Glucose-negative Mutants of *Pachysolen tannophilus*

By D. N. WEDLOCK, A. P. JAMES† AND R. J. THORNTON*

Microbiology and Genetics Department, Massey University, Palmerston North, New Zealand

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Mutants of the yeast *Pachysolen tannophilus*, exhibiting decreased ability to utilize D-glucose as the sole carbon source, were obtained by selecting for resistance to 2-deoxyglucose. Enzyme studies confirmed that these strains are defective in the hexose-phosphorylating enzymes and are unable to phosphorylate D-glucose to D-glucose 6-phosphate. The results confirmed the presence of two hexokinases, A and B, with ratios of D-glucose to D-fructose phosphorylation activity of 1.3/1.0 and 3.0/1.0, respectively, and a D-glucose-specific glucokinase. The behaviour of a hexose-negative strain, able to ferment D-xylose in the presence of D-glucose, is described.

INTRODUCTION

The yeast *Pachysolen tannophilus* has the ability to ferment hexose and pentose sugars to ethanol. It has, therefore, potential use in the production of ethanol from lignocellulose hydrolysates which are comprised of a mixture of hexose and pentose sugars. Utilization of D-xylose, the major pentose in lignocellulose hydrolysates, by *P. tannophilus* is subject to hexose catabolite repression (Slininger *et al.*, 1987; Bicho *et al.*, 1988). *P. tannophilus* has low ethanol tolerance and the ethanol produced from the hexoses in lignocellulose hydrolysates prevents the complete utilization of D-xylose. Mutations which affect the hexokinase and glucokinase enzyme activities of *P. tannophilus* should result in hexose-negative strains. D-Xylose utilization by hexose-negative strains should be unaffected since the initial metabolism of D-xylose follows a different pathway from that of D-glucose and other hexoses (Maleszka *et al.*, 1983; Slininger *et al.*, 1987). Glucose-negative mutants could be used to ferment the xylose component of hydrolysates and a more ethanol-tolerant yeast, e.g. *Saccharomyces cerevisiae* used subsequently to ferment the hexose sugars. Hexokinase and glucokinase mutants of *Saccharomyces cerevisiae* have been isolated by selecting for resistance to the toxic analogue of D-glucose, 2-deoxyglucose (Maitra, 1970; Lobo & Maitra, 1977*a, b*). This paper reports the isolation, by resistance to 2-deoxyglucose, and characterization of hexokinase- and glucokinase-defective mutants of *P. tannophilus* which are unable to grow on D-glucose as sole carbon source. The characteristics of these mutants in the fermentation of mixtures of D-glucose and D-xylose are also described.

METHODS

Yeast strains. The 'glucose-negative' strains described in this study were derived from *Pachysolen tannophilus* P17-1A, a methionine-requiring auxotroph of NRRL Y-2460. Strain P471-5D *ade2* was used in genetic crosses with several of the glucose-negative strains. P444-3D, a wild-type segregant, was used for comparison with the mutants in enzyme assays and growth/sugar utilization experiments.

Media. The medium for vegetative growth contained yeast nitrogen base (YNB), either supplemented with amino acids or minimal. The complete composition (% w/v) was yeast nitrogen base (Difco) (0.67), agar (Difco) (2) and either D-xylose (2), D-glucose (2), D-mannose (2) or D-fructose (2) as the carbon source. For hybridization of haploid strains, malt extract medium (YM-YE) was used; it contained (% w/v), yeast extract (Difco) (1), malt extract (Difco) (1), glucose (0.4), agar (Difco) (2), pH 5.0. The media for the determination of growth and sugar utilization in liquid culture contained 0.67% YNB and 2% (w/v) of the appropriate carbon source or a 2%/2%

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† Present address: National Research Council, Ottawa, Canada.

mixture of two carbon sources. Strains were grown in yeast extract peptone medium (YEP) [composition (% w/v): yeast extract (Difco) (1), peptone (Difco) (2), sugar (2)] for enzyme analyses, initial screening of mutants and fermentation trials. Sugars and other carbon sources were prepared as 20% stock solutions, filter-sterilized and added to the rest of the media, which was sterilized at 121 °C for 15 min. The L-malic acid stock was adjusted to pH 6.0 with KOH.

Isolation of glucose-negative strains. Strain P17-1A was grown to exponential phase on complete YNB-xylose medium and plated (10^7 – 10^8 cells per plate) onto complete xylose medium supplemented with 6 mM or 8 mM-2-deoxyglucose (Sigma). Mutants that were resistant to these levels of 2-deoxyglucose were further plated onto complete xylose medium supplemented with 30 mM, 40 mM or 80 mM 2-deoxyglucose. Mutants were tested for growth on solid media using various hexoses as carbon sources by replica-plating onto complete medium containing either D-glucose, D-mannose or D-fructose as the carbon source. The amount of growth was assessed visually at 12–24 hr periods. Mutants showing a reduction of growth on any of these carbohydrates were further checked for stability of this characteristic.

Cells of strain D/X A were mutagenized with UV light. Cells, precultured on minimal YNB-glycerol, were grown in this medium to exponential phase. The cells were diluted in water and plated out (approximately 10^3 cells per plate) onto YNB-glucose/xylose plates and exposed to UV light for 30 s and incubated in the dark at 30 °C for 3–4 d. The survival rate was about 5%. The fastest growing colonies were picked and cultured on YNB-glycerol and replica-plated onto YNB-glucose/xylose plates. Several fast-growing strains were checked for utilization of D-glucose and D-xylose in liquid YEP media.

Genetic techniques. The genetics of *P. tannophilus* have been described by James & Zahab (1982, 1983). Haploid strains carrying auxotrophic markers were hybridized by plating on YM-YE media, incubating at 30 °C for 4 d, followed by incubation at 30 °C on minimal media, with appearance of prototrophic colonies after 2 d. The sporulation of diploid strains was achieved by incubation on YM for 4–5 d at 30 °C.

Enzyme analyses and chromatography of cell-free extracts. Methods used to prepare cell-free extracts and to measure the activities of hexokinase (EC 2.7.1.1), phosphoglucose isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), xylose reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9) enzymes are described in the accompanying paper (Wedlock & Thornton, 1989).

Cell-free extracts were prepared for chromatography as described by Wedlock & Thornton (1989) except that the cells were suspended in 50 mM-potassium phosphate buffer, pH 7.5, containing either 0.2 or 2 mM-PMSF prior to breakage. Extracts were desalted by passage through a Sephadex G-25 column (Wedlock & Thornton, 1989) or by overnight dialysis against 10 mM-potassium phosphate buffer, pH 7.5. The extract (15 units D-glucose phosphorylating activity) was chromatographed on a 1.6×20 cm DEAE-cellulose column (Whatman DE-52) equilibrated with the same buffer containing 2 mM-PMSF. The column was washed with two column volumes of buffer and the enzyme eluted with 250 ml of a 0–0.2 M-NaCl gradient at a flow rate of 30 ml h⁻¹. Fractions (5 ml) were collected and the D-glucose- and D-fructose-phosphorylating activities measured. The shape of the gradient was checked with a conductivity meter.

Analysis of cell-free extracts by HPLC. The enzymic conversion of D-glucose and sorbitol by cell-free extracts, in *in vitro* reaction mixtures, was followed by HPLC. Reaction mixtures (1 ml total volume) contained 50 mM-D-glucose or sorbitol, 50 mM-cofactor (NADPH or NAD⁺ or NADP⁺ and NAD⁺, pH 7.0), 100 mM-Tris/HCl buffer, pH 7.0, and extract (3 mg protein) which had been dialysed against 10 mM-Tris/HCl, pH 7.0, prior to use. Samples (0.45 ml) were removed at zero time and after 24 h incubation at 30 °C. Protein was precipitated by the addition of 10 µl TCA (50%, w/v), the samples centrifuged in an Eppendorf microfuge at 12,000 g for 10 min and frozen at –20 °C until required for HPLC analysis. Controls lacked either cofactor or extract.

RESULTS AND DISCUSSION

Isolation of glucose-negative mutants

Initially, mutants resistant to 8 mM-2-deoxyglucose were isolated. Growth on D-xylose, D-glucose and D-fructose was similar to the wild-type but was greatly reduced on D-mannose. A 2:2 segregation both for growth on D-mannose and for resistance to 10 mM-2-deoxyglucose was observed when diploids of crosses of two of these strains with P471-5D *ade2* were sporulated. This segregation pattern suggested mutation in a single gene which was designated *hxx2*.

A further set of mutants, which exhibited reduced growth on both D-glucose and D-mannose and was resistant to 40 mM-2-deoxyglucose, was isolated. This additional lesion responsible for reduced growth on D-glucose was designated *glu1*. Crosses of these strains with P471-5D *ade2* give the diploids P509 and P510. Three segregants, P509-3C *hxx2*, P510-5A *glu1* and P509-1B *hxx2 glu1*, mutant at either one or two independent loci, were recovered. The resistance of these strains to 2-deoxyglucose is shown in Table 1. The wild-type strains, P444-3D and P510-5A were

Table 1. *Resistance of strains to 2-deoxyglucose*

+, Good growth observed within 1–3 d; (+), inhibition of growth; –, no growth.

Strain/genotype	Growth on D-xylose (2%, w/v)						
	2-Deoxyglucose concn (mM):						
	2	4	10	20	40	80	120
P444-3D (wild-type)	+	(+)	–	–	–	–	–
P510-5A (<i>glu1</i>)	+	(+)	–	–	–	–	–
P509-3C (<i>hxx2</i>)	+	+	(+)	–	–	–	–
P509-1B (<i>hxx2 glu1</i>)	+	+	+	+	(+)	(+)	(+)
D/X A (<i>hxx1 hxx2 glu1</i>)	+	+	+	+	+	+	+

Table 2. *Growth of the wild-type and mutants of P. tannophilus on different carbon sources*

+, Confluent growth after 2 d; +/-, growth evident after 3–4 d; –, no growth after 6d.

Strain/genotype	Growth (minimal YNB plates, 30 °C) on:			
	D-Xylose	D-Glucose	D-Mannose	D-Fructose
P444-3D (wild-type)	+	+	+	+
P510-5A (<i>glu1</i>)	+	+	+	+
P509-3C (<i>hxx2</i>)	+	+	+/-	+
P509-1B (<i>hxx2 glu1</i>)	+	–	–	+/-
D/X A (<i>hxx1 hxx2 glu1</i>)	+	–	–	–

inhibited at 4 mM, while strains P509-3C and P509-1B were able to grow relatively well in the presence of 4 mM and 20 mM-2-deoxyglucose, respectively.

A third phenotypically distinct mutant was obtained by selecting for increased resistance to 2-deoxyglucose in liquid media. Although the segregant P509-1B was apparently capable of growth on solid YNB-xylose supplemented with 40 mM-2-deoxyglucose, only slow growth was found to occur in liquid medium at this concentration of analogue. Growth ceased after 24 h but resumed after about 96 h of further incubation. Two mutant strains were isolated from two independent cultures. These strains had identical growth and sugar utilization characteristics. One of these strains, D/X A, was studied further; it was capable of normal growth in the presence of 40 mM-2-deoxyglucose and a slightly reduced rate of growth at 120 mM. From this strain, a further mutant was derived, by treatment with UV light and selecting for rapid growth on YEP-glucose/xylose medium. This latter strain was designated F/G 2.

Growth and sugar utilization

The growth of the wild-type and four 2-deoxyglucose-resistant mutant strains on a number of carbon sources is summarized in Table 2. Growth of strains P510-5A and P509-3C differed little from the wild-type except that P509-3C exhibited greatly reduced growth on D-mannose. Strains P509-1B and D/X A exhibited the most reduction in growth and were unable to grow on D-mannose and D-glucose. Growth on D-fructose was slow with the former strain but was evident after incubation for 3–4 d. The latter strain did not grow on D-fructose. Neither P509-1B nor D/X A utilized D-glucose when the wild-type and the four mutants were inoculated into YNB-glucose at the standard cell density level (0.5 ml inoculum of OD 34–36 at 600 nm). At a high cell density (10 ml inoculum of OD 34–36 at 600 nm) strain P509-1B grew and utilized glucose more slowly than the wild-type (Fig. 1*a, b*). Strain D/X A did not grow when inoculated at high cell density, but there was a slow apparent utilization of glucose (Fig. 1*a, b*). Most of the D-glucose consumption was accounted for by the conversion of D-glucose to D-fructose. After 96 h, 3.3 g glucose l⁻¹ had been consumed and 2.4 g fructose l⁻¹ had accumulated in the medium.

Fig. 1. (a) Growth of the wild-type, P444-3D (□) and mutants P509-1B (○) and D/X A (▲) on D-glucose (2%, w/v) at high cell density. (b) Utilization of D-glucose (2%, w/v) at high cell density by the wild-type, P444-3D (□) and mutants P509-1B (○) and D/X A (▲). ▼, D-Fructose production by strain D/X A.

The identity of the fructose was confirmed by HPLC separation followed by reduction with sodium borohydride (P. Bicho, personal communication). An approximate 1:1 ratio of sorbitol and D-mannitol was obtained after reduction with sodium borohydride, as would be expected from the reduction of D-fructose. Further confirmation was by ¹³C-NMR (P. Bicho, personal communication).

The conversion of D-glucose to D-fructose was thought to be catalysed by the xylose pathway enzymes, xylose reductase and xylitol dehydrogenase, which are known to have broad substrate specificities (Ditzelmüller *et al.*, 1984*a, b*; Morimoto *et al.*, 1986, 1987). D-Glucose could be converted by a reduction step to sorbitol, catalysed by xylose reductase, and then oxidized to D-fructose, catalysed by xylitol dehydrogenase. To test this hypothesis we examined cell-free extracts, prepared from strain D/X A, for the conversion of D-glucose and sorbitol to D-fructose *in vitro*. When the extract was incubated with D-glucose in the presence of NADPH and NAD⁺, a small amount of D-fructose was detected after 24 h incubation. A small amount of sorbitol was detected when NADPH alone was used as the cofactor. The conversion of sorbitol to D-fructose, with NAD⁺ as the cofactor, was readily observed and more than 50% was converted during the 24 h period. These results agreed with enzyme analyses done on the cell-free extracts of strain D/X A. When D-glucose was used as a substrate, the NADPH-dependent reductase activity was about 6% of the activity measured with D-xylose. Approximately 71% of the NAD⁺-dependent activity with xylitol was measured when sorbitol was substituted. These observations are consistent with the proposition that D-glucose can be converted to D-fructose by xylose pathway enzymes. Furthermore, a mutant defective in the xylitol dehydrogenase enzyme and unable to grow on D-xylose, although normal for growth on D-glucose, cannot grow on sorbitol. Mutants such as D/X A, defective in all three hexokinases, have lost the ability to grow on sorbitol also. Although the sorbitol presumably could be converted to D-fructose in these strains, subsequent metabolism of the D-fructose is blocked by the lesions within the hexokinase genes.

Growth in media containing two carbon sources

All the mutant strains with the exception of D/X A and F/G 2 were found to utilize D-glucose readily when inoculated into media containing both D-glucose and D-xylose. The wild-type strain and mutant strains P510-5A and P509-3C utilized the D-glucose preferentially, D-xylose utilization only commencing after most of the D-glucose was utilized. Mutant P509-1B was found to utilize D-glucose and D-xylose simultaneously when inoculated into minimal YNB-glucose/xylose at the standard cell density. The 'co-utilization' of D-glucose and D-xylose by this strain was also found to occur in media which contained either D-glucose and glycerol or D-glucose and L-malic acid as carbon sources. D-Glucose utilization by P509-1B was slow initially, but after 48 h was faster than the rate of D-xylose utilization, which was inhibited. At least part of this observed utilization was due to the larger population of cells present in the media after growth on D-xylose or glycerol had occurred. The D-glucose was converted to ethanol, since the ethanol concentration in the medium was greater than the amount produced from the fermentation of D-xylose as a sole carbon source.

Fig. 2

Fig. 3

Fig. 2. Utilization of D-glucose and D-xylose (both 2%, w/v) by strain D/X A in YNB media. ○, D-glucose; ■, D-xylose; △, growth.

Fig. 3. Fermentation of D-glucose and D-xylose (both 2%, w/v) by strain F/G 2 in YEP media. ○, D-glucose; ■, D-xylose; ●, D-fructose; ▲, ethanol; △, growth.

Table 3. *Hexokinase, phosphoglucose isomerase and D-glucose-6-phosphate dehydrogenase activities of wild-type and mutants*

For each assay, the strains were grown in YEP-glucose/xylose (both 2%, w/v) for 42 h, except that for the hexokinase assay, P444-3D, P510-5A and P509-3C were grown in YEP-glucose (2%, w/v) for 42 h.

Strain/genotype	Specific activity [$\mu\text{mol NADP reduced min}^{-1} (\text{mg protein})^{-1}$]			
	Hexokinase		Phosphoglucose isomerase	Glucose-6-phosphate dehydrogenase
	D-Glucose as substrate	D-Fructose as substrate		
P444-3D (wild-type)	0.80	0.87	3.3	0.17
P510-5A (<i>glu1</i>)	0.63	0.78	4.4	0.12
P509-3C (<i>hxx2</i>)	0.38	0.11	3.9	0.24
P509-1B (<i>hxx2 glu1</i>)	0.050	0.15	2.0	0.31
D/X A (<i>hxx1 hxx2 glu1</i>)	0.0061	0.0096	1.1	0.34

A different pattern of sugar utilization was displayed by mutant D/X A. D-Glucose utilization in the minimal YNB-glucose/xylose media by this strain was greatly reduced but utilization of the D-xylose was markedly inhibited (Fig. 2). The utilization of D-xylose slowed after 72 h incubation, whereas the sugar was typically almost completely utilized at this time when presented as the sole carbon source. Strain F/G 2, which was derived from D/X A and presently uncharacterized, showed negligible D-glucose utilization when inoculated into the same minimal YNB-glucose/xylose medium, but utilization of the D-xylose was incomplete. However, in YEP-glucose/xylose medium, there was complete utilization of the D-xylose to ethanol (Fig. 3). The maximum ethanol concentration in the medium reached 4.1 g l^{-1} , with a yield of 0.19 g per g sugar consumed. D-Glucose utilization was minimal until 48 h, after which D-fructose started to appear in the medium. At the point of maximum ethanol concentration (72 h), only $3.0 \text{ g D-glucose l}^{-1}$ had been consumed, whereas all the D-xylose had been depleted. Further work is continuing to determine the suitability of this mutant to ferment D-xylose in mixtures of D-glucose and D-xylose.

Enzyme activities and chromatography of cell-free extracts

Hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase activities were measured in the mutants and compared with the wild-type activities (Table 3). Small

reductions of D-glucose-phosphorylating activity were seen with mutants defective in one gene. The double mutant, P509-1B, exhibited only about 6% of the wild-type activity on D-glucose; greater activity was measured with D-fructose as the substrate. This correlated with the ability of strain P509-1B to grow slowly on D-fructose. Strain D/X A had the lowest measured hexokinase activity, both with D-glucose and D-fructose as the substrate. Although phosphoglucose isomerase and glucose-6-phosphate dehydrogenase activities did vary between the mutants and the wild-type, the differences were not great. This further supported the view that the resistance of the mutants to 2-deoxyglucose was due to mutations within the hexokinase genes.

Cell-free extracts prepared from the wild-type strain and each of the mutants were chromatographed on DEAE-cellulose. The wild-type extracts were desalted by filtration on Sephadex G-25, whereas extracts prepared from the mutants were desalted by dialysis. Three major peaks of hexose-phosphorylating activity were measured in extracts of the wild-type strain P444-3D (Fig. 4a). These peaks differed in their ratio of D-glucose to D-fructose phosphorylation (F/G) activity, being 3.0/1.0, 0.6/1.0 and 1.2/1.0, respectively. These activities corresponded to hexokinase B, a glucokinase and hexokinase A, respectively. The latter two enzymes were only partially separated.

Cell-free extracts prepared from strains P510-5A, P509-3C, P509-1B and D/X A were also subjected to chromatography. Two hexose-phosphorylating enzymes were present in mutant P510-5A, with F/G ratios of 3.0/1.0 and 1.3/1.0 (Fig. 4b). Hence, hexokinases A and B but not the glucokinase were active. In addition to hexokinase B, a glucokinase with activity specific to D-glucose was present in strain P509-3C (Fig. 4c). Both hexokinase A and the glucokinase were absent from the double mutant, P509-1B, but hexokinase B (F/G ratio 3.0/1.0) was present (Fig. 4d). In order to chromatograph 5 units of D-glucose-phosphorylating activity from a cell-free extract of strain P509-1B about five times as much protein, compared to the wild-type extract, had to be loaded onto the column. Thus it would appear that this enzyme is present at only low levels in this yeast. Negligible activity of any of the three enzymes was measured when an extract of D/X A was chromatographed.

Chromatography of the wild-type extract, prepared from cells grown on either D-xylose or glycerol, revealed lower levels of hexokinase A compared with cells grown on D-glucose (Fig. 4e). The activities of hexokinase B and the glucokinase were similar in cells grown on all three carbon sources.

These results suggest some similarities to the enzymes found in *S. cerevisiae*. The latter possesses two hexokinases, PI and PII, with F/G ratios of 2.5–3.5/1.0 and 1.0–1.5/1.0, respectively (Ramel *et al.*, 1971; Colowick, 1973; Barnard, 1975; Gancedo *et al.*, 1977; Entian & Mecke, 1982; Frohlich *et al.*, 1984; Fernández *et al.*, 1985) and a D-glucose specific glucokinase (Maitra, 1970; Gancedo *et al.*, 1977). Both hexokinase PI and the glucokinase are constitutive, while the hexokinase PII isoenzyme is inducible. In *S. cerevisiae* the PII hexokinase enzyme constitutes the major hexose-phosphorylating activity within the cells during the exponential growth phase (Gancedo *et al.*, 1977; Fernández *et al.*, 1985). In *P. tannophilus*, both the glucokinase and the hexokinase A appeared to contribute the major activity. The glucokinase was constitutive, whereas the hexokinase A was inducible. The constitutive hexokinase B was only present at low levels and its sole presence within the cell could only support a slow rate of growth.

From results in this study, it appears probable that, in the absence of hexose:ATP-kinase activity, D-glucose is converted to sorbitol as a first step in the conversion of D-glucose to D-fructose. In the wild-type, D-glucose would follow the normal pathway of catabolism and none would be converted to sorbitol and D-fructose, since both xylose reductase and xylitol dehydrogenase are repressed by D-glucose (Maleszka *et al.*, 1983; Bicho *et al.*, 1988; Wedlock & Thornton, 1989). The existence of such a pathway has important implications for the acquisition of glucose-negative strains capable of D-xylose utilization in the presence of D-glucose. In the accompanying paper (Wedlock & Thornton, 1989) we demonstrated that xylose reductase and xylitol dehydrogenase are not repressed in mutants which are defective in hexokinase A. However, in this study, strain D/X A, which is defective in hexokinase A as well as hexokinase B and glucokinase, failed to efficiently utilize D-xylose in a mixture of D-glucose and D-xylose. This

Fig. 4. DEAE-cellulose chromatography of cell-free extracts from the wild-type strain, P444-3D (a, cells grown on D-glucose; e, cells grown on D-xylose), and mutants P510-5A (b), P509-3C (c) and P509-1B (d). ○, D-glucose-phosphorylation; ■, D-fructose phosphorylation; □, conductivity.

apparent inhibition of D-xylose utilization may be due to substrate competition for the xylose reductase and xylitol dehydrogenase enzymes and for NADPH and NAD⁺ cofactors. Alternatively, D-glucose and D-xylose may compete for carrier-mediated transport into the cell. The inhibition of D-xylose utilization by D-glucose is alleviated in the F/G 2 mutant. The exact nature of the mutation conferring the ability to ferment D-xylose to ethanol in the presence of D-glucose needs to be investigated further.

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