Regulation of pyruvate catabolism in Escherichia coli: the role of redox environment

degraef, M.R.

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Chapter 5

Cloning and sequencing of the lipoamide dehydrogenase of the pyruvate dehydrogenase complex of Enterococcus faecalis NCTC 775
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ABSTRACT
The E3 component (dihydrolipoamide dehydrogenase) of the pyruvate dehydrogenase complex of *Enterococcus faecalis* was cloned and sequenced. The enzyme was cloned on a plasmid in *Escherichia coli* and in *E. coli* high overexpression could be obtained (22 mg l⁻¹). The sequence showed homology with dihydrolipoamide dehydrogenases from other sources. The enzyme consists of 469 amino acids and has a molecular weight of 49158 Da.
INTRODUCTION

One of the central enzymes in aerobic pyruvate catabolism is the pyruvate dehydrogenase complex (PDHc). It catalyses the reaction from pyruvate to acetyl CoA with concomitant reduction of NAD (for a review see Mattevi et al., 1992; Guest et al., 1989). The pyruvate dehydrogenase complex consists of three enzymes: a pyruvate decarboxylase (pyruvate dehydrogenase, E1), a dihydrolipoamide transacetylase (E2) and a dihydrolipoamide dehydrogenase (E3).

For a long time, it has been thought, that the enzyme complex is active only aerobically, but recent studies (chapter 2; Snoep et al. 1990) have revealed that this multienzyme complex can also be active anaerobically. In anaerobic cultures of Enterococcus faecalis, high levels of pyruvate dehydrogenase were observed. The complex of E. faecalis was purified from these anaerobic cultures (Snoep et al., 1992). The E1:E2:E3 polypeptide ratios are 1.2-1.5:1.0:0.6-0.8 (M, of the complex is appr. 5·10^6) (Reed, 1974; Packman et al., 1984; Snoep et al., 1992). The pyruvate dehydrogenase complex is regulated both at the level of gene expression and at the level of enzyme activity (Dietrich and Henning, 1970). More recently, it has been shown that the synthesis of the complex in E. coli is regulated by the Arc two component regulatory system (Quail et al., 1994; see General Introduction). The operon consists of three genes: aceEF for resp. E1 and E2 and lpd encoding E3. E. coli has a separate promoter for E3, but a transcript of all three genes is possible from the aceEF promoter. In E. faecalis there is no additional promoter for E3 (Allen and Perham, 1991).

Snoep et al. (1993) observed that the PDHc from several bacterial species showed a different sensitivity towards NADH. In earlier studies (Snoep et al. 1990) it was concluded that this was due to the sensitivity of the E3 component towards NADH. Of the studied strains, the PDHc from Enterococcus faecalis showed the lowest sensitivity towards NADH and Escherichia coli the highest. This difference probably explains why the complex of E. faecalis can be active anaerobically without an electron acceptor and the complex of E. coli cannot (chapter 2). This feature makes the lipoamide dehydrogenase of E. faecalis interesting for cloning and overexpression, in order to resolve the structure. Until now lpd genes from several species have been cloned and sequenced: a.o. Azotobacter
vinelandii, Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens (fig 5.4). The dihydrolipoamide dehydrogenase oxidises the lipoyl groups on the E2 component with concomitant reduction of NAD. These lipoyl groups have an interesting feature: they can act as swinging arms interacting sequentially with the active sites of the E1 and E2 enzymes (Bleile et al., 1979). The enzyme belongs to the family of FAD-containing pyridine nucleotide oxidoreductases, which include glutathione reductase, thioredoxin reductase, mercuric reductase and tryptanthion reductase. These enzymes all contain a redox-active disulphide bridge, which participates in catalysis.

The lpd gene is also used for the 2-oxoglutarate dehydrogenase multienzyme complex (ODHc, sucAB operon). A lpd mutant lacks the activity of the pyruvate dehydrogenase complex and the 2-oxoglutarate dehydrogenase complex and requires acetate for aerobic growth (Guest et al., 1989).

In this chapter we report the studies of cloning and sequencing the lpd gene of E. faecalis.

**MATERIALS AND METHODS**

*Bacterial strains and vectors, culture conditions*

Bacterial strains: Enterococcus faecalis NCTC 775, Escherichia coli TG2 (Gibson (1984)) recA' Δ(lac-pro) thi supE hsdM' hsdR' F' (traD36proA' B' lacZ lacZAM15)

Plasmids: pUC18, pUCBM20 (Boehringer Mannheim), pBluescript KS’ (Promega), pES18 (personal comm. E. Schulze and A. Westphal) pUC18 containing AsuI/Aval fragment from the PDHc of E. faecalis ligated into Smal site of pUC18 (this fragment carries the gene for E2 and part of the gene for E3, see fig 5.1), pAM1 (this study, 3.5 kb clal fragment of E. faecalis in Pluekescript KS’), pAM2 (this study, as pAM1 but in pUC18 reversed orientation), pAM4 (this study, asel fragment of pAM1 in pUCBM20), pAM5 (this study, as pAM4, reversed orientation)
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Fig 5.1. Position of the probe and pES18 on the PDHc operon of E. faecalis

E. faecalis was grown in medium described previously (Snoep et al., 1990). E. coli was grown in TY medium (10 g tryptone, 5 g Yeast Extract, 5 g NaCl, 11 distilled water).

Materials
Restriction endonucleases were obtained from Gibco-BRL or New England Biolabs. T4-DNA ligase and Klenow fragment of E. coli DNA polymerase I were obtained from Gibco-BRL. Calf intestinal alkaline phosphatase and universal sequencing primer were obtained from Boehringer.

Lipoamide dehydrogenase assay
Activity of lipoamide dehydrogenase was measured spectrophotometrically at 340 nm by the formation of NADH at 25°C as described by Westphal and de Kok (1988). The standard mix contained 50 mM sodium pyrophosphate (pH 8.0), 0.5 mM EDTA, 1 mM reduced dihydrolipoamide and 1 mM NAD. The reaction was started by the addition of enzyme.

Isolation of E. faecalis chromosomal DNA and plasmid DNA
Cells of E. faecalis were harvested by centrifugation (10 min, 6000xg) and washed once with 0.1 M Tris/acetate, pH 8.0, 0.2 M NaCl. The pellet was resuspended in 5 ml 10 mM Tris/Cl, pH 8.0 containing 10% (w/v) sucrose, 0.1 M EDTA. Lysozyme (5 mg) was added followed by incubation for 1 h at 37°C. Next 2.5 mg proteinase K was added followed by another incubation for 1 h at 37°C. This is the same procedure as used by Westphal and de Kok (1988). The lysate was extracted as described by Sambrook et al. (1989).
Plasmid DNA was isolated using a kit (Magic Miniprep, Promega).
Cloning and sequencing

Standard techniques according to Sambrook et al. (1989) and Ausubel (1987) were used for plasmid preparation, restrictions and ligations. Restriction enzymes and buffers etc. were used from GibcoBRL.

Southern blot analysis

Chromosomal DNA of *E. faecalis* was restricted by several restriction enzymes and the fragments were separated on an agarose gel (0.8% agarose in TBE). The fragments were transferred to a nylon filter (Mobilon). Filters were hybridised with radioactive homologous DNA probes at 65°C, according to Sambrook et al. (1989). Probes were radiolabelled by the nick translation method and purified through a Sephadex column (Sambrook et al., 1989). The probe contained a 197 bp HindIII/HindIII fragment of pES18. After hybridisation filters were washed in 2xSSC and 0.1% SDS at 65°C and then exposed to X-ray film (Kodak).

Sequence analysis

Sequencing was performed by standard sequencing methods on an Applied Biosystems 373 DNA Sequencer Strech wrt 48 cm. An ABI prism dye terminator sequencing ready reaction kit with amplitaq DNA polymerase was used. The following primers were used: the standard forward sequencing primer, GAAACCGGGTGAAACACC, CAGCACCTGGTACGATG, CCGCCAAATTTGAATCC and for the reversed sequencing: the standard reverse sequencing primer, GTCATCGGTGCTGAATTAG, GGATTCATGCGTTAG.

RESULTS AND DISCUSSION

*Enterococcus faecalis* chromosomal DNA was digested by various restriction enzymes and separated on a 0.8% agarose gel. pES18 was digested by HindIII yielding a 197bp fragment, which was used as a probe in Southern blot analysis of the digested chromosomal DNA of *E. faecalis* (see fig. 5.1 and 5.2).

Hybridised fragments, digested by Clal, of ca. 3.5 kb size, were purified from the gel and ligated into pBluescript II KS+ *E. coli* TG2 cells were transformed with the ligation mixture and using the blue/white screening, positive colonies were blotted on a nylon membrane. On a southern blot of
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Fig 5.2. Southern blot analysis of the restricted chromosomal DNA of *E. faecalis*. On top of the lanes the different restriction enzymes are shown. Marker is λ DNA restricted with HindIII. Pes18 was restricted with HindIII.

This membrane, using the same 197 bp probe, clones containing the dihydrolipoamide dehydrogenase were selected. These clones, however, showed no lipoamide dehydrogenase activity, according to the assay used. There are two explanations for this: (i) the gene is ligated in reversed orientation in the plasmid, (ii) the 467 basepairs in front of the start codon of the *lpd* gene prevent the *lac* promotor to induce the *lpd* gene.

To overcome this problem two different strategies were followed. First the fragment was ligated in the reverse orientation into pUC18; this new plasmid (pAM2) was then transformed to TG2 cells. Again, these cells showed no significant overexpression of the E3 (table 5.1).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.42 mg l⁻¹</td>
</tr>
<tr>
<td>PAM2</td>
<td>1.5 mg l⁻¹</td>
</tr>
<tr>
<td>PAM4</td>
<td>22 mg l⁻¹</td>
</tr>
<tr>
<td>PAM5</td>
<td>1.1 mg l⁻¹</td>
</tr>
</tbody>
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Table 5.1. Expression of the E3 unit of the PDHc of *E. faecalis* in *E. coli* TG2, using different plasmids. Cells were grown to the same optical density in TY medium.
Subsequently, the fragment was subcloned by using the restriction enzyme asel, which was used to cut the original 3.5 kb Clal fragment and the new fragment was ligated into pUCBM20, yielding pAM4 and pAM5 (reverse orientation). This new clone (pAM4) showed high expression (compared to the wild type) of the dihydrolipoamide dehydrogenase. (Table 5.1).

The sequence of the subcloned fragment (4777 bp) was analysed. Complete overlap was obtained and 95% of the sequence was derived from both strands. The analysis of the sequence is given in fig 5.3.
Cloning of the lipoamide dehydrogenase

Fig. 5.3. Sequence analysis of the cloned fragment of pAM-t. The E3 fragment is beginning at bp 24. The stop codon is indicated with the asterisk.

The part that is coding for E3, codes for 469 amino acids and the calculated molecular weight is 49158 Da, FAD excluded. The sequence shows 100% homology with the part of the E3 sequence that was published by Allen and Perham (1991). The homology with other published E3 sequences are given in fig. 5.4.
Cloning of the lipoamide dehydrogenase

Azogi 303 ATSVPGVYAI GDVVRG-AML AKHSKEGQV VAERIAGHKXQMN

Ecoli 297 RTNVPHIFAI GDVVRG-QLM AKHSVHEGQV AAVIAGHKXQYPD

Psefl 304 KTVSVGVPAI GDVVRG-AML AKHSVSEGQV VAERIAGHKXQMN

Enttal 301 RTNVPHIFAI GDVVRG-PGAL AKHSASYAQI AAIRSEGKVXQAVD

Entfa2 310 YTTEGHIYAI GDVVRG-AML AKHSASYEGQVAIRIAGHKXQMN

Bacsu 300 RTNYPNYAI GDVVRG-APAL AKHSASYEGQV AAREAGHPXQIN

Bacst 299 RTSPNIPFAL GDVRRG-PGAL AKHASYEAGV AEEIRAGPHXQAVD

Azogi 349 YDLIPAVIYT HPEIAAYEGT EQAQIAGSV---AINVGVF PFAASAAGT

Ecoli 334 PEPVPSIAYT EPEVAVGBT EFAKASGQV---SYETATF PFAASAAGT

Psefl 348 YDLIPAVIYT HPEIAAYEKT EQAQIAGSV---AINVGVF PFAASAAGT

Entfal 337 ----AVD YKAMPAVAFN DEKLAYEGQV AAIKAYEAT XQAYAKFY

Entfa2 339 ----AVD YKAIPAVAFN DEKLAYEGQV AAIKAYEAT XQAYAKFY

Bacsu 336 ----AVD YGSPAVAFN DEKLAYEGQV AAIKAYEAT XQAYAKFY

Bacst 333 ----AVD YVISPVAFN DEKLAYEGQV AAIKAYEAT XQAYAKFY

Azogi 393 LTEAMNT AGFVKVIADA KTDRVLGVHV IGPSAAELVQ QGAIAMEF GT

Ecoli 378 LTEAMNT AGFVKVIADA KTDRVLGVHV IGPSAAELVQ QGAIAMEF GT

Psefl 392 LTEAMNT AGFVKVIADA KTDRVLGVHV IGPSAAELVQ QGAIAMEF GT

Enttal 381 LTEAMNT AGFVKVIADA KTDRVLGVHV IGPSAAELVQ QGAIAMEF GT

Entfa2 381 LTEAMNT AGFVKVIADA KTDRVLGVHV IGPSAAELVQ QGAIAMEF GT

Bacsu 380 LTEAMNT AGFVKVIADA KTDRVLGVHV IGPSAAELVQ QGAIAMEF GT

Bacst 377 LTEAMNT AGFVKVIADA KTDRVLGVHV IGPSAAELVQ QGAIAMEF GT

Azogi 439 SAEILQ--MQ VFAHPALSEA LHEAALAVG QAHLVNNKRX

Ecoli 424 SAEILQ--MQ VFAHPALSEA LHEAALAVG QAHLVNNKRX

Psefl 438 SAEILQ--MQ VFAHPALSEA LHEAALAVG QAHLVNNKRX

Enttal 427 SAEILQ--MQ VFAHPALSEA LHEAALAVG QAHLVNNKRX

Entfa2 427 SAEILQ--MQ VFAHPALSEA LHEAALAVG QAHLVNNKRX

Bacsu 426 SAEILQ--MQ VFAHPALSEA LHEAALAVG QAHLVNNKRX

Bacst 423 SAEILQ--MQ VFAHPALSEA LHEAALAVG QAHLVNNKRX

Fig 5.4. Homology between discriminant E3s from different species: Azogi, Azotobacter vinelandii (Westphal and de Kok, 1988); Ecoli, Escherichia coli (Stephens et al., 1983); Psefl, Pseudomonas fluorescens (Benen et al., 1988); Entfa, Enterococcus faecalis (Ward, Bowman School of Medicine, North Carolina, USA; personal communication; this report); Bacsu, Bacillus subtilis (Hamila et al., 1990); Bacst, Bacillus stearothermophilus (Borges et al., 1990); Enttal is the sequence on which we report here.

The other E3 enzyme from E. faecalis in this comparison is a component of the branched chain amino acid oxidation complex. (personal comm. D. Ward, Bowman School of Medicine, North Carolina, USA). There is not much homology between the two lipoamide dehydrogenases (fig. 5.4) of E. faecalis, but this gene was cloned by using a nonhomologous probe against E3. The sequencing of the E3 gene of E. faecalis makes it possible to produce a mutant lacking this enzyme. This mutant could play an important role in studying further the distribution of the pyruvate flux over the PFL and the PDHc branch. A mutant of E. coli carrying instead of the wild type E3, the E3 from E. faecalis would be of interest to such a study, since it would possess a PDHc, which is much less sensitive towards NADH. It
could very well be possible that this mutant is active under anaerobic conditions without an external electron acceptor. The use of constructs like these could help to determine whether the NADH/NAD ratio observed under steady state conditions is a resultant of other regulatory systems or serves as an effector.

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REFERENCES


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The use of reductants like these would help to determine whether the NADH/NAD ratio changes during anaerobic fermentation. A formula for these other regulatory systems and enzymes is required.

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