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Research Letter

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Secretion of M2e:HBc fusion protein by *Lactobacillus casei* using Cwh signal peptide

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One sentence summary: The novel Cwh signal peptide selected from the *Lactobacillus casei* genome by using a bioinformatics approach was successfully used in the expression–secretion vector for heterologous protein secretion.

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ABSTRACT

The ability to serve as a delivery vehicle for various interesting biomolecules makes lactic acid bacteria (LAB) very useful in several applications. In the medical field, recombinant LAB expressing pathogenic antigens at different cellular locations have been used to elicit both mucosal and systemic immune responses. Expression–secretion vectors (ESVs) with a signal peptide (SP) are pivotal for protein expression and secretion. In this study, the genome sequence of *Lactobacillus casei* ATCC334 was explored for new SPs using bioinformatics tools. Three new SPs of the proteins Cwh, SurA and SP6565 were identified and used to construct an ESV based on our *Escherichia coli*–*L. casei* shuttle vector, pRCEID-LC13.9. Functional testing of these constructs with the green fluorescence protein (GFP) gene showed that they could secrete the GFP. The construct with CwhSP showed the highest GFP secretion. Consequently, CwhSP was selected to develop an ESV construct carrying a synthetic gene encoding the extracellular domain of the matrix 2 protein fused with the hepatitis B core antigen (M2e:HBc). This ESV was shown to efficiently express and secrete the M2e:HBc fusion protein. The identified SPs and the developed ESVs can be exploited for expression and secretion of homologous and heterologous proteins in *L. casei*.

Keywords: Lactic acid bacteria; *Lactobacillus casei*; expression and secretion vector; signal peptide

INTRODUCTION

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria that are used in various biotechnological applications. With respect to human and animal health, many LAB are being used as mucosal delivery vehicles for therapeutic and prophylactic molecules because of their GRAS (Generally Regarded As Safe) status (Eijsink *et al.* 2002), their probiotic properties and the ease with which they can be engineered to express heterologous proteins (Ouwehand *et al.* 2002) to elicit an effective immune response (Lee *et al.* 2006). Up to now, a definite conclusion on the best cellular location for optimal immunization, i.e. intracellular, cell wall anchored or secreted, has yet to be reported. This seems to depend on parameters such as the bacterial type, the amount and localization of the antigen, the route of

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administration, etc. (Wells and Mercenier 2008). Many expression and secretion systems for LAB to deliver protective antigens have already been developed (Kruger *et al.* 2002; Pusch *et al.* 2005). Such systems require the use of expression–secretion vectors (ESVs) with a signal peptide (SP) to translocate the protein after synthesis across the bacterial cell membrane. Several SPs have been identified and incorporated into ESVs (Hols *et al.* 1997; Hazebrouck, Pothelune and Azevedo 2007). The secretion efficiency of different SPs seems to be host specific. For this reason, homologous SPs are thought to drive protein secretion more efficiently than heterologous SPs (Mathiesen *et al.* 2008). The availability of the complete genome sequences of many *Lactobacillus casei* strains (Makarova *et al.* 2006) makes it easy to search for new and efficient SPs that can be used in the construction of new ESVs for this species and maybe other lactobacilli.

In this study, putative SPs were selected from the genome of *L. casei* ATCC334 (accession no. CC000423.1) using bioinformatics analysis. The selected SPs were tested for the secretion of a gene product encoding a green fluorescent protein (GFP) as a reporter protein and using the ESV based on *Escherichia coli*–*L. casei* shuttle vectors previously developed in our group (Panya *et al.* 2012; Suebwongsa *et al.* 2013). The SP with the highest efficiency was then used to drive the secretion of a synthetic gene coding for an M2e:HBc fusion protein. M2e is a highly conserved protein of the influenza A virus that has been incorporated as an antigen into many influenza vaccine formulations. The immunogenicity of M2e has been reported to be enhanced when fused with the hepatitis B core antigen (HBc) (De Filette *et al.* 2005). The final construct, pLC-Cwh:M2e:HBc, successfully expressed and secreted the fused antigenic protein.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains, plasmid vectors and oligonucleotide primers used in this study are listed in Table 1. *Lactobacillus* strains were grown statically in de Man Rogosa and Sharpe (MRS) medium (LabM, Heywood, Lancashire, UK) at 37°C. *Escherichia coli* DH5α was cultured in Luria–Bertani (LB) broth (LabM) at 37°C with shaking. Agarified media were prepared by adding 15 g l^{-1} bacteriological agar to the corresponding broth. When required, appropriate antibiotics (Sigma-Aldrich, St Louis, MO, USA) were added to the media as follows: erythromycin for *L. casei* at 2.5 μg ml^{-1}, and ampicillin for *E.coli* at 100 μg ml^{-1}.

DNA manipulation

All DNA manipulation procedures were performed as described by Sambrook and Russell (2001). Plasmids from *E. coli* were isolated and purified using the HiYield plasmid mini Kit (RBC Bioscience, New Taipei City, Taiwan). Plasmids from *L. casei* were extracted as described by O'Sullivan and Klaenhammer (1993). Genomic DNA from *L. casei* was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). PCR amplification was performed using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Amplicons were purified from agarose gels using the HiYield Gel PCR DNA Fragments Extraction kit (RBC Bioscience).

Analysis of candidate signal peptides

The nucleotide sequences of cell surface-associated proteins of *L. casei* ATCC334 were retrieved from the NCBI microbial genomes database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The SignalP4.1 program (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the putative signal peptides. The likely location of the proteins was predicted with the programs PSORTb v 3.0 (http://www.psort.org/psortb/), SubLoc v 1.0 (http://www.bioinfo.tsinghua.edu.cn/SubLoc/pro_predict.htm) and Gpos-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/Gpos-multi/). DNA sequences encoding SPs of cell surface-associated proteins with a clear SP cleavage site and an extracellular location were selected for the construction of ESVs.

Construction of an expression–secretion vector (ESV) for GFP

To determine whether the three selected SPs (i.e. CwhSP, SurASP and SP6565) could drive heterologous protein secretion in *L. casei*, an ESV based on each of these was constructed. SP strength was analyzed using the GFPuv-encoding gene as a reporter. Sequences encoding the three SPs were amplified from genomic DNA of *L. casei* ATCC334 under the following conditions: one cycle of 95°C for 3 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 14 s; and 72°C for 3 min. The lengths of the amplicons, CwhSP, SurASP and SP6565, were 129 bp, 105 bp and 126 bp, respectively. The *gfp*uv gene fragment, recovered as a *Kpn*I/*Spe*I-digested fragment from pGFPuv, was cloned into pCwh, pSurA and pSP6565 to generate pCwhSP:GFPuv, pSurSP:GFPuv and pSP6565:GFPuv, respectively. The Cwh:GFPuv fusion gene was obtained by double digestion of the pCwh:GFPuv with *Hin*dIII/*Spe*I and cloned into *Hin*dIII/*Spe*I-digested pLdh-Pro1, a vector containing the constitutive promoter (P_{ldh}) and ribosome binding site (RBS_{ldh}) of the lactate dehydrogenase gene (GenBank accession no. D12591.1), resulting in pLdh:Cwh:GFPuv. Using the same procedure, pLdh:Sur:GFPuv and pLdh:SP6565:GFPuv were also obtained. The Ldh:Cwh:GFPuv DNA fragment obtained as a *Aat*II/*Spe*I-digested pLdh:Cwh:GFPuv was cloned into *Aat*II/*Spe*I-digested pRCEID-LC13.9 resulting in pLC-Cwh:GFPuv. The same strategy was followed to obtain the recombinant ESVs containing GFPuv with SurSP and SP6556, designated as pLC-Sur:GFPuv and pLC-SP6565:GFPuv. Figure 1 shows the construction diagram of pLC-Cwh:GFPuv and a similar procedure was used for the generation of the pLdh:Sur:GFPuv and pLdh:SP6565:GFPuv. All three recombinant plasmids were verified by DNA sequencing (data not shown) and were independently electrotransformed into *L. casei* RCEID02. The preparation of competent cells and the electroporation protocol were as described elsewhere (Chassy and Flickinger 1987).

Determination of the secreted GFP by measurement of fluorescence intensity

To determine the secretion efficiency, the fluorescence intensity of culture supernatants derived from recombinant *L. casei* RCEID02 harboring pLC-Cwh:GFP, pLC-Sur:GFP and pLC-SP6565:GFP was determined in triplicate. Cultures of the above three recombinant constructs were incubated at 37°C for 20 h with shaking at 200 rpm until an optical density of 3.0 at 600 nm (OD600) was reached. Supernatants of the cultures were harvested and the fluorescence intensity was measured with a fluorometer (Cary Eclipse, Victoria, Australia), as previously described (Wu and Chung 2006). As a negative control, the culture supernatant of *L. casei* RCEID02 harboring the expression vector without signal peptide (pLC-GFPuv) was used.

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

Materials	Relevant characteristics	Source or reference
Bacterial strains		
E. coli DH5α	Transformation host	Taylor, Walker and McInnes *et al.* (1993)
L. casei RCEID02	Plasmid-free strain of *L.casei* TISTR1341 and protein expression host	RCEID
L. casei ATCC334	Source of signal peptide-encoding genes	ATCC
Plasmids		
pGEM-T Easy vector	Ap^r, M13*ori*, T-overhang cloning vector	Promega
pGFPuv	Ap^r, plasmid containing the GFPuv gene from *Aequorea victoria*	Clontech
pCwh	Ap^r, M13*ori*, a pGEM-T-derived vector containing a promoter-less part of the *cwh* gene (*cwhSP*) encoding the Cwh signal peptide (SP)	This study
pSur	Ap^r, M13*ori*, pGEM-T-derived vector containing a promoter-less part of the SurSP gene encoding SurSP signal peptide	This study
pSP6565	Ap^r, M13*ori* pGEM-T-derived vector containing a promoter-less part of the SP6565 gene encoding SP6565 signal peptide	This study
pCwh:GFPuv	Ap^r, M13*ori*, pCwh derivative containing GFPuv-encoding gene downstream of *cwh* gene	This study
pSur:GFPuv	Ap^r, M13*ori*, pSur containing GFPuv-encoding gene downstream of Sur gene	This study
pSP6565:GFPuv	Ap^r, M13*ori*, p6565 containing GFPuv-encoding gene downstream of 6565gene	This study
pLdh-Pro1	Ap^r, M13*ori* pGEM-T vector containing the promoter of the lactate dehydrogenase (Ldh) gene and transcription terminator (TT)	This study
pLdh:Cwh:GFPuv	Ap^r, M13*ori*, pLdh-Pro1-derived vector containing Cwh:GFPuv downstream of Ldh promoter	This study
pLdh:Sur:GFPuv	Ap^r, M13*ori*, pLdh-Pro1-derived vector containing Sur:GFPuv downstream of Ldh promoter	This study
pLdh:SP6565:GFPuv	Ap^r, M13*ori*, pLdh-Pro1-derived vector containing SP6565:GFPuv downstream of Ldh promoter	This study
pRCEID-LC13.9	Ap^r, Em^r, *E. coli–L. casei* shuttle vector	Panya *et al.* (2012)
pLC-GFPuv	pRCEID-LC13.9 containing the GFPuv-encoding gene downstream of *ldh* gene promoter	This study
pLC-Cwh:GFPuv	pRCEID-LC13.9 containing a cassette expressing the fusion protein CwhSP:GFPuv from the Ldh promoter	This study
pLC-Sur:GFPuv	pRCEID-LC13.9 containing gene expression cassette consisting of Ldh:SurSP:GFPuv	This study
pLC-SP6565:GFPuv	pRCEID-LC13.9 containing gene expression cassette consisting of Ldh: SP6565:GFPuv	This study
pLC-M2e:HBc	pRCEID-LC13.9 containing gene expression cassette consisting of Ldh: M2e:HBc:TT	This study
pLC-Cwh:M2e:HBc	pLC-M2e:HBc containing Cwh upstream of Ldh:M2e:HBc	This study
Oligonucleotide primers	Sequences (5′–3′)/(restriction site)	Target gene (GenBank accession no.)
CwhSP-F CwhSP-R	ACGTAAGCTTCATGGTAGATGCAAAGAA (*Hind*III) ATTAGGTACCGCACCCGATTTGTAATTG (*Kpn*I)	Signal peptidase (SP) region of the *cwh* gene (YP_805583.1)
SurASP-F SurASP-R	GGGCAAGCTTAATGAAATTCAATAAAGT (*Hind*III) ATTTGGTACCGCGATACTGTCGCTTGTA (*Kpn*I)	Surface antigen encoding gene (YP_805328.1)
SP6565-F SP6565-R	ACGTAAGCTTTATGAAGCATTTGAGACG (*Hind*III) GACAGGTACCACCAACGTACCTTTTTGT (*Kpn*I)	Surface protein 6565 encoding gene (YP_806565.1)
Cwh-ex-F Cwh-ex-R	GCCGCGGCCCGGGATGGTAGATGCAAA (*Xma*I) AACGCCCGGGCCGCACCCGATTT (*Xma*I)	*cwh* gene in pLC-Cwh:M2e:HBc
M13 (-40) forward M13 (-48) reverse	GTTTTCCCAGTCACGAC AGCGGATAACAATTTCACACAGGA	Primer for sequencing of cloned fragments in TA cloning vectors
Ldh:M2e:HBc-F Ldh:M2e:HBc-R	GGGAATAAGGGCGACACGGAAATGTTG TGGTTTCTGGCAAGGTTGACAAGATTG	Ldh promoter and M2e:HBc
M2e:HBc:TT-F M2e:HBc:TT-R	TCAGTTGAATTGTTGTCATTCTTGCCAT GCCTGATGCGGTATTTTCTCCTTA	M2e:HBc and transcription terminator

TISTR: Thailand Institute of Scientific and Technological Research; ATCC: American Type Culture Collection; RCEID: Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University; Ap^r: ampicillin resistance; Em^r: erythromycin resistance; *Ori*: origin of replication. Underlined nucleotides show sequences in the primers to introduce restriction enzyme sites (indicated in parentheses).

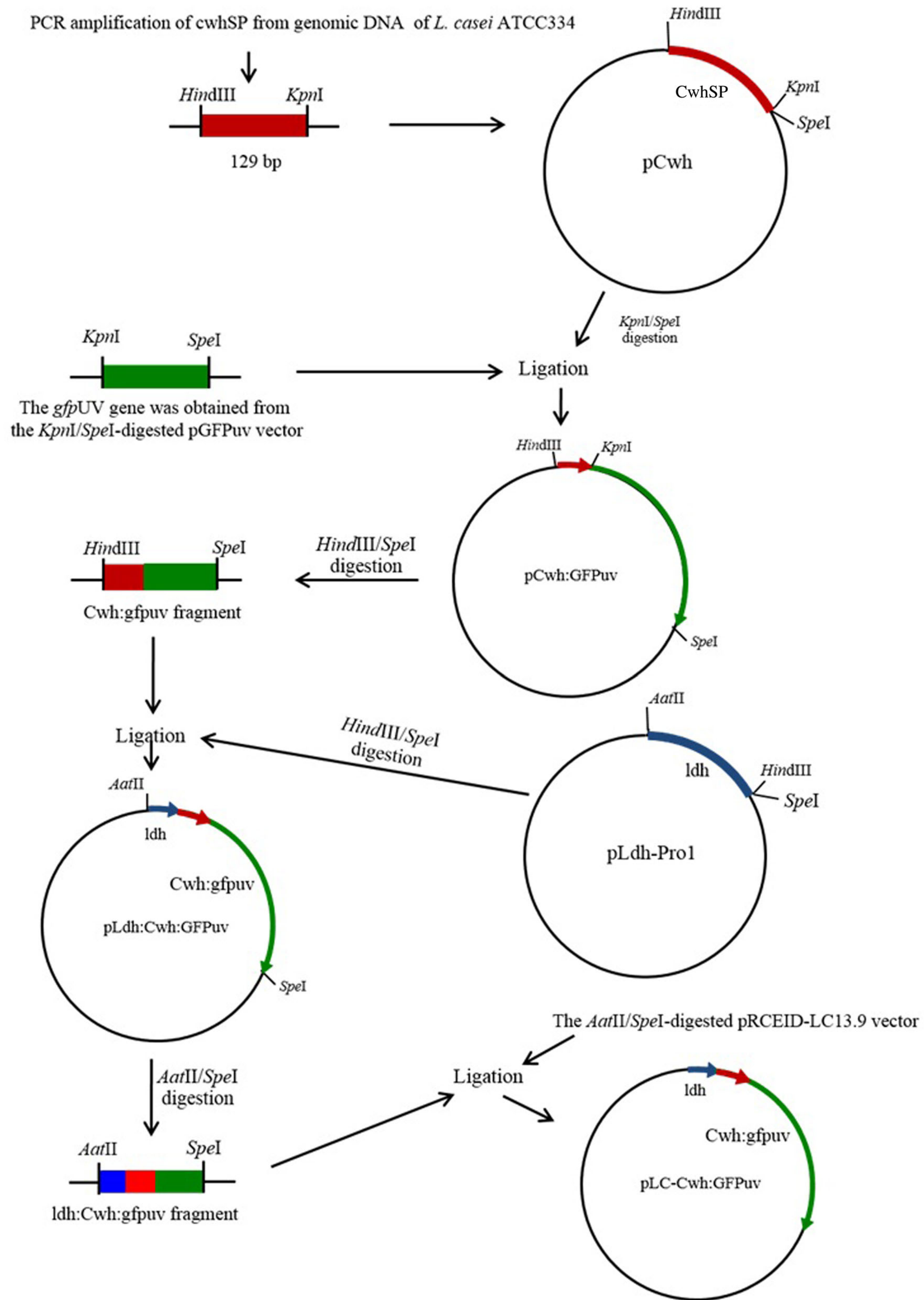

Figure 1. Diagram depicting the construction of pLC-Cwh:GFPuv.

Figure 2. Diagram of pLC-Cwh:M2e:HBc, the expression cassette for the fusion protein M2e:HBc containing the constitutive promoter and ribosome binding site (RBS) of lactate dehydrogenase gene (*ldh*) of *L. casei* ATCC393, the Cwh signal peptide (CwhSP), M2e:HBc fusion gene and the transcription terminator (TT) of *Lactococcus lactis* subsp. *cremoris* amino peptidase N (*pepN*) gene (GenBank accession no. M87840.1).

Construction of recombinant *L. casei* expressing M2e:HBc fusion protein in a secreted form

To construct the recombinant *L. casei* expressing the secreted M2e:HBc fusion protein, the CwhSP fragment with *Xma*I flanking recognition sequences was amplified using pLC-Cwh:GFP as the template. The CwhSP-coding region was inserted upstream of the M2e:HBc gene in pLC-M2e:HBc, a vector containing a gene cassette composed of the *ldh*-derived promoter, the fusion gene encoding M2e:HBc and the transcription terminator (TT) of the *Lactococcus lactis* subsp. *cremoris* amino peptidase N (*pepN*)-encoding gene (GenBank accession no. M87840.1). Figure 2 shows the expression cassette of pLC-Cwh:M2e:HBc. The recombinant plasmid pLC-CwhSP:M2e:HBc was verified by DNA sequencing (data not shown) and was electrotransformed into *L. casei* RCEID02. The transformants were checked for secretion of the M2e:HBc fusion protein.

Determination of the secreted M2e:HBc fusion protein by western blot analysis

Transformants containing pLC-Cwh:M2e:HBc were cultured at 37°C for 20 h with shaking at 200 rpm until an OD600 of 3.0 was reached. The supernatant and cell pellet were collected. The supernatant was concentrated 10-fold using centricons with a molecular weight cut-off of 10 kDa (Pall Life Sciences, NY, USA). The total cell extract and concentrated supernatant were electrophoresed in 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blotting. The M2e:HBc fusion protein on the membrane was detected with mouse anti-M2 monoclonal antibody (Abcam, Cambridge, UK) at a dilution of 1:1000. Horseradish peroxidase-conjugated goat anti-mouse IgG (Abcam) was used as a secondary antibody at a dilution of 1:10 000. The signal was developed with a chemiluminescent substrate reagent (Thermo Fisher Scientific, Rockford, IL, USA) and recorded with a digital camera (Image Quant TM LAS 4000, Uppsala, Sweden). Total cell extracts from the transformants with pLC-Cwh:M2e:HBc and those with pLC-M2e:HBc were used as positive controls for M2e:HBc expression. Supernatants from the transformants with the pLC-M2e:HBc construct and those with pRCEID-LC13.9 were used as negative controls.

RESULTS

Selection of the candidate signal peptides

Seventy-seven deduced amino acid sequences of *L. casei* ATCC334 genes encoding cell surface-associated proteins were retrieved from the NCBI database and analyzed with the SignalP 4.1 program to predict likely SP sequences (data not shown). The program generates a parameter called the D score, which is used to discriminate signal peptide from non-signal peptide sequences. It also indicates the presumptive location of the signal peptide cleavage site. Six proteins with a D score above the cut-off value of 0.450 contained good predictions for SP sequences (Table 2). The D score of these proteins and the likely cleavage sites are summarized in Table 2. Cellular location of each of the six proteins were predicted using three different programs. Five, three and one of six proteins were predicted to be extracellular by Gpos-mPLoc, PSORTb and SubLoc programs, respectively. Based on this bioinformatics analysis, SPs of the proteins encoded by the ORFs YP˙805583.1, YP_805328.1 and YP_806565.1 were selected. These were designated as CwhSP, SurSP and SP6565, respectively.

Secretion of GFPuv in recombinant *L. casei* using different SP-containing ESV

To determine the secretion efficiency of the selected sequences, ESVs based on each SP with the GFPuv gene as a reporter were constructed and designated pLC-Cwh:GFPuv, pLC-Sur:GFPuv and pLC-Sp665:GFP, respectively. These ESVs were independently transformed into *L. casei* RCEID02, resulting in the recombinant strains RCEID02:CwhSP, RCEID02:SurSP and RCEID02:SP6556. The fluorescence intensity of culture supernatants of these strains, and that of *L. casei* RCEID02 pLC-GFPuv (used as a negative control), was determined. The highest fluorescence intensity value was shown by the CwhSP (164.80), followed by that of SurASP (118.80) and SP6565 (113.49) (Fig. 3).

Table 2. Main characteristics of signal peptides (SPs) from the genome of *L. casei*, as predicted from bioinformatics analysis.

		Predicted signal peptide function		Predicted cellular location		
Signal peptide	Protein	D score[a]	Cleavage site	PSORTb	Gpos-mPLoc	SubLoc
YP_805583.1 (CwhSP)	Cell wall-associated hydrolase	0.696	VSA-ST	Extracellular	Extracellular	Periplasm
YP_805328.1 (SurSP)	Surface antigen	0.864	VFA-DT	Extracellular	Extracellular	Extracellular
YP_806565.1 (SP6565)	Cell surface protein	0.798	VHA-DD	Extracellular	Extracellular	Periplasm
YP_805464.1	Cell surface protein	0.758	VGA-TT	Unknown	Extracellular	Periplasm
YP_805465.1	Cell surface protein	0.834	VLA-LQ	Unknown	Extracellular	Periplasm
YP_805466.1	Cell surface protein	0.777	VFA-SE	Unknown	Plasma membrane	Periplasm

[a]The D cut-off value for cell surface associate protein is 0.450.

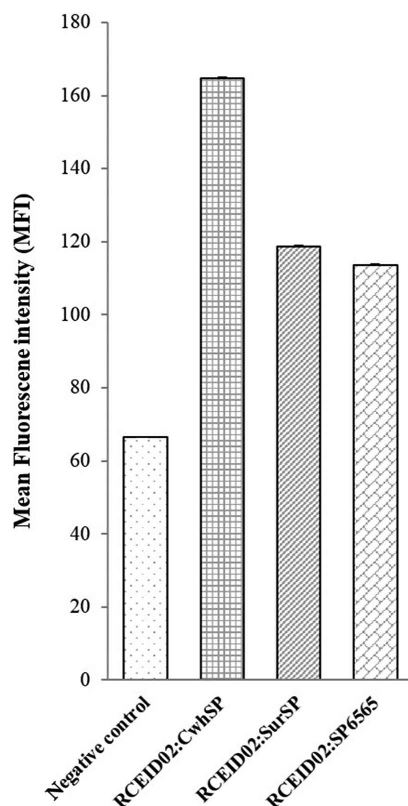

Figure 3. Mean fluorescence intensity (MFI) of culture supernatants from the recombinant *L. casei* RCEID02 harboring different SP-containing constructs. The supernatant from *L. casei* RCEID02 harboring pLC-GFPuv was used as a negative control.

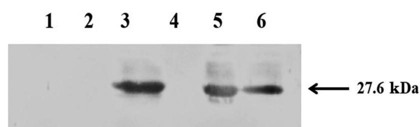

Figure 4. Western blot analysis of M2e:HBc fusion protein expressed in recombinant *L. casei* RCEID02. Lanes 1 and 2 = total cell extracts and supernatant from *L. casei* RCEID02 containing pRCEID-LC13.9; lanes 3 and 4 = total cell extracts and supernatant from *L. casei* RCEID02 containing pLC-M2e:HBc; lanes 5 and 6 = total cell extracts and supernatant from *L. casei* RCEID02 containing pLC-CwhSP:M2e:HBc.

Expression and secretion of the M2e:HBc fusion protein by *L. casei* RCEID02

The CwhSP was selected for constructing the ESV containing the M2e:HBc fusion gene, i.e. pLC-Cwh:M2e:HBc. Western blot analysis of the supernatant from *L. casei* cells containing pLC-Cwh:M2e:HBc showed a prominent band with an apparent molecular weight of around 27.6 kDa (Fig. 4, lane 6), which presumably corresponded to the expected fusion protein. The supernatant from an intermediate construct lacking CwhSP showed no band (Fig. 4, lane 4). The M2e:HBc fusion protein was detected in total cell extracts of both *L. casei* carrying pLC-M2e:HBc (Fig. 4 lane 3) and pLC-Cwh:M2e:HBc (Fig. 4 lane 5). No protein band was detected in either total cell extracts (Fig. 4, lane 1) or supernatant (Fig. 4, lane 2) of the negative control strain.

DISCUSSION

Engineered LAB strains constitute bacterial systems that may serve as alternative mucosal delivery vehicles for a variety of biomolecules (Wells and Mercenier 2008). Recombinant LAB can be used as vaccine vehicles to elicit both secretory IgA and systemic antibody responses against the delivered antigen (Neutra and Kozlowski 2006). In this study we constructed recombinant *L. casei* cells expressing M2e:HBc in a secreted form with the use of novel SPs derived from the *L. casei* genome. Several SPs derived from cell surface-associated proteins and secreted proteins of LAB have been identified and used to construct distinct ESVs (Wu and Chung 2006; Mathiesen *et al.* 2008). Of these, the SP of Usp45SP *from Lactococcus lactis* (Daniel *et al.* 2011) is the most widely used heterologous SP for protein secretion in LAB species (Dieye *et al.* 2001; Mathiesen *et al.* 2008). Previous studies in Gram-positive bacteria have shown that the secretion efficiency depends not only on the SP but also on the protein to be secreted and on bacterial host (Dieye *et al.* 2001; Mathiesen *et al.* 2008). Nonetheless, studies on the SP functionality for heterologous protein secretion in *Lactobacillus plantarum* WCFS1 found that the homologous SPs have similar or higher secretion efficiencies than those of heterologous origin (Mathiesen *et al.* 2008). Aimed to express and secrete M2e:HBc in *L. casei*, we screened for homologous SPs using a bioinformatics approach. In our hands, the best SP predictor is SignalP4.1 (Petersen *et al.* 2011). This program has been successfully used to select efficient SPs from the protein pool of *L. plantarum* WCSF1 (Mathiesen *et al.* 2008). The SubLoc, PSORTb and Gpos-mPLoc programs were also used to predict the localization of the proteins produced. Using these programs, three putative SPs (CwhSP, SurASP and SP6565) were selected from cell surface-associated proteins of *L. casei* ATCC334 and used for construction of ESVs with GFP as reporter protein. The reason that the SubLoc program predicts CwhSP and SP6565 as periplasmic might be due to the fact that this program uses a dataset based on that of Reinhardt and Hubbard (1998). This dataset contains a small number of sequences of both extracellular and periplasmic groups for training the neural network, which may result in a less accurate prediction. Based on these ESV constructs, it was found that all three SPs can function for GFP secretion in *L. casei* RCEID02, and that the construct using CwhSP provides the highest GFP secretion. Previous studies found that an efficient signal peptide in Gram-positive bacteria generally contains the consensus sequence Vla-X-Ala↓Ala or Ala-X-Ala↓Ala as the SP cleavage site (Nielsen *et al.* 1997; Mathiesen *et al.* 2008). In this study, all three selected SPs have the consensus motif Val-X-Ala at position −3 to −1 relative to the putative cleavage site. However, none of the selected SPs contained Ala at position +1. In addition, the most efficient SP, CwhSP, harbors Ser at position +1, which indicates Ala at this position is not required. CwhSP was further used to construct an ESV containing M2e:HBc. Recombinant *L. casei* carrying this construct expressed and secreted M2e:HBc successfully, demonstrating the usefulness of bioinformatics to predict and select novel SPs for *L. casei*. However, this approach does not guarantee that the SPs selected will function, since protein secretion depends on the SP itself, the genetic background of the host strain and the target protein. In addition, secretion efficiency may be affected by the extent to which protein production levels and rates are adapted to the capacity of the translocation machinery, the influence of SP sequence on mRNA stability and its translation efficiency (Mathiesen *et al.* 2008). For these reasons, to maximize the production of a secreted protein in *Lactobacillus*, and before optimization and balancing of other factors,

it might be worthwhile selecting an optimal SP for each individual protein.

Besides conferring only short-term immunity, a major drawback of the current influenza virus A vaccines is that they confer only short-term subtype-specific protection. Thus, keeping vaccines up-to-date with influenza A antigens requires constant monitoring of the subtypes of the circulating viruses (Johansson and Brett 2007). This inconvenience has led to much discussion about the development of a universal influenza A vaccine, able to provide protection against all virus subtypes (Du, Zhou and Jiang 2010). Such a vaccine would require identification of a viral component conserved through all subtypes of influenza A virus for eliciting a broad-spectrum immunity (Stanekova and Vareckova 2010). The extracellular domain of the M2 protein (M2e) of the influenza virus is one such conserved component (Ebrahimi and Tebianian 2011). However, this is a short peptide of only 24 amino-acid residues. In this study, the M2e-encoding gene was fused with the gene coding for the hepatitis B core antigen (HBc) protein in order to increase its immunogenicity. In a previous study, strong immunogenicity and full protection were obtained in mice after either intraperitoneal or intranasal administration of the M2e:HBc fusion protein (De Filette *et al.* 2005). Considering their GRAS status, the use of *L. casei* as an antigen delivery vehicle can confer great advantage for the expression of the M2e:HBc fusion protein.

As a conclusion, in this study, we used a bioinformatics approach to select potential SPs from the *L. casei* ATCC334 genome. The three selected SPs, which include those of Cwh, SurA and 6565 proteins, proved to direct secretion of the GFP in *L. casei*. CwhSP, which showed the highest secretion efficiency, was then used to develop an ESV construct expressing and secreting the fused M2e:HBc protein. This SP and the ESV developed may further serve for the expression and secretion of a variety of homologous and heterologous proteins in *L. casei* and surely in other LAB species.

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Conflict of interest. None declared.

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