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Two lipoyl domains in the dihydrolipoamide acetyltransferase chain of the pyruvate dehydrogenase multienzyme complex of *Streptococcus faecalis*

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A fragment of DNA incorporating the gene, *pdhC*, that encodes the dihydrolipoamide acetyltransferase (E2) chain of the pyruvate dehydrogenase multienzyme complex of *Streptococcus faecalis* was cloned and a DNA sequence of 2360 bp was determined. The *pdhC* gene (1620 bp) corresponds to an E2 chain of 539 amino acid residues, M_r 56 466, comprising two lipoyl domains, a peripheral subunit-binding domain and an acetyltransferase domain, linked together by regions of polypeptide chain rich in alanine, proline and charged amino acids. The *S. faecalis* E2 chain differs in the number of its lipoyl domains from the E2 chains of all bacterial pyruvate dehydrogenase complexes hitherto described.

Pyruvate dehydrogenase complex; Lipoyl domain; Dihydrolipoamide acetyltransferase; DNA sequence; *Streptococcus faecalis*

1. INTRODUCTION

The 2-oxo acid dehydrogenase multienzyme complexes are built round a core of multiple subunits of the dihydrolipoamide acyltransferase (E2; EC 2.3.1.12) component. The E2 chain has an unusual domain-and-linker structure; from the N-terminus, it comprises one or more lipoyl domains, a peripheral subunit-binding domain and a C-terminal domain which contains the acyltransferase active site and which aggregates with octahedral or icosahedral symmetry to form the inner core of the enzyme complex (for reviews, see [1–4]). The domains are linked by long segments (20–30 amino acid residues) of polypeptide chain that, on the basis of ^{1}H-NMR spectroscopic evidence [5,6], are conformationally flexible. Antibodies raised against an inter-domain linker region of the E2 chain of the pyruvate dehydrogenase (PDH) complex of *Escherichia coli* inhibit the overall catalytic activity of the complex without affecting any of the successive partreactions catalysed by the E1 (pyruvate dehydrogenase (lipoamide), EC 1.2.4.1), E2 and E3 (dihydrolipoamide dehydrogenase, EC 1.8.1.4) components [7], lending substance to the view that conformational flexibility in the inter-domain segments of the E2 chains facilitates lipoyl domain movements that are essential to the coupling of the three active sites [1–4].

The number of lipoyl domains in the E2 chain varies with the source of the 2-oxo acid dehydrogenase complex [1–4]. However, thus far no bacterial E2 chain has been found to accommodate other than one or three lipoyl domains. We describe here the cloning of a fragment of DNA carrying the structural gene for the E2 chain of the PDH complex of the Gram-positive bacterium *Streptococcus faecalis*. From the amino acid sequence of the protein deduced from the DNA sequence of the gene, we infer that it has two lipoyl domains, in addition to a peripheral subunit-binding domain and an acetyltransferase domain.

Abbreviations: PDH, pyruvate dehydrogenase; SDS, sodium dodecylsulphate.

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2. MATERIALS AND METHODS

2.1. *Generation of antiserum*

The PDH complex was purified from *S. faecalis* (ATCC 29212) essentially as described [8] for the complex from *E. coli*. An antiserum against the *S. faecalis* complex was raised in a New Zealand White rabbit by Dr L. Hederstedt, Department of Microbiology, University of Lund, Sweden.

2.2. *Cloning of the gene encoding the* S. faecalis *E2 chain*

Antibodies raised against purified *S. faecalis* PDH complex were used to screen a library of *S. faecalis* chromosomal DNA in bacteriophage λgt11 (Clonetech) plated on *E. coli* strain Y1090*hsdR* [9]. Approx. 5000 plaques were screened by the method of Mierendorf et al. [10], using HYBOND C nitrocellulose filters (Amersham). Binding of the polyclonal primary antibody to antigens was visualized using alkaline phosphatase-conjugated goat anti-rabbit IgG (Tago Inc., USA). The substrates used in the colour reaction were 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in combination with nitro blue tetrazolium (NBT) [11].

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2.3. *DNA sequencing*

An immunoreactive recombinant phage, λPDH-6, was isolated. For unknown reasons, the DNA insert in λPDH-6 could not be released by digestion with *Eco*RI. However, digestion with *Hin*dIII released two contiguous fragments (2.1 kbp and 279 bp) derived from the insert. Their sequences were overlapped by sequencing λPDH-6 directly, using an oligonucleotide (O1-1851; 5'-TTGTGGGACGCATGATG-3') designed to hybridize to the 3' end of the 2.1 kpb fragment. The two fragments were digested with other restriction endonucleases (*Hae*III, *Alu*I, *Sau*3A, *Taq*I or *Rsa*I) to generate smaller overlapping fragments which were end-filled and ligated into dephosphorylated, *Sma*I-cut M13mp18 for sequence analysis.

DNA sequence was determined by the dideoxy chain-termination method [12] using either the T7 Sequencing Kit (Pharmacia) and [α-^{35}S]dATP (Amersham) or the Autoread Sequencing Kit in conjugation with a fluorescein-labelled universal primer and the ALF Sequencer (Pharmacia). Specific oligonucleotide primers based on the N-terminal sequence of the E2 chain, determined by Dr I.C. Packman, were synthesized by Mr M. Weldon, both of the Department of Biochemistry, University of Cambridge. The nucleotide sequence of the 3'-region of the *pdhB* gene, the reconstructed *pdhC* gene, and the 5'-region of the *pdhD* gene was established in both directions and all restriction sites were overlapped by sequencing across them. Bacterial strains, sources of enzymes and all other methods of molecular biology were as described in detail elsewhere [13,14].

2.4. *Expression of genes from λPDH-6*

The genes in the λgt11 recombinant phage, λPDH-6, were expressed from the lysogenic *E. coli* strain Y1089 [15,16]. The protein products were analysed by SDS-polyacrylamide gel electrophoresis [13,14], followed by transfer onto cellulose nitrate (BA85, 0.45 μm, Schleicher and Schull) and immunoblotting with the antiserum directed against the *S. faecalis* PDH complex.

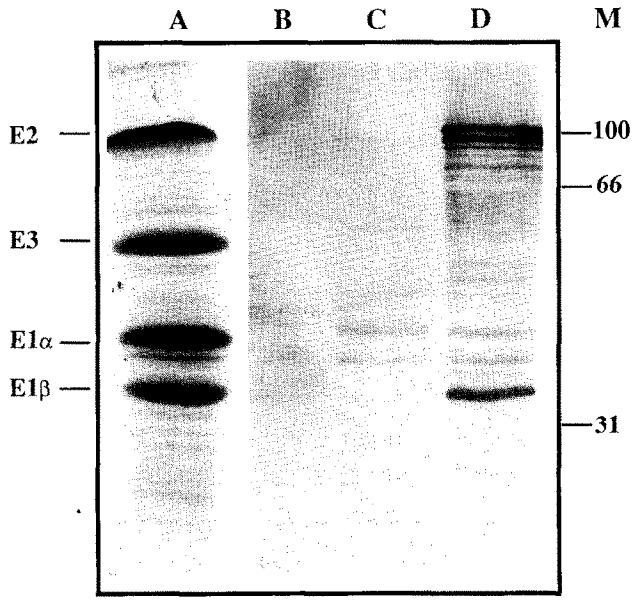


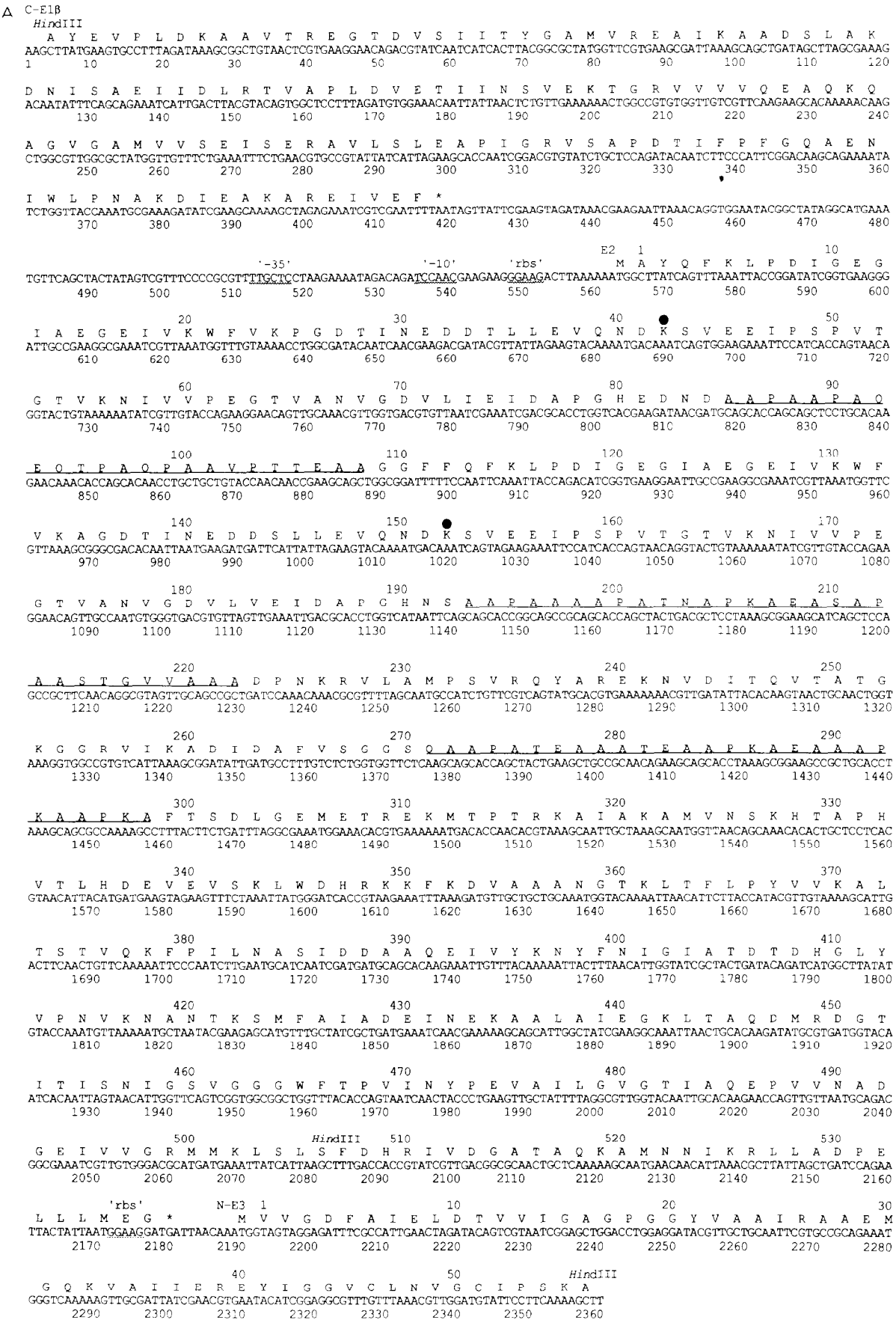
Fig. 1. Separation of component subunits from purified *S. faecalis* PDH complex by SDS-polyacrylamide gel electrophoresis and expression in *E. coli* of proteins encoded by λPDH-6. Track A, purified *S. faecalis* PDH complex stained with Coomassie brilliant blue. Tracks B, C and D; immunoblots of cell-free extracts of *E. coli* probed with antibodies directed against purified *S. faecalis* PDH complex. Track B, cell-free extract of *E. coli* strain Y1089 (uninduced); track C, cell-free extract of *E. coli* strain Y1089 (induced); track D, cell-free extract of λPDH-6 lysogenic *E. coli* strain Y1089 (induced). Track M; M_r markers, kDa.

3. RESULTS

An immunoreactive recombinant phage, λPDH-6, was isolated from a λgt11 library of chromosomal DNA from *S. faecalis* by using antibodies directed against purified *S. faecalis* PDH complex. Genes in the recombinant phage were expressed in the lysogenic *E. coli* strain Y1089, and the products were examined by means of SDS-polyacrylamide gel electrophoresis followed by immunoblotting with the antiserum directed against the PDH complex purified from *S. faecalis*. This revealed the existence of two immunoreactive polypeptides, with M_r of approx. 100 000 and 34 000, respectively (Fig. 1). These proteins comigrated with the E2 and E1β components of the purified *S. faecalis* PDH complex.

Digestion of the λPDH-6DNA with *Hin*dIII released two contiguous fragments (2.1 kbp and 279 bp) from the insert. The nucleotide sequences of these fragments and the amino acid sequences deduced from them are shown in Fig. 2A. One open reading frame of 1620 bp, designated *pdhC*, encodes a protein of 539 amino acids, which incorporates the N-terminal sequence (25 residues) determined for the E2 component of the purified *S. faecalis* PDH complex. The 3'-end of another open reading frame (designated *pdhB*) is located 141 bp upstream of the *pdhC* gene, and the amino acid sequence derived from it is similar to the C-terminal regions of the E1β chains of numerous PDH complexes from other organisms [13,17]. Another open reading frame (designated *pdhD*) starts 7 bp downstream of the *pdhC* gene. The amino acid sequence deduced from this is similar to the N-terminal sequences of the E3 component of the *B. stearothermophilus* [14] and many other [18] PDH complexes. Therefore, assuming that there is another gene (*pdhA*) encoding the E1α chain located upstream of the *pdhB* gene, the PDH complex of *S. faecalis* appears to be encoded by a gene cluster much like that found in *B. stearothermophilus* [14] and *B. subtilis* [19].

The M_r of the *S. faecalis* E2 chain calculated from the DNA sequence of the *pdhC* gene is 56 466 (excluding the N-terminal methionine residue which is post-translationally removed), whereas the apparent M_r of the E2 chain of the PDH complex estimated from SDS-polyacrylamide gel electrophoresis is 100 000. This anomalous electrophoretic mobility is also observed in the migration of the E2 polypeptide chain encoded by the λPDH-6 lysogen (Fig. 1). Over-estimation of the M_r values of the E2 chains of 2-oxo acid dehydrogenase complexes has been noted widely [1–4] and attributed to the elongated or swollen nature of the lipoyl domains [20] and/or to anomalies in the electrophoretic mobility induced by the inter-domain linkers which are generally rich in alanine and proline residues [21,22]. The


A
C-E1β
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'-35'
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E2
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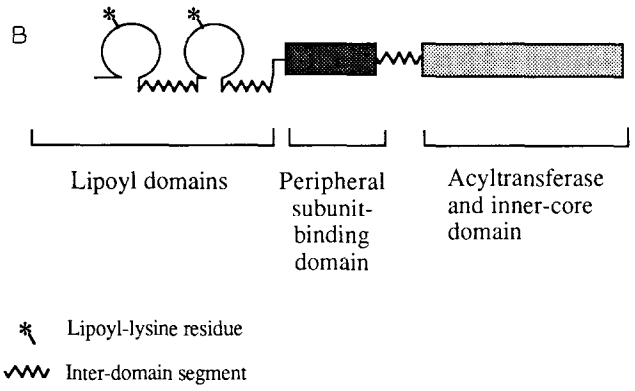

Fig. 2. (A) Nucleotide sequence of the *pdhC* gene from *S. faecalis*. A putative promoter (−35 and −10) and possible ribosome-binding sites (rbs) are marked; termination codons are indicated (*); potentially lipoylatable-lysine residues are indicated (●); alanine/proline-rich interdomain linkers are underlined. (B) Schematic diagram of the domain structure of the *S. faecalis* E2 chain.

discrepancy between the true and apparent M_r values of the *S. faecalis* E2 chain is unusually large.

The domain organization of the *S. faecalis* E2 chain can readily be inferred by comparison of its amino acid sequence (Fig. 2A) with the sequences of E2 chains from other 2-oxo acid dehydrogenase complexes [1-4]. It evidently contains (Fig. 2B) two lipoyl domains (residues 1-84 and 109-192) in a tandem repeat, a peripheral (E1/E3) subunit-binding domain (residues 223-270), and an acetyltransferase and inner-core domain (residues 299-538). All residue numbers quoted are of course approximate. The domains are separated by linker sequences rich in alanine, proline and charged amino acids. These linkers are similar in amino acid content and length to those found in the E2 chains of many other 2-oxo acid dehydrogenase complexes, although significant differences (for example, fewer alanine and proline residues and a preponderance of charged amino acids) have been reported in some instances (reviewed in [1]).

Considerable sequence similarity is evident between the two lipoyl domains of the *S. faecalis* E2 chain, first (90% identity) when compared with each other, and also (approx. 62% identity) when compared with the single lipoyl domains of the E2 chains of the PDH complexes from other Gram-positive bacteria, *B. stearothermophilus* [14] and *B. subtilis* [19]. The lipoyl domains from these Gram-positive bacteria show much less sequence similarity (approx. 23% identity) with the equivalent domains from the PDH complexes of Gram-negative bacteria, such as *E. coli* [23] and *A. vinelandii* [24], respectively. Similarly, the peripheral (E1/E3) subunit-binding domain of the *S. faecalis* E2 chain shares approx. 64% sequence identity with the corresponding domains from the *B. stearothermophilus* and *B. subtilis* PDH complexes, but only about 30% identity when compared with the E3-binding domains of the *E. coli* and *A. vinelandii* PDH complexes. The acetyltransferase domain from *S. faecalis* E2 shows about 58% and 26% sequence identity, respectively, when compared with the corresponding domains from the PDH complexes of the Gram-positive and Gram-negative bacteria described above.

4. DISCUSSION

Hitherto it seemed that PDH complexes isolated from Gram-negative bacteria such as *E. coli* [23,25] and *A. vinelandii* [24] possess three lipoyl domains per E2 chain and a core of octahedral symmetry, whereas PDH complexes isolated from Gram-positive bacteria such as *B. stearothermophilus* [14] and *B. subtilis* [19] have a single lipoyl domain and icosahedral symmetry. On the other hand, the E2 chains of the PDH complexes from human [26,27] and probably rat liver [28] mitochondria have two lipoyl domains, whereas that of yeast enzyme [29] contains only one. All the eukaryotic PDH complexes are based on E2 cores of icosahedral symmetry. As pointed out in detail elsewhere [1], there is no simple correlation between the number of lipoyl domains per E_2 chain, the symmetry of the E2 core or the phylogeny of the source of the enzyme complex. With the discovery that the E2 chain of the PDH complex from *S. faecalis* possesses two lipoyl domains, any correlation with respect to core symmetry and number of lipoyl domains between the PDH complexes from Gram-positive and Gram-negative organisms disappears.

Up to two of the three lipoyl domains can be deleted from the E2 chain of the *E. coli* PDH complex without major effect on the catalytic activity of the enzyme [21] and only one of the three domains needs be functional to confer essentially full catalytic activity on the complex [30]. Thus, it is far from clear why the Gram-negative bacteria elaborate an E2 chain with the additional two lipoyl domains. By the same token, it is not clear what useful advantage the mammalian and *S. faecalis* PDH complexes derive from the possession of two lipoyl domains in each E2 chain. It is an apparent contravention of the normal principle of parsimony [31] in the assembly of biomolecular structures.

A common feature in all dihydrolipoamide acyltransferase catalytic domains is the sequence motif -HXXXDG- found near the C-terminus of the protein, the histidine residue of which may act to promote the nucleophilic attack of coenzyme A on 8-*S*-acyldihydrolipoamide as part of the catalytic mechanism [32]. There is some evidence from directed mutagenesis in favour of this [33,34] but conflicting evidence has been reported [35]. It is worth noting that the motif is conserved (residues 503-515) in the E2 chain of the PDH complex of *S. faecalis* (Fig. 2A). It is also worth noting that there is considerable sequence similarity between the acyltransferase domains of all E2 chains thus far

analysed. However, no systematic differences have yet been identified between the amino acid sequences of those E2 chains that aggregate with octrahedral symmetry and those that do so with icosahedral symmetry. The key to this aspect of assembly remains to be determined.

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