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Improved mannitol production by a random mutant of *Leuconostoc pseudomesenteroides*

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Abstract

A mutant of *Leuconostoc pseudomesenteroides* ATCC12291 that was unable to grow on fructose was constructed by chemical mutagenesis. The fructose uptake of this mutant, designated as BPT143, was unaltered and allowed fructose still to be converted into mannitol when glucose was present in the growth medium. The mutant grew and consumed fructose faster than the parent strain when grown in a medium containing both glucose and fructose. The specific activity of fructokinase, the enzyme involved in phosphorylation of fructose to fructose-6-phosphate, was decreased to about 10% of that of the parent strain, and resulted in a reduced leakage of fructose into the phosphoketolase (PK) pathway. The yield of mannitol from fructose was improved from 74 to 86 mol%. The increased fructose consumption rate and higher mannitol yield of the mutant also resulted in improvement of volumetric mannitol productivity. In addition, isolation and characterization of the wild type *L. pseudomesenteroides* fructokinase gene (*fruK*) was performed. DNA sequence analysis of the *fruK* gene region of BPT143 revealed only one silent mutation which does not explain the highly reduced fructokinase activity of the mutant. The genetic characterization of *fruK* was completed by analyzing the expression, size and 5′ end of *fruK* transcripts. Expression data with BPT143, revealing absence of *fruK* transcripts, was in accordance with the reduced fructokinase activity of the mutant.

Keywords: Lactic acid bacteria; Leuconostoc; Fructose metabolism; Fructokinase; Mannitol

Abbreviations: CDM, chemically defined medium; cdw, cell dry weight; DTT, dithiotreitol; EtOH, ethanol; FK, fructokinase; fru, fructose; glu, glucose; HAc, acetic acid; Hlac, lactic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; LAB, lactic acid bacteria; MDH, mannitol dehydrogenase; mtol, mannitol; PGI, phoshoglucose isomerase; PK, phoshoketolase pathway; PTS, sugar specific phosphoenolpyruvate sugar transferase system; SP, simplified production medium

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1. Introduction

Mannitol is a naturally occurring six-carbon sugar alcohol (polyol), which is widely used in the food, chemical and pharmaceutical industry, as well as in medicine (Soetaert et al., 1995). It has a sweet cool taste and it is only partially metabolized by humans and does not induce hyperglycemia, which makes it a suitable component of diabetic food products (Griffin and Lynch, 1972). Mannitol has also reduced physiological caloric value, which makes it ideal for low calorie (light) products. Mannitol and other sugar alcohols reduce the crystallization tendency of sugars, and for this reason they are also used for increasing the shelf-life of foodstuffs and cosmetic products. In medicine, mannitol is used as a powerful osmotic diuretic and in many types of surgery it is used for prevention of kidney failure and for reduction of dye and brain edema (Saha and Nakamura, 2002).

Mannitol is currently produced industrially by catalytic hydrogenation of glucose/fructose mixtures using Raney nickel as the catalyst (Makkee et al., 1985). The hydrogenation reaction of glucose/fructose (50:50) mixtures typically results in a 25:75 mixture of mannitol and sorbitol. This means that when fructose is catalytically hydrogenated, only about 50% of it is converted into mannitol; the rest is converted to sorbitol (Soetaert et al., 1999). In addition, all glucose present is reduced to sorbitol. Mannitol is also relatively difficult to separate from sorbitol and ultrapure raw materials are required for efficient conversion, which results in even higher production costs (Wisselink et al., 2001).

Microbial production of mannitol using food grade microorganisms may therefore be an interesting alternative to chemical processes. Microbes that are able to produce mannitol include many yeasts (Oinishi and Suzuki, 1970; Lee et al., 2003), filamentous fungi (Smiley et al., 1967) and lactic acid bacteria (LAB) (Soetaert, 1990; Aarnikunnas et al., 2002a, 2002b; Hahn et al., 2003; Saha, 2004). Certain LAB can convert fructose into mannitol with yields up to 100% when fructose and glucose are present in the medium in a ratio of 2:1 (Korakli et al., 2000). In heterofermentative LAB fructose is fermented via the phosphoketolase (PK) pathway to produce a mixture of CO_2, ethanol, acetate and lactate. A significant part of fructose is simultaneously reduced to mannitol by a NAD^+:mannitol dehydrogenase (MDH) (Dols et al., 1997). For some lactobacilli the growth rate on fructose is higher than on glucose, although fructose fermentation is less efficient than glucose fermentation in terms of ATP formed per sugar consumed. This indicates that under the conditions of excess substrate, priority is given to growth rate rather than efficiency of substrate utilization. The reduction of fructose to mannitol could play a role in this. The co-metabolism of fructose results in decreased ethanol production, because in this case the acetylphoshate is not reduced to ethanol in order to regenerate NAD^+. Instead, acetic acid is produced from acetyl-phosphate with the formation of ATP (Axelsson, 1998).

We have recently compared different mannitol producing LAB (von Weymarn et al., 2001) and optimized a production process using a membrane cell recycle bioreactor system (von Weymarn et al., 2002). However, a quantitative yield of mannitol from fructose was not achieved. Korakli et al. (2000) have reported that with *Lactobacillus sanfranciscensis* the yield of mannitol from fructose can reach 100%. These results suggest that this species either lacks alternative pathways for fructose catabolism or that these pathways are strongly repressed by glucose. However, it has been shown that *Lb. sanfranciscencis* is not an optimal strain for industrial production of mannitol due to its slow growth and fructose consumption rate (von Weymarn et al., 2001). To be channeled into the PK pathway, intracellular fructose is first phosphorylated into fructose-6-phosphate by fructokinase (FK) (EC 2.7.2.4) and then isomerized to glucose-6-phosphate by phosphoglucose isomerase (PGI) (EC 5.3.1.9). The hypothesis of the present work is that inactivation of a gene encoding for one of these enzymes would prevent the leakage of fructose into the PK pathway and give improved yield of mannitol from fructose.

In this study, we describe the construction and characterization of a random mutant of *Leuconostoc pseudomesenteroides* that is unable to grow on fructose and the positive effects of the mutation on mannitol production. Fructokinase gene and its putative promoter were also characterized in this study.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All *L. pseudomesenteroides* strains were maintained in standard MRS growth medium (Difco Laboratories, Detroit, MI) supplemented with 13% (w/v) glycerol and stored at −80 °C. Bioreactor cultivations and cultivation of cells for enzyme activity assays were performed using a simplified production (SP) medium (von Weymarn et al., 2001) containing 3% (w/v) of various sugars. The sugars were autoclaved separately as stock solutions. *Escherichia coli* XL1-Blue strain (Stratagene, La Jolla, CA) was used as the cloning host in the sequencing studies. It was grown in standard LB medium (Difco) at 37 °C using 100 μg ml^{-1} ampicillin for selection of the pGM-T Easy plasmid (Promega Corporation, Madison, WI).

2.2. Inactivation of fructokinase activity by random mutagenesis

Chemical mutagenesis of *L. pseudomesenteroides* ATCC12291 was performed using cells at the exponential growth phase (optical density (OD) at 600 nm = 1.0) grown in M17 growth medium (Difco) supplemented with 1% (w/v) glucose (GM17). The initial pH was 6.9. Cells were washed with 50 mM sodium phosphate buffer, pH 7.0 and treated with 1-methyl-3-nitro-1-nitrosoguanidine (0.5 mg ml^{-1}) for 40–50 min at room temperature. The washing procedure was repeated three times. Washed cells were incubated in GM17 (1 h and 30 °C), plated on GM17 agar and incubated for 2 days at 30 °C. Colonies on GM17 plates were replica-plated on a chemically defined medium (CDM) (Poolman and Konings, 1988) supplemented with either 1% glucose or 1% fructose. After 2 days of incubation at 30 °C colonies growing on glucose, but not on fructose, were selected.

2.3. Enzyme activity assays

Cell free extracts for measuring enzyme activities were prepared as follows. *L. pseudomesenteroides* cells, grown in SP medium containing 2% (w/v) fructose and 1% (w/v) glucose at 30 °C for 5 h, were harvested by centrifugation (5000 × g, 15 min), washed with 50 mM Tris–HCl, pH 7.0 and resuspended in 1/10 of culture volume of cold sonication buffer consisting of 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol and 1 tablet/20 ml Protease inhibitor (EDTA-free, Roche Diagnostics GmbH, Germany). Cells were disrupted by sonication, and the cell debris was removed by centrifugation (20,000 × *g*, 30 min). The supernatants were used in fructokinase and mannitol dehydrogenase assays.

Enzyme activities were assayed at 30 °C by measuring the change in absorbance at 340 nm. The reaction mixture for the fructokinase activity assays was a modification of the one used by Thomson et al. (1991) and contained 125 mM Tris–HCl, pH 7.5, 12.5 mM $MgCl_2$, 1.25 mM NAD^+, 1.25 mM ATP, 20 U ml^{-1} glucose-6-phosphate dehydrogenase (Roche), 20 U ml^{-1} glucose-6-phosphate isomerase (Roche) and 100 mM D-fructose. Mannitol dehydrogenase assays were performed as described by Aarnikunnas et al. (2002a). Protein concentrations were determined by the Bradford method using BIO-RAD protein assay reagent with bovine serum albumin as the protein standard (Bradford, 1976).

2.4. Isolation of chromosomal DNA

L. pseudomesenteroides chromosomal DNA was isolated as follows: cells were grown overnight in GM17 growth medium supplemented with 1% glycine, harvested by centrifugation (10,000 × *g*, 10 min) and resuspended in B1-buffer (Qiagen Ltd., UK) containing 2 mg ml^{-1} lysozyme (Sigma) and 12 U ml^{-1} mutanolysin (Sigma). The suspension was incubated at 37 °C for 40 min. Proteinase K (Qiagen) was added to a final concentration of 1 mg ml^{-1} and the solution was incubated for further 40 min. Chromosomal DNA was isolated with the Genomic-tip kit 100/G (Qiagen).

2.5. Cloning and sequencing a part of fruK and genome walking

The primers used in this study are listed in Table 1. Closely related bacterial fructokinase sequences from *Lactococcus lactis* sp. *lactis*, *L. lactis* sp. *cremoris*, *Streptococcus mutans*, *Pediococcus pentosaceus* and *Bacillus subtilis* were selected using the Blast search program on the NCBI server (http://www.ncbi.nlm.nih.gov/blast/). Conserved regions of these fructokinases were aligned using

Table 1
Oligonucleotides used in this study

Primer	Sequence (5′→3′)	Specific use
FRK-1F	GARGCTGGYGGWACAAAATTTGT	Degenerated primer for cloning of *fruK*
FRK-2R	HGGICCIGCNGCWAIICCTTC	Degenerated primer for cloning of *fruK*
LPFRK1F	TTGTTGTGGCGGTAGCGGAT	Sequencing of *fruK*
LPFRK2F	TGGCGAACAAACACTAAAACAAGTCA	Sequencing of *fruK* and primer for
LPFRK3F	TGGTATCGCAGCATTTGGTCC	Sequencing of *fruK*
LPFRK4F	GTGGTTGGTCAGGATACGACTTTTTAG	Sequencing of *fruK*
LPFRK5F	TTGAAACTGGTGCCGCTAAAGA	Sequencing of *fruK* and inverse primer for Vectorette II
LPFRK6F	GGTGGTCGTTTTGTCAGCGG	Sequencing of *fruK*
LPFRK7R	GGACCAAATGCTGCGATACCAATAG	Sequencing of *fruK*
LPFRK8R	TGCCCGTATGTTTTTGACCCCT	Sequencing of *fruK* and inverse primer for Vectorette II
LPFRK9R	ACCTAAAAAGTCGTATCCTGACCAACCAC	Sequencing of *fruK*
LPFRK10R	ACATCTTTAGCGGCACCAGTTTCAA	Sequencing of *fruK*
LPFRK11R	CCGCTGACAAAACGACCACC	Sequencing of *fruK*
LPFRK12R	TTATCGCCGTGGAATGGGTAATG	Sequencing of *fruK* and primer for *fruK*-specific probe for hybridisation
LPFRKsekvstart	TGCCAAACATCCTGTAAACGGTTA	Sequencing of *fruK*
LPFRKsekvend	GGTTGTTTGAGATAACTTGCTTTGTTGA	Sequencing of *fruK*
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing of pGM-T Easy insert
RP	TTTCACACAGGAAACAGCTATGAC	Sequencing of pGM-T Easy insert
1315	TCCTACGGGAGGCAGCAGT	Universal primer for *L. pseudomesenteroides* 16S RNA
1316	GGACTACCAGGGTATCTAATCCTGTT	Universal primer for *L. pseudomesenteroides* 16S RNA
LPFRKPROMSD	AAACACGAACCCGATGAGCTAACGC	Sequencing of *fruK*
LPFRKPROMSU	AACCGTTTACAGGATGTTTGGCAAT	Sequencing of *fruK*
LPFRKprom2D	AGTCAACTTAGCGGGTTGTTTGAGA	Sequencing of *fruK*
LPFRKprom2U	GTGTTGGAAATGATGTCCGCTCTAC	Sequencing of *fruK*
Cy5LPFRK	TGTTGGAAATGATGTCCGCTCTAC	Cy5-labelled oligonucleotide

ClustalW with default settings (http://www.npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl). Degenerated primers were designed according to the most conserved segments of the alignment as described by Bartl (1997). A 0.5 kbp fragment of the *L. pseudomesenteroides* fructokinase gene was amplified by PCR using the degenerated primer pair FRK-1F and FRK-2R (Table 1). The PCR fragment was subcloned into the cloning vector pGEM-T Easy. Blue-white screening for positive colonies was carried out with the final concentrations of 80 μg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (Promega) and 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside) (Sigma Chemicals, UK) on LB-agar plates. Plasmids were sequenced at the Institute of Biotechnology and Faculty of Veterinary Medicine (University of Helsinki, Finland).

The Vectorette II system (Sigma Genosys, Ltd., UK) was used for creating a genomic library of *L. pseudomesenteroides*. Chromosomal DNA of *L. pseudomesenteroides* was digested either with *Bam*HI, *Cla*I, *Eco*RI or *Hind*III (New England Biolabs, Beverly, MA), and ligated with the respective Vectorette units. Vectorette amplicons were amplified with PCR using sequence specific (Table 1) and Vectorette unit specific primers, subcloned into the cloning vector pGM-T Easy, and sequenced using the Sanger dideoxy method (Sanger et al., 1977). The set of sequencing primers is indicated in Table 1. Sequence analyses were performed with GCG Wisconsin package.

2.6. RNA isolation, northern hybridization and primer extensions

For isolation of total RNA, *L. pseudomesenteroides* cells were grown in SP medium at 37 °C to an OD_{600} of 1.2. Cells from 5 ml of culture were harvested by centrifugation (5000 × g, 10 min) and incubated at 37 °C for 30 min in SP medium containing 3% (w/v) of glucose. Cells were harvested by centrifugation (5000 × g, 10 min), washed with RNase free water and disrupted for 2 × 2.5 min with glass bead in a mill homogenizer (Edmund Bühler), followed by RNA isolation with the RNeasy® Mini Kit (Qiagen).

The RNA (5 μg) used for northern blotting was denaturated with 2 × Loading Dye Solution containing formamide (MBI Fermentas, Lithuania). Agarose gel electrophoresis was performed using the Reliant® gel system (1.25% agarose gel) according to the instructions by the manufacturer (Cambrex). The gel was blotted on a Zeta-probe membrane (Bio-Rad Laboratories, Hercules, CA) and hybridized with [α-^{33}P]dATP-labeled probe, generated by PCR amplification with the *fruK* specific primer pair LPFRK2F and LPFRK12 (Table 1), at 50 °C for 18 h. The filter was washed twice in 2 × SSC, 0.1% SDS and twice in 0.5 × SSC, 0.1% SDS at room temperature for 15 min. The membranes were visualized using a molecular imaging system (Model GS-525, Bio-Rad). Quantification of RNA on the membrane was performed by hybridization of 16S rRNA specific PCR probes. The 5′-end determination of *fruK* transcripts was performed at the Institute of Biotechnology by using 5 pmol Cy5-labelled oligonucleotide Cy5LPFRK and an ALF Express DNA Sequencer as described previously (Vesanto et al., 1995).

2.7. Bioreactor cultivations

For the first pre-culture, 10 ml of standard MRS growth medium (Difco Laboratories, Detroit, MI) containing 20 g l^{-1} fructose was inoculated from a frozen glycerol stock and incubated for 10 h at 30 °C without mixing. For the second pre-culture, 100 ml of MRS growth medium containing 20 g l^{-1} fructose was inoculated with 5% (v/v) of the broth from the first pre-culture and incubated at 30 °C without mixing until the cells reached late exponential growth phase. The initial pH of the media was 6.2.

The bioreactor cultivations were conducted in a 2 l glass-vessel bioreactor (Biostat MD, B. Braun Biotech International, Germany) equipped with three 6-blade Rushton turbines for mixing. The working volume was 1.5 l. The experiments were performed in SP medium at 30 °C and pH 5.0 (pH controlled with 3 M NaOH). The agitation rate was 200 rpm. The growth medium was sparged with pure nitrogen gas for 30 min prior to inoculation. Flushing of the culture with nitrogen was continued throughout the experiments at a rate of 0.2 l min^{-1}. The experiments were started by addition of the total second culture broth into the bioreactor. The experiments were performed in duplicate and samples were withdrawn hourly. The results are presented as averages and the errors as standard deviations.

The optical density of the culture broths was measured at 600 nm against distilled water. The samples were diluted in such a way that the OD values were in the range of 0.1–0.6. The cell dry weight (cdw) was measured as follows: a sample of the culture broth was pipetted into pre-weighed centrifuge tubes followed by centrifugation (6000 × *g*, 5 min). The cell pellet was washed with sterile saline (0.9% (w/v) NaCl), the centrifugation was repeated, after which the centrifuge tube was dried at 80 °C until a constant cdw was achieved. The correlation factor between the cell dry weights and optical densities was 0.5.

2.8. HPLC analyses

Concentrations of organic acids, sugars, ethanol and mannitol were determined by high-performance liquid chromatography (HPLC). Mannitol concentrations were analyzed using an Aminex HPX-87P column (Bio-Rad Laboratories, USA) at 70 °C with distilled water as the mobile phase. Glucose and fructose concentrations were measured using an Aminex HPX-87C column at 70 °C with distilled water as the mobile phase. Organic acids and ethanol concentrations were measured using Aminex HPX-87H ion exclusion column at 60 °C with 5 mM H_2SO_4 as the mobile phase. All components were analyzed with a refractive index (RI) detector. A Deashing Micro-Guard pre-column (Bio-Rad Laboratories, USA) was used in analysis of mannitol and the sugars, while a Cation H Micro-Guard pre-column (Bio-Rad Laboratories, USA) was used in analysis of organic acids and ethanol. The elution rate in all systems was 0.6 ml min^{-1}.

2.9. Calculations

The maximum specific growth rates (μ_{max}) were calculated as follows: A chart for the natural logarithm of cell dry weights versus time was plotted. The maximum specific growth rate was the steepest slope of a linear trendline (three to five successive values) in the exponential growth phase.

The following substrate and product abbreviations are used in the equations below: fru is fructose, glu is glucose, mtol is mannitol, HAc is acetic acid, HLac is lactic acid, EtOH is ethanol and X is biomass.

The efficiency of the fructose to mannitol reaction at different timepoints was characterized either by yield of mannitol from fructose (Y_{mtol}) or by conversion of fructose to mannitol (x_{mtol}), and calculated as presented below:

$$Y_{mtol} = (n_{mtol} - n_{mtol,t=0})/(n_{fru} - n_{fru,t=t}) \times 100\ \% \quad (1)$$

where n_{mtol} is mole of mannitol and n_{fru} is mole of fructose.

$$x_{mtol} = n_{mtol}/n_{fru} \quad (2)$$

The latter equation assumes that no mannitol was present at $t = 0$ h. If present, the amount was subtracted from the concentration of mannitol at $t > 0$ h.

The specific fructose consumption rates (q_{fru}) were calculated as presented below:

$$q_{fru} = \left(\frac{C_{t1} - C_{t2}}{X' \times t}\right) \quad (3)$$

where C is fructose concentration ($g\,l^{-1}$) and t is cultivation time (h).

The logarithmic mean of the biomass (X') was calculated as presented below:

$$X' = \left(\frac{X_{t2} - X_{t1}}{\ln X_{t2} - \ln X_{t1}}\right) \quad (4)$$

where X is the biomass concentration (grams of cell dry weight per liter) and t is time.

The carbon-balances were calculated on a C-molar basis as the ratio between the sum of the end products and consumption of sugars as described by Curic et al. (1999). The formation of carbon dioxide was calculated as the sum of the experimentally determined amounts of acetic acid and ethanol produced.

The elemental composition of the biomass, $C_{4.63}H_{7.89}O_{2.35}N_{1.0}$, was taken from Novak et al. (1997). The fraction of glucose-6-phosphate channeled into formation of biomass was calculated with the following equation:

$$n_X = \frac{6}{4.63}(n_{fru} - n_{mtol} + n_{glu} - n_{HAc} - n_{EtOH}) \quad (5)$$

where n_X is mol of biomass produced, n_{fru} is mol of fructose consumed, n_{mtol} is mol of mannitol produced and n_{glu} is mol of glucose consumed. The redox balances were as follows:

$$\frac{NAD}{NADH} = \left(\frac{3 \times n_{HAc} + 3 \times n_{EtOH}}{r_{mtol} + 2 \times n_{EtOH} + n_{HLac}}\right) \quad (6)$$

The NADH formation/consumption from biomass produced was assumed to be negligible and the equation considers only the NADH formed and consumed in the primary sugar metabolism of the cells.

2.10. Computer analysis

Sequence fragments were joined together and analyzed by the GCG Wisconsin Package. Secondary structure of mRNA was analyzed using the mFold web server (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) to predict transcription terminator sites (Zuker, 2003).

2.11. Nucleotide sequence accession number

Sequence data reported in this work have been deposited in the GenBank database under the accession number AJ431694.

3. Results

3.1. Isolation of L. pseudomesenteroides mutants unable to grow on fructose

To improve the efficiency of mannitol production in *L. pseudomesenteroides*, inactivation of its fructokinase activity was performed with random mutagenesis followed by screening of mutants unable to grow on fructose. Fructose-negative mutants were found by replica plating colonies from GM17 plates to CDM plates supplemented with either 1% glucose or 1% fructose and by screening for colonies growing on glucose, but not on fructose. Conversion of fructose to mannitol by these mutants was tested to ensure that the fructose permease was not affected by the mutagen. Three colonies out of 1000 screened were found to possess lowered fructokinase activity in the range of 10–60% of that of the wild type. The mutant with the lowest fructokinase activity (10% of that of the parent strain), named BPT143, was selected for further studies. There

were no significant difference in mannitol dehydrogenase activities between the mutant and the wild type. *L. pseudomesenteroides* BPT143 strain was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen with the accession number DSM14613.

3.2. Sequence analysis of the fruK gene

To characterize the fructokinase gene *fruK* from wild type *L. pseudomesenteroides* ATCC12291 sequence data of the corresponding genes from other bacteria were utilized. A fragment of the *L. pseudomesenteroides* fructokinase gene (*fruK*) was isolated by PCR amplification using degenerate oligonucleotides FRK1F and FRK2R (Table 1) which were designed according to the conserved regions of fructokinase genes from various LAB and *B. subtilis*. Sequence analysis of the 0.5 kbp PCR fragment revealed high similarity with other bacterial fructokinases. The unknown upstream (0.5 kbp) and downstream (0.8 kbp) regions of the *fruK* gene were isolated using the Vectorette II system and specific inverse primers (Table 1), followed by sequencing and sequence assembling of the three *fruK* fragments.

The coding sequence of the *L. pseudomesenteroides fruK* gene was found to be 862 bp in size and preceded by a putative-35 and -10 promoter region (TTG CCA-17 bp-TACAAT) starting 94 bp upstream of the translation initiation site (Fig. 1). A *cre*-like consensus sequence (TGWAARCGYTWNCW) (Inacio et al., 2000) was found in promoter region (Fig. 1). A putative ribosome binding (AGGAG) site was located six base pairs upstream of the translation initiation codon. A putative stem-loop transcription terminator with $\Delta G = -84$ kJ was located 290–325 bp downstream of the translation stop codon (Fig. 1). In addition, an upstream stem-loop terminator with $\Delta G = -84$ kJ was found 490–453 bp upstream of the translation initiation codon of *fruK* (data not shown). No additional open reading frames were found 450 bp upstream or 460 bp downstream of the *fruK* coding sequence by sequence analyses performed (data not shown). The sequence analyses further showed that the *fruK* gene region is flanked by approximately 350 bp long inverted repeats (IR) (Fig. 2).

The genomic DNA from the fructose-negative strain BPT143 was isolated and possible mutations in the *fruK* gene sequence were analyzed by sequencing the *fruK* gene region after PCR amplification. A silent point mutation in Thr104 was identified in the *fruK* sequence of the mutant. This was not, however, likely to be the reason for the reduced fructokinase activity. No mutations could be found in the 450 bp upstream region of *fruK*, suggesting that the putative *fruK* promoter sequence was not directly affected by the mutagen.

3.3. Analyses of L. pseudomesenteroides fruK transcripts

Northern analysis revealed that the *fruK* specific probe hybridized with the total RNA isolated from wild-type *L. pseudomesenteroides* (Figs. 3 and 4, lane 1), whereas *fruK* transcripts in the total RNA from the BPT143 mutant were below the detection level (Fig. 3, lane 2).

The size of the hybridized *fruK* transcript was determined to be approximately 1.3 kbp, which is in good agreement with the size of the putative *fruK* mRNA deduced from the gene sequence (Fig. 1). Determination of the 5′-end of the 1.3 kbp transcript, using primer extension with an *fruK*-specific oligonucleotide, located the transcription start site seven nucleotides downstream of the -10 region of the *fruK* promoter and 59 nucleotides upstream of the initiation codon (Fig. 1). According to these results and the transcription terminator analysis the actual size of the *fruK* transcript was verified to be 1257 bp.

3.4. Bioreactor cultivations

Comparison of some key properties of wild type *L. pseudomesenteroides* ATCC12291 and its fructokinase mutant BPT143 revealed that the mutant grew faster than the parent strain (Table 2). In addition, the fructose consumption rate was slightly increased in BPT143. More importantly, the specific fructokinase activity was significantly reduced resulting in an improved mannitol yield (from 74 to 86 mol%). A faster fructose consumption rate and an improved yield subsequently resulted in a slightly better volumetric mannitol productivity by the mutant (from 2.1 to 2.8 $g\,l^{-1}\,h^{-1}$).

Both the parent strain and the mutant consumed approximately the same amount of glucose in relation to fructose (Fig. 5). Due to a greater leakage of

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     -35                    -10         V
1    TTGCCAAACATCCTGTAAACGGTTACAATATATTTGTAAACAACTGATTAACGTTTATCATTAA 65
                      RBS                        fruK
60   CGCGATAAAATCGCAAAAAAGGAGTAACTCATGGGATTATTAGGCGCAATTGAAGCCGGTGGTA 130
                                  M  G  L  L  G  A  I  E  A  G  G  T
131  CAAAATTTGTTGTGGCGGTAGCGGATCACGACTATAACATTGTAGAGCGGACATCATTTCCAAC 195
       K  F  V  V  A  V  A  D  H  D  Y  N  I  V  E  R  T  S  F  P  T
196  ACTTGATGGCGAACAAACACTAAAACAAGTCATTGCTTTTTTTGACCAATTTGAAAATATTGAT 260
      L  D  G  E  Q  T  L  K  Q  V  I  A  F  F  D  Q  F  E  N  I  D
261  GCTATTGGTATCGCAGCATTTGGTCCAATTGATATTGTCGAGGGGTCAAAAACATACGGGCACG 325
     A  I  G  I  A  A  F  G  P  I  D  I  V  E  G  S  K  T  Y  G  H  V
326  TATTGGACACGCCAAAGCGTGGTTGGTCAGGATACGACTTTTTAGGTGCTATGAAGGCTTGGCG 390
       L  D  T  P  K  R  G  W  S  G  Y  D  F  L  G  A  M  K  A  W  R
391  TGATATTCCTTATTATTGGACAACAGATGTCAACGGTGCAGGTTGGGCAGAATTTGAAACTGGT 455
      D  I  P  Y  Y  W  T  T  D  V  N  G  A  G  W  A  E  F  E  T  G
456  GCCGCTAAAGATGTACAAAATATGGTTTATTTGACAGTTGGTACAGGCGTTGGTGCAGGAATTG 520
     A  A  K  D  V  Q  N  M  V  Y  L  T  V  G  T  G  V  G  A  G  I  V
521  TTTCCGGTGGTCGTTTTGTCAGCGGCTATGGACATCCTGAAGCAGGACACATTTTCTTGCAGAA 585
       S  G  G  R  F  V  S  G  Y  G  H  P  E  A  G  H  I  F  L  Q  K
586  GCATCCTAAAGACACTTACGCTGGTCATTGCCCATTCCACGGCGATAATTGTTTGGAAGGCTTA 650
      H  P  K  D  T  Y  A  G  H  C  P  F  H  G  D  N  C  L  E  G  L
651  GCCGCTGGTCCAGCAATTGAAGAACGCTGGCAAATGAGCGCTAAGGAACTACCAGACGATCATT 715
     A  A  G  P  A  I  E  E  R  W  Q  M  S  A  K  E  L  P  D  D  H  L
716  TAGCTTGGGAAATTGAAGCCTTTTATTTGGCGCAAGCAGCTTTGGACTATACAATGATTTTGCG 780
       A  W  E  I  E  A  F  Y  L  A  Q  A  A  L  D  Y  T  M  I  L  R
781  TCCAGAAAAGATTGTGTTTGGTGGTGGTGTGCCACATCGTGAAATCCTATTTCCACTGATTCGC 845
      P  E  K  I  V  F  G  G  G  V  P  H  R  E  I  L  F  P  L  I  R
846  GAGCAATTTGCAAAGTTGATGAGTGATTATCTGGCTGTTCCAGACTTGGATGATTATATTGTGC 910
     E  Q  F  A  K  L  M  S  D  Y  L  A  V  P  D  L  D  D  Y  I  V  P
911  CTGTTGCCAATGGTGACAATGCCGGGATTCTTGGTTGCTTCTATCTTGCCAAGACCCTAATTTA 975
       V  A  N  G  D  N  A  G  I  L  G  C  F  Y  L  A  K  T  L  I  *
976  ATTTTCAGTTTATCTCCGATTTATCTCGTTTTAATTTTCAATCGTTATATTATAAACATCAACA 1040

1041 AAGCAAGTTATCTCAAACAACCCGCTAAGTTGACTTAAAGTAGACATTGAATAAAAATTAGACT 1105

1106 TTCGGAGGGCTTGTCTCATAAGTGGTATAACTGATGACCATCACATCAGAAGCACGAAACCCAT 1170

1171 GGCAAAAAGGAGCCAAAGTCGCGTTCTCCCCGCGACTCTAAATAATTCATATTCTCTCCCGAAT 1235

1236 ATATTCTCTTCTCTCTCAAATATTCTCTCAAAAATATTTCCCGATGAACGTGTTAGTTCATCGG 1300
                                  --------  ---------->      <-------
1301 GTTTCGTGTTTTTATGAGTTGTAAATTTGTCTTGTTCATTGTTTTGAGAAAAATACAGGCGTAA 1366
     -    --------
1367 ATGTGAAAAAGTTCC 1381
```

Fig. 1. Northern hybridization of *L. pseudomesenteroides* ATCC12191 and its fructose-negative mutant BPT143. Total RNA isolated from wild-type *L. pseudomesenteroides* (lane 1) and fructose-negative strain BPT143 (lane 2) was hybridized with a 0.5 kbp *fruK* specific probe. Sizes of a RNA ladder are shown on the left hand side.

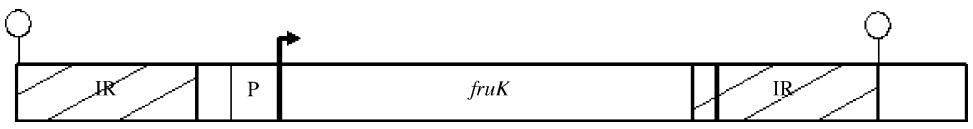

Fig. 2. Map of the *L. pseudomesenteroides* ATCC12291 *fruK* gene and its flanking areas. The coding sequence is marked with *fruK*, promoter area with P and 350 bp long inverted repeat sequences with IR. Putative transcription terminators are marked with hairpin loops.

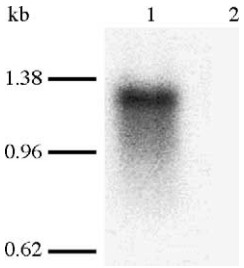

Fig. 3. Quantification of RNA on the membrane was performed by Northern hybridization. Total RNA isolated from wild-type *L. pseudomesenteroides* ATCC12191 (lane 1) and fructose-negative strain BPT143 (lane 2) was hybridized with a 16S rRNA specific probe.

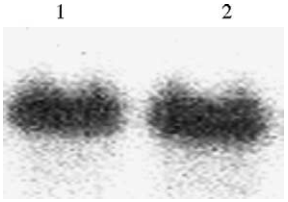

Fig. 4. The primary metabolism of *L. pseudomesenteroides* ATCC12291 and its fructokinase mutant (BPT143) at 30 °C and pH 5.0. The values represent yields on fructose (mol/mol × 100). Fructose, glucose, mannitol, lactic acid, acetic acid and ethanol were measured with HPLC, whilst the biomass and carbon dioxide were calculated as described in Appendix A. The respective values for the parent strain are shown in brackets.

fructose into the PK pathway, the parent cells produced clearly more carbon dioxide, lactic acid and ethanol than the mutant cells did. To balance the increased NADH oxidation due to increased mannitol production, the mutant cells produced less ethanol and more acetic acid. However, the yield of ATP per mol of fructose consumed was still approximately the same with both strains: 112 mol/mol for the parent strain and 110 mol/mol for the mutant. Hence, the yields of ATP correlated well with the final cell dry weights observed with both strains (Table 2).

4. Discussion

In this work, we aimed at improving mannitol production of *L. pseudomesenteroides* by constructing random mutants lacking fructokinase activity by chemical mutagenesis. Three mutants with reduced fructokinase activity were found. The best mutant, BPT143, showing fructokinase activity of only 10% of that of the wild type strain, was further analyzed for the *fruK* gene and *fruK* expression.

No detectable amounts of *fruK* transcripts were synthesized in the BPT143 mutant. However, no mutations

Table 2
Comparison of *L. pseudomesenteroides* ATCC12291 and its fructokinase mutant BPT143 grown in a medium containing both fructose and glucose at 30 °C and pH 5.0

	Parent	Mutant
Time to fructose depletion (h)	8.5	8
Final cell dry weight ($g\,l^{-1}$)	1.3 ± 0.0	1.2 ± 0.0
Maximum specific growth rate (h^{-1})	0.59 ± 0.04	0.69 ± 0.01
Volumetric fructose consumption rate ($g\,l^{-1}\,h^{-1}$)[a]	2.1 ± 0.3	2.8 ± 0.3
Specific fructose consumption rate ($g\,g\,cdw^{-1}\,h^{-1}$)[a]	6.0 ± 0.2	6.5 ± 0.1
Yield of mannitol from fructose (mol%)	73.7 ± 0.6	85.7 ± 0.4
Volumetric mannitol productivity ($g\,l^{-1}\,h^{-1}$)	1.6 ± 0.0	2.0 ± 0.0
Specific fructokinase activity ($U\,mg^{-1}$)	0.49 ± 0.11	0.06 ± 0.00
Specific mannitol dehydrogenase activity ($U\,mg^{-1}$)	0.058 ± 0.003	0.067 ± 0.001
Mannitol (C-mol C-mol^{-1} sugar)	0.465 ± 0.002	0.537 ± 0.001
Lactic acid (C-mol C-mol^{-1} sugar)	0.256 ± 0.004	0.218 ± 0.003
Acetic acid (C-mol C-mol^{-1} sugar)	0.071 ± 0.001	0.095 ± 0.002
Ethanol (C-mol C-mol^{-1} sugar)	0.103 ± 0.001	0.054 ± 0.002
Carbon dioxide (C-mol C-mol^{-1} sugar)	0.087 ± 0.000	0.074 ± 0.000
Biomass (C-mol C-mol^{-1} sugar)	0.015 ± 0.004	0.023 ± 0.001
Carbon balance	0.998 ± 0.003	1.000 ± 0.003
NAD/NADH balance	0.98 ± 0.00	1.03 ± 0.00

[a] Between $t = 2$ and 6 h. The specific consumption rates were calculated using a logarithmic mean of the biomass.

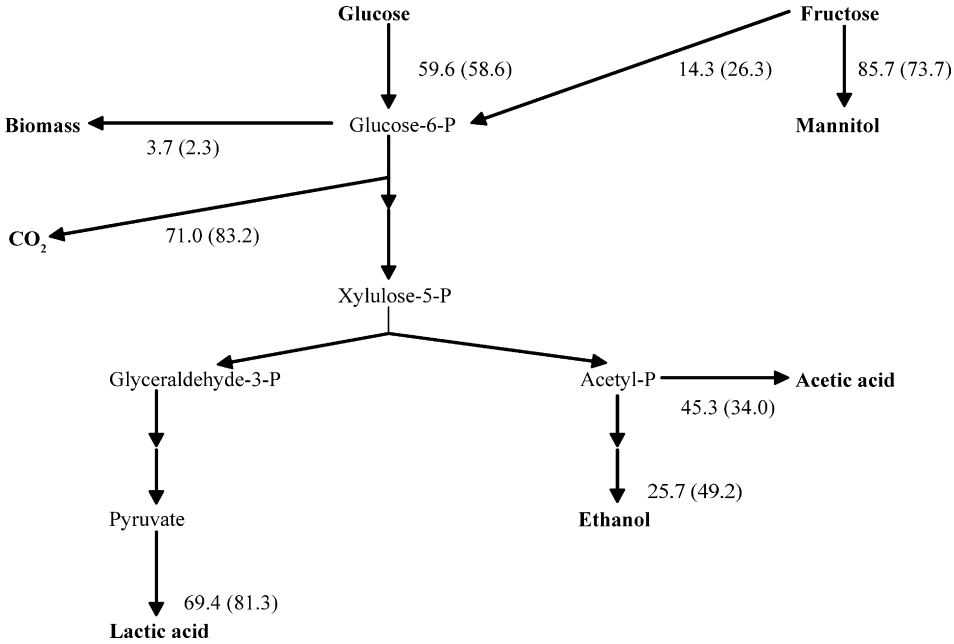

Fig. 5. Structure of the *L. pseudomesenteroides* ATCC12291 *fruK* gene and its putative expression signals. The -35 and -10 region of the putative promoter is underlined. RBS refers to the putative ribosome binding site. Transcription start site is marked with a vertical arrow head. The transcription terminator is marked with underline arrows. A *cre*-like consensus sequence is highlighted in grey.

directly affecting the expression of *fruK* could be found in the *fruK* gene sequence or in its upstream region of 450 bp. It would therefore seem likely that some more distant sequences involved in the regulation of *fruK* transcription were mutated. For example, a regulatory protein may have been affected by the mutagen. In an activator based system, inactivation of this protein or its synthesis would have directly resulted in the phenotype observed. On the other hand, if the *fruK* gene is controlled by a repressor, e.g. either a constitutive repressor mutation or a non-inducible repressor mutation, could then result in the phenotype found. It is neither possible to completely rule out DNA conformation rearrangement effects as the cause of the change in the BPT143 phenotype.

In homofermentative LAB, uptake of fructose and other sugars is generally carried out using the sugar specific phosphoenolpyruvate sugar transferase system (PTS), with phosphorylation of the target sugar during the translocation across the cell membrane. However, heterofermentative LAB are assumed to generally use non-phosphorylating sugar permease systems for sugar uptake followed by intracellular phosphorylation by kinases to channel hexoses to the PK pathway and energy metabolism. Indeed, this seems to be the case also with heterofermentative *Lactobacillus fermentum* NRRL-B-1932 (Helanto, unpublished results). However, sugar uptake via PTS systems has been reported in some heterofermentative strains. From heterofermentative *L. brevis* and *L. buchneri* strains Reizer et al. (1988) found a protein similar to Hpr (heat stable protein), a part of PTS system, but no enzyme I, concluding that a complete PTS could not operate in these organisms. Nagasaki et al. (1992) found complete PTS systems in two *L. fermentum* strains, a sucrose-PTS in one strain and sucrose- and mannose-PTSs in the other. In addition, Saier et al. (1996) have described a complete fructose-PTS functioning under anaerobic conditions in *L. brevis*.

The PTS dependent fructokinases are generally defined as type I (fructokinases). In well characterized PTS these type I fructokinases are also genetically linked to other components of PTS (Schmid et al., 1988; Thomson et al., 1991; Luesink et al., 1999; Reid et al., 1999; Tangney and Mitchell, 2000). Instead the *fruK* genes from *L. pseudomesenteroides* and *L. fermentum*

appear not to be linked to any components of the putative PTS in these strains. The *fruK* gene of *L. fermentum* was recently found to be located in an operon structure with a phosphoglucose isomerase *fruI* gene and regulated by FruR repressor protein (Helanto, unpublished results). Our results with the *L. pseudomesenteroides fruK* gene, showing that *fruK* is monocistronic and flanked by approximately 350 bp long inverted repeats, suggests instead, that the ability of *L. pseudomesenteroides* to use unphosporylated intracellular fructose has been evolved via lateral Tn-transposition during evolution.

The effect of the random mutation on mannitol production was also studied in bioreactors under process conditions. The mutant strain grew and consumed sugars faster than the parent strain. Less side products (acids, carbon dioxide and ethanol) were synthesized by the mutant cells and an increased fraction of fructose was reduced to mannitol by mannitol dehydrogenase. This was elucidated by an increased yield of mannitol from fructose (from 74 to 86 mol%). These results can be explained by the decreased channeling of fructose into the PK pathway that is caused by the decreased fructokinase activity. The mutation also resulted in a somewhat improved volumetric mannitol productivity (from 2.1 to 2.8 $g\,l^{-1}\,h^{-1}$). This was expected because both the mannitol yield and the fructose consumption rate were improved in the mutant.

Despite the 90% decrease in fructokinase activity a 100% yield of mannitol from fructose was not achieved. A possible explanation for this is that the fructose phoshorylation pathway is simply more efficient than the fructose reduction reaction which makes it difficult to direct the flux to mannitol. Nevertheless, our results indicate that the yield can be improved by modifying the fructokinase. It is possible that a quantitative yield would be achieved, if the fructokinase activity was completely disrupted and the mutant strain did not have PTS transporter for fructose. However, the genetic engineering tools available did not allow the use of directed inactivation techniques on *L. pseudomesenteroides*. Further studies are planned with a more suitable species as the target, namely *Lactobacillus fermentum*.

It has been reported for some native LAB strains that only negligible amounts of fructose leak into the PK pathway when grown in a 2:1 mixture of fructose and glucose (e.g. Korakli et al., 2000; Saha and Nakamura, 2002). This is usually achieved at low pH. However, acidic process conditions result in decreased cell metabolism, which subsequently cause decreased volumetric mannitol productivity. A fructokinase-negative mutant could enable higher pH to be used in the production process without lowering the yield. With such a mutant both yield and productivity could be maximized.

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