

## Shuttle Vectors Developed from *Streptococcus thermophilus* Native Plasmid

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pER8 (2.2 kb), a native plasmid of *Streptococcus thermophilus* ST108, was used to develop pMEU-series shuttle vectors. In addition to the replication function of the pER8, these vectors contain origin of replication and  $\beta$ -lactamase gene (*bla*) of *Escherichia coli* vector pUC18/19, the *cat* gene of pC194, and the pPV141-borne *erm* determinant of *Staphylococcus hyicus* ssp. *chromogenes* 3688. pMEU5a, pMEU5b, pMEU6a, and pMEU6b (all 5.7 kb in size) contain *bla* and *erm* markers, are capable of transforming *E. coli* and *S. thermophilus* at frequencies in the order of  $10^5$ – $10^6$  and  $10^3$  colony forming units (CFU)/ $\mu$ g DNA, respectively, and are highly stable in the two host systems. pMEU9 and pMEU10 (both 6.9 kb) contain the *cat* marker in addition to the DNA elements found in pMEU5-6. These plasmids are also highly effective in transforming *E. coli* (at ca.  $6 \times 10^5$  CFU/ $\mu$ g DNA) and *S. thermophilus* (ca.  $10^3$  CFU/ $\mu$ g DNA). Although expression of the resistance markers is not completely consistent, pMEU9 and pMEU10 remain important shuttle vectors for clonal selection by an insertional inactivation method. © 1993 Academic Press, Inc.

*Streptococcus thermophilus* (ST) is a thermophilic lactic acid bacterium used in the dairy fermentation industry for the production of yogurt and certain cooked cheeses (Rosenthal, 1991). The main function of this starter culture in the fermentation process is to serve as the producer of lactic acid. As with the other lactic acid bacteria (LAB), certain properties of ST important to its function are not always stable. Moreover, LAB as a group are in general very susceptible to phage infection. Consequently, research efforts abound which attempt culture improvement of this organism by genetic engineering approaches in order to stabilize certain desirable traits and to provide phage resistance mechanism(s). Because of its generally recognized

as safe ("GRAS") status, *S. thermophilus* (as well as the other lactic acid bacteria) is also an attractive host system for the production of recombinant gene products. This provides an additional incentive for the dairy research community to develop further the molecular biology and genetic engineering methodology of this organism.

Crucial to the genetic engineering of an organism is the availability of cloning vectors. Earlier efforts to attempt gene transfer in *S. thermophilus* invariably involved the use of vectors containing replication sequences of a heterologous bacterial species (Somkuti and Steinberg, 1988; Mercenier, 1990; Somkuti *et al.*, 1991). As a result, the recombinant plasmids often were unstable and their transformation efficiency was low (Solaiman *et al.*, 1992). Furthermore, the introduction of these additional "foreign" replication sequences into the "GRAS" organism would only add to the regulatory hurdles faced by investigators.

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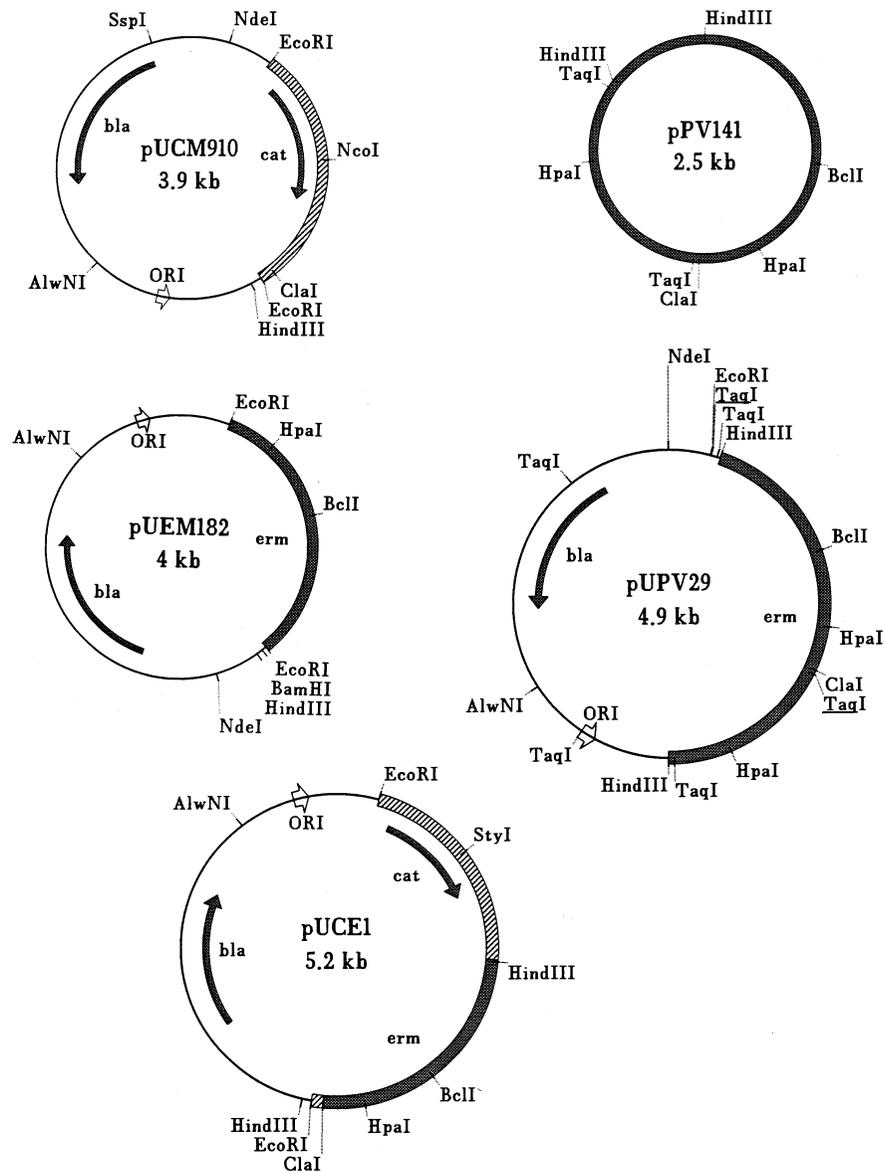


FIG. 1. Plasmids involved in the subcloning of the staphylococcal *cat* and *erm* determinants. Sequences originated from *E. coli* pUC vectors, *S. hyicus* ssp. *chromogenes* 3688 plasmid pPV141, and *S. aureus* plasmid pC194 (Ehrlich, 1977) were represented by single line (—), shaded box (■), and hatched box (▨), respectively. The symbols suggested by Novick *et al.* (1976) were used to label the antibiotic resistance markers. Thus *bla*, *cat*, and *erm* represent Ap<sup>R</sup>, Cm<sup>R</sup>, and Em<sup>R</sup> determinants, respectively. The underlined *TaqI* sites of pUPV29 indicated the ca. 1.3-kb fragment that was incorporated into pUCE1.

In this communication, we describe the construction of the pMEU series of shuttle vectors by using pER8, a native plasmid of *S. thermophilus* (Somkuti and Steinberg,

1986a). As evidenced by their stability and high transformation efficiency, these plasmids can apparently serve as gene shuttle vectors for *Escherichia coli* and *S. thermo-*

*philus*. This effort represents an important first step to the development of "all food grade" cloning vectors for *S. thermophilus* and possibly other lactic acid bacteria.

## MATERIALS AND METHODS

### *Bacterial Strains, Plasmids, and Growth Conditions*

*E. coli* DH5 $\alpha$ , HB101, DH5 $\alpha$ FIQ, and SURE (*recB*, *recJ*, *sbcC*, *umuC*, *uvrC* genotypes useful for the cloning of unstable DNA structures) were purchased as competent cell preparations from BRL Life Technologies (Gaithersburg, MD) and Stratagene Cloning Systems (La Jolla, CA). *B. subtilis* BD170 harboring pC194 was obtained from ATCC (Rockville, MD). *S. thermophilus* ST108 and ST128 and *Staphylococcus hyicus* ssp. *chromogenes* 3688 were from our laboratory collection.

The *E. coli* vectors pUC18 and pUC19 were from BRL Life Technologies Inc. pER8 is a native plasmid of *S. thermophilus* ST108 (Somkuti and Steinberg, 1986a). pC194 was isolated from *B. subtilis* BD170. pPV141 is a resident plasmid of *S. hyicus* ssp. *chromogenes* 3688.

*E. coli* and *B. subtilis* were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C with vigorous agitation. *S. thermophilus* was maintained in tryptone-yeast extract-lactose (TYL) broth (Somkuti and Steinberg, 1979). *S. hyicus* was cultured in tryptic soy broth (Difco Laboratories, Detroit, MI).

### *Molecular Cloning Procedures*

Large-scale plasmid isolation from *E. coli* and *B. subtilis* was carried out by the alkaline lysis method (Ausubel *et al.*, 1987a). Further purification of the plasmid preparations was affected by CsCl/ethidium bromide gradient ultracentrifugation in a VTi-65 vertical rotor (Beckman Instruments, Palo Alto, CA). Alternatively, crude plasmid preparations were di-

gested sequentially with RNase A (0.2 mg/ml) and proteinase K (0.2 mg/ml) at 37°C for 1 hr. The DNA samples were then collected and washed with TE buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA) on a Centricon-100 concentrator (Amicon, Farmington, MA). Plasmids obtained by this alternative method were deemed pure based on  $A_{260\text{nm}}/A_{280\text{nm}} > 1.8$  and were suitable for use in restriction analysis and bacterial transformation. An occasional problem with complete digestibility by restriction enzymes was alleviated by processing the plasmid preparations through Elutip-d columns (Schleicher & Schuell, Inc., Keene, NH). Plasmid purification from *S. thermophilus* and *S. hyicus* was performed according to the method of Somkuti and Steinberg (1986b).

DNA concentration of the plasmid preparations was estimated spectrophotometrically by using an extinction coefficient of  $\epsilon_{260\text{nm}}$  (1 mg/ml) = 20. Agarose gel electrophoresis was carried out in TBE buffer (0.089 M Tris base; 0.089 M boric acid; 0.002 M Na-EDTA). When needed, DNA fragments were purified from agarose gel by an electroelution procedure using a GE200 Sixpac gel eluter (Hoefer Scientific Instruments, San Francisco, CA). PhiX174 RF DNA/*Hae*III and  $\lambda$  DNA/*Hind*III fragments, and *E. coli* V517 plasmids (Macrina *et al.*, 1978) were used as size markers for the digested and the uncutted plasmid samples, respectively, in gel electrophoresis.

Transformation of *E. coli* by the heat shock method was carried out according to the suppliers' protocols. The electrotransformation procedure for *S. thermophilus* had been detailed elsewhere (Somkuti and Steinberg, 1987, 1988). *E. coli* transformants were identified by spreading transformation cell mixtures on LB + 1% agar plates containing 50–100  $\mu\text{g/ml}$  ampicillin (Ap), 30–50  $\mu\text{g/ml}$  chloramphenicol (Cm), or 150–200  $\mu\text{g/ml}$  erythromycin (Em). Electrotransformed *S. thermophilus* clones were selected on solid TYL medium containing 5–15  $\mu\text{g/ml}$  Cm or 7.5–15  $\mu\text{g/ml}$  Em.

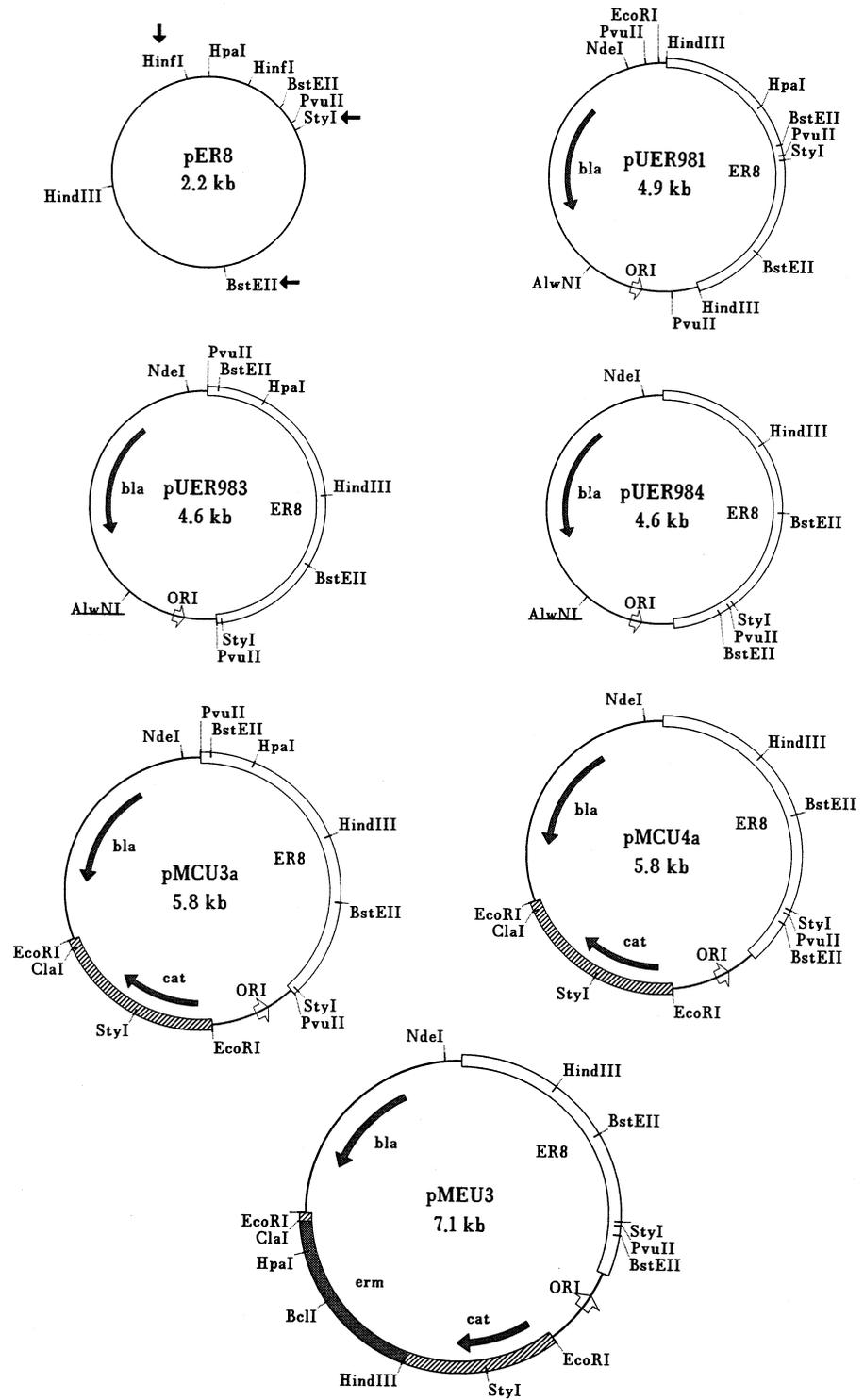


FIG. 2. First generation plasmids constructed with linearized pER8. Open box region (□) represents the

Reactions of plasmid DNAs with restriction enzymes were performed under the conditions described in BRL Life Technologies' 1992 Catalogue & Reference Guide. Ligation reactions with T4 DNA ligase, dephosphorylation reactions using calf intestinal alkaline phosphatase (CIP), filling in 3'-recessive ends with Klenow enzyme, and removal of 3'-overhangs by T4 DNA polymerase were carried out as described by Ausubel *et al.* (1987b). Attachment of oligonucleotide linkers to DNA fragments was performed as described by Sambrook *et al.* (1989).

#### *Subcloning of cat and erm Genes in E. coli*

Plasmid pUCM910 (3.9 kb) was constructed to serve as the source of *cat* gene cartridge for subsequent clonings. A 1.2-kb *SspI* fragment containing the gene was purified from the *SspI*-digested pC194 sample, and the terminals were fitted with 10-mer *EcoRI* linkers (New England Biolabs, Beverly, MA). This fragment was inserted into the *EcoRI* site of pUC19 to yield pUCM910 (Fig. 1).

The *erm* gene used in this study originated from pPV141 (2.5 kb; Fig. 1). The location of the gene had previously been mapped to the 1.3-kb *Clal-HindIII* fragment of the plasmid. For convenience, we first subcloned the 2.2-kb *HindIII* fragment of pPV141 into *HindIII* site of pUC19 to produce pUPV29 (4.9 kb; Fig. 1). Recombinant plasmid pUEM182 (4 kb; Fig. 1) carrying an *erm* gene cartridge was next constructed as follows: The 1.3-kb *Clal-HindIII* fragment containing the *erm* determinant was first isolated

from an appropriately digested pUPV29 sample. The fragment was treated with Klenow enzyme to fill in the 3'-recessive termini, ligated with the 10-mer *EcoRI* linker, and finally inserted into the unique *EcoRI* site of pUC18.

Plasmid pUCE1 (5.2 kb; Fig. 1) containing a *cat erm* gene cartridge was also constructed. Here, the *erm* determinant on a 1.3-kb *TaqI* fragment was first isolated from pUPV29. After filling in the 3'-recessive ends with the Klenow enzyme, the DNA piece was inserted at a previously blunt-ended *Clal* site of the 1.2-kb *cat* gene cartridge described above. The entire 2.5-kb *cat erm* cartridge was spliced into the *EcoRI* site of pUC18 to yield pUCE1.

#### *Plasmid Biology*

Plasmid copy number was estimated by fluorescence densitometry according to the previously described protocol (Solaiman *et al.*, 1992) developed from Projan's method (Projan *et al.*, 1983).

Plasmid stability was assessed essentially as described earlier (Solaiman *et al.*, 1992). Briefly, bacteria harboring the plasmids were subcultured daily through LB medium for 3 days. A portion of the cultures was used to plate LB and LB + Ap (50 µg/ml) solid medium to determine the percentage of cell population that retained the Ap<sup>R</sup> trait. Another portion of the cultures was added to LB medium containing Ap (50 µg/ml), Em (200 µg/ml), or Cm (15 and 30 µg/ml) to register the continued expression of the corresponding antibiotic resistance markers. Finally, the

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sequence derived from pER8. All other symbols were used as described in the legend of Fig. 1. Plasmids pUER982, pUER985, and pUER986 (not shown) are counterparts of pUER981, pUER983, and pUER984, respectively, where the pER8 sequence is oriented in the reverse direction. Similarly, pMCU3b, pMCU4b, and pMEU4 (not shown) are counterparts of pMCU3a, pMCU4a, and pMEU3, respectively, in which the *cat* (for pMCUs) or *erm* (for pMEUs) cartridge runs in the opposite direction. For pMCU1a and pMCU2b (not shown), the direction of the cloned *cat* gene is opposite to that of the *bla* gene of the constructs. Arrows on the pER8 map indicate restriction sites involved in the construction of the second generation vectors (Fig. 3). The underlined *AlwNI* of pUER983/pUER984 was modified to an *EcoRI* site during the construction of pMCU3a/pMCU4a.

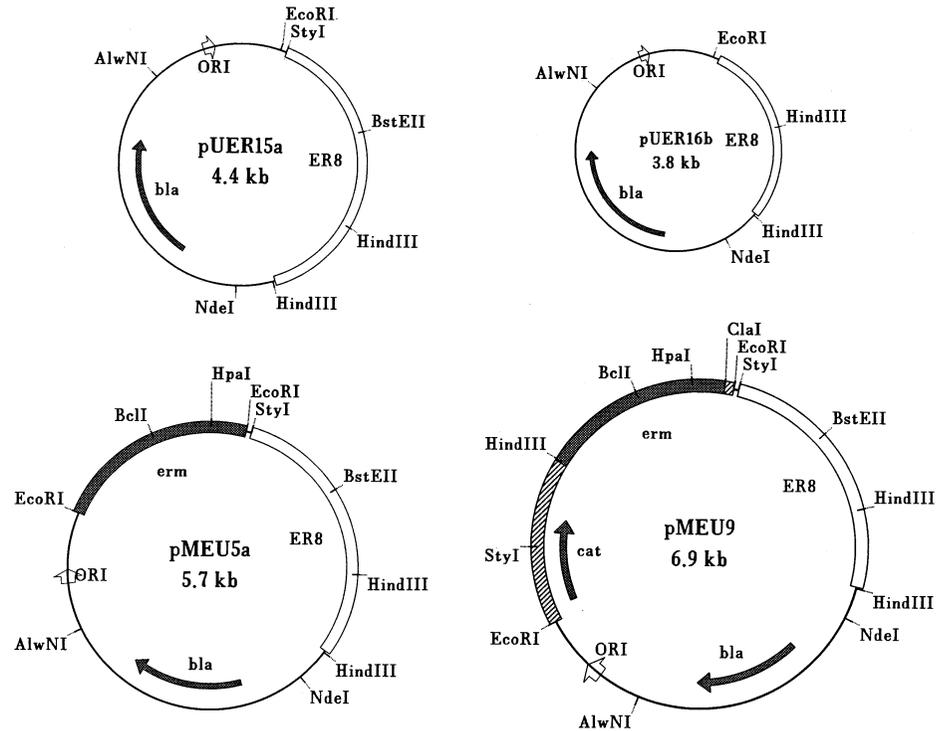


FIG. 3. Second generation shuttle vectors. These vectors were constructed using only fragments of pER8. In pUER15b and pUER16a (not shown), the pER8 sequence runs opposite to that found in their corresponding counterparts pUER15a and pUER16b. pMEU5b (not shown) contained the *erm* cassette in the reverse direction in comparison to that of the pMEU5a plasmid. For pMEU6a/pMEU6b and pMEU8a/pMEU8b (not shown), the relative orientation of all components in the constructs is analogous to that found in the corresponding pMEU5a/pMEU5b pair. pMEU9 and pMEU10 are related constructs in which the ER8 sequence runs in the opposite direction.

plasmid miniprep procedure was performed using the remaining cultures to evaluate the structural integrity of the vectors. For comparison, a duplicate set of transformants subcultured through LB + Ap (50  $\mu$ g/ml) medium was similarly processed.

## RESULTS AND DISCUSSION

### *Bifunctional Plasmids Containing Linearized pER8*

In order to make preliminary identification of restriction site(s) located inside the replication region of pER8 (2.2 kb; Fig. 2), we first constructed three sets of bifunctional plasmids using the streptococcal plasmid linearized at *Hind*III, *Pvu*II, or *Hpa*I site. The

*Hind*III-linearized pER8 was directly inserted into the unique *Hind*III site of pUC19 in the two possible orientations to yield the pUER981/pUER982 plasmid set (4.9 kb; Fig. 2). The *Pvu*II- or *Hpa*I-digested pER8 was ligated with the large pUC19/*Pvu*II fragment (2.4 kb) to generate pUER983/pUER985 (4.6 kb; Fig. 2) and pUER984/pUER986 (4.6 kb; Fig. 2) sets, respectively. The *bla* gene of these recombinants was not expected to function in *S. thermophilus* (Solaiman *et al.*, 1992). Accordingly, we spliced the staphylococcal *cat* gene cartridge into these pUER plasmids so that ST transformants could be scored using Cm. The *cat* determinant isolated from pUCM910 by *Eco*RI digestion was conveniently inserted into

TABLE 1  
TRANSFORMATION FREQUENCY  
OF PLASMID CONSTRUCTS

Plasmid	Transformation frequency (cfu/ $\mu$ g DNA) <sup>a</sup>	
	<i>E. coli</i> DH5 $\alpha$	<i>S. thermophilus</i> ST128
pMEU5a	$6.6 \times 10^5$	$1.5 \times 10^3$
pMEU5b	$7.7 \times 10^5$	$1.2 \times 10^3$
pMEU6a	$1.0 \times 10^6$	$1.7 \times 10^3$
pMEU6b	$8.2 \times 10^5$	$1.2 \times 10^3$
pMEU9	$6.3 \times 10^5$	$8.5 \times 10^2$
pMEU10	$5.6 \times 10^5$	$1.3 \times 10^3$
pUC19	$1.5 \times 10^6$	NA <sup>b</sup>
pVA736 <sup>c</sup>	NA <sup>b</sup>	$1.1 \times 10^3$

<sup>a</sup> Transformants were scored on LB/1% agar plates containing Ap (50  $\mu$ g/ml) for DH5 $\alpha$  and on TYL/1% agar plates containing Em (15  $\mu$ g/ml) for ST128. Values were the average counts from 2 to 3 plates.

<sup>b</sup> NA, Not applicable.

<sup>c</sup> Determined from a separate experiment (Solaiman *et al.*, 1992).

pUER981/982 at their unique *EcoRI* site. This procedure produced pMCU1b (6.1 kb) and pMCU2a (6.1 kb) from pUER981 and pUER982, respectively. This direct splicing procedure, however, could not be carried out with pUER983 to 986 plasmids because of the absence of an *EcoRI* site. Accordingly, we created an *EcoRI* site in these plasmids by first linearizing them with *AlwNI*, then removing the 3'-overhangs with T4 DNA polymerase and adding the 10-mer *EcoRI* linker to the blunt ends, and finally recircularizing the plasmids using T4 DNA ligase. The *cat* gene cartridge flanked by *EcoRI* cohesive ends could now be conveniently inserted into these modified pUER983 to 986 plasmids. Using this strategy, we obtained pMCU3a/pMCU3b (5.8 kb; Fig. 2) and pMCU4a (5.8 kb; Fig. 2) from pUER983 and pUER984, respectively. Repeated attempts to generate the other pMCU combinations not mentioned here were met with failure. The relative orientation of the various DNA compo-

nents of the pMCU plasmids apparently determines their transformation efficiencies/survival in the *E. coli* host. Furthermore, these *cat*-containing constructs consistently showed signs of instability in *E. coli*. Plasmid preparations of pMCU3a and pMCU4a, for example, always contained large amounts of deletion species where the entire *cat* cartridge had been lost. These events occurred regardless of whether *E. coli* strain DH5 $\alpha$ , HB101, or SURE was used as host system. The inherent instability of pC194 sequence present in the *cat* gene cartridge certainly contribute to the observed deletion mutations (Niaudet *et al.*, 1984; Michel *et al.*, 1989).

We next tested the ability of the pMCU plasmids to transform *S. thermophilus* ST128. Among the five pMCU plasmids, only pMCU3a and pMCU4a could electrotransform ST128 to the Cm<sup>R</sup> phenotype with high transformation frequencies; the estimated values were  $1.8 \times 10^3$  and  $2.1 \times 10^3$  colony forming units (CFU)/ $\mu$ g DNA for pMCU3a and pMCU4a, respectively. These results showed that linearization of pER8 with *PvuII* or *HpaI* during the construction of pMCU3a and pMCU4a, respectively, did not destroy the replication function of the streptococcal plasmid; the two restriction sites are apparently located outside of the region essential to the replication of pER8. The failure of pMCU3b, whose *cat* sequence is opposite to that of pMCU3a, to transform *S. thermophilus* to Cm<sup>R</sup> suggested that the expression of the *cat* determinant was highly dependent on its relative orientation in the plasmid. Since the relative orientation of *cat* in pMCU2b is similar to that found in the transforming pMCU3a and pMCU4a, its inability to electrotransform *S. thermophilus* indicated that the *HindIII* site of pER8 is located in the replication function.

Using the newly constructed shuttle vector pMCU4a, we then tested the suitability of the *erm* gene from *S. hyicus* to serve as an alternative selection marker in *S. thermophilus*. For this purpose, pMEU3/pMEU4 (7.1 kb; Fig. 2) were constructed by inserting the 1.3-

TABLE 2  
STABILITY OF pMEUS IN INTERMEDIATE HOST *E. coli* DH5 $\alpha$

Plasmid	Ap in Medium <sup>a</sup>	CFU <sup>b</sup>		% Ap <sup>r</sup> Cell <sup>c</sup>	Growth <sup>d</sup> in			
		LB	LB + Ap		Ap	Em	Cm <sub>15</sub>	Cm <sub>30</sub>
pMEU5a	–	212 ± 26	189 ± 11	89 ± 16	+	+	ND	ND
	+	168 ± 22	168 ± 12	100 ± 20	+	+	–	–
pMEU5b	–	145 ± 4	153 ± 1	106 ± 4	+	+	ND	ND
	+	152 ± 32	138 ± 21	91 ± 33	+	+	ND	ND
pMEU6a	–	152 ± 11	198 ± 22	130 ± 24	+	+	ND	ND
	+	152 ± 2	129 ± 0	88 ± 1	+	+	–	–
pMEU6b	–	128 ± 7	130 ± 9	102 ± 13	+	+	ND	ND
	+	120 ± 3	125 ± 10	104 ± 11	+	+	ND	ND
pMEU9	–	76 ± 8	36 ± 5	47 ± 12	+	+	+	+
	+	86 ± 0	71 ± 4	83 ± 5	+	+	+	+
pMEU10	–	155 ± 7	104 ± 6	67 ± 7	+	+	+	+
	+	103 ± 1	65 ± 1	63 ± 2	+	+	+	+

<sup>a</sup> Cells were transferred daily at 1/10 dilution for 3 days in LB without (–) or with (+) 50  $\mu$ g/ml Ap.

<sup>b</sup> Number of colonies that appeared on LB or LB + Ap (50  $\mu$ g/ml) agar plates after 100  $\mu$ l of  $3 \times 10^{-6}$  diluted culture were plated. Values represented the average colony counts from 2 plates.

<sup>c</sup> Calculated as [CFU (LB + Ap)]/[CFU (LB)]  $\times$  100%.

<sup>d</sup> Growth (+) or no growth (–) in LB containing Ap (50  $\mu$ g/ml), Em (200  $\mu$ g/ml), Cm<sub>15</sub> (15  $\mu$ g/ml) or Cm<sub>30</sub> (30  $\mu$ g/ml). ND, Not determined.

kb pUPV29/*TaqI* fragment containing the *erm* gene into the unique *ClaI* site of the vector pMCU4a. When *S. thermophilus* ST128 was electroporated with these pMEU plasmids, Em<sup>R</sup> clones were obtained at transformation frequencies of  $2.6 \times 10^2$  and  $6.8 \times 10^2$  CFU/ $\mu$ g DNA for pMEU3 and pMEU4, respectively. These results showed that the *erm* gene from pPV141 of *S. hyicus* ssp. *chromogenes* was expressed in *S. thermophilus* and could thus serve as a selection marker. Interestingly, as with the parental pMCU4a plasmid, the pMEU3/pMEU4 vectors in which the *erm* determinant had been inserted into the *cat* cartridge were susceptible to deletion where the entire *cat* + *erm* unit would be lost.

#### Shuttle Vectors Containing Shortened pER8

Since larger-size plasmids often suffer from low transformation frequency and high instability, we constructed the next series of shuttle vectors using only fragments of pER8. Based on the observation made with pMCU1

to 4 mentioned above, the essential *HindIII* site was retained in these fragments. Two pER8 fragments, namely, the 1.7-kb *StyI/HinI* (S/H) and the 1.1-kb *BstEII/HinI* (B/H) pieces (Fig. 2), were used in our initial attempt. After converting the termini to blunt ends by a reaction with Klenow enzyme, these fragments were separately ligated with a *HincII*-linearized pUC18 vector and cloned in *E. coli* DH5 $\alpha$ . Screening of Ap<sup>R</sup> transformants yielded recombinants pUER15a/pUER15b (4.4 kb; Fig. 3) and pUER16b (3.8 kb; Fig. 3) containing the S/H and B/H fragments of pER8, respectively. As with the pUER980-series plasmids, the orientation of the cloned pER8 sequence governed the transformation efficiency and plasmid yield of the recombinants. With these pUER15 and pUER16 plasmids, the recombinants with configurations designated by an “a” suffix were less frequently identified among the screened transformants than the “b” type plasmids, indicating that fewer cells were successfully transformed by the “a”

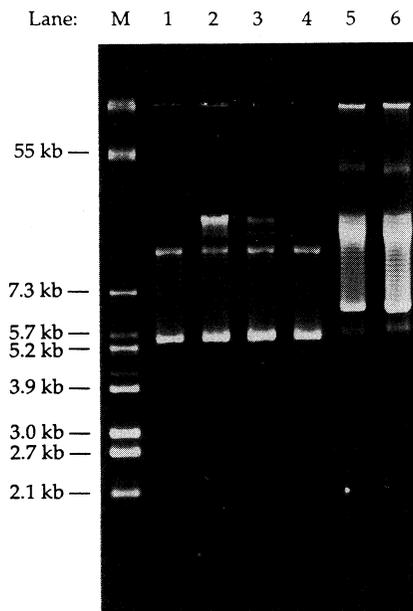


FIG. 4. Gel electrophoresis of pMEU's isolated from *S. thermophilus* ST128 transformants. Plasmids were isolated for ST as described in the text. pMEU5a (lane 1), pMEU5b (lane 2), pMEU6a (lane 3), and pMEU6b (lane 4) had been further purified with an Elutip-d column. For each sample, about 15  $\mu$ g of DNA (as estimated by  $A_{260\text{nm}}$ ) was loaded and run on 0.8% agarose gel (TBE buffer system). Lane M, plasmids of *E. coli* V517 as size markers; lanes 5 and 6, pMEU9 and pMEU10, respectively. Note that the topological isomers of the plasmids and traces of the chromosomal DNA are apparent in the sample lanes 1-6.

type recombinants. Moreover, plasmid isolation consistently yielded smaller amounts of DNA with the "a" than the "b" type pUER's. These phenomena were observed even in *E. coli* DH5 $\alpha$ F'IQ (*lacI*<sup>q</sup> genotype) regardless of the absence or presence of 2 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) (data not shown). These results ruled out the possibility that the subcloned pER8 sequences coded for any *trans*-acting factor capable of affecting pUER's replication in *E. coli*.

We next inserted selection marker(s) expressible in *S. thermophilus* into these plasmids. The *erm* marker was initially used because we had noticed earlier that the *cat* gene cartridge tended to undergo recombination

in *E. coli* leading to its deletion from the plasmid. The ca. 1.2-kb *erm* gene cartridge excised from pUEM182 by *Eco*RI digestion was ligated with *Eco*RI-linearized pUER15a, pUER15b, or pUER16b. Using *E. coli* DH5 $\alpha$  as the cloning host, we obtained pMEU5a/pMEU5b (5.7 kb; Fig. 3), pMEU6a/pMEU6b (5.7 kb), and pMEU8a/pMEU8b (5.1 kb) from pUER15a, pUER15b, and pUER16b, respectively. When these newly constructed pMEUs were used to electrotransform *S. thermophilus*, Em<sup>R</sup> clones were obtained at high transformation frequencies with pMEU5a/pMEU5b and pMEU6a/pMEU6b (Table 1). The failure of pMEU8 plasmids to transform *S. thermophilus* indicated that the affected *Bst*EII site was located within the region essential for the plasmid replication. These results thus identified the 1.7-kb *Sty*I/*Hin*I fragment of pER8 as containing the sequence sufficient for plasmid replication in *S. thermophilus*. This replication region by itself, however, did not appear to support plasmid propagation in *E. coli*. When the *E. coli* ORI region was deleted from pUER983 (Fig. 2) by the removal of the appropriate 0.6-kb *Sty*I/*Alw*NI fragment, the resulting plasmid failed to transform *E. coli* DH5 $\alpha$  or HB101, clearly suggesting that the streptococcal replication elements of the pER8 were not recognized in this gram-negative host.

To improve the versatility of the pMEU5- and pMEU6-class plasmids, shuttle vectors containing two markers selectable in *S. thermophilus* were next constructed. Such vectors will allow for molecular cloning in this bacterial host using the insertional inactivation method. Accordingly, the *cat erm* gene cartridge was isolated from *Eco*RI-digested pUCE1 and cloned into the *Eco*RI site of pUER15a and pUER15b. Recombinants pMEU9 (6.9 kb; Fig. 3) and pMEU10 (6.9 kb) were obtained. Both plasmids electrotransformed *S. thermophilus* to Em<sup>R</sup> at frequencies of  $8.5 \times 10^2$  and  $1.3 \times 10^3$  CFU/ $\mu$ g of pMEU9 and pMEU10, respectively (Table 1).

### Characteristics of the pMEU Vectors

Since initial plasmid manipulation and cloning procedures were expected to be performed extensively in *E. coli*, the properties of the pMEU vectors were characterized in depth in this host system. In this respect, only the relatively more sophisticated second generation vectors (pMEU5 to pMEU10) were studied. Implicit to the transformant selection procedures, all three selection markers used in this study were expressed in this gram-negative host. Thus, *E. coli* transformants containing these vectors were capable of growing in LB medium containing 100 µg/ml Ap, 30 µg/ml Cm, or 200 µg/ml Em. Nevertheless, the *bla* gene remained the preferred selection marker because of the relatively poor expression of the staphylococcal *cat* gene and the high inherent Em<sup>R</sup> of *E. coli*.

Table 1 shows the transformation frequencies of the pMEUs in *E. coli* DH5α. These values range from  $5.6 \times 10^5$  (for pMEU10) to  $10 \times 10^5$  (for pMEU6a) CFU/µg DNA. In the same experiment, pUC19 gave a transformation frequency of  $1.49 \times 10^6$  CFU/µg DNA. These results showed that the pMEUs were indeed very effective in transforming *E. coli*.

The pMEU plasmids exhibited high segregational stability in the *E. coli* host. Results presented in Table 2 showed that transformants of pMEU5's and pMEU6's continued to display Ap<sup>R</sup> traits even after being propagated for generations without selection pressure. The *cat*-containing pMEU9 and pMEU10, however, appeared to be less stable; nearly half of the cell population became Ap<sup>S</sup>. Unexpectedly, this loss of antibiotic resistance occurred in spite of the presence of Ap during culture propagation. This observation reinforced the earlier conclusion that the *cat* gene cartridge underwent undefined recombination event(s) leading to plasmid loss.

The structural stability of the pMEU's was assessed by isolating and characterizing plasmids from the same repeatedly transferred cultures. The results showed that none of the plasmid preparations contained any deleted

species (data not shown), indicating that the pMEU vectors were structurally stable in *E. coli*. Apparently, the aforementioned loss of Ap<sup>R</sup> phenotype seen with the transformants of pMEU9/pMEU10 was due to segregational instability.

In addition to the Ap<sup>R</sup> trait, the continued expression of Cm<sup>R</sup> and Em<sup>R</sup> after generations of growth without selective pressure was also examined. Accordingly, the repeatedly transferred cultures from the preceding experiment were seeded at 1/40–1/30 dilution into fresh LB medium containing Ap (50 µg/ml), Em (200 µg/ml), or Cm (15 and 30 µg/ml). After an overnight incubation at 37°C with vigorous shaking, cell growth in LB + Em medium was observed with all samples as indicated by the turbidity of the cultures (Table 2). Growth in Cm-containing medium was also observed with the pMEU9- and pMEU10-containing cells. The pMEU5 and pMEU6 transformed clones did not grow in the LB + Cm medium as expected. The staphylococcal *erm* gene was obviously maintained stably in *E. coli*. As for the *cat* gene, segregants that still contained plasmid apparently retained the function of this gene.

Similar to their parental pUC vectors, the pMEU shuttle plasmids seemed to replicate under relaxed control in *E. coli*. Preliminary estimation indicated that these constructs were maintained in this Gram-negative host at copy numbers similar to that of pUC19 measured in the same experiment (data not shown).

As had been alluded to earlier, the pMEU's could electrotransform *S. thermophilus* to Em<sup>R</sup> at high transformation frequencies (Table 1). Plasmids isolated from these transformants contained only the transforming DNA's (Fig. 4), indicating that deletion events had not occurred with these vectors. The transformation frequencies and stability of these plasmids in the two host systems *E. coli* and *S. thermophilus* are thus comparable with, or even superior to, those seen with some well-known cloning vectors for these

bacteria such as the pUC, pVA736 (Macrina *et al.*, 1980; Somkuti and Steinberg, 1988), pNZ, (De Vos, 1987; Solaiman *et al.*, 1992) and pBN (Solaiman *et al.*, 1992). These properties underscore the usefulness of these plasmids to function as shuttle vectors for the two species. In fact, we have used pMEU5a to first clone a streptomycete tyrosinase gene in *E. coli* and subsequently shuttle the recombinants into *S. thermophilus* (unpublished data).

We had also categorized the functioning in *S. thermophilus* of the three selection markers presently used in the pMEU's construction. An earlier study had shown that the *bla* gene was not functioning in this Gram-positive host (Solaiman *et al.*, 1992). The staphylococcal *erm* determinant was fully expressed in all transformants; cells were resistant to Em at concentrations as high as 15  $\mu\text{g/ml}$ . The expression of *cat* present in the pMEU9 and pMEU10 plasmids, however, was not easily explained. When 126 Em<sup>R</sup> clones containing pMEU9 were grown in TYL + Cm (5  $\mu\text{g/ml}$ ), only 69 of them (54%) survived and grew after an overnight incubation. When 124 pMEU10-containing Em<sup>R</sup> transformants were tested for Cm<sup>R</sup> in a similar fashion, all of the cultures spotted growth. Since plasmid preparations from pMEU9-transformed cells did not contain any deleted DNA species indicative of the loss of *cat*, it seemed that the orientation of the gene relative to the other sequences in the vectors affected its expression.

In summary, a series of pMEU vectors useful for interspecies gene cloning and shuttling have been constructed. Some of these vectors such as pMEU5 and pMEU6 are remarkably stable in *E. coli* and *S. thermophilus*. Others including pMEU9 and pMEU10 allow for transformant selection by the insertional inactivation method because of the presence of multiple markers. The use of the native plasmid pER8 from *S. thermophilus* in these construction efforts paves the way for the development of "all-food-grade" cloning vehicles for this industrially important bacterium.

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