Microbiology (2002), **148**, 433–442 Printed in Great Britain

A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components

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A gene from *Lactobacillus reuteri* 1063 encoding a cell-surface protein, designated Mub, that adheres to mucus components *in vitro* has been cloned and sequenced. The deduced amino acid sequence of Mub (358 kDa) shows the presence of 14 approximately 200 aa repeats and features typical for other cell-surface proteins of Gram-positive bacteria. Fusion proteins consisting of different repeats of Mub and the maltose-binding protein (MBP) were produced. These proteins adhered to pig mucus components, with molecular masses ranging from <0·1 to >2 MDa, to pig gastric mucin and to hen intestinal mucus. The binding of Mub to mucus components occurred in the pH range 3–7·4, with maximum binding at pH 4–5 and could be partly inhibited by the glycoprotein fetuin. Affinity-purified antibodies against recombinant Mub were used in immunofluorescence microscopy to demonstrate the presence of Mub on the cell surface of strain 1063. By using the antibodies in a Western blot analysis, Mub could also be detected in the growth medium. The results implicate Mub as a cell-surface protein that is involved in *Lactobacillus* interactions with mucin and in colonization of the digestive tract.

Keywords: adhesion, mucin

INTRODUCTION

Members of the genus *Lactobacillus* are often found in the gastrointestinal tract of birds and mammals (Finegold *et al*., 1983; Drasar & Barrow, 1985). For bacteria to be able to colonize this open flow environment, adhesion to the mucosa is considered to be a prerequisite (Berg, 1996; Brassart & Schiffrin, 1997). The epithelial cells of the intestine are covered by a protective layer of mucus, which is a complex mixture of glycoproteins and glycolipids with the large glycoprotein mucin being the main component. Bacteria colonizing the mucosa can be found both in the mucus layer and adhering to the epithelial cells (Savage, 1980). During recent decades, several reports describing adhesion of lactobacilli to epithelial cells and mucus have been published (Conway & Kjelleberg, 1989; Coconnier *et al*., 1992; Adlerberth *et al*., 1996; Rojas & Conway, 1996). Relatively recently, Kirjavainen *et al*. (1998) showed that most lactobacilli used in probiotic products adhered to human intestinal mucus. In most cases, the adhesion has been reported to be mediated by proteins, but saccharide moieties on the cell surface of lactobacilli have also been described to interact with components of the mucosa (Henriksson & Conway, 1992; Mukai & Arihara, 1994). Despite several reports of the phenomenon, the identities of the bacterial adhesins involved are unknown, except for a collagen-binding protein (CnBP) of *Lactobacillus reuteri* (Aleljung *et al*., 1994; Roos *et al*., 1996). The binding activity of this protein is blocked by porcine intestinal mucin and by a lectin with specificity for α-D-galactose (Aleljung, 1994), suggesting that CnBP also adheres to mucin via a lectin-like interaction.

Lactobacillus reuteri frequently occurs in the gastrointestinal tract of various mammals, including man (Molin *et al*., 1993; Naito *et al*., 1995). Strains of this species have been described to possess several properties believed to be important for its capacity to colonize. These are adherence to epithelial cells (Wadström *et al*., 1987), binding of fibronectin (Lindgren *et al*., 1992), expression of the cell-surface CnBP (Aleljung *et al*., 1994), production of an autoaggregation-promoting protein (Roos *et al*., 1999) and production of the

Abbreviations: CnBP, collagen-binding protein; MBP, maltose-binding protein; NANB, non-A, non-B.

The GenBank accession number for the sequence reported in this paper is AF120104.

antimicrobial substance reuterin (Axelsson *et al*., 1989). Recently, Jonsson *et al*. (2001) reported that many strains of *Lb. reuteri* adhere to components in mucus. Interestingly, for some of the strains the adhesion to mucus was triggered by addition of mucin to the growth medium.

In this report we have characterized the adhesion of *Lb. reuteri* strain 1063 to mucus material *in vitro*. The results presented show that a high-molecular-mass protein, which possesses the typical features of cell-surface proteins from Gram-positive bacteria, adheres to components in mucus.

METHODS

Bacterial strains and growth conditions. *Lactobacillus reuteri* 1063 (ATCC 53608) and 1068 were previously isolated from the small intestine of a pig (Wadström *et al*., 1987). *Escherichia coli* LE392 was used as a λ phage host strain and *E. coli* TG1 was used for subcloning (Sambrook *et al*., 1989). Lactobacilli were grown at 37 °C in MRS broth (Oxoid). When growing lactobacilli for preparation of extracellular proteins, the semi-defined substrate LDM II was used (Kotarski & Savage, 1979). *E. coli* was grown at 37 °C in LB broth (Sambrook *et al*., 1989) on a rotary shaker, or on LB agar. Ampicillin (Amp) was used at 50 μg ml^{-1} for selection.

Proteins and reagents. *Lb. reuteri* 1063 was grown for 16 h in 500 ml LDM II broth and the cells were harvested by centrifugation at 10000 ***g***. Proteins in the spent culture medium were precipitated by adding $(NH_4)_2SO_4$ to 40% saturation and pelleted by centrifugation at 15000 ***g***. The proteins were dissolved in 50 mM acetic acid (HAc), dialysed (cut-off 10000 Da) against the same buffer and subsequently lyophilized. The protein material was finally dissolved in 1 ml PBS (g l^{-1}: NaCl, 8·0; KCl, 0·2; $Na_2HPO_4.2H_2O$, 1·44; KH_2PO_4, 0·2; pH 7·3) and stored at −20 °C. To remove loosely bound surface material, the pelleted bacteria were washed five times with distilled water (50 ml each time). Thereafter, the wash solution was lyophilized, the material was dissolved in PBS (1 ml) and stored at −20 °C. Antibodies were raised against material from the culture medium and from cell-surface water wash in a rabbit immunized with approximately 100 μg protein and given three booster doses at 2-week intervals. The animal was sacrificed 8 weeks after the first immunization. To raise the specific activity of the antiserum (P108) against the mucus adhesion factor, it was preadsorbed against cells of non-adhering *Lb. reuteri* 1068. The bacteria were grown in 200 ml MRS for 16 h and washed twice in PBS supplemented with 0·05% Tween 20 (PBST) after which they were suspended in 20 ml PBST. One millilitre of antiserum was mixed with 1 ml bacterial suspension and incubated at room temperature for 2 h. After centrifugation, the adsorbed antiserum was passed through a 0·2 μm filter. The IgG fraction from the adsorbed antiserum was purified on a Protein A-Sepharose CL-4B column (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Mucus from pig or hen was prepared from the small intestine of freshly slaughtered animals. The intestine was rinsed with cold PBST after which the mucus was released by gently scraping the mucosa and washing with PBST. Particles were pelleted by centrifugation and the mucus was stored at −20 °C. Urea was added (final concentration 8 M) to some of the mucus material after which it was fractionated on a Superose 6 PC 3.2/30 column in a Smart System (Amersham Pharmacia Biotech) at a flow rate of 50 μl min^{-1}. Fractions (50 μl) were collected and stored at −20 °C. The relative molecular masses of the eluted fractions were estimated by calibration of the column with a Gel Filtration HMW Calibration Kit (Amersham Pharmacia Biotech).

Binding of bacteria to immobilized mucus. Mucus material, fractionated mucus material, pig gastric mucin (Sigma; M1778) and BSA were dissolved and diluted in 50 mM Na_2CO_3 buffer, pH 9·7, and immobilized in microtitre wells (Greiner) by incubation of 150 μl solution overnight at 4 °C with slow rotation. The final concentrations or amounts used were an OD_{280} of 0·1 for the mucus material, 20 × dilution for the fractionated mucus and 100 μg ml^{-1} for mucin and BSA. The wells were blocked with 0·2 ml PBS supplemented with 1% Tween 20 for 1 h and then washed with PBST. *Lb. reuteri* 1063 was grown in MRS broth for 16 h at 37 °C, washed once in PBST and diluted to an OD_{600} of 0·5 in the same buffer. A portion of the bacterial suspension was treated with proteinase K (100 μg ml^{-1}) for 1 h at 37 °C and PMSF was added to a final concentration of 1 mM. Another portion was incubated with P108 antiserum IgG (30 μg ml^{-1}), αMub1 (30 μg ml^{-1}), αMub2 (30 μg ml^{-1}) (αMub1 and αMub2 are described below) or IgG from preimmune serum (30 μg ml^{-1}) for 1 h at 37 °C. The bacteria from the different treatments and untreated bacteria were washed four times with PBST and diluted to an OD_{600} of 0·5 in the same buffer. Bacterial suspensions (0·15 ml) were added to each well and incubated for 1 h at room temperature. The wells were washed with PBST and the degree of binding was examined with an inverted microscope. The buffer was poured off and, after the wells had dried, OD_{403} was measured in an ELISA plate reader. All measurements were done in triplicate.

Construction and screening of a λ library from *Lb. reuteri* 1063. *Lb. reuteri* 1063 was grown in 100 ml MRS broth and DNA was extracted according to Axelsson & Lindgren (1987). The DNA was partially digested with *Sau*3AI and ligated into λ EMBL3 *Bam*HI arms. Packaging into phage particles was performed according to the manufacturer's instructions (Promega). After infection of *E. coli* LE392, the resulting plaques were screened with the adsorbed IgG-fraction from P108, according to the procedure described previously (Roos *et al*., 1996).

Subcloning and isolation of positive clones. DNA from the λ clones 108:21 and 108:34 was isolated according to Sambrook *et al*. (1989) and cleaved in separate reactions with *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, *Sca*I (complete cleavages) or *Sau*3AI (partial cleavage). The material from the five first cleavages was pooled, treated with T4 DNA polymerase to generate blunt ends and then ligated into a *Sma*I-cleaved pUC18 vector. The *Sau*3AI-cleaved material was ligated into a *Bam*HI-cleaved pUC18 vector. Ligation mixes were electrotransformed into *E. coli* TG1 cells and the resulting clones were selected on LB agar plates containing ampicillin, followed by screening with the IgG fraction of the P108 antiserum as described previously (Roos *et al*., 1996). Plasmid DNA from positive clones was purified with the Wizard Minipreps DNA purification system (Promega) and characterized by restriction enzyme analyses and sequencing. Deletion clones of 108.21:3 were constructed by using the EraseABase kit (Promega), according to instructions of the manufacturer, and subsequently used in the sequencing work.

DNA sequencing and analysis of the DNA and deduced protein sequences. The λ clones and different plasmid subclones were used to determine the nucleotide sequence of the gene. Sequencing was performed by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin

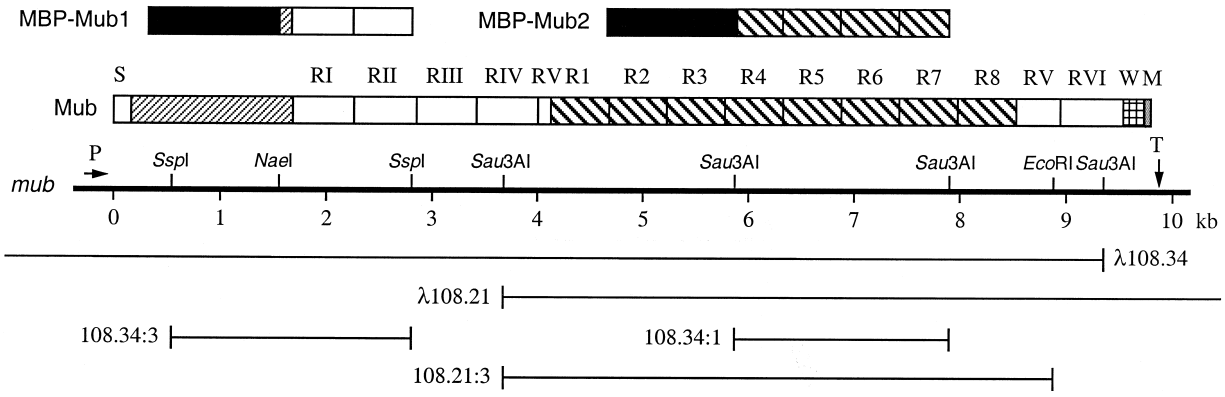

Fig. 1. Map of protein Mub, the *mub* gene, the fusion proteins MBP-Mub1 and MBP-Mub2, and the different clones used in the work. S, Signal sequence; RI–VI, type 1 repeats; R1–8, type 2 repeats; W, cell-wall-spanning region; M, membrane-spanning region; P, promoter; T, transcription terminator.

Elmer) and commercial standard and customized sequencing primers. The sequencing samples were analysed on an automatic sequencing machine (ABI 377; Perkin Elmer). The PC/GENE DNA and protein data-handling package was used for analysis of the DNA and deduced protein sequence. The SignalP server (http://www.cds.dtu.dk/services/SignalP) was used for prediction of signal peptides. NCBI's search tool BLAST (http://www.ncbi.nlm.nih.gov/blast/) was used for similarity searches. Alignments of protein sequences were done with the CLUSTAL W program (Thompson *et al*., 1994) and phylogenetic analyses with programs of the PHYLIP package (Felsenstein, 1993).

Production of fusion proteins and affinity-purified antibodies. Plasmid DNA from 108.34:1 (encoding Mub2 repeats) was cleaved at *Sma*I and *Pst*I sites in the multilinker of the vector and 108.34:3 (encoding Mub1 repeats) with the *Nae*I site in the gene and the *Pst*I site in the multilinker (Fig. 1). The first cleavage resulted in a 2031 bp fragment and the second in a 1247 bp fragment that were purified and ligated into an *Xmn*I/*Pst*I-cleaved pMAL-c2 vector (encoding the maltose-binding protein, MBP; New England Biolabs). The constructs were electrotransformed into TG1 cells and transformants were selected on LB plates with 50 μg ampicillin ml^{-1}. A number of clones were analysed by restriction enzyme digestion and DNA sequence determination. Production of fusion protein was verified by SDS-PAGE and Western blot analysis. Fusion proteins (MBP-Mub1 and MBP-Mub2) were produced and affinity-purified according to New England Biolabs' instructions. The concentrations of the proteins were estimated by measuring A_{280} and the purity by SDS-PAGE analyses. The fusion proteins were coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) and used for affinity purification of the P108 antiserum mentioned above. The purified antibodies (αMub1 and αMub2) were transferred to PBS buffer supplemented with 1% BSA and 0·05% NaN_3 and stored at 4 °C. Antibodies against MBP (αMBP) were purchased from New England Biolabs.

Purification of Mub from strain 1063. *Lb. reuteri* 1063 cells were cultivated for 20 h at 37 °C in 3 l LDM II broth supplemented with 2% glucose. Bacteria were pelleted and the supernatant proteins precipitated with $(NH_4)_2SO_4$ (40% saturation). The precipitate was pelleted at 15000 ***g***, dissolved in 10 mM HAc and dialysed twice against the same buffer, after which the solution was lyophilized. The dry material was dissolved in 0·1 ml PBS and stored at −20 °C. Fifty microlitres of the sample was fractionated on a Superose 6 PC 3.2/30 column in a SMART chromatography system (Amersham Pharmacia Biotech) at a flow rate of 50 μl min^{-1}. Fractions (50 μl) were collected, analysed by SDS-PAGE and stored at −20 °C. The relative molecular masses of the eluted fractions were estimated by calibration of the column with the Gel Filtration HMW Calibration Kit (Amersham Pharmacia Biotech). The amino acid sequence of the purified protein Mub was determined with an ABI 476A protein sequencer (Perkin-Elmer).

SDS-PAGE and Western blotting of Mub. The protein preparations from the spent growth medium and the cell surface of strain 1063, Mub purified from strain 1063 and the two affinity-purified fusion proteins were mixed 1:1 with sample buffer containing SDS and 2-mercaptoethanol and separated by SDS-PAGE. Electrophoresis was performed on PhastGel Gradient 8–25% gels with the PhastSystem (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The proteins were blotted to a Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech) by diffusion at 65 °C for 45 min (PhastSystem Development Technique File No. 220). The membrane was blocked in PBST for 1 h at 37 °C followed by an incubation overnight at 4 °C with the P108 antiserum (diluted 1/1000) or affinity-purified antibodies αMub1 and αMub2 (both diluted 1/1000). After washing with PBST, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (Bio-Rad), diluted 1/1000, at 37 °C for 1 h. After washing, the membranes were developed with 4-chloro-1-naphthol as substrate. The gels were stained with Coomassie blue after the blotting procedure.

Immunofluorescence detection of Mub on the bacterial surface. Strains 1063 and 1068 were grown overnight in MRS broth and washed once in PBST. The bacteria were attached onto Poly Prep slides (Sigma) by drying 10 μl bacterial suspension (OD_{600} of 1·0) onto the slide. The slides were washed with PBST and blocked with PBST containing 5% goat serum for 1 h at room temperature with slow rotation in a humidity chamber. After washing with PBST, the slides were incubated with a mixture of the affinity-purified antibodies αMub1 and αMub2, both diluted 1/30 in PBST. The slides were washed and incubated with FITC-labelled goat anti-rabbit $F(ab')_2$ fragments (Sigma), diluted 1/40 in PBST, for 1 h in a humidity chamber. After washing, the presence of Mub

was visualized by fluorescence microscopy. Controls where the primary antibodies were excluded were done in parallel.

Adhesion of Mub to immobilized mucus. Mucus material, mucus fractions, mucin or BSA were immobilized in microtitre wells as described above. ELISA was performed by incubation of 150 µl λ lysate (produced according to Sambrook *et al*., 1989), diluted to an A_{280} of 0·2, 150 µl purified Mub, diluted to an A_{280} of 4×10^{-4}, or 150 µl fusion protein or MBP diluted to an A_{280} of 10^{-3}, per microtitre well for 1 h at room temperature. Normally the proteins were diluted in PBST, but 10 mM citrate buffers, pH 3–6, with 0·15 M NaCl or 10 mM phosphate buffers, pH 6–7·4, with 0·15 M NaCl were used in some experiments. The wells were washed and incubated with the αMub1, αMub2 or αMBP antibodies, diluted 1/2000 in PBST, for 1 h at room temperature. After washing, the wells were incubated with an anti-rabbit IgG peroxidase conjugate (Bio-Rad), diluted 1/5000 in PBST, for 1 h at room temperature, washed and developed with 3,3′,5,5′-tetramethylbenzidine as substrate, according to Bos *et al*. (1981). A_{450} was measured in an ELISA plate reader. All assays were performed in triplicate. Inhibition experiments were done by addition of different components to the microtitre wells together with the fusion proteins MBP-Mub1 and MBP-Mub2. The different components were mucin, fetuin, asialofetuin, BSA (each 1 mg ml^{-1}) or maltose, mannose, glucose, fucose, raffinose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid (each 10 mg ml^{-1}).

SDS-PAGE and Western blotting of mucus material. The mucus material was mixed with sample buffer, separated by SDS-PAGE and blotted as described above. After blocking in PBST for 1 h at 37 °C the membrane was incubated overnight at 4 °C with MBP-Mub2, MBP-Mub1 fusion proteins or MBP (diluted to an A_{280} of 5×10^{-3} in PBST) followed by incubation for 1 h at 37 °C with the affinity-purified antibodies αMub2 or αMBP (diluted 1/1000 in PBST). The incubation with the secondary antibody and development was performed as above.

RESULTS

Adhesion of *Lb. reuteri* 1063 to mucus

Lactobacillus reuteri strain 1063 adhered efficiently to both pig and hen small intestinal mucus immobilized in microtitre wells. The bacteria also adhered to pig gastric mucin and to fractions of pig mucus material with molecular masses ranging from < 0·1 to > 2 MDa with maximum adhesion to the 0·1–0·25 MDa fraction. Treatment of strain 1063 with Proteinase K eliminated adhesion to mucus, suggesting that the binding was mediated by proteinaceous structures. The IgG fraction from P108, an antiserum raised against extracellular and cell-surface proteins from strain 1063, inhibited adhesion to pig and hen mucus by > 95 and 85 %, respectively (Table 1). IgG from preimmune serum had no effect on the binding of the bacteria (data not shown). Thus, it was concluded that the antibodies could recognize the bacterial protein(s) involved in binding to mucus material.

Cloning and characterization of *mub*

Using the P108 antibodies, raised against secreted proteins and cell-surface proteins from strain 1063, a λ library generated from the parental strain was screened. A number of clones that were reactive with the antiserum were identified. Examination of the recombinant proteins produced by these clones showed that they were of different classes, as indicated by a comparison of band patterns in a Western blot analysis. Most of the clones, among them the two clones λ 108:21 and λ 108:34, were expressing proteins of > 0·2 MDa (data not shown). Functional studies with an ELISA revealed that both clones expressed proteins that adhered to mucus (Table 2). Subcloning of the λ clones into a plasmid vector allowed identification of clones 108.21:3, 108.34:1 and 108.34:3, reacting with the antiserum and expressing peptides of different sizes. Sequence determination revealed that both λ clones indeed harboured the same gene, here designated *mub* (mucus binding). The gene in λ 108:21 is truncated at the 5′ end, but its putative 3′ end is present (Fig. 1). Expression of recombinant protein was possible because the gene was fused in-frame to the 5′ region of another unrelated gene, as a coincidental result of the cloning procedure. This was confirmed by PCR analysis of chromosomal DNA from strain 1063 using primers hybridizing to both sides of the fusion site in clone λ 108:21 and also with primers designed from λ 108:34. Clone λ 108:34 contains the start of *mub*, but lacks the 3′-terminal 456 nt of the gene. The plasmid clones derived from λ 108:34 expressed parts of the gene, fused to *lacZ* in the plasmid vector. The combined sequence revealed an initiation codon, ATG, followed by an ORF of 9807 nt (GenBank accession no. AF120104) encoding a polypeptide, Mub, containing 3269 aa and with a deduced molecular mass of 358 kDa. The initiation codon is preceded by a possible Shine–Dalgarno sequence and, further upstream, by putative transcription initiation signals. A tentative transcription terminator is located downstream of the stop codon.

Mub is predicted to have a 49 aa N-terminal secretion signal peptide. Thus, the mature protein has a predicted molecular mass of 353 kDa. In the C-terminal part of Mub, a cell-wall anchoring motif (LPQTG), a putative membrane-spanning region and a cell-membrane anchor are located. Mub is highly repetitive and contains two types of related amino acid repeats. Six copies (RI–VI) of a type 1 repeat (Mub1), consisting of 183–197 aa each, and eight copies (R1–8) of a type 2 repeat (Mub2), all 184 aa in length, were identified. These are organized in an interesting manner, with the Mub2 repeats R1–8 inserted into the Mub1 repeat RV (Fig. 1). The six Mub1 repeats are rather diverse, with amino acid sequence identities ranging from 15 to 85 %. The Mub2 repeats, on the other hand, are well conserved (> 91 % sequence identity), with R1, R3 and R5 being identical, and R2, R4 and R6 being identical. The highest sequence identity between Mub1 and Mub2 repeats was 35 %.

The best match in a database search using the BLAST search engine was an unknown protein from *Lactococcus lactis* subsp. *lactis* encoded by the gene *ywfG* (GenBank accession no. AAK06278). The protein has previously not been assigned any features, but it is possible to predict an N-terminal signal sequence and a

Table 1. Adhesion of *Lb. reuteri* strain 1063 with different treatments to immobilized mucus material

Each column represents the mean of triplicate determinations ± SD.

Adhesion to wells coated with:	Adhesion (OD_{403}) of:		
	Untreated bacteria	Proteinase K-treated bacteria	P108 IgG-treated bacteria
Pig mucus	0·169 ± 0·023	0·007 ± 0·004	0·001 ± 0·001
Hen mucus	0·136 ± 0·004	0·007 ± 0·003	0·020 ± 0·006
BSA	0·012 ± 0·007	0·002 ± 0·001	0·002 ± 0·001
Blocked wells	0·002 ± 0·001	0·001 ± 0·001	0·001 ± 0·001
Pig gastric mucin	0·120 ± 0·002		
Pig mucus material (> 2 MDa)	0·077 ± 0·019		
Pig mucus material (1–2 MDa)	0·060 ± 0·016		
Pig mucus material (0·5–1 MDa)	0·138 ± 0·001		
Pig mucus material (0·25–0·5 MDa)	0·106 ± 0·005		
Pig mucus material (0·1–0·25 MDa)	0·181 ± 0·003		
Pig mucus material (< 0·1 MDa)	0·127 ± 0·014		

Table 2. Adhesion of Mub and different segments of the protein to mucus material and mucin

Each value represents the mean ± SD of triplicate determinations in an ELISA.

Adhesion to wells coated with:	Adhesion (A_{450}) of:						
	λ108.21 lysate	λ108.34 lysate	Negative λ-lysate	Mub from strain 1063	MBP-Mub1	MBP-Mub2	MBP
Pig mucus	0·627 ± 0·010	1·536 ± 0·069	0·183 ± 0·013	1·018 ± 0·011	1·080 ± 0·077	1·378 ± 0·086	0·206 ± 0·006
Hen mucus		1·111 ± 0·051	0·039 ± 0·006	0·716 ± 0·076			
BSA					0·110 ± 0·006	0·119 ± 0·009	0·015 ± 0·002
Blocked wells	0·171 ± 0·002	0·175 ± 0·010	0·007 ± 0·004	0·020 ± 0·003	0·122 ± 0·020	0·118 ± 0·011	0·016 ± 0·002
Pig gastric mucin					0·970 ± 0·017	1·880 ± 0·036	0·125 ± 0·015
Pig mucus material (> 2 MDa)					1·102 ± 0·341	1·984 ± 0·194	0·084 ± 0·004
Pig mucus material (1–2 MDa)					0·892 ± 0·224	2·422 ± 0·163	0·120 ± 0·002
Pig mucus material (0·5–1 MDa)					1·306 ± 0·121	2·698 ± 0·110	0·332 ± 0·006
Pig mucus material (0·25–0·5 MDa)					0·872 ± 0·023	1·549 ± 0·137	0·188 ± 0·003
Pig mucus material (0·1–0·25 MDa)					0·649 ± 0·098	1·420 ± 0·134	0·149 ± 0·003
Pig mucus material (< 0·1 MDa)					0·912 ± 0·037	2·150 ± 0·205	0·077 ± 0·007

C-terminal cell-wall anchoring motif (LPXTG), putative membrane-spanning region and a cell-membrane anchor. The protein consists of 926 aa and has four repeats of approximately 175 aa each. In a phylogenetic analysis, the repeats of this protein were shown to be more related to the Mub1 repeats (37 % sequence identity) than the Mub2 repeats are. The Mub repeats also showed similarities to a non-A, non-B (NANB) hepatitis virus antigen (Reyes *et al*., 1990) and the human ocular component hr44 (Braun *et al*., 1995). Some less pronounced similarities were also found with a family of high-molecular-mass adhesion proteins from *Haemophilus influenzae* (Barenkamp & St Geme, 1996).

Production and purification of the MBP-Mub fusion protein

To produce and purify recombinant Mub, two constructs encoding fusions of the MBP with Mub1 and Mub2 repeats, respectively, were constructed in the

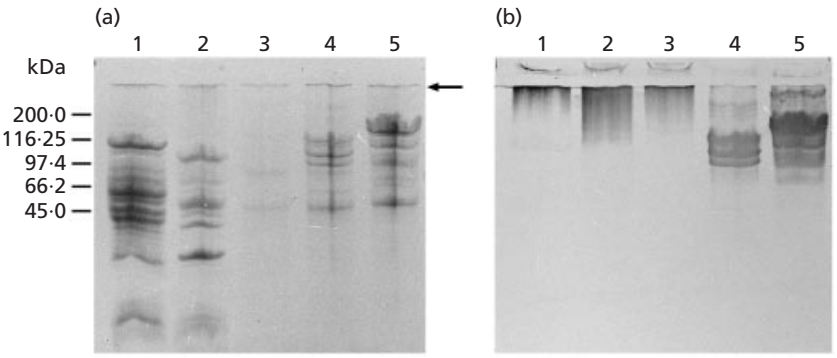

Fig. 2. SDS-PAGE/Western immunoblot analysis. (a) Coomassie-stained gel. (b) Immunoblot with αMub1 and αMub2 antibodies. Lanes: 1, proteins extracted from the cell surface of *Lb. reuteri* strain 1063; 2, proteins secreted from strain 1063; 3, Mub purified from growth medium; 4, purified MBP-Mub1; 5, purified MBP-Mub2. An arrow indicates the position of full length Mub.

protein expression vector pMAL-c2. MBP-Mub1 contains approximately two repeats whereas MBP-Mub2 contains almost four repeats and the molecular masses of the proteins are 94·4 and 123·2 kDa, respectively (Fig. 1). The purified fusion proteins were immobilized on Sepharose beads and used for affinity purification of the P108 antiserum. The antibodies obtained, αMub1 and αMub2, were used in Western immunoblot analyses and shown to react specifically with MBP-Mub1 and MBP-Mub2, respectively (data not shown). Both fusion proteins were subjected to degradation as shown on immunoblots (Fig. 2, lanes 4 and 5).

Purification and characterization of Mub from *Lb. reuteri*

Mub could be detected in both spent culture medium and cell-surface extract from strain 1063 by immunoblot analysis (Fig. 2, lanes 1 and 2). Only one obvious band of such size was visible in the preparations and hence it could be purified in one step by gel filtration (Fig. 2, lane 3). The protein was eluted from the Superose 6 column at 0·93 ml, suggestive of a mass of approximately 2 MDa. The reason for this exceedingly high apparent molecular mass is not known, but it is possible that the protein forms aggregates or is linked to other structures of the bacterial cell wall. Two smaller proteins that were co-eluted with Mub might also be components of these supposed aggregates or structures.

The N terminus of the purified protein was sequenced. The result showed a heterogeneous population of N-terminal sequences. However, by also looking at the second and third choice in some of the positions, the sequence of the predicted mature protein, ATTESNASAK, could be identified. This indicates that the predicted cleavage site is correct, but also that the protein is easily degraded.

By using affinity-purified antibodies against recombinant Mub in an SDS-PAGE analysis (Fig. 2), it was evident that the large protein purified from strain 1063 was recognized by the αMub1 and αMub2 antibodies and most probably represents the full-length protein encoded by *mub*. By using the antibodies in an immunofluorescence microscopy experiment, Mub could also be detected on the cell surface of strain 1063, but not on strain 1068 (Fig. 3).

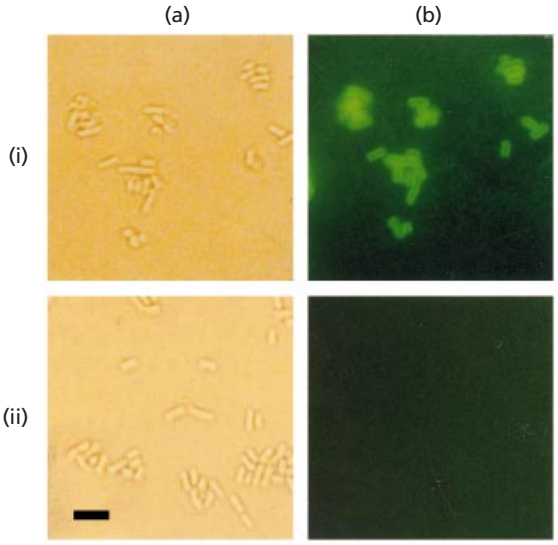

Fig. 3. Immunofluorescence detection of Mub on the surface of *Lb. reuteri* 1063 (i) or 1068 (ii). (a) Light microscopy and (b) immunofluorescence microscopy. Bar, 3 μm.

Adhesion of protein Mub to mucus material

In ELISA, recombinant Mub proteins as well as Mub purified from strain 1063 were shown to adhere to mucus (Table 2). To see to which component(s) Mub binds, adhesion to pig gastric mucin and fractionated pig mucus was measured. Both the MBP-Mub1 and the MBP-Mub2 fusions adhered to pig gastric mucin and to all tested mucus fractions with molecular masses ranging from $<0{\cdot}1$ to >2 MDa, with maximum adhesion to the 0·5–1·0 MDa fraction. pH was shown to be an important parameter for adhesion. Although the interaction is efficient at pH 7·4, it is stronger at lower pH values between 3 and 6. MBP-Mub1 was more affected than MBP-Mub2 by a low pH and showed a maximum adhesion at pH 5 with a value more than 3·4 times higher than at pH 7·4. MBP-Mub2 showed maximum adhesion at pH 4 with a value more than 1·7 times

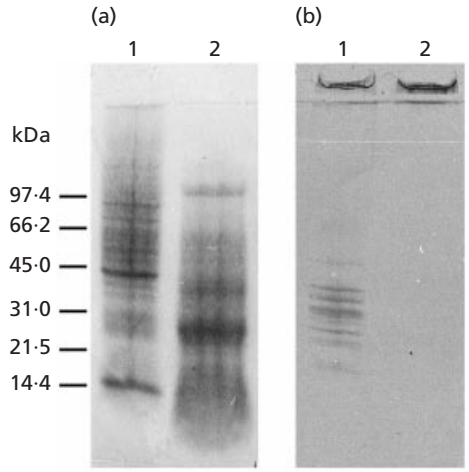

Fig. 4. Adhesion of MBP-Mub2 to mucus material from pig or hen intestine. (a) SDS-PAGE; (b) Western blot analysis. Lanes: 1, pig intestinal mucus; 2, hen intestinal mucus.

Table 3. Inhibition of adhesion to mucus after addition of different substances (expressed as percentage of adhesion in absence of inhibition)

Addition of:	Adhesion (%) to mucus by:	
	MBP-Mub1	MBP-Mub2
No addition	100	100
Mucin	49	21
Fetuin	38	7·5
Asialofetuin	33	6·0
BSA	99	81
Maltose	133	121
Glucose	121	109
Fucose	91	91
Raffinose	108	102
N-Acetylgalactosamine	106	107
Sialic acid	168	118
N-Acetylglucosamine	111	118
Mannose	119	95

higher than at pH 7·4. Additionally, changing from phosphate to citrate buffer in general increased the adhesion by approximately 40 % (data not shown).

Adhesion of the MBP-Mub2 (Fig. 4) and MBP-Mub1 (data not shown) fusion proteins to mucus material was also demonstrated in Western blot analyses. Both fusion proteins showed a similar adhesion pattern, while the fusion partner MBP itself did not adhere. The fusion proteins were shown to adhere to more than 10 different components of pig mucus with molecular masses between 15 and 45 kDa (Fig. 4b), but also to components with very high molecular mass that could not enter the gel. Interestingly, they seemed to adhere only to a high-molecular-mass component in the hen mucus.

Inhibition of adhesion of Mub to mucus material

To further characterize the interaction between Mub and mucus components, different glycoproteins and carbohydrates were tested for inhibition of the interaction (Table 3). Mucin, fetuin and asialofetuin substantially inhibited the adhesion of both MBP-Mub1 and MBP-Mub2 to mucus, whereas the interaction was only slighted affected by the same concentration of BSA. This suggests that Mub interacts with carbohydrates on the mucus components. The inhibition by asialofetuin was of the same order of magnitude as the inhibition by fetuin, which shows that sialic acid is not involved in the interaction. The sugars maltose, glucose, fucose, raffinose, *N*-acetylgalactosamine, sialic acid, *N*-acetylglucosamine and mannose were tested for their abilities to inhibit the interaction and most of them did not affect adhesion. The exceptions were fucose that reduced both MBP-Mub1 and MBP-Mub2 adhesion by approximately 10 % and mannose that slightly inhibited MBP-Mub2 adhesion.

To investigate the role of Mub in the adhesion of strain 1063 to mucus components, an inhibition experiment with αMub1 and αMub2 was conducted. The antibodies were shown to reduce the binding of strain 1063 to mucus by 69 % from an OD_{403} of $0{\cdot}169 \pm 0{\cdot}023$ ($\pm$ SD) to $0{\cdot}053 \pm 0{\cdot}019$, but the inhibition could only be seen if antibodies against both types of repeats of Mub were used together. This strongly indicates that Mub contributes to the adhesion of strain 1063 to mucus *in vitro*.

DISCUSSION

Mub is the first described *Lactobacillus* adhesion protein that features the same domain organization as most of the characterized adhesins from other Gram-positive bacteria (Fischetti, 2000). The architecture thus includes a signal peptide, C-terminal anchoring sequences, repeated regions with adhesion properties and an N-terminal region that often has an unknown function. However, there are some unusual features. Mub protein is one of the largest bacterial cell-surface proteins identified. Besides that, the corresponding *mub* gene has two possible translation start sites. A second site is predicted to generate a 35 aa secretion signal peptide with the same predicted cleavage site as the proposed 49 aa signal peptide. The second start is located in the wrong reading frame, with a possible −1 frameshift site located just behind it. Five nucleotides after the second start a TTTC site, known to be highly frameshift-prone (Schwartz & Curran, 1997), is located. Furthermore, the site is followed by a stem–loop structure, which is required for many programmed frameshifts (Farabaugh, 1996). Although speculative, a protein with two possible signal peptides where the second might be regulated by a frameshifting mechanism could represent an interesting mechanism for regulation and localization of secreted proteins. Another uncommon feature is that the membrane anchor sequence at the C terminus of Mub contains a cysteine residue. Rocha & Fischetti (1999) identified and characterized a fibronectin-binding pro-

tein from *Streptococcus pyogenes* that contained three cysteine residues, one of which was located in the membrane anchor. However, the authors did not postulate any function of the cysteine residues. In *Staphylococcus aureus*, proteins with the consensus cell-wall-anchoring sequence have been shown to be cleaved within the motif LPXTG after transport through the membrane (Schneewind *et al*., 1995). The presence of a highly functional amino acid such as cysteine in this region could suggest an alternative mechanism for the anchoring of Mub to the cell surface.

The fact that strain 1063 adhered efficiently to both pig and hen mucus indicates that the bacterium has little or no host specificity regarding adhesion to mucus. No inhibition of adhesion of strain 1063 cells could be seen when αMub1 or αMub2 antibodies were used separately. However, the significant inhibition observed when using the antibodies together suggests that both types of repeats are important for binding of the bacteria to mucus components. Moreover, since the P108 antibodies, raised against a mix of cell-surface and secreted proteins from strain 1063, blocked binding more efficiently than the mixture of αMub1 and αMub2, other proteins may also be involved in the binding.

The presence of two types of amino acid residue repeats and a >500 aa N-terminal region makes it likely that Mub is a multifunctional protein, in common with many described adhesins from Gram-positive organisms (Fischetti, 2000). The two types of repeats both adhere to mucus, mucus fractions and to mucin (Table 2). Western blot experiments showed that the Mub1 and Mub2 repeats adhere to the same components in pig intestinal mucus and to the same components in hen mucus. Furthermore, the inhibition of binding by the glycoproteins fetuin and asialofetuin suggests that Mub adheres to carbohydrate structures in mucus. However, the binding of MBP-Mub1 was less sensitive to the addition of glycoproteins, indicating that it also interacts with other components in mucus. Of the simple carbohydrates tested, only fucose gave a reduction in binding of MBP-Mub1 and MBP-Mub2, indicating that it might form part of a more complex carbohydrate receptor structure. There was also a pH dependence in the binding with maximum binding below neutral pH. Interestingly, MBP-Mub1 and MBP-Mub2 had different pH optima for binding, which could reflect an adaptation to the interaction with different niches in the gastrointestinal tract. Mukai *et al*. (1998) showed that three *Lb*. *reuteri* strains adhered to different sugar residues of glycoconjugates. Specificity of binding was determined for only one of the strains that, in contrast to Mub, exhibited binding that was sensitive to addition of *N*-acetylgalactosamine.

The type 1 and 2 repeats of Mub display extensive sequence similarities with each other and also to the repeats of the *Lc*. *lactis* protein YwfG. Lactococci are not usually considered as members of the normal intestinal microflora, but in studies during the 1970s *Lc*. *lactis* was commonly isolated from faeces from many different groups of humans (Finegold *et al*., 1983). The presence of a protein with strong similarities to Mub suggests that *Lc*. *lactis* actually is adapted to the gastrointestinal tract environment. The Mub repeats also share similarities with an NANB hepatitis virus antigen (Reyes *et al*., 1990) and the human ocular component hr44 (Braun *et al*., 1995). The hepatitis peptide is described as a peptide that is recognized by antiserum from a human infected with the virus and hr44 is a protein located on the cell surface of epithelial cells of the eye. The functions of these proteins are not known, but both might be present at surfaces that are in contact with a mucus layer. The finding that proteins from such distant organisms appear related is intriguing. It could be speculated that the similarities between the proteins reflect some common mechanism for interaction with mucus components.

In most cases, the initial step in infection by any micro-organism is adhesion or association with host mucosal surfaces. Therefore, blocking of this step is a potential point of disease control. In several reports, probiotic lactobacilli have been shown to inhibit the *in vitro* attachment of enterovirulent bacteria to mucus and enterocytes and also to prevent invasion of the pathogen (Craven & Williams 1998; Tuomola *et al*., 1999). Whether the mechanism of inhibition is competition for the same receptor structures or an effect of steric hindrance by *Lactobacillus* cells is not known. *Lb*. *reuteri* has been tested for its probiotic activities in different animal systems and has been found to have protecting effects against pathogenic micro-organisms such as *Salmonella* (Edens *et al*., 1997) and *Cryptosporidium* (Alak *et al*., 1997). Furthermore, children suffering from rotavirus diarrhoea were found to have a significantly reduced duration of the disease when treated with *Lb*. *reuteri* (Shornikova *et al*., 1997). The importance of the mucus-binding properties of lactobacilli in this context has not yet been evaluated. Preliminary results show that *mub* is not present in a majority of other *Lb*. *reuteri* strains tested. However, tested strains of the species *Lactobacillus mucosae* harboured it (Roos *et al*., 2000). Construction of a *mub* mutant is planned in the near future. This is the key for a thorough evaluation of the role of Mub in colonization and in the control of pathogenic micro-organisms.

ACKNOWLEDGEMENTS

This study was supported by grants from The Swedish National Board for Industrial and Technical Development, NUTEK, (P5974) in a co-operation project with BioGaia AB, Stockholm, Sweden, and from The Swedish Council for Forestry and Agricultural Research, SJFR, (31.0243/91).

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Received 17 April 2001; revised 24 September 2001; accepted 10 October 2001.