Construction of the Recombinant Probiotic *Lactobacillus casei* and *Lactobacillus fermentum* Expressing the Codon-Optimized M2e: HBc Fusion Gene

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Objective: *To construct the recombinant Lactobacillus casei and Lactobacillus fermentum for the expression of codon-optimized M2e: HBc fusion gene.*
Material and Method: *The ectodomain of the Matrix 2 (M2e), a conserved gene of influenza viruses, was fused with the gene encoding hepatitis core protein (HBc) to generate M2e: HBc fusion gene. The M2e: HBc fusion gene was changed to obtain the optimized codon usage in L. casei. The codon-optimized M2e: HBc fusion gene was cloned into an E. coli/L. casei shuttle vector under the control of nisA-inducible promoter for the expression in L. casei EM116, and under the control of ldhL strong constitutive promoter for expression in two probiotic strains L. casei RCEID02 and L. fermentum RCEID01. The expression of M2e: HBc fusion gene in lactobacilli was determined by western blotting using a specific antibody against M2e.*
Results: *Codon optimization of the M2e: HBc fusion gene in L. casei was performed on 14 rare codons, consisting of 12 AGA and 2 AGG. The optimized M2e: HBc fusion gene with the size of 638 bp was successfully generated. Two recombinant expression plasmids based on nisA inducible- and ldhL constitutive-promoter were successfully constructed and electro transformed into lactobacilli. Western blotting analysis revealed that the codon-optimized M2e: HBc fusion gene under the control by those two bacterial promoters was successfully expressed in Lactobacillus species.*
Conclusion: *In this study, the codon optimization strategy can be used for the expression of M2e: HBc fusion gene in two lactobacilli prototypes, L. casei and L. fermentum. The expression of the M2e: HBc fusion gene in both lactobacilli would provide the opportunity to apply these two bacteria as an alternative broad-spectrum vaccine candidate for influenza A virus.*

Keywords: *Lactic acid bacteria, Lactobacillus casei, Lactobacillus fermentum, Ectodomain Matrix 2 protein, Influenza virus, Heterologous protein expression*

J Med Assoc Thai 2016; 99 (Suppl. 9): S9-S18
Full text. e-Journal: *http://www.jmatonline.com*

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore-forming bacteria that can be found in a variety of environments including many living organisms. LAB have been widely used in food, such as cheese and yogurt, due to their ability to ferment a range of raw materials and because they are safe for both humans and animals to consume. Several species of LAB are important members of the human microbiota associated with various mucosal parts of the human body. The human gastrointestinal tract comprises a complex microbiota with lactobacilli and streptococci in the ileum and jejunum at 10^3-10^5 organisms per gram of luminal content and at 10^6-10^8 per gram in the colon. Most of LAB are probiotics which are defined as live microorganisms that confer health benefits on the host when administered in adequate amounts[1]. Many LAB with probiotic properties have been isolated from human and animal intestines[2,3]. There is now increasing interest in manipulating LAB as mucosal delivery vehicles for therapeutic and prophylactic molecules. It was found that a mucosal delivery system for vaccine antigens is required to minimize degradation and promote uptake of the antigen in the gastro-intestinal tract, and effectively stimulate the adaptive

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immune response, as opposed to tolerance responses seen in cases in which soluble antigens are administered[4]. There are a number of advantages in using LAB for this purpose, such as the fact that they are generally regarded as safe (GRAS)[5], are resistant to acid and bile salt (allowing them to survive in the gastrointestinal tract after oral or nasal administration), are able to elicit both mucosal and systemic immunity[4,5] and can be engineered to express several heterologous proteins. LAB, especially *Lactobacillus* and *Lactococcus* species, have been successfully engineered to express pathogenic antigens and serve as live oral vaccines against various infectious diseases[6].

Influenza, the respiratory disease caused by human influenza viruses, is a major health problem throughout the world. Currently available influenza vaccines can elicit neutralizing antibodies against viral hemagglutin in (HA) and neuraminidase (NA)[7], which can confer only subtype-specific protective immunity. This is a major drawback, as influenza undergoes continuous antigenic change, making continuous influenza surveillance necessary in order to update the influenza vaccine against the circulating subtype[8]. For this reason, there is a need to develop an alternative universal influenza vaccine containing conserved viral component(s) for stimulating broad-spectrum immunity against all influenza subtypes. The extracellular domain of the Matrix 2 (M2e) protein is one of the most conserved influenza components among the different influenza subtypes. Due to its small size and low immunogenicity, M2e is often fused with a larger carrier to enhance anti-M2e immune protection in vaccine experiments[9,10]. One attractive carrier for fusion with M2e is hepatitis B core antigen (HBc)[11]. However, the low expression level of heterologous proteins is a major disadvantage and might be a critical constraint in using LAB as an alternative vaccine. One factor is codon-usage bias in LAB, which was shown to be an important factor affecting the translational efficiency of heterologous protein[12]. This problem can be solved through modification of the coding sequence of the inserted gene according to the sequence frequently used by the bacterial host[13].

In this study, we aim to construct a recombinant *E. coli/L. casei* shuttle vector[14] containing codon-optimized M2e fused with the HBc gene (M2e: HBc) and engineer this construct in probiotic *L. casei* isolated from a chicken and in probiotic *L. fermentum* isolated from a healthy child for expression of the M2e: HBc fusion protein.

Material and Method

Bacterial strains and growth conditions

Table 1 lists bacterial strains, cloning vectors, and primers used in this study. *L. casei* and *L. fermentum* strains were cultured statically in de Man Rogosa and Sharpe (MRS) medium (Difco, USA) at 37°C. Agarified media were prepared by adding 15 g/l of biological grade agar (Difco) to the corresponding broth. When necessary, erythromycin (2.5 µg/ml) antibiotics (Sigma-Aldrich, USA) were added to the media for the selection of transformants in the *Lactobacillus* species.

Isolation and purification of DNA

Plasmid DNA from the *Lactobacillus* species was isolated and purified as previously described by O'Sullivan and Klaenhammer[15]. Plasmid DNA from *E. coli* was isolated and purified with the Hi Yield Plasmid Mini Kit according to the manufacturer's instructions (RBC Bioscience, Taiwan). The PCR products and DNA fragments, embedded in agarose gels, were purified using the Hi Yield Gel PCR DNA Fragments Extraction Kit (RBC Bioscience).

Amplification of M2e: HBc: TT

The M2e: HBc: TT fusion gene fragment was amplified through *Pfu* DNA polymerase-based reaction using pnisA: M2e: HBc: TT (unpublished) as the template. The reaction mixture contained 10 mM KCl, 2 mM $MgCl_2$, 20 mMTris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 µg/ul BSA, 0.1 µM of each dNTP, 0.2 µM of each primer (pnisNdeI and M2e: HBcsynF1) and 2.5 units of *pfu* DNA polymerase. The PCR condition consisted of 94°C for 5 min followed by 30 cycles of 94°C for 30s, 60°C for 30 s, 68°C for 90s each and an additional 7 min at 68°C.

Electro-transformation

Preparation of competent cells and electro-transformation of *L. casei* and *L. fermentum* was performed as described by Chassy and Flickinger with slight modification[16]. A single colony of *L. casei* RCEID02 or *L. fermentum* RCEID01 on an MRS plate was inoculated in 10 ml of MRS broth supplemented with 1% glycine. After 16 h of incubation, the bacterial pellet was collected using centrifugation at 13,000 xg for 5 min and was transferred into 100 mL of the pre-warmed MRS broth containing 1% glycine and incubated until the OD 600 reached 0.6. The bacterial cells were harvested, washed three times in cold sterilized deionized water and resuspended in ice-cold

Table 1. Bacterial strains, plasmids and oligonucleotide primers used in this study

Materials	Relevant properties	Source/reference
Bacteria		
Lactobacillus casei RCEID02	Plasmid-free strain of *L. casei* TISTR1341	RCEID[a], (14)
L. casei EM116	*L. casei* ATCC393 derivative containing a chromosome *nisRK* gene	(14)
L. fermentum 47-7	Human isolate	This study
L. fermentum RCEID01	Plasmid-free strain of *L. fermentum* 47-7	This study
Plasmid		
pIDTSMART-AMP-M2e: HBc	*Amp*, vector harboring synthetic M2e: HBc gene	IDT
pnisA-*Aat*II$^-$	pGEM-T-Easy vector containing *nis*A promoter, multiple cloning site (MCS), and the transcription terminator signals (TT)	RCEID
pnisA: M2e: HBc: TT	pnisA-*Aat* II containing M2e: HBc downstream of *nis*A promoter	This study
pRCEID-LC13.9 shuttle vector	*Amp*, *Ery*, *E. coli-L. casei* shuttle vector based on pRCEID13.9	RCEID[a], (14)
pLC13.9: nisA: M2e: HBc: TT	pRCEID-LC13.9 containg M2e: HBc gene downstream of *nis*A promoter	This study
pGEM: LdhL-PRO: GFPuv	*Amp*, plasmid containing *Ldh*L promoter and GFPuv gene	(14)
pLC13.9: LDH: PRO1: M2e: HBc: TT	pRCEID-LC13.9 containing M2e: HBc gene downstream of *Ldh*L promoter	This study
Oligonucleotide primers	Sequence (5'-3')	
pnisNdeI	attacatatgaagctcgcgttatcggtc (*Nde*I)	This study
M2e: HBcsynF1	aataggtaccaatggcctcacaaggca (*Kpn*I)	This study

[a] Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University.
Underlined nucleotides show introduced restriction enzyme sites, which are indicated in parenthesis.
Amp and *Ery* indicate the ampicillin and erythromycin resistance genes.

30% polyethylene glycol 8000. These competent cells can be used immediately or stored at -80°C until use. For electro-transformation, the frozen competent cells were thawed on ice, mixed with 1 µL (100 ng/µL) of desired plasmid DNA and poured into ice-chilled electroporation cuvette. An electric pulse with a voltage of 2.5 kV, capacitance of 25 mF and parallel resistance of 400W was applied using the Gene Pulser MXCell™ (BioRad, USA). Following the pulse, the electroporated cells were incubated in MRS broth at 37°C for 3 h and plated onto MRS agar plates containing 2.5 µg/mL of erythromycin and incubated at 37°C for 48 h.

Verification of the constructs

The correct nucleotide sequence and orientation of the cloned genes were confirmed by DNA sequencing at 1st BASE Company, Malaysia.

Codon optimization of M2e: HBc fusion gene in L. casei and L. fermentum

To construct recombinant plasmid containing codon-optimized M2e: HBc fusion gene, the native sequence of both ectodomain matrix2 (M2e) gene (GenBank accession No. CY000370) derived from influenza A virus (A/New York/31/2004 (H3N2) and hepatitis B virus core antigen (HBc) gene (GenBank accession No. EU562217) derived from hepatitis B virus with genotype C was retrieved from the NCBI database athttp://www.ncbi.nlm.nih.gov. The M2e was designed to fuse to the N-terminal part of HBc gene in order to create M2e: HBc fusion gene. To optimize the codon of M2e: HBc fusion gene that is used preferentially by two *lactobacillus* species, this fusion gene was optimized using the OPTIMIZER program at http://genomes.urv.es/OPTIMIZER and was compared with a reference codon usage database *L. casei* ATCC334 (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=321967). The codon-optimized M2e: HBc fusion gene was synthesized (Integrated DNA technologies, Co. Ltd., USA) with 5'- and 3'- end having *Nco*I sequence (CCATGG) and *Xba*I (TCTAGA) sites, respectively. The synthetic gene was supplied

as a clone into pUC19, designated as pIDTSMART AMP: M2e-HBc.

Determination of M2e: HBc expression in recombinant L. casei and L. fermentum

For the *nisA*-based inducible expression system, recombinant *L. casei* EM116 carrying pLC13.9: NisA: M2e: HBc: TT designated as EM116: M2e: HBc+ was statically cultured in an MRS medium supplemented with 2.5 μg/ml erythromycin at 37°C. After the OD600 reached 0.3, nisin was added to the culture at the final concentration of 10 ng/ml. M2e expression was monitored by sampling the bacterial cultures at 1, 2 and 3 h after induction. The bacterial samples were washed twice with phosphate-buffered saline (PBS, pH7.0), and suspended in a lysis buffer (0.5M Na_2HPO_4, 5 M NaCl, 1M imidazole, 100 mg/ml lysozyme, 1X protease inhibitor cocktail [Amresco, USA], 1 M dithiothreitol). The bacterial cells were further broken up using sonication with 10 pulses of 30s each with intermittent cooling. The whole cell lysate was subjected to SDS-PAGE using 15% polyacrylamide gels and run at 100V for 2 h, followed by Western blot. The proteins were blotted to PVDF membrane (Bio-Rad) using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) with the voltage setting at 25V for 30 min. For immuno-detection of M2e: HBc, mouse monoclonal anti-M2 (Abcam, UK) and goat anti-mouse monoclonal IgG conjugated-HRP (Santa Cruz Biotechnology, USA) were used as primary and secondary antibodies respectively. For signal detection, the membrane was applied with SuperSignal West Pico Chemiluminescent Substrate (Thermoscientific, USA) and exposed to CL-Xposure Film (Thermoscientific). After brief exposure, the film was incubated in developing solution for generating the specific protein band and finally fixed in fixative solution.

For the *ldh*L-based constitutive expression system, recombinant *L. casei* RCEID02 and *L. fermentum* RCEID01 containing pLC13.9: LDH-PRO1: M2e: HBc: TT designated as r *L. casei* 02: M2e: HBc+ and r *L. fermentum* 01: M2e: HBc+ were statistically cultured in MRS media supplemented with 2.5 μg/ml erythromycin at 37°C until the O.D. 600 reach 3.0. After incubation, the cells were harvested and the processes for sample preparation and detection for the expressed M2e were followed as described above.

Results

Codon-optimization of M2e: HBc fusion gene for expression in lactobacilli

Studies on heterologous protein expression have found that codon usage is one factor that affects translational efficiency and that replacing native codons of the introduced gene with those naturally used by the host could enhance the protein expression. In this study, the native codons of the M2e: HBc fusion gene were replaced with those preferentially used by *L. casei* and *L. fermentum*. It has been reported that the codon usage bias in bacteria including lactobacilli is one important factor that affects the translational efficiency of the heterologous protein[17]. There are three codons composed of AGG and AGA coding for Arginine (Arg), and ATA coding for Isoleucine (Ile) that are rare codons in lactobacilli including *L. casei* and *L. fermentum*. To optimize the usage of codons that is frequently used in both *Lactobacillus* species, the native nucleotide sequence of M2e: HBc was analyzed by comparing it with the codon-usage preference of *L. casei* ATCC334. The M2e: HBc fusion gene is 638 bp long, with 209 codons that are translated into 209 amino acids. It was found that 14 rare codons, consisting of 12 AGA and 2 AGG, were presented in the M2e: HBc nucleotide sequence, (Table 2). All of these rare codons were replaced with CGT. Therefore, the codon-optimized M2e: HBc fusion gene was externally synthesized with 5'- and 3'- end having *Nco*I (CCATGG) and *Xba*I (TCTAGA) sites. The synthetic gene was supplied as a clone into pUC19, designated as pIDTSMART AMP: M2e-HBc.

Construction of recombinant expression plasmid containing the M2e: HBc fusion gene under the control of nisA-inducible promoter

In this study, two M2e: HBc expression systems were constructed, one based on the nisin gene (*nisA*)-inducible promoter and the other on the constitutive promoter of the lactate dehydrogenase gene (*ldh*L). The codon-optimized M2e: HBc fusion gene was separated from *Nco*I/*Xba*I-digested pIDT SMARTAMP: M2e-HBc, isolated and purified from an agarose gel. The purified M2e: HBc fragment was cloned into *Nco*I/*Xba*I-digested pnisA-*Aat*II, which resulted in pnisA: M2e: HBc: TT. The pnisA-*Aat*II is the pGEM-T-Easy vector containing *nisA* gene promoter, multiple cloning sites (MCS), and the transcription terminator signals (TT). The gene fragment containing nisA: M2e: HBc: TT derived from *Aat*II/*Nde*I-digested pnisA: M2e: HBc: TT was isolated, purified from a gel and subcloned into *Aat*II/*Nde*I-digested pRCEID-LC13.9 resulting in the new

Table 2. Native- and codon-optimized M2e: HBc gene

Amino acid	Codon		Amino acid	Codon	
	Native	Optimized		Native	Optimized
Arg (R)	**AGA (12), AGG (2),** CGA (4), CGC (3), CGG (4), CGT (1)	CGT	Ser (S)	AGC (2), AGT (2), TCA (4), TCC (3), TCG (2), TCT (10)	TCA
Leu (L)	CTA (3), CTC (2), CTG (4), CTT (5), TTA (4), TTG (4)	TTG	Ala (A)	GCA (2), GCC (4), GCG (0), GCT (4)	GCC
Cys (C)	TGC (2), TGT (4)	TGC	His (H)	CAC (2),CAT (2)	CAT
Phe (F)	TTC (1), TTT (7)	TTC	Tyr (Y)	TAC (1), TAT (4)	TAT
Gly (G)	GGA (4), GGC (2), GCG (1), GCT (1)	GGC	Val (V)	GTA (1), GTC (4), GTG (4), GTT (3)	GTT
Pro (P)	CCA (4), CCC (2), CCG (3), CCU (8)	CCA	Ile (I)	ATA (0), ATC (3), ATT (5)	ATC
Thr (T)	ACA (2), ACC (3), ACG (0), ACT (7)	Thr (T)	Asn (N)	AAC (2), AAT (5)	AAC
Asp (D)	GAC (6), GAT (2)	GAT	Glu (E)	GAA (10), CGA (6)	GAA
Lys (K)	AAA (2), AAG (0)	AAA	Met (M)	ATG (5)	ATG
Gln (Q)	CAA (5), CAG (0)	CAA	Trp (W)	TGG (5)	TGG

[a] In parenthesis is the number of each codon found in the native M2eHBc sequence.
Rare codons found in the native M2eHBc gene are shown in bold.

recombinant expression vector containing the M2e: HBc fusion gene under the control of *nisA*-inducible promoter, designated as pLC13.9: nisA: M2e: HBc: TT. This construct was electro-transformed into *L. casei* EM116. The resulting transformant, designated as r*L. casei* EM116: M2e: HBc+, was further used for expression of the M2e: HBc fusion protein. Fig. 1 shows a schematic diagram of the construction of the recombinant expression plasmid pLC13.9: nisA: M2e: HBc: TT.

Construction of recombinant expression plasmid containing the M2e: HBc fusion gene under the control of ldhL-constitutive promoter

For the construction of the *ldh*L-based expression system (Fig. 2), the M2e: HBc: TT fragment was amplified using pnisA: M2e: HBc: TT as a template and using pnisNdeI and M2e: HBcsynF1 as a primer pair (Table 1). The amplified M2e: HBc: TT fragment was digested with *Kpn*I and *Nde*I and cloned into *Kpn*I/*Nde*I-digested pGEM: LDH-PRO1: GFPuv, resulting in pGEM: LDH-PRO1: M2e: HBc: TT. The fragment containing LDH-PRO1: M2e: HBc: TT, derived from *Aat*II/*Nde*I-digested pGEM: LDH-PRO1: M2e: HBc: TT, was cloned into *Aat*II/*Nde*I-digested pRCEID-LC13.9, resulting in the recombinant expression vector containing the M2eHBc fusion gene under the control of the *ldh*L promoter designated as pLC13.9: LDH-PRO1: M2e: HBc: TT. This construct was electro-transformed into *L. casei* RCEID02 and into *L. fermentum* RCEID01, resulting in the transformants designated as r*L. casei*02: M2e: HBc+ and r*L. fermentum*01: M2e: HBc+. *L. casei* RCEID02 is the plasmid-free strain of the probiotic *L. casei* TISTR1341 isolated from a chicken while *L. fermentum* RCEID01 is the plasmid-free strain derived from *L.fermentum* 47-7 isolated from a healthy infant. Fig. 2 shows the schematic diagram of the construction of the recombinant-expression plasmid pLC13.9: LDH-PRO1: M2e: HBc: TT.

Expression of M2e: HBc fusion protein in L. casei and L. fermentum

Western blotting using a specific antibody against M2e showed r*L. casei* EM116: M2e: HBc+ (Fig. 3), r*L. casei*02: M2e: HBc+ (Fig. 4), and r*L. fermentum*01: M2e: HBc+ could express M2e: HBc fusion protein (Fig. 5). The expression of the M2e: HBc fusion protein in the nisin inducible system of *L. casei* was confirmed by the detection of a protein with the expected size of 27.6 kDa after 2 and 3 h of nisin induction (Fig. 3, lane 3 and 4) but not observed at 1h after nisin induction. Fig. 4 shows the M2e: HBc fusion protein expression by r*L. casei*02: M2e: HBc+ under the control of the constitutive *ldh*L promoter. We further analyzed the expression of the codon-optimized M2e: HBc fusion

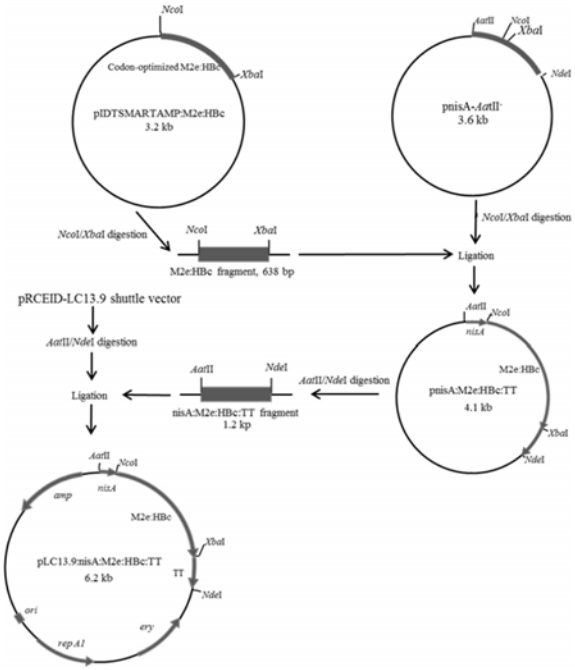

Fig. 1 Schematic diagram of the construction of the recombinant plasmid pLC13.9: nisA: M2e: HBc: TT. Position *amp, ery, ori,* and *repA1* indicate ampicillin resistance gene, erythromycin resistance gene, origin of replication, and replicon A1, respectively.

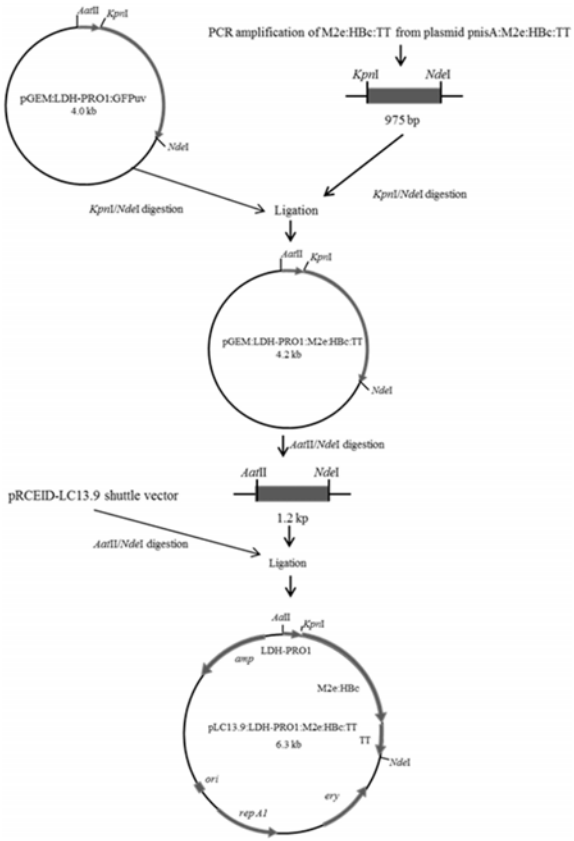

Fig. 2 Schematic diagram of the construction of the recombinant expression plasmid ppLC13.9: LDH-PRO1: M2e: HBc: TT. Position *amp, ery, ori,* and *repA1* indicate ampicillin resistance gene, erythromycin resistance gene, origin of replication, and replicon A1, respectively.

gene in *L. fermentum* RCEID01 as shown in Fig. 5. Taken together, our results demonstrated that the codon-optimized M2e: HBc fusion gene can successfully express in *L. casei* both under the *nisA* and the *ldh*L promoters and in *L. fermentum* under the control of the later promoter.

Discussion and Conclusion

Vaccination is accepted as the most effective approach for controlling the spread of influenza. Currently, the licensed influenza vaccines induce antibodies that are strain-specific to hemagglutinin (HA) and neuraminidase (NA)[7]. Thus, it is necessary to update the influenza vaccines annually in order for them to be effective against circulating viral strains. For this reason, there is an attempt to develop universal influenza vaccine to elicit broad-spectrum immunity against all subtypes of influenza viruses. The extracellular domain of Matrix 2 (M2e) is an evolutionarily conserved region in influenza A viruses and a promising component for designing a universal influenza vaccine. M2e has low immunogenicity due to its small size consisting of only 23 amino-acid residues and is often fused with a number of suitable carriers either through genetic fusion or chemical linkage to enhance anti-M2e responses in vaccination experiments. Chemical conjugates between M2e and carriers include the use of bovine serum albumin (BSA), KLH and *Neisseria meningitides* outer membrane protein complex (OMPC), all of which are able to induce anti-M2e protective antibodies[17]. Nyrynck et al[11] demonstrated an efficient and cost-effective method to make M2e immunogenic by fusing M2e to the hepatitis B virus core protein (HBc), which forms a virus-like particle (VLP) with M2e radiating from the surface. Besides HBc, many other viral proteins have been fused with M2e to generate VLP and to evaluate their efficacy as vaccines[18,19].

At present, heterologous protein expression is being intensively studied in LAB as live mucosal

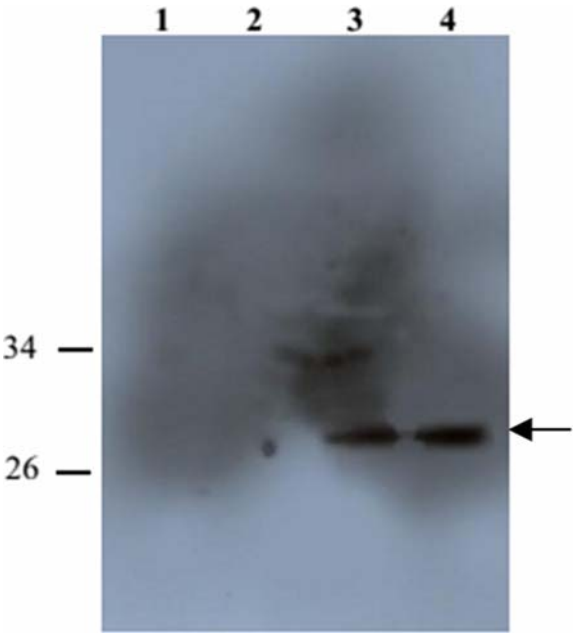

Fig. 3 Immuno detection of the expressed M2e: HBc fusion protein in crude lysate of *L. casei* EM116 at different nisin induction time. Mouse anti-M2 monoclonal antibody was used to detect M2e. Lane 1 = *L. casei* EM116 harboring pRCEID-LC13.9. Lane 2, 3, and 4 = nisin-induced r*L. casei* EM116: M2e: HBc+ cells at 1, 2, and 3 h after nisin induction, respectively. The arrow indicates the M2e: HBc band with the molecular weigth of 27.6 kDa.

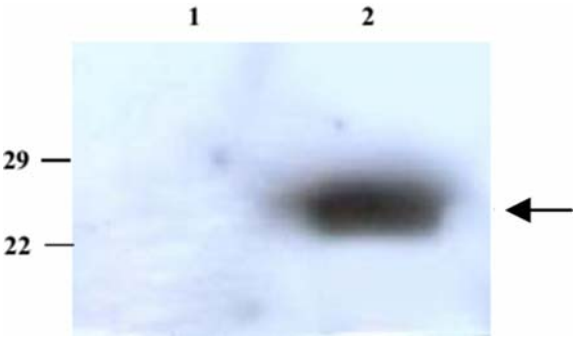

Fig. 4 Immuno detection of the expressed M2e: HBc fusion protein in crude lysate of *L. casei* RCEID 02. Mouse anti-M2 monoclonal antibody was used to detect M2e. Lane 1 = *L. casei* RCEID 02 harboring pRCEID-LC13.9. Lane 2 = r*L. casei* 02: M2e: HBc+. The arrow indicates the M2e: HBc band with the molecular weight of 27.6 kDa.

delivery vehicles for therapeutic and prophylactic proteins. However, the low expression level of heterologous in LAB is a major drawback that restricts

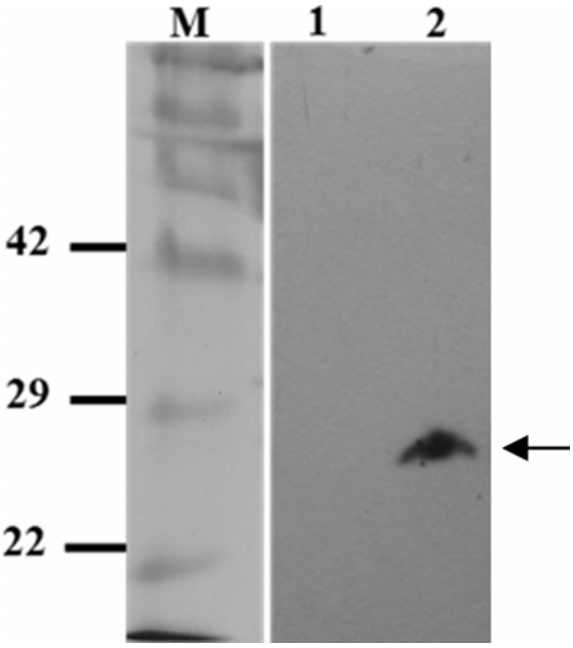

Fig. 5 Immuno detection of the expressed M2e: HBc fusion protein in crude lysate of *L. fermentum* RCEID 01. Mouse anti-M2 monoclonal antibody was used to detect M2e. Lane 1 = *L. fermentum* RCEID 01 harboring pRCEID-LC13.9. Lane 2 = r*L. fermentum* 01: M2e: HBc+. The arrow indicates the M2e: HBc band with the molecular weigth of 27.6 kDa.

their application for such purposes. It was found that several factors could possibly improve the yield of the expressed proteins such as the use of strong promoters (of both constitutive and inducible types), high-copy number plasmid vectors, and efficient Shine-Dalgarno sequences[20].

It was found that codon usage bias in LAB dramatically reduces the translational efficiency[12]. The expression level of genes containing codons matching those of the bacterial hosts is higher than that of genes containing native codons[21]. Bioinformatic analysis showed that the M2e: HBc fusion gene contains 14 rare codons consisting of 12 AGA and 2 AGG codons. All of these rare codons were replaced by the codons used by *L. casei* ATCC344 (Table 2). Our previous study was unable to gain the expression of nucleoprotein (NP) of the influenza A virus with the use of the native NP coding sequence in *L. casei.* This problem was successfully overcome by using a codon optimization strategy[13]. It has been reported previously that the translation of heterologous genes containing a high percentage of rare codons is generally stalled or

prematurely terminated, resulting in a low level of expression[22].

Besides expression of M2e: HBc in our LAB prototype, *L. casei* RCEID02, we have also expressed this codon-optimized M2e: HBc construct in a probiotic *L. fermentum* RCEID01 isolated from a healthy infant in our laboratory and the codon usage in *L. fermentum* is similar to that of *L. casei*. For *L. fermentum*, it is the major hetero-fermentative *Lactobacillus* species in the human intestine, possessing antagonistic activity and contributing to intestinal health in mammals[23,24].

The safety and health benefits of *L. fermentum* have been reported in several studies. Long-term safety of early consumption of the probiotic *L. fermentum* CECT5716 has recently been demonstrated by Maldonado-Lobon et al. Olivares et al demonstrated that oral administration of the strain *L. fermentum* CECT5716 potentiates the immunologic response against influenza in subjects receiving an influenza vaccine[25,26]. Maldonado et al found that infants receiving a follow-on formula supplemented with *L. fermentum* have a significant reduction in the incidence rate of community-acquired gastrointestinal and respiratory infection[27]. Our previous studies[28] have demonstrated that our *L. fermentum* isolate, *L. fermentum* 47-7, displays tolerance to acid and bile salt, which implies that it can survive passage through the intestine and is viable in the large intestine. It also displays antimicrobial activity against the bacteriocin sensitive *L. sakei* CECT906 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 13311 (*S. thyphimurium*). Aside from its in vitro probiotic properties, it was found that this isolate is suitable for use as a bacterial host for heterologous protein expression, as it can acquire and maintain the heterologous plasmid, the *E. coli/L. casei* shuttle vector-pRCEID-LC13.9 and can be used for expressing the green fluorescent protein (GFP) with the use of an expression vector based on pRCEID-LC13.9 under the control of the lactate dehydrogenase (*ldh*L) promoter. All of these findings indicate that *L. fermentum* 47-7 could be a good candidate as a delivery vehicle for biologically useful molecules.

It was also found that the recombinant plasmid based on the pRCEID-LC13.9 *E. coli/L. casei* shuttle vector introduced to either *L. casei* or *L. fermentum* show highly segregational (no plasmid loss during growth) and structural stability (no gene rearrangements) even after 80 generations (data not shown). Such stability is a necessary requirement for the development of recombinant LAB as live vaccines. In addition, the growth rate of both *L. casei* and *L. fermentum* harboring the recombinant plasmid were similar to those of wild-type bacteria.

In this study, we successfully cloned and expressed the codon-optimized M2e: HBc in both our LAB prototype, *L. casei* and probiotic human LAB isolate, *L. fermentum*, with the use of the *E. coli/L. casei* shuttle vector pRCEID-LC13.9. In addition, the codon optimization can be used for the expression of M2e:HBc fusion gene in both *L. casei* and *L. fermentum*. The expression of the M2e: HBc fusion gene in both LAB species would provide the opportunity to apply these two bacteria as an alternative broad-spectrum vaccine candidate for influenza A virus.

What is already known on this topic?

Probiotic bacteria, including lactobacilli, have been engineered to express many reactive antigens derived from pathogen for using as vaccine carrier.

What this study adds?

This study demonstrate that codon optimization strategy can be used for the expression of viral antigen gene, M2e: HBc, in two lactobacilli prototypes, *L. casei* and *L. fermentum*. The expression systems, nisA-inducible system and ldh promoter-based constitutive system were successfully used in both lactobacilli.

Acknowledgements

This study was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Thailand and invitation research grant (I56318) from the Faculty of Medicine, Khon Kaen University. Special thanks to Mr. Dylan Southard for the English revision of the manuscript.

Potential conflicts of interest

None.

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การสร้างเชื้อ Lactobacillus casei และ Lactobacillus fermentum ที่มีการแสดงออกของ codon-optimized M2e: HBc fusion gene

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วัตถุประสงค์: *เพื่อสร้างเชื้อ Lactobacillus casei และ Lactobacillus fermentum ที่มีการแสดงออกของ codon-optimized M2e: HBc fusion gene*

วัสดุและวิธีการ: *ทำการออกแบบจีน M2e: HBc fusion โดยการเชื่อมต่อระหว่างจีนอนุรักษ์ M2e ของเชื้อไวรัสไข้หวัดใหญ่กับจีน HBc ของเชื้อไวรัสตับอักเสบบี จากนั้นนำจีนดังกล่าวมาปรับโคดอนเพื่อให้เหมาะสมสำหรับการแสดงออกในเชื้อ L. casei แล้วโคลนจีน codon-optimized M2e: HBc เข้าสู่เวคเตอร์ E. coli/L. casei shuttle vector ภายใต้การควบคุมของโปรโมเตอร์ nisA-inducible promoter สำหรับการแสดงออกในเชื้อ L. casei EM116 และภายใต้การควบคุมของ ldhL strong constitutive promoter สำหรับการแสดงออกในเชื้อ L. casei RCEID02 และ L. fermentum RCEID01 จากนั้นทำการทดสอบการแสดงออกของจีน M2e: HBc fusion ด้วยวิธี western blotting โดยใช้แอนติบอดีที่จำเพาะต่อโปรตีน M2e*

ผลการศึกษา: *การวิเคราะห์ codon usage ของจีน M2e: HBc fusion ในเชื้อ L. casei พบ 14 โคดอนมีคุณสมบัติเป็น rare codon ได้แก่ AGA และ AGG จำนวน 12 และ 2 โคดอนตามลำดับ ซึ่งทั้งหมดเป็นโคดอนสำหรับกรดอะมิโนอาร์จินีน ผลการปรับโคดอนและสังเคราะห์ codon-optimized M2e: HBc ได้จีนขนาด 638 bp เพื่อนำไปสร้าง recombinant expression plasmids ภายใต้การควบคุมของโปรโมเตอร์ nisA-inducible promoter และ ldhL strong constitutive promoter ได้สำเร็จผลการทดสอบการสร้างโปรตีนด้วยเทคนิค western blotting พบว่าเชื้อที่มี recombinant expression plasmids สามารถควบคุมการสร้างโปรตีน M2e: HBc fusion ได้*

สรุป: *การใช้จีนที่ผ่านการปรับโคดอนให้เหมาะสมกับเซลล์แบคทีเรียเจ้าบ้าน สามารถนำมาประยุกต์ใช้ในการแสดงออกของจีน M2e: HBc fusion ในเชื้อ L. casei และ L. fermentum ได้นอกจากนี้การแสดงออกของจีน M2e: HBc fusion ในเชื้อกลุ่ม lactobacilli สามารถนำไปพัฒนาต่อยอดการพัฒนาวัคซีนสำหรับไวรัสไข้หวัดใหญ่สายพันธุ์ เอ ได้*
