GENETIC ANALYSIS OF MUTATIONS AFFECTING GROWTH OF *SACCHAROMYCES CEREVISIAE* AT LOW TEMPERATURE

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ABSTRACT

A large number of genes control growth of the yeast *Saccharomyces cerevisiae* at low temperatures (< 10°). Approximately 47 percent of the mutants selected for inability to grow at 4–5°C show increased sensitivity to cycloheximide. In 3 of 4 cases tested, supersensitivity to cycloheximide and inability to grow at the low temperature segregate together and thus appear to be effects of the same mutation. Since many cold-sensitive mutants of bacteria have been found to have altered ribosomes and since cycloheximide resistance in yeast can be caused by ribosomal changes, this suggests that the mutants having low-temperature-sensitive growth may be defective in ribosome-assembly processes at the low temperatures. Two of the *lts* loci, *lts1* and *lts3* have been located on chromosome *VII* and another two, *lts4* and *lts10* on chromosome *IV*. A mutation, *cyh10*, conferring cycloheximide resistance, but not cold sensitivity, has been located close to the centromere on chromosome *II*.

COLD-sensitive mutants of *Escherichia coli* (GUTHRIE, NASHIMOTO and NOMURA 1969) and of *Salmonella typhimurium* (TAI, KESSLER and INGRAHAM 1969) have contributed significantly toward the elucidation of ribosome synthesis in these organisms. In this paper we present a genetic analysis of mutants of the yeast, *Saccharomyces cerevisiae* that are unable to grow on complete media below 10°. A large proportion of the mutants described show an altered level of resistance to the antibiotic cycloheximide, an inhibitor of protein synthesis. It has been established that resistance to cycloheximide is effected through altered ribosomes in *Saccharomyces pastorianus* (SIEGEL and SISLER 1965), in *Saccharomyces fragilis* (RAO and GROLLMAN 1967), and in *Saccharomyces cerevisiae* (COOPER, BANTHORPE and WILKIE 1967). The property of cold sensitivity coupled with modified level of cycloheximide resistance of a number of mutants described herein indicates that some of these mutants may be defective in ribosome assembly at low temperatures.

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MATERIALS AND METHODS

Strains: Most of the strains employed in these studies were from the collection of DR. R. K. MORTIMER, University of California, Berkeley as such, or were derivatives thereof. The genotypes of the strains obtained from R. K. MORTIMER are as follows:

X2180–1A: *a gal2*
X2180–1B: *α gal2*
XS144: *a/α leu1/+ trp5/+ cyh2/+ met13/+ aro2/+ lys5/+ ade5,7/+*
X2956–2C: *a ade1 gal1 trp1 ura3 his2 leu1 arg4 met14 lys7 lts10*
X901–35C: *a thr2 tyr4 trp5 leu1 ade6 lys1 his6 ura1 arg4–1 thr1 CUP1*

The following strains were derived by mutation and by hybridization and sporulation from the above:

XT1177–S24: *a trp5–18 leu1 gal2 lts1* (derived from X901–35C)
MH2: *α cyhx gal2 lts2* (derived from X2180–1B)
XS144–S84: *α trp5 cyh2 met13 aro2 lys5 ade5,7 lts3*
XA6: XT1177–S24/MH2
XA6–45B: *a leu1*

The *lts* genes designated in these genotypes were apparently of spontaneous origin. The remaining *lts* genes were isolated from XA6–45B following mutagenesis.

Media and Scoring: Temperature-sensitive growth was scored at 4–5°C. The media used were yeast extract-peptone-dextrose (YEPD) and synthetic complete (SC) agar media described previously (MANNEY 1964). After it was established that final scoring of ability to grow at low temperature was the same on both SC and YEPD and that longer periods of incubation were required in order to score on SC, YEPD was routinely used to score the low-temperature sensitivity. Nutritional requirements were scored on SC lacking the particular supplement. Cycloheximide was scored at 30° on SC containing 1 μg/ml cycloheximide.

Four-spore asci were dissected by the method described by JOHNSTON and MORTIMER (1959). Replica plating technique was used for scoring the phenotypes.

Mutagenesis: Cold-sensitive mutants were induced by treatment with 3.0% ethylmethane sulfonate for 1 hour at 30°. This treatment resulted in 35% survival of treated cells. After the treatment EMS was inactivated with 5% solution of sodium thiosulfate. Cells were then washed and appropriate dilutions were plated on YEPD containing only 1% dextrose. After 4 days of incubation at 30° these plates were replica-plated onto fresh YEPD. The replica plates were incubated at 4–5°C for 3 weeks. Colonies that failed to grow on the replica at the low temperature were isolated from the master plates and tested further.

Cycloheximide-resistant mutants were induced by UV irradiation from a germicidal lamp with a dose of 500 ergs/mm² at the surface of the plates. The medium used was SC agar containing 1, 2 or 5 mg cycloheximide per liter.

RESULTS

Discovery of mutants affecting growth at low temperature: Detailed genetic analysis of low-temperature-sensitive (*lts*) growth was undertaken when incubation of spore clones from 90 dissected tetrads at 4° for four weeks revealed the following: ten tetrads segregated 2:2 for growth and failure of growth under these conditions, and 63 and 17 tetrads segregated 1:3 and 0:4 respectively. The growth medium was YEPD agar, on which all segregants grew well at the usual temperature of incubation (30°). The lack of ambiguity in distinguishing sensitive from normal strains may be appraised from Figure 1A in which each row represents the four spores from one tetrad. The parent diploid was heterozygous for one gene (*lts5*) affecting the low-temperature-sensitive growth.

The ratios of various tetrad types given above are consistent with a hypothesis that complementary action of two independently segregating genes are required

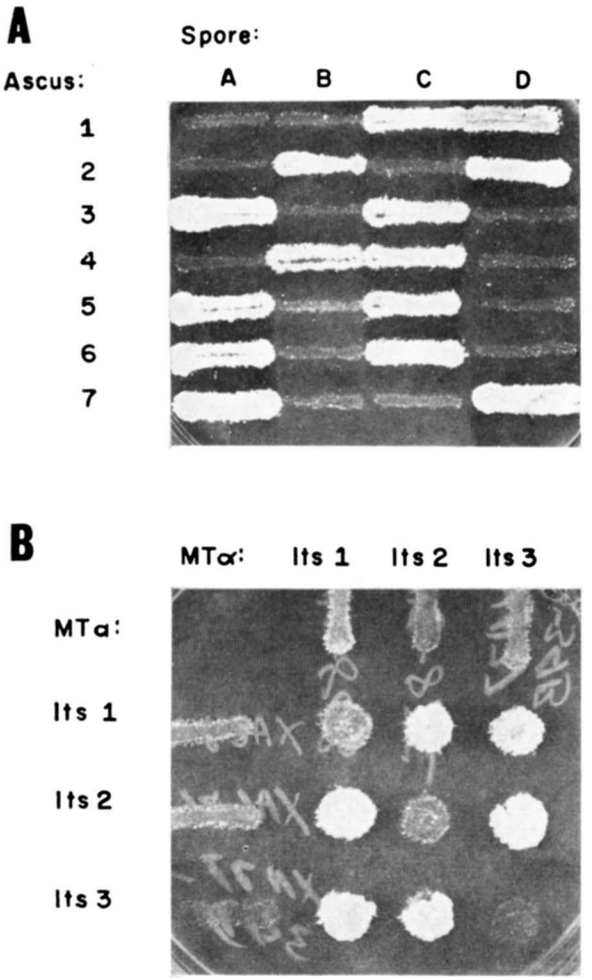

FIGURE 1.—Low-temperature-sensitive growth phenotype (*lts*) exhibited by cells stored for three weeks at 4–5°C on YEPD medium. A. Ascospore cultures from a diploid that was heterozygous for *lts5*. The four cultures in each row were grown at 30° from the four spores from a single ascus. They were then replica plated to the plate shown in the photograph and incubated at the low temperature. B. Haploid mutants and mating mixtures containing diploids and unmated haploids. Haploid strains in each mating type, bearing the mutations *lts1*, *lts2* and *lts3* were mixed on YEPD agar and incubated overnight at 30° to allow mating and growth of the resulting diploids. They were then replica plated to the plate shown and incubated at the low temperature. The faint streaks at the left side and at the top are the mutant haploid strains. The nine round spots in the center are the mating mixtures containing diploids.

for growth at the low temperature. The parent diploid was fortuitously heterozygous for *leu1*, *trp5* and an unidentified locus for resistance to cycloheximide *cyhx*, unlinked to *trp5*. It was found that all spore clones capable of growth at the low temperature were sensitive to cycloheximide and that all but three of these carried the *TRP5* allele. This indicates that one locus (*lts1*) affecting low-temperature-sensitive growth is linked to *trp5* and the other, *lts2*, is linked to *cyhx*. That

the above is, in fact, the case was confirmed as follows. Making use of the indicated linkage relationships, the genotype with respect to *lts* loci of each of the 4 spores in a tetrad segregating 1:3 for growth: non-growth at low temperature, could be deduced. Four types of diploids carrying *lts1* and *lts2* in various combinations were constructed:

$$1.\ \frac{LTS1}{lts1}\ \frac{LTS2}{LTS2} \qquad 2.\ \frac{LTS1}{LTS1}\ \frac{LTS2}{lts2} \qquad 3.\ \frac{LTS1}{lts1}\ \frac{lts2}{lts2} \text{ and } 4.\ \frac{lts1}{lts1}\ \frac{LTS2}{lts2}$$

The diploids were sporulated and tetrads were dissected and analyzed for growth at low temperature. The segregation of other pertinent markers was also followed.

The data for diploid of type 1 are given in Table 1. All 45 tetrads analyzed segregated 2+:2−. There was no recombination between *trp5* and *lts1* in this sample. Eleven three-spored asci analyzed from this diploid also gave results consistent with the above. Six tetrads from another diploid heterozygous for *lts1* and *trp5* segregated 2+:2− and all tetrads were of PD type with respect to these two genes. Thirty-four four-spored tetrads from a type 2 diploid were analyzed. Thirty-three of 34 segregated 2+:2− and all *LTS* spores were cycloheximide-sensitive. The remaining tetrad segregated 1+:3−. The one aberrant tetrad included a spore which was *lts* and sensitive to cycloheximide. This spore appears to have mutated at another *lts* locus, because it was also more cold sensitive than the other *lts* spores from the cross.

Ten four-spored asci from diploids of types 3 and 4 each were analyzed. All produced 0+:4− ratios of spores for growth and failure of growth at 4°.

From the data described above, we conclude that the original diploid was heterozygous for two loci affecting growth at low temperatures. Presence of the dominant alleles at both of these loci is necessary for growth at the low temperature. Further, *lts1* is closely linked to *trp5* and *lts2* is closely linked to *cyhx*. *Cyhx* and *lts2* may, in fact, be allelic.

Association of cold sensitivity with loci conferring resistance to cycloheximide: Since failure to obtain any spore that was recombinant between *lts2* and *cyhx* in a sample of 123 complete asci indicated possible allelism of these two mutations, two series of mutants were induced to determine if other *lts* mutations also exhibit a pleiotropic effect with respect to cycloheximide resistance and *vice versa*. In the first series 70 independent UV-induced cycloheximide-resistant mutants were isolated and checked for ability to grow at 5°. Only 4 of the 70 mutants failed to

TABLE 1

Segregation of lts1

XA22: $\frac{a}{\alpha}\ \frac{leu1}{+}\left(\frac{+}{lts1}\ \frac{+}{trp5}\right)$

Gene pairs	PD	NPD	T	Total
leu1–trp5	27	0	19	46
leu1–lts1	27	0	18	45
lts1–trp5	45	0	0	45

grow at the low temperature. In the second series 360 low-temperature-sensitive mutants were obtained following EMS mutagenesis. The frequency of such mutations was surprisingly high. There was considerable variation in the extent of growth on the replica plates that were employed for selection of the mutants. Only those colonies which were unambiguously *lts* were scored as such. Twenty plates contained a total of 3414 colonies among which 360 (10.5%) were *lts*.

One hundred thirty-four mutants having the *lts* phenotype, no growth at 5°, normal growth at 30°, were checked for cycloheximide resistance. None were more resistant to the antibiotic than the parent strain, however, 63 of 134 mutants had become significantly more sensitive to it. They produced no appreciable growth when replica plated on agar media containing 0.5 mg cycloheximide per liter, whereas the parent strain produced almost confluent growth. The level of resistance of the parent strain is the same as that of the wild-type strains which are customarily classified as cycloheximide-sensitive.

One of the four UV-induced mutants that were resistant to cycloheximide and had low-temperature-sensitive growth, and 4 of the 63 EMS-induced *lts* mutants that were supersensitive to cycloheximide were subjected to recombination analysis to determine if the two phenotypes were an effect of the same mutation. The results of this analysis are given in Table 2. The two properties segregated independently in the hybrid of the UV-induced mutant (SA5) and in one (SB9) of the four EMS-induced mutations. It appears that the two phenotypes are determined by the same mutation in the remaining three cases. Incidently, the gene, designated here as *cyh10*, responsible for resistance to cycloheximide of the mutant SA5, has been located on chromosome II. It has a second division segregation frequency of approximately 3 percent. In 70 asci analyzed from hybrids that were heterozygous for a number of centromere-linked genes, *cyh10* segregated at the second division in only two asci.

Complementation among lts *mutants:* Complementation tests were carried out among 9 *lts* mutants. Three spontaneous, one UV-induced, and five EMS-induced mutants were tested in all possible diploid combinations. Each of the mutants tested complemented all the remaining mutants. An appreciation of the extent of complementation may be obtained from Figure 1B, which shows the growth of *lts1*, *lts2* and *lts3* and of all possible pair-wise mating mixtures of these mutants. One of the mutants (SB21) complements the eight other mutants only poorly. Unless growth of some of the cultures is due to interallelic complemen-

TABLE 2

Segregation of cycloheximide resistance and lts *phenotype in crosses involving various* lts *mutants*

Mutant	Origin	Phenotype	No. spores examined	No. recombinant between *lts* and *cyh*	Percent recombinant
SA5	UV-induced	Cycloheximide resistant	60	24	40
SB9	EMS-induced	Cycloheximide supersensitive	32	12	37.5
SB20	EMS-induced	Cycloheximide supersensitive	34	0	0
SB34	EMS-induced	Cycloheximide supersensitive	27	0	0
SB101	EMS-induced	Cycloheximide supersensitive	192	0	0

TABLE 3

Data bearing on location of lts3

Gene pairs	PD	NPD	T	Total
lts3–leu1	5	7	26	38
lts3–cyh2	17	0	22	39
lts3–met13	34	0	5	39
lts3–aro2	39	0	0	39
lts3–lys5	32	0	7	39
lts3–ade5,7	11	2	24	37
met13–aro2	34	0	5	39
met13–lys5	28	0	12	40

tation, the 9 mutants represent 9 different loci. Even if interallelic complementation is involved in some cases, recombination data indicate that the 9 mutants represent at least 5 different loci. Data have already been given that *lts1*, *lts2* are located in different portions of the genome and, as will be shown in the following sections, *lts3*, *lts4* and *lts5* are three distinct loci, each different from *lts1* and *lts2*.

Location of lts3: A mutation (*lts3*), of spontaneous origin, affecting low-temperature-sensitive growth, has been located on chromosome VII. It is closely linked to *aro2* (see Table 3) with which it did not recombine in 39 complete asci that have been analyzed.

The lts4 *and* lts10 *loci are linked to each other and are located in Chromosome* IV: After a preliminary set of data from crosses involving *lts4* and *lts10* indicated centromere linkage of both of these two mutations, crosses were made to identify the centromeres to which the genes were linked. Diploids that were heterozygous for *lts4* or *lts10* or both and simultaneously heterozygous for various centromere-linked genes were constructed and complete tetrads derived from these diploids were analyzed. As shown in Table 4 these two *lts* loci are located very close to the *trp1* locus, which is linked to the centromere of chromosome IV and segregates at second division with a frequency of about one percent (see MORTIMER and HAWTHORNE 1966). *lts4* and *lts10* segregated independently of a number of other centromere markers.

From crosses in which a number of known centromere-linked genes were segregating, the first division spore arrays could be determined unequivocally. These hybrids were heterozygous for a minimum of 9 centromere-linked genes. *lts4* segregated during the second division in 3 of 71 asci, whereas no second division segregation tetrads were obtained in 35 asci analyzed for *lts10*. Thus, the second division segregation frequency of *lts4* is approximately 4 percent. One

TABLE 4

Linkage of lts4 *and* lts10 *to* trp1 *locus*

lts gene	Tetrad type with respect to *trp1* PD	NPD	T	Total
lts4	34	0	1	35
lts10	35	0	0	35

TABLE 5

Data showing centromere linkage of lts5

Centromere marker and chromosome tested	Tetrad types obtained PD	NPD	T	Total
leu2, III	14	15	6	35
lts4, IV	7	7	0	14
leu1, VII	24	32	4	60
his6, IX	15	10	12	37
met14, XI	19	19	0	38
pet8, XIV	17	21	0	38

of the hybrids was heterozygous for *lts4* segregated at the second division both *lts10* and *trp1* segregated at the first division. From this information two of 6 possible arrangements of these 3 loci can be ruled out. The order of these loci can be *lts4*–centromere–*lts10–trp1* or *lts4*–centromere–*trp1–lts10* or *lts10*–centromere–*trp1–lts4* or centromere–*trp1–lts10–lts4*; but not *lts lts4 trp1* and *trp1 lts4 lts10*, regardless of the centromere location. The last two arrangements would involve invoking multiple crossing over to explain the mode of segregation described above.

Centromere linkage of lts5: It has been established that this mutation is closely linked to its centromere. Though it has not yet been possible to assign it to a particular chromosome, it has been shown (see Table 5) that this gene is not in chromosomes *III, IV, VII, IX, XI* or *XIV*. Thirty-eight complete asci were analyzed from a diploid that was heterozygous for 5 known centromere-linked genes. In these tetrads it was possible to determine at which division of meiosis *lts5* segregated. In all 38 tetrads this locus segregated at the first division. If we consider the relation

$$T = w + y - \frac{3}{2}wy \quad \text{(PERKINS 1949)}$$

where T is tetratype frequency and w and y are the second division segregation frequencies of 2 genes, and we substitute the value for *leu1* which was involved in the maximum number of asci studied, then, taking T as 6.67 (Table 5) and second division segregation frequency of *leu1* as 4.9 (MORTIMER and HAWTHORNE 1966) we obtain a negative value (~ -0.3) for the second division segregation frequency of *lts5*. This simply means that *lts5* is very close to its centromere and that all tetratype asci with respect to *lts5* and *leu1* were due to crossovers between *leu1* and its centromere.

DISCUSSION

It appears that there are a large number of genes which can mutate to prevent growth of this organism at low temperatures (<10°). In the foregoing section data on segregation of a minimum of 6 loci have been presented. Two loci, *lts1* and *lts3*, have been located in chromosome *VII*. Another two loci, *lts4* and *lts10* are in chromosome *IV*, both closely linked to each other and to the centromere. That *lts2* and *lts5* are different from each other and each distinct from the four

genes mentioned above is evident when we take into account the following facts: The *lts5* locus is tightly linked to its centromere but is not in chromosome *IV* or *VII* and *lts2* is not centromere-linked, and also is not linked to *lts1* or *lts3*. Thus, these six mutations are definitely at six different loci. On the basis of complementation, all 9 mutants tested are at 9 different loci. However, the possibility of interallelic complementation in some cases is not ruled out.

The involvement of a large number of loci is also borne out by the frequency with which such mutations arise following mutagenesis. We have found, as indicated before, that following a 1 hour treatment with 3.0% ethylmethanesulfonate approximately 10.5% of the surviving cells were low-temperature sensitive. Approximately 35% of the cells survive after this treatment. As a basis of comparison, it may be pointed out that under the same conditions of treatment, the frequency of mutations at the *trp5* locus is about 0.3%. This indicates that there are a large number of loci which influence growth of this yeast at temperatures below 10°. In this connection it is important to note that all 4 spontaneous mutants and 6 induced mutants tested by complementation and/or segregation analysis appear to be at different loci.

A significant proportion of genes determining the low-temperature-sensitive growth also express themselves by an altered sensitivity to the antibiotic cycloheximide. This aspect was extensively explored when it was found that the spontaneous mutation *lts2* is allelic to a locus conferring resistance to cycloheximide. However, only four of seventy induced cycloheximide-resistant mutants failed to grow at low temperatures and in one mutant which was further analyzed, the properties segregated independently. A strong case for the association of modified level of sensitivity with the inability to grow at the low temperature is found when *lts* mutants are selected and tested for growth on the media with the inhibitor. Thus, 63 of 134 such mutants were more sensitive to the antibiotic than the parent strain. Analysis of 4 of these supersensitive mutants revealed that in three of them the two properties segregate as if they result from an alteration in the same gene (see Table 2). Reversion analysis has strengthened the conclusion drawn from the segregation data. Four spontaneous *LTS* revertants were recovered from each of the *lts6* and *lts7* mutants; all had simultaneously reverted for the cycloheximide effect. It is perhaps worth noting that all the induced *lts* mutants that exhibited any change in the cycloheximide sensitivity were in one direction—all were more sensitive to the drug than the parent strain, whereas in the case of spontaneous mutant *lts2*, it is the allele which determines an enhanced level of resistance that does not allow growth at the low temperature.

It has been shown that it is the modified ribosomes that confer resistance to cycloheximide in *Saccharomyces fragilis* (SIEGEL and SISLER 1965) and in those cycloheximide resistant mutants of *Saccharomyces cerevisiae* that affect protein synthesis (COOPER, BANTHORPE and WILKIE 1967). It has further been established that the alteration is in the 60s ribosomal subunit (RAO and GROLLMAN 1967). These considerations coupled with the fact that cold-sensitive mutants of *Escherichia coli* (GUTHRIE, NASHIMOTO and NOMURA 1969) and of *Salmonella typhimurium* (TAI, KESSLER and INGRAHAM 1969) have been a rich source of mutants defective in ribosome assembly in these organisms, prompts us to specu-

late that the cold-sensitive mutants that have an altered level of sensitivity to cycloheximide may be defective in ribosome assembly at low temperatures.

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LITERATURE CITED

COOPER, D., D. V. BANTHORPE and D. WILKIE, 1967 Modified ribosomes conferring resistance to cycloheximide in mutants of *Saccharomyces cerevisiae*. J. Mol. Biol. **26**: 347–350.

GUTHRIE, C., H. NASHIMOTO and M. NOMURA, 1969 Structure and function of E. *coli* ribosomes. VIII. Cold-sensitive mutants defective in ribosome assembly. Proc. Natl. Acad. Sci. U.S. **63**: 384–391.

JOHNSTON, J. R. and R. K. MORTIMER, 1959 Use of snail digestive juice in isolation of yeast spore tetrads. J. Bacteriol. **72**: 292.

MANNEY, T. R., 1964 Action of a super-suppressor in yeast in relation to allelic mapping and complementation. Genetics **50**: 109–121.

MORTIMER, R. K. and D. C. HAWTHORNE, 1966 Genetic mapping in Saccharomyces. Genetics **53**: 165–173.

PERKINS, D. D., 1949 Biochemical mutants of smut fungus *Ustilago maydis*. Genetics **34**: 607–626.

RAO, S. S. and A. P. GROLLMAN, 1967 Cycloheximide resistance in yeast: A property of 60s ribosomal subunit. Biochem. Biophys. Res. Commun. **29**: 696–704.

SIEGEL, M. R. and H. D. SISLER, 1965 Site of action of cycloheximide in cells of *Saccharomyces pastorianus*. III. Further studies on the mechanism of action and the mechanism of resistance in *Saccharomyces* species. Biochim. Biophys. Acta **103**: 558–567.

TAI, PHANG-CHENG, D. P. KESSLER and J. INGRAHAM, 1969 Cold-sensitive mutations in *Salmonella typhimurium* which affect ribosome synthesis. J. Bacteriol. **97**: 1298–1304.

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