D-Glucose Isomerase: Constitutive and Catabolite Repression-Resistant Mutants of Streptomyces phaeochromogenes

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Received for publication 16 July 1975

As in other Streptomyces species, the enzymatic conversion of D-glucose to D-fructose is carried out in Streptomyces phaeochromogenes NRRL B-3559 by the inducible enzyme, D-xylose keto isomerase (EC 5.3.1.5.). Mutants of this microorganism were selected for their ability to grow on D-lyxose (2-epimer of D-xylose). As a result of the mutational event, the microorganism constitutively produced D-xylose isomerase. In the parent strain, the constitutive formation of the isomerase was repressed by D-glucose. The fact that this mutant was unable to grow in low D-xylose concentrations in the presence of the D-glucose analogue, 3-O-methylglucose, permitted the isolation of D-xylose isomerase constitutive mutants which were insensitive to D-glucose repression.

In the genus Streptomyces, the enzymatic conversion of D-glucose to D-fructose by D-xylose keto isomerase (EC 5.3.1.5.) was initially reported by Tsumura and Sato in 1965 (10). Subsequent studies on the streptomycete enzyme revealed that it possesses high thermal stability (7–10) which permits its extraction and manipulation for long periods of time at room temperature. Additionally, it is strongly activated by low Mg2+ concentrations (7, 9) and it exhibits optimum activity at temperatures around 70 °C; the high temperature prevents the further metabolism of the product by unrelated enzymes when crude extracts are used as enzyme source (7–10). Streptomyces isomerase shows a high affinity for D-glucose ranging from 86 to 250 mM (7–10). Finally, since 80 to 90% of the total enzyme content can be fixed inside the cells by heat treatment (9), cells can be used without disruption as an enzyme source. These qualities give this genus a high value in the industrial production of corn sugar syrups containing high D fructose concentrations.

Streptomyces isomerase is an inducible enzyme requiring the presence of D-xylose in the culture medium for its production (7, 9, 10). This characteristic leads to an increase in the cost of enzyme production. Takasaki and Kossugi (9) found one means to decrease this cost by isolating a mutant of Streptomyces albus capable of using xylans (D-xylose polymer), thereby producing the inducer in the medium from the less expensive polysaccharide.

We are interested in determining new mechanisms concerned with the economical and efficient production of isomerase. For this reason, constitutive D-xylose keto isomerase mutants of Streptomyces phaeochromogenes NRRL B-3559 were obtained. The selection and characterization of the mutants are described, and their possible utility is discussed in the present paper.

MATERIALS AND METHODS

Organism and cultural conditions. S. phaeochromogenes NRRL B-3559 was kindly supplied by the Agricultural Research Service Culture Collection, maintained at the Northern Regional Research Laboratory, Peoria, Ill.

All cultures were grown in the RM medium previously described (7). The cultures were incubated at 29 C on a rotary shaker at 100 rpm. Stock cultures were maintained on RM medium solidified with 1.5% agar.

For mycelial propagation and enzyme formation, a 250-ml Erlenmeyer flask with 100 ml of RM medium (supplemented with D-xylose and D-glucose, 0.7 and 0.3%, respectively) was inoculated with spores from a 2-day-old slant and incubated for 36 h. The total content of the flask was added to a 2-liter Florence flask containing 1 liter of the same supplemented RM medium. After 36 h of incubation, the mycelium was harvested by filtration on Whatman no. 41 paper, washed twice with distilled water, and suspended in 0.2 M potassium phosphate buffer (pH 7.2) at room temperature, maintaining a ratio of 2 g (wet weight) of mycelium to 5 ml of buffer.

Enzyme preparation. Enzyme solutions were prepared as reported earlier (7). The final enzyme preparation from the parent strain and the mutants had specific activities for D-glucose ranging from 0.3 to 0.55 μmol/min per mg of protein.

The enzyme was assayed using 80 μmol of D-
glucose per ml as substrate, as reported earlier (7), with the modification that the reaction was stopped with 1 ml of 0.5 M perchloric acid. The d-fructose formed was determined by the cysteine carbazole method (2), by comparison with a standard curve prepared with d-fructose.

Protein was determined by the method of Lowry et al. (3), using bovine serum albumin as standard.

Spore preparation and mutant selection. Spores were harvested from a 2-day-old slant by agitation in distilled water. The spores were then separated from the mycelia by filtration through cotton wool and washed twice with distilled water by centrifugation. The spores were resuspended in distilled water (5 ml) and placed in a 9-cm glass petri dish. They were then incubated with a germicidal 15-W ultraviolet lamp for 30 s at a distance of 15 cm. This treatment resulted in a 90% kill. These spores were plated (1.5 × 10⁶ viable cells per plate) under the desired selective conditions on the inorganic salts agar medium (MM) of Pridham et al. (6).

Constitutive mutants for d-xylose keto isomerase were selected for their ability to grow in the presence of 3.3 μmol of d-lyxose per ml as sole carbon source. Constitutive d-xylose isomerase mutants, resistant to d-glucose repression, were selected in the presence of 0.5 μmol of d-xylose per ml plus 6.18 μmol of 3-O-methylglucose per ml.

Spot inoculations for preliminary characterization were done in petri dishes containing solid MM medium supplemented with appropriate selective conditions. The plates were incubated at 29 C. d-Lyxose and 3-O-methylglucose were filter sterilized through a 0.45-μm membrane filter (Millipore Corp.) before use.

Chemicals. All carbohydrates were purchased from Sigma Chemical Co.

RESULTS

Spores of S. phaeochromogenes NRRL B-3559 are not able to germinate and grow in MM medium when d-lyxose (2-epimer of d-xylose) is used as sole carbon source (Table 1). On the other hand, either d-glucose or d-xylose support good growth. Varying the d-lyxose concentration has no effect. However, when the spores are preincubated in the presence of D-lyxose plus d-glucose and then transferred to D-lyxose, rapid growth is observed. Spores transferred from D-lyxose plus d-glucose to a medium devoid of any carbon source do not grow. Similarly, when the spores are preincubated in MM medium plus d-glucose and then transferred to d-lyxose, they fail to grow (not shown). After mutagenesis with ultraviolet radiation, mutants were selected for their ability to grow in 3.3 μmol of D-lyxose per ml as sole carbon source without being exposed to d-lyxose. Two mutants (lyx-1 and lyx-2) were obtained after 72 h of incubation under these selective conditions. Their phenotypes are presented in Table 2.

Table 1. Effect of carbohydrates on the growth of Streptomyces phaeochromogenes NRRL B-3559

| Carbohydrate | Spores not preincubated | Spores preincubated in D-xylose plus D-glucose |
|--------------|--------------------------|-----------------------------------------------|
| D-Glucose    | +                        | +                                             |
| D-Xylose     | +                        | +                                             |
| D-Lyxose     | -                        | +                                             |
| None         | -                        | -                                             |

* The carbohydrates were added to solid MM medium at the following final concentrations: 26 μmol of d-glucose per ml; 11 μmol of d-xylose per ml; and 3.3 μmol of d-lyxose per ml.

b The plates were observed after 24 h of incubation.

c The spores were preincubated for 20 h in liquid MM medium supplemented with 16 μmol of d-glucose per ml and 46 μmol of d-xylose per ml. Subsequently the cells were washed twice with distilled water and plated under the desired conditions.

d +, Ability of the inoculum to form a dense and confluent colony, producing aerial mycelium.

Table 2. Effect of carbohydrates* on the growth of Streptomyces phaeochromogenes strains with and without the lyx" mutation

| Strains   | No addition | d-Glucose (26 μmol/ml) | D-Xylose (0.5 μmol/ml) | D-Lyxose (3.3 μmol/ml) |
|-----------|-------------|------------------------|------------------------|------------------------|
| Parent    | -           | +                      | +                      | -                      |
| lyx-1     | -           | +                      | +                      | +                      |
| lyx-2     | -           | +                      | +                      | +                      |

* The carbohydrates were added to solid MM medium.

b The plates were observed after 24 h of incubation.

When the mutants are grown in RM medium in the absence of inducer, a five- and tenfold increase in the specific activity of the isomerase is obtained for the lyx-2 and lyx-1 strains, respectively (Table 3), as compared to the parent strain also grown without inducer.

When the parent strain is grown in RM medium, either in d-glucose or glycerol as sole carbon source, only basal levels of the isomerase can be detected (Fig. 1). However, a 10-fold increase in the specific activity of the enzyme is obtained when 46 μmol of D-xylose per ml is added as inducer to the culture medium in the presence of 16 μmol of D-glucose per ml. A greater (35-fold) increase is obtained when the
 TABLE 3. Glucose isomerase activity in Streptomyces phaeochromogenes strains

| Strain   | Condition | Relative activity (%) |
|----------|-----------|-----------------------|
| Parent   | NI        | 10                    |
| lxy-1    | NI        | 97                    |
| lxy-2    | NI        | 50                    |
| Parent   | W1d       | 100                   |

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The cultures were grown in liquid RM medium as described.

After 36 h of growth, the isomerization was measured as described.

The RM medium was supplemented with 16 μmol of D-glucose per ml. No inducer.

The activity was induced with 46 μmol of D-xylose per ml. WI. With inducer.

The activity obtained in the parent strain in the presence of inducer (micromoles of ketose formed per minute per milligram of protein) was defined as 100%.

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**Fig. 1.** Glucose isomerase activity in the parent strain previously induced in liquid medium with 46 μmol of D-xylose per ml in the presence of 16 μmol of D-glucose per ml (△) or of 18.4 μmol of glycerol per ml (●) and in the absence of inducer at the same D-glucose (△) or glycerol (●) concentrations. The activity was measured in a 2-ml mixture containing 16 μmol of MgSO₄·7H₂O, 100 μmol of potassium phosphate buffer, pH 7.2, 160 μmol of D-glucose, and 2 to 4 mg of protein with isomerizing activity.

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**Fig. 2.** Glucose isomerase activity in the mutant strain lxy-1 previously grown without inducer in liquid medium in the presence of 16 μmol of D-glucose per ml (△) or 18.4 μmol of glycerol per ml (○). The activity was measured as described in Fig. 1.

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**Table 4. Effect of carbohydrates on the growth of constitutive mutants with and without the mgluc^-m mutation**

| Conditions   | Growth on spot plates at 29°C |
|--------------|-------------------------------|
|              | lyx^-1 | mgluc^-m |
| No addition  |       |         |
| D-Glucose    | +      | +        |
| D-Xylose     | +      | +        |
| 3-O-methylglucose | -    | -        |
| D-Xylose plus 3-O-methyl-glucose | - | + |
present in the culture medium. Mutants of the 
\( lyx^-1 \) strain previously irradiated with ultravi-
iolet were selected for their ability to grow in 0.5 
\( \mu \)mol of \( \text{D-xylose} \) plus 6.18 \( \mu \)mol of 3-O-methyl-
\( \text{D-glucose} \) per ml. The phenotypic characteristics 
of one of these mutants (\( lyx^-\text{mgluc}^-1 \)) isolated 
after 48 h of incubation under the selective 
conditions is shown in Table 4. As shown in 
Table 5, the isomerase of the double mutant was 
resistant to repression by \( \text{D-glucose} \).

Among the kinetic characteristics measured, 
the optimum pH and temperature values of the 
isomerase in the double mutant are the same 
as those reported for the parent strain (8). The \( K_m 
\) and \( V_{\text{max}} \) values for \( \text{D-glucose} \), determined 
graphically, are 250 mM and 0.55 \( \mu \)mol/min per 
\text{mg} of protein, respectively.

**DISCUSSION**

Mutants of *S. phaeochromogenes* NRRL B-
3559, constitutive for \( \text{D-xylose} \) keto isomerase, 
were selected for their ability to grow in \( \text{D-xylose} \).
This sugar is an analogue of \( \text{D-xylose} \) which 
must be isomerized to \( \text{D-xyulose} \) before 
it can be used as carbon source (5). The observation 
that only those cells previously incubated 
in the presence of \( \text{D-xylose} \) are able to utilize 
\( \text{D-xylose} \) as sole carbon source suggested that \( \text{D-}
\text{xylose} \) itself is not an inducer of the isomerizing 
activity in this microorganism. As a conse-
quence, when \( \text{D-xylose} \) was used as sole carbon 
source for the germination and growth of spores 
that had not been previously incubated in \( \text{D-
xylose} \), constitutivity of the isomerase repre-
sented a means by which a strain could germi-
nate and grow. It seems that in this microorga-
nism, the same isomerase is responsible for the 
utilization of both \( \text{D-xylose} \) and \( \text{D-xyulose} \). It is 
possible also that the degradation pathway for 
\( \text{D-xylose} \) can be constructed in this way from 
the enzymes already present in the cell machi-
ery.

Carbon sources such as corn starch, glucose, 
and molasses are usually used in the fermenta-
tion industry as growth substrates in the pro-
duction of several catabolic enzymes. Such pro-
duction, however, is frequently limited due to 
catabolite repression by \( \text{D-glucose} \) (4). In the 
present study, \( \text{D-xylose} \) isomerase production 
was similarly limited when \( \text{D-glucose} \) was pre-
set in the culture medium. Even the synthesis 
of isomerase in a constitutive mutant (\( lyx^-1 \)) 
was repressed by \( \text{D-glucose} \). Since this mutant 
is unable to grow in low \( \text{D-xylose} \) concentrations 
when \( 3-O\)-methylglucose is present in the me-
dium, constitutive \( \text{D-xylose} \) isomerase mutants, 
insensitive to catabolite repression, could be 
isolated. As a result of a second mutational 
event, a new mutant (\( lyx^-\text{mgluc}^-1 \)) was 
obtained which was able to constitutively produce 
almost threefold more enzymes per \text{mg} of pro-
tein than its parent in the presence of \( \text{D-glucose} \) 
(Table 5). These two characteristics, the consti-
tutivity and the insensitivity to catabolite 
repression, make the double mutant a poten-
tially useful industrial strain.

**ACKNOWLEDGMENTS**

We are indebted to A. L. Demain, J. Piret, and 
Y. Aharonowitz for critical reading of the manusc-
rupt and for helpful suggestions. Particular thanks 
are due to L. Blanco, E. Mazariogies, and H. Zepeda 
for technical assistance in the initial part of this 
work.

**LITERATURE CITED**

1. Bhattacharya, A. K., and M. Chakravorty. 1971. Induc-
tion and repression of \( L\)-arabinose isomerase in Salmo-
ella typhimurium. J. Bacteriol. 106:107-112.

2. Dische, Z., and E. J. Borenfreund. 1961. A new spectro-
photometric method for the detection and determina-
ion of keto sugar and trioses. J. Biol. Chem. 
192:583-587.

3. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. 
Randall. 1961. Protein measurement with the Folin 
phenol reagent. J. Biol. Chem. 193:265-275.

4. Magasanik, B. 1961. Catabolite repression. Cold Spring 
Harbor Symp. Quant. Biol. 26:249-256.

5. Mortlock, R. P., and W. A. Wood. 1964. Metabolism of 
pentoses and pentitols by *Aerobacter aerogenes*. I. 
Demonstration of pentose isomerase, pentulokinase, 
and pentitol dehydrogenase enzyme families. J. Bact-
eriol. 88:838-84.

6. Pridham, T. G., P. Anderson, C. Foley, L. A. Lindenfel-
sen, C. W. Hesselteine, and R. G. Benedict. 1967. A 
selection of media for maintenance and taxonomic 
study of *Streptomyces*. Antibiot. Annu. 57:947-983.

7. Sanchez, S., and K. L. Smiley. 1975. Properties of \( \text{D-}
\text{xylose} \) isomerase from *Streptomyces albus*. Appl. Mi-
crobiol. 29:745-750.
8. Strandberg, G. W., and K. L. Smiley. 1971. Free and immobilized glucose isomerase from *Streptomyces phaeochromogenes*. Appl. Microbiol. 21:588–593.

9. Takasaki, Y., and Y. Kosugi. 1969. *Streptomyces* glucose isomerase, p. 561–570. In D. Perlman (ed.), Fermentation advances. Academic Press Inc., New York.

10. Tsumura, N., and T. Sato. 1965. Enzymatic conversion of D-glucose to D-fructose. VI. Properties of the enzyme from *Streptomyces phaeochromogenes*. Agric. Biol. Chem. 29:1129–1134.