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Biochemical and Molecular Characterization of the *Clostridium magnum* Acetoin Dehydrogenase Enzyme System

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E2 (dihydrolipoamide acetyltransferase) and E3 (dihydrolipoamide dehydrogenase) of the *Clostridium magnum* acetoin dehydrogenase enzyme system were copurified in a three-step procedure from acetoin-grown cells. The denatured E2-E3 preparation comprised two polypeptides with M_rs of 49,000 and 67,000, respectively. Microsequencing of both proteins revealed identical amino acid sequences. By use of oligonucleotide probes based on the N-terminal sequences of the α and β subunits of E1 (acetoin dehydrogenase, thymine PP_i dependent), which were purified recently (H. Lorenzl, F. B. Oppermann, B. Schmidt, and A. Steinbüchel, Antonie van Leeuwenhoek 63:219–225, 1993), and of E2-E3, structural genes *acoA* (encoding E1α), *acoB* (encoding E1β), *acoC* (encoding E2), and *acoL* (encoding E3) were identified on a single *Cla*I restriction fragment and expressed in *Escherichia coli*. The nucleotide sequences of *acoA* (978 bp), *acoB* (999 bp), *acoC* (1,332 bp), and *acoL* (1,734 bp), as well as those of *acoX* (996 bp) and *acoR* (1,956 bp), were determined. The amino acid sequences deduced from *acoA*, *acoB*, *acoC*, and *acoL* for E1α (M_r, 35,532), E1β (M_r, 35,541), E2 (M_r, 48,149), and E3 (M_r, 61,255) exhibited striking similarities to the amino acid sequences of the corresponding components of the *Pelobacter carbinolicus* acetoin dehydrogenase enzyme system and the *Alcaligenes eutrophus* acetoin-cleaving system, respectively. Significant homologies to the enzyme components of various 2-oxo acid dehydrogenase complexes were also found, indicating a close relationship between the two enzyme systems. As a result of the partial repetition of the 5′ coding region of *acoC* into the corresponding part of *acoL*, the E3 component of the *C. magnum* acetoin dehydrogenase enzyme system contains an N-terminal lipoyl domain, which is unique among dihydrolipoamide dehydrogenases. We found strong similarities between the AcoR and AcoX sequences and the *A. eutrophus acoR* gene product, which is a regulatory protein required for expression of the *A. eutrophus aco* genes, and the *A. eutrophus acoX* gene product, which has an unknown function, respectively. The *aco* genes of *C. magnum* are probably organized in one single operon (*acoABXCL*); *acoR* maps upstream of this operon.

Within the last 5 years, significant advances have been made in the knowledge of the catabolism of acetoin (3-hydroxy-2-butanone), resulting from studies that focused on the acetoin-utilizing, gram-negative bacteria *Pelobacter carbinolicus* (50–53) and *Alcaligenes eutrophus* (22, 39, 57–59, 68). In the strictly fermentative and anaerobic bacterium *P. carbinolicus*, the key and initial step of the degradation of acetoin is the thiamine PP_i (TPP)-, coenzyme A-, and NAD-dependent cleavage of acetoin into acetaldehyde and acetyl coenzyme A, which is catalyzed by the acetoin dehydrogenase enzyme system (50). This type of reaction is analogous to the oxidative decarboxylation of 2-oxo acids (pyruvate, 2-oxoglutarate, and branched-chain 2-oxo acids, respectively), which are catalyzed by the respective 2-oxo acid dehydrogenase complexes (55, 56). Like the latter, the acetoin dehydrogenase enzyme system is composed of three enzyme components, which are E1 (acetoin dehydrogenase, TPP dependent), a heterotetramer ($\alpha_2\beta_2$); E2 (dihydrolipoamide acetyltransferase), a homomultimer; and E3 (dihydrolipoamide dehydrogenase), a homodimer. Recent studies (51) identified the structural genes for E1α (*acoA*), E1β (*acoB*), E2 (*acoC*), and E3 (*acoL*) of the *P. carbinolicus* acetoin dehydrogenase enzyme system, which were clustered on a 6.1-kbp region of the genome. The amino acid sequences deduced from *acoA*, *acoB*, *acoC*, and *acoL* exhibited remarkable similarities to the primary structures of the corresponding components of 2-oxo acid dehydrogenase complexes which possess heteromeric E1 components. In addition, the genes of the acetoin dehydrogenase enzyme system and the respective genes of 2-oxo acid dehydrogenase complexes were organized similarly, indicating a close relationship of the *P. carbinolicus* acetoin dehydrogenase enzyme system to 2-oxo acid dehydrogenase complexes.

In the strictly respiratory bacterium *A. eutrophus*, acetoin breakdown is catalyzed in a similar way (57). A heterodimeric, TPP-dependent acetoin dehydrogenase and a dihydrolipoamide acetyltransferase were also identified as putative E1 and E2 components, respectively, of an *A. eutrophus* acetoin-cleaving system; both revealed striking homologies to the corresponding E1 and E2 components of the *P. carbinolicus* acetoin dehydrogenase enzyme system (51). The participation of a dihydrolipoamide dehydrogenase (E3) in the acetoin cleavage reaction of *A. eutrophus* is obscure and is still being investigated.

Although the strictly anaerobic gram-positive acetoin-degrading bacterium *Clostridium magnum* is, on the basis of its homoacetogenic type of fermentation (66) and its phylogenetic status, distinct from both *P. carbinolicus* and *A. eutrophus*, synthesis of E1, E2, and E3 occurred during growth on acetoin (50), indicating that an acetoin dehydrogenase enzyme system of a similar type is also involved in acetoin breakdown in this bacterium. *C. magnum* E1, which was recently purified, exhibited an M_r of 138,000 and consisted of two different subunits, α (M_r, 38,500) and β (M_r, 34,000) (43). The N-terminal amino

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
C. magnum Wo BD P1	Wild type, strictly anaerobic, prototrophic	66; DSM2767[a]
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 ΔlacU169* (ϕ80 *lacZΔM15*)	26
Plasmids		
pBluescript SK$^+$	Ap^r *lacPOZ'*; T7 and T3 promoter	Stratagene
pUC18 or pUC19	Ap^r *lacPOZ'*	27
pSK::Cla85	8.5-kbp insert from *C. magnum* DNA in pBluescript SK$^+$ harboring *acoA*, *acoB*, *acoX*, *acoC*, and *acoL*	This study
pSK::Xba46	4.6-kbp insert from *C. magnum* DNA in pBluescript SK$^+$ harboring *acoR* and *acoA*	This study
pSK::Xba38	3.9-kbp insert from *C. magnum* DNA in pBluescript SK$^+$ harboring *acoX* and *acoC*	This study
pSK::Hpa54-0	5.4-kbp insert from *C. magnum* DNA in pBluescript SK$^+$ harboring *acoA*, *acoB*, *acoX*, and *acoC* colinear with respect to *lacZ'*	This study
pSK::Hpa54-1	5.4-kbp insert from *C. magnum* DNA in pBluescript SK$^+$ harboring *acoA*, *acoB*, *acoX*, and *acoC* antilinear with respect to *lacZ'*	This study
pSK::SC39	derivative from pSK::Cla85 containing 3.9-kbp insert harboring *acoC* and *acoL* colinear with respect to *lacZ'*	This study
pUC19::SK34	derivative from pSK::SC39 containing 3.4-kbp insert harboring *acoC* and *acoL* colinear with respect to *lacZ'*	This study
pUC18::SK34	derivative from pSK::SC39 containing 3.4-kbp insert harboring *acoC* and *acoL* antilinear with respect to *lacZ'*	This study

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, FRG.

acid sequences of both subunits revealed significant homologies to the N termini of the corresponding E1 subunits from *P. carbinolicus* and *A. eutrophus*. In this report, we describe the purification of E2 and E3 and the identification and characterization of the structural genes of the acetoin dehydrogenase enzyme system of *C. magnum*.

MATERIALS AND METHODS

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

Growth of bacteria. *C. magnum* was grown anaerobically in the presence of 0.3% (wt/vol) acetoin or glucose under a gas atmosphere of 80% (vol/vol) nitrogen and 20% (vol/vol) carbon dioxide at 30°C in modified GV medium (described in the 1992 Deutsche Sammlung von Mikroorganismen catalog of strains), which had the following composition (per liter): K_2HPO_4, 0.348 g; KH_2PO_4, 0.227 g; NH_4Cl, 0.5 g; NaCl, 2.25 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 2.0 mg; trace element solution SL-7 (82), 1.0 ml; vitamin solution (82), 10.0 ml; yeast extract, 1.0 g; $NaHCO_3$, 3.0 g. After the medium was reduced by addition of 0.3 g of cysteine hydrochloride per liter and 0.3 g of $Na_2S \cdot 9H_2O$ per liter, the pH was finally adjusted to 7.2 *Escherichia coli* was grown at 37°C in Luria-Bertani medium (64).

Preparation of cell extract. Cells of *C. magnum* were disrupted by threefold passage through a French press at 96 MPa. Cells of *E. coli* were disrupted by sonication for 1 min/ml of cell suspension at an amplitude of 14 μm. Cell disruption was performed in the presence of 10 mM EDTA–1 mM phenylmethylsulfonyl fluoride–1 μM leupeptin–1 μM pepstatin. Soluble cell fractions were obtained as supernatants from 50 min of centrifugation at 100,000 × *g* and 4°C. Protein was determined as described by Lowry et al. (44).

Enzyme assays. E1, E2 (EC 2.3.1.12), and E3 (EC 1.8.1.4) were determined spectrophotometrically as described previously (50), 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.

Purification of E2 and E3. All steps were carried out at 4°C. A 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MOPS) buffer (pH 7.2) containing 1% (wt/vol) ammonium sulfate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin was used throughout the purification procedure. Phenylmethylsulfonyl fluoride was added from a 200 mM stock solution in 2-propanol immediately before the buffer was used. The soluble cell fraction (approximately 0.5 g of protein) derived from about 5 g (wet weight) of cells was applied to a column (2.6 by 89 cm; 470-ml bed volume [BV]) of Sephadex G-200 equilibrated with buffer. Protein was eluted with a constant flow rate of 0.019 BV/h. Fractions containing high enzyme activity were combined and applied to a column (2.6 by 10 cm; 53-ml BV) of DEAE-Sephacel equilibrated with buffer. After the column was washed with 2 BV of buffer, the protein was eluted with a KCl gradient (0 to 400 mM) at a constant flow rate of 0.5 BV/h. Fractions containing high enzyme activity were combined, concentrated, and washed by ultrafiltration in a Diaflo chamber with a PM 30 membrane. The enzyme solution was applied to a Mono Q HR5/5 column which had been equilibrated with buffer supplemented with 0.1% (vol/vol) Triton X-100. After the column was washed with 2 BV of buffer, the protein was eluted with a linear KCl gradient (0 to 600 mM) at a flow rate of 1 ml/min. Fractions with high enzyme activity were combined, concentrated, and washed by ultrafiltration. The enzyme preparation was stored at −20°C in the presence of 10% (vol/vol) glycerol.

Preparation of triacine dye media. Procion Brown MX-5BR, Procion Red HE-3B, and Procion Blue MX-2G were coupled to Sepharose CL-4B by the procedure of Atkinson et al. (4).

Electrophoretic methods. Native polyacrylamide gel electrophoresis (PAGE) was performed with 7% (wt/vol) gels in a flat gel apparatus for vertical slab electrophoresis with 125 mM Tris–19 mM borate buffer, pH 8.9 (67). Linear polyacrylamide gradient gels (5 to 27.5%, wt/vol) were run at a constant power of 7 W for 48 h (2). Sodium dodecyl sulfate (SDS)-PAGE was performed with 11.5% (wt/vol) gels as described by Laemmli (40). E2 was visualized in polyacrylamide gels by precipitation of $Ca_3(PO_4)_2$ in the presence of 10 mM acetylphosphate–0.1 mM coenzyme A–5 U of phosphotransacetylase from *Bacillus*

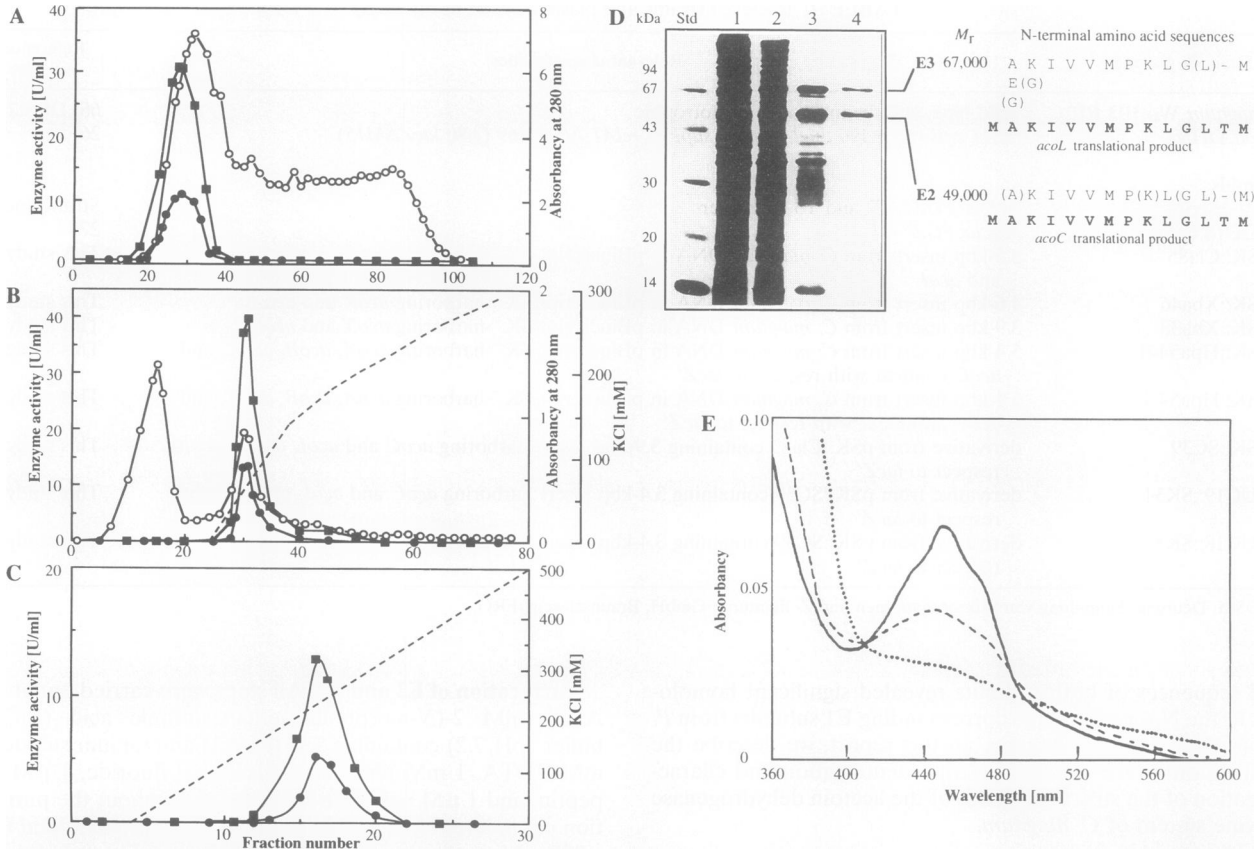

FIG. 1. Copurification of E2 and E3 of the *C. magnum* acetoin dehydrogenase enzyme system from acetoin-grown cells. (A) Gel filtration on Sephadex G-200. Protein (351 mg) from the soluble cell fraction was applied to a column (2.6 by 89 cm; 473-ml BV) of Sephadex G-200 equilibrated with buffer (pH 7.2) containing 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MOPS), 1% (wt/vol) ammonium sulfate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin. Protein was eluted at a constant flow rate of 0.019 BV/h, and 5.0-ml fractions were collected. (B) Chromatography on DEAE-Sephacel. Protein (183 mg) from the E2-E3-containing protein preparation after gel filtration on Sephadex G-200 was applied to a column (2.6 by 10 cm; 53-ml BV) of DEAE-Sephacel equilibrated with buffer. After the column was washed with 2 BV of buffer, the protein was eluted with a KCl gradient (0 to 400 mM) at a constant flow rate of 0.5 BV/h and 3.0-ml fractions were collected. (C) Chromatography on Mono Q HR. Protein (19.9 mg) from the E2-E3-containing pool obtained from chromatography on DEAE-Sephacel was applied to a Mono Q HR5/5 column equilibrated with buffer supplemented with 0.1% (vol/vol) Triton X-100. After the column was washed with 2 BV of buffer, the protein was eluted with a linear KCl gradient (0 to 600 mM) at a flow rate of 1 ml/min. Fractions of 1.0 ml each were collected. Symbols: ■, dihydrolipoamide acetyltransferase (E2); ●, dihydrolipoamide dehydrogenase (E3); ○, A_{280}; – – –, KCl. (D) SDS-PAGE of E2 and E3. Samples were separated in SDS–11.5% (wt/vol) polyacrylamide gels and stained with Serva Blue R. Lanes: 1, 30 μg of protein from the soluble cell fraction; 2, 10 μg of protein after gel filtration on Sephadex G-200; 3, 9 μg of protein after chromatography on DEAE-Sephacel; 4, 5 μg of protein after chromatography on Mono Q HR. On the right side, the N-terminal sequences obtained for the two major proteins of the E2-E3 preparation after chromatography on Mono Q HR (upper lines) are aligned with those of the *acoL* and *acoC* translational products. On the left side, the sizes of molecular mass standard proteins (lane Std) are provided. (E) Absorption spectra of the E2-E3 preparation after chromatography on Mono Q HR. Spectra were determined anaerobically under an N_2 atmosphere in 25 mM MOPS (pH 7.0)–1% (wt/vol) ammonium sulfate with the purified E2-E3 preparation after chromatography on Mono Q HR (1.4 mg/ml of protein). The oxidized protein (—) was reduced by addition of 0.01 (– – –) or 0.1 (· · ·) mM NADH.

stearothermophilus–5 mM dihydrolipoamide during incubation of the gel at 30°C in 100 mM Tris/HCl (pH 7.0) containing 50 mM $CaCl_2$. Activity staining of gels for E3 was done in the presence of NADH, lipoamide, and 5-nitroblue tetrazolium chloride as described previously (53).

Flavin identification. Absorption spectra of purified E2-E3 preparations were obtained with a Lambda 3 UV/VIS double-beam spectrophotometer (Perkin Elmer Bodenseewerk, Überlingen, Federal Republic of Germany [FRG]). Samples were measured anaerobically under an atmosphere of nitrogen at room temperature against a reference containing the sample buffer. Enzyme-bound flavin was liberated by thermal denaturation of the protein in a boiling water bath for 15 min in the dark and centrifugation for 15 min at 15,000 × *g* (18). Flavins were separated and identified by high-performance liquid chromatography on a reversed-phase column as described previously (50).

N-terminal sequence analysis. For determination of N-terminal amino acid sequences, purified SDS-denatured E2 and E3 were electroblotted from SDS-polyacrylamide gels in 25 mM Tris–192 mM glycine–5% (vol/vol) methanol (pH 8.4) onto a polyvinylidene difluoride membrane (75) using a semi-dry Fast Blot B33 apparatus (Biometra GmbH, Göttingen, FRG) at a constant 5 mA/cm^2, in accordance with the instruc-

TABLE 2. Purification of the E2 and E3 components of the *C. magnum* acetoin dehydrogenase enzyme system

Step	Vol (ml)	Protein concn (mg/ml)	Total protein (mg)	E2				E3			
				Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Crude extract	6.5	54.0	351.0	1,170	3.33	1.0	100	585	1.71	1.0	100
Sephadex G-200	61.0	3.0	183.0	1,220	6.67	2.0	104	580	3.17	1.9	99.1
DEAE-Sephacel	11.7	1.7	19.9	516	25.9	7.8	44.1	294	14.8	8.7	50.3
Mono Q	8.4	1.4	11.8	285	24.2	7.3	24.4	174	14.8	8.7	29.7

tions of the manufacturer. Proteins on the membrane were stained with Serva Blue R. Areas of the membrane corresponding to E2 and E3, respectively, were cut out and subjected to sequence analysis with a 477A pulsed liquid-phase protein-peptide sequencer (33) and a 120A on-line phenylthiohydantoin amino acid analyzer (62) (both from Applied Biosystems, Weiterstadt, FRG) in accordance with the instructions of the manufacturer. At positions where no signal was obtained, cysteine might be the actual amino acid present, since the protein was not carboxymethylated before being sequenced.

Immunological methods. Double-immunodiffusion tests and Western blot immunoblot analysis of soluble cell fractions and E2-E3 preparations were performed as described before (51).

Isolation and manipulation of DNA. Total genomic DNA from *C. magnum* was obtained as described by Saito and Miura (63). Plasmid DNA was isolated from *E. coli* by the alkaline extraction procedure (7). Restrictions of DNA were performed with various restriction endonucleases under the conditions described by Sambrook et al. (64) or by the manufacturer. Other DNA-manipulating enzymes were used as described by the manufacturer. DNA fragments were isolated from agarose gels either by using a GeneClean kit (Bio 101, La Jolla, Calif.) (76) or by the centrifugation technique (79).

Transformation. For transformation, *E. coli* was grown in LB medium containing 10 mM $MgCl_2$ and 10 mM $MgSO_4$ (26). Competent cells were prepared and transformed by the calcium chloride procedure (64).

Synthesis of oligonucleotides. Synthesis of oligonucleotides was performed in 0.2-μmol portions from deoxynucleotide phosphoramidites (5) with a Gene Assembler Plus apparatus (Pharmacia-LKB Biotechnology, Uppsala, Sweden) in accordance with the instructions of the manufacturer. Release of the oligonucleotides from the supports and removal of the protection groups were achieved by 15 h of incubation at 55°C in a 32% (vol/vol) ammonia solution. Oligonucleotides were purified by gel filtration in NAP-5 columns. The following oligonucleotides were synthesized in accordance with the N-terminal amino acid sequences of purified components of the *C. magnum* acetoin dehydrogenase enzyme system (at wobble positions, the different bases which are bracketed were used considering the low G+C content of *C. magnum* genomic DNA [66]): probe Iα, 5′-AA[AG]AT[AT]AT[AT]GATATG TATAA[AG]-3′ (in accordance with the amino acids at positions 6 to 12 of E1α [43]); probe Iβ, 5′-ATGAA[AG]A C[AT]ATGAC[AT]TATATGGAA-3′ (in accordance with the amino acids at positions 1 to 8 of E1β [43]); probe II/III, 5′-AAAAT[AT]GT[AT]GT[AT]ATGCC[AT]AA-3′ (in accordance with the amino acid sequence KIVVMPK, which was determined for both polypeptides of the purified E2-E3 preparation). For DNA-DNA hybridizations, oligonucleotides were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (64).

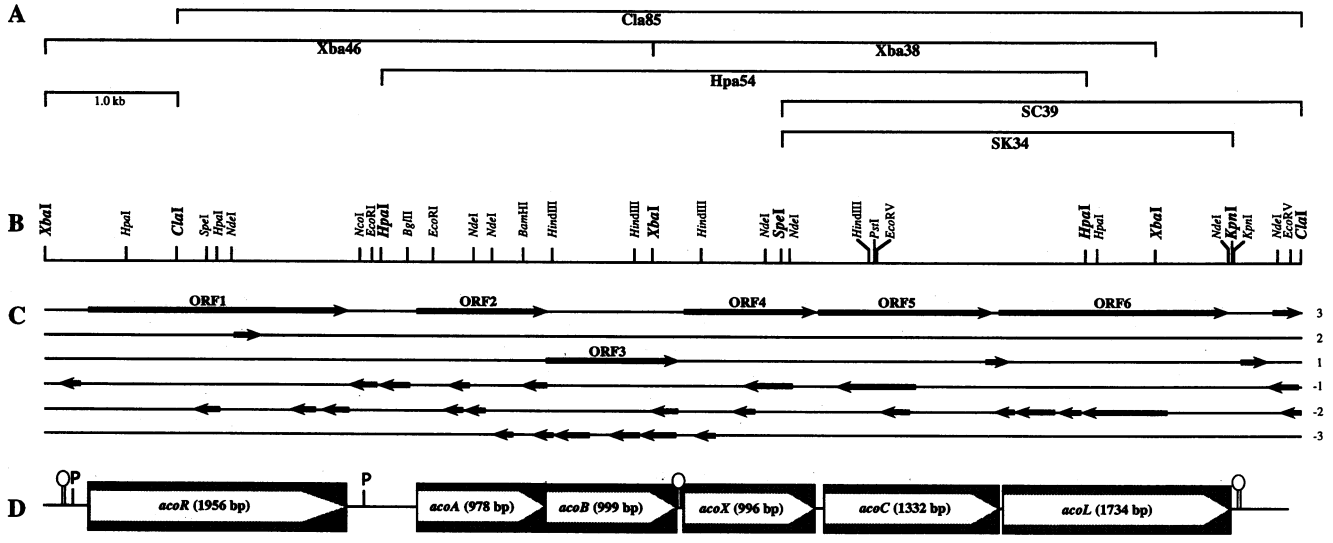

FIG. 2. Molecular organization of the *C. magnum aco* gene cluster. (A) Restriction map of the region analyzed in this study and of relevant fragments. (B) Relevant restriction sites of the sequenced region (sites also shown in panel A are in boldface). (C) ORFs detected in the sequenced region. ORFs comprising more than 150 bp, which were identified in the six reading frames of both orientations, are symbolized by arrows. (D) Structural genes of the *aco* gene cluster. The positions of putative promoters (P) and hairpin-like structures are indicated.

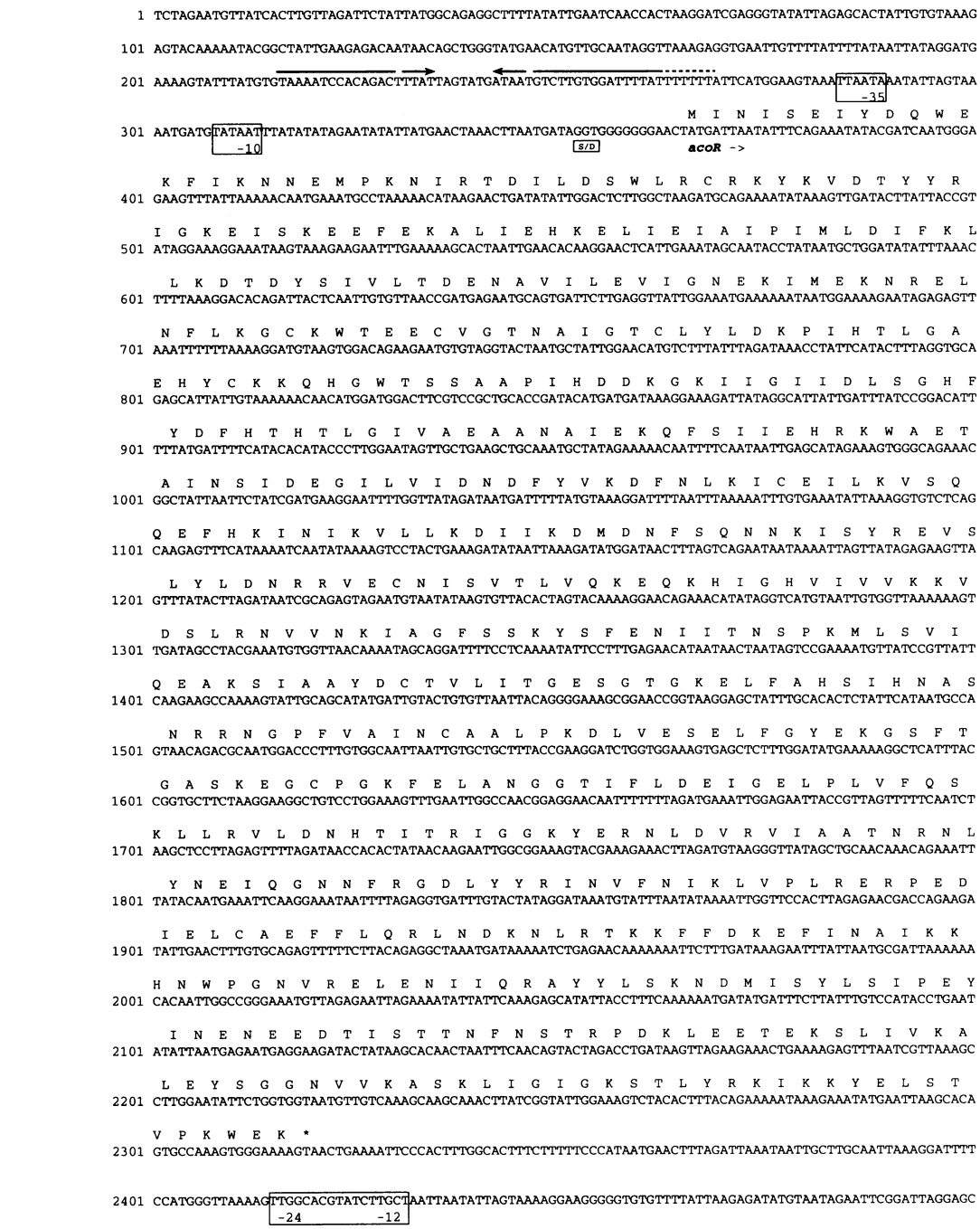

FIG. 3. Nucleotide sequence of the *C. magnum aco* gene cluster. Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations. The amino acid sequences determined for the N termini of purified *C. magnum* E1α, E1β, E2, and E3 are overlined. The positions of putative −35/−10 and −24/−12 promoters, the putative integrated host factor-binding site (IHF-BS), and putative ribosomal binding sites (S/D) are indicated. The positions of hairpin-like structures are marked by inverted arrows.

Cloning of the *aco* gene locus. *C. magnum* genomic DNA was digested with various restriction enzymes and separated by 0.8% (wt/vol) agarose gel electrophoresis. Southern blot analysis (64) was performed at 30°C in 6× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) with ^{32}P-labeled oligonucleotide probes Iα, Iβ, and II/III. Following hybridization, the membranes were washed at 43°C in 2× SSC containing 1% SDS. Chromosomal *Xba*I fragments of 3 to 5 kbp and *Cla*I fragments of 6 to 10 kbp corresponding in size to fragments which hybridized with the labeled oligonucleotides were recovered from Tris-acetate-agarose gels by using the GeneClean kit, inserted into *Xba*I- or *Cla*I-treated pBluescript SK$^+$, and transformed into *E. coli* DH5α. For selection of positive clones, colony hybridization (24) was performed with the same probes.

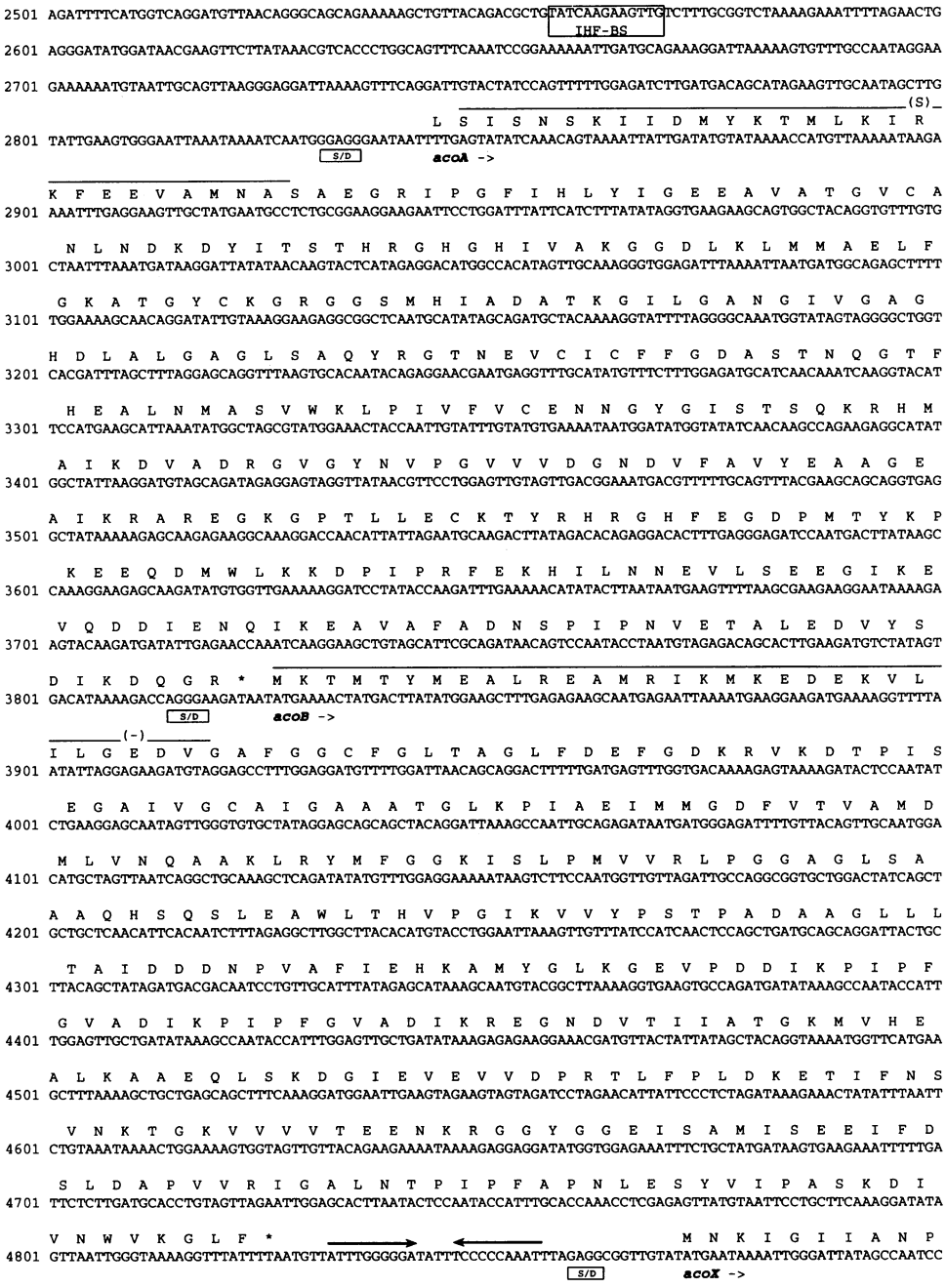

FIG. 3—*Continued.*

DNA sequence analysis. DNA sequences were determined from double-stranded, alkali-denatured plasmid DNA by the dideoxy-chain termination method (65) with the T7 polymerase sequencing kit and [α-^{35}S]dATP in accordance with the instructions of the manufacturer (Pharmacia-LKB Biotechnology). Synthetic oligonucleotides were used as primers (71). 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_2 EDTA, 50% [wt/vol] urea) in an S2 sequencing apparatus (GIBCO/BRL GmbH, Eggenstein, FRG) and visualized on X-ray films.

Analysis of sequence data. Nucleic and amino acid sequences were analyzed with computer programs from the Genetics Computer Group Sequence Analysis Software Package (17).

Chemicals. DNA-modifying enzymes were obtained from GIBCO/BRL GmbH; coenzyme A, FMN, and M_r standard proteins were obtained from Sigma Chemie (Deisenhofen, FRG); DL-lipoamide was purchased from Serva Feinbiochemica (Heidelberg, FRG); all other coenzymes and phosphotransacetylase were obtained from C. F. Boehringer & Söhne (Mannheim, FRG); RNase-free DNase, phosphoramidites, agarose NA, NAP-5 columns, DEAE-Sephacel, Sephadex G-200, Superose 12, phenyl Sepharose CL-4B, and a Mono Q HR5/5 column were from Pharmacia (Freiburg, FRG); Pro-

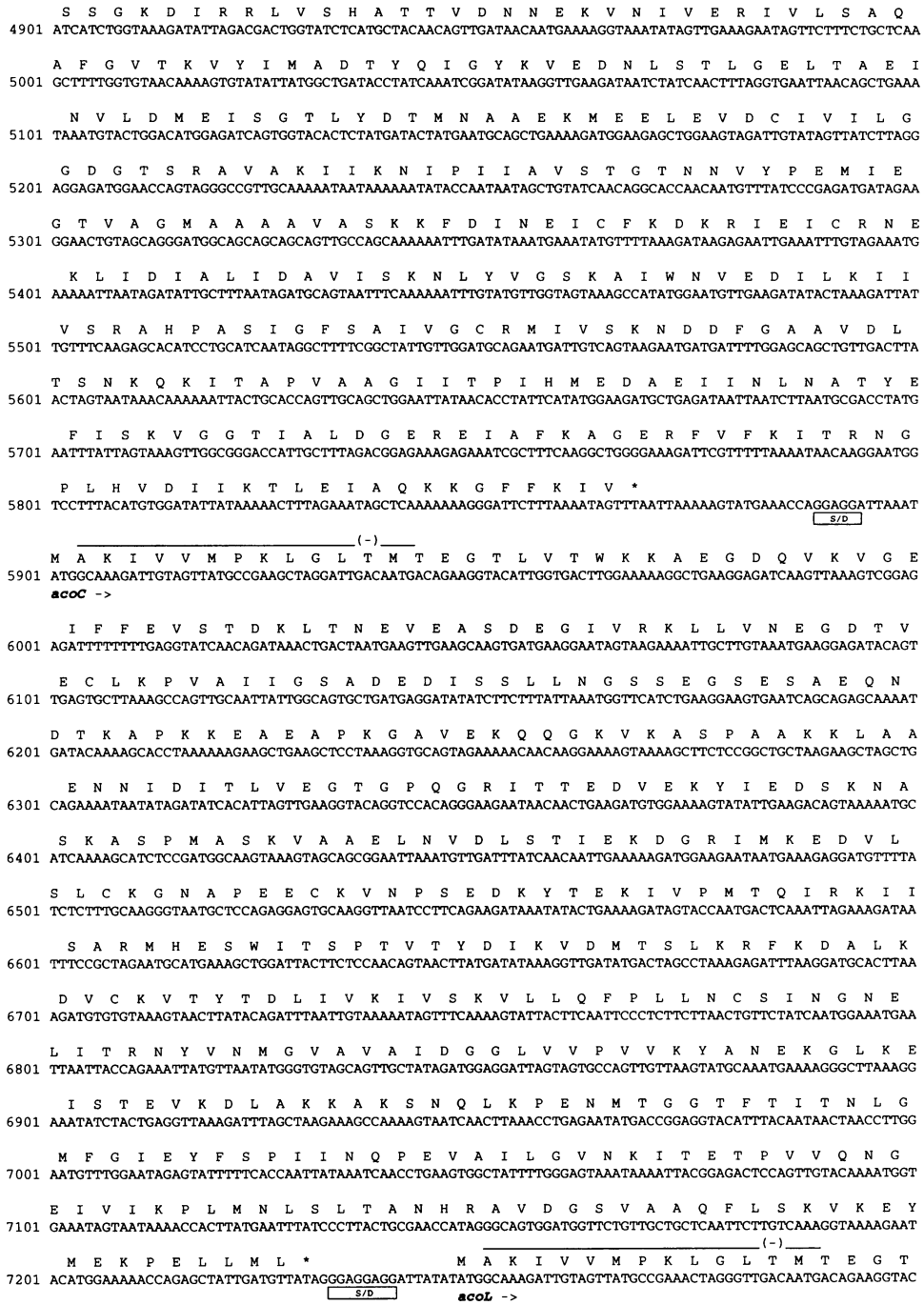

FIG. 3–*Continued.*

cion Brown MX-5BR, Procion Red HE-3B, and Procion Blue MX-2G were from Deutsche ICI (Frankfurt, FRG); polyvinylidene difluoride membranes were obtained from Millipore (Bedford, Mass.); radioisotopes were from Amersham/Buchler (Braunschweig, FRG); acetoin was from Fluka Chemie (Buchs, Switzerland); and complex media were from Difco Laboratories (Detroit, Mich.). All other chemicals were from E. Merck AG (Darmstadt, FRG), Fluka Chemie, Serva Feinbiochemica, or Sigma Chemie.

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported in this study have been submitted to the Genome Sequence Database at the Los Alamos National Laboratory under accession number L31844.

RESULTS

Purification of E2 and E3 of the *C. magnum* acetoin dehydrogenase enzyme system. The E2 and E3 components of the *C. magnum* acetoin dehydrogenase enzyme system were copurified from acetoin-grown cells by a three-step procedure: coelution of both activities occurred during gel filtration on Sephadex G-200 in the void volume (Fig. 1A), during chroma-

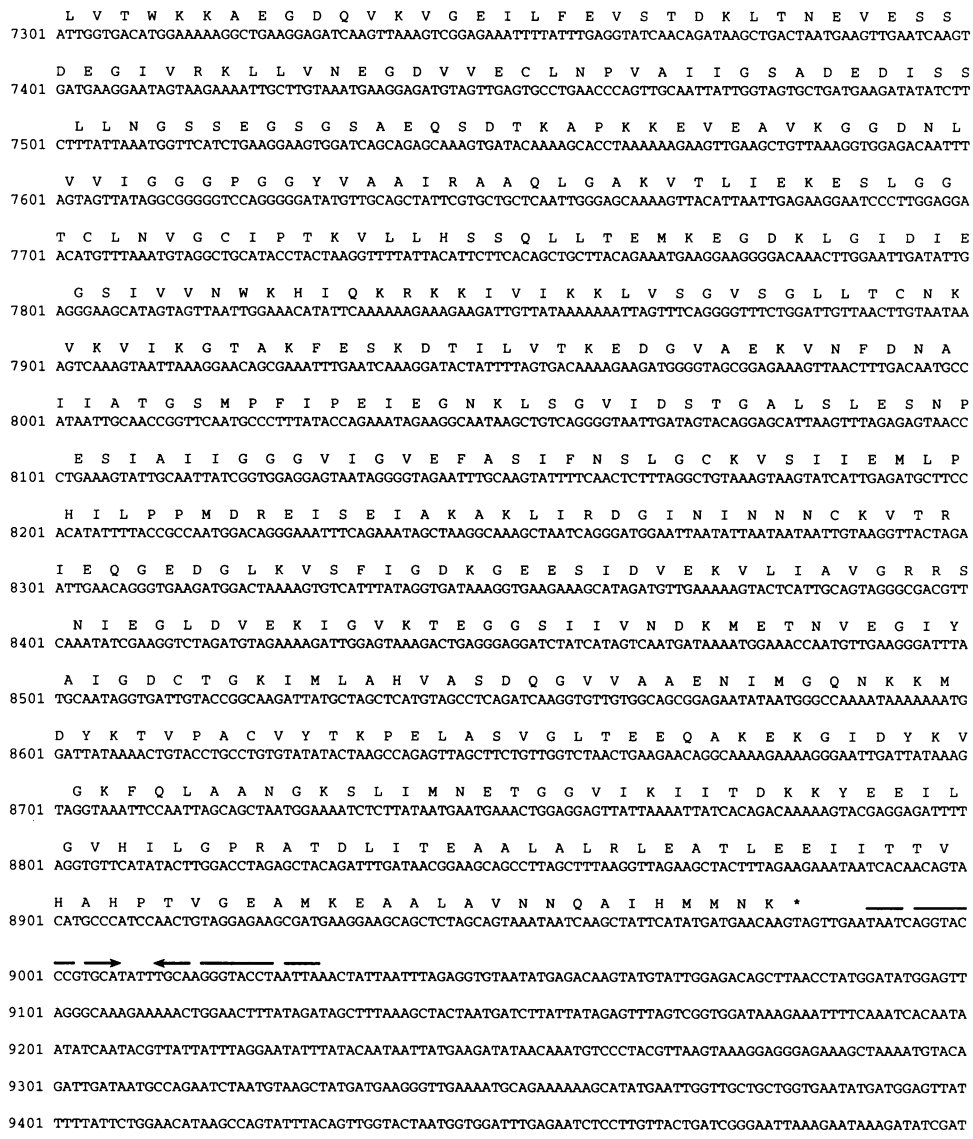

FIG. 3—*Continued.*

tography on DEAE-Sephacel at 50 to 80 mM KCl (Fig. 1B), and during chromatography on Mono Q HR at 200 to 300 mM KCl (Fig. 1C). The ratio between the two activities remained almost constant over final 7.3-fold and 8.7-fold purifications, respectively (Table 2).

SDS-PAGE of the E2-E3 preparation after chromatography on Mono Q HR resulted in two protein bands, representing proteins with M_rs of 49,000 and 67,000 (±1,000) (Fig. 1D). Determination of the N termini revealed almost identical amino acid sequences for the two SDS-denatured polypeptides (Fig. 1D). We found significant homologies between these sequences and the N termini of the E2 component of the *P. carbinolicus* acetoin dehydrogenase enzyme system (51), as well as to the E2 component of the *A. eutrophus* acetoin-cleaving system (57), whereas similarities to the corresponding highly conservative N-terminal regions of prokaryotic dihydrolipoamide dehydrogenases (12) were not found. As the conserved part of the N-terminal amino acid sequence of dihydrolipoamide dehydrogenases is known to be involved mainly in binding of the cofactor flavin adenin dinucleotide (FAD) (12), the E2-E3 preparation was examined for the presence of FAD. The absorption spectrum from 360 to 600 nm of the yellowish E2-E3 preparation exhibited the typical absorption profile of flavoproteins (83), with one maximum at 454 nm and with two shoulders at 430 and 470 nm, respectively, which decreased during anaerobic titration with NADH (Fig. 1E). The flavin component was liberated from the E2-E3 preparation by heat treatment and was identified as FAD by high-performance liquid chromatography.

The molecular weight(s) of the protein(s) of the E2-E3 preparation under native conditions could not be determined exactly either by linear gradient (5 to 27.5%, wt/vol) PAGE or by gel filtration on Superose 12 or Sephadex G-200 (Fig. 1A). However, from the data obtained, it was concluded that the native M_r of both enzymes was higher than 600,000. In double-immunodiffusion tests and Western blots performed with the purified E2-E3 preparation, as well as with the soluble fraction of acetoin-grown cells of *C. magnum*, no immunolog-

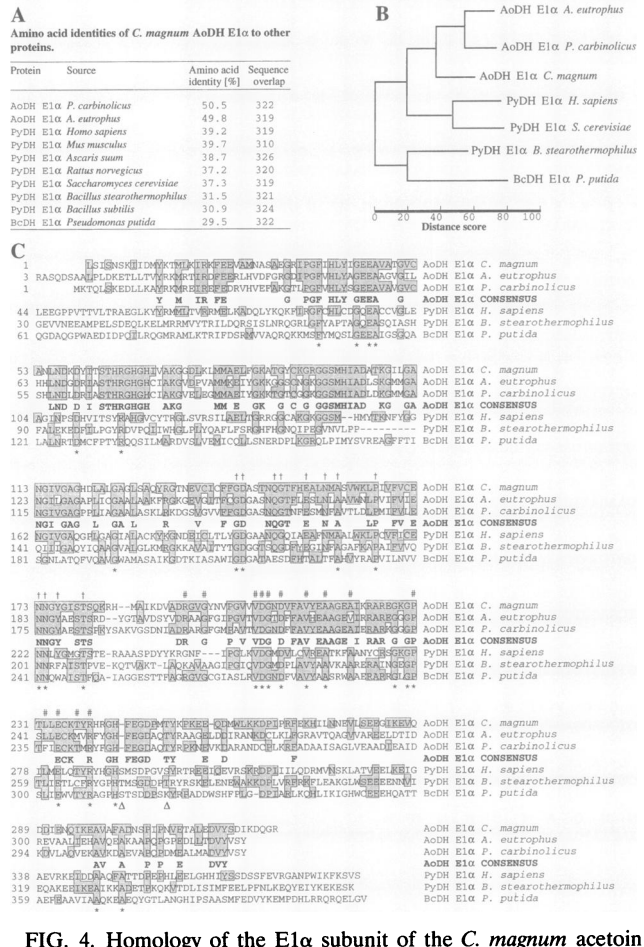

FIG. 4. Homology of the E1α subunit of the *C. magnum* acetoin dehydrogenase enzyme system to other proteins. AoDH, *P. carbinolicus* acetoin dehydrogenase enzyme system or *A. eutrophus* acetoin-cleaving system; PyDH, pyruvate dehydrogenase complex; BcDH, branched-chain 2-oxo acid dehydrogenase complex. (A) Similarities of the *C. magnum* AoDH E1α subunit to other proteins. The amino acid sequence of *C. magnum* AoDH E1α was compared to the primary structures of proteins stored in the SWISSPROT data library (6, 11, 14, 20, 29, 32, 34, 37, 45, 51, 57) by using the program FASTA (17). (B) Phylogenetic tree for the AoDH E1α subunits and the E1α subunits of 2-oxo acid dehydrogenase complexes from different relevant sources. The branching order and distance score were calculated by the program TREE as described by Feng and Doolittle (19). (C) Multialignment of the AoDH E1α subunits and E1α subunits of 2-oxo acid dehydrogenase complexes from different relevant sources. Sequences were aligned by using the program MULTALIGN (38). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate the positions of the respective amino acids within the protein. Regions with identity to the *C. magnum* E1α amino acid sequence shown in the upper line are shaded. Amino acids conserved in all three AoDH E1α subunits are labeled AoDH E1α CONSENSUS; amino acids conserved among all of the aligned sequences are marked by asterisks below the aligned sequences. Conserved amino acids of the putative TPP-binding region (28, 41, 80) and the putative E1β subunit binding site (80) are marked by the symbols † and ≠, respectively, above the aligned sequences. The positions of serine residues, which represent phosphorylation sites in *H. sapiens* PyDH E1α (14, 80), are marked by triangles.

ical reactions were obtained with immunoglobulins G specific for E2 and E3 of the *P. carbinolicus* acetoin dehydrogenase enzyme system (51).

Different methods were used to separate the two activities under native conditions. During ammonium sulfate precipitation, both activities were coprecipitated at 40% (wt/vol) ammonium sulfate. From hydrophobic interaction chromatography on phenyl Sepharose CL-4B, less than 15% of both activities could be recovered, even with 80% (vol/vol) ethylene glycol in the elution buffer. It seems that both proteins were tightly bound to the column. Both activities also bound to the triazine dye affinity media Procion Brown MX-5BR–Sepharose, Procion Red HE-3B–Sepharose, and Procion Blue MX-2G–Sepharose and coeluted at approximately 500 mM KCl. Heat treatment with subsequent centrifugation, which was previously used as the final step in the purification of the E3 component from the *P. carbinolicus* acetoin dehydrogenase enzyme system (50), resulted in the coprecipitation of both proteins and almost complete loss of the enzyme activities. These results indicated a tight association of the E2 and E3 components of the *C. magnum* acetoin dehydrogenase enzyme system under native conditions.

Identification of the *C. magnum aco* gene cluster. Southern hybridization analysis of *Cla*I-digested *C. magnum* genomic DNA identified a fragment of 8.5 kbp (Cla85; Fig. 2) which hybridized with oligonucleotide probes Iα, Iβ, and II/III, which corresponded to the N-terminal amino acid sequences obtained from E1α, E1β (43), and the E2-E3 preparation, respectively. Hybridization of *Xba*I-digested DNA resulted in a 4.6-kbp signal (Xba46; Fig. 2) specific for the Iα and Iβ probes and in a 3.8-kbp probe II/III-specific signal (Xba38; Fig. 2). *Cla*I and *Xba*I restriction fragments from 7 to 10 kbp and from 3 to 5 kbp, respectively, were isolated from an agarose gel and ligated into *Cla*I- or *Xba*I-digested pBluescript SK$^+$ and used to transform *E. coli* DH5α. Colony hybridization with oligonucleotide probes Iα and Iβ identified four positive *Xba*I clones specific for Iα, two positive *Xba*I clones specific for Iβ, and one *Cla*I clone specific for both probes.

As depicted in Fig. 2, sequence analysis revealed that these fragments comprised 9,500 bp exhibiting a G+C content of 34.1 mol%, which corresponded to the G+C content of 29.1 mol% determined for the total genomic DNA (66). Four open reading frames (ORFs) among 37 with a minimum length of 150 bp (ORF2, ORF3, ORF5, and ORF6 in Fig. 2) were identified as the structural genes encoding the four known subunits of the *C. magnum* acetoin dehydrogenase system.

DNA sequence analysis of *acoA* and *acoB*. The N terminus of the E1α subunit of the *C. magnum* acetoin dehydrogenase enzyme system (43) was identical to the amino acid sequence from positions 9 to 35 deduced from ORF2, which corresponded to the structural gene of E1α and was therefore designated *acoA*. Position 8 would be represented by a leucine residue, which is encoded by the putative start codon TTG (position 2844 in Fig. 3). TTG occurs as an alternative start codon in approximately 1% of prokaryotic genes (46). The notion that TTG also functions as the start codon of *acoA* was strengthened by the presence of a reliable Shine-Dalgarno sequence at a distance of 8 bp upstream of the TTG codon. Since a serine residue at the second position of a nascent polypeptide often causes removal of the N-terminal amino acid (21), the formylmethionine residue of the isolated E1α protein was presumably removed during posttranslational processing. The E1α polypeptide encoded by *acoA* consisted of 326 amino acid residues and had a calculated M_r of 35,532, which corresponded to the value of 38,500 (±1,000) that had been

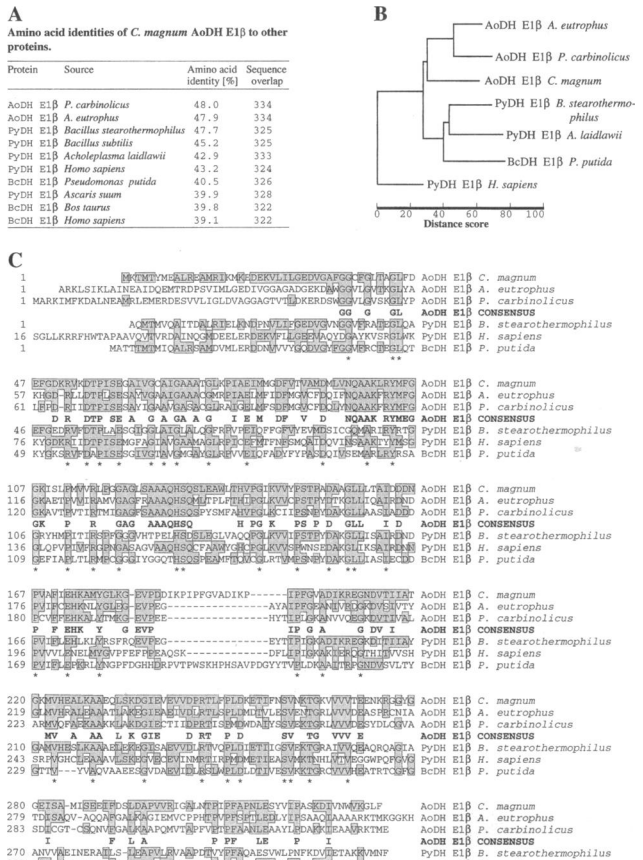

FIG. 5. Homology of the E1β subunit of the *C. magnum* acetoin dehydrogenase enzyme system to other proteins. AoDH, *P. carbinolicus* acetoin dehydrogenase enzyme system or *A. eutrophus* acetoin-cleaving system; PyDH, pyruvate dehydrogenase complex; BcDH, branched-chain 2-oxo acid dehydrogenase complex. (A) Similarities of the *C. magnum* AoDH E1β subunit to other proteins. The amino acid sequence of *C. magnum* AoDH E1β was compared with the primary structures of proteins stored in the SWISSPROT data library (11, 29, 32, 35, 48, 49, 51, 57, 77, 81) by using the program FASTA (17). (B) Phylogenetic tree for the AoDH E1α subunits and the E1β subunits of 2-oxo acid dehydrogenase complexes from different relevant sources. The branching order and distance score were calculated by the program TREE as described by Feng and Doolittle (19). (C) Multialignment of AoDH E1α subunits and E1α subunits of 2-oxo acid dehydrogenase complexes from different relevant sources. Sequences were aligned by using the program MULTALIGN (38). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate the positions of the respective amino acids within the protein. Regions with identity to the *C. magnum* E1β amino acid sequence shown in the upper line are shaded. Amino acids conserved in all three AoDH E1β subunits are labeled AoDH E1β CONSENSUS; amino acids conserved among all of the aligned sequences are marked by asterisks below the aligned sequences.

previously determined for the α subunit of SDS-denatured E1 from *C. magnum* (43).

The amino acid sequence deduced from ORF3 corresponded exactly to the N terminus of the E1β subunit of the *C. magnum* acetoin dehydrogenase enzyme system and was referred to as *acoB*. The start codon of *acoB* (ATG at position 3826 in Fig. 3) is separated by only 1 bp from the stop codon of *acoA*. At a distance of 7 bp, the start codon of *acoB* is preceded by a reliable Shine-Dalgarno sequence. The polypeptide deduced from the nucleotide sequence of *acoB* comprised 333 amino acid residues and corresponded to a calculated M_r of 35,541, which was in agreement with the M_r of 34,000 (±1,000) that had been estimated from SDS-denatured *C. magnum* E1β (43).

Comparison of the amino acid sequences of the *C. magnum* E1 subunits deduced from *acoA* (Fig. 4) and *acoB* (Fig. 5), respectively, with the primary structures of proteins collected in the data libraries revealed approximately 50% amino acid identity to the corresponding E1 subunits of the *P. carbinolicus* acetoin dehydrogenase enzyme system (51), as well as those of the *A. eutrophus* acetoin-cleaving system (57) (Fig. 4A and 5A). We also found strong similarities to the corresponding subunits of the heteromeric type of E1 components of 2-oxo acid dehydrogenase multienzyme complexes from various eukaryotic and prokaryotic sources (Fig. 4A and 5A), i.e., the pyruvate dehydrogenase complexes from *Homo sapiens* (14, 48) and *B. stearothermophilus* (29) and the branched-chain 2-oxo acid dehydrogenase complex from *Pseudomonas putida* (11). We found no significant homologies to the E1 components of 2-oxo acid dehydrogenase complexes, which are composed of only one type of subunit, i.e., the *E. coli* pyruvate dehydrogenase complex (69). The E1β polypeptide of *C. magnum* exhibited the strongest homologies to the E1β subunits of the pyruvate dehydrogenase complexes of two *Bacillus* species and *Acholeplasma laidlawii*, which is phylogenetically related to the *Clostridium* and *Bacillus* line of gram-positive bacteria (77). The E1α polypeptide of *C. magnum* revealed the strongest homologies to the E1α subunits of the pyruvate dehydrogenase complexes from eukaryotic sources, whereas the similarities to the corresponding prokaryotic proteins were relatively weak.

The central region of the amino acid sequence of *C. magnum* E1α matched a fingerprint sequence motif (Fig. 4C) which had been previously found in the sequences of various TPP-dependent enzymes (28, 80) and which is probably involved in the binding of the metal ion (i.e., Mg^{2+}) and the diphosphate group of the cofactor (41). This putative TPP-binding region was previously also identified in the primary structures of the E1α subunits of the acetoin dehydrogenases from *A. eutrophus* (57) and *P. carbinolicus* (51). In addition, we obtained strong homologies of the α subunits of all three acetoin utilizers towards a second conserved site of heteromeric E1 components from 2-oxo acid dehydrogenase complexes (Fig. 4C), which has been reported to be involved in the binding of the respective β subunit (80). In contrast, potential sites for regulatory phosphorylation (Fig. 4C), which had been identified adjacent to the C terminus of the subunit interaction region in animal and some prokaryotic E1α subunits (14, 78, 80), were absent. The sequence homologies among the β subunits of the acetoin dehydrogenase and the 2-oxo acid dehydrogenases, which covered the four known regions of extensive homology of 2-oxo acid dehydrogenase β subunits (80), were found to be stronger than homologies revealed by the corresponding α subunits (Fig. 5). This is consistent with the proposed main functions of the β subunits of 2-oxo acid dehydrogenases, which is stabilization the α subunit and mediation of the contact between E1 and E2 in the enzyme complex (15, 28, 80).

Phylogenetic trees (Fig. 4B and 5B) calculated from progressively aligned sequences revealed common branch points of the corresponding subunits of the acetoin dehydrogenases from *C. magnum* and the subclusters of the corresponding subunits from *A. eutrophus* and *P. carbinolicus*, indicating a close phylogenetic relationship of the acetoin dehydrogenases from

TABLE 3. Amino acid identicalness of the E2 and E3 components of the *C. magnum* acetoin dehydrogenase enzyme system to other proteins[a]

Protein	Source	Reference	Identicalness to analogous *C. magnum* component (mol% amino acids)	Overlap (no. of amino acids)[b]
E2 component from:				
AoDH	*Pelobacter carbinolicus*	51	32.0	451
PyDH	*Bacillus subtilis*	32	30.6	444
PyDH	*Enterococcus faecalis*	1	32.3	427
PyDH	*Saccharomyces cerevisiae*	47	27.1	451
AoDH	*Alcaligenes eutrophus*	57	18.8	400
E3 component from:				
AoDH	*Pelobacter carbinolicus*	51	42.3	465
PyDH	*Bacillus subtilis*	32	43.9	462
PyDH	*Staphylococcus aureus*	31	43.0	460
PyDH	*Saccharomyces cerevisiae*	10	41.4	478
(LPD-3)?[c]	*Pseudomonas putida*	54	39.3	463

[a] Amino acid sequences from E2 and E3 of the *C. magnum* acetoin dehydrogenase enzyme system were compared to the corresponding primary structures of various pyruvate dehydrogenase (PyDH) complexes, the *P. carbinolicus* acetoin dehydrogenase (AoDH) enzyme system, and the *A. eutrophus* acetoin-cleaving system (AoDH).
[b] The lengths of the *C. magnum* AoDH E2 and E3 sequences are 443 and 578 amino acids, respectively.
[c] The function of the third dihydrolipoamide dehydrogenase (LPD-3) of *P. putida* and the affiliation to a dehydrogenase complex are unknown (54).

the three acetoin-degrading bacteria to 2-oxo acid dehydrogenases.

DNA sequence analysis of *acoC* and *acoL*. The N-terminal parts of both of the amino acid sequences deduced from ORF5 and ORF6 (Fig. 2), which were designated *acoC* and *acoL*, respectively, corresponded to the N-terminal amino acid sequences, which had been found to be identical for both SDS-denatured proteins of the purified E2-E3 preparation

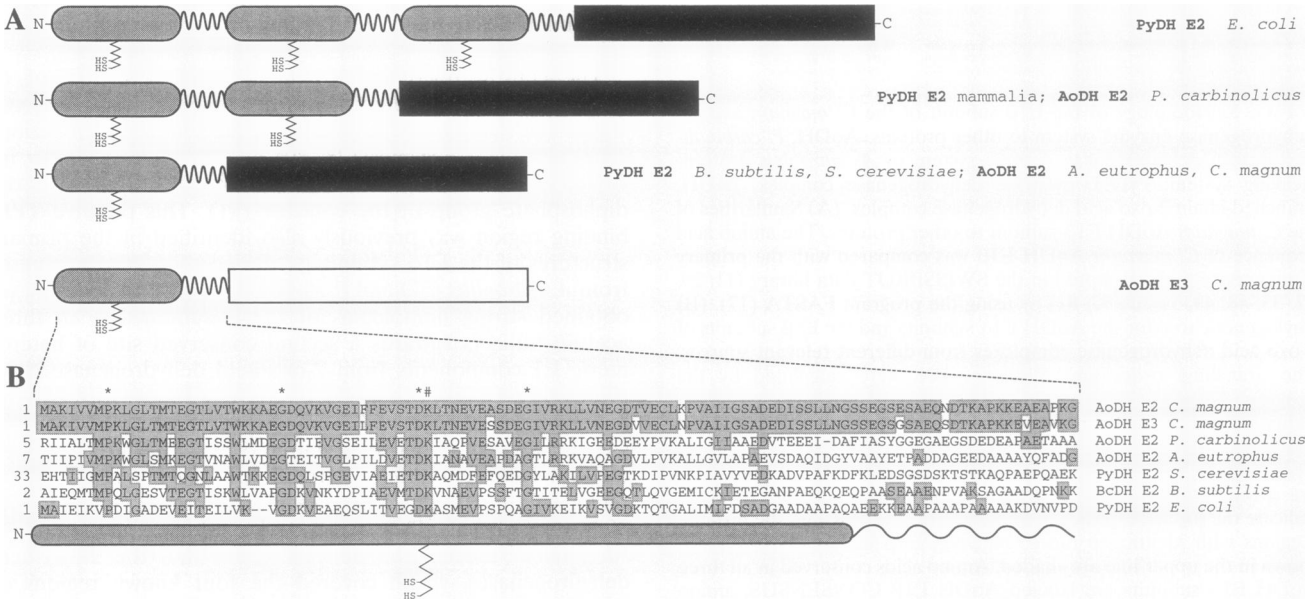

FIG. 6. Putative domain structure of the E2 and E3 components of the *C. magnum* acetoin dehydrogenase enzyme system and comparison to E2 components of 2-oxo acid dehydrogenase complexes from different sources. PyDH, pyruvate dehydrogenase complex; AoDH, *P. carbinolicus* acetoin dehydrogenase enzyme system or *A. eutrophus* acetoin-cleaving system; BcDH, branched-chain 2-oxo acid dehydrogenase complex. (A) Comparison of the putative domain structures of *C. magnum* AoDH E2 and E3 to other proteins (according to Reed and Hackert [60]). Catalytic domains of dihydrolipoamide acetyltransferases (E2) are depicted as black rectangles. The C-terminal part of *C. magnum* AoDH E3, which exhibited complete sequence overlap with other dihydrolipoamide dehydrogenases, is symbolized by the open rectangle. Putative lipoyl domains are depicted as greyish segments. The wavy lines indicate potential flexible regions in the C-terminal flanking sequences of the putative lipoyl domains. Lipoyl moieties bound to the lipoyl domains are symbolized in their reduced (HS) form by zigzag lines. The relative sizes of the respective domains are not to scale. (B) Sequence comparisons between the putative lipoyl domains and their C-terminal flanking regions from *C. magnum* AoDH E2 and E3 with corresponding regions of dihydrolipoamide acyltransferases from different relevant sources (47, 51, 57, 70, 78). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate the positions of the respective amino acids within the protein. Regions of identity to the *C. magnum* AoDH E2 amino acid sequence shown in the upper line are shaded. The positions of lysine residues, which are presumably lipoylated, and of conserved glycine residues which flank those lysine residues (9) are indicated by the symbols # and *, respectively.

from *C. magnum*. Most surprisingly, an almost perfect amino acid sequence overlap was found, even up to amino acid position 114, between the two deduced polypeptides (93% amino acid identity; Fig. 6B). This obviously results from an almost complete (94.15% nucleotide identity) and gapless repetition of a 359-bp nucleotide sequence ranging from positions 5888 to 6247 (Fig. 3), which corresponds to the 5′ coding region of *acoC*, into the 5′ coding region of *acoL* ranging from positions 7235 to 7594. Only 16 nucleotide positions were different (11 transitions and five transversions); 6 are silent and 10 led to the few amino acid sequence differences (Fig. 6B).

At a distance of 7 bp, the start codon of *acoC* (ATG at position 5901 in Fig. 3) is preceded by a reliable Shine-Dalgarno sequence. The *acoC*-encoded polypeptide consisted of 443 amino acid residues and had a calculated M_r of 48,149, which corresponded to the size of 49,000 (±1,000) that had been determined for the smaller protein of the SDS-denatured E2-E3 preparation (Fig. 1D). The total amino acid sequence deduced from *acoC* exhibited striking homologies to the dihydrolipoamide acetyltransferases (E2 components) of the *P. carbinolicus* acetoin dehydrogenase enzyme system (51) and of the *A. eutrophus* acetoin-cleaving system, as well as to the E2 components of pyruvate dehydrogenase complexes from different sources (Table 3). Amino acid sequence analysis identified the N-terminal portion of the *C. magnum* E2 polypeptide up to amino acid position 80 as a typical lipoyl domain (Fig. 6B), which had been found to be a characteristic structural prerequisite of dihydrolipoamide acyltransferases facilitating the acyl transfer within the corresponding enzyme complexes (60). Like most dihydrolipoamide acyltransferases (55) and like the E2 component of the *A. eutrophus* acetoin-cleaving system (57), the *C. magnum* protein contains only one lipoyl domain, whereas the E2 component of the *P. carbinolicus* acetoin dehydrogenase enzyme system (51) and the E2 component of mammalian pyruvate dehydrogenase complexes contain two lipoyl domains (60) (Fig. 6A). The C-terminal remainder of the *acoC*-encoded protein, which comprised significant homology to the C-terminal catalytic domains of various dihydrolipoamide acetyltransferases (Table 3) and included the conserved putative active-site histidine-aspartate couple (His-416 and Asp-420) (25), is separated from the putative lipoyl domain by a region of approximately 30 amino acid residues. This region contains many charged amino acid and alanine residues and therefore resembles the interdomain linker segments of dihydrolipoamide acyltransferases from other gram-positive bacteria (8, 55).

The start codon of *acoL* (ATG at position 7248 in Fig. 3) is also preceded by a reliable Shine-Dalgarno sequence at a distance of 7 bp. The polypeptide encoded by *acoL* consisted of 578 amino acid residues and had a calculated M_r of 61,255, which slightly differs from the value of 67,000 that had been estimated for the larger SDS-denatured protein of the purified E2-E3 preparation (Fig. 1D). The main part of the amino acid sequence deduced from *acoL*, which starts at amino acid position 114 and extends to the C terminus, comprises striking homologies to the total amino acid sequences of various dihydrolipoamide dehydrogenases (Table 3); in addition, it contains the characteristic sequence motifs which are conserved within the pyridine nucleotide-disulfide oxidoreductase enzyme family (12), i.e., the FAD-binding region (amino acid positions 122 to 145), the disulfide-active site surrounding both redox-active cysteine residues (Cys-153 and Cys-158), the NAD(H)-binding region (amino acids from positions 285 to 315), and the interface region (amino acid position 543 and the sequence downstream). In contrast to all other primary structures of dihydrolipoamide dehydrogenases that have been published and that contain a FAD-binding domain in the very N-terminal region (12), the FAD-binding region of the *acoL*-encoded protein of *C. magnum* is preceded by a typical lipoyl domain (Fig. 6). This indicates the presence of a new structural type of dihydrolipoamide dehydrogenase in *C. magnum*.

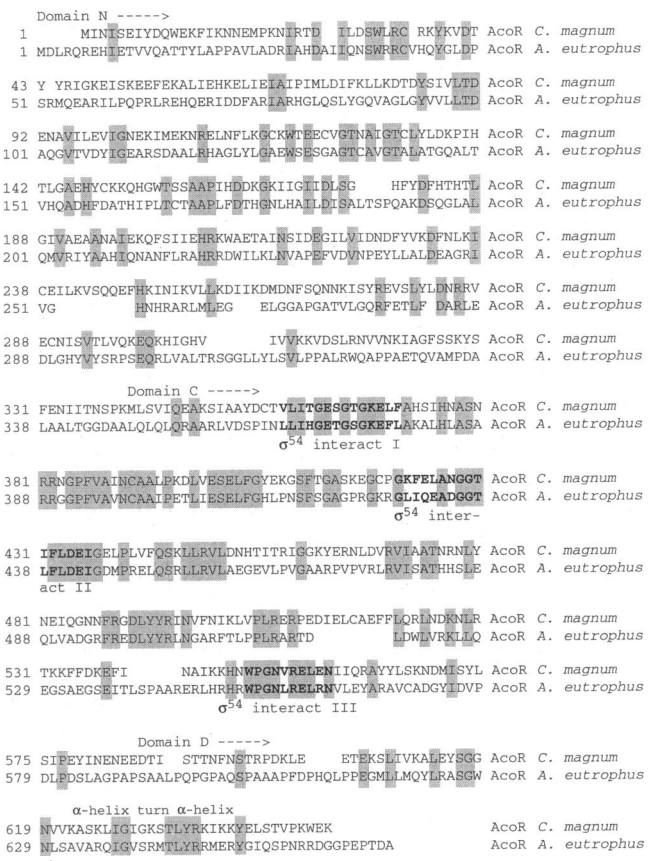

FIG. 7. Homology of the *acoR* gene products from acetoin-utilizing bacteria. The amino acid sequences encoded by *C. magnum acoR* and *A. eutrophus acoR* (39) were compared by using the program GAP (17). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate the positions of the respective amino acids within the protein. Regions of identity are shaded. Domains were designated C and D in accordance with the notation of Thöny and Hennecke (73). Conserved motifs of domain C were localized by using the program MOTIFS (17) and are in boldface and indicated as σ^{54} interact I, II, and III. The secondary structure of domain D shown was obtained with the program PREDICT (17).

DNA sequence analysis of *acoR* and *acoX*. Upstream from *acoA*, we identified ORF1, which is preceded by a Shine-Dalgarno sequence at a distance of 11 bp (Fig. 3) and is referred to as *acoR*. The polypeptide deduced from the nucleotide sequence of *acoR* consisted of 651 amino acid residues and had a calculated M_r of 74,715. Comparison of the deduced amino acid sequence with primary structures stored in the data libraries exhibited striking homology, with complete sequence overlap, to AcoR of *A. eutrophus* (Fig. 7), which was recently identified as a regulatory protein belonging to the NifA family of transcriptional activators and is required for expression of the genes of the *A. eutrophus acoXABC* operon (39). In addition, we found significant homologies with the central C domain (73) of other −24/−12 promoter-activating

TABLE 4. Heterologous expression of the E1, E2, and E3 components of the *C. magnum* acetoin dehydrogenase enzyme system in *E. coli*[a]

Plasmid	Relevant genes	Orientation[b]	Sp act (U/mg)		
			E1	E2	E3
pSK::Hpa54-0	*acoA acoB acoX acoC*	Co	0.046	0.09	0.01
pSK::Hpa54-1	*acoA acoB acoX acoC*	Anti	<0.003	0.08	0.01
pSK::SC39	*acoC acoL*	Co	<0.003	0.18	0.21
pUC19::SK34	*acoC acoL*	Co	<0.003	0.61	0.18
pUC18::SK34	*acoC acoL*	Anti	<0.003	0.07	0.01
Vector[c]			<0.003	0.08	0.01

[a] Activities of E1, E2, and E3 were determined in soluble cell fractions from recombinant *E. coli* DH5α, which was grown for 10 h in LB medium containing 1.0 mM isopropyl-β-D-thiogalactopyranoside.
[b] Co, colinear orientation with respect to *lacZ'*; Anti, antilinear orientation with respect to *lacZ'*.
[c] Activities were determined in soluble cell fractions from *E. coli* DHα harboring either pBluescript SK$^+$ or pUC19.

proteins. The identities within the respective sequence overlaps covering approximately 250 amino acids were 41.6% with NifA of *Klebsiella pneumoniae* (3), 42.5% with VnfA of *Azotobacter vinelandii* (36), 42.7% with HydG of *Salmonella typhimurium* (13), and 49.0% with NtrC of *Rhizobium meliloti* (72). Domains C of these activator proteins contained several highly conserved amino acid sequence motifs, which are involved in the ATP-dependent interaction of the proteins with σ^{54}-dependent RNA polymerases (Fig. 7). The C-terminal region of the *acoR*-deduced polypeptide contained a typical helix-turn-helix element, which is believed to interact directly with specific upstream activator sequences of the respective −24/−12 promoter (73).

Interjacent to *acoB* and *acoC*, the colinear ORF4 was identified and designated *acoX* (Fig. 2). *acoX* corresponded to the codon preference of the *C. magnum* structural *aco* genes mentioned above and was preceded by a reliable Shine-Dalgarno sequence at a distance of 9 bp (Fig. 3). The deduced polypeptide consisted of 332 amino acid residues and had a calculated M_r of 36,027. We found striking homologies (30.4% amino acid identity with complete overlap) solely to the *acoX* gene product of *A. eutrophus*, which consists of 359 amino acids (57). In *A. eutrophus*, *acoX* is located upstream of *acoA* and is part of the *acoXABC* operon. Although it is known that *acoX* is essential for acetoin catabolism in *A. eutrophus*, the exact physiological function of the *acoX* gene product is still unclear (57).

Putative transcriptional and regulatory signals. At a distance of 52 bp upstream of *acoR*, we localized a sequence (5'-TTAATA-N_{18}-TATAAT-3'; Fig. 3) which exhibited strong homology to the *E. coli* σ^{70} (−35/−10) promoter consensus sequence (5'-TTGACA-N_{17}-TATAAT-3' [30]). Furthermore, in the region 410 bp upstream of *acoA*, we identified a sequence (5'-TTGGCACGTATCTTGCT-3'; Fig. 3) which matched the enterobacterial σ^{54} (−24/−12) promoter consensus sequence (5'-CTGGYAYR-N_4-TTGCA-3' [61]) at 11 of 13 conserved positions. At 125 bp downstream of the latter, we found a sequence motif (5'-TATCAAGAAGTT-3'; Fig. 3) which matches the consensus sequence of the integrated host factor-binding site (5'-WATCAANNNNTTR-3' [85]). As an integrated host factor-binding site is generally localized in the upstream regions of −24/−12 promoters, where the bound integrated host factor is believed to facilitate DNA loop formation and activator-RNA polymerase interaction (16), the physiological significance of this motif for initiation of transcription in *C. magnum* is obscure.

Upstream of *acoR*, we found an inverted repeat with a free energy of 69.9 kcal (1 cal = 4.184 J)/mol (74), which is followed by a run of seven U residues in the RNA; this region may represent a factor-independent transcriptional terminator (84). In addition, closely downstream of *acoB* and *acoL*, we found inverted repeats with free-energy values of 52.3 and 75.8 kcal/mol (Fig. 3), respectively, which may represent factor-dependent transcriptional terminators (84).

Heterologous expression of *acoA*, *acoB*, *acoC*, and *acoL* in *E. coli*. Weak expression of the E1 component of the *C. magnum* acetoin dehydrogenase enzyme system was achieved in strains of *E. coli*(pSK::Hpa54-0) which contained *acoA*, *acoB*, *acoX*, *acoC*, and the 5' coding region of *acoL* in colinear orientation with respect to the *lacZ* promotor (Table 4). However, the activity measured in the recombinant *E. coli* amounted to only 12% of the activity that had been previously determined in the soluble cell fraction of acetoin-grown *C. magnum* (43). No activity was detected in the soluble cell fractions from *E. coli*(pSK::Hpa54), which contained the respective genes in antilinear orientation with respect to *lacZ'* (Table 4).

Expression of E2 and E3 of the *C. magnum* acetoin dehydrogenase enzyme system was demonstrated in the soluble cell fraction of LB medium-grown *E. coli*(pSK::SC39), as well as in *E. coli*(pUC19::SK34); both plasmids harbor *acoC* and *acoL* in colinear orientation with respect to the *lacZ* promoter but differ in the length of the region downstream of *acoL* (Table 4 and Fig. 2). Whereas dihydrolipoamide dehydrogenase specific activities were similar in the two recombinant strains and were approximately 20-fold higher than in the control, dihydrolipoamide acetyltransferase activity was considerable higher in *E. coli* harboring pUC19::SK34 than in *E. coli* harboring pSK::SC39. No expression of *C. magnum* E2 and E3 occurred in *E. coli* cells harboring pSK::Hpa54-1 or pUC18::SK34, indicating the absence of promoters functioning in *E. coli* in the respective regions upstream of *acoC* and *acoL*. Activity staining for E2 and E3 of the soluble proteins of *E. coli*(pSK::SC39) and *E. coli*(pUC19::SK34), which were separated in native polyacrylamide gels, resulted in staining with nitroblue tetrazolium chloride (E3) and precipitation of calcium phosphate (E2) at the bottom of the wells in the polyacrylamide gels, which was also obtained with the soluble cell fraction of acetoin-grown *C. magnum*. Since the staining was not obtained with the soluble cell fraction of *E. coli* (pUC18::SK34), E2 and E3 are most probably expressed in *C. magnum*, as well as in the recombinant *E. coli* cells, as high-molecular-weight aggregates. This is consistent with the tight association of E2 and E3 during purification from *C. magnum* extracts. In the protein pattern of the SDS-denatured soluble cell fraction of LB medium-grown *E. coli*(pUC19::SK34), one distinct protein band appeared, which exhibited the same electrophoretic mobility as the 49,000-Da protein of the purified E2-E3 preparation of *C. magnum* (Fig. 8). No such protein band was visible in the protein pattern of *E. coli*(pUC18::SK34) or *E. coli*(pSK::SC39); this confirmed that

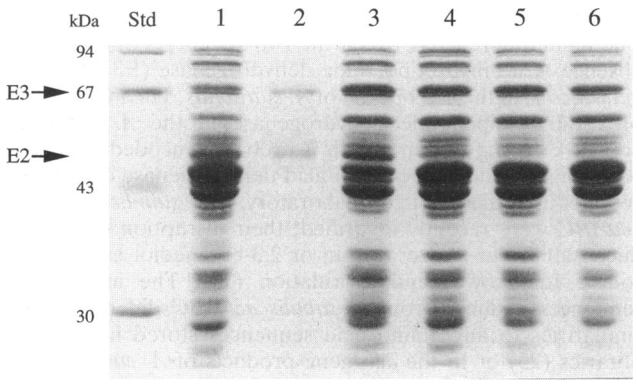

FIG. 8. Expression of *C. magnum* E3 in *E. coli*. Soluble cell extracts from recombinant *E. coli* DH5α were obtained from cells grown for 10 h in LB 1.0 mM medium containing isopropyl-β-D-thiogalactopyranoside. Cell extracts and purified *C. magnum* enzymes were separated in SDS–11.5% (wt/vol) polyacrylamide gels and stained with Serva Blue R. Molecular masses of standard proteins (lane Std) are given. The positions of protein bands corresponding to purified *C. magnum* E2 and E3 are marked. Lanes: 1, 21 μg of protein of the soluble cell fraction from *E. coli* DH5α(pSK::SC39); 2, 5.5 μg of protein of the purified E2-E3 preparation from *C. magnum*; 3, 30 μg of protein of the soluble cell fraction from *E. coli* DH5α(pUC19::SK34); 4, 25 μg of protein of the soluble cell fraction from *E. coli* DH5α(pUC18::SK34); 5, 25 μg of protein of the soluble cell fraction from *E. coli* DH5α (pUC19); 6, 24 μg of protein of the soluble cell fraction from *E. coli* DH5α(pBluescript SK^+).

the E2 component is the smaller polypeptide of the E2-E3 preparation (Fig. 1D) from *C. magnum*. As expected from the low E3 activity in the recombinant cells, no distinct protein band was visible that corresponded to the 67,000-Da protein of the SDS-denatured E2-E3 preparation in the electropherograms obtained from the corresponding crude extracts, which is presumably the E3 component of the *C. magnum* acetoin dehydrogenase enzyme system (Fig. 1D).

DISCUSSION

This study identified and characterized *C. magnum* structural genes *acoA*, *acoB*, *acoC*, and *acoL* for the enzyme components E1α, E1β, E2, and E3 of the acetoin dehydrogenase enzyme system. These genes and two additional genes—*acoR* and *acoX*—are clustered on an 8.7-kbp region in the *C. magnum* genome. Although *C. magnum* is taxonomically and physiologically not related to *P. carbinolicus* and *A. eutrophus*, the physiology and molecular genetics of the acetoin catabolism in this homoacetogenic, gram-positive bacterium strikingly resemble those previously found in both gram-negative acetoin utilizers (39, 50, 51, 57). The amino acid sequences of the α and β subunits of *C. magnum* E1 (acetoin dehydrogenase) deduced from *acoA* and *acoB*, respectively, exhibited striking similarities to the corresponding subunits of the acetoin dehydrogenases of *P. carbinolicus* (51) and *A. eutrophus* (57). In addition, we obtained strong homologies to the corresponding polypeptides of various 2-oxo acid dehydrogenases which belong to the major group of heteromeric E1 components. We obtained no significant homologies to homomeric E1 components of 2-oxo acid dehydrogenase complexes from other sources, e.g., the E1 component of the pyruvate dehydrogenase complex of *E. coli* (69). As phylogenetic trees of the respective primary structures revealed a close resemblance between the corresponding subunits of the three acetoin dehydrogenases, as well as common branch points with the respective subunits of pyruvate dehydrogenases from other prokaryotic and eukaryotic organisms, the acetoin dehydrogenase presumably evolved from a common pyruvate dehydrogenase ancestor. Since the acetoin dehydrogenase enzyme system does not accept 2-oxo acids as substrates (50), the evolution of this system did not simply result in a pyruvate dehydrogenase with a broader substrate spectrum.

Little is known about the function of the two different E1 subunits of 2-oxo acid dehydrogenases. According to the proposed catalytic function of the 2-oxo acid dehydrogenase α subunits (28, 29), the E1α subunit of the acetoin dehydrogenase enzyme system catalyzes the initial TPP-dependent nucleophilic attack on acetoin. This is consistent with the localization of the TPP-binding region within the sequences of the α subunits of the three known acetoin dehydrogenases. Sequence comparisons with other conserved motifs of the α subunits of 2-oxo acid dehydrogenases (80) revealed the presence of a putative β subunit-binding domain localized in the C-terminal region flanking the TPP-binding region and the absence of regulatory phosphorylation sites in the α subunits of the three acetoin dehydrogenases. As the sequence region comprising the N-terminal approximately 120 to 150 amino acid residues of the aligned E1α subunits is, on the one hand, highly homologous within the three acetoin dehydrogenases and as there is, on the other hand, a relatively weak similarity to the corresponding regions of 2-oxo acid dehydrogenase sequences, this region may harbor a domain involved in the recognition and binding of the respective substrates. Similarities of the β subunits of the three acetoin dehydrogenases to the corresponding subunits of 2-oxo acid dehydrogenases were greater, and they covered the entire sequence overlap. This finding coincides with previous findings (28) that the homologies between β subunits of different 2-oxo acid dehydrogenases were higher than those between α subunits, and it is consistent with their proposed main function in the binding of E1 and E2 and the stabilization of the respective E1α subunits (15, 28, 80).

The amino acid sequence deduced from *acoC* for the E2 component of the *C. magnum* acetoin dehydrogenase enzyme system revealed striking similarities to most dihydrolipoamide acyltransferases. One major difference of these E2 components is the number of lipoyl domains at the N terminus (60). Whereas the E2 component of the *A. eutrophus* acetoin-cleaving system contains one lipoyl domain (57), the E2 component of the *P. carbinolicus* acetoin dehydrogenase enzyme system harbors two lipoyl domains (51) and the E2 components of the pyruvate dehydrogenase complexes of *E. coli* and *A. vinelandii* even contain three very similar lipoyl domains (55).

The translation of *acoL* from *C. magnum* revealed an amino acid sequence of a new type of dihydrolipoamide dehydrogenase which differs from all other known members of this particularly conservative enzyme family of pyridine nucleotide-disulfide oxidoreductases, including the *P. carbinolicus* enzyme (12, 18, 51), in possessing an N-terminal lipoyl domain. Lipoyl domains are known as a typical structural attribute of dihydrolipoamide acyltransferases, facilitating acyl transfer within the different catalytic sites of the enzyme complexes and providing attachment sites for binding of E3 and/or E1 (60). The presence of a lipoyl domain at the N terminus of E3 is obviously a result of an almost complete sequence repetition of the 5′ region of *acoC* which encodes the lipoyl domain of E2. As the corresponding DNA sequences were confirmed by examination of independent *E. coli* clones, and as both proteins of the E2-E3 preparation obtained from acetoin-grown

cells of *C. magnum* exhibited the same N terminus, this almost perfect DNA duplication is obviously not a cloning artifact. The presence of a lipoyl domain in the *C. magnum* enzyme may, rather, reflect a structural peculiarity of the *C. magnum* acetoin dehydrogenase enzyme system, i.e., formation of a tightly associated high-molecular-weight E2-E3 subcomplex (43). This is consistent with the copurification of an E2-E3 subcomplex and with the data from the gel electrophoretic analysis under native conditions.

The amino acid sequence deduced from *acoX*, which is interjacent to *acoB* and *acoC*, revealed a striking similarity solely to the gene product of *acoX* from *A. eutrophus*, which was previously identified in the upstream region of *A. eutrophus acoA* (57). The polar effect of Tn*5* insertions in *A. eutrophus acoX* on the expression of the downstream *aco* genes, as well as the identification of the transcriptional start site of the *aco* genes upstream of *acoX*, had demonstrated an important, although previously unknown, function of *acoX* in the catabolism of acetoin in *A. eutrophus* (57). As, in contrast to *C. magnum* and *A. eutrophus*, no *acoX*-equivalent genetic information was identified in the genome of *P. carbinolicus* (51), the physiological function of *acoX* in acetoin cleavage remains obscure.

In addition to the structural genes of the acetoin dehydrogenase enzyme system and *acoX*, we identified *acoR*, which is located upstream of the putative −24/−12 promoter preceding *acoA*. The amino acid sequence of the putative *acoR* gene product exhibited striking homologies to the conserved C domains of various −24/−12 promoter-activating proteins which are involved in the interaction with σ^{54}-dependent RNA polymerases (73). We found no significant homologies to the N-terminal amino acid sequences of the activator proteins belonging to the different homologous groups of regulatory two-component systems, which are controlled by sensor protein-mediated modifications of the respective N-terminal regions (comprising the A, F, and E domains [73]). However, there was a complete sequence overlap, covering the whole N-terminal region, solely with −24/−12 promoter-activating protein AcoR of *A. eutrophus*, which was previously identified in the upstream region of *A. eutrophus acoX* and is required for expression of the genes of the *A. eutrophus acoXABC* operon (39). The high degree of similarity of the domains at the N termini of the two regulatory proteins may indicate that AcoR is modified by the same signal molecule, which might be acetoin itself, in the two taxonomically nonrelated bacteria.

Besides *P. carbinolicus*, *C. magnum* is the second anaerobic acetoin-degrading bacterium for which the complete structural genes encoding a functioning acetoin dehydrogenase enzyme system were localized in a close cluster, which is flanked by *acoA* and *acoL*. Differences in organization between the respective genes occur in the presence or absence of *acoS* (located interjacent to *acoC* and *acoL* and encoding the *P. carbinolicus* lipoate synthase; 51) and *acoX*. From the localization of the genes and of hairpin-like structures, it is likely that in *P. carbinolicus*, *acoA*, *acoB*, and *acoC* constitute one single operon which is transcribed from an unidentified promoter upstream of *acoA*, whereas *acoS* and *acoL* constitute a second operon (51). The molecular data presented in this report provided evidence that in *C. magnum*, all four *aco* genes downstream of *acoR* are organized in one operon (*acoABXCL*). According to this hypothesis, transcription of *acoABXCL* is switched on in the presence of acetoin by the activated *acoR* gene product and is started at the −24/−12 promoter upstream of *acoA*; termination at the hairpin-like structure downstream of *acoC* would then result in the formation of a 6.5-kb transcript. This resembles the situation previously found in the aerobic acetoin utilizer *A. eutrophus* (39, 57). The main difference between the two systems is obviously the absence of a dihydrolipoamide dehydrogenase (E3)-encoding gene, *acoL*, in the *aco* operon of *A. eutrophus*. The involvement of a dihydrolipoamide dehydrogenase in the *A. eutrophus* acetoin-cleaving system, which is probably encoded by a gene belonging to one of the 2-oxo acid dehydrogenase complexes, is under investigation in our laboratory. In *B. subtilis*, the genes *acuABC* were recently identified; their disruption caused reduced ability to utilize acetoin or 2,3-butanediol as a carbon source for growth and sporulation (23). The amino acid sequences deduced from *B. subtilis acuABC* did not exhibit similarities to any amino acid sequences stored in the data libraries (23) or to the *aco* gene products of *A. eutrophus*, *P. carbinolicus*, and *C. magnum*. As it is known from previous studies (42) that *B. subtilis* cell extracts contain TPP-dependent acetoin dehydrogenase, it remains to be elucidated whether two different pathways for acetoin utilization are present in *B. subtilis*.

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