JOURNAL OF BACTERIOLOGY, July 1991, p. 4056–4071
0021-9193/91/134056-16$02.00/0

Vol. 173, No. 13

Identification and Molecular Characterization of the *Alcaligenes eutrophus* H16 *aco* Operon Genes Involved in Acetoin Catabolism

HORST PRIEFERT,[1] SILKE HEIN,[1] NIELS KRÜGER,[1] KARIN ZEH,[1] BERNHARD SCHMIDT,[2] AND ALEXANDER STEINBÜCHEL[1]*

Institut für Mikrobiologie der Georg-August-Universität zu Göttingen, Grisebachstraße 8,[1] and Zentrum Biochemie, Abteilung Biochemie II der Georg-August-Universität zu Göttingen, Goßlerstraße 12d,[2] Germany

Received 17 January 1991/Accepted 22 April 1991

Acetoin:dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR) and the fast-migrating protein (FMP) were purified to homogeneity from crude extracts of acetoin-grown cells of *Alcaligenes eutrophus*. Ao:DCPIP OR consisted of α and β subunits (M_rs, 35,500 and 36,000, respectively), and a tetrameric $\alpha_2\beta_2$ structure was most likely for the native protein. The molecular weight of FMP subunits was 39,000. The N-terminal amino acid sequences of the three proteins were determined, and oligonucleotides were synthesized on the basis of the codon usage of *A. eutrophus*. With these, the structural genes for the α and β subunits of Ao:DCPIP OR and FMP, which were referred to as *acoA*, *acoB*, and *acoC*, respectively, were localized on one single *Eco*RI restriction fragment which has been cloned recently (C. Fründ, H. Priefert, A. Steinbüchel, and H. G. Schlegel, J. Bacteriol. 171:6539–6548, 1989). The nucleotide sequences of a 5.3-kbp region of this fragment and one adjacent fragment were determined, and the structural genes for *acoA* (1,002 bp), *acoB* (1,017 bp), and *acoC* (1,125 bp) were identified. Together with the gene *acoX*, whose function is still unknown and which is represented by a 1,080-bp open reading frame, these genes are probably organized in one single operon (*acoXABC*). The transcription start site was identified 27 bp upstream of *acoX*; this site was preceded by a region which exhibited complete homology to the enterobacterial σ^{54}-dependent promoter consensus sequence. The amino acid sequences deduced from *acoA* and *acoB* for the α subunit (M_r, 35,243) and the β subunit (M_r, 35,788) exhibited significant homologies to the primary structures of the dehydrogenase components of various 2-oxo acid dehydrogenase complexes, whereas those deduced from *acoC* for FMP (M_r, 38,941) revealed homology to the dihydrolipoamide acetyltransferase of *Escherichia coli*. The occurrence of a new enzyme type for the degradation of acetoin is discussed.

Alcaligenes eutrophus has been studied with respect to the degradation of acetoin. Acetoin-grown cells of *A. eutrophus* are devoid of 2,3-butanediol dehydrogenase and acetoin dehydrogenase (diacetyl forming; EC 1.1.1.5), and mutants which lack 2,3-butanediol dehydrogenase (73) are still able to utilize acetoin as the sole carbon source for growth (72). Therefore, this bacterium lacks the 2,3-butanediol cycle which was described by Juni and Heym (36) in *Acinetobacter calcoaceticus*. In contrast, *A. eutrophus* degrades acetoin directly by cleavage into two C_2 compounds (23), like *Bacillus subtilis* (35, 43, 44) or *Pelobacter carbinolicus* (48–50).

During growth of *A. eutrophus* on acetoin, formation of at least three different proteins is induced: (i) an acetoin:2,6-dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR), (ii) a fast-migrating protein (FMP) which exhibits a high mobility toward amido black in polyacrylamide gels, and (iii) acetaldehyde dehydrogenase II (EC 1.2.1.3). Ao:DCPIP OR catalyzes 2,6-dichlorophenolindophenol-dependent cleavage of acetoin into acetate and acetaldehyde. However, it is not known whether this reaction is an artifact that occurs only in vitro and whether in vivo coenzyme A (CoA)-dependent cleavage of acetoin to acetyl-CoA and acetaldehyde occurs as in *P. carbinolicus* (48–50). Acetaldehyde is presumably oxidized to acetate by acetaldehyde dehydrogenase II, which is one of three acetaldehyde-oxidizing enzymes in *A. eutrophus* and exhibits a high affinity towards acetaldehyde (33); acetate is subsequently converted to acetyl-CoA by acetate thiokinase. The function of FMP is still unknown.

Recently, the gene loci which are essential for catabolism of acetoin in *A. eutrophus* have been located on five different genomic *Eco*RI restriction fragments (A, B, C, D, and E [23]). The gene locus on fragment B was identical to the *hno* gene and represents an *rpoN*-like gene (23, 59). As insertional inactivation of this gene caused a pleiotropic effect and as these mutants were unable to synthesize proteins which are essential for degradation of acetoin, it was concluded that expression of the corresponding genes is *rpoN* dependent (23).

Physiological studies localized the genes for Ao:DCPIP OR and FMP on restriction fragments A and C, which are closely linked in the genome (23), whereas the gene for acetaldehyde dehydrogenase II was localized on restriction fragment D (52a). The present study was aimed at isolation of FMP and Ao:DCPIP OR and identification and characterization of their structural genes. The results of this study are consistent with our assumption that FMP has a catalytic rather than a regulatory function (23), and the results indicated striking similarities of the *A. eutrophus* and *P. carbinolicus* acetoin-catabolic enzyme systems, which both represent a new type of dehydrogenase enzyme system.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *A. eutrophus* and *Escherichia coli* and the plasmids, DNA fragments, and bacteriophage used in this study are listed in Table 1.

Growth of bacteria. *E. coli* was grown at 37°C in Luria-

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, bacteriophage, and DNA fragments used in this study

Strain, plasmid, bacteriophage, or DNA fragment	Relevant characteristic(s)	Source or reference
A. eutrophus		
H16	Wild type, autotrophic, prototrophic	DSM 428, ATCC 17699; 83
N9A	Wild type, autotrophic, prototrophic	DSM 518
E. coli		
DH1	*recA1 thi-1*	24
S17-1	*recA*; harbors the *tra* genes of plasmid RP4 in the chromosome; *proA thi-1*	69
JM83	*ara* Δ(*lac-proAB*) *rpsL* (Sm^r) *thi-1*, φ80 *lacZ*Δ*15*	79
WL87	*recBC*	Amersham Buchler
WL95	*metB supE supF hsdRK trpR* P2	Amersham Buchler
BHB2688	N205 *recA*(λ *imm*434 *c*Its857 *b2 red*3 *E*am4 *S*am7/λ)	28, 30
BHB2690	N205 *recA*(λ *imm*434 *c*Its857 *b2 red*3 *D*am15 *S*am7/λ)	28, 30
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ$^-$ *lac* (F′ *proAB lacI*^{q}ZΔM15 Tn*10* [Tet])	13
Plasmids		
pSUP5011	Cm^r Ap^r Km^r; harbors Tn*5*::*mob*	68
pSUP202	Tc^r Cm^r Ap^r	69
pHC79	Cosmid; Tc^r Ap^r	29
pUC9-1	Ap^r *lacPOZ′*	25
pBluescript KS$^-$	Ap^r *lacPOZ′*; T_7 and T_3 promoter	Stratagene[a]
pBluescript SK$^-$	Ap^r *lacPOZ′*; T_7 and T_3 promoter	Stratagene
pVK101	Tc^r Km^r	37
pHP1016	Tc^r Km^r Cm^r	23
Bacteriophage λL47		42
DNA fragments		
A, B, C, D, E	Genomic *Eco*RI restriction fragments of *A. eutrophus* harboring genes essential for acetoin catabolism	23
AT^{H1098}, AT^{N1230}, AT^{N1236}, AT^{N1239}, AT^{N1240}, AT^{N1244}, AT^{N1246}, AT^{N1247}, BT^{H1070}, BT^{N1242}, CT^{H1053}, CT^{N1233}, CT^{N1245}, CT^{N1248}, DT^{H1074}, ET^{H1094}	Tn*5*-harboring genomic *Eco*RI restriction fragments A, B, C, D, and E from mutants of *A. eutrophus* affected in acetoin catabolism	23

[a] San Diego, Calif.

Bertani medium or in M9 mineral salts medium (60). *A. eutrophus* was grown aerobically at 30°C in either a nutrient broth medium (0.8% [wt/vol]) or a mineral salts medium (63). To obtain bulk amounts of cells of *A. eutrophus* H16 as a source for FMP and Ao:DCPIP OR, cells were cultivated in 4 or 280 liters of mineral salts medium with 0.3% (wt/vol) acetoin as the carbon source in 5-liter glass fermentors (E. Schütt, Göttingen, Germany) or a 365-liter steel fermentor (Giovaola Freres SA, Monthey, Switzerland), respectively. The vessel was stirred at 500 rpm, and the cells were harvested at the late exponential growth phase in a GLT continuous centrifuge (Carl Padberg, Lahr, Germany). Cells used as a source for purification of FMP or Ao:DCPIP OR were washed with 50 mM potassium phosphate buffer, pH 7.0, or 100 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride, respectively; the cells were stored at −20°C.

Purification of FMP. All steps were carried out at 4°C. Approximately 30 g of wet acetoin-grown cells of strain H16 were suspended in 60 ml of 50 mM potassium phosphate buffer, pH 7.0, and disrupted by sonication (2 × 3 min) by using an MSE (150 W) ultrasonic disintegrator with a probe 19.0 mm in diameter. Crude extracts were obtained by 60 min of centrifugation at 130,000 × *g* in a Sorvall OTD 50B ultracentrifuge (Dupont, Newton, Conn.). The resulting supernatant was dialyzed for 2 h against 2 × 5 liters of buffer.

In step 1, the dialyzed crude extract was applied to a column (5.0 by 10.0 cm; 20-ml bed volume [BV]) of DEAE-Sephacel equilibrated with the buffer used for dialysis containing 100 mM KCl. After the column was washed with 3 BV of buffer, the protein was eluted with a linear KCl gradient (100 to 500 mM KCl; 2 × 800 ml). Fractions (each 6.0 ml) containing FMP were combined; the proteins in this pool were concentrated by ultrafiltration in a diaflow chamber by using a PM10 membrane and washed with buffer to decrease the concentration of salts.

In step 2, approximately 5 ml of the concentrated DEAE-Sephacel eluate was applied to a column (2.5 by 100 cm; 500-ml BV) of Sephadex G75 which was equilibrated with 50 mM potassium phosphate buffer containing 300 mM KCl. Proteins were eluted with the same buffer at a flow rate of 15 ml/h. Fractions (each 5 ml) containing FMP were combined and concentrated by ultrafiltration to a volume of 6 ml.

In step 3, the Sephadex G75 eluate was layered on top of a column (1.5 by 29 cm; 50-ml BV) of Procion Brown MX-5BR-Sephadex 4B-CL which was equilibrated with 20 mM potassium phosphate buffer, pH 7.0. After the column was washed with 3 BV of buffer, the protein was eluted with a linear KCl gradient (0 to 200 mM KCl; 2 × 100 ml). Fractions (each 3 ml) which contained FMP at a high concentration but were devoid of Ao:DCPIP OR activity were combined, and the proteins were concentrated by ultrafiltration as described above.

Separation of two forms of FMP. To separate FMP-I and

FMP-II, 0.7 ml of the concentrated Procion Brown eluate was applied to a Mono Q HR5/5 column (5-ml BV), which was equilibrated with 50 mM potassium phosphate buffer and connected to an LCC-500 controlled fast protein liquid chromatography apparatus (Pharamcia LKB Biotechnologie GmbH, Freiburg, Germany). After the column was washed with 3 BV of buffer, the protein was eluted by washing with (i) a linear KCl gradient (0 to 210 mM KCl; 5 BV), (ii) 5 BV of buffer containing 210 mM KCl, and (iii) a linear KCl gradient (210 to 1,000 mM KCl; 5 BV) at a flow rate of 0.4 BV/min. Elution of the protein was monitored by measuring the A_{280}. Fractions of 1 ml were collected and analyzed for FMP content in polyacrylamide gels.

Purification of Ao:DCPIP OR. All steps were carried out at 4°C in 20 mM potassium phosphate buffer, pH 7.2, which contained 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride if not indicated otherwise. Ultracentrifugational supernatants were obtained as described for purification of FMP.

In step 1, the dialyzed crude extract was applied to a column (2.6 by 15 cm; 80-ml BV) of DEAE-Sephacel. After the column was washed with 5 BV of buffer, the protein was eluted with a linear KCl gradient (50 to 350 mM KCl; 2 × 600 ml). Fractions (each 10 ml) containing Ao:DCPIP OR activity were combined, and the proteins were concentrated by ultrafiltration by using a PM30 membrane.

In step 2, the concentrated DEAE-Sephacel eluate was applied to a column (2.6 by 94 cm; 500-ml BV) of Sephadex G-200 equilibrated with 100 mM potassium phosphate buffer, pH 7.2. Proteins were eluted at a flow rate of 9 ml/h. Fractions (each 5 ml) containing Ao:DCPIP OR activity were combined.

In step 3, the resulting Sephadex G-200 eluate was dialyzed for 14 h against 6 × 2 liters of 20 mM potassium phosphate buffer, pH 7.2, and layered on top of a column (2.6 by 9.7 cm; 52-ml BV) of Q-Sepharose. After the column was washed with 2 BV of buffer, the protein was eluted with a linear KCl gradient (50 to 350 mM KCl; 2 × 230 ml). Fractions (each 5 ml) containing Ao:DCPIP OR activity were combined, and the proteins were concentrated by ultrafiltration.

Preparation of triacine affinity media. Procion Brown MX-5BR, Procion Blue MX-2G, Procion Green HE-4BD, Procion Red HE-3B, Procion Yellow MX-8G, and Procion Orange A were coupled to Sepharose 4B-CL by the procedure of Atkinson et al. (5).

Determination of enzyme activities. Ao:DCPIP OR activity was determined by measuring DCPIP reduction as described recently (72). Dihydrolipoamide acetyltransferase (EC 2.3.1.12) activity was measured with a UV spectroscopic assay at 240 nm as described by Schwarz and Reed (67). For *S*-acetyldihydrolipoamide, an ε_{240} of 1.46 mM^{-1} cm^{-1} was determined by using the hydroxamate assay described by Reed and Willms (55), and DL-dihydrolipoamide was prepared by reduction of DL-lipoamide (54). In addition, dihydrolipoamide acetyltransferase was determined by employing the hydroxamate assay described by Reed and Willms (55).

Electrophoretic methods. Polyacrylamide gel electrophoresis under nondenaturing conditions was performed in 7.4% (wt/vol) gels as described by Stegemann et al. (71) and described in detail recently (72) or in linear polyacrylamide gradient (5 to 27.5% [wt/vol]) gels as described by Andersson et al. (4). Sodium dodecyl sulfate (SDS)- and mercaptoethanol-denatured proteins were separated in 11.5% (wt/vol) polyacrylamide gels in Tris-glycine buffer (25 mM Tris, 190 mM glycine, 0.1% [wt/vol] SDS [41]). Alternatively, 10 mM sodium phosphate buffer, pH 7.2, was used (82). The size of the gel was 1.5 by 140 by 140 mm, and electrophoresis was done for 5 to 6 h at a constant current of 40 mA.

Proteins were stained with Coomassie brilliant blue (81) or by the silver stain procedure (11). Standard proteins from commercially available molecular weight marker kits were used as references. Dihydrolipoamide acetyltransferase (DHLTA) was visualized in polyacrylamide gels by precipitation of Ca_3 $(PO_4)_2$ in the presence of acetylphosphate, CoA, phosphotransacetylase of *Bacillus stearothermophilus*, and dihydrolipoamide during incubation of the gel at 30°C in 100 mM Tris hydrochloride buffer, pH 7.0, containing 50 mM $CaCl_2$ (47a).

Determination of protein. Soluble protein was determined as described by Lowry et al. (45).

N-terminal sequence analysis. Sequence analysis was performed with a 477A pulsed liquid-phase protein-peptide sequencer (27) and a 120A on-line phenylthiohydantoin amino acid analyzer (58) (both from Applied Biosystems, Weiterstadt, Germany) as recommended by the manufacturer. Prior to sequencing, disulfide bonds between cysteine residues of the protein were reduced and alkylated by treatment with dithioerythritol and iodoacetic acid in one experiment as described by Allen (2). In one experiment, the protein was treated with *Staphylococcus aureus* V8 protease as recommended by the manufacturer (C. F. Boehringer & Soehne, Mannheim, Germany).

Isolation and manipulation of DNA. Plasmid DNA was isolated from crude lysates by the alkaline extraction procedure (10, 60). λ DNA from bacteriophage particles were prepared by the method of Sambrook et al. (60) from plates exhibiting confluent lysis of *E. coli* WL87, as described in detail recently (65). Isolated plasmid DNA was digested with various restriction endonucleases under the conditions described by Sambrook et al. (60) or the manufacturer. DNA restriction fragments were isolated from agarose gels by using the Geneclean kit (80) or by electroelution into a sodium acetate solution in an apparatus obtained from Biometra (Göttingen, Germany). All other DNA-manipulating enzymes were used as described by the manufacturer.

Construction of an *A. eutrophus* genomic library. Genomic DNA isolated from *A. eutrophus* H16 was partially digested with *Bam*HI and ligated to λL47-*Bam*HI arms. The products were packaged with λ coat proteins by using an in vitro packaging kit, which was prepared from *E. coli* BHB2688 and BHB2690, and transfected into *E. coli* WL95 and WL87 by established methods (29, 62).

Hybridization experiments. DNA restriction fragments were separated electrophoretically in 0.8 or 2.0% (wt/vol) agarose gels in TBE buffer (50 mM Tris hydrochloride, 50 mM boric acid, 1.25 mM disodium EDTA [pH 8.5] [60]). For transfer of denatured DNAs from agarose gels or plaques to positively charged nylon membranes (pore size, 0.45 μm; Pall Filtrationstechnik, Dreieich, Germany), for their hybridization with probes (labeled with biotin-16-dUTP or ^{32}P), and for detection of DNA, standard procedures (60) or recently described methods (47) were employed.

Synthesis of oligonucleotides. Synthetic nucleotide oligomers were synthesized in 0.2-μmol portions from deoxynucleoside phosphoramidites (6) in a Gene Assembler Plus apparatus as recommended by the manufacturer (Pharmacia-LKB, Uppsala, Sweden). Oligonucleotides were released from the support matrix, and protection groups were removed by 15 h of incubation at 55°C in 25% (vol/vol)

TABLE 2. Purification of Ao:DCPIP OR

Step	Vol (ml)	Protein concn (mg/ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Crude extract	138.0	24.8	3,428	552.0	0.16	1	100
DEAE-Sephacel (PM 30)	4.7	60.5	248.1	265.0	0.93	5.8	48
Sephadex G-200	27.0	1.08	29.2	55.3	1.90	11.9	10
Q-Sepharose (PM 30)	2.0	2.37	4.74	10.2	2.15	13.4	1.9

ammonium. Oligonucleotides were finally purified by passage through NAP-5 or NAP-10 columns (Pharmacia-LKB).

DNA sequence analysis. DNA sequencing was done by the dideoxy-chain termination method of Sanger et al. (61) with single- or double-stranded alkali-denatured plasmid DNA, 7-deazaguanosine 5'-triphosphate instead of dGTP (46), and [α-^{35}S]dATP with a T7 polymerase sequencing kit as recommended by the manufacturer (Pharmacia-LKB). Synthetic oligonucleotides were used as primers, and the primer-hopping strategy was employed. Products of the sequencing reactions were separated in 6% (wt/vol) acrylamide gels in buffer (pH 8.3) containing 100 mM Tris hydrochloride, 83 mM boric acid, 1 mM EDTA, and 42% (wt/vol) urea in an S2 sequencing apparatus (GIBCO/BRL, Bethesda Research Laboratories GmbH, Eggenstein, Germany) and visualized on X-ray films.

Analysis of sequence data. Nucleic acid sequence data and deduced amino acid sequences were analyzed with the Genetic Computer Group Sequence Analysis Software Package (GCG Package, version 6.2, June 1990) as described by Devereux et al. (20).

Determination of the transcription start site. To determine the transcription start site, a nuclease protection assay was used. The hybridization conditions were as described in detail by Berk and Sharp (8) and Sambrook et al. (60), and the S1 nuclease reactions were conducted by the method described by Aldea et al. (1). Total RNA was isolated as described by Oelmüller et al. (47). DNA probes and dideoxynucleotide sequencing reactions for sizing the signals were performed with Bluescript SK$^-$::EE35 DNA as the template. In the annealing reaction, an oligonucleotide (5'-GGCCAGCTGCAGGCTGT-3') which was complementary to positions 227 to 211 was used for ^{35}S labeling. For all mapping experiments, 40 μg of RNA was mixed with the labeled DNA fragments; the specific labeling rate was higher than 10^7 cpm/μg of DNA.

Chemicals. Restriction endonucleases, biotin-16-dUTP, the nick translation kit, the DNA detection kit, T4 DNA ligase, λ DNA, S1 nuclease, *S. aureus* V8 protease, phosphotransacetylase, and the substrates used in the enzyme assays were obtained from C. F. Boehringer & Soehne or GIBCO/BRL-Bethesda Research Laboratories. DEAE-Sephacel, Sephadex G200, Q-Sepharose, RNase-free DNase, and phospoamidites were obtained from Pharmacia-LKB, and radioisotopes were from Amersham/Buchler (Braunschweig, Germany).

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported here have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession no. M66060.

RESULTS

Purification of Ao:DCPIP OR. The Ao:DCPIP OR of *A. eutrophus* H16 was purified 13.4-fold to homogeneity from cells harvested in the late-exponential growth phase (Table 2) in a three-step procedure by chromatography on DEAE-Sephacel, Sephadex G200, and Q-Sepharose. Enzyme activity was eluted from DEAE-Sephacel and Q-Sepharose at approximately 100 to 200 mM KCl (maximum activity at 140 mM KCl) or 210 to 260 mM KCl (maximum activity at 240 mM KCl), respectively. As indicated by the recovery of only 1.9% of the activity, the Ao:DCPIP OR of *A. eutrophus* seems to be very labile. Instability of the Ao:DCPIP OR of *P. carbinolicus* during purification was also reported recently (48). The Ao:DCPIP OR preparation appeared homogeneous in polyacrylamide gels under nondenaturing conditions, as well as in SDS-polyacrylamide gels prepared in sodium phosphate buffer. Amino acid sequence analysis of the protein transferred from an SDS-polyacrylamide gel to a polyvinylidene difluoride membrane revealed the N-terminal sequence 1-Ala-(Arg)-Lys-Leu-Ser-Ile-Lys-Leu-Ala-Ile-Asn - Glu - Ala - Ile - Asp - Gln - Glu - Met - (Thr) - ? - (Asp) - Pro-22 (NTAAS1). Little background signals appeared, indicating the presence of a second protein species on the polyvinylidene difluoride membrane.

In contrast, in SDS-polyacrylamide gels prepared in Tris-glycine buffer and with a stacking gel, two protein bands of identical intensity appeared after staining with, e.g., Coomassie brilliant blue. These bands represented proteins with M_rs of 35,500 and 36,000 ($\pm$1,000), which were referred to as the α and β subunits of Ao:DCPIP OR, respectively (Fig. 1). In contrast, the nondenatured protein exhibited an M_r of 136,000 $\pm$ 10,000 in linear polyacrylamide gels. By centrifugation in sucrose density gradients, an M_r of 165,000 was determined recently for the partially purified enzyme (22a). From these data it was concluded that Ao:DCPIP OR of *A. eutrophus* was isolated as a tetrameric protein with an $\alpha_2\beta_2$ structure.

As it was not possible to separate both subunits sufficiently and to obtain partial amino acid sequences of each subunit, an alkylated sample of the Q-Sepharose pool was treated with *S. aureus* V8 protease, which hydrolyzes at glutamic acid residues. After 18 h of incubation, two major protein species with M_rs of 32,000 (V8/1 protein) and 16,000 (V8/2 protein) appeared as major products of partial cleavage in SDS-polyacrylamide gels besides some minor species. Amino acid sequence analysis revealed identical N-terminal sequences 1-(Thr)-Ala-Arg-Ala-Ser-Gln-Asp-Ser-Ala-Ala-Leu-Pro-Leu-Asp-Lys-Glu-Thr-Leu-Leu-Thr-?-(Tyr)-Arg-Lys-Met-?-?-Ile-Arg-Asp-Phe-31 (NTAAS2) for the V8/1 and V8/2 proteins. NTAAS2 had appeared partly as weak background during determination of NTAAS1. Later it was shown that NTAAS1 and NTAAS2 represent parts of the β and α subunit primary structures, respectively.

Purification of FMP. FMP often appeared as a double band in polyacrylamide gels close to the tracking dye amido black (Fig. 1 [72]). As the function of FMP was not known, no enzymatic assay was available for this protein. However, as FMP was distinguished from most other proteins by its high electrophoretic mobility, electrophoresis in minigels in combination with silver staining provided a rapid and sensitive

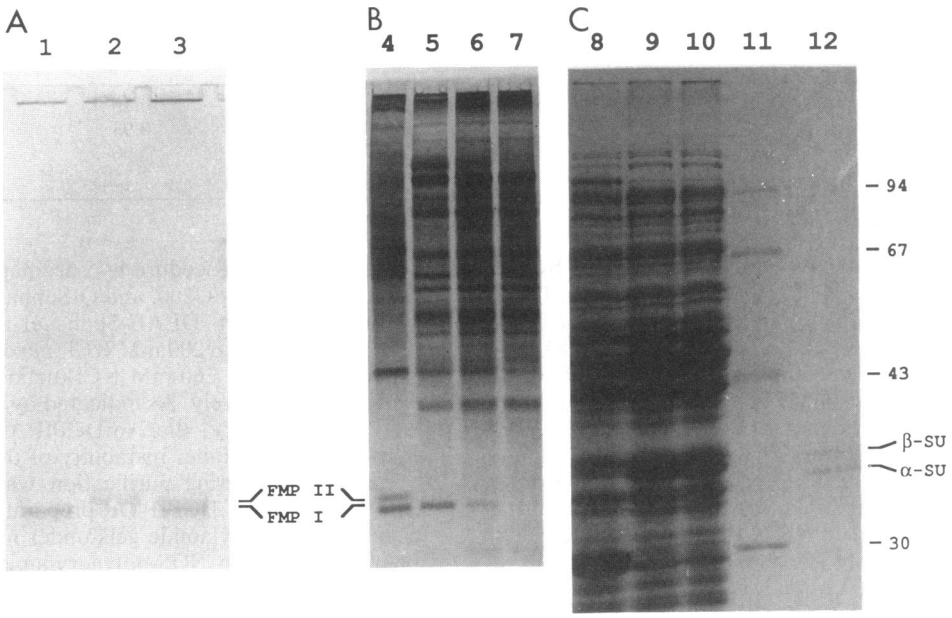

FIG. 1. Electropherograms of purified proteins of *A. eutrophus* and of cytoplasmic fractions obtained from recombinant strains of *E. coli*. (A) Purified FMP. A 7.4-μg sample of FMP-I (lane 1) and 5.5 μg of FMP-II, which had been separated by fast protein liquid chromatography, and 11.5 μg of protein of the Sephadex G75 eluate containing both FMP species (lane 3) were separated in a polyacrylamide gel under nondenaturing conditions. (B) Heterologous expression of FMP in *E. coli*. Crude extracts from acetoin-grown cells of *A. eutrophus* H16 (lane 4), *E. coli* XL1-Blue (KS^-::EB30) (lanes 5 and 6), and *E. coli* XL1-Blue (SK^-::EB30) were separated in a polyacrylamide gel under nondenaturating conditions. *E. coli* was grown in LBApTc medium in the absence (lane 6) or presence (lanes 5 and 7) of 10 mM isopropyl-β-D-thiogalactopyranoside. (C) Heterologous expression of Ao:DCPIP OR in *E. coli*. Crude extracts from *E. coli* XL1-Blue (SK^-) (lane 8) and *E. coli* XL1-Blue (SK^-::ES25) (lanes 9 and 10) were separated in an SDS-polyacrylamide gel. *E. coli* was grown in LBApTc medium in the absence (lane 9) or presence of 10 mM isopropyl-β-D-thiogalactopyranoside (lanes 8 and 10). The molecular masses of standard proteins (lane 11) are given on the right in kilodaltons. Purified Ao:DCPIP OR from *A. eutrophus* was separated in lane 12. SU, subunit.

method for detection of even small quantities of FMP and for rough and rapid estimation of its concentration. A three-step procedure was developed to purify FMP to homogeneity. FMP was eluted from DEAE-Sephacel in close relation to Ao:DCPIP OR at 170 to 220 mM KCl (maximum at approximately 190 mM). Fractions which contained high amounts of FMP were combined. The 5.5-ml pool contained approximately 600 mg of protein and a high proportion of the Ao:DCPIP OR activity. As the molecular weight of FMP was assumed to be rather low, gel filtration on Sephadex G75 was chosen as a second purification step. When 50 mM potassium phosphate buffer, pH 7.0, was used, FMP was already eluted with the voided volume of the column together with Ao:DCPIP OR. Separation of FMP and Ao:DCPIP OR was achieved when KCl was added to the buffer at a concentration of 300 mM.

Of six different triazine affinity media, only Procion Brown MX-5BR retarded FMP. Procion Blue MX-2G, Procion Green HE-4BD, Procion Red HE-3B, Procion Yellow MX-8G, and Procion Orange A did not bind FMP when the Sephadex G75 pool was applied. Although binding was very weak, purification of FMP to approximately 90% homogeneity and its separation from Ao:DCPIP OR were achieved when the Sephadex G75 eluate was subjected to chromatography on Procion Brown MX-5BR Sepharose 4B-CL. Elution of FMP started after the column was washed with one BV of buffer and continued until the second BV of buffer had been applied. Fractions containing mainly FMP but no other proteins were combined and used for further experiments.

Separation of FMP-I and FMP-II. During the purification of FMP, there was no indication for separation of the two forms of FMP. It was not possible to separate FMP-I and FMP-II on a preparative scale by varying the acrylamide concentration in the gels from 4 to 10% (wt/vol). Even in linear polyacrylamide gradient gels (5 to 27.5% monomer, wt/vol), separation was not achieved. However, separation of FMP-I and FMP-II occurred during fast protein liquid chromatography on Mono Q HR5/5. Whereas FMP-I was eluted from the column at 200 mM KCl, FMP-II was eluted immediately after the KCl concentration in the elution buffer had increased slightly (Fig. 1).

Differences between FMP-I and FMP-II. A slight difference in migration in native polyacrylamide gels (relative mobilities towards amido black of 0.95 and 0.93 for FMP-I and FMP-II, respectively) and a difference in binding to Mono-Q HR5/5 were the only differences between the two forms detected in this study. Various electrophoretic techniques revealed no differences between the two forms of FMP. Electrophoresis in linear polyacrylamide gradient gels (5 to 27.5% monomer, wt/vol), in linear SDS-polyacrylamide gels (5 to 30% monomer, wt/vol), or in uniform SDS-polyacrylamide gels (7.4% monomer, wt/vol) revealed M_rs of 36,500 ± 2,000, 46,500 ± 2,000, and 36,500 ± 2,000 for the native or denatured proteins. Isoelectric focusing in agarose gels revealed an isoelectric point of 4.4 for both forms of FMP. In addition, the N-terminal amino acid sequences determined for FMP-I and FMP-II by Edman degradation were identical: 1-Ala-Thr-Glu-Ile-Ser-Pro-Thr-Ile-Ile-Pro-Ile-Val-Met-Pro-Lys-?-Gly-Leu-Ser-Met-Lys-(Glu)-Gly-Thr-(Val)-(Asn)-(Ala)-27 (NTAAS3).

Identification of the structural gene for FMP. On the basis of the primary structure of FMP, a 47-mer oligonucleotide

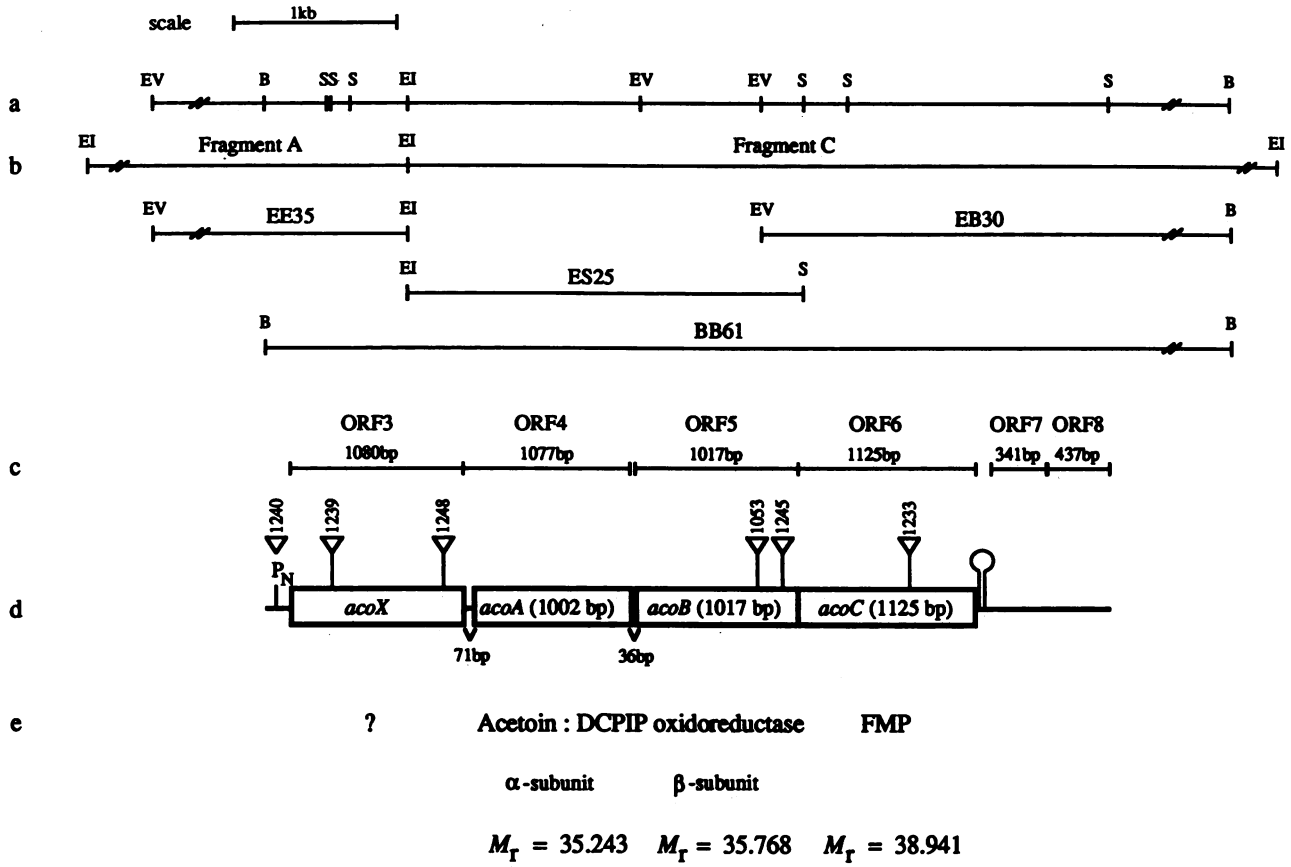

FIG. 2. Molecular organization of the *A. eutrophus aco* operon. (a) Physical map of the region analyzed in this study. Abbreviations: B, *Bam*HI; S, *Sal*I; EI, *Eco*RI; EV, *Eco*RV. (b) Relevant restriction fragments used in this study. (c) ORFs detected in this study. (d) Structural genes of the *aco* operon. Triangles indicate the positions of Tn*5*::*mob* in acetoin-negative mutants. The promoter (P_N) upstream of *acoX* and the hairpinlike structure downstream of *acoC* are indicated. (e) Proteins encoded by the genes of the *aco* operon. The molecular masses were calculated from the deduced amino acid sequences.

(3′-TGGTAGTAGGGGTAGCACTACGGGTTCTTCCCGG ACAGGTACTTCCT-5′) and a 17-mer oligonucleotide (3′-TA[TGA]GG[TGAC]TA[TGA]CA[TGCA]TACGG-5′) (variations of nucleotides at a single position are shown in brackets), which exhibited less variability in the wobble position and were based on the codon usage of *A. eutrophus*, were synthesized and labeled with ^{32}P by the T4 polynucleotide kinase reaction. These oligonucleotides were hybridized at 37°C with DNA restriction fragments A, B, C, D, and E, which had been cloned recently in plasmids pSUP202, pVK101, and pHP1016 (23), and with *Eco*RI digests of genomic DNA of *A. eutrophus* which had been transferred to a nylon membrane. With both oligonucleotides, specific hybridization signals were obtained with fragment C and a genomic fragment of identical size after the membrane was washed at 42°C with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% (wt/vol) SDS to diminish heterologous hybridizations. Fragment C was isolated from a pHC79 hybrid cosmid harbored by a recombinant clone of *E. coli* DH1 (23). With the 47-mer oligonucleotide, the structural gene of FMP was localized on various subfragments of C. One of these subfragments was the 3.0-kbp *Eco*RV-*Bam*HI restriction fragment EB30 (Fig. 2), which was ligated to *Eco*RV-*Bam*HI-treated plasmids Bluescript KS^- and SK^- and transformed into *E. coli* XL1-Blue.

Identification of the structural gene for the β subunit of Ao:DCPIP OR. On the basis of the N-terminal amino acid sequence of the β subunit of Ao:DCPIP OR, a mixture of 18-mer oligonucleotides (3′-CG[AGTC]TA[AGT]CT[AG]GT [TC]CT[TC]TAC-5′) was synthesized and labeled with ^{32}P. This probe was hybridized at identical conditions to the same membranes, which were used for identification of the structural gene of FMP. Again, only with fragment C was a signal obtained. In a separate hybridization experiment, the structural gene was located on various subfragments of fragment C, e.g., on the 2.5-kbp *Eco*RI-*Sal*I restriction fragment ES25 (Fig. 2). This fragment was ligated to Bluescript KS^- and SK^- DNAs which had been treated with *Eco*RI plus *Sal*I and transformed into *E. coli* XL1-Blue.

Nucleotide sequences of *acoA*, *acoB*, *acoC*, and adjacent regions. To determine the nucleotide sequences of the structural gene for FMP, which was referred to as *acoC*, and the structural genes for the subunits of Ao:DCPIP OR, which were referred to as *acoA* and *acoB*, the above-mentioned hybrid plasmids Bluescript KS^- and SK^-, harboring DNA fragments EB30 and ES25, were used as DNA templates. By employing universal primers and then synthetic primers, the nucleotide sequences of both strands of these fragments were determined by the dideoxy-chain termination method and the primer-hopping strategy. Several open reading frames (ORFs) were identified (Fig. 2).

The amino acid sequences deduced from the 5′ regions of

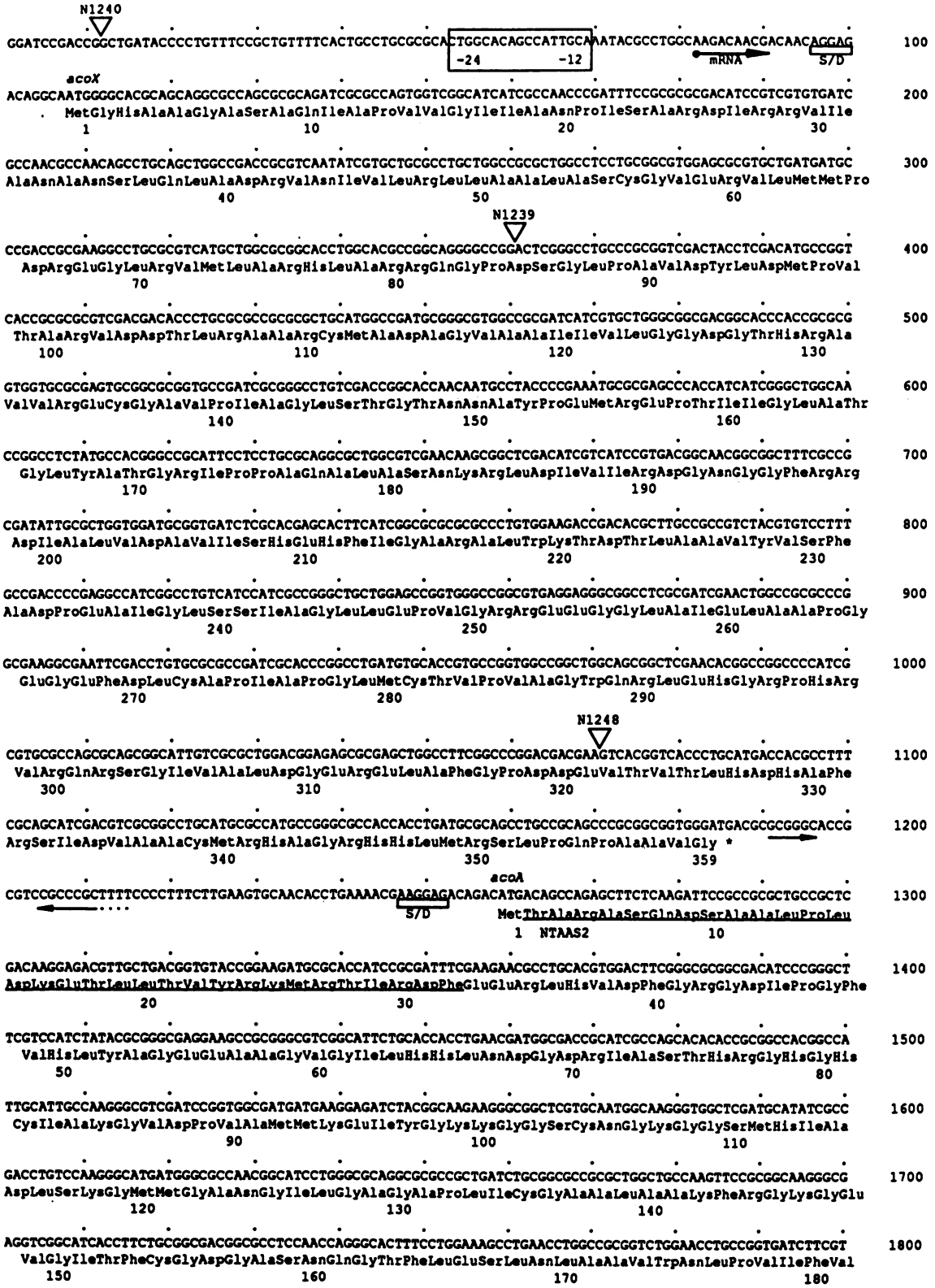

FIG. 3. Nucleotide sequence of the *A. eutrophus aco* operon. Amino acids deduced from the nucleotide sequence are specified by standard three-letter abbreviations. The amino acid sequences determined for the N termini of the isolated α and β subunits of Ao:DCPIP OR (NTAAS2 and NTAAS1) and FMP (NTAAS3) are underlined. Putative ribosome-binding sites are indicated by boxes labeled S/D. The positions of hairpinlike structures downstream of *acoX* and *acoC* are indicated by opposed arrows. Triangles indicate insertions of Tn*5*::*mob* in acetoin-negative mutants. The putative promoter is boxed, and highly conserved nucleotides (−12 and −24) are indicated by boldface letters. A dot and arrow labeled mRNA indicate the transcription start site, as determined by S1 nuclease protection assay, and the direction of transcription.

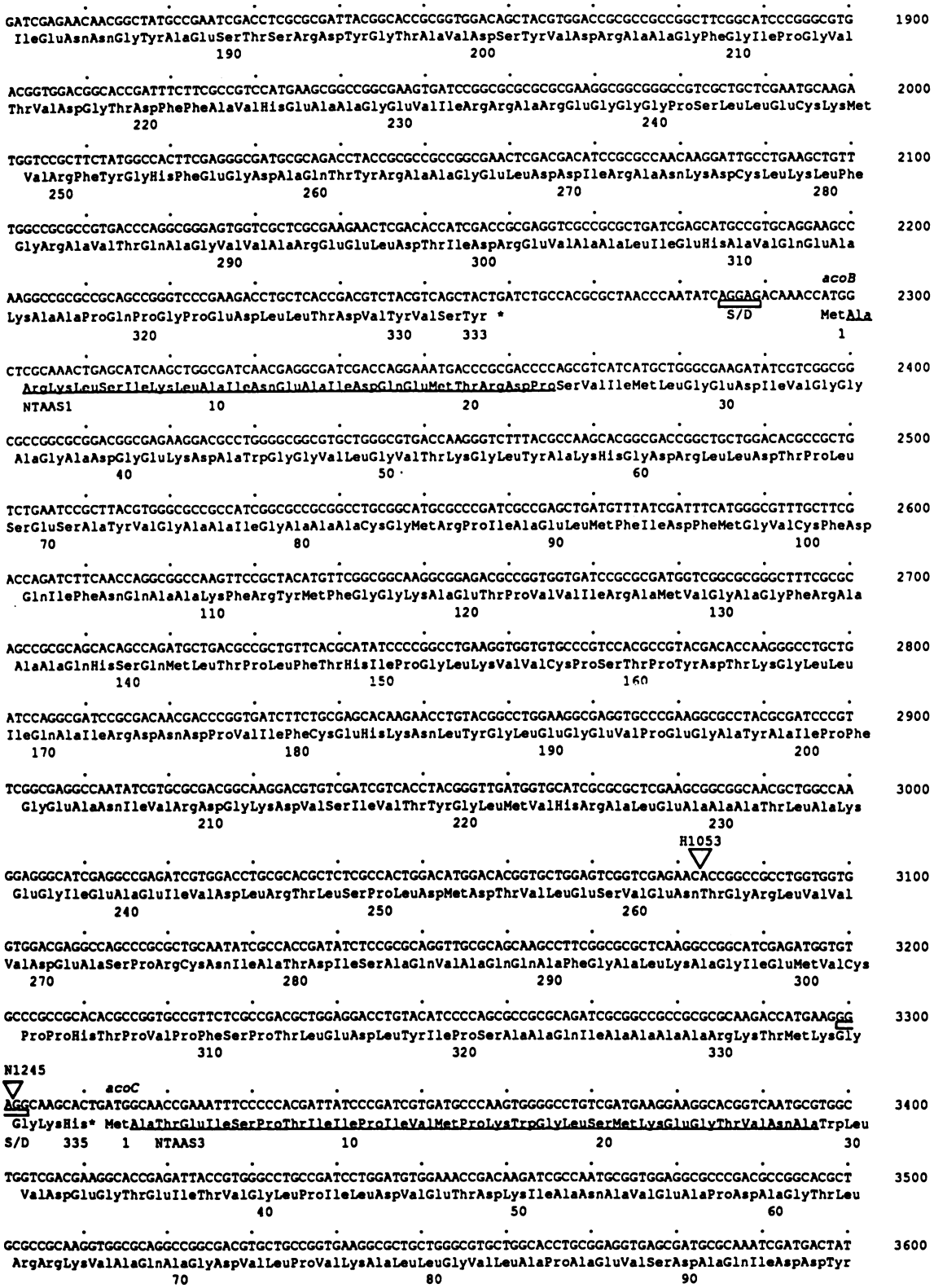

FIG. 3—*Continued*

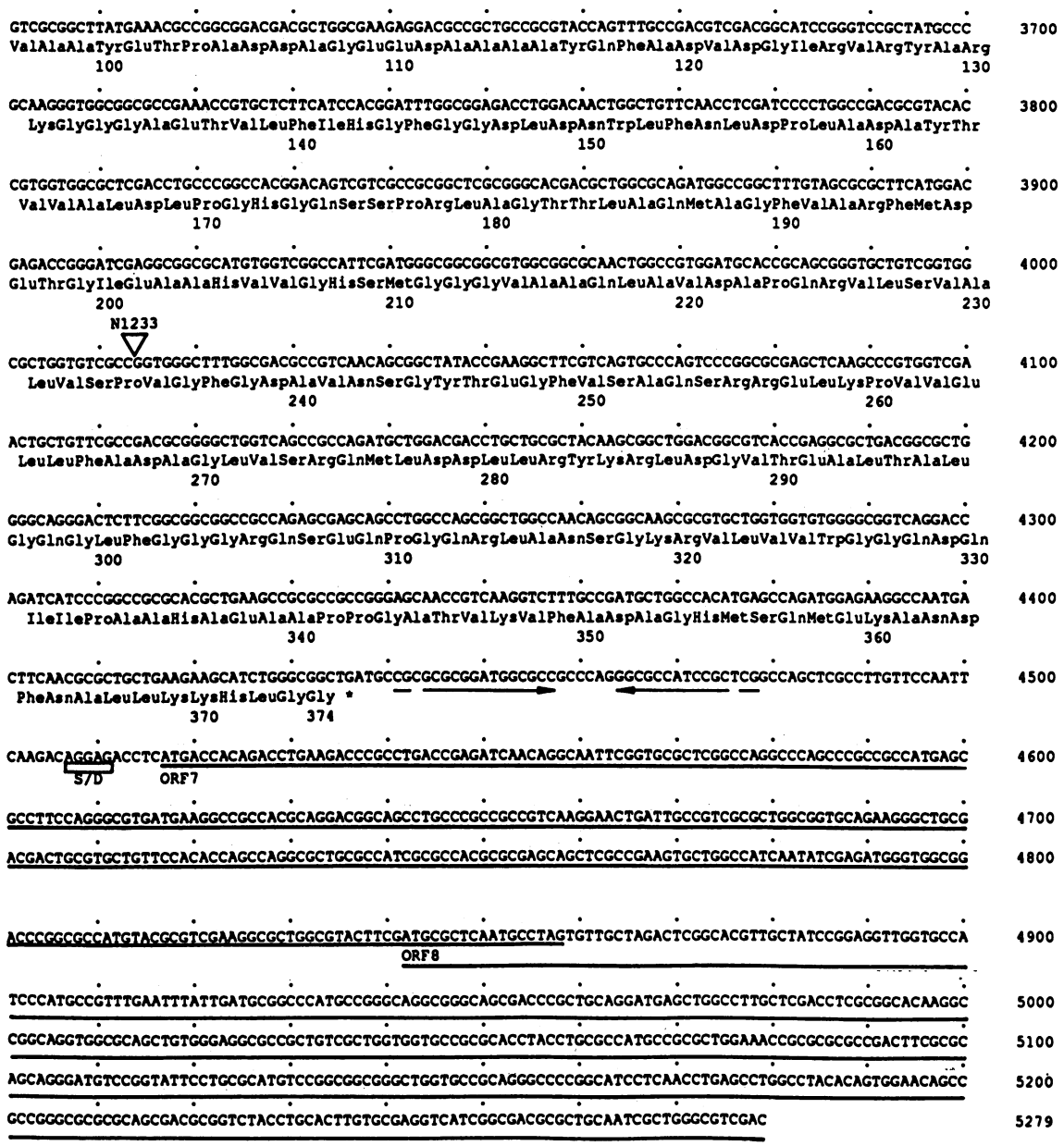

FIG. 3—*Continued*

ORF5 and ORF6 were identical to the amino acid sequences, which had been determined by Edman degradation of purified β subunits of Ao:DCPIP OR and purified FMP, respectively, except that they lacked the methionine residues (Fig. 3). Therefore, ORF5 represents *acoB* and ORF6 represents *acoC*. The translation start codons of both genes were preceded by reliable Shine-Dalgarno sequences which were located 7 or 9 nucleotides upstream of *acoB* or *acoC*, respectively.

The amino acid sequence, which had been determined by Edman degradation of the purified α subunit of Ao:DCPIP OR, corresponded to the amino acid sequence deduced from the nucleotide sequence downstream of the second ATG codon of ORF4 (position 1259 in Fig. 3). As only this and not the first ATG codon of ORF4 (position 1184) was preceded by a reliable Shine-Dalgarno sequence and as the second ATG codon was not preceded by a glutamate codon, *acoA* starts at position 1259. This also indicates that during treatment of Ao:DCPIP OR with *S. aureus* V8 protease peptides were removed from the C terminus rather than from the N terminus of the α subunit.

Cloning of the intact *aco* operon. The cocloning of fragment A and Tn*5*-labeled fragments C (23) had already provided first evidence that fragments A and C are closely linked in the genome of *A. eutrophus*. Further evidence for linkage of the two fragments was obtained in this study when *Bam*HI-digested wild-type genomic DNA was hybridized either with ES25 DNA (subfragment of C) or with the 0.9-kbp *Eco*RI-*Bam*HI restriction fragment (subfragment of A) as DNA probes and when only one signal appeared respectively,

representing a restriction fragment of identical size (6.1 kbp [BB61 in Fig. 2], results not shown in detail). The polar effect exerted by insertions of Tn*5* in fragment A on expression of the genes for Ao:DCPIP OR and FMP (23), which were clearly localized on fragment C in this study, provided further evidence. These results also indicated that the *aco* genes are organized in one single operon whose transcriptional start site is located on fragment A. This assumption was confirmed by the lack of sequences typical for σ^{54}- or σ^{70}-dependent promoter sequences upstream of *acoA*, *acoB*, or *acoC*.

To clone the region, which comprises the entire *aco* operon, plaques of a λL47 gene bank of *Bam*HI-digested genomic DNA from *A. eutrophus* H16 were hybridized with biotinylated ES25 DNA. From one recombinant phage, a 6.1-kbp *Bam*HI restriction fragment (BB61 [Fig. 2]) was isolated and ligated to *Bam*HI-digested Bluescript SK$^-$ vector DNA. By DNA sequencing, it was demonstrated that this fragment contained only one *Eco*RI restriction site and that fragments A and C are therefore directly linked in the genome.

Nucleotide sequence of the 5′-upstream region of the gene cluster. To obtain information about the region upstream of *acoA*, fragment A was isolated from a clone of *E. coli* S17-1 harboring a hybrid plasmid of pSUP202 (23). A 3.5-kbp *Eco*RV-*Eco*RI subfragment of fragment A, which was referred to as EE35, was ligated with Bluescript SK$^-$ vector DNA which had been treated with *Eco*RV plus *Eco*RI and was transformed into *E. coli* XL1-Blue. The primer-hopping strategy and synthetic oligonucleotides were applied to obtain the nucleotide sequence of this fragment. In Fig. 3, only the nucleotide sequence of fragment A between the *Bam*HI and *Eco*RI sites is shown. A 1,080-bp ORF, referred to as ORF3, was identified. ORF3 was separated by 71 nucleotides from *acoA* (Fig. 2). It was preceded by a tentative Shine-Dalgarno sequence which was separated by 6 nucleotides from the putative translational start codon. The deduced amino acid sequence exhibited no significant homologies to the primary structures of approximately 25,000 proteins included in the NBRF data bank. Hydrophobicity plots provided no evidence that the gene product is a membrane protein indicating a function as a protein for the uptake of, e.g., acetoin.

Codon usage. For the region which was sequenced in this study, a G+C content of 67.4 mol% was determined, which was close to the G+C content determined for the total genomic DNA of this bacterium (66.3 to 66.9 mol% [18]). The G+C contents of ORF3, *acoA*, *acoB*, *acoC*, ORF7, and ORF8 were 70.8, 66.1, 66.6, 67.7, 67.3, and 70.2 mol%, respectively. A highly biased choice between codons with either G or C at position 3 occurred for *acoA* (86.3 mol%), *acoB* (90.1 mol%), and *acoC* (84.8 mol%), as well as for ORF3 (86.5 mol%), ORF7 (89.5 mol%), and ORF8 (82.0 mol%). According to Bibb et al. (9), the theoretical value is 84.5 mol%. In contrast, the G+C contents at position 2 were 45.8 (*acoA*), 43.7 (*acoB*), 44.8 (*acoC*), 50.8 (ORF3), 45.6 (ORF7), 48.7 (ORF8), and 46.0 (theoretical value) mol%; the G+C contents at position 1 were 67.4 (*acoA*), 64.8 (*acoB*), 73.6 (*acoC*), 75.0 (ORF3), 66.7 (ORF7), 75.3 (ORF8), and 67.0 (theoretical value) mol%.

As with the *rbcL* (3) and *adh* (34) genes of *A. eutrophus*, the bias toward the use of codons with G or C in position 3 of all of the genes and ORFs investigated in this study was not as extreme for the glutamate codons. Furthermore, GGC is preferred to GGG as a codon for glycine, and CGC is preferred to CGG for arginine, whereas CUG is preferred to CUC for leucine in all of the genes and ORFs investigated in this study.

```
a  CTGGYAYRNNNNTTGCA
   IIIIIIII    IIIII
b  CTGGCACAGCCATTGCA
    IIII II     III
c  TTGGCGCACATCCTGCG
```

FIG. 4. Comparison of the putative promoter of the *aco* operon with other σ^{54}-dependent promoters. The *E. coli* σ^{54} promoter consensus sequence (a [56]), the putative promoter of the *acoXABC* operon (b [this study]), and the putative promoter of the *hoxFUYH* operon (c [78]) were aligned. Vertical lines (I) connect identical nucleotides. The invariant base pairs at positions −24 and −12 are in boldface.

Identification of the promoter. A region which exhibited maximum possible homology (13 of 13 possible matches) to the enterobacterial σ^{54} promoter consensus sequence (56) was located 38 bp upstream of ORF3 (Fig. 4). By an S1 nuclease protection assay with total RNA isolated from acetoin-grown cells, the transcription start site was located at nucleotide 82 (Fig. 3 and 5).

Putative termination signals. Immediately downstream of *acoC*, an inverted repeat which may represent a factor-dependent transcription terminator was localized (Fig. 3). According to Tinoco et al. (77), the free energy of this structure is approximately −104.2 kJ/mol. A short inverted repeat with four T residues was identified immediately downstream from ORF3 (Fig. 3). The free energy of this structure is approximately −86.9 kJ/mol; the function of this hairpinlike structure is not known.

Mapping of Tn*5* insertions. The insertions of Tn*5*::*mob* in various acetoin-negative mutants had already been mapped roughly on *Eco*RI restriction fragments A and C (23). In this study, we ligated the *Sal*I restriction fragments of fragments AT^{N1240}, AT^{N1239}, CT^{H1053}, CT^{N1248}, CT^{N1245}, and CT^{N1233} to pUC9-1 DNA and transformed the ligation mixtures into *E. coli* JM83. Recombinant clones exhibiting kanamycin resistance, mediated by the respective fraction of Tn*5* in one of these *Sal*I restriction fragments, were selected. By employing a synthetic oligonucleotide (5′-GTTAGGAGGTCA CATGG-3′) which hybridized at a distance of 63 to 79 bp from the end of IS*50*L for nucleotide sequencing, the exact locations of Tn*5* in each of the mutant fragments mentioned above were identified (Fig. 3). In mutant strain N1233, Tn*5*::*mob* had inserted into *acoC*, whereas Tn*5*::*mob* was localized in *acoB* in mutant strains H1053 and N1245. No Tn*5* insertion was identified in the gene for the α subunit of Ao:DCPIP OR. Two additional insertions of Tn*5* of acetoin-negative mutants were localized in ORF3, which encodes a still unknown protein. In mutant N1240, Tn*5*::*mob* had inserted approximately 40 bp upstream of the promoter of the *aco* operon. This mutant synthesized, for instance, active Ao:DCPIP OR but was impaired in utilization of acetoin as the sole carbon source for growth. The reason for this is still unknown, and the mutant has to be studied further.

Properties of the *acoA* and *acoB* gene products. The molecular weights of the proteins, which were calculated from the amino acid sequences deduced from *acoA* and *acoB*, were 35,243 and 35,788, respectively. These values correlated well with the molecular weights determined for the purified proteins by SDS-polyacrylamide gel electrophoresis (35,500 and 36,000, respectively). If fragment ES25 was ligated to

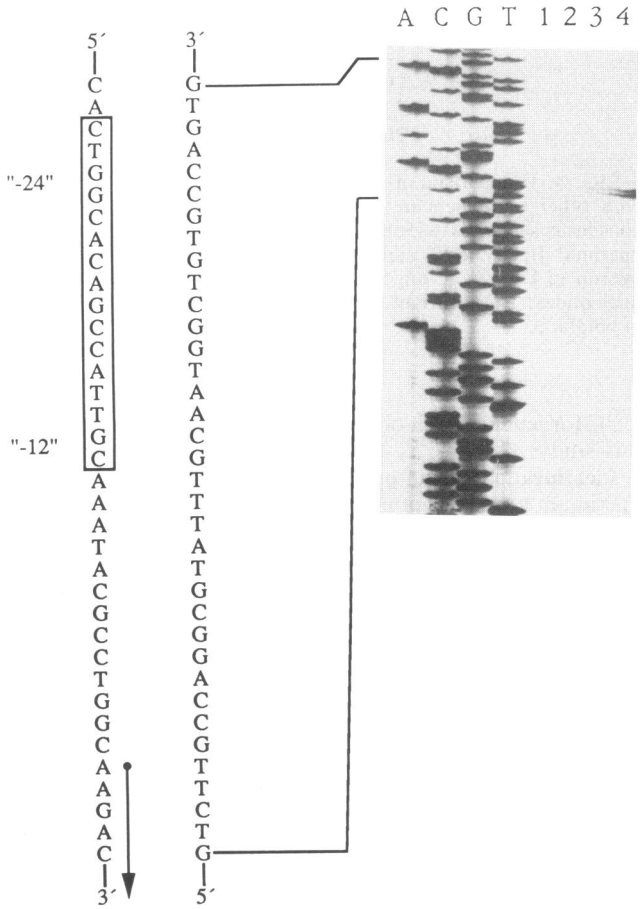

FIG. 5. S1 nuclease protection assay of the transcript of the *aco* operon. Lanes: A, C, G, and T, standard sequencing reactions used to size the mapping signals; 1, RNA from *A. eutrophus* H16 (gluconate-grown cells); 2, control without RNA; 3, RNA from *A. eutrophus* (acetoin-grown cells, exponential growth phase); 4, RNA from *A. eutrophus* (acetoin-grown cells, early stationary growth phase). The dot and arrow show the origin and direction of transcription. The −12 and the −24 regions are boxed.

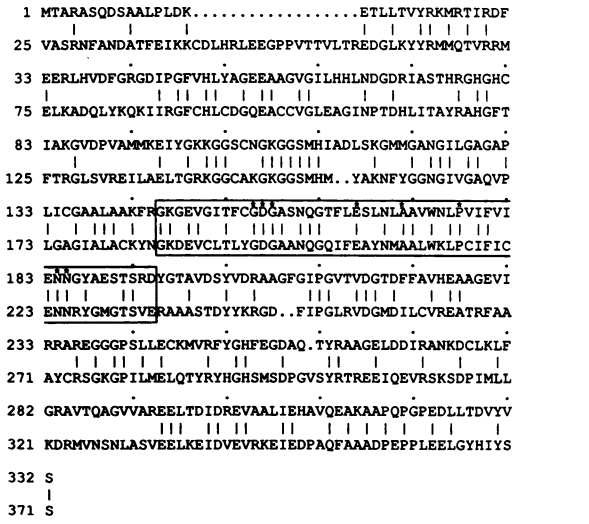

FIG. 6. Pairwise alignments of the deduced amino acid sequences of the α subunit of Ao:DCPIP OR of *A. eutrophus* (upper line) and the human α-subunit precursor protein of the pyruvate dehydrogenase complex (bottom line). Amino acids are specified by standard one-letter abbreviations, and the numbers indicate the positions of the respective amino acids within the proteins. The putative thiamine PP_i-binding region is boxed, and the positions of highly conserved amino acids in thiamine PP_i-binding regions of other 2-oxo acid dehydrogenases (26) are marked by asterisks.

Bluescript SK^- vector DNA with *acoA* and *acoB* downstream and colinear with the *lacZ* promoter, recombinant strains of *E. coli* XL1-Blue expressed Ao:DCPIP OR activity. In SDS-polyacrylamide gels, two protein bands appeared which exhibited the same electrophoretic mobility as the proteins of the Ao:DCPIP OR purified from *A. eutrophus* (Fig. 1). No expression occurred when *acoA* and *acoB* and the *lacZ* promoter were ligated antilinearly in the Bluescript KS^- vector.

The amino acid sequences deduced from *acoA* and *acoB* were compared with the primary structures of the proteins collected in the NBRF data bank. These studies revealed that the α and β subunits of *A. eutrophus* Ao:DCPIP OR exhibit striking homologies to the α and β subunits of 2-oxo acid dehydrogenase components of various multienzyme complexes (Table 3). The greatest homology for the α subunit was obtained with the α chain of human pyruvate dehydrogenase (38.7% identical amino acids). The alignment of the two peptide chains is shown in Fig. 6. The β subunit exhibited the greatest homology with the β subunit of the dehydrogenase component of the branched-chain 2-oxo acid dehydrogenase complex of *Pseudomonas putida* (41.4% identical amino acids). Interestingly, the N termini of both subunits of *A. eutrophus* Ao:DCPIP OR also exhibited homologies to the corresponding subunits of Ao:DCPIP OR of *P. carbinolicus* (Fig. 7). Like other thiamine PP_i-dependent enzymes, the α subunit of Ao:DCPIP OR also has the

TABLE 3. Comparison of the α and β subunits of *A. eutrophus* Ao::DCPIP OR with the subunits of E1 components of 2-oxo acid dehydrogenase complexes from different sources

Protein[a]	Source	Reference	α-Subunit % identity	β-Subunit % identity
E1 of PDHC (EC 1.2.4.1), precursor of the α subunit	Human	19	38.7	18.8
E1 of PDHC (EC 1.2.4.1), precursor of the β subunit	Human	38	15.4	40.9
E1 of BCOADHC (EC 1.2.1.25), α subunit	*P. putida*	14	28.4	20.0
E1 of BCOADHC (EC 1.2.1.25), β subunit	*P. putida*	14	17.1	41.4
E1 of BCOADHC (EC 1.2.4.4), precursor of the α subunit	Cattle	31	26.4	21.0
E1 of BCOADHC (EC 1.2.4.1), precursor of the α subunit	Norwegian rat	84	26.4	22.0
E1 of OGDHC (EC 1.2.4.2)	*E. coli* K-12	17	21.8	18.5

[a] PDHC, pyruvate dehydrogenase complex; BCOADHC, branched-chain oxo acid dehydrogenase complex; OGDHC, oxoglutarate dehydrogenase complex.

```
a  13 Pro Leu Asp Lys Glu Thr Leu Leu Thr Val Tyr Arg Lys Met Arg Thr Ile Arg Asp Phe Glu Glu Arg Leu His  Val  Asp 39
          I       I   I       I   I         I   I   I   I   I       I   I       I   I       I       I    I
    4 Lys Leu Ser Lys Glu Asp Leu Leu Lys Ala Tyr Arg Lys Met Arg Glu Ile Arg Glu Phe Glu Asp Arg Val His (Val) Glu 30

b   2 Ala Arg Lys Leu Ser Ile Lys Leu Ala Ile Asn Glu Ala Ile Asp Gln Glu Met Thr Arg Asp Pro Ser  Val   Ile  Met Leu Gly 29
      I   I   I               I       I       I   I   I               I   I       I   I       I   I                   I
    1 Ala Arg Lys Ile Met Phe Lys Asp Ala Leu Asn Glu Ala Met Arg Leu Glu Met Glu Arg Asp Glu Ser (Val) (Val) Leu Ile Gly 28

c   6 Ser Pro Thr Ile Ile Pro Ile Val Met Pro  Lys  Trp Gly Leu Ser Met Lys Glu Gly Thr Val 26
              I   I               I   I   I   I   I       I       I   I
    2 Asp Asn Arg Ile Ile Ala Leu Thr Met Pro (Lys) Trp Gly Leu Thr Met Glu Glu Gly Leu Ile 22
```

FIG. 7. Comparison of the N termini of proteins involved in acetoin catabolism in *A. eutrophus* and *P. carbinolicus*. The N-terminal amino acid sequences of the proteins of *P. carbinolicus* (bottom lines) had been determined recently by Edman degradation (48), whereas those of *A. eutrophus* (upper lines) were deduced from the nucleotide sequences of the corresponding genes and were aligned pairwise. Vertical lines (I) connect identical amino acids. Amino acids are specified by standard three-letter abbreviations, and the numbers indicate the positions of the respective amino acids within the proteins. a, α subunits of Ao:DCPIP OR; b, β subunits of Ao:DCPIP OR; c, DHLTA and FMP.

GDG-n_{7-8}-E-n_4-A-n_{5-6}-P-n_6-NN motif for a thiamine-binding region (26; Fig. 6). This motif was not detected in the sequence of the β subunit, indicating that only the α subunit is the catalytic active subunit; the function of the β subunit remains to be elucidated.

Properties of the *acoC* gene product. The molecular weight of FMP calculated from the amino acid sequence deduced from *acoC* was 38,941, and it correlated well with the molecular weight obtained under denaturing and nondenaturing conditions by electrophoretic methods. This indicated that FMP was isolated as a monomeric protein. From the deduced amino acid sequence, an isoelectric point of 4.55 was calculated.

A computer-assisted search for homologies with proteins collected in the NBRF data bank revealed weak similarities between FMP and the DHLTA of *E. coli* (19% identical amino acids). Further evidence for the function of FMP as a DHLTA was provided by detection of a region close to the N terminus of the protein which was very similar to the lipoyl-binding regions (12) of other proteins that include Lys-50 (Fig. 8) and by significant homology of the N-terminal amino acid sequences of FMP and DHLTA of *P. carbinolicus* (Fig. 7). Only weak similarities were obtained with the alanine-proline-rich region located downstream of the lipoyl-binding region of DHLTAs.

For heterologous expression of FMP in *E. coli*, fragment EB30 was cloned in both orientations in Bluescript vectors. Recombinant strains of *E. coli* XL1-Blue harboring KS^-:: EB30 (*acoC* downstream from and colinear with the *lacZ* promoter) synthesized a protein which, e.g., in nondenaturing polyacrylamide gels exhibited the same electrophoretic mobility as FMP-I in *A. eutrophus* (Fig. 1). Expression of *acoC* was stimulated by isopropyl-β-D-thiogalactopyranoside and did not occur in strains harboring plasmid SK^-:: EB30 (*acoC* antilinear towards the *lacZ* promoter). FMP-II never did appear. The occurrence of only FMP-I might indicate that FMP-I represents the unlipoated form of this protein, whereas FMP-II represents the lipoated form.

In crude extracts from recombinant strains of *E. coli* which expressed *acoC* and in crude extracts of acetoin-grown cells of *A. eutrophus*, DHLTA activity was not detectable with the UV spectroscopic assay or the hydroxamate assay. However, when these crude extracts were separated in native polyacrylamide gels and the gels were subjected to activity staining for DHLTA, faint precipitin bands of calcium phosphate were visible at the positions in the gels were FMP-I and FMP-II were located, as revealed by subsequent protein staining. Three and five additional faint bands became visible with crude extracts of *A. eutrophus* and *E. coli*, respectively, indicating the presence of further proteins exhibiting DHLTA activity. Unfortunately, evidence that FMP represents a DHLTA came up when purified FMP was completely used up for N-terminal amino acid sequencing and other experiments. Therefore, it was not possible to assign DHLTA activity directly to the purified protein.

```
a    43 Ile Leu Asp Val Glu Thr Asp Lys* Ile Ala Asn Ala Val Glu Ala Pro Asp Ala Gly Thr Leu  63
b   136 Leu Ile Thr Val Glu Gly Asp Lys* Ala Ser Met Glu Val Pro Ala Pro Phe Ala Gly Thr Val 156
c    39 Ile Ala Glu Val Glu Thr Asp Lys* Ala Thr Val Gly Phe Glu Ser Leu Glu Glu Cys Tyr Met  59
d    35 Leu Leu Glu Leu Glu Thr Asp Lys* Val Asn Val Glu Leu Thr Ala Glu Glu Ser Gly Val Leu  55
e    36 Val Ala Asp Val Met Thr Asp Lys* Ala Thr Val Glu Ile Pro Ser Pro Val Ser Gly Lys Val  56
```

FIG. 8. Alignments of the putative lipoyl-binding site of FMP with approved sites of selected dihydrolipoamide acyltransferases from other sources. a, FMP of *A. eutrophus* (this study); b, site LII of the E2 component of the *E. coli* pyruvate dehydrogenase complex (75); c, site LI of the E2 component of the human liver pyruvate dehydrogenase complex (76); d, E2 component of the *B. subtilis* 2-oxoglutarate dehydrogenase complex (15); e, E2 component of the branched-chain 2-oxo acid dehydrogenase complex of *P. putida* (14). Amino acids are specified by standard three-letter abbreviations, and the numbers indicate the positions of the respective amino acids within the proteins. The amino acids of E2 components which are identical to the residues at the corresponding positions of FMP are underlined. Putative lipoyllysyl residues are indicated by asterisks.

Probing for structural genes of DHLDH. In dihydrolipoamide dehydrogenases (DHLDH), the FAD-binding region (G-n_1-GP-n_1-GY-n_2-A-n_5LG [21]) and the catalytic domain (TLGGTCLNVGCIPSK [16]) are highly conserved. The amino acid sequences of the putative proteins deduced from the ORFs detected in the region sequenced in this study exhibited no homology to these domains. To map a structural gene(s) for DHLDH, we synthesized a mixture of oligonucleotides (3'-GGGATGCA[83% G+17% C][67% C+33% G]AC[87% G+13% G]TT[87% C+13% G]AGGCA-5') whose sequence was deduced from the consensus amino acid sequence for the catalytic domain of DHLDH on the basis of the codon usage of *A. eutrophus*. These oligonucleotides were labeled with ^{32}P, and a Southern blot containing *Eco*RI restriction fragments A, B, C, D, and E, which are essential for catabolism of acetoin in *A. eutrophus*, and *Eco*RI-digested genomic DNAs of strains H16 and N9A was hybridized. Whereas fragments A, B, C, D, and E did not give specific hybridization signals, two genomic fragments (6.8 and 3.5 kbp) gave strong hybridization signals. In addition, two weaker signals appeared which represented genomic fragments of 5.2 and 4.9 kbp. These results indicated that no gene for dihydrolipoamide dehydrogenase is located on one of the fragments analyzed.

Growth of acetoin-negative mutants on branched amino acids. The growth of mutant strains N1236, N1230, H1098 (subclass A), N1248, N1245, H1053 (subclass C), H1074 (subclass D), and H1094 (subclass E) on mineral agar plates containing valine, leucine, or isoleucine (each 0.2%, wt/vol), pyruvate (0.3%, wt/vol), or succinate (0.5%, wt/vol) as the sole carbon source was not distinguished from the growth of the wild type. As these mutants exhibit no pleiotropic effects, it is unlikely that Ao:DCPIP OR is a side activity of one of the enzymes for oxidative decarboxylation of 2-oxo acids.

Interestingly, the colony growth of mutants H1070 and N1242 (subclass B) on isoleucine was much slower than that of the wild type. In contrast, the growth of these mutants on valine or leucine was not affected. These results indicate that in *A. eutrophus* one protein for uptake of isoleucine or its conversion to acetyl-CoA is encoded by an *ntrA*-dependent gene. Therefore, an additional marker different from those described by Römermann et al. (59) was detected.

DISCUSSION

The *A. eutrophus* structural genes for Ao:DCPIP OR (*acoA* and *acoB*), which is composed of two different subunits, and for FMP (*acoC*) were identified and sequenced. These genes and three additional ORFs (ORF3, ORF7, and ORF8) are clustered on a 5.3-kbp region of the genome. The codon usage and a strong bias for G+C in codon position 3 of *acoA*, *acoB*, *acoC*, ORF3, ORF7, and OFR8 are in good agreement with corresponding data on other genes of *A. eutrophus* (3, 34, 51, 52, 78). Therefore, the data confirm that ORF3, ORF7, and ORF8 represent protein-coding sequences. The functions of the putative genes which are represented by these ORFs are still unknown, as comparison of the deduced amino acid sequences revealed no significant homologies to known proteins. ORF7 and ORF8 are probably not relevant for catabolism of acetoin; no insertions of Tn*5* were identified in these two ORFs, and they are separated from ORF3 and the genes by a strong hairpinlike structure. In contrast, the gene which is represented by ORF3 and which was referred to as *acoX* is essential for acetoin catabolism in *A. eutrophus*. The molecular data and the polar effect of Tn*5* insertions within *acoX* and *acoB* on the expression of Ao:DCPIP OR and/or FMP provided multiple evidence that all four *aco* genes are organized in one operon (*acoXABC*).

All four *aco* genes are preceded by tentative ribosome-binding sites. Whereas *acoX* and *acoA* and *acoA* and *acoB* are separated by 71 and 36 nucleotides, respectively, the translational stop codon of *acoB* and the translational start codon of *acoC* share the A. Similar close relationships were also detected in *A. eutrophus* between *hoxF* and *hoxU* and *hoxU* and *hoxY* (78). Only *acoX* was preceded by a region which exhibited maximum homology with the *E. coli* consensus sequence for σ^{54}-dependent promoters. By S1 nuclease protection assay, the transcription start site was identified downstream of this promoter sequence. Such a promoter was expected, since it was shown that expression of the genes essential for catabolism of acetoin was dependent on an *ntrA*-like gene (23). σ^{54}-dependent promoters were also identified upstream of the *hoxFUYH* operon (78) and the structural gene for acetaldehyde dehydrogenase II (52a). These promoters are distinguished from σ^{70}-dependent promoters which were recently identified for the alcohol dehydrogenase gene (32) and the poly(β-hydroxybutyrate) synthesis operon (64) in *A. eutrophus*. Transcription of *acoXABC* is probably terminated at the hairpinlike structure downstream of *acoC*, and formation of a 4.4-kb transcript is most likely to occur.

The deduced amino acid sequences for both subunits of Ao:DCPIP OR exhibited striking homologies to the corresponding subunits of dehydrogenase components of various 2-oxo acid (pyruvate, 2-oxoglutarate, and branched-chain 2-oxo acids) dehydrogenase complexes from other bacteria and even eukaryotes (Table 3). Detailed sequence analysis identified a putative thiamine-binding domain in the *acoA* gene product. This is consistent with the observation that 2,6-dichlorophenolindophenol-dependent oxidative cleavage of acetoin is thiamine PP_i dependent (23, 72). No extended homology was observed with, e.g., the pyruvate dehydrogenase component of the *E. coli* pyruvate dehydrogenase complex, which is composed of one rather than two types of subunits (74).

The amino acid sequence deduced from *acoC* for FMP exhibited weak homology to DHLTA of *E. coli* (75), including the presence of a lipoyl-binding domain (12). DHLTA activity was conferred directly on the protein by activity staining for DHLTA after separation of the proteins in a polyacrylamide gel. This and the low molecular mass indicate that FMP represents DHLTAs which possess only one lipoyl domain (53). That FMP represents a DHLTA is also obvious from the similarity in molecular organization between the *aco* genes and the genes of 2-oxo acid dehydrogenase complexes. The genes of the pyruvate dehydrogenase complex from *E. coli* (74, 75) or the 2-oxoglutarate dehydrogenase complex from *E. coli* (17, 70), *B. subtilis* (15), *Azotobacter vinelandii* (66), or *Pseudomonas fluorescens* (7) and the genes of the branched-chain 2-oxo acid dehydrogenase complex from *P. putida* (14) are clustered. Whereas the gene for DHLDH is not always included in these clusters, the genes for the 2-oxo acid dehydrogenase components and the acyltransferases seem to be organized principally in one operon, with the latter as the distal gene.

Recently, a mechanism for oxidative cleavage of acetoin related to the oxidative decarboxylation of 2-oxo acids was proposed for the strictly anaerobic bacterium *P. carbinolicus* (49). In this bacterium, formation of Ao:DCPIP OR, which likely also has an $\alpha_2\beta_2$ structure, a DHLTA and a DHLDH

are induced during growth on acetoin (50). CoA-, thiamine PP_i-, and NAD-dependent oxidative cleavage of acetoin concomitant with the formation of acetyl-CoA has been demonstrated to occur with crude extracts or with the purified protein components (48). A similar reaction was suggested for the cleavage of diacetyl by *Streptococcus faecalis* (22). Similarities between the components of the two enzyme systems from *P. carbinolicus* and *A. eutrophus* support our assumption that acetoin is oxidatively cleaved by a similar mechanism in *A. eutrophus*.

Future studies will focus on several interesting questions. (i) It has to be shown whether a DHLDH is also essentially involved in the cleavage of acetoin in *A. eutrophus*. As *A. eutrophus* possesses dehydrogenase complexes for pyruvate, 2-oxoglutarate, and branched-chain 2-oxo acids, in addition to acetoin dehydrogenase, this bacterium may need more than one DHLDH. On the other hand, the product of the gene(s) may be shared by several enzyme systems. In *E. coli*, for instance, only one DHLDH is present for the pyruvate and the 2-oxoglutarate dehydrogenase complex, although a second DHLDH was detected recently (57). As a consequence, insertional inactivation of such a gene may cause major pleiotropic effects and may even be lethal to the cells. If a DHLDH is involved in the cleavage of acetoin in *A. eutrophus* at all, then we expect that its structural gene will most probably be clustered with the genes for the pyruvate or 2-oxoglutarate dehydrogenase complex. Otherwise our extended analysis of Tn*5*-induced acetoin-negative mutants (23, 39) should have revealed the identification of a DHLDH gene specific for catabolism of acetoin. (ii) The function of the *acoX* gene product has to be evaluated. (iii) It has to be shown whether the proteins involved in oxidative cleavage of acetoin are structurally organized in a multienzyme complex such as 2-oxo acid dehydrogenase complexes. During purification of the components, we observed some weak association of the components of the acetoin dehydrogenase enzyme system, as it also occurred in *P. carbinolicus* (48). (iv) Initiation of transcription of *ntrA*-dependent genes usually requires an activator protein (for a review, see reference 40). For the *A. eutrophus hox* genes, such an activator (HoxA) was identified recently (cited in reference 40). In our laboratory, we have molecular evidence for the essential involvement of an activator protein in the expression of the *acoXABC* operon (38a).

ACKNOWLEDGMENTS

The assistance of Rolf Brinkmann during mass cultivation of *A. eutrophus* is gratefully acknowledged. We thank H. G. Schlegel for carefully reading the manuscript.

This study was supported by a grant (Ste 386/3-1) provided by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Aldea, M., F. Claverie-Martin, M. R. Diaz-Torres, and S. R. Kushner.** 1988. Transcript mapping using [^{35}S]DNA probes, trichloroacetate solvent and dideoxy sequencing ladders: a rapid method for identification of transcriptional start points. Gene **65**:101–110.
2. **Allen, G.** 1981. Sequencing of proteins and peptides, p. 28–31. *In* T. S. Work and R. H. Burdon (ed.), Laboratory techniques in biochemistry and molecular biology, vol. 9. Elsevier Science Publisher, Amsterdam.
3. **Andersen, K., and J. Caton.** 1987. Sequence analysis of the *Alcaligenes eutrophus* chromosomally encoded ribulose bisphosphate carboxylase large and small subunit genes and their gene products. J. Bacteriol. **169**:4547–4558.
4. **Andersson, L. O., H. Borg, and M. Mikaelsson.** 1972. Molecular weight estimations of proteins by electrophoresis in polyacrylamide gels of graded porosity. FEBS Lett. **20**:199–202.
5. **Atkinson, T., P. M. Hammond, R. D. Hartwell, P. Hughes, M. D. Scawen, R. F. Sherwood, D. A. P. Small, C. J. Bruton, M. J. Harvey, and C. R. Lowe.** 1981. Triazine-dye affinity chromatography. Biochem. Soc. Trans. **9**:290–293.
6. **Beaucage, S. L., and M. H. Caruthers.** 1981. Deoxynucleoside phosphoramidites—a new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Lett. **22**:1859–1862.
7. **Benen, J. A. E., W. J. H. van Berkel, W. M. A. M. van Dongen, F. Müller, and A. de Kok.** 1989. Molecular cloning and sequence determination of the *lpd* gene encoding lipoamide dehydrogenase from *Pseudomonas fluorescens*. J. Gen. Microbiol. **135**: 1787–1797.
8. **Berk, A., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
9. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30**:157–166.
10. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
11. **Blum, H., H. Beier, and H. J. Gross.** 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8**:93–98.
12. **Bradford, A. P., A. Aitken, F. Beg, K. G. Cook, and S. J. Yeaman.** 1987. Amino acid sequence surrounding the lipoic acid cofactor of bovine kidney 2-oxoglutarate dehydrogenase complex. FEBS Lett. **222**:211–214.
13. **Bullock, W. O., J. M. Fernandez, and J. M. Stuart.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5**:376–379.
14. **Burns, G., T. Brown, K. Hatter, and J. R. Sokatch.** 1988. Comparison of the amino acid sequences of the transacylase components of branched chain oxoacid dehydrogenase of *Pseudomonas putida*, and the pyruvate and 2-oxoglutarate dehydrogenases of *Escherichia coli*. Eur. J. Biochem. **176**:165–169.
15. **Carlsson, P., and L. Hederstedt.** 1989. Genetic characterization of *Bacillus subtilis odhA* and *odhB*, encoding 2-oxoglutarate dehydrogenase and dihydrolipoamide transsuccinylase, respectively. J. Bacteriol. **171**:3667–3672.
16. **Carothers, D. J., G. Pons, and M. S. Patel.** 1989. Dihydrolipoamide dehydrogenase: functional similarities and divergent evolution of the pyridine nucleotide-disulfide oxidoreductases. Arch. Biochem. Biophys. **268**:409–425.
17. **Darlison, M. G., M. E. Spencer, and J. R. Guest.** 1984. Nucleotide sequence of the *sucA* gene encoding the 2-oxoglutarate dehydrogenase of *Escherichia coli* K12. Eur. J. Biochem. **141**:351–359.
18. **Davis, D. H., M. Doudoroff, and R. Y. Stanier.** 1969. Proposal to reject the genus *Hydrogenomonas*: taxonomic implications. Int. J. Syst. Bacteriol. **19**:375–390.
19. **De Meierleir, L., N. MacKay, A. M. L. H. Wah, and B. H. Robinson.** 1988. Isolation of a full-length complementary DNA coding for human Elot subunit of the pyruvate dehydrogenase complex. J. Biol. Chem. **263**:1991–1995.
20. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
21. **Dietrichs, D., M. Meyer, B. Schmidt, and J. R. Andreesen.** 1990. Purification of NADPH-dependent electron-transferring flavoproteins and N-terminal protein sequence data of dihydrolipoamide dehydrogenases from anaerobic, glycine-utilizing bacteria. J. Bacteriol. **172**:2088–2095.
22. **Dolin, M. I.** 1955. Diacetyl oxidation by *Streptococcus faecalis*, a lipoic acid dependent reaction. J. Bacteriol. **69**:51–58.

22a. **Fründ, C.** 1989. Ph.D. thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

23. **Fründ, C., H. Priefert, A. Steinbüchel, and H. G. Schlegel.** 1989. Biochemical and genetic analysis of acetoin catabolism in *Al-*

caligenes eutrophus. J. Bacteriol. **171**:6539–6548.
24. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
25. **Hanna, Z., C. Fregeau, G. Prefontaine, and R. Brousseau.** 1984. Construction of a family of universal expression plasmid vectors. Gene **30**:247–250.
26. **Hawkins, C. F., A. Borges, and R. N. Perham.** 1989. A common structural motif in thiamine pyrophosphate-binding enzymes. FEBS Lett. **255**:77–82.
27. **Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256**:7990–7997.
28. **Hohn, B.** 1979. *In vitro* packaging of lambda and cosmid DNA. Methods Enzymol. **68**:299–309.
29. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11**:291–298.
30. **Hohn, B., and K. Murray.** 1977. Packaging recombinant DNA molecules into bacteriophage particles *in vitro*. Proc. Natl. Acad. Sci. USA **74**:3259–3263.
31. **Hu, C.-W. C., K. S. Lau, T. A. Griffin, J. L. Chuang, C. W. Fisher, R. P. Cox, and D. T. Chuang.** 1988. Isolation and sequencing of a cDNA encoding the decarboxylase (E1) α precursor of bovine branched-chain α-keto acid dehydrogenase. J. Biol. Chem. **263**:9007–9014.
32. **Jendrossek, D., N. Krüger, and A. Steinbüchel.** 1990. Characterization of alcohol dehydrogenase genes of derepressible wild-type *Alcaligenes eutrophus* H16 and constitutive mutants. J. Bacteriol. **172**:4844–4851.
33. **Jendrossek, D., A. Steinbüchel, and H. G. Schlegel.** 1987. Three different proteins exhibiting NAD-dependent acetaldehyde dehydrogenase activity from *Alcaligenes eutrophus*. Eur. J. Biochem. **167**:541–548.
34. **Jendrossek, D., A. Steinbüchel, and H. G. Schlegel.** 1988. Alcohol dehydrogenase gene from *Alcaligenes eutrophus*: subcloning, heterologous expression in *Escherichia coli*, sequencing, and location of Tn*5* insertions. J. Bacteriol. **170**:5248–5256.
35. **Juni, E.** 1978. Genetics and physiology of Acinetobacter. Annu. Rev. Microbiol. **32**:349–371.
36. **Juni, E., and G. A. Heym.** 1956. A cyclic pathway for the bacterial dissimilation of 2,3-butanediol, acetylmethylcarbinol, and diacetyl. I. General aspects of the 2,3-butanediol cycle. J. Bacteriol. **71**:425–432.
37. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8**:45–54.
38. **Koike, K., S. Ohta, Y. Urata, Y. Kagawa, and M. Koike.** 1988. Cloning and sequencing of cDNAs encoding α and β subunits of human pyruvate dehydrogenase. Proc. Natl. Acad. Sci. USA **85**:41–45.
38a. **Krüger, N., and A. Steinbüchel.** Unpublished data.
39. **Kuhn, M., D. Jendrossek, C. Fründ, A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* alcohol dehydrogenase gene. J. Bacteriol. **170**:685–692.
40. **Kustu, S., E. Santero, J. Keener, D. Opham, and D. Weiss.** 1989. Expression of σ54 (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53**:367–376.
41. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
42. **Loenen, W. A. M., and W. J. Brammar.** 1980. A bacteriophage lambda vector for cloning large fragments made with several restriction enzymes. Gene **10**:249–259.
43. **Lopez, J., B. Thoms, and P. Fortnagel.** 1973. Mutants of *Bacillus subtilis* blocked in acetoin reductase. Eur. J. Biochem. **40**:479–483.
44. **Lopez, J. M., B. Thoms, and H. Rehbein.** 1975. Acetoin degradation in *Bacillus subtilis* by direct oxidative cleavage. Eur. J. Biochem. **57**:425–430.
45. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
46. **Mizusawa, S., S. Nishimura, and F. Seela.** 1986. Improvement of the dideoxychain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Res. **14**:1319–1324.
47. **Oelmüller, U., N. Krüger, A. Steinbüchel, and C. G. Friedrich.** 1990. Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. J. Microbiol. Methods **11**:73–84.
47a. **Oppermann, F. B.** 1990. Ph.D. thesis, Georg-August-Universität Göttingen, Göttingen, Germany.
48. **Oppermann, F. B., B. Schmidt, and A. Steinbüchel.** 1991. Purification and characterization of acetoin:2,6-dichlorophenolindophenol oxidoreductase, dihydrolipoamide dehydrogenase, and dihydrolipoamide acetyltransferase of the *Pelobacter carbinolicus* acetoin dehydrogenase enzyme system. J. Bacteriol. **173**:757–767.
49. **Oppermann, F. B., A. Steinbüchel, and H. G. Schlegel.** 1988. Utilization of methylacetoin by the strict anaerobe *Pelobacter carbenolicus* and consequences for the catabolism of acetoin. FEMS Microbiol. Lett. **55**:47–52.
50. **Oppermann, F. B., A. Steinbüchel, and H. G. Schlegel.** 1989. Evidence for oxidative thiolytic cleavage of acetoin in *Pelobacter carbinolicus* analogous to aerobic oxidative decarboxylation of pyruvate. FEMS Microbiol. Lett. **60**:113–118.
51. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. **264**:15293–15297.
52. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. **264**:15298–15303.
52a. **Priefert, H., et al.** Unpublished data.
53. **Reed, L. J., and M. L. Hackert.** 1990. Structure-function relationships in dihydrolipoamide acyltransferases. J. Biol. Chem. **265**:8971–8974.
54. **Reed, L. J., M. Koike, M. E. Lewitch, and F. R. Leach.** 1958. Studies on the nature and reactions of protein-bound lipoic acid. J. Biol. Chem. **232**:143–158.
55. **Reed, L. J., and C. R. Willms.** 1966. Purification and resolution of the pyruvate dehydrogenase complex (*Escherichia coli*). Methods Enzymol. **9**:247–265.
56. **Reitzer, L. J., and B. Magasanik.** 1985. Expression of *glnA* in *Escherichia coli* is regulated at tandem promoters. Proc. Natl. Acad. Sci. USA **82**:1979–1983.
57. **Richarme, G.** 1989. Purification of a new dihydrolipoamide dehydrogenase from *Escherichia coli*. J. Bacteriol. **171**:6580–6585.
58. **Rodriguez, H., W. J. Kohr, and R. M. Harkins.** 1984. Design and operation of a completely automated Beckman microsequencer. Anal. Biochem. **140**:538–547.
59. **Römermann, D., J. Warrelmann, R. A. Bender, and B. Friedrich.** 1989. An *rpoN*-like gene of *Alcaligenes eutrophus* and *Pseudomonas facilis* controls expression of diverse metabolic pathways, including hydrogen oxidation. J. Bacteriol. **171**:1093–1099.
60. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
61. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
62. **Scalenghe, F., E. Turco, J. E. Edström, V. Pirotta, and M. Melli.** 1981. Microdissection and cloning of DNA from specific region of *Drosophila melanogaster* polytene chromosomes. Chromosoma **82**:205–216.
63. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38**:209–222.
64. **Schubert, P., Krüger, N., and A. Steinbüchel.** 1991. Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthetic operon: identification of the N terminus of poly(3-hydroxybutyrate) synthase and identification of the promoter. J. Bacteriol. **173**:168–175.

65. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170**:5837–5847.
66. **Schulze, E., A. H. Westphal, R. Hanemaaijer, and A. de Kok.** 1990. The 2-oxoglutarate dehydrogenase complex from *Azotobacter vinelandii*. 1. Molecular cloning and sequence analysis of the gene encoding the 2-oxoglutarate dehydrogenase component. Eur. J. Biochem. **187**:229–234.
67. **Schwarz, E. R., and L. J. Reed.** 1969. α-Keto acid dehydrogenase complexes. XII. Effects of acetylation on the activity and structure of the dihydrolipoyl transacetylase of *Escherichia coli*. J. Biol. Chem. **244**:6074–6079.
68. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the *in vitro* constructed Tn*5*-mob transposon. Mol. Gen. Genet. **196**:413–420.
69. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering : transposon mutagenesis in gram-negative bacteria. Bio/Technology **1**:784–791.
70. **Spencer, M. E., M. G. Darlison, P. E. Stephens, I. K. Duckenfield, and J. R. Guest.** 1984. Nucleotide sequence of the *sucB* gene encoding the dihydrolipoamide succinyltransferase of *Escherichia coli* K12 and homology with the corresponding acetyltransferase. Eur. J. Biochem. **141**:361–374.
71. **Stegemann, H., H. Francksen, and V. Macko.** 1973. Potato proteins: genetic and physiological changes, evaluated by one or two-dimensional PAA-gel-techniques. Z. Naturforsch. **28c**:722–732.
72. **Steinbüchel, A., C. Fründ, D. Jendrossek, and H. G. Schlegel.** 1987. Isolation of mutants of *Alcaligenes eutrophus* unable to derepress the fermentative alcohol dehydrogenase. Arch. Microbiol. **148**:178–186.
73. **Steinbüchel, A., and H. G. Schlegel.** 1984. A multifunctional fermentative alcohol dehydrogenase from the strict aerobe *Alcaligenes eutrophus*: purification and properties. Eur. J. Biochem. **141**:555–564.
74. **Stephens, P. E., M. G. Darlison, H. M. Lewis, and J. R. Guest.** 1983. The pyruvate dehydrogenase complex of *Escherichia coli* K12. Nucleotide sequence encoding the pyruvate dehydrogenase component. Eur. J. Biochem. **133**:155–162.
75. **Stephens, P. E., M. G. Darlison, H. M. Lewis, and J. R. Guest.** 1983. The pyruvate dehydrogenase complex of *Escherichia coli* K12. Nucleotide sequence encoding the dihydrolipoamide acetyltransferase component. Eur. J. Biochem. **133**:481–489.
76. **Thekkumkara, T. J., L. Ho, I. D. Wexler, G. Pons, T.-C. Liu, and M. S. Patel.** 1988. Nucleotide sequence of a cDNA for the dihydrolipoamide acetyltransferase component of human pyruvate dehydrogenase complex. FEBS Lett. **240**:45–48.
77. **Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246**:40–41.
78. **Tran-Betcke, A., U. Warnecke, C. Böcker, C. Zaborosch, and B. Friedrich.** 1990. Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16. J. Bacteriol. **172**:2920–2929.
79. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
80. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76**:615–619.
81. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
82. **Weber, K., J. R. Pringle, and M. Osborne.** 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Methods Enzymol. **26**:3–27.
83. **Wilde, E.** 1962. Untersuchungen über Wachstum und Speicherstoffsynthese von *Hydrogenomonas*. Arch. Mikrobiol. **43**:109–137.
84. **Zhang, B., M. J. Kuntz, G. W. Goodwin, R. A. Harris, and D. W. Crabb.** 1987. Molecular cloning of a cDNA for the E1α subunit of rat liver branched chain α-ketoacid dehydrogenase. J. Biol. Chem. **262**:15220–15224.