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Use of the Cellulase Gene as a Selection Marker of Food-grade Integration System in Lactic Acid Bacteria

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Abstract The application of the cellulase gene (*celA*) as a selection marker of food-grade integration system was investigated in *Lactobacillus* (*Lb.*) *casei*, *Lactococcus lactis*, and *Leuconostoc* (*Leu.*) *mesenteroides*. The 6.0-kb vector pOC13 containing *celA* from *Clostridium thermocellum* with an integrase gene and a phage attachment site originating from bacteriophage A2 was used for site-specific recombination into chromosomal DNA of lactic acid bacteria (LAB). pOC13 was also equipped with a broad host range plus replication origin from the *lactococcal* plasmid pWV01, and a controllable promoter of *nisA* (P_{nisA}) for the production of foreign proteins. pOC13 was integrated successfully into *Lb. casei* EM116, and pOC13 integrants were easily detectable by the formation of halo zone on plates containing cellulose. Recombinant *Lb. casei* EM116::pOC13 maintained these traits in the absence of selection pressure during 100 generations. pOC13 was integrated into the chromosome of *L. lactis* and *Leu. mesenteroides*, and *celA* acted as an efficient selection marker. These results show that *celA* can be used as a food-grade selection marker, and that the new integrative vector could be used for the production of foreign proteins in LAB.

Keywords: integration vector, cellulase, lactic acid bacteria, *Lactobacillus casei*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, site-specific recombination

Introduction

Lactic acid bacteria (LAB) have been used worldwide for fermented foods such as milk products (e.g., cheese and yogurt), beverages, and vegetables (e.g., sauerkraut and *kimchi*). LAB also reportedly exhibit probiotic, antitumor, and immunopotentiating activities (1-6). This has resulted in extensive investigations of the genetics and physiology of lactococci, and genetically modified LAB have been developed for various industrial applications such as dairy products, fermented foods, and live vaccines (1,7,8). Since improved LAB and their products are widely used in the production of foods and even medicines, the host strains should be composed solely of DNA from food-grade organisms and devoid of any antibiotic-resistance markers. Several food-grade selection markers have been developed for LAB, and these can be categorized into dominant (e.g., bacteriocin or heavy metal resistance) and complementary (e.g., lactose or pyrimidine metabolism) markers (8-11). The dominant markers do not affect the gene expression in hosts, and hence they can be used in most wild-type strains (12-14). However, when using dominant markers, the phenotypes used in the selection process naturally occur in numerous lactococcal strains, which consequently limit their host applicability. Meanwhile, the complementation markers require the development of a bacterial mutant with a specific deficiency as a prerequisite, which is complemented by the marker to restore the original phenotype (15,16). Accordingly, a food-grade selection marker based on the phenotype of fermenting rare sugars appears to be an attractive choice. Cellulose is not readily fermented by all species of LAB, and hence genes involved in their utilization can be introduced into nonfermenting hosts that do not have any homologues of these genes. Cellulose fermentation depends critically on the activity of cellulases. Therefore, the cellulase-activity phenotype is regarded as a prospective dominant selection marker for cloning vectors, and it is not necessary to construct the cellulase-deficient hosts.

Several food-grade cloning systems have already been developed for LAB, most of which are based on plasmids (8,13,14). However, plasmids often lose their characteristic genetic traits due to segregational and structural instability under nonselective conditions (17). An alternative strategy for gene stabilization is to integrate appropriate genes into the chromosome. Until recently, integration systems constructed for LAB were based on homologous chromosomal targeting (16,18-20). However, these integration systems have drawbacks including low integration efficiency, instability, and unpredictable integration sites (21-23). 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 method for implementing chromosomal insertions in many bacterial species, particularly where integrants with high integration efficiency, high specificity, and high stability are required (24-26).

A 6.0-kb integration vector pOC13 was constructed using the cellulase gene (*celA*) from *Clostridium thermocellum*

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(27), and the integrase gene (*int*) and attachment site of the phage (*attP*) from temperate bacteriophage A2 (24) as an integration element for site-specific recombination into the chromosomal DNA of LAB. Integrants could be easily distinguished by the presence of clear halo colonies on plates containing CM-cellulase, and were successfully replicated in *Lactobacillus* (*Lb.*), *Lactococcus*, and *Leuconostoc* (*Leu.*) species. The results showed that *celA* can indeed be used as a food-grade selection marker for LAB.

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, *Lactococcus lactis* NZ9800, *Lactobacillus casei* EM116, and *Leuconostoc mesenteroides* C7 were used as hosts. *E. coli* EC101 was grown in Luria-Bertani (LB) medium at 37°C. *Lb. casei* EM116 and *Leu. mesenteroides* C7 were cultivated in deMan Rogosa Sharpe (MRS) medium (Difco, Franklin Lake, NJ, USA) at 37 and 30°C, respectively. *L. lactis* NZ9800 was grown in M17 medium supplemented with 0.5%(w/v) glucose (M17G) at 30°C. The activation of cellulase activity by recombinant LAB was detected on EL1 plates (1% tryptone, 0.4% NaCl, 0.15% sodium-acetate, 0.5% glucose, and 1.5% agar) containing 1% carboxymethylcellulose (CMC) by staining with Congo red solution. Ampicillin (100 μg/mL) and erythromycin (200 μg/mL) were employed as antibiotics for *E. coli*, with erythromycin (5 μg/mL) and chloramphenicol (5 μg/mL) used for LAB.

DNA manipulation procedures Plasmid DNA was isolated using the DNA-spin™ Plasmid DNA purification kit (iNtRON, Seongnam, Korea). Genomic DNA from LAB was extracted using the G-spin™ Genomic DNA extraction kit for Bacteria (iNtRON). *E. coli* was transformed according to the method of Hanahan (28), and LAB were transformed by electroporation as described by Holo and Nes (29) using a Gene Pulser® (Bio-Rad, Hercules, CA, USA), with some modifications. Cells were electroporated in a 0.2-cm cuvette with a Gene Pulser® (Bio-Rad) at 25 μF, 200 Ω, and 2.5 kV for *Lb. casei* and *L. lactis*, and at 25 μF, 400 Ω, and 1.0 kV for *Leu. mesenteroides*. After the single pulse, the DNA was recovered with 0.96 mL of recovery medium (MRS medium containing 0.5 M sucrose and 20 mM $MgCl_2$ for *Lb. casei* and *Leu. mesenteroides*, and M17G medium containing 0.5 M sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ for *L. lactis*) for 2 hr, and then spread on appropriate agar plates for 24-48 hr.

Polymerase chain reaction (PCR) primer and amplification PCR primers were designed based on known DNA sequences, and relevant restriction enzyme sites were introduced when needed (Table 2). PCR amplification comprising 30 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and elongation at 72°C for 3 min was performed with a commercial PCR system (GeneAmp 2700; Applied Biosystems, Foster, CA, USA) using standard procedures in the reaction conditions as recommended by the manufacturer of Ex *taq* polymerase (Takara, Kyoto, Japan).

Plasmid and integrant construction Figure 1 shows the construction scheme of the integration vector pOC13 containing *celA* as a selection marker. The constructed plasmids were verified by enzyme digestion and DNA sequencing.

celA was amplified from pJC5H containing *celA* from *C. thermocellum* (personal communication) with celA-won-F and celA-won-R, and the 1.5-kb amplified fragment containing *celA* was inserted into pGEM-T Easy vector (Promega, Madison, WI, USA), generating pGC1. The pGC1 was digested with *Sac*I and *Sph*I, and inserted into the same site of pORI19, and the resulting plasmid was called pOC1.

The nisin-inducible promoter (P_{nisA}) and multiple cloning

Table 1. Bacterial strains and plasmids used in the present study

Strain or plasmid	Relevant features	Reference
Bacteria		
E. coli EC101	*E. coli* JM101 with *repA* gene from pWV01 integrated into the chromosome	(35)
Lb. casei EM116	*Lb. casei* ATCC393 containing *nisR* and *nisK*	(31)
L. lactis NZ9800	NZ9700 derivative; Δ*nisA*	(36)
Leu. mesenteroides C7	Wild type	(37)
Plasmids		
pORI19	Em^{r} [1], ori+ of pWV01, replicates only in strains providing *repA in trans*	(35)
pJC5H	pJC4 derivative	(personal communication)
pNZ8020	pNZ8010 derivative without the *gusA* gene carrying MCS	(30)
pEM76	pUC19E bearing *six1* A2 *attP-int six2*	(31)
P13C	pBV5030 derivative containing clone 13C	(10)
PGAA	pGEM-T Easy vector containing *attP-int*	
pOC1	pORI19 derivative containing *celA*	This study
pOC11	pOC1 derivative containing P_{nisA}	This study
pOC12	pOC11 derivative containing *attP-int*	This study
pOC13	pOC12 derivative containing P13C	This study

[1] Em^{r}, erythromycin-resistance gene.

Table 2. Oligonucleotides utilized in this study

Primer	Sequence (5′ → 3′)	Specificity	Reference
celA-won-F	AGG AGG AAA AAA CCA TGA AGA ACG TAA AAA AAA	celA	This study
celA-won-R	AAA AAC CCA TTA CAC TAA TAA GGT AGG TGG	celA	This study
Pnis-F	GGA TCA GTC CTA ATT CTA TCT TGA GAA AG	P_{nisA}	This study
Pnis-R	GCG TCG ACG TCC TGA ATC TTT CTT CGA AAC	PnisA	This study
P13C-F	TCC CCG CGG ATC AAA TTG TTA ATG TAA TCA	P13	This study
P13C-R	TCC CCG CGG TCG ACT CTA GAG GAT CTT TAA	P13C	This study
att-F	GCT GGA TAC AAA ATA AAA AGC GCC T	attP	(24)
att-R	TTG TGT GCC CAT ATT TCT GAA CTC T	Int	(24)
b1	TCT CTG ATA GAC AGT ATA GAG GAG		(24)
int2	CTG GGA TCC CCA AGG CTT ACT TT		(31)

site (MCS) was recovered from pNZ8020 (30) with the digestion of *Sal*I and *Spe*I, and inserted into pOC1 after being digested with the same enzymes. The resulting plasmid was named pOC11.

The *attP* and *int* derived from *Lb. casei* temperate bacteriophage A2 were amplified using primers att-F and att-R containing restriction enzyme site *Not*I from pEM76 (31) for the site-specific recombination. The amplified 1.7-kb PCR fragment of the *attP-int* region was digested with *Not*I, and inserted into the same site of pOC11, producing pOC12.

Fragment containing P13C promoter was amplified with P13C-F and P13C-R containing restriction enzyme site *Sac*II from pB13C (32), and digested with *Sac*II. The digested fragment was inserted into the same site of pOC12. The resulting plasmid pOC13 was an integrative food-grade expression/secretion vector.

To integrate pOC12 and pOC13 into chromosomal DNA, the plasmids were introduced into LAB by electroporation, and the cellulose-utilizing and erythromycin-resistance colonies were selected, respectively.

Assays of cellulase The cellulase activity was qualitatively determined on agar plates containing 1% CMC. After overnight incubation, the plates were stained with 0.1% Congo red solution for 15 min and washed with 1 M NaCl. *E. coli* and LAB conferring cellulase activity formed a yellowish zone in a red background.

Southern blot hybridization analysis For Southern hybridization, LAB chromosomal DNA was digested with *Stu*I or *Pvu*II and blotted on a Hybond nylon membrane (Amersham, Uppsala, Sweden). The *Hin*dIII-digested pOC12 or pOC13 was used as a probe DNA in the hybridization experiments. The DNA probe preparations, hybridization, washing, and staining were performed using enhanced chemiluminescence direct nucleic acid labeling and detection systems (Amersham) as recommended by the manufacturer.

Evaluation of the stability of integrants The segregational stability was determined using *Lb. casei* EM116::pOC13 as a described by Roberts *et al.* (33). These integrants carry *celA* - which confers cellulose fermentation ability - as a selection marker. A single colony was used to inoculate the selection-pressure-free MRS medium, and the culture was grown at 37°C overnight. The saturated culture was diluted 1,000-fold using fresh MRS medium in the absence of selection pressure. This consecutive transfer of culture into fresh MRS was performed every 24 hr using the same dilution ratio. Diluted culture samples were plated as single cells on MRS agars at various intervals and incubated at 37°C overnight. Colonies were plated onto EL1 medium containing 1% CMC to check for the presence of integrants. Southern hybridizations were then performed on samples from several independent colonies to confirm the integration.

Results and Discussion

Construction of an integration vector containing *celA* as a selection marker The integration vectors, pOC12 and pOC13, were constructed for the transfer of a specific gene into the chromosome via site-specific recombination (Fig. 1). pOC12 and pOC13 contained *celA* as a selection marker, a broad-host-range replication origin site (ori^+) from the *L. lactis* Wg2 promiscuous plasmid pWV01 (34), the *attP* site and *int* gene from bacteriophage A2 (24), P_{nisA} (30) for the expression of the foreign gene, and MCS. In addition, in order to enhance the transcription of the *celA*, the constitutive promoter P13C (32) was located upstream of *celA* in pOC12 and pOC13.

Generation of a recombinant *Lb. casei* strain by pOC1 series Southern hybridization using *Hin*dIII-digested pOC12 and pOC13 confirmed that the integration of pOC12 and pOC13 occurred in an orientation-dependent manner in the genomic DNA of *Lb. casei* EM116.

In the hybridization with *Hin*dIII-digested pOC12, a 3.6-kb hybridized band resulted from the *Stu*I-digested total DNA from *Lb. casei* EM116::pOC12, and a 3.0-kb band that did not hybridize was detected. In addition, a 1.9-kb hybridized band was detected from *Pvu*II-digested total DNA from *Lb. casei* EM116::pOC12, and a 3.7-kb band was not hybridized (data not shown), from which it was assumed that pOC12 was integrated into the chromosome of *Lb. casei* EM116. However, the activity of *celA* from *Lb. casei* EM116::pOC12 was low, indicating that it was not an efficient selection marker (Fig. 2C).

To enhance *celA* activity, pOC13 containing *celA* under promoter P13C was integrated into the chromosome of *Lb. casei* EM116 (Fig. 2A). In the hybridization with *Hin*dIII-

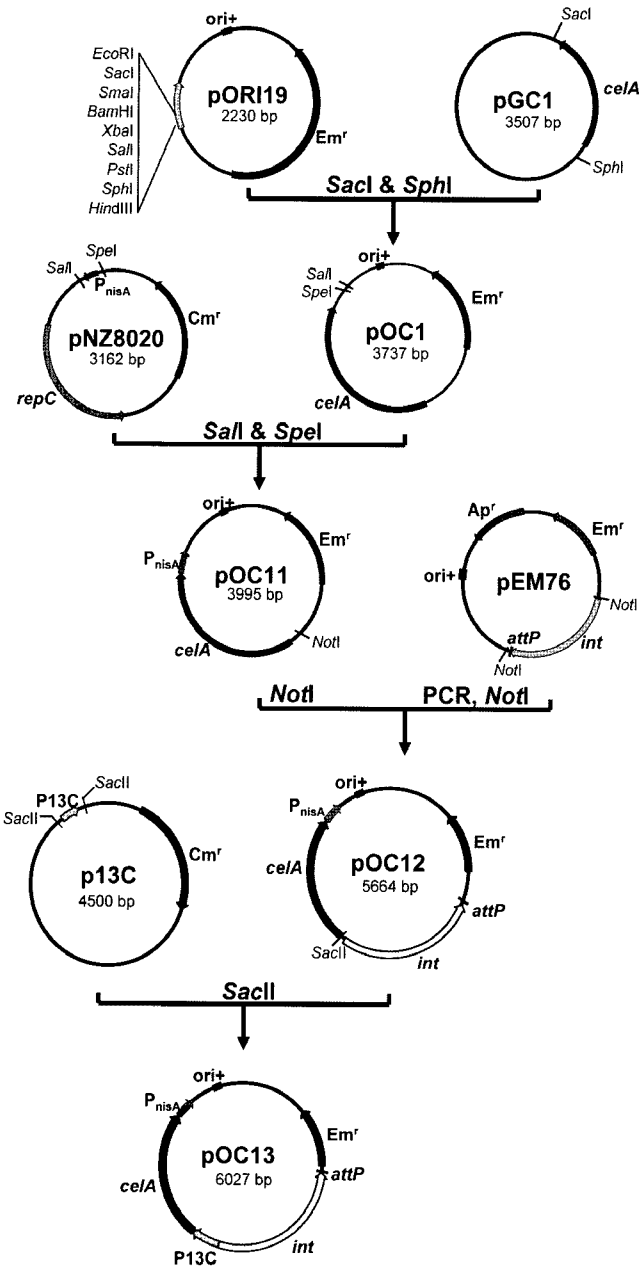

Fig. 1. Construction of an integrative vector using *celA* as a selection marker. ori$^+$, Replication origin of Gram-positive bacteria; repC, replication protein gene; Em^r, erythromycin-resistance gene; Ap^r, ampicillin-resistance gene.

digested pOC13, no hybridization band was detected in control DNA extracted from *Lb. casei* EM116. However, a 3.6-kb band corresponding to *Stu*I-digested *Lb. casei* EM116::pOC13 was detected, as were hybridization bands at 1.9-, 4.1-, and 6.0-kb digested with *Pvu*II. It can be assumed that the unexpected bands were the 4.1- and 6.0-kb *Pvu*II fragments, since the *Pvu*II sites were flanking the site where pOC13 inserted into the chromosome of *Lb. casei* EM116 (Fig. 2B).

The constructed pOC12 and pOC13 - which do not replicate without complementation of *repA* - were introduced into *Lb. casei* EM116. Suicide plasmids from pORI19 such as pOC12 and pOC13 were previously integrated under RepA complementation (35). However, we managed to integrate pOC12 and pOC13 without RepA complementation by using the *attP-int* gene based on site-specific recombination. pOC12 and pOC13 were integrated into the chromosome of LAB via site-specific recombination, which occurs via a single recombination step between *attP* (31) and the attachment site of its host (*attB*) from bacteriophage A2. This integration system has several advantages: (i) the integration vectors mediate recombination more efficiently than homologous recombination, (ii) the integration sites are predictable because they are directed towards one specific chromosomal site, and (iii) the integration vectors do not generate destabilizing terminal DNA repeats.

Evaluation of *celA* as a selection marker The presence of pOC13 in the chromosome meant that *Lb. casei* EM116 was able to ferment cellulose. This phenotype was easily observable on EL1 plates containing CMC because the recombinants formed halos on the red background (Fig. 2C).

The cellulase activity of *Lb. casei* EM116::pOC13 was measured after growing at 37°C in the presence of various sugars. Cells grown on cellobiose and cellulose were 2 to 3 times more active than cells grown on glucose. Also, the absence of detectable activity in the parental strain *Lb. casei* EM116 clearly indicated that *celA* was responsible for the cellulase activity in the transformant.

celA has the principal advantage as a selection marker of not requiring specific mutations in the host chromosome, which may be readily obtainable from the industrial strains. Moreover, transformants can be easily distinguished through the formation of a clear halo without the formation of false transformants.

Segregational and structural stability of *Lb. casei* EM116::pOC13 The segregational and structural stability of integrants containing pOC13 were examined. The CelA-positive phenotype of *Lb. casei* EM116::pOC13 was not lost after 100 generations in the absence of selection pressure in cellulose (data not shown). Also, the structure of integrants did not change (Fig. 3). These results indicated that pOC13 was stably maintained for 100 generations in *Lb. casei* EM116, since no obvious detectable changes in the structure of any plasmids were observed.

Evaluation of *celA* as a selection marker in other LAB pOC13 was introduced into *L. lactis* and *Leu. mesenteroides* to evaluate the use of *celA* as a selection marker. The results in *L. lactis* NZ9800 were similar to those in *Lb. casei* EM116. The activities of *celA* in *L. lactis* NZ9800::pOC13 and *Lb. casei* EM116::pOC13 were compared in terms of the formation of a clear halo on the plates (Fig. 4). PCR analysis of these integrants was performed using the primers Pnis-R and P13C-F. As expected, the DNA of *L. lactis* NZ9800 was not amplified. Then, a 2.0-kb DNA fragment containing P_{nisA} and P13C flanking *celA* was amplified. Lastly, the integration of plasmid DNA into the chromosomal DNA of *L. lactis* NZ9800 was confirmed by Southern hybridization. The 4.1-kb *Stu*I fragment of *L. lactis* NZ9800::pOC13 was evident as bands of the same size as in the *Lb. casei*

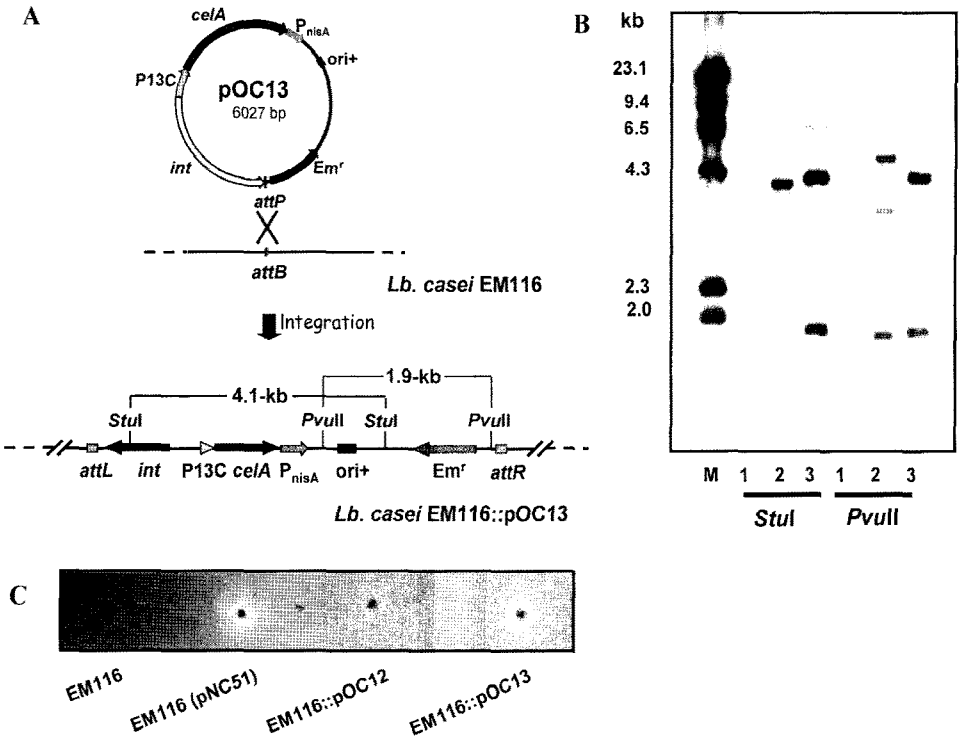

Fig. 2. Generation of recombinant *Lb. casei* EM116::pOC13. A, Mechanism of integration of pOC13 into the genome of *Lb. casei* EM116 to produce *Lb. casei* EM116::pOC13. B, Confirmation of the integration of XCP12 into the chromosomal DNA of *Lb. casei* ATCC 393 by Southern hybridization. Lanes: M, molecular size marker; 1, *Lb. casei* EM116; 2, *Lb. casei* EM116::pOC13; 3, pOC13. C, Qualitative cellulase activity of *Lb. casei* EM116::pOC13 by agar assay using Congo red solution.

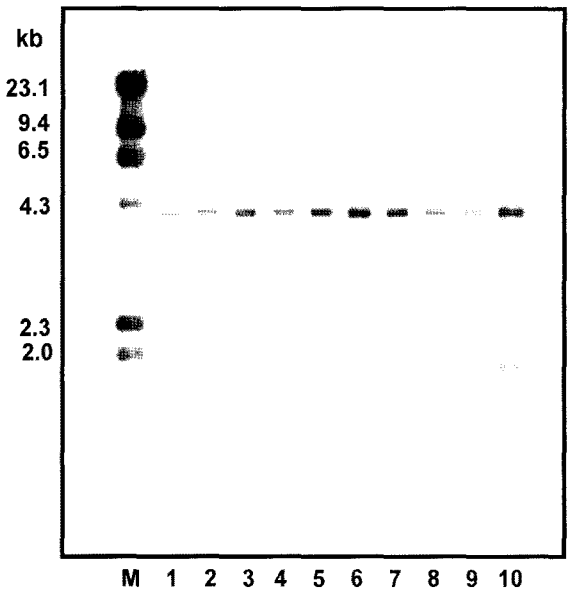

Fig. 3. Structural stability of *Lb. casei* EM116::pOC13 in the absence of selection pressure during 100 generations. Lanes: M, molecular size marker; 1–8, randomly selected *L. lactis* NZ9800 (pVE6007) colonies after 100 generations; 9, *Lb. casei* EM116::pOC13 after 0 generations; 10, pOC13.

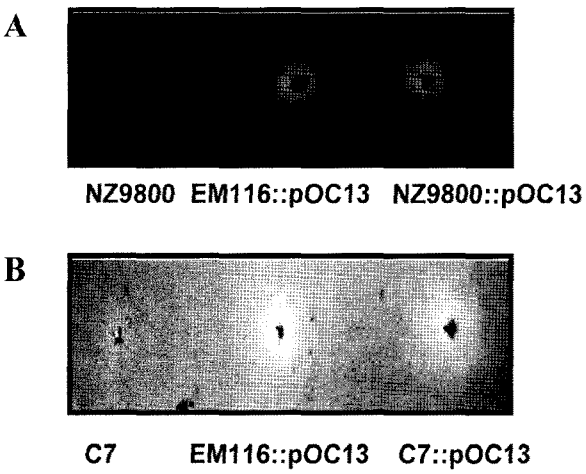

Fig. 4. Influence of *celA* as a selection marker in LAB. A, Cellulase activity from *L. lactis* NZ9800::pOC13. B, Cellulase activity from *Leu. mesenteroides* C7::pOC13.

EM116::pOC13 positive control.

pOC13 was integrated into *Leu. mesenteroides* chromosomal DNA. Also, *celA* activity was evident in *Leu. mesenteroides* C7::pOC13 integrants as clear halos on a cellulose-containing plate (Fig. 4). In addition, the optical density (600 nm) of recombinants was 3-4 times higher than them of host LAB after 10 hr incubation under CMC as a sole carbon source.

These results suggest that the integration vector pOC13 has the ability to transform and directly integrate into *Lb. casei*, *L. lactis*, and *Leu. mesenteroides*. Also, the integrants are easily detectable as a CelA-positive phenotype by the formation of clear halos of colonies around plates containing cellulose.

In conclusion, integration vector pOC13 containing the *attP-int* gene as a site-specific element, the ori+ site of

pWV01, and the P_{nisA} was constructed using *celA* as a selection marker for LAB. This vector was integrated successfully by site-specific recombination into *Lb. casei* EM116, *L. lactis* NZ9800, and *Leu. mesenteroides* C7 genomic DNA. In addition, *celA* was evaluated as a selection marker for LAB with primary selection by the formation of a clear halo on plates. The *Lb. casei* EM116::pOC13 integrant was stably maintained in a nonselective condition for 100 generations. Because pOC13 contains the P_{nisA} gene, it can efficiently indicate the extracellular concentration of nisin. In conclusion, we have evaluated *celA* as a food-grade selection marker and constructed an integrative vector based on site-specific recombination containing *celA*. This will allow the use of a food-grade selection marker for LAB recombinants, and the plasmid pOC13 will allow the further development of LAB as acceptable hosts for the production of various proteins, peptides, and metabolites.

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