

## Native Promoter-Plasmid Vector System for Heterologous Cholesterol Oxidase Synthesis in *Streptococcus thermophilus*

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The cholesterol oxidase gene (*choA*) of a streptomycete was used as a model for studying heterologous gene expression in *Streptococcus thermophilus*, an essential bacterium in dairy food fermentations. The vectors pER82 and pER82P were developed from the 2.2-kb indigenous plasmid (pER8) of *S. thermophilus* ST108, and sP1, a 51-bp synthetic promoter patterned after a chromosomal sequence of *S. thermophilus*. The presence of sP1 promoter in pER82PbCOB with the *choA* insert aligned with the *cat* gene was essential for the intracellular production of cholesterol oxidase. The pER82PbCOB was apparently stable in *S. thermophilus* with no detectable evidence of deletion mutational events. © 1995 Academic Press, Inc.

*Streptococcus thermophilus* (ST)<sup>3</sup> is an important thermotolerant lactic acid bacterium used extensively as a biocatalyst in cheese and yogurt fermentation processes, usually in combination with other bacteria (lactobacilli, propionibacteria, lactococci). Interest in the genetic development of lactic acid bacteria (LAB) with the capacity to impart to foods novel or improved compositional, textural, or storage characteristics has been stimulated by their long-established safety for human consumption. Recent advances in the field include the cloning in LAB of heterologous endoglucanases (Bates *et al.*, 1989; Baik and Pack, 1990), lipase (Vogel *et al.*, 1990),  $\alpha$ -amylase (Jones and Warner, 1990),  $\beta$ -glucanase (Thompson and Collins, 1991), chole-

sterol oxidase (Somkuti *et al.*, 1991, 1992; Brigidi *et al.*, 1993), lysostaphin (Gaier *et al.*, 1992), and tyrosinase (Somkuti *et al.*, 1993).

DNA delivery to LAB at present is done most efficiently by electrotransformation (Chassy and Flickinger, 1987; Somkuti and Steinberg, 1987; Luchansky *et al.*, 1988) with homologous or heterologous genes incorporated in a variety of plasmid constructs. Among the problems encountered in heterologous gene expression in LABs are the low level recognition or nonrecognition of promoters (Brigidi *et al.*, 1993; Somkuti *et al.*, 1993) and the instability of heterologous genes in the new host (Somkuti *et al.*, 1991). These problems may be alleviated by using indigenous promoter sequences positioned close to the 5' terminus of the heterologous gene to be expressed and cloning vectors developed from native plasmids of LABs.

In this report, we discuss the use of a synthetic 51-bp promoter (sP1) modeled after a chromosomal sequence and a cloning vector (pER82) developed from a native ST plasmid (pER8, 2.2 kb) as a model system for the maintenance and expression of a streptomycete cholesterol oxidase (*choA*) gene in *S. thermophilus*.

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<sup>2</sup> Reference of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

<sup>3</sup> Abbreviations used: ST, *Streptococcus thermophilus*; LAB, lactic acid bacteria; TYL, tryptone-yeast extract-lactose; Em, erythromycin; AGE, agarose gel electrophoresis; TLC, thin layer chromatographic.

## MATERIALS AND METHODS

### *Bacteria and Growth Conditions*

*S. thermophilus* ST128, a strain free of plasmids in our culture collection, was used as the host for cholesterol oxidase (Cho) production. The culture was maintained at 37°C in liquid tryptone–yeast extract–lactose (TYL) medium (Somkuti and Steinberg, 1986a) and transferred every 3 days. For the selection of transformants, erythromycin (Em) was used in TYL at 15 µg/ml final concentration.

*Escherichia coli* DH5α was purchased as a competent cell preparation from BRL Life Technologies (Gaithersburg, MD). Transformed cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C, with shaking, for 16 h in the presence of 100 µg/ml ampicillin and 200 µg/ml Em when needed.

### *Transforming DNAs*

Starting materials for the transforming DNA species included pUCE1 (5.2 kb, Cm<sup>R</sup>·Em<sup>R</sup>) and pUER983 (4.6 kb) described earlier (Solaiman and Somkuti, 1993). Insertion of the *cat-erm* cartridge into pUER983 yielded pMCU20a (7.1 kb), followed by the removal of the pUC19 sequence via *Pvu*II digestion and self-ligation of the larger fragment leading to pER82 (4.7 kb). Alternatively, sP1, a 51-bp synthetic promoter (Gen-set, La Jolla, CA) based on the ST promoter P25 (Slos *et al.*, 1991), was recovered from the pSTP25 (2.8 kb) maintenance plasmid and inserted into pMCU20a/*Bam*HI, followed by *Pvu*II digestion and self-ligation of the larger fragment to yield pER82P (4.8 kb). Orientation of sP1 with respect to *cat* was determined by *Alu*I digestion of an 84-bp *Sma*I/*Hind*III fragment followed by agarose gel electrophoretic analysis.

Plasmid DNAs used for electrotransformation were prepared by ligation (T4 DNA ligase) of pER82/*Pst*I and pER82P/*Pst*I with the ca. 2.7-kb promoterless *choA* gene excised from pNC0937/*Pst*I (Somkuti *et al.*,

1991). Conditions of restrictions and ligations were based on recommendations of the supplier (BRL Life Technologies). Ligation mixtures containing pER82CO and pER82PCO ligation products were used directly for transformation.

### *Electrotransformation*

*S. thermophilus* ST128 was grown overnight at 37°C, subcultured in TYL supplemented with 40 mM DL-threonine, and allowed to reach OD<sub>660</sub> = 0.2. The cell pellet was washed 2× with 300 mM raffinose–5 mM KH<sub>2</sub>PO<sub>4</sub>–1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O (RPM, pH 4.5) between centrifugations at 10,000g at 4°C for 10 min. The final pellet was dispersed in 1 ml of ice-cold RPM, mixed with 15 µg DNA, and subjected to a single electric pulse at 4.0 kV/cm in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) set at 25 µF capacitance. Reaction mixtures were immediately mixed with  $\frac{1}{5}$  vol of 5× TYL and kept at 37°C for 3 h to allow gene replication under nonselective conditions. This was followed by plating 100-µl samples of the transformation mixture on TYL agar (1.2%) supplemented with Em at 15 µg/ml. Erythromycin resistant (Em<sup>R</sup>) transformants were scored after incubating plates for 48 h at 37°C.

### *Plasmid Analysis and Southern Blots*

Plasmid species with ca. 7.2 kb molecular mass putatively identified as pER82CO or pER82PCO were isolated from genetic transformants and purified (Somkuti and Steinberg, 1986b). To establish the orientation of the *choA* gene, restriction products obtained by *Sal*I digestion were analyzed in 1.2% agarose gels in TBE buffer (0.089 M Tris base; 0.089 M boric acid; 0.002 M Na-EDTA). The presence of *choA* in recombinant plasmids was established by a Southern hybridization procedure described previously (Somkuti *et al.*, 1991). Agarose gel electrophoresis (AGE) of intact plasmids was performed in 0.7% agarose gels at 100 V for 3 h.

### Cholesterol Oxidase Measurements

*S. thermophilus* transformants with pER82CO (7.1 kb) or pER82PCO (7.2 kb) were grown in 200 ml TYL for 16 h at 37°C. Cells were washed 2× with 50 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> (pH 7.4)–1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O buffer (POM), resuspended in 5.4 ml POM buffer, and mixed with 0.6 ml of a 10 mg/ml lysozyme (Sigma Chemical Co., St. Louis, MO) solution. After holding for 60 min at 37°C, the mixture was sonically disrupted at 4°C (W-225 sonicator, Heat Ultrasonics, Farmingdale, NY). To each 1-ml sample of the crude enzyme extract, 80 µl of a 5 mg/ml cholesterol solution in *n*-propanol was added and the reaction mixtures were incubated at 40°C. Reaction was stopped by adding 2 ml of 95% ethanol and 2 ml of 50% KOH. Saponification was allowed to proceed for 15 min at 60°C. After cooling to 28°C, each sample was extracted once with 6 ml of ethyl acetate and phase separation was facilitated by centrifugation in a tabletop centrifuge for 5 min. Five milliliters of the solvent phase was removed and evaporated to dryness under a stream of N<sub>2</sub>. Cholesterol content of the residues was measured by the ferric chloride assay (Thomas and Stevens, 1960).

For thin layer chromatographic (TLC) analysis, samples were dissolved in ethyl acetate, spotted on SIL G-25 plates, and developed in a chloroform–ethyl acetate (95/5, v/v) solvent system. Cholesterol and its oxidation products were detected as described previously (Somkuti *et al.*, 1991).

## RESULTS AND DISCUSSION

### Construction of pER82 and pER82P

The transfer of the *cat-erm* cartridge from pUCE1 to the *Hpa*I site of the pER8 span (2.2 kb) in pUER983 by blunt-end ligation resulted in pMCU20a (Fig. 1). Restriction of purified pMCU20a with *Pvu*II and resolution of digestion products in agarose permitted the electroelution of the 4.7-kb fragment which now included the *cat-erm* cartridge at *Hpa*I. Self-ligation of this fragment with T4

DNA ligase resulted in pER82 which could electrotransform *S. thermophilus* ST128 at a frequency of 10<sup>2</sup> CFU/µg DNA.

In constructing pER82P, the 51-bp synthetic promoter sP1 was first inserted into the *Bam*HI site of pUC18 with the orientation of the insert being opposite to that of the *lacZ* gene (Fig. 2), and the resulting pSTP25 was maintained in *E. coli* DH5α. Transfer of the *Bam*HI-flanked sP1 sequence to the unique *Bam*HI site of pMCU20a resulted in pMCU20aPa and pMCU20aPb with the sP1 being opposite to or aligned with the *cat-erm* cartridge, respectively. Subsequent isolation by AGE and self-ligation of the larger (4.8 kb) fragment produced both pER82Pa and pER82Pb, as determined by *Alu*I digestion. However, electrotransformation of ST128 was successful only with pER82Pb at a frequency of 10<sup>2</sup> CFU/µg DNA.

The strategy described above yielded, for the first time, transforming vectors with or without an ST promoter (sP1) that made use of pER8, a small indigenous cryptic plasmid (2.2 kb) of *S. thermophilus* ST108 (Somkuti and Steinberg, 1986a). The new constructs were equipped with easily traceable markers (*erm*, *cat*) and restriction sites for the insertion of heterologous genes and were suitable for direct electrotransformation of *S. thermophilus* and possibly other LABs.

### Construction of pER82CO and pER82PCO

The *choA* gene present in the ca. 2.7-kb fragment of pNCO937 (8.1 kb) was excised by *Pst*I digestion, resolved by AGE, electroeluted, and ligated with pER82 or pER82Pb (Fig. 3). In each instance, both orientations (alignment or opposition with respect to *cat*) of the *choA* gene were possible (Fig. 4). However, restriction analysis of recombinant plasmids from *S. thermophilus* ST128 electrotransformants with *Sal*I showed that only pER82COa and pER82PbCOa in which the *choA* was positioned opposite to the *cat* gene were formed. To generate the corresponding pER82COb and pER82PbCOb constructs,

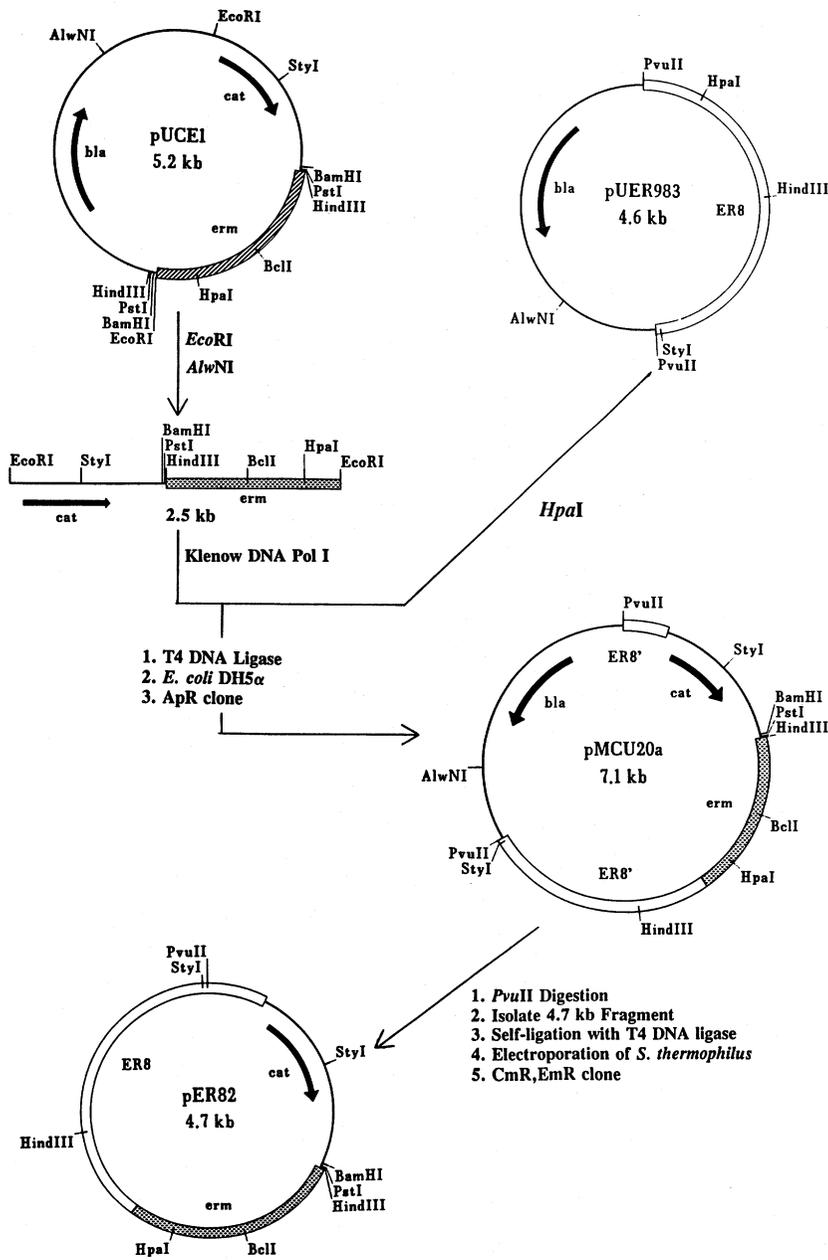


FIG. 1. Construction strategy for pER82|cat.erm.

the original plasmids were digested with *Pst*I, self-ligated, and retransformed into *S. thermophilus* ST128. Screening of transformants and plasmid analysis led to the isolation of transformants with pER82COB or pER82PbCOB. The presence of sP1 in

pER82Pb was verified by Southern hybridization analysis (Fig. 4).

#### Production of Cholesterol Oxidase

*S. thermophilus* ST128 transformants carrying pER82COa, pER82COB, or

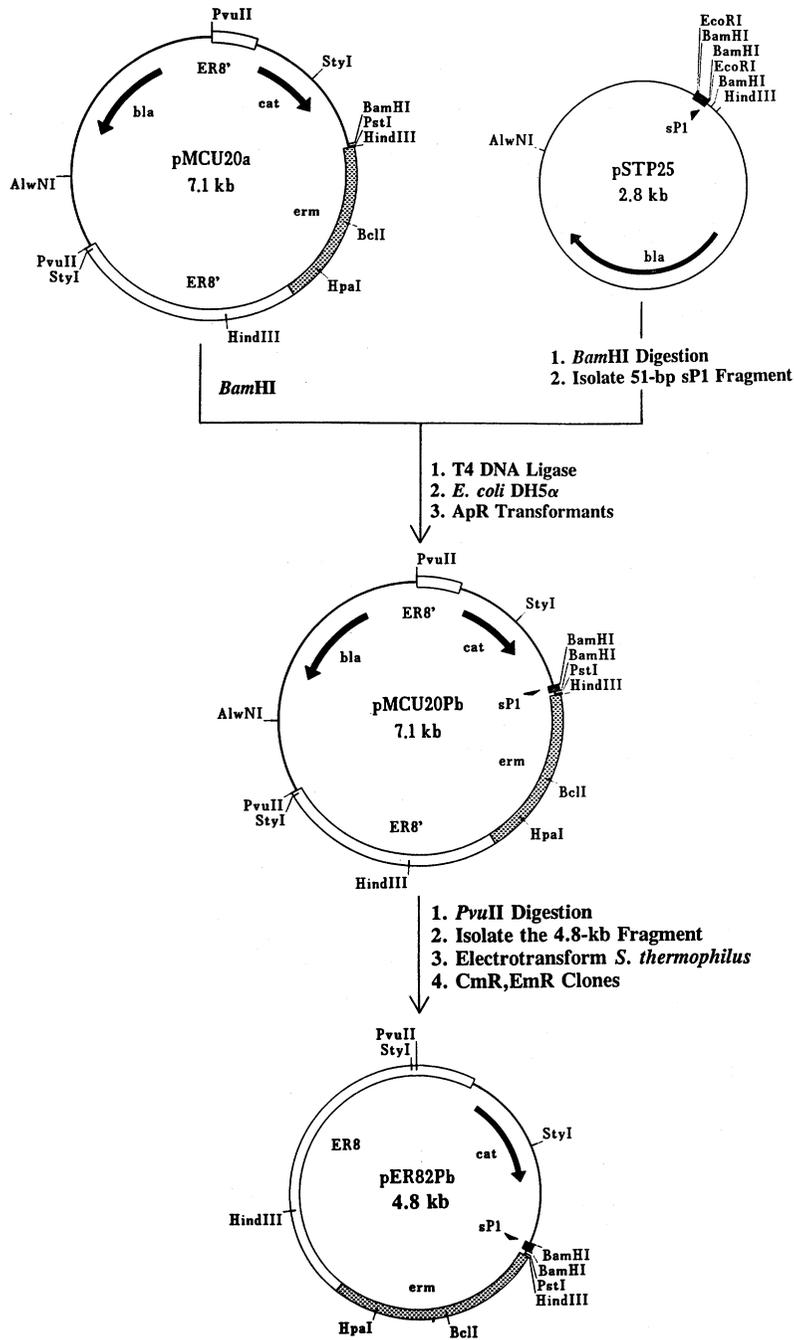


FIG. 2. Construction strategy for pER82Pb with the sP1 promoter in correct alignment with *cat*.

pER82PbCOa failed to produce measurable cholesterol oxidase activity. This was anticipated even in the case of pER82COb with the *choA* gene in alignment with *cat* since other workers reported that *choA* expression, which is apparently polycistronic in *Strepto-*

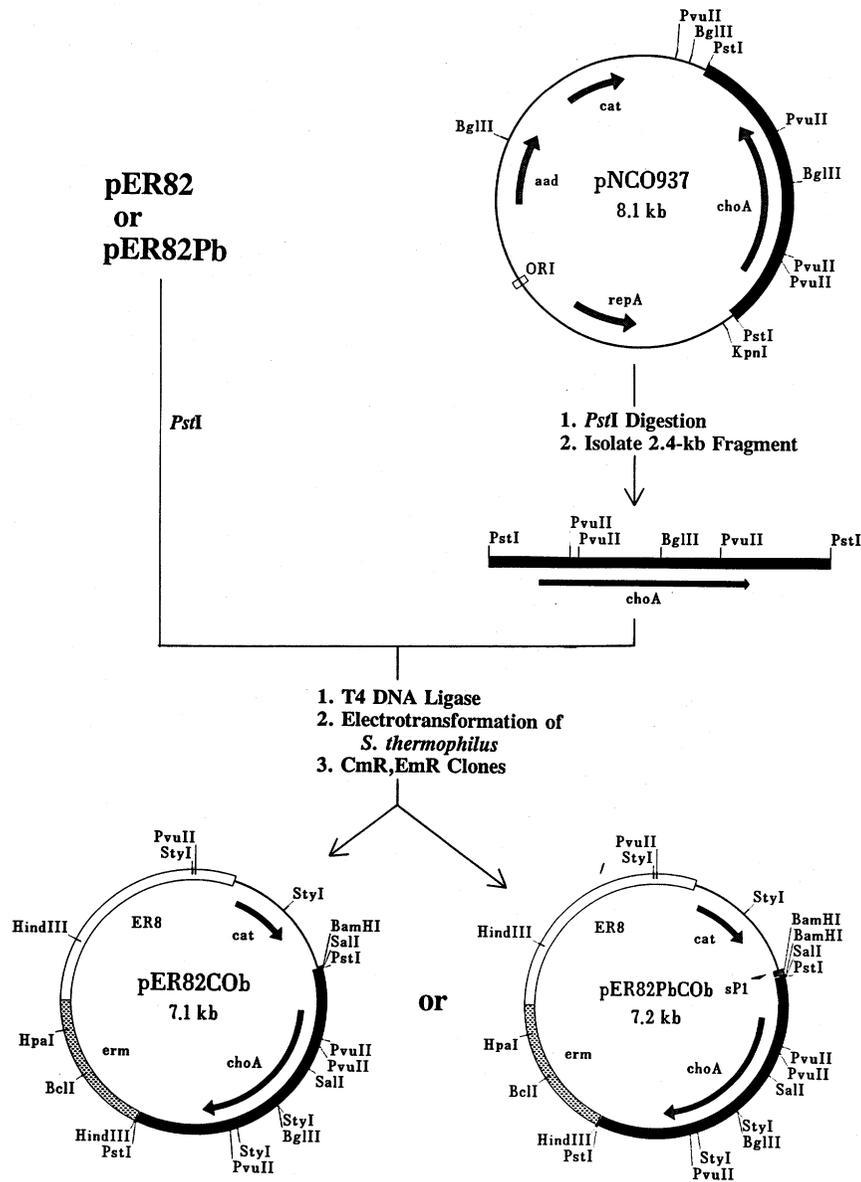


FIG. 3. Cloning of the *choA* gene into pER8-derived vectors. Only plasmids with *choA* in the correct alignment with *cat* are shown.

*myces* sp. SA-COO, also requires the presence of the *choP* gene and its promoter (Horii *et al.*, 1990). These elements are either partially present (*choP*) or totally absent (*choP* promoter) in the *Pst*I fragment containing the *choA* in pNCO937. Thus, previously observed expression of cholesterol oxidase in *S. thermophilus* harboring pNCO937 must

have been influenced by the activity of some other promoter sequence in the cloning vector pNZ19 which had a lactococcal origin (De Vos, 1987) and was used in the construction of pNCO937 (Somkuti *et al.*, 1991).

On the other hand, the presence of pER82PbCOb in transformants resulted in cholesterol oxidase synthesis, as evidenced by

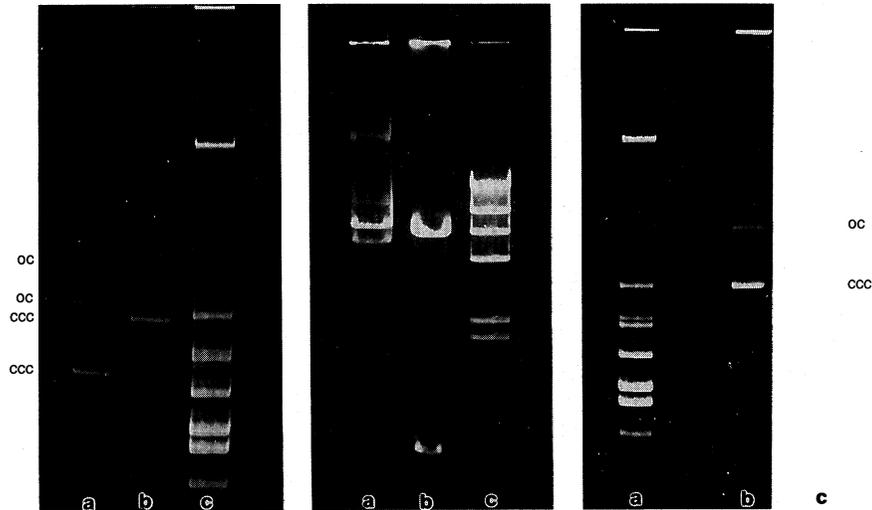


FIG. 4. AGE analysis of pER8-derived plasmid constructs. (Left) (a) pER82Pb (4.8 kb), (b) pER82PbCOB (7.2 kb), (c) *E. coli* V517 standards. (Center) (a) pER82PbCOa/SalI (1.74 kb)\*; (b) pER82PbCOB/SalI (0.92 kb)\*; (c)  $\lambda$ DNA/HindIII. (Right) (a) *E. coli* V517 standards, (b) pER82PbCOB (7.2 kb), (c) Southern hybridization with biotin-labeled sP1 probe. \*Lower band.

TLC analysis (Fig. 5) confirming the functionality of sP1, which was patterned after a known ST chromosomal promoter. Under the experimental conditions used, ca. 80% of

cholesterol in the reaction mixture was depleted (Table 1).

The pER82PbCOB construct was stable in *S. thermophilus* even after daily retransfers for 10 days with no deletion mutations being evident. This was in contrast with earlier data obtained with pNCO937, which underwent deletion mutations in ST128 apparently involving the *choA* insert (Somkuti *et al.*, 1991).

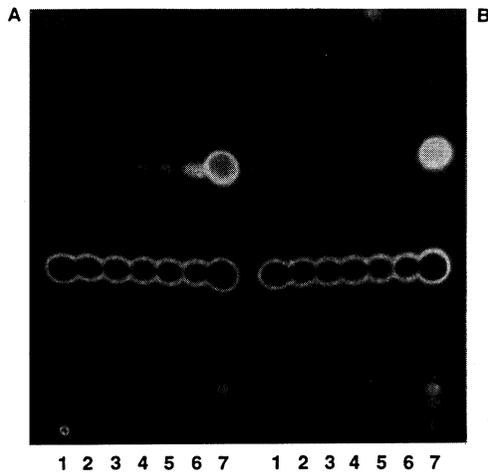


FIG. 5. TLC analysis of reaction products following the incubation of cholesterol with sonicated cell extracts of ST128 transformants. (A) ST128/pER82PbCOB cell extract + cholesterol at 37°C. Lanes 1–6, 0, 1, 2, 4, 6, and 24 h; lane 7, cholesterol and 4-cholestene-3-one standards. (B) ST128/pER82PbCOa (*choA* in incorrect alignment).

TABLE 1

CHOLESTEROL OXIDASE IN ST128 TRANSFORMANTS

| Culture <sup>a</sup> /plasmid | Percentage cholesterol depleted |     |     |      |
|-------------------------------|---------------------------------|-----|-----|------|
|                               | 2 h                             | 4 h | 6 h | 20 h |
| ST128/pER82COa                | 0                               | 0   | 0   | 0    |
| ST128/pER82COB                | 0                               | 0   | 0   | 0    |
| ST128/pER82PbCOa              | 0                               | 0   | 0   | 0    |
| ST128/pER82PbCOB              | 9                               | 30  | 76  | 81   |

<sup>a</sup> Each reaction mixture contained the sonicated extract of a cell mass equivalent to a cell density of 40 OD units per ml at 660 nm or ca.  $5 \times 10^9$  CFU (colony forming unit)/ml.

The results showed the usefulness of pER8, a native plasmid of *S. thermophilus* ST108, in the construction of cloning vectors suitable for use in the direct electrotransformation of this species. Further, the synthetic sP1 promoter that apparently functioned well in the pER82 (Em<sup>R</sup>, Cm<sup>R</sup>) vector had the capacity to facilitate the transcription of the streptomycete cholesterol oxidase gene in *S. thermophilus*. There is reason to hope for similarly successful sP1-mediated expression of other heterologous genes in this and possibly other dairy industrial microbes to expand their breadth of metabolic functions.

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