STUDY OF SUBTELOMERIC RECOMBINATION AND STRESS RESPONSE IN THE YEASTS

CANDIDA ALBICANS* AND *KLUYVEROMYCES LACTIS

by

PAUL MICHAEL GRIFFITH

(Under the Direction of Michael McEachern)

ABSTRACT

Subtelomeres are specialized regions adjacent to the chromosome end of DNA/protein structures known as telomeres. These regions experience distinct differences in mutation rate, recombination rate, gene density, and gene silencing which provide a unique environment in which to promote rapid evolution. Subtelomeres may utilize adjacent telomeres as drivers of evolution in response to stress conditions, which may generate transient telomeric dysfunction through sequence modification or protein association modifications and in turn promote telomere recombination. Telomeric recombination has been shown to promote subtelomeric recombination and this process may be used to drive subtelomeric evolution. In this way, the rapid evolution of contingency gene families found at subtelomeric sites may occur through gene duplication and subsequence diversification to provide means of adapting to the surrounding environments. The Adaptive Telomere Failure hypothesis

provides a model by which an organism may utilize programmed stress-related telomeric dysfunction to drive the subtelomeric architecture into a evolutionarily active state by promoting increased rates of recombination. To study the role in which subtelomeric recombination plays as part of the milk yeast *Kluyveromyces lactis* and the human pathogen *Candida albicans* and its response to stress conditions, *URA3* biosynthetic markers and a naturally occurring β-galactosidase were utilized to study single strand annealing (SSA) and break induced replication (BIR) recombinational pathways at subtelomeric and non-subtelomeric loci. *K. lactis* was found to demonstrate a significant increase in recombination at subtelomeric sites in response to certain stress conditions including exposure to arsenate, but not other stresses. Additionally, we found that exposure to sodium arsenate stress produced reversible slower growing colony morphologies even after removal from arsenate. Finally, studies in *C. albicans* provided evidence to support a varied rate of subtelomeric recombination among multiple members of the subtelomeric *TLO* gene family that is transcriptionally modulated during stress conditions.

INDEX WORDS: subtelomere, telomere, recombination, *Kluyveromyces lactis, Candida albicans*, *TLO,* arsenic, petite, Adaptive Telomere Failure hypothesis

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A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2016

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May 2016

Acknowledgements

The culmination of the journey of my PhD brings to light the many people who have supported me and helped make this dream a reality. Through all of the complications, project changes, life changes (including the birth of my two children) and personal adventures there have been a wonderful group of supporters who I feel are key reasons I have found success. There are not many times in your life where you get the opportunity to thanks those who have been there through thick and thin, and I am glad to have this brief opportunity.

First and foremost, I'd like to thank my family and friends for their support and eternal guidance through this process and my education. I want to extend many thanks to my wife for the help with my writing, emotional support through my research, and helping me through the long nights in the lab and at home. A thank you also goes out to my parents who have allowed me to follow my dreams in science and have supported my education through their personal sacrifices.

These projects, scientific successes and my experience during graduate school would not be possible without the guidance, mentoring and support of Dr. Michael McEachern. Words cannot express my thanks for the times spent troubleshooting, listening and providing support through the trials and tribulations of my projects and the projects within projects which have arisen over the past six years. Your guidance and how you have provided insights into the exciting field of cancer, telomeres and subtelomeres are all things which will stay with me throughout my future careers.

In collaboration with Dr. McEachern, I would like to thank my committee: Dr. Dave Hall, Dr. Kelly Dawe, Dr. Robert Sabatini and Dr. Richard Meagher. Your guidance, support and suggestions through my projects from the early iterations to late stage complexities have positively shaped my scientific career and projects while at the University of Georgia.

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Dissertation Overview

To adapt to environmental stresses, cells can respond in many ways including inducing signaling pathways, transcriptional differences and genetic mutations. Stress responses are diverse and can occur at the cellular or genetic level in single celled eukaryotes through a wide variety of mechanisms including cell cycle checkpoints, protein modifications, recombination events and mutations. The focus of this dissertation is, using the yeast *Kluyveromyces lactis*, to determine the potential role that telomeres and their adjoining subtelomeric regions may play in adaptive response to stress conditions through recombination and how this may play a part in the rapid evolution of subtelomeric genes. Chapter 2 details studies of recombination assays to measure subtelomeric and internal recombination rate variation during stress conditions. This work uncovers potential subtelomere-specific recombination increases that occur in response to certain stresses including the presence of arsenate. Chapter 3 covers details related to arsenic-induced semi-stable colony morphologies in *Kluyveromyces lactis*. Chapter 4 focuses on recombination assays at subtelomeric loci in the pathogenic yeast *Candida albicans*. Chapter 1 will introduce details about some of what is known about the stress conditions used in this study as well as discuss features of telomeres, the protective DNA-protein complexes at the ends of the chromosomes. It will also describe the unusual characteristics of the regions adjacent to telomeres known as the subtelomeres and the gene/sequence information contained therein. Furthermore I will detail the Adaptive Telomere Failure hypothesis and how it may pertain to growth-related responses to stress. Through the studies here, I have attempted to determine how eukaryotic microbes respond to adverse conditions using recombination and parse details about possible preferential treatment of subtelomeric sites in regard to these responses.

CHAPTER 1

Introduction and Literature Review

This study of stress response in the yeasts *Kluyveromyces lactis* and *Candida albicans* is focused on analyzing how chromosome ends experience recombination during abnormal environmental conditions. More specifically, I focus on how stress can drive telomeres to influence genic regions adjacent to them, dubbed the subtelomeres, and potentially drive mitotic recombination events that alter subtelomeric gene structure and content. Furthermore, the concept of adaptive telomere failure will be tested in this study by establishing the rates of recombination at subtelomeric loci that occur in response to stress conditions and studying the differences between two yeast species to examine how different environmental origins can further influence chromosomal instability.

Stress can be defined in many ways, the simplest being the requirement to adjust or adapt to deviations from a normal condition. The normal state does not have to be without complications, but once an organism has adapted to its environment, it is no longer considered stressful. These studies will focus on environmental stresses which encompass complications arising from the modifications to the environment which often require a cellular or metabolic response to properly adapt and survive changes such as arsenic exposure, high temperatures or high salt conditions. When speaking about stress related responses or stress conditions for the majority of this dissertation and within each defense chapter, the reference is almost exclusively referencing environmental perturbations as a stress.

This introduction will first briefly cover stress conditions and cellular responses to them as the concept of cellular stress is central to this research. Telomeric structure and function will follow this as they are regions of the chromosome which are the subject of this work and which are important regulators of genomic stability and, we predict, may also be genetically destabilized by specific types of

stresses. Telomeric structure, function and dynamics help to dictate how subtelomeric DNA behaves. Subtelomeres are regions of high diversity within species and yet interesting gene and sequence commonalities exist between divergent species. This point will be elaborated on along with their other noteworthy characteristics such as their increased mutation and recombination rates. The focus on chromosomal ends during stress response in this study is due to the desire to begin to experimentally address the Adaptive Telomere Failure hypothesis. This hypothesis is introduced and the supports for its assumptions are laid out along with its potential consequences for chromosomal evolution. Although the bulk of the information provided in this chapter is related to subtelomeres, stresses, and the Adaptive Telomere Failure hypothesis, additional information regarding how organisms may respond in a cellular or genic level to attempt to survive harsh conditions, will be covered to address additional findings in this dissertation.

Genetic and physiological responses to stress

Organisms exist in an imperfect world which requires adaptation and response to variables in order to survive. These variables may be environmental such as osmotic pressures or radiation, or they may internal such as replication problems and chromosomal instability. How a cell responds to each stress condition or genetic variable helps to determine the likelihood of successful adaptation by allowing a cell to progress through that stress. What stresses entail and how they illicit a cellular, proteomic or genetic response is an important factor to establish before attempting to determine how they can affect a cell at the recombinational level, and may help explain how this may happen.

Environmental stresses elicit a response by conserved stress genes

Stress may be considered any condition which perturbs the natural being or state of things and often generates a response to help right or overcome the negative consequences generated by the stress. In yeast, stress induced genes are diverse: heat shock proteins including the well studied *Hsp70*

and *Hsp 90* families, cell membrane proteins, the *RAD52* epistasis family, and a diverse array of other stress specific genes (*1-5*). As heat, high salt, and arsenate stresses are used in my studies, particular discussion of their effects will be described. Specific signal cascades and biochemical stability mechanisms exist which trigger stress responses. It is important to note that some gene families, known as stress-response genes, promote cellular responses and adaptations to a stress condition. These are inherently different from cellular stress responses resulting in short term biochemical or cellular modifications to protect against damaging stress effects. Microorganisms are particularly adept at adapting to stress conditions and have evolved the ability to respond to specific conditions in their environment by transcriptionally activating their stress response genes in an effort to survive, but activation requires appropriate signaling to respond to environmental cues. One of the largest and most widely conserved groups of proteins associated with stress responses are heat shock proteins. These proteins are synthesized in response a multitude of stresses including temperature, osmotic stresses , heavy metals and ionizing radiation following appropriate signaling from the environment-sensing machinery of the cell (*6*). Signaling of environmental conditions in most eukaryotes is often performed by multiple MAP kinases, initiating signal cascades through phosphorylation of target proteins which in turn may activate other kinases, transcription factors or genes in order to promote an appropriate stress response. These stress responses are conserved among many species, but the modes of stimulation, signaling and response are diverse. The responses of the yeast S. cerevisiae, a reasonably close relative of *K. lactis*, will be the focus here.

S. cerevisiae has two well studied pathways using signaling kinases in the HOG1 pathway (responding to osmotic stress) and the Ras-cAMP PKA (involved in starvation response) which converge to stimulate a cluster of stress related genes (*7*). Stress response genes are predicated by a stress-regulated *Msn2*- or *Msn4*- targeted STRE promoter which responds to both HOG1 and Ras-cAMP and activates cytoplasmic catalases, DNA damage proteins, polyubiquitin and the temperature response

sugar trehalose in addition to heat shock proteins (*7*). The expression of *Msn2* is highly regulated at the transcriptional level and quickly diminishes when stress conditions are not maintained, providing a cell with the ability to create a rapid response by activation of dozens of *Msn2* targets without sustained energy-intensive synthesis of stress genes (*8*). Beyond the activation of many stress response genes designated by the presence of STRE promoter sites, repression of these genes during normal conditions is vital to successful growth. Therefore many of the stress response genes are normally transcriptionally inactive. The kinase *Srb10* leads to rapid degradation of *Msn2* after a spike in activity, providing the cell with a means to counter the stress response spike in gene expression by removing the stimulating protein (*8*). The high level of conserved regulation over dozens of genes through a simple pathway implies that the quick transcriptional response and the rapid transcriptional repression of stress-related genes is an evolutionarily advantageous process. This also suggests that the transcriptionally inactive state may be beneficial for stress response genes in most conditions.

The most studied stress response proteins that are transcriptionally activated by these cascades are the heat shock proteins (HSPs), which occur in all organisms, but these are far from the only responses that yeast have. The widely conserved heat shock proteins fall into two major large classes: the cytoplasmic *Hsp70* and *Hsp90* families, which can account for up to 5% of total proteins found within a cell following heat stress (*9*). During stress conditions, HSPs perform a wide range of tasks including the modification of mis-folded proteins and the prevention of denatured protein aggregates. HSPs play a key role in stabilizing cellular functions and structure while cells are attempting to respond to stress. Some members also act in the nucleus to activate transcription of other stress related genes. Given their diverse range of actions, it is unsurprising that heat shock proteins have also been found to function under normal growth conditions to assist in protein folding and chaperonin-like functions to support proper post-translational modifications (*5*).

Thermal tolerance is an important factor for unicellular organisms, as their environmental conditions dictate the effectiveness of protein synthesis, stability, DNA damage repair, and cell membrane stability while temperature fluctuations can negatively affect all of these aspects of the cell. Yeast heat shock proteins are responsive to environmental conditions throughout the cell cycle, preventing a cell cycle arrest from prohibiting the appropriate stress response and nuclear information degradation through mRNA decay (*10, 11*). These proteins have been found associated with the cytoplasm, mitochondria, ER and nucleus which suggest the wide array of targets and roles which stress response proteins influence providing a wide range of actions to protect stressed cells (*6*). Heat shock proteins retain many different roles, as their many cellular locations would suggest, including modulating protein folding, proteolysis, and protecting the nuclei during stress conditions (*11*). In the nucleus, HSPs do not only activate stress-related genes but are also essential for repair of DSBs by supporting *Rad51* protein with its active ATPase to promote repair (*12*).

HSPs are responsible for promoting or providing rapid protective measures during temperature stress and their activation is dependent on organism-specific conditions (*5*). This is important to note as it suggests that each organism's expression of these highly conserved protein families is augmented so that their activation coincides with an organism's ranges of habitation. For example, *C. albicans*, which can survive at 55℃, does not illicit the same response as *K. lactis* does as it approaches 40℃ which is considered the upper range of the latter's thermal tolerance (*13, 14*). This characteristic suggests that proper signaling of HSPs is an important regulatory step during evolution of species in unique environmental conditions. Furthermore, the diversity of signaling among species and the subsequent adjustment of signaling following adaptation to a new environment occurs at the MAPK level, which shows more distinct variation compared to the highly conserved HSPs, and provides a noticeable difference in acceptable growth conditions.

Osmotic stress is also a catalyst that signals HOG1 which initiates heat shock proteins activation to help stabilize the cell membrane through modulation of osmotic pressure and solute concentrations (*3-6*). The variance in osmotic concentrations can destabilize cellular membranes and interfere with common processes in both the cytoplasm and mitochondria. *S. cerevisiae* modulates cellular membranes with aquaporins specific to the type of solute stress, providing a controlled means for molecule transport and maintenance of desired concentrations (*15*). Other responses generated by nutrient deficiencies may also activate the Ras-cAMP pathway in addition to HOG1 and induce transcription of cytoplasmic catalase *CTT1*, mitochondrial superoxide dismutase *SOD1* and trehalose, all of which provide protection within the cell and its membrane structure (*7*). Although these genes are a small subset of those which are activated through these pathways, they provide insight into a cellular response addressing oxidative stresses in both the cytoplasm and mitochondria and the maintenance of the cellular membrane as well as the importance of these processes.

Arsenic is a widely studied source of stress due to its relevance to human health and its prevalence in the environment. Arsenic acts through currently unknown means to illicit an oxidative response from the cell, reduce available sulfur compounds, damage proteins involved in DNA repair, and cause cell cycle arrests in G_1 (*16, 17*). One of the methods in which arsenic elicits a response is by inhibition of ubiquitination resulting in phosphorylation of multiple proteins including the DNA repair protein *RAD53 (16)*. Given the wide reaching effects of heavy metals within the cell, the presence of multiple signaling pathways, the conservation of cellular responses is expected. Heavy metals stimulate oxidative stress response transcription factor *Yap1* which in turn activates a large number of HSPs and antioxidants such as catalases and thioreductases (*17*). While many different genes respond to environmental stresses, however, there is wide variation in the species which can respond to specific cues such as heavy metals. This may suggest that there is more to stress response than the directly influenced signaling pathways and their targets, and that a rapid evolutionary process may also be

promoting adaptation by providing a wide range of genes with high rates of mutation. High mutation rates may predate the adaptation to new environmental stresses and the study of gene families which respond to common environmental conditions is important in understanding how these pathways may benefit the cell.

Stress can promote mutation and diversity

In yeast cells, there is a diverse array of physiological and transcriptional responses to stress. Focus thus far has included the signaling and cascade of MAPK, HSPs and the activation of their targets which are considered stress response genes. Stabilizing compounds such as trehalose are synthesized during stress response to help stabilize the cell membrane. A significant number of stress response genes in *S. cerevisiae* are cell membrane proteins associated including the *FLO* and *PAU* gene families which may be utilized to protect cultures from harsh environments or utilize complex sugars (*18, 19*). *FLO* family members are adhesion genes, sharing similarities with pathogenic adhesins associated with biofilm formation and pathogenicity in *Candida albicans* and *Candida glabrata (20-22).* These adhesins support attachment to surfaces and biofilm formation which can be a key method of survival by providing altered environments within the biofilm and protecting member cells. Furthermore, *FLO* family members are responsive to the stress signaling from RAS and are normally transcriptionally inactive, suggesting their role may be important in response to stress conditions (*23*). Sugar utilization gene families *PAU*, *MAL* and *MEL* are members of rapidly evolving families which likely benefit from decreased expression during non-stress conditions while they experience high rates of mutation prior to expression during nutrient deprivation (*19, 24*). *PAU* genes are specifically associated with stress response to anaerobic conditions and low temperatures in *S. cerevisiae*, and their study has elucidated a divergent and large gene family which experiences repeated duplications and mutations but is often repressed so these modifications go unnoticed (*19*).

Previous work has shown that *S. cerevisiae,* when exposed briefly to 50℃, increases its rate of chromosomal recombination (*2*). Alternatively, exposure to 37℃ for three days leads to a growth reduction and increase in subtelomeric recombination rates in *K. lactis* (*25*). Temperature stresses are believed to cause breakdown in cellular membranes and denaturation of proteins, thus resulting in widespread potential sources of cellular stress, which does not directly affect genetic information.

Osmotic stresses are a normal environmental variance which illicit a different response than heat shocks, primarily by altering cell membrane structure, fluidity and turbidity (*15, 26*). Initial studies have suggested that NaCl and KCl concentrations of 0.8-1.5 M can be significantly stressful to *S. cerevisiae* and *K.* (*15, 27, 28*). NaCl and KCl stress in *K. lactis* translates to growth repression by the inhibition of biosynthesis and inhibition of activity of invertase during de-repression when glucose concentration is low (*28*). Additionally, salt stresses can affect the respiratory network in the mitochondria by affecting multiple genes including cytochromes or ATPases, as suggested by knock-out mutants (*27*). The focus on the respiratory and sugar utilization pathways as a limiting factor suggest that sodium and potassium chloride stresses do not directly impact DNA, and instead illicit responses by interfering with other cellular structures and processes.

S. cerevisiae has been shown to experience reductions in cell viability and growth rates during sodium arsenate stress exceeding 0.25 mM, providing a strong response at 0.5 mM (*29*). Arsenic toxicity affects many different aspects of the cell; including the mitochondria, mutation of cellular genes, as well as modifications to telomere length and chromosome stability (*30, 31*). How arsenic affects the cell is believed to be through a combination of oxidative stress and prevention of DNA repair mechanisms (*32*). *S. cerevisiae* possess multiple subtelomeric genes associated with arsenic resistance (*33, 34*). *K. lactis* possesses subtelomeric genes similar to *S. cerevisiae* arsenic resistance genes, and has been shown to grow at reduced rates on 0.5 mM sodium arsenate plates (*35*). 0.5mM sodium arsenate was therefore

used as a starting point to evaluate recombination at subtelomeric and non-subtelomeric loci in response to stress for the final stress in an effort to parse support for the Adaptive Telomere Failure Hypothesis by demonstrating a chromosomal preference for subtelomeric recombination during stress conditions.

Additionally, the *S. cerevisiae ACR/ARR* arsenate resistance genes are transcriptionally activated through a mechanism similar to oxidative stress response. The three genes (*ARR1, 2,* and *3*) act in concert with *ARC1* responding to stress as a transcription regulator which imparts resistance through the activation of *ARR2* (arsenate reductase) and *ARR3* (arsenite transporter) which confer resistance to both arsenite and arsenate (*33, 36*). Although these genes are part of a larger contingent of heavy metal resistance genes, their specificity for arsenic resistance is a key factor in the study of arsenic herein. Although these stresses entail a wide range of environmental concerns, they are external in focus and only experienced by a cell on occasion. However, there are stresses involved in the replication and repair of DNA which generate genomic stress during normal conditions within the cell.

Genome related stress

Details thus far described have addressed conditions in regard to the stresses applied at the cellular level by modification of the cell membrane, proteins and potentially DNA by exogenous agents. However, much of the stress experienced within a cell is at the chromosomal level. Chromosomal instability, DNA damage and breakage all contribute to stress and require considerable effort to modulate, and therefore have multiple mechanisms by which they act on the cell and are repaired. Chromosomal instability can be caused by errors in meiotic and mitotic cell cycle progression leading to chromosome loss or duplication, unrepaired double strand breaks (DSBs), or failures in replication leading to DNA damage and cell cycle arrest. DNA damage can be related to normal DNA repair gone awry, replication errors, unstable DNA such as long tracts of repeats or transposons, and failure of the

stability of telomeres or centromeres. In concert, these forces, which are always present and acting against replication and transcriptional fidelity, lead to a constant state of genetic stress which the cell must reconcile and manage in order to promote proper growth and replication.

Mutation can drive adaptation during stress

Although mutations usually occur at a basal rate during the lifetime of a cell, there have been studies which have implicated stress as an enhancer of gene-specific changes in the rates of mutation. *E. coli* demonstrates an increased rate of mutation in response to stress; however, there is a significant increase in the rate of beneficial mutations, generated through a unique means compared to genome-wide mutation rate increases, which allow the cell to adaptively respond to environmental stress. Originally described in 1993 (*37*) this concept has opened the idea of adaptive mutations which suggest that there may be a specific series of responses by cells which allow them to more rapidly adapt by selecting for beneficial mutations at specific sites at a higher rate. The mechanism by which this occurs is believed to be based on protein targeted nicking of DNA which in turn promotes replication fork failure, and subsequent utilization of stationary phase polymerases (*38*). Following resectioning of the break, preferential utilization of error-prone DNA polymerases promotes rapid mutations at the site of the break, introducing multiple point mutations. The bases incorrectly inserted are mostly corrected by the mismatch repair pathway, which can specifically reduce adaptive mutations when its components are over-expressed. The presence of both mechanisms, which differ from standard hyper-mutation mechanisms and specific repair pathways, suggests that adaptive mutations are a directed and preferentially controlled pathway in prokaryotes (*38, 39*).

S. cerevisiae has also been shown to utilize directed mutagenesis to promote adaptive mutations in response to stress conditions at higher rates than prokaryotes, suggesting a means for yeasts to promote rapid adaptation. One method which yeast use to adaptively mutate their genome is

similar to prokaryotes in that they use stationary phase error-prone polymerases to repair DNA breaks and promote rapid mutations (*40*). *S. cerevisiae* has established mutation-prone and inherently unstable variable nucleotide tandem repeats (VNTRs) present within multiple stress-related genes. These may act in concert with error prone polymerases and DNA repair pathways to selectively and adaptively promote mutations in stress related genes (*18*). This process is especially profound within the *FLO* genes, which are cell-cell and cell-surface adhesins, as well as at the *PIR* genes, which are essential in yeast response to temperature and antifungals. Both sets of genes utilize VNTRs to experience high rates of mutation and polymorphism (*18*). These genes are potentially representative of a wide range of cell wall-associated proteins that utilize large repeat regions encoding GPI cell wall anchors. Another method that can promote adaptive mutations at a genome wide level, which *S. cerevisiae* can use effectively, is genomic amplifications and chromosomal rearrangements. One of the methods in which *S. cerevisiae* is believed to have effectively performed rapid chromosomal rearrangements is through failed or dysfunctional recombinational processes. In an alternative method to adaptive modification of chromosomes, the pathogen *Candida albicans* utilizes loss of heterozygosity as a means for rapid evolution and response to environmental pressures such as the presence of antifungals. This, in conjunction with *S. cerevisiae*'s utilization of chromosome variation and duplications, suggests that large scale modification of yeast chromosomes may be a prevalent means of adaptive evolution (*18, 41-44*)

Another eukaryotic cell type which demonstrates elevated rates of mutation, some of which may be considered advantageous and adaptive (at least to the affected cells) occurs in cancer. Cancer cells result from accumulations of mutations in a wide range of genes including, but not limited to, those regulating cell growth checkpoints, chromosomal instability checkpoints and programmed cell death. The predispositions to mutation or chromosomal instability, which cancer cells often exhibit, could be considered an adaptive response in their environment, attempting to promote unregulated growth, respond to drug treatments or to successfully metastasize or invade the surrounding tissue. Cancer cells

can utilize certain regions of the chromosome to promote mutagenesis such as common fragile sites and microsatellites. These regions promote both replicative instability and can be used as inter or intra-chromosomal recombination points, potentially by inducing DSBs (*45, 46*). Cancer cells also regularly experience chromosome rearrangements, with large and small tracts of DNA sequence shifted among chromosomes or locations within a chromosome. This can cause issues with regulation or copy number and also generate new fragile sites in previously stable regions (*47-49*). Small scale modifications and rearrangements to the chromosome, unstable regions, and increased rates of mutation are all compounding factors which can rapidly contribute to cellular malfunction and cancer formation. But most cancers demonstrate large-scale genomic instabilities and rearrangements which are predicated on different types of mutational forces (*45, 46, 48-53*).

Cancer cells were originally recognized as often being chromosomal aneuploidies that resulted from segregation defects during cell division by Boveri in 1914 (*54*). These defects are often multi-step and include failures in centrosome function and attachment to the spindle as well as checkpoints associated with centrosome defects (*55*). While centromeric malfunctions can produce significant errors in the faithful segregation of chromosomes, malfunctions with the structures at the ends of chromosomes, telomeres, may produce a more varied and detrimental series of mutations. When telomeres fail due to protein dysfunction or critical shortening of their length (covered in greater detail below) they no longer provide the required end protection against degradation and DSB repair mechanisms (*56-58*) Without proper protection, cells often attempt to rapidly repair defective chromosome ends through non-homologous end joining which results in telomere-telomere fusions (*56, 59*). These fusions often result in dicentric chromosomes which will randomly break during a subsequent mitosis and result in severely damaged chromosomes and re-initiation of non-homologous end joining to repair the newly broken ends in a process known as a Breakage-Fusion-Bridge cycle which results in widespread chromosomal instability and loss. Telomeric dysfunction can also be related to

overexpression of the reverse transcriptase telomerase, which is activated in the majority of cancer cells, but is normally inactivated in human somatic cells (*60, 61*). Telomerase over-activity is implicated in an increase in genomic tetrapoloidy when cells are exposed to heavy metals and other DNA damaging agents (*62*). Telomerase-negative cells do not regularly experience tetraploidy under the same conditions.

Another means of significant DNA modification in cancer is known as chromothripsis (chromosome shattering). In this process, chromosomes that fail to properly segregate are instead separated into micronuclei, within which they experience drastic breakage and rearrangement of the mis-segregated chromosome. Chromothripsis is believed to be a single large scale event. In recognizing the rapidly adaptive nature of recombination and chromosomal rearrangements in cancer cells, the focus of understanding how stress can drive these events in a beneficial manner becomes apparent, as do the ramifications of uncontrolled events within the cell (*59, 62-66*).

DNA replication is a common generator of genetic stress

DNA replication in bacteria, yeasts and humans inherently causes errors through a low rate of incorrectly inserted base pairs, with yeast replication achieving an error rate of one mismatch in 5.5 x 10^7 bases (*67*). These incorrectly inserted base pairs are often excised by either the polymerase itself through a proofreading exonuclease activity or by mismatch repair proteins (including *Pms1* in yeast) which post-replicationally remove the incorrect base (*67*). Mutations in either a replicative DNA polymerase's proofreading exonuclease or in *Pms1* elevate mutation rates 150-240 fold while a mutation in both can increase the rate of mutation by 38,000 fold (*67*). This is one small example of the importance of DNA fidelity and the importance of the proteins maintaining it. However, there are other aspects of replication which can cause mutations. Replication errors can be independent of the

sequence or protein compliment of the cell, and instead directly related to replication timing, with late replicating regions experiencing higher rates of mutation (*68*)

The function of proof-reading proteins and polymerases has its bounds, with some types of DNA such as highly repetitive elements and GC-rich sequences providing additional complexity during replication which can result in increased rates of mutation (*69*). These regions of instability occur naturally, and the errors contained therein may beget more complex insertion/deletion events following nucleotide incorporation errors, suggesting the steeply sloped risk of simple mutations cascading into much more significant errors if not corrected (*69*). Prokaryotes have tracts of simple sequence repeats which are unstable and prone to expansion or deletion due to replicative slippage (*70*). The wide range of organisms which utilize these simple sequence repeats suggest that their instability is important, and in some prokaryotic pathogens these repeats are found associated with antigenic variation and immune evasion (*70*). Repetitive sequence is not limited to prokaryotes, as simple sequence repeats exist in eukaryotes as well, albeit often in larger sizes both in size of the repeat and the size of the repeated region (*70*). There is also an abundance of trinuclotide sequence such as CAG, GAA and CTG repeats in *E. coli*, *S. cerevisiae*, and *H. sapiens* respectively are known to rapidly incur large deletions and expansions leading to chromosomal instability and may cause genetic disease such as Huntington's chorea and Fragile X syndrome (*71-75*). Repeat regions can be utilized to introduce diversity, provide homology between chromosomes, and destabilize regions of chromosomes by introducing common break points or recombination initiation sites. However, they are still found throughout all domains of life both in and between genes. In yeast, variable number tandem repeats (VNTRs) are present in genes associated with stress and adaptive responses to the environment, suggesting that the variability provided by the repeats is evolutionarily beneficial (*18*). Stress-related VNTRs are often located within key phosphorylation sites of stress-related genes such as the temperature stress proteins of the *SRP, TIP,* and *DAN* families (*18*). The causes for these rapid expansions and deletions are two-fold:

polymerase slippage across simple and trinucleotide repeats, and the use of homology to expand or delete large tracts of repeats (*76-78*). These sequences are believed to often be exposed to selection pressures, with variation in repeat regions providing key advantages to rapid evolution through homeologous recombination (*18*). The *FLO* family of adhesions utilizes VNTRs to increase adhesive capabilities, and present a model for how these repeat regions may be utilized to promote rapid adaptation of stress-related genes (*18*).

Base-pair mutations are another commonly seen DNA alteration which can cause significant genomic stress. There are multiple mechanisms, conserved from prokaryotes to eukaryotes, by which the cell can repair them such as mismatch repair, trans-lesion synthesis and nucleotide excision repair; all of which are stressful processes which can themselves promote mutation. Mismatch repair typically acts after DNA synthesis; improving fidelity by up to 3-fold in yeast and 100-fold in bacteria, as a secondary measure to correct incorrectly incorporated (*79, 80*). This repair process is also a potentially potent evolutionary lever, which can be used by pathogenic bacteria to increase the rate of mutation over 1000-fold by using specific mutations knocking out proteins used in mismatch repair (*81*). The generation of such mutator strains can also be induced by trans-lesion DNA polymerases which are capable of DNA synthesis in a highly error-prone manner (*82, 83*). Nucleotide excision repair is utilized as a method to correct both small and significantly sized errors in DNA structure reaching up to hundreds of base-pairs, including erroneous products of trans-lesion synthesis (*84*). This process is often utilized to remove RNA from DNA strands following replication by utilizing a RNase or *Top1*-like protein and is capable of removing mis-paired bases (*84*). Although correcting small stretches of DNA errors is a critical part of preventing genomic stress related to DNA damage, these regions of repair are single stranded and limited in size. Cellular mechanisms, notably homologous recombination and non-homologous end joining, also exist which have evolved to repair double strand breaks and help prevent chromosomal instability.

The *RAD52* epistasis group of proteins is involved in homologous recombination and DNA damage repair in eukaryotes and their activity can act to relieve genomic stress caused by DNA damage. The activity of DNA repair utilizing Rad proteins may increase stress due to errors in recombination resolution resulting in incomplete repairs. Using DSBs, duplications and replicative stress as factors initiating recombination, the *RAD52* epistasis group's purpose is diverse and it can perform different types of recombination dependent on different subsets of *RAD* proteins including: Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59 as well as MRX (*85*). Recombination in eukaryotes requires multiple members of this set of proteins, but depending on the substrate, not all proteins may be required. Response to double strand breaks is crucial to relieve genetic stress as the loss of sequence or chromosomal regions are a potential outcome of unrepaired breaks. The entire Rad52 group of proteins is utilized for conservative homologous recombination (HR/SDSA Figure 1.1-1); a process which utilizes tracts of homology shared between sister chromatids or sometimes between homologs or to accurately repair DNA breaks (*85*). Alternatively, break induced replication (BIR) utilizes Rad52, other Rad proteins, and DNA polymerases in utilizing another homologous sequence as a template for synthesis of lost regions of DNA (Figure 1.1-2) (*86*). Unlike other DSB repair mechanisms discussed here, BIR occurs when only a single end is available for recombination. It typically results in a non-reciprocal duplication of sequence, often copying all the way to a telomere (Figure 1.1-2C). It also, unusually, utilizes conservative rather than semi-conservative DNA synthesis, and has a highly elevated point mutation rate REFS. Another method of recombination present in yeast is the single strand annealing (SSA) pathway which utilizes nearby direct repeats as substrates for recombination. This process doesn't utilize *Rad51*, which functions in strand invasion, as complementary single strand sequences on the two ends are able to anneal together using Rad52 alone (Figure 1.1-3)(*76*). These three recombinational processes all depend on *Rad52* and supporting elements of other *RAD* proteins and promote successful maintenance of unstable chromosomes by repairing damaged sequence. Rad proteins sometimes can

also function outside of DNA repair. For example, *RAD55* activity can function in signaling arrest of the cell cycle as part of a checkpoint (*1*).

SDSA, SSA and BIR can be beneficial to damaged regions of DNA, however, they may also act in a destabilizing manner by modifying repetitive regions or providing a manner for sequence amplification/deletion. Common DNA structures such as LTRs, tandem duplications and repeat elements, which are prevalent in eukaryotic genomes, can act as templates for recombination proteins. Recombination proteins interact with the DNA structure and with each other to promote recognition of homologous sites between sister chromatids, homologs or unrelated chromosomes sharing some homologous DNA sequence (*24, 87-89*). Recombination at these sites can sometimes cause expansion of repeated sequence or deletion of the sequence if repeats are found in a direct orientation (*90, 91*). Given the propensity for recombination to take place at repeat regions, it is possible that genes may be included in the intervening sequence, and thereby be amplified or deleted. This places significant amounts of stress on yeast cells, as they often respond to gene duplications through rapid mutation or removal of the second gene copy (*91*). This activity can be co-opted into a beneficial evolution of the duplication following mutation, if the gene is transcriptionally silenced, which may occur at high frequency in regions where gene duplication is common. This allows for the potential sub-functionalization or neo-functionalization of duplicated sequences which then may act to select against future recombination between the duplicated regions (*91*).

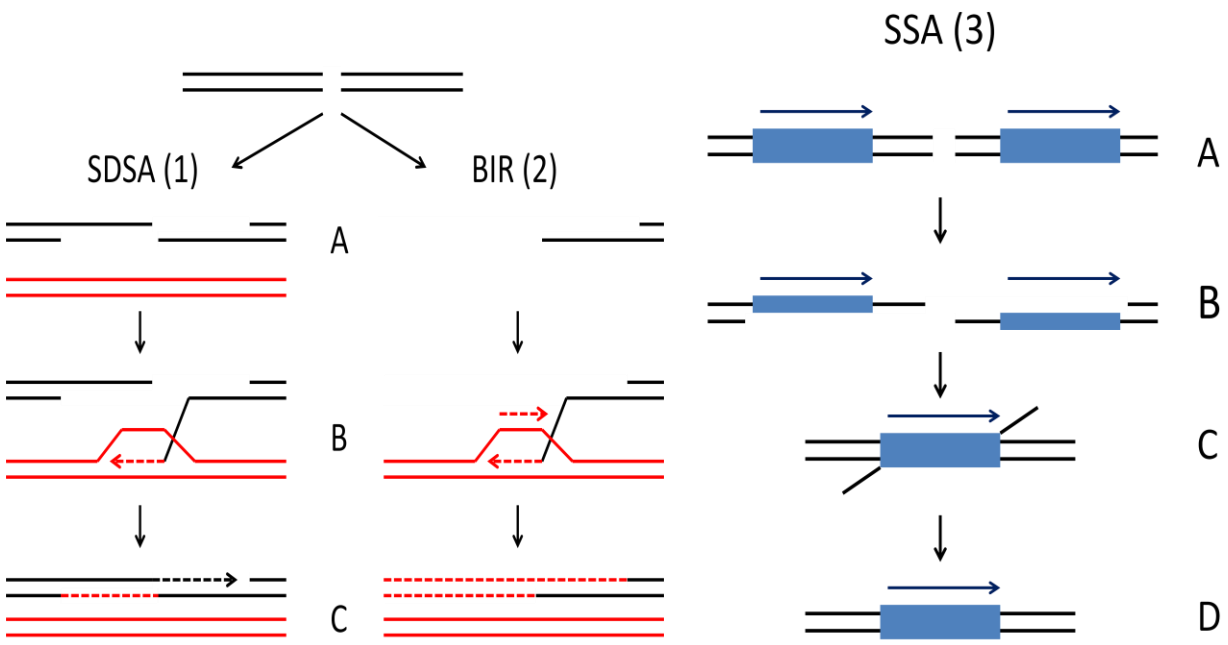

Figure 1.1 Modes of double strand break repair using recombination. Following a double strand break (DSB) both Synthesis Dependent Strand Annealing (SDSA) and Break Induced Replication (BIR) can be utilized to repair the break. If the break is flanked by two direct repeats flank a DSB, Single Strand Annealing (SSA) may take place as well. SDSA (1) proceeds through resectioning of the 5' ends (A) followed by strand invasion of a homologous sequence on another chromosome and DNA synthesis, (B) unwinding of the invaded end(s), annealing of the two ends gap-filling DNA synthesis and lastly, ligation break (C). BIR (2) proceeds with a single end of the original chromosome following 5' resectioning (A) with strand invasion of a homologous region on another chromosome followed by DNA synthesis, potentially all the way to the end of the chromosome (B) resulting in duplication of the copied chromosome and loss of native sequence (C). SSA (3) proceeds following a DSB between two direct repeats (A) followed by 5' resectioning exposing single strand sequence of the repeats (B), subsequent annealing and ligation of the single strand repeats (C) resulting in deletion of sequence between the repeats and leaving a single copy of the original repeats remaining (D).

Telomere and chromosome structure

DNA can be packaged into in two primary chromosome formats: circular and linear. Circular chromosomes are present in most prokaryotes and present a simplified method of replication and gene orientations when compared to linear chromosomes. Circular chromosomes often utilize a single origin of replication (*Ori)* which initiates bi-directionally and culminates at a terminus region where replication fork movement is actively arrested, thereby preventing replication (*92*). Highly transcribed genes on circular chromosomes are preferentially oriented in the same direction as replication (*92*). Additionally, complete circular chromosomes do not have any natural DNA ends that might have the potential to illicit DNA damage checkpoints or other DNA damage responses. Circular chromosomes present a key advantage in that their lack of telomeres permits a simpler mechanism for complete replication. Complications arising from replication termination and separation of newly synthesized chromosomes which are attached at the terminus region are resolved using topoisomerases and site-specific recombination systems to decatenate and resolve dimers of the two sister chromosomes and allow for cell cycle progression and complete replication (*92*). However, as circular chromosomes form dimers from crossovers, they would appear poorly suited to function during the meiotic recombination that drives genetic diversity in eukaryotes.

Contrasting the simpler nature of circular chromosomes, linear chromosomes are beguiled by difficulties in replication due to the end replication problem and require protection from double-strand break repair mechanisms. They also have multiple origins of replication (in eukaryotes) perhaps due to their often larger size. Although prokaryotes such as *Streptomyces sp.* and *Borellia burgdorferi*, a well studied spirochete, can sometimes have linear chromosomes, the focus of this brief overview will be on eukaryotic linear chromosomes due to the relevance of the studies herein (*93, 94*). The structure of linear chromosomes in eukaryotes is complex with various proteins utilized in the formation of

chromatin and other DNA/protein complexes. Linear chromosomes contain centromeres, essential DNA/protein complexes that associate with the cellular spindle during cell division and promote faithful segregation of chromosomal material (*95*). These complexes utilize species-specific DNA sequences and large repeats subject to heterochromatic silencing to recruit centromeric proteins which in turn form a large kinetochore complex which attaches to the mitotic spindle (*96*). Linear chromosomes often utilize multiple origins of replication which do not all initiate in concert and require higher levels of regulation based on the cell cycle in order to prevent inappropriate re-replication of DNA (*97*).

The ends of linear chromosomes demonstrate the most significant difference in structure resolution at the end of replication in comparison to circular chromosomes. They are, by nature, double-strand ends which can potentially appear as DNA damage and trigger checkpoints and attempted repair – often by ligation to other chromosome ends, to the detriment of the cell (*98*). Additional issues with linear chromosomes arise during replication. Whereas circular chromosomes can complete replication in its entirety as they can remove any RNA primers and fill in DNA sequence utilizing sequence from the 3' end of the adjacent 5' side Okazaki fragment, linear chromosomes do not have any DNA adjacent to one side of their ends. The inability of replicative DNA polymerases to be able to fully replicate DNA ends, following the removal of the RNA primer at the 5' strand end of the chromosome end, causes the loss of a small amount of DNA sequence following each replication of the chromosome (*98*). Thus, preventing natural ends from resembling double-strand breaks and preventing, or compensating for, the inevitable sequence loss from the end replication problem are crucial factors in the structure and evolution of linear chromosomes (*93, 99, 100*). Organisms have evolved different ways to produce these protective effects ranging from covalent protein attachment to chromosome ends, conserved sequence structures, secondary structures and RNA association. These end structures that exist at the end of all linear chromosomes have been termed telomeres (*93, 99, 101-107*).

Telomeres are critically important DNA/protein features of linear chromosomes which protect chromosome ends from degradation, prevent their recognition as DNA damage points, prevent terminal sequence loss, and promote chromosomal stability. Telomeres are made of repeating elements in eukaryotes which act as binding sites for a complex set of proteins which provide protection against degradation, telomere length regulation, and prevention of inappropriate DSB repair. They may also promote secondary features of telomeres such as t-loops, recombination, and subtelomeric gene silencing (*61, 101, 108-113*). These telomeric protein structures have been demonstrated to play roles in genetic disease, cancer and aging in higher eukaryotes and are vital to successful growth in all eukaryotes (*56, 59, 60, 65, 114-116*).

Linear chromosomes and telomeres in prokaryotes

Prokaryotes are ancient organisms which have existed and been subject to evolution longer than eukaryotic organisms. Some prokaryotes contain non-circular chromosomes such as the soil bacterial family *Streptomyces sp.* and the causative agent of Lyme's disease *Borellia burgdorferi.* Even some bacterial phages such as N-15 have linear genomes that require the function of telomeres (*93, 94, 117*). There are two known forms of prokaryotic telomeres: AT-rich inverted repeats which form hairpins and another structure which has both inverted repeats as well as an associated protein to form the telomere (*118*). *B. burgdorferi* and N-15, as well as members of the eukaryotic *Varicella* viruses, utilize AT-rich inverted repeats at the ends of their chromosomes which allow chromosomes and plasmids to form concatemers between hairpin/repeat regions to allow for complete replication of the chromosome (*118, 119*). Interestingly, these structures are also found within some eukaryotic mitochondria as well as some eukaryotic extra-chromosomal DNA structures (*118*). The prokaryotic telomeric structure using terminal proteins associated with inverted repeats is utilized by *Streptomyces sp.* and is of particular interest because it perhaps more closely resembles eukaryotic telomeres; not just

a DNA sequence, but a DNA/protein complex (*108, 118*). *Streptomyces* utilizes its telomeric sequences differently than eukaryotes, as the telomere-associated terminal protein binds a nucleotide and that functions as a primer for DNA synthesis (*118*). These two different methods of end protection of linear chromosomes in prokaryotes require specific proteins to assist in stability and unwinding following replication. *Varicella* viruses utilize a topoisomerase to properly resolve telomeric structures following replication and subsequent formation of cruciform DNA, unwinding DNA through cleavage which results in two stable linear progeny with independent prokaryotic-like telomeres (*120, 121*). *B. burgdorferi* and phage N-15 both utilize a resolvase protein (*ResT* and *TelN* respectively) to perform a unique combination of DNA cuts to resolve the telomeres and generate their hairpin structures (*118, 122*).

Distinct prokaryotic telomeres and their maintenance present an interesting point in studying linear chromosome ends: what is required to generate such structures? Although eukaryote telomeres have been studied in great detail and their multiple modes of replication and elongation are comparatively well understood, how prokaryotic telomeric sequence arose and is maintained has been more difficult to elucidate. Although the exact cause behind the generation of telomeres is uncertain, its purpose to protect the ends is clear – as it appears that all linear DNA (eukaryotic, prokaryotic and viral) requires some form of end protection. Prokaryotes and viruses appear to have co-opted a series of processes, primarily replication of the genome, possibly using concatemers, to synthesize their telomeric sequence. The complex structures associated with hairpins appear to be maintained by the proteins similar to those which resolve cruciform structures through direct cleavage or recombination and separate newly synthesized dimeric chromosomes. Alternatively, proteins may play the role in initiating DNA synthesis that accounts for short stretches of replication at the telomere. Although these two methods of maintenance are not conserved in eukaryotic chromosome ends, the utilization of repeats, proteins and recombination to maintain or resolve telomeres is conserved while the manner in which they are utilized and their functions are highly divergent.

Telomeres in eukaryotes

Contrasting with prokaryotic genomes, eukaryotic chromosomes are almost exclusively linear, and thereby the requirement for telomeric protection of chromosome ends is important. The commonality of telomeres among eukaryotic genomes also presents an opportunity for observation of a diverse range of methods by which they are generated and maintained as well as the differences between the telomeric proteins in well studied organisms such as *H. sapiens, S. cerevisiae, Drosophila melanogaster* , *K. lactis* and *C. albicans*. In subsequent sections I will review details about the function of telomeres in linear chromosomes; their associated proteins and functions along with the idea of capping; telomeric repair and recombination; telomeric dynamics and finally the attributes of telomeres specific to the study organisms *K. lactis* and *C. albicans*.

Typical telomeric DNA consists of tandemly repeated TG-rich repeats, usually between 5-8 bp in length, with the 5'-3' strand running toward the end having a G-bias (*123*). The sequence of repeats is usually conserved within a species, with human telomeres using TTAGGG repeats, *S. cerevisiae* using $TG_{1\text{-}3}$ repeats, *K. lactis* a unique 25 base pair repeat (GGTATGTGGTGTACGGATTTGATTA) and *C. albicans* using a 23 base pair repeat (ACGGATGTCTAACTTCTTGGTGT) (*124-126*). Telomeric sequence exists as both double stranded (centromere proximal) and single stranded (distal) G-strand regions which have unique proteins and secondary characteristics which include stably maintained 3' overhangs (*127*). The telomeric repeats are maintained by the reverse transcriptase telomerase in human stem and germ-line cells as well as yeasts and the great majority of other eukaryotes. However, in humans and many other mammals, telomerase activity is often absent in somatic cells (*128, 129*). The repeating structure of telomeric DNA is a key factor in their association with telomeric proteins, as each repeat, or pair of repeats, function as binding sites for telomeric proteins. Which proteins bind depends on whether it is single stranded or double stranded telomeric DNA.

Although telomeric sequences in different yeasts vary, the proteins associated with them are often well conserved. Double stranded telomere repeats are directly bound by Rap1, inhibits telomere resection by exonucleases and blocks telomere-telomere fusions through prevention of the non-homologous end joining pathway (NHEJ) from acting on telomeres (*123, 130*). Additionally, Rap1 acts as a recruiter for telomeric proteins Rif1 and Rif2 and the chromatin silencing mediators Sir3 and Sir4 which promote heterochromatin formation into neighboring subtelomeric sequence (*123*). Single strand telomeric repeats are bound by Cdc13, which recruits Ten1 and Stn1 (*123*). As a complex, these proteins regulate telomere length, prevent recognition of the telomere as a DNA break, recruit telomerase, and recruit DNA polymerase α for synthesis of the second telomeric strand (*123, 130, 131*). The DNA damage associated proteins Ku70/80 are also present at telomeric double strand/single strand junctions and helps regulate proper C-strand resectioning (*132*). The C-strand resectioning results in G-strand overhangs of about 14 nucleotides in yeast, although this is believed to increase during S-phase to upwards of 100 nucleotides (*132*). This may also induce a t-loop structure where the G-strand overhand strand invades double stranded telomeric as a protective measure, as occurs in telomeres in humans. The telomeric protein assortment at *S. cerevisiae* telomeres also appears to apply to *K. lactis* and *C. albicans* with the notable exception of Rif2 which has not been detected and *C. albicans* and with *Cdc13* being a heterodimer instead of a monomer (*123, 127*).

Telomere proteins provide a capping function for telomeric ends of linear chromosomes, shielding them from degradation, aberrant repair mechanisms and recognition as DSBs. Single strand DNA telomere binding proteins play a pivotal role in this by masking the DNA sequence and competing for single strand binding with RPA, a signal for DNA damage and initiator of repair mechanisms (*133*). These telomere binding proteins may also help prevent the formation of complex secondary structures known as G-quadruplexes (*124*). Cellular recognition of telomeric length seems primarily to be related to amounts of telomere-associated proteins, not necessarily the physical length of the sequence per se.

This introduces another potential issue regarding telomere capping proteins: whether mutation or dysfunction affecting telomeric proteins can illicit DNA damage checkpoints without physical disruption of the DNA sequence, instead by disruption through modification of telomeric proteins causing misregulation of one or more telomeric function. In *K. lactis* and *S. cerevisiae*, modification of telomeric proteins can modulate telomeric length to a significant degree, implying great importance in the proper function of telomeric proteins (*134-137*). The importance of capping can be understood in looking at how telomere length is regulated by telomeric proteins and their interactions with two primary forms of telomere length change: telomerase and recombination.

Telomeres length in yeast is highly regulated and deviations from a normal length range are often quickly remedied using different means to shorten or lengthen telomeric sequence. Yeast telomere elongation occurs primarily by the reverse transcriptase telomerase which is a complex of Est1, Est2, Est3 and TLC1 (TER1 in *K. lactis*) (*138*). Telomerase interacts with both Cdc13 (recruitment) and Rif1/2 (negative regulators) to properly modulate telomere length during S phase following DNA replication of telomeric sequence. Telomerase's ability to add telomeric repeats to telomeres is highly regulated. Repeats are added at a higher rate at shorter telomeres as telomerase is more often recruited to shortened telomeres compared to those of normal or longer than normal lengths. The sensitivity to telomere length is believed to be related to the number of repeats bound by Rap1 and subsequent Rif 1/2 accumulation which negatively regulates telomerase activity (*123*). A protein believed to contribute to negative regulation of telomerase is Pif1, a helicase that is capable of removing telomerase from telomeric sequences (*139*). An RNA product of telomere transcription, TERRA, is another means of telomere length regulation which is independent of telomerase activity (*140-142*). The production of TERRA is believed to disrupt telomeric proteins, specifically Ku70/80 which are known to protect telomeric sequences from exonucleolytic degradation, potentially exposing telomeres to these forces. TERRA has not been described in either *K. lactis* or *C. albicans*.

Telomeric recombination and Alternative Lengthening of Telomeres (ALT)

Telomeres are preferentially maintained by telomerase in concert with, and under regulation of, telomeric proteins Rap1, Rif1/2, Cdc13, Stn1, and Ten1 in yeast. There are also recombination proteins of the Rad52 family, Mre11 complex, Ku70/80 and multiple topoisomerases that associate with telomeric sequences. In the absence of telomerase in *K. lactis* and *S. cerevisiae*, growth senescence occurs and most cells die. However, there are a small proportion of cells that survive this senescence (*143-145*). These post-senescent survivors utilize recombination to generate telomeres longer than the critically short lengths associated with senescence (*143, 145, 146*). This method of telomere rescue is also common in the up to 10% of cancer cells, which are telomerase negative, where recombination produces long and heterogeneous length telomeres (*126*).

It is notable that the yeast *C. albicans*, which utilizes its *Rap1* to modulate telomeric recombination that occurs even in cells with telomerase, does not undergo cellular senescence in the absence of telomerase. This presumably occurs due to very high rates of recombination at telomeres, possibly all sizes (*147*). Interestingly, *C. albicans* appears to normally require the recombination proteins Ku70/80 and Rad52 in order to properly regulate telomere length, with abnormally long telomeres occurring in *rad52*Δ mutants (*147, 148*). This unique telomere dynamic may make *C. albicans*' telomeres highly resistant to significantly deleterious effects to disruptions affecting either single one of its maintenance pathways.

Telomeric recombination can play an important role as another means of maintaining proper telomere length in cells with telomerase. Telomeres can be subject to telomere rapid deletion (TRD) events in which single stranded telomeric ends may initiate intramolecular strand invasion and recombination resolution which results in the loss of a significant portion of the end of the telomere (*149*). This appears to more directly affect elongated telomeres, and can produce shortened or normal

length telomeres rapidly (*149, 150*). Though first described in *S. cerevisiae*, TRD has been shown to also occur in *K. lactis* where it appears to influence telomeric repeat turnover even in wild type cells (*150*). TRD is a mostly Rad52-dependent process that is sensitive to the Hpr1 recombination associated protein, utilized in intra-chromatid excision (*149*). In *S. cerevisiae*, TRD events are highly regulated and specific, preferentially shortening elongated telomeres under the regulation of the G-strand ssDNA binding protein Cdc13 and the telomere-associated Sir3 (*149*). Telomeric recombination resulting from t-loop formation may generate extra-chromosomal telomeric circles from the elongated telomeres. Such t-circles can be utilized as templates for telomere elongation in *K. lactis* cells lacking telomerase (*151-154*). In this method of Rad52-dependent extension of telomere sequence, the extra chromosomal telomeric circles can be double or single stranded DNA and presumably act as a template for DNA polymerase to utilize in order to rapidly extend the G-strand of the telomere (*153*). Once a telomere is extended using this process, the lengthened sequence can be spread through break-induced replication events, to other short telomeres in process known as Roll and Spread (*153, 155, 156*).

Break induced replication (BIR) events are believed to be occasional forms of recombination which occur at telomeres and their adjoining sequences that can act at both collapsed replication forks and in telomere maintenance/rescue. BIR is unique compared to other recombination events and can be utilized to maintained telomeres. It requires all three replicative DNA polymerases as well as the associated replication proteins including the Mcm2-7 helicase, Cdc45, GINS complex and (*157*). BIR events utilize homologous regions on other chromosomes and, through using Rad51-dependent strand invasion, generates a D-loop that can, after a delay, initiate a DNA replication fork (*157, 158*). There are also BIR events which are capable of strand invading homologous sequences without the use of Rad51 at specific sites along the genome, some of which allow for up to 150 kb of DNA sequence to be duplicated (*158*). This latter process utilizes additional members of the Rad family including Rad54*,* Rad59*,* and MRX (*158*). Although there are multiple pathways by which BIR can be initiated, they all can

generate replication forks capable of extending from the DSB all the way to the end of the invaded chromosome, duplicating an arm of the template chromosome and repairing the original breakage. BIR events at telomeres can duplicate the length of neighboring telomeres, and, as previously mentioned, can act in concert with a roll and spread mechanism to spread the sequence of an elongated telomere to other telomeres in *K. lactis* cells lacking telomerase. If this process initiates in genic regions, the genes present will be duplicated or deleted, a potent generator of rapid duplications or deletions. Although it is possible for BIR events to initiate a great distance away from chromosome ends, the loss or duplication of a significant amount of sequence, often precludes this event from occurring or generating viable products (*158*). Therefore, it is likely that BIR events in normal cells are enriched in regions including or adjacent to the telomere.

BIR-like events are of great interest in cancer studies, both as a general mechanism of genetic instability, and also as they relate to a pathway of telomerase independent telomere length maintenance and extension known as ALT (alternative lengthening of telomeres) (*159*). ALT extension of telomeres may involve BIR-like strand invasion and duplication of adjacent telomeres, potentially from non-homologous chromosomes or even from intramolecular recombination. ALT cancers are resistant to telomerase inhibiting therapies, as they typically lack telomerase and solely extend their telomeres through recombination. The recombinational extension of telomeres in ALT cancers may also help contribute to increased mutations and heterochromatin variation at regions adjacent to telomeres (*160, 161*). Although the ALT pathways are not as well studied or understood as yeast BIR events, the likely similarities in the mechanisms are of note in relating yeast telomeric behaviors to cancer biology and the arising awareness of telomere's importance in cancer cell immortality. (*159*)

Homologous recombination is inhibited by telomeric proteins at fully functional telomeres, but is able to act on telomeres that become critically short. In *K. lactis*, telomeres can become capable of

recombining once they shorten to ~100 bp in length, a size still long enough to be resistant to NHEJ (*156*). While telomeres may be susceptible to homologous recombination, they are also subject to Ku70/80-mediated NHEJ, which attempts to repair DNA breaks by preventing resectioning and joining two breaks products. This process is effective if the two ends of the DSB are joined, but if NHEJ acts on telomeric ends it may join them together to form a telomere-telomere fusion, thereby initiating a Bridge-Fusion-Bridge cycle, as previously covered. The constant threat of action from these forces on telomeric sequence highlights the importance of telomeric proteins in protecting the ends of chromosomes from double strand break repair (*162*).

The recombinational processes discussed thus far detail mitotic recombination events in yeast cells, however, meiotic recombination is a more studied and often better understood form of recombination. Meiotic crossovers are often initiated by Spo11-generated DSBs at specific sites across the chromosome prior to 5' end resection, strand invasion, and crossing over (*163*). Telomeres play an important role in meiotic recombination by forming directed clusters known as bouquets, which may facilitate sister chromatid crossovers by maintaining close physical distances between them (*164*). Although they play a role in assisting crossovers by stimulating the homolog pairing process, the telomeres themselves do not contain *Spo11* sites and are not likely initiation points for meiotic recombination. This may prevent unwanted crossover events between non-homologous chromosome ends being initiated by homologous sequence shared between different chromosome ends (*165*).

Functional telomere complexes inhibit homologous recombination among normal length telomeres as part of promoting genomic stability (*166, 167*). The increase in recombination when telomeres become critically short suggests that there is a correlation between telomeric stability and subtelomeric recombination rates (*56, 146*). Modification of telomeric proteins, resulting in variation from normal lengths (elongation or critical shortening) can drastically increase recombination rates

within the subtelomere, while not affecting non-subtelomeric recombination rates (*168*). Therefore, modifications in telomere lengths are expected to play a significant role in both subtelomeric transcriptional repression and in modulating telomeric and subtelomeric recombination rates. This link between subtelomeric recombination and telomeric stability has the ability to modulate the behavior and adaptive potential of genic regions near to telomeres (*169*).

Subtelomeres: Broadly defined regions near the ends of chromosomes

Subtelomeres are the chromosomal regions adjacent to telomeres. They have a variety of unusual characteristics including being rich in repetitive sequences, being populated with genes and gene families that allow growth in niche conditions (hereafter referred to as contingency genes) and having elevated mutation and mitotic recombination rates (*24, 170-176*). Subtelomeric genes are also commonly subject to epigenetic regulation in the form of transcriptional silencing (*170, 177, 178*). Although their specific gene content and homology organization differ dramatically between organisms, their basic features are often similar across vastly divergent species from *Saccharomyces cerevisiae* to *Trypanosoma brucei* to *Homo sapiens* (Figure 1.2) (*171, 174, 179-184*). This begs the questions, why are these features so commonly seen and what implications might they have for evolution, adaptation and species divergence?

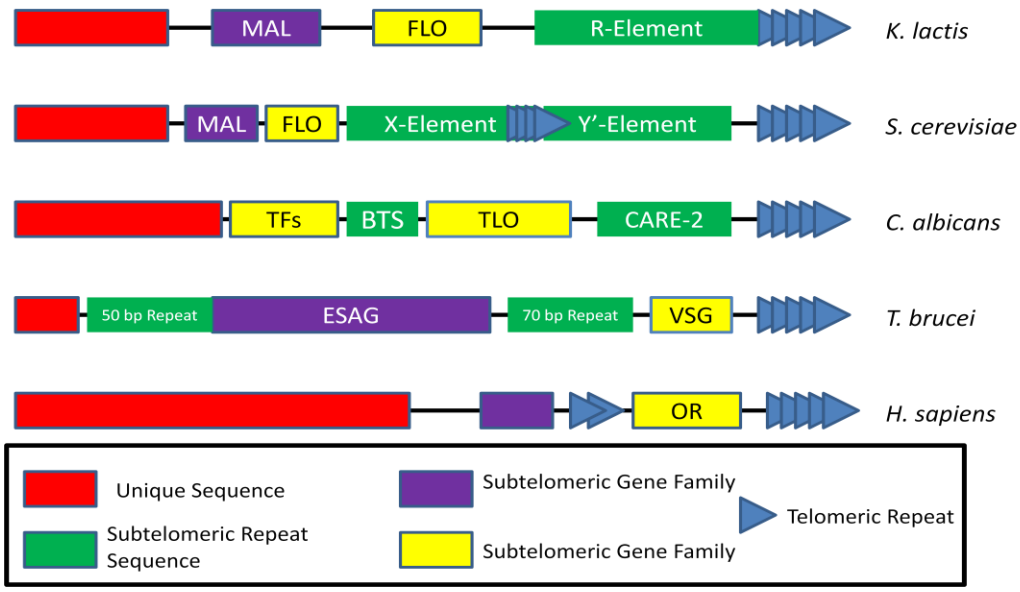

Figure 1.2. Illustration of shared subtelomeric structure across diverse species. Subtelomeres in a wide range of species often possess common sequence elements with shared homology among multiple chromosome ends specific to their species (green). Additionally they often have species-specific subtelomeric gene families (purple, yellow), which may act as points of homologous recombination between subtelomeres. Subtelomeres sometimes also contain interstitial telomeric repeats (blue). Outside of the subtelomere is sequence unique to the chromosome (red).

Subtelomeric sequence falls under the purview of many different genetic forces due to their presence adjacent to telomeres. In *S. cerevisiae*, the Rap1 telomere binding proteins recruits Sir2-4 to the telomere which in turn generate a heterochromatic-like state that can spread into subtelomeric regions (*185*). The Ku 70/80 DNA repair proteins also bind to telomeres and influence the spread of heterochromatin into adjacent subtelomeric sequences (*137*). Telomeric heterochromatin formation is brought about by Sir2 recruitment and subsequence de-acetylation of histone H3, a modification which can extend up to 8 kb into the adjacent subtelomere (*186*). Subtelomeric silencing is diminished when

the adjacent telomere is shortened, and can be extended further in the presence of long telomeric sequence, potentially due to changes in associated protein concentrations (*178*). Spreading of heterochromatin into subtelomeric sequence is regulated in *S. cerevisiae* by X and Y' elements, two conserved subtelomeric elements which are present within most subtelomeres. These elements buffer the spread of heterochromatin, with the X elements acting as anti-silencers (*175, 187*). This may suggest the potential for other organisms to regulate the occurrence and spread subtelomeric heterochromatin, albeit using different means (*188*). The presence of silencing regulators within subtelomeric sequences provides an organism with a controlled location in which changes to the sequence or local genes may go unexpressed and undetected, providing a unique situation for duplications and subsequent mutation to generate diversity. Silenced regions where mutations and duplications go unnoticed may play a role in the tolerance for increased mutation rates in the subtelomere.

Mutations occur at a markedly higher rate within subtelomeric loci when compared to non-subtelomeric loci (*189, 190*). Utilizing a *URA3* marker, the rate of mutational loss due to BIR events at subtelomeric sites <15 kb from *S. cerevisiae* telomeres were greater than 2-fold higher than at non-subtelomeric loci, suggesting a unique behavior of the subtelomeric region (*168*). Furthermore, it was shown that the increase in subtelomeric mutations were amplified by mutation of either the telomere associated Stn1 protein or DNA polymerase alpha (*168*). The combination of increased mutation rates and their sensitivity to dysfunction of a telomeric protein implies that telomeres' influence on their adjacent sequence extends past silencing and includes the fidelity of replication and DNA maintenance near the chromosome ends. Increased subtelomeric mutation rates may also be due to Sir protein interference with repair mechanisms. UV DNA damage repair proteins are unable to properly bind and repair thymine dimers in Sir2-4-silenced subtelomeres leading to increased mutation accumulation (*191*). In addition to base-pair changes, rearrangements of subtelomeric sequence and exchange between chromosome ends has been shown to contribute to diversity within a species (*24, 192*).

Chromosomal rearrangements in yeast occur at higher frequency at subtelomeres when compared to the genome as a whole, potentially modifying gene numbers and spreading subtelomeric repeat elements (*193*). Increased mutation rate, regional instability due to chromosomal rearrangements, and the inefficiency of repair mechanisms in silenced subtelomeric sequence may all contribute to a hostile environment for genes. These factors likely play a role in the general absence of essential genes in the subtelomeric regions, while promoting the presence of genes that may benefit from duplication and rapid diversification due to increased mutation rates.

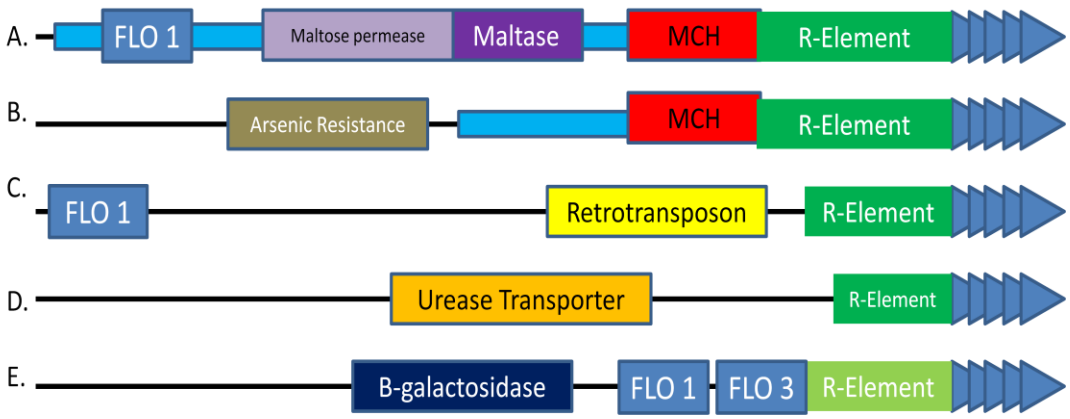

Figure 1.3. Generalization of *K. lactis* subtelomeres including β-galactosidase (*LAC4*), arsenic resistance genes (*ARR*) and flocculation genes (*FLO*), adapted from Nickles et. al 2004. and Fairhead et. al 2006. Illustration of non-genic homology (light blue), R-element (green) and shared genes (other colors) found in subtelomeric sequences of *K. lactis*. A. View of the 2L and 4L chromosome ends, sharing the largest subtelomeric homology (15 kb). B. Illustration of conserved elements across 4R, 5L, 3L, 6L and 1L; arsenic resistance gene ARR is found on three of the 5 subtelomeres. C, D and E represent 1R, 6R and 2R respectively. It is of note that 1R and 6R have diminished R-elements and do not share additional significant sequence homology with other subtelomeric regions. 2R has a degenerate R-element, which may act to prevent BIR events from occurring between itself and other R-elements.

The abundant presence of silencing and contingency genes may suggest that subtelomeric regions are not well suited to essential genes, while also implying that potentially beneficial, but rarely utilized genes, might have less selection against them being located at the subtelomeres (*174*). *Kluyveromyces lactis* subtelomeres contain *ARR* (Arsenic resistance), *MAL* (Maltases), *FLO* (Flocculation) gene families, and an unusual β-galactosidase (*LAC4*), all of which may provide evolutionary benefits when cells are in less than optimal conditions (Figure 1.3) *(88, 171). Saccharomyces cerevisiae* typically also possesses sugar utilization genes such as *SUC* (invertase)*, MAL, MEL* (α-galactosidase), *RTM* (resistance to molasses toxicity) as well as *FLO* genes marking multiple similarities with *K. lactis* (*192, 194, 195*). The pathogenic yeasts *Candida glabrata* and *Candida albicans* both possess gene families that are key aspects to their pathogenicity in regions adjacent to the telomere. *EPA* genes in *C. glabrata* are adhesins, similar to *S. cerevisiae FLO* genes, found in tandem and in inverted duplications at high numbers within 10 kb of a telomere (*196*). In *C. albicans*, the *TLO* genes are a family present at most chromosome ends which has seen the largest copy number expansion compared to its less pathogenic relatives (*197*).

Journeying beyond yeasts, the eukaryotic pathogen *Trypanosoma brucei* utilizes its subtelomeric regions to great effect during host invasion through its *VSG* genes. This immense gene family allows the parasite to evade the human immune system through rapid mutation, formation of chimeras, and recombination that alternates the active VSG with a store of alternatives present at other sites, largely at other subtelomeres (*198, 199*). *Plasmodium falciparum* also contains gene duplications in its subtelomere with 10 copies of an *ACS* (acyl-CoA synthetase) ortholog, diversifying it from other members of the *Plasmodium sp.* (*200*). Even *Homo sapiens* contains regions of shared homology at chromosome ends some of which consist of multiple gene families shared in various copy numbers across different human lineages such as a subset of the *OR* genes, encoding for olfactory receptors (*201*). The similarities in duplications of subtelomeric gene families from divergent organisms

demonstrate the potential for rapid gene diversification and utilization of subtelomeric regions ro enhance genetic diversity. The common usage of subtelomeric regions in both non-invasive and pathogenic eukaryotes, suggest that this region of the chromosome may play an important role in this diversity.

A common theme among subtelomeric gene families is the presence of multiple subtelomeric copies of an ancestral or non-subtelomeric gene, often found interspersed among repeat elements that share homology between multiple non-homologous chromosome ends (*24, 192, 199-203*). Repeated duplication in such varied organisms suggests conserved mechanisms may underlie them. These regions of homology may be conserved elements, such as the R-element in *K. lactis,* which is found adjacent to telomeres, or the subtelomeric Y' and X elements in *S. cerevisiae.* Y' elements may include interspersed telomeric repeats which, like R-elements and X elements, may be sites for inter-chromosomal recombination (*171, 188, 192*). The subtelomeric sequences of *C. albicans* often contain long repeat-rich regions such as LTR graveyards and CARE-2 elements. These sequences and the widespread subtelomeric TLO genes may serve as points of homology that promote subtelomeric recombination (*183, 197*). In some cases, homologous sequences may be more obviously purposeful. The 70 bp repeats found near *T. brucei* subtelomeric VSG genes act as conserved regions of recombination initiation to induce VSG switching (*203, 204*). *H. sapiens* also posses regions of homology as well as degenerate telomeric repeats across their subtelomeric regions (*201*). The conservation of the presence of homology tracts within subtelomeric regions may suggest an important role that they play as mitotic recombination hot spots (*87, 143, 150, 170, 182, 205-207*). Mitotic subtelomeric recombination is influenced by local proteins associated with heterochromatin and the length of adjacent telomeres (*170, 176, 186, 208*). Meiotic recombination in *S. cerevisiae* occurs at a 2-3 fold decreased rate at subtelomere compared to non-subtelomeric loci (*182*). This is especially striking considering mitotic

recombination rates within 200 kb of the telomere occur at a higher overall rate when compared to the chromosome as a whole (*182*).

The Adaptive Telomere Failure hypothesis

DSBs occurring in subtelomeric regions break off acentric fragments with fewer genes than DSBs occurring more internally. This makes subtelomeric regions more intrinsically tolerant of terminal deletions and certain other rearrangements compared to more internal chromosomal locations. This characteristic is further heightened by the absence of essential genes and gene silencing that typifies subtelomeric regions. McEachern has proposed that an organism, when exposed to adverse environmental conditions, may utilize instances of telomere failure, programmed or unprogrammed, to elevate recombination in subtelomeric regions and thereby potentially drive rapid evolution of subtelomeric genes and gene families (*169*). This could include duplication of subtelomeric regions through BIR events, formation of gene family chimeras, increased base change rates, and potentially acquisition of non-native sequence into a subtelomere.

The adaptive telomere failure hypothesis stands on three primary pillars. The first is that subtelomeres are inherently tolerant of loss/duplication. Second, telomere failure or dysfunction can promote recombination that sometimes spreads into and affects subtelomeric DNA. As described in the sections above, these points are clearly true. The third pillar, which is essentially the hypothesis itself, suggests that cells may induce limited telomere dysfunction as a means to produce higher rates of subtelomeric recombination in response to environmental stress, and thereby potentially foster evolutionary change beneficial to overcoming the stress (*169*). However, it has not been demonstrated that subtelomeric recombination increases at a higher rate than non-subtelomeric sites during stress conditions. Testing this idea will be a major focus of this dissertation.

Stress conditions for yeast are highly varied and methods of studying such conditions are diverse, ranging from pulsed stress conditions, to mild stresses invoking minimal growth defects, to conditions which are lethal to the majority of stressed cells. This study utilizes longer term stress conditions that significantly slow growth on solid media and asks whether they illicit a recombinational response. Temperature, osmotic and arsenic stresses have been studied and can be considered potential environmental stresses for yeast (*26*). These stresses were chosen in part for their different methods of affecting cellular growth rates and used to study which stresses alter subtelomeric recombination rates and whether those alterations are specific to subtelomeres or may be genome-wide.

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CHAPTER 2

Subtelomere-specific recombination induced by KCl and sodium arsenate exposure in the yeast *Kluyveromyces lactis*

To be submitted to Journal of Molecular Cell Biology

Abstract

Subtelomeres are rapidly evolving regions that are enriched in genes involved in adaptation to environmental changes. The adaptive telomere failure hypothesis predicts that some organisms may utilize programmed telomere dysfunction in some stressful environments as a mechanism to elevate subtelomeric recombination rates and thereby generate potentially adaptive duplications or modifications of subtelomeric genes. In this study, we utilized a series of *URA3* gene cassettes in the milk yeast *Kluyveromyces lactis* to study recombination rates in subtelomeric and non-subtelomeric loci in cells grown in stressed and unstressed environments. Our results demonstrated that while growth under some stresses led to some elevation recombination rates at both subtelomeric and internal loci, other stresses, most notably growth on sublethal arsenate levels, elevated recombination only near telomeres. We further showed that mutation of the native *LAC4* subtelomeric gene is also elevated by arsenate stress. Interestingly, genes for arsenate resistance are located in two *K. lactis* subtelomeric regions, providing a potential evolutionary rationale for arsenate's effect on subtelomeres.

Introduction

Subtelomeres are from the chromosomal regions adjacent to telomeres. They have a number of unusual characteristics including having high levels of repeats, being enriched for genes allowing adaptation to niches, having increased mutation and mitotic recombination rates, and frequently being subject to epigenetic silencing (Barry, Ginger et al. 2003, Lebrun, Fourel et al. 2003, Ricchetti, Dujon et al. 2003, Fairhead and Dujon 2006, Mak, Pillus et al. 2009, Ng, Cropley et al. 2009, Brown, Murray et al. 2010, Power, Jeffery et al. 2011). Subtelomeres tend to contain few essential genes but are often enriched for genes and gene families involved in adaptation to niche environments, referred to as contingency genes (Mak, Pillus et al. 2009). One example is the variant surface glycoprotein gene family of Trypanosomes, most of the hundreds of copies of which are present near telomeres (Berriman, Hall et al. 2002, Hertz-Fowler, Figueiredo et al. 2008). Switching which gene is expressed combined with frequent recombination between family members provide *T. brucei* with a mechanism to evade killing by the host's immune system (Berriman, Hall et al. 2002, Glover, Alsford et al. 2013). A variety of other eukaryotic pathogens also have gene families associated with pathogenesis located near telomeres (Bain, Stubberfield et al. 2001, Halme, Bumgarner et al. 2004, Castano, Pan et al. 2005, Bethke, Zilversmit et al. 2006). Subtelomeric regions of non-pathogenic eukaryotic microbes are also often enriched in contingency genes. Examples in yeasts include genes encoding extracellular and cell surface proteins, genes involved in the utilization of different sugars, and genes involved in detoxification of arsenic (Nickles and McEachern 2004, Fabre 2005, Fairhead and Dujon 2006)?. Genetic differences in *S. cerevisiae* subtelomeric regions are known to play a disproportionately important role in explaining phenotypic variation between natural strains (Cubillos, Billi et al. 2011).

Although the gene content and homology organization differ between organisms, the basic structure of subtelomeres is similar across species as divergent as *Saccharomyces cerevisiae*, *Trypanosoma brucei* and *Homo sapiens* (Fairhead and Dujon 2006, Rehmeyer, Li et al. 2006, Ambrosini,

Paul et al. 2007, van het Hoog, Rast et al. 2007, Anderson, Song et al. 2008, Barton, Pekosz et al. 2008, Mak, Pillus et al. 2009, Glover, Jun et al. 2011). Subtelomeric regions can be described as having two general domains. The more terminal domain, often gene-free, is often shared among many or most chromosome ends in the same species. The X and Y elements of *Saccharomyces cerevisiae* and the the R element of the milk yeast *Kluyveromyces* lactis are examples of these (Louis and Haber 1992, Nickles and McEachern 2004, Power, Daniel et al. 2011). The more internal domain is larger and contains genic regions that, if duplicated at all, are shared across a smaller number of chromosome ends. Their conservation of general features suggests that subtelomeric evolution and function are subject to influences common to most organisms. Perhaps the simplest and most important of these is that subtelomeric regions are intrinsically more tolerant of terminal deletions and duplication than other chromosomal regions. This arises simply because double strand breaks (DSBs) in subtelomeric regions break off less DNA (and therefore fewer genes) than DSBs located more internally. This tolerance of terminal rearrangements has been proposed to drive the acquisition of other subtelomeric characteristics such as the unusual gene content, rapid evolution, and epigenetic flexibility (McEachern 2008).

The functioning of telomeres is central to subtelomeric and genomic stability. The primary function of telomeres is to protect chromosome ends from being subjected to the double strand break (DSB) repair processes of homologous recombination (HR) and non-homologous end joining (NHEJ) (Louis 1995, Teixeira and Gilson 2005, Bhattacharyya and Lustig 2006). Telomeres subject to NHEJ become fused together, destroying the telomeres and creating dicentric chromosomes that are subject to breakage/fusion cycles that are lethal or detrimental to cells (Liti and Louis 2003, Boukamp, Popp et al. 2005, Almeida and Godinho Ferreira 2013). Telomeres subject to HR typically undergo less drastic alterations. These include telomere truncations and break-induced replication (BIR) events that may lengthen telomeres or replace subtelomeric/telomeric regions of one chromosome end with the

terminal regions of another chromosome end (McEachern and Iyer 2001, McEachern and Haber 2006, Royle, Foxon et al. 2008). Both yeast mutants deleted for telomerase as well as certain human cancers are able to utilize HR as a mechanism to lengthen and indefinitely maintain telomeric repeat arrays at chromosome ends (Dreesen and Cross 2006, Grandin and Charbonneau 2007, Basenko, Cesare et al. 2010, Basenko, Topcu et al. 2011, Bechard, Jamieson et al. 2011).

While severe telomere dysfunction has dramatic and often lethal consequences for cells, more modest perturbations of telomere function can also be consequential. The silencing of subtelomeric genes can be influenced by telomere length (Pryde and Louis 1999, Marvin, Griffin et al. 2009, Ng, Cropley et al. 2009, Decottignies 2014). *K. lactis* mutants with telomeres stable at substantially below-normal lengths can elevate recombination rates in the telomere-adjacent R-elements(Iyer, Chadha et al. 2005). The adaptive telomere failure hypothesis (McEachern 2008) proposes that, in some organisms, the protective capping function of telomeres may have evolved to have an increased failure rate in response to certain environmental stresses. According to this idea, occasional telomere failure would promote homologous recombination events, some of which would recombine subtelomeric gene family members or generate terminal duplications or deletions through break-induced replication (BIR) events. As BIR has recently been shown to be due to highly error prone DNA synthesis (Davis and Symington 2004, Anand, Lovett et al. 2013) elevated telomere failure rates would also be expected to generate high levels of point mutations that were focused in subtelomeric regions. A limited degree of telomere dysfunction induced by certain deleterious environments could therefore be adaptive by specifically mutating regions rich in genes that function in allowing growth in stressful and novel environments. In this study, we tested several environmental stresses for their effects on recombination rates at subtelomeric and non-subtelomeric sites in *K. lactis*. Our results show that at least one stress, growth on sublethal arsenate levels, specifically increased recombination next to telomeres as well as the rate of mutation in a native subtelomeric gene.

Materials and Methods

Strains and growth conditions

All yeast cultures utilized in this study were maintained from a single colony every 3 days and grown at 30°C, unless otherwise noted, on YPD (1% yeast extract, 2% peptone, 2% glucose) medium. Transformants were all generated utilizing K. *lactis* strain 7B250 (*ura3-1 his2-2 trp1*) (Wray, Witte et al. 1987, McEachern and Blackburn 1996). STU transformants STU-6 and STU-9 are *K.* lactis 7B520 derivatives with *URA3* inserted into the subtelomeric R-element as previously described (McEachern and Iyer 2001). GG1958 (*ade2*) which was used as an early strain for stress identification was provided as a gift from Ben Zonneveld.

K. lactis cells from independent single colonies freshly grown at 30°C on SD plates lacking uracil were used to inoculate into the various stress conditions used. Stress conditions using YPD medium supplemented with salts or arsenate or grown at 35°C. Growth under stress conditions was done at 30°C for 5 days. *K. lactis* subtelomeric and non-subtelomeric *HISG-URA3-HISG* constructs were grown for 5 days for YPD, 5 days for 35°C, 5 days for 1.2 M NaCl, 5 days for 1.5 KCl and 5 days for 0.5 mM sodium arsenate. For STU constructs, cells were grown for 2 days on YPD, 4 days at 35°C, 5 days on 1.2 M NaCl, 7 days on 1.5 M KCL, and 7 days on 0.5 mM sodium arsenate prior to assaying recombination.

DNA Isolation from *K. lactis*

DNA isolation was performed on yeast cells grown in liquid culture at 30°C overnight and then spun at 5000 rpm in a microfuge for 2 minutes prior to resuspension in 180 µl SEB/Zymolyase at 37°C for 30 minutes. Cells were then spun at 13,000 rpm for 10 minutes and the supernatant was then transferred to a new microfuge tube. 150 µl 0.5 M EDS (2.5 mM EDTA, 0.1% SDS, 0.125 M NaOH) was added and tubes were incubated at 65°C for 15 minutes followed by the addition of 15 µl 5M CH_3CO_2K

and incubation on ice for 1 hour. Samples were then centrifuged 10 min. at 13,000 rpm with the supernatants transferred to new tubes. 200 µl EtOH was added, mixed and centrifuged at 2,000 rpm for 1 minute, decanted and allowed to dry. DNA was then resuspended in TE (10 mM Tris pH 8, 1 mM EDTA) and 26 µL 10M $(NH_4)_2SO_4$ was added and the tube was incubated for 20 minutes on ice prior to centrifugation at 13,000 rpm for 10 minutes. Supernatants were transferred to new tubes and 150 µl of cold EtOH was added gently to rinse tubes and centrifugation was done again at 2,000 rpm for 1 minute. Supernatants were decanted and DNA was again allowed to dry prior to final resuspension in TE.

Southern hybridization

All Southern blots were done following gel electrophoresis on 0.8% agarose gels as detailed for each batch of isolated DNA. Following DNA electrophoresis gels were blotted using 0.4 M NaOH and Hybond N+ membranes and blotted membranes were immersed in buffer containing 500 mM Na_2HPO_4, 7% SDS, and 5 mM EDS (Gilbert 1984). Membranes were hybridized to probes overnight at 60°C for telomeric probes or 50°C for gene fragment probes. Washing was done with 100 mM Na_2HPO_4 and 2% SDS for gene fragment probes at 45°Cor washed with 200 mM Na_2HPO_4 and 2% SDS at 55°C for telomeric probes. Labeling of telomeric probes was done using T4 kinase (NEB) to label telomeric G-strand oligos with γ-phosphate labeled $AT^{32}P$.

Generation and confirmation of subtelomeric *HISG-URA3-HISG* constructs

Target sites for *K. lactis* subtelomeric *HisG-URA3-HisG* cassette integration were selected by their natural presence of MfeI and NheI sites within 1 kb of each other. These sites were then amplified with 250-400 base pairs of flanking sequence on either side of the targeted restriction sites by PCR using New England Biolabs Taq polymerase (Figure 2.3). The 1R locus had 975 bp between its primers

(CACCTCTGTTAACACCACTTTAC and ATCAGCCTTGTGACACCTC) while the 6R locus had 1373 bp between its primers (CACTTGGTTGTGAAGCATAGAAG and CGGAGTCGCTATGACATGGATGG). The amplified target loci were then integrated into Invitrogen Topo TA plasmids through TA cloning and transformed into *E. coli* DH5α through electroporation. Transformants were grown on ampicillin-LB plates for 2 days and successful integration in Topo TA vectors (creating p1R and p6R) was confirmed through plasmid isolation and EcoRI digestion. The p1R and p6R plasmids were then each digested with NEB MfeI and NheI, treated with recombinant Shrimp Alkali Phosphatase (rSAP) 37°C for 2 hours, and then heat killed at 65°C for 15 minutes.

The *HisG-URA3-HisG* cassette was isolated from the pBS-URA3-1 plasmid (McEachern)). It contained the *S. cerevisiae URA3* flanked by *Salmonella sp. HisG* direct repeats, analogous in structure to the *C. albicans* Ura-Blaster cassette (Wilson, Davis et al. 2000). pBS-URA3-1 was digested with NEB EcoRI and XbaI simultaneously (Figure 2.1) and 3.9 kb *HisG-URA3-HisG* cassette was gel purified prior to ligation into MfeI and NheI digested p1R and p6R. Ligation was performed with NEB T4 ligase at room temperature for 30 minutes, and the resulting ligations were then transformed into *E. coli* DH5α and grown on plates containing ampicillin for 48 hours. p1R and p6R plasmids containing the *HisG-URA3-HisG* inserted cassette (designated pU1R and pU6R respectively) were identified. The plasmids pU1R and pU6R were then digested with BsaXI and transformed into *K. lactis* 7B520 through electroporation and grown on plates of synthetic defined (SD) medium lacking uracil plates for 2 days Isolated colonies were re-streaked onto fresh plates of the same medium prior to growth in liquid YPD for genomic DNA extraction. Confirmation of plasmid integration was done through Southern hybridization of SacI plus BamHI digested DNA with *URA3* and *HISG* probe.

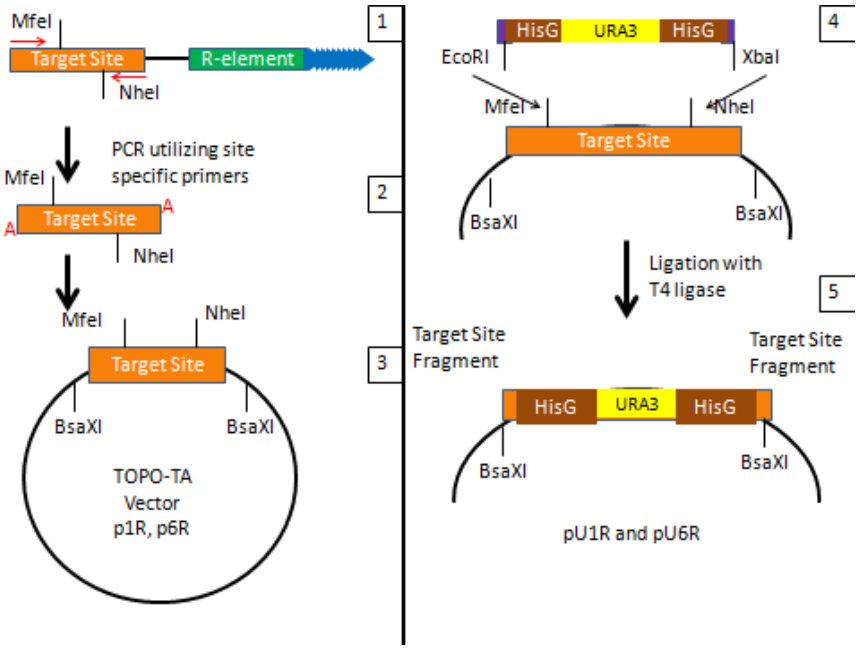


Figure 2.1. Generation of pU1R and pU6R *HisG-URA3-HisG* targeted sites. Construction of *HisG-URA3-HisG* target sites through PCR of target sites (1,2) and incorporation into Topo-TA plasmid (3). Generated plasmids p1R and 16R were digested with MfeI and NheI in conjunction with digestion of *HisG-URA3-HisG* containing pBS-URA3-1 with EcoRI and XbaI (4) prior to ligation (4→5) to generate pU1R and pU6R (5).

Generation and confirmation of non-subtelomeric *HISG-URA3-HISG* constructs

Plasmid pBS-URA-3-1 was digested with EcoRI and SacII, treated with rSAP for 2 hours at 37°C, generating a linear 3.9 kb *HISG-URA3-HISG* fragment with 34 bp of plasmid sequence beyond the *HisG* repeats. The DNA was transformed into *K. lactis* 7B520 through electroporation. And, following two rounds of growth on SD plates lacking uracil, colonies were inoculated into liquid YPD culture for the isolation of genomic DNA. Genomic DNA was digested with EcoRI and electrophoresed before

performing a Southern Blot. Hybridization was done utilizing a URA3 probe and imaged using an Amersham STORM phosphorimager.

Recombination assays

Following growth under stress conditions, colonies of both *HISG-URA3-HISG* constructs and STR constructs were removed from plates individually and in their entirety, suspended in 100 µL of water and 10-fold serially diluted five times in 100 µl aliquots of water. These aliquots were observed under a microscope to confirm that there was not significant aggregation of the cells, potentially reducing the number of cells analyzed. The dilution series were then plated onto both YPD plates containing 0.5 mg/L of 5-fluro-orotic-acid media and synthetic complete medium and then grown for 2 days at 30°C. Colonies were then counted to determine the percent of 5-FOA resistant cells present in the original colonies. Mutation rate estimations were done first by normalizing all mutation frequencies to a total cfu/colony count of 30,000 and establishment of the median number of mutants (m) per culture was calculated using Jones Median Indicator (Jones, Thomas et al. 1994). Following the estimation of median mutants per colony (m) for each condition, the estimation of mutation rate per cell per generation (µ) was calculated by the equation $\mu = m/1.44N_t$ using N_t = 30,000 as a normalized cells per colony size. Significance was determined utilizing adjusted colony frequencies as values for Mann Whitney U Test for p, with $p<0.05$ considered significant.

Confirmation of 5-FOA^R colonies

Following isolation of 5-FOA^R colonies, loss of *URA3* was confirmed through Southern blotting using a *URA3* probe. For RN-HUH 10 of 10 5-FOA^R colonies for the control, 35°C , 1.2 M NaCl and 1.5 M KCl demonstrated loss of the appropriate *URA3* band. For RN-HUH1 0.5 mM arsenate 7 of 10 5-FOA^R colonies had not lost *URA3*, while 3 of 10 RN-HUH7 clones had not lost *URA3* which resulted in a decrease their reported of mutation rates. ST-HUH 5-FOA^R colonies were analyzed for all stress

conditions and 10 out of 10 analyzed colonies had lost *URA3*. STU6 and STU9 5-FOA^R colonies were analyzed for all stress conditions and 5 out of 5 analyzed colonies for each stress demonstrated the loss of the original *URA3* band found in the original clone.

Results

Identification of stresses that significantly inhibit growth of *K. lactis*.

The Adaptive Telomere Failure hypothesis predicts that telomere function in unicellular organisms may partially fail in response to cellular stresses thereby potentially promoting adaptive recombination events in subtelomeric regions. To test this, we chose to examine the effects of salt, temperature and arsenate stress on the frequency of subtelomeric recombination in the yeast *K. lactis*. As a first step, we determined levels of each of these stresses that led to large but not extreme decreases in average colony size of *K. lactis* strain 7B520. Initial screening was done with cells plated onto YPD plates for three days with 31, 33, 35 and 37°C, 0.5 M, 1.0, 1.2 and 1.5 M NaCl, 0.5 mM and 1mM sodium arsenate, and 0.5, 1.0 and 1.5 M KCl utilized as stress ranges. Based on the results of these initial tests, specific stress conditions chosen for further testing were: temperature at 35°C, NaCl at 1.2 M, sodium arsenate at 0.5 mM, and KCl at 1.5 M. Representative colonies grown under these conditions are shown in Figure 2.2 A-D. As indicated in Figure 2.4F, the stresses resulted in reductions in average colony diameter of 54% (35°C), 67% (NaCl), 71% (KCl) and 90% (arsenate).

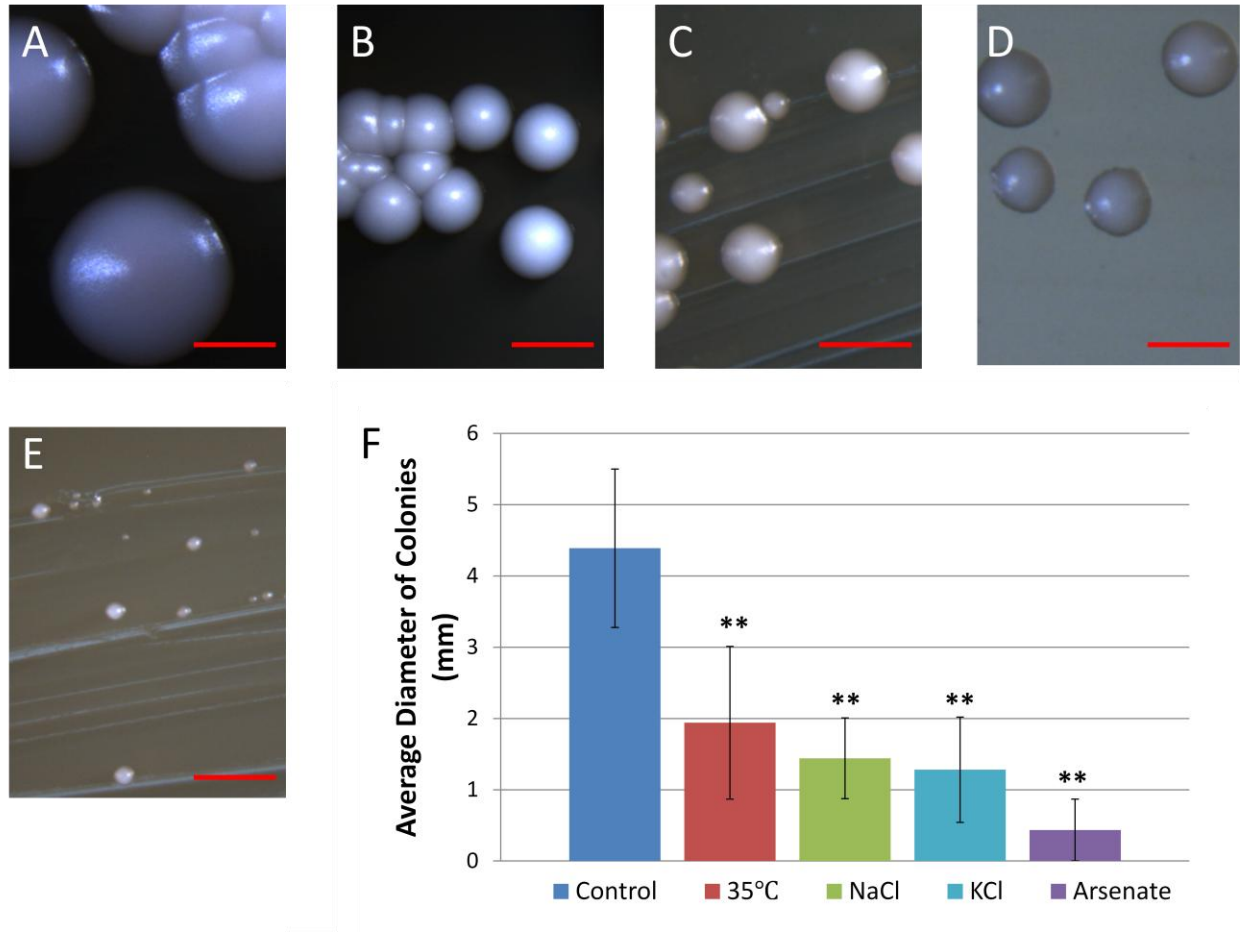


Figure 2.2 Evaluation of *K. lactis* growth inhibition in four stress conditions. Photos showing typical colonies for *K. lactis* 7B520 grown either without stress on YPD at 30° C (A), (B) or grown on YPD at 35°C (B), on YPD containing 1.2 M NaCl at 30°C. (C), on YPD containing 1.5 M KCl at 30°C (D), or on YPD containing 0.5 mM arsenate at 30°C (E). All panels show colonies after five days growth with bar indicating 2 mm (F) Comparison of mean colony diameter for the above stress conditions measured in mm. All stress conditions resulted in significantly smaller average colony diameters with ** = $p<0.01$ (2-tailed Student's T-test). Error bars represent standard error.

NaCl and temperature stresses result in moderate increases in recombination rates at both subtelomeric and non-subtelomeric loci.

To assess recombination rates at subtelomeric and internal locations, two types of *URA3* marker gene constructs were initially utilized. Subtelomeric *URA3* (STU) strains have an *S. cerevisiae URA3* gene inserted into one of the R-elements located immediately next to 11 of 12 telomeres in the parental 7B520 strain (Fig 2.3A). Plating cells onto medium containing 5-fluoro orotic acid (5-FOA) selects for loss of *URA3* function. It has been shown previously that 5-FOA^r derivatives of STU strains have not undergone transcriptional silencing but instead have deleted *URA3* via break induced replication (BIR) events that replace part of the *URA3*-tagged R element with sequence duplicated from one of the other R elements (Iyer, Chadha et al. 2005). These events clearly involve homologous recombination as indicated by their dependency on both *RAD51* and *RAD52* (Natarajan and McEachern 2002). Extensive past work has shown that telomere dysfunction from very short or otherwise abnormal telomeres often results in large increases in loss of *URA3* from STU strains (McEachern and Iyer 2001, Natarajan, Nickles et al. 2006, Xu and McEachern 2012). Two STU clones, designated STU6 and STU9, were used for our recombination assays. STU6 contains *URA3* inserted in the BL (2L) subtelomeric R element and STU9 contains *URA3* in the AR (1R) subtelomeric R element (Nickles and McEachern 2004, Fairhead and Dujon 2006)

To assess recombination rates at internal sites away from telomeres, we utilized *HisG-URA3-HisG* (HUH) cassettes. These contain an *S. cerevisiae URA3* gene located between two *Salmonella HisG* fragments. The HisG repeats are functionally inert in *K. lactis* but homologous recombination between them results in deletion of *URA3* and one of the HisG copies (Figure 2.3B). Genomic insertions of the HUH cassettes were generated by transforming a 3.9 kb fragment containing the cassette into *K. lactis* 7B520. This fragment lacked homology to the *K. lactis* genome and was therefore expected to integrate

randomly via non-homologous end joining (NHEJ), which occurs much more efficiently in *K. lactis* than in *S. cerevisiae*. Twenty Ura$^+$ transformants were examined by and Southern blot analysis utilizing *URA3* and *HisG* sequences as probes and found to contain patterns consistent with cassette integration at various different positions (data not shown). Two of these randomly inserted clones, designated RN-HUH2 and RN-HUH7, were chosen for use in recombination assays. Attempts using inverse PCR to identify the insertion points of these HUH inserts were unsuccessful. However, given the haploid genome of *K. lactis* and the apparently normal growth of both clones, we conclude that neither insertion disrupts the functioning of any vital gene.

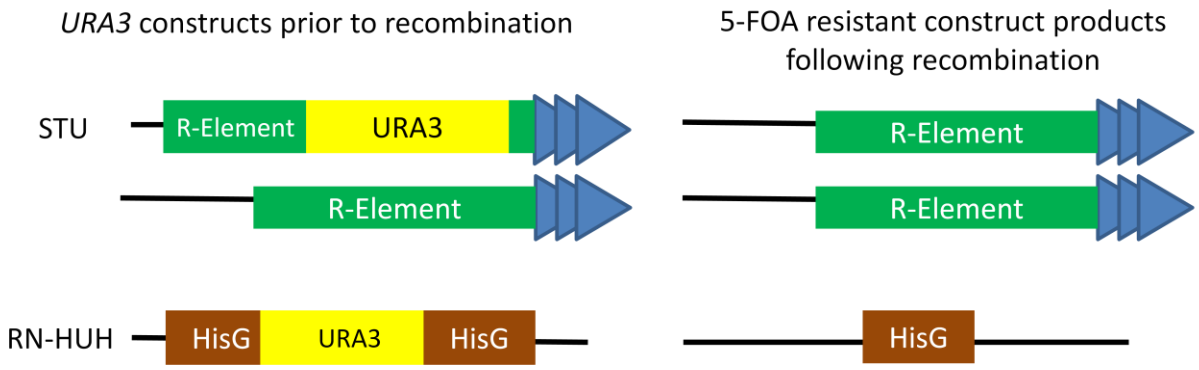


Figure 2.3. Recombination assays utilized in this study. A) Subtelomeric *URA3* (STU) constructs utilize *URA3* inserted into a subtelomeric R-element (present in highly homologous form immediately next to 11 of 12 telomeres in *K. lactis* 7B520) which, following gene conversion with another R-element (likely through BIR), results in the loss of *URA3* and production of a 5-FOA resistant colony. B) HUH cassettes utilize *HisG* direct repeats to promote recombination events resulting in the loop-out of a *URA3* leaving one remaining *HisG* and creating a 5-FOA-resistant colony.

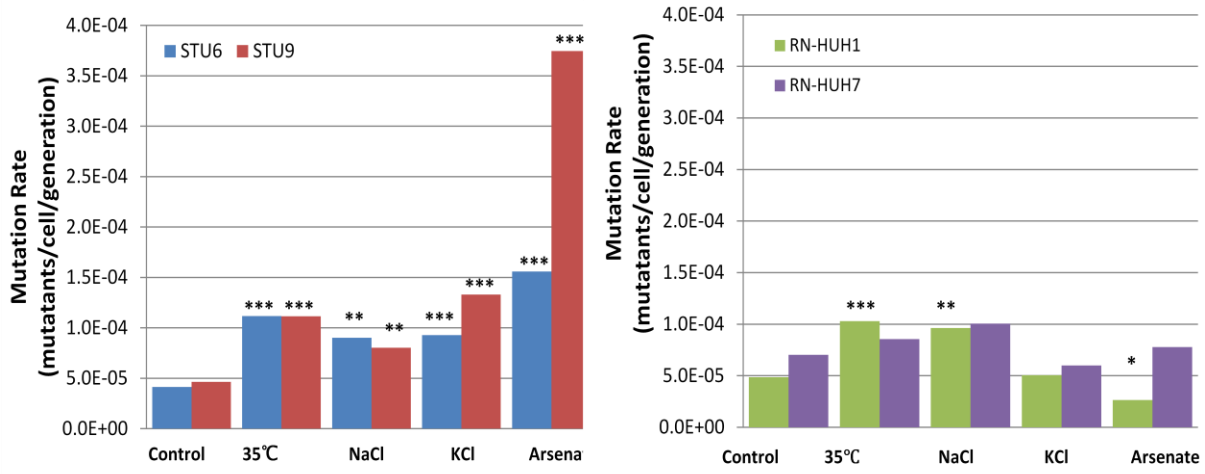
To estimate subtelomeric and internal recombination rates under stress conditions, the two STU and two RN-HUH strains were each streaked onto YPD plates and incubated at either 30° C (no stress

control) or 35°C (high temperature stress) or onto YPD plates containing 1.2 M NaCl. At least 20 individual colonies formed under each condition and were harvested after 3 days (control) or 5 days (35° C and NaCl stresses) and were then serially diluted and plated onto 5-FOA and synthetic defined complete plates in order to determine their rate of *URA3* loss. Confirmation that 5-FOA^R clones incurred the expected deletion of *URA3* was done by Southern blot analysis. 10 5-FOA^R RN-HUH clones were analyzed for the control, 35° C and NaCl stress conditions through Southern analysis. 5 samples for STU6 for each condition and 5 samples for STU9 for each condition were also analyzed by Southern blot, measuring the loss of *URA3* by deletion of the *URA3* band found in the original transformants.

Our results, summarized in Figure 2.4A and with all data points shown in Fig 2.5C and Fig 2.5D, showed that the rate of *URA3* deletion for STU6 and STU9 grown in control unstressed conditions was very similar, 4.1-4.7 x 10^{-5} mutations/cell/generation, respectively. These rates are higher than previously described mutation rates for STU constructs ranging from 1 x 10^{-5} – 6.5 x 10^{-6} mutations/division using Lea Coulson's Method of the Median, comparable to Jones' Median Indicator used here (Rosche and Foster 2000, McEachern and Iyer 2001, Natarajan, Nickles et al. 2006). Our results also showed that the *URA3* deletion rates for STU6 and STU9 cells grown at 35° C were significantly higher each at 1.1 x 10^{-4} ($p<0.001$) mutations/cell/generation. Similarly, the *URA3* deletion rates for STU6 and STU9 cells grown on 1.2 M NaCl were also significantly elevated, experiencing rates of 8.0 - 9.0 x 10^{-5} ($p<0.001$) mutations/cell/generation. These results demonstrate that stressing *K. lactis* with growth at 35°C or on medium with 1.2M NaCl leads to moderate but significant increases in subtelomeric recombination rates.

Cells containing the RN-HUH1 and RN-HUH7 cassette constructs were then used to estimate the effects of the same temperature and NaCl stresses on recombination rates at more chromosome internal positions. Mutation rates for cells grown on non-stressed YPD 30° C conditions for RN-HUH1

and RN-HUH7 were measured at 4.9- and 7 x 10^{-5} mutations/cell/generation, respectively (Fig. 2.4B). Mutation rates for cells grown at 35°C showed an increase to 8.6 x 10-5 – 1.0 x 10^{-4} ($p<0.01$)mutations/cell/generation (Fig. 2.4B). Growth of cells containing RN-HUH1 and RN-HUH7 on 1.2 M NaCl resulted in mutation rates of 9.6 x 10^{-5} and 1 x 10^{-4} ($p<0.05$) mutations/cell/generation, respectively. These results demonstrate that the 35°C and 1.2M NaCl stresses lead to modest elevations of recombination rates at the tested non-subtelomeric locations. We conclude that the 35°C and 1.2M NaCl stresses are likely to increase recombination rates in a non-specific way across the genome.



2.4 Recombination rate increases specifically at subtelomeric loci during KCl and arsenate stress. The rate of mutation resulting from recombination at two subtelomeric (A) STU6 (blue) and STU9 (red) and non-subtelomeric loci (B) RN-HUH1 (green) and RN-HUH7 (purple) for each stress (Left to Right) Control, YPD grown at 35°C, 1.2 M NaCl, 1.5 M KCl and 0.5 mM sodium arsenate. Significance was determined using the Mann-Whitney test for P with * representing $p<0.05$, ** representing $p<0.01$ and *** representing $p<0.001$.

Sodium arsenate and KCl stresses preferentially increases recombination rates at subtelomeric loci.

Our experiments showed that exposure to a 1.5 M KCl stress led to 1.2 and 1.9 fold increases of recombination with the STU6 and STU9 constructs, respectively (Fig. 2.4), consistent with this stress also leading to a modest elevation in subtelomeric recombination. Interestingly, however, there was no increase, or even a trend toward increase, in the recombination rates experienced by the RN-HUH1 or RN-HUH7 constructs. These results suggest that KCl-induced recombination may be limited to regions near telomeres.

Exposure to sodium arsenate stress generated even stronger evidence of subtelomere specific recombination. The mutation rate for RN-HUH1 and RN-HUH7 on arsenate was estimated at 2.7×10^{-5} and 7.77×10^{-5} mutations/cell/generation, respectively. This represented no significant change in recombination rate relative to unstressed controls for RN-HUH7 and, surprisingly, a significant 45% lower recombination rate ($p<0.05$) for arsenate-stressed RN-HUH1. In contrast, both STU constructs exhibited large increases in recombination rates after exposure to arsenate. STU6 showed a 3.7 fold increase in mutation rate in response to arsenate exposure while STU9 showed an 8.1 fold increase ($p<0.001$). These data indicate that the subtelomeric loci experience significantly higher rates of recombination from sodium arsenate stress while non-subtelomeric loci experience no change or even a decrease in recombination rates.

Median mutation rates previously described provide significant insight into recombination rate variations in both subtelomeric and non-subtelomeric loci during normal and stressed growth conditions. Mutation rates obtained by control and stress conditions as previously described were analyzed further by calculating mutation rates for each colony analyzed for each stress conditions and comparing their standard deviations. This allowed for a visual comparison of trends in mutation rates

following stress exposure for RN-HUH1, RN-HUH7, STU6 and STU9. Aside from the readily visible increases in the medians for each stress condition for STU6 and STU9 compared to the relatively minimal variation in medians of RN-HUH1 and RN-HUH7, variation in mutation rates are more obvious.

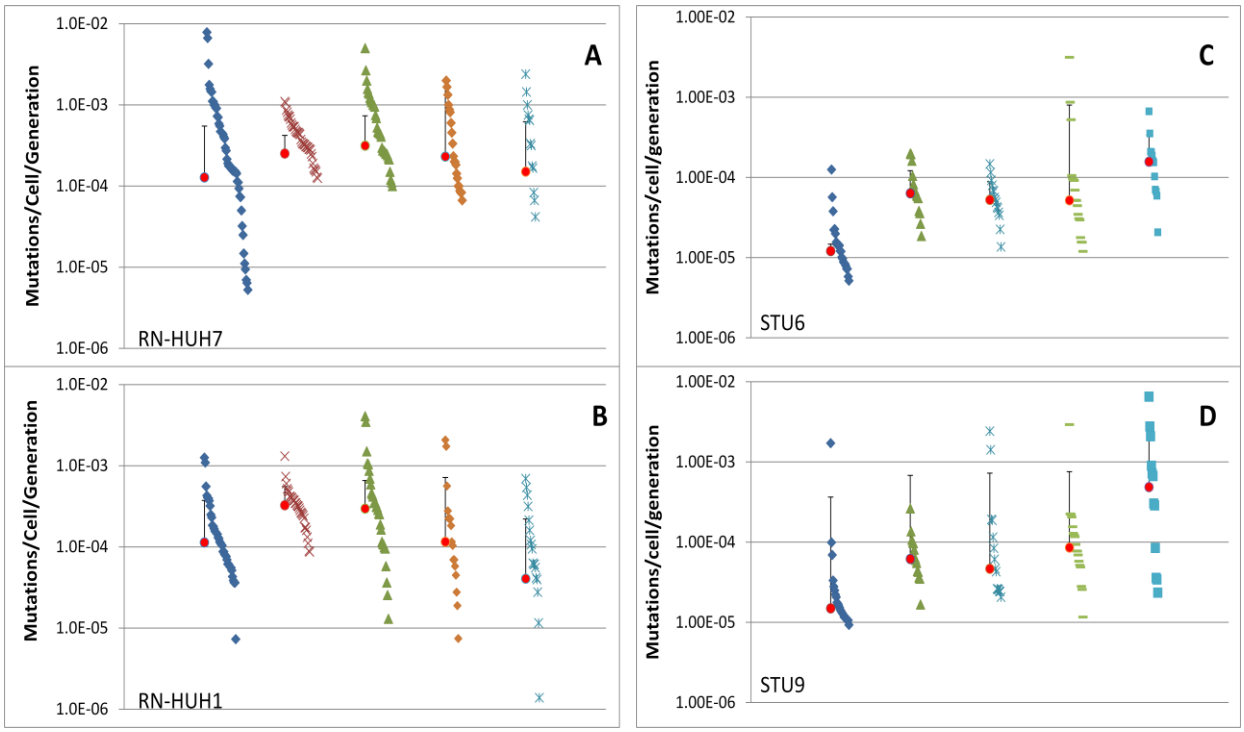


Figure 2.5 Visualization of individual mutation rates generated following stress exposure. Mutation rates for each stress condition (left to right): control, YPD @ 35°C, 1.2 M NaCl, 1.5 M KCl and 0.5 mM arsenate for (A) RN-HUH1, (B) RN-HUH7, (C) STU6 and (D) STU9. 0.5 mM arsenate exposure for panels A and B produce a median which appears skewed to the low end due to the presence of multiple assays where no mutants were observed which are not shown on the graphs.

Recombination at HUH cassettes placed in genic regions of subtelomeres (ST-HUH constructs) largely mirror results with RN-HUH constructs

We next were interested to determine if recombination was also elevated in the genic regions adjacent to the R-elements. As a first test of this, we first constructed two *HisG-URA3-HisG* constructs targeted to subtelomeric loci. As was done for RN-HUH construction, this was done using pBS-URA3-1 inserted into the Topo-TA plasmid containing PCR products of two loci from the of 1R and 6R subtelomeres. This resulted in the HUH cassette being inserted ~5 kb and ~ 3 kb from the 1R and 6R telomeres, respectively, of the 7B520 strain to create ST-HUH1 (1R) and ST-HUH7 (6R). These insert locations were confirmed through Southern blotting (data not shown).

We found that the mutation rates for ST-HUH1 and ST-HUH7 under unstressed conditions were 4.7×10^{-5} and 5.6×10^{-5} mutations/cell/generation, respectively (Fig. 2.6), values very similar to those seen with the RN-HUH constructs. During 35° C and 1.2 M NaCl stresses, recombination showed modest but significant increases with one ST-HUH construct (ST-HUH1 at 35° C) or both ST-HUH constructs (NaCl). In contrast, the 1.5 M KCl stress produced no significant change in recombination rates with either ST-HUH construct while the 0.5 mM arsenate stress showed a significant reduction 43-55% in recombination rates for both ST-HUH constructs. Altogether, these results are very similar to what we observed with the RN-HUH constructs and do not show any subtelomeric increase in recombination for either the KCl or the arsenate stress.

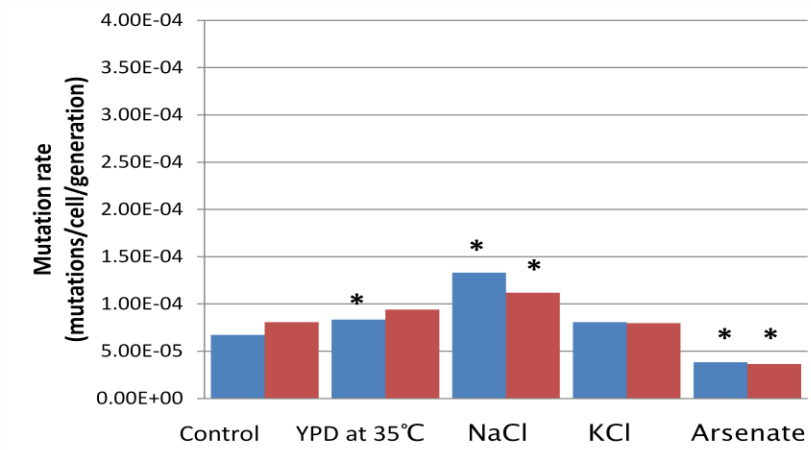


Figure 2.6 HUH constructs located within the genic regions of the subtelomere demonstrate similar behaviors to non-subtelomeric constructs. The rate of mutation resulting from recombination at subtelomeric sites: ST-HUH1 (blue) and ST-HUH7 (red) for each stress (Left to Right) 35°C, 1.2 NaCl, 1.5 KCl and 0.5 sodium arsenate. All * values shown are significant with * = $p<0.05$.

Rate of *β*-galactosidase (*LAC4*) gene loss following exposure to 0.5 mM sodium arsenate suggests that BIR events may provide a better measure of subtelomeric recombination and mutation

K. lactis 7B520 naturally possesses a β-galactosidase gene (*LAC4*) about 8 kb from the 2R telomere. This gene has been shown to be lost at elevated frequency in the presence of telomeric dysfunction, resulting in formation of white colonies on X-gal plates (Olmstead 2012). Analysis of these *LAC4* deletions has implicated BIR events, commonly involving duplication of the 1R telomere, are often the source of these white colonies (Olmstead 2012). We therefore reasoned that if exposure to arsenate caused telomere dysfunction that elevated subtelomeric recombination, it would manifest itself as an increase in the frequency of white colonies on X-gal plates. 5 independent colonies were plated onto 0.5 mM arsenate for seven days prior to dilution plating onto X-gal plates for analysis of β-galactosidase activity. Plates were visualized under magnification and white colonies counted against

total colonies. From a total cell population of 72,000 colonies, 41 isolates appeared white from twenty separate parent colonies. 15 colonies were re-streaked onto X-gal plates and grown for two days to confirm growth as a white colony. Some of these colonies produced white colonies while others generated variations of blue colonies with inconsistent and unpredictable results, potentially mimicking a 'pale blue' phenotype previously described by Olmstead and McEachern.

Further analysis of potential white colonies produced following arsenate exposure demonstrated that a subset of these colonies may be *LAC4* cells which experience mutations or silencing resulting in a white-like phenotype. These colonies produce white sectoring colonies, blue spotted colonies, and dark blue colonies which in turn produce the same range of phenotypes. Further analysis of these diverse colonies demonstrate that the white, blue and dark blue phenotypes are one set of phenotypes while the other differences are a medium sectoring colony and a large (faster growing) colony type (Figure 2.6). More details regarding these alternate morphologies and their potential impacts on *LAC4* expression will be detailed in chapter 3.

Table 2.1. Wild-type *K. lactis* exposed to arsenate result in a large increase in the frequency of white colonies compared to unstressed growth conditions. Wild-type 7B520 colonies (possessing normal telomeres) rarely produce white colonies. In contrast, cells of the short telomere mutant *ter1-28C(Taq)* mutant produce white colonies with notably higher frequency (both values (*) produced by Olmstead and McEachern, manuscript in preparation). Colonies of 7B520 grown on 0.5 mM arsenate for 7 days produce white colonies at a frequency at least 30 fold elevated compared to cells not exposed to arsenate.

Cell Condition	Frequency of White Colonies
WT 7B520	<1.7E-05*
ter1-Taq28C	6.7E-04*
WT 7B520 - arsenate	5.7E-04

Discussion

The work in these studies focused on investigating the impact of stress on subtelomeric recombination in *K. lactis* 7B520. Two different *URA3* gene cassettes, inserted in subtelomeric or chromosome internal locations, were utilized in recombination assays for cells grown under stressful conditions including high temperature (35°C), 1.2 M NaCl, 1.5 M KCl and 0.5 mM arsenate. Our results showed that the NaCl and 35°C stresses led to moderate increases in recombination at both subtelomeric and non-subtelomeric locations. However, the arsenate and, to a lesser degree, the KCl stresses led to increases the rate of subtelomere specific recombination in the STU assays while not increasing, or even decreasing, recombination at internal loci These results argue that some stresses experienced by *K. lactis* cells can preferentially promote recombination within subtelomeric regions, as predicted by the adaptive telomere failure hypothesis.

One question our results raise is why arsenate and KCl raised subtelomeric recombination as measured with STU cassettes at two subtelomeres but failed do so at either subtelomeric ST-HUH cassette. Two factors are likely to explain much, if not all, of this difference. First, the STU cassettes are located within R element sequence less than 150 bp from their respective telomeres while the ST-HUH are located many kbs distant from their respective telomeres. Any type of homologous recombination event stemming from telomere failure is expected to less frequent as a function of distance from that telomere. Second, the background rate of recombination occurring between the two HisG repeats of the HUH cassettes is quite high at all genomic locations and recombination stemming from an additional source such as telomere failure would have to be appreciably more frequent still to be detectable above this background. Although our HUH cassettes detected increased recombination due to certain environmental stresses, these increases were never large relative to the high background of recombination they experience.

Previous work in *K. lactis* has shown that a variety of mutations that perturb telomere function lead to increases in subtelomeric recombination as measured by loss of STU cassettes identical to those used in this study (McEachern and Iyer 2001, Underwood, Carroll et al. 2004, Iyer, Chadha et al. 2005, Carter, Iyer et al. 2007). In extreme cases where there is severe telomere dysfunction accompanied by strongly deleterious consequences to host cell growth, such as in senescing telomerase deletion mutants, STU deletion rates through BIR events may be elevated ~1000 fold or more above rates in wild type cells while loop-out rates from an internal direct repeat cassette similar to the HUH cassettes used in this study showed no increase in recombination (McEachern and Iyer 2001, Iyer, Chadha et al. 2005).Even the much milder telomere dysfunction of telomerase RNA gene template mutant *ter1-28C(Taq)*, that generates telomeres stable at ~1/4 normal length and virtually no cell growth impairment, can lead to ~100 fold elevation in STU deletion rates (McEachern and Iyer 2001).

More recent studies with the *ter1-28C(Taq)* mutant showed that it was prone to a large increase in the rate of deletion of the subtelomeric *LAC4* gene (Olmstead and McEachern, manuscript in preparation). These deletions were accompanied by BIR-like events that copied sequence from another chromosome end onto the truncated 2R end. This evidence shows that even mild telomere dysfunction can have a large effect of recombination rates affecting actual subtelomeric genes in *K. lactis*. The above study also found that *lac4* mutants generated in the presence of severe telomere dysfunction were largely or entirely deletions but that the *lac4* mutants generated in the presence of the mild telomere dysfunction of the *ter1-28C(Taq)* mutant were equal mixes of deletions and point mutations. The point mutations were speculated to come from BIR-like copying of a sister chromatid as DNA synthesis during BIR is known to be highly error prone ((Carr and Lambert 2013). If mild telomere dysfunction caused by an environmental stress such as arsenate also leads to a similar mild telomere dysfunction, we would predict that white colonies formed from arsenate exposure would to due to a mix of *lac4* deletions from BIR events and *lac4* point mutations.

Arsenic is considered a significant human carcinogen (Mahata, Basu et al. 2003, Xiong, He et al. 2012, Chakraborty, Alam et al. 2014). However, arsenicals do not react with DNA and are not considered mutagenic at levels that do not severely affect cell growth (Rossman 2003). The mode of toxicity for arsenic is highly dependent on the type of arsenic compound which is studied. Arsenic compounds have been shown to cause acute toxicity through a very broad set of compounds and pathways including: interactions with sulfur, phosphate, reactive oxygen species, modification of DNA methylation, cell proliferation and signal transduction (Hughes, Beck et al. 2011). Carcinogenesis related to arsenic exposure is related to complications arising from DNA ligase inhibition as well as the promotion of chromosomal aberrations and increased recombination events (Gebel 2001, Ying, Myers et al. 2009, Ali and Ali 2010, Hughes, Beck et al. 2011, Nagai, Davoodi et al. 2011, Litwin, Bocer et al. 2013). In mammalian cells, arsenic specifically triggers degradation of promyelocytic leukaemia (PML) nuclear bodies (Nagai, Davoodi et al. 2011). A subset of PML bodies known as ALT-associated PML bodies (APBs) appear to be sites where telomeres undergo recombination in cancers using alternative lengthening of telomeres (ALT) instead of telomerase to maintain telomeres (Nagai, Davoodi et al. 2011). Any equivalent connection between arsenic and telomere function in *K. lactis* conceivably could promote increases in subtelomeric recombination events, as seen in our studies.

How arsenate specifically increases recombination in subtelomeric regions remains unknown. It is conceivable that it generates DNA damage at specific subtelomeric sites and does not directly affect telomere function per se. An example of loss of telomere function from subtelomeric DNA damage is known in *Trypanosoma brucei*, where subtelomeric double strand breaks trigger recombination that leads to antigenic variation (Glover, Alsford et al. 2013). Alternatively, there might be a mechanism by which arsenate directly affects a telomeric structure or a telomeric protein that then leads to an increase in telomere capping errors. Increasing the frequency of telomere rapid deletion (TRD) events that greatly truncate normal length telomeres is an attractive particular possibility. TRD events appear

to be relatively common in *K. lactis* and are proposed to be an important mechanism that underlies turnover of the outer regions of telomeric repeat arrays (Bechard, Jamieson et al. 2011)). Another key question is whether the recombinational response of subtelomeric DNA to arsenate evolved to be adaptive. Any particular physiological change such as perturbation of telomere function might simply be the accidental byproduct of a particular environmental stresses rather than being an adaptive response. Although we cannot rule this out, it is notable that three genes (*ARR1-3*) encoding proteins encoding resistance to arsenate and arsenite are clustered in *K. lactis* subtelomeric regions. Thus, arsenate's ability to increase subtelomeric recombination could potentially foster adaptive changes directly linked to arsenate resistance. Similar to what has been shown in *S. cerevisiae* and *Cryptococcus neoformans* (Maciaszczyk, Wysocki et al. 2004, Chow, Morrow et al. 2012) increased resistance to arsenate in *K. lactis* is often associated with amplification of the *ARR1-3* genes (Olmstead and McEachern, manuscript in preparation). More work will be needed to determine whether arsenate's effect on *K. lactis* telomeres represents an example of adaptive telomere failure.

LAC4 studies provide further insight into recombination within genic regions of the subtelomere and potential silencing behaviors of the subtelomere. The increased in the frequency of white colonies originating from exposure to arsenate is significantly higher than a non-stressed control. Previously described works utilizing *ter1Δ*-Taq mutants with stably shortened telomeres demonstrate a similar increase in the frequency of white mutants. The similarity between the frequency of *lac4* cells arising from arsenate exposure and previously reported *lac4* frequencies for cells with stably short telomeres suggests that these two results may be due to a similar root cause: shortened telomeres. It is theorized by the Adaptive Telomere Failure hypothesis that subtelomeric recombination rates are influenced by adjacent telomere lengths, and that during stress conditions cells may purposely cause their telomeres to dysfunction to promote subtelomeric recombination. The evidence that genic regions of subtelomeres increase recombination rates during significant stresses, such as arsenate exposure, in a

manner similar to cells with shortened telomeres lends credence to the Adaptive Telomere Failure hypothesis.

Acknowledgements:

This research was in part funded by a generous donation from Mr. Paul Truex and family. I also want to acknowledge undergraduates Bradley Parke and Christopher Walsh who both contributed significantly to the information collected for this chapter.

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CHAPTER 3

Kluyveromyces lactis produces semi-stable smaller colony phenotypes during arsenate stress

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To be submitted to Yeast

Abstract

We report that *Kluyveromyces lactis* cells exposed to a sublethal concentration of arsenate produced colonies mostly composed of cells with heritably altered slower growth rates. At least two slow growing phenotypes were observed, one producing much smaller colonies with a cell division rate double that of normal cells and the second with a more modest growth rate reduction. Both of these altered colony types produced at high frequency more rapidly growing sectors that contained cells with growth rates indistinguishable from the original wild type cells. Cells from the smallest colony form were found to have distinctly higher plating efficiency on plates containing arsenate than either fast-growing cells never exposed to arsenate or fast-growing cells derived from sectors of the slow-growing form. These results indicate that *K. lactis* is able to efficiently and reversibly switch between its normal rapid growth phase and a slower-growing phase with increased resistance to arsenate.

Introduction

Organisms adapt to severe environmental conditions through different means depending on the condition to which the organism was exposed and its impact on the cell. For example, bacteria are known to produce small subsets of cells, known as persistors, which are capable of surviving harsh or bactericidal conditions for an extended period by being in a temporary non-growing state (Michele, Ko et al. 1999, Mukherjee and Chatterji 2008, Lewis 2010). The persistor phenotype is not stable or inherited over multiple generations, as the persisting cells present after exposure to an antibiotic remain as susceptible to killing by the antibiotic as the original cells were, once growth is resumed (Lewis 2010). Persistors have been described in response to a multitude of antibiotics, desiccation, and UV irradiation, suggesting that persistor formation is an effective means for survival under a wide range of stress conditions (Lewis 2010).

Although persistors have not been described in eukaryotes, a similar concept exists. Biofilms are another example of differentiated grown that occurs in response to harsh environments has (Lewis 2008, Cho 2009, Desai, Mitchell et al. 2014). Biofilms are clusters of differentiated cells that express a series of genes which often not expressed in other conditions. These growths are highly resistant to many antibiotics and antifungals. This resistance can arise from the production of a waxy surface layer or from cells in interior layers of the biofilm having reduced exposure to drugs (Ramage, Wickes et al. 2001, Nobile and Mitchell 2006, Verstrepen and Klis 2006). Biofilm-associated cells are similar to persistors in the fact that they grow more slowly and that their resistance to environmental or antimicrobial compounds is fleeting (Verstrepen and Klis 2006, Lewis 2010). Persistors and biofilms demonstrate that epigenetic or alternative activation of transcriptional programs to produce non-growing or alternatively growing states can be a frequent and effective response to environmental stresses for microbes.

Yeast, most notably many strains of *S. cerevisiae*, demonstrate the ability to form an alternative colony form, known as petites, at relative high frequency during normal growth, and the frequency of petite colony formation is amplified by exposure to DNA damaging agents (Chen and Clark-Walker 2000). Petites differ from persistors and biofilms in being caused by irreversible mitochondrial deletions mutations that disrupt oxidative phosphorylation (Chen and Clark-Walker 2000). During certain stresses causing high lethality, the preponderance of petite colonies increases from 1-2% to frequencies sometimes greater than 75%, suggesting that the petite phenotype may be an adaptive response to specific environmental conditions (Davermann, Martinez et al. 2002). Resistance of petite colonies to reactive oxygen species (ROS) and oxidative stress has shown that the malfunction of mitochondrial genes is beneficial during exposure to elements or compounds which cause oxygen related stresses (Davermann, Martinez et al. 2002). This could suggest that petite mutations are maintained in small numbers, much like persistors, as an advantageous variation in cultures to promote survival during ROS exposure. Although irreversible in mitotically dividing cells, the petite phenotype is fully reversible through mating and meiosis. In the pathogenic yeast *Candida glabrata*, the presence of petite colonies generated through mitochondrial DNA deletions has been documented as a method to evade antifungal azole killing through modulation of ABC transporters and a wide range of mutations in mitochondrial DNA (Brun, Berges et al. 2004, Ferrari, Sanguinetti et al. 2011). *C. glabrata* petite isolates were later found to be significantly more virulent than normal growing strains, suggesting that the petite colony form may be advantageous during specific environmental conditions.

Examples in yeast of reversible cell type switching include yeast/hyphal switching and white/opaque switching of *C. albicans*. The rapid shift between yeast and filamentous hyphal growth is a key factor in successful pathogenesis (Jackson, Gamble et al. 2009). White and opaque cells are morphologically distinct yeast cell types with many different properties (Slutsky, Staebell et al. 1987). For example, opaque cells are capable of mating but incapable of producing hyphae whereas white cells

cannot mate but can produce hyphae (Strauss, Michel et al. 2001, Si, Hernday et al. 2013). Another means of rapidly shifting phenotypes in yeast is transcriptional silencing, which can rapidly activate or silence subtelomeric genes (Gotta and Gasser 1996, Pryde and Louis 1999, Martin, Pouchnik et al. 2004, Castano, Pan et al. 2005, Marvin, Becker et al. 2009, Power, Jeffery et al. 2011). Subtelomeric silencing provides means to mask surface antigens during infection, inhibit transcription of unnecessary sugar utilization genes, or allow for expression of key contingency genes during stress conditions (Maciaszczyk, Wysocki et al. 2004, Orlandi, Bettiga et al. 2004, Verstrepen and Klis 2006, Mak, Pillus et al. 2009, Yang, Figueiredo et al. 2009, Power, Jeffery et al. 2011).

Arsenic is a toxin and carcinogen which is widespread in the environment (Vujcic, Shroff et al. 2007, Chakraborty, Alam et al. 2014, Sun, Rathinasabapathi et al. 2014). Arsenic exposure promotes the damaging activities of ROS as well as reducing the production and availability of antioxidant compounds (Acharyya, Deb et al. 2015). Resistance to arsenic in yeasts is provided primarily by inducible genes for an arsenate reductase and an arsenite transporter (Bobrowicz, Wysocki et al. 1997, Maciaszczyk, Wysocki et al. 2004, Vujcic, Shroff et al. 2007). Additionally, petite colonies can exhibit increased arsenic resistance potentially by diffusing the burst of arsenic-related ROS (Day 2013). In this study, we show that the aerobic and petite-negative yeast *Kluyveromyces lactis* is capable of the efficient formation of two reversible small colony phenotypes following exposure to sodium arsenate. At least one of these small colony forms exhibits increased resistance to arsenate.

Materials and Methods

Strains and growth conditions

K. *lactis* strain 7B250 (*ura3-1 his2-2 trp1*) utilized in this study was maintained from a single colony every 3 days and grown at 30°C, unless otherwise noted, on YPD (1% yeast extract, 2% peptone, 2% glucose) medium. *K. lactis* cells from independent single colonies freshly grown at 30°C on SD plates lacking uracil were used to inoculate YPD plates containing 0.5 mM sodium arsenate and grown at 30°C for 7 days. Growth on YPD following arsenate exposure was done for 3 days at 30°C. GG1958 (*ade2*) was provided as a gift from Ben Zonneveld.

Analysis of small colony phenotypes

Following growth on 0.5 mM sodium arsenate for 7 days, colonies were randomly selected and suspended in 100 µL of water prior to four 10-fold serial dilutions. Following dilution, 10 µL of cells were spotted onto YPD and grown at 30℃ for 3 days to allow counting of colonies. For colony size determination, cells were grown overnight, analyzed and sorted according to size, and then returned to 30℃ for further growth before secondary analysis after 2 days growth. Small colonies were confirmed through streaking onto YPD and observation of large sectors (faster growing) which, when streaked onto YPD again, would produce large colonies. Analysis of cell type within small and large colonies was done by randomly selecting either large or small colonies and suspending them in 100µL of water prior to 10-fold dilution four times and plating of 10 µL of the cell suspension onto YPD and grown at 30℃ for 3 days. Significance of size differences in colonies was done using a 2-tailed T-Test, significance established at $p<0.05$.

Results

Semi-stable smaller colony morphologies arise at very high frequency following exposure to 0.5 mM arsenate

Plating of *K. lactis* cells onto YPD plates containing 0.5 mM sodium arsenate leads to a decreased plating efficiency and considerably slowed colony grow (P. Griffith, chapter 2, this dissertation). When cells from arsenate-exposed colonies were serially diluted onto plates lacking arsenate we observed that the resulting colonies after overnight growth were typically in three largely discrete size classes, which we termed small, medium and large (Fig. 3.1A). The large colonies were equivalent in size to those from *K. lactis* cells unexposed to arsenate grown under equivalent time and culture conditions. When several representative examples of each size category were restreaked onto YPD medium without arsenate we observed that the colony size categories were maintained. This indicated that the smaller colony sizes present on the initial plating were heritable and not simply due to temporary toxicity from exposure to arsenate.

To quantitate this colony size alteration, 25 independent *K. lactis* colonies were picked from plates containing 0.5 mM arsenate and diluted and plated on medium without arsenate. The results from this, shown in Fig. 3.1B, indicated that a majority (55-100%) of cells derived from all 25 arsenate-grown colonies produced grew into small or medium sized colonies. All but two of the 25 arsenate-grown colonies produced both small and medium colonies and the two that didn't had no more than 26 total colonies that were scored. Among all colonies scored, medium-sized colonies represented 42% of the total and small-sized colonies represented 35% of the total. Despite the nearly even proportion of small-sized and medium-sized colonies, individual arsenate-grown colonies sometimes produced ratios skewed >10:1 in one direction or the other. Altogether, our results demonstrate that exposure to

arsenate in the conditions we used is highly efficient at generating both the small-sized and medium-sized colony forms.

Restreaked small-sized and medium-sized colonies grown for three revealed that, while colonies derived from the large-sized original colonies produced new colonies that were uniformly round and without sectors, most colonies derived from both the small-sized and the medium-sized original colonies had faster-growing sectors on them (Fig. 3.1C-E). Representative fast-growing sectors their corresponding unsectored colony regions from both small and medium colonies were then restreaked onto YPD lacking arsenate. The results from this showed that the faster-growing sectors from both small and medium colonies uniformly produced large unsectored colonies indistinguishable from colonies of the original *K. lactis* isolate. In contrast, the unsectored regions of small-sized colonies produced new streaks composed of similarly small-sized colonies with faster-growing sectors. Similarly, the unsectored regions of medium-sized colonies produced new streaks composed of medium-sized colonies with faster-growing sectors. These data indicate the small-sized and medium-sized colony phenotypes, though heritable, were not stable and both apparently switched back to the original large colony phenotype.

Although small and large colonies form discrete size morphologies, medium colonies are highly varied and only confirmed through subsequent re-streaking to observe fast growing sectors. Therefore, these studies will focus on well defined small colonies, their frequency and growth behaviors.

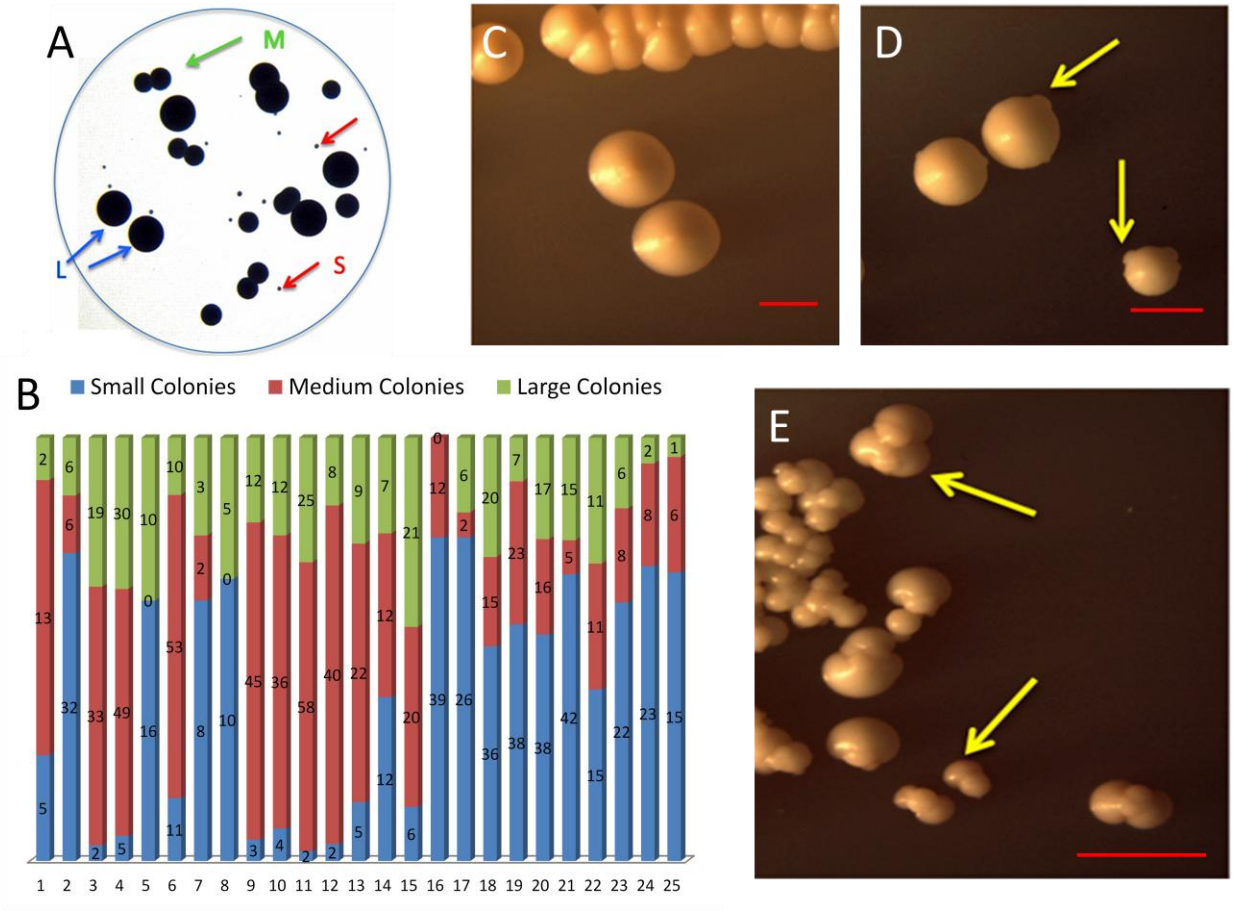

Figure 3.1. Alternative size morphologies spontaneously arise in *K. lactis* following arsenate exposure. (A) Backlit photo of three discrete colony sizes, large (L) medium (M) and small (S), formed after exposure to 0.5 mM arsenate. A colony from a plate containing arsenate was diluted and plated on medium lacking arsenate producing the colonies shown after overnight growth. Width of circle is ~1 cm. (B) Observed frequencies of large, medium and small colonies observed after diluting and plating 25 independent colonies isolated from a plate containing 0.5 mM arsenate.(C) Typical large colonies, lacking any sectors. (D) Medium-sized colonies generate faster growing sectors (yellow arrows). (E) Small colonies generate rapidly growing sectors (yellow arrows) which quickly overtake the colony. Bars in C-E indicate 2 mm. Colonies in C-E were grown for 72 hours prior to observation.

Small colonies have a doubling time approximately twice that of large colonies

We next compared the rate of growth of the small and large colonies. As the rapid switching back to the large colony phenotype precluded meaningful measurements of growth rates in liquid media, we chose instead to compare growth rates of normal and small colony phenotypes by measuring the average colony size at different times after plating on solid YPD medium. As shown in Fig. 3.2, colonies, after 1 day of growth on YPD, demonstrated a significant difference in the size of small and large colonies with small and large colonies measuring an average of 0.041 mm and 0.29 mm, respectively. After 48 hours of growth, the difference in colony sizes increased further with small colonies averaging 0.28 mm and large colonies averaging of 1.42 mm. As the small colonies took 48 hours to reach an average size just below that achieved by the normal large colonies at 24 hours, we conclude that the doubling time of the cells in the small colonies is likely very close to one half that of cells in the large colonies

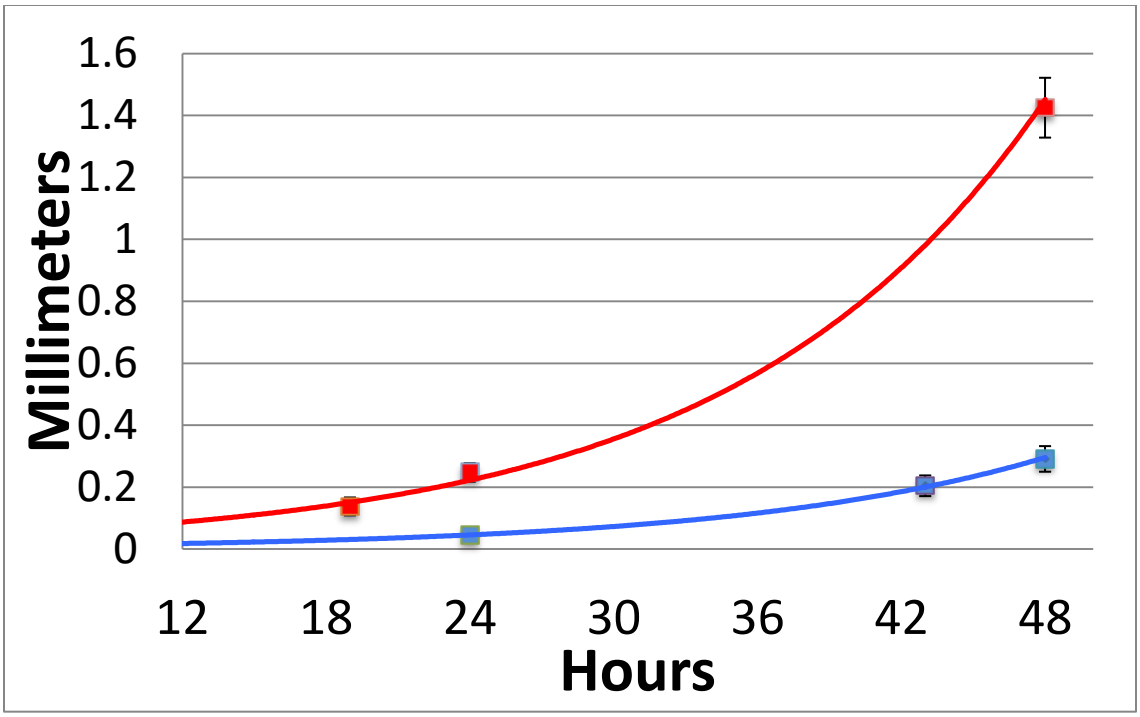

Figure 3.2. The small colonies grow at half the rate of large/normal colonies on YPD. Colonies were plated on YPD and analyzed following 19, 24 and 48 hours for large colonies (red, top line) and 24, 43 and 48 hours for small colonies (blue, bottom) and an exponential line of best fit was added. 24 hour and 48 hour time point differences are significant ($p<0.05$ 2 tailed T-test) between colony types. Error bars represent standard deviations.

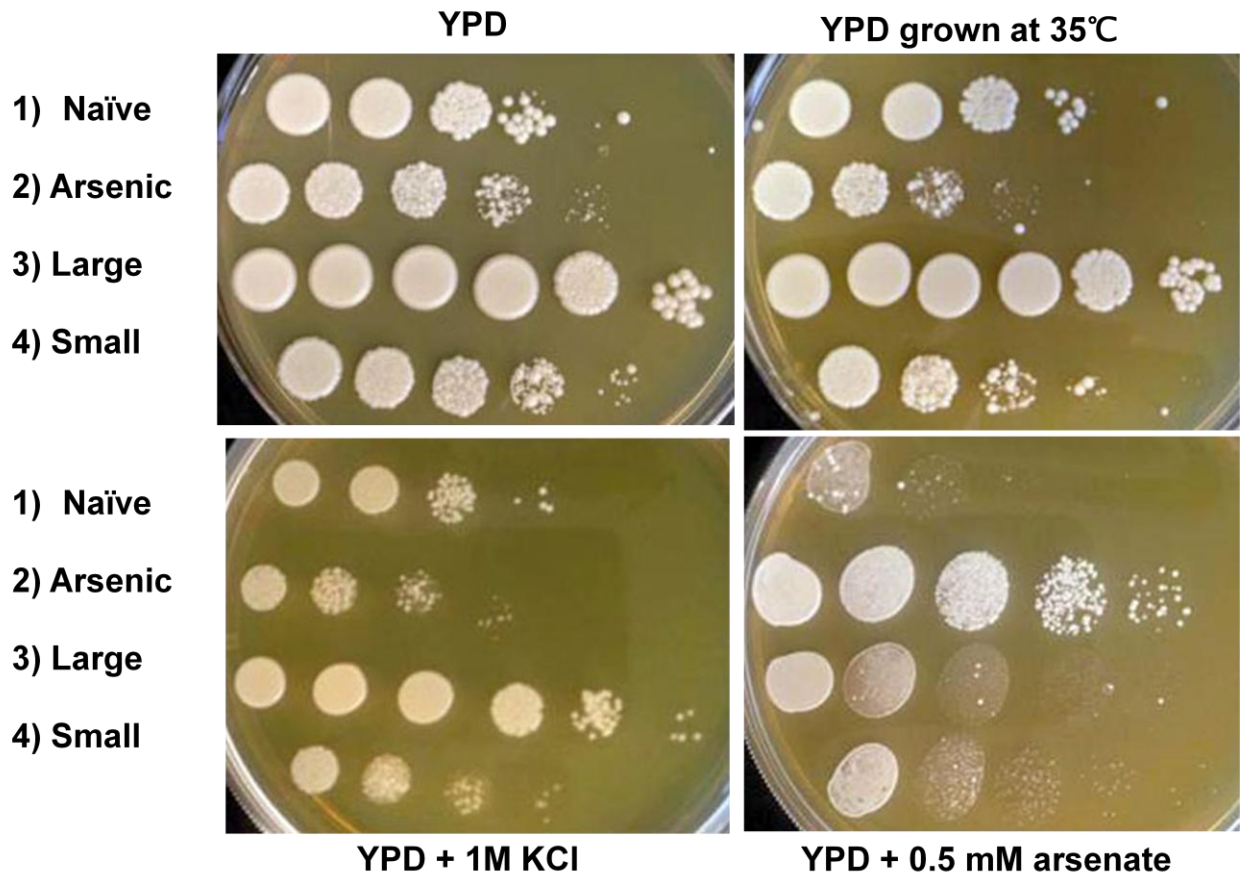

Figure 3.3. Small colonies show increased resistance to arsenate but not to other stresses. Naïve cells (not previously exposed to arsenate), cells grown immediately prior on arsenate), large (derived from arsenate exposure) and small (derived from arsenate exposure) colonies plated onto YPD grown at 30℃, YPD grown at 35℃, YPD with 1.0 M KCl and YPD with 0.5 mM arsenate and grown for 3 days.

The prevalence of the small colony cell type after selection on arsenate suggested that this cell type might have greater resistance to arsenate. To test this, we performed serial dilutions with cells from different cell types and grew them under arsenate, KCl, and heat stresses as well as under unstressed control conditions. The four types of colonies chosen were small and large colonies derived from arsenate exposure but grown immediately prior on YPD without arsenate, a colony isolated

directly from first time exposure to YPD containing 0.5 mM arsenate, and a naïve large 7B520 colony grown on YPD that had no prior history of arsenate exposure. Our results, shown in Fig. 3.3, showed that naive 7B520 experienced a ~50-fold reduction in colony formation following exposure to 0.5 mM sodium arsenate. In contrast, cells from the colony taken from 0.5 mM sodium arsenate showed no reduction in viability upon replating at that arsenate concentration. Cells from the large colony, previously exposed to 0.5 sodium arsenate but grown without arsenate prior to this experiment behaved like naïve cells, showing an overall drop in viability approaching 100-fold. Notably, however, cells from the small colony, though slowly growing, did not show a significant reduction in plating efficiency on 0.5 mM arsenate. This suggests that cells from small colonies have a greater resistance to arsenate than naïve cells or similarly treated cells from large colonies. In contrast, cells from small colonies showed ~10 reduction in plating efficiency when grown at 35° C compared to cells from naïve or large colonies. This indicates that cells from small colonies were actually less heat resistant than naïve cells. Interestingly, cells from a colony taken directly from 0.5 mM sodium arsenate also experienced an ~10-fold decrease in plating efficiency at 35° C. Plating efficiency on 35℃ and 1M KCl stress did not result in any apparent killing of plated cells, while arsenate exposure resulted in a significant number of growing colonies.

***LAC4* can be differentially expressed in sectoring colonies**

Previously described studies have shown that *K. lactis* cells exposed to 0.5 mM arsenate leads to an increased percentage of white colonies on plates containing X-gal apparently due to recombinational deletion or other mutation of the subtelomeric *LAC4* gene (Chapter 2 of this dissertation). Very recent analysis of one such colony, upon restreaking onto YPD X-gal medium without arsenate, produced colonies that were mostly whitish in color but that had blue spots and sectors (Fig 3.4A). This phenotype was maintained upon further streaking (Fig 3.6B). Whether this sectoring phenotype is related to the medium-sized sectoring colonies described above has not yet been determined. These findings,

however, do suggest that alternate colony morphologies may at times also demonstrate different levels of subtelomeric gene expression. Separate tests of normal (large), medium-sized, and small-sized colonies derived from the arsenate-exposed cells described above showed that all had similar blue color on YPD X-gal plates (data not shown).

Faster growing sectors observed on *K. lactis* cells grown on KCl

During the course of earlier work to test the effect of KCl stress on subtelomeric recombination (Chapter 2, this dissertation), we noticed that two colonies out of ~200 of *K. lactis* GG1958 (*ade2*) that had grown on YPD containing 1 M KCl had smaller colonies with numerous sectors along their edges (Figure 3.5A, arrows). Restreaking one of these colonies onto YPD without KCl showed that the resulting colonies typically had one to several faster growing sectors (Figure 3.5B). Restreaking the main body of such colonies produced similarly sectoring colonies (data not shown). However, restreaking from sectors produced somewhat faster-growing colonies that never had sectors, resembling the normal GG1958 phenotype. These results demonstrate that a sectoring phenotype highly reminiscent of the "medium-sized" sectoring colonies seen after arsenate exposure can be seen in another *K. lactis* strain and that they can be produced independently of arsenate. Whether this sectoring phenotype in GG1958 was produced by the KCl stress or not is not known. If so, it must have arisen in the very first cell division after being placed on the medium with KCl in order to produce colonies already uniformly composed of the slower-growing, sectoring phenotype.

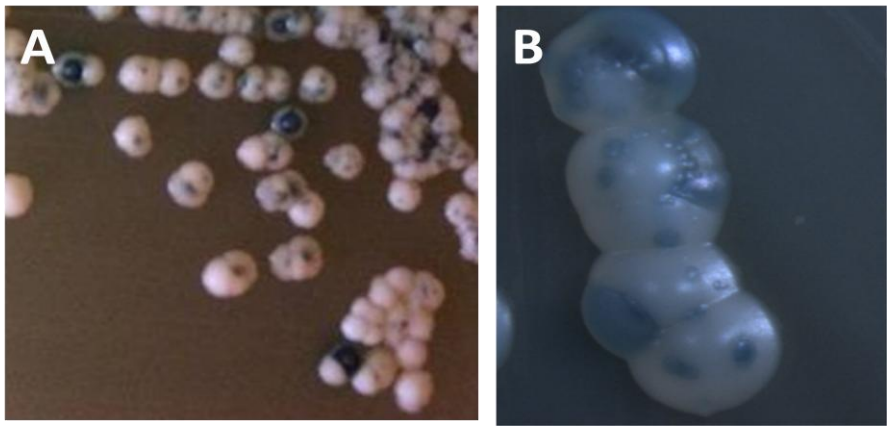

Figure 3.4 Alternate colony size morphologies may experience different transcription levels of subtelomeric *LAC4*. (A) Shown is a photo of a white-appearing colony after being restreaked onto a new YPD X-gal plate. The original colony was isolated from a YPD-X-gal plate after exposure to 0.5 mM arsenate. (B) Close-up photo of colonies restreaked from a single colony of the streak shown in (A).

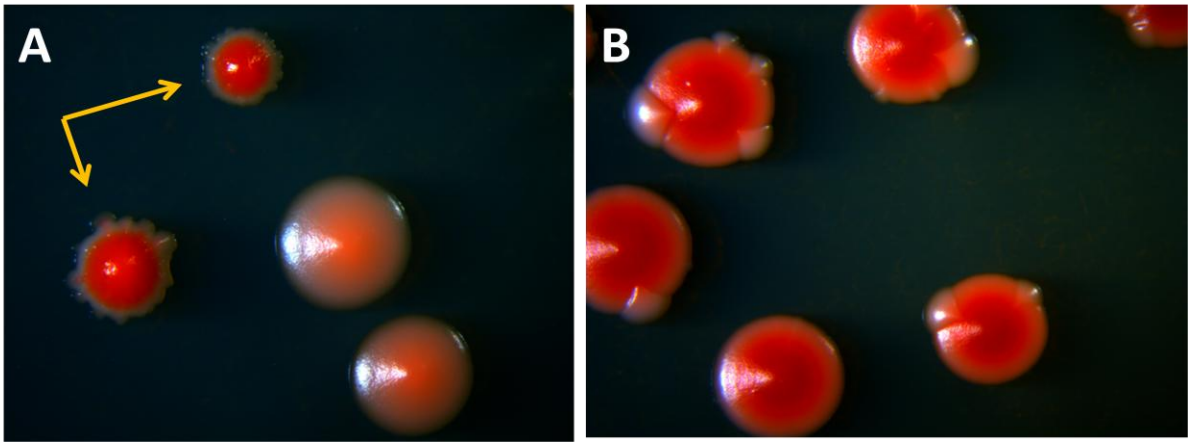

Figure 3.5 *K. lactis* growth rate-switching phenotype isolated from plates containing 1M KCl. (A) Photograph showing two slower growing colonies (marked with arrows) of the red *ade2* strain GG1958 as they were originally found after 3 days growth on a YPD plate containing 1M KCl. Fine sectors are visible along the edges of both colonies. (B) Photos showing restreaks of one of the smaller colonies from (A) onto YPD without KCl. Faster-growing lighter red sectors are plainly visible on most of the resulting colonies.

Discussion

Our results in this work show that at least two different slower-growing and sectoring forms of the yeast *K. lactis* are generated after exposure to arsenate. Both slow-growing forms are able to switch back to the normal faster-growing mode at high frequency. These slow-growing forms constituted 55-100% of the cells of colonies recovered from YPD plates containing 0.5 mM arsenate. The high frequencies in which the slow-growing forms both arise and switch back to normal suggests that the switches are much more likely to be due to epigenetic rather than mutational changes. The basis of the cell type switching behavior in either direction was not explored and remains unknown. The small-sized colonies were directly demonstrated to have a higher plating efficiency of medium containing 0.5 mM sodium arsenate compared to either normal cells not previously exposed to arsenate or to cells produced after the slower-growing forms have sectored back to normal growth rates. Although not tested, we would hypothesize that the medium-sized colonies might also have elevated resistance to arsenate. These observations support the idea that smaller-sized sectoring phenotypes convey resistance to 0.5 mM sodium arsenate. Whether the slower-growing forms are induced to form by arsenic or instead are always present in *K. lactis* cell populations but were selected by growth on arsenate remains uncertain. However, given the apparently high rate of conversion to the slow-growing, sectoring states and our lack of ever noticing such phenotypes in other experiments on media lacking arsenate makes us favor the former possibility.

A small colony phenotype has been associated with resistance to a toxin at least once before in *K. lactis*. Marmiroli et. al demonstrated that *K. lactis* CBS 2359 produced small colony phenotypes following exposure to both erythromycin and chloramphenicol (Marmiroli and Puglisi 1980). This drug resistance was conveyed through modification of the mitochondrial genomes. The antibiotic-resistant small colonies maintained mtDNA, but were sensitive to increased temperatures through reduced

mitochondrial protein function (Marmiroli and Puglisi 1980, Zubko and Zubko 2014). The colonies described by Marmiroli were only present following antibiotic exposure (at frequencies<17% of colonies analyzed) and were stably inherited (Marmiroli and Puglisi 1980).

The petite mutants of some yeasts, including *S. cerevisiae*, are common forms of mutants that lead to small colony size. Petite colonies in *S. cerevisiae* are generated by the deletion of large portions of or complete loss of mitochondrial DNA (mtDNA). Such mutations appear not to be possible in *K. lactis* without two nuclear mutations (Bianchi, Tizzani et al. 1996, ClarkWalker and Chen 1996, Chen and Clark-Walker 2000). Interestingly, studies have demonstrated a significant benefit from the reduced oxidative phosphorylation associated with the loss of mitochondrial function in order to combat ROS bursts, such as those created by arsenic exposure (Chen and Clark-Walker 2000, Aoki, Ito-Kuwa et al. 2005, Day 2013). It is therefore possible to speculate that the slow-growing phenotypes we observed might produce their effects via some alteration in mitochondrial function. However, the high frequency reversibility of the slow-growing colony forms described here argues strongly against them being mtDNA deletions or other mutants. Although some mtDNA defects may be reversible by restoring proper mtDNA copy number (Chen and Clark-Walker 2000, Day 2013), the apparently discrete restoration of normal growth rates from the slower-growing forms in our study tends to argue against that possible mechanism.

A key question about the slow-growing sectoring colony forms we discovered is whether they are a specific adaptive response to arsenate or whether they have a more general adaptive role. Our tests with the small-sized sectoring form showed that it did not convey any obvious selective advantage against stress with high temperature or high KCl. This does not rule out the possibility, however, that the small-sized sectoring form might provide some protection against environmental insults in addition to arsenate. Our observation that a sectoring form very reminiscent of the medium-sized sectoring form

we isolated from arsenate was isolated during an experiment plating *K. lactis* on plates with 1M KCl. Whether this form is identical to the medium-sized sectoring form we isolated from arsenate is not at all certain. However, this result does indicate that arsenate is not absolutely required to generate slower-growing sectoring forms. Other examples of 7B520 derivatives forming slower-growing sectoring colony forms similar to those produced by arsenate have been noted under conditions of selection for ura3 phenotypes on 5-FOA concentrations slightly higher than optimal (M. McEachern, unpublished observations). In some cases, the slower-growing colonies may have been comparable in size to the small-sized colonies described in this work. We conclude that it is likely that *K. lactis* can produce slow-growing alternate colony morphologies under a variety of circumstances and speculate that these may be resistant to a variety of stresses or toxins. It is possible, that the slow-growing forms of *K. lactis* bear some resemblance to bacterial persistors, which achieve temporary resistance to some anti-bacterial compounds by virtue of being in a non-growing state (Lewis 2000).

Acknowledgements:

This research was in part funded by a generous donation from Mr. Paul Truex and family. I also want to acknowledge the Georgia REU program and its two students Shavonte Anderson (2015) and Dominique Lacey (2014) who both contributed to the information collected for this chapter.

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CHAPTER 4

Study of recombination adjacent to a *de novo* telomere affecting the subtelomeric *TLO* family of transcription factors in *Candida albicans*

Not to be submitted: Preliminary body of work

Abstract

Candida albicans is the most prominent pathogenic yeast known to infect humans. *TLO* genes are a subtelomeric family of genes encoding transcriptional activators that influence hyphal growth, cell type switching and sugar utilization. *TLOs* experience drastically higher rates of mutation and recombination compared to other regions of the genome, and this recombination may play a role in the rapid evolution of the family. Here we show that the loss of *URA3* marker genes, presumably from recombination, that are located TLO genes can be affected by growth in certain stress conditions. We also show that subtelomeric recombination near a de novo telomere in the *C. albicans* strain WO-1 A+4 can be greatly affected by growth temperature. These results suggest that functioning of *C. albicans* telomeres to block homologous recombination in subtelomeric regions may be malleable, and responsive to different growth conditions. This may have important consequences with regard to the evolution of *TLO* genes and other genes in *C. albicans* subtelomeres.

Introduction

The *Candida* clade possess multiple members who exist as commensal members of human flora as well as opportunistic pathogens such as *Candida glabrata, Candida dubliniensis* and *Candida albicans* (Barns, Lane et al. 1991, Rossignol, Lechat et al. 2008, McManus and Coleman 2014). *C. glabrata* has been studied extensively as a model organism for yeast pathogenesis due to its relatively simple genetics as a haploid yeast and its relatively close relationship to *S. cerevisiae* (Verstrepen, Reynolds et al. 2004, Ho and Haynes 2015). *C. glabrata* has shown how pathogenic yeast may utilize its subtelomeres to promote diversity of pathogenicity genes through subtelomeric recombination and also shown how subtelomeric genes may be regulated through telomere-associated silencing (Verstrepen and Klis 2006, Rosas-Hernandez, Juarez-Reyes et al. 2008). *C. glabrata* utilizes subtelomeric *EPA* genes, similar to *S. cerevisiae FLO* genes, to encode adhesions required for adherence to human cells and for virulence (Verstrepen and Klis 2006). *C dubliniensis* is another member of this clade, known to cause a small percentage of clinical *Candida* infections. It has been studied in detail due to its close relation with *C. albicans (Padmanabhan, Thakur et al. 2008)*. The two species have few genetic differences, to the point where they are capable of mating in a lab setting (Butler, Rasmussen et al. 2009). The study of *C. dubliniensis* suggested particular genes that may have helped *C. albicans* become a more efficient and potent pathogen (Jackson, Gamble et al. 2009). One of the most striking differences between *C. dubliniensis* and *C. albicans* is the presence of a large family of subtelomeric *TLO* genes in *C. albicans* that exist as only two non-subtelomeric genes in *C. dubliniensis* (Jackson, Gamble et al. 2009). These studies have revealed that *TLO*s are involved in hyphal/filamentous growth, white-opaque switching, biofilm formation and sugar utilization (Anderson 2015).

C. albicans infections often arise as opportunistic invasions of weakened hosts such as immune compromised individuals or through breaks in host defenses such as a break in the skin or modification

of throat or vaginal environments which promote yeast growth over normal bacterial growth. A key factor in successful host invasion is the cell type switching from yeast growth to hyphal/filamentous growth and the formation of drug resistant biofilms (Akins 2005). Although there are multiple genes involved in these pathways, a conserved set of transcription activators, the *TLO* genes, are essential activators of them that act in concert with the Med complex of transcriptional activators in *C albicans* (Anderson 2015). Members of the *TLO* family are found at the ends of almost every chromosome in *C. albicans*, with transcription running from the telomere-proximal promoter towards the centromere, often as the most telomere-proximal gene and the only conserved subtelomeric gene family (van het Hoog, Rast et al. 2007). The subtelomeric regions of *C. albicans* exhibit similar characteristics to other yeasts such as *S. cerevisiae* in that they experience increased rates of mutation, silencing and recombination (Pryde, Gorham et al. 1997). The architecture of *C. albicans* subtelomeres may explain how the subtelomeric presence of *TLO*s in *C. albicans* has led to their rapid expansion and divergence (Anderson, Baller et al. 2012) .

Subtelomeres in *C. albicans* are prone to high levels of rearrangement, which explains the lack of complete and annotated sequence data that provides a consensus of subtelomeres between strains (Sadhu, McEachern et al. 1991, McEachern and Hicks 1993). In one well-documented strain, SC5317, derivatives of which are utilized in this study, the subtelomeres possess three distinct classes of *TLO* genes α, β and γ, which are distinct in their size and subtelomeric localization. These clades are separated by their size and preference for use during subtelomeric recombination events, with TLOα genes presenting a significantly higher rate of subtelomeric recombination than the other two clades combined. Extensive study of the TLO genes has shown that they experience recombinational rearrangements, duplications and mutations at a higher rate than any other region or pathogenicity related gene in the *C. albicans* genome. These rearrangements are likely break-induced replication (BIR)-like events, which cause exchange of TLO genes among subtelomeres as often as one in 5500 cell

divisions (Anderson 2015). Although there is a preference for recombination break points within the 3' end of TLO genes, there are also events which occur at a measurable rate at the 5' end as well as further towards the centromere. The prominent mutational forces acting on this specific gene family suggests that *C. albicans* may be driving the TLO genes in evolutionary directions so as to better adapt it to its surroundings. It is currently unknown if the various TLO genes or clades possess notably different sets of targets, or which targets they affect, but it is reasonable to assume that their modification, duplication and evolution are significant aspects to *C. albicans'* pathogenic prowess.

TLO genes may help provide insight into high rates of subtelomeric recombination in *C. albicans* during the normal growth of the organism. The adaptive telomere failure hypothesis proposes that telomeres and their occasional dysfunction can be important drivers of subtelomeric recombination (McEachern 2008). This suggests that telomere length could potentially be an important regulator of subtelomeric recombination rates. Studies in *Saccharomyces cerevisiae* and *Kluyveromyces lactis* have shown that elongated telomeres can result in high rates of telomere rapid deletion (TRD) events (Lustig and Petes 1986, Bechard, Jamieson et al. 2011). TRD events that deleted sufficient numbers of telomeric repeats potentially could trigger recombination events that, in occasional cases, might alter subtelomeric sequence via deletions or BIR events (Sadhu, McEachern et al. 1991, Singh, Steinberg-Neifach et al. 2002, Iyer, Chadha et al. 2005). Previous work showed that telomeres in the *C. albicans* strain WO-1 increase two to four fold in response to protracted growth at 37℃ and 42℃ compared to cells grown at 30℃ or lower (McEachern and Hicks 1993). This telomere length plasticity suggests that C. albicans may be particularly useful for investigating the potential role of telomere length on subtelomeric recombination.

In this study, we utilized an *ADE2* sequence duplication next to a de novo telomere in *C. albicans* WO-1 to examine the effect of cell growth temperature on subtelomeric recombination rates. We also

utilized a series of *URA3*-tagged *TLO* genes to begin to study the effects of environmental stresses on subtelomeric recombination in *C. albicans*.

Materials and Methods

Strains utilized

Yeast cultures utilized in this study were maintained from a single colony every 3 days and grown at 30℃ unless otherwise noted, on YPD (1% yeast extract, 2% peptone, 2% glucose) medium. *C. albicans* WO-1 A+4(1) and WO-1 A+4(2) were generated previously (McEachern and Hicks 1993). *C. albicans* WO-1 A+4 Alt30 has telomere lengths comparable to typical *C. albicans* telomeres and WO-1 A+4 Alt37 presenting stably longer telomeres (McEachern and Hicks 1993). Additional *C. albicans* strains were obtained from ATCC LifeSciences, its subsidiaries, or previously acquired companies. All *URA3*-tagged strains used in this work were generously provided by Matthew Anderson (Anderson, Baller et al. 2012). Strains were stored at -80℃ and then grown on plates lacking uridine for three days prior to plating on stress media. Positioning of *URA3* for each strain can be found in Table 4.1.

Table 4.1 Identification of *C. albicans* strains and associated TLO *URA3* tags.

Strain Name	TLO and *URA3* location (D-telomere distal or P-telomere proximal)
CA12002	*TLOβ2* (D)
CA12005	*TLOα12* (D)
CA12655	*TLOα9* (P)
CA12657	*TLOγ16* (P)
CA12658	*TLOα12* (P)

C. albicans cells from independent single colonies freshly grown at 30℃ on SD plates lacking uridine were used to inoculate onto the various stress conditions used. *C. albicans* subtelomeric *URA3* and *ADE2* constructs were grown for 5 days on YPD at 30°C as a control. Growth under stress conditions was done using a single colony grown at 30℃ for 5 days for 1.2 M NaCl and 1.5 M KCl, 7 days for 1 mM sodium arsenate and 4 days at 37℃/42℃/50℃ on YPD for temperature stress prior to assaying recombination rates.

DNA isolation was performed on yeast cells grown in liquid culture at 30℃ overnight and then spun at 5000 rpm in a microfuge for 2 minutes prior to resuspension in 360 µl SEB/Zymolyase (3mg/mL) at 37℃ for 1 hour. Cells were then spun at 13,000 rpm for 10 minutes and the supernatant was then transferred to a new microfuge tube. 150 µl EDS (2.5 mM EDTA, 0.1% SDS, 0.125 M NaOH) was added and tubes were incubated at 65℃ for 15 minutes followed by the addition of 15 µl 5M CH_3CO_2K and incubation on ice for 1 hour. Samples were then centrifuged 10 min. at 13,000 rpm with the supernatants transferred to new tubes. 200 µl EtOH was added, mixed and samples were centrifuged at 2,000 rpm for 1 minute, decanted and allowed to dry. DNA was then resuspended in TE (10 mM Tris pH 8, 1 mM EDTA) and 26 µL 10 M $(NH_4)_2SO_4$ was added and the tube was incubated for 20 minutes on ice prior to centrifugation at 13,000 rpm for 10 minutes. Supernatants were transferred to new tubes and 150 µl of cold EtOH was added gently to rinse tubes and centrifugation was done again at 2,000 rpm for 1 minute. Supernatants were decanted and DNA was again allowed to dry prior to final resuspension in TE.

Analysis of telomere length variation

C. albicans strains were streaked from single colonies onto YPD and grown for 2 days prior to experimentation. After two days of growth, isolates from each colony were grown in liquid YPD and DNA isolation was performed on half of a colony. The other half of each colony analyzed was

transferred to three separate plates and incubated at 30℃, 42℃ and 50℃ for three weeks. Every three days colonies were re-streaked onto new media. After three weeks single colonies were isolated from each temperature for each strain studied and DNA isolation was performed. Following DNA isolation, EcoRI digests were performed for 8 hours prior to running the samples on a 0.8% agarose gel for 16 hours at 28V. Gels were treated with 0.5 M HCl and blotted onto Amersham nitrocellulose paper in 0.4 M NaOH. After blotting, DNA was fixed to the nitrocellulose paper by irradiation with UV light for 1 minute. Fixed nitrocellulose was incubated with γ-^{32}P ATP-labeled CA23 repeat (McEachern and Hicks 1993) prior to washing and exposure to Amersham photovoltaic plates, which were imaged using an Amersham STORM scanner.

Analysis of *ade2* sectoring and red/tan colonies following growth.

Cultures of *C. albicans* WO-1 A+4 1, WO-1 A+4 2, WO-1 A+4 Alt30, and WO-1 A+4 Alt37 were grown at 22℃, 30℃ and 37℃ as for three days prior to analysis. Colonies were analyzed by isolating a single colony grown at specified temperatures, suspending it in 100 µL of water and then diluting it 1000x twice prior to plating on YPD. YPD plates were then grown at 30℃ for two days and analyzed. Colonies which appeared red or tan were recognized as an *ade2* colony while the appearance of sectors were counted as colonies which were experiencing sectors due to difficulties in accurately identifying individual *ade2* sectors as they grew at a reduced rate compared to *ADE2* cells.

5-FOA plating of stressed colonies to analyze loss of *URA3.*

Following growth on media, colonies were removed from plates individually and in their entirety. Isolated colonies were suspended in 100 µL of water and 10-fold serially diluted five times in 100 µL aliquots of water. Dilution series were then plated simultaneously onto 0.5 mg/L of 5-fluro-orotic-acid (5-FOA) medium and synthetic complete medium and grown for 2 days at 30℃. Plated colonies were then counted to determine the percent of 5-FOA-resistant cells present in the original

colony. A sebset of 30 5-FOA resistant colonies were re-streaked as individual colonies onto YPD and grown for 2 days prior to plating onto plate lacking uridine. Colonies on plates lacking uridine were grown for three days and growth of isolated colonies was analyzed to check for loss of *URA3*.

Estimation of 5-FOA resistant mutation rate (loss of *URA3)* using total mutants.

Mutation rate estimations were done first by normalizing all mutation frequencies to a total cfu/colony count of 30,000. Following normalization the median number of mutants (m) per culture was calculated using Jones Median Indicator (Rosche and Foster 2000). Jones Median Indicator (JMI) was utilized due to its accuracy above 1.5 mutations per culture and its existence as a static equation, and therefore was utilized in Microsoft Excel. Following the estimation of median mutants per colony (m) for each condition, the estimation of mutation rate per cell per generation (μ) was calculated by the equation $\mu = m/1.44N_t$ using N_t = 15,000 as a normalized cells per colony size. Significance was determined utilizing adjusted colony frequencies as values for Mann-Whitney U Test for p, with $p<0.05$ considered significant.

Results

***C. albicans* strains demonstrate variable length phenotypes in response to prolonged growth at different temperatures.**

The adaptive telomere failure hypothesis predicts that altered telomere function may occur in some circumstances and lead to elevated recombination in subtelomeric regions that could potentially contribute to adaptive change (McEachern 2008). The length of telomeres is an important parameter that can contribute to alterations in telomere function including their abilities to suppress subtelomeric recombination or transcriptionally silence subtelomeric genes (Castano, Pan et al. 2005). Previous work has shown that the WO-1 strain of *C. albicans* and its derivatives developed up to four fold longer

telomeres after two to three weeks of growth at 37° or 42° C, when compared to the same cells grown at room temperature or 30° C (McEachern and Hicks 1993). As long telomeres only very gradually reduce back to their starting shorter lengths, such temperature shifts provide a means of generating genetically identical cells with different telomere lengths. Finding that cells with naturally generated differences in telomere lengths had different rates of subtelomeric recombination could provide evidence suggestive of adaptive telomere failure.

We first chose to examine whether *C. albicans* strains other than WO-1 were capable of generating significantly elongated telomeres when grown at elevated temperatures. To do this, clonal isolates of each of several *C. albicans* strains, including WO-1 and its subtypes A+4(1), A+4(2), were passaged for three weeks by serial streaking on YPD plates and incubated at each of three temperatures, 30°, 37°, and 42° C. After this growth period at the different temperatures, genomic DNAs were isolated, digested with EcoRI, and their telomere lengths were visualized by Southern blotting. The results of this are shown in Figure 4.1. We observed that, as expected, WO-1 and its subtypes A+4(1), A+4(2) all demonstrated elongated telomeres following 3 weeks of growth at 37℃ and 42℃. For WO-1 and A+4(2), the telomeres from 30℃-grown cells were dramatically shorter. Surprisingly, however, for A+4(1) cells, the 30℃-grown cells had telomeres that were only slightly shorter than the telomeres from cells grown at 37℃ and 42℃. Among the other strains examined, some, including ATCC32032, 3153a and B4201 all demonstrated substantial increases in the length of their telomeric sequences in 42℃ and sometimes to 37℃. Strains 3153a and B4201 both showed an increase in their telomere sizes in response to 37℃, although 3153a's increase was significantly less than B4201. Both, however, showed large increases in the sizes of their telomeres in response to 42℃. In contrast, strains ATCC18814 and ATCC32077 showed similar average telomere sizes in cells grown at all three temperatures. Strikingly, ATCC32033 showed a significant drop in the length of its telomeres in response to 42℃ growth conditions. We conclude that increased telomere length is common but not universal among *C. albicans*

strains in response to prolonged growth at higher temperatures. The variation in telomere length behavior between the two A+4 subclones, which should be genetically identical, indicates that telomere lengths in *C. albicans* cells can also be altered by large degrees by factors other than temperature.

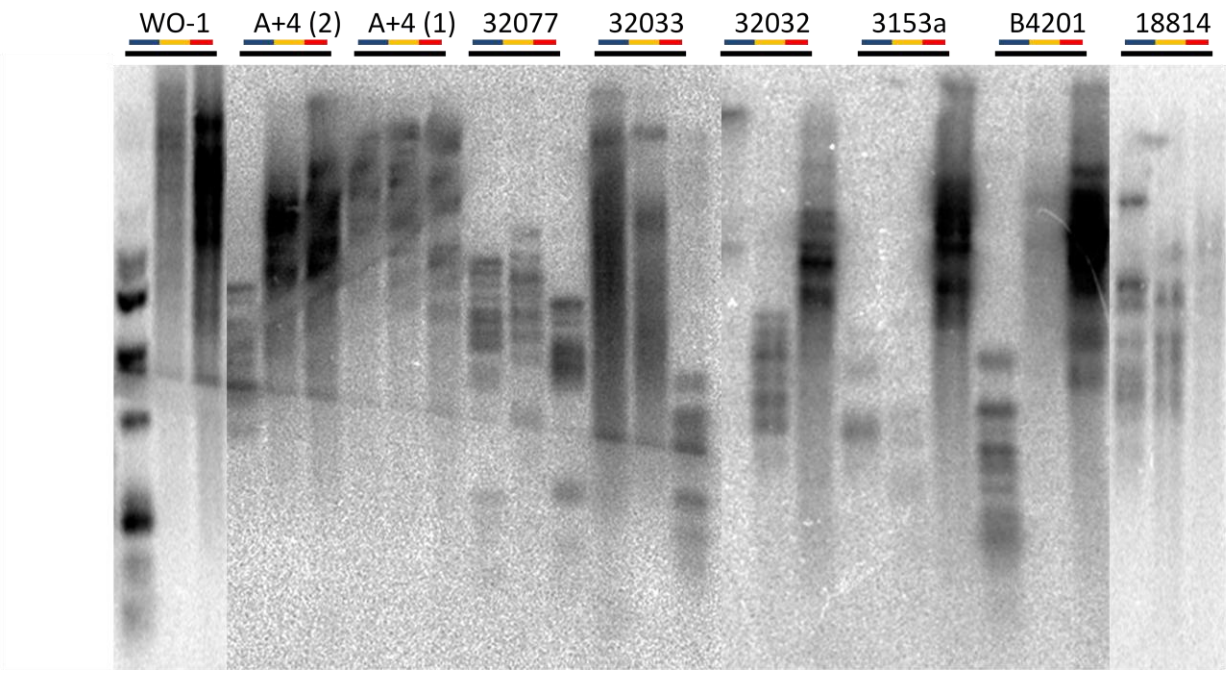

Figure 4.1. The response of telomere lengths to prolonged growth at different temperatures was variable across 7 *C. albicans* strains. Shown is a Southern blot of EcoRI-digested genomic DNA from seven *C. albicans* isolates hybridized to a telomeric probe that had been grown extensively at each of three temperatures: 30℃, (lanes marked with blue) 37℃ (lanes marked with yellow) and 42℃ **(**lanes marked with red). Strains ATCC32077, ATCC32033, ATCC32032 and ATCC18814 are all indicated with just their number designations.

Effects of altered growth temperature on a subtelomeric recombination event in *C. albicans* A+4.

A *de novo* subtelomere tagged with *ADE2* was previously generated in *C. albicans* WO-1 (McEachern and Hicks 1993). This *ADE2* insert resulted from an aberrant homologous gene knock-out experiment which truncated the target chromosome and, as diagrammed in Fig. 4.2A, resulted in a partial duplication of *ADE2* (the selectable marker gene in the knock-out construct) as well as a de novo telomere added on the truncated *ade2* partial copy. This clone, designated A+4, helped demonstrate that the 23 bp repeats adjoining *ade2* sequence comprised the actual telomeric repeats of *C. albicans*. The presence of one complete and one partial *ADE2* copies as tandem repeats next to a telomere led to the A+4 clone experiencing high rates of homologous recombination between the repeats and loss of *ADE2* function (McEachern and Hicks 1993). Thus, monitoring deletion of *ADE2* from the A+4 clone provides a potential assay to measure subtelomeric recombination rates in *C. albicans*.

We used a WO-1 A+4 subclone that had been propagated at 37℃ for three weeks to lengthen its telomeres (designated A+4 Alt37) to measure how growth at different temperatures might affect recombination of the subtelomeric *ADE2* sequences. This was done by taking a single colony of A+4 Alt37 which had been maintained at 37℃, streaking it onto YPD plates and incubating those plates at 30℃, 37℃ or 42℃, followed by plating dilutions made from multiple examples of the resulting colonies, and then, finally, determining the frequency of red/brown colonies (characteristic of the *ade2* phenotype) or colonies with red/brown sectors. We found that WO-1 A+4 Alt37 grown at 22℃ had a frequency of 1.7×10^{-4} red/brown colonies or red/brown sectored colonies (Fig. 4.2B). In contrast, growth at 30℃ generated a 6.3-fold increase in the frequency of colored or sectored colonies while growth at 37℃ resulted in a dramatic 18-fold increase in colored or sectored colonies. These results demonstrate that growth temperature can have a profound effect on the frequency of a subtelomeric recombination event in *C. albicans*. Although telomere lengths were not measured in colonies taken

from the three temperatures, the brief period of growth at the different temperatures makes it unlikely that telomere lengths were significantly different between the different situations examined.

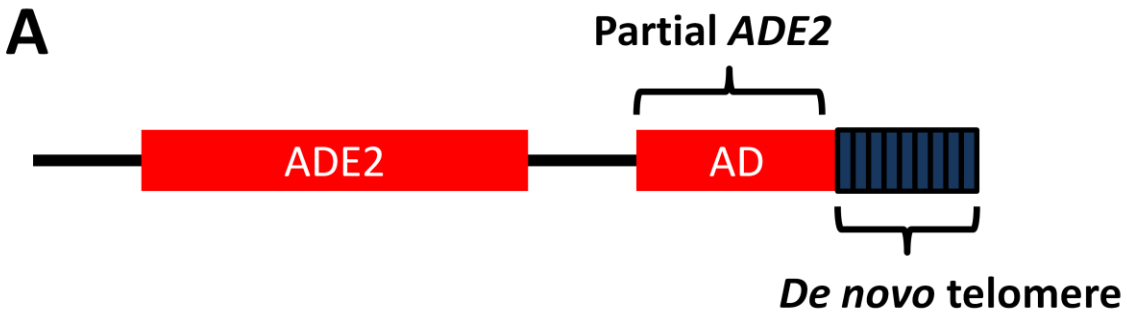

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Figure 4.2. Frequency of red-sectored colonies increase in response to temperature stress. (A) A diagram of the *de novo* telomere and subtelomeric *ADE2* studied, as originally generated by McEachern and Hicks (McEachern and Hicks 1993). The shorter, non-functional *ade2* copy is nearer to the telomere. (B) Graphs showing the frequency of appearance of red/brown colonies or red/brown sectored colonies in A+4 Alt 37 clone grown at each of three temperatures; from left to right: 22℃, 30℃ and 37℃.

Rates of subtelomeric recombination near *TLO genes* in *C. albicans* during growth on YPD.

The adaptive telomere failure hypothesis suggests that subtelomeric recombination may increase in response to environmental stress. This would be expected to be advantageous for pathogens, which often experience environmental stresses involved with host invasion and immune responses. *C. albicans* subtelomeres are populated with a crucial series of transcription factors involved in hyphal growth and successful pathogenesis (Anderson, Baller et al. 2012). *C. albicans* strain Rm 1000 experiences a higher rate of subtelomeric recombination at markers on the telomere-proximal region of *TLO* genes compared to the telomere-distal region and present a preference for recombination near α-

clade members (Anderson 2015). We attempted to follow-up on this increased recombinational preference by studying recombination rates adjacent to *TLO* gene family members during environmental stress conditions.

We first re-examined *t*he rate of *URA3* loss at subtelomeric *TLO* sites at the 3' (telomere distal) and 5' (telomere proximal) UTRs by growing *C. albicans* strains carrying each of five *URA3* inserts, illustrated in Fig. 4.3A, for two days on YPD followed by plating serial dilutions onto 5-FOA from multiple examples of the colonies that resulted. Our results, shown in Fig 4.3B, showed that the rate of mutation at three sites on the telomere proximal side of the *TLOs* varied depending on the classification of the *TLO* (α/γ) with *TLO*α9 (CA12655) and *TLO*α*12* (CA12658) generating 8.4 x 10^{-5} and 5.7 x 10^{-5} mutations/cell/generation, respectively. The α-clade demonstrated a 2.8 to 4-fold higher rate of recombination compared to a telomere-proximal *URA3* at TLOγ16 (CA12657) (Fig 4.3B). *URA3* located at the telomere-distal side of *TLO*α*12* (CA12005) showed 27-fold increase in its loss rate compared to a telomere-distal locus of *TLOβ2* (CA12002) (Fig 4.3B).

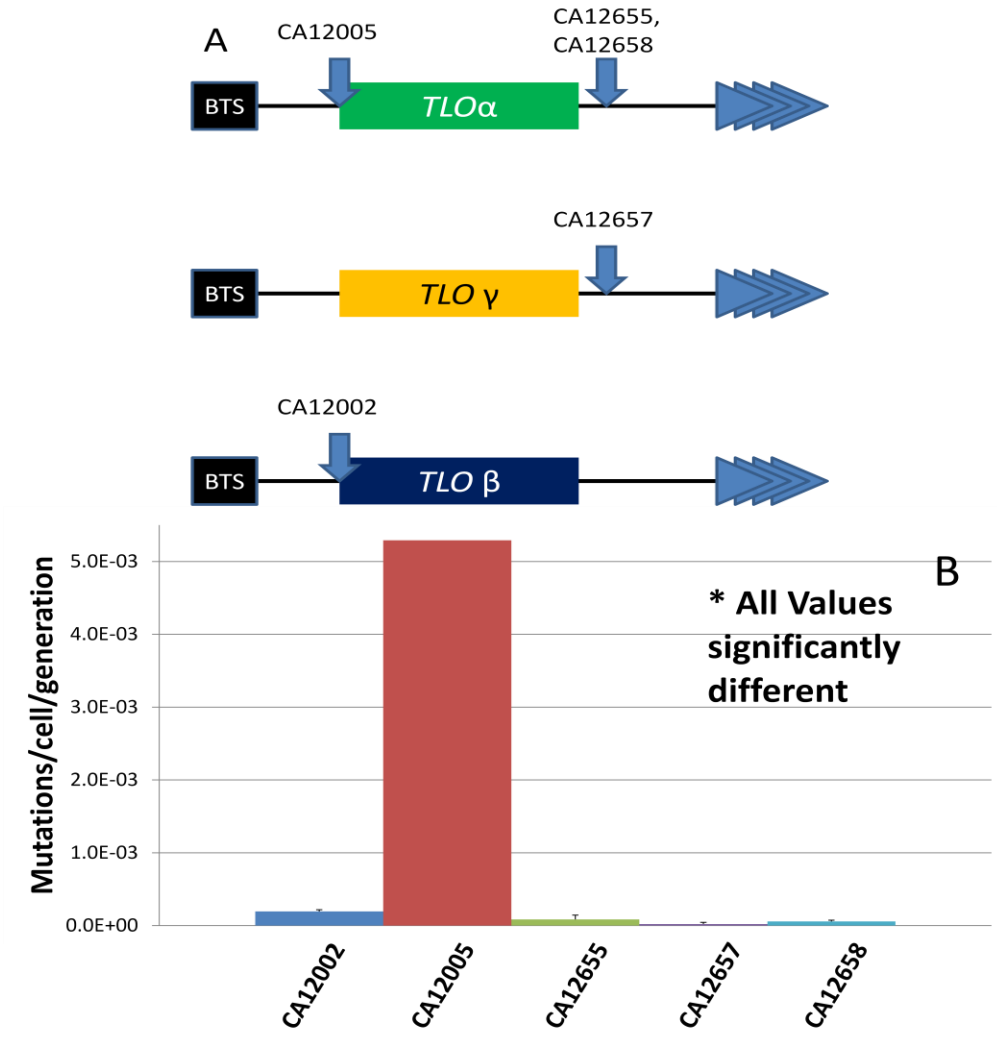

Figure 4.3 Five *URA3* inserts near subtelomeric *C. albicans TLO* genesand their mutational loss rates during growth on YPD. A) Illustration of the location of *URA3* inserts adjacent to three clades of subtelomeric *TLOs*. Five subtelomeric *URA3* loci used in this study flank the *TLO* genes belonging to the α, β, and γ clades. Although CA12005 and CA12655 are pictured with CA12658, the latter is present on a different alpha clade *TLO* but the location shown is identical, and therefore presented as such for simplicity. B) Loss rates of five subtelomeric *TLO* associated *URA3* loci in *C. albicans*. Analysis of mutation rates at five loci are indicated; (Left to right) CA12002, CA12005, CA12655, CA12657 and CA12658. * denotes $p<0.05$. Significance was calculated between values for each locus compared to each other locus and resulted in p-values of <0.05 in all pair-wise comparisons.

Effects of environmental stresses on subtelomeric mutation rates in *C. albicans*

To begin to study the effect of environmental stresses on subtelomeric recombination, we performed preliminary experiments to test the effect of different NaCl, KCl, and sodium arsenate levels on colony growth of *C. albicans*. These experiments showed that *C. albicans* RM1000 experiences significant growth stress, as indicated by significantly smaller colony sizes, when grown in the presence of 1.2 M NaCl or 1.5 M KCl, the same stress conditions previously studied in our work with *Kluyveromyces lactis*. However, we found that *C. albicans* was naturally slightly more resistant to arsenate than *K. lactis* and growth stress at sodium arsenate concentrations of 1 mM was chosen for use. Using these levels of stress, studies of the rates of recombination at a telomere-proximal locus of *TLOβ2* (CA12002) were performed. The proximal locus of *TLOα9* (CA12655) was also used to further analyze the mutational response to 1 mM sodium arsenate, as previous studies in *K. lactis* suggested that different loci at subtelomeres may respond to stresses differently. The analysis of a proximal locus of the *TLO* genes may provide insight into how *TLOs* may act as promoters of recombination in conjunction with the telomeres during stress conditions.

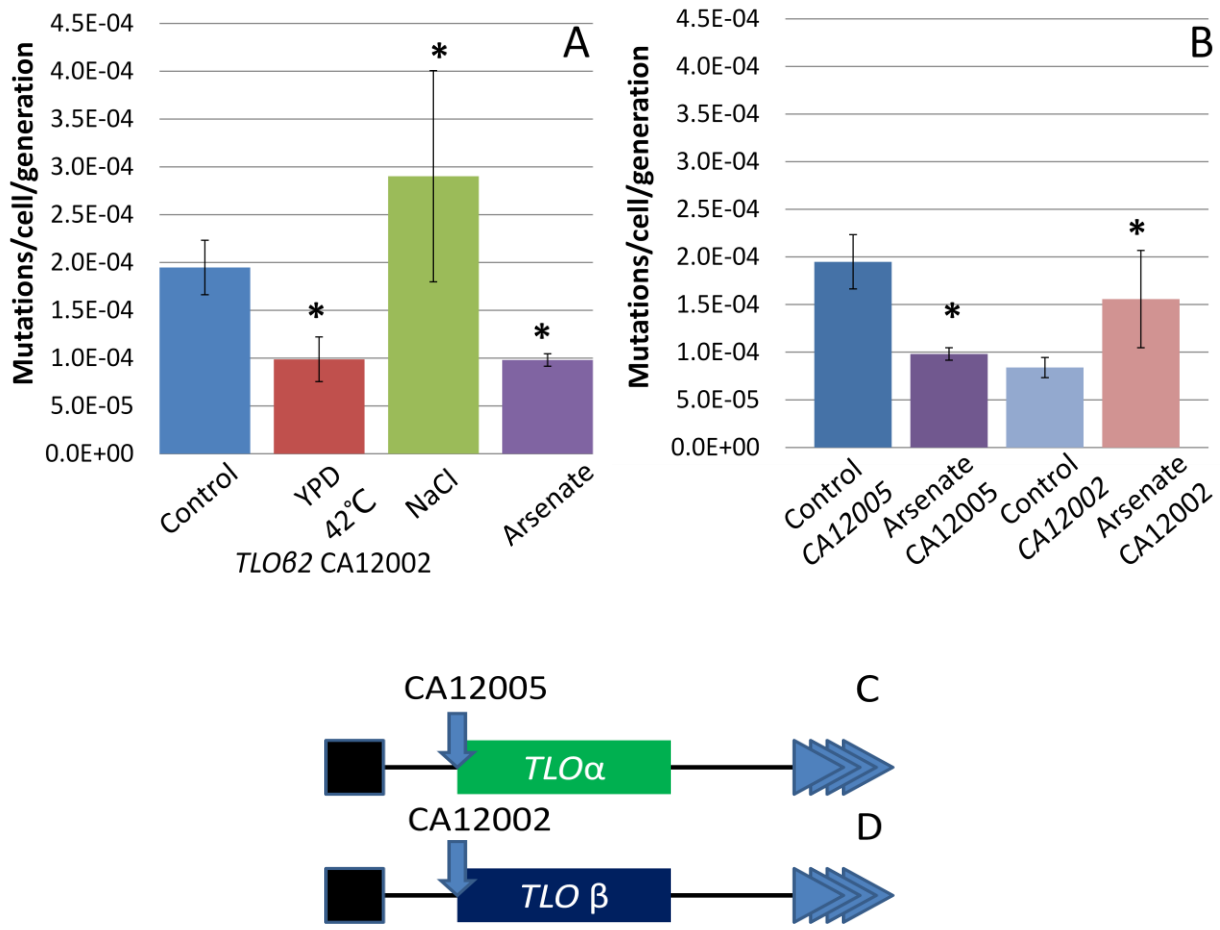

Figure 4.4. Mutation rates at two *C. albicans* subtelomeric loci during stress conditions. (A) The mutation rate at the telomere-distal *URA3* insert site of *TLOβ2* (CA12002), shown in (D), after grown on, YPD, YPD grown at 42℃, YPD with 1.2 M NaCl and YPD with 1 mM sodium arsenate exposure. Except where indicated, cells were grown at 30℃. (B) Comparison of the loss rates at two different subtelomeric *URA3* inserts next to *TLO* genes (left pair – *TLOα12* (CA12005) (C), right pair *TLOβ2* (CA12002) (D)) after growth on YPD or growth on YPD containing 1 mM sodium arsenate. * denotes significance of $p<0.05$.

The initial focus was on the telomere proximal locus of *TLOβ2* (CA 12002) due to its lower rate of movement between subtelomeres (Anderson 2015) and to the fact that the locus has not demonstrated issues with high rates of apparent transcriptional silencing that were seen during studies at the telomere-proximal locus of *TLOα12* (data not shown). As mentioned above, CA 12002 experiences a mutation rate of 2.0 x 10^{-4} mutations/cell/generation when grown on a YPD control. Exposure to 42℃ or 1 mM sodium arsenate both reduced the mutation rate at *TLOβ2* by ~2-fold ($p < 0.001$) (Fig 4.4A). Exposure to 1.2 M NaCl, on the other hand, increased the rate of mutation by ~2-fold ($P<0.05$) compared to the control (Fig 4.4A). With cells containing the *URA3* tag on the telomere proximal side of *TLOα9,* the rate of loss of *URA3* from 1 mM sodium arsenate exposure increased the mutation rate by 1.8-fold. We conclude that the stress conditions tested could produce modest but significant alterations in *URA3* loss.

Discussion

C. albicans is an important human fungal pathogen which possesses a significant compliment of subtelomeric transcription factors which are important for pathogenicity. The adaptive telomere failure hypothesis may predict a means by which *C. albicans* can rapidly promote variation and recombination within its subtelomeric *TLO* gene family. Modification of telomere lengths in response to environmental cues is a possible mechanism by which telomere function could be modified and we have demonstrated that multiple *C. albicans* strains have telomeres that become altered in length due to growth temperature variations. Telomere length variation in response to increased temperatures was initially described in 1993 using the lab strain WO-1 (McEachern and Hicks 1993). The majority of strains we studied here demonstrated a clear increase in the overall lengths of their telomeres during growth at high temperatures. Telomeres in *C. albicans* are readily maintained by both telomerase and recombination, and the responsiveness of their lengths to changes in temperature may be an indicator

that changes in recombination rates, sometimes affecting subtelomeric regions and may be sensitive to environmental factors (Ciudad, Andaluz et al. 2004, Chico, Ciudad et al. 2011). These differences in telomere length variations demonstrate, importantly, that WO-1 is not the only *C. albicans* strain to fluctuate the length of its telomeres in response to temperature. Consistent with the significant genetic variation in *C. albicans*, the telomere length variation due to temperature is not a uniform one in *C. albicans*, with some strains elongating their telomeres in response to temperature increases while others showed no obvious changes or different types of responses.

Studying the frequency of recombination events at a subtelomeric *ADE2* adjacent to a *de novo* telomere in *C. albicans* WO-1 A+4 (2) indicated that the rate of subtelomeric recombination in response to higher temperatures could be up to 18 fold higher than seen at a lower temperature. This strongly suggests that subtelomeric recombination events in *C. albicans* strain WO-1 A+4 (2) can be influenced in response to environmental stimuli. This effect of temperature appears not to be uniform across all subtelomeric recombination in all *C. albicans* strains. In another experiment, we observed that growth at 42℃ led to a modest decrease in the loss rate of *URA3* at a subtelomeric TLO gene (Fig. 4.4A) in *C. albicans* strain Rm 1000. Whether these differences are due to different strains, different subtelomeres, different assays or some other factor, is not known.

Previously detailed work by Anderson (Anderson, Baller et al. 2012, Anderson 2015) provided guidance regarding the preference for recombination at the three *TLO* clades as well as estimations regarding the rate of *TLO*-related recombinational exchange between non-homologous chromosomes that resulted in hybridization of *TLOs* or loss of a specific *TLO*. Anderson focused on the propensity of *TLO*s to be lost or gained at particular positions and showed that there was a significantly likelihood that *TLOα9* (CA12655) would be lost during extended passage while *TLOβ2* (CA12002) was likely the most stable and with *TLOα12* (CA12005/CA12658) and *TLOγ16* (CA12657) experiencing few duplications or

deletions. The movement of these *TLOs* was due to recombinational events with break points at the 3' telomeric end of the genes in either the UTR or a shared 50 bp repeat known as the BTS. But there were notable variations in the sites of this recombination which could also include break points within the *TLOs* themselves (50% of the time). It is notable then that CA12002 and CA12005 are sites which are expected to represent half of the potential recombination events at any given *TLO* while CA12655, CA12657 and CA12658 should provide a different picture, one of potential *TLO* chimerization instead of deletion or BIR events (Anderson 2015).

The mutation rate resulting in loss of *URA3* at CA12005 represents a level 27-fold increase above that seen with CA12002. CA12005 is adjacent to a γ member of the *TLO* family and is located telomere-proximal to a BTS sequence. Anderson has previously described the BTS sequence as a common hot-spot for subtelomeric recombination (both BIR and gene conversions), and these results support the hypothesis that *TLO* genes demonstrate varied, but significant, recombination rates with *TLOβ2* being the most stable *TLO* studied (Anderson 2015). Although recombination involving *TLO* members on non-homologous chromosomes occurs, often with *TLOγ* members as sequence donors, this is not known to occur in the lone *TLO*β2 gene, providing an unexpected measurement of regular loss of *URA3* at CA12002. The mutation rate we recorded in this work at CA12005 was more than 10-fold higher than those recorded by Anderson regarding all *TLO* movements on average. This may suggest that our results may be representative of *URA3* loss due to recombination with the homologous chromosome of the tagged subtelomere instead of loss due to exchange with a non-homologous chromosome. Our studies did not attempt to measure the proportion of *URA3* loss events that are due to gene conversion events or to BIR events copying a non-homologous subtelomere.

Comparing the telomere-distal 3' UTR locus of *TLOα12* CA12005 to a telomere-proximal locus within proximal promoter of *TLOα12* (CA12655), a 9-fold drop in the mutation rate is observed ($p<0.01$).

This suggests that although recombination within a *TLO* locus occurring between homologous chromosomes may occur outside of the *TLO*, the *TLO* itself provides homology that can be utilized to promote gene conversion.

CA12655, CA12667 and CA12658 represent two different clades of *TLOs*, the α (CA12655, CA12658) and γ (CA12657) which are known to recombine with each other at a measurable rate (Anderson 2015). The higher rates of *URA3* loss at the two α *TLO* loci agrees with previously published works, as does the difference between CA12655 (*TLOα9*) and CA12658 (*TLO*α12) . *TLOα9* is the most frequently lost *TLO* in *C. albicans* which may explain why, in our tests, its mutation rate at CA12655 was the highest.

Recombination rates at telomere-distal sites associated with *TLOβ2* (CA12002) suggest that *C. albicans* Rm 1000 subtelomeres are recombinationally responsive to environmental stresses. 42℃ and 1 mM sodium arsenate exposure both resulted in the reduction in the mutation rate of ~50% ($p<0.01$). Conversely, exposure to 1.2 M NaCl resulted in the increase in the rate of recombination by 1.5-fold. The decrease in recombination in response to temperature suggests that subtelomeres in *C. albicans* Rm 1000 may experience a significant reduction in the rate of recombination during 42℃ temperature stress. The increase in mutation rates in response to 1.2 M NaCl is consistent with our *K. lactis* experiments (Chapter 2, this dissertation) although our results cannot distinguish whether the increase is genome-wide as it appears to be in *K. lactis*. Sodium arsenate also resulted in a 2-fold reduction in recombination at CA12002, which is similar to results in the genic subtelomeres of *K. lactis* with *HisG-Ura3-HisG* cassettes. The sharp decrease in recombination in response to sodium arsenate prompted a follow-on experiment utilizing CA12005, which contains *URA3* adjacent to *TLOα12.* This construct, showed the opposing trend, with a 1.8 fold increase in the rate of mutation of *URA3* in the presence of the same level of arsenate. The divergence in results for sodium arsenate exposure suggests that the

recombination rate may vary between subtelomeres. This would agree with notion that *C. albicans* subtelomeres are far from equal in their behaviors and recombination rates.

Acknowledgements:

This research was in part funded by a generous donation from Mr. Paul Truex and family. I also want to acknowledge Dr. Matthew Anderson for providing the subtelomerically tagged strains utilized in part of this study.

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CHAPTER 5

Perspectives

Organisms experience stress due to both environmental conditions and complications regarding replication, transcription and reproduction involved in the maintenance of life (Attfield 1997, Estruch 2000, Folch-Mallol, Garay-Arroyo et al. 2004, Forche, Abbey et al. 2011, Aguilera and Garcia-Muse 2013, Carr and Lambert 2013, Brown, Budge et al. 2014). Cellular responses to these stress conditions are highly depended on the stress and often result in modifications to cell morphology or its genome. Rapid genetic responses to stress conditions may involve recombination to promote deletions, duplications or chimerization of specific genes or gene families which can help the cell adapt to stresses (Letavayova, Markova et al. 2006, Forche, Abbey et al. 2011, Lindstrom, Leverich et al. 2011, Aguilera and Garcia-Muse 2013). Subtelomeres are regions of linear chromosomes adjacent to telomeres which are theorized to provide a locale which benefits from recombinational forces (McEachern 2008). Subtelomeres are often devoid of essential genes, prone to silencing and experiencing higher rates of mitotic recombination compared to other regions of the genome (McEachern 2008). Another means of rapid adaptation to environmental stresses is a physiological change such as entering a slow growth state (Day 2013). Gaining a stronger understanding as to how an organism attempts to adapt to its environment through recombination and growth modulation may provide insight into how cells can

provide a means for rapid evolution. The concept of utilizing subtelomeric recombination to promote rapid evolution is one the tenants of stress adaptation supported by the adaptive telomere failure hypothesis (McEachern 2008).

The adaptive telomere failure hypothesis proposes that subtelomeres provide an optimal environment for rapid evolution (McEachern 2008). This environment is augmented by the proximity to telomeres, which can promote silencing and recombination through modulation of their length (Gotta, Laroche et al. 1996, McEachern and Iyer 2001, Barry, Ginger et al. 2003, Gurevich, Smolikov et al. 2003, Juarez-Reyes, De Las Penas et al. 2011, Juarez-Reyes, Ramirez-Zavaleta et al. 2012). The adaptive telomere failure hypothesis proposed that subtelomeres experience higher rates of recombination in response to stress conditions. Subtelomeric recombination would take advantage of subtelomeric contingency genes, by causing telomeres to dysfunction and thereby increasing local recombination which can extend into the subtelomere (McEachern 2008). Increases in subtelomeric recombination allows for rapid evolution in response to stress conditions and potentially increase fitness. The results of the studies contained herein provide evidence to support the theory that organisms may respond to stress conditions by increasing recombination rates at the subtelomeres.

Cell types and colony growth morphologies are another means by which cells can rapidly adapt to environmental stresses. These alternate growth phenotypes were studied as a part of better understanding how organisms respond to stresses, and potentially understanding if these alternate phenotypes provide additional means for increasing fitness during stress. Some yeast are capable to generating a petite colony phenotype during stress conditions, which promotes significant changes in metabolism and sensitivity to the environment (Day 2013). These slower growing cell types can provide significant advantages to growth during oxidative stresses and during infections. *K. lactis* does not form petite colonies, but does experience differences in colony size following stress conditions (Chapter 3).

Further analysis of these colony morphologies has provided insight into some of the complex responses which cells undergo following exposure to arsenate.

Conclusions of Results

These studies have provided insight into stress responses in both *K. lactis* and *C. albicans*. Analysis of subtelomeric recombination in *K. lactis* using STU, RN-HUH and ST-HUH constructs in addition to a natural subtelomeric *LAC4* demonstrated that subtelomeres increase recombination rates in response to 35℃, 1.2 M NaCl, 1.5 M KCl and 0.5 mM arsenate exposure. STU constructs demonstrated that recombination specifically increases at the subtelomeric R-element during KCl and arsenate stress. While the R-element increases recombination in response to stress, two non-subtelomeric loci demonstrate no significant changes for KCl exposure and a significant decrease in recombination for arsenate exposure. Recombination in the genic regions of the subtelomere using ST-HUH constructs was not conclusively different from non-subtelomeric loci, potentially due to subtelomere-specific BIR events being relatively infrequent compared to single strand annealing (SSA) events which are expected to cause the majority of ST-HUH mutation events. *LAC4* studies showed an increase in white colony frequency following exposure to arsenate. White colonies were not confirmed to be subtelomeric recombination; however they did provide results suggesting that consistent with mutation rates increase significantly during arsenate exposure.

These results support the component of the adaptive telomere failure that argues that subtelomeric regions can uniquely experience increases in mutation rates in response to environmental stress conditions. Specifically, this theory finds support in the results from STU vs. RN-HUH, wherein subtelomeres specifically demonstrate increases in recombination rates while non-subtelomeric loci see decreases in recombination rates in the presence of arsenate. This suggests that the subtelomeres might experience a directed response during some stress conditions. The increase in subtelomeric

recombination can provide a means for rapid evolution by modifying local gene content through duplications or deletions. The function of telomeric length and the role that it plays in stress-related subtelomeric recombination is not yet clear. The studies here using *LAC4* imply that there is a similarity between stress-related behaviors of the subtelomere and subtelomeric behaviors when telomeres are shortened. The results from these *LAC4* studies may also suggest different means for subtelomeric adaptation during stress conditions, such as point mutations or substitutions, are experienced when telomeres are compromised. Although it is relegated to conjecture at this point, the similarities between these two conditions implies that some stress conditions may modulate telomere lengths to produce subtelomeric modifications and recombination rate variation. This would bridge the largest complication of the adaptive telomere failure hypothesis, providing evidence that telomeres can be the drivers of subtelomeric behaviors directly in response to stress.

Studies of telomeres and subtelomeric recombination in *C. albicans* provided insight into how a pathogen may utilize its telomeres and subtelomeres in conjunction with one another to promote rapid adaptation to environmental conditions and potential host defenses. Prolonged growth at different temperatures has a variable but often dramatic effect on *C. albicans* telomere lengths, however, in strains where the telomeres appear responsive to temperatures, the majority of them present a significant increase in telomere length over the course of a 3-week exposure to temperatures of 37℃ and 42℃. These temperature length changes are suggested to be linked to subtelomeric recombination at an *ADE2* tagged telomere which demonstrated a significant increase in loss through recombination or mutation when the *C. albicans* was exposed to 37℃. Studying mutations at the subtelomeric *TLOβ2* gene in cells exposed to 35℃, NaCl or arsenate stress suggests that *C. albicans* subtelomeres experience a decrease in mutation during temperature and arsenate exposure, but demonstrate an increase in

mutation rate during NaCl exposure. This is markedly different from *K. lactis* subtelomeres, but may also provide insight into how different environmental conditions weigh differently on different species.

Analysis of the rate of *URA3* mutation at three different subtelomeric *TLO*s provided mixed results regarding the subtelomeric behaviors of *C. albicans*. The results suggest, but do not prove, that the telomere-distal region of *TLO*s are more likely to experience mutations or silencing than the telomere-proximal region, which is surprising for two reasons. Firstly, mutations via recombination are expected to occur most frequency at the telomere-distal BTS, within the *TLO* and nearer the telomeres, providing more opportunities for telomere-proximal regions to experience recombination. Secondly, if these mutations were a by-product of silenced regions experiencing point mutations, the silencing is also expected to emanate from the telomere, which would suggest a different result. Ultimately, these results are striking because they are unusual and unexpected. Further analysis of these outcomes through sequencing may provide insight into the complicated behaviors of *C. albicans* subtelomeres and how these regions may take advantage of their locale to increase the diversity of this pathogenic gene family.

While subtelomeric recombination rates and telomeric variation are a newly studied means of rapid adaptation to the environment, a previously analyzed method of adaptation to oxidative stress is the generation of smaller, slower growing colonies in yeast. In this body of work we have provided evidence building on ideas first suggested in 1980 that *K. lactis* is capable of producing a petite-like colony growth morphology when exposed to significant oxidative stress. Although this colony morphology is not a petite colony, as it is readily reversible, it does have similar hallmarks of petite colonies such as a heritable resistance to oxidative stress, sensitivity to high temperature and a heritable growth phenotype, which rapidly reverts back to normal growth rates. The potential cause of this phenotype are currently only speculated as involving silencing of cytochromes to reduce the

effectiveness of the oxidative phosphorylation pathway, mutation in metabolic pathways or modification of mitochondria or total number of mitochondria in the cell. Sequencing of these pathways, analyzing the transcriptional variations in normal and smaller colony morphologies or counting the number of mitochondrial genomes present within the cell are potential ways to address the root causes of these small colonies.

Future Directions

These works have addressed a wide range of manners in which the yeasts *K. lactis* and *C. albicans* respond to stress conditions. *K. lactis* studies have provided support for the adaptive telomere failure hypothesis by suggesting that subtelomeres respond by increasing recombination during stress. These studies require a clearer connection between the increases in recombination and telomere length, which can be done by using telomere mutants and analyzing telomere length variations due to the different stress conditions. Studies in *K. lactis* of the generation of semi-stable small colonies following exposure to arsenate suggest that yeast may utilize a slow growth phenotype as a means of adapting to some oxidative stresses. These small colonies require further analysis through establishment of the mitochondrial content of the cells, sequencing of the transcriptome and potentially analyzing metabolic changes within the small and large cells. This project provided the tip of the iceberg with these analyses, and further experimentation may show that non-petite yeast may have another similar, while less stable, manner for adapting to oxidative stress. Finally, *C. albicans* studies brought further insight into the behaviors of both telomere lengths during stress and mutations within the subtelomeres of a pathogen. Although these studies did not answer what types of the *URA3* mutations generated in this study, they to provide suggestions as to behaviors of the subtelomeres surrounding the *TLO* gene family. Gaining a better understanding as to the dynamics of *TLOs* during stress conditions and how they adapt through mutation and recombination may help provide information as to how this

pathogen is capable of rapidly responding to its environment. These experiments have opened many doors and provided as many questions as they have attempted to provide answers. The support for the adaptive telomere failure hypothesis suggests that a new picture of how the genome is organized and how rapid evolution during stress conditions is viewed requires further analysis. The previous idea that selection removes less-fit organisms may not be iron-clad with regard to single-celled organisms or cancers which may promote rapid evolution during selection in an attempt to promote fitness increases during stress instead of always preparing for it.

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