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Cloning and Expression of *Serratia marcescens* Coenzyme A(CoA) Transferase Gene *in E. coli*

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Abstract We have got several clones from *Serratia marcescens* which stimulated the cells to use maltose as a carbon source in *E. coli* TP2139 (*Δlac*, *Δcrp*). One of the cloned genes, pCKB13, was further analyzed. In order to find whether the increased expression of the gene under the direction of maltose metabolism, we constructed several recombinant subclones. We have confirmed that the clone, pCKB13 codes Coenzyme A transferase gene by partial nucleotide sequencing in the terminal region. The enzyme activity of Coenzyme A transferase increased after introduction of the multicopy of the cloned gene in *E. coli*. The recombinant proteins expressed by multicopy and induction with IPTG, two polypeptide of 26- and 28-kDa, were confirmed by SDS-PAGE. Southern hybridization analysis confirmed that the cloned DNA fragment was originated from *S. marcescens* chromosomal DNA.

Key words: *Serratia marcescens*, CoA transferase gene, gene expression

Introduction

CoenzymeA (CoA) transferase are enzymes catalyzing the reversible transfer of CoA from one carboxylic acid to another. They have been identified in many prokaryotes. Although the CoA-transferases appear to be mechanistically and functianally very similar, their substrate ranges and activities differ [17].

The acetate (succinate)-acetoacetate CoA-transferase (EC 2.8.3.9) found among *Clostridia* acts mainly to detoxify the medium by removing the acetate and butyrate excreted earlier in the fermentation. This enzyme therefore has a role fundamentally different from other CoA-transferases, usually involved in the uptake of substrates for energy and structural use [3,6,8,11].

The succinyl CoA:3-oxoacid CoA transferase (SCOT, EC 2.8.3.5) is responsible for the formation of acetoacetyl CoA by transfer of a CoA moiety from succinyl CoA to a 3-oxoacid, usually acetoacetate. This enzyme has been found to have the highest activity in heart and kidney of various mammals. In the mitochondrion of these tissues, acetoacetate is converted to acetoacetyl CoA, which is further broken down to two acetyl CoA molecules capable of entering the tricarboxylic acid cycle [4,12]. And also this enzyme is characterized in some bacteria.

The best characterized CoA-transferases are the β-ketoadipate CoA-transferase of *Pseudomonas putida* and *Acinetobacter calcoaceticus*, the butyrate-acetoacetate CoA transferase from *Clostridium acetobutylicum*, and the succinyl CoA:3-oxoacid CoA transferase found in mammalian mitochondria. The β-ketoadipate CoA-transferase (β-ketoadipate: succinyl CoA transferase, EC 2.8.3.6) carries out the penultimate step in the conversion of benzoate and 4-hydroxybenzoate to tricarboxylic acid cycle intermediates in bacteria utilizing the β-ketoadipate pathway and several genes of CoA transferase in bacteria were characterized as well [4,6,8,12,15,17,19].

In this study, we report the cloning and identification of the genes which designated *aco*, encoding the subunits of CoA transferase from *S. marcescens*.

Materials and Methods

Bactarial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were routinely grown and maintained. Cultures for enzyme assay were grown in minimal medium with 0.5% potassium acetate and 0.2% casamino acids.

DNA isolation and manipulation

Total cellular DNA from *S. marcescens* was prepared as previously described [20]. Rapid, small-scale plasmid DNA isolation was performed by the method of Birnbaum and Doly [2] and Wizard kit of Promega Biotech. All restriction

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Table 1. Bacterial strains and plasmid.

strans & plasmid	genotype	source and ref.
STRAINS		
W3110	wild type	Lab. stock
JM 109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, △(*lac-pro*AB)/F′ [*tra*D36, *pro*AB$^+$, *lacI*^{q},*lacZ*△M15]	Lab. stock
TP 2139	*crp*, *lacX74*, *xyl*, *ilvA*, *argH*, *recA1*	A. Danchin, [9]
Serratia marceescens KTCC 2172		[7]
PLASMIDS		
pBluescript KS(+)	Amp^r	
pKK223-4	*tac*, Amp^r	[5]
pCKB13	CoA-transferase gene, Amp^r	this study
pACO13	CoA-transferase gene, Amp^r, *tac*	this study

enzymes, T4 DNA ligase, and polynucleotide kinase were purchased from Takara shuzo Co. and were used according to the recommendation of the suppliers. Double-stranded DNA was used as templates for sequencing reactions carried out by the dideoxy-chain termination method with an Autocycle DNA sequencing kit and A.L.F. DNA sequencer (Pharmacia) [14].

Southern hybridization

Total cellular DNA from *S. marcescens* was digested to completion with the desired restriction enzymes and separated by electrophoresis on 0.7% agarose gels. The DNA was transfered to the nitrocellulose filters by the method of Southern [16]. Double stranded DNA probe was prepared by using the random primer DNA labeling system (BRL) with [α-^{32}P] dATP (3,000 Ci/mmol) and the 1.5 kb insert fragment DNA of pCKB13 [13].

Preparation of cell extracts and enzyme assays

Cultures for enzyme assay were grown in minimal medium with 0.5% potassium acetate and 0.2% casamino acids. The cultured cells were harvested and suspended in cell suspension buffer (50 mM MOPS, pH 7.0, 0.5 M $(NH_4)_2SO_4$, 20 % [vol/vol] glycerol, 1 mM EDTA). The suspension was sonicated and cell debris was removed by centrifugation. The protein content of the cell extracts was determined by the method of Barford [1] with bovine serum albumin as the standard (Bio-Rad).

CoA-transferase activity was analyzed aerobically at room temperature in the carboxylic acid conversion direction by monitering the decrease in A_{310} as a indication of the disappearance of the enolate from acetoacetyl-CoA [3,18]. The assay contained the following, in a final volume of 1.0 ml: 100 mM HCl (pH 7.5), 150 mM carboxylic acid (potassium salt of acetate), pH 7.5, 40 mM $MgCl_2$, 0.1 mM acetoacetyl-CoA, 5% (vol/vol) glycerol, and 50 $\mu\ell$ of enzyme solution appropriately diluted in cell suspension buffer. The addition of the carboxylic acid initiated the reaction. Enzyme unit was defined as the amount of enzyme which could convert 1 μm of acetoacetyl-CoA per min under these conditions.

Analysis of protein

The crude enzyme extracts were prepared cells grown overnight in L-broth. The cells were suspended in cell suspension buffer, 25 mM phosphate buffer (pH 7.5) containing 0.5% Triton X-100. The cell suspension was sonicated, and cell debris was removed by centrifugation. The crude cell proteins were fractionated on 12% SDS-polyacrylamaide gels and stained with either Coomassie Blue by the method of Laemmli [10].

Results and Discussion

Identification of the *S. marcescens aco* gene

In a previous study, we have got several clones from *Serratia marcescens* which stimulate the cells to use maltose as a carbon source in *E. coli* TP2139 [20]. One of the cloned genes, pCKB13. was further analyzed. In order to find whether the increased expression of the gene under the direction of maltose metabolism, we constructed a restriction endonuclease cleavage map of the cloned DNA fragment and several recombinant subclones (Fig. 1). The clone, pACO13 was positive upon stimulating the cells to use maltose as a sole carbon source in *E. coli* TP2139 and pCKB13P was negative. The nucleotide sequence of the end region of pCKB13 was determined (data not shown). On analyzing the nucleotide sequence of the region with a gene analysis program, we found two truncated ORFs. The sequencing data was compared with GeneBank, EMBL, and SWISS-PROT data bases. The ORFs were highly homologous to the several bacteria genes encoding acetate and butyrate-acetoacetyl Coenzyme A transferase.

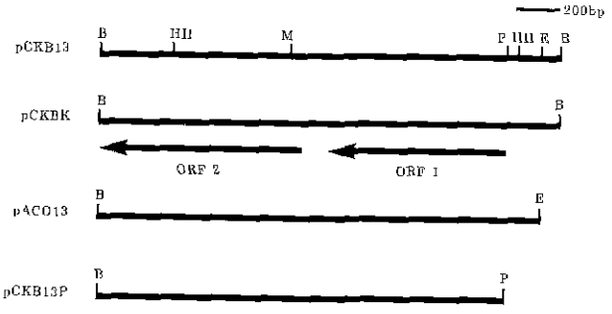

Fig. 1. Physical map of the plasmid pCKB13 of *S. marcescens* and derivative plasmids.
The transcriptional direction of the CoA transferase gene is shown by the thick arrow. Abbreviations : B; *Bam*HI, E; *Eco*RI, H; *Hinc*II, M; *Mlu*I, P; *Pst*I.

Therefore, we have found that the clone, pCKB13 codes Coenzyme A transferase gene by partial nucleotide sequencing on the terminal region.

Expression of the *S. marcescens aco* gene in *E. coli*

Genes for the putative of *S. marcescens* CoA-transferase were introduced into the bacterial high expression vector pKK223-4 [5]. The putative CoA-transferase gene region of pCKB13 was cut with *Eco*RI-*Bam*HI and ligated into the same site of pKK223-4 to construct pACO13. The enzymatic activity base line was determined using cellular lysates of *E. coli* transferase with pKK223-4 as a control. *E. coli* crude lysates expressing pACO13 showed a 22-fold increase in acetate-acetoacetyl CoA activity, respectively. In plasmid pACO14, the gene is cloned in the opposite direction and therefore is transcribed in the antidirection to pACO13. The result of assay showed low level activity in the same set of *E. coli*. This result showed that the plasmid pACO13 did not contain the full promoter region.

After induction of CoA transferase with isopropyl-thio-β-D-galactopyronoside, crude extracts were prepared from cultured bacterial cells and were analyzed by SDS-PAGE. As shown in Fig. 2, distinct bands corresponding to proteins of approximately 26- and 28-kDa was detected by SDS-PAGE. To determine whether the two CoA-transferase subunits were required for protein function, we deleted the region which containing a ORF, one of the putative CoA-transferase subunits. Although synthesis of one subunit was detected by SDS-PAGE (data not shown), no CoA transferase activity could be detected (Table 2). These results suggest that the two subunits of *S. marcescens* are requried for enzyme activity. The proximity and orientation of the genes suggest that the genes encoding the two subunits of CoA transferase may form an operon similar to other bacteria [3].

Southern blot analyis

To confirm the cloned gene was originated from the chromosomal DNA of *S. marcescens*, a hybridization experiment was performed (Fig. 3). *S. marcescens* chromosomal DNA was digested with *Bam*HI, *Eco*RI and *Hind*III and hybridized with [α-^{32}P] dATP (3,000 Ci/ mmol)-labeled 1.5 kb insert fragment of the pCKB13. The strong signal bands which hybridized with the *S. marcescens* chromosomal DNA corresponded to the 2.1 kb DNA size of the insert fragment of the pCKB13. The result confirmed that the cloned DNA fragment was originated from *S. marcescens* chromosomal DNA.

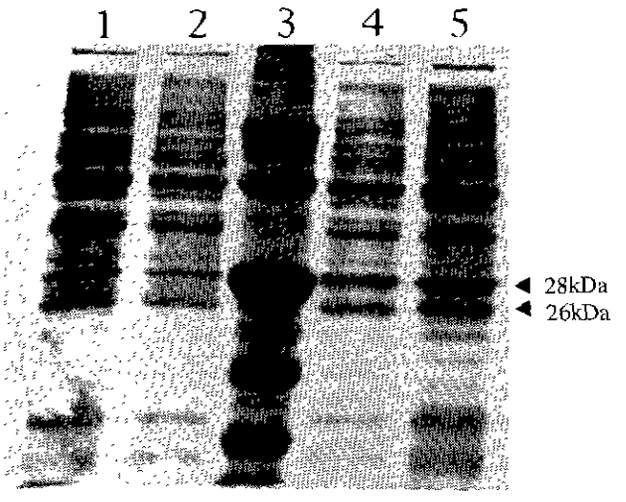

Fig. 2. SDS-polyacrylamide gel electrophoretic patterns showing expression of CoA transferase gene in *E. coli*.
Staining was done with Coomassie brilliant blue. The black triangle arrows indicate the position of the CoA transferase proteins. Lanes : 1; pKK223-4/TP 2139, 2; pKK223-4/TP 2139/1 mM IPTG, 3; size marker, 4; pACO13/TP 2139, 5; pACO13/TP 2139/ 1 mM IPTG.

Table. 2. Coenzyme A transferae activities in *E. coli* TP 2139 cells transformed with recombinant plasmids[1)]

plasmid	specific activity[2)]
pKK223-4	0.022
pACO13	0.491
pACO14	0.101
pCKB13P	0.029

[1)]Cultures for enzyme assay were grown in minimal medium with 0.5% potassium acetate and 0.2% casamino acids.
[2)]Transferase activity is expressed as nanomoles of acetoacetyl CoA formed for per milligram of protein. Activities reported are average of three assays of two independentyl prepared extracts

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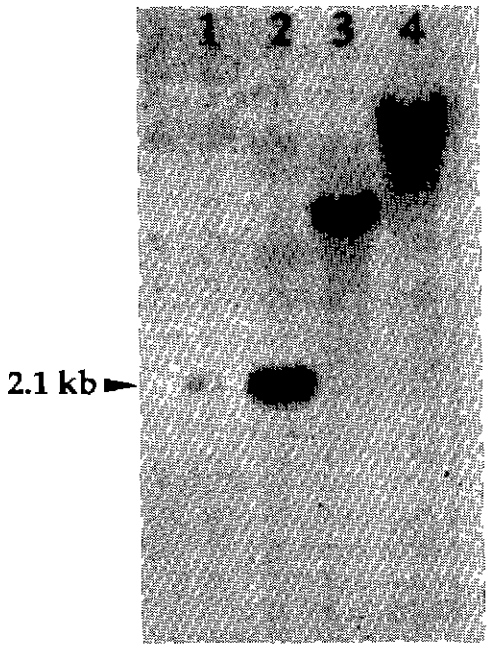

Fig. 3. Southern hybridization analysis of the enzyme-digested *S. marcescens* chromosomal DNA.
The 1.5 kb insert fragment of pCKB13 was used as a probe. Lanes : 1; size marker, 2; *S. marcescens* chromosomal DNA digested with *Bam*HI, 3; *Eco*RI, 4; *Hind*III

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