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Pleiotropic Glucose Repression-Resistant Mutation in *Saccharomyces carlesbergensis*

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We describe the characterization of a mutation of the locus *GLR1*. This mutation allowed for (i) the glucose repression-insensitive synthesis of the enzymes maltase, galactokinase, α-galactosidase, reduced nicotinamide adenine dinucleotide-cytochrome *c* reductase, and cytochrome *c* oxidase and (ii) growth on maltose in the presence of the gratuitous glucose repressor D-glucosamine. The glucosamine resistance cosegregated with the glucose-insensitive synthesis of the enzymes listed above. In addition, crosses between the glucosamine-resistant mutant and isogenic sensitive strains gave only tetrads containing two resistant and two sensitive spores. Thus, a single pleiotropic mutation is responsible for both phenotypes. We call the locus *GLR1*, for glucose regulation, and the glucose repression-insensitive mutation *glr1-1*.

In yeasts, the level of activity of a wide variety of enzymes is regulated by glucose. This so-called "glucose effect" consists of two distinct phenomena: glucose inactivation and glucose repression. Glucose inactivation describes the proteolytic inactivation of specific enzymes which occurs after the addition of glucose to the growth medium (13). Among those enzymes known to be glucose inactivated are maltose permease (26), fructose 1,6-diphosphatase (25), isocitrate lyase (4), and cytoplasmic malate dehydrogenase (26). Glucose repression, on the other hand, describes the inhibition of enzyme synthesis by glucose. Among those functions sensitive to glucose repression are the sugar fermentation enzymes maltase (27), galactokinase (1), invertase (11), and α-galactosidase (unpublished data); mitochondrial functions including the Krebs cycle enzymes, the electron transport chain, and mitochondrial ATPase (20, 21), as well as some of the enzymes of gluconeogenesis (21).

A number of glucose analogs have been studied to determine their ability to mimic glucose repression (30, 31). Of these, D-glucosamine shows the greatest potential as a gratuitous glucose repressor in yeast and therefore is most useful for the isolation of glucose repression-insensitive mutants. D-Glucosamine is taken up by yeast cells and phosphorylated but is not metabolized further at any appreciable rate (3). Thus, D-glucosamine cannot be utilized for growth. Nevertheless, glucosamine does produce a repression which is similar to glucose repression in yeast. It represses the synthesis of maltase (10), cytochrome *c* oxidase (10), galactokinase (unpublished data), and α-galactosidase (unpublished data). Growth on an energy source such as maltose, galactose, or lactate is therefore inhibited in the presence of D-glucosamine, but growth on glucose is unaffected. D-Glucosamine also has no generalized growth-inhibiting effects as does 2-deoxyglucose, another glucose analog that has been used for isolating glucose repression-insensitive mutants in yeast (33).

We have used D-glucosamine to isolate mutants in which the synthesis of maltase is insensitive to glucose repression by isolating mutants able to grow on maltose in the presence of repressing levels of D-glucosamine. One glucosamine-resistant strain has been studied in detail. In this mutant, M1, the synthesis of all of the following enzymes is insensitive to glucose repression: maltase, galactokinase, α-galactosidase, NADH-cytochrome *c* reductase, and cytochrome *c* oxidase. A single pleiotropic mutation is responsible for the D-glucosamine resistance and for the glucose insensitivity of enzyme synthesis. The locus has been called *GLR1* for glucose repression.

MATERIALS AND METHODS

Strains. The strains of *Saccharomyces carlesbergensis* used are as follows: E56-2C (α *ade1 ura3 MAL6^c GAL MEL*), E6-1 (**a** *lys MAL6^c GAL MEL*), and E6-2 (**a** *lys MAL6^c GAL MET*). E6-1 and E6-2 were derived from E56-2C and CB1100 (**a** *lys MAL6 GAL MEL*) by six backcrosses and therefore are isogenic to E56-2C at approximately 94% of the loci. The strains originate from the collection of A. M. A. ten Berge, Utrecht, The Netherlands.

Media. YEP medium containing 1% yeast extract (Difco Laboratories) and 2% peptone (Difco) was used as the basic culture medium. To this were added various carbon sources and 0.1% D-glucosamine as appropriate for each experiment.

Genetic analysis. Standard techniques of mating, sporulation, dissection, and tetrad analysis were used (19).

Isolation of mutants. The mutants were isolated, using nitrous acid as a mutagen, in the following manner. E56-2C was grown to saturation in YEP plus 2% glucose. The cells were harvested and suspended in an equal volume of water and starved at 4°C for 1 to 4 days. These cells were then concentrated 10-fold into water. A 0.3-ml portion of this cell suspension was added to 3.0 ml of a freshly prepared solution of 5-μg/ml sodium nitrite in 0.1 M sodium citrate, pH 4.5. The time of exposure to the mutagen was adjusted to give 10 to 20% survival. After mutagenesis, the cells were washed with potassium phosphate buffer, pH 6.8, resuspended in liquid YEP plus 10% glucose, and divided into a number of separate cultures. Each culture was allowed to grow to saturation at 30°C and, from each, a heavy inoculum of cells was plated onto plates containing YEP plus 2% maltose plus 0.1% glucosamine. The plates were incubated at 30°C until colonies appeared. One colony from each culture was selected for study.

Growth conditions. The cells were grown aerobically at 30°C in a New Brunswick gyratory water bath or in a tube roller. Growth was followed by reading the optical density of the cells at 520 nm. The optical density values ranging from 0.4 to 0.8 were selected, corresponding to the log phase of cellular growth.

Cell breakage and subcellular fractionation. A highly concentrated suspension of cells was prepared in 3.5 ml of MTE buffer (0.25 M mannitol, 0.05 M Tris-acetate buffer, pH 7.5, and 1 mM EDTA, pH 7.2) for the respiratory enzymes and 0.05 M phosphate buffer, pH 6.8, for the maltase assay. The cells were broken in screw-cap test tubes, containing glass beads (0.45 to 0.5 mm in diameter), by vigorously shaking the mixture on a Vortex Genie Mixer for 2.5 min. The cells were chilled at intervals of 30 s. Cell extracts were obtained by centrifuging the homogenate at 3,000 rpm for 10 min and saving the supernatant fluid. This total cell extract was used for the maltase assay. The mitochondrial particles were obtained from the cell extract by centrifuging the 3,000 rpm supernatant at 15,000 rpm for 20 min. The mitochondrial pellet obtained was suspended in 0.3 to 0.5 ml of MTE buffer to give a final protein concentration of 2 to 3 mg/ml. This mitochondrial fraction was used for assaying the respiratory enzymes.

(i) Respiratory enzymes. All enzyme assays were performed in a Beckman DB spectrophotometer (model 25) at room temperature. The enzymatic assays were performed in triplicate, and the standard deviation was from 20 to 25% on samples run on different days.

For the NADH-cytochrome *c* reductase assay, the rate of reduction of cytochrome *c* was monitored at 550 nm. The reaction was started by addition of 25 to 100 μg of mitochondrial protein to the cuvette containing 100 μg of cytochrome *c*, 0.9 ml of phosphate buffer, pH 7.2 (0.05 M sodium phosphate-2 mM EDTA), 2 μg of KCN, and 0.4 mg of NADH (24). The absorbance coefficient of 18.5 mM^{-1} cm^{-1} was used.

Cytochrome *c* oxidase was assayed according to the method of Smith (23). Reduced cytochrome *c* was prepared by addition of sodium dithionate to give a pale orange color. The rate of oxidation of cytochrome *c* was followed at 550 nm after addition of 25 to 100 μg of mitochondrial protein to the cuvette containing 20 μg of reduced cytochrome *c*, in phosphate buffer (0.05 M sodium phosphate-2 mM EDTA, pH 7.2), in 1 ml of reaction mixture. The absorbance coefficient of 18.5 mM^{-1} cm^{-1} was used.

(ii) Fermentation enzymes. Galactokinase assay measures the phosphorylation of [^{14}C]galactose (28). The procedure used to permeabilize the cells is described by Adams (2).

Maltase activity was measured by following the rate of splitting of *p*-nitrophenyl-α-glucoside as described by Halvorson and Ellias (12) and Khan and Eaton (15), using a cell extract prepared by the procedure described above. α-Galactosidase activity was measured according to the method of Kew and Douglas (14).

Protein determination. Protein was measured by the method of Lowry et al. (16), using bovine serum albumin as the standard.

RESULTS

Isolation of D-glucosamine-resistant mutants. D-Glucosamine-resistant mutants were isolated in E56-2C, a strain which ferments maltose, galactose, and melibiose and carries a *MAL6*^{c} allele. The mutants were obtained by nitrous acid mutagenesis and growth of the mutagenized cells in YEP medium containing 10% glucose before selection on plates containing YEP plus 2% maltose and 0.1% D-glucosamine. This concentration of D-glucosamine inhibits growth on maltose and represses maltase synthesis but does not affect growth on glucose. Growth of the mutagenized cells on 10% glucose before selection was used for two reasons. First, at this high glucose concentration the cells grow to saturation without utilizing all of the glucose, and the remaining glucose concentration is sufficient to prevent derepression of the constitutive *MAL6* allele. Preinduction of maltase might have interfered with the selection of the desired class of mutations. Second, this procedure increases the probability of selecting mutants capable of glucose fermentation. Many of the glucose repression-insensitive mutants reported in the literature are in fact defective in their glucose uptake system and are not able to ferment glucose (33).

Twenty-four independent glucosamine-resistant mutants were obtained. Each of these was screened by determining the level of maltase activity under glucose-repressed conditions. In the parent strain, E56-2C, derepression produced a 10-fold increase in the level of maltase activity. Of the 24 tested, 12 showed at least a fourfold increase in the level of synthesis of maltase under glucose-repressed conditions. One

mutant, M1, exhibited almost no glucose sensitivity and was selected for detailed study.

Glucose sensitivity of enzyme activity in the D-glucosamine-resistant mutant M1. Table 1 shows the level of maltase activity in mutant M1 and in the parent strain, E56-2C. In E56-2C, the level of maltase synthesized under repressed conditions was about 10% of the level found when fully induced. In M1, the amount of maltase synthesized when grown on maltose was increased compared with E56-2C and the synthesis of maltase was completely insensitive to glucose repression.

To determine whether the glucose insensitivity was restricted to maltase synthesis only, the level of activity of a number of other enzymes whose synthesis is normally sensitive to glucose repression was determined. The levels of activity of galactokinase, α-galactosidase, NADH-cytochrome *c* reductase, and cytochrome *c* oxidase in M1 were also insensitive to glucose repression (Table 2). These results imply that the mutation in M1 is not simply an alteration at the *MAL6* locus or some other locus involving only maltase synthesis. Instead, M1 appears to carry a mutation (or mutations) affecting a primary step in glucose repression.

Other pleiotropic mutants which show a decreased glucose sensitivity for a number of repressible enzymes have been reported. Among these, most have altered rates of glucose uptake and have an increased doubling time when utilizing glucose (8, 33). Table 3 compares the growth rates of M1 and E56-2C on different fermentable and nonfermentable carbon sources. Clearly, the ability to utilize glucose as well as the other sugars was unaffected in M1. The glucose insensitivity therefore did not result from an altered glucose uptake system.

TABLE 1. *Level of maltase activity of E56-2C and mutant M1 grown under repressing and nonrepressing growth conditions*

Strain	Maltase activity (U/mg of protein)		
	5% Glucose	2% Maltose	2% Galactose
E56-2C	467	4,783	3,317
M1	9,583	8,717	3,200

Genetic analysis of M1. To characterize M1 genetically, it was crossed to the D-glucosamine-sensitive strains E6-1 and E6-2. Both were derived from the parent strain E56-2C by repeated backcrosses. The results of the tetrad analysis are shown in Table 4. Clearly, a single locus was responsible for the D-glucosamine-resistant phenotype. In addition, the glucosamine-resistant phenotype had no affect on germination or spore viability.

The gene producing resistance to glucosamine was also responsible for the glucose insensitivity of the synthesis of the enzymes maltase, galactokinase, α-galactosidase, NADH-cytochrome *c* reductase, and cytochrome *c* oxidase. Five tetrads were selected at random from the cross between M1 and E6-2, and the level of synthesis of each of the above enzymes was determined under induced but glucose-repressed growth conditions (Table 5). It is quite evident from these results that the glucosamine-resistant phenotype segregated with glucose insensitivity for each enzyme studied. Thus, the mutation found in strain M1 is pleiotropic and regulates the synthesis of all the glucose-sensitive enzymes tested here and possibly others we have not yet studied. We have called this gene *GLR1*, for glucose regulation, and the allele found in strain M1 is called *glr1-1*.

The *glr1-1* allele was recessive to the wild-type *GLR1*. The heterozygous diploid from crossing M1 with E6-2 was glucosamine sensitive, and maltase synthesis in this diploid was glucose repression sensitive (Table 6). A homozygous *glr1-1/glr1-1* diploid obtained by mating M1 with the glucosamine-resistant spore D3-5D (see Table 4) was glucosamine resistant, and synthesis of maltase was glucose repression insensitive. Dissection of this diploid gave only glucosamine-resistant spores.

TABLE 2. *Comparison of the induced level of activity of various glucose-sensitive enzymes in E56-2C and M1 under repressed and nonrepressed growth conditions*

Strain	Galactokinase (nmol/min per OD_{650})[a]		α-Galactosidase (ΔOD_{400}/min per OD_{650})		NADH-cytochrome *c* reductase (nmol/min per mg of protein)		Cytochrome *c* oxidase (nmol/min per mg of protein)	
	Repressed, 5% glucose + 2% galactose	Derepressed, 2% galactose	Repressed, 5% glucose + 2% galactose	Derepressed, 2% galactose	Repressed, 5% glucose	Derepressed, 2% lactate	Repressed, 5% glucose	Derepressed, 2% lactate
E56-2C	0.1	2.07	0.29	0.91	0.510	1.85	0.425	2.02
M1	1.77	4.80	0.83	0.85	1.51	1.63	1.06	1.53

[a] OD_{650}, Optical density at 650 nm.

DISCUSSION

We describe the isolation of a pleiotropic mutation of the locus *GLR1*. This mutation allows for (i) the glucose repression-insensitive synthesis of the enzymes maltase, galactokinase, α-galactosidase, NADH-cytochrome *c* reductase, and cytochrome *c* oxidase and (ii) growth on maltose in the presence of the gratuitous glucose repressor D-glucosamine. The glucosamine resistance is seen to cosegregate with the glucose-insensitive synthesis of the enzymes listed above. Genetic analysis shows that a single pleiotropic mutation is responsible for both phenotyes. We call the locus *GLR1*, for glucose regulation, and the glucose repression insensitive mutation is called *glr1-1*.

Before discussing the *GLR1* locus, a brief description of the previously reported pleiotropic mutations affecting glucose repression is necessary. Montenecourt et al. (18) reported a mutant, FH4C, in which the synthesis of invertase and maltase was insensitive to glucose repression, but the mutant did not mate, thus making genetic analysis impossible. A mutant similar to FH4C has been isolated by Schamhart et al. (22) called *flaky* (*flk*). *flaky* has a single nuclear mutation producing resistance to glucose repression of maltase, invertase, and succinic dehydrogenase synthesis. Like FH4C, its growth is extremely flocculant, making biochemical analysis difficult. Zimmermann and co-workers have reported a large number of mutants showing alterations in the glucose effect. Zimmermann et al. (32) characterized a mutant, *cat1*, which they believe controls the process of derepression from growth on glucose. The mutant *cat1* does not grow on glycerol or ferment maltose. Derepression of maltose permease, fructose 1,6-diphosphatase, isocitrate lyase, and malate dehydrogenase is prevented, but maltase and invertase synthesis and respiration are normally glucose regulated. Zimmermann et al. (32) suggest that the *cat1* mutation controls derepression since revertants accelerate the time of onset of derepression of the affected enzymes. Another interpretation of these results is possible. The phenotypic effect of *cat1* is reserved for only those enzymes known to be glucose inactivated. The action of *cat1* may be on this aspect of the glucose effect and not glucose repression as we have defined it. Using the *CAT1-2*^{d} and *cat1-1* mutants, Zimmermann and Scheel (33) isolated a large number of 2-deoxyglucose-resistant mutants. All were found to decrease the glucose sensitivity of maltase and invertase synthesis. The mutations mapped in three genes. Mutations in one gene, called *HEX1* by Entian et al. (8), showed reduced hexokinase activity. All *HEX1* mutants grew poorly on glucose, apparently causing glucose insensitivity as a result of reduced levels of phosphorylated glucose. All three classes of mutants isolated by Zimmermann and Scheel (33) are disappointing because they all exhibit reduced rates of growth on glucose or maltose, especially at high concentrations. Ciriacy (5) has isolated mutations in three loci, *CCR1, CCR2,* and *CCR3,* which do not grow on ethanol or glycerol. They resemble the *cat1* mutation described by Zimmermann et al. (32) in that the derepression of isocitrate lyase, fructose 1,6-diphosphatase, alcohol dehydrogenase II, and malate dehydrogenase is prevented. The Krebs cycle enzymes and succinic dehydrogenase are unaffected. As discussed above, these mutations may involve the regulation of glucose inactivation. In another report, Ciriacy (6) describes mutations in a locus designated *CCR80.* These mutants strongly resemble the *HEX1* mutants in that they have a reduced rate of glucose fermentation.

The *glr1-1* mutant described in this paper appears to be distinct from all the pleiotropic carbon catabolite repression mutants previously reported. It does not flocculate under any growth conditions. It ferments glucose (2 and 8%), mal-

TABLE 3. *Comparison of the doubling time of E56-2C and M1 grown on various carbon sources*

Carbon source	Doubling time (h)	
	E56-2C	M1
2% Glucose	2.3	2.3
8% Glucose	2.5	2.6
2% Maltose	2.1	2.0
2% Galactose	2.3	2.3
3% Lactate	4.2	4.3

TABLE 4. *Analyses of crosses between glucosamine-resistant mutant M1 and isogenic glucosamine-sensitive strains*

Diploid	Parents	No. of asci analyzed	Segregation of glucosamine resistance/glucosamine sensitivity											
			4-Spored asci					3-Spored asci				2-Spored asci		
			4:0	3:1	2:2	1:3	0:4	3:0	2:1	1:2	0:3	2:0	1:1	0:2
D2	M1 × E6-1	6	0	0	3	0	0	0	0	1	0	2	0	0
D3	M1 × E6-2	19	0	0	11	0	0	0	3	1	0	4	0	0

TABLE 5. *Level of enzyme activity in tetrads from the diploid M1 × E6-2 after growth under repressed condition of various glucose repression-sensitive enzymes*

Tetrad/spore	Glucosamine phenotype	Maltase (U/mg of protein)	Galactokinase (nmol/min per OD_{650})[a]	α-Galactosidase (ΔOD_{400}/min per OD_{650})	NADH-cytochrome c reductase (nmol/min per mg of protein)	Cytochrome c oxidase (nmol/min per mg of protein)
1						
A	Sensitive	16	0.11	0.10	0.27	0.20
B	Resistant	9,183	2.59	0.70	1.20	1.08
C	Resistant	7,967	0.95	0.88	1.66	1.59
D	Sensitive	350	0.21	0.21	0.35	0.17
2						
A	Resistant	9,833	2.21	1.18	1.55	1.55
B	Sensitive	1,100	0.06	0.22	0.44	0.65
C	Sensitive	116	0.07	0.13	0.36	0.47
D	Resistant	13,317	0.83	0.98	1.17	1.24
13						
A	Resistant	6,500	1.39	0.73	1.24	1.41
B	Sensitive	316	0.05	0.22	0.38	0.53
C	Sensitive	416	0.02	0.11	0.47	0.61
D	Resistant	9,500	0.67	0.77	1.85	1.13
14						
A	Sensitive	650	0.34	0.21	0.19	0.28
B	Sensitive	800	0.29	0.32	0.20	0.20
C	Resistant	6,467	1.77	1.01	0.88	1.35
D	Resistant	8,100	1.11	0.86	1.50	0.91
15						
A	Resistant	7,933	1.21	0.85	2.19	2.64
B	Resistant	7,517	1.13	0.72	1.79	1.35
C	Sensitive	600	0.10	0.13	0.30	0.18
D	Sensitive	517	0.05	0.23	0.55	0.47

[a] OD_{650}, Optical density at 650 nm.

TABLE 6. *Glucose sensitivity of maltase synthesis in diploids homozygous and heterozygous for the glr1-1 allele*

Diploid	Haploid parents	Phenotype	Maltase activity (U/mg of protein)	
			5% glucose	2% galactose
D1	E56-2C × E6-2 (*GLR1* × *GLR1*)	Sensitive	500	5,717
D3	M1 × E6-2 (*glr1-1* × *GLR1*)	Sensitive	1,017	8,683
D4	M1 × D3-5D (*glr1-1* × *glr1-1*)	Resistant	6,783	7,600

tose (2%) and galactose (2%) well and thus is clearly distinct from the three classes of 2-deoxyglucose-resistant mutants isolated by Zimmermann and Scheel (33). The enzyme functions which are glucose insensitive in *glr1-1* (maltase, galactokinase, α-galactosidase, NADH-cytochrome *c* reductase, and cytochrome *c* oxidase) are functions which are normally glucose repressed but not glucose inactivated. These glucose-repressible activities were unaffected in the *ccr-1, -2,* and *-3* mutations described by Ciriacy (5) and in the *cat1* mutation described by Zimmermann et al. (32) and therefore *glr1-1* is not related to these mutations. In addition, *glr1-1* grows normally on glycerol, whereas the *cat1* and *ccr* mutant do not.

Recent evidence suggests that, in yeast, glucose itself or glucose phosphate (and not a catabolite of glucose as in *Escherichia coli* [17]) produces glucose repression. First, the glucose analog D-glucosamine, which is not metabolized beyond the initial phosphorylation, produces a repression similar to that exerted by glucose (3). Also, the effects of mutants which block glycolysis at a variety of steps indicate that glycolysis intermediates are not the control molecules in glucose repression (7). Since *glr1-1* has the same doubling time as the parent strain on all of the fermentable sugars tested, it is strongly suggested that internal glucose and glucose phosphate levels are normal. Thus, the altered function in *glr1-1* is likely to be in a primary regulatory step which is sensitive to the level of internal glucose or glucose phosphate. In yeast, as in *E. coli,* cAMP has been implicated as a primary effector molecule in glucose repression

(9). The role of the *GLR1* locus may be in the regulation of internal cAMP levels or in a regulatory protein which binds to cAMP. We are presently involved in determining the cAMP levels in *glr1-1* under various growth conditions. In addition, we are continuing to analyze other mutants with a phenotype similar to that of *glr1-1* in order to determine the number of loci involved in the pleiotropic control of glucose repression in yeast.

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