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High-Level Production of the Low-Calorie Sugar Sorbitol by *Lactobacillus plantarum* through Metabolic Engineering▽

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Sorbitol is a low-calorie sugar alcohol that is largely used as an ingredient in the food industry, based on its sweetness and its high solubility. Here, we investigated the capacity of *Lactobacillus plantarum*, a lactic acid bacterium found in many fermented food products and in the gastrointestinal tract of mammals, to produce sorbitol from fructose-6-phosphate by reverting the sorbitol catabolic pathway in a mutant strain deficient for both L- and D-lactate dehydrogenase activities. The two sorbitol-6-phosphate dehydrogenase (Stl6PDH) genes (*srlD1* and *srlD2*) identified in the genome sequence were constitutively expressed at a high level in this mutant strain. Both Stl6PDH enzymes were shown to be active, and high specific activity could be detected in the overexpressing strains. Using resting cells under pH control with glucose as a substrate, both Stl6PDHs were capable of rerouting the glycolytic flux from fructose-6-phosphate toward sorbitol production with a remarkably high efficiency (61 to 65% glucose conversion), which is close to the maximal theoretical value of 67%. Mannitol production was also detected, albeit at a lower level than the control strain (9 to 13% glucose conversion), indicating competition for fructose-6-phosphate rerouting by natively expressed mannitol-1-phosphate dehydrogenase. By analogy, low levels of this enzyme were detected in both the wild-type and the lactate dehydrogenase-deficient strain backgrounds. After optimization, 25% of sugar conversion into sorbitol was achieved with cells grown under pH control. The role of intracellular NADH pools in the determination of the maximal sorbitol production is discussed.

Obesity is a growing problem in Western countries. Therefore, special diets and dietary ingredients for body weight control are of major interest to the food industry. Belonging to the family of low-calorie sugars, polyols such as mannitol and sorbitol are nonmetabolized sugar alcohols that can replace sucrose or lactose in food products, with a nearly equivalent sweetness and taste (28). Moreover, these compounds have a stabilizing effect on food by partially mimicking fat (7). The range of potential applications of polyols goes far beyond their use as low-calorie sweeteners or texturing agents. For instance, they have been shown to display an in vivo anticariogenic effect since they are not fermented by *Streptococcus mutans*, the most potent cariogenic bacterium (15). Taking into consideration health benefits and industrial applications, the development of novel dairy products naturally enriched in polyols during fermentation processes offers interesting perspectives (16).

In the context of polyol production, *Lactobacillus plantarum* possesses some relevant characteristics. It is a food-grade microorganism belonging to the group of lactic acid bacteria. *L. plantarum* is a normal member of the human intestinal microbiota and can also be isolated from the oral cavity (27, 32). It is largely found as the dominant species in the last step of natural food raw-material fermentation, including a variety of vegetables, meat, and milk (6, 14). Its sugar metabolism is dedicated to lactic acid production. The construction of a mutant strain deficient in both lactate dehydrogenases (L- and D-LDH) revealed interesting features for polyol production by metabolic engineering (10). The sugar metabolism of this genetically engineered strain was previously examined using resting cells (10) and a range of fermentation products, such as acetate, succinate, ethanol, acetoin, and 2,3-butanediol, were identified in various fermentation conditions (Fig. 1A). Notably, low concentrations of mannitol were detected in all conditions tested. The most probable metabolic pathway for mannitol production is the reversion of mannitol catabolism via the mannitol-1-phosphate (mannitol-1P) dehydrogenase, an enzyme activity that has been detected in *L. plantarum* (4, 10) (Fig. 1A). Analogously, mannitol production was also reported for *Lactococcus lactis* strains deficient in L-lactate dehydrogenase activity (24, 25). Metabolic engineering strategies aiming to enhance mannitol production by the overproduction of mannitol-1P dehydrogenase have been explored in multiple organisms. Production of this polyol was achieved in bacteria (11, 35–37), higher plants (34), and *Saccharomyces cerevisiae* (5). These observations underline the potential for the produc-

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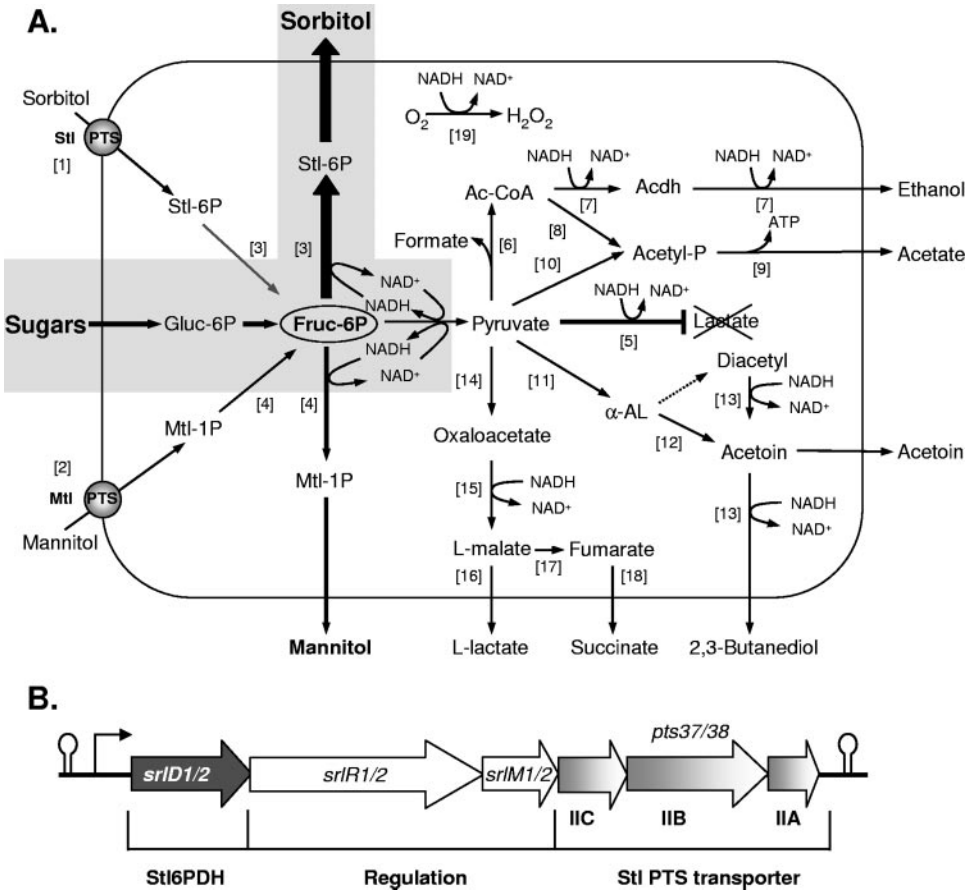

FIG. 1. Metabolic engineering strategy to activate sorbitol production and structure of the putative sorbitol operons in *L. plantarum*. (A) Sorbitol and mannitol degradation pathways for the LDH-deficient strain of *L. plantarum* (VL103) and scheme (gray area) of the metabolic engineering strategy used to activate sorbitol production (see the text for a detailed explanation). 1, sorbitol phosphate transport system (PTS); 2, mannitol PTS; 3, Stl6P dehydrogenase; 4, Mtl1P dehydrogenase; 5, lactate dehydrogenase; 6, pyruvate-formate lyase; 7, acetaldehyde (Acdh)/alcohol dehydrogenase; 8, phosphotransacetylase; 9, acetate kinase; 10, pyruvate oxidase; 11, α-acetolactate (α-AL) synthase; 12, α-AL decarboxylase; 13, diacetyl/acetoin reductase; 14, pyruvate carboxylase; 15, malate dehydrogenase; 16, malolactic enzyme; 17, fumarase; 18, fumarate reductase; 19, NADH oxidase. (B) Genetic organization scheme of the two sorbitol (*srl*) operons in *Lactobacillus plantarum* NCIMB8826. *srlD*, sorbitol-6P dehydrogenase; *srlR*, transcriptional repressor; *srlM*, transcriptional activator; *pts*, sorbitol phosphotransferase transport system (PTS subunits IIA, IIB, and IIC). Numbers accompanying gene names refer either to the first (1 and 37) or the second (2 and 38) *srl* operon. Putative promoters (arrow segments) and transcriptional terminators (hairpins) are also indicated.

tion of other polyols through engineering of these organisms (16, 18).

Sorbitol, also referred to as D-glucitol, is naturally found in many fruits (e.g., berries, cherries, and apples) (3). The worldwide production of sorbitol is estimated to be higher than 500,000 tons/year, and the market is continuously increasing (30). This polyol has a relative sweetness of around 60% compared to that of sucrose and displays a 20-fold higher solubility in water than mannitol (8, 30). Based on these properties, sorbitol is widely used in a range of food products such as confectionery, chewing gum, candy, desserts, ice cream, and diabetic foods. In these products, it fulfills a role not only as a sweetener but also as a humectant, a texturizer, and a softener (8, 30). In addition, sorbitol is the starting material for the production of pharmaceutical compounds such as sorbose and ascorbic acid (3). Several industrial processes have been described for the production of sorbitol (8). However, only a few microorganisms have been suggested as potential sorbitol producers, including three yeast strains and the ethanol-producing bacterium *Zymomonas mobilis* (19, 30, 31).

Here we describe a metabolic engineering approach to achieving high-level sorbitol production from *L. plantarum* by reversing the catabolic pathway for sorbitol utilization. Two operons potentially involved in sorbitol catabolism were identified in the genome of *L. plantarum* (20). The corresponding sorbitol-6-phosphate (sorbitol-6P) dehydrogenase genes were expressed at a high level, and sorbitol production was evaluated using both resting and growing cells. Analysis of the impact of culture conditions on sorbitol production, such as the carbon source, pH, and aeration, enabled optimization of production, which reached a maximum of 65% of sugar rerouting with resting cells, while a level of 25% was achieved with growing cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* TG1 was grown in Luria broth with aeration at 37°C (29). *L. plantarum* NCIMB8826 (National

Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland) and its derivatives were routinely grown in MRS medium (Becton Dickinson, Cockeysville, MD) at 30 or 37°C. Batch fermentation experiments under controlled conditions were performed in modified MRS broth (1% tryptone, 0.8% beef extract, 0.4% yeast extract, 0.2% K_2HPO_4, 0.1% Tween 80, 0.041% $MgSO_4 \cdot 7H_2O$, 0.0066% $MnCl_2$, 0.2% ammonium citrate, 0.5% sodium acetate) supplemented with 2% (wt/vol) of various sugars (glucose, fructose, maltose, sucrose). In several instances (mentioned in the text), fermentations were performed in modified MRS that lacked acetate (MRS − Ac) to prevent its metabolic conversion to ethanol. Erythromycin was used at the following concentrations: 250 μg/ml for *E. coli* and 10 μg/ml for *L. plantarum*.

DNA techniques and transformation. DNA manipulations were performed according to standard procedures (29) and instructions from manufacturers. *L. plantarum* was electroporated as reported previously (1).

Plasmid and strain constructions. The *srlD* genes of *L. plantarum* NCIMB8826 were amplified by PCR using the following primer pairs: Stl1Nsi (5′-AGTATG CATACAGATTGGTTGGG-3′) and Stl1Xba (5′-ACGTCTAGATTGATTAT TCAACTACCTC-3′) for *srlD1*, and Stl2Nsi (5′-TTGATGCATAATTCATGG ATTAATATTTCG-3′) and Stl2Xba (5′-TTGTCTAGACATTGCCTCACCAT GC-3′) for *srlD2*, containing NsiI and XbaI restriction sites (underlined). The NsiI/XbaI-digested PCR products were cloned into plasmid pGIZ906 (12), digested with the same enzymes, yielding plasmids pGIVL201 (*srlD1*) and pGIVL202 (*srlD2*). In both constructs, the *srlD* open reading frame was translationally fused with the expression signals of the *L. plantarum ldhL* gene. The absence of mutations in the translational fusions was confirmed by DNA sequencing. Both plasmids were transformed in *L. plantarum* strains NCIMB8826 (wild type) and VL103(Δ*ldhL* Δ*ldhD*). The VL103 strain is a derivative of TF103 (Δ*ldhL ldhD*::*cat*) (9), obtained by removing the chloramphenicol resistance marker from its genome (V. Ladero, unpublished data).

Enzymatic assays. Cells were grown in MRS broth until mid-exponential phase (an optical density at 600 nm [OD_{600}] of 2.0), harvested by centrifugation, and mechanically broken with glass beads, as previously described (13). Sorbitol-6P oxidation by sorbitol-6P dehydrogenase (Stl6PDH) was determined with sorbitol-6P as a substrate, as reported by Yebra et al. (39). Mannitol-1P dehydrogenase (Mtl1PDH) activity was assayed with mannitol-1P as a substrate, as described by Wisselink et al. (35). Mtl1PDH and Stl6PDH activities were determined from the rate of NADH formation by measuring the absorbance at 340 nm. One unit corresponds to 1 nmol of NAD^+ reduced min^{-1} mg total $protein^{-1}$. Total protein concentration in the crude cell extracts was measured using the Bradford method (2).

Small-scale cell suspensions without pH control. Cells were grown in MRS medium under microaerobic conditions (static cultures) until mid-exponential phase (OD_{600}, 2.0), harvested by centrifugation, washed twice with either potassium phosphate buffer (50 mM) or Tris-maleate buffer (50 mM), and resuspended in 1/10 the initial culture volume of the washing buffer supplemented with 50 mM sugar (glucose, fructose, or an equimolar mixture of both) at an initial pH ranging from 5.0 to 8.0. After 2 hours of fermentation, culture supernatants were collected and analyzed either by high-performance liquid chromatography (HPLC) or by ^{13}C nuclear magnetic resonance (NMR). For ^{13}C NMR analyses, the fermentation buffer was supplemented with 30 mM [1-^{13}C]glucose.

Large-scale cell suspensions under pH control for in vivo NMR. Cells were collected at the mid-exponential growth phase, harvested, washed, and resuspended to a protein concentration of approximately 10 mg ml^{-1} in potassium phosphate buffer (pH 6.5) as described for small-scale suspensions (see above). In vivo NMR experiments were performed under controlled pH (6.5) and gas atmosphere (argon), using the experimental system described previously (23). Twenty or 30 millimolar of [1-^{13}C]glucose was supplied to the cell suspension, and the time course for its consumption and product formation was monitored in vivo. After substrate exhaustion and when no changes in the resonances due to end products were observed, an NMR total extract was prepared as reported previously (24). End products of glucose catabolism were quantified in the NMR total extract by ^1H and ^{13}C NMR assays.

Fermentation with growing cells. Fermentation experiments were carried out in a 1-liter batch reactor controlled by Bioprocessor ADI 1020 (Applikon Biotechnology, Schiedam, The Netherlands) software. Fermentation data were processed using BioXpert NT software (version 2.60.113; Applikon Biotechnology). In all experiments, a culture volume of 500 ml (of modified MRS broth) was used. During fermentation, the pH was controlled with 2 M NaOH, and the culture was stirred at 120 rpm. During the course of the fermentation, samples were collected for sugar and organic acid analyses by HPLC.

NMR spectroscopy. ^{13}C spectra were acquired at 125.77 MHz on a Bruker DRX500 spectrometer. All in vivo experiments were run using a quadruple-nucleus probe head as described previously (23). For the quantitative analysis of end products in the NMR total extracts by ^{13}C NMR, a repetition delay of 60.5 s was used. Carbon chemical shifts were referenced to the resonances of external methanol, designated at 49.3 ppm. ^1H NMR analysis of the fermentation products in total extracts was performed with a Bruker AMX300 spectrometer, using formate as a concentration standard as described by Neves et al. (23).

HPLC analyses. Organic acids were analyzed by HPLC as previously reported (33). Sugars were analyzed by HPLC using a chromatographic system consisting of a precolumn packed with a cation exchange resin, AG50W-X4, 400 mesh (Bio-Rad, Hercules, CA) and AG3-X4A, 200/400 mesh (in a proportion of 35:65; Bio-Rad), and a cation exchanger in a prepacked column (RT 300-7.8 Polyspher CHPb, 300 by 7.8 mm; Merck, Darmstadt, Germany). The samples were eluted with an isocratic pump system (Shimadzu Corporation, Kyoto, Japan) using water as the mobile phase. Detection was carried out using a refractive index detector, ERC-7512 (Erma).

RESULTS

Cloning and overexpression of the sorbitol dehydrogenase genes. Glycolytic conversion of the available carbon source by the *L. plantarum* mutant strain deficient for both L-LDH and D-LDH generates an excess of NADH that is dissipated by the activation of metabolic routes capable of NADH oxidation in order to maintain an equilibrated redox balance (Fig. 1A) (10). Although many activated metabolic routes use pyruvate as the initial substrate, production of mannitol from fructose-6-phosphate (fructose-6P) opens the possibility of producing other related compounds from this glycolytic intermediate. One such possibility is the overproduction of sorbitol-6P dehydrogenase, with the aim of deviating the glycolytic flux from fructose-6P toward the end product sorbitol, while at the same time providing an additional NADH sink (Fig. 1A).

The genome of *L. plantarum* WCFS1 (a single-colony isolate of strain NCIMB8826) contains two putative operons that could be involved in sorbitol catabolism (20). A sequence comparison of the two operons revealed a 65% identity at both the DNA and the protein levels. Both sorbitol operons have highly similar genetic organizations. The first gene (*srlD*) encodes sorbitol-6P dehydrogenase, followed by two regulatory genes (*srlR* and *srlM*) that encode a putative repressor and activator, respectively, and the components of a complete phosphotransferase sugar uptake system (*pts37* and *pts38*, components IIA, IIB, and IIC, encoded by separate genes). Both *srl* operons are preceded by a putative promoter sequence and are enclosed by predicted transcription termination sequences (Fig. 1B).

In order to evaluate their function as specific sorbitol-6P dehydrogenases, the two *srlD* coding regions were constitutively overexpressed by translational fusion to the strong expression signals of the *L. plantarum ldhL* gene (plasmids pGIVL201 and pGIVL202, containing *srlD1* and *srlD2*, respectively). The recombinant plasmids were introduced into *L. plantarum* NCIBM8826 (wild type) and its LDH-deficient derivative, VL103. Stl6PDH overproduction was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein gel showed an additional band with the expected molecular mass of 29 kDa in crude cell extracts of VL103 (pGIVL201) and VL103(pGIVL202) that was absent from crude extracts of the control strain containing the empty overexpression vector [VL103(pGIZ906)] (data not shown). In order to confirm Stl6DH overproduction, Stl6PDH-specific activity was measured in crude extracts of the recombinant strains. High Stl6PDH-specific activity levels were detected in the overexpressing strains VL103(pGIVL201) (250.6 U/mg to-

TABLE 1. Production of sorbitol and mannitol from glucose by small-scale cell suspensions[a]

Strain	Glucose consumed (mM)	Polyol production[b]			Polyol activity[c]	
		Sorbitol (mM)	Mannitol (mM)	% of sorbitol	Stl6PDH activity (U mg^{-1})	Mtl1PDH activity (U mg^{-1})
NCIMB8826(pGIZ906) (wild type)	20	ND	ND		ND	135.0 ± 3.0
VL103(pGIZ906) (Δ*ldhL* Δ*ldhD*)	18.5	ND	7.9 (42.3)		ND	157.0 ± 3.0
VL103(pGIVL201) (Δ*ldhL* Δ*ldhD* P_{ldhL}::*srlD1*)	19.6	5.2 (26.7)	2.0 (10.1)	72	250.6 ± 15.9	145.0 ± 5.0
VL103(pGIVL202) (Δ*ldhL* Δ*ldhD* P_{ldhL}::*srlD2*)	37.6	5.5 (14.5)	2.0 (5.4)	73	459.0 ± 31.7	119.0 ± 15.0

[a] Sorbitol and mannitol were produced from glucose by small-scale cell suspensions of *L. plantarum* strains NCIMB8826(pGIZ906), VL103(pGIZ906), VL103(pGIVL201), and VL103(pGIVL202). Samples were collected after 2 h of incubation at 37°C under low aeration and without pH control (initial glucose concentration, 50 mM; initial pH, 5.5).

[b] Data are representative of at least two experiments. Values in parentheses are percentages of glucose converted to the product. The percentage of sorbitol is from total polyol. ND, not detected.

[c] Stl6PDH and Mtl1PDH activities were measured from crude extracts at the start of the experiment. These data represent average values from three individual measurements with their SD.

tal proteins) and VL103(pGIVL202) (457.0 U/mg), while no activity could be detected in the wild-type and VL103 strains harboring the empty vector (Table 1). Additionally, significant Mtl1PDH activity could be measured in all four strains (between 119.0 and 157.0 U/mg) (Table 1).

Sorbitol production using cell suspensions without pH control. In order to evaluate sorbitol production, small-scale cell suspensions (20 ml) of the wild-type and LDH-deficient strains carrying the expression vector with each of the *srlD* genes or the empty vector were incubated with a fermentable substrate. Cells were prepared and collected as described in Materials and Methods and resuspended in buffer containing 50 mM glucose at an initial pH value ranging from 5.5 to 8.0. After 2 hours of fermentation, the presence of sorbitol in the supernatants of cell suspensions was measured by HPLC. Sorbitol production was detected in all cell suspensions of the LDH-deficient strains overexpressing the *srlD* genes [VL103 (pGIVL201) and VL103(pGIVL202)], while no sorbitol production could be detected in suspensions of the corresponding control strain [VL103(pGIZ906)] or any of the wild-type strain derivatives with or without the *srlD* overexpression plasmid (Table 1 and data not shown). As an example, results from cell suspensions performed with an initial pH value of 5.5 are shown in Table 1. In this experiment, sorbitol production from VL103(pGIVL201) and VL103(pGIVL202) reached 27 and 14.5% of glucose rerouting, respectively. The presence of mannitol was detected in both fermentations, but mannitol production was strongly reduced in comparison to that for the VL103(pGIZ906) control strain (Table 1). This reduction in mannitol production is indicative of competition between the Mtl1PDH and Stl6PDH enzymes for their common substrate fructose-6P (Fig. 1A). However, this competition largely favors sorbitol production, which accounts for 70% of the total polyol detected in the *srlD*-overexpressing strains. Globally, modifications of parameters such as the initial pH and the carbon source (glucose, fructose, and an equimolar mixture of both) showed that sorbitol production by cell suspensions of VL103(pGIVL201) and VL103(pGIVL202) varied from 9 to 47% of sugar rerouting and that the relative fraction of sorbitol over total polyols produced ranged from 50 to 98% (data not shown).

During this preliminary evaluation, aeration had a strong negative impact on the production of sorbitol and mannitol. Analysis of the fermentation products by ^{13}C NMR using 30 mM of [1-^{13}C]glucose as the carbon source revealed that aeration resulted in a complete absence of the production of sorbitol and mannitol in suspensions of the three LDH-deficient strains, which is illustrated for strain VL103(pGIVL201) in Fig. 2. A low amount of lactate was detected in aerated cell suspensions as reported previously (10). Since no LDH activity was present in the double-LDH-deficient strain, the most probable pathway for lactate production could be oxaloacetate reduction to L-malate, followed by malolactic conversion to L-lactate, as previously suggested (Fig. 1A) (10).

Analysis of sorbitol production by in vivo NMR with cell suspensions under pH control. In order to gain better insights into the kinetics of sugar consumption and formation of fermentation end products, a range of fermentations with large-

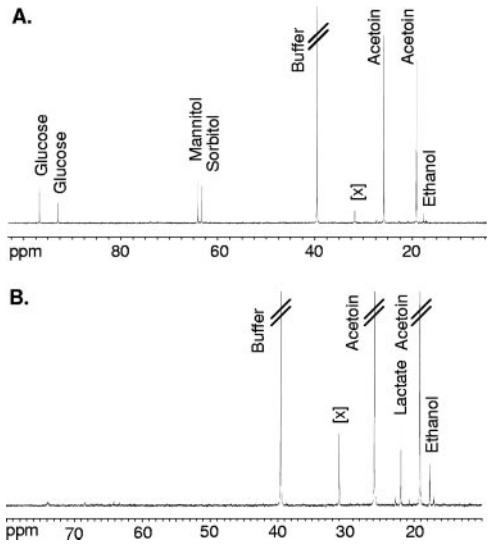

FIG. 2. ^{13}C NMR spectra of fermentation products from supernatants of small-scale cell suspensions (Tris-maleate buffer; 50 mM; initial pH, 5.5) of the VL103(pGIVL201) strain performed in the presence of 30 mM [1-^{13}C]glucose under low (A) and high (B) aeration. Samples were analyzed after 2 hours of fermentation at 37°C. Spectra are presented from 10 to 95 ppm. x denotes an unidentified compound.

TABLE 2. End-product amounts from large-scale cell suspensions[a]

Strain	Glucose consumption (mM)	End-product amounts (mM) of[b]:								Carbon balance (mM)
		Sorbitol	Mannitol/ mannitol-1P (%)	Acetoin	2,3-Butanediol	Ethanol	Acetate	Lactate	Succinate	
NCIMB8826(pGIZ906)	28	ND	ND	ND	ND	ND	1.1 (2.0)	55.7 (99.4)	0.3 (0.5)	1.02
VL103(pGIZ906)	20	ND	4.0 (20.0)	2.6 (13.0)	8.8 (44.0)	4.8 (12.0)	2.6 (6.5)	0.7 (1.7)	1.8 (4.5)	1.02
VL103(pGIVL201)	20	12.2 (61.0)	1.8 (9.0)	6.4 (32.0)	ND	1.0 (2.5)	1.6 (4.0)	0.4 (1.0)	1.0 (2.5)	1.12
VL103(pGIVL202)	20	13.1 (65.5)	2.7 (13.5)	6.6 (33.0)	ND	0.7 (1.7)	1.8 (4.5)	0.5 (1.2)	0.3 (0.7)	1.20

[a] End-product concentration values (mM) from [1-^{13}C]glucose (mM) fermentation as determined by ^{1}H and ^{13}C NMR analyses from large-scale cell suspensions under controlled conditions (pH 6.5, 30°C, argon atmosphere) of *L. plantarum* strains NCIMB8826(pGIZ906), VL103(pGIZ906), VL103(pGIVL201), and VL103(pGIVL202). Samples were collected after glucose exhaustion. Data are representative of at least two experiments.
[b] Values in parentheses are percentages of glucose converted to the product. ND, not detected.

scale cell suspensions was performed under pH control (pH 6.5) with continuous measurement of metabolites by ^{13}C NMR (in vivo NMR).

The glucose consumption rate of the wild-type strain was very high (0.08 μmol min^{-1} mg^{-1} dry weight) in comparison to that of the three LDH-deficient strains, which metabolized glucose at a rate of 0.01 μmol min^{-1} mg^{-1} dry weight (data not shown).

In the wild type, [1-^{13}C]glucose catabolism by resting cells under pH control resulted almost exclusively in lactate production, with only minor amounts of acetate and succinate (Table 2 and data not shown). For strain VL103(pGIZ906), [1-^{13}C]glucose (20 mM) was fermented with a mixture of 2,3-butanediol (8.8 mM), acetoin (2.6 mM), mannitol/mannitol-1P (4.0 mM), ethanol (4.8 mM), acetate (2.6 mM), and minor amounts of succinate (1.8 mM) and lactate (0.7 mM) (Table 2). For strain VL103(pGIVL202), the kinetics of glucose consumption and product formation are shown in Fig. 3. Similar results were obtained with VL103(pGIVL201) (data not shown). With VL103(pGIVL202), the major fermentation end products formed from [1-^{13}C]glucose (20 mM) were sorbitol (13.1 mM) and acetoin (6.6 mM) (Fig. 3A), while minor amounts of mannitol/mannitol-1P (2.7 mM), acetate (1.8 mM), ethanol (0.7 mM), lactate (0.5 mM), succinate (0.3 mM), and pyruvate (0.3 mM) were detected (Fig. 3B and Table 2). The resonances of [1-^{13}C]mannitol and [1-^{13}C]mannitol-1P overlap in the in vivo ^{13}C NMR spectra, and therefore their individual concentrations could not be calculated (24).

Comparison of the end-product formations measured by in vivo NMR for VL103(pGIVL201) and VL103(pGIVL202) revealed very similar profiles in which sorbitol (61 to 65% glucose rerouting) and acetoin (32 to 33%) were the major fermentation products (Table 2). In contrast, strain VL103 (pGIZ906) produced a considerable amount of mannitol (20%) but did not display sorbitol production, which corroborates the observations with small-scale cell suspensions (Table 2). In the latter strain, the redox balance appeared to be maintained mainly via the production of mannitol, 2,3-butanediol, and, to a lesser extent, ethanol. In strains VL103(pGIVL201) and VL103(pGIVL202), the NADH used to produce sorbitol via the Stl6PDH was derived mainly from the carbon that flowed through the glycolytic pathway, leading to 2,3-butanediol and ethanol (Table 2).

Optimization of sorbitol production with growing cells. In order to optimize sorbitol production with growing cells, different fermentations were performed under pH-controlled conditions (pH 6.5). Using modified MRS supplemented with 2% glucose, the final cell yield obtained with *srlD*-overexpressing strains was 36% relative to that of the LDH-positive wild type but equivalent to the LDH-deficient parental strain

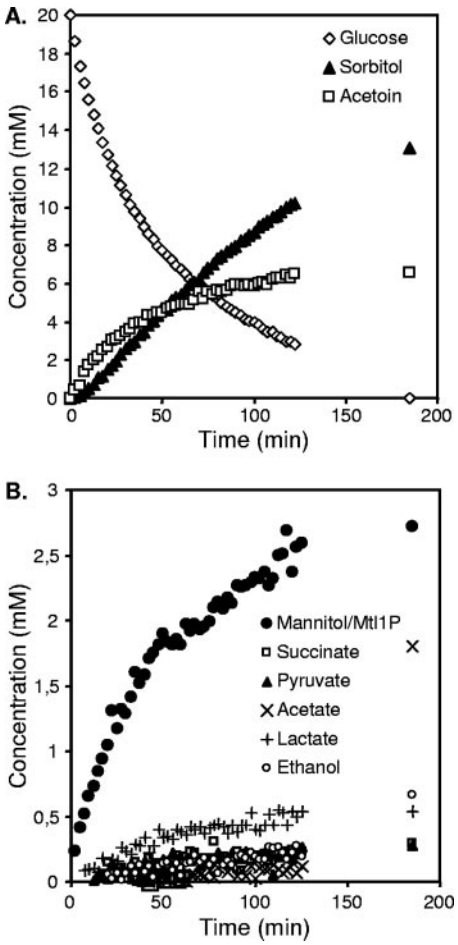

FIG. 3. Kinetics of glucose consumption and end-product formation by cell suspensions (30°C, controlled pH 6.5, argon atmosphere) of strain VL103(pGIVL202) as determined by in vivo ^{13}C NMR using 20 mM [1-^{13}C]glucose as a substrate. (A) Major metabolites were sorbitol and acetoin. (B) Minor metabolites were mannitol/mannitol-1P (Mtl-1P), succinate, pyruvate, acetate, lactate, and ethanol.

TABLE 3. Influence of the carbon source and acetate on the production of polyols during fermentations performed with growing cells[a]

Carbon source(s) (2% wt/vol)[b]	Maximum polyol formation (mM) from strain[c]								
	VL103(pGIVL201)			VL103(pGIVL202)			VL103(pGIVL201)[d]		
	Sorbitol	Mannitol	OD_{600} max	Sorbitol	Mannitol	OD_{600} max	Sorbitol	Mannitol	OD_{600} max
Glucose	0.2 (0.2)	1.2 (1.1)	1.6	0.2 (0.1)	0.9 (0.9)	2.1	15.8 (14.4)	2.7 (2.4)	3.2
Glucose + fructose	1.2 (1.1)	0.5 (0.5)	1.8	0.1 (0.1)	0.6 (0.5)	2.4	10.2 (9.3)	2.7 (2.4)	3.0
Fructose	ND	0.6 (0.6)	2.6	ND	0.5 (0.4)	3.4	2.7 (2.5)	ND	3.9
Maltose	6.0 (5.5)	1.1 (1.0)	2.8	5.0 (4.5)	3.7 (3.4)	4.7	25.2 (23.0)	3.5 (3.2)	2.4
Sucrose	0.8 (0.7)	0.2 (0.15)	0.9	ND	1.7 (1.6)	3.3	2.7 (2.5)	1.0 (0.9)	5.1

[a] Data show influences of the carbon source and acetate on the production of polyols (sorbitol and mannitol) during fermentations performed with growing cells of *L. plantarum* strains VL103(pGIVL201) and VL103(pGIVL202). Cells were grown in reconstituted MRS broth in the presence or absence of acetate at 37°C under low aeration (120 rpm) and pH control (pH 6.5) conditions.

[b] Glucose + fructose, equimolar mixture.

[c] Values in parentheses are percentages of sugar(s) converted to the product. The maximum (max) value of OD_{600} was measured at the stationary growth phase. ND, not detected.

[d] Grown in modified MRS broth without acetate supplemented with 2% (wt/vol) sugar.

[VL103(pGIZ906)] (data not shown). The generation time of the three LDH-deficient strains (between 120 and 144 min) was twofold higher than that of the LDH-positive wild type (57 min). Sorbitol production of strains VL103(pGIVL201) and VL103(pGIVL202) was evaluated with modified MRS supplemented with 2% each of different carbon sources (glucose, fructose, a mixture of glucose and fructose, maltose, and sucrose) (Table 3). The relative conversion rate of the available carbon source to sorbitol from strain VL103(pGIVL201) appeared to be consistently higher than that from strain VL103(pGIVL202). Both strains produced the highest sorbitol levels when grown on maltose. VL103(pGIVL201) could convert up to 5.5% of the maltose consumed into sorbitol (6.0 mM) (Table 3). Besides sorbitol, these strains also produced minor amounts of mannitol. VL103(pGIVL202) converted 3.4% of the maltose consumed to mannitol (3.7 mM), while in VL103(pGIVL201), mannitol production levels appeared to be lower (1.1 mM) (Table 3).

For all fermentations performed during the optimization procedure, the formation of other fermentation end products was monitored. The metabolic end-product profiles showed that part of the acetate that was present in modified MRS was consumed, while concomitant and equimolar production of ethanol was observed (Fig. 4A). Similar results were previously reported in an LDH-deficient strain of *L. lactis* that converted acetate to ethanol via acetyl-P and acetyl coenzyme A (Fig. 1A), thereby contributing to the redox balance via NADH consumption (17). Since the electron sink provided by acetate conversion to ethanol could reduce the efficiency of polyol production, the effect of acetate on sorbitol production in strain VL103(pGIVL201) was investigated using MRS − Ac supplemented with various sugars (Table 3 and Fig. 4B). Notably, higher biomass yields were obtained for all carbon sources analyzed when acetate was omitted from the media, with the exception of maltose (Table 3 and Fig. 4B). With maltose as the carbon source, sorbitol production was more than fourfold higher in MRS − Ac compared to that in MRS with acetate. Analogously, fermentations performed in the absence of acetate dramatically improved sorbitol production for all carbohydrate sources evaluated (Table 3). Sorbitol and mannitol formation during the course of the fermentation on MRS − Ac supplemented with 2% maltose was monitored

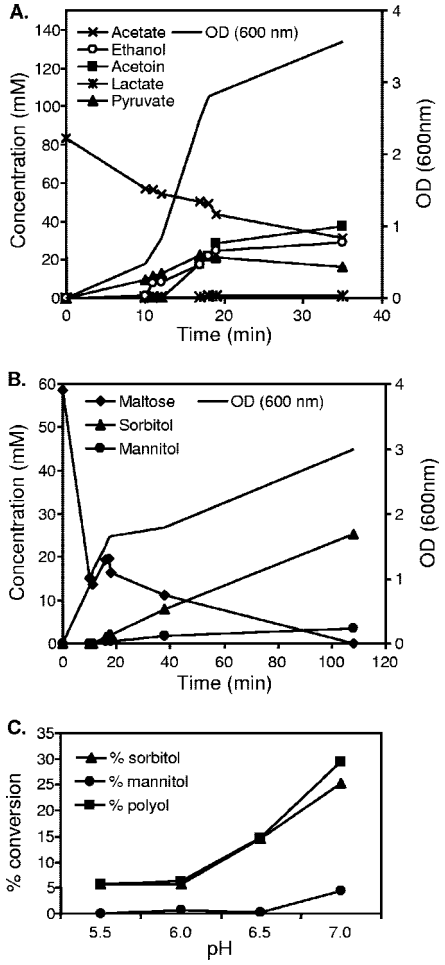

FIG. 4. Batch fermentations of strain VL103(pGIVL201) performed under pH control in modified MRS medium at 37°C. (A) Product formation and consumption at a controlled pH of 6.5 from MRS supplemented with 0.5% (wt/vol) acetate and 2% (wt/vol) glucose. Acetate consumption and the formation of acetoin, ethanol, pyruvate, and lactate were monitored during the course of the fermentation. Growth was monitored by measuring the OD_{600}. (B) Time course of the conversion of maltose into sorbitol and mannitol at pH 6.5 in MRS − Ac supplemented with 2% (wt/vol) maltose. (C) pH dependency of the percentage of maltose conversion into sorbitol and mannitol (polyols) in growing cultures performed in MRS − Ac supplemented with 2% (wt/vol) maltose.

TABLE 4. Influence of pH on the maximal production of polyols during fermentation with growing cells[a]

pH value	Maximum polyol formation (mM)[b]		OD_{600} max[c]
	Sorbitol	Mannitol	
5.5	6.1 (5.6)[b]	0.05 (0.05)	4.2
6.0	6.3 (5.7)	0.6 (0.55)	3.9
6.5	16.0 (14.5)	0.2 (0.15)	3.0
7.0	27.7 (25.2)	4.8 (4.4)	2.0

[a] Data show the influence of pH on the maximal production of polyols (sorbitol and mannitol) during fermentation experiments performed with growing cells of *L. plantarum* VL103(pGIVL201). Cells were grown in modified MRS medium supplemented with maltose (2% wt/vol) in the absence of acetate at 37°C under low aeration (120 rpm) and pH control.

[b] Values in parentheses are percentages of maltose converted to the product.

[c] Maximum (max) values of OD_{600} were measured at the stationary growth phase.

(Fig. 4B). Sorbitol production appeared to start at the end of the exponential growth phase and coincided with the time at which more than 75% of the available maltose had been consumed.

For further optimization, a range of pH values (5.5, 6.0, 6.5, 7.0 and 7.5) was evaluated using the most effective sorbitol production strain, VL103(pGIVL201), in MRS − Ac supplemented with 2% maltose. This strain was unable to grow at pH 7.5, and the highest sorbitol production was observed at pH 7.0. Under these conditions, some mannitol was also detected (Fig. 4C and Table 4), but the production was largely in favor of sorbitol (85% of total polyols), amounting to a 25% conversion of the initial carbon source to sorbitol (with a final yield of 5 g/liter; 27.7 mM). Moreover, sorbitol and mannitol were the only sugars left at the end of fermentation (data no shown).

DISCUSSION

The *L. plantarum* genome contains two operons potentially involved in sorbitol catabolism that display the same genetic organization. This redundancy could indicate that one of them is involved in the catabolism of other related compounds, like sorbose, as was recently demonstrated in *Lactobacillus casei* (38). However, the absence of a predicted sorbose reductase-encoding gene in the *L. plantarum* genome suggests that both operons may be involved in sorbitol catabolism but could potentially be differentially expressed under different environmental conditions. Here, we show that both *srlD* genes code for active Stl6PDHs capable of reversing the metabolic pathway from fructose-6P toward sorbitol production in *L. plantarum*.

High-level constitutive expression of both *srlD* genes in the LDH-deficient background led to sorbitol production under a range of different conditions. The LDH deficiency of the production host appeared essential, since similar experiments using an LDH-positive production host did not result in detectable sorbitol production under any of the conditions analyzed. Moreover, *srlD* expression in the wild-type background did not lead to significant changes in the fermentation profile, in which lactate was the major fermentation end product. Most probably, the high glycolytic flux in these LDH-positive strains results in the limited availability of the glycolytic intermediate fructose-6P, which is the substrate of the primary glycolysis-branching reaction leading to sorbitol production. In addition, lactate production will reduce the levels of available NADH, which is a cofactor that is also required for sorbitol production.

Remarkably, 61 to 65% rerouting of glucose toward sorbitol production was observed for *srlD*-overexpressing strains in cell suspensions under pH control. Concomitant production of sorbitol and mannitol was observed, but the competition is clearly in favor of sorbitol (up to 87% of total polyols). Notably, considerable amounts of mannitol (20% rerouting) were produced by the parental LDH-deficient strain VL103, while sorbitol production depended strictly on the plasmid-based expression of the Stl6PDH enzyme. By analogy, no intrinsic Stl6PDH activity could be detected, suggesting tight control of *srlD* expression, which may involve one or more of the two putative transcription regulators that are present in both *srl* operons and/or the *mtlR*-encoded transcriptional regulator identified in the mannitol catabolic operon (20). Interestingly, inactivation of the *ldh* gene in *L. lactis* resulted in an enhanced Mtl1PDH activity (24), while similar levels of Mtl1PDH activity were present in all *L. plantarum* strains used in this study, including the wild-type strain. This observation suggests that mannitol production is not subjected to a strict control, which is in apparent contrast to sorbitol production.

Interestingly, production of sorbitol and/or mannitol by cell suspensions was not observed under conditions of strong aeration. Since sorbitol production depends strictly on the availability of NADH as a cofactor, this effect is most likely explained by oxidation of the NADH pool by the NADH oxidase in the presence of molecular oxygen (NADH-oxidase reaction: $O_2 + NADH \rightarrow 2H_2O + NAD^+$). A similar effect of high aeration was previously shown to strongly reduce mannitol production by an LDH-deficient strain of *L. lactis*, which was suggested to be the consequence of NADH oxidase activation (24). Analogously, NADH oxidase activity is known to be strongly induced in *L. plantarum* under aerobic conditions (22) and effectively dissipates NADH in the presence of molecular oxygen and thereby interferes with polyol production via the Mtl1PDH and Stl6PDH enzymes by competing for their mutual cofactor NADH (Fig. 1A). The importance of the availability of high levels of NADH for the production of sorbitol was corroborated by the negative effect of NAD^+ regeneration via the acetate-to-ethanol conversion, which was observed for growing cells. Taken together, these observations indicate that a relatively high level of NADH accumulation is a prerequisite for activation of the Stl6PDH enzyme and sorbitol formation.

Although a reasonably high level of sugar rerouting toward polyol (sorbitol and mannitol) was achieved with growing cells (up to 30%), this level is significantly lower than the maximal rerouting level obtained with resting cells, which corresponds to the theoretical maximum percentage of conversion (67%) (36). This difference may be caused by a higher ATP demand for biomass production in growing cells (21). In resting cells as well as in the stationary phase of growth, NAD^+ regeneration and maintenance of redox balance probably exert a more dominant metabolic control than ATP generation.

The high rerouting levels obtained show that *L. plantarum* is a promising candidate host for polyol production. By comparison, higher mannitol production levels (50%) were recently reported with growing cells of *L. lactis* (36). However, the

metabolic engineering strategy employed in that study was relatively complicated and included multiple gene overexpressions and deletions, which were required to avoid mannitol consumption and to increase mannitol-1P dephosphorylation (11, 35, 36). Notably, such complex engineering strategies are not required to achieve relatively effective polyol production in *L. plantarum*. Nevertheless, a high capacity for polyol production does not seem to be general among lactobacilli, since only low levels of sorbitol production (3% compared to 65% in *L. plantarum*) were recently obtained with resting cells of *Lactobacillus casei* using a similar strategy (26).

Our results show that metabolic engineering of *L. plantarum* for high sorbitol production was successfully achieved by a simple two-step strategy that does not require any heterologous gene expression. However, the use of *L. plantarum* as a cell factory for polyol production at this stage would be restricted to that of a resting cell bioreactor, while production in growing cultures would require further optimization of conversion efficacy. Nevertheless, considering the consumer health-related properties of sorbitol, the moderate level of polyol production obtained here offers opportunities for the future use of *L. plantarum* for in situ sorbitol production in fermented food products, since in that case, a highly efficient polyol production would not be necessarily required.

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