Characterization of the Genes Encoding β -Ketoacid: Succinyl-Coenzyme A Transferase in *Pseudomonas putida*

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β -Ketoacid:succinyl-coenzyme A transferase (β -ketoacid: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 -ketoacid pathway. This report describes the characterization of a DNA fragment from *Pseudomonas putida* that encodes this enzyme. The fragment complemented mutants defective in the synthesis of the CoA transferase, and two proteins of sizes appropriate to encode the two nonidentical subunits of the enzyme were produced in *Escherichia coli* when the fragment was placed under the control of a phage T7 promoter. DNA sequence analysis revealed two open reading frames, designated *pcaI* and *pcaJ*, that were separated by 8 bp, suggesting that they may comprise an operon. A comparison of the deduced amino acid sequence of the *P. putida* CoA transferase genes with the sequences of two other bacterial CoA transferases and that of succinyl-CoA:3-ketoacid CoA transferase from pig heart suggests that the homodimeric structure of the mammalian enzyme may have resulted from a gene fusion of the bacterial α and β subunit genes during evolution. Conserved functional groups important to the catalytic activity of CoA transferases were also identified.

The β -ketoacid pathway is widely distributed among bacteria (53). It consists of two parallel branches for the dissimilation of benzoate and 4-hydroxybenzoate (Fig. 1). Although numerous studies have shown that the enzymes of the pathway from different microorganisms share common ancestry (13, 19, 65, 66), many different gene organizations and forms of regulation have evolved (7, 34, 37, 41, 53). For example, in *Acinetobacter calcoaceticus*, protocatechuate is the inducer of a single gene cluster (*pcaEFDBCHG*) encoding most of the enzymes required to convert 4-hydroxybenzoate to tricarboxylic acid cycle intermediates (7, 12). In *Pseudomonas putida*, synthesis of the second enzyme (Fig. 1) necessary for the dissimilation of 4-hydroxybenzoate is induced by protocatechuate (25), but the synthesis of the remaining five enzymes is induced by β -ketoacid (33, 40) (Fig. 1) in conjunction with the positive regulatory element *pcaR* (27). The genes encoding these five enzymes are located in at least three separate transcriptional units (27), and there is evidence that gene(s) involved in chemotactic responses to aromatic compounds may constitute a fourth transcriptional unit in the *pca* regulon (11, 20).

The molecular basis for the regulation of the *pca* genes from any bacterium is an aspect of the β -ketoacid pathway that has received very little attention. As a starting point for understanding how these genes are regulated in *P. putida*, we have initiated a study of the *pcaI* and *pcaJ* genes (previously designated *pcaE* [35]) encoding β -ketoacid:succinyl-coenzyme A transferase (β -ketoacid:succinyl-CoA transferase) (EC 2.8.3.6). This enzyme catalyzes the transfer of CoA from succinyl-CoA to the central metabolite of the pathway, β -ketoacid, to form β -ketoacyl-CoA.

CoA thioesterification is a commonly used metabolic reaction. In many bacteria, carboxylic acids are activated by

the addition of CoA before they are further metabolized and used as carbon and energy sources. A similar metabolic route occurs in mammals, in which, under conditions of starvation or diabetes, when glucose levels are low or unusable, acetoacetate and β -hydroxybutyrate (ketone bodies) are released from the liver and provide a major source of metabolic energy for certain tissues, including the brain, heart, and kidney. Activation by CoA thioesterification is the first step in the metabolism of ketone bodies.

One type of enzyme that carries out CoA thioesterification reactions is the CoA transferase. CoA transferases that catalyze the reversible transfer of CoA from one carboxylic acid to another have been identified in many prokaryotes (2, 6, 46, 52, 57, 62, 66) and mammalian tissues (3, 44, 48, 59). Although the substrate ranges and subunit structures may differ, CoA transferases appear to be mechanistically and functionally very similar (2, 24, 51, 57, 62).

We report here the identification and characterization of the genes, designated *pcaI* and *pcaJ*, encoding the two subunits of β -ketoacid:succinyl-CoA transferase from *P. putida*. When the deduced amino acid sequences of the *P. putida* *pcaI* and *pcaJ* genes were compared with the sequences of two bacterial CoA transferases as well as with a eukaryotic CoA transferase sequence, all of which have very recently become available, a high degree of amino acid identity was seen. These comparisons, combined with previously published protein structure and mechanistic data, allowed assignment of probable catalytic domains as well as the identification of individual amino acids that are likely to be critical for catalysis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Cultures of *P. putida* were grown at 30°C in defined mineral medium as described previously (38) except that media containing tetracycline were made with 1% rather than 10% mineral salts solution.

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FIG. 1. The β -ketoadipate pathway. *P. putida* gene designations for each enzymatic step and known inducer molecules are indicated. Brackets indicate known gene clusters in *P. putida* (5, 27).

Carbon sources were sterilized separately and added at the time of inoculation (final concentrations: benzoate and 4-hydroxybenzoate, 5 mM; succinate, 10 mM). *Escherichia coli* strains were grown in Luria broth (LB) (9) at 37°C. For *P. putida*, antibiotics were used at the following final concentrations: tetracycline, 50 μ g/ml, and kanamycin, 100 μ g/ml. For *E. coli*, the following antibiotics were used: ampicillin, 100 μ g/ml; tetracycline, 25 μ g/ml; and kanamycin, 100 μ g/ml. For Lac⁺ colony screening, isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) were added to final concentrations of 0.1 mM and 40 μ g/ml, respectively. Solid media contained 1.5% agar.

DNA manipulations. Plasmid DNA to be used for cloning and sequencing was purified by the method of Lee and Rasheed (30). Restriction endonuclease digestions were performed according to manufacturer's instructions (New England Biolabs, Beverly, Mass.). Dephosphorylation reactions with calf intestinal phosphatase (Boehringer Mannheim,

Indianapolis, Ind.) were carried out as described previously (1). Ligation reactions and agarose gel electrophoresis were done as described previously (45). DNA fragments for subcloning were purified from gel slices with GeneClean (Bio 101, La Jolla, Calif.).

DNA sequencing and analysis. DNA sequences were determined by the dideoxy chain termination method with a commercial kit from United States Biochemical Corp. (Cleveland, Ohio) and ³⁵S-dATP purchased from Amersham (Arlington Heights, Ill.). Sequencing of the *pcaI* region was done by using synthetic oligonucleotide primers (typically 17 to 20 nucleotides in length) and a combination of DNA templates, including pPX202, pHRP110, and pHRP122, a clone that contains more DNA upstream of *pcaI* (Fig. 2). Plasmid DNA was denatured and annealed to primers as described by Hattori and Sakaki (21). Oligonucleotide primers were purchased from the Northwestern University Biotechnology Facility. DNA sequence data were analyzed by using DNA Inspector IIe, version 3.15 (Textco, Inc., W.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>P. putida</i> strains		
PRS2000	Wild type	36
PRS2241	<i>pcaE</i> ^a	36
PRS3004	<i>pcaJ</i> ::Tn5	27, this work
<i>E. coli</i> strains		
DH5 α	F ⁻ λ^- <i>recA1</i> Δ (<i>lacZYA-argF</i>)U169 <i>hsdR17 thi-1 gyrA96 supE44 endA1 relA1</i>	GIBCO-Bethesda Research Laboratories
S17-1	ϕ 80/ <i>lacZ</i> Δ M15 <i>thi pro hsdR hsdM⁺ recA</i> , chromosomal insertion of RP4-2(Tc::Mu, Km::Tn7)	50
Plasmids		
pUC19	Ap ^r ; ColE1 replicon	64
pHG165	Ap ^r ; ColE1 replicon, controlled copy number (<i>rop⁺</i>)	54
pRK415	Tc ^r ; IncP, mobilizable cloning vector	28
pT7-5	Ap ^r ; T7 promoter	55
pGP1-2	Km ^r ; <i>cI856</i> (Ts) <i>p_L</i> : <i>polT7</i>	55
pPX202	pUC19 with 8.5-kb <i>EcoRI</i> fragment carrying <i>pcaIJ</i> ; Ap ^r	27
pHRP100	pRK415 with 8.5-kb <i>EcoRI</i> fragment from pPX202; Tc ^r	This work
pHRP110	pHG165 with 2.9-kb <i>PstI</i> fragment from pPX202 carrying <i>pcaIJ</i> downstream of <i>lac</i> promoter; Ap ^r	This work
pHRP111	Same as pHRP110, but insert in opposite orientation	This work
pHRP122	pHG165 with 3.3-kb <i>PstI</i> fragment from PRS2000 carrying <i>pcaIJ</i> and upstream DNA; Ap ^r	This work
pHRP124	pT7-5 with 4-kb <i>HindIII-EcoRI</i> fragment from pPX202 carrying <i>pcaIJ</i> downstream of T7 promoter; Ap ^r	This work

^a *pcaE* has been redesignated *pcaI* and *pcaJ* to account for the two genes (35). The precise location of the mutation in PRS2241 is not known.

Lebanon, N.H.), and the University of Wisconsin Genetics Computer Group (UWGCG) software package, version 7.0 (10). The FASTA program (42) was used to search for similar sequences. Pairwise sequence comparisons were carried out by using the Gap program in the UWGCG software package, and multiple sequence alignments were done by using the Pileup program, also in the UWGCG package.

Primer extension. A synthetic oligonucleotide primer (5' TGTTCTGGAGTATGGTTGTCGGGCTGGCC 3') was end labelled with [γ -³²P]ATP (Amersham) by using T4

polynucleotide kinase (New England Biolabs) as described previously (1). It was partially purified from unincorporated radiolabel by using a Centricon 3 filter (Amicon, Danvers, Mass.). Previously described methods for RNA purification (1) and primer extension reactions (45) were used. Reverse transcriptase (Moloney murine leukemia virus; New England Biolabs) reactions were carried out at 42°C to reduce the frequency of nonspecific stops (1).

Bacterial transformations and conjugations. *E. coli* strains were transformed with plasmid DNA according to the

FIG. 2. Restriction maps of pPX202 and other constructions carrying the *pcaI* and *pcaJ* genes. The figure shows vectors used, the identity and orientation of relevant vector promoters, the restriction maps of the inserts, and the names of the plasmids. E, *EcoRI*; H, *HindIII*; N, *NruI*; P, *PstI*; S, *SalI*; Sm, *SmaI*; Xh, *XhoI*; Plac, *lac* promoter; T7, T7 promoter.

TABLE 2. β -Ketoacidate:succinyl-CoA transferase activities in complemented transferase mutants

Strain (plasmid)	Phenotype ^a	Growth condition(s) ^b	Transferase activity ^c (mU/mg)
PRS2000	Wild type	Succinate	3.6
		Benzoate + succinate	54.0
PRS2241	Ben ⁻ POB ⁻ TR ⁻	Succinate	4.5
		Benzoate + succinate	6.3
PRS2241(pHRP100)	Ben ⁺ POB ⁺ TR ⁺	Succinate	3.9
		Benzoate + succinate	71.0
PRS3004	Ben ⁻ POB ⁻ TR ⁻	Succinate	4.2
		Benzoate + succinate	7.3
PRS3004(pHRP100)	Ben ⁺ POB ⁺ TR ⁺	Succinate	3.0
		Benzoate + succinate	106.0

^a Ben, growth with benzoate; POB, growth with 4-hydroxybenzoate; TR, β -ketoacidate:succinyl-CoA transferase activity.

^b Cultures were grown in mineral medium with 10 mM succinate (uninduced) or 10 mM succinate plus 5 mM benzoate (induced).

^c Transferase activity is expressed as nanomoles of β -ketoacidate:CoA formed per minute per milligram of protein. Activities reported are averages of at least three assays of two independently prepared extracts.

method of Hanahan (18). Broad-host-range plasmids (pRK415 and derivatives) were mobilized from *E. coli* S17-1 into *P. putida* by patch matings on LB agar plates incubated overnight at 30°C. The resulting cell culture was suspended in mineral medium, washed once, and plated on appropriate selective media.

Measurement of enzyme activities. Cell extracts were prepared from *E. coli* or *P. putida* cultures (50 ml) that were harvested in the exponential phase of growth (A_{660} of 0.25 to 0.5). Cells were washed once with minimal medium, and pellets were stored at -20°C until just prior to disruption. Thawed cell pellets were resuspended in 1 ml of a solution of 50 mM sodium phosphate buffer (pH 6.8) and 1 mM dithiothreitol. Cell suspensions were disrupted by sonication, and cell debris was removed by centrifugation. The β -ketoacidate:succinyl-CoA transferase assay has been described previously (66). Briefly, the assay mix contained 10 mM β -ketoacidate, 400 μ M succinyl-CoA, and 40 mM magnesium ion in 200 mM Tris-HCl buffer, pH 8.0. After addition of cell extract, the increase in A_{305} (corresponding to the formation of the β -ketoacidate:CoA-Mg²⁺ complex) was measured. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μ mol of substrate to product in 1 min under the assay conditions used. Protein concentrations were determined by using the method of Bradford (4) with reagents from BioRad (Richmond, Calif.) and bovine serum albumin as a standard.

³⁵S labelling of *P. putida* gene products in *E. coli*. The method used to express the *P. putida* genes in *E. coli* was done as described previously (1, 55) with the following changes. Duplicate cultures of *E. coli* DH5 α (pGP1-2) harboring either pT7-5 or pHRP124 (Table 1) were grown as described previously (1). One set of cultures was treated with rifampin (200 μ g/ml, final concentration) for 20 min at 30°C prior to pulse-labelling with L-[³⁵S]methionine (Amersham) at room temperature for 5 min. Samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (1), and radiolabelled proteins were visualized by autoradiography (1).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned the accession number M88763.

RESULTS

Complementation of *P. putida* β -ketoacidate:succinyl-CoA transferase mutants. The plasmid pPX202 (Fig. 2) contains an 8.5-kb *Eco*RI fragment from *P. putida* cloned into pUC19. *E. coli* carrying pPX202 had been reported to express β -ketoacidate:succinyl-CoA transferase (27). We verified this expression by complementing two *P. putida* CoA transferase mutants as follows. The 8.5-kb *Eco*RI fragment from pPX202 was cloned into the broad-host-range plasmid pRK415. The resulting plasmid, designated pHRP100, was mobilized into PRS2241 and PRS3004, two previously described derivatives of PRS2000 that are unable to grow on either benzoate or 4-hydroxybenzoate and which have been shown to be defective in the synthesis of β -ketoacidate:succinyl-CoA transferase (27, 36). Plasmid pHRP100 restored the ability of both mutants to express β -ketoacidate:succinyl-CoA transferase (Table 2) and to grow on both benzoate and 4-hydroxybenzoate.

Localization of the *pcaI* and *pcaJ* genes. Several restriction fragments from pPX202 were subcloned into the vector pHG165 (54), and culture extracts of *E. coli* harboring the resulting plasmids were assayed for CoA transferase activity. The smallest subclone that expressed β -ketoacidate:succinyl-CoA transferase activity contained a 2.9-kb *Eco*RI-*Pst*I fragment (pHRP110; Fig. 2). CoA transferase activity was detected in *E. coli* cell extracts only when this 2.9-kb fragment was located downstream of the *lac* promoter of pHG165 (pHRP110; Table 3). No activity was detected when the fragment was cloned in the opposite orientation (pHRP111; Table 3). The lower activity in extracts of *E. coli* carrying pHRP110 compared with those carrying pPX202 is probably due to the fact that pHG165 is a lower-copy-number plasmid than pUC19 (54).

Identification of the *pcaI* and *pcaJ* gene products. The Tabor and Richardson T7 expression system (55) allows selective expression of cloned genes in *E. coli*. Genes are cloned downstream of a phage T7 promoter in the vector pT7-5, and a heat-inducible T7 RNA polymerase gene is supplied on a second plasmid (pGP1-2). Addition of rifampin just prior to pulse-labelling eliminates background *E. coli* protein synthesis (unlike *E. coli* RNA polymerase, T7 RNA

TABLE 3. β -Ketoadipate:succinyl-CoA transferase activities in *E. coli* DH5 α cell extracts

Plasmid	Plac orientation ^a	Transferase activity (mU/mg) ^b
pUC19		1.1
pPX202	+	63.0
pHG165		2.3
pHRP110	+	18.0
pHRP111	-	1.1

^a + indicates that *pcaI* and *pcaJ* are cloned in the correct orientation to be expressed from the *lac* promoter (Plac) of the vector; - indicates the opposite orientation.

^b Transferase activity is expressed as nanomoles of β -ketoadipyl-CoA formed per minute per milligram of protein. Activity of fully induced wild-type *P. putida* is in the range of 30 to 100 mU/mg. *E. coli* strains were grown in LB medium with 100 μ g of ampicillin per ml. Activities are averages of at least three assays of two independently prepared extracts.

polymerase is insensitive to rifampin). When the 4-kb *EcoRI-HindIII* fragment from pPX202 was subcloned into the T7 expression vector pT7-5 such that the phage T7 promoter was in the correct orientation to drive *pcaIJ* transcription (pHRP124, Fig. 2), two proteins with apparent molecular weights of 25,000 and 24,000 were produced following induction of the T7 RNA polymerase (Fig. 3). These proteins corresponded very closely in size to the reported sizes of the two subunits of purified *P. putida* β -ketoadipate:succinyl-CoA transferase, 25,300 and 24,200 Da (66).

Sequence analysis of the *pcaIJ* gene region. Computer analysis of the DNA sequence of the *pcaIJ* region (Fig. 4) revealed two open reading frames (ORFs) predicted to encode proteins with molecular weights of 24,400 and 22,500. Both ORFs were oriented in the same direction and were separated by only 8 bp, suggesting that the two genes may constitute an operon. By comparing N-terminal amino acid sequence data (66) with our deduced amino acid sequences, we identified each translational start site (Table 4). ORF1 (*pcaI*) encodes a polypeptide 231 amino acids in length and starts at nucleotide 744, with the unusual initiation codon UUG (16), which normally codes for leucine.

FIG. 3. Expression of the *pcaI* and *pcaJ* genes in *E. coli*. A T7 RNA polymerase expression system was used as described in Materials and Methods. An autoradiogram of an SDS-12% polyacrylamide gel loaded with heat-induced samples is shown. Lanes: 1 and 2, DH5 α /pGP1-2/pHRP124 (pT7-5 with *pcaIJ* gene region); 3 and 4, DH5 α /pGP1-2/pT7-5 (vector control); 2 and 3, rifampin treated; 1 and 4, no rifampin. The sizes (in kilodaltons) of protein standards are indicated on the right.

ORF2 (*pcaJ*) encodes a polypeptide of 213 amino acids and begins at nucleotide 1448, with AUG as the start codon. Putative ribosome binding sites were located 6 bp upstream of each ORF (Fig. 4). The previously reported amino acid composition of the native enzyme (66) also correlated well with our deduced amino acid sequences.

To determine the site of the Tn5 insertion in the CoA transferase mutant PRS3004 (27), cloned DNA from that strain was sequenced by using a primer complementary to the ends of Tn5. Additional primers complementary to *pcaI* DNA were used to extend and verify the sequence. The insertion was located in the second half of the *pcaI* coding region (after the G at position 1844 of the sequence, Fig. 4).

Codon usage. The *pcaIJ* region has a G+C content of 63%, consistent with the overall G+C content of *P. putida*, 62.5% (39). Codon usage is typical of *Pseudomonas* species (8, 58). With few exceptions, G or C was preferred in the third position for amino acids with multiple codons. As previously noted for other *Pseudomonas* genes (8, 17, 58), GGG (glycine) and CCC (proline) were avoided. Also, the use of leucine and arginine codons with C in the first position was favored. The preferred termination codon UGA (58) was also used in both ORFs.

Primer extension analysis. To determine the 5' end of the *pcaI* transcript, a 30-mer oligonucleotide primer complementary to nucleotides 700 to 729 (Fig. 4) was hybridized to total RNA purified from 4-hydroxybenzoate-grown PRS2000. The primer was extended with reverse transcriptase, and two major bands were consistently observed, one corresponding to the T at position 599 of the DNA sequence and one slightly weaker band corresponding to the C at position 600 (Fig. 5). A search of upstream DNA revealed a possible *E. coli* sigma-70-type -10 region (GATAAT), but the corresponding -35 region sequence exhibited very poor similarity to the *E. coli* consensus sequence. However, evidence indicates that *pcaI* is under positive control and that this type of promoter typically shows poor homology with the consensus sequence, especially in the -35 region (43).

Sequence comparisons. The predicted amino acid sequences were used to search the Swiss-Prot (release 20.0) and GenPept (release 70.0) data bases by using the FASTA program (42). Three sequences with significant similarity were identified: β -ketoadipate:succinyl-CoA transferase from *A. calcoaceticus* (29, 47), a putative subunit of the *Clostridium acetobutylicum* acetoacetyl-CoA:acetate/butyrate:CoA transferase (15), and pig heart succinyl-CoA:3-ketoacid transferase (31). Comparisons by using the Gap program in the UWGCG software package (10) gave the following results. The *P. putida* and *A. calcoaceticus* enzymes were 67% identical over the entire lengths of both subunits. The β subunits of each of these two enzymes were 43% identical to the putative CoA transferase subunit from *C. acetobutylicum*. Amino acid sequence alignments prepared by using the Pileup program of the UWGCG package (Fig. 6) showed that the bacterial α and β subunits align with the N and C termini of the pig heart protein, respectively, with high amino acid sequence identity (*PcaIJ* and the mammalian enzyme are about 44% identical) and very few gaps. In many cases, amino acid differences involve conserved substitutions.

DISCUSSION

We identified a 2.9-kb DNA fragment from *P. putida* that directed the synthesis of β -ketoadipate:succinyl-CoA trans-

1 GCGGTACCACGTTGTGGCCAGGTGAGGCGATGATGCTGCCGCGAGCATCGACGACAAACAGCTCGGAAAAGTCTTCGGCCTGTGCCAGCTTGTCCCGCA
 101 GCTGCTGTCCGGGTGAGCGAACCCCTTGGCGGAGTTGCTCCGCAAGTTCCGCCAAGTCTCCAGTGTCTGCACGCCCATTCATGCAACAAACGTACCGGGG
 201 TCTGGGCGATGGCTTCGAAGGTTTGTCTGATCTGGGTAATTCGCCGTTTCAAGTACATGCCAGGCCATTTCGGAGCTTTCGGGTTTACCGAACCGAG
 301 GCAGCCAGCCCTTCTGCTGGATGACAACTGCATGGAACCACTGGAAGCGACATTATCTTACCATACCGGACATCGGATGCAATCTGATAGCAAGTC
 EcoRI
 401 GCACGCCAACCGCGGAGCGGTGATTATGGGGAATTCAGTCATTGAATGCACAGCACTTGTGCAAGAAAGTTATGGCAGGCCCTGAATGCACCAACG
 +1
 501 AGGC CGCAATCACATGAAATCAGGCCCTTATCTGGAATGTGAAGTAACACCAGAACTGCTGCACATCGCACAAAGTTGATAATCGCAAAATTC
 601 CGCTAAACCCCTCTACTGTTGCCCTATCTTGGCGCAGCTATAGCGAAAGTCAGCAAAACAATTTAAGCGCTGATTGGCCCCATCGCCTCACACGCCGC
 701 GCCAGCCGACAAACCATACTCCAGGAAC ACCAGGAGCTCGCCTTGATCAATAAAACGTACGAGTCCATCGCCAGCGCGGTGGAAGGGATCAACGACGGT
pcal M I N K T Y E S I A S A V E G I T D G
 801 TGAACCATCATGGTTCGGTGGCTTCGGCACAGCCGCATGCCGTCGAGCTGATCGATGGCCTGATCGCAACCGGTGCGCGGACCTGACCATTATCAGCA
 S T I M V G G F G T A G M P S E L I D G L I A T G A R D L T I I S N
 901 ACAACGCCGCAACCGCGAGATCGGCCCTGGCCGCGCTGCTCATGGCGGCGAGTGTGCGCAAGGTGGTCTGCTCGTTCGCCGCGCAGTCCGACTCTACGT
 N A G N G E I G L A A L L M A G S V R K V V C S F P R Q S D S Y V
 1001 GTTCGACGAACTGTACCGCGCCGCAAGATCGAGCTGGAAGTGGTCCGCAAGGCAACCTGGCCGAGCGCATCGCCGCGAGCGGGTTCGGGCATTTGGTGG
 F D E L Y R A G K I E L E V V P Q G N L A E R I A A A G S G I G A
 1101 TTCTTCTCGCGACTGGCTACGGCACCTGTTGGCCGAAGTAAAGAAACCCGTGAAATCGACGCGCGCATGTAAGTGTGGAATGCCGCTGCAACCGG
 F F S P T G Y G T L L A E G K E T R E I D G R M Y V L E M P L H A D
 1201 ACTTCGCGCTGATTAAGGCGCACAGGGTGAACGTTGGGGCAACCTGACCTACCGCAAGCGCGCGCAACTTCGGCCCGATCATGGCCATGGCTGCCAA
 F A L I K A H K G D R W G N L T Y R K A A R N F G P I M A M A A K
 SalI SmaI
 1301 GACCCCATCGCCCGAGTTCGACAGGTTGTGCAACTCGGTGAATGGACCCGGAACACATCATCACCCCGGTATCTTCGTCCAGCGCGTGGTTCGCCGTT
 T A I A Q V D Q V V E L G E L D P E H I I T P G I F V Q R V V A V
 1401 TCCGCGCTGCCGCTTCTTCGATTGCCAAAGCTATCTGAGGCCCGCATGACCATCACAAAAAGCTCTCCCGCACCAGATGGCCAGCGCGTGGCCGC
 S G A A A S S I A K A I * *pcal* M T I T K K L S R T E M A Q R V A A
 1501 AGACATCCAGGAAGCGCGTACGTAAACCTGGGCATTGGCGCACCGACCTGGTGGCAACTACCTGGCGACAAGGAAGTGTCTGCACAGCGAGAAC
 D I Q E G A Y V N L G I G A P T L V A N Y L G D K E V F L H S E N
 1601 GGCCTGCTGGCGATGGGCCAAGCCCTGCGCGGGCGAGGAAGCATGACCTGATCAACGCCGCAACAGCAGCTACCCCTGCTTACCGGTGGCGCCT
 G L L G M G P S P A P G E E D D D L I N A G K Q H V T L L T G G A F
 1701 TCTTCCACACGCGGACTGCTTCTCGATGATGCGTGGCGGCCACCTGGACATCGCAGTACTGGCGCCTTCCAGGTGTGCGTCAAGGGCGACCTGGCCAA
 F H H A D S F S M M R G G H L D I A V L G A F Q V S V K G D L A N
 1801 CTGGCACACCGGTGCCGAAGGTTGATCCCGGCGTAGCGGCGCTGAGCACTCGCCACCGCGCTCGCCAAAGTGTTCGTGATGATGGACCACTGACC
 W H T G A E G S I P A V G G A M D L A T G A R Q V F V M M D H L T
 1901 AAGACCGCGCAAGCAAGCTGGTGGCGAGTGCACTACCGCTGACCGGCATTGCGTGGCTGAGCCGATCTACACCGAAGTGGCGTACTGGAAGTGA
 K T G E S K L V P E C T Y P L T G I A C V S R I Y T D L A V L E V T
 2001 CGCCTGAAGGGTGAAGTGGTAGAAATCTGCGCGGATATCGACTTTGACGAGTTGCGAAGCTGAGCGGTGTGCCGCTGATCAAGTATCGCGGGAAC
 P E G L K V V E I C A D I D F D E L Q K L S G V P L I K *
 NruI
 2101 GGGGGCAAGCGCCCGCTTCCGATGTTGCATGGCTACTGTGGAACTGCCATTGCGCGGCGCTCGACTGCCCATTTTGTGGAGCGGCGCTTGTTCGGG
 2201 AAACTGGCAAGACGCCCCAGCAACAGTTGATGTAACCCCAAAATCCGGGGGCGCTTTGCGGCAGGACTAGCCACGCTGGCATCACAGATGCTCAGA
 2301 CTCGCCAGCGACGGTCCAACATCAGCCCTAGAAATCCACCTTGCCTCCGACCGGCGCTTGACAGTGTCTCCGCTGGCTTTGGCCCTCAAGCCGGCGGCTCTC
 2401 GAGCCAAAGGTGCGCTTGGTGGGCGGCGCTTCTTCTCGGTCTTGGCGGCGAGTATCAACTCGGCAAGACGCGCATGCGTTCAGCACGGTTCGAT
 2501 CTTGAGTGGGTATTTGCTGGGCTTGTGATGATCAACACACCGTCCGCGGTGATGCGACTGTACGACGCGCAGCAGCCGCTCTTGTAGAACTCGGGCAA
 2601 TGAAGAGGCGCGATGTGGAAGCGCAGGTGCACGCGCTGGACACCTTGTGACATTCTGCCACCCGCGCTTGGCGCGGATGTAGGTGAGTTGATT
 2701 TCGGCATCCGGCAGATGCACGTTATTGGAGATGGTCAGCATGGCATGGCCCTTGTGATTGCGGCCATTATCACAGCTTGGCCGCGCGCCCATAGAACAA
 2801 TGACAGATTCTTGTATCCCCAACAGATGATGCTATCTGTGTCGAAAAATCAGAATTTTTCGCTTCTTGTAGGGCATTTCCCAAGATAGCTGTG
 2901 CCGCCTATTTTCGCTGCGGCTGCCCTATTGGGCTCTAGTCTGGCGCGGTGCTGCTCATCAGCGGCAGCGCTTTGACGGGCGGTAAACGGTATATAGGT
 3001 TCTGCGGCGCCCTCAATGGCGGCTGCGCGTGGCAGCGCTTGGCTGCGCGGAACTTCTATATCCGCGGCTGTGCAACCCACGACAGCTGCCACCTAA
 3101 CCTGTTTGAACGCGGATCGGCGCAGCTATCTGGAGATATAGGAATGTTAAAGATGGTCCCGACCCACCCACAGCTCCCTCGAAGACACTC
 3201 TCATCAAAGCCGCGAATATCCGCTCTGCGGCTAGCGATTTCGCAACAGCGATGCTGATG

FIG. 4. Nucleotide and deduced amino acid sequences of the *pcalI* gene region from *P. putida* PRS2000. The transcriptional start site is labelled +1, and the boxed sequence is the site of primer binding for primer extension analysis (see Fig. 5 and Materials and Methods). Translational start sites and potential ribosome binding sites (49) are underlined, and relevant restriction sites are underlined and labelled above the DNA sequence. The arrow indicates the site of the Tn5 insertion in the β -ketoadipate:succinyl-CoA transferase mutant strain PRS3004 (see the text).

TABLE 4. N-terminal amino acid sequence of the β -ketoadipate:succinyl-CoA transferase subunits

Residue no.	N-terminal amino acid sequence ($\alpha + \beta$) ^a	Predicted amino acid sequence ^b	
		α	β
1	Thr + Met	Met	(Met) ^c
2	Ile	Ile	Ile
3	Asn + Thr	Asn	Thr
4	Lys	Lys	Lys
5	Thr + Lys	Thr	Lys
6	Tyr + Leu	Tyr	Leu

^a N-terminal amino acid sequence of the native enzyme as reported previously (66).

^b Predicted from the DNA sequence of the *pcaI* and *pcaJ* genes.

^c The initial methionine is presumably cleaved in the mature peptide (66).

ferase in *E. coli* when cloned in a vector downstream of the *lac* promoter. The two genes encoding β -ketoadipate:succinyl-CoA transferase are closely linked and may constitute an operon, although this has not been demonstrated directly. The fact that β -ketoadipate:succinyl-CoA transferase was not expressed in *E. coli* unless adjacent to the *lac* promoter suggests either that *E. coli* did not recognize the *P. putida* promoter or that the promoter is not on the 2.9-kb fragment. A number of reports have indicated that *Pseudomonas* genes are not well expressed in *E. coli* (14, 22, 56). The fact that *pcaI* and *pcaJ* appear to be positively controlled by a specific activator protein probably not present in *E. coli* may account for the poor expression.

This is the first detailed report of a bacterial CoA trans-

FIG. 5. Autoradiogram showing the *pcaI* transcription start site. Primer extension was carried out as described in Materials and Methods. The sample (lane 1) was loaded on a 6% sequencing gel next to the dideoxy sequencing ladder (lanes labelled G, A, T, and C) generated by using the same primer.

PcaI	MAALTLLSSR	LRLCASAYRS	GGAWSQCCAG	YFSTSTRRHT	MIN	KTYESIASAV	13
CatI					MID	KSATLITEAL	
PigTr						KFYTDAVEAV	50
PcaI	EGITDGSTIM	VGGGTAGMP	SELIDGLIAT	GARDLTIISH	MAONGEIGLA		63
CatI	SQTHDQATIL	IGGFTAGQP	AELIDGLIEL	GRKNLTIVSN	MAONGDYOLA		
PigTr	KDIPNGATVL	VGGFGLCGIP	ENLIGALLKT	GVKELTAVSN	MAOVNDFGLG		100
PcaI	ALLMAGSVRK	VVCSFPRQSD	SYVFDELYRA	GKIELEVVPQ	GNLAERIAAA		113
CatI	KLKRTGAVKK	IICSPRRQAD	SYVFDELYRA	GKIELEIVPQ	GNLACRIQAA		
PigTr	LLQSKQIKR	MISSYV..GE	NAEFERQYLA	GELEVELTPQ	GTLAERIRAG		148
PcaI	GSQIGAFFSP	TOYOTLLAEG		KE	TREIDORMYV		145
CatI	GMGLGPIYTP	TGFTLLAEG		KP	TLNFDGKDYV		
PigTr	GAGVPAPYTS	TOYOTLVQEG	GSPIRYNKDG	SIATASKPRE	VREFNQHFPI		198
PcaI	LEMPLHADFA	LKAKHKDRW	GNLTYRKAAR	NFGPIDAMAA	KTAIAQVDQV		195
CatI	LENPIKADFA	LKAYKGRW	GNLVYRKSAR	NFGPIDAMAA	NVTIAQVSEV		
PigTr	LEEAIRGDFA	LVKAWKADQA	GNVTFRKSAR	NFNLPCKKAA	ETTVEVEVEI		248
PcaI/J	VELGELDPKH	IITPGIFVQR	VVAVSGAAAS	SIAKAI	MTITK		5
CatI/J	VALGELDPEN	VVTPGIFVQH	VVFVQSTPAS	AAP	MSYH		
ClosTr					MINDK		
PigTr	VDIGSFAPED	IHIPKIYVHR	LVKGEKYEKR	IERLSVRKEE	DVKTRSGKLG		298
PcaJ	KLSRTEMAQR	VAADIQEGAY	VNLGIGAPTL	VANYL.GDKE	VFLHSENGLL		54
CatJ	KLTRDQIAQR	VAQDIPEGSY	VNLGIGLPTK	IASYLPADKQ	VFLHSENGLL		
ClosTr	NLAKEIIAKR	VARELKNQQL	VNLGVGLPTM	VADYIPKNFK	ITPQSENGIV		
PigTr	DNVRERIIKR	AALFEDOMY	ANLIGIPIPL	ASNFIISPNT	VHLQSENGIL		348
PcaJ	GMQSPAPGE	EDDDLINAGK	QHVTLTGGGA	FFHHADSFMS	MROGHLDIAV		104
CatJ	AFQPPPAAGE	EDPELINAGK	EYVTMLGGCC	FFHHGDSFAM	MROGHLDICV		
ClosTr	GMQASPKINE	ADKDVVWAGG	DYTTVLDPGT	FDDSSVSFSL	IROGHVDVTV		
PigTr	GLQPYPLQNE	VDADLINAGK	ETVTVLPQAS	YFSSDESFAF	IROGHVNLTM		398
PcaJ	LGAPQVSVKG	DLANWHTGAE	GSIPAVGGAM	DLATGAR.QV	FVMMDLTKTK		153
CatJ	LGAPQIAANG	DLANWHTGAP	DAIPSVGGAM	DLAVGAK.KV	FVTTDBVTKK		
ClosTr	LGALQVDEKG	NLANWIVPGK	.MLSGMGGAM	DLVNGAK.KV	IIAMRHTNKG		
PigTr	LGAMQVSKYG	DLANWIMPGK	.LVKGMGGAM	DLVSSAKTKV	VVTMEBSAKG		447
PcaJ	GESKLVPPECT	YPLTGIACVS	RIYTDLAVAL	VTP.EGLKVV	EICADIDFDE		202
CatJ	GEPKIVAELE	YPATGQKQVD	RIYTDLCIID	VVP.EGLKVI	EKVEGLSFEE		
ClosTr	QP.KILKKCT	LPLTAKSQAN	LIVTELGVIE	VI..NDGLLT	EINKNTTIDE		
PigTr	NAHKIMEKCT	LPLTGKQCVN	RIITEKAVFD	VDRKGLTLI	ELNEGLTVDD		497
PcaJ	LQKLSGVPLI	K			213		
CatJ	LQRLTGATLI	DATQG					
ClosTr	IRSLTAADLL	ISNELRPMVA					
PigTr	IKKSTGCDA	VSPKLIPMQQ	VTT				520

FIG. 6. Alignment of the deduced amino acid sequences of the CoA transferase subunits from *P. putida* (PcaI and PcaJ), *A. calcoaceticus* (CatI and CatJ), the pig heart monomer (PigTr), and the one available subunit from *C. acetobutylicum* (ClosTr). Identical residues among all aligned proteins are in bold type. The conserved active site glutamate is indicated by an E above the sequence. The glycine cluster of each protein is underlined. Numbers refer to the amino acid residues of the *P. putida* CoA transferase (PcaI and PcaJ, top lines) and the pig heart CoA transferase (bottom lines). The arrow indicates the end of the pig heart protein mitochondrial signal sequence.

ferase sequence. By analyzing our sequence together with the recently published pig heart CoA transferase sequence (31), the *A. calcoaceticus* sequence (47), and the partial sequence from *C. acetobutylicum* (15), several important points can be made about the evolution and structure-function relationships of CoA transferases.

CoA transferases from very diverse organisms show remarkable amino acid sequence conservation (Fig. 6). The β subunit from *C. acetobutylicum*, a gram-positive organism, is very similar to the β subunits of the two gram-negative bacteria. The α subunits of the prokaryotic proteins align with the N-terminal half of the mammalian protein, the β subunits align with the C-terminal portion, and strong sequence similarity is seen throughout the length of the alignment with very few gaps. The pig heart enzyme is located in the mitochondrion and has a typical N-terminal mitochondrial signal sequence that is presumably cleaved (31) at a site approximately corresponding to the N terminus of the bac-

terial α subunits (Fig. 6). The major gap in the alignment is located in a region of the pig heart protein that is highly hydrophilic (31) and falls between the alignments of the bacterial α and β subunits (Fig. 6). This region of the pig heart protein is susceptible to a proteolytic event (31, 32, 63) that cleaves the protein into two fragments but does not affect catalytic activity (31, 32). The pig heart CoA transferase thus appears to consist of an N terminus corresponding to the α subunits and a C terminus corresponding to the β subunits of the bacterial enzymes, suggesting that a gene fusion probably occurred at some time in the evolution of CoA transferases. Bacterial CoA transferases with homodimeric structures similar to that of the pig heart enzyme have been purified from *Peptostreptococcus elsdenii* (57) and *Clostridium aminobutyricum* (46). However, the sequences of these proteins have not been determined.

Enzyme-CoA intermediates have been detected in the reactions of several CoA transferases (23, 51, 57). In the *E. coli* and pig heart enzymes, a glutamate residue appears to be the site of CoA thioesterification (23, 26, 51, 63). Amino acid sequence alignments of the pig heart and the three bacterial CoA transferases revealed two conserved glutamate residues (corresponding to positions 344 and 358 of the pig heart enzyme [Fig. 6]) in the region proposed to contain the site of thioesterification (26, 31). Williams (63) determined the amino acid sequence of a short CoA-peptide fragment of the pig heart enzyme-CoA intermediate and located the site of CoA modification directly. Comparison of his peptide sequence to the sequences presented here allowed us to identify glutamate-344 of the pig heart sequence as the site of thioesterification (Fig. 6). The sequence alignments indicate that glutamate-50 of PcaJ is the probable site of CoA thioesterification in the *P. putida* enzyme.

Thiol-modifying reagents have been shown to inactivate CoA transferases from pig heart (60) and various bacterial sources, including the *P. putida* and *A. calcoaceticus* enzymes (67), suggesting the presence of an active site cysteine residue. According to the Pileup alignment (Fig. 6), there are no conserved cysteines that occur in all of the available sequences, suggesting that an essential active site cysteine is not necessarily a general feature of CoA transferases.

Glycine clusters are part of ADP-binding $\beta\alpha\beta$ folds (61) that have been implicated in binding ADP-containing substrates or cofactors such as NAD or FAD. Williams (63) noticed the presence of a glycine cluster in the N-terminal amino acid sequence of the pig heart enzyme, with strong similarity to the consensus sequence described by Wierenga et al. (61). He suggested that it may be the first example of a glycine cluster involved in binding the ADP moiety of CoA. Both bacterial β -ketoacyl-CoA transferases have glycine clusters in the α subunit (Fig. 6), suggesting that this part of the protein may be important to the function of CoA transferases by binding CoA.

This work not only sets the stage for a detailed molecular analysis of the *pcaIJ* gene region but also targets specific amino acid residues for future site-directed mutagenesis studies to directly identify catalytically important regions of the CoA transferases.

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