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Saccharomyces Mutants with Invertase Formation Resistant to Repression by Hexoses

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Production of invertase by many strains of yeast is repressed in the presence of hexoses. This phenomenon interferes with studies on the secretion of invertase and with the preparation of large quantities of the enzyme for examination of its chemical and physical characteristics. *Saccharomyces* strain 303-67, a diploid carrying the single gene SUC-2 for (hexose repressible) invertase production, was subjected to ultraviolet irradiation. No single-step mutations to high level resistance were detected. By a two-step irradiation process mutants were obtained with differing degrees of resistance. The biochemical and genetic characteristics of these mutants are summarized with particular emphasis on FH4C (the most resistant). Although the steady state level of cyclic 3',5'-adenosine monophosphate (cyclic AMP) was usually slightly higher in cells grown in low- rather than in high-glucose media, the level of cyclic AMP was not correlated with the sensitivity of invertase synthesis to glucose repression. In mutant FH4C, 1 to 2% of the total cell protein is present as invertase; synthesis of alpha-glucosidase is also resistant to repression by hexoses. This mutant does not sporulate and is probably a haploid of *a*-mating type with low frequency of conjugation and poor viability of conjugants. Mutants 1016 and 1710 are substantially resistant to hexose repression and still sporulate well. They may be useful for genetic analysis of hexose resistance.

Hexose repression of invertase formation has been described in yeast (2, 3, 4, 7) and in *Neurospora crassa* (19). Early attempts to study secretion of invertase by yeast protoplasts (10, 24) were limited by the repression of invertase synthesis in the presence of even moderate levels of glucose, fructose, or mannose. Incubation with sucrose or raffinose, both of which are invertase substrates, merely delayed the onset of repression, since the initial rapid synthesis of invertase soon fell to the repressed rate as free hexose accumulated (10, 24). A preliminary report on mutant FH4C was presented at the IX International Congress of Microbiology, Moscow, Russia (1966).

MATERIALS AND METHODS

Organisms and culture conditions. The parent organism *Saccharomyces* strains 303-67 (28) contains a single gene for invertase formation (R_2; equivalent to SUC-2 [7]). This strain was reported to lack the genes for maltose fermentation (28), but, when a culture is plated on maltose agar, maltose-positive colonies appear with a frequency of 1 per 10^6 cells. The culture that was irradiated to produce the hexose-resistant mutants had become maltose positive, thus all mutants described here ferment this sugar. Cultures for inoculum were usually grown at 27 C in 0.3% yeast extract and 0.5% peptone (YEP medium) with 2% glucose. Stock cultures were maintained on the same medium with 1.5% agar.

When invertase formation was to be followed, we used Vogel's defined medium N (27) modified to contain 2.0 mg of inositol, 0.2 mg of calcium pantothenate, 0.2 mg of pyridoxine hydrochloride, and 0.2 mg of thiamine hydrochloride per liter. All carbon sources were autoclaved separately.

Growth was measured as turbidity by using a Klett-Summerson photoelectric colorimeter (filter no. 66). A reading of 150 corresponds to about 1 mg (dry weight equivalent) of organisms per ml. Mutant FH4C grows as clumps and sediments rapidly; this can be minimized by adding disodium ethylenediaminetetraacetic acid (EDTA) to 10 mM. Under these circumstances stable turbidity readings can be obtained.

Strain 303-67 sporulates poorly on the usual media, but can be induced to sporulate by a procedure suggested by Marcelle Grenson (Institut de Recherches du C.E.R.I.A., Brussels, Belgium): a rapidly growing culture was streaked on a medium containing 0.25% yeast extract, 0.1% glucose, 0.1

M potassium acetate, and 1.5% agar. After 48 h about 90% of the organisms had formed spores. The same conditions were used to detect sporulation by the various mutants.

The tested strains used in the mating experiments (*Saccharomyces* strains D-310-4D α, D-310-2a a, D-332-4A α, and D-344-2c a) were kindly provided by Charlotte Avers of Rutgers University. Cells to be mated were grown overnight on YEP slants with 2% glucose. The young cells were mixed on fresh solid medium and incubated for 4 to 24 h. Conjugation figures were detected by microscope examination.

Enzyme assays. Invertase was measured on intact cells (95 to 97% of the invertase is external and available to substrate [5]) and in culture supernatant fluids by methods described previously (13, 21). If a culture contained residual glucose, the supernatant fluid was dialyzed overnight at 4 C against running tap water before analysis. One unit of invertase is the amount that will hydrolyze 1 μmol of sucrose per min at pH 4.7 and 30 C. (Pure external invertase is assumed to have a specific activity of about 4,500 units per mg of protein [16]). Alpha-glucosidase was assayed with p-nitrophenyl-α-D-glucopyranoside as substrate (13) in cell-free extracts prepared by grinding cells with Virtis glass beads (0.2-mm diameter, Arthur H. Thomas). Cleavage of maltose and of methyl-α-D-glucopyranoside under the same conditions was detected by measurement of the formation of glucose (as in the invertase assay).

Determinations. Protein and deoxyribonucleic acid were extracted by a modification of Schneider's procedure (23). The method of Lowry et al. (18) was used for estimation of protein and that of Burton (1) for deoxyribonucleic acid (DNA).

For assay of cyclic 3',5'-adenosine monophosphate (cyclic AMP), packed cell samples (about 8×10^8 cells), without washing, were suspended in 1.5 ml of 6% ice-cold trichloroacetic acid and centrifuged at $5,000 \times g$ for 3 min. The supernatant fraction was acidified with 0.1 ml of 1 N HCl, extracted six times with 4-ml volumes of water-saturated ether, and then lyophilized. The residue was dissolved in 0.5 ml of 50 mM acetate buffer (pH 4.0) and a 50-μliter sample was assayed for cylic AMP by the method of Gilman (8).

Detection of hexose-resistant mutants. The succinate-YEP medium of Hu et al. (9) was modified to contain 10% fructose and 1.5% agar (succinate-fructose agar). After growth of isolated colonies of strain 303-67 on this highly repressive medium for 24 to 36 h, no invertase could be detected.

A culture of strain 303-67 grown on YEP with 2% glucose to early stationary phase (single cells) was centrifuged, and the cells were suspended in 0.2 M potassium phosphate buffer, pH 6.5. The suspension was irradiated with an ultraviolet light source until about 99.99% of the cells were killed and then plated on the succinate-fructose agar in the dark and incubated for 24 to 36 h.

Colonies producing large amounts of invertase were detected as follows. The succinate-fructose plates were overlaid with Whatman No. 2 filter paper and sprayed with a solution containing 1 vial of Glucostat Special enzyme mixture and 1 vial of Chromogen (Worthington Biochemical Corp., Freehold, N.J.) in 60 ml of 0.2 M sucrose and 0.06 M acetate buffer, pH 5.0. A mixture adapted from the invertase assay of Gascon and Lampen (5) has also been used with satisfactory results: 80 μg of glucose oxidase, 4 μg of peroxidase, and 240 μg of o-dianisidine per ml of 0.06 M acetate buffer, pH 5.0, containing 0.2 M sucrose. The plates were incubated at 50 C for 15 min. Invertase-positive colonies formed distinct red spots. The colonies were picked into liquid succinate-fructose medium and incubated overnight, and the culture was assayed for invertase. Mutants FHi and FH13, produced by separate irradiations (Fig. 1), formed substantial amounts of invertase. These mutants were subsequently irradiated and plated as described above. Mutant FH4C was obtained from FHi; mutants 1015, 1016, and 1710 from FH13.

RESULTS

Growth and production of invertase. The production of invertase by strain 303-67 and by the various mutants under highly repressive (glucose medium) and mildly repressive (raffinose medium) conditions is illustrated in Table 1. Synthesis of invertase by strain 303-67 is almost completely repressed in both media. The single-step mutants FHi and FH13 produced greater quantities of invertase but are still repressed, especially in glucose medium. Second-step mutants to relatively high-level resistance were obtained from both FHi and FH13; FH4C (from FHi) appears to be the most resistant of these mutants to repression by hexoses (Table 1).

The results, presented in Table 2 on invertase production under conditions strongly repressive for strain 303-67, again illustrate the superior resistance of mutant FH4C. Under most circumstances, FH4C forms 10 to 20 units of invertase per mg (dry weight equivalent) of cells, but values essentially double these levels can be attained (Table 1). Thus invertase can represent 1 to 2% of the protein of this mutant.

It should also be noted that 90% or more of the enzyme is cell bound in most cultures of 303-67, whereas 25 to 35% of the invertase formed by FH4C is usually found in the medium.

Effect of carbon source. Strain 303-67 grows readily on glucose, fructose, or mannose, and on sucrose and raffinose which can be utilized only after cleavage by invertase. However, production of the enzyme is low on all of these substrates (<1 unit per mg of cells).

Mutant FH4C grows relatively well on glucose, sucrose, or raffinose until it has exhausted the sugar; at this point growth ceases. There is

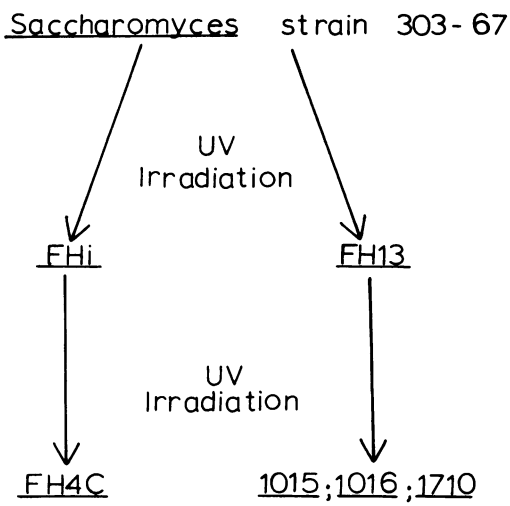

FIG. 1. *Derivation of hexose-resistant mutants.*

no indication that the mutant has formed any product inhibitory to further growth. In contrast, strain 303-67 will at least double in cell mass following exhaustion of the free hexose. Small inocula of FH4C grow on fructose and mannose only after a lag period, although large inocula (1% vol/vol or more) grow without delay. Once good growth has been attained, invertase production on these substrates is comparable to that on glucose (data not presented in detail).

The cessation of growth by FH4C when all the hexose had been utilized indicated that FH4C could not grow in this minimal salts medium on the breakdown products of the hexoses, i.e., ethanol, acetic and succinic acids, and glycerol. The two strains were therefore compared for growth on these compounds (1%) in the presence of 0.5% casein hydrolysate. Neither organism could grow on the combination of succinate and amino acids. In addition, FH4C did not grow on acetate and grew slowly on ethanol as compared with 303-67. Mutant FH4C grew moderately well on glycerol, but the growth of strain 303-67 was limited, probably because more than 90% of the cells sporulated within 6 to 8 h.

Mutant FH4C is not respiratory deficient. It grows well on glycerol and casein hydrolysate and oxidizes hexoses as readily as does the parent 303-67 (B. S. Montenecourt, Ph.D. thesis, Rutgers University, 1968). The parent and mutant were tested by the tetrazolium overlay technique of Nagai (20) and both reduced the dye rapidly.

Specificity of hexose resistance in mutant FH4C. The formation of α-glucosidase (defined as hydrolysis of *p*-nitrophenyl-α-D-glucoside), maltase, and α-methylglucosidase was examined since formation of these activities by yeast is known to be subject to repression by glucose (11, 12). The enzymes cleaving maltose and α-methylglucoside can be separated (11); both act on *p*-nitrophenylglucoside. Mutant FH4C and a maltose-positive clone of 303-67 were grown in glucose and maltose media and harvested in exponential phase, and the cell-free extracts were prepared. Strain 303-67 showed the expected repressibility, i.e., it produced the enzymes on maltose but not on glucose medium (Table 3). In contrast, mutant FH4C formed substantial levels of the enzymes

TABLE 1. *Invertase production by hexose-resistant mutants of Saccharomyces strain 303-67*[a]

Strain	Glucose (2%)		Raffinose (6%)	
	Turbidity (Klett units)	Invertase (units/mg of cells)	Turbidity (Klett units)	Invertase (units/mg of cells)
303-67	480	0.7	435	0.9[b]
FHi	440	3	420	9
FH4C	271	25	308	39
FH13	465	2	410	9.5
1015	380	10	235	46
1016	630	5	480	20
1710	630	16	640	23

[a] Vogel's medium N modified as in Materials and Methods, with the indicated carbon source, was inoculated with 1% (vol/vol) of a late exponential-phase culture (glucose medium) and incubated for 24 h. Cycloheximide (5 μg/ml) was then added to halt enzyme synthesis, and invertase was determined on the total culture.

[b] By slow addition of sucrose or raffinose, partial derepression can be obtained with specific activities as high as 4.7 units/mg of cells.

TABLE 2. *Comparison of invertase production under repressive conditions*[a]

Strain	Invertase formed (units/mg of cells)	
	4 h	8 h
303-67	0.15	0.3
FH4C	7.2	9.4
FH4C (nonclumping variant)	1.2	2.0
1016	0.9	4.1
1710	0.6	2.9

[a] Vogel's medium N modified as in Materials and Methods, with 0.1 M glucose, was inoculated with 10% (vol/vol) of an early exponential-phase culture in the same medium. After 4 h and 8 h of incubation, invertase was determined on the total culture.

TABLE 3. *Resistance of alpha-glucosidase production in mutant FH4C to hexose repression*[a]

Substrate	Activity (units/mg of protein)			
	Glucose		Maltose	
	Strain 303-67	Strain FH4C	Strain 303-67	Strain FH4C
PNPG[b]	0	225	450	700
Maltose	0	675	405	1,000
α-Methylglucoside	0	98	880	490

[a] The strains were grown on modified Vogel's medium N with either 100 mM glucose or 50 mM maltose for 6 h (approximately three doublings). The cells were harvested, and cell-free extracts were prepared and assayed for α-glucosidase (PNPG cleavage), maltase, and α-methylglucosidase (see Materials and Methods). Activities are expressed as units (cleavage of 1 nmole of substrate/min) per mg of protein in the cell-free extract.

[b] PNPG: *p*-nitrophenyl-α-D-glucopyranoside.

in the presence of glucose, although some response to maltose was evident. Formation of these enzymes by mutant FH4C had clearly become resistant in parallel with invertase production.

Intracellular cyclic AMP content. Van Wijk and Konijn (26) and Sy and Richter (25) have recently reported that intracellular cyclic AMP is increased in derepressed yeast cells. It was of interest to see whether there was any correlation between the sensitivity of invertase formation to catabolite repression and the intracellular cyclic AMP levels in strains 303-67, 1016, and FH4C. Cultures were grown in high- (300 mM) and low-glucose (50 mM) media and harvested during the exponential phase of growth; invertase and cyclic AMP were then determined (Table 4). The cyclic AMP level was slightly higher in cells from low than from high-glucose media, but there was no correlation between these levels and the amount of invertase synthesized. FH4C produced 20 to 30 times as much invertase as 303-67, yet their cyclic AMP levels were approximately the same. Surprisingly, cyclic AMP was extremely low in mutant 1016, even though formation of invertase was much greater than in the parental strain 303-67. The low level of cyclic AMP in 1016 probably reflects the presence of a highly active cyclic AMP phosphodiesterase previously demonstrated in protoplast lysates and cell free extracts of this strain (S.-C. Kuo, unpublished results).

Flocculation of FH4C. One of the most striking differences between mutant FH4C and the parent is its tendency to flocculate. In a liquid culture of FH4C left standing for 30 s, 70 to 80% of the cells sediments in clumps of 50 to 100. In 10 mM ethylenediaminetetraacetic acid, the clumps are reduced to 5 to 20 cells; higher concentrations do not produce single cells. Electron micrographs (B. K. Ghosh and B. S. Montenecourt, unpublished data) show that the clumps of 5 to 20 cells contain completely formed daughter cells held to the mother cell by the outer layer of the cell wall which is continuous around the cluster.

After repeated serial transfer, mutant FH4C no longer exhibited an unusual tendency to clump and now resembled 303-67 under electron microscopic examination. This nonclumping variant had lost most of its resistance to hexose repression, both for production of invertase (Table 2) or for α-glucosidase (S.-C. Kuo, unpublished results). A similar variant has been observed by others (P. Liras and S. Gascon, personal communication).

Genetic studies. Since mutant FH4C exhibits many characteristics not found in the parent organism, it was of interest to determine whether these were the result of one or several mutations. Strain 303-67 contains the D gene for self-diploidization (28) so that matings of the haploid spores are necessary for genetic analysis. Attempts to induce sporulation of FH4C under a variety of conditions which caused rapid sporulation of the parent were unsuccessful. The mutant appeared to be smaller and rounder than the oval-shaped parent and was therefore suspected to be haploid.

Attempts were made to mate strain FH4C with *Saccharomyces* tester strains D-310-4D α,

TABLE 4. *Cyclic AMP levels in organisms grown in high- and low-glucose media*[a]

Strain	Invertase[b]		Cyclic AMP[c]	
	300 mM Glucose	50 mM Glucose	300 mM Glucose	50 mM Glucose
303-67	0.37	0.51	8.4	14.3
1016	0.97	1.80	2.0	2.8
FH4C	8.2	10.8	10.8	12.8

[a] A light inoculum from a fresh slant culture was added to Vogel's medium N containing 300 mM or 50 mM glucose. After 15 to 17 h at 30 C, the cell density corresponded to 4×10^7 cells per ml. The levels of residual glucose were about 200 mM and 10 mM from the original 300 mM and 50 mM. The cells were harvested, and invertase and cyclic AMP content was determined as described under Materials and Methods.

[b] Units per milligram of cells.

[c] Picomoles per milligram of cells.

D-310-2a *a*, D-332-4A α, and D-344-2c *a*, but conjugation was not observed. An adenine-requiring mutant, produced by irradiation of 303-67, was also smaller and rounder than the parent. This mutant conjugated readily with the *Saccharomyces a* mating type tester strains and at low frequency with FH4C. Thus FH4C must also be the *a* mating type. After conjugation with FH4C the mixture of cells was spread on sporulation medium to obtain asci for tetrad analysis; however, less than 0.1% of the cells sporulated. This difficulty and the tendency of strain FH4C to grow as clusters prevented isolation of individual asci from unstained preparations.

The apparent haploid nature of mutant FH4C was substantiated by determining the DNA content of stationary-phase cells of strain 303-67 and FH4C. The cell counts for FH4C are less reliable than those for 303-67 since FH4C grows as clusters and these frequently contain dead cells. Despite this variability, the results of two separate experiments (Table 5) showed that the mutant contains approximately 0.5 as much DNA per cell as does the parent strain.

Comparison of the hexose resistant mutants. The properties of the various mutants (Fig. 1) are compared in Table 6 with those described in detail for FH4C. Mutants FHi and FH13 (from separate single irradiations of 303-67) differ from the parent primarily in their ability to produce increased amounts of invertase while growing in high concentrations of glucose. Mutant FHi (but not FH13) has lost the ability to sporulate. Of the resistant mutants obtained from FH13, mutant 1015 resembles FH4C closely. Mutants 1016 and 1710 have lesser degrees of resistance to hexose repression and differ from FH4C in sporulation, relative lack of flocculation and growth on acetate. It is obvious that none of the physiological changes detected in mutant FH4C can be correlated with the sensitivities of the various mutants to repression by hexoses.

DISCUSSION

By ultraviolet irradiation of a hexose-repressible *Saccharomyces* strain carrying a single gene (SUC-2) for invertase production, a series of mutants has been obtained whose synthesis of invertase is resistant in varying degrees to repression by hexoses. In two mutants, FH4C and 1015, formation of α-glucosidase is also resistant. Additional information has been published (5, 17) on the production of invertase by mutant FH4C and strain 303-67 in high- and low-glucose media and on the relative amounts of the external glycoprotein form and of the internal carbohydrate-free enzyme formed under these conditions. Kuo and Lampen (13) have reported that mutant 1016 produces much higher levels of invertase at low hexose concentrations than does 303-67, and forms substantial amounts of invertase even when growing on 0.05 M maltose.

Since mutant FH4C produces 20 to 50 times as much invertase per mg of cell protein as the parent strain, it has been valuable for purification of the external invertase (16, 21) and of the internal enzyme (5, 6), as well as for studies of invertase secretion by yeast protoplasts (17). Mutant 1016 has been used to investigate the modification of invertase formation and secretion by osmotic conditions (13) and by 2-deoxy-D-glucose (14).

TABLE 5. *Deoxyribonucleic acid content of strain 303-67 and mutant FH4C*[a]

Strain	μg of DNA per 5×10^9 cells		
	Preparation[b]		Avg
	I	II	
303-67	172	176	174
FH4C	79	60	70

[a] Samples of freeze-dried stationary-phase organisms (200 mg) were extracted with 0.5 N perchloric acid at 100 C for 30 min and analyzed for DNA. Initial cell count was determined with a Petroff-Hauser counter.

[b] Organisms were harvested from separate cultures. DNA analyses were done in duplicate.

TABLE 6. *Comparison of the hexose-resistant mutants and the parent strain 303-67*

Strain	Repressibility by hexoses		Delayed growth on mannose	Flocculation	Sporulation	Growth on acetate
	Invertase[a]	α-glucosidase				
303-67	++++[b]	+	−	−[b]	+	+
FHi	+++	+	−	−	−	+
FH4C	±	−	+	++++	−	−
FH13	+++	+	−	−	+	+
1015	+	−	+	++++	−	+
1016	++	+	+	++	+	+
1710	++	+	−	−	+	+

[a] Qualitative assignments based on several experiments of the type presented in Table 1.

[b] These characteristics are graded from − (absent) to ++++; the others are designated only as − or +.

The physiological or enzymatic basis for the resistance of these mutants to repression by hexoses is not known. The highly resistant mutants all carry at least two mutations. The degree of resistance does not correlate directly with the defects in growth on mannose (or fructose) and acetate observed in mutant FH4C, or with growth as clumps, or with the ability to sporulate (Table 5); however, the nonclumping variant of FH4C has also lost much of its resistance to repression. Also there was no correlation between the steady-state levels of cyclic AMP in several of the mutants and their sensitivity to hexose repression.

Mutants 1016 and 1710 are of special interest in that they have substantial degrees of resistance to hexoses, yet grow well and can sporulate; they may prove suitable for genetic analysis of the resistance to hexose repression.

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