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Development of *Leuconostoc* sp. Host Vector System

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Abstract

Leuconostoc citreum CBUE isolated from kimchi proved to harbor a small cryptic plasmid, pNS75. The complete nucleotide sequence of pNS75 was 1,821 bp and had a low G+C content of 39.2%. Computer analysis using DNASIS revealed one open reading frame (ORF), having ATG as putatitive start condon and potentially encoding proteins with molecular mass of 38 kDa. The chimeric plasmid pLeuCM was first constructed wih pNS75, pUC19 and chroamphenicol acetyltransferase (CAT) from *Staphylococcus* sp.. pLeuCM replicated and expressed chroamphenicol acetyltransferase in *Leuconostoc citerum* CBNF after transformation. To test the availability of shuttle vector as cloning vehicle of foreign gene, α-amylase gene of *Streptococcus bovis* was cloned and all transformants secreated the α-amylase successfully. The result indicates that pLeuCM is a potential shuttle vector for *Leuconostoc* spp. and lactic acid bacteria.

Introduction

In recent years there has been an increase of interest in the genetic manipulation of lactic acid bacteria. Numerous plasmid vectors have been constructed using the origin of replication of cryptic plasmids of *Lactobacillus* and *Lactococcus* species. But, no host-vector system has been described for *Leuconostoc* strains. *Leuconostoc* spp. are G(+) hetero-fermentative bacteria. They are widely used in the food industry, involved in the fermentation of vegetables (Kimchi, sauerkraut and pickle), wine and milk products. As many other LAB, *Leuconostoc* species harbor one or several plasmids of various sizes without any known function for most of them. But to date, only a few researches have reported about size, nucleotide sequences and restriction endonuclease site of native plasmids. In this report, we describe isolation and sequence analysis of pNS75 which is a plasmid of *Leuconostoc citreum* CBUE isolated from Kimchi and expression of α-amylase wsing this plasmid in a recombinant *Leuconostoc citreum*.

Materials and Methods

Bacterial strains, plasmids, and culture media

Bacterial strains and plasmids used in this work are listed in Table 1. *Leuconostoc* strains were routinely

grown on MRS medium (Difco) broth or agar (1.5%, w/v) at 28℃. *E. coli* were propagated at 37℃ in LB broth or on agar (1.5%, w/v). Ampicillin and chloramphenicol were used at final concentration of 50 and 5 μg/mL, respectively.

Table1. Bacterial strains and plasmids

Strains or plasmids	Genotype/relevant features	Source or reference
Strains		
Escherichia coli	Φ80lacZ∧(lacZYA-argF)U169recA1 endA1bsdR17supE44thi-1gyrA96relA1	Gibco-BBL
Leuconostoc citreum CBUE	wild-type	This study
Leuconostoc citreum CBUF	wild-type	This study
Plasmids		
pUC19	*E. coli* vector, 2.68kb, Amp^R, lacZ	Gibco-BBL
pEK104	pUC19 with 1.3kb Cm^R	This study
pNS75	1.8kb cryptic plasmid from *Leuc. citreum* CBHE	This study
pLeuCM	pEK104::1.8kb *Sac*I pNS75, 5.8kb	This study
pLeuCMamy59	pLeuCM with 2.4kb *Bam*HI α-amylase from *S. bovis*	This study

General cloning techniques and sequence analysis

The plasmid DNA preparation from *E. coli*, restriction enzyme digestion, ligation and transformation of *E. coli* was carried out according to the procedures of Sambrook *et al.*,[(1)]. The plasmid DNA from the *Leuconostoc* strains was prepared by the method of Anderson *et al.*[(2)]. DNA sequences were determined using the Auto-read sequencing kit (Pharmacia) of Genome Research Center in Seoul National University. Sequence analysis of DNA and amino acid were performed using the DNASIS programs (HITACHI software Engineering Co. Japan).

Shuttle vector construction and transformation of *Leuconostoc* sp.

For the construction of shuttle vectors, pEK104 containing staphylococcal chloramphenicol acetyltrasnsferase (CAT) was ligated with 1.8 kb pNS75 (Fig. 1). The electrocompetent cells of *Leuc. citreum* CBNF were prepared according to the method of Wyckoff *et al.*,[(3)], and *E. coli- Leuconostoc* shuttle vector, pLeuCM, was transformed. The electroporation condition was set at 8 kV/cm, 400 Ω and 40 μF.

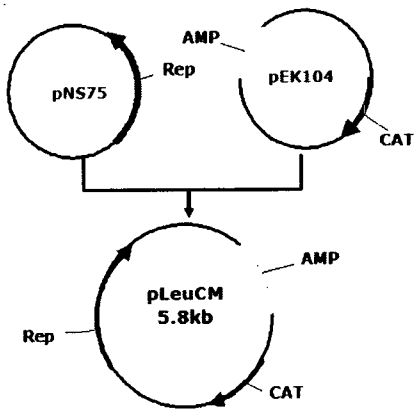

Fig. 1. Construction of *E. coli-Leuconostoc* shuttle vector, pLeuCM.

Expression of α-amylase

α-Amylase gene from *Streptococcus bovis* was PCR-amplified, cloned into pLeuCM, and transformed into *Leuconostoc* sp. colonies containing α-amylase gene were screened by the formation of a clear zone when poured with iodine solution onto plates.

Results and Discussion

Isolation of pNS75 and derivatives construction

Leuc. citreum CBUE was proved to harbor several plasmids including a 1.8 kb one, named as pNS75. This plasmid was clone into the unique *Sac* I site of pUC19 for sequence determination.

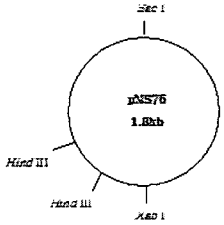

Fig. 2. Restriction enzyme digestion maps of pNS75.

Sequence analysis of pNS75

The complete nucleotide sequence of pNS75 was 1,821 bp and had a low G+C content of 39.2%. Computer analysis with DNASIS revealed one open reading frame. The putative proteins coded by one ORF were designated as replication protein. ORF corresponded to a polypeptide similar to the replication protein of pFR18[4] suggesting that pNS75 replicates by the rolling circle mechanism.

Fig. 3. Complete nucleotide sequence of pNS75.

replication nick-site
↓

pT181	122	GACAATTTTTCTAAAA CCGGC TACTCTAATAGCCGGTTGG
pC223	454	GACAATTTTTCTAAAA CCGGC TACTCTAATAGCCGGTTAG
pC221	1246	GACAATTTTTCTAAAA CCGGC TACTCTAATAGCCGGTTAA
pCW7	54	GACAATTTTTCTAAAA CCGGC TACTCTAATAGCCGGTTAA
pS194	3424	TCAAATTTTTCTAAAA CCGGC TACTCTAATAGCCGGTTAA
pUB112	1323	TCAAATTTTGCTAAAA CCGGC TACTCTAATAGCCGGTTAA
pOg32	204	AACA TCTATTGAAAAGCCGGCCCCTTCTAACAGCCGGCTAA
pFR18	388	CAGTCGACCAGAAAAAGCGGGCTTTTACTAACAGCCCGCTAACAGCCCGCT
pNS75	36	CAGCCGACCAGAAAAAAGCGTGCTGTACTAACAGCACGCTAACAGCACGCT

Fig. 4. Nucelotide sequence comparison of the DSO of pT181-type plasmids an putative DSO of pNS75. The stem-loop structure that includes the replication nick site.

Shuttle vector construction and electroporation condition

The whole plasmid pNS75 was ligated into *Sac* I site of pEK104. It was transformed into *Leuc. citreum* CBNF by electorpoaration and the successful transformation was confirmed by plasmid isolation from transformant (Fig. 5).

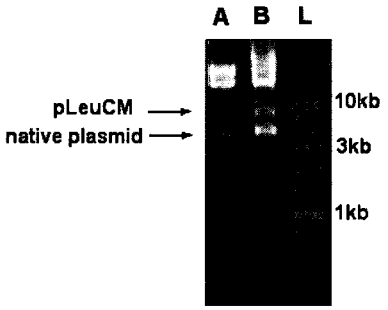

Fig. 5. Confirmation of the transformation of *Leuc. citreum* CBNF with shuttle vector pLeuCM after plasmid isolation. A : native plasmid from *Leuc. citreum* CBNF, B : pLeuCM from *Leuc. citreum* CBNF.

Cloning and expression of α-amylase in *Leuconostoc* sp.

pLeuCMamy59 was created using *E. coli-Leuconsotoc* shuttle vector and α-amylase gene from *S. bovis*. α-Amylase-positive halos were detected around the colonies of all transformants on agar plastes supplemented with 0.5% soluble starch by exposure to iodine vapor (Fig. 6).

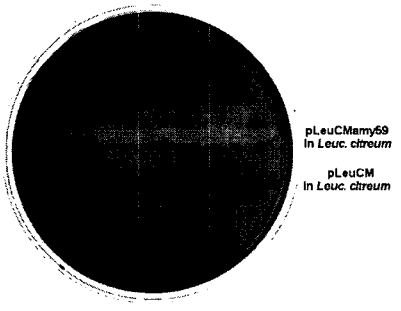

Fig. 6. Expression of α-amylase in *Leuc. citreum* CBNF.

References

1. Sambrood, J., Fritsch, E. F., and Maniatis, T., "Molecular Cloning: A Laboratory Manual, 2nd ed." (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Anderson, D. G., and Mckay, L. L., "Simple and rapid method for isolating large plasmid DNA from lactic streptococci" (1983), Appl. Environ. Microbiol., 46, 549~552
3. Wyckoff, H. A., and Sandine, W. E., "Transformation of dairy *Leuconostoc* using plasmid vectors from *Bacillus*, *Escherichia*, and *Lactococcus* hosts"(1990), J. Dairy Sci., 74, 1454~1460
4. Biet, F., Cenatiempo, Y., and Fremaux, C., "Characterization of pFR18, a small cryptic plasmid from *Leuconostoc mesenteroides ssp. mesenteroides* FR52, and its use as a food grade vector."(1999), FEMS Microbiol. Lett. 179, 375~383
5. Park, M., Shin, D., Lee, K., and Ji, G., "Characterization of plasmid pKJ36 from *Bifidobacterium longum* and construction of an *E. coli-Bifidobacterium* shuttle vector"(2000), J. Microbiol. Biotechnol, 10, 312~320