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Engineering *Lactococcus lactis* for Production of Mannitol: High Yields from Food-Grade Strains Deficient in Lactate Dehydrogenase and the Mannitol Transport System

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Mannitol is a sugar polyol claimed to have health-promoting properties. A mannitol-producing strain of *Lactococcus lactis* was obtained by disruption of two genes of the phosphoenolpyruvate (PEP)-mannitol phosphotransferase system (PTS^{Mtl}). Genes *mtlA* and *mtlF* were independently deleted by double-crossover recombination in strain *L. lactis* FI9630 (a food-grade lactate dehydrogenase-deficient strain derived from MG1363), yielding two mutant (Δ*ldh*Δ*mtlA* and Δ*ldh*Δ*mtlF*) strains. The new strains, FI10091 and FI10089, respectively, do not possess any selection marker and are suitable for use in the food industry. The metabolism of glucose in nongrowing cell suspensions of the mutant strains was characterized by in vivo ^{13}C-nuclear magnetic resonance. The intermediate metabolite, mannitol-1-phosphate, accumulated intracellularly to high levels (up to 76 mM). Mannitol was a major end product, one-third of glucose being converted to this hexitol. The double mutants, in contrast to the parent strain, were unable to utilize mannitol even after glucose depletion, showing that mannitol was taken up exclusively by PEP-PTS^{Mtl}. Disruption of this system completely blocked mannitol transport in *L. lactis*, as intended. In addition to mannitol, approximately equimolar amounts of ethanol, 2,3-butanediol, and lactate were produced. A mixed-acid fermentation (formate, ethanol, and acetate) was also observed during growth under controlled conditions of pH and temperature, but mannitol production was low. The reasons for the alteration in the pattern of end products under nongrowing and growing conditions are discussed, and strategies to improve mannitol production during growth are proposed.

Mannitol, a six-carbon sugar polyol, is synthesized by many eukaryotes and a few bacteria, but apparently not by archaea. Mannitol is also a well-known compatible solute that accumulates mainly in yeast, fungi, and plants, but rarely in bacteria, in response to osmotic and/or heat stresses (5, 16, 21, 23). Like several other compatible solutes, mannitol protects proteins and cells against different stressing conditions. Recently, its ability to enhance survival of starter cultures of *Lactococcus lactis* subjected to drying has been demonstrated (6).

Mannitol is assumed to have several health-promoting properties; thus the enrichment of foods with mannitol by in situ production during fermentation could be a positive, clean strategy to obtain healthier fermented food products. In the human gut, mannitol can be converted to short-chain fatty acids (such as butyrate), which have been claimed to confer protection against the development of colon cancer (42). Moreover, mannitol is a scavenger of hydroxyl radicals and a low-calorie sweetener that is partially and slowly absorbed in the small intestine (8, 40).

Lactic acid bacteria (LAB) are a group of microorganisms widely used as starter cultures in dairy fermentations. Hence, these food-associated organisms could be ideal agents for the in situ production of mannitol in foods. The ability of heterofermentative LAB to produce mannitol during fructose fermentation, via the enzyme mannitol dehydrogenase, has been known for many years and is very well documented (11, 37, 38, 43, 44). On the other hand, production of mannitol from sugar substrates other than fructose or sucrose has not been reported.

Mannitol formation in homofermentative LAB, in contrast to the heterofermentative LAB, has been detected only in strains whose ability to regenerate NAD^+ is severely impaired (7, 28, 30). Our team has reported the synthesis of mannitol and mannitol-1-phosphate (Mtl1P) in *L. lactis* strains deficient in lactate dehydrogenase (LDH) (28). Production of mannitol in these strains was rationalized as an alternative way to fulfill the redox balance during glucose catabolism, since conversion of fructose-6-phosphate (F6P) to Mtl1P is associated with regeneration of NAD^+. We also observed that the mannitol produced was taken up and rapidly metabolized after glucose depletion; therefore, it was apparent that the design of a mannitol-producing strain would have to consider the ability of *L. lactis* to utilize mannitol as an energy source for growth (27). Thus, the disruption of the mannitol transport system in *L. lactis* was envisaged in the metabolic strategy pursued herein.

Transport of mannitol in *L. lactis* is most likely mediated by a phosphoenolpyruvate (PEP)-mannitol phosphotransfer-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source
L. lactis		
MG1363	Plasmid-free *L. lactis* subsp. *cremoris* NCDO712	10
FI9630	MG1363 Δ*ldh,* food-grade strain	30
FI10021	FI9630 carrying pVE6007, Cm^r	This work
FI10022	FI10021 carrying pFI2425, Ery^r Cm^r, blue on X-Gal plates	This work
FI10089	FI9630 Δ*mtlF,* food-grade strain	This work
FI10090	FI10021 carrying pFI2429, Ery^r Cm^r, blue on X-Gal plates	This work
FI10091	FI9630 Δ*mtlA,* food-grade strain	This work
Plasmids		
pGEM-T	Amp^r, cloning vector	Promega
pVE6007	Cm^r plasmid providing RepA, temperature sensitive, only replicates at 30°C	24
pORI280	Ery^r *lacZ*$^+$ *ori*$^+$ of pWV01, replicates only in strains providing *repA* in *trans*	19
pFI2421	Amp^r derivative of pGEM-T vector containing *mtlF* upstream fragment generated by using primers MtlF1 and MtlF2	This work
pFI2422	Amp^r derivative of pGEM-T vector containing *mtlF* downstream fragment generated by using primers MtlF3 and MtlF4	This work
pFI2425	Ery^r, specific for deletion of the *L. lactis mtlF* gene	This work
pFI2426	Amp^r derivative of pGEM-T vector containing 3.11-kb *Bam*HI-*Bgl*II fragment generated by using primers MtlA1 and MtlA2	This work
pFI2427	Amp^r derivative of pFI2426 vector containing 2.04-kb *Bam*HI-*Bgl*II fragment generated by deletion of 1.07-kb *Eco*RI-*Eco*RI portion inside the 3.11-kb fragment	This work
pFI2428	Ery^r Amp^r derivative of pORI280 containing pFI2427 cloned into *Bam*HI site	This work
pFI2429	Ery^r derivative of pFI2428 where pGEM-T portion has been deleted, specific for deletion of the *L. lactis mtlA* gene	This work

ase system (PTS^{Mtl}), since the genome sequence of *L. lactis* IL1403 revealed the existence of an operon (*mtlARFD*) encoding proteins with high sequence similarity to subunits B and C of EII^{Mtl}, MtlR (regulatory protein), subunit A of EII^{Mtl}, and mannitol-1-phosphate dehydrogenase (Mtl1PDH) of other organisms (1). A similar organization of the mannitol operon is found in *L. lactis* MG1363 (N. Mansour, personal communication). The protein $EIICB^{Mtl}$, encoded by the *mtlA* gene, is involved in the phosphorylation and mannitol permeation across the membrane, whereas protein $EIIA^{Mtl}$ (encoded by *mtlF*) is responsible for phosphotransfer between the histidine protein (HPr) and $EIIBC^{Mtl}$ (34). The role of the regulatory protein, MtlR, in the expression of the mannitol operon is not entirely clear. In *Bacillus stearothermophilus*, MtlR is probably involved in the regulation of this operon, and its affinity for DNA is controlled via phosphorylation by HPr and $EIICB^{Mtl}$ (12). However, in *Streptococcus mutans*, the *mtlR* gene product is not necessary for growth on mannitol or for expression of the operon (15).

In this paper, we describe the construction of *L. lactis* strains able to form mannitol as an end product of glucose metabolism, using a food-grade LDH-deficient strain as genetic basis for knocking out the gene *mtlA* (encoding $EIICB^{Mtl}$) or *mtlF* (encoding $EIIA^{Mtl}$). Nongrowing cells of the double mutants (Δ*ldh*/Δ*mtlA*) and (Δ*ldh*Δ*mtlF*) yielded mannitol, ethanol, 2,3-butanediol, and lactate as major end products, with approximately one-third of the carbon from glucose being successfully channeled to the production of mannitol.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All plasmids produced in this study were constructed and maintained in *Escherichia coli* (Table 1). MC1022 (3) or TOP10 competent cells (Invitrogen, United Kingdom) were used for transformation as the general *E. coli* background strains. *E. coli* EC1000 was the specific host for pORI280-derived plasmids, which require the chromosomal *repA* gene for replication (19). The *L. lactis* strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown in Luria broth (39) at 37°C. *L. lactis* strains were routinely grown at 30°C in M17 medium (41) supplemented with 0.5% glucose (GM17). The ability to ferment mannitol was tested in McKay's indicator medium (17) containing 0.5% (wt/vol) mannitol and 0.005% (wt/vol) bromocresol purple (BCP) indicator. Antibiotic selection was used when appropriate: for *E. coli* (in milliliters), 400 μg of erythromycin, 100 μg of ampicillin, 200 μg of streptomycin, and 25 μg of kanamycin; and for *L. lactis* (in milliliters), 5 μg of erythromycin and 5 μg of chloramphenicol. When necessary, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at a concentration of 50 μg/ml (*E. coli*) or 175 μg/ml (*L. lactis*) and IPTG (isopropyl-β-D-thiogalactopyranoside) was added at 0.5 mM (*E. coli*).

Molecular techniques. Chromosomal DNA was isolated from *L. lactis* strains according to the procedure of Lewington et al. (20). Plasmid DNA isolation was carried out with the QIAprep Spin Miniprep or QIAGEN Plasmid Mini kits for small-scale purifications (adding 5 mg of lysozyme per ml and 100 U of mutanolysin per ml to buffer P1 for lactococcal strains) and the HiSpeed Plasmid Midi kit for large-scale purifications. Restriction enzymes and other DNA-modifying enzymes were used according to the suppliers' recommendations. Recombinant plasmids were introduced into *E. coli* by transformation by the calcium chloride method (25) or by electrotransformation (4) and introduced into *L. lactis* by electrotransformation according to the method of Holo and Nes (13). Chromosomal DNA or colony PCR was performed in 50 μl by using *Taq* polymerase (5 U μl^{-1}) and 10× PCR buffer (Amersham, Little Chalfont, United Kingdom). Twenty picomoles of each primer was used, and deoxynucleoside triphosphates (dNTPs) (Bioline, London, United Kingdom) were used at a concentration of 250 μM each. PCR amplifications were carried out in a DNA thermal cycler for 25 cycles (each consisting of 2 min at 94°C, 2 min at the annealing temperature, and 3 min at 72°C). Specific PCR fragments used for construction of deletion vectors were amplified by using Bio-X-Act DNA polymerase (Bioline). Oligonucleotides were obtained from Sigma Genosys (Haverhill, United Kingdom). Ligation reactions were performed according to Sambrook et al. (39). DNA sequencing reactions were performed with the DNA sequencing kit (ABI PRISM Big Dye) supplied by Applied Biosystems.

Construction of gene replacement vectors. The pORI280/pVE6007 two-plasmid system (18, 19) was used to generate food-grade deletions of the *mtlF* or

mtlA genes in the Δ*ldh* FI9630 strain (30). For deletion of the *mtlF* gene, the upstream and downstream flanking fragments were amplified from *L. lactis* MG1363 (10) genomic DNA by using the primers MtlF1 (5′ CTTATCTAGAT TCGGTCTATG 3′) plus MtlF2 (5′ GTAAAAAGTTCAAATAGGATCCCAC ACGCCGTAAAC 3′) and MtlF3 (5′ GTTTACGGCGTGTGGGATCCTATT TGAACTTTTTAC 3′) plus MtlF4 (5′ GTTAGAATTCAAGTCAGAAAA TCCA 3′), respectively. The primers were designed on the basis of available sequence data for the mannitol operon from strain MG1363, and the specific restriction sites *Xba*I, *Bam*HI, and *Eco*RI were added (underlined in primer sequences). The upstream and downstream amplified fragments were cloned into pGEM-T (Promega), giving pFI2421 and pFI2422, respectively, and their sequences were confirmed. The upstream and downstream fragments were cloned sequentially into pORI280 to give pFI2425. The correct orientation of the fragments cloned in pFI2425 was confirmed by PCR analysis.

Inactivation of the *mtlA* gene was achieved by deletion of the 1.07-kb *Eco*RI-*Eco*RI region present inside the gene. For this, MG1363 genomic DNA was amplified with MtlA1 (5′ ACTGGATCCATTTGGTATTTATTGCATAG 3′, based on the IL1403 genome sequence) and MtlA2 (5′ CAAGATCTGACGA ATTTTATAGGAGAA 3′, based on the MG1363 genome sequence) primers, generating a 3.11-kb *Bam*HI-*Bgl*II fragment containing the complete *mtlA* gene and flanking regions, where the *Bam*HI and *Bgl*II restriction sites have been engineered (underlined in primer sequences). This fragment was cloned into pGEM-T, giving pFI2426. Digestion of this construct with *Eco*RI followed by religation resulted in plasmid pFI2427, in which the internal 1.07-kb *Eco*RI fragment within the *mtlA* gene has been deleted, leaving a 2.04-kb *Bam*HI-*Bgl*II fragment that covers 750 bp of the *mtlA* gene and flanking regions. pFI2427 and pORI280 were digested with *Bam*HI and ligated together, giving pFI2428. Further digestion of this construct with *Bgl*II followed by religation gave pFI2429 (pORI280 containing the 2.04-kb *Bam*HI-*Bgl*II fragment), a Δ*mtlA* gene replacement vector.

Gene replacement protocol. The pFI2425 and pFI2429 constructs were electroporated into strain FI10021 (FI9630/pVE6007), giving FI10022 and FI10090, respectively. These strains were taken through the temperature shift protocol for single and double crossovers (19), yielding *L. lactis* strains FI10089 (Δ*ldh*Δ*mtlF* double mutant) and FI10091 (Δ*ldh*Δ*mtlA* double mutant), respectively. The correctness of both mutants was confirmed by PCR analysis.

Quantification of fermentation products during growth. Strains were grown in a 2-liter fermentor in chemically defined medium (CDM) (33) containing 1% (wt/vol) glucose at 30°C and pH 6.5. The pH was kept constant by automatic addition of NaOH. Growth was evaluated by measuring the turbidity (optical density of the culture at 600 nm [OD_{600}]) and calibrating against cell dry mass measurements. Culture samples (5 ml) were taken at different growth stages and centrifuged (2,000 ×*g*, 5 min, 4°C), and the supernatant solutions were stored at −20°C until analyzed by high-performance liquid chromatography (HPLC). Glucose, mannitol, acetate, ethanol, formate, lactate, acetoin, and 2,3-butanediol were quantified in an HPLC apparatus equipped with a refractive index detector (Shodex RI-101, Showa Denko K.K., Oita, Japan) using an HPX-87H anion-exchange column (Bio-Rad Laboratories, Inc., Richmond, Calif.) at 60°C, with 5 mM H_2SO_4 as the elution fluid and a flow rate of 0.5 ml min^{-1}.

Quantification of intracellular metabolites in cell extracts by ^{13}C-NMR. Mutant FI10089 was grown under controlled conditions as described above, except that a mixture of [1-^{13}C]glucose and nonlabeled glucose (1:10) was used. At mid-exponential and stationary growth phases, samples were collected and extracted with ethanol as described previously (36). Samples were analyzed by ^{13}C nuclear magnetic resonance (^{13}C-NMR); assignment of resonances was done as in previous studies (28, 35). In particular, this methodology was used to assess intracellular mannitol in growing cultures.

In vivo NMR experiments and quantification of metabolites. Cells were grown in CDM as described above and harvested in the mid-exponential growth phase, washed twice in 5 mM potassium phosphate (KP_i), and suspended in 50 mM KP_i buffer (pH 6.5), to a protein concentration in the range 13 to 15 mg of protein ml^{-1}. In vivo NMR experiments were performed with the system described previously (29). [1-^{13}C]glucose or [6-^{13}C]glucose (40 mM) was supplied to the cell suspension, and the time courses for substrate consumption, product formation, and intracellular metabolite pools were monitored in vivo. After substrate exhaustion and when no changes in the resonances due to end products and intracellular metabolites were observed, a 10-ml aliquot was passed through a French press, and an NMR sample extract was prepared as reported previously (30). Lactate, acetoin, acetate, and formate were quantified in the NMR sample extract by ^{1}H-NMR in a Bruker AMX300 (29). The concentrations of mannitol, 2,3-butanediol, ethanol, minor products, and metabolic intermediates that remained inside the cells (phosphoenolpyruvate, 3-phosphoglycerate) were determined in fully relaxed ^{13}C spectra of the NMR sample extracts as described by Neves et al. (28).

Due to the fast pulsing conditions used for acquiring in vivo ^{13}C spectra, quantification of the intracellular metabolites required the use of correction factors that allow the conversion of resonance areas into concentrations. Correction factors of 0.73 ± 0.04, 0.65 ± 0.03, and 0.71 ± 0.04 were used for resonances of fructose-1,6-bisphosphate, mannitol or Mtl1P, and PEP or 3-phosphoglycerate, respectively (28). Intracellular metabolite concentrations were calculated by using a value of 2.9 μl mg of $protein^{-1}$ for the intracellular volume (32). The concentration limit for detection of intracellular metabolites under the conditions used to acquire in vivo spectra (30-s total acquisition time for each spectrum) was 3 to 4 mM. Although individual experiments are illustrated in each figure, each type of in vivo NMR experiment was repeated at least twice. The values reported are averages of two to three experiments, and the accuracy varied from 2% (end products) to 15% in the case of intracellular metabolites.

Quantification of intracellular and extracellular mannitol in nongrowing cells. Intracellular and extracellular pools of mannitol were quantified in independent experiments performed with the experimental setup used for in vivo NMR measurements. After glucose addition, 1-ml samples were withdrawn at 5-min intervals. The samples were centrifuged, the pellets were discarded, and the supernatants were used for quantification of extracellular mannitol. For the determination of total mannitol (intracellular plus extracellular), 5-ml samples were taken at 10, 20, 30, and 60 min after addition of [1-^{13}C]glucose. Perchloric acid (final concentration, 0.6 M) was added to the samples. After stirring for 20 min on ice, the pH of the samples was adjusted to neutrality with 2 M KOH and centrifuged. The supernatant solutions were used for mannitol quantification by ^{13}C-NMR.

NMR spectroscopy. ^{13}C spectra were acquired at 125.77 MHz on a Bruker DRX500 spectrometer. All in vivo experiments were run with a quadruple-nucleus probe head at 30°C, as described before (29). For the quantitative analysis of the NMR sample extracts by ^{13}C-NMR, a repetition delay of 60.5 s was used. The ^{13}C-NMR spectra of the perchloric and ethanol extracts were obtained with a quadruple-nucleus probe head with a pulse width of 13 μs (flip angle, 60°) and recycle delays of 3.5 and 1.5 s, respectively. Correction factors to take into account incomplete relaxation of resonances were calculated by comparison with spectra acquired under fully relaxed conditions (recycle delay, 60.5 s). ^{13}C-NMR spectra of supernatant solutions were acquired with a 5-mm-diameter selective probe head using a pulse width corresponding to a 60° flip angle and a recycle delay of 5.5 s. Chemical shifts were referenced to the resonance of external methanol designated at 49.3 ppm.

Enzyme activity measurements. Cells were grown and harvested in the mid-exponential phase as described for in vivo NMR experiments, and crude extracts were prepared as described by Neves et al. (28). Enzyme activities were assayed in a spectrophotometer (Shimadzu UV-1601) equipped with a cell compartment thermostatted at 30°C, in a total volume of 1 ml. One unit of enzyme activity is the amount of enzyme catalyzing the conversion of 1 μmol of substrate per min under the experimental conditions used. LDH activity was assayed as described by Garrigues et al. (9). The reverse reaction (F6P→Mtl1P) catalyzed by Mtl1PDH was assayed as reported before (28).

Chemicals. [1-^{13}C]glucose and [6-^{13}C]glucose (99% enrichment) were supplied by Campro Scientific (Veenendaal, The Netherlands). All other chemicals were reagent grade and were obtained from Merck or Aldrich.

RESULTS

Inactivation of *mtlA* and *mtlF* genes in *L. lactis* FI9630 by double-crossover recombination. To obtain a mannitol-producing *L. lactis* strain, the genes *mtlA* and *mtlF*, involved in the transport of mannitol, were deleted from the genome of FI9630 by double-crossover recombination, creating strains FI10091 and FI10089, respectively (see Materials and Methods for details). FI10091 carries a double deletion in the *ldh* and *mtlA* genes, whereas FI10089 carries deletions in the *ldh* and *mtlF* genes (Fig. 1). The chromosomal deletions of *mtlA* and *mtlF* performed in *L. lactis* FI9630 were confirmed by PCR analysis using primers in the regions flanking the genes deleted.

Growth of the mutant strains in McKay's indicator medium

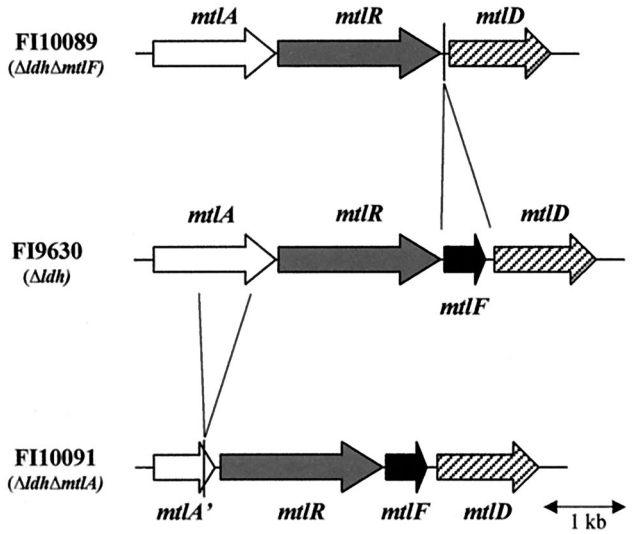

FIG. 1. Schematic representation of the mannitol operon in the chromosome of the parent (FI9630) and mutant strains obtained by gene replacement. The deletions were confirmed by PCR analysis. *mtlA'*, truncated *mtlA*.

containing mannitol gave white colonies, indicating that both strains were unable to metabolize this polyol.

Characterization of growth of strains FI10089 and FI9630 on glucose. The profiles of growth, glucose consumption, and product formation by strains FI10089 (Δ*ldh*Δ*mtlF* double-deletion mutant) and FI9630 (parent strain) under anaerobic conditions are shown in Fig. 2. The major end products from the metabolism of 59 mM glucose by the Δ*ldh*Δ*mtlF* double mutant were formate (77.6 mM), ethanol (47.5 mM), acetate (26.1 mM), and acetoin (9.3 mM); minor amounts of lactate (5.2 mM), 2,3-butanediol (5.7 mM), and mannitol (2.3 mM) were also detected. The fermentation profiles of the two strains were very similar, except for mannitol: a transient production of this polyol was observed in the Δ*ldh* strain (maximal concentration, 0.7 mM), whereas in the double mutant, the level of mannitol increased to 2.3 mM and remained constant even after glucose depletion. Quantification of mannitol in an ethanol extract derived from Δ*ldh*Δ*mtlF* cells in the stationary growth phase revealed that 95% of the total mannitol present at that stage was in the extracellular medium; a value of 25 mM was determined for the intracellular concentration of mannitol. The growth features of the Δ*ldh*Δ*mtlA* strain (FI10091) were similar to those of the Δ*ldh*Δ*mtlF* strain (data not shown). In particular, the growth rates were identical ($0.87 \pm 0.02\ h^{-1}$). A comparison of the relevant growth and bioenergetic parameters for strains FI10089 (Δ*ldh*Δ*mtlF*) and FI9630 (parent strain) is shown in Table 2.

Characterization of glucose metabolism in nongrowing cells of FI10089 and FI10091 by in vivo ^{13}C-NMR. To identify possible bottlenecks in mannitol production, we resorted to in vivo ^{13}C-NMR studies of glucose metabolism in nongrowing cells, since this technique allows the noninvasive quantification of intracellular metabolite pools. The pools of the phosphorylated metabolites Mtl1P, fructose-1,6-bisphosphate (FBP), and 3-phosphoglycerate (3-PGA), PEP, as well as end product formation and glucose consumption, were monitored. A typical sequence of ^{13}C spectra acquired during the metabolism of 40 mM [6-^{13}C]glucose by a cell suspension of FI10089 is shown in Fig. 3. Resonances due to lactate (at 20.4 ppm), acetoin (18.8 and 25.4 ppm), ethanol (17.3 ppm), and 2,3-butanediol (17.2 ppm) were clearly detected. Additionally, resonances due to the intracellular metabolites [1-^{13}C]FBP (66.4 ppm) and [6-^{13}C]FBP (65.1 ppm) (β-furanose forms), 3-PGA (67.4 ppm), and PEP (101.2 ppm) were present. The resonance at 66.1 ppm was assigned to [1-^{13}C]Mtl1P, whereas the resonance at 63.8 ppm comprised contributions of overlapping resonances due to [6-^{13}C]Mtl1P and [1-^{13}C]mannitol (28). Mtl1P is labeled either on C1 or C6, because FBP is also labeled on C1 or C6 due to scrambling of the label at the level of triose phosphate isomerase and backflux through aldolase. The overlapping of resonances due to [6-^{13}C]Mtl1P and [1-^{13}C]mannitol prevented the determination of the individual concentra-

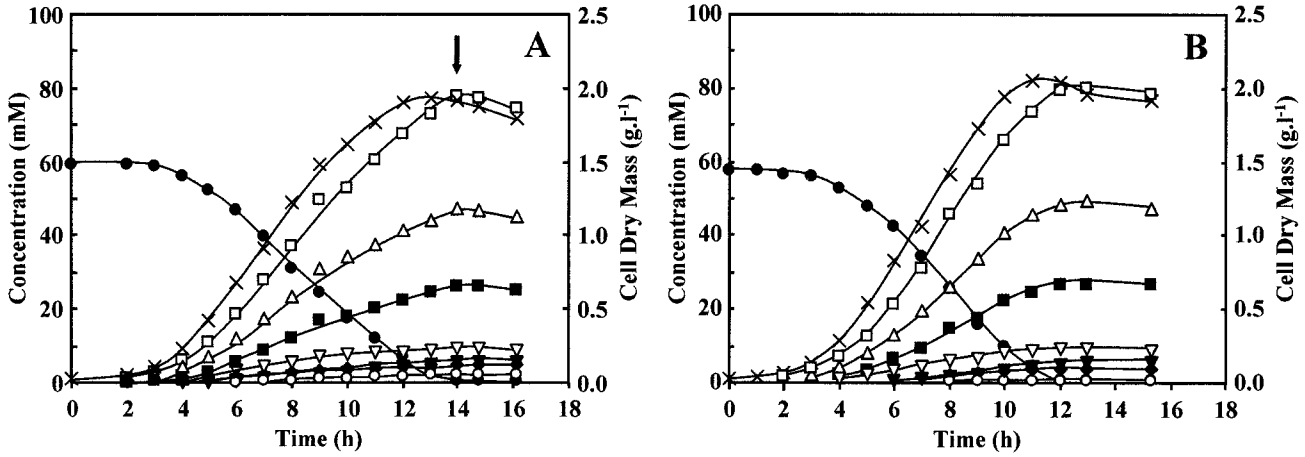

FIG. 2. Growth, glucose consumption, and end product formation by FI10089 (A) and FI9630 (B). ●, glucose; □, formate; △, ethanol; ■, acetate; ▽, acetoin; ▼, 2,3-butanediol; ◆, lactate; ○, mannitol; x, biomass. At the time indicated by the arrow, a culture sample was withdrawn for ethanol extraction and quantification of intracellular mannitol. Data are from a representative experiment in which the error for each data point is ≤15%.

TABLE 2. Carbon and redox balances, specific growth rate, and growth and energetic parameters for FI10089, FI9630, and MG1363 cultured on glucose[a]

Strain	Carbon balance[b]	Redox balance[c]	μ_{max} (h^{-1})	Biomass yield (g mol of substrate^{-1})	ATP yield (mol mol of substrate^{-1})	Y_{ATP} (g of biomass mol of ATP^{-1})	q_s^{max} (mmol g^{-1} h^{-1})
FI10089	98	96	0.89	34.7	2.3	14.2	5.5
FI9630	98	96	0.94	37.6	2.4	14.5	5.7
MG1363[d]	95	97	1.15	34.2	1.89	18.1	18.3

[a] Values are from representative experiments with each strain. μ_{max}, specific growth rate, Y_{ATP}, biomass yield, q_s^{max}, substrate consumption rate.
[b] Carbon balance is the percentage of carbon in metabolized glucose that is recovered in the fermentation products (lactate, ethanol, acetate, 2,3-butanediol, acetoin, and mannitol).
[c] Redox balance is the ratio between [mannitol] + [lactate] + [2,3-butanediol] + 2 × [ethanol] and 2 × ([glucose] − [mannitol]) × 100.
[d] Data from Neves et al. (27).

tions of Mtl1P and mannitol from the data of this experiment alone. Therefore, additional experiments were performed with [1-^{13}C]glucose. The pool of [6-^{13}C]Mtl1P derived from [6-^{13}C]glucose is identical to the pool of [1-^{13}C]Mtl1P in cells that metabolized [1-^{13}C]glucose. By measuring the intensity of resonance at 66.1 ppm, due to [1-^{13}C]Mtl1P, in an experiment with a supply of [1-^{13}C]glucose, it is possible to determine the contribution of [6-^{13}C]Mtl1P to the resonance at 63.8 ppm in the experiment using [6-^{13}C]glucose as a substrate.

The kinetics of glucose consumption, end product formation, and the buildup of pools of intracellular metabolites during [6-^{13}C]glucose catabolism by FI10089 cells were determined (Fig. 4). The glucose consumption rate was 0.06 ± 0.01 μmol min^{-1} mg of protein^{-1}, similar to the value found for the parental strain FI9630 determined under the same experimental conditions (30). The buildup of the Mtl1P pool was very fast during the first minutes and continued at a slower rate while glucose was available, reaching a maximal concentration of 76 ± 1.8 mM (Fig. 4A). The FBP pool became detectable concomitantly with Mtl1P, reaching a maximal concentration of 34 ± 4.7 mM. After glucose exhaustion, Mtl1P and FBP dropped to undetectable levels at a similar rate, and the 3-PGA and

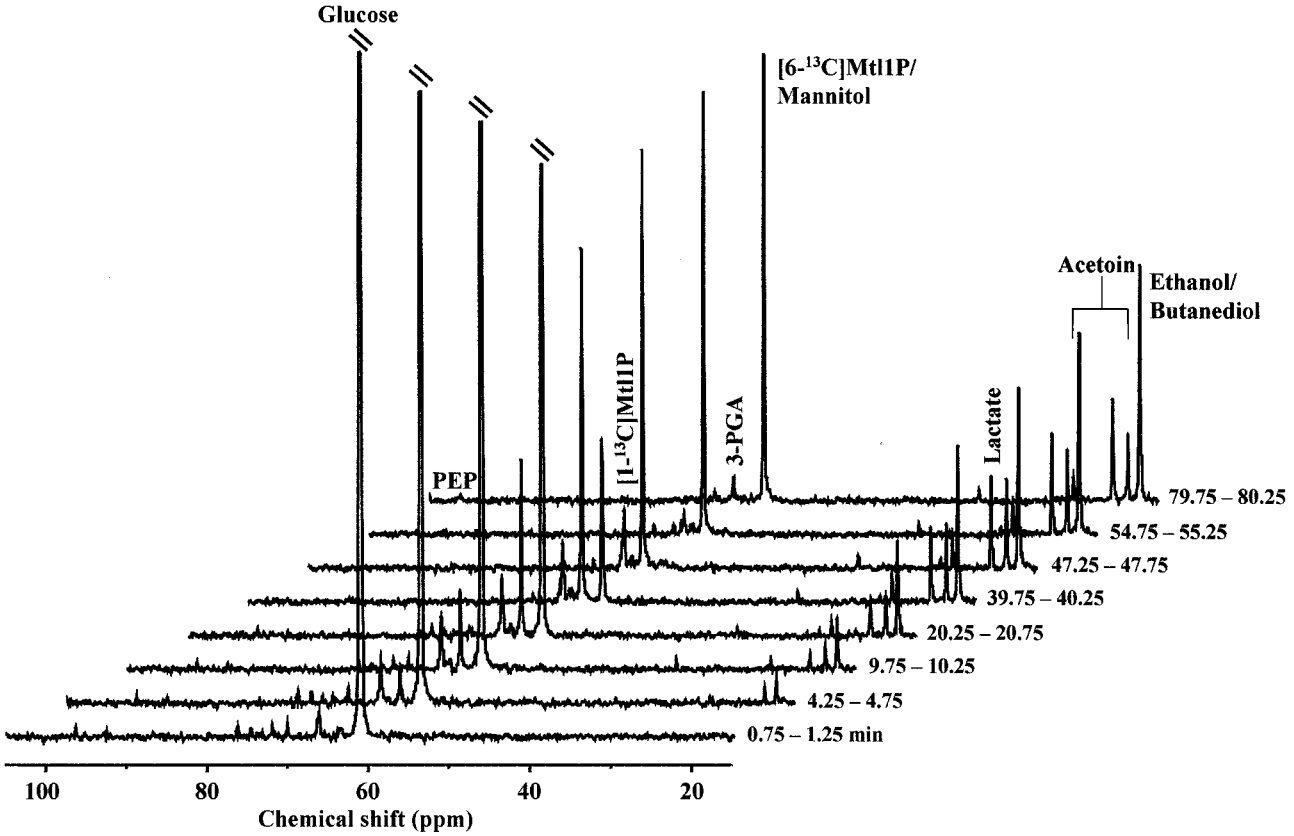

FIG. 3. Sequence of ^{13}C-NMR spectra acquired during the metabolism of 40 mM [6-^{13}C]glucose by a cell suspension of FI10089 under anaerobic conditions at 30°C. Cells were suspended in 50 mM KP_i (pH 6.5) at a concentration of 15 mg of protein ml^{-1}. The pH was kept constant by automatic addition of NaOH. Each spectrum represents 30 s of acquisition. Glucose was added at time zero, and each spectrum was acquired during the indicated interval and processed with a 12-Hz line broadening.

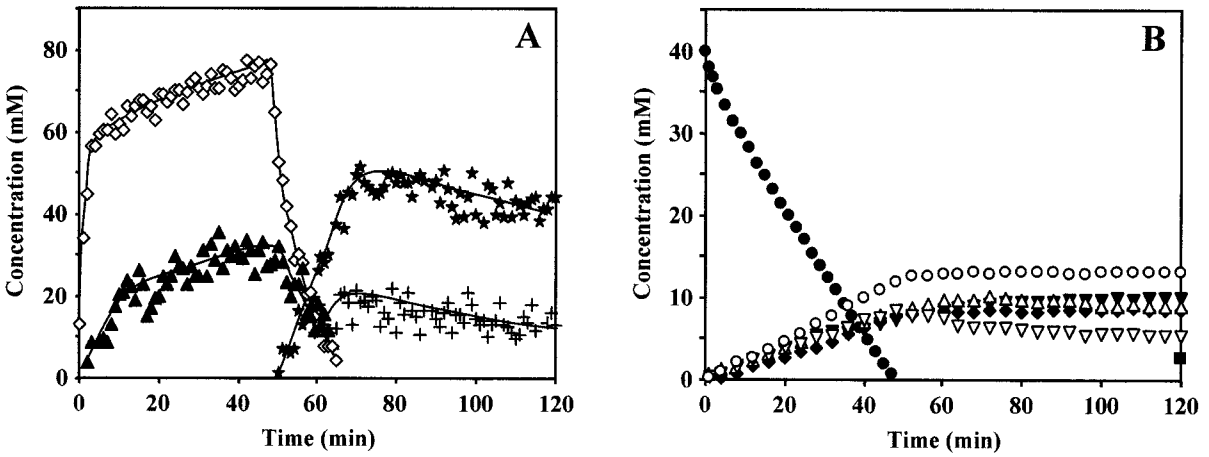

FIG. 4. Kinetics of glucose consumption by nongrowing cells of *L. lactis* FI10089 as determined in vivo by ^{13}C-NMR. (A) Pools of intracellular metabolites. (B) Kinetics of glucose consumption and end product formation. Data are from an individual experiment, except for those representing mannitol and mannitol-1-phosphate pools, which were estimated by combining data from parallel ^{13}C-NMR experiments with a supply of [6-^{13}C]glucose or [1-^{13}C]glucose, as described in the Results section. The error varied from 2% (end products) to 15% in the case of intracellular metabolites. ●, glucose; ▼, 2,3-butanediol; △, ethanol; ◆, lactate; ▽, acetoin; ■, acetate; ○, mannitol; ◇, Mtl1P; ▲, FBP; ★, 3-PGA; +, PEP.

PEP pools started to increase, reaching maximal values of 45.2 ± 2.0 and 17.3 ± 1.4 mM, respectively.

With respect to end products, mannitol (13.1 ± 0.03 mM) was the major metabolite. The determination of the extracellular and intracellular pools of mannitol (as described in Materials and Methods) showed that while glucose was available, the amount of extracellular mannitol continuously increased; after glucose exhaustion, 90% of the mannitol produced was in the extracellular medium (Fig. 5). Other end products of glucose metabolism were 2,3-butanediol (10.4 ± 0.3 mM), ethanol (10.1 ± 0.8 mM), lactate (8.1 ± 0.5 mM), acetoin (5.3 ± 0.7 mM), and acetate (3.1 ± 0.03 mM) (Fig. 4B). In addition, formate (14.1 ± 1.2 mM) was measured by ^{1}H-NMR in NMR sample extracts resulting from these experiments. In all of the experiments performed with nongrowing cells, the carbon and redox balances were on average 95% ± 2% and 98% ± 3%, respectively. Comparisons of the product yields derived from the metabolism of glucose during growth and in nongrowing cells are presented in Table 3.

The characterization of glucose metabolism in FI10091 by in vivo ^{13}C-NMR was also performed (data not shown), yielding results similar to those obtained with FI10089.

Measurements of enzyme activities. Specific activities of LDH and Mtl1PDH were measured in crude extracts derived from cultures of FI10089. LDH activity was not detected, confirming that the *ldh* gene encoded by the *las* operon has been deleted. An activity of 2.5 ± 0.4 U mg of protein^{-1} was determined for Mtl1PDH (reverse reaction, F6P→Mtl1P), a value similar to that measured in the parent strain FI9630 grown under similar conditions (30).

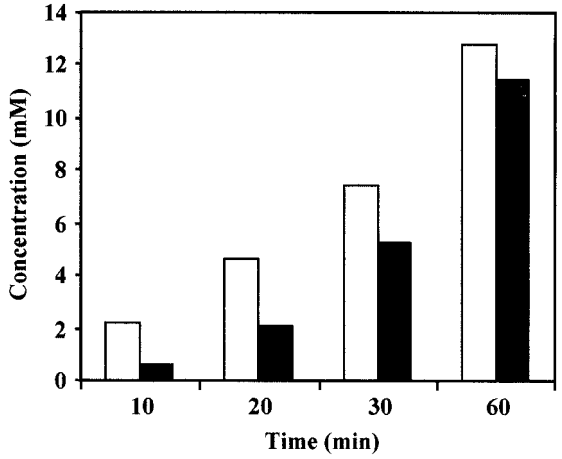

FIG. 5. Total and extracellular mannitol during glucose metabolism in nongrowing cells of *L. lactis* FI10089 as determined by ^{13}C-NMR. For the calculations, the total mannitol was assumed as extracellular. □, total mannitol; ■, extracellular mannitol.

DISCUSSION

The range of potential applications of mannitol in the food industry, given the recently claimed health-promoting properties of this hexitol, goes far beyond its use as a low-calorie sweetener and texturing agent. In this respect, the development of dairy products naturally enriched in mannitol during the process of fermentation should be highly beneficial.

Our previous work on the metabolic characterization of *L. lactis* MG1363 strains deficient in LDH revealed formation of mannitol during glucose catabolism. However, these strains were able to metabolize mannitol, and this metabolite was consumed once glucose was depleted (27, 28, 30). To obtain an effective mannitol-producing strain, the mannitol transport system of an LDH-deficient strain was disrupted: the *mtlA* or *mtlF* genes were deleted by gene replacement in *L. lactis* FI9630, a food-grade LDH-deficient strain derived from MG1363. The new constructs, FI10091 and FI10089, do not possess any selection marker and are suitable for use in the food industry.

Nongrowing cells of these double mutants produced manni-

TABLE 3. Comparison of product yields from glucose metabolism by the double mutant FI10089 and the parent strain FI9630 during growth or in nongrowing cells

Cells	% Product yield (mol of product/mol of glucose consumed)						
	Mannitol	Lactate	Formate	Acetate	Ethanol	2,3-Butanediol	Acetoin
FI10089 (nongrowing)[a]	32.8	22.3	31.6	7.8	25.3	25.9	13.3
FI9630 (nongrowing)[b]		44.3	45.3	8.0	39.8	41.0	6.3
FI10089 (growing)[c]	3.9	8.8	132.8	44.3	81.3	9.7	15.9
FI9630 (growing)[d]	1.3	6.9	139.7	47.1	86.5	9.1	15.8

[a] Average values of three independent experiments; carbon and redox balances were 95% ± 2% and 98% ± 3%, respectively. Data from individual experiments do not differ by more than 15%.
[b] Data from Neves et al. (30).
[c] Values of a representative experiment. Carbon and redox balances were 98 and 96%, respectively.
[d] Values of a representative experiment. Carbon and redox balances were 98 and 96%, respectively.

tol as an end product of glucose metabolism with high yield. Our NMR results showed conversion of Mtl1P to mannitol (Fig. 4). Thus, synthesis of mannitol from glucose proceeds via Mtl1P and implies the involvement of an enzyme responsible for converting Mtl1P to mannitol (Fig. 6). It is intriguing that no genes coding for mannitol-1-phosphatase have been identified in *L. lactis* or in any other organism, with the exception of *Eimeria tenella* (22).

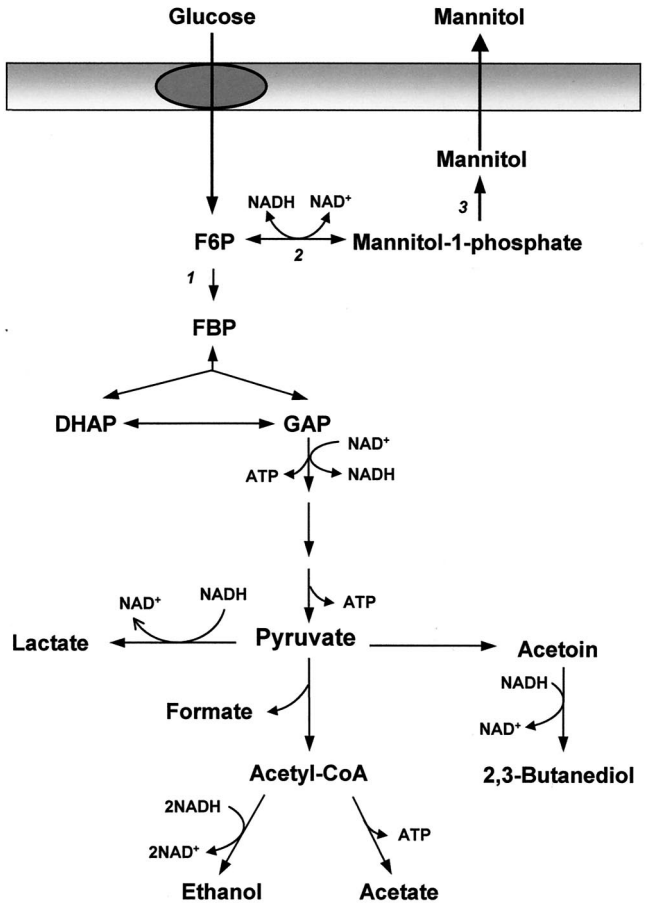

FIG. 6. Pathways proposed for glucose metabolism and mannitol synthesis in *L. lactis* FI10089 carrying a double deletion of the genes *ldh* and *mtlF*. 1, phosphofructokinase; 2, mannitol-1-phosphate dehydrogenase; 3, mannitol-1-phosphatase.

In *Lactobacillus plantarum*, Ferain et al. (7) proposed that the conversion of Mtl1P to mannitol is mediated by a hypothetical phosphatase activity of EII^{Mtl} (encoded by *mtlA*), which is additional to its role in mannitol transport. The observed production of mannitol in strain FI10091 shows that this hypothesis does not hold for *L. lactis*, since the deletion of the *mtlA* gene would eliminate both the mannitol transport and the mannitol-1-phosphatase activities of EII^{Mtl}. Therefore, an enzyme other that EII^{Mtl} is responsible for dephosphorylation of Mtl1P.

It is well known that in *L. lactis*, glucose exerts a strong exclusion or expulsion effect on some PTS substrates, including mannitol (26). During inducer expulsion, accumulated sugar-P is hydrolyzed by an intracellular phosphatase and expelled from the cells. A membrane-associated phosphatase that initiates the inducer expulsion process has been reported to show affinity for Mtl1P (45). Although BLASTP searches of the available sequences of *L. lactis* IL1403 or MG1363 gave no hits for mannitol-1-phosphatase, there are at least four genes assigned as putative protein phosphatases in the *L. lactis* IL1403 genome. So there are a number of potential candidates to catalyze the dephosphorylation of Mtl1P to mannitol.

LDH-deficient strains of *L. lactis* metabolize sugars via a mixed-acid fermentation as a way to fulfill the redox balance (2, 14, 27, 28, 30, 31). In the FI10089 strain studied in this work, as in other LDH-deficient strains, glucose catabolism under anaerobic conditions proceeded via a mixed-acid fermentation in either growing or resting cells. Growth on glucose yielded formate, ethanol, and acetate as the main end products. In contrast, glucose metabolism by nongrowing cells produced mannitol as a major end product and approximately equimolar amounts of lactate, ethanol, and 2,3-butanediol. The comparison of the pattern of end products in nongrowing cells of the strain deficient in the transport of mannitol (FI10089) and the parent strain (FI9630) reflects the expected increased utilization of NADH-oxidizing reactions, other than Mtl1PDH, for balancing redox equivalents (Table 3). Interestingly, the relative proportions of the reduced products derived from pyruvate (lactate, ethanol, and 2,3-butanediol) were similar in both strains, but the acetoin/2,3-butanediol ratio was considerably enhanced in the mannitol-producing mutant. This is explained by the efficient utilization of the Mtl1PDH pathway for the regeneration of NAD^+, which is expected to decrease the $NADH/NAD^+$ ratio in the cell and thus alleviate the pressure

on the NAD^+-regenerating reactions downstream of the pyruvate node.

Lactate production was observed despite a stable LDH deletion in these strains, suggesting the existence of an alternative *ldh* gene that is expressed when the *ldh* gene in the *las* operon is deleted. In fact, four genes homologous to *ldh* genes from other organisms are present in the genome of *L. lactis* IL1403 (1), and an alternative LDH has been isolated from an LDH-deficient strain obtained by single-crossover deletion of the *ldh* gene in *L. lactis* MG1614 (P. Gaspar, P. Coelho, A. R. Neves, C. A. Shearman, M. J. Gasson, A. Ramos, and H. Santos, unpublished results).

Mannitol production was drastically depressed during growth as compared to the level in nongrowing cells. Several lines of evidence suggest three different aspects that could control mannitol production during growth, namely: (i) the F6P pool, (ii) ATP demand, and (iii) pressure to regenerate NAD^+. The K_m values of Mtl1PDH for F6P in *L. lactis* have not been reported; however, a K_m value of 1.7 mM in *Streptococcus mutans* has been found (BRENDA enzyme database at http://www.brenda.uni-koeln.de). Assuming a similar K_m for *L. lactis* Mtl1PDH, the F6P concentration could constitute a limiting factor for mannitol production, since the F6P pools are generally low. In nongrowing cells, we were unable to detect ^{13}C-NMR resonances due to F6P during the metabolism of glucose, meaning that the pool of F6P is below the detection limit (3 mM); moreover, a maximum of 0.7 mM F6P was measured for the intracellular pool in cell extracts derived from *L. lactis* MG5267 during the metabolism of glucose under nongrowing conditions (29). Because F6P is also a substrate of phosphofructokinase (PFK), the affinity of this enzyme should be taken into account in this discussion. A K_m of 0.25 mM was reported for the *L. lactis* enzyme (BRENDA database), and therefore, as far as affinity for the substrate is concerned, PFK can compete efficiently for the common substrate. The activities of these enzymes are comparable: 2.5 U mg of $protein^{-1}$ for Mtl1PDH in cell extracts of FI10089 (this work), and 1 U mg of $protein^{-1}$ for PFK in cell extracts of MG1363 (27). Therefore, the overexpression of the gene encoding Mtl1PDH could be potentially useful to improve mannitol production during growth.

The ATP demand for anabolic processes could limit mannitol synthesis during growth. One could consider increasing the ATP supply through the operation of alternative ATP-generating pathways, such as citrate or arginine catabolism, thus allowing conversion of more sugar carbon to mannitol.

On the other hand, if mannitol production depends on the $NADH/NAD^+$ pools, an increased pressure to oxidize NADH by pathways other than ethanol synthesis could improve accumulation of mannitol in actively growing cells. A comparison of ethanol production in growing and resting cells suggests that enhanced pressure to regenerate NAD^+ (obtained by inactivation of acetaldehyde dehydrogenase) is required to achieve higher yields of mannitol. The ATP yield is expected to decrease if considerable amounts of glucose are deviated to mannitol, but it appears that there is still a reasonable working range to reduce the ATP yield without affecting severely the growing ability of *L. lactis*, since the ATP yield and the specific growth rate of strain FI10089 were not considerably lower than those for the model strain MG1363 (Table 2).

The present work demonstrates that in *L. lactis*, mannitol is transported exclusively by the $PEP\text{-}PTS^{Mtl}$ encoded in *L. lactis* by *mtlARFD*, since deletions of the *mtlA* or *mtlF* genes led to an inability to utilize mannitol. However, blockage of mannitol transport is not enough to obtain high levels of mannitol synthesis during growth of *L. lactis*, which would be an essential feature of a mannitol overproducer in dairy fermentations. A combination of strategies that increase the pressure to regenerate NAD^+, such as deletion of acetaldehyde dehydrogenase and overproduction of Mtl1PDH (to enhance flux via this enzyme in detriment of PFK), will be attempted. Furthermore, insight into how the transcription of the mannitol operon in *L. lactis* is regulated will provide useful clues for manipulating mannitol production in this organism.

In conclusion, the engineering strategy followed here led to the construction of *L. lactis* strains able to produce a high yield of mannitol (33%) from glucose. These features make FI10089 a strain with potential interest for the production of this polyol by resting cells in processes involving, for example, cell recycling or cell immobilization. Moreover, the yield of mannitol can be further improved by implementing genetic manipulations along the lines discussed above. Work is in progress to enhance mannitol production by *L. lactis*, especially during growth.

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