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Cloning and Sequencing of a Cluster of Genes Encoding Branched-Chain α-Keto Acid Dehydrogenase from *Streptomyces avermitilis* and the Production of a Functional E1[αβ] Component in *Escherichia coli*

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A cluster of genes encoding the E1α, E1β, and E2 subunits of branched-chain α-keto acid dehydrogenase (BCDH) of *Streptomyces avermitilis* has been cloned and sequenced. Open reading frame 1 (ORF1) (E1α), 1,146 nucleotides long, would encode a polypeptide of 40,969 Da (381 amino acids). ORF2 (E1β), 1,005 nucleotides long, would encode a polypeptide of 35,577 Da (334 amino acids). The intergenic distance between ORF1 and ORF2 is 73 bp. The putative ATG start codon of the incomplete ORF3 (E2) overlaps the stop codon of ORF2. Computer-aided searches showed that the deduced products of ORF1 and ORF2 resembled the corresponding E1 subunit (α or β) of several prokaryotic and eukaryotic BCDH complexes. When these ORFs were overexpressed in *Escherichia coli*, proteins of about 41 and 34 kDa, which are the approximate masses of the predicted *S. avermitilis* ORF1 and ORF2 products, respectively, were detected. In addition, specific E1[αβ] BCDH activity was detected in *E. coli* cells carrying the *S. avermitilis* ORF1 (E1α) and ORF2 (E1β) coexpressed under the control of the T7 promoter.

The branched-chain α-keto acid dehydrogenase (BCDH) complex catalyzes the oxidative decarboxylations of α-ketoisovalerate, α-keto-β-methylvalerate, and α-ketoisocaproate (the deamination products of the branched-chain amino acids valine, isoleucine, and leucine, respectively), releasing CO_2 and generating the corresponding acyl-coenzyme A and NADH (36). The enzyme has been characterized from several sources, including *Pseudomonas putida* (55), *Pseudomonas aeruginosa* (39), *Bacillus subtilis* (37), rabbit liver (46), and bovine and rat kidneys (43, 49). The purified complexes from *P. putida*, *P. aeruginosa*, *B. subtilis*, and several mammals are all composed of four polypeptides, E1α, E1β, E2, and E3, with three different catalytic activities: a BCDH and decarboxylase (E1[αβ]), a dihydrolipoamide acyltransferase (E2), and a dihydrolipoamide dehydrogenase (E3). The E1[αβ] component requires thiamine pyrophosphate (TPP) as a cofactor and possesses a structural binding motif that resembles those of many of the TPP-binding enzymes (25). The BCDH complex has structural and enzymatic properties similar to those of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase complexes (48, 69). Interestingly, a dual-purpose α-keto acid dehydrogenase complex which has both pyruvate and BCDH activities has been isolated from *B. subtilis* (37). In addition, an exclusive BCDH which is essential for branched-chain fatty acid synthesis has been isolated from *B. subtilis* (44).

Cloning of prokaryotic BCDH genes has been reported for *Pseudomonas* and *Bacillus* species but not for *Streptomyces* species. In these systems, it was found that the genes encoding the BCDH complex were clustered in an operon. The genes encoding the BCDH complex of *P. putida* (*bkd* genes) and the PDH/BCDH dual complex of *B. subtilis* and *Bacillus stearothermophilus* are clustered in the sequence E1α, E1β, E2, and E3 (10, 11, 26, 27, 59). Recently, the genes for the branched-chain fatty acid-specific BCDH from *B. subtilis* were cloned and sequenced (64). This operon consisted of only three genes, E1α, E1β, and E2. Additionally, the sequences of numerous eukaryotic E1α and E1β BCDH subunits have been reported (20, 68, 71, 72).

Streptomyces avermitilis is a gram-positive, filamentous, soil microorganism which produces eight closely related polyketide compounds named avermectins that have anthelmintic and insecticidal activity (9). The avermectin polyketide backbone is derived from seven acetate and five propionate extender units added to one α-branched-chain fatty acid starter, which is either *S*(+)-α-methylbutyric acid or isobutyric acid (45). The acyl-coenzyme A forms of the latter two molecules are the products of the BCDH step of isoleucine and valine catabolism and are the precursors of the two possible substituents present at the C-25 position of naturally occurring avermectins: a *sec*-butyl substituent derived from isoleucine (a avermectins) or an isopropyl substituent derived from valine (b avermectins). A mutant of *S. avermitilis* with no detectable BCDH activity was previously isolated (24). The mutant could synthesize the natural avermectins only when the α-branched-chain fatty acid or a precursor bearing the isopropyl or *sec*-butyl (*S*-form) group was added to the medium in which the mutants were fermented. To further understand the involvement of the BCDH activity in avermectin production, we decided to clone and analyze the genes encoding BCDH from *S. avermitilis*. Recently, an amino acid sequence comparison of all of the published sequences for both E1α and E1β subunits of the PDH and the BCDH complexes from multiple species was performed by computer analysis (65). Interestingly, several regions of the α and β subunits were identified that are highly conserved not only in all PDHs so far described but also in both prokaryotic and eukaryotic BCDH complexes. The extensive homology observed in both the α and β subunits of BCDH prompted us to attempt a homology probing approach to clone

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the genes encoding BCDH from *S. avermitilis*. Here, we report the cloning and sequencing of a cluster of genes encoding the E1α, E1β, and E2 subunits of a BCDH complex. We have also produced the E1[αβ] component of the *S. avermitilis* BCDH in *E. coli* in an active form.

MATERIALS AND METHODS

Microorganisms and growth conditions. *S. avermitilis* ATCC 31272 was grown in liquid YEME medium (31) at 29°C for 96 h to prepare chromosomal DNA. The components of the T7 RNA polymerase/promoter system, namely, *Escherichia coli* strains DH1 and C600, harboring plasmids pT7-7 and pGP1-2, respectively, were kindly provided by S. Tabor (Harvard Medical School, Boston, Mass.) and grown as previously described (60). *E. coli* UH-Ac-2 (35) was obtained from the *E. coli* Genetic Stock Center (New Haven, Conn.), and *E. coli* DH5α competent cells were purchased from Life Technologies (Gaithersburg, Md.). *E. coli* cultures were grown in Luria-Bertani medium (52) supplemented, when needed, with ampicillin (50 μg/ml).

DNA isolation and manipulation. Total chromosomal DNA from *S. avermitilis* was prepared by cesium chloride gradient centrifugation (31). Plasmids were isolated from *E. coli* by a modification of the method of Birnboim and Doly (7), as described by Denoya et al. (17). General DNA manipulations were performed as described previously (52). DNA probes were prepared by nick translation (52) by using [α-^{32}P]dCTP purchased from NEN (DuPont, Boston, Mass.) and the BRL Nick Translation System purchased from Life Technologies, following the instructions obtained from the supplier. A cosmid library of *S. avermitilis* chromosomal DNA, kindly provided by E.L. McCormick and K. Stutzman-Engwall, was prepared with total genomic DNA that was partially digested with *Sau*3A and was ligated into the *Bam*HI site in pKC505 (50), essentially as described previously (6).

DNA amplification and cloning. *S. avermitilis* genomic DNA was amplified essentially as described previously (54). Briefly, the PCR was carried out with *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.) and the buffer provided by the manufacturer in the presence of 200 μM deoxynucleoside triphosphate, 0.5 μM (each) primer, 50 ng of template DNA, and 2.5 U of enzyme in a final volume of 100 μl for 30 cycles with a thermal cycler. The first cycle of amplification consisted of 3 min of denaturation at 95°C, 2 min of annealing at 55°C, and 2 min of extension at 72°C. The subsequent 29 cycles had the denaturation step shortened to 1.5 min. In some experiments, 15% glycerol (29) was added to the reaction mixture. DNA primers (Genosys, The Woodlands, Tex.) were 5′-GAATTCGGCGACGGCGCCACCTCCGAGGGCGAC-3′ (rightward) and 5′-TCTAGACCGCAGGTGGTCCGGCATCTC-3′ (leftward). The amplification products were size fractionated by agarose gel electrophoresis. A 0.2-kb PCR-amplified fragment was recovered by electroelution, treated with the Klenow fragment of the DNA polymerase I to produce blunt ends (52), and cloned into the *Sma*I site of pGEM-3Z (Promega, Madison, Wis.) to produce pCD503.

Colony and Southern hybridizations. The genomic library was replicated and screened as described previously (6). Southern blots (57) and hybridizations to [^{32}P]DNA probes were carried out as previously described (16, 61). The 0.2-kb E1α *bkd*-specific *S. avermitilis* cloned insert (pCD503) was isolated as an *Eco*RI-*Bam*HI fragment, labeled, and used to probe the cosmid library. Four positively hybridizing cosmid clones (>2,200 screened) were found to contain overlapping sequences by restriction mapping and Southern blot hybridizations. The following genomic fragments were subcloned from the cosmid clones into pGEM-3Z (see Fig. 2): 7-kb *Bam*HI (pCD528), 2.3-kb *Sph*I-*Bam*HI (pCD545), 4.2-kb *Sph*I-*Bgl*II (pCD574), 6-kb *Sph*I (pCD550), and 7-kb *Bam*HI (pCD559).

DNA sequencing and computer analyses. Relevant subfragments were subcloned into M13mp18 and M13mp19 bacteriophages to determine the sequences of both DNA strands (41). Nested sets of deletion mutants were generated with ExoIII according to procedures previously described (28). The nucleotide sequence was determined by the dideoxy sequencing method (53) as described previously (16). The Genetics Computer Group software (Madison, Wis.) (version 7.3) (18) was used for sequence analysis. Deduced amino acid sequence data were compared with those from available databases (GenBank [release 80.0], EMBL [release 36.0], PIR-Protein [release 38.0], and SWISS-PROT [release 27.0]).

Construction and use of *E. coli* expression plasmids. Several derivatives of pT7-7 (60) were constructed for this study (Fig. 1) as follows.

(i) E1α. An *Nde*I site was created at the open reading frame 1 (ORF1) translational start codon by PCR with pCD528 (see previous sections) as template. The DNA primers were 5′-AAGAGATCTCATATGACGGTCATGGAG CAGCGG-3′ (mutagenic, rightward), and 5′-AAGGATCCTGCAGACAGCTA TGACCATGATTACGCCA-3′ (universal, leftward). The PCR-amplified DNA fragment was electroeluted from a 0.8% agarose gel, digested with *Nde*I and *Bam*HI, and ligated into pT7-7 (60) to give pCD663 (Fig. 1). Finally, the entire ORF1 was assembled by subcloning the 1.1-kb *Bam*HI genomic fragment (prepared from pCD550) into the unique *Bam*HI site present in pCD663, to give pCD670.

(ii) E1β. An *Nde*I site was introduced into ORF2 by PCR with pCD574 as template and the primers 5′-AAGAGATCTCATATGACCACCGTTGCCCT GAAG-3′ (mutagenic, rightward) and 5′-AAGGATCCTGCAGCCCAGTCAC GACGTTGTAAAACGA-3′ (universal, leftward). The PCR-amplified DNA fragment was digested with *Nde*I and *Eco*RI and ligated into pT7-7 to give pCD666.

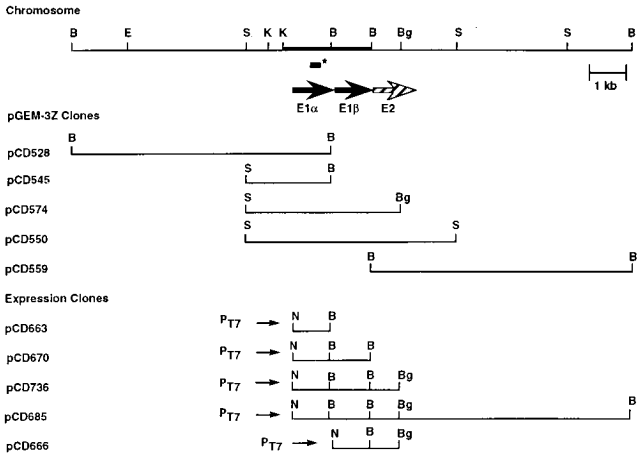

FIG. 1. Restriction map of the region of the *S. avermitilis* genome containing the genes encoding the components of the BCDH complex. The positions of restriction sites are indicated above the line (B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Sph*I). The sequenced region is marked as a bold black line. The arrows below the map indicate the location and orientation of the ORFs (black represents completely sequenced ORFs, and cross-hatching represents a partially sequenced ORF). Overlapping *S. avermitilis* inserts present in pGEM-3Z derivatives and expression clones constructed in pT7-7 are also shown. P_{T7} indicates the orientation of the T7 RNA polymerase promoters.

(iii) E1α+E1β. The 0.8-kb *Bam*HI-*Bgl*II fragment (prepared from pCD559), containing the 3′ end of ORF2 and the 5′ end of ORF3, was cloned into a *Bam*HI-linearized pCD670 (prepared by partial *Bam*HI digestion), to give pCD736. Another construct, pCD705, carrying the 0.8-kb *Bam*HI-*Bgl*II fragment in the wrong orientation, was used as a negative control in expression experiments.

(iv) E1α+E1β+E2. The 7-kb *Bam*HI fragment (prepared from pCD559), containing the 3′ end of ORF2 and the putative ORF3, was subcloned into a *Bam*HI-linearized pCD670 (prepared as before by partial *Bam*HI digestion), to give pCD685. Each construct was used to transform either *E. coli* C600 carrying plasmid pGP1-2 (containing the T7 RNA polymerase gene) (60) or *E. coli* UH-Ac-2 (pGP1-2). Transformants were grown at 30°C in Luria-Bertani medium containing 60 μg (each) of kanamycin and ampicillin per ml. When the cultures reached an optical density at 590 nm of 0.3 to 0.4, they were induced by raising the temperature to 42°C for 30 min and then were incubated at 37°C for a further 90 min. Uninduced control cultures were always kept at 30°C. Proteins were analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (15). Cell extracts were prepared as described in the next section.

Preparation of *E. coli* cell extracts. *E. coli* cells (derived from 8-ml cultures) were collected by centrifugation, resuspended in 5 ml of breakage buffer (0.05 M potassium phosphate buffer [pH 7.0], containing 3% Triton X-100, 15% glycerol, 3 mM dithiothreitol, 1-mg/ml turkey egg white trypsin inhibitor, 5 mM EDTA, and 0.04 mM TPP), and transferred to a French press. The cells were ruptured by one passage at 5,000 lb/in^2. A 1.5-ml aliquot of the French pressate was then transferred to a microcentrifuge tube and clarified by 30 s of centrifugation at 14,000 × *g*. Aliquots of 100 μl of each supernatant were used per enzyme assay. Protein contents in French-pressed samples of each culture were determined by the method of Bradford (8).

E1 BCDH and PDH assays. E1 BCDH activity was determined by measuring the production of $^{14}CO_2$ from 1-^{14}C-labeled α-ketoisocaproic acid by a modified version of the radiochemical assay described previously (24, 32). To the bottom of a 20-ml glass scintillation vial were added 148 μl of 0.25 M potassium phosphate buffer (pH 6.5), 2 μl of 0.1 M EDTA (disodium salt), 4 μl of 0.1 M $MgCl_2$, 20 μl of 3.7 mM TPP, 20 μl of 37 mM $NaAsO_2$, 10 μl of 37 mM 2,6-dichlorophenolindophenol (sodium salt), 8 μl of α-[1-^{14}C]ketoisocaproate stock solution (prepared as described below), 58 μl of H_2O, and 0.1 ml of clarified cell extract. The mouth of the vial was immediately covered with Whatman 4CHR paper (Whatman International, Maidstone, United Kingdom) that had been impregnated with Solvable (DuPont NEN). A plastic cap was then firmly placed on the vial, and both the cap and the upper half of the vial were wrapped with parafilm and incubated with gentle shaking for 2 h at 30°C. At the completion of the incubation, the filter paper was transferred to a 7-ml glass scintillation vial containing 4 ml of Ready Safe (Beckman, Fullerton, Calif.) liquid scintillation

cocktail to determine radioactivity. The α-[1-^{14}C]ketoisocaproate stock solution was prepared by mixing 5.6 μl of 20 mM α-ketoisocaproate (sodium salt), 50 μl of α-[1-^{14}C]ketoisocaproate (55 mCi/mmol; 50 μCi/ml) (Amersham, Arlington Heights, Ill.) and H_2O to a final volume of 1 ml. E1 PDH activity was determined by a radiochemical assay similar to that described above but with [1-^{14}C]pyruvate as substrate in place of α-[1-^{14}C]ketoisocaproate in the reaction mixture (24). The [1-^{14}C]pyruvate stock solution was prepared by mixing 5.6 μl of 20 mM pyruvate (sodium salt), 50 μl of [1-^{14}C]pyruvate (28 mCi/mmol; 81 μCi/ml) (Amersham), and H_2O to a final volume of 1 ml.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited at GenBank under accession number U17169.

RESULTS AND DISCUSSION

PCR amplification and cloning of a *S. avermitilis* gene fragment encoding a portion of the E1α subunit of the BCDH complex by using a homology probing approach. Early attempts to detect and clone *bkd*-like sequences from *S. avermitilis* by using the *P. putida bkd* genes (59) (GC content approximately 65%, compared with approximately 72% for *Streptomyces* species) were not successful. An alternative approach involving the use of PCR was therefore investigated.

Three E1α BCDH peptide sequences from human (20), rat (72), and *Pseudomonas putida* (10) cells and the E1α subunit of the dual PDH/BCDH complex from *B. stearothermophilus* (26) were aligned to identify conserved regions that could serve as candidate sequences to design corresponding PCR primers. Several regions of extended resemblance (65) were chosen, and minimal homology requirements for PCR primer design were followed as previously discussed (19, 23, 56). One PCR primer was based on a region encompassing amino acids 212 to 220 of the *P. putida* E1α BCDH protein (10), which was used as a representative model of an E1α BCDH subunit. These amino acids are located within the TPP-binding motif (25). The second primer was based on a region encompassing amino acids 396 to 402 of the same E1α BCDH subunit (C-terminal region). *Streptomyces* gene codon assignments (67) were used to design the PCR primers (see Materials and Methods).

S. avermitilis genomic DNA was amplified first by using the two primers described above and the standard PCR conditions described in Materials and Methods. Under these reaction conditions, multiple PCR products were observed. However, a single band of approximately 0.2 kb was obtained when glycerol (29) was incorporated into the PCR mixture to eliminate spurious priming (not shown). Despite the fact that the latter PCR product was smaller than the expected size of 0.6 kb, the amplified fragment was cloned and sequenced. CodonPreference analysis (18) of the corresponding 138-bp DNA sequence (excluding the primers) revealed an ORF presenting the codon usage and G+C third-position bias characteristic of a *Streptomyces* gene (5, 67). Computer search analysis revealed that the deduced amino acid sequence of the cloned PCR product was highly similar to those of all of the E1α BCDH subunits obtained to date (identity up to 57%). The alignment of the amino acid sequences suggested that the rightward primer hybridized to the expected target region of a typical E1α gene, but the leftward primer hybridized considerably upstream of the corresponding target (see next section and Fig. 2).

Cloning, mapping, and sequencing of a cluster of genes encoding BCDH from *S. avermitilis*. The cloned 0.2-kb PCR product was then used as a probe to screen a *S. avermitilis* chromosomal library (see Materials and Methods). The genomic region containing the E1α determinant was cloned, and a restriction map is shown in Fig. 1. The DNA sequence of the complete 1.2-kb *Kpn*I-*Bam*HI fragment and the adjacent 1.2-kb *Bam*HI fragment (Fig. 1) is presented in Fig. 2. The figure also shows more than 100 bp sequenced from the *Bam*HI end of the adjacent 0.8-kb *Bam*HI-*Bgl*II fragment. The G+C content of the sequence was 72%. The CodonPreference program (18) revealed two complete ORFs (ORF1 and 2) and the 5′ end of a third ORF (ORF3), all with the same orientation (Fig. 2). ORF1 would encode a polypeptide of 381 amino acids with an estimated molecular mass of 40,969. ORF2 would encode a polypeptide of 35,577 Da (334 amino acids). The intergenic distance between ORF1 and ORF2 is 73 bp. The putative ATG start codon of ORF3 overlaps the stop codon of ORF2. Figure 2 also shows that the complementary nucleotide sequence of the leftward primer matched better with its experimental hybridization site (nucleotides [nt] 909 to 929) than with its originally anticipated target site (nt 1275 to 1295), providing an explanation for why the cloned PCR product was shorter than expected (see previous section).

Similarity of the deduced gene products to the components of the BCDH complex. A computer homology search of translated nucleotide and peptide sequence databases by using the deduced amino acid sequence of the *S. avermitilis* ORF1 and ORF2 sequences showed the best scores with E1α and E1β subunits of several BCDH and PDH complexes from different species (Table 1). A multiple amino acid sequence alignment of the *S. avermitilis* ORF1 product with those of the E1α BCDH and E1α PDH subunits in Table 1 showed similarity to structural motifs found in all E1α subunits of α-keto acid dehydrogenase complexes examined so far, such as the TPP-binding motif (25), a putative subunit interaction site (65), and the phosphorylation sites (I and II) of the E1α chains of the mammalian BCDH and PDH complexes (12, 47, 70) (Fig. 2). Similarly, alignment of the ORF2 product with the E1β BCDH and E1β PDH subunits revealed four regions of extensive similarity characteristic of this protein family (65) (Fig. 2). Finally, the deduced product of the incomplete ORF3 (only 34 amino acids of the N terminus [Fig. 2]) showed significant similarity to several dihydrolipoamide acyltransferases (the E2 component of the BCDH complex), including *B. subtilis* (64) (44% identity) and *P. putida* (11) (41% identity) E2 BCDHs (not shown). These results suggest that ORF1, ORF2, and the putative ORF3 would encode three components of the BCDH complex in *S. avermitilis*. This contention was substantiated by the experiments discussed below.

Overexpression of the putative *S. avermitilis* E1α and E1β *bkd* genes in *E. coli*. Expression plasmids pCD670 (ORF1, E1α) and pCD666 (ORF2, E1β) were constructed by the strategies described in Materials and Methods. To attain optimal expression of ORF2, the third position of the seventh codon was changed from C to G to reflect the most frequently used synonymous codon found in 941 genes from *E. coli* (62). *E. coli* C600 cells containing the plasmids pGP1-2, carrying the structural gene for T7 RNA polymerase under the control of a temperature-sensitive repressor, and pCD670 or pCD666 were analyzed for expression by SDS-PAGE. Upon heat induction (42°C), proteins of about 41 kDa (Fig. 3, lane 5) and 34 kDa (Fig. 3, lane 6), which are the approximate masses of the predicted *S. avermitilis* ORF1 and ORF2 products, respectively, were detected in protein extracts from C600(pCD670 [ORF1, E1α]) and C600(pCD666 [ORF2, E1β]). As is evident in Fig. 3 (lane 6), ORF2 was strongly expressed, and for unknown reasons, accumulation of the 34-kDa ORF2 product caused cell lysis that was particularly evident 2 h after induction. Neither the 41-kDa protein nor the 34-kDa protein was found in the vector-containing control strain.

Detection of a functional *S. avermitilis* E1 component of the BCDH complex in *E. coli*. While the close similarity between the *S. avermitilis* gene products described here and the previously described BCDH proteins strongly suggests that the former are indeed part of the family of BCDH proteins, con-

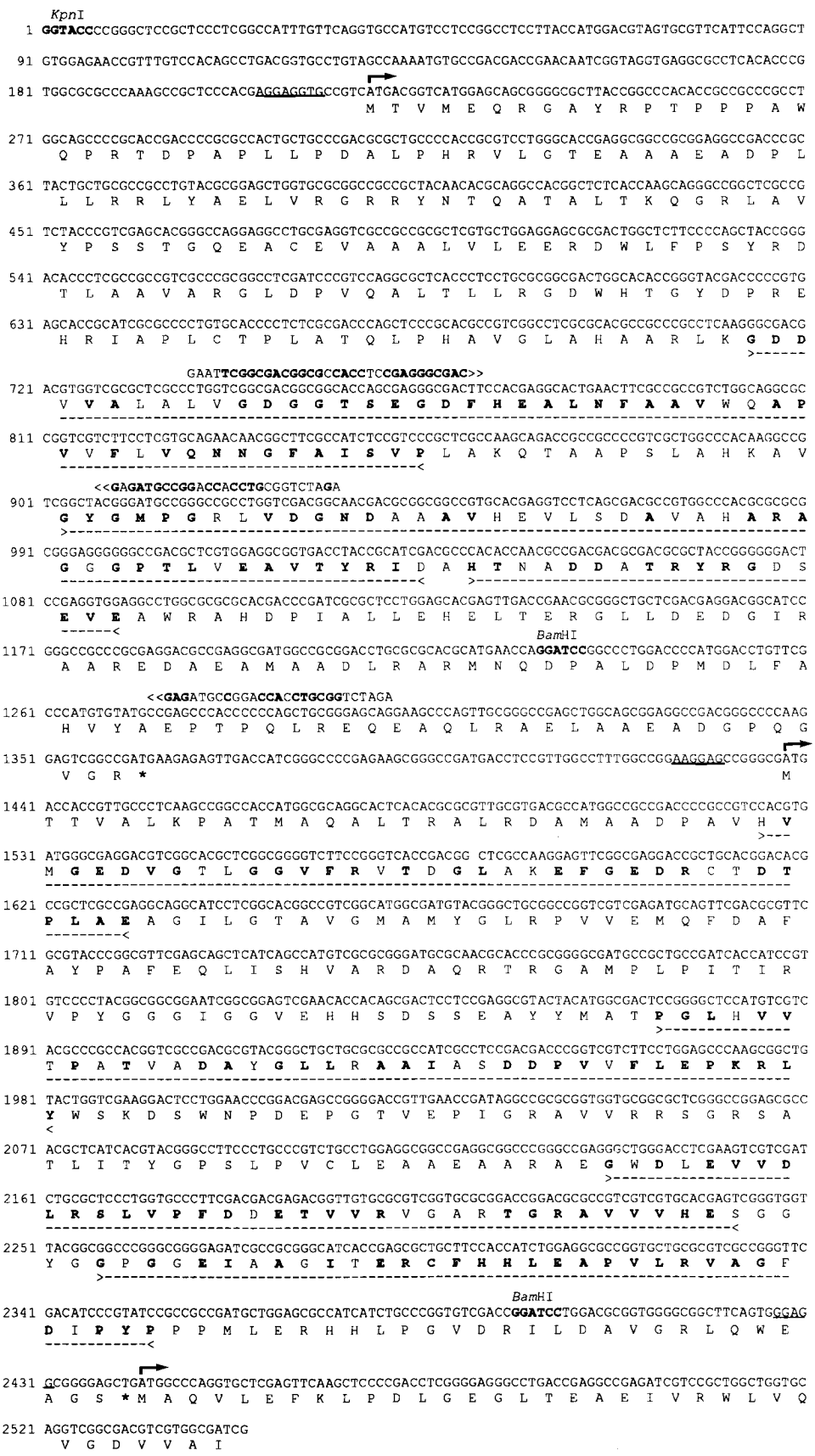
KpnI
BamHI
BamHI

TABLE 1. Amino acid identity between proteins encoded by *S. avermitilis* ORF1 and ORF2 and the subunits of E1 components of α-keto acid dehydrogenase complexes from different sources

S. avermitilis protein size (kDa)	Homologous protein	Source	Identical amino acids (%)	Reference(s)
41	E1α BCDH	*Bacillus subtilis*	33.4	64
	E1α BCDH	*Pseudomonas putida*	33.0	10
	E1α BCDH	Bovine	33.9	32
	E1α BCDH	Human	32.7	20, 40, 71
	E1α PDH	*Bacillus stearothermophilus*	35.1	26
	E1α PDH	*Bacillus subtilis*	32.0	27
	E1α PDH	*Acholeplasma laidlawii*	33.1	63
	E1α PDH	*Saccharomyces cerevisiae*	27.7	2
	E1α PDH	Human	28.0	13, 34
	E1α PDH	Mouse	27.7	22
	E1α PDH	*Ascaris suum*	25.0	33
35	E1β BCDH	*Bacillus subtilis*	43.1	64
	E1β BCDH	*Pseudomonas putida*	43.6	10
	E1β BCDH	Bovine	36.4	68
	E1β BCDH	Human	37.1	38
	E1β PDH	*Bacillus stearothermophilus*	45.8	26
	E1β PDH	*Bacillus subtilis*	44.0	27
	E1β PDH	*Acholeplasma laidlawii*	42.6	63
	E1β PDH	*Saccharomyces cerevisiae*	32.7	42
	E1β PDH	Human	35.3	30
	E1β PDH	*Ascaris suum*	33.5	66

firmatory evidence was obtained by assaying for E1 BCDH and PDH activities in crude extracts of *E. coli* cells harboring pGP1-2 and either pT7-7 (vector, no insert) or a pT7-7 derivative (see Fig. 1, bottom, and Materials and Methods). As shown in Table 2, E1-specific BCDH assays performed in vitro on crude extracts of *E. coli* C600 cells carrying either pCD736 (E1α+E1β) or pCD685 (whole gene cluster) showed significant E1 activity upon induction of the T7 promoter. Cell extracts from uninduced cultures of these clones also showed a significant basal level of activity. The latter result is expected because the T7 system is known to allow a low level of expression of the cloned genes even under uninduced conditions. Crude extracts prepared from cells carrying either pCD670 (E1α), pCD666 (E1β), pCD705 (a construct carrying a normal E1α gene and an impaired E1β gene), or pT7-7 showed no E1 BCDH activity (Table 2). Attempts to reconstitute a functional E1 component in vitro by mixing crude extracts from cells carrying pCD670 (E1α) and pCD666 (E1β) failed to demonstrate any significant activity. This result was not surprising, because Davie et al. (14) demonstrated that association of E1α and E1β mammalian BCDH subunits in recombinant *E. coli* is dependent on coexpression in the same host. In contrast, there were no differences in the level of E1 PDH activity detected when the crude extracts of *E. coli* C600 cells carrying either pT7-7 or any of the pT7-7 derivatives were retested with pyruvate in place of α-ketoisocaproate in the reaction mixtures (not shown). Because *E. coli* C600 cells have a normally high level of endogenous PDH activity, a better evaluation of E1 PDH activity was carried out with *E. coli* UH-Ac-2 (*aceE2*) (35), which lacks E1 PDH activity, as a host of the T7 dual-plasmid system. Cell extracts prepared from either induced or uninduced *E. coli* UH-Ac-2 cells harboring pGP1-2 and either pT7-7, pCD685, or pCD736 showed no significant E1 PDH activity. In addition, levels of E1 BCDH activities similar to those shown in Table 2 for the *E. coli* C600 cultures were detected in *E. coli* UH-Ac-2 crude extracts of induced cells carrying either pCD736 or pCD685 (not shown). The latter E1 BCDH assays also served as a control, indicating that the T7 constructs were expressing functional products in the *E. coli aceE2* host as they were in the C600 cells. The results presented in this section are consistent with the sequence similarity analysis results discussed above, suggesting that the cloned *S. avermitilis* genes encode components of the BCDH.

Concluding remarks. We have cloned and sequenced a cluster of *S. avermitilis* genes encoding proteins highly similar to the E1α, E1β, and E2 components of reported BCDH complexes. In addition, specific E1 BCDH activity was detected in *E. coli* cells carrying *S. avermitilis* ORF1 (E1α) and ORF2 (E1β) coexpressed under the control of the T7 promoter. Preliminary DNA sequencing analysis of transposon mγδ-1 (3) insertions allowed us to estimate that the putative ORF3 (E2) probably has a length of approximately 1.2 kb, which is typical for a gene encoding E2 (51). Moreover, CodonPreference analysis (18) of sequence data from mγδ-1 insertions randomly distributed at either side of the *S. avermitilis* gene cluster failed to demonstrate any additional ORF (up to a distance of 0.5 kb)

FIG. 2. Nucleotide sequence of the 2.5-kb *S. avermitilis* genomic region containing the E1α and E1β ORFs and its surrounding regions. The deduced amino acid sequence is given in the single-letter code below the nucleotide sequence. Three regions of homology in the α subunit and four regions of homology in the β subunit of the E1 component of the BCDH complex, as identified by Wexler et al. (65), are indicated by dashed lines below the deduced amino acid sequences. Amino acid residues identical in the *S. avermitilis* E1α and E1β proteins and at least one other E1α or E1β protein (see Table 1 for references) are indicated in boldface. Presumed Shine-Dalgarno ribosome binding sites (1, 4, 58) are underlined, and the potential translation terminator codons are indicated by an asterisk. Selected restriction sites are listed above their recognition sequences (which are highlighted as boldface letters). The nucleotide sequence of the rightward primer is indicated above its corresponding target site (nt 744 to 770), and the complementary nucleotide sequence of the leftward primer is indicated above its originally anticipated target site (nt 1275 to 1295) and above its corresponding experimental hybridization site (nt 909 to 929). Nucleotides identical to the primer sequences and either the theoretical target or the experimental priming sites are highlighted as boldface letters.

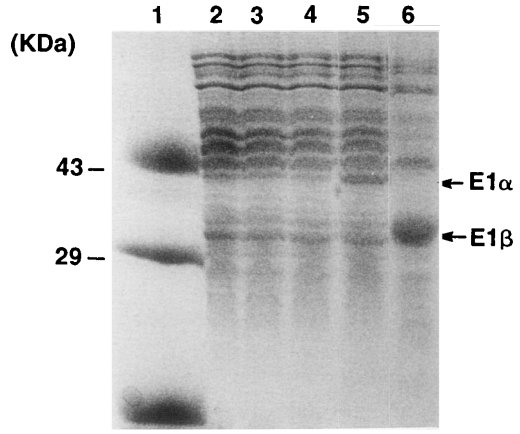

FIG. 3. Synthesis of *S. avermitilis* E1α and E1β subunits of the BCDH complex in *E. coli* C600 examined by SDS-PAGE. Whole-cell extracts of *E. coli* C600(pGP1-2) containing the expression vector pT7-7 (lane 2, uninduced, and lane 3, induced) and *E. coli* C600(pGP1-2) containing the pT7-7 construction carrying the E1α BCDH gene (pCD670) (lane 4, uninduced, and lane 5, induced) or E1β BCDH gene (pCD666) (lane 6, induced) were analyzed. The sizes of two prestained protein markers (ovalbumin and α-chymotrypsinogen) (lane 1) are indicated in kilodaltons on the left.

(not shown), suggesting that a gene encoding an E3 component of the BCDH complex is not present in the vicinity of this cluster. Many species seem to have a single lipoamide dehydrogenase which functions as the E3 component of pyruvate, α-ketoglutarate, and BCDHs. In humans and *B. subtilis*, the E3 component of the PDH complex is shared by the three α-keto acid dehydrogenases (27, 48). The data suggest that the *S. avermitilis* genes reported here may have an organization similar to that of the *B. subtilis* cluster of genes encoding the BCDH responsible for branched-chain fatty acid synthesis, which consists of E1α, E1β, and E2 genes in the same orientation (64).

Because little information is available about the degradative pathway for many amino acids and other nitrogen-containing compounds utilized by *Streptomyces* species and other gram-positive bacteria (21), the isolation of the gene cluster encoding a BCDH complex from *S. avermitilis* reported here should contribute to a better understanding of the catabolism of amino acids in these microorganisms and its relationship to secondary metabolism.

TABLE 2. *S. avermitilis* E1 BCDH activity in crude extracts of recombinant *E. coli* C600 cells

Construction	Plasmid	Induction	E1 BCDH sp act[a]
No insert	pT7-7	+	0.4
E1α	pCD670	−	0.2
		+	0.3
E1β	pCD666	−	0.1
		+	0.3
E1[α+β]	pCD736	−	1.6
		+	13.3
E1[α+β][b]	pCD705	−	0.5
		+	0.1
E1[α+β]+E2	pCD685	−	2.5
		+	5.6

[a] The specific activity of the E1 component of BCDH is given as picomoles of CO_2 evolved per minute per milligram of protein. The values shown are averages of results from three separate experiments, with the assays performed in duplicate.

[b] This construct carries the C-terminal part of the E1β ORF in the wrong orientation; it was used as a negative control.

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