IDENTIFICATION OF THE STRUCTURAL GENE FOR β-GLUCOSIDASE IN *SACCHAROMYCES LACTIS*

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HERMAN, ALBERTA (University of Wisconsin, Madison) AND HARLYN HALVORSON. Identification of the structural gene for β-glucosidase in *Saccharomyces lactis*. J. Bacteriol. **85**:895–900. 1963.—Three allelic forms ($B^{h, m, l}$) of the structural gene for β-glucosidase have been identified in the yeast *Saccharomyces lactis*. Evidence that these are structural gene alleles includes the independent expression of the alleles in homozygous and heterozygous diploids and differences in the specificity and in the physical properties of the enzyme produced in response to the various allelic mutations. Two factors, one controlling production of the pulcherrimin-like pigment, the other β-galactosidase activity, are linked to the B locus. The β-glucosidase in these strains hydrolyzes the chromogenic substrate, *p*-nitrophenyl-β-D-glucoside, arbutin, salicin, and esculin. Cellobiose, on the other hand, is hydrolyzed by another enzyme.

The synthesis of β-glucosidase in yeast is under the control of several factors. In *Rhodotorula minute*, β-glucosidase is inducible by a variety of β-glucosides (Duerkson and Halvorson, 1959), whereas in the hybrid *Saccharomyces fragilis* × *S. dobzhanskii* enzyme synthesis is paraconstitutive (MacQuillan and Halvorson, 1962). That is, this organism produces high basic levels of enzyme which can be further increased by induction. In addition, the response of paraconstitutive synthesis to various inducers and to catabolic repression suggests the existence of further multiple controls. One analogous example has been described in *Escherichia coli*, where mutations in an operator gene (O^c) regulating β-galactosidase synthesis lead to paraconstitutive enzyme synthesis (Jacob et al., 1960).

To characterize further the regulation of paraconstitutive synthesis of β-glucosidase in yeast, a genetic analysis was undertaken. Investigations of the genetic controls in *R. minuta* and in the hybrid were not possible, since these yeast are not amenable to genetic study. After a survey of yeasts containing β-glucosidase, two heterothallic strains of *S. lactis* were selected as the most satisfactory system for carrying out this aspect of the problem.

This paper is concerned with the identification, in *S. lactis*, of the nature of the genetic loci affecting paraconstitutive levels of β-glucosidase. Comparisons relating β-glucosidase levels and rates of hydrolysis of several natural β-glucosides were also examined. An analysis of the genetic controls regulating induced β-glucosidase synthesis is reported in the accompanying paper (Herman and Halvorson, 1963).

MATERIALS AND METHODS

Organisms used and production of spores. The two original haploid heterothallic parental strains of *S. lactis* Y14 and Y123 were obtained from L. J. Wickerham (strains Y1140 and Y1118, respectively). All subsequent studies involved them or strains derived from matings in which they served as the original parents.

Spore isolates and stock cultures were maintained on yeast malt agar slants (YMA) described by Wickerham (1951). The mass-mating method (Lindegren and Lindegren, 1943) was followed, using malt extract agar (ME; Wickerham, 1951) as both mating and sporulation medium.

Tetrad dissections. Spore isolation involved digestion (10 to 12 hr at room temperature) of the sporulated cultures with the gut juices of *Helix pomatia* followed by dissection on YMA blocks using the De Fonbrune micromanipulator. Genetic analyses were carried out only on asci in which all four spores survived.

Isolation of diploids. In *S. lactis*, the diploid state is transitory for the majority of cells in the culture. To enrich for diploids, selected strains were mass-mated on ME agar and incubated at

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30 C for a prolonged period of time (2 to 5 weeks). Stable diploids would be expected to grow vegetatively, and unstable diploids and haploid cells would mate and sporulate without producing many cells vegetatively.

Periodically, inocula were transferred to fresh ME slants and examined microscopically, after 24 to 48 hr of incubation at 30 C, for four-spored, nonconjugated asci. After the presence of such asci was verified, inocula from the original-mating ME slant was plated for isolated colonies on YMA plates. After 24 to 48 hr of incubation at 30 C to detect the presence of diploids, the colonies on YMA plates were replica plated onto ME plates, and after incubation the replicated colonies were examined microscopically for the presence of four-spored, nonconjugated asci. The corresponding diploids on the YMA plates were selected and transferred to YMA stock slants.

Analytical procedures. β-Glucosidase activity was determined by measuring the rate of hydrolysis of the chromogenic substrate *p*-nitrophenyl-β-D-glucoside (PNPG). Two methods of assay were employed. In the first, the differential rate of enzyme synthesis (ΔE/ΔOD) was determined in cultures growing in a succinate synthetic medium as previously described (MacQuillan and Halvorson, 1962). With the organism one optical density $(OD)_{600\ m\mu}$ unit is equivalent to 0.46 mg (dry weight) of cells per ml. In the second assay, a qualitative plate assay was developed for measuring the enzyme level in isolated colonies. Cells were grown at 30 C for 2 to 3 days on succinate synthetic medium (pH 5.4) containing 2% agar. The plates were then placed in an atmosphere saturated with benzene vapor and held at 37 C for 2.5 to 3 hr to disrupt the cells. In practice, this was accomplished by placing approximately 3 ml of benzene in the lids of the inverted petri plates and incubating the inverted plates in a closed container saturated with benzene vapor. Other organic solvents, such as toluene, acetone, chloroform, and ethyl acetate, could also be used; however, longer periods of incubation (4 to 6 hr) were required to give equivalent results. After incubation, the plates were overlaid with 10 ml of 0.067 M potassium phosphate buffer (pH 6.8) containing 3 mg of PNPG and 2% agar and incubated at 30 C for 30 to 60 min. β-Glucosidase activity of the colonies or streaks was scored by the intensity of the yellow color from PNPG hydrolysis. Strains, which assayed as high-activity strains, intermediate-activity strains, or low-activity strains by direct measurement of ΔE/ΔOD, consistently agreed with the same assays by the plate method.

β-Galactosidase was measured by a plate assay in a manner analogous to that described above. All materials and concentrations were the same, except that 3 mg of *o*-nitrophenyl-β-D-galactoside (ONPGal) were substituted for PNPG.

The rate of hydrolysis of the natural β-glucosides (arbutin, esculin, salicin, and cellobiose) was measured by modifications of the above two methods (Herman and Halvorson, 1963). In each case, the liberated glucose was estimated by means of the commercial glucose oxidase (Glucostat). In the plate assay, cells were grown and pretreated with benzene as described above. Plates were then overlaid with 10 ml of 2% agar in 0.067 M potassium phosphate buffer (pH 6.8) to which 1 ml of 0.25 M natural β-glucoside and 2 ml of Glucostat (diluted 1:25 from the original concentrate) had been added. Plates were incubated at room temperature for 2 to 4 hr and then scored as having high, intermediate, or low rates of hydrolysis.

Protein determinations were made by the method of Lowry et al. (1951), using bovine crystalline serum albumin as standard.

Reagents. PNPG, ONPGal, arbutin, salicin, esculin, and cellobiose were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Glucose oxidase (Glucostat) was obtained from Worthington Biochemical Corp., Harrison, N.J. Bovine crystalline serum albumin was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio. Snail gut juice (suc d'helix pomatia) was purchased from Industrie Biologique Francaise.

RESULTS

Segregation of β-glucosidase levels. The two parental strains of *S. lactis* were paraconstitutive for β-glucosidase synthesis. As reported in the accompanying paper (Herman and Halvorson, 1963), β-methyl glucoside (2×10^{-2} M) induced the enzyme level of Y123 4-fold, and glucose (1×10^{-3} M) led to a 60-fold induction of Y14.

The two parental strains also differed in their basic enzyme levels. The differential rate of enzyme synthesis (ΔE/ΔOD) for Y123 ranged from 3 to 5, while that of Y14 ranged from 0.7 to 1.7. In the plate method of assay, Y123 was scored as

a high-activity strain and Y14 as an intermediate-activity strain. A spontaneous mutant was derived from an intermediate-activity strain, which had a $\Delta E/\Delta OD$ of less than 0.7 (plate assay low). This mutant was not an absolute negative, since cell extracts contained 13 units of enzyme per mg of protein compared with 609 units per mg of protein for extracts from strain Y123.

The segregation of the factors affecting the high, intermediate, and low activity levels of β-glucosidase are shown in Table 1. The segregation ratios (2:2) in heterozygous crosses and the ratios (4:0) in homozygous crosses indicated that these basic enzyme levels are expressions either of closely linked loci or of allelic forms of a locus affecting β-glucosidase activity.

Since no recombinants were observed in 92 tetrads from high-activity strains × intermediate-activity strains, or in 16 tetrads from low-activity strains by either intermediate- or high-activity strains, the high, intermediate, and low activities of β-glucosidase are undoubtedly expressions of allelic forms of a single locus (B) affecting β-glucosidase activity. These alleles have been designated B^h, B^m, and B^l for high, intermediate, and low enzyme activities, respectively.

Evidence that the B locus is the structural gene for β-glucosidase. The properties of the B locus are consistent with either a structural or regulatory gene for β-glucosidase synthesis. If the B alleles are alternative forms of the structural gene, then some of the properties of the enzyme formed under the influence of these alleles might be expected to differ. If, on the other hand, the B alleles are alternative forms of a control gene, no significant differences in the properties of the enzyme should be detected. Consequently, a comparison of the specificity and physical properties of the B-mutant enzymes was undertaken.

TABLE 1. *Segregation ratios of $B^{h,m,l}$ alleles in homozygous and heterozygous crosses*

Parental cross	No. of asci	Segregation ratio (B^h-B^m-B^l)*
$B^h \times B^m$	92	2:2:0
$B^m \times B^l$	7	0:2:2
$B^h \times B^l$	9	2:0:2
$B^h \times B^h$	12	4:0:0
$B^m \times B^m$	14	0:4:0
$B^l \times B^l$	11	0:0:4

* β-Glucosidase activity was measured by the plate assays; see Materials and Methods.

TABLE 2. *Comparison of the β-glucosidases produced by the various B alleles**

Source of enzyme	PNPG	Inhibition by mannose	Stability time for 50% inactivation at 20 C†	Inhibition by citrate‡
	$K_m \times 10^{-5}$		*hr*	%
B^h	10.4	Competitive	12	0
B^m	4.5	No effect	4	55
B^l	4.5	No effect	1	5

* Measurements were carried out on crude enzyme extracts. PNPG = *p*-nitrophenyl-β-D-glucoside.

† Enzyme extracts were incubated at 20 C in a buffer containing 4.5×10^{-2} M tris(hydroxymethyl)aminomethane, 2.5×10^{-2} M borate, 10^{-3} M ethylenediaminetetraacetic acid, and 10^{-2} M mercaptoethanol at pH 8.48.

‡ Enzyme extracts were incubated in 0.3 M citrate-phosphate buffer at pH 6.6. The per cent inhibition was calculated by comparison with controls incubated in 0.3 M potassium phosphate (pH 6.6).

Crude enzyme extracts were prepared by breaking yeasts in a French pressure cell. Using PNPG as substrate, the apparent K_m values (by the standard Lineweaver-Burke plot) in 0.067 M phosphate buffer (pH 6.8) were estimated. The concentration of carbohydrates which results in a 50% inhibition (K_i) was calculated from measurements in which the inhibition of PNPG hydrolysis by a fixed concentration (5×10^{-3} M) of carbohydrate was estimated at various PNPG concentrations. The results of these and other comparisons are summarized in Table 2. K_m values, using PNPG as substrate, ranged from 4.5×10^{-5} M for the B^m and B^l enzyme extracts to 10.4×10^{-5} M for the B^h extract. These differences have been reproduced several times, and it was concluded they were beyond experimental error. Mannose is a competitive inhibitor for the B^h enzyme, but had no effect on the B^m or B^l enzymes. On the other hand, the $B^{h,m,l}$ enzymes were all competitively inhibited to a similar degree by glucose. The K_i values for glucose on the $B^{h,m,l}$ enzymes were 3.1×10^{-3}, 2.1×10^{-3}, and 2.2×10^{-3}, respectively.

The β-glucosidase formed under the influence of the B alleles also showed significant differences in their stability at room temperature. The time

required for 50% inactivation of the B^h enzyme was approximately 12 hr, that for the B^m enzyme 4 hr, and that for the B^l enzyme was about 1 hr. The optimal pH for the B mutant enzymes in 0.3 M phosphate buffer were similar and ranged from pH 6.2 to 6.7. However, citrate ion showed a specific inhibitory effect on the B^m enzyme. In the presence of citrate, B^m enzyme activity was reduced approximately two-thirds over the control. B^h enzyme, on the other hand, showed no change in activity in the presence of this ion.

These differences in the specificity and physical properties of the B mutant enzymes led to the conclusion that the B alleles are alternate forms of the structural gene for β-glucosidase. This conclusion is further supported by the findings in Table 3 that, in diploids heterozygous for the B alleles, the β-glucosidase level is the arithmetical mean of the two parental alleles. Thus, each allele finds expression in the diploid state, and, therefore, the observed differences in enzyme levels cannot be attributed to variations in the kind or amount of repressor formed in these strains.

Linkage factors. Wickerham and Burton (1956) had previously shown that the B^m parent Y14 possessed the ability to produce a pink pulcherrimin pigment. This pigment is produced when the cells are grown in media containing ferric citrate and glucose. The B^h parent Y123 was unable to synthesize this pigment.

When progeny from a cross of these two parental strains were assayed for pigment-producing ability, a 2:2 segregation of pigment-nonpigment was observed in each of 22 tetrads examined, indicating a single locus was involved.

TABLE 3. *Diploid paraconstitutive enzyme levels**

Diploid genotype	β-Glucosidase (ΔE/ΔOD)
$B^h(4) \times B^h(3.7)$	4
$B^m(1.7) \times B^m(1.7)$	1.7
$B^l(<0.7) \times B^l(<0.7)$	<0.7
$B^h(4) \times B^m(1)$	2.3
$B^m(1) \times B^l(<0.7)$	<0.7
$B^h(4) \times B^l(0.7)$	2.3

* The differential rate of enzyme synthesis (ΔE/ΔOD) was measured on cells growing exponentially in synthetic succinate medium. Numbers in parentheses represent differential rate of β-glucosidase synthesis (ΔE/ΔOD) for each parent from which the individual diploids were derived.

Since the B^l strain was derived from a B^m mutant, all of the B^l strains also possessed pigment-forming ability. In heterozygous diploids, pigment production was dominant over nonproduction. More interestingly, pulcherrimin production was found to segregate with the B^m allele in each of the 22 tetrads examined. Isolation of an ultraviolet-induced mutant, lacking ability to produce this pink pigment but still possessing medium β-glucosidase levels, indicated that these two activities were controlled by independent but closely linked loci.

Both parental strains Y14 and Y123 possess β-galactosidase activity when grown on synthetic succinate plates. When ascospore sets derived from a cross of these two strains were assayed for β-galactosidase activity (plate assay using ONPGal), the following types of segregation ratios for activity-nonactivity were obtained: 4:0, 2:2, and 3:1. Such ratios suggest the presence of two polymeric genes for β-galactosidase. Segregation ratios in 19 tetrads derived from crosses of each parent with strains lacking β-galactosidase activity were always 2-β-galactosidase-positive, 2-β-galactosidase-negative spores thus supporting the previous observations of two polymeric genes. The gene present in Y123 was designated B_{gal_1} and that in Y14 was designated B_{gal_2}.

The percentages of parental ditype-nonparental ditype-tetratype for tetrads derived from a $B_{gal_1} \times B_{gal_2}$ cross were found experimentally to be 20%:33%:47%. Such percentages fall well within the ranges predicted, if the two polymeric genes were unlinked.

A study of the segregation ratios in 15 tetrads derived from a cross of $B^hB_{gal_1} \times B^mB_{gal_2}$ showed that the B_{gal_1} gene always segregated with the B^h allele, and the B_{gal_2} gene segregated independently of the B alleles. These results imply that B_{gal_1} is linked to the B locus. However, the relationship between these two loci, as well as that controlling pulcherrimin production, cannot be definitely established until additional studies are carried out.

Hydrolysis of natural β-glucosides by B-mutant strains. The experiments of Barnett, Ingram, and Swain (1956) suggest that yeast may produce more than one type of β-glucosidase. However, β-glucosidase hydrolyzed the three natural β-glucosides, arbutin, esculin, and salicin (Hu et al., 1960). It was of interest, therefore, to

investigate the specificity of the β-glucosidase in *S. lactis* toward the natural β-glucosides and to determine whether changes in the structural gene led to changes in the rates of hydrolysis of the natural β-glucosides.

Following the procedure described in Materials and Methods, 34 selected ascospore sets containing B^h, B^m, and B^1 alleles were assayed for their ability to hydrolyze arbutin, salicin, esculin, and cellobiose. Rates of hydrolysis were recorded as high, intermediate, or low, depending upon the intensity of the yellow-brown product formed in the assay mixture. In all of the cases examined, there was a close correlation between the rate of hydrolysis of PNPG, arbutin, salicin, and esculin, thus suggesting that the same enzyme hydrolyzed all four compounds. Cellobiose hydrolysis, on the other hand, showed some variability. Several B^m strains showed high cellobiose activity while a few B^h strains showed only an intermediate to low activity toward cellobiose.

These observations imply that a second β-glucosidase active against cellobiose may be present in some of the strains examined. Observations suggesting the presence of more than one β-glucosidase in *S. lactis* are similar to those reported by Barnett et al. (1956), who studied the β-glucosidase activity of some 119 strains of yeast which had been grown in the presence of the natural β-glucosides, arbutin, esculin, salicin, and cellobiose. In their study, several strains of *S. lactis* were examined. All of these strains were able to hydrolyze arbutin, esculin, and salicin with approximately equal efficiency. On the other hand, ability to hydrolyze and grow on cellobiose varied considerably in these strains. From these, as well as the results from other strains, Barnett et al. (1956) suggested that yeast may contain different β-glucosidases.

Discussion

The β-glucosidases produced in response to the three allelic forms of the B locus are very similar. As described here and elsewhere (Herman and Halvorson, 1963), they have the same relative specificity to PNPG and the natural β-glucosides and the same pH optimum, and are all competitively inhibited by glucose in an identical manner. The description in this paper of differences in both the specificity of the active center and physical properties signify these three alleles as alternate forms of the structural gene for β-glucosidase. In the parental natural stocks, the B^h locus differed from the B^m locus in producing an enzyme with an increased K_m for the substrate PNPG, an increased sensitivity to citrate ions, a loss of complexing properties with mannose, and an increased lability. In a B^1 mutant derived from a strain carrying B^m, a more labile form of the enzyme was produced. The independent function of each of these alleles in the diploid state is further evidence that the B locus is a structural gene.

Two aspects concerning the nature of the controls regulating paraconstitutive synthesis of β-glucosidase in *S. lactis* warrant consideration. First, the paraconstitutive synthesis of β-glucosidase implies that some control mechanism is present which permits synthesis of this enzyme even in the absence of inducer. Second, the results of this study indicate that the B alleles in some way influence the basic level of β-glucosidase found in individual strains of *S. lactis*. The independent expression of the B alleles in homozygous and heterozygous diploids shows that the difference in basic levels does not result from formation of different amounts or kind of repressor. Therefore, these alleles must regulate paraconstitutive levels through some other mechanism.

There are at least two mechanisms for altering the basal, constitutive level of enzyme activity in cells: (i) mutations in the structural gene, which alter the turnover number of the enzyme but not its rate of synthesis; and (ii) operator mutations, which regulate the rate of enzyme synthesis.

The nature of the genetic controls regulating paraconstitutive enzyme formation has been especially well-studied in β-galactosidase mutants of *E. coli* (Jacob et al., 1960). In this system, mutants were isolated which contained enzyme levels which varied from 10 to 50% of the level formed under conditions of induction. In heterogenotes, the paraconstitutive condition was dominant to inducibility. Genetic studies showed that these paraconstitutive mutants all mapped in the operator region of the genome and were designated as o^c mutants. In some o^c mutants, the mutations appeared to overlap the structural gene, and the β-galactosidase formed by these mutants showed altered physical properties (Jacob et al., 1960). From the properties exhibited by these o^c mutants, it was suggested that the paraconstitutive conditions resulted

from mutation in the operator region, which lead to a decreased sensitivity of the operator for repressor. It is, therefore, clear that mutations in the structural gene can affect both the activity and rate of synthesis of an enzyme, and, consequently, the present data are insufficient to decide between these two possibilities.

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LITERATURE CITED

BARNETT, J. A., M. INGRAM, AND T. SWAIN. 1956. The use of β-glucosides in classifying yeasts. J. Gen. Microbiol. **15**:529–555.

DUERKSON, J. D., AND H. O. HALVORSON. 1959. The specificity of induction of β-glucosidase in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **36**:47–55.

HERMAN, A., AND H. HALVORSON. 1963. Genetic control of β-glucosidase synthesis in *Saccharomyces lactis*. J. Bacteriol. **85**:901-910.

HU, A. S. L., R. EPSTEIN, H. O. HALVORSON, AND R. M. BOCK. 1960. Yeast β-glucosidase: comparison of the physical-chemical properties of purified constitutive and inducible enzymes. Arch. Biochem. Biophys. **91**:210–218.

JACOB, F., D. PERRIN, C. SANCHEZ, AND J. MONOD. 1960. L'operon: groupe de genes à expression coordonnée par un operateur. Compt. Rend. **250**:1727–1729.

LINDEGREN, C. C., AND G. LINDEGREN. 1943. A new method of hybridizing yeast. Proc. Natl. Acad. Sci. U.S. **29**:306–308.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

MACQUILLAN, A. M., AND H. O. HALVORSON. 1962. Metabolic control of β-glucosidase synthesis in yeast. J. Bacteriol. **84**:23–30.

WICKERHAM, L. J. 1951. Taxonomy of yeasts. U.S. Dept. Agr. Tech. Bull. No. 1029.

WICKERHAM, L. J., AND K. BURTON. 1956. Hybridization studies involving *Saccharomyces lactis* and *Zygosaccharomyces ashbyi*. J. Bacteriol. **71**:290–295.