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Cell Division Cycle Mutants in *Kluyveromyces lactis*

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Abstract

Cell division cycle mutants, cells with malfunctioning genes that disrupt cell replication and division, have been studied and used in research to better understand the genetic aspect of cancer. The goal of the this research was to create and study novel temperature-sensitive cell division cycle (*ts/cdc*) mutants that have not been previously identified or studied in the budding yeast *Kluyveromyces lactis*. Previous work from Redlands students has yielded ten *cdc* mutants, created by ethyl methane sulfonate (EMS) mutagenesis, which arrested with large buds and were placed into eight complementation groups. To expand the number and variety of *cdc* mutants, ultraviolet (UV) light was used to create additional *ts/cdc* mutants in *K. lactis* because UV has a broad mutagenic spectrum, unlike ethyl methane sulfonate. The screening for *ts* conditional mutants was performed by overlaying the images of replica plates at the restrictive temperature onto replica plate images at the permissive temperature.

Using UV mutagenesis, 61 *K. lactis ts* mutants were generated. Screening nine of our *ts* mutants and eight previously identified *ts* mutants for the characteristic uniform morphology, bud-size and nuclear configuration revealed five mutants of the cell division cycle. Four of the five *cdc* mutants found revealed novel phenotypes in this experiment. One, UV1BS4.32, displayed the previously seen large-budded phenotype. Two mutants, UV1BS3.27 and UV1BS5.36, showed the novel phenotype of small-budded morphology. The other two *cdc* mutants UV1BS1.4 and RCY1110 had unbudded phenotypes, which are also new to this research, and possibly new to this species.

Introduction

The process through which cells grow and replicate themselves is called the cell division cycle. It is the key process to the continuity of life and reproduction, because cells can only be made from previously existing cells (Alberts *et al.,* 2007). The cell cycle is separated into four stages: G1 phase, S phase, G2 phase, and M phase. G1 phase is the stage in which the cell assesses its internal and external conditions and responds to certain factors like nutrients, size, or mating factors to make sure that it is ready to commit to the replication process. S phase is when DNA is replicated. In G2 phase, the cell again assesses itself for factors like completion of DNA synthesis, size, appropriate protein synthesis, etc. to determine if it is qualified to progress into M phase. M phase is when mitosis, the separation of nuclei, and cytokinesis, the division of cytoplasm, occurs to generate two separate cells. To restrict the time and number of replications and ensure fidelity of events, cells have a control system for regulating the progression through events in the cell cycle. This regulatory system of checks works to ensure that replicated cells are viable and that the environment is suitable and capable of supporting the number of replicated cells.

Regulation of the cell cycle is controlled through a combination of different types of regulators that form a complex network of biochemical machinery for controlled growth based on different signals in the cell. A main tool in this process is CDKs, or cyclin-dependent kinases. CDKs are enzymes that phosphorylate targets to activate or deactivate them by covalent modification. These regulatory enzymes are activated by cyclin, a class of proteins with many types that vary in concentration throughout the cell cycle. CDKs can also be inhibited by CKIs, cyclin-dependent kinase inhibitors. Counteracting the work of kinases there are phosphatases, which remove phosphate groups to activate or inhibit

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proteins. Another type of regulatory machinery is ubiquitin ligase. Ubiquitin ligases add ubiquitin, a protein, to target proteins, which then marks the targeted protein for degradation by proteosomes. The interactions between these enzymes and proteins regulate the cell cycle and cause transitions of phases (Alberts *et al.*, 2007). For example, in G1 phase the G1-cyclin is bound to an active G1-CDK, while a S-cyclin is bound to the S-CDK that is inhibited by a bound CKI called Sic1. When the cell senses the appropriate conditions to transition to S phase, a different G1-CDK will phosphorylate the G1-cyclin and the Sic1 (CKI) attached to the S-CDK. This causes the ubiquitin ligase SCF to add ubiquitin chains to the phosphorylated components, G1-cyclin and Sic1. Proteosomes then digest the tagged proteins so that G1-CDK is inactive without its G1-cyclin and the S-CDK is not inhibited by the Sic1, its CKI. The cell can then proceed into the S phase where S-CDK can phosphorylate its targets to turn on DNA replication genes (Alberts *et al.*, 2007). When these regulatory processes do not function properly, the cell division cycle can lead to abnormal cell growth or cell death.

Regulatory machinery such as CDKs and ubiquitin ligases are examples of positive regulators that push the cell cycle forward through different transitions. In contrast to these positive regulators, there are also negative regulators that halt the cell cycle at three main checkpoints. Checkpoints in the cell cycle are points in the cycle where the cell monitors regulation of the cycle for competency or task completion and accuracy. The main checkpoints of the cell cycle are the Start, G2/M, and metaphase-to-anaphase checkpoints. The Start checkpoint occurs in late G1 and stops the cell to monitor if the environmental conditions are favourable for cell division. The G2/M checkpoint stops the cell cycle at the transition of G2 phase to M phase to assess the fidelity and completion of

DNA replication. The metaphase-to-anaphase checkpoint occurs in M phase and ensures that the mitotic spindles are attached correctly to the chromosomes aligned at the metaphase plate. If a cell cycle task is not completed or is not completed correctly, the cycle will halt at these checkpoints because negative regulators will target and inactive the positive regulators that would act to push the cycle forward. The cell cycle uses these checkpoints to ensure high fidelity of replication and functionality by stopping the cell cycle when an error is detected and allowing for repair systems to correct the error or for the cycle task to complete. If the environment is unfavourable for replication, for example, the cell will fail to pass the Start checkpoint, which would leave the cell in G1 phase. Cells that have an error with DNA replication or mitotic spindle attachment would halt midway through the cell cycle and result with a protruding bud from the mother cell (Alberts *et al.*, 2007).

A main method in learning what the "normal" or functional role of cell division cycle genes is to study the mutants or disrupted genes of the cell division cycle, referred to as *cdc* genes. Mutations are changes in the sequence of genetic material (DNA) that can later affect protein production or function, leading to abnormal or non-functioning cells. Cells that have a mutation disabling the cell cycle are difficult to study because they are unable to complete replication and cannot be manipulated or studied once found. To address this issue, conditional mutants are created, and then *cdc* mutants are selected from among the conditional. Conditional mutations are mutations that are expressed within certain parameters or conditions. *cdc* mutants are studied by creating conditional, temperature-sensitive (*ts*) mutants that have functioning mutant proteins that allow growth at a lower, permissive temperature but then cause the cell to arrest at a higher, restrictive

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temperature. The higher temperature causes the mutant protein to fail to function and makes the cell unable to complete the cell cycle (Hartwell, 1967). The *ts* mutation must be a change in an amino acid that, when exposed to the higher temperature, causes the structure to become unstable and denature, thereby losing function (Alberts *et al.*, 2007).

Budding yeast has been used for the study of the cell division cycle specifically, because, in haploids, uniform cellular morphology, bud-size and shape, correlate with the *cdc* mutation. A "bud" refers to the protruding daughter cell that grows from a mother cell. From previous work, it was known that the size of the bud correlated to position in the cell cycle. Leland H. Hartwell exploited this observation and searched for mutants that when shifted to the restrictive temperature, displayed uniform termination morphology, or bud-size at time of arrest. Most *ts* mutants that are involved in non-cell cycle processes arrest with the bud-size correlating to the phase of the cell cycle wherever they happen to be when placed at the restrictive temperature or where their proteins are no longer functional. In contrast, cells that are both *ts* and *cdc* mutants have uniform bud morphology and size at arrest, because *ts*/cdc mutants continue, usually to a checkpoint or transition, and arrest there (Alberts *et al.*, 2007). This uniformity of arrest can be observed and screened for. This process looks at cell transition regulators that normally drive the cycle forward. Uniformity of the *cdc* mutants, with respect to bud morphology, is a result of defective transition regulators or cell cycle components, such as DNA polymerase, that cause the cell cycle to halt at the same point, as what would normally propel the cell forward is non-functional.

Mutations occur naturally within cells, but the frequency of this spontaneous event is not high enough that natural mutants can easily be studied. To increase the frequency of

mutation, mutagens are used. Mutagens are chemical or radiation agents that induce mutation within cells by altering a cell's DNA. Ethyl methane sulfonate (EMS) is a chemical mutagen that causes a point mutation, or a change in a single base pair. EMS causes a G-C base pair to be replaced with an A-T base pair and does so by transferring the ethyl group from EMS to guanine to make O-6-ethylguanine, which pairs with thymine instead of cytosine (Lodish *et al.*, 2000; Griffiths, 1996; Coulondre & Miller, 1977). UV light is a radiation mutagen that causes a "wide spectrum of mutational changes"(Ausubel *et al.*, 1993). UV mutagenesis also favors G-C → A-T mutations, but can also cause A-T → C-G, G-C → T-A, and A-T→ T-A. UV light can also cause tandem double mutations to occur, which could possibly cause two codons to change in mutants thereby creating different types of mutants. EMS and other chemical agents do not cause this type of mutation (Coulondre & Miller, 1977). UV light creates mutants by causing pyrimidine-pyrimidine photodimers, usually thymine-thymine, that form cyclobutyl rings or cross-linked 6-4 photoproducts. These photodimers alter the structure of DNA, which can interfere with DNA repair or site recognition (Alberts *et al.*, 2007). UV-induced mutations rely on DNA polymerase η, which ignores some pyrimidine photodimers and allows region carrying the mutation to be correctly replicated and transcribed. However, at some sites, or for some types of photoproducts, this DNA polymerase fails to correct the photodimers, which leads to misinsertion of nucleotides opposite the site of UV-induced damage (Zhang & Siede, 2002). In mutant screens, UV has many advantages over EMS, such as less specificity of mutation. However, UV has site specificity for secondary structures of DNA, such as hairpins, and can cause hotspots of mutagenesis for UV (Todd & Glickman, 1982).

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The study of *cdc* mutants has been explored using different species of yeast. The advantages of yeast for scientific research are abundant; it is inexpensive, readily available, and easily manipulated (Botstein, 1997). Extensive studies with yeast in the last forty years have led to the genomes of many species of yeast being sequenced (Dujon *et al.*, 2004; Goffeau *et al.*, 1996; Wood *et al.*, 2002), many tools of analysis created, and a plethora of resources for comparison and reference. In addition, yeast multiplies within a reasonable timeframe that depends upon the cell concentration and temperature and also generates a sufficient number of colonies for statistical analysis. Yeast, in general, has been the genetic model of choice because the characteristics of yeast make it an ideal organism for experimentation. Yeast can exist as haploid, containing only one set of chromosomes, which allows for identification of recessive mutations that would be masked by the second chromosome in diploid cells. Genetic analysis using different crosses of yeast is possible because yeast mate easily, sporulate, and have two forms of ploidy.

Despite the abundance of research done with yeast, we continue to study it because there is still much more to be learned, especially in regards to *cdc* mutants. *Saccharomyces cerevisiae*, a budding yeast, has been studied extensively but as of 1997 "60% of its genes still have no experimentally determined function," which calls for more research (Botstein, 1997). Leland Hartwell, one of the prominent researchers of *cdc* mutants in *S. cerevisiae*, was able to analyze a rather large group of *cdc* mutants only to find that the mutations fell into only 32 genes. From his research, Hartwell concluded there should be more genes that regulate the cell division cycle because his screen showed non-random bias by having a profusion of certain mutants while a lack of others. If a screen of *cdc* mutants revealed a saturated distribution, meaning a complete exploration of all the *cdc* mutants possible with

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this method, then we would see mutations falling on a few genes no matter how many additional mutants were created (Hartwell *et al.*, 1973). A comparison of the 50 known *CDC* genes with the much greater number of proteins that have been sequenced reveals that there are still more *CDC* genes to be studied because of this "gene number paradox"(Kaback *et al.*, 1984). In fact, using a genomic approach Yu *et al.* found that more than 65% of the strains with damaged essential genes showed the defects in the cell cycle progression (2006), which is a much higher number than that seen in Hartwell's work.

In order to expand the knowledge of the cell division cycle, we are using a species of yeast called *Kluyveromyces lactis*. *K. lactis* is a budding yeast that is similar to *S. cerevisiae*, the species used by Hartwell, which means that previously determined information and techniques for *S. cerevisiae* can be applied to this similar species of yeast. While they are similar, *K. lactis* and *S. cerevisiae* differ on a genomic level; an ancestor of *S. cerevisiae* had an entire genome duplication, which causes an increased amount of redundancy of genes in present day *S. cerevisiae*. *K. lactis* lacks this ancestral genomic duplication, which makes it better suited for screening of recessive *cdc* mutants (Wolfe & Shields, 1997: Dujon *et al.*, 2004). This species of yeast also has not been studied to the extent that *S. cerevisiae* has, so screening *K. lactis* for *cdc* mutants could lead to a discovery of new cell division cycle genes. Hartwell's earlier research with *cdc* mutants in *S. cerevisiae* resulted in a saturated screen of the mutants that showed a non-random bias. Although Hartwell started to average 4.6 mutations per complementation group, the complementation groups did not have an even distribution of mutations or number of members close to the average. Most groups had only one member while other groups had very high numbers (Hartwell *et al.*, 1973). This indicates that some *CDC* genes were left unmutated and unidentified, judging from the

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large number of complementation groups with only one member. In contrast to these complementation groups, the few groups with many members indicate that further screening of this species of yeast would result in identifying more members of the "crowded" complementation groups.

David Kaback attempted to address the limited screen found by Hartwell by focusing on chromosome *I* of *S. cerevisiae* to find *ts* mutants in the hopes that he would reveal more *cdc* mutants. Unfortunately, Kaback's screen of 32 *ts* mutants fell into three complementation groups, or three different genes on chromosome *I*, and also showed the non-random bias Hartwell experienced (1984). Kaback proposed that this bias was due to the fact it is not possible to get *ts* mutations for all genes because the nature of the temperature sensitivity is too stringent a condition to be achieved for all proteins. The nature of temperature-sensitivity means that *ts* mutants are difficult to make, in terms of mutation, because only certain amino acid changes will yield the stringent characteristics of denaturation at high temperatures and functionality at lower temperatures. This stringency eliminates many possible mutations for study. The limitations of the mutagen EMS were a possible source of bias because the limited mutational spectrum of this mutagen creates a restricted amount of amino acid changes (Kaback *et al.*, 1984). EMS primarily causes G C → A T transitions and is not the most effective tool to create temperature-sensitive mutants.

To find novel *cdc* mutations and have different non-random bias as seen in the work of Hartwell and Kaback, we will use UV irradiation, a different form of mutagenesis, and *Kluyveromyces lactis*, a different species of yeast. Using a different mutagen that causes a broader spectrum of mutations, we attempt to minimize this bias in screening by creating

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more opportunities for temperature-sensitive proteins. By using a different species of yeast than *S. cerevisiae*, we will hopefully provide a different non-random bias in the identification of *cdc* mutants that provides new classes of mutants to study. We use a different species of yeast to avoid the same bias because the specific DNA sequences and proteins will differ from species to species. This means that in our species of yeast, *K. lactis*, there might be easier or harder ways to alter a protein to create a temperature-sensitive protein. We would expect to have the same constraints with the temperature-sensitivity, but with a different set of proteins. We use a different species of yeast to also find novel *cdc* mutations because *K. lactis* has already displayed a new class of *cdc* mutants. In *cdc* screens with *S. cerevisiae*, there have not been any mitotic spindle mutants found. From previous research on this project, RCY597, a *cdc* mutant, displayed a large-budded phenotype and a single nucleus at arrest and was found to be a mitotic spindle mutant. RCY597 was found to be complemented by a segment of DNA encoding *TUB2*, a β-tubulin which is a subunit of microtubules (Fonseca, 2008; Fonseca & Molinos, personal communication).

Previous screens of *cdc* mutants have revealed different classes of *cdc* mutants that we should expect to see in our UV mutagenesis. One class of mutants deals with genes that have regulatory functions in the cell cycle, such as the gene for the CDK, *CDC28*. The second class of *cdc* mutants has mutations in genes that are required for checkpoint-monitored events, such as DNA replication, DNA damage, or spindle formation. The third class of mutants has mutations in genes involved in environmental sensing, such as nutrient assessment or pheromone detection. The last type of *cdc* mutants is mutants whose genes for morphology determination or cytokinesis mechanisms are not functional (Lew *et al.*, 1997). The different morphological classes of *cdc* mutants include unbudded, small-

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budded, large-budded, and undivided chains of cells. From Hartwell's work, unbudded *cdc* mutants were found to fail to pass the Start checkpoint because a variety of reasons (1973). Unbudded mutants could fail to pass Start because they cannot sense nutrient levels (*CDC25*), lack the appropriate transition regulators to pass Start (*CDC28*), or lack a gene product required in bud formation (*CDC24/42*) (Lew *et al.*, 1997). Small-budded *cdc* mutants were not found by Hartwell but could deal with a mutation that causes the cell to halt in early S phase as a result of defective replication early on or a mutation that causes arrest as it starts to build a bud. Hartwell's work did show that large-budded mutants halted in S, G2, or M phase (1973) because of complications with DNA replication, DNA damage/repair, spindle attachment, or mitotic exit. Arrest occurred in some of these large-budded mutants because a transition regulator was not functional for the G2/M transition or the metaphase-to-anaphase checkpoint (*CDC20*), machinery for DNA replication checkpoint was not functional (*CDC2*)(Booher *et al., 1998)*, or cells could not synthesize a component needed for cytokinesis (*CDC30*) (Lew *et al.*, 1973).

The cell division cycle affects all forms of life. Therefore it is important to study and understand how cells replicate at a genetic level. Learning the functions and mechanisms of the genes that affect the cell cycle leads to a better understanding of the events that occur during cell replication. In eukaryotes, this process of cell replication has been conserved evolutionarily, which means knowledge of one species can be applicable to other species. Further applications of our work would take advantage of the observed homology and conserved genetics of eukaryotes, as exhibited by yeast, and use this knowledge to study other species. For example, Lee & Nurse were able to clone the human homologue of the cell division cycle gene *cdc2* in *Schizosaccharomyces pombe*, a fission yeast, and rescue

the mutant phenotype in *S. pombe* with the human homologue *CDC2* (1987). This experiment showed that what was learned from yeast could be applied to humans because conservation of sequence and function (Lee & Nurse, 1987). By comparing sequences of human DNA to other species that show a homology, the function of the human gene can be proposed if the function is known for the homologous species. This use of homology was used to study human genes that were similar to genes of *S. cerevisiae* and later found to cause cancer and aging (Botstein, 1997; Weinert & Hartwell, 1988).

Studying the factors that regulate the cell cycle and can stop defective or mutated cells from growing allows insight into conditions or diseases where these monitoring processes have become defective. This work could be applied in studying and providing new understanding of human diseases, disorders, and conditions that are a result of genetic mutations or malfunctions of the cell division cycle. Overexpression of certain gene products can also cause abnormal, uncontrolled growth and division by driving the cell cycle forward. Cancer is an example of this type of failure to monitor replication and overstimulation of cell cycle activators. Studying the cell division cycle in the yeast *K. lactis* will hopefully lead to understanding and possible treatments of human conditions that involve mutations of cell cycle controls or monitoring, like cancer.

By studying the cell division cycle in *K. lactis*, we are working towards gathering additional knowledge that is important scientifically and can be applicable to real-world problems. A main goal of this project was to identify new *cdc* mutants that can lead to new information or factors regarding cell cycle control and regulation. If this goal was not met, meaning only previously determined *cdc* mutants are identified, then we could explore our findings across species by comparing *K. lactis* to *S. cerevisiae* and *S. pombe*. As mentioned

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before *K. lactis* is similar to *S. cerevisiae* in that both are budding yeast. *K. lactis* is also similar to *S. pombe* because both exist mostly as haploids in nature (Hendriks *et al.*, 1992) while *S. cerevisiae* is mainly diploid in nature. This similarity to both species of yeast, in different ways, allows *K. lactis* to act like an intermediate species that can be studied, through *cdc* mutants, to relate lifestyle characteristics to genetic regulation of the cell cycle and answer why there are differences in cell cycle control between the species. This comparison would serve to relate function and role in the cell cycle to gene expression based on the differences between the species of yeast. For example, the role of tyrosine phosphorylation of the CDK, *cdc2*⁺ protein kinase in *S. pombe* was thought to be a universal regulator for entry into M phase by inhibition of the CDK (Gould & Nurse, 1989) but in further studies of the homologue *(CDC28)* in *S. cerevisiae* showed that, while tyrosine phosphorylation did occur, it did not regulate the cell cycle in the same way (Sorger & Murray, 1992). In *S. cerevisiae* the lack of phosphoryl removal only caused a delay in growth, while in *S. pombe* it caused cell arrest (Sorger & Murray, 1992). By studying *K. lactis*, a species of yeast similar to both *S. cerevisiae* and *S. pombe*, the differences in gene function and expression may be explored and the benefits of a particular cell cycle regulatory strategy may be inferred by correlating to lifestyle or growth habits of the yeast in which it appears.

From prior work on this project there have been ten *cdc* mutants isolated from the *K. lactis* strain GG1888, using EMS as a mutagen. They were all found to arrest in a large-budded state at non-permissive temperatures and fit into eight complementation groups. A majority of the complementation groups have only one member while the largest complementation groups have two mutants. Hartwell's screen of *cdc* mutants resulted in

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148 *cdc* mutants that had different sizes of buds at arrest and fell into 32 complementation groups. Of the 32 complementation groups, ten of the complementation groups had only one mutant, four complementation groups contained two members, two complementation groups had eight members, and the largest complementation group had sixteen mutants (Hartwell *et al.*, 1973). The comparison between the two studies shows that previous research has yet to achieve a saturated screen of the *cdc* mutants possible in *K. lactis* because the number of mutants found are but a fraction of the mutants found in Hartwell's work with *S. cerevisiae*, and we do not have complementation groups with as many members. The lack of variation in the arrest morphology also indicates that the project is incomplete in the aspect of *cdc* mutant creation and isolation. For example, no unbudded or cytokinesis phenotypic mutants have been found in past research. We are addressing this problem by using UV mutagenesis to create mutants in *K. lactis* in order to isolate new *cdc* mutants that have not been previously found in this project, or in previous screens of other species of yeast.

Materials & Methods

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Media and Strains

YPD plates and liquid media along with the minimal SD plates were prepared according to the protocol from Ausubel *et al.* (1993). To determine the growth of the cultures, the optical density at 600nm (OD_{600}) was taken for liquid cultures using the Jasco V-530 UV/VIS Spectrometer. The optical density was used in combination with the conversion factor 2.0×10^7cells/OD to give the cell count of the samples. The *K. lactis* strains used for this experiment are listed in Table 1.

Table 1. Characterization of strains used in this experiment.

Strain	Genotype	Source	Use
GG1888 (LSY 17)	*MAT a, ura3*	Zonneveld and Van der Zanden (1995)	Wild type strain for mutagenesis
RCY1043	*MAT a, ade1, ts, his7, ura3**	Product of RCY290 x LSY25 (Bohmer, 2009)	Control for *ts* screen and cdc screen
RCY303	*MAT a, ura3, ts/cdc*	EMS-derived mutant from GG1888 found by L. Silveira	Control for *cdc* screen
RCY1110-RCY1117**	*MAT a, ura3, ts*	EMS-derived mutants from GG1888 found by N. Dror (2009)	*Cdc* screen
RCY1120-1180**	*MAT a, ura3, ts*	UV-derived mutants from GG1888 found in this study by S. Heitz and B. Callaghan	

*The mutations in *his7* and *ura3* are only possible and not confirmed.
** RCY1110, RCY1120, RCY1123, RCY1124, and RCY1125 were confirmed as *cdc* mutants in this study.

Pilot Study for creation of temperature-sensitive mutants

In 5 mL of liquid YPD, the strains GG1888 and RCY1043 were inoculated separately and allowed to grow on a spinning culture wheel for two days at room temperature. The OD_{600} was taken for the samples, using the Jasco V-530 UV/VIS Spectrometer (Easton, MD). Using the conversion factor 2.0×10^7 cells/OD and the OD_{600}, the concentration of the GG1888 cells was adjusted so that the optical density would be 5.0×10^{-5} OD/mL and 30 μL were used per plate, which would give an initial count of 1000 cells per plate for the irradiated plates. This number was targeted so that at a 70% kill rate there would be approximately 300 cells left on the irradiated plates after treatment with ultraviolet light. The dilution of GG1888 cells was then further diluted by a factor of three in order to test what dilution of cells works optimally for this process. For the control plates the GG1888 concentration was adjusted so that the optical density would be 1.0×10^{-5} OD/mL and 30 μL applied to each plate, allowing for 200 cells per plate. The concentration of RCY1043 was diluted so that 50 cells were plated along with 150 cells of the GG1888 strain.

The treatment plates were then irradiated with UV light using the Stratalinker UV Crosslinker 1800 (La Jolla, CA) at a dosage of 10,000 μJ (10 mJ) for a 70% kill rate, as determined by previous work (Dror, 2011). Both the control plates and the irradiated plates were allowed to grow at room temperature for four days. After the four days, the colonies were counted and the kill rate was calculated. The kill rate was determined by comparing the growth on the control plates that was corrected for dilution to the average number of colonies on the irradiated plates. The irradiated plates were then replica plated, and one of each replicate plate was placed at the permissive temperature (24°C) and one at the restrictive temperature (36°C). The replica plates were made by first creating the

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restrictive plate and then the permissive plate. These plates were allowed to grow for four days.

To screen for possible *ts* mutants an image overlay process was used, utilizing the Epson Expression 1600 Artist scanner (Long Beach, CA), GIMP imaging program (Kimball & Mattis, 2011), and printer quality transparencies. This was done by first scanning all of the plates using greyscale. The plates were scanned without lids, open side facing up away from the scanner glass, and covered with black velvet. The restrictive (36°C) plate images were opened in GIMP in order to invert the greyscale and adjust brightness to 15 and the contrast to 70, which resulted in a white background with black colonies. The permissive (24°C) plate images were also opened in GIMP but only the contrast was adjusted to 70 so that the image had the original black background with white colonies. The restrictive plates were printed on printer-quality transparencies while the permissive plates were printed on regular printer paper. The restrictive plate images were overlaid onto the matching permissive plate images, and the possible *ts* mutants were selected as the colonies that failed to grow or had reduced growth on the restrictive plates but had corresponding colonies that grew on the permissive plates. These appeared as white colonies not overshadowed by black colonies in the overlay screening.

The possible *ts* mutants were patched onto YPD plates, one plate incubated at the permissive (24°C) temperature and the other at the restrictive (36°C) temperature. These plates were allowed to grow for six days and then re-examined for no growth on the restrictive plate and growth on the corresponding permissive plate. The mutants were named UV1BSX.Y, where UV1 is the method and round of mutagenesis, BS was the designation for the experimenters (myself Samantha Heitz and my research partner Blair

Callaghan), X was the plate number the mutant originally came from, and Y was the number of the possible *ts* mutant screened from that plate.

UV Mutagenesis

The *K. lactis* strain GG1888 was prepared in the same way as the pilot study but with no added control strain RCY1043. As with the pilot study of *ts* mutants, an image overlay process was used to screen for possible *ts* mutants. Colonies that failed to grow on the restrictive plates but grew on the respective permissive plates, as identified by white colonies not covered with black colonies, were selected as possible *ts* mutants. These colonies were then patched onto two YPD plates, one plate incubated at the permissive temperature and one plate incubated at the restrictive temperature, and allowed to grow for three days at their respective temperatures. The patch plates were then examined for lack of growth at the restrictive temperature and growth at the permissive to confirm temperature-sensitivity. The mutants were named UV2BSX.Y, where UV2 designates the second round of UV mutagenesis, BS represented who created the mutants, X was the plate number the mutant originally came from, and Y was the number of the possible *ts* mutant screened.

Storage of *ts* mutants

For each confirmed *ts* mutant derived from this screening process, a single colony was patched on a YPD plate and allowed to grow for four days at room temperature. The cells from the *cdc* mutant patch were resuspended in 0.8 mL of 15% sterile glycerol in a cryotube and immediately placed at -70°C in the freezer for future use (Oldroyd, 2002).

The strains were renamed RCY1120-1128 and RCY1129-1180 as noted on the Tables 2 and

3.

Screen for cell division cycle mutants from pilot screen of *ts* mutants

From the freezer, the strains GG1888, RCY303, RCY1043, and RCY1110-1117 were

plated on YPD plates and allowed to grow at room temperature for four days. The wild

type GG1888 was used as a negative control. The RCY303 strain was used as a control for a

known *ts/cdc* mutant that arrests with uniform bud morphology that is large-budded. The

RCY1043 strain was used as a control as a known *ts* mutant. The strains RCY1110-1117

were previously isolated *ts* mutants that had not been screened for cell division

phenotypes (Dror, 2011).

The newly created *ts* mutants, the previously found *ts* mutants, and the control

strains were inoculated in 4 mL liquid YPD by placing a pinhead-sized colony in the liquid

media. The inoculated cultures were then allowed to grow for 24 hours at the permissive

temperature on a spinning culture wheel. This time frame was used to ensure that the

cultures were in log phase, still actively dividing. After this time, the OD_{600} was taken for

the culture with the most growth (cloudiest) and the culture with medium growth to check

that the optical density was between 0.5-2.0 OD. The cultures with the highest amount of

growth were diluted so that the OD fell within the appropriate range mentioned previously.

The GG1888 culture was diluted 1:5 and 1:10 to account for the non-arresting cell growth

at the restrictive temperature because this strain is wild type with respect to the cell

division cycle. The samples were then shifted to the restrictive temperature by placing the

samples in the shaking water bath at a slant. The water bath was set at 250 rpm and at

36.2°C, as measured by the external thermometer. The samples were allowed to grow for seven hours before they were fixed with ethanol to a final concentration of 50% ethanol.

To screen for *cdc* mutants, the samples were prepared and then scored for uniform bud-size at arrest. The samples were transferred to 15 mL disposable centrifuge tubes and centrifuged at top speed for one minute using a tabletop clinical centrifuge. The supernatant containing the ethanol and YPD liquid was removed and the samples were washed with 1 mL of sterile water. After washing, the cultures were resuspended in 2 mL of sterile water. To break apart clumps of cells for viewing, the GG1888 sample was sonicated with a microtip on the Branson Sonifier 450 (Danbury, CT) at a 30% cycle duty and an output control of 3 (Bohmer, 2009; Guthrie & Fink, 1991) for 40 pulses. Immediately after sonication, 6 μL of the GG1888 sample was placed on a microscope slide and topped with a covered slip. The cells were examined using a phase microscope at 400x magnification under phase optics. About 100 cells were scored for bud-size and morphology and *cdc* mutants were characterized as having 70-80% arrest uniformity (Hartwell *et al.*, 1973). The process of sonication and observation with a microscope was then repeated, one at a time, for the other controls and *ts* mutants.

DAPI and Slide Preparation

To observe nuclear morphology and to preserve the samples, *cdc* mutants were treated with mounting media and then placed on slides. The microscope slides were first treated with about 10 μL of 0.1% (w/v) polylysine solution (Sigma Chemical Company, St. Louis, MI) for one minute. The slide was washed free of the polylysine with deionized water and then allowed to dry. About 20 μL of sonicated *cdc* mutant sample was then

placed on the treated slide and the excess aspirated off. A drop of ProLong Gold (Invitrogen, Paisley, UK), a mounting medium that contained DAPI stain, was then added to the sample and covered with a cover slip. The slide was stored overnight under heavy weight in order to cure the mounting media (Guthrie & Fink, 1991). The edges of the cover slip were then sealed with nail polish and allowed to dry. The image and the DAPI stain were visualized using the Olympus BX41 fluorescence microscope with attached camera.

Results

<u>Pilot Screen for *ts*</u>

In this pilot study, the goal was to create a functional procedure to create and isolate *ts/cdc* mutants, using the UV dosage found by a previous researcher to give a 70% kill rate (Dror, 2011). This was done by treating the wild type *K. lactis* strain GG1888 with ultraviolet light to create temperature-sensitive mutants. The *ts* mutants were then selected from among the other colonies as colonies that grew at the permissive temperature (24°C) but failed to grow at the restrictive temperature (36°C). The kill rate was determined to be 71% by comparing the average number of surviving colonies on the irradiated plates to the average total number of colonies from the control plates (after accounting for the increased dilution for the control plates). From among twenty plates treated with UV light, only eleven were scored and screened as the others lacked sufficient growth or were contaminated beyond rescue. The eleven scored plates contained 1243 colonies, 55 of which were possible *ts* mutants. The possible *ts* mutants were selected using the image overlay procedure as described in the Methods. Figure 1 shows some of the images of the replica plates and the possible *ts* mutants that lacked growth at the restrictive temperature.

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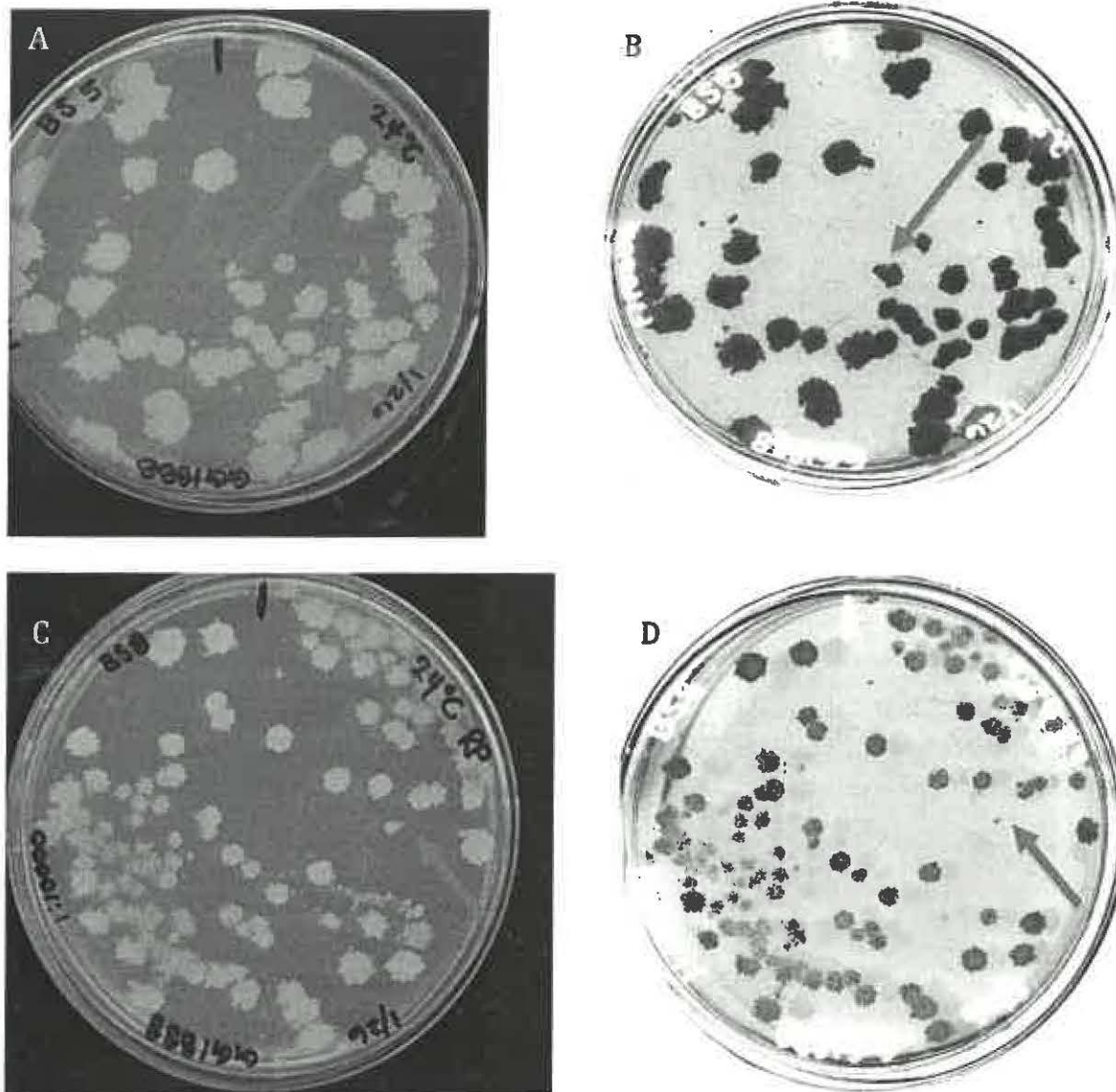
Figure 1: Replica plate images used for overlay procedure for screening of possible *ts* mutants.

A) Replica of plate UV1BS5 grown at the permissive temperature 24°C. B) Replica of plate UV1BS5 grown at the restrictive temperature 36°C. C) Replica of plate UV1BS8 grown at the permissive temperature 24°C. D) Replica of plate UV1BS8 grown at the restrictive temperature 36°C. Arrows indicate possible *ts* mutants as indicated by growth on the permissive plates A and C and lack of growth of the same colony at the restrictive temperature replica plates B and D. The contrast of the images in B and D has been inverted in order to have the growing colonies on these plates block the image of the growing colonies on the 24°C plate images when images are overlaid.

The 55 possible *ts* mutants were patched to two YPD plates. One of each patch plate was then placed at the permissive temperature and the restrictive temperature and allowed to grow. Examination of the plates showed that nine of the 55 patches were confirmed as *ts* mutants. Thus, of the original colonies plated, only 0.73% were confirmed to be *ts* mutants. Table 2 shows the names of the identified *ts* mutants and from whence the mutants came. The *ts* mutants were then frozen for storage and given the designation of RCY1120-1128 as shown below.

**Table 2: Confirmed *ts* mutants from the pilot study for *ts* mutants**
Replica plate name is the name each mutagenesis plate was given during the experiment. UV1 indicates the mutagen and the round of mutagenesis. BS represents the experimenters (Blair and Sam) and the number represents the consecutive number for each plate. The numbering of the ts mutants represents the individual colonies on those plates that were selected as ts mutants from among the possible mutants. The RCY label is the designation of strains that were frozen and saved for future research under the direction of Dr. L. Silveira.

Replica Plate Name	Numbering of *ts* mutant	RCY Label
UV1BS1	4	RCY1120
UV1BS1	9	RCY1121
UV1BS3	25	RCY1122
UV1BS3	27	RCY1123
UV1BS4	32	RCY1124
UV1BS5	36	RCY1125
UV1BS6	16	RCY1126
UV1BS8	42	RCY1127
UV1BS14	50	RCY1128

UV Mutagenesis

Using the method determined by the pilot screen of *ts* mutants, we created and screened for *ts* mutants. Because the pilot study gave the desired kill rate we did not adjust the UV dosage or the concentration of the cells plated. After treatment with UV light, the kill rate was determined to be 85% by comparing the average number of surviving cells,

after adjusting for dilution, to the average total number of cells as calculated from the control plates. The 25 irradiated plates yielded a total of 4025 surviving colonies. From among these colonies 204 possible *ts* mutants were found using the image overlay method. Figure 2 shows some of the images of the replica plates grown at the permissive temperature (on the left) and the restrictive temperature (on the right) and the possible *ts* mutants with no growth on the restrictive plate.
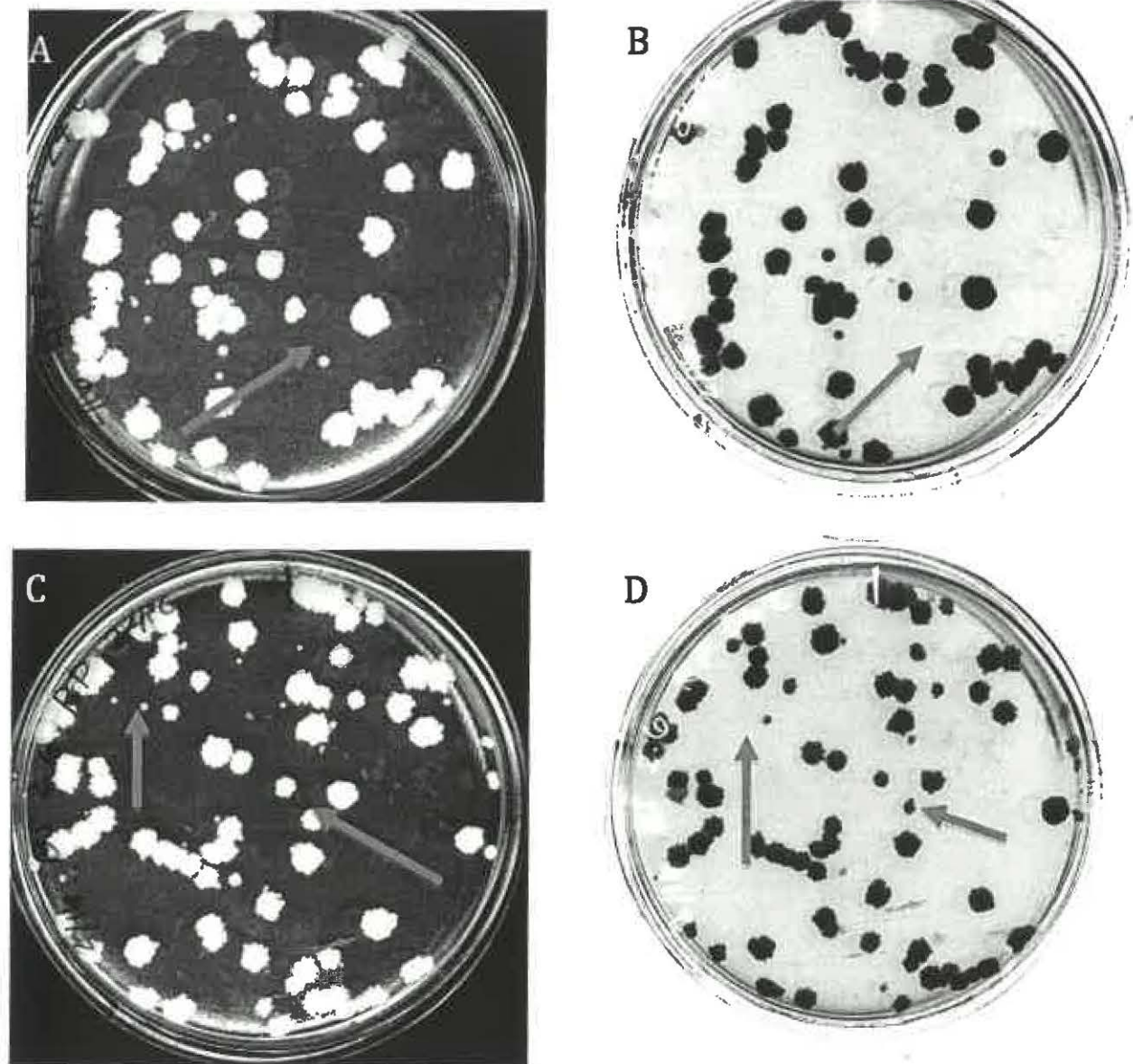
Figure 2: Replica plate images used for overlay procedure for UV mutagenesis screening of possible *ts* mutants. A) Replica of plate UV2BS1 grown at the permissive temperature 24°C. B) Replica of plate UV2BS1 grown at the restrictive temperature 36°C. C) Replica of plate UV2BS6 grown at the permissive temperature 24°C. D) Replica of plate UV2BS6 grown at the restrictive temperature 36°C. The arrows indicate possible *ts* mutants that show no growth or reduced growth at the restrictive temperature.

To confirm temperature-sensitivity, the 204 possible *ts* mutants were patched onto YPD plates in duplicate so that half were allowed to grow at the permissive temperature (24°C) while the other half were incubated at the restrictive temperature (36°C). The patching revealed that of the 204 possible mutants screened, 52 of them were confirmed to be *ts* mutants. Of all the initially plated colonies (4025), only 1.29% were *ts* mutants. Table 3 shows the numbering and origin of the possible and confirmed *ts* mutants. The *ts* mutants were frozen and stored and renamed with the label RCY1129-1180. The designations are shown on Table 3 with respect to each *ts* mutant.

Table 3: Possible and confirmed *ts* mutants from UV mutagenesis.

Replica Plate Name	Numbering of Possible *ts* Mutants	Numbering of Confirmed *ts* mutants	RCY Label
UV2BS1	1-11	-	-
UV2BS2	12-17	12	RCY1129
UV2BS3	18-24	21, 23	RCY1130-1131
UV2BS4	25-35	33, 35	RCY1132-1133
UV2BS5	46-49	46, 47	RCY1134-1135
UV2BS6	36-45	44, 45	RCY1136-1137
UV2BS7	50-56	54, 56	RCY1138-1139
UV2BS8	57-71	59, 61, 71	RCY1140-1142
UV2BS9	72-76	72, 73	RCY1143-1144
UV2BS10	77-87	81, 86, 87	RCY1145-1147
UV2BS11	88-99	88, 94	RCY1148-1149
UV2BS12	100-117	106, 107	RCY1150-1151
UV2BS13	118-121	121	RCY1152
UV2BS14	122-125	122, 124, 125	RCY1153-1155
UV2BS15**	N/A	-	-
UV2BS16	126-132	128, 132	RCY1156-1157

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UV2BS17	133-141	133, 135, 136	RCY1158-1160
UV2BS18	142-145	144, 145	RCY1161-1162
UV2BS19	146-155	147, 153, 155	RCY1163-1165
UV2BS20	156-163	158, 159, 161, 163	RCY1166-1169
UV2BS21	164-181	166, 167, 171, 173, 180*	RCY1170-1174
UV2BS22	182-193	190*, 193	RCY1175-1176
UV2BS23	194-195	195	RCY1177
UV2BS24	196-197	197	RCY1178
UV2BS25	198-204	198, 204	RCY1179-1180

* Designate *ts* mutants that were pink and could have mutations in *ade*.
**Plate UV2BS15 did not have any possible *ts* mutants.

As shown in Table 3, the *ts* mutants UV2BS21.180 (RCY1174) and UV2BS22.190 (RCY1175) were observed to have a pinkish-red color. This could indicate that a mutation is in an *ade* gene (Zonneveld & Van der Zanden, 1995) or that the yeast is about to produce a red pigment called pulcherrimin. Pulcherrimin production has been exhibited when yeast have been plated on media containing iron salts, which made the yeast a deep maroon color (Kluyver *et al.*, 1953). To distinguish between the two possible mutations, we could examine whether the mutants regained color on adenine-supplemented plates (for *ade* mutation) or on iron salt deficient plates (mutation involving pulcherrimin). From observation of the original colonies, it is not likely though that the mutation involves pulcherrimin because the color was more pink than a deep red color.

Screen for *cdc* mutants from the *ts* mutants identified in the pilot screen

The confirmed *ts* mutants identified from the pilot screen and the controls were grown to log phase at the permissive temperature and then shifted to the restrictive temperature for 7 hours before being fixed with ethanol. The samples of *ts* mutants and the control strains were examined with a phase microscope to score for terminal

morphology, such as unbudded, small-budded, large-budded, and unique budding. This screen was used to look for *ts/cdc* mutants that displayed a uniform bud-size at arrest of 70% or greater uniformity. The bud-size and morphology was scored for about 100 cells per sample. Figure 3 shows the size and morphology of the buds at arrest for the controls and the *ts* mutants that were observed by using the phase microscope.
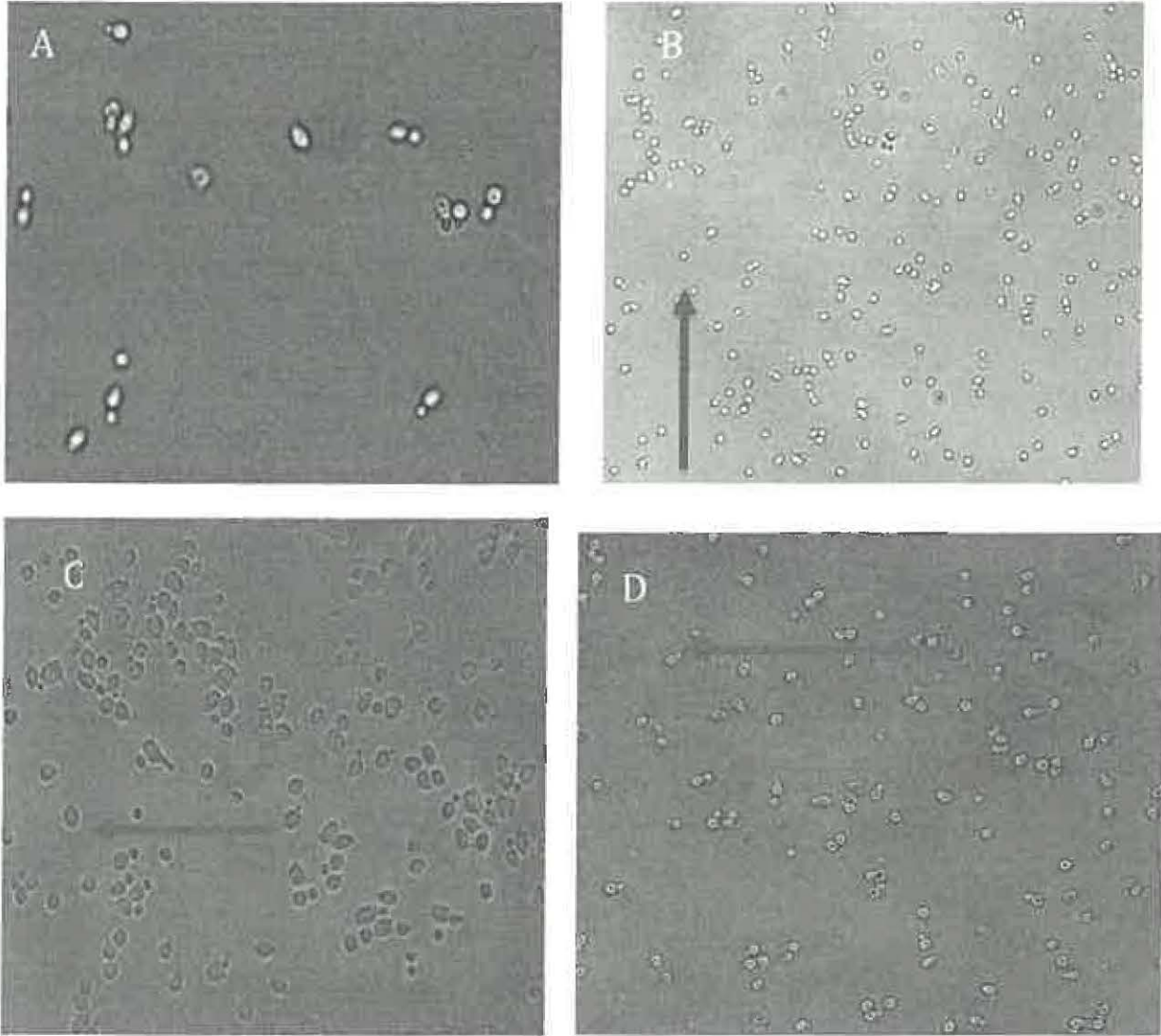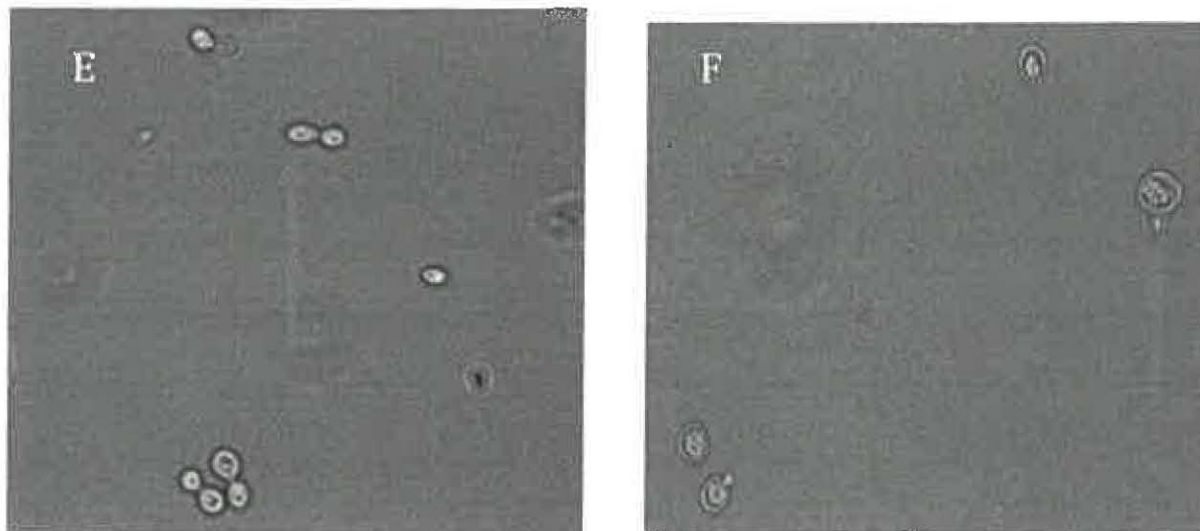
Figure 3: Size and morphology of buds at arrest for the *cdc* screen. A) Negative control non-uniform bud-size of the strain GG1888. B) Bud-size pattern at arrest for the *ts* mutant UV1BS1.4, which displays no buds at arrest. C) Bud-size pattern at arrest for the *ts* mutant UV1BS1.4, which displays tiny buds. D) Bud-size pattern at arrest for the *ts* mutant UV1BS3.27, which displays small-budded arrest. E) Bud-size pattern at arrest for the *ts* mutant UV1BS4.32, which displays a large-budded arrest. F) Bud-size pattern at arrest for the *ts* mutant UV1BS5.36, which displays a small-bud arrest. Arrows indicate the phenotype(s) described. All images were taken at 1000X magnification except for the photo in panel B and D, which were taken at 400X.

The negative control strain GG1888 population did not appear uniform with respect to bud-size because it displayed a mixture of unbudded, small-budded, and large-budded cells. The positive control strain RCY303, a known *cdc* mutant that displays large-budded arrest morphology (Oldroyd, 2002), showed a 69.91% uniform large-budded arrest morphology (not shown). The newly created *ts* mutant UV1BS1.4 showed a 69.91% uniform unbudded arrest morphology upon the first examination at 400X magnification (Figure 3B). When the image from Figure 3C was taken, the magnification at 1000X allowed better visualization to show that the unbudded phenotype was actually tiny buds. Despite this, the classification as a *cdc* mutant still applies to this mutant. The phenotype of tiny-budded was characterized differently than from that of small-budded. The *ts* mutant

UV1BS3.27 showed a 87.16% uniform small-budded morphology at arrest. Both of these phenotypes are new for this study as all previous *K. lactis cdc* mutants were characterized as large-budded. However the small-budded *cdc* mutant is an especially significant find because no small-budded mutants (differentiated from tiny-budded mutants) were found in Hartwell's research with *S. cerevisiae* (Hartwell *et al.*, 1973). The *ts* mutant UV1BS4.32 displayed a 76.81% uniform large-budded morphology at arrest, which classifies it as a *cdc* mutant but with a phenotype already found in this study. The next *ts* mutant UV1BS5.36 displayed a uniform small-budded morphology at arrest that was below our 70% standard for *cdc* mutant classification. However, there were not a lot of cells to be screened initially meaning we had to hunt to find cells we scored. The number of cells screened was higher for this strain than the others because we scored extra cells to ensure the uniformity of the morphology. In comparison to the 25.5% small-budded morphology scored from the negative control wild type strain GG1888, we can conclude that the strain UV1BS5.36 is a small-budded *cdc* mutant. The previously isolated *ts* mutant RCY1110 displayed a 68% uniform unbudded morphology at arrest (image not shown). While this percentage may be slightly lower than the accepted cut off for *cdc* mutant uniformity, we still classify it as a possible *cdc* mutant. Table 4 displays the total number of cells scored along with the percentage of each of the bud morphologies.

Table 4: Bud morphologies at arrest from *cdc* mutant screen.

Strain	Unbudded	Small-Budded	Large-Budded	Other*	Total # of Cells Screened
GG1888	93 (47.49%)	50 (25.5%)	52 (26.5%)	1 (0.51%)	196
RCY303	18 (15.93%)	16 (14.16%)	79 (69.91%)	0	113
RCY1043	61 (39.10%)	65 (41.67%)	29 (18.59%)	1 (0.64%)	156
UV1BS1.4**	79 (69.91%)	3 (2.65%)	31 (27.44%)	0	113

UV1BS1.9	29 (22.14%)	59 (45.04%)	39 (29.77%)	4 (3.05%)	131
UV1BS3.25***	-	-	-	-	-
UV1BS3.27	13 (11.93%)	95 (87.16%)	0	1 (0.91%)	109
UV1BS4.32	29 (21.01%)	1 (0.73%)	106 (76.81%)	2 (1.45%)	138
UV1BS5.36	44 (23.78%)	110 (59.46%)	31(16.76%)	0	185
UV1BS6.16					
UV1BS8.42	36 (27.07%)	67 (50.37%)	30 (22.56%)	0	133
UV1BS14.50	50 (36.49%)	58 (42.34%)	29 (21.17%)	0	137
RCY1110	85 (68.00%)	32 (25.60%)	8 (6.40%)	0	125

* Unique bud-size and morphologies at arrest were separately scored when multiple morphologies arose.
**This mutant originally appeared unbudded but upon better magnification displayed a tiny-budded morphology.
***The cells for this sample were over-sonicated (at an 80% duty cycle) and consequently were unable to be scored.

While preparing the different strains for observation, the *ts* mutant UV1BS3.25 was over-sonicated because the sonicator was set at an 80% duty cycle. This caused the cells to fragment and we were not able to screen for uniform morphology or lack thereof. In the initial screening of the *ts* mutant UV1BS6.16 a majority of the cells appeared to be unbudded, but upon further examination the cells appeared to have a "weird" phenotype, which we called "frakenyeast." The phenotype of this mutant was unbudded but larger than the other cells. Hartwell observed a similar phenotype of *cdc* mutants because it is common for the cells to swell in size after arrest; the cells continue to grow in size even though they are no longer able to progress in the cell cycle (Hartwell *et al.*, 1973). We were unable to rescore the cells for UV1BS6.16, due to time constraints, but we can predict that re-examination will show this strain to be a *cdc* mutant.

DAPI staining

Some of the *ts* mutants that also displayed the characteristic uniform termination point were prepared on permanent slides and examined for nuclear morphology using

DAPI staining. Figure 4 shows the DAPI staining of identified *ts/cdc* mutants. DAPI staining of the unbudded/tiny-budded mutant UV1BS1.4 showed that the nucleus was intact and was not divided or in fragments (Figure 4A). Examination of the large-budded UV1BS4.32 for nuclear morphology proved inconclusive because the DAPI stain adhered to the plasma membrane, and thus the nucleus lacked contrast or distinction from the cell body.
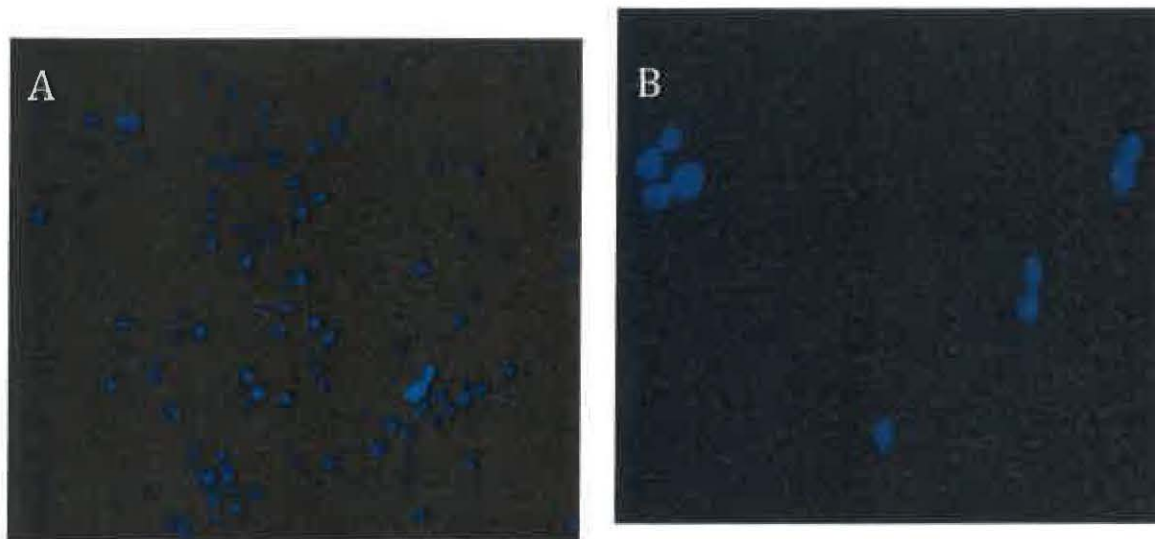
Figure 4: Nuclear morphology of *cdc* mutants, visualized using DAPI and a fluorescence microscope at 1000x. A) UV1BS1.4 unbudded/tiny-budded phenotypic *cdc* mutant that displays a single nucleus. B) UV1BS4.32 large-budded *cdc* mutant that displays inconclusive nuclear information.

Discussion

The purpose of this work was to use UV mutagenesis to create *ts/cdc* mutants and then to isolate and screen the resultant mutants in order to discover new *cdc* mutants not previously found. Using ultraviolet light as the mutagen, we were able to find four *ts/cdc* mutants from among the 61 *ts* mutants generated. An additional *ts/cdc* mutant was found from a previously isolated *ts* mutant (Dror, 2011), making a total of five *ts/cdc* mutants found. Our *cdc* mutants showed unbudded/tiny-budded, small-budded, and large-budded morphologies at arrest. The unbudded/tiny-budded and small-budded morphologies are novel *cdc* mutant phenotypes for this research project.

From the pilot study we saw 0.73% of cells that survived were *ts* mutants, and of those *ts* mutants, 44.4% were also *cdc* mutants. From the scaled up UV mutagenesis study, we saw 1.27% of the cells were *ts* mutants. We were unable to perform *cdc* screening on the second batch of isolated *ts* mutants because of time constraints. We expected to see about 5% of all cells to be *ts* mutants (Novick & Schekman, 1979) and 15% of the *ts* mutants to also be *cdc* mutants (Hartwell *et al.,* 1973). We did not get the expected yield for the *ts* mutants for either round of mutagenesis, which could have been attributed to any number of factors. One possibility as to why we did not see the expected percentage of *ts* mutants was that the expected ratios were generated from a much larger screen of *S. cerevisiae* cells. Besides representing ratios that pertain to a different species of yeast, the expected percentages derived from a study using EMS mutagenesis. Using a different species of yeast and a different type of mutagen on a much smaller scale may have caused our results to differ from the expected results. Naama Dror, a past researcher who also used UV mutagenesis to create *ts* mutants in *K. lactis*, found only eight mutants from among

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twenty-three plates (the counts per plate or total count of colonies was not reported)(2011), which is more similar to our results, as we found nine *ts* mutants from among eleven plates.

Other explanations for the subpar ratios of *ts* mutants may be from human error and miscalculation. The method used to spread the cells in liquid YPD onto the YPD plates may have caused the cells to be pushed towards the edges of the plates where the UV light was less effective because the plastic edges may have shielded the colonies from the mutagenic UV light. The replica plating method also have may attributed to the small number of *ts* mutants found because the velvets and replicating block did not transfer possible mutants located near the edge of the plates or growing on the sides to the replica plates used for temperature-sensitivity screening. As we scored the original plates for total colony counts this would lower the observed percentage of *ts* mutants. It is also possible that some *ts* mutants were missed in the image overlay process because of error and inconsistency. The level of colony separation could have contributed to our human error in visualization of *ts* mutants because the higher the density of colonies and the closer the colonies were meant less definition and more smudging of individual colonies. When the colonies were congregated and abutting each other the growth of individual colonies was difficult to compare between the two plates, which may have lead to undercounting the *ts* mutants. The level of contrast for the replica plate images may also have contributed to our dearth of observed *ts* mutants because they could not be optimally visualized. We could also have missed some *ts* mutants on the patch plates because when possible *ts* mutants were clustered near wild type cells and then patched onto the new plates, some of the wild type cells may have come along. This would create a false negative because the wild type would

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be able to show some growth, and the *ts* mutant would have been disregarded. One way to validate that the screening method was effective would be to have a control plate that contained a wild type culture spiked with a marked *ts* strain. An auxotrophic mutant that required some nutritional supplement could be used to calculate the effectiveness of locating *ts* mutants by knowing what percentage of the mutants should be auxotrophs.

We did not get the expected results for the *cdc* screening. The vast contrast in our results to those found by Hartwell in regards to *cdc* mutants could be a result of the different mutagenesis procedures. Hartwell found 15 *cdc* mutants from among 100 *ts* mutants, amounting to 15% (1970). These *ts* mutants were created by chemical mutagenesis at a kill rate of 99-99.8% (Hartwell, 1967). This extremely high kill rate created a lot of *ts* mutants but also created multiple mutations within the temperature-sensitive mutants (Hartwell *et al.*, 1970). Multiple *ts* mutations within a cell could create a non-*cdc/ts* mutation that would be epistatic to a *cdc/ts* mutation because the non-*cdc/ts* mutation would cause the cell to die before it could reach the termination point that would be characteristic of the *cdc/ts* mutation (Silveira, personal communication, 2012). This could explain why he found a lesser number of *cdc* mutants than we did. We used a 70-85% kill rate because this ensures the optimal levels of mutagenesis while preventing multiple mutations (Ausubel *et al.*, 1993).

The control strains GG1888 and RCY303 used for the *cdc* screen showed the expected results. The control strain RCY1043 did not show the expected results. The GG1888 strain showed a non-uniform bud morphology because it is wild type with respect to the cell division cycle and was used as a negative control. The screen of RCY303 resulted in a 70% uniform large-budded morphology at arrest that was expected for this known *cdc*

mutant derived from past research. The screen of RCY1043 showed a non-uniform bud arrest morphology, like the wild type strain GG1888. From previous work, RCY1043 was determined to be a *cdc* mutant that arrested with a large-budded morphology (Bohmer, 2009). This was a deviation from the expected but within reason, because the nature of the mutation(s) of this strain and its parent derivative RCY290 have not been explored to explain the unusual inheritance of RCY290's progeny (Bohmer, 2009).

The screening of our initial set of *ts* mutants from the pilot study revealed an unbudded/tiny-budded *cdc* mutant UV1BS1.4 (RCY1120), which is unique to this research project because it has not yet been found in *K. lactis*. This bud arrest morphology is similar to the *cdc1* mutant detected by Hartwell in *S. cerevisiae*. The *cdc1* mutant was seen to arrest with no bud or a tiny bud and a single nucleus and terminated at its execution point, the point in the cell cycle in which the affected gene stops functioning (Hartwell *et al.*, 1970). This *cdc* mutant was later found to be associated with defects in actin cytoskeleton organization (Rossanese *et al.*, 2001), but the direct connection to the cell cycle is not yet clear.

We also found two small-budded *cdc* mutants UV1BS3.27 (RCY1123) and UV1BS5.36 (RCY1125). This phenotype is novel to both this study and previous research with *S. cerevisiae* (Hartwell *et al.*, 1973). Finding a phenotype that is unique to the major species of yeast studied, suggests that there may be a different mechanism of control or regulation of the cell cycle in *K. lactis*, a further justification for continuing research in this species of yeast.

In our search for novel *cdc* mutants, we found UV1BS4.32, a large-budded mutant that may or may not be novel. Large-budded mutants may arise from complications in DNA

replication, spindle-to-chromosome attachment, the Anaphase Promoting Complex/ Cyclosome (APC/C), or mitotic exit. To distinguish between an error in mitotic exit and the other possible problems, nuclear staining using DAPI could be performed. If the mutation affected mitotic exit, the DAPI image would show two masses of DNA because the cells were able to complete anaphase, became stuck in telophase, and were unable to complete cytokinesis. The other possible mutations would result in two joined buds but there would only be one DNA mass, because the checkpoint would halt at the metaphase-to-anaphase stage.

While we were able to find a novel *cdc* mutant for this research, our screen of *cdc* mutants is not done. Compiling previous research with the research we conducted, there was a total of 15 *cdc* mutants that fell into at least ten complementation groups based on previous knowledge and bud-size only. The screen shows at least ten complementation groups with only a few groups that have more than one member, which means the screen of *cdc* mutants in *K. lactis* is not saturated. If the screen were saturated we would see results that had some complementation groups with many *cdc* mutants and most complementation groups with only one mutant (Hartwell *et al.*, 1973). From our UV mutagenesis of *K. lactis* and our screen of the resultant *cdc* mutants, we should see additional novel categories of mutants as compared to the previous screens of *cdc* mutants of *S. cerevisiae* because one such mutant RCY597 has a spindle mutation that has not been found in *S. cerevisiae* (Fonseca, 2008).

To continue the creation, isolation, and characterization of *cdc* mutants, continuing with the path of this project would be advised. First, screening of the rest of the *ts* mutants isolated from this study along with the ones from a previous study (Dror, 2011) for

mutations that are also mutations of cell cycle genes would need to be finished. The next step would be to backcross the *cdc* mutants to the wild type strain of opposite mating type to determine the nature of the mutation and the number of defective genes. To tell if the mutation was recessive or dominant one would observe the resultant diploid created in a backcross for temperature-sensitivity. Knowing the mutations are recessive is important in further genetic analysis like cloning by complementation, because it utilizes the principle that the mutant phenotype can be rescued by the wild type gene. Even though we have not yet performed the backcross of the *ts* mutants to the wild type strain we can predict that they are recessive mutations because dominant *ts* mutations only occur 1% of the time in *S. cerevisiae* (Hartwell, 1967). Dominant mutations are not as common, especially in *cdc* mutants because dominant mutations usually confer a gain of function rather than a loss of function like recessive mutations (Lodish *et al.,* 2000). To determine the number of genes mutated, the spores produced by the backcross diploid would be examined for temperature-sensitivity. If the spores displayed a 2:2 segregation pattern for temperature-sensitive to wild type cells, then only one gene was mutated. If the spores segregated with a higher ratio of temperature-sensitive to wild type cells, then it is possible multiple genes were mutated.

To confirm that the *cdc* mutants found were different from previously screened mutants, further work would include complementation testing. Complementation testing is achieved by crossing mutants of opposite mating types and examining the resultant diploid for rescue of the mutant phenotype. If a cross of the two mutants results in a diploid that is temperature-sensitive, then the two mutants fail to complement each other. This means the two mutants are of the same complementation groups and are alleles of the

same gene. If a cross of the two mutants results in a diploid that is wild type, then the two mutants complement each other. The two mutants are in separate complementation groups and are mutations of different genes. Complementation testing would also show whether or not our screen of *cdc* mutants was saturated or not, depending on the number and members of the complementation groups. Until the screen is saturated, future researchers should continue the process of creating *ts/cdc* mutants with UV mutagenesis and using the image overlay method to screen for *ts* mutants in order to complete the screen of *cdc* mutants for the budding yeast *K. lactis.*

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