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Identification of a Second *trans*-Acting Gene Controlling Maltose Fermentation in *Saccharomyces carlsbergensis*

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Maltose fermentation in *Saccharomyces* spp. requires the presence of a dominant *MAL* locus. The *MAL6* locus has been cloned and shown to encode the structural genes for maltose permease (*MAL61*), maltase (*MAL62*), and a positively acting regulatory gene (*MAL63*). Induction of the *MAL61* and *MAL62* gene products requires the presence of maltose and the *MAL63* gene. Mutations within the *MAL63* gene produce nonfermenting strains unable to induce the two structural gene products. Reversion of these *mal63* nonfermenters to maltose fermenters nearly always leads to the constitutive expression of maltase and maltose permease, and constitutivity is always linked to *MAL6*. We demonstrated that for one such revertant, strain C2, constitutivity did not require the *MAL63* gene, since deletion disruption of this gene did not affect the constitutive expression of the structural genes. In addition, constitutivity was *trans* acting. Deletion disruption of the *MAL6*-linked structural genes for maltase and maltose permease in this strain did not affect the constitutive expression of a second, unlinked maltase structural gene. We isolated new maltose-fermenting revertants of a nonfermenting strain which carried a deletion disruption of the *MAL63* gene. All 16 revertants isolated expressed maltase constitutively. In one revertant studied in detail, strain R10, constitutive expression was demonstrated to be linked to *MAL6*, semidominant, *trans* acting, and residing outside the *MAL63-MAL61-MAL62* genes. From these studies we propose the existence of a second *trans*-acting regulatory gene at the *MAL6* locus. We call this new gene *MAL64*. We mapped the *MAL64* gene 2.3 centimorgans to the left of *MAL63*. The role of the *MAL64* gene product in maltose fermentation is discussed.

Maltose fermentation in *Saccharomyces* spp. requires the presence of at least one of five unlinked *MAL* loci: *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6* (1). All of the *MAL* loci show significant homology to each other both on the sequence level as determined by Southern blot analysis (10, 11, 17) and on the functional level as determined by complementation analysis (3, 13, 16; M. Charron, personal communication). To understand the mechanisms controlling maltose fermentation and to determine the functions encoded by a *MAL* locus, we undertook an analysis of the *MAL6* locus.

The genetic organization of *MAL6* is shown in Fig. 1. The locus is a cluster of three genes: *MAL61*, *MAL62*, and *MAL63* (16). All three genes are required for maltose fermentation. The *MAL62* gene codes for the enzyme maltase (3, 5). *MAL61* encodes maltose permease (2, 3; Y. S. Chang, R. A. Dubin, C. A. Michels, and R. B. Needleman, submitted for publication). Transcripts encoded by *MAL61* and *MAL62* are highly induced by maltose and repressed by glucose (5, 16; Chang et al., submitted). The genes are divergently transcribed from an approximately 700-base-pair region which contains the promoters for both genes and, most likely, any controlling sequences.

The *MAL63* gene encodes a regulatory function. After mutagenesis of a *MAL6* fermenting strain, ten Berge et al. (26) isolated a number of nonfermenting mutants which were uninducible for maltase and maltose permease but did synthesize uninduced levels of these enzymes. Genetic analysis showed the mutations to be recessive and linked to *MAL6*. Chang et al. (submitted) mapped these mutations to within the *MAL63* gene and clearly showed that the *MAL63* gene product is *trans* acting and required for the induction of maltase, maltose permease, and the *MAL61* and *MAL62* transcripts.

ten Berge et al. (25, 27) also isolated maltose-fermenting revertants of the *mal63* nonfermenting mutants. Most of these revertants were constitutive for the synthesis of both maltase and maltose permease and were recessive to the wild type and to the nonfermenting *mal6* mutants; all were linked to *MAL6*. In their analysis of these constitutive mutations, ten Berge et al. (25) proposed that they were alleles of the regulatory gene. We show here that constitutivity in one of these revertants, C2, lies in a gene outside the *MAL63-MAL61-MAL62* gene cluster. In addition, we isolated other *MAL6*-linked constitutive revertants and demonstrated that in one strain, R10, constitutivity similarly resides in a gene outside the *MAL63-MAL61-MAL62* gene cluster. This new gene, which we refer to as *MAL64*, maps 2.3 centimorgans to the left of the *MAL63* gene. The role of the *MAL64* gene product in the regulation of maltose fermentation is discussed.

MATERIALS AND METHODS

Strains and growth conditions. Yeast strains used in this study are listed in Table 1. Plasmids were propagated in strain RR1. Strain 8-2B was derived from the constitutive revertant C2 isolated by ten Berge et al. (27). Since the original C2 mutant was obtained in an *S. carlsbergensis* strain, it was likely to contain cryptic *MAL1g* and *MAL3g* loci in its background (17). Using the type of genetic analysis described by Michels and Needleman (10), we found that the *MAL1g* and *MAL3g* loci were present and were later crossed out of the strain. The *MAL* genotype of 8-2B is *MAL6-C2*

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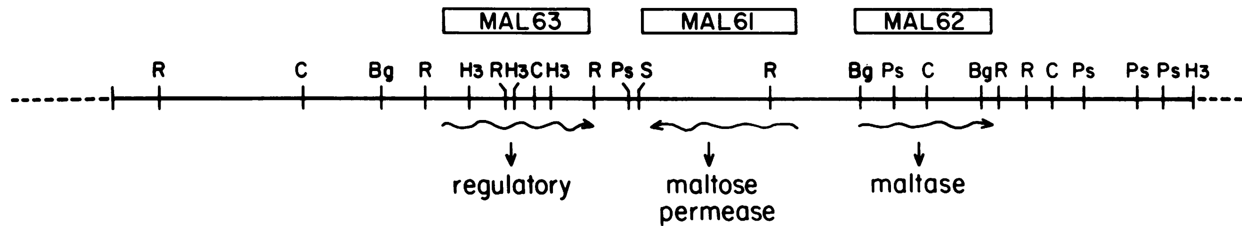

FIG. 1. *MAL6* locus of strain CB11. The restriction map of the *MAL6* locus of CB11 is shown (—), along with the locations of the three transcribed gene regions *MAL61*, *MAL62*, and *MAL63*, the directions of their transcription (〰), and functions.

MAL12. *MAL6-C2*, because of its derivation, contains both the *MAL6*-linked constitutive mutation C2 and the original *mal6-13* mutation from which it was isolated. Additionally, this strain carries in its background a partially functional allele of the *MAL1* locus which has been shown to contain only a functional *MAL12* gene encoding maltase. Isogenic strains were constructed as previously described (5). Yeast strains were grown on yeast extract-peptone (YEP) medium (1% [wt/vol] yeast extract plus 1% [wt/vol] peptone) plus the indicated amount of a specified carbon source. Maltose fermentation is defined as the production of acid and gas 1 to 3 days after inoculation and determined in 5 ml of YEP plus 2% (wt/vol) maltose medium in Durham tubes.

Reversion of A9. Approximately 4×10^7 cells of strain A9 were plated onto a plate containing YEP plus 2% glucose. A solution of nitrosoguanidine in acetone-ethanol-water was prepared, and 1 drop of this mixture was placed in the center of the plate. After growth overnight at 30°C, the plate was replica plated onto YEP plus 0.5% maltose (filter sterilized) and incubated at 30°C. Cells growing on this plate were purified and tested for maltose fermentation in Durham tubes.

Measurement of PNPGase activity. *p*-Nitrophenyl-α-D-glucopyranosidase (PNPGase) activity, measured as the rate of release of *p*-nitrophenol from PNPG, was determined by the method described by Dubin et al. (5). (Growth conditions are described below.) The rate of hydrolysis of maltose and alpha-methylglucoside into glucose was measured either by the glucostat assay or by the production of NADPH from NADP by using hexokinase and glucose-6-phosphate dehydrogenase (9, 21). Based upon a comparison of the PNPGase, maltase, and alpha-methylglucosidase activities found, it is clear that nearly all PNPGase levels are an accurate measure of the amount of maltase present in strain 332-5A. The small amount of alpha-methylglucoside cleavage observed results from the slight activity of maltase on this alternate substrate (15).

Permease assay. The rate of maltose transport was determined by the method of Serrano (24). The values reported represent the picomoles of [^{14}C]maltose transported in 2 min/mg (dry weight) of cells. The cells were grown to mid-log phase in the designated media.

Yeast transformation. Yeast transformation was done by the method of Ito et al. (7), using lithium acetate. All transformants were screened for stability of the selective marker to determine whether the selective marker was being maintained in an integrated state or episomally. For gene disruptions (23) and for site-directed plasmid integration (20), transformants which stably maintained the selective marker were further screened by Southern gel transfer analysis with techniques described by Dubin et al. (5), or by standard genetic analysis (12), or both.

Gel transfer analysis of DNA and RNA. DNA and RNA isolation and Southern and Northern blot analyses were performed as previously described (5, 16).

RESULTS

Constitutivity in C2 does not require *MAL63*. The previously reported genetic analysis regarding the *MAL6* locus (3, 5, 6, 16; Chang et al., submitted) is summarized in Fig. 1. The restriction map of the *MAL6* locus of strain CB11 and the approximate positions of the genes that constitute this complex locus, their directions of transcription, and functions are shown.

Strain 8-2B is a derivative of the constitutive strain C2 isolated by ten Berge et al. (27) as a Mal$^+$ revertant of the *mal6-13* mutation. It was constructed as outlined above. Strain 8-2B is genetically *MAL6-C2 MAL12*. Strain 8-2B is

TABLE 1. Yeast strains

Strain	Genotype	Source
8-2B	*MATa MAL6-C2 MAL12 ura3-52 leu2-3,112 trp1 ade*	This study
8-2BΔ63-1 and -2	Isogenic to 8-2B except *MAL63::URA3*	This study
8-2BΔ61/Δ62-5	Isogenic to 8-2B except *MAL61/MAL62::LEU2*	This study
8-2BΔ12-6	Isogenic to 8-2B except *MAL12::LEU2*	This study
RDY101-1B	Isogenic to 8-2BΔ12-6 except *MATα*	This study
332-5A	*MATa MAL6 MAL12 ura3-52 leu2-3,112 trp1 his*	Dubin et al. (5)
612-1D	Isogenic to 332-5A except *MATα*	This study
612-1D-T	Spontaneous Trp$^+$ revertant of 612-1D	This study
332-5AΔ61/Δ62-9	Isogenic to 332-5A except *MAL61/MAL62::LEU2*	This study
2b-46	Isogenic to 332-5A except *MAL62::LEU2*	Dubin et al. (5)
LB34 and LB35	Isogenic to 332-5A except *MAL61/MAL62/ MAL63::LEU2*	This study
A9	Isogenic to 332-5A except *MAL63::URA3*	Chang et al. (submitted)
654-5D	Isogenic to A9 except *MATα*	This study
R10	Isogenic to A9 except *MAL64-10*	This study
618-2B	Isogenic to R10 except *MATα*	This study
618-2B-T	Spontaneous Trp$^+$ revertant of 618-2B	This study
R10Δ61/Δ62-12	Isogenic to R10 except *MAL61/MAL62::LEU2*	This study

constitutive for maltase and maltose permease (Table 2). Northern blot analysis of RNA isolated from 8-2B (Fig. 2) demonstrates that *MAL61*- and *MAL62*-specific transcripts are expressed at high levels under uninduced (YEP plus 2% galactose) growth conditions. This is in contrast to the situation in inducible strains, where maltase, maltose permease, and the *MAL61*- and *MAL62*-specific transcripts require the presence of maltose for high levels of expression (4, 16, 28; Chang et al., submitted).

Constitutivity may arise through the loss of a repressor, the alteration of a positive activator, or changes in specific binding sites for such effectors. Chang et al. (submitted) demonstrated that *mal6* nonfermenters of the type isolated by ten Berge et al. (26) have mutations within *MAL63*. Maltose-fermenting constitutive revertants of these *mal6* strains, like C2, were demonstrated to be linked to *MAL6*, and from complementation studies, it was concluded that *mal6* and the *MAL6-C* constitutive revertants are allelic (25, 27). Thus, both would contain mutations only within the *MAL63* gene. *MAL63* is a *trans*-acting positive regulatory protein (Chang et al., submitted). If *MAL6-C* were an allele of *MAL63*, it would likely encode an activator which no longer requires maltose. To test this directly, we deleted *MAL63* in the *MAL6-C2* derivative 8-2B.

MAL63 was deleted from strain 8-2B by the one-step gene disruption technique of Rothstein (23). Plasmid pDM3 deletes the two *Hin*dIII fragments within *MAL63* and replaces them with *URA3*, as described by Chang et al. (submitted). *Eco*RI-restricted pDM3 was used to transform strain 8-2B to Ura$^+$. Five stable transformants were isolated, and integrative disruption was confirmed by Southern blot analysis (data not shown). All five were maltose fermenters. Two isolates, 8-2BΔ63-1 and 8-2BΔ63-2, were subjected to further analysis. Both were genetically shown now to contain *URA3* linked to *MAL6*. The deletion of *MAL63* does not alter the constitutive expression of maltase and maltose permease in these strains (Table 2). Northern blot analysis of 8-2BΔ63-1 (Fig. 2) and 8-2BΔ63-2 (data not shown) demonstrates that the *MAL61* and *MAL62* transcripts remain constitutively expressed. These results are in contrast with those obtained in an identical deletion in a wild-type *MAL6* strain. Chang et al. (submitted) showed that deletion of *MAL63* in *MAL6 MAL12* strain 332-5A results in a nonfermenting strain that is uninducible for maltase, maltose permease, and the *MAL61* and *MAL62* transcripts. Taken together, these results strongly demonstrate that *MAL63* is not required for *MAL6* constitutivity and that constitutivity lies outside the *MAL63* gene.

Constitutivity is *trans* acting and does not lie in the *MAL61*-*MAL62* gene region. *MAL6*-linked constitutivity could result from an alteration in a common upstream region controlling both structural genes. Because the *MAL61* and *MAL62* genes are divergently transcribed and because both transcripts initiate within an approximately 700-base-pair region, it is possible that a single *cis*-acting alteration in this region could lead to the constitutive expression of both genes. Alternatively, the constitutive mutation could reside within either structural gene, or within a new, as yet uncharacterized regulatory site or gene.

To begin to localize the C2 mutation within the *MAL6* locus, we isolated the region containing the *MAL61* and *MAL62* genes from strain 8-2B. This was done by integrating a selectable plasmid at the *MAL6-C2* locus and recovering this plasmid from the genome, along with its flanking *MAL6* DNA. With probes derived from the wild-type *MAL6* locus of strain CB11, we demonstrated extensive sequence homology between the wild-type *MAL6* locus and the constitutive locus present in strain 8-2B. Southern blot analysis of strain 8-2B (data not shown) showed that the maltase and maltose permease structural genes are contained on the same 7.3-kilobase (kb) *Hin*dIII fragment as are those of the wild-type *MAL6* locus (17). Based upon the demonstrated homology between the 7.3-kb fragment in the wild type and that of the constitutive mutant, a subclone of the wild-type locus was used to direct the integration of a yeast-selectable plasmid at the *MAL6* locus of constitutive strain 8-2B. A 1.6-kb *Cla*I-*Hin*dIII fragment derived from the wild-type *MAL6* locus and homologous to the region flanking the 3′ end of the *MAL62* gene (16; Fig. 3) was cloned into YIp5. The *Hin*dIII site was deleted by filling in with T4 DNA polymerase, and integration was directed to *MAL6* by digesting the plasmid with *Hpa*I before transforming strain 8-2B. Stable Ura$^+$ transformants were selected, and integration at *MAL6* was confirmed for one strain, 8-2BΔCΔH-6, by Southern blot and genetic analyses. Total genomic DNA was prepared from this strain, digested with *Hin*dIII, and ligated under dilute

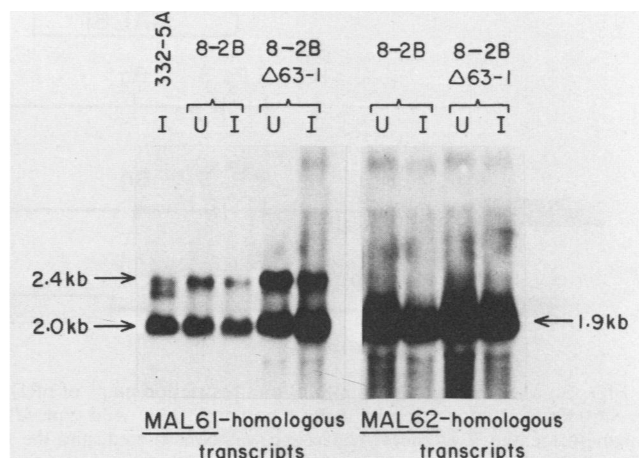

FIG. 2. Northern blot analysis of constitutive strains 8-2B and 8-2BΔ63-1. Poly(A$^+$) RNA was prepared after growth on either YEP medium plus 2% galactose (uninduced) or YEP medium plus 2% galactose and 2% maltose (induced). The RNA was size fractionated on a formaldehyde-containing agarose gel, transferred to nitrocellulose, and probed with *MAL61*- or *MAL62*-specific plasmids labeled with [^{32}P]dCTP by nick translation. The *MAL61*-specific probe contains the 1.5-kb *Pst*I-*Eco*RI fragment internal to the *MAL61* gene. The *MAL62*-specific probe contains the 1.0-kb *Pst*I-*Eco*RI fragment internal to the *MAL62* gene.

TABLE 2. Maltase and maltose permease in constitutive strains 8-2B and 8-2BΔ63[a]

Strain	Maltase activity		Maltose transport	
	Uninduced	Induced	Uninduced	Induced
332-5A	5	310	10	460
8-2B	152	427	365	381
8-2BΔ63-1	172	392	ND[b]	ND
8-2BΔ63-2	137	412	268	432

[a] Cells were grown about 14 h to mid-log phase in either uninducing medium (YEP plus 2% galactose) or inducing medium (YEP plus 2% maltose). Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C. Maltose transport is reported as picomoles of [^{14}C]maltose transported per 2 min/mg (dry weight) of cells.

[b] ND, Not determined.

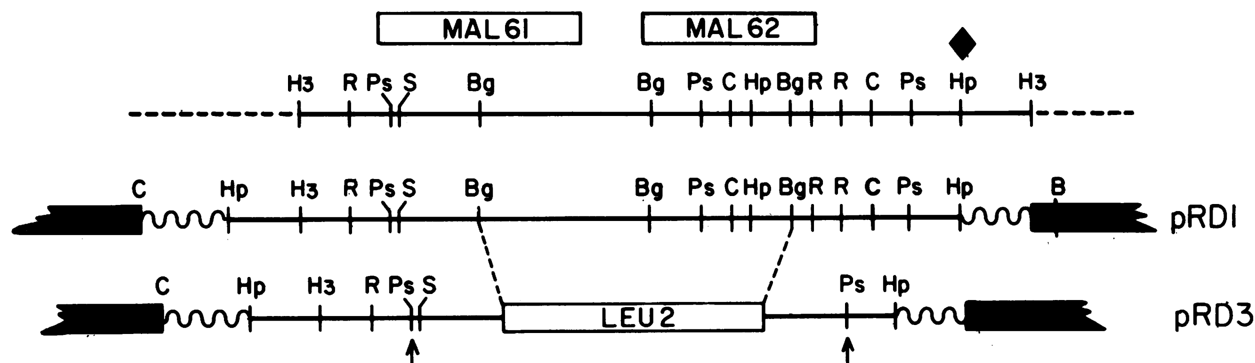

FIG. 3. *MAL6* locus of strain 8-2B and restriction maps of pRD1 and pRD3. The *MAL6* locus of the constitutive strain 8-2B was cloned by site-directed integration by using homology to the wild-type *MAL6* locus of strain CB11. A plasmid containing the 1.6-kb *ClaI-HindIII* fragment located 3′ to the *MAL62* gene was constructed, and the *HindIII* site was deleted as described in the text. The resulting plasmid, pY6ΔCΔH was used to transform strain 8-2B. Integration was directed to *MAL6* by restricting pY6ΔCΔH with *HpaI* (marked by ◆ on the map) before transformation. Integration at *MAL6* was confirmed in one transformant. Total genomic DNA prepared from this transformant was restricted with *HindIII*, ligated under dilute conditions, and used to transform RR1 to ampicillin resistance. pRD1 was recovered, and its restriction map is presented. Regions of homology to the wild-type *MAL6* locus were determined by Southern blot analysis with *MAL6*-specific probes derived from strain CB11. The gene disruption plasmid pRD3 was constructed by replacing the two *BglII* fragments present in pRD1 with the *LEU2*-containing *BglII* fragment of CV9. pRD3 was restricted with *PstI* and used to transform various *MAL* strains to disrupt both the *MAL61* and *MAL62* genes; its restriction map is presented.

conditions; the ligated DNA was used to transform bacterial strain RR1 selecting for ampicillin resistance. Plasmid pRD1 was recovered (Fig. 3). Restriction mapping of this cloned region showed that, for the enzymes tested, it is largely identical to the *MAL6* locus of CB11 with only two changes: the *EcoRI* site near the 5′ end of the *MAL61* transcript has been lost, and a new *BglII* site within the *MAL61* gene has been acquired. These site differences, as will be seen below, appear to represent polymorphisms between the *MAL6* loci of the *S. carlsbergensis* strains used by ten Berge et al. (25–27) and Needleman and Michels (17) and are not functionally significant. Hybridization between cloned probes derived from the *MAL6* locus of CB11 and the *MAL6* locus derived from strain 8-2B confirmed the organizational similarity (data not shown).

Strain 8-2B carries, in addition to the *MAL6* locus, a partially functional allele of the *MAL1* locus which contains a functional copy of the maltase structural gene (*MAL12*). Since strain 8-2B contains two maltase structural genes, we attempted to determine whether the *MAL12* gene is constitutively expressed in strains carrying the *MAL6*-linked constitutive mutation C2. If the *MAL12* gene were constitutively expressed, then constitutivity would be a *trans*-acting function. To determine this, we deleted the *MAL61* and *MAL62* genes from strain 8-2B as follows. The two *BglII* fragments spanning the region from the *MAL61* to the *MAL62* genes and including the 5′ ends of both genes were deleted in pRD1 and replaced with the *BglII* fragment containing the *LEU2*-selectable marker from CV9 (Fig. 3). The resulting new plasmid, pRD3, was digested with *PstI* and used to transform strain 8-2B. Stable Leu$^+$, maltose-nonfermenting transformants were isolated and shown by Southern blot analysis to have disrupted the *MAL6* locus. One transformant, strain 8-2BΔ61/Δ62-5, was genetically analyzed, shown to carry the *MAL6::LEU2* disruption, and selected for further analysis. A deletion disruption of the *MAL12* gene in strain 8-2B was also constructed with plasmid pDM2b, as previously described (5), and strain 8-2BΔ12-6 was isolated. Disruption at *MAL12* in 8-2BΔ12-6 was confirmed by Southern blot analysis. Strain RDY101-1B was constructed and is isogenic to 8-2BΔ12-6 except at the mating type locus. The *MAL12::LEU2* disruption in strain RDY101-1B was confirmed by genetic analysis. The results of determining maltase activity in these disruption strains are shown in Table 3. Although deletion of the functional *MAL12* gene (strain RDY101-1B) has little effect on the level of constitutively synthesized maltase or on the ability of the strain to ferment, deletion of the *MAL61* and *MAL62* genes (strain 8-2BΔ61/Δ62-5) produces a nonfermenter with reduced, but significant, levels of constitutively expressed maltase activity. To demonstrate that the residual maltase activity in strain 8-2BΔ61/Δ62-5 in fact results from the constitutive expression of the *MAL12* gene, we mated the two single disruption strains, 8-2BΔ61/Δ62-5 and RDY101-1B, to produce diploid RDY103. RDY103 was sporulated, and the resulting tetrads were analyzed. Five tetrads from this diploid are shown in Table 4. By monitoring the leucine phenotype of the segregants and their ability to ferment maltose, one can determine whether the disrupted or undisrupted alleles of the *MAL6* and *MAL12* loci are present. Tetrad 1 is a nonparental ditype, tetrad 14 is a parental ditype, and tetrads 5, 8, and 10 are tetratypes.

TABLE 3. Effects of gene disruptions on the uninduced levels of maltase activity in wild-type and constitutive strains[a]

Strain[b]	*MAL* genotype	Uninduced maltase activity[c]
8-2B	*MAL6-C2 MAL12*	221
8-2BΔ61/Δ62-5	*MAL6-C2 MAL61/MAL62::LEU2 MAL12*	60
RDY101-1B	*MAL6-C2 MAL12::LEU2*	192
332-5A	*MAL6 MAL12*	3
332-5AΔ61/Δ62-9	*MAL6 MAL61/MAL62::LEU2 MAL12*	2

[a] Cells were pregrown in YEP medium plus 2% galactose, diluted into fresh YEP medium plus 2% galactose, and allowed to grow 8 to 9 h (mid- to late-log phase).

[b] Strains 8-2B, RDY101-1B, 332-5A, and R10 are maltose fermenters. Strains 8-2BΔ61/Δ62-5 and 332-5AΔ61/Δ62-9 are maltose nonfermenters.

[c] Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C.

Comparison of the constitutive (2% galactose grown) level of maltase synthesis of the various segregants clearly indicates that the *MAL12* maltase gene at the *MAL1* locus is responsible for the constitutively expressed maltase activity in 8-2BΔ61/Δ62-5.

Deletion of the *MAL61* and *MAL62* genes, using pRD3, in the inducible *MAL6 MAL12* strain 332-5A also produces nonfermenters; however, these do not express maltase activity constitutively. One such transformant, 332-5AΔ61/Δ62-9, was analyzed by Southern blotting and genetic analysis, and the *MAL6*::*LEU2* disruption was confirmed. Disruption of this region in the inducible strain 332-5A does not lead to the constitutive expression of maltase at the *MAL12* locus (Table 3).

Together, these results clearly indicate that the *MAL6*-linked constitutive mutation C2 is controlling the expression of the unlinked *MAL12* gene located at the *MAL1* locus and thus strongly supports the existence of a second *trans*-acting regulatory gene controlling the expression of maltase and maltose permease. It is also clear that this *trans*-acting function is neither the *MAL61* nor *MAL62* gene. We refer to this second *MAL6*-linked regulatory gene as *MAL64*.

Isolation and characterization of new *MAL6*-linked constitutive strains. We previously demonstrated that deletion disruption of the *MAL63* gene with plasmid pDM3, in an inducible strain, 332-5A, produces a nonfermenting strain unable to induce maltase, maltose permease, or the *MAL61* and *MAL62* transcripts (Chang et al., submitted). A9 is one such strain that carries the *MAL63*::*URA3* deletion disruption.

Revertants of A9 were obtained by using nitrosoguanidine mutagenesis followed by selection for growth on YEP plates containing 0.5% maltose. Revertants appeared at a frequency of approximately 10^{-6}. For 16 revertants, the specific activity of maltase was determined under induced (YEP plus 2% maltose) and uninduced (YEP plus 2% galactose) growth conditions. All 16 revertants were found to synthesize significant levels of maltase, ranging from 50 to 250% of induced levels under noninducing conditions. Thus, by using this selection method, it appears that constitutivity is the most common result of the reversion of A9.

We selected one revertant, R10, for further study. Strain R10 expresses maltase and maltose permease constitutively (Table 5). R10 retains the Ura^+ phenotype of the original *MAL63*::*URA3* disruption, Southern blot analysis confirmed the continued presence of the original disruption, and both Ura^+ and constitutivity segregate with *MAL6* (see below). R10 was crossed to a series of isogenic strains carrying either the wild-type *MAL6*, the *MAL63*::*URA3* deletion, or *MAL6C-R10*. Strain R10 is partially dominant to wild type (Table 5). This is apparent from the cross R10 × 612-1D, where the uninduced level of maltase in the *MAL6C-R10*/*MAL6* heterozygous diploid is slightly higher than that in the homozygous wild-type diploid (332-5A × 612-1D). In addition, the *MAL6C-R10*/*MAL6* heterozygous diploid is constitutive for maltose permease. R10 is also partially dominant to the *MAL63*::*URA3* deletion. This is evidenced from the cross between the noninducing strain A9 and the constitutive revertant 618-2B (which is isogenic to R10 except at *MAT*), where it is demonstrated that the diploid is partially constitutive for maltase and fully constitutive for maltose permease.

Clearly, constitutivity in strain R10 must lie outside *MAL63*. Constitutivity in strain R10 is also a *trans*-acting function. This was demonstrated by deleting the *MAL61* and *MAL62* genes in this strain and determining whether the *MAL12* gene was constitutively expressed. Strain R10 was transformed with *Pst*I-digested pRD3 DNA, and Leu^+ maltose nonfermenters were isolated. Strain R10Δ61/Δ62-12 was isolated, and the *MAL6*::*LEU2* disruption was confirmed by Southern blot and genetic analyses. When grown under noninducing conditions (YEP plus 2% galactose), strain R10Δ61/Δ62-12 produces nearly 570 U of maltase. This strain thus continues to express maltase constitutively, confirming that constitutivity in strain R10, as in the C2 mutant, is *trans* acting. It also demonstrates that neither the *MAL61* nor the *MAL62* gene is required for constitutivity in strain R10. Since the constitutive mutations in both strains C2 and R10 are linked to *MAL6*, are epistatic to *mal63*, and express *trans*-acting constitutivity, it is likely that both mutations lie within the *MAL64* gene. Thus, strains C2 and R10 would carry the *MAL64-C2* and *MAL64-10* alleles, respectively. It is not possible to confirm allelism by standard complementation tests, due to the partial dominance of strain R10. When the two constitutive strains are crossed (8-2B × 618-2B), the *MAL6-C2*/*MAL6C-R10* diploid is partially constitutive for maltase (Table 5), making it difficult to interpret whether the effect is due to dominance between alleles or between genes.

As additional evidence that the constitutive mutation in R10 lies outside the *MAL61*, *MAL62*, and *MAL63* coding regions, these three genes were deleted from the genome of the *MAL6 MAL12* wild-type strain 332-5A, and the heterozygous diploid between this deletion strain and the R10 constitutive mutant was shown to be partially constitutive for maltase. The deletion of the *MAL61*, *MAL62*, and

TABLE 4. Uninduced maltase activity in segregants from diploid RDY103[a]

Tetrad segregant	Leucine phenotype	Maltose fermentation	Uninduced maltase activity[b]
1 A	+	−	2
B	−	+	133
C	+	−	2
D	−	+	176
5 A	−	+	159
B	+	+	103
C	+	−	2
D	+	−	149
8 A	−	+	263
B	+	−	3
C	+	−	83
D	+	+	218
10 A	+	−	4
B	−	+	311
C	+	−	70
D	+	+	199
14 A	+	−	87
B	+	+	200
C	+	+	226
D	+	−	91

[a] Diploid RDY103 was constructed by mating strain 8-2BΔ61/Δ62-5 (*MAL6-C2 MAL61*/*MAL62::LEU2 MAL12*) with RDY101-1B (*MAL6-C2 MAL12::LEU2*). Cells were pregrown in YEP medium plus 2% galactose, diluted into fresh YEP medium plus 2% galacose, and allowed to grow 8 to 9 h (mid- to late-log phase).

[b] Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C.

TABLE 5. Maltase activity and maltose transport in strains 332-5A, A9, R10, and various R10 diploid strains[a]

Strain	MAL genotype or description	Maltase activity[b]		Maltose transport[c]	
		Uninduced	Induced	Uninduced	Induced
332-5A	*MAL6 MAL12*	88	742	9	490
A9	*MAL63::URA3 MAL12*	2	2	23	19
R10	*MAL63::URA3 MAL6C-R10 MAL12*	1,200	1,570	980	665
Diploid strains					
332-5A × 612-1D	WT[d] × WT	41	535	11	281
R10 × 612-1D	const[e] × WT	153	943	340	359
R10 × 618-2B	const × const	1,120	891	445	460
A9 × 654-5D	Δ*MAL63* × Δ*MAL63*	32	27	14	17
A9 × 612-1D	Δ*MAL63* × WT	23	185	18	307
A9 × 618-2B	Δ*MAL63* × const	411	1,047	315	359
8-2B × 618-2B	*MAL6-C2* × *MAL6C-R10*	254	1,263	ND[f]	ND

[a] Cells were pregrown in YEP medium plus 2% dextrose, diluted into fresh uninducing medium (YEP plus 2% galactose) or inducing medium (YEP plus 2% maltose), and allowed to grow to mid-log phase.

[b] Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C.

[c] Maltose transport is reported as picomoles of [^{14}C]maltose transported per 2 min/mg (dry weight) of cells.

[d] WT, Wild type.

[e] const, Constitutive.

[f] ND, Not determined.

MAL63 coding regions was accomplished as follows. Plasmid YEpMAL6 (Fig. 1) contains an approximately 13-kb yeast DNA fragment including these three genes and was derived from the wild-type *MAL6* locus (16). The two *Bgl*II fragments containing the three transcribed regions were replaced by a *Bgl*II fragment carrying the *LEU2*-selectable marker from CV9. This plasmid is called pLB (Fig. 4). Plasmid pLB was restricted with *Hin*dIII and used to transform strain 332-5A. Stable Leu$^+$ transformants were selected, and integration at the *MAL6* locus was confirmed by Southern blot and tetrad analyses. Two transformants, LB34 and LB35, were selected for further study.

LB34 and LB35 are themselves uninducible nonfermenters. Nonetheless, when crossed to strains carrying the R10 constitutive revertant, the diploids LB34 × 618-2B-T and LB35 × 618-2B-T are partially constitutive for maltase, producing levels similar to those found in the diploid between the wild-type *MAL6* and the constitutive revertant R10 (612-1D-T × R10) (Table 6). If the constitutive mutation did lie within this region, the LB34 × 618-2B-T and LB35 × 618-2B-T diploids would be expected to be fully constitutive for maltase. This result strongly suggests that the constitutive mutation lies outside the coding regions defined by the *MAL61*, *MAL62*, and *MAL63* genes.

It is interesting that the diploids from crosses between strain LB34 or LB35 and strain 612-1D-T, which carries the wild-type *MAL6* locus, induce maltase only to very low levels (Table 6). Similar results were found for the diploid from a cross between A9 and 612-1D. The meaning of this repressing effect on the induction of maltase is unclear but could result from the increased ratio of the number of *MAL64* genes to *MAL63* genes in these diploids.

Linkage of the *MAL63* gene to the constitutive mutation *MAL64*. Constitutive revertant R10 was crossed to strain 612-1D-T, a spontaneous Trp$^+$ revertant of strain 612-1D, which is genotypically *MAL6 MAL12* (Table 1). The diploid was sporulated and dissected, and 142 tetrads were analyzed. The parental *MAL* genotypes (and phenotypes) are R10 *MAL61 MAL62 MAL63::URA3 MAL64-10* (Ura$^+$ Mal$^+$, constitutive) × 612-1D-T *MAL61 MAL62 MAL63 MAL64* (ura$^-$ Mal$^+$, inducible). Segregants resulting from recombination between the *MAL63* and *MAL64* genes will have the genotypes (and phenotypes) *MAL61 MAL62 MAL63::URA3 MAL64* (Ura$^+$ mal$^-$, uninducible) and *MAL61 MAL62 MAL63 MAL64-10* (ura$^-$ Mal$^+$, constitutive). A total of 13 such recombinants were obtained. Of these, six were reciprocal recombinants found in three tetrads, and the remaining seven were gene convertants. Isolation of the ura$^-$ Mal$^+$ constitutive class of recombinants indicates that *MAL64-10* is epistatic to both *mal63* and *MAL63*. A total of 10 segreg-

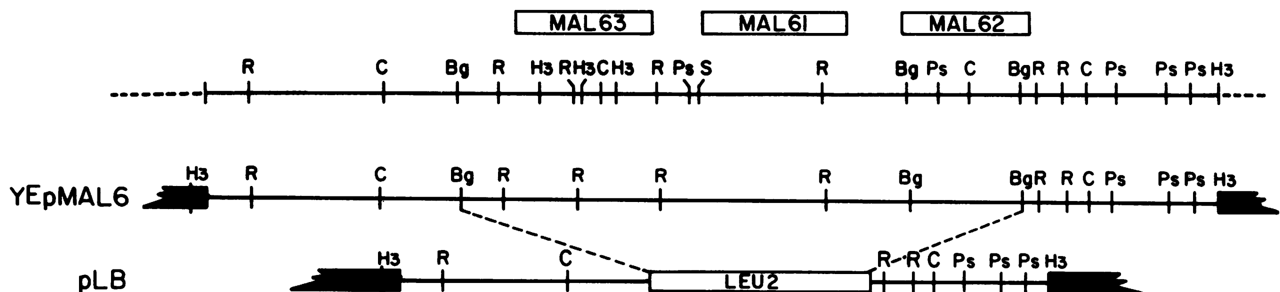

FIG. 4. Construction of the disruption plasmid pLB. The gene disruption plasmid pLB was constructed by replacing the two *Bgl*II fragments in YEpMAL6 with the *LEU2*-containing *Bgl*II fragment of CV9. pLB was restricted with *Hin*dIII and used to transform strain 332-5A to disrupt the *MAL61*, *MAL62*, and *MAL63* genes.

ants having an unusual phenotype which could not be explained by simple recombination were also obtained. The phenotype of eight of these was Ura$^+$ Mal$^+$ and inducible, and they appear to be gene conversions induced at the *ura3* locus. The phenotype of the remaining two unusual segregants was ura$^-$ mal$^-$ and uninducible. The explanation for these is presently under analysis, but we suspect they could have been the result of a rearrangement in this region. These 10 unusual segregants were not considered when calculating map distance. Thus, 13 recombinants were obtained in 558 segregants giving a map distance between *MAL63* and *MAL64* of 2.3 centimorgans.

The *MAL64* gene was shown to lie to the left of the *MAL63* gene (Fig. 1) by the following cross. Strain 618-2B-T carrying the *MAL64*-constitutive mutation from strain R10 and the *URA3* deletion disruption of *MAL63* was mated to strain 2b-46, which contains a *LEU2* deletion disruption of *MAL62* (5): 618-2B-T *MAL61 MAL62 MAL63::URA3 MAL64-10* (Ura$^+$ leu$^-$ Mal$^+$, constitutive) × 2b-46 *MAL61 MAL-62::LEU2 MAL63 MAL64* (ura$^-$ Leu$^+$ Mal$^+$, inducible). (Strain 2b-46 is a maltose fermenter despite the deletion in the *MAL62* gene, because of the presence of the *MAL12* gene, a *MAL1*-linked copy of the gene encoding maltase [5].) The haploid segregants of this cross were analyzed by random spore analysis selecting for Ura$^+$ Leu$^+$ haploid segregants. It is predicted that if *MAL64* lies to the left of *MAL63*, all the Ura$^+$ Leu$^+$ segregants should be Mal$^+$ and constitutive; if *MAL64* lies to the right of both *MAL63* and *MAL62*, all the Ura$^+$ Leu$^+$ segregants should be mal$^-$ and uninducible; if *MAL64* lies between *MAL63* and *MAL62*, the Ura$^+$ Leu$^+$ segregants should fall into two classes: Mal$^+$ and constitutive, and mal$^-$ and uninducible. A total of 151 Ura$^+$ Leu$^+$ segregants were obtained, all of which were maltose fermenters. Of these 151 segregants, 23 were assayed; all of these were found to synthesize maltase constitutively. This result, along with the previous cross, places the *MAL64* gene 2.3 centimorgans to the left of *MAL63* (Fig. 1).

TABLE 6. Constitutivity in strain R10 does not reside within the *MAL63-MAL61-MAL62* gene cluster[a]

Strain	*MAL* genotype or description	Maltase activity[b]	
		Uninduced	Induced
332-5A	*MAL6 MAL12*	44	648
612-1D-T	*MAL6 MAL12*	63	861
R10	*MAL63::URA3 MAL61 MAL62 MAL64-10 MAL12*	1,200	1,570
618-2B-T	*MAL63::URA3 MAL61 MAL62 MAL64-10 MAL12*	1,108	1,272
LB34	*MAL63/MAL61/ MAL62::LEU2 MAL64 MAL12*	24	10
LB35	*MAL63/MAL61/ MAL62::LEU2 MAL64 MAL12*	25	19
332-5A × 612-1D-T	WT[c] × WT	15	731
R10 × 618-2B-T	const[d] × const	1,802	1,896
R10 × 612-1D-T	const × WT	125	1,446
LB34 × 612-1D-T	Δ[*MAL61/62/63*] × WT	11	160
LB35 × 612-1D-T	Δ[*MAL61/62/63*] × WT	10	184
A9 × 612-1D	Δ*MAL63* × WT	23	185
LB34 × 618-2B-T	Δ[*MAL61/62/63*] × const	85	1,264
LB35 × 618-2B-T	Δ[*MAL61/62/63*] × const	74	700

[a] Cells were pregrown in YEP medium plus 2% dextrose, diluted into fresh uninducing medium (YEP plus 2% galactose) or inducing medium (YEP plus 2% maltose), and allowed to grow to mid-log phase.
[b] Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C.
[c] WT, Wild type.
[d] const, Constitutive.

DISCUSSION

We previously demonstrated that induction of the maltose fermentative enzymes is controlled by the *MAL63* gene product (Chang et al., submitted). Mutations in this gene, including several isolated by ten Berge et al. (26), as well as a deletion disruption of *MAL63*, lead to an uninducible phenotype that is recessive to the wild type. The *MAL63* gene product, therefore, is a positive regulator. Ten Berge et al. (25, 27) found that their uninducible *mal6* mutant strains were easily reverted to maltose fermenters that constitutively expressed maltase and maltose permease. These constitutive mutations were linked to *MAL6* and nearly always recessive to the wild-type *MAL6* locus, and when mated to strains carrying different *mal6* alleles, the diploids exhibited various degrees of inducibility. We undertook the analysis described in the present report to localize the site of the alteration in these constitutive revertants and to understand the recessive nature of the mutations. Such information would enable us to define the regulatory mechanisms controlling maltose fermentation.

The results described here clearly demonstrate that the *MAL6*-linked constitutive mutations C2 (27) and R10 (isolated in the present report) each represent alterations in a *trans*-acting function, as evidenced from our demonstration of the constitutive expression of a second maltase structural gene located outside the *MAL6* locus at a partially functional *MAL1*-linked locus. This *trans*-acting function is not an allele of the previously described positive regulatory gene *MAL63* because, as shown in the present report, the *MAL63* gene is dispensable for constitutivity in a derivative of constitutive strain C2 and because the constitutive R10 mutant was in fact isolated from a noninducible strain carrying a deletion of the *MAL63* gene. Neither the *MAL61* and *MAL62* gene products nor their common upstream sequences are involved in *MAL6*-linked constitutivity. Deletion of both genes in constitutive strains does not affect the constitutive expression of an unlinked maltase structural gene. This result is supported by the fact that the *MAL6*-linked structural genes isolated from constitutive strain 8-2B are unable to complement strains lacking a functional *MAL63* gene or its equivalent (unpublished data). Based upon these results, we propose that in the constitutive revertants a second *MAL6*-linked regulatory gene, the *MAL64* gene, controls the expression of the maltose fermentative enzymes. Although we have not demonstrated allelism between the constitutive mutations in strains C2 and R10, it is likely that both lie within the *MAL64* gene. We have mapped the *MAL64-10* mutation in strain R10 to be 2.3 centimorgans to the left of the *MAL63* gene (Fig. 1). The role of the *MAL64* gene product is not apparent from the results reported here. Analysis of diploids presented in Tables 5 and 6 was originally undertaken in an attempt to understand the regulatory role of the *MAL64* gene product and to elucidate possible interactions with the *MAL63* gene product. The results appear inconsistent and are open to several interpretations, including that the wild-type *MAL64* gene product may not play a major role in the regulation of maltose

fermentation. The exact nature of the *MAL64* and *MAL64*-constitutive functions must await the cloning and characterization of the genes. This work is currently under way.

The *MAL* loci are highly homologous with regard to both the sequence of the locus and the functions encoded by these loci. The most extensive comparison has been made between the *MAL1* and the *MAL6* loci. Naturally occurring mutant alleles of the *MAL1* locus are complemented by various strains carrying mutations in the genes of the *MAL6* locus (13; Chang et al., submitted). Additionally, plasmid subclones of *MAL6* complement point mutations and deletion disruptions of the genes of the *MAL1* locus (3; M. J. Charron, R. A. Dubin, and C. A. Michels, submitted for publication). The *MAL1* locus has been isolated along with several kilobases of flanking DNA (Charron et al., submitted). Structural comparisons between *MAL6* and *MAL1* show that the homology between these two loci is extensive within the 9.0-kb region containing the *MAL61-62-63* gene cluster, but beyond this region homology is largely absent. Preliminary studies on the other *MAL* loci (Charron and Michels, unpublished data) provide similar evidence supporting the proposal that only three functions are required to ferment maltose: the two structural genes encoding maltase and maltose permease and the regulatory gene encoding the positive regulator. This comparative study underscores the possible unique nature of the *MAL64* gene and argues against a required role for its gene product in the regulation of maltose fermentation.

Constitutive mutations have been isolated in other maltose-fermenting strains carrying other *MAL* loci. Zimmerman and Eaton (29) isolated a series of constitutive revertants of a *mal2* nonfermenting mutant. All the revertants were tightly linked to *MAL2*, all were dominant to the wild type, and about 50% were glucose repression insensitive. Khan and Eaton (8) describe a constitutive allele of the *MAL4* locus which they show to be closely linked to *MAL4*, dominant to the wild-type *MAL4* allele, and glucose repression insensitive. In another study, constitutive mutations were isolated directly from maltose-fermenting strains containing either the *MAL1* or the *MAL3* loci, and these were linked to their respective *MAL* loci (14). It will be interesting to localize the site and nature of these constitutive mutations.

Several of the *MAL*-constitutive mutations in the appropriate genetic backgrounds have been shown to be pleiotropic and affect other enzymes involved in sugar metabolism. The *MAL6*-linked constitutive mutation C2, originally isolated in a strain inducible for the alpha-methylglucoside-fermenting enzymes, resulted not only in the constitutive synthesis of maltase but also in the partially constitutive synthesis of alpha-methylglucosidase (25, 27). Additionally, the *MAL2*-linked constitutive mutations isolated in a strain unable to ferment alpha-methylglucoside led to the constitutive synthesis of alpha-methylglucosidase (29). Trehalose accumulation is also controlled by *MAL*-constitutive loci. The accumulation of trehalose in glucose-grown cells depends upon the presence of a *MAL*-constitutive gene in the background. This has been demonstrated for the *MAL2*-, *MAL4*-, and *MAL6*-linked constitutive loci (18, 22). This phenotype is independent of the presence of trehalose synthetase, suggesting that *MAL*-constitutive loci control a second system involved in trehalose accumulation (19). It would appear that the *MAL* regulatory genes represent master controlling genes coordinating the expression of many functions involved in the metabolism of the various alpha-glucosides (such as maltose, alpha-methylglucoside, and trehalose) used or stored by this yeast.

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LITERATURE CITED

1. **Barnett, J. A.** 1976. The utilization of sugars by yeasts. Adv. Carbohydr. Chem. Biochem. **32**:126–234.
2. **Cohen, J. D., M. J. Goldenthal, B. Buchferer, and J. Marmur.** 1984. Mutational analysis of the *MAL1* locus of *Saccharomyces*: identification and functional characterization of three genes. Mol. Gen. Genet. **196**:208–216.
3. **Cohen, J. D., M. J. Goldenthal, T. Chow, B. Buchferer, and J. Marmur.** 1985. Organization of the *MAL* loci of *Saccharomyces*: physical identification and functional characterization of three genes at the *MAL6* locus. Mol. Gen. Genet. **200**:1–8.
4. **de Kroon, R. A., and V. V. Kroningsberger.** 1970. An inducible transport system for α-glucosidase in protoplasts of *Saccharomyces carlsbergensis*. Biochim. Biophys. Acta **204**:590–609.
5. **Dubin, R. A., R. B. Needleman, D. Gossett, and C. A. Michels.** 1985. Identification of the structural gene encoding maltase within the *MAL6* locus of *Saccharomyces carlsbergensis*. J. Bacteriol. **164**:605–610.
6. **Federoff, H. J., J. D. Cohen, T. R. Eccleshall, R. B. Needleman, B. A. Buchferer, J. Giacalone, and J. Marmur.** 1982. Isolation of a maltase structural gene from *Saccharomyces carlsbergensis*. J. Bacteriol. **149**:1064–1070.
7. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
8. **Khan, N. A., and N. R. Eaton.** 1974. Genetic control of maltase formation in yeast. I. Strains producing high and low basal levels of enzyme. Mol. Gen. Genet. **112**:317–322.
9. **Lloyd, J. B., and W. J. Whelan.** 1969. An improved method for enzymatic determination of glucose in the presence of maltose. Anal. Biochem. **30**:467–470.
10. **Michels, C. A., and R. B. Needleman.** 1983. A genetic and physical analysis of the *MAL1* and *MAL3* standard strains of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **191**:225–230.
11. **Michels, C. A., and R. B. Needleman.** 1984. The dispersed, repeated family of *MAL* loci in *Saccharomyces* spp. J. Bacteriol. **157**:949–952.
12. **Mortimer, R. K., and D. C. Hawthorne.** 1975. Genetic mapping in yeast. Methods Cell Biol. **6**:221–233.
13. **Naumov, G. J.** 1976. Comparative genetics of yeast. XVI. Genes for maltose fermentation in *Saccharomyces carlsbergensis* N.C.Y.C. 74. Genetika **12**:87–100.
14. **Needleman, R. B., and N. R. Eaton.** 1974. Selection of yeast mutants constitutive for maltase synthesis. Mol. Gen. Genet. **133**:135–140.
15. **Needleman, R. B., H. J. Federoff, T. R. Eccheshall, B. Buchferer, and J. Marmur.** 1978. Purification and characterization of an α-glucosidase from *Saccharomyces carlsbergensis*. Biochemistry **17**:4657–4661.
16. **Needleman, R. B., D. B. Kaback, R. A. Dubin, E. L. Perkins, N. G. Rosenberg, K. A. Sutherland, D. B. Forrest, and C. A. Michels.** 1984. *MAL6* of *Saccharomyces*: a complex genetic locus containing three genes required for maltose fermentation. Proc. Natl. Acad. Sci. USA **81**:2811–2815.
17. **Needleman, R. B., and C. Michels.** 1983. Repeated family of genes controlling maltose fermentation in *Saccharomyces carlsbergensis*. Mol. Cell. Biol. **3**:796–802.
18. **Oliveira, D. E., E. G. C. Rodrigues, J. R. Mattoon, and A. D. Panek.** 1981. Relationships between trehalose metabolism and maltose utilization in *Saccharomyces cerevisiae*. II. Effect of constitutive *MAL* genes. Curr. Genet. **3**:235–242.
19. **Operti, M. S., D. E. Oliveira, A. B. Freitas-Valle, E. G.**

Oestricher, J. R. Mattoon, and A. D. Panek. 1982. Relationships between trehalose metabolism and maltose utilization in *Saccharomyces cerevisiae*. III. Evidence for alternative pathways of trehalose synthesis. Curr. Genet. **5**:69–76.

20. **Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein.** 1983. Genetic applications of yeast transformation with linear and gapped plasmids. Methods Enzymol. **101**:228–245.
21. **Ouewehand, J., and R. van Wijk.** 1972. Regulation of maltase and alpha-methylglucosidase synthesis in genetically defined strains of *Saccharomyces carlsbergensis*. Mol. Gen. Genet. **117**:30–38.
22. **Panek, A. D., A. L. Sampaio, G. C. Braz, S. J. Baker, and J. R. Mattoon.** 1979. Genetic and metabolic control of trehalose and glycogen synthesis: new relationships between energy reserves, catabolite repression and maltose utilization. Cell. Mol. Biol. **25**:345–354.
23. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–211.
24. **Serrano, R.** 1977. Energy requirements for maltose transport in yeast. Eur. J. Biochem. **80**:97–102.
25. **ten Berge, A. M. A., G. Zoutewelle, and R. B. Needleman.** 1974. Regulation of maltose fermentation in *Saccharomyces carlsbergensis*. III. Constitutive mutations at the *MAL6*-locus and suppressors changing a constitutive phenotype into a maltose negative phenotype. Mol. Gen. Genet. **131**:113–121.
26. **ten Berge, A. M. A., G. Zoutewelle, and K. W. van de Poll.** 1973. Regulations of maltose fermentation in *Saccharamyces carlsbergensis*. I. The function of the gene *MAL6*, as recognized by *mal6*-mutants. Mol. Gen. Genet. **123**:233–246.
27. **ten Berge, A. M. A., G. Zoutewelle, K. W. van de Poll, and H. P. J. Bloemers.** 1973. Regulation of maltose fermentation in *Saccharomyces carlsbergensis*. II. Properties of a constitutive *MAL6*-mutant. Mol. Gen. Genet. **125**:139–146.
28. **van Wijk, R., J. Ouwehand, T. van de Bos, and V. V. Kroningsberger.** 1969. Induction and catabolite repression of α-glucosidase synthesis in protoplasts of *Saccharomyces carlsbergensis*. Biochim. Biophys. Acta **186**:178–191.
29. **Zimmerman, F. K., and N. R. Eaton.** 1974. Genetics of induction and catabolite repression of maltase synthesis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **134**:261–272.