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Expression of streptomycete cholesterol oxidase in *Escherichia coli*

Daniel K.Y. Solaiman and George A. Somkuti

U.S. Department of Agriculture, ARS, Eastern Regional Research Center, Philadelphia, Pennsylvania, U.S.A.

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SUMMARY

A streptomycete gene coding for extracellular cholesterol oxidase (*choA*) was subcloned and expressed in *Escherichia coli*. The pUCO series recombinants were obtained by inserting the *choA* gene into the unique *Kpn*I site of pUC19 vector. Expression was observed with pUCO192A and pUCO193 constructs in which the cloned gene(s) were aligned with the upstream *lac*Z promoter. Isopropyl β-D-thioglucopyranoside (IPTG) enhanced this expression up to 2.5-fold. Specific Cho activity in the cell extracts of the stable pUCO193 transformant were 0.004 U and 0.007 U per mg protein without and with IPTG induction, respectively. Cho activity was detected in the spent medium of this culture, suggesting possible secretion of the enzyme.

INTRODUCTION

Cholesterol oxidase (Cholesterol : oxygen oxidoreductase; EC 1.1.3.6) is the first enzyme of the cholesterol degradation pathway found in many microorganisms [22]. It catalyses the oxidation of cholesterol (5-cholesten-3β-ol) to 4-cholesten-3-one. Concomitantly, molecular oxygen is stoichiometrically reduced to hydrogen peroxide. Since its first description in a soil bacterium [24], the enzyme has been identified and purified from various microbial sources [4,12,25].

Cholesterol oxidase (Cho) is an important ingredient in the majority of commercially available assay kits used in cholesterol screening. In these assays, the stoichiometric H_2O_2 generated during enzymatic oxidation of the cholesterol substrate, is quantitated after coupling to chromogenic reactions [1,18]. Because of its commercial value, there is widespread interest in producing this enzyme in high yields through genetic engineering. To this end, Murooka et al. [7,9,14] have cloned, sequenced, and expressed in *Streptomyces lividans*, a *choA* gene from *Streptomyces* sp. strain SA-COO. In this paper, we describe our studies on the subcloning and expression of this gene in *E. coli*, a well-studied microorganism used extensively in the commercial production of cloned gene products such as insulin and chymosin [6,15,23].

MATERIALS AND METHODS

Bacteria, growth media and plasmids

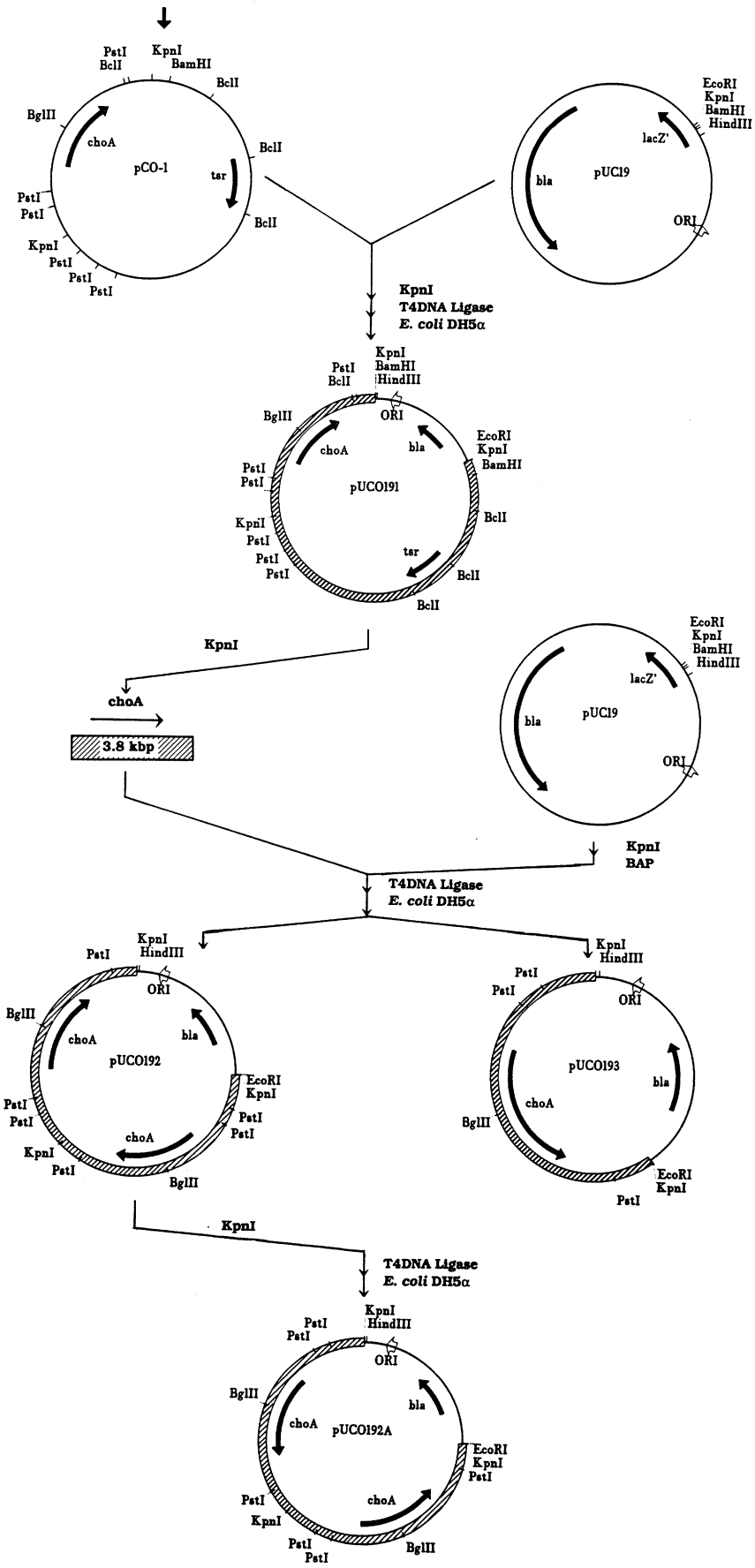
Escherichia coli DH5α (BRL Life Technologies, Gaithersburg, MD) and JM109 (Stratagene, La Jolla, CA) were used as host strains. Cells were maintained in LB medium {1% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl} supplemented with 50–100 μg/ml ampicillin (Ap) or 30–50 μg/ml chloramphenicol (Cm). Solid LB medium contained 1–1.25% (w/v) agar. When needed, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal) was included in the medium at 50 μg/ml.

Plasmid pUC19 was purchased from BRL Life Technologies. *S. lividans* 1326 harboring the pCO-1 plasmid that contained the cloned *choA* gene, was obtained from Y. Murooka (Hiroshima University). *S. lividans* 1326 was grown in brain heart infusion broth (Difco) containing thiostrepton (5 μg/ml).

Molecular biology procedures

Transformable *E. coli* DH5α and JM109 were purchased from commercial sources (see above). Transformation was carried out by the heat shock procedure [13]. Plasmids were isolated from *E. coli* as described [2], and from *S. lividans* according to the method of Somkuti and Steinberg [20].

Restriction endonucleases, bacterial alkaline phosphatase and T4 DNA ligase (BRL Life Technologies) were used under the conditions prescribed by the supplier. Agarose gel electrophoresis and DNA electroelution were performed in Tris-Borate-EDTA buffer system as described [13].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Determination of ChoA expression

Cytoplasmic Cho levels were determined with cell extracts prepared from 2.0–4.0 h cultures grown in the absence or presence of 0.2 mM isopropyl-β-D-thiogluco-pyranoside (IPTG). Cells from 15-ml cultures were harvested by centrifugation and resuspended in 2 ml buffer A (50 mM Tris · HCl, pH 7.6; 20 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 10 mM DTT; 10 mM EDTA; 500 mM NaCl; 5% (v/v) glycerol). Cytoplasmic contents were released by sonication using a cell disruptor (Sonicator Model W-225; Heat Systems, Ultrasonics, Farmingdale, NY) equipped with a stepped microtip probe (power setting of 3; 5 × 30 s bursts; 50% duty cycle; 4 °C). Cell debris was removed by centrifugation (10000 × *g* RCF; 45 min; 4 °C). The Cho activity in cell extracts was assayed by determining the depletion of extraneously added cholesterol. In these assays, 75–400 μg (in 10–15 μl) of cholesterol (in *n*-propanol) were added to 0.2–1.0 ml cell extracts (0.02–0.8 mg protein) in buffer A or 50 mM potassium phosphate buffer (pH 7.4). After a period of incubation at 37 °C, cholesterol in reaction mixtures was extracted with ethyl acetate and quantitated with ferric chloride reagents as described [11]. The production of 4-cholesten-3-one in these reactions was confirmed by thin layer chromatography (TLC) [11].

The specific Cho activity of the cell extracts of pUCO193 transformant was quantitated spectrophotometrically by monitoring the increase in absorbance at 240 nm due to the production of 4-cholesten-3-one [21]. An extinction coefficient value of $\varepsilon_{240\ nm} = 12.2\ mM^{-1}\ cm^{-1}$ was used to quantitate the reaction product [4]. One unit (U) of enzyme activity catalyzed the production of 1 μmol of 4-cholesten-3-one in 30 min.

The presence of extracellular Cho was assessed by adding 0.2–0.4 mg of cholesterol to either cell free spent medium or newly inoculated growing cultures. After a period of incubation at 37 °C, the residual cholesterol in the reaction mixtures was extracted and quantitated as described above.

Protein determination

Protein concentration was determined by the Coomassie Brilliant Blue G-250 dye-binding assay [3] using a kit purchased from Bio-Rad Laboratories (Rockville Centre, NY). Bovine serum albumin was used as a standard.

Materials

Bactotryptone, yeast extract and bactoagar were purchased from Difco (Detroit, MI). Cholesterol, 4-cholesten-3-one, Xgal, IPTG, Ap, and Cm were obtained from Sigma (St. Louis, MO). All other reagent grade chemicals were from commercial sources.

RESULTS

Plasmid construction

For the convenience of large scale DNA purification, we first subcloned the entire pCO-1 plasmid [14] into the unique *Kpn*I site of pUC19 vector to yield the recombinant DNA, pUCO191 (Fig. 1). In this subcloning, pCO-1 was linearized at the *Kpn*I site appropriately marked in Fig. 1. The recombinant plasmid pUCO191 was maintained in *E. coli* DH5α and was the source of *choA*-containing fragments used for subsequent subcloning.

The pUCO192 and pUCO193 were obtained from the ligation of a 3.8-kbp *Kpn*I fragment of pUCO191 with *Kpn*I-linearized pUC19. This *Kpn*I fragment was used because it contained the entire operon consisting of the promoter and the *choP* and *choA* genes [7]. As shown in Fig. 1, pUCO192 contained two copies of the cloned fragment tandemly ligated to pUC19. The orientation of the *choA* genes in pUCO192 was opposite to that of the *lac*Z promoter of pUC19. The other recombinant, pUCO193, represented a 1 : 1 ligation product with the orientation of the *choA* gene aligned with that of the *lac*Z promoter (Fig. 1). In an attempt to generate more recombinants with different *choA* orientations, pUCO192 was again digested with *Kpn*I and re-ligated in situ with T4 DNA ligase. Upon transformation into DH5α, screening of the Ap^r transformants only yielded pUCO192A in which two tandemly repeated *choA*-gene fragments were aligned with the *lac*Z promoter (Fig. 1).

ChoA expression with pUCO series plasmids

The expression of *choA* gene was first assessed by the ability of cell extracts to deplete cholesterol on incubation. Cell extracts were obtained from *E. coli* JM109 that harbored the recombinant DNAs. The choice of this *lac*I^q host strain enabled us to examine the effect of IPTG on gene expression. The results showed that the crude extracts of cells containing pUCO192A and pUCO193 depleted cholesterol at a rate of $4.05 \pm 1.50\ \mu g$ and $6.28 \pm 0.66\ \mu g$ per μg protein, respectively, on 24 h

Fig. 1. Construction of the pUCO series plasmids. Only restriction sites pertinent to the present study were shown. Plasmids shown are not proportionate to size. pCO-1 was linearized at the *Kpn*I site marked with arrow (↓). BAP, bacterial alkaline phosphatase; *bla*, β-lactamase gene; *str*, thiostrepton resistance marker; *choA*, coding region of ChoA; *lacZ'*, gene coding for α-fragment of β-galactosidase; ORI, origin of replication of pUC19.

TABLE 1

Cholesterol depletion by cell extracts from *E. coli* JM109 Carrying pUC19 or pUCO series plasmids

Plasmid	IPTG (2 mM)	Cholesterol depletion [a]
pUC19	–	(0)
(Baseline control)	+	(0)
pUCO192	–	-3.03 ± 2.10 [b]
	+	2.79 ± 2.03 [b]
pUCO192A	–	4.05 ± 1.50
	+	10.35 ± 1.92
pUCO193	–	6.28 ± 0.66
	+	9.63 ± 1.31

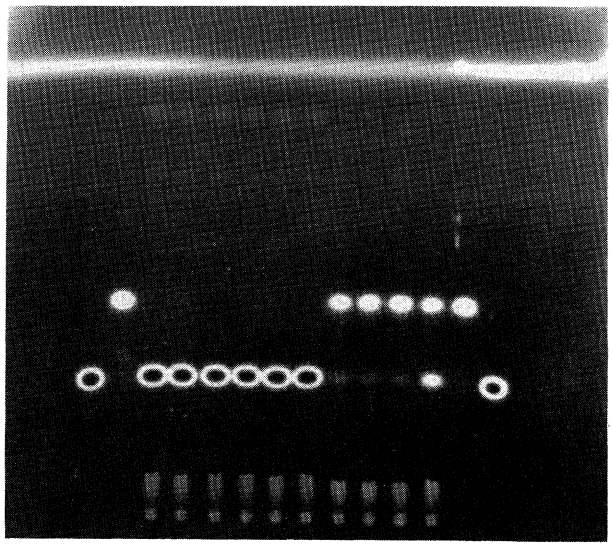
[a] Expressed in μg cholesterol depleted per μg protein per 24-h incubation at 37°C.

[b] The large standard deviations and the negative value indicated lack of cholesterol depletion within experimental error.

incubation with the substrate (Table 1). Cell extracts of pUCO192 transformants, on the other hand, did not exhibit measurable cholesterol depletion activity. As expected, extracts of pUC19-containing *E. coli* was incapable of oxidizing cholesterol. Incubation of cells harboring pUCO192A or pUCO193 with 2 mM IPTG for 2 h prior to cell harvest, increased the cholesterol depletion activity of the cell extracts. The rates of substrate utilization were $10.35 \pm 1.92\,\mu$g and $9.36 \pm 1.31\,\mu$g cholesterol depleted per μg protein for IPTG-induced pUCO192A and pUCO193 transformants, respectively. The gratuitous inducer, however, did not induce cholesterol metabolizing activity in cells containing pUCO192. These results indicated that the expression of the *choA* gene in pUCO series plasmids was mediated by the *lacZ* promoter of the pUC19 vector, and was thus responsive to IPTG induction.

Analysis of reaction products with TLC confirmed that cholesterol depletion by cell extracts of the pUCO192A and pUCO193 transformants was indeed due to the expressed Cho activity. Fig. 2 shows that cholesterol incubated with these cell extracts was converted to 4-cholesten-3-one that comigrated with the genuine standard. Similar oxidation of cholesterol to 4-cholesten-3-one was not observed with the pUCO192 and pUC19 samples. Fig. 2 also shows that one of the two pUCO192A samples (lane 12) displayed lower ChoA activity than the other (lane 11); this was attributed to the instability of the plasmid in some *E. coli* JM109 transformants (see DISCUSSION).

Since the pUCO193 transformant appeared to be a stable clone, we further characterized its *choA* expression properties. The specific Cho activity in this transformant



1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 2. Cholesterol oxidation by cell extracts of *E. coli* DH5α containing pUCO and the related plasmids. Cell extracts (1.5 ml; in 50 mM potassium phosphate, pH 7, and 1 mM $MgCl_2$) from overnight cultures (35 ml) were incubated (24 hr, 37°C) with 0.36 mg cholesterol. Reaction mixtures were extracted and analysed on TLC plate (see MATERIALS AND METHODS). Lanes 1 & 14, cholesterol (0.36 mg); lanes 2 & 13, 4-cholesten-3-one (0.36 mg); lanes 3 & 4, pUC19; lanes 5 & 6, pUCO191; lanes 7 & 8, pUCO192; lanes 9 & 10, pUCO193; lanes 11 & 12, pUCO192A.

was first determined as described [4] in order to facilitate comparison with the other Cho-producing microbes [4,12]. The results showed that this transformant produced 0.004 U of Cho per mg protein of crude extracts under non-induced conditions. With IPTG induction, the Cho yield was increased by 1.5-fold to 0.007 U per mg protein. The possible occurrence of extracellular enzyme in the *choA*-expressing *E. coli* transformants was next investigated, because the cloned gene used in the present study codes for a secreted form of the streptomycete Cho [14]. Spent medium from a 2-h-old culture of JM109 containing pUCO193, grown in the absence or presence of IPTG (2 mM), was collected by centrifugation. When 2 ml of these cell-free media were incubated with 0.2 mg cholesterol for 22 h at 37°C, the amounts of the sterol were reduced by 10% and 15% with the uninduced and IPTG-induced samples, respectively. These results suggested that some of the Cho produced by the transformants was apparently released into the growth medium. This conclusion was supported by the results of another experiment in which 2-ml whole-cell cultures of the pUCO193-containing clone nearly depleted all the cholesterol (0.4 mg) in the medium after 3 days of growth at

37 °C. A parallel TLC study verified that the sterol substrate was indeed oxidized to 4-cholesten-3-one (data not shown).

Discussion

A streptomycete *choA* gene was successfully subcloned and expressed in *E. coli* by using pUC19 plasmid. Expression of the cloned gene required that its orientation was aligned with the *lac*Z promoter, as in the pUCO192A and pUCO193 recombinants. ChoA activity was not detected with pUCO192 where the *choA* genes were opposed to the *lac*Z promoter. Expression was enhanced in the presence of inducer IPTG, indicating that the entire *lac*Z promoter/operator region was involved. The incomplete suppression of Cho production in the absence of IPTG likely was due to the presence of unidentified substance(s) in the complex LB medium, as was also observed by Doran et al. [5] in the expression of cloned streptomycete isopenicillin N synthase. The finding that the *lac*Z promoter/operator region of pUC19 was needed for the expression of the cloned *choA* gene, was not surprising. Although the cloned *Kpn*I fragment contained the entire *cho* operon, it was apparent that the *Streptomyces* promoter was not recognized by the *E. coli* transcription system. Other studies also had indicated that such intergenic expression occurred infrequently [8,16].

The expressed Cho activity in the pUCO192A-transformed cells was lower than that found in pUCO193 transformants, in spite of the presence of two copies of the cloned gene. The relatively large size of pUCO192A, as well as the presence of repeated sequences possibly promoted plasmid instability through recombination events [10], which may have resulted in a lower copy number of this plasmid. In fact, plasmid isolation procedures consistently yielded smaller amounts of pUCO192A than the pUCO193 (data not shown).

The specific yields of Cho by pUCO193 transformants were estimated at 0.004 U and 0.007 U enzyme per mg protein of crude extracts under non-induced and IPTG-induced conditions, respectively. Although these values were comparable to those reported for Cho-producing *Pseudomonas* sp. and *Nocardia* sp. [4,12], the total yields per unit volume of culture should be considerably higher for this genetically engineered *E. coli* in view of its ability to achieve high density growth [6]. Some of the Cho produced by pUCO193 transformant appeared to be secreted, suggesting that the putative leader sequence of the streptomycete *choA* gene [9] was in part recognized by the *E. coli* protein secretion system(s) [17]. The exact localization of the secreted Cho was not investigated, nor was its levels relative to the intracellular enzyme determined. Potentially high total yields and the likely secretion of Cho rendered the pUCO193-containing *E. coli* transformant an attractive candidate for further development into a Cho production strain.

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