Metabolic Engineering of Mannitol Production in *Lactococcus lactis*

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Metabolic Engineering of Mannitol Production in *Lactococcus lactis*

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CONTENTS

ABSTRACT

Mannitol is a sugar alcohol that is produced by a wide variety of (micro) organisms. It is assumed to have several beneficial effects as a food additive. It can serve as an antioxidant and as a low-calorie sweetener which can replace high-calorie sugars such as sucrose, lactose, glucose and fructose. In addition, it has been shown that mannitol has a protective effect for lactic acid bacteria, such as *Lactococcus lactis*, when they are subjected to drying and/or freezing. The viability of starter cultures containing these lactic acid bacteria may thus be enhanced by inducing mannitol production in these strains. In addition, the use of a mannitol producing *L. lactis* may result in fermented products with extra nutritional value.

The metabolic engineering of mannitol production in *L. lactis,* using a combined approach of predicting the control points for mannitol production in *L. lactis* by a kinetic glycolysis model, and experimental metabolic engineering steps, is described in this thesis. Based on the model predictions, we combined engineering steps, such as knocking out lactate dehydrogenase, reduction of phosphofructokinase and fructokinase activity, and overexpression of genes involved in mannitol biosynthesis, such as mannitol 1-phosphate dehydrogenase, mannitol 1-phosphatase, and mannitol dehydrogenase. This resulted in different levels of mannitol production in several *L. lactis* strains. Especially the combination of overexpression of genes encoding mannitol 1-phosphate dehydrogenase and a mannitol-1-phosphatase resulted in high mannitol production by *L. lactis*. Moreover, it was found that mannitol 1-phosphatase has a high control on the mannitol production, and a clear correlation between the activity of this enzyme and the mannitol production was shown.

The research described in his thesis is a text book example of metabolic engineering. It contributes to the understanding of mannitol biosynthesis in *L. lactis*, and how the mannitol production can be improved by metabolic engineering. Moreover, it provides suggestions for food-grade applications, especially in the field of the production of low-calorie sweeteners.

OUTLINE OF THIS THESIS

Mannitol, a low calorie sweetener and antioxidant, is believed to have several health-promoting and protective properties. In situ mannitol production by *Lactococcus lactis*, a lactic acid bacterium extensively used in the dairy industry, may therefore result in fermented food products with extra value. Moreover, the viability of starter cultures may be enhanced by the production of mannitol, due to the protective properties of mannitol.

The aim of the research described in this thesis is to increase the mannitol production in *L. lactis*. Metabolic engineering strategies were designed with the use of kinetic models, and are described in the different chapters.

In **chapter 1**, an overview of mannitol production by lactic acid bacteria is presented. Genes, enzymes and pathways involved in mannitol biosynthesis in homofermentative and heterofermentative lactic acid bacteria are discussed. Moreover, possible metabolic engineering strategies to aim for mannitol production in *L. lactis* are suggested.

Chapter 2 focuses on the overexpression of the *Leuconostoc mesenteroides mdh* gene, encoding mannitol dehydrogenase (MDH), an enzyme which converts fructose into mannitol in a single step. Overproduction of MDH in *L. lactis* cells growing on sucrose was expected to result in high mannitol production in a strain able to grow on sucrose, and its fructokinase-deficient derivative. However, due to the presence of alternative pathways to metabolize the fructose moiety of sucrose in these *L. lactis* strains, production of high amounts of mannitol was prevented.

Chapter 3 describes the overexpression of the *Lactobacillus plantarum mtlD* gene, encoding mannitol 1-phosphate dehydrogenase (M1PDH), in different *L. lactis* strains. It was shown that increasing the M1PDH activity, resulted in low mannitol production in growing cells. In addition, resting cells of a LDH-deficient strain overproducing M1PDH, showed a much higher mannitol production than growing cells of this strain.

In **chapter 4**, the development and application of a kinetic model for mannitol production in *L. lactis* is described. A *L. lactis* glycolysis model was extended with two enzymes involved in mannitol synthesis in *L. lactis*, M1PDH and mannitol 1-phosphatase (M1Pase). The model predicted a high control of M1Pase on the mannitol production in *L. lactis*, and high glucose to mannitol conversions were expected by overproducing both M1Pase and M1PDH.

Chapter 5 describes the overexpression of the *Eimeria tenella* M1Pase gene *in L. lactis*. High glucose to mannitol conversions up to 50% were reached by the overproduction of M1PDH and M1Pase in a LDH-deficient *L. lactis* strain. Moreover, a clear correlation between the mannitol production and the M1Pase activity was shown.

Chapter 6 summarizes the research described in this thesis. Moreover, additional data is discussed and conclusions coming from this thesis are drawn.

CHAPTER 1

General Introduction

Mannitol Production by Lactic Acid Bacteria: a Review

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ABSTRACT

Mannitol is a polyol or sugar alcohol that is produced by several organisms. Mannitol is assumed to have several beneficial effects, as an antioxidant (protection against oxidative damage by oxygen radicals) and as a non-metabolizable sweetener. Mannitol can therefore be applied to foods leading to health-promoting effects (functional foods). Mannitol-producing lactic acid bacteria may directly be applied in the manufacture of foods and this may lead to fermented food products with an extra value.

In this article, the production of mannitol by lactic acid bacteria is reviewed. Several heterofermentative lactic acid bacteria produce mannitol in large amounts, using fructose as an electron acceptor, whereas homofermentative lactic acid bacteria only produce small amounts of mannitol, often only when the bacteria are defective in lactate dehydrogenase activity. The physiology, pathways and genetics of mannitol production in both homofermentative and heterofermentative lactic acid bacteria are presented and discussed.

INTRODUCTION

(D-)Mannitol is a naturally occurring six-carbon sugar alcohol or polyol. It is the most abundant polyol in nature occurring in bacteria, yeasts, fungi, algae, lichens and several plants like pumpkins, celery, onions, grasses, olives and mistletoe.

Jennings (30) stated that polyols including mannitol could play several roles in fungi: (I) as carbohydrate reserves, (II) as translocatory compounds, (III) as an osmoregulatory compound, and (IV) in coenzyme regulation and storage of reducing power. Mannitol is a well-known compatible solute, i.e. a component that protects organisms that are exposed to a number of stress situations. It was observed that mannitol protects plants, fungi, yeasts and bacteria during conditions of stress (9, 62). Mannitol has been reported to accumulate in response to environmental stresses like osmotic stress (33, 61). Efiuvwevwere et al. (15) showed that mannitol has an osmoprotecting and antioxidant effect on the dairy lactic acid bacterium *Lactococcus lactis* subjected to decreased water activity and that mannitol enhances the survival of dried starter cells.

Mannitol is claimed to have several health-promoting effects, so the addition of mannitol to foods could lead to products with an extra nutritional value. Mannitol could either be added directly to foods, or the use of mannitol-producing bacteria, yeasts or fungi might direct lead to “natural” mannitol-containing foods. Lactic acid bacteria, which are used as starter cultures in many fermented foods and feeds, form one group of such food-associated microorganisms. Besides that, several health-promoting properties have been attributed to lactic acid bacteria themselves. The utilization of mannitol-producing probiotic lactic acid bacteria might therefore be of considerable interest.

In this paper, we review the microbial production and properties of mannitol with a focus on mannitol production by lactic acid bacteria. The mannitol production in lactic acid bacteria is strongly dependent on the pathway of carbohydrate fermentation: lactic acid bacteria possess either a homofermentative or a heterofermentative pathway. The physiology, enzymology and genetics of mannitol production in both fermentation pathways are discussed. Furthermore, possible approaches are presented for the achievement of mannitol overproduction in *L. lactis*, a lactic acid bacterium that is frequently used in the dairy industry and which is a model organism in a large number of research fields.

MECHANISMS OF PROTECTION BY MANNITOL

The mechanism of protecting living cells by polyols like mannitol is not fully understood. Three hypotheses have been proposed so far for the mechanism of protection, which all might play a role:

1. Maintenance of turgor. At low water activity, water is taken up by the cell and turgor is restored due to the accumulation of mannitol (30, 32).

2. Stabilization of the structures of membrane lipids and proteins at low water activity (35).

3. Preventing oxidative damage by scavenging off free reactive oxygen radicals . Water stress may cause an increased generation of oxygen radicals. Mannitol is known in chemistry as an effective hydroxyl radical scavenger (9, 10, 52, 55).

The antioxidant effect of mannitol has also been reported for a number of human systems. For instance, air pollution particles less than 10 μm in diameter were shown to have free hydroxyl radical activity by its ability to deplete supercoiled DNA, an effect which could be reversed by the addition of mannitol (36); hydroxyl radicals were also found to be responsible for the asbestos-mediated DNA damage, which could be inhibited by addition of mannitol (20). Significant tissue protection was achieved by different doses of mannitol.

APPLICATIONS AND COMMERCIAL PRODUCTION OF MANNITOL

Because of its assumed health-promoting effects, the addition of mannitol to foods, or a direct application of mannitol overproducing lactic acid bacteria in particular, could result in the manufacture of food products with an extra nutritional value, so called functional foods. Several interesting applications for mannitol-forming lactic acid bacteria may arise from this hypothesis.

The protective effect of mannitol as a compatible solute on microorganisms subjected to low water activity may result in an enhanced viability of dried starter cells. This results not only in reduced transport and storage costs, but also in an enhanced viability of the starter cells leading to a higher residual activity.

Mannitol is also an interesting product from another point of view. Mannitol is a valuable nutritive sweetener because it is non-toxic, non-hygroscopic in its crystalline form and has no teeth decaying effects. It has a sweet, cool taste and it is about half as sweet as sucrose (13, 14).

Mannitol is only partially metabolized by humans and it does not induce hyperglycemia, which makes it useful for diabetics (21). Mannitol is applied as a food additive (E421) as a sweet tasting bodying and texturing agent and it is used as a sweet builder in “sugar free” chewing gum and in pharmaceutical preparations (57). Mannitol has some laxative properties and the daily intake of mannitol should therefore not exceed 20 grams (14).

Manna, the exudate produced by the manna ash tree *Fraxinus ornus*, was the commercial source for mannitol for many years until the 1920’s (56). Nowadays, mannitol is industrially produced by the catalytic hydrogenation of a glucose/fructose (1:1) mixture using a nickel catalyst and hydrogen gas at a high temperature and a high pressure (39). In this reaction, glucose is completely converted into sorbitol and fructose into mannitol as well as its isomer sorbitol, due to the poor selectivity of the nickel catalyst used. The composition of the hydrogenated mixture is about 25% mannitol and 75% sorbitol. Mannitol is recovered by crystallization since it is less soluble than sorbitol. These production and purification processes make the manufacture costs for mannitol relatively high. Enzymatic production of mannitol from fructose with mannitol dehydrogenase is also possible, but this may lead to an incomplete conversion due to, for instance, product inhibition and furthermore this reaction is requiring a high-priced co-factor such as NAD(P)H that also needs to be regenerated (29, 53).

Mannitol production by fermentation with microorganisms, and food-grade microorganisms in particular, may therefore be an interesting alternative. A fermentation process could have several advantages compared to the chemical synthesis, such as a complete conversion of fructose to mannitol, absence of side products (like sorbitol) that are difficult to remove, moderate production conditions and no requirement of highly purified substrates (58).

MANNITOL PRODUCTION BY MICROORGANISMS

Jennings (30) reviewed the production of mannitol and other polyols by several yeasts and fungi, together with different biosynthetic pathways. In the yeast *Cryptococcus neoformans*, large amounts of mannitol are formed in yeast nitrogen base plus 1% glucose, the presence of mannitol was used as a marker for cryptococcal meningitis in rabbits (68). In the mushroom *Agaricus bisporus* mannitol is the main storage carbon. The fruit body dry weight may consist of up to 50% mannitol and the mycelium up to 20% (44, 45). In *Aspergillus candidus*, approximately 50% of the glucose consumed was converted to mannitol (54). In *Pseudomonas putida*, mannitol is produced as a consequence of the exposure to high concentrations of salts or sucrose (33).

Lactic acid bacteria producing mannitol

Mannitol production by lactic acid bacteria and other food-grade microorganisms offer several important advantages. Firstly, food-grade microorganisms and their products are directly applicable in food products, without any restriction. Secondly, there is no need for a careful separation of products and microorganisms, which would be the case if microorganisms are not food-grade. Thirdly, some lactic acid bacteria are claimed as beneficial in the gastrointestinal tract. Mannitol production by those bacteria may strengthen their health-promoting ability.

Several lactic acid bacterial strains are known to produce mannitol. Up to now two biosynthetic pathways for mannitol in lactic acid bacteria were described, depending on the pathway for hexose fermentation used by the organism. There are two major pathways for hexose fermentation occurring within lactic acid bacteria: homolactic fermentation and heterolactic fermentation. Both pathways will be discussed below, in relation to mannitol formation.

Some homofermentative lactic acid bacteria were found to produce small amounts of mannitol. In the presence of large amounts of glucose or sucrose, cell suspensions of *Streptococcus mutans* produce approximately 0.1 – 0.3 mg mannitol (37). *Lactobacillus leichmanii* also showed mannitol production (8). Since in homofermentative lactic acid bacteria the carbon flow from carbohydrates is directed mainly to lactate production, the formation of other fermentation products like mannitol is often only possible when strains are more or less hampered in the lactate production pathway. A lactate dehydrogenase-negative mutant of *Lactobacillus plantarum* produced 6 mM mannitol from 50 mM glucose whereas the wild type was not able to produce any mannitol from glucose (17). Until now no mannitol production was observed by *L. lactis* strains, although Neves et

al. (41) showed that a lactate dehydrogenase deficient mutant transiently accumulates high amounts of intracellular mannitol (up to 90 mM) and mannitol 1-phosphate (up to 80 mM) in succession.

The natural mannitol-producing lactic acid bacteria show a heterofermentative metabolism. In 1920, it was already noted that fructose fermentation by the heterofermentative lactic acid bacterium *Lactobacillus pentoaceticus* resulted in mannitol formation (43). In the presence of fructose or sucrose *Leuconostoc (pseudo)mesenteroides* produces high levels of mannitol (22, 56, 57, 67). Mannitol production was found in concentrations up to 150 g L^{-1}, close to its solubility limit of 180 g L^{-1} at 25°C, indicating that mannitol has no toxic effects on the organism (58). Two other heterofermentative lactic acid bacteria, *Lactobacillus* sp. and *Leuconostoc* sp., also produced mannitol from fructose and sucrose (69, 70).

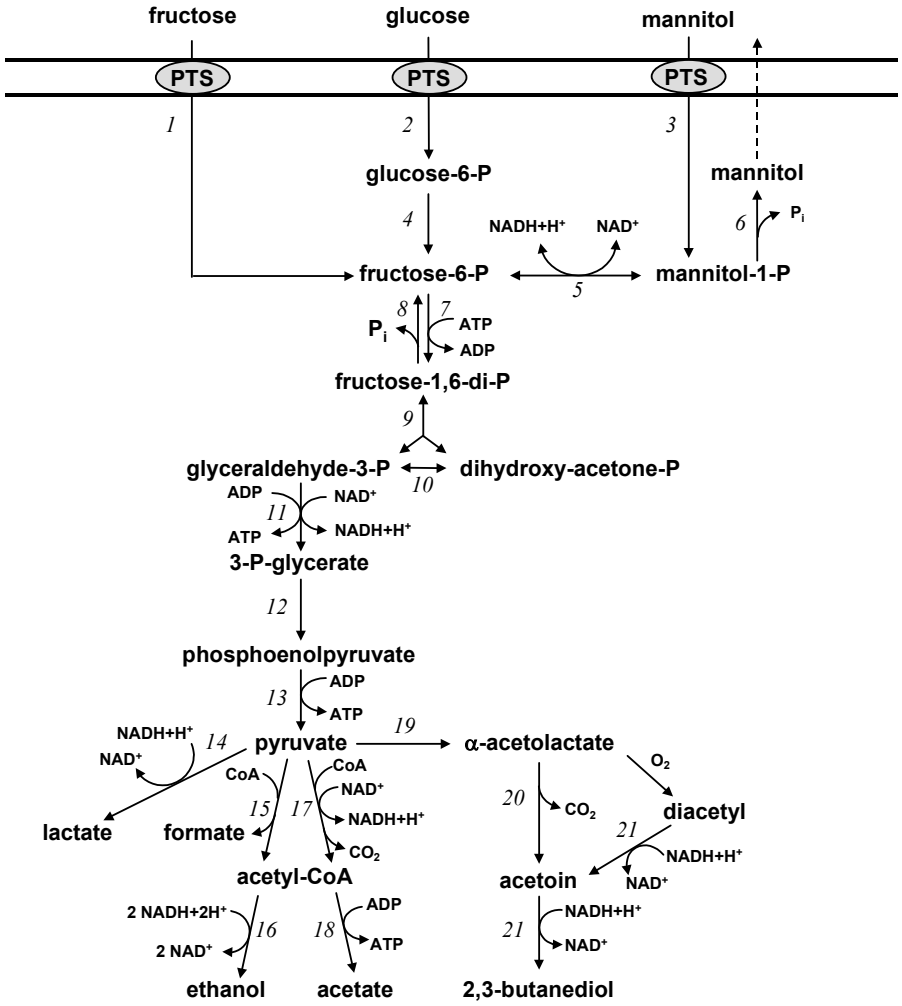

FIG. 1. Proposed pathway for hexose metabolism of homofermentative lactic acid bacteria

1 and *2*. Phospho*enol*pyruvate (PEP)-dependent sugar phosphotransferase system (PTS); *3*. Mannitol-specific PTS; *4*. Phosphoglucose isomerase; *5*. Mannitol 1-phosphate dehydrogenase; *6*. Mannitol 1-phosphatase; *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*. Lactate dehydrogenase; *15*. Pyruvate-formate lyase; *16*. Acetaldehyde dehydrogenase and alcohol dehydrogenase; *17*. Pyruvate dehydrogenase; *18*. Acetate kinase; *19*. α-Acetolactate synthase; *20*. α-Acetolactate decarboxylase; *21*. 2,3-Butanediol dehydrogenase.

BIOSYNTHETIC PATHWAYS OF MANNITOL

Homofermentative lactic acid bacteria

Homofermentative lactic acid bacteria use the glycolysis, also known as the Embden-Meyerhof-Parnas pathway, for hexose fermentation (Fig. 1; (2)). The group consists of the *Lactobacillus* group I and II (31), enterococci, lactococci, pediococci, streptococci, tetragenococci and vagococci. The pathway is characterized by the formation of fructose 1,6-diphosphate (FDP) that is split by a FDP aldolase into dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate. Fermentation of 1 mol of glucose results in the formation of 2 mol of lactic acid and 2 mol of ATP. Before entering the glycolysis, monosaccharides like glucose and fructose are generally phosphorylated when translocated into the cell by a phospho*enol*pyruvate (PEP)-dependent sugar:phosphotransferase system (PTS) (65). Some lactic acid bacteria such as *Streptococcus thermophilus* transport lactose by a *lac* permease transport system, followed by an intracellular hydrolysis and phosphorylation (47).

When homofermentative lactic acid bacteria grow on substrates such as glucose, a typical homolactic fermentation pattern can be observed. Pyruvate is reduced to lactic acid by a NAD^+-dependent lactate dehydrogenase, thereby reoxidizing the NADH formed during the early glycolytic steps. Under certain conditions, glycolysis may also lead to a mixed acid fermentation (2), leading to end products such as acetate, ethanol, diacetyl, acetoin, 2,3-butanediol, and in some cases, mannitol (Fig. 1). Mannitol biosynthesis in homofermentative lactic aid bacteria starts with the glycolysis intermediate fructose 6-phosphate. Mannitol 1-phosphate dehydrogenase (EC 1.1.1.17) catalyses the reduction of fructose 6-phosphate, and also the reverse reaction, the oxidation of mannitol 1-phosphate (7, 17). Mannitol 1-phosphate is dephosphorylated to mannitol by mannitol phosphatase. When grown on glucose or other hexoses, no or very low mannitol 1-phosphate dehydrogenase activity can be found in cell extracts of *L. lactis*. Under these conditions, no mannitol is formed. Inactivation of lactate dehydrogenase in this species led transiently to intracellular mannitol accumulation in cell suspensions incubated anaerobically with glucose (41). Also a lactate dehydrogenase-negative mutant of *Lactobacillus plantarum* produced small amounts of mannitol (17). In these cases, mannitol production was believed to be an alternative pathway instead of lactate formation to regenerate NAD^+ .

In contrast to the mannitol production, its utilization is more common among homofermentative lactic acid bacteria (31). For the transport of mannitol, homofermentative lactic acid bacteria use the mannitol-specific PTS. This has been described for *S. mutans* (27, 28) and non-lactic acid bacteria such as *Escherichia coli* (34), *Staphylococcus carnosus* (18), *Enterococcus faecalis* (19) and *Bacillus stearothermophilus* (24). During transport across the cytoplasmic membrane by a specific mannitol transporter, mannitol is phosphorylated to form mannitol 1-phosphate. Mannitol 1-phosphate is oxidized by mannitol 1-phosphate dehydrogenase to give the glycolysis-intermediate fructose 6-phosphate. When *L. lactis* was grown on mannitol, mannitol 1-phosphate dehydrogenase activity was found in cell-free extracts.

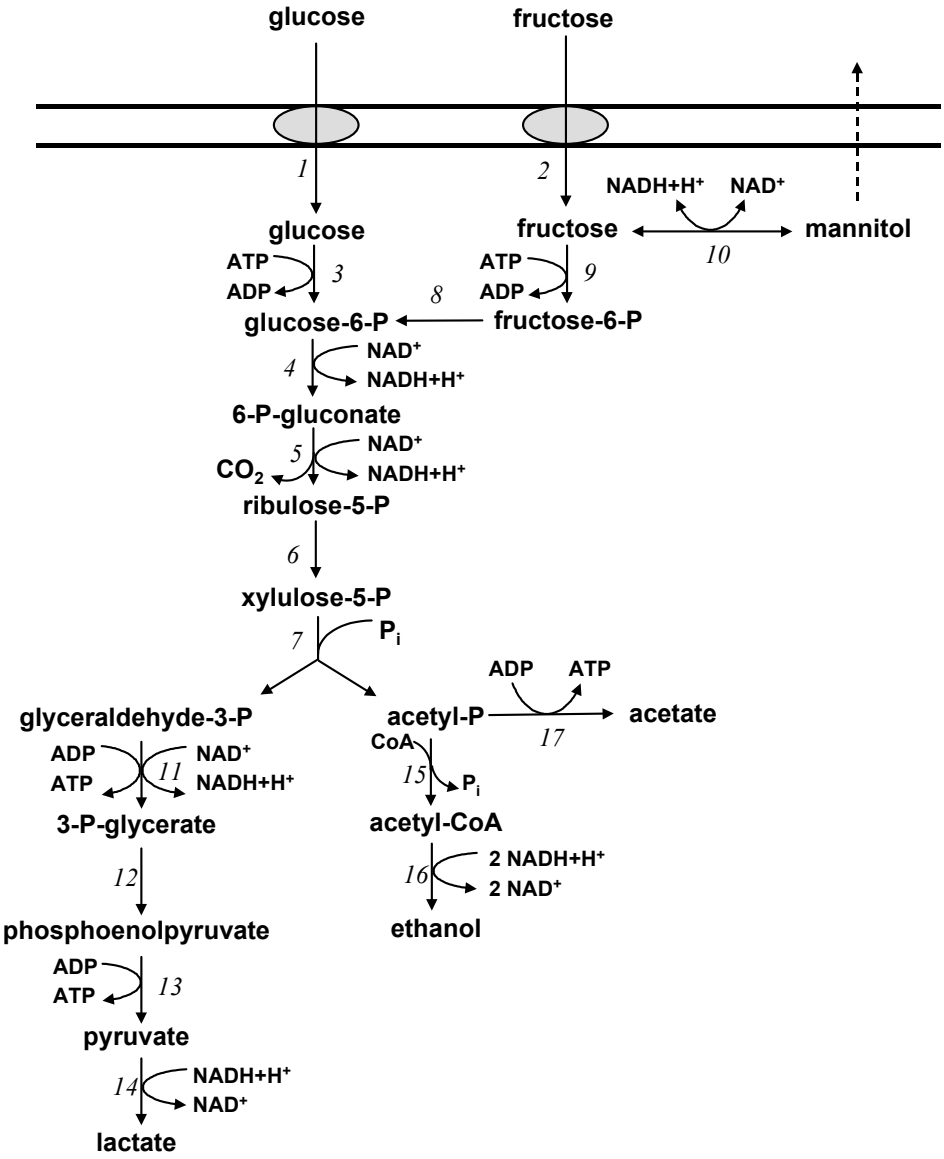

FIG. 2. Proposed pathway for hexose metabolism of heterofermentative lactic acid bacteria. *1* and *2*. Glucose and fructose permease; *3*. Glucokinase; *4*. Glucose 6-phosphate dehydrogenase; *5*. 6-Phosphogluconate dehydrogenase; *6*. Epimerase; *7*. Phosphoketolase; *8*. Glucose phosphate isomerase; *9*. Fructokinase; *10*. Mannitol dehydrogenase; *11*. Glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase; *12*. Phosphoglyceromutase and enolase; *13*. Pyruvate kinase; *14*. Lactate dehyrogenase; *15*. Phosphate acetyltransferase; *16*. Acetaldehyde dehydrogenase and alcohol dehydrogenase; *17*. Acetate kinase.

Heterofermentative lactic acid bacteria

Heterofermentative lactic acid bacteria, consisting of *Leuconostoc* and lactobacilli Group III (obligately heterofermentative), use the 6-phosphogluconate/phosphoketolase pathway for hexose fermentation (31). They lack the enzyme fructose 1,6-diphosphate aldolase. Under anaerobic conditions, glucose is converted to equimolar amounts of lactic acid, ethanol and carbon dioxide; per mol of glucose fermented net 1 mol of ATP is produced (2, 11). With the conversion of acetyl phosphate to acetate instead of ethanol, an additional ATP can be produced. Then the regeneration of NAD^+ must be achieved by using an alternative electron acceptor. Under aerobic conditions, oxygen may serve as an electron acceptor (12). Under anaerobic conditions, compounds like pyruvate (42), glycerol (63) and fructose may be reduced. Mannitol is the end product of fructose reduction, catalyzed by the enzyme mannitol dehydrogenase (Fig. 2). In contrast to homofermentative lactic acid bacteria, heterofermentative lactic acid bacteria do not use fructose 6-phosphate for mannitol biosynthesis. With a mixture of glucose and fructose as the carbohydrate sources, mannitol production by *Leuc. mesenteroides* was observed (16) It was shown that per mol of glucose fermented, approximately two mol of fructose are reduced to mannitol (22). A conversion equation of this conversion is as follows (equation I):

$$1 \text{ glucose} + 2 \text{ fructose} \rightarrow 1\ CO_2 + 1 \text{ lactate} + 1 \text{ acetate} + 2 \text{ mannitol} \qquad \text{(I)}$$

At optimal conditions, a maximal yield of 2 mol of ATP per mol of glucose fermented is found, and there is a complete reduction of fructose to mannitol.

If only fructose is available as the carbohydrate source for *Leuc. mesenteroides*, fructose is both a fermentable substrate and an electron acceptor. This results in the following conversion equation (II):

$$3 \text{ fructose} \rightarrow 1\ CO_2 + 1 \text{ lactate} + 1 \text{ acetate} + 2 \text{ mannitol} \qquad \text{(II)}$$

Here one mol of fructose is fermented and 2 moles converted to mannitol, so the conversion efficiency of fructose to mannitol is therefore 67 per cent at most. The net ATP gain is 2 mol of ATP per mol of fructose fermented. Grobben et al. (22) showed that the maximum growth rate on fructose of a mannitol-producing strain of *Leuc. pseudomesenteroides* was 0.55 h^{-1}, significantly

higher than that of a strain that was only able to ferment fructose (0.39 h^{-1}) . *Lactobacillus brevis* showed an identical fermentation pattern on fructose compared to that of *Leuc. mesenteroides* (40).

Busse et al. (6) and Erten (16) found a lower mannitol yield from fructose in *Leuc. mesenteroides*. The fermentation balance approximated (equation III):

3 fructose → 2 CO_2 + 2 lactate + 0.5 acetate + 1.5 ethanol + 1 mannitol (III)

The fermentation pattern in equation (III) leads to only 1.25 mol of ATP per mol of fructose fermented. This might be caused by a limiting availability of fructose. The fermentation balance mentioned in equation (II) is only favorable at a fructose excess. This pattern requires 3 mol of fructose, but only one mol fructose is fermented, so the net ATP gain is only 0.67 mol of ATP per mol of fructose utilized. Although mannitol production enhances the growth rate of *Leuc. pseudomesenteroides*, the growth yield, defined as the amount of energy produced from fructose consumption, becomes lower (22). Grobben et al. (unpublished results) indeed showed that when the initial fructose concentration decreased, both the growth rate and the fructose to mannitol conversion efficiency decreased. A regulatory tool for an efficient energy household might be the activity of mannitol dehydrogenase and its affinity (K_m) for fructose, which was found to be 35 mM in *Leuc. mesenteroides* (50). Since a high mannitol dehydrogenase activity is only beneficial at a fructose excess, a high conversion efficiency of fructose to mannitol becomes energetically more disadvantageous at decreasing fructose concentrations. Therefore, at decreasing fructose concentrations a shift towards fructose fermentation becomes more economical. The high K_m value of mannitol dehydrogenase makes that relatively more fructose is available for fermentation.

Leuc. pseudomesenteroides DSM 20193 initially did not show any mannitol production, a mannitol producing phenotype only appeared after prolonged growth in the presence of fructose. The change in phenotype was correlated with the appearance of the mannitol dehydrogenase enzyme. Furthermore, the original strain showed a diauxic growth pattern when grown with a mixture of glucose plus fructose, whereas the mannitol-producing variant strain used both carbohydrates simultaneously. The mannitol-producing phenotype was shown to be stable, since a reconversion to the original strain was not observed when the strain was grown in the absence of fructose (22).

The reduction of fructose to mannitol in heterofermentative lactic acid bacteria is catalyzed by an NADH-linked mannitol dehydrogenase (EC 1.1.1.67). It was observed that this enzyme reduces fructose exclusively to mannitol and with a high activity. No concomitant production of sorbitol is found. This enzyme is also able to oxidize mannitol to fructose, but with a fourfold lower maximum activity (49, 50). Sorbitol could not be oxidized (22). In *Leuc. pseudomesenteroides* the activity of mannitol dehydrogenase was found to be independent of the carbohydrate source for growth, so the enzyme appears to be constitutively synthesized (22). In *Lactobacillus brevis* the enzyme is induced by growth on fructose, glucose-grown cells show only little mannitol dehydrogenase activity (40).

In contrast to homofermentative lactic acid bacteria, heterofermentative lactic acid bacteria do not possess any mannitol phosphate dehydrogenase activity, but they only show mannitol dehydrogenase activity using intracellular fructose or mannitol as a substrate. It was observed that the mannitol dehydrogenase of *Leuc. pseudomesenteroides* could not convert mannitol 1-phosphate, fructose 1-phosphate and fructose 6-phosphate (22). Therefore it is clear that these enzymes differ strongly from the mannitol-phosphate dehydrogenases found in homofermentative lactic acid bacteria that only convert fructose-phosphate and mannitol-phosphate, as mentioned above. Up to now, no mannitol dehydrogenase activity has been found in homofermentative lactic acid bacteria and no mannitol phosphate dehydrogenase in obligately heterofermentative lactic acid bacteria.

The substrate spectrum of mannitol dehydrogenase of lactic acid bacteria is much smaller compared to other microorganisms. Several NAD(H)-dependent mannitol dehydrogenases from other microorganisms such as *Pseudomonas fluorescens* (4), and *Rhodobacter sphaeroides* (51), have a broad substrate spectrum, oxidizing mannitol, sorbitol, glucitol and arabitol, and reducing fructose and xylulose. Furthermore, also NADP(H)-dependent mannitol dehydrogenases (EC 1.1.1.138) have been described for *Gluconobacter* (1) and *Agaricus bisporus* (60).

ORGANIZATION OF GENES INVOLVED IN MANNITOL METABOLISM

In general, the genes encoding mannitol 1-phosphate dehydrogenase (*mtlD*) and the mannitol-specific proteins of the PEP-dependent PTS (*mtlA*, *mtlR* and *mtlF*) are clustered in a single operon (Fig. 3). In the mannitol-PTS of *Staphylococcus carnosus*, *Enterococcus faecalis* (18, 19) and *B. stearothermophilus* (25), the soluble IIA^{Mtl} protein, encoded by the *mtlF* gene, is phosphorylated by the general cytoplasmic proteins EI and HPr, whereafter the B domain of the mannitol transport protein $IICB^{Mtl}$ is phosphorylated by IIA^{Mtl} (Fig. 4). In the $IICB^{Mtl}$ protein, encoded by the *mtlA* gene, the B domain is responsible for the phosphorylation of mannitol that is transported by the transmembrane C domain. In *E. coli*, IIA is linked covalently to IICB to form a $IICBA^{Mtl}$ protein (34). In some mannitol operons, also a *mtlR* gene can be found, encoding a regulatory protein. In *B. stearothermophilus,* transcription of the *mtl* genes is regulated by the transcriptional regulator mtlR, a DNA binding protein whose affinity for DNA is controlled by phosphorylation by the PTS proteins HPr and $IICB^{Mtl}$ (23, 25). Furthermore, the promoter of the mannitol operon in *B. stearothermophilus* is sensitive to catabolite repression. When rapidly metabolizable carbohydrates such as glucose are transported by their PTS, the mannitol operon is no longer stimulated. In *L. lactis* IL1403 a similar mannitol operon as observed in *B. stearothermophilus* has been found (Fig. 3) (3). Probably, also in this homofermentative lactic acid bacterium, mannitol utilization is regulated in a similar way.

Since mannitol dehydrogenase oxidizes mannitol but not mannitol 1-phosphate, heterofermentative lactic acid bacteria must have other transport mechanisms for mannitol than the previously described mannitol-specific PTS for homofermentative lactic acid bacteria. So far, no mannitol operon such as that in homofermentative lactic acid bacteria has been described for heterofermentative lactic acid bacteria. In some non-lactic acid bacteria however, such gene clusters involving the uptake and subsequent oxidation of mannitol have been found. In *Pseudomonas fluorescens*, a gene cluster was present, containing a gene for a polyol dehydrogenase and genes similar to components of the maltose transport system of *E. coli* and *Salmonella typhimurium* (5). In *Rhodobacter sphaeroides*, a polyol operon was recognized, encoding an ATP-binding cassette (ABC) transport system and two polyol dehydrogenases (59).

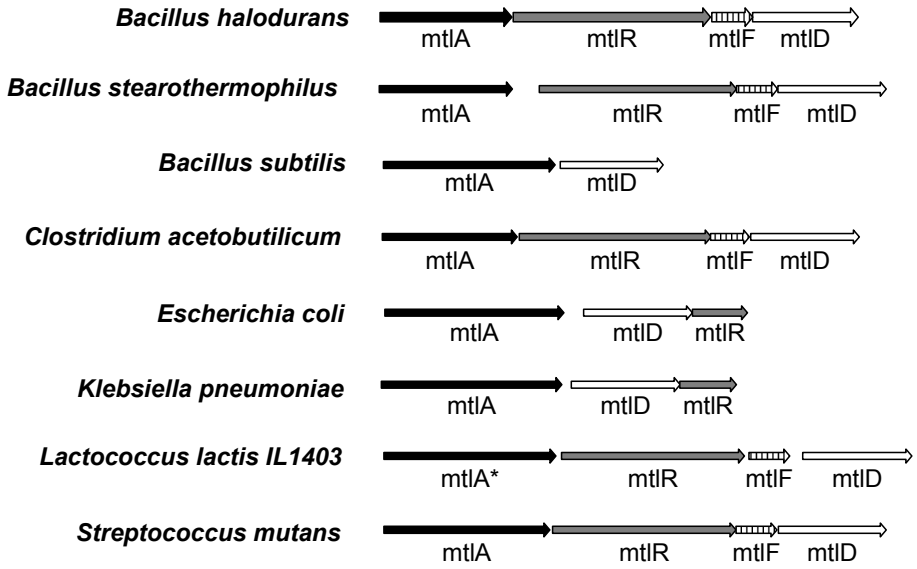

FIG. 3. Mannitol operons found by BLAST search (website: http://www.ncbi.nlm.nih.gov/) on the nucleotide databases. Accession nrs: *Bacillus halodurans* (AP001520; BA000004); *Bacillus subtilis* (Z99106; AL009126); *Clostridium acetobutilicum* (U53868); *Escherichia coli* (AE000438; U00096); *Klebsiella pneumoniae* (AF166095); *Lactococcus lactis* IL1403 (AE006241; AE005176; AE006242; AE005176), *Streptococcus mutans* (AF210133; M94225).

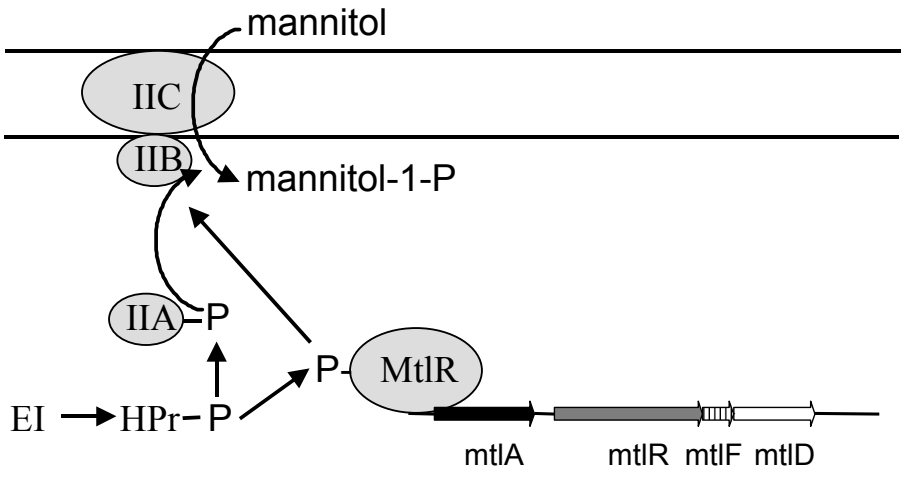

FIG. 4. Simplified model for the uptake of mannitol by the mannitol-specific PTS of *Bacillus stearothermophilus*. The regulatory protein MtlR can be phosphorylated on several domains, leading to an increased or decreased affinity for the promoter region of the mannitol operon. A more detailed model is described by Henstra, Duurkens & Robillard (2000).

STRATEGIES FOR ENGINEERING MANNITOL PRODUCTION IN *L. LACTIS*

Starter cultures of lactic acid bacteria are often prepared by drying cell cultures. During this process, the cells are subjected to a combination of hostile conditions, including osmotic and oxidative stress. For *L. lactis*, a homofermentative lactic acid bacterium that is extensively used in the dairy industry, mannitol was observed to be a good protectant during these stress conditions (15). The viability of starter cultures of *L. lactis* may be enhanced by inducing mannitol production in these strains. Furthermore, the use of a mannitol producing *L. lactis* may result in fermented products with extra value, as discussed before. This species is also readily approachable to genetic engineering and it is considered to belong to the best characterized model systems for engineering strategies.

As described before, mannitol production by homofermentative lactic acid bacteria is not very common. Catabolite repression of the mannitol operon by favorable substrates (23, 25) may result in very low mannitol 1-phosphate dehydrogenase activities, and therefore no mannitol production can take place. To achieve mannitol production in *L. lactis*, several strategies can be followed.

Overexpression of mannitol 1-phosphate dehydrogenase in *L. lactis*

In this strategy, mannitol 1-phosphate dehydrogenase from *L. lactis* or other homofermentative lactic acid bacteria can be cloned and overexpressed in *L. lactis*, which might result in an increasing flux from fructose 6-phosphate to mannitol 1-phosphate. Examples of expression of *E. coli* mannitol 1-phosphate dehydrogenase in *Saccharomyces cerevisiae* and tobacco, resulting in mannitol-producing mutants, capable of growing in the presence of high salt concentrations are known (9, 64).

Possibly, the overexpression of mannitol 1-phosphate dehydrogenase alone will not be sufficient to get mannitol production. In general, the intracellular concentration of fructose 6-phosphate will be very low as a result of the high flux of hexoses through glycolysis, and therefore the flux towards mannitol might be hampered. Another strategy to direct the hexose flux towards mannitol, is to knock out NAD^+-regenerating enzymes such as lactate dehydrogenase and alcohol dehydrogenase. These lactate dehydrogenase- or alcohol dehydrogenase-deficient cells must find another way to regenerate NAD^+, and this may lead to NAD^+-regeneration via mannitol 1-phosphate dehydrogenase, resulting in mannitol synthesis.

A few examples of lactate dehydrogenase knockouts among lactic acid bacteria are known. ^{13}C-NMR analysis of end products in an ldhL-ldhD double-knockout mutant of *Lactobacillus plantarum* (17) revealed that this mutant produced small amounts of mannitol. Although two papers report lactate dehydrogenase deficient *L. lactis* strains which do not produce mannitol but instead a mixture of formate, ethanol, acetoin and 2,3-butanediol (46), and exclusively ethanol (26), transient mannitol production by another lactate dehydrogenase deficient *L. lactis* has been observed (41). In cell suspensions, high levels of intracellular mannitol were produced, but upon glucose depletion, mannitol was re-metabolized.

Although mannitol accumulated intracellularly in the *L. lactis cells*, no extracellular mannitol was detected (41). Glucose might inhibit mannitol excretion and only after glucose depletion mannitol might be transported out of the cell. However, mannitol is not converted into mannitol 1-phosphate until glucose is depleted, but the mechanism is not clear yet. Since mannitol kinase has not been reported in bacteria (48), this may imply that mannitol cannot be phosphorylated unless it is excreted and subsequently re-taken up by the mannitol-specific PTS. Since no extracellular mannitol was found, the mannitol uptake might take place at a higher rate. It has to be taken into account that for a high mannitol production process the uptake and subsequent conversion of mannitol is undesirable.

(Over)expression of mannitol dehydrogenase in *L. lactis*

Another possibility to produce mannitol in *L. lactis* is the (over)expression of the gene encoding the heterofermentative lactic acid bacterial enzyme mannitol dehydrogenase. To ensure mannitol production, intracellular fructose has to be formed, since fructose and not fructose 6-phosphate is the substrate for mannitol dehydrogenase. To accomplish this, a *L. lactis* strain capable of growing on sucrose can be used. Sucrose will be taken up by a sucrose-specific PTS (38), whereafter the resulting sucrose 6-phosphate is hydrolyzed by sucrose 6-phosphate hydrolase. The products of this hydrolysis are glucose 6-phophate, which enters glycolysis, and fructose (66). Subsequently, fructose might be reduced to mannitol by mannitol dehydrogenase. However, it has to be taken into account that fructose may also be phosphorylated by an ATP-dependent fructokinase, which means a competition of fructokinase and mannitol dehydrogenase for fructose. In general, K_m values of mannitol dehydrogenase for fructose are higher than the K_m value of fructokinases (K_m values were found via the Internet website http://srs.ebi.ac.uk in the BRENDA enzyme database), so this might be another hurdle in mannitol synthesis.

CONCLUSIONS

In literature, mannitol production has been described for both homo- and heterofermentative lactic acid bacteria. In general, homofermentative species produce no or only small amounts of mannitol, which in most cases remain intracellularly, whereas some heterofermentative lactic acid bacteria form and export substantial amounts of this sugar alcohol. Two different key enzymes are involved in mannitol production, mannitol 1-phosphate dehydrogenase for homofermentative, and mannitol dehydrogenase for heterofermentative lactic acid bacteria. Overexpression of genes encoding these enzymes might help in enhancing mannitol biosynthesis in *L. lactis*. Knocking out genes of NAD^+ regenerating enzymes such as lactate dehydrogenase may lead to a regeneration of NAD^+ via the reduction of fructose 6-phoshate to mannitol 1-phosphate and so redirect the glycolytic flux towards mannitol production.

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CHAPTER 2

Mannitol Production from Sucrose by Overproduction of Mannitol Dehydrogenase in *Lactococcus lactis*

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ABSTRACT

The mannitol dehydrogenase gene (*mdh*) of *Leuconostoc mesenteroides* was cloned and overexpressed in *Lactococcus lactis* strain NZ9800 and its derivative NZ9841, deficient in fructokinase I. Overexpression of *mdh* during growth on sucrose of these strains was expected to result in mannitol production by the conversion of the fructose moiety. Despite high expression of *mdh*, only very low mannitol production was observed in both strains. HPLC analysis and *in vitro* enzyme activity measurements indicated that both strains NZ9800 and NZ9841 contain an alternative route for fructose utilization. By expulsion of the fructose and subsequent re-uptake by the PTS, both strains are able to utilize intracellular fructose.

INTRODUCTION

Lactococcus lactis has been proven to be an ideal organism for the application of metabolic engineering. Efficient diacetyl, alanine, and folic acid production have been achieved by the controlled overexpression of genes using the NICE system, and inactivation of undesired genes (16, 17, 29). Also for the production of mannitol, a low calorie sweetener and antioxidant (6-9, 11), successful engineering strategies have been developed. Recently, it has been reported that lactate dehydrogenase deficiency in *L. lactis* has resulted in transient mannitol production in resting cells (26), and that inactivation of the mannitol transport system prevents re-utilization of the produced mannitol in this *L. lactis* strain (12). Moreover, with the aid of metabolic control analysis using a detailed glycolysis model of *L. lactis*, we developed a metabolic engineering strategy which resulted in maximal 50% glucose conversion into mannitol in a lactate dehydrogenase-deficient strain, by the combined overproduction of a mannitol 1-phosphatase (M1Pase) and mannitol 1-phosphate dehydrogenase (M1PDH) (35). However, a disadvantage of the mannitol production via M1PDH and M1Pase, is that mannitol is not produced directly but via the formation of phosphorylated intermediates. One of these intermediates, fructose 6-phosphate, is also part of the glycolytic pathway, thus creating a direct competition between mannitol production and glycolysis. Uncoupling the mannitol production pathway from the glycolysis would favor the production of mannitol. This could be realized by introducing an alternative mannitol pathway in *L. lactis*.

In contrast to homofermentative lactic acid bacteria (LAB) such as *L. lactis*, many heterofermentative LAB are able to produce high amounts of mannitol, using a single enzyme step, the conversion of fructose into mannitol by mannitol dehydrogenase (MDH). When glucose and fructose are supplied to growing cells of *Leuconostoc mesenteroides* or *Lactobacillus fermentum* in a 1:2 ratio, glucose is converted in the 6-phosphogluconate/ phosphoketolase pathway, while more than 90% of the fructose can be converted into mannitol (2, 13, 28, 33). By introduction of a functional MDH in *L. lactis*, the mannitol production pathway is not competitive with glycolysis. Consequently, another hurdle to take is supplying intracellular fructose, since fructose is phosphorylated by a phosphotranferase system (PTS) upon transport across the membrane in *L. lactis* (31). This can be achieved by using a *L. lactis* strain capable of growing on sucrose (19, 23). Sucrose is transported by a PTS in such a strain, phosphorylated to sucrose 6-phosphate, and hydrolyzed by sucrose 6-phosphate hydrolase into glucose 6-phosphate and fructose. The glucose 6-phosphate part of sucrose enters glycolysis, and the fructose moiety can be converted into mannitol (Fig. 1).

In this work, we describe the cloning of the *mdh* gene, encoding MDH of *Leuconostoc mesenteroides*, into *L. lactis* NZ9800 (19), which is able to grow on sucrose, and strain NZ9841 (21), carrying an inactivated *sacK* gene, encoding the sucrose-inducible fructokinase I (23). The ability to produce mannitol by both of the *mdh* overexpressing strains is investigated and compared, and some apparent obstacles in efficient mannitol production are discussed.

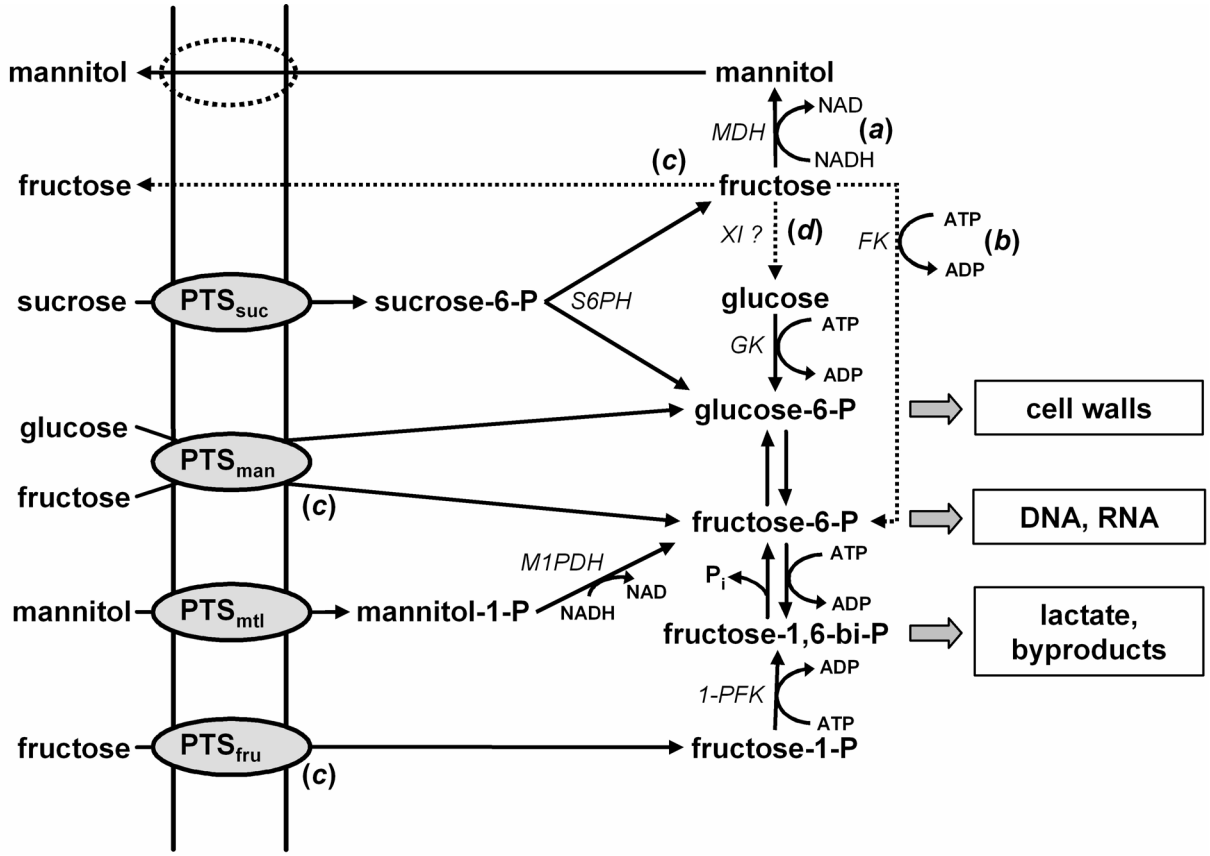

FIG. 1. Proposed sucrose metabolism of *L. lactis* NZ9800/NZ9841 overproducing MDH from *Leuconostoc mesenteroides*. The possible pathways of the metabolization of intracellular fructose are indicated: conversion of fructose into mannitol by the introduced mannitol dehydrogenase of *Leuconostoc mesenteroides* (a), phosphorylation of fructose into fructose 6-phosphate by fructokinase (b), export and the subsequent import of fructose by the fructose PTS (PTS_{fru}) or the mannose PTS (PTS_{man}) (c), and the conversion of fructose into glucose by a xylose isomerase-like enzyme activity (d). Mannitol is exported by an unknown mechanism, and is possibly re-utilized by the mannitol PTS (PTS_{mtl}) (27). Enzyme abbreviations: sucrose 6-phosphate hydrolase (S6PH), mannitol dehydrogenase (MDH), fructokinase (FK), glucokinase (GK), 1-phosphofructokinase (1-PFK), xylose isomerase (XI), phosphotransferase system (PTS).

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

Strains and plasmids	Characteristics	References or source
Strains		
NZ9800	NZ9700 derivative; Δ*nisA*; Tn*5276* with deletion in nisA, growth on sucrose	(19)
NZ9841	NZ9800 derivative, Δ*sacK*; *sacK::ery,* Ery^r.	(21)
NZ9800 (pNZ8148)	NZ9800 containing pNZ8148 plasmid	This work
NZ9841 (pNZ8148)	NZ9841 containing pNZ8148 plasmid	This work
NZ9800 (pWW004*)*	NZ9800 containing pWW004 plasmid	This work
NZ9841 (pWW004*)*	NZ9841 containing pWW004 plasmid	This work
Plasmids		
pNZ8148	pNZ8048 derivative; Cm^r, lactococcal cloning and expression vector with *nisA* promoter upstream of a multiple cloning site	(20)
pWW004	pNZ8148 carrying *Leuconostoc mesenteroides mdh* gene fused to *nisA* promoter	This work

MATERIALS & 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 sucrose, fructose, and glucose. Concentrations of the supplied sugars are indicated in the results. For (semi)anaerobically cultivations, cells were grown in batch cultures in 50 ml tubes without aeration. When applicable, chloramphenicol and erythromycin were supplemented at 10 and 5 µg/ml, respectively. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a Ultrospec 2000 spectrofotometer (Pharmacia Biotech). For inducing MDH activity, 3 ng/ml nisin was added to a growing culture at an OD_{600} of 0.1. Maximal *mdh* expression was reached at these conditions.

Construction of plasmid pWW004. The *mdh* gene coding for mannitol dehydrogenase of *Leuconostoc mesenteroides* ATTC-9135 (1) was cloned in the lactococcal expression vector pNZ8148. For this, *mdh* was amplified by PCR from *Leuconostoc mesenteroides* genomic DNA, using the primers mdhlm-1FW (5'- ATTAACCATGGAAGCACTTGTTCTAAC-3') and mdhlm-1RV (5'- GTCAGTCTAGATTATGCCTCTTCGCCGCCAA-3'), with introduced *Nco*I and *Xba*I digestion sites, respectively (underlined). The sequence of *mdh* was verified by sequencing the PCR product (Baseclear, The Netherlands). *Nco*I-*Xba*I digested *mdh* was cloned into pNZ8148, resulting in pWW004, containing *mdh* fused to the *nisA* promoter. pWW004 was transformed into the *L. lactis* strains NZ9800 (19) and NZ9841 (21).

Analysis of fermentation products and sugar consumption. During growth, samples were taken from the *L. lactis* cultures, centrifuged for 1 min at 10,000 × g and the supernatants were stored at –20°C until analysis. Lactate, acetate, formate, mannitol, ethanol, were detected in the supernatants by a refractive index detector (Waters 2414), after separation by HPLC using a 30 cm IOA-1000 ion exclusion column (Alltech, Breda, the Netherlands), with 3 mM sulfuric acid as eluens, at a flow rate of 0.4 ml/min and a temperature of 90°C. A Dionex Carbopac PA1 anion exchange column was used for determining the supernatant concentrations of sucrose, fructose, and glucose. After separation on this column using a sodium hydroxide gradient of 0 to 45 mM NaOH in 20 min, at 1 ml min^{-1}, and a temperature of 35°C, the sugar compounds were detected by a Dionex ED50 BioLC electrochemical detector (pulsed amperometric detector). Fluxes (in mmol min^{-1} mg $protein^{-1}$) for the different sugars and lactate were calculated with the assumption that 50% of the dry weight consists of protein, as in *E. coli* (25), and that an OD_{600} of 1 is equal to 0.455 mg dry weight ml^{-1} (18).

Preparation of cell extracts. Cell-free extracts were prepared by disruption of cells by glass beads. For this, 50 ml of cell culture was harvested at an OD_{600} of approximately 1.1, centrifuged (4°C, 20 min at 2000 × g), and the cell pellets were washed with 50 mM MES buffer (pH 7.0) and resuspended in 2 ml 50 mM MES buffer (pH 7.0). Then 1 ml of cell suspension was added to 1.0 g of 0.1 mm zirconia/silica beads (BioSpec products, Inc.) in a 2 ml eppendorf cup, and cells were disrupted by vigorously 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.

Enzyme activities. All in vitro enzyme assays were performed in a volume of 200 µl in wells of a 96-wells microtiter plate at 30°C in a microplate reader (Tecan Safire, Austria) with freshly prepared cell extracts. The protein content of the cell extracts was determined using the BCA protein assay (Pierce, USA), with bovine serum albumin as the standard. In all assays, the enzyme activity was determined by monitoring the increase or decrease of NAD(P)H by measuring the absorbance at 340 nm. All enzymes used in the combined enzyme assays were obtained from Roche.

Fructokinase (FK) activity was assayed in a reaction mixture containing 0.1 M triethanolamine pH 7.0, 5 mM ATP, 10 mM $MgCl_2$, 1 mM NAD, 5 U/ml glucose 6-phosphate dehydrogenase (G6PDH, from *Leuconostoc mesenteroides*), 3.5 U/ml phosphoglucose isomerase (from yeast), and 50 µg/ml cell-free extract. The reaction was started by adding D-fructose to the reaction mixture to a final concentration of 10 mM. FK activity in extracts of the NZ9800 (pWW004) transformant could not be determined, due to the disturbing high MDH activity in these samples, which converts the formed NADH in the assay into NAD again.

The 1-phosphofructokinase (1-PFK) activity in the cell extracts was determined according to Grobben et al. (14), with the modification that the assay contained 5 mM ATP and 0.4 mM NADH. 50 µg/ml cell-free extract was added to the assay and the reaction was started by the addition of fructose 1-phosphate to a final concentration of 1 mM.

Mannitol dehydrogenase (MDH) was assayed in forward direction. The reduction of fructose by MDH was determined in a reaction mixture containing 50 mM sodium acetate buffer pH 5.5, and 0.4 mM NADH. Approximately 5 µg/ml of cell-free extract was added to the reaction mixtures, and the reaction was started by the addition of D-fructose to a final concentration of 100 mM.

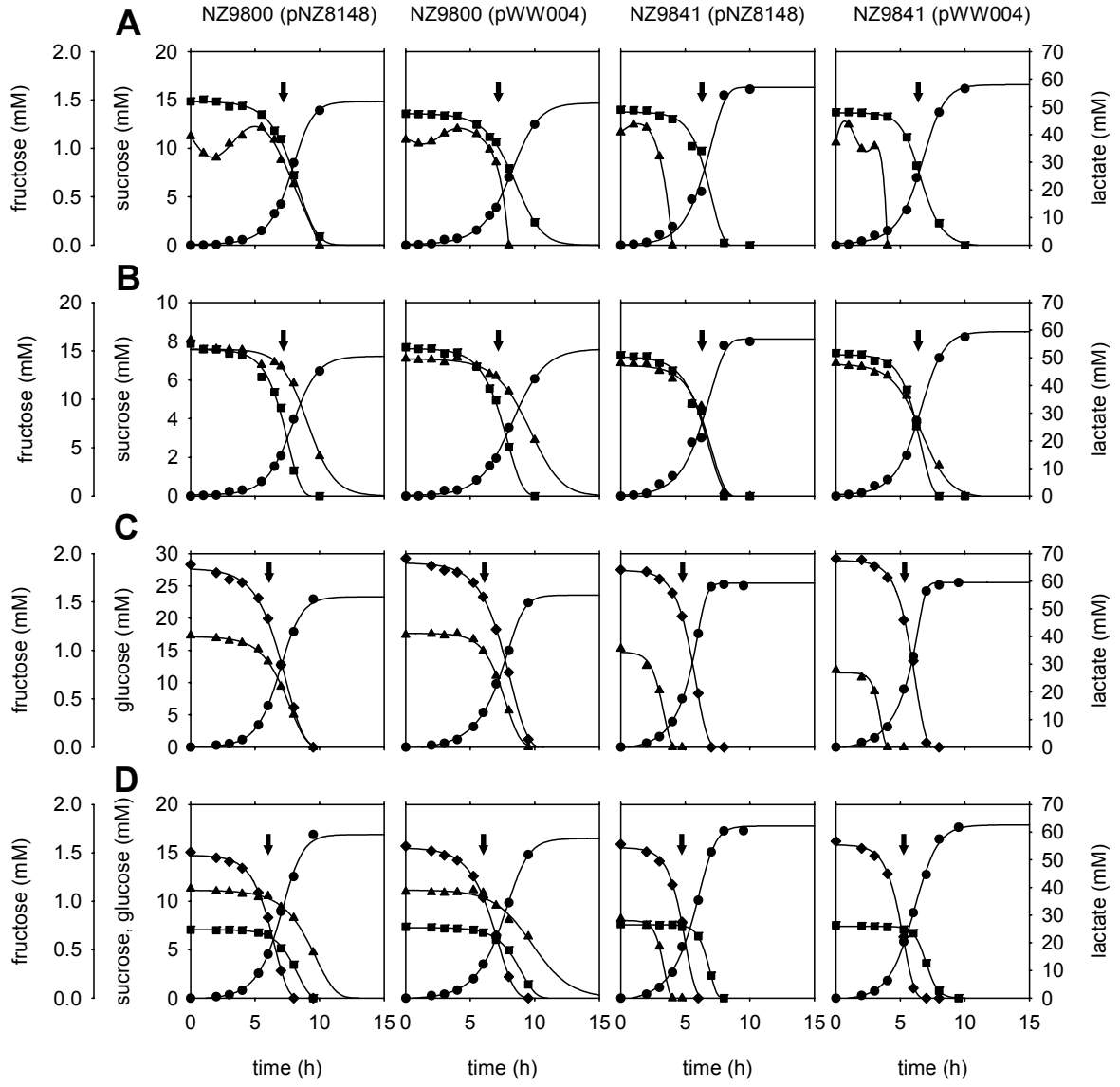

FIG 2. Sugar consumption and lactate formation of strains NZ9800 and NZ9841 strains, harboring either pNZ8148 or pWW004. Cells were cultured anaerobically in M17 broth supplemented with 0.5% sucrose (A), 0.25% sucrose + 0.25% fructose (B), 0.5% glucose (C), and 0.25% glucose + 0.25% sucrose (D), and induced by the addition of 3 ng/ml of nisin at an OD_{600} of 0.1. The arrows indicate the time point at which cells were harvested for in vitro enzyme activity measurements. Symbols: sucrose (■), fructose (▲), glucose (◆), and lactate (●).

RESULTS

The *mdh* gene of *Leuconostoc mesenteroides* was cloned into the nisin-controlled lactococcal expression vector pNZ8148, and the resulting pWW004 was transformed to *L. lactis* NZ9800 and the FK-deficient strain NZ9841. As a control, pNZ8148 was transformed to both strains. Strains NZ9800 and NZ9841 harboring either pNZ8148 or pWW004 were grown on M17 broth supplemented with sucrose, fructose, and glucose (Fig. 2). Sugar consumption, lactate and mannitol formation, and enzyme activities were determined for growing cultures induced with nisin.

Mannitol production. Overproduction of nisin-induced MDH in strains NZ9800 and NZ9841 was expected to result in mannitol production during growth on sucrose. Growth of strain NZ9800 (pWW004) on sucrose resulted in 0.2 mM of mannitol at the end of growth (Table 2). No mannitol was produced when this strain was grown on glucose. When grown on both glucose and sucrose, a low concentration of 0.3 mM of mannitol was found to be present in the supernatant after sugar depletion. Equal amounts of mannitol were produced by the nisin-induced FK-deficient strain NZ9841 (pWW004) during growth on sucrose.

Sugar consumption and lactate production. Since only low amounts of mannitol were formed during growth of strains NZ9800 (pWW004) and NZ9841 (pWW004) in the presence of sucrose, the fructose moiety of sucrose is metabolized in an alternative way by these strains. Strains NZ9800 and NZ9841 harboring pWW004 produce equal amounts of approximately 60 mM of lactate, compared to the control strains harboring pNZ8148 (Fig. 2). Thus, in all strains investigated, sucrose is metabolized completely into lactate in a homolactic fermentation, including the fructose moiety of sucrose. In addition to the consumption of sucrose, it was observed that a residual amount of fructose present in the M17 broth, was consumed by both strains NZ9800 and NZ9841 harboring either the empty pNZ8148 or the *mdh* overexpression plasmid pWW004 (Fig 2, row A). A remarkable observation, was the slight but reproducible increase in the extracellular fructose concentration observed for strains NZ9800 (pNZ8148) and NZ9800 (pWW004). This is followed by a decrease of the fructose in time, implying that fructose is exported and subsequently taken up again by a transport system. This phenomenon was also observed for strains NZ9841 (pNZ8148) and NZ9841 (pWW004), but the increase of the extracellular fructose here was less pronounced and the fructose was consumed more rapidly than for strains NZ9800 (pNZ8148) and NZ9800 (pWW004) (Fig. 2, row A). Under all conditions investigated, strains NZ9841 (pNZ8148) and NZ9841 (pWW004) displayed a more rapid fructose uptake, compared to strains NZ9800 (pNZ8148) and NZ9800 (pWW004) (Fig. 2).

This was reflected in the different fructose fluxes calculated for strains NZ9800 and NZ9841, harboring either pNZ8148 or pWW004 (Table 2). In the presence of sucrose, fructose fluxes of 0.02-0.07 μmol min^{-1} (mg protein)$^{-1}$ were calculated for the strains NZ9800 (pNZ8148) and (pWW004), whereas strains NZ9841 (pNZ8148) and NZ9841 (pWW004) displayed much higher fluxes of 0.17-0.21 μmol min^{-1} (mg protein)$^{-1}$ (Table 2). Also in the presence of glucose, the fructose fluxes in strains NZ9841 (pNZ8148) and NZ9841 (pWW004) were found to be higher than in strains NZ9800 (pNZ8148) and NZ9800 (pWW004) (0.11-0.16 versus 0.01-0.02 μmol (mg protein)$^{-1}$ min^{-1}, respectively). The higher fructose fluxes of the FK-deficient strain NZ9841 may imply that either a different fructose uptake system is active in strain NZ9841 in comparison with strain NZ9800, or the fructose transport system is more active in strain NZ9841. The observed differences in fructose uptake between the two strains during growth on M17 broth containing both glucose and sucrose, suggests involvement of two different fructose uptake systems in strains NZ9800 and NZ9841. The residual fructose was consumed simultaneously with the sucrose in strains NZ9800 (pNZ8148) and NZ9800 (pWW004), while in strain NZ9841 (pNZ8148) fructose was consumed rapidly immediately after inoculation, simultaneously with the glucose (Fig. 2, row D).

Enzyme activities. Analysis of the supernatants showed that only small amounts of mannitol were produced by strains NZ9800 and NZ9841 overexpressing *mdh*. To investigate the fate of fructose and the fructose moiety of sucrose in both NZ9800 and NZ9841 strains, extracts were prepared of growing cells of the various NZ9800 and NZ9841 transformants harvested at different time points (Fig. 2). Subsequently, the activities of fructokinase (FK), 1-phosphofructokinase (1-PFK), and mannitol dehydrogenase (MDH) were determined and compared (Table 3).

TABLE 2. Fluxes, growth rate, and mannitol production determined for strains NZ9800 and NZ9841, harboring either pNZ8148 (empty vector) or pWW004 (*mdh* expression plasmid). Cells were grown anaerobically in M17 broth supplemented with 0.5% of sucrose (S), 0.25% sucrose + 0.25% fructose (S+F), 0.5% glucose (G), or 0.25% glucose + 0.25% sucrose (G+S), and induced by the addition of 3 ng/ml of nisin at an OD_{600} of 0.1. Fluxes were calculated for the exponential growth phase, except for the sucrose flux of cultures grown on glucose and sucrose, as sucrose consumption started upon depletion of glucose in the late growth phase. The growth rate was determined during exponential growth. The final mannitol concentration was measured after sugar depletion. The detection limit of mannitol in the supernatants was approximately 0.1 mM

		flux (μmol (mg protein)$^{-1}$ min^{-1})					Final conc.
L. lactis strain	C-source	sucrose	glucose	fructose	lactate	μ (h^{-1})	mannitol (mM)
NZ9800 (pNZ8148)	S	-0.15	ND	-0.02	0.58	0.62	<0.1
	S+F	-0.13	ND	-0.06	0.56	0.60	<0.1
	G	ND	-0.67	-0.02	1.38	0.71	<0.1
	G+S	-0.08	-0.46	-0.01	1.24	0.69	<0.1
NZ9800 (pWW004)	S	-0.13	ND	-0.02	0.57	0.59	0.2
	S+F	-0.12	ND	-0.07	0.59	0.59	0.1
	G	ND	-0.32	-0.02	0.74	0.61	<0.1
	G+S	-0.04	-0.28	-0.01	0.68	0.60	0.3
NZ9841 (pNZ8148)	S	-0.20	ND	-0.21	0.85	0.76	<0.1
	S+F	-0.12	ND	-0.21	0.94	0.75	<0.1
	G	ND	-0.52	-0.13	1.22	0.80	<0.1
	G+S	-0.07	-0.52	-0.11	1.22	0.78	<0.1
NZ9841 (pWW004)	S	-0.17	ND	-0.20	0.77	0.71	0.3
	S+F	-0.10	ND	-0.17	0.78	0.70	0.2
	G	ND	-0.65	-0.16	1.30	0.77	<0.1
	G+S	-0.08	-0.53	ND	1.10	0.75	0.2

ND: Not determined

Induction with nisin of strains NZ9800 (pWW004) and NZ9841 (pWW004) resulted in high MDH activity in cell-extracts, as determined in the direction of fructose reduction (Table 3). No MDH activity was detected in extracts of the control strains with the empty pNZ8148 vector. Thus, despite the high MDH activity in both strains harboring pWW004 (7.7-17.4 μmol min^{-1} (mg protein)$^{-1}$), only small amounts of mannitol were produced during growth on sucrose (Table 2).

As already mentioned, the fructose moiety of sucrose is apparently metabolized in an alternative way. In *L. lactis* NZ9800, intracellular fructose is phosphorylated by FK I (23, 32), and the resulting fructose 6-phosphate is metabolized via glycolysis. Indeed, significant FK activity was found in extracts of strains NZ9800 (pNZ8148) and NZ9800 (pWW004) grown in the presence of sucrose, but no FK activity was detected in glucose-grown cells. However, in extracts of the FK-deficient strain NZ9841 harboring pNZ8148 or pWW004, no FK activity could be detected for cells grown on all sugars tested here.

Analysis of the sugars in the supernatants already showed that fructose is possibly exported and subsequently taken up by a transport system in both strains NZ9800 and NZ9841 (Fig. 2). In the case of fructose transport by a specific fructose PTS (PTS_{fru}), fructose is phosphorylated during transport into fructose 1-phosphate (31). To enter glycolysis at the level of fructose 1,6-bi-phosphate, 1-PFK activity is needed. For all strains investigated, 1-PFK was detected in extracts of cells grown on sucrose, and grown on the combination of sucrose and fructose. Growth on glucose and glucose with sucrose did not result in induction of 1-PFK activity. The higher 1-PFK activities observed for strains NZ9841 (pNZ8148) and NZ9841 (pWW004), compared to strains NZ9800 (pNZ8148) and NZ9800 (pWW004) (0.15-0.23 versus 0.03-0.06 μmol min^{-1} (mg protein)$^{-1}$), is in agreement with the higher fructose fluxes measured for the NZ9841 strains.

TABLE 3. *In vitro* enzyme activities in cell extracts of the strains NZ9800 and NZ9841, harboring either pNZ8148 or pWW004. Cells were grown in M17 broth supplemented with 0.5% of sucrose (S), 0.25% sucrose + 0.25% fructose (S+F), 0.5% glucose (G), or 0.25% glucose + 0.25% sucrose (G+S). The time points at which the cells were harvested during growth are indicated in Figure 2. Abbreviations: FK, fructokinase; 1-PFK, 1-phosphofructokinase; MDH, mannitol dehydrogenase; -, below detection limit of approximately 0.01 μmol min^{-1} (mg protein)$^{-1}$; ND, not determined.

		enzyme activities (μmol min^{-1} (mg protein)$^{-1}$)		
L. lactis strain	C-source	FK	1-PFK	MDH
NZ9800 (pNZ8148)	S	0.12	0.03	-
	S+F	0.10	0.03	-
	G	0.01	-	-
	G+S	0.06	-	-
NZ9800 (pWW004)	S	ND	0.06	13.0
	S+F	ND	0.05	17.4
	G	ND	-	10.0
	G+S	ND	-	12.8
NZ9841 (pNZ8148)	S	-	0.15	-
	S+F	-	0.23	-
	G	-	-	-
	G+S	-	-	-
NZ9841 (pWW004)	S	ND	0.22	13.9
	S+F	ND	0.23	13.2
	G	ND	-	7.7
	G+S	ND	-	9.6

DISCUSSION

An alternative mannitol production pathway was introduced in *L. lactis* NZ9800, by the controlled expression of the *Leuconostoc mesenteroides mdh* gene. *L. lactis* NZ9800, capable of growing on sucrose, was expected to produce large amounts of mannitol when *mdh* is introduced, by conversion of the fructose moiety of sucrose directly into mannitol. The *mdh* overexpression in both strains NZ9800 and NZ9841 did not result in high mannitol production from sucrose (Table 2). Approximately 60 mM lactate is produced from 15 mM of sucrose, indicating that both the glucose and the fructose part of sucrose are metabolized to lactate. During growth on sucrose, the intracellular fructose is phosphorylated by the sucrose-induced FK I (32), encoded by the *sacK* gene located on the Tn*5276* transposon present in *L. lactis* NZ9800. Indeed, significant FK activity was detected in extracts of the NZ9800 transformants (Table 3). Apparently, despite the high MDH activity in strain NZ9800 (pWW004), FK outcompetes MDH for fructose, due to the K_m of MDH (44-71 mM) (1, 15), which is much higher than the K_m of FK (0.3 mM) (32). To avoid this problem, *mdh* was expressed in *L. lactis* NZ9841, a NZ9800 derivative lacking FK I. However, in this *L. lactis* host, *mdh* expression did also not result in high mannitol production (Table 2). A second enzyme with FK activity, as has been shown to be present in *L. lactis* K1 (32), might complement the *sacK* deficiency in the NZ9841 strain. Indeed, a *scrK* gene coding for a FK, has been annotated in the *L. lactis* IL1403 genome (5), and could well be also present in the MG1363-derivative strains used here. However, the alternative fructokinase II reported for *L. lactis* K1 was only detected in cells grown on ribose, galactose, maltose, and lactulose (32). In agreement with this, no FK activity was detected in extracts of strains NZ9841 (pNZ8148) and NZ9841 (pWW004) (Table 3), so the possibility of a second fructokinase activity can be ruled out. Since strain NZ9841 harboring either pNZ8148 or pWW004 shows a homolactic fermentation pattern comparable to that of its parental strain NZ9800 (Fig. 2), and has a growth rate even higher than that of its parental strain NZ9800 (Table 2), the fructose moiety of sucrose was definitely metabolized in an alternative way in strain NZ9841.

The most likely alternative for the utilization of intracellular fructose is the export of fructose, and its subsequent uptake by a PTS system (22), as proposed in Figure 1. This process was observed for the sucrose metabolism in *Corynebacterium glutamicum* (10), and is similar to the process described as galactose expulsion by *L. lactis* subsp. *cremoris* FD1 during growth on lactose (3). In all *L. lactis* strains investigated, fructose was possibly exported during growth on sucrose in a process similar to inducer expulsion (24, 36), which is supported by the increasing external

fructose concentration of strains NZ9800 (pNZ8148) and NZ9800 (pWW004) during growth on sucrose (Fig. 2, row A). The subsequent fructose uptake in *L. lactis* may occur via either the inducible PTS_{fru} to give fructose 1-phosphate, or via the mannose PTS (PTS_{man})which has a broad substrate specificity, transporting fructose to give fructose 6-phosphate (30) (Fig. 1). When fructose is imported via the PTS_{fru}, the resulting fructose 1-phosphate is subsequently converted into the glycolysis intermediate fructose 1,6-bi-phosphate by the action of 1-PFK. Table 3 shows that 1-PFK activity is present in cell extracts of all strains investigated, grown in the presence of sucrose. These results imply that the specific PTS_{fru} was induced in both strains during growth on sucrose. In strain NZ9800, one would not be sure what the role of the fructose efflux and the subsequent uptake is, noting the presence of FK activity. Assuming that no sucrose 6-phosphate is accumulating during sucrose uptake because of its toxicity, it is possible that in strain NZ9800 the phosphorylation of the intracellular fructose by FK cannot keep up with the sucrose uptake and the hydrolysis of sucrose 6-phosphate into glucose 6-phosphate and fructose by sucrose 6-phosphate hydrolase (S6PH), resulting in accumulation of intracellular fructose. The export and the subsequent uptake of the fructose by the induced PTS_{fru} may assist in the metabolization of the fructose moiety of sucrose in strain NZ9800.

In strain NZ9841, the efflux and the subsequent uptake of fructose would compensate the FK deficiency. As in strain NZ9800, the presence of 1-PFK activity in extracts of strain NZ9841 harboring either pNZ8148 or pWW004, also suggests the induction of the specific PTS_{fru} during growth on sucrose (Table 3). Moreover, the higher fructose uptake rates and 1-PFK activities in strains NZ9841 (pNZ8148) and NZ9841 (pWW004), in comparison with the parental NZ9800 strains (Table 2 and 3), suggest that in the FK-deficient strain NZ9841 the specific PTS_{fru} and 1-PFK are induced at a higher level by growth on sucrose. In addition, the simultaneous glucose and fructose consumption in strain NZ9841 (Fig. 2, row C and D), suggest the induction of the specific PTS_{fru} from the start of the growth experiment. When only the PTS_{man} would be active, glucose would competitively inhibit the fructose transport, since the K_m of the PTS_{man} for glucose is much lower than the K_m for fructose (<11 μM versus 890 μM).

An alternative explanation for the higher fructose fluxes of strain NZ9841 in comparison with strain NZ9800, may be the activation of another fructose transport system, invoked by the disruption of *sacK* in strain NZ9841. In *L. lactis* subsp. *cremoris* FD1, the fructose transport is mediated by two PTS systems: the highly specific PTS_{fru} with a very small fructose saturation constant (< 17μM), which is induced by fructose; and the PTS_{man} with broad specificity and a fructose saturation constant of 0.89 mM, which can transport both glucose and fructose (4).

It is likely that also the PTS_{man} mediates the fructose transport in *L. lactis* NZ9841, in addition to the specific PTS_{fru}. Fructose transport via the PTS_{man} would also explain the higher growth rates of strain NZ9841 than that of strain NZ9800 (Table 2). When fructose is transported exclusively via the PTS_{fru}, the fructose 1-phosphate is mainly used for metabolic energy via the formation of lactate and other fermentation products, and only for a small part for biomass production via fructose 1,6-diphosphatase (4) (Fig. 1), resulting in a lower growth rate. If fructose is transported by the PTS_{man}, fructose 6-phosphate is formed, which is converted by phosphogluco-isomerase (PGI) into glucose 6-phosphate, both good precursors for cell wall and DNA/RNA formation (Fig. 1). In addition, the absence of 1-PFK activity in extracts of NZ9841 cells grown in the presence of glucose, also suggests that fructose was transported via the broad specifity PTS_{man} during growth on glucose and glucose with sucrose. An alternative explanation for the lack of 1-PFK activity, is that fructose was already consumed, or that sucrose consumption and the induction of the specific PTS_{fru} had not yet started, at the time points at which the samples for enzymes activity measurements were taken (Fig. 2).

A possibility we have not examined here, is the conversion of intracellular fructose into glucose by xylose isomerase (XI) (Fig. 1). This alternative is supported by the presence of an annotated *xylA* gene in the *L. lactis* IL1403 genome (5), which is supposed to code for XI. However, to the best of our knowledge, the conversion of fructose into glucose by xylose isomerase has not been reported in *L. lactis* MG1363 derivatives. Another possibility, is the re-utilization of the formed mannitol by the action of the mannitol PTS and mannitol 1-phosphate dehydrogenase, as described by Neves et al. (27) (Fig. 1). However, upon the production of mannitol, its re-utilization was not observed earlier than after 24 h in the LDH-deficient *L. lactis* NZ9010 (34). Moreover, fermentation products such as ethanol and formate are expected to be formed during growth on mannitol (27, 34), which was not observed in the fermentation patterns of the *L. lactis* strains investigated here. Hence, it is not likely that the produced mannitol was re-utilized by strains NZ9800 and NZ9841 harboring pWW004.

In conclusion, *mdh* overexpression in strains NZ9800 and NZ9841 strains did not result in high mannitol production, in contrast to the high mannitol production observed for the mannitol 1-phosphatase overproducing *L. lactis* strains (35). Presumably, the metabolism of the fructose moiety of sucrose in both strains via the efflux of intracellular fructose and the subsequent transport via a PTS system, and the high K_m value of MDH for fructose, have obviously hampered the mannitol production in the investigated *L. lactis* strains. Overproduction of an alternative NAD-dependent MDH (EC 1.1.1.67) with a lower K_m for fructose would be an option for mannitol production in *L. lactis*, but no such MDH was found in the BRENDA enzyme database (http://www.brenda.uni-koeln.de). Hence, it is likely that multiple engineering steps, such as inactivation of the fructose transport, will be needed to counteract the undesired fructose utilization.

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CHAPTER 3

Metabolic Engineering of Mannitol Production in *Lactococcus lactis*: Influence of the Overproduction of Mannitol 1-Phosphate Dehydrogenase in Different Genetic Backgrounds

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ABSTRACT

To obtain a mannitol producing *Lactococcus lactis*, the mannitol 1-phosphate dehydrogenase gene (*mtlD*) from *Lactobacillus plantarum* was overexpressed in a wild type strain, a lactate dehydrogenase deficient strain, and a strain with reduced phosphofructokinase activity. HPLC and ^{13}C NMR analysis revealed that small amounts of less than 1% of mannitol were formed by growing cells of *mtlD* overexpressing LDH-deficient and PFK reduced strains, whereas resting cells of the LDH-deficient transformant converted 25 % of glucose into mannitol. Moreover, the formed mannitol was not re-utilized upon glucose depletion. Of the investigated metabolic engineering strategies in this work, the *mtlD* overexpressing LDH-deficient *L. lactis* seemed to be the most promising strain for mannitol production.

INTRODUCTION

Mannitol is a sugar alcohol that is produced by a wide variety of (micro) organisms. It is assumed to have several beneficial effects as a food additive. It can serve as an antioxidant (5, 6, 26, 27) and as a low-calorie sweetener which can replace sucrose (9, 10). Efiuvwevwere and coworkers (11) showed that mannitol has an osmoprotecting and antioxidant effect on the dairy lactic acid bacterium *Lactococcus lactis* subjected to decreased water activity and that mannitol enhances the survival during drying of starter cells. The viability of starter cultures of *L. lactis*, which are extensively used in dairy industry, may thus be enhanced by mannitol production in these strains. In addition the use of a mannitol producing *L. lactis* may result in fermented products with extra nutritional value.

Mannitol biosynthesis in homofermentative lactic acid bacteria such as *L. lactis* starts with the glycolysis intermediate fructose 6-phosphate (Fig. 1). Mannitol 1-phosphate dehydrogenase (M1PDH) (EC 1.1.1.17) catalyses the reduction of fructose 6-phosphate, and also the reverse reaction, the oxidation of mannitol 1-phosphate (4, 12). Mannitol 1-phosphate is dephosphorylated to mannitol by mannitol phosphatase activity. Although the gene encoding M1PDH (*mtlD*) is reported for *L. lactis* IL1403 (2), mannitol production by *L. lactis* and other homofermentative lactic acid bacteria is not very likely. Presumably, the *mtlD* gene which is located in a mannitol operon, is not transcribed due to catabolite repression as described for *Bacillus stearothermophilus* (14, 15) and no mannitol production can take place during growth on certain sugar substrates. Therefore, overexpression of M1PDH might be an important step for mannitol synthesis in *L. lactis*.

In contrast to some heterofermentative lactic acid bacteria, mannitol production by homofermentative lactic acid bacteria is not very common. However, mannitol production by *L. lactis* was observed by Neves and coworkers (24). In resting high-density cell suspensions of a lactate dehydrogenase deficient *L. lactis*, high levels of intracellular mannitol were produced. Upon glucose depletion, mannitol was re-metabolized. Also a lactate dehydrogenase-negative mutant of *Lactobacillus plantarum* produced small amounts of mannitol (12). In these cases, mannitol production was believed to be an alternative pathway to regenerate NAD^+ instead of lactate formation.

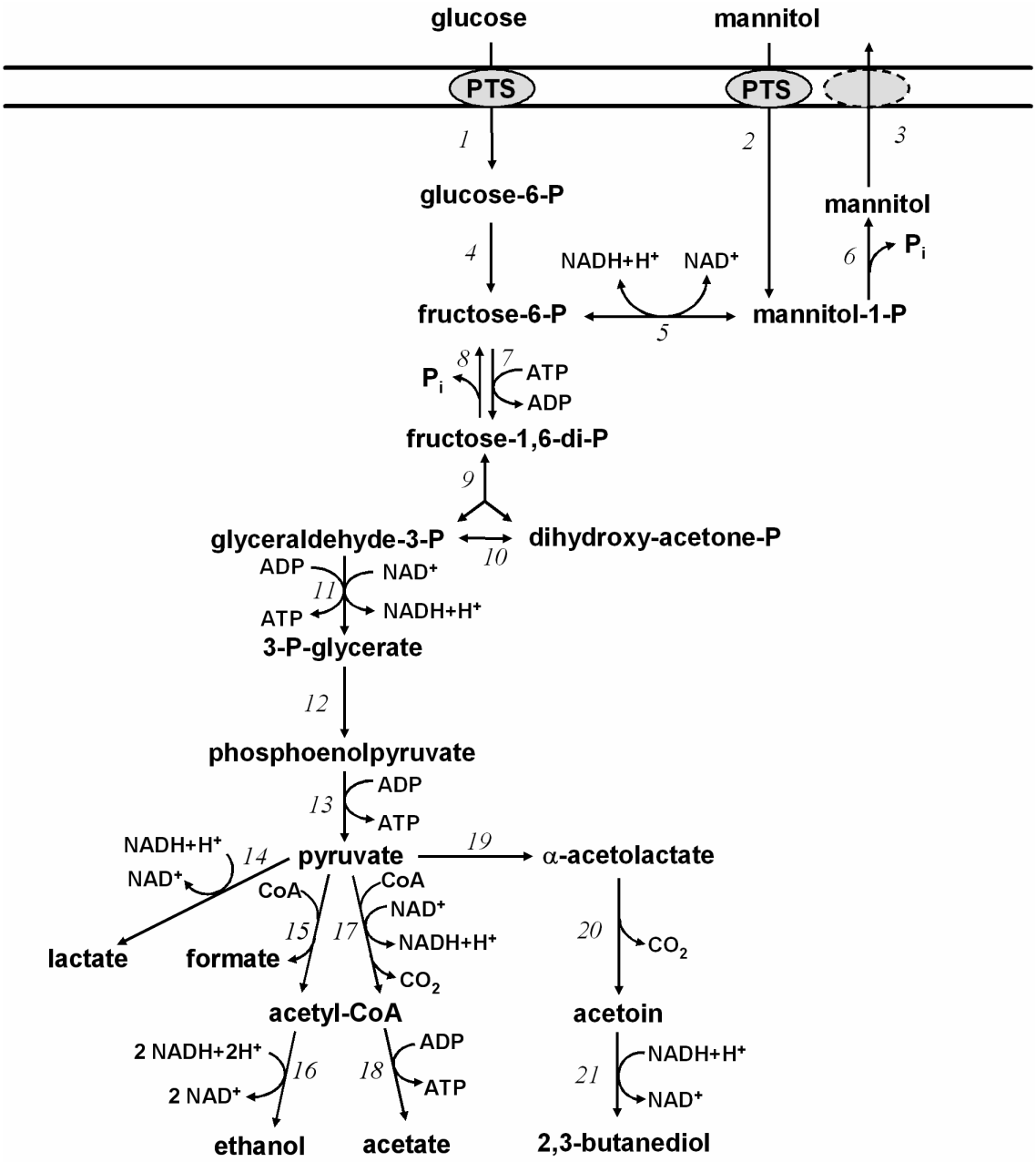

FIG. 1. Proposed pathway for hexose metabolism of homofermentative lactic acid bacteria. *1* Phospho*enol*pyruvate (PEP)-dependent sugar phosphotransferase system (PTS); *2*. Mannitol-specific ; *3*. mannitol export by unknown mechanism; PTS; *4*. Phosphoglucose isomerase; *5*. Mannitol 1-phosphate dehydrogenase; *6*. Mannitol 1-phosphatase; *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*. Lactate dehydrogenase; *15*. Pyruvate-formate lyase; *16*. Acetaldehyde dehydrogenase and alcohol dehydrogenase; *17*. Pyruvate dehydrogenase; *18*. Acetate kinase; *19*. α-Acetolactate synthase; *20*. α-Acetolactate decarboxylase; *21*. 2,3-Butanediol dehydrogenase.

Metabolic engineering can be a helpful tool to achieve mannitol production in *L. lactis*. In this report, *mtlD* from *Lactobacillus plantarum* was cloned and overexpressed in *L. lactis.* The involvement of fructose 6-phosphate as substrate of M1PDH could imply that the accumulation of fructose 6-phosphate, such as reported in *L. lactis* with reduced phosphofructokinase (PFK) activity (1), could coincide with mannitol production. Also alternative NAD^+ regeneration via M1PDH, as reported in a LDH-deficient *L. lactis* (24), could contribute to mannitol production. Therefore, *mtlD* was overexpressed in different genetic backgrounds: the parental *L. lactis* strain NZ9000, a LDH-deficient strain NZ9010, and a strain with reduced PFK activity (HWA217). Also, a comparison was made between mannitol production by growing cells and high-density resting cells.

TABLE 1. Bacterial Strains and plasmids used in this study

Strains and plasmids	Characteristics	References or source
Strains		
L. lactis NZ9000	MG1363 *pepN::nisRK*	(22)
L. lactis NZ9010	NZ9000 *ldh::ery,* Ery^r	(3, 17)
L. lactis HWA217	reduced phosphofructokinase activity	(1)
Plasmids		
pNZ8148	pNZ8048 derivative; Cm^r, lactococcal cloning and expression vector with nisA promoter upstream of a multiple cloning site	(22)
pNZmtlD	pNZ8148 carrying *Lactobacillus plantarum mtlD* gene, fused to the *nisA* promoter	this work
pNZ9530	Ery^r, *nisRK*	(19)

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)anaerobically cultivations, cells were grown in batch cultures in 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 µg/ml, respectively. Growth was followed by measuring the optical density at 600 nm (OD_{600}) with a Ultrospec 2000 spectrofotometer (Pharmacia Biotech). For inducing M1PDH activity, 1 ng/ml nisin was added to a growing culture at an OD_{600} of 0.5.

Construction of plasmid pNZmtlD. The gene encoding mannitol 1-phosphate dehydrogenase (*mtlD*) from *Lactobacillus plantarum* was cloned into the nisin inducible expression vector pNZ8148 (Table 1). For this, *mtlD* was amplified by PCR from *L. plantarum* genomic DNA (accession no NP 784055) (20) using the primers MPDHLP-1FW (5'-TCGTACCATGGTAGACG-TACATTTTG-3') and MPDHLP-3RV (5'-GTCAGTCTAGACTACTTTGCTGCA-GCTAAG-3'), with introduced *NcoI* and *XbaI* digestion sites, respectively (underlined). *NcoI – XbaI* digested *mtlD* was cloned into pNZ8148, resulting in pNZmtlD, containing the *mtlD* fused to the *nisA* promoter. The sequence of *mtlD* was verified by sequencing the cloned PCR product (Eurogentec, Seraing, Belgium). pNZmtlD was cloned into the *L. lactis* strains NZ9000, NZ9010 and HWA217 (Table 1). Plasmid pNZ9530, containing *nisR* and *nisK* genes, was co-transformed in *L. lactis* HWA217, to make nisin induction of *mtlD* possible. The *NisRK* genes, coding for the histidine protein kinase

NisK and the response regulator NisR, are the only nis genes required for *nisA* promoter activation on the pNZ*mtlD* plasmid. (19).

Analysis of fermentation products and glucose consumption. During the growth experiments, samples were taken from the *L. lactis* cultures, centrifuged for 1 min at 10.000 × g and the supernatants were stored at –20°C until analysis. In the supernatant, lactate, acetate, formate, glucose, mannitol, ethanol, 2,3-butanediol and acetoin were analysed by HPLC. Separation was performed with a ION-300 ion exclusion column (30 cm length) (Alltech, Breda, the Netherlands), at a flow rate of 0.4 ml/min and a temperature of 90°C. The eluens consisted of 3 mM sulfuric acid. Products were detected on a refractive index detector (Waters 410). The distribution of glucose to different fermentation products was calculated as the slope of the product concentration vs. the consumed glucose.

Preparation of cell extracts. Cell free extracts were prepared by disruption of cells by glass beads. 50 ml of cell culture was centrifuged (4°C, 20 min at 2000 × g) and washed with 50 mM MES buffer (pH 7.0). Cells were resuspended in 2 ml 50 mM MES buffer (pH 7.0). For cell disruption, 1 ml of cell suspension was added to 1.0 g of 0.1 mm zirconia/silica beads (BioSpec products, Inc.) in a 2 ml eppendorf cup, and cells were disrupted by vigorously 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, USA), with bovine serum albumin as the standard.

Enzyme assays. Cell cultures (50 ml) were harvested for enzyme assays at an OD600 of ± 1.3 or 2 h after induction with nisin, and cell extracts were prepared as described above. L-lactate dehydrogenase (LDH) activity was determined by the method of Hillier and Jago (16). Phosphofructokinase (PFK) activity was assayed according to Grobben et al. (13), with the modification that 5 mM fructose 6-phosphate was used to initiate the reaction. PFK activity of extracts with high mannitol 1-phosphate dehydrogenase (M1PDH) activity could not be determined, as fructose 6-phosphate is a substrate for both PFK and M1PDH. Mannitol 1-phosphate oxidation by M1PDH was determined in a reaction mixture containing 25 mM Tris.HCl pH 8.0 and 1.5 mM NAD. The reaction was started with the addition of 1 mM mannitol 1-phosphate. The reduction of fructose 6-phosphate was assayed in 25 mM sodium phosphate buffer pH 6 with 0.15 mM NADH. 1 mM fructose 6-phosphate was used to initiate the reaction. LDH, PFK and M1PDH activities were determined from the rate of NADH oxidation or formation at 30°C by measuring the absorbance at 340 nm (Ultrospec 2000, Pharmacia Biotech).

Mannitol 1-phosphatase activity was determined in a 1 ml reaction mixture containing 0.1-0.2 mg/ml protein enzyme extract, 50 mM MES buffer (pH 7.0), 10 mM $MgCl_2$ and 3 mM mannitol 1-phosphate. The formed inorganic phosphate was determined at 0, 60, 120 and 180 minutes incubation at 30°C by a modified protocol of the Sigma inorganic phosphate kit (Sigma Diagnostics). At the given time points, 200 µL samples were taken and the reaction was stopped with the addition of 40 µL acid molybdate solution. 10 µL Fiske & Subbarrow reducer solution was added to 200 µL of the clear centrifuged solution in a 96 wells microplate, and the absorbance at 655 nm was measured with a micro plate reader (3550-UV, Biorad).

NMR experiments. Cells grown on M17 broth were harvested at an OD600 of approximately 1.5, centrifuged, washed and resuspended in 50 mM potassium phosphate buffer (pH 6.5) to an OD_{600} of ± 35. ^{13}C-NMR spectra were taken using a Bruker AMX-400Wb Spectrometer at 100.62 MHz. All experiments were carried out at 30°C in a 10 mm NMR tube. 4 ml cell suspension was placed in the NMR tube and a initial spectrum was acquired. At time 0, 20 mM 1-^{13}C-glucose (Campro Scientific, The Netherlands) was supplied in the NMR tube. ^{13}C-NMR spectra were acquired during 104 seconds (64 scans). Chemical shifts were referred to the β-C1 of D-glucose (96.6 ppm). Resonances in the spectra (Fig. 2) were identified by spiking with the pure (unlabelled) materials.

Quantification of products by ^{13}C-NMR. Glucose, lactate, acetoin, 2,3-butanediol, ethanol and mannitol were quantified during the consumption of [1-^{13}C] glucose. Due to the fast pulsing conditions and short repetition times, the in vivo NMR spectra were not fully relaxed, and therefore there was not a direct correlation between peak intensities and concentrations. To correct for saturation, the saturation recovery method was used, by performing relaxation measurements.

RESULTS

In this work, we used different metabolic engineering approaches to induce mannitol production in *Lactococcus lactis*. We, basically, used the glycolytic model of *L. lactis* (18) (jjj.biochem.sun.ac.za) to predict the most efficient metabolic engineering strategy. The model predicted that knocking out the LDH gene and decreasing the PFK activity could play a significant role in mannitol production in *L. lactis* by increasing the intracellular NADH and fructose 6-phosphate levels. Mannitol production in three *L. lactis* strains was determined: the wild type NZ9000, the LDH-deficient NZ9010 (3) and the PFK reduced strain HWA217 (1). Enzyme activities in mid-log grown cultures were measured, fermentation products in supernatants of batch grown cultures were analysed, and ^{13}C-NMR spectra were recorded during [1-^{13}C]glucose consumption by cell suspensions.

M1PDH enzyme activity in *L. lactis.* Both *L. lactis* NZ9000 and HWA217 that were grown on glucose did not show any M1PDH activity while cell extracts of *L. lactis* NZ9010 contained a very low M1PDH activity (Table 2). Since M1PDH activity is essential for mannitol production, the corresponding gene of *Lactobacillus plantarum* (*mtlD*) was overexpressed in all three *L. lactis* strains using pNZmtlD (Table 1). The transformants were grown on glucose and induced with nisin, and the cells were harvested after 2 h of induction. In all three hosts, the introduction of pNZmtlD led to a large increase of M1PDH activity (Table 2). The low M1PDH activity exhibited by the non-induced culture can be explained by the very low residual activity of the nisin promoter in the pNZ8148 expression vector under non-induced conditions (7, 8).

TABLE 2. Enzyme activities determined in crude cell extracts of the various *L. lactis* strains grown on glucose

L. lactis strain	Nisin (ng/mL)	Activity [a]				
		LDH	PFK	M1PDH		MP
				(F6P → M1P)	(M1P → F6P)	
NZ9000	0	13.5 ± 3.5	1.6 ± 0.6	ND	ND	4.2 ± 1.5
NZ9000 (pNZmtlD)	0	15.8 ± 2.7	1.8 ± 0.6	ND	0.03 ± 0.01	5.2 ± 1.0
NZ9000 (pNZmtlD)	1	16.1 ± 2.8	- [b]	6.2 ± 0.4	20.9 ± 0.3	3.5 ± 0.3
NZ9010	0	ND	1.5 ± 0.5	0.02 ± 0.02	≤ 0.01	4.9 ± 0.4
NZ9010 (pNZmtlD)	0	ND	1.2 ± 0.6	0.03 ± 0.02	0.03 ± 0.02	5.6 ± 0.3
NZ9010 (pNZmtlD)	1	ND	- [b]	9.8 ± 1.6	30.3 ± 8.3	3.6 ± 0.2
HWA217 (pNZ9530)	0	17.0 ± 5.2	0.6 ± 0.4	ND	ND	8.6 ± 1.9
HWA217 (pNZ9530, pNZmtlD)	0	19.2 ± 1.2	0.3 ± 0.2	0.04 ± 0.01	0.12 ± 0.09	7.5 ± 0.8
HWA217 (pNZ9530, pNZmtlD)	1	17.7 ± 6.0	- [b]	2.5 ± 0.9	8.4 ± 2.3	6.7 ± 2.4

[a] In micromoles per minute per milligram of protein (nanomoles per minute per milligram of protein for mannitol 1-phosphatase [MP]). ND, not detected. Cell extracts were made of cultures during exponential growth at an OD_{600} of 1.3-1.6 or after 2 h induction with nisin (*mtlD* overexpression strains).

[b] -, not determined

Mannitol production by *mtlD* constructs. The effect of overexpression of *mtlD* on the formation of fermentation products was determined by cultivating the strains in batch cultures on M17 medium under oxic or anoxic conditions. To induce M1PDH activity, 1 ng/ml nisin was added to growing cultures of the strains containing the pNZmtlD plasmid, and the fermentation products were determined over time in the culture supernatant (Table 3). Besides, *in vivo* ^{13}C-NMR measurements were performed with suspensions of glucose-grown cells (Fig. 2, Table 4). The consumption of [1-^{13}C]glucose by the suspensions was monitored until no changes in the spectra were observed. Resonances in the spectra of these measurements (Fig. 2) were identified as lactate (20.7 ppm), acetoin (18.8 ppm), ethanol (17.5 ppm), 2,3-butanediol (17.4 ppm) and mannitol (63.9 ppm) by spiking with the pure (unlabeled) materials.

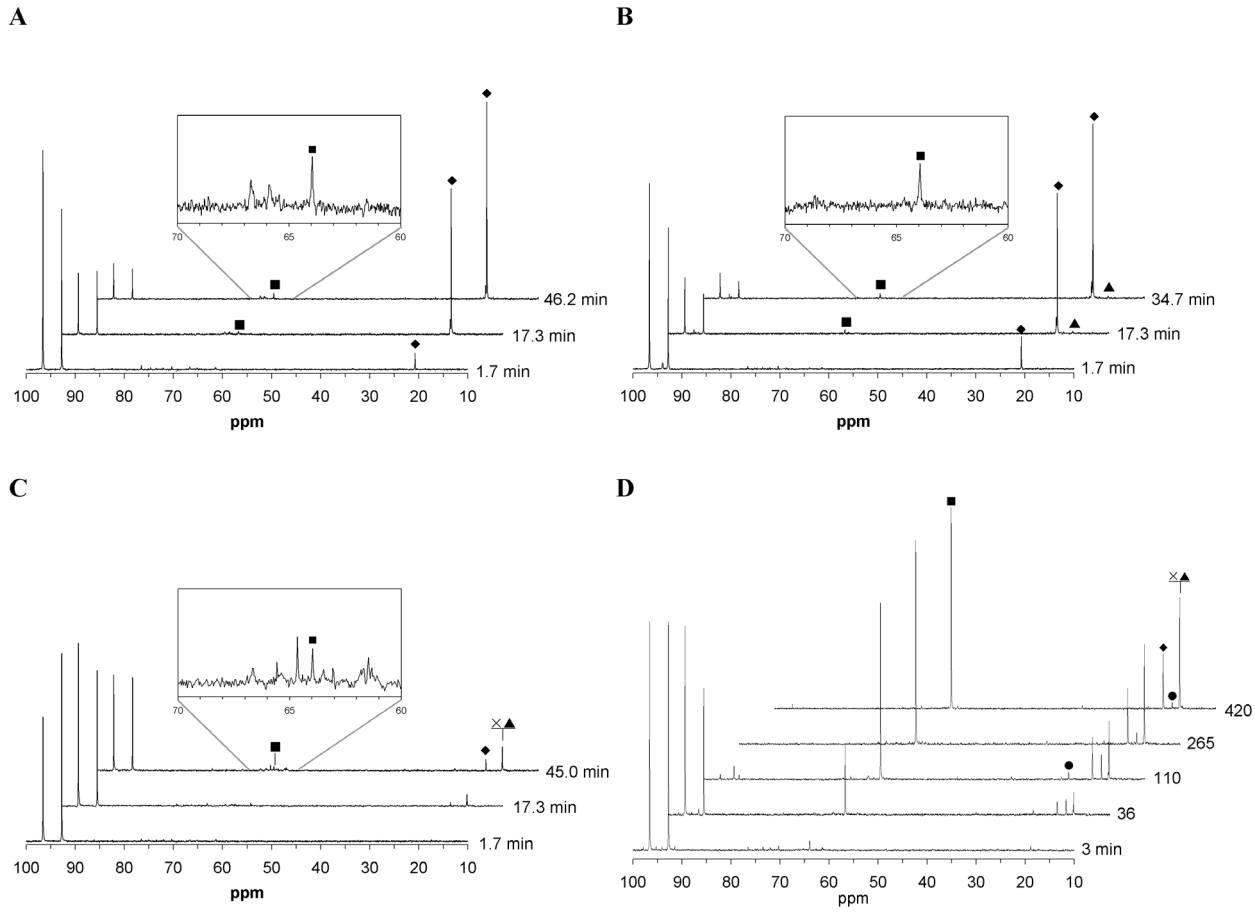

FIG. 2. ^{13}C-NMR spectra during metabolism of 1-^{13}C-glucose by high density suspensions of non-growing cells of the *L. lactis* strains NZ9000 (pNZmtlD) (A), HWA217 (pNZ9530, pNZmtlD) (B), NZ9010 (C), and NZ9010 (pNZmtlD) (D). Spectra were recorded until no changes could be detected. The resonances of α and β C1 of D-glucose-were detected at 92.8 and 96.6 ppm. Symbols: ^{13}C-labeled lactate (◆), acetoin (●), 2,3-butanediol (×), ethanol (▲), mannitol (■).

Overexpression of mtlD in parental *L. lactis.* Wild-type *L. lactis* NZ9000 showed a typical homolactic fermentation pattern, with lactate as main fermentation product (Table 3). Overexpression of *mtlD* in NZ9000 resulted in the same homolactic pattern (Table 3) and no mannitol was detected during growth. However, ^{13}C-NMR analysis of [1-^{13}C]glucose-consuming cell suspensions of nisin-induced *L. lactis* NZ9000 (pNZmtlD) revealed the accumulation of a small amount of mannitol (Fig. 2A). The conversion rate of glucose into mannitol was 0.02 mol mannitol per mol of glucose (Table 4).

TABLE 3. fermentation products formed per mol of consumed glucose during growth on 0.5% glucose.

			Amt of product (mol product mol glucose^{-1})								
L. lactis	ox	nisin (ng/ml)	lactate	formate	acetate	ethanol	acetoin	2,3-butanediol	pyruvate	mannitol	C recovery
NZ9000			1.71								0.86
NZ9000 (pNZmtlD)			1.74								0.87
NZ9000 (pNZmtlD)		1	1.74								0.87
NZ9010			0.13	1.1	0.15	1.1	0.28	0.07			1.04
NZ9010	+		0.02		0.09	0.12	0.57	0.03	0.26		0.85
NZ9010 (pNZmtlD)			0.06	1.0	0.37	0.77	0.17	0.16		0.006	0.94
NZ9010 (pNZmtlD)		1	0.08	0.93	0.15	0.87	0.24	0.15		0.011	0.95
NZ9010 (pNZmtlD)	+		0.02		0.22		0.58		0.16		0.78
NZ9010 (pNZmtlD)	+	1	0.02		0.21		0.60		0.15		0.79
HWA217 (pNZ9530)			1.86	0.12	0.01						0.94
HWA217 (pNZ9530, pNZmtlD)			1.83		0.01					0.005	0.93
HWA217 (pNZ9530, pNZmtlD)		1	1.87		0.01					0.008	0.95

TABLE 4. Formation of ^{13}C labeled fermentation products during [1-^{13}C] glucose consumption at 30°C by high density suspensions of non-growing *L. lactis* cells.

	[^{13}C]glucose consumed (%)	Amt of product (mol mol [1-^{13}C]glucose^{-1})[a]						
L. lactis strain		lactate	acetate	ethanol	acetoin	2,3-butanediol	mannitol	^{13}C recovery
NZ9000	70	0.68						0.68
NZ9000 (pNZmtlD)	85	0.62					0.02	0.64
NZ9010	44	0.17		0.22		0.24	0.02	0.65
NZ9010 (pNZmtlD)	100	0.07 (0.3)	(0.14)	0.03 (0.11)	0.14 (0.22)	0.07 (0.15)	0.15 (0.25)	0.46 (0.90)
HWA217 (pNZ9530)	65	0.49						0.49
HWA217 (pNZ9530, pNZmtlD)	88	0.53		0.02			0.01	0.56

[a] Spectra were recorded for 46.2 min, except for the spectrum of NZ9010 (pNZmtlD), which was recorded for 420 min. Values in parentheses are concentrations measured in the supernatants by HPLC

MtlD overexpression in a *L. lactis* strain with reduced PFK activity. Growth of *L. lactis* with reduced phosphofructokinase activity (strain HWA217) resulted in a mainly homolactic fermentation pattern with small amounts of acetate and formate as side products, as described by Andersen et al. (1). Introduction of pNZmtlD in HWA217 resulted in a mannitol production during growth of 0.005 and 0.008 mol mannitol per mol of consumed glucose (Table 3). The mannitol production was not dramatically improved when the M1PDH activity was increased by nisin induction (Table 2). Analysis of glucose consumption by non-growing cell suspension confirmed the homolactic pattern of HWA217 (spectra not shown). ^{13}C-NMR analysis of suspensions of *L. lactis* HWA217 (pNZ9530, pNZmtlD) showed that mannitol and ethanol accumulated (spectra not shown). In this strain, 1% of the [1-^{13}C]glucose is converted into [1-^{13}C] mannitol by cell suspensions. (Table 4).

Overexpression of *mtlD* in a LDH-deficient *L. lactis*. HPLC analysis of the LDH-deficient strain *L. lactis* NZ9010 showed a mixed type fermentation pattern when grown on glucose under aerobic and (semi) anaerobic conditions (Table 3), similar to Bongers et al. (3). No mannitol could be detected during growth. Introduction of pNZmtlD in strain NZ9010, resulted in conversion rates of 0.6 and 1.1 % , for the uninduced and the induced growing cultures, respectively (Table 3). Production of mannitol was not observed during growth under aerobic conditions (Table 3).

^{13}C-NMR analysis of the glucose metabolism of *L. lactis* NZ9010 suspensions did not result in high amounts of mannitol (Fig. 2C), only 2% of the ^{13}C-labeled glucose was converted into mannitol (Table 4). Furthermore, the glucose consumption by the cell suspension of *L. lactis* NZ9010 was much slower compared to the other strains since only 44 % of the glucose was metabolized during 45 minutes, whereas the others strains converted 65 to 88% of glucose in the same time or less (Table 4). In contrast, a much larger amount of mannitol was produced by the nisin induced *L. lactis* NZ9010 (pNZmtlD) cell suspension (Fig. 2D). A conversion rate as high as 0.15 mol of ^{13}C labeled-mannitol per mol of [1-^{13}C]glucose was obtained (Table 4). During glucose consumption, a rapid increase in mannitol was observed, and upon glucose exhaustion, the produced mannitol was not re-utilized (Fig. 3).

The large difference in mannitol production between growing and non-growing cells might be explained by intracellular accumulation of mannitol in the resting cells. To determine this, supernatant samples from cell suspensions converting [1-^{13}C]glucose were analysed by HPLC. The measured concentrations of all fermentation products in the supernatants were higher than the concentrations that were measured with ^{13}C-NMR. The extracellular mannitol production was corrected to 25% (Fig. 4, Table 4). This suggested the loss of ^{13}C label during the experiment, for example via CO_2 formation. HPLC analysis of cell extracts of glucose-consuming cell suspensions confirmed that mannitol was excreted and not accumulated in the cells (data not shown).

To investigate the mannitol-utilizing abilities of *L. lactis* NZ9010 (pNZmtlD), glucose pre-cultured NZ9000 and NZ9010 (pNZmtlD) cells were grown anaerobically on M17 broth supplemented with mannitol. Both strains NZ9000 and NZ9010 (pNZmtlD) showed a lag-phase of about 24 h before growth on mannitol was observed (Fig. 5).

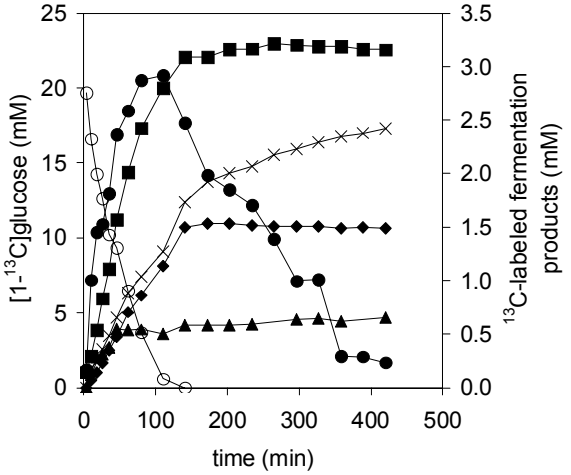

FIG. 3. Formation of ^{13}C-labeled fermentation products during [1-^{13}C]glucose consumption by high density suspensions of non-growing cells of *L. lactis* NZ9010 (pNZmtlD). The starting concentration of 1-^{13}C-glucose is 20 mM. Symbols: glucose (○), lactate (◆), acetoin (●), 2,3-butanediol (×), ethanol (▲), and mannitol (■).

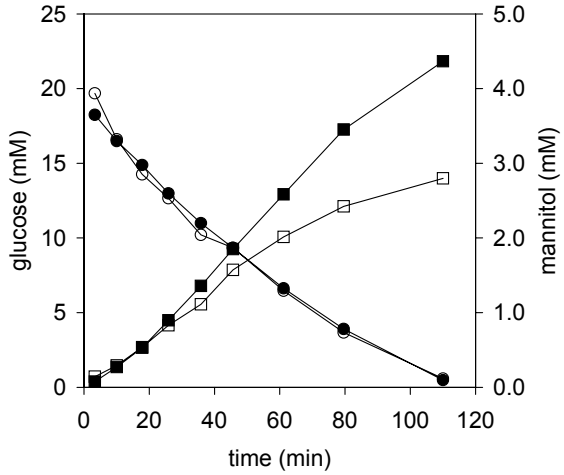

FIG. 4. Mannitol production and glucose consumption by high density suspensions of non-growing cells of *L. lactis* NZ9010 (pNZmtlD). Symbols: mannitol (■) and glucose (●) measured by HPLC in the supernatant; ^{13}C-labeled mannitol (□) and glucose (○) measured by ^{13}C-NMR analysis.

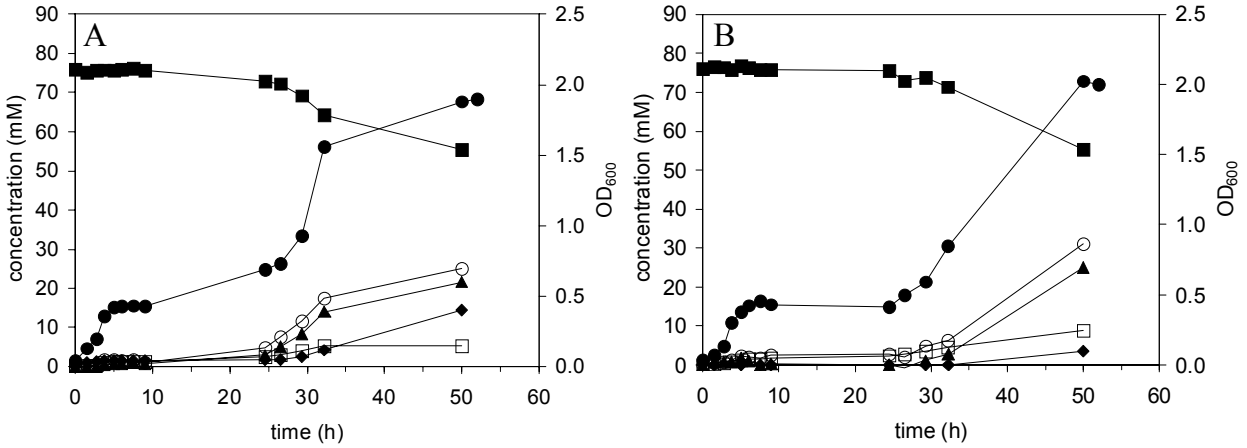

FIG. 5. Growth curves, mannitol consumption and product formation by *L. lactis* NZ9000 (A) and NZ9010 (pNZ-mtlD) (B), grown under anaerobic conditions. M17 broth, supplemented with 75 mM mannitol, was inoculated with 2 (v/v)% glucose-grown O.N. culture. Symbols: mannitol (■), lactate (◆), ethanol (▲), formate (○), acetate (□), and OD_{600} (●).

DISCUSSION

We investigated the effect of overexpression of the *Lactobacillus plantarum mtlD* gene in three *L. lactis* hosts: the parental NZ9000, the LDH-deficient NZ9010 and the PFK reduced HWA217. The mannitol producing capacities of the constructed transformants were determined by HPLC analysis of supernatants of growing cultures, in vivo ^{13}C-NMR analysis of glucose-consuming cell suspensions, and enzyme activity measurements. Combining these data, the effect of overexpression of *mtlD* in different genetic backgrounds on the mannitol producing capacity of *L. lactis* could be determined.

When *mtlD* was overexpressed in the wild type *L. lactis* NZ9000, no mannitol was detected in the supernatant of growing cells (Table 3). Although both M1PDH and MP activities were present in cell extracts (Table 2), no mannitol production could be observed. Apparently, the glycolytic flux in the growing cells is too high and subsequently the fructose 6-phosphate concentration too low to enable a flux to mannitol. Also NAD is regenerated by LDH, so the organism has no need to regenerate NAD by an alternative pathway such as mannitol synthesis via M1PDH. Under non-growing conditions, *mtlD* overexpression resulted in a small amount of mannitol (Fig. 2A). About 2% of the glucose was converted to mannitol by this transformant (Table 4). Due to the low glycolytic flux in the non-growing cells (21), the fructose 6-phosphate concentration might just have been high enough for M1PDH to convert fructose 6-phosphate into mannitol 1-phosphate.

Increasing the intracellular fructose 6-phosphate concentration by lowering the phosphofructokinase activity might be a strategy to enable mannitol production in *L. lactis*. Andersen and coworkers (1) showed that glycolytic fluxes were reduced in growing cells of a *L. lactis* mutant with 40% of PFK activity compared to the MG1363 wild type. In this strain, sugar phosphates such as glucose 6-phosphate and fructose 6-phosphate accumulated. The increase of the fructose 6-phosphate concentration might contribute to a flux towards mannitol 1-phosphate via M1PDH. Indeed, when *mtlD* is overexpressed in the PFK reduced *L. lactis* HWA217, mannitol production is observed in the supernatant of growing cultures (Table 3). Since no mannitol was produced by the *mtlD* overexpressing parental strain NZ9000, the results may imply that the reduction of PFK has resulted in the increased flux towards mannitol. However, non-growing cells of M1PDH overexpressing HWA217 produced similar amounts of mannitol compared to the parental strain with high M1PDH overexpression. Since PFK reduction increases the fructose 6-

phosphate pool (1), it can be assumed that further increase of the fructose 6-phosphate pool does not enlarge the conversion of glucose into mannitol by non-growing cells.

Another strategy to increase the flux to mannitol is to increase the NADH pool by knocking out the LDH activity, which is mainly responsible for the regeneration of NAD^+ in *L. lactis*. Neves et al. (24) showed that non-growing cells of a LDH-deficient *L. lactis* transiently produced mannitol intracellulary under anaerobic conditions, to relieve the pressure to regenerate NAD^+. In contrast to the LDH-deficient strain of Neves and coworkers (24), no high (intracellular) mannitol levels were produced by non-growing cells of NZ9010 (Fig. 2C, Table 4). Moreover, both anaerobic and aerobic growing cultures of NZ9010 produced no mannitol at all. The formation of ethanol and 2,3-butanediol under anaerobic conditions suggest that the LDH-deficient strain has used these pathways to regenerate NAD^+. Analysis of the fermentation products of the LDH-deficient strains showed that under anaerobic conditions lactate is formed. This can be attributed to transcriptional activation of the alternative LDH gene *ldhB,* under anaerobic conditions (3).

When *mtlD* was overexpressed in NZ9010, mannitol production could be observed by growing cultures (Table 3). Both nisin-induced and uninduced cultures produced mannitol in small amounts, about 1% of the glucose was converted into mannitol. Apparently, the low M1PDH activity in the non-induced cultures, due to residual activity of the nisin promoter in pNZ8148, is sufficient to accomplish a flux to mannitol. Nisin-induced overexpression of *mtlD* did not result in a much higher mannitol production. Apparently, M1PDH activity is not rate limiting in the mannitol production. Noting that mannitol 1-phosphatase activity is rather low in the different *L. lactis* strains (Table 2), this enzyme might have the highest control on the mannitol synthesis pathway.

Higher amounts of mannitol were detected by ^{13}C-NMR in the NZ9010 (pNZmtlD) cell suspensions (Fig. 2D, Table 4). About 25% of the glucose was converted into mannitol, and conversion rates of glucose into acetoin, ethanol and 2,3-butanediol were lower compared to the NZ9010 strain. This implies that NAD^+ regeneration has resorted to mannitol production via the introduced M1PDH activity. In theory, up to 66.7 % of the glucose can be converted into mannitol, when no NAD^+ is regenerated through lactate, ethanol or 2,3-butanediol formation. These high conversion rates can not be expected, noting the regained lactate production and the high ethanol production by the LDH-deficient *L. lactis* strain. In addition, the fairly low mannitol 1-phosphatase activities in all three strains might be limiting for high glucose-mannitol conversions.

We show that the mannitol concentration remains constant (figure 3) even after glucose exhaustion. This is in contrast with the observations of Neves et al. (24) that clearly demonstrated re-utilization of the produced mannitol.

Also in the supernatant of growing cultures used in this work, mannitol was still present after 24 hours, when glucose was already depleted for at least 10 hours (data not shown). For *L. lactis* IL1403, a putative CRE site in the promoter region of the mannitol operon was identified (accession no. AE006241 and AE006242), which suggest a possible involvement of ccpA in the regulation of the mannitol operon (23) and thus transcription of the genes involved in mannitol transport and metabolism will be derepressed when glucose is absent and mannitol is present. Moreover, *L. lactis* MG1363 and the LDH-deficient variant of Neves et al. is capable of growing on mannitol (25), so one would expect that mannitol is consumed when glucose is depleted. Apparently, the disruption of the LDH gene in the LDH-deficient strain used in this work, did not induce the expression of genes coding for mannitol transport and utilization in contrast to the mutant of Neves et al. (24, 25). In contrast to the findings of Neves and co-workers (25), the LDH-deficient strain did not grow better than the parental strain (Fig. 5). The 24 h lag phase of *L. lactis* NZ9010 (pNZmtlD) growing on mannitol (Fig. 5) emphasizes that mannitol utilization genes have to be induced prior to growth on mannitol. NZ9010 (pNZmtlD)Hence, immediate re-utilization of mannitol by high-density resting cell suspensions of NZ9010 (pNZmtlD) would not be expected

The great difference in mannitol production between the growing and the non-growing cells may be caused by accumulation of NADH in the resting cells. In the resting cells, where no ATP is needed for biomass production, the ATP demand is low (21), so ATP generating steps such as the conversion of PEP to pyruvate are less important. Still, NADH is generated in glycolysis, and hence the non-growing cells might give priority to regeneration of NAD^+ above ATP generation. Because of the LDH deficiency, M1PDH takes care of a major part in the NADH oxidation in the NZ9010 (pNZmtlD) strain.

Our work presented here showed that *mtlD* overexpression, PFK reduction, and LDH deficiency have contributed to mannitol production in *L. lactis*. The most promising combination for mannitol production in *L. lactis* was the *mtlD* overexpression in an LDH-deficient background. Although the capability of growing on mannitol, no concomitant mannitol re-utilization was observed in the LDH-deficient mutant, which is very desirable regarding the possibilities of mannitol in food products.

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CHAPTER 4

Metabolic Engineering of *Lactococcus lactis* for Mannitol Production, A Combined Experimental and Modeling Approach

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ABSTRACT

In this work, we expanded the detailed kinetic model on glycolysis of *Lactococcus lactis* with two steps involved in mannitol biosynthesis, mannitol 1-phosphate dehydrogenase (M1PDH) and mannitol 1-phosphatase (M1Pase) activity. The resulting model was used to predict the control points in the flux to mannitol. Metabolic control analysis on the model, based on *in vitro* enzymatic data of the strains used, showed that in particular M1Pase, catalyzing the last step in the mannitol synthesis, has a high control on the mannitol flux. Increasing the activity of this enzyme in combination with that of M1PDH should result in significant mannitol production in *L. lactis*. Furthermore, reduction of lactate dehydrogenase (LDH) activity is predicted to exert a strong effect on the mannitol production. A 42% glucose-mannitol conversion (theoretical maximum is 67%) is predicted for a LDH-deficient *L. lactis* when both M1PDH and M1Pase are overexpressed.

INTRODUCTION

Lactococcus lactis, a homofermentative lactic acid bacterium, which is extensively used in the dairy industry, is very efficient in the conversion of sugars into lactate through glycolysis. However, *L. lactis* is also capable of producing other metabolic products such as acetate and ethanol during mixed acid fermentations (4, 28). Moreover, genetic engineering of *L. lactis* has resulted in the overproduction of existing and new fermentation products (5). Construction of lactate dehydrogenase (LDH)-deficient strains has resulted in redistribution to products other than lactate, such as acetoin, 2,3-butanediol, diacetyl, ethanol, formate, and acetate (3, 13, 14, 21), and even mannitol (20). Furthermore, alanine (12) and folate (27) production was achieved by overexpression of single and multiple genes in *L. lactis*. In these examples, the choice of engineering strategies or fermentation conditions for product formation by *L. lactis* have mostly been based on intuition and experience. The development of a predictive tool would make the choice for a certain strategy more rational. Based on kinetic data, a model has been developed for the flux distribution over the pyruvate branches in *L. lactis* (10). Metabolic control analysis (MCA) clearly indicated the key control points in the flux to acetoin and diacetyl, the latter an important flavour compound. Moreover, experiments confirmed the predictions of the model, i.e. inactivating LDH and overexpressing NADH oxidase (NOX) increased the flux through the acetolactate synthase branch (14). The use of such a detailed kinetic model can be very helpful in determining which engineering step should be taken, and how much of a certain product can be expected. Recently, the *L. lactis* pyruvate distribution model was extended to a more detailed *L. lactis* glycolytic model (9, 11).

In this work, the detailed kinetic model of the *L. lactis* glycolysis has been extended with a mannitol production pathway, to design a engineering strategy for the production of this polyol by *L. lactis*. Mannitol is a low-calorie sugar that could replace sugars such as sucrose, glucose, and lactose in food products. Mannitol can serve as anti-oxidant in cells and can protect cells in stress situations as shown in the enhanced survival of *L. lactis* cells during drying in the presence of mannitol (6). In previous work we have shown that overproduction of mannitol 1-phosphate dehydrogenase (M1PDH) in an LDH-deficient *L. lactis* strain and in a strain with reduced PFK activity, resulted in mannitol production by growing cells, although production was very low (29). This metabolic engineering work was partly based on an intuitive approach and on literature data: M1PDH is needed for mannitol production and LDH-deficiency in *L. lactis* is likely to lead to higher NADH/NAD ratios in the cell needed for the formation of mannitol.

Moreover, some LDH-deficient *L. lactis* strains are known to produce considerable amounts mannitol in resting cells (8, 20). Hence, we report here on the development and use of a kinetic model for mannitol production, based on kinetic data of enzymes in the glycolytic and the mannitol production pathway in the M1PDH-overproducing *L. lactis* strains, were included in the model. MCA was performed to determine the control points on the mannitol pathway, resulting in the prediction of an efficient metabolic engineering strategy leading to high mannitol production by *L. lactis*.

MATERIALS AND METHODS

***L. lactis* strains, growth and media.** The control *L. lactis* strain NZ9000, and the mannitol-producing strains NZ9010 (pNZmtlD) and HWA217 (pNZmtlD) from our previous work (29), were grown in a 1.5 l Applicon fermenter (Applicon Dependable Instruments) containing 1 l of CDM (22), supplemented with 1 % glucose. The pH was maintained at 6.5 by addition of 2 M NaOH, the temperature was set at 30°C, and the cultures were stirred at 200 rpm. Anaerobic conditions in the cultures were maintained by gassing with nitrogen gas. When applicable, erythromycin and chloramphenicol were supplied at 5 and 10 μg/ml, respectively. Nisin was added at 1 ng/ml to induce M1PDH activity. Growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Analysis of fermentation products. Glucose, mannitol, lactate, formate, acetate, acetoin, 2,3-butanediol, and ethanol in supernatants were analysed according to earlier work (29). Additional to the HPLC analysis, mannitol was also determined using a colorimetric mannitol assay (24). Flux distributions of the various strains were determined during exponential growth (pseudo steady-state).

Enzyme activities. When growing cultures had reached an OD_{600} of approximately 1.0, 50 ml samples were taken. Cell extracts were prepared, and LDH, PFK, M1PDH, and M1Pase activities were measured as described previously (29).

Intracellular metabolites and cofactors. The concentrations of the cofactors NAD and NADH were determined in growing *L. lactis* cultures with an OD_{600} of approximately 1.0. For rapid inactivation of the metabolism in the cells, 10 ml of sample was transferred directly in 0.33 ml 5 M HCl (NAD) or 0.33 ml 5 M KOH (NADH) to give a pH of approximately 1.2 and 12 respectively. An internal standard of 0, 1, 2 and 5 μM NAD or NADH was added to the samples. NAD was extracted by incubating the HCl-treated cultures at 50°C for 8 minutes, NADH was extracted by incubating the KOH-treated samples at room temperature (7). The HCl and KOH treated samples were neutralized to pH 6.5 and 7.5 respectively, and centrifuged (4°C, 20 min at 2000 × g). The supernatant was used immediately for NAD and NADH measurements by the method described by Snoep et al. (26), based on a NAD cycling assay of Bernofsky and Swan (2).

For the determination of the sugar phosphates, 20 ml samples from a *L. lactis* culture were transferred directly in 20 ml –80°C precooled methanol to inactivate the metabolism in the cells, and samples were stored at –80°C. For the analysis, samples were thawed, and the methanol was evaporated at 50°C. The pH of the cell culture was set to 1.2 with 5 M HCl and metabolites were extracted by incubating at 50°C for 8 min (7). The samples were neutralized to pH 6.5 by addition of 5 M KOH and centrifuged to remove cell debris (4°C, 20 min at 2000 × g). The metabolites glucose 6-phosphate (G6P), fructose 6–phosphate (F6P) and mannitol 1-phosphate (M1P) were measured by coupled enzyme assays with fluorometric determination of NADH, as described by (7). With a fluorescence spectrofotometer (Perkin Elmer LS 50B) emission was measured at 460 nm after excitation at 350 nm. A reaction mixture was prepared (7) with the modification that 100 μl 15 mM NAD was used instead of NADP. The measurements were started by G6P consumption by addition of 2 μl of G6P dehydrogenase (1000 U/ml, Roche, from *Leuconostoc mesenteroides*). After completion of this reaction, 2 μl of phosphoglucose isomerase (700 U/ml, Roche, from yeast) was added to determine the F6P concentration. When the reaction was completed, the M1P concentration was measured by M1PDH, purified from *Aspergillus niger* (23).

Intracellular concentrations were calculated with the assumption that the internal cell volume is 1.5 μl (mg dry weight)$^{-1}$ (15), 50% of the dry weight consists of protein, as in *E. coli* (19), and that an OD_{600} of 1 is equal to 0.455 mg dry weight ml^{-1}.

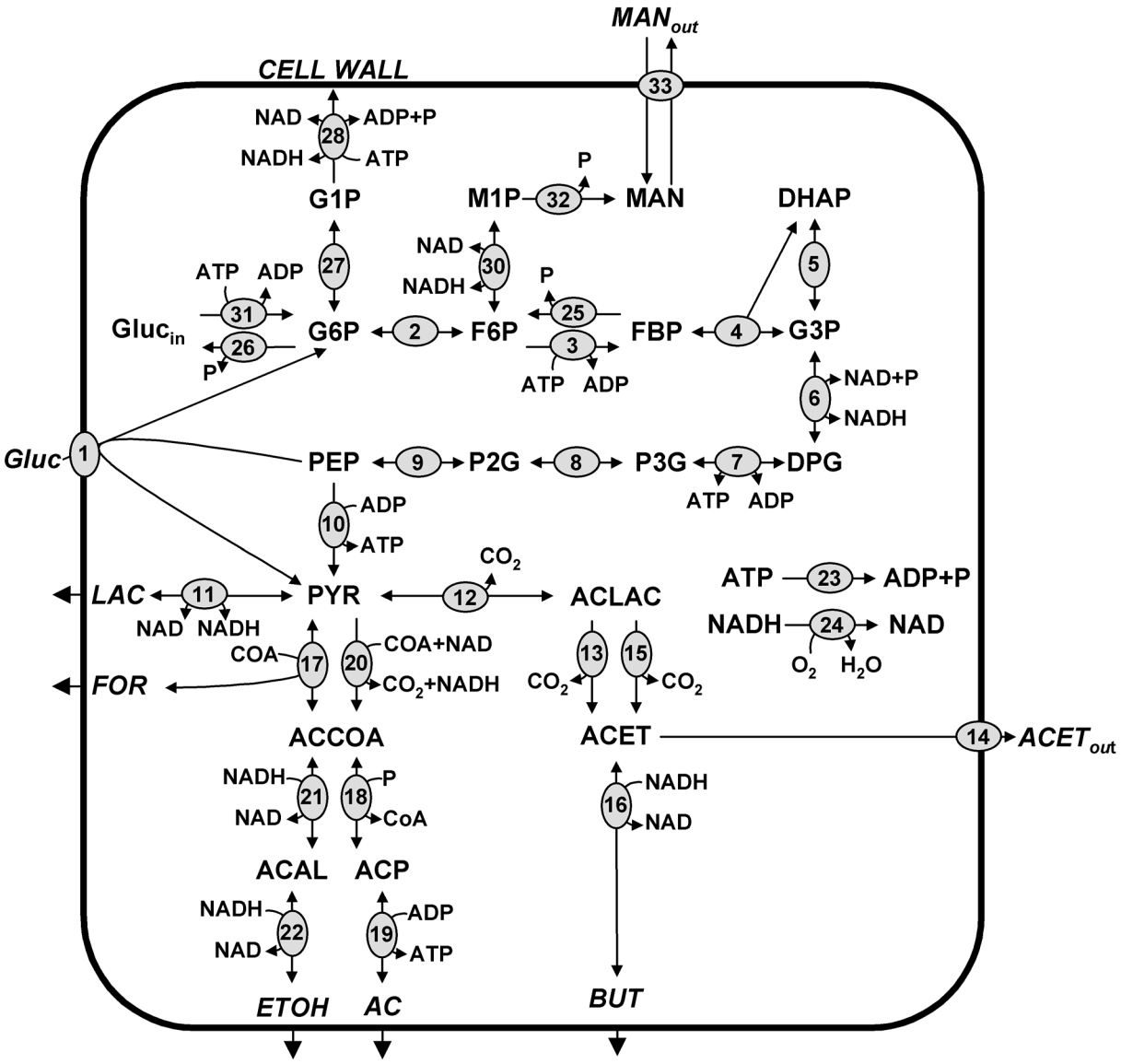

FIG. 1. Reactions included in the *L. lactis* glycolysis/mannitol model. The numbers indicating the enzymes or steps, and the abbreviations for the metabolites, are listed in table 1. Substrates and products with a fixed level in the model are indicated in italics.

Kinetic modeling. A mannitol production branch with the enzymes M1PDH and M1Pase, and a mannitol efflux step, was added to the *L. lactis* glycoytic model described by Hoefnagel et al. (9, 11). Figure 1 shows the complete *L. lactis* glycolytic model used in this study, including the mannitol branch. Table 1 shows the definitions of the mathematical symbols and abbreviations in the equations and kinetic models in this study.

The kinetic parameters of the overproduced M1PDH, in *L. lactis* NZ9010 and HWA217 were determined by measuring M1PDH enzyme activities at variable substrate levels in cell extracts of a *L . lactis* NZ9000 (pNZmtlD) culture, grown anaerobically in 50 ml M17 medium with 0.5% glucose, and induced with 1 ng/ml of nisin (29). To estimate the kinetic parameters of the different substrates, curve-fitting by Mathematica (http://www.wolfram.com) was performed on the enzyme activity data at variable substrate levels. The K_m for F6P was determined in an assay with a saturating NADH concentration of 0.15 mM. The K_m for M1P was determined in an assay with a saturating concentration of 1.5 mM NAD, while the K_m for NAD and NADH were determined in a reaction mix assay containing 1 mM M1P and 1 mM F6P, respectively. M1PDH activity was modelled using an equation for a reversible reaction with substrate inhibition by fructose 6-phosphate, resulting in the equation in Table 2.

M1Pase, has been modelled as an irreversible reaction with Michaelis-Menten kinetics with product sensitivity. Kinetic parameters for M1Pase were obtained from experimental data in literature for *Eimeria tenella* (25). The mannitol efflux was modelled as a diffusion process. The parameters for the diffusion of mannitol were guessed, making sure that the efflux would not significantly limit the flow to mannitol.

The kinetic parameters of M1PDH, M1Pase and the mannitol efflux are listed in Table 3. Here we only give details of the enzymes that were no part of the glycolysis model.

TABLE 1. Mathematical symbols and abbreviations used in this study. The numbers for the enzymes or conversions correspond to the numbers of the reactions used in the kinetic model.

Mathematical symbols	**Definition**
K_{eq}	Equilibrium constant
K_i	Inhibition constant
K_m	Affinity constant
V	Predicted enzyme activities
V^+	Maximal enzyme activities under saturating substrate and activator conditions, and in the absence of inhibitors
k	Rate constant

Abbreviations and numbers used in Fig. 1

Metabolites		*Enzymes/steps*	
2PG	2-Phosphoglycerate	1.	Glucose transport (PEP dependent PTS)
3PG	3-Phosphoglycerate	2.	Phosphoglucose isomerase
AC	Acetate	3.	6-Phosphofructokinase
ACAL	Acetaldehyde	4.	Fructose 1,6-diphosphate aldolase
ACCOA	Acetyl coenzyme A	5.	Triosephosphate isomerase
ACET	Acetoin	6.	Glyceraldehyde 3-phosphate dehydrogenase
$ACET_{out}$	Acetoin (extracellular)	7.	3-Phosphoglycerate kinase
ACLAC	Acetolactate	8.	Phosphoglycerate mutase
ACP	Acetyl phosphate	9.	Enolase
BUT	2,3-Butanediol	10.	Pyruvate kinase
COA	Coenzyme A	11.	Lactate dehydrogenase
DHAP	Di-hydroxy acetone phosphate	12.	Acetolactate synthase
DPG	Di-phoshoglycerate	13.	Acetolactate decarboxylase
ETOH	Ethanol	14.	Acetoin efflux
F6P	Fructose 6-phosphate	15.	Non-enzymic acetolactate decarboxylase
FBP	Fructose 1,6-bis-phosphate	16.	Acetoin dehydrogenase
FOR	Formate	17.	Pyruvate formate lyase
G1P	Glucose 1-phosphate	18.	Phosphotransacetylase
G3P	Glyceraldehyde 3-phosphate	19.	Acetate kinase
G6P	Glucose 6-phosphate	20.	Pyruvate dehydrogenase
Gluc	Glucose (extracellular)	21.	Acetaldehyde dehydrogenase
$Gluc_{in}$	Glucose (intracellular)	22.	Alcohol dehydrogenase
LAC	Lactate	23.	ATP-ase
M1P	Mannitol 1-phosphate	24.	NADH oxidase
MAN	Mannitol (intracellular)	25.	Fructose diphosphatase
MAN_{out}	Mannitol (extracellular)	26.	Glucose 6-phosphatase
O	Oxygen	27.	Phosphoglucomutase
P	Inorganic phosphate	28.	Biomass formation
PEP	Phosphoenol pyruvate	30.	Mannitol 1-phosphate dehydrogenase
PYR	Pyruvate	31.	Hexokinase
		32.	Mannitol 1-phosphatase
		33.	Mannitol efflux

TABLE 2. Rate equations of the steps involved in the mannitol synthesis in the *L. lactis* glycolysis/mannitol model. Abbreviations in this table are listed in Table 1.

$$\upsilon_{\mathrm{M1PDH}} = \frac{V^{+}\left(\frac{\mathrm{F6P}}{K_{\mathrm{m,F6P}}}\right)\times\left(\frac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}\right)\times\left(1-\frac{\mathrm{M1P}\times\mathrm{NAD}}{\mathrm{F6P}\times\mathrm{NADH}\times K_{\mathrm{eq}}}\right)}{\left(1+\frac{\mathrm{F6P}}{K_{\mathrm{m,F6P}}}+\frac{\mathrm{M1P}}{K_{\mathrm{m,M1P}}}\right)\times\left(1+\frac{\mathrm{NADH}}{K_{\mathrm{m,NADH}}}+\frac{\mathrm{NAD}}{K_{\mathrm{m,NAD}}}\right)\times\left(1+\frac{\mathrm{F6P}}{K_{\mathrm{m,F6P}}\times K_{\mathrm{i}}}\right)}$$

$$\upsilon_{\mathrm{M1Pase}} = \frac{V^{+}\left(\frac{\mathrm{M1P}}{K_{\mathrm{m,M1P}}}\right)}{1+\frac{\mathrm{M1P}}{K_{\mathrm{m,M1P}}}+\frac{\mathrm{MAN}}{K_{\mathrm{m,MAN}}}}$$

$$\upsilon_{\mathrm{MAN,out}} = k\times\left(\frac{\mathrm{MAN}}{\mathrm{V_{in}}}-\frac{\mathrm{MAN_{out}}}{\mathrm{V_{out}}}\right)$$

TABLE 3. Kinetic parameters of the conversions involved in the mannitol synthesis in the kinetic model. The specific conversions included mannitol 1-phosphate dehydrogenase (M1PDH), mannitol 1-phosphatase (M1Pase), and a mannitol efflux.

Reaction	Kinetic parameter	Value [a]	Reference
$\upsilon_{\mathrm{M1PDH}}$	$K_{m,\ F6P}$	0.25	This work
	$K_{m,\ NADH}$	4×10^{-3}	This work
	$K_{m,\ M1P}$	0.045	This work
	$K_{m,\ NAD}$	0.145	This work
	$K_{i,\ F6P}$	3.2	This work
	K_{eq}	200	*E. coli* (31)
$\upsilon_{\mathrm{M1Pase}}$	$K_{m,\ M1P}$	0.5	*Eimeria tenella* (25)
	$K_{m,\ MAN}$	1	Estimated
$\upsilon_{\mathrm{MAN,\ out}}$	K	5	Estimated
	V_{in}	1	Estimated
	V_{out}	100	Estimated

[a] K_m values are given in mM; V_{in} and V_{out} are given in mmol (l internal vol.)$^{-1}$ min^{-1}

A set of ordinary differential equations was used to describe the time dependence of the metabolite concentrations. Details of the differential equations concerning the glycolysis model can be found at http://jjj.biochem.sun.ac.za (9, 11).

$d[M1P]/dt = v_{M1PDH} - v_{M1Pase}$

$d[MAN]/dt = v_{M1Pase} - v_{MAN,\ out}$

$d[MAN,out]/dt = v_{MAN,\ out}$

We used GEPASI (17) for the prediction of metabolic fluxes and metabolite concentrations and for metabolic control analysis (MCA). The concentrations of the following metabolites were assumed to be constant: glucose, 2 mM; lactate, 0.1 mM; formate, 0.02 mM; acetate, 0.02 mM; ethanol, 0 mM; acetoin (outside), 0.1 mM; 2,3-butanediol, 0 mM; oxygen, 0 mM; mannitol (outside), 10^{-5} mM. The sums of [ATP]+[ADP], [NAD]+[NADH], and [CoA]+[AcCoA] were assumed to be constant at the values of 10, 5.55, and 1 mM, respectively.

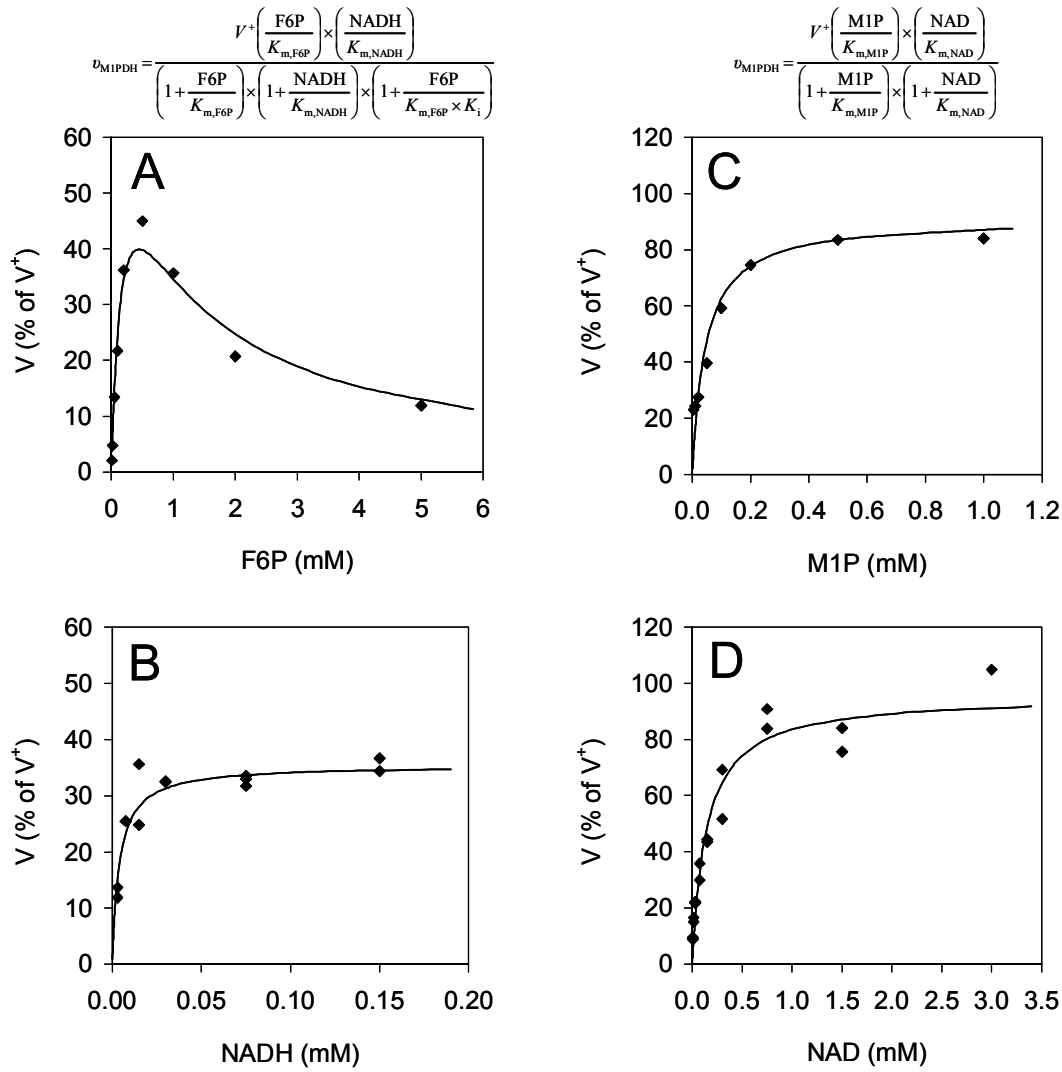

FIG. 2. Determination of the kinetics parameters of the overproduced M1PDH from *Lactobacillus plantarum*. The K_m and K_i parameters for F6P (A) and the K_m for NADH (B) were estimated by fitting the data with the equation for the forward reaction of M1PDH, above Figure 2A. The K_m for M1P and NAD were estimated by fitting the activity data with the equation for the backward reaction of M1PDH, above Figure 2C. Both equations were derived from the equation for V_{M1PDH} used in the model (Table 2).

RESULTS

Modeling the mannitol production in *L. lactis.* The kinetic *L. lactis* glycolysis model (9, 11) was extended with a mannitol branch with the two enzymes M1PDH and M1Pase, and a mannitol efflux step. The kinetic parameters of M1PDH were determined using *in vitro* enzyme activity measurements in cell-free extracts of *L. lactis* NZ9000 overproducing M1PDH (Fig. 2, Table 3).

In Figure 2A is shown that the M1PDH activity with F6P as variable substrate could be fitted with a substrate-inhibited Michaelis-Menten like curve (Fig 2A). The K_m and the inhibition constant K_i for F6P were estimated to be 0.25 and 3.2 mM, respectively. Activity curves for M1P, NAD, and NADH were fitted with a regular Michaelis-Menten type curve (Fig 2B, C, and D). The K_m estimated for M1P and NADH were rather low, 45 and 4 μM, respectively, while the K_m for NAD was determined to be 0.15 mM.

For modeling the glycolysis and the mannitol pathway in the strains NZ9010 and HWA217, the V^+ values in the model for LDH, PFK, M1PDH, and M1Pase were required. Hence, the activities were assayed *in vitro* in cell-free extracts of these strains (Table 4). Cell-extracts of strain HWA217 (pNZmtlD) showed a reduced PFK activity and an increased LDH activity, compared to the parental strain NZ9000. No LDH activity was detected in cell-extracts of NZ9010 (pNZmtlD). The M1PDH activity was much lower than observed in our previous work (29), although we used the same nisin concentration of 1 ng/ml to induce M1PDH activity. This might be explained by the different fermentation conditions: M17 broth versus CDM, and non-controlled conditions versus controlled conditions. Also, the M1PDH activity in strain HWA217 (pNZmtlD) is 12 times lower than in the LDH-deficient strain, compared to a factor 4 in our previous work (29). The V^+ values of the forward reaction of M1PDH in the model, were corrected by multiplying the measured activities by a factor 100/35, as the measured activities of the forward reaction do not reach the V^+ in the assays due to substrate inhibition by F6P (Fig. 2A). At the assay conditions (1 mM F6P, 0.15 mM NADH), the activity is approximately 35% of the V^+. The M1Pase activity in the three strains is low, and varies from 1 to 2 nmol min^{-1} (mg protein)$^{-1}$.

TABLE 4. Enzyme activities of lactate dehydrogenase (LDH), phosphofructokinase (PFK), mannitol 1-phosphate dehydrogenase (M1PDH) for the three *L. lactis* strains used in this work. Entries represent enzyme activities, while the V^+ values in mmol (l internal vol.)$^{-1}$ min^{-1} are given in italics, as they are used in the kinetic model.

	Enzyme activities (µmol min^{-1}mg^{-1} protein)				
L. lactis strain	LDH	PFK	M1PDH ^{forw}	M1PDH ^{rev}	M1Pase
NZ9000	19.2 ± 5.1	1.9 ± 0.1	-	-	$4.0\cdot10^{-3} \pm 0.5\cdot10^{-3}$
	6394	*633*	*0*	*0*	*1.3*
HWA217 (pNZmtlD)	29.9 ± 1.3	0.65 ± 0	0.15 ± 0	0.58 ± 0.1	$6.6\cdot10^{-3} \pm 0.6\cdot10^{-3}$
	9957	*216*	*143*		*2.2*
NZ9010 (pNZmtlD)	-	2.5 ± 0.2	1.9 ± 0.3	8.5 ± 1.6	$4.1\cdot10^{-3} \pm 0.3\cdot10^{-3}$
	0	*833*	*1809*		*1.4*

TABLE 5. Experimental and predicted flux distribution over the different fermentation products produced by the *L. lactis* strains NZ9000, NZ9010 (pNZmtlD), and HWA217 (pNZmtlD).

		flux distribution (mmol (l internal vol.)$^{-1}$ min^{-1})							
L. lactis strain		glucose	lactate	acetate	formate	ethanol	acetoin	2,3-butanediol	mannitol
NZ9000		185 (100)	365 (99)						
		97 (100)	*189 (97)*	*0.6 (0.3)*	*0.3 (0.2)*	*0.5 (0.3)*			
HWA217 (pNZmtlD)		180 (100)	344 (96)						0.5 (0.3)
		109 (100)	*209 (96)*	*1.5 (0.7)*	*0.4 (0.2)*	*0.4 (0.2)*			*1.7 (1.5)*
NZ9010 (pNZmtlD)		205 (100)	11 (3)	76 (19)	242 (59)	190 (47)	49 (24)	3 (1)	0.7 (0.3)
		7.0 (100)	*0 (0)*	*0.5 (3.5)*	*3.1 (22)*	*2.6 (19)*	*0.1 (1.2)*	*3.5 (50)*	*1.4 (20)*
	[a, b]	*17 (100)*	*7.7 (24)*	*0.9 (2.5)*	*7.1 (21)*	*6.3 (19)*	*0.2 (1.0)*	*6.8 (41)*	*1.4 (8.3)*
	[a, c]	*59 (100)*	*8.7 (7.4)*	*13 (11)*	*50 (43)*	*39 (33)*	*1.5 (2.5)*	*24 (41)*	*1.3 (2.2)*

Experimental and predicted fluxes are presented in regular and italics numbers, respectively. In parentheses, the percentage of glucose converted into the product is given.

Based on the experimental flux data of NZ9010 (pNZmtlD), the model prediction assumes a:

[a] V^+ for LDH of 11 mmol (l internal vol.)$^{-1}$ min^{-1};

[b] V^+ for PFL of 242 mmol (l internal vol.)$^{-1}$ min^{-1}.

[c] V^+ for PFL of 2000 mmol (l internal vol.)$^{-1}$ min^{-1}.

Flux distributions, experimental data versus model predictions. Previously, we found that overexpression of *mtlD* in the LDH-deficient *L. lactis* strain NZ9010, and in strain HWA217, a strain with reduced PFK activity, resulted in very low mannitol production by growing cells (29). Here, we have quantified the fluxes to mannitol and other fermentation products of the two mannitol-producing strains and the parental strain NZ9000. The experimental fluxes were compared with the fluxes predicted by the model (Table 5).

Both the parental strain and strain HWA217 (pNZmtlD) showed a mainly homolactic fermentation pattern. Both experimentally determined and predicted fluxes showed a 96 to 99% glucose to lactate conversion (Table 5). In the experimental data and the model predictions, no substantial differences could be observed between the glucose and lactate fluxes of the HWA217 strain and the parental strain. The model prediction of the steady state glycolytic flux was about 50% lower than the flux experimentally determined. This, however, is acceptable for such a detailed kinetic model largely based on kinetic parameters from literature, that have not been determined in the same strain under the same conditions. The amount of mannitol produced by strain HWA217 (pNZmtlD) was very low [0.5 mmol (l internal vol.)$^{-1}$ min^{-1}], comparable to the mannitol production by the LDH-deficient strain NZ9010 (pNZmtlD) [0.7 mmol (l internal vol.)$^{-1}$ min^{-1}]. The measured mannitol flux of 0.5 mmol (l internal vol.)$^{-1}$ min^{-1} corresponds to a conversion of glucose to mannitol of 0.3%, which was lower than the 1.5% conversion predicted by the model. In agreement with the model prediction the LDH-deficient strain NZ9010 (pNZmtlD) showed a mixed-acid pattern, with a strongly reduced lactate production, and production of mainly ethanol, formate, acetate, and acetoin. Furthermore, the experiments show a very small flux to mannitol, not more than 0.7 mmol (l internal vol.)$^{-1}$ min^{-1}, corresponding to a flux distribution of 0.3% of the glucose flux during the exponential growth phase. In accordance with the experimental mannitol flux, the model predicted a very small flux to mannitol of 1.4 mmol (l internal vol.)$^{-1}$ min^{-1}. However, the predicted amount of mannitol produced per mole of glucose is much higher (20%). This is most likely the result of the very low glycolytic/glucose flux that is predicted by the model. The actual glycolytic flux was much higher and it appeared that the flux to formate was higher than the V^+ used for pyruvate formate lyase (PFL) in the model. Obviously, the V^+ of PFL in this strain had to be much higher. It is likely that the V^+ should be at least 242 mmol (l internal vol.)$^{-1}$ min^{-1}, which is the experimentally determined formate flux. Increasing the PFL activity in the model to this level (Table 5, note [a]) and an assumed PFL level of 2000 mmol (l internal vol.)$^{-1}$ min^{-1} (Table 5, note [b]), is indeed predicted to result in a higher glucose flux, and in a lower flux distribution (8.3% and 2.2% versus 20%) to mannitol. By increasing the V^+ of PFL, the model prediction of the conversion

to ethanol (33%), is also more in agreement with the experimental results (47%).. On the other hand, the model predicts that about 40% of the glucose would be converted to 2,3-butanediol, whereas in the experiments this is only 1%. Moreover, a considerable lactate flux of 11 mmol (l internal vol.)$^{-1}$ min^{-1} was observed for the LDH-deficient strain. This was probably the result of activation of an alternative LDH in strain NZ9010, with different kinetic parameters than that of the original LDH, as has been reported before (3). This implies that the alternative LDH has a V^+ of at least 11 mmol (l internal vol.)$^{-1}$ min^{-1}, and insertion of this activity in the model resulted in a flux to lactate (Table 5, note [c]).

In both mannitol-producing batch cultures of strains HWA217 (pNZmtlD) and NZ9010 (pNZmtlD), the low mannitol concentrations remained constant after exhaustion of glucose for at least 24 h, as described previously (29).

Intracellular metabolites and cofactors. The model not only predicts the metabolic fluxes, but also metabolite concentrations that can be indicative of control exerted by certain parts of the metabolism. Therefore, the intracellular metabolites G6P, F6P, and M1P, and the cofactors NAD and NADH were determined in cell-extracts of the different *L. lactis* strains used in this work (Table 6). In agreement with the model predictions, strain HWA217 (pNZmtlD) has increased G6P and F6P levels in comparison to the parental strain NZ9000, as described by Andersen et al. (1). In the LDH-deficient strain NZ9010 (pNZmtlD), the G6P and F6P concentrations were similar to the parental strain. The model predicts an increased NADH/NAD ratio for strain NZ9010 (pNZmtlD), in comparison to strain NZ9000 (Table 6). Indeed, the NADH/NAD ratio in strain NZ9010 (pNZmtlD) was a factor 2 higher than in the control strain NZ9000. The M1P concentrations in strains NZ9010 (pNZmtlD) and HWA217 (pNZmlD) were rather low. Only 0.81 mM of M1P was detected in the extracts of strain HWA217 (pNZmtlD). In contrast to the predicted accumulation of M1P in strain NZ9010 (pNZmtlD), a low M1P concentration of 0.14 mM was detected in extracts of this strain. Thus despite the high M1PDH activity in these strains overexpressing *mtlD*, and unlike the model predictions, M1P did not accumulate to high concentrations.

Control coefficients on the mannitol flux. In agreement with the experimental results, the model predicted very low fluxes to mannitol (Table 5). MCA analysis was performed on the model for the different strains (parental, LDH-deficient, reduced PFK) to determine the distribution of control on the flux to mannitol.

MCA analysis of the models of all three strains with M1PDH activity, shows that in particular M1Pase has a high control on the flux to mannitol. For the experimental situation, when the V^+ of M1PDH is set at 1809 and 143 mmol (l internal volume)$^{-1}$min^{-1}for strains NZ9010 (pNZmtlD) and HWA217 (pNZmtlD) respectively, the control coefficients of M1Pase on the mannitol flux are 0.98 and 0.78, respectively. Also in the parental strain NZ9000, the control of M1Pase is high (0.93) when a M1PDH is assumed to have a V^+ of 2000 mmol (l internal volume)$^{-1}$ min^{-1} (Table 7).

TABLE 6. Intracellular metabolites and cofactors in extracts of three *L. lactis*. Experimental and predicted concentrations are presented in regular and italics numbers, respectively.

	intracellular metabolites and cofactors (mM)					
L. lactis strain	G6P	F6P	M1P	NAD	NADH	NADH/NAD
NZ9000	4.6 ± 0.1	1.6 ± 0.3	-	6.6	3.7	0.56
	2.3	*0.45*		*5.2*	*0.32*	*0.06*
HWA217 (pNZmtlD)	11.3 ± 3.0	2.1 ± 0.2	0.81 ± 0.1	5.1	2.6	0.51
	5.2	*1.0*	*2.0*	*5.4*	*0.16*	*0.03*
NZ9010 (pNZmtlD)	4.9 ± 2.1	1.6 ± 0.6	0.14 ± 0.03	6.7	7.9	1.2
[a]	*0.64*	*0.18*	*29*	*0.99*	*4.6*	*4.6*
[b]	*1.5*	*0.33*	*10*	*4.6*	*0.92*	*0.20*

[a] The model assumes V^+ values of 11 and 242 mmol (l internal vol.)$^{-1}$ min^{-1} for LDH and PFL, respectively.

[b] The model assumes V^+ values of 11 and 2000 mmol (l internal vol.)$^{-1}$ min^{-1} for LDH and PFL, respectively.

TABLE 7. Control coefficients on the flux through M1Pase as predicted by the model for the different *L. lactis* genetic backgrounds. Regular and italics numbers represent measured and assumed M1PDH and M1Pase V^+ values, respectively. The LDH and PFK activities for the different genetic backgrounds are listed in Table 4. For strain NZ9010, LDH and PFL are assumed to have V^+ values of 11 and 242 mmol (l internal vol.)$^{-1}$ min^{-1}, respectively.

	L. lactis strain						
	parental (NZ9000)		reduced PFK (HWA217)			LDH deficient (NZ9010)	
step/enzyme	V^+ (mmol (l internal vol.)$^{-1}$ min^{-1})						
M1PDH	*2000*	*2000*	143	143	*2000*	1809	1809
M1Pase	1.3	*100*	2.2	*100*	*100*	1.4	*100*
step/enzyme	control coefficient on mannitol flux						
PFK	-0.06	-0.14	-0.19	0.15	-0.12	-0.01	-0.08
LDH	-0.06	-0.20	-0.06	-0.24	-0.20	0.00	0.00
Acetate kinase	0.03	0.24	0.02	0.11	0.26	0.00	0.00
Pyruvate dehydrogenase	0.06	0.21	0.06	0.25	0.21	0.00	0.00
M1PDH	0.01	0.11	0.13	0.42	0.19	0.01	0.29
M1Pase	0.93	0.28	0.78	0.15	0.21	0.98	0.44
Mannitol efflux	0.03	0.18	0.05	0.08	0.13	0.0	0.26
	flux (mmol (l internal vol.)$^{-1}$ min^{-1})						
glucose	102	126	109	121	125	16	78
mannitol	1.1	14.9	1.7	8.2	18.9	1.4	33
mannitol/glucose (%)	1.1	12	1.5	6.8	15	8.3	42

Predicted mannitol flux by increased M1Pase activity. The predicted control coefficient of M1Pase on the mannitol flux of 0.93, implies a 0.93% increase of the mannitol flux as a result of a 1% increase of M1Pase. To predict the effect of increasing the M1Pase activity on the mannitol flux, the V^+ of M1Pase was increased to a level of 100 mmol (l internal volume)$^{-1}$min^{-1} in the models of the parental strain (NZ9000), the LDH-deficient strain (NZ9010), and the strain with reduced PFK activity (HWA217) (Table 7).

Table 7 shows that the model predicted an increase of the mannitol flux from 1.1 to 14.9 mmol (l internal volume)$^{-1}$min^{-1} for strain NZ9000, corresponding with an increase of the glucose to mannitol conversion from 1.1% to 14.9%. Increase of the V^+ of M1Pase in the model of strain HWA217, resulted in an increase of the mannitol flux from 1.7 to 8.2 mmol (l internal vol)$^{-1}$ min^{-1}, corresponding with an increase of the glucose to mannitol conversion from 1.5% to 6.8%.. The increased control coefficient of M1PDH from 0.13 to 0.42 in the model of strain HWA217, indicated that an increased V^+ of M1PDH would increase the mannitol flux. Indeed, when a V^+ of 2000 mmol (l internal vol.)$^{-1}$ min^{-1} was assumed for M1PDH, an increase for the mannitol flux from 8.2 (6.8%) to 18.9 (15%) mmol (l internal vol.)$^{-1}$ min^{-1} was predicted for strain HWA217 (Table 7). In Figure 3 is shown that a higher glucose to mannitol conversion can be expected for the strain with reduced PFK activity (HWA217), in comparison to the wild-type stain (NZ9000).

Furthermore, an increased control on the mannitol flux was predicted for acetate kinase and pyruvate dehydrogenase in the models of strains NZ9000 and HWA217, when M1Pase activity was increased (Table 7). Indeed, when these activities were doubled in the models, a small increase in the glucose to mannitol conversion ratio of 1-2% was observed (data not shown).

Remarkably, the increased negative flux control of LDH on the mannitol flux increased when the V^+ of M1Pase is increased in the models of strains NZ9000 and HWA217, suggesting that a decrease or even a deletion of LDH may be an important step to further increase the mannitol production. Indeed, the model predicted that an LDH deficiency has a strong effect on the mannitol flux. When the V^+ of M1Pase was set to 100 mmol (l internal volume)$^{-1}$min^{-1} in the model of the LDH-deficient strain (NZ9010), the mannitol flux increased to 33 mmol (l internal volume)$^{-1}$min^{-1}, corresponding with a glucose to mannitol conversion of 42 % (Table 7). In addition to the increased mannitol flux, also the glycolytic (glucose) flux in the model of strain NZ9010 was increased from 16 to 78 mmol (l internal volume)$^{-1}$min^{-1}, when the V^+ of M1Pase was increased to 100 mmol (l internal volume)$^{-1}$min^{-1}. From the three analyzed genetic backgrounds (parental, reduced PFK, and LDH deficiency), the highest mannitol production is expected for the LDH-deficient *L. lactis* strain (NZ9010), and glucose to mannitol conversion percentages up to 50% can be expected when M1Pase is further increased in the LDH-deficient strain (Fig. 3).

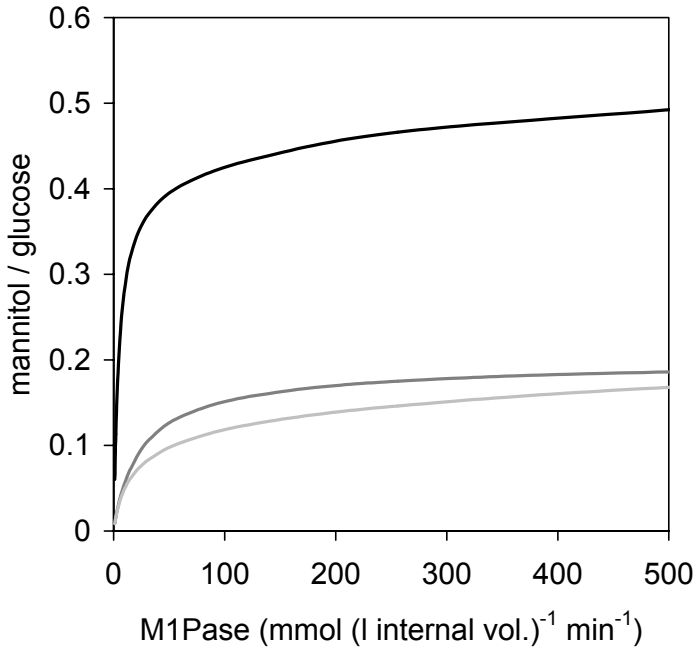

FIG. 3. Mannitol production as a function of the M1Pase activity as predicted by the model for (over)production of M1Pase in strains NZ9000, HWA217, and NZ9010. Mannitol production is presented as mol mannitol (mol glucose)$^{-1}$.The model assumes an equal M1PDH activity of 2000 mmol (l internal vol.)$^{-1}$ min^{-1} for all three *L. lactis* strains: NZ9000 (light grey line), HWA217 (dark grey line), and NZ9010 (black line).

DISCUSSION

Modeling of the *L. lactis* glycolysis and pyruvate metabolism (10, 11) has provided us with a very useful tool to predict the effects of alterations in the metabolism of *L. lactis*. In this work we used the *L. lactis* glycolytic model as a basis for a kinetic model for mannitol production by *L. lactis*. The model was extended with a mannitol branch and used to analyze the fermentation products of growing cultures of *mtlD* overexpressing *L. lactis* strains. Previously we reported that some, but very low mannitol production (less than 1% of the initial glucose) was observed for growing cultures of the *mtlD* overexpressing *L. lactis* strains NZ9010 (pNZmtlD) and HWA217 (pNZmtlD) (29). The mannitol production by these strains was critically investigated, by 1) comparison of experimental flux data with predicted fluxes by the model, and by 2) a control analysis of the system.

In agreement with the experimental results, the model predicted the homolactic fermentation for strain HWA217 (pNZmtlD), and the mixed acid fermentation for strain NZ9010 (pNZmtlD) (Table 5). In addition, the model predicted low mannitol fluxes, just as observed in the two *L. lactis* strains. Clearly, the model predicted correctly that increasing the M1PDH activity alone is not sufficient to get a reasonable flux to mannitol. In contrast to the experiments, the model predicted a very low glycolytic flux for the LDH-deficient strain NZ9010, and underestimated the ratio of the conversion into the reduced fermentation product ethanol. This is most likely to be the result of too low V^+ values in the model for PFL, and alcohol dehydrogenase in this LDH-deficient strain. No kinetic data are available, and hence in the model levels of these enzymes were assumed not to change as a result of abolishing LDH activity. Increasing the V^+ of PFL in the model to the measured formate flux in strain NZ9010 (pNZmtlD), resulted in a slight increase of the predicted glycolytic (glucose) flux (Table 5).

A decreased glucose flux in comparison with the parental strain, as a result of the lowered PFK activity in strain HWA217, was not observed in the experimental set-up, nor predicted by the model (Table 5). This contrasts to the findings of Andersen et al. (1), who described a reduced growth and glycolytic flux for this strain (1). The observation that repeatedly sub-culturing of this strain in CDM resulted in an increasing growth rate (unpublished data) suggests that the strain has adapted and that higher glycolytic fluxes can be reached in the adapted strain. This was supported by the findings of Andersen et al., who described that by slowly increasing the dilution rate in a continuous culture of strain HWA217, the growth rate could be almost doubled compared to that in batch culture (1).

MCA was performed on the model to determine which steps exert the highest control on the mannitol production in *L. lactis*. These steps are likely to be the targets for efficient engineering of the mannitol production. It was assumed that the efflux of mannitol would not significantly limit the mannitol production, as it was shown in our earlier work that the mannitol produced by resting cells of *L. lactis* NZ9010 (pNZmtlD) was found extracellularly (29). In the investigated strains HWA217 (pNZmtlD) and NZ9010 (pNZmtlD) M1Pase has the highest control coefficient on the flux to mannitol, 0.78 and 0.98, respectively (Table 7). This means that the mannitol production is strongly controlled by M1Pase activity. The experimental M1Pase enzyme activity levels show that no higher mannitol fluxes could be reached, because the measured V^+ values (Table 4) are in the same order of the measured mannitol fluxes (Table 5), while the M1PDH activities are not limiting for the mannitol flux. However, the bottleneck at the level of M1Pase was not reflected in the intracellular M1P concentration (Table 6). No substantial intracellular accumulation of M1P was observed for both strains NZ9010 (pNZmtlD) and HWA217 (pNZmtlD), although an accumulation of M1P was predicted by the model for strain NZ9010 (pNZmtlD).

MCA indicated a very large positive control (approximately 0.9) of M1Pase on the mannitol flux. Therefore, an increased M1Pase activity combined with a high M1PDH activity in the models of the parental *L. lactis* strain, the LDH-deficient derivative and that with reduced PFK activity, was likely to increase the mannitol flux. Indeed, the model predicted an increased mannitol production in all three situations (Table 7). For strain NZ9000, a glucose-mannitol conversion of about 13% was expected when the V^+ of M1Pase was increased approximately 100-fold. With a V^+ value of 100 mmol (l internal vol.)$^{-1}$ min^{-1}, strain HWA217 is expected to convert 6.8 % of glucose into mannitol. When the V^+ of M1PDH was increased to a level of 2000 mmol (l internal vol.)$^{-1}$ min^{-1}, a glucose to mannitol conversion of 15% was predicted. The positive effect of the reduced PFK activity in HWA217 on the glucose to mannitol conversion, is shown in the predicted M1Pase-dependent mannitol production curve (Fig. 3). When V^+ values of M1PDH and M1Pase are equal in the models of NZ9000 and HWA217, a clearly higher mannitol production is expected for strain HWA217, in comparison to strain NZ9000 (Fig. 3). Most likely, the predicted increased F6P concentration in strain HWA217 (Table 6) has contributed to a higher predicted glucose to mannitol conversion, in comparison to strain NZ9000. In agreement with the negative control coefficient of PFK on the mannitol flux (-0.12), a further decrease of the PFK activity in the model of strain HWA217 did result in a higher glucose-mannitol conversion percentage. At the same time, this resulted in a decreased glycolytic flux (not shown).

MCA indicated a large negative control of LDH (-0.20) on the mannitol flux for strains NZ9000 and HWA217 (Table 7), indicating that elimination of LDH activity would lead to an significantly increased mannitol flux. Indeed, in contrast to the moderate mannitol production predicted for strains NZ9000 and HWA217, the model predicted that much higher glucose-mannitol conversion ratios should be obtained by the LDH-deficient *L. lactis* strain NZ9010. Increase of M1Pase to a level of 100 mmol (l internal vol)$^{-1}$ min^{-1} should result in 42% conversion of glucose into mannitol (Table 7), and conversions of up to 50% glucose into mannitol are expected by further increasing the M1Pase activity (Fig. 3). So far, resting cells of LDH-deficient strains are the only published examples of *L. lactis* strains known to produce fairly high amounts of mannitol (8, 20, 29). To cope with the increased NADH/NAD ratio, M1PDH activity is induced in these strains, and NAD regenerated by mannitol production. It is likely that the higher intracellular NADH/NAD ratio in the NZ9010 cells (Table 6) contributes to the higher mannitol production in this strain.

Despite the high glucose-mannitol conversion percentages predicted by our model as a result of the increased M1Pase activity, mannitol production could be hampered by subsequent catabolism of the produced mannitol. Neves and co-workers showed that mannitol produced by LDH-deficient *L. lactis* cells was re-utilized (20). Very recently, Gaspar et al. reported high conversion rates of glucose to mannitol without re-metabolization of mannitol by resting *L. lactis* cells, by inactivation of the mannitol transport system in the LDH-deficient *L. lactis* strain (8). Our experimental results presented here and in our previous work (29) show that mannitol is not re-utilized upon glucose exhaustion by the *L. lactis* strains used, and that growth on mannitol was only observed after a long lag-phase of more than 24 h, probably as a result of adaptation. Therefore, we have not included a mannitol uptake system in our model.

In conclusion, our model predicts that an increased M1Pase activity should dramatically improve the mannitol flux in *L. lactis*. In addition, LDH deficiency is predicted to contribute significantly to mannitol production. The conversion of M1P into mannitol by *L. lactis* is probably mediated by aspecific phosphatases, in a process called inducer expulsion (18), since no annotated M1Pase sequences were found in the genome of *L. lactis* IL1403 and MG1363. This process has been described for other sugar phosphates, but also a membrane-located phosphatase with affinity for M1P has been reported (32). Increasing the activity of such an aspecific phosphatase would increase the dephosphorylation of M1P, but would probably also result in increased expulsion of other sugars and decreased intracellular sugar phosphate concentrations. Therefore, an increase in specific M1Pase activity is preferred. This could be achieved by expression of a heterologeous M1Pase gene in *L. lactis*. To our best knowledge, the only known gene coding for a specific M1Pase is that from the apicomplexan parasite *Eimeria tenella,* coding for a highly specific M1Pase with limited overall homology to members of the phosphohistidine family of phosphatases (16). This *E. tenella* M1Pase gene was cloned and expressed at high levels in *E.coli*, and M1Pase was enzymatically active in this bacterial host. Preliminary expression experiments of the *Eimeria tenella* M1Pase gene indicate that M1Pase is also active in *L. lactis,* (30). Currently, we are investigating the role of M1Pase in the mannitol synthesis by *L. lactis*.

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CHAPTER 5

Mannitol 1-phosphatase: A Key Enzyme for Engineering Mannitol Production by *Lactococcus lactis*

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ABSTRACT

To achieve high mannitol production by *Lactococcus lactis,* the mannitol 1-phosphatase gene of *Eimeria tenella* and the mannitol 1-phosphate dehydrogenase gene *mtlD* of *Lactobacillus plantarum* were cloned in the nisin-dependent NICE *L. lactis* overexpression system. As predicted by a kinetic *L. lactis* glycolysis model, increase of 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 the mannitol 1-phosphatase activity and the mannitol production was shown, demonstrating the usefulness of this metabolic engineering approach.

INTRODUCTION

Mannitol is a reduced form of fructose and is produced by a variety of micro- organisms 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 micro organisms 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 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 may serve as an antioxidant (4, 5, 25, 26) and as a low-calorie sweetener which can replace sucrose (7, 8).

In heterofermentative lactic acid bacteria (LAB) such as *Leuconostoc mesenteroides*, mannitol is formed in a single conversion from fructose by mannitol dehydrogenase, and fructose to mannitol conversions of more than 90% are common (13, 24, 27). In contrast, most homofermentative LAB, such as *L. lactis,* do not normally produce mannitol. Mannitol formation in homofermentative LAB 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 non-growing cell suspensions of a lactate dehydrogenase (LDH)-deficient *L. lactis* strain (22). During growth, only small amounts of mannitol (less than 0.4 mM) were transiently produced extracellularly (23). Recently, inactivation of the mannitol transport system in a LDH-deficient *L. lactis* strain resulted in high extracellular mannitol production. About one-third of glucose was converted into mannitol by non-growing 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 explore the limits of mannitol production by *L. lactis*, we further employed a metabolic engineering strategy. Recently, we reported that overexpression of the *mtlD* 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://jjj.biochem.sun.ac.za) and expanded by introducing a mannitol branch (29). Moreover, the metabolic model predicted that M1Pase has a high control on 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 LAB (2, 18) or other bacteria. However, a specific M1Pase gene has been described in *Eimeria tenella*, a protozoan parasite (20). In these parasites, and some fungi, M1Pase has an important role in the mannitol cycle. In this work we investigated the effect of overexpression of the M1Pase gene from *Eimeria tenella* on the mannitol-producing capacities of *L. lactis* NZ9000, a LDH-deficient strain NZ9010 (3, 14), and strain HWA217 with reduced phophofructokinase 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.

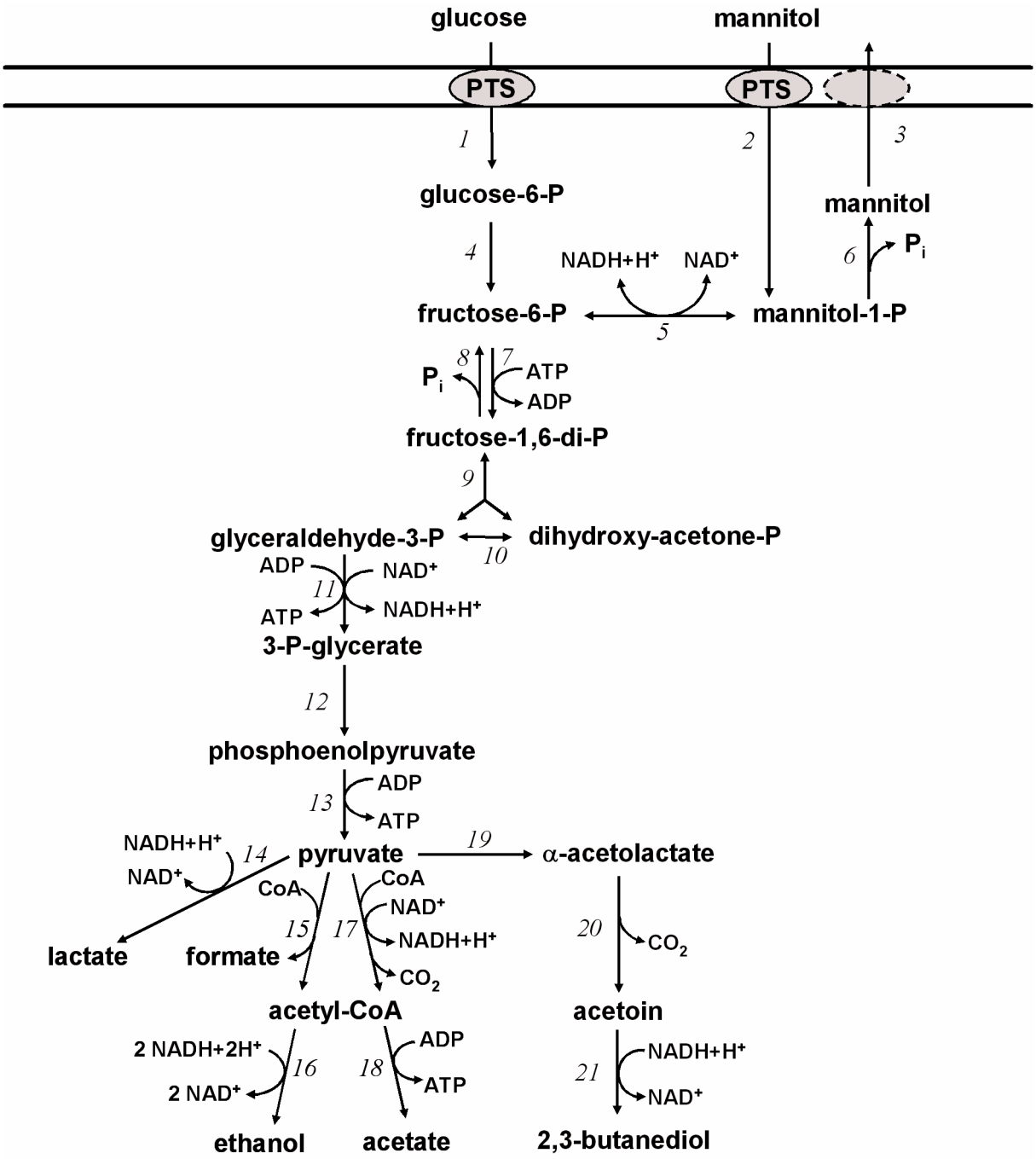

FIG. 1. Proposed pathway for hexose metabolism of homofermentative lactic acid bacteria. *1* Phospho*enol*pyruvate (PEP)-dependent sugar phosphotransferase system (PTS); *2*. Mannitol-specific PTS; *3*. Mannitol export (mechanism unknown); *4*. Phosphoglucose isomerase; *5*. Mannitol 1-phosphate dehydrogenase; *6*. Mannitol 1-phosphatase; *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*. Lactate dehydrogenase; *15*. Pyruvate-formate lyase; *16*. Acetaldehyde dehydrogenase and alcohol dehydrogenase; *17*. Pyruvate dehydrogenase; *18*. Acetate kinase; *19*. α-Acetolactate synthase; *20*. α-Acetolactate decarboxylase; *21*. 2,3-Butanediol dehydrogenase.

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)anaerobically 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 µg/ml, respectively. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a Ultrospec 2000 spectrofotometer (Pharmacia Biotech). For inducing M1PDH and M1Pase activity, 0.1 to 10 ng/ml nisin was added to a growing culture at an OD_{600} of 0.5, or at an other OD_{600} when indicated.

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

Strains and plasmids	Characteristics	References or source
Strains		
NZ9000	MG1363 *pep*N*::nis*RK	(19)
NZ9010	NZ9000 *ldh::ery,* 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 plasmid	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 plasmid	This work
Plasmids		
PQE60-M1Pase	*E. coli* expression vector pQE60 carrying the *E. tenella* M1Pase gene	(20)
pNZ8148	pNZ8048 derivative; Cm^r, lactococcal cloning and expression vector with *nis*A promoter upstream of a multiple cloning site	(19)
pNZ9530	Ery^r, *nis*RK	(17)
pNZ-*mtlD*	pNZ8148 carrying *L. plantarum mtlD* gene fused to *nis*A promoter	(30)
pWW002	pNZ8148 carrying *E. tenella* M1Pase gene fused to *nisA* promoter	This work
pWW003	pNZ-*mtlD* with *E. tenella* M1Pase gene cloned downstream of *mtlD*	This work

Construction of plasmid pWW002 and pWW003. In our previous work, the *mtlD* gene encoding M1PDH from *Lb. 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 an *E. coli* expression vector pQE-60 (Qiagen) containing the M1Pase gene from *E. tenella* (20), using the primers M1PaseET-1FW (5'-GGGTCTAGAAGCCATGGCAGAGACTGAGTGG-3') and M1PaseET-1RV (5'-GGCCGAGCTCTTAGGGTTTAGCGTTTGG-3'), with introduced *Xba*I, *Nco*I and *Sac*I 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, The Netherlands). The *Nco*I-*Sac*I digested PCR product of the M1Pase gene was cloned into pNZ8148, resulting in pWW002, containing the M1Pase gene fused to the *nis*A promoter. The M1Pase gene was also cloned downstream of *mtlD* in pNZ-*mtlD* by ligation of *Xba*I-*Sac*I digested M1Pase PCR product into *Xba*I-*Sac*I digested pNZ-*mtlD*, resulting in pWW003.

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

Analysis of fermentation products and glucose consumption. During growth, samples were taken from the *L. lactis* cultures, centrifuged for 1 min at 10,000 × g and 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 HPLC using a 30 cm IOA-1000 ion exclusion column (Alltech, Breda, the Netherlands), with 3 mM sulfuric acid as eluens, at a flow rate of 0.4 ml/min and a temperature of 90°C.

Preparation of cell extracts. Cell-free 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 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 zirconia/silica beads (BioSpec products, Inc.) in a 2 ml eppendorf cup, and cells were disrupted by vigorously 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 with the BCA protein assay (Pierce, USA), with bovine serum albumin as the standard.

Enzyme assays. Cell cultures (50 ml) were harvested for enzyme assays at an OD_{600} of approximately 1.2, or 2 h after induction with nisin, and cell extracts were prepared as described above.

The reduction of fructose 6-phosphate by M1PDH was assayed in a reaction mixture containing 50 mM sodium phosphate buffer pH 6.0 with 0.5 mM NADH. The reaction was initiated by the addition of 1 mM of 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 using a microplate reader (Tecan Safire, Austria).

M1Pase activity was determined at RT in a reaction mixture containing cell extract (20-50 µg/ml protein), 50 mM MES buffer (pH 7.0), 10 mM $MgCl_2$ and 3 mM mannitol 1-phosphate. The formed inorganic phosphate was determined using a modified protocol of the Sigma inorganic phosphate kit (Sigma Diagnostics). At several time points, 100 µl samples were taken and the reaction was stopped with the addition of 20 µl acid molybdate solution. 5 µl Fiske & SubbaRow reducer solution was added to 100 µl of the clear centrifuged solution in a 96 wells microplate, and the absorbance at 660 nm was measured with a micro plate reader (Tecan Safire, Austria).

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 plasmids pNZ8148, resulting in pWW002. To overproduce M1Pase and M1PDH simultaneously in *L. lactis*, the M1Pase gene was also cloned downstream 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/ml nisin resulted in 0.9 μmol min^{-1} mg^{-1} protein 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). An increased M1Pase activity was also detected in extracts of nisin-induced cells of strains NZ9010 (pWW002) and HWA217 (pWW002), but also no mannitol production was observed (data not shown).

Thus, overexpression of the M1Pase gene alone did not result in mannitol production. However, simultaneous expression of both the M1Pase gene and *mtlD* in *L. lactis* NZ9000 harboring pWW003, resulted in production of 2.3 mM of mannitol in the supernatant, corresponding to a glucose-mannitol conversion of 10% (Table 2). A M1PDH activity of 2.2 and a M1Pase activity of 0.27 μmol min^{-1} mg^{-1} protein were detected in the cell extracts of strain NZ9000 (pWW003) (Table 2). The lower M1Pase activity in strain NZ9000 (pWW003), compared to the 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.

TABLE 2. M1Pase and M1PDH activities in cell free 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 free extract were prepared of cells harvested after 2 h of nisin induction. The final concentration of mannitol (mM) produced from 0.5% of glucose is given. <0.1 mM of mannitol means that no mannitol was detected above the detection limit of 0.1 mM.

	nisin	enzyme activity (µmol $min^{-1}mg^{-1}$ protein)		mannitol
L. lactis strain	(ng/ml)	M1Pase	M1PDH	(mM)
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

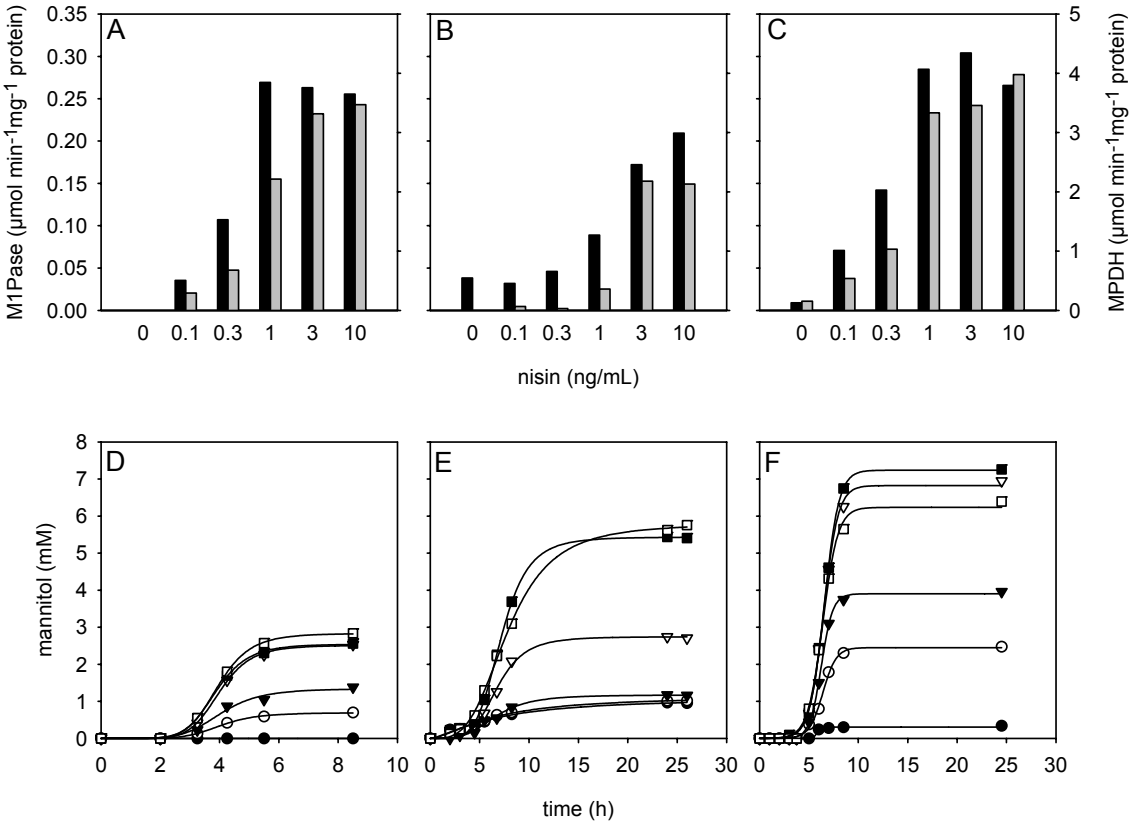

FIG. 2. M1Pase and M1PDH activities and mannitol production of *L. lactis* overexpressing the M1Pase gene of *E. tenella* and the M1PDH gene *mtlD* from *Lb. plantarum.* Cells were grown anaerobically on M17 broth supplemented with 0.5% glucose, M1PDH and M1Pase production was induced with 0, 0.1, 0.3,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

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/ml nisin. For the HWA217 (pWW003) strain, the highest enzyme activities were reached with 10 ng/ml nisin. Concomitant to the increasing M1PDH and M1Pase activities, also a significant increase of mannitol production was observed in all three strains (Fig. 2). Moreover, increasing nisin concentrations resulted in higher final concentrations of mannitol. In agreement with the measured M1Pase activities, higher concentrations than 1.0 ng/ml of nisin did not result in much higher mannitol production in strains NZ9000 (pWW003) and NZ9010 (pWW003). Maximal mannitol production by the *L. lactis* strain HWA217 (pWW003) with reduced PFK activity was reached at 10 ng/ml nisin, 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 highest 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-mannitol conversion of 27% (Table 3). To investigate whether the observed mannitol production serves as an alternative redox sink, the mannitol-producing strains were grown under aerobic conditions. The activation of NADH oxidase under aerobic conditions was expected to decrease the availability of NADH for M1PDH and thus a decreased mannitol production was expected. Indeed, less mannitol was produced compared to the anaerobic conditions. Strain NZ9000 (pWW003) still converted 8% of the glucose into mannitol (Table 3), whereas the mannitol production by the LDH-deficient strain NZ9010 (pWW003) and strain HWA217 (pWW003) with reduced PFK activity, was severely decreased to conversion ratios of 10% and 3%, respectively.

TABLE 3. Product formation by the *L. lactis* strains overproducing the *E. tenella* M1Pase and the *Lb. Plantarum* M1PDH. The initial glucose concentration, amount of nisin used for induction (ng/ml), induction OD_{600}, culture conditions (oxic/anoxic), and the product concentrations in moles produced per mole of glucose are presented.

					end products (mol mol^{-1} glucose)							
L. lactis strain	initial glucose (%)	nisin (ng/ml)	Induction OD_{600}	ox	lactate	formate	acetate	acetoin	2,3-butanediol	ethanol	mannitol	recovery
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 [a]	0	0.1	-	0.95	0.59	0.07	0.10	0.19	0.48	0.03	1.1
	2.0 [a]	3	0.1	-	0.20	0.32	0.28	0.04	0.16	0.15	0.50	1.0

[a] Glucose was not completely consumed (see Fig. 3).

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 is accompanied by a change in the amount of redox neutral products such as acetate and acetoin. In strain NZ9000 (pWW003) and HWA217 (pWW003), the increased mannitol production resulted in a decreased production of lactate and an increase of acetate, while the LDH-deficient strain NZ9010 (pWW003) showed a slight decreased production of acetate, ethanol, formate and 2,3-butanediol, together with an increase of 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. The M1Pase activity in this strain was induced by the addition of various concentrations of nisin. The endogenous M1PDH was induced in this strain by pre-culturing 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 of these mannitol-induced cells during growth on glucose, resulted in a stable and high level of M1PDH activity, and an increasing M1Pase activity (Fig 3A). Concomitant to the M1Pase activity, also the mannitol production increased (Fig 3B), with a maximum of 0.11 mol mannitol per mole of glucose. M1Pase activities higher than 1.2 μmol min^{-1} mg^{-1} protein 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/ml nisin to a growing culture at an OD_{600} of 0.1 in stead of 0.5. This resulted in an increased glucose to mannitol conversion of 39%, compared to 27% conversion rate by 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 compared to the uninduced culture (Fig 4A), and after 120 h the glucose was almost completely consumed, while the glucose consumption of uninduced culture ceased after 60 h.

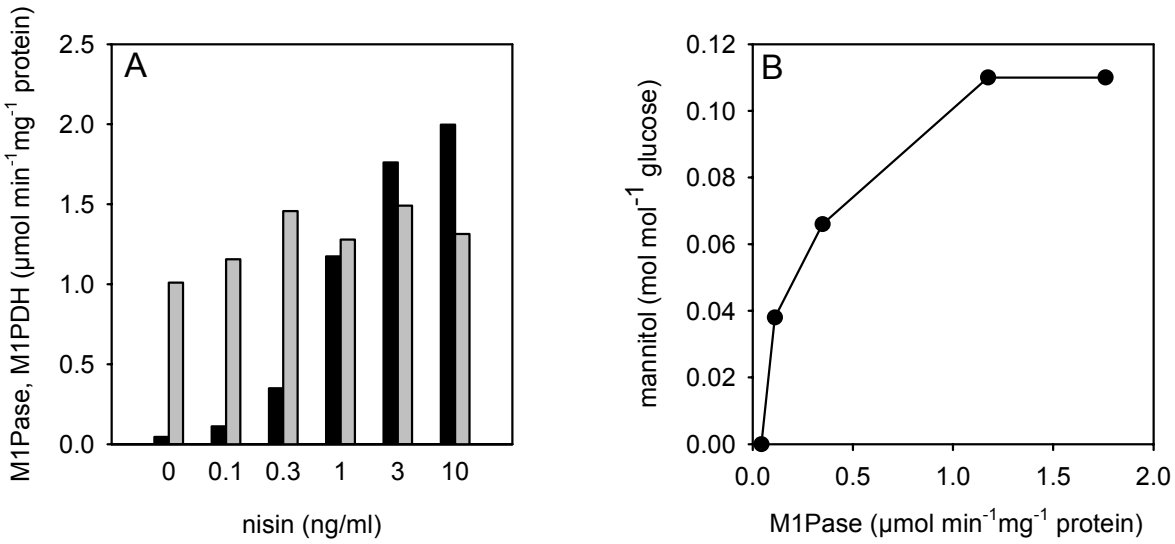

FIG. 3. M1Pase-dependent mannitol production by *L. lactis* NZ9000 (pWW002). Cells, precultured on 0.5% of mannitol, were grown anaerobically on M17 broth containing 0.5% of glucose, induced with 0, 0.1, 0.3, 1.0, 3.0, and 10 ng/ml of nisin at an OD_{600} of 0.1 and cells were harvested at an OD_{600}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

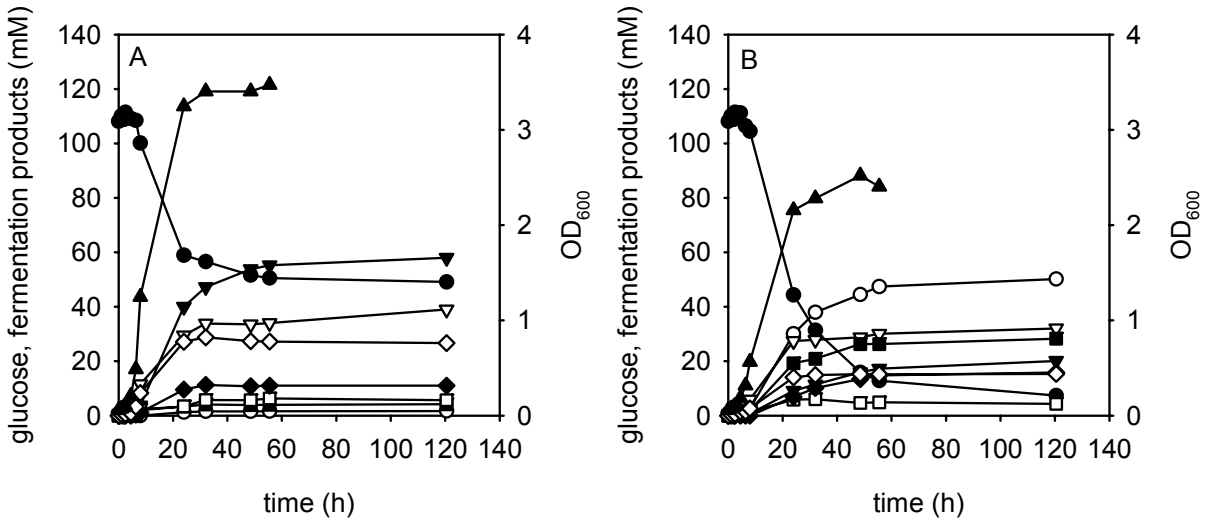

FIG. 4. Glucose consumption and product formation during growth by uninduced (A) and nisin-induced (B) *L. lactis* NZ9010 (pWW003) cells. Cells were grown anaerobically on M17 broth supplemented with 2% glucose. At an OD_{600} of 0.1, 3 ng/ml nisin was added to the culture to induce M1PDH and M1Pase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

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). In *L. lactis* and *Lb. 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 LAB (6). Our present approach focused on the overexpression of genes involved in 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 *Lb. 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 GC 60% versus 28% GC in wobble nucleotide, respectively), M1Pase was overproduced at a high level in *L. lactis* NZ9000 (pWW002) (Table 2). 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) is lacking M1PDH activity (Table 2), and no mannitol production can 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 higher mannitol yields than 11% were reached. The high activity and similar high affinity of M1PDH for NADH ($K_{m,\ NADH}$ of 4 μM) (29) in comparison to that of LDH ($K_{m,\ NADH}$ of 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, compared to the 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 NADH oxidase activity during aerobic growth, resulted in decreased mannitol production (Table 3) compared to the anaerobic cultures. This confirms that NAD^+ is regenerated partly via mannitol production by the *L. lactis* strains overproducing M1Pase and M1PDH.

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 mannitol production (30), it was expected that the mannitol production is largely dependent on the M1Pase activity. This was confirmed by the mannitol production from glucose by the NZ9000 (pWW002) cells, pre-cultured on mannitol (Fig. 3). The increased M1Pase activities and stable levels of M1PDH activities, observed for cells induced with an increasing amount of nisin, suggest a direct correlation between the M1Pase activity and mannitol production, with a maximum mannitol production at a M1Pase activity higher than 1.2 μmol min^{-1} mg^{-1} protein. The most likely explanation for this maximum mannitol production is that substrates such as fructose 6-phosphate and NADH become limiting at these high M1Pase activities. Compared to strain NZ9000 (pWW003), NZ9000 (pWW002) displayed higher M1Pase activities at equal mannitol production. Possibly, the lower affinity of the endogenous *L. lactis* M1PDH for the substrate NADH ($K_{m,\ NADH}$ of 23 μM, unpublished results) compared to that of the *Lb. plantarum* M1PDH ($K_{m,\ NADH}$ of 4 μM) (29), results 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, M1PDH was possibly only induced during preparation of the pre-culture on mannitol, 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 the parental strain NZ9000 (pWW003) and strain HWA217 (pWW003), a decreased ethanol and 2,3-butanediol production was the result of mannitol production by the LDH-deficient strain, and also the residual lactate production was 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 mol of mannitol formed. Keeping a neutral redox balance, maximal two-third (67%) of glucose can be converted into mannitol, assuming that no lactate is formed.

The results (Table 3) indicate that indeed an increased acetate production coincides with mannitol production for these two strains. For the 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 to obtain 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 cells of *L. lactis* (12, 22). The results shown here emphasize the importance of M1Pase activity for mannitol production by *L. lactis*, and 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 LAB 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 LAB. Further research on mannitol production by *L. lactis* could be focused 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 re-utilization of mannitol would occur eventually, 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 re-utilization of mannitol in strains overproducing the high amounts of mannitol as presented here.

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CHAPTER 6

Summary and Concluding Remarks

Mannitol is a sugar alcohol that is produced by a wide variety of (micro) organisms. Mannitol has been reported to accumulate in response to environmental stresses like osmotic stress (26, 27, 45, 46), and can protect the organism by 1) maintenance of turgor (24, 25), 2) stabilizing the structures of membrane lipids and proteins at low water activity (30), and 3) acting as an antioxidant (7, 8, 39-41). Efiuvwevwere and coworkers showed that mannitol has an osmoprotecting and antioxidant effect on the dairy lactic acid bacterium *Lactococcus lactis* subjected to decreased water activity and that mannitol enhances the survival during drying of starter cells (13). Starter cultures of lactic acid bacteria are often prepared by drying cell cultures, and during this process, the cells are subjected to a combination of hostile conditions, including osmotic and oxidative stress. The viability of starter cultures of *L. lactis* may thus be enhanced by inducing mannitol production. Since mannitol is also assumed to have several health-promoting properties, *in situ* mannitol production may result in fermented products with extra value. Since mannitol is not metabolized by humans, mannitol can be converted to short-chain fatty acids (such as butyrate) by colonic fermentation, which have been claimed to confer protection against the development of colon cancer (49). Moreover, mannitol is assumed to have several beneficial effects as a low-calorie sweetener (11, 12).

MANNITOL PRODUCTION BY *LACTOCOCCUS LACTIS*

L. lactis, a homofermentative lactic acid bacterium which is extensively used in the dairy industry, could be an ideal organism for *in situ* mannitol production in dairy fermentations. By using the homofermentative *L. lactis*, mannitol can be produced from lactose or other sugar substrates, in contrast to heterofermentative lactic acid bacteria (LAB), which produce mannitol from fructose exclusively (51). Food-grade microorganisms such as *L. lactis* and their products are directly applicable in food products. Moreover, *L. lactis* has proven to be an efficient host for genetic engineering. Overexpression of genes and deletion of undesired genes has led to high production of products in *L. lactis* such as alanine (22), diacetyl (23), and folate (47). Also the production of mannitol in *L. lactis* could be improved by applying metabolic engineering of steps involved in the mannitol biosynthesis. Mannitol formation in homofermentative lactic acid bacteria (LAB) such as *L. lactis* has been detected only in strains whose ability to regenerate NAD is severely hampered. In these mannitol producing strains, mannitol is produced via mannitol 1-phosphate dehydrogenase (M1PDH), which converts the glycolytic intermediate fructose 6-phosphate into mannitol 1-phosphate, while mannitol 1-phosphatase (M1Pase) dephosphorylates

mannitol 1-phosphate to form mannitol. A lactate dehydrogenase (LDH)-negative mutant of *Lactobacillus plantarum* produced 6 mM mannitol from 50 mM glucose whereas the wild-type was not able to produce any mannitol from glucose (14). Neves and coworkers reported mannitol production by *L. lactis* for the first time (34). They showed that resting cells of a LDH-deficient *L. lactis* strain transiently accumulates high amounts of intracellular mannitol (up to 90 mM) and mannitol 1-phosphate (up to 80 mM). However, upon glucose depletion, the produced mannitol was re-utilized. Very recently, Gaspar and coworkers reported extracellular mannitol production by resting cells of a *L. lactis* strain in which both the LDH gene and the mannitol permease gene were inactivated (15). About one third of the initial glucose could be converted into mannitol by resting cells of this transformant. In these strains, mannitol was produced to fulfill the redox balance during sugar metabolism.

In this thesis, different strategies are described to engineer mannitol production in *L. lactis*. To increase mannitol production, i) the mannitol dehydrogenase gene of the heterofermentative *Leuconostoc mesenteroides*, which is known to produce high amounts of mannitol, is introduced and overexpressed in *L. lactis* (**Chapter 2**), ii) the effect of increasing mannitol 1-phosphate dehydrogenase activity on mannitol production is investigated (**Chapter 3**), iii) the development and application of a kinetic mannitol production model is described, which predicts the effect of engineering steps on mannitol production, and suggests strategies for improved mannitol production (**Chapter 4**), and iv) a heterologous mannitol 1-phosphatase gene is overexpressed in *L. lactis* (**Chapter 5**). The results and successes of these approaches are summarized here and discussed in a wider perspective.

OVERPRODUCTION OF MANNITOL DEHYDROGENASE IN *LACTOCOCCUS LACTIS*

Many heterofermentative LAB such as *Lactobacillus* and *Leuconostoc* species, are known to produce high amounts of mannitol. In the heterofermentative metabolism, the enzyme mannitol dehydrogenase (MDH) can convert fructose into mannitol in a single step. In 1920, it was already noted that fructose fermentation by the heterofermentative lactic acid bacterium *Lactobacillus pentoaceticus* resulted in mannitol formation (36). High yields of mannitol from fructose have been reported for *Lactobacillus* species such as *Lactobacillus sanfranciscus* (28)*, Lactobacillus intermedius* (38)*, and Lactobacillus fermentum* (2, 51). In the presence of fructose or sucrose, also *Leuconostoc (pseudo)mesenteroides* produces high levels of mannitol (16, 42, 43, 50, 51). With a mixture of glucose and fructose as carbohydrate sources, approximately two mol of fructose are reduced to mannitol per mol of glucose fermented, resulting in mannitol yields from fructose of more than 90%.

A disadvantage of the mannitol production via M1PDH and M1Pase, as in the mannitol producing LDH-deficient *L. lactis* strains (15, 34), is that mannitol is not produced directly but via the formation of phosphorylated intermediates. One of these intermediates, fructose 6-phosphate, is also part of the glycolytic pathway, thus creating a direct competition between mannitol production and glycolysis. Uncoupling the sugar pool meant for mannitol synthesis from the glycolysis would favor the production of mannitol. By introducing a functional MDH in *L. lactis*, which has fructose as substrate for mannitol formation in stead of fructose 6-phosphate, the mannitol pathway is not competitive with glycolysis. Consequently, another hurdle to take is supplying intracellular fructose, since fructose is phosphorylated by a phosphotranferase system upon transport across the membrane. This can be achieved by using a *L. lactis* strain capable of growing on sucrose (29, 33). Sucrose is transported by a PTS by such a strain, phosphorylated to sucrose 6-phosphate, and hydrolyzed by sucrose 6-phosphate hydrolase into glucose 6-phosphate and fructose. The glucose 6-phosphate part of sucrose enters glycolysis, and the fructose moiety can be converted into mannitol.

To aim for high mannitol production in *L. lactis*, the *mdh* gene of *Leuconostoc mesenteroides* (1) was introduced into *L. lactis* NZ9800, a strain that is able to grow on sucrose. Flux analysis applied on a kinetic model of strain NZ9800 overexpressing *mdh*, predicted that only low amounts of mannitol are expected to be produced during sucrose metabolism (Table 1) (M. H. N. Hoefnagel, unpublished results). The model predicted that fructokinase I (FK I) outcompetes the high MDH activity in the *mdh* overexpressing NZ9800 strain, due to the high K_m of MDH (44-71 mM) (1, 17), compared to the K_m of FK I (0.3 mM) for the substrate fructose (48). When fructokinase (FK) activity was excluded from the model, as in *L. lactis* NZ9841, the model predicted fructose to accumulate, resulting in the conversion into mannitol of 72% of the fructose moiety of sucrose (Table 1). As a result of these predictions, the *mdh* gene was also introduced and overexpressed in the FK-deficient derivative of NZ9800, strain NZ9841 (32).

TABLE 1. Predicted intracellular concentrations and flux distributions during sucrose metabolism, when MDH is overproduced in *L. lactis* NZ9800 and its FK-deficient derivative NZ9841. The model is based on a detailed *L. lactis* glycolysis model ((21), and available at http://jjj.biochem.sun.ac.za), and extended with a sucrose-PTS, sucrose 6-phosphate hydrolase, FK, and MDH (M. H. N. Hoefnagel, unpublished result).

[a] In the model, no steady state could be reached with FK activity set at 0. Therefore, a residual FK activity of 10 mmol min^{-1} l internal vol^{-1} was used in the model of the FK-deficient strain NZ9841

	NZ9800	NZ9841
Enzyme activities in the model (mmol min^{-1} l internal vol^{-1})		
Sucrose-PTS	60	60
Sucrose 6-phosphate hydrolase	200	200
FK	330	10[a]
MDH	1565	1565
Predicted intracellular concentrations (mM)		
Sucrose 6-phosphate	0.08	92
Fructose	0.06	960
Mannitol	0.04	4.6
Predicted fluxes (mmol min^{-1} l internal vol^{-1})		
Sucrose-PTS	43	32
Sucrose 6-phosphate hydrolase	43	32
FK	43	8.9[a]
MDH	0.08	23
Sucrose to mannitol conversion (%)	0.5	72

Despite high expression of *mdh*, only low mannitol production was observed for the wild-type strain NZ9800. Less than 1% of the sucrose was converted into mannitol, and both glucose 6-phosphate and fructose moieties of sucrose are converted into lactate. The experiments confirmed the expectations from the model predictions (Table 1) that the fructose moiety of sucrose is phosphorylated by FK in the NZ9800 strain, and that only low amounts of mannitol are produced. Overexpression of *mdh* in the FK-deficient *L. lactis* NZ9841 was expected to avoid this problem. However, in this *L. lactis* host, *mdh* expression did not result in the expected high mannitol production. HPLC analysis and *in vitro* enzyme activity measurements indicated that the presence of an alternative pathway for fructose utilization - fructose efflux and subsequent transport by a PTS - and the high K_m value of MDH for fructose, possibly have hampered the mannitol production in both strains NZ9800 and NZ9841. Also disruption of the *ldh* gene in *L. lactis* NZ9800, which has proven to be a successful strategy in combination with overproduction of M1PDH and M1Pase, did not result in high mannitol production when *mdh* was overexpressed (Wisselink et al., unpublished results). This suggests that not NADH but fructose is the limiting substrate for mannitol production by MDH, presumably due the high K_m of the introduced MDH for fructose (44-71 mM) (1, 17), and the alternative fructose metabolization pathways.

OVERPRODUCTION OF MANNITOL 1-PHOSPHATE DEHYDROGENASE IN *L. LACTIS*

Mannitol biosynthesis in homofermentative lactic aid bacteria such as *L. lactis* starts with the glycolysis intermediate fructose 6-phosphate. Mannitol 1-phosphate dehydrogenase (EC 1.1.1.17) (M1PDH) catalyses the conversion of fructose 6-phosphate into mannitol 1-phosphate (6, 14). Mannitol 1-phosphate is dephosphorylated to mannitol by a (mannitol) phosphatase. Although the presence of a *mtlD* gene encoding M1PDH is reported for *L. lactis* IL1403 (4), mannitol production by *L. lactis* and other homofermentative lactic acid bacteria is not very likely. Presumably, the annotated *mtlD* gene which is located in a mannitol operon, is not transcribed due to catabolite repression as described for *Bacillus stearothermophilus* (18, 19) and no mannitol production can take place during growth on favorable sugars such as glucose. Therefore, production of M1PDH might be an effective strategy to realize mannitol synthesis in *L. lactis*. The involvement of fructose 6-phosphate as substrate of M1PDH could imply that the accumulation of fructose 6-phosphate, such as reported in *L. lactis* with reduced phosphofructokinase (PFK) activity (3), could coincide with mannitol production. Also alternative NAD regeneration via M1PDH, as reported in an LDH-deficient *L. lactis* (15, 34), could contribute to mannitol production.

To investigate the effect of an increased M1PDH activity on the mannitol production in *L. lactis*, the *Lactobacillus plantarum mtlD* gene was overexpressed in different genetic backgrounds: the parental *L. lactis* strain (NZ9000), a LDH-deficient strain (NZ9010), and a strain with reduced PFK activity (HWA217) (**Chapter 3**). Overexpression of *mtlD* in the three isogenic backgrounds resulted in production of small amounts of extracellular mannitol by growing cells. HPLC analysis showed that not more than 1% of the initial glucose was converted into mannitol by the growing cultures. However, resting cells of the LDH-deficient strain expressing *mtlD*, converted approximately 25% of the glucose into mannitol, indicating that a LDH-deficient background is favoring mannitol production in *L. lactis*. Moreover, it was shown that the mannitol was detected extracellularly in this strain, and no mannitol was re-utilized. In contrast to previous findings for a LDH-deficient *L. lactis* strain (34), it was found that growth on mannitol only started after a lag phase of approximately 24 h, and not immediately after inoculation. Possibly, different growth conditions or repeated subculturing have induced the mannitol utilization genes in the LDH-deficient strain of Neves et al. (34, 35), unlike the more stable LDH-deficient strain NZ9010 used in our work.

KINETIC MODELING OF MANNITOL PRODUCTION IN *L. LACTIS*

The choice of engineering strategies or fermentation conditions for product formation by *L. lactis* have been based mostly on intuition and experience, even for the presented *mtlD* overexpression in the different genetic backgrounds (**Chapter 3**). The development and use of a detailed kinetic model, as reported by Hoefnagel et al. (20, 21), would make the choice for a strategy for an improved mannitol production more rational. Such a model could be very helpful in determining the choice of engineering steps, and the level of mannitol production that can be expected. To develop an engineering strategy for improved mannitol production by *L. lactis*, the detailed kinetic model of the *L. lactis* glycolysis ((21), and available at http://jjj.biochem.sun.ac.za) was extended with a mannitol production pathway, represented by the enzymes M1PDH and mannitol 1-phosphatase (M1Pase) (**Chapter 4**). In this model, enzyme data of two constructed strains, overexpressing M1PDH in an LDH-deficient background and a background with reduced PFK activity, were included in the model. Metabolic control analysis (MCA) was performed to determine the control points on the mannitol pathway in these *L. lactis* strains, and the results of the analysis predict that M1Pase, the second step in the homofermentative mannitol biosynthesis route, has a high control of approximately 0.9 on the mannitol flux in *L. lactis*. Moreover, flux analysis on the model predicted that an LDH-deficient genetic background is favorable for mannitol production in *L. lactis*, and that glucose-mannitol conversions of 42% can be reached in a LDH-deficient strain with high M1PDH activity, by increasing the M1Pase activity 100-fold.

OVERPRODUCTION OF MANNITOL 1-PHOSPHATASE IN *L. LACTIS*

The presented kinetic model (**Chapter 4**) predicted a strong control of M1Pase on the mannitol production in *L. lactis*, and indicated that high mannitol yields could be expected if this activity is increased, in combination with high M1PDH activity. Noting the predictions of the mannitol model and previous results (**Chapter 3**), the cloning and (over)expression of a M1Pase gene in *L. lactis* would be a logical step. To increase M1PDH and M1Pase enzyme activity in *L. lactis*, both the *Lactobacillus plantarum mtlD* gene and the gene encoding M1Pase of *Eimeria tenella* (31) were cloned in the lactococcal nisin-controlled expression (NICE) system, and overexpressed by nisin induction in a parental strain, a strain with reduced PFK activity, and a

LDH-deficient *L. lactis* strain, resulting in strains NZ9000 (pWW003), HWA217 (pWW003), and NZ9010 (pWW003) (**Chapter 5**). Increasing the amount of nisin used for induction resulted in increased M1PDH and M1Pase enzyme activities, and concomitant mannitol production. Maximum induction of both enzymes activities in growing cells resulted in glucose-mannitol conversions of 11%, 21% and 27% by strains NZ9000 ,HWA217, and NZ9010, all harboring pWW003, respectively (Table 2).

Altered induction conditions and initial substrate concentrations in the LDH-deficient *L. lactis* strain resulted in an even higher glucose-mannitol conversion of 50%, close to the theoretical mannitol yield of 67%. Moreover, a clear correlation between the M1Pase activity and the mannitol production was shown for *L. lactis* strain NZ9000 (pWW002) in which only the M1Pase gene expression was induced by the addition of nisin, and the M1PDH activity was kept at a constant level by pre-culturing on mannitol as carbon source. The nisin-controlled increase of the M1Pase activity resulted in increased conversion of glucose into mannitol, indicating a clear correlation between the M1Pase activity and the mannitol production.

The effect of the metabolic engineering steps on the mannitol production was also supported by the predictions of the kinetic *L. lactis* mannitol production model (**Chapter 4**). Figure 1 shows that our experimental data match the predicted mannitol flux distributions quite well. As the model expected, the highest mannitol production was observed for the LDH-deficient strain. Moreover, the effect of reduced PFK activity on the mannitol production in strain HWA217 confirmed the model predictions. Both the model predictions and experimental data of strain HWA217 (pWW003) showed an increased mannitol production (18 and 20% respectively), in comparison with strain NZ9000 (pWW003) (13 and 11%, respectively). A critical comment on the model may be added for the production of high amounts of ethanol by the LDH-deficient strain NZ9010 (pWW003). The experimental data show that ethanol is the main product, whereas the model predicts 2,3-butanediol to be the main product. This discrepancy may have its origin in the assumption in the model, that when a genetic modification step is simulated, enzyme levels different from the target enzyme do not change. Possibly, levels of enzymes involved in the ethanol formation, such as alcohol dehydrogenase and pyruvate formate lyase, have increased in the LDH-deficient strain. To overcome underestimation of the effect of changes of enzyme levels, activities of more than only the target enzyme(s) should be determined and included in the model.

TABLE 2. Mannitol production by *L. lactis*. Intracellular (I) or extracellular (e) mannitol production by growing or nongrowing cells of different *L .lactis* strains. Percentages glucose converted into mannitol (%) and final concentrations (mM) are indicated.

L. lactis strain	characteristics	Mannitol		note	reference
		(%)	(mM)		
FI7851	LDH-deficient	-	88 (i)	transient mannitol production by nongrowing cells	Neves et al. (34, 35)
FI9630	Δ*ldh*	1.3	0.7 (e)	transient mannitol production by growing cells	Gaspar et al. (15)
FI10089	Δ*ldh* Δ*mtlF*	33	13 (e)	Continuous mannitol production by nongrowing cells	Gaspar et al. (15)
		3.9	2.3 (e)	Continuous mannitol production by growing cells	
FI10091	Δ*ldh* Δ*mtlA*	33	13 (e)	Continuous mannitol production by nongrowing cells	Gaspar et al. (15)
		3.9	2.3 (e)	Continuous mannitol production by growing cells	
NZ9000 (pNZmtlD)	M1PDH overproduction	0		No mannitol produced by growing cells	Wisselink et al. (53)
NZ9000 (pWW003)	M1PDH and M1Pase overproduction	11	2.8 (e)	Continuous mannitol production by growing cells	Wisselink et al. (55)
HWA217 (pNZmtlD)	reduced PFK activity, M1PDH overproduction	<1	0.1 (e)	Continuous mannitol production by growing cells	Wisselink et al. (53)
HWA217 (pWW003)	reduced PFK activity, M1PDH and M1Pase overproduction	21	5.8 (e)	Continuous mannitol production by growing cells	Wisselink et al. (55)
NZ9010 (pNZmtlD)	LDH-deficient, M1PDH overproduction	1	0.3 (e)	Continuous mannitol production by growing cells	Wisselink et al. (53)
		25	5 (e)	Continuous mannitol production by nongrowing cells	
NZ9010 (pWW003)	LDH-deficient, M1PDH and M1Pase overproduction	27-50	7.3-50 (e)	Continuous mannitol production by growing cells	Wisselink et al. (55)
NZ9800 (pWW004)	sucrose growth, MDH overproduction	<1	0.3 (e)	Continuous mannitol production by growing cells	Wisselink et al. (54)
NZ9841 (pWW004)	sucrose growth, FK-deficient, MDH overproduction	<1	0.3 (e)	Continuous mannitol production by growing cells	Wisselink et al. (54)

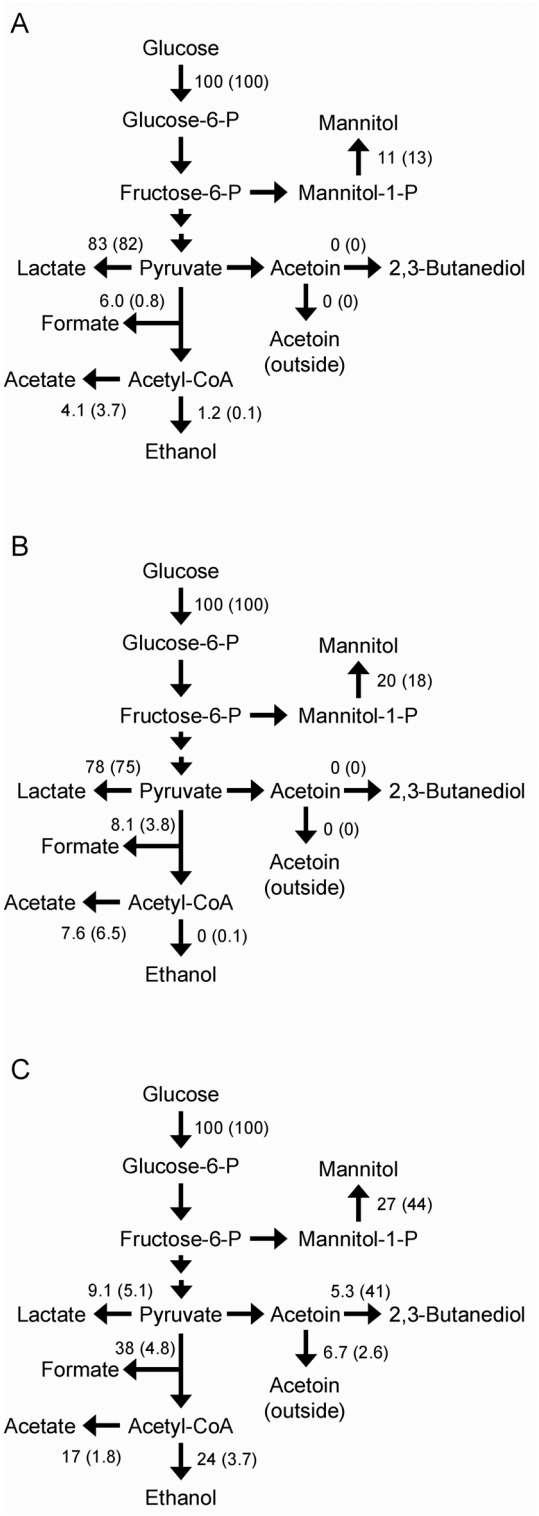

FIG. 1. Distribution of product formation by *L. lactis* NZ9000 (pWW003) (A), HWA217 (pWW003) (B), and NZ9010 (pWW003) (C). Experimental results are shown in plain numbers, whereas the model predictions are shown in parentheses. The experimental data represent the percentage of the initial glucose that is converted into a product. The model predictions are flux distributions in % during steady state conditions

CONCLUSIONS

Several strategies have been described here that aim to increase mannitol production in *L. lactis*. For determining these strategies, kinetic models of the *L. lactis* sugar metabolism have proven to be helpful tools. However, the model predictions did not always match the experimental data. Simulating high MDH activity in a kinetic *L. lactis* glycolysis model, high mannitol production was predicted for a FK-deficient strain fermenting sucrose. However, despite the prediction of high mannitol production by the model, the introduction of *mdh* in a FK-deficient *L. lactis* in the experimental set-up only resulted in low amounts of less than 1% mannitol from sucrose. Presumably, the fructose moiety of sucrose was metabolized in this strain via expulsion and subsequent transport by a PTS, implying that multiple engineering steps are needed to improve the mannitol production by such a strain. In addition, the usage of the alternative fructose pathway could not be predicted by the kinetic model, indicating the limitations of such a model.

For predicting the effect of increasing activities of M1PDH and M1Pase on the mannitol production in *L. lactis*, a kinetic model predicted that M1Pase has the highest control on the mannitol production, and that high glucose-mannitol conversions up to 44% could be expected when both enzyme activities are increased. Indeed, increase of both M1PDH and M1Pase activities resulted in the production of high amounts of mannitol. A glucose-mannitol conversion of 50% could be reached by growing cells of a LDH-deficient *L. lactis* strain (Chapter 5). This is significantly higher than the mannitol production presented by Gaspar et al. (15, 34), who showed mannitol yields of 4% and 30%, produced by growing and resting cells respectively, of a *L. lactis* strain deficient in LDH and the mannitol transport system (Table 2). Moreover, the mannitol productions observed for a wild-type strain, a strain with reduced PFK activity, and an LDH-deficient strain, all overproducing M1PDH and M1Pase, did answer to the expectations of the kinetic model.

Homologous overexpression of the *L. lactis mtlD* gene and a gene encoding mannitol 1-phosphatase activity by a food-grade expression system such as the NICE system (9) in combination with the *lacF* complementation system (37) could provide a food-grade mannitol-producing *L. lactis* strain. Such a strain would be a good candidate for the *in situ* production of mannitol as sweetener or antioxidant in food products. An advantage of such a *L. lactis* above a mannitol-producing heterofermentative lactic acid bacteria such as *Leuconostoc mesenteroides* (16, 44, 51), includes the capability of *L. lactis* to use a variety of cheap sugar substrates for mannitol

synthesis, whereas mannitol is exclusively formed from fructose by the heterofermentative lactic acid bacteria.

To further optimize mannitol production in *L. lactis*, additional engineering steps could be applied. Due to activation of the alternative *ldh*B gene in the LDH-deficient NZ9010 strain under anaerobic conditions (5), some lactate is still produced by the mannitol-producing strain NZ9010 (pWW003), although the lactate production decreased with increasing mannitol production in this strain. The affinity of the LDH encoded by the *ldhB* gene for the substrate NADH is lower than the affinity of the original LDH (K_m of 6 μM) (5), and also lower than the affinity of the overproduced M1PDH (K_m of 4 μM) (52). This indicates that M1PDH outcompetes the alternative LDH for NADH in strain NZ9010, and this might explain the decreased lactate production with increasing mannitol production. Nevertheless, noting the urge of NAD regeneration in *L. lactis*, the use of a robust LDH-deficient *L. lactis* strain is likely to contribute to higher mannitol conversions. Although no direct mannitol re-utilization by *L. lactis* upon glucose depletion was observed in our studies, inactivation of the mannitol-PTS as presented by Gaspar et al. (15) could be an additional step to optimize mannitol production in *L. lactis.* Moreover, use can be made of *L. lactis* strains deficient in NADH-consuming enzymes such as 2,3-butanediol dehydrogenase and alcohol dehydrogenase. Another alternative strategy could be the construction of a PFK-deficient strain, capable of fermenting lactose (10). In such a strain, the galactose moiety of lactose could be metabolized by the tagatose pathway, while the glucose moiety could be converted completely into mannitol, with a maximum of 50% lactose-mannitol conversion.

The work presented in this thesis has focused on the metabolic engineering of the production of a single end product, mannitol. Basically, the relatively simple approach of increasing the activities of two enzymes involved in the mannitol synthesis, M1PDH and M1Pase, resulted in significant mannitol production in *L. lactis*. More and more, metabolic engineering is focusing on global effects of modulating complex pathways involved in the secundary metabolism of an organism, such as the production pathways of folate, and other vitamins. In stead of analyzing the expression of single genes, enzyme activities, or metabolites, analyses of the transcriptome, proteome, and metabolome, should provide give a global insight in expected bottlenecks in the production of these compounds.

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SAMENVATTING

Productie van Mannitol in *Lactococcus lactis* met behulp van Metabolic Engineering

Wereldwijd worden melkzuurbacteriën toegepast in de industrie voor de productie van gefermenteerde voedselproducten. In deze productieprocessen worden melksuikers door de melkzuurbacteriën voornamelijk omgezet in melkzuur (lactaat). Naast de productie van lactaat, zijn melkzuurbacteriën ook in staat om uit de melksuikers andere (bij)producten te maken, zoals geur- en smaakstoffen, organische zuren, alcoholische verbindingen, en suiker-alcoholen. Een voorbeeld van zo'n suiker-alcohol is mannitol. Mannitol is een verbinding die bescherming kan bieden aan verschillende soorten micro-organismen in stress-situaties zoals droogte en hoge zoutconcentraties. In de levensmiddelenindustrie wordt mannitol geproduceerd voor het gebruik als alternatieve zoetstof, en wordt het in de farmaceutische industrie gebruikt in tabletten en poeders om de onplezierige smaak van medicijnen te maskeren en om actieve componenten te beschermen. Mannitol wordt momenteel vooral met behulp van chemische processen geproduceerd, maar ook zijn er al processen beschreven waarin micro-organismen zoals melkzuurbacteriën gebruikt worden voor mannitol productie.

In de zuivelindustrie wordt de melkzuurbacterie *Lactococcus lactis* veelvuldig gebruikt om gefermenteerde zuivelproducten te produceren zoals kaas, karnemelk en kwark. Productie van mannitol door *L. lactis* zou kunnen leiden tot nieuwe gefermenteerde producten met een toegevoegde waarde, vanwege de bovengenoemde karakteristieken van mannitol. Het doel van het beschreven onderzoek is om een mannitol-producerende *L. lactis* te verkrijgen met behulp van metabolic engineering, d.w.z. het aanpassen van enzymactiviteiten in de mannitol synthese route, in het suikermetabolisme (glycolyse), en de pyruvaat distributieroute.

In **hoofdstuk 1** wordt een overzicht gegeven van de producteigenschappen van mannitol, er wordt beschreven door welke micro-organismen het geproduceerd wordt, met de nadruk op melkzuurbacteriën, en de genen/enzymen die betrokken zijn bij de productie van mannitol worden vermeld. Tevens worden er suggesties gedaan voor strategieën om via metabolic engineering de productie van mannitol door *L. lactis* te verhogen.

Hoofdstuk 2 beschrijft een metabolic engineeringstrategie waarin het mannitol dehydrogenase gen van de heterofermentatieve melkzuurbacterie *Leuconostoc mesenteroides* geïntroduceerd wordt en tot expressie wordt gebracht in *L. lactis*. Het enzym mannitol dehydrogenase kan in één enkele stap de suiker fructose omzetten in mannitol.

Verwacht werd dat de expressie van dit enzym in *L. lactis* tijdens groei op sucrose, een suiker bestaande uit een glucose en fructose, zou resulteren in mannitol productie. Het bleek echter dat het fructose-deel van sucrose niet in mannitol werd omgezet, maar via de glycolyse werd omgezet in lactaat.

Hoofdstuk 3 concentreert zich op de (over)productie van het enzym mannitol 1-fosfaat dehydrogenase (M1PDH) in *L. lactis*. Dit enzym katalyseert de reductie van fructose 6-fosfaat, een intermediair van de glycolyse, tot mannitol-1-fosfaat. Om mannitol te vormen moet de fosfaatgroep van mannitol-1-fosfaat verwijderd worden door een fosfatase enzym. Overexpressie van M1PDH in groeiende cellen van *L. lactis* resulteerde slechts in zeer lage mannitol productie. Echter, productie van M1PDH in niet-groeiende cellen van een *L. lactis* stam, waarin het enzym lactaat dehydrogenase uitgeschakeld was, resulteerde in een omzetting van glucose tot mannitol van 25%.

Uit hoofdstuk 3 bleek dat de expressie van alleen M1PDH in *L. lactis* niet resulteerde in hoge mannitol productie. Om voorspellingen te kunnen doen over welke stappen belangrijk zijn voor mannitol productie in *L. lactis*, is er een simulatie model gemaakt voor mannitol productie in *L. lactis*. Dit model omvat alle kinetische parameters voor de enzymen betrokken in de glycolyse en de mannitol productie route, en wordt beschreven in **hoofdstuk 4**. Het model voorspelde dat de mannitol productie in *L. lactis* in grote mate afhankelijk is van de tweede stap in de mannitol productie, het afsplitsen van de fosfaatgroep van mannitol-1-fosfaat door het enzym mannitol-1-fosfatase (M1Pase). Tevens wordt een hoge glucose-mannitol omzetting van bijna 50 % verwacht wanneer M1PDH en M1Pase tot hoge expressie worden gebracht in *L. lactis* stam waarin lactaat dehydrogenase uitgeschakeld is.

In **hoofdstuk 5** wordt de voorspelling dat M1Pase een belangrijke rol speelt in de mannitol-productie route in *L. lactis* experimenteel getoetst. Hiervoor werd het gen coderend voor M1Pase uit *Eimeria tenella* geïntroduceerd in *L. lactis*. Gelijktijdige productie van de enzymen M1PDH en M1Pase leverde inderdaad hoge mannitol productie op in groeiende *L. lactis* cellen. Met name de *L. lactis* stam waarin lactaat dehydrogenase uitgeschakeld was, bleek een goede mannitol-producent. Deze stam was in staat tot 50% van het substraat glucose om te zetten in mannitol.

Concluderend kan gesteld worden dat M1Pase de meeste controle heeft op de mannitol productie in *L. lactis*. De hoge mannitol productie die bereikt is door de overproductie van dit enzym in combinatie met M1PDH biedt wellicht goede perspectieven voor toepassing in gefermenteerde zuivelproducten, mits dit ook gerealiseerd kan worden door klonering van melkzuurbacterie-eigen genen (**hoofdstuk 6**).

NAWOORD

Dit proefschrift omvat veel meer dan alleen de wetenschappelijke beproeving die ik als promovendus zo'n vijf jaar ondergaan heb. Voor mijzelf, en voor iedereen die er dicht bij betrokken was, staat er tussen de regels door een verhaal van successen, tegenslagen, blijdschap en verdriet. Nu ik het boekje nog eens doorblader, op zoek naar de laatste foutjes, denk ik terug aan een nogal roerige periode als WCFS AiO bij het ATO (A&F). Na een wat moeizame start begonnen de eerste resultaten zichtbaar te worden, en kon er al wat voorzichtig nagedacht worden over de grove indeling van het uiteindelijke proefschrift. Vol met nieuwe plannen en ideeën, kwam er abrupt een voorlopig einde aan de opmars naar nieuwe resultaten. In de zomer van 2002 werd ik met een ziekte geconfronteerd, en het werd me duidelijk dat dit wel even kon duren. Tijdens mijn herstel heb ik goed kunnen nadenken of en hoe ik verder wilde gaan met mijn promotieonderzoek. Waar de grote wijsgeer J. Cruijff zei: "ieder voordeel hep z'n nadeel", gold dit voor mij in zekere zin dan ook andersom, ondanks de ernst van de situatie. In een tijd waarin van alles door je hoofd spookt, was het 'werken' aan mijn promotie een goede afleiding, en ik durf zelfs te stellen dat dit mijn herstel bespoedigd heeft.

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Wouter

CURRICULUM VITAE

Hendrik Wouter Wisselink werd geboren op 5 september 1972 in Doetinchem. Na de basisschool in zijn woonplaats Varsseveld, behaalde hij in 1990 zijn Atheneum diploma aan de Gemeentelijke Scholengemeenschap Doetinchem. Hierna begon hij aan de Hogere Laboratorium Opleiding, studierichting biotechnologie, aan de toenmalige Hogeschool Gelderland in Nijmegen, waarvan hij het diploma behaalde in 1994. De afstudeeropdracht werd uitgevoerd bij Diosynth b.v. te Oss. In datzelfde jaar besloot hij verder te gaan studeren in Nijmegen, en wel biologie aan de Katholieke Universiteit Nijmegen (huidige Radboud Universiteit). Tijdens de doctoraalfase van deze studie deed hij afstudeeronderzoeken bij de vakgroepen Microbiologie (Prof. G.D. Vogels; Dr. W.J.B. Wannet), en Aquatische Oecologie (Prof. G. v.d. Velde, Dr. J.H.P. Hackstein). Eind 1997 werd de Drs. bul behaald. In 1998 begon hij bij het toenmalige ATO (nu Agrotechnology & Food Innovations) als projectmedewerker (Dr. F.A. de Wolf), waarna hij in 1999 bij ATO begon aan zijn promotieonderzoek voor Wageningen Centre for Food Sciences. Dit promotieonderzoek werd uitgevoerd onder begeleiding van promotor Prof. dr. W.M. de Vos en co-promotor Dr. J. Hugenholtz en heeft geresulteerd in dit proefschrift.

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