Molecular Analysis of the Anaerobic Succinate Degradation Pathway in *Clostridium kluyveri*

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A region of genomic DNA from *Clostridium kluyveri* was cloned in *Escherichia coli* by a screening strategy which was based on heterologous expression of the clostridial 4-hydroxybutyrate dehydrogenase gene. The gene region (6,575 bp) contained several open reading frames which encoded the coenzyme A (CoA)- and NADP⁺-dependent succinate-semialdehyde dehydrogenase (*sucD*), the 4-hydroxybutyrate dehydrogenase (*4hbD*), and a succinyl-CoA:CoA transferase (*cat1*), as analyzed by heterologous expression in *E. coli*. An open reading frame encoding a putative membrane protein (*orfY*) and the 5' region of a gene encoding a σ^{54} -homologous sigma factor (*sigL*) were identified as well. Transcription was investigated by Northern (RNA) blot analysis. Protein sequence comparisons of SucD and 4HbD revealed similarities to the *adhE* (*aad*) gene products from *E. coli* and *Clostridium acetobutylicum* and to enzymes of the novel class (III) of alcohol dehydrogenases. A comparison of CoA-dependent aldehyde dehydrogenases is presented.

The gram-positive anaerobic bacterium Clostridium kluyveri (3) ferments ethanol and acetate to butyrate, caproate, and molecular hydrogen (7). ATP, required for growth, is gained by substrate-level phosphorylation from acetyl phosphate, and the quantity is proportional to the amount of hydrogen produced (46, 50). Investigations of additional metabolic abilities revealed that this organism can utilize crotonate, vinylacetate, and 4-hydroxybutyrate as substrates (4, 5) and is able to ferment the unusual substrate combination of succinate plus ethanol (27). A pathway was proposed, one in which succinate is first activated and then reduced by a two-step reaction to give 4-hydroxybutyrate, which is then further metabolized to crotonyl-coenzyme A (CoA) (Fig. 1) (27). In a previous study, we discussed enzymes involved in the anaerobic breakdown of succinate by C. kluyveri, specifically, a succinyl-CoA:CoA transferase, a CoA- and NADP+-dependent succinate-semialdehyde dehydrogenase, and a 4-hydroxybutyrate dehydrogenase (49). Wolff et al. (55) independently confirmed these data by ¹³C-nuclear magnetic resonance studies as well as enzymatic investigations on the dehydrogenases. 4-Hydroxybutyryl-CoA dehydratase, which catalyzes the last step of the succinatespecific pathway, the dehydration and isomerization of 4-hydroxybutyryl-CoA to crotonyl-CoA, was recently identified, purified, and characterized (45). We present here some molecular aspects of this pathway, including the cloning, sequencing, and heterologous expression of a C. kluyveri DNA region which encodes a succinyl-CoA:CoA transferase, the succinatesemialdehyde dehydrogenase, and the 4-hydroxybutyrate dehydrogenase.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *C. kluyveri* (DSM 555) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *Escherichia coli* JM109 (58) and the pBluescript SK vector (Stratagene, San Diego, Calif.) were from the laboratory collection. *C. kluyveri* cells used for DNA isolation were grown at 37°C

under strictly anaerobic conditions on ethanol (300 mM) and succinate (100 mM) as previously described (49). Cells for RNA preparation were cultured in the same medium except that succinate was replaced by acetate (100 mM). *E. coli* cultures were routinely grown at 30°C in Luria-Bertani (LB) medium (42) on a rotary shaker. Tetrazolium indicator plates (6) containing 4 g of 4-hydroxybutyrate per liter were employed for the screening procedure (oxidation of 4-hydroxybutyrate). Utilization of 4-hydroxybutyrate (4 g/liter) as a carbon source for recombinant *E. coli* clones was investigated in M9 medium (42), supplemented with a small amount of yeast extract (0.2 g/liter), MgSO₄ (2 mM), and CaCl₂ (0.1 mM). Ampicillin (75 mg/liter) was added to the media for *E. coli* as a selection marker, when needed.

Nucleic acids isolation and recombinant DNA techniques. Chromosomal DNA from C. kluyveri was isolated by the method of Saito and Miura (41). Total RNA from C. kluyveri was isolated by the hot phenol-chloroform procedure as described by Gerischer and Dürre (19). DNA was manipulated by standard methods (42); restriction enzymes and T4 DNA ligase were purchased from GIBCO/BRL (Eggenstein, Germany). For plasmid isolation from E. coli, the Quiagen Midi Kit (Diagen GmbH, Düsseldorf, Germany) was used. For cloning purposes, genomic C. kluyveri DNA was partially HindIII digested and fractionated on a sucrose gradient (10 to 40% [wt/vol]). Fractions of approximately 3 to 5 and 4 to 7 kb were ligated into HindIII-digested pBluescript SK vector, and the product was used to transform E. coli JM109. Recombinant clones were screened for their ability to oxidize 4-hydroxybutyrate (see above). Nested deletion subclones were prepared from pCK1 and pCK3 (the clostridial inserts were in a different orientation within the vector) by using the KpnI and ClaI sites of the vector to generate the exonuclease III-resistant and -sensitive ends, respectively. Exonuclease III digestion and all further steps were performed with the Erasea-Base system (Promega, Madison, Wis.) according to the manufacturer's instructions.

DNA sequencing and sequence analysis. Double-stranded DNA was sequenced by the dideoxy chain termination method (43) with $\alpha^{-35}S$ -dATP (DuPont, NEN Research Products) and the Sequenase T7 DNA polymerase kit from U.S. Biochemical (Bad Homburg, Germany) according to the corresponding protocol. The entire sequence (6,575 nucleotides) of the C. kluyveri insert of pCK1 was determined for both strands with the nested deletion subclones generated from pCK1 and pCK3 and the commercially available M13/pUC universal and reversal sequencing primers. In addition, some synthetic oligonucleotides (17-mers) complementary to the already sequenced templates were employed. These primers were prepared with a Gene Assembler Plus (Pharmacia Biotech Europe, Freiburg, Germany) according to the manufacturer's instructions.

Computer sequence analysis. The DNA sequence data and the deduced amino acid sequences were analyzed with the Genetics Computer Group Inc. sequence analysis software package, version 6.2 (13), on a VAX 9000 computer. Database searches were performed with the National Biomedical Research Foundation Protein Information Resource Network Server according to the algorithm of Pearson and Lipman (38).

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Hybridization. Total chromosomal DNA from *C. kluyveri* or *E. coli* plasmid DNA was digested to completion with the appropriate restriction enzyme and separated on agarose gels. Southern blots on nylon membranes (GeneScreen Plus; DuPont, NEN Research Products) were prepared according to the manufacturer's protocol. DNA fragments used as probes were isolated from agarose gels with the Gene Clean Kit (Bio 101, La Jolla, Calif.). The probes were labeled



FIG. 1. Schematic pathway for the ethanol-succinate fermentation of *C. kluyveri.* 1, alcohol dehydrogenase; 2, acetaldehyde dehydrogenase; 3, thiolase; 4, β -hydroxybutyryl-CoA dehydrogenase; 5, crotonase; 6, butyryl-CoA dehydrogenase; 7, CoA-transferase (probably several enzymes with different substrate specificities); 8, succinate-semialdehyde dehydrogenase; 9, 4-hydroxybutyrate de-hydrogenase; 10, 4-hydroxybutyryl-CoA dehydratase-vinylacetyl-CoA Δ^3 - Δ^2 -isomerase, as described elsewhere (21, 45, 49, 55). The formation of acetate, hydrogen, and caproate is not shown.

either with $[\alpha^{-32}P]$ dATP (DuPont, NEN Research Products) by using the Random Primed DNA Labeling Kit (U.S. Biochemical) or with digoxigenin by using the DIG DNA Labeling Kit (Boehringer GmbH, Mannheim, Germany). Membranes were prehybridized in 0.15% (wt/vol) polyvinylpyrrolidone–0.15% (wt/ vol) bovine albumin-0.15% (wt/vol) Ficoll 400-0.9 M NaCl-10% (wt/vol) dextran sulfate-1% (wt/vol) sodium dodecyl sulfate (SDS)-6 mM EDTA-90 mM Tris-HCl (pH 7.5)-100 µg of herring sperm DNA per ml (80 ml/cm²) for 1 to 3 h at 55°C. The appropriate probe was added to the prehybridization solution and incubated for 10 to 15 h at 55°C. Membranes were then washed twice in $2 \times$ SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at room temperature and once in 2× SSC plus 1% (wt/vol) SDS at hybridization temperature. If necessary, additional washing was performed in SSC solutions, containing 1% (wt/vol) SDS, of decreasing ionic strength (1× SSC, 0.1× SSC) at hybridization temperature. Membranes were then subjected to autoradiography or, in the case of the digoxigenin-labeled probes, manipulated with the appropriate detection kit (Boehringer Mannheim GmbH). RNA for Northern blot analysis was separated in denaturing formaldehyde gels and transferred to nylon membranes (GeneScreen Plus; DuPont, NEN Research Products) as described in the manufacturer's manual. An RNA ladder (0.24, 1.4, 2.4, 4.4, 7.5, and 9.5 kb; GIBCO/BRL) was included as standard for size determination. Hybridization and washing were performed according to the protocol described above for the radiolabeled DNA hybridization procedure.

Determination of enzyme activity. Cells from recombinant *E. coli* clones (pCK1, pCK2, pCK3, pCK4, and pSK) were grown at 30°C in LB medium on a rotary shaker and harvested by centrifugation. If necessary, isopropyl-B-D-thiogalactopyranoside (IPTG; 1 mM) was added at an optical density of 0.5 (580 nm) and cells were grown for an additional 3 h and harvested as described above. In order to prevent enzyme inactivation by oxygen, crude extracts were prepared anaerobically in 50 mM potassium phosphate buffer (pH 7.5) containing 3 mM dithioerythritol, with a French pressure cell (80 MPa; Amicon, Silver Springs, Fla.). Protein concentration was determined according to the method of Bradford (8). 4-Hydroxybutyrate dehydrogenase was assayed in anaerobic glass cuvettes at 30°C in 90 mM 2-amino-2-methyl-1,3-propanediol-HCl (pH 8.5), containing 3 mM dithioerythritol, 1 mM MgSO4, and 1 mM NAD+ in a final volume of 1 ml. The reaction was started by the addition of 4-hydroxybutyrate (10 mM) and monitored at 340 nm ($\epsilon = 6.3 \text{ mM}^{-1} \text{ cm}^{-1}$). Activity of succinate-semialdehyde dehydrogenase was assayed in anaerobic glass cuvettes in 50 mM TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid)-HCl buffer (pH 8.5) containing 1 mM dithioerythritol-1 mM NADP+ at 30°C in a final volume of 1 ml. The reduction of NADP⁺ was monitored at 340 nm. To determine the CoA-independent enzyme activity, the reaction was initiated by the addition of succinate-semialdehyde (10 mM). In the case of the CoA-dependent enzyme activity, the assay was initiated with succinate-semialdehyde (10 mM) and then started with CoA (0.1 mM). CoA-dependent succinate-semialdehyde dehydrogenase activity was defined as the difference between the CoA-dependent and



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FIG. 2. (A) Schematic map of the pCK1 to pCK4 inserts and the ORFs deduced from the pCK1 nucleotide sequence. Vertical bars indicate *Hind*III restriction sites. (B) Schematic drawing of some of the subclones generated from pCK1 and pCK3 by the nested deletion method. The *E. coli* host phenotype (oxidation of 4-hydroxybutyrate) is indicated by continuous lines (positive) and dashed lines (negative).

CoA-independent enzyme activity. Succinyl-CoA:CoA transferase was determined with succinyl-CoA (0.1 mM) and acetate (0.2 M) as substrates as described by Scherf and Buckel (44). The reaction was initiated with succinyl-CoA, and the acetyl-CoA product was condensed with oxaloacetate-liberating CoASH. Formation of the latter was determined with 5,5'-dithio-bis(2-nitrobenzoic acid) (NbS₂) at 412 nm (ϵ = 13.6 mM⁻¹ cm⁻¹).

Determination of the N-terminal amino acid sequence. The N-terminal amino acid sequence of the purified succinate-semialdehyde dehydrogenase was determined directly from the electroblotted polyvinylidene difluoride membrane by using a Protein Peptide Sequencer 477A (Applied Biosystems, Foster City, Calif.). Detection was performed on-line with a phenylthiohydantoin analyzer.

Nucleotide sequence accession number. The sequence data reported here were submitted to the EMBL database and assigned accession no. L21902.

RESULTS

Cloning of the C. kluyveri gene region. Of 10⁴ transformants screened, four E. coli clones formed red colonies; they were able to reduce the tetrazolium salt added at the expense of 4-hydroxybutyrate oxidation. pCK1, which was chosen for further analysis, harbored, as shown by sequence analysis, a 6,575-bp insert, which contained seven HindIII restriction endonuclease recognition sites (Fig. 2). pCK2 and pCK4 covered only a part of pCK1 (four HindIII sites; 4.458 bp) whereas the insert of pCK3 (about 7,500 bp) exceeded the pCK1 region at the left-hand end (Fig. 2). The inserts of pCK1 and pCK2 were in the orientation of the pBluescript lac promoter, while those from pCK3 and pCK4 were in opposite orientation. All clones containing the recombinant plasmids pCK1, pCK2, pCK3, and pCK4 were able to grow on 4-hydroxybutyrate as the sole carbon and energy source, which is not a substrate for the E. coli wild type. 4-Hydroxybutyrate dehydrogenase activity was detected in all four clones (50 to 160 mU/mg).

To confirm an identical arrangement of the *Hin*dIII fragments within the genomic DNA of *C. kluyveri*, Southern blot analysis was performed. With a 1,087-bp *Eco*RI and an 864-bp *Hin*dIII fragment (bp 900 to 1987 and 2117 to 2981, respectively) as probes for chromosomal *C. kluyveri* DNA, digested with *PstI*, *Hin*dIII, or *Eco*RI, hybridization signals yielded the expected size of fragments (data not shown). From these data as well as from the identical nucleotide sequence present in pCK3, it was concluded that the cloned *Hin*dIII fragments in pCK1 represent a contiguous *C. kluyveri* genomic DNA fragment and not a multiple ligation of *Hin*dIII fragments.

Nucleotide sequence analysis. For sequencing purposes and for localization of the 4-hydroxybutyrate dehydrogenase-encoding gene, nested deletion subclones were generated from pCK1 and pCK3 as described in Materials and Methods. The complete nucleotide sequence encompassing the insert of pCK1 (6,575 bp) was submitted to the EMBL data base (see Materials and Methods). DNA sequence analysis revealed four complete open reading frames (ORFs) (referred to as orfY, cat1, sucD, and 4hbD) and two truncated ORFs at the 5' and 3' ends, respectively (orfZ and sigL), in the same transcriptional orientation (Fig. 2 and 3). An additional reading frame, encoding 36 amino acids, was located in the intergenic region between orfZ and orfY (bp 856 to 966), but it is likely that it has no coding function, since its G+C content (20.7%) was very low compared with that of the other ORFs (31.1 to 36.2%) and with that reported for genomic DNA (29.8%) from C. kluyveri (1). In addition, the nucleotide sequence downstream of the corresponding AUG start codon contained an inverted repeat with a calculated free energy of -66.5 kJ/mol (17), which could inhibit ribosome binding. Two other inverted repeats were found downstream of the 4hbD gene. A free energy of -62.8kJ/mol was calculated for the first repeat, which is 33 bp downstream of the 4hbD stop codon (bp 6252 to 6285). The corresponding mRNA stem-loop would be followed by several U's as is typical for a rho-independent terminator (39). The second repeat had a calculated free energy of -44 kJ/mol and was located 133 bp further downstream. The AUG start codons of cat1, sucD, and 4hbD were preceded by putative ribosome binding sites at a distance of 7, 6, and 7 bases, respectively, with reasonable homology to those described for E. coli (47) and for clostridia (60). In the case of the 3'-truncated ORF (sigL), the Shine-Dalgarno sequence was at a distance of 17 bases from the AUG codon. However, there was another possible start codon (GUG) in frame which would reduce the distance to the ribosome binding site (9 bases). For orfY, two possible sites for translation initiation were found. Upstream of the usual start codon, AUG (bp 1,050), with the putative ribosome binding site at a distance of 11 bp (AGGAG), there was a second possible start codon (UUG, bp 981), which also had a putative Shine-Dalgarno sequence at a distance of 8 bp (GGAGG).

Identification and expression of 4hbD, sucD, and cat1. By analyzing the phenotype of the nested deletion subclones, the DNA region encoding the 4-hydroxybutyrate dehydrogenase gene was identified. Subclones having a deletion within the last complete ORF (4hbD) were no longer able to oxidize 4-hydroxybutyrate. That this gene (371 amino acids [aa], 41,755 Da) encodes the 4-hydroxybutyrate dehydrogenase was further confirmed by homologies to several class III alcohol dehydrogenases (see below).

The adjacent ORF upstream had an N-terminal amino acid sequence which was identical to that determined for the purified CoA-dependent succinate-semialdehyde dehydrogenase (M-[S]-N-E-V-S-I-K-E-L-I-E-K-A-K-V-A-Q-K-K-L-E-[A]-Y), except for one mismatch in position 16 where a valine was found in the protein but an alanine was deduced from the nucleotide sequence. Therefore, that ORF was designated *sucD*. For the *sucD* gene product (472 aa), a molecular mass of 50,915 Da was calculated, which was somewhat smaller than that determined for the purified enzyme by SDS-polyacrylamide gel electrophoresis (PAGE) (55 kDa [49]). CoA-dependent succinate-semialdehyde dehydrogenase activities in recombinant *E. coli* clones harboring the *sucD* gene (pCK1 to 4) were very low (10 to 20 mU/mg).

cat1, which was located directly upstream of *sucD*, encoded a 538-aa protein with a calculated molecular mass of 58,852 Da. A database search showed significant homologies (41.8 and 40.2% identity and 62.4 and 61.4% similarity) to an acetyl-CoA hydrolase (Ach1) from *Saccharomyces cerevisiae* (31) and to a gene product from *Neurospora crassa* (Acu-8) which is essential for growth on acetate (34), respectively. For bioenergetic reasons, we assume that in *C. kluyveri* this ORF encodes a CoA transferase rather than an acetyl-CoA-hydrolyzing enzyme. However, as with the CoA-dependent succinate-semialdehyde dehydrogenase, the specific activities of a succinyl-CoA:CoA transferase in recombinant *E. coli* clones were very low (5 to 16 mU/mg).

To induce transcription of cat1, sucD, and 4hbD starting from the pBluescript lac promoter, recombinant clones harboring either a part (pCK169 ['orfZ orfY cat1 sucD'; bp 1 to 3772], pCK2 ['cat1 sucD 4hbD sigL'; bp 2117 to 6575]) or the complete gene region (pCK1, bp 1 to 6575) were grown in the presence of IPTG (1 mM), and cell extracts were prepared and analyzed for enzyme activity as described in Materials and Methods (Table 1). Cell extracts from E. coli harboring either pCK1 or pCK169 revealed significant succinyl-CoA:CoA transferase activity. Since the release of CoASH (monitored by the reduction of NbS₂) was dependent on all assay components (succinyl-CoA, oxaloacetate, citrate synthase, and acetate), a simple CoA-hydrolyzing activity like Ach1 from S. cerevisiae could be excluded. CoA-dependent succinate-semialdehyde dehydrogenase and 4-hydroxybutyrate dehydrogenase activities were present in E. coli (pCK1) and E. coli (pCK2) (Table 1). In the case of succinate-semialdehyde dehydrogenase, the assay was performed both in the presence and in the absence of CoA in order to differentiate between the CoA-independent E. coli enzyme, which occurred in all clones, and the recombinant CoA-dependent enzyme from C. kluyveri (Table 1). SDS-PAGE analysis during induction of the recombinant E. coli clones revealed an increase of protein bands corresponding to molecular masses of 66, 55, and 37 kDa (not shown). It is apparent that the 66-, 55-, and 37-kDa protein bands correspond to the cat1, sucD, and 4hbD gene products. The 66-kDa protein occurred only in recombinant clones harboring the cat1 gene (pCK1 and pCK169), whereas the 55- and 37-kDa proteins were present only in clones harboring the *sucD* and *4hbD* genes

SucD and 4HbD sequence comparisons. A database search with the amino acid sequences of SucD (472 aa) and 4HbD (371 aa) revealed homologies to the *adhE* gene product from *E. coli* (891 aa) and to homologous proteins from two *Clostridium acetobutylicum* strains (DSM792, AdhE, 862 aa; ATCC 824, Aad, 873 aa) and an anaerobic protozoan (*Entamoeba histolytica*, Adh2, 870 aa), respectively (16, 20, 35, 57). The protein sequences from the *C. acetobutylicum* strains are almost identical and differ only with respect to the last 11 aa. Whereas the *C. kluyveri* SucD protein showed high similarity to the AdhE (Aad, Adh2) N-terminal region, the 4HbD protein corresponded to the C-terminal region (Table 2). The *E. coli adhE* gene product has both CoA-dependent aldehyde dehydrogenase activity and alcohol dehydrogenase activity (20). These functions are also proposed for the *adhE* and *aad* gene

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FIG. 3. Nucleotide sequence of the 6,575-bp insert of pCK1 representing the region of chromosomal DNA from *C. kluyveri* that contains orfZ (3'-terminal fragment), orfY, cat1, sucD, 4hbD, and sigL (5'-terminal fragment). The genes have been translated with the one-letter amino acid code with the symbols below the first nucleotide of the corresponding codon. Putative ribosome binding sites are underlined, and inverted repeats are indicated by open arrows representing the length and orientation of the stems. Bars above the sequence indicate the -35 and -10 regions of a putative promoter motif.

TABLE 1. Enzyme activities of recombinant <i>E. coli</i> clones
harboring the C. kluyveri cat1, sucD, and 4hbD genes after induction
from the pBluescript <i>lac</i> promoter ^a

	Sp act (mU/mg)				
Strain and plasmid	Succinyl-CoA: CoA-transferase	Succinate- semialdehyde dehydrogenase		4-Hydroxybutyrate dehydrogenase	
		$-CoA^b$	$+CoA^{b}$		
E. coli JM109	≤1	19 ± 1	≤1	≤2	
pBluescript					
<i>E. coli</i> JM109 pCK1	184 ± 10	51 ± 3	80 ± 17	273 ± 66	
(cat1 sucD 4hbD)					
E. coli JM109	220 ± 13	23 ± 2	≤1	22 ± 5	
pCK169 (cat1)					
E. coli JM109 pCK2	≤1	49 ± 11	101 ± 15	475 ± 87	
(sucD 4hbD)					

^a Cells were grown in LB medium containing ampicillin (75 mg/liter), and IPTG (1 mM) was added at an optical density of 0.5. Crude extracts were prepared and analyzed for enzyme activity as described in Materials and Methods. Data represent average values and standard deviations from two independent experiments.

^b –CoA and +CoA refer to the CoA-dependent and -independent succinatesemialdehyde dehydrogenase activities.

products from *C. acetobutylicum* DSM792 and ATCC 824, respectively (16, 35). Significant similarities were also found between the *C. kluyveri* 4HbD protein and other alcohol dehydrogenases (Table 2). These enzymes as well as the *E. coli* and *C. acetobutylicum adhE (aad)* gene products represent a new class (III) of alcohol dehydrogenases, which differ from both the long-chain zinc-containing (type I) and the short-chain zinc-lacking (type II) enzymes (2, 26, 40).

Analysis of orfY, orfZ, and sigL. As mentioned above, two possible sites for translation initiation of orfY were found. Hydropathy analysis of the deduced protein sequence revealed several highly hydrophobic regions. A translation start with AUG (as indicated by the arrow) would result in a protein of 288 amino acids (30,873 Da) with nine hydrophobic and probably membrane-spanning regions (Fig. 4). A translation start with UUG (311 aa, 33,507 Da) would produce a signal sequence with a basic N terminus (two lysine residues) followed by 12 hydrophobic amino acids. A $\phi(lacZ' \cdot 'orfY)hyb30$ fusion containing the first 30 aa of the $lacZ \alpha$ -peptide and 279 aa of orfY (starting with Pro-33 at bp 1073) was constructed from pCK1 with the *Bam*HI restriction endonuclease site (pCK7). Recombinant *E. coli* clones were able to grow in LB medium, but if expression of the fusion protein was induced by the addition of IPTG (1 mM), cell lysis occurred, probably because of a disintegration of the cell membrane (not shown).

Database searches revealed similarities (about 50%) to other putative membrane-spanning proteins from *Desulfurolobus ambivalens* (29), *Haemophilus influenzae* (52), and *E. coli* (DDBJ:D13267). However, the function of these proteins is yet to be determined, and most of the similarities between them might be due to their common membrane-spanning function.

Whereas the amino acid sequence deduced from the 5'truncated ORF orfZ showed no significant homology to protein sequences in the databases, the N-terminal amino acid sequence deduced from the last reading frame (*sigL*), which was located at the 3' end of the pCK1 insert, revealed significant similarities to the family of σ^{54} -related proteins (not shown). Since the N terminus of these proteins is highly conserved, it seems likely that *sigL*, which was designated in analogy to the corresponding gene from another gram-positive organism (*Bacillus subtilis*), encodes a σ^{54} -homologous sigma factor.

Northern blot analysis. To analyze the expression of the cloned genes in C. kluyveri, RNA was isolated from cells grown on ethanol plus succinate and ethanol plus acetate and prepared for Northern blot hybridization. With radiolabeled probes complementary to the 4hbD (bp 5668 to 6025) and the cat1 (bp 2117 to 2981) genes, significant hybridization predominantly occurred with RNA isolated from cells grown on ethanol plus succinate (Fig. 5). A strong signal corresponding to approximately 2,700 nucleotides in length was obtained with the 4hbD-complementary probe. In addition, two weak signals of about 5,500 and 9,500 nucleotides and a small transcript of about 1,800 nucleotides could be detected after prolonged exposure (Fig. 5). The cat1-complementary probe gave similar results: only low hybridization occurred with RNA from ethanol-acetate-grown cells, whereas with RNA from cells grown on ethanol plus succinate, signals corresponding to a length of

 TABLE 2. Comparison of the C. kluyveri gene products SucD (succinate-semialdehyde dehydrogenase) and 4HbD (4-hydroxybutyrate dehydrogenase) with aldehyde and alcohol dehydrogenases from other organisms

	c. kluy		veri SucD ^b	C. kluyveri 4HbD ^b	
Organism and gene product (reference[s])	Sequence positions"	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
C. acetobutylicum AdhE-Aad (16, 35)	2–458	41.8	58.8		
	449-860			26.1	51.9
E. coli AdhE (20)	3–474	37.2	57.7		
	451-862			26.2	51.4
Entamoeba histolytica AdhE2 (57)	7–491	38.0	60.8		
Entamoeba histolytica AdhE2 (57) C. acetobutylicum Adh1 (61)	462-862			26.1	51.9
C. acetobutylicum Adh1 (61)	1–381			28.2	55.2
Saccharomyces cerevisiae Adh4 (54)	4-380			26.4	51.4
Zymomonas mobilis ATCC 10988 AdhB (59)	1–383			27.0	50.1
Żymomonas mobilis ZM4 AdhB (11)	1-383			26.6	49.7
Citrobacter freundii (12)	25-393			26.2	50.0
Bacillus methanolicus Mdh (14)	3–381			25.9	49.3
E. coli FucO (10)	5–383			23.6	51.9
C. acetobutylicum BdhA (51)	6–389			22.9	47.9
C. acetobutylicum BdhB (51)	18-388			21.2	46.9

^a Numbers refer to the first and last amino acids of the resulting sequence comparison.

^b Sequence comparison was performed with the Bestfit algorithm (Genetics Computer Group package [13]) with a gap weight of 3.0 and a length weight of 0.1.



FIG. 4. Hydropathy plot of the *orfY* gene product according to the work of Kyte and Doolittle (30). The amino acid sequence was deduced from a translation start with UUG (bp 981 [Fig. 3]), and the arrow indicates the first amino acid from a translation start with AUG (bp 1050 [Fig. 3]). Positive values represent high hydrophobicity and negative values indicate low hydrophobicity, averaged over a window of 7 aa (30).

about 2,700, 5,500, and 9,500 nucleotides and some small transcripts were obtained (Fig. 5).

DISCUSSION

We screened recombinant *E. coli* clones for an oxidative utilization of 4-hydroxybutyrate and could isolate a DNA region from *C. kluyveri* encoding the 4-hydroxybutyrate dehydrogenase, the CoA-dependent succinate-semialdehyde dehydrogenase, and a succinyl-CoA:CoA transferase. Since *E. coli* has two CoA-independent succinate semialdehyde dehydrogenases which directly catalyze the oxidation of succinate semialdehyde to succinate (15), the use of 4-hydroxybutyrate as a carbon and energy source by *E. coli* depends only on the



FIG. 5. Northern blot hybridization with radiolabeled fragments which are complementary to the *C. kluyveri* (*A*) (A) and *cat1* (B) genes. Lanes 1, 8 μ g of total RNA from *C. kluyveri* cells, grown on ethanol plus succinate; lanes 2, 8 μ g of total RNA from *C. kluyveri* cells, grown on ethanol plus acetate. The cells were harvested in the logarithmic growth state. The sizes of selected marker bands (RNA ladder) are indicated in kilobases at the left side of the figure. (A) Results obtained after hybridization with a *4hbD*-complementary probe and autoradiography for 9 and 21.5 h. (B) The blot was hybridized against a *cat1*-complementary probe and subjected to autoradiography for 16 h, as indicated.

presence and expression of the clostridial 4-hydroxybutyrate dehydrogenase-encoding gene (4hbD) as shown by the nested deletion subclone analysis.

Thus far, the only known protein sequences of CoA-acylating bacterial aldehyde dehydrogenases were those from the multifunctional AdhE enzymes from E. coli and the homologous gene from C. acetobutylicum, which have both CoA-dependent aldehyde dehydrogenase and an alcohol dehydrogenase activity (16, 20, 35). From the tandem arrangement of the C. kluyveri sucD and 4hbD genes and the similarities to the Nand C-terminal regions of the E. coli and C. acetobutylicum adhE (aad) gene products, it is evident that the AdhE proteins are composed of two catalytic domains: the N terminus exhibits a CoA-acylating aldehyde dehydrogenase activity whereas the C-terminal part is an alcohol dehydrogenase. This has also been suggested by Goodlove et al. (20), Fischer et al. (16), and Nair et al. (35). However, it should be noted that the E. coli AdhE protein has a third function, the deactivation of the pyruvate:formate-lyase (28). Primary protein sequence comparison of the C. kluyveri SucD-4HbD proteins and the adhE (aad) gene products of C. acetobutylicum, E. coli, and the protozoan E. histolytica reveals some interesting features (Fig. 6). The N-terminal region which corresponds to the C. kluyveri SucD protein (positions 1 to 472) allows now a first comparison of CoA-acylating aldehyde dehydrogenases (Fig. 6A). The sequence is highly conserved: from a consensus length of 490, 138 aa are strictly conserved in the bacterial enzymes (99 aa including the protozoan AdhE protein sequence) with 321 aa being present in two of the three microbial species. The SucD homologous sequence includes one highly conserved motif for nucleotide binding (G-x-G-x-x-G; site 4) (53) and another region with G-rich motifs (G-G-x-G; G-x-G-x-G; site 2), one of which also might contribute to ADP binding. Since CoA, like $NAD(P)^+$, contains the ADP moiety, two nucleotide binding sites are reasonable. Another highly conserved region (site 3) contains a glycine and a cysteine residue and shows limited similarity to the active center proposed for CoA-independent aldehyde dehydrogenases (2, 23). It is suggested for these enzymes as well as for the CoA-acylating aldehyde dehydrogenases that an enzyme-bound thiol group (cysteine) is required at the catalytic site for the formation of a hemithioacetal intermediate (25, 48). Database searches performed with the AdhE (Aad) proteins from C. acetobutylicum revealed significant but low homology to aldehyde dehydrogenases (CoA independent) from different sources (<25% identity). In addition to the active center described above, a conserved dodecapeptide of CoA-independent aldehyde dehydrogenases (G-V-TC-TGV-GQ-I-LIS-P-W-N-FY-P [24]) would correspond to positions 111 to 122 of the CoA-dependent enzymes (site 1 [Fig. 6A]). The proposed nucleotide binding site of CoA-independent aldehyde dehydrogenases would match positions 174 to 179 and is thus not conserved. In addition, a typical decapeptide (V-TC-L-E-L-G-G-K-AS-P) of these enzymes is missing. Since there is no similarity of CoA-dependent and CoA-independent aldehyde dehydrogenases in the C-terminal region, the highly conserved nucleotide binding site of CoA-dependent enzymes (site 4) might be involved in CoA binding.

The *C. kluyveri* 4HbD protein as well as the AdhE proteins belongs to the class III alcohol dehydrogenases (2, 26, 40). Sequence comparison revealed a more or less common motif, which comprises several histidine residues (Fig. 6B, sites 1 and 2) and might be involved in divalent cation (iron) binding (2, 16, 61). The *C. kluyveri* 4HbD protein contains three of the four histidine residues proposed to be involved in iron binding. However, a glutamate and an aspartate residue (site 1) are

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	CkSucD CaAdhE CaAad EcAdhE EhAdhE	MSNEVSIKELIEKAKAAQKKLEAYSQEQVDVLVKALGKVVYDNAEMFAKEAVEETEMGVYEDKVAKCHLKSGAIWNHIKDKKTVGIIKEEPE MKVTTVKELDEKLKVIKEAQKKFSCYSQEMVDEIFRNAAMAAIDARIELAKAAVLETGMGLVEDKVIKNHFAGEYIYNKYKDEKTCGIIERNEP MKVTTVKELDEKLKVIKEAQKKFSCYSQEMVDEIFRNAAMAAIDARIELAKAAVLETGMGLVEDKVIKNHFAGEYIYNKYKDEKTCGIIERNEP MAVTNVAELNALVERVKKAQREYSCYSQEMVDEIFRNAAMAAIDARIELAKAAVLETGMGIVEDKVIKNHFAGEYIYNKYKDEKTCGIIESNEP MSTQQTMTVDEHINQLVRKAQVALKEYLKPEYTQEKIDYIVKKASVAALDQHCALAAAAVEETGRGIFEDKATKNIFACEHVTHEMRHAKTVGIINVDPL	92 94 94 94 100 100
	CkSucD CaAdhE CaAad EcAdhE EhAdhE	F ^{GV} P→P _I RALVYVAKPK GV VAATT P ITN P VTPMCNAMAAIKGRNTIIVAPHPKAKKVSAHTVELMNAELKKLGAPENIIQIVEAPSREAAKELMESADVVIATG YGITKIAEPI GV VAAII P VTN P TSTTIFKSLISLKTRNGIFFSPHPRAKKSTILAAKTILDAAVKSGAPENIIGWIDEPSIELTQYLMQKADITLATG FGTITIAEPI G VVAAII P VTN P TSTIFKSLISLKTRNGIFFSPHPRAKKSTILAAKTILDAAVKSGAPENIIGWIDEPSIELTQYLMQKADITLATG YGITEIAEPI G VVACGVTPVTN P TSTAIFKSLISLKTRNAIIFSPHPRAKDATNKAADIVLQAAIAAGAPKDLIGWIDOPSVELSNALMHHPDINLILATG YGITEIAEPV GV VCGVT P VTN P TSTAIFKSLISIKTRNPIVFSFHPSALKCSIMAAKIVRDAAIAAGAPENCIQWIEFGGIEASNKLMNHPGVATILLATG	190 192 192 194 200 200
	CkSucD CaAdhE CaAad EcAdhE EhAdhE	G=G=G=G=G=G=G=G=G=G=G=G=G=G=G=G=G=G=G=	283 285 285 287 300 300
	CkSucD CaAdhE CaAad EcAdhE EhAdhE	- FKDGKINSKIIGKSVQIIADLAGVKVPEGTKVIVLKGKGAGEKDVLCKEKMCPVLVALKYDTFEEAVEIAMANYMYEGAGHTAGIHSDNDENIRYARTV - KDGSVNPKIVGQSAYTIAAMAGIKVPKTTRILIGEVTSLGEEEPFAHEKLSPVLAMYEADNFDDALKKAVTLINLGGLGHTSGIYADEIKA - KDGSVNPKIVGQSAYTIAAMAGIKVPKTTRILIGEVTSLGEEEPFAHEKLSPVLAMYEADNFDDALKKAVTLINLGGLGHTSGIYADEIKA - KNGALNAAIVGQPAYKIAELAGFSVPENTKILIGEVTVVDESEPFAHEKLSPTLAMYRAKDFEDAVEKAEKLVAMGGIGHTSCLYTDQDNQ DVNNARLNPKCPGMSPQWFAEQVGIKVPEDCNIICAVCKEVGPNEPLTREKLSPVLAILKAENTQDGIDKAEAMVEFNGRGHSAAIHSND	382 376 376 378 390 400
	CkSucD CaAdhE CaAad EcAdhE EhAdhE	FG=G==G LPISRLVVNQPATTAGGTVLPISRLVVNQPATTAGGSFNNGFNPTTTLGCGSWGRNSISENLTYEHLINVSRIGYFNKEAKVPSYEEIWG 472 RDKIDRFSSAMKTVRTFVNIPTSQGASGDLYNFRIPPSFTLGCGFWGGNSVSENVGPKHLLNIKTVAERRENMLWFRVPHKVY 459 RDKIDRFSSAMKTVRTFVNIPTSQGASGDLYNFRIPPSFTLGCGFWGGNSVSENVGPKHLLNIKTVAERRENMLWFRVPHKVY 459 PARVSYFGQKMKTARILINTPASQGGIGDLYNFKLAPSLTLGCGSWGGNSISENVGPKHLINKKTVAKRAENMLWHKLPKSIY 461 KAVVEKYALTMKACRILHNTPSSQGGIGSIYNY-IWPSFTLGCGSYGGNSVSANVTYHNLLNIKRLADRRNNLQWFRVPPKIF 472 490	
В	Ck4HbD CaAdhE CaAad EcAdhE EhAdhE	MKLLKLAPDVYKFDTAEEFMKYFKVGKGDFILTNEFLYKPFLEKFNDGADAVFQEKYGLGEPSDEMINNIIKDIGDKQYNRIIAVGGGSVIDIAKILSLK FKFGCLQFALKDLKDLKKKRAFIVTDSDPYNLNYVDSIIKILEHLDIDFKVFNKVG-READLKTIKKATEEMSSFMPDTIIALGGTPEMSSAKLMWVL FKFGCLQFALKDLKDLKKKRAFIVTDSDPYNLNYVDSIIKILEHLDIDFKVFNKVG-READLKTIKKATEEMSSFMPDTIIALGGTPEMSSAKLMWVL FRGSLPIALDEVITDGHKRALIVTDRFLFNNGYADQITSVLKAAGVETEVFFEVE-ADPTLSIVRKGAELANSFKPDVIIALGGSPMDAAKIMWVM FEPHSIRYLAELKELSKIFIVSDRMMYKLGYVDRVMDVLKRRSNEVEIEIFIDVE-PDPSIQTVQKGLAVMNTFGPDNIIAIGGGSAMDAAKIMWL	100 556 556 558 568 100
	Ck4HbD CaAdhE CaAad EcAdhE EhAdhE	FD===H= YTDDSLDLFEGKVPLVKNKELIIVPTTCGTGSEVTNVSVAELKRRHTKKGIASDELYATYAVLVPEFIKGLPYKFFVTSSVDALIHA YEHPEVKFEDLAIKFMDIRKRIYTFPKLGKKAMLVAITTSAGSGSEVTPFALVTDNNTGNKYMLADYEMTPNMAIVDAELMMKMPKGLTAYSGIDALVNS YEHPETHFEELALRFMDIRKRIYTFPKLGKKAMLVAITTSGTGSEVTPFAVVTDDATGCKYPLADYALTPDMAIVDAELMMKMPKGLTAYSGIDALVNS YEHPETHFEELALRFMDIRKRIYKFPKMGVKAKMIAVTTTGGTGSEVTPFAVVTDDATGCKYPLADYALTPDMAIVDANLVMDMPKSLCAFGGLDAVTHA YEHPEADFFAMKQKFIDLRKRAFKFPTMGKKARLICIPTTSGTGSEVTPFAVISDHETGKKYPLADYSLTPSVAIVDPMFTMSLPKRAIADTGLDVLVHA ====================================	187 656 658 668 200
	Ck4HbD CaAdhE CaAad EcAdhE EhAdhE	EG H AH C PHG TEAYVSPNANPYTDMFSVKAMELILNGYMQMVEKGNDYRVEIIEDFVIGSNYAGIAFGNAGVGAVHALSYPIGGNYHPHGEANYLFFTEIFKTYYEKNP IEAYTSVYASEYTNGLALEAIRLIFK-YLPEAYKNGRTNEKAREKMAHASTMAGMASANAFLGLCHSMAIKLSSEHNIPSGIANALLIEEVIKFNAVDNP IEAYTSVYASEYTNGLALEAIRLIFK-YLPEAYKNGRTNEKAREKMAHASTMAGMASANAFLGLCHSMAIKLSSEHNIPSGIANALLIEEVIKFNAVDNP MEAYVSVLASEFSDGQALQALKLLKE-YLPASYHEGSKNPVARERVHSAATIAGIAFANAFLGVCHSMAIKLGSQFHIPHGLANALLIEVIKFNAVDNP TEAYVSVMANEYTDGLAREAVKLVFE-NLLKSYNGDLEAREKMHNAATIAGMAFASAFLGMDHSMAHKVGAAFHLPHGRCVAVLLPHVIRYNG-QKP 	277 755 755 757 763 300
	Ck4HbD CaAdhE CaAad EcAdhE EhAdhE	NGKIKDVNKLLAGILKCDESEAYDSLSQLLDKLLSRKPLREYGMKEEEIETFADSVIEGQQRLLVNNYEP VKQAPCPQYKYPNTIFRYARIADYIKLGGNTDEEKVDLLINKIHELKKALNIPTSIKDAGVLEENFYSSLDRISELALDDQCTGANPRFPLTSEIKEM VKQAPCPQYKYPNTIFRYARIADYIKLGGNTDEEKVDLLINKIHELKKALNIPTSIKDAGVLEENFYSSLDRISELALDDQCTGANPRFPLTSEIKEM TKQTAFSQYDRPQARRYAEIADHLGLSAPGDRTAAKIEKLLAWLETLKAELGIPKSIREAGVQEADFLANVDKLSEDAFDDQCTGANPRYPLISELKQI RKLAMWPKYNFYKADQRYMELAQMVGLKCNTPAEGVEAFAKACEELMKATETITGFKKANIDEAAWMSKVPEMALLAFEDQCSPANPRVPMVKDMEKI	347 846 853 857 858 400
	Ck4HbD CaAdhE CaAad EcAdhE EhAdhE		

FIG. 6. Amino acid alignment of the *sucD* and *4hbD* gene products from *C. kluyveri* (CkSucD [A] and Ck4HbD [B], respectively) with the *adhE* gene products from *C. acetobutylicum* (CaAdhE [16] and CaAad [35]), *E. coli* (EcAdhE [20]), and *E. histolytica* (EhAdhE [57]). The *C. kluyveri* SucD and 4HbD proteins are depicted in the first line and cover the positions 1 to 472 and 1 to 371, as indicated. (A) The first site indicates a conserved dodecapeptide for aldehyde dehydrogenases (24). Glycine-rich motifs (sites 2 and 4) indicate putative nucleotide binding sites of the aldehyde dehydrogenase domain. Site 3 depicts the proposed active center of the aldehyde dehydrogenase domain with a conserved cysteine residue. (B) Sites 1 and 2 represent the proposed iron binding motif for the class III alcohol dehydrogenases (2, 61) comprising several histidine residues.

CkCat1 NcAcu8 ScAch1	MSKGIKNSQLKKKNVKASNVAEKIEEKVEKTDKVVEKAAEVTEKRIRNLKLQEKVVTADVAADMIENGMIVAISGFTPSGYPKEVPKALTKKVNALEEE- MASPIASAALRARQGPSMLKKLCNPEDMLQHFPNGAYIGWSGFTASATPRR-SYYLADHVEKNASRP MTISNLLKQRVRYAPYLKKVKEAHELIPLFKNGQYLGWSGFTGVGTPKAVPEALIDHVEKNNLQG	99 66 65 100
CkCat1 NcAcu8 ScAch1	FKVTLYTGSSTGADIDGEWAKAGIIERRIPYQTNSDMRKKINDGSIKYADMHLSHMAQYINYSVIPKVDIAIIEAVAITEEGDIIPSTGI AQVQPLRRCLRRRRDRERWAALDMIAHDRRRAPHQVGKNIAKGINEGRINFFDKHLSMFPVDLVYGYYTKDR-QNKNLDVVCVEATEIKEDGSIVLGASV KLRFNLFVGASAGPEENRWAEHDMIIKRAPHQVGKPIAKAINQGRIEFFDKHLSMFPQDLTYGFYTRERKDNKILDYTIIEATAIKEDGSIVPGPSV	189 165 162 200
CkCat1 NcAcu8 ScAch1	GNTATFVENADKVIVEINEAQPLELEGMADIYTLKNPPRREPIPIVNAGNRIGTTYVTCGSEKICAIVMTNTQDKTRPLTEVSPVSQAISDNLIGFLNKE GATPELIQMADKVIIEVNTAIP-SFDGLHDITFSDLPPNRKPYLIQQCRDRIGTTSVPVDPEKVVGIIECTTPDQTLPNSPADETATAIAGHLIEFFEHE GGSPEFITVSDKVIIEVNTATP-SFEGIHDIDMPVNPPFRKPYPYLKVDDKCGVDSIPVDPEKVVAIVESTMRDQVPPNTPSDDMSRAIAGHLVEFFRNE	289 264 261 300
CkCat1 NcAcu8 ScAch1	VEEGKLPKNLLPIQSGVGSVANAVLAGLCESNFKNLSCYTEVIQDSMLKLIKCGKADVVSGTSISPSPEMLPEFIKDINFFREKIVLRPQEISNNPEIAR VAHGRLPKNLLPIQSGIGNIANAVIGGLETSNFKNLNVWTEVIQDTFLDLFDSGKLDFATATSIRFSPTGFERFYKNWDNYYDKLLRSQSVSNAPEIIR VKHGRLPENLLPLQSGIGNIANAVIEGLAGAQFKHLTVWTEVLQDSLLDLFENGSLDYSTATSVRLTEKGFDRAFANWENFKHRLCLRSQVVSNNPEMIR	389 364 361 400
CkCat1 NcAcu8 ScAch1	RIGVISINTALEVDIYGNVNSTHVMGSKMMMGIGSGDFARNAYLTIFTTESIAKKGDISSIVPMVSHVDHTEHDVMVIVTEQGVADLRGLSPREK RLGVIGMNTPVEVDIYAHANSTNVMGSRMLNGLGGSADFLRNSKYSIMHTPSTRPSKTDAHGVSCIVPMCTHVDQTEHDLDVIVTENGLADVRGLSPRER RFPVIAMNTPVEVDIYAHANSTNVNGSRMLNGLGGSADFLRNAKLSIMHAPSARPTKVDPTGISTIVPMASHVDQTEHDLDILVTDQGLADLRGLSPKER	485 464 461 500
CkCat1 NcAcu8 ScAch1	AVAIIENCVHPDYKDMLMEYFEEACKSSGGNTPHNLEKALSWHTKFIKTGSMK 538 ARVIIDKCAHDVYKPILKAYFEKAEFECLRKGMGHEPHLLFNSFDMHKALVEEGSMAKVKF 525 AREIINKCAHPDYQALLTDYLDRAEHYAKKHNCLHEPHMLKNAFKFHTNLAEKGTMKVDSWEPVD 526 565	
FIG. 7	Amino acid alignment of the C. kluweri succinvl-CoA:CoA transferase (CkCat1) with a protein necessary for acetate utilization in N. crassa (NcAcu	18) and

strictly conserved among all class III alcohol dehydrogenases and might also be involved in metal binding. It is evident that the clostridial 4HbD enzyme requires divalent cations for activity; our assay includes Mg^{2+} (1 mM) as described elsewhere (22, 49). However, the ion specificity of the enzyme has not yet been investigated.

with an acetyl-CoA hydrolase from S. cerevisiae (ScAch1). Blocks indicate putative sites for nucleotide (CoA) binding.

The *E. coli* AdhE is a homodimeric protein composed of two 96-kDa subunits (9). Interestingly, an AdhE-like aldehydealcohol dehydrogenase enzyme complex has also been purified and characterized from *C. kluyveri*, which is composed of two 55- and two 42-kDa subunits (33). From the data presented here, a gene arrangement similar to *sucD-4hbD* might be expected. Cloning and sequencing of the corresponding genes could be an important step for the elucidation of evolutionary relationships within this class of enzymes. A dimeric aldehyde dehydrogenase domain also corresponds to the composition of several CoA-acylating enzymes: the succinate-semialdehyde dehydrogenase from *C. kluyveri* (2 × 55 kDa [49]), the butyraldehyde dehydrogenase from *C. acetobutylicum* (2 × 55 kDa [36]), and the aldehyde dehydrogenase from *Clostridium beijerinckii* (2 × 56 kDa [56]).

During growth on ethanol and succinate, a succinyl-CoA: CoA transferase reaction is required for the initial activation of the substrate (49). Heterologous expression of the clostridial *cat1* gene in *E. coli* from the pBluescript *lac* promoter identified its gene product as a succinyl-CoA:CoA transferase. Northern blot analysis revealed that *cat1* is efficiently transcribed only during growth on ethanol plus succinate. We could not detect sequence similarities between Cat1 and the heterodimeric CoA transferases from *Pseudomonas putida*, *Acinetobacter calcoaceticus*, and *C. acetobutylicum* or the monomeric enzyme from pig heart, which is presumably cleaved after translation (18, 32, 37). It is somewhat surprising that the *C. kluyveri* succinyl-CoA:CoA transferase (Cat1) and the Acu-8 protein sequence from *N. crassa* indicate high homologies to an acetyl-CoA hydrolase from *S. cerevisiae* (Fig. 7). However, the conserved CoA (ADP) binding site from the heterodimeric CoA transferases (-G-x-G-x-x-G- [37]) is also abundant (-G-x-G-x-x-G/A-) in the amino acid sequences of Ach1 (*S. cerevisiae*), Acu-8 (*N. crassa*), and Cat1 (*C. kluyveri* [Fig. 7]). As in *C. kluyveri*, growth of *N. crassa* on acetate should not involve an acetyl-CoA hydrolase, but a succinyl-CoA:CoA transferase, which catalyzes the activation of acetate, might be required.

Northern blot analysis revealed that the cloned gene region is efficiently transcribed during growth on ethanol plus succinate (Fig. 5). The signal of 2,700 nucleotides obtained with the 4hbD-complementary probe most likely represents a common transcription of sucD and 4hbD, which together are 2,590 bp in length. The presence of a rho-independent terminator and an additional stem-loop downstream of the 4hbD gene further supports this hypothesis. The signals of 5,500 and 9,500 nucleotides might represent a common transcription of orfY, cat1, sucD, and 4hbD (which together cover 5,042 bp) and point to an additional transcription start point upstream of the cloned gene region. Because of the high A+T content of the clostridial DNA, especially in the intergenic regions, identification of putative promoter structures by sequence comparison alone is often ambiguous. However, a more or less significant motif (TGGTAG-21 bp-TATAAT) is located 44 bp upstream of sucD. Clearly, more experiments (including primer extension) are needed for a detailed transcription analysis.

The data on heterologous expression of *cat1*, *sucD*, and *4hbD* are in good agreement with the results from the work of Wolff et al. (55), who recently reported on several protein bands in cell extracts from *C. kluyveri*, which occurred only during growth on ethanol plus succinate (100, 66, 58, 55, 37, and 30 kDa). The 30-kDa protein band would correspond to *orfY*, but it could not be identified during expression of the cloned genes in *E. coli*, probably because of poor translation initiation.

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