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Construction of Recombinant *Lactobacillus casei* Strains Using Splicing by Overlap Extension

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Recombinant *Lactobacillus* strains have been constructed using gene splicing by overlap extension (SOE). Primers were designed of which one end of an amplified product contained complementary sequences for an end of other amplified fragment. For efficient matching, we used an asymmetric PCR step that was effective at generating an excess of strands that would anneal in the final PCR. CP12, a recombinant fragment consisting of the integrase gene and attachment site of the bacteriophage A2, was constructed and inserted into the genome of *Lactobacillus casei* ATCC 393, yielding *Lb. casei* ATCC 393::XCP12. Another recombinant *Lb. casei* strain was constructed, where the *egfp* gene was a part of the construction. The EGFP produced from *Lb. casei* ATCC 393::XCEGFP14 was detected by Western blot hybridization. This simple and widely applicable approach has significant advantages over standard recombinant DNA techniques for *Lactobacillus* species.

Keywords: Splicing by overlap extension, *Lactobacillus*, recombination, integration

Lactic acid bacteria (LAB) are industrially important microorganisms in the food industry as starter cultures for fermented foods, food preservatives, and flavor enhancers. LAB such as *Lactococcus* and *Lactobacillus* species are most commonly given a GRAS (generally recognized as safe) status. *Lactobacillus* species are used in food fermentation and form part of the commensal microbial flora of the intestinal tracts of humans and other mammals. The importance of lactobacilli has resulted in extensive investigations of their genetics and physiology [8, 13, 14]. Genetic engineering for lactobacilli required the development of new genetics tools, including plasmid vectors and transformation protocols [8, 12–15]. Genetics tools involving plasmids have been used to construct recombinant lactococci [8, 13, 15]. However, in many cases, it is very difficult to construct recombinant vectors using these tools [4, 7–9, 11, 16]. A food-grade vector comprising only a homologous DNA containing replicon, selection marker, promoter, and secretion signal was constructed using various cloning steps [4, 9, 11, 16].

Standard methods for constructing recombinant plasmid or lactobacilli are dependent on the use of a restriction enzyme to cut DNA into specific fragments, which can then be rejoined into the new recombinant lactobacilli. Horton and colleagues [5] described the technique of splicing by overlap extension (SOE) by the polymerase chain reaction (PCR), which was not limited by the presence of restriction sites at appropriate locations. SOE by PCR generates intermediate products of each of the components of the hybrid tipped with a small sequence of the other, and then mixes these products in a subsequent PCR to produce the final spliced product. Recombinant DNA and fused genes, without using restriction enzymes, using SOE by PCR have been reported [2, 3, 10, 17, 18]. The technique has mostly been used to construct the fusion gene for the expression of a protein of interest, and to develop the associated recombinant antibody [3, 10, 17, 18]. Here, we describe the construction of recombinant lactobacilli based on SOE by PCR. This is the first report of the use of SOE to construct recombinant lactobacilli. The SOE approach is a fast, simple, and extremely powerful way of recombining and modifying nucleotide sequences.

Materials and Methods

Bacterial Strains and Growth Conditions

Lactobacillus casei ATCC 393 was used in this study as the cloning host, and was grown at 37°C in MRS (deMan-Rogosa-Sharpe) broth

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(Difco, Franklin Lakes, NJ, U.S.A.) supplemented with chloramphenicol (170 μg/ml) as required.

pEM76 [11], pNZ8020 [1], pB13C [6], and pEGFP (BD Biosciences Clontech, Mountain View, CA, U.S.A.) were used as sources for the integrase gene (*int*) and attachment site of the phage (*attP*), chloramphenicol resistance gene (*cat-86*), promoter P13C sequence, and enhanced green fluorescent protein (*egfp*) gene, respectively.

SOE by PCR

Fig. 1 shows the construction procedures for CP and CEGFP using SOE by PCR. The constructed PCR fragments were verified by PCR and DNA sequencing. PCR primers were designed based on known DNA sequences, and relevant restriction enzyme sites were introduced when needed (Table 1).

The construction of CP using SOE by PCR involves three separate reactions, where the two DNA fragments produced in the first two reactions are mixed for the third reaction. Four primers (SOE1, SOE2, SOE3, and SOE4) are required for each construct. In the first stage, CP1 containing *int* and the *attP* site was amplified from pEM76 with SOE1 and SOE2. Additionally, CP2 containing *cat-86* was obtained from pNZ8020 using SOE3 (whose sequence overlaps that of SOE2) and SOE4. To construct a final PCR product CP12, asymmetric PCR was performed with CP1 and CP2 but without primers. Using the resulting asymmetric-PCR products as a template, the CP12 was amplified with SOE1 and SOE4.

The construction of CEGFP using SOE by PCR involves seven separate reactions. The four DNA fragments produced in the first four reactions were amplified as follows: CP1 and CEGFP2 containing *cat-86* amplified from pNZ8020 using SOE3 and SOE5, CEGFP3 containing promoter P13C amplified from pB13C using SOE6 and SOE7, and CEGFP4 containing *egfp* amplified from pEGFP using SOE8 and SOE9. SOE5 and SOE7, like SOE3, have sequences that overlap with those of neighboring primers SOE6 and SOE8, respectively. CEGFP12 and CEGFP34 were obtained from CP1/CEGFP2 and CEGFP3/CEGFP4 after the asymmetric PCR. To construct a final PCR product CEGFP14, asymmetric PCR was performed with CEGFP12 and CEGFP34 without primers. Using the resulting asymmetric-PCR products as a template, the CEGFP14 was amplified with SOE1 and SOE9.

All PCRs in the first stage were conducted using 30 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 1 min, and elongation at 72°C for 2 min with a commercial PCR system (GeneAmp 2700; Applied Biosystems, Foster City, CA, U.S.A.), using standard procedures in the reaction conditions recommended by the manufacturer of Ex *Taq* polymerase (Takara, Kyoto, Japan). PCR products obtained from the first stage were preheated for 3 min at 96°C, and then amplified using 10 cycles of 30 sec at 94°C, 10 min at 60°C, and 5 min at 72°C for asymmetric PCR. SOE by PCR was conducted using 35 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 1 min, and elongation at 72°C for 3 min.

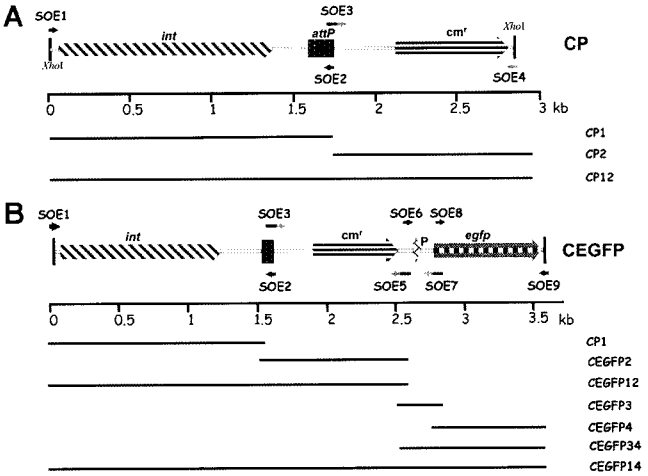

Fig. 1. Scheme of gene splicing by overlap extension (SOE).
A. Recombinant CP12 fragment. Four PCR primers (SOE1-4) were used in three PCRs. **B.** Recombinant CEGFP14 fragment. Eight PCR primers (SOE1-3 and SOE5-9) were used in seven PCRs. Primers SOE3, SOE5, and SOE7 contained the neighboring gene fragment at the 5' sequence. Primers SOE2/SOE3, SOE5/SOE6, and SOE7/SOE8 match their template genes, respectively. cm^r, chloramphenicol resistance gene

Gene Integration

CP12 and CEGFP14 were digested with restriction enzyme XhoI. The circular-form DNA was obtained through self-ligation. Circular XCP12 and XCEGFP14 DNA was introduced into *Lb. casei* ATCC 393 by electroporation. The transformants in the recovery medium after electroporation were grown in MRS broth containing chloramphenicol at 37°C for 18 h. Transformants were spread on MRS agar containing chloramphenicol, and the formed colonies were selected.

Southern Blot Hybridization

For Southern hybridization, *Lb. casei* DNA was digested with AccI or ClaI and blotted on a Hybond nylon membrane (Amersham,

Table 1. Oligonucleotides used to generate the constructs in this study.

Primer	Sequence (5'→3')	Template	Specificity
SOE1	CCTCGAGGCTGGATCCAAAATAAAAAGCGCCT	pEM76	*int* and *attP* site, forward
SOE2	TTGTGTGCCCATATTTCTGAACTCT	pEM76	*int* and *attP* site, reversed
SOE3	AGAGTTCAGAAATATGGGCACACAAGACGAAAGTCGACGGCAATA	pNZ8020	*cat-86*, forward
SOE4	GGCTCGAGTACAGTCGGCATTATCTCAT	pNZ8020	*cat-86*, reversed
SOE5	TTAGTAGTATGAAGGCACTTGACAGTCGGCATTATCTCAT	pNZ8020	*cat-86*, reversed
SOE6	CAAGTGCCTTCATACTACTAAAC	pB13C	Promoter P13C, forward
SOE7	GAGTCGACCTGCAGGCATGTCTAATCACCTCCTGTTTTA	pB13C	promoter P13C, reversed
SOE8	CATGCCTGCAGGTCGACTCTAGAGG	pEGFP	*egfp*, forward
SOE9	CCTCGAGTGGAATTCTAGAGTCGCGGCCGC	pEGFP	*egfp*, reversed

Underlined sequence is the XhoI restriction site.

Uppsala, Sweden). The DNA probe preparations, hybridization, washing, and staining were performed using enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection systems (Amersham) and protocols provided by the manufacturer.

Western Blot Hybridization

For Western blotting, proteins were transferred from 10% 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, Hercules, CA, U.S.A.). Bands corresponding to EGFP were detected with mouse anti-GFP (B-2) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and goat antimouse IgG horseradish-peroxidase conjugate (Zymed, San Francisco, CA, U.S.A.) using an ECL detection kit (Amersham).

RESULTS AND DISCUSSION

Generation of Recombinant *Lactobacillus* by SOE by PCR

The PCRs that included the amplified 1,684-bp CP1 with the SOE1/SOE2 primer set and the amplified 1,004-bp CP2 with the SOE3/SOE4 primer set were performed separately. They included *int*, the *attP* site, and *cat-86*. In addition, because the 5' sequence of SOE3 contains a sequence that complements SOE2, CP2 of the first-stage PCR produced a DNA fragment with the sequence 5' to the splice point, and the other produced a CP1 fragment with the sequence 3' to the splice point (Fig. 1). However, since the hybrid oligonucleotides span the splice point, each of the first-stage products is tipped with a short sequence derived from the other. For the efficient SOE by PCR, each product was used as the primer for the asymmetric PCR. The PCR product was then used as the template for SOE by PCR, which generated the expected 2,651-bp fragment of CP12 (Fig. 2), with both ends containing the restriction enzyme site XhoI.

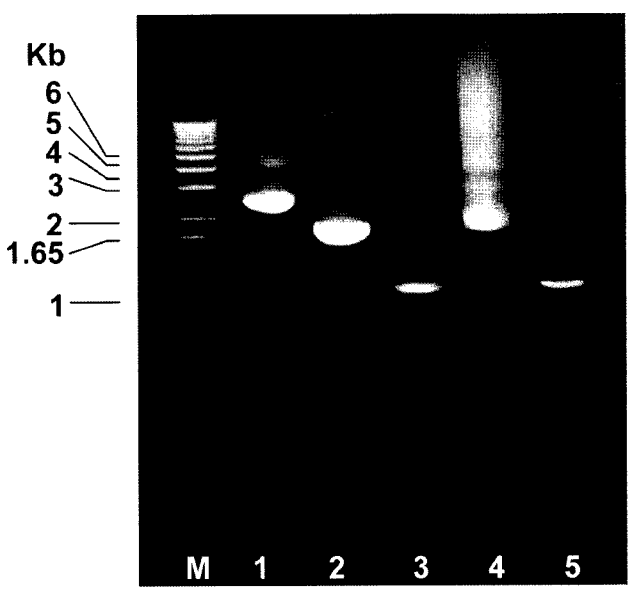

Fig. 2. Recombinant CP12 fragment obtained by SOE by PCR.
Lanes: M, molecular size marker; 1, CP12 amplified fragment from the asymmetric-PCR mixture of CP1 and CP2; 2 and 3, CP1 and CP2 amplified fragments from pEM76 and pNZ8020, respectively; 4 and 5, CP1 and CP2 amplified fragments from CP12, respectively.

XCP12, a self-ligated fragment of XhoI-digested CP12, was introduced into *Lb. casei* ATCC 393. The *int* and the *attP* site of XCP12 were integrated into the chromosomal DNA of *Lb. casei* ATCC 393. Transformants were selected on MRS agar containing chloramphenicol. Fig. 3A shows a schematic of the transformation and integration of XCP12.

The integration of XCP12 occurred in an orientation-dependent manner at the genomic DNA of *Lb. casei* ATCC 393, as confirmed by PCR and Southern hybridization (Figs. 3B and 3C). To identify the integration of XCP12 into *Lb. casei* ATCC 393, PCR amplification was performed with an int2 and b1 primer set, which are complementary to *int* and *attR*. No DNA amplification was observed from

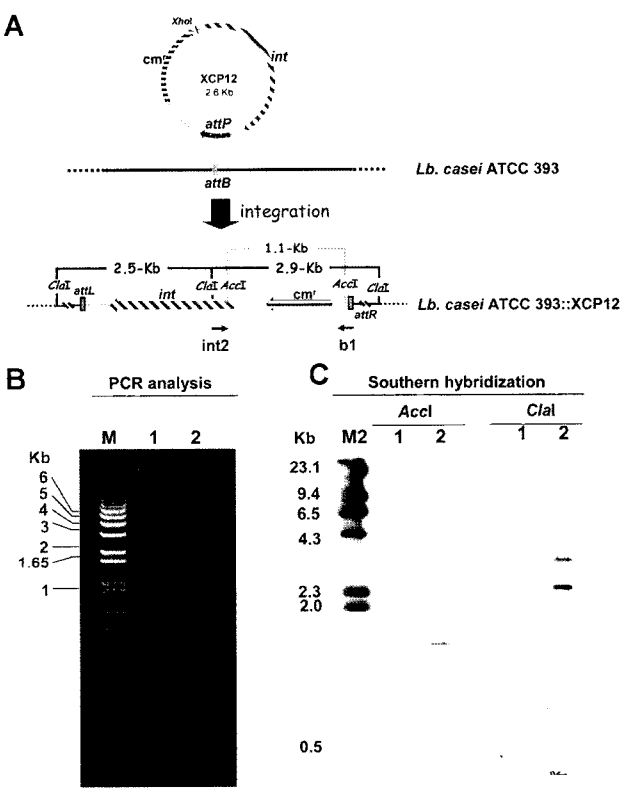

Fig. 3. Generation of recombinant *Lactobacillus* species using SOE by PCR.
A. Mechanism of integration of XCP12 into the genome of *Lb. casei* ATCC 393 to produce *Lb. casei* ATCC 393::XCP12. XCP12 is introduced by transformation into *Lb. casei* ATCC 393, and the Int protein catalyzes site-specific integration between *attP* and *attB* to yield *Lb. casei* ATCC 393::XCP12. **B, C.** Confirmation of the integration of XCP12 into the chromosomal DNA of *Lb. casei* ATCC 393 by PCR (**B**) and Southern hybridization (**C**). Lanes: M, molecular size marker; 1, *Lb. casei* ATCC 393; 2, *Lb. casei* ATCC 393::XCP12.

the total DNA extracted from *Lb. casei* ATCC 393 (Fig. 3B). CP12 was used as a probe to identify the XCP12 integration. The 1.1-kb hybridized band resulted from the AccI-digested total DNA integrating XCP12 into *Lb. casei* ATCC 393, and a 1.1-kb band that did not hybridize from *Lb. casei* ATCC 393 was also detected (Fig. 3C). The integration of XCP12 into the *Lb. casei* ATCC 393 chromosome was confirmed by the detection of 2.5-kb and 2.9-kb hybridized bands from the ClaI-digested DNA of *Lb. casei* ATCC 393::XCP12.

Evaluation of Recombination Method by SOE by PCR

To evaluate the usefulness of gene recombination for lactobacilli using SOE by PCR, and the expression of foreign protein, recombinant lactobacilli were constructed using promoter P13C [6] and *egfp*. Fig. 1 presents a schematic of CEGFP using SOE by PCR. The PCRs that included CP1, 1,034-bp CEGFP2, 329-bp CEGFP3, and 803-bp CEGFP4 were performed separately. The amplified fragments contained *int* and the *attP* site, *cat-86*, promoter P13C, and *egfp*, respectively. Primers SOE5 and SOE7 obtained by amplifying CEGFP2 and CEGFP3 contained the complimentary sequences SOE6 and SOE8, respectively.

SOE by PCR produced CEGFP12, the hybrid of CP1 and CEGFP2, and CEGFP34, the hybrid of CEGFP3 and CEGFP4. CEGFP12 and CEGFP34 were then used as the template for SOE by PCR generating the expected 3,763-bp fragment CEGFP14, both ends of which contained restriction enzyme site XhoI. XCEGFP14, a self-ligated fragment of XhoI-digested CEGFP14, was introduced into *Lb. casei* ATCC 393. Transformants of XCEGFP14 were selected on MRS agar containing chloramphenicol (Fig. 4A).

Total DNA from *Lb. casei* ATCC 393::XCEGFP14 was digested with AccI and ClaI probed with the CEGFP14 as a probe DNA, and Southern hybridization was performed (Fig. 4B). In the case of AccI-digested DNA, the hybridized 2.4-kb band was shifted up, as was that of the promoter P13C and *egfp* from the 1.1-kb DNA fragment. Using the ClaI-digested DNA for hybridization produced only 2.5-kb and 4.1-kb bands hybridized with the total DNA from *Lb. casei* ATCC 393::XCEGFP14.

EGFP Production

The function of the P13C in recombinant cells was tested based on EGFP production (Fig. 4C). The expressed EGFP was analyzed by Western blot hybridization using anti-GFP antibody. Bands corresponding to EGFP were detected in *Lb. casei* ATCC 393::XCEGFP14 (Fig. 4C), implying the successful production of EGFP in recombinant *Lb. casei* ATCC 393::XCEGFP14 by SOE by PCR.

The goal of this study was to construct useful *Lb. casei* strains, *Lb. casei* ATCC 393::XCP12 and *Lb. casei* ATCC 393::XCEGFP14, using SOE by PCR. Our results showed that cloning by SOE by PCR is a more simple and efficient

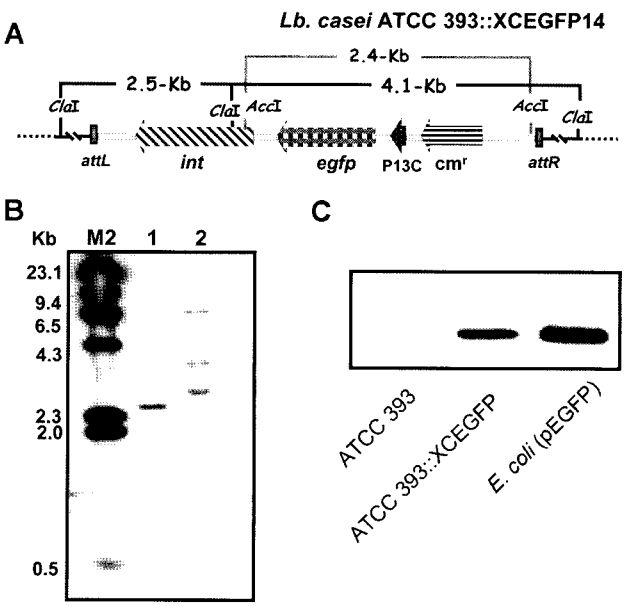

Fig. 4. Confirmation of integration of XCEGPF14 into *Lb. casei* by SOE by PCR.

A. Integration of XCEGFP14 into the genome of *Lb. casei* ATCC 393 to produce *Lb. casei* ATCC 393::XCEGFP14. **B.** Confirmation of the integration of XCEGFP12 into the chromosomal DNA of *Lb. casei* ATCC 393 by Southern hybridization. Lanes: M, molecular size marker; 1 and 2, *Lb. casei* ATCC 393::XCEGFP14 digested with AccI and ClaI, respectively. **C.** Detection of EGFP from *Lb. casei* ATCC 393::XCEGFP14 by Western blot hybridization.

method than the classic cloning procedure employing restriction enzymes. This new method will allow the further development of *Lactobacillus* strains as acceptable hosts for the production of proteins, peptides, and metabolites for the food industry.

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