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Identification and Molecular Characterization of the *aco* Genes Encoding the *Pelobacter carbinolicus* Acetoin Dehydrogenase Enzyme System

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Use of oligonucleotide probes, which were deduced from the N-terminal sequences of the purified enzyme components, identified the structural genes for the α and β subunits of E1 (acetoin:2,6-dichlorophenolindophenol oxidoreductase), E2 (dihydrolipoamide acetyltransferase), and E3 (dihydrolipoamide dehydrogenase) of the *Pelobacter carbinolicus* acetoin dehydrogenase enzyme system, which were designated *acoA*, *acoB*, *acoC*, and *acoL*, respectively. The nucleotide sequences of *acoA* (979 bp), *acoB* (1,014 bp), *acoC* (1,353 bp), and *acoL* (1,413 bp) as well as of *acoS* (933 bp), which encodes a protein with an M_r of 34,421 exhibiting 64.7% amino acid identity to the *Escherichia coli lipA* gene product, were determined. These genes are clustered on a 6.1-kbp region. Heterologous expression of *acoA*, *acoB*, *acoC*, *acoL*, and *acoS* in *E. coli* was demonstrated. The amino acid sequences deduced from *acoA*, *acoB*, *acoC*, and *acoL* for E1α (M_r, 34,854), E1β (M_r, 36,184), E2 (M_r, 47,281), and E3 (M_r, 49,394) exhibited striking similarities to the amino acid sequences of the components of the *Alcaligenes eutrophus* acetoin-cleaving system. Homologies of up to 48.7% amino acid identity to the primary structures of the enzyme components of various 2-oxo acid dehydrogenase complexes also were found. In addition, the respective genes of the 2-oxo acid dehydrogenase complexes and of the acetoin dehydrogenase enzyme system were organized very similarly, indicating a close relationship of the *P. carbinolicus* acetoin dehydrogenase enzyme system to 2-oxo acid dehydrogenase complexes.

Whereas much is known about the synthesis of 3-hydroxy-2-butanone (acetoin or acetylmethylcarbinol), and although it has been known for over 65 years (86) that many bacteria are able to grow on acetoin, little is known about its catabolism. For *Acinetobacter calcoaceticus*, a cyclic pathway for the degradation of 2,3-butanediol, acetoin, and diacetyl to acetate was postulated (42). In *Bacillus subtilis* and *Alcaligenes eutrophus*, this 2,3-butanediol cycle is not present since mutants lacking 2,3-butanediol dehydrogenase, which is one of the key enzymes of the cycle (48, 74), grew on acetoin. Instead, both aerobic bacteria catalyze a direct oxidative cleavage of acetoin (21, 49), as do acetoin-degrading bacteria that rely on a fermentative metabolism, such as *Streptococcus faecalis* (19) and *Pelobacter carbinolicus* (57).

The substrate range for growth of the strict anaerobe *P. carbinolicus* Gra Bd 1 is restricted to acetoin, methylacetoin, 2,3-butanediol, and ethylene glycol (57, 71). During fermentation, acetoin is degraded to equimolar amounts of acetate and ethanol (71). Recent studies revealed a thiamine pyrophosphate-, coenzyme A-, and NAD-dependent cleavage of acetoin to acetaldehyde and acetyl coenzyme A; in *P. carbinolicus*, this key catabolic reaction is catalyzed by the acetoin dehydrogenase enzyme system (56–58). This reaction was reconstituted in vitro by combination of the purified components of the enzyme system, i.e., E1 (acetoin:2,6-dichlorophenolindophenol [DCPIP] oxidoreductase), E2 (dihydrolipoamide acetyltransferase), and E3 (dihydrolipoamide dehydrogenase). The formation of these proteins is induced during growth on acetoin not only in *P. carbinolicus* (56–58) but also in the other anaerobic bacteria *Pelobacter venetianus*, *Pelobacter acetylenicus*, *Pelobacter propionicus*, *Acetobacterium carbinolicum*, and *Clostridium magnum* (50, 56). The three enzyme components of the anaerobic acetoin dehydrogenase enzyme system are similar to the corresponding components of 2-oxo acid dehydrogenase multienzyme complexes (56, 63–65). The difference between oxidative acetoin cleavage and oxidative decarboxylation of 2-oxo acids seems to be based mainly on the different substrate specificities of the respective E1 components (56).

The E1 component, which is a tetraheteromer of two different subunits, and the E2 component from *P. carbinolicus* resemble the acetoin:DCPIP oxidoreductase and the dihydrolipoamide acetyltransferase, respectively, of the *A. eutrophus* acetoin-cleaving system (56, 65). In *A. eutrophus*, the structural genes for these proteins (*acoA*, *acoB*, and *acoC*) as well as for a transcriptional activator protein (*acoR*) and for a protein of unknown function (*acoX*) are clustered (45, 65). The goal of the present study was to identify and characterize the structural genes for the components of the acetoin dehydrogenase enzyme system of *P. carbinolicus*. The molecular data obtained will enable much more detailed comparisons with the acetoin-cleaving system of *A. eutrophus* and with 2-oxo acid dehydrogenase complexes.

MATERIALS AND METHODS

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

Growth of bacteria. *P. carbinolicus* cells were grown anaerobically in a mineral salts medium supplemented with 0.5% (wt/vol) filter-sterilized acetoin (56). *Escherichia coli* cells were grown at 37°C in Luria-Bertani (LB) (68) or lipoate-deficient medium as described previously (32). Growth was monitored spectroscopically by using a Klett-Summerson photometer.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference(s) or source[a]
P. carbinolicus Gra Bd 1	Wild type, strictly anaerobic, prototrophic	71 DSM2380
E. coli		
BHB2688	N205 *recA*(λ *imm*^{434} cIts857 *b2 red3 E*am4 *S*am7/λ)	38, 39
BHB2690	N205 *recA*(λ *imm*^{434} cIts857 *b2 red3 D*am15 *S*am7/λ)	38, 39
S17-1	*recA*; harboring the *tra* genes of plasmid RP4 in the chromosome, *proA thi-1*	73
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- *lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10*(Tet)]	12
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lacU169* (φ80 *lacZ*Δ*M15*)	24
JRG26 (W1485-lip2)	*lip-2 supE42* λ^-	36 CGSC5782
JRG33 (AT1325 lip9)	Δ(*gpt-proA*)*62 lacY1 lip-9 supE44*? *galK2* λ^- *purB15 hisG4*(Oc) *rpsL35 xyl-5 mtl-1 thi-1*	36 CGSC4286
Plasmids		
pVK100	Cosmid; Tc^r Km^r	43
pBluescript KS$^-$ and pBluescript SK$^-$	Ap^r *lacPOZ′*, T7 and T3 promoters	Stratagene
pUC8, pUC8-1, and pUC8-2	Ap^r *lacPOZ′*	27
pVKH	14.6-kbp insert from *P. carbinolicus* DNA in pVK100 containing *acoB*, *acoC*, *acoS*, *acoL*, and ORF5	This study
pKSH48 and pKSH48-1	4.8-kbp insert from pVKH in pBluescript KS$^-$ containing *acoB*, *acoC*, and *acoS* colinear and antilinear to *lacZ′*, respectively	This study
pKSH64	6.4-kbp insert from pVKH in pBluescript KS$^-$ containing ORF5 colinear to *lacZ′*	This study
pKSE67	6.4-kbp insert from *P. carbinolicus* DNA in pBluescript KS$^-$ containing ORF7, *acoA*, and *acoB* antilinear to *lacZ′*	This study
pUC8BP25, pUC8-1BP25, and pUC8-2BP25	2.5-kbp insert from pKSE67 in pUC8, pUC8-1, and pUC8-2, respectively, containing *acoA* and *acoB* colinear to *lacZ′*	This study
pUC8BN13	1.3-kbp insert from pUC8BP25 in pUC8 containing *acoA*	This study
pUC8BP25Δ*Sal*I	2.4-kbp insert from pUC8BP25 in pUC8 containing *acoB*	This study
pUC8BP25Δ*Sst*II	2.1-kbp insert from pUC8BP25 in pUC8 containing *acoB*	This study
pKSH48ΔExoIII	1.5-kbp insert from pKSH48 containing *acoS*	This study
pSKD12	1.2-kbp insert from pKSH48 in pBluescript KS$^-$ containing *acoS* antilinear to *lacZ′*	This study
pKSHP12	1.2-kbp insert from pKSD12 in pBluescript KS$^-$ containing *acoS* colinear to *lacZ′*	This study

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. CGSC, *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, Conn.

Preparation of cell extracts. *P. carbinolicus* or *E. coli* cells were disrupted by a twofold passage through a French press at 96 MPa or by sonication (for 1 min/ml of cell suspension with an amplitude of 14 μm) by using an MSE (150-W) ultrasonic disintegrator, respectively. The resulting extract was referred to as cell extract. Soluble cell fractions were obtained as supernatants from 50-min centrifugations at 100,000 × *g* and 4°C. Protein was determined as described by Lowry et al. (51).

Determination of enzyme activities. E1 (acetoin:DCPIP oxidoreductase; acetoin dehydrogenase, thiamine pyrophosphate dependent), E2 (dihydrolipoamide acetyltransferase; EC 2.3.1.12), and E3 (dihydrolipoamide dehydrogenase; EC 1.8.1.4) were determined spectrophotometrically as described previously (56) by monitoring the initial rates of DCPIP reduction at 578 nm, formation of *S*-acetyldihydrolipoamide at 240 nm, and oxidation of NADH at 365 nm, respectively.

Electrophoretic methods. Sodium dodecyl sulfate (SDS)- and 2-mercaptoethanol-denatured proteins were separated in 11.5% (wt/vol) polyacrylamide gels in Tris-glycine buffer (25 mM Tris, 190 mM glycine, 0.1% [wt/vol] SDS) as described by Laemmli (46). Proteins were stained with Serva Blue R. DNA fragments were separated in 0.8% (wt/vol) agarose gels in Tris-borate-EDTA buffer (50 mM Tris, 50 mM boric acid, 1.25 mM Na_2EDTA [pH 8.5]) (68). DNA bands were stained with ethidium bromide and visualized on a UV transilluminator.

Immunological methods. Antisera against purified E1 were raised in a rabbit as described previously (56). Immunoglobulins were purified from the serum by chromatography on protein A–Sepharose CL-6B (37). Double-immunodiffusion tests were performed in 1% (wt/vol) agarose gels in 50 mM diethylbarbiturate-acetate buffer (pH 8.2) as described previously (55). For Western blot (immunoblot) analysis of soluble cell fractions, electroblots were prepared with a semidry Fast Blot B33 (Biometra GmbH, Göttingen, Germany) at constant 5 mA/cm^2 as instructed by the manufacturer, and antigenic proteins were stained with anti-rabbit immunoglobulin G (IgG)–alkaline phosphatase conjugate (81).

Isolation and manipulation of DNA. Total genomic DNA from *P. carbinolicus* was obtained as described by Marmur (52). Plasmid DNA was isolated from *E. coli* crude lysates by the alkaline extraction procedure (7). Restrictions of DNA were performed with various restriction endonucleases under the conditions described by Sambrook et al. (68) or the manufacturer. For introducing unidirectional deletions, the Stratagene Bluescript Exo/Mung DNA system was used according to the instructions of the manufacturer. DNA fragments were isolated from agarose gels by using the Geneclean kit (84). All other DNA-manipulating enzymes were used as described by the manufacturer.

Transformation. For transformation, *E. coli* was grown in LB medium containing 10 mM $MgCl_2$ and 10 mM $MgSO_4$ (24).

Competent cells were prepared and transformed by the calcium chloride procedure (68).

Construction of *P. carbinolicus* genomic libraries. *P. carbinolicus* genomic DNA was partially digested with *Hin*dIII and ligated to pVK100. The products were packaged with phage λ coat proteins by using an in vitro packaging kit, which was prepared from *E. coli* BHB2688 and BHB2690 (38, 39, 43), and transfected into *E. coli* S17-1. For the preparation of a partial *Eco*RI library, fragments with desired length from totally digested genomic DNA were separated electrophoretically and were isolated from the agarose gel by using the Geneclean kit. The fragments were subsequently ligated with *Eco*RI-restricted plasmid pBluescript KS^- and transformed into *E. coli* XL1-Blue.

Synthesis of oligonucleotides. Synthesis of oligonucleotides was performed in 0.2-μmol portions from deoxynucleoside phosphoramidites (3) with a Gene Assembler Plus apparatus (Pharmacia-LKB Biotechnology, Uppsala, Sweden) according to the instructions of the manufacturer. Release of the oligonucleotides from the supports and removal of protection groups were achieved by 15 h of incubation at 55°C in 32% (vol/vol) ammonia solution. Oligonucleotides were purified by gel filtration in NAP-5 columns. The following oligonucleotides were synthesized according to the N-terminal amino acid sequences of purified components of the *P. carbinolicus* acetoin dehydrogenase enzyme system (wobble positions are shown in brackets; equimolar fractions of the different bases were used at these positions): probe Iα, 3′-TT[CT]TAC[TG]C[ACGT]CT[CT]TA[AGT][GT]C-5′ (corresponding to amino acid positions 16 to 21 of E1α); probe Iβ, 3′-TA[AGT]TACAA[AG]TT[CT]CT[AG]CG-5′ (corresponding to amino acid positions 5 to 10 of E1β); probe II, 3′-TG[ACGT]TACCT[CT]CT[CT]CC[ACGT][AG]A-5′ (corresponding to amino acid positions 17 to 22 of E2); and probe III, 3′-CT[AG]CT[CT]TA[AGT]AA[AG]CT[AG][AG]A-5′ (corresponding to amino acid positions 2 to 7 of E3). For DNA-DNA hybridizations, oligonucleotides were labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP (68).

DNA-DNA hybridization. Transfer of denatured DNA from agarose gels or cell colonies to nylon membranes, hybridization with ^{32}P- or biotin-16-dUTP-labeled probes, and detection of DNA were performed by standard procedures (22, 47, 68).

DNA sequence analysis. Plasmids pKSH48 and pKSH48-1 were digested with *Apa*I and *Xho*I and subsequently treated with exonuclease III and mung bean nuclease to introduce unidirectional deletions. By using pKSH48, pKSH48-1, and their deleted derivatives as template DNAs, and by using universal and reverse primers, the nucleotide sequence of the 4.8-kbp *Hin*dIII fragment was determined. In addition, parts of pKSH64 and pKSH34 were also sequenced by using the primer-hopping strategy with synthetic oligonucleotides as primers (77). DNA sequences were determined from double-stranded alkali-denatured plasmid DNA by using the dideoxy-chain termination method (69) with the T7 polymerase sequencing kit and [α-^{35}S]dATP as instructed by the manufacturer (Pharmacia-LKB Biotechnology). Synthetic oligonucleotides were used as primers. Products of the sequencing reactions were separated in 6% (wt/vol) polyacrylamide gels in Tris-borate-EDTA-urea buffer (100 mM Tris, 83 mM boric acid, 1 mM Na_2EDTA, 42% [wt/vol] urea) in an S2 sequencing apparatus (GIBCO/BRL GmbH, Eggenstein, Germany) and visualized on X-ray films.

Analysis of sequence data. Nucleic and amino acid sequences were analyzed with computer programs from the Genetic Computer Group sequence analysis software package (17).

Insertion of deletions into *acoA* and *acoB*. Deletions in *acoB* were introduced by restriction with *Nar*I, for which two restriction sites were located in *acoB* and one was located in the vector DNA downstream of *lacZ′*. Religation resulted in plasmid pUCBN13. To delete *acoA*, pUC8BP25 was digested either with *Sal*I, resulting in the loss of 85 bp from the central region of *acoA* with a concomitant downstream frameshift, or with *Sst*II, resulting in a 396-bp deletion from the central region of *acoA* including the thiamine pyrophosphate-binding region. Religations of these derivatives resulted in plasmids pUC8BP25Δ*Sal*I and pUC8BP25Δ*Sst*II, respectively.

Chemicals. Restriction endonucleases, biotin-16-dUTP, the nick translation kit, the DNA detection kit, T4 DNA ligase, S1 nuclease, anti-rabbit IgG–alkaline phosphatase conjugate, and the substrates used for enzyme assays were obtained from GIBCO/BRL, C. F. Boehringer & Soehne (Mannheim, Germany), or Stratagene Cloning Systems (San Diego, Calif.). RNase-free DNase, phosphoramidites, NAP-5 columns, and protein A–Sepharose CL-6B were obtained from Pharmacia-LKB Biotechnology, radioisotopes were from Amersham/Buchler (Braunschweig, Germany), and polyvinylidene fluoride membranes were obtained from Millipore (Bedford, Mass.). All other chemicals were from E. Merck AG (Darmstadt, Germany), Fluka Chemie (Buchs, Switzerland), Serva Feinbiochemica (Heidelberg, Germany), or Sigma Chemie (Deisenhofen, Germany).

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported in this study have been submitted to the National Center for Biotechnology Information under accession number U01100.

RESULTS

Identification and cloning of *acoA*, *acoB*, *acoC*, and *acoL*. Oligonucleotide probe Iβ, which was synthesized on the basis of the N-terminal amino acid sequence of the E1β component of the *P. carbinolicus* acetoin dehydrogenase enzyme system, was used to screen a *P. carbinolicus Hin*dIII genomic library in the cosmid pVK100 by colony hybridization. From approximately 1,500 colonies, three clones gave strong signals. Each hybrid cosmid (designated pVKH) contained three genomic *Hin*dIII fragments of 6.4, 3.4, and 4.8 kbp, respectively. The latter fragment gave hybridization signals with any of the four probes (Fig. 1), indicating that the corresponding N-terminal regions of both subunits of the E1 component, as well as the E2 and E3 components of the acetoin dehydrogenase enzyme system, were encoded on the 4.8-kbp *Hin*dIII fragment. The 4.8-kbp *Hin*dIII fragment and the 6.4- and 3.4-kbp *Hin*dIII fragments, which are presumably linked in the genome, were ligated to pBluescript KS^-, resulting in plasmids pKSH48, pKSH64, and pKSH34 (Fig. 2A).

Nucleotide sequences of *acoA*, *acoB*, *acoC*, *acoL*, and adjacent regions. The nucleotide sequence of a region of 7,251 bp, which was obtained from both strands of pKSH48, pKSH64, and pKSH34, revealed 50 open reading frames (ORFs) with a minimum length of 120 bp (Fig. 2B). The sequenced region exhibited a G+C content of 55.3 mol%, which was close to 52.3 mol% G+C determined for the total genomic DNA (71). Knowledge of the N-terminal amino acid sequences of the acetoin dehydrogenase enzyme system components allowed identification of the corresponding structural genes. The amino acid sequence deduced from the 5′ region of ORF1 was identical to the N-terminal amino acid sequence of purified E2 (56) except for two previously uncertain amino acid residues and the terminal methionine residue, which was removed by

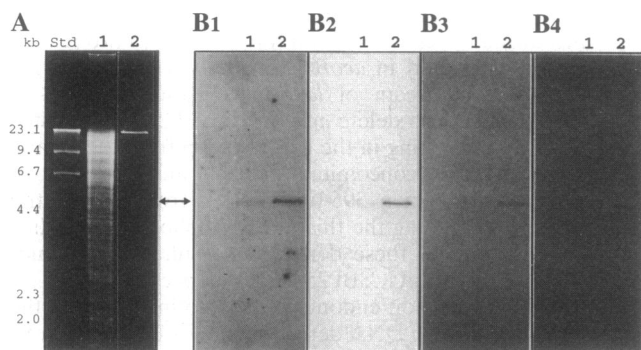

FIG. 1. Localization of *P. carbinolicus aco* genes. *Hin*dIII digests of hybrid plasmid pVKH and of *P. carbinolicus* genomic DNA were separated in 0.8% (wt/vol) agarose gels, blotted onto nylon membranes, and hybridized with the ^{32}P-labeled oligonucleotide probe Iα, Iβ, II, or III (see Materials and Methods). (A) Agarose gel stained with ethidium bromide; (B) autoradiograms of blots hybridized with probe Iα (B1), Iβ (B2), II (B3), or III (B4). Lanes: 1, *Hin*dIII-restricted *P. carbinolicus* genomic DNA; 2, *Hin*dIII-restricted pVKH; Std, λ *Hin*dIII standard. The 4.8-kbp fragment, which gave signals with any of the four probes, is indicated by an arrow. The sizes of standard fragments are indicated at the left.

posttranslational modification (Fig. 3). The latter occurred also for the N termini of E3 and of E1β, which matched exactly the amino acid sequences deduced from the 5′ regions of ORF2 and ORF6, respectively. The translational start codons of these genes were preceded by putative Shine-Dalgarno sequences (Fig. 3). Therefore, ORF1, ORF2, and ORF6 represent the structural genes for E2 (*acoC*), E3 (*acoL*), and E1β (*acoB*), respectively. Plasmid pKSH48 contained the entire sequences of *acoB* and *acoC* but only 243 nucleotides of the 5′ region of *acoL*. The 3′ region missing from *acoL* was identified at one end of the 6.4-kbp *Hin*dIII fragment in pKSH64. Plasmid pKSH48 also lacked the 5′ region of the structural gene for E1α (*acoA*) encoding the N-terminal 12 amino acid residues. Since this region was also not detectable in pKSH34 or pKSH64, a partial *Eco*RI genomic library was screened with a biotinylated 2.8-kbp *Eco*RI fragment of pKSH48-1, which included the 3′ coding region of *acoA*, the entire coding sequence of *acoB*, and part of the 5′ coding region of *acoC*. Four of 200 transformants gave strong signals after hybridization, and each of them harbored a single 6.7-kbp *Eco*RI fragment, which was oriented antilinearly to *lacZ′*. From the resulting plasmid, pKSE67 (Fig. 2A), a region of approximately 700 bp including the 5′ region of *acoA* was sequenced. The amino acid sequence of purified E1α corresponded to the amino acid sequence deduced from the nucleotide sequence downstream of the third ATG codon of ORF3 (position 683 in Fig. 3). Because, in addition, only this and not the first (position 422) and second (position 650) ATG codons of ORF3 was preceded by a reliable Shine-Dalgarno sequence, *acoA* starts at position 683.

The codon preferences of *acoA*, *acoB*, *acoC*, and *acoL* corresponded to that of the colinearly oriented ORF4 (Fig. 2B), which is preceded by a tentative Shine-Dalgarno sequence (Fig. 3). The G+C contents of *acoA*, *acoB*, *acoC*, *acoL*, and ORF4 were 56.7, 56.9, 56.3, 58.3, and 56.2%, respectively. The G+C contents for the different codon positions followed the rules of Bibb et al. (6): the G+C contents for the first codon position of *acoA*, *acoB*, *acoC*, *acoL*, and ORF4 were 64.1 (theoretical value, 61.6), 59.9 (61.8), 63.6 (61.3), 63.7 (62.7), and 62.3 (61.3) mol%, respectively. For the second position, the following values were calculated: 42.5 (42.3), 43.4 (42.3), 42.9 (42.1), 39.6 (42.9), and 44.7 (42.1) mol%. For the third position, the values were 64.7 (66.4), 68.0 (66.8), 63.4 (65.7), 71.1 (69.5), and 63.7 (65.5) mol%. GGU and GGC were the preferred codons for glycine, whereas UUA (leucine), CUA (leucine), CGA (arginine), and CCA (proline) were avoided; UAG was not used as a stop codon. These data provided evidence that ORF4, like *acoA*, *acoB*, *acoC*, and *acoL*, represents a coding DNA sequence.

Nucleotide sequence of the downstream region of *acoL*. At a distance of 308 bp downstream and colinear to *acoL*, ORF5 was identified (Fig. 2B). A tentative Shine-Dalgarno sequence was located upstream of the putative start codon (Fig. 3). The protein deduced from the nucleotide sequence comprised 58 amino acid residues, and the primary structure showed strong similarities to several low-molecular-weight 2[4Fe-4S] ferredoxins, such as those from *Clostridium pasteurianum* (79) (33.3% identical amino acids at 54 positions), *C. perfringens* (72) (31.5% identity at 54 positions), and *C. butyricum* (5) (32.7% identity at 52 positions). Therefore, ORF5 presumably encodes a 2[4Fe-4S] ferredoxin with a calculated M_r of 6,381. A putative ferredoxin with an estimated M_r of 4,900 was previously identified in the soluble cell fraction of *P. carbinolicus* in both acetoin- and ethylene glycol-grown cells (58).

Putative transcriptional termination signals. In the 5′ flanking regions of *acoA*, *acoB*, *acoC*, and *acoL* as well as of ORF4, ORF5, and ORF7 (the latter did not exhibit significant resemblance to any protein stored in the data libraries), no sequences which exhibited significant homologies to the enterobacterial −35/−10 or −24/−12 promoter consensus sequences (1, 30) were identified. In cell extracts of recombinant LB-grown *E. coli* harboring plasmid pKSE67 with *acoA* and *acoB* in an antilinear orientation to *lacZ′* (Fig. 2A), no E1 proteins and no activity were detected, indicating that no functional promoter was present for *acoA* and *acoB* transcription in the host cells. Closely downstream of *acoC*, an inverted repeat which may represent a factor-dependent transcriptional terminator (88) was found (Fig. 3). According to Tinoco et al. (80), the free energy of this structure is 81.6 kJ/mol. In addition, two inverted repeats were identified at distances of 58 and 131 bp downstream of *acoL* (exact positions are shown in Fig. 3); the values for free energy were 82.5 and 75.8 kJ/mol, respectively. As both hairpin-like structures were followed by a run of 5 or 6 U residues in the RNA, which is sufficient to destabilize the RNA-DNA hybrid, they may represent factor-independent transcription factors (88).

Properties of the *acoA* and *acoB* gene products. The M_r of 37,500, which had been determined previously for E1β (56), corresponded with the calculated value of the *acoB* product (36,184), whereas the calculated value of 34,854 for the *acoA* gene product differs slightly from the M_r of 38,500 determined for E1α (56).

Comparisons of the amino acid sequences deduced from *acoA* and *acoB* with the primary structures of proteins collected in the data libraries revealed striking homologies of *P. carbinolicus* E1α and E1β to the corresponding subunits of the *A. eutrophus* acetoin:DCPIP oxidoreductase (65). Strong similarities to the corresponding subunits of E1 components of 2-oxo acid dehydrogenase multienzyme complexes from different sources were also found (Table 2; Fig. 4). Similarities of *P. carbinolicus* E1β to the corresponding subunits of different 2-oxo acid dehydrogenases were found to be greater than those of E1α to the corresponding subunits of 2-oxo acid dehydrogenases. This coincides with previous findings (29) that the homologies between α subunits of different 2-oxo acid dehydrogenases were weaker than those between β subunits. The

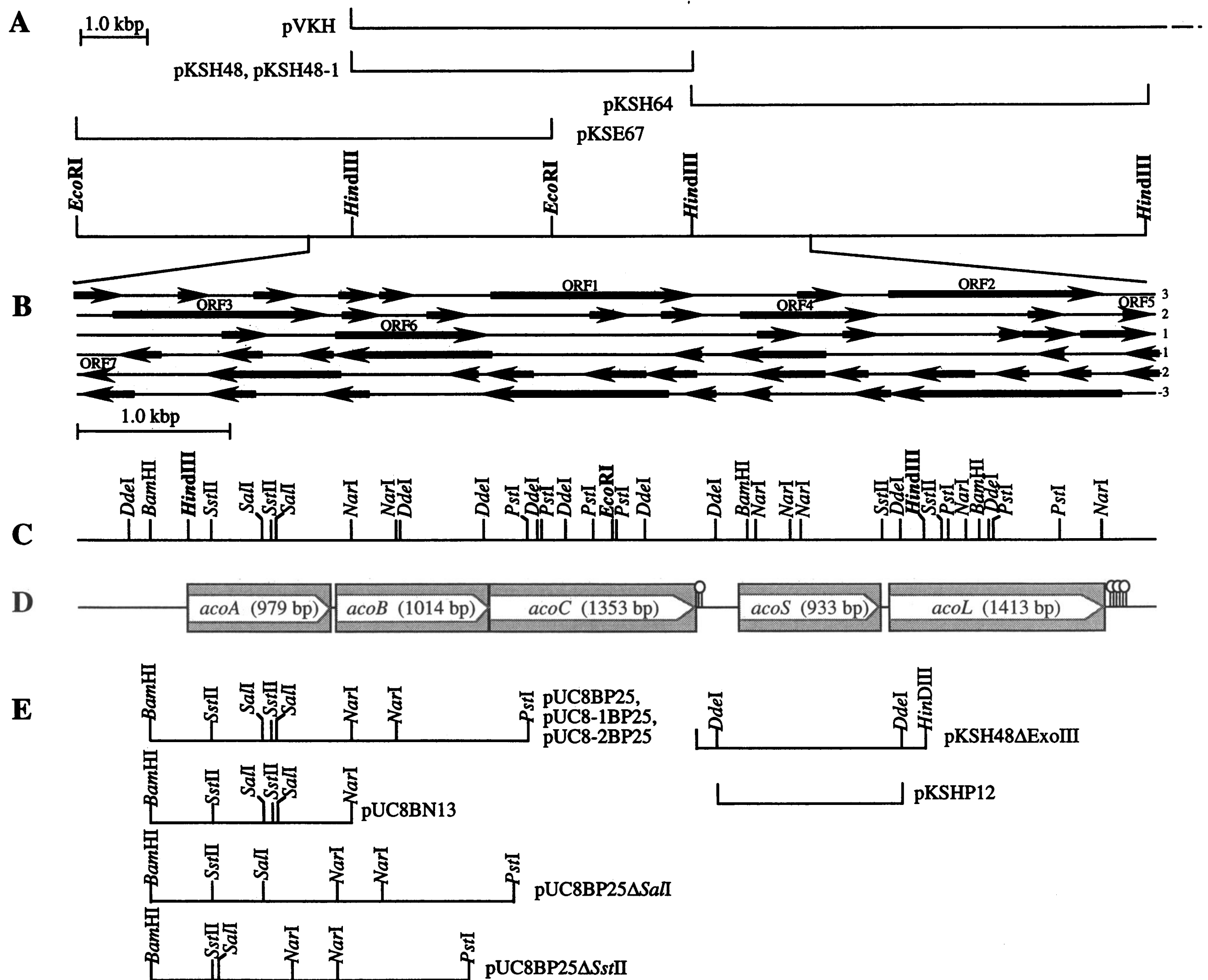

FIG. 2. Molecular organization of the *P. carbinolicus aco* gene cluster. (A) Restriction map of the region analyzed in this study and of relevant inserts of plasmids. (B) ORFs detected in the sequenced region comprising more than 120 bp. (C) Relevant restriction sites of the sequenced region (sites which are also shown in panel A are in boldface). (D) Structural genes of the *aco* gene cluster. The positions of hairpin-like structures are symbolized by loops. (E) Plasmid clones of subfragments used for heterologous expression of *acoA*, *acoB*, and ORF4 (*acoS*).

amino acid sequence of *P. carbinolicus* E1α contained the putative thiamine pyrophosphate-binding motif (20, 28), which is a central region of the consensus sequence depicted in Fig. 4A.

Heterologous expression of *acoA* and *acoB* in *E. coli*. To delete most of the regions upstream and downstream of *acoA* and *acoB*, pKSE67 was restricted with *Bam*HI and *Pst*I (Fig. 2C and D). The resulting 2.5-kbp fragment was ligated with *Bam*HI-plus-*Pst*I-restricted pUC8, pUC8-1, and pUC8-2 (27, 83) and transformed into *E. coli* DH5α, resulting in plasmids pUC8BP25, pUC8-1BP25, and pUC8-2BP25, respectively (Fig. 2E). After incubation for 12 h in LB medium in the presence of either 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) or 0.6% (wt/vol) glucose, soluble cell fractions from all transformants were subjected to SDS-polyacrylamide gel electrophoresis and tested for E1 enzyme activity and for immunological reaction with anti-E1 IgG. In the presence of IPTG, plasmids pUC8BP25, pUC8-1BP25, and pUC8-2BP25 conferred E1 activity to the recombinant cells. The specific activities in the soluble cell fractions of *E. coli*(pUC8BP25), *E. coli*(pUC8-1BP25), and *E. coli*(pUC8-2BP25) were 0.20, 0.004, and 0.15 U/mg of protein, respectively, which were much lower than in acetoin-grown *P. carbinolicus* (2.85 U/mg) (56). In the presence of glucose, only residual enzyme activities (<0.001 U/mg) were measured. Two dominant protein bands appeared in the protein pattern of the IPTG-induced recombinant *E. coli* cells, which exhibited the same electrophoretic mobility as E1α and E1β purified from *P. carbinolicus* (Fig. 5A1, lanes 1 to 5). In IPTG-induced cells of *E. coli*(pUC8BP25), the *acoA* and *acoB* products represented the major fraction (>50%) of the soluble proteins (Fig. 5A1, lane 2). This recombinant *E. coli* synthesized even more E1α and E1β than did *P. carbinolicus* during growth on acetoin (56). The reason for the relatively low specific activity of expressed E1 in the recombinant *E. coli* cells is not clear. The reaction with anti-E1 IgGs, which was strongest with soluble cell fractions from IPTG-induced *E. coli*(pUC8BP25), revealed that heterologous expressed E1 was

```
    40                              30                              20                              10
     G   H   T   F   Q   V   T   L   A   A   G   E   N   L   G   K   A   T   L   P   S   S   R   R   L   N   Q   R   G   L   K   E   Q
   1 TACCGTGTGTGAACTGGACCGTCAGTGCCGCGCCCTCATTTAAGCCTTTCGCTGTGAGTGGCGAACTTCGGCGAAGGTTTTGTCTGCCAAGTTTTTCCTG

     W   T   V   E   P   I   M
 101 CCACGTTACTTCCGGGATCATTCTCTTCTTTCCCTGGATGACATTTCTCTCTAGTCCTTACGCAGGCCAGGCCGTTGTGCTTTGCCTGTTTTTTTGCTTT
                 <- ORF7      [S/D]

 201 TCTACGAGCATTGCCCGGGGTGAAAAGCGAAGCCTCGCAAACCTGTCTCAATTCCTCAACCGGGGTTTTGCGACAGATGTTTGTCTTGCCGTAAGACACT

 301 TGTATCAAAATTATCAAAACCTCTGAGAACCAGTGCTCTCAAATACTTGCAATGCAAACATTCAGGATTGTTCAGATCTTTTTTTGAAAAACTTTTACAT

 401 CTGAAATCCCTCGCAAAAACAATGTTTTGCGAATTGCTTTTAGGGATTTGGGAGTGTTTGTTTTTTGCGATCCGGATCCTTGGCACCTGTTGTGCTCTAA
                                                                              ↑BamHI

 501 CACATGACCGTGAGACCAAGCGCGGCCGTATCAAGAGCCTGTCTTGCGGTCATCGATTCAGCAGCGGCTTGCCACCTCCAGGCGGGCGTGAGGCAGGTCG

                                                                                       M   K   T   Q   L   S
 601 GGATCGACTATCACAGGTGAAGATCGGTCAATCGTTACCCTATCGTTTTATGGCACGATCCAAAGGAAGAAGGGAGAAGCAAATGAAGACACAACTGTCG
                                                                          [S/D]    acoA ->

              10                              20                              30                      40
     K   E   D   L   L   K   A   Y   R   K   M   R   E   I   R   E   F   E   D   R   V   H   V   E   F   A   K   G   T   L   P   G   F   V
 701 AAAGAGGATCTTCTCAAAGCTTATCGCAAGATGCGGGAGATTCGGGAGTTTGAAGACCGTGTGCATGTTGAGTTCGCGAAAGGGACTCTCCCCGGCTTCG
                 ↑HindIII
                                      50                              60                              70
     H   L   Y   S   G   E   E   A   V   A   V   G   V   C   S   H   L   N   D   L   D   R   I   A   S   T   H   R   G   H   G   H   C
 801 TTCATCTGTATTCAGGTGAGGAAGCTGTGGCTGTTGGTGTTTGTTCACACCTCAACGATCTGGACCGTATCGCCAGTACCCACCGCGGCCATGGACACTG
                                                                                                 ↑SstII
                  80                              90                             100
     I   A   K   G   V   E   L   E   G   M   M   A   E   I   Y   G   K   K   T   G   T   C   G   G   K   G   G   S   M   H   I   A   D
 901 CATCGCCAAGGGTGTTGAGCTCGAAGGCATGATGGCCGAGATCTACGGCAAGAAAACCGGTACCTGCGGCGGTAAGGGCGGTTCCATGCATATTGCCGAC

         110                             120                             130                             140
     L   D   K   G   M   M   G   A   N   G   I   V   G   A   G   P   P   L   I   A   G   A   A   L   A   S   K   L   R   K   D   G   S   V
1001 CTCGACAAAGGTATGATGGGTGCAAACGGTATCGTCGGTGCCGGTCCCCCGCTGATCGCTGGTGCTGCTCTGGCTTCCAAACTGCGCAAAGACGGCAGCG

                                     150                             160                             170
     G   V   V   F   F   G   D   G   A   S   N   Q   G   T   N   F   E   S   M   N   F   A   V   T   L   D   L   P   M   I   F   V   L
1101 TCGGTGTGGTTTTCTTCGGCGACGGTGCTTCCAACCAGGGTACCAACTTCGAGTCGATGAACTTCGCCGTTACCCTGGATCTGCCGATGATCTTCGTTCT

                 180                             190                             200
     E   N   N   G   Y   A   E   S   T   S   P   K   Y   S   A   K   V   G   S   D   N   I   A   D   R   A   R   G   F   G   M   P   A
1201 GGAGAACAACGGTTACGCCGAGTCGACTTCTCCCAAGTACTCCGCCAAAGTCGGTTCCGACAATATCGCTGACCGCGCCCGCGGTTTCGGCATGCCCGCA
                          ↑SalI                                                          ↑SstII
         210                             220                             230                             240
     V   T   V   D   G   N   D   F   F   A   V   Y   E   A   A   G   E   A   I   E   R   A   R   K   G   G   G   P   T   F   I   E   C   K
1301 GTCACCGTCGACGGCAACGATTTCTTCGCCGTTTACGAAGCTGCCGGGGAAGCTATTGAGCGCGCCCGCAAAGGCGGCGGCCCTACCTTCATCGAGTGCA
          ↑SalI
                                     250                             260                             270
     T   M   R   Y   F   G   H   F   E   G   D   A   Q   T   Y   R   P   K   N   E   V   K   D   A   R   A   N   D   C   P   L   K   R
1401 AGACCATGCGTTATTTCGGTCACTTCGAAGGTGATGCCCAGACTTATCGTCCCAAGAACGAAGTTAAGGACGCCCGTGCCAATGATTGCCCTCTGAAGCG

                 280                             290                             300
     F   A   D   A   A   I   S   A   G   L   V   E   A   A   D   I   E   A   I   D   K   D   V   L   A   Q   V   E   K   A   V   K   D
1501 CTTTGCTGACGCTGCGATCTCCGCCGGCCTGGTGGAAGCCGCTGATATCGAGGCCATCGATAAGGATGTTCTGGCCCAGGTCGAAAAGGCTGTCAAGGAC

         310                             320
     A   E   V   A   P   Q   P   D   M   E   A   L   M   A   D   V   Y   Y   V   S   Y   *
1601 GCCGAGGTCGCACCGCAGCCTGATATGGAAGCGTTGATGGCCGACGTGTATGTATCTTACTGATCCCTTTTTGACGAAAAGATATAGAAAGGAATAAGAC
                                                                                             [S/D]

              10                              20                              30
     M   A   R   K   I   M   F   K   D   A   L   N   E   A   M   R   L   E   M   E   R   D   E   S   V   V   L   I   G   L   D   V   A
1701 GATGGCTAGAAAGATTATGTTCAAGGACGCACTGAACGAAGCGATGCGTTTGGAGATGGAACGTGACGAGTCCGTTGTCCTCATCGGGCTCGACGTTGCC
     acoB ->

                  40                              50                              60
     G   G   A   G   T   V   T   L   D   K   E   R   D   S   W   G   G   V   L   G   V   S   K   G   L   Y   P   L   F   P   D   R   I   I
1810 GGCGGCGCCGGTACCGTTACTCTGGACAAGGAGCGCGATTCCTGGGGCGGCGTTCTCGGCGTCAGCAAGGGTCTGTATCCGCTGTTCCCCGACCGTATCA
        ↑NarI
         70                              80                              90                             100
     D   T   P   I   S   E   S   A   Y   I   G   A   A   V   G   A   S   A   C   G   L   R   A   I   G   E   L   M   F   S   D   F   M
1901 TCGATACTCCGATCTCCGAGTCCGCTTATATCGGTGCTGCCGTCGGCGCTTCGGCTTGTGGATTGCGCGCCATCGGCGAGCTGATGTTCTCCGACTTCAT

                                     110                             120                             130
     G   V   C   F   D   Q   L   Y   N   Q   A   A   K   F   R   Y   M   F   G   G   K   A   V   T   P   V   T   I   R   T   M   I   G
2001 GGGCGTCTGCTTTGACCAGCTGTACAACCAGGCTGCCAAGTTCCGTTACATGTTCGGTGGCAAGGCTGTTACCCCGGTAACCATCCGCACCATGATCGGC
                                                                                                   NarI↑
                 140                             150                             160
     A   G   F   S   A   A   A   Q   H   S   Q   S   P   Y   S   M   F   A   H   V   P   G   L·  K   C   I   I   P   S   N   P   Y   D   A
2101 GCCGGTTTCAGCGCCGCCGCTCAGCATTCCCAGAGTCCTTACTCGATGTTCGCTCATGTGCCGGGTCTGAAGTGCATCATCCCCTCCAACCCCTACGATG

         170                             180                             190                             200
     K   G   L   L   A   A   S   I   A   D   D   D   P   C   V   F   F   E   H   K   A   L   Y   T   M   K   G   E   V   P   E   E   H
2201 CCAAAGGTCTGCTGGCCGCTTCCATTGCGGATGACGATCCCTGCGTGTTCTTCGAGCACAAAGCTCTTTACACCATGAAGGGCGAGGTTCCTGAAGAGCA

                                     210                             220                             230
     Y   T   I   P   L   G   K   A   N   V   V   Q   E   G   K   D   V   T   I   V   A   L   A   R   M   V   Q   F   A   E   K   A   A
2301 CTACACCATTCCTCTGGGCAAAGCCAATGTTGTTCAGGAAGGTAAGGACGTTACCATCGTTGCTCTGGCCCGCATGGTTCAGTTCGCCGAAAAGGCTGCC

                 240                             250                             260
     K   K   L   A   K   D   G   I   E   C   T   I   I   D   P   R   T   I   S   P   M   D   W   D   A   I   Y   S   S   V   E   K   T   G
2401 AAGAAGCTGGCCAAAGACGGTATCGAGTGCACCATTATCGATCCCCGTACCATCTCGCCGATGGACTGGGACGCCATCTACTCCAGCGTCGAGAAGACCG
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FIG. 3. Nucleotide sequence of the *P. carbinolicus aco* gene cluster. Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations. The amino acid sequences determined for the N termini of purified *P. carbinolicus* E1α, E1β, E2, and E3 are overlined (differences between the amino acid sequence obtained by microsequencing of E2 and E3 and those deduced from the nucleotide sequence are indicated by parentheses). Putative ribosome-binding sites (S/D) are symbolized by boxes. The positions of hairpin-like structures are marked by inverted arrows. Relevant restriction sites are indicated below the nucleotide sequence.

```
            270                                      280                                       290                                     300
       R   L   V   V   V   D   E   S   Y   D   L   C   G   V   A   S   D   I   C   G   T   C   S   Q   N   V   F   G   A   L   K   A   A
2501 GTCGCCTGGTGGTTGTTGACGAAAGCTATGACCTCTGCGGCGTTGCTTCCGATATCTGCGGCACGTGTTCCCAGAATGTGTTCGGCGCTTTGAAGGCAGC

                                310                                      320                                       330
       P   Q   M   V   T   A   P   F   V   P   T   P   F   A   A   N   L   E   A   A   Y   L   P   D   A   K   K   I   E   A   A   V   R
2601 TCCCCAGATGGTAACGGCTCCGTTCGTTCCTACGCCTTTTGCAGCCAATCTTGAGGCCGCTTACCTGCCTGACGCTAAGAAAATCGAAGCGGCTGTACGT

                                               ____________________10__________(K)_______________20_____(L)_____
       K   T   M   E   *           M   S   D   N   R   I   I   A   L   T   M   P   K   W   G   L   T   M   E   E   G   T   I   S   S   W   L
2701 AAAACCATGGAGTAAGTATCATGAGTGACAATAGAATCATAGCTCTCACCATGCCCAAGTGGGGCCTGACCATGGAAGAAGGCACCATCTCTTCCTGGCT
              [S/D]           acoC ->

            30                                       40                                        50                                      60
       M   D   E   G   D   T   I   E   V   G   S   E   I   L   E   V   E   T   D   K   I   A   Q   P   V   E   S   A   V   E   G   I   L
2801 GATGGACGAGGGCGACACTATCGAAGTCGGGAGTGAAATTCTCGAAGTCGAAACCGACAAGATCGCGCAGCCCGTTGAAAGTGCTGTCGAAGGTATCCTG

                                70                                       80                                        90
       R   R   K   I   G   E   E   E   D   E   E   Y   P   V   K   A   L   I   G   I   I   A   A   E   D   V   T   E   E   E   I   D   A   F   I
2901 CGTCGCAAGATCGGTGAAGAAGACGAAGAATACCCTGTGAAGGCTCTGATCGGGATCATCGCTGCAGAGGATGTCACCGAAGAGGAAATCGACGCGTTTA
                                                                          ↑PstI
                  100                                      110                                       120
       A   S   Y   G   G   E   G   A   E   G   S   D   E   D   E   A   P   A   E   T   A   A   A   P   E   G   I   Y   E   L   T   M   P
3001 TCGCCAGCTACGGCGGCGAAGGTGCTGAGGGTTCCGATGAAGATGAAGCTCCTGCAGAAACCGCTGCTGCACCTGAAGGTATCTATGAGCTGACCATGCC

            130                                      140                                       150                                     160
       K   W   G   L   T   M   E   E   G   T   I   S   S   W   L   I   D   E   G   D   E   V   E   V   G   T   E   I   M   E   V   E   T
3101 CAAGTGGGGCCTGACCATGGAAGAAGGCACCATCTCTTCCTGGCTCATCGACGAAGGGGATGAAGTCGAAGTCGGTACCGAGATCATGGAAGTTGAGACG

                                170                                      180                                       190
       D   K   I   A   Q   P   V   E   S   T   V   A   G   V   L   R   R   K   I   G   E   E   D   E   E   Y   P   V   K   A   L   I   G   I
3201 GATAAGATCGCTCAGCCGGTTGAAAGTACCGTGGCAGGTGTGTTGCGTCGCAAGATCGGTGAAGAGGACGAGGAATATCCGGTAAAAGCCTTGATCGGTA

                  200                                      210                                       220
       I   A   D   A   S   V   S   D   A   D   I   D   A   Y   L   A   S   R   G   G   E   A   A   S   G   D   E   E   E   E   A   A   A
3301 TTATTGCCGATGCCTCGGTATCCGATGCCGATATCGATGCTTACCTTGCCAGCCGTGGTGGCGAAGCCGCCTCGGGTGACGAGGAAGAAGAAGCTGCAGC

            230                                      240                                       250                                     260
       P   A   Q   P   T   S   K   P   M   S   A   I   G   A   A   I   S   N   T   V   T   N   S   W   T   I   P   Q   F   P   V   T   M
3401 TCCGGCGCAGCCGACCAGCAAGCCGATGTCGGCCATCGGTGCCGCCATCTCCAATACGGTGACCAACTCGTGGACTATTCCCCAGTTCCCGGTCACCATG

                                270                                      280                                       290
       G   I   E   M   G   A   A   K   E   F   R   A   G   L   K   A   A   G   K   A   V   S   M   N   D   M   V   I   R   A   C   G   K   A
3501 GGCATCGAGATGGGTGCGGCCAAGGAATTCCGTGCCGGTCTCAAGGCTGCAGGCAAGGCTGTTTCCATGAACGATATGGTAATCAGGGCCTGTGGCAAGG
                                 ↑EcoRI
                  300                                      310                                       320
       I   E   Q   Y   P   M   V   N   A   T   L   G   G   K   E   Y   G   L   N   A   D   V   N   I   A   V   A   V   G   T   D   D   A
3601 CCATCGAGCAGTACCCGATGGTTAACGCCACTCTGGGCGGCAAGGAATATGGCCTTAATGCCGATGTAAACATCGCCGTTGCCGTCGGCACCGACGATGC

            330                                      340                                       350                                     360
       L   M   M   P   V   V   K   G   C   Q   A   L   S   L   E   E   V   A   S   A   S   R   A   V   I   D   K   V   K   A   G   T   C
3701 TCTGATGATGCCGGTTGTCAAGGGCTGTCAAGCCCTCAGCCTTGAAGAAGTTGCCAGCGCTTCCCGCGCTGTAATCGACAAGGTTAAGGCCGGTACCTGC

                                370                                      380                                       390
       G   P   A   E   M   A   G   G   N   F   A   I   S   N   L   G   M   L   G   V   D   S   F   G   A   L   V   P   P   G   M   S   A   I
3801 GGCCCTGCTGAAATGGCTGGTGGTAACTTCGCCATTTCCAACCTGGGTATGCTCGGGGTCGATTCATTCGGTGCCCTGGTGCCCCCGGGAATGTCTGCGA

                  400                                      410                                       420
       L   A   V   G   G   I   K   D   E   V   V   V   K   D   G   E   M   V   P   V   S   T   M   K   V   T   L   V   A   D   H   R   V
3901 TCCTGGCAGTTGGCGGCATCAAAGACGAAGTCGTAGTGAAAGATGGCGAAATGGTTCCCGTGTCAACGATGAAGGTGACCCTGGTAGCCGACCACCGGGT

            430                                      440                                       450
       V   D   G   L   Y   S   A   Q   F   L   V   E   L   K   R   L   L   E   N   P   E   E   L   *                                 ------->
4001 GGTGGACGGGTTGTACTCCGCCCAGTTCCTGGTGGAACTGAAGCGTCTTCTGGAAAATCCCGAAGAGCTTTAATTTTAGATAGCAAAAAGCCGGCACCCC

          <-------
4101 CTCAAAGGGGTGCCGGTCGATAAAAGATTTCGCGGAATAGCGTTTGTGTATATTTGGTATAGTCGGATCATGAAAAAAAGAGTCAGGGGTTGTTGGTCTTG

4201 CTCAGGATTTCATGTGCCGGGGATGACAGCCCATGTTGAGCTGCTGATCGAAGGCGATTGGGTCGGTCAGGTTCGAAGAGTTGTGATGATTCTGCACGAG
      ↑DdeI
                                                                                                                10
                                                                          M   D   V   G   Q   K   K   R   G   A   D   K   T   A   L
4301 ACGGTATTACGGGTTTGATGCCGCACAAAGGGCATCGGGAAGATAAGGGTCACGATCATGGATGTTGGACAAAAAAAACGTGGTGCCGATAAAACGGCCC
                                                       [S/D]              ORF4(acoS)->

                  20                                       30                                        40
       A   T   T   D   E   Q   G   A   L   S   K   P   T   W   I   R   A   K   A   P   I   S   P   E   V   G   R   L   T   G   I   L   R
4401 TAGCGACGACTGATGAGCAAGGCGCTTTGTCCAAGCCGACCTGGATCCGCGCCAAGGCGCCGATCTCGCCGGAGGTTGGCAGGTTGACGGGAATCCTGCG

            50                                       60                                        70                                      80
       D   L   H   L   H   T   V   C   E   E   A   S   C   P   N   L   G   E   C   F   K   R   G   T   A   T   F   M   I   M   G   D   V
4501 TGATTTGCATCTGCATACGGTGTGCGAAGAGGCCAGCTGTCCCAACCTCGGCGAATGTTTCAAGCGTGGGACGGCAACCTTCATGATCATGGGCGATGTC

                                90                                       100                                       110
       C   T   R   R   C   P   F   C   D   V   A   H   G   R   P   A   A   L   D   T   E   E   P   G   H   L   A   D   A   I   G   A   M   K
4601 TGTACGCGTCGTTGTCCCTTTTGCGACGTGGCACATGGCCGGCCTGCTGCCTTGGATACCGAAGAGCCGGGACATCTGGCGGACGCCATCGGCGCCATGA

                  120                                      130                                       140
       L   K   Y   V   V   I   T   S   V   T   R   D   D   L   E   D   G   G   A   A   H   F   A   Q   C   I   E   S   I   R   K   K   T
4701 AGCTCAAATACGTGGTGATCACCTCGGTGACCAGGGACGATCTGGAAGATGGCGGCGCCGCACATTTCGCACAGTGTATCGAATCCATCCGCAAGAAAAC

            150                                      160                                       170                                     180
       R   R   V   Y   K   V   E   I   L   V   P   D   F   R   G   H   V   D   A   A   L   K   N   L   G   N   C   L   P   D   V   F   N
4801 CCGTAGGGTGTACAAGGTGGAGATCCTGGTGCCTGATTTCCGGGGGCATGTCGATGCCGCCCTCAAGAACCTCGGCAACTGTCTGCCCGACGTGTTTAAC
```

FIG. 3—*Continued.*

```
                            190                           200                           210
     H  N  L  E  T  V  P  R  L  Y  A  E  S  R  P  G  A  R  Y  H  E  S  L  R  L  L  Q  R  F  K  E  T  Y  P
4901 CATAATCTTGAAACGGTACCGCGTCTTTATGCGGAGTCACGACCTGGTGCCCGTTATCACGAATCTTTGCGGTTGTTGCAGCGGTTTAAGGAAACCTATC

                  220                           230                           240
       G  I  P  T  K  S  G  L  M  L  G  L  G  E  T  D  E  E  I  L  E  V  M  R  D  L  R  V  H  G  C  D  M
5001 CCGGTATACCGACCAAATCCGGCCTGATGCTGGGATTGGGCGAAACAGACGAAGAGATCCTTGAAGTTATGCGGGATTTGCGGGTGCACGGTTGCGATAT

        250                           260                           270                           280
      L  T  I  G  Q  Y  L  R  P  S  R  H  H  L  P  V  Q  R  Y  V  T  P  E  Q  F  E  A  F  R  V  A  G  L
5101 GCTGACCATCGGACAGTATCTGCGACCCAGTCGCCATCATCTGCCGGTTCAGCGCTATGTAACCCCGGAGCAGTTCGAGGCGTTTCGGGTTGCCGGTTTA

                            290                           300                           310
     K  M  G  F  S  Q  V  A  S  G  P  L  V  R  S  S  Y  H  A  D  L  Q  A  K  E  V  L  H  T  *
5201 AAGATGGGCTTTTCCCAGGTCGCTTCAGGGCCGCTGGTGCGTTCTTCCTATCACGCCGATCTACAGGCAAAAGAAGTTTTGCATACATGACAAGCCGCGG

                                               _______________________10_____________________________20
                                            M  A  D  E  I  F  D  L  I  V  L  G  A  G  P  G  G  Y  V  G  A
5301 TTAAAACCCAGGAGTTTTGCCGCATAAGGAGAATATAAAATGGCTGACGAAATTTTCGATCTTATCGTATTGGGTGCCGGTCCCGGAGGTTATGTCGGGG
                              [S/D]   acoL ->

     _________________________30_________________(?)___(?)____ 40                         50
       I  R  A  A  Q  L  G  M  K  V  A  V  V  E  S  R  P  T  L  G  G  V  C  L  N  E  G  C  I  P  S  K  A
5401 CGATCCGCGCAGCTCAGCTCGGCATGAAGGTCGCCGTGGTGGAAAGTCGTCCGACCCTCGGCGGTGTGTGCCTTAACGAAGGCTGCATTCCGAGTAAAGC
                  ↑DdeI
                     60                            70                            80
      L  L  D  S  S  E  H  F  A  L  A  R  D  K  F  D  M  H  G  I  E  I  P  A  P  K  L  N  L  A  K  M  M
5501 GCTGCTCGACTCCAGCGAACATTTCGCTCTGGCGCGGACAAGTTCGATATGCACGGCATCGAGATCCCTGCACCGAAGCTTAACCTGGCCAAGATGATG
                                                                                 ↑HindIII
           90                            100                           110                           120
     E  R  K  E  G  V  V  S  D  L  T  G  G  I  A  F  L  F  K  K  N  K  V  T  W  I  K  G  R  G  K  L  L  G
5601 GAGCGCAAGGAAGGCGTTGTCAGCGATCTTACCGGCGGCATCGCGTTCCTTTTCAAAAAGAATAAAGTTACCTGGATCAAGGGCCGCGGCAAGCTGCTCG

                              130                           140                           150
       A  G  G  D  G  L  Q  Q  V  E  V  T  G  K  N  A  G  V  V  K  G  K  N  V  L  L  A  T  G  G  K  V  A
5701 GAGCCGGTGGCGACGGTCTGCAGCAGGTTGAAGTGACCGGTAAGAACGCCGGCGTGGTCAAGGGCAAGAATGTTCTGCTGGCTACCGGCGGCAAGGTTGC

                    160                           170                           180
      Q  V  P  G  I  T  V  D  N  D  V  I  I  D  N  V  G  A  L  S  I  D  K  V  P  E  H  L  M  I  I  G  A
5801 GCAGGTTCCCGGCATTACCGTAGACAACGATGTGATCATCGACAACGTCGGCGCCCTGAGCATTGATAAAGTCCCCGAACATCTGATGATCATCGGCGCC

          190                           200                           210                           220
     G  Y  I  G  L  E  L  G  S  V  W  L  R  L  G  S  K  V  T  V  V  E  M  L  P  K  M  L  P  K  T  D  A  D
5901 GGCTATATCGGTCTGGAGCTCGGATCCGTGTGGTTGCGCCTCGGCTCCAAGGTTACCGTGGTCGAAATGTTGCCCAAAATGCTGCCTAAGACCGACGCCG

                              230                           240                           250
       T  T  Q  A  L  Q  R  S  L  K  K  Q  G  M  T  F  N  M  G  T  T  V  G  G  I  E  V  S  G  G  K  A  T
6001 ATACCACTCAGGCGCTGCAGCGCTCCCTGAAAAAACAGGGCATGACCTTCAACATGGGCACCACCGTCGGCGGTATCGAAGTGTCCGGCGGTAAGGCGAC

                    260                           270                           280
      V  K  L  V  K  N  D  K  E  K  E  V  V  C  D  K  V  L  M  S  I  G  R  K  P  N  T  D  G  L  G  L  E
6101 CGTGAAATTGGTCAAAAACGATAAAGAAAAAGAAGTTGTGTGCGACAAGGTGCTCATGTCCATCGGTCGCAAGCCCAATACCGACGGCCTCGGTCTGGAA

          290                           300                           310                           320
     E  L  G  V  E  M  G  E  R  G  T  I  K  V  D  D  N  Y  A  T  N  V  P  G  I  Y  A  I  G  D  L  I  P  G
6201 GAACTCGGTGTGGAAATGGGCGAGCGCGGAACCATCAAGGTCGATGACAATTACGCCACCAATGTCCCGGGTATCTACGCTATCGGCGACCTGATCCCCG

                              330                           340                           350
       P  M  L  A  H  K  A  S  E  E  A  V  V  F  V  E  R  L  V  G  K  N  S  E  V  H  Y  G  T  I  P  G  V
6301 GCCCCATGCTGGCGCACAAAGCCTCCGAAGAAGCCGTGGTTTTCGTGGAGCGCCTGGTCGGGAAAAATTCCGAAGTTCACTACGGTACCATCCCCGGTGT

                    360                           370                           380
      C  Y  T  W  P  E  V  A  S  V  G  K  T  E  Q  Q  L  Q  E  E  G  T  P  V  K  V  G  K  F  N  F  V  G
6401 CTGCTATACCTGGCCGGAAGTGGCTTCCGTCGGCAAAACCGAGCAGCAGCTGCAGGAAGAGGGTACCCCCGTCAAAGTCGGTAAATTCAATTTCGTCGGC

          390                           400                           410                           420
     N  G  R  A  R  A  M  A  E  T  E  G  F  V  K  I  I  A  H  A  E  N  G  Q  V  L  G  V  H  I  F  G  P  R
6501 AACGGTCGTGCCCGCGCCATGGCGGAAACCGAAGGTTTCGTCAAGATCATCGCCCATGCCGAGAACGGGCAGGTGCTGGGTGTGCACATCTTCGGGCCTC

                              430                           440                           450
       A  S  D  M  I  A  E  A  V  A  V  M  S  Y  G  G  T  A  H  D  I  G  A  M  F  H  G  H  P  T  L  S  E
6601 GCGCTTCCGATATGATTGCTGAAGCCGTGGCGGTTATGAGCTACGGCGGTACGGCCCACGACATCGGTGCCATGTTCCACGGTCATCCGACCCTGTCCGA

                    460                           470
      A  V  K  E  A  A  L  D  V  D  G  A  A  V  H  C  *
6701 AGCGGTCAAGGAAGCCGCTCTGGATGTGGACGGCGCCGCCGTACACTGCTGATAAATCTAAACGTGAAAATAACTGTTTTAGGCTAGTCCTGCGAGGGCT

                 -------  ->          <-  --------                                        ------------->
6801 GACCTGCTTGCCGGGAGGTGGACCTCCTAGCCGCCTCCCGGTCTTTTTACCGTTTTATTCTTCCTCTCCAAGTTCCTCCTGTACCAAGTGGAAACGATTC

          <------------
6901 GGGTCCGTTTCCACTTGGATTTTTTCCATGGGGAGATGGGTAGAGGACAGGTTGTTTGTGGAAATCCGGCAAGCGTATCGTGGCGCTTGCCTTCATGGCC

                                                                                            10
                                                                 M  K  Q  F  K  V  D  K  S  R  C  T  Q  C
7001 GGGGTGATTGTCATGCCGCCGATGCCATGACCATAATTCAAACGGGAAACAGGGGAGACCATGAAACAGTTCAAGGTGGATAAATCACGTTGTACCCAAT
                                                         [S/D]   ORF5 ->

                      20                            30                            40
       G  E  C  I  Q  D  C  V  F  G  L  L  S  M  E  Q  G  Y  P  A  L  P  A  D  K  E  S  V  C  I  E  C  Q
7101 GTGGCGAATGTATTCAGGATTGTGTGTTCGGGTTGCTCAGCATGGAGCAAGGTTATCCGGCATTGCCCGCCGATAAAGAGTCTGTCTGCATCGAATGCCA

            50
      H  C  M  A  V  C  K  P  G  A  E  *
7201 GCACTGTATGGCGGTTTGCAAACCCGGTGCCGAATAAGTATTTTAGGATTG
```

FIG. 3—*Continued.*

A

```
   1 -----------MKTQLSKEDLLKAYRKMREIREFEDRVHVEFAKGTLPGFVHLYSGEEAV AoDH P.car.
   1 --MTARASQDSAALPLDKETLLTVYRKMRTIRDFEERLHVDFGRGDIPGFVHLYAGEEAA AoDH A.eut.
  42 CDLYRLEEGPPTSTVLTRAEALKYYRTMQVIRRMELKADQLYKQKFIRGFCHLCDGQEAC PDH mouse
  61 SFESYMLEPPDLSYESSKATLLQMYKDMVIIRRMEMACDALYKAKKIRGFCHLSVGQEAI PDH S.cere.
  28 ILNEKGEVVNEAAMPDLTDDQLKELMRRMVFTRVLDQRSISLNRQGRLGFYAPTAGQEAS PDH B.subt.
                    L K  LLK YR M  IRR E           I GF HL  GQEA  CONSENSUS

  50 AVGVCSHLNDLDRIASTHRGHGHCIAKGVELEGMMAEIYGKKTGTCGGKGGSMHIADLDK AoDH P.car.
  59 GVGILHHLNDGDRIASTHRGHGHCIAKGVDPVAMMKEIYGKKGGSCNGKGGSMHIADLSK AoDH A.eut.
 102 CVGLEAGINPTDHVITSYRAHGFCYTRGLSVKSILAELTGRKGGCAKGKGGSMHM--YGK PDH Mouse
 121 AVGIENAITKLDSIITSYRCHGFTFMRGASVKAVLAELMGRRAGVSYGKGGSMHL--YAP PDH S.cere.
  88 QIATHFALEKEDFVLPGYRDVPQLIWHGLPLYQAFLFSRGHFRG---------NQMPDDV PDH B.subt.
      VG    LN  D I   YR HG CI  G       AE  G K G   GKGGSMH     K CONSENSUS

 110 GMMGANGIVGAGPPLIAGAALASKLRKDGSVGVVFFGDGASNQGTNFESMNFAVTLDLPM AoDH P.car.
 119 GMMGANGILGAGAPLICGAALAAKFRGKGEVGITFCGDGASNQGTFLESLNLAAVWNLPV AoDH A.eut.
 160 NFYGGNGIVGAQVPLGAGVAFACKYLKNGQVCLALYGDGAANQGQVFEAYNMSALWKLPC PDH mouse
 179 GFYGGNGIVGAQVPLGAGLAFAHQYKNEDACSFTLYGDGASNQGQVFESFNMAKLWNLPV PDH S.cere.
 139 NALSPQIIIGAQYIQTAGVALGLKKRGKKAVAITYTGDGGRSQGDFYEGINFAGAYKAPA PDH B.subt.
     G  G NGIVGAQ PL  AG ALA K R  G V  T  GDGASNQG  FES N A    LP  CONSENSUS
                                         ***        *    *     *

 170 IFVLENNGYAESTSPKYSAKVGSDNIADRARGFGMPAVTVDGNDFFAVYEAAGEAIERAR AoDH P.car.
 179 IFVIENNGYAESTSRDYGTAV--DSYVDRAAGFGIPGVTVDGTDFFAVHEAAGEVIRRAR AoDH A.eut.
 220 VFICENNLYGMGTSNERSAASTDY----HKKGFIIPGLRVNGMDILCVREATKFAADHCR PDH mouse
 239 VFCCENNKYGMGTAASRSSAMTEYFKRGQY----IPGLKVNGMDILAVYQASKFAKDWCL PDH S.cere.
 199 IFVVQNNRYAISTPVEKQSAAET--IAQKAVAAGIVGVQVDGMDPLAVYAATAEARERAI PDH B.subt.
     IFV ENN YA STS   S A         A GFGIPGV VDGMD LAVYEA  EA  RAR CONSENSUS
          **

 230 KGGGPTFIECKTMRYFGH-FEGDAQTYRPKNEVKDARANDCPLKRFADAAISAGLVEAAD AoDH P.car.
 237 EGGGPSLLECKMVRFYGH-FEGDAQTYRAAGELDDIRANKDCLKLFGRAVTQAGVVAREE AoDH A.eut.
 276 SGKGPIVMELQTYRYHGHSMSDPGISYRSREEVHNVRSKSDPIMLLRERIISNNLSNIEE PDH mouse
 295 SGKGPLVLEYETYRYGGHSMSDPGTTYRTRDEIQHMRSKNDPIAGLKMHLIDLGIATEAE PDH S.cere.
 257 NGEGPTLIETLTFRYGPHTMAGDDPTKYRTKEIENEWEQKDPLVRFRAFLENKGLWSEEE PDH B.subt.
      G GP    E  T RY GH M GD  TYR   E    R   DPL  F    I  GL   EE CONSENSUS

 289 IEAIDKDVLAQVEKAVKDAEVAPQPDMEALMADVYVSY---------------------- AoDH P.car.
 296 LDTIDREVAALIEHAVQEAKAAPQPGPEDLLTDVYVSY---------------------- AoDH A.eut.
 336 LKEIDADVKKEVEDAAQFATTDPEPAVEDIANYLYHQDPPFEVRGAHKWLKYKSHS---- PDH mouse
 355 VKAYDKSARKYVDEQVELADAAPPPEAKLSILFEDVYVKGTETPTLRGRIPEDTWDFKKQ PDH S.cere.
 317 EAKVIEDAKEEIKQAIKKADAEPKQKVTDLMKIMYEKMPHNLEEQFEIYTQKESK----- PDH B.subt.
        ID DV   VE AV  A AAP P  EDL    YV                        CONSENSUS
```

B

```
   1 ---------------MARKIMFKDALNEAMRLEMERDESVVLIGLDVAGGAGTVTLDKER AoDH P.car.
   1 ---------------MARKLSIKLAINEAIDQEMTRDPSVIMLGEDIVGGAGA---DGEK AoDH A.eut.
   1 -----------------------------MAQMTMVQAITDALRIELKNDPNVLIFGEDV PDH B.stea.
   1 -----------------------------MAQMTMIQAITDALRTELKNDENVLVFGEDV PDH B.subt.
   1 MAAVSGLVRRPLREVSGLLKRRFHWTAPAAVQVTVRDAINQGMDEELERDEKVFLLGEEV PDH Human
                                 AM Q T RDAI   L  EL  D  V   GEEV CONSENSUS

  46 DSWGGVLGVSKGLYPLFP-DRIIDTPISESAYIGAAVGASACGLRAIGELMFSDFMGVCF AoDH P.car.
  43 DAWGGVLGVTKGLYAKHG-DRLLDTPLSESAYVGAAIGAAACGMRPIAELMFIDFMGVCF AoDH A.eut.
  32 GVNGGVFRATEGLQAEFGEDRVFDTPLAESGIGGLAIGLALQGFRPVPEIQFFGFVYEVM PDH B.stea.
  32 GVNGGVFRATEGLQKEFGEDRVFDTPLAESGIGGLALGLGLNGFRPVMEIQFFGFVYEVM PDH B.subt.
  61 AQYDGAYKVSRGLWKKYGDKRIIDTPISEMGFAGIAVGAAMAGLRPICEFMTFNFSMQAI PDH Human
        GGV  VT GL   FG DR  DTPLSESG  G A GAA  G RPI E MFF F      CONSENSUS

 105 DQLYNQAAKFRYMFGGKAVTPVTIRTMIGAGFSAAAQHSQSPYSMFAHVPGLKCIIPSNP AoDH P.car.
 102 DQIFNQAAKFRYMFGGKAETPVVIRAMVGAGFRAAAQHSQMLTPLFTHIPGLKVVCPSTP AoDH A.eut.
  92 DSICGQMARIRYRTGGRYHMPITIRSPFGGGVHTPELHSDSLEGLVAQQPGLKVVIPSTP PDH B.stea.
  92 DSVSGQMARMRYRSGGRWTSPVTIRSPFGGGVHTPELHADSLEGLVAQQPGIKVVIPSTP PDH B.subt.
 121 DQVINSAAKTYYMSGGLQPVPIVFRGPNGASAGVAAQHSQCFAAWYGHCPGLKVVSPWNS PDH Human
     DQ  NQAAK RYM GG     PVTIR P GAG   AAQHSQSL  L AH PGLKVVIPSTP CONSENSUS

 165 YDAKGLLAASIADDDPCVFFEHKALY----TMKGEVPEEHYTIPLGKANVVQEGKDVTIV AoDH P.car.
 162 YDTKGLLIQAIRDNDPVIFCEHKNLY----GLEGEVPEGAYAIPFGEANIVRDGKDVSIV AoDH A.eut.
 152 YDAKGLLISAIRDNDPVIFLEHLKLYR---SFRQEVPEGEYTIPIGKADIKREGKDITII PDH B.stea.
 152 YDAKGLLISAIRDNDPVVFLEHMKLYR---SFRQEVPEEEYTIELGKADVKREGTDLSII PDH B.subt.
 181 EDAKGLIKSAIRDNNPVVVLENELMYGVPFEFPPEAQSKDFLIPIGKAKIERQGTHITVV PDH Human
     YDAKGLLISAIRDNDPVVFLEH  LY     F  EVPE  YTIP GKA I REGKD TIV CONSENSUS

 221 ALARMVQFAEKAAKKLAKDGIECTIIDPRTISPMDWDAIYSSVEKTGRLVVVDESYDLCG AoDH P.car.
 218 TYGLMVHRALEAAATLAKEGIEAEIVDLRTLSPLDMDTVLESVENTGRLVVVDEASPRCN AoDH A.eut.
 209 AYGAMVHESLKAAAELEKEGISAEVVDLRTVQPLDIETIIGSVEKTGRAIVVQEAQRQAG PDH B.stea.
 209 TYGAMVHESLKAADELEKDGISAEVVDLRTVSPLDIDTIIASVEKTGRAIVVQEAQKQAG PDH B.subt.
 241 SHSRPVGHCLEAAAVLSKEGVECEVINMRTIRPMDMETIEASVMKTNHLVTVEGGWPQFG PDH Human
      YG MVH  LKAAA L KEGIEAEVVDLRT SPLD DTI  SVEKTGRLVVV EA  Q G CONSENSUS

 281 VASDICGTC-SQNVFGALKAAPQMVTAPFVPTPFAANLEAAYLPDAKKIEAAVRKTME-- AoDH P.car.
 278 IATDISAQV-AQQAFGALKAGIEMVCPPHTPVPFSPTLEDLYIPSAAQIAAAARKTMKGG AoDH A.eut.
 269 IAANVVAEI-NERAILSLEAPVLRVAAPDTVYPFAQA-ESVWLPNFKDVIETAKKVMNF- PDH B.stea.
 269 IAANVVAEI-NDRAILSLEAPVLRVAAPDTVFPFSQA-ESVWLPNHKDVLETARKVLEF- PDH B.subt.
 301 VGAEICARIMEGPAFNFLDAPAVRVTGADVPMPYAKILEDNSIPQVKDIIRAIKKTL--- PDH Human
     IAA I A I    AF   L AP  RV  APDTP PFA  LE   LP  KDI  AARKTM    CONSENSUS
```

FIG. 4. Amino acid sequence comparisons of *P. carbinolicus* E1α and E1β with other proteins. Sequences have been aligned by the program Multalign (44). Amino acids are specified by standard one-letter abbreviations, and numbers on the left side indicate positions of the respective amino acids within the protein. Regions of identity to *P. carbinolicus* amino acid sequences shown in the corresponding top line are shaded. Amino acids present in three of five aligned sequences are written as consensus. Amino acids conserved among all aligned sequences are shown in boldface. Conserved amino acids of the thiamine pyrophosphate-binding region (28) are marked by asterisks. (A) Multialignment of *P. carbinolicus* E1α with E1α subunits from different sources (4, 20, 35, 65); (B) multialignment of *P. carbinolicus* E1β with E1β subunits from different sources (29, 35, 41, 65). AoDH, E1 of the *P. carbinolicus* acetoin dehydrogenase enzyme system or the *A. eutrophus* acetoin-cleaving system; PDH, E1 of the pyruvate dehydrogenase complex; *P. car.*, *P. carbinolicus*; *A. eut.*, *A. eutrophus*; *S. cere.*, *S. cerevisiae*; *B. subt.*, *B. subtilis*; *B. stea.*, *B. stearothermophilus*; mouse, house mouse testis; human, human liver.

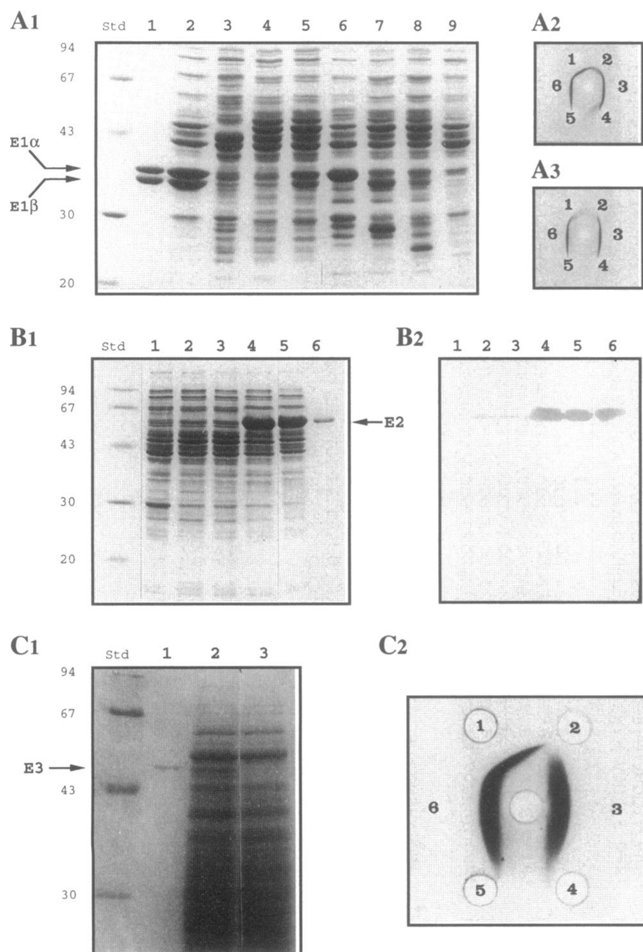

FIG. 5. Expression of *P. carbinolicus aco* genes in *E. coli*. If not otherwise stated, extracts from recombinant *E. coli* were obtained from cells grown for 12 h in LB in the presence of 1.0 mM IPTG. Cell extracts and purified *P. carbinolicus* enzymes were separated in SDS–11.5% (wt/vol) polyacrylamide gels and were also subjected to immunological tests. Polyacrylamide and agarose gels were stained with Serva Blue R. Molecular masses of standard proteins (Std) are given in kilodaltons. Protein bands corresponding to purified *P. carbinolicus* proteins are marked by arrows. (A1 to A3) Expression of *acoA* and *acoB*, encoding E1α and E1β, respectively, in *E. coli* DH5α. (A1) SDS-polyacrylamide gel. Lanes: 1, purified *P. carbinolicus* E1, 7.5 μg of protein; 2, soluble cell fraction from *E. coli* DH5α(pUC8BP25), 20 μg; 3, soluble cell fraction from *E. coli* DH5α(pUCB8P25) grown in the absence of IPTG, 20 μg; 4, soluble cell fraction from *E. coli* DH5α(pUC8-1BP25), 20 μg; 5, soluble cell fraction from *E. coli* DH5α (pUC8-2BP25), 20 μg; 6, cell extract from *E. coli* DH5α(pUC8BN13), 20 μg; 7, cell extract from *E. coli* DH5α(pUC8BP25Δ*Sal*I), 20 μg; 8, cell extract from *E. coli* DH5α(pUC8BP25Δ*Sst*II), 20 μg; 9, soluble cell fraction from *E. coli* DH5α(pUC8BN13), 20 μg. (A2) Immunodiffusion tests with soluble cell fractions from recombinant *E. coli*. (A3) Immunodiffusion tests with soluble cell fractions from recombinant *E. coli* grown in the absence of IPTG. Central well, anti-E1 IgGs (38 μg of protein); well 1, *E. coli* DH5α(pUC8BP25), 10 μg; well 2, *E. coli* DH5α(pUC8-2BP25), 20 μg; well 4, *E. coli* DH5α(pUC8-1BP25), 20 μg; well 5, *E. coli* DH5α(pUC8), 30 μg; wells 3 and 6, purified *P. carbinolicus* E1 (12 μg each). (B1 and B2) Expression of *acoC*, encoding E2, in *E. coli* XL1-Blue. Soluble cell fractions from recombinant *E. coli* and purified *P. carbinolicus* E2 were separated in an SDS-polyacrylamide gel, blotted onto polyvinylidene difluoride membranes, and incubated with anti-E2 IgGs. (B1) SDS-polyacrylamide gel; (B2) Western blot stained with anti-rabbit IgG-alkaline phosphatase conjugate. Lanes: 1, *E. coli* XL1-Blue(pBluescript KS$^-$), 18 μg of protein; 2, *E. coli* XL1-Blue(pKSH48-1), 15 μg; 3, *E. coli* XL1-Blue(pKSH48-1) grown in the absence of IPTG, 15 μg; 4, *E. coli* XL1-Blue(pKSH48), 20 μg; 5, *E. coli* XL1-Blue(pKSH48) grown in the absence of IPTG, 20 μg; 6, purified *P. carbinolicus* E2 (2.5 μg). (C1 and C2) Expression of *acoL*, encoding E3, in *E. coli* S17-1. Soluble cell fractions were obtained from cells grown in the absence of IPTG. (C1) SDS-polyacrylamide gel. Lanes: 1, purified *P. carbinolicus* E3 (1.5 μg of protein); 2, *E. coli* S17-1(pVKH), 35 μg; 3, *E. coli* S17-1(pVK100), 35 μg. (C2) Immunodiffusion test against anti-E3 IgGs. Central well, anti-E3 IgGs (80 μg of protein); well 1, *E. coli* S17-1(pVKH), 220 μg; well 2, *E. coli* S17-1(pVK100), 300 μg; wells 3 and 6, purified *P. carbinolicus* E3 (20 μg).

identical with purified *P. carbinolicus* enzyme (Fig. 5A2 and A3).

Separate heterologous expression of *acoA* and *acoB* in *E. coli*. To determine whether both subunits of E1 or only one is needed for enzyme activity, *acoA* and *acoB* were expressed separately in *E. coli*. For this purpose, deletions were introduced in both genes in pUC8BP25 as described in Materials and Methods. Transformants of *E. coli* DH5α harboring pUCBN13, pUC8BP25Δ*Sal*I, or pUC8BP25Δ*Sst*II (Fig. 2E) were incubated for 12 h in the presence of IPTG. Cell extract from *E. coli*(pUC8BN13) contained large amounts of protein, which exhibited the same electrophoretic mobility as *P. carbinolicus* E1α (Fig. 5A1, lane 6). No protein band representing E1β was visible. In the soluble cell fraction from the same cells of *E. coli*(pUC8BN13), only traces of the *acoA* gene product were visible (Fig. 5A1, lane 9), indicating that most of the synthesized E1α protein had presumably aggregated to inclusion bodies. In SDS-denatured cell extracts of *E. coli*(pUC8BP25Δ*Sal*I) and of *E. coli*(pUC8BP25Δ*Sst*II), a protein band which had the same electrophoretic mobility as *P. carbinolicus* E1β appeared in significant amounts. Concomitant with the loss of a protein band representing E1α, new protein bands which corresponded to proteins with M_rs of 26,500 and 24,500 ± 2,000 were visible (Fig. 5A1, lane 7 and 8). These M_rs agreed with those calculated for the deleted *acoA* gene products, which were 25,554 (*Sal*I deletion) and 21,425 (*Sst*II deletion). E1 activity was not detectable in the cell extract or in the soluble cell fraction of either transformant. Mixing of the different cell extracts from the three IPTG-induced transformants and preincubation under various conditions did not reconstitute E1 activity. Addition of cell extract from the transformants to that of the parent *E. coli* strain, which expressed active E1, or to soluble cell fractions from acetoin-grown *P. carbinolicus* had no effect on E1 activity. These results and the high portion of E1α aggregated in inclusion bodies during separate expression indicated that both subunits of E1 must be coexpressed for correct assembly into functional E1 tetraheteromers, as was previously described for the E1 subunits of mammalian branched-chain 2-oxo acid dehydrogenase complex (16, 87).

Properties of the *acoC* gene product. The E2 polypeptide, as deduced from the nucleotide sequence of *acoC*, consisted of 450 amino acid residues and was highly acidic, with a net charge of −51 at pH 7.0. The calculated molecular weight of 47,281 was significantly lower than the M_r of 60,000, which had been estimated from SDS-denatured purified *P. carbinolicus* E2 (56). Similar discrepancies had been observed for dihydrolipoamide acyltransferases from different sources and had been explained by the anomalous electrophoretic migration of SDS-denatured E2, which is caused by elongated or swollen lipoyl domains (9, 26, 53, 76).

Further analysis of the primary structure of the N-terminal region of E2 revealed two repeating units (amino acid posi-

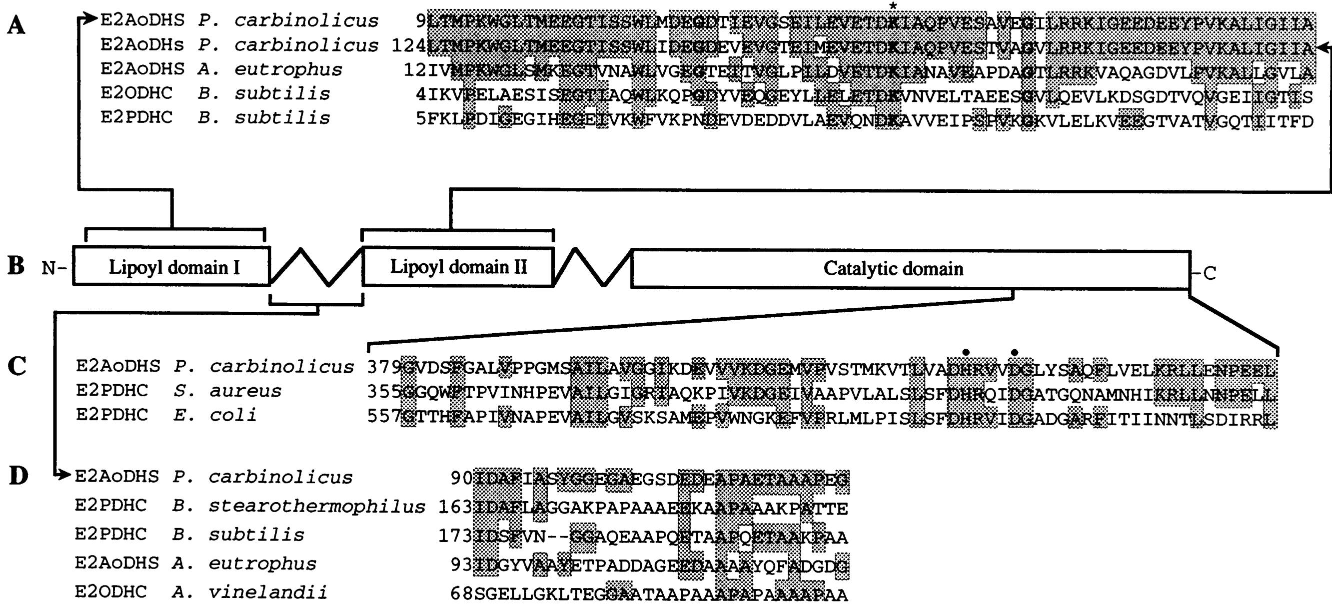

FIG. 6. Putative domain structure of *P. carbinolicus* E2 and partial amino acid sequence comparisons. Sequences were aligned by the program Gap (17). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate positions of the respective amino acids within the protein. Regions of identity to *P. carbinolicus* amino acid sequences shown in the corresponding upper line are shaded. (A) Sequence comparisons between both putative lipoyl domains from *P. carbinolicus* E2 and with the lipoyl domains of other dihydrolipoamide acyltransferases (13, 35, 65). Lysine residues, which are according to the literature (11) presumably lipoylated, are marked by asterisks. Conserved glycine residues, which flank covalently modified lysine residues (11), are in boldface. (B) Putative domain structure of *P. carbinolicus* E2. The relative size of each domain is according to the length of the corresponding amino acid sequences. Zigzag lines indicate potential flexible regions in the C-terminal flanking sequences of the putative lipoyl domains as deduced from the program Peptidestructure (17). (C) Sequence comparison with the catalytic sites of different dihydrolipoamide acetyltransferases (34, 76). Putative active-site histidine and aspartate residues (23, 64, 67) are indicated by dots. (D) Sequence comparison with the interdomain linker segments of different dihydrolipoamide acyltransferases (13, 63, 65, 85). AoDHS, *P. carbinolicus* acetoin dehydrogenase enzyme system or *A. eutrophus* acetoin-cleaving system; ODHC, 2-oxoglutarate dehydrogenase complex; PDHC, pyruvate dehydrogenase complex.

tions 9 to 82 and 124 to 197, respectively; Fig. 3 and 6A), both of which were flanked at their C termini by segments rich in alanine, proline, and charged amino acid residues (Fig. 6D). A comparison with the consensus sequence for the attachment site of lipoate (11) revealed one lysine residue in the center of each repeating unit (Fig. 6A), which is lipoylated in E2 components of 2-oxo acid dehydrogenase complexes. Both repeating units are highly homologous to the putative lipoylation site of the E2 component from the *A. eutrophus* acetoin-cleaving system (65). In addition, homologies to the sequences of the lipoyl domains of the E2 component of the 2-oxoglutarate dehydrogenase complex (13) and of the pyruvate dehydrogenase complex (35) from *B. subtilis* were found (Fig. 6A). The C-terminal flanking regions of the putative lipoyl domains

TABLE 2. Similarities of *P. carbinolicus* E1 subunits to other proteins[a]

Subunit[b]	Source	Reference	Identity to analogous *P. carbinolicus* subunit (mol% amino acids)	Overlap (no. of amino acids)[c]
E1α subunit from:				
AoDHS	*Alcaligenes eutrophus*	65	64.9	322
PDHC	House mouse testis	20	36.3	322
PDHC	*Saccharomyces cerevisiae*	4	36.0	322
PDHC	*Bacillus subtilis*	35	30.7	322
BCDHC	Human	52a	31.8	321
BCDHC	Bovine liver	40	31.2	321
E1β subunit from:				
AoDHS	*A. eutrophus*	65	60.1	336
PDHC	*B. stearothermophilus*	29	39.0	310
PDHC	*B. subtilis*	35	39.8	309
PDHC	Human liver	41	40.6	303
BCDHC	Human placenta	54	41.6	298
BCDHC	Bovine liver	87	41.3	298

[a] Amino acid sequences from E1α and E1β of the *P. carbinolicus* acetoin dehydrogenase enzyme system were compared with those from the corresponding E1 subunits of the *A. eutrophus* acetoin-cleaving system and of 2-oxo acid dehydrogenase complexes from different sources.
[b] AoDHS, acetoin-cleaving system; PDHC, pyruvate dehydrogenase complex; BCDH, branched-chain 2-oxo acid dehydrogenase complex.
[c] The lengths of the amino acid sequences of *P. carbinolicus* E1α and E1β are 326 and 337, respectively.

```
        |-----------FAD-binding----------|          |Disulfide active site|
  1 MADEIFDLIVLGAGPGGYVGAIRAAQLGMKVAVVE------SRPTLGGVCLNEGCIPSKA E3AoDHS P.carb.
  1 MSQK-FDVIVIGAGPGGYVAAIKSAQLGLKTALIEKYKGKEGKTALGGTCLNVGCIPSKA E3P/ODHC A.vin.
  1 --MKSYDVVIIGGGPGGYNAAIRAGQLGLTVACVE------GRSTLGGTCLNVGCMPSKA LPD-3 P.put.
 37 DQPIDADVTVIGSGPGGYVAAIKAAQLGFKTVCIEKNE------TLGGTCLNVGCIPSKA E3P/O/BCDHC Hu.
  1 -MTQKFDVVVIGAGPGGYVAAIKAAQLGLKTACIEKYTDAEGKLALGGTCLNVGCIPSKA E3P/ODHC P.put.
         FDV VIGAGPGGYVAAIKAAQLGLK A  EK    EG  TLGGTCLNVGCIPSKA CONSENSUS
                                                     *    *

 55 LLDSSEHFALARD-KFDMHGIEIPAPKLNLAKMMERKEGVVSDLTGGIAFLFKKNKVTWI E3AoDHS P.carb.
 60 LLDSSYKFHEAHE-SFKLHGISTGEVAIDVPTMIARKDQIVRNLTGGVASLIKANGVTLF E3P/ODHC A.vin.
 53 LLHASELYEAASGDEFAHLGIEV-KPTLNLAQMMKQKDESVTGLAKGIEYLFRKNKVDWI LPD-3 P.put.
 91 LLNNSHYYHMAHGTDFASRGIEMSEVRLNLDKMMEQKSTAVKALTGGIAHLFKQNKVVHV E3P/O/BCDHC Hu.
 60 LLDSSWKYKEAKES-FNVHGISTGEVKMDVAAMVGRKAGIVKNLTGGVATLFKANAFTSF E3P/ODHC P.put.
    LLDSS  Y  A    F  HGI   EV LNLA MM RK   V  LTGGIA LFK NKVT   CONSENSUS

114 KGRGKLLGAGGDGLQQVEVTGKNAGVVKGKNVLLATGGKVAQVPGITVDNDVIIDNVGAL E3AoDHS P.carb.
119 EGHGKLLAGKK---VEVTAADGSSQVLDTENVILASGSKPVEIPPAPVDQDVIVDSTGAL E3P/ODHC A.vin.
112 KGWGRL---DGVGKVVVKAEDGSETALQAKDIVIATGSEPTPLPGVTIDNQRIIDSTGAL LPD-3 P.put.
151 NGYGKITG---KNQVTATKADGGTQVIDTKNILIATGSEVTPFPGITIDEDTIVSSTGAL E3P/O/BCDHC Hu.
119 QGTGKLLAGK---KVEVTKADGTTEVIEAENVILASGSRPIDIPPAPVDQNVIVDSTGAL E3P/ODHC P.put.
     G GKLL        V VT ADG   V   KNV LATGS P   PG TVD DVIVDSTGAL CONSENSUS

        |---------NADH-binding---------|
174 SIDKVPEHLMIIGAGYIGLELGSVWLRLGSKVTVVEMLPKML-PKTDADTTQALQKSLKK E3AoDHS P.carb.
176 DFQNVPGKLGVIGAGVIGLELGSVWARLGAEVTVLEAMDKFL-PAVDEQVAKEAQKILTK E3P/ODHC A.vin.
169 SLPQVPKHLVVIGAGVIGLELGSVWRRLGSQVTVIEYLDRIC-PGTDTETAKTLQKALAK LPD-3 P.put.
208 SLKKVPEKMVVIGAGVIGVELGSVWQRLGADVTAVEFLGHVGGVGIDMEISKNFQRILQK E3P/O/BCDHC Hu.
176 EFQAVPKRLGVIGAGVIGLELGSVWARLGAEVTVLEALDTF-LMAADTAVSKEAQKTLTK E3P/ODHC P.put.
    S   VP   L VIGAGVIGLELGSVW RLGA VTV E LD     P D     K  QK L K CONSENSUS

233 QGMTFNMGTTVGGIEVSGG-KATVKLVKNDKEKEVVCDK--VLMSIGRKPNTDGLGLEEL E3AoDHS P.carb.
235 QGLKILLGARVTGTEVKNK-QVTVKFVDAEGEKSQAFDK--LIVAVGRRPVTTDLLAADS E3P/ODHC A.vin.
228 QGMVFKLGSKVTQATASAD-GVSLVLEPAAGGTAESLQADYVLVAIGRRPYTKGLNLESV LPD-3 P.put.
268 QGFKFKLNTKVTGATKKSDGKIDVSIEAASGGKAEVITCDVLLVCIGRRPFTKNLGLEEL E3P/O/BCDHC Hu.
235 QGLDIKLGARVTGSKVNGNEVE----VTYTNAEGQKITFDKLIVAVGRRPVTTDLLAADS E3P/ODHC P.put.
    QG  FKLG  VTG  V         V  V A G K       D LLVAIGRRP T  L LE  CONSENSUS

290 GVEMGERGTIKVDDNYATNVPGIYAIGDLIPGPMLAHKASEEAVVFVERLVGKNSEVHYG E3AoDHS P.carb.
292 GVTLDERGFIYVDDYCATSVPGVYAIGDVVRGAMLAHKASEEGVVVAERIAGHKAQMNYD E3P/ODHC A.vin.
287 GLETDKRGML-AQRTPPTSVPGVWVIGDVTSGPMLAHKAEDEAVACIERIAGKPHEVNYN LPD-3 P.put.
328 GIELDPRGRIPVNTRFQTKIPNIYAIGDVVAGPMLAHKAEDEGIICVEGMAGGAVHIDYN E3P/O/BCDHC Hu.
291 GVTIDERGYIFVDDYCATSVPGVYASVTWYAGMMLAHKASEEGIMVVERIKGHKAQMNYD E3P/ODHC P.put.
    GVE DERG I VDD  ATSVPGVYAIGDV  GPMLAHKASEEGV  VERIAG      NY  CONSENSUS

350 TIPGVCYTWPEVASVGKTEQQLQEEGTPVKVGKFNFVGNGRARAMAETEGFVKIIAHAEN E3AoDHS P.carb.
352 LIPAVIYTHPEIAGVGKTEQALKAEGVAINVGVFPFAASGRAMAANDTAGFVKVIADAKT E3P/ODHC A.vin.
346 LIPGVIYTRPELATVGKTEEQLKAEGRAYKVGKFPFTANSRAKINHETEGFAKVIADAET LPD-3 P.put.
388 CVPSVIYTHPEVAWVGKSEEQLKEEGIEYKVGKFPFAANSRAKTNADTDGMVKILGQKST E3P/O/BCDHC Hu.
351 LIPSVVYTHPEIAWVGKTEQALKAK-VENVGTFPFAASGRAMAANDTGGFVKVIADAKT E3P/ODHC P.put.
    LIP VIYTHPE A VGKTEQQLKAEG   KVGKFPFAANGRA A  DT GFVKVIADA T CONSENSUS

                                 |-----------Interface region-----------|
410 GQVLGVHIFGPRASDMIAEAVAVMSYGGTAHDIGAMFHGHPTLSEAVKEAALDVDGAAVHC- E3AoDHS P.carb.
412 DRVLGVHVIGPSAAELVQQGAIAMEFGTSAEDLGMMVFAHPALSEALHEAALAVSGHAIHVA E3P/ODHC A.vin.
406 DEVLGVHLVGPSVSEMIGEFCVAMEFSASAEDIALTCHPHPTRSEALRQAAMNVDGMAMQI- LPD-3 P.put.
448 DRVLGAHILGPGAGEMVNEAALALEYGASCEDIARVCHAHPTLSEAFREANLAASFGKSINF E3P/O/BCDHC Hu.
410 DRVLGVHVIGPSAAELVQQGAIAMEFGTSAEDLGMMVFSHPTLSEALHEAALAVNGGAIHVA E3P/ODHC P.put.
    DRVLGVH  GPSA EMV E A AMEFG SAEDIG M H HPTLSEAL EAALAV G A H   CONSENSUS
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FIG. 7. Amino acid sequence comparisons of *P. carbinolicus* E3 with other proteins. Sequences have been aligned by the program Multalign (44). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate positions of the respective amino acids within the protein. Regions of identity to *P. carbinolicus* E3 amino acid sequence shown in the corresponding top line are shaded. Amino acids present in three of five aligned sequences are written as consensus. Amino acids conserved among all aligned sequences are in boldface. Putative regions for the redox-active disulfide site, for the interface, and for flavin adenine dinucleotide (FAD)- or NADH-binding sites are indicated above the aligned sequences. Conserved active-site cysteine, histidine, and glutamate residues are marked by asterisks and dots. Sequences are from references 59, 61, 62, and 85. AoDHS, acetoin dehydrogenase enzyme system; E3P/O/BCDHC, common E3 component of pyruvate, 2-oxoglutarate, and branched-chain 2-oxo acid dehydrogenase complexes; *P. carb.*, *P. carbinolicus*; *A. vin.*, *A. vinelandii*; *P. put.*, *P. putida*; Hu, human liver.

resembled those of the interdomain linker segments of dihydrolipoamide acyltransferases from gram-positive bacteria (Fig. 6D), which in addition to the characteristic alanine and proline residues contain many charged and polar residues (13, 35, 63). Similarities to the corresponding linker segments of the E2 components from the *A. eutrophus* acetoin-cleaving system (65) and from the *Azotobacter vinelandii* 2-oxoglutarate dehydrogenase complex (85) were also found. Because of their elongated lengthy structure, these linker segments are excellent substrates for proteases, resulting (under the condition of limited proteolysis) in the cleavage of the protein into distinct fragments (8, 25, 67). This had been also demonstrated for *P. carbinolicus* E2 as a result of limited tryptic digestion (56). The C-terminal region of *P. carbinolicus* E2 exhibited significant homologies to the corresponding regions of the catalytic domain of E2 components, such as from the pyruvate dehydrogenase complexes of *Staphylococcus aureus* (34) and *E. coli* (76), including the putative active-site histidine-aspartate couple (Fig. 6C).

Heterologous expression of *acoC* in *E. coli*. Overexpression of E2 was achieved in strains of *E. coli* XL1-Blue(pKSH48), which contained *acoC* in a colinear orientation to *lacZ'* (Fig. 2A). In the soluble fraction from cells which were grown in LB medium for 12 h, E2 activity was determined to be 146 U/mg of protein, which was sevenfold higher than the activity determined in the soluble cell fraction from acetoin-grown *P.*

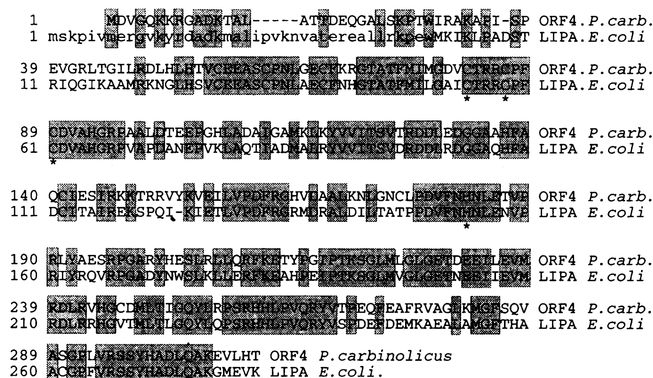

FIG. 8. Pairwise comparison of the deduced amino acid sequence of *P. carbinolicus* ORF4 and the sequence of the *E. coli lipA* gene product (LIPA). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate positions of the respective amino acids within the deduced amino acid sequences (for LIPA, positions are indicated as published by Hayden et al. [31]). Lowercase letters represent the N-terminal amino acid sequence as published by Reed and Cronan (66). Identical amino acids are shaded. Potential conserved active-site amino acid residues of LIPA (31) are marked by asterisks. *P. carb.*, *P. carbinolicus.*

carbinolicus (56). The protein pattern of the SDS-denatured soluble cell fraction of *E. coli* XL1-Blue(pKSH48) revealed one dominant protein band, which exhibited the same electrophoretic mobility as E2 purified from *P. carbinolicus* and gave a strong reaction with anti-E2 IgGs on a corresponding Western blot (Fig. 5B1 and B2). Only low activity (5.3 U/mg) and weak immunological reaction were obtained with the soluble cell fractions from *E. coli* XL1-Blue(pKSH48-1), which contained *acoC* in an antilinear orientation to *lacZ'*.

Properties of the *acoL* gene product. Analysis of the nucleotide sequence revealed that *acoL* encoded a protein with an M_r of 49,394 comprising 470 amino acid residues. Recently an M_r of 54,000 had been determined for E3 purified from *P. carbinolicus* (56). Comparisons of the deduced amino acid sequence with the primary structures of proteins collected in the data libraries revealed striking similarities to various dihydrolipoamide dehydrogenases. Greatest homologies were obtained with the E3 component, which is shared by the pyruvate and 2-oxoglutarate dehydrogenase complex in *A. vinelandii* (85) (48.7% identity in a 470-amino-acid overlap), with the third dihydrolipoamide dehydrogenase (LPD-3) of *Pseudomonas putida* (62) (50.1% identity in a 467-amino-acid overlap), with the E3 component, which is shared by the three human 2-oxo acid dehydrogenase complexes (59) (48.7% identity in a 460-amino-acid overlap), and with LPD-glc, which is the E3 component shared by the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complex in *P. putida* (61) (46.6% identity in a 470-amino-acid overlap). The consensus sequence deduced from the multialigned amino acid sequences (Fig. 7) contains several sequence motifs of known function (14, 70). The N-terminal region of *P. carbinolicus* E3 contained, for example, the flavine adenine dinucleotide-binding motif (from Asp-7 to Glu-35), whereas the NADH-binding domain, which is similar to the former, was identified in the central region (from His-181 to Glu-209). Furthermore, the conserved disulfide active-site motif including both essential cysteine residues was localized between Thr-39 and Ala-54. The redox-active cysteine residues of one subunit from the dimeric enzymes are thought to interact during catalytic action with a histidine-glutamate couple from the conserved interface region at the C terminus of the other subunit (14, 70). Both were also identified as His-449 and Glu-454 in the corresponding region of *P. carbinolicus* E3.

Heterologous expression of *acoL* in *E. coli*. Expression of *P. carbinolicus* E3 was determined in the soluble cell fraction of *E. coli* S17-1 harboring plasmid pVKH after 12 h of growth in LB with 0.35 U/mg of protein, which was significantly higher than the activity of the host enzyme (0.01 U/mg). On SDS-acrylamide gels, one faint protein band appeared in the protein pattern of the SDS-denatured soluble cell fraction of *E. coli* S17-1(pVKH), which exhibited the same electrophoretic mobility as purified *P. carbinolicus* E3 (Fig. 5C1); an immunodiffusion test revealed an immunologically identical protein (Fig. 5C2).

Properties of the ORF4 gene product. The polypeptide deduced from the nucleotide sequence of ORF4 (Fig. 2B) comprised 310 amino acids, and an M_r of 34,421 was calculated. In contrast to the *aco* gene products mentioned above, it is slightly basic, with a net charge of +2 at pH 7.0. The primary structure of the putative protein encoded by ORF4 is strikingly homologous to that deduced from the *E. coli lipA* gene (31) (64.7% identity in a 278-amino-acid overlap). The pairwise alignment exhibited an almost complete overlap, with only some minor discrepancies in the N-terminal region (Fig. 8), which may be caused by some uncertainties regarding the translational initiation site of *E. coli lipA* (31, 32, 66). Significant homologies to any other protein sequence stored in the data libraries were not obtained. Molecular genetic studies provided strong evidence that the *E. coli lipA* gene product is involved in the conversion of octanoate into lipoic acid (32, 66).

To investigate the protein-coding function of ORF4, plasmid pKSH48ΔExoIII (Fig. 2E) was digested with *Dde*I, resulting in a 1.2-kbp fragment harboring ORF4. The fragment was treated with mung bean nuclease and ligated with *Eco*RV-linearized pBluescript SK$^-$. Four transformants of *E. coli* XL1-Blue harboring plasmid pSKD12, which contained the 1.2-kbp fragment in an antilinear orientation to *lacZ'*, were obtained. To express ORF4 from the *lacZ* promoter, pSKD12 was restricted with *Hin*dIII plus *Pst*I. The resulting 1.2-kbp fragment was ligated to *Hin*dIII-plus-*Pst*I-restricted pBluescript KS$^-$, and the resulting plasmid, pKSHP12 (Fig. 2E), was transformed into *E. coli* XL1-Blue. The transformants were incubated for 12 h in LB medium in the presence of either 1.0 mM IPTG or 0.6% (wt/vol) glucose. On polyacrylamide gels with SDS-denatured cell extract obtained from cells grown in the presence of IPTG, a faint but distinct protein band appeared with an estimated molecular weight of 34,000 ± 2,000, which corresponded well to the value calculated from ORF4 (data not shown). Such a protein band appeared neither in cell extract from glucose-repressed recombinants nor in cell extract from *E. coli* harboring only the vector. To investigate the physiological function of the putative ORF4 gene product in lipoate biosynthesis, pKSHP12 and pSKD12 were transformed into cells of the lipoate auxotrophic *E. coli* strains JRG33 and JRG26 (36), which both are affected in *lipA* (32). After lag phases of 10 and 22 h, respectively, *E. coli* JRG33(pKSHP12) and *E. coli* JRG26(pKSHP12) grew in lipoate-deficient medium, whereas transformants harboring pSKD12 or the vector did not grow without DL-lipoate as a supplement. Growth of the pKSHP12-harboring strains in lipoate-deficient medium was only slightly slower (with growth rates of 0.28 and 0.27 h^{-1}, respectively) than growth in medium supplemented with 35 or 70 nM DL-lipoate (0.29 h^{-1}). The restoration of lipoate prototrophy in pSKHP12-harboring transformants demon-

strated that ORF4 encodes a protein that is most probably involved in lipoic acid biosynthesis of *P. carbinolicus.*

DISCUSSION

The structural genes for the E1 component (*acoA* and *acoB*), which is composed of two different subunits, for the E2 component (*acoC*), and for the E3 component (*acoL*) of the *P. carbinolicus* acetoin dehydrogenase enzyme system were identified, sequenced, and expressed in *E. coli*. These four genes and ORF4 are clustered on a 6.1-kbp region of the genome. The codon preferences of *acoA*, *acoB*, *acoC*, *acoL*, and ORF4 are in good agreement, and all *aco* genes and ORF4 are preceded by tentative ribosome-binding sites. The organization of *P. carbinolicus acoA*, *acoB*, and *acoC* is similar to that of the corresponding structural genes of the acetoin-cleaving system from the strictly respiratory *A. eutrophus* (65). In *A. eutrophus*, upstream of *acoA*, the structural gene *acoX*, which encodes a protein of unknown function, a −24/−12 promoter, and *acoR*, which encodes presumably a regulatory protein for the σ^{54}-dependent transcription of *acoXABC* (45, 65), were identified. Similar genes or promoter-like structures were not identified in the *aco* region of *P. carbinolicus*. The regions downstream of the respective *acoC* genes were also different in both acetoin-utilizing bacteria: whereas in *A. eutrophus* no other genes relevant for acetoin catabolism were identified in this region (65), in *P. carbinolicus*, ORF4 and *acoL* were localized in the downstream region. The molecular organization of both *aco* gene clusters is similar to that of the genes for the components of 2-oxo acid dehydrogenase complexes, especially those that are composed of E1 components with a heteromeric structure, e.g., the pyruvate dehydrogenase complexes from gram-positive bacteria (13, 29, 34). Whereas the gene for the respective E3 component is not in all cases included in those clusters, the genes for the respective E1 and E2 components were found to be organized in one operon, with the latter as the distal gene.

The deduced amino acid sequences of both subunits of *P. carbinolicus* E1 exhibited striking homologies to the corresponding E1 subunits of the *A. eutrophus* acetoin-cleaving system (65). Furthermore, high degrees of homologies occurred with the corresponding subunits of various 2-oxo acid dehydrogenases. The amino acid sequence of *P. carbinolicus* E1α contains the thiamine pyrophosphate-binding motif (28), which is consistent with previous findings that the DCPIP-dependent oxidative hydrolytical cleavage of acetoin and the physiological overall reaction of the acetoin dehydrogenase enzyme system are thiamine pyrophosphate-dependent reactions (56–58). In analogy to the proposed catalytic function of 2-oxo acid dehydrogenase α subunits (29), *P. carbinolicus* E1α may catalyze the initial nucleophilic attack on acetoin. Less is known about the function of 2-oxo acid dehydrogenase β subunits. The increased formation of E1α-containing inclusion bodies during separate heterologous expression of *acoA* may indicate that the β subunit is required for correct assembly of the E1 heterotetramers or for binding of E1 to the E2 core (16, 29, 87). No extended homology was obtained with the second structural type of E1 components from 2-oxo acid dehydrogenase complexes, which is composed of one rather than two types of subunits, e.g., the E1 component of the pyruvate dehydrogenase complex of *E. coli* (75).

The amino acid sequence deduced from *acoC* for the E2 component revealed striking similarities to the unique multidomain structure of dihydrolipoamide acyltransferases (67). The N-terminal portion is dominated by two highly homologous domains, which each presumably contain one lipoylated lysine residue. The C-terminal region of the remainder is similar to the corresponding regions from different dihydrolipoamide acyltransferases, including a conserved histidine-aspartate couple, which is thought to be involved in the E2-catalyzed acetyl transfer (23, 64, 67). Between the putative catalytic domain and the N-terminal remainder as well as between both putative lipoyl domains, there are regions which show significant resemblance to the interdomain linker segments (hinge regions) of different dihydrolipoamide acyltransferases (Fig. 6D). These hinge regions are thought to provide flexibility to the lipoyl domains, facilitating active-site coupling within the multienzyme complexes (67). From the number of lipoyl domains, the enzyme from *P. carbinolicus* resembles the E2 components of the mammalian pyruvate dehydrogenase complexes (63, 64). Most dihydrolipoamide acyltransferases contain only one lipoyl domain per subunit, whereas the E2 components of the pyruvate dehydrogenase complexes of *E. coli* and *A. vinelandii* contain three highly similar lipoyl-bearing domains (63). The high degree of homology of parts of the amino acid sequences of *P. carbinolicus* E2 to corresponding regions of *A. eutrophus* E2 confirms the postulated catalytic function of the latter as a dihydrolipoamide acetyltransferase (65).

The amino acid sequence deduced from *acoL* for *P. carbinolicus* E3 exhibited striking homologies to dihydrolipoamide dehydrogenases from various prokaryotic and eukaryotic sources and shares with them the characteristic features of the enzyme group of pyridine nucleotide-disulfide oxidoreductases (14, 18). Interestingly, a very high degree of homology was obtained with the third dihydrolipoamide dehydrogenase of *P. putida* (LPD-3), the physiological function of which is unknown (62). As it is known that strains of *P. putida* grow on 2,3-butanediol (60) and on acetoin (33), LPD-3 may be involved in the oxidative cleavage of acetoin. The involvement of a dihydrolipoamide dehydrogenase in the *A. eutrophus* acetoin-cleaving system is still under investigation in our laboratory.

A comparison of the amino acid sequence deduced from ORF4 revealed an almost perfect overlap to the amino acid sequence of the *E. coli lipA* gene product. Together with *lipB*, *lipA* belongs to the *lip* locus (66, 82), which is involved in lipoic acid biosynthesis. *lipA* was independently sequenced and characterized by two groups (31, 66), resulting in slightly different interpretations concerning the size and the function of the *lipA* gene product. Hayden et al. (32) suggested that *lipA* codes for a protein of 281 amino acids and that it catalyzes the two-step incorporation of both sulfur atoms into octanoic acid. In contrast, Reed and Cronan (66) had determined a translational start site 40 codons upstream of that published by Hayden et al. (Fig. 8), and the authors suggested that the *lipA* gene product is involved in the incorporation of only one sulfur atom either at C-6 or at C-8 of octanoic acid. The *lip* locus is located on the *E. coli* chromosome at ca. 14.5 min (15) between *rna* (14 min, encoding RNase I [78]) and *dacA* (15 min, encoding D-alanine carboxypeptidase [10]), which is part of a gene cluster involved in peptidoglycan synthesis. Therefore, *lipA* is located far from *aceF* (3 min) and *sucA* (17 min [2]), which encode the final target proteins of lipoic acid biosynthetic enzymes in *E. coli*. In contrast, in *P. carbinolicus*, ORF4 is located in a colinear orientation downstream of *acoC*, which encodes a potential target protein of the *P. carbinolicus* lipoic acid biosynthetic apparatus. The localization of a gene for lipoate synthesis within the *aco* genes in *P. carbinolicus* may indicate that the restricted catabolic metabolism of this bacterium requires no further enzyme system, which contain lipoylated proteins (56–58, 71). As ORF4 encodes information able to restore lipoate synthesis in different *E. coli* strains mutated

in *lipA*, and as ORF4 is located between *acoC* and *acoL* in a colinear orientation to the *aco* genes, ORF4 was referred to as *acoS*.

Further studies must focus on the following. (i) From the data presented for the *P. carbinolicus* acetoin dehydrogenase enzyme, it is obvious that a close relationship exists between this and the acetoin-cleaving system of *A. eutrophus* as well as between the acetoin-degrading enzyme systems and the 2-oxo acid dehydrogenase complexes, which consist of heteromeric E1 components. As the *P. carbinolicus* acetoin dehydrogenase enzyme system does not accept 2-oxo acids as substrates (56), this system did not evolve from 2-oxo acid dehydrogenase complexes by a simple broadening of the substrate spectrum of the E1 component. For investigation of the phylogenetic relationship to the 2-oxo acid dehydrogenase complexes, it is necessary to obtain molecular data about acetoin-degrading enzyme systems from other phylogenetically more distant organisms, i.e., gram-positive bacteria. For this reason, molecular analysis of the genes of the acetoin dehydrogenase enzyme system from *Clostridium magnum* (50) is in progress. (ii) As significant heterologous expression of the *P. carbinolicus aco* genes occurred only if these genes were localized in a colinear orientation close to an *E. coli* promoter, and as no homologies to known promoter structures were obtained in the sequenced region, the initiation site(s) of transcription of the *aco* genes from this strictly anaerobic bacterium remains to be identified. From the localization of the genes and of hairpin-like structures, it is likely that *acoA*, *acoB*, and *acoC* constitute a single operon that is transcribed from a promoter upstream of *acoA*, whereas *acoS* and *acoL* constitute a second operon. (iii) The molecular data presented in this and in a previous study (65) offer the possibility of investigating the compatibility of the components of the anaerobic *P. carbinolicus* acetoin dehydrogenase enzyme system and of the aerobic *A. eutrophus* acetoin-cleaving system. This effort will also contribute to an understanding of the enzymology of acetoin cleavage in *A. eutrophus* and the genetic control of the *P. carbinolicus* acetoin dehydrogenase enzyme system.

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