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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED DEVELOPMENT OF A GENETIC SYSTEM TO ISOLATE DELETION

MUTATIONS AT THE CAN1 LOCUS OF SACCHAROMYCES CEREVISIAE

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DEVELOPMENT OF A GENETIC SYSTEM TO ISOLATE DELETION MUTATIONS AT THE *CAN1* LOCUS OF *SACCHAROMYCES CEREVISIAE*

By

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1 INTRODUCTION

The molecular mechanisms of induction of spontaneous deletion mutations in eukaryotes are not well understood. In prokaryotes palindromic models for deletions have been proposed by Weston-Hafer and Berg (1989), Glickman and Ripley (1984), and Shapper et al. (1986) (Figure 1). My research involved the development of a genetic system to isolate deletion mutations in the *CAN1* gene of the yeast *Saccharomyces cerevisiae*. These deletion mutants will be eventually studied by DNA sequence analysis to determine whether palindromic or quasipalindromic structures in the DNA lead to the formation of deletions.

The *CAN1* gene is located on chromosome V of *S. cerevisiae* and codes for arginine permease. This protein has 12 proposed membrane spanning regions of the type seen in channel-forming permeases (Ahmad and Bussey, 1986).

CAN1 was chosen as the target gene because of the ease of isolating spontaneous forward mutants at this locus. Spontaneous *can1* mutants can easily be selected by their insensitivity to the toxic arginine analog L-canavanine. L-canavanine competitively inhibits arginine uptake and can be incorporated into protein, leading to loss of viability to the cell (Grenson et al., 1966). A *can1* cell, defective in arginine permease (Grenson et al., 1966; Whelan et al., 1979), can survive in the presence of canavanine by transporting arginine by another mechanism or by relying on its own arginine biosynthesis pathway to make its own arginine.

In order to study mutations in *CAN1*, arginine transport and arginine biosynthesis must be controlled. The general amino acid permease (GAP) of *S. cerevisiae* can transport all amino acids.

including citrulline and D-amino acids (for a review see Cooper, 1982). This transport system is under the control of nitrogen repression and transport will be suppressed in cases of nitrogen excess. Arginine biosynthesis is regulated by the introduction of two mutations (*arg6* and *arg8*) in the cell's arginine biosynthetic pathway. A *can1*, *arg6*, *arg8* cell (which is unable to transport arginine or synthesize its own arginine) can survive in the presence of canavanine if the arginine biosynthetic block is displaced by the addition of ornithine to the medium.

It was important to construct a haploid yeast strain containing the genotype *CAN1*, *arg6*, *arg8*, and *leu2-3* (as a selective marker for future plasmid transformation) in order to study deletion mutations at *CAN1*. The double mutation in the arginine biosynthetic pathway was needed to ensure that there would be no reversion to wild type in the ability to synthesize arginine.

The isolation of deletions involved collecting several hundred spontaneous *can1* mutants by plating the yeast strain STT110 (*CAN1*, *arg6*, *arg8*, and *leu2-3*) onto M110 + canavanine medium (minimal medium containing the required amino acids and canavanine) (Figure 2). A *can1* mutant was able to grow because both the transport of canavanine was blocked and arginine biosynthesis occurred with the addition of ornithine via the urea cycle. Under conditions of excess nitrogen, the general amino acid permease was repressed and arginine biosynthesis must occur for cell survival. Due to the *arg6* and *arg8* mutation, the most likely route of arginine biosynthesis occurred only under conditions where ornithine is added to the medium. Whelan et al. (1979) described that ornithine was able to be imported into the cell under excess nitrogen conditions independent of arginine permease. Under M110 + canavanine selection a *CAN1*, *arg6*, *arg8* cell cannot grow while a *can1*, *arg6*, *arg8* cell forms a colony. Each *can1* mutant was screened for the ability to revert to *CAN1* on

synthetic complete medium (SC), which does not contain ornithine. Those *can1* mutants that did not revert were labeled as presumptive deletions and isolated for further study (Figure 3).

My research involved the construction of the yeast strains STT27 and STT110, the isolation and characterization of several hundred spontaneous *can1* mutants, and the determination of the growth characteristics of the yeast strain STT110. Strain STT27 was used to isolate several hundred independently arising spontaneous *can1* mutants, which were frozen away for future study. The new strain, STT110, was constructed because of the need for a strain with a double mutation in the arginine biosynthesis pathway, which STT27 did not have. The growth characteristics of M110 were defined because of the need to know how many viable cells are plated in each mutant cycle and the least amount of incubation time before stationary phase was reached.

2 MATERIALS AND METHODS

2.1 Construction of *S. cerevisiae* Strain STT27

Cells from freshly grown colonies from the haploid parents STX406-1B (*MATα, arg8, trp1, ura3, his3, asp5*) and XY729-5A (*MATa, leu2-3, his4-38, ade2-1*) were mixed with a sterile toothpick in a 0.5 cm circle on a YEPD plate and incubated for at least 4 h at 30 C. The mating mixture was selected for diploids on synthetic media lacking arginine and leucine (SC arg-leu). The resulting diploids were streaked onto sporulation media and incubated for 4 days at 25 C. The cells were then washed by suspending several toothpicks-full in 5 ml water in a 50 ml flask and vortexed. Ten microliters of diluted glusulase (DuPont NEN diluted 10 fold) were added to the spore preparation and mixed gently by vortexing. The preparation was incubated for 2 minutes at room temperature. An equal volume of ether was added--to kill any remaining diploids--and vortexed. The aqueous layer was removed and a ten fold dilution of the treated spores was counted with a hemacytometer. The spores were diluted to 1000 cells ml. 100 microliters were plated on YEPD plates and incubated for 3 days. Colonies were replica plated onto SC-arg, SC-leu, and YEPD plates to select colonies that were *arg8* and *leu2-3*.

2.2 Construction of *S. cerevisiae* Strain STT110

Cells from the haploid parents STT27 (*MATα, arg8, leu2-3*) and D100-4D (*MATa, arg6, ura3, hom3, ilv1, his1, trp2, ade1, met1, gal2*) were mated, sporulated, and random spore analysis was carried out as above to select for leucine and arginine auxotrophs. Complementation experiments were carried out against known *arg6* and *arg8* tester strains of both mating types to select for a *leu2-3, arg6, arg8* strain (Figure 4). The resulting strain, STT110, contained the genotype CAN1, *arg6, arg8*, and *leu2-3*.

2.3 Isolation of Independent Spontaneous *can1* Mutants

Several hundred independently arising spontaneous *can1* mutants were isolated, using strain STT27, for future study. Single colony isolates of STT27 were inoculated into 72 culture tubes each containing 1 ml MOLH liquid medium and incubated at 30 C for 72 h in a shaking incubator. After 72 h, there was a small pellet of cells at the bottom of each tube. The cells were allowed to fully settle for approximately 15 min. 0.8 ml of the supernatant was removed and remaining fluid containing the pellets was vortexed and the entire 0.2 ml from each tube were plated onto 72 separate MOLCH plates and incubated for 4 to 5 days at 30 C. A single random colony from each plate was inoculated into a culture tube containing 3 ml MOLH liquid medium, incubated for 48 h at 30C and 0.85 ml of this culture was placed in a sterile cryovial along with 0.15 ml glycerol. The cryovials were vortexed, frozen, and stored at -80 C.

2.4 Isolation of *can1* Deletion Mutants

Several hundred spontaneous *can1* mutants were isolated from STT110. An isolated colony of STT110 was inoculated into 20 ml M110 media and incubated at 30 C in a shaking incubator for 72 h. 20, 1 ml fractions were centrifuged at 14,000 rpm in a microcentrifuge. 0.8 ml of the supernatant was discarded from each tube and the remaining 0.2 ml containing the pellet was plated onto an M110 + canavanine plate for a total of approximately 20 plates (usually 18 or 19 due to evaporation during incubation). The plated cells were incubated at 30 C for 4 to 5 days and all of the *can1* colonies were picked with sterile toothpicks. Each colony isolate was patched onto an SC plate (as a primary test for reversion) and an M110 + canavanine plate and incubated at 30 C for 4 to 5 days. The *can1* mutants that did not show reversion (no colonies on the SC patch) were inoculated from the M110 + canavanine patch plate into individual culture tubes containing 1.5 ml M110 liquid medium and incubated for 48 h at 30 C in a shaking incubator. The resulting cells were

spun in a microcentrifuge as before and the pellet was washed in 0.9% saline and plated onto SC plates. Those *can1* mutants that did not show reversion were labeled as presumptive deletions.

2.5 Growth Curve for Strain STT110 in M110 Medium

The growth curve was measured in viable cells/ml vs. time (Figure 5). 30 ml M110 liquid medium was inoculated with STT110 in a sterile flask and incubated in a shaking incubator at 30 C. At time intervals of 0, 11.2, 20, 25.5, 31.25, 42, 49.75, 59.25, 68.25 and 89.25 h, a 100 microliter sample of culture was removed and diluted ten fold. Serial dilutions were made and 0.1 ml of these dilutions were plated onto YEPD plates and incubated at 30 C. When the colonies were of sufficient size, they were counted and the viable cells/ml for each time interval was determined. In a separate experiment a standard curve was made relating the absorbance at 660 nm to the number of viable cells/ml of strain STT110 in M110 liquid medium (Figure 6). This was accomplished in a similar manner by growing STT110 in 30 ml M110 liquid medium and measuring the absorbance of the culture at 660 nm using sterile M110 liquid medium as a blank at different time intervals until stationary phase was reached (determined by examining the growth curve). At the same time that the absorbance was read, an aliquot was taken out and serially diluted as before and plated onto a YEPD plate. The colonies were counted and the viable cells/ml for each absorbance reading was determined. This was used to make a standard curve plotting the absorbance at 660 nm vs. viable cells/ml.

3 RESULTS

3.1 Isolation of Independently Arising Spontaneous *can1* Mutants

363 independently arising spontaneous *can1* mutants were isolated from strain STT27. All of them reverted on SC-L was soon realized, however, that colony formation on SC media might have been due to a reversion from *arg8* to *ARG8*, and not just from *can1* to *CAN1*, since a functional arginine biosynthesis pathway would allow a *can1* cell to grow on SC media and give a false indication of reversion at the *CAN1* locus. This complicated the procedure unnecessarily, so a new strain that had a double block in the arginine biosynthesis pathway was needed (because a double reversion in the arginine biosynthesis pathway was very unlikely). Nevertheless these mutants were still important because they were a random collection of completely independently arising spontaneous forward *can1* mutants (because only one mutant per culture was collected). These *can1* mutants were stored at -80 C for future use in the laboratory.

3.2 Isolation of *can1* Deletion Mutants

To date our data reveal two presumptive deletions identified. Approximately 4×10^7 STT110 cells were screened and over 1,000 *can1* mutants were isolated. Each *can1* mutant was screened for the ability to revert to *CAN1* on SC media. Two *can1* mutants were shown not to revert on SC media. Those two *can1* mutants that did not revert were labeled as presumptive deletions and isolated for further study. More mutants are currently being isolated and checked for their ability to revert, and a deletion frequency will be established once several deletions have been isolated. The results so far have shown that deletion mutations are fairly rare events.

3.3 Growth Curve of Strain STT110 in M110 Medium

The need to establish a growth curve for STT110 in M110 medium was twofold. First, the number of viable cells per milliliter of culture needed to be known to determine a deletion frequency. Second, the time that stationary phase began for STT110 in M110 medium was important in reducing the time it takes to isolate a set of *can1* mutants. The growth curve illustrated in Figure 5 demonstrates that stationary phase began after approximately 36 hours, with 3.4×10^{7} viable cells per milliliter. A standard curve (Figure 6) relating the absorbance at 600 nm to the number of viable cells per milliliter of culture was generated to allow a researcher to quickly determine the cell density of a particular culture by reading its absorbance at 600 nm and applying the standard curve to that reading. This was necessary as the cell density varies slightly from culture to culture.

4 DISCUSSION

The goal of this project was to develop a genetic system to isolate deletion mutations on an unaltered eukaryotic chromosome. The molecular spectrum of these deletions will be determined to test the hypothesis that palindromic or quasipalindromic DNA sequences are associated with the induction of deletion mutations in eukaryotes. Once several deletions have been isolated and confirmed by PCR amplification and agarose gel electrophoresis, a low resolution map of the deletions will be made by generating a series of plasmids containing the *CAN1* gene, each of them gapped in a different region of *CAN1*. Figure 7 illustrates the effect of a gapped plasmid recovering sequences from a non-mutated region or a mutated region of a *can1* mutant. The plasmid that has a double strand gap that includes the mutant region within the *CAN1* locus will allow the cell to survive in the presence of canavanine. By determining the general region within *CAN1* that each deletion is located, these regions can be sequenced and their molecular spectra established.

Several factors made the search for isolating deletions at *CAN1* in *S. cerevisiae* very challenging and often frustrating. One was the need to control arginine biosynthesis and arginine transport. It was realized that after unsuccessfully trying to isolate *can1* mutants for several weeks, we needed to switch to a medium that did not repress the general amino acid permease (one with a low nitrogen content). This led to the search for a medium with the minimal growth requirements that would still allow the cells to grow fairly quickly. We also needed to identify a strain that grew the best in such a medium among the many strains we isolated. This led to strain ST127, which was grown in MOLH (minimal + ornithine, leucine, and histidine) and then plated onto MOLCH (MOLH + canavanine) to select for *can1* mutants.

The problem with STT27 was that it had only one mutation in its arginine biosynthesis pathway, and this interfered with our screen for *can1* mutants that lost their ability to revert to *CAN1*; some of the "*CAN1*" revertants might actually be *ARG8* revertants. Thus the we generated a strain that was *arg6 arg8*. This led to strain STT110.

Another factor in the search for deletions was the fact that deletions are rare events, and in order to isolate many of them, we needed to develop a method to screen hundreds of millions of cells per cycle. This led to the method of inoculating 20 ml of M110 with strain STT110 and plating all of the resulting culture onto several M110 + canavanine plates and checking every *can1* colony for its ability to revert to *CAN1*, initially by patching a *can1* colony onto SC and then plating approximately 1×10^8 cells onto SC. Although this is not a way to isolate independently arising mutants, it is a relatively fast way to screen approximately 1×10^7 cells at one time. In any event all of the deletions will be sequenced, and if two are found that came from the same culture to be identical, only one will be counted as an independently arising deletion. This is important in determining the correlation between DNA secondary structure (palindromes and quasipalindromes) and deletion formation. If the same deletion event were counted twice, it would skew the statistical analysis of this correlation.

The system that we developed to study deletions has many useful characteristics. One of them is the ability to screen approximately 1×10^7 cells per cycle. This is important because of the rare occurrence of spontaneous deletions in *S. cerevisiae*. Bitoun and Zamir (1988) estimated that spontaneous deletions occur in yeast at a frequency that ranges from 1×10^{-5} to 1×10^{-10}. The fact that deletions are rare in *S. cerevisiae* required us to develop the system of growing a *CAN1, arg6,*

arg8, leu2-3 strain (STT110) in 20 ml of M110 medium and plating the entire cell culture onto a medium that selects for *can1* mutants (M110 + canavanine).

Another useful characteristic of this system is that we can know the number of viable cells that are being screened in each cycle by measuring the absorbance at 600 nm and converting this number to viable cells ml using the standard curve. Also, the growth curve showed that stationary phase was reached earlier than was previously thought, and this was used to reduce the time spent on each cycle.

One very important aspect of this system is the ability to study deletion events that occur in a gene on an unaltered chromosome. Bitoun and Zamir had developed a system to isolate deletions in *S. cerevisiae*, but this system utilizes a tandem duplication of a gene on a plasmid and the minimal length of a deletion that can be studied is 2.8 kilobase pairs (Bitoun and Zamir, 1988). Theoretically, our system can be used to study deletions that are as small as 2 base pairs to those that delete the entire gene. Preliminary evidence indicates that 2 deletions have arisen from a total population of approximately 4×10^7 cells.

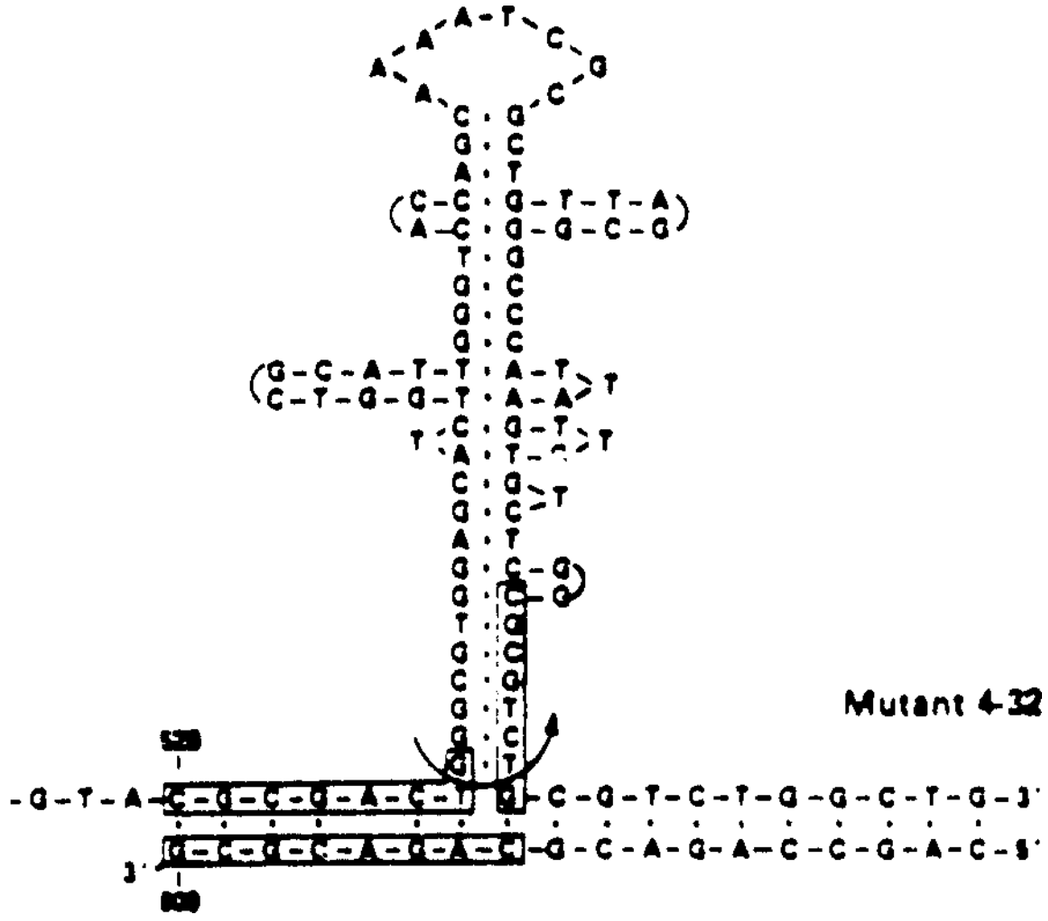


Figure 1. Quasipalindrome model for deletion formation at *LacI* in *E. coli* (Shapper et al., 1986).

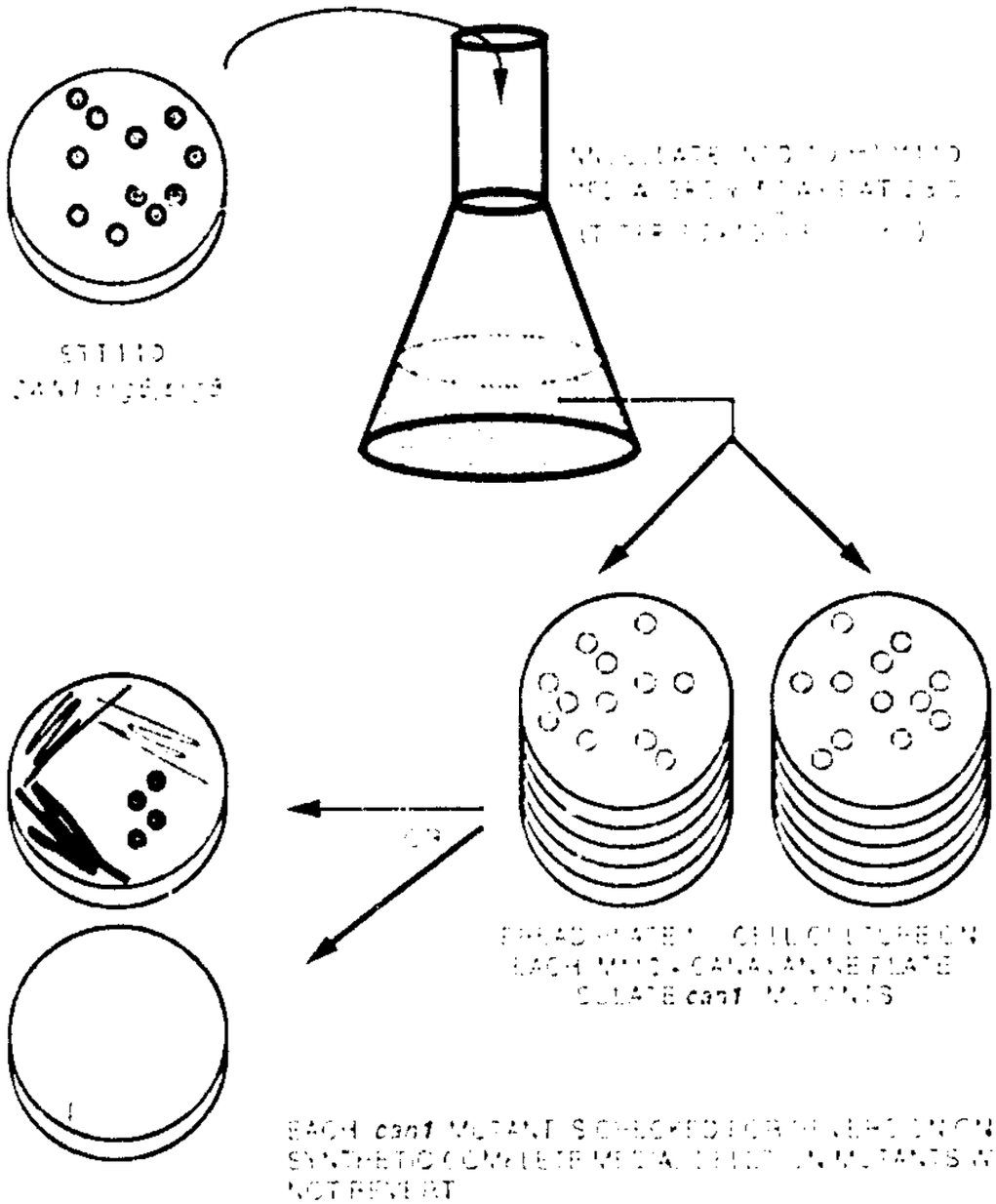
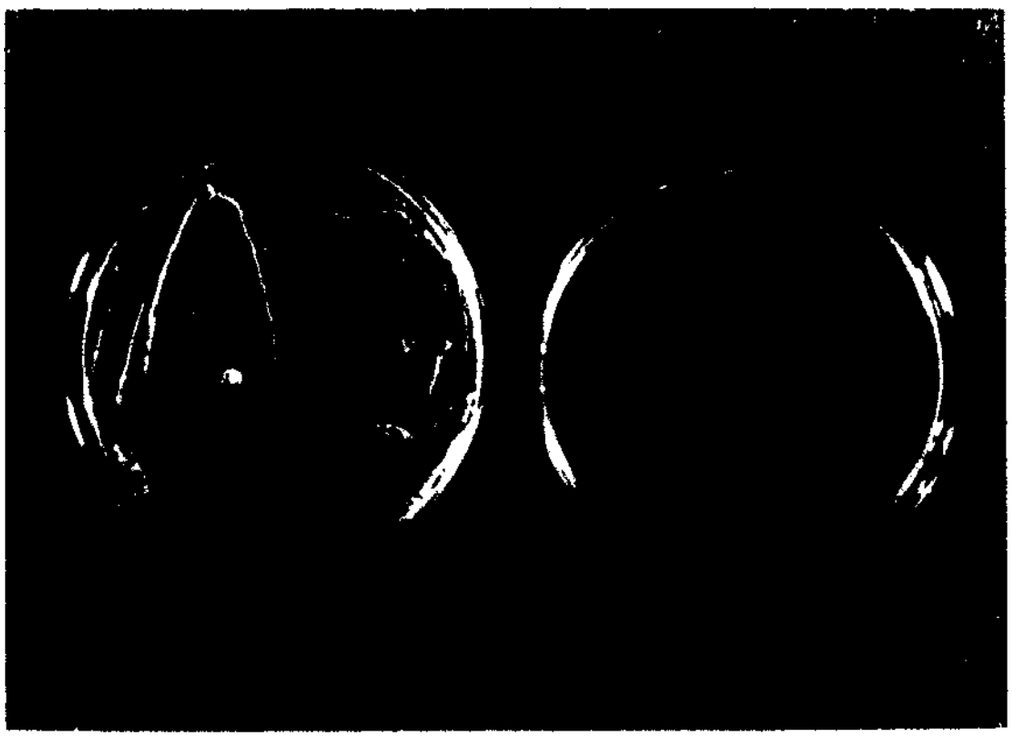


Figure 2. Isolation of spontaneous *can1* deletion mutants in strain STT110 (Taylor et al., 1990).



COMPLEMENTATION EXPERIMENT

STRAIN	*MATa, arg6*	*MATα, arg6*	*MATa, arg8*	*MATα, arg8*
STT104		+		
STT106	+			
STT105				+
STT110				

Figure 4. Complementation test results. STT110 does not complement with an *arg6* or *arg8* strain of either mating type (Taylor et al., 1990).

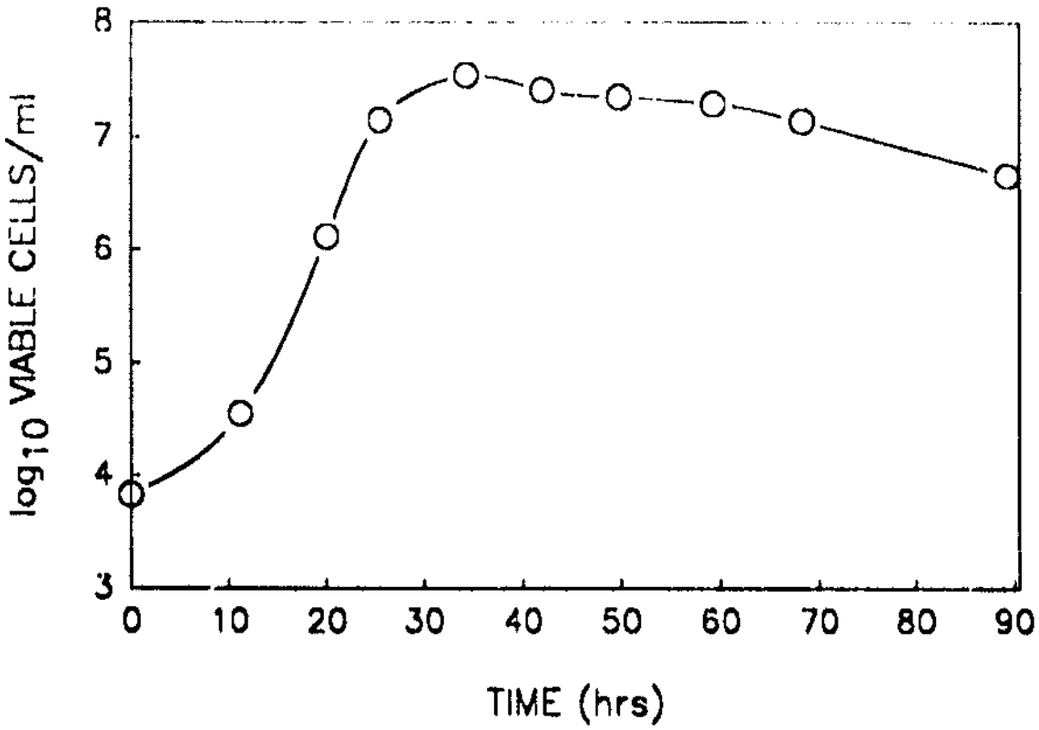


Figure 5. Growth curve for STT110 in M110 medium.

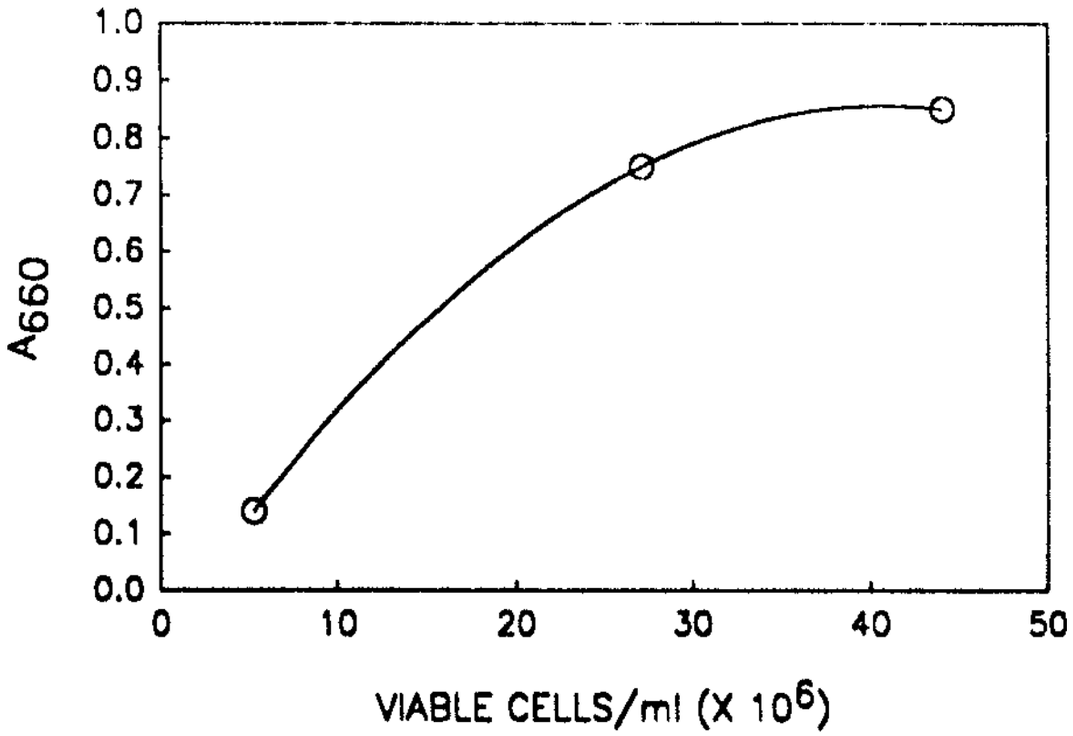


Figure 6. Standard curve relating A_{660} to viable cells/ml for strain STT110 in M110 medium.

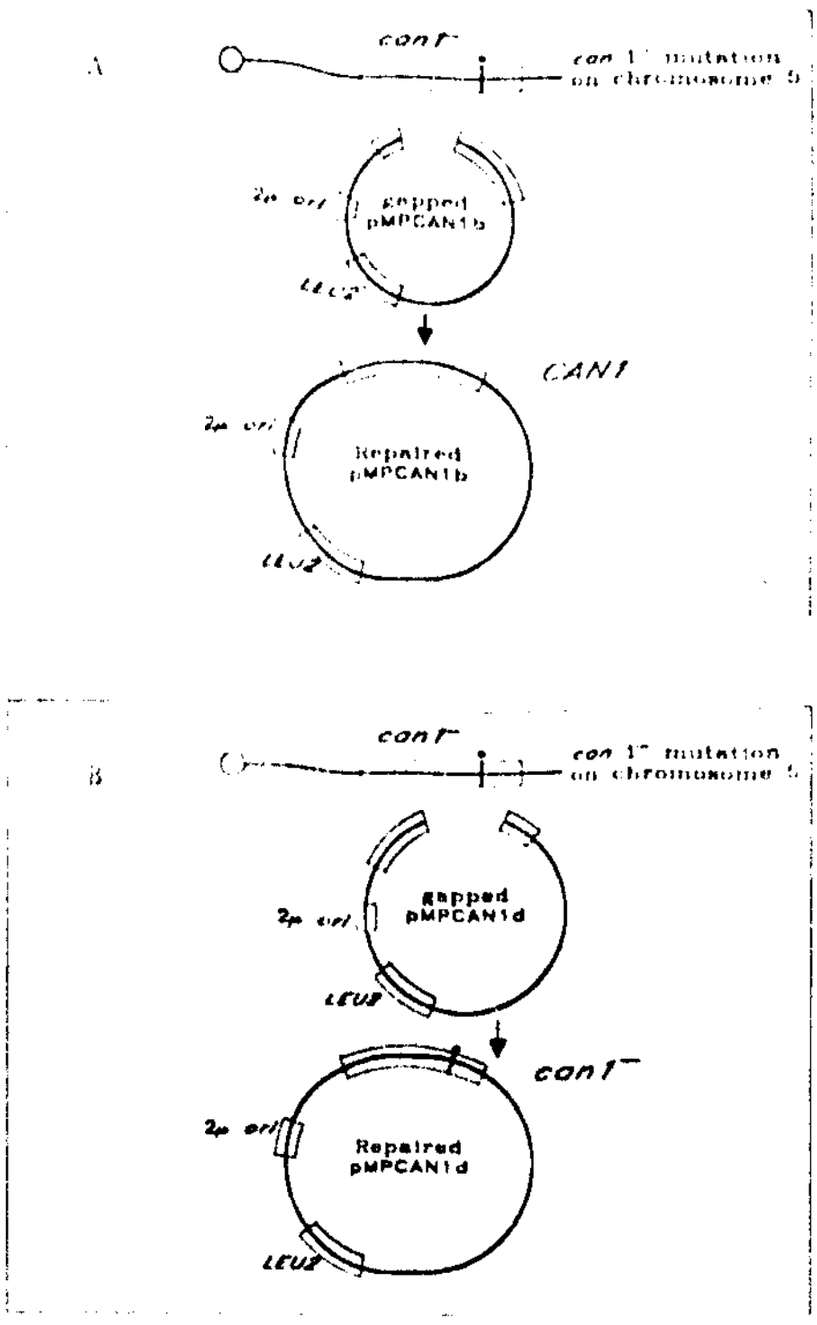


Figure 7. An illustration of the effect of a gapped plasmid recovering chromosomal sequences from a non-mutated region (panel A) or a mutated region (panel B) of a *can1* mutant.

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APPENDIX

Media for CAN1

YEPD Media - Plates and Broth

	1 liter
Bacto Peptone	20 g
Bacto Yeast Extract	10 g
Dextrose	20 g
Agar (for plates only)	20 g
dH_2O	1000 ml

SC Media - Plates and Broth

	1 liter
Bacto Yeast Nitrogen Base (w o Amino Acids)	6 g
Dextrose	20 g
dH_2O	960 ml
Threonine Stock Solution	7.5 ml
Tryptophan Stock Solution	10 ml
SC Stock Solution	40 ml
Agar (for plates only)	20 g

Note: To make SC-arg or any other SC-___ solution, use appropriate SC ___ stock solution in place of SC stock (or in the case of SC-thr or SC-trp, leave out threonine or tryptophan stock).

Threonine Stock Solution

	200 ml
L-Threonine	4 g
dH_2O	200 ml

Filter sterilize and refrigerate.

Tryptophan Stock Solution

	200 ml
L-tryptophan	800 mg
dH_2O	200 ml

Filter sterilize and refrigerate.

SC Stock Solution

	360 ml
Adenine	0.24 g
Arginine	0.24 g
Histidine	0.24 g
Leucine	0.36 g
Lysine	0.36 g
Methionine	0.24 g
Uracil	0.24 g
dH_2O	360 ml

Filter sterilize and refrigerate.

Note: For an SC-___ stock, leave out the desired amino acid or nucleotide.

MOLH Liquid Media

	1.0 L
Bacto Yeast Nitrogen Base (w.o amino acids)	6 g
Dextrose	20 g
dH_2O	1000 ml
Orn-Leu-His Stock Solution	10 ml

MOLCH Media

	1.0 L
Bacto Yeast Nitrogen Base (w.o amino acids)	6 g
Dextrose	20 g
Agar (for plates only)	20 g
dH_2O	1000 ml
Orn-Leu-His Stock Solution	10 ml
L-canavanine Stock Solution	5 ml

MI10 Liquid Media

	1.0 l
Dextrose	20 g
Bacto Yeast Nitrogen Base (w o amino acids)	6 g
Orn-Leu-His Stock Solution	10 ml
Methionine Stock Solution	4 ml
Threonine Stock Solution	7.5 ml
Tryptophan Stock Solution	10 ml
Adenine Stock Solution	40 ml
Uracil Stock Solution	40 ml
dH$_2$O	890 ml

MI10 + Canavanine Plates

	1.0 l
Dextrose	20 g
Agar	20 g
Bacto Yeast Nitrogen Base (w o amino acids)	6 g
Orn-Leu His Stock Solution	10 ml
Methionine Stock Solution	4 ml
Threonine Stock Solution	7.5 ml
Tryptophan Stock Solution	10 ml
Adenine Stock Solution	40 ml
Uracil Stock Solution	40 ml
L-canavanine Stock Solution	5 ml
dH$_2$O	890 ml

Orn-Leu-His Stock Solution

	400 ml
L-ornithine	8 g
L-histidine	0.4 g
L-Leucine	1.2 g
dH$_2$O	400 ml

Filter sterilize and refrigerate.

L-canavanine Stock Solution

400 ml

L-canavanine sulfate 1.6 g
dH$_2$O 400 ml

Filter sterilize and refrigerate.

Methionine Stock Solution

360 ml

L-methionine 2.4 g
dH$_2$O 360 ml

Filter sterilize and refrigerate.

Adenine Stock Solution

360 ml

Adenine 0.24 g
dH$_2$O 360 ml

Filter sterilize and refrigerate.

Uracil Stock Solution

360 ml

Uracil 0.24 g
dH$_2$O 360 ml

Filter sterilize and refrigerate.

Sporulation Media

	1.0 L
Potassium Acetate	10 g
Yeast Extract	1 g
Dextrose	0.5 g
Agar	20 g
L-leucine Stock Solution	3 ml
NaOH Pellet	1
dH$_2$O	1000 ml

L-leucine Stock Solution

	100 ml
L-leucine	1 g
dH$_2$O	100 ml

Filter sterilize and refrigerate.