APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2001, p. 3033–3040 Vol. 67, No. 7
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.7.3033–3040.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Functional Analysis of the *Lactococcus lactis galU* and *galE* Genes and Their Impact on Sugar Nucleotide and Exopolysaccharide Biosynthesis

INGEBORG C. BOELS,[1,2] ANA RAMOS,[3] MICHIEL KLEEREBEZEM,[1,2]* AND WILLEM M. DE VOS[1]

Wageningen Centre for Food Sciences, Wageningen,[1] and NIZO Food Research, Ede,[2] The Netherlands, and Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa and Instituto de Biologia Experimental e Technólogica, Oeiras, Portugal[3]

Received 8 November 2000/Accepted 20 April 2001

We studied the UDP-glucose pyrophosphorylase (*galU*) and UDP-galactose epimerase (*galE*) genes of *Lactococcus lactis* MG1363 to investigate their involvement in biosynthesis of UDP-glucose and UDP-galactose, which are precursors of glucose- and galactose-containing exopolysaccharides (EPS) in *L. lactis*. The lactococcal *galU* gene was identified by a PCR approach using degenerate primers and was found by Northern blot analysis to be transcribed in a monocistronic RNA. The *L. lactis galU* gene could complement an *Escherichia coli galU* mutant, and overexpression of this gene in *L. lactis* under control of the inducible *nisA* promoter resulted in a 20-fold increase in GalU activity. Remarkably, this resulted in approximately eightfold increases in the levels of both UDP-glucose and UDP-galactose. This indicated that the endogenous GalE activity is not limiting and that the GalU activity level in wild-type cells controls the biosynthesis of intracellular UDP-glucose and UDP-galactose. The increased GalU activity did not significantly increase NIZO B40 EPS production. Disruption of the *galE* gene resulted in poor growth, undetectable intracellular levels of UDP-galactose, and elimination of EPS production in strain NIZO B40 when cells were grown in media with glucose as the sole carbon source. Addition of galactose restored wild-type growth in the *galE* disruption mutant, while the level of EPS production was approximately one-half the wild-type level.

Lactic acid bacteria are widely used for production of fermented foods, where they are responsible not only for production of lactic acid as a food preservative but also for generation of flavor and texture. Several lactic acid bacteria produce exopolysaccharides (EPS). These EPS contribute to the rheological properties and texture of fermented products and are therefore of interest for food applications as natural biothickeners (46). Moreover, it has been suggested that EPS may confer health benefits to the consumer, and mouse model studies have indicated that EPS may have immunostimulatory (22), antitumoral (23), or cholesterol-lowering activity (34).

Microbial polysaccharides can be present as constituents of cell walls, as parts of lipopolysaccharides (LPS) often referred to as O-antigens, or as capsular polysaccharides (CPS) associated with the cell surface, or they can be secreted as EPS in the environment of the cell. Detailed knowledge concerning microbial polysaccharide biosynthesis and the biophysical characteristics of these molecules has accumulated over the years (46). Different classes of EPS can be distinguished on basis of their biosynthesis mechanisms and the precursors required (45). They can be synthesized either extracellularly from exogenous substrates or intracellularly from sugar nucleotide precursors. Many EPS contain repeating units, the biosynthesis of which involves glycosyltransferases that sequentially link sugars from intracellular nucleotide sugars to a lipid carrier. This mechanism resembles the mechanism of production of O-antigens and several types of CPS (39). It is closely related to the mechanism of biosynthesis of cell envelope components like peptidoglycan (36) and teichoic acid (1), since in all of these mechanisms assembly takes place on a common lipid carrier that is situated in the cell membrane.

Genes involved in EPS biosynthesis are organized in gene clusters which appear to be highly conserved. The gene clusters that direct EPS biosynthesis in *Lactococcus lactis* NIZO B35, NIZO B40, and NIZO B891 (49, 50) and *Streptococcus thermophilus* Sfi6 (43) are comparable to the gene clusters in *Streptococcus pneumoniae* (33) and *Streptococcus agalactiae* (53) involved in CPS biosynthesis (51, 43). These gene clusters encode enzymes which are involved in formation of polysaccharides by sequential addition of sugars to a membrane-anchored repeating unit, followed by export and polymerization. One of the best-studied EPS-producing lactic acid bacterial strains is *L. lactis* NIZO B40 (for a recent review see reference 24), which produces a polymer with the regular repeating unit →4)[α-L-Rha*p*-(1→2)][α-D-Gal*p*-1-PO_4-3]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→ (34, 49). A variety of studies have shown that the backbone of the NIZO B40 repeating unit is assembled from two precursors, UDP-glucose and UDP-galactose, by the activity of specific glycosyltransferases encoded in an *eps* gene cluster that is encoded on a plasmid (49, 51). The subsequent steps in the synthesis of the repeating unit include coupling of the side chain sugars rhamnose and galactosylphosphate to the galactose of the backbone. Addition of the rhamnose involves a third precursor, dTDP-rhamnose, which is catalyzed by a putative rhamnosyltransferase, and addition of the galactosylphosphate is thought to be coupled by

* Corresponding author. Mailing address: NIZO Food Research, Department of Flavor and Natural Ingredients, P.O. Box 20, 6710 BA Ede, The Netherlands. Phone: 31-318-659511. Fax: 31-318-650400. E-mail: kleerebe@nizo.nl.

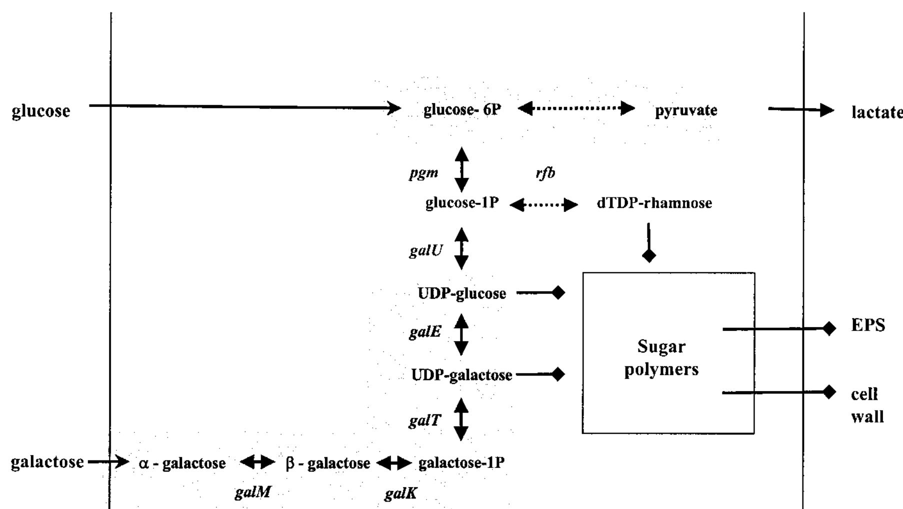

FIG. 1. Schematic representation of pathways involved in glucose fermentation via glycolysis (upper grey box), galactose fermentation via the Leloir pathway (lower grey box), and biosynthesis of EPS in *L. lactis*. Cell membranes are indicated by vertical lines. The following enzymes are involved (encoding genes are indicated): phosphoglucomutase (*pgm*); UDP-glucose pyrophosphorylase (*galU*); UDP-galactose epimerase (*galE*); galactose-1-phosphate uridyltransferease (*galT*); galactokinase (*galK*); galactose mutarotase (*galM*); and dTDP-rhamnose biosynthetic enzyme system (*rfb*). Multiple-step reactions are lumped together and are indicated by dotted arrows. The multiple reactions involved in synthesis of sugar polymers are indicated by diamonds.

a putative glycerophosphotransferase. Both transferases are potentially also encoded in the *eps* cluster (51).

In addition to specific Eps enzymes encoded in the *eps* gene cluster, EPS biosynthesis requires chromosomally encoded housekeeping enzymes involved in synthesis of the EPS building blocks, the sugar nucleotide precursors. Not only do these sugar nucleotides probably function as precursors for EPS biosynthesis, but they also are involved in biosynthesis of several cell wall components and are therefore considered essential for growth. The biosynthetic pathways starting from glucose or galactose for formation of the NIZO B40 EPS precursors in *L. lactis*, the sugar nucleotides UDP-glucose, UDP-galactose, and dTDP-rhamnose, are shown in Fig. 1 (47, 51). Glucose is fermented through the glycolysis step to pyruvate, which in turn is converted to lactate. The glycolytic intermediate glucose 6-phosphate is converted to glucose 1-phosphate by phosphoglucomutase activity, and this metabolite is subsequently converted to UDP-glucose by UDP-glucose pyrophosphorylase (GalU) activity. Galactose is degraded via the Leloir pathway, leading to the formation of UDP-glucose and UDP-galactose; this involves the gene products encoded by the *galAMKTE* operon (19). The *galE* gene encodes UDP-galactose epimerase (GalE), which interconverts these nucleotide sugars.

The potential for using EPS in food is determined by their physical and rheological properties. The factors that influence these properties are structural characteristics, including degree of polymerization, length of side chains, presence of substituents, type of linkages, and sugar composition. Engineering of polysaccharide biosynthesis at the level of the chemical structure of the repeating unit has been performed successfully and has resulted in changes in EPS (44) and also CPS (7). However, the levels of production of the altered polymers were reduced (7) or even extremely low (44). Metabolic engineering may be used as a tool to increase EPS production, which requires a detailed understanding of the physiology and genetics of EPS biosynthesis (14). Although several reports have described the effect of modulation of enzyme activity and its effect on polysaccharide biosynthesis, to our knowledge no study has described the effect on biosynthesis of the sugar nucleotides. For the first time, in this study we evaluated the role of the lactococcal GalU and GalE enzymes in biosynthesis of NIZO B40 EPS and the EPS precursors UDP-glucose and UDP-galactose by performing overexpression and disruption analyses of the corresponding genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The lactococcal strains and plasmids used in this study are listed in Table 1. *Escherichia coli* MC1061 (6), used as a host in cloning experiments, was grown with aeration in TY broth at 37°C. *L. lactis* was grown without aeration at 30°C in M17 broth (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose or in a chemically defined medium (CDM) (27). If appropriate, the media contained chloramphenicol (10 $\mu g\ ml^{-1}$), erythromycin (10 $\mu g\ ml^{-1}$), tetracycline (2 $\mu g\ ml^{-1}$), or ampicillin (100 $\mu g\ ml^{-1}$). To analyze the effect of gene overexpression, the nisin-controlled expression system was used (10, 25). Briefly, *L. lactis* cells were grown to an optical density at 600 nm of about 0.5 and then split into two cultures. One nanogram of nisin ml^{-1} was added to one culture, and both cultures were grown for an additional 2 h.

DNA techniques and DNA sequence analysis. Small-scale isolation of *E. coli* plasmid DNA was performed as described by Sambrook et al. (40). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with JetStar columns by following the instructions of the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). Isolation and transformation of *L. lactis* DNA were performed as previously described (12).

Southern blots were hybridized at 65°C with homologous DNA probes, which were labeled by nick translation using established procedures (40), and the blots were then washed with a solution containing 0.015 M NaCl and 0.0015 M sodium citrate at 65°C before exposure.

RNA was isolated from *L. lactis* cultures, and Northern analysis was performed as described by Luesink et al. (29). Blots were probed with an internal fragment of the *galU* gene. To amplify this fragment of the lactococcal *galU* gene, primers 5′-CATTGCCAAAGAAATGTTGCC-3′ and 5′-GTCAAGAGGTAAC

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
L. lactis strains		
MG1363		17
NZ8460	MG1363 *galE*::*Em*	20
NZ9000	MG1363 *pepN*::*nisRK*	25
E. coli CGSC4973		*E. coli* Genetic Stock Center[b]
Plasmids		
pCI182	Tet^r	21
pGEM-T	Ap^r	Promega
pUC18	Ap^r	54
pUC18Ery	Em^r, integration vector	49
pG$^+$host9	Em^r	30
pNZ4000	Eps$^+$	49
pNZ4030	Em^r Eps$^+$	49
pNZ4101	Ap^r, pUC18 derivative containing an MG1363 1.8-kb *Bam*HI-*Pst*I chromosomal DNA fragment carrying the *galU* gene	This study
pNZ4102	Cm^r, pNZ8048 derivative containing a functional lactococcal *galU* gene	This study
pNZ4103	Em^r, pG$^+$host9 derivative containing a 0.6-kb internal fragment of the *galU* gene	This study
pNZ4130	Tet^{r}Eps$^+$, pNZ4030 derivative carrying the *tetM* gene from Tn*919*	This study
pNZ8020	Cm^r, inducible expression vector carrying the *nisA* promoter	11
pNZ8048	Cm^r, inducible expression vector carrying the *nisA* promoter	25
pNZ8421	Cm^r, pNZ8020 derivative containing a functional lactococcal *galE* gene	20
pNZ8460	Em^r, pUC18 derivative containing a 0.6-kb internal fragment of the *galE* gene	20

[a] Eps$^+$, EPS-producing phenotype; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tet^r, tetracycline resistant.
[b] Strain CGSC4973 was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

GACCGAT-3′ were used in a PCR with *Taq* polymerase and with chromosomal DNA from *L. lactis* MG1363 (17) as the template.

Automatic double-stranded DNA sequence analysis was performed 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 Cy13-dATP by following the instructions of the manufacturer (Pharmacia Biotech). Sequence data were assembled and analyzed by using the PC/GENE program, version 6.70 (Intelli-Genetics).

Construction of strains and plasmids. To amplify an internal fragment of the lactococcal *galU* gene, degenerate primers 5′-ATHCCNGCNGCNGGNYTNG GNACNMGNTTYYTNCCNGCNACNAARGC-3′ and 5′-RTCCATNTRRTC RTCNCCNARCATNACNACRAANGG-3′ (where H is A, C, or T; N is A, C, G, or T; Y is C or T; M is A or C; and R is A or G) were used in a PCR performed with *Taq* polymerase and with chromosomal DNA from *L. lactis* MG1363 (17) as the template. The 0.29-kb PCR product generated was sequenced and used as a probe in Southern analysis; it was hybridized with a 1.8-kb *Bam*HI-*Pst*I fragment of the *L. lactis* MG1363 chromosomal DNA, which was cloned in similarly digested pUC18 (54), yielding pNZ4101.

To overexpress the *galU* gene, this gene was amplified by PCR performed with *Tth* polymerase, with pNZ4101 as the template DNA, and with primers 5′-AT GCCATGGCAAAACAAACTACTATACCTAACAAAG-3′ and 5′-GCGCTC TAGAGCATCAAAAAGAAAAAGCCAATAGGC-3′. The 1-kb PCR product was digested with *Nco*I and *Xba*I (sites underlined in the primer sequences) and inserted under control of the inducible *nisA* promoter into similarly digested pNZ8048; this resulted in pNZ4102, which was transformed into NZ9000 (25).

To inactivate the *galU* gene by single cross-over recombination, an internal *galU* fragment was obtained by PCR performed with *Taq* polymerase, with pNZ4101 as the template, and with primers 5′-CATTGCCAAAGAAATGTTG CC-3′ and 5′-TTTATCACCAACATCATAACG-3′. The 690-bp PCR product was cloned into pGEM-T (Promega Biotech, Roosendaal, The Netherlands). From the resulting plasmid the *galU* internal fragment was isolated as an *Apa*I-*Sal*I fragment and cloned in similarly digested pG$^+$host9 (30); this resulted in pNZ4103, which contained a temperature-sensitive replicon which is not functional at 37°C. pNZ4103 was transformed into strains MG1363 and NZ9000, and transformants were subsequently cultured at 37°C. Several erythromycin-resistant (Em^r) single-crossover transformants were selected and were analyzed by Southern analysis. Upon integration, the resulting strain would contain two disrupted copies, one of these copies lacking the 3′ end of *galU* that encodes the 44 C-terminal amino acids and the second copy lacking both the 5′ translational signals and the first 33 N-terminal amino acids.

galE disruption strain NZ8460 was constructed as described by Grossiord (20). Briefly, pNZ8460, a pUC18Ery variant containing the *Em* gene of pAMβ and a 600-bp internal PCR fragment of the *galE* gene fragment, was transformed into strain MG1363. Em^r colonies were obtained and were examined by Southern analysis. One of the colonies, designated NZ8460, was selected and contained a disrupted copy of the *galE* gene encoding a truncated protein lacking 36 C-terminal amino acids. Since NZ8460 is Em^r, the EPS-producing capacity could not be introduced into this strain by transformation with pNZ4030 (49) carrying the *eps* operon, which also contains an *Em* gene. Therefore, a tetracycline (*tetM*) derivative of pNZ4030 was constructed. To obtain convenient flanking restriction sites, the 4.2-kb *Hin*cII fragment of pCI182 (21), containing the *tetM* gene, was subcloned in pUC18 (54) digested with *Sma*I. The *tetM* gene was excised from the resulting plasmid by cutting with *Eco*RI, blunting with the Klenow fragment, and then digesting with *Sph*I. The insert was then ligated to pNZ8020 (11) that had been digested with *Xba*I, end filled with the Klenow fragment, and digested with *Sph*I. Finally, the *tetM* gene was isolated from the resulting plasmid as an *Xho*I-*Sph*I fragment and was cloned in similarly digested pNZ4000, yielding pNZ4130.

Preparation of cell extracts, protein analysis, and enzyme assays. Exponentially grown lactococcal cells (50 ml) were harvested by centrifugation and resuspended in 1 ml of 20 mM sodium phosphate (pH 6.5) containing 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The resulting suspension was mechanically disrupted in the presence of zirconium beads (48). Cell debris was removed by centrifugation. The cell extracts (CE) were each mixed with an equal volume of twofold-concentrated Laemmli buffer, and after boiling 15 μg of each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26). The protein content of the CE was determined by the method of Bradford (3) using bovine serum albumin as the standard.

Enzyme reactions were performed at 30°C in 1-ml mixtures. The UDP-glucose pyrophosphorylase (EC 2.7.7.9) reverse reaction assay was performed as described by Bernstein (2). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.8), 14 mM $MgCl_2$, 0.3 mM $NADP^+$, 0.1 mM UDP-glucose, 2.1 U of α-phosphoglucomutase (Sigma Aldrich Chemie GmbH, Steinheim, Germany), 4 U of glucose-6-phosphate dehydrogenase (Sigma Aldrich Chemie GmbH), and CE. The reaction was started by adding 4 mM inorganic pyrophosphate, and the increase in absorbance at 340 nm was determined. UDP-galactose 4-epimerase (EC 5.1.3.2) activity was assayed as described previously (28). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 0.5 mM NAD^+,

0.015 U of UDP-glucose dehydrogenase (Sigma Aldrich Chemie Gmbh), and CE. The reaction was started by adding 0.2 mM UDP-galactose, and the increase in absorbance at 340 nm was determined.

Intracellular sugar phosphate analysis by ^{31}P-NMR spectroscopy. *L. lactis* NZ8460 harboring pNZ4130 was grown on CDM supplemented with 1% (wt/vol) glucose in a fermentor (New Brunswick Bioflo 2C) at pH 6.5. Ethanol extracts were obtained from a mid-exponential-phase sample (corresponding to 30 mg of protein), and the intracellular sugar phosphate contents of these extracts were determined by ^{31}P nuclear magnetic resonance (^{31}P-NMR) analysis as previously described by Ramos et al. (38). The intracellular metabolite concentrations were calculated by using a value of 2.9 μl per mg of protein for the intracellular volume of *L. lactis* (37).

Sugar nucleotide and EPS analysis. Sugar nucleotides were separated from CE, and sugar nucleotide contents were determined by high-performance liquid chromatography (HPLC) analysis as previously described by Looijesteijn et al. (28). EPS were isolated, quantified, and characterized as described by Looijesteijn and Hugenholtz (27). Since the EPS isolation process cannot be strictly controlled, the variation in quantification for individual samples in the same experiment is 5 to 10%, and the variation for individual samples in experiments separated by time is 10 to 20%.

Nucleotide sequence accession numbers. The nucleotide sequences of the *galU* and *galE* genes have been deposited in the GenBank database under accession no. AF304368 and AJ011653, respectively.

RESULTS

Cloning and characterization of the *galU* gene. To identify the *L. lactis* MG1363 *galU* gene, an internal DNA fragment of this gene was obtained by PCR using degenerate primers based on conserved regions an alignment (PC/GENE package; Intelligenetics, Inc.) of amino acid sequences of the GalU analogues GtaB (accession no. L12272), HasC (U33452), and Cps3U (U15171) from the gram-positive bacteria *Bacillus subtilis, Streptococcus pyogenes*, and *Streptococcus pneumoniae*, respectively. Sequence analysis of the 0.29-kb PCR product generated revealed a continuous open reading frame (ORF) predicted to encode a protein exhibiting high sequence homology with GalU proteins. A 1.8-kb *Bam*HI-*Pst*I chromosomal DNA fragment of *L. lactis* MG1363 was found to hybridize with this PCR fragment and was subsequently cloned into pUC18, resulting in pNZ4101. Sequence analysis of the insert in pNZ4101 revealed the presence of a 5′ truncated ORF and a complete ORF. The truncated ORF putatively encodes a glycerol-3-phosphate dehydrogenase based on homology to the C-terminal part of *B. subtilis* GpdA (45% identity). Translation of the complete ORF resulted in a predicted 313-amino-acid protein with a calculated molecular mass of 35,002 Da; this protein is referred to here as *L. lactis* GalU, since it exhibited 73% amino acid identity with the GalU homologue of *S. pyogenes* (8). The first ATG of the *galU* ORF was preceded by a typical lactococcal Shine-Dalgarno sequence (5′-AAGGAG-3′) (13). A putative promoter region, containing possible −10 (5′-TAATAA-3′) and −35 (5′-CTGAA-3′) sequences, was found to precede the *galU* coding sequence. An inverted repeat sequence (5′-AAGAAAGAGCCTATTGGCTTTTTCTT-3′) and a stretch of six T residues downstream the *galU* coding sequence could function as a rho-independent transcriptional terminator.

To assess the transcriptional organization of the *galU* gene, RNA was isolated from strain MG1363 and used for Northern analysis. An internal fragment of the *galU* gene was generated by PCR, labeled, and used as a DNA probe. This probe hybridized with an approximately 0.9-kb transcript (data not shown). The size of the transcript suggests that the *galU* gene is transcribed as a single monocistronic mRNA from a putative promoter upstream of the *galU* gene and terminates at the putative terminator (see above).

To ascertain that the lactococcal *galU* gene codes for a UDP-glucose pyrophosphorylase, pNZ4101 was introduced into *E. coli galU* mutant CGSC4973, which lacks UDP-glucose pyrophosphorylase activity due to a single base pair substitution in the *galU* gene. While this strain has been shown to be able to grow on glucose, it was not able to grow on galactose (42). In contrast, the pNZ4101 transformants could grow on both glucose and galactose, indicating that the *galU* gene of *L. lactis* encodes a functional UDP-glucose pyrophosphorylase.

Modulation of GalU activity. To evaluate the role of reduced GalU activity in UDP-sugar formation and EPS biosynthesis in *L. lactis*, we repeatedly tried to inactivate *galU*. Using a strategy based on a temperature-sensitive replicon, we obtained several integrants, but none of these integrants showed the correct Southern hybridization pattern. These results indicate that integration did not occur in a site-specific manner and left the *galU* gene intact. Moreover, Southern blot analysis of chromosomal DNA obtained from the integrants, using integration vector-based probes, revealed that the integration plasmid had been incorporated into the chromosome in a random manner, which eliminated the possibility that a pseudo-*galU* locus or a highly homologous additional copy of the *galU* gene is present in *L. lactis*. Since *galU* appeared to be transcribed as a monocistronic mRNA (see above), polar effects of the intended *galU* disruption were not expected, suggesting that this gene has an essential role in *L. lactis*.

To evaluate the role of increased GalU activity in UDP-sugar formation and EPS biosynthesis in *L. lactis*, we studied the effect of controlled *galU* overexpression by using the nisin-controlled expression system (10, 25). Thus, pNZ8048 derivative pNZ4102 carrying the lactococcal *galU* gene under control of the lactococcal *nisA* promoter was transformed into strain NZ9000. Strain NZ9000 harboring pNZ4102 was grown under inducing and noninducing conditions, and CE of the cultures were prepared and analyzed by SDS-PAGE (Fig. 2). Growth in the presence of nisin resulted in the appearance of an extra protein band at an apparent molecular mass of approximately 35 kDa, which was the expected size of GalU (see above).

Twenty-fold-higher GalU specific activity was obtained with CE of the induced cultures than with CE of the control cultures, demonstrating that controlled and functional overexpression of the *galU* gene occurred (Table 2).

Effect of *galU* overexpression on UDP-sugar formation and EPS biosynthesis. To study the effect of GalU activity on production of UDP-sugars, which is necessary for production of the NIZO B40 EPS, the concentrations of UDP-glucose and UDP-galactose were determined by performing an HPLC analysis of NZ9000 harboring pNZ4102 grown in the presence and in the absence of nisin. Since the absolute levels of the UDP-sugars were found to be variable, the results of a typical experiment are shown in Table 2. Overexpression of *galU* resulted in eightfold increases in the levels of both UDP-glucose and UDP-galactose. Apparently, the endogenous UDP-galactose epimerase (GalE) activity is sufficient to maintain an almost stable ratio of the UDP-sugars in this strain. Although the increased level of GalU activity did not have a significant effect on the level of EPS production (Table 2), the results

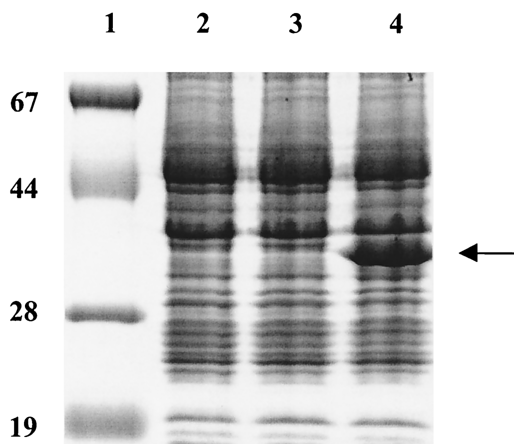

FIG. 2. Coomassie blue-stained gel after SDS-PAGE of CE of *L. lactis* subsp. *cremoris* NZ9000 (lane 2) and NZ9000 harboring pNZ4102 grown in the absence (lane 3) or in the presence (lane 4) of nisin. Lane 1 contained a molecular mass marker (molecular masses [in kilodaltons] are indicated on the left). The arrow indicates the position of the overproduced GalU protein.

indicate that GalU plays an important role in control of UDP-glucose and UDP-galactose sugar biosynthesis in *L. lactis*.

Effect of *galE* disruption on growth and EPS biosynthesis. The role of the GalE enzyme in growth and EPS biosynthesis was evaluated by analyzing NIZO B40 EPS biosynthesis in *galE* disruption strain NZ8460. As expected, strain NZ8460 was not able to ferment galactose, and CE of this strain grown on a mixture of glucose and galactose showed no detectable GalE activity (data not shown). The galactose-negative phenotype could be complemented by transforming NZ8460 with pNZ8421 containing the *L. lactis galE* gene under control of its own promoter (data not shown). These results indicate that the *galE* gene was functionally disrupted.

EPS-producing capacity was introduced into strain NZ8460 by transformation with pNZ4130, which contained the *eps* gene cluster required for biosynthesis of NIZO B40 EPS. Growth of NZ8460 harboring pNZ4130 was analyzed on CDM with different carbon sources (Table 3). No difference in growth rate between NZ8460 and its parental strain was observed when cells were grown on a mixture of glucose and galactose. However, NZ8460 was not able to grow on galactose and exhibited a reduced growth rate when it was grown on glucose as the sole carbon source (data not shown). Under the latter culture conditions cell division was also affected, suggesting that GalE has an important role in cell wall biosynthesis, as first reported by Grossiord (20). Remarkably, EPS production was completely absent during growth on glucose (Table 3). Moreover, ^{31}P-NMR analysis of extracts obtained from glucose-grown cells of NZ8460 harboring pNZ4130 showed that the intracellular concentration of UDP-galactose was below the level of detection (<0.2 mM). The concentration of UDP-glucose (0.6 ± 0.12 mM) was similar to that obtained with an EPS-producing strain derived from MG1363 (38). Therefore, it is likely that the lack of UDP-galactose leads to an EPS-negative phenotype. However, both EPS production and the growth rate could be restored to intermediate levels by adding galactose to the medium (Table 3). The sugar composition of the EPS produced in a medium containing a mixture of glucose and galactose consisted of glucose, galactose, and rhamnose at a ratio of 0.7 (± 0.1):1:1.9 ± 0.6), which is the expected ratio for NIZO B40 EPS (49), indicating that the *galE* disruption did not alter specific incorporation of sugar nucleotides.

TABLE 3. Maximum growth rates and levels of EPS production for *L. lactis* subsp. *cremoris* MG1363 (wild type) and *galE* mutant strain NZ8404 (GalE$^-$)

Strain[a]	Carbon source(s)	Growth rate (h^{-1})	EPS production (mg liter^{-1})[b]
MG1363 (wild type)	Glc	0.66 ± 0.01[c]	128 ± 6
	Gal	0.19 ± 0.01	91 ± 1
	Glc + Gal	0.66 ± 0.01	107 ± 6
NZ8460 (GalE$^-$)	Glc	0.18 ± 0.01	<1
	Gal	NG[d]	NG
	Glc + Gal	0.52 ± 0.01	44 ± 3

[a] Both strains harbored pNZ4130 and were grown in CDM supplemented with glucose, galactose, or glucose plus galactose.
[b] Levels of EPS production were determined after pNZ4130 was transformed.
[c] The values are averages based on at least two independent experiments.
[d] NG, no growth.

Taken together, the data show that GalE activity is essential for normal growth and EPS production by cells grown on media in the absence of galactose.

DISCUSSION

EPS contribute to the rheology and texture of fermented products and are therefore of interest for food applications as natural biothickeners. We studied EPS biosynthesis in *L. lactis* NIZO B40, a strain which originated from a very viscous Scandinavian fermented dairy product (49). EPS biosynthesis in *L. lactis* is mediated by proteins encoded by a plasmid-located cluster of *eps* genes that are involved in formation of EPS by sequential addition of sugars to a membrane-anchored repeating unit, export, and polymerization (49–51). Besides these specific functions, EPS production also requires household

TABLE 2. UDP-sugar levels as determined by HPLC and GalU enzyme activities of *L. lactis* subsp. *cremoris* NZ9000 (wild type) and of NZ9000 harboring pNZ4102 (GalU) grown in the presence and in the absence of nisin

Strain	Nisin	GalU activity (μmol g of protein^{-1})	Concn (nmol min^{-1} mg of protein^{-1}) of: UDP-glucose	UDP-galactose	UDP-glucose/ UDP-galactose ratio	EPS production (mg liter^{-1})[a]
NZ9000 (wild type)	−	4.8 ± 0.1	7.6 ± 1	2.8 ± 0.3	2.7	147 ± 7
NZ9000 (pNZ4102) (GalU)	−	4.9 ± 0.1	7.5 ± 0.3	2.6 ± 0.1	2.9	156 ± 9
	+	94 ± 4	60 ± 20	17.5 ± 6	3.4	158 ± 5

[a] The levels of EPS production were determined after pNZ4030 was transformed.

proteins which are involved in biosynthesis of the EPS building blocks, the nucleotide sugars. The model for synthesis of NIZO B40 EPS predicts a requirement for the precursors UDP-glucose, UDP-galactose, and dTDP-rhamnose, which are formed from a central intermediate, glucose 1-phosphate (Fig. 1). To generate tools to target these specific endogenous enzymatic activities, which are potential bottlenecks in sugar nucleotide biosynthesis and subsequent EPS biosynthesis; we cloned and characterized several of the encoding genes. In this study, we evaluated the model for EPS biosynthesis (Fig. 1) by analyzing the roles of the *galU* and *galE* gene products in production of UDP-glucose and UDP-galactose from glucose 1-phosphate.

Previously, the *galE* gene was cloned from *L. lactis* MG1363 (20). Here we describe cloning of the *galU* gene from the same strain. The predicted gene product showed strong homology to UDP-glucose pyrophosphorylases from several bacteria. Evidence for this function was obtained by functional overexpression of the *galU* gene and by functional complementation of an *E. coli galU* mutant. The *galU* gene of *L. lactis* MG1363 is linked to a putative glycerol-3-phosphate dehydrogenase gene. Since these genes are not linked metabolically, it is not thought that their genetic linkage has any significance. This hypothesis is supported by the finding that the *galU* gene is expressed as a monocistronic transcript. The *galU* gene in other bacteria has been shown to be genetically linked to genes encoding related metabolic functions. In *S. pyogenes* and *S. pneumoniae* type 3, the *galU* gene is immediately preceded by the *gpsA* gene, which is presumably involved in synthesis of membrane lipids for cell wall formation. Moreover, in these streptococci another copy of a *galU*-like gene was located in the chromosomal CPS biosynthesis locus (32). Hence, in *S. pneumoniae* type 3 residual GalU activity could be measured when the *galU* gene was functionally disrupted, which was probably caused by the second copy of a *galU*-like gene. Interestingly, the *galU* gene of *S. pneumoniae* has been shown to be essential for type 1 or 3 CPS biosynthesis in this organism (32). Since our attempts to inactivate the *galU* gene in *L. lactis* were unsuccessful, it is likely that the absence of a second *galU* gene copy in *L. lactis* explains the lack of success when we tried to isolate a *galU* mutant.

The role of the Leloir enzyme GalE, which catalyzes interconversion of UDP-galactose and UDP-glucose, in EPS biosynthesis was evaluated by using a *galE* mutant strain. *L. lactis galE* mutant NZ8460 harboring pNZ4130, encoding EPS production, did not produce detectable amounts of EPS when it was cultured on glucose as the sole carbon source. Similar results were obtained for EPS production in *Rhizobium meliloti exoB* (*galE* analogue) (4) and *Erwinia stewartii galE* (15) strains. The EPS-negative phenotype of *L. lactis* could be complemented by adding galactose to the medium; a similar finding was reported for the *E. stewartii galE* strain (15). These results indicate that *galE* plays an essential role in synthesis of UDP-galactose from glucose and thus in EPS biosynthesis. Moreover, the *galE* mutant strain was affected in cell division, which led to formation of long chains of cells when the organism was cultured in medium with glucose as the sole carbon source, as reported previously by Grossiord (20). These results indicate that the *galE* gene is essential not only for EPS production but also for normal cell growth when cells are cultured in media with glucose alone. The effect of *galE* disruption on both EPS production and cell division can probably be explained by a crucial role for UDP-galactose in cell wall biosynthesis. In glucose-grown wild-type *L. lactis* cells the sugar composition of the polysaccharide fraction in the cell wall is 29 mol% glucose, 15 mol% galactose, and 55 mol% rhamnose (28). UDP-glucose is involved in formation of membrane anchors of the lipoteichoic acids (LTA) which are decorated with galactosyl units from UDP-galactose (9). In contrast to UDP-glucose, the precursor UDP-galactose could not be detected in extracts of a glucose-grown culture, suggesting that the lack of UDP-galactose may be a limiting factor for both growth and EPS production. It is possible that the lack of UDP-galactose in the *galE* mutant strain has a significant impact on LTA galactosylation and could therefore lead to inhibition of cell division. Remarkably, a similar mode of growth, long chains of cells, was also observed for *L. lactis* strains that were deficient in autolysin (AcmA) (5) or the LTA D-alanylation (DltD) (16). The similarities suggest that the essential role of AcmA in normal cell division (5) could depend on alanylation and galactosylation of LTA.

Although growth of the *galE* mutant was completely restored in media containing a mixture of glucose and galactose, the levels of EPS production were not restored to the wild-type level. Since this observation was also made with other independent isolates of the *galE* mutant strain (data not shown), it is not likely that the reduced level of EPS production is caused by mutations in the Eps plasmid. It has been shown that in *L. lactis* the *gal* genes are subject to CcpA-mediated catabolite repression, which results in lower levels of transcription of the *gal* operon when cells are grown in the presence of glucose (29). It seems likely that the repressed galactose fermentation in the *galE* mutant strain would lead to reduced availability of UDP-galactose which is preferentially used for growth rather than EPS formation, resulting in an intermediate level of EPS production. This type of control was also found when an EPS-producing *L. lactis* strain was grown in a medium with fructose as the sole carbon source (28).

It has been shown that mutations in the *galE* gene affect the EPS composition of *Rhizobium leguminosarum*, which produces an altered EPS lacking the galactose residue and the substitutions attached to it (41). In addition, *Erwinia amylovora galE* strains were deficient in EPS production but were able to produce LPS although the LPS had an altered side chain structure (31). These results indicate that in these bacteria it might be possible to change the EPS composition by inactivating the polysaccharide-precursor-forming enzymes. Nevertheless, the sugar composition of the EPS produced by the *galE* strain consisted of glucose, galactose, and rhamnose at the ratio expected for NIZO B40 EPS, indicating that this is not the case for *L. lactis*.

Functional overexpression of the *galU* gene resulted in a 20-fold increase in enzyme activity and eightfold increases in UDP-glucose and UDP-galactose levels. These results imply that the level of GalU enzyme activity controls the levels of production of UDP-glucose and UDP-galactose in wild-type cells. Apparently, increased GalU activity leading to increased precursor availability did not result in production of more NIZO B40 EPS. This contrasts with a recent report on expression of the *cps3D* and *cps3S* genes from *S. pneumoniae* type 3 in *L. lactis*. This expression resulted in a low level of production of type 3 polysaccharide, which could be increased sub-

stantially by coexpression of the *cps3U* gene that encodes a GalU analogue (18). It has been shown for several bacteria that enzymes involved in sugar nucleotide biosynthesis control EPS production. However, this correlation seems to depend on the type of polysaccharide produced with regard to GalU activity by *L. lactis*. One possible explanation is that the availability of dTDP-rhamnose, which is incorporated as a side chain in NIZO B40 EPS, limits NIZO B40 EPS production. Alternatively, the maximal level of EPS production could also be determined by the activity of the specific EPS biosynthesis machinery encoded by the EPS plasmid rather than by the level of sugar nucleotides. This hypothesis is supported by the finding that overexpression of the priming glycosyltransferase gene *epsD* resulted in an increase in EPS production (49, 52). Elevated expression of all of the biosynthetic *eps* genes would allow evaluation of this alternative for increasing EPS production.

Evaluation of the EPS biosynthesis model described here allowed us to assess the role of the GalU and GalE activities in *L. lactis* by using overexpression and disruption studies. We could significantly influence the internal levels of UDP-glucose and UDP-galactose, both of which are precursors for EPS biosynthesis as well as for growth. This knowledge is important for targeting bottlenecks in EPS biosynthesis or for creating EPS with novel properties.

ACKNOWLEDGMENTS

We thank Benoît Grossiord for providing the *L. lactis galE* gene disruption plasmid, Marke Beerthuyzen for assistance with the transcription analysis, Jan van Riel for determining the sugar nucleotide contents, and Ellen Looijesteijn for assistance with the EPS analysis. Furthermore, we thank Richard van Kranenburg, Jeroen Hugenholtz, and Roland Siezen for critically reading the manuscript.

This work was supported by EC research grant BIOT-CT96-0498.

REFERENCES

1. **Archibald, A. R., I. C. Hancock, and C. R. Harwood.** 1993. Cell wall structure, synthesis and turnover, p. 381–410. *In* J. A. Hoch and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
2. **Bernstein, R. L.** 1965. Control aspects of uridine 5′-diphosphate glucose and thymidine 5′-diphosphate glucose synthesis by microbial enzymes. J. Biol. Chem. **240:**391–397.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Buendia, A. M., B. Enenkel, R. Köplin, K. Niehaus, W. Arnold, and A. Pühler.** 1991. The *Rhizobium meliloti exoZ/exoB* fragment of megaplasmid 2: ExoB functions as a UDP-glucose-4-epimerase and ExoZ shows homology to NodX of *Rhizobium leguminosarum* biovar. *viciae* TOM. Mol. Microbiol. **5:**1519–1530.
5. **Buist G., J. Kok, K. J. Leenhouts, M. Dabrowska, G. Venema, and A. J. Haandrikman.** 1995. Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J. Bacteriol. **177:**1554–1563.
6. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *E. coli.* J. Mol. Biol. **138:**179–207.
7. **Chaffin, D. O., S. B. Beres, H. H. Yim, and C. E. Rubens.** 2000. The serotype of type Ia and III group B streptococci is determined by the polymerase gene within the polycistronic capsule operon. J. Bacteriol. **182:**4466–4477.
8. **Carter, D. L., B. A. Dougherty, and I van de Rijn.** 1995. Molecular characterization of hasC from an operon required for hyaluronic acid synthesis in group A streptococci. Demonstration of UDP-glucose pyrophosphorylase activity. J. Biol. Chem. **270:**28676–28680.
9. **Delcour, J., T. Ferain, M. Deghorain, E. Palumbo, and P. Hols.** 1999. The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Antonie Leeuwenhoek **76:**159–184.
10. **De Ruyter, P. G. G. A., O. P. Kuipers, and W. M. De Vos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Eviron. Microbiol. **62:**3662–3667.
11. **De Ruyter, P. G. G. A., O. P. Kuipers, M. M. Beerthuyzen, I. J. Van Alen-Boerrigter, and W. M. De Vos.** 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J. Bacteriol. **178:**3434–3439.
12. **De Vos, W. M., P. Vos, H. De Haard, and I. Boerrigter.** 1989. Cloning and expression of the *Lactococcus lactis* ssp. *cremoris* SK11 gene encoding an extracellular serine protease. Gene **85:**169–176.
13. **De Vos, W. M., and G. Simons.** 1994. Gene cloning and expression systems in lactococci, p. 52–105. *In* M. J. Gasson, and W. M. de Vos, (ed.), Genetics and biotechnology of lactic acid bacteria. Chapman and Hall, London, United Kingdom.
14. **De Vos, W. M.** 1996. Metabolic engineering of sugar catabolism in lactic acid bacteria. Antonie Leeuwenhoek **70:**223–242.
15. **Dolph P. J., D. R. Majerczak, and D. L. Coplin.** 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. J. Bacteriol. **170:**865–871.
16. **Duwat, P., A. Cochu, S. D. Ehrlich, and A. Gruss.** 1997. Characterization of *Lactococcus lactis* UV-sensitive mutants obtained by ISS*1* transposition. J. Bacteriol. **179:**4473–4479.
17. **Gasson, M. J.** 1983. Plasmid complements of NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
18. **Gilbert, C., K. Robinson, R. W. F. Le Page, and J. M. Wells.** 2000. Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in *Lactococcus lactis*. Infect. Immun. **68:**3251–3260.
19. **Grossiord, B., E. E. Vaughan, E. Luesink, and W. M. De Vos.** 1998. Genetics of galactose utilisation via the Leloir pathway in lactic acid bacteria. Lait **78:**77–84.
20. **Grossiord, B.** 1998. Metabolisme du galactose par la voie de Leloir l'operon *gal* de *Lactococcus lactis*. Ph.D. thesis. 1′ École Nationale Supérieure Agronomique de Montpellier, Montpellier, France.
21. **Hill, C., G. Venema, C. Daly, and G. F. Fitzgerald.** 1988. Cloning and characterization of the tetracycline resistance determinant of and several promoters from within the conjugative transposon Tn*919*. Appl. Environ. Microbiol. **54:**1230–1236.
22. **Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S. Kaminogawa.** 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101–4. Biosci. Biochem. **61:**312–316.
23. **Kitazawa, H., T. Toba, T. Itoh, N. Kumano, S. Adachi, and T. Yamaguchi.** 1991. Antitumoral activity of slime-forming, encapsulated *Lactococcus lactis* subsp. *cremoris* isolated from Scandinavian ropy sour milk, 'viili.' Anim. Sci. Technol. **62:**277–283.
24. **Kleerebezem, M., R. Van Kranenburg, R. Tuinier, I. C. Boels, P. Zoon, E. Looijesteijn, J. Hugenholtz, and W. M. De Vos.** 1999. Exopolysaccharides produced by *Lactococcus lactis*: from genetic engineering to improved rheological properties? Antonie Leeuwenhoek **76:**357–365.
25. **Kuipers, O. P., P. G. G. A. De Ruyter, M. Kleerebezem, and W. M. De Vos.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. **64:**15–21.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Looijesteijn, P. J., and J. Hugenholtz.** 1999. Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and optimisation of its exopolysaccharide synthesis. J. Biosci. Bioeng. **88:**178–182.
28. **Looijesteijn, P. J., I. C. Boels, M. Kleerebezem, and J. Hugenholtz.** 1999. Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl. Environ. Microbiol. **65:**5003–5008.
29. **Luesink, E. J., R. E. M. A. Van Herpen, B. P. Grossiord, O. P. Kuipers, and W. M. De Vos.** 1998. Transcriptional activation of the glycolytic *las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. Mol. Microbiol. **30:**789–798.
30. **Maguin, E., H. Prévost, S. D. Ehrlich, and A. Gruss.** 1996. Efficient insertional mutagenesis in lactococci and other Gram-positive bacteria. J. Bacteriol. **178:**931–935.
31. **Metzer, M., P. Bellemann, P. Bugert, and K. Geider.** 1994. Genetics of galactose metabolism of *Erwinia amylovora* and its influence on polysaccharide synthesis and virulence of the fire blight pathogen. J. Bacteriol. **176:** 450–459.
32. **Mollerach, M., R. López, and E. García.** 1998. Characterization of the *galU* gene of *Streptococcus pneumoniae* encoding a uridine diphosphoglucose pyrophosphorylase: a gene essential for capsular polysaccharides biosynthesis. J. Exp. Med. **188:**2047–2056.
33. **Morona, J. K., R. Morona, and J. C. Paton.** 1997. Characterization of the locus encoding the *Streptococcus pneumoniae* type 19F capsular polysaccharide biosynthetic pathway. Mol. Microbiol. **23:**751–763.
34. **Nakajima, H., Y. Suzuki, H. Kaizu, and T. Hirota.** 1992. Cholesterol lowering activity of ropy fermented milk. J. Food Sci. **57:**1327–1329.
35. **Nakajima, H., T. Hirota, T. Toba, T. Itoh, and S. Adachi.** 1992. Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subsp. *cremoris* SBT 0495. Carbohydr. Res. **224:**245–253.
36. **Park, J. T.** 1987. Murein synthesis, p. 663–671. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and M. E. Umbarger

(ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
37. **Poolman, B., E. J. Smid, H. Veldkamp, and W. N. Konings.** 1987. Bioenergetic consequences of lactose starvation for continuously cultured *Streptococcus cremoris*. J. Bacteriol. **169:**1460–1468.
38. **Ramos, A., I. C. Boels, W. M. de Vos, and H. Santos.** 2001. Relationship between glycolysis and exopolysaccharide biosynthesis in *Lactococcus lactis*. Appl. Environ. Microbiol. **67:**33–41.
39. **Roberts, I. S.** 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu. Rev. Micobiol. **50:**285–315.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Sánchez-Andújar, B., C. Coronado, S. Philip-Hollingsworth, F. B. Dazzo, and A. J. Palomares.** 1997. Structure and role in symbiosis of the *exoB* gene of *Rhizobium leguminosarum* by. *trifolii*. Mol. Gen. Genet. **255:**131–140.
42. **Shapiro, J. A.** 1966. Chromosomal location of the gene determining uridine diphosphoglucose formation in *Escherichia coli* K-12. J. Bacteriol. **92:**518.
43. **Stingele, F., J. R Neeser, and B. Mollet.** 1996. Identification of the *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. J. Bacteriol. **178:**1680–1690.
44. **Stingele, F., S. J. F. Vincent, E. J. Faber, J. W. Newel, J. P. Kamerling, and J. R. Neeser.** 1999. Introduction of the exopolysaccharide gene cluster from *Streptococcus thermophilus* Sfi6 into *Lactococcus lactis* MG1363: production and characterization of an altered polysaccharide. Mol. Microbiol. **32:**1287–1295.
45. **Sutherland, I. W.** 1993. Microbial polysaccharides, p. 69–85. *In* R. L. Whistler and J. N. Miller (ed.), Industrial gums; polysaccharides and their derivatives, 3rd ed. Academic Press, San Diego, Calif.
46. **Sutherland, I. W.** 1998. Novel and established applications of microbial polysaccharides. Trends Biotechnol. **16:**41–46.
47. **Thompson, J.** 1987. Regulation of sugar transport and metabolism in lactic acid bacteria. FEMS Microbiol. Rev. **46:**221–231.
48. **Van der Meer, J. R., J. Polman, M. M. Beerthuyzen, R. J. Siezen, O. P. Kuipers, and W. M. De Vos.** 1993. Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. J. Bacteriol. **175:**2578–2588.
49. **Van Kranenburg, R., J. D. Marugg, I. I. Van Swam, J. Willem, and W. M. De Vos.** 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. **24:**387–397.
50. **Van Kranenburg, R., H. R. Vos, I. I. Van Swam, M. Kleerebezem, and W. M. De Vos.** 1999. Functional analysis of glycosyltransferase genes from *Lactococcus lactis* and other gram-positive cocci: complementation, expression, and diversity. J. Bacteriol. **181:**6347–6353.
51. **Van Kranenburg, R., I. I. Van Swam, J. D. Marrug, M. Kleerebezem, and W. M. De Vos.** 1999. Exopolysaccharide biosynthesis in *Lactococcus lactis* NIZO B40: functional analysis of glycosyltransferase genes involved in synthesis of the polysaccharide backbone. J. Bacteriol. **181:**338–340.
52. **Van Kranenburg, R., I. C. Boels, M. Kleerebezem, and W. M. de Vos.** 1999. Genetics and engineering of microbial exopolysaccharides for food: approaches for the production of existing and novel polysaccharides. Curr. Opin. Biotechnol. **10:**498–504.
53. **Yamamoto, Y., K. Miyake, Y. Koike, M. Watanabe, Y. Machida, M. Ohta, and S. Iijima.** 1999. Molecular characterization of type-specific capsular polysaccharide biosynthesis genes of *Streptococcus agalactiae*. J. Bacteriol. **181:**5176–5184.
54. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.