

# Exopolysaccharides of *Lactobacillus rhamnosus* GG form a protective shield against innate immune factors in the intestine

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## Summary

Probiotic bacteria are administered as live microorganisms to provide a health benefit to the host. Insight into the adaptation factors that promote the survival and persistence of probiotics in the gastrointestinal tract (GIT) is important to understand their performance. In this study, the role of the long galactose-rich exopolysaccharides (EPS) of the prototypical probiotic strain *Lactobacillus rhamnosus* GG (LGG) was investigated. In a competition experiment with wild type, the isogenic EPS mutant CMPG5351 exhibited a reduced persistence in the murine GIT, especially in the lower parts of the intestine. This was surprising as our previous *in vitro* studies had shown an increased adhesion capacity for this EPS mutant. Follow-up assays indicated that this mutant is more sensitive towards host innate defence molecules, such as the LL-37 antimicrobial peptide and complement factors. This suggests that EPS forms a protective shield for LGG against these molecules in the GIT. Moreover, culturing LGG wild-type in subinhibitory concentrations of host defence factors such as LL-37 resulted in increased production of EPS, indicating that bacterial EPS production is modulated in the host to fine-tune the balance between adhesion and immune evasion. These observations are of interest in understanding the dynamics of adaptation of probiotics to the host environments.

## Introduction

Bacterial surface polysaccharides of pathogens are known to exert important functions at several stages

during the infection process. These functions include tissue adherence, receptor interaction, biofilm formation and evasion of host defences such as phagocytosis (Hooper and Gordon, 2001; Lerouge and Vanderleyden, 2002; Comstock and Kasper, 2006). Also for gut commensals, an important biological role has been recently suggested for capsular polysaccharides (CPS) and glycoproteins in the ecological performance of *Bacteroides* bacteria (Fletcher *et al.*, 2007; Coyne *et al.*, 2008; Liu *et al.*, 2008), although the molecular details are not yet fully elucidated. Conversely, the role of surface polysaccharides in probiotic–host interactions has not been studied in great detail. Members of the genus *Lactobacillus* are commonly studied for their health-promoting probiotic capacities, but their probiotic efficacy can differ largely depending on the purpose of application (Klaenhammer *et al.*, 2005; Marco *et al.*, 2006; Lebeer *et al.*, 2008a) and depending on the physiological status of the applied probiotic (van Baarlen *et al.*, 2009). Recent large-scale genome sequencing projects highlight the diversity among lactobacilli in gene clusters coding for surface polysaccharides (Makarova *et al.*, 2006; Claesson *et al.*, 2007; Kankainen *et al.*, 2009). This high diversity among lactobacilli makes surface polysaccharides potential candidates to confer strain-specific properties to probiotic microbes. For instance, exopolysaccharides (EPS) have been recently linked to the differential mucosal host response provoked by *Lactobacillus plantarum* applied in mid-logarithmic versus stationary phase (van Baarlen *et al.*, 2009). Nevertheless, dedicated mutant analyses that allow the study of these polysaccharides *in situ* on live bacteria are needed for functional validation of their postulated roles.

*Lactobacillus rhamnosus* GG (LGG) was isolated more than 20 years ago by Goldin and Gorbach from a faecal sample of a healthy adult based on several selection criteria (Doron *et al.*, 2005). These criteria included a high adhesion capacity *in vitro*, a high resistance against gastric acidity and high antimicrobial activity against pathogens such as *Salmonella* (Gorbach, 1996). *In vivo* studies have also shown the good persistence capacity of LGG in the human gastrointestinal tract (GIT) (Alander *et al.*, 1999). Since its isolation, LGG has become one of the clinically best-documented probiotic strains (Doron

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et al., 2005). Proven benefits include prevention and relieve of certain types of diarrhoea (Vanderhoof et al., 1999; Guandalini et al., 2000), treatment of relapsing *Clostridium difficile* colitis (Gorbach et al., 1987) and prevention of atopic disease (Kalliomäki et al., 2001; 2003). Thus, both with respect to adaptation to the GIT and probiotic effects, LGG is often regarded as a model probiotic strain. However, much remains to be understood about the molecular details of its adaptation and probiotic factors. We recently identified the gene cluster that is involved in the biosynthesis of a unique galactose-rich EPS of this prototypical probiotic (Lebeer et al., 2009). Concomitantly, an EPS knock-out mutant CMPG5351 was constructed that showed increased adhesion to mucus and intestinal epithelial cells *in vitro*, suggesting that EPS negatively impacts on adherence by shielding adhesins (Lebeer et al., 2009). Here, we investigate the importance of EPS as an adaptation factor for the *in vivo* survival of LGG inside the GIT.

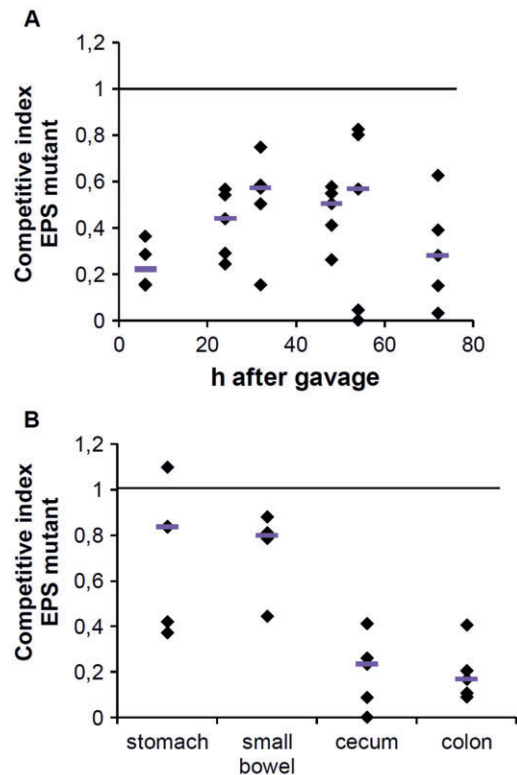
## Results

### *EPS promotes the survival and persistence of LGG in the murine GIT*

The survival and persistence capacity in the murine GIT of the LGG EPS mutant was investigated in a competition experiment. A 1:1 mixture of LGG wild-type control and EPS mutant CMPG5351 was administered by oral gavage on 3 consecutive days to five female BALB/c mice. Faecal samples were collected between 6 and 72 h after the first gavage. A marked proportional decrease of the mutant population could be observed in all faecal samples collected, resulting in a competitive index (C.I.) lower than 1 (Fig. 1A). Some variation in the C.I. values between individual mice was observed, related to the differences in the transit time of LGG bacteria in individual mice, and hence differences in contact time with the harsh conditions of the GIT, as also previously observed (Lebeer et al., 2008b). To obtain more information on the relative contribution of adhesion to the appearance of the LGG strains in the faeces, the mice were sacrificed 24 h after the last gavage, and tissue samples (mucosal scrapings) were collected from different parts of the GIT (stomach, duodenum, caecum and colon). Replica-plating indicated that the relative proportion of the EPS mutant CMPG5351 was particularly reduced in the mucus and mucosa layer of the caecum and colon (Fig. 1B).

### *EPS is not required for stress resistance in gastric juice*

The survival of bacteria inside the GIT is challenged by various stress factors, of which gastric acidity is generally regarded as one of the most important stressors (Lebeer



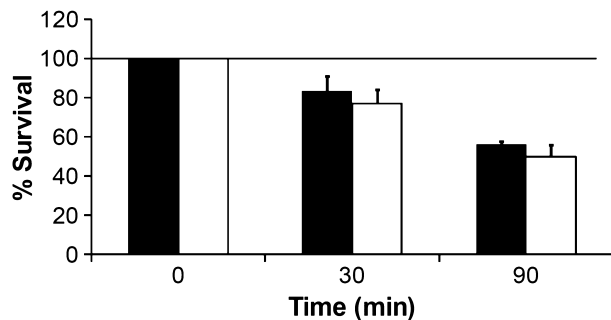
**Fig. 1.** Analysis of the gastrointestinal survival of the LGG EPS mutant. A 1:1 ratio (indicated by the line) of wild-type control and EPS mutant CMPG5351 was administered at 3 consecutive days (at 0, 24 and 48 h) to five female BALB/c mice (8 weeks old). A. The relative presence of the mutant (expressed as competitive indices) in faecal samples, taken at different time points after the first gavage, is shown. B. Data for the competitive indices for the EPS mutant based on mucosal scrapings from different locations along the GIT are shown.

et al., 2008a). The *in vivo* data for the murine GIT (Fig. 1B) suggest, however, that the main cause for the reduced capacity of the LGG EPS mutant to survive is not the acidic stomach or presence of bile in the small intestine. In order to substantiate this, we subsequently compared the survival of wild type and EPS mutant CMPG5351 in simulated gastric juice containing bile and having pH 2. Indeed, the results indicated highly similar survival rates for the wild type and mutant (Fig. 2), suggesting EPS does not play a major role in this capacity.

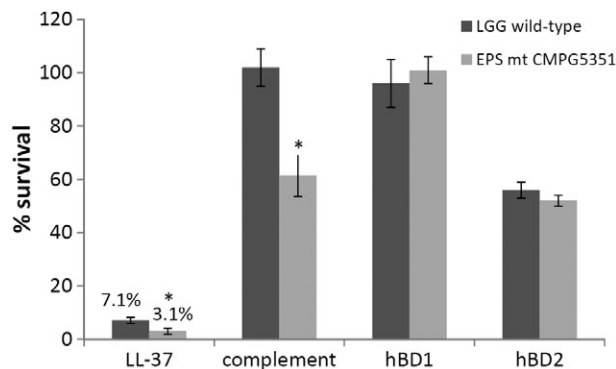
### *EPS protects against innate immune factors of the intestine*

The role of stress factors other than gastric acidity and bile in the survival of probiotic bacteria is not often addressed. However, it can be postulated that interaction with the various antimicrobial factors of the innate immune system also imposes a serious challenge for the probiotic microbes (Lebeer et al., 2008a). Therefore,

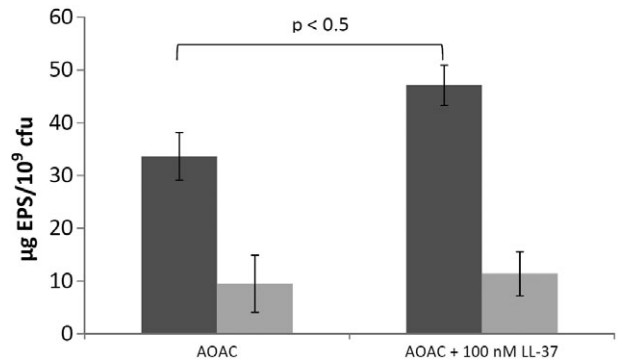
we subsequently compared the resistance of LGG wild type and EPS mutant CMPG5351 against various antimicrobial factors produced by intestinal epithelial cells. These factors were used in physiologically relevant concentrations from different protein/peptide families: small antimicrobial peptides belonging to the  $\beta$ -defensin family (hBD1 and hBD2) (Schneider *et al.*, 2005), LL-37/human cationic protein 18 kDa (CAP18) from the cathelicidin family of cationic antimicrobial peptides (Limura *et al.*, 2005), and complement factors previously reported to be induced by LGG in small bowel mucosa (Di Caro *et al.*, 2005). Of these antimicrobial factors, LGG wild type was most sensitive to LL-37 ( $100 \mu\text{g ml}^{-1}$ ), showing a 10- to 15-fold reduction in viable counts after 3 h (Fig. 3). The long galactose-rich EPS molecules seem to provide some protection against LL-37, as the EPS mutant



**Fig. 2.** Analysis of the role of EPS for survival in simulated gastric juice. Wild-type LGG (black bars), the EPS mutant CMPG5351 (white bars) were grown to stationary phase in AOAC medium, washed and then incubated in simulated gastric juice at pH 2. Samples were taken at different time points and the percentage of viable cells compared with the initial time point is shown based on plating experiments.



**Fig. 3.** Analysis of the role of EPS in protection against host defence molecules. Comparison of the survival capacity of LGG wild type (dark grey bars) and EPS mutant CMPG5351 (light grey bars) after incubation with  $100 \mu\text{g ml}^{-1}$  LL-37, freshly isolated normal human serum (containing complement factors),  $12 \mu\text{g ml}^{-1}$  human  $\beta$ -defensin-1 (hBD1) and  $12 \mu\text{g ml}^{-1}$  human  $\beta$ -defensin-2 (hBD2). The data set comparisons (mutant pairwise to wild type) that are considered significant (see *Experimental procedures*) ( $P < 0.05$ ) are indicated with an asterisk.



**Fig. 4.** Analysis of the dynamics of EPS production in subinhibitory concentrations of LL-37. LGG wild type (dark grey bars) and EPS mutant CMPG5351 (light grey bars) were grown overnight in lactobacilli AOAC medium that was supplemented with subinhibitory concentrations of LL-37 (100 nM). Standard AOAC medium was used as a control. EPS was isolated and quantified as described in *Experimental procedures*.

CMPG5351 showed a greater reduction (20- to 30-fold) in viability under the same conditions. Similarly, EPS was found to protect against complement-mediated lysis, as the EPS mutant demonstrated a twofold reduction in viable counts after 3 h incubation in freshly isolated normal human serum, while LGG wild type was found to be resistant (Fig. 3). No difference in resistance or susceptibility was observed between LGG wild type and EPS mutant for the human  $\beta$ -defensins (Fig. 3).

#### EPS production is increased in subinhibitory concentrations of LL-37

As EPS of LGG negatively impacts on the adherence capacity of LGG *in vitro* (Lebeer *et al.*, 2009), but is required for optimal resistance against host antimicrobial compounds (Figs 1B and 3A), it can be hypothesized that EPS production needs to be dynamically regulated. Therefore, we investigated whether subinhibitory concentrations of LL-37 could induce the expression of EPS by LGG. Hereto, EPS production was compared after overnight growth in lactobacilli AOAC medium with or without 100 nM LL-37 (200-fold less than the inhibitory concentration). A 1.4-fold increase in EPS production by LGG wild type was observed in these subinhibitory concentrations. This increase was not observed in the *welE* mutant that lacks the main long galactose-rich EPS molecules (Fig. 4), suggesting that LL-37 specifically induces the long galactose-rich EPS molecules.

#### Discussion

With the increasing availability of genome sequences of probiotic lactobacilli, mutant approaches are needed for functional validation in order to achieve improved strain

selection procedures, improved product quality control criteria and molecular science-based health claims (Boesten and de Vos, 2008; Kleerebezem *et al.*, 2010). In this study, we investigated the role of the long galactose-rich EPS molecules of LGG in its survival in the GIT, by phenotypic analysis of a dedicated EPS mutant CMPG5351 that we have previously constructed and characterized (Lebeer *et al.*, 2009). *In vitro* studies with this mutant suggested that the long galactose-rich EPS molecules of LGG have a negative impact on *in vitro* adhesion to mucus and intestinal epithelial cells by shielding adhesins (Lebeer *et al.*, 2009). Here, *in vivo* persistence studies in the murine GIT and stress tests with various innate immune factors of the human and murine GIT suggest that the long galactose-rich EPS molecules of LGG are required for optimal survival inside the GIT by forming a protective shield against complement factors and cathelicidins. To our knowledge, this is the first direct evidence on such a role for EPS molecules of probiotic bacteria.

The isogenic EPS mutant CMPG5351 that showed a markedly increased *in vitro* adherence capacity (Lebeer *et al.*, 2009) provided us with the unique opportunity to critically evaluate in this study the importance of two commonly applied selection criteria for probiotics. Current selection assays for new probiotic strains usually include *in vitro* adhesion assays (Tuomola *et al.*, 2001; Sanders, 2008). Adhesion is thought to promote colonization, pathogen exclusion and beneficial interactions of the probiotics with host cells (Marco *et al.*, 2006). Here, we showed that enhanced adhesion can also result in enhanced killing by host defence molecules, if not properly protected by EPS, because of a closer contact with the host cells producing these molecules. Another selection criterion is the resistance against acid. Gastric acidity is often regarded as the most important antimicrobial host factor for probiotics inside the GIT (Tuomola *et al.*, 2001; Sanders, 2008). LGG was indeed selected based on its excellent gastric juice survival capacities (Gorbach, 1996; Doron *et al.*, 2005). In contrast, the effect of antimicrobial proteins on the survival of probiotic strains has not been investigated thoroughly. Our EPS mutant study clearly demonstrates that these stress factors also play a role. In addition, although our *in vivo* colonization studies with the EPS mutant were performed in mice due to ethical reasons of working with genetically modified strains, the subsequent *in vitro* validation experiments were performed with various innate immune factors produced by both human and murine epithelial cells, suggesting that our findings can be relevant for the human GIT. Nevertheless, additional mutant studies, preferably with additional probiotic strains, are needed to further validate the importance of several selection criteria for probiotic effects. Strain differences with respect to sensitivity towards host

antimicrobial proteins and individual host differences with respect to the production of antimicrobial proteins need to be taken into account when evaluating the adaptation capabilities of administered probiotics.

The mechanisms by which the EPS molecules of LGG can provide protection against innate immune factors are not fully understood, but seem to be related to their structural properties. For instance, we reported in this study that LGG is sensitive to the cathelicidin LL-37. The cationic peptide LL-37 binds to negatively charged cell wall polymers such as LTA (Weidenmaier *et al.*, 2003). The long and neutral EPS molecules of LGG (Landersjö *et al.*, 2002; Lebeer *et al.*, 2009) appear to provide a protective shield against LL-37 as the sensitivity was even increased in the EPS mutant CMPG5351. However, EPS was found not to protect against the cationic peptide human  $\beta$ -defensin-2, which is also suggested to target negatively charged residues in the LGG bacterial cell wall and membrane (De Keersmaecker *et al.*, 2006; Perea Vélez *et al.*, 2007). Probably, subtle structural differences and differences in mode of action between this cathelicidin and defensin, as reviewed by Dann and Eckmann (2007), are underlying the different protective capacity of the EPS molecules of LGG. In addition to LL-37, our results suggest that EPS of LGG plays a role in protection against complement-mediated lysis. Complement has been studied largely as a serum factor, but components of the complement cascade are synthesized by intestinal epithelial cells (Andoh *et al.*, 1998) and may also play a role in maintaining homeostasis in the gut. This is of interest, as LGG was previously reported to induce complement factors such as C3 and C4b in human small bowel mucosa (Di Caro *et al.*, 2005). Complement activation is often triggered by mannose residues on bacterial cell walls (Zipfel and Skerka, 2009). We have previously demonstrated by single molecule force spectroscopy that LGG has, in addition to EPS, also shorter mannose-containing polysaccharides (Francius *et al.*, 2008). These polysaccharides are good candidates to induce the mannose-binding lectin alternative complement pathway when the EPS shield is absent. The structural characteristics of the long galactose-rich EPS of LGG, which are composed of galactose, rhamnose and *N*-acetylglucosamine, but lack mannose (Landersjö *et al.*, 2002; Lebeer *et al.*, 2009), are on the other hand probably important in the protection against the complement-mediated lysis.

In terms of providing a molecular framework for selection criteria for probiotic strains based on the presence of certain genetic characteristics, it is yet difficult to pinpoint whether EPS production as such is an essential feature. Based on our data, it can be envisioned that EPS production must be balanced between optimal protection against innate immune defences and optimal adherence. Kanainen and colleagues (2009) recently identified the

fimbriae or pili of LGG as crucial adhesion factors to mucus. We have also shown that fimbriae can be shielded by EPS (Lebeer *et al.*, 2009). Here, we showed that sub-inhibitory concentrations of innate immune factors such as LL-37 induce EPS production of LGG. Similar results were recently reported for pathogens such as group A *Streptococcus* (Gryllos *et al.*, 2008). Future experiments are aimed at understanding the regulation of the dynamic behaviour of EPS expression.

In conclusion, our data indicate that the long galactose-rich EPS molecules of LGG serve an important protective function for LGG in the GIT. EPS is thus an adaptation factor of LGG that promotes its survival and persistence inside the host. Interestingly, similar biological functions for EPS/CPS have been described for pathogenic and symbiotic bacteria (e.g. Magee and Yother, 2001; Coyne *et al.*, 2008). This is in agreement with the fact that adaptation factors are often 'shared' by pathogenic, commensal and probiotic bacteria as factors that contribute to their persistence in the host. This is in contrast to the 'genuine' virulence factors such as toxins that cause disease, versus the 'genuine' probiotic factors that directly mediate the health-promoting effects. Sometimes, the same molecules can be both adaptation and probiotic factors. Therefore, in addition to deciphering the details of the role of EPS as adaptation factor of LGG, future studies should bring more insight into the potential role of EPS as a probiotic factor involved in some of the reported health benefits of LGG.

## Experimental procedures

### *Bacterial strains and culture conditions*

Lactobacilli were grown at 37°C in de Man-Rogosa-Sharpe (MRS) medium (Difco, Erembodegem, Belgium) or in Lactobacilli AOAC medium (Difco) in non-shaking conditions. *Escherichia coli* cells were grown in Luria-Bertani (LB) medium with aeration at 37°C (Sambrook *et al.*, 1989). *Lactococcus lactis* was grown in M17 medium (Difco) containing 0.5% glucose. When appropriate, antibiotics were obtained from Sigma-Aldrich (Bornem, Belgium) and used at the following concentrations: 10 µg ml<sup>-1</sup> tetracycline (Tc), 100 µg ml<sup>-1</sup> ampicillin, 50 µg ml<sup>-1</sup> rifampicin (Rif) and erythromycin (Ery) at 10 µg ml<sup>-1</sup> for LGG and at 100 µg ml<sup>-1</sup> for *E. coli*.

### *In vivo analysis of the gastrointestinal survival and persistence*

The competitive ability of the EPS mutant CMPG5351 in the murine GIT was determined as described previously (Lebeer *et al.*, 2008b). Pathogen-free female BALB/c, 6–8 weeks, weighing 16–22 g, were obtained from Harlan (Zeist, the Netherlands). All experiments were performed under the approval of the K.U.Leuven Animal Experimentation Ethics Committee (Project approval number P05045). Five female

BALB/c mice (8 weeks old) received 100 µl of a 1:1 mixture of 10<sup>9</sup> cfu of wild-type control (CMPG5340-Rif<sup>R</sup>) (Lebeer *et al.*, 2008b) and EPS mutant CMPG5351 (Lebeer *et al.*, 2009) by gavage for 3 consecutive days. The bacteria had been grown to late stationary phase (24 h) in AOAC medium, washed and dissolved in PBS to 10<sup>10</sup> cfu ml<sup>-1</sup>. Faecal samples were collected at 6, 24, 32, 48, 54 and 72 h after the first gavage and replica-plated on MRS plates containing 10 µg ml<sup>-1</sup> Tc (total number of wild-type control and mutant cells) and 50 µg ml<sup>-1</sup> Rif (wild-type control only). To confirm the colony counts, 200 colonies were transferred from MRS plates with 10 µg ml<sup>-1</sup> Tc to MRS plates with 5 µg ml<sup>-1</sup> Ery (wild-type control only) and the percentage of mutants was calculated. This ratio was compared with the exact initial ratio of the EPS mutant upon gavage, resulting in a C.I. This C.I. value is thus defined as the output ratio of mutant to wild type divided by the input ratio of mutant to wild type. Additionally, a control PCR reaction was performed on randomly picked colonies to confirm their identity as wild-type control CMPG5340 versus *welE* mutant CMPG5351. Hereto, we used the primer couples Pro-564 (5'-AGCAGGACGAGAAAGCAATGAATGT-3') and Pro-565 (5'-GCCGGTGTGGCGGAATTGGCAG-3') specific for CMPG5340 versus Pro-0788 (5'-CAAAGGCGGTCTTGAT TGAAGG-3') and Pro-0789 (5'-CTTCTATGCTACACGATG ATAG-3') specific for integration of the tetracycline resistance marker in the *welE* gene. Additionally, the mice were sacrificed 72 h after the first gavage by cervical dislocation, and their GIT was isolated and washed. Subsequently, mucosal scrapings were collected from stomach, small intestine (duodenum), caecum and colon, resuspended in 1 ml of PBS and replica-plated as described for the faeces samples. C.I. values were calculated.

### *Survival in simulated gastric juice*

To investigate the role of EPS in gastric acid resistance, survival tests were performed as previously described (Lebeer *et al.*, 2008b), with the strains grown in AOAC medium. Simulated gastric juice was formulated using glucose (3.5 g l<sup>-1</sup>), NaCl (2.05 g l<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (0.60 g l<sup>-1</sup>), CaCl<sub>2</sub> (0.11 g l<sup>-1</sup>), KCl (0.37 g l<sup>-1</sup>), adjusted to pH 2.0 using 1 M HCl, and autoclaved at 121°C for 15 min. Porcine bile (Sigma) (0.05 g l<sup>-1</sup>), lysozyme (Sigma) (0.1 g l<sup>-1</sup>) and pepsin (Sigma) (13.3 mg l<sup>-1</sup>) were added as stock solutions prior to analysis. 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.

### *Challenge with defensins and cathelicidins*

Sensitivity to human β-defensins was assessed as previously described (De Keersmaecker *et al.*, 2006). Briefly, LGG wild-type and mutant cells were grown to mid-exponential phase and resuspended at c. 10<sup>6</sup> cfu in 45 µl of sixfold-diluted Dulbecco's modified Eagle's medium (Invitrogen, Merelbeke, Belgium) supplemented with 16.67 mM glucose. Subsequently, 5 µl of human β-defensins (Peptides International, Louisville, KY) dissolved in 10 mM acetic acid was added. For human β-defensin-1 (hBD1) and -2 (hBD2), a final

concentration of 12 µg ml<sup>-1</sup> was used. After 3 h of incubation at 37°C, bacterial viability was measured by plating serial dilutions on MRS agar. Each experiment was performed in triplicate. Sensitivity to the cathelicidin LL-37 (Anaspec, distributed by TEBU-bio, Boechout, Belgium) was assessed in a similar way. LL-37 was applied at 100 µg ml<sup>-1</sup> and viable cell counts were determined after 0, 1, 2 and 3 h of incubation.

#### Complement bactericidal assay

These assays were performed as previously described (Coyne *et al.*, 2008). Briefly, 10 ml aliquots of normal human serum (NHS) were collected from three different donors. Half of these aliquots were heated by incubation for 30 min at 56°C to inactivate complement factors. LGG wild-type and mutant cells were incubated in NHS or heat-inactivated NHS at a concentration of  $2 \times 10^5$  cfu ml<sup>-1</sup> at 37°C for 1, 2 or 3 h and plated to determine viable counts. The percentage survival is reported as the number of cfu from wild type or mutants incubated in NHS compared with the same strain incubated in heat-inactivated NHS.

#### EPS isolation

Exopolysaccharide molecules were isolated from LGG wild-type and mutant cells as previously described (Lebeer *et al.*, 2007; 2009), after overnight growth in lactobacilli AOAC medium supplemented with 0.5 µg ml<sup>-1</sup> LL-37 or standard AOAC medium.

#### Statistical analysis

Data sets are based on at least triplicate determinations and were averaged and expressed as one data point. All data are expressed as means ± standard error of the means (SEM). Comparison between experimental groups was performed using Student's *t*-test. Results were considered statistically significant for  $P < 0.05$ .

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