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A mutation causing constitutive synthesis of the pyruvate dehydrogenase complex in *Escherichia coli* is located within the *pdhR* gene

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The *aceE-aceF-lpd* genes encoding the pyruvate dehydrogenase (PDH) complex of *Escherichia coli* are preceded by a gene encoding a putative transcriptional regulator, PdhR (formerly designated GenA). Enzymological tests and studies with *pdhR-lacZ* and *aceE-lacZ* translational fusions have shown that a constitutive mutation (*ace^{c}816*), which increases PDH complex synthesis to the pyruvate-induced level in the absence of inducer, is recessive to the wild-type *pdhR* gene in *trans*. Sequence comparisons further showed that the *ace^{c}816* mutation affects a single site in the *pdhR* gene leading to an Arg^{118} (CGU) → Cys (UGU) substitution in the PdhR protein. The results support the view that synthesis of the PDH complex is regulated from the *pdhR* promoter of a *pdhR-aceEF-lpd* operon.

Pyruvate dehydrogenase complex; Transcriptional regulation; PdhR regulatory protein; Gene expression; *Escherichia coli*

1. INTRODUCTION

The pyruvate dehydrogenase (PDH) complex of *Escherichia coli* catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA + CO_2 with concomitant reduction of NAD^+. Synthesis of the complex is induced by pyruvate or thiamine starvation, repressed during growth on acetate and partially repressed during fermentative growth (where activity is inhibited), but not repressed during anaerobic nitrate respiration [1–3]. The complex contains multiple copies of three enzymatic subunits (E1p, E2p and E3) encoded by three adjacent genes, *aceE-aceF-lpd* (Fig. 1), which have been cloned, sequenced, and subjected to transcript analysis and other regulatory studies [4,5]. Regulatory mutants (*ace^c*) responsible for constitutive synthesis of the PDH complex have been selected by their ability to render phosphopyruvate synthetase mutants (*pps*) resistant to lactate during growth on acetate minimal medium [6]. This phenotype arises because the inhibitory effects of added lactate (and the pyruvate derived therefrom) can be relieved by accelerating its removal by increasing synthesis of the PDH complex. In previous studies, one *ace^c* mutation was tentatively located within the *aceE* gene [7], whereas in an independent deletion analysis, the *ace^{c}816* mutation (studied here) was located upstream of the *aceE* structural gene [8]. The *pdhR* gene (formerly *genA*) located immediately upstream of *aceE* [9], was recently identified as a member of the *gntR* family of transcriptional regulatory genes [10,11]. This, together with in vivo and in vitro expression studies on the *aceEF* and *lpd* genes, which show that *pdhR* is the proximal gene of the *pdhR-aceEF-lpd* operon [5,12], suggested that the *ace^{c}816* mutation might lie within the *pdhR* gene. The present work reports that a single base change in the *trans*-recessive *pdhR* gene is responsible for constitutive synthesis of the PDH complex.

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Abbreviations: PDH, pyruvate dehydrogenase; E1p, pyruvate dehydrogenase (decarboxylating); E2p, lipoate acetyltransferase; E3, lipoamide dehydrogenase.

2. EXPERIMENTAL

2.1. *Bacteria, plasmids and phages*

The bacterial strains are listed in Table I. The *ace^{c}816* mutation had previously been incorporated into JRG747 [1] by P1 transduction from its original source, K1–1LR8–18 [6]. Here it was incorporated into the MC1000 background by a two step conjugation procedure, producing JRG2547 (*Δlac ace^c*). The *guaC-nadC-aceF* deletion of JRG590 was first transferred to MC1000 to generate a Leu^+ Nad^- Ace^- Sm^R intermediate strain (MCΔ10), which was then mated with JRG747 to mediate high frequency transfer of the *ace^{c}816* mutation to $Nad^+Ace^+Sm^R$ exconjugants.

The *pdhR* gene (formerly *genA*) was cloned by ligating the 1.25 kb *Aha*III–*Bam*HI fragment of pGS14 [14] into *Sma*I–*Bam*HI digested pUC119 to generate the Ap^R derivative, pGS346 (Fig. 1). A plasmid (pGS623) containing the entire *pdhR-aceEF-lpd* region was constructed by first cloning the 1.25 kb *Eco*RI–*Bam*HI *pdhR* fragment of pGS346 into the corresponding sites of pBR322 (producing pGS622) and the *aceEF* and *lpd* genes were then inserted between the *Bam*HI and *Sal*I sites using the corresponding fragment from pGS400 [15] (Fig. 1). The *aceF* gene from pGS400 encodes an E2p subunit with one lipoyl domain rather than three, but this has no detectable effect on PDH complex activity [16]. The *pdhR* gene was also subcloned between the *Eco*RI and *Pst*I sites of pBR322 as a 1.29 kb *Eco*RI–*Pst*I fragment from pGS346, to give the Tc^R derivative, pGS613. An 'in-phase' *pdhR-lacZ* translational fusion was constructed in pGS612 by

ligating the 0.38 kb *Eco*RI–*Sau*3A fragment of pGS346 into *Eco*RI–*Bam*HI digested pNM481 [17], and an analogous *aceE-lacZ* fusion was constructed in pGS342 by ligating the 2.1 kb *Hin*dIII fragment of pGS17 [14] into *Hin*dIII-digested pNM481 in the desired orientation (Fig. 1). Plasmids pGS612 and pGS342 encode hybrid proteins comprising 22 or 502 N-terminal residues of PdhR or E1p (respectively), fused to a β-galactosidase lacking 8 N-terminal residues. The respective inserts extend 290 bp upstream of the *pdhR* or 682 bp upstream of the *aceE* coding region, and should contain the sequences necessary for transcriptional regulation. Each *lacZ* fusion was transferred to λRZ5 by in vivo recombination in MC1000 [18] and representative fusion phages, λG216 (*λaceE-lacZ*) and λG238 (*λpdhR-lacZ*), were established as prophages in the monolysogenic ($\lambda cI90c17^S$) derivatives of MC1000 and JRG2547, as listed in Table I.

2.2. *Microbiological, enzymological and other methods*

Cultures were grown routinely in L broth with appropriate antibiotics, whereas nutritional tests and genetic selections were performed in glucose minimal medium supplemented with acetate (2 mM) and nicotinic acid (10 μg/ml) as required [1,13]. The pyruvate dehydrogenase complex specific activities (mmol APAD reduced/mg protein/h) were assayed in ultrasonic extracts of organisms grown aerobically in L broth from a 2% inoculum of fresh overnight culture and harvested at A_{650nm} = 0.8 [7]. Protein was estimated by the Lowry method [19] and β-galactosidase activity was assayed according to Miller [20]. Standard methods were used for cloning, plasmid isolation and transformation [21].

2.3. *Cloning and sequencing of pdhR gene from ace⁺ and ace^c strains*

Chromosomal DNA was isolated from MC1000, K1–1LR8–16, and JRG2547 [22]. The *pdhR* genes were isolated from independent PCR reactions containing 300 ng DNA, 100 pmol of each primer (PCR-1 and PCR-2, see Fig. 4), and 2U *pfu* polymerase (Stratagene), over 30 cycles of 95°C for 30 s, 52°C for 1 min, and 72°C for 1.5 min. The PCR products were resolved by agarose gel electrophoresis and the single 1.2 kb bands of the expected size were excised and purified by a double Geneclean II procedure according to the manufacturers instruction's (BIO 101 Inc.). Each fragment was treated with DNA polymerase I (Klenow fragment), ligated into *Sma*I-digested and phosphatased pUC118, and single-stranded template DNA prepared for sequencing [21]. The cloned DNA was sequenced initially with a -40 'set-back' primer to ascertain its orientation, and then fully sequenced using specific oligonucleotide primers SEQ-1 to SEQ-5 (Fig. 4).

3. RESULTS AND DISCUSSION

3.1. *Effects of pdhR plasmids on PDH complex activity in the ace^c mutant*

The PDH complex activities in crude extracts of different strains grown in the presence or absence of 40 mM pyruvate are summarised in Fig. 2. The activity of the wild-type strain (MC1000) was induced by pyruvate, whereas the *ace^c* mutant (JRG2547) exhibited a high basal activity which was not significantly enhanced by growth in the presence of pyruvate. However, inducibility was restored to the *ace^c* strain when the *pdhR* gene was provided in *trans*, i.e. in transformants containing the *pdhR* (pGS622) or *pdhR-aceEF-lpd* (pGS623) plasmids. In contrast, the *aceEF-lpd* plasmid (pGS400) failed to lower the PDH complex activity of the *ace^c* mutant in the absence of pyruvate (Fig. 2). These results show that the constitutive mutation is complemented by, and recessive to, the wild-type *pdhR* gene supplied

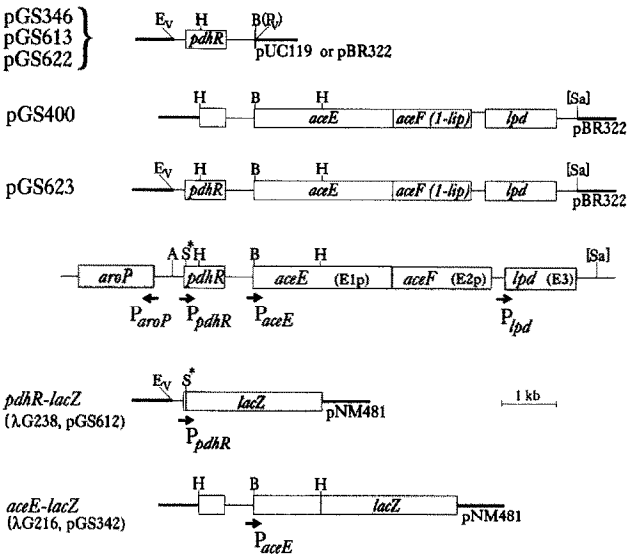

Fig. 1. Diagram showing the organization of the operon encoding the PDH complex of *E. coli* (*pdhR-aceEF-lpd*) and the segments cloned in relevant plasmids and phages. Coding regions are represented by open or shaded boxes connected to vector and bacterial DNA (thick and thin lines, respectively). The positions and transcription polarities of known promoters [5,12] are arrowed. Relevant restriction sites are abbreviated as follows: A, *Aha*III; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sau*3A; and Sa, *Sal*I. Vector sites (v subscript), a non-unique *Sau*3A site (*) and an engineered *Sal*I site (square brackets), are also indicated.

in *trans*, and they further indicate that the *ace^c816* mutation probably lies within the *pdhR* gene. The same plasmids had little effect on the inducibility or absolute PDH complex activities in the wild-type background. Indeed, a disappointing feature of the PDH complex is the very poor enzyme amplification obtained with multicopy *aceEF-lpd* plasmids [14], and this was not improved with the *pdhR-aceEF-lpd* plasmid (pGS623). Even in the *ace^c* host, the higher basal activity was not greatly enhanced by the pyruvate-inducible (*pdhR-aceEF-lpd*) or non-inducible (*aceEF-lpd*) plasmids, as if some other factor prevents expression at a level commensurate with copy-number.

Table I

Strains of *Escherichia coli* K12

Strain	Relevant genotype	Source
JRG590	HfrH *Δ(guaC-nadC-aroP-aceEF)10* (formerly HΔ10)	[13]
K1-1LR8-16	F⁻ *metB thy pps ace^c816*	[6]
JRG747	HfrH *ace^c816* (formerly H-LR8-16)	[1]
MC1000	*Δ(araABC-leu) Δ(lacIPOZYA)X74 rpsL*	S.T. Cole
MCΔ10	*Δ(lacIPOZYA)X74 Δ(nad-aroP-aceEF) rpsL*	This work
JRG2547	*ace^c816 Δ(lacIPOZYA)X74 rpsL*	This work
JRG2137	MC1000 (λG216)	This work
JRG2562	MC1000 (λG238)	This work
JRG2580	JRG2547 (λG216)	This work
JRG2581	JRG2547 (λG238)	This work

3.2. *Gene expression studies with pdhR-lacZ and aceE-lacZ translational fusions*

Studies with a single-copy *pdhR-lacZ* translational fusion confirmed that the *pdhR* promoter is induced by pyruvate in the wild-type background, MC1000 (Fig. 3). They also showed that *pdhR* expression increases to the pyruvate-induced level in the *ace*^{c} mutant (JRG2547) both in the presence and absence of pyruvate. However, this constitutive expression was abolished by introducing a multicopy *pdhR* plasmid (pGS613), whereupon the pyruvate-inducible pattern is restored, albeit at a lower level than in the wild-type (Fig. 3). Clearly, the mutation causing constitutive synthesis of the PDH complex has the same effect on the expression of the *pdhR-lacZ* fusion, and in both cases, pyruvate-inducibility is restored by the wild-type *pdhR* gene. This further indicates that the *ace*^{c}*816* mutation is located in the *pdhR* gene and is recessive to the wild-type *pdhR* gene in *trans*. It would also appear that the *pdhR* gene is autogenously regulated with pyruvate as the inducing coeffector.

In sharp contrast, the activity of the *aceE-lacZ* fusion was extremely low and unaffected by the presence of either pyruvate or the *ace*^{c}*816* mutation (2 Miller units approximately, under all conditions). This confirms earlier findings with two different *aceE-galK* fusions [5,23]. These results strongly indicate that the behaviour of the *aceE-lacZ* fusion does not reflect the normal pattern of

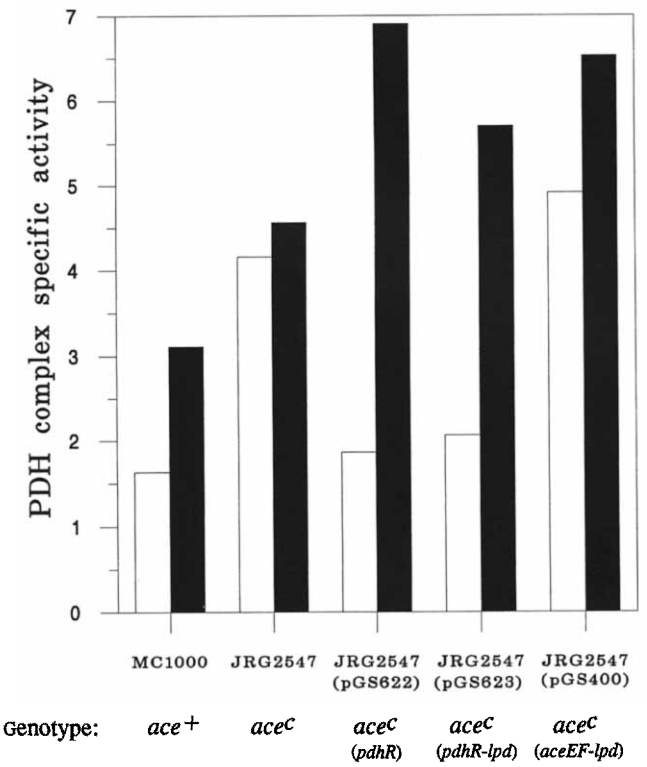

Fig. 2. Effects of multicopy *pdhR* and other plasmids on PDH complex synthesis in *ace*$^+$ (MC1000) and *ace*^{c} (JRG2547) strains. The specific activities (μmol/mg protein/h) are averages from at least three independent extracts prepared from aerobic cultures grown in L broth: □, without pyruvate; ■, with pyruvate (40 mM).

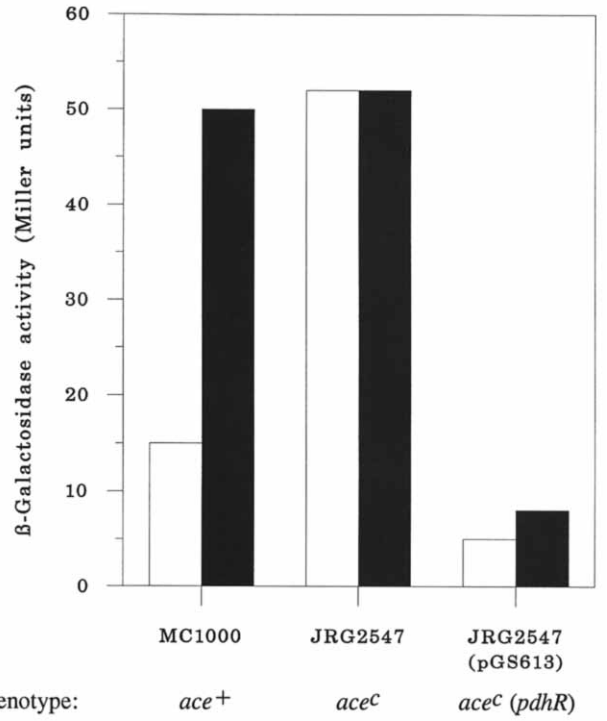

Fig. 3. Expression of a *pdhR-lacZ* translational fusion in *ace*$^+$, *ace*^{c}, and *ace*^{c} with multicopy *pdhR*, backgrounds. The β-galactosidase specific activities (Miller units) are averaged from at least three independent aerobic cultures grown to A_{650} = 0.8 in L broth: □, without pyruvate; ■, with pyruvate (40 mM).

PDH complex synthesis, viz. induction by pyruvate and derepression in the *ace*^{c} strain. The DNA fragment cloned in the *aceE-lacZ* fusion extends some 635 bp upstream of the transcription start point identified for the *aceE* promoter [5,12] and should contain all the corresponding regulatory elements (Fig. 1). However, it would now appear that the normal regulatory pattern for PDH complex synthesis is imposed at the *pdhR* promoter, which not only controls the *pdhR* gene but also the downstream *aceEF-lpd* genes possibly by a read-through mechanism. Such a mechanism has now been corroborated by in vivo and in vitro transcriptional studies involving the entire *pdhR-aceEF-lpd* operon and the purified PdhR protein [12], without invalidating previous transcript analyses which focused specifically on the *aceEF-lpd* region and identified the independent *aceE* and *lpd* promoters [4].

3.3. *The molecular basis of the ace*^{c}*816 mutation*

The nucleotide sequences of the *pdhR* genes amplified from two independent DNA samples from the original *ace*^{c}*816* mutant (K1–1LR8–16), the *ace*$^+$ strain (MC1000) and its *ace*^{c}*816* derivative (JRG2547), showed that the *ace*^{c}*816* mutants have suffered a single C→T substitution at position 537 relative to wild-type (Fig. 4). This generates an Arg (CGU) → Cys (UGU) substitution at residue 118 in the *pdhR* gene product.

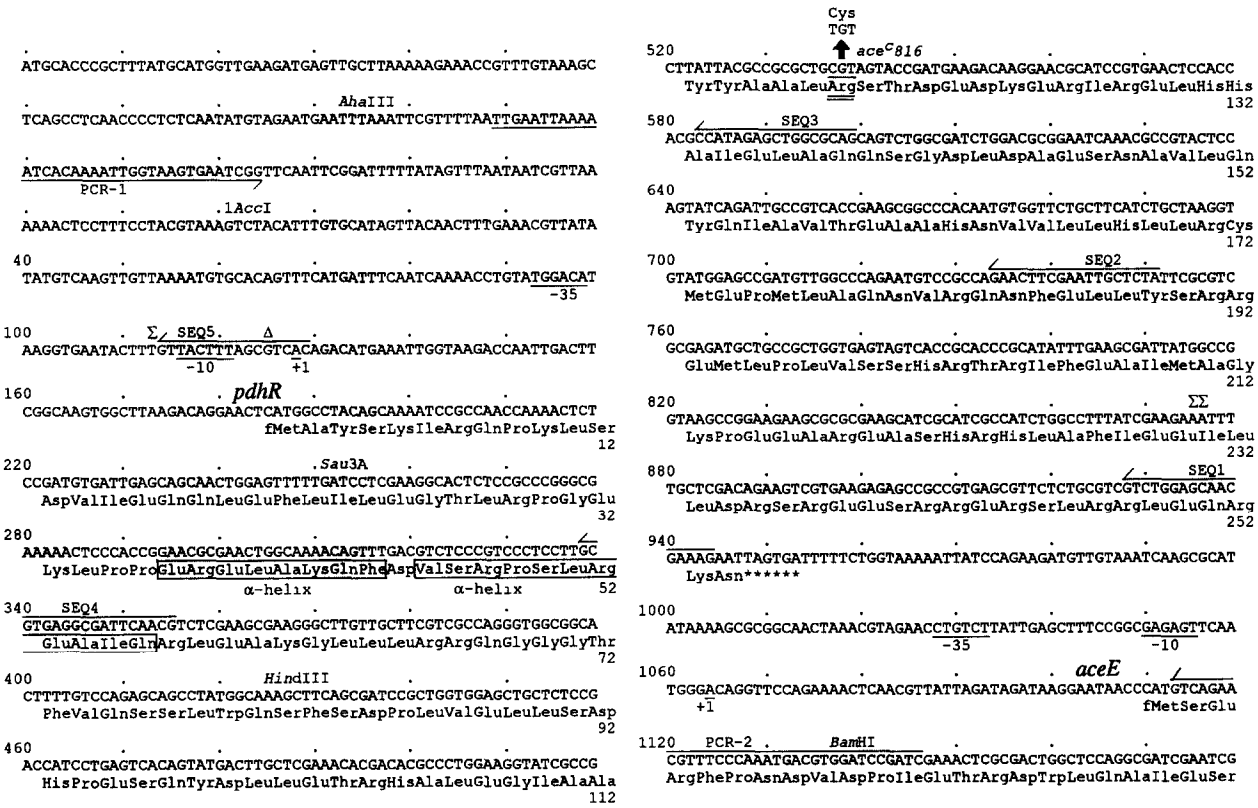

Fig. 4. Nucleotide sequence of the *pdhR* gene and flanking regions. The nucleotide sequence of the non-coding strand of the *pdhR* gene, revised in the *Aha*III–*Bam*HI region but still numbered from the *Acc*I site [9], is based on previous data [9,24]. The promoter sequences (−35 and −10) and corresponding start-sites (+1) identified for *pdhR* and *aceE* [4,12] are underlined. The positions of the PCR primers, specific sequencing primers and relevant restriction sites, are marked, as are the sites of nucleotide insertion (*Σ*) and deletion (*Δ*) relative to the original sequence [9] (see text). The putative helix–turn–helix motif of the DNA-binding domain of the PdhR protein is boxed [10,11], and the codon and amino acid substitutions associated with the *ace^c816* mutation are shown.

The substitution is located 62 residues downstream from the proposed helix–turn–helix motif of the putative DNA-binding region (residues 37–56) [10,11], so it may not affect DNA-binding specificity per se. However, it could inactivate DNA-binding in some other way, and this will be investigated by amplifying the mutant protein and comparing its DNA-binding activity with that of wild-type PdhR, in the presence and absence of pyruvate.

The sequence analysis revealed four deviations from the original sequence [9] in all of the mutant and wild-type isolates, and these were subsequently validated by resequencing the entire *pdhR* insert of pGS346 (Fig. 1). Two of the revisions, +T at 113 and −G at 126 (Fig. 4), confirmed the sequence reported by Chye and Pittard [24] for the overlapping *aroP* region. The other revisions (+2A at 874) have the effect of extending the *pdhR* reading frame by 18 codons up to the tandem stop codons at 948–953 (Fig. 4). As a result the M_r deduced for PdhR increases from 27,050 (236 residues) to 29,425 (254 residues), which is closer to the value of 30,000 estimated from the SDS-PAGE mobility of overproduced and purified pdhR protein [12].

It is concluded that the *pdhR* gene encodes a regulatory protein (PdhR), which represses synthesis of the PDH complex in response to pyruvate-limitation, by acting at the *pdhR* promoter of the *pdhR-aceEF-lpd* operon. By inactivating the PdhR protein, the *trans*-recessive *ace^c816* mutation, derepresses PDH complex synthesis.

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