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Lactococcus lactis IBB477 presenting adhesive and muco-adhesive properties as a candidate carrier strain for oral vaccination against influenza virus

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In the gastrointestinal tract (GIT), adhesion is a prerequisite for bacterial colonization. Lactococci can be used in functional food (probiotics) and health-related applications (mucosal vaccines, therapeutic drug delivery), both potentially involving adhesive properties. A candidate lactic acid bacterium for influenza antigen delivery through the GIT should display the ability to adhere. The present work probes the interactions between *Lactococcus lactis* and mucins using pig gastric mucin (PGM) as a model. Two strains were used for the optimization of the screening method for adhesion: *L. lactis* subsp. cremoris IBB477 persistent in the GIT of germ-free rats, and the low-adhering control strain MG1820. High adhesion to bare and mucin-coated polystyrene of IBB477 in comparison with MG1820 was observed. We searched for genetic determinants potentially involved in the adhesion/muco-adhesion of IBB477, identifying two such genes: *prtP* and a gene coding for a protein with MUB and MucBP domains. Based on its persistence in the GIT and adhesive properties, *L. lactis* IBB477 is a candidate carrier strain for expression of influenza haemagglutinin (HA) protein for induction of mucosal immune response.

Key words: *Lactococcus lactis*, mucin, adhesive properties, screening, mucosal vaccine, therapeutic drug delivery

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INTRODUCTION

Lactococci can be used in functional food (probiotics) and health-related applications (mucosal vaccines, therapeutic drug delivery), both potentially involving adhesive properties. The main reason for using the mucosal route of vaccination is that most infections (gastrointestinal, respiratory or genital infections), including influenza, affect a mucosal surface or start from one. Thus, adhering to the mucosal surface of the gastrointestinal tract (GIT) is tightly correlated with bacterial pathogenic activity and at the same time reflects the protective capabilities of "good" bacteria, e.g., lactic acid bacteria (LAB), including those serving as carriers for delivery of antigens and immunomodulatory factors. Furthermore, this route of vaccine application is often required for induction of a protective immune response. The mucosal vaccine should be targeted to the mucosal inductive sites and should be protected from physical elimination and enzymatic digestion (Holmgren & Czerkinsky, 2005). The mucosal delivery systems include live bacterial vectors and among them those that use commensal bacteria, such as lactobacilli or certain streptococci and staphylococci. We have shown that using *Lactococcus* as a producer of an influenza virus antigenic protein, haemagglutinin, is a promising approach (Szatraj *et al.,* 2014).

Surface proteins of Gram-positive bacteria play a crucial role in the bacterial adhesion to host tissues. In *L. lactis*, only three surface proteins with a direct implication in adhesion have been described to date: the chromosomally-encoded sex factor aggregation protein CluA (Godon *et al.,* 1994), and two plasmid-encoded proteins, serine proteinase PrtP (Reid & Coolbear, 1999) and protein YghE2 displaying pilin characteristics (Meyrand *et al.,* 2013). The PrtP proteinase is responsible for enhanced cell surface hydrophobicity and adhesion to solid surfaces (Habimana *et al.,* 2007), whereas CluA is involved in mediating cell-to-cell contact and DNA transport during conjugation (Stentz *et al.,* 2006). Recently, the role of pilin in adhesion has been demonstrated by inactivation of the pilin gene *yhgE2*, which led to the loss of adhesion of *L. lactis* to Caco-2 cells (Meyrand *et al.,* 2013). In lactobacilli, the most common proteins that have been shown to promote adhesion to mucus are those containing MUB and MucBP domains (Van Tassell & Miller, 2011; Roos & Jonsson, 2002). Based on bioinformatics studies, MUB domain-containing proteins have been identified and characterized in nine LAB species, including two *Lactococcus* representatives (Boekhorst *et al.,* 2006).

The present work was focused on the adhesive and muco-adhesive properties of *L. lactis* subsp. *cremoris* IBB477. This strain, originally isolated from raw cow milk in Poland, persists in the GIT of germ-free rats and was shown to be tetracycline-resistant (Boguslawska *et al.,* 2009). The adhesion of the IBB477 strain to an abiotic polystyrene surface was quantified and genetic determinants encoding putative adhesion/muco-adhesion capacity of IBB477 were identified. These studies were carried out with an intention to use IBB477 as a candidate for

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Abbreviations: GIT, gastrointestinal tract; LAB, lactic acid bacteria; PBS, phosphate buffered saline; PGM, pig gastric mucin; PS, polystyrene.

development of an oral protective vaccine against avian influenza virus infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The IBB477 strain was originally isolated in Poland from samples of raw cow milk (Boguslawska *et al.,* 2009), while *L. lactis* subsp. *cremoris* MG1820 is a laboratory strain presenting low level of adhesion to mucins (Dague *et al.,* 2010). Bellow-mentioned strains were used as positive controls in PCR amplification experiments: *L. lactis* subsp. *cremoris* MG1363 (*cluA*$^+$, gene coding for MucBP domain-containing protein$^+$) (Godon *et al.*, 1994), *L. lactis* subsp. *lactis* (*yhgE2*$^+$) (Meyrand *et al.,* 2013) and *L. lactis* subsp. *cremoris* J60011 (*prtP*$^+$) (Rochat *et al.*, 2005). Bacteria were cultured on M17-glucose (0.5% w/v) at 30°C (M17; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). For the IBB477 strain the medium was supplemented with 10 μg/mL of tetracycline (Sigma-Aldrich, Inc., St. Louis, MO). Bacterial stock cultures were kept at –80°C in M17 broth (Oxoid) containing 20% (v/v) glycerol and 0.5% (w/v) glucose.

Mucins and PGM-coated polystyrene plates. Type III mucin from porcine stomach (PGM) (lyophilized powder, cat. no. M1778, Sigma-Aldrich) was dissolved at 10 mg/mL in phosphate buffered saline (PBS) pH=7.5 just before use. Adhesion of *L. lactis* to PGM was determined on polystyrene microtiter 96-well plates (cat. no. 163320, Thermo Fischer Scientific Nunc A/S, Roskilde, Denmark) coated with 200 μL of PGM solution (10 mg/mL) and incubated overnight at 4°C, with gentle agitation on a platform rocker shaker. After incubation, the wells were washed three times with PBS and five times with sterile MilliQ-grade water to remove loosely bound material. The plates were air-dried and used directly after preparation.

Adhesion assay under static conditions. Adhesion of bacterial cells to bare polystyrene (PS) or PGM-coated polystyrene (PS+PGM) was tested on the microtiter plates, using the technique described by Christensen *et al.* (1985; 1995), as follows. Bacteria from overnight cultures diluted to $OD_{660\ nm}$ of 1.0 were harvested by centrifugation at $9\,000 \times g$ for 1 min and resuspended in an equal volume of PBS. A volume of 100 μL of bacterial suspension was added to each well (eight for each strain). After a 3-h incubation under static conditions at 30°C the wells were carefully washed three times with 200 μL of sterile MilliQ-grade water to remove unbound bacteria. Bound cells were stained with crystal violet (cat. no. 109218, Merck, Darmstadt, Germany) (100 μL per well) at room temperature for 10 min and rinsed three times with water as above, and once with 200 μL of 96% ethanol to remove excess stain. Finally, stained bacteria were suspended in 200 μL of 96% ethanol and optical density was determined at 583 nm on a Synergy HT Multi-Detection Reader (BioTek Instruments Inc., Winooski, VT). The average value of eight measurements was calculated after rejecting extreme results. Bacterial adhesion was determined in three independent experiments and the results are presented as means ± standard deviations. A statistical analysis was performed using Welch *t*-test. Each microtiter plate included the negative control strain *L. lactis* MG1820, low-adhesive to PS and PS+PGM (Dague *et al.,* 2010; Le *et al.,* 2011) and blank wells with PBS.

Primer design and PCR amplification. Identification of genes *cluA*, *prtP*, *yhgE2* and the gene coding for a MucBP domain-containing protein in *L. lactis* IBB477 was performed by PCR in a 25-μL reaction volume, with 0.625 U of Taq polymerase (Fermentas), 200 μM deoxynucleoside triphosphate mix (Fermentas), 0.4 μM specific primers (Table 1) and 2 μL of the template DNA. DNA templates for PCR amplification were obtained as follows: supernatants were obtained from a single bacterial colony suspended in 100 μL of sterile MilliQ-grade water, mixed with 50 mg of sterile glass beads (Sartorius Stedim Biotech, Goettingen, Germany), and disrupted in a Mini-Beadbeater apparatus (BioSpec Products, Inc., Bartlesville, OK, USA). The PCR mixture was pre-denaturated at 94°C for 5 min and incubated for 30 cycles, each consisting of 3 steps (94°C for 30 s, 55°C for 1 min, and 70°C for 2 min), with a final cycle of 70°C for 7 min.

DNA sequencing. The complete gene coding for the MucBP domain-containing protein from IBB477 was amplified with primers dopUC19F and dopUC19R (Table 1) and cloned into pUC19 vector. In order to facilitate sequence analysis of long cloned DNA fragment, the Deletion Kit for Kilo-Sequencing (TaKaRa Bio Inc., Dalian, China) was used according to manufacturer's instructions. The sequencing products were analysed on an ABI 3730/xl Genetic Analyzer in the Laboratory of DNA Sequencing and Oligonucleotide

Table 1. Oligonucleotide primers used in PCR reactions

Name	Oligonucleotide	Fragment coordinates (bp)	Reference
*cluA*F	5′-GAATCAACCCAAAGCCCTAC-3′	1372016–1369995	llmg_1398[a]
*cluA*R	5′-GGAGCGACAATCTTACCTTC-3′		
*prtP*F	5′-TTCAGCGGAAGCAACTGTGG-3′	50843–49949	LACR_C42[b]
*prtP*R	5′-TAAAGTGATCGCGGCTCAGG-3′		
*yhgE2*F	5′-CAGGTAGTGCAAATGATGG-3′	N/A	(Meyrand *et al.,* 2013)
*yhgE2*R	5′-TAGCGATACCGAATACAGC-3′		
MucBPF	5′-CGTCAACTGGTGCCAATATG-3′	2416894–2415037	llmg_2465[a]
MucBPR	5′-ACAATGATTCCCGCAGGAAC-3′		
dopUC19F	5′-GCGGATCCATTCGCTCGATTTCGAGTTG-3′	2414550–2417608	llmg_2465[a]
dopUC19R	5′-GCGGATCCACCATGGGAACCTTAACG-3′		

[a](Wegmann *et al.,* 2007) (GeneBank: AM40667.1) [b](Makarova *et al.,* 2006) (GenBank: CP000428.1). N/A not available

Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. Nucleotide sequences were compared with those referenced in the GenBank database using the online BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Nucleotide sequence analysis. Sequence similarity was detected with BLAST program. Signal peptides were predicted with SignalP 4.0 (Petersen *et al.,* 2011), sortase recognition sites with LPxTG-type motifs were predicted with the CW-PRED method (Litou *et al.,* 2008) (http://biophysics.biol.uoa.gr/CW-PRED/index.jsp) and MUB domains were predicted with a developed HMMs (Boekhorst *et al.,* 2005; 2006). Other domains in MucBP domain-containing proteins were identified using Pfam database (Punta *et al.,* 2012).

Nucleotide sequence accession number. The complete nucleotide sequence of the *mub* gene coding for the LPXTG-anchored mucus-binding protein (in this work also mentioned as the MucBP domain-containing protein) from IBB477 determined in this study has been deposited in the GeneBank under accession number JX845572.

RESULTS

Adhesion of *L. lactis* IBB477 to bare and PGM-coated polystyrene plates

As the *L. lactis* IBB477 strain persists in the GIT of germ-free mice (Boguslawska *et al.,* 2009) we speculated that it should display a strong adhering potential to the mucous layer of the GIT. The binding ability of IBB477 strain to PS was tested from early to late exponential growth phases, corresponding to $OD_{660\ nm}$ of 0.4, 0.7 and 1, and also in the post-exponential (stationary) growth phase (overnight culture) (Fig. 1). Irrespective of the growth phase the adhesion level was the same. Therefore, *L. lactis* cells from overnight cultures diluted to $OD_{660\ nm}$ of 1 were used in subsequent experiments.

Next, the microtiter plate method was used to quantitate the adhesive properties of IBB477 to PS used as a standard abiotic surface, and to PS+PGM. Adhesion was expressed as the optical density ($OD_{583\ nm}$) of stained cells (Fig. 2). As expected, the IBB477 strain adhered significantly better than the low-adhering MG1820 control strain, with the p-value < e^{-11} (95% CI = 0.54–0.76) and p-value < e^{-16} (95% CI = 0.077-0.080) for PS and PS+PGM, respectively. IBB477 showed about 2-fold better adherence compared with MG1820 strain to PS and ca. 9-fold better adherence to PS+PGM.

Searching for genetic determinants encoding adhesion and muco-adhesion capacity of *L. lactis* IBB477

The genes were selected for analysis based on the current knowledge pertinent to adhesion of bacteria from the genus *Lactococcus* (Giaouris *et al.,* 2009). Amplification of *cluA* and *yhgE2* genes gave the expected DNA products of 2022 bp and 813 bp only for positive controls, MG1363 (*cluA*$^+$) and TIL448 (*yhgE2*$^+$), which suggested that these genes are absent in the IBB477 genome. The PCR reaction with the *prtP*F and *prtP*R primers resulted in the generation of DNA products of 895 bp for the control strain J60011 (*prtP*$^+$) as well as for IBB477. The PCR product obtained for IBB477 was positively verified as *prtP* by DNA sequencing and comparison of the sequence against the NCBI nucleotide database.

In addition, amplification of the gene coding for the MucBP domain-containing protein resulted in the formation of the expected PCR product of 1858 bp for the control strain MG1363. However, for IBB477 this DNA product was approximately 500 bp larger. Its sequence analysis confirmed amplification of a gene coding for a protein containing MucBP domains. The complete sequence of this gene was determined with the aid of the Deletion Kit for Kilo-sequencing. The amino acid (aa) sequence was subsequently analyzed and compared with aa sequences of corresponding genes or pseudo-genes in sequenced lactococcal genomes (Table 2). It appears that the protein encoded by the gene of 3240 bp from the IBB477 genome contains four MucBP domains as well as four partly overlapping but larger MUB domains, postulated to be present only in LAB (Boekhorst *et al.,* 2006). Furthermore, other structures such as an LPxTG-type motif, C-terminal anchor and Gram-positive anchor, predicted to be involved in binding to extracellular components, were identified in this protein as well (Table 2). The complete nucleotide sequence of the gene has been deposited in the GeneBank as *mub* gene coding for the LPXTG-anchored mucus-binding protein.

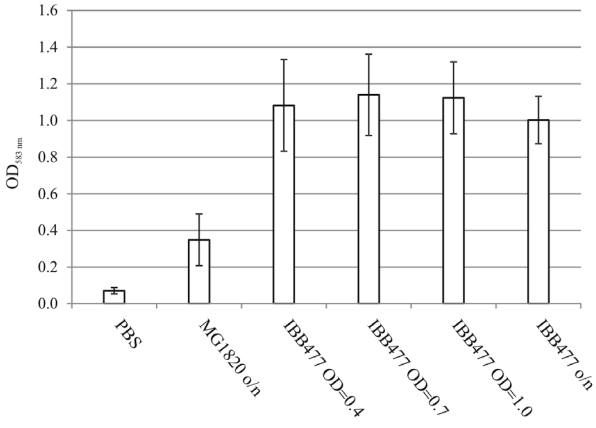

Figure 1. Adhesion of IBB477 at different growth phases.
Adhesion to bare polystyrene (PS) under static conditions was measured for bacteria from cultures at different growth phases. Adhesion is expressed as optical density ($OD_{583\ nm}$) of stained cells. For each condition, mean ± standard deviation from three independent experiments is shown.

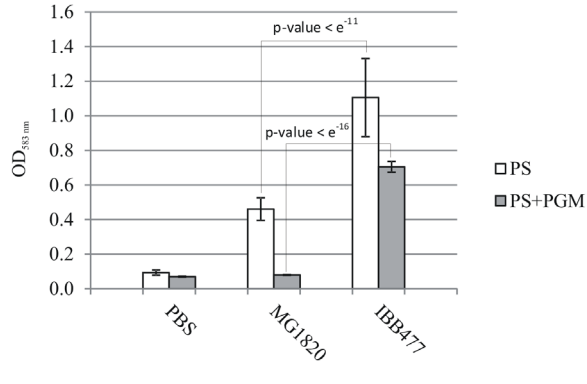

Figure 2. Adhesion of MG1820 and IBB477 to bare polystyrene (PS) and PGM-coated polystyrene (PS+PGM).
Adhesion was measured under static conditions, using the microtiter plate method. Means ± standard deviations from three independent experiments are shown. The p-values were calculated using Welch *t*-test.

Table 2. Domains and cell wall sorting signals of lactococcal MucBP domain-containing proteins

Strain	Locus	Length (aa)	SP	LPxTG	MucBP	MUB	C-term. anchor	Gram pos. anchor
L. lactis subsp. *lactis*								
IL1403	L39650	926	-	+	4	4	1/0	1
KF147	LLKF_2426	759	-	+	2	2	1	1
CV56	pseudo_CVCAS_2209	578	-	-	1	0	1	0
IO-1	lilo_2152	933	-	+	3	3	1	1
L. lactis subsp. *cremoris*								
MG1363	llmg_2465	925	-	+	3	3	1	1
SK11	pseudo_LACR_2488	502	-	-	2	2	1	0
NZ9000	LLNZ_12740	925	-	+	3	3	1	1
A76	llh_12650	721	-	+	3	3	1	0/1
IBB477	JX845572	1079	-	+	4	4	1	1

SP, signal peptide; LPxTG, sortase recognition site; MucBP, MucBP domain; MUB, MUB domain; C-term. anchor, C-terminal anchor; Gram pos. anchor, Gram-positive anchor

DISCUSSION

Adhesion to mucosa is essential for the bacterial persistence in the host and may promote beneficial health effects such as stimulation of the innate immune system to generate effective adaptive immunity in the case of vaccines against infections. Thus a candidate lactic acid bacterium for influenza antigen delivery through the GIT should display adhering properties. The *L. lactis* IBB477 strain was selected due to its unexpected ability to persist in the GIT of germ-free rats (Boguslawska *et al.,* 2009) indicating that it should possess adhesive properties. We focused on the interaction between *L. lactis* and mucins. To this end, the microtiter plate method, aiming at fast screening of adhesive properties on a large panel of strains, was first optimized and then used to analyze adhesion of IBB477 to bare and PGM-coated polystyrene plates. Under both conditions the adhesion of IBB477 was much higher than that observed for the low-adherent MG1820; however, the difference was more pronounced on PS+PGM (ca. 9-fold), than on PS (ca. 2-fold).

Next we focused on potential genetic determinants involved in adhesion/muco-adhesion of *L. lactis* IBB477. To this end, we selected for PCR amplification all the genes coding for surface proteins, comprising adhesins and aggregation proteins that have been described to date in *L. lactis*. We found that the *cluA* and *yhgE2* were absent in the IBB477 genome, while the *prtP* gene, coding for a serine proteinase shown to enhance cell surface hydrophobicity and mediate adhesion to solid surfaces, was present (Habimana *et al.,* 2007). This gene has been found in only a few strains out of the 22 sequenced LAB (Siezen *et al.,* 2005; Liu *et al.,* 2010). The presence of PrtP was also explored using pangenome comparative genome hybridization analysis in 39 *L. lactis* strains (Liu *et al.,* 2010). According to the authors, *prtP* is mainly present in *L. lactis* subsp. *cremoris*, although several *L. lactis* subsp. *lactis* strains may also harbour this gene. This statement corroborates the taxonomic position of IBB477 that has been identified as *L. lactis* subsp. *cremoris*. Furthermore, we revealed the presence of a gene coding for a MucBP domain-containing protein in the genome of IBB477, which was named *mub* gene coding for the LPXTG-anchored mucus-binding protein. The nucleotide sequence analysis demonstrated that this gene is longer than that found in other *L. lactis* subsp. *cremoris* genomes. Indeed, this gene from IBB477 contains four MucBP and four partly overlapping but larger MUB domains, which are postulated to play an important role in the adherence to mucus. It is proposed that each additional copy tends to increase the cell affinity for mucins (Boekhorst *et al.,* 2006). Furthermore, bioinformatics analysis showed that the protein encoded by this gene contains an LPxTG anchoring motif, and C-terminal and Gram-positive anchors, all predicted to be involved in binding to extracellular components. The role in adhesion of the *prtP* and *mub* genes in IBB477 will be further analyzed with knockout mutants. The highest number of MUB and MucBP domains among the *L. lactis* subsp. *cremoris* strains sequenced so far seems likely to enhance the muco-adhesion capacity of IBB477 and its potential to induce a protective immune response when applied as an oral vaccine against avian flu.

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