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Development of a Food-Grade Integration Vector for Heterologous Gene Expression and Protein Secretion in *Lactococcus lactis*

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Abstract A food-grade integration vector based on site-specific recombination was constructed. The 5.7-kb vector, pIMA20, contained an integrase gene and a phage attachment site originating from bacteriophage A2, with the α-galactosidase gene from *Lactobacillus plantarum* KCTC 3104 as a selection marker. pIMA20 was also equipped with a controllable promoter of *nisA* (P_{nisA}) and a signal peptide-encoding sequence of *usp45* (SP_{usp45}) for the production and secretion of foreign proteins. pIMA20 and its derivatives mediated site-specific integration into the *attB*-like site on the *Lactococcus lactis* NZ9800 chromosome. The vector-integrated recombinant lactococci were easily detected by the appearance of blue colonies on a medium containing X-α-gal and also by their ability to grow on a medium containing melibiose as the sole carbon source. Recombinant lactococci maintained these traits in the absence of selection pressure during 100 generations. The α-amylase gene from *Bacillus licheniformis*, lacking a signal peptide-encoding sequence, was inserted downstream of P_{nisA} and SP_{usp45} in pIMA20, and the plasmid was integrated into the *L. lactis* chromosome. α-Amylase was successfully produced and secreted by the recombinant *L. lactis*, controlled by the addition and concentration of nisin.

Key words: *Lactococcus lactis*, food-grade integration expression/secretion vector, site-specific integration, α-galactosidase, *nisA* promoter

The industrially important *Lactococcus lactis*, which is used worldwide for producing a variety of fermented dairy products, biobased industrial products, and probiotics [8, 17], imparts important product qualities such as organoleptic properties, digestibility, texture characteristics, and food value. Therefore, the genetics and physiology of lactococci have been studied extensively, and genetic tools involving plasmids have been used for the construction of recombinant lactococci [5, 8]. However, plasmids often lose their characteristic gene traits because of the presence of segregational instability in the lactococcal plasmids [32]. This genetic instability may result in starter culture failure, which can cause significant financial costs in the dairy industry and can act as a potential obstacle to the industrial use of genetically modified lactococcal strains.

One strategy for achieving genetic stabilization is integrating foreign genes into the lactococcal chromosome of the corresponding genes. However, the recombination systems that target homologous chromosomes [6, 22, 37] have drawbacks, such as low integration efficiency, instability, and unpredictable integration sites. In contrast, integration systems based on site-specific recombination can often avoid these unwanted features of homologous recombination. Therefore, site-specific recombination has been the optimal choice for implementing chromosomal insertions in many bacterial species, in particular where integrants with high integration efficiency, high specificity, and high stability are demanded [1, 2, 4, 10, 13, 27].

Only a few integrative food-grade systems have been constructed for lactococci [12, 24, 38], all of which were based on homologous recombination. Moreover, substitutive selection markers adopted by these integrative food-grade systems for lactococci showed limited efficacy since they were based on bacteriocin resistance, sugar utilization, or nonsense suppressors [9, 24]. The phenotypes of these markers are widespread in several *Lactococcus* strains. Therefore, fermentation phenotypes of the metabolism of rare sugars such as melibiose and raffinose may represent attractive food-grade selection markers.

In this study, we used site-specific integration to construct more efficient and industrially feasible food-grade vectors

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for lactococci. The constructed food-grade integration vector, pIMA20, contained the α-galactosidase gene (*melA*) from *Lactobacillus plantarum* KCTC 3104 as a selection marker. In addition, to ensure the efficient expression and secretion of a relevant gene, the pIMA20 contained a nisin-inducible promoter of *nisA* (P_{nisA}) [7], a signal peptide-encoding sequence of *usp45* (SP_{usp45}) [39], and multiple coning sites (MCS). Finally, we have demonstrated that a foreign protein was successfully expressed and secreted using the pIMA20 vector system.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* EC101 and *L. lactis* NZ9800 were used as hosts and grown in Luria-Bertani broth [26] and M17 broth (Difco, Sparks, MD, U.S.A.) supplemented with the appropriate sugar, respectively. Food-grade *L. lactis* recombinants activating *melA* as a selection marker were detected on BCP medium (2% tryptone, 0.5% yeast extract, 0.4% NaCl, 0.15% Na-acetate, and 40 mg/l bromocresol purple) containing 0.5% melibiose and on M17 agar containing X-α-gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside; 4 mg/ml). Ampicillin (100 μg/ml) and erythromycin (200 μg/ml) were employed as antibiotics for *E. coli*, and erythromycin (5 μg/ml) and chloramphenicol (5 μg/ml) used for *L. lactis*.

DNA Manipulation Procedures

Genomic DNA from *L. lactis* strains was extracted using a DNeasy Tissue kit (Qiagen, Hilden, Germany). *E. coli* was transformed according to the method of Hanahan and Meselson [11], and *L. lactis* was transformed by electroporation as described by Holo and Nes [14] with a gene pulser (Bio-Rad, Hercules, CA, U.S.A.).

PCR Primers and Amplification

PCR primers were designed on the basis of known DNA sequences, and relevant restriction enzyme sites were introduced when needed (Table 2). PCR was conducted with 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and elongation at 72°C for 3 min, with a commercial PCR system (GeneAmp 2700, Foster, CA, U.S.A.), using standard procedures in the reaction conditions as recommended by the manufacturer for Ex *taq* polymerase (Takara, Kyoto, Japan).

Plasmid and Integrants Construction

A schematic of the construction of the integrative food-grade vector pIMA20 is presented in Fig. 1. The constructed plasmids were verified by enzyme digestion and DNA sequencing.

The *melA* gene was amplified from pFM2 using the melA2F and melA2R primers designed to have an SphI site [16]. The amplicon was digested with SphI and inserted into the same site of pORI19, and the resulting plasmid was called pIM1.

pEM76 [31] containing the bacteriophage attachment site (*attP*) and the integrase gene (*int*) originating from bacteriophage A2 were amplified with primers attPF and attPR containing the ApaLI site and inserted into pIM1 after being digested with the same enzyme. The resulting plasmid was named pIMA.

Then, the fragment containing P_{nisA} [7], SP_{usp45} [39] for the secretion of an expressed foreign protein, and MCS for the easy insertion of foreign protein genes were amplified

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Origin/ reference
E. coli EC101	*E. coli* JM101 with *repA* from pWV01 integrated in chromosome	[21]
L. lactis NZ9800	Nisin-producing transconjugant containing Tn*5276*; Δ*nisA*, Lac^-, Mel^-	[18]
L. lactis NZ9800::pIMA20	*L. lactis* NZ9800 with pIMA20 integrated in chromosome	This study
L. lactis NZ9800::pIMA20:BLA	*L. lactis* NZ9800 with pIMA20:BLA integrated in chromosome	This study
pORI19	Suicide vector, ORI^+, $RepA^-$	[21]
pVE6007	Temperature-sensitive derivative of pWV01, $RepA^+$, Cm^r	[29]
pEM76	pUC19E-borne *six1* A2 int-*attP six2*	[31]
pFM2	*L. lactis*/*E. coli* shuttle vector containing the *melA* gene	[16]
pFMN20	*L. lactis*/*E. coli* shuttle vector containing P_{nisA}, SP_{usp45}, and MCS	[16]
pGS40	*L. lactis*/*E. coli* shuttle vector containing an α-amylase gene lacking signal peptide	[15]
pIM1	pORI19 derivatives containing the *melA* gene	This study
pIMA	pIM1 derivatives containing the *attP-int* gene	This study
pIMA10	pIMA derivatives containing P_{nisA}, SP_{usp45}, and MCS	This study
pIMA20	Integrative food-grade expression/secretion vector, pIMA20 derivatives eliminating Em^r gene	This study
pIMA20:BLA	pIMA20 derivatives containing an α-amylase gene lacking signal peptide	This study

Am^r, ampicillin-resistance gene; Em^r, erythromycin-resistance gene; *melA*, α-galactosidase gene; *int*, integrase gene; *attP*, attachment site of the phage.

Table 2. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'→3')	Specificity	Source
melA2F	GGCATGCGGTCCTCGGGATATGATAAGATTAA	*melA* gene	This study
melA2R	TGCATGCTCCCGGCCGCCATGGCGGCC	*melA* gene	This study
attPF	CCGTGCACGCTGGATACAAAATAAAAAGCGCCT	*int* gene	This study
attPR	CCGTGCACTTGTGTGCCCATATTTCTGAACTCT	*attP* gene	This study
nis2F	GGAAGCTTGTTAACGGCTCTGATTAAATTC	P_{nisA}	This study
nis2R	CCAAGCTTGGATCCCGGGCGCCGGCTCG	MCS	This study
attS	CCCTCTAGGGGCTTTGATACCGGTGATC	attR	This study
int2	CTGGGATCCCCAAGGCTTACTTT	*int* gene	[31]
NcoI-1	TCAGTACGTATACATTCCGTGATACCCCGATC	Upstream of *attR* gene	This study
NcoI-2	GATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACG	Upstream of *attR* gene	This study
NcoI-3	TCAGATTAGTTTAGAACGGGACCCAAT	Downstream of *attL* gene	This study
NcoI-4	TGCAAGAATGCCGGTTTAAAGCCATTTCTGAGCAC	Downpstream of *attL* gene	This study
BLAF	ATCTAGAAGCTGCGGCGGCAAATCTTA	α-Amylase gene lacking signal peptide	[16]
BLA3R	CAGTACTCTATCTTTGAACATAGATCG	α-Amylase gene lacking signal peptide	This study
BLA2F	AGCTGCGGCGGCAAATCTTA	α-Amylase gene lacking signal peptide	This study
BLA2R	CTATCTTTGAACATAGATCG	α-Amylase gene lacking signal peptide	This study

Underlines indicate nucleotides participating in restriction sites.

with HindIII site-containing primers nis2F and nis2R from pFMN20 [16]. The amplicon was digested with HindIII and inserted into the same site of pIMA, and the resulting plasmid was named pIMA10. The erythromycin-resistance gene was eliminated from pIMA10 by digestion with HpaI and ligation. Consequently, plasmid pIMA20 without an antibiotic-resistance marker was constructed as an integrative food-grade expression/secretion vector.

To integrate pIMA20 into chromosomal DNA, the plasmid was introduced into *L. lactis* NZ9800 (pVE6007), and the melibiose-utilizing and chloramphenicol-resistance colonies were obtained. The transformants were incubated in M17 broth containing 0.5% melibiose at 30°C for 90 min, and then they were incubated at a nonpermissive temperature for pVE6007 (*i.e.*, 37°C) overnight. Temperature-shift-treated transformants were first spread on M17 agar containing 0.5% melibiose, and forming colonies were moved to M17 agar containing 0.5% melibiose and to M17 agar containing chloramphenicol, from which the chloramphenicol-sensitive and melibiose-utilizing colonies were selected (Fig. 2).

For Southern hybridization, *L. lactis* DNA was digested with NcoI or XbaI and blotted on a Hybond nylon membrane (Amersham, Uppsala, Sweden). The DNA probe preparations, hybridization, washing, and staining were performed using enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection systems (Amersham, Uppsala, Sweden) as recommended by the manufacturer.

Inverse PCR

An inverse PCR strategy was used to obtain the integrated site of the *L. lactis* chromosome by an integration vector based on *attP* obtained from bacteriophage A2. The total DNA of the integrant pIMA20 was digested with the restriction enzyme NcoI. This restriction fragment was converted into circles by intramolecular ligation, with the circularized DNA then being used as a template (at 1 ng/ml) in the PCR. The positions and sequences of the primers (NcoI-1, NcoI-2, NcoI-3, and NcoI-4; Table 2, Fig. 2) were constructed from pIMA20 nucleotide sequences. The inverse PCR was conducted with 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 2.5 min with Ex *taq* polymerase (Takara, Kyoto, Japan).

Assays of α-Amylase

The α-amylase activity was qualitatively determined on agar plates containing soluble starch with iodine solution (0.3% I_2+0.6% KI), with the formation of clear zones (halos) examined around colonies of transformants. The quantitative enzyme activity was assayed at 60°C in 50 mM sodium phosphate buffer (pH 7.0) using 3,5-dinitrosalicyclic acid according to Miller [33]. Soluble starch (0.5%) was used as a substrate in the hydrolysis reaction for the quantitiative enzyme assay, with one unit of α-amylase activity quantified as the amount of enzyme that produced 1 μmol of glucose from the soluble starch at 60°C in 1 min. Protein concentrations were measured by the BCA protein assay (Pierce, Erembodegem, Belgium) using BSA as a standard.

Western Blot Hybridization

For Western blotting, proteins were transferred from SDS-PAGE gels onto nitrocellulose membranes (0.45 μm pore size; Life Science, Pensacola, FL, U.S.A.). The protein concentration was determined using the DC assay kit (Bio-Rad). Bands corresponding to α-amylase were detected

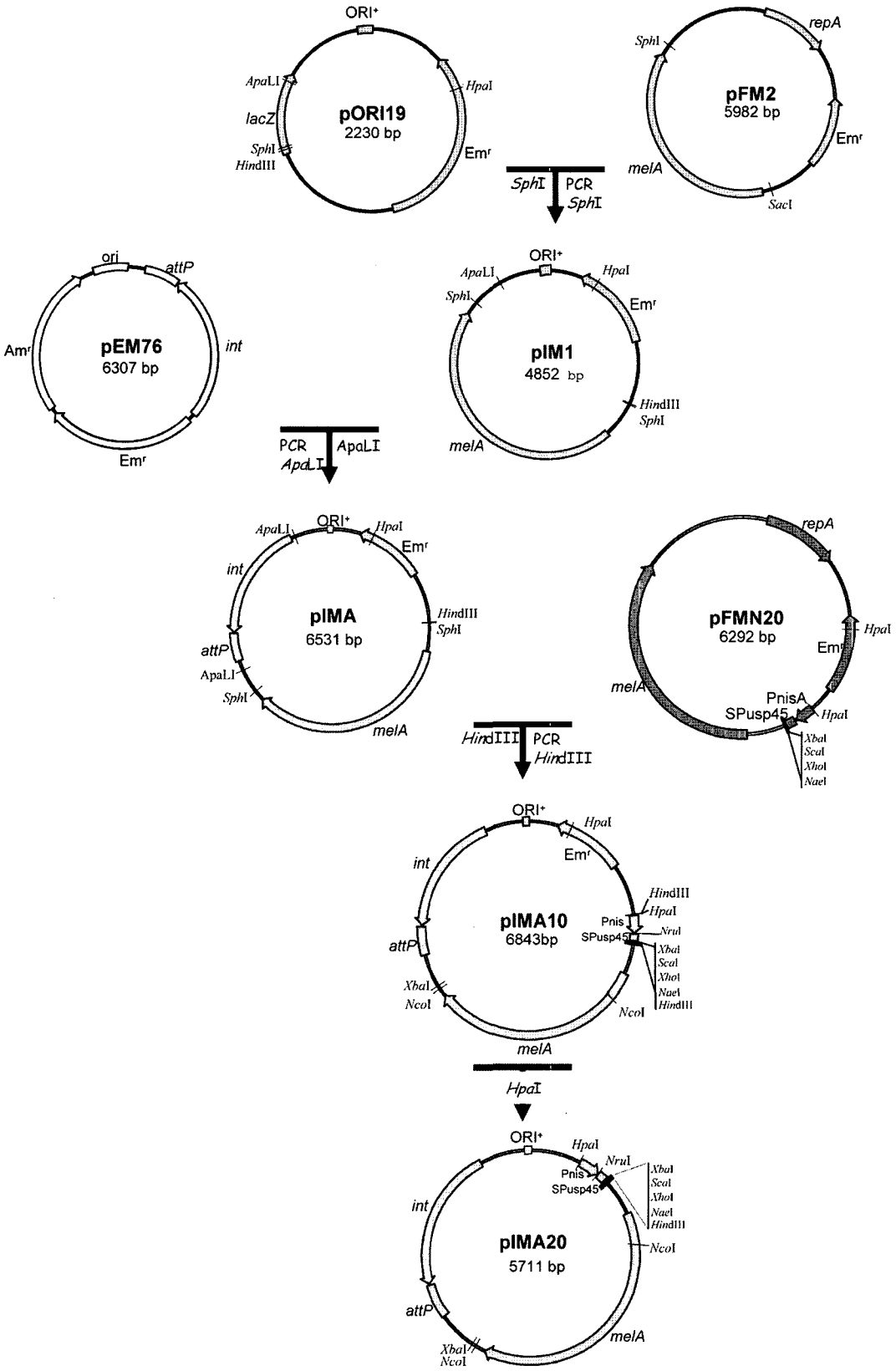

Fig. 1. Construction of an integrative food-grade expression/secretion vector using *melA* as a selection marker.
Symbols: ORI$^+$, replication origin of Gram-positive bacteria; Em^r, erythromycin-resistance gene; Am^r, ampicillin-resistance gene; *melA*, α-galactosidase gene; *attP*, attachment site of the phage; *int*, integrase gene; P_{nisA}, nisin-inducible promoter; SP_{usp45}, signal peptide-encoding sequence of *usp45*.

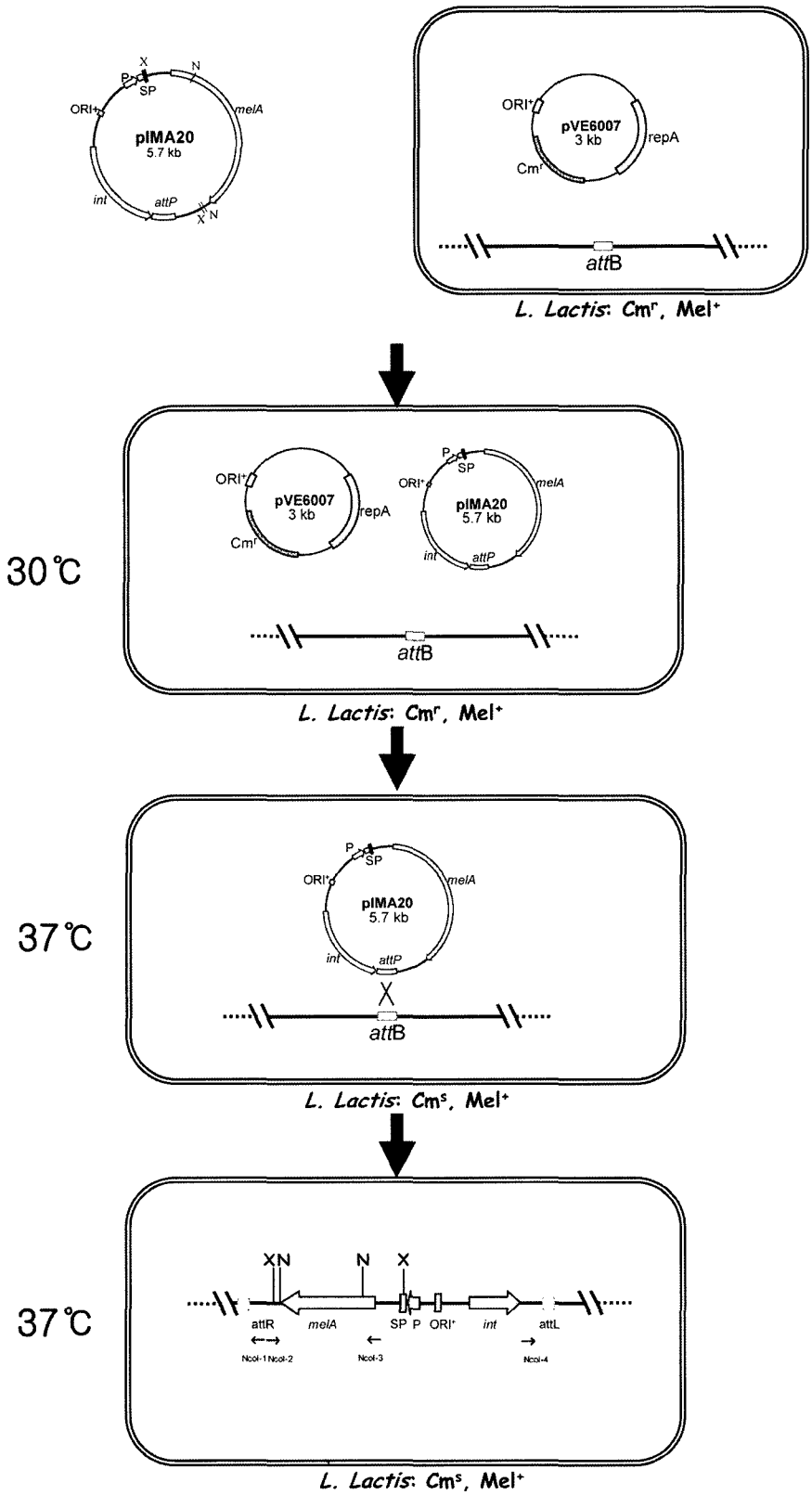

Fig. 2. Schematic of the site-specific integration of pIMA20 into the chromosomal DNA of *L. lactis* NZ9800.
Symbols: ORI$^+$, replication origin of Gram-positive bacteria; Cm^r, chloramphenicol-resistance gene; *melA*, α-galactosidase gene; P, nisin-inducible promoter; SP, signal peptide-encoding sequence of *usp45*; *attP*, attachment site of the phage; *attB*, attachment site of bacteria; *int*, integrase gene; *attL* and *attR*, regions flanking the inserted prophage on the left and right, respectively; X, XbaI; N, NcoI.

with rabbit anti-α-amylase polyclonal antiserum (Takara Korea Biomedical, Sungnam, Korea) and anti-rabbit-horseradish-peroxidase conjugate using an ECL detection kit (Amersham).

Evaluation of the Stability of the Integrants

The segregational stability was determined using *L. lactis* NZ9800::pIMA20 and *L. lactis* NZ9800::pIMA20:BLA. These integrants carry *melA*, which confers melibiose fermentation ability, as a selection marker. A single colony was used to inoculate the selection-pressure-free M17 medium containing 0.5% glucose (GM17), and the culture was grown at 30°C overnight. The saturated culture was diluted 1,000-fold using fresh GM17 medium without selection pressure. This consecutive transfer of culture into fresh GM17 was performed every 24 h using the same dilution ratio. Diluted culture samples were plated for single cells on M17 agars at various intervals and incubated at 30°C overnight. Colonies were plated onto M17 medium containing 0.5% melibiose to check for the presence of integrants. Southern hybridizations were then performed on samples from several independent colonies to confirm the integration.

RESULTS

Construction of an Integrative Food-Grade Expression/Secretion Vector

The integrative food-grade expression/secretion vector pIMA20, consisting entirely of DNA sequences derived from lactic acid bacteria (LAB) except for MCS, was constructed for the transfer of a specific gene into chromosome through site-specific recombination (Fig. 1). pIMA20 was comprised of a *melA* gene as a selection marker, a broad-host-range replication origin site (ORI^+) from the *L. lactis* Wg2 promiscuous plasmid pWV01 [23], the *attP* site and *int* gene from bacteriophage A2, P_{nisA} [7] for the expression of foreign gene, SP_{usp45} [39] for the secretion of a synthesized foreign protein, and MCS.

Generation of a Food-Grade Recombinant *L. lactis* Strain

The constructed pIMA20 plasmid – which does not replicate without complementation of *repA*, the gene for the pWV01 replication protein [23] – was introduced into *L. lactis* NZ9800 harboring the thermally sensitive plasmid pVE6007 containing *repA* under the control of promoter P23 [29]. Transformants containing both plasmids were selected on M17 agar containing melibiose and chloramphenicol. pVE6007 was cured by changing the temperature from 30°C to 37°C, which led to the integration of a suicide vector (of the pORI19 derivatives) into chromosomal DNA [21]. A schematic presentation of the transformation and integration of pIMA20 is shown in Fig. 2.

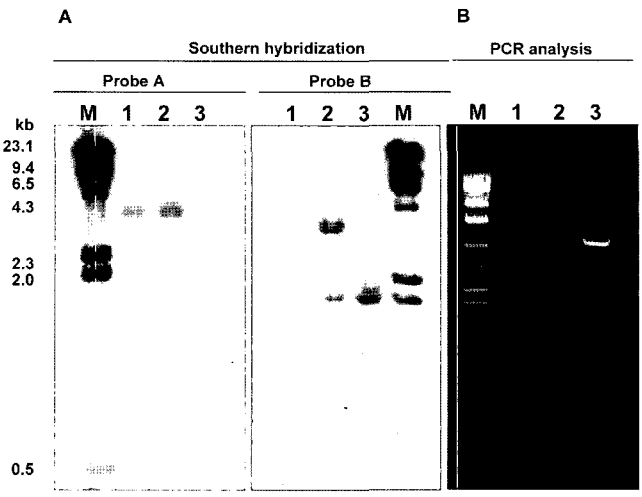

Fig. 3. Confirmation of the integration of pIMA20 into the chromosomal DNA of *L. lactis* NZ9800 by Southern hybridization (**A**) and PCR (**B**).
Lanes: M, molecular size marker; 1, *L. lactis* NZ9800 (pVE6007); 2, *L. lactis* NZ9800 (pVE6007 and pIMA20); 3, *L. lactis* NZ9800::pIMA20.

The integration of pIMA20 occurred in an orientation-dependent manner in the genomic DNA of *L. lactis* NZ9800, as confirmed by Southern hybridization (Fig. 3A). EcoRI-digested pVE6007 (Probe A) was used to identify helper-plasmid curing, and *melA* containing DNA fragment retrieved from the XbaI-digested pIMA20 (Probe B) was used to identify the plasmid integration.

Probe A was hybridized with the DNA from *L. lactis* NZ9800 (pVE6007) and *L. lactis* NZ9800 (pVE6007 and pIMA20), and the size of the hybridized band was consistent with that of pVE6007. However, the probe was not complementary to the NcoI-digested total DNA from the pIMA20 integrant (Fig. 3A).

In the hybridization with probe B, the 2-kb hybridized bands resulted from the NcoI-digested total DNA from *L. lactis* NZ9800 (pVE6007 and pIMA20) and the pIMA20 integrant, and another 3.7-kb band that did not hybridize was detected from *L. lactis* NZ9800 (pVE6007 and pIMA20), which corresponds to the XbaI fragment of pIMA20 that does not contain *melA*. The two bands were due to the NcoI digestion of pIMA20 yielding two DNA fragments, both of which contained a partial fragment of *melA* (Fig. 3A).

To identify the integrated position of pIMA20 in *L. lactis* NZ9800, inverse PCR experiments were performed using the primer pairs NcoI-1/NcoI-2 and NcoI-3/NcoI-4 (Fig. 2). Bands at 3.6 and 6.3-kb were obtained from each PCR amplification, and the corresponding nucleotide sequences were determined after cloning. These sequences showed 100% homology with transposase and inactivated derivatives from *L. lactis* subsp. *cremoris* SK11 (NCBI accession no: ZP_00383099.1) and 96% homology with an unknown of protein *L. lactis* (accession no: AAX19714.1).

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TGATAACGGTGTAGGGGGG   LcLN
TGATAcCGGt gat cGGgGt Consensus
TGATACCGGTGATCGGGGT   LbCa
TGATACCGGTGATCGGGGT   LbPc
TGATACCGGTGATCGGGGT   LbPt
TGATACCGGCTGTTGGAGC   LcLI
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Fig. 4. The *attB* core sequence of *L. lactis* NZ9800 that was integrated by the *attP-int* gene of bacteriophage A2.
LcLN, *L. lactis* NZ9800; LbCa, *Lb. casei* ATCC 393; LbPc, *Lb. paracasei* ATCC 27092; LbPt, *Lb. plantarum* CECT 3801; LcLI, *L. lactis* IL1403.

Moreover, the integrated core sequence from pIMA20 exhibited high similarity with another known integrated 19-bp core sequence from bacteriophage A2 (Fig. 4). No DNA amplification was observed from the total DNA extracted from *L. lactis* NZ9800 (pVE6007) and *L. lactis* NZ9800 (pVE6007 and pIMA20) by PCRs using primers attS and int2, which are complementary to *attR* and *int* (Fig. 3B). A 4.8-kb DNA fragment was amplified when the *L. lactis* NZ9800::pIMA20 DNA was used as the template. These results confirmed the integration of pIMA20 into *L. lactis* NZ9800 chromosome and the curing of pVE6007 in the integrant simultaneously in a nonpermissive condition.

Evaluation of a Food-Grade Recombinant *L. lactis* Strain

To evaluate the usefulness of pIMA20 in gene cloning, controlled expression, and secretion, an α-amylase gene lacking the signal peptide from *Bacillus licheniformis* was amplified from pGS40 [15] with BLAF and BLA3R primers containing XbaI and ScaI restriction sites, respectively, and inserted into the same sites of pIMA20. The resulting plasmid pIMA20:BLA was introduced into *L. lactis* NZ9800 (pVE6007), and then recombinant *L. lactis*::pIMA20:BLA was selected on melibiose-containing M17 agar by increasing the temperature from 30°C to 37°C. The selected colonies formed halos on plates containing soluble starch (Fig. 5A). Total DNA was extracted from relevant strains and PCR amplification was done with primers attS and int2. As expected, a 6.3-kb DNA fragment was amplified (Fig. 5B).

Total DNA from *L. lactis* NZ9800::pIMA20 and *L. lactis* NZ9800::pIMA20:BLA was digested with XbaI and probed with the *melA*-containing DNA fragment retrieved from the XbaI-digested pIMA20 (Probe B) and the α-amylase gene amplicon (Probe C) with BLA2F and BLA2R from pGS40 by Southern hybridization (Fig. 5C). In the case of *L. lactis* NZ9800::pIMA20:BLA, the band hybridized with probe B (*i.e.*, 4.3-kb) was shifted up as that of the α-amylase gene from the 2.8-kb DNA fragment. When the probe C was used for hybridization, a 4.3-kb band hybridized with the total DNA appeared only from *L. lactis* NZ9800::pIMA20:BLA, which corresponds to the total size of the α-amylase gene and *melA*.

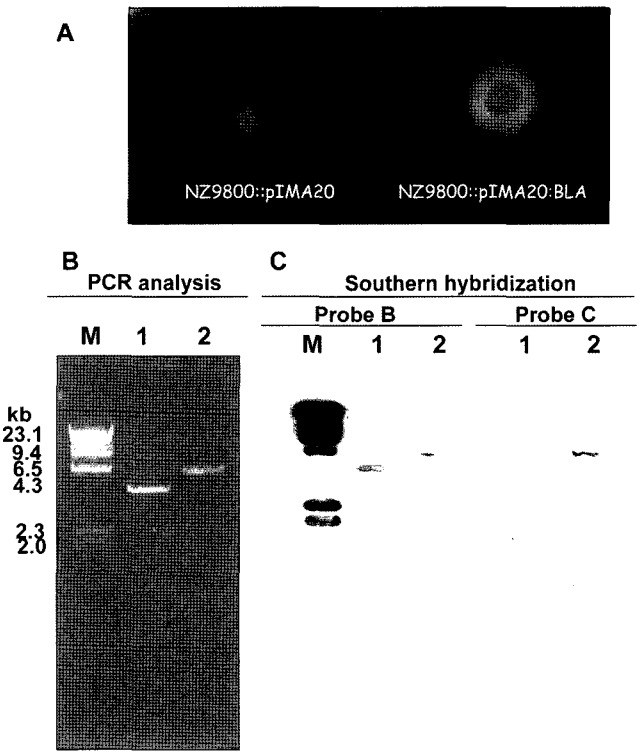

Fig. 5. Confirmation of the integration of pIMA20:BLA into the chromosomal DNA of *L. lactis* NZ9800 by agar assay using the iodine solution (**A**), PCR (**B**), and Southern hybridization (**C**).
Lanes: M, molecular size marker; 1, *L. lactis* NZ9800::pIMA20; 2, *L. lactis* NZ9800::pIMA20:BLA.

The function of the P_{nisA} and SP_{usp45} in the recombinant cell was tested on the basis of nisin induction and α-amylase secretion (Fig. 6). The results of the qualitative assay of α-amylase secretion from *L. lactis* NZ9800::pIMA20:BLA are shown in Fig. 5A. *L. lactis* NZ9800::pIMA20 did not form haloed colonies, whereas *L. lactis* NZ9800::pIMA20:BLA exhibited clear halos around the colonies, indicating the amylolytic activity. The α-amylase gene expression under P_{nisA} from *L. lactis* NZ9800::pIMA20:BLA was strongly nisin concentration dependent up to 10 ng/ml, and increased only slightly at higher concentrations (Fig. 6A). This suggests the presence of controllable activity of the *nisA* promoter. Moreover, α-amylase was efficiently secreted into the medium, as judged by the fraction of enzyme activity in the supernatant relative to the enzyme activity of the whole-cell fraction. The secretion efficiencies of *L. lactis* NZ9800::pIMA20:BLA were higher than 90% at all five nisin concentrations tested (Fig. 6A). Moreover, the expressed and secreted α-amylase was analyzed by Western blot hybridization using anti-α-amylase antibodies. The corresponding bands of α-amylase were detected at different nisin concentrations in both the whole-cell and supernatant fractions (Fig. 6B), which implies that SP_{usp45} successfully directed the secretion of α-amylase in *L. lactis*.

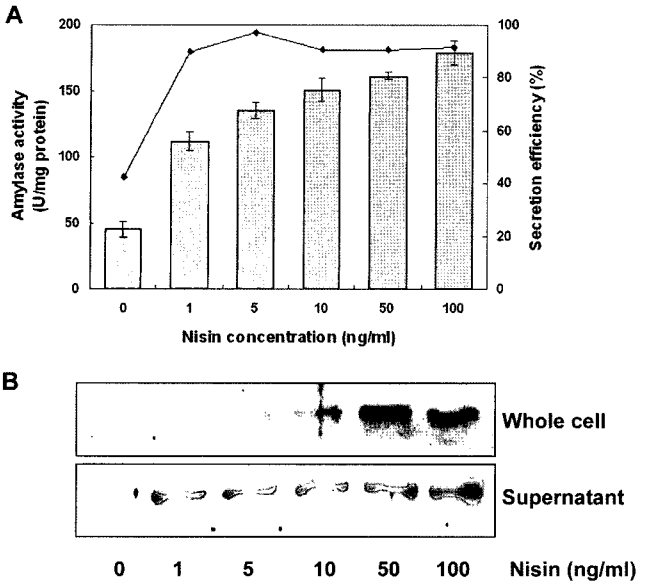

Fig. 6. The influence of SP_{usp45} and P_{nisA}.
Quantitative α-amylase activity (black bar) and the secretion efficiency (line) (**A**), and Western blot hybridization (**B**) of *L. lactis* NZ9800:: pIMA20:BLA grown for 18 h in an M17 broth containing 0.5% melibiose in the presence of nisin at different concentrations.

DISCUSSION

The safe use of genetically modified LAB in the dairy industry requires the use of cloning systems composed solely of DNA from food-grade organisms, and without the use of antibiotic-resistance genes in the selection process. The sugar fermentation phenotypes such as sucrose and lactose metabolism of LAB have mostly been used as an alternative to an antibiotic marker in the construction of food-grade cloning systems [9, 24]. However, the widespread existence of both phenotypes among lactococcal strains limits their usefulness.

LAB however have an exceptional reservoir of peculiar sugar fermentation phenotypes that have not been employed as selection markers in the construction of cloning vectors. Despite the diversity of organic compounds that collectively can be metabolized by LAB, most strains and species can only grow on a small number of substrates [28]. Therefore, a dominant marker based on the fermentation phenotype of rare sugars (*e.g.*, melibiose and raffinose) represents an ideal option in the construction of a novel food-grade vector [31]. We have previously constructed the food-grade vector pFMN30 using *melA*, which is responsible for the hydrolysis of melibiose, as a selection marker [16]. The lactococcal transformants containing the plasmid outgrew nontransformants on a medium containing melibiose, and formed blue colonies on a medium containing X-α-gal.

In the previous study, the loss of pFMN30 was approximately 0.15% after 20 generations under nonselective pressure, and the significant instability of the plasmid hindered its application. To resolve this problem, we have constructed an integrative food-grade vector, pIMA20, containing the *melA* gene as a selection marker. The integrants *L. lactis* NZ9800::pIMA20 and *L. lactis* NZ9800::pIMA20:BLA did not lose their newly acquired traits during 100 generations of growth in the absence of selective pressure.

A few controllable gene expression systems for lactococci have been developed [7, 20, 38]. One of the most reliable systems is based on the food-grade inducer nisin, in which subinhibitory doses of nisin increase the expression level by up to 1,000-fold [7, 20]. Nisin production in lactococci is autoregulated by nisin and 11 genes, involved in nisin synthesis, posttranslational modification, transport, and immunity, from three operons. Expression of *nisA* and *nisF* are induced by nisin via a two-component regulatory system involving histidine protein kinase (NisK) and response regulator (NisR). In some *L. lactis* strains, such as the NZ9800 used in this work, the endogenous genetic elements needed for the control of gene expression by nisin are found to be conserved [19].

Although heterologous proteins have been synthesized in *L. lactis* by using nisin-inducible promoters, without an appropriate secretion system for the foreign protein, continuous high-level production of a foreign protein could lead to its intracellular accumulation, aggregation, or degradation in the cytoplasm [30]. Signal peptides for lactococci have been characterized and employed to solve the problem [15, 34, 35]. *L. lactis* secretes a few proteins, but until now, only Usp45 has been fully characterized for its secretion signal and the sequence employed for the secretion of heterologous proteins [39]. Several expression/ secretion vectors using P_{nisA} and SP_{usp45} have recently been reported [3, 25, 36]. The pFMN30 constructed previously was equipped with P_{nisA}, SP_{usp45}, and MCS for the expression and secretion of foreign proteins. It was shown that the synthesis and secretion of the foreign protein, α-amylase from *B. licheniformis*, was successfully achieved by using P_{nisA} and SP_{usp45}. The pIMA20 developed in this study is also composed of P_{nisA}, SP_{usp45}, and MCS, so as to ensure efficient gene expression and secretion of proteins. pIMA20:BLA was tried to be expressed at different nisin concentrations. The α-amylase activity of cells harboring pIMA20:BLA was lower than that of pFMN30:BLA, which is attributable to the low copy number of integrated pIMA20:BLA. However, the range of nisin concentrations at which α-amylase expression was induced was broader for pIMA20:BLA than for pFMN30:BLA. In addition, α-amylase was secreted by the Usp45 signal peptide, with a secretion efficiency of higher than 90% from the cytoplasm, when determined by the enzymatic assay.

One of the recently developed food-grade gene delivery systems for lactococci is the utilization of the *attP* site and *int* gene from bacteriophage A2. This method, a site-

specific recombination system for in *Lactococcus* and *Lactobacillus* [1, 31], has demonstrated a high efficiency of integration and high specificity and stability. We constructed an integration vector based on site-specific recombination using the *attP* site and *int* gene from a bacteriophage A2 as an alternative, and a more reliable approach for maintaining the desired genetic elements. pIMA20 was inserted into the unknown gene (not the 3' end of $tRNA^{Leu}$) in *L. lactis* NZ9800. Nevertheless, the *attB* core sequence was identified. In summary, we successfully obtained site-specific recombinants by using a suicide-derived plasmid, without involving the use of thermosensitive vectors.

The nucleotide sequence of the developed vector shows that it consists entirely of *L. lactis* DNA except for MCS, thereby it can be regarded as a GRAS-class vector to be used to improve lactococci by self-cloning. This will allow the further development of lactococci strains as acceptable hosts for the production of proteins, peptides, or metabolites for the food industry.

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