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Functional Analysis of D-Alanylation of Lipoteichoic Acid in the Probiotic Strain *Lactobacillus rhamnosus* GG[▽]

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Lipoteichoic acid (LTA) is a macroamphiphile molecule which performs several functions in gram-positive bacteria, such as maintenance of cell wall homeostasis. D-Alanylation of LTA requires the proteins encoded by the *dlt* operon, and this process is directly related to the charge properties of this polymer strongly contributing to its function. The insertional inactivation of *dltD* of the probiotic strain *Lactobacillus rhamnosus* GG (ATCC 53103) resulted in the complete absence of D-alanyl esters in the LTA as confirmed by nuclear magnetic resonance analysis. This was reflected in modifications of the bacterial cell surface properties. The *dltD* strain showed 2.4-fold-increased cell length, a low survival capacity in response to gastric juice challenge, an increased sensitivity to human beta-defensin-2, an increased rate of autolysis, an increased capacity to initiate growth in the presence of an anionic detergent, and a decreased capacity to initiate growth in the presence of cationic peptides compared to wild-type results. However, in vitro experiments revealed no major differences for adhesion to human intestinal epithelial cells, biofilm formation, and immunomodulation. These properties are considered to be important for probiotics. The role of the *dlt* operon in lactobacilli is discussed in view of these results.

The cell wall of gram-positive bacteria constitutes a protective barrier essential for survival, shape, and integrity. Proteins and teichoic acids (TAs) composed of wall teichoic acid (WTA) and/or lipoteichoic acid (LTA) are associated with this peptidoglycan-containing wall (33, 40). WTA is covalently linked to the peptidoglycan, whereas LTA is a macroamphiphile molecule with its glycolipid moiety anchored to the membrane and its polyglycerophosphate (Gro-P) chain extending into the wall (40). Together, TAs are the most abundant polyanions of gram-positive bacteria and represent up to 50% of the cell wall dry weight (24). Glycosyl substitutions in WTA and D-alanyl ester (D-Ala ester) substitutions in LTA are directly related to the charge properties of these polymers and strongly contribute to their function, as evidenced by the phenotypes of strains with genetic changes altering the glycosyl substitution and D-alanylation process (17, 33, 45, 57).

The D-Ala ester substitution of LTA requires four proteins encoded by the *dlt* operon. Two of these proteins are the D-alanyl carrier protein ligase (Dcl, encoded by *dltA*), which activates D-alanine by use of ATP, and the D-alanyl carrier protein (Dcp), which is encoded by *dltC*. DltB is a putative transmembrane protein predicted to be involved in the passage of the activated D-alanyl-Dcp complex across the glycerol phosphate backbone of LTA. Finally, the DltD membrane protein facilitates the binding of Dcp for ligation with D-Ala and additionally has thioesterase activity for removing mischarged D-alanyl carrier proteins (13, 40). Inactivation of genes within this operon in various gram-positive bacteria results in the decrease or complete absence of D-Ala esters from LTA (1, 18, 35, 46, 47). D-Ala-deficient mutants are found to exhibit a variety of phenotypic changes that could be attributed to the resulting charge modification of their cell surface. For instance, alterations of cell morphology associated with defects in septum formation (9, 41, 45), variations in modulation of the activity of autolysins (6, 19, 55, 58), differences in cation binding to the cell envelope required for enzyme function (33), alterations of the electromechanical properties of the cell wall (40), altered resistance to antimicrobial cationic peptides (8, 40), modified adhesion, epithelial cell invasion, and virulence (1, 35), effects on biofilm formation (7, 18, 23), and alterations in immune response (14, 22, 38, 39, 51) have been reported. While it is apparent that the D-Ala esters of LTA play an essential role in the physiology and properties of the cell surface of gram-positive bacteria, the genotype-phenotype relation of the *dlt* operon is complex and appears to be species dependent.

By construction of a *dltD* knockout mutant, this study aimed to determine first the role of the *dltD* gene in the D-alanylation of the LTA in the probiotic strain *Lactobacillus rhamnosus* GG. Probiotic bacteria are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (20). *L. rhamnosus* GG is a well-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
E. coli		
DH5α	F⁻ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44* λ⁻ *thi-1 gyrA96 relA1*	Gibco-BRL
S. enterica serovar Typhimurium		
SL1344	*xyl hisG rpsL*; virulent; Sm^r	26
L. rhamnosus GG		
Wild type	Human isolate	ATCC 53103 (54)
CMPG5540	*dltD* knockout mutant of *L. rhamnosus* GG; *dltD*::*tetR*	This study
CMPG5541	CMPG5540 complemented by pCMPG5227	This study
Plasmids		
pCRII-TOPO	Cloning vector, ampicillin and kanamycin resistance	Invitrogen
pFAJ5301	Cloning vector, pUC18 derivative, erythromycin resistance	Unpublished results
pLAB1301	*E. coli-Lactobacillus* shuttle vector, ampicillin and erythromycin resistance	28
pMD5057	Tetracycline resistance plasmid from *Lactobacillus plantarum* 5057	11
pCMPG5221	pCRII-TOPO vector containing the 2,682-bp *dltD* gene and flanking regions from *L. rhamnosus* GG (Pro-230 and Pro-231)	This study
pCMPG5222	pFAJ5301 containing an HindIII-EcoRV fragment (*dltD* gene and flanking regions)	This study
pCMPG5223	Suicide vector to knock out the *L. rhamnosus* GG *dltD* gene through insertion of *tetR* gene from pMD5057 in the BbsI-NcoI site	This study
pCMPG5226	pCMPG5901 containing the functional *dltD* gene driven by the *L. rhamnosus* GG *ldhL* promoter	This study
pCMPG5227	PLAB1301 containing the functional *dltD* gene driven by the *ldhL* promoter	This study
pCMPG5901	PCRII-TOPO vector containing the *ldhL* promoter (176 bp) from *L. rhamnosus* GG amplified using primers Pro-127 and Pro-128	Unpublished results

studied probiotic strain (4, 21). For this reason, the second aim of this study was to investigate the role of the D-alanylation of the *L. rhamnosus* GG LTA in some morphological, membrane charge property, and probiotic characteristics, such as adherence to human intestinal epithelial cells, biofilm formation, resistance to gastric juice challenge, and immunomodulation of human intestinal epithelial cells and peripheral blood mononuclear cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *L. rhamnosus* GG was routinely grown in nonshaken MRS medium (Difco) at 37°C. *Escherichia coli* cells were grown in Luria-Bertani (LB) medium with aeration at 37°C. When required, antibiotics were used at the following concentrations: 10 μg/ml tetracycline, 100 μg/ml ampicillin, and 5 μg/ml (*L. rhamnosus* GG) or 100 μg/ml (*E. coli*) erythromycin.

DNA manipulations. Routine molecular biology techniques were employed as described before (49). Primer sequences used in this study are listed in Table 2 (Eurogentec). Enzymes for molecular biology were purchased from New England Biolabs and used according to the instructions of the suppliers. Plasmid DNA from *E. coli* was prepared using QIAGEN Miniprep kits. Chromosomal DNA from *L. rhamnosus* GG was isolated as previously described (15).

Construction and analysis of the *L. rhamnosus* GG *dltD* mutant (CMPG5540). The complete genome sequence of the *dlt* operon of *Lactobacillus rhamnosus* ATCC 7469 is published in the NCBI database under accession number AF192553 (13). Based on this sequence, a fragment of 2,682 bp containing the *L. rhamnosus* GG *dltD* gene and its 779-bp upstream and 635-bp downstream regions was amplified using primers Pro-230 and Pro-231 and cloned in pCRII-TOPO, yielding pCMPG5221. Subsequently, the *dltD* gene with its flanking regions was subcloned as a HindIII-EcoRV fragment in pFAJ5301, resulting in pCMPG5222. To inactivate the *dltD* gene, a BbsI-NcoI fragment from pCMPG5522 was replaced by the tetracycline resistance cassette *tet*(M) previously amplified from plasmid pMD5057 of *L. plantarum* 5057 (11) by use of primers Pro-221 and Pro-222. The resulting suicide vector, pCMPG5223, was electroporated into *L. rhamnosus* GG (15). Transformants were selected for resistance to 10 μg/ml of tetracycline. Confirmation of DNA recombination was performed by PCR using primers Pro-261 and Pro-231 and Southern hybridization using a *dltD* probe synthesized with primers Pro-261 and Pro-262. The *dltD* mutant was designated CMPG5540 and was further analyzed.

Complementation of the knockout *dltD* mutant. For the construction of the complemented strain, the functional *dltD* gene was cloned under the control of the *L. rhamnosus* GG *ldhL* promoter in pCMPG5901, yielding pCMPG5226. Subsequently, an EclII136-NotI fragment containing the functional *dltD* gene and the *ldhL* promoter was subcloned into pLAB1301 (28). This construct was

TABLE 2. Primer sequences used in this study

Bacterial primer	Sequence (5′ to 3′)[a]
Pro-128 (NdeI)	GTCATATGGATATCATCCTTTCTTATGTGCATGC
Pro-127 (SacI)	CTGAGCTCCTTGTCACAGGATTCACAAGTCTTGC
Pro-221 (EcoRI)	GAATTCGAGATTCCTTTACAAATATGCTCTTAC
Pro-222 (EcoRI)	CGAATTCGTTCGGAATAGGTTATACTAGACAAAAG
Pro-230	GCCGGTTACAGTTTGTTCGCGG
Pro-231	ACAGGAGGCACAACATGGCAAAATC
Pro-261	TCCATGTCGGCCAACGTGCT
Pro-262	ATTGGCTTCCCGCCATTCCC
Pro-443	TCAAGTTTGTTAAGATGGAT
Pro-444	GGATAATTTTCCTGCTTGAA
Pro-445	TTTGTCGAAATTGCGTTACT
Pro-446	TCGAAACACCGATGAACTTT

[a] Underlined sequences represent restriction sites indicated parenthetically.

designated pCMPG5227 and introduced in CMPG5540 by electroporation as previously described (15). Transformants were selected for resistance to 10 μg/ml of tetracycline and to 5 μg/ml of erythromycin. The complemented strain, CMPG5541, was confirmed by PCR using primers Pro-127, Pro-262, and Pro-261 and included in all assays performed.

Cloning and analysis of the *L. rhamnosus* GG *dlt* operon. Concomitantly, in order to sequence the additional genes within the *dlt* operon of *L. rhamnosus* GG, primers Pro-443, Pro-444, Pro-445, and Pro-446 were designed after selection on the DNA sequence of the *dlt* operon of *L. rhamnosus* ATCC 7469. The PCRs were carried out with *Pfx* (Invitrogen), a DNA polymerase with proofreading activity. The PCR fragments were cloned in pCRII-TOPO and sequenced by the chain termination dideoxynucleoside triphosphate method (50) with a BigDye Terminator V3.1 cycle sequencing kit, using an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems, Lennik, Belgium). Databases were screened for similarities by using BLAST (2, 3), and alignment of overlapping fragments was performed with Vector NTI Advance 10 ContigExpress software (Informax, Oxford, United Kingdom).

LTA purification. For the LTA isolation, 11.83 g of lyophilized *L. rhamnosus* GG cells and 22.12 g of the *dltD* mutant (CMPG5540) cells were extracted using butanol and hydrophobic interaction chromatography as previously described (38), with minor modifications. Briefly, bacterial cells were disrupted for 15 min by sonication and resuspended in 50 ml of 0.1 M citrate buffer (pH 4.7). The bacterial lysate was mixed while being stirred with an equal volume of *n*-butanol (Merck, Darmstadt, Germany) for 20 min at room temperature. The suspension was then centrifuged at 8,300 × *g* for 40 min, resulting in a two-phase system. The lower aqueous phase was lyophilized and subsequently resuspended in 35 ml of chromatography start buffer (15% *n*-propanol in 0.1 M ammonium acetate; pH 4.7) followed by 60 min of centrifugation at 26,900 × *g* and sterilization by filtration (0.2 μm). The supernatant was subjected to hydrophobic interaction chromatography on an octyl-Sepharose column (2.5 by 11 cm) using a linear gradient of from 15% to 60% *n*-propanol in 0.1 M ammonium acetate (pH 4.7). LTA-containing fractions were identified by their phosphate content based on the formation of phosphomolybdenum blue from phosphate (52). The endotoxin contamination of the LTA preparations was assessed by the kinetic *Limulus* amoebocyte lysate assay (Charles River, Charleston, SC).

LTA structure analysis by NMR spectroscopy. Nuclear magnetic resonance (NMR) spectra of the LTA samples were determined with a Bruker Avance 600 MHz spectrometer with a 5-mm probe at 300 K. Spectra were obtained using D_2O solutions and 3-(trimethylsilyl)3,3,2,2-tetradeuteropropionic acid sodium salt (d_4-TSPA) as an internal standard for ^1H NMR (δ_H, 0.00 ppm) and acetone for chemical shifts of ^{13}C (δ_C, 30.02 ppm). Assignments were taken from two-dimensional homonuclear double-quantum-filtered correlation spectroscopy, total correlated spectroscopy, and rotational nuclear Overhauser effect spectroscopy experiments using a water suppression technique and two-dimensional heteronuclear single-quantum correlation (^1H to ^{13}C) spectra. In the total correlated spectroscopy experiments the mixing times were 100 ms, and the rotational nuclear Overhauser effect spectroscopy experiments were performed with 200 ms of spinlock time. Data acquisition and processing were done using standard Bruker software. The average number of repeating units in the polyglycerophosphate backbone, the percentage of substitution, and the chain length of the fatty acids in the membrane anchor were calculated directly from the integrals of the proton spectra.

Transmission electron microscopy. Bacteria were grown overnight (16 h at 37°C). Uncoated grids were used as a probe to adsorb bacterial cells. The grids were placed on a drop of bacterial suspension for 15 s, incubated in 0.25% phosphotungstenic acid (pH = 7) for 30 s, and washed three times, and excess liquid was drained. The bacteria were observed with a Philips EM 208S transmission electron microscope at 80 kV. Images were digitalized using an SIS image analysis system.

Analysis of autolysis. Triton X-100 was used to study induced autolysis under nongrowing conditions as previously described (48), with minor modifications. Briefly, cells were grown overnight, harvested by centrifugation (4,000 × *g* for 20 min at 4°C), washed three times with equal volumes of phosphate buffer (pH 6.5; 10 mM), and resuspended (optical density at 600 nm [OD_{600}] of ~1.6) in phosphate buffer (pH 6.5; 200 mM) containing 0.05% (vol/vol) Triton X-100. The cell suspension was incubated at 37°C under agitation, and autolysis was monitored by examining the decrease of OD_{600} in time.

Growth in the presence of the cationic peptides nisin and polymyxin B. Nisin and polymyxin B were purchased from Sigma-Aldrich. Overnight cultures containing about 10^8 to 10^9 CFU/ml of *L. rhamnosus* GG wild-type, *dltD* mutant, and complemented strain cells were diluted 15,000-fold in MRS medium containing either nisin at a concentration between 0.2 and 2.5 μg/ml or polymyxin B at a concentration between 8 and 1,000 μg/ml. Bacteria were grown at 37°C with continuous shaking in order to avoid cell aggregation, and the OD_{600} was measured automatically each 30 min during at least 80 h using a Bioscreen C instrument (Labsystems Ltd. Oy). Concomitantly, growth of *L. rhamnosus* GG, the *dltD* mutant, and the complemented strain in the presence of an anionic detergent (sodium dodecyl sulfate [SDS] at 0.01% and 0.015%) was assessed. Each run was performed at least in triplicate.

Human beta-defensin sensitivity assay. *L. rhamnosus* GG wild-type and *dltD* mutant cells were tested for their sensitivity to 3 μg/ml human beta-defensin 1 (hBD1) and hBD2. After 3 h of incubation at 37°C, bacterial viability was measured by plating serial dilutions on MRS agar as previously described (15). The experiment was performed in triplicate.

Survival in simulated gastric juice. Simulated gastric juice was prepared and survival tests were performed as previously described (10). The percentages of survival were calculated by comparing the cell numbers before and after addition to simulated gastric juice at 0, 30, 60, and 90 min. The experiment was performed in triplicate.

In vitro adhesion assay to Caco-2 and HT-29 human intestinal epithelial cell lines. Caco-2 and HT-29 cells were purchased from ATCC (Rockville, MD). Cells were routinely grown in 75-cm^2 culture flasks under conditions of 37°C, 5% CO_2, and 90% relative humidity. For both cell lines, Dulbecco modified Eagle medium (DMEM)/F-12 (GibcoBRL) (1:1) supplemented with 10% fetal bovine serum (FBS; HyClone) but without antibiotics was used as the culture medium. Cells were passaged every 3 days (at 70 to 80% confluence) at a split ratio of 1 to 7. For adhesion experiments, epithelial cells were plated at a density of 40,000 cells/cm^2 in 12-well plates (Cellstar). Confluence was reached within 3 to 4 days after seeding, and monolayers were used for the experiments 15 days after seeding. Adherence of *L. rhamnosus* GG wild-type, *dltD* mutant, and complemented strain cells to the epithelial cells was examined by adding 1.5 ml of DMEM containing 10^9 CFU/ml. After incubation at 37°C for 45 min, epithelial cells were washed two times with prewarmed phosphate-buffered saline (PBS). Subsequently, 100 μl of 1× trypsin-EDTA (Invitrogen) was added to each well and incubated for 10 min at 37°C. Finally, 900 μl of PBS was added and mixed, and serial dilutions were plated out. Plates were incubated at 37°C for 72 h. Adhesion of *L. rhamnosus* GG wild-type, *dltD* mutant, and complemented strain cells was tested in triplicate in three independent experiments.

In vitro biofilm assay. In vitro biofilm formation was determined as previously described (16, 36), with minor modifications. Briefly, biofilm formation was assayed on polystyrene pegs hanging into microtiter plate wells. The pegs were placed in wells containing a bacterial concentration of 3×10^7 CFU in 200 μl AOAC medium (Difco) (36) and incubated in anaerobic jars for 72 h at 37°C. Medium was changed every day. Wells containing sterile AOAC medium and *L. rhamnosus* GG wild-type cells were included as negative and positive controls, respectively. Each experiment was performed in triplicate.

Immunomodulation of HT-29 cell line. HT-29 cells were maintained as described above. *L. rhamnosus* GG wild-type and *dltD* mutant cells were grown overnight, centrifuged at 4,000 × *g* and 4°C for 20 min, and washed with cold PBS. Immunomodulation of HT-29 cells was examined by adding 1.5 ml of DMEM without FBS containing 5×10^6 CFU/ml of either *L. rhamnosus* GG wild-type or *dltD* mutant cells. *Salmonella enterica* serovar Typhimurium SL1344 cells in a concentration of 5×10^6 CFU/ml and interleukin-1 beta (IL-1β) (Sigma-Aldrich) (10 ng/ml) were used as positive controls, and DMEM without FBS was used as a negative control. After incubation at 37°C for 3.5 h (5% CO_2 and 90% humidity), epithelial cells were washed two times with prewarmed PBS. Subsequently, 200 μl of PBS was added to each well and RNA extraction was performed by using a High Pure RNA isolation kit (Roche Molecular Biochemicals) and following the manufacturer's instructions. After isolation, RNA integrity was analyzed using an Agilent RNA 600 kit and 2100 bioanalyzer expert software. Cytokine gene expression was determined by real-time reverse transcriptase PCR using primers and probes as previously described (43).

Cytokine induction in peripheral blood mononuclear cells (PBMC). Cytokine induction using a 24-h culture of bacterial cells, and using *Streptococcus gordonii* LMG17843 and *E. coli* TG1 cells as internal controls, was performed as previously described (22).

Nucleotide sequence accession number. The *dlt* sequence of *L. rhamnosus* GG has been deposited in the NCBI database under GenBank accession number DQ906101.

RESULTS

Analysis of the *dlt* operon of *L. rhamnosus* GG. Since the genome sequence of *L. rhamnosus* GG is unavailable, a homology strategy based on the *dlt* sequence of *L. rhamnosus*

FIG. 1. Genetic organization of the *L. rhamnosus* GG *dlt* operon. A schematic representation of the *dlt* operon (~5.8 kb) of *L. rhamnosus* GG as revealed by sequencing and BLAST analysis is presented. Open reading frames corresponding to *dltA*, *dltB*, *dltC*, and *dltD* are indicated with arrows. The putative promoter region (5′ end), represented in the figure by a flag, contains a −10 "TATTAA" region and a −35 "TGGTTT" region separated by 19 bp. The potential ribosome binding site "GGGGG" is located 8 bp upstream of the putative ATG start codon of *dltA* (not shown). A second potential ribosomal binding site, "AAAGAGG," was found 9 bp upstream of the putative start codon of the *dltC* gene (not shown). *dltB* overlaps the stop codon of *dltA* by 1 bp, and *dltD* overlaps the stop codon of *dltC* by 4 bp. *dltC* and *dltB* are separated by a 69-bp intergenic region. The 3′ end of the operon reveals a putative terminator 99 bp downstream of the *dltD* stop codon, represented in the figure by a stem-loop. It is a 12-bp inverted repeat followed by a series of T residues (TTTATTTT).

ATCC 7469 (AF192553) (13) was used to isolate the *dlt* genes within the *dlt* operon of *L. rhamnosus* GG cells. Based on this strategy, a continuous 5.8-kb sequence of *L. rhamnosus* GG genomic DNA was determined. The analysis of the sequence revealed four open reading frames (*dltA*, *dltB*, *dltC*, and *dltD*), as depicted in Fig. 1. Additionally, the in silico-translated protein sequences encoded by the *L. rhamnosus* GG *dlt* operon, i.e., those corresponding to DltA protein (506 amino acids [aa]), DltB protein (405 aa), DltC protein (81 aa), and DltD protein (423 aa), were used for screening databases using BLAST (2, 3). Homologies of these *L. rhamnosus* GG Dlt protein sequences to the Dlt protein sequences of *L. rhamnosus* ATCC 7469 and *L. plantarum* WCFS1 are summarized in Table 3.

Finally, the *dltD* gene of *L. rhamnosus* GG which encodes a putative protein of 423 aa with a theoretical mass and pI of 47.987 kDa and 9.57, respectively, was successfully knocked out by the insertion of the tetracycline cassette from *L. plantarum* 5057. The correct insertion was confirmed by PCR and Southern hybridization as described in Materials and Methods (data not shown).

LTA purification and structure analysis. After purification, different LTA-containing fractions were identified. For *L. rhamnosus* GG wild-type cells (Fig. 2A), fractions 41 to 49 were pooled, resulting in 14.81 mg LTA. For the *dltD* mutant cells (Fig. 2B), the phosphate determination revealed fractions 41 to 46 (represented by peak 1) and fractions 48 to 88 (represented by peak 2) with 9.56 mg LTA in peak 1 and 9.26 mg LTA in peak 2, respectively. Endotoxin contamination of all LTA extractions showed lipopolysaccharide contamination below 0.6 endotoxin units/mg LTA, i.e., less than 60 pg lipopolysaccharide per mg LTA.

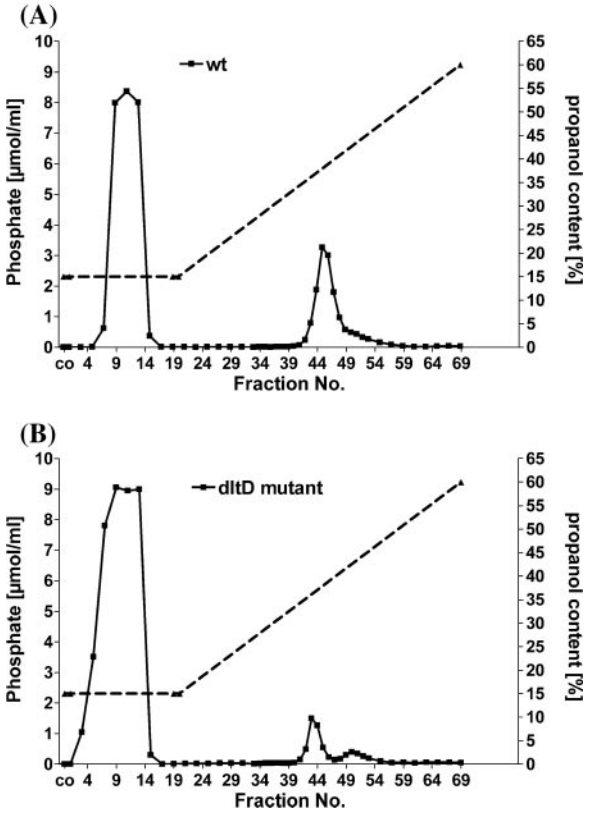

FIG. 2. LTA elution profiles and phosphate content of *L. rhamnosus* GG wild-type and mutant cells. The phosphate determination of the LTA fractions from *L. rhamnosus* GG wild-type (A) and *dltD* mutant (B) cells is plotted together with the correspondent propanol gradient of the fast protein liquid chromatography.

The molecular structure determined by NMR spectroscopy revealed that LTA from *L. rhamnosus* GG wild-type cells is constituted of Gro-P with D-alanyl esters as unique detectable substituents (74% D-Ala:Gro-P). LTA from the *L. rhamnosus* GG wild type is formed by average chains of 50 glycerophosphate residues (Fig. 3). For the *dltD* mutant, two fractions were eluted from the chromatography column as previously mentioned. The major peak (fractions 41 to 46) and the second peak (fractions 48 to 55) contain LTA molecules with average lengths of only 29 and 7 glycerophosphate residues, respectively. D-Alanyl ester substitution in the *dltD* mutant was completely abolished in both peaks. The analysis of the glycolipic moiety in the wild type reveals an average fatty acid chain of C_{14}, with one double bond per fatty acid (two double bonds in every membrane anchor). The fatty acid chain in the *dltD* mutant was on average two carbon atoms longer (C_{16}) in comparison to that seen in the parental strain. Likewise, as in the

TABLE 3. Homology analysis of the translated *L. rhamnosus* GG Dlt proteins

Strain	% Homology (GenBank accession no.)				Reference
	DltA	DltB	DltC	DltD	
L. rhamnosus ATCC 7469	99 (AAF09201)	95 (AAF09202)	100 (AAF09203)	99 (AAF09204)	12
L. plantarum WCFS1	62 (NP_785546)	64 (NP_785546)	64 (NP_785544)	51 (NP_785543)	30

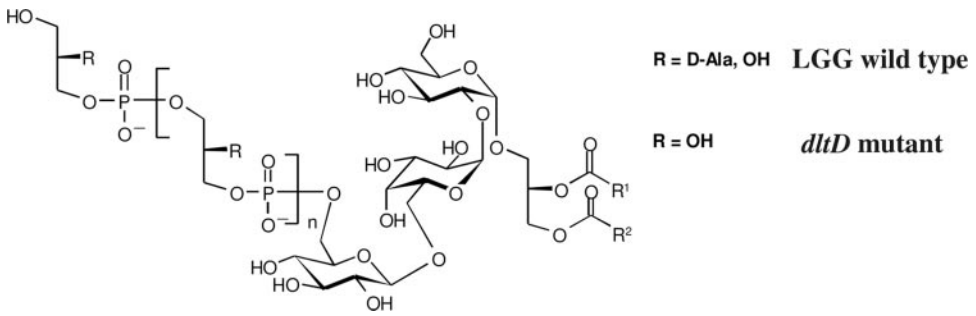

FIG. 3. Schematic representation of the structure of the LTA from the *L. rhamnosus* GG wild type determined from NMR spectroscopy analysis. The average number of repeating units in the polyglycerophosphate backbone, the average percentage of substitution, and the average chain length of the fatty acids in the membrane anchor were calculated directly from the integrals of the proton spectra. R^1 and R^2, fatty acid chains. D-Ala ester substitution of the wild type (R = D-Ala, OH) is replaced by OH groups in the *dltD* mutant.

wild type, in the *dltD* mutant one double bond per fatty acid was present (data not shown).

Increase in cell length and defects in septum formation assessed by transmission electron microscopy. Comparison of transmission electron micrographs of the *L. rhamnosus* GG wild-type and *dltD* mutant cells showed an increase in the length of the mutant of about 2.4-fold in comparison to the parental strain results (Fig. 4A). Additionally, morphological alterations at the level of the septum and defective cell separation in the *dltD* mutant were observed (Fig. 4B).

Analysis of autolysis. As shown in Fig. 5, the *dltD* mutant lyses to a greater extent than the parental strain. An OD_{600} decrease of 0.2 units for the *dltD* mutant corresponding to $\sim 10^8$ CFU/ml was observed during the first hour of incuba-

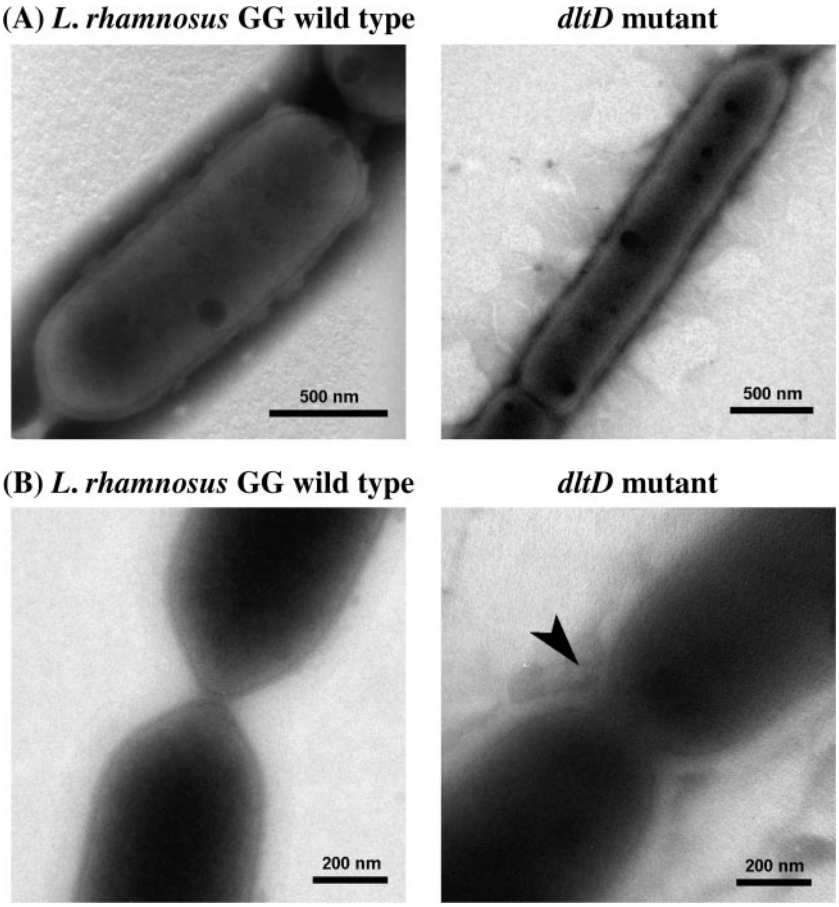

FIG. 4. Effect of D-alanylation on cell length and septum formation. (A) Ultrastructural analysis of the cell morphology of *L. rhamnosus* GG wild-type and *dltD* mutant cells grown overnight in MRS medium and visualized with transmission electron microscopy at 80 kV. The cell length of the complemented strain was restored to wild-type values. (B) Defect in septum morphology. The arrow shows the altered cell wall around the septum in the *dltD* mutant.

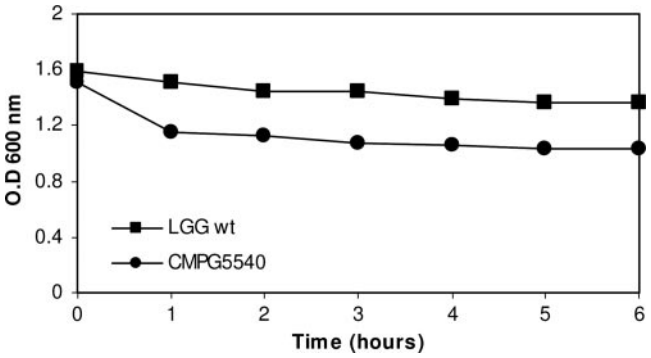

FIG. 5. Effects of the mutation of the *dltD* gene on autolysis. Autolysis of the *L. rhamnosus* GG wild type (squares) and the *dltD* mutant (CMPG5540) (circles) was followed in time by incubation of the cells with a lysis inducer (Triton X-100). The values reported in the experiment did not differ by more than 5%. Therefore, single datum points are presented in the figure without standard deviation bars. OD_{600} values comparable to wild-type values were obtained for the complemented strain (CMPG5541).

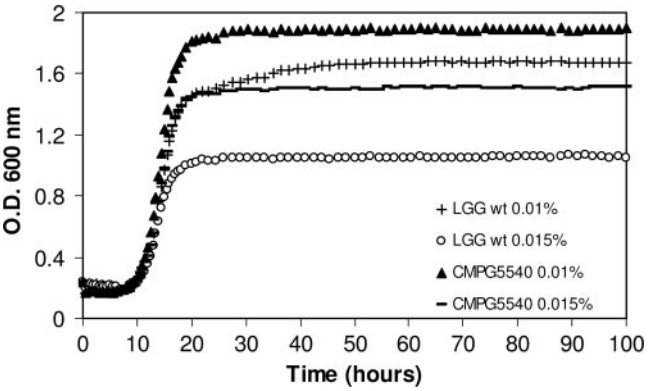

FIG. 7. Effect of SDS on the growth of the *L. rhamnosus* GG wild type and the *dltD* mutant. Values obtained for the growth of the *L. rhamnosus* GG wild type (cross and lines) versus that of the *dltD* mutant (CMPG5540) (triangles and squares) in medium containing either 0.01% or 0.015% SDS represent the average results of three independent runs.

tion in comparison to the OD_{600} value obtained for the wild type.

Effects of the cationic peptides nisin and polymyxin B and the anionic detergent SDS on the growth of *L. rhamnosus* GG wild-type and *dltD* mutant cells. The capacity of *L. rhamnosus* GG and the *dltD* mutant cells to grow in medium supplemented with the cationic peptides nisin and polymyxin B was evaluated. In general, a minimum of ~10 h of lag-phase retardation was observed for the mutant grown in the presence of nisin at different concentrations compared to the wild-type results. However, the final cell densities reached were similar for the two strains (Fig. 6). For polymyxin B, the *dltD* mutant was found to have ~10 h of lag-phase retardation even with a concentration of polymyxin B 100 times lower than that used for the wild type. Again, no differences were observed regarding final cell density (data not shown). Additionally, the ability of *L. rhamnosus* GG and the *dltD* mutant to grow in medium containing low concentrations of a strong anionic detergent (SDS) was evaluated. In this case, the *dltD* mutant was shown to be less affected by the action of SDS than the parental strain, reaching a higher optical density in the stationary phase (Fig. 7). The sensitivity of the *dltD* mutant to the tested cationic peptides and SDS was restored to wild-type levels when the mutant was complemented with the *dltD* gene (data not shown).

Sensitivity of *L. rhamnosus* GG wild type and *dltD* mutant cells to human beta-defensins. We investigated the effect of hBD1 and hBD2 on *L. rhamnosus* GG wild-type and *dltD* mutant cells (Fig. 8). While hBD1 showed no effect on *L. rhamnosus* GG wild-type or *dltD* mutant cells after 3 h of incubation, the cells were sensitive to hBD2. The *dltD* mutant was on average ~50% more sensitive to cationic hBD2 than the parental strain (Fig. 8).

Survival in simulated gastric juice. The *dltD* mutant showed a strong reduction in the capacity to survive the in vitro gastric juice challenge, resulting in complete loss of viability after 30 min of incubation (Fig. 9). Additionally, the capacity to initiate

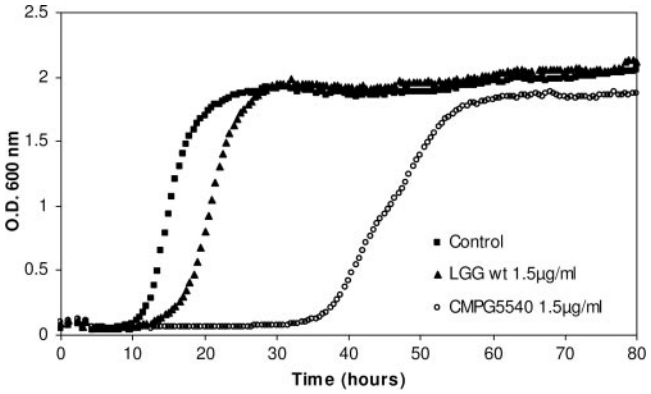

FIG. 6. Effect of the cationic peptide nisin on the growth of *L. rhamnosus* GG wild-type and *dltD* mutant cells. No differences in growth in MRS medium between the *L. rhamnosus* GG wild type and the *dltD* mutant were observed. Therefore, average growth values for both strains are represented by squares (control). Values obtained for the growth of the *L. rhamnosus* GG wild type (triangles) versus that of the *dltD* mutant (CMPG5540) (circles) in medium containing 1.5 μg/ml nisin represent the average results of three independent runs.

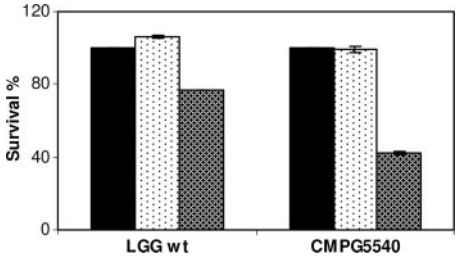

FIG. 8. Sensitivity of the *L. rhamnosus* GG wild type (wt) and the *dltD* mutant to human beta-defensins. Bacterial viability of *L. rhamnosus* GG wild-type and *dltD* mutant cells after 3 h of contact with 3 μg/ml hBD1 (stippled white bars) or hBD2 (stippled gray bars) was determined by plate counting. The values represent percentages of survival of the initial numbers of *L. rhamnosus* cells. No human beta-defensins were added to the controls (black bars). The error bars indicate standard deviations of the results of three independent measurements.

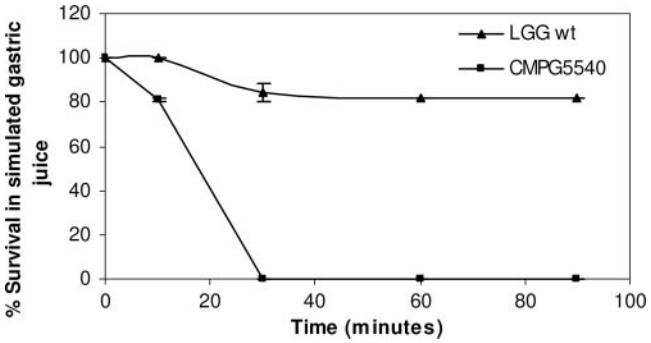

FIG. 9. Comparison of survival of *L. rhamnosus* GG wild-type (wt) and *dltD* mutant cells in simulated gastric juice. Percentages of survival of *L. rhamnosus* GG (triangles) compared to the *dltD* mutant (CMPG5540) (squares) were calculated by comparing the cell numbers before and after addition to simulated gastric juice at 30-min intervals. Recovery of acid tolerance was restored to wild-type values in the complemented strain.

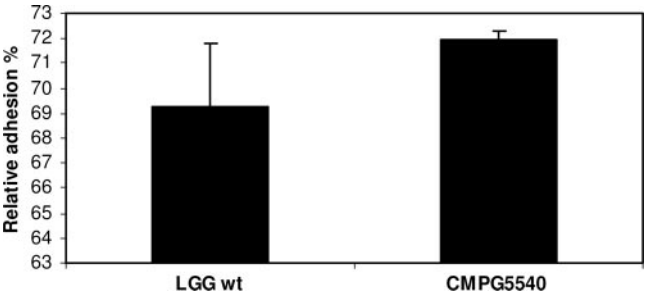

FIG. 10. Adhesion of *L. rhamnosus* GG and the *dltD* mutant to human intestinal epithelial cells. The levels of bacteria initially added to the Caco-2 cells were set to 100%. Data shown are relative to this percentage. Similar percentages of adhesion were observed for the HT-29 and Caco-2 cell lines. Graphs show the values obtained for Caco-2 cells only.

growth under conditions of low pH (3.5, 4.0, and 4.5) was evaluated. Results confirmed that the acid tolerance of the *dltD* mutant was strongly diminished compared to that of the wild type (data not shown).

In vitro adhesion to Caco-2 and HT-29 human intestinal epithelial cell lines and biofilm formation. No significant differences in adhesion to human intestinal epithelial cells (Fig. 10) and capacity to form biofilm in vitro were observed between the wild type and the *dltD* mutant (data not shown).

Cytokine induction by HT-29 cells and PBMCs after *L. rhamnosus* GG and *dltD* challenge. An increase in cytokine induction in HT-29 was observed after either bacterial challenge (*L. rhamnosus* GG wild type, *dltD* mutant, and *S. enterica* serovar Typhimurium SL1344) or cytokine stimulation (IL-1β) compared to the values obtained using the negative control (data not shown). However, no significant differences in cytokine induction were observed for the mutant and the parental strain challenge (Fig. 11A). As expected, the proinflammatory cytokines tested (IL-8 and tumor necrosis factor alpha [TNF-α]) were highly induced by the positive controls (IL-1β and *S. enterica* serovar Typhimurium SL1344) in comparison to the low levels detected for the negative control (medium) and for *L. rhamnosus* GG or the *dltD* mutant. Lack of D-Ala residues in the LTA of the *L. rhamnosus* GG *dltD* mutant did not result in significant differences in the levels of IL-10, IL-12, gamma interferon, and TNF-α released from PBMC in comparison to the wild-type strain results (Fig. 11B). The gram-negative control strain (*E. coli*) induced more IL-10 than the gram-positive bacteria (*L. rhamnosus* GG and *S. gordonii*), as previously reported (25) (data not shown).

DISCUSSION

Inactivation of the *dltD* gene in *L. rhamnosus* GG has a strong impact on LTA composition, resulting in a complete absence of D-alanyl ester content. This is in contrast to other lactic acid bacteria so far analyzed with respect to the *dlt* operon. A reduction in D-alanylation of LTA was previously reported for *Lactococcus lactis* (fivefold lower for the *dltD* mutant) and *Lactobacillus plantarum* NCIMB8826 (8- to 40-fold lower for the *dltB* mutant) (45, 55). A complete absence of D-alanylation of LTA has been reported for the *dltA* mutants of a number of gram-positive pathogens (1, 18, 35, 46, 47).

Recently, the function of the D-Ala ester substitution in LTA has been the subject of investigation for several genera of gram-positive bacteria. Previous results with D-Ala ester-deficient mutants have shown changes in cell morphology, in some cases associated with defective cell separation (41). For *L. rhamnosus* GG, electron micrographs showed an increase in cell length for the *dltD* mutant compared to the wild type. This observation can probably be related to defects in the septal region. For another *L. rhamnosus* strain (the ATCC 7469 *dltD* mutant), an increase in cell length was also reported compared to parental strain results. However, in this case no obvious alteration at the septum was reported (13). In a recent publication, elongated *L. plantarum* NCIMB8826 cells were observed as a consequence of the mutation in the *dltB* gene (45). Taking all of these data together, it is clear that for all the bacteria studied thus far, D-alanylation of LTA plays an important role in determining cell shape and cell septation.

In addition to the role of D-alanylation in determining morphology, this process also allows gram-positive bacteria to modulate surface charge. For example, LTA appears to play a crucial role in the control of autolysin activity (55), and D-Ala ester content seems to determine the number of anionic sites on LTA for autolysin binding (58). In accordance with these data, it was expected that the *L. rhamnosus* GG *dltD* mutant would show an increased rate of autolysis in the presence of Triton X-100 as a consequence of the complete absence of D-Ala ester residues in the LTA. Similar results have been reported for the *L. lactis* MG1363 *dltD* mutant (55). In addition to the role in autolysis, autolysins are also involved in cell division and separation (32). Therefore, the observation of elongated *L. rhamnosus* GG *dltD* mutant cells having defects in septum formation and showing increased autolysis is suggested to be the result of changes in electromechanical properties of the cell wall.

A second clear example of the role of D-alanylation in the modification of the surface charge is the correlation between D-Ala ester content and the action of cationic antimicrobial peptides (34, 40). For this reason, the capacity of *L. rhamnosus* GG wild-type and *dltD* mutant cells to initiate growth in the

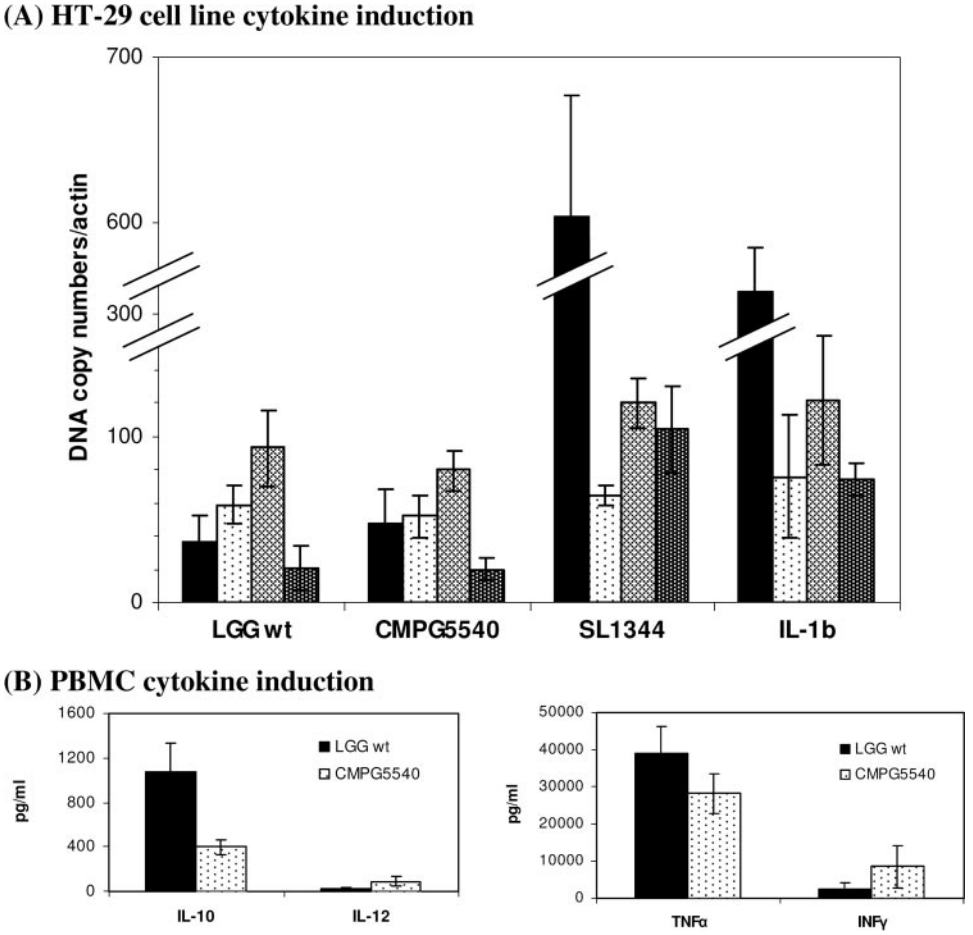

FIG. 11. Cytokine response of human HT-29 intestinal cells and PBMC to stimulation with *L. rhamnosus* GG and *dltD* mutant. (A) Bars represent the averages of triplicate results of three independent reverse transcriptase PCR experiments. Black bars, IL-8; stippled white bars, IL-15; stippled light gray bars, transforming growth factor β; stippled dark gray bars, TNF-α. (B) Results represent the cytokine responses of at least six individual donors as determined by enzyme-linked immunosorbent assays. Black bars, *L. rhamnosus* GG wild type; stippled white bars, *dltD* mutant.

presence of two cationic peptides was evaluated. As expected, a significant difference between the *dltD* mutant and the parental strain was observed. On the other hand, it can be speculated that negatively charged compounds might be repelled to a greater extent in bacteria with a lower degree of D-alanylation. Our results indeed show that inactivation of the *dltD* gene in *L. rhamnosus* GG increases the capacity of the bacterium to grow in a medium containing SDS, a potent anionic detergent.

Human beta-defensins are cationic host defense peptides expressed by epithelial cells. It has been reported that constitutively expressed hBD1 can mediate epithelial interactions with the commensal flora whereas hBD2 may participate in the host defense response to enteric microbes that can breach the epithelial barrier (15). We have previously demonstrated that *L. rhamnosus* GG is not sensitive to hBD1, whereas it is very sensitive to hBD2 (15). Consequently, considering these data together with the charge-mediated mode of action of this antimicrobial peptide, it can be speculated that the increased sensitivity of the *L. rhamnosus* GG *dltD* mutant to hBD2 is likely the result of an increased net negative charge of the bacterial cell envelope as a consequence of the modification in the D-Ala content of the LTA (40).

In general, probiotic bacteria should survive gastric transit in order to confer beneficial effects on the host. A mutation in the *dltD* gene of *L. rhamnosus* GG causes a dramatic decrease in acid tolerance. This particular phenotype is interesting, taking into account that cell wall components, soluble factors, and genomic DNA from *L. rhamnosus* GG have been found to have a strong immunostimulatory capacity (27, 56). Consequently, after gastric transit, the *dltD* mutant could still exert immunological benefits as a result of release of cell wall components and DNA.

After colonization of the gastrointestinal tract, in vivo biofilm formation on the intact intestinal mucosa represents for the host an additional part of the mucosal barrier (31, 37, 44). In the present study we found no differences either in adhesion to the tested human intestinal epithelial cells or in biofilm formation between *L. rhamnosus* GG wild-type and *dltD* mutant cells.

The capacity of probiotic bacteria to stimulate or regulate the mucosal immune system and therefore maintain the gut immunological barrier has been widely studied (5, 22, 42, 53). Furthermore, the role of D-alanylation of the LTA in the anti-inflammatory properties of the probiotic strain *L. plantarum* NCIMB8826 was recently described (22). The immunomodu-

lation of an *L. plantarum dltB* mutant was significantly different from that of the parental strain in the in vivo and in vitro model systems studied (IL-10/IL-12 ratio, 1.1 for wild type and 160.8 for the *dltB* mutant) (22). In the case of *L. rhamnosus* GG, cytokine stimulation of human intestinal epithelial cells and peripheral blood mononuclear cells was not significantly altered by the lack of D-Ala ester substitution in the *dltD* mutant of *L. rhamnosus* GG (IL-10/IL-12 ratio, 51.09 for the wild type and 4.33 for the *dltD* mutant), although the level of D-Ala content of the *L. rhamnosus* GG wild type is 1.8 times higher than that of the D-Ala content of the LTA from *L. plantarum*. These results are in agreement with the findings of B. Pot and C. Grangette that a *dltD* mutant of *Lactococcus lactis* MG1363 did not yield a considerable increase in anti-inflammatory potential either (unpublished data).

In addition to the differences in LTA D-Ala substitution results observed, the cell wall of *L. rhamnosus* contains only one type of TA, LTA (29), in contrast to *L. plantarum*, which contains two types of TAs, LTA and WTA. Although it has been reported that WTA from *L. plantarum* carries D-Ala and glucose residues in a strain-dependent ratio (40), no reports about the substitution of WTA in the particular strain NCIMB8826 have been published. However, it can be speculated that the degree of D-alanylation of WTA from *L. plantarum* NCIMB8826 is affected by the mutation in the *dltB* gene, since the D-Ala ester substituents of WTA are derived from those of LTA (40). Additionally, the LTA from the *L. plantarum* NCIMB8826 *dltB* mutant was shown to contain a large amount of glucose substitutions, whereas glucose substitutions were nearly undetectable in the LTA from the NCIMB8826 wild type (22). These differences in the LTA (and WTA) of *L. plantarum* and *L. rhamnosus* GG strains might well offer an explanation for the different immunological responses in challenges with the two different species.

Moreover, other structural features of the LTA need to be considered as key factors for immune stimulation. For instance, the LTA glycolipid anchor and the length of the Gro-P backbone, as they have been reported to have immunostimulatory potential in other gram-positive bacteria (14, 38), should be taken into account. Interestingly, the fatty acid chains of the glycolipid anchor of the *L. rhamnosus* GG *dltD* mutant showed an average increase in length of two carbon atoms compared to the length of fatty acid chains of the lipid anchor in the wild type. Additionally, the polyglycerophosphate chains, containing an average of 50 Gro-P residues in the *L. rhamnosus* GG wild type, were reduced to averages of 29 Gro-P and 7 Gro-P residues for the major and minor peaks of the *dltD* mutant, respectively. In contrast, the Gro-P backbone of the *L. plantarum dltB* mutant increased threefold in length compared to the backbone in the wild type. It can therefore be concluded from both studies that altering the D-Ala substitution of LTA, by either a *dltD* (*L. rhamnosus* GG) or a *dltB* (*L. plantarum*) mutation, also affects the other building blocks (glycolipid anchor and/or polyglycerophosphate chains) of LTA. This is of interest and needs to be taken into account for future studies of the immunomodulatory properties of LTA.

Conclusively, our study showed the importance of DltD of the probiotic strain *Lactobacillus rhamnosus* GG in the biosynthesis of the LTA. Lack of D-alanylation of the LTA affects a number of cell morphology and surface properties but does not affect important probiotic characteristics, including immunomodulation properties. On the other hand, it is very likely that the *dltD* mutant of *L. rhamnosus* GG will more easily lyse after administration than will the wild type. Therefore, as a follow-up study it will be interesting to compare immunomodulation effects in vivo.

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