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Engineering of Carbon Distribution between Glycolysis and Sugar Nucleotide Biosynthesis in *Lactococcus lactis*

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We describe the effects of modulating the activities of glucokinase, phosphofructokinase, and phosphoglucomutase on the branching point between sugar degradation and the biosynthesis of sugar nucleotides involved in the production of exopolysaccharide biosynthesis by *Lactococcus lactis*. This was realized by using a described isogenic *L. lactis* mutant with reduced enzyme activities or by controlled expression of the well-characterized genes for phosphoglucomutase or glucokinase from *Escherichia coli* or *Bacillus subtilis*, respectively. The role of decreased metabolic flux was studied in *L. lactis* strains with decreased phosphofructokinase activities. The concomitant reduction of the activities of phosphofructokinase and other enzymes encoded by the *las* operon (lactate dehydrogenase and pyruvate kinase) resulted in significant changes in the concentrations of sugar-phosphates. In contrast, a >25-fold overproduction of glucokinase resulted in 7-fold-increased fructose-6-phosphate levels and 2-fold-reduced glucose-1-phosphate and glucose-6-phosphate levels. However, these increased sugar-phosphate concentrations did not affect the levels of sugar nucleotides. Finally, an ~100-fold overproduction of phosphoglucomutase resulted in 5-fold-increased levels of both UDP-glucose and UDP-galactose. While the increased concentrations of sugar-phosphates or sugar nucleotides did not significantly affect the production of exopolysaccharides, they demonstrate the metabolic flexibility of *L. lactis*.

Exopolysaccharides (EPS) include a range of diverse polymers that play vital roles in a variety of biological processes. In addition, EPS have a variety of industrial applications, including their use as biothickeners in foods. Notably, EPS produced by lactic acid bacteria (LAB) contribute significantly to the structure and viscosity of fermented milk products. Furthermore, several reports indicate that they can confer health benefits on consumers arising from their immunogenic and cholesterol-lowering properties. Although the production of EPS by LAB in milk is relatively low (<1 mg liter^{-1}) in comparison with the milk sugar concentration (>40 g liter^{-1}), some EPS appear to be effective thickeners (19), especially since they interact with milk proteins (40).

To study EPS biosynthesis by LAB, we have focused on *Lactococcus lactis* NIZO B40, since it produces a phosphopolysaccharide with known structure (for a recent review, see reference 2). Moreover, the NIZO B40 EPS-producing capacity is encoded by a 42,180-bp plasmid, pNZ4000, that can be transferred to genetically well-studied model strains of *L. lactis* (38, 39). The EPS plasmid pNZ4000 contains 14 *eps* genes involved in the formation of a repeating unit by sequential addition of sugars to a membrane-anchored lipid carrier and in export and polymerization of these repeating units. Upon its transfer to model strains, the NIZO B40 phosphopolysaccharide, which contains glucose, galactose, and rhamnose moieties, is produced. Therefore, their production requires the sugar nucleotides UDP-glucose, UDP-galactose, and dTDP-rhamnose, which are formed by enzymes encoded by genes on the chromosome (Fig. 1). The central intermediate, glucose-1-phosphate (glucose-1P), is converted to dTDP-rhamnose by the sequential activities of the *rfbACBD* gene products (2). GalU activity catalyzes the conversion of glucose-1P into UDP-glucose, which is subsequently converted into UDP-galactose by GalE activity (3). All the genes that encode the enzymes involved in the biosynthesis of these sugar nucleotides from glucose-1P (*galU*, *galE*, and *rfbACBD* [Fig. 1]) have been cloned from *L. lactis* MG1363, and their roles in controlling sugar nucleotide levels have been investigated (2, 3).

Insight into the biosynthesis of EPS is crucial for the exploitation of microorganisms for the production of EPS of industrial or medical importance. For the design of metabolic-engineering strategies that aim at increased fluxes to EPS production, it is relevant to include control factors in sugar degradation and EPS formation pathways (13). In *L. lactis* used in dairy fermentation, sugar degradation starts with lactose uptake via a phosphotransferase system, which yields, after hydrolysis, galactose-6P and glucose moieties. Subsequently, the galactose-6P moiety can be catabolized completely via the tagatose pathway and glycolysis for the generation of biomass and energy, while the glucose moiety can be used for EPS production (13). The uncoupling of lactose-derived glucose and galactose metabolism has been established in an *L. lactis* mutant impaired in glucokinase (Glk; EC 2.7.1.2) and glucose phosphotransferase system activity that accumulated glucose (35). Assuming that the linkage between glycolysis and EPS formation occurs at the branching point starting from the glycolysis intermediate glucose-6P, it is tempting to speculate that it might be possible to engineer EPS overproduction by increasing the pool of this sugar-phosphate.

The availability of glucose-6P can be affected by the modulation of glycolytic activity (1). It has been reported that the lactococcal glycolytic flux can be affected by the activities of phosphofructokinase (Pfk) (1) and the global catabolite control protein CcpA (28). The latter protein acts as an activator

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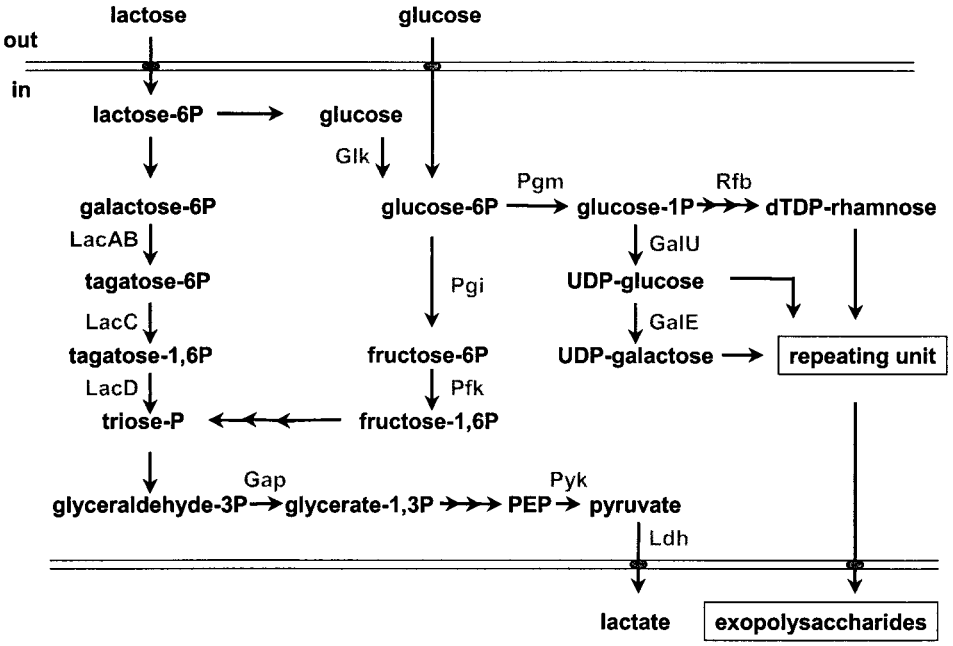

FIG. 1. Schematic representation of pathways involved in sugar fermentation via glycolysis to lactate and/or other acids and biosynthesis of EPS in *L. lactis*. Glucose and lactose are transported via phosphotranseferase systems. The following enzymes are involved: Glk, Pgi, Pfk, Gap (glyceraldehyde 3-phosphate dehydrogenase), LacABCD (tagatose-6-phosphate pathway), Pyk (pyruvate kinase), Ldh (lactate dehydrogenase), Pgm, GalU (UDP-glucose pyrophosphorylase), GalE (UDP-galactose epimerase), Rfb (dTDP-rhamnose biosynthetic system consisting of RfbA and glucose-1P thymidylyltransferase), RfbB (dTDP-glucose-4,6 dehydratase), RfbC (dTDP-4-keto-6-deoxy-*D*-glucose-3,5 epimerase), and RfbD (dTDP-4-keto-*L*-rhamnose reductase).

of transcription of the *las* operon (24) encoding the glycolytic enzymes Pfk and pyruvate kinase and the lactate-forming enzyme lactate dehydrogenase. Furthermore, modulation of glucose-6P could potentially be achieved by engineering the enzyme activities that are involved in the formation of this intermediate, such as Glk (Fig. 1).

Another possible key step in sugar nucleotide biosynthesis is the interconversion of the glycolysis intermediate glucose-6P and glucose-1P, which can be regarded as the central precursor in sugar nucleotide biosynthesis (34), performed by phosphoglucomutase (Pgm; E.C. 2.7.5.1) (Fig. 1). *L. lactis* contains two distinct forms of Pgm, one specific for β-glucose-6P (i.e., β-Pgm) and the other specific for α-glucose-6P (i.e., α-Pgm) (30). Since the phosphotransferase sugar uptake or the Glk-mediated phosphorylation of glucose yields α-glucose-6P (32), α-Pgm could be a key enzyme in sugar nucleotide biosynthesis. However, only the gene encoding β-Pgm has been identified in *L. lactis* (31).

We studied the effects of the modulation of enzyme activities at the branching point between sugar degradation and sugar nucleotide and/or EPS biosynthesis. By influencing the expression of the corresponding genes, we evaluated the roles of CcpA (28) and controlled overproduction of heterologous Pgm and Glk in the biosynthesis of NIZO B40 EPS in the plasmid-free model strain *L. lactis* MG1363. It could be established that the overproduction of *Bacillus subtilis* Glk and *Escherichia coli* Pgm significantly increased the levels of sugar-phosphates and sugar nucleotides, respectively. However, this did not affect the NIZO B40 EPS production level. Furthermore, although the *ccpA* mutant strain did not show any changes in the levels of the sugar nucleotides, the mutant produced significantly less EPS than the wild-type strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The lactococcal strains and plasmids used in this study are listed in Table 1. *E. coli* MC1061 (6), which was used as a host in cloning experiments, was grown with aeration in tryptone-yeast extract broth at 37°C. *L. lactis* was grown without aeration at 30°C in a chemically defined medium (CDM) (25) or in M17 broth (Merck, Darmstadt, Germany) supplemented with 0.5% (wt vol^{-1}) glucose or lactose. When appropriate, the media contained chloramphenicol (10 μg ml^{-1}), erythromycin (10 μg ml^{-1}), tetracycline (2 μg ml^{-1}), or ampicillin (100 μg ml^{-1}). To analyze the effect of gene overexpression, the nisin-controlled expression (NICE) system was used (10, 20). For enzyme activity analysis, *L. lactis* cells were grown to an optical density at 600 nm (OD_{600}) of ~0.5, and for EPS analysis the cells were grown to an OD_{600} of ~0.1. Subsequently, the culture was split into two cultures. Nisin (1 ng ml^{-1}) was added to one of the cultures, and both cultures were grown for an additional 2 to 24 h.

DNA manipulations and DNA sequence analysis. Small-scale isolation of *E. coli* plasmid and chromosomal DNAs and standard recombinant-DNA techniques were performed as described by Sambrook et al. (33). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with JetStar columns (Genomed GmbH, Bad Oberhausen, Germany) by following the instructions of the manufacturer. Isolation and transformation of *L. lactis* DNA were performed as previously described (12). Isolation of chromosomal DNA of *B. subtilis* was performed as described previously (5).

Automatic double-stranded DNA sequence analysis was performed on both strains with an ALF*red* DNA sequencer (Pharmacia Biotech, Roosendaal, The Netherlands). Sequence reactions were performed with an Autoread kit, were initiated by using Cy5-labeled universal and reverse primers, and were continued with synthetic primers purchased from Pharmacia Biotech in combination with fluorescein-15-dATP by following the instructions of the manufacturer. Sequence data were assembled and analyzed by using the PC/GENE program version 6.70 (Intelli-Genetics).

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
L. lactis		
MG1363		16
MG5267		41
NZ3800	MG5267 *pepN*::*nisRK*	This work
NZ9870	MG1363 *ccpA*::*ery*	28
NZ9000	MG1363 *pepN*::*nisRK*	20
E. coli MC1061		6
B. subtilis ATCC 6633		ATCC[b]
Plasmids		
pNZ4123	Cm^r; pNZ8048 derivative containing a functional *E. coli pgmU* gene	This work
pNZ4124	Cm^r; pNZ8048 derivative containing a functional *B. subtilis glk* gene	This work
pNZ4030	Ery^r Eps^+	38
pNZ4130	Tet^r Eps^+	3
pNZ9573	Ery^r; nonreplicative lactococcal plasmid; *pepN*::*nisRK*	10
pNZ8048	Cm^r; inducible expression vector carrying the *nisA* promoter	20

[a] EPS^+, EPS-producing phenotype; Cm^r, chloramphenicol resistant; Ery^r, erythromycin resistant; Tet^r, tetracycline resistant.
[b] ATCC, American Type Culture Collection.

Construction of strains and plasmids. To make the *L. lactis* MG1363 derivative MG5267, a suitable host for the use of the NICE system, the lactococcal *nisRK* genes were introduced into the *pepN* locus as described by De Ruyter et al. (11). The resulting strain, designated NZ3800, contains the *nisRK* genes under the control of their own promoter integrated in the *pepN* locus. The expected genetic configuration of the *pepN*::*nisRK* locus was confirmed by PCR analysis.

The EPS-producing capacity was introduced into the *L. lactis* CcpA mutant strain NZ9870 (28) by transformation of the plasmid pNZ4130 (3), a tetracycline-resistant derivative of pNZ4000.

The *E. coli* α-Pgm gene (*pgmU*) was amplified by PCR using *Tth* polymerase and chromosomal DNA of *E. coli* MC1061 (6) as template DNA with the primers 5′-CATGCCATGGCAATCCACAATCGTGCAG-3′ and 5′-CTAGTCTAGATTACGCGTTTTTCAGAACTTCGC-3′. The 1.64-kb PCR product generated was cloned into pNZ8048 (20) using the *Nco*I and *Xba*I restriction sites that were introduced by the primers used (underlined), yielding the *pgm* overexpression plasmid pNZ4123.

The *B. subtilis* Glk gene (*glk*) was amplified by PCR using *Tth* polymerase and chromosomal DNA of *B. subtilis* ATCC 6633 as template DNA with the primers 5′-CATGCCATGGACGAGATATGGTTTGCG-3′ and 5′-CTAGTCTAGATTAACAATTTTGATGTTTCA-3′. The 0.98-kb PCR product generated was cloned into pNZ8048 (20) using the *Nco*I and *Xba*I restriction sites that were introduced by the primers used (underlined), yielding the *glk* overexpression plasmid pNZ4124. The plasmid pNZ4123 or pNZ4124 was introduced into *L. lactis* strain NZ3800 by transformation. Subsequently, the EPS-producing capacity was introduced into *L. lactis* NZ3800 harboring pNZ4123 or pNZ4124 by electroporation of plasmid pNZ4030 (38).

Preparation of CEs and protein analysis. Lactococcal cells (50 ml) were harvested by centrifugation (3,500 × *g*; 10 min; 4°C), and the cell pellets were resuspended in 1 ml of 20 mM sodium-phosphate buffer (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. These suspensions were mechanically disrupted by bead beating in the presence of zirconium beads (37), and cell debris was removed by centrifugation (3,500 × *g*; 10 min; 4°C), resulting in the cell extract (CE). The protein content of the CE was determined by the method of Bradford (4) using bovine serum albumin as the standard.

For protein analysis, lactococcal CE was mixed with an equal amount of twofold-concentrated Laemmli buffer and, after the mixture was boiled, 10 μg of each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21).

Enzyme assays. Enzyme reactions were performed at 30°C in a total volume of 1 ml containing various amounts of freshly prepared CE. The formation of NADH or NADPH was determined by measuring the change in absorbency at 340 nm. The values given are the means of at least two independent measurements. The control contained the reaction buffer, cofactors, and the substrate but lacked the CE.

The Glk reaction assay mixture contained 100 mM triethanolamine hydrochloride buffer (pH 7.8), 5 mM $MgCl_2$, 1 mM $NADP^+$, 4 U of glucose-6P dehydrogenase, 2 mM ATP, and CE. The reaction was initiated by the addition of 10 mM glucose (29).

The α-Pgm reaction assay mixture contained 50 mM triethanolamine buffer (pH 7.2), 5 mM $MgCl_2$, 0.4 mM $NADP^+$, 50 μM glucose-1,6P, 4 U of glucose-6P dehydrogenase, and CE. The reaction was initiated by the addition of 1.4 mM α-glucose-1P (30).

Estimation of intracellular metabolites. The intracellular metabolites glucose-6P, glucose-1P, and fructose-6P were fixed by rapid inactivation of cell metabolism. Therefore, 4 ml of cell culture in the logarithmic growth phase was mixed with an equal amount of cold (−80°C) methanol. The methanol was removed by evaporation in a heating block, and the cell metabolites were measured by coupling appropriate enzyme assays with fluorimetric determination of NADPH as described by Garrigues et al. (15).

Sugar nucleotide and EPS analyses. Sugar nucleotides were separated from CEs, and individual sugar nucleotide contents were determined by high-performance liquid chromatography as previously described by Looijesteijn et al. (26). The values reported are the averages of at least two independent determinations. EPS was isolated, quantified, and characterized as described by Looijesteijn and Hugenholtz (25).

RESULTS

Glycolytic-intermediate modulation. By modulation of the pools of glycolytic intermediates, we investigated the possibilities for increasing the pool of glucose-6P, the levels of sugar nucleotides, and subsequently the level of EPS production. We evaluated the effect of reduced glycolytic flux on EPS biosynthesis by analyzing NIZO B40 EPS biosynthesis in the *L. lactis ccpA* disruption strain NZ9870. In this strain, the transcription levels of the *las* operon genes (*pfk*, *pyk*, and *ldh*) are reduced fourfold compared to those in the parental strain (28). Moreover, the activities of other enzymes may be affected as well, since CcpA is a global control protein. The *ccpA* disruption strain NZ9870 showed a reduced growth rate, reduced glycolytic flux, and a switch from homolactic to mixed-acid fermentation (28). In this strain, the levels of the first two glycolytic intermediates, glucose-6P and fructose-6P, appeared to be two- and sevenfold increased relative to the levels measured in the wild-type strain (Table 2). However, neither the level of glucose-1P (Table 2) nor the levels of the sugar nucleotides UDP-glucose, UDP-galactose, and dTDP-rhamnose (data not

TABLE 2. Enzyme activities and sugar nucleotide and sugar-phosphate concentrations in *Lactococcus lactis* subsp. *cremoris* MG1363 derivatives grown in M17 medium with Glc or Lac as sole carbon source

Strain (plasmid)	Sugar source	Nisin (ng ml^{-1})	Relevant enzyme activity (mU of protein^{-1}[a])	Sugar-phosphates (μM mg of protein^{-1})		
				Glucose-6P	Fructose-6P	Glucose-1P
NZ3800	Glc	0	ND	23	9.7	39
NZ3800	Lac	0	ND	31	44	37
NZ9870	Glc	0	ND	47	68	46
NZ3800(pNZ4123)	Lac	0	222 ± 42[b]	30	25	35
	Lac	1	24,767 ± 506[b]	30	33	33
NZ3800(pNZ4124)	Lac	0	352 ± 51[c]	5.2	74	10
	Lac	1	9,482 ± 93[c]	13	290	10

[a] Values (± standard deviations) are based on at least two independent experiments. ND, not determined.
[b] Pgm-specific activity.
[c] Glk-specific activity.

shown) were influenced by the changed level of glucose-6P. Consequently, no effect of the *ccpA* mutation on the EPS production level was expected. However, EPS production in this strain appeared to be reduced twofold relative to that of the wild-type strain (Table 3), although this could suggest that CcpA exerts control on EPS production at the level of the EPS machinery itself. To evaluate the effect of stimulated phosphorylated-sugar intermediates in glycolysis on EPS biosynthesis in *L. lactis*, we studied the effect of controlled *glk* overexpression, using the NICE system (10, 20). Since the sequence of the *L. lactis glk* gene was not available, we used a heterologous approach as a proof of principle. Therefore, we cloned the *B. subtilis* ATCC 6633 *glk* gene under the control of the lactococcal *nisA* promoter, and the resulting plasmid (pNZ4124) was introduced into strain NZ3800. Strain NZ3800 harboring pNZ4124 was grown under inducing and noninducing conditions, and CEs of the cultures were prepared and analyzed by SDS-PAGE (Fig. 2). Growth in the presence of nisin resulted in the appearance of an additional protein band with an apparent molecular mass of ~34-kDa, which is the expected size of Glk. Moreover, >25-fold-increased Glk specific activity was obtained with CEs of the induced cultures compared to that in the control cultures (Table 2). Functional overexpression of *glk* resulted in an almost sevenfold-increased fructose-6P level compared to that of the wild-type strain (Table 2). Remarkably, both the level of glucose-6P and the level of glucose-1P were reduced, suggesting that substrate activation may take the place of phosphoglucoisomerase (Pgi) activity. Pgm apparently maintains a constant ratio between glucose-6P and glucose-1P which is independent of the glucose-6P concentration. The increased Glk activity did not affect the sugar nucleotide levels (data not shown) or the NIZO B40 EPS production level (Table 3), suggesting that Glk does not play an important role in the control of sugar nucleotide and EPS biosynthesis in *L. lactis*.

Branching-point modulation. To evaluate the effect of increased Pgm activity on UDP-sugar levels and EPS production in *L. lactis*, we studied the effect of controlled *pgm* overexpression by using the NICE system (10, 20). Similar to our study of *glk* overexpression, we used a heterologous approach and cloned the *E. coli* K-12 *pgmU* gene under the control of the lactococcal *nisA* promoter (pNZ4123). Strain NZ3800 harboring pNZ4123 was grown under inducing and noninducing conditions, and CEs of the cultures were analyzed by SDS-PAGE (Fig. 2). Nisin induction resulted in the appearance of a protein band with an apparent molecular mass of ~58-kDa, corresponding to the expected size of PgmU. An ~100-fold-increased Pgm specific activity was determined in the CEs of the induced cultures (Table 2). These results demonstrate the

TABLE 3. EPS production by *L. lactis* subsp. *cremoris* NZ9000 derivatives harboring pNZ4030 grown in CDM with Glc or Lac as sole carbon source

Strain (plasmid)	Nisin (ng ml^{-1})	EPS[a] (mg liter^{-1} · OD_{600}^{-1})	
		Glc	Lac
MG1363(pNZ4030)	0	36 ± 1	ND
NZ9870(pNZ4130)	0	16 ± 2	ND
NZ3800(pNZ4030, pN8048)	0	46 ± 1	39 ± 1
NZ3800(pNZ4030, pNZ4123)	0	42 ± 1	38 ± 1
	1	49 ± 1	39 ± 1
NZ3800(pNZ4030, pNZ4124)	0	46 ± 1	37 ± 3
	1	50 ± 1	39 ± 1

[a] Values (± standard deviations) are averages based on at least two independent experiments. ND, not determined.

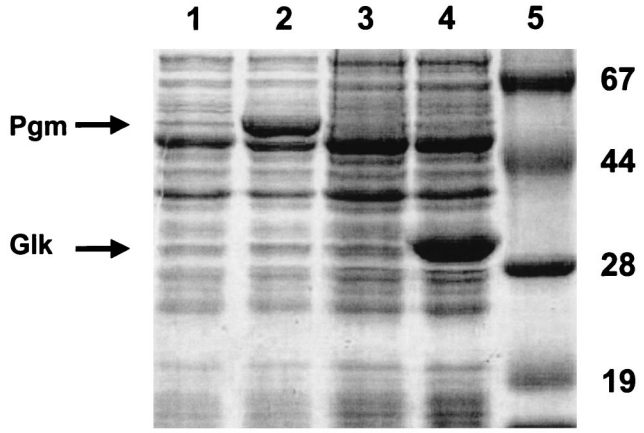

FIG. 2. Coomassie blue-stained gel after SDS-PAGE of CE of *L. lactis* NZ3800 harboring pNZ4123 (lanes 1 and 2) or pNZ4124 (lanes 3 and 4) grown in the absence (lanes 1 and 3) and in the presence (lanes 2 and 4) of nisin. Lane 5 contained a set of protein standards, whose molecular masses (in kilodaltons) are indicated on the right. Additional bands resulting from nisin induction and representing the Pgm protein (lane 2) and Glk protein (lane 4) are indicated.

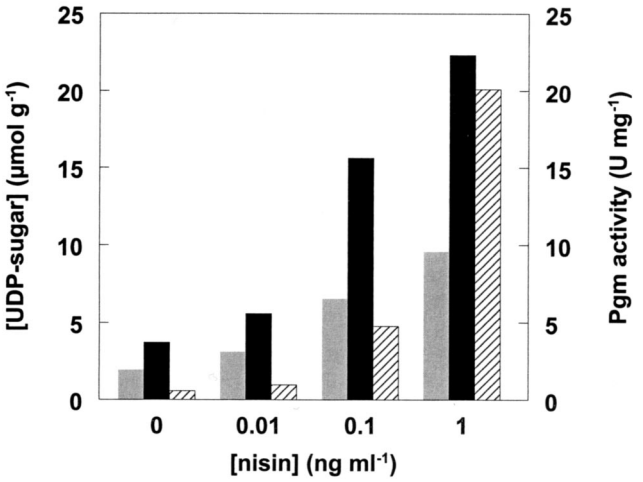

FIG. 3. UDP-glucose (solid bars) and UDP-galactose (shaded bars) levels and Pgm activity (hatched bar) of *L. lactis* NZ9000 harboring pNZ4123 and grown in the presence of different levels of nisin.

functional overexpression of the *pgmU* gene. Despite this high-level Pgm production, no differences in growth rates between nisin-induced NZ3800 harboring pNZ4123 and the uninduced strain were observed (data not shown).

To study the effect of *pgmU* overexpression on sugar nucleotide levels, the concentrations of UDP-glucose and UDP-galactose were determined in NZ3800 harboring pNZ4123 grown in the presence of nisin. Overexpression of *pgmU* resulted in a fivefold increase in the levels of both UDP-glucose and UDP-galactose (Fig. 3), while dTDP-rhamnose levels remained the same (data not shown). These results indicate that the activity level of Pgm to some extent affects UDP-glucose and UDP-galactose levels in *L. lactis*. In contrast, the increased level of PgmU activity did not have a significant effect on the level of EPS production when cells were grown in CDM with glucose or lactose as a sole carbon source (Table 3). These results indicate that the increased UDP-glucose and UDP-galactose levels in NZ3800 harboring pNZ4123 have no effect on EPS production efficiency in this strain.

DISCUSSION

The biosynthesis of EPS involves a large number of housekeeping enzymes required for the production of EPS precursors and EPS-specific enzymes that are encoded in *eps* gene clusters. In this study, we focused on the regulation of EPS production by three household enzymes, Glk, Pgm, and Pfk, all acting at the branching point between glycolysis and EPS precursor biosynthesis (Fig. 1), which is a potential bottleneck in sugar nucleotide and subsequent EPS biosyntheses. In this respect, increasing the intracellular glucose-6P pool, which is the central branching-point intermediate, might push metabolism toward sugar nucleotide biosynthesis and EPS production. It has been reported that glucose-6P could be toxic at enhanced levels (1, 14), although accumulation of lactose-phosphate was tolerated by *L. lactis* deficient in LacG activity (8). Therefore, it is feasible that concomitant enhancement of sugar anabolism, leading to the increased production of sugar nucleotides and subsequently EPS, might relieve glucose-6P toxic effects by decreasing the pool of glucose-6P in the cell and could lead to enhanced EPS production.

We evaluated the effect of reduced glycolytic flux on EPS biosynthesis by analyzing a *ccpA* mutant. Such mutants are known to have reduced glycolytic flux, and the *L. lactis ccpA* mutant has a fourfold-reduced transcription level of the *las* operon, containing the *pfk*, *pyk*, and *ldh* genes (28). However, it is also known that CcpA is a global regulator and has pleiotropic effects. The concentrations, as well as the ratio, of fructose-6P and glucose-6P were increased in the *ccpA* mutant strain. These results confirm reported data on a lactococcal strain with reduced Pfk activity (1). Andersen et al. (1) showed clearly that Pfk activity had a very high negative control of the level of upstream metabolites, and by decreasing the Pfk activity merely twofold, the sugar phosphates glucose-6P and fructose-6P increased two- to fourfold. In contrast, we determined a fivefold-higher glucose-6P/fructose-6P ratio for the wild-type strain. We suspect that this may be a consequence of the different stages of growth when the cells were harvested, since we collected the cells later in the exponential phase than Andersen et al. did (1). The results establish that overall reduction of the enzymatic activities encoded by the *las* operon leads to accumulation of intermediates high up in the glycolytic pathway. Although the *ccpA* mutation led to an increase of the pool of glucose-6P, the EPS production level was approximately twofold reduced compared to that in the wild type. Since no catabolite control elements were found in any of the sequences of genes involved in sugar nucleotide or EPS biosynthesis (data not shown), it seems unlikely that CcpA regulates the expression of any of these genes directly. The lack of success with respect to the increase of EPS flux may well be a consequence of the pleiotropic effects resulting from the *ccpA* mutation. Since the *ccpA* mutation caused a shift to mixed-acid fermentation (28), it is possible that the overall reduced metabolic rate in the *ccpA* mutant results in reduced energy availability, which could negatively influence EPS production. In another approach to increase EPS production, we aimed at glucose-6P accumulation as a result of increased Glk activity. Heterologous overexpression of *B. subtilis glk* in *L. lactis* resulted in a >25-fold increase in the enzyme activity level. Remarkably, the levels of glucose-6P and glucose-1P were decreased while the level of fructose-6P was increased almost sevenfold. These observations suggest that Pgi activity is subject to substrate glucose-6P activation. However, the increased Glk activity level did not result in more EPS precursors and NIZO B40 EPS. These results imply that the level of Glk enzyme activity does not control NIZO B40 EPS production levels.

It has been shown that mutations in the *pgm* gene affect the morphology of *E. coli* (27) and that they affected the polysaccharide composition of *Pseudomonas aeruginosa* (7, 42) and *Streptococcus pneumoniae* (17, 18). However, in *L. lactis*, α-Pgm is likely to be indispensable for sugar nucleotide formation and thus essential for the growth and viability of the species (32). To investigate the role of Pgm at the branching point between sugar catabolism and anabolism in *L. lactis*, we evaluated the effect of increased levels of Pgm activity. Heterologous overexpression of *E. coli pgmU* in *L. lactis* resulted in a >100-fold increase in the enzyme activity level and fivefold

increases in the UDP-glucose and UDP-galactose levels. These results show that the level of PgmU enzyme activity controls the level of production of UDP-glucose and UDP-galactose in wild-type cells. However, the sugar-phosphate analysis of the Glk overproduction strain suggests that the endogenous Pgm activity maintains a stable glucose-6P/glucose-1P ratio. These results suggest that Pgm overproduction will have no effect on sugar nucleotide synthesis. Therefore, it is likely that the increased sugar nucleotide levels are the result of favorable kinetic parameters of *E. coli* PgmU. However, increased Pgm activity did not affect NIZO B40 EPS production (Table 3). Analogously, GalU overproduction led to the same result (3). Similar findings were reported for *Sphingomomas*, in which a sixfold increase of Pgm activity did not affect EPS production (36). Contrasting results were reported for *Streptococcus thermophilus* strain LY03. Degeest and de Vuyst (9) showed a linear relationship between Pgm activity and EPS production, while Levander and Rådström (22) reported that overexpression of *pgm* in the same strain did not change EPS production. It is suggested that precursors for EPS biosynthesis in a galactose-fermenting strain of *S. thermophilus* originate from fermentation of the galactose moiety of lactose, thereby circumventing the need for a functional Pgm in these cells (22). Interestingly, when *pgm* was overexpressed in combination with *galU*, the EPS yield was shown to increase twofold in this strain (23).

Evaluation of the EPS biosynthesis model described here allowed us to assess the roles of Glk and Pfk in *L. lactis* by modulating their enzyme activity levels. Although early glycolytic intermediates like glucose-6P and fructose-6P accumulated in some cases, these modulations did not affect the sugar nucleotide levels and, eventually, the EPS production level. In contrast, modulation of Pgm activity resulted in significantly increased internal pools of both UDP-glucose and UDP-galactose. However, Pgm overproduction did not result in a significant increase of NIZO B40 EPS. Remarkably, a *ccpA* mutant strain produced only half the amount of EPS that wild-type cells did, which might be caused by the overall reduced metabolic rate in the *ccpA* mutant, resulting in reduced energy availability for EPS biosynthesis. The results presented here provide insight into enzymatic control factors that determine the carbon distribution between catabolic and anabolic reactions involved in conversion of the central glycolytic intermediate glucose-6P. Such insight is essential for the design of metabolic-engineering strategies that aim at resolving bottlenecks in EPS biosynthesis in *L. lactis*. Moreover, the possibility of stably overproducing significant amounts of phosphorylated glycolytic intermediates in *L. lactis* demonstrates the flexibility of its metabolism, which may be instrumental in further optimizing its metabolic activities in dairy and other food fermentations.

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