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ORIGINAL ARTICLE

Expression of a leptospiral leucine-rich repeat protein using a food-grade vector in *Lactobacillus plantarum*, as a strategy for vaccine delivery

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Abstract

In this study, a first food-grade mucosal vaccine against leptospirosis was developed without the use of antibiotic resistance gene. This expression system is based on a food-grade host/vector system of *Lactobacillus plantarum* and a new vaccine candidate antigen, a leucine-rich repeat (LRR) protein of *Leptospira borgpetersenii*. The LRR of interest from serovar Sejroe is encoded by two overlapping genes and these genes were fused together by site-directed mutagenesis. The mutant gene thus obtained could be successfully expressed in this system as was shown by western blot analysis and liquid chromatography–mass spectrometry (LC–MS/MS) analysis. In addition, this analysis showed that the mutant LRR protein fused to a homologous signal peptide of *L. plantarum* could be exported to the cell surface as a result of the native LPXAG motif of the heterologous LRR protein, which presumably is responsible for anchoring the protein to the cell wall of *L. plantarum.* This new strategy could be an essential tool for further studies of leptospirosis mucosal vaccine delivery.

Keywords *Lactobacillus plantarum* · Leucine-rich repeat · Leptospiral protein · Food-grade expression system · Mucosal vaccine

Introduction

One of the most wide spread zoonosis worldwide is leptospirosis caused by pathogenic *Leptospira* species. It is considered to be a re-emerging disease which impacts public health globally. Commercial leptospiral vaccines based on induction of antibodies against leptospiral lipopolysaccharide (LPS) can protect only against homologous or closely related serovars because of the diversity in the LPS carbohydrate structures of *Leptospira*, while more than 260 serovars of these pathogenic spirochetes were identified (Adler and de la Peña 2010; Brown et al. 2003). Therefore, several potential alternative vaccine candidates for heterologous protection have been studied. The outer membrane proteins are highly conserved across pathogenic *Leptospira* species (Branger et al. 2001); hence, many component proteins of the leptospiral outer membrane, including lipoproteins (such as LipL32 and LipL41), the leptospiral immunoglobulin-like proteins (such as LigA and LigB) and porins (such as OmpL1), have been studied as target antigens to induce protective immunity (Branger et al. 2001; Deveson Lucas et al.

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2011; Haake et al. 1999; Palaniappan et al. 2006; Silva et al. 2007).

An interesting target of antigen candidates is the leucine-rich repeat (LRR). This structural motif is found in a number of proteins that appear to be involved in protein–protein interactions (Enkhbayar et al. 2004; Kobe and Kajava 2001). Previous studies have shown that the LRR protein has considerable potential as a vaccine candidate antigen. The LRR surface protein of *Streptococcus agalactiae* was tested as a vaccine antigen and shown to elicit protection against lethal challenge of virulent streptococci in mice (Seepersaud et al. 2005). An analysis of leptospiral LRR-containing genes has been performed, indicating that pathogenic *Leptospira* show a higher frequency of LRR genes than nonpathogenic species (Hniman and Prapong 2007). Furthermore, LRR proteins containing immunogenic epitopes were identified as well by Nitipan et al. (2013).

Since some pathogens, including *Leptospires*, enter the body via mucosal surfaces, using a mucosal vaccine to administer the antigen directly to the mucosal surface is the effective way to induce mucosal immunity instead of using conventional vaccine based on injection which lacks stimulation of local immunity. Gram-positive lactic acid bacteria (LAB) such as *Lactobacillus* species, which often carry the 'generally recognized as safe (GRAS)' status, are attractive for the development of mucosal vaccines. Several components of *Lactobacillus* spp., including their peptidoglycan, lipoteichoic acids, and bacterial oligodeoxynucleotides, elicit innate immune responses through interaction with molecular pattern recognition receptors of mammalian cells (Kim et al. 2006; Shimosato et al. 2005). Several strains of *Lactobacillus* have been studied for vaccine delivery, including *Lactobacillus casei, Lactobacillus acidophilus,* and *Lactobacillus plantarum* (del Rio et al. 2010; Mohamadzadeh et al. 2009; Shaw et al. 2000; Yigang and Yijing 2008).

In this study, we chose *L. plantarum* for reasons of survival in the gut, persistence in the host, and proven safety for consumption. Several strains of *L. plantarum* are marketed as probiotics, which confer various health benefits to humans (de Vries et al. 2006). *L. plantarum* is also considered a better agent for oral vaccination than *L. casei* or *Lactococcus lactis* (Cortes-Perez et al. 2007; Grangette et al. 2002; Shaw et al. 2000). Moreover, the complete genome sequence of *L. plantarum* WCFS1, a single colony isolate from *L. plantarum* NCIMB8826, which was originally isolated from human saliva, has been reported (Kleerebezem et al. 2003) and an inducible expression system for *Lactobacillus* hosts, namely the pSIP system, was developed and analyzed in some detail (Diep et al. 2009; Nguyen et al. 2011a, b; Sørvig et al. 2003, 2005). A food-grade system for the pSIP expression vector series was recently developed (Nguyen et al. 2011a, b). To construct a food-grade complementation-based system, the erythromycin antibiotic resistance gene (*erm*) was replaced by the alanine racemase gene (*alr*) as selection marker complementing the corresponding gene deletion in the chromosome of the host *L. plantarum* WCFS1. Thus, it is suitable for food-associated application.

The objective of the present study was to develop an expression system for leptospiral genes in nonpathogenic LAB using a food-grade system as a new strategy for leptospirosis vaccine delivery. Here, we describe the construction of recombinant *L. plantarum* expressing a leptospiral LRR antigen. Furthermore, the food-grade pSIP system containing an *L. plantarum* homologous signal peptide was also developed to direct the target antigen to the cell surface as well. These recombinant bacteria carrying the LRR antigen would be the first mucosal vaccine development against leptospirosis which is not a conventional needle-based vaccine and does not contain any antibiotic resistance gene.

Methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* MB2159 (D-alanine auxotroph) was used as cloning hosts, whereas *L. plantarum* TLG02 (WCFS1 derivative, D-alanine auxotroph) was used as hosts for expression. *E. coli* was grown in Luria–Bertani medium at 37 °C with shaking. *L. plantarum* was grown in deMan–Rogosa–Sharpe (MRS) medium at 30 °C in tightly capped flasks and without agitation, D-alanine (Sigma, St. Louis, MO, USA) was added at a final concentration of 200 µg/mL for negative control (*L. plantarum* TLG02 without vector).

Construction of leptospiral LRR protein expression vectors and transformation

Primers used in this study were purchased from VBC-Biotech Service, Austria (Table 2). To construct pSIP vectors containing the Sejroe LRR genes for *L. plantarum* expression, the plasmid KU_pET160_R21F_2012, carrying LRR of *Leptospira borgpetersenii* serovar Sejroe (NCBI accession number JN627495), was used as a template to amplify two overlapping genes, *KU_Sej_R21N_2012* gene (NCBI accession number JN627491) and *KU_Sej_R21C_2012* gene (NCBI accession number JN627492). For full-length LRR protein expression, site-directed mutagenesis of these LRR overlapping genes was performed from plasmid KU_pET160_R21F_2012 using the overlap extension PCR method (Ho et al. 1989). The R21_*Nco*I and R21N_Mut primers were used to create the N-terminal fragment (*KU_Sej_R21N_2012*), while the R21C_Mut and R21C_*Eco*RI_St primers were used to create the C-terminal fragment

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics[a]	Source/references
Strains		
E. coli MB2159	Host strain for cloning, D-alanine auxotroph	Nguyen et al. (2011a, b)
L. plantarum TLG02	Host strain for expression, WCFS1 derivative, D-alanine auxotroph	Nguyen et al. (2011a, b)
Plasmids		
KU_pET160_R21F_2012	pET160/GW/D-TOPO derivative, containing *KU_Sej_R21F_2012* from *Leptospira borgpetersenii* serovar Sejroe, Am^r	Nitipan (2013)
pSIP609	pSIP409 derivative, *erm* replaced by *alr*	Nguyen et al. (2011a, b)
pSIP609-KU_R21M_2012	pSIP609 derivative, *gusA* replaced by *KU_Sej_R21M_2012*	This work
pLp_2145sAmy	pSIP401 derivative, P_{sppA}, sp_{Lp_2145} fused to *amyA*, Em^r	Mathiesen et al. (2009)
pLp_2145sAlr-KU_R21M_2012	pLp_2145sAmy derivative, *erm* replaced by *alr, amyA* replaced by *KU_Sej_R21M_2012*	This work

[a]*alr* alanine racemase encoding gene, *erm* erythromycin resistance gene, *amyA* alpha-amylase encoding gene, Am^r, Em^r ampicillin and erythromycin resistance, respectively

Table 2 Primers used in this study

Primers	Sequences (5′→3′)[a]
R21_*Nco*I	GGCGCCATGGGAAAAAATGAATG (*Nco*I)
R21N_Mut	GGAAGTTTTGACAAAAGACCGAGATC
R21C_Mut	GATCTCGGTCTTTTGTCAAAACTTCC
R21C_*Eco*RI_St	CCGGGAATTCTTAAAATGAGATTTTCAC ATCG (*Eco*RI)
Fsig_*Pst*I	TATGCTGCAGCACGCCAAATGATC (*Pst*I)
R2145_*Sal*I	GTAAGTCGACGGCGCTAGCAT (*Sal*I)
R21F_*Sal*I	CGTTGTCGACCAGGGAAAAAATGAA (*Sal*I)

[a]The restriction sites are underlined

(*KU_Sej_R21C_2012*) during the first round of PCR. These fragments were mixed for the second round of PCR using primers R21_*Nco*I and R21C_*Eco*RI_St to create the mutant LRR gene, named *KU_Sej_R21M_2012* (Fig. 1a). The final fragment was digested with *Nco*I and *Eco*RI, and ligated into the ~ 6 kb *Nco*I–*Eco*RI digested fragment of plasmid pSIP609, yielding the pSIP609-KU_R21M_2012 plasmid for expression in *L. plantarum* (Fig. 1b).

To construct expression vectors carrying a homologous signal peptide of *L. plantarum*, a fragment containing the *L. plantarum* signal peptide *Lp_2145* fused to the promoter P_{sppA} was amplified from plasmid pLp_2145sAmy using the Fsig_*Pst*I and R2145_*Sal*I primers, and digested with *Pst*I and *Sal*I. pSIP609-KU_R21M_2012 was used as a template for PCR amplification of the *KU_Sej_R21M_2012* fragment using the R21F_*Sal*I and R21C_*Eco*RI_St primers. The gene fragment was digested with *Sal*I and *Eco*RI. The signal peptide and gene fragments were then ligated into a ~ 5.5 kb *Pst*I–*Eco*RI digested fragment of pSIP609, resulting in pLp_2145sAlr-KU_R21M_2012 (Fig. 1b). The correct nucleotide sequence of all inserts was verified by DNA sequencing. All pSIP vectors were constructed in *E. coli* MB2159 before electroporation into *L. plantarum* TLG02 according to the method described previously (Josson et al. 1989).

The nucleotide sequence of *KU_Sej_R21M_2012* was translated into the amino acid sequence using a web-based tool, ExPASy Proteomics Server (http://web.expasy.org/translate/) and was compared with the LRR of *Lp. borgpetersenii* serovar Hardjo-bovis strain JB197 using a web-based tool, Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The potential T cell and B cell epitopes were predicted by MHCPred (https://www.jener.ac.uk/MHCPred) and antigenic (http://emboss.bioinformatics.nl/cgi-bin/emboss/antigenic) programs, respectively.

Expression of mutant LRR protein in *L. plantarum*

Overnight culture of *L. plantarum* carrying the respective plasmids was diluted in fresh prewarmed MRS medium to an OD_{600} of ~ 0.1. Then, 50 mL of bacterial culture was incubated at 30 °C without shaking until the absorbance reached an OD_{600} of ~ 0.3. The cells were induced by adding 50 ng/mL of the 19-residue peptide pheromone (IP) IP-673, the specific inducer for an inducible promoter P_{sppA} of *Lactobacillus* sp., and harvested at OD_{600} of ~ 6–7 by centrifugation at 3200*g* for 15 min at 4 °C.

Cell extract preparation and western blot analysis

The supernatant was collected and concentrated 50 times to examine secreted proteins by centrifugal filters. The cell pellet was washed once with LEW buffer (50 mM of sodium phosphate, 300 mM of sodium chloride, pH 8.0) and resuspended in 10 mL of the same buffer. The cells were disrupted by glass beads using a Precellys 24 glass bead mill (PEQLAB Biotechnologie GmbH, Germany).

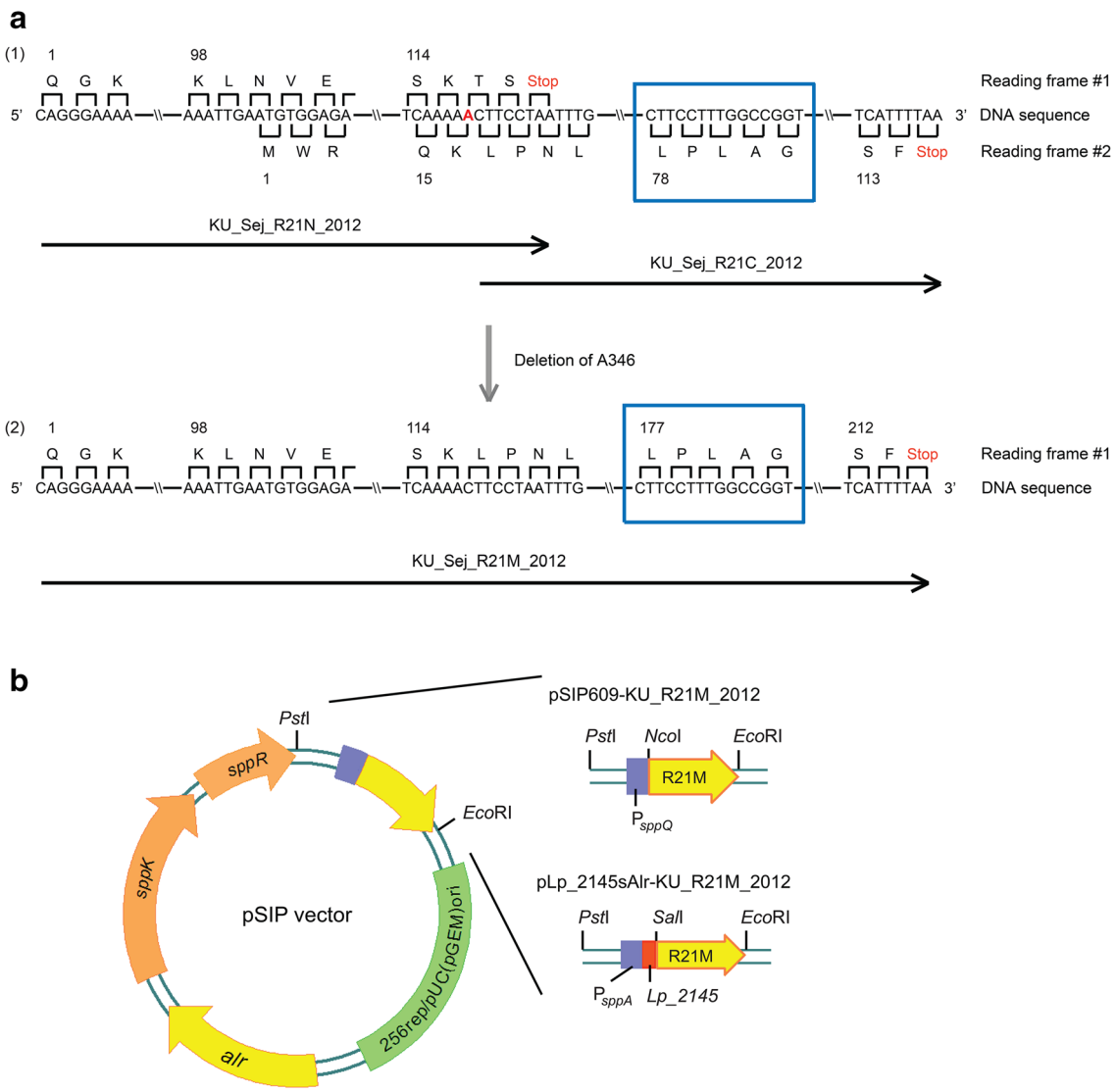

Fig. 1 Schematic diagram of DNA sequences (**a**) and expression vectors (**b**). DNA sequences and reading frames of the *Lp. borgpetersenii* serovar Sejroe LRR genes before (1) and after (2) a single base-pair deletion at A346 (marked by bold red A). The LPXTG motif (LPXAG) is marked by the blue box. The black arrows represent the gene fragments. Expression vectors for the full-length LRR protein, using the alanine racemase (*alr*) gene as selection marker. *SppK* and *sppR*, histidine kinase and response regulator, respectively; 256rep/pUC(pGEM)ori, replication determinant. The structural gene (*KU_Sej_R21M_2012* gene of leptospiral LRR, indicated by 'R21M') is fused to the inducible promoter P_{sppQ} in the pSIP609-KU_R21M_2012 vector (6654 bp) and fused to P_{sppA} with a signal peptide of *L. plantarum*, *Lp_2145*, in the pLp_2145sAlr-KU_R21M_2012 vector (6755 bp)

For total protein extraction, 1 mL of the whole cell lysate was heated with 1 mL of 2 × SDS-PAGE sample buffer (0.2 M dithiothreitol, 4% SDS, 0.1 M Tris, pH 6.8, 20% glycerol and 0.2% bromophenyl blue) at 95 °C for 5 min, and cell debris was removed by centrifugation at 16,000*g* for 5 min at 4 °C to obtain the total protein extract.

To separate insoluble proteins, cytosolic proteins and surface proteins, the remaining whole cell lysate was centrifuged at 16,000*g* for 5 min at 4 °C. The pellet was collected for insoluble proteins extraction, while supernatant was kept for soluble protein extraction. The pellet for insoluble proteins extraction was washed once with LEW buffer and resuspended in 2 mL denaturing solubilization buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 8 M urea, pH 8.0) per g wet cells, stirred on ice for 60 min and centrifuged at 16,000*g* for 5 min at 20 °C to remove cell debris. The supernatant was kept at 4 °C as an insoluble protein extract.

Then, the soluble protein fraction was centrifuged at 80,000*g* for 90 min at 4 °C using a Beckman L-70 Ultracentrifuge to separate cytosolic proteins and surface proteins. The supernatant was collected as cytosolic proteins extract, whereas the pellet was resuspended in LEW buffer

and collected as surface protein extract. Protein concentration was determined using the Bradford protein assay with bovine serum albumin (BSA) as standard.

Each protein extract was mixed with SDS-PAGE sample buffer and denatured at 95 °C for 5 min. Twelve micrograms of the surface proteins and 15 µg of the proteins from other extracts were applied to the individual lanes of SDS-PAGE gels. SDS-PAGE was carried out on 14% acrylamide gels and the protein bands were transferred electrophoretically onto nitrocellulose membranes for western blot using a Bio-Rad electrophoresis unit. Rabbit hyperimmune serum against the KU_Sej_LRR_2012N protein (1:5000 dilution) was used as primary antibody for the blot. The secondary antibody was a peroxidase-conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) at a dilution of 1:10,000. Blot was developed using the Bio-Rad Clarity™ western ECL substrate kit, according to the manufacturer's instructions.

To acquire rabbit hyperimmune serum as primary antibody, the gel fragment approach was used for immunization (Harlow and Lane 1988). Briefly, the gel slice containing the KU_Sej_LRR_2012N protein from the previous study (Nitipan 2013) was rinsed in deionized water for a few minutes and was lyophilized for 48 h. Then, the dried gel slice was moved to a mortar and was ground into a fine powder. A volume of PBS equal to one-half of the original gel volume was added and incubated at room temperature for 1 h. Complete Freund's adjuvant (FCA) and incomplete Freund's adjuvant (FIA) were mixed with an equal volume of the protein antigen for the primary injection and all boosts, respectively. A New Zealand White rabbit received four 1.2 mL doses of the sample containing 0.6 mg protein antigen on days 0, 14, 176 and 363. Rabbit serum was collected twice a month. Animal experiments were approved by the Kasetsart University Institutional Animal Care and Use Committee (Kasetsart University-IACUC). To calculate antibody titer, the serum sample was diluted in serial ratios and tested for the presence of detectable levels of antibody. The titer value was indicative of the last dilution in which the antibody was detected. The serum with a high titer of more than 12,800 was collected and absorbed with *E. coli* BL21 (DE3) acetone powder before use as primary antibody.

Flow cytometric analysis

A million cells of *L. plantarum* carrying pLp_2145sAlr-KU_R21M_2012 were incubated with rabbit hyperimmune serum against KU_Sej_LRR_2012N protein at a dilution of 1:600 and were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (AbD Serotec) at a dilution of 1:600 as secondary antibody. At least 100,000 events were analyzed using the CytoFLEX flow cytometer running with CytExpert software (Beckman Coulter). Negative controls were *L. plantarum* cells harboring plasmid and without plasmid, which were stained with only FITC-conjugated goat anti-rabbit IgG and with both rabbit hyperimmune serum and FITC-conjugated goat anti-rabbit IgG, respectively. Fluorescent signals were represented by histograms.

Liquid chromatography–mass spectrometry analysis (LC–MS/MS)

The gel slices containing the KU_Sej_LRR_2012M protein from gel electrophoresis were subjected to in-gel digestion and dehydrated twice by 100% acetonitrile (ACN). Sulfhydryl bonds were reduced by 10 mM tributylphosphine (TBP) in 10 mM ammonium bicarbonate for 1 h at 60 °C. Then, sulfhydryl groups were alkylated with 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate and left in the dark for 45 min at room temperature. The gel slices were dehydrated twice again and digested by adding 100–200 ng of sequencing grade trypsin. The gel slices were incubated for 1 h at 4 °C followed by 37 °C overnight. The digested peptide fragments were extracted by adding 30 µL of 50% ACN in 0.1% formic acid and incubated for 10 min at room temperature with shaking. The peptide fragments were dried at 44 °C and protonated with 0.1% formic acid before injection into a NanoAcquity system. The peptide fragments were analyzed using an Ultimate3000 Nano/Capillary LC System (Dionex, UK) coupled to a hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics GmbH, Germany) with a nano-captive spray ion source. Sample (500 nL) was loaded into the trapping column (Thermo Scientific), using full loop injection, and resolved on an analytical column (PepSwift Monolithic Nano Column) at a column temperature of 60 °C. A linear gradient method was used to elute peptides into the mass spectrometer at a constant flow rate of 1 µL/min of the mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in 80% ACN). Electrospray ionization was carried out at 1.6 kV using the CaptiveSpray. Mass spectra were acquired in the positive-ion mode over the range m/z 150–2200 (Compass 1.9 for otofSeries software, Bruker Daltonics) and the MS/MS data were analyzed using MASCOT software (Matrix Science, UK) with the target protein database. Ion score was $-10 \times \mathrm{Log}(P)$, where P was the probability that the observed match was a random event. Protein score was derived from ion scores as a non-probabilistic basis for ranking protein hits. The peptide sequences from mass spectrometry were aligned with the KU_Sej_LRR_2012M sequence using a web-based tool, Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

Leptospiral LRR gene expression in *L. plantarum* with the food-grade pSIP system after fusion of the two overlapping genes

Since the LRR protein of interest of *Lp. borgpetersenii* serovar Sejroe is encoded by two overlapping genes, *KU_Sej_R21N_2012* (NCBI accession number JN627491) and *KU_Sej_R21C_2012* (NCBI accession number JN627492), we decided to fuse the two overlapping genes of the Sejroe LRR for full-length LRR protein expression. PCR site-directed mutagenesis was performed for a single base-pair deletion (A346), resulting in *KU_Sej_R21M_2012,* which consists of both the *KU_Sej_R21N_2012* and the *KU_Sej_R21C_2012* genes in frame (Fig. 1a). The recombinant pSIP vector containing *KU_Sej_R21M_2012* was constructed and named pSIP609-KU_R21M_2012 (Fig. 1b).

From in silico identification, an amino acid sequence of the mutant LRR protein of Sejroe was compared to that of the LRR of serovar Hardjo-bovis strain JB197 (LBJ_2012 gene, NCBI accession number ABJ76523) and showed 85% similarity (Fig. 2). This fused protein contained sequence encoding each of the four target epitopes for T cell and B cell. In cell-mediated immunity (CMI) system, T cell activation requires the antigen to bind to major histocompatibility complex (MHC) molecules. According to this prediction, the result showed epitopes for HLA-A0101, -A3101 and -A0203 alleles as MHC class I and for HLA-DRB0401 allele as MHC class II. For humoral immunity (HI), B cell epitope is the antigen portion recognized by B cells and binds to the immunoglobulin. The scores of predicted epitopes were 1.121, 1.137, 1.145 and 1.140, respectively (Fig. 2). The epitopes having more than 1.0 are potentially antigenic. These conserved epitopes were also found in serovar Ballum, Javanica and Mini (Nitipan 2013).

L. plantarum TLG02 was used as the host of the vector and analyzed after induction with IP-673. The formation of the mutant LRR protein in *L. plantarum* could be detected by western blot analysis (Fig. 3a). The target protein was apparently expressed in both soluble and insoluble forms.

LRR protein expression with an *L. plantarum* signal peptide

To develop an expression system for cell surface display of the LRR protein, we tested whether it is possible to secrete the recombinant mutated LRR protein. *KU_Sej_R21M_2012* was first fused to the signal peptide Lp_2145 of *L. plantarum* before ligation into the pSIP vector for *L. plantarum* expression, resulting in pLp_2145sAlr-KU_R21M_2012 (Fig. 1b). Western blot analysis of different samples obtained from induced *L. plantarum* cells showed target protein bands in several fractions, including insoluble protein, cytoplasmic protein and surface protein fractions (Fig. 3a). No mutant LRR protein was found in the culture supernatant (exhausted media).

Analysis of protein localization

Expression of the *KU_Sej_R21M_2012* gene as a surface-anchored protein on *L. plantarum* carrying

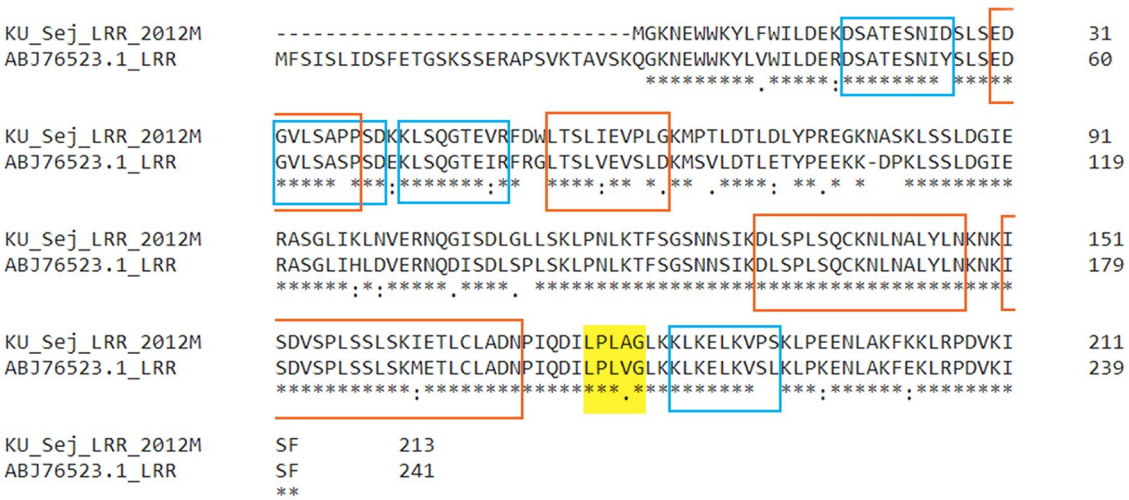

Fig. 2 Amino acid sequence alignment of the KU_Sej_LRR_2012M sequence with the LRR of *Lp. borgpetersenii* serovar Hardjo-bovis strain JB197. The LPXTG motif (LPXAG) is marked by the yellow box. The KU_Sej_LRR_2012M sequence encoding target epitopes for MHC HLA alleles and B cell is marked by blue and red boxes, respectively

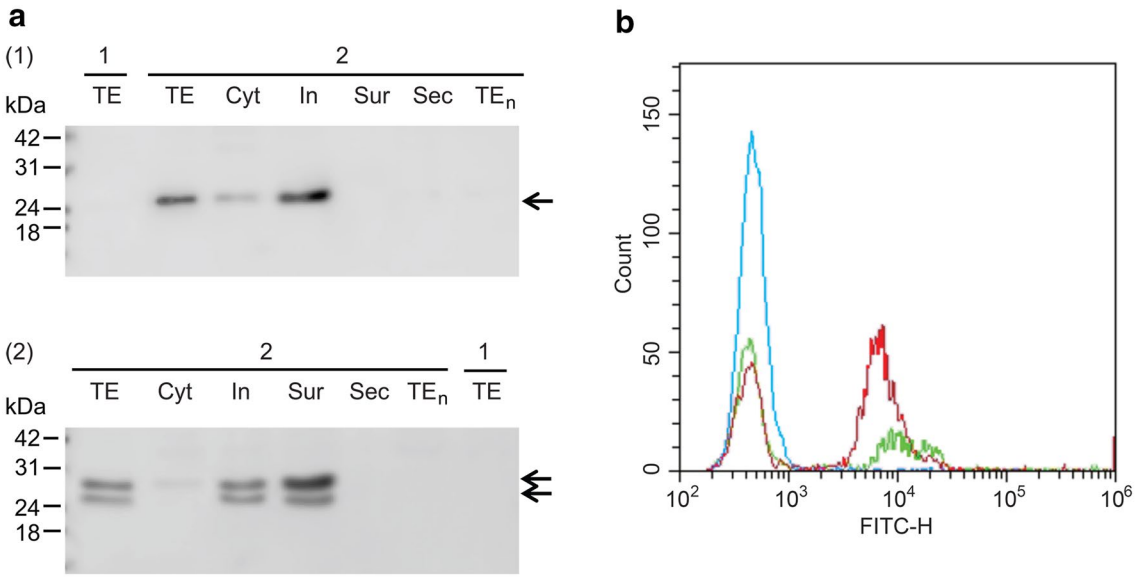

Fig. 3 Western blot (**a**) and flow cytometric analysis (**b**) of KU_Sej_LRR_2012M protein. The target protein from *L. plantarum* transformants containing pSIP609-KU_R21M_2012 (1) and pLp_2145sAlr-KU_R21M_2012 (2) plasmids was detected using rabbit hyperimmune serum against KU_Sej_LRR_2012N protein. *Cyt* cytoplasmic protein, *In* insoluble protein, *Sur* surface protein, *Sec* secreted protein fraction, *TE* total protein extract, TE_n total protein extract of uninduced cells, *lane* 1 negative control (*L. plantarum* without vector), *lane* 2 recombinant *L. plantarum*. All protein fractions were loaded at 15 µg/lane, except for the surface fractions which were loaded at 12 µg/lane. Panel 1: the arrow indicates the mass of the target protein (23.5 kDa). Panel 2: the arrows indicate the mass of the mutated LRR protein fused to the Lp_2145 signal peptide, with 27.5 kDa and 24 kDa for the unprocessed and processed proteins, respectively. Lactobacilli were gated by forward and side scatter and surface-anchored expression of the LRR protein was analyzed as histogram. The fluorescent signals from *L. plantarum* harboring the pLp_2145sAlr-KU_R21M_2012 plasmid and stained with both antibodies, rabbit hyperimmune serum against KU_Sej_LRR_2012N protein and FITC-conjugated goat anti-rabbit IgG (red line), *L. plantarum* harboring the pLp_2145sAlr-KU_R21M_2012 plasmid and stained with only FITC-conjugated goat anti-rabbit IgG (blue line), and *L. plantarum* without plasmid with both antibodies (green line) are shown

pLp_2145sAlr-KU_R21M_2012 was demonstrated by flow cytometry as shown in Fig. 3b. The signals from the cell surface-anchored protein, KU_Sej_LRR_2012M fused with the signal peptide Lp_2154, on the recombinant *Lactobacillus* cells were significantly higher than those of the control (*L. plantarum* without plasmid). Therefore, the results of flow cytometric analysis strongly suggest that the KU_Sej_LRR_2012M protein is both attached to the cell wall of *L. plantarum* and can be accessed by the antibodies used.

Protein identification by LC–MS/MS analysis

To provide additional evidence of the successful target gene expression and confirm the western blot results, the protein band proposed as the KU_Sej_LRR_2012M protein (23.5 kDa) was analyzed by LC–MS/MS. After comparison of the MS/MS data with the protein sequence database of KU_Sej_LRR_2012M, a Mascot protein score of 1466 was obtained (Fig. 4a). The Mascot protein score is a statistical score that represents the similarity between the experimental data and the database sequence. The higher value shows the higher level of similarity. The threshold of acceptable similarity at 95% confidence level is above a score of 90. Since our result showed a Mascot protein score of 1466, it is very likely that the expressed protein is the KU_Sej_LRR_2012M protein.

Furthermore, 11 peptide matches were found (Table 3) showing 65.7% of sequence coverage (Fig. 4b). This analysis corroborates that the expressed protein recognized by the rabbit hyperimmune serum is indeed the LRR protein.

Discussion

In this study, we expressed the leptospiral LRR in *L. plantarum* aiming at the development of a mucosal vaccine candidate against leptospirosis. The LRR of *Lp. borgpetersenii* serovar Sejroe is of interest for vaccine candidates as it would be a surface protein and an immunoreactive antigen. The LRR from one of these two overlapping genes, *KU_Sej_R21N_2012* (NCBI accession number JN627491), showed immunoreactivity to rabbit hyperimmune serum anti-Sejroe, *Lp. borgpetersenii* and anti-Ballico, *Lp. interrogans* antibodies provided by the National Institute of Animal Health, Department of Livestock Development, Thailand, whereas the latter, the LRR from *KU_Sej_R21C_2012*

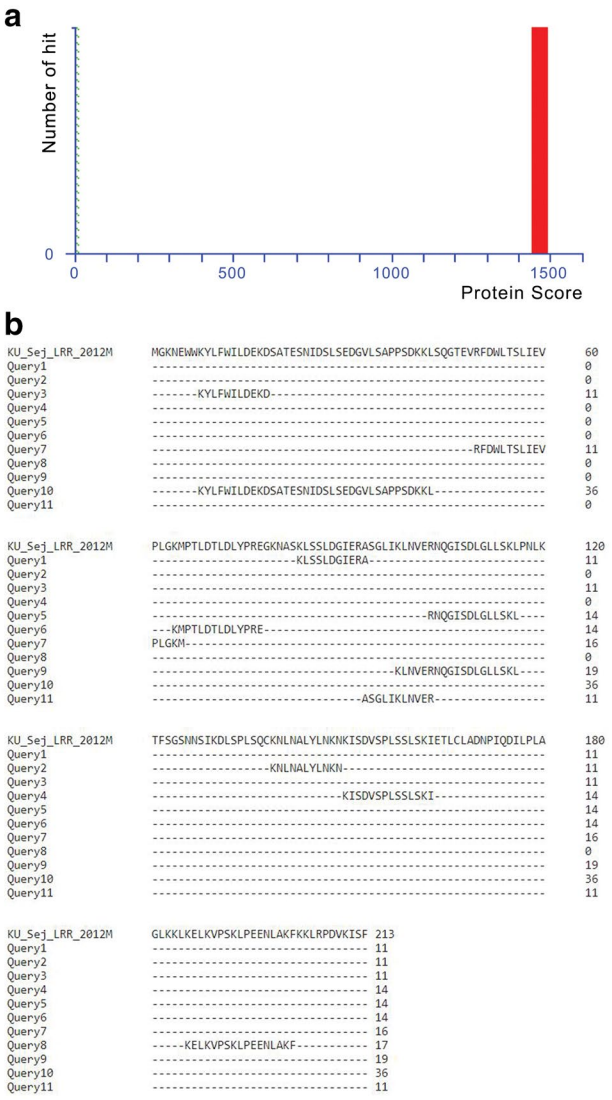

Fig. 4 Mascot score histogram (**a**) and alignment of peptide sequences (**b**) from LC–MS/MS analysis of the protein band at 23.5 kDa expected as the KU_Sej_LRR_2012M protein using in-gel trypsin digestion method

(NCBI accession number JN627492), was detected with rabbit hyperimmune serum anti-Javanica and anti-Ballico, *Lp. borgpetersenii* (Nitipan 2013). Since we would like to express both of them in lactic acid bacteria, similar observations have been made for other heteromeric proteins such as for the β-galactosidase from *Lactobacillus sakei*. This enzyme is encoded by two overlapping genes (*LacLM*). The active β-galactosidase could not be produced when only expressing one of the two subunits (Obst et al. 1995). Moreover, the full-length LRR protein of another serovar, *Lp. borgpetersenii* serovar Hardjo-bovis strain JBL197, is encoded by only one gene (LBJ_2012 gene, NCBI accession number ABJ76523). The LRR gene sequence of this serovar is quite similar to that of serovar Sejroe (91.4%),

Table 3 Peptide matches from the protein band at 23.5 kDa identified by mass spectrometry

Peptide sequences from Mascot	Ion score[a]	Number of queries
KLSSLDGIERA	54–69	3
KNLNALYLNKN	37	2
KYLFWILDEKD	38	2
KISDVSPLSSLSKI	25–26	2
RNQGISDLGLLSKL	17–54	9
KMPTLDTLDLYPRE	77–88	6
RFDWLTSLIEVPLGKM	53–102	6
KELKVPSKLPEENLAKF	42	1
KLNVERNQGISDLGLLSKL	31–36	2
KYLFWILDEKDSATESNIDSLSEDGVLSAPPSDKKL	83	1
ASGLIKLNVER	5[b]	1

[a]Individual ion scores > 13 indicate identity or extensive homology ($P < 0.05$)

[b]Peptide match is not assigned to protein hits

except that the complete LBJ_2012 gene is in one reading frame and does not separate into two overlapping genes. In other serovars, LRR amino acid sequence of serovar Pomona (LEP1GSC133_2670, NCBI accession number EMO64909) is encoded by one gene same as serovar Hardjo-bovis (98% peptide identity with Hardjo-bovis). However, two overlapping genes of LRR were also found in serovar Ballum and Javanica (Nitipan 2013). Based on the information given above, we decided to fuse the two overlapping LRR genes, *KU_Sej_R21N_2012* and *KU_Sej_R21C_2012*, obtaining the mutated gene *KU_Sej_R21M_2012*.

The corresponding protein was detected via western blot analysis, indicating that this mutant LRR protein derived from the fused genes was expressed as both soluble and insoluble forms in *L. plantarum*. The insoluble LRR protein may cause a problem for in vivo experiments due to the pathogenic misfolded proteins (for example, amyloid-β plaques). However, in some cases, the insoluble proteins do not cause disease (Trimpin and Brizzard 2009). Further experiments on its property would be necessary. As an in-house specific antibody was used in this study, it seemed necessary to further confirm that the expressed protein was indeed the target LRR protein by determination of the amino acid sequence of the expressed protein using LC–MS/MS analysis. Identified peptides were accepted with a total ion score of over 30 and more than five peptide matches. The data obtained by LC–MS/MS method corroborate the result obtained from western blot analysis. Therefore, the mutated gene *KU_Sej_R21M_2012* was successfully expressed in the lactobacillal host and the in-house rabbit hyperimmune serum can be used to recognize the target protein.

Previous studies on vaccine development reported that an antigen displayed on the surface of LAB can induce mucosal and systemic immune responses (del Rio et al. 2008; Maassen et al. 1999; Medaglini et al. 1995). In addition, membrane-associated leptospiral antigens could induce significant protection in hamsters challenged with *Leptospira*, while non-membrane-associated forms of them were not protective. This suggests that the system, in which the antigens associate with the cell surface, is important for the induction of a protective immune response (Haake et al. 1999). Our LRR protein contains the pentapeptide LPXAG, which has a similar function as the LPXTG motif, a cell wall anchor recognized by sortase (Roche et al. 2003). Various signal peptides of *L. plantarum* WCFS1 have been analyzed with respect to protein secretion and were shown to export different heterologous proteins to the cell wall or the culture medium (Fredriksen et al. 2010; Mathiesen et al. 2008, 2009). Lp_2145 is one of the signal peptides of *L. plantarum* that was found to be very effective in these previous studies and we fused this signal peptide to the N-terminus of the mutant LRR gene. After expression the target protein was, however, not detected as a secreted soluble protein in the culture supernatant, but we found a strong signal for the LRR protein associated with the cell surface fraction of *L. plantarum*. Presumably, the native LPXAG motif in KU_Sej_LRR_2012M resulted in anchoring of the recombinant protein on the cell wall of the expression host. Our data thus support the finding that a homologous signal peptide can be useful in food-grade systems to direct a heterologous protein to the Sec translocation machinery. Normally, Sec machinery recognizes the signal peptide on the target protein, cleaves the signal peptide off and exports the secreted protein outside the cell. In this step, our protein was not secreted due to the presence of a hydrophobic region terminated by a positively charged tail at the C-terminus. As sortase anchoring system in Gram-positive bacteria, the protein remains embedded in the membrane because of its tail. Then, the enzyme cleaves the target protein between the glycine and alanine residues of LPXAG. The residue links to the peptide cross bridge in the peptidoglycan of the cell surface. Therefore, the LRR protein from *Lp. borgpetersenii*, Gram-negative bacteria, could be anchored to the cell surface of *L. plantarum* by its own, native sequence LPXAG.

Normally, leptospiral vaccine has been developed based on conventional injection so far, which can induce only systemic immunity and lack stimulation of local immunity. The effective way to induce both local and systemic immunity is the mucosal vaccine system which recombinant bacteria carrying the antigen are administered directly to the body via needle-free oral injection. This study showed the first construction of mucosal vaccine against leptospirosis. In addition, *Lactobacillus* spp. has been used for mucosal vaccines against several diseases. However, most of them used an antibiotic resistant gene as selection marker of the vector. In this study, we used the food-grade complementation-based system instead of antibiotics to prevent the release of antibiotic resistant bacteria to the environment. Using the pSIP expression vectors with the novel mutant LRR gene described here, the protein of interest could be produced and anchored to the surface of recombinant *L. plantarum* by the anchoring domain of leptospiral native motif. The new method of leptospiral antigen production using the food-grade expression system of LAB could be a first and important step in the development of a mucosal vaccine against leptospirosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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