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Isolation of a Catabolite Repression Mutant of Yeast as a Revertant of a Strain That Is Maltose Negative in the Respiratory-Deficient State

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A character originating from *Saccharomyces cerevisiae* 1403-7A is described which interferes with maltose growth in the respiratory-deficient state. This character is inherited in an apparently non-Mendelian way, but at present no statement can be made concerning the localization of this character on a plasmid or the involvement of multiple genes. As a revertant of this character, a flaky mutant was isolated, showing a heavy flocculation during growth on liquid medium and resistance to catabolite repression for maltase, α-methyl-glucosidase, invertase, and succinate dehydrogenase. In wild-type cells, repression (caused by growth on 2% glucose) and derepression (caused by growth on 2% galactose) can be correlated with a lower and a higher level of cyclic 3',5'-adenosine monophosphate (cAMP), respectively. In cells of flaky mutant, growth on these carbon sources results in the same levels of cAMP as observed for the wild type. Consequently, in this mutant derepression in the presence of 2% glucose is not reflected in a higher level of cAMP.

Catabolite repression of a number of enzymes has been described in prokaryotes and in simple eukaryotes (for reviews see 8 and 28). It is generally assumed that in *Escherichia coli* catabolite repression is mediated by the cyclic 3',5'-adenosine monophosphate (cAMP) system (4, 15, 19, 20). On the other hand, a recent report by Ullmann (29) summarizes a number of observations that are difficult to reconcile with the view that cAMP is the (sole) mediator of catabolite repression in prokaryotes.

In yeast, a correlation was found between the degree of catabolite repression and the intracellular level of cAMP (23, 33), and the existence of phosphodiesterase (6, 21), cAMP-binding protein (22), protein kinase (24, 25), and adenyl cyclase (12, 23) was shown. Furthermore, cAMP (and other nucleotides) stimulate respiratory adaptation (5), and cAMP has a small stimulatory effect on α-glucosidase synthesis in protoplasts of *Saccharomyces cerevisiae* under strong repression (S. Haarasilta and E. Oura, Proceedings of the Third International Specialized Symposium on Yeasts, Helsinki, 1973). On the other hand, cAMP can influence carbon metabolism by stimulating trehalose degradation (30). Consequently, it is not clear whether the observed effects of cAMP are really direct effects on the degree of catabolite repression. Recently, Montenecourt et al. (16) described mutants of *Saccharomyces* in which the formation of invertase and α-glucosidase is insensitive to catabolite repression. However, in these mutants no correlation was found between the degree of derepression and the intracellular level of cAMP.

S. carlsbergensis, carrying the gene *MAL6* for maltose fermentation, and *rho*$^-$ derivatives of this strain are able to grow on maltose (26). However, among other *Saccharomyces* strains that grow on maltose in the respiratory competent state, some can be found that do not grow on maltose in the respiratory-deficient state (A. M. A. ten Berge, Ph.D. thesis, University of Utrecht, Utrecht, The Netherlands, 1973). In this paper it is demonstrated, that such a *rho*$^-$-dependent maltose-negative character, originating from *S. cerevisiae* 1403-7A, is inherited in an apparently non-Mendelian way. The results do not allow determination of the location of this character on a plasmid or the involvement of multiple genes. However, it appeared that a catabolite repression-negative mutant could be isolated as a spontaneous revertant of this *rho*$^-$-dependent maltose-negative character.

MATERIALS AND METHODS

Strains. The strains used in these experiments are summarized in Table 1.

TABLE 1. *Strains*

Strain	Genotype
1403-7A[a]	*S. cerevisiae* *a gal3 MAL4 trp1 ura3*
Wild type (2n)	*S. carlsbergensis a/α MAL6 MAL6*, ATCC 24904 (NCYC 74)
CB-6	*S. carlsbergensis α MAL6 ade1*
CB-8	*S. carlsbergensis α MAL6 trp5*
CB-99	*S. carlsbergensis α mal6-13 trp5*
99-C[b]	*a mal gal3 trp1 ura3*
CF-1[b]	*a MAL6 ade1 ura3*
CF-3[b]	*a MAL6 ade1 ura3*
CF-19[b]	*a MAL6 ura3*
Flaky (n)[b]	*a MAL6 ade1 ura3 flk1*
Flaky (2n)[b]	*a/α MAL6 MAL6 flk1 flk1*

[a] This strain was kindly provided by N. Eaton, Brooklyn College, New York.

[b] Hybrid between *S. cerevisiae* and *S. carlsbergensis*.

Petites. Cytoplasmic petites were routinely induced by growing cells in the presence of 5 mg of acriflavine per liter of medium containing 1% yeast extract (Difco), 2% peptone (Difco) and 2% glucose. In one experiment petites were induced by using 10 mg of ethidium bromide per liter of the same medium by the method of Goldring et al. (7).

Growth conditions. For testing growth on maltose, cells were plated on a medium containing 1% yeast extract, 2% peptone (Difco), 2% maltose, and 1% agar. Growth was followed up to 12 days. For the determination of enzyme levels and cAMP, cells were grown until the early log phase on liquid medium containing 0.7% yeast nitrogen base (without amino acids; Difco) and a carbon source as indicated in the text.

Enzyme determinations. A 100-ml volume of cells was centrifuged at 3,000 × *g*, suspended in 10 ml of water, centrifuged again, and suspended in 1 ml of water. Subsequently, crude extracts were prepared by ultrasonic oscillation. Cell debris was removed by centrifugation at 3,000 × *g*.

Succinate dehydrogenase (EC 1.3.99.1) was determined by the method of Mackler et al. (14). The enzyme activities are expressed as nanomoles of cytochrome *c* reduced per minute per milligram of protein at 30 C.

Maltase (EC 3.2.1.20) and α-D-methylglucosidase (EC 3.2.1.20) activities were assayed as described by Ouwehand and van Wijk (18). Enzyme activities are expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein at 30 C.

Invertase (EC 3.2.1.26) activity on intact cells was measured, by a slightly modified procedure of Neumann and Lampen (17), by estimating the amount of glucose released with a coupled glucose oxidase-peroxidase system (Glucostat, Worthington Biochemicals). The samples containing (per milliliter) 0.15 nmol of sucrose, 0.04 mmol of sodium acetate buffer (pH 5.0), and 0.2 ml of a yeast suspension were incubated at 30 C. The enzyme reaction was stopped by adding 1 ml of 1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0) to 1 ml of the incubation mixture; subsequently, the invertase was inactivated by heating at 100 C for 5 min. The addition of tris(hydroxymethyl)aminomethane, which we found to be a strong inhibitor of invertase activity, was included in the procedure to prevent the enzyme from acting with unknown velocities at elevated temperatures prior to the heat denaturation. Glucose standard solutions were treated in the same way. When a culture contained residual glucose, it was dialyzed overnight at 4 C against 0.04 M sodium acetate buffer (pH 5.0). Activities are expressed as micromoles of sucrose hydrolyzed per minute per milligram of protein at 30 C. For the determination of protein, cells were centrifuged, washed with water, and dissolved in 1 N NaOH at 100 C.

Protein was determined by the method of Lowry et al. (13).

Determination of cAMP. For the determination of cAMP, cells were collected by centrifugation and suspended in a solution containing 5% trichloroacetic acid. This suspension was immediately frozen in liquid nitrogen. The procedure was completed within 1 min. The extracts were neutralized as described by Montenecourt et al. (16), and cAMP was determined as described by Brown et al. (3).

RESULTS

Growth on maltose in the rho⁻ state. *S. cerevisiae* 1403-7A carries the *MAL4* gene, which is responsible for constitutive maltase synthesis (9, 10), and strain CB-6, a derivative of *S. carlsbergensis* ATCC 24904, harbors the inducible gene *MAL6* (26). Both strain 1403-7A and strain CB-6 are able to grow on maltose as a petite. From a cross of these strains a number of random spores was isolated and tested for constitutivity versus inducibility and for maltose growth in the *rho⁻* state. Most of the constitutive (*MAL4*) spores grew on maltose as petites. Surprisingly, none of the inducible (*MAL6*) spores showed normal growth on maltose as a petite, varying from the complete absence of growth to the occurrence of a lag time of at least 3 days. This impaired growth is specific for maltose, since normal growth of these petite strains was observed on glucose, fructose, mannose, sucrose, galactose, melibiose, and raffinose. When a lag time was observed, the growth response was the same for all cells of a population. Diploids obtained from a cross between these *rho⁻* ascosporal clones (aberrant growth on maltose as petite strain) and *rho⁻* strains of *S. carlsbergensis* (normal growth on maltose as a petite) showed the same aberrant growth characteristics on maltose.

Strain 99-C is a derivative of strain 1403-7A and, therefore, can harbor the maltose-negative character, but a direct test is impossible since this strain contains no *MAL* gene. Tetrad anal-

ysis was performed after crossing strain 99-C with a strain of *S. carlsbergensis* (CB-6) showing normal growth on maltose as a petite. Among the maltose-positive spores (50%) of 11 complete tetrads, none was found showing normal growth on maltose as a petite, indicating again a non-Mendelian inheritance of this negative character, independent of the presence of a *MAL* gene.

A non-Mendelian inheritance can be due either to the localization of the determinant on a plasmid or to the involvement of multiple non-linked nuclear genes. To see whether the non-Mendelian inherited maltose-negative phenotype is a mitochondrial determinant, petites were induced in strain CF-19 by repeatedly growing cells in the presence of ethidium bromide, a procedure that depletes cells from any mitochondrial deoxyribonucleic acid (7; for a review, see 2). It appeared that the maltose-negative character is also observed in, and inherited by, these petites. Consequently, the maltose-negative phenotype is probably not a mitochondrial determinant.

As mentioned before, in those petite spores showing impaired growth on maltose, a growth response was observed varying between a completely negative phenotype and some leakiness (a lag time of at least 3 days). It appeared that in a backcross between one of these leaky spores (CF-1) and *S. carlsbergensis* CB-8 most spores showed normal growth on maltose as a petite. These observations could indicate that the non-Mendelian inheritance is caused by a dependence of the phenotype on multiple non-linked nuclear genes.

Isolation of a catabolite repression mutant. Clones (100) of a petite *MAL6* strain (CF-3), carrying the maltose-negative character, were subjected to long-term adaptation. Among these, one was found to be a stable revertant that always showed an immediate growth on maltose as a petite. This revertant had a very high maltase activity when grown on 0.3% glucose (derepressed conditions). Moreover, when it was grown on 2% glucose (repressed conditions), the same high maltase activity was found. Since the revertant showed a very fast flocculation in liquid medium it was termed "flaky" (*flk1*).

Characterization of the flaky mutation. The petite flaky mutant was crossed with a grande strain of *S. carlsbergensis* (CB-99). The latter strain is completely maltose negative since it contains the *mal6-13* mutation (27). The (grande) hybrid was neither flaky nor glucose repression insensitive for maltase, proving that for both effects the *flk1* mutation is recessive. From this hybrid, tetrads were analyzed and tested for growth on maltose, for flocculation, and for glucose repression-insensitive maltase activity. The results (Table 2), indicate that *flk1* is a single mutation that is responsible both for the flocculation and for the glucose repression-insensitive maltase activity. Apparently this mutation is linked to neither *MAL6* nor to the petite phenotype locus. Note the absence of growth on maltose in spite of a high level of maltase in the *mal6-13 flk1* spores.

In *Saccharomyces* the synthesis of a number of enzymes, such as maltase, α-methylglucosidase, succinate dehydrogenase (18, 32), and invertase (16), is sensitive to catabolite repression. To determine whether the *flk1* mutation causes catabolite repression insensitivity as a general effect, specific activities of these enzymes were determined in the diploid flaky strain and in the wild-type diploid *S. carlsbergensis* after growth under repressed conditions (in the presence of 2% glucose) and derepressed conditions (in the presence of 0.3% glucose or 2% galactose) (Table 3). In wild-type cells a repression by glucose of about 90% is observed on all enzymes tested. In the flaky mutant, differential sensitivities to glucose are observed, since the percentage of repression varies from 0% (maltase) to 59% (succinate dehydrogenase).

Intracellular levels of cAMP. It was of interest to see whether the cAMP level in a catabolite repression-insensitive mutant had changed. Consequently, this level was determined in the diploid flaky strain and the

TABLE 2. *Genotypic and phenotypic segregation among spores[a] from the hybrid of the haploid flaky strain with CB-99*

No. of spores	Genotype	Phenotype		
		Growth on maltose	Sp act of maltase (nmol of maltose hydrolyzed/min per mg of protein) after growth on 2% glucose	Flocculation
31	*MAL6 FLK1*	+	0.5	−
31	*MAL6 flk1*	+	65	+
31	*mal6-13 FLK1*	−	0.4	−
31	*mal6-13 flk1*	−	65	+

[a] Thirty-one complete tetrads were analyzed. Parental ditypes, nonparental ditypes, and tetratypes were found in the ratio of 7:7:17, respectively.

TABLE 3. *Resistance of various enzymes to catabolite repression in the diploid wild-type S. carlsbergensis and in the diploid flaky mutant*[a]

Strain	Carbon source in the growth medium	Sp act of:			
		Maltase (nmol of maltose hydrolyzed/min per mg of protein)	α-Methylglucosidase (nmol of α-methylglucoside hydrolyzed/min per mg of protein)	Invertase (μmol of sucrose hydrolyzed/min per mg of protein)	Succinate dehydrogenase (nmol of cytochrome *c* reduced/min per mg of protein)
Wild type (2n)	Glucose, 2%	0.5 (95%)[a]	1 (87%)	0.4 (91%)	0.9 (93%)
	Glucose, 0.3%			4.3	12.2
	Galactose, 2%	10	8		
Flaky (2n)	Glucose, 2%	65 (0%)	110 (31%)	7.7 (36%)	2.8 (59%)
	Glucose, 0.3%			12.0	6.9
	Galactose, 2%	65	160		

[a] Percent repression, comparing repressed conditions (growth on 2% glucose) and derepressed conditions (growth on 0.3% glucose or 2% galactose).

wild-type diploid *S. carlsbergensis* after growth on 2% glucose (repressed conditions) and 2% galactose (derepressed conditions) (Table 4). It is clear that a small but significant difference in the level of cAMP is observed between the glucose- and galactose-grown cultures of both strains. Consequently, the derepression in the presence of glucose in the flaky mutant is not reflected in a higher level of cAMP.

TABLE 4. *Intracellular level of cAMP in the diploid wild-type strain and in the diploid flaky mutant*

Strain	cAMP[a]	
	Growth on 2% glucose	Growth on 2% galactose
Wild type (2n)	1.8 ± 0.3	3.2 ± 0.4
Flaky (2n)	2.2 ± 0.3	3.7 ± 0.5

[a] Expressed as picomoles per milligram (dry weight) of cells.

DISCUSSION

Both *S. cerevisiae* 1403-7A (*MAL4*) and *S. carlsbergensis* strains containing *MAL6* grow on maltose as petites. However, in spores isolated from a cross between these strains, this same growth response is observed only if they contain the gene *MAL4*. In spores containing gene *MAL6*, an aberrant growth response is observed, varying between complete absence of growth and long-term adaptation (a lag time of at least 3 days). Diploids isolated from crosses between ascosporal clones showing the maltose-negative character and wild-type strains of *S. carlsbergensis*, and all spores isolated after tetrad analysis of such diploids, show this maltose-negative character, indicating a non-Mendelian inheritance.

Prolonged treatment of a yeast cell with ethidium bromide results in a cytoplasmic petite that contains no mitochondrial deoxyribonucleic acid (2). The non-Mendelian inheritance of the maltose-negative character is probably not caused by a mitochondrial localization, since this character is still observed in and inherited by petites that are isolated after repeated treatment with ethidium bromide. However, at least two other hypotheses can explain the non-Mendelian inheritance. It could be due to a cytoplasmic factor located on a type of plasmid other than the mitochondrion, as is possibly the case for the killer factor (1), the *ure3* mutation (11), and the extrachromosomal element ψ (34). Alternatively, the inheritance of impaired growth on maltose in petites could be only apparently non-Mendelian, due to the fact that multiple nonlinked genes are involved. The fact that maltose-positive *rho*$^-$ spores are observed after a leaky strain is crossed with wild-type *S. carlsbergensis* could point in the direction of this latter alternative. If it is assumed that these multiple genes show some additive effect, the variation in the growth response (complete absence of growth to long-term adaptation) could be explained.

The flaky mutant was isolated as a spontaneous revertant of the maltose-negative character. This *flk1* mutation causes a heavy flocculation of the cells and a decrease in repression, in the presence of 2% glucose, of all enzymes tested that are sensitive to catabolite repression. It is interesting to note that this decrease is not the same for all enzymes: maltase shows a complete abolition of repression, whereas succinate dehy-

drogenase, invertase, and α-methylglucosidase show only a partial decrease in repression. Apparently, the *flk1* mutation results in an incomplete abolition of catabolite repression. The absence of a coordinate effect is probably caused by differential sensitivities to (residual) catabolite repression, a phenomenon known to occur in yeast (31).

The flaky mutant partly resembles mutant FH4C, described by Montenecourt et al. (16). This mutant also shows a heavy flocculation, impaired growth on some carbon sources, poor mating and sporulation behavior, and insensitivity to catabolite repression of α-glucosidase and invertase. If both strains indeed contain the same mutation, the impaired growth on some carbon sources and the poor mating and sporulation behavior of strain FH4C must be caused by additional mutations. This does not seem unlikely, since strain FH4C was isolated in a two-step mutational procedure with a low survival. Unfortunately, because of the latter characteristics, genetic manipulation of strain FH4C is impossible. Therefore, we cannot test whether strain FH4C and the flaky mutant are (partly) identical.

In our wild-type strain a small, but significant, difference was found between the intracellular levels of cAMP, comparing repressed and derepressed conditions, as was observed before (16, 23, 33). However, the change in the degree of catabolite repression in the flaky mutant in the presence of 2% glucose is not reflected in a change in the intracellular level of cAMP, a result also observed in strain FH4C. Consequently, from these observations and from those described in the Introduction, it can be concluded that until now there has been no clear evidence in favor of the view that catabolite repression in yeast is mediated by the cAMP system.

Until now, only one type of maltose-negative mutant of grande strains containing the *MAL6* gene could be isolated (27). It was tentatively concluded that these mutants are regulatory mutants, and not structural mutants of maltase, since a noninduced and, therefore, low level of maltase was observed in this mutant. In a maltose-negative strain containing both the *mal6-13* and the *flk1* mutation, a high derepressed level of maltase is observed (Table 2). This definitely proves that these mutants do not bear structural gene mutations affecting the maltase system. On the other hand, the maltose transport remains low in this strain (ten Berge, Ph.D. thesis). This explains the observation that this strain does not grow on maltose in spite of a high level of maltase.

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