

Identification of a Gene Cluster for the Biosynthesis of a Long, Galactose-Rich Exopolysaccharide in *Lactobacillus rhamnosus* GG and Functional Analysis of the Priming Glycosyltransferase^{∇†}

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Cell surface polysaccharides have an established role as virulence factors in human bacterial pathogens. Less documented are the biosynthesis and biological functions of surface polysaccharides in beneficial bacteria. We identified a gene cluster that encodes the enzymes and regulatory and transporter proteins for the different steps in the biosynthesis of extracellular polysaccharides (EPS) of the well-documented probiotic strain *Lactobacillus rhamnosus* GG. Subsequent mutation of the *welE* gene, encoding the priming glycosyltransferase within this cluster, and comparative phenotypic analyses of wild-type versus mutant strains confirmed the specific function of this gene cluster in the biosynthesis of high-molecular-weight, galactose-rich heteropolymeric EPS molecules. The phenotypic analyses included monomer composition determination, estimation of the polymer length of the isolated EPS molecules, and single-molecule force spectroscopy of the surface polysaccharides. Further characterization of the *welE* mutant also showed that deprivation of these long, galactose-rich EPS molecules results in an increased adherence and biofilm formation capacity of *L. rhamnosus* GG, possibly because of less shielding of adhesins such as fimbria-like structures.

Bacterial surface polysaccharides are considered to be key macromolecules in determining microbe-host interactions, as they display a high degree of variety and diversity among bacterial species in terms of composition, monomer linkages, branching degree, polymer size, production level, etc. (24, 46). Since most bacteria contain more than one type of surface polysaccharides, such as lipopolysaccharides (O antigens), capsular polysaccharides (CPS), exopolysaccharides (EPS), and/or glycan chains as part of glycoproteins, the elucidation of their exact role is complex. Nevertheless, surface polysaccharides are now known to exert important functions at several stages during pathogenesis, including tissue adherence, biofilm formation, and evasion of host defenses such as phagocytosis (9, 24, 33). In addition to their role in pathogens, an important biological role for CPS and glycoproteins has also recently been shown in colonization of the gut by bacteria of the genus *Bacteroides* (10, 34).

Conversely, the role of surface polysaccharides in probiotic-host interactions has not yet been studied in great detail. A probiotic bacterium is defined as “a live microorganism that, when administered or ingested in adequate amounts, confers a health benefit on the host” (18). Members of the genus *Lactobacillus* are commonly studied for their health-promoting capacities (26, 31, 37). As polysaccharides display a high diver-

sity among lactobacilli, they are thought to be involved in determining strain-specific properties important for probiotic action, such as adhesion, stress resistance, and interactions with specific receptors and effectors of the host defense system (13, 56). Moreover, these EPS molecules are of interest in the dairy industry for conferring textural and rheological properties to fermented products such as yogurt and soft cheese (56). Nevertheless, detailed genetic and functional studies of EPS molecules of lactobacilli are currently limited (26, 56).

Lactobacillus rhamnosus GG (ATCC 53103) is one of the probiotic strains with the largest number of proven health benefits (15). Several clinical trials have reported that *L. rhamnosus* GG can prevent and relieve certain types of diarrhea (22) and atopic disease (25) and reduce inflammation in some milder states of inflammatory bowel diseases (60). However, the cell surface factors or specific characteristics of *L. rhamnosus* GG that underlie these health benefits are largely unknown.

We recently showed by single-molecule force spectroscopy (SMFS) with specific lectin tips that the cell surface of *L. rhamnosus* GG wild-type cells contains two major types of cell wall-associated polysaccharides (CW-PS) (21). The longest and most abundant polysaccharides are galactose-rich and seem to correspond with the EPS molecules of *L. rhamnosus* GG, which were previously structurally identified by Landersjö et al. (27) using nuclear magnetic resonance spectroscopy. Additionally, shorter, yet-uncharacterized glucose-rich polysaccharides are present on the *L. rhamnosus* GG surface (21). In the current study, we describe the identification and annotation of the *L. rhamnosus* GG gene cluster that encodes the enzymes and transporter and regulatory proteins involved in the biosynthesis of long, galactose-rich EPS molecules. This

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> strains		
DH5 α	F ⁻ ϕ 80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r _K ⁻ m _K ⁻) supE44 λ ⁻ thi-1 girA96 relA1	Gibco-BRL
LE392	hsdR574 (r _K ⁻ m _K ⁺) supE44 supF58 lacY1 [or Δ (lac1ZY)6] galK2 galT22 metB1 trpR55 λ ⁻ mcrB	ATCC 33572
<i>L. rhamnosus</i> GG strains		
Wild type	Human isolate; wild-type strain GG	ATCC 53103
CMPG5351	welE knockout mutant of strain GG; welE::tet(M)	This work
CMPG5354	CMPG5351 complemented by genomic integration of pCMPG5353	This work
Plasmids		
pFAJ5301	Cloning vector, pUC18 derivative, Ery ^r	30
pMD5057	Plasmid from <i>L. plantarum</i> 5057 containing a tet(M) marker	11
pEM40	pUC19E-derived integration vector (<i>attB</i> located at the 3' end of tRNA ^{Leu} locus) containing a 1.6-kb <i>int-attP</i> cassette of phage A2, Ap ^r Ery ^r	2
pCMPG5351	pFAJ5301 derivative used to inactivate <i>welE</i> gene by insertion of a tet(M) marker via double homologous recombination (for details, see text)	This work
pCMPG5353	pEM40 derivative containing a fragment with the <i>welE</i> gene in the Ecl136II/EcoRI site (amplified with primers Pro-0722 and Pro-0723), Ap ^r Ery ^r	This work

was experimentally confirmed by the construction of a knockout mutant of the corresponding priming glycosyltransferase and subsequent characterization of the surface polysaccharides of wild-type and mutant strains. We also studied the specific role of these EPS molecules in adherence to mucus and gut epithelial cells and in biofilm formation by *L. rhamnosus* GG.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. rhamnosus* GG and its derivatives were grown at 37°C in MRS (Difco) or Lactobacilli AOAC medium (Difco) in nonshaking conditions (Table 1). *Escherichia coli* cells, used as cloning hosts, were grown in Luria-Bertani medium with aeration at 37°C (48). When appropriate, antibiotics (Sigma-Aldrich) were used at the following concentrations: tetracycline (Tc) at 10 μ g/ml, ampicillin (Ap) at 100 μ g/ml, and erythromycin (Ery) at 10 μ g/ml for *L. rhamnosus* GG and at 100 μ g/ml for *E. coli*.

DNA manipulations. Routine molecular biology techniques were performed according to standard procedures (48). Restriction and modification enzymes (from New England Biolabs or Roche) were used as recommended by the manufacturer. Plasmid DNA was prepared from *E. coli* cells with Qiagen mini-prep kits. Chromosomal DNA and plasmid DNA were isolated from *L. rhamnosus* GG as previously described (12).

Sequencing and annotation of the putative EPS biosynthetic cluster. The *wzb* gene of *L. rhamnosus* GG (accession no. EF690379) was identified as previously described (32), starting from a PCR with primers based on the published *wzb* sequence of the closely related strain *L. rhamnosus* ATCC 9595 (43). This gene provided the starting point for the sequencing of the remainder of the EPS gene cluster of *L. rhamnosus* GG. Primers Pro-0224, Pro-0225, Pro-249, and Pro-250 (see Table S1 in the supplemental material) were designed for chromosome walking. DNA sequencing was performed using the chain termination dideoxynucleoside triphosphate method (48) (BigDye Terminator V3.1 Cycle-Sequencing kit, ABI 3100-Avant Genetic Analyzer; Applied Biosystems). This yielded a 2.8-kb fragment including the sequences for the *wzb* gene and the *wzr* gene. This information was used to develop a digoxigenin-labeled probe with PCR primers Pro-0300 and Pro-0278 (see Table S1 in the supplemental material) based on an internal fragment of the *wzr* gene to screen a lambda phage genomic library of *L. rhamnosus* GG (λ CMPG5317). This library was made with the Packagene lambda DNA packaging system (Promega) with *E. coli* LE392 as a host and phage EMBL3 containing inserts of ca. 15 to 20 kb of *L. rhamnosus* GG genomic DNA. Southern hybridization resulted in the identification of several phages containing a fragment that hybridized with the *wzr* probe. One phage that contained a ~15-kb fragment upstream of *wzr* was selected. Phage DNA was prepared with the Qiagen lambda DNA extraction minikit and subsequently digested with SalI, BamHI, KpnI, and HindIII. The restriction fragments obtained were subcloned and sequenced. This yielded the DNA sequence of a

15-kb fragment containing the genes encoding glycosyltransferases (*welIIHGFE*), proteins involved in dTDP-rhamnose precursor biosynthesis (*rmlACB*), the oligosaccharide transporter (*wzx*), and polymerase (*wzy*). To complete the EPS gene cluster, primers Pro-0708, Pro-0839, and Pro-0989 (see Table S1 in the supplemental material) were subsequently used to sequence *wzd-wze* with genomic DNA of *L. rhamnosus* GG as a template. Finally, all fragments were assembled in a ca. 20-kb contig containing the DNA sequence for the putative EPS gene cluster of *L. rhamnosus* GG. The Entrez nucleotide database was screened for similarities using BLASTx (1), and alignment of overlapping fragments was performed with the VectorNTI Advance 10 ContigExpress software (Informax, Oxford, United Kingdom). Annotation of the open reading frames (ORFs) was performed with the Vector NTI Advance 10 software (Informax, Oxford, United Kingdom) and the Sequin sequence submission software. Membrane-spanning regions of translated gene products were predicted using the TMpred program (23). Additionally, the Pfam database (19) was searched for conserved motifs in the translated gene products. The genes were annotated according to Péant et al. (43). These genes were named based on the bacterial polysaccharide gene nomenclature system (45).

Insertional inactivation of the *welE* gene (CMPG5351). A knockout mutant of the *welE* gene, encoding the priming glycosyltransferase, was constructed by double homologous recombination as previously described (30). DNA fragments containing ca. 1 kb up- and downstream of *welE* were amplified by PCR with primers Pro-0722 and Pro-0723 (see Table S1 in the supplemental material). These fragments were cloned into the pFAJ5301 suicide vector (30). Subsequently, *welE* was inactivated by insertion of a tet(M) Tc^r cassette from plasmid pMD5057 of *Lactobacillus plantarum* 5057 (11), amplified with primers Pro-221 and Pro-222 containing EcoRI sites. The tet(M) marker gene fragment was blunted and ligated into the blunted BglII site of the *welE* gene. The resulting suicide vector pCMPG5351 was transformed to *L. rhamnosus* GG wild-type cells. At 3 days after the electroporation, transformants were screened for resistance to 10 μ g/ml Tc and sensitivity to 10 μ g/ml Ery to select for the occurrence of a double homologous recombination event. Confirmation of DNA recombination was obtained by PCR using different primer pairs (Pro-249/Pro-0722 and Pro-0788/Pro-0789) (see Table S1 in the supplemental material). One colony showing the correct insertion pattern of the tet(M) marker and absence of the suicide vector was selected for further analyses and designated CMPG5351. The insertion of the tet(M) marker by double homologous recombination was shown to be 100% stable after 100 generations. Therefore, the mutant was grown in medium without antibiotics for all phenotypic assays, to limit possible interference of Tc with observed phenotypes.

Construction of the complemented strain (CMPG5354). The *welE* knockout mutant was complemented by insertion of a wild-type copy including the genuine promoter in a single copy at another locus in the chromosome of *L. rhamnosus* GG. The self-integrating plasmid pEM40 (2) was used, which integrates at the tRNA^{Leu} locus in the *L. rhamnosus* GG genome (12). The *welE* gene of *L. rhamnosus* GG was amplified with primers Pro-0722 and Pro-0723 (see Table S1

in the supplemental material). The resulting PCR fragment was digested with the restriction enzymes *Ecl*136II/*Eco*RI and ligated in the *Ecl*136II/*Eco*RI sites of pEM40, resulting in plasmid pCMPG5353. pCMPG5353 was electroporated to the *welE* knockout mutant CMPG5351 for complementation, resulting in strain CMPG5354.

Isolation and characterization of CW-PS. CW-PS were isolated and quantified as described previously (32, 51). Briefly, total CW-PS was extracted from *L. rhamnosus* GG cells by mild sonication followed by ethanol precipitation and dialysis against water (6,000- to 8,000-Da dialysis membrane [Spectra/Por, VWR International]). The total amount of carbohydrate was estimated by the phenol-sulfuric acid method (16). The sugar monomer composition of the isolated polysaccharides was determined according to the method of Englyst and Cummings by gas chromatography after hydrolysis and derivatization to alditol acetates (17). β -D-Allose was used as an internal standard, and calibration samples (glucose, galactose, rhamnose, and mannose) containing the expected monosaccharides were included with each set of samples. For an estimation of the molecular weights of the isolated polysaccharides, samples were analyzed by size exclusion chromatography as previously described (6). A volume of 50 μ l of EPS sample (1 mg/ml) was injected, and the detection was performed with a refractive index detector. The results were compared using a dextran standard series of 8×10^4 , 1.5×10^5 , 2.7×10^5 , 6.7×10^5 , and 1.4×10^6 Da (Sigma-Aldrich). Alternatively, differences in molecular weight profiles of the isolated polysaccharides were compared on 5% polyacrylamide gels (10 by 10 cm) (Hoefer miniVE; GE Healthcare) using Tris-borate-EDTA buffer (50 mM Tris, 13 mM EDTA, 15 mM boric acid). Samples were run for 120 min at 350 V. The polysaccharides were stained with the Pro-Q Emerald 488 glycoprotein stain (Molecular Probes, Invitrogen). Gels were imaged with a Typhoon 9400 variable-mode imager (GE Healthcare) using an excitation laser at 488 nm and an emission filter at 520 nm (band-pass).

TEM and SMFS. To observe the polysaccharides on the cell surface produced in situ, cell surface structures of *L. rhamnosus* GG were visualized by negative staining in transmission electron microscopy (TEM) experiments using published methods (29). Additionally, SMFS experiments were performed to detect and localize single polysaccharide molecules on live bacteria as described recently (21). Briefly, atomic force microscopy (AFM) tips functionalized with the carbohydrate-binding lectins concanavalin A (mannose and glucose specificity) and PA-I (galactose specificity) were used to scan the surfaces of wild-type *L. rhamnosus* GG and mutant CMPG5351 for the localization, abundance, and polymer length of mannose/glucose-rich and galactose-rich polysaccharides, respectively. Adhesion forces and rupture distances between the functionalized AFM tips and surface polysaccharides were recorded as described previously (20).

In vitro biofilm formation and adhesion assays. In vitro biofilm formation by *L. rhamnosus* GG was assessed by crystal violet staining after 72 h as previously described (32). Additionally, adhesion to immobilized mucins (pig mucin type II; Sigma-Aldrich) was tested in a microtiter plate-based assay (3). Cells were labeled with *N*-hydroxysuccinimidobiotin (Sigma-Aldrich), followed by detection with streptavidin conjugated to alkaline phosphatase (Roche) and addition of 4-nitrophenylphosphate (Fluka) as a substrate. The percentage of adherence was calculated based on the optical density at 405 nm of the labeled adherent bacteria compared to the optical density at 405 nm of the original labeled suspension before adhesion. Adhesion was tested each time in eight replicates, and each experiment was repeated at least twice. Adhesion to bovine serum albumin (Sigma-Aldrich) was included to account for nonspecific adhesion. Finally, adhesion to Caco-2 cell line was investigated as previously described (29). The adhesion ratio (percent) was calculated by comparing the number of adherent cells to the cell number of the added original bacterial suspension (10^7 CFU/ml). All strains were tested in triplicate in three independent experiments.

Nucleotide sequence accession number. The entire sequence determined in this study was submitted to the NCBI database under GenBank accession number FJ428614.

RESULTS

Identification of the EPS biosynthetic gene cluster of *L. rhamnosus* GG. As the genome sequence of *L. rhamnosus* GG is not publicly available, the data for the EPS gene cluster of the phylogenetically related strain *L. rhamnosus* ATCC 9595 (43) were used as starting point to determine the DNA sequence of the putative *L. rhamnosus* GG EPS gene cluster. Following the strategy described in Materials and Methods, we obtained an *L. rhamnosus* GG EPS gene cluster containing 17

putative ORFs (Fig. 1A), of which 16 putatively encode proteins involved in the biosynthesis of various bacterial polysaccharides and 1 (*orf1*) encodes a putative transposase (Table 2). All ORFs except *wzr* are located in the same orientation. The EPS gene cluster of *L. rhamnosus* GG has a modular organization, which is typical for surface heteropolysaccharides (26). The genes putatively encoding regulatory proteins are located at both extremities of the cluster. The first two ORFs (*wzd* and *wze*) encode proteins that have predicted amino acid sequences with homology to the putative Wzd-Wze tyrosine kinase complex of *L. rhamnosus* ATCC 9595 (43) (Table 2). At the other end of the cluster, the *wzb* gene is located 145 bp downstream of *wzr* in reverse orientation. The Wzb protein of *L. rhamnosus* GG shows 99% amino acid identity with Wzb of *L. rhamnosus* ATCC 9595, which was recently biochemically characterized as a copper-dependent *O*-phosphatase (28). A regulatory role in CPS biosynthesis and polymer export has been experimentally demonstrated in streptococci for the Wzb ortholog CpsB (48% amino acid similarity) in combination with the autophosphorylating tyrosine kinase complex Wzd-Wze, designated CpsC-CpsD in streptococci (44 to 55% amino acid similarity) (4, 40–42). *Wzr* of *L. rhamnosus* GG shows homology to transcriptional regulators of the *LytR*-CpsA-*Psr* family involved in cell envelope-related functions, and it contains a short putative N-terminal cytoplasmic domain and a transmembrane domain forming a signal-anchor. Mutation of *cpsA*, the ortholog of *wzr* (encoded proteins show 61% similarity), has been shown to affect CPS production in certain streptococci (8, 40).

In addition to regulatory proteins, the EPS cluster of *L. rhamnosus* GG contains the genes encoding the enzymes for the actual biosynthesis of EPS. Glycosyltransferases synthesize blocks of repeating units that are linked to a lipid carrier at the inner side of the cytoplasmic membrane (Fig. 1B). Six genes (*welE* to *-J*) encoding putative glycosyltransferases are contained in the central portion of the putative EPS locus of *L. rhamnosus* GG. The protein encoded by the *welE* gene displays 61% identity with YP_001271961 from *Lactobacillus reuteri* F275, annotated as a galactose phosphotransferase (Table 2). The *welE* gene putatively encodes the priming glycosyltransferase that transfers the first sugar of each subunit of an EPS molecule of *L. rhamnosus* GG. *welF* to *welJ* probably encode the glycosyltransferases transferring the other sugars of the EPS subunit in an ordered and sugar- and glycosidic linkage-dependent fashion (Fig. 1B). In general, heteropolymeric polysaccharide subunits are flipped across the cytoplasmic membrane by a *Wzx*-type exporter and polymerized into long polysaccharides by a *Wzy*-type polymerase at the outer side of the cytoplasmic membrane (Fig. 1B) (13, 56, 58), which has been experimentally best studied in *E. coli* (57). The *wzx* and *wzy* genes of *L. rhamnosus* GG are located in the 5' region of the putative EPS cluster (Fig. 1A). In contrast to the conserved *Wzd/Wze* and *Wzb* homologs, *Wzx* and *Wzy* proteins are transmembrane proteins that are specific for the associated EPS repeating unit (13, 56, 58), as reflected by their lower level of similarity among orthologs (Table 2).

The EPS cluster of *L. rhamnosus* GG also contains genes for the synthesis of specific nucleotide sugars that cannot be obtained from the central metabolism. The *glf* gene putatively encodes a UDP-galactopyranose mutase for the conversion of

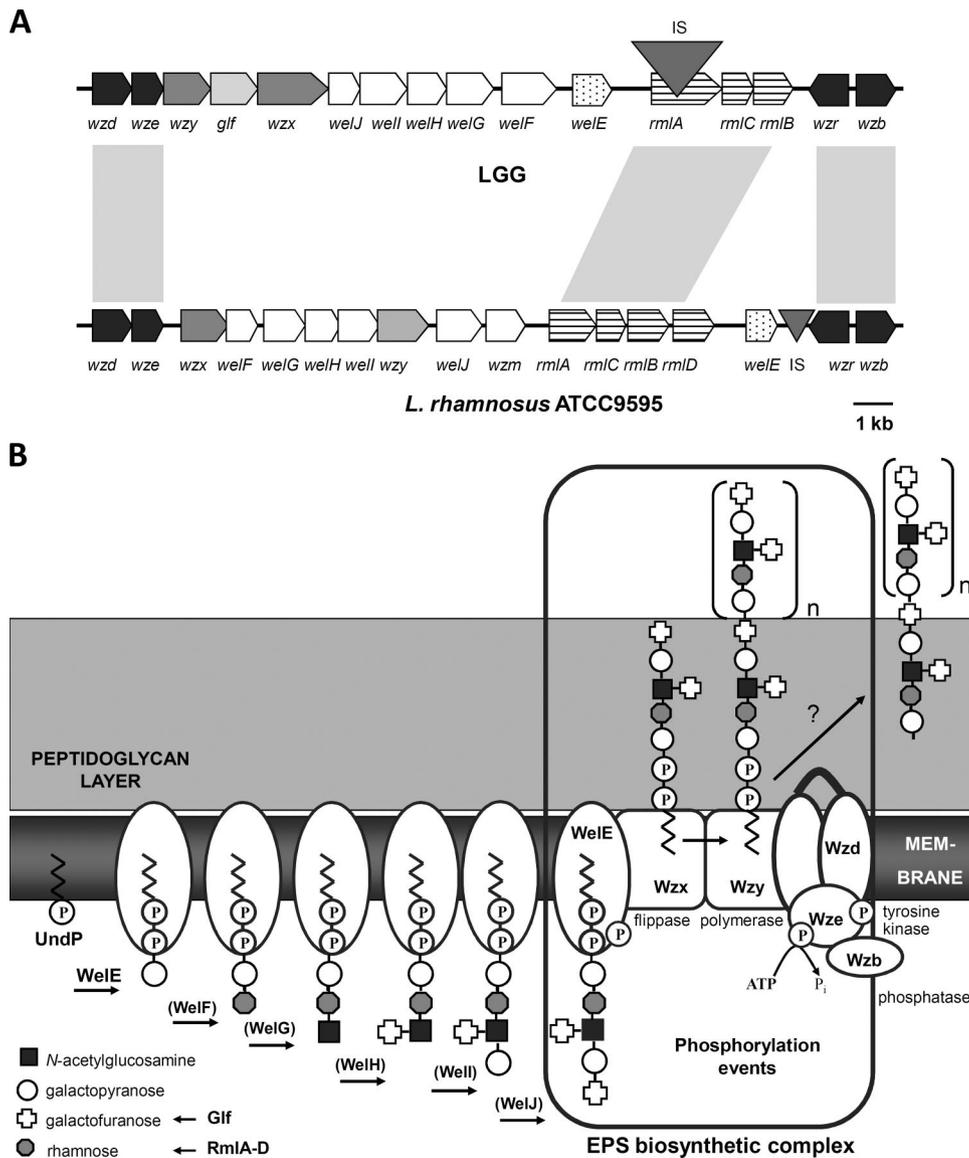


FIG. 1. (A) Organization of the EPS gene cluster of *L. rhamnosus* GG. The organization of the EPS gene cluster of *L. rhamnosus* GG is compared by BLASTx analysis with that of the EPS gene cluster of the phylogenetically related strain *L. rhamnosus* ATCC 9595 (43). Genes encoding similar functions in EPS biosynthesis have a similar gray scaling code. Genes indicated in dark gray encode proteins putatively involved in the regulation of EPS production and polymerization. *wzx* and *wzy* (light gray) encode the putative polysaccharide transporter and polymerase, respectively. Genes indicated in white encode the putative glycosyltransferases, with *wzm* in *L. rhamnosus* ATCC 9595 encoding a putative pyruvyltransferase. Long stripes indicate genes encoding the proteins involved in the biosynthesis of the dTDP-rhamnose precursor. The lightest gray indicates the *glf* gene, encoding the UDP-galactopyranose mutase for the biosynthesis of the sugar nucleotide precursor for galactofuranose present in the EPS molecules of *L. rhamnosus* GG. The triangles indicate insertion sequence elements (IS). The insertion sequence of the *L. rhamnosus* GG EPS gene cluster is indicated with *orf1* present on *ISLrh2* in Table 2. Finally, genes sharing high nucleotide identity are linked by light gray boxes. Cutoff values for the displayed degrees of similarity are >88% identity. (B) Schematic representation of the putative steps in EPS biosynthesis by *L. rhamnosus* GG that are encoded within the EPS gene cluster. First, a phosphogalactosyl residue is transferred from an activated nucleotide sugar to the undecaprenyl phosphate (UndP)-lipid carrier on the cytoplasmic face of the membrane. This step is catalyzed by the membrane-associated priming glycosyltransferase *WelE*. Subsequently, unique glycosyltransferases *WelF* to *-J* add the remaining sugars in a sugar and glycosidic linkage-dependent manner, of which the exact order remains to be determined (indicated by brackets). The substrates for these glycosyltransferases are sugar nucleotides, which are available from existing cellular pools (e.g., UDP-galactose, UDP-*N*-acetylglucosamine) or synthesized by EPS-specific enzymes encoded within the EPS gene cluster (e.g., UDP-galactofuranose by the *glf* gene). Once an EPS subunit is completed, it needs to be translocated across the cytoplasmic membrane by a *Wzx* flippase, followed by linkage of the repeating units into long polysaccharides by a specific *Wzy* polymerase. A phosphorylation complex including a *Wze* autophosphorylating tyrosine kinase and a *Wzb* phosphotyrosine protein phosphatase is thought to be involved in the regulation of EPS biosynthesis. This figure is partly based on the model for *Streptococcus pneumoniae* (5), with modifications based on reference 27 and the gene cluster and phenotypic analyses presented here.

TABLE 2. ORFs identified in the EPS gene cluster of *L. rhamnosus* GG

ORF	Size (bp)	Predicted encoded function	Predicted domain(s) present in encoded ORF	Best BLASTx hit (accession no.)		% Amino acid identity
				Protein	Organism	
<i>wzd</i>	920	Auxiliary protein for polysaccharide export and chain length determination	2 transmembrane domains	Wzd (AAW22433)	<i>L. rhamnosus</i> ATCC 9595	72
<i>wze</i>	756	Autophosphorylating tyrosine-protein kinase	Walker A and B motif (nucleotide binding); C-terminal tyrosine-rich region	Wze (AAW22434)	<i>L. rhamnosus</i> ATCC 9595	88
<i>wzy</i>	1,221	Repeat unit polymerase	10 putative transmembrane helices	Wzy (CAI33441)	<i>Streptococcus pneumoniae</i> serotype 16a	29
<i>glf</i>	1,131	UDP-galactopyranose mutase	1 putative transmembrane helix	Glif (YP_536435)	<i>L. salivarius</i> UCC118	73
<i>wzx</i>	1,437	Repeat unit transporter	12 putative transmembrane helices	Wzx (YP_1988153)	<i>L. casei</i> BL23	55
<i>welJ</i>	600	Glycosyltransferase	No hit in Pfam database; no transmembrane helix	ZP_1274133	<i>L. reuteri</i> 100-23	46
<i>welI</i>	1,137	Glycosyltransferase	Glycosyltransferase family 1 (PF00534)	NP_784849	<i>L. plantarum</i> WCFS1	35
<i>welH</i>	936	Glycosyltransferase	Glycosyltransferase family 2 (PF00535)	NP_784850	<i>L. plantarum</i> WCFS1	40
<i>welG</i>	1,017	Glycosyltransferase	No hit in Pfam database	YP_1271955	<i>L. reuteri</i> F275	32
<i>welF</i>	1,068	Glycosyltransferase	Glycosyltransferase family 1 (PF00534)	WfU (ACA24914)	<i>Shigella dysenteriae</i>	28
<i>welE</i>	669	Undecaprenyl-phosphate galactose phosphotransferase; priming glycosyltransferase	Bacterial sugar transferases (PF02397 domain)	YP_1842290	<i>L. reuteri</i> F275	61
<i>rmlA^a</i>	795	dTDP-glucose pyrophosphorylase	Nucleotidyl transferase	RmlA (AAW22443)	<i>L. rhamnosus</i> ATCC 9595	90
<i>orf1</i>	1,494	Putative transposase	Transposase DDE domain	YP_806463	<i>L. casei</i> ATCC 334	62
<i>rmlC</i>	597	dTDP-4-dehydro-rhamnose-3,5-epimerase	dTDP-4 dehydro-rhamnose-3,5-epimerase	RmlC (AAW22444)	<i>L. rhamnosus</i> ATCC 9595	98
<i>rmlB</i>	936	dTDP-D-glucose-4,6-dehydratase	NAD-dependent epimerase/dehydratase family	RmlB (AAW22445)	<i>L. rhamnosus</i> ATCC 9595	99
<i>wzr</i>	891	Transcriptional regulator of polysaccharide biosynthesis	LytR-CspA-Psr superfamily	Wzr (AAW22447)	<i>L. rhamnosus</i> ATCC 9595	97
<i>wzb</i>	765	Phosphotyrosine protein phosphatase	Polymerase and histidinol phosphatase domain	Wzb (AAW22466)	<i>L. rhamnosus</i> RW-6541 M	100

^a Interrupted by *orf1* encoded on *ISLrh2*.

UDP-galactopyranose to UDP-galactofuranose (Table 2). This is in agreement with the presence of galactofuranose residues in the repeating units of the EPS molecules of *L. rhamnosus* GG, as previously reported (27). Genes for dTDP-rhamnose biosynthesis (*rmlACB*) are also located in the EPS gene cluster (Fig. 1A). However, the *rmlA* gene, putatively encoding the glucose-1-phosphate thymidyl transferase, appears to be inactivated by insertion of *ISLrh2*. The *rmlB* and *rmlC* genes putatively encode a dTDP-glucose-4,6-dehydratase and a dTDP-4-keto-rhamnose-3,5-epimerase, respectively. No *rmlD* gene encoding the final-acting dTDP-rhamnose synthase was found within the *L. rhamnosus* GG EPS gene cluster, in contrast to the case for the previously identified EPS gene cluster of the phylogenetically related strain *L. rhamnosus* ATCC 9595 (43) (Fig. 1A). Nevertheless, Southern hybridization experiments suggest the presence of a complete *rmlACBD* operon at another locus in the *L. rhamnosus* GG genome distinct from the EPS gene cluster (data not shown).

Mutation of the priming glycosyltransferase reduces the total level of CW-PS. As *welE* putatively encodes the priming glycosyltransferase, i.e., an important control point of EPS biosynthesis, we hypothesized that deletion of this gene should result in a drastic reduction of EPS production. To test this hypothesis, a *welE* insertion mutant of *L. rhamnosus* GG, designated CMPG5351, was constructed. The growth capacity of CMPG5351 was not significantly different from that of wild-type *L. rhamnosus* GG. Wild-type *L. rhamnosus* GG and *welE* mutant CMPG5351 both showed a generation time of 1.6 ± 0.1 h and 1.9 ± 0.1 h in MRS and AOAC media, respectively. For subsequent analyses, cells were grown in AOAC medium, as we previously observed that this medium induces a high level of EPS production by *L. rhamnosus* GG (32). *L. rham-*

nosus GG wild-type cells that are grown in AOAC medium have a typical outer layer formed by CW-PS (29). TEM analysis showed that this layer is absent in the CMPG5351 mutant, although some isolated zones with accumulation of, presumably, polysaccharides could still be detected (Fig. 2A). Additionally, fimbria-like structures appear to become exposed in *welE* mutant cells at the poles opposite the septum (Fig. 2A). Subsequently, total CW-PS material was extracted from *L. rhamnosus* GG wild-type and CMPG5351 cells as described in Materials and Methods. The quantity of CW-PS that could be extracted from *welE* mutant cells was ca. 3-fold lower than that from *L. rhamnosus* GG wild-type cells (Fig. 2B). Importantly, *cis* complementation of CMPG5351 with a functional *welE* gene containing its upstream promoter region (corresponding to strain CMPG5354) could restore CW-PS production to the wild-type level (Fig. 2B), indicating that the *welE* mutation is not polar and that the *welE* gene in the wild-type strain is transcribed from its own promoter (see also below).

Mutation of the priming glycosyltransferase specifically abolishes the production of the long, galactose-rich EPS molecules. Having established that the *L. rhamnosus* GG *welE* mutant CMPG5351 contains less CW-PS than the wild-type strain, we subsequently investigated whether the *welE* mutation specifically affects the production of the long, galactose-rich type of *L. rhamnosus* GG CW-PS molecules. First, the sugar monomer composition of the CW-PS extracts was determined by gas chromatography as described in Materials and Methods. The extract of wild-type *L. rhamnosus* GG was found to be composed of approximately 70% galactose, 19% rhamnose, and 10% glucose (Fig. 3A). On the basis of the *L. rhamnosus* GG EPS structure determined by Landersjö et al. (27), this implies that the CW-PS extract of *L. rhamnosus* GG wild-

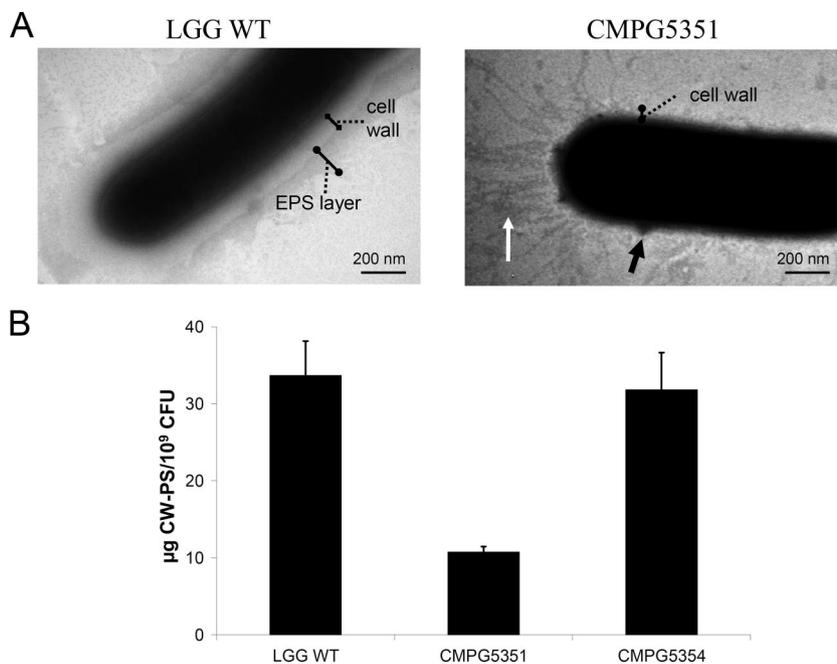


FIG. 2. Mutation of the *welE* gene in *L. rhamnosus* GG reduces the level of CW-PS. (A) TEM analysis of *L. rhamnosus* GG wild-type and *welE* mutant CMPG5351 strains grown in AOAC medium. For wild-type *L. rhamnosus* GG, the cell-bound EPS layer is indicated. While this layer is absent in the *welE* mutant CMPG5351, some patches of presumably polysaccharide accumulations could still be detected (black arrow). Additionally, fimbria-like appendages appear to become exposed in *welE* mutant cells (white arrow). (B) CW-PS were extracted from cell pellets from wild-type *L. rhamnosus* GG (WT), *welE* mutant CMPG5351, and the complemented strain CMPG5354. Error bars indicate standard deviations.

type cells contains mainly the known galactose-rich EPS molecules. The sugar monomer composition of the CW-PS extract of the CMPG5351 mutant was drastically changed to approximately 63% glucose, 31.5% rhamnose, 3% galactose, and 2.5% mannose (Fig. 3A). This indicates that the galactose-rich CW-PS molecules are strongly reduced in the *welE* mutant CMPG5351, while the relative proportion of the (yet-unknown) glucose-rich CW-PS has increased. Importantly, sugar monomer analysis also showed that complementation of the *welE* mutation in strain CMPG5354 could fully restore synthesis of the galactose-rich type of CW-PS at the *L. rhamnosus* GG surface (Fig. 3A). Additionally, the size of the CW-PS molecules was estimated by gel electrophoresis of the CW-PS extracts on 5% polyacrylamide gels. These analyses revealed the absence of the largest band in the CW-PS extract of the *welE* mutant (Fig. 3B), corresponding to the high-molecular-mass ($>1.4 \times 10^6$ Da) CW-PS molecules based on size exclusion chromatography (data not shown). Finally, SMFS for surface polysaccharides was applied on live bacteria, using functionalized AFM tips containing single lectins specific for end-standing galactose (PA-I lectin) as recently described (21). In comparison to the wild type, the *welE* mutant CMPG5351 showed a marked reduction in the galactose-rich CW-PS molecules (reduced adhesion frequency with the PA-I functionalized AFM tip) and a marked reduction in polymer length (reduced elongation and rupture distance) (Fig. 3C). On the other hand, SMFS experiments with mutant CMPG5351 and concanavalin A-functionalized AFM tips showed an increase in the glucose-rich type of polysaccharides, with especially an increase in elongation and rupture length (data not shown).

Taken together, the data on monomer composition, polymer size, and SMFS for the *L. rhamnosus* GG wild-type, *welE* mutant, and complemented *welE* mutant strains demonstrate that the *WelE* enzyme has a crucial and specific role in the biosynthesis of the high-molecular-weight, galactose-rich type of CW-PS molecules of *L. rhamnosus* GG, referred to as the EPS molecules of *L. rhamnosus* GG in this study. Moreover, abolishment of the galactose-rich EPS molecules in *welE* mutant CMPG5351 seems to increase the presence of the glucose-rich type of CW-PS molecules, which were described for the first time in reference 21.

Inactivation of the priming glycosyltransferase *WelE* results in increased biofilm formation and adhesion to mucus and epithelial cells by *L. rhamnosus* GG. Prior experiments showed that *L. rhamnosus* GG has an intrinsic high biofilm formation capacity (29, 32). The *welE* mutant with a specific reduction in the long, galactose-rich EPS molecules allowed us to investigate the relative contribution of these EPS molecules to the biofilm-forming capacity of *L. rhamnosus* GG. Surprisingly, biofilm formation by the EPS mutant CMPG5351 was increased threefold compared to that by the wild type in AOAC medium (Fig. 4A). Importantly, *cis* complementation with a functional *welE* gene in CMPG5351 (strain CMPG5354), restored wild-type levels of biofilm formation (Fig. 4A). Additionally, the *in vitro* adherence capacity of the EPS mutant CMPG5351 was investigated with mucus as a substrate, as *L. rhamnosus* GG has been previously reported to display high mucus-adhering properties (52). In our assay, the *welE* mutant CMPG5351 grown in AOAC medium showed a ca. 1.5-fold-increased adhesion to commercially available pig gastric mucus

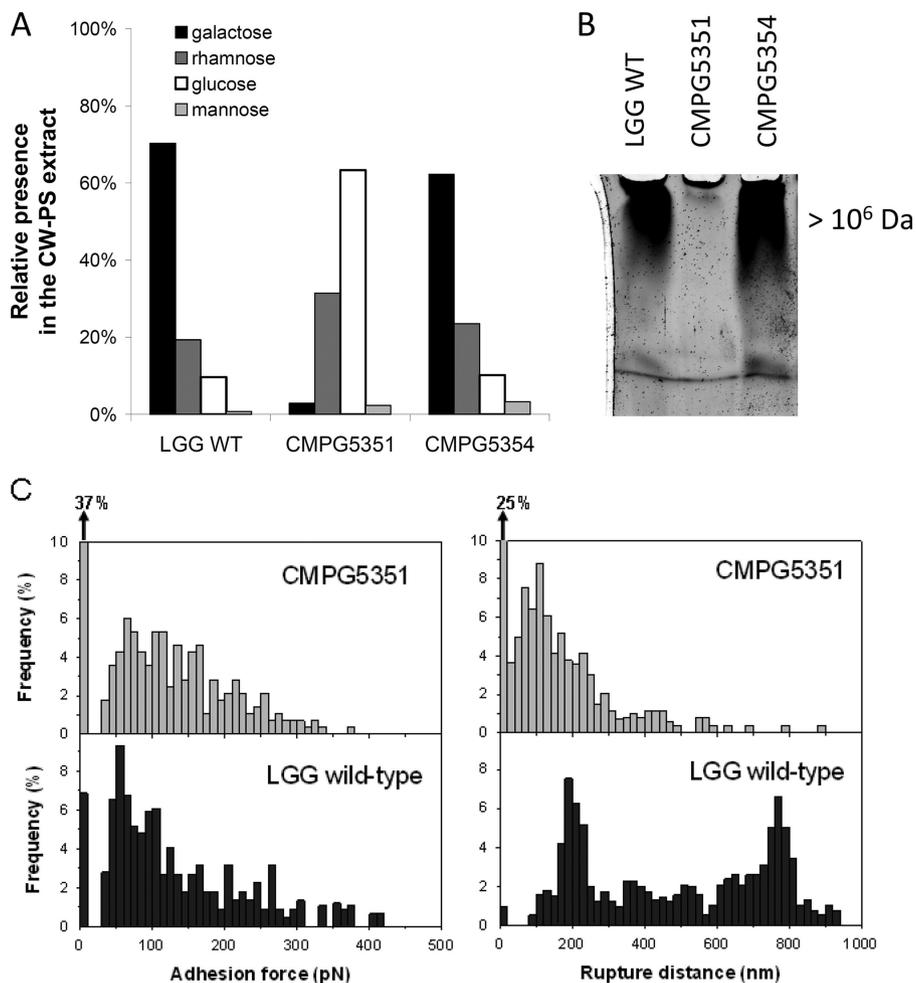


FIG. 3. Mutation of the *welE* gene in *L. rhamnosus* GG specifically attenuates the high-molecular-mass, galactose-rich EPS molecules. (A) Analysis of the sugar monomer compositions of the CW-PS extracts of wild-type *L. rhamnosus* GG (WT), *welE* mutant CMPG5351, and the complemented strain CMPG5354. The data are expressed in relative amounts, taking the total amount of detected monomeric sugars as 100%. (B) Polyacrylamide gel electrophoresis of the CW-PS extract of wild-type *L. rhamnosus* GG, *welE* mutant CMPG5351, and complemented strain CMPG5354. Equal amounts of material were loaded. The upper band corresponds to polysaccharides with a polymer size of $>1.4 \times 10^6$ Da based on size exclusion chromatography (data not shown). (C) Detection of individual galactose-rich polysaccharides on wild-type *L. rhamnosus* GG and *welE* mutant CMPG5351 by SMFS with PA-1 lectin-functionalized tips. Histograms ($n = 1,024$) of adhesion forces (left) and rupture distances (right) are shown.

compared to wild-type *L. rhamnosus* GG grown under the same conditions (Fig. 4B). Again, complementation restored the adherence capacity to wild-type levels (Fig. 4B). Finally, adhesion of wild-type *L. rhamnosus* GG, the *welE* mutant CMPG5351, and the complemented strain CMPG5354 grown in AOAC medium was studied using the human gut epithelial cell line Caco-2. A marked increase in adherence by the *welE* mutant could be observed, while complementation restored wild-type levels of adherence (Fig. 4C). Overall, these results indicate that the EPS-deficient mutant CMPG5351 has an increased in vitro adherence capacity to several substrates.

DISCUSSION

In this study, we identified and annotated the EPS gene cluster of the probiotic strain *L. rhamnosus* GG. This cluster shows a modular organization that is characteristic for gene clusters involved in the biosynthesis of heteropolymeric EPS or

CPS molecules (26, 45, 56). In comparison with the previously reported EPS gene clusters of four closely related *L. rhamnosus* strains (43), considerable differences can be observed, in terms of both the DNA sequences of the orthologs and the organization of the genes within the EPS gene cluster (Fig. 1A). Only the ORFs for regulatory proteins (Wzd, Wze, Wzr, and Wzb) and dTDP-rhamnose precursor biosynthesis (RmlA to -C) are highly conserved. The low level of conservation between the *L. rhamnosus* GG EPS gene cluster and the cluster of the other *L. rhamnosus* strains is in agreement with the different EPS structures of these strains: galactose-rich EPS molecules are synthesized by *L. rhamnosus* GG cells (27), while rhamnose-rich EPS molecules are synthesized by the *L. rhamnosus* ATCC 9595-related strains (54). The EPS gene cluster described in this paper seems to be unique and strain specific to *L. rhamnosus* GG. The mosaic organization of the EPS gene cluster of *L. rhamnosus* GG probably results from

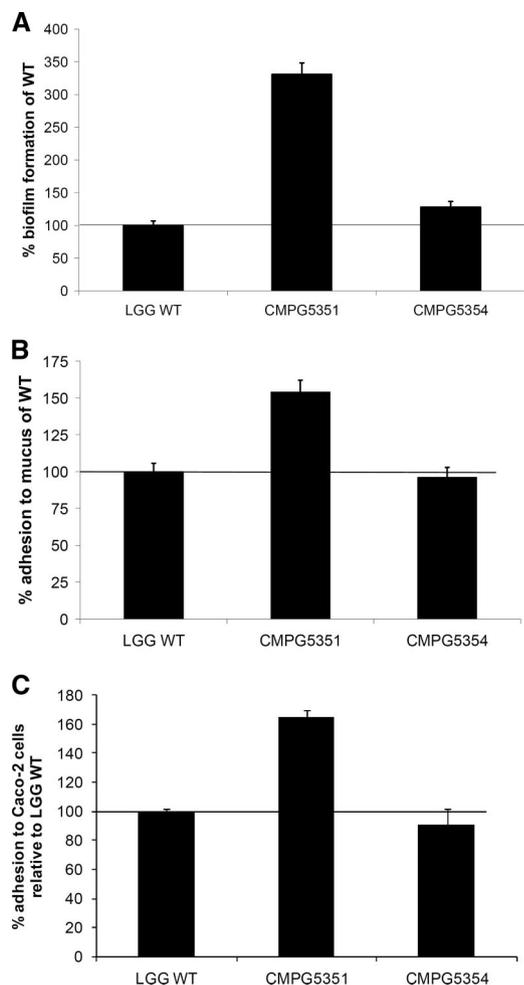


FIG. 4. Biofilm formation and adhesion to mucus and Caco-2 cells by wild-type *L. rhamnosus* GG (WT), the EPS *weIE* mutant CMPG5351, and complemented strain CMPG5354. (A) Biofilm formation (in AOAC medium) is expressed relative to the amount formed by wild-type *L. rhamnosus* GG. (B) The adhesion capacity to mucus is expressed relative to wild-type *L. rhamnosus* GG (set as 100%), of which ca. 22% of the added cells adhered. (C) The adhesion capacity to Caco-2 cells is expressed relative to wild-type *L. rhamnosus* GG, of which ca. 4% of the added cells adhered. Error bars indicate standard deviations.

horizontal gene transfer and homologous recombination events, as is common for EPS/CPS gene clusters (see, e.g., reference 38). Interestingly, the *rml* genes within the EPS cluster of *L. rhamnosus* GG seem to be inactivated due to an insertion sequence element (*ISLrh2* containing *orf1*) in *rmlA*, while Southern hybridization experiments suggest the presence of a complete *rmlABCD* operon at another locus in the *L. rhamnosus* GG genome.

The function of the identified EPS gene cluster in *L. rhamnosus* GG was confirmed by mutation of the *weIE* gene, encoding the putative priming glycosyltransferase. Detailed phenotypic analyses of the cell wall polysaccharides (quantity, monomer composition, polymer size, and SMFS) of *weIE* mutant CMPG5351 versus the wild type indicate a crucial and specific role for the priming galactosyltransferase *WeIE* in the biosynthesis of the high-molecular-weight, galactose-rich EPS

molecules of *L. rhamnosus* GG. Similarly, the priming glycosyltransferase encoded within the EPS cluster was shown to be crucial for EPS biosynthesis in the gram-positive cocci *Lactococcus lactis* (55) and *Streptococcus thermophilus* (39). However, such a key function has, to the best of our knowledge, not yet been established in gram-positive rod-shaped bacteria such as lactobacilli. Additionally, comparative phenotypic analyses of the *L. rhamnosus* GG wild-type and mutant CMPG5351 strains confirm the previous SMFS experiments (21) indicating that the *L. rhamnosus* GG surface contains, besides the long, galactose-rich type of EPS, also shorter, glucose-rich CW-PS. Of note, CW-PS quantification and monosaccharide analyses revealed that the glucose-rich CW-PS molecules become more exposed in the EPS mutant CMPG5351. As a consequence, only a ca. 3-fold decrease in the total extractable CW-PS amount in this mutant was observed, whereby the monomer composition and polymer length drastically changed. The characterization of these other CW-PS molecules and their biosynthetic genes is ongoing.

In comparison with other *Lactobacillus* strains, *L. rhamnosus* GG is known for its intrinsic high in vitro adherence capacity in several experimental setups (32, 52, 53). The construction of the *L. rhamnosus* GG EPS mutant CMPG5351 allowed us to study the specific contribution of the strain-specific long, galactose-rich EPS molecules to this adherence capacity. Interestingly, the EPS mutant CMPG5351 showed an increased adherence to commercially available pig mucus and Caco-2 epithelial cells. This suggests that the long, galactose-rich EPS molecules of *L. rhamnosus* GG are not required for its high adherence capacity. On the contrary, EPS seems to have a negative impact, possibly by shielding off adhesins. These data are in agreement with studies of streptococci showing that surface polysaccharides such as CPS can shield adhesins and reduce the adhesion capacity (35, 40, 50). Similarly, an EPS mutant of *Lactobacillus johnsonii* NCC533 was reported to have a slightly increased residence time in the murine gut, possibly because of enhanced exposure of adhesins (14). The characterization of the specific adhesins of *L. rhamnosus* GG should further substantiate this “shielding hypothesis.” Besides a putative role for the increased expression of glucose-rich polysaccharides in the adherence capacity of *L. rhamnosus* GG, TEM analyses suggest also an increased exposure of fimbria-like structures in the EPS mutant. Fimbriae or pili are well studied in certain bacterial species for their adhesive and biofilm-promoting properties (36), and some contain a considerable glycan chain (59). Fimbrial genes have been described for *L. johnsonii* NCC533 (44), but they are not common in lactobacilli for which the genome sequences have been determined to date (31). We are currently investigating further these fimbria-like structures on the *L. rhamnosus* GG surface.

Adhesion of probiotic strains is usually assessed in short-term assays (31). However, in most natural niches, adherent bacteria can form multicellular structures recognized as biofilm-like communities (7). Small biofilms or microcolonies have also been documented for bacteria residing in the gastrointestinal tract, and this is suggested to enhance their residence time (49). Interestingly, the EPS mutant of *L. rhamnosus* GG showed an increased biofilm formation capacity when grown in AOAC medium. The fact that the long, galactose-rich EPS molecules are not required for its biofilm formation is remark-

able given the well-documented role for extracellular polysaccharides in biofilm matrices of various bacterial species (7). Nevertheless, these findings do not rule out that polysaccharides as such are implicated in the extracellular matrix of *L. rhamnosus* GG biofilms. The shorter, yet-unknown glucose-rich CW-PS molecules of *L. rhamnosus* GG could be important constituents of *L. rhamnosus* GG biofilms together with proteinaceous compounds, such as the fimbria-like structures. This would imply that the specific type of CW-PS is important for *L. rhamnosus* GG biofilm formation. Of interest is that a divergent role for surface polysaccharides in biofilm formation has also been shown in other bacteria, such as *Pseudomonas aeruginosa* (47). Future analyses of the biofilm matrix of *L. rhamnosus* GG are aimed to shed light on its exact composition under various conditions.

In conclusion, the current report describes the identification and annotation of the gene cluster involved in the biosynthesis of specific galactose-rich EPS molecules of the clinically well documented probiotic strain *L. rhamnosus* GG, as well as the functional analysis of the corresponding priming glycosyltransferase *welE*. Phenotypic analysis of the *welE* mutant indicate that the long, galactose-rich EPS molecules of *L. rhamnosus* GG play an important role in determining cell surface properties such as adhesion and biofilm formation. Future studies should provide more insight into the potential role of EPS as a probiotic factor important for some of the reported health benefits of *L. rhamnosus* GG.

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