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Functional Analysis of Glycosyltransferase Genes from *Lactococcus lactis* and Other Gram-Positive Cocci: Complementation, Expression, and Diversity

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Sixteen exopolysaccharide (EPS)-producing *Lactococcus lactis* strains were analyzed for the chemical compositions of their EPSs and the locations, sequences, and organization of the *eps* genes involved in EPS biosynthesis. This allowed the grouping of these strains into three major groups, representatives of which were studied in detail. Previously, we have characterized the *eps* gene cluster of strain NIZO B40 (group I) and determined the function of three of its glycosyltransferase (GTF) genes. Fragments of the *eps* gene clusters of strains NIZO B35 (group II) and NIZO B891 (group III) were cloned, and these encoded the NIZO B35 priming galactosyltransferase, the NIZO B891 priming glucosyltransferase, and the NIZO B891 galactosyltransferase involved in the second step of repeating-unit synthesis. The NIZO B40 priming glucosyltransferase gene *epsD* was replaced with an erythromycin resistance gene, and this resulted in loss of EPS production. This *epsD* deletion was complemented with priming GTF genes from gram-positive organisms with known function and substrate specificity. Although no EPS production was found with priming galactosyltransferase genes from *L. lactis* or *Streptococcus thermophilus*, complementation with priming glucosyltransferase genes involved in *L. lactis* EPS and *Streptococcus pneumoniae* capsule biosynthesis could completely restore or even increase EPS production in *L. lactis*.

Many gram-positive bacteria produce significant amounts of capsular polysaccharides (CPSs) or exopolysaccharides (EPSs). Most molecular studies have focused on the CPSs from strains of *Streptococcus pneumoniae*, group B streptococci, and *Staphylococcus aureus* (23). These CPSs have unique structures that determine the serotype and virulence of these pathogens. Their biosynthesis is encoded by large clusters of genes that often show unidirectional organization, are transcribed into single polycistronic mRNAs, and appear to be coordinately expressed (15, 16, 20, 26). In these clusters, the serotype-specific genes encoding the glycosyltransferases (GTFs) are flanked by genes that are common to all serotypes and are likely to be involved in processes like chain length determination, polymerization, and export (12, 15, 16, 27). Several lactic acid bacteria are known to produce EPSs that are of industrial importance, as they are beneficial for the structure of dairy products (2). Recently, the genes encoding EPS production in the dairy starters *Streptococcus thermophilus* Sfi6 and *Lactococcus lactis* NIZO B40 were characterized and their organization was found to be similar to that of the CPS biosynthesis gene clusters of the gram-positive pathogens (29, 34). Functional analysis of the NIZO B40 *eps* genes demonstrated that the *epsDEF* genes are functional homologues of the *cps14EFG* genes from *S. pneumoniae* serotype 14 and code for GTFs that are involved in identical steps of the polysaccharide biosynthesis route (35). In general, the GTF involved in linking the first sugar of the repeating unit to the lipid carrier, here referred to as the priming GTF, is highly homologous in gram-positive bacteria, while other GTFs are often unique or have very little homology to others (12, 15, 27, 29, 34).

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In spite of the increasing sequence information on the CPS or EPS gene clusters in gram-positive cocci, very little is known about the function of the predicted GTF genes and even less is known about their specificities. By investigation of the GTF genes expressed in *Escherichia coli*, the substrate specificities of GTFs involved in the biosynthesis of *S. pneumoniae* serotype 14, *L. lactis* NIZO B40, and *S. thermophilus* Sfi6 were determined (12, 30, 34). However, it was reported that GTF genes expressed in a heterologous host could result in a different composition of the EPS (30). Therefore, we have used a recently developed homologous expression system to demonstrate the substrate specificity of the *epsDEFG* genes of *L. lactis* NIZO B40 (35). Here we describe a screening approach used to identify new GTF genes in *L. lactis* and show the diversity of GTF genes in *L. lactis* and their EPSs, resulting in a classification of three major groups. Two new priming GTF genes were selected, and their function and substrate specificity were determined. Finally, a transcomplementation of a knockout of the NIZO B40 *epsD* gene encoding the priming GTF was realized by controlled expression of several homologous GTF genes derived from different gram-positive cocci.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in L-broth-based medium at 37°C (24). *L. lactis* was grown at 30°C in M17 broth (Difco Laboratories) supplemented with 0.5% glucose (GM17) or in a chemically defined medium (22). If appropriate, the media contained chloramphenicol (10 μg/ml), erythromycin (10 μg/ml), or ampicillin (100 μg/ml).

DNA isolation and manipulation. Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook et al. (24). 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). Isolation and transformation of *L. lactis* plasmid DNA were performed as previously described (6). Southern blots were hybridized with *eps* gene probes at 45°C and washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 45°C before exposure.

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli DH5α		9
L. lactis		
NIZO B35	Lac^+ Eps^+ multiplasmid strain	NIZO collection
NIZO B36	Lac^+ Eps^+ multiplasmid strain	NIZO collection
NIZO B39	Lac^+ Eps^+ multiplasmid strain	NIZO collection
NIZO B40	Lac^+ Eps^+ multiplasmid strain harboring pNZ4000	34
NIZO B891	Lac^+ Eps^+ multiplasmid strain	NIZO collection
NIZO B1136	Lac^+ Eps^+ multiplasmid strain	NIZO collection
NIZO B1137	Lac^+ Eps^+ multiplasmid strain	NIZO collection
SBT 0495	Lac^+ Eps^+ multiplasmid strain	17
H414	Lac^+ Eps^+ multiplasmid strain	8
SD8	Lac^+ Eps^+ multiplasmid strain	19
SD11	Lac^+ Eps^+ multiplasmid strain	19
VI6	Lac^+ Eps^+ multiplasmid strain	19
VI8	Lac^+ Eps^+ multiplasmid strain	19
MLT1	Lac^+ Eps^+ multiplasmid strain	Quest collection
MLT2	Lac^+ Eps^+ multiplasmid strain	Quest collection
MLT3	Lac^+ Eps^+ multiplasmid strain	Quest collection
NZ3900	*pepN*::*nisRnisK*	4
Plasmids		
pNZ4080	Ap^r, 3.8-kb pUC19 derivative carrying NIZO B35 *orfU*	This study
pNZ4081	Ap^r, 5.5-kb pUC19 derivative carrying NIZO B35 *orfU* and *epsD*	This study
pNZ4082	Ap^r, 4.0-kb pUC18 derivative carrying NIZO B35 *epsD*	This study
pNZ4083	Cm^r, 4.4-kb pNZ8020 derivative carrying NIZO B35 *epsD*	This study
pNZ4085	Ap^r, 6.3-kb p119HE derivative carrying NIZO B891 *epsD*	This study
pNZ4086	Ap^r, 7.2-kb p119HE derivative carrying NIZO B891 *epsDEF*	This study
pNZ4087	Cm^r, 4.2-kb pNZ8020 derivative carrying NIZO B891 *epsD*	This study
pNZ4090	Cm^r, 4.5-kb pNZ8020 derivative carrying *cps14E*	This study
pNZ4091	Cm^r, 5.8-kb pNZ8020 derivative carrying *cps14EFG*	This study
pNZ4055	Ery^r Eps^-, pNZ4000Δ*epsD* derivative carrying *ery* from pIL253	This study
pNZ4030	Ery^r Eps^+, 27-kb pNZ4000 derivative carrying *ery* from pIL253	34
pNZ4070	Cm^r, 4.6-kb pNZ8020 fragment carrying NIZO B40 *epsD*	35

[a] Lac^+, lactose-fermenting phenotype; Eps^+, EPS-producing phenotype; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Ery^r, erythromycin resistant.

Nucleotide sequence analysis. Automatic double-stranded DNA sequence analysis was performed on both strands with an ALF*red* DNA sequencer (Pharmacia Biotech). Sequencing reactions were accomplished by using the AutoRead sequencing kit, initiated by using Cy5-labelled universal and reverse primers, and continued with synthetic primers in combination with Cy5-13-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 (IntelliGenetics).

Construction of plasmids. For expression of the NIZO B35 *eps* genes in *E. coli*, a 1.0-kb *Sca*I-*Hinc*II fragment containing *orfU*, a 2.7-kb *Sca*I-*Kpn*I fragment containing *orfU-epsD*, and a 1.3-kb *Sca*I fragment containing *epsD* were cloned under control of the *lac* promoter in pUC18 or pUC19 (41). To express the NIZO B891 *eps* genes in *E. coli*, a 1.0-kb *Sca*I-*Bal*I fragment containing *epsD* and a 1.9-kb *Sca*I-*Eco*RI fragment containing *epsDEF* were cloned under control of the *lac* promoter in pJF119HE (7). For expression of the NIZO B35 and NIZO B891 *epsD* genes in *L. lactis*, a 1.3-kb *Sca*I fragment and a 1.0-kb *Sca*I-*Bal*I fragment, respectively, were cloned under control of the *nisA* promoter in pNZ8020 (5). To express the streptococcal *cps14* GTF genes in *L. lactis*, a 1.3-kb *Xba*I-*Pvu*II fragment containing the GTF part of *cps14E* and a 2.6-kb *Xba*I fragment containing *cps14EFG* were cloned from pMK100 (10) under control of the *nisA* promoter in pNZ8020. To express the streptococcal *epsE* GTF gene in *L. lactis*, a 1.8-kb *Eco*RV-*Xba*I fragment containing *epsE* was cloned from pFS30 (29) under control of the *nisA* promoter in pNZ8020. To construct a NIZO B40 *epsD* gene disruption, a PCR was used to clone the flanking regions containing *epsC* (by using the primers 5′-AGCAGCAAGCTTTTCAAGTTATATATTGA-3′ and 5′-TTCAGAGGATCCCTCAAAAACTTCCAT-3′) and *epsEF* (by using the primers 5′-CTACATGGATCCGATGCTTATTAAAGTAA-3′ and 5′-ATTATTGAATTCATCAGAATAATTCCCCTA-3′) in pUC18, making use of the *Eco*RI, *Bam*HI, and *Hin*dIII sites of the primers (underlined). The *ery* gene of pIL253 was cloned from pUC18Ery (34) into the *Bam*HI site between the *epsC* and *epsEF* fragments in the same orientation as the *eps* genes. The complete *Eco*RI-*Hin*dIII insert was transferred to pG^+host8 (14), resulting in a tetracycline-resistant (Tet^r), erythromycin-resistant (Ery^r) construct containing a temperature-sensitive replicon which is not functional at 37°C. The resulting plasmid was transformed to strain NZ4010 harboring EPS plasmid pNZ4000 (34), and transformants were subsequently cultured at 37°C. A Tet^s Eps^- Ery^r double-crossover mutant of pNZ4000 was obtained in which *epsD* was exchanged for the *ery* gene (pNZ4055). The pUC, pJF119HE, and pG^+host derivatives were constructed in *E. coli* DH5α, and the pNZ8020 derivatives were constructed in *L. lactis* NZ3900.

EPS purification and characterization. *L. lactis* was grown in 50 ml of defined medium containing 2% glucose for 48 h at 30°C, and after pelleting of the cells, EPS was purified by dialysis and lyophilization and quantified by gel permeation high-performance liquid chromatography analysis using dextran 500 as a standard as described before (34). Sugar analysis was performed by high-performance liquid chromatography analysis of the monosaccharide units after complete hydrolysis with 4 N HCl (37). To analyze the EPS in overproducing strain NZ3900 harboring pNZ4055 and pNZ8020 derivatives, induction was performed with nisin A (1 ng ml^{-1}) at an optical density at 600 nm of 0.5 (4).

GTF activity assays and TLC analysis. GTF activity assays and thin-layer chromatography (TLC) analysis were performed with permeabilized *E. coli* cells as described before (34). Permeabilized *L. lactis* cells were prepared like those of *E. coli* after a 30-min incubation with lysozyme (10 mg ml^{-1}) on ice. After incubation with UDP-[^{14}C]glucose and/or UDP-[^{14}C]galactose, the extracted lipid fractions were subjected to complete and mild acid hydrolysis and analyzed by TLC and autoradiography to detect ^{14}C-labelled monosaccharides (complete acid hydrolysis) and oligosaccharides (mild acid hydrolysis), respectively.

Nucleotide sequence accession numbers. The nucleotide sequences of the NIZO B35 and NIZO B891 *eps* gene cluster fragments are available under GenBank accession no. AF100297 and AF100298.

RESULTS

Diversity of lactococcal GTF genes and EPSs. In a search for new GTF genes, we screened a collection of 16 different EPS-

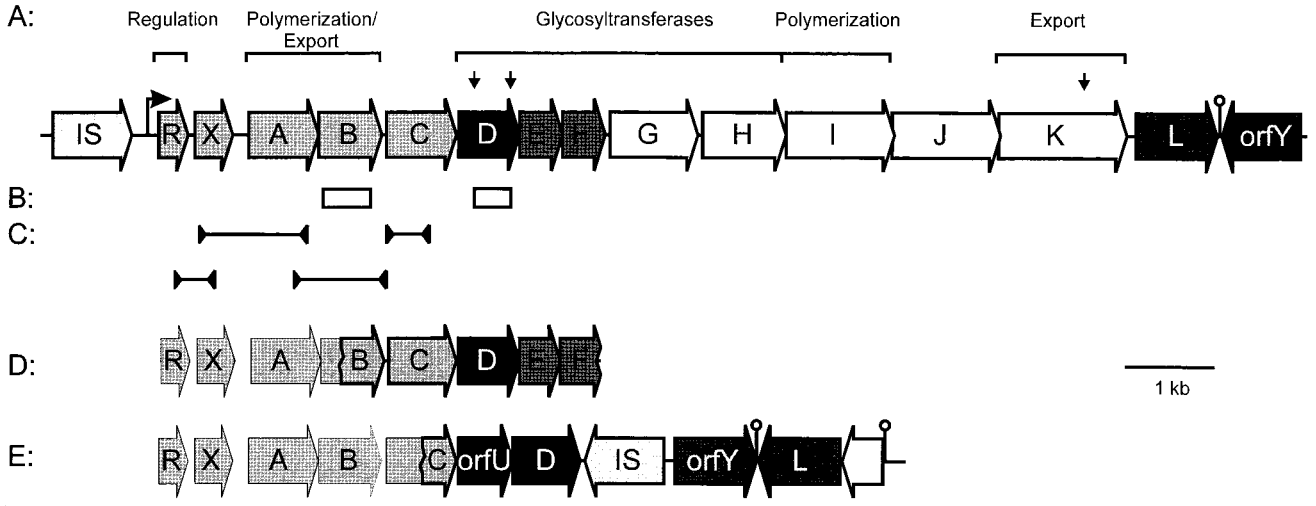

FIG. 1. (A) Genetic map of the *eps* gene cluster of *L. lactis* NIZO B40. The *Sst*I recognition sites are indicated by downward-pointing arrows. The predicted functions of the gene products are depicted above the map (34). (B) DNA fragments of the NIZO B40 *eps* gene cluster used for hybridization. (C) PCR fragments generated by the primers indicated by the arrowheads used to determine the order of the conserved *eps* genes of various strains (see text). (D and E) Genetic maps of the *eps* gene cluster of *L. lactis* NIZO B891 and NIZO B35 based on DNA sequences of cloned fragments and on PCR analysis.

producing *L. lactis* strains at the genetic and biochemical levels. To localize putative *eps* gene clusters, DNA from the strains was probed with an internal fragment of the *L. lactis* NIZO B40 *epsB* gene (Fig. 1B), which is highly conserved and has homologues in all studied EPS or CPS gene clusters of gram-positive cocci (34). All of the *L. lactis* strains tested contained a single plasmid (>20 kb) that hybridized with the *epsB* probe (results not shown). This confirms previous suggestions that EPS production in *L. lactis* is plasmid encoded (19, 38, 39). The diversity of the plasmid-encoded GTF genes was studied by analyzing their hybridization to the NIZO B40 *epsB* and *epsD* genes (Fig. 1B). This *epsD* gene codes for the priming glucosyltransferase and shows homology to other priming GTF genes (34). For this purpose, plasmid DNA of all strains was digested with *Sst*I, which has three sites within the NIZO B40 *eps* gene cluster, two of which are present in the *epsD* gene (Fig. 1A). All strains hybridized with both *epsB* and *epsD* probes, but the sizes of the hybridizing *Sst*I bands differed considerably, allowing genetic differentiation (Table 2).

The biochemical diversity of the EPSs isolated from the 16 strains was studied by determining the nature and molar ratio of the sugar monosaccharides (Table 2). No sugars other than glucose, galactose, or rhamnose were present in these polymers. Based on the genetic and biochemical diversity of the putative GTF genes and the EPSs, the *L. lactis* strains could be classified into three main groups (Table 2). Group I contains six strains that produced EPS containing the monosaccharides galactose, glucose, and rhamnose and includes strains SBT 0495 and NIZO B40, which produce EPSs with repeating units consisting of →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→ (18, 31, 34). Group II comprises five strains that produced EPS with only galactose and includes strain H414, the EPS repeating unit of which is known to be →4)-[β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)]-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→ (8). This group shows restriction fragment length polymorphism for *epsD*. Group III contains three strains that produced EPS composed of both galactose and glucose in a molar ratio of approximately 2 to 3. In addition to these three major groups, there are two strains (NIZO B39 and NIZO B1137) that show a unique combination of hybridization pattern and EPS sugar composition.

Genetic variety of *eps* gene clusters. From the three major groups of EPS-producing lactococci, strains NIZO B40, NIZO B35, and NIZO B891 were selected as representatives and further characterized together with the unique strains NIZO B39 and NIZO B1137, as the structure of their EPS is known

TABLE 2. Hybridization patterns of *Sst*I-digested lactococcal plasmid DNA and sugar composition of EPS produced

Strain	Fragment size (kb)[a]		Molar ratio[b]		
	epsB	*epsD*	Gal	Glc	Rha
Group I					
NIZO B40	7.5	0.4	1.00	1.82	0.88
SBT 0495	7.5	0.4	1.00	1.70	0.82
NIZO B1136	7.5	0.4	1.00	1.70	0.82
VI6	7.5	0.4	1.00	1.46	0.73
VI8	7.5	0.4	1.00	1.80	0.89
MLT3	7.5	0.4	1.00	1.46	0.73
Group II					
NIZO B35	7.5	15	1.00		
NIZO B36	7.5	15	1.00		
H414	7.5	15	1.00		
SD8	7.5	13	1.00		
SD11	7.5	13	1.00		
Group III					
NIZO B891	6.5	12	1.00	1.50	
MLT1	6.5	12	1.00	1.46	
MLT2	6.5	12	1.00	1.44	
Unique					
NIZO B39	7.5	12	1.00	0.60	0.55
NIZO B1137	15	18	1.00	1.79	

[a] Approximate sizes of fragments hybridizing with NIZO B40 probes are listed.

[b] Abbreviations: Rha, rhamnose; Gal, galactose; Glc, glucose. Molar ratios relative to galactose content are listed.

TABLE 3. Homologies of the *L. lactis* NIZO B35 and NIZO B891 *eps* gene products to those from *L. lactis* NIZO B40

Strain and gene	No. of amino acids of protein	Proposed function of gene product[a]	Homology (% identity) to:		
			NIZO B40	*S. thermophilus* Sfi6	*S. pneumoniae* serotype 14
NIZO B35					
epsC[b]	125	Unknown	EpsC (93.6)	EpsB (33.9)	Cps14B (33.0)
orfU	199	Unknown	EpsD (85.1)	EpsE (42.1)	Cps14E (39.1)
epsD	251	Gal-P-TF	EpsD (39.2)	EpsE (41.9)	Cps14E (36.3)
orf982	296	Transposase	*orf982* (98.0)		
orfY	300	Unknown	OrfY (95.7)		
epsL	300	Unknown	EpsL (88.6)		
NIZO B891					
epsB[b]	155	Chain length determination	EpsB (93.5)	EpsD (31.8)	Cps14D (33.1)
epsC	230	Unknown	EpsC (96.9)	EpsB (26.7)	Cps14B (29.2)
epsD	228	Glc-P-TF	EpsD (88.1)	EpsE (40.0)	Cps14E (39.1)
epsE	149	Gal-TF[c]	EpsE (40.4)		Cps14F (83.8)
epsF[d]	150	Gal-TF	EpsF (36.5)		Cps14G (53.0)

[a] Gal-P-TF, priming galactosyltransferase; Glc-P-TF, priming glucosyltransferase; Gal-TF, galactosyltransferase.
[b] Incomplete at 5′ end.
[c] Accessory function to EpsF (see text).
[d] Incomplete at 3′ end.

(NIZO B40) or is being analyzed (NIZO B35 and NIZO B891) (32, 33). Plasmid DNA of these strains was analyzed by Southern blot analysis with specific probes for each of the genes of the *epsRXABCDEFGHIJKLorfY* operon from NIZO B40 plasmid pNZ4000. The genes *epsR*, *epsX*, *epsA*, *epsB*, *epsC*, and *epsD* hybridized with the EPS plasmids of all five strains, and *epsL* and *orfY* hybridized with those of NIZO B40, NIZO B35, NIZO B39, and NIZO 1137, indicating their conservation in all gene clusters. The other *eps* genes of NIZO B40 only hybridized with NIZO B40 plasmid pNZ4000.

To further determine the organization of the different *eps* gene clusters, specific primers based on the NIZO B40 *eps* gene cluster were used for PCRs to detect fragments overlapping *epsRX*, *epsXA*, *epsAB*, or *epsBC* (Fig. 1C). For the *epsRX*, *epsAB*, and *epsBC* fragments, all of the strains yielded PCR products identical in size (results not shown). For the *epsXA* fragments, NIZO B39, NIZO B891, and NIZO B1137 yielded PCR products that were 165 bp larger than those of NIZO B35 and NIZO B40 (results not shown). These results confirm the homologies found by the Southern blot analysis and indicate that all of the gene clusters contain a conserved region with the same organization i.e., *epsRXABC*.

NIZO B35 and NIZO B891 *eps* genes. To study the function of the priming GTF genes, strains NIZO B35 and NIZO B891 were selected because they represent the two major groups with an EPS structure that differs markedly from that of strain NIZO B40 (Table 2). Overlapping fragments of the *eps* gene clusters of NIZO B35 and NIZO B891 that hybridized with the NIZO B40 *epsD* probe were cloned and sequenced (Fig. 1). The homologies of the deduced gene products are listed in Table 3. Unexpectedly, the NIZO B35 gene cluster contained two different genes that are homologous to NIZO B40 *epsD* (*orfU* and *epsD*, respectively). To test which of these *epsD*-like genes encodes the priming GTF activity, each of these was cloned under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter in pUC18 and GTF activities were determined in *E. coli*. When NIZO B35 *epsD* was induced in *E. coli*, galactosyltransferase activity could be detected (Fig. 2A). However, when *orfU* was induced, no GTF activity could be detected (data not shown). Simultaneous induction of both *orfU* and *epsD* from NIZO B35 resulted in the same galactosyltransferase activity as that found with NIZO B35 *epsD* alone (data not shown). These results indicate that NIZO B35 *epsD* encodes a priming GTF activity and *orfU* is either not involved in these synthetic steps, poorly expressed, or unstable.

The products of the NIZO B891 *epsD*, *epsE*, and *epsF* genes are expected to be the GTFs involved in the first two steps of EPS biosynthesis in this strain, as they are homologous to NIZO B40 *epsD*, *epsE*, and *epsF*. Fragments containing NIZO B891 *epsD* and *epsDEF* were cloned under control of the *lac* promoter in medium-copy-number expression vector pJF119HE, since attempts to clone them in pUC18 were unsuccessful. When NIZO B891 *epsD* was expressed in *E. coli*, only glucosyltransferase activity could be detected (Fig. 2B, lanes 1 and 2). When *epsDEF* was expressed, both glucosyltransferase and galactosyltransferase activities could be de-

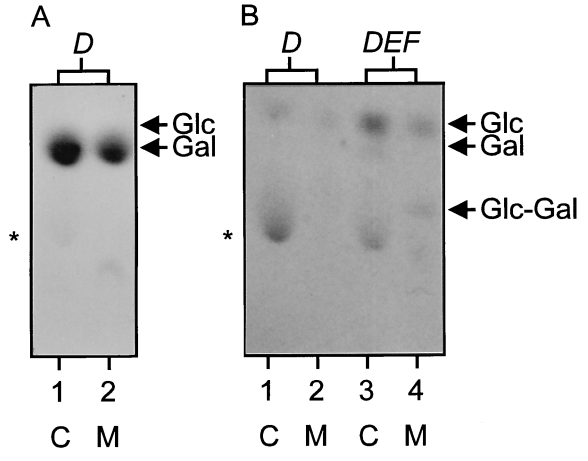

FIG. 2. TLC of ^{14}C-labelled intermediates isolated from the lipid fraction of permeabilized *E. coli* cells. (A) *E. coli* expressing NIZO B35 *epsD* incubated with UDP-[^{14}C]galactose. (B) *E. coli* expressing NIZO B891 *epsD* (1, 2) or NIZO B891 *epsDEF* incubated with a combination of UDP-[^{14}C]glucose and UDP-[^{14}C]galactose (3, 4). The positions of the standard sugars glucose (Glc), galactose (Gal), and lactose (Glc-Gal) are indicated on the right. The products which are nonspecific for lactococcal GTF activity are indicated by the asterisk on the left. C, complete acid hydrolysis; M, mild acid hydrolysis.

TABLE 4. Functional expression of streptococcal GTF genes to complement an *epsD* knockout in *L. lactis* NZ3900

Original host	Gene(s)	Specificity[a]	EPS production (mg liter^{-1})[b]
L. lactis NIZO B40	*epsD*	Glc	133
L. lactis NIZO B891	*epsD*	Glc	7.5
L. lactis NIZO B35	*epsD*	Gal	<0.5
S. pneumoniae type 14	*cps14E*	Glc	102
S. pneumoniae type 14	*cps14EFG*	Glc + Gal	3.9
S. thermophilus Sfi6	*epsE*	Gal	<0.5

[a] Glc, glucosyltransferase; Gal, galactosyltransferase.

[b] Amounts of EPS are the mean values of data from two independent cultures. The EPS production of *L. lactis* NZ3900 harboring pNZ4030 (wild type) is 113 mg liter^{-1}, and that of NZ3900 harboring pNZ4055 (Δ*epsD*) is <0.5 mg liter^{-1}. All EPSs had a monosaccharide composition identical to that produced by the wild type.

tected (Fig. 2B, lane 3) and the lipid-linked oligosaccharide had the same mobility on TLC as lactose (Fig. 2B, lane 4). The incorporation of ^{14}C-labelled sugars was approximately fivefold lower than that of cells expressing NIZO B40 or NIZO B35 *eps* genes (data not shown), and this lower GTF activity resulted in an increase in the appearance of a product in the complete acid hydrolysates which is nonspecific for lactococcal GTF activity (Fig. 2B, lanes 1 and 3). These results demonstrate that NIZO B891 *epsD* encodes a glucosyltransferase linking glucose to the lipid carrier and *epsE* and/or *epsF* encode a galactosyltransferase linking galactose via a β-1,4 linkage to lipid-linked glucose, resulting in lipid-linked lactose. Methylation analysis of NIZO B891 EPS has confirmed the presence of 1,4-linked glucose and galactose residues (32). In analogy to the homologous pneumococcal proteins Cps14F and Cps14G (11), EpsF is expected to contain GTF activity while EpsE is expected to have an accessory function.

Homologous and heterologous complementation of a NIZO B40 *epsD* mutant. To analyze the function of the GTFs in a gram-positive host, we constructed pNZ4055, a pNZ4000 derivative in which the *epsD* gene was replaced with an erythromycin resistance (*ery*) gene. This was achieved through a double crossover with a pGhost8 derivative containing the *ery* gene from pIL252 flanked by NIZO B40 *epsC* and *epsEF*. The *ery* gene has no terminator, ensuring expression of the downstream genes (34). *L. lactis* harboring pNZ4055 was erythromycin resistant and produced no EPS. To test whether the *epsD* knockout could be complemented, the pNZ8020 derivative pNZ4070 carrying the NIZO B40 *epsD* gene under control of the lactococcal *nisA* promoter was cotransformed with pNZ4055 into *L. lactis* NZ3900, which allows the use of the NICE (nisin-controlled expression) system (5, 13). Upon induction with nisin A, the EPS production of the resulting heteroplasmid strain was even higher than that of the wild-type strain, demonstrating that controlled overexpression of the *epsD* gene was achieved (Table 4). To test their heterologous complementation ability, various priming GTF genes from *L. lactis*, *S. thermophilus*, and *S. pneumoniae* were cloned in pNZ8020. The EPS produced by cultures of *L. lactis* harboring pNZ4055 and pNZ8020 derivatives was quantified, and the monosaccharide composition was determined (Table 4). The NIZO B40 and NIZO B891 genes encoding glucosyltransferases were able to complement the EPS-deficient phenotype. While expression of the NIZO B40 *epsD* gene restored EPS production completely, the amount of EPS produced by expression of NIZO B891 *epsD* was dramatically lower. A low GTF activity of the NIZO B891 EpsD compared to that of NIZO B40 EpsD was also found in *E. coli* (see above). In contrast, complete restoration of wild-type EPS production by heterologous complementation was achieved by using the *cps14E* gene of *S. pneumoniae* type 14 (Table 4). This gene is involved in pneumococcal capsule synthesis, encoding the priming glucosyltransferase (11), and is homologous to NIZO B40 *epsD* (34). Expression of the NIZO B35 *epsD* or the *S. thermophilus* Sfi6 *epsE* gene (30), both encoding a galactosyltransferase, did not complement the EPS-deficient phenotype (Table 4), indicating that a matching sugar specificity is required for transcomplementation. Although expression of *cps14E* restored EPS production completely, complementation with the pneumococcal *cps14EFG* genes resulted in reduced production of wild-type EPS compared to complementation with *cps14E* alone. The products of the *cps14F* and *cps14G* genes are involved in the second step of serotype 14 CPS biosynthesis linking galactose to lipid-linked glucose (11). Therefore, it is likely that they will compete for the lipid-linked glucose as the acceptor molecule with the products of the NIZO B40 *epsE* and *epsF* genes that link glucose to it, resulting in lipid-linked cellobiose (35). If so, it may be assumed that the lipid-linked lactose resulting from Cps14F and Cps14G activity cannot be used for NIZO B40 EPS biosynthesis, hence lowering NIZO B40 EPS production. These results demonstrate that functional expression of gram-positive GTFs in *L. lactis* is possible and may result in heterologous complementation when the enzymes are alike in sugar specificity.

DISCUSSION

We have analyzed the diversity of GTF genes of 16 different ropy *L. lactis* strains and the EPSs they produced, allowing division into three major groups and two individual strains. The grouping observed is in agreement with the known structural EPS information, as the EPSs produced by group I strains NIZO B40 and SBT 0495 are identical and differ from those of strains H414 (group II) and NIZO B891 (group III) (8, 18, 32, 34). Furthermore, methylation analysis of the EPS produced by strain NIZO B35 (group II) demonstrated that it contains the same galactose linkages as the H414 EPS and it is expected to have an identical EPS repeating unit (33). The sugar specificity of the GTFs needed for EPS biosynthesis in the different groups can be predicted according to the sugars present in the EPSs. The results suggest that EPS biosynthesis in all groups requires active galactosyltransferases, while groups I and III also need glucosyltransferases and only group I needs rhamnosyltransferases.

The genetic organization of the lactococcal *eps* gene clusters is conserved with respect to the first genes *epsRXABC*, which seem to be highly homologous for all strains. Furthermore, these genes share the most homology with those of other gram-positive polysaccharide biosynthesis gene clusters, including those of *S. aureus*, *S. pneumoniae*, *S. agalactiae*, and *S. thermophilus* (34). These homologies are confirmed for the NIZO B891 *epsB* and *epsC* and NIZO B35 *epsC* gene products by analysis of their nucleotide sequences, demonstrating that these genes are common to gene clusters involved in the biosynthesis of many gram-positive polysaccharide types (Table 3). It is likely that they will be involved in general functions and not directly related to the composition of the polymer produced (16, 25, 29).

The *epsL* and *orfY* genes have homologues in all of the lactococcal gene clusters tested. The function of these genes is unknown. OrfY is homologous to the regulator protein LytR from *Bacillus subtilis* (34). NIZO B40 *epsL* can be disrupted by single crossover using an internal gene fragment or overproduced without any effect on EPS production (36). Nonetheless,

epsL- and *orfY*-like genes are also found at the end of the *eps* gene cluster from *S. thermophilus* CNRZ368 adjacent to an IS element (1).

The genetic organization of the NIZO B35 *eps* gene cluster differs from that of NIZO B40 and NIZO B891 by an interruption of the gene cluster by an IS*982* element after the first GTF gene. An almost identical IS element is located upstream of the NIZO B40 *eps* gene cluster (Fig. 1A). Furthermore, the NIZO B35 gene cluster differs by containing two *epsD*-like genes, of which only one is actively involved in the first step of EPS biosynthesis, as was shown by the analysis of the products formed in the GTF activity assays of *E. coli* cells expressing NIZO B35 *epsD*, *orfU*, or *epsD* and *orfU*. A possible explanation for the differences in organization of the NIZO B35 *eps* gene cluster is that it has undergone rearrangement mediated by the IS element and received an additional *epsD* gene from another *eps* gene cluster. Horizontal gene transfer of parts of polysaccharide gene clusters has been observed in various bacteria, including *S. pneumoniae* (3).

All 16 of the *L. lactis* strains studied carry an *epsD* homologue which was cloned and subjected to functional analysis for strains NIZO B35 and NIZO B891. The product of the NIZO B891 *epsD* gene is a glucosyltransferase that is more homologous to NIZO B40 EpsD than to the product of the NIZO B35 *epsD* gene, which is a galactosyltransferase (Table 3). Sequence alignment of several EpsD-like proteins from different polysaccharide biosynthesis systems with known glucosyl- or galactosyltransferase activity showed three blocks that are conserved in all of the proteins (40). An alignment of the EpsD-like gram-positive GTFs with known sugar specificity shows that the three blocks are also conserved in these proteins (Fig. 3). Blocks A and B are predicted to interact with the lipid carrier, and block C is supposed to contain specific conserved residues for each type of transferase (40). From these, only a galactosyltransferase-specific tyrosine was observed (Fig. 3) and different residues appeared to be conserved for the gram-positive GTFs, demonstrating that the previously reported residues are not critical in determining sugar specificity. GTF activity involves amino acids that can catalyze an acid-base reaction. Hydrophobic cluster analysis of various β-GTFs has shown two aspartic acid residues with a spacing of approximately 50 amino acids to be conserved, and these are predicted to be the catalytic residues (28). Four conserved aspartate residues (D) and two conserved glutamate residues (E) were found for the gram-positive GTFs (Fig. 3), two of which are likely to be the catalytic residues. Two possible candidates are the conserved E residue in block C in combination with the conserved D residue in the C terminus just outside block C, which are separated by 50 amino acids (51 in Cps14E). The amino acid sequence of NIZO B35 OrfU lacks 30 amino acids at its C terminus compared to the other priming GTFs, including this conserved aspartate.

Disruption of the NIZO B40 *epsD* gene could be complemented by homologous expression of NIZO B40 *epsD* and heterologous expression of NIZO B891 *epsD* or the streptococcal capsule biosynthesis gene *cps14E*, which is known to be involved in a similar reaction (10). The use of a controlled expression system enabled the expression of GTFs that did not complement the mutation and could be toxic to the cell as a result of the accumulation of lipid-linked intermediates (NIZO B35 *epsD*, *S. thermophilus epsE*, and *S. pneumoniae cps14EFG*), as has been reported for the heterologous expression of several gram-negative GTFs (21). Moreover, to the best of our knowledge, this is the first demonstration of functional heterologous expression of a GTF gene in a gram-positive host allowing the expression of GTF genes from different origins by the shotgun

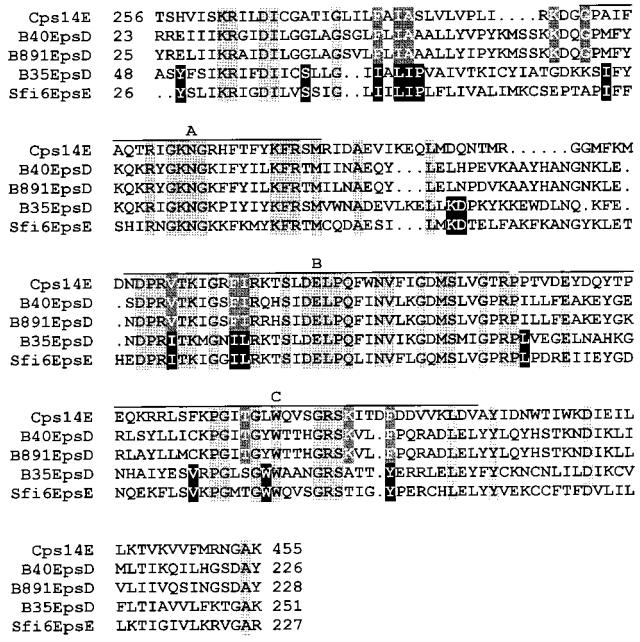

FIG. 3. Multiple-sequence alignment of priming GTFs with known sugar specificity from gram-positive bacteria. Cps14E, B40EpsD, and B891EpsD are glucosyltransferases from *S. pneumoniae* serotype 14 (11) and *L. lactis* NIZO B40 and NIZO B891, respectively. B35EpsD and Sfi6EpsE are galactosyltransferases from *L. lactis* NIZO B35 and *S. thermophilus* Sfi6 (30), respectively. Residues conserved in all five sequences, residues conserved only in glucosyltransferases, and residues conserved only in galactosyltransferases are shaded light grey, dark grey, and black, respectively. The three conserved blocks (A, B, and C) described by Wang et al. (40) are indicated.

or directed-cloning approach in *L. lactis*. Furthermore, these results demonstrate that the enzymes involved in the biosynthesis of different polysaccharides can be functionally coupled, although the *eps* genes are located on different transcriptional units. The possibility of constructing clean deletion mutations in the lactococcal *eps* gene cluster combined with the use of the NICE expression system, enabling induced expression of GTF genes, opens the way to polysaccharide engineering in *L. lactis* and provides a new approach to the study of polysaccharide biosynthesis genes of gram-positive cocci.

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