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Identification and Analysis of the Genes Coding for the Putative Pyruvate Dehydrogenase Enzyme Complex in *Acholeplasma laidlawii*

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A monospecific antibody recognizing two membrane proteins in *Acholeplasma laidlawii* identified a plasmid clone from a genomic library. The nucleotide sequence of the 4.6-kbp insert contained four sequential genes coding for proteins of 39 kDa (E1α; N terminus not cloned), 36 kDa (E1β), 57 kDa (E2), and 36 kDa (E3; C terminus not cloned). The N termini of the cloned E2, E1β, and native *A. laidlawii* E2 proteins were verified by amino acid sequencing. Computer-aided searches showed that the translated DNA sequences were homologous to the four subenzymes of the pyruvate dehydrogenase complexes from gram-positive bacteria and humans. The plasmid-encoded 57-kDa (E2) protein was recognized by antibodies against the E2 subenzymes of the pyruvate and oxoglutarate dehydrogenase complexes from *Bacillus subtilis*. A substantial fraction of the E2 protein as well as part of the pyruvate dehydrogenase enzymatic activity was associated with the cytoplasmic membrane in *A. laidlawii*. In vivo complementation with three different *Escherichia coli* pyruvate dehydrogenase-defective mutants showed that the four plasmid-encoded proteins were able to restore pyruvate dehydrogenase enzyme activity in *E. coli*. Since *A. laidlawii* lacks oxoglutarate dehydrogenase and most likely branched-chain dehydrogenase enzyme complex activities, these results strongly suggest that the sequenced genes code for the pyruvate dehydrogenase complex.

Mycoplasmas are the smallest free-living cells known and are often found as surface parasites on eukaryotic cells. They lack a bacterial cell wall but are related to the *Clostridium* and *Bacillus* line of gram-positive bacteria (68). The species *Acholeplasma laidlawii* probably has the most well characterized membrane of all cells and organelles with respect to the physical properties and phase equilibria of the membrane lipids (39, 44, 71).

The membrane of *A. laidlawii*, similarly to several other mycoplasmas, also has a larger number of fatty acid-modified membrane proteins than membranes from common eubacteria (46, 47). These proteins are enriched in hydrophilic amino acid residues and have pIs lower than average for the membrane proteins (47). The acyl proteins are strongly enriched in saturated acyl chains, preferably of endogenous origin, in comparison with the acyl chains of the polar membrane lipids (46, 47).

A monospecific antibody prepared against a carefully purified *A. laidlawii* membrane acyl protein (37) recognized two membrane proteins in *A. laidlawii*. A plasmid clone that reacted strongly with the antibody was isolated from an *A. laidlawii* genomic library (64). We show here that the clone contained four genes coding for proteins homologous to the pyruvate dehydrogenase (PDH) complex and to the branched-chain dehydrogenase (BCDH) enzyme complex in certain gram-positive bacteria and humans. The identity of the proteins was corroborated by genetic complementation studies with *Escherichia coli* mutants known to be defective in PDH.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For preparation of protein for amino acid sequencing and immunoblotting, *A. laidlawii* A-EF22 was grown in a bovine serum albumin (BSA)-tryptose medium (per liter: 20 g of tryptose [Difco], 4 g of BSA, 5 g of NaCl, 5 g of Tris, 7 g of glucose, 60 mg of penicillin G) supplemented with 10% yeast extract (Difco). Fatty acids, i.e., 120 μM oleic acid (18:1c) and 30 μM myristic acid (14:0), were added from sterile ethanolic stock solutions.

E. coli MM 294 and plasmid vector pAT 153 were used for cloning of partially *Sau*3A-digested *A. laidlawii* DNA (64). Strain MM 294 and plasmid pB15 or strain TG 1 and vectors M13mp18 and -mp19 were used for DNA sequencing. Cells were grown in Luria-Bertani medium or on agar plates supplemented with carbenicillin (100 μg/ml) and vitamins (41). Phage M13 was propagated in 2× YT medium (41).

E. coli mutant strains JRG 1174 (*trp aceE2*), JRG 1176 (*aceF10*), and JRG 301 (*trpA trpE lpd1*) were used for complementation studies. The mutant strains, a kind gift from J. R. Guest (University of Sheffield, Sheffield, United Kingdom), were kept in minimal medium E (66) with 10 mM glucose. Supplements of 2 mM acetate, 2 mM succinate, and 30 μg of L-tryptophan per ml were added when required. Transformed mutant cells were selected on 100-μg/ml carbenicillin plates.

Sample preparation. *A. laidlawii* cells in late log phase were harvested by centrifugation, washed in β^- buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4]), frozen, thawed, and lysed, and membranes were centrifuged as described previously (46). The soluble fraction was either diluted in sodium dodecyl sulfate (SDS) cocktail or freeze-dried and dissolved in gel filtration elution buffer. Whole-cell samples for SDS-polyacrylamide gel electrophoresis (PAGE) were withdrawn before cell lysis, and the membrane fractions were washed three times in β^- buffer before addition of SDS-sample cocktail. *E. coli* cells were grown overnight and processed for SDS-PAGE (64).

SDS-PAGE and immunoblotting. Proteins were analyzed

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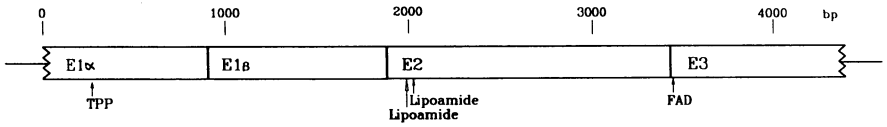


FIG. 1. ORFs of the 4.6-kbp *A. laidlawii* DNA insert in plasmid pB15. The orientation of the ORFs is from left to right. TPP, consensus sequence for TPP binding; lipoamide, consensus sequence for lipoyl binding domain; FAD, consensus FAD-binding fold.

by the discontinuous SDS-PAGE system of Neville (46) with 0.1 mM sodium thioglycolate added to the cathode buffer for protein sequencing samples. Acrylamide (13%) was used for *A. laidlawii*, and 15% acrylamide was used for *E. coli* samples. The proteins in SDS-acrylamide gels were electroblotted onto polyvinylidenedifluoride filters (Immobilon; Millipore) with the semidry technique, 0.8 mA/cm^2 for 1 h at 22°C. The electroblotting was performed in a modified Laemmli buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% [vol/vol] methanol). Proteins were detected with monospecific antibodies or alkaline phosphatase- or peroxidase-conjugated secondary antibodies (46).

The membrane protein T2 from *A. laidlawii* was carefully purified, and monospecific antibodies were prepared (37). The purified immunoglobulin G fraction of a rabbit antiserum to the 64-kDa protein of the *Bacillus subtilis* secretory complex (S complex) was kindly donated by P. C. Tai (34). The 64-kDa S complex protein has been shown to be identical to the PDH-E2 subenzyme of *B. subtilis* (30) and will be referred to as so herein. Antiserum raised against the E2 subenzyme of the oxoglutarate dehydrogenase (OGDH) complex from *B. subtilis* was a kind gift from L. Hederstedt (11). The antibodies directed against PDH-E2 from *Streptococcus faecalis* were kindly donated by A. Allen (1). Processing of SDS-PAGE-separated proteins for amino acid sequencing was performed essentially as previously described (43) with the addition of 0.5 mM dithiothreitol in all solutions.

Separation of soluble proteins by gel filtration. *A. laidlawii* soluble, cytoplasmic proteins were separated by gel filtration on Sepharose 6B in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and 0.15 mM phenylmethanesulfonyl fluoride. Fractions containing putative PDH-E2 were identified by anti-T2 antibodies, precipitated by 80% acetone, dissolved in SDS cocktail, and separated by SDS-PAGE.

N-terminal amino acid sequencing. Amino acid sequencing was performed with a gas-phase sequencer (Applied Biosystems 477 A) equipped with an on-line phenylthiohydantoin analyzer (Applied Biosystems 120 A). Repetitive yields were 97%.

Cloning and DNA sequencing. The cloning and detection of several *A. laidlawii* membrane proteins in *E. coli* plasmid vector pAT153 have been described (64). Plasmid pB15 was isolated by using the alkaline lysis method (4), purified, and digested with *Hin*dIII-*Sal*I or *Eco*RI essentially as previously described (41). Excised fragments were subcloned in M13mp18 and -mp19 in reverse orientations, and clones with partially overlapping DNA sequences were generated by a deletion subcloning method (14) (Cyclone 1; IBI).

DNA sequencing of subclones in phage M13 or plasmid sequencing was carried out by the Sanger dideoxy chain-termination method (58) by using ^{35}S-labelled nucleotide. The polymerase used was Sequenase (version 2.0; U.S. Biochemicals) or *Taq* (*Taq*Track Sequencing Systems; Promega Corporation).

Computer analysis. Sorting of overlapping sequences and pattern recognition were achieved by using the GENEUS version 2.0 program SEQPROJ (27) and the Genetics Computer Group Sequence Analysis Software Package programs CODONFREQUENCY and COMPOSITION (16). Protein similarity searches and sequence comparison were performed with the Genetics Computer Group programs WORDSEARCH, TFASTA, and BESTFIT (16). NBRF and GenEMBL were used as protein and nucleotide sequence data banks, respectively.

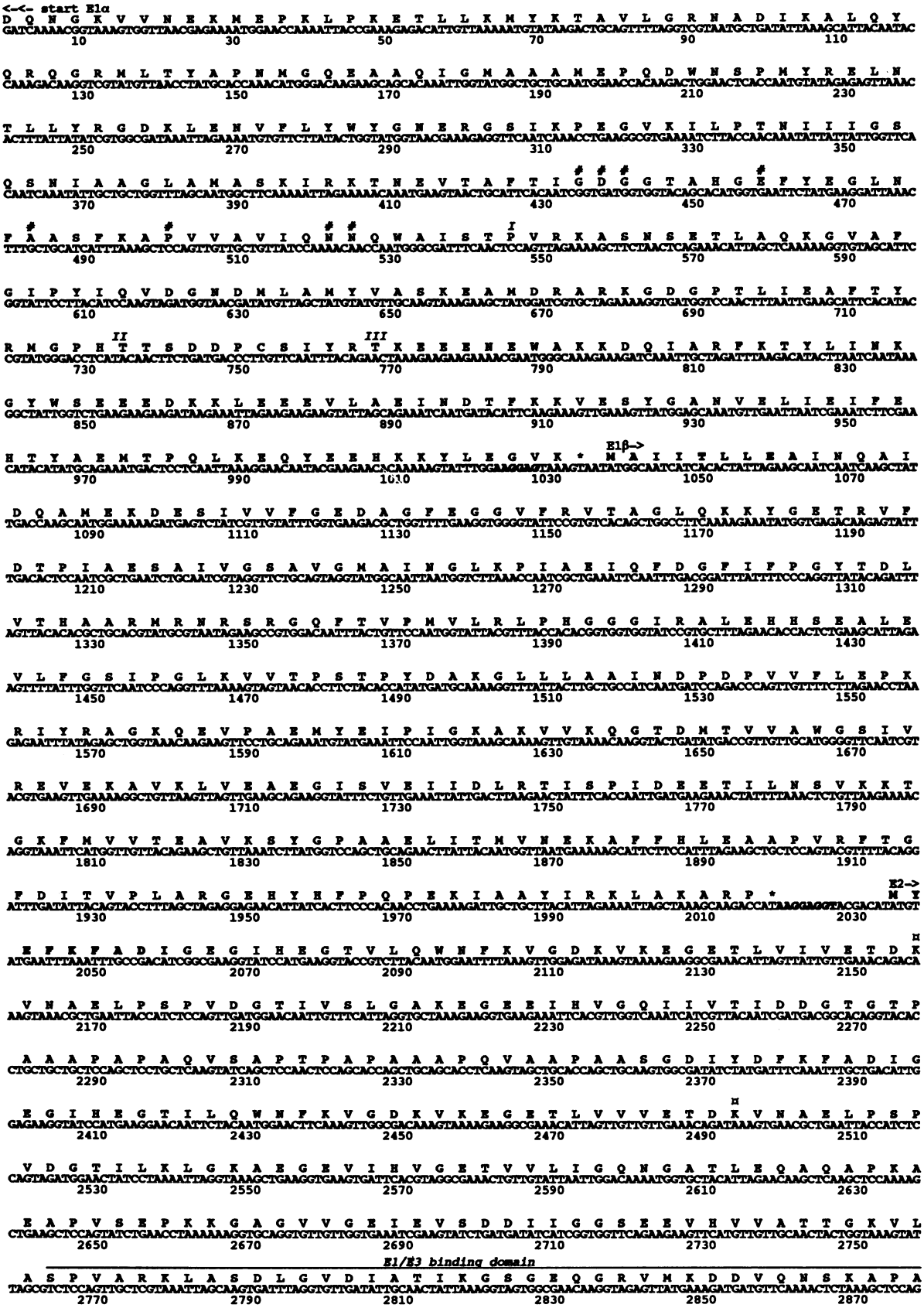
Complementation studies. For in vivo complementation studies, *E. coli* mutant cells (see above) were transformed with pB15 and pAT153. Cells were grown overnight on glucose minimal medium plates supplemented with growth factors when required. Otherwise, preparation and use of competent cells were performed mainly as previously described (25) but with selective media for incubation and plating. Transformants were checked for complementarity on selective agar plates supplemented with 100 μg of carbenicillin per ml.

Nucleotide sequence accession number. The GenBank accession number for the genes of the putative *A. laidlawii* PDH-enzyme complex is M81753.

RESULTS

Gene analysis of the DNA insert in pB15. The cloning of several *A. laidlawii* membrane proteins in *E. coli* has been described (64). The entire 4.5-kbp insert in plasmid pB15 was sequenced from both strands. The cloned fragment was excised with *Eco*RI or *Hin*dIII-*Sal*I and subcloned in different orientations in M13mp18 and -mp19. Clones with different insert sizes were created by treatment with exonuclease III, and initial DNA sequences were determined by using M13mp19 primers. Sequence gaps and overlaps were determined by using specifically designed oligonucleotides as primers for double-stranded DNA sequencing directly from pB15. The DNA sequence revealed four open reading frames (ORFs) encoding putative proteins of 39 kDa (ORF1, the N-terminal part not included), 36 kDa (ORF2), 57 kDa (ORF3), and 36 kDa (ORF4, the C-terminal part not included) (Fig. 1). ORF3 and ORF4 are translated from the same frame, and the intergenic regions between ORF1, ORF2, ORF3, and ORF4 are 1, 13, and 15 bp, respectively (Fig. 2). The start codons are preceded by correctly spaced ribosomal binding sites which share good complementarity with the 3′ end of the 16S rRNA from *A. laidlawii* (72). The genes of ORF1 and ORF2 are closely linked; thus, the ribosomal binding site of ORF2 occurs within the coding frame of ORF1.

The G+C content of the four ORFs is about 37%, which is slightly higher than the G+C content (32%) reported for *A. laidlawii* genomic DNA (68). The codon frequency table showed a preference for codons with A or T in the first and third positions (data not shown). No CGG codon (arginine), shown to be an unassigned codon in *Mycoplasma capri-*


<-<- start E1α
E1β->
E2->
E1/E3 binding domain

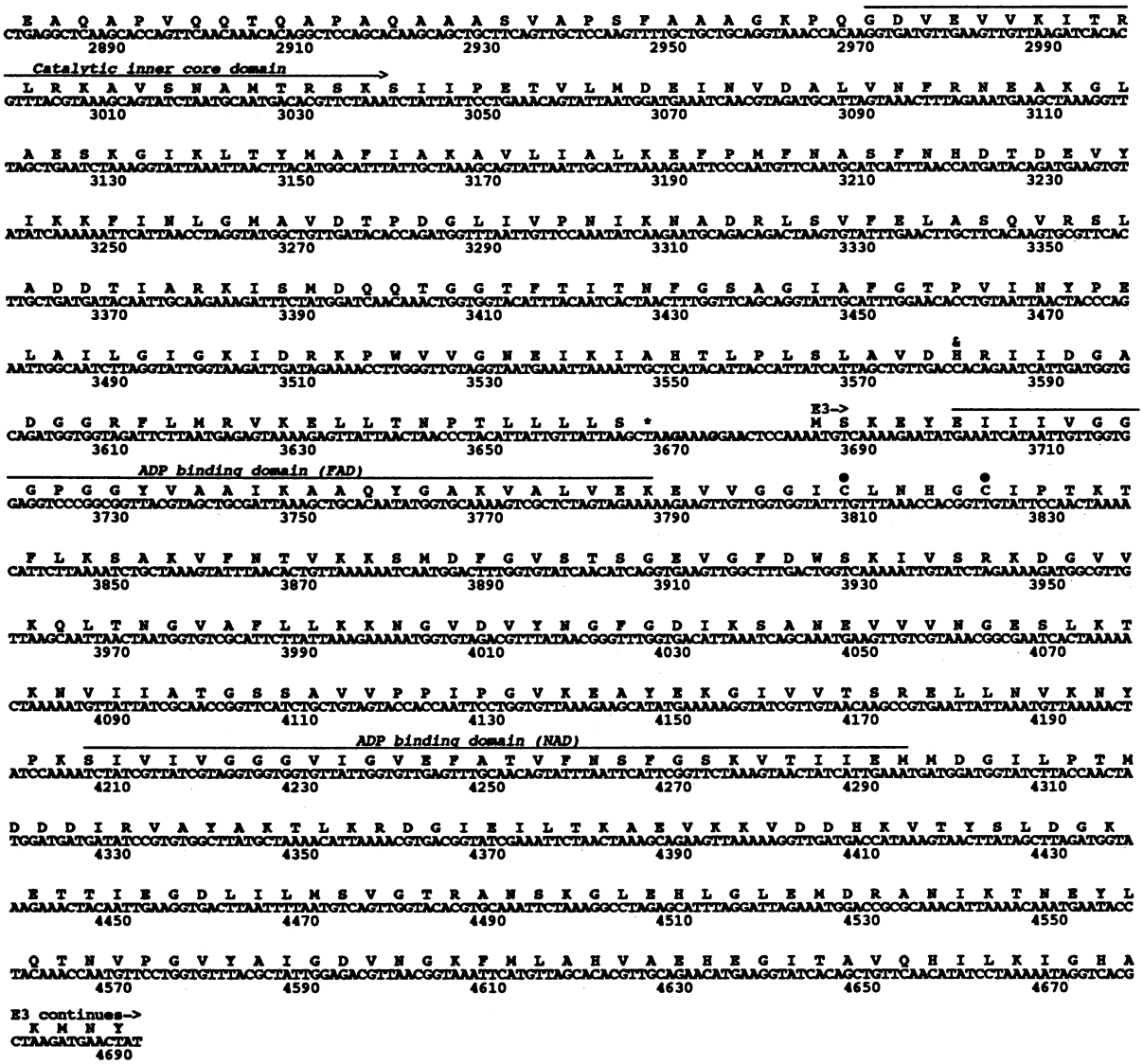


FIG. 2. Nucleotide sequence of the *A. laidlawii* DNA insert in pB15. Consensus sequences for ribosomal binding sites are in boldface type, and termination sites are marked by asterisks. The sequence reveals four ORFs, named E1α (the N terminus not cloned), E1β, E2, and E3 (the C terminus not cloned). Amino acids determined by sequencing of purified proteins are in boldface type. The conserved amino acid residues within the putative TPP-binding motif (#) are marked, and the three phosphorylation sites for the human PDH complex E1α chains are denoted *I*, *II*, and *III*. The lipoyl lysine residues (○) and the putative active site histidine (&) are marked. The limits for the domains within the E2 subunit are approximate and refer to *E. coli* as previously proposed (21). The consensus ADP binding sites for FAD and NAD binding are overlined, and the cysteine residues forming the redox-active disulfide bridge (●) are indicated.

colum (48), was found within the coding regions. In at least three different genera of the mycoplasma class (*Mollicutes*), tryptophan is coded for by the TGA (stop) codon (73). In *A. laidlawii*, only the normal Trp tRNA has been identified so far (12). In accordance with this, no TGA stop codons were found within the coding regions of the pB15 insert. A normal tryptophan codon was corroborated by the incorporation of ^{14}C-labelled tryptophan into the cloned 34- and 57-kDa proteins when expressed in *E. coli* grown in a minimal medium (data not shown).

Identification of proteins. Plasmid pB15 was originally isolated because it encodes a protein that reacts with antibodies prepared against an *A. laidlawii* membrane protein (64). The monospecific antibody directed against the 80-kDa acylated membrane protein T2 (37) recognized an apparent 75-kDa protein encoded by pB15. In *A. laidlawii*, the anti-T2 antibody also recognized a second 75-kDa protein (Fig. 3).

The central part of the DNA insert in pB15 was subcloned in plasmid pUC19, and the encoded proteins were overexpressed in *E. coli* by induction with isopropylthiogalactoside (data not shown). After induction, the 75-kDa protein constituted 3% and a 34-kDa protein constituted 8% of the total *E. coli* proteins. The recombinant 75-kDa protein was purified by SDS-PAGE as described in Materials and Methods, and antibodies in rabbits were prepared. These antibodies recognized the 75-kDa protein in *A. laidlawii* but not protein T2 (data not shown).

Computer-aided homology searches of the translated

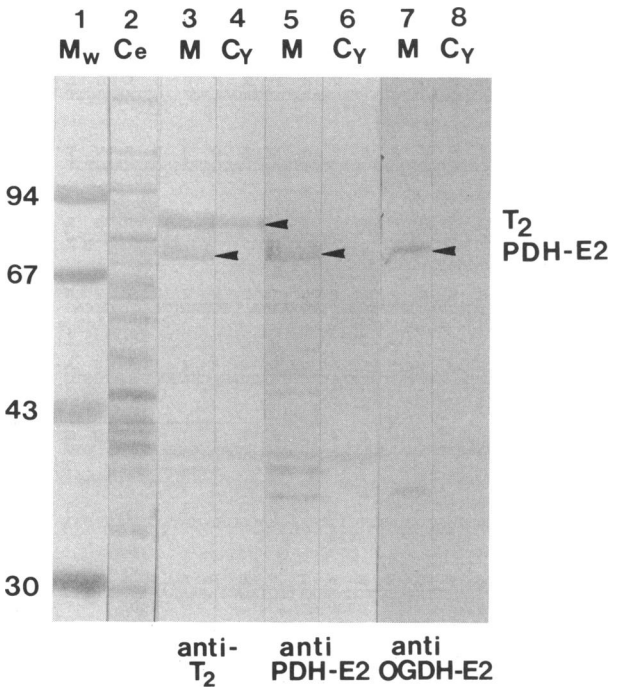


FIG. 3. Immunoblotting of *A. laidlawii* proteins. Proteins were detected with monospecific antibodies against membrane acyl protein T2 (lanes 3 and 4), *B. subtilis* PDH-E2 subunit (lanes 5 and 6), and *B. subtilis* OGDH-E2 subunit (lanes 7 and 8). Lane 1 and 2 show Coomassie brilliant blue-stained proteins. A total amount of 25 μg of protein was loaded in lanes 2 to 8, and 5 μg was loaded in lane 1. Lane 1, molecular mass marker (M_w) in kilodaltons; lane 2, *A. laidlawii* whole-cell (Ce) proteins; lanes 3, 5, and 7, *A. laidlawii* membrane (M) proteins; lanes 4, 6, and 8, *A. laidlawii* cytoplasmic (Cy) proteins.

DNA sequence (see below) from the region of pB15 that encodes the 75-kDa protein indicated homology between the 75-kDa protein and the E2 subenzymes of several 2-oxo acid dehydrogenase complexes. Antibodies directed against the E2 subenzyme of the PDH and OGDH complexes from *B. subtilis* cross-reacted with the 75-kDa *A. laidlawii* protein (Fig. 3). It should be emphasized that *A. laidlawii* has no OGDH (13, 42). In addition to the 75-kDa protein, a 34-kDa protein was also expressed from pB15 and the pUC derivative (see above) as observed with SDS-PAGE (64). This protein was not recognized by any of the *Bacillus* antibodies. Antibodies prepared against the 34-kDa protein overexpressed in *E. coli* (see above) recognized a protein of similar size in *A. laidlawii* membranes (data not shown).

The starts of ORF2 and ORF3 (Fig. 2) were verified by N-terminal amino acid sequencing of the cloned proteins purified by SDS-PAGE. The N-terminal sequence of the *A. laidlawii* 75-kDa protein purified by gel filtration (see Materials and Methods) was identical to that of the ORF3 protein, which has a predicted molecular mass of 57 kDa, according to the DNA sequence (Fig. 2).

The size of the complete (ORF2) 36-kDa protein, as deduced from the DNA sequence (Fig. 2), is with reasonable accuracy the same as that of the 34-kDa protein observed by SDS-PAGE (64). In contrast, the (ORF3) 57-kDa protein has an abnormally low mobility in the SDS-PAGE system, resulting in an apparent molecular mass of 75 kDa.

Similarity searches. Computer-aided searches for homologous proteins showed significant sequence identities (>40%) and similarities (>60%) between the pB15-encoded proteins and components of 2-oxo acid dehydrogenase complexes from different species (Table 1). The 2-oxo acid dehydrogenase complexes, the PDH, the BCDH, and the OGDH multiprotein complexes, consist of subunits termed E1 (or E1α and E1β), E2, and E3 and catalyze the oxidative decarboxylation of 2-oxo acids. The E1 subunits bind thiamine PP_i (TPP) as a prostethic group, react with the primary substrate, and catalyze the decarboxylation step. A consensus sequence for TPP binding is found in all E1α subunits known hitherto (28), whereas little is known about the function of the E1β subunit. The E2 subenzyme has one, two, or three lipoyl domains which contain covalently bound lipoyl chains as prostethic groups. The E3 subenzymes are usually the same for the three 2-oxo acid enzyme complexes in an organism.

TABLE 1. Amino acid identity between proteins encoded by plasmid pB15 and related proteins

A. laidlawii protein (kDa)	Homologous protein	Organism or source	% Identical amino acids	Reference
39[a]	PDH-E1α	*B. stearothermophilus*	46	29
		B. subtilis	44	30
		Human	30	38
		S. cerevisiae	29	3
	BCDH-E1α	Rat	37	75
		Human	36	17
		Bovine	36	35
36	PDH-E1β	*B. stearothermophilus*	47	29
		B. subtilis	45	30
		Human	38	38
	BCDH-E1β	*P. putida*	43	8
57	PDH-E2	*B. subtilis*	41	30
		B. stearothermophilus	38	6
		S. faecalis	38	1
		E. coli	30	61
		Azotobacter vinelandii	30	26
		Human	28	65
	BCDH-E2	Bovine	33	19
		Human	33	15, 36
		P. putida	27	9
	OGDH-E2	*B. subtilis*	32	11
		E. coli	36	60
36[b]	E3	*B. stearothermophilus*	41	6
		B. subtilis	39	30
		S. cerevisiae	38	7
		P. putida	38	10
		A. vinelandii	37	69
		Pig	37	50
		Human	37	53
		E. coli	34	63

[a] N-terminal part not cloned.
[b] C-terminal part not cloned.

The 39-kDa (ORF1) protein (the N terminus not cloned) was found to be homologous to the PDH-E1α subenzymes from *B. subtilis* and *Bacillus stearothermophilus*. Alignment of the sequence with PDH-E1α from humans (data not shown) showed high similarity in the primary sequence surrounding the phosphorylation sites (74), although the serine residues are replaced by Phe, Thr, and Thr, respec-

TABLE 2. In vivo complementation of *E. coli aceE*, *aceF*, and *lpd* mutants with cloned proteins from *A. laidlawii*

Mutant and characteristic	Plasmid	Growth on:	
		Selective media	Nonselective media
JRG 1174 (*trp aceE2*), lacks PDH-E1 enzyme activity	pAT 153	−	+
	pB15	+[a]	+
JRG 1176 (*aceF10*), lacks PDH-E2 enzyme activity	pAT 153	−	+
	pB15	+[a]	+
JRG 301 (*trpA trpE lpd1*), lacks PDH-E3 enzyme activity	pAT 153	−	+
	pB15	+[b]	+

[a] Small colonies.
[b] Small, almost transparent colonies.

tively (Fig. 2). This has also been observed for *B. stearothermophilus*, *Saccharomyces cerevisiae*, and *Pseudomonas putida*. A consensus sequence similar to the structural motif found for TPP-binding enzymes (28) was also observed within the sequence (Fig. 2). The amino acid sequence derived from the 36-kDa protein (ORF2) showed a remarkable similarity to that of the *B. subtilis* and *B. stearothermophilus* PDH-E1β, with the homology extending over the entire lengths of the proteins. The highest degree of similarity to the 57-kDa protein (ORF3) was found with the PDH-E2 subenzymes from *B. stearothermophilus*, *B. subtilis*, and *Streptococcus faecalis*. However, antibodies against the *S. faecalis* PDH-E2 subenzyme did not cross-react with the corresponding *A. laidlawii* protein (data not shown). The sequence revealed two putative lipoyl-bearing domains which are similar to the PDH-E2 subenzymes from human and *S. faecalis*. In addition, a consensus E1-E3 binding and a catalytic inner core domain were found within the protein sequence (Fig. 2). The amino acid sequences of the translated ORF4 (the C terminus not cloned), the PDH-E3 subenzyme from *B. subtilis*, and the OGDH-E3 subenzyme from *P. putida* resembled each other strongly. The homologies of the proteins are especially high around the consensus flavin adenine dinucleotide (FAD) and NAD binding sites (70) and around the two cysteine residues forming the redox-active disulfide bridges (Fig. 2).

In vivo complementation of *E. coli* mutants with the proteins encoded by plasmid pB15. In order to establish the function of the cloned proteins, *E. coli* mutant strains lacking different functional subenzymes of the PDH complex were transformed with plasmid pB15 and checked for growth on a selective medium (minimal medium E without acetate) (Table 2). The mutant strains JRG 1174 (E1 defective) and JRG 1176 (E2 defective) require acetate for aerobic growth on glucose (33), whereas the mutant strain JRG 301 (E3 defective) requires acetate and succinate (22). All three mutant strains lack overall PDH complex activity which can be restored by addition of wild-type cell extract or purified complementing subenzymes (22, 33). Mutant strain JRG 1174 produces an immunologically detectable but dysfunctional PDH-E1 subenzyme with a mutation in the 5′ part of the gene. Please note that this E1 is probably twice as large as the putative *A. laidlawii* E1α (62). Complementation studies performed by mixing cell extract from this mutant with that from another PDH-E1 functionally defective mutant (mutation in the 3′ part of the gene) showed that the two differently defective E1 subenzymes could restore overall PDH complex activity (31, 33). The JRG 1176 mutant lacks PDH-E2 enzyme activity (mutation in 5′ part of the gene) (5, 32). The mutant strain JRG 301 (mutation in the 5′ part of the gene) produces a dysfunctional although immunologically detectable E3 subenzyme (20, 23). PDH and OGDH complex activities in this mutant could be restored by addition of pig heart or wild-type *E. coli* PDH-E3 subenzyme (22).

The three different mutant strains harboring the plasmid pB15 were able to grow on selective media, whereas cells without recombinant plasmid were not. Colonies of transformed mutant cells were smaller and, for the mutant strain JRG 301, transparent, compared with growth on nonselective medium. Successful complementation of mutant strain JRG 1174 with the N-terminally truncated E1α subenzyme from *A. laidlawii* indicated that the truncated protein was transcribed and translated in *E. coli*, perhaps with help from the vector *tet* promoter. The results indicate that the proteins encoded by plasmid pB15 could form an at least partially functional PDH enzyme in *E. coli*, and the abnormal colony morphology observed for mutant JRG 301 may be explained by the lack of the C-terminal part of the *A. laidlawii* E3 subenzyme.

DISCUSSION

A plasmid clone encoding four proteins of 39 kDa (N terminus not cloned), 36 kDa, 57 kDa, and 36 kDa (C terminus not cloned) has been isolated from an *A. laidlawii* genomic library. The cloned 57-kDa protein was recognized by a monospecific antibody directed against a major acylated membrane protein from *A. laidlawii* and also by antibodies prepared against the PDH-E2 and OGDH-E2 subenzymes from *B. subtilis*. Homology searches revealed sequence similarities between the plasmid-encoded proteins and the subenzymes of the PDH and BCDH complexes from different organisms. The strongest similarities were found with the subenzymes of the PDH complex from the gram-positive *B. subtilis* and *B. stearothermophilus*. Studies of different *E. coli* PDH-defective mutant strains corroborated the homology.

The clone expressing the putative PDH complex from *A. laidlawii* was isolated by the use of a monospecific antibody against an 80-kDa acylated membrane protein, T2. The anti-T2 antibody also reacted with a 75-kDa *A. laidlawii* protein, i.e., the plasmid-encoded 57-kDa E2 protein, and with smaller fragments of the latter prepared by partial proteolytic cleavage with endoproteinase Glu-C (data not shown). This indicates that the 57- and 80-kDa proteins share some antigenic domains. However, the endoproteinase digestion maps of these two proteins are not similar (data not shown). The 80- and 57-kDa proteins have calculated pIs of 4.9 (47) and 4.8, respectively, both contain covalently bound hydrophobic chains (the 80-kDa protein contains a fatty acid and the 57-kDa protein contains two lipoamides), and it is possible that the antigenic similarities are found in those regions. Acylated peptide fragments from protein T2 released by endoproteinase Glu-C are recognized by the monospecific T2 antibodies (47), and it has been shown that the lipoyl domains of human PDH-E2 are strongly immunogenic (18).

Are there any functional relationships between proteins E2 and T2? *A. laidlawii* contains no detectable OGDH activity (13, 42). In *B. subtilis*, PDH and BCDH activities are confined to the same proteins (40). The BCDH enzyme

complex is thought to supply the cell with short branched-chain starter units for the synthesis of branched-chain saturated fatty acids, the dominating acyl chains in membrane lipids (29, 40, 49). The *A. laidlawii* growth medium is rich in branched-chain amino acids (manufacturer specification), but branched-chain fatty acids are not synthesized unless proper branched precursors are supplied (54, 57). This indicates that neither E2 nor T2 is part of a BCDH complex, since the enzyme activity is obviously absent in *A. laidlawii*. A recent sequence comparison of dihydrolipoamide acyltransferases (55) plus the PDH-E2 sequences from *B. subtilis*, *B. stearothermophilus*, and *S. faecalis* reveals only two amino acid positions unique for PDH-E2 subenzymes, but the present E2 (ORF3) is at variance with these sequences (data not shown). This also holds for the unique BCDH and OGDH positions previously revealed (55).

The PDH-E2 from *E. coli* has three lipoyl binding domains. Each lipoyl domain has been isolated as a separate functional segment (51). Partial in vivo complementation of PDH-deficient *E. coli* mutants with independently expressed lipoyl and catalytic domains has also been observed (56). In addition, two of the three lipoyl binding domains have been genetically removed without any detectable loss of enzyme activity (24). Also after removal of all three lipoyl binding domains, an assembled PDH enzyme complex with normal E1 and E3 activities but lacking overall enzyme activity was formed (2). This flexibility may explain why the overall PDH enzyme activity could be restored by the pB15-encoded putative PDH proteins in the different *E. coli* defective mutants (Table 2).

The PDH enzyme activity detected in *A. laidlawii* (42, 45) is, at least partially, associated with the membrane fraction (13, 67). In accordance, the putative PDH-E2 subenzyme is detected in the membrane fraction by immunoblotting (Fig. 3). However, the percentage of E2 subenzyme detected in the membrane and cytoplasmic fractions varied depending on growth conditions and harvesting methods (data not shown). Similar results have been found for the PDH-E2 subenzyme from *B. subtilis* (30), which is found associated with membrane-free ribosomes, with membrane-ribosomal complexes, and in the cytosol.

A. laidlawii ferments glucose to lactate, pyruvate, and acetate but lacks the tricarboxylic acid cycle and cytochromes (42, 52). Saturated but not unsaturated fatty acids can be synthesized from acetyl coenzyme A by *A. laidlawii*, and no β-oxidation is present (59). Acetyl coenzyme A is synthesized from pyruvate by the PDH complex (45, 52). The latter enzyme thus connects the basic carbohydrate-utilizing pathway (i.e., glycolysis) in *A. laidlawii* with the synthesis of fatty acyl chains for the membrane polar lipids and acyl proteins.

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