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Two Uptake Systems for Fructose in *Lactococcus lactis* subsp. *cremoris* FD1 Produce Glycolytic and Gluconeogenic Fructose Phosphates and Induce Oscillations in Growth and Lactic Acid Formation

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Fructose transport in lactococci is mediated by two phosphotransferase systems (PTS). The constitutive mannose PTS has a broad specificity and may be used for uptake of fructose with a fructose saturation constant (K_{Fru}) of 0.89 mM, giving intracellular fructose 6-phosphate. The inducible fructose PTS has a very small saturation constant (K_{Fru}, <17 μM), and the fructose 1-phosphate produced enters the Embden-Meyerhof-Parnas (EMP) pathway as fructose 1,6-diphosphate. Growth in batch cultures of *Lactococcus lactis* subsp. *cremoris* FD1 in a yeast extract medium with fructose as the only sugar is poor both with respect to specific growth rate and biomass yield, whereas the specific lactic acid production rate is higher than those in similar fermentations on other sugars metabolized via the EMP pathway, e.g., glucose. In fructose-limited chemostat cultures, the biomass concentration exhibits a strong correlation with the dilution rate, and starting a continuous culture at the end of a batch fermentation leads to large and persistent oscillations in the biomass concentration and specific lactic acid production rate. Two proposed mechanisms underlying this strange growth pattern follow. (i) Fructose transported via the fructose PTS cannot be converted into essential biomass precursors (glucose 6-phosphate or fructose 6-phosphate), because *L. lactis* subsp. *cremoris* FD1 is devoid of fructose 1,6-diphosphatase activity. (ii) The fructose PTS apparently produces a metabolite (presumably fructose 1-phosphate) which exerts catabolite repression of both mannose PTS and lactose PTS. Since the repressed mannose PTS and lactose PTS are shown to have identical maximum molar transport rates, the results indicate that it is the general PTS proteins which are repressed.

Sugar transport in bacteria often occurs via phosphotransferase systems (PTS) which catalyze concomitant phosphorylation and translocation across the membrane via a chain of enzymatic reactions (Fig. 1) (15). In lactococci, most metabolizable sugars are transported via the PTS, e.g., glucose and lactose via the constitutive mannose PTS (Man-PTS) and lactose PTS (Lac-PTS), respectively, and fructose via the inducible fructose PTS (Fru-PTS) (21).

Fructose transport in bacteria may occur via the inducible Fru-PTS to give fructose 1-phosphate (Fru-1P) or via the Man-PTS which has a broad substrate specificity and translocates fructose to give fructose 6-phosphate (Fru-6P [12–15, 21]). Fru-1P enters the Embden-Meyerhof-Parnas (EMP) pathway as fructose 1,6-diphosphate (FDP), and fructose should therefore be considered a gluconeogenic substrate when it is transported via the Fru-PTS.

Metabolism of fructose by *Lactococcus lactis* seems to have received little attention (23, 24), probably because lactococci are used in the dairy industry, where the metabolism of other sugars, e.g., lactose, glucose, and galactose, is more relevant. Fructose is, however, an important sugar in the food industry, and it is therefore pertinent to investigate the metabolism of fructose.

This study describes the unusual pattern of growth and lactic acid formation of *L. lactis* subsp. *cremoris* FD1 in batch and continuous cultures with fructose and with mixtures of fructose and glucose.

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MATERIALS AND METHODS

Microorganisms and growth conditions. *L. lactis* subsp. *cremoris* FD1 was obtained from the Danish Institute of Dairy Research, Hillerød, Denmark (closed since 1990). In a few experiments, another strain, *L. lactis* subsp. *cremoris* Wg2, obtained from the Dutch Institute of Dairy Research (NIZO), Ede, The Netherlands, was used.

The inoculation medium contained 5 g of yeast extract per liter, 5 g of casein peptone per liter, 10 g of disodium-β-glycerophosphate-pentahydrate per liter, 12.5 ml of a salt solution (0.23 M NaH_2PO_4, 1.15 M NH_4Cl, 58 mM $MgSO_4$, 58 mM KCl) per liter, and 10 g of lactose per liter and was adjusted to pH 7.0. Inocula for the batch fermentations were prepared by transferring colonies from LM-17 agar plates (Merck 15108) to test tubes containing the inoculation medium and incubating them overnight on a rotary shaker at 30°C.

The fermentation media were inoculated with 1 mg of biomass per liter, and the bacteria were cultivated unaerated in laboratory fermentors (0.7- to 1.8-liter working volume). The media contained a mixture of equal amounts of yeast extract and casein peptone (YECP) and 1.25 ml of salt solution per g of YECP. The concentrations of YECP and sugar (autoclaved separately) are given in the figure legends. During the fermentations, the temperature was maintained at 30°C and pH was maintained at 6.30 by the addition of 2 M NaOH.

The percentage of glucose contamination in the fructose (Sigma F-0127) was measured by flow injection analysis by

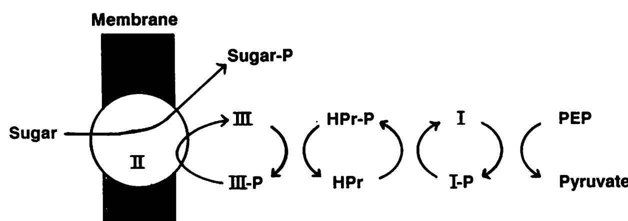

FIG. 1. Sugar transport by the PTS where a general phosphate transfer chain (enzyme I and HPr) catalyzes the transfer of phosphate groups from phosphoenolpyruvate (PEP) to sugar-specific proteins (enzyme III and the integral membrane protein II).

applying glucose oxidase (3) and was found to be 0.015% (wt/wt).

Analytical methods. During the fermentations, the concentrations of glucose, lactic acid, and biomass were measured on-line every 6 to 12 min by three flow injection analyzers as described previously (2). The substrate addition rate and the acid production rate of the cultures were measured on-line every 6 to 12 min by electronic balances (Sartorius F32000S-D2 and E8100P). During conditions of homofermentative metabolism, the specific lactic acid formation rate, r_{LA} (in grams of lactic acid gram of biomass^{-1} hour^{-1}), was calculated from the measured acid production rate, biomass concentration, and culture volume. The specific growth rate was calculated by numerical differentiation of biomass measurements during 1 h (correcting for the effect of the net flow through the fermentor). Fructose was measured as reducing sugar by the dinitrosalicylic acid assay.

Disrupted cells. Disrupted cells were prepared by washing the bacteria twice in a solution of 50 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.0, followed by disruption in a ball mill (19) at 4°C for 40 min. Cell debris was removed by centrifugation at 5,000 × g for 15 min at 4°C, and the clear supernatants were used for the fructose 1,6-diphosphatase (FDPase) assays.

FDPase assay. FDPase activity was measured by hydrolysis of FDP and quantification of the NADPH formed from isomerization of Fru-6P and oxidation by glucose 6-phosphate dehydrogenase as described elsewhere (16). The only modification of the procedure was to maintain the temperature at 30°C during the assays.

RESULTS

Batch fermentations. In a batch fermentation with fructose as the only sugar, growth was very slow compared with growth on glucose (Fig. 2). This fermentation lasted 40 h, whereas a similar fermentation on glucose lasted only 17 h (not shown). As the biomass concentration increased above 0.025 g/liter, the specific growth rate (μ) decreased from 0.8 h^{-1} to less than 0.2 h^{-1}. The short period at high μ is attributed to the consumption of sugars other than fructose. The biomass concentration during a similar fermentation on glucose is indicated as a continuous line in Fig. 2.

When a small fraction of the fructose was replaced by glucose, growth improved markedly, as seen in Table 1, where the integral yield coefficient, Y_{sx}, is listed for several batch fermentations with mixtures of glucose and fructose. With only fructose present, the value of Y_{sx} was 28% of that obtained in the fermentation with only glucose. Glucose and fructose were used simultaneously. Higher glucose content

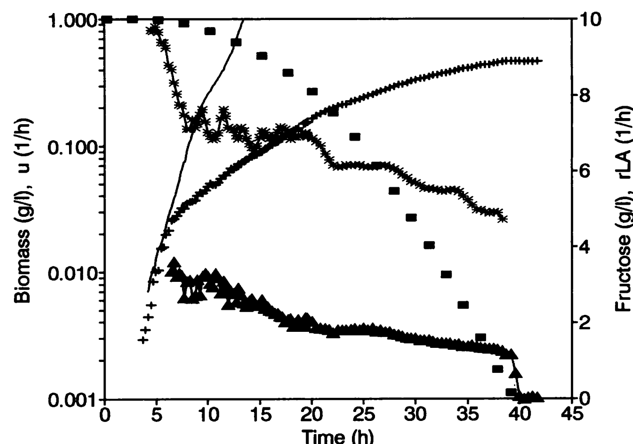

FIG. 2. Batch fermentation with a medium containing fructose (10 g/liter) and YECP (10 g/liter). Curves for biomass concentration (+), specific growth rate (✱), specific lactic acid production rate (rLA) (▲), and estimated fructose concentration (■) are shown. The biomass concentration (——) in a similar fermentation where glucose was used instead of fructose is also shown.

in the sugar mixture resulted in an increased fraction of glucose metabolized. However, there was no simple correlation between the distribution of the two sugars in the medium and the relative rates of their utilization.

Start-up of a chemostat. Figure 3A shows the biomass concentration and the specific lactic acid production rate after the batch fermentation (Fig. 2) with fructose as the only sugar was changed to chemostat operation (dilution rate [D] = 0.10 h^{-1}). When a batch fermentation on a mixture of glucose and fructose was changed to fructose-limited continuous operation (D = 0.35 h^{-1}), the steady state was attained after 110 h (40 residence times [Fig. 3B]). It was not possible to start a chemostat experiment with fructose as the limiting substrate without these prolonged and often oscillatory transient states.

Steady-state cultures. Continuous fermentations at different specific growth rates (μ) confirmed the unusual growth pattern on fructose. The biomass concentration and the specific lactic acid production rate depended almost linearly on the specific growth rate as follows.

$$x = 0.26 \text{ g liter}^{-1} + 1.88\mu \text{ g liter}^{-1} \text{ h}^{-1} \quad (1)$$

$$r_{LA} = 1.08 \text{ h}^{-1} + 4.14\mu \quad (2)$$

Equation 1 is strictly empirical, whereas the two parameters

TABLE 1. Integral yield coefficient, Y_{sx}, and the percentage of glucose in the consumed sugar in batch fermentations with mixtures of fructose and glucose

% of glucose in initial sugar mixture	Total initial sugar concn (g/liter)	Y_{SX} (g of biomass/g of sugar)[a]	% of glucose in consumed sugar[b]
0	10.0	0.050	0
6	11.7	0.119	39
19	13.8	0.157	48
64	14.0	0.176	64
100	10.0	0.180	100

[a] Calculated at the end of the fermentation, when both sugars were depleted.
[b] Calculated when glucose was depleted from the medium.

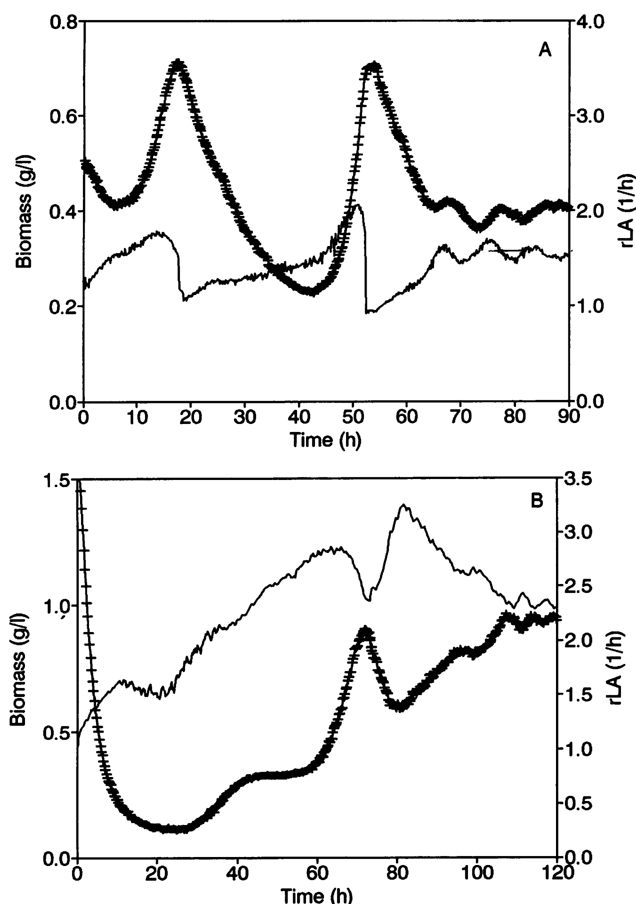

FIG. 3. Biomass concentration (+) and specific lactic acid production rate (rLA) (——) during start-up of a continuous culture with fructose limitation (7 g of fructose per liter and 10 g of YECP per liter). (A) D = 0.10 h^{-1} imposed on the batch fermentation shown in Fig. 2. (B) D = 0.35 h^{-1} imposed on a batch culture grown on a mixture of fructose and glucose (19% glucose in the mixture [see Table 1]).

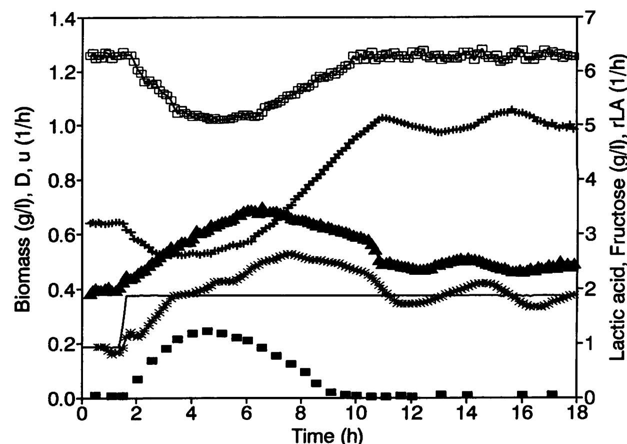

FIG. 4. Step change of the dilution rate from 0.19 to 0.37 h^{-1} in a fructose-limited chemostat culture (7 g of fructose per liter and 10 g of YECP per liter). Symbols: □, lactic acid; ■, fructose; +, biomass; ✳, specific growth rate; ▲, specific lactic acid production rate. ——, dilution rate.

in equation 2 are the maintenance coefficient (in grams of lactic acid gram of biomass^{-1} hour^{-1}) and the true yield coefficient (in grams of lactic acid per gram of biomass). The fructose concentration was very low (<0.1 mM) at all dilution rates, and lactic acid was the only metabolic end product seen. In a similar medium with glucose as the sugar, formic acid, acetic acid, and ethanol were also formed when the D was less than 0.3 h^{-1}. Whereas equation 2 expresses the well-known Luedeking-Piret relationship between the specific rate of lactic acid production and the specific growth rate, the linear relationship between biomass concentration and growth rate in equation 1 is highly surprising. Steady-state cultures (data not shown) with glucose or lactose as the limiting substrate exhibited a decreasing biomass concentration as μ (= D) increased.

Pulse addition of sugars. The response of the cells in a fructose-limited culture was tested by the addition of various sugars in pulses (all sugar solutions in mutarotational equilibrium) (5). During metabolism of a fructose pulse to a chemostat at a D of 0.46 h^{-1}, the rate of fructose consumption could be fitted to a Michaelis-Menten expression with a maximum consumption rate of fructose ($r_{Fru,max}$) of 2.9 h^{-1} and a fructose saturation constant (K_{Fru}) of <17 μM. This shows that a fructose-transporting enzyme has been induced, since the saturation constant for the Man-PTS was determined to be K_{Fru} = 0.89 mM (from a fructose pulse to a glucose-limited chemostat in which the glucose concentration is very low) (5, 6). To fructose-limited cultures with a D of 0.46 h^{-1}, pulses of glucose or lactose were added and the fructose feed was stopped. The added sugars were metabolized at constant rates, $r_{Glc,max}$ = 0.46 h^{-1} and $r_{Lac,max}$ = 0.87 h^{-1}. These values are much smaller than those observed when pulses were added to similar cultures which were glucose limited ($r_{Glc,max}$ = 3.0 h^{-1} and $r_{Lac,max}$ = 1.8 h^{-1}).

Step change of dilution rate in chemostat. Analysis of the transient state between two steady states in a chemostat may also give information on fructose-supported growth. A step change of the dilution rate from 0.19 to 0.37 h^{-1} was seen to give rise to oscillations which dampened as the system approached the new steady state (Fig. 4). There was an incipient washout of the biomass and a corresponding accumulation of fructose, but the increased fructose concentration gave rise to an increase of μ (from 0.2 to 0.5 h^{-1}), and the biomass concentration again started to increase. After 9 h, when the fructose concentration had decreased below the saturation limit, the biomass concentration oscillated about a new and much higher steady-state level (see equation 1). The time courses of r_{LA} and μ were almost the same, and throughout the transient state, the linear relationship (equation 2) between the two specific rates was closely followed. The lactic acid concentrations at the start and end of the transient state were virtually identical (6.36 g/liter), and since the fructose concentration at both ends of the transient state was less than 20 mg/liter, the higher biomass concentration must be attributed to a higher yield of biomass on the YECP substrate.

In another experiment with a step change of D from 0.075 to 0.153 h^{-1}, the oscillations in biomass concentration and acid production rate were more stable and dampened less rapidly (Fig. 5). As the dilution rate was increased, there was incipient washout of biomass and a complex, seemingly irregular oscillatory transient state followed. The sudden decrease in the acid production rate at 8 h suggested that the

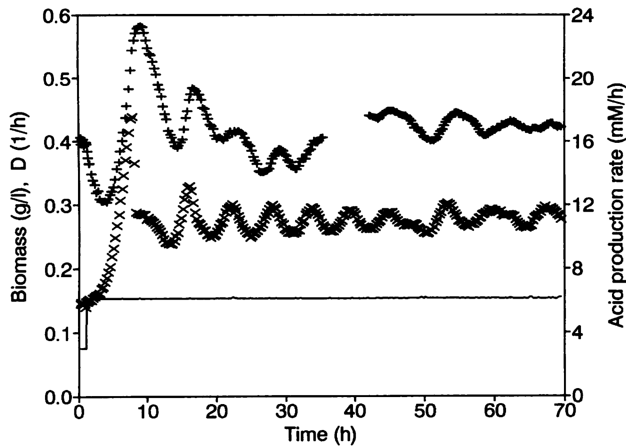

FIG. 5. Step change of the dilution rate from 0.075 to 0.153 h^{-1} in a fructose-limited chemostat culture (7 g of fructose per liter and 10 g of YECP per liter). +, biomass concentration; ×, acid production rate; ——, dilution rate.

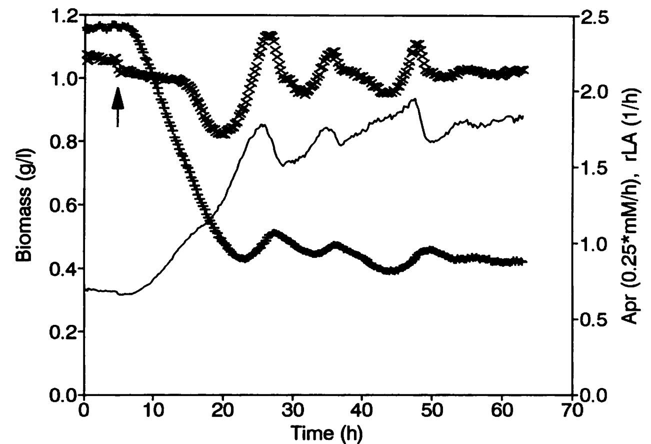

FIG. 7. Step change (arrow) from a glucose-limited culture to a fructose-limited culture in a chemostat (D = 0.10 h^{-1}; cultures as described in the legend to Fig. 6). +, biomass; ×, measured lactic acid production rate (Apr); ——, specific lactic acid production rate.

accumulated fructose was virtually depleted at this time. During the rest of the fermentation, such large jumps in the acid production rate were not observed. This means that fructose must also be transported via the Man-PTS because the Fru-PTS, with a saturation constant of less than 17 μM, cannot give rise to the observed amplitude of the acid production rate. The oscillations in acid production rate are regular, and the period is 5.8 h, which is somewhat smaller than the residence time (6.5 h). The disturbance of the oscillations in acid production rate at 48 h is caused by changing the substrate flask.

Change in feed sugar. The adaptation of bacteria cultivated on one sugar to growth on another sugar was investigated by step changing the substrate in a chemostat where the steady-state concentrations of glucose (~5 mg/liter) and fructose (<20 mg/liter) were small. A step change from fructose-limited medium (7 g of fructose per liter) to a glucose-limited medium (7 g of glucose per liter) led to an immediate

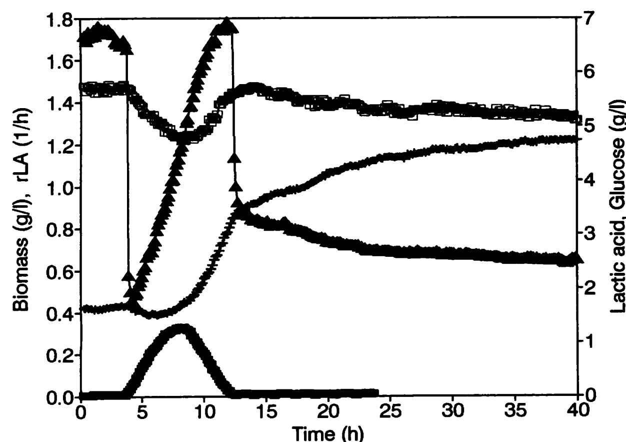

FIG. 6. Step change (t = 4 h) from a fructose-limited culture (7 g of fructose per liter and 10 g of YECP per liter) to a glucose-limited culture (7 g of glucose per liter and 10 g of YECP per liter) in a chemostat (D = 0.10 h^{-1}). Symbols: □, lactic acid; ■, glucose; +, biomass; ▲, specific lactic acid production rate (rLA).

decrease of the specific lactic acid production rate (Fig. 6). Apparently the bacteria which had adapted to growth on fructose could not at first metabolize glucose as fast as they could metabolize fructose. This led to accumulation of glucose during a period of 8 h in which the specific glucose consumption rate increased with time. The biomass concentration also increased markedly during the adaptation period. After 12 h, the glucose concentration was very low and the lactic acid concentration slowly decreased, since byproducts (acetic acid, formic acid, and ethanol) are formed under conditions with very low glucose concentrations (<7 mg/liter) (4, 5). Since the formation of byproducts (acetate) gives rise to the formation of more ATP than homofermentative metabolism does (10), the biomass concentration increased also after the excess glucose was depleted.

In a similar experiment, the medium was changed from glucose to fructose (Fig. 7). Thus, the feed to a glucose-limited culture at D = 0.10 h^{-1} was step changed to fructose. The biomass concentration started to decrease 1 h after the medium shift, and after about 20 h, the biomass concentration had decreased to about 35% of its initial value. Steady-state conditions for the biomass concentration and lactic acid production rate were reached after about 60 h following a series of damped oscillations. Since the acid production rate in the new steady state was almost the same as the production rate before the medium change, the specific rate of acid production had increased by a factor of 3.

No FDPase activity could be detected in extracts from *L. lactis* subsp. *cremoris* FD1 grown on fructose or glucose, whereas in accordance with a previous study (16), FDPase was found to be present in extracts of *L. lactis* subsp. *cremoris* Wg2 grown on fructose or glucose. The batch fermentation with fructose as the only sugar (Fig. 2) was also carried out with *L. lactis* subsp. *cremoris* Wg2, and indeed, the fermentation was completed within 16 h and with a high biomass yield of Y_{SX} = 0.14 g/g (data not shown).

DISCUSSION

In *L. lactis* subsp. *cremoris* FD1, the metabolism of fructose differs fundamentally from the metabolism of other sugars, e.g., glucose and lactose, even though all these

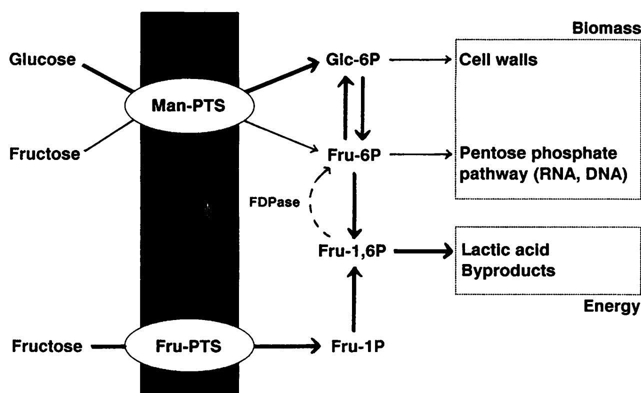

FIG. 8. Schematic illustration of the reactions involved in the uptake and metabolism of fructose for energy formation and biomass synthesis. The inducible fructose-PTS yields Fru-1P, and the only way that biomass precursors (Glc-6P or Fru-6P) can be formed is by fructose uptake via the mannose-PTS or by FDPase activity.

sugars are catabolized via the EMP pathway. When fructose is metabolized in the presence of glucose, the growth curve looks similar to that on pure glucose. However, when fructose is the only sugar, an unusual pattern of growth and product formation is observed: little biomass is synthesized, but the specific lactic acid production rate is high.

The inducible Fru-PTS in lactococci has not been described in the literature, but the results described here imply that, as in many other bacteria (8, 9, 11–14, 17, 18), it phosphorylates fructose at C-1. A schematic illustration of the two pathways for fructose metabolism (Fig. 8) shows that when fructose 6-phosphate is produced by uptake via the Man-PTS, both biomass precursors and metabolic energy can be formed. However, when fructose is transported and phosphorylated via the Fru-PTS, the resulting Fru-1P enters glycolysis as Fru-6P and would therefore have to be a gluconeogenic substrate. In the absence of FDPase, Fru-1P can be used only for generation of metabolic energy, and certain biomass precursors (Glc-6P or Fru-6P) cannot be formed from Fru-1P.

The presence of FDPase in lactococci was described as surprising (16), since lactococci catabolize and grow on a very limited number of hexoses. It is evident that this enzyme is important in the formation of essential biomass precursors during growth on fructose (even in a complex medium as used here). Also, if galactose is phosphorylated at C-6 during transport (by galactose PTS or lactose PTS [see, e.g., reference 20]), FDPase is required to produce biomass precursors from galactose. FDPase therefore catalyzes a pivotal step required for growth on certain sugars (fructose and galactose). It seems as if it is the strain used in the present work which is atypical since it lacks FDPase, but it remains to be shown whether this is also the case for other lactococci.

Since fructose-grown *L. lactis* subsp. *cremoris* FD1 is shown to be limited by the rate of formation of biomass precursors (the Glc-6P and Fru-6P pool), one can conclude that intracellular dephosphorylation of Fru-1P followed by phosphorylation at C-6 to give Fru-6P does not occur to an extent which can alleviate the insufficiency of Glc-6P/Fru-6P. This is consistent with Fru-1P being a very poor substrate of hexose-6P phosphohydrolase in lactococci (22).

Several of the fermentations clearly show that the oscillations during growth on fructose cannot be attributed only to a variable activity of the inducible Fru-PTS. As shown in the pulse experiments and in the substrate shift experiments, the Man-PTS is either inhibited or repressed. The substrate change experiment from fructose to glucose limitation (Fig. 6) shows a linear increase of the specific lactic acid production rate as a function of time during 7 h after the substrate shift. Inhibition of the Man-PTS can therefore be ruled out, and the low initial rate of catabolism of glucose is due to either repression of the Man-PTS (e.g., by Fru-1P) or inducible regulation of the Man-PTS (e.g., by Glc-6P or Fru-6P). The pulse experiments with glucose or lactose addition to fructose-limited chemostats show that the molar uptake rates are identical ($r_{Glc,max}$ = 0.46/180 = 2.56 mmol g^{-1} h^{-1} and $r_{Lac,max}$ = 0.87/342 = 2.54 mmol g^{-1} h^{-1}), and this suggests that it is a general PTS protein catalyzing the transfer of the phosphate group from phosphoenolpyruvate to the sugar-specific proteins (Fig. 1) which is repressed. In many bacteria, the Fru-PTS does not contain the general PTS proteins HPr and enzyme I (13–15); this might also be the case for lactococci, thus explaining the apparent repression of other PTS after the Fru-PTS has been induced.

The different K_{Fru} of the two transport systems combined with the interaction between these can explain the oscillations in a chemostat. When the fructose concentration is high, the Fru-PTS is synthesized, and fructose is also transported via the Man-PTS, since the concentration is much larger than the saturation constant. Prolonged exposure of the cells to a high fructose concentration (or in general to a high ratio of fructose to glucose in the medium) will repress transport of any of the sugars via the Man-PTS. When the fructose concentration decreases to the range of the saturation constant of the Man-PTS, transport via this system is further decreased. Hence, little biomass can be synthesized, and this causes incipient washout of the biomass. The low fructose concentration also reduces the synthesis of Fru-PTS, which again leads to a decrease of the repression of Man-PTS. As the flux of fructose via the Man-PTS increases, the rate of biomass synthesis increases and the biomass concentration in the broth increases. The next cycle in the oscillations then starts.

The metabolism of a mixture of fructose and glucose by *L. lactis* subsp. *cremoris* FD1 is very different from that observed in other bacteria. *Escherichia coli* preferentially metabolizes glucose from a mixture of glucose and fructose, but growth is not diauxic (7). In *Vibrio cholerae*, glucose inhibits the Fru-PTS, and growth in a mixture of glucose and fructose is diauxic (1).

In *L. lactis* subsp. *cremoris* FD1, glucose and fructose are metabolized in parallel and the Fru-PTS is induced from the start of the fermentations (otherwise glucose would competitively inhibit the fructose transport via the Man-PTS, since K_{Glc} is <11 μM and K_{Fru} is 890 μM) (5, 6). The specificities of the Man-PTS and Fru-PTS toward the four tautomers of fructose are likely to be different (4, 6), and at low fructose concentrations, the mutarotation of fructose may therefore also influence the fructose flux distribution through the Man-PTS and Fru-PTS.

The absence of FDPase in *L. lactis* subsp. *cremoris* FD1 is the reason for the extreme sensitivity of growth to the fructose flux distribution through the Man-PTS and Fru-PTS. This sensitivity makes it possible to perform an in vivo analysis of the function and regulation of both transport systems.

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