

Expression of *Streptomyces* sp. cholesterol oxidase in *Lactobacillus casei*

Summary. Three strains of *Lactobacillus casei* were electrotransformed with pNCO937, an 8.1-kb recombinant plasmid carrier of a *Streptomyces* sp. cholesterol oxidase gene. Transformation frequency was generally low and strain-dependent, ranging from 6 to 40 transformants/ μ g DNA. *L. casei* transformants stably maintained pNCO937 with no indication of deletion mutational events. Transformants produced active cholesterol oxidase and sonicated cells formed 4-cholesten-3-one from both free and lipoprotein-bound cholesterol. *L. casei* shows promise as a host suitable for studying heterologous gene expression in lactobacilli.

Introduction

Bacteria of the genus *Lactobacillus* find extensive use around the world in the preparation of fermented foods (dairy, meats, vegetables) and animal feed (silage). Through the synthesis of lactic acid and other small metabolites as well as enzyme systems and antimicrobial agents, lactobacilli contribute to the development of the characteristic flavor, texture and storage properties of the finished products. In addition to these primary metabolic functions, several *Lactobacillus* species have been reported to possess therapeutically useful properties, which for the most part still await confirmation and application on a large scale. These properties include the synthesis of antimicrobials and the alleviation of lactose intolerance (Kilara and Treki 1984), detoxification of potential carcinogens (Goldin and Gorbach 1984), deconjugation of bile acids and cholesterol assimilation (Gilliland et al. 1985), stimulation of immune response (Perdigon et al. 1986), anticollitic effect (Gorbach et al. 1987), hypocholesterolemic effect (Gilliland and Walker 1990), antitumor effect (Macleod et al. 1990) and the synthesis of an antihypertensive compound (Furushiro et al. 1990).

Because of their non-pathogenic and non-toxic nature, food-fermenting lactobacilli also attract increased attention as potential expression systems for heterologous gene products, which would expand their breadth of metabolic capabilities and widen the scope of industrial applications. Recent reports on the genetic manipulation of lactobacilli have included the cloning and expression of *Clostridium thermocellum* endoglucanase (Bates et al. 1989), *Staphylococcus hyicus* lipase (Vogel et al. 1990), *Bacillus subtilis* endoglucanase (Baik and Pack 1990), and *B. amyloliquefaciens* α -amylase (Jones and Warner 1990) and β -glucanase (Thompson and Collins 1991) in these microbes.

The present investigation sought to introduce the cholesterol oxidase (CO) gene (*cho*) of *Streptomyces* sp. (Murooka et al. 1986) into *L. casei* and to study the expression of the novel heterologous gene in this economically important bacterium.

Materials and methods

Strains of bacteria and growth conditions. The *L. casei* LC1, LC2, LC3 strains were from our laboratory collection and were cultured in MRS broth (Difco Laboratories, Detroit, Mich., USA) without agitation in a 5% (v/v) CO₂ atmosphere. *Escherichia coli* HB101 was cultured aerobically in Luria Bertani broth (Sambrook et al. 1989). When needed, chloramphenicol (Cm) was used in media at 15 μ g/ml concentration.

Plasmid DNA isolation and characterization procedures. The *cho* gene of *Streptomyces* sp. SA-COO (Murooka et al. 1986) was stably maintained in *E. coli* HB101 as part of the 8.1-kb plasmid vector pNCO937 as reported previously (Somkuti et al. 1991). Plasmid DNA used in transformation experiments was isolated according to the procedure of Birnboim and Doly (1979) and purified by a rapid cesium chloride density gradient centrifugation method (Stougaard and Molin 1981).

Plasmid DNA from *L. casei* strains was isolated by a standard procedure developed for lactic acid bacteria and purified by a minicolumn adsorption-desorption procedure (Somkuti and Steinberg 1986).

Restriction endonucleases were purchased from BRL Life Technologies (Gaithersburg, Md., USA) and used according to the manufacturer's instructions.

Agarose gel electrophoresis (AGE) of intact plasmids and restriction fragments was carried out in 0.8% and 1.2% vertical agarose (Seakem, FMC Corporation, Rockland, Me., USA) gels in TBE buffer system (0.089 M TRIS, 0.089 M boric acid, 0.002 M EDTA, pH 8.2).

Genetic transformation of *L. casei* strains. *L. casei* was transformed with pNCO937 by an electrotransformation procedure (ET) developed for lactic acid bacteria (Somkuti and Steinberg 1987). Overnight cultures of *L. casei* strains LC1, LC2 and LC3 grown in MRS broth were transferred to fresh medium (0.5% inoculum, v/v) and incubated at 37°C until an optical density at 660 nm (OD₆₆₀) of 0.2 reached. Pellets from 1-ml samples were washed twice with PO buffer (50 mM K₂HPO₄-KH₂PO₄, 1 mM MgCl₂, pH 7.4) and resuspended in electrotransformation medium (ETM), (5 mM K₂HPO₄-KH₂PO₄, 1 mM MgCl₂, 0.3 M raffinose, pH 4.5).

Electroporation of *L. casei* strains was done in a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif., USA) after mixing 0.8 ml of chilled (4°C) cell suspensions with the transforming plasmid pNCO937 (1–10 µg) in less than 50 µl TE [10 mM TRIS-HCl, 0.1 mM ethylenediaminetetraacetate (EDTA), pH 8.0] in a Gene Pulser cuvette (0.4-cm interelectrode distance). The cells were exposed to a single electropulse at 4 kV/cm at a 25 µF capacitance setting. Immediately after electropulsing, cell suspensions were diluted with 0.2 vol. of fivefold concentrated MRS broth and incubated for 3 h at 37°C to allow gene expression. Cell suspensions were plated in 1.5% MRS-agar plates with Cm at 15 µg/ml and incubated in a 5% CO₂ atmosphere for 2 days.

Detection of the *cho* gene and cholesterol oxidase activity. Samples of total plasmid DNA extracted from *L. casei* transformants scored on MRS-Cm agar plates were analyzed by AGE for the presence of an 8.1-kb entity as putative evidence for the uptake of the *cho*-bearing vector pNCO937. In agarose gels, plasmid bands were visualized by UV light after staining with 1 µg ethidium bromide/ml.

Presence of the *Streptomyces* sp. *cho* gene in *L. casei* transformants was also established by Southern blot hybridization. Total plasmid DNA samples were digested with *Pst*I and digestion mixtures were resolved by AGE in 1.2% gels. After vacuum transfer to nylon membranes, a 2.4-kb biotinylated *Pst*I fragment of *Streptomyces* sp. DNA (Ishizaki et al. 1989) incorporating the *cho* gene was used as the probe to detect homologous sequences in plasmid DNA digests of *L. casei* transformants. The development of the biotinylated probe and other details of the DNA-DNA hybridization procedure have been described previously (Somkuti et al. 1991).

Expression of the *cho* gene in *L. casei* transformants was checked by assaying for CO activity associated with sonicated cell preparations. Cells were collected from 35 ml of MRS-Cm broth, washed repeatedly and resuspended in 1.5 ml PO buffer. Sonication was done with a microtip cell disrupter (Heat Systems Ultrasonics, Farmingdale, N.Y., USA) at maximum output, 20 times for 10 s, with intermittent cooling in crushed ice. To each sonicate was added 200 µl of a cholesterol stock solution (2 mg/ml) in *n*-propanol. Alternatively, 1.5 ml lipoprotein-bound cholesterol (PPLO) serum fraction (Difco) containing 1.2 mg/ml cholesterol in the form of lipoprotein complexes was used as the substrate. In all cases, reaction mixtures were supplemented with chloramphenicol at 20 µg/ml to retard microbial contamination. Incubation was for 2 days at 37°C with agitation on an orbital shaker. At the end, reaction mixtures were extracted with 3 × 5 ml ethyl acetate. Separation of phases was by centrifugation at 1000 rpm for 3 min. Solvent phases were evaporated to dryness and the dry residue was dissolved in *n*-propanol.

Cholesterol transformation products were detected by TLC on SIL G-25 silica plates (Brinkman Instruments, Westbury, N.Y., USA) after development in chloroform-ethyl acetate (95:5, v/v) and spraying with an H₃PO₄-CuSO₄ reagent (Bitman and Wood 1982). TLC plates were heated at 130°C for 5 min and photographed under UV light.

The extent of cholesterol depletion in incubation mixtures was also measured by determining the amount of residual cholesterol after 2 days at 37°C with the colorimetric ferric chloride assay (Thomas and Stevens 1960).

Results

Transformation of *L. casei* with pNCO937 to Cm^r phenotype

The transforming DNA was pNCO937, an 8.1-kb recombinant plasmid that incorporates the *cho* gene of *Streptomyces* sp. on a 2.4-kb *Pst*I fragment (Fig. 1). Its construction protocol has been reported previously (Somkuti et al. 1991) and involved the use of pNZ19, a 5.7-kb bifunctional vector with the chloramphenicol resistance (Cm^r) gene of pC194 as selection marker (de Vos 1987), which permitted the use of *E. coli* HB101 as a convenient intermediate host for producing pNCO937 for electrotransformation experiments.

The ET procedure facilitated the uptake of pNCO937 by *L. casei* and agarose electrophoresis established the presence of a new 8.1-kb plasmid in the plasmid DNA complement of every clone that grew in MRS-Cm agar within 48 h (data not shown). The presence of pNCO937 in transformants was also taken as putative evidence for the acquisition of the heterologous *cho* gene.

The efficiency of transformation was low, with *L. casei* strains LC1, LC2 and LC3 yielding 6, 11, and 40 Cm^r transformants, respectively, per microgram of transforming DNA.

Detection of *Streptomyces* sp. *cho* gene

The putative presence of the *Streptomyces* sp. *cho* gene in *L. casei* LC1 transformants with Cm^r phenotype was confirmed by Southern blot hybridization (Fig. 2) with a 2.4-kb *Pst*I fragment of pCO1 (Murooka et al. 1986) as the probe. Hybridization signals were given only by the intact cloning vector pNCO937 harboring the *cho* gene, its 2.4-kb *Pst*I digestion fragment and an identical size fragment found in *Pst*I digests of total plasmid DNA extracted from Cm^r transformants. Except for differences in the number and size distribution of repetitive *Pst*I digestion fragments, *L. casei* LC2 and LC3 transformants also showed only a single 2.4-kb entity that hybridized with the biotinylated probe (data not shown). The re-

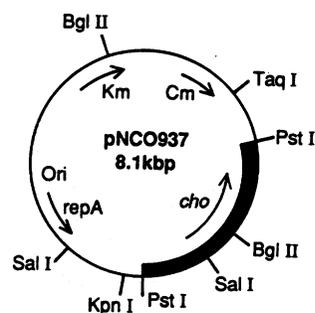


Fig. 1. The structure of pNCO937 used in the electrotransformation of *Lactobacillus casei* to cholesterol-oxidase-producing (CO⁺) phenotype

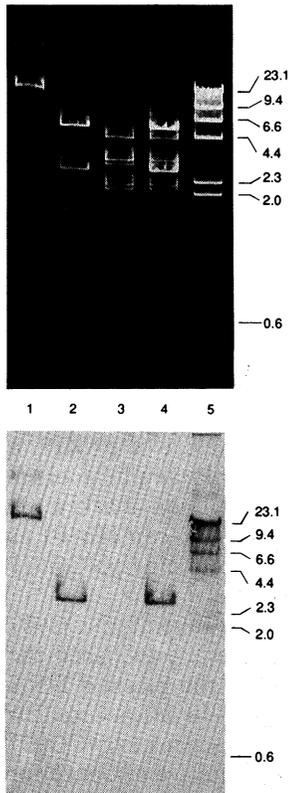


Fig. 2. Presence of the *Streptomyces* sp. CO gene (*cho*) in *L. casei* transformants. *Top*: agarose gel electrophoresis of *Pst*I digests of total plasmid DNA isolated from *L. casei* LC1. *Bottom*: Southern hybridization patterns with the 2.4-kb biotinylated *Pst*I fragments of pCO1 on nylon membrane. *Lane 1*, intact pNCO937; *lane 2*, *Pst*I digest of pNCO937; *lane 3*, *Pst*I digest of plasmid DNA from *L. casei* LC1; *lane 4*, *Pst*I digest of plasmid DNA from *L. casei* LC1 Cm^r transformant; *lane 5*, *Hind*III fragments of λ DNA (multicomponent biotinylated probe was developed from *Hind*III fragments)

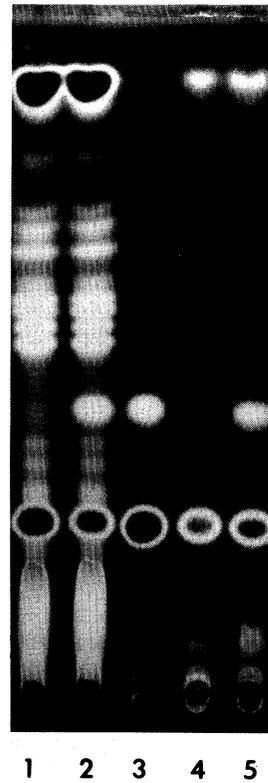


Fig. 3. Detection of CO in cell sonicates of *L. casei* LC1. Conditions of the TLC analysis are described in Materials and methods. Lipoprotein-bound cholesterol (PPLO) serum (*lane 1*) and free cholesterol (*lane 4*) incubated with *L. casei* LC1; PPLO serum (*lane 2*) and free cholesterol (*lane 5*) incubated with *L. casei* LC1 chloramphenicol-resistant (Cm^r) transformant; *lane 3*, 4-cholesten-3-one (fast spot) and cholesterol (slow spot) standards

sults of Southern hybridization also confirmed the absence of any substantial molecular rearrangements or deletions involving the *cho* gene in the new hosts.

Expression of cholesterol oxidase activity

Cell-free culture fluids of *L. casei* transformants did not have measurable CO activity indicating that the *cho* gene product remained intracellular. When whole-cell sonicates of genetic transformants were incubated with cholesterol for up to 2 days, solvent extracts contained 4-cholesten-3-one, the reaction product of CO, as indicated by TLC (Fig. 3). Either free cholesterol dispersed in *n*-propanol or PPLO serum was suitable as a substrate for the enzyme. Quantitative determination of residual cholesterol content of reaction mixtures indicated that, depending on the strain used, 40–50% cholesterol supplied in water-soluble form (PPLO serum) was converted to 4-cholesten-3-one over a 2-day incubation period at 37°C (Table 1).

Discussion

This report describes the suitability of the recombinant plasmid pNCO937 for introducing a heterologous *cho* gene into *L. casei*, an important dairy fermentation bacterium. The work also demonstrates that the shuttle vector pNZ19, designed originally for use in lactococci (de Vos 1987), may be useful in the molecular cloning of lactobacilli.

Table 1. Depletion of cholesterol from lipoprotein-bound cholesterol (PPLO) serum by cell extracts of *Lactobacillus casei* transformants

Strain	Cholesterol extractable (μ g) ^a		Loss (%)
	0 h	48 h	
<i>L. casei</i> LC1-2	188.5	110	41.7
<i>L. casei</i> LC2-6	161.3	78	51.7
<i>L. casei</i> LC3-9	230.0	109	52.6

Conditions of assay were as described in Materials and methods
^a Average of triplicate determinations

Formerly, conjugation was the only effective way of delivering DNA into this group of bacteria, with most studies involving antibiotic resistance genes (Vescovo et al. 1983; Shrago et al. 1986; Tannock 1987; Thompson and Collins 1988; Langella and Chopin 1989; Takemoto et al. 1989), although the conjugal transfer of bacteriocin genes has also been reported (Muriana and Klaenhammer 1987). A significant achievement in the conjugal transfer of truly heterologous genes into lactobacilli has been the cloning and expression of β -glucanase from *B. amyloliquefaciens* in *L. helveticus*, which made use of pSA3 as the cloning vector and pVA797 as the mobilizing plasmid via co-integrate formation (Thompson and Collins 1991).

Since the first successful application of electrotransformation (electroporation) to lactobacilli by Chassy and Flickinger (1987), several research groups have reported the introduction of single or double antibiotic re-

sistance marker-bearing plasmids into a variety of *Lactobacillus* species. Thus, pNZ12 (4.3-kb, Cm^r) was used in *L. curvatus* (Gaier et al. 1990), pGK12 [4.5-kb, Em^r (erythromycin-resistant) and Cm^r] in *L. acidophilus* (Luchansky et al. 1988), *L. plantarum* (Bringel and Hubert 1990), and *L. delbrueckii* ssp. *lactis* (Zink et al. 1991). Larger plasmids such as pLHR (8.5 kb, Em^r), pSA3 (10.2 kb, Em^r, Cm^r), pTV1 (12.4 kb, Em^r, Cm^r) and pAM β 1-1 (17 kb, Em^r) were used in *L. helveticus* ssp. *jugurti* (Hashiba et al. 1990), *L. fermentum* (Vescovo et al. 1991), *L. plantarum* (Aukrust and Nes 1988) and *L. casei* (Natori et al. 1990), respectively.

Our success with the uptake and expression of a *Streptomyces* sp. CO in *L. casei* adds to the growing number of reports on the expression of heterologous genes encoding novel metabolic enzymes in lactobacilli. *L. casei* as a host was apparently able to recognize gene expression signals or structures of replicons of different origins as they exist in the recombinant plasmid pNCO937. No evidence was found for deletion mutations involving the DNA fragment carrying the streptomycete *cho* gene. However, CO, which is extracellular in *Streptomyces* sp. and remains so when cloned in *S. lividans* 1326 (Murooka et al. 1986) was detectable only in sonicated cells of *L. casei*. This implies that the protein transport machinery of the three *L. casei* strains studied failed to recognize efficiently the *Streptomyces* sp. CO signal peptide sequence. On the other hand, reports from other laboratories indicated that *C. thermocellum* endoglucanase and *B. amyloliquefaciens* α -amylase remained extracellular in electrotransformants of *L. plantarum* (Bates et al. 1989; Jones and Warner 1990), and most of the *B. subtilis* endoglucanase was released into the medium by recombinant cultures of *L. acidophilus* obtained by electrotransformation (Baik and Pack 1990). Similar results were obtained following the conjugal transfer and expression of the *B. amyloliquefaciens* β -glucanase gene in *L. helveticus* (Thompson and Collins 1991). Whether inability to secrete CO is a problem limited only to *L. casei* remains to be decided.

Although the conditions of electrotransformation and enzyme production were not optimized, the results of this study confirmed that *L. casei* and other lactobacilli are promising hosts for the expression of novel heterologous gene products. The expansion of the metabolic diversity of lactobacilli will increase their potential utility for bringing about desirable compositional changes in dairy foods.

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