spun down at 3000rpm for three minutes. The water was poured off and the cells were resuspended in pretreating buffer (YPD, 25mM DTT, 20mM HEPES, pH 8.0) at 2×10⁸ cells/ml using the conversion factor 1 O.D. = 2×10⁷ cells/ml. The cells were incubated at 24°C for 30 minutes at 100rpm. The cells were spun down at 13,000rpm for two minutes and resuspended in electroporation buffer (10mM TrisHCl, pH 7.5, 270mM sucrose, 1mM lithium acetate) at 6×10⁹ cells/ml. A 50µl aliquot was transferred to a chilled eppendorf and 100ng of K. lactis DNA library was added. The solution was put on ice for 15 minutes and then electroporated in a 0.2cm gap cuvette in a Bio-Rad Gene Pulser (San Francisco, CA) at 1000V, 400Ω, and 25µF with a time constant of approximately 5ms. The cells were transferred to a tube containing 1ml of ice-cold YPD liquid media and the tube was placed on ice for 15 minutes. The sample was rotated for one hour at room temperature. The cells were plated on SD minimal media in 200µl aliquots and placed at 24°C for 2-3 days.

**Replica Plating**
To determine which transformants, if any, contain the complementing plasmid to the mutation in RCY435, the cells were replica plated onto two SD and YPD plates according to the protocol in Ausubel et al. (1993). One plate was placed at the permissive temperature and one at the restrictive temperature.

**Extraction of proposed complementing plasmid of RCY435, from RCY947**

The likely complementing plasmid is extracted from *K. lactis* in order to amplify it in *E. coli*. There are two methods of extraction, the first of which is the yeast boiling DNA miniprep developed by Robzyk & Kassir (1992). An RCY947 transformant colony was grown overnight in 1.5ml of YPD liquid media. This cell culture was centrifuged for five minutes at 5000 x g. The supernatant was discarded and the cells were resuspended in 100μl STET (8% sucrose, 50nM Tris pH 8, 50mM EDTA, 5% Triton X-100). To the resuspension, 0.2g of 0.45mm glass beads were added and the solution was vortexed for five minutes. An additional 100μl STET was added to the mixture and the tube was placed in a 95°C heat block for three minutes. The mix was briefly cooled on ice and then centrifuged at 4°C for ten minutes. In a new tube containing 60μl of 7.5M ammonium acetate, 120μl of the mixture supernatant was added. The solution was incubated for one hour at -20°C and then centrifuged at 4°C for ten minutes. A 120μl aliquot of the supernatant was added to 2 volumes of ice-cold 100% ethanol. This solution was incubated at 20°C for 30 minutes and then centrifuged at 4°C for ten minutes. The pellet was washed with 70% ethanol, left to air-dry overnight, and then resuspended in 25μl TE.
The second method used to extract the RCY947 plasmid DNA was the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) per a user-developed protocol (http://www1.qiagen.com/literature/protocols/pdf/PR04.pdf).

Electroporation transformation of *E. coli*

For amplification, previously isolated proposed complementing plasmids of RCY246 and RCY435 were transformed into *E. coli* using electroporation. *E. coli* cells had been made competent by Jenna Woo (2006) according to the Sharma & Schimke (2006) protocol and were kept at -70°C in 40μl aliquots. A 1μl aliquot of DNA isolated using the yeast boiling prep was added to the 40μl of *E. coli* cells. Additionally, a 1μl aliquot of DNA isolated using the QIAprep Spin Miniprep Kit was added to another 40μl sample of *E. coli*. Two controls were used to indicate if the transformation was successful; *E. coli* only, negative control, and *E. coli* plus *pTZ*, the positive control. All samples were kept on ice. The solutions were kept on ice for one minute and then added to ice-cold 0.2cm electroporation cuvettes and left on ice. The samples were electroporated in a Bio-Rad Gene Pulser (San Francisco, CA) at 200Ω, 25μF, and 2.5kV and produced a time constant of approximately 4ms. Immediately following electroporation, 1ml of SOC medium (Invitrogen, Carlsbad, CA) was added to each sample. Samples were transferred to sterile microcentrifuge tubes and rotated for one hour at 37°C. The cells were spun down at 15,000 x g for two minutes. All but one drop of the supernatant was discarded and the cells were resuspended in this remaining drop. Cells were plated on LB+amp (150μg/ml) plates in 100μl aliquots and grown at 37°C overnight.
Extraction of plasmid from *E. coli*

The proposed complementing plasmids of RCY246 and RCY435, RCY946 and RCY947 respectively, were amplified in *E. coli* and must be extracted to retransform into the original mutants to confirm complementation. The plasmids were isolated from *E. coli* using the QIAprep HiSpeed Maxi Kit for low-copy plasmids protocol made by QIAGEN (Valencia, CA).

Verifying presence of DNA

Four *E. coli* transformant colonies with RCY946 were chosen and their plasmid DNA was isolated using the HiSpeed Maxi QIAGEN Kit. To verify the presence of plasmid DNA, 3μl of each sample of expected isolated DNA was run on an agarose gel against a 1kb molecular DNA ladder (New England Biolabs, Ipswich, MA).

Confirming plasmid similarity

To verify that each sample of isolated DNA contained the same plasmid, the DNA was cut with three different restriction endonucleases. The three restriction enzymes used were HindIII, XbaI and EcoRI, which were all chosen at random but would cut at different points within the plasmid. Three 20μl reactions were made, for each of the three chosen colonies, containing 2μl of RCY946 plasmid DNA, 1 X restriction buffer and 0.5 X BSA. After mixing, 10U of each restriction enzyme was added to its respective tubes. The samples were incubated at 37°C for two hours. Each sample was run on a 0.8% TAE
agarose gel (Sambrook and Russell, 2001) and the band patterns were compared across samples.

**Electroporation transformation of RCY246 with complementing plasmid**

To confirm complementation of RCY946, the original mutant, RCY246, was transformed with the complementing plasmid using the electroporation protocol according to Wesolowski-Louvel (personal communication). This protocol is the same as for the *K. lactis* library transformation of RCY435 above. For this particular transformation, 1μl of DNA was added at an approximate concentration of 150ng/μl.

**Confirming plasmid complementation**

Transformants were chosen at random, since they should contain the same plasmid, and were restreaked on YPD plates against positive control, LSY17, and negative control, RCY246. One plate was placed at the permissive temperature and one at the restrictive temperature.

**Ethanol Precipitation of RCY946 isolated plasmid**

Ethanol precipitation of the isolated plasmid DNA from RCY946 was done in order to concentrate the DNA. A 300μl aliquot of the isolated plasmid DNA was transferred to an eppendorf tube. To the DNA, 7.5M ammonium acetate was added to a final concentration of 2.0-2.5M. To this mixture, 2 volumes of 100% ethanol was added and the tube was placed on ice for 15 minutes. The sample was spun down at 15,000 x g for 15 minutes. After discarding the supernatant, 70% ethanol was added to wash the
pellet. The solution was centrifuged at 15,000 x g for one minute and the supernatant was discarded. The tube was allowed to air-dry overnight and then 30µl of electroporation buffer (10mM TrisHCl, pH 8.0, 270mM sucrose, 1mM lithium acetate) was added.

Sequencing

The KCp491 Wesolowski-Louvel library plasmid in RCY946 contains an insert in the Bam site located within the tetracycline resistance gene. The Bam site and tet gene were derived from pBR322, but are also located in KCp491. The two primers chosen for sequencing were designated tet forward and pBRrevSph, since they bind within the pBR322 tetracycline gene and direct sequencing toward the Bam site from either side. The RCY946 plasmid was sent to the DNA sequencing facility at Cal State Northridge for sequencing. The plasmid DNA was sent at a concentration of 100-150ng/µl estimated from a gel. Both tet forward and pBRrevSph were sent at 1µM with a total volume of 25µl. The primer sequences are shown below:

\[
\text{Tet Forward:} \\
AGCCACTATCGACTACGC \\
pBRrevSph: \\
GCGACTCCTGCATTAGGAAG
\]

High Fidelity Polymerase Chain Reaction (PCR)

In order to determine if there are primer binding sites present on the plasmid insert, a PCR reaction was completed on the RCY946 plasmid DNA. Due to the large size of our intended product, the plasmid insert (8-10kb according to Weslowski-Louvel), the Phusion High Fidelity PCR Kit protocol made by FINNZYMES was used (New England Biolabs, Ipswich, MA). Two 50µl reactions were made containing the 1µl
plasmid DNA, 1X Phusion HF Buffer, 200μM dNTPs, 0.5μM of each primer and 1U of Phusion DNA polymerase. A 20μl reaction was made containing 1μl of the plasmid DNA preparation (prepared by A. Oldroyd and A. Munson) that complements the ts mutation of RCY303, a plasmid from the same K. lactis library with a characterized insert, as a control. Each reaction was run according to the general cycling instructions in the Phusion kit. An initial denaturation temperature of 98°C was run for thirty seconds. In one cycle, the DNA was denatured at 98°C for another seven sec., annealed at 50°C for thirty seconds, and extended at 72°C for 15-30sec/1 kb. The inserts in the Wesolowski-Louvel library plasmids are 8-10 kb (personal communication) so 3.5 minutes (210 sec.) were used for extension. This cycle procedure was repeated 35 times. After the cycles, a final extension was run at 72°C for ten minutes and the samples were then held at 4°C.

To examine the results, 47μl of each 50μl reaction was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) protocol for a microcentrifuge. The remaining 3μl of each initial reaction was run on a 0.8% TAE agarose gel (Sambrook and Russell, 2001) against the purified PCR product and RCY303 PCR product. The gel would indicate the presence of primer binding sites.

**Touchdown PCR**

The touchdown PCR method was used to increase specificity of priming and bypass determination of the optimal annealing temperature for the RCY946 plasmid sample (Don et al., 1991). The samples were prepared according to the Phusion High-Fidelity PCR kit protocol developed by FINNZYMES (New England Biolabs, Ipswich, MA). Each sample was initially denatured at 98°C for thirty seconds. This was followed
by 19 cycles of denaturation at 98°C for seven seconds, annealing starting at 60°C decreasing 0.5°C per cycle, and extending at 72°C for 3.5 minutes. After this, an additional 19 cycles were run at the same denaturation and extension temperatures and durations, but the annealing temperature was set at 50°C for thirty seconds each cycle. The samples were extended one final time at 72°C and then held at 4°C.

**PCR product purification**

The touchdown PCR samples were run on a 0.8% TAE agarose gel (Sambrook & Russell, 2001) against a 1 kb molecular DNA ladder. Specific bands of interest were chosen and cut out of the gel. Each band was purified from the gel using the QIAquick Gel Extraction Kit protocol (Qiagen, Valencia, CA) for a microcentrifuge. Samples were run on a 0.8% TAE agarose gel to quantify DNA.

**Results:**

Both RCY246 and RCY435 had previously proposed complementing transformants, RCY946 and RCY947 respectively (Louie, 2008; Molinos, 2008). The plasmid DNA from RCY946 had previously been isolated and frozen by Rebekah Louie in 2008. Even though RCY435 had a likely complementing plasmid, the mutant strain was transformed with the *K. lactis* genomic DNA library to check for additional complemented transformants. The results are listed in Table 2 below.

**Table 2: Number of RCY435 colonies transformed with *K. lactis* genomic DNA library**

<table>
<thead>
<tr>
<th>Plate #</th>
<th># of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>
RCY435 was transformed with the *K. lactis* genomic DNA library by electroporation. Cells (200µl) were plated on SD medium and incubated at 24°C for three days. After incubation the cells were counted; any growth indicated successfully transformed cells. To test for complementation, transformants were replica plated on YPD and grown at 24°C and 36°C.

All replica plated transformants grew at the permissive temperature, however, none were able to grow at the restrictive temperature. In addition, replica plated transformants were unable to grow on minimal media. Thus the transformation was unable to identify any new complementing factors. Instead of continuing to transform RCY435 with a *K. lactis* library, the previously proposed complement, RCY947, was used for each additional study of this mutant strain.

In order to amplify the likely complementing plasmid to RCY435, the plasmid DNA had to be taken out of RCY947, using both the Qiagen and yeast boiling prep methods. Once the plasmid DNA was extracted from both *K. lactis* complements, electroporation was used to transform *E. coli* for plasmid amplification. Table 3 displays the results of the transformations for both RCY246 and RCY435 mutant strains.

<table>
<thead>
<tr>
<th>Sample</th>
<th># Colonies for RCY246 complementing plasmid</th>
<th># Colonies for RCY435 complementing plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (- control)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em> + pTZ (+ control)</td>
<td>1590</td>
<td>Plate Covered</td>
</tr>
<tr>
<td><em>E. coli</em> + QIA isolated DNA</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td><em>E. coli</em> + YB isolated DNA</td>
<td>18</td>
<td>227</td>
</tr>
</tbody>
</table>

Competent *E. coli* cells were transformed by electroporation with the proposed complementing plasmid for RCY246 and RCY435 isolated with either the QIAprep Qiagen kit (QIA) or the Yeast Boiling prep (YB). *E. coli* was grown alone as a negative control. As a positive control, *E. coli* was transformed with *pTZ*. Each sample was grown...
on a separate LB + ampicillin plate to select for transformants. The plates were grown at 37°C for two days and then transformants were counted.

The controls indicate that the transformations worked for both mutant strains. In order to confirm that the isolated plasmids do complement their respective original mutant strains, RCY246 and RCY435 must be transformed with their proposed complementing plasmid DNA. To do this, the plasmid from the transformants was first reisolated from E. coli using a Qiagen kit.

Since only the yeast boiling prep isolated DNA for RCY246 yielded colonies, four of these colonies were chosen for further plasmid analysis. These colonies were chosen at random since they are likely to contain the same plasmid, although it is possible that they may differ if RCY946 contained multiple plasmids. Using the Qiagen kit, the plasmid DNA was isolated from each of the four colonies and a 3μl aliquot of each sample was run on an agarose gel to ensure the presence of DNA. During plasmid isolation from E. coli, one of the Qiagen buffers appeared contaminated and thus one colony sample was thrown out. The gel results from the other three samples suggested that DNA was successfully isolated from each E. coli colony (Figure 3).

Figure 3. Isolated plasmid DNA from E. coli transformants of RCY246
RCY246 plasmid DNA was isolated from four E. coli transformants using the QIAprep Maxi Qiagen kit. Colony 1 was thrown out for suspected contamination. To determine
the presence of DNA, 3μl of each isolated sample was run on a 0.8% TAE agarose gel. Colonies 2, 3 and 4 were split into two samples (A & B) because of their large volume. Lanes: 1) 1 kb molecular DNA ladder – the bright middle band is 3 kb, 2) Colony 2A, 3) Colony 2B, 4) Colony 3A, 5) Colony 3B, 6) Colony 4A, 7) Colony 4B. Bands from each colony appear around 8 kb.

To verify that each isolated plasmid was identical, the plasmids were cut with three different restriction endonucleases; Xba1, EcoR1 and HindIII. These restriction enzymes were chosen at random. The reactions were run on an agarose gel to analyze the band patterns (Figure 4). The results indicate similar band patterns within a restriction enzyme across all three colonies. This suggests that the isolated plasmids are the same.

Figure 4. Isolated Plasmids with Restriction Endonucleases
Each of the three isolated plasmids (2, 3 and 4 A colonies) was cut with three restriction endonucleases, HindIII, Xba1 and EcoR1, to determine if they were identical. Lanes: 1) 1 kb molecular DNA ladder – the bright middle band is 3 kb, descending from this band are 2 kb, 1.5 kb, 1 kb, and 0.5 kb markers, 2) Colony 2A plasmid cut with HindIII, 3) Colony 2A plasmid cut with Xba1, 4) Colony 2A plasmid cut with EcoR1, 5) Colony 3A plasmid cut with HindIII, 6) Colony 3A plasmid cut with Xba1, 7) Colony 3A plasmid cut with EcoR1, 8) Colony 4A plasmid cut with HindIII, 9) Colony 4A plasmid cut with Xba1, 10) Colony 4A plasmid cut with EcoR1.

To confirm that the plasmid found is the complement to the mutation in RCY246, the plasmid was transformed back into RCY246 by electroporation (Table 4). Any colony that grows should contain the plasmid of interest since the cells are plated on minimal media. The plasmid vector is known to contain a URA3 marker which allows the cells to grow on media lacking uracil.
Table 4. RCY246 transformation results

<table>
<thead>
<tr>
<th>Plate #</th>
<th>Approximate # of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>381</td>
</tr>
<tr>
<td>2</td>
<td>763</td>
</tr>
<tr>
<td>3</td>
<td>890</td>
</tr>
<tr>
<td>4</td>
<td>636</td>
</tr>
<tr>
<td>5</td>
<td>699</td>
</tr>
</tbody>
</table>

RCY246 was transformed with its proposed complementing plasmid by electroporation. Cells were plated (200μl) on SD medium to select for transformants and incubated at 24°C for three days. After incubation the cells were counted, any growth indicated successfully transformed cells.

Given that each colony should be the same, two colonies were chosen at random, labeled T1 & T2, to check complementation to the RCY246 mutation. These two colonies were restreaked on YPD plates against LSY17 (wild type parent of RCY246), the positive control, and RCY246, the negative control. Rich YPD medium was used, even though it would not select for the plasmid marker, because RCY246 is not temperature sensitive on minimal SD medium (Louie, 2008). One plate was placed at the permissive temperature, while the other was placed at the restrictive temperature. If the isolated plasmid complemented the mutation, the cells should grow at the higher temperature. When comparing the growth of the transformants at the restrictive temperature to that of LSY17, the results indicate partial complementation of RCY246 (Figure 5).
Figure 5. RCY246 transformants: Verifying complementation.
Two RCY246 colonies (T1 & T2) transformed with the proposed complementary plasmid were grown on YPD at 24°C and 36°C to check for complementation. LSY17 was used as a positive control and RCY246 served as the negative control.

In the attempt to obtain reproducible results, the transformants from the original transformation plate were restreaked onto YPD against the same controls. In addition, transformant colonies grown at 24°C in Figure 5, were streaked out again on YPD. One plate from each set was put at the permissive temperature, and the other was put at the restrictive temperature (Figure 6).

Figure 6. RCY246 transformants plated on YPD.
To confirm partial complementation, RCY246 transformants (T1 & T2) were regrown on YPD media and placed at both 24°C and 36°C. LSY17 acted as the positive control and RCY246 served as the negative control. A) RCY246 transformants (T1 & T2) from the original SD transformant plates. B) RCY246 transformants (T1 & T2) previously grown on YPD at 24°C (Figure 5). Large circular spots in part B are a contamination.

The transformant T1 displayed greater complementation on the second streak from the original transformation plates. However, T2 didn’t grow on either plate, suggesting that the cells may have died or there weren’t many cells left to plate having previously been streaked out. Figure 6B shows that both T1 and T2 are unable to grow at the restrictive temperature. Yet, the cells are alive as proven by their growth at the permissive temperature. These results suggest that the RCY246 transformants lose their ability to grow at the restrictive temperature with each successive plating on rich media. Possibly, the colonies chosen could have lost their plasmid prior to replating, and thus couldn’t grow at the restrictive temperature. To examine if the cells had lost their plasmid, each set of previously grown transformants was grown on minimal media at the permissive temperature. Again only transformants are able to grow on minimal media since they contain the *URA3* marker needed to grow on media lacking uracil. No growth was seen for any of the transformants (not shown).

Due to their inability to grow on minimal media, the transformants appeared to have lost their plasmid, but they also could have been dead cells, as was seen in the experiment in Figure 6A for transformant T2. In order to verify that the plasmid was lost, all transformants were restreaked onto a minimal media plate, and a minimal media plate containing uracil (Figure 7). The transformants were compared to LSY17, which cannot grow on minimal media because it lacks uracil, and RCY947, which is known to carry a
URA3 marked plasmid. All transformants were able to grow on minimal media once uracil was supplied (Figure 7).

**Figure 7. RCY246 transformants on minimal media & minimal media + uracil**
To determine if transformants have lost their plasmid, all previously grown transformants were grown on SD only and SD + uracil plates at 24°C. Transformants were grown against LSY17, negative control, and RCY947 known to contain a URA3 plasmid, the positive control. Transformant (A)s are from the transformants grown on YPD at 24°C in Figure 5. Transformant (B)s are from the transformants grown at 24°C in Figure 6A. Transformant (C)s are from the transformants grown at 24°C in Figure 6B prior to contamination.

Since the RCY 246 transformant cells appear capable of only growing on minimal media with uracil, this indicates that the transformants lost their URA3 plasmid. One possible explanation is that when grown on rich media at the permissive temperature, the transformants don’t need the plasmid insert and thus, may experience selection against the plasmid. Therefore, when regrown on additional plates, the colonies chosen have lost their plasmids and cannot grow at either the restrictive temperature on rich media or on minimal media alone. Since it is known that colonies do progressively lose plasmids with each replating, two different transformants (Table 2), labeled T3 & T4, were chosen from
the original RCY246 transformation and grown on minimal media and rich media at both
the restrictive and permissive temperatures (Figure 8).

Figure 8. Two new RCY246 transformants on minimal media & rich media at 24°
and 36°C. Two different transformants (T3 & T4) were taken from the original RCY246
transformation SD plates and regrown on A) SD at 24°C and B) YPD at 36°C and 24°C.
LSY17 served as the positive control on YPD and the negative control on SD medium
because it lacks the uracil needed to grown on minimal media. RCY246 was the negative
control on both YPD plates. Extreme contamination of the YPD plate at 24°C after
storage conceals the results; T4 had no growth and both controls grew as normal.

Both T3 and T4 were unable to grow on minimal media or at either temperature
on rich media. Results from these additional transformants indicate that the cells have
died from a long duration in storage. Since both T1 and T2 grew at the restrictive
temperature after the original RCY246 transformation, they must have carried the
plasmid at that point. Thus both T1 and T2 from Figure 5 at the restrictive temperature
were restreaked onto YPD and grown again at the higher temperature. Neither of the
transformants grew at either temperature concluding that the transformants die over time.
Taken together, these results suggest that RCY946 carried the proposed complementary plasmid for RCY246, but transformants may lose their plasmids over time and die. To ensure that RCY946 still contained the plasmid, it was grown on minimal media and rich media at the restrictive temperature. RCY946 was able to grow under both conditions indicating that it still contains the desired plasmid (results not shown).

Due to these complications, the plasmid was sent off for sequencing to try to find answers that would explain the plasmid instability in addition to identifying what gene might be affected in RCY246. In constructing the library the pieces from the *K. lactis* genome were inserted at the BamHI site on the KCp491 plasmid. The two chosen primers should bind to either side of this region and promote sequencing of the insert ends. However, the sequencing attempt did not produce a clear sequence. This result was thought to be the due to a small quantity of plasmid. The plasmid DNA was reisolated using the HiSpeed Plasmid Maxi Qiagen Kit to increase the amount of plasmid available for sequencing. In addition, the DNA was concentrated through an ethanol precipitation before sending the plasmid for sequencing a second time.

Unfortunately, no sequence was obtained which may have been due to the primers not being able to bind the plasmid. The sequence peaks observed were of low intensity and appeared to overlap when magnified. This might indicate a plasmid rearrangement which could potentially lead to the lack of sequencing observed if the primer binding sites were lost or duplicated. Seeing as both sequencing attempts failed, the likely next step was to confirm the presence of primer binding sites on the plasmid. To do this, a
High Fidelity PCR was used in hopes of amplifying the plasmid insert region. The same primers used in sequencing were used for this PCR reaction.

After the reaction, most of each sample was purified using the QIAquick PCR Purification Qiagen Kit. The samples were run on an agarose gel to determine the presence of primer binding sites. In order to amplify a specific region in a PCR reaction, the 3' ends of two different bound primers must be facing one another. The typical expected result for this Wesolowski-Louvel library plasmid would be one amplified product between 8-10 kb, the size of the plasmid insert (Wesolowski-Louvel, personal communication). However, if the primer binding sites had been lost or rearranged, it would be difficult to observe any specific amplification.

**Figure 9. PCR product for RCY946 and RCY303**

A polymerase chain reaction (PCR) was run on RCY946, the proposed complementary plasmid to RCY246 using the tet forward and pBRrevSph primers. Both RCY946 samples were purified using the QIAquick Purification kit. RCY303 was used as a control for its characterized insert that complements its mutation. All samples were run on a 0.8% TAE agarose gel to analyze amplification patterns. Lanes: 1) 1 kb molecular DNA ladder, 2) RCY946 50μl rxn, 3) Second RCY946 50μl rxn, 4) Empty lane, 5) Purified RCY946 PCR product, 6) Second purified RCY946 PCR product, 7) RCY303 PCR product.

As shown, both of the samples before and after purification display the same amplification pattern. A plasmid complementing RCY303, pictured in lane seven of
Fig.9, was used as a control since it was known to contain the KCp491 vector and a genomic DNA insert of approximately 7 kb. Since the plasmid inserts were expected to be 8-10 kb, a large amount to amplify in a PCR reaction, this control was used to show that the reaction was capable of amplifying a band of this size. Most often, Taq polymerase is used for PCR reactions; however, a different Phusion polymerase, proven capable of amplifying longer products, was used in these samples.

The results for RCY303 show the band pattern normally seen when a single specific region of interest is amplified. The samples of the plasmid from RCY946 suggest that there are amplified fragments of various sizes. This strongly indicates that there may be numerous primer binding sites on the plasmid and thus many different regions are amplified. Since each band is amplified in relatively equal amounts, it is difficult to determine which region is more prominent. Instead of isolating each band and purifying the DNA within each fragment, another PCR method was used.

Touchdown PCR is a technique used to find the optimal annealing temperature for a given sample of DNA. In this protocol, the annealing temperature is raised above the Tm's of each of the two chosen primers and then progressively lowered with each cycle. This method was chosen for the RCY946 plasmid in hopes that the region surrounding the plasmid insert would contain the sites the primers would most likely to bind at higher temperatures. If this is true, a brighter band would be seen in one area of the gel because this region would be amplified from the highest to the lowest annealing temperatures and would therefore be most abundant. If other regions are amplified, this suggests primer binding of suboptimal primer binding sites. Hopefully other plasmid regions would not be amplified until lower annealing temperatures, and thus lead to fainter bands on a gel.
The touchdown PCR products yielded the same band pattern as the previous PCR results, but some bands were more amplified due to this method (Figure 10). For example, the top band seems relatively more intense than in the same samples in Figure 9. This suggests that the primers were binding most often to this region. The multiple bands seen from the single primer controls indicate at least two primer binding sites per primer.

The bands specified in the figure, were cut from the gel and purified. The large band (lanes 2 & 3) was selected because it seemed to have the most faithful primer binding. Also, the 1 kb molecular DNA ladder indicates that this large band is approximately 4.5 kb. While the plasmid insert was expected to be larger, this is a plausible size for the insert in this Wesolowski-Louvel library. When compared to the single primer controls, the smaller bands (lane 2 & 3) appear to be the products of proper binding by both primers. Its small size suggests that it may lack duplicate primer sites that would cause sequencing at multiple locations. The samples boxed in red were sent for sequencing (Figure 10).

![Figure 10. Touchdown PCR results](image)
Touchdown PCR was run on RCY946 with the tet forward and pBRrevSph primers. Each primer was run alone with RCY946 to serve as controls. Samples were run on a 0.8% TAE agarose gel to examine amplification patterns. Lanes: 1) 1 kb molecular DNA ladder, 2) 50µl RCY946 Touchdown PCR product, 3) Second 50µl RCY946 Touchdown PCR product, 4) Empty lane, 5) RCY946 with tet forward primer only, 6) RCY946 with pBRrevSph primer only. The bands boxed in the figure were cut from the gel and purified using the QIAquick Gel Extraction kit. The purified DNA from the red boxed samples was sent for sequencing.

Sequencing of the larger band yielded a clear sequence from the tet forward primer only. When input into the BLAST program, the sequence turned out to be homologous to DH10B E. coli genomic DNA. The small band, which was sequenced in its entirety in the tet forward reaction, yielded two different sequences from each individual primer. This suggests that the original band was not a pure sample. When put into the BLAST program, the tet forward sequence was found to be homologous to the DH10B E. coli genomic DNA.

<table>
<thead>
<tr>
<th>Bands from Gel</th>
<th>Primer Used</th>
<th>Location in Genome</th>
<th>Genes within the sequence</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large (4.5 kb)</td>
<td>tet forward</td>
<td>812037-811700</td>
<td>2-oxoglutarate decarboxylase</td>
<td>332/338 (98%)</td>
</tr>
<tr>
<td>Small (500bp)</td>
<td>tet forward</td>
<td>361442-361020</td>
<td>maltodextrin glucosidase</td>
<td>423/423 (100%)</td>
</tr>
<tr>
<td>Small (500bp)</td>
<td>pBRrevSph</td>
<td>4311776-4311547</td>
<td>acetyltransferase</td>
<td>207/230 (90%)</td>
</tr>
</tbody>
</table>

The specified bands were purified from the gel in Figure 10 and sequenced. BLAST program analysis indicated multiple sequences homologous to the DH10B E. coli genomic DNA. This table summarizes the details of the sequences from the large band with the tet forward primer only and the small band with both primers sequencing different regions. The locations within the E. coli genome are indicated along with genes present within these specified regions of the genome.

It is unclear whether pieces of the E. coli chromosome were somehow now incorporated into the K. lactis plasmid or if our samples were contaminated with E. coli genomic DNA.


Discussion:

The *K. lactis* strains in this study, RCY246 and RCY435, have both been previously identified as *ts cdc* mutants. Work done by previous students at the University of Redlands have transformed both RCY246 and RCY435 with the Wesolowski-Louvel *K. lactis* genomic library. This specific library was used because it is constructed in a low copy vector and thus can help avoid the overexpression of genes by preventing multiple copies of the plasmid in a cell (Guthrie *et al.*, 1991). This decreases the probability of identifying multicopy suppressors, rather than the authentic complementing gene.

The transformations led to a complementing plasmid that appeared to rescue the mutant phenotype in each strain. The proposed complemented transformant for RCY246 was RCY946. The plasmid DNA from RCY946 had previously been isolated and was then amplified in *E. coli*. The first *E. coli* transformation yielded 18 successful transformants, four of which were analyzed and determined to carry an identical plasmid. To confirm complementation, the plasmid was transformed back into RCY246, a method that selects for cells carrying the plasmid of interest. When two chosen transformants were streaked onto rich media to test complementation, the results indicated a partial complementation to the mutant phenotype present in RCY246. As the transformants were continually replated on rich media, the transformants became unable to grow at the restrictive temperature. Due to this lack of growth, the cells appeared to have lost their plasmid. To confirm this, the cells were grown on minimal media and minimal media with uracil. As the Wesolowski-Louvel library contains a *URA3* marker, the cells that
carry the plasmid should grow on minimal media. However, the results indicate that all transformants were incapable of growing on minimal media unless uracil was present.

Because the cells were able to grow on minimal media with uracil, the cells don’t appear dead, but over the course of these experiments it seems that these transformants lost their plasmid and eventually died. It is evident that with successive platings on rich media, the cells were unable to grow at the restrictive temperature. In addition, since most of the transformants streaked on the minimal media had previously been plated on rich media, the lack of growth may have been due to cell selectiveness to certain environmental conditions. For example, the cells don’t need the plasmid to grow on rich media at the permissive temperature. Therefore, when grown under these conditions, cells that lose their plasmid may have a selective growth advantage in the event that a gene on the plasmid is deleterious when present in multiple copies. Also, under these conditions, losing the plasmid may not lead to negative effects and thus the cells remain viable without a plasmid; at least at the permissive temperature. When these cells are restreaked on to other plates, their loss of plasmid prevents them from growing at the restrictive temperature. When two new transformants were streaked on both minimal and rich media, neither grew under any condition. This result is indicative of cell death.

The partial complementation seen in the original RCY246 transformant cells could have been due to the plasmid loss seen in subsequent generations, followed by cell death. At one point, the plasmid may fully complement, but as the cells lose the plasmid they also lose their ability to grow at the restrictive temperature and thus their colony growth rate is slowed. However, partial complementation may also be due to suppression of the mutation. Past research has shown that genes of overlapping function can suppress
mutations in other genes (Fitch *et al.*, 1992). In the RCY246 transformants, overexpression of other genes may partially compensate for the mutation and allow cell growth at the restrictive temperature.

Past research on a protein kinase C (PKC1) *ts cdc* mutant in *S. cerevisiae* has shown that its *ts* phenotype was suppressed by the addition of CaCl₂. In the presence of this osmotic stabilizing agent, *pkc1* mutants were able to grow exponentially. However, when transferred to a medium lacking osmotic stabilizers, *pkc1* mutants underwent cell lysis. The study concluded that mutations in *PKCI* resulted in an osmotic stability defect specific to the cell cycle (Levin, 1992). If RCY246 had an osmotic defect, cell death could be due to cell lysis after extended periods of storage.

The KCp491 Wesolowski-Louvell library is a centromeric plasmid that contains the *CEN2* locus, the centromere from *K. lactis* chromosome B. The presence of this centromere allows the plasmid to essentially function as a chromosome within the cell; both meiotically and mitotically (Clarke *et al.*, 1980). Even though the plasmid carries a centromeric sequence, the plasmid is still relatively unstable and is lost at a frequency of 1% per generation (Cormack *et al.*, 2002). However, the plasmid loss rate seen for RCY246, while not measured, appears much more severe as no cells retain the plasmid after only one-time growth on rich medium.

One theory for the apparent plasmid rejection is that the plasmid carries a second copy of wild type loci already present within the cells. The cells may become unstable with the presence of a second copy of particular genes. By losing their plasmids, the cells have better growth opportunities. Previous research has shown an example of this phenomenon in dicentric plasmids. A study by Mann & Davis (1982) has shown that a
plasmid with a second centromere undergoes extreme plasmid rearrangement in yeast. Their results show that in each rearrangement, at least one or both of the centromere sequences were deleted. In many cases, if one centromere was deleted, the cells had a growth advantage, but those cells with larger deletions weren't as stable. Their study also indicates that in some cases the URA3 marker was lost in the rearrangement, which could be an occurrence in the RCY946 plasmid, thus leading to loss of growth on minimal media. Since KCp491 already contains a centromere, the plasmid rearrangement proposed could result in a duplication of the centromere sequence or a deletion of this sequence, both of which cause plasmid instability. Alternatively, the plasmid insert may contain a centromere, which would also result in an unstable dicentric plasmid.

Another study by Burke et al. looked at the effects of tubulin overexpression. They found that an overexpression of specific types of tubulin caused cell cycle arrest and proved lethal to the cells (Burke et al., 1989). Even though this might not be the case in the RCY246 complementing plasmid, it provides evidence for selective pressures against overexpression of certain genes.

Sequencing the plasmid insert may help to explain the apparent plasmid rejection. Unfortunately, both sequencing attempts were in vain. The sequencing difficulties may have been due to a lack of primer binding sites or multiple primer binding sites. Prior sequencing attempts in previous years at the University of Redlands have used a different tet reverse primer, pBRrevBam to sequence the Bam insert region. Sequencing results continued to show results from the tet forward primer region, however the reverse sequences weren't as clear, containing overlapping peaks. Analysis of the plasmid and its origin led to the conclusion that the pBRrevBam primer was binding both adjacent to the
insert and to a duplicated tet vector region adjacent to CEN2 in the plasmid. During the construction of the KCp491 vector the region between the Bam and Sph site on the tetracycline gene was duplicated when CEN2, along with a portion of the tet gene, was inserted into the plasmid (Fukuhara, 2007). Thus in the attempt to improve sequencing results, a primer was chosen that would bind near the Sph site distal to the insert, that would hopefully bind outside of the duplicated section of the vector. The pBRrevSph was used in this research for that reason. The PCR product of the plasmid complementing RCY303 proved that pBRrevSph only bound one primer binding site, otherwise there would have been at least two amplified bands on the gel (Figure 9, lane 7). Thus this primer was used in subsequent sequencing attempts because it is known to bind outside of the duplicated tetracycline region.

Due to this small duplication in the vector, plasmid rearrangement could have occurred between these duplicated regions. A rearrangement of this sort may have caused duplicate CEN sequences and thus plasmid instability in yeast. This type of rearrangement is not often seen as Wesolowski-Louvel, among others, have used KCp491 without problems (see for example, Betina et al., 2001).

A PCR reaction was completed to prove the presence of primer binding sites (Figure 9). As there were many bands seen on the gel, this indicated an abundance of primer binding sites. This would explain the complication in sequencing; if multiple regions were getting amplified, multiple regions could also have been sequenced, leading to the overlapping sequencing patterns that resulted from each attempt. Another explanation is that the annealing temperature used during PCR was too low and may have caused difficulties with primer binding to near matches, resulting in mispriming.

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Therefore, the touchdown PCR method was chosen to decrease mispriming. Single primer controls were used to indicate multiple primer binding sites. If multiple bands appeared on the gel for each of these controls, then the primers must be binding in more than one region.

The results for this method show similar amplification patterns to those seen in the normal PCR reaction (Figure 10). However, some of the bands were more intense which hopefully indicated a primary location of amplification. Since the primers were both run alone to examine their amplification patterns, the bands chosen for further analysis were those that didn’t correspond to bands seen in the single primer control lanes. The large band was chosen for its size since it was the most likely band to contain most, if not all of the plasmid insert. The smallest product was also chosen because it was least likely to contain extra primer binding sites and thus would most likely yield a clear sequence.

The multiple bands seen from the PCR product prove the presence of multiple primer binding sites. Initially this was thought to be the result of a drastically rearranged plasmid. As noted above, originally, the KCp491 vector was made with a direct repeat of the sequence around the insert, with a second copy near the CEN2 locus (Fukuhara et al., 2007, Silveira, personal communication). When the primers were run alone, their resulting multiple amplifications suggest that at least some duplicated primer binding sites are arranged so that the 3’ ends of the primers are pointed toward one another. Thus, even though the plasmid didn’t start out this way, it seemed to gain inverted repeats as well. Previous studies have shown that although inverted repeats are common in eukaryotes,
when on plasmids they can lead to instability (Henderson et al., 1993). Henderson et al. showed that inverted repeats led to multiple deletions due to plasmid recombination.

Some possible plasmid rearrangements are pictured below:

**Figure 11. Possible KCp491 plasmid rearrangement.** A) The KCp491 shown on the left was somehow previously rearranged to form some inverted repeats. Thus further rearrangement of the plasmid would lead to the scenario on the right causing a loss of complementation and URA3. B) The insert region contains a second CEN2. This duplication would cause recombination between the CEN2 on the plasmid and the CEN2 in the insert leading to the scenario pictured on the right.

This diagram suggests that once a plasmid rearranges to generate inverted repeats, two separate plasmid pieces result. The cell can only duplicate DNA that has an origin of replication (Ori). However, this smaller plasmid no longer contains the identifiable URA3 marker. Even though the insert may be present on the larger plasmid containing URA3 and CEN2, the cell cannot replicate this DNA and thus will lose its complementing insert in subsequent generations.
Figure 12. Hypothesized duplication of KCp491 plasmid insert region. The KCp491 with a plasmid rearrangement that causes multiple duplications of the plasmid insert region, some of which are inverted duplications. Therefore, there is an excess of primer binding sites causing amplification of multiple regions of the plasmid.

In addition to rearrangement possibly causing loss of complementing activity, it could also explain the large distribution of amplified bands on the gel; this could be due to duplications of primer binding sites. If a rearrangement somehow caused duplication of parts of the vector, then there would be multiple primer binding sites leading to many amplified regions of varying sizes.

The plasmid rearrangement could have occurred before the first transformation of the plasmid into E. coli. This is likely because restriction digest proved that my three plasmid isolates from E. coli were identical (Figure 4). To determine if this were a possibility, comparing restriction maps of plasmid isolated before and after E. coli transformation could indicate the occurrence of a plasmid rearrangement.

In the attempt to find the sequence of the plasmid insert, the large and small bands in lanes 2 & 3 from Figure 10 were purified and sent for sequencing. Fortunately, a sequence was produced for both the large and small band samples. However, when the
sequences were put into BLAST, they were homologous to portions of *E. coli* genomic DNA.

Since there was no *K. lactis* vector or plasmid sequence identified, it brings a question to mind. Is there any yeast DNA in the plasmid at all? Since RCY246 was complemented, if only for a short period, this indicates that *K. lactis* DNA was present at one point. Therefore, there could be an *E. coli* genomic DNA contamination of the plasmid preparation. Each PCR reaction yielded the same amplification patterns, yet this could have been because the primers were binding to the *E. coli* DNA sequence instead of the *K. lactis* plasmid. In the plasmid isolation process from *E. coli* there are bound to be small amounts of *E. coli* genomic DNA present in the template preparation. On the other hand, pieces of the *E. coli* chromosome could be present on the plasmid itself and somehow were copied during rearrangement. Thus it must be determined whether the *E. coli* DNA is on the plasmid or a genomic contamination.

Since both the large and small purified bands showed sequences in *E. coli*, two of the three sequences were analyzed for their locations within the *E. coli* genome. The results indicated that the sequences were located far away from one another highly suggesting *E. coli* DNA contamination and not simply a portion of an *E. coli* chromosome in the plasmid. Due to this, no information could be concluded about the RCY246 complementing plasmid. However, these results do indicate that at least one of the primer binding sites is missing or altered on the plasmid. In the PCR reaction if both primers were able to bind to the plasmid, the plasmid would have had greater amplification than any contamination, as was the case in the RCY303 control. Therefore,
it’s highly likely that the plasmid rearranged but instead of gaining multiple primer binding sites, it lost primer binding sites.

Since this plasmid does seem to contain complementing activity, in the future, the plasmid could be digested with restriction enzymes and each piece could act as a probe in a Southern Blot analysis against the *K. lactis* genomic library. The pieces giving rise to probes that correlate to the *K. lactis* genome could be further analyzed to determine which portion of the plasmid they comprise. Also, random digest of the plasmid could be done with EcoRI or HindIII, which cuts the plasmid into multiple pieces (Figure 4) but only cuts the vector once or not at all (Wesolowski-Louvel, personal communication). This would be followed by subcloning pieces of the digest into a *K. lactis* vector and transforming them into RCY246 to check for complementary activity. The new found complementary plasmid could be sequenced using primers specific to the new vector that flank the subcloned inserts. Again, since RCY246 arrests pre-anaphase, genes involved in mitotic spindle formation, DNA replication or the APC may cause this phenotype if mutated in the cell.

The second mutant strain of interest, RCY435 also had a proposed complemented transformant RCY947. The plasmid DNA was isolated from RCY947 and transformed into *E. coli* for amplification. The transformation was incredibly successful and thus the plasmid was reisolated from *E. coli*. Time constraints have prevented further findings for this strain. Therefore, RCY947 was not proven to be the complement to the mutations in RCY435. Future research should retransform the plasmid back into RCY435 to confirm complementation. If the plasmid appears to rescue the mutant phenotype in RCY435, the plasmid should be sequenced. Once a sequence is obtained, it can be put in the BLAST
program to look for a gene on that sequence with a known role that may cause the
phenotype observed. If this isn’t possible, the sequence can be compared to genes of
unknown function; if one of these genes were mutated and could lead to the observable
mutant phenotype then it is likely to be the complementing gene. In the event that these
options weren’t available, each gene in the insert could be cloned into a *K. lactis* vector
and put back into RCY435 to see which gene complements its mutation.
References


