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The Frequency of Survivorship in Heterozygous Diploids of Cdc13-1exo1Δ Mutants of *S. cerevisiae* Is One Survivor Cell in 72 Cells/Generation at 36˚C

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Abstract

Telomeres cap ends of eukaryotic chromosomes prevent them from degradation and ensure genomic stability. Cdc13 is an essential telomere recruitment and maintenance protein. A temperature-sensitive point mutation in cdc13 gene leads to telomere impairment, giving rise to cdc13-1 mutants that suffer lethality at enhanced temperatures. Deleting Exo1 gene from these mutants, however, leads to the emergence of temperature-tolerant mutants called survivors. Yeasts are known to exist as either diploids or haploids. These yeast genotypes generate survivors. The frequency of survivorship in the haploid genotype is one cell in 104 cells/generation at 36˚C, however, the frequency at which they emerge in their diploid counterparts at the same temperature is not known. In this study, we investigated the frequency of Survivorship in heterozygous diploids of cdc13-1exo1Δ mutants of *S. cerevisiae* at 36˚C. Diploids were constructed by mating haploid strains of opposite mating type cdc13-1 exo1:LEU strains with strains of cdc13-1 exo1:HIS. The crosses were 1296 × 3181, 2561 × 3182, 1296 × 3182 and 2561 × 3181. Genetic markers and phenotypic appearance were considered while mating the mutant cells. Using a stick, a smear of one haploid strain was made on each YEPD plate labelled C2, C8, C9, D1, D14, and D15. A smear of another opposite mating type was made on the previous strain. They were mixed and allowed to mate overnight, before culturing on media lacking Luecine and Histidine (–L and –H). Survivors were generated by culturing these diploids at 36˚C. Using SPSS 20.0 software for windows SPSS, 2011, the frequency was determined as one

Survivor cell in 72 cells/generation, as their frequency of survivorship averaged $5.9 \times 10^{-5} \pm 0.04$.

Keywords

Telomere, Diploids, Haploids, Mutation, Survivors, Frequency, Cdc13

1. Introduction

1.1. Role of Telomere in Maintaining Genomic Stability

Telomere cap ends of eukaryotic chromosomes [1] prevent chromosome fusions and ensure genomic stability [2] [3]. Mutations in the gene for the main human telomere capping protein POT1 could lead to impairments of telomeres [4], genomic instability and different pathological disorders, including cancer [4]. Many aspects of telomeric biology are investigated in budding yeast (*Saccharomyces cerevisiae*) as a simple eukaryotic model [5] [6] in which many molecular processes are similar to those of humans [6].

1.2. Cdc13 Protein of *S. cerevisiae*

Cdc 13 is an essential protein of telomere capping CST complex in *S. cerevisiae* and it is an athologue of POT1 human protein [4]. A temperature-sensitive point mutation in cdc13 gene that encodes this protein cdc13-1 makes mutants' cells cdc13-1 of *S. cerevisiae* suffer lethality and cell arrest at the G2 phase of the cell cycle [7], as they accumulate single-stranded DNA (ssDNA) near their telomere [8], which triggers the G2/M DNA damage checkpoint [9]. This cell arrest inhibits entry to mitosis and results to senesce and subsequent death of the cell [9]. In vertebrates, the DNA damage signals cause the activation of the transcription factor p53, which initiates the P-53-dependent G2 cell arrest, by directly inhibiting CDk1 [10].

1.3. Activation of DNA Damage Checkpoint in *S. cerevisiae*

In *S. cerevisiae*, however, G2 DNA damage checkpoint is activated through the p53-independent pathway [9] [10] [11], and involves the Rad3 and Mec1 which activates Chk1 and Chk2. Chk1 thus mediates the degradation of cdc25A [10] [12]. The insertion of intact *YG*100 or *YG*102 gene into the yeast genome, though, helps to overcome the temperature-sensitive phenotype [13] [14] [15].

1.4. Emergence of Cdc13-1 Survivor Clones in *S. cerevisiae*

The deletion of *EXO*1 gene in cdc13-1 temperature-sensitive mutants of *S. cerevisiae* allows the incidence of cdc13-1 survivors when cultured at an enhanced temperature [4], the mechanism for this survivorship is not known. These survivors become resistant, and could grow at the enhanced temperature [16]. The progenies of these temperature-resistant mutants inherit the temperature-resistant

allele from their parents.

2. Materials and Methods

2.1. Generation of Study Organisms from the Stock

Old culture plates tend to contain organisms at various phases of growth. To obtain young culture at the exponential phase which is ideal for this experimental work, sub-cultures were made from the stock material stored at −80˚C by streaking onto separate YEPD media plates, loop full aliquot of four strains of cdc13-1 *EXO*1Δ (DLY 3181, 3182, 1296 and 2561) *Saccharomyces cerevisiae*. The plates were properly labelled and cultured at 23˚C for 3 days. The organism was sourced from the National Centre for Disease Control (NCDC) Nigeria, where it was stored at −80˚C.

2.2. Construction of Diploids

Diploids were constructed by mating strains of the opposite mating type. We mate cdc13-1 exo1:LEU strains with strains of cdc13-1 exo1:HIS. The crosses were 1296 × 3181, 2561 × 3182, 1296 × 3182 and 2561 × 3181. Genetic markers and phenotypic appearance were considered while mating the mutant cells. Using a stick, a smear of one haploid strain was made on a well-labelled YEPD (Yeast Extract Peptone Dextrose Agar sourced from Oxoid UK) plate, and a smear of another opposite mating type strain was made on the previous strain. They were mixed and allowed to mate overnight, before culturing on media lacking Luecine and Histidine (−L and −H). Temperature-resistant mutants were used to mate with corresponding temperature-resistant mutants to generate diploids with duplex temperature-resistant alleles and also temperature-resistant mutants were used to mate with temperature-sensitive mutants, to generate diploids with a single allele each of temperature-resistant and temperature sensitivity.

2.3. Confirmation of Genetic Markers in Diploids

Upon mating, presumptive diploids were confirmed by culturing them and their haploid parents on media lacking Histidine and Leucine, (−L, −H) the diploids cells grew on the limiting media, while each parent that were also streaking on the same plate failed to grow. This confirmed that the colonies that grew were diploids as they were selected based on their ability to grow on a medium lacking the amino acids Histidine and Leucine.

2.4. Generation of Survivors from Diploid Cells at 36˚C

2 ml of sterile autoclaved water was placed into 6 sterile labelled bijoux bottles. Using a stick, colonies were picked from the various diploids on the culture plate, each diploid was put into a labelled bijoux bottle and mixed using a vortex mixer, and the Optical Density (OD) was measured for each suspension. 0.9 ml of sterile water was measured into 6 sets of 6 bijoux bottles. Each of the 6 sets of bottles was for each heterozygous diploid. The bottles were labelled from 10^{-1} to

10^{-6} to indicate dilution ratios. 10-fold serial dilutions were made from each suspension into the labelled bottles.

Each dilution bottle was mixed properly using a vortex mixer, while 50 µl aliquots were measured from the concentrated suspension, 10^{-1}, 10^{-2} and 10^{-3} dilutions, using sterile spreader, the aliquots were spread onto a separate labelled Yeast Extract Peptone Dextrose Agar (YEPD) plates for generation of survivors, 25 µl aliquots were also measured from dilutions 10^{-4} to 10^{-6} and were spread onto a separate YEPD media plates, as count plates. The concentrated, 10^{-1}, 10^{-2} and 10^{-3} dilutions were cultured at 36˚C to generate survivors, while the 10^{-4} to 10^{-6} dilutions were cultured at 23˚C to generate viable cells.

2.5. PCR Reaction

PCR reaction was carried out to confirm that the colonies generated at the restrictive temperature were not reverse mutants but survivors. Ten samples were prepared, including one reaction each for the wild strains DLY 640 and 641, one positive and one negative control reaction. The wells were loaded with the ladder followed by one digested sample, and the next an undigested sample, the sequence of digested sample, followed by an undigested sample was repeated, till all the samples were loaded, then the controls were finally loaded and run for 32 cycles.

2.6. Loading of Gel and Electrophoresis

After digestion of the DNA, 6 µl of ×6 loading dye was added to each reaction mixture, while 15 µl from each reaction mixture was loaded onto 2.5% agarose gel (sourced from Oxoid UK) that contained ethidium bromide (sourced from Oxoid UK) and was allowed to electrophorese for one hour after which the DNA bands were visualized using illuminator.

2.7. Statistical Analysis

Obtained data were statistically analyzed and the significance in difference of mean were obtained by Duncan's Multiple Range (DMR) test using SPSS 20.0 software for windows SPSS, 2011.

3. Results

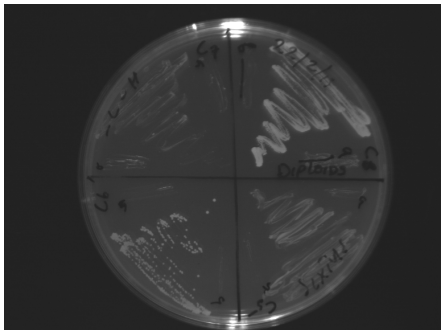

Figure 1. Cdc13-1 exo1Δ diploids selected at 23˚C.

Table 1. Mean and standard deviation of the time of arrival for diploid survivors with single temperature-resistant allele generated at 36˚C.

Diploids	Time of arrival (days)
C9	3 ± 0.04
C2	2 ± 0.05
C8	3 ± 0.06

Table 2. Mean and standard deviation of the time of arrival for diploid survivors with duplex temperature-resistant allele generated at 36˚C.

Diploids	Time of arrival (days)
D14	6 ± 0.02
D1	8 ± 0.02
D15	9 ± 0.03

Table 3. Mean and standard deviation of the frequency of diploid survivors with single temperature-resistant allele generated at 36˚C.

Diploids	Survivors (36^0) 10^{-1} (50 µl plated)	Viable count (23˚C) 10^{-4} (25 µl plated)	Frequency of survivorship
C9	3 ± 0.04	42 ± 0.04	7.1×10^{-5} ± 0.02
C2	2 ± 0.05	36 ± 0.03	5.6×10^{-5} ± 0.06
C8	4 ± 0.06	60 ± 0.03	6.7×10^{-5} ± 0.04

Table 4. Mean and standard deviation of the frequency of diploid survivors with duplex temperature-resistant allele generated at 36˚C.

Diploids	Survivors (36^0) 10^{-1} (50 µl plated)	Viable count (23˚C) 10^{-4} (25 µl plated)	Frequency of survivorship
D14	3 ± 0.02	58 ± 0.06	5.2×10^{-5} ± 0.03
D1	4 ± 0.02	53 ± 0.05	7.5×10^{-5} ± 0.06
D15	2 ± 0.03	64 ± 0.02	3.1×10^{-5} ± 0.04

4. Discussion

4.1. The Time of Arrival of Survivor Colonies Varies in Diploids with Duplex Temperature-Resistant Allele and Their Counterparts with Single Temperature-Resistant Allele When Generated at 36˚C

When cultured at enhanced temperature, cells of *Saccharomyces cerevisiae*, with temperature-sensitive point mutation (cdc13-1 mutation), suffer lethality and undergo cell cycle arrest at the G2 phase [7], later, they undergo senescence,

loose their replicative capacity [17] and die. This cell cycle arrest occurs due to the degradation of the telomeric DNA [18] occasioned by a conditional telomere uncapping, resulting in genomic instability [19] [20]. However, some mutant cells can undergo reverse mutation, and revert to the wild type, which have cdc13 protection, and survive the action of the enhanced temperatures.

When *EXO*1 gene is deleted, some of the mutant cells with the cdc13-1 temperature-sensitive mutation, through mechanism yet to be elucidated [21] [22] survive the restrictive temperature, and emerge into survivor colonies. In this study, diploid survivors at the restrictive temperature of 36˚C from C9, C2, C8, D14, D1 and D15 of *Sacharomyces cerevisiae* with cdc13-1 mutation were generated.

The cells were cultured in YEPD media. After day 2 ± 0.05 of culture, tiny colonies were seen emerging on the 1/10 dilution plate of C2 plates, on day 3 ± 0.04 and 3 ± 0.06, survivors arrived on C9 and C8 plates (see **Figure 1**), such that, within approximately three days all plates of diploids with duplex copy of temperature-resistant allele generated survivors, suggesting that survivors arrive faster from those with duplex allele, while those with single copy of temperature-resistant allele: D14 generated survivors on day 6 ± 0.02, D1 on day 8 ± 0.02 and D15 generated on day 9 ± 0.03 in the same concentration. This indicates that survivors from this genotype arrive slowly, see **Table 1** and **Table 2**.

Other dilutions, though, showed no growth, except the concentrated plates of all the diploids, which showed evidence of expanding suppressed growth, indicating inability to grow, as a result of cell arrest [4] [23]. Although it is noted that DNA degradation-induced G2 phase cell cycle arrest, occurs in *S. cerevisiae*, when exposed to a restrictive temperature [7] [24], observation that cells over-expressing cdc20, a β-transducin homologue, no longer undergo cell cycle arrests at G2 at the restrictive temperature but instead undergo a nuclear division, exit from mitosis and enter a subsequent division cycle, could explain why in the duplex genotype colonies emerge so early contrary to studies suggesting that survivors emerge slowly.

These survivor colonies were tested for the presence of cdc13-1 mutation, by PCR and were confirmed to be survivors and not mutants that reverted to the wild type. However, after 24 hours of culture, the count plate or dilutions cultured at 23˚C showed emergence of viable colonies, this is expected, as the organisms were cultured at the optimal temperature regime of 23˚C, without any thermal restrictions that could be lethal and cause damage to their telomeric DNA.

This result showed variations in the arrival time for survivors from the various diploid genotypes. Although, slow arrival for cdc13-1 exo1Δ had been reported [25], this work had shown that there is a significant variation in the arrival time of survivorship between diploids with single copy temperature-resistant allele and those with duplex copy with p-value of < 0.005 and therefore suggests that those with duplex copy allele have a better mechanism of survivorship than with

single copy do, despite that higher inoculum concentration of the single copy allele genotype was plated. Implying that, to generate survivors from this genotype, higher inoculum concentrations of the diploid cells/cfu should be plated.

[4] observed that the survival of cdc13-1 cells at the restrictive temperature of 36˚C segregates as a multigenic trait. This, therefore, suggests that genes may influence the incidence of survivorship. However, gene regulation could therefore be used to interpret the above result, as it could be suggested that, in the various diploid genotypes, the switching on an nd switching off timing of the individual gene could play a role on the time of arrival of viable survivor colonies. Since, the exact mechanism of survivorship has remained obscured, mutation, however, has not been ruled out.

4.2. The Frequency of Survivorship in Diploids of Cdc13-1exo1Δ Mutants of *S. cerevisiae* Is One Survivor Cell in 72 Cells/Generation at 36˚C

Diploids were constructed by mating strain with known genetic marker that were determined by replica plating with a corresponding strain with also known genetic marker and opposite mating type. Cdc13-1 exo1:LEU strains were mated with strains of cdc13-1 exo1:HIS. The crosses were 1296 × 3181, 2561 × 3182, 1296 × 3182 and 2561 × 3181 phenotypic traits of the strains, such as colony size were considered when mating the strains. Large colony was crossed with corresponding large colony, small colony crossed with corresponding small colony, large colony was also crossed with corresponding small colony and small colony was crossed with corresponding large colony of opposite mating type. Combinations of diploids were generated by mating both survivors and survivor and non-survivors and survivors.

Upon mating, streak of the crosses that were plated on media lacking Histidine and Leucine, (−L, −H) grew into diploids cells, while each parent that were also streak on the same plate failed to grow. This confirmed that the colonies that grew were diploids [26]; this method, however is mostly used in recent studies, as it is easy, and gives accurate result in confirming if the prospective diploids are actually diploids by selecting them based on their ability grow on medium lacking the amino acids Histidine and Leucine [27].

Six diploids: C2, C8, C9 (see **Figure 1**), D1, D14, and D15 were selected at random from among the diploids, for generation of survivors. Interestingly, in all the diploids, survivors could only emerge from the undiluted and the 10^{-1} dilution [28] plate when cultured at 36˚C, while other plates showed no growth and hence failed to generate survivors. Although, [29] [30] have already observed the frequency of survivorship in haploid cdc13-1 exo1Δ mutants of *S. cerevisiae* but the frequency at which survivors emerge from their diploids counterparts is not known. This study, however, showed that the frequency of survivorship in diploids is one survivor cell in about 72 cells/generation, as their frequency of survivorship averaged $5.9 \times 10^{-5} \pm 0.04$, see **Table 3** and **Table 4**.

4.3. There Is No Variation in the Frequency of Survivorship in Diploids with Duplex Temperature-Resistant Allele and Their Counterparts with Single Temperature-Resistant Allele When Generated at 36˚C

The frequency at which survivors emerge from haploid cdc13-1 exo1Δ temperature-resistant mutants of *S. cerevisiae* when cultured at a restrictive temperature has continued to be a topic of debate among scholars and researchers, however, recent studies have shown the frequency of one survivor cell in about 104 cells/generation [29] [30] [31]. Although, there is variation in the time of arrival of survivors in the various diploid genotypes, the frequency at which they emerge shows no variation (see **Table 3** and **Table 4**). In this study, it is shown that, the frequency of survivorship at the restrictive temperature of 36˚C for C9 was $1.7 \times 10^{-5} \pm 0.02$, C2 was $5.6 \times 10^{-5} \pm 0.06$, C8 was $6.7 \times 10^{-5} \pm 0.04$. D14 was $5.2 \times 10^{-5} \pm 0.03$, D1 was $7.5 \times 10^{-5} \pm 0.06$ and that D15 was $3.1 \times 10^{-5} \pm 0.04$.

5. Conclusion

The ease with which yeast can be maintained as either haploid or diploid cells, or its genes transformed, its rapid growth, the similarity of its cellular activity with that of humans, and the fact that its entire genome has been sequenced are the characteristics that make them useful as a model organism for genetic research. In this study, we investigated the frequency at which survivors emerge from diploid species of *S. cerevisiae*, and found it to be 72 cells/generation, with variation in the arrival time for the survivors in diploids with single and duplex temperature-resistant alleles.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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