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Biochemical and Molecular Characterization of the *Alcaligenes eutrophus* Pyruvate Dehydrogenase Complex and Identification of a New Type of Dihydrolipoamide Dehydrogenase

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Sequence analysis of a 6.3-kbp genomic *Eco*RI-fragment of *Alcaligenes eutrophus*, which was recently identified by using a dihydrolipoamide dehydrogenase-specific DNA probe (A. Pries, S. Hein, and A. Steinbüchel, FEMS Microbiol. Lett. 97:227–234, 1992), and of an adjacent 1.0-kbp *Eco*RI fragment revealed the structural genes of the *A. eutrophus* pyruvate dehydrogenase complex, *pdhA* (2,685 bp), *pdhB* (1,659 bp), and *pdhL* (1,782 bp), encoding the pyruvate dehydrogenase (E1), the dihydrolipoamide acetyltransferase (E2), and the dihydrolipoamide dehydrogenase (E3) components, respectively. Together with a 675-bp open reading frame (ORF3), the function of which remained unknown, these genes occur colinearly in one gene cluster in the order *pdhA*, *pdhB*, ORF3, and *pdhL*. The *A. eutrophus pdhA*, *pdhB*, and *pdhL* gene products exhibited significant homologies to the E1, E2, and E3 components, respectively, of the pyruvate dehydrogenase complexes of *Escherichia coli* and other organisms. Heterologous expression of *pdhA*, *pdhB*, and *pdhL* in *E. coli* K38(pGP1-2) and in the *aceEF* deletion mutant *E. coli* YYC202 was demonstrated by the occurrence of radiolabeled proteins in electropherograms, by spectrometric detection of enzyme activities, and by phenotypic complementation, respectively. A three-step procedure using chromatography on DEAE-Sephacel, chromatography on the triazine dye affinity medium Procion Blue H-ERD, and heat precipitation purified the E3 component of the *A. eutrophus* pyruvate dehydrogenase complex from the recombinant *E. coli* K38(pGP1-2, pT7-4SH7.3) 60-fold, recovering 41.5% of dihydrolipoamide dehydrogenase activity. Microsequencing of the purified E3 component revealed an amino acid sequence which corresponded to the N-terminal amino acid sequence deduced from the nucleotide sequence of *pdhL*. The N-terminal region of PdhL comprising amino acids 1 to 112 was distinguished from all other known dihydrolipoamide dehydrogenases. It resembled the N terminus of dihydrolipoamide acyltransferases, and it contained one single lipoyl domain which was separated by an adjacent hinge region from the C-terminal region of the protein that exhibited high homology to classical dihydrolipoamide dehydrogenases.

In the strictly fermentative bacteria *Pelobacter carbinolicus* (45–48) and *Clostridium magnum* (34, 39), the acetoin dehydrogenase enzyme system is the key enzyme for the catabolism of acetoin. This enzyme initiates the degradation of acetoin by the coenzyme A-, thiamine pyrophosphate (TPP)-, and NAD-dependent cleavage of acetoin into acetaldehyde and acetyl coenzyme A. Not only is the reaction formally analogous to the oxidative decarboxylation of 2-oxo acids that is catalyzed by the 2-oxo acid dehydrogenase complexes, but also the protein components of this system and even the organization of their genes are similar (50, 51). The acetoin dehydrogenase enzyme systems of both fermentative bacteria are composed of three enzyme components, E1 (a TPP-dependent acetoin dehydrogenase, $\alpha_2\beta_2$), E2 (dihydrolipoamide acetyltransferase [DHLTA]), and E3 (dihydrolipoamide dehydrogenase [DHLDH]); the structural genes encoding these proteins are clustered in the genomes of *P. carbinolicus* (46) and of *C. magnum* (34). Oxidative cleavage of acetoin is an alternative to the cyclic pathway for the degradation of acetoin to acetate via the 2,3-butanediol cycle, which was postulated by Juni and Heym (31) for *Acinetobacter calcoaceticus*.

In acetoin-grown cells of the strictly respiratory bacterium *Alcaligenes eutrophus* also, a heterodimeric TPP-dependent acetoin dehydrogenase as well as a DHLTA was identified, which exhibited striking similarities to the respective E1 and E2 components of the acetoin dehydrogenase enzyme systems of both fermentative bacteria mentioned above (52). Therefore, the participation of an analogous enzyme system in aerobic degradation of acetoin in *A. eutrophus* was postulated (16, 52, 64). The structural genes for the *A. eutrophus* acetoin dehydrogenase (α and β subunits) and DHLTA, *acoA*, *acoB*, and *acoC*, respectively, are organized in one single operon. In contrast to both fermentative acetoin utilizers, a structural gene encoding a DHLDH was not identified downstream or upstream of the known *aco* genes in *A. eutrophus* (52). Therefore, the participation of a DHLDH in the cleavage of acetoin in this bacterium remained obscure.

In *A. eutrophus*, two gene loci for a DHLDH were identified previously on two different nonrelated chromosomal *Eco*RI fragments (54). This indicated the occurrence of at least two distinct DHLDHs in *A. eutrophus*. This bacterium possesses dehydrogenase complexes for pyruvate (17, 59) and 2-oxoglutarate (72), and since it is able to utilize leucine, valine, isoleucine, and glycine as carbon sources for growth, it most probably possesses a branched-chain 2-oxo acid dehydrogenase complex and a glycine decarboxylase complex (15, 52) in addition to the acetoin dehydrogenase cleavage system. Therefore, *A. eutrophus* has several enzyme systems which require a DHLDH. In principle, one particular DHLDH may be shared by more than one of these enzyme systems. In *Escherichia coli*, for instance, only one DHLDH is present for the pyruvate and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Strain no., reference, or source
A. eutrophus H16	Wild-type autotrophic prototrophic	ATCC 17699, DSM 428
E. coli		
S17-1	*recA* harboring the *tra* genes of plasmid RP4 in the chromosome *proA thi-1*	62
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- *lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(*tet*)]	9
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 ΔlacU169* (ϕ80 *lacZΔM15*)	22
K38	F$^-$ *thi-1 leuB6 thr-1 supE44 tonA21* λ^-	1
YYC202	Hfr *zbi*::Tn*10 poxB1* Δ(*aceEF*) *rpsL pps-4 pfl-1*	11, 12
Plasmids		
pBluescript KS$^-$ or pBluescript SK$^-$	Ap^r, *lacPOZ′*, T7 and T3 promoters	Stratagene
pHC79	Cosmid Tc^r, Ap^r	29
pGP1-2	Km^r, P_{lac}/cI857 p_L/T7 gene 1	68
pT7-4	Ap^r, T7 promoter	68
pSKE6.3	6.3-kbp insert (genomic *Eco*RI fragment) from *A. eutrophus* H16 DNA in pBluescript SK$^-$	54
pHC79S11.4	11.4-kbp insert (genomic *Sma*I fragment) from *A. eutrophus* H16 DNA in pHC79	This study
pSKB1.25	1.25-kbp insert (*Bam*HI subfragment of *S*11.4) from *A. eutrophus* H16 DNA in pBluescript SK$^-$	This study
pSKE1.0	1.0-kbp insert (*Eco*RI subfragment of *S*11.4) from *A. eutrophus* H16 DNA in pBluescript SK$^-$	This study
pSKEE7.3	7.3-kbp insert (two adjacent genomic *Eco*RI fragments [*E*1.0 and *E*6.3]) from *A. eutrophus* H16 DNA in pBluescript SK$^-$ harboring *pdhA*, *pdhB*, ORF3, and *pdhL* antilinear to *lacZ′*	This study
pKSEE7.3	7.3-kbp insert (two adjacent genomic *Eco*RI fragments [*E*1.0 and *E*6.3]) from *A. eutrophus* H16 DNA in pBluescript KS$^-$ harboring *pdhA*, *pdhB*, ORF3, and *pdhL* colinear to *lacZ′*	This study
pT7-4SH7.3	7.3-kbp insert (two adjacent genomic *Eco*RI fragments [*E*1.0 and *E*6.3]) from *A. eutrophus* H16 DNA in pT7-4 harboring *pdhA*, *pdhB*, ORF3, and *pdhL* colinear to the T7 promoter	This study

2-oxoglutarate dehydrogenase complex and the glycine cleavage enzyme system (19, 21, 49). Recent analysis of a Tn*5*-induced mutant, which exhibited the phenotype poly(3-hydroxybutyric acid)-leaky and which harbored the transposon inserted into a DHLDH structural gene (54), gave the first evidence that DHLDHs are also interchangeable between different enzyme systems in *A. eutrophus*. If an DHLDH is involved in the cleavage of acetoin also in *A. eutrophus*, its structural gene will most probably be clustered with the genes for the pyruvate or 2-oxoglutarate dehydrogenase complex. This study aimed at the identification and characterization of the *A. eutrophus* pyruvate dehydrogenase complex and focused on the DHLDH component. Molecular and biochemical characterization of this protein revealed a second example of a DHLDH possessing an N-terminal lipoyl domain (34).

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. *A. eutrophus* was grown at 30°C in a complex medium of nutrient broth (0.8% [wt/vol]) or in a mineral salts medium (MM1) as described by Schlegel et al. (60). Different media were used to cultivate *E. coli*, as indicated in the text: Luria-Bertani (LB) medium, Terrific broth (TB), M9 medium (57), or mineral salts medium (MM2) of Tanaka et al. (69), supplemented with 20 μg of thiamine per ml. Growth was monitored spectroscopically by using a Klett-Summerson photometer.

Isolation and manipulation of DNA. Total genomic DNA of *A. eutrophus* was isolated from cells grown in MM1 containing fructose (0.2% [wt/vol]) as the sole carbon source at 30°C by the method of Marmur (42). Plasmid DNA was prepared from crude lysates by the alkaline extraction procedure (6). Restrictions of DNA were performed with various restriction endonucleases under the conditions described by Sambrook et al. (57) or by the manufacturer. For introducing unidirectional deletions, the Stratagene Bluescript Exo/Mung DNA system was used as instructed by the manufacturer (Stratagene Cloning Systems, San Diego, Calif.). DNA fragments were isolated from agarose gels by using either a GeneClean kit (Bio 101, La Jolla, Calif.) (73) or the centrifugation technique described by Weichenhan (74). 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 $MgSO_4$ (22). Competent cells were prepared and transformed by the calcium chloride procedure (57).

Construction of a cosmid library. Total genomic DNA of *A. eutrophus* was partially digested by *Sma*I and ligated to *Eco*RV-digested pHC79. The ligation products were subsequently packaged with phage λ coat proteins by using an in vitro packaging kit which was prepared from *E. coli* BHB2688 and *E. coli* BHB2690 (28, 30, 32) and were transfected into *E. coli* S17-1.

DNA-DNA hybridization. Transfer of denatured DNA from agarose gels or from bacterial colonies to nylon membranes, hybridization with biotin-16-dUTP-labeled probes, and detection of DNA were performed by standard procedures (18, 37, 57).

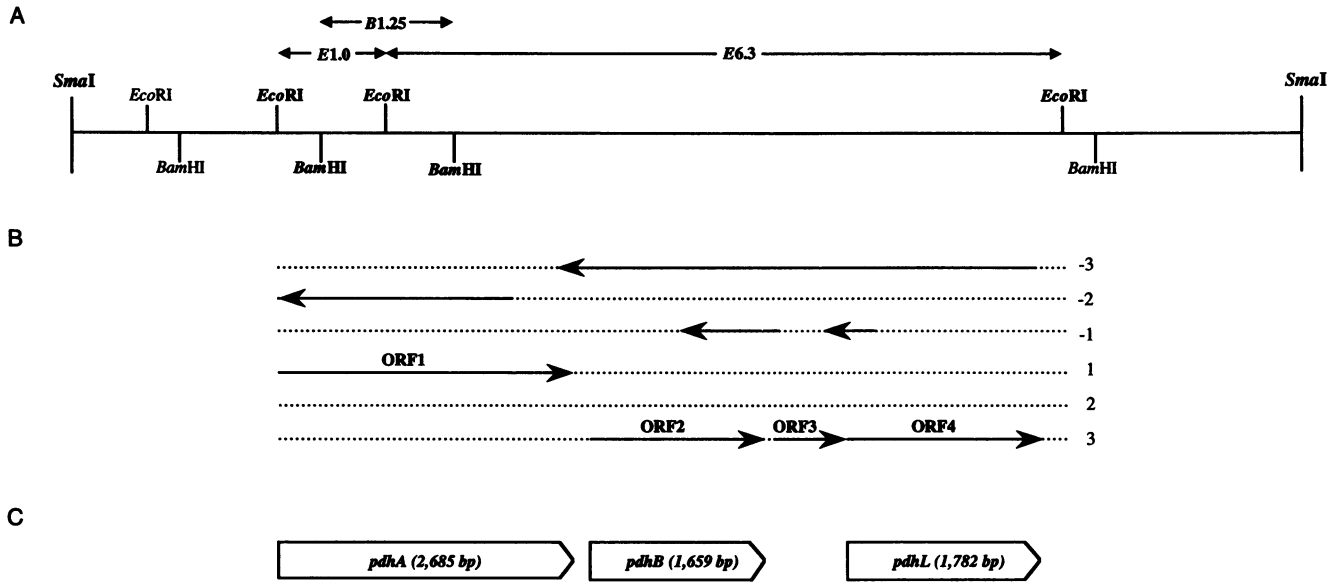

FIG. 1. Molecular organization of the *A. eutrophus pdh* gene cluster. (A) Restriction map of the region analyzed in this study. Relevant *Bam*HI and *Eco*RI fragments are indicated. (B) ORFs of the sequenced region which started with ATG and comprised more than 450 bp. (C) Structural genes of the *pdh* gene cluster.

Synthesis of oligonucleotides. Synthesis of oligonucleotides was performed in 0.2-μmol portions from deoxynucleoside phosphoramidites (4) with a Gene Assembler Plus apparatus (Pharmacia-LKB Biotechnology, Uppsala, Sweden) as instructed by 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.

DNA sequence analysis. Plasmid pSKE6.3 was digested with *Apa*I and *Hin*dIII, and the linearized DNA was subsequently treated with exonuclease III and mung bean nuclease to introduce unidirectional deletions. By using pSKE6.3 and its deleted derivatives as template DNAs, and by using universal and reverse primers, the nucleotide sequence of the 6.3-kbp *Eco*RI fragment was determined. pSKE6.3 was also sequenced by using the primer-hopping strategy with synthetic oligonucleotides as primers (67). DNA sequencing was carried out by the dideoxy-chain termination method of Sanger et al. (58) with double-stranded alkali-denaturated plasmid DNA, with d7-deaza-GTP instead of dGTP (43), and with α-^{35}S-dATP by using a T7 polymerase sequencing kit as instructed by the manufacturer (Pharmacia-LKB). 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, 50% [wt/vol] urea) in an S2 sequencing apparatus (GIBCO/BRL GmbH, Eggenstein, Germany) and were 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 (14).

Heterologous expression of *pdhA*, *pdhB*, and *pdhL* in *E. coli*. The coupled T7 RNA polymerase promoter system of Tabor and Richardson (68) was used. Plasmid pSKEE7.3 harboring *pdhA*, *pdhB*, open reading frame 3 (ORF3), and *pdhL* was restricted with *Sma*I and *Hin*dIII. The resulting 7.3-kbp *Sma*I-*Hin*dIII fragment was isolated and ligated with pT7-4 DNA which was restricted with *Sma*I plus *Hin*dIII. The ligation products were transformed into *E. coli* K38(pGP1-2). Transformants were labeled with L-[^{35}S]methionine by the protocol of Tabor and Richardson (68). Proteins were separated by electrophoresis in an 11.5% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel and subjected to autoradiography.

Preparation of cell extract. Cells of *E. coli* were disrupted by sonication in an MSE (150-W) ultrasonic disintegrator at an amplitude of 14 μm for 1 min/ml of cell suspension. Soluble cell fractions were obtained as supernatants from a 1-h centrifugation at 100,000 × *g* and at 4°C. Protein content was determined as described by Lowry et al. (40).

Enzyme assays. The assay mixture for the determination of pyruvate dehydrogenase complex activity contained 100 mM Tris hydrochloride (pH 7.5), 0.8 mM TPP, 0.5 mM $MgCl_2$, 1.2 mM NAD, 6 mM dithiothreitol, 0.13 mM coenzyme A, 20 to 50 μg of protein from the crude cellular extract, and 1.5 mM pyruvate in a total volume of 1 ml. The activity was measured under anaerobic conditions by monitoring NAD reduction spectrophotometrically at 340 nm. The assay mixture for the determination of pyruvate dehydrogenase (E1) activity contained 100 mM Tris hydrochloride (pH 7.5), 0.8 mM TPP, 0.5 mM $MgCl_2$, 0.07 mM dichlorophenolindophenol (DCPIP) or ferricyanide, 50 to 100 μg of protein from crude cellular extract, and 2.5 mM pyruvate in a total volume of 3 ml. The activity was measured under anaerobic conditions by monitoring DCPIP or ferricyanide reduction spectrophotometrically at 578 or 420 nm, respectively. Amounts of E2 (DHLTA) and E3 (DHLDH) were determined spectrophotometrically in 100 mM Tris hydrochloride (pH 7.5) as described previously (45) by monitoring the initial rates of the formation of *S*-acetyldihydrolipoamide at 240 nm and of the oxidation of NADH at 365 nm, respectively.

Purification of DHLDH. The *pdhL* gene product was purified from recombinant *E. coli* K38 harboring pGP1-2 and pT7-4SH7.3. To maximize protein production, the T7 RNA polymerase promoter system was used. The cells were grown in TB in the presence of ampicillin and kanamycin (each at 100

TABLE 2. Similarities of the E1, E2, and E3 components of the *A. eutrophus* pyruvate dehydrogenase complex to the corresponding components of 2-oxo acid dehydrogenase complexes from different sources[a]

Component	Source	Accession no.	Identity to *A. eutrophus* PDH counterpart (mol% amino acids)	Overlap (no. of amino acids)[b]
E1 from PDH	*E. coli*	P06958	62.4	884
	Azotobacter vinelandii[c]	P10801	62.2	45
E2 from:				
PDH	*E. coli*	P06959	58.3	549
	A. vinelandii	P10802	53.2	546
	Bacillus subtilis	P21883	37.7	449
ODH	*A. vinelandii*	P20708	34.9	427
	B. subtilis	P16263	33.1	429
	E. coli	P07016	33.0	426
BCDH	*Pseudomonas putida*	P09062	32.7	432
ODH	Rat	Q01205	29.2	523
BCDH	Bovine	P11181	28.7	519
	Human	P11182	28.5	498
DHLDH components				
DHLDH	*E. coli*	P00391	66.8	478
	B. subtilis	P21880	44.5	475
	B. stearothermophilus	P11959	43.3	476
	P. fluorescens	P14218	42.0	474
	A. vinelandii	P18925	41.8	473
Lpd-glc	*P. putida*	P31052	40.0	474
DHLDH	Human	P09622	39.6	536
	Pig	P09623	39.3	514
	Pisum sativum	P31023	38.2	507
Lpd-3	*P. putida*	P31046	37.8	467
DHLDH	*Saccharomyces cerevisiae*	P09624	37.6	532
Lpd-val	*P. putida*	P09063	36.0	472
AcoL	*Clostridium magnum*	L31844	35.8	575

[a] PDH, pyruvate dehydrogenase complex; ODH, 2-oxoglutarate dehydrogenase complex; BCDH, branched-chain 2-oxo acid dehydrogenase complex.
[b] The lengths of the amino acid sequences of *A. eutrophus* E1, E2, and E3 are 895, 553, and 594, respectively.
[c] Only a 45-amino-acid sequence is available in the data libraries.

μg/ml) at 30°C. At an optical density of 250 Klett units, the temperature was raised to 42°C for 20 min. After rifampin was added to a final concentration of 400 μg/ml, the cells were shifted to 37°C for 2 h and then harvested. The cell pellet was washed two times, and the cells were resuspended in 100 mM Tris hydrochloride buffer (pH 7.5). The soluble cell fraction (approximately 0.17 g of protein) derived from about 2.6 g (wet weight) of cells was applied onto a column (2.6 by 11 cm; 58-ml bed volume [BV]) of DEAE-Sephacel equilibrated with buffer. After the column was washed with 5.5 BV of buffer, the proteins were eluted with an NaCl gradient (0 to 500 mM; two times, 250 ml) 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, using a PM30 membrane. The enzyme solution was applied onto a column (2.6 by 6 cm; 32-ml BV) of Sepharose 4B-CL–Procion Blue H-ERD equilibrated with buffer. After the column was washed with 2 BV of buffer, the proteins were eluted with an NaCl gradient (0 to 2 M; two times, 500 ml) at a constant flow rate of 0.5 BV/h. Fractions containing high enzyme activity were combined, concentrated, and washed by ultrafiltration. The enzyme preparation was incubated at 75°C for 10 min, precipitated proteins were removed by centrifugation (11,000 × *g*, 10 min), and the supernatant containing the active enzyme was stored at −20°C.

Preparation of triazine affinity media. Procion Blue H-ERD, Procion Blue MX-2G, Procion Orange A, Procion Yellow MX-8G, Procion Green A, Procion Brown MX-5BR, and Procion Green HE-4BD were coupled to Sepharose 4B-CL by the procedure of Atkinson et al. (2).

Electrophoretic methods. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in 11.5% (wt/vol) polyacrylamide gels as described by Laemmli (36).

N-terminal sequence analysis. For determination of the N-terminal amino acid sequence, purified DHLDH protein was electroblotted from an SDS-polyacrylamide gel in 25 mM Tris–192 mM glycine–5% (vol/vol) methanol (pH 8.4) onto a polyvinylidene fluoride membrane (71), using a semidry Fast Blot B33 apparatus (Biometra GmbH, Göttingen, Germany) at constant 5 mA/cm^2 as instructed by the manufacturer. Proteins on the membrane were stained with Serva Blue R. Areas of the membrane corresponding to DHLDH were cut out and subjected to sequence analysis using a 477A pulsed liquid-phase protein/peptide sequencer (27) and a 120A on-line phosphothiohydantoin amino acid analyzer (56) (both from Applied Biosystems, Weiterstadt, Germany) as instructed by the manufacturer.

Chemicals. Restriction endonucleases, biotin-16-dUTP, the nick translation kit, the DNA detection kit, T4 DNA ligase, and the substrates used for enzyme assays were obtained from GIBCO/BRL, C. F. Boehringer & Soehne (Mannheim, Germany), or Stratagene; phosphoramidites, NAP-5 columns, agarose NA, and DEAE-Sephacel were obtained from Pharmacia (Freiburg, Germany). Coenzyme A and relative molecular mass standard proteins were obtained from Sigma Chemie (Deisenhofen, Germany). Procion Blue H-ERD, Procion Blue MX-2G, Procion Orange A, Procion Yellow MX-8G, Procion Green A, Procion Brown MX-5BR, and Procion Green HE-4BD were from Deutsche ICI (Frankfurt, Germany). DL-Lipoamide was purchased from Serva Feinbiochemica (Hei-

delberg, Germany); polyvinylidene fluoride membranes were obtained from Millipore (Bedford, Mass.); radioisotopes were from Amersham/Buchler (Braunschweig, Germany). All other chemicals were from E. Merck AG (Darmstadt, Germany), Fluka Chemie (Buchs, Switzerland), Serva Feinbiochemica, Sigma Chemie, or Difco Laboratories (Detroit, Mich.).

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 U09865.

RESULTS

Identification and cloning of *pdhA*, *pdhB*, ORF3, and *pdhL*. From a genomic library of *A. eutrophus* H16, a 6.3-kbp *Eco*RI fragment which restored the wild-type phenotype in transposon-induced poly(3-hydroxybutyric acid)-leaky mutants of *A. eutrophus* was cloned recently (54). This 6.3-kbp *Eco*RI fragment (referred to as *E*6.3) conferred expression of DHLDH activity to *E. coli*, and it gave a strong hybridization signal with a DHLDH-specific DNA-probe representing the 5′ region encoding a highly conserved part of the N-terminal region of DHLDHs (54), indicating the presence of a DHLDH-encoding gene. The nucleotide sequence of *E*6.3 revealed eight ORFs, which are shown in Fig. 1B. The amino acid sequences deduced from the nucleotide sequences of ORF2 and ORF4 exhibited significant homologies to the amino acid sequences of the enzyme components DHLTA (E2) and DHLDH (E3), respectively, of the pyruvate dehydrogenase complex of *E. coli* and other organisms. This indicated that the structural genes of the E2 and E3 components of the *A. eutrophus* pyruvate dehydrogenase complex map on *E*6.3 (Fig. 1C and Table 2). Therefore, ORF2 and ORF4 were referred to as *pdhB* and *pdhL*, respectively.

To clone the 5′ upstream region of ORF1, which was not located on *E*6.3, a *Sma*I genomic library of *A. eutrophus* in the cosmid pHC79 was screened with the biotinylated 6.3-kbp *Eco*RI fragment. Four of 650 clones hybridized with this probe, and each harbored an 11.4-kbp *Sma*I fragment (Fig. 1A). A 1.25-kbp *Bam*HI (referred to as *B*1.25) and a 1.0-kbp *Eco*RI (*E*1.0) subfragment, which overlap and which were obtained from one clone, were cloned into pBluescript SK$^-$; the resulting hybrid plasmids were pSKB1.25 and pSKE1.0, respectively. Sequence analysis of both DNA fragments led to the identification of the 5′ region of ORF1 (Fig. 1B). Comparison of the amino acid sequence deduced from the nucleotide sequence of ORF1 with primary structures of proteins stored in data libraries revealed striking homologies to the pyruvate dehydrogenases of *E. coli* and *Azotobacter vinelandii* (Table 2). Therefore, ORF1 was referred to as *pdhA*.

Nucleotide sequences of *pdhA*, *pdhB*, ORF3, and *pdhL*. The nucleotide sequence of a region of 7,291 kbp was obtained from both strands of *E*1.0 and *E*6.3 (Fig. 1A). It exhibited a G+C content of 66.5 mol%, which corresponded almost exactly to the value determined previously for total genomic DNA of *A. eutrophus* (13). The G+C contents of *pdhA*, *pdhB*, *pdhL*, and ORF3 were 65.6, 67.8, 66.2, and 67.0 mol%, respectively. The G+C contents for the different codon positions followed the rules of Bibb et al. (5): the G+C contents for the first codon position of *pdhA*, *pdhB*, *pdhL*, and ORF3 were 63.4, 69.9, 66.2, and 65.5 mol%, respectively, whereas they were 40.6, 48.7, 43.4, or 50.1 mol% for the second codon position and 91.8, 84.6, 87.0, and 86.1 mol% for the third codon position. The codon preferences of *pdhA*, *pdhB*, *pdhL*, and ORF3 corresponded to those of other genes of *A. eutrophus* (35, 52, 53, 61). The putative translational start codons of *pdhA* (ATG in position 22), *pdhB* (ATG in position 2877), ORF3 (ATG in position 4611), and *pdhL* (ATG in position 5322) were preceded by reliable Shine-Dalgarno sequences (Fig. 2). These data provided evidence that not only *pdhA*, *pdhB*, and *pdhL* but also ORF3 represent protein-encoding information and that ORF3 is not only a very large noncoding region which separates *pdhL* from the rest of the operon such as in the *E. coli ace* operon (21). However, no significant homologies to proteins stored in the data libraries were obtained with the amino acid sequence deduced from the nucleotide sequence of ORF3, not even with the deduced amino acid sequences of genes of unknown function occurring in the *aco* gene clusters of *C. magnum* or *A. eutrophus*, such as *acoX* (34, 52). Since the other four ORFs, which were orientated antilinearly to the *pdh* genes, did not match the *A. eutrophus* codon preference, and since they were not preceded by reliable Shine-Dalgarno sequences, these ORFs most probably do not represent protein-encoding DNA sequences.

Sequences which exhibited significant homologies to the enterobacterial −35/−10 or −24/−12 promoter consensus sequence (3, 25) could not be identified in the 5′ flanking regions of *pdhA*, *pdhB*, ORF3, or *pdhL*. Between *pdhB* and ORF3, an inverted repeat was found (Fig. 2). According to Tinoco et al. (70), the free energy of this structure is 178 kJ/mol. In addition, an inverted repeat which included five U residues was identified at a distance of 17 bp downstream of *pdhL* (Fig. 2). The free energy of this structure, which might represent a putative transcriptional termination signal, was 84 kJ/mol.

Properties of the *pdhA*, *pdhB*, and *pdhL* gene products. The polypeptide deduced from the nucleotide sequence of *pdhA* consisted of 895 amino acid residues (Fig. 2) and had a calculated relative molecular weight (M_r) of 100,251. Comparison of this amino acid sequence with the primary structures of proteins collected in the data library revealed approximately 62% amino acid identity to the pyruvate dehydrogenases of *E. coli* and *Azotobacter vinelandii* (Table 2), which are also composed of only one type of subunit. No significant homologies were found to the corresponding subunits of heteromeric E1 components of 2-oxo acid dehydrogenase multienzyme complexes from various eukaryotic and prokaryotic sources which are composed of two different subunits (α and β) with lower M_rs. The N-terminal region of the *pdhA* gene product matched a fingerprint sequence motif (amino acid residues 236 to 265; Fig. 2), which had been previously found in the sequences of various TPP-dependent enzymes (24, 75) and is

FIG. 2. Nucleotide sequence of the *A. eutrophus pdh* gene cluster. Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations, and the numbers indicate positions of amino acids within the protein. Highly conserved amino acids within characteristic sequence motifs are shown in boldface. Conserved amino acids of the putative TPP-binding region (24, 75) of the *pdhA* gene product are marked with asterisks. The positions of lysine residues in the gene products of *pdhB* and *pdhL*, which are presumably lipoylated (8), are indicated by #. The putative active-site histidine-aspartate couples of the putative catalytic domain (20, 50, 55) of the *pdhB* gene product are marked with +. The amino acid sequences determined for the N terminus of purified *A. eutrophus* PdhL are underlined. Putative ribosome-binding sites are underlined and indicated by S/D. The positions of hairpin-like structures are marked by inverted arrows. A triangle indicates the insertion of Tn*5*::*mob* in a PHB-leaky mutant of *A. eutrophus*.

```
                S/D      -> pdhA
1     GAATTCACCCAGGAGACAGTCATGTCCGCCGTACCAGAGCAGATTCTCGGCGCCTCGAGCGCCAACGACGCCGATCCCCAGGAAACCCATGAATGGCTCG
                       M  S  A  V  P  E  Q  I  L  G  A  S  S  A  N  D  A  D  P  Q  E  T  H  E  W  L  D
                       1                 10                 20

101   ACGCCCTGCAGGGCGTCCTGGCCGCCGAAGGTCCGGCGCGCGCCGCTTTCCTGATCGACAAGCAGATCGAATACGCACGCGTGAACGGCGTGACCCAGCC
        A  L  Q  G  V  L  A  A  E  G  P  A  R  A  A  F  L  I  D  K  Q  I  E  Y  A  R  V  N  G  V  T  Q  P
              30                40                50                60

201   GTTCCACGCCGAGACGCAGTACATCAACACGATTCCGGTCGAGCAGCAGGCCCGCATCCCCGGCGACCAGGACATCGAGCACCGGATCCGCTCGTACACC
        F  H  A  E  T  Q  Y  I  N  T  I  P  V  E  Q  Q  A  R  I  P  G  D  Q  D  I  E  H  R  I  R  S  Y  T
                          70                80                90

301   CGCTGGAACGCCATGGCGATGGTGCTGCGCGCCAACAAGCACACCAACGTCGGCGGCCACATCTCGTCGTTCGCCTCGGCGGCCACGCTGTATGACGTCG
        R  W  N  A  M  A  M  V  L  R  A  N  K  H  T  N  V  G  G  H  I  S  S  F  A  S  A  A  T  L  Y  D  V  G
                    100               110               120

401   GCTACAACCACTTCTGGCGCGCCCCGTCGGAAGCCGGCGGCGGCGACCTGGTGTTCGTGCAGGGCCACTCGGCTCCGGGCGTGTATTCGCGCGCCTTCCT
        Y  N  H  F  W  R  A  P  S  E  A  G  G  G  D  L  V  F  V  Q  G  H  S  A  P  G  V  Y  S  R  A  F  L
              130               140               150               160

501   GCTCGGCCGCCTGACCCAGGACCAGCTCGACAACTTCCGCCAGGAGGTGGACGGCAAGGGCATCTCGTCCTACCCGCACCCGTGGCTGATGCCGGACTTC
        L  G  R  L  T  Q  D  Q  L  D  N  F  R  Q  E  V  D  G  K  G  I  S  S  Y  P  H  P  W  L  M  P  D  F
                          170               180               190

601   TGGCAGTTCCCGACGGTGTCGATGGGCCTGGGCCCGATCATGGCCATCTACCAGGCCCGCTTCATGAAGTACCTGGACAGCCGCGGCCTGGCCAAGGCCG
        W  Q  F  P  T  V  S  M  G  L  G  P  I  M  A  I  Y  Q  A  R  F  M  K  Y  L  D  S  R  G  L  A  K  A  G
                    200               210               220

701   GCGACCGCAAGGTCTGGGCCTTCCTGGGCGACGGCGAGACCGACGAGCCGGAATCGCTGGGTGCGATCGGCATGGCCGGCCGCGAGAAGCTCGACAACCT
        D  R  K  V  W  A  F  L  G  D  G  E  T  D  E  P  E  S  L  G  A  I  G  M  A  G  R  E  K  L  D  N  L
              230            * *  *    240               250  *
260
801   GGTCTTCGTCATCAACTGCAACCTCCAGCGCCTGGATGGCCCGGTGCGCGGCAACGGCAAGATCATCCAGGAACTGGAATCCGAATTCCGCGGTGCCGGC
        V  F  V  I  N  C  N  L  Q  R  L  D  G  P  V  R  G  N  G  K  I  I  Q  E  L  E  S  E  F  R  G  A  G
                 *             270               280               290

901   TGGAACGTGATCAAGGTGGTGTGGGGCAGCAAGTGGGATTCGCTGCTGGCGCGCGACACCAAGGGCCTGCTGATGAAGCGCATGATGGAATGCGTCGACG
        W  N  V  I  K  V  V  W  G  S  K  W  D  S  L  L  A  R  D  T  K  G  L  L  M  K  R  M  M  E  C  V  D  G
                    300               310               320

1001  GCGAGTACCAGACCATGAAGGCCAAGGACGGCGCCTATGTCCGCGAGCACTTCTTCAACACGCCTGAGCTGAAGGCGATGGTGGCCGACTGGTCCGACGA
        E  Y  Q  T  M  K  A  K  D  G  A  Y  V  R  E  H  F  F  N  T  P  E  L  K  A  M  V  A  D  W  S  D  D
              330               340               350               360

1101  CGACATCTGGCGCCTGAACCGCGGCGGCCACGATCCGCACAAGATCTACGCCGCCTACAAGGCCGCCAGCGAGCACAAGGGCCAGCCCACGCTGATCCTG
        D  I  W  R  L  N  R  G  G  H  D  P  H  K  I  Y  A  A  Y  K  A  A  S  E  H  K  G  Q  P  T  L  I  L
                          370               380               390

1201  GCCAAGACCATCAAGGGCTATGGCATGGGCGACGCCGGCCAGGCCATGAACGTGGCCCACCAGCAGAAGAAGATGCCGGTGGACGCGATCCGCAAGTTCC
        A  K  T  I  K  G  Y  G  M  G  D  A  G  Q  A  M  N  V  A  H  Q  Q  K  K  M  P  V  D  A  I  R  K  F  R
                    400               410               420

1301  GCGACCAGTTCAATCTTCCCGTTGCCGACGACCAGCTCGAAGAGGTGCCGTACATCACCTTCCCGGAAGGTTCGAAGGAACTGGAATACATGCGCCAGGC
        D  Q  F  N  L  P  V  A  D  D  Q  L  E  E  V  P  Y  I  T  F  P  E  G  S  K  E  L  E  Y  M  R  Q  A
              430               440               450               460

1401  GCGGCAGAACCTGGGCGGCTACCTGCCGGCCCGCCGCCAGAAGGCCGAAGCGCTGCCGGTCCCGCAGCTGTCCGCGTTCGACGCGCTGCTGAAGGCCACC
        R  Q  N  L  G  G  Y  L  P  A  R  R  Q  K  A  E  A  L  P  V  P  Q  L  S  A  F  D  A  L  L  K  A  T
                          470               480               490

1501  GGCGAAGGCCGCGAAGTGTCCACCACCATGGCCTTCGTGCGGATCCTGAACACGCTGCTGAAAGACAAGCAGATCGGCAAGCACGTGGTGCCCATCGTGC
        G  E  G  R  E  V  S  T  T  M  A  F  V  R  I  L  N  T  L  L  K  D  K  Q  I  G  K  H  V  V  P  I  V  P
                    500               510               520

1601  CGGACGAGTCGCGCACCTTCGGCATGGAAGGCCTGTTCCGCCAGGTCGGCATCTGGAACCAGGAAGGCCAGAAGTACGTGCCGGAAGACCATGACCAGCT
        D  E  S  R  T  F  G  M  E  G  L  F  R  Q  V  G  I  W  N  Q  E  G  Q  K  Y  V  P  E  D  H  D  Q  L
              530               540               550               560

1701  GATGTTCTACAAGGAATCGCAGACTGGCCAGGTGCTGCAGGAGGGCATCAACGAAGCAGGCGCCATGTGCGACTGGATCGCGGCCGCCACGTCGTACTCG
        M  F  Y  K  E  S  Q  T  G  Q  V  L  Q  E  G  I  N  E  A  G  A  M  C  D  W  I  A  A  A  T  S  Y  S
                          570               580               590

1801  ACGCACGGCGTGCAGATGATCCCGTTCTACATCTACTACTCGATGTTCGGCATCCAGCGTATCGGCGACCTGTGCTGGGCCGCTGCCGACATGCGCTCGC
        T  H  G  V  Q  M  I  P  F  Y  I  Y  Y  S  M  F  G  I  Q  R  I  G  D  L  C  W  A  A  A  D  M  R  S  R
                    600               610               620

1901  GCGGCTTCCTGCTGGGCGGCACCGCCGGCCGCACCACGCTGAACGGCGAAGGCCTGCAGCATGAAGACGGCCACTCGCACGTGTTCCACGCCGCGATCCC
        G  F  L  L  G  G  T  A  G  R  T  T  L  N  G  E  G  L  Q  H  E  D  G  H  S  H  V  F  H  A  A  I  P
              630               640               650               660

2001  GAACTGCATCTCGTACGACCCGACCTTCCAGTACGAACTGGCGGTGGTCATGCAGGACGGCCTGCGCCGCATGTACGCCGAGCAGGAAGACGTCTACTAC
        N  C  I  S  Y  D  P  T  F  Q  Y  E  L  A  V  V  M  Q  D  G  L  R  R  M  Y  A  E  Q  E  D  V  Y  Y
                          670               680               690

2101  TACCTGACGGTGATGAACGAGAACTACGAGCATCCGGAAATGCCGGCTGGCGTGGAGCAGGACATCGTCAAGGGCATGTACCAGTTCCGCAAGGGTGTCG
        Y  L  T  V  M  N  E  N  Y  E  H  P  E  M  P  A  G  V  E  Q  D  I  V  K  G  M  Y  Q  F  R  K  G  V  E
                    700               710               720

2201  AGAACAGCAACGCGCCGCGCGTGCAGCTGCTGGGCTCGGGCACGATCTTCCGCGAGGTGATCGCCGCTGCCGACCTGCTCAAGAAGGACTGGGGCGTGGA
        N  S  N  A  P  R  V  Q  L  L  G  S  G  T  I  F  R  E  V  I  A  A  A  D  L  L  K  K  D  W  G  V  E
              730               740               750               760

2301  GTCGGACCTGTGGGGCTGCCCGAGCTTCACCGAACTGGCCCGCGAAGGCCACGACGTCGAGCGCTTCAACCTGCTGCACCCGACCGAGACCCCGCGCGAA
        S  D  L  W  G  C  P  S  F  T  E  L  A  R  E  G  H  D  V  E  R  F  N  L  L  H  P  T  E  T  P  R  E
                          770               780               790

2401  TCGCACGTGGCCAAGAGCCTGAAGTCGGCCCGCGGCCCGGTGATCGCCTCCACCGACTACGTGCGTGCGTTTGCCGAGCAGATCCGTCCGTTCGTGCCGC
        S  H  V  A  K  S  L  K  S  A  R  G  P  V  I  A  S  T  D  Y  V  R  A  F  A  E  Q  I  R  P  F  V  P  R
                    800               810               820
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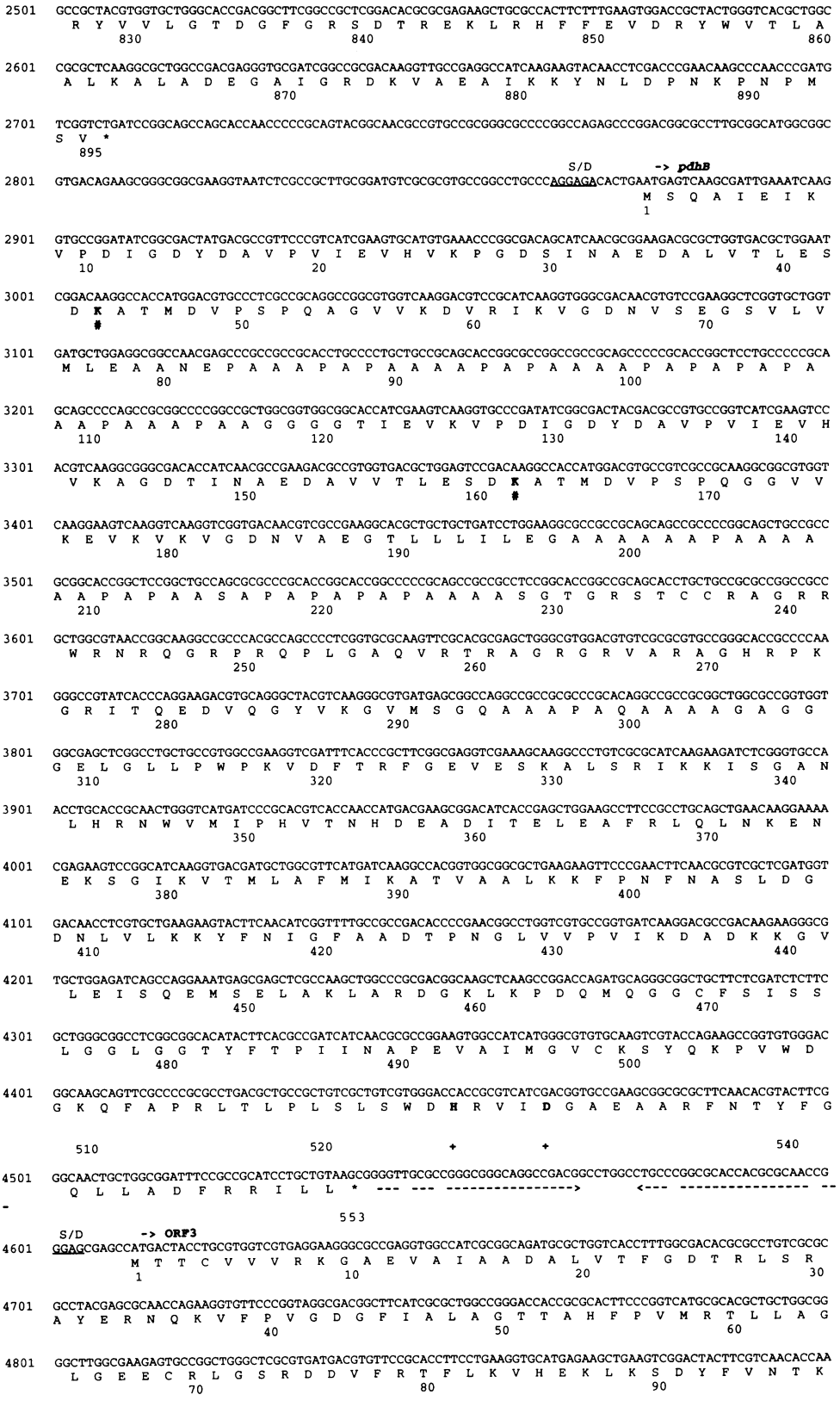

FIG. 2—*Continued.*

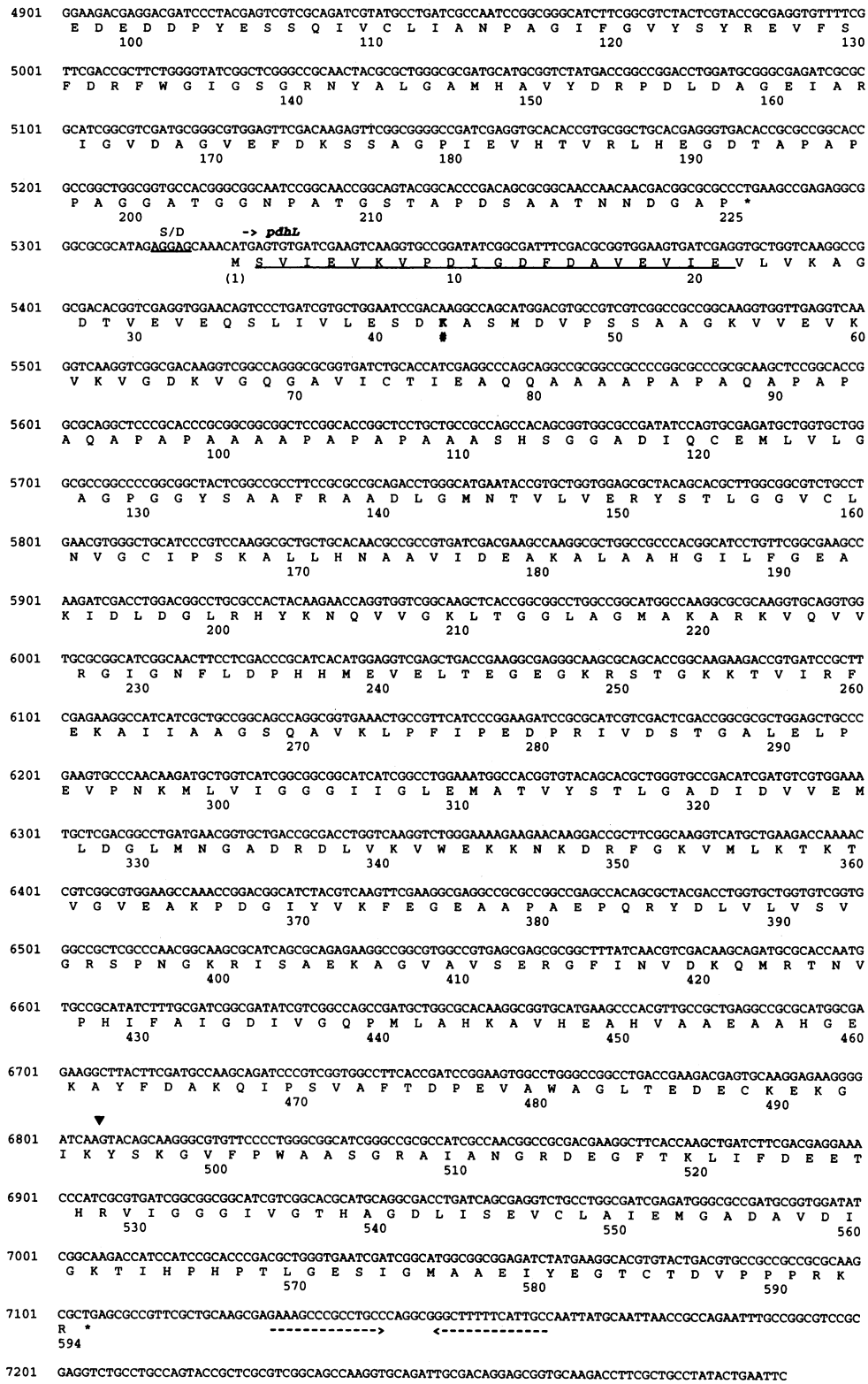

FIG. 2—*Continued.*

probably involved in the binding of the metal ion (i.e., Mg^{2+}) as well as of the diphosphate group of the cofactor (38).

The *pdhB* gene product consisted of 553 amino acid residues (Fig. 2) with a calculated M_r of 57,273. The DHLTA of *A. eutrophus* exhibited significant homologies with up to 58.3% amino acid identity to E2 components of various 2-oxo acid dehydrogenase complexes from different sources (Table 2). Amino acid sequence analysis of the N-terminal region of this protein revealed two repeating units (amino acid positions 5 to 71 and 123 to 189; Fig. 2), which exhibited homologies to the consensus sequence for the attachment sites of lipoate (8). One lysine residue was identified in the center of each repeating unit (amino acid positions 44 and 62, respectively; Fig. 2), which is lipoylated in the E2 components of 2-oxo acid dehydrogenase complexes (8). Both repeating units were flanked at their C termini by segments composed mainly of alanine and proline residues (Fig. 2). Therefore, the C-terminal flanking regions of the putative lipoyl domains resembled those of the interdomain linker segments of DHLTA from *E. coli* (66) and other organisms. The interdomain linker segments are thought to provide flexibility to the lipoyl domains, facilitating active-site coupling within the multienzyme complexes (55). The C-terminal region of the *pdhB* gene product exhibited strong homologies to the corresponding regions of the catalytic domains of dihydrolipoamide acyltransferases, e.g., of the E2 component of the *E. coli* pyruvate dehydrogenase complex (66), including the conserved putative active-site histidine-aspartate couple (His-526 and Asp-530, Fig. 2) (20).

The polypeptide encoded by *pdhL* consisted of 594 amino acid residues and had a calculated M_r of 62,039. The main part of the amino acid sequence deduced from the nucleotide sequence of *pdhL*, which starts at amino acid position 112 and extends to the C terminus, exhibited up to 66.8% amino acid identity to the total amino acid sequences of DHLDHs from various sources (Table 2). This main portion of the polypeptide contains the characteristic sequence motifs which are conserved within the family of pyridine nucleotide-disulfide oxidoreductases (10) (Fig. 3), i.e., the flavin adenine dinucleotide (FAD)-binding region (amino acid positions 125 to 150), the disulfide-active site surrounding both redox-active cysteine residues (Cys-159 and Cys-164), the NAD(H)-binding region (amino acid positions 297 to 325), and the interface region (downstream from amino acid position 554). In contrast to the classical type of DHLDH, which contains a FAD-binding domain close to the N terminus, the FAD-binding region of the *pdhL*-encoded protein of *A. eutrophus* is preceded by a typical lipoyl domain (Fig. 3) that is normally only found in the N-terminal region of dihydrolipoamide acyltransferases (this study and reference 55). To our knowledge, only the *acoL*-encoded DHLDH, which was recently identified and characterized as the E3 component of the acetoin dehydrogenase enzyme system of the strictly anaerobic, gram-positive bacterium *C. magnum* (34), exhibited analogous domain structures and had significant homology to the total amino acid sequence deduced from the nucleotide sequence of *pdhL* (35.8% amino acid identity; Table 2 and Fig. 3). This indicated that the *acoL* and *pdhL* gene products of *C. magnum* and *A. eutrophus*, respectively, represent a new type of DHLDH.

Heterologous expression of *pdhA*, *pdhB*, and *pdhL* in *E. coli*. To confirm the molecular data, the *A. eutrophus pdh* genes should be expressed in *E. coli*, and independent evidence for the existence of this unusual DHLDH should be obtained, e.g., by purification and subsequent analysis of the DHLDH protein. The fragments *E*1.0 and *E*6.3 harboring *pdhA*, *pdhB*, ORF3, and *pdhL* were isolated from pSKE1.0 and pSKE6.3, respectively, and ligated with *Eco*RI-digested pBluescript KS^- and SK^- DNA. One of the resulting plasmids, pKSEE7.3, harbored *pdhA*, *pdhB*, ORF3, and *pdhL* in the original order and colinear to as well as downstream of *lacZ'*. Transformants of *E. coli* DH5α, harboring pKSEE7.3, were incubated for 12 h in LB medium or TB in the presence of either 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) or 1.0% (wt/vol) glucose. Crude protein extracts obtained from the transformants were separated by SDS-PAGE; however, the electropherograms revealed no evidence for the expression of proteins, which corresponded to the predicted sizes of the *pdh* gene products. The rationale for this remains unclear.

To achieve expression under the control of the T7 RNA polymerase promoter, pSKEE7.3 was restricted with *Sma*I and *Hin*dIII, and the resulting 7.3-kbp *Sma*I-*Hin*dIII fragment, carrying *pdhA*, *pdhB*, ORF3, and *pdhL*, was inserted into the T7 RNA polymerase promoter plasmid pT7-4. The resulting plasmid, pT7-4SH7.3, which harbored the β-lactamase gene and the *pdh* genes plus ORF3 colinear to the T7 promoter, was introduced into *E. coli* K38(pGP1-2), which contained a heat-inducible copy of the gene for T7 RNA polymerase on plasmid pGP1-2. At 30°C, the T7 promoter was repressed by the *c*I857 repressor. Raising the temperature to 42°C induced the synthesis of the T7 RNA polymerase, and this in turn initiated transcription of the genes inserted into pT7-4 by the T7 promoter (68). Rifampin, which was added to a final concentration of 400 μg/ml, inhibited the bacterial RNA polymerase, whereas the T7 RNA polymerase was not affected. Plasmid pT7-4SH7.3 directed the synthesis of three proteins with apparent M_rs of 94,000 ± 10,000, 73,300 ± 2,000, and 69,700 ± 2,000), respectively, in addition to the β-lactamase (M_r, 29,000 ± 1,000) (Fig. 4, lanes b and c). These proteins were absent in transformants harboring only the vector (Fig. 4, lanes a and d). An M_r of 94,000 was in good agreement with the theoretical M_r of 100,251 calculated for the *pdhA* gene product. In contrast, the M_rs calculated for PdhB and PdhL were 57,273 and 62,039, respectively, and were significantly lower than those estimated for the two other proteins of the autoradiogram of the SDS-polyacrylamide gel. This coincided with the anomalous electrophoretic migration behavior of SDS-denaturated dihydrolipoamide acyltransferases from different sources, which has been repeatly reported in the literature and is caused by elongated or swollen lipoyl domains (7, 23, 41, 44, 46, 66). It therefore corresponded with the putative presence of lipoyl domains at the N termini of the gene products of *pdhB* and *pdhL*. A protein band, which would correspond to the putative ORF3 gene product and would represent a protein comprising 225 amino acids (Fig. 2) and exhibiting a calculated M_r of 25,000, was not detected in the electropherograms of the recombinant transformants.

Enzyme activities of the pyruvate dehydrogenase complex and of its components were assayed in ultrasonic extracts obtained from cells of recombinant strains which had been grown in TB or M9 medium supplemented with 0.5% (wt/vol) sodium succinate or acetate. Pyruvate dehydrogenase (E1) activity was not detectable in either *E. coli* strain, using either DCPIP or ferricyanide as the nonphysiological electron acceptor. The specific activities of DHLTA (E2) and of DHLDH (E3) in the recombinant *E. coli* K38(pGP1-2, pT7-4SH7.3) were significantly higher than in the control *E. coli* K38, which harbored only plasmid pGP1-2 (Table 3), indicating the expression of *A. eutrophus pdhB* as well as of *pdhL*. No differences were obtained for the specific activity of the overall reaction of the pyruvate dehydrogenase complex in both strains of *E. coli*, using NAD^+ as the physiological electron acceptor.

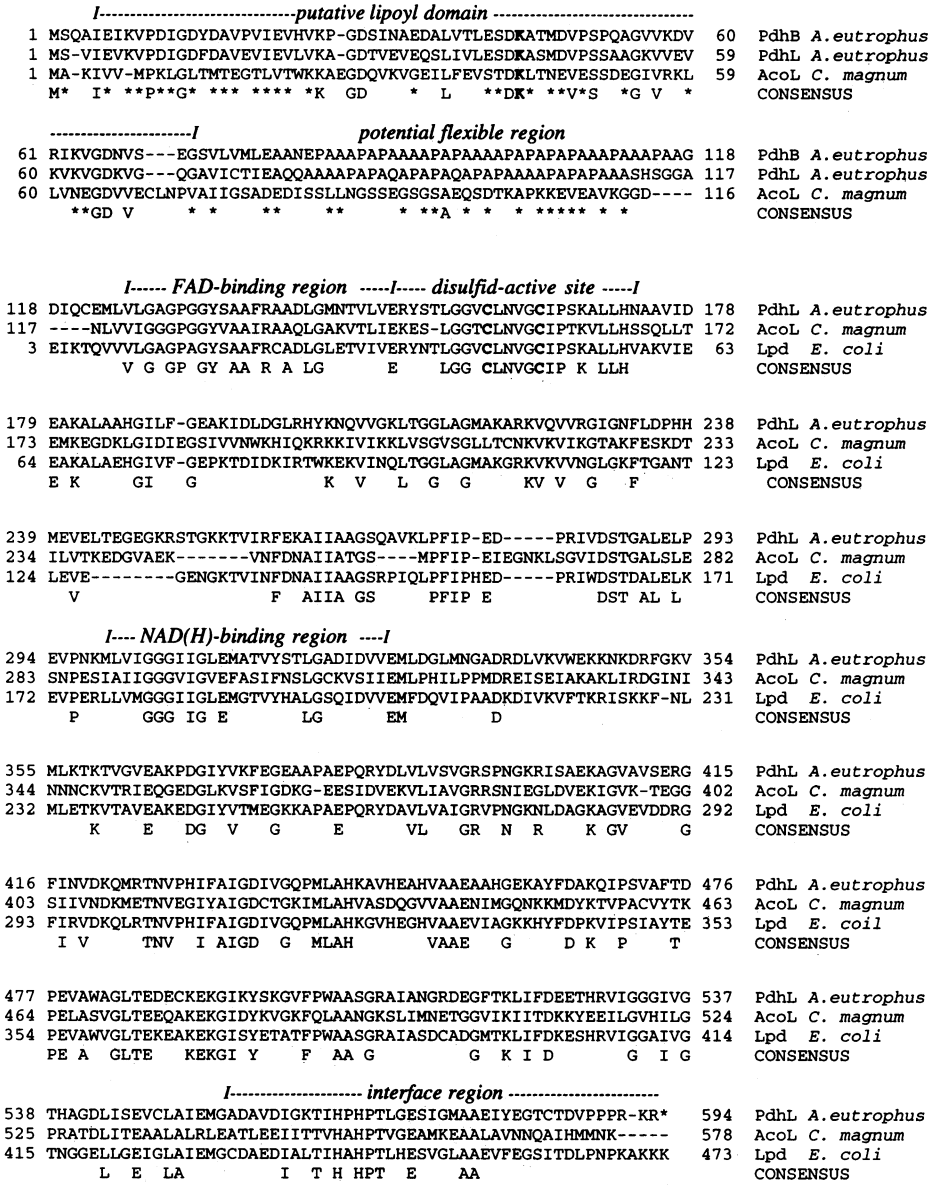

FIG. 3. Amino acid sequence comparison of the E3 component of the *A. eutrophus* pyruvate dehydrogenase complex with other proteins. Sequences have been aligned by the program Multalign (33). Amino acids are specified by standard one-letter abbreviations, and numbers on the left and right side indicate positions of amino acids within the protein. Amino acids conserved among all aligned sequences are compiled in the line CONSENSUS. Amino acids which are identical in PdhB and PdhL are marked by asterisks. Conserved lysine residues of the putative lipoyl domains and cysteine residues of the putative disulfide-active sites are shown in boldface. Abbreviations: PdhB and PdhL, E2 and E3 components, respectively, of the *A. eutrophus* pyruvate dehydrogenase complex; AcoL, E3 component of the *C. magnum* acetoin dehydrogenase enzyme system; Lpd, DHLDH of *E. coli*.

Purification of the DHLDH of the *A. eutrophus* pyruvate dehydrogenase complex. The *pdhL* gene product was purified 60-fold to approximately 90% electrophoretic homogeneity from the recombinant *E. coli* K38(pGP1-2, pT7-4SH7.3) in a three-step procedure (Table 4). At pH 7.5 in 100 mM Tris hydrochloride buffer, DHLDH activity bound to DEAE-Sephacel and was eluted at 150 mM NaCl by using a linear gradient of sodium chloride. Chromatography on DEAE-Sephacel resulted in a 4.9-fold increase of the specific activity, and 83% of the enzyme activity was recovered. The enzyme preparation obtained was tested for binding of DHLDH to several triazine dye affinity media which had been equilibrated with 100 mM Tris hydrochloride buffer (pH 7.5). DHLDH bound to Procion Blue H-ERD, Procion Brown MX-5BR, and Procion Green HE-4BD, whereas it did not bind to Procion Blue MX-2G, Procion Orange A, Procion Yellow MX-8G or Procion Green A, each coupled to Sepharose 4B-CL. Because of the strength of binding, Sepharose 4B-CL–Procion Blue H-ERD was chosen for further purification. DHLDH was eluted from the column at 250 mM NaCl. The enzyme was enriched 13-fold by this step, and 64% of the activity was recovered. Because of the considerable insensitivity of DHLDH to high temperatures (15, 45) that was also exhibited by the enzyme of *A. eutrophus*, some contaminating proteins could be removed by heat precipitation.

Properties of DHLDH. SDS-PAGE of the DHLDH prepa-

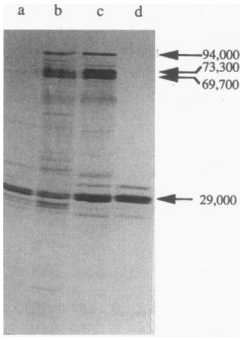

FIG. 4. T7 polymerase promoter-dependent expression of the *A. eutrophus pdh* genes. L-[^{35}S]methionine-labeled proteins (68) were separated electrophoretically in an 11.5% (wt/vol) SDS-polyacrylamide gel and autoradiographed. Lanes contained crude extracts of different transformants of *E. coli* K38(pGP1-2) with the following plasmids: a, pT7-4 (cultivated without rifampin); b, pT7-4SH7.3 (without rifampin); c, pT7-4SH7.3 (plus rifampin); and d, pT7-4 (plus rifampin). Proteins expressed under the direction of the T7 promoter are indicated by arrows, and the apparent M_rs are given at the right.

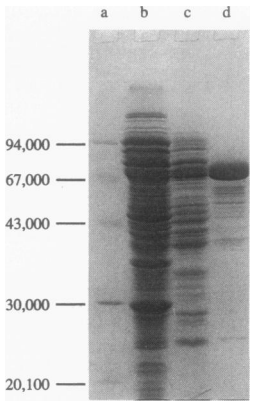

FIG. 5. Purification of the DHLDH (E3) component of the *A. eutrophus* pyruvate dehydrogenase complex from the recombinant *E. coli* K38(pGP1-2, pT7-4SH7.3). Samples were separated in an 11.5% (wt/vol) SDS-polyacrylamide gel and were stained with Serva Blue R. Lanes: a, molecular mass standard proteins; b, 100 μg of protein from the soluble cell fraction; c, 38 μg of protein after chromatography on DEAE-Sephacel; d, 14 μg of protein after chromatography on Procion Blue H-ERD. The M_rs of molecular mass standard proteins are given at the left.

ration resulted in one major protein band, indicating the presence of only one type of subunit (Fig. 5, lane d) with an M_r of 69,200 ± 1,000. This apparent M_r agreed with the M_r (69,700 ± 1,000) of one of the L-[^{35}S]methionine-labeled major protein bands from the crude extract of the recombinant *E. coli* K38(pGP1-2, pT7-4SH7.3) (Fig. 4, lanes b and c). The DHLDH from *A. eutrophus* proved to be highly specific for NAD(H); no reaction was observed with NADP(H). The N-terminal amino acid sequence determined from the highly purified DHLDH (SVIEVKVPDIGDFDAVEVIE) was identical to the N-terminal amino acid sequence deduced from the nucleotide sequence of *pdhL* except for the terminal methionine residue, which was presumably removed by posttranslational modification (Fig. 2).

TABLE 3. Expression of the *A. eutrophus* pyruvate dehydrogenase complex and of its components in *E. coli*

Growth substrate (concn)	Enzyme sp act[a] (U/mg)		
	PDH complex	E2	E3
E. coli K38(pGP1-2)[b]			
M9, succinate (0.5% [wt/vol])	0.05	0.014	<0.001
M9, acetate (0.5% [wt/vol])	0.05	0.015	<0.001
TB	0.12	<0.001	<0.001
E. coli K38(pGP1-2, pT7-4SH7.3)[b]			
M9, succinate (0.5% [wt/vol])	0.05	0.094	1.60
M9, acetate (0.5% [wt/vol])	0.03	0.035	0.26
TB	0.09	0.300	11.26
E. coli YYC202(pKSEE7.3)[c]			
MM2, glucose (0.2% [wt/vol])	0.04	0.090	0.21
MM2, glucose (0.2% [wt/vol]) plus IPTG (0.2 mM)	0.08	0.160	0.36
MM2, glucose (0.2% [wt/vol]) plus acetate (0.01% [wt/vol])	0.03	0.100	0.24
E. coli YYC202[c]			
MM2, glucose (0.2% [wt/vol]) plus acetate (0.01% [wt/vol])	<0.001	<0.001	<0.001

[a] Assayed in ultrasonic extracts under anaerobic conditions except for the DHLTA assay. PDH, pyruvate dehydrogenase.

[b] *E. coli* K38(pGP1-2) and the recombinant *E. coli* strain were grown at 30°C in 50 ml of TB or M9, supplemented with thiamine (1 μg/ml). Ampicillin and kanamycin were each added to a final concentration of 100 μg/ml. In the late exponential growth phase, the temperature was raised to 42°C for 20 min. After rifampin had been added (400 μg/ml), the cells were shifted to 37°C and were harvested after 2 h of cultivation at this temperature.

[c] *E. coli* YYC202 (pKSEE7.3) and *E. coli* YYC202 were grown at 37°C in 50 ml of MM2. Ampicillin and tetracycline were added to final concentrations of 100 and 12.5 μg/ml, respectively. Cells were harvested at the beginning of the stationary growth phase (Fig. 6).

Complementation of the *aceEF* deletion mutant *E. coli* YYC202. *E. coli* YYC202 is affected in *aceE* (encoding pyruvate dehydrogenase) and *aceF* (encoding DHLTA) and is unable to grow in media lacking acetate (11, 12). To investigate the ability to complement this mutation, plasmid pKSEE7.3 harboring *pdhA*, *pdhB*, ORF3, and *pdhL* colinear to *lacZ'* was transformed into *E. coli* YYC202. *E. coli* YYC202 and the recombinant strain were incubated at 37°C in MM2 containing glucose (0.2% [wt/vol]), glucose (0.2% [wt/vol]) plus IPTG (0.2 mM), or glucose plus acetate (0.2 and 0.01% [wt/vol], respectively). The recombinant *E. coli* YYC202(pKSEE7.3) grew well under all three conditions, whereas the *aceEF* mutant grew only in MM2 supplemented with acetate (Fig. 6). DHLDH and DHLTA activities as well as the overall reaction of the pyruvate dehydrogenase complex were detected only in the recombinant strain of *E. coli* (Table 3) and not in the parent strain. Although E1 activity was not detectable, the restoration of prototrophy in transformants harboring pKSEE7.3 clearly demonstrated that a functionally active pyruvate dehydrogenase was expressed from *pdhA*.

DISCUSSION

This study identified and characterized the *A. eutrophus* structural genes *pdhA*, *pdhB*, and *pdhL* for the enzyme components E1, E2, and E3, respectively, of the pyruvate dehydrogenase complex. Together with ORF3, the function of which remained unknown, these genes occur as a cluster that was colinear in the order *pdhA*, *pdhB*, ORF3, and *pdhL* on a 7.3-kbp chromosomal fragment. It has to be shown whether all *pdh* genes and ORF3 constitute a single operon, which is transcribed from an as yet unidentified promoter upstream of *pdhA*, or if *pdhL* can be independently expressed, such as in *E. coli* (21). The genes for the E1 and E2 components of other

TABLE 4. Purification of the *A. eutrophus* DHLDH from recombinant *E. coli*

Step	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery of activity (%)
Soluble fraction	6.5	26.0	169	333	1.97	1.0	100
DEAE-Sephacel	7.4	3.9	28.5	277	9.73	4.9	83.2
Procion Blue H-ERD	1.0	1.4	1.4	178	126	64.1	53.4
Heat precipitation	0.9	1.3	1.2	138	118	60.1	41.5

2-oxo acid dehydrogenase complexes were found to be organized in one operon with the latter as the distal gene, whereas the gene for the respective E3 component is not in all cases included.

The putative amino acid sequence of the *A. eutrophus* pyruvate dehydrogenase (E1) revealed this enzyme to be the third example of E1 components, which are composed of monomeric E1 components, in addition to the pyruvate dehydrogenases of *E. coli* and *Azotobacter vinelandii* (23, 65). Significant homologies to 2-oxo acid dehydrogenases, which are composed of heteromeric E1 components, did not occur. The *pdhB* gene product revealed striking homologies to dihydrolipoamide acyltransferases from various sources. One major difference of these E2 components is the number of lipoyl domains at their N termini (50, 55). The *A. eutrophus* enzyme, which was investigated in this study, contained two putative lipoyl domains. The DHLTAs of *E. coli* and *Azotobacter vinelandii*, which each contain three very similar lipoyl domains (23, 66), exhibited highest homologies to the *A. eutrophus* enzyme, whereas the similarities to the DHLTAs which contain only one lipoyl domain (55) were significantly weaker.

The most remarkable result of this study was the detection of a new type of DHLDH. The DHLDH of the *A. eutrophus* pyruvate dehydrogenase complex investigated in this study possessed a lipoyl domain at the N-terminal region, and as in DHLTAs, the lipoyl domain was separated from the other part of the enzyme by a flexible hinge region (Fig. 7). The major C-terminal part of the protein was highly homologous to the classical type of DHLDHs. The *pdhL* gene product shared this unique property only with the E3 component (*acoL* gene product) of the acetoin dehydrogenase enzyme system of the strictly fermentative, gram-positive bacterium *C. magnum* (34). Therefore, these two DHLDHs are distinguished from all other known members of the highly conservative enzyme family of pyridine nucleotide-disulfide oxidoreductases (10, 15, 34, 46). Since the DNA sequence of the 5′ region of *pdhL* was obtained also from three independent *E. coli* clones, it was ensured that the occurrence of the N-terminal lipoyl domain in the *A. eutrophus* DHLDH did not result from a cloning artifact. The nucleotide sequence encoding the lipoyl domain of the *pdhL* gene product was highly similar (approximately 70% identical base pairs), but not identical, to those regions of *pdhB* encoding the two lipoyl domains of DHLTA. Therefore, the lipoyl domain at the N terminus of the *pdhL* gene product might have originated during the evolution from a sequence repetition of one of the 5′ regions of *pdhB* encoding the corresponding lipoyl domain.

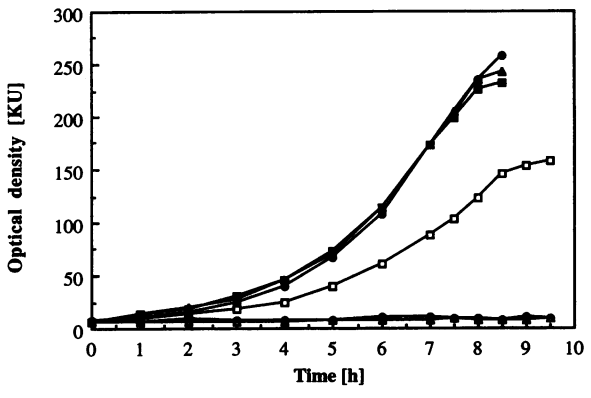

FIG. 6. Phenotypic complementation of the *aceEF* deletion of the mutant *E. coli* YYC202. *E. coli* YYC202 (open symbols) and a recombinant strain harboring pKSEE7.3 (closed symbols) were grown at 37°C in 50 ml MM2 supplemented with ampicillin and tetracycline (100 and 12.5 μg/ml, respectively) and containing glucose (0.2% [wt/vol]) (circles), glucose (0.2% [wt/vol]) plus IPTG (0.2 mM) (triangles), or glucose plus acetate (0.2 and 0.01% [wt/vol], respectively) (squares) as carbon sources. Growth was monitored spectroscopically by using a Klett-Summerson photometer. KU, Klett units.

The codon preferences and a strong bias for G+C in codon position 3 of all three *pdh* genes and of ORF3 are in good agreement with those of other genes of *A. eutrophus* (35, 52, 53, 61), and all *pdh* genes as well as ORF3 are preceded by tentative ribosome-binding sites. Since the analysis of the corresponding 7.3-kbp nucleotide sequence revealed no evidence for potential promoter structures, expression of the *pdh* genes was dependent on promoters located on the vectors. The heterologous expression of *pdhA*, *pdhB*, and *pdhL* under the control of the T7 RNA polymerase promoter in *E. coli* K38(pGP1-2, pT7-4SH7.3) was demonstrated by the occurrence of radiolabeled protein bands in electropherograms as well as by spectrometric detection of DHLDH and DHLTA enzyme activities. Pyruvate dehydrogenase activity was not detectable in this clone. The expression of a functionally active pyruvate dehydrogenase was, however, clearly demonstrated by the phenotypic complementation of the *aceEF* deletion mutant *E. coli* YYC202 as well as by the detection of the overall reaction of the pyruvate dehydrogenase complex in crude protein extracts of transformants of *E. coli* YYC202 harboring pKSEE7.3. Heterologous expression of the *A. eutrophus pdh* genes under the control of the *lac* promoter restored, for example, prototrophy in transformants of *E. coli* YYC202. These data revealed evidence for a very labile pyruvate dehydrogenase (E1) enzyme component.

Besides the acetoin-cleaving system (52), the pyruvate dehydrogenase complex is the second dehydrogenase enzyme system in *A. eutrophus* for which the structural genes have been identified and characterized. We recently also analyzed the genes for the 2-oxoglutarate dehydrogenase complex and identified an additional structural gene for a second DHLDH downstream of the corresponding genes for the E1 and E2 components (26). Further studies will identify the branched chain 2-oxo acid dehydrogenase complex as well as a putative glycine decarboxylase complex of *A. eutrophus*. All five enzyme systems are potentially targets of the *pdhL* gene product. It

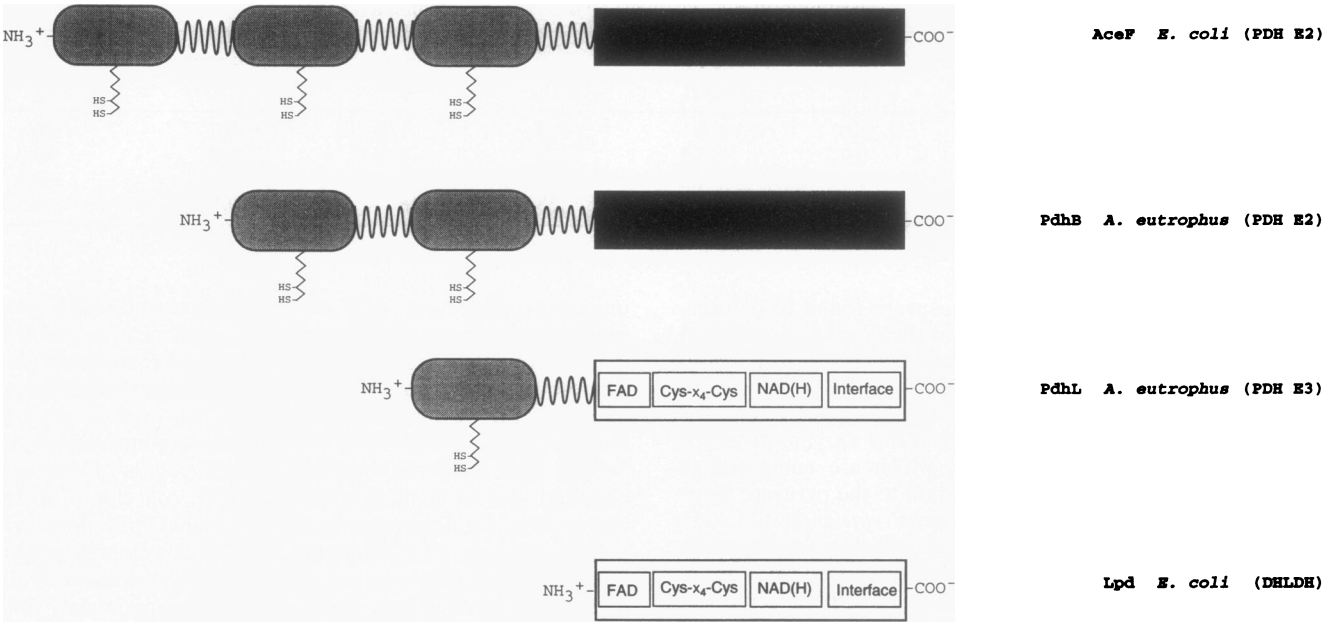

FIG. 7. Comparison of the putative domain structures of the E2 and E3 components of the *A. eutrophus* pyruvate dehydrogenase complex with the corresponding components of the *E. coli* pyruvate dehydrogenase complex. Catalytic domains of DHLTAs (E2) are shown as black rectangles. The C-terminal part of *A. eutrophus* PdhL, which exhibited complete sequence overlap to classical DHLDHs and which includes the characteristic sequence motifs that are conserved in the family of pyridine nucleotide-disulfide oxidoreductases, and the DHLDH of *E. coli* are shown as open rectangles. Putative lipoyl domains are depicted as grey segments. Wavy lines indicate the putative flexible regions in the C-terminal flanking regions of the putative lipoyl domains. The relative sizes of the respective domains are not drawn to scale. Notation: PDH, pyruvate dehydrogenase complex; DHLDH, dihydrolipoamide dehydrogenase; FAD, FAD-binding site; Cys-x_4-Cys, disulfide-active site; NAD(H), NAD(H)-binding region; Interface, interface region.

remains to be elucidated whether the *pdhL* gene product or the second DHLDH is shared by several enzyme systems. In *E. coli* and *Pseudomonas putida*, only one DHLDH is present for the pyruvate and 2-oxoglutarate dehydrogenase complex and the glycine cleavage enzyme system (19, 21, 49, 63). Besides LPD-glc, *P. putida* contains two additional separate DHLDHs: LPD-val, the E3 component of the branched-chain oxo acid dehydrogenase complex, and LPD-3, which was isolated from a pseudorevertant of a mutant affected in the LPD-glc structural gene. The LPD-3 gene is not part of an oxo acid dehydrogenase operon, and the gene product replaces LPD-glc in mutants with insertionally inactivated *lpd*-glc (49).

A Tn*5*-induced PHB-leaky mutant of *A. eutrophus*, which harbored the transposon inserted into *pdhL*, expressed less DHLDH activity than the wild type but grew like the parent strain on pyruvate, fructose, gluconate, acetoin, or succinate (54). Since the transposon had inserted into the C-terminal part of *pdhL* (Fig. 2), in this mutant the major portion of this DHLDH (amino acids 1 to 493), which includes the characteristic sequence motifs such as an FAD-binding site, disulfide-active site, and NAD(H)-binding region, remained unaffected. Therefore, it is obscure whether the modified *pdhL* gene still expresses partially active DHLDH or whether the second DHLDH substitutes for the *pdhL* gene product. Studies with insertionally inactivated *pdhL* and the characterization of the second DHLDH (26, 54) will reveal additional features of DHLDHs in *A. eutrophus*.

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