Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of *Lactobacillus reuteri*

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Mucus-binding proteins (MUBs) have been revealed as one of the effector molecules involved in mechanisms of the adherence of lactobacilli to the host; *mub*, or *mub*-like, genes are found in all of the six genomes of *Lactobacillus reuteri* that are available. We recently reported the crystal structure of a Mub repeat from *L. reuteri* ATCC 53608 (also designated strain 1063), revealing an unexpected recognition of immunoglobulins. In the current study, we explored the diversity of the ATCC 53608 *mub* gene, and MUB expression levels in a large collection of *L. reuteri* strains isolated from a range of vertebrate hosts. This analysis revealed that the MUB was only detectable on the cell surface of two highly related isolates when using antibodies that were raised against the protein. There was considerable variation in quantitative mucus adhesion *in vitro* among *L. reuteri* strains, and mucus binding showed excellent correlation with the presence of cell-surface ATCC 53608 MUB. ATCC 53608 MUB presence was further highly associated with the autoaggregation of *L. reuteri* strains in washed cell suspensions, suggesting a novel role of this surface protein in cell aggregation. We also characterized MUB expression in representative *L. reuteri* strains. This analysis revealed that one derivative of strain 1063 was a spontaneous mutant that expressed a C-terminally truncated version of MUB. This frameshift mutation was caused by the insertion of a duplicated 13 nt sequence at position 4867 nt in the *mub* gene, producing a truncated MUB also lacking the C-terminal LPxTG region, and thus unable to anchor to the cell wall. This mutant, designated 1063N (*mub*-4867¹), displayed low mucus-binding and aggregation capacities, further providing evidence for the contribution of cell-wall-anchored MUB to such phenotypes. In conclusion, this study provided novel information on the functional attributes of MUB in *L. reuteri*, and further demonstrated that MUB and MUB-like proteins, although present in many *L. reuteri* isolates, show a high genetic heterogeneity among strains.

Received 6 July 2010

Revised 9 September 2010

Accepted 10 September 2010

INTRODUCTION

The adult human intestine hosts 10^{13} to 10^{14} bacteria belonging to at least 500 different species or strains (Dethlefsen *et al.*, 2008). Up to nine different bacterial

phyla are usually found; however, the *Firmicutes* and *Bacteroidetes* account for over 90 % of all bacteria in the gut (Ley *et al.*, 2008). Despite the striking conservation at a higher phylogenetic level, the abundance of bacteria at species or strain level varies extensively between non-related individuals. Nevertheless, a core gut microbiome that is shared among different individuals ensures conservation of the metabolic functions provided by the microbiota (Turnbaugh *et al.*, 2009). It is assumed that this intricate association required significant coevolution of the host and its microbiota, guided by positive selection for factors that result in a state of both mutual tolerance and benefit. The Gram-positive bacterium *Lactobacillus reuteri*

Abbreviations: EPS, exopolysaccharide(s); FCM, flow cytometry; FSC, forward scatter; GIT, gastrointestinal tract; LaCOG, *Lactobacillales*-specific clusters of orthologous protein coding genes; MCM, mouse colonic mucus; MWCO, molecular weight (mass) cut-off; MUB, mucus-binding protein; PI, propidium iodide; PMT, photomultiplier tube; qRT-PCR, quantitative real-time PCR; SSC, side scatter.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

is an excellent model organism to study the evolutionary mechanisms of a vertebrate gut symbiont and to test the hypothesis of coevolution, as this species stably inhabits the gastrointestinal tract (GIT) of mammals as diverse as humans, pigs, mice and rats as well as different species of birds (Oh *et al.*, 2010). *L. reuteri* is one of the dominant species in the GIT of rodents, pigs and chickens (Leser *et al.*, 2002; Salzman *et al.*, 2002; Brooks *et al.*, 2003; Abbas Hilmi *et al.*, 2007) and is considered autochthonous to the human gut in certain individuals, although it is not detectable in most human subjects (Reuter, 2001; Walter *et al.*, 2001; Heilig *et al.*, 2002; Walter, 2008). *L. reuteri* is widely documented to benefit humans and animals (Connolly, 2009). The phylogenetic relationships reported by Oh *et al.* (2010) suggest that the beneficial attributes of *L. reuteri* strains could be the outcome of a long-term evolutionary process that resulted in a mutualistic relationship between microbe and host.

A possible mechanism for bacterial adherence and colonization of the host involves the binding of microbial cell-surface molecules to the protective mucus layer covering the epithelial cells of the GIT. An example is provided by the distribution of mucus-binding proteins (MUB) encoded by *Lactobacillales*-specific clusters of orthologous protein coding genes (LaCOG) in *Lactobacillus* genomes (Kleerebezem *et al.*, 2010). In total, the 47 proteins with one or more Mub repeat found in the exoproteomes of six *Lactobacillus* genomes are distributed over six separate LaCOG. The largest cluster, LaCOG 01470, encodes 14 proteins that possess either the MucBP (Pfam PF06458) domain, the recently defined extended Mub repeat (Boekhorst *et al.*, 2006; Mackenzie *et al.*, 2009), or combinations of both domains. These Mub-repeat-containing proteins are most abundant in lactobacilli of the GIT, strongly suggesting that the Mub repeat is a functional unit that could fulfil an important function in host-microbe interactions. Most of the proteins with multiple Mub repeats contain, in addition to a signal peptide, a C-terminal anchoring motif called LPxTG, which is recognized by a family of enzymes called sortases for covalent attachment to the peptidoglycan of the bacterial cell wall (Navarre & Schneewind, 1999).

The extracellular 353 kDa MUB from *L. reuteri* 1063 (ATCC 53608) contains two types of related amino acid repeats (Mub1 and Mub2); six copies (RI–RVI) of the type 1 repeat (Mub1) and eight copies (R1–R8) of the type 2 repeat (Mub2) (Roos & Jonsson, 2002). The six Mub1 repeats are rather diverse (31–87% aa identity) whereas the Mub2 repeats show very low sequence variation (83–100% aa identity). We recently reported the first three-dimensional structure of a type 2 Mub repeat from the *L. reuteri* 1063 (ATCC 53608) MUB, providing insight into a previously undetected immunoglobulin (Ig)-binding activity for the repeat structural unit of MUBs (MacKenzie *et al.*, 2009).

Interestingly, sequences corresponding to MucBP domains are present in all the currently available genomes of sequenced *L. reuteri* strains (100-23, DSM 20016^T, MM2-3,

MM4-1, ATCC 55730 and CF48-3A), suggesting an important role in the evolution of this species. However, little is known about the diversity of MUBs in *L. reuteri* and their contribution to phenotypes. In this study, we investigated the mucus-binding properties of a large collection of *L. reuteri* strains isolated from a range of vertebrate hosts and correlated the degree of adherence of a subset of strains to the presence and expression of MUB to gain an understanding of the evolution of these proteins and their effect on the adhesion properties of this vertebrate symbiont.

METHODS

Strains, media and growth conditions. The strains of *L. reuteri* used in this study are listed in Table 1. Pure cultures were obtained by streaking out for single colonies on modified MRS (mMRS) agar, as described by Oh *et al.* (2010), after anaerobic growth in 5% (v/v) CO₂, 10% (v/v) H₂ and 85% (v/v) N₂ at 37 °C for 3–4 days. Liquid cultures were incubated without shaking at 37 °C in 20 ml standard MRS broth (2%, w/v, glucose) for up to 24 h depending on the

Table 1. *L. reuteri* strains

Strain*	Host
ATCC 53608	Pig
DSM 20016 ^T	Human
MM4-1a	Human
MF2-3	Human
CF4-6g	Human
FJ1	Human
MF14-C	Human
LMS11-3	Human
sr11	Human
1063N†	Pig
JW2015	Pig
Lp167-67	Pig
20-2	Pig
3c6	Pig
CR	Rat
One-One	Rat
ATCC 55739	Rat
DSM 17509 (=100-23)	Rat
N2D	Rat
R2LC	Rat
ML1	Mouse
#20	Mouse
r13	Mouse
Lr4020	Mouse
LB54	Chicken

*All strains were kindly supplied by Jens Walter, University of Nebraska, USA, except for ATCC 53608, which came from the ATCC, and 1063N, which is a derivative of 1063 (this study). For further information on host and provenance please refer to Oh *et al.* (2010).

†1063N is a spontaneous mutant containing the *mub*-4867ⁱ insertion described in this paper.

application. Strains were maintained as frozen stocks held at -80°C in MRS broth containing 50 % (v/v) glycerol.

Production of anti-MUB antibodies. Purified recombinant Mub-R5 from *L. reuteri* ATCC 53608 was prepared as described previously (MacKenzie *et al.*, 2009). Polyclonal anti-MUB antibodies were raised in rabbits by BioGenes to a titre of $>1:200\,000$. The specificity of the antibody was tested by ELISA against recombinant Mub repeats from *L. reuteri* ATCC 53608 and BSA as negative control. Microtitre plate wells were coated with target protein ($10\,\mu\text{g ml}^{-1}$ in 50 mM sodium carbonate buffer, pH 9.6) overnight at 4°C . All subsequent washing steps between incubations were performed with PBST (PBS containing 0.05 %, v/v, Tween 20) (MacKenzie *et al.*, 2009). Wells were blocked with 300 μl protein-free (PBS) blocking buffer (Thermo Scientific) for 2 h at room temperature. Volumes of anti-MUB antibody in dilutions ranging from 1:900 to 1:218 700 in PBS (100 μl) were then added to the coated wells and incubated for 2 h at room temperature. Pre-immune sera, in dilutions ranging from 1:100 to 1:900 in PBS, were included as negative controls. Antibody binding was detected after 1 h incubation with 100 μl alkaline-phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (1:30 000) at room temperature and 30 min incubation with 100 μl *p*-nitrophenyl phosphate ($1\,\text{mg ml}^{-1}$) in 0.2 M Tris, pH 9.6–10.5 (SIGMAFAST, Sigma) as substrate. The colorimetric assay was stopped with the addition of 3 M NaOH (100 μl per well) and measured at 410 nm in an MRX II (Dynex) microtitre plate reader. All assays were performed in triplicate.

Immunodetection of MUB on the bacterial surface by ELISA. Bacteria were grown to stationary phase at 37°C (20 h), washed three times with PBS (pH 7.4) using 2 min centrifugation steps at 17 000 *g*, and resuspended with PBS to OD_{600} 0.5. Cells were transferred (100 μl per well) onto a high-binding microtitre plate (Greiner Bio-One, Microlon 96); BSA ($1\,\text{mg ml}^{-1}$ in PBS) was included as negative control. Bacteria were incubated overnight at 4°C and unbound cells were removed by washing with 300 μl PBST three times using a plate washer (Applied Quality Systems). Each well was blocked with 300 μl protein-free (PBS) blocking buffer (Thermo Scientific) for 3 h at 4°C . After washing with PBST, 100 μl anti-MUB antibody (1:2430 in PBS) was added per well and incubated at room temperature for 2 h. After further washing with PBST, alkaline-phosphatase-conjugated goat anti-rabbit IgG (whole molecule) was added and enzyme activity was measured as described above. All assays were performed in triplicate.

Microscopic immunolocalization of MUB on the bacterial surface. MUB was visualized on the surface of whole bacteria by immunogold electron microscopy. Bacteria were grown to early stationary phase in MRS broth (20 h incubation at 37°C), washed once with PBS, as described above, and resuspended in PBS to OD_{600} 3.0 (approx. 1×10^9 cells ml^{-1}). PBS-washed bacterial strains were air-dried on to carbon-coated nickel grids and fixed by exposure to 25 % (v/v) glutaraldehyde vapour. Residual free aldehydes were blocked with PBS buffer (pH 7.4) containing 50 mM glycine for 15 min. The grids were incubated in goat blocking solution (Aurion) for 1 h, washed three times for 5 min with incubation buffer (PBS containing 0.1 %, w/v, BSA-c; Aurion), then incubated overnight at 4°C with anti-MUB polyclonal antibody (1:1000) in incubation buffer. Grids were washed six times for 10 min with incubation buffer and then incubated for 2 h at room temperature with goat anti-rabbit IgG conjugated with 15 nm gold particles (GAR-15, Aurion), diluted 1:50 in incubation buffer. Following six 5 min washes with incubation buffer and three 5 min washes with PBS, the bacteria were refixed in PBS containing 2 % (v/v) glutaraldehyde. After one 5 min wash with PBS and a final two 5 min washes with ultrapure water, the grids were examined and photographed in an FEI Tecnai

G2 20 Twin transmission electron microscope. Control grids which were treated only with the secondary antibody were included.

PCR amplification and DNA sequencing of *mub* and 16S rRNA gene fragments. Bacteria from mid- to late-exponential phase MRS cultures were harvested as described above and resuspended in sterile ultrapure water to OD_{600} 5.0. PCRs (50 μl) were carried out with 10 μl of washed cells as template using HotStarTaq (Qiagen) and the gene-specific primers listed in Table 2 for 35 cycles. Amplified DNA fragments were purified by using PCR purification or gel extraction kits (Qiagen); their purity was confirmed by agarose gel electrophoresis and their concentration was measured in a NanoDrop ND-1000 spectrophotometer. DNA sequences were determined at the BBSRC Genome Analysis Centre (TGAC), Norwich, UK.

RNA isolation and quantification of *mub* gene expression by qRT-PCR. Total RNA was isolated from early- to mid-exponential phase bacterial cultures (OD_{600} ~0.6–1.0 in MRS) using the SV Total RNA Isolation System (Promega) and following a modified protocol for Gram-positive bacteria (N. Horn, IFR, personal communication). Briefly, the cell pellet from 1 ml culture was washed twice with 1 ml STE buffer (0.1 M NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and, after centrifugation for 2 min at 14 000 *g*, the cells were resuspended in 100 μl TE buffer containing 30 mg hen egg white lysozyme ml^{-1} (93 072 U mg^{-1} , Fluka). Cell wall digestion was carried out at 37°C for 30 min. The integrity and purity of the RNA preparation was determined by agarose gel electrophoresis and by spectrophotometry in a NanoDrop ND-1000. First-strand cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen) with random primers and qRT-PCR was carried out using the QuantiFast SYBR Green PCR kit in a 7300 Real-time PCR System (Applied Biosystems) with *mub* gene-specific primer pairs MucB1-R1f/MucB1-R1r and MucB2-R4f/MucB2-R4r (Table 2). Signals were normalized to cDNA amplified from 16S rDNA generated with primer pair 16S-341f/16S-534r (Table 2).

Determination of adherence to mucus. Mouse colonic mucus (MCM) was extracted from the large intestines of C57BL/6 wild-type mice. After washing with cold sterile PBS buffer (pH 7.4) containing a complete protease inhibitor cocktail tablet (Roche), the mucosal surface was scraped with a glass slide to remove the mucus layer. Mucus scrapings were collected in ice-cold PBS (1 ml) containing 4 M guanidine hydrochloride (Sigma-Aldrich) and protease inhibitors. The tissue was homogenized with an Ultra-Turrax polytron (IKA) and a Status 70 ultrasonicator (Philip Harris Scientific) at 4°C , then centrifuged (17 000 *g* for 1 h at 4°C) to remove cell debris. *L. reuteri* cells, grown in MRS overnight, were collected by centrifugation (1342 *g*, 5 min, 15°C), washed twice with PBS and resuspended in PBS to OD_{600} 0.5. For the labelling of bacteria, cell suspensions were incubated with 10 μM carboxyfluorescein diacetate (cFDA) (Sigma-Aldrich) at 37°C for 40 min. Cell labelling and viability were checked by flow cytometry (FCM) analysis. Cells were then washed twice and resuspended in the same volume of PBS. MCM was diluted in PBS ($1\,\text{mg ml}^{-1}$) and 200 μl was transferred into high-binding polystyrene microtitre plate wells (Greiner Bio-One) and incubated overnight at 4°C . BSA ($1\,\text{mg ml}^{-1}$ in PBS) was coated on to separate wells of the same plate as a negative control for adhesion. After washing three times with PBST (200 μl) using a plate washer (Applied Quality Systems) to remove the excess mucus, 300 μl protein-free blocking reagent (Thermo) was added per well and the plates were incubated at room temperature for 1 h. After further washing, 200 μl of the labelled bacterial suspension was added per well and the plates were incubated at 4°C for 4 h. After washing to remove unbound bacteria, bacteria bound to the mucus were lysed by incubation at 37°C for 1 h with 1 % (w/v) SDS in 0.1 M NaOH (200 μl). The released fluorescence was measured in a FLUOstar OPTIMA fluorometer (BMG Labtech) with excitation and emission

Table 2. Oligonucleotide primers used

Primer	Sequence (3'–5')	Gene (nt position)*
16S-341f	CCTACGGGAGGCAGCAG	16S rRNA (341–357)
16S-534r	ATTACCGCGGCTGCTGG	16S rRNA (518–534)
16S-10f	AGAGTTTGATCCTGGCTCAG	16S rRNA (10–29)
16S-1577r	AAGGAGGTGATCCAGCCGCA	16S rRNA (1558–1577)
MucB1-RIIf	ATGCAAGAAGCTGCCATCAG	Mub-RI (1789–1805) and Mub-RIII (2959–2975)†
MucB1-RIr	TTATGCCTTTGAGCTAGTTG	Mub-RI (2039–2055)
MucB2-RIIIr	TTAGGCATTTCCCAACATAAAC	Mub-RIII (3505–3522)
MucB1-RVIf	ATGCAAGAAGCTCAAGCCATC	Mub-RVI (9064–9081)
MucB2-RVIf	TTAATCAAGCTTCTTGTAGGT	Mub-RVI (9664–9681)
MucB2-R4f	ATGGGTACGAAGACGCTGAC	Mub-R2 (4921–4937), Mub-R4 (6025–6041), Mub-R5 (6577–6593), Mub-R6 (7129–7145), Mub-R7 (7681–7697), Mub-R8 (8233–8249)
MucB2-R4r	TTAGGCATCAGCCGTGTAGA	Mub-R2 (5186–5202), Mub-R4 (6290–6306), Mub-R5 (6842–6858), Mub-R6 (7394–7410), Mub-R7 (7946–7962), Mub-R8 (8498–8514)
1063Mub-3'r	CACGCCCTTTCATTTGTTTTC	<i>mub</i> 3' region (10050–10070)‡
1063Mub-R1f	CAAACAGTTGGAAGCCAAGGG	Mub-R1 (4209–4229)
1063Mub-R24r	TCAGCGGCCTTGGTACCATCT	Mub-R2 (4962–4982), Mub-R4 (6066–6086)

*Nucleotide positions are given relative to the complete 16S rRNA (NC_009513; Lreu_R0003) and *mub* (AF120104) genes.

†Includes a 1 bp mismatch at the 3' end.

‡Primer 1063Mub-3'r anneals in the 3' region downstream from the published *mub* genomic sequence (AF120104) and is based on the draft genome sequence of strain ATCC 53608 (unpublished data).

wavelengths of 485 nm and 520 nm, respectively. Adhesion was calculated as the percentage of fluorescence recovered after binding relative to the fluorescence of the bacterial suspension added to the mucus or BSA and the values were normalized to the percentage adhesion values of ATCC 53608, which was used as an internal control on each plate. Each assay was performed in triplicate for each strain and on at least three separate occasions. The effect of antisera on bacterial adhesion to the mucus was studied by pre-incubating the labelled bacterial suspension with anti-MUB antibody, or pre-immune serum as a control, at appropriate concentrations (0–1%, v/v, antibody/serum) at room temperature for 1 h prior to addition to the mucus-coated wells.

Characterization of cell-surface extracts and spent media by gel electrophoresis, Western blotting and mass spectrometry.

Bacteria were cultured in 20 ml MRS broth until early stationary phase and harvested by centrifugation at 1342 *g* for 10 min. Spent, centrifuged growth medium in either 2 ml or 10 ml volumes was retained for concentration using Vivaspin-2 or Vivaspin-6 ultrafiltration 100 kDa molecular weight (mass) cut-off (MWCO) spin columns, respectively (Sartorius Stedim Biotech), at 4 °C, following the manufacturer's instructions. The concentrated spent media samples were then buffer-exchanged with PBS (1 ml) and centrifuged to give a final concentration approximately 50 × to 70 ×. Cell-surface extracts were prepared from PBS-washed bacteria following the method described by Kankainen *et al.* (2009) with modifications. Briefly, cells were disrupted by vortexing with an equal volume of glass beads (425–600 µm diameter, Sigma) and 17 ml PBS per g wet weight of cells for three 2 min pulses at full speed with 2 min intervals on ice between pulses. Samples of cell-surface extracts, soluble cytoplasmic extracts and concentrated spent media were analysed by SDS-PAGE using NuPAGE 4–12% BisTris gels (Invitrogen) with MOPS SDS running buffer (50 min at 200 V constant voltage) and HiMark Unstained High Molecular Weight Protein Standard (Invitrogen) as a marker. Gels were stained with the Colloidal Blue staining kit (Invitrogen) and scanned in a GS-800 calibrated densitometer (Bio-Rad). When required, proteins were electroblotted

onto an Immobilon-P membrane (0.45 µm pore size, Millipore) for Western blot detection with anti-MUB polyclonal antibody (1:1000), alkaline-phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (1:30 000) and 100 µg nitro blue tetrazolium ml⁻¹, 50 µg 5-bromo-4-chloro-3-indolyl phosphate-toluidine ml⁻¹ in 4 mM MgCl₂ and 100 mM Tris/HCl (pH 9.6) as substrate. Trypsinized samples of proteins excised from SDS-PAGE gels were analysed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) and the MS data were used to search against the relevant sequence databases as described previously (MacKenzie *et al.*, 2009) using the Mascot 2.2.06 search engine (Matrix Science).

Cell aggregation and cell-surface MUB quantification by FCM.

Bacterial cultures were grown in triplicate to early stationary phase [20 h growth in 20 ml filtered (0.2 µm) MRS broth at 37 °C]. Aliquots (1 ml) were harvested and washed once with filtered (0.2 µm) PBS buffer, which was also used for all subsequent washing and resuspension steps. The cell suspensions were adjusted to OD₆₀₀ ~2.5 in PBS.

Cell aggregation of PBS-washed cells was quantified by measuring forward scatter (FSC) and side scatter (SSC) signals detected by photodiode and photomultiplier tube (PMT) sensors, respectively. In addition, cell aggregation of unwashed cells was determined using a more gentle approach by minimizing the number of sample-handling steps whereby 5 µl of unwashed MRS culture was gently mixed with 1 ml PBS. During data analysis, a gate for events with high FSC and high SSC was used to determine the percentage of aggregating cells.

To determine the production of MUB, aliquots (50 µl) of PBS-washed cells were incubated with polyclonal anti-MUB antibody (1:100) in the dark, on ice for 30 min. Following a wash with 1 ml PBS, the cells were resuspended in 25 µl PBS containing fluorescein-conjugated goat anti-rabbit IgG (whole molecule) antibody (1:26) in the dark, at room temperature for 15 min. Subsequently, 20 µl of cell suspension was diluted in 1 ml PBS, 1 µl propidium iodide (PI, 1.0 mg ml⁻¹, Invitrogen Molecular Probes) was added and the sample was analysed in a FACSCalibur flow cytometer (Becton

Dickinson) using CellQuestPro software. A total of at least 20 000 events per sample were acquired at low flow rate. PMT sensors in channels FL1 (530/30 nm) and FL2 (585/42 nm) were used to quantify green fluorescein and orange PI fluorescence, respectively. FCM data were analysed using FlowJo (TreeStar).

RESULTS AND DISCUSSION

Binding of *L. reuteri* strains to mucus *in vitro* is strain-specific

The adhesion values of the different *L. reuteri* strains tested are reported in Fig. 1. All the strains tested display an identity of at least 99.5% to the 16S rRNA sequence of the type strain (DSM 20016^T) (Oh *et al.*, 2010). The *L. reuteri* strains varied markedly in their ability to bind to mouse mucus *in vitro*, with substantial differences reflected in percentages of adhesion, ranging from 0.16% ($\pm 0.07\%$) to 34.66% ($\pm 0.52\%$). *L. reuteri* ATCC 53608 and ATCC 55739 showed the highest binding to mouse mucus (Fig. 1); both strains were also shown to adhere most efficiently to pig gastric mucin-coated wells (not shown). In contrast, strains MF14-C, MF2-3 and LB54 showed a lower percentage of adhesion to mucus than to BSA, with only the latter two being significantly different ($P < 0.05$) (Fig.

1). The adhesion values of strains 1063N, Lp167-67, 20-2, 3c6, #20, r13, Lr4020, ML1, CR, One-One, DSM 17509, N2D and R2LC to mucus-coated wells were similar to those detected in BSA-coated wells ($< 0.8\%$; Fig. 1). These results agree with previous reports showing that the binding properties of lactobacilli to host intestinal mucus *in vitro* are strain-specific, although those studies were limited to a much smaller number of strains (Ouwehand *et al.*, 2001; Uchida *et al.*, 2004; Vesterlund *et al.*, 2006; Collado *et al.*, 2007; Kinoshita *et al.*, 2007; Li *et al.*, 2008; Tallon *et al.*, 2007; Muñoz-Provencio *et al.*, 2009).

Host-specific adherence is regarded as a desirable property for probiotic bacteria and is recommended as one of the selection criteria. A remarkable feature of the *L. reuteri* population is how it has diversified to form ecotypes that are highly host-specific (Oh *et al.*, 2010). However, our *in vitro* binding assay showed no clear correlation between the host of origin of the bacteria and the origin of the intestinal mucus (Fig. 1). For example, strains ATCC 53608 and ATCC 55739, originating from pig and rat, respectively, adhered most efficiently to mouse colonic mucus (Fig. 1) and adhered without discrimination to pig gastric mucin (data not shown). This lack of host specificity in relation to mucus adhesion *in vitro* had been reported for *L. reuteri*

Fig. 1. Adhesion of *L. reuteri* strains to MCM (black bars) and BSA (white bars). *L. reuteri* strains are grouped in relation to the host from which they were isolated. Microtitre plate adhesion assays were carried out in triplicate for each strain and on at least three separate occasions as described in Methods. Results from different plates are expressed as percentage binding values normalized to the mean value for strain ATCC 53608, used as an internal control on each plate. sd ($n \geq 3$) is indicated by error bars. Strains where binding to MCM was significantly higher than to BSA, as determined by the Student's *t*-test ($P < 0.05$), are indicated by a single asterisk, while strains showing significantly higher binding to BSA than to MCM are indicated with a double asterisk.

strain ATCC 53608, which showed binding to pig but also to hen small intestinal mucus (Roos & Jonsson, 2002). Similarly, the binding properties of probiotic lactic acid bacteria to host intestinal mucus were considered to be more dependent on the strain than on the host (Rinkinen *et al.*, 2003). In contrast, *in vivo* competitive colonization using gnotobiotic mouse models revealed that most rodent *L. reuteri* isolates, including strain R2LC from a rat, showed elevated ecological performance in the murine gut (Schreiber *et al.*, 2009; Oh *et al.*, 2010). This absence of correlation between *in vitro* adhesion assays and *in vivo* colonization studies highlights the multifactorial nature of the colonization process and the need for caution when using mucus adhesion as a measure of colonization performance.

In our assay, adhesion is a reflection of both the mucus recognition abilities and the aggregation behaviour of the bacterial strains. The apparent mucus-binding property reported here for strains ATCC 53608 and ATCC 55739 could be related to the ability of these strains to form multicellular aggregates. Indeed, FCM revealed that the aggregation levels of PBS-washed cells of *L. reuteri* ATCC 53608 and ATCC 55739 were significantly higher than those of strains 1063N and DSM 20016^T (Fig. 2). It should be noted here that strain DSM 20016^T shows high aggregation during growth in MRS broth but, even when handled gently, this strain displayed only about 4% cell aggregates when diluted directly into PBS (data not shown), indicating that aggregation is a multifactorial

process affected by ionic strength and/or pH, and is strain-dependent. High levels of autoaggregation of PBS-washed cells have previously been reported in some *L. reuteri* strains, and a protein (AggH) from strain ATCC 53608 with homology to ATP-dependent RNA helicases was identified as one of the effector molecules mediating aggregation (Roos *et al.*, 1999). Similarly, exopolysaccharides (EPS), which contribute to cell-surface physico-chemical properties, have been implicated in the *L. reuteri* aggregation phenotype, as supported by the implication of EPS-producing enzymes (GtfA and Inu) in cell aggregation and biofilm formation (Walter *et al.*, 2008).

The expression and production of MUB in *L. reuteri* is strain-dependent

The *L. reuteri* MUB and MUB-like sequences have characteristics typical of cell-surface proteins of Gram-positive bacteria: an N-terminal signal peptide (YSIRK motif), targeting the protein for secretion, and a C-terminal sortase recognition site (LPxTG), targeting the protein for covalent attachment to the peptidoglycan layer on the outside of the bacterial cell (Ton-That *et al.*, 2004). The diversity of MUBs among *L. reuteri* strains was first explored at the protein level using immunodetection assays. Of the 25 strains screened by ELISA, MUB was detected on the cell surface of strains ATCC 53608 and ATCC 55739 (Fig. 3). FCM was used to further quantify the level of MUB present on the surface of live and dead

Fig. 2. Autoaggregation of *L. reuteri* strains. PBS-washed bacterial cells were analysed using FCM and cell aggregation was quantified by determining the percentage of events with a high SSC and FSC. These cell clumps or aggregates consist of multiple cells and can be homogenized into a single cell suspension using sonication (data not shown).

Fig. 3. Immunodetection of MUBs by ELISA. *L. reuteri* strains are grouped according to the host from which they were isolated. Bacteria were allowed to adhere to the surface of microtitre plates and were probed with polyclonal anti-MUB antibody and fluorescein-conjugated secondary antibody (black bars) or with the secondary antibody only (white bars). Negative controls with BSA-coated wells were also included. SD is indicated by error bars; for all strains $n=3$, except for ATCC 53608 ($n=9$) and DSM 20016^T ($n=12$).

cells in a subset of selected *L. reuteri* strains with varying mucus-adhesion capacities and isolated from a variety of hosts: strains ATCC 53608 (pig), ATCC 55739 (rat), 1063N (pig), and the type strain DSM 20016^T (human) (Supplementary Fig. S1, available with the online version of this paper). The results were in agreement with the ELISA data, with strains ATCC 53608 and ATCC 55739 showing the highest MUB levels and having a significant 150–200-fold difference in fluorescence intensity for live and dead cells compared to strains 1063N and DSM 20016^T. Surprisingly, although strains 1063N and ATCC 53608 originate from the same porcine isolate (Roos & Jonsson, 2002; Wadström *et al.*, 1987) and the *mub* gene could be detected by PCR in both strains, no MUB was detected on the surface of 1063N.

To further characterize the postulated relationship between variation in MUB production and mucus adhesion and/or aggregation capacity, the diversity of *mub* genes among *L. reuteri* strains was further explored by PCR amplification and DNA sequencing. The presence of the *mub* gene was confirmed in strains ATCC 53608 and ATCC 55739, both of which produce MUB, showing 100% identity to the published sequence in GenBank (AF120104), while no *mub* gene was amplified from DSM 20016^T, as was expected from its genome sequence (NCBI reference sequence: NC_009513.1). Surprisingly, strain 1063N was also shown

to contain the *mub* gene. qRT-PCR was therefore carried out to determine whether the lack of MUB expression was occurring at the transcriptional level in this strain. As shown in Supplementary Table S1, the level of *mub* transcripts produced by strain 1063N was comparable to that of ATCC 53608, when expressed relative to that of 16S rRNA.

To confirm these results at the protein level, the production of MUB and other high-molecular-mass cell-wall proteins was investigated by analysing spent culture medium and cell-wall extracts from strains ATCC 53608, ATCC 55739, 1063N and DSM 20016^T by gel electrophoresis (Fig. 4a). As reported previously by Roos & Jonsson (2002), and confirmed here, a substantial amount of MUB was released into the growth medium by strain ATCC 53608. An apparent lower level of MUB was produced by strain ATCC 55739, in both the medium and the cell wall extract, in agreement with the FCM data. The production of a high-molecular-mass protein by strain DSM 20016^T was also observed in the growth medium and identified by MALDI-TOF-MS as the cell-wall LPxTG protein LAR_0958 from DSM 20016^T. Although not classified as a MucBP protein, this protein has similarities with members of this protein family and could possibly be involved in the relatively high mucus binding detected in strain DSM 20016 (Fig. 1).

Interestingly, strain 1063N did not produce a protein of the same size as full-length MUB but released a substantial

Fig. 4. (a) SDS-PAGE of cell wall extracts (CWE) and spent medium (SM) samples from *L. reuteri* strains. Lane 1, HiMark MW marker; lane 2, DSM 20016^T CWE; lane 3, DSM 2006^T SM; lane 4, 1063N CWE; lane 5, 1063N SM; lane 6, ATCC 55739 CWE; lane 7, ATCC 55739 SM; lane 8, ATCC 53608 CWE; lane 9, ATCC 53608 SM. The ATCC 53608 and ATCC 55739 MUBs are indicated by the arrowheads, the truncated MUB from 1063N by the open triangle and the DSM 20016^T lar_0958 protein by the solid triangle. (b) SDS-PAGE and anti-MUB Western blot of spent medium (SM) and soluble cytoplasmic extracts (SCE) from *L. reuteri* strains 1063N and ATCC 53608. Lane 1, HiMark MW marker; lanes 2 and 6, 1063N SM; lanes 3 and 7, 1063N SCE; lanes 4 and 8, ATCC 53608 SM; lanes 5 and 9, ATCC 53608 SCE. MUBs are indicated using the same symbols as in (a).

amount of a protein with a size of approximately 200 kDa into the medium (Fig. 4a). This shortened version of MUB still reacted with the anti-MUB polyclonal antiserum and weak signals could also be seen in the soluble cytoplasmic extract from strain 1063N, perhaps due to protein that was transiently present during translation, prior to secretion through the cell wall (Fig. 4b). After DNA sequencing of the PCR fragment amplified from strain 1063N using primers 1063Mub-R1f/1063Mub-R24r, the MUB truncation was shown to be due to a frameshift caused by the insertion of a direct repeat sequence of 13 bp (5'-CACGGTAATCTTC-3') at position 4867 nt in the wild-type *mub* gene (Supplementary Table S2). This frameshift created a premature stop codon that resulted in a truncated mature protein (after removal of the putative N-terminal 49 aa secretion signal) of 1541 aa with an estimated mass of 169 kDa, which is close to the apparent mass of ~200 kDa observed in SDS-PAGE and predicted from MALDI-TOF-MS data. The equivalent PCR fragment amplified from ATCC 53608 lacked the 13 bp insertion as expected. However, the 3'-terminal 1 kb of the *mub* gene from strain 1063N, including the Mub-RVI- and LPxTG-encoding regions, was 100 % identical at the DNA level to the wild-type *mub* gene from ATCC 53608 (Supplementary Table S2). This is consistent with the mutant *mub* gene (and cDNA) in strain 1063N being full-length, but its mRNA being translated to give a truncated MUB that only contains Mub repeats RI, RII, RIII, RIV, R1 and part of R2, compared with the 14 Mub repeats in the full-length MUB,

which is in excellent agreement with MALDI-TOF-MS results. The truncated protein also lacks the LPxTG cell-wall anchor motif and thus the ability to attach to the cell surface, which is in agreement with the ELISA and FCM data. This spontaneous mutation may be the result of an adaptive response to the *in vitro* environment as is postulated for lactobacilli and other bacteria (Bth *et al.*, 2005). Indeed, a putative, highly truncated YSIRK-containing protein (Lr1997) with 89 % similarity to the N-terminal 39 aa of the 12 MucBP repeat protein Lreu23DRAFT_4946 from *L. reuteri* strain 100-23 was identified on the pLR581 plasmid of *L. reuteri* ATCC 55730, although the gene product was not characterized further (Bth *et al.*, 2005; Rosander *et al.*, 2008). We propose to designate the mutated *mub* gene in strain 1063N as *mub*-4867ⁱ. The location of MUB on the bacterial cell surface of ATCC 53608 and ATCC 55739 strains was confirmed by immunogold electron microscopy, while negligible or no MUB could be detected in the cell walls of strains 1063N and DSM 20016^T, respectively (Supplementary Fig. S2).

MUB contributes to the *in vitro* mucus-binding and aggregation properties of *L. reuteri* strains

From the results presented above, the mucus-binding properties of the *L. reuteri* strains tested in this study appear to correlate with the presence of MUB at the bacterial cell surface. Individual recombinant Mub repeats have been shown to adhere to mucus (Roos & Jonsson,

Fig. 5. Adhesion of *L. reuteri* strains to mucus in presence or absence of anti-MUB or pre-immune (PI) serum. The results are expressed relative to strain ATCC 53608. SD ($n \geq 3$) is indicated by error bars.

2002, and unpublished data). To further characterize the contribution of MUB to the interaction of the whole bacteria with mucus, a polyclonal antibody that recognized Mub repeats was tested for inhibition of the interaction using strains ATCC 53608, ATCC 55739, 1063N and DSM 20016^T. The antibodies were shown to reduce the binding of strains ATCC 53608 and ATCC 55739 to mucus by 55.4 % and 42.3 %, respectively, while IgG from pre-immune serum had no effect on the binding of the bacteria, showing that MUB contributes to the binding of the bacteria to mucus (Fig. 5). Strain 1063N, which did not exhibit a significant mucus-binding ability, remained unaffected by the antibody treatment, whereas the binding of strain DSM 20016^T to mucus was completely inhibited by the pre-immune serum treatment, lending evidence to the multifactorial nature of mucus adhesion. The contribution of MUB to bacterial adherence to mucus can be explained by its strain-specific role in the recognition of mucus elements and/or in its ability to promote aggregation. It has been suggested that the majority of the aggregation interactions between gut isolates are mediated by lectin-carbohydrate interactions (Ledder *et al.*, 2008). Although a direct interaction of MUB with specific glycans remains to be demonstrated, competitive adhesion assays showed that such an interaction can be significantly reduced by the addition of specific sugars (Bumbaca *et al.*, 2007; Pretzer *et al.*, 2005; Roos & Jonsson, 2002), suggesting a lectin-type mediated interaction and a potential role for MUB in cell aggregation.

Concluding remarks

In conclusion, our study has shown that MUB contributes to the strain-specific ability of *L. reuteri* to bind to mucus and autoaggregate *in vitro*, although the mechanisms need to be investigated further. This property is considered to provide an ecological advantage for extended persistence in the gut and to ensure the optimal functionality and

expression of the health-promoting physiological functions of probiotics (Kleerebezem & Vaughan, 2009). The spontaneous (non-GM) mutant expressing truncated MUB characterized here will serve as a valuable tool to assess the impact of MUB on gut colonization *in vivo*. Interestingly, the MUB under study was only detectable in *L. reuteri* strains ATCC 53608, ATCC 55739 and 1063N (albeit in truncated form in the latter). These strains are indistinguishable by amplified fragment length polymorphism and multi-locus sequence typing analysis (Oh *et al.*, 2010), and the *mub* gene sequences are also 100 % identical, except for the 13 bp insertion in *mub*-4867ⁱ (Supplementary Table S2). Therefore, this study has revealed that the particular MUB investigated is highly specific to a very small set of closely related strains of *L. reuteri*. This is despite the fact that 17 proteins with a putative MucBP domain can be found in the available genomes of *L. reuteri* strains 100-23, DSM 20016^T, MM2-3, MM4-1, ATCC 55730 and CF48-3A, nine of which were present in the rodent isolate 100-23. However, all these proteins show <25 % homology to the ATCC 53608 MUB, and several of the MUB in *L. reuteri* strains DSM 20016^T, MM2-3, MM4-1 are probably truncated through pseudo-gene formation. Therefore, it appears that although proteins with mucus-binding domains are important in the biology of *L. reuteri* or played important roles during the evolution of the species, they show a remarkable degree of variability. The ecological and evolutionary forces that cause this variation remain an important area for future research.

ACKNOWLEDGEMENTS

The authors would like to thank Fran Mulholland (IFR) for MALDI-TOF-MS protein analysis, Phaik Lyn Oh (University of Nebraska) for supplying *L. reuteri* strains and advising on culture conditions, and Christine Fuell, Maddy Houchen, Ola Symes and Kathryn Cross (IFR) for excellent technical support. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC).

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Edited by: V. Eijsink