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Regulation of xylose and alpha-xyloside transport and metabolism in Lactobacilli

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Chapter 5

Contribution of the Phosphoenolpyruvate:Mannose Phosphotransferase System and of CcpA to Carbon Catabolite Repression in *Lactobacillus pentosus*[1]

5.1 – SUMMARY

We have investigated the role of the phosphoenolpyruvate:mannose phosphotransferase system of *Lactobacillus pentosus* in sugar transport and control of sugar utilization. Three 2-deoxy-D-glucose-resistant ($2DG^R$) mutants (LPE5, LPE6 and LPE8) were isolated. The mutants lacked EII^{Man} activity and were unable to phosphorylate D-glucose, D-mannose and *N*-acetyl-D-glucosamine in a PEP-dependent reaction. In wild-type bacteria, the activity of an inducible, high-affinity fructose-specific PTS was decreased by the presence of several PTS sugars in the growth medium, but this regulation was absent in the three $2DG^R$ mutants. The regulation of EII^{Fru} activity in wild-type bacteria was independent of the *trans*-factor CcpA. In contrast, the strength at which EII^{Fru} activity was decreased correlated with the level of EII^{Man} activity. Using the multi-copy plasmid pMJ18, expressing the *manB* gene encoding the $EIIB^{Man}$ subunit of the *Lactobacillus curvatus* EII^{Man} complex, the defective activity of EII^{Man} was complemented in LPE6 but not in LPE5 and LPE8. The $EIIB^{Man}$ subunit of *L. curvatus* was found to mediate strong decrease of EII^{Fru}

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[1] This chapter is submitted for publication: Chaillou, S., B. C. Lokman, Pieter W. Postma and Peter H. Pouwels. (1998).

activity in the three $2DG^R$ mutants, suggesting a critical role of the $EIIB^{Man}$ subunit in the mechanism regulating EII^{Fru} activity. A defect in EII^{Man} also resulted in a strong relief of catabolite repression exerted by EII^{Man} substrates on two catabolic enzyme activities, β-galactosidase and β-glucosidase, whose expression was negatively regulated by CcpA. Mutations in EII^{Man} did not relieve the D-fructose-mediated catabolite repression of β-galactosidase and β-glucosidase activities, however. The extent of the repression mediated by D-fructose was dependent on the activity of a high-affinity inducible D-fructose PTS.

5.2 – INTRODUCTION

The low-GC-content Gram-positive lactic acid bacteria represent a group of microorganisms, which are widely used in the food-industry for their ability to rapidly ferment sugars into lactic acid. During sugar fermentation, some metabolic pathways leading to the production of desired flavour compounds in the final food-product, may be negatively regulated. This inhibitory effect is due to regulatory mechanisms, triggered in the presence of readily fermentable carbon sources such as D-glucose, and which regulate the synthesis of enzymes involved in the catabolism of other carbon sources. This global regulatory phenomenon, present in many if not all bacteria, is commonly termed carbon catabolite repression (CR).

CR pathways occurring in low-GC-content Gram-positive bacteria have been extensively studied in the species *Bacillus subtilis*. The dominant CR pathway of this microorganism involves one of the components of the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS), the HPr protein. HPr can be phosphorylated at two distinct sites: at the catalytic His-15 in a phosphoenolpyruvate-dependent reaction, catalysed by Enzyme I (EI), and at the regulatory Ser-46 by an ATP-dependent HPr kinase, activated by fructose 1,6-bisphosphate (FBP) (34, 49, 132, 133). HPr(Ser-P) can interact with the *trans*-factor CcpA, a member of the GalR-LacI family of transcriptional regulators (182), resulting in a protein complex which can bind to *cis*-acting catabolite responsive elements (*cre*) located in the promoter regions of many catabolic operons (34, 47, 73), thereby preventing transcription.

Unlike in *B. subtilis*, the molecular mechanisms of CR in lactic acid bacteria are still poorly understood. However, it has been proposed that the HPr(Ser-P)/CcpA

pathway could represent a global strategy among Gram-positive bacteria to achieve repression of many genes (67). Indeed, the role of CcpA in the D-glucose-mediated repression of some catabolic pathways has already been demonstrated for *Bacillus megaterium* (69), *Staphylococcus xylosus* (40), *Lactobacillus casei* (103), *Lactobacillus pentosus* (16, 95) and has been suggested for *Lactobacillus plantarum* and *Lactococcus lactis* (84).

Several studies have indicated that components of the mannose PTS of certain lactic acid bacteria may play an important role in the regulation of sugar utilization. The EII^{Man} complex of the mannose PTS is the main transport system for glucose in *Lb. casei* (175), *Lactobacillus sake* (86), *Lactobacillus curvatus* (174), *Lc. lactis* (166, 167), *Tetragenococcus halophila* (1) and several species of oral streptococci (for a review, see ref. 171). Mutations rendering the EII^{Man} complex inactive resulted in the loss of the preferential use of D-glucose over secondary carbon sources such as lactose or D-ribose in *L. casei* (56, 175) or D-xylose in *P. halophilus* (1). Additional evidence for a regulatory role of EII^{Man} was obtained in *Streptococcus salivarius*, in which a relationship between defects in components of the EII^{Man} complex and derepression of the synthesis of several sugar catabolic-enzymes (51, 85), as well as derepression of an inducible fructose PTS (10) was demonstrated. However, the mechanism by which the EII^{Man} is implicated in regulatory functions is poorly defined.

We have previously demonstrated a role of CcpA in the regulation of D-xylose catabolism in *Lb. pentosus* (16, 95), a model organism which we are using to study the mechanisms regulating the utilization of the homolactic pathway versus the heterolactic pathway of sugar fermentation. To further understand CR mechanisms in facultative heterofermentative species of Lactobacilli, in this study we have investigated in more details the regulatory functions of EII^{Man} and of CcpA in *Lb. pentosus*.

5.3 – MATERIAL AND METHODS

Bacterial strains and plasmid. The following *Lb. pentosus* strains were used: MD353 (wild-type, Lokman *et al.,* 1991), MD363 (wild-type, ref. 95), LPE4 (MD363-derived *ΔccpA* mutant; Ap^R, Em^R; ref. 95), LPE5, LPE6 and LPE8 (MD353-derived $2DG^R$ mutants; this study). The following plasmid was used: pMJ18 (containing the

manB gene from *L. curvatus;* Ap^R, Em^R; ref. 174). This plasmid was introduced in the three $2DG^R$ mutants by electroporation as described (ref. 96).

Growth conditions. During all experiments described in this study, cells were invariably grown on the *Lactobacillus* synthetic rich MCD medium (86), supplemented with 50 mg l^{-1} L-aspartic and L-glutamic acid which are essential amino acids for species related to *Lb. plantarum* (89). All carbohydrates were added at a final concentration of 0.5% (wt/vol) and erythromycin (5 μg ml^{-1}) was added when necessary. All incubations were carried out at 37 °C in non-shaking tubes containing either 25 ml (growth, phosphorylation and uptake studies) or 5 ml (enzyme assays) MCD medium. Inoculations were performed by diluting an MCD culture (OD_{600nm}=1.0; obtained after 8 to 24 hours incubation, depending on the energy source used) 1/100 into fresh medium.

Isolation of 2DG-resistant mutants. Spontaneous mutants, resistant to 2DG, were isolated by plating 100 μl of a late exponential culture grown in M-medium plus sucrose on an M medium (96) agar plate containing 25 mM sucrose and 10 mM 2DG. Resistant clones were purified by streaking two times on the same selective medium.

Preparation of permeabilized cells. Bacterial cultures were grown as described above, washed two times with ice-cold 50 mM potassium phosphate buffer (pH 6.5) containing 2 mM $MgSO_4$ (KPM buffer), resuspended in 1/100 of culture volume in KPM containing 20% (wt/vol) glycerol and then rapidly frozen in liquid nitrogen and kept at –80 °C until use. After thawing, cells were washed once with KPM buffer and then resuspended to a final OD_{600nm}=50 in 50 mM potassium phosphate buffer (pH 6.5) containing 12.5 mM NaF, 5mM $MgCl_2$ and 2.5 mM dithiothreitol. Cells were permeabilized as follows: 2.5 μl toluene-acetone (1:9, vol/vol) was added per 250 μl cell suspension and the mixture was vortexed for 5 min at 4 °C. Cells were then rapidly centrifuged (150 × *g*, at 4 °C for 2 min) and the supernatant was discarded. This step was necessary to remove PEP originating from the intracellular pool. The cell pellet was resuspended again in the same buffer (OD_{600nm}=50) and treated once more with toluene-acetone as described above. After 5 min of vortexing, permeabilized cells were kept on ice.

PEP- and ATP-dependent ^{14}C-labelled carbohydrate phosphorylation assay. For each assay, 2.5 μl, 5 μl and 10 μl of permeabilized cells (OD_{600nm}=50) were incubated in a final volume of 100 μl of 50 mM potassium phosphate buffer (pH 6.5) containing 12.5 mM NaF, 5mM $MgCl_2$, 2.5 mM dithiothreitol, 10 mM of PEP or ATP

and 10 mM ^{14}C-labelled carbohydrate (specific activity 0.1μCi/nmol). After an incubation for 15 to 30 minutes at 37 °C, the phosphorylated carbohydrates were separated on Dowex AG 1-X2 columns as described previously (122). The radioactivity was determined by liquid scintillation counting. The rate of PEP- or ATP-dependent phosphorylation was calculated by estimating the rate of phosphorylation from the three values obtained and substracting the rate of phosphorylation obtained in permeabilized cells without phosphoryl donor (background).

Uptake of D-[U-^{14}C]fructose. Cells for uptake studies were grown, harvested, washed and frozen as described above for the preparation of permeabilized cells. For the transport measurements, cells were first washed once with KPM buffer and resuspended at a concentration of 0.5 mg dry wt ml^{-1} in 100 μl KPM buffer. This cell suspension was incubated for 2 min at 37 °C and transport was initiated by addition of D-[U-^{14}C]fructose (concentration as indicated and specific activity ranged from 0.1μCi/nmol to 1μCi/nmol). After 15 s of uptake, 2 ml ice-cold 0.1 M LiCl was added to the cells and the samples were rapidly filtered through glass fiber filters (Whatman GF/F) and washed with an equal amount of ice-cold 0.1 M LiCl. The radioactivity was determined by liquid scintillation counting.

Enzyme assays. β-glucosidase and β-galactosidase activity was determined at 37 °C in 750 μl KPM buffer, containing 10 to 20 μl permeabilized cells (OD_{600nm}= 50) and 5 mM *p*-nitro-phenyl-β-D-glucopyranoside and *o*-nitrophenyl-β-D-galactopyranoside, respectively. The reaction was stopped by adding 250 μl 1M Na_2CO_3 and the optical density at 410 nm was measured.

Radiochemicals. D-[U-^{14}C]glucose (11.5 GBq/mmol), D-[U-^{14}C]mannose (10.6 GBq/mmol), D-[U-^{14}C]fructose (11.9 GBq/mmol), [U-^{14}C]methyl-α-D-glucopyranoside (10.8 GBq/mmol), 2-deoxy-D-[U-^{14}C]glucose (11.1 GBq/mmol), D-[U-^{14}C]galactose (2.3 GBq/mmol), [D-glucose-1-^{14}C]lactose (1.9 GBq/mmol), [U^{14}C] sucrose (27.4 GBq/mmol), D-[U-^{14}C]ribose (10.6 GBq/mmol), D-[U-^{14}C]gluconic acid (10.6 GBq/mmol) and *N*-acetyl-D-[1-^{14}C]glucosamine (2.11 GBq/mmol) were obtained from Amersham International, Amersham, U.K.

5.4 – RESULTS

Determination of PTS activities in *Lb. pentosus* MD353. *Lb. pentosus* can ferment a wide range of mono-, di- and trisaccharides by either the homo- or hetero-lactic pathway. However, little is known about the mechanisms by which this bacterium is taking up readily fermentable sugars such as hexoses. To determine whether a particular sugar is transported by the PTS, we measured the rate of phosphorylation of several mono- and disaccharides in the presence of PEP or ATP, or a mixture of PEP and ATP in permeabilized cells of *Lb. pentosus* MD353. The results are summarized in Table 5.1. PEP-dependent phosphorylation could be detected for D-glucose, D-mannose, *N*-acetyl-D-glucosamine (Nag), and D-fructose. A very low rate of PEP-dependent phosphorylation could also be detected for sucrose. In contrast, ATP-dependent phosphorylation was detected for all sugars, although the rates were very low for D-fructose and sucrose. In the case of sucrose, the reason for the low rates of both PEP- and ATP-dependent phosphorylation is not yet known.

TABLE 5.1. Phosphorylation activities in permeabilized cells of MD353[a]

^{14}C-labelled sugar	Rate of phosphorylation (nmol min^{-1} mg dry wt^{-1})[b]		
	Phosphoryl donor		
	PEP	ATP	PEP + ATP
D-glucose	63 ± 7	30 ± 6	129 ± 8
D-mannose	75 ± 12	17 ± 4	116 ± 8
Nag	47 ± 4	55 ± 2	111 ± 9
D-fructose	81 ± 11	3 ± 1	82 ± 15
Sucrose	2 ± 0.5	4 ± 1	8 ± 1
Lactose	< 0.5	43 ± 7	45 ± 3
D-xylose	< 0.5	45 ± 8	51 ± 9
D-galactose	< 0.5	107 ± 12	118 ± 12
D-ribose	< 0.5	95 ± 15	96 ± 9
D-gluconate	<0.5	43 ± 8	42 ± 5

[a] MD353 cells were grown in the presence of the sugar used in the assay. The reactions were carried out as described in Materials and Methods.

[b] Standard deviation values were calculated from three independent experiments.

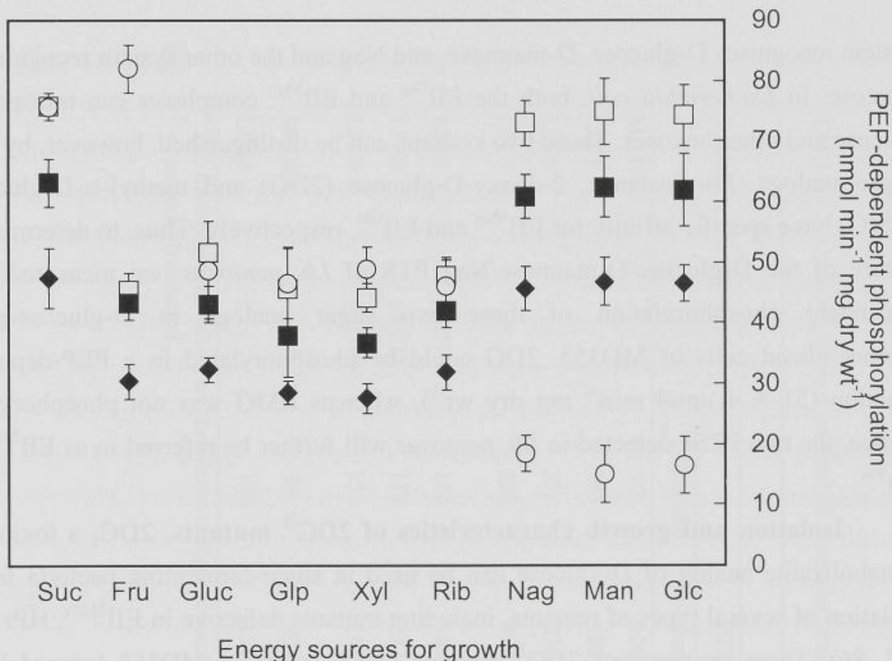


FIGURE. 5.1: Effect of the growth substrate on the rate of PEP-dependent phosphorylation in *Lb. pentosus* MD353 permeabilized cells: D-glucose (■), D-mannose (□), Nag (◆) and D-fructose (○). Values are the means ± standard deviation (error bars) for three separate experiments. The reactions were carried out with 10 mM PEP, 10 mM ^{14}C-labelled sugar (specific activity 0.1 μCi/nmol) under conditions described in Materials and Methods. Cells were grown in MCD medium supplemented with 0.5% (wt/vol) of the corresponding sugar, and harvested at midlog phase of growth (OD_{600nm}= 0.5). Phosphorylation rates are expressed in nmol min^{-1} mg dry wt^{-1}. Abbreviations used: Suc, sucrose; Fru, D-fructose; Gluc, D-gluconate; Glp, glycerol; Xyl, D-xylose; Rib, D-ribose; Nag, *N*-acetyl-D-glucosamine; Man, D-mannose; Glc, D-glucose.

Effect of the growth substrate on PTS activities in *Lb. pentosus* MD353. Next we determined the effect of the growth conditions on the four PTS activities (Fig. 5.1). A similar pattern of activities was observed for D-glucose, D-mannose, and Nag. In each case the activities were increased about 1.6-fold when *Lb. pentosus* cells were grown on these substrates or on sucrose, as compared to the activities in cells cultivated on other energy sources. A different pattern was observed in the case of the D-fructose PTS activity. PEP-dependent phosphorylation of D-fructose increased 1.8-fold in cells grown on D-fructose or sucrose compared to that of cells grown on D-ribose, D-xylose, glycerol or D-gluconate. Furthermore, the D-fructose PTS activity was strongly decreased in cells grown on D-glucose, D-mannose, and Nag. From these results, we conclude that at least two different PTSs are present in *Lb. pentosus*. One

system recognises D-glucose, D-mannose, and Nag and the other system recognises D-fructose. In *Escherichia coli*, both the EII^{Glc} and EII^{Man} complexes can transport D-glucose and other hexoses. These two systems can be distinguished, however, by using sugar analogs. For instance, 2-deoxy-D-glucose (2DG) and methyl-α-D-glucoside (αMG) have specific affinity for EII^{Man} and EII^{Glc}, respectively. Thus, to determine the nature of the D-glucose/D-mannose/Nag PTS of *Lb. pentosus*, we measured PEP-dependent phosphorylation of these two sugar analogs in D-glucose-grown permeabilized cells of MD353. 2DG could be phosphorylated in a PEP-dependent reaction (51 ± 4 nmol min^{-1} mg dry wt^{-1}), whereas αMG was not phosphorylated. Hence, the two PTSs detected in *Lb. pentosus* will further be referred to as EII^{Man} and EII^{Fru}.

Isolation and growth characteristics of $2DG^R$ mutants. 2DG, a toxic non-metabolizable analog of D-glucose can be used in sugar-fermenting bacteria for the isolation of several types of mutants, including mutants defective in EII^{Man} , HPr or EI (52, 86). Three spontaneous $2DG^R$ mutants of *Lb. pentosus* MD353 (named LPE5, LPE6 and LPE8), were isolated on complex medium (M medium, ref. 96) agar plates containing 25 mM sucrose and 10 mM 2DG. To further characterize the mutations in LPE5, LPE6 and LPE8, we first tested their growth on MCD medium containing either PTS or non-PTS sugars. Results are shown in Table 5.2. The three $2DG^R$ mutants had doubling times on D-glucose, D-mannose, and Nag which were approximately 2 to 3 times longer than those determined for the wild-type strain MD353, whereas the four strains exhibited the same doubling time on sucrose and D-ribose. These results were in agreement with the above mentioned observation that D-glucose, D-mannose, and Nag are taken up by a common transporter, and suggested that LPE5, LPE6 and LPE8 were altered in the activity of EII^{Man}. However, the three mutants were still able to grow, albeit slowly, on these compounds, indicating the presence of another pathway for transport and phosphorylation of these sugars. Interestingly, LPE5, LPE6 and LPE8 grew 1.5 fold faster on D-fructose than MD353. Thus, it was unlikely that the mutations in LPE5, LPE6 and LPE8 occurred in the genes encoding the general enzymes of the PTS, HPr and EI. Furthermore, the faster growth of the mutants on D-fructose suggested that the utilization of this hexose is negatively regulated in wild-type bacteria. Another unexpected result was observed with D-xylose. Two of the mutants, LPE5 and LPE8, were unable to grow on this compound, whereas the third mutant, LPE6, was still able to grow, although more slowly than MD353.

TABLE 5.2. Doubling times of several *Lb. pentosus* strains in MCD medium supplemented with 0.5% (wt/vol) of different energy sources

Strains	Plasmid	Doubling time (min):[a]						
		PTS energy sources				Non-PTS energy sources		
		D-glucose	D-mannose	Nag	D-fructose	Sucrose	D-ribose	D-xylose
MD353	-	97	104	96	135	95	122	455
LPE5	-	205	188	169	89	92	126	∞[b]
LPE6	-	278	230	250	90	96	123	729
LPE8	-	228	199	188	89	96	129	∞
LPE5	pMJ18	164	169	156	191	94	122	∞
LPE6	pMJ18	118	127	118	228	92	127	533
LPE8	pMJ18	156	165	162	197	95	120	∞
MD363	-	95	101	103	171	95	301	462
LPE4	-	149	151	163	169	156	315	475

[a] The doubling time values are the means of three separate experiments and their standard deviations never exceeded 15%.

[b] ∞, no growth detected.

Complementation of LPE6 with plasmid pMJ18, expressing the *manB* gene from *Lb. curvatus*. In order to determine whether the mutations in LPE5, LPE6 and LPE8 were located in the membrane-bound or in the cytoplasmic domains of EII^{Man}, we attempted to measure *in vitro* mannose PTS activity in cell-free extracts. Unfortunately, we were unsuccessful since no PEP-dependent D-mannose phosphorylation could be detected even in the wild-type strain MD353 (data not shown), although such an activity is present in permeabilized cells (Table 5.1).

We then tried to complement LPE5, LPE6 and LPE8 mutants with plasmid pMJ18, a multi-copy plasmid expressing the *manB* gene from *Lb. curvatus* encoding the subunit $EIIB^{Man}$ of the EII^{Man} complex from this microorganism (174). The growth characteristics of the transformants were determined (see Table 5.2). Three important observations could be made from these growth studies. First, LPE6/pMJ18 grew faster on D-glucose, D-mannose, and Nag than the strain without plasmid, although the doubling times remained somewhat higher (20%) than those of the parental strain MD353. Nevertheless, the faster growth showed that the mutation in LPE6 must have affected $EIIB^{Man}$ activity and that it could be complemented by the *manB* gene from *Lb. curvatus*. We also observed that LPE6/pMJ18 grew faster on D-xylose which supported a possible role of EII^{Man} in D-xylose fermentation in *Lb. pentosus*. The involvement of EII^{Man} in the utilization of D-xylose in *Lb. pentosus* will be analyzed in Chapter 6 of this thesis. Secondly, the doubling times of LPE5/pMJ18 and LPE8/pMJ18 on D-glucose, D-mannose, and Nag were not restored to the levels of MD353, though a slight decrease (10% to 20%) was observed compared to those determined for the untransformed mutants. Moreover, LPE5/pMJ18 and LPE8/pMJ18 transformants were still unable to grow on D-xylose. This suggested that $EIIB^{Man}$ did not complement the defect of mutants LPE5 and LPE8. Third, the three mutants transformed with plasmid pMJ18 grew about 2.6-fold slower on D-fructose than the untransformed mutants, the effect being more pronounced for LPE6/pMJ18.

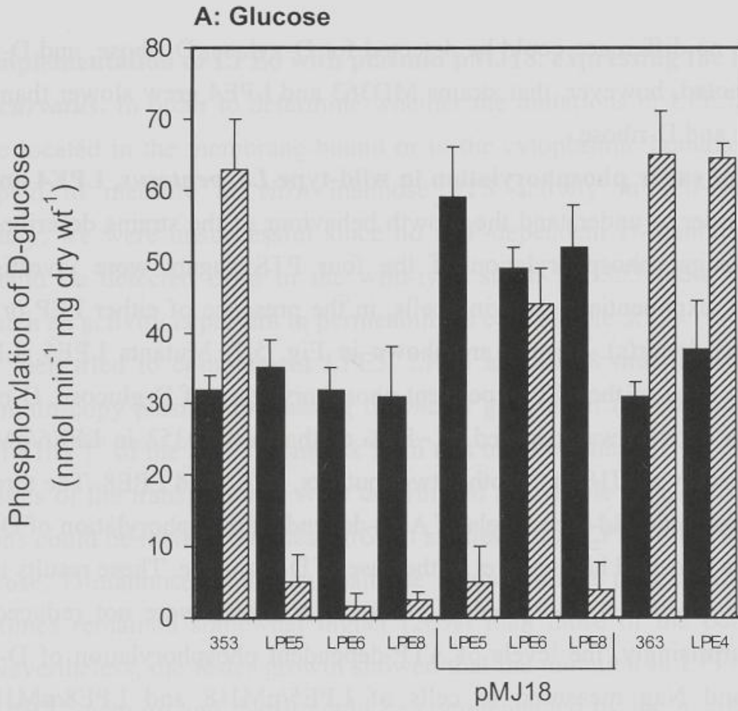
Growth characteristics of the *ccpA* deletion mutant LPE4. In order to determine whether CcpA, a global regulator of CR in *Lb. pentosus* (95), was also involved in the regulation of D-fructose utilization, the influence of a *ccpA* disruption on the utilization of the various sugars mentioned previously was studied (Table 5.2). We found that the phenotype of mutant LPE4 was different from that of the $2DG^R$ mutants. Mutant LPE4 grew at least 1.5-fold slower than its parental wild-type strain MD363 on various energy sources, including sucrose and substrates of the mannose

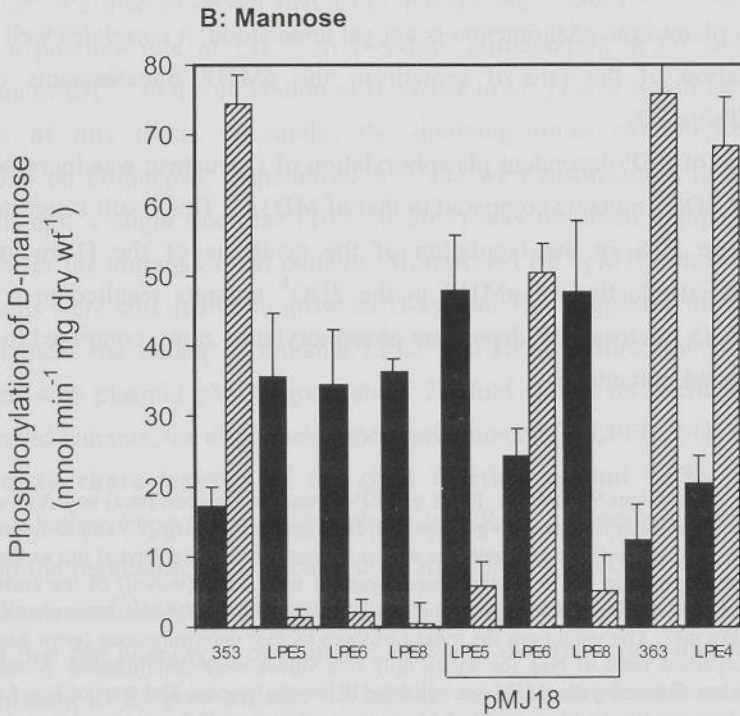
PTS, whereas no difference could be detected for D-xylose, D-ribose, and D-fructose. It should be noted, however, that strains MD363 and LPE4 grew slower than MD353 on D-fructose and D-ribose.

Rates of sugar phosphorylation in wild-type *Lb. pentosus*, LPE4 and $2DG^R$ mutants. In order to understand the growth behaviour of the strains described above, the rates of sugar phosphorylation of the four PTS sugars were investigated in permeabilized, exponentially-growing cells, in the presence of either PEP or ATP as the phosphoryl donor(s). Results are shown in Fig. 5.2. Mutants LPE5, LPE6 and LPE8 had lost most of the PEP-dependent phosphorylation of D-glucose, D-mannose, and Nag. This activity was restored to ~80% of that of MD353 in LPE6/pMJ18 but was not restored by pMJ18 in the other two mutants, LPE5 and LPE8. The three $2DG^R$ mutants still showed wild-type levels of ATP-dependent phosphorylation of D-glucose and Nag, and a two-fold higher level in the case of D-mannose. These results indicated that the sugar-specific ATP-dependent kinase(s) activities were not reduced by the mutations. Surprisingly, the levels of ATP-dependent phosphorylation of D-glucose, D-mannose and Nag measured in cells of LPE5/pMJ18 and LPE8/pMJ18 were somewhat higher than those determined in cells of the untransformed mutants. Although this pleiotropic phenomenon is not yet understood, it correlates well with the slight stimulation of the rate of growth of the pMJ18 transformants on these compounds (Table 5.2).

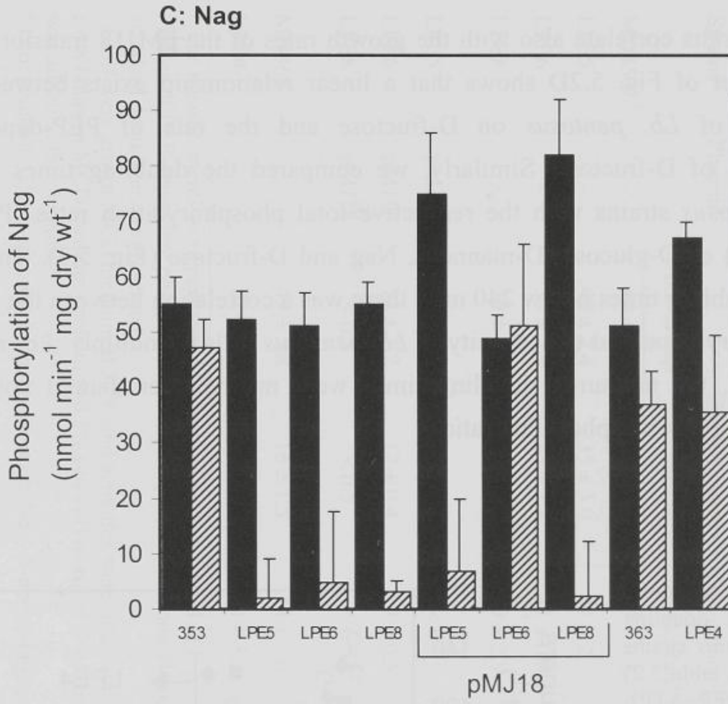
The rate of PEP-dependent phosphorylation of D-fructose was increased about 1.7-fold in the $2DG^R$ mutants compared to that of MD353. This result suggested a role of the mannose PTS in the regulation of the synthesis of the D-fructose PTS. Moreover, the introduction of pMJ18 in the $2DG^R$ mutants resulted in a 2.5-fold decrease of the D-fructose PEP-dependent phosphorylation rates, compared to those of the untransformed mutants.

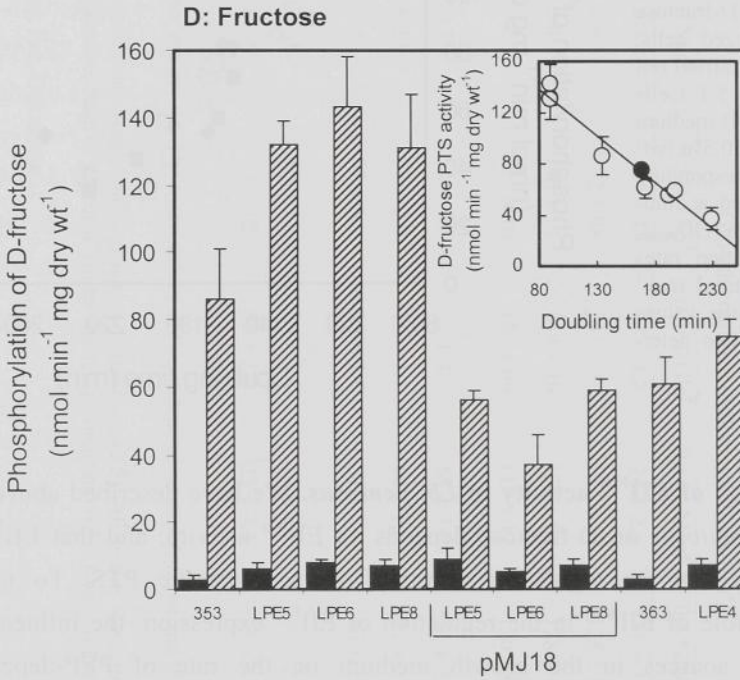
FIGURE. 5.2 ⇨ (next pages 90 and 91): Rates of PEP-dependent (hatched bars) and ATP-dependent (dark shaded bars) phosphorylation of D-glucose (A), D-mannose (B), Nag (C) and D-fructose (D) in permeabilized cells of the various *Lb. pentosus* strains. Experiments were carried out as described in Fig 5.1. Cells were grown in MCD medium supplemented with 0.5% (wt/vol) of the corresponding sugar, and harvested at midlog phase of growth (OD_{600nm}= 0.5). Phosphorylation rates are expressed in nmol min^{-1} mg dry wt^{-1}. The values are the means of three to four determinations (error bars), except for the phosphorylation rates of Nag for which only two values were determined. Mutants LPE5, LPE6 and LPE8 transformed with pMJ18 are indicated below the graphs. The inset of Fig. 5.2D shows the relationship between the doubling times of *Lb. pentosus* strains on D-fructose and the rates of PEP-dependent phosphorylation of D-fructose (D-fructose PTS activity) in permeabilized cells.





(⇧ FIGURE 5.2, see legend on page 89)





(⇧ Following of FIGURE 5.2)

These results correlate also with the growth rates of the pMJ18 transformants. Indeed, the inset of Fig. 5.2D shows that a linear relationship exists between the doubling time of *Lb. pentosus* on D-fructose and the rate of PEP-dependent phosphorylation of D-fructose. Similarly, we compared the doubling times of the various *L. pentosus* strains with the respective total phosphorylation rates (PEP- + ATP-dependent) of D-glucose, D-mannose, Nag and D-fructose (Fig. 5.3). The data show that at doubling times below 240 min, there was a correlation between the rate of hexose phosphorylation and the capacity of *Lb. pentosus* cells to multiply. For mutant LPE4, however, the measured doubling times were much lower than it could be expected from the rates of phosphorylation.

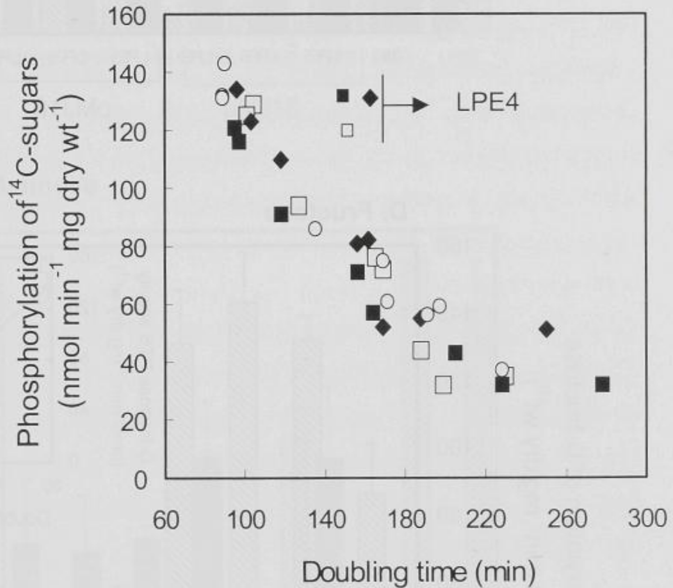


FIGURE. 5.3 ⇨: Relationship between the doubling times of *Lb. pentosus* strains (average values of table 5.2) and the rates of (PEP+ATP)-dependent phosphorylation of D-glucose (■), D-mannose (□), Nag (◆) and D-fructose (○) in permeabilized cells. Experiments were carried out as described in Fig. 5.1. Cells were grown in MCD medium supplemented with 0.5% (wt/vol) of the corresponding sugar, and harvested at mid-log phase of growth (OD_{600nm} =0.5). Phosphorylation rates are expressed in nmol min^{-1} mg dry wt^{-1}, and the values are the means of two determinations.

Regulation of EII^{Fru} activity in *Lb. pentosus*. We have described above that growth of *Lb. pentosus* on D-fructose depends on EII^{Fru} activity, and that EII^{Man} is possibly involved in the regulation of this D-fructose-specific PTS. To further investigate the role of EII^{Man} in the regulation of EII^{Fru} expression, the influence of several energy sources in the growth medium on the rate of PEP-dependent phosphorylation of D-fructose was tested for various *Lb. pentosus* strains.

TABLE 5.3. Regulation of PEP-dependent phosphorylation of D-fructose (EII^{Fru} activity) in permeabilized cells of *Lb. pentosus* [a].

Strains	Plasmid	Rate of PEP-dependent phosphorylation of D-fructose in[b]: Growth condition (non-inducing)[c]: 0.5 % Glp[d]	0.5 % Glc[d]	0.5 % Man[d]	0.5 % Nag[d]	Growth condition (inducing)[c]: 0.5% Fru[d]+ 0.5 % Glp[d]	0.5 % Glc[d]	0.5 % Man[d]	0.5 % Nag[d]
MD353	-	49 ± 4.0	16 ± 1.0	15 ± 0.8	18 ± 3.4	85 ± 15	28 ± 3.2	24 ± 2.1	31 ± 3.1
LPE5	-	40 ± 2.4	35 ± 3.0	41 ± 2.3	37 ± 0.5	132 ± 08	143 ± 12	127 ± 12	125 ± 13
LPE6	-	38 ± 3.9	43 ± 2.7	41 ± 0.8	38 ± 4.3	143 ± 11	149 ± 10	138 ± 08	128 ± 11
LPE8	-	39 ± 1.7	41 ± 5.0	39 ± 2.9	41 ± 1.5	131 ± 10	153 ± 09	119 ± 11	120 ± 09
LPE5	pMJ18	< 0.5	< 0.5	< 0.5	< 0.5	56 ± 5.9	57 ± 4.7	58 ± 7.0	51 ± 2.6
LPE6	pMJ18	12 ± 1.2	12 ± 0.4	13 ± 0.5	13 ± 2.9	37 ± 2.8	18 ± 1.1	14 ± 1.3	21 ± 1.0
LPE8	pMJ18	< 0.5	< 0.5	< 0.5	< 0.5	59 ± 5.1	55 ± 5.6	58 ± 5.1	63 ± 2.9
MD363	-	49 ± 5.8	16 ± 1.5	14 ± 1.0	23 ± 3.6	61 ± 11	26 ± 3.1	19 ± 2.1	31 ± 2.1
LPE4	-	51 ± 4.9	17 ± 1.0	14 ± 3.2	22 ± 1.9	73 ± 12	25 ± 2.9	23 ± 3.3	35 ± 4.8

[a] The measurements were performed as described in the legend of Fig. 1.

[b] Rates of PEP-dependent phosphorylation are expressed in nanomole per min per mg dry wt. The mean values of two independent experiments and their standard deviations are presented.

[c] Cells were grown MCD medium at 37°C and activities were measured in permeabilized exponentially-grown cells ($OD_{600nm} \approx 0.5$). All strains were pre-grown for 8 hours in MCD medium containing 0.5% (wt/vol) of either D-glucose, D-mannose, Nag, or glycerol. Under inducing condition, 0.5% (wt/vol) D-fructose was added to the medium.

[d] Glp, glycerol; Glc, D-glucose; Man, D-mannose; Nag, *N*-acetyl-D-glucosamine; Fru, D-fructose.

In these experiments, all strains were grown either in the presence or in the absence of D-fructose (inducing or non-inducing conditions). Results are shown in Table 5.3. Under both conditions, the presence of D-glucose, D-mannose, and Nag in the growth medium caused a strong decrease of the rate of PEP-dependent phosphorylation of D-fructose in cells of MD353 and MD363, compared to the rate determined in cells of the same strains grown on a non-PTS energy source, glycerol. This repression effect was absent in the $2DG^R$ mutants, but was still present in the Δ*ccpA* mutant, LPE4. Furthermore, the rate of PEP-dependent phosphorylation of D-fructose in cells of MD353, grown on glycerol plus D-fructose, was 35 to 40% lower than that of the $2DG^R$ mutants (85 versus 131-143 nmol min^{-1} mg dry wt^{-1}), but about 20% higher when cells were grown in glycerol (49 versus 38-40 nmol min^{-1} mg dry wt^{-1}). This observation suggests that the inducer, D-fructose, may also be responsible for a decreased activity of EII^{Fru} in wild-type bacteria.

In strains LPE5/pMJ18 and LPE8/pMJ18, no PEP-dependent phosphorylation of D-fructose could be detected when cells were grown under non-inducing conditions. Under similar conditions, however, an average rate of PEP-dependent phosphorylation of D-fructose (12.5 ± 0.5 nmol min^{-1} mg dry wt^{-1}) was measured in LPE6/pMJ18 cells, in which EII^{Man} activity was complemented. This observation suggested that D-fructose could be phosphorylated via EII^{Man}. When LPE5/pMJ18 and LPE8/pMJ18 cells were grown under inducing conditions, D-fructose phosphorylating activity was present, although it remained lower than that of the untransformed bacteria. In LPE6/pMJ18 cells grown under inducing conditions the activity of EII^{Fru} was lower than that found in LPE5/pMJ18 and LPE8/pMJ18 cells. This result indicates that the activity of EII^{Man} in LPE6/pMJ18 has restored the repressive effect of D-glucose, D-mannose, Nag and D-fructose on the activity of EII^{Fru}.

Kinetics of D-fructose uptake in cells of MD353 and LPE6. Previous reports have shown that D-fructose could be a substrate for PTS transporters of the mannose class in various microorganisms (9, 42, 99, 181). If D-fructose could also be a substrate of EII^{Man} in *Lb. pentosus*, possibly its transport and phosphorylation via EII^{Man} could be responsible, to a certain extent, for the reduced activity of EII^{Fru} in cells of MD353 grown on D-fructose compared to that of the $2DG^R$ mutants. Thus, we measured the kinetics of D-fructose uptake in cells of MD353 and LPE6 grown on either D-glucose or D-fructose.

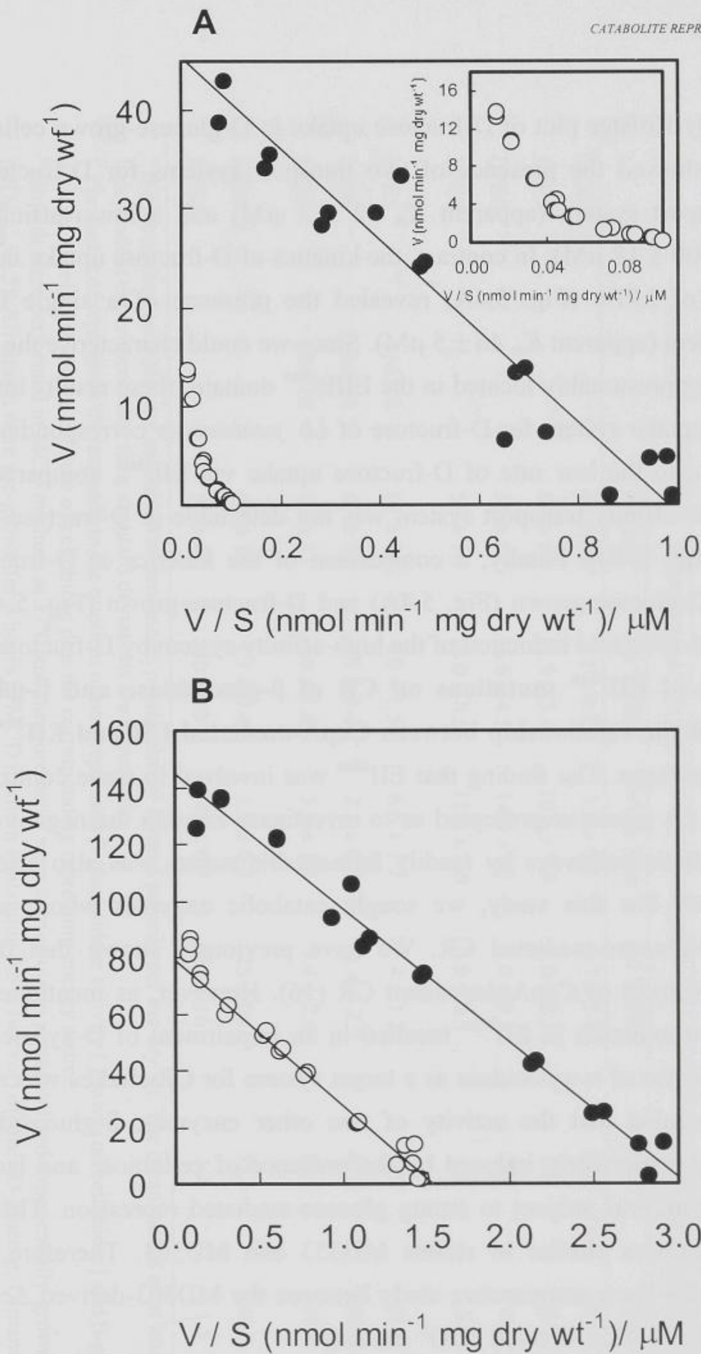


FIGURE. 5.4⇧: Eadie-Hofstee plot of initial rate of D-[U-^{14}C]fructose uptake in starved cells of LPE6 (●) and MD353 (○) grown in either D-glucose (A) or D-fructose (B). The data obtained with cells of MD353 grown in D-glucose are enlarged in the inset of graph A. Initial rates of uptake were determined by taking a sample at 15 s after the start of the transport experiments as described in Materials and Methods. The sugar concentration ranged from 1 μM to 1 mM. The inial rate of uptake is expressed in nmol min^{-1} mg dry wt^{-1} and the substrate concentration in μM.

A Eady-Hofstee plot of D-fructose uptake in D-glucose-grown cells of MD353 (Fig. 5.4A) showed the presence of two transport systems for D-fructose, a high-affinity transport system (apparent K_m 52 ± 1 μM) and a lower-affinity transport system (K_m 300 ± 18 μM). In contrast, the kinetics of D-fructose uptake in D-glucose-grown cells of LPE6 (Fig. 5.4A) revealed the presence of a single high-affinity transport system (apparent K_m 46 ± 5 μM). Since we could characterise the mutation of LPE6 as being presumably located in the $EIIB^{Man}$ domain, these results imply that the low-affinity uptake system for D-fructose of *Lb. pentosus* is corresponding to EII^{Man}. However, due to the low rate of D-fructose uptake via EII^{Man}, compared to that of EII^{Fru}, the low-affinity transport system was not detectable in D-fructose-grown cells of MD353 (fig. 5.4B). Finally, a comparison of the kinetics of D-fructose uptake obtained in D-glucose-grown (Fig. 5.4A) and D-fructose-grown (Fig. 5.4B) cells of LPE6 showed a 3.2-fold induction of the high-affinity system by D-fructose.

Effect of EII^{Man} mutations on CR of β-glucosidase and β-galactosidase activities and the relationship between CcpA-mediated CR and EII^{Man}-mediated CR in *Lb. pentosus*. The finding that EII^{Man} was involved in some control of carbon utilization in *Lb. pentosus* prompted us to investigate whether the negative regulation of other catabolic pathways by readily fermentable sugars was also affected in the $2DG^R$ mutants. For this study, we sought catabolic enzymes whose activity was subject to D-glucose-mediated CR. We have previously shown that α-xylosidase activity was subject to CcpA-dependent CR (16). However, as mentioned earlier in this chapter, a mutation in EII^{Man} resulted in an impairment of D-xylose utilization. Therefore, the use of α-xylosidase as a target system for CR studies was not suitable. Screening revealed that the activity of two other enzymes, β-glucosidase and β-galactosidase, respectively induced by the presence of cellobiose and lactose in the growth medium, was subject to strong glucose-mediated repression. The activity of these enzymes was similar in strains MD353 and MD363. Therefore, they were suitable systems for a comparative study between the MD363-derived Δ*ccpA* mutant (LPE4) and the MD353-derived $2DG^R$ mutants.

The influence of three energy sources, D-glucose, D-mannose and D-fructose on the activity of the two enzymes was determined for all strains described above, including the pMJ18 transformants of LPE5, LPE6 and LPE8. Results are shown in Table 5.4 and Table 5.5. The three hexoses showed a repressive effect on the expression of the two catabolic enzymes in the wild-type strain. The repressive effect

TABLE 5.4. β-glucosidase activities on *p*-nitrophenyl-β-D-glucopyranoside measured in permeabilized cells of *Lb. pentosus*.

Strains	Plasmid	β-glucosidase activity:[a]				Repression factor:[b]		
		Growth cond.[c] : 0.5 % (wt/v) of cellobiose +				Repressing energy sources		
		No add	0.5 % Glc	0.5 % Man	0.5 % Fru	Glc	Man	Fru
MD353	-	61 ± 11	1.1 ± 0.7	1.3 ± 0.8	2.7 ± 1.1	55.5	46.9	22.6
LPE5	-	60 ± 06	11.9 ± 3.4	10.6 ± 3.3	1.0 ± 0.3	5.0	5.7	60.0
LPE6	-	58 ± 09	16.6 ± 4.3	14.0 ± 03	1.0 ± 0.3	3.5	4.1	58.0
LPE8	-	59 ± 16	13.0 ± 4.5	11.9 ± 2.0	0.9 ± 0.3	4.5	5.0	66.0
LPE5	pMJ18	61 ± 10	4.8 ± 1.5	6.6 ± 1.2	7.4 ± 1.2	12.7	9.2	8.2
LPE6	pMJ18	63 ± 12	1.5 ± 0.4	2.1 ± 0.5	11.4 ± 2.9	42.0	30.0	5.5
LPE8	pMJ18	61 ± 11	4.0 ± 1.6	5.9 ± 1.8	9.4 ± 2.4	15.3	10.3	6.5
MD363	-	61 ± 18	1.1 ± 0.4	1.3 ± 0.8	25 ± 06	55.5	46.9	2.5
LPE4	-	57 ± 09	40 ± 10	42 ± 03	51 ± 16	1.4	1.3	1.1

[a] β-glucosidase activities are expressed in nanomoles *p*-nitrophenol formed per min per mg dry wt. The mean values of at least five independent experiments and their standard deviations are presented.
[b] The factor of repression corresponds to the quotient of non-repressed β-glucosidase activity (growth on cellobiose) and β-glucosidase activity in cells grown in a mixture of cellobiose plus the corresponding repressing energy source.
[c] Growth were performed in MCD medium at 37°C and measurements were carried out in permeabilized exponentially-grown cells (OD_{600nm}≈0.5). All strains were pre-grown for 8 hours in MCD medium containing 0.5% (wt/vol) ofeither D-glucose, D-mannose or D-fructose. For the induced and non-repressed condition (column 1), cells were pre-grown overnight in 0.5% (wt/vol) cellobiose.

TABLE 5.5. β-galactosidase activities on *o*-nitrophenyl-β-D-galactopyranoside measured in permeabilized cells of *Lb. pentosus*.

Strains	Plasmid	β-galactosidase activity:[a] Growth cond.[c] : 0.5 % (wt/v) of lacose +				Repression factor:[b] Repressing energy sources		
		No add	0.5 % Glc	0.5 % Man	0.5 % Fru	Glc	Man	Fru
MD353	-	44 ± 7	0.7 ± 0.2	0.9 ± 0.5	2.0 ± 0.7	62.8	49.0	22.0
LPE5	-	39 ± 5	6.9 ± 1.7	5.8 ± 1.0	0.6 ± 0.2	5.7	6.7	65.0
LPE6	-	38 ± 6	10.6 ± 3.0	8.1 ± 3.9	0.6 ± 0.1	3.6	4.7	63.3
LPE8	-	41 ± 4	8.5 ± 1.9	6.6 ± 2.1	0.6 ± 0.2	4.8	6.2	65.0
LPE5	pMJ18	39 ± 7	2.7 ± 1.5	4.2 ± 1.6	6.8 ± 2.2	14.4	9.3	5.7
LPE6	pMJ18	39 ± 4	1.1 ± 0.5	1.4 ± 0.4	8.6 ± 2.0	35.5	27.9	4.5
LPE8	pMJ18	36 ± 3	2.6 ± 0.7	4.3 ± 2.1	6.4 ± 2.1	13.8	8.4	5.6
MD363	-	45 ± 5	0.7 ± 0.2	0.8 ± 0.4	9.6 ± 1.6	64.3	56.3	4.7
LPE4	-	45 ± 4	18.7 ± 4.2	16.0 ± 5.5	15.9 ± 2.5	2.4	2.8	2.8

[a] β-galactosidase activities are expressed in nanomoles *o*-nitrophenol formed per min per mg dry wt. The mean values of at least three independent experiments and their standard deviations are presented.
[b] The factor of repression corresponds to the quotient of non-repressed β-galactosidase activity (growth on lactose) and β-galactosidase activity in cells grown in a mixture of lactose plus the corresponding repressing energy source.
[c] Growth were performed in MCD medium at 37ºC and measurements were carried out in permeabilized exponentially-grown cells ($OD_{600nm} \approx 0.5$). All strains were pre-grown for 8 hours in MCD medium containing 0.5% (wt/vol) OF either D-glucose, D-mannose or D-fructose according to the repressing energy tested. For the induced and non-repressed condition (column 1), cells were pre-grown overnight in 0.5% (wt/vol) lactose.

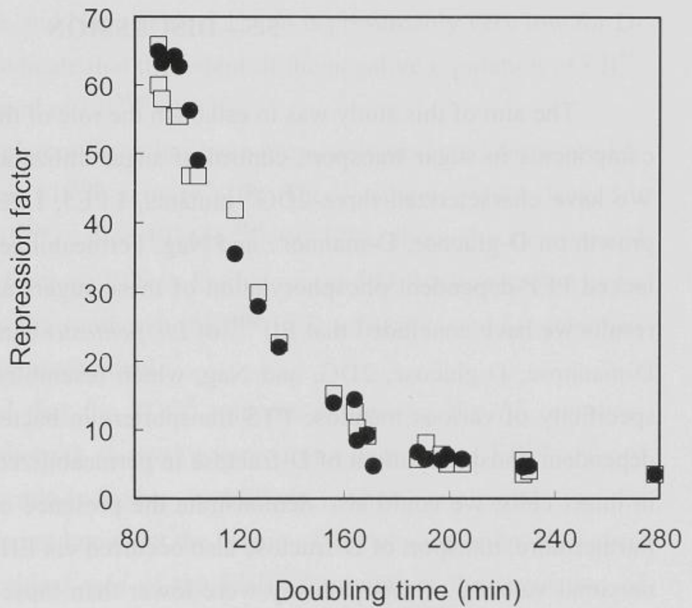


FIGURE. 5.5: Plot of the catabolite repression factor (R) of β-glucosidase (□) and β-galactosidase (●) activities in *Lb. pentosus* (values of Tables 5.4 and 5.5) versus doubling times of the various *Lb. pentosus* strains on D-glucose, D-mannose or D-fructose (values of Table 5.2).

mediated by D-fructose was less pronounced in MD353, and strongly reduced in MD363, compared to the repression mediated by D-glucose or D-mannose. In the Δ*ccpA* mutant, the repression which D-glucose, D-mannose and D-fructose exerted on the β-glucosidase activity was relieved for more than 80%, but β-galactosidase activity was still repressed two- to threefold by these sugars. Nevertheless, these results indicated that the expression of both enzymes was subject to strong CcpA-dependent CR in *Lb. pentosus*. The expression of both enzymes in LPE5, LPE6 and LPE8 was partially released from CR mediated by D-glucose and D-mannose, but an increased extend of D-fructose-mediated CR could be detected in these mutant strains. In contrast, all three pMJ18 transformants had lost most of the strong D-fructose-mediated CR observed in the untransformed mutants. Fig. 5.5 shows the relationship between the doubling times of *Lb. pentosus* strains used in this study on various energy sources (values of Table 5.2) and the corresponding repression factor of both β-glucosidase and β-galactosidase activities (values of Table 5.4 and Table 5.5). At doubling times higher than 180 min, however, the influence of the doubling times on the repression factor was less pronounced than for doubling times shorter than 180 min.

5.5 – DISCUSSION

The aim of this study was to establish the role of the *Lb. pentosus* mannose PTS components in sugar transport, control of sugar utilization and catabolite repression. We have characterized three $2DG^R$ mutants, LPE5, LPE6 and LPE8, with impaired growth on D-glucose, D-mannose and Nag. Permeabilized cells of the $2DG^R$ mutants lacked PEP-dependent phosphorylation of these sugars and also of 2DG. From these results we have concluded that EII^{Man} of *Lb. pentosus* can transport and phosphorylate D-mannose, D-glucose, 2DG, and Nag, which resembles the typical broad substrate specificity of various mannose PTS transporters in bacteria (125). By studying PEP-dependent phosphorylation of D-fructose in permeabilized cells and D-fructose uptake in intact cells, we could also demonstrate the presence of a D-fructose-specific PTS. Furthermore, transport of D-fructose also occurred via EII^{Man}, although the affinity and maximal velocity of this pathway were lower than those determined for EII^{Fru}. PEP-dependent phosphorylation of D-fructose measured in cells of *Lb. pentosus* wild-type and $2DG^R$ mutants, grown on various energy sources, allowed us to demonstrate that the synthesis of EII^{Fru} is regulated at two levels: one resulting in a 3-fold induction by D-fructose, and another one involving repression mediated by the presence of several energy sources in the growth medium.

EII^{Man} regulates the synthesis of EII^{Fru} independently of CcpA. The three *Lb. pentosus* $2DG^R$ mutants appeared to lack the negative regulation of EII^{Fru} synthesis observed in the wild-type strain. To further study the regulation of EII^{Fru} synthesis, we have investigated the individual contributions of EII^{Man} and of CcpA to this phenomenon. Data in Table 5.3 show that the *ccpA* mutation did not relieve the repressive effect of several sugars on the synthesis of EII^{Fru} when compared to the $2DG^R$ mutants. Therefore, EII^{Man} but not CcpA, is an important component in the mechanism regulating EII^{Fru} synthesis in *Lb. pentosus*. Furthermore, two lines of evidence suggest that transport of PTS sugars via EII^{Man}, is a prerequisite condition for this regulation. First, no decreased EII^{Fru} activity was observed when MD353 cells were grown on substrates such as glycerol, which are not transported by the PTS. Second, we found that the decrease of EII^{Fru} activity caused by D-mannose was slightly higher than that caused by D-glucose, and that the decrease in the activity of EII^{Fru} was even less for Nag and D-fructose (see Table 5.3). Our results also show that the PEP-dependent phosphorylating activity of EII^{Man} is the highest with D-mannose,

then decreases with D-glucose, and Nag (Table 5.1) and is presumably very low for D-fructose. These observations indicate that the extent of the negative regulation of EII^{Fru} synthesis correlates with the level of EII^{Man} activity.

Interestingly, a similar phenomenon has been previously observed for several *S. salivarius* mutants deficient in EII^{Man} activity (10). These mutants which lacked a cytoplasmic component of EII^{Man}, called $EIIAB_L^{Man}$ (9, 170), showed a derepressed expression of an inducible D-fructose PTS in D-glucose- and D-fructose-grown cells. This observation indicates that the synthesis of EII^{Fru} in *S. salivarius* and *Lb. pentosus* might be regulated via a similar mechanism.

Surprisingly, we found that the $2DG^R$ mutants expressing the *Lb. curvatus manB* gene on a multi-copy plasmid, showed a very strong decrease of EII^{Fru} activity under both inducing and non-inducing conditions, when compared to the untransformed mutants. We do not know yet the reason of this phenomenon. However, this result would suggest a critical role of the $EIIB^{Man}$ subunit in the regulation of EII^{Fru} synthesis, as already claimed for the $EIIAB_L^{Man}$ subunit of *S. salivarius*. How does EII^{Man} regulate the synthesis of EII^{Fru}? The expression of several bacterial genes, encoding substrate-specific PTSs, have been shown to be regulated by an antiterminator protein. The mechanism involves phosphorylation of the antiterminator by one of the EII components, belonging to the specific PTS (for reviews, see 33 and 161). Thus, it is possible that the synthesis of EII^{Fru} in *Lb.* pentosus is regulated via a mechanism which involves phosphorylation of an antiterminator protein by one or more component(s) of the EII^{Man} complex. A molecular genetic analysis of the *Lb. pentosus* EII^{Man} and EII^{Fru} encoding genes is needed to unravel in more detail this regulatory mechanism.

Role of EII^{Man} and EII^{Fru} in CcpA-dependent CR of two catabolic enzymes in *L. pentosus*. In this paper, we show that the expression of β-glucosidase and β-galactosidase in *Lb. pentosus* is negatively regulated by CcpA. In addition, the repression exerted by D-glucose and D-mannose on the activities of these two enzymes in MD353 was partially relieved in the $2DG^R$ mutants. These results are in good agreement with previous observations made for $2DG^R$ mutants of *S. salivarius*, *Lb. casei* or *T. halophila*, which displayed a relief of D-glucose-mediated repression of several metabolic enzymes (1, 51, 56, 85, 175).

However, we found that mutations in EII^{Man} did not relieve the D-fructose-mediated repression of β-glucosidase and β-galactosidase activities in *Lb. pentosus*.

The extent of the repression mediated by D-fructose was dependent on the rate at which *Lb. pentosus* grew on this compound (Fig. 5.5, or Table 5.2 and Table 5.4). Since we could show that the growth rate of *Lb. pentosus* correlates with the activity of EII^{Fru} (inset of Fig. 5.2D), these results demonstrate that the repression mediated by D-fructose on β-glucosidase and β-galactosidase activities is dependent on EII^{Fru} activity. Therefore, both EII^{Man} and EII^{Fru} are important components of CcpA-mediated CR in *Lb. pentosus*. Indeed, from our data it appears that CcpA-dependent repression of the two enzymes is correlated with the doubling times of *Lb. pentosus* on the three hexoses tested: D-glucose, D-mannose and D-fructose (Fig. 5.5). Moreover, the rate at which hexose phosphate is provided to the glycolytic pathway reflects the capacity of *Lb. pentosus* cells to divide (Fig. 5.3). Therefore, these results suggest that the degree of CcpA-dependent repression of β-glucosidase and β-galactosidase activities may correlate with the rate of hexose catabolism via the glycolytic pathway. Such a hypothesis would agree with the finding that HPr(Ser-P)/CcpA-mediated CR is activated by phosphorylated glycolytic intermediates in *B. subtilis*. In the light of this and considering the predominant role of EII^{Man} and EII^{Fru} in the transport and phosphorylation of D-glucose, D-mannose and D-fructose, it can be concluded that these two PTS provide key signals in the CcpA-dependent repression mediated by these sugars in *Lb. pentosus*. Interestingly, the data shown in Fig. 5.5 indicate that when the doubling time of *Lb. pentosus* is more than 180 min, CcpA-mediated repression of β-glucosidase and β-galactosidase activities is weakly influenced by changes in the doubling time. This phenomenon suggests that a certain threshold, in doubling time and/or in the rate of hexose fermentation, may be necessary for the stimulation of CcpA-dependent CR.

To summarize, we show in this report that EII^{Man} of *Lb. pentosus* is involved in two distinct pathways of carbon catabolite repression. First, the activity of EII^{Man} regulates negatively the synthesis of EII^{Fru} by a mechanism which does not involve the global *trans*-factor of CR, CcpA. Secondly, we also demonstrate the role of EII^{Man} and EII^{Fru} in the CcpA-dependent CR of the expression of two catabolic enzyme activities.

5.6 – ACKNOWLEDGEMENTS

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