Metabolic engineering in *Escherichia coli*: lowering the lipoyl domain content of the pyruvate dehydrogenase complex adversely affects the growth rate and yield

Emma Dave, John R. Guest and Margaret M. Attwood

The Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, PO Box 594, Firth Court, Western Bank, Sheffield S10 2UH, UK

Isogenic strains of *Escherichia coli* W3110 containing pyruvate dehydrogenase complexes with three (wild-type), two or one lipoyl domains per lipoate acetyltransferase (E2p) chain, were constructed. The maximum growth rates (μ\text{max}) for batch cultures growing in minimal medium containing different carbon sources showed that reducing the number of lipoyl domains adversely affects cell growth. The lower μ\text{max} value of the mutant containing one lipoyl domain per E2p chain was restored by the presence of compatible multicopy plasmids encoding PDH complexes with either one or three lipoyl domains per E2p chain. In glucose-limited chemostat cultures the protein contents of all strains were similar and substrate carbon was totally accounted for in the biomass and CO₂ produced. However, the carbon efficiencies (percentage carbon conversion to biomass) were significantly lower when the lipoyl domain content of the E2p subunit was reduced from three to one. Similarly, the cellular maintenance energy (mₐ) and the maximum growth yield (Y\text{max}) were lower in bacteria containing PDH complexes with fewer than three lipoyl domains per E2p chain. Wild-type values were restored by supplementing the medium with either casamino acids (0.01%) or acetate (up to 0.1 mM). The lower growth efficiencies of the mutants were further confirmed in competition experiments where equal numbers of genetically marked (NaIR) mutant and wild-type bacteria were used to inoculate glucose-limited chemostat cultures (dilution rate 0.075 h⁻¹). The mutants with one or two lipoyl domains per E2p chain were washed out, whereas in controls, the initial ratio of wild-type (NaI⁺) to reconstructed wild-type (NaIR) bacteria was maintained over 50 generations.

**Keywords**: pyruvate dehydrogenase complex, metabolic engineering, *Escherichia coli*, lipoate acetyltransferase, lipoyl domains

INTRODUCTION

The pyruvate dehydrogenase (PDH) complex of *Escherichia coli* catalyses the NAD-dependent oxidative decarboxylation of pyruvate to acetyl-CoA by three integrated reactions (Guest *et al.*, 1989; Perham, 1991; Mattevi *et al.*, 1992). It contains multiple copies of the pyruvate dehydrogenase (E1p) and lipoamide dehydrogenase (E3) components assembled on a 24-meric lipoate acetyltransferase (E2p) core with an approximate polypeptide stoichiometry of 24:24:12 (E1p:E2p:E3). The covalently bound lipoyl cofactors of the E2p subunits perform a central role in the catalytic mechanism, being in turn reductively acetylated, transacetylated and re-oxidized at the respective E1p, E2p and E3 active sites. The PDH complex is expressed from the *pdbR–aceEF–lpd* operon (Fig. 1) which encodes a pyruvate-responsive repressor, PdhR (*pdbR*), and the three enzymic subunits, E1p (*aceE*), E2p (*aceF*) and E3 (*lpd*). The operon is transcribed from the *pdb* promoter located upstream of the *pdbR* gene (Quail *et al.*, 1994). There is a very weak

**Abbreviations**: D, dilution rate; E1p, pyruvate dehydrogenase; E2p, lipoate acetyltransferase; E3, lipoamide dehydrogenase; PDH, pyruvate dehydrogenase (complex).
promoter of unknown physiological significance in the pdhR-aceE intergenic region, and the distal gene (lpd) is additionally transcribed from its own promoter, presumably to meet the E3 requirements of the 2-oxoglutarate dehydrogenase complex and the glycine cleavage system. Mutations in the aceE and aceF genes lead to a requirement for acetate during aerobic growth on glucose, lpd mutants require acetate plus succinate for best aerobic growth, and pdhR mutants synthesize the PDH complex at a constitutive (pyruvate non-inducible) level.

The E2p chain contains five independently folded domains joined by flexible linkers. The domains include: three highly homologous 80-residue lipoyl domains (each with a lipoylatable lysine residue); an internal 50-residue E3-binding domain; and a C-terminal 250-residue core-forming and E1p-binding domain containing the lipote acetyltransferase catalytic site (Guest et al., 1989; Perham, 1991). A similar segmented organization occurs in the respective E2p, E2o and E2b subunits of the pyruvate, 2-oxoglutarate and branched chain 2-oxo acid dehydrogenase complexes from a variety of sources, but there are differences in the lipoyl domain contents. Thus, whereas the E. coli and Azotobacter vinelandii PDH complexes contain three lipoyl domains per E2p chain, the E2p chains of Streptococcus faecalis and mammalian mitochondria contain two, and the E2p chains of Bacilli and yeast contain only one, like the E2o and E2b chains from both prokaryotic and eukaryotic sources (Perham, 1991). It is not clear why the E2p chains of E. coli and A. vinelandii have three lipoyl domains. Indeed, the selective deletion of aceF sub-segments (1lip or 2lip) encoding one or two net lipoyl domains so as to generate operons that express PDH complexes containing two or one hybrid lipoyl domains per E2p chain (2lip- and 1lip-PDH complexes), has little effect on the specific activities of the purified complexes relative to the wild-type, 3lip-PDH complex (Guest et al., 1985; Graham et al., 1986). It is also apparent that multicopy aceEF-lpd plasmids containing aceF genes with 1lip and 2lip segments, fully complement the nutritional lesions of a pdhR-aceEF-lpd deletion strain. It has likewise been found that about half of the lipoyl domains can be proteolytically cleaved from the PDH complex without significant loss of catalytic activity, and that chemical inactivation or enzymic removal of the lipoyl groups has a non-linear (disproportionately lower) effect on catalytic activity (Stepp et al., 1981; Berman et al., 1981). This is attributed to the process of active-site coupling in which the rate-limiting E1p subunits can interact with more than one of the many lipoyl domains, which themselves participate in an extensive network of intramolecular transacetylation reactions. The acetyl groups and reducing equivalents can thus take many routes through the complex before transfer to CoA and NAD, and the function of a particular lipoyl domain can be adopted by others of the apparently superfluous number of such domains (reviewed by Perham, 1991). More recently, studies with PDH complexes containing four to nine lipoyl domains per E2p chain have shown that activity decreases with increasing numbers of lipoyl domains, possibly due to under-lipoylation of the domains participating in catalysis and to interference from unlipoylated domains (Machado et al., 1992).

An important facilitator of active-site coupling and hence catalytic efficiency, is the mobility conferred by the interdomain linkers. This is apparent from the loss of activity which ultimately occurs when the linkers in a 1lip-PDH complex are shortened or replaced (Miles et al., 1988; Turner et al., 1993). High-field NMR studies with PDH complexes containing zero to nine lipoyl domains per E2p chain, have recently indicated that the wild-type arrangement (3lip-PDH complex) provides the highest degree of mobility, in terms of the ratio of lipoyl linker mobility to lipoyl domain mobility, and presumably the highest catalytic efficiency (Machado et al., 1993). It would appear that the predicted higher catalytic efficiency of the 3lip-PDH complex relative to the 1lip- and 2lip-PDH complexes is not reflected in the observed active-site coupling abilities (an equilibrium assay) nor in the specific activities measured under substrate-saturating conditions, but it may be revealed by detailed kinetic studies under substrate-limiting conditions. The latter is made difficult by the need to amplify and purify different complexes so that they each have identical subunit stoichiometries and identical degrees of lipoylation, in order to make valid comparisons.

Here, an alternative approach has been used to investigate whether, contrary to evolutionary pressure or the normal principle of parsimony in biomolecular assembly (Perham, 1991), E. coli elaborates a PDH complex containing more than sufficient lipoyl domains to satisfy its requirements under a range of growth conditions. This involved constructing stable isogenic strains which have chromosomal pdhR-aceEF-lpd operons that encode 1lip-, 2lip- and 3lip-PDH complexes. Physiological comparisons with these strains has provided evidence that the molecular-genetic lowering of the lipoyl domain content of the PDH complex adversely affects the growth rate and yield of E. coli.

**METHODS**

**Bacterial strains and plasmids.** The prototrophic strain of E. coli K12 used as the wild-type or parental strain was W3110 (deR trpr), and JRG2930 (aceF::kan) is a derivative in which the aceF gene was inactivated by the net deletion of two lip segments (encoding two net lipoyl domains) and the insertion of a kan cassette into the residual lip segment. JRG2930N is a spontaneous Nah mutant of JRG2930, selected on L agar plus nalidixic acid. Further isogenic derivatives of JRG2930N in which the aceF::kan region was replaced by aceF genes encoding E2p subunits with one to three lipoyl domains are: JRG2931 (1lip); JRG2932 (2lip); and JRG2933 (3lip). Some details of key bacterial strains and relevant plasmids are given in Table 1 and Fig. 1. Several pBR322 derivatives (pGS101, pGS102 and pGS104) were used as sources of the Kpn-SphI fragments containing aceEF cassettes with different numbers of lip segments, that were transferred to the thermosensitive replication (pMAK705) prior to allelic replacement. Other plasmids (pGS87 and pGS110) that express wild-type and modified aceEF-lpd genes from the weak ace promoter in the...
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>Prototroph; Na⁺</td>
</tr>
<tr>
<td>JRG2930N</td>
<td>Derivative of W3110 with a disrupted 1lip aceF gene (aceF::kan⁺); Na⁺ This work</td>
</tr>
<tr>
<td>JRG2931-3</td>
<td>Derivatives of JRG2930N with reconstructed 1lip aceF genes; Na⁺ This work</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
</tr>
<tr>
<td>pGS8</td>
<td>pBR322 derivative containing aceEF—lip operon encoding a 3lip—E2p; Ap⁺ Guest et al. (1985)</td>
</tr>
<tr>
<td>pGS101</td>
<td>pBR322 with 3lip aceEF' fragment; Ap⁺ Guest et al. (1985)</td>
</tr>
<tr>
<td>pGS102</td>
<td>pBR322 with 2lip aceEF' fragment; Ap⁺ Guest et al. (1985)</td>
</tr>
<tr>
<td>pGS104</td>
<td>pBR322 with 1lip aceEF' fragment; Ap⁺ Guest et al. (1985)</td>
</tr>
<tr>
<td>pGS110</td>
<td>pBR322 containing aceEF—lip operon encoding a 1lip—E2p; Ap⁺ Guest et al. (1985)</td>
</tr>
<tr>
<td>pGS459</td>
<td>pGS104 with aceEF': kan⁺ fragment; Ap⁺ Km⁺ This work</td>
</tr>
<tr>
<td>pMAK705</td>
<td>Rep⁺ Cm⁺ Km⁺ Hamilton et al. (1989)</td>
</tr>
<tr>
<td>pGS522</td>
<td>pMAK705 with aceEF': kan⁺ fragment from pGS459; rep⁺ Cm⁺ Km⁺ This work</td>
</tr>
<tr>
<td>pGS741</td>
<td>pMAK705 with 1lip aceEF' from pGS104; rep⁺ Cm⁺ This work</td>
</tr>
<tr>
<td>pGS742</td>
<td>pMAK705 with 2lip aceEF' from pGS102; rep⁺ Cm⁺ This work</td>
</tr>
<tr>
<td>pGS743</td>
<td>pMAK705 with 3lip aceEF' from pGS101; rep⁺ Cm⁺ This work</td>
</tr>
</tbody>
</table>

* lip denotes a segment of the aceF gene that encodes a lipoyl domain.

**pdbR—aceE intergenic region**. The rich medium used for routine subcultures was L broth (Lennox, 1955) which contains glucose (0.1%), supplemented with chloramphenicol, kanamycin, naldixic acid or ampicillin (each at 20 μg ml⁻¹), as required. The minimal medium used in nutritional tests was the citrate-containing medium E of Vogel & Bonner (1956) with glucose (20 mM) or potassium succinate (40 mM) as carbon source, with or without a supplement of sodium acetate (2 or 4 mM), and agar 1.5% (w/v). Cultures were streaked to single colonies (one culture per plate) and growth was recorded at intervals for up to 72 h. The minimal medium used for batch cultures had the following composition (g l⁻¹); (NH₄)₂SO₄, 2.0; K₂HPO₄, 1.0; NaH₂PO₄·2H₂O, 1.3; MgSO₄·7H₂O, 0.2; with trace elements solution (Vishniac & Santer, 1957), 20 ml; and the stated carbon source. The standard medium used for carbon (glucose)-limited chemostat cultures was that described by Brooke et al. (1989) without the vitamin solution. It differs from the medium used for batch cultures only in containing less phosphate (K₂HPO₄, 1.0 g l⁻¹) since less buffering capacity is required.

Batch cultures were used to determine doubling times and hence μmax values of different cultures by monitoring the OD₄₉₀ (Pye Unicam SP6-250) of triplicate cultures (50 ml in 250 ml flasks) shaken at 37 °C. The inocula were pre-grown in the same medium, washed with carbon-free medium and resuspended in the growth medium. The slopes of log OD₄₉₀ versus time plots were determined by regression analysis in the exponential regions and used to calculate μmax values.

Chemostat cultures were grown in chemostat vessels (1 litre nominal; working volumes 700 and 750 ml) maintained at 37 °C. The pH was adjusted to 6.9 ± 0.1 by automatic titration with 2 M KOH. Dissolved O₂ levels were monitored with galvanic oxygen electrodes. The culture agitation speed (500–750 r.p.m.) and air flow (500–1000 ml min⁻¹) were adjusted to maintain a dissolved O₂ concentration above 50% air saturation. The inocula were grown overnight in the same medium.

**DNA manipulation and Southern blotting.** DNA was prepared and manipulated by standard procedures (Sambrook et al., 1989). For Southern blotting, (HindIII or PvuII) digests of bacterial DNA were fractionated by electrophoresis in 0.7% agarose-Tris/acetate-EDTA gels and transferred to nitrocellulose membranes (Sambrook et al., 1989). The blots were hybridized for 18 h at 68 °C with 5× SSC and washed twice for 5 min at 20 °C with 2× SSC+0.1% SDS and then twice for 15 min at 65 °C with 0.1× SSC+0.1% SDS (1× SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0). Digoxigenin-labelled probes containing the 1.26 kb Km⁺ cassette (PstI digest of pGS459), the 1.68 kb, KpnI–SpbI fragment from pGS104 encoding part of the E1p chain linked to a 1lip—E2p chain (see Fig. 1), or the 2.28 kb KpnI–SpbI fragment from pGS101 encoding an analogous segment with a 3lip—E2 chain, were used. The corresponding restriction fragments were extracted from low-melting-point agarose gel after electrophoresis, and used in hybridization and detection procedures, as described by the manufacturers.

**Preparation of cell-free extracts and enzyme assays.** Cultures were grown to mid-exponential phase, harvested, washed and resuspended at 1 g per 4 ml cold potassium phosphate buffer (20 mM, pH 7.0) containing disodium EDTA (2 mM), PMSF (1 mM) and benzamidine hydrochloride (1 mM). The cell suspension was cooled to 4 °C and then disrupted by ultrasonic treatment (MSE disintegrator 150W) of 15×30 s pulses interspersed with 30 s cooling periods. intact bacteria and cell debris were removed by centrifugation (25000 g, 30 min, 4 °C). The clear supernatant fluid was used as the crude cell-free extract.

The specific activities of the PDH complex and individual components were measured by standard procedures (Russell & Guest, 1990). The observed rates were proportional to the amounts of added cell-free extract and linear for at least 3 min. One enzyme unit (U) is defined as the amount of enzyme that catalyses the formation of 1 μmol product or the reduction of 1 μmol coenzyme per h at 37 °C; specific enzyme activities are defined as U (mg protein)⁻¹.

Three lipoyl domains are better than two or one
Analytical methods. Glucose in feed medium and culture filtrates was measured enzymically using glucose oxidase (Bergmeyer & Bernt, 1974). Low-molecular-mass overmetabolites in culture filtrates were measured by HPLC with a Shodex Ion pakKC-811 column (8 x 300 mm) at 50 °C. Samples were eluted with 0·1 % phosphoric acid at a flow rate of 2 ml min⁻¹ through detectors for UV (210 nm) and refractive index (Waters 490E and 410, respectively) coupled in tandem to a data module (Millennium 2010); the peak areas were proportional to concentration. Protein concentrations in whole cells and in cell-free extracts were measured by the Lowry method with BSA as the standard.

Metabolic rates and carbon recovery. Glucose consumption, O₂ consumption and CO₂ production rates (qₜₐₜ, qₜ₈, and qₜ₁₀, respectively), carbon conversion efficiencies and carbon balances were calculated as described by Brooke et al. (1989). The specific growth rates of the out-competed population in the competition experiments were calculated using a modified washout growth rate equation (Jannasch, 1969).

Materials. Restriction enzymes were from Northumbria Biochemicals, the KmR GenBlock was from Pharmacia, and the Non-radioactive Digoxygenin Labelling Kit was from Boehringer Mannheim.

RESULTS
Construction of isogenic strains with modified aceF genes encoding E2p subunits with one, two or three lipoyl domains

An isogenic set of strains containing restructured aceF genes was obtained by a combination of site-specific mutation (in vitro) and allelic replacement (in vivo) using a temperature-sensitive replicon (pMAK705; Hamilton et al., 1989). In a three-phase strategy, the lip region of an aceF gene encoding a 1lip–E2p subunit was first disrupted by inserting a kanR cassette in vitro. The corresponding chromosomal aceF segment (3lip) was then replaced by the disrupted aceF::kanR segment in vivo, and finally the chromosomal aceF::kanR region was replaced by segments of DNA encoding E2p subunits with one, two or three lipoyl domains. The plasmid (pGS104) encoding part of a 1lip–E2p subunit was first cleaved at a unique BclI site in the lip region and the kanR gene (1.27 kb BglII–SphI fragment from KmR GenBlock) was inserted with known orientation, to generate pGS459 (Fig. 1). The aceF::kanR region was then transferred to pMAK705 by inserting the KmR::Spbl fragment into the corresponding sites of the polyclinker to generate pGS522 (Fig. 1).

In the second stage, plasmid co-integrants arising by the homologous recombination between plasmid and chromosome were obtained by transforming E. coli W3110 with pGS522 and selecting CmR and KmR colonies at 42 °C (Hamilton et al., 1989). The co-integrants represented 1·4 % of comparable transformants selected at 30 °C and after purification, five to ten independent co-integrants were inoculated into L broth (100 ml) containing both antibiotics and grown at 30 °C to allow resolution by a second recombination event. KmR colonies were then selected at 42 °C and screened first for CmS at 30 °C to ensure that the pMAK705 replicon had been eliminated, and then nutritionally for Ace⁻ resolution products. After enzymological tests and Southern blotting with ace and kan probes to ensure that the aceF::kanR cassette had been incorporated at the desired chromosomal site (see below), a representative mutant (JRG2930) and a spontaneous nalidixic acid resistant derivative thereof (JRG2930N, Fig. 1), were retained. The NalR marker served in later studies to distinguish between engineered and wild-type strains.

In the final stage, the chromosomal aceF::kanR region was replaced by analogous aceF segments from plasmids encoding one (pGS741), two (pGS742) or three (pGS743) lipoyl domains (Fig. 1). The same replacement strategy involving the thermosensitive CmR replicon was adopted, except that batches of transformant colonies (≥30) were grown for two successive periods of 16 h at 42 °C in L broth containing both antibiotics, before inoculating into L broth plus chloramphenicol (0·5 ml inoculum in 50 ml culture) and growing at 30 °C to allow co-integrate resolution. Nalidixic acid resistant colonies, selected on L agar at 42 °C to ensure elimination of the thermosensitive replicon, were screened first for CmS KmS products at 30 °C, and then for CmS KmS Ace⁺ derivatives. The desired Ace⁺ strains, potentially having one, two and three lipoyl domains per E2p chain, were recovered at rather low frequencies (1/195, 3/12119 and 3/13500, respectively). These low frequencies probably reflect the relatively short regions of homology between the incoming and resident segments of DNA (1·54 and 0·44 kb). The predicted replacements were confirmed for all of the Ace⁺ derivatives by Southern blotting using kan and ace probes with diagnostic digests of chromosomal DNA (see Methods). Positive hybridization of the ace probe to chromosomal PMII fragments of 2·05, 0·7, 1·07 or 1·38 kb (L₁, L₂ or L₃ in Fig. 1) was diagnostic either for the retention of the aceF::kanR region of JRG2930N, or for the presence of an aceF gene with one, two or three lip segments (respectively). Likewise with the kan probe, hybridization was observed with two HindIII fragments (2·05 and 0·6 kb; K₁ and K₂ in Fig. 1) using DNA from JRG2930N, whereas the lack of hybridization with the other strains confirmed that the kanR cassette had been eliminated. Ultimately, this yielded the representative set of isogenic JRG2930N derivatives having chromosomal pdbR–aceEF–lpd operons encoding 1lip⁻ (JRG2931), 2lip⁻ (JRG2932) and 3lip⁻ (JRG2933) PDH complexes, as listed in Fig. 1.

Characterization of strains

The physiological consequences of lowering the lipoyl domain content of the PDH complex was investigated by nutritional and enzymological tests. Plate growth tests at temperatures ranging from 30 to 44 °C, with the wild-type (W3110), the insertion mutant (JRG2930N) and the isogenic set of 1–3lip derivatives (JRG2931–3), were monitored over a period of 72 h (Table 2). As expected, the aceF::kanR insertion mutant was unable to grow in medium containing glucose or succinate as sole carbon source unless supplemented with acetate, and the ability to grow on unsupplemented medium was restored when
Three lipoyl domains are better than two or one

PdhR E3

0 2 4 kb

j

f

B/Bg K Bc S BPs PsB

pGS104

B/Bg K Bc/B B/BcS K S

pGS459

pGS522

pGS741

pGS742

pGS743

AceF::kan IPd

JRG2930N

JRG2931

JRG2932

JRG2933

Fig. 1. Organization of the pdhR-aceEF-lpd operon of E. coli and strategy for constructing isogenic derivatives with one, two and three lipoyl domains per lipoate acetyltransferase chain (E2p) in the corresponding PDH complexes. The genes, gene-products and promoters in the wild-type operon, where the aceF gene has 3lip segments, are shown at the top. The lip segments of the aceF genes are marked (L). Derivatives of the thermosensitive replicon, MAK705 (rep, cml), containing modified ace'EF' fragments used for inactivating and reactivating the chromosomal aceF gene are shown on the left. Segments of the restructured operons in the corresponding bacterial strains are shown on the right. The DNA fragments used as kan and ace probes in Southern blotting, and the diagnostic fragments to which they hybridize, are indicated (KA, K, and L,-3). Relevant restriction sites: B, BamHI; Bc, Bcll; Bg, Bglll; H, Hindilll; K, Kpnl; P, Pvuill; Ps, Pstl, S, Sphl.

the disrupted aceF gene was replaced by those encoding E2p chains with one, two or three lipoyl domains. Temperature-sensitive growth was observed with JRG2930N and its derivatives, in some media at or above 42 °C (Table 2). The reason for this is unknown, especially as it was not detected with W3110 (except in succinate minimal medium), and it was not specifically correlated with the lipoyl domain complement. Nevertheless, at the temperature used in this study (37 °C) all of the strains except for JRG2930N exhibited the same growth patterns. It can also be concluded that the nutritional complementation of the Ace'Lpd' phenotypes of an aroP-lpd deletion strain by plasmids expressing 1lip- and 2lip-PDH complexes, observed in previous growth tests (Guest et al., 1985), is not dependent on the presence of multiple rather than single copies of the restructured operons. Enzymological tests with crude cell-free extracts of mid-exponential phase cultures grown at 37 °C, confirmed that all of the Ace' strains contain active PDH complexes and component enzymes (E1p, E2p and E3), whereas the aceF::kan insertion mutant (JRG2930N) lacks both lipoate acetyltransferase (E2p) and PDH complex activities (Table 2). The specific activities were measured in the presence of excess substrates under optimal conditions, and although directly comparable with each other and other related studies, the absolute values do not necessarily reflect the corresponding in vivo activities.
Table 2. Nutritional and enzymological characterization of isogenic strains with different lipoyl domain contents in their PDH complexes

Growth of cultures on solid media was observed at a range of temperatures (30-44 °C) over 72 h. The relative extents of growth in different media was recorded as (−) no growth, (+) some growth, and (++) good growth; no growth was observed at or above the temperature shown in parentheses. The enzyme specific activities of crude cell-free extracts are each based on measurements taken at three rate-determining protein concentrations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source (and supplement)</th>
<th>Specific activity [U (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>PDH complex</td>
</tr>
<tr>
<td></td>
<td>Glucose (acetate)</td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>+ +</td>
<td>4:2</td>
</tr>
<tr>
<td>JRG2930N</td>
<td>−</td>
<td>&lt; 0:001</td>
</tr>
<tr>
<td>JRG2931 (1lip)</td>
<td>+ + (44 °C)</td>
<td>9:0</td>
</tr>
<tr>
<td>JRG2932 (2lip)</td>
<td>+ + (42 °C)</td>
<td>4:3</td>
</tr>
<tr>
<td>JRG2933 (3lip)</td>
<td>+ + (44 °C)</td>
<td>3:0</td>
</tr>
</tbody>
</table>

Table 3. Maximum growth rates (μmax) of E. coli W3110 and isogenic mutants

The μmax values were calculated from at least three batch cultures and the SD values were no more than ±0.03.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>μmax (h⁻¹)</th>
<th>W3110</th>
<th>JRG2931</th>
<th>JRG2932</th>
<th>JRG2933</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (20 mM)</td>
<td>0.67</td>
<td>0.31</td>
<td>0.45</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>dL-Lactate (10 mM)</td>
<td>0.43</td>
<td>0.36</td>
<td>0.41</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Succinate (40 mM)</td>
<td>0.23</td>
<td>0.14</td>
<td>0.13</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Pyruvate (40 mM)</td>
<td>0.33</td>
<td>0.30</td>
<td>0.30</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Acetate (10 mM)</td>
<td>0.13</td>
<td>0.13</td>
<td>0.12</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Casamino acids (0.3 %)</td>
<td>0.49</td>
<td>0.43</td>
<td>0.42</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Yeast extract (0.3 %)</td>
<td>0.85</td>
<td>0.75</td>
<td>0.78</td>
<td>0.82</td>
<td></td>
</tr>
</tbody>
</table>

Growth studies with batch cultures

To investigate whether or not the lipoyl content of PDH complex affects the carbon flux into biomass and energy generation, the μmax values of the different strains were measured during growth in medium containing different carbon sources. It was predicted that the growth rates of different strains in media containing carbon sources which can be metabolized without the involvement of the PDH complex (e.g. acetate) should be similar. On the other hand, where the PDH complex performs a key role in the metabolism of a carbon source (e.g. glucose, succinate and pyruvate) the μmax values of the different strains might be affected. Growth was monitored for 10 h on medium containing the carbon source of interest. As expected, the μmax values of the wild-type (W3110) and reconstructed wild-type (JRG2933) were similar in all of the test media, but those of the isogenic 1lip and 2lip mutants (JRG2931 and JRG2932) growing in glucose, succinate and possibly lactate, exhibited lower μmax values (Table 3). This was particularly evident with the glucose-grown cultures, where the μmax values decreased sequentially with decreasing numbers of lipoyl domains per E2p chain. The number of lipoyl domains had little effect with acetate as the sole carbon source (Table 3). The μmax values were similar for all strains during growth on pyruvate, which is somewhat surprising in view of the central role of the PDH complex in pyruvate metabolism (Table 3). Possible reasons based on the inducing effects of exogenous pyruvate are considered in the Discussion.

Complementation studies

The possibility that the defective chromosomally encoded PDH complexes might be complemented by plasmid-encoded 1lip- and 3lip-PDH complexes supplied in trans, was investigated by transforming JRG2931 (1lip) and JRG2933 (3lip) hosts with the multicopy plasmids pGS110 (1lip), pGS87 (3lip) and pBR322 (vector). The transformants were grown as batch cultures with 20 mM glucose as sole carbon source and their growth rates are...
Three lipoyl domains are better than two or one

Table 4. Effects of plasmids encoding different PDH complexes on the maximum growth rates of isogenic E. coli strains

<table>
<thead>
<tr>
<th>Host strain</th>
<th>μmax for plasmid-containing derivatives (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110 (3lip)</td>
<td>No plasmid: 0.67, pBR322 (vector): 0.55, pGS87 (3lip): 0.65, pGS110 (1lip): 0.36</td>
</tr>
<tr>
<td>JRG2933 (3lip)</td>
<td>0.64, 0.60, 0.55, 0.45</td>
</tr>
<tr>
<td>JRG2931 (1lip)</td>
<td>0.30, 0.29, 0.65, 0.36</td>
</tr>
</tbody>
</table>

compared in Table 4. Most strikingly, the μmax of JRG2931 (1lip) was restored to the wild-type level by pGS587 (3lip) but only marginally increased by pGS110 (1lip). Clearly, the metabolic debility of JRG2931 (1lip) is fully complemented by the presence of 3lip-E2p subunits, but only partially complemented by the presence of extra 1lip-E2p subunits. In contrast, the growth of JRG2933 (3lip) was impaired by pGS587 (3lip) and more so by pGS110 (1lip), relative to pBR322 (Table 4). When interpreting these observations it has to be remembered that the chromosomally encoded E2p subunits are expressed from the pyruvate-sensitive pdh promoter, whereas those encoded by the multicopy plasmids are expressed from the weak and apparently unregulated ace promoter (Fig. 1). Both sources presumably contribute, in different proportions depending on the strain, to the pool of E2p subunits from which 1lip-, 3lip- and a wide variety of hybrid-PDH complexes are assembled. Nevertheless, it appears that the unregulated contribution of extra 3lip-PDH complex subunits by pGS87 is somewhat debilitating for JRG2933 (3lip), whereas it enhances the growth of JRG2931 (1lip). The latter effect is presumably due both to the provision of wild-type subunits and to the ensuing limitation in the formation of chromosomally encoded 1lip-E2p subunits, caused by an intensification in repression of the pyruvate-sensitive pdh promoter that will occur as pyruvate is processed more efficiently by the plasmid-encoded wild-type complex. It would be interesting to determine the total amounts of PDH complex and the relative contributions of chromosome- and plasmid-encoded E2p subunits in different transformant strains.

Growth studies using continuous culture

To investigate in more detail the physiological consequences imposed by selectively deleting lipoyl domains from the E2p chain, all strains were grown aerobically under carbon (glucose) limitation with and without a supplement of casamino acids (0.01%). The cellular protein contents were essentially constant at all steady states and the substrate carbon was quantitatively recovered in biomass and CO₂, i.e. 100% carbon balance was obtained. This was confirmed by the absence of metabolites in culture filtrates. All of the strains except JRG2931 (1lip) grew in unsupplemented and supplemented media, maintaining steady-states over a wide range of D up to 0.32 h⁻¹ (Fig. 2). The same applied to JRG2931 (1lip) in supplemented medium, but without the supplement steady-states could only be maintained over a narrower range of D (up to 0.09 h⁻¹), wash-out occurring at 0.10 h⁻¹.

The carbon efficiencies (percentage carbon input into biomass) were relatively similar (56.2–60.4%) for the wild-type and mutant strains under all conditions except for JRG2931 (1lip) growing in unsupplemented medium, where a significant decrease (48.4%) was observed (Table 5). Since the carbon balances at each of the steady-states maintained by JRG2931 (1lip) showed that substrate carbon is quantitatively recovered in the biomass and CO₂ produced, the lower carbon efficiency indicates that there is a significant increase in the proportion of substrate carbon being used for energy generation. The glucose consumption rates (gGlc) of all of the strains under all conditions, increased linearly with D (Fig. 2). However, in the absence of the supplement, the maintenance energies (mₑ) and the maximum growth yields (Ymax) decreased with decreasing numbers of lipoyl domains per E2p chain (JRG2931 1lip and JRG2932 2lip), but both parameters could be restored to those of the wild-type (W3110) and the reconstructed wild-type (JRG2933), by adding the casamino acids supplement (Fig. 2; Table 5). Similar results were obtained with JRG2931 (1lip) in glucose-limited medium supplemented with low concentrations of acetate (up to 0.1 mM; data not shown); steady-states could not be maintained at higher acetate concentrations.

Competition experiments

To confirm that bacteria with E2p subunits containing only one or two lipoyl domains per E2p chain are at a physiological disadvantage, equal numbers of wild-type E. coli W3110 (3lip Nal) and either JRG2931 (1lip Nal),
Fig. 2. Glucose consumption rates of *E. coli* W3110 and isogenic mutants. Steady-states were obtained over a range of *D* values in the standard glucose-limited medium in the presence (■) and the absence (□) of casamino acid (0·01%); pH 6·9; dissolved O₂ ≥ 50% air saturation; 37 °C. (a) wild-type W3110 (3lip); (b) reconstructed wild-type JRG2933 (3lip); (c) isogenic mutant JRG2932 (2lip); (d) isogenic mutant JRG2931 (1lip).

JRG2932 (2lip *Nal*<sup>R</sup>) or JRG2933 (3lip *Nal*<sup>R</sup>) were inoculated into glucose-limiting medium in the same chemostat vessel. Growth under standard conditions of pH, temperature and dissolved O₂ was maintained for several hours to ensure that both bacterial strains had entered the exponential phase. The feed pump was then turned on and maintained at *D* 0·075 h<sup>−1</sup>. The pairwise cultures were sampled immediately (zero generations) and at five-generation intervals thereafter, up to 50 generations. The fraction of the mutants (*Nal*<sup>R</sup>) to the total number of bacteria (*Nal*<sup>R</sup> + *Nal*<sup>+</sup>) was plotted versus the number of generations to assess the retention of the
Three lipoyl domains are better than two or one

Table 5. Physiological parameters of glucose-limited chemostat cultures

Chemostat cultures were grown in minimal medium with limiting glucose (20 mM), at pH 6.9 and 37 °C with dissolved O2 at ≥ 50% air saturation, in the presence and the absence of casamino acids (0.01%). The rate of wash-out of non-competitive mutants (NalR) with increasing generations is indicated by the decline in the fraction of NalR bacteria relative to the total (NalR+NalS). The fraction expressed as a percentage is plotted logarithmically versus the number of generations. Starting populations contained equal numbers of wild-type, W3110 (3lip) and either: [square], the reconstructed wild-type JRG2933 (3lip); [triangle], JRG2932 (2lip); or [circle], JRG2931 (1lip).

Fig. 3. Pairwise competition between wild-type and isogenic mutants in glucose-limited chemostat cultures (D 0.075 h−1). The rate of wash-out of non-competitive mutants (NalR) with increasing generations is indicated by the decline in the fraction of NalR bacteria relative to the total (NalR+NalS). The fraction expressed as a percentage is plotted logarithmically versus the number of generations. Starting populations contained equal numbers of wild-type, W3110 (3lip) and either: [square], the reconstructed wild-type JRG2933 (3lip); [triangle], JRG2932 (2lip); or [circle], JRG2931 (1lip).
glucose and lactate, whose catabolism requires an active PDH complex, were reduced significantly when the lipoyl domain content was lowered. In contrast, similar values for $m_{\text{max}}$ were observed with all strains during growth on acetate, the only substrate tested whose catabolism is not dependent on PDH complex activity.

The observation that all strains exhibit similar growth rates on pyruvate, was unexpected in view of the central role of the PDH complex in pyruvate metabolism, but it is explicable in terms of the established role of pyruvate as inducer of the $\text{pdhR-aceEF-lpd}$ operon. It has been observed previously that expression from the $\text{pdh}$ promoter is 3- to 4-fold higher during growth on pyruvate (40 mM) compared to glucose (Quail et al., 1994). In vitro studies have further shown that transcription of the operon is repressed by the PdhR protein and relieved by pyruvate in a concentration-dependent manner over a $>1000$-fold range (Quail & Guest, 1995). It is therefore quite conceivable that in the mutant strains, any limitation in C-flux to biomass or energy generation may be compensated by inducing higher levels of PDH complex expression when high concentrations of pyruvate are supplied exogenously, without affecting growth rate. During growth on glucose and succinate the intracellular pyruvate concentrations are presumably insufficient to induce the compensating level of induction. Lactate probably represents an intermediate case, and higher lactate concentrations may have given results similar to those observed with pyruvate. Alternatively, or additionally, pyruvate might induce another pathway for the conversion of pyruvate to acetyl-CoA. These possibilities are being investigated using $\text{pdhR-lacZ}$ and $\text{pdhR-aceE-lacZ}$ reporter fusions in the mutant strains, as well as direct tests with pyruvate-limited continuous cultures.

The different effects of subunits from the plasmid-encoded 1lip- and 3lip–PDH complexes on the growth rates of mutant and wild-type strains (Table 3), were also explicable in terms of an impairment conferred by the 1lip-E2p subunits and their expression from either pyruvate-regulated or unregulated promoters.

A more detailed study of the C-flux in different strains growing under controlled carbon (glucose)-limited conditions over a range of growth rates in the chemostat, supported and extended the results obtained with batch cultures. Steady-states were maintained over a wide range of $D$ (up to 0.32 h$^{-1}$) except for JRG2931 (1lip). Despite the $m_{\text{max}}$ of the latter strain being 0.31 h$^{-1}$ in batch culture, steady-states could only be maintained at $D \leq 0.09$ h$^{-1}$ in a comparable medium. There is no obvious explanation for this apparent discrepancy. Studies now in progress with single-copy reporter fusions in the mutant strains should provide further insights into the degree of operon expression under different conditions, and a clearer understanding of the C-flux through the modified PDH complexes and its effect on overall C-flux. Despite the anomaly, substrate carbon was quantitatively recovered in an anomaly, substrate carbon was quantitatively recovered in biomass and CO$_2$, the total cellular protein contents were similar, and there was no evidence for C-flux into overflow metabolites or storage compounds, at all of the steady-states examined. However, the fraction of substrate carbon incorporated into biomass was lower in bacteria containing one lipoyl domain per E2p chain, JRG2931 (1lip), but not significantly lower in JRG2932 (2lip). This suggests that the C-flux distribution between biomass formation and energy generation in JRG2931 (1lip) is readjusted in order to maximise the growth efficiency under carbon-limiting conditions. A corresponding increase in the rate of CO$_2$ formation ($\psi_{\text{CO}_2}$) further supported this conclusion (data not shown). The glucose consumption rates ($\psi_{\text{GS}}$) were linear with increasing dilution (growth) rate for all strains (Fig. 2). However, the values of $m_{\text{max}}$ and $Y_{\text{max}}$ fell as the lipoyl domain contents of the E2p chains were reduced, and the lower values could be restored to wild-type levels by the addition of casamino acids or acetate supplements (Table 5). Thus it is concluded that lowering the lipoyl domain content of the PDH complex has an adverse effect on growth under some conditions. This was confirmed in competition experiments in the chemostat where it was found that the wild-type (3lip) out-competes the isogenic mutants JRG2932 (2lip) and JRG2931 (1lip) in mixed populations. The wash-out rate was higher for the 1lip derivative than the 2lip derivative, further confirming that the progressive removal of lipoyl domains (from three to two to one per E2p chain) is accompanied by a corresponding stepwise decline in the physiological proficiency of the host.

These results do not explain why the PDH complex of E. coli has three lipoyl domains per E2p chain, but they clearly demonstrate that all three lipoyl domains are required for efficient balanced growth on carbon substrates requiring the PDH complex for their catabolism. Because no gross effects on the specific activities of the isolated PDH complexes were observed previously, it is concluded that the debility imposed by lowering the lipoyl domain content is quite subtle, and may only be detected by sensitive kinetic analyses using substrate-limiting conditions with carefully standardized complexes. Further studies on the physiological and metabolic effects of genetic modification of the structure and expression of the PDH complex are in progress.

ACKNOWLEDGEMENTS

The work was supported by a project grant from the Biotechnology Directorate of the Science and Engineering Research Council and a research studentship from the SERC. We are very grateful to Dr G. C. Russell for help and advice in constructing the $\text{aceF::kan}^R$ insertion mutant (JRG2930).

REFERENCES


Three lipoyl domains are better than two or one tolerant methylohotrophic Bacillus strains. *Arch Microbiol* 151, 268–273.


Received 28 March 1995; accepted 19 April 1995.