

Exopolysaccharide Production and Ropy Phenotype Are Determined by Two Gene Clusters in Putative Probiotic Strain *Lactobacillus paraplantarum* BGCG11

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Lactobacillus paraplantarum BGCG11, a putative probiotic strain isolated from a soft, white, artisanal cheese, produces a high-molecular-weight heteropolysaccharide, exopolysaccharide (EPS)-CG11, responsible for the ropy phenotype and immunomodulatory activity of the strain. In this study, a 26.4-kb region originating from the pCG1 plasmid, previously shown to be responsible for the production of EPS-CG11 and a ropy phenotype, was cloned, sequenced, and functionally characterized. In this region 16 putative open reading frames (ORFs), encoding enzymes for the production of EPS-CG11, were organized in specific loci involved in the biosynthesis of the repeat unit, polymerization, export, regulation, and chain length determination. Interestingly, downstream of the *eps* gene cluster, a putative transposase gene was identified, followed by an additional *rfb* gene cluster containing the *rfbACBD* genes, the ones most probably responsible for dTDP-L-rhamnose biosynthesis. The functional analysis showed that the production of the high-molecular-weight fraction of EPS-CG11 was absent in two knockout mutants, one in the *eps* and the other in the *rfb* gene cluster, as confirmed by size exclusion chromatography analysis. Therefore, both *eps* and *rfb* genes clusters are prerequisites for the production of high-molecular-weight EPS-CG11 and for the ropy phenotype of strain *L. paraplantarum* BGCG11.

A “functional starter” is defined as “cultures that possess properties which contribute to food safety and/or offer one or more organoleptic, technological, nutritional, or health advantages” (1). Accordingly, some strains of lactic acid bacteria (LAB) that produce exopolysaccharides (EPSs) meet both technological and health aspect criteria. Recently, it has been shown that EPS-producing LAB strains might be capable of improving the rheological characteristics of fermented milk products, as well as having immunomodulatory potential (2, 3).

Bacterial exopolysaccharides (EPSs) represent a large group of carbohydrate polymers which can be either covalently associated with the cell surface, forming a capsule, or loosely attached or even totally secreted into the environment of the cell (2). EPSs are classified as homo- or heteropolysaccharides, depending on whether they are composed, respectively, of one or more than one type of sugar. EPSs are widely distributed among bacteria. They can have a protective function in the natural environment against phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds, and osmotic stress. Moreover, EPSs also have a role in cell recognition, adhesion to surfaces, and biofilm formation (4). EPSs can interfere with adhesion of pathogens and probiotics to intestinal mucus or have an immunomodulatory effect (3, 5–9). Furthermore, EPSs can have the ability to lower the cholesterol and triglyceride levels in the serum and liver of rats (10). For that reason, detailed characterization of the genes involved in EPS production is of great importance.

The genetic information for heteropolymer-type EPS production is organized into gene clusters, located in chromosomal or plasmid DNA. Sometimes more than one EPS operon can be found in the genome of bacteria (11, 12). In the *eps* operons several genes with specific functions are present: genes involved in the assembling of repeating units, chain length determination, polymerization, and export and those involved in regulation (13).

Heteropolysaccharides are made by polymerizing repeating-unit precursors formed in the cytoplasm. The sugar nucleotides, such as UDP-glucose, UDP-galactose, and dTDP-rhamnose, play an essential role in heteropolysaccharide biosynthesis due to their role in the sugar activation necessary for monosaccharide polymerization (13). It has been shown previously that a 9-fold increase in the number of EPS plasmid copies can lead to an almost 3-fold increase in the *eps* expression level, which results in an almost 4-fold increase in the EPS production level in *Lactococcus lactis* NIZO B40. In the same study the authors indicated that the maximum amount of NIZO B40 EPS production is limited by the level of the expression products of the *eps* gene cluster rather than by the level of EPS precursors (14).

In lactobacilli, *eps* gene clusters have been identified in *Lactobacillus helveticus* NCC2745, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus rhamnosus*, and *Lactobacillus plantarum* (15–18), among others. In *Lactobacillus paraplantarum* BGCG11, formerly classified as *Lactobacillus casei* CG11, isolated from a soft,

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TABLE 1 Plasmid and constructs used in this study

Name	Relevant characteristic(s) ^a	Source or reference
pAZIL	Shuttle cloning vector, 7,109 bp, Em ^r	28
pAZIL-BH18.5	18,466-bp BamHI fragment from pCG1 cloned in BamHI site of pAZIL, Em ^r	This work
pAZIL-BH3	3,018-bp BamHI fragment from pCG1 cloned in BamHI site of pAZIL, Em ^r	This work
pAZIL-Bg4.27	4,266-bp BglII fragment from pCG1 cloned in BglII site of pAZIL, Em ^r	This work
pAZIL-E650	656-bp EcoRI fragment from pCG1 cloned in EcoRI site of pAZIL, Em ^r	This work
pAZIL-BHBc1.3	1,328-bp BamHI/BclI fragment from pCG1 cloned in BamHI/BclI site of pAZIL, Em ^r	This work
pAZIL-BgBH1	996-bp BglII/BamHI fragment from pCG1 cloned in BglII/BamHI site of pAZIL, Em ^r	This work
pGhost9	Em ^r , vector for insertional mutagenesis with a temperature-sensitive origin of replication	31
pGhost/31	pGhost9 derivative carrying part of the <i>orf2</i> gene	This work
pGhost/32	pGhost9 derivative carrying part of the <i>rfbB</i> gene	This work
p-GEM-T-Easy	3,015 bp, Amp ^r , PCR cloning vector	Promega

^a Em^r, resistance to erythromycin; Amp^r, resistance to ampicillin.

white, artisanal cheese, the assumption of plasmid localization of the *eps* gene cluster was proposed after plasmid-curing experiments when some derivatives lacking the pCG1 plasmid had lost their EPS-CG11 production and ropy phenotype (19). In previous work it was shown that when *L. paraplantarum* BGCG11 was grown in minimal medium supplemented with glucose as the only sugar source, EPS-CG11 was composed of glucose (75.7%), rhamnose (20.5%), galactose (2.1%), and mannose (1.7%). In the same medium, novobiocin (NB) derivatives which had lost the ropy phenotype produced residual EPS in an amount almost 10 times lower and with a different composition of sugars: glucose (86.6%), mannose (6.2%), galactose (4.1%), and rhamnose (3.1%) (20). The biosynthesis of more than one EPS polymer by one strain was noticed previously (21–24). Recently, it has been shown that the EPS-CG11 polymer is responsible for *in vitro* anti-inflammatory or immunosuppressive effects (3). Taking these findings into consideration, together with the fact that there have been no data on functional *eps* genes clusters in *L. paraplantarum* until now, the aim of this study was to decipher the genetic organization of the *eps* gene cluster in the strain *L. paraplantarum* BGCG11.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. paraplantarum* BGCG11 and its derivative NB1 were grown at 30°C in deMan-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany). *Escherichia coli* DH5 α cells, used as cloning hosts, were grown in LB medium with aeration at 37°C. When appropriate, erythromycin (Em) (Serva, Heidelberg, Germany) was added at 300 $\mu\text{g ml}^{-1}$ for *E. coli*.

DNA manipulations. Plasmid DNA from *E. coli* cells was isolated using a Qiagen Plasmid Mini-Prep kit (Qiagen, Hilden, Germany). Total DNA from *L. paraplantarum* BGCG11 and NB1 was extracted as previously described (25) with a slight modification (cells were treated with lysozyme before lysis with SDS). Plasmid DNA was isolated and purified as previously described (26). Restriction enzymes (Fermentas UAB, Vilnius, Lithuania) were used as recommended by the manufacturer. The plasmids and DNA fragments were analyzed by horizontal electrophoresis on 1% (wt/vol) agarose gels, in 1 \times TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) buffer with ethidium bromide (0.5 $\mu\text{g ml}^{-1}$), at a constant voltage of 2.5 V cm^{-1} and visualized using a charge-coupled-device (CCD) camera (Biometra BDR2/5/6; Bio Doc Analyze GmbH, Göttingen, Germany). GeneRuler DNA ladder mix (Fermentas UAB) was used for comparison of the sizes of obtained DNA fragments.

Southern blot hybridization. Hybridization experiments were carried out essentially as previously described (27). DNA probes were labeled following the instructions of the manufacturer using a digoxigenin (DIG) DNA labeling and detection kit (Roche Applied Science, Mannheim, Germany). Hybridizations were carried out at 65°C.

Plasmid construction. The plasmids and constructs used in the study are listed in Table 1. In order to obtain the nucleotide sequence(s) of the *eps* gene cluster, total plasmids from *L. paraplantarum* BGCG11, as well as from the BGCG11 EPS-negative (EPS⁻) derivative NB1 (for comparison), were subjected to BamHI restriction enzyme digestion. The DNA fragments obtained were ligated into pAZIL vector (28) predigested with BamHI, using T4 DNA ligase (Promega, Madison, USA) in the buffer as recommended by the manufacturer. Nucleotide sequencing of the 18,466-bp BamHI fragment revealed that it contained part of the *eps* gene cluster. In order to search the neighboring DNA region and to complete the cloning of the entire *eps* gene cluster, the next step was performed by constructing new plasmid libraries in the pAZIL vector using BclI and BglII restriction enzymes. Overlapping the BclI clone with the previously cloned 18,466-bp BamHI fragment was done by Southern blot hybridization using an E650 DNA probe to map in the overlapping region at the end of the pAZIL-BH18.5 clone, resulting in the clone pAZIL-Bc7.25. The downstream part of the *eps* gene cluster was selected by screening the already constructed BamHI plasmid library using the BHBc1.3 probe, resulting in the overlapping clone pAZIL-BH3. To complete the cloning of the *eps* gene cluster located in the pCG1 plasmid, an additional clone, pAZIL-Bg4.27, was selected using the probe BgBH1 from the constructed BglII plasmid library. The complete *eps* and *rfb* gene clusters were assembled by selecting overlapping clones obtained from three independent plasmid libraries (constructed by BamHI, BclI, and BglII restriction enzymes) of the pCG1 plasmid. The construct pAZIL-BH18.5 carrying the BamHI fragment (18,466 bp), was selected for further work. The BamHI fragment (18,466 bp) was originally from the plasmid pCG1, which is present only in the parental strain BGCG11. That was confirmed by Southern blot DNA-DNA hybridization using a 656-bp EcoRI DNA fragment subcloned from the pAZIL-BH18.5 into the pAZIL vector (named pAZIL-E650) as a probe. The total plasmids pCG11 and pNB1, digested with several restriction enzymes (see Fig. S1a in the supplemental material), were used for hybridization (see Fig. S1b). It was confirmed that pAZIL-BH18.5 contains a DNA fragment from the plasmid not present in the derivative NB1. Based on the nucleotide sequence analysis of the DNA fragment in the pAZIL-E650 construct (98% identity with the part of the sequence belonging to the polysaccharide biosynthesis protein [GenBank accession number YP_008122774.1] from *L. plantarum* 16), the whole DNA fragment in pAZIL-BH18.5 (Fig. 1) was analyzed. Since an 18,466-bp BamHI fragment in pAZIL-BH18.5 did not contain the complete information for the *eps* gene cluster, additional cloning of neighboring DNA regions from pCG1 was performed (Fig. 1). The continuation of the genetic information for EPS-CG11 production was mapped on the construct pAZIL-Bc7.25, selected from the BclI plasmid library. In the next two steps, overlapping fragments with additional DNA were selected, first from the BamHI (the clone pAZIL-BH3) and then from the BglII (the clone pAZIL-Bg4.27) plasmid libraries. Complete information for EPS-CG11 production, consisting of two gene clusters, was cloned by

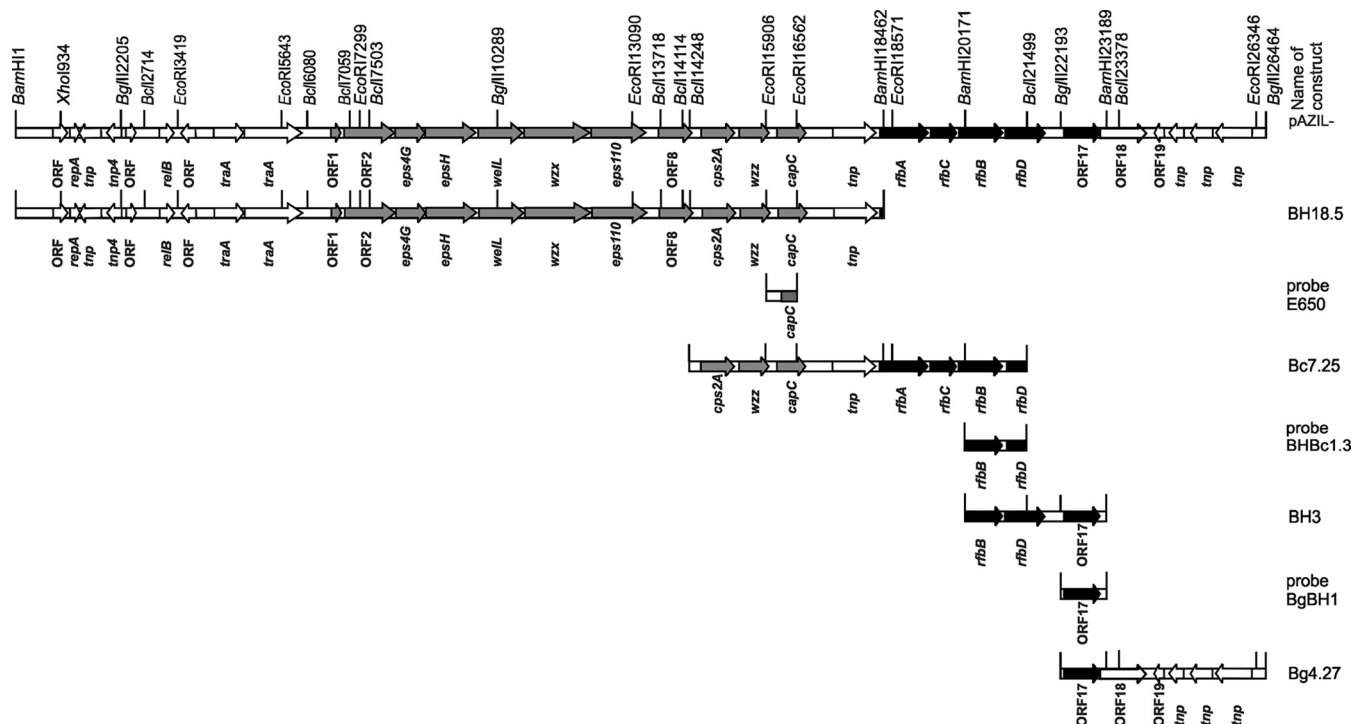


FIG 1 Linear gene map of the pCG1 region carrying *eps* gene clusters of 26,464 bp and the scheme of construction of overlapping clones used for sequencing and probes for Southern blot hybridization. Positions of relevant restriction sites are indicated. The sizes and orientations of predicted ORFs are indicated by arrows. Genes representing the *eps* gene cluster are indicated by gray arrows; those for specific *rfb* gene clusters are indicated by black arrows.

combining clones from the three constructed plasmid libraries of the pCG1 plasmid.

Sequencing and annotation of the putative EPS biosynthetic cluster.

DNA sequencing reactions were performed by Macrogen Europe (Amsterdam, The Netherlands), a nucleotide sequencing service. The obtained nucleotide sequences were assembled into a ca. 17-kb contig containing the complete nucleotide sequence of the putative *eps* gene clusters of *L. paraplantarum* BGCG11. The NCBI Entrez nucleotide database was searched for similar sequences using BLASTx, and alignment of overlapping fragments was performed with BLASTn (29). Membrane-spanning regions of translated gene products were predicted using the Phobius program (<http://phobius.sbc.su.se/>). Additionally, the Pfam database (30) was searched for conserved motifs in the translated gene products.

Construction of the knockout mutants. The genes *orf2* (GenBank accession number [HG316787](#), position 6949 to 7917) and *rfbB* (GenBank accession number [HG316787](#), position 19902 to 20930) were inactivated in pCG1 by homologous integration using a protocol described by Maguin et al. (31). For insertional inactivation, a part of the *orf2* gene and a part of the *rfbB* gene were amplified using the primer pair ORF2F (5'-C TAAGACTAGTAAGGATGC-3') and ORF2R (5'-GCTTAGCTCCCAAA ATCCAGG-3') and the pair RFBBF (5'-GGTAATCGGGCCAACCTGG-3') and RFBRR (5'-CGTAAATCATGGCCGGGACG-3'), respectively. KapaTaq DNA polymerase (Kapa Biosystems, INC., Boston, MA, USA) was used to amplify DNA fragments by PCR using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with a QiaQuick PCR purification kit (Qiagen) according to the protocol of the supplier. The commercial vector p-GEM-T-Easy (Promega) was used for cloning the PCR products. Standard heat shock transformation was used for plasmid transfer into *E. coli*. For plasmid isolation from *E. coli*, a QIAprep Spin Miniprep kit was used according to the manufacturer's recommendations (Qiagen, Hilden, Germany). A fragment from the *orf2* gene (NcoI-NcoI, 674 bp, position 7070 to 7744) was cloned into the pGhost9 vector, resulting in the construct pGhost/31.

A fragment from the *rfbB* gene (NcoI-NcoI, 742 bp, position 20019 to 20761) was cloned into the pGhost9 vector, resulting in the construct pGhost/32. Plasmid constructs were sequenced by the Macrogen Sequencing Service (Macrogen, Netherlands). In the next step, strain BGCG11 was transformed with pGhost/31 and pGhost/32, individually, and grown on MRS plates containing 7.5 µg/ml of erythromycin at 28°C for 48 h. Obtained transformants were transferred to 37°C and incubated for 48 h, resulting in the knockout mutants MUT1 for the *orf2* gene and MUT2 for the *rfbB* gene.

EPS extraction, purification, and analysis by high performance liquid chromatography (HPLC). EPS was isolated and purified from BGCG11, its derivative NB1, and both knockout mutants (MUT1 and MUT2) from cultures grown in basal minimum medium (BMM). The purified EPS was analyzed by means of size exclusion chromatography (SEC) coupled with a multiangle laser light scattering (MALLS) detector. These procedures were done as described by Nikolic et al. (3). In short, EPS fractions were separated in size exclusion columns (TSKgel G3000 PW_{XL} plus TSKgel G5000 PW_{XL} protected with a TSKgel guard column; Supelco-Sigma) at 40°C with a flow rate 0.45 ml min⁻¹ using 0.1 M NaNO₃ as the mobile phase. The chromatographic equipment (Waters, Milford, MA) was coupled to a photodiode array (PDA) 996 detector, set at 280 nm for protein detection, and to a 410 refractive index (RI) detector, which allows the quantification of each EPS fraction by using calibration equations made with dextran standards. The Empower software (Waters) was used for analysis. Additionally, a third static MALLS detector (Dawn Heleos II; Wyatt Europe GmbH, Dembach, Germany) was coupled in series to analyze the molar mass distribution of the EPS fractions using the software Astra, version 3.5 (Wyatt Europe GmbH).

Nucleotide sequence accession number. The complete *eps* and *rfb* gene clusters with surrounding genes were submitted to the European Nucleotide Archive (ENA) and GenBank under accession number [HG316787](#).

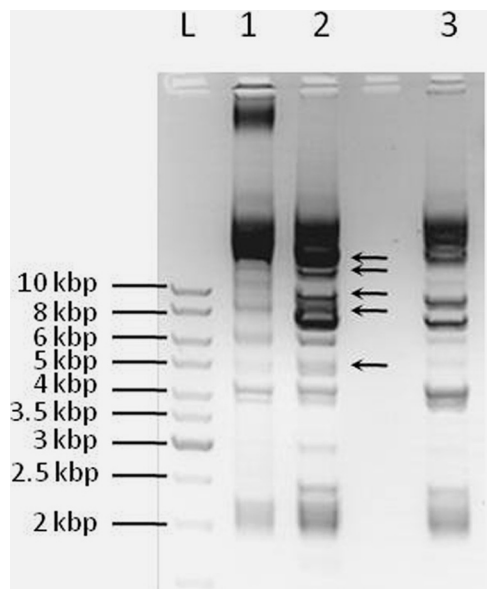


FIG 2 The comparison of the total plasmid profile of *L. paraplantarum* BGCG11 (pCG11) and the EPS-CG11-negative derivative NB1 (pNB1). Lane L, GeneRuler DNA ladder mix; lane 1, pCG11; lane 2, BamHI restriction digestion of total pCG11; lane 3, BamHI restriction digestion of total pNB1. The arrows indicate plasmid bands present only in BGCG11.

RESULTS AND DISCUSSION

Exopolysaccharides produced by LAB display a wide variety of composition, structure, and size of molecules (22). Although these macromolecules are synthesized in representatives of different LAB, such as *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, organization of the genes involved in EPS synthesis is conserved constitutively. Until now, a large number of operons for EPS production have been characterized, and it has been noticed that *eps* clusters in LAB have a conserved functional structure, with most genes found in the same position (except for in *L. rhamnosus*) (17, 22). In this work we have identified, sequenced, and functionally characterized a 26,464-bp region from the pCG1 plasmid present in *L. paraplantarum* BGCG11 containing 16 open reading frames (ORFs) assumed to be involved in EPS-CG11 biosynthesis.

Cloning of the EPS-CG11 gene clusters. Total plasmids isolated from *L. paraplantarum* BGCG11 (pCG11) and its EPS-CG11-negative derivative NB1 (pNB1) were subjected to BamHI digestion in order to distinguish the DNA fragments belonging to the pCG1 plasmid (responsible for the EPS-CG11 production), present in BGCG11 but not in the NB1 derivative (Fig. 2). It was noticed that the resulting BamHI restriction profile of the pCG1 plasmid showed extra BamHI plasmid bands originating from the pCG1 plasmid. A pCG1 plasmid library consisting of BamHI fragments from the pCG11 plasmid cloned into the pAZIL vector was constructed as described in Materials and Methods. Afterwards, restriction analysis of the obtained clones was performed with the same enzyme, as described above.

Identification of the *eps* and *rfb* gene clusters and putative functions of predicted ORFs. It was found that the *eps* gene cluster, located in a 17-kb region of the pCG1 plasmid as proposed previously (19), has a unique structure and is divided into two gene clusters by a transposase gene. The functional structure of the

eps gene cluster was similar to that of previously known operons for EPS production as it has genes for enzymes responsible for regulation, polymerization, export, assembling repeating units, and chain length determination (32). The complete sequence of 26,464 bp consists of 31 predicted ORFs, 16 of which represent the gene cluster responsible for EPS-CG11 production (Table 2 and Fig. 3). The other genes are involved in plasmid replication, mobilization, and transposition.

The ORFs thought to be involved in the glycosyltransferase (GTF) function are located at the beginning of the *eps* gene cluster. EPS-CG11 biosynthesis starts with a priming *gtf* gene, *orf1*, at the beginning of *eps* gene cluster. ORF1 has 77% identity at the amino acid level with a putative priming glycosyltransferase (p-GTF) of *L. plantarum* 16 (Table 2). The product of this gene has a role in the first (priming) step in the synthesis of an oligosaccharide “block” since it is a member of the family of EPS biosynthesis polyprenyl glycosylphosphotransferases which transfer the sugar from, for instance, UDP-glucose or UDP-galactose to a lipid carrier, such as undecaprenyl phosphate. This gene is highly evolutionarily conserved (33). In comparison to previously described *eps* gene clusters, the GTF that initiates EPS biosynthesis could be located at the beginning of the *eps* gene cluster, as in *Lactobacillus helveticus* NCC2745 (15), downstream from the other genes in the *eps* gene cluster, in the central region of the *eps* clusters, as in *L. lactis* NIZO B40 (34), *Streptococcus thermophilus* strains (35, 36), and *L. delbrueckii* subsp. *bulgaricus* Lfi5 (16), or even near the end of 3' region, as in the case of *L. rhamnosus* (17). Downstream of *orf1* there are five genes assigned as GTFs, and their function should be in transferring the other sugars of the EPS subunit in an ordered way. Among them, the genes *orf2*, *epsH*, and *well* encode proteins belonging to different GTF superfamilies (families 8, 11, and A, respectively), while *eps4G* encodes a putative ribitol phosphotransferase. Among other GTFs encoded by the *eps* gene cluster, *Eps4G* showed more than 50% amino acid identity with a putative ribitol phosphotransferase in *Streptococcus pneumoniae*, while others (ORF2, *EpsH*, and *Well*) share less than 40% amino acid identity with GTF gene products. ORF8, as a putative 1,6-D-galactosyltransferase, might have a role in the addition of galactose to the EPS-CG11 polymer. ORF8 has 42% identity at the amino acid level with UDP-D-galactose, for example, the (glucosyl) lipopolysaccharide-1,6-D-galactosyltransferase from *Lactobacillus fermentum* IFO 3956 (Table 2). These GTF gene products are involved in the synthesis of repeating-unit blocks that are linked to the lipid carrier at the inner side of the cytoplasmic membrane.

Interestingly, *orf8* is separated from the ORFs encoding other GTFs by two genes, *wzx* and the *eps110*. The flippase (*Wzx*) is potentially involved in translocation of the EPS subunits across the cytoplasmic membrane while the other protein, assigned as *Eps110*, is a polysaccharide polymerase and could be involved in the polymerization process of EPS into long polysaccharides on the outer side of the cytoplasmic membrane, as similarly described for *L. rhamnosus* GG (22). The *wzx* gene of BGCG11 is presumed to encode a polysaccharide biosynthesis protein (36% amino acid identity with *Lactobacillus reuteri*), a member of a family of integral membrane proteins, and shows 27% amino acid identity to the flippase from *Streptococcus sanguinis* (Table 2). Both *Wzx* and *Eps110* from the *eps* gene cluster could be integral membrane proteins since the prediction (<http://phobius.sbc.su.se/>) showed 13 transmembrane domains for *Wzx* and 10 transmembrane domains for *Eps110* (data not shown). The supposed function of

TABLE 2 Results of BLASTx comparison of predicted ORFs from *eps* and *rfb* gene clusters with the NCBI Entrez nucleotide database

ORF (no. of aa) ^a	Position (nt) ^b	Protein(s) with highest homology (GenBank accession no.)	Predicted domain(s) or superfamily in encoded ORF (identification no.) ^c	Organism	Amino acid identity (%)
<i>orf1</i> (62)	6746–6934	Putative priming glycosyltransferase (YP_008122775.1)	Bacterial sugar transferase (Pfam 02397); exopolysaccharide biosynthesis polyprenyl glycosylphosphotransferase (TIGR03025)	<i>Lactobacillus plantarum</i> 16	77
<i>orf2</i> (322)	6949–7917	Glycosyltransferase family 8 (WP_003663531)	Glycosyltransferase family 8 (Pfam 01501)	<i>Lactobacillus reuteri</i>	36
<i>eps4G</i> (232)	7956–8654	Putative ribitol phosphotransferase (CAI34193.1) Eps4G (AAN63683)	CDP-alcohol phosphatidyltransferase (cl00453)	<i>Streptococcus pneumoniae</i> <i>Streptococcus thermophilus</i>	51 48
<i>epsH</i> (341)	8680–9705	EpsH (NP_053026)	Glycosyltransferase family 11 (Pfam 01531)	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	33
<i>welL</i> (323)	9744–10715	Putative glycosyltransferase (WP_003566665) Well (CCC15461)	Glycosyltransferase family A (cl11394)	<i>Lactobacillus casei</i> <i>Lactobacillus pentosus</i> IG1	35 34
<i>wzx</i> (463)	10778–12169	Putative polysaccharide biosynthesis protein (WP_003676723) Flippase (WP_002911476)	Wzx, a subfamily of the multidrug and toxic compound extrusion (MATE)-like proteins (cd13128); polysaccharide biosynthesis protein (pfam01943), RfbX (COG2244)	<i>Lactobacillus reuteri</i> <i>Streptococcus sanguinis</i>	36 27
<i>eps11O</i> (369)	12195–13304	Eps11O, putative polysaccharide polymerase (AAN63798)	No domains	<i>Streptococcus thermophilus</i>	27
<i>orf8</i> (226)	13596–14276	UDP-D-galactose: (glucosyl)lipopolysaccharide-1, 6-D-galactosyltransferase (YP_001844233)	Glycosyltransferase_GTB_type (cl10013)	<i>Lactobacillus fermentum</i> IFO 3956	42
<i>cps2A</i> (256)	14522–15292	Exopolysaccharide biosynthesis protein (YP_008122772); Exopolysaccharide biosynthesis protein Cps2A	Chain length determinant protein (pfam02706); capsular polysaccharide biosynthesis protein (COG3944)	<i>Lactobacillus plantarum</i> 16 <i>Lactobacillus plantarum</i> WCFS1	95 92
<i>wzz</i> (239)	15304–16023	Exopolysaccharide biosynthesis protein, chain length determinant Wzz (CCC15467)	Capsular exopolysaccharide family (TIGR01007); chain length determinant protein tyrosine kinase EpsG (TIGR03029); exopolysaccharide transport protein family (TIGR01005); exopolysaccharide/PEP-CTERM locus tyrosine autokinase (TIGR03018)	<i>Lactobacillus pentosus</i> IG1	97
<i>capC</i> (257)	16010–16783	Capsular polysaccharide biosynthesis protein (CCC15468) Polysaccharide biosynthesis protein, phosphotyrosine-protein phosphatase (YP_008122774)	Capsular polysaccharide biosynthesis protein CapC (COG4464)	<i>Lactobacillus pentosus</i> IG1 <i>Lactobacillus plantarum</i> 16	91 94
<i>tnp</i> (309)	17355–18284	IS30 family transposase (YP_795436)	Transposase and inactivated derivatives, IS30 family (COG2826)	<i>Lactobacillus brevis</i> ATCC 367	99
<i>rfaA</i> (289)	18438–19307	Glucose-1-phosphate thymidyltransferase	Glucose-1-phosphate thymidyltransferase, short form, RmlA (TIGR01207); dTDP-glucose pyrophosphorylase RfbA (COG1209)	<i>Lactobacillus plantarum</i>	99
<i>rfaC</i> (193)	19311–19892	dTDP-4-dehydrorhamnose 3,5-epimerase (YP_003062617)	dTDP-4-dehydrorhamnose 3,5-epimerase, RmlC (TIGR01221); RfbC (COG1898)	<i>Lactobacillus plantarum</i> JDM1	99
<i>rfaB</i> (342)	19902–20930	dTDP-glucose 4,6-dehydratase (YP_004889090)	dTDP-D-glucose 4,6-dehydratase, RfbB (COG1088)	<i>Lactobacillus plantarum</i> WCFS1	99
<i>rfaD</i> (280)	20963–21805	dTDP-4-dehydrorhamnose reductase (WP_003646252)	RmlD substrate binding domain (Pfam 04321); dTDP-4-dehydrorhamnose reductase RfbD (COG1091)	<i>Lactobacillus plantarum</i>	95

(Continued on following page)

TABLE 2 (Continued)

ORF (no. of aa) ^a	Position (nt) ^b	Protein(s) with highest homology (GenBank accession no.)	Predicted domain(s) or superfamily in encoded ORF (identification no.) ^c	Organism	Amino acid identity (%)
<i>orf17</i> (233)	22271–22972	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (YP_003063113)	Glycosyltransferase family A (cl11394), UDP-N-acetylglucosamine diphosphorylase/glucosamine-1-phosphate N-acetyltransferase (TIGR01173); glucose-1-phosphate thymidyltransferase, long form (TIGR01208)	<i>Lactobacillus plantarum</i> JDM1	100
<i>orf18</i> (341)	22974–23999	Ribitol-5-phosphate 2-dehydrogenase (YP_003063114)	Medium chain reductase/dehydrogenase (MDR)/zinc-dependent alcohol dehydrogenase-like family (cl16912); threonine dehydrogenase and related Zn-dependent dehydrogenases (COG1063)	<i>Lactobacillus plantarum</i> JDM1	100

^a The number of amino acids (aa) encoded.

^b The position from the beginning of the sequence of pCG1 plasmid (from the BamHI restriction site). nt, nucleotides.

^c Identification numbers are from databases from Pfam protein families, clusters of orthologous groups of proteins (COG), The Institute for Genomic Research (TIGR), and NCBI Conserved Domains Database.

these two proteins is the polymerization and export process in EPS-CG11 biosynthesis since the proteins show similar homology to *S. thermophilus* and the *wzx* and the *wzy* genes in *L. rhamnosus* RW-9595M (17).

With high (95%) identity at the amino acid level with the EPS biosynthesis protein from *L. plantarum* 16, Cps2A belongs to a family of chain length determinant proteins (or Wzz protein) with a region of bacterial tyrosine kinases (Table 2). The product of the gene *wzx* showed 97% amino acid identity with the *Lactobacillus pentosus* IG1 chain length determiner Wzz, a member of the family of the chain length determinant protein-tyrosine kinase EpsG and also a member of the exopolysaccharide/PEP-CTERM locus tyrosine autokinase, with C-terminal tyrosine residues (likely to be autophosphorylation sites). The next gene product, CapC, has 94% identity at the amino acid level with a polysaccharide biosynthesis protein, phosphotyrosine-protein phosphatase, from *L. plantarum* 16 and 91% identity at the amino acid level with a capsular polysaccharide biosynthesis protein from *L. pentosus*. Gene products of *cps2A*, *wzx*, and *capC* have more than 90% identity at the amino acid level with previously described proteins involved in the regulation of EPS biosynthesis, strongly indicating their role in the regulation of the EPS-CG11 biosynthesis process. Cps2A can be located in the membrane since it has two transmembrane domains on the N and C termini of the predicted protein. With this characteristic, Cps2A resembles export proteins (Wzx, Wzy, Wzz, and homologues to ExoP-like proteins) that consist of a transmembrane domain containing two putative transmembrane helices and a domain containing a nucleotide-binding motif (32). In the previously described models for EPS biosynthesis in *L. rhamnosus* GG (22) and strains of *S. pneumoniae* (37), Wzz and Wzb (phosphotyrosine-protein phosphatase) are thought to be involved in the regulation of EPS biosynthesis. Taking this into

consideration, Wzz and CapC from the *eps* gene cluster might be part of the phosphorylation complex. Wzz is a member of the family of the chain length determinant protein-tyrosine kinase, with C-terminal tyrosine residues, which could be autophosphorylation sites, while CapC is a phosphotyrosine-protein phosphatase. The organization of the previously described *eps* gene clusters is similar to that of the *cps* gene cluster *cps1A-I* in *L. plantarum* WCFS1 (18).

Following the mentioned genes there is a *tnp* gene, and its product showed 99% amino acid identity with an IS30 family transposase from *Lactobacillus brevis* ATCC 367. The *tnp* gene separates the above-mentioned preceding genes in the *eps* gene cluster from the four genes putatively involved in dTDP-L-rhamnose biosynthesis (*rfbA*, *rfbC*, *rfbB*, and *rfbD*). Using the IS Finder reference database for insertion sequences (IS) (<https://www-isi.biotoul.fr/>), the presence of complete IS30 sequences was detected at positions between 17256 and 17305 nucleotides (left end) and between 18253 and 18298 nucleotides (right end). Also, partial IS sequences were found, as follows: a transposase of the IS30 family (position 24475 to 24771) had only the left end of the IS sequence at position 24461 to 24500; a transposase of the IS3 family (position 25456 to 26223) had only the left end of the insertion sequence at the position between 25424 and 25452 nucleotides; and partial IS sequences for transposases of the IS5 family were found for ISLho2 at the position between nucleotides 1207 and 1229 and for ISAb15 at the position between nucleotides 1841 and 1868. However, the *tnp* gene is not involved in EPS-CG11 biosynthesis. Interestingly, in the strain *L. plantarum* WCFS1 there are four functional *cps* gene clusters, but only after *cps1A-I* are there *rmlACBD* genes responsible for the presence of rhamnose in the polysaccharide polymer.

The *rfbACBD* genes represent a specific *rfb* gene cluster encod-

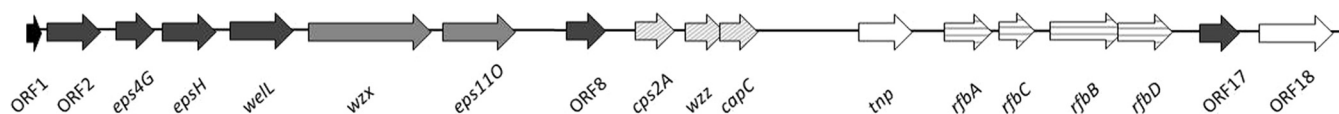


FIG 3 Schematic genetic organization of polysaccharide biosynthesis of *eps* gene clusters in *L. paraplantarum* BGCG11. ORF1 is a putative priming glycosyltransferase (black arrow). Other glycosyltransferases are indicated with dark gray arrows (ORF2, *eps4G*, *epsH*, *well*, and ORF8); lighter-gray arrows indicate genes putatively involved in polymerization and the export process in EPS-CG11 biosynthesis (*wzx* and *eps110*); arrows with hatched lines indicate genes putatively involved in the regulation of EPS-CG11 biosynthesis (*cps2A*, *wzx*, and *capC*). The transposase *tnp* separates the above-mentioned genes from the *rfbACBD* gene cluster (indicated by arrows with horizontal stripes) putatively involved in the synthesis of dTDP-rhamnose.

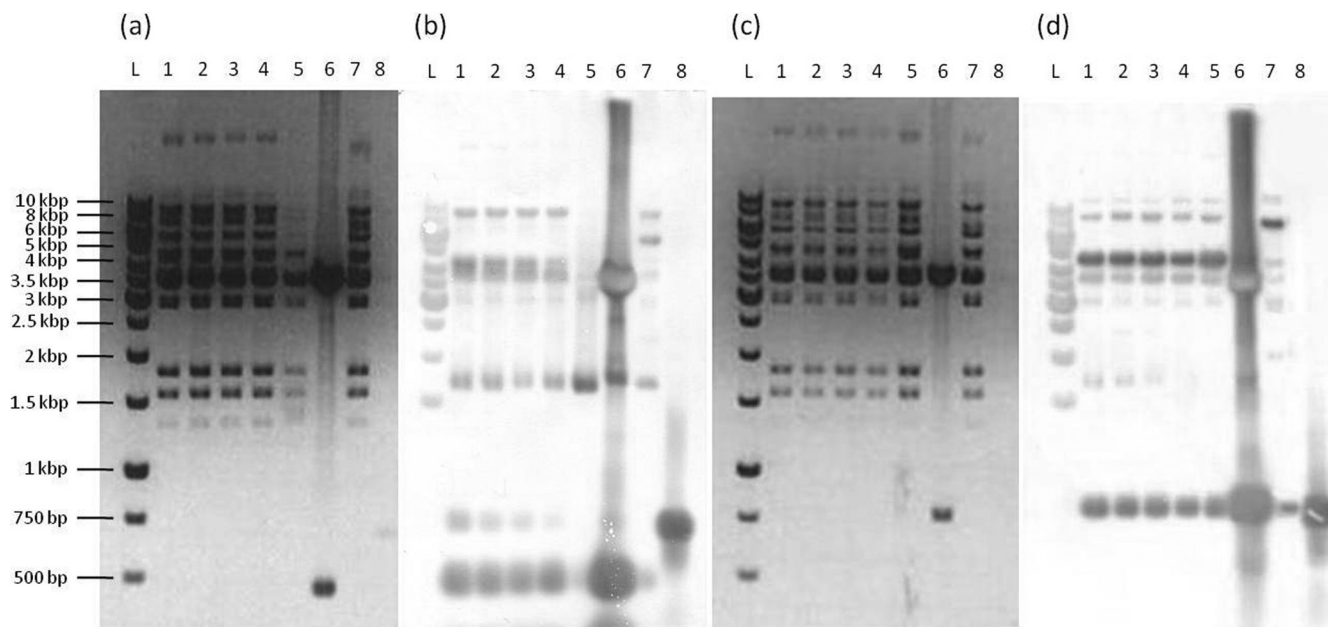


FIG 4 The electrophoresis of total plasmid DNA of the strain *L. paraplantarum* BGCG11 (pCG11) and its MUT1 (a) or MUT2 (c) mutant digested with EcoRI restriction enzyme. (b) Southern blot hybridization with MUT1. Lane L, GeneRuler 1-kb DNA ladder; lanes 1, 2, 3, 4, 5, and 7, digested pCG11 (different isolations); lane 6, digested MUT1; lane 8, DNA probe from the *orf2* gene. (d) Southern blot hybridization with MUT2. Lane L, GeneRuler 1-kb DNA ladder; lanes 1, 2, 3, 4, 5, and 7, digested pCG11 (different isolations); lane 6, digested MUT2; lane 8, DNA probe from the *rfbB* gene.

ing proteins with a very high percentage of amino acid identity (over 95%) with the ones in the *L. plantarum* strains, indicating that they are highly conserved, as was shown previously in *L. rhamnosus* (22). RfbA, with 99% amino acid identity to glucose-1-phosphate thymidyltransferase from *L. plantarum*, is the first of four enzymes thought to be involved in the biosynthesis of dTDP-L-rhamnose. The next, RfbC, with 99% amino acid identity to dTDP-4-dehydrorhamnose 3,5-epimerase from *L. plantarum* JDM1, is a member of a family which catalyzes the isomerization of dTDP-4-dehydro-6-deoxy-D-glucose with dTDP-4-dehydro-6-deoxy-L-mannose. The RfbB showed 99% identity at the amino acid level with dTDP-glucose 4,6-dehydratase from *L. plantarum* WCFS1 and most likely functions as the second of four steps in the dTDP-L-rhamnose pathway (the dehydration of dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose) in the synthesis of L-rhamnose. The fourth, RfbD, with 95% amino acid identity with the dTDP-4-dehydrorhamnose reductase from *L. plantarum*, is assumed to synthesize dTDP-L-rhamnose from alpha-D-glucose-1-phosphate. This protein also has an RmlD substrate binding domain, responsible for binding a sugar nucleotide. A similar *rfb* gene cluster separated by insertional elements was also found in *Lactobacillus gasseri* ATCC 33323^T and *Lactobacillus johnsonii* NCC533 and NCC2767 strains, suggesting that the *eps* clusters could be a hot spot of recombination events, which under selective pressure result in diverse EPS structures (38).

Since rhamnose represents more than 20% of the total monosaccharide composition in EPS-CG11, it could be expected that the *rfb* gene cluster has its own promoter upstream from *rfbA*. According to the literature on four *L. rhamnosus* strains, genes involved in biosynthesis of the sugar nucleotide precursor dTDP-L-rhamnose (designated *rmlACBD*) are expressed from their own promoters, as well as cotranscribed with the upstream *eps* genes (17). The presence of the *rfbACBD* genes in the *rfb* gene cluster

could be responsible for the specific composition, and are possibly involved in the physicochemical characteristics, of the EPS-CG11 polymer.

In order to ensure that there is no other *rfb* gene cluster on the chromosome (since the presence of 3% rhamnose was revealed in the low-molecular-weight residual EPS in the NB1 derivative), Southern blot hybridization using chromosomal DNA with a probe complementary to the *rfb* gene was performed (see Fig. S1c and S1d in the supplemental material). The results clearly showed that only the BGCG11 wild-type strain gave specific hybridization signals with this probe. This result strongly indicates that the obtained signal corresponds to the pCG1 plasmid present only in BGCG11 and not to a gene present at the bacterial chromosome.

The last two genes in the *rfb* gene cluster are *orf17* and *orf18*, encoding proteins with 100% amino acid identity with two proteins from *L. plantarum* JDM1. The former gene, *orf17*, encodes a protein thought to be involved in UDP-N-acetylglucosamine and peptidoglycan biosynthesis and shows identity with 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase, a member of GTF family A. It has predicted domains of UDP-N-acetylglucosamine diphosphorylase/glucosamine-1-phosphate N-acetyltransferase and possibly a bifunctional enzyme, GlmU, which catalyzes the last two reactions in the four-step pathway of UDP-N-acetylglucosamine biosynthesis from fructose-6-phosphate. Hence, *orf17* might be involved in the biosynthesis and degradation of surface polysaccharides. The product of the latter gene, *orf18*, is a putative ribitol-5-phosphate 2-dehydrogenase, part of a medium-chain reductase/dehydrogenase (MDR)/zinc-dependent alcohol dehydrogenase-like family, and has predicted domains of threonine dehydrogenase and related Zn-dependent dehydrogenases. ORF18 might not be directly related to EPS-CG11 biosynthesis since, as a putative ribitol-5-phosphate 2-dehydrogenase, it participates in pentose and glucuronate interconversions (39).

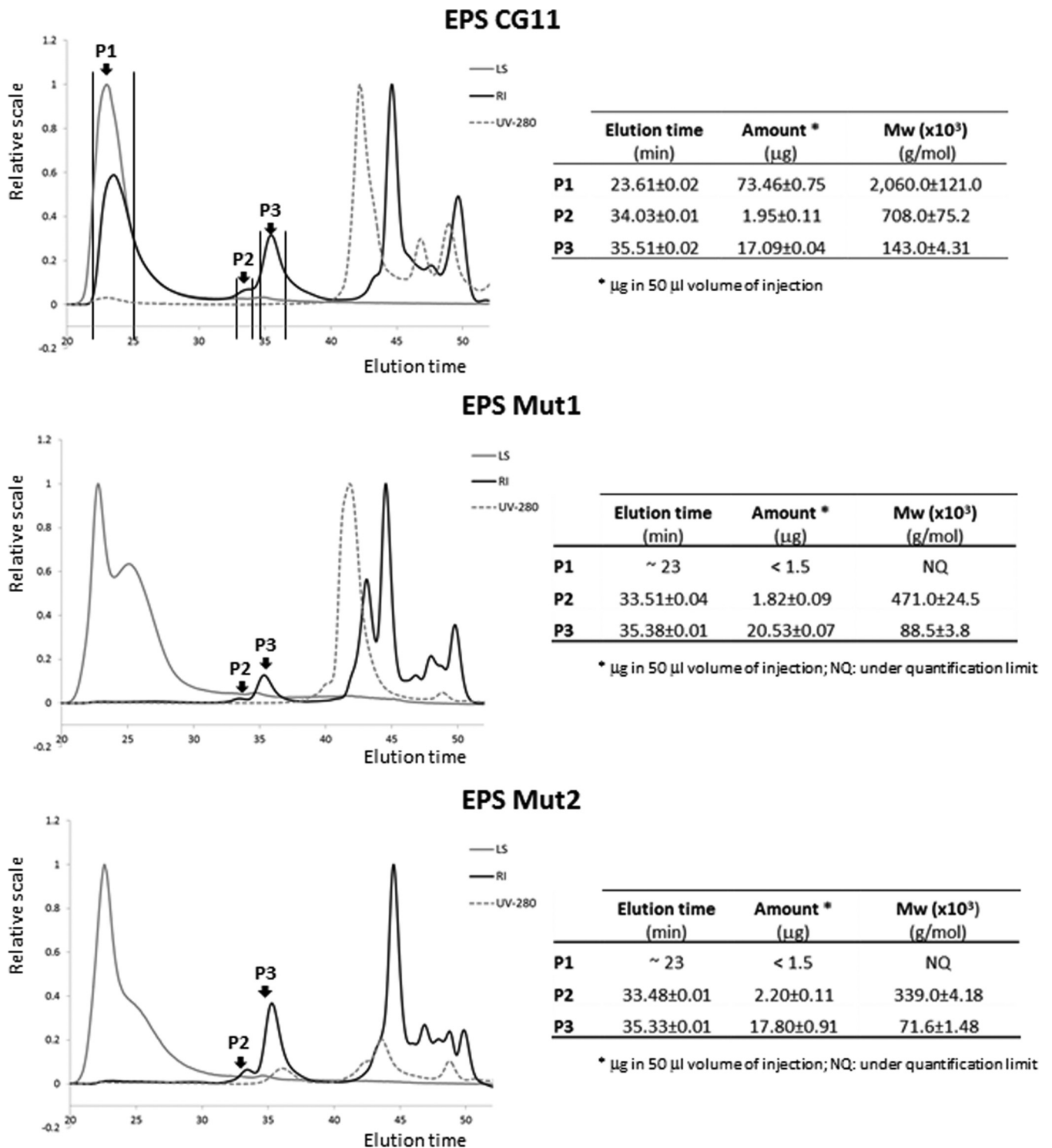


FIG 5 Analysis of the EPSs from BCG11, NB1, MUT1, and MUT2 strains by size exclusion chromatography (SEC) coupled to multiangle laser light scattering (MALLS) detection. LS, light scattering detector set at 90°; RI, refraction index; UV-280, PDA detector set at 280 nm; Mw, weighted average molar mass; P, peak.

Subsequently, downstream from the *eps* gene cluster, a hypothetical protein and three putative transposases in opposite directions are present in the pCG1 plasmid. This has been noticed previously in other *eps* operons, where transposases and insertion elements are also present, indicating that possible horizontal gene

transfers and homologous recombination events contribute to the ubiquity and diversity of EPSs among bacteria (22, 40).

Insertional inactivation of the *orf2* and the *rfbB* genes. In order to test the functionality of the *eps* and *rfb* gene clusters and their role in the production of high-molecular-weight (HMW)

polymer, the MUT1 mutant with an inactivated glycosyltransferase (the *orf2* gene) and the MUT2 mutant with an inactivated *rfbB* gene from the *rfb* mutant gene cluster were constructed as described in Materials and Methods. The insertional inactivation was checked by Southern blotting hybridization (Fig. 4). Both mutants have lost the ropy phenotype (observed visually in comparison to the BGCG11 wild-type strain in Fig. S2 in the supplemental material).

In the case of BGCG11, mutations in the *eps* mutant gene cluster (MUT1 strain) as well as in the *rfb* gene cluster (MUT2 strain) lead to the loss of the ropiness in comparison with BGCG11 cultivated in medium with glucose as the sugar source. Similarly, the NB1 strain, derived from BGCG11 without the plasmid pCG1, also lacked the ropy phenotype (3). It has been reported that the ropy phenotype could be related to the size (molar mass) of the polymer (41). In this regard, the EPS fractions purified from the parental and the two mutant strains were characterized by SEC-MALLS (Fig. 5). The results revealed that peak 1, corresponding to EPS-CG11 with the highest molecular mass ($\sim 2 \times 10^6$ g/mol), was present in the wild-type BGCG11, whereas it was under the quantification level in the mutants MUT1 and MUT2. However, the other two fractions of smaller molar mass (peaks 2 and 3) were produced in similar amounts by the three strains. Thus, it seems that both mutants, MUT1 and MUT2, have lost the capability to synthesize the HMW EPS fraction detected in the wild type and, therefore, the ropy phenotype. In this regard, it was shown that the $\Delta cps1A-I$ strain, a derivative of *L. plantarum* WCFS1, produced surface polysaccharides in amounts equal to those of the wild-type strain although the molar mass was reduced and there is a lack of rhamnose (18). The molar mass and the composition of polysaccharides were not affected by *cps2A-J*, *cps3A-J*, or *cps4A-J* mutants, but the genes produced decreased levels of surface polysaccharides. In the quadruple mutant, the amount of surface polysaccharides was strongly reduced (18).

Although we cannot undoubtedly conclude which genes are responsible for the ropiness, it was shown that the mutant in which the *rfb* gene cluster is inactivated does not retain the ropy phenotype. The ropiness is lost in both mutants obtained in this work by insertional inactivation (inactivation of the *orf2*, glycosyltransferase family 8, and the *rfbB* genes) in which the production of EPS-CG11 high-molecular-weight polymer is disrupted. This ropy phenotype is affected by the presence of sugar in the growth medium; e.g., in medium supplemented with fructose as the only sugar source, there is no visible ropiness of BGCG11 colonies (unpublished data). The influence of using fructose as the sole sugar source on the lack of EPS production of high molecular weight was also noticed in *L. delbrueckii* subsp. *bulgaricus* NCFM 2772, but this strain retained the production of EPS of low molecular weight (21). Similarly, *L. pentosus* LPS26 lost the production of high-molecular-weight EPS A in medium with fructose as the sole sugar source (24). It is possible that if fructose is present in the medium as the sole sugar, there is no available glucose-1-phosphate for the beginning of dTDP-L-rhamnose biosynthesis (42).

Interestingly, none of the sequenced genes was annotated as a transcriptional regulator, indicating that the transcriptional regulatory elements for EPS-CG11 biosynthesis are probably located at the chromosome, and this will be the subject of our further research.

In conclusion, we have described here complete gene information with respect to two gene clusters for high-molecular-weight

EPS (EPS-CG11) biosynthesis in the *L. paraplantarum* BGCG11 strain, which was previously reported to have immunomodulatory effects (3). In the last 10 years EPS operons from different lactobacilli were sequenced, and only a few were partially functionally characterized. Our future work will be focused on the role of BGCG11 and its EPS in the beneficial effects in treatment or prevention of intestinal infections or other diseases where its immunomodulatory effect can be beneficial.

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