Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 contribute to cell aggregation, *in vitro* biofilm formation, and colonization of the mouse gastrointestinal tract

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Members of the genus *Lactobacillus* are common inhabitants of the proximal gastrointestinal tract of animals such as mice, rats, chickens and pigs, where they form epithelial biofilms. Little is known about the traits that facilitate biofilm formation and gut colonization. This study investigated the ecological role of a glucosyltransferase (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 and a fructosyltransferase (FtfA) of *L. reuteri* LTH5448. *In vitro* experiments using isogenic mutants revealed that GtfA was essential for sucrose-dependent autoaggregation of *L. reuteri* TMW1.106 cells under acidic conditions, while inactivation of Inu slowed the formation of cell aggregates. Experiments using an *in vitro* biofilm assay showed that GtfA and Inu contributed to biofilm formation of *L. reuteri* TMW1.106. Experiments using ex-*Lactobacillus*-free mice revealed that the ecological performance of the *inu* mutant, but not of the *gtfA* or *ftfA* mutant, was reduced in the gastrointestinal tract when in competition with the parental strain. In the absence of competition, the *gtfA* mutant showed delayed colonization of the murine gut relative to the wild-type. In addition, the *gtfA* mutant showed reduced ecological performance in competition experiments with *Lactobacillus johnsonii* #21. From the evidence provided in this study we conclude that GtfA and Inu confer important ecological attributes of *L. reuteri* TMW1.106 and contribute to colonization of the mouse gastrointestinal tract.

INTRODUCTION

The gastrointestinal tract of mammals is colonized by a complex collection of micro-organisms (the gut microbiota) that influences biochemical, physiological, immunological and non-specific disease-resistance characteristics of the host (Gordon & Pesti, 1971). Bacteria of the genus *Lactobacillus* are commonly detected as inhabitants of gut ecosystems and predominate in the proximal (gastric) regions of animals such as pigs, chickens, mice and rats (Tannock, 1992; Walter, 2005). The high population levels of lactobacilli in the gut of these animals are facilitated through bacterial adherence to the non-secretory, stratified, squamous epithelia present in the forestomach, pars oesophagea and crop of mice and rats, pigs and chickens, respectively. The epithelial associations formed by lactobacilli show characteristics of bacterial biofilms (Donlan &

Costerton, 2002) because the bacteria are firmly attached to a surface (epithelium) and are embedded in a matrix of extracellular polymeric substances (Fuller & Brooker, 1974; Savage *et al.*, 1968). The mechanisms by which lactobacilli form these epithelial associations are not well understood, but preliminary *in vitro* investigations have shown that carbohydrate molecules are likely to be involved, while a large surface protein (Lsp) appears to initiate adherence *in vivo* (Tannock, 1997; Walter *et al.*, 2005).

Extracellular polysaccharides (exopolysaccharides, EPS) are synthesized by a wide variety of bacteria including lactic acid bacteria, and they have been shown to contribute to dental biofilm formation and cell aggregation of streptococci (Burne *et al.*, 1996; Munro *et al.*, 1991; Yamashita *et al.*, 1993). Many strains of lactobacilli produce homopolysaccharides (HoPS) and oligosaccharides (OS) consisting of either glucose residues (glucans and gluco-oligosaccharides, GOS) or fructose residues (fructans and fructo-oligosaccharides, FOS) (Gänzle & Schwab, 2005; Korakli & Vogel, 2006; van Hijum *et al.*, 2006). These

compounds are synthesized from sucrose by the single action of extracellular enzymes termed glycosyltransferases, or more specifically glucosyltransferases and fructosyltransferases, respectively. Research on EPS of lactobacilli has focused on their properties as viscosifying, stabilizing, emulsifying, gelling, water-binding and prebiotic agents in the food industry (Bello *et al.*, 2001; Korakli & Vogel, 2006; van Hijum *et al.*, 2006). However, whereas the importance of streptococcal glycosyltransferases for colonization of the oral cavity is clearly established, the ecological importance of these enzymes and their products has not been revealed for lactobacilli (Korakli & Vogel, 2006).

Strains of *Lactobacillus reuteri* are commonly detected in the gastrointestinal tract of humans, pigs, chickens, mice and rats (Reuter, 2001; Walter, 2005). They often produce glucans and fructans of different linkage types, and some glycosyltransferases responsible for their production have been biochemically characterized (Kralj *et al.*, 2002, 2004; Tieking *et al.*, 2005; van Hijum *et al.*, 2002, 2006). HoPS and OS formation of *L. reuteri* TMW1.106 and LTH5448 and its regulation under different environmental conditions has been previously investigated in detail (Gänzle & Schwab, 2005; Schwab & Gänzle, 2006; Schwab *et al.*, 2007). *L. reuteri* TMW1.106 forms large amounts of a high molecular mass glucan and GOS and low amounts of FOS from sucrose and expresses the *gtfA* and *inu* genes encoding a glucosyltransferase and an inulosucrase, respectively (Schwab & Gänzle, 2006; Tieking *et al.*, 2003). *L. reuteri* LTH5448 produces a high molecular mass fructan and FOS and expresses the *ftfA* gene encoding a levansucrase (Schwab & Gänzle, 2006). Insertional inactivation of these genes eliminated the synthesis of corresponding poly- and oligosaccharides in *L. reuteri* TMW1.106 and *L. reuteri* LTH5448, but did not impair the growth and metabolism of maltose and glucose (Schwab *et al.*, 2007).

The prevalence of HoPS and OS production among lactobacilli isolated from gut ecosystems and the importance of these compounds in cell aggregation and biofilm formation of bacteria indigenous to the oral cavity led us to hypothesize that EPS production might constitute an important phenotypic trait of lactobacilli in colonization of the gastrointestinal tract. The availability of well-characterized, isogenic *gtfA*, *inu* and *ftfA* mutants of *L. reuteri* (Schwab *et al.*, 2007) and the *Lactobacillus*-free mouse model (Tannock *et al.*, 1988) provided an excellent opportunity to test this hypothesis.

METHODS

Bacterial strains, media and incubation conditions. The bacterial strains used in this study are listed in Table 1. The mutants were derived by insertional mutagenesis of genes encoding the respective glycosyltransferases by site-specific integration of pORI28 into the chromosome (Schwab *et al.*, 2007). Cultures were propagated anaerobically at 37 °C in Lactobacilli MRS medium (Difco) unless otherwise stated. Erythromycin (5 µg ml^{-1}) was added to the growth medium for culture and maintenance of the mutant strains. Given that the plasmid insertion of the mutants showed high stability in the absence of antibiotics (Schwab *et al.*, 2007), functional assays and phenotypic comparisons were performed using media without erythromycin. Bacterial growth *in vitro* was investigated by monitoring optical densities (OD$_{600}$) as described previously (Tannock *et al.*, 2005).

Aggregation assays. Strains listed in Table 1 were grown for 16 h (0.5 % inoculum) in either MRS medium or MRS medium supplemented with 1 % (w/v) sucrose. Cells were harvested from 1 ml of culture by centrifugation at 6000 ***g*** for 7 min at room temperature, washed once in 1 ml phosphate-buffered saline (PBS) adjusted to either pH 4 or 7, and resuspended in the initial volume of the same solution. Cell suspensions were kept at room temperature and autoaggregation was monitored visually. For determination of coaggregation, two tubes containing 1 ml cell suspension were prepared as described above using PBS (pH 4). Then 100 µl of a 10× concentrated cell suspension of the second strain was added to one of the tubes. After 1 h at room temperature, the optical densities were determined in both tubes. The degree of coaggregation between the two strains was recorded as the difference in optical densities following addition of cells of the second strain.

Biofilm experiments. *L. reuteri* TMW1.106, the *gtfA* mutant and the *inu* mutant were grown for 12 h in MRS medium. Cells were

Table 1. *Lactobacillus* strains used in this study

Strain	Relevant characteristics	Reference
L. reuteri		
TMW1.106	Sourdough isolate; produces large amounts of glucan HoPS and GOS and small amounts of FOS; GtfA$^+$ Inu$^+$, Em^s	Schwab & Gänzle (2006)
TMW1.106 *gtfA* mutant	TMW1.106 mutated in the *gtfA* gene; does not produce glucan; *gtfA*::pORI28, Em^r	Schwab *et al.* (2007)
TMW1.106 *inu* mutant	TMW1.106 mutated in the *inu* gene; large amounts of glucan and GOS without FOS production; *inu*::pORI28, Em^r	Schwab *et al.* (2007)
LTH5448	Sourdough isolate producing fructan HoPS and FOS; Ftf$^+$, Em^s	Schwab & Gänzle (2006)
LTH5448 *ftfA* mutant	LTH5448 mutated in the *ftfA* gene; absence of fructan/FOS production; *ftfA*::pORI28, Em^r	Schwab *et al.* (2007)
100-23	Rodent gut isolate; weak EPS production on sucMRS; Ftf$^+$, Em^s	Wesney & Tannock (1979)
L. johnsonii		
#21	Rodent gut isolate; weak EPS production on sucMRS; Em^s	Bateup *et al.* (1995)
100-100	Rodent gut isolate; absence of EPS production on sucMRS; Em^s	Lundeen & Savage (1990)

harvested from 0.5 ml culture by centrifugation at 6000 g for 7 min at room temperature and resuspended in 1 ml prewarmed (37 °C) half-strength MRS medium containing 0.5 % sucrose (pH 4.5). Disposable flow cells (Stovall Life Science) were connected to a sterile reservoir of the same medium stored at 37 °C. Flow was generated using a Barnant Manostat Multichannel Pump (Carter). The flow rate was adjusted individually for each channel to 12 ml h^{-1}. The flow condition used was at least 50 times faster than the doubling time of *L. reuteri* TMW1.106. Medium flow was stopped, and the flow cell was aseptically inoculated with bacterial cultures using sterile syringes as described by the manufacturer of the flow cell. After 1 h, flow was started again, and biofilm formation was monitored at different time points using a Nikon Optiphot microscope. Images of mature biofilms were acquired using an Olympus SZX12 dissecting microscope.

Experiments with mice. *Lactobacillus*-free mice (males and females) were maintained throughout the experiments in isolators using gnotobiotic technology (Tannock *et al.*, 1988). The *Lactobacillus*-free status was verified on a regular basis. The animals had free access to water and a standard rat diet (NRM Diet 86, Tegel). The feed contained about 1.25 % sucrose from molasses. The animals were 3–6 weeks of age at the start of the experiments. All animal experiments were conducted with approval from the University of Otago Animal Ethics Committee (approval number 104/02).

Testing the ability of *L. reuteri* TMW1.106 and LTH5448 to colonize *Lactobacillus*-free mice. The ability of *L. reuteri* strains TMW1.106 and LTH5448 to colonize the gastrointestinal tract of *Lactobacillus*-free mice was tested by distributing 10 ml of overnight culture ($\sim 5 \times 10^9$ bacteria) per cage over the feed and fur of the animals (three mice per bacterial strain). Animals were killed 14 days later, and gut samples (forestomach, jejunum, caecum) were collected for bacteriological culture of serial dilutions of organ homogenates on Rogosa SL agar (Difco), as described previously (Walter *et al.*, 2003).

Ecological competitiveness of the *gtfA*, *inu* and *ftfA* mutants. Measurements of ecological competitiveness of strains were made by inoculating *Lactobacillus*-free mice (intragastric gavage) with 1 : 1 mixtures (total dose of 10^6 lactobacilli) of mutant and respective wild-type strains as described previously (Walter *et al.*, 2005). Quantification of the mutant strain and the total *Lactobacillus* populations in faecal (after 7 days colonization), forestomach and caecal (14 days colonization) samples was achieved by determining counts on Rogosa SL agar plates with or without erythromycin as described previously (Walter *et al.*, 2005). In competition experiments with *L. reuteri* TMW1.106 and *Lactobacillus johnsonii* #21 (both erythromycin-sensitive), differentiation of strains was achieved by plating gut samples (forestomach and caecum) after 7 days on Rogosa SL agar containing 0.05 % bromocresol green (Sigma). Each strain was quantified based on its distinctive colony morphologies. Correct differentiation of TMW1.106 and #21 was confirmed by picking representative colonies to MRS agar supplemented with 5 % sucrose, where TMW1.106 but not #21 produced large amounts of EPS (slimy phenotype). Ninety-eight per cent of colonies were identified correctly based on colony morphology on bromocresol green agar.

Experiments in *Lactobacillus*-free mice with pure cultures of *L. reuteri* TMW1.106, *gtfA* mutant and *inu* mutant to study colonization dynamics, *in vivo* metabolism and biofilm formation. *Lactobacillus*-free mice were inoculated by intragastric gavage with pure cultures (dose of 10^5 lactobacilli) of either TMW1.106, or the *gtfA* or *inu* mutant strain. Bacterial populations were quantified in faecal samples 2 days after inoculation by plating dilutions of homogenates on Rogosa SL agar (Difco) with or without erythromycin. The animals were killed 7 days after inoculation, and the size of *Lactobacillus* populations in the caecum was determined.

Comparison of colony counts on media with or without erythromycin indicated that the mutational insert of both mutants was stably maintained throughout the experiment (data not shown).

Stomach contents of seven *Lactobacillus*-free mice and of five mice colonized for 7 days by either *L. reuteri* TMW1.106 or the *gtfA* or *inu* mutant were collected, pooled and freeze-dried. Carbohydrates in stomach contents were extracted using double-distilled H$_2$O at 80 °C for 2 h. Maltose, glucose and lactate were separated using an Aminex HPX 87H column (Bio-Rad) with 5 mM H$_2$SO$_4$ as solvent at 0.4 ml min^{-1} and detected with a refractive index detector as described previously (Schwab *et al.*, 2007).

Bacterial associations formed on the epithelial surface of the murine forestomach were investigated by transmission electron microscopy (TEM) as described previously (Walter *et al.*, 2007). Briefly, stomachs from two mice colonized by either *L. reuteri* TMW1.106, the *inu* mutant or the *gtfA* mutant (see above), excised from the animals, were fixed in a 0.1 M cacodylate buffer (pH 7) containing 3 mg ruthenium red ml^{-1} and 3 % glutaraldehyde for 4 h at room temperature and then at 4 °C for 4–5 days. Forestomach pieces of ~ 1 mm^3 were successively fixed, dried and washed before embedding in resin. Sections of 80 nm thickness were examined by TEM.

RESULTS

Autoaggregation and coaggregation

L. reuteri TMW1.106 was the only strain shown in Table 1 whose cells autoaggregated. At pH 7, cells aggregated whether sucrose was present in the growth medium or not, and autoaggregation was not affected by inactivation of the *gtfA* and *inu* genes (data not shown). At pH 4, *L. reuteri* TMW1.106 cells aggregated when grown with sucrose, and autoaggregation of the *gtfA* and the *inu* mutant was adversely affected. Aggregation of the *inu* mutant cells was slower, while the *gtfA* mutant did not aggregate (Fig. 1a, b). None of the strains aggregated at pH 4 when grown in the absence of sucrose, but autoaggregation could be initiated for *L. reuteri* TMW1.106 and the *inu* mutant, but not for the *gtfA* mutant, by the addition of $\geqslant 1$ mg sucrose ml^{-1} to the cell suspensions and incubation at 37 °C for ~ 5 h (data not shown). These findings indicated that glucan production mediated by GtfA was involved in autoaggregation of TMW1.106 cells.

To gain information about the aggregative mechanism of *L. reuteri* TMW1.106, the ability of the *gtfA* mutant to coaggregate with cells producing glucan at pH 4 was tested. Addition of wild-type and *inu* mutant cells, but not of the *gtfA* mutant, resulted in a reduction in optical density of the *gtfA* mutant cell suspension (Fig. 1c). Therefore, *gtfA* cells could still coaggregate with *L. reuteri* TMW1.106 and the *inu* mutant. The ability of the *gtfA* mutant to coaggregate was not dependent on sucrose, but required sucrose in the growth medium of the wild-type strain (data not shown).

Biofilms in the proximal gut are composed of different *Lactobacillus* strains and species. Therefore the effect of mutation of *gtfA* and *inu* on coaggregation with other
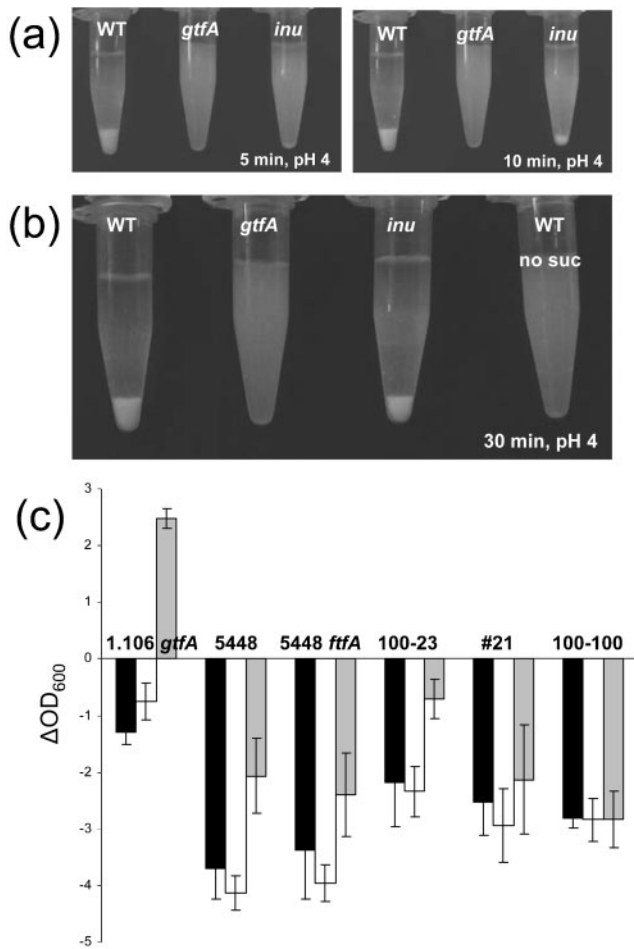
Fig. 1. Autoaggregation and coaggregation of *L. reuteri* TMW1.106 and its *gtfA* and *inu* mutants. (a) Autoaggregation under acidic conditions (pH 4) of *L. reuteri* TMW1.106, *gtfA* and *inu* mutants grown in MRS medium with an additional 1 % sucrose at 5 min and 10 min. One representative of five individual experiments is shown. (b) Autoaggregation after 30 min under acidic conditions (pH 4) of *L. reuteri* TMW1.106, *gtfA* and *inu* mutants grown in MRS medium with an additional 1 % sucrose and *L. reuteri* TMW1.106 grown without sucrose. One representative of five individual experiments is shown. (c) Characterization of coaggregation at pH 4 of *Lactobacillus* strains (Table 1) that do not autoaggregate with *L. reuteri* TMW1.106 (black bars), *gtfA* (grey bars) and *inu* (white bars) mutants by determining the difference in optical density arising from the addition of the latter strains. All strains were grown in MRS medium with an additional 1 % sucrose. Means±SEM (error bars) of three individual experiments are shown.

strains of *L. reuteri* (LTH5448, its *ftfA* mutant and 100-23) and *L. johnsonii* (strains #21 and 100-100) was tested. All the strains coaggregated with *L. reuteri* TMW1.106 at pH 4, resulting in a reduction in optical density (Fig. 1c). Inactivation of *gtfA,* but not *inu,* adversely affected coaggregation with strains of *L. reuteri*. Inactivation of *ftfA* of *L. reuteri* LTH5448 had no effect. Coaggregation of

L. reuteri TMW1.106 with strains of *L. johnsonii* (#21 and 100-100) was not affected by inactivation of *gtfA*.

Evaluation of *in vitro* biofilm formation of *L. reuteri* TMW1.106, the *gtfA* mutant and the *inu* mutant

Biofilm formation was assessed by direct, non-destructive examination of the cells adhering to a glass surface. As shown in Fig. 2(a), wild-type TMW1.106 formed a biofilm that was visible under a light microscope within 18 h. The *gtfA* and the *inu* mutant showed impaired biofilm formation. Intense cell aggregation could be observed for the wild-type and the *inu* mutant. In contrast, cell aggregation of the *gtfA* mutant was virtually absent at 12 h, and greatly reduced at 18 h. Biofilms became macroscopically visible after around 30 h. When observed with a dissecting microscope, the biofilms formed by the mutants showed reduced density compared to the wild-type (Fig. 2b). Although clearly impaired when compared with the wild-type, the *inu* mutant performed slightly better than the *gtfA* mutant in two independent experiments (Fig. 2).

Ecological performance of the *gtfA*, *inu* and *ftfA* mutants in the murine gut in competition experiments with the respective wild-type strain

L. reuteri TMW1.106 and LTH5448 colonized the murine gastrointestinal tract of *Lactobacillus*-free mice ($n=3$) after a single inoculation (data not shown), and formed population sizes comparable to the rodent strain *L. reuteri* 100-23 used in previous studies (Tannock *et al.*, 2005; Walter *et al.*, 2003, 2005, 2007). Further animal experiments were carried out to determine whether the *L. reuteri* glycosyltransferase mutant strains exhibit an impaired competitiveness in the murine gut. Competitive colonization experiments using 1:1 mixtures of wild-type and mutant strains as inoculum ($n=9$–11) revealed that the *inu* mutant competed poorly with *L. reuteri* TMW1.106 (see Fig. 3b). The *gtfA* and *ftfA* mutant populations were not different from that of the wild-type TMW1.106 and LTH5448, respectively (Fig. 3a, c).

Colonization dynamics of *L. reuteri* TMW1.106, *gtfA* and *inu* mutants in the gut of mice

As the *ftfA* mutant of *L. reuteri* LTH5448 was not adversely affected in aggregation or gut colonization, we focused further studies on strain *L. reuteri* TMW1.106 and its *gtfA* and *inu* mutants. Groups of *Lactobacillus*-free mice were inoculated with a single strain to determine the population sizes in faecal (2 days) and caecal (7 days) samples, as well as to detect growth substrates, bacterial metabolites and biofilm formation in the stomach (see below). Inactivation of *gtfA* and *inu* of *L. reuteri* TMW1.106 did not result in decreased population levels in the caecal samples when the bacteria were given time to establish (data not shown).
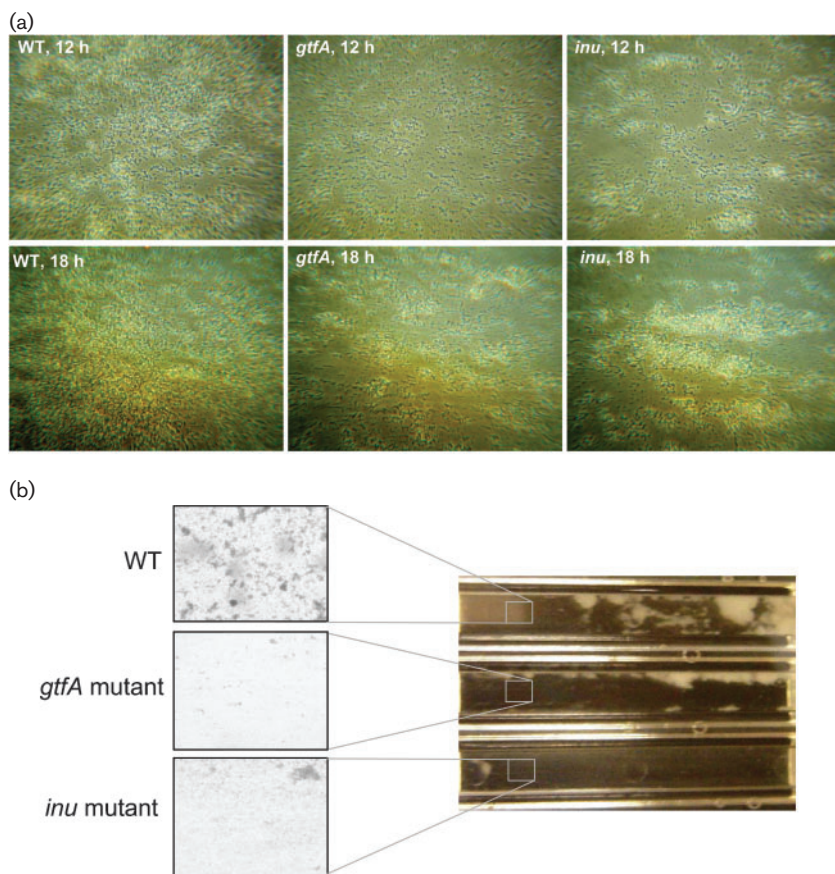
(a)

(b)

Fig. 2. Examination of biofilm formation of *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants using an *in vitro* biofilm assay. (a) Light micrographs (taken at 800× magnification, shown at 500× magification) of biofilms of wild-type TMW1.106 (WT), the *gtfA* mutant and the *inu* mutant after 12 and 18 h. (b) Examination of macroscopic biofilms after 38 h. Enlarged photographs were taken at 90× magnification (shown at 60× magification) using a dissecting microscope. Representative findings of two independent experiments are shown.

However, colonization by the *gtfA* mutant was delayed because mice ($n=7$) colonized with this strain had statistically significant (Student's *t*-test, $P=0.013$) lower numbers of lactobacilli (6.91 $\log_{10}$ c.f.u.) in faecal samples 2 days after inoculation compared to mice inoculated with the wild-type (7.63 $\log_{10}$ c.f.u.; $n=7$). Counts of the *inu* mutant were lower than wild-type counts (7.40 $\log_{10}$ c.f.u.; $n=6$), but the difference was not statistically significant.

Metabolism of lactobacilli during growth in the stomach of mice

Lactobacilli inhabiting the forestomach utilized maltose and glucose and produced lactate (Table 2). Sucrose was present in the stomach of mice not colonized with lactobacilli but the peak was too small for quantification (data not shown). Interestingly, stomach contents of mice colonized with the *inu* mutant contained higher maltose and lower lactate concentrations compared to stomach contents of mice colonized with the wild-type or the *gtfA* mutant, indicating that the utilization of maltose by the *inu* mutant was reduced *in vivo*. We compared the *in vitro* growth of *L. reuteri* TMW1.106, the *gtfA* and the *inu* mutant in MRS broth containing glucose or maltose as the only carbohydrates, but the mutant strains did not differ from the wild-type with respect to growth rate (data not shown).

Epithelial association studied by electron microscopy

TEM of forestomach epithelium after 7 days of colonization revealed that *L. reuteri* TMW1.106 adhered to the surface of the stratified squamous epithelium of the forestomach (Fig. 4). The organisms formed cell layers and aggregates on the epithelium, and bacterial densities were highest in inversions (infoldings) of the epithelium and on epithelial cells that had been shed into the lumen. The *gtfA* mutant and the *inu* mutant formed epithelial associations that were qualitatively similar to the wild-type (Fig. 4b, c).

Competition experiments with *L. johnsonii*

Given the possibility that glucan produced by wild-type cells might have complemented the *gtfA* mutant in competition experiments with the mice, we tested if GtfA contributes to the ecological performance of *L. reuteri* TMW1.106 when colonizing the gut in competition with an organism that does not produce glucan. Competitive colonization assays (six mice) with *L. johnsonii* #21 revealed that *L. reuteri* TMW1.106 formed an average of 39.7 % (SD 27.4) and 36 % (SD 6.0) of the total population in the forestomach and caecum, respectively. The proportion of the total population formed by the *gtfA* mutant was
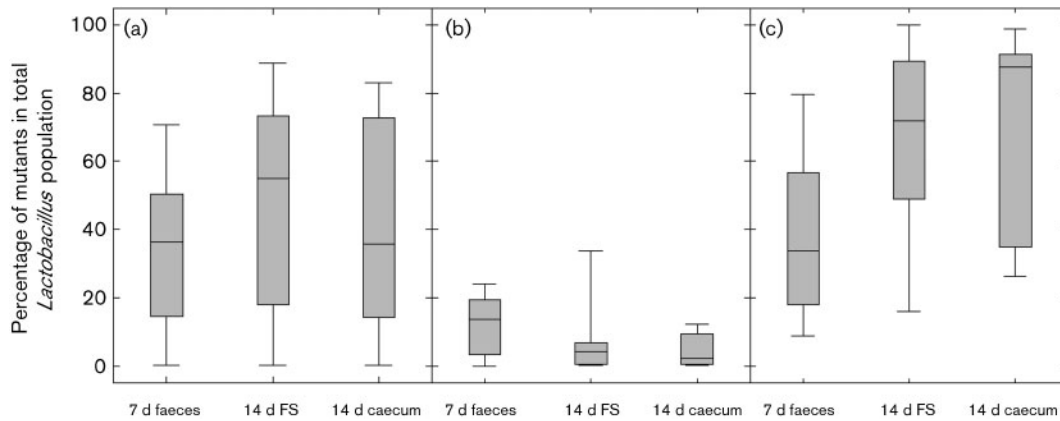
Fig. 3. Competition between wild-type and mutant strains in the gastrointestinal tract of mice. Mixtures of mutant and the respective wild-type (1:1) were used to inoculate *Lactobacillus*-free mice, and the percentage of mutants in the total *Lactobacillus* population was determined in faecal samples (7 days) and forestomach (FS) and caecum (14 days). The bars show the range of values obtained with different animals (n=9–11), with the box indicating the 25 and 75 % percentile. The bars in the boxes indicate medians. (a) *gtfA* mutant, (b) *inu* mutant, and (c) *ftfA* mutant.

9.8 % (SD 6.9) and 22.2 % (SD 20.2) respectively (n=6). The difference in population levels between *L. reuteri* TMW1.106 and the *gtfA* mutant was statistically significant in the forestomach (Student's *t*-test, P=0.027).

DISCUSSION

Mechanisms involved in attachment and coaggregation that may be relevant to biofilm formation remain largely unexplored for commensal gut bacteria (Flint *et al.*, 2007). Here we present evidence that *gtfA* and *inu* contribute to cell aggregation and *in vitro* biofilm formation of *L. reuteri* TMW1.106. Furthermore, by using the well-characterized *Lactobacillus*-free mouse model, we could show that both genes contribute to the ecological performance of the organism during gut colonization. In contrast, inactivation of *ftfA* did not affect coaggregation or ecological performance of *L. reuteri* LTH5448 in the experiments presented here.

According to Rickard *et al.* (2003), bacterial aggregation is an integral process of biofilm formation which proceeds in

Table 2. Concentrations (mmol per g dried stomach content) of fermentable sugars and lactate in pooled stomach contents of *Lactobacillus*-free (n=8) and ex-*Lactobacillus*-free (n=5) mice colonized with *L. reuteri* TMW1.106, the *gtfA* and the *inu* mutant

	L. reuteri TMW1.106	*gtfA* mutant	*inu* mutant	No lactobacilli
Maltose	109	73	194	759
Glucose	75	49	101	253
Lactate	168	216	83	34

the form of a succession of adhesion and multiplication events. Autoaggregation of *L. reuteri* TMW1.106 at neutral pH was independent of sucrose, and inactivation of *gtfA* and *inu* had no effect. In contrast, under acidic conditions (pH 4), autoaggregation required both sucrose and GtfA. These findings indicate that the glucan produced by GtfA from sucrose mediates aggregation of *L. reuteri* TMW1.106. Glucan production has been shown to play an important role in cell aggregation in Gram-positive bacteria (Banas & Vickerman, 2003; Gibbons & Fitzgerald, 1969; Lynch *et al.*, 2007). A possible explanation for the impaired aggregation of the *inu* mutant is that Inu functions as a glucan-binding protein in *L. reuteri* TMW1.106. Accordingly, homologues of Inu function as glucan-binding proteins in *Streptococcus mutans* (Rozen *et al.*, 2004; Russell *et al.*, 1983). The *gtfA* mutant could still coaggregate with wild-type cells that had been grown in medium containing sucrose, showing that the ability to bind glucan was not mediated by GtfA. Coaggregation with non-aggregating strains of *L. reuteri* (100-23, LTH5448) was adversely affected (but not eliminated) by inactivation of *gtfA*. These findings indicated that some *L. reuteri* strains possess glucan-binding proteins that contribute to coaggregation, even though they do not produce glucan.

We tested the ability of *L. reuteri* TMW1.106, the *gtfA* mutant and the *inu* mutant to form biofilms under acidic conditions (pH 4.5) on a glass surface. Biofilm formation was adversely affected for both mutants. Microscopic evaluation revealed that cell aggregation on the glass slides was basically absent for the *gtfA* mutant after 12 h, while the *inu* mutant showed clear signs of cell aggregation. These findings are consistent with those obtained in the aggregation tests. We conclude that GtfA and Inu contribute to biofilm formation by allowing individual cells to form cell aggregates.
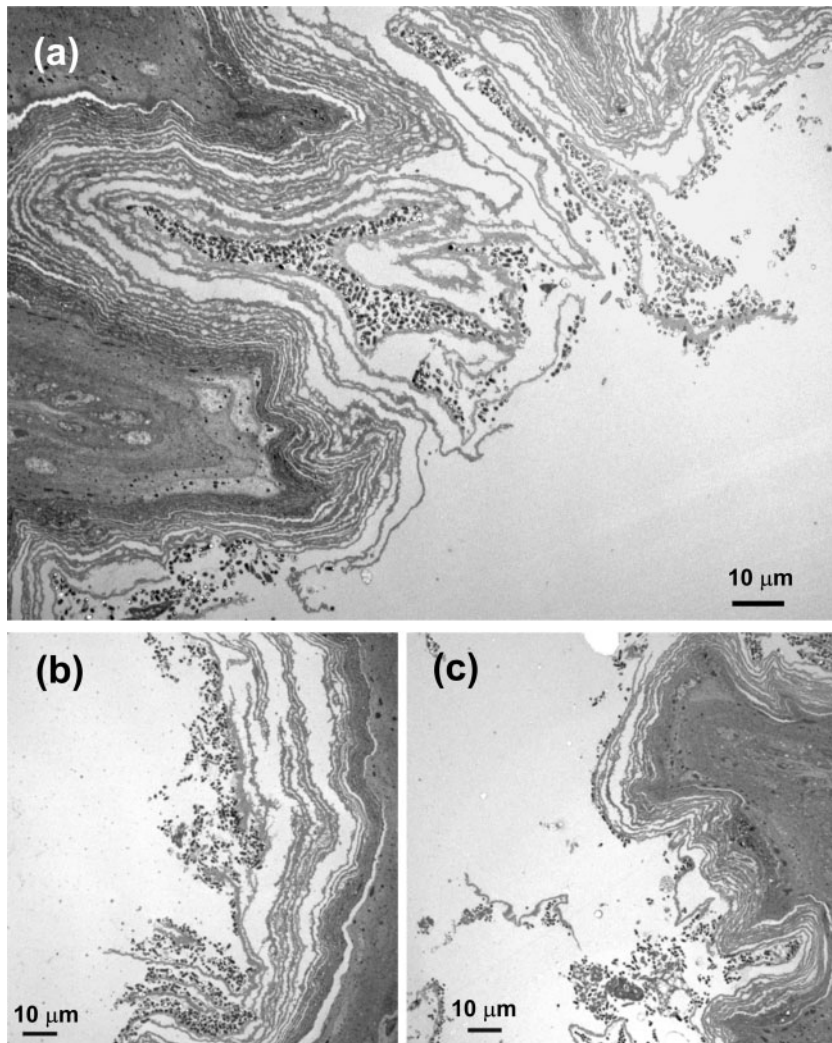
Fig. 4. Characterization of epithelial associations of *L. reuteri* TMW1.106 (a), the *gtfA* mutant (b) and the *inu* mutant (c) on the forestomach epithelium of ex-*Lactobacillus*-free mice 7 days after inoculation, assessed by TEM.

Due to production of lactic acid by the bacteria and host acid secretion, the milieu encountered by lactobacilli during colonization of the forestomach is likely to be acidic (Baumgartner & Montrose, 2004). The lactate levels in the forestomach of mice (Table 2) correspond to those that are typical for stationary cultures of lactobacilli on cereal substrates, which show a pH of around 4 (Gänzle *et al.*, 1998). The importance of GtfA and Inu for cell aggregation and biofilm formation under acidic conditions therefore provides a potential explanation for the decreased ecological performance of the two mutant strains when colonizing the gut of *Lactobacillus*-free mice. As shown in Fig. 4, *L. reuteri* TMW1.106 forms epithelial associations that are characterized by the formation of cell aggregates while colonizing the forestomach. These formations resemble natural biofilms detected on forestomach (rodents), crop (chicken), and pars oesophagea (pigs) epithelia, which are dominated by lactobacilli (Fuller & Brooker, 1974; Fuller *et al.*, 1978; Savage *et al.*, 1968). Although the *gtfA* and *inu* mutants were still able to form epithelial associations that were qualitatively similar to the wild-type, our *in vitro* data imply that GtfA and Inu enhance the ability to form these cell aggregates on the forestomach epithelium.

A striking finding of this study was that the disruption of *inu* impaired colonization of mice by *L. reuteri* TMW1.106 in competition experiments using the wild-type strain, while disruption of *gtfA* had no adverse effect. On the other hand, GtfA did contribute to ecological performance when TMW1.106 colonized the gut alone or in competition with *L. johnsonii* #21. Thus both enzymes contribute to the colonization phenotype of *L. reuteri* TMW1.106 but they appear to play different roles. A possible explanation is that Inu is a glucan-binding protein and a receptor for the glucan produced by GtfA. Inactivation of Inu would then impair binding to the glucan matrix of the biofilm, resulting in decreased ecological performance in competition experiments with the wild-type. In contrast, assuming that GtfA is only involved in glucan synthesis but not in binding, the *gtfA* mutant would remain competitive when extracellular glucan is provided by the wild-type strain.

Determination of the exact role of GtfA and Inu in cell aggregation and biofilm formation requires further experimentation.

Although we clearly show the importance of GtfA and Inu for cell aggregation and biofilm formation, we cannot exclude other functions for these enzymes in the murine gut. Previous work showed that glycosyltransferases of *L. reuteri* could have several functions under different environmental conditions (Gänzle & Schwab, 2005; Schwab *et al.*, 2007). For example, the *gtfA* and *inu* mutants showed a lower resistance to lactic acid when compared to wild-type TMW1.106 (Schwab *et al.*, 2007). This could account for the reduced metabolic turnover of the *inu* mutant in the murine stomach (Table 2). However, growth rates of both mutants were not impaired in media containing maltose and glucose (Schwab *et al.*, 2007), which are the main growth substrates available in the gut, and the preferred carbon sources for *L. reuteri*.

In conclusion, our study has shown the importance of two EPS-producing enzymes, GtfA and Inu, for the ecological performance of a gut commensal. Moreover, this is to our knowledge the first report on the importance of glycosyltransferases for cell aggregation and biofilm formation in the genus *Lactobacillus*. A high proportion of *Lactobacillus* isolates from gut environments produce EPS and possess orthologues of GtfA and Inu (Gänzle & Schwab, 2005; Korakli & Vogel, 2006; Tieking *et al.*, 2003, 2005; van Hijum *et al.*, 2006). Taken together, this study and the literature data indicate that GtfA and Inu confer important ecological attributes on *L. reuteri* TMW1.106 and contribute to colonization of the gastrointestinal tract.

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