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## Construction and Characterization of Shuttle Plasmids for Lactic Acid Bacteria and *Escherichia coli*

The chimeric plasmid pBN183 was first constructed in *Escherichia coli* by ligating the *Bam*HI-digested *E. coli* plasmid pBR322 and a *Bgl*II-linearized streptococcal plasmid, pNZ18. The pBN183 transformed *E. coli* to Ap<sup>R</sup> at a frequency of  $(8.2 \pm 1.2) \times 10^5$  colony forming units (CFU)/ $\mu$ g DNA. Electrotransformation of *Streptococcus thermophilus* with pBN183 yielded Cm<sup>R</sup>, Ap<sup>S</sup> clones at a frequency of  $(2.6 \pm 0.3) \times 10^1$  CFU/ $\mu$ g DNA. Plasmid screening with pBN183-transformed *S. thermophilus* clones revealed that ca. 70% of these transformants contained deleted plasmids. Plasmid pBN183A, a pBN183 deletion mutant lacking one copy of a tandemly arranged, highly homologous DNA sequence, was isolated for further study. It transformed *E. coli* to Ap<sup>R</sup> and *S. thermophilus* to Cm<sup>R</sup> with frequencies of  $(4.8 \pm 0.1) \times 10^5$  and  $(8.1 \pm 0.2) \times 10^2$  CFU/ $\mu$ g DNA, respectively. Screening of *S. thermophilus* transformants did not show the presence of deleted plasmids. Based on the structure of pBN183A, a new shuttle plasmid, pDBN183, was constructed from pBN183 by removal of the small (1.2 kb) *Sa*I fragment. Transformation frequencies of pDBN183 were  $(5.0 \pm 1.3) \times 10^5$  and  $(4.6 \pm 0.2) \times 10^2$  CFU/ $\mu$ g DNA with *E. coli* and *S. thermophilus*, respectively. In contrast to the parent pBN183, only 17% of the pDBN183-transformed *S. thermophilus* contained deleted plasmids. Plasmid copy numbers of the three vectors in *E. coli* were estimated at 17–18 per chromosome. The three plasmids conferred Ap<sup>R</sup> and Cm<sup>R</sup> to *E. coli*, but only Cm<sup>R</sup> to *S. thermophilus*. The insertion of a *Streptomyces* cholesterol oxidase gene (*choA*) into pDBN183 did not affect the plasmid's stability in *Lactobacillus casei*, but resulted in deletion of the recombinant DNA in *S. thermophilus*. © 1992 Academic Press, Inc.

Lactic acid bacteria (LAB)<sup>1</sup> constitute a group of microorganisms used extensively as starter cultures in the dairy industry. They impart flavor and texture characteristics to the fermented dairy products such as cheeses and yogurt. Over the years, attempts have been made to improve the desired traits of these important organisms, and current research efforts focus on the application of genetic engineering for strain improvement (Sandine, 1987; Mercenier and Lemoine, 1989; Harlander, 1990). To this end, plasmid vectors have been constructed for the cloning and expression of genes (De Vos, 1986;

Chassy, 1987) and newer DNA transfer techniques have been developed for the introduction of DNA into these organisms (Chassy and Flickinger, 1987; Harlander, 1987; Somkuti and Steinberg, 1987). In addition, DNA restriction-modification systems capable of degrading incoming DNAs and thus influencing gene product synthesis in LAB have been characterized (Klaenhammer, 1989; Solaiman and Somkuti, 1990, 1991a).

In the construction of LAB cloning vectors, the origin of replication of a cryptic lactic streptococcal plasmid, pSH71 (Gasson, 1983), was among the most widely used replication functions. Thus, De Vos (1987) constructed pNZ18 and pNZ19 plasmids by using: (1) the replication region of the pSH71; (2) the *cat* (Cm<sup>R</sup>) and *aad* (Km<sup>R</sup>) genes of the *Staphylococcus aureus* plasmids pC194 (Ehrlich, 1977) and pUB110 (Gryczan *et al.*,

<sup>1</sup> Abbreviations used: LAB, lactic acid bacteria; Ap, ampicillin; Tc, tetracyclin; Cm, chloramphenicol; *choA*, cholesterol oxidase gene; CIP, calf intestinal alkaline phosphatase; TBE, Tris–borate, boric acid, and EDTA; TE, Tris–HCl and EDTA.

1978), respectively; and (3) a fragment of the *Escherichia coli* plasmid pJRD158 (Heusterspreute and Davison, 1984) containing a restriction site bank. The pNZ plasmids replicated in *E. coli* and in certain gram-positive bacteria such as *S. aureus*, *Bacillus subtilis*, and *S. lactis* (De Vos, personal communication) and were maintained in high copy number in some of these hosts. Successful cloning and expression of several genes in lactic acid bacteria have been reported by using these pNZ-series plasmids (De Vos, 1987; Somkuti *et al.*, 1991). In this communication, we report the development of the pBN series shuttle plasmids with the use of pNZ18 and pBR322. The plasmid chimeras transformed *E. coli* with considerably higher transformation frequency than the parent pNZ18, thus allowing the convenient manipulation of recombinant DNA prior to introduction into a variety of LAB. We also describe the use of these plasmids for the introduction of a streptomycete cholesterol oxidase gene, *choA* (Ishizaki *et al.*, 1989), into *S. thermophilus* and *Lactobacillus casei*. The *choA* gene was used as a model gene here because of our interest in developing cholesterol-metabolizing lactic acid bacteria.

## MATERIALS AND METHODS

### *Bacteria, Plasmids, and Growth Conditions*

*E. coli* HB101, DH5 $\alpha$ , and JM109 were purchased as competent cell preparations from BRL Life Technologies (Gaithersburg, MD) or Stratagene Cloning Systems (La Jolla, CA). *S. thermophilus* ST128 and *L. casei* LC2 were from our culture collection.

Plasmids pBR322 and pUCO191 (Somkuti *et al.*, 1991) were from our laboratory stocks. pNZ18 (De Vos, 1987) was obtained from G. Simons (Netherlands Institute for Dairy Research). pUC19 was purchased from BRL Life Technologies. Plasmid pVA736 (Macrina *et al.*, 1980) was a gift from F. Macrina (Virginia Commonwealth University).

*E. coli* cultures were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C with vigorous shaking. *S. thermophi-*

*lus* and *L. casei* were maintained in lactose/sucrose (Somkuti and Steinberg, 1979) and MRS (Difco, Detroit, MI) broth, respectively, at 37°C without agitation. When needed, ampicillin (Ap), tetracycline (Tc), or chloramphenicol (Cm) was added to a final concentration of 50–100, 15, or 10–30  $\mu\text{g}/\text{ml}$ , respectively.

### *Plasmid Construction*

pBN183 was constructed in *E. coli* HB101 by ligating *Bam*HI-digested pBR322 and the pNZ18 linearized at the *Bg*II site of the multiple-cloning-site region (marked with an arrow in Fig. 1). Transformants were selected for Ap<sup>R</sup>, Tc<sup>S</sup> phenotype with RepliPlate colony transfer pads (FMC BioProducts, Rockland, ME). The configuration of the recombinant DNA was determined by single and double restriction analyses.

pBN183A, a deletion derivative of pBN183, was obtained from a Cm<sup>R</sup> clone of *S. thermophilus* ST128 electrotransformed with the parental plasmid. Restriction analyses of pBN183A showed that a ca. 1.4-kb DNA fragment originally located between 5.1- and 6.5-kb coordinates of the parental plasmid pBN183 (Fig. 1) was absent in the truncated derivative. The precise sequence of the deleted fragment was not determined in this study.

pDBN183 was derived from pBN183 by *in vitro* excision of the 1.2-kb *Sal*I fragment from the parental plasmid.

The *Streptomyces* cholesterol oxidase gene (*choA*) used in the construction of *choA*-containing recombinants was obtained as a 2.4-kb *Eco*RI fragment from pUCO196 (Fig. 1). The latter was constructed as follows: The *choA*-containing, 2.4-kb *Pst*I fragment of pUCO191 (Somkuti *et al.*, 1991) was isolated by gel electrophoresis and electroelution, treated with T4 DNA polymerase to remove the 3' overhang, ligated by T4 DNA ligase to the 5'-phosphorylated 10-mer *Eco*RI oligonucleotide linker, and finally inserted into the unique <sup>1</sup>*Eco*RI site of pUC19 to yield pUCO196.

The *choA*-carrying derivatives of pBN183 were obtained by ligating the purified 2.4-kb *EcoRI* fragment of pUCO196 with the *EcoRI*-linearized and calf intestinal alkaline phosphatase (CIP)-dephosphorylated pBN183. Recombinants were isolated from Ap<sup>R</sup> clones of *E. coli* DH5 $\alpha$  transformed with the ligation mixture and were labeled pBCO1 and pBCO2 based on the orientation of the DNA insert (Fig. 1). Similar protocol was followed for the construction of pACO1 and pACO2 from pBN183A and of pDCO2 and pDCO3 from pDBN183.

#### DNA Manipulation

Restriction digestions were carried out in buffer systems and under the conditions described in BRL Life Technologies' 1990 *Catalogue and Reference Guide*. For double digestion reactions involving enzymes with different optimal buffer systems, reaction conditions with the least side effects (star activity, nonspecific nuclease activity, etc.) were used. Ligation with T4 DNA ligase, dephosphorylation with CIP, and removal of 3'-protruding end with T4 DNA polymerase were performed as described by Ausubel *et al.* (1987a). Attachment of oligonucleotide linker to DNA fragment was carried out as outlined by Maniatis *et al.* (1982).

Agarose gel electrophoresis of DNA was performed in TBE buffer system (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA).  $\phi$ X174 RF DNA/*Hae*III and  $\lambda$  DNA/*Hind*III fragments were used as size markers for DNA fragment analysis, and *E. coli* pV517 plasmids (Macrina *et al.*, 1978) were employed as the standards for the sizing of undigested plasmids. DNA fragments were isolated by electroelution (Maniatis *et al.*, 1982). The eluted DNA was purified and simultaneously concentrated by subjecting the solution to Elutip-d column (Schleicher & Schuell, Inc., Keene, NH) treatment.

#### Transformation of Bacteria

Competent *E. coli* cells were either prepared by using the CaCl<sub>2</sub> challenge method

(Mandel and Higa, 1970) or purchased from commercial sources as described above. Transformation was performed by the heat shock method (Maniatis *et al.*, 1982). Cells were allowed to recover in 1 ml SOC medium (Hanahan, 1983). Transformants were selected by spreading aliquots (usually 20–100  $\mu$ l) of the transformation mixture on LB/1% agar plates containing the appropriate antibiotic.

Electrotransformation of *S. thermophilus* was performed according to the method developed in this laboratory (Somkuti and Steinberg, 1987). The Gene Pulser electroporation system from Bio-Rad Laboratories (Richmond, CA) was used. Similar protocol was used to electrotransform *L. casei*.

#### Plasmid Isolation

Miniprep of *E. coli* plasmid was prepared from 1 to 1.5 ml overnight culture by the alkaline lysis method described by Ausubel *et al.* (1987b). Nonspecific nuclease contaminants that interfered with restriction analysis were removed by phenol extraction (Maniatis *et al.*, 1982) or by treatment with Strataclean resin according to the supplier's procedure. Screening of *S. thermophilus* plasmids was routinely carried out by the method of Somkuti and Steinberg (1986) which yielded high-quality preparations. For rapid plasmid screening involving as many as 72 *S. thermophilus* cultures, the alkaline lysis miniprep protocol described for *E. coli* (see above) was adopted. In this case, *S. thermophilus* cells from 4-ml stationary phase cultures (1–3 days old) were collected by centrifugation in a bench-top clinical centrifuge operated at top speed. Cell pellets were washed once with cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) and resuspended in 50 mM Tris-HCl (pH 7.0). Lysozyme and mutanolysin were added to a final concentration of 2 mg/ml and 0.2 unit/ $\mu$ l, respectively. The lysis mixtures were incubated at 37°C for 30 min. Subsequent alkaline lysis and plasmid isolation were performed as described for *E. coli* (Ausubel *et al.*, 1987b).

Large-scale preparation of *E. coli* plasmid was performed by the alkaline lysis method (Ausubel *et al.*, 1987b), and of the *S. thermophilus* and *L. casei* plasmid according to the method of Somkuti and Steinberg (1986). CsCl/ethidium bromide gradient ultracentrifugation in a vertical rotor (Ausubel *et al.*, 1987b) or Elutip-d column treatment after RNase A (0.1 mg/ml) and Proteinase K (0.1 mg/ml) digestions was performed to purify plasmid preparations.

The DNA concentration of plasmid preparations obtained from CsCl gradients was estimated spectrophotometrically based on the  $A_{260\text{ nm}}$  of the samples by using an extinction coefficient of  $\epsilon_{260\text{ nm}}$  (1 mg/ml) = 20. For the specific estimation of DNA contents of the Elutip-d purified preparations, the bis-benzimidazole (or Hoechst 33258) fluorescent dye binding assay was performed using a DNA fluorometer (Model TKO100, Hoefer Scientific Instruments, San Francisco, CA) according to the manufacturer's instructions. All plasmid samples were virtually protein free as evidenced by the  $A_{260\text{ nm}}/A_{280\text{ nm}}$  ratio of >1.8.

#### Plasmid Copy Number Estimation

The copy number of plasmids in *E. coli* was estimated essentially as described by Projan *et al.* (1983). Cells harvested from 1.5-ml overnight cultures were resuspended in 100  $\mu\text{l}$  of a buffer containing 25 mM Tris-HCl (pH 8), 50 mM glucose, 10 mM EDTA, 5 mg/ml lysozyme, and 0.1 mg/ml RNase A. After the suspension was incubated at 37°C for 30 min, 100  $\mu\text{l}$  of 2% (w/v) sodium dodecyl sulfate solution was then added to induce cell lysis. Solutions were mixed gently to avoid shearing of the chromosomal DNA. Two freeze-thaw cycles (at -70 and +37°C, respectively) were performed to complete cell breakage. Proteinase K (100  $\mu\text{g}/\text{ml}$ ) was then added, and the mixtures were incubated at 37°C for 30 min. The cell lysates were subjected to agarose gel electrophoresis to separate the chromosomal and plasmid DNAs for subsequent quantitation. Cell lysate samples

containing ca. 0.2, 1.0, and 2.0  $\mu\text{g}$  total DNA were loaded on 5-mm-thick, 0.8% (w/v) agarose gel. Electrophoresis was carried out in TBE buffer at 3.5 V/cm for 17 h. The gel was stained with ethidium bromide solution (2  $\mu\text{g}/\text{ml}$ ) for 30 min and washed in excess  $\text{H}_2\text{O}$  for 90 min. The gel, placed on an ultraviolet transilluminator, was photographed with Polaroid Type 55 film. The film negatives were scanned and documented with a Bio-Rad video densitometer (Model 620, Bio-Rad Laboratories), and the densitometry profiles were analyzed with the 1-D Analyst II/PC Data Analysis Software program (Bio-Rad Laboratories). Copy numbers of plasmids were calculated as described by Projan *et al.* (1983). The size of the *E. coli* chromosomal DNA used in the calculation was 4700 kb (Kohara *et al.*, 1987).

#### Assay of ChoA Expression

Expression of *choA* in *E. coli* transformants harboring pACO, pBCO, or pDCO plasmid was evaluated based on the depletion of added cholesterol by the growing cultures. In this experiment, 20- $\mu\text{l}$  aliquots of a cholesterol stock solution (7.5 mg/ml, in *n*-propanol) were added to culture tubes containing 2.5 ml LB medium (100  $\mu\text{g}/\text{ml}$  Ap), freshly inoculated with 0.2 ml of stationary-phase freshly thawed stocks. The cultures containing the cholesterol were grown at 37°C in an incubator-shaker operated at 250 rpm. After ca. 3 days of growth, cholesterol in each of the stationary phase cultures was extracted twice with 2 ml of ethyl acetate. The solvent was subsequently removed by flowing  $\text{N}_2$  gas through the solution heated to 85°C. The amounts of cholesterol in the dry residues were estimated by colorimetric assay with  $\text{FeCl}_3\text{-H}_2\text{SO}_4$ -acetic acid reagent (Johnson and Somkuti, 1990).

The ChoA activity of *S. thermophilus* and *L. casei* transformants was determined as detailed elsewhere (Somkuti *et al.*, 1991).

#### Biochemicals

Growth medium components such as yeast extract, bactotryptone, beef extract, and

bactoagar were purchased from Difco Laboratories (Detroit, MI). Ampicillin and tetracycline were from Sigma Chemical Co. (St. Louis, MO), and chloramphenicol was from Calbiochem (San Diego, CA). Cholesterol and 4-cholesten-3-one were purchased from Sigma.

Restriction endonucleases were from commercial sources such as BRL Life Technologies, New England BioLabs (Beverly, MA), Sigma, and U.S. Biochemicals (Cleveland, OH). T4 DNA ligase and T4 DNA polymerase were purchased from BRL Life Technologies. CIP was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). New England BioLabs was the supplier of the phosphorylated 10-mer *EcoRI* oligonucleotide linker. Lysozyme, mutanolysin, and RNase A were from Sigma; Proteinase K was from IBI (New Haven, CT).

SeaKem ME agarose for gel electrophoresis was from FMC Corp. (Rockland, ME).  $\phi$ X174 RF DNA/*HaeIII* fragments and  $\lambda$  DNA/*HindIII* digests were from New England BioLabs and BRL Life Technologies, respectively. Elutip-d columns were purchased from Schleicher & Schuell, Inc. (Keene, NH), and Stratagene resin was obtained from Stratagene Cloning Systems (La Jolla, CA).

All other reagent-grade biochemicals and chemicals were obtained from common commercial sources. The mention and/or use of any commercial sources and/or products does not constitute official endorsement by the USDA.

## RESULTS

### *Construction of the pBN-Series Plasmids*

Transformation of *E. coli* HB101 with the ligation mixture containing 0.4  $\mu$ g *Bam*HI-linearized pBR322 and 0.8  $\mu$ g pNZ18/*Bgl*II partial digest produced ca. 100 transformants with Ap<sup>R</sup> phenotype. Replica plating of these transformants on LB + Ap (50  $\mu$ g/ml) and LB + Tc (15  $\mu$ g/ml) resulted in the identification of an Ap<sup>R</sup>, Tc<sup>S</sup> clone that contained plasmid pBN183. Restriction analyses showed

that pBN183 yielded 2.8- and 7.3-kb fragments on *Pvu*II digestion and 3.4- and 6.7-kb DNA pieces on reaction with *Pst*I. Based on these results and the available restriction maps of pBR322 and pNZ18, the configuration of pBN183 was deduced as shown in Fig. 1. In this construct, the direction of the *cat* gene and the Nm<sup>R</sup> (or Km<sup>R</sup>) determinant was opposite to that of the *repA* gene, the  $\beta$ -lactamase (or Ap<sup>R</sup>) determinant, and the pBR322 replication function region.

Electrotransformation of *S. thermophilus* ST128 with pBN183 yielded Cm<sup>R</sup> clones; 10 of these transformants were randomly chosen for further study. Repeated transfers of these cells in Cm-containing medium resulted in the loss of five cultures. Plasmids were prepared from the remaining five surviving cultures. Gel electrophoretic analysis revealed that each of the five plasmid preparations contained a host of deleted pBN183 derivatives. To characterize these deletion plasmids, we transformed *E. coli* HB101 with the five plasmid preparations. Miniplasmid screening of the Cm<sup>R</sup> *E. coli* transformants allowed us to identify one deletion species with the least DNA sequence missing. We labeled this pBN183 deletion derivative as pBN183A. Subsequent electrotransformation of *S. thermophilus* ST128 with this plasmid gave Cm<sup>R</sup> transformants that apparently contained undeleted pBN183A. Results of restriction analyses of pBN183A (Table 1) indicated that a ca. 1.4-kb sequence was deleted between coordinates 5.1 and 6.5 kb of the original pBN183 plasmid. The precise sites of the deletion were not determined. Computer analysis of the available DNA sequence in this region of pBN183 revealed two tandem repeats (ca. 0.6 kb each) with complete homology. These repeating sequences started at the two *Sal*I sites at coordinates 5.12 and 6.35 kb and extended in a clockwise direction.

Based on the observation that pBN183A lacking the direct repeats as seen in pBN183 appeared to escape DNA deletion in *S. thermophilus*, we proceeded to construct pDBN183 vector (Fig. 1) from the parental

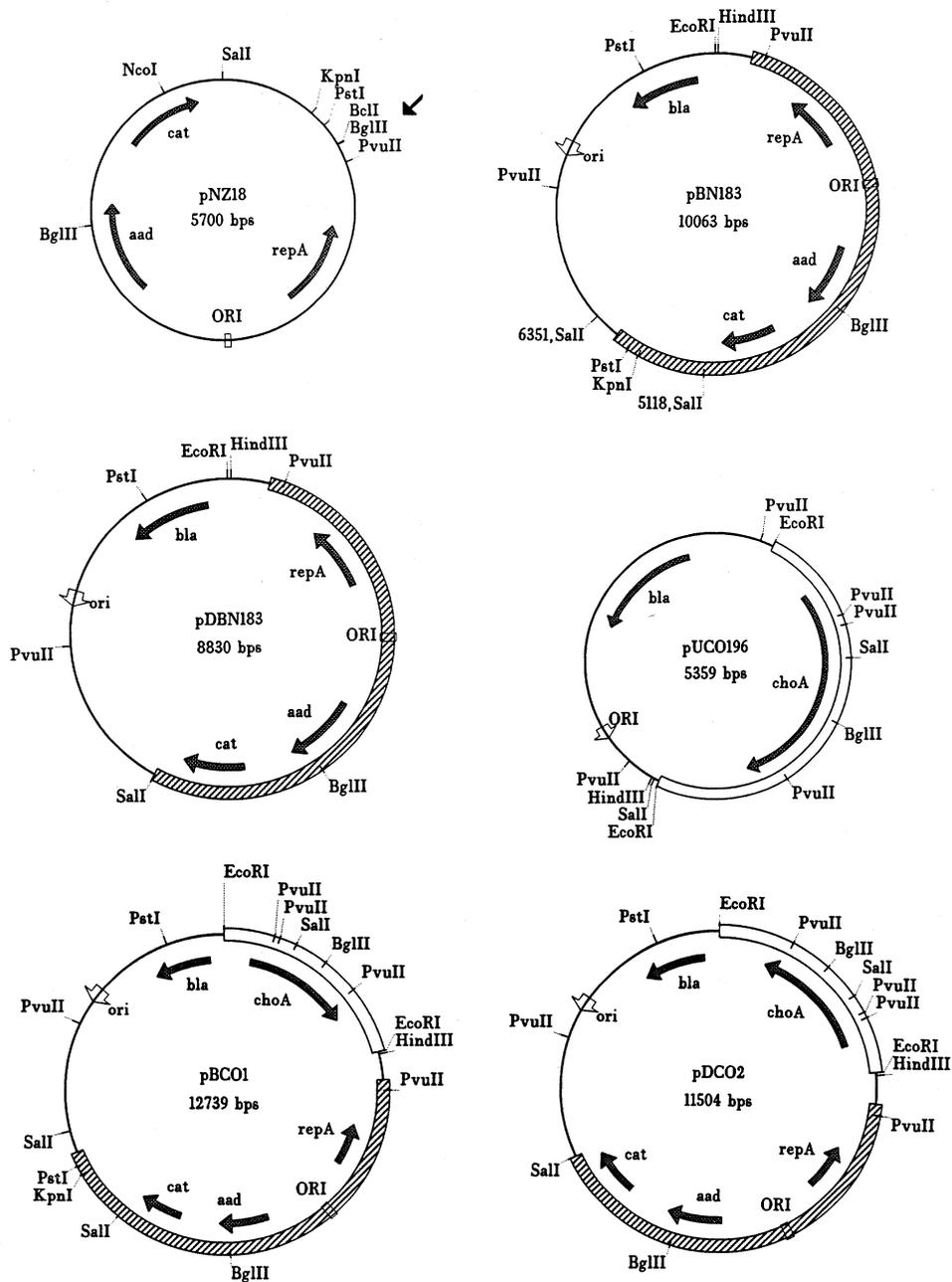


FIG. 1. Plasmid maps. The structure of pNZ18 was based on nucleotide sequence information kindly provided by G. Simons. Linearization of pNZ18 occurred at the *BglII* site marked with an arrow. pBCO2 and pDCO3 (not shown) are sister plasmids of pBCO1 and pDCO2, respectively; the orientation of the small (2.4 kb) *EcoRI* fragment in pBCO2 and pDCO3 is opposite to that of their respective sister plasmids shown here. The structures of pBN183A and its *choA*-containing derivatives are not shown, because the precise deletion sites have not been determined. Antibiotic-resistance genes are labeled as recommended by Novick *et al.* (1976). Thus, the *bla*, *cat*, and *aad* genes confer resistance to Ap, Cm, and Km, respectively. *ori* and *repA* are the replication functions from pNZ18, and *ori* represents the origin of replication of pBR322. Restriction sites are shown only for endonucleases pertinent to this study. Plasmids were not drawn proportionately to size.

TABLE 1

RESTRICTION ANALYSIS OF pBN183 AND pBN183A		
Restriction endonuclease	Fragment size (kb)	
	pBN183	pBN183A
A. Single digestion <sup>a</sup>		
<i>Hind</i> III, <i>Eco</i> RI, or		
<i>Bgl</i> II	10.1	8.7
<i>Kpn</i> I	10.1	NR <sup>b</sup>
<i>Pvu</i> II	7.3; 2.8	5.9; 2.8
<i>Sal</i> I	8.9; 1.2	8.7
<i>Pst</i> I	6.7; 3.4	8.7
B. Double Digestion <sup>a</sup>		
<i>Pvu</i> II + <i>Sal</i> I	4.6; 2.8; 1.4; 1.2 <sup>c</sup>	4.5; 2.8; 1.4
<i>Bgl</i> II + <i>Sal</i> I	7.3; 1.6; 1.2 <sup>c</sup>	7.3; 1.4

<sup>a</sup> Reaction products were analyzed on 0.7 and 1.5% agarose gels, respectively, for single- and double-digestion experiments.

<sup>b</sup> No reaction occurred, indicating the loss of the unique *Kpn*I site.

<sup>c</sup> Fragment size was discerned from the restriction map in Fig. 1.

pBN183 by the removal of the small, 1.4-kb *Sal*I fragment.

#### Characteristics of pBN-Series Plasmids in *E. coli*

We first studied the expression of the *cat* and  $\beta$ -lactamase genes of the three plasmids to ascertain their suitability as selection markers in *E. coli*. As expected, *E. coli* harboring these plasmids exhibited Cm<sup>R</sup> and Ap<sup>R</sup> phenotypes, as indicated by their ability to grow and form colonies on LB + Ap (as high as 100  $\mu$ g/ml) and LB + Cm (up to 50  $\mu$ g/ml) solid media.

We further studied the antibiotic-resistance properties of the transformants containing pBN183A and pDBN183, since these plasmids were potentially useful shuttle vectors owing to their apparent stability in *S. thermophilus*. Overnight cultures of *E. coli* harboring pBN183A or pDBN183 were prepared. Inoculations into fresh LB media containing 30  $\mu$ g/ml Cm, 50  $\mu$ g/ml Cm, 50  $\mu$ g/ml

Ap, or 100  $\mu$ g/ml Ap were performed using these overnight cultures. Cell growth was monitored by recording the OD<sub>590</sub> nm of the cultures. The results, depicted in Figs. 2A and 2B, showed that both plasmids conferred Ap<sup>R</sup> and Cm<sup>R</sup> to *E. coli*. Cell growth in Cm-containing medium, however, was not as optimal as that in Ap medium, as evidenced by the occurrence of an initial lag period, the longer cell-doubling time, and the lower final cell mass.

The segregation stability of pBN183A- or pDBN183-transformed *E. coli* was next examined. Overnight cultures of the transformants were prepared in LB + Cm (50  $\mu$ g/ml) or LB + Ap (100  $\mu$ g/ml). Four consecutive transfers into fresh growth media, with and without the same antibiotic, were carried out at 4-h intervals (except the last transfer which took place after an overnight growth) at 1/20

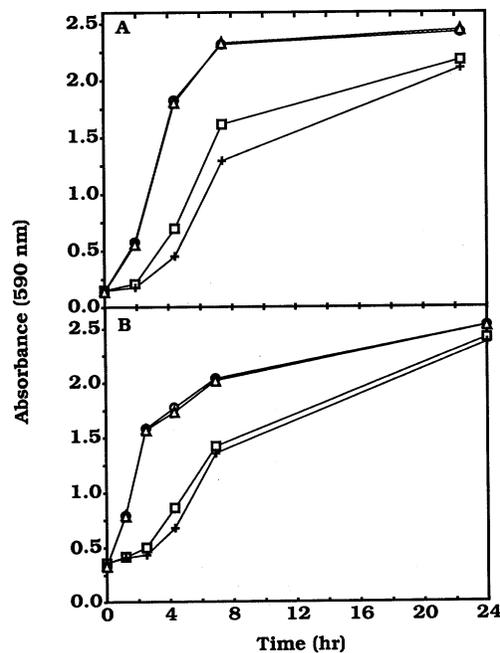


FIG. 2. Growth curve of *E. coli* transformants in Ap- or Cm-containing medium. Overnight culture of transformants was prepared in LB + Cm (30  $\mu$ g/ml) medium. Inoculation (4–5%) was performed into LB containing 50  $\mu$ g/ml Ap (O), 100  $\mu$ g/ml Ap ( $\Delta$ ), 30  $\mu$ g/ml Cm ( $\square$ ), or 50  $\mu$ g/ml Cm (+). (A) *E. coli* HB101 harboring pBN183A; (B) *E. coli* HB101 containing pDBN183.

to 1/10 dilution. Cells at the end of the four passages were used to: (1) perform plasmid miniprep procedures and (2) spread on LB + Cm (30  $\mu\text{g}/\text{ml}$ ) and LB + Ap (50  $\mu\text{g}/\text{ml}$ ) agar plates. The results showed that regardless of the absence or presence of antibiotic acting as selection pressure, the plasmids remained stably maintained in the cells after generations of growth. Thus, gel electrophoretic analyses of the plasmid minipreps did not reveal any apparent changes in vector size. Furthermore, the numbers of colonies spotted on the spread agar plates were comparable regardless of whether the transformants had been passaged through LB only, LB + Cm, or LB + Ap growth medium.

The transformation frequency of the pBN-series plasmids was determined using the commercially available competent *E. coli* DH5 $\alpha$  as recipient. pBR322 was used as a standard for comparison. Since cells harboring the pBN-series plasmids exhibited more optimal growth in Ap medium than in Cm broth, this study was performed using agar plates containing Ap. The results (Table 2) showed that the three chimeric plasmids had comparable transformation frequencies in the order of  $10^5$  CFU/ $\mu\text{g}$  DNA. These values were 1–2 orders of magnitude lower than that of pBR322, but were considerably higher than that obtained with pNZ18.

Similar to the parental pBR322, the three chimeric plasmids seemed to replicate in *E. coli* under relaxed copy number control. The results of copy number estimation showed that the three plasmids were maintained at ca. 17–18 copies/chromosome (Table 2).

#### *Characteristics of pBN-Series Plasmids in S. thermophilus*

When pBN183 plasmid was electroporated into *S. thermophilus* ST128, we obtained Cm<sup>R</sup> clones at a transformation frequency of  $(2.6 \pm 0.3) \times 10^4$  CFU/ $\mu\text{g}$  DNA. However, miniscale plasmid screening with randomly chosen transformants revealed that 17 out of these 24 clones contained various deleted forms of pBN183. The deletion-prone

pBN183 was apparently not an ideal shuttle vector for *S. thermophilus*.

The second generation chimeric vectors, pBN183A and pDBN183, transformed ST128 by electroporation with transformation frequency of  $(8.1 \pm 0.2) \times 10^2$  and  $(4.6 \pm 0.2) \times 10^2$  Cm<sup>R</sup> CFU/ $\mu\text{g}$  DNA, respectively. Unlike pBN183, these vectors appeared to be more resistant to deletion in *S. thermophilus*. Miniscale plasmid screening of the Cm<sup>R</sup> transformants showed that 0 and 4, respectively, out of 24 clones from the pBN183A- and pDBN183-transformed ST128 harbored deleted plasmid species. Obviously, these vectors were more suitable shuttle vectors than pBN183 for *E. coli* and *S. thermophilus*.

The  $\beta$ -lactamase gene carried on the pBN-series plasmids was not expressed in ST128, as evidenced by the results of a study with pBN183. *S. thermophilus* transformed to Cm<sup>R</sup> by this plasmid failed to grow when placed in medium containing 15  $\mu\text{g}/\text{ml}$  Ap, even though restriction analyses of plasmids (intact or deleted) isolated from these transformants indicated the continued presence of the  $\beta$ -lactamase gene.

#### *Evaluation of pBN Plasmids as Cloning Vehicles*

The streptomycete *choA* gene was used as a passenger DNA in this evaluation, because it had previously been expressed in *E. coli* (Solaiman and Somkuti, 1991b), *S. thermophilus* (Somkuti *et al.*, 1991), and *L. casei* (Somkuti *et al.*, 1992) in our laboratory.

The insertion of the *choA* gene into the pBN-series plasmids did not affect their stability in *E. coli* host. The recombinant plasmids (pBCO, pACO, and pDCO) could be recovered from *E. coli* transformants with no detectable deletion. Expression of ChoA activity, however, was not detected in the transformants. This result agreed with our earlier observation (unpublished data) that a functional *E. coli* promoter was required for the expression of the apparently promoterless *choA* gene (Ishizaki *et al.*, 1989).

TABLE 2  
TRANSFORMATION FREQUENCY AND COPY NUMBER OF THE PLASMID CONSTRUCTS

Plasmid	Transformation frequency		Copy number <sup>b</sup> (in <i>E. coli</i> DH5 $\alpha$ )
	<i>E. coli</i> DH5 $\alpha$	<i>S. thermophilus</i> ST128	
pBN183	$(8.2 \pm 1.2) \times 10^5$	$(2.6 \pm 0.3) \times 10^{1c}$	18
pBN183A	$(4.8 \pm 0.1) \times 10^5$	$(8.1 \pm 0.2) \times 10^2$	18
pDBN183	$(5.0 \pm 1.3) \times 10^5$	$(4.6 \pm 0.2) \times 10^2$	17
pNZ18	$6 \times 10^{2d}$	$1.2 \times 10^2$	ND <sup>e</sup>
pBR322	$1.8 \times 10^7$	NA <sup>e</sup>	18 <sup>f</sup>
pVA736	NA <sup>e</sup>	$1.1 \times 10^{3g}$	NA <sup>e</sup>

<sup>a</sup> Unless otherwise specified, transformants were scored on LB/1% agar plates containing Ap (100  $\mu$ g/mg) for *E. coli* and on lactose or sucrose broth (Somkuti and Steinberg, 1979)/1% agar plates containing Cm (10  $\mu$ g/ml) for *S. thermophilus*.

<sup>b</sup> Copy number was estimated using cells grown in LB + Ap (100  $\mu$ g/ml).

<sup>c</sup> Miniprep plasmid screening showed that ca. 70% of the transformants contained deleted pBN183.

<sup>d</sup> Determined in a separate experiment with *E. coli* HB101. Transformants were selected on LB/1% agar plate containing Cm (30  $\mu$ g/ml). In the same experiment, pBR322 yielded  $\geq 2.2 \times 10^5$  CFU/ $\mu$ g DNA on LB + Ap (50  $\mu$ g/ml) agar plate.

<sup>e</sup> ND, Not determined; NA, not applicable.

<sup>f</sup> Reported by Covarrubias *et al.* (1981).

<sup>g</sup> Scored on lactose broth agar plate containing Em (15  $\mu$ g/ml).

When pBCO-family plasmids electrotransformed ST128, they were degraded into smaller-size DNAs by the host organism. This was anticipated because the parental vector, pBN183, itself underwent deletion in *S. thermophilus*. Unexpectedly, the pACO- and pDCO-series recombinant DNAs were also deleted to various extents in ST128. It appeared that the *choA* insert contained sequence(s) detrimental to the stability of the parental pBN183A and pDBN183 vectors.

We had observed earlier that recombinant DNA, specifically the pNCO937 plasmid where *choA* was carried on pNZ19 vector, appeared to be more stably maintained in another lactic acid bacterium, *L. casei*, than in *S. thermophilus*. Consequently, the fate of the pDCO-series plasmids in *L. casei* was studied. The results showed that these plasmids indeed did not undergo detectable deletion when electroporated into *L. casei* (Fig. 3). Although the transformants contained undeleted plasmids, we failed to detect cholesterol oxidase activity in these cells, suggesting

again that the *choA* gene required a functional promoter for expression.

## DISCUSSION

The pBN-series shuttle vectors containing the replication functions of *E. coli* plasmid (pBR322) and gram-positive streptococcal plasmid (pNZ18) were constructed and characterized in this study. The first-generation construct, pBN183, was deleted to varying extents when introduced into the lactic acid bacterium, *S. thermophilus*. Sequence analyses of pBN183 and a truncated mutant, pBN183A, revealed that the region susceptible to deletion contained direct repeats of ca. 0.6 kb long. Sequence repeats had previously been shown (Peeters *et al.*, 1988; Janniére and Ehrlich, 1987; Niaudet *et al.*, 1984; Brunier *et al.*, 1988) to promote plasmid recombinations. These rearrangement events occur most frequently with plasmids that replicate by rolling-circle (RC) mechanism involving single-stranded DNA as intermediate

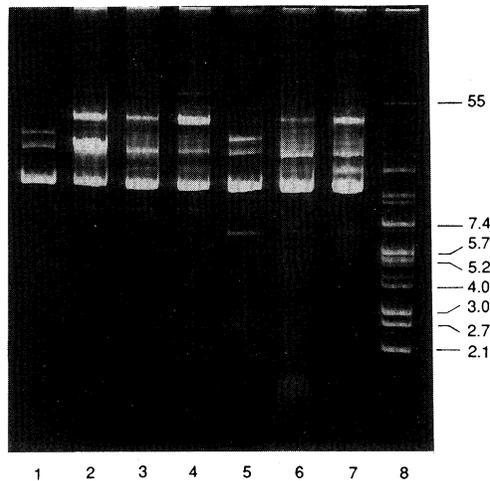


FIG. 3. Agarose gel electrophoresis of pDCO2 and pDCO3 isolated from *Lactobacillus casei*. Plasmid DNA was isolated from *L. casei* LC2 transformed with pDCO2 and pDCO3 and electrophoresed in 0.7% agarose-TBE gels. Lanes 1 and 5: pDCO2 and pDCO3, respectively, isolated from *E. coli*. Lanes 2-4: plasmids of *L. casei*/pDCO2 transformants. Lanes 5-7: plasmids of *L. casei*/pDCO3 transformants. Lane 8: *E. coli* V517 plasmid standards. *L. casei* transformants also harbor a large, 35.4-kb native plasmid.

(Gruss and Ehrlich, 1989). Since the replication function most probably used by pBN183 in *S. thermophilus* is that of the pNZ18 [which in turn originates from single-stranded DNA plasmid, pSH71 (Gruss and Ehrlich, 1989)], the direct repeats in pBN183 apparently promote its recombination in this gram-positive host. In agreement with this explanation, when one copy of the 0.6-kb repeating units was removed from pBN183, the resulted pDBN183 plasmid replicates with dramatically improved stability in *S. thermophilus*.

The second-generation plasmid constructs, pBN183A and pDBN183, are attractive shuttle vectors for use in *E. coli* and lactic acid bacteria. These plasmids transform *E. coli* with frequencies in the order of  $10^5$  CFU/ $\mu$ g DNA. These values are ca. 3 orders of magnitude higher than that seen with the parent pNZ18 broad-host-range plasmid. Thus, molecular cloning experiments in the well-

studied *E. coli* can be performed more conveniently with the pBN-series plasmids than with the pNZ18. The large difference in transformation frequencies exhibited by the two classes of vectors is likely due to the selection pressure used. For the new shuttle vectors, the  $\beta$ -lactamase gene can be used as selection marker. This gene is well expressed in *E. coli*. In contrast, the *cat* gene that does not express well in *E. coli* (De Vos, personal communication) is the only available selection marker for pNZ18-transformed cells. In addition to the choice of selection marker, the presence of an *E. coli* origin of replication in the new chimeric plasmids may contribute to their higher transformation frequencies in comparison with pNZ18.

The copy number of these pBN183 derivatives in *E. coli* is as high as that reported for the widely used *E. coli* cloning vector, pBR322 (Covarrubias *et al.*, 1981). Furthermore, these bifunctional shuttle vectors also appear to remain stably maintained in *E. coli* even after generations of growth without selection pressure. These attributes, together with the reasonably high transformation frequencies mentioned above, further justify the suitability of these bifunctional vectors for use as cloning vehicles in *E. coli*.

As shuttle vectors for lactic acid bacteria such as *S. thermophilus*, these more stable recombinant plasmids (pBN183A and pDBN183) are 5 to 10 times more efficient in transforming the host cells than the parent pNZ18. In fact, pBN183A seems to transform *S. thermophilus* with similar efficiency as pVA736—a 7.6-kb model vector for the electrotransformation of this microbe (Somkuti and Steinberg, 1988). In the case of pDBN183, however, the advantage of higher transformation frequency over pNZ18 is partially compromised by a slight plasmid instability; plasmid screening of transformants showed that ca. 17% of them contained deletion mutants. Rearrangement of pDBN183 lacking direct repeats suggests that additional sequence element or structure may be operative here in inducing recombinations (Gruss and Ehrlich, 1989). Studies are in progress to

identify these recombination-inducing DNA sequences.

Using the streptomycete *choA* gene as model, an attempt to demonstrate the versatility of the newly constructed vectors for cloning and expression purposes in the intended host cells was met with mixed results. As expected of an *E. coli* shuttle vector, the *choA*-carrying derivatives of pBN-series plasmids could be conveniently constructed and stably maintained in this host. In the two lactic acid bacteria tested, however, only in *L. casei* were the plasmids stably maintained; *S. thermophilus* deleted the recombinants to various extents. These results suggested that the *choA* gene might contain sequences detrimental to plasmid stability in *S. thermophilus*. Identification of these plasmid-destabilizing sequence elements is currently underway with the goal of achieving stable maintenance of recombinant DNA in *S. thermophilus*.

The present studies also show that the *choA* gene in the recombinant plasmids was not expressed in the host cells. This is not completely unexpected, since the shuttle vectors were constructed primarily for gene cloning and shuttling purposes. As a result, gene expression is not expected if the passenger DNA does not contain functional promoter, as is the case with *choA* (Ishizaki *et al.*, 1989). Research is in progress to build expression elements (such as promoter and ribosomal binding site) into these shuttle plasmids so that they may function as expression vectors as well.

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