

Transfer and Expression of a *Streptomyces* Cholesterol Oxidase Gene in *Streptococcus thermophilus*

GEORGE A. SOMKUTI,¹ DANIEL K. Y. SOLAIMAN, TERRANCE L. JOHNSON,
AND DENNIS H. STEINBERG

*U.S. Department of Agriculture, ARS, Eastern Regional Research Center,
600 East Mermaid Lane, Philadelphia, Pennsylvania 19118*

SOMKUTI, G. A., SOLAIMAN, D. K. Y., JOHNSON, T. L., AND STEINBERG, D. H. Transfer and Expression of a *Streptomyces* Cholesterol Oxidase Gene in *Streptococcus thermophilus*. *Biotechnol. Appl. Biochem.* 13, 238-245 (1991).

The recombinant plasmid pNCO937 (8.1 kbp) containing a *Streptomyces* sp. cholesterol oxidase gene was introduced into *Streptococcus thermophilus* by electrotransformation. Transformation frequency was 7.2×10^5 colony forming units/ μ g of DNA. The presence of the cholesterol oxidase gene in *S. thermophilus* was confirmed with Southern blot analysis using a biotinylated probe. Thin-layer chromatographic analysis showed the expression of the *Streptomyces* cholesterol oxidase gene resulting in the oxidation of cholesterol to 4-cholesten-3-one. *S. thermophilus* may be a suitable host for the expression of other genes regulating prokaryotic cholesterol metabolism. © 1991 Academic Press, Inc.

Attempts to establish a direct linkage between dietary cholesterol, blood cholesterol, and mortality from coronary heart disease have not provided consistent data (1, 2, 3). Nevertheless, the publicly perceived connection between dairy products and the dietary cholesterol problem has continued to fuel interest in the removal of cholesterol from milk.

A biotechnological approach to reducing the cholesterol content of milk involves the genetic engineering of starter culture bacteria (lactococci, lactobacilli, streptococci) leading to the expression of phenotypic traits which are related to cholesterol metabolism, and the use of these cultures in the production of fermented dairy foods (yogurt and cheeses). We have investigated the possibility of introducing a known prokaryotic gene controlling the synthesis of a cholesterol-modifying enzyme into a common dairy starter culture. In this study, we describe the cloning and expression of the cholesterol oxidase gene of *Streptomyces* sp. SA-COO (4) into *Streptococcus thermophilus*, a thermophilic bacterium used primarily for lactic acid synthesis in dairy fermentations (5).

MATERIALS AND METHODS

Bacterial Strains and Plasmids

S. thermophilus ST128 (plasmid-free) was from our laboratory collection. *S. sanguis* Challis harboring pVA736 (7.6 kbp) was a gift of F. Macrina (Virginia Common-

wealth University). Streptococci were cultured in lactose broth (6). *Streptomyces lividans* 1326, host of pCO-1 (11.6 kbp) carrying the cholesterol oxidase gene (*cho*) of *Streptomyces* sp. SA-COO, was provided by Y. Murooka (Hiroshima University) and was maintained in brain–heart infusion broth (Difco) containing 5 µg of thiostrepton/ml. LB broth (7) was used for culturing *Escherichia coli* strains HB101 and DH5α which were purchased from BRL Life Technologies (Gaithersburg, MD). Incubation of all microbial cultures was at 37°C with (*E. coli*, *S. lividans*) or without (streptococci) agitation. Solid media used in the detection of genetic transformants contained 1.5% agar (Difco). Chloramphenicol (Cm) or erythromycin (Em) was added at a final concentration of 15 µg/ml and ampicillin (Ap) was used at 25 µg/ml concentration. The plasmid pUC19 (Ap^r, 2.68 kbp) was purchased from BRL Life Technologies, and pNZ19 (Cm^r, 5.7 kbp) was a gift from G. Simons (Netherlands Institute for Dairy Research).

Isolation of DNA

Plasmids from *S. sanguis* Challis, *S. thermophilus* ST128, and *S. lividans* 1326 were isolated after 20 h of growth according to a protocol previously developed for lactic acid bacteria (8). Plasmids from *E. coli* strains were isolated by the method of Birnboim and Doly (9). Plasmid DNAs were purified by a rapid cesium chloride density gradient centrifugation method (10). Prior to subcloning, specific DNA fragments were fractionated by agarose gel electrophoresis, electroeluted using an Elutrap apparatus (Schleicher and Schuell, Inc., Keene, NH) and further purified by a mini-column adsorption–desorption procedure (Schleicher and Schuell, Inc.) as described previously (11).

DNA Manipulation

Restriction endonucleases and T4 DNA ligase were purchased from BRL Life Technologies (Gaithersburg, MD) and used according to the recommendations of the supplier. Restriction maps were constructed by analyses of single and double digests of recombinant plasmids after electrophoresis on 1.2% agarose gels in Tris borate–EDTA (pH 8.2) buffer (11).

Transformation and Electrotransformation

Competent cells of *E. coli* HB101 and DH5α were prepared according to Cohen *et al.* (12). Electrotransformation of *S. thermophilus* ST128 was performed using a gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA) according to a procedure reported previously (13).

DNA–DNA Hybridization

A biotinylated probe was developed from the 2.4-kbp *Pst*I fragment of pCO-1 which incorporates the *cho* gene of *Streptomyces* sp. SA-COO (14). The nick-translation kit and biotin-labeled nucleotides were used as recommended by the manufacturer (Oncor, Inc., Gaithersburg, MD). The biotinylation procedure was based on the method of Leary *et al.* (15). The presence of the streptomycete *cho* gene in plasmids isolated from electrotransformants of *S. thermophilus* ST128 was detected by the Automated Southern Blot System (Oncor, Inc., Gaithersburg, MD). Intact plasmid DNAs were electrophoresed in 0.7% agarose in Tris borate–EDTA buffer (11), and

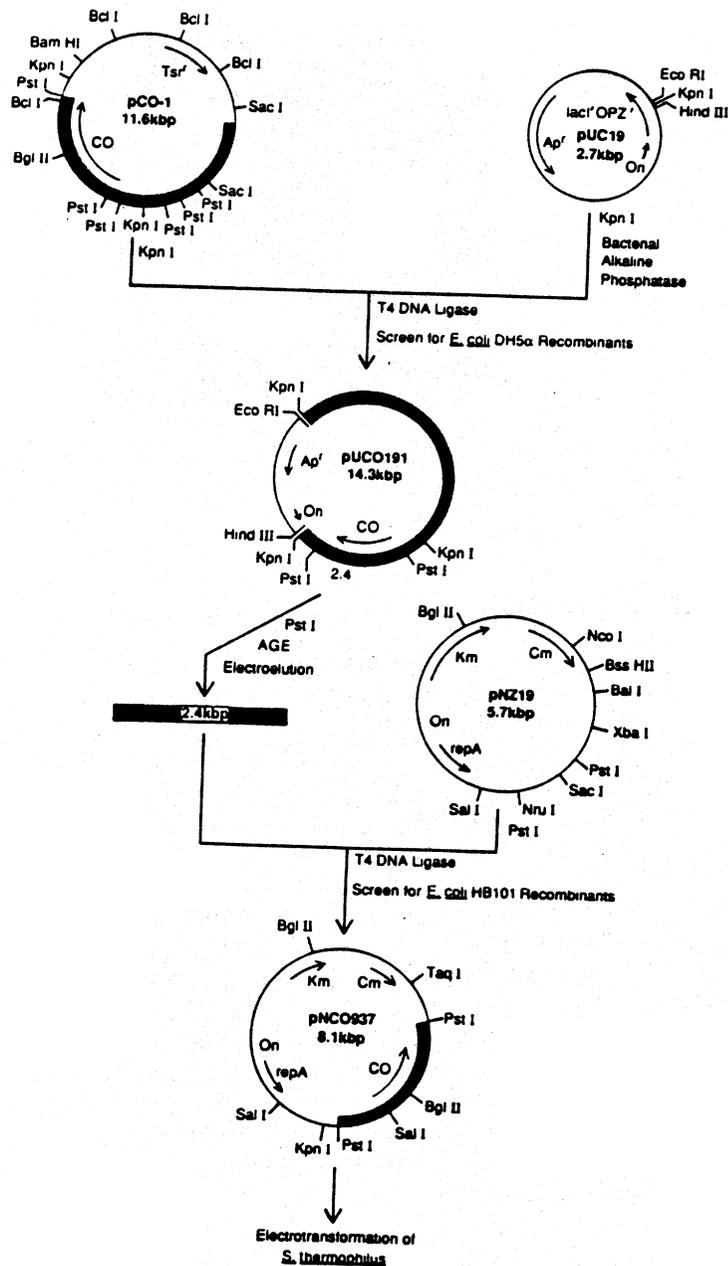


FIG. 1. Strategy of construction and production of pNCO937 (8.1 kbp) used in the transformation of *S. thermophilus* ST128 to Cho⁺ phenotype.

vacuum transferred to nylon membranes (16). Hybridization with the biotin-labeled 2.4-kbp *Pst*I fragment of pCO-1 (12.5 ng/ml) was carried out at 45% formamide concentration in sealed bags for 18 h at 42°C. Posthybridization procedures (washes, filter blocking, streptavidin and alkaline phosphatase treatment, and staining with nitroterazolium blue and 5-bromo-4-chloro-3-indolyl phosphate) were carried out according to the protocol of the manufacturer (Oncor, Inc).

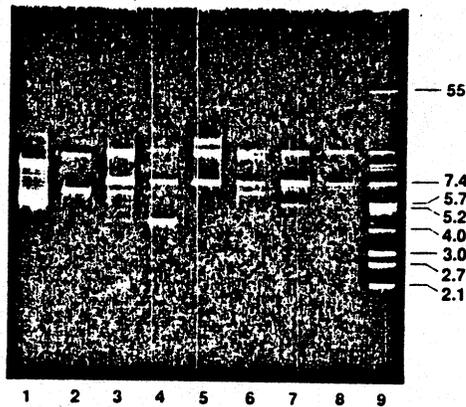


FIG. 2. Plasmid profiles of *S. thermophilus* electrotransformants. Lanes 1-7, plasmid DNA content of randomly selected electrotransformants; lane 8, pNCO937 (8.1 kbp) control; lane 9, *E. coli* V517 plasmid standards (kbp).

Expression of Cholesterol Oxidase Activity in S. thermophilus

S. thermophilus electrotransformants isolated from Cm-agar plates were tested for cholesterol oxidase activity after growth in Cm-lactose broth for 16 h at 37°C. Cultures (30 ml) were centrifuged at 10,000 rpm for 10 min at 4°C and washed twice with 50 mM K₂HPO₄ (PO buffer, pH 7.4) buffer. Pellets were resuspended in 1.5 ml PO buffer and 5 µl of phenylmethylsulfonyl fluoride was added before sonic disruption in a Model W-225 sonicator (Heat Systems Ultrasonics, Farmingdale, NY). The microtip probe was used at 6.5 power setting, 50% pulse duty cycle, and 4°C. After 20× 20-s bursts with intermittent cooling the cell sonicates constituted the crude cell extracts. To each extract, 200 µl of a 2 mg/ml cholesterol solution in *n*-propanol was added and after sealing reaction vessels with Parafilm, reaction mixtures were incubated at 37°C on an orbital shaker set at 200 rpm. After 24 h, reactions were stopped by extraction with 2× 5 ml of ethyl acetate. The combined solvent phases were evaporated to dryness and residues were dissolved in 500 µl of *n*-propanol. Samples were spotted on SIL G-25 silica gel plates and developed in a chloroform-ethyl acetate (95:5 (v/v)) solvent system at room temperature. Plates were sprayed with a phosphoric acid (8.5%)-copper sulfate (10% (w/v)) reagent (17) and heated at 130°C for 5 min. Fluorescent spots were visualized on an ultraviolet transilluminator and photographed with Polaroid Type 57 film.

Cholesterol Determination

After incubating cholesterol with cell sonicates of Cm^r transformants of *S. thermophilus* for 24 h, residual cholesterol content of ethyl acetate extracts was determined by a colorimetric enzyme assay kit (Cholesterol CII, Wako Pure Chemical Industries Ltd., Osaka, Japan), according to the manufacturer's recommendations.

RESULTS

Cloning of the cho Gene in pVA736

Attempts were made to use pVA736, a known streptococcal Em^r cloning vector (18) for the direct introduction of the *Streptomyces cho* gene into *S. thermophilus*

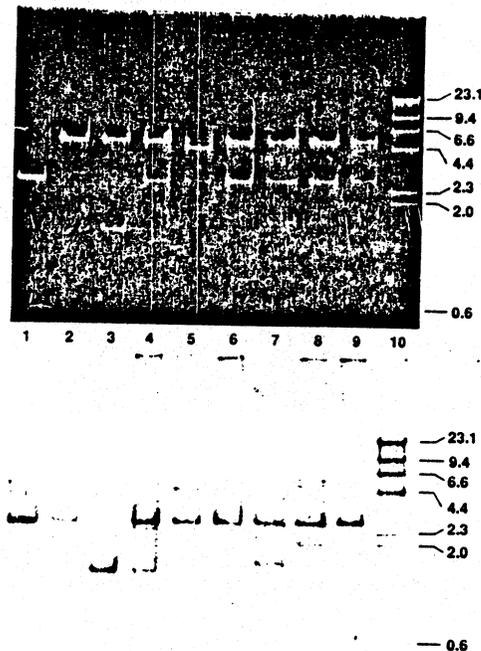


FIG. 3. Hybridization of biotinylated 2.4-kbp *Pst*I fragment (*cho* gene) of pCO-1 to *Pst*I-digested plasmids isolated from *S. thermophilus* electrotransformants. Top: Agarose gel electrophoresis; Lane 1, 2.4-kbp *Pst*I fragment of pCO-1 (positive control 1); lanes 2–8, restriction fragments of electrotransformant plasmids; lane 9, *Pst*I digest of pNCO937 (positive control 2); lane 10, *Hind*III fragments of λ DNA (kbp). Bottom: Corresponding Southern hybridizations on a nylon membrane. Some deletion fragments shown by Southern hybridizations which are poorly detectable or apparently absent in agarose gel plates (lanes 4, 5, and 8) is due to the extreme sensitivity of the biotinylated probe to minute amounts of DNA.

ST128 by electrotransformation. The simultaneous *Bcl*I/*Kpn*I digestion of plasmids pCO-1 and pVA736 yielded the corresponding 3.5- and 7.2-kbp fragments which were resolved on agarose gels and equal amounts of each (ca. 250 ng) were ligated for 16 h at 8°C using standard methods (7). Agarose gel electrophoresis of the reaction mixture showed a faint band in the 10-kbp banding area, the anticipated size of the ligation product (data not shown). However, direct use of the ligation mixture in electrotransformation trials failed to transform *S. thermophilus* to the Em^r phenotype. This may have been due to the insufficient amount of transforming DNA present in the ligation mixture.

E. coli as Intermediate Host for the *cho* Gene

To prepare the *Streptomyces cho* gene in larger quantities needed for the electrotransformation of *S. thermophilus* ST128, *E. coli* strains were used as intermediate hosts (Fig. 1). First, pCO-1 (11.6 kbp) isolated from *S. lividans* 1326, and pUC19 (2.7 kbp) were digested with *Kpn*I. One of the ligation products designated as pUCO191 (14.3 kbp) which contained the entire length of pCO-1, was transformed into competent cells of *E. coli* DH5 α . Recombinants (white colonies) were selected on LB agar plates with 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal). The presence of

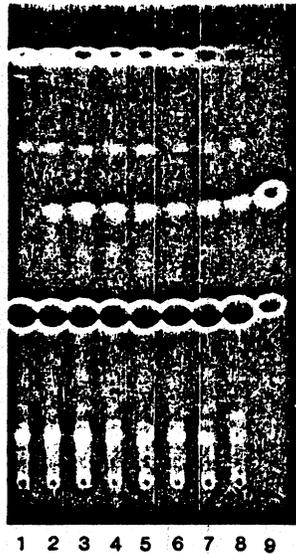


FIG. 4. Expression of *cho* gene in *S. thermophilus* cell extracts. TLC analysis of reaction products was in chloroform-ethyl acetate (95:5 (v/v)). Lane 1, ST128/pNZ19; lanes 2-8, ST128/pNCO937; lane 9, 4-cholesten-3-one (fast-moving spot) and cholesterol (slow-moving spot) standards.

pUCO191 in randomly selected transformants was verified by agarose gel electrophoresis. Larger amounts of pUCO191 were isolated from broth cultures and further digested with *Pst*I. One of the digestion products, a 2.4-kbp fragment that contained the *cho* gene (14), was purified and introduced with T₄ DNA ligase into the bifunctional 5.7-kbp streptococcal cloning vector pNZ19 (19). The ligation mixture was efficient in transforming competent *E. coli* HB101 to the Cm^r phenotype. Analysis of restriction fragments generated after single and double digestions of plasmid DNA from Cm^r *E. coli* HB101 transformants allowed the construction of the restriction map of the recombinant plasmid pNCO937 (8.1 kbp) as seen in Fig. 1.

In addition to pUCO191 (14.3 kbp), the ligation of *Kpn*I fragments of pCO-1 with *Kpn*I-linearized pUC19 gave rise to several recombinant plasmids which, depending on the orientation of the *cho* gene, provided for a high level of expression of cholesterol oxidase in *E. coli* (D. K. Y. Solaiman and G. A. Somkuti, manuscript in preparation).

Transformation of S. thermophilus ST128 with pNCO937

The plasmid pNCO937 was introduced into *S. thermophilus* ST128 by electrotransformation. Cm^r transformants occurred at a frequency of $7.2 \times 10^5/\mu\text{g}$ of DNA. The analysis of plasmid profiles of randomly selected transformants indicated the occurrence of intact pNCO937 (8.1 kbp) as well as different types of deletion mutant plasmids that varied in molecular mass (Fig. 2).

The digestion of several recombinant plasmids with *Pst*I and Southern blot hybridization tests with the biotinylated 2.4-kbp *Pst*I fragment of pCO-1 as a probe revealed that deletions, or molecular rearrangements involved primarily the 2.4-kbp fragment

of pCO-1 (Fig. 3, bottom; lanes 3, 4, 7 and 8), although occasionally, the pNZ19 (5.7 kbp) component of the hybrid pNCO937 was also affected (Fig. 3, top; lane 5).

Expression of the cho Gene in S. thermophilus

The thin-layer chromatographic analysis of reaction products obtained after incubating cell extracts of *S. thermophilus* ST128 (pNCO937) with cholesterol clearly showed the transformation of the substrate to 4-cholesten-3-one (Fig. 4) which indicated the presence of a functional cholesterol oxidase in the host organism. Quantitative assays showed approximately 20% conversion of the substrate under the experimental conditions used during the 24-h incubation period. Cholesterol oxidase activity was not detectable in cell free culture fluids of *S. thermophilus*.

Stability of the Streptomyces cho Gene in S. thermophilus

The expression of the *Streptomyces cho* gene was checked in *S. thermophilus* ST128 transformants after repeated passages in Cm-lactose broth. After three to four passages, most of the fifty Cm^rCho⁺ transformants originally selected were Cho⁻ but still retained the Cm^r phenotype. This trend indicated ongoing mutational events (deletions) involving the *cho* gene, as already shown in Fig. 3, leading to the loss of Cho activity in Cm^r transformants.

DISCUSSION

We demonstrated for the first time the potential of *S. thermophilus* as a host for heterologous gene expression and the synthesis of an active enzyme involved in prokaryotic cholesterol metabolism. The transfer of the cholesterol oxidase gene into *S. thermophilus* ST128 involved construction of the recombinant plasmid pNCO937 from the *Streptomyces* sp. SA-COO *cho* gene and the bifunctional shuttle vector pNZ19. Electroporation yielded Cm^r clones that harbored plasmids identical in molecular size (8.1 kbp) with pNCO937 and Southern hybridization experiments confirmed the presence of a 2.4-kbp *Pst*I fragment that contains the *cho* gene. The biosynthesis of a functional cholesterol oxidase in *S. thermophilus* transformants was also confirmed by TLC analysis of reaction products following incubation of sonically disrupted cells with cholesterol. However, unlike *S. lividans* 1326, (4), *S. thermophilus* did not secrete cholesterol oxidase into the culture medium.

The data presented here provided evidence that the promoter sequences of the *Streptomyces cho* gene were recognized in *S. thermophilus* ST128. However, instability of the insert was obvious, as indicated by the loss of cholesterol oxidase activity from transformants after repeated transfers. Agarose gels clearly showed that mutational events involved primarily the 2.4-kbp component of pNCO937 which contained the *cho* gene. This was also confirmed by the DNA-DNA hybridization data. Current studies in our laboratory on the mechanism of the loss of Cho⁺ phenotype may lead to the stabilization of this trait in *S. thermophilus*.

Our results obtained with cholesterol oxidase as a model system indicated that the possibility exists for the transfer of other heterologous genes controlling cholesterol metabolism in other prokaryotes into *S. thermophilus*, an industrial microbe used extensively in the production of fermented dairy foods. The stable expression of cholesterol modifying or degrading enzyme systems in *S. thermophilus* and other

dairy fermentation bacteria may ultimately lead to the development of milk products with reduced cholesterol content.

RECEIVED: September 5, 1990; REVISED: November 13, 1990

REFERENCES

1. MATTSON, F. H., ERICKSON, B. A., AND KLINGMAN, A. M. (1972) *Am. J. Clin. Nutr.* **25**, 589-594.
2. FLAIM, E., FERREI, L. F., THYE, F. W., HILL, J. E., AND RITCHEY, S. J. (1981) *Am. J. Clin. Nutr.* **34**, 1103-1108.
3. MCGILL, H. C., MCMAHAN, C. A., AND WENE, J. D. (1981) *Arteriosclerosis* **1**, 164-176.
4. MUROOKA, Y., ISHIZAKI, T., NIMI, O., AND MAEKAWA, N. (1986) *Appl. Environ. Microbiol.* **52**, 1382-1385.
5. HUTKINS, R. W., AND MORRIS, H. A. (1987) *J. Food Prot.* **50**, 876-884.
6. SOMKUTI, G. A., AND STEINBERG, D. H. (1979) *J. Food Prot.* **11**, 885-887.
7. SAMBROOK, J., FRITSCH, E. F., AND MANIATIS, T. E. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
8. SOMKUTI, G. A., AND STEINBERG, D. H. (1986) *J. Biotechnol.* **3**, 323-332.
9. BIRNBOIM, H. C., AND DOLY, J. (1979) *Nucleic Acids Res.* **7**, 1513-1523.
10. STOUGAARD, P., AND MOLIN, S. (1981) *Anal. Biochem.* **118**, 191-193.
11. SOMKUTI, G. A., AND STEINBERG, D. H. (1986) *J. Ind. Microbiol.* **1**, 157-163.
12. COHEN, S. N., CHANG, A. C. Y., AND HSU, L. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110-2114.
13. SOMKUTI, G. A., AND STEINBERG, D. H. (1988) *Biochimie* **70**, 579-585.
14. ISHIZAKI, T., HIRAYAMA, N., SHINKAWA, H., NIMI, O., AND MUROOKA, Y. (1989) *J. Bacteriol.* **171**, 596-601.
15. LEARY, J. J., BRIGATI, D. J., AND WARD, D. C. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4045-4049.
16. REED, K., AND MANN, D. (1985) *Nucleic Acids Res.* **13**, 7207-7221.
17. BITMAN, J., AND WOOD, D. L. (1982) *J. Liq. Chromatogr.* **5**, 1155-1162.
18. MACRINA, F. L., JONES, K. R., AND WOOD, P. H. (1980) *J. Bacteriol.* **143**, 1425-1435.
19. DE VOS, W. M. (1987) *FEMS Microbiol. Rev.* **46**, 281-295.