

Overproduction of Heterologous Mannitol 1-Phosphatase: a Key Factor for Engineering Mannitol Production by *Lactococcus lactis*

H. Wouter Wisselink,^{1,2} Antoine P. H. A. Moers,² Astrid E. Mars,^{1,2} Marcel H. N. Hoefnagel,³
Willem M. de Vos,¹ and Jeroen Hugenholtz^{1,4*}

Wageningen Centre for Food Sciences (WCFS)¹ and Wageningen University and Research Centre—Agrotechnology and Food Innovations,² Wageningen, NIZO Food Research, Ede,⁴ and Centre for Biological Medicines and Medical Technology, RIVM, Bilthoven,³ The Netherlands

Received 26 July 2004/Accepted 18 October 2004

To achieve high mannitol production by *Lactococcus lactis*, the mannitol 1-phosphatase gene of *Eimeria tenella* and the mannitol 1-phosphate dehydrogenase gene *mitD* of *Lactobacillus plantarum* were cloned in the nisin-dependent *L. lactis* NICE overexpression system. As predicted by a kinetic *L. lactis* glycolysis model, increase in mannitol 1-phosphate dehydrogenase and mannitol 1-phosphatase activities resulted in increased mannitol production. Overexpression of both genes in growing cells resulted in glucose-mannitol conversions of 11, 21, and 27% by the *L. lactis* parental strain, a strain with reduced phosphofructokinase activity, and a lactate dehydrogenase-deficient strain, respectively. Improved induction conditions and increased substrate concentrations resulted in an even higher glucose-to-mannitol conversion of 50% by the lactate dehydrogenase-deficient *L. lactis* strain, close to the theoretical mannitol yield of 67%. Moreover, a clear correlation between mannitol 1-phosphatase activity and mannitol production was shown, demonstrating the usefulness of this metabolic engineering approach.

Mannitol is a reduced form of fructose and is produced by a variety of microorganisms including bacteria, yeasts, and fungi. Besides the ability of several organisms to maintain their redox balance by the production of mannitol (9, 21, 22), mannitol has a physiological function in microorganisms as an osmolyte (16) and can serve as a protecting agent. It has been reported that mannitol enhances survival of *Lactococcus lactis* cells during drying processes (10). The viability of starter cultures of *L. lactis*, a lactic acid bacterium (LAB) extensively used in dairy industry, may thus be enhanced by mannitol production. In addition, the use of a mannitol-producing *L. lactis* may result in fermented products with extra value, since mannitol is assumed to have several beneficial effects as a food additive. It can serve as an antioxidant (4, 5, 25, 26) and as a low-calorie sweetener that can replace sucrose (6, 8).

In heterofermentative LABs such as *Leuconostoc mesenteroides*, mannitol is formed from fructose in a single conversion by mannitol dehydrogenase, and fructose-to-mannitol conversion rates of >90% are common (13, 24, 27). In contrast, most homofermentative LABs, such as *Lactococcus lactis*, do not normally produce mannitol. Mannitol formation in homofermentative LABs is limited to strains whose ability to regenerate NAD to fulfill the redox balance is severely hampered. In these strains, mannitol 1-phosphate dehydrogenase (M1PDH) and mannitol 1-phosphatase (M1Pase) are the enzymes involved in the mannitol biosynthesis route (Fig. 1). Transient formation of high concentrations of intracellular mannitol (90 mM) and mannitol 1-phosphate (76 mM) were detected in high-density nongrowing cell suspensions of a lactate dehydrogenase (LDH)-deficient *L. lactis* strain (22). During growth, only small amounts of mannitol (<0.4 mM) were transiently

produced extracellularly (23). Recently, inactivation of the mannitol transport system in an LDH-deficient *L. lactis* strain resulted in high extracellular mannitol production. About one-third of glucose was converted into mannitol by nongrowing cells, and no undesired mannitol utilization after glucose depletion was observed (12). In these strains, mannitol was produced to fulfill the redox balance during sugar metabolism, since NAD is regenerated in the conversion of fructose 6-phosphate into mannitol 1-phosphate by M1PDH.

To improve mannitol production by *L. lactis*, we further employed a metabolic engineering strategy. Recently, we reported that overexpression of the *mitD* gene, encoding M1PDH from *Lactobacillus plantarum*, resulted in low mannitol production by growing cultures of *L. lactis* (30). This was supported by a kinetic mannitol production model of *L. lactis* (29), which was based on a *L. lactis* glycolysis model (15) (available at <http://jij.biochem.sun.ac.za>) and expanded by introducing a mannitol branch (29). Moreover, the metabolic model predicted that M1Pase has a high level of control of the mannitol flux and that increasing its activity in *L. lactis* would result in substantial mannitol production.

Based on the predictions of the mannitol model and taking into account previous engineering results, the cloning and expression of a M1Pase gene in *L. lactis* would be a logical step to increase mannitol production. To the best of our knowledge, there are no M1Pase genes annotated in the genome databases of LABs (2, 18; <http://genome.jgi-psf.org>) or other bacteria. However, a specific M1Pase gene in *Eimeria tenella*, a protozoan parasite (20), has been described. In these parasites and some fungi, M1Pase has an important role in the mannitol cycle. In the mannitol cycle of *E. tenella*, mannitol is produced as reserve carbohydrate source for spores; during sporulation, mannitol is consumed. Storage of mannitol is considered to be essential for parasite viability (20).

In this work, we investigated the effect of overexpression of

* Corresponding author. Mailing address: NIZO Food Research, Kernhemseweg 2, 6718 ZB Ede, The Netherlands. Phone: (31) 318 659511. Fax: (31) 317 650400. E-mail: Jeroen.Hugenholtz@nizo.nl.

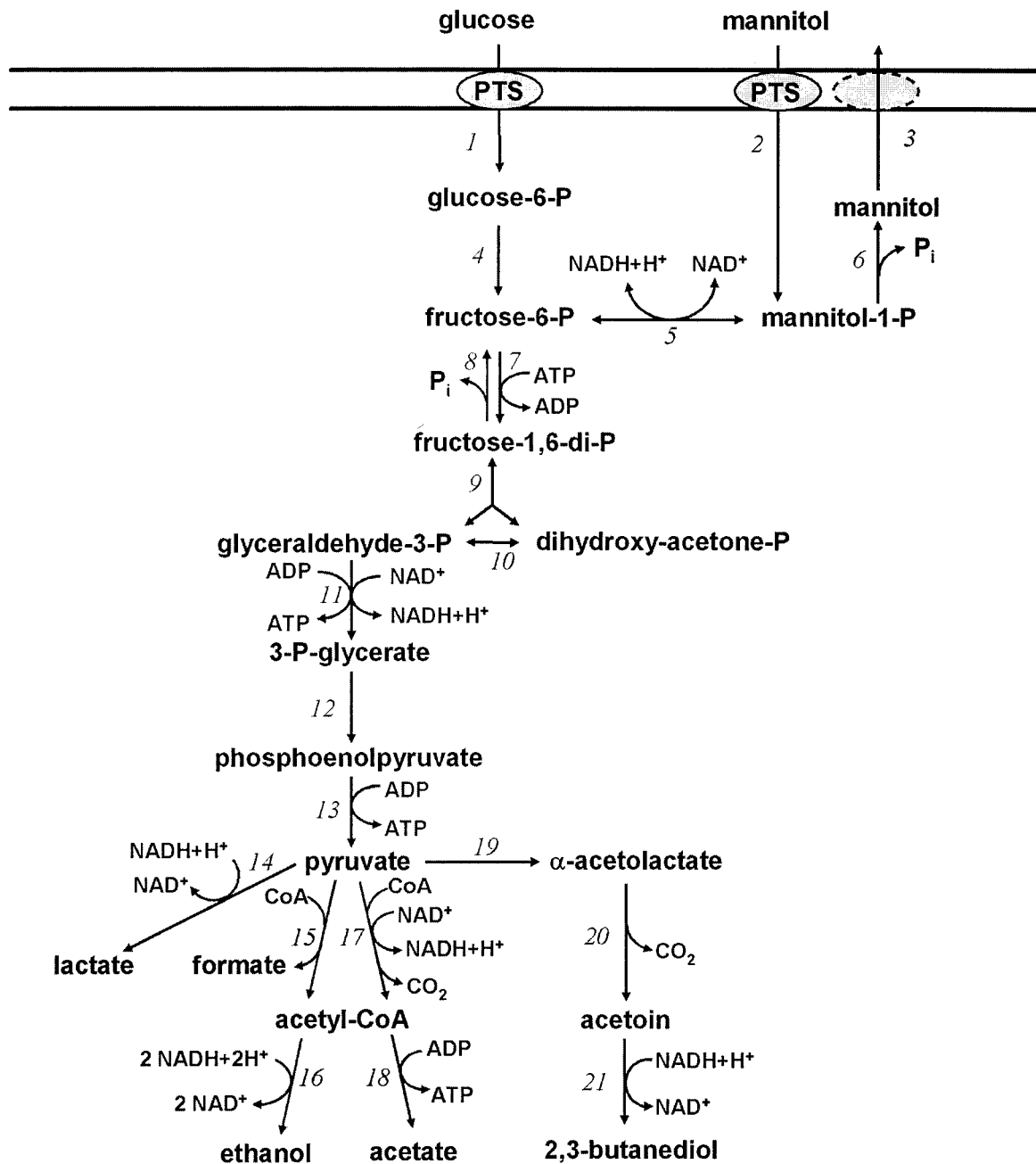


FIG. 1. Proposed pathway for glucose and mannitol metabolism of *Lactococcus lactis*. The steps are as follows: 1, phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS); 2, mannitol-specific PTS; 3, mannitol export (mechanism unknown); 4, phosphoglucose isomerase; 5, M1PDH; 6, M1Pase; 7, 6-phosphofructokinase; 8, fructose-diphosphatase; 9, fructose 1,6-diphosphate aldolase; 10, triosephosphate isomerase; 11, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase; 12, phosphoglyceromutase and enolase; 13, pyruvate kinase; 14, LDH; 15, pyruvate-formate lyase; 16, acetaldehyde dehydrogenase and alcohol dehydrogenase; 17, pyruvate dehydrogenase; 18, acetate kinase; 19, α -acetolactate synthase; 20, α -acetolactate decarboxylase; and 21, 2,3-butanediol dehydrogenase.

the M1Pase gene from *E. tenella* on the mannitol-producing capacities of *L. lactis* NZ9000, LDH-deficient strain NZ9010 (3, 14), and strain HWA217 with reduced phosphofructokinase activity (1). High mannitol production by growing cells of the *L. lactis* strains overexpressing the M1Pase gene and *mtlD* gene was observed, and a correlation between the M1Pase activity and the mannitol production was shown.

MATERIALS AND METHODS

***L. lactis* strains, plasmids, and media.** The *L. lactis* strains and plasmids used in this report are listed in Table 1. *L. lactis* strains were grown at 30°C in M17 broth (Oxoid), supplemented with 0.5% glucose. For (semi)anaerobic cultivations, cells were grown in batch cultures in closed 50-ml tubes without aeration. When cells were grown aerobically, shaking flasks with baffles were used. When applicable, chloramphenicol and erythromycin were supplemented at 10 and 5 $\mu\text{g/ml}$, respectively. Growth was monitored by measuring the optical density at

TABLE 1. *L. lactis* strains and plasmids used in this work

Strain or plasmid	Characteristics	Reference(s) or source
Strains		
NZ9000	MG1363 <i>pepN::hisRK</i>	19
NZ9010	NZ9000 <i>ldh::ery</i> ; Ery ^r	3, 14
HWA217	Reduced phosphofructokinase activity	1
NZ9000(pNZ8148)	NZ9000 containing pNZ8148 plasmid	This work
NZ9000(pWW002)	NZ9000 containing pWW002 plasmid	This work
NZ9010(pWW002)	NZ9010 containing pWW002 plasmid	This work
HWA217(pWW002)	HWA217 containing pWW002 and pNZ9530 plasmids	This work
NZ9000(pWW003)	NZ9000 containing pWW003 plasmid	This work
NZ9010(pWW003)	NZ9010 containing pWW003 plasmid	This work
HWA217(pWW003)	HWA217 containing pWW003 and pNZ9530 plasmids	This work
Plasmids		
PQE60-M1Pase	<i>E. coli</i> expression vector pQE60 carrying the <i>E. tenella</i> M1Pase gene	20
pNZ8148	pNZ8048 derivative; Cm ^r , lactococcal cloning and expression vector with <i>nisA</i> promoter upstream of a multiple cloning site	19
pNZ9530	Ery ^r , <i>hisRK</i>	17
pNZ- <i>mtlD</i>	pNZ8148 carrying <i>L. plantarum mtlD</i> gene fused to <i>nisA</i> promoter	30
pWW002	pNZ8148 carrying <i>E. tenella</i> M1Pase gene fused to <i>nisA</i> promoter	This work
pWW003	pNZ- <i>mtlD</i> with <i>E. tenella</i> M1Pase gene cloned downstream of <i>mtlD</i>	This work

600 nm (OD₆₀₀) with an Ultrospec 2000 spectrophotometer (Pharmacia Biotech). For induction of M1PDH and M1Pase activity, 0.1 to 10 ng of nisin/ml was added to a growing culture at an OD₆₀₀ of 0.5 or at another OD₆₀₀ level when indicated.

To induce M1PDH activity in strain NZ9000(pWW002), cells were precultured (semi)anaerobically in M17 broth containing 0.5% mannitol prior to growth experiments in M17 broth supplemented with glucose, as described above.

Construction of plasmid pWW002 and pWW003. In our previous work, the *mtlD* gene encoding M1PDH from *L. plantarum* was cloned into the nisin-inducible expression vector pNZ8148 (30).

The M1Pase gene of *E. tenella* was cloned in the lactococcal expression vector pNZ8148. For this, the M1Pase gene was amplified by PCR from *Escherichia coli* expression vector pQE-60 (QIAGEN) containing the M1Pase gene from *E. tenella* (20), using the primers M1PaseET-1FW (5'-GGGTCTAGAAAGCCATG GCAGAGACTGAGTGG-3') and M1PaseET-1RV (5'-GGCCGAGCTCTTA GGGTTTAGCGTTTGG-3'), with introduced XbaI, NcoI, and SacI digestion sites, respectively (underlined). The M1Pase gene was cloned into *E. coli* cloning vector pCR4-TOPO and the resulting plasmid was transformed to *E. coli* (TOPO TA cloning kit; Invitrogen). The sequence of the *E. tenella* M1Pase gene (20) was verified by sequencing the cloned PCR product (Baseclear, Leiden, The Netherlands). The NcoI-SacI-digested PCR product of the M1Pase gene was cloned into pNZ8148, resulting in pWW002 containing the M1Pase gene fused to the *nisA* promoter. The M1Pase gene was also cloned downstream of *mtlD* in pNZ-*mtlD* by ligation of XbaI-SacI-digested M1Pase PCR product into XbaI-SacI-digested pNZ-*mtlD*, resulting in pWW003.

The plasmids pWW002 and pWW003 were transformed into the *L. lactis* strains NZ9000, NZ9010, and HWA217 (Table 1) by electroporation. Plasmid pNZ9530, containing the *nisR* and *nisK* genes, was cotransformed in *L. lactis* HWA217 to allow nisin-induced expression of *mtlD* and the M1Pase gene in strain HWA217 (17).

Analysis of fermentation products and glucose consumption. During growth, samples were taken from the *L. lactis* cultures and centrifuged for 1 min at 10,000 × g; the supernatants were stored at -20°C until further analysis. Lactate, acetate, formate, glucose, mannitol, ethanol, 2,3-butanediol, and acetoin were detected in the supernatants by a refractive index detector (Waters 2414) after separation by high-performance liquid chromatography with a 30-cm IOA-1000 ion exclusion column (Alltech, Breda, The Netherlands) with 3 mM sulfuric acid as an eluent, at a flow rate of 0.4 ml/min and a temperature of 90°C.

Preparation of cell extracts. Cell cultures (each, 50 ml) were harvested for enzyme assays at an OD₆₀₀ of approximately 1.2 or 2 h after induction with nisin. Cell extracts were prepared by disruption of cells by glass beads. For this, 50 ml of cell culture was centrifuged (4°C; 20 min at 2000 × g), and the cell pellets were washed with 50 mM MES (morpholineethanesulfonic acid) buffer (pH 7.0) and resuspended in 2 ml 50 mM MES buffer (pH 7.0). Subsequently, 1 ml of cell suspension was added to 1.0 g of 0.1-mm-diameter zirconia-silica beads (BioSpec Products, Inc.) in a 2-ml Eppendorf cup, and cells were disrupted by vigorous

shaking at 4°C for 5 min. Cell debris was removed by centrifugation (4°C; 2 min at 10,000 × g), and the supernatant was used for all enzyme assays. The protein content of the extracts was determined by the BCA protein assay (Pierce), with bovine serum albumin as the standard.

Enzyme assays. The reduction of fructose 6-phosphate by M1PDH was assayed as reported previously (30), with the following modifications: a reaction mixture containing 50 mM sodium phosphate buffer (pH 6.0) with 0.5 mM NADH was used. For the strains containing plasmid pWW003 and overproducing M1PDH, cell extract was added to the reaction mixture to give a final concentration of 5 to 10 µg of protein/ml. For strains not overproducing M1PDH, cell extract was added to a final concentration of 250 µg of protein/ml. The reaction was initiated by the addition of 1 mM fructose 6-phosphate. M1PDH activities were determined from the rate of NADH oxidation or formation at 30°C by measuring the absorbance at 340 nm with a microplate reader (Tecan Safire, Salzburg, Austria).

M1Pase activity was basically determined in a reaction mixture as reported previously (30), with the only modification in the amount of cell extract used (20 to 50 µg of protein/ml). The inorganic phosphate formed was determined by a modified protocol from the Sigma Diagnostics inorganic phosphate kit. The absorbance at 660 nm was measured with a microplate reader (Tecan Safire).

RESULTS

Overexpression of the M1Pase gene and *mtlD*. To investigate whether overproduction of M1Pase contributes to mannitol production in *L. lactis*, the M1Pase gene of *E. tenella* was cloned in the nisin-inducible expression plasmid pNZ8148, resulting in pWW002. To overproduce M1Pase and M1PDH simultaneously in *L. lactis*, the M1Pase gene was also cloned downstream of the *mtlD* gene in pNZ-*mtlD*, resulting in pWW003. Both plasmids were transformed to *L. lactis* strains NZ9000, HWA217, and NZ9010 (Table 1). Induction of strain NZ9000 harboring pWW002 with 1.0 ng of nisin/ml resulted in 0.9 µmol min⁻¹ mg of protein⁻¹ of M1Pase activity, while no M1Pase activity was detected in extracts of the control strain NZ9000 harboring pNZ8148 (Table 2). Despite the presence of M1Pase activity, strain NZ9000(pWW002) did not produce any mannitol, and analysis of the fermentation products showed a typical homolactic pattern (data not shown). Increased M1Pase activity was also detected in extracts of nisin-induced cells of strains NZ9010(pWW002) and HWA217

TABLE 2. Mannitol productions related to M1Pase and M1PDH activity in *L. lactis*^a

<i>L. lactis</i> strain	Nisin (ng/ml)	Enzyme activity ($\mu\text{mol min}^{-1} \text{mg}$ of protein ⁻¹)		Mannitol (mM)
		M1Pase	M1PDH	
NZ9000(pNZ8148)	0	<0.01	<0.01	<0.1
	1	<0.01	<0.01	<0.1
NZ9000(pWW002)	0	0.02	<0.01	<0.1
	1	0.90	<0.01	<0.1
NZ9000(pWW003)	0	<0.01	<0.01	<0.1
	1	0.27	2.2	2.5

^a M1Pase and M1PDH activities in cell extracts of *L. lactis* NZ9000 containing pWW002 and pWW003. *L. lactis* was grown anaerobically on 0.5% glucose, nisin was added at an OD_{600} of 0.5, and cell extract was prepared from cells harvested after 2 h of nisin induction. The final concentration of mannitol (in micromoles) produced from 0.5% of glucose is given. Values of mannitol or <0.1 mM indicate that no mannitol was detected above the detection limit of 0.1 mM.

(pWW002), but no mannitol production was observed (data not shown).

Thus, overexpression of the M1Pase gene alone did not result in mannitol production. However, simultaneous expres-

sion of both the M1Pase gene and *mtlD* in *L. lactis* NZ9000 harboring pWW003 resulted in production of 2.3 mM mannitol in the supernatant, corresponding to a glucose-mannitol conversion rate of 10% (Table 2). Forward M1PDH activity of 2.2 and M1Pase activity of $0.27 \mu\text{mol min}^{-1} \text{mg}$ of protein⁻¹ were detected in the cell extracts of strain NZ9000(pWW003) (Table 2). The lower M1Pase activity in strain NZ9000(pWW003), compared to M1Pase activity in strain NZ9000(pWW002), was presumably caused by the absence of a good ribosome binding site upstream of the M1Pase gene in plasmid pWW003.

Nisin-dependent mannitol production by overproduction of M1PDH and M1Pase. The *L. lactis* strains harboring pWW003 were grown anaerobically on glucose, and M1Pase and M1PDH production was induced with increasing nisin concentrations, which resulted in increasing enzyme activities of both enzymes (Fig. 2). For strains NZ9000(pWW003) and NZ9010(pWW003), maximum M1Pase activity was reached at 1 ng of nisin/ml. For the HWA217(pWW003) strain, the highest enzyme activities were reached with 10 ng of nisin/ml. Concomitant to the increasing M1PDH and M1Pase activities, a significant increase

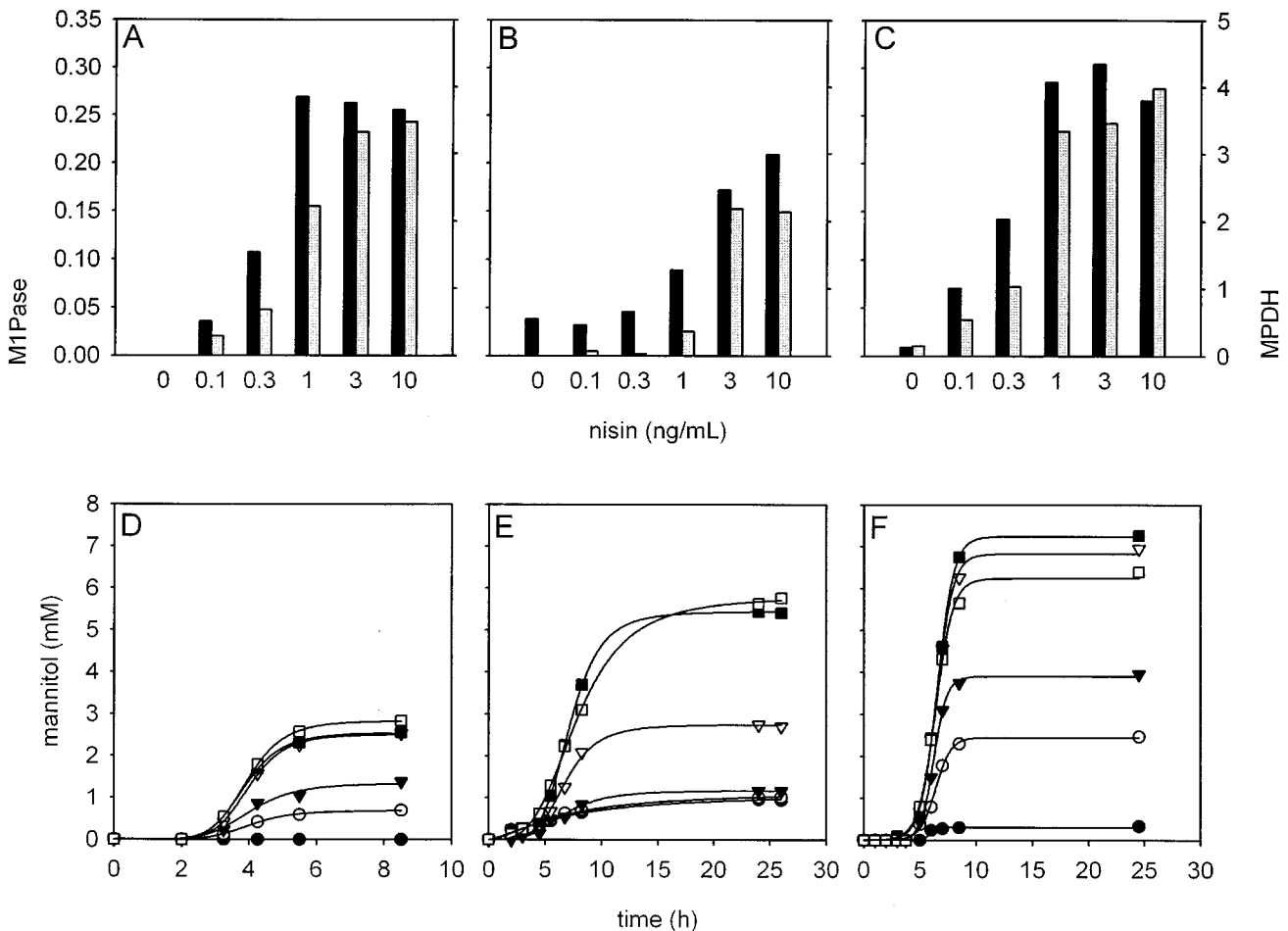


FIG. 2. M1Pase and M1PDH activities (in micromoles per minute per milligram of protein) and mannitol production of *L. lactis* overexpressing the M1Pase gene of *E. tenella* and the M1PDH gene *mtlD* from *L. plantarum*. Cells were grown anaerobically on M17 broth supplemented with 0.5% glucose; M1PDH and M1Pase production was induced with 0, 0.1, 0.3, 1.0, 3.0, and 10 ng of nisin/ml at an OD_{600} of 0.5; and cells were harvested 2 h after induction. Mannitol production during growth on glucose was recorded. *L. lactis* strains used are as follows: NZ9000(pWW003) (A and D), HWA217(pWW003) (B and E), and NZ9010(pWW003) (C and F). Black bar, M1Pase; grey bar, M1PDH (A to C); ●, ○, 0.1; ▼, ▽, 1.0; ■, ▽, 3.0; and □, 10 ng of nisin/ml.

TABLE 3. Product formation by *L. lactis* strains overproducing *E. tenella* M1Pase and *L. plantarum* M1PDH^a

<i>L. lactis</i> strain	Initial glucose (%)	Nisin (ng/ml)	Induction (OD ₆₀₀)	Ox	End products							Recovery
					Lactate	Formate	Acetate	Acetoin	2,3-Butanediol	Ethanol	Mannitol	
NZ9000(pNZ8148)	0.5	0	0.5	-	1.96	0.11	0.01	<0.02	<0.01	0.08	<0.003	1.0
	0.5	1	0.5	-	1.93	0.09	0.02	<0.02	<0.01	0.07	<0.003	1.0
NZ9000(pWW003)	0.5	0	0.5	-	1.93	0.10	0.04	<0.02	<0.01	0.04	<0.003	1.0
	0.5	0.1	0.5	-	1.85	0.15	0.06	<0.02	<0.01	0.05	0.03	1.0
	0.5	0.3	0.5	-	1.79	0.16	0.08	<0.02	<0.01	0.06	0.05	1.0
	0.5	1	0.5	-	1.66	0.14	0.08	<0.02	<0.01	0.03	0.10	0.98
	0.5	3	0.5	-	1.65	0.12	0.09	<0.02	<0.01	0.02	0.10	0.98
	0.5	10	0.5	-	1.63	0.10	0.08	<0.02	<0.01	0.02	0.11	0.98
	0.5	3	0.5	+	1.39	<0.02	0.27	0.02	<0.01	<0.02	0.08	0.94
HWA217(pWW003)	0.5	0	0.5	-	2.01	0.22	0.08	<0.02	<0.01	0.03	0.036	1.1
	0.5	0.1	0.5	-	1.95	0.18	0.08	<0.02	<0.01	<0.02	0.038	1.1
	0.5	0.3	0.5	-	1.92	0.19	0.08	<0.02	<0.01	<0.02	0.043	1.1
	0.5	1	0.5	-	1.79	0.19	0.11	<0.02	<0.01	<0.02	0.10	1.1
	0.5	3	0.5	-	1.55	0.16	0.15	<0.02	<0.01	<0.02	0.20	1.0
	0.5	10	0.5	-	1.59	0.15	0.16	<0.02	<0.01	<0.02	0.21	1.0
	0.5	3	0.5	+	1.33	<0.02	0.53	0.02	<0.01	<0.02	0.03	0.97
NZ9010(pWW003)	0.5	0	0.5	-	0.28	1.14	0.40	0.04	0.22	0.78	0.01	1.0
	0.5	0.1	0.5	-	0.20	1.03	0.42	0.04	0.18	0.67	0.09	0.96
	0.5	0.3	0.5	-	0.17	1.00	0.43	0.05	0.16	0.61	0.14	0.96
	0.5	1	0.5	-	0.16	0.73	0.31	0.11	0.11	0.45	0.26	0.94
	0.5	3	0.5	-	0.18	0.76	0.33	0.13	0.11	0.48	0.27	1.0
	0.5	10	0.5	-	0.19	0.79	0.34	0.12	0.10	0.47	0.25	0.98
	0.5	3	0.5	+	0.02	<0.02	0.41	0.57	<0.01	<0.02	0.10	0.88
	0.5	3	0.1	-	0.12	0.71	0.46	0.04	0.07	0.31	0.39	0.94
	2.0 ^b	0	0.1	-	0.95	0.59	0.07	0.10	0.19	0.48	0.03	1.1
	2.0 ^b	3	0.1	-	0.20	0.32	0.28	0.04	0.16	0.15	0.50	1.0

^a The initial glucose concentration, amount of nisin used for induction (in nanograms per milliliter), induction OD₆₀₀ values, culture conditions (oxic or anoxic), and the product concentrations in moles produced per mole of glucose are presented. Ox, oxic conditions.

^b Glucose was not completely consumed (see Fig. 3).

in mannitol production in all three strains was also observed (Fig. 2). Moreover, increasing nisin concentrations resulted in higher final concentrations of mannitol. In agreement with the measured M1Pase activities, concentrations higher than 1.0 ng of nisin/ml did not result in much higher mannitol production in strains NZ9000(pWW003) and NZ9010(pWW003). Maximal mannitol production by *L. lactis* strain HWA217 (pWW003) with reduced PFK activity was reached at 10 ng of nisin/ml, although growth and glucose consumption were slightly inhibited at this nisin concentration (data not shown). At maximum induction conditions, glucose-to-mannitol conversions of 11 and 21% were observed for strains NZ9000 (pWW003) and HWA217(pWW003) (Table 3). The largest amount of mannitol was produced by the LDH-deficient strain NZ9010(pWW003). Up to 7.3 mM mannitol was produced from 26.8 mM glucose, corresponding to a glucose-to-mannitol conversion of 27% (Table 3). To investigate whether the observed mannitol production served as an alternative redox sink, the mannitol-producing strains were grown under aerobic conditions. The activation of NADH oxidase (NOX) under aerobic conditions was expected to decrease the availability of NADH for M1PDH, and thus a decreased level of mannitol production was expected. Indeed, less mannitol was produced than under anaerobic conditions. Strain NZ9000(pWW003) still converted 8% of the glucose into mannitol (Table 3), whereas mannitol production by the LDH-deficient strain NZ9010(pWW003) and strain HWA217(pWW003) with re-

duced PFK activity was severely decreased to conversion ratios of 10 and 3%, respectively.

Fermentation patterns. The increased mannitol production by the different *L. lactis* strains was accompanied by changes in the pattern of fermentation products (Table 3). It was observed that mannitol is produced at the expense of other reduced fermentation products and that the production of mannitol was accompanied by a change in the amount of redox neutral products such as acetate and acetoin. In strain NZ9000 (pWW003) and HWA217(pWW003), increased mannitol production resulted in a decreased production of lactate and an increase in acetate, while the LDH-deficient strain NZ9010 (pWW003) showed a slight decrease in production of acetate, ethanol, formate, and 2,3-butanediol, together with an increase in acetoin production.

M1Pase-dependent mannitol production. To determine the direct effect on the mannitol production by modulating solely the M1Pase activity, *L. lactis* NZ9000(pWW002) was used. M1Pase activity in this strain was induced by the addition of various concentrations of nisin. The endogenous M1PDH was induced in this strain by preculturing cells on M17 broth containing mannitol, since the mannitol utilization genes, including the *mtlD* gene encoding M1PDH, are activated during growth on mannitol (23, 30). Induction with increasing levels of nisin in these mannitol-induced cells during growth on glucose resulted in a stable and high level of M1PDH activity, and an increasing level of M1Pase activity (Fig. 3A). Concomitant

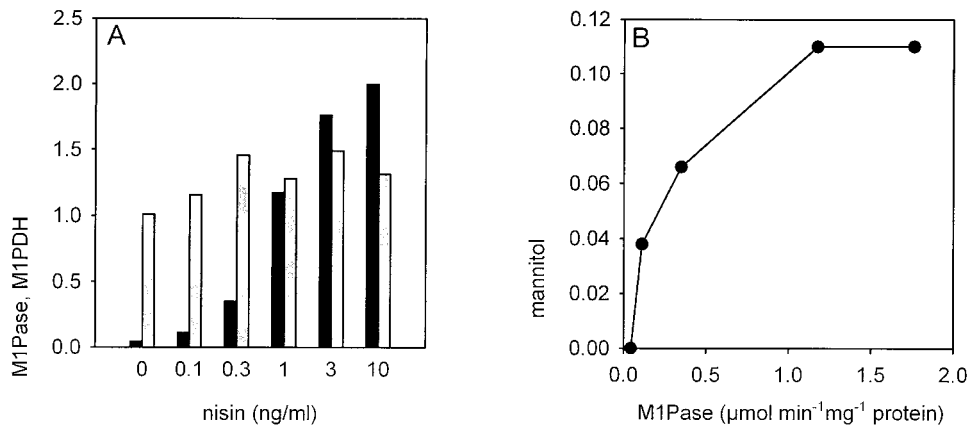


FIG. 3. M1Pase-dependent mannitol production by *L. lactis* NZ9000(pWW002). Cells precultured on 0.5% of mannitol were grown anaerobically on M17 broth containing 0.5% of glucose and induced with 0, 0.1, 0.3, 1.0, 3.0, and 10 ng of nisin/ml at an OD_{600} of 0.1; cells were harvested at an OD_{600} of 1.2. The activities of the *E. tenella* M1Pase and the *L. lactis* M1PDH in the cell extracts were measured in micromoles per minute per milligram of protein (A) and the final amount of mannitol produced in moles per mole of glucose was plotted against M1Pase activity (B). Black bars, M1Pase; grey bars, M1PDH.

with the M1Pase activity, mannitol production also increased (Fig. 3B), with a maximum of 0.11 mol mannitol per mole of glucose. M1Pase activities higher than 1.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein⁻¹ did not result in higher glucose-to-mannitol conversions.

Improved mannitol production by *L. lactis* NZ9010(pWW003).

To increase mannitol production by the LDH-deficient strain NZ9010(pWW003), both M1PDH and M1Pase activities were induced with the addition of 3 ng of nisin/ml to a growing culture at an OD_{600} value of 0.1 instead of 0.5. This resulted in an increased glucose-to-mannitol conversion of 39%, compared to a 27% conversion rate in the culture induced at an OD_{600} of 0.5 (Table 3). Besides the earlier nisin induction, increase of the initial glucose concentration in the batch culture to 2% resulted in a final concentration of 50 mM extracellular mannitol (Fig. 4B), corresponding to a glucose-to-mannitol conversion rate of 50% (Table 3). Moreover,

less lactate, formate, and ethanol were produced than with the uninduced culture (Fig. 4A); after 120 h, the glucose was almost completely consumed, while the glucose consumption of the uninduced culture ceased after 60 h.

DISCUSSION

To create a mannitol-producing *L. lactis* strain, several metabolic engineering strategies have been applied, such as inactivation of LDH activity and a mannitol transport system (12, 22, 23). With *L. lactis* and *L. plantarum*, inactivation of NADH consuming pathways such as LDH has been proven to be a successful strategy to invoke alternative NAD⁺ regeneration through mannitol production (11, 12, 22). Similar redox engineering has resulted in the overproduction of various flavor compounds in *L. lactis* and other LABs (7). Our present approach focused on the overexpression of genes involved in

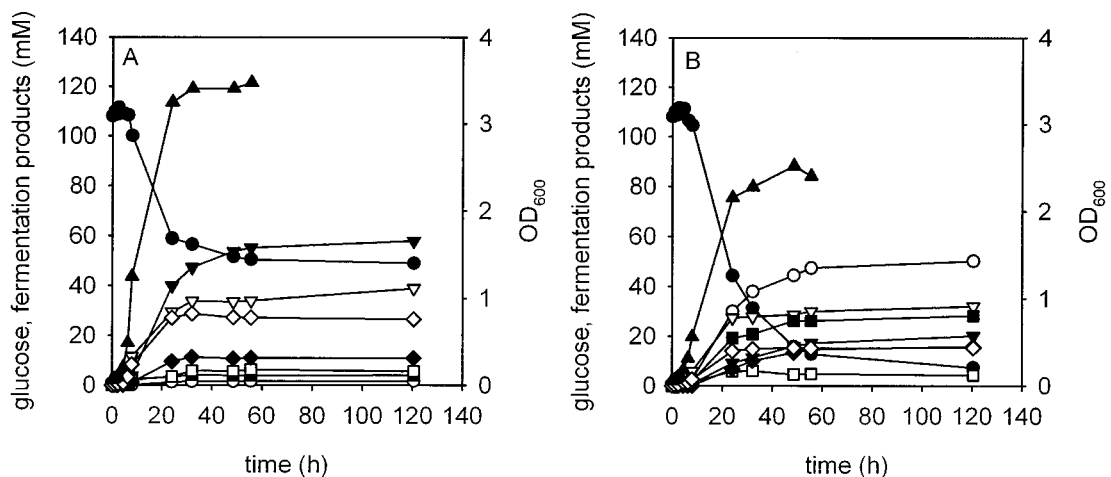


FIG. 4. Glucose consumption and product formation during growth by uninduced (A) and nisin-induced (B) *L. lactis* NZ9010(pWW003) cells. Cells were grown anaerobically on M17 broth supplemented with 2% glucose. At an OD_{600} of 0.1, 3 ng of nisin/ml was added to the culture to induce M1PDH and M1Pase production. Symbols: ▲, OD_{600} ; ●, glucose; ○, mannitol; ▼, lactate; ▽, formate; ■, acetate; □, acetoin; ◆, 2,3-butanediol; and ◇, ethanol.

mannitol biosynthesis. Metabolic flux control analysis of a kinetic mannitol production model of *L. lactis* predicted that increase of M1PDH and, in particular, M1Pase would result in a large increase of the mannitol flux (29). Previous studies showed that the overexpression of *mtlD* gene of *L. plantarum* in *L. lactis* did not result in high mannitol production in growing batch cultures (30). In this work, we demonstrate the effect of overproducing M1Pase in *L. lactis* on the mannitol production by this *L. lactis*.

Since no bacterial genes encoding M1Pase are known, the *E. tenella* M1Pase gene was cloned and overexpressed in *L. lactis* under the control of the *nisA* promoter of the *L. lactis* NICE expression system (19). Despite the difference in the overall GC content and codon usage between the *E. tenella* M1Pase gene and the lactococcal host (53 versus 37% GC, and 60 versus 28 GC in the wobble nucleotide, respectively), M1Pase was overproduced at a high level in *L. lactis* NZ9000(pWW002) (Table 2) compared to the low M1Pase activities measured in the strains described in our previous work (29, 30). Not surprisingly, the nisin-controlled overproduction of M1Pase in *L. lactis* NZ9000(pWW002) did not result in mannitol production (Table 2). Grown on glucose, *L. lactis* NZ9000(pWW002) lacked M1PDH activity (Table 2), and no mannitol production could take place. However, simultaneous overexpression of *mtlD* and the M1Pase gene in strains NZ9000(pWW003), HWA217(pWW003), and NZ9010(pWW003) resulted in high glucose-mannitol conversion rates of 11 to 50% (Table 3). In the parental strain NZ9000(pWW003), no mannitol yields higher than 11% were reached. The high activity and similar high affinity of M1PDH for NADH (NADH K_m , of 4 μM) (29) in comparison to that of LDH (NADH K_m , 6 μM) (3) suggests that M1PDH can compete with LDH for NADH and that higher glucose-to-mannitol conversions can be reached. Most likely, fructose 6-phosphate is limiting for M1PDH in the parental strain, due to a high glycolytic flux. Hence, an increased fructose 6-phosphate level in strain HWA217(pWW003) with reduced PFK activity (1) would explain the higher conversion rate of glucose to mannitol than with parental strain NZ9000(pWW003) (Table 3). Although the LDH-deficient strain NZ9010(pWW003) partly recovered its lactate production capacity, probably due to transcriptional activation of the alternative LDH gene *ldhB* (3), the requirement for an alternative redox sink contributed strongly to the production of mannitol by this strain. Decreasing the NADH availability by NOX activity during aerobic growth resulted in decreased mannitol production (Table 3) compared to the (semi)anaerobic cultures. This confirms that NAD^+ is regenerated partly via mannitol production by the *L. lactis* strains overproducing M1Pase and M1PDH. Since our experiments were not performed under strictly anaerobic conditions, it may be possible to produce more mannitol by inactivation of NOX.

Noting that the M1Pase/M1PDH activity ratios were about 1:40 in the cell extracts and that overexpression of *mtlD* alone did not result in high levels of mannitol production (30), it was expected that mannitol production was largely dependent on M1Pase activity. This was confirmed by the production of mannitol from glucose by the NZ9000(pWW002) cells precultured on mannitol (Fig. 3). The increased M1Pase activities and stable levels of M1PDH activities observed for cells induced with an increasing amount of nisin suggested a direct correla-

tion between the M1Pase activity and mannitol production, with a maximum mannitol production at a M1Pase activity $>1.2 \mu\text{mol min}^{-1} \text{mg of protein}^{-1}$. The most likely explanation for this maximum mannitol production is substrates such as fructose 6-phosphate and NADH become limiting at these high M1Pase activity levels. Compared to strain NZ9000(pWW003), NZ9000(pWW002) displayed higher M1Pase activity at equal levels of mannitol production. Possibly, the lower affinity of the endogenous *L. lactis* M1PDH for the substrate NADH (NADH K_m , 23 μM) (unpublished results) than that of the *L. plantarum* M1PDH (NADH K_m , 4 μM) (29), resulted in a lower flux towards mannitol. Another explanation could be the decreasing specific M1PDH activity in cells of strain NZ9000(pWW002) during the growth on glucose. Although M1PDH activity was detected in cell extracts of the glucose-grown cells, possibly M1PDH was only induced during preparation of the mannitol preculture and not during the growth on glucose.

Since the mannitol pathway branches off from glycolysis at the level of fructose 6-phosphate, less NADH is formed by GAPDH when mannitol is formed. Consequently, mannitol is produced at the expense of the NADH-consuming pathways of the pyruvate metabolism, namely, lactate, ethanol, and 2,3-butanediol formation. While mannitol is produced at the expense of lactate by parental strain NZ9000(pWW003) and strain HWA217(pWW003), decreased ethanol and 2,3-butanediol production was the result of mannitol production by the LDH-deficient strain; residual lactate production also decreased with increasing mannitol formation (Table 3). To keep the redox balance neutral in the mainly lactate-producing strains NZ9000(pWW003) and HWA217(pWW003), acetate or another redox neutral product, such as acetoin, has to be formed for each mole of mannitol formed. Keeping a neutral redox balance, a maximum of two-thirds (67%) of glucose can be converted into mannitol, assuming that no lactate is formed. The results (Table 3) indicated that an increased acetate production indeed coincides with mannitol production for these two strains. For LDH-deficient strain NZ9010(pWW003) producing both ethanol and 2,3-butanediol in addition to mannitol to regenerate NAD^+ (Table 3), both acetate and acetoin were expected to be produced. In fact, a small decrease of the redox-neutral acetate and an increase in acetoin production coincided with increased mannitol production.

The combined overproduction of M1PDH and M1Pase has proven to be a successful strategy for obtaining a mannitol-producing *L. lactis*. To the best of our knowledge, this work presents the first example of high and stable mannitol production in growing *L. lactis* cells, in contrast to the mannitol production observed for resting *L. lactis* cells (12, 22). The results shown here emphasize the importance of M1Pase activity for mannitol production by *L. lactis* and indicate that a *L. lactis* strain deficient in LDH activity and with high M1PDH and M1Pase activity would be a good candidate for in situ mannitol production in food products. Regarding the possibility of the use of such a *L. lactis* in a microbial mannitol production process, an advantage of mannitol production by a *L. lactis* strain in comparison with heterofermentative LABs such as *Leuconostoc mesenteroides* (13, 28) might be the capability of *L. lactis* to use several sugar substrates to synthesize mannitol, whereas mannitol is exclusively formed from fructose by the

heterofermentative LABs. Further research on mannitol production by *L. lactis* could focus on the uptake system of mannitol. Although we have shown that no consumption of mannitol was observed after glucose exhaustion, *L. lactis* is capable of growing on mannitol (23, 30). Therefore, it is not unlikely that reutilization of mannitol would occur eventually after repeated subculturing, due to induction of the mannitol transport system by mannitol (22, 23). Inactivation of such a mannitol transport system, as recently published (12), could prevent the reutilization of mannitol in strains overproducing the large amounts of mannitol as presented here.

ACKNOWLEDGMENT

We gratefully acknowledge Paul Liberator for supplying the pQE60 plasmid containing the *Eimeria tenella* M1Pase gene.

REFERENCES

- Andersen, H. W., C. Solem, K. Hammer, and P. R. Jensen. 2001. Twofold reduction of phosphofructokinase activity in *Lactococcus lactis* results in strong decreases in growth rate and in glycolytic flux. *J. Bacteriol.* **183**: 3458–3467.
- Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res.* **11**: 731–753.
- Bongers, R. S., M. H. N. Hoefnagel, M. J. C. Starrenburg, M. A. J. Siemerink, J. G. A. Arends, J. Hugenoltz, and M. Kleerebezem. 2003. IS981-mediated adaptive evolution recovers lactate production by *ldhB* transcription activation in a lactate dehydrogenase-deficient strain of *Lactococcus lactis*. *J. Bacteriol.* **185**:4499–4507.
- Chaturvedi, V., A. Bartiss, and B. Wong. 1997. Expression of bacterial *mtlD* in *Saccharomyces cerevisiae* results in mannitol synthesis and protects a glycerol-defective mutant from high-salt and oxidative stress. *J. Bacteriol.* **179**:157–162.
- Chaturvedi, V., B. Wong, and S. L. Newman. 1996. Oxidative killing of *Cryptococcus neoformans* by human neutrophils. Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates. *J. Immunol.* **156**: 3836–3840.
- Debord, B., C. Lefebvre, A. M. Guyot-Hermann, J. Hubert, R. Bouche, and J. C. Guyot. 1987. Study of different forms of mannitol: comparative behaviour under compression. *Drug Dev. Ind. Pharm.* **13**:1533–1546.
- de Vos, W. M., and J. Hugenoltz. 2004. Engineering metabolic highways in lactococci and other lactic acid bacteria. *Trends Biotechnol.* **22**:72–79.
- Dwivedi, B. K. 1978. Low calorie and special dietary foods. CRC Press, Inc., West Palm Beach, Fla.
- Edwards, K. G., H. J. Blumenthal, M. Khan, and M. E. Slodki. 1981. Intracellular mannitol, a product of glucose metabolism in staphylococci. *J. Bacteriol.* **146**:1020–1029.
- Efiuvwevere, B. J. O., L. G. M. Gorris, E. J. Smid, and E. P. W. Kets. 1999. Mannitol-enhanced survival of *Lactococcus lactis* subjected to drying. *Appl. Microbiol. Biotechnol.* **51**:100–104.
- Ferain, T., A. N. Schanck, and J. Delcour. 1996. ¹³C nuclear magnetic resonance analysis of glucose and citrate end products in an *ldhL*-*ldhD* double-knockout strain of *Lactobacillus plantarum*. *J. Bacteriol.* **178**:7311–7315.
- Gaspar, P., A. R. Neves, A. Ramos, M. J. Gasson, C. A. Shearman, and H. Santos. 2004. Engineering *Lactococcus lactis* for production of mannitol: high yields from food-grade strains deficient in lactate dehydrogenase and the mannitol transport system. *Appl. Environ. Microbiol.* **70**:1466–1474.
- Grobben, G. J., S. W. P. G. Peters, H. W. Wisselink, R. A. Weusthuis, M. H. N. Hoefnagel, J. Hugenoltz, and G. Eggink. 2001. Spontaneous formation of a mannitol-producing variant of *Leuconostoc pseudomesenteroides* grown in the presence of fructose. *Appl. Environ. Microbiol.* **67**: 2867–2870.
- Hoefnagel, M. H. N., M. J. C. Starrenburg, D. E. Martens, J. Hugenoltz, M. Kleerebezem, I. I. Van Swam, R. Bongers, H. V. Westerhoff, and J. L. Snoep. 2002. Metabolic engineering of lactic acid bacteria, the combined approach: kinetic modelling, metabolic control and experimental analysis. *Microbiol-ogy* **148**:1003–1013.
- Hoefnagel, M. H. N., A. van der Burgt, D. E. Martens, J. Hugenoltz, and J. L. Snoep. 2002. Time dependent responses of glycolytic intermediates in a detailed glycolytic model of *Lactococcus lactis* during glucose run-out experiments. *Mol. Biol. Rep.* **29**:157–161.
- Kets, E. P. W., J. A. M. de Bont, and H. J. Heipieper. 1996. Physiological response of *Pseudomonas putida* S12 subjected to reduced water activity. *FEMS Microbiol. Lett.* **139**:133–137.
- Kleerebezem, M., M. M. Beerthuyzen, E. E. Vaughan, W. M. de Vos, and O. P. Kuipers. 1997. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. *Appl. Environ. Microbiol.* **63**:4581–4584.
- Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Turchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. Klein Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Nierop Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* **100**:1990–1995.
- Kuipers, O. P., P. G. G. A. de Ruyter, M. Kleerebezem, and W. M. de Vos. 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* **64**:15–21.
- Liberator, P., J. Anderson, M. Feiglin, M. Sardana, P. Griffin, D. Schmatz, and R. W. Myers. 1998. Molecular cloning and functional expression of mannitol-1-phosphatase from the apicomplexan parasite *Eimeria tenella*. *J. Biol. Chem.* **273**:4237–4244.
- Loesche, W. J., and K. S. Kornman. 1976. Production of mannitol by *Streptococcus mutans*. *Arch. Oral Biol.* **21**:551–553.
- Neves, A. R., A. Ramos, C. Shearman, M. J. Gasson, J. S. Almeida, and H. Santos. 2000. Metabolic characterization of *Lactococcus lactis* deficient in lactate dehydrogenase using in vivo ¹³C-NMR. *Eur. J. Biochem.* **267**:3859–3868.
- Neves, A. R., A. Ramos, C. Shearman, M. J. Gasson, and H. Santos. 2002. Catabolism of mannitol in *Lactococcus lactis* MG1363 and a mutant defective in lactate dehydrogenase. *Microbiology* **148**:3467–3476.
- Sakai, S., and K. Yamanaka. 1968. Crystalline D-mannitol:NAD⁺ oxidoreductase from *Leuconostoc mesenteroides*. *Biochim. Biophys. Acta* **151**: 684–686.
- Shen, B., R. G. Jensen, and H. J. Bohnert. 1997. Mannitol protects against oxidation by hydroxyl radicals. *Plant Physiol.* **115**:527–532.
- Smirnov, N., and Q. J. Cumbes. 1989. Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* **28**:1057–1060.
- Soetaert, W., K. Buchholz, and E. J. Vandamme. 1995. Production of D-mannitol and D-lactic acid by fermentation with *Leuconostoc mesenteroides*. *Agro Food Ind. Hi Technol.* **6**:41–44.
- von Weyarn, N., M. Hujanen, and M. Leisola. 2002. Production of D-mannitol by heterofermentative lactic acid bacteria. *Process Biochem.* **37**: 1207–1213.
- Wisselink, H. W., R. Heinen, D. Martens, J. L. Snoep, W. M. de Vos, J. Hugenoltz, and M. H. N. Hoefnagel. Kinetic modelling of mannitol production in *Lactococcus lactis*, submitted for publication.
- Wisselink, H. W., A. E. Mars, P. van der Meer, G. Eggink, and J. Hugenoltz. 2004. Metabolic engineering of mannitol production in *Lactococcus lactis*: influence of the overexpression of mannitol 1-phosphate dehydrogenase in different genetic backgrounds. *Appl. Environ. Microbiol.* **70**:4286–4292.