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Role of *Lactobacillus reuteri* cell and mucus-binding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus *in vitro*

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Lactobacillus reuteri, a symbiotic inhabitant of the gastrointestinal tract in humans and animals, is marketed as a probiotic. The ability to adhere to intestinal epithelial cells and mucus is an interesting property with regard to probiotic features such as colonization of the gastrointestinal tract and interaction with the host. Here, we present a study performed to elucidate the role of sortase (SrtA), four putative sortase-dependent proteins (SDPs), and one C-terminal membrane-anchored cell surface protein of *Lactobacillus reuteri* ATCC PTA 6475 in adhesion to Caco-2 cells and mucus *in vitro*. This included mutagenesis of the genes encoding these proteins and complementation of mutants. A null mutation in *hmpref0536_10255* encoding *srtA* resulted in significantly reduced adhesion to Caco-2 cells and mucus, indicating involvement of SDPs in adhesion. Evaluation of the bacterial adhesion revealed that of the five putative surface protein mutants tested, only a null mutation in the *hmpref0536_10633* gene, encoding a putative SDP with an LPxTG motif, resulted in a significant loss of adhesion to both Caco-2 cells and mucus. Complementation with the functional gene on a plasmid restored adhesion to Caco-2 cells. However, complete restoration of adhesion to mucus was not achieved. Overexpression of *hmpref0536_10633* in strain ATCC PTA 6475 resulted in an increased adhesion to Caco-2 cells and mucus compared with the WT strain. We conclude from these results that, among the putative surface proteins tested, the protein encoded by *hmpref0536_10633* plays a critical role in binding of *Lactobacillus reuteri* ATCC PTA 6475 to Caco-2 cells and mucus. Based on this, we propose that this LPxTG motif containing protein should be referred to as cell and mucus binding protein A (CmbA).

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INTRODUCTION

Strains of *Lactobacillus reuteri* have been isolated from the gastrointestinal tract, mother's milk and vagina of humans and animals (Oh *et al.*, 2010; Walter *et al.*, 2011). However, the primary habitat of the species appears to be the gastrointestinal tract, and the species has been designated a universal entero-*Lactobacillus* (Casas & Dobrogosz, 2000) and a vertebrate symbiont of the gastrointestinal tract (Walter *et al.*, 2011). *Lactobacillus reuteri* is marketed as a probiotic for humans and several clinical studies indicate positive effects (Weizman *et al.*, 2005; Agustina *et al.*, 2012; Hunter *et al.*, 2012; Szajewska *et al.*, 2013). *Lactobacillus reuteri* ATCC PTA 6475, isolated from human mother's milk, is a candidate probiotic with anti-inflammatory properties (Lin *et al.*, 2008; Jones *et al.*, 2011; Thomas *et al.*, 2012), which has shown promising effects in animal studies

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Abbreviations: IEC; intestinal epithelial cell, SDP; sortase-dependent protein.

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(Eaton *et al.*, 2011; Preidis *et al.*, 2012; McCabe *et al.*, 2013).

Bacterial adherence to intestinal epithelial cells (IECs) and/or mucus is frequently considered to be a desirable feature for a probiotic strain as it can promote the gut residence time and interaction with host epithelial and immune cells (Lebeer *et al.*, 2008; Kleerebezem *et al.*, 2010; Juge, 2012). In general, adhesins of lactobacilli can be classified according to their targets in the intestine (e.g. mucus components, extracellular matrix proteins), according to their localization on the bacterial surface (e.g. surface layer proteins) or according to how they are anchored to the bacterial surface [e.g. sortase-dependent proteins (SDPs)] (Vélez *et al.*, 2007). The mechanisms of adhesion are not fully understood; however, several reports have shed light on the mechanisms for a variety of *Lactobacillus* species (Roos *et al.*, 1996; Rojas *et al.*, 2002; Roos & Jonsson, 2002; Granato *et al.*, 2004; Buck *et al.*, 2005, 2009; Pretzer *et al.*, 2005; Bergonzelli *et al.*, 2006; van Pijkeren *et al.*, 2006; Kankainen *et al.*, 2009; Vélez *et al.*, 2010; Sánchez *et al.*, 2011; von Ossowski *et al.*, 2011). In addition to specific bacterial adhesins, other cell surface molecules, such as S-layer proteins, lipoteichoic acid and exopolysaccharides (Lebeer *et al.*, 2008), and extracellular appendages, such as flagella, fimbriae and pili (Juge, 2012), can also contribute to adhesion to host epithelial cells and mucus. See Vélez *et al.* (2007), Juge (2012) or Lebeer *et al.* (2008) for comprehensive reviews on adherence factors.

Several adhesins of *Lactobacillus reuteri* have been described. The first *Lactobacillus reuteri* surface protein involved in adhesion to be described was CnBP of *Lactobacillus reuteri* NCIB 11951 (Aleljung *et al.*, 1994; Roos *et al.*, 1996), which binds to collagen type I. Later, the adhesion-promoting protein MapA of *Lactobacillus reuteri* 140R (Rojas *et al.*, 2002) was described. This protein binds to both mucus (Rojas *et al.*, 2002) and Caco-2 cells (Miyoshi *et al.*, 2006), and two receptor-like molecules for MapA have been identified on Caco-2 cells (Miyoshi *et al.*, 2006). CnBP and MapA are considered homologues in light of their similarity at the amino acid level (94 %). Roos & Jonsson (2002) were the first to describe a mucus-binding protein of *Lactobacillus reuteri* when they described an extracellular mucus-binding protein (Mub) in *Lactobacillus reuteri* 1063. The crystal structure of the Mub protein has been determined, which revealed an unexpected immunoglobulin binding activity (MacKenzie *et al.*, 2009). Other surface proteins of *Lactobacillus reuteri* that contribute to adhesion include a high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB), which both contribute to adherence and ecological performance of *Lactobacillus reuteri* 100-23 in the murine gut (Walter *et al.*, 2005).

SDPs are a group of surface-associated proteins in Gram-positive bacteria, many of which have been shown to impact the adhesive ability of several lactobacilli. SDPs have a common molecular structure that includes an N-terminal signal peptide, often with an YSIRK-G/S motif that promotes secretion (Bae & Schneewind, 2003) and directs the protein to a specific surface localization (DeDent *et al.*, 2008), a C-terminal LPxTG motif, followed by a C-terminal transmembrane helix and a positively charged tail (Lebeer *et al.*, 2008; Call & Klaenhammer, 2013). Examples are the mannose-specific adhesin (Msa) in *Lactobacillus plantarum* WCFS1 (Pretzer *et al.*, 2005), Mub in *Lactobacillus reuteri* ATCC 53608 (strain 1063) (Roos & Jonsson, 2002) and *Lactobacillus* epithelium adhesin of *Lactobacillus crispatus* ST1 (Edelman *et al.*, 2012). Sortase A (SrtA) cleaves the LPxTG motif between the threonine and glycine residues, and covalently links the threonine carboxyl group to amino groups provided by the cell wall cross-bridges of peptidoglycan precursors (Marraffini *et al.*, 2006). Thus, a SDP is linked to the cell wall and displayed on the bacterial surface.

Lactobacillus reuteri ATCC PTA 6475 has a single gene encoding SrtA, five putative SDPs and one putative C-terminal membrane-anchored cell surface protein with similarities to SDPs but lacking the LPxTG motif. Among the human *Lactobacillus reuteri* strains, ATCC PTA 6475 is highly adherent to mucus (MacKenzie *et al.*, 2010) and various intestinal human cell lines (Wang *et al.*, 2008; Jensen *et al.*, 2012). Here, we present a study performed to elucidate whether SrtA and five of the six above-mentioned putative surface proteins of *Lactobacillus reuteri* ATCC PTA 6475 play a role in the ability of the strain to adhere to IECs and mucus. The characterization of the functionality of these proteins includes adhesion to IECs and mucus *in vitro*, mutagenesis of specific genes, and complementation of mutants. We found that the putative SDP encoded by the gene *hmpref0563_10633* plays a significant role in the ability to adhere to IECs and mucus, and propose that this protein should be referred to as cell and mucus-binding protein A (CmbA).

METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. Strains were maintained at –80 °C in 20 % (v/v) glycerol. *Lactobacillus reuteri* strains were grown at 37 °C in Man–Rogosa–Sharpe (MRS) broth or on MRS agar. *Lactococcus lactis* MG1363 was grown at 30 °C in M17 supplemented with glucose [0.5 % (w/v)]. Strains harbouring the pSIP411 vector or its derivatives were cultured in the presence of 10 μg erythromycin ml^{-1} (Sigma-Aldrich). All culture media were from Oxoid.

Cell culture. The human colorectal adenocarcinoma cell line Caco-2 (HTB-37) was obtained from the American Type Culture Collection. Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 20 % heat-inactivated FBS, 1 % non-essential amino acids, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. All solutions were obtained from Invitrogen. The cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and subcultivated at 70–90 % confluence.

Sequence analysis of *Lactobacillus reuteri* ATCC PTA 6475. In order to identify genes encoding cell wall anchor domain proteins (Marraffini *et al.*, 2006), the genome sequence and identified proteins of strain ATCC PTA 6475, hereafter called 6475 (previously named

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Strains		
Lactobacillus reuteri		
6475	ATCC PTA 6475 (earlier designated MM4-1A); WT, host strain; human breast milk (a kind gift from BioGaia AB, Stockholm, Sweden)	Oh *et al.* (2010)
6475 *cmbA*$^-$	Derivative of 6475; nonsense mutation in *cmbA* (*hmpref0536_10633*) encoding a hypothetical LPxTG motif containing protein*	This work
6475 *10146*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_10146* encoding a hypothetical LPxTG motif containing protein*	This work
6475 *11993*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_11993* encoding a putative C-terminal membrane-anchored cell surface protein*	This work
6475 *10802*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_10802* encoding a LPxTG motif containing amidase*	This work
6475 *10154*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_10154* encoding a LPxTG motif containing Ser/Thr protein phosphatase*	This work
6475 *srtA*$^-$	Derivative of 6475; nonsense mutation in *srtA* (*hmpref0536_10255*) encoding sortase A*	This work
6475(pSIPΔ)	Derivative of 6475 containing pSIPΔ	This work
6475(pSIP-*cmbA*)	Derivative of 6475 containing pSIP-*cmbA*	This work
6475 *cmbA*$^-$(pSIPΔ)	Derivative of 6475 *cmbA*$^-$ containing pSIPΔ	This work
6475 *cmbA*$^-$(pSIP-*cmbA*)	Derivative of 6475 *cmbA*$^-$ containing pSIP-*cmbA*	This work
6475 *srtA*$^-$(pSIPΔ)	Derivative of 6475 *srtA*$^-$ containing pSIPΔ	This work
6475 *srtA*$^-$(pSIP-*srtA*)	Derivative of 6475 *srtA*$^-$ containing pSIP-*srtA*	This work
Lactococcus lactis		
MG1363	Intermediate cloning host	Gasson (1983)
Plasmids		
pSIP411	Em^r; SppIP-based expression vector with P_{sppQ}::*gusA*	Sørvig *et al.* (2005)
pSIPΔ	Em^r; pSIP411 derivative without *gusA*	This work
pSIP-*cmbA*	Em^r; pSIP411 derivative containing *cmbA* under control of P_{sppQ}	This work
pSIP-*srtA*	Em^r; pSIP411 derivative containing *srtA* under control of P_{sppQ}	This work

*Details given in Table S1.

MM4-1A; GenBank accession number ACGX02000000, sequences ACGX02000001–ACGX02000007), were reanalysed after the preliminary analysis made by Saulnier *et al.* (2011). The sorting motif LPxTG was searched for manually in the protein sequences, and YSIRK-G/S signal sequences (pfam04650), cell wall anchor domains (TIGR01167) and other protein domains were searched for in GenBank and with BLASTP at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Secretion signal peptides were predicted with SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) (Petersen *et al.*, 2011) and transmembrane helices were predicted with TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Repeats in the protein sequences were identified using RADAR (http://www.ebi.ac.uk/Tools/pfa/radar).

Construction of *Lactobacillus reuteri* mutants. Mutagenesis in *Lactobacillus reuteri* 6475 was performed using ssDNA recombineering as described previously (van Pijkeren & Britton, 2012). Defined nonsense mutations were established within the *Lactobacillus reuteri* 6475 genes *hmpref0536_10633*, *hmpref0536_10255*, *hmpref0536_10146*, *hmpref0536_11993*, *hmpref0536_10154* and *hmpref0536_10802* (Table S1, available in the online Supplementary Material); hereafter referred to as *cmbA*, *srtA*, *10146*, *11993*, *10154* and *10802*, respectively.

Reagents and enzymes. Total DNA was isolated using the DNeasy Tissue kit (Qiagen) and the Bacterial Genomic DNA Purification kit (EdgeBio). Plasmid DNA was isolated using the QIAprep Miniprep kit (Qiagen). Lysozyme (20 mg ml^{-1}) and mutanolysin (40 U ml^{-1}) (Sigma-Aldrich) were used in all lysis steps. Restriction endonucleases *Nco*I, *Xba*I and *Xho*I, and T4 DNA ligase were obtained from New England Biolabs. Phusion High-Fidelity DNA Polymerase (Finnzymes/Thermo Fisher Scientific) and Maxima Hot Start polymerase (Thermo Fisher Scientific) were used for PCR. PCR fragments required for cloning were recovered from 0.7 % agarose gels using the QIAquick Gel Extraction kit (Qiagen). All kits were used according to the manufacturers' instructions.

Cloning of *Lactobacillus reuteri* ATCC PTA 6475 *cmbA* and *srtA*. Plasmid pSIP411 (Sørvig *et al.*, 2005), known to yield inducible and titratable gene expression in *Lactobacillus reuteri* 6475 (van Pijkeren & Britton, 2012) through the P_{sppQ} (previously P_{orfX}) promoter, was used as cloning vector. Plasmid constructions were performed with *Lactococcus lactis* MG1363 as an intermediate host. *Lactococcus lactis* MG1363 and *Lactobacillus reuteri* were transformed by electroporation using the Gene Pulser system (Bio-Rad) as described previously (Holo & Nes, 1989; Ahrné *et al.*, 1992), except that 0.5 % sucrose in 10 % glycerol was used as electroporation buffer for *Lactobacillus reuteri*. The *Lactobacillus reuteri* 6475 *cmbA* gene contains an internal *Nco*I site. In order to clone the gene at the *Nco*I/*Xba*I sites in pSIP411 (at the ATG start codon), replacing the reporter gene *gusA*, the internal *Nco*I site was removed and the fourth base of the gene was changed from C to G to create an *Nco*I site at the 5′ end of the gene. This was accomplished by fusion PCR (Horton & Pease, 1991), where the complete coding region of *cmbA* was amplified in two parts and assembled, including the base changes, before ligation

into the pSIP411 vector at the *Nco*I and *Xba*I sites (primers used for fusion PCR are listed in Table S2). The base changes resulted in codon changes at the positions corresponding to aa 2 (CTA to GTA) and aa 407 (TCC to TCG) in *cmbA*. The former results in an amino acid residue change (Leu to Val), which was anticipated not to affect the function of the protein. Cloning of the *Lactobacillus reuteri* 6475 *srtA* gene was performed using synthetically manufactured DNA (GenScript). The synthetic fragment consisted of the P_{sppQ} promoter and ribosome binding site, as present in pSIP411, and the *srtA* gene with the following base changes: A instead of G at position 1 (changing the start codon from GTG to ATG to ensure expression in the pSIP system) and T instead of C at positions 202 and 405 (removing internal *Bgl*II and *Nco*I sites to minimize problems in future cloning in the pSIP plasmids). None of the codon changes resulted in an amino acid residue change in the corresponding protein. The synthetic fragment was flanked by *Bgl*II and *Xho*I sites, which were used for cloning in pSIP411, replacing the P_{sppQ}::*gusA* region. pSIP411 with the *gusA* reporter gene deleted, designated pSIPΔ, was also used as a control. Sanger sequencing of the final constructions was performed to ensure correct sequences. Molecular cloning and gel electrophoresis procedures were performed using standard procedures. All primers used in this study are listed in Table S2.

Adhesion assays. The initial adhesion assays (for strain 6475 and mutants) were performed as follows. Before adhesion experiments, strains were taken from frozen stock and grown in MRS broth at 37 °C. The strains were subcultured once before experiments. On the day of experiment, the strains were inoculated to OD_{600} 0.1 in MRS broth and grown at 37 °C. At OD_{600} 1.0±0.1, the cells were harvested by centrifugation at 4000 r.p.m. for 10 min, washed in Dulbecco's PBS (DPBS) and resuspended in 1 vol. DPBS (Sigma-Aldrich). In the complementation/overexpression adhesion assays, where the pSIP-inducible gene expression system (Sørvig *et al.*, 2005) was used, the strains were inoculated to OD_{600} 0.1 in two separate tubes for each strain. At OD_{600} 0.3, SppIP-inducing peptide (Molecular Biology Unit, University of Newcastle, UK) was added to one set of cultures. For *Lactobacillus reuteri* 6475, 6475 *cmbA*$^-$ and their derivative strains, 50 ng SppIP ml^{-1} was used. For 6475 *srtA*$^-$ and derivative strains, 1 ng SppIP ml^{-1} was used due to severe growth inhibition at higher concentrations for the strain containing pSIP-*srtA*. At 1.5 h after induction (OD_{600} 1.0±0.1), both induced and non-induced cultures were harvested as described above. *Lactobacillus reuteri* adhesion to Caco-2 cells was tested as described previously (Jensen *et al.*, 2012). Briefly, *Lactobacillus reuteri* strains were added (~5×10^6 c.f.u.) to confluent cell layers in 1 ml antibiotic-free cell media per well. After 1 h incubation, the cell layer was washed to remove non-adherent bacteria and lysed by addition of 0.1 % Triton X-100 (Sigma-Aldrich) in DPBS. The remaining suspensions with viable adhered bacteria were plated onto MRS agar and the number of c.f.u. was counted after 48 h incubation. Adhesion to Caco-2 cells was calculated as per cent of adhered bacteria in relation to the total number of bacteria added. Experiments were performed with triplicate determinations and repeated three to six times. The bacterial adhesion to mucus for *Lactobacillus reuteri* strains (Table 1) was tested as described previously (Roos & Jonsson, 2002). Briefly, mucus from pig small intestine (obtained from slaughterhouse material at Uppsala, Sweden) was prepared and coated in microtitre wells (MaxiSorp; Nunc). Strains were prepared as described above and washed once in PBS pH 6.0 (PBS) supplemented with 0.05 % Tween 20 (PBST) and resuspended in an equal volume of the same buffer. An aliquot of 100 µl bacterial suspension was added to each well and incubated overnight at 4 °C. The wells were washed three times with PBST, the buffer was poured off and OD_{405} was measured using a plate reader after the wells had dried. Wells coated with BSA were used as control. Experiments were performed with triplicate determinations and repeated three times.

Statistics. ANOVA of the bacterial adhesion to Caco-2 cells was performed in Minitab version 16 (Minitab) using the General Linear Model and Tukey's or Dunnet's post-hoc test. Differences were considered statistically significant at $P\leqslant0.05$. Illustrations were created in Prism version 5.0 (GraphPad).

RESULTS

Sequence analysis of *Lactobacillus reuteri* ATCC PTA 6475

The sequences of the proteins encoded by the draft genome sequence of *Lactobacillus reuteri* ATCC PTA 6475 were analysed, and one sortase and five cell wall anchor domain proteins with LPxTG motifs were identified. One protein with a similar architecture, but lacking the actual LPxTG motif, thus predicted to be anchored to the cell envelope by a C-terminal membrane anchor, was also found. Furthermore, two putative pseudogenes encoding domains related to cell wall anchoring were identified. The genetic loci in strain 6475, the corresponding loci in the fully sequenced and highly related strain JCM 1112 (Walter *et al.*, 2011), the identity of the proteins, and some of their features are shown in Table 2.

Adhesion of *Lactobacillus reuteri* mutants to Caco-2 cells and mucus

Bacterial adhesion to the human colorectal cell line Caco-2 and mucus was investigated initially for *Lactobacillus reuteri* 6475, the *srtA* mutant and the five mutants for genes encoding putative surface proteins (Table 1). The growth of the strains was followed as a part of the Caco-2 cell adhesion assay (see Methods). The growth rates were very similar (results not shown). *Lactobacillus reuteri* 6475 *srtA*$^-$ revealed a significantly lower adhesion to Caco-2 cells and mucus compared with 6475 ($P=0.0057$ and $P=0.00017$, respectively) (Fig. 1), indicating involvement of SDPs in adhesion. Of the LPxTG protein mutants, *Lactobacillus reuteri* 6475 *cmbA*$^-$ revealed a significant loss of adhesion to Caco-2 cells, whereas the other mutants did not show significantly reduced adhesion compared with 6475 (1.3 vs 4.8 %, $P<0.0001$) (Fig. 1a). *Lactobacillus reuteri* 6475 *cmbA*$^-$ also showed a total loss of adhesion to mucus ($P<0.0001$). *Lactobacillus reuteri* 6475 *10802*$^-$ had a significant loss of adhesion to mucus compared with 6475 ($P=0.0161$), whereas the other mutants did not show significantly reduced adhesion to mucus (Fig. 1b). Only *Lactobacillus reuteri* 6475 *srtA*$^-$ and 6475 *cmbA*$^-$ thus revealed significantly reduced adhesion to both Caco-2 cells and mucus, and only those strains were selected for complementation.

Cloning of *cmbA*

The annotated *hmpref0536_10633* (*cmbA*) gene of strain 6475 is reported to be 3093 bp (Fig. S1). However, the cloning procedure yielded a gene of 2517 bp. Sequencing of

Table 2. Genes encoding cell wall anchor domain proteins and related proteins in *Lactobacillus reuteri* 6475

Locus tag HMPREF0536	Homologue in strain JCM 1112	Annotation/features/comments	Size (aa)	Mutant analysed
10255	LAR_0227	Sortase	234	Yes
10146	LAR_0989	LPxTG protein, Pilus_PilP and Rib regions	630	Yes
10154	LAR_0983	LPxTG protein, 5′-nucleotidase/2′,3′-cyclic phosphodiesterase	752	Yes
10633	LAR_0958	LPxTG protein, YSIRK-G/S signal sequence	1030	Yes
10706	LAR_0903	LPxTG protein, a second putative start 87 bp downstream (size 272 aa)	301	NA
10802	LAR_0813	LPxTG protein, amidase, a second putative start 25 bp downstream (size 637 aa)	645	Yes
11242-11241	LAR_1193-1192	11242: YSIRK-G/S signal sequence; 11241: LPxTG motif; ORFs separated by frame shift, putative pseudogene	328*	NA
12042	LAR_0089	YSIRK-G/S signal sequence, truncated, no LPxTG motif, putative pseudogene	607*	NA
11993	LAR_0044	C-terminal membrane-anchored cell surface protein	951	Yes

NA, Not available.
*Truncated genes.

the cloned gene revealed that the difference was due to a part where the annotated gene shows the presence of three identical tandem repeat regions of 288 bp (Fig. S1), whereas the cloned gene only has one such region. Several control PCRs with different primer pairs were performed, verifying that the *cmbA* gene in our culture of strain 6475 was ~0.6 kbp shorter than expected from the annotated genome sequence (not shown). Whether this represents a sequencing error and/or artefact in the reported 6475 genome sequence, or that variants of the gene exist, is not known at present.

Complementation of mutants: adhesion to Caco-2

Based on the initial adhesion experiments (Fig. 1), *Lactobacillus reuteri* 6475 *cmbA*$^-$ and 6475 *srtA*$^-$ were complemented with the corresponding functional gene using the pSIP411 vector and its inducible gene expression system. Gene expression in complemented strains was first validated for *cmbA* using quantitative real-time PCR. This showed that *cmbA* expression increased in the complemented strain ~400-fold during induction compared with the WT expression from the chromosome (Table S3 and accompanying text). Together with controls, the adhesion of the complemented strains to Caco-2 cells was tested (Fig. 2). Similar to the initial experiments with the original mutants (see above), the growth was monitored for the different variant strains as a part of the adhesion assay. The growth rates of the strains again appeared similar (not shown). After SppIP induction of *cmbA* expression in *Lactobacillus reuteri* 6475 *cmbA*$^-$(pSIP-*cmbA*) the adhesion was restored to that of *Lactobacillus reuteri* 6475 (4.7 vs 5.2 %), significantly higher than the corresponding non-induced strain (4.7 vs 0.9 %, P=0.0017) (Fig. 2). Furthermore, SppIP induction of vector *cmbA* in *Lactobacillus reuteri* 6475(pSIP-*cmbA*), i.e. overexpression of *cmbA* in the WT strain, resulted in a significantly higher adhesion compared with the corresponding non-induced strain (8.5 vs 3.6 %, P=0.0001) and the WT strain (8.5 vs 5.2 %, P=0.0095) (Fig. 2). The SppIP-induced *Lactobacillus reuteri* 6475 *srtA*$^-$(pSIP-*srtA*) did not show a higher adhesion than the corresponding non-induced strain (2.7 vs 3.0 %). However, both these strains showed a somewhat higher adhesion than *Lactobacillus reuteri* 6475 *srtA*$^-$ and 6475 *srtA*$^-$(pSIPΔ) (~1.7 %) (Fig. 2), although the difference was not statistically significant. The presence of the pSIPΔ vector alone did not influence the adhesion with statistical significance. However, non-induced *Lactobacillus reuteri* 6475(pSIPΔ) and 6475(pSIP-*cmbA*) did show a somewhat poorer adhesion compared with *Lactobacillus reuteri* 6475 (3.8 and 3.6 vs 5.2 %, respectively) (Fig. 2). Furthermore, the induction peptide SppIP (1 or 50 ng ml^{-1}) did not influence the growth of the strains (results not shown) or the adhesion to Caco-2 cells (Fig. 2).

Complementation of mutants: adhesion to mucus

Bacterial adhesion to immobilized mucus was investigated for *Lactobacillus reuteri* 6475, 6475 *cmbA*$^-$, 6475 *srtA*$^-$ and their derivative strains (Fig. 3). SppIP induction of vector *cmbA* in 6475(pSIP-*cmbA*) resulted in a significantly higher adhesion compared with both the corresponding non-induced strain (P<0.0001) and 6475 (P<0.0001) (Fig. 3). On the contrary, SppIP induction of vector *cmbA* in 6475 *cmbA*$^-$(pSIP-*cmbA*), i.e. complementation of the *cmbA* mutant, did not restore fully the adhesion to that of 6475. A clear trend towards increased adhesion for the complemented strain compared with 6475 *cmbA*$^-$ was observed (Fig. 3), although the difference was not statistically significant in the ANOVA. After complementation with vector *srtA* in *Lactobacillus reuteri* 6475 *srtA*$^-$, the adhesion was restored to that of 6475 for both SppIP-induced and non-induced cultures, and was significantly higher than 6475 *srtA*$^-$ (P<0.0001 for both SppIP-induced and non-induced cultures). Although showing clearly higher adhesion than

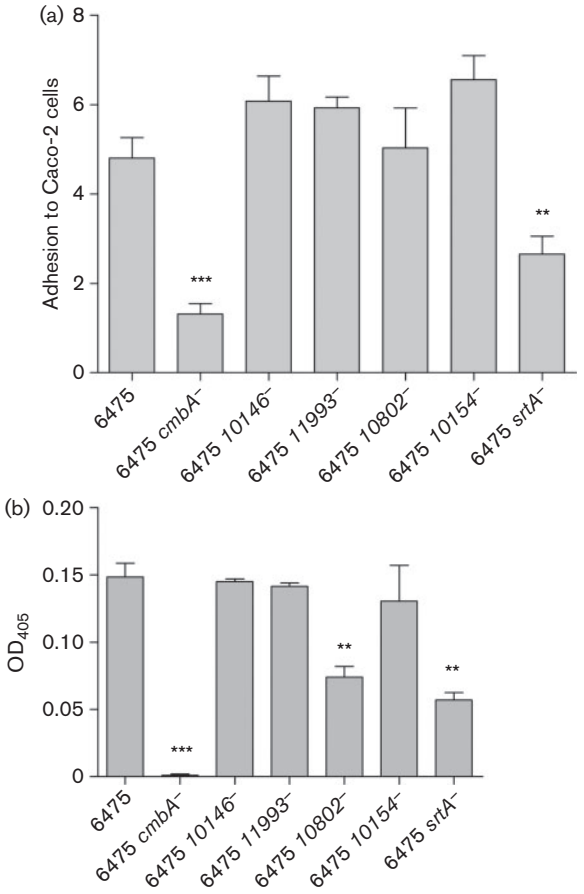

Fig. 1. (a) Adhesion of *Lactobacillus reuteri* 6475 and mutant strains to Caco-2 cells. *Lactobacillus reuteri* strains were added (~5×10^6 c.f.u.) to confluent cell layers in 1 ml antibiotic-free cell media per well. After 1 h incubation, the cell layer was washed to remove non-adherent bacteria and lysed by addition of 0.1 % Triton X-100 (Sigma-Aldrich) in DPBS. The remaining suspensions with viable adhered bacteria were plated onto MRS agar and the number of c.f.u. counted after 48 h incubation. Adhesion to Caco-2 cells was calculated as per cent of adhered bacteria in relation to the total number of bacteria added. At least three independent biological replicates were performed with each strain in triplicate. (b) Adhesion of *Lactobacillus reuteri* 6475 and mutant strains to mucus. Mucus from pig small intestine was prepared and coated in microtitre wells. An aliquot of 100 µl bacterial suspension was added to each well and incubated overnight at 4 °C. The wells were washed three times with PBST, the buffer was poured off and OD_{405} was measured using a plate reader after the wells had dried. At least three independent biological replicates were performed with each strain in triplicate. All results are expressed as means; error bars, SEM. *Lactobacillus reuteri* mutants were compared with *Lactobacillus reuteri* 6475 (ANOVA, General Linear Model, Dunnet's post-hoc test), $*P\leqslant0.05$, $**P\leqslant0.01$, $***P\leqslant0.001$.

the mutant, non-induced *Lactobacillus reuteri* 6475(pSIP-*cmbA*) revealed, for unknown reasons, a significantly lower adhesion compared with non-induced 6745 ($P=0.0002$), whereas *Lactobacillus reuteri* 6475 harbouring pSIPΔ did not show reduced adhesion under these conditions (Fig. 3).

DISCUSSION

In this study, we addressed the effect of inactivating sortase (SrtA), four putative SDPs and one C-terminal membrane-anchored cell surface protein of *Lactobacillus reuteri* ATCC PTA 6475 on adhesion to Caco-2 cells and mucus. The putative SDP, encoded by the gene *hmpref0536_10633*, proved to be highly important for adhesion to both Caco-2 cells and mucus *in vitro* as shown by various mutant and complemented strains (Figs 1, 2 and 3). We therefore propose the protein encoded by this gene to be named and referred to as cell and mucus binding protein A (CmbA).

According to the genome sequence of *Lactobacillus reuteri* 6475, CmbA encodes a polypeptide of 1030 aa containing an N-terminal YSIRK-G/S type signal peptide and a C-terminal LPxTG motif followed by a hydrophobic region predicted to be a transmembrane helix and a positively charged tail (Fig. S1). The LPxTG motif is recognized by SrtA (Marraffini *et al.*, 2006; Spirig *et al.*, 2011; Call & Klaenhammer, 2013), which in turn is responsible for anchoring of the protein to the cell wall. When using the C-terminal 42 aa of the predicted protein (including the LPxTG motif, the hydrophobic region and the positively charged tail) as the search string in a Pfam search (http://pfam.sanger.ac.uk), a very significant similarity for this region was obtained with the protein family 'Gram positive anchor' (Pfam: PF00746). CmbA thus has all the features that define SDPs (Schneewind *et al.*, 1992; Navarre & Schneewind, 1994, 1999; Bae & Schneewind, 2003; van Pijkeren *et al.*, 2006; Schneewind & Missiakas, 2012; Call & Klaenhammer, 2013; Remus *et al.*, 2013). CmbA is not classified as a 'MucBP' protein (Pfam: PF06458); however, BLASTP searches revealed that the best similarity scores were obtained with mucus binding proteins and LPxTG proteins in other *Lactobacillus* species, especially those of the so-called '*acidophilus* complex' (Kullen *et al.*, 2000), e.g. *Lactobacillus gasseri* MV-22 mucus binding protein (GenBank accession number ZP_07711536), *Lactobacillus crispatus* ST1 mucus binding protein (GenBank accession number YP_003602126), *Lactobacillus johnsonii* NCC 533 MucBP region protein (GenBank accession number NP_964406) and *Lactobacillus acidophilus* NCFM mucus binding protein (GenBank accession number YP_194552). The homologies to these proteins were in the range of 30–35 % identity (45–55 % similarity considering conservative amino acid changes). Among analysed and sequenced *Lactobacillus reuteri* strains, CmbA is unique to *Lactobacillus reuteri* 6475, JCM 1112 (DSM 20016), ATCC PTA 4659 (MM2-3) and ATCC PTA 5289 (FJ1). These strains are almost identical at the genome level with only a maximum of nine SNP differences between them (Walter *et al.*, 2011) and are all clustered in one phylogenetic group, lineage II, of *Lactobacillus reuteri* (Oh *et al.*, 2010), which essentially contains *Lactobacillus reuteri* strains of human origin. In

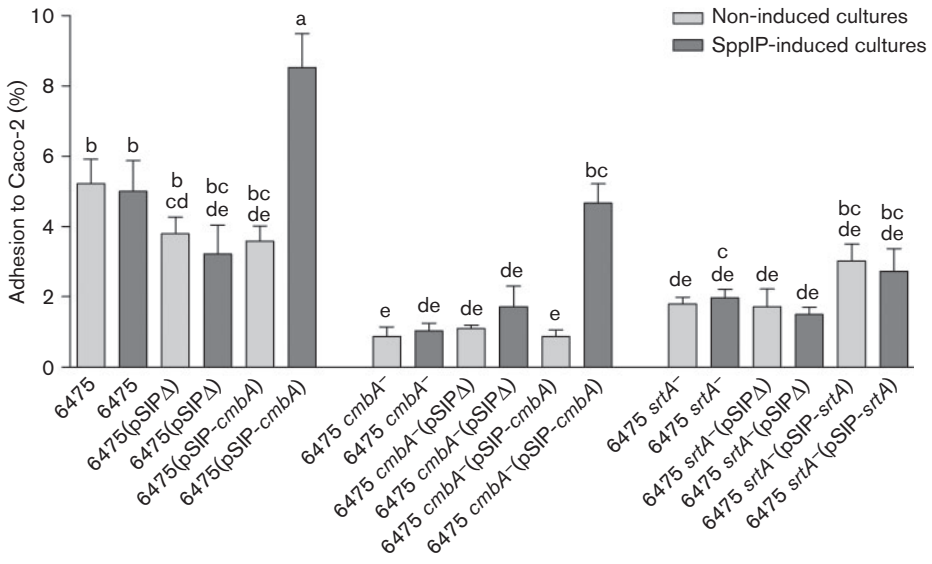

Fig. 2. Adhesion of *Lactobacillus reuteri* strains to Caco-2 cells with and without SppIP induction. *Lactobacillus reuteri* strains, either induced or non-induced with SppIP, were added (~5×10^6 c.f.u.) to confluent cell layers in 1 ml antibiotic-free cell media per well. After 1 h incubation, the cell layer was washed to remove non-adherent bacteria and lysed by addition of 0.1 % Triton X-100 (Sigma-Aldrich) in DPBS. The remaining suspensions with viable adhered bacteria were plated onto MRS agar and the number of c.f.u. was counted after 48 h incubation. Adhesion to Caco-2 cells was calculated as per cent of adhered bacteria in relation to the total number of bacteria added. At least three independent biological replicates were performed with each strain in triplicate. The results are expressed as means; error bars, SEM. Letters above columns refer to the ANOVA: means that do not share a letter are significantly different (ANOVA, General Linear Model, Tukey's post-hoc test accounting for the interaction between strains and level of induction), $P \leqslant 0.05$.

addition, and notably, the *cmbA* gene is differently reported in GenBank for some of these strains with regard to the tandem repeat region (Fig. S1): the JCM 1112 sequence (GenBank accession number NC_010609.1) contains five repeats, the 6475 draft sequence (GenBank accession number ACGX02000000) reports three (with a comment: 'unresolved tandem repeat'), and the ATCC PTA 4659 draft sequence (GenBank accession number ACLB01000000) has the repeat region as a separate contig. The cloning of *cmbA* from the 6475 strain in our collection yielded a gene with only one of these repeat regions. This might indicate that variants of the gene exist. Whether this is the case and if this has any bearing on the function of CmbA remains to be investigated. Worthy of note is that repeat regions, with variable numbers in different strains, have been found in other adhesins from lactobacilli (Boekhorst *et al.*, 2006; Gross *et al.*, 2010). Human *Lactobacillus reuteri* strains are also found in another phylogenetic group (lineage VI). One representative of this latter group is the commercial strain DSM 17938, which lacks CmbA. This strain has been shown to adhere significantly less to IECs than the strongly adhering strains 6475, DSM 20016 and ATCC PTA 5289 (FJ1) in the same assay system as used here (Jensen *et al.*, 2012). One might therefore speculate that CmbA has a specific interaction with structures on human IECs that renders the strains possessing the protein to be highly adherent.

The *cmbA* mutant showed a significant reduction in adhesion to Caco-2 cells and a total loss of adhesion to mucus. This effect was reversible for adhesion to Caco-2 cells upon complementation with *cmbA* expressed from a vector. In addition, overexpression of *cmbA* in the WT strain [i.e. strain 6475(pSIP-*cmbA*)] did increase adhesion to both Caco-2 cells and mucus. In light of this, it was somewhat surprising that the adhesion to mucus was poorly restored with complemented *cmbA*. It should be noted, however, that there was a relatively clear trend towards increased adhesion for the complemented strain compared with the mutant. The lack of statistical significance in the ANOVA may in part be a result of uncertainties in the measurements at these low levels of adhesion (in the case of the *cmbA* mutant, no adhesion could be measured). Lack of full complementation cannot be copy number or pSIP system related, as such effects would also have been evident in studies of the same culture in adhesion to Caco-2 cells. The effect must therefore be specific for the mucus binding property of CmbA. Some possibilities exist for obtaining poor complementation, e.g. improper folding of the overexpressed protein that specifically affects mucus binding or improper co-expression of CmbA in relation to additional unidentified factors specifically involved in adhesion to mucus. This remains to be investigated, and has to await a more thorough characterization of CmbA and possible interactions with

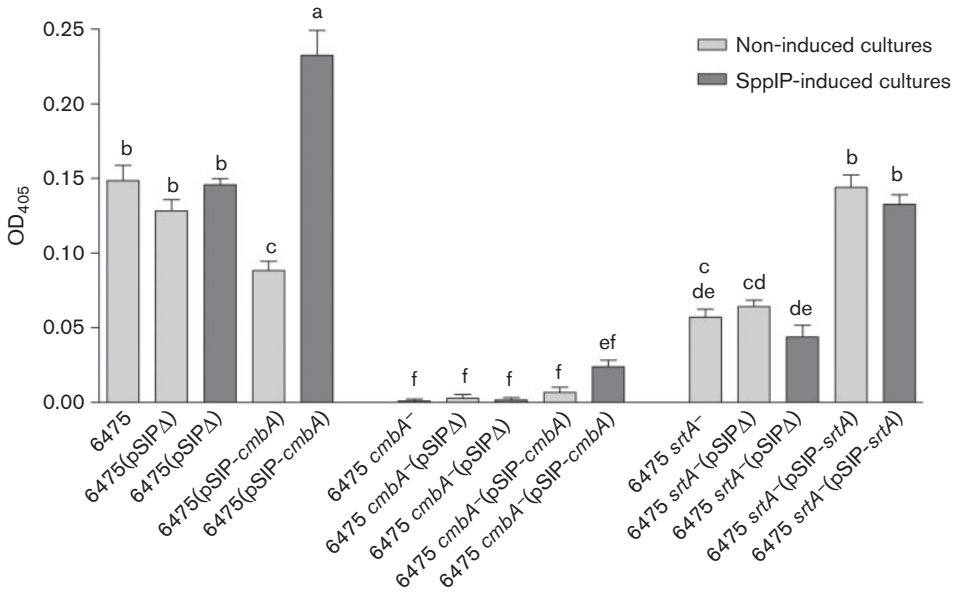

Fig. 3. Adhesion of *Lactobacillus reuteri* strains to mucus with and without SppIP induction. Mucus from pig small intestine was prepared and coated in microtitre wells. An aliquot of 100 µl bacterial suspension, either induced or non-induced with SppIP, was added to each well and incubated overnight at 4 °C. The wells were washed three times with PBST, the buffer was poured off and OD_{405} was measured using a plate reader after the wells had dried. At least three independent biological replicates were performed with each strain in triplicate. The results are expressed as means; error bars, SEM. Letters above columns refer to the ANOVA: means that do not share a letter are significantly different (ANOVA, General Linear Model, Tukey's post-hoc test), $P \leqslant 0.05$.

other proteins. Other adhesion factors to IECs and mucus have been described previously for *Lactobacillus reuteri* (Miyoshi *et al.*, 2006; MacKenzie *et al.*, 2010), and the present study also indicated that the putative SDP encoded by the gene *hmpref0536_10802* was involved in mucus binding (Fig. 1b), but not in IEC binding. Nevertheless, the results indicate a significant role of CmbA in the adhesive properties of *Lactobacillus reuteri* 6475.

The *srtA* mutant showed a significantly reduced adhesion compared with *Lactobacillus reuteri* 6475, but a somewhat higher adhesion than the *cmbA* mutant. This supports the strong *in silico* evidence that CmbA is an SDP, and is in line with previous studies on SDPs and sortase-deficient mutants. SDPs may still be found as surface proteins in *srtA* mutants (Bierne *et al.*, 2002; Nobbs *et al.*, 2007; Remus *et al.*, 2013), although significantly decreased in abundance and possibly displayed in a non-optimal fashion. The reason for the increased adhesion for the *srtA* mutant compared with the *cmbA* mutant may thus be explained by the physical properties of the C-terminal end of the CmbA protein and the fact that *cmbA* is still expressed in the *srtA* mutant. In the *srtA* mutant, the protein will be exported through the Sec machinery by way of the signal sequence (Ton-That *et al.*, 2004). It is, however, no longer cleaved at the LPxTG site and therefore not covalently bound to the peptidoglycan, but the C-terminal transmembrane helix and the positively charged tail may anchor some of the expressed protein to the membrane. Thus, it is likely that whilst CmbA is not coupled to the peptidoglycan in the absence of SrtA, and therefore not optimally displayed, it remains surface associated in the cytoplasmic membrane by the C-terminal anchor and promotes some adhesion of *Lactobacillus reuteri* to IECs and mucus. The adhesion of the *srtA* mutant was essentially restored by introducing pSIP-*srtA* without induction. This was particularly evident with regard to mucus adhesion. Induction with 1 ng SppIP ml^{-1} did not increase adhesion further. This level of SppIP is well below the saturation level, but still promotes measurable and higher expression in *Lactobacillus reuteri* compared with the non-induced state (unpublished observations). Higher induction levels were tested, but this resulted in severe growth inhibition of the strain. This may indicate that *srtA* expression in the WT is at a relatively low level due to possible toxic effects of high SrtA concentrations, consistent with the protein being membrane located. The GTG start codon, used in the native *srtA* gene, but changed to ATG in the cloned version, is generally also an indication of downregulation of expression (Vellanoweth & Rabinowitz, 1992; O'Donnell & Janssen, 2001). The P_{sppQ} promoter in the pSIP411 vector was shown previously to be not inactive completely in the non-induced state, i.e. a very minor, but still detectable, degree of gene expression occurred also without the presence of the induction peptide SppIP (Sørvig *et al.*, 2005). This minor expression of *srtA*, together with a more

effective translational start, could thus be enough to complement the mutant. Due to the sensitivity of *srtA* expression in *Lactobacillus reuteri* 6475 as described above, overexpression of *srtA* in the WT strain was anticipated to also give severe growth inhibition and therefore not tested.

Of the five putative surface protein mutants tested, only a null mutation in the *hmpref0536_10633* gene (*cmbA*), encoding a putative surface protein with an LPxTG motif, had a significant loss of adhesion to both Caco-2 cells and mucus. The *hmpref0536_10802* gene might play a role in adhesion of *Lactobacillus reuteri* 6475 to mucus, although the effect of inactivation of this gene was not as large as inactivation of *cmbA*. None of the other putative cell wall/membrane-anchored proteins investigated in the present study appeared to be important for adhesion to Caco-2 cells or mucus. Whether these proteins are expressed and, if so, their role in the surface properties of *Lactobacillus reuteri* 6475 remain to be investigated. The automatic bioinformatic analysis initially did not designate *hmpref0536_10706* as SDP encoding (due to a possible start codon 87 bp downstream, leading to a protein without a signal sequence; Table 2). A mutant for this remaining putative SDP gene was therefore not available for this study. This SDP also remains to be evaluated for any role in the adhesive properties of strain 6475.

The importance of CmbA in adhesion to both IECs and mucus adds to the complexity of the interactions that mediate the adhesion of gut bacteria to the intestine. As the mucus layer of the intestine is renewed continuously, it would probably be advantageous for probiotic bacteria to have the ability to bind to various intestinal surfaces as this would allow for prolonged time in the intestine for interactions with IECs and immune cells. The importance of CmbA in colonization of *Lactobacillus reuteri* 6475 in the intestine *in vivo* will have to be validated in future studies, but as this strain of *Lactobacillus reuteri* most probably does not bind to intestinal surfaces of mice (Oh *et al.*, 2010; Frese *et al.*, 2011; Walter *et al.*, 2011), such studies also require the development of suitable models.

In conclusion, we have identified a novel cell and mucus-binding protein, CmbA, of *Lactobacillus reuteri* 6475. Other surface proteins of *Lactobacillus reuteri* 6475 may contribute to the adhesion properties of the strain. However, since the *cmbA* mutant had a very significant loss of adhesion to Caco-2 cells and a total loss of adhesion to mucus, effects that were partially reversible by complementation of mutants, CmbA is clearly a highly important protein for the adhesive properties of *Lactobacillus reuteri* 6475.

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