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Biochemical Genetics of the α-Keto Acid Dehydrogenase Complexes of *Escherichia coli* K12: Isolation and Biochemical Properties of Deletion Mutants

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SUMMARY

Mutants of *Escherichia coli* K12 with deletions in the *nadC–lpd* region of the chromosome were obtained for use in studies on the expression of the *ace* (pyruvate dehydrogenase complex, specific components) and *lpd* (lipoamide dehydrogenase) genes. These were isolated by selecting spontaneous *aroP* mutants (lacking the general aromatic amino-acid permease and thus resistant to inhibitory aromatic amino-acid analogues) and screening for auxotrophy due to deletions extending into neighbouring genes. From 2892 isolates tested, the AroP$^-$ phenotypes of 2322 were confirmed and, of these, 28 stable and independently-derived auxotrophs were designated as deletion mutants.

Six nutritionally-distinct categories were recognized: Nad$^-$ (8 strains); Nad$^-$Ace$^-$ (7); Nad$^-$ 'Ace$^-$' (3); Ace$^-$ (8); 'Ace$^-$' (1); Lpd$^-$ (1). The Ace$^-$ phenotypes of four isolates designated 'Ace$^-$' were leaky and enzymological studies confirmed that they had less than 7 % of parental pyruvate dehydrogenase complex activity.

Enzymological studies showed that the 15 Ace$^-$ or Nad$^-$Ace$^-$ strains all lacked the pyruvate dehydrogenase complex and pyruvate dehydrogenase (E1p) activities and only three retained detectable dihydrolipoamide acetyltransferase (E2p). The one Lpd$^-$ strain lacked pyruvate dehydrogenase, dihydrolipoamide acetyltransferase and lipoamide dehydrogenase (E3) activities as well as the activities of the pyruvate and α-ketoglutarate dehydrogenase complexes.

The results confirmed the gene order *nadC–aroP–aceE–aceF–lpd* and indicated that no other essential functions are determined by genes within the *nadC–lpd* region. Resistance to lactate during growth of *pps* mutants on acetate was directly related to the specific activity of the pyruvate dehydrogenase complex. None of the deletions promoted the high degree of resistance characteristically associated with constitutive expression of the dehydrogenase complex. Six *pps* mutants having Ace$^+$ or 'Ace$^-$' phenotypes were more sensitive than the parental strains and expression of their *ace* operons appeared to be affected; most sensitive were the Ace$^-$ strains which lacked pyruvate dehydrogenase complex and phosphoenolpyruvate synthetase activities.

The lipoamide dehydrogenase activities of the deletion strains (Lpd$^+$) ranged between 30 % and 100 % of parental levels indicating that expression of the *lpd* gene may be affected by the *ace* operon but can be independent.

INTRODUCTION

The pyruvate dehydrogenase (E1p) and dihydrolipoamide acetyltransferase (E2p) components of the pyruvate dehydrogenase complex are specified by a pair of closely-

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linked genes, *aceE* and *aceF* respectively, at 2 min on the *Escherichia coli* K12 linkage map (Henning & Herz, 1964). Mutants lacking pyruvate dehydrogenase complex activity require acetate for aerobic growth on glucose or succinate, but they can grow on unsupplemented acetate medium. The corresponding dehydrogenase (E1kg) and succinyltransferase (E2kg) components of the α-ketoglutarate dehydrogenase complex are specified by a second pair of closely-linked genes, *sucA* and *sucB*, at 16 min (Herbert & Guest, 1969). Mutants lacking α-ketoglutarate dehydrogenase complex activity require succinate or a dual supplement of lysine plus methionine for aerobic growth on glucose, and they cannot grow on acetate minimal medium. The lipoamide dehydrogenase (E3) components of both complexes are specified by a single gene (*lpd*) which is located very close to the *aceF* gene at the distal end of the *ace* operon (Guest & Creaghan, 1972, 1973; Alwine, Russell & Murray, 1973; Guest, 1974). The *ace* and *lpd* genes are also very close to the *nadC* (quinolinate phosphoribosyltransferase) and *aroP* (general aromatic amino-acid permease) genes and the segment of chromosome containing the sequence *nadC–aroP–aceE–aceF–lpd* corresponds to approximately 0·2 min or 8 kilobases. Lipoamide dehydrogenase mutants lack the overall activities of both multienzyme complexes and consequently require nutritional supplements of both acetate and succinate for best aerobic growth on glucose. They cannot grow on acetate or succinate minimal media. All *ace*, *suc* and *lpd* mutants can grow anaerobically on unsupplemented glucose medium. Under these conditions the corresponding functions are not essential and other mechanisms for the synthesis of acetate and succinate are induced (or derepressed).

Studies with polar mutants have established that expression of both the *ace* and *suc* operons is polarized from the dehydrogenase to acyltransferase genes in a clockwise direction, $\overrightarrow{aceEF}$ and $\overrightarrow{sucAB}$ relative to the linkage map (Henning *et al.*, 1966, 1968, 1969; Creaghan & Guest, 1972). A regulatory element linked to the *ace* genes and controlling their expression has also been detected (Flatgaard, Hoehn & Henning, 1971). The existence of a single *lpd* gene raises the problem of how its expression is controlled relative to the syntheses of the two enzyme complexes, which appear to be independently regulated (Langley & Guest, unpublished observations). Lipoamide dehydrogenase synthesis is reduced by polar *ace* mutations and, to a lesser extent, by polar *suc* mutations, but *lpd* expression is never less than 30 % of normal in strains combining the most polar *aceE* and *sucA* mutations (Creaghan & Guest, 1972).

Studies with *Salmonella typhimurium* LT2 indicate that the genes specifying its α-keto acid dehydrogenase complexes are organized in the same way as in *E. coli* (Langley & Guest, 1974). Mutants of *S. typhimurium* analogous to the *aceE* and *sucA* mutants of *E. coli* have been examined. Two mutants were found which possessed deletions in the *ace–lpd* region extending to the *aroP* gene (strain SM16) and to the *nadC* plus *aroP* genes (strain SM51). Mutation or deletion of the *aroP* gene affects the transport of aromatic amino acids and thus generates resistance to analogues which would normally inhibit growth (Ames & Roth, 1968; Brown, 1970).

Strains of *E. coli* with deletions in or near the *ace* and *lpd* genes could be useful for studying factors controlling the expression of the *ace* operon and the *lpd* gene. The close proximities of the *aroP*, *ace* and *lpd* genes in *E. coli* (as in *S. typhimurium*) offered the possibility of isolating such deletion strains by screening spontaneous *aroP* mutants selected in the presence of appropriate supplements (acetate, succinate and nicotinate) for auxotrophy due to deletions extending into the neighbouring genes. This paper describes the isolation and biochemical properties of 28 auxotrophic *aroP* deletion strains representing six nutri-

Table 1. *Strains of Escherichia coli* K12

Strain	Characteristics	Source
H	Hfr; *thiA, azi*	W. Hayes
KI	*metB, thy, azi*	H. L. Kornberg
KI-1	*metB, thy, azi, ton, pps*	H. L. Kornberg
KI-ILR8-16	*metB, thy, azi, ton, pps, ace*^{c}*	U. Henning
A2T3	*aceE2, trpE61* (λ)	U. Henning
A6	*aceE6* (λ)?	U. Henning
A10	*aceF10* (λ)	U. Henning
W1485A1	*aceE1, supE42*	J. R. Guest
T3A58*lpd1*	*trpE61, trpA58, lpd-1*	J. R. Guest

* *ace*^{c} denotes the presence of a mutation which leads to constitutive expression of the *ace* genes.

tionally-distinct groups. A preliminary report of some of this work has been published previously (Langley & Guest, 1975).

METHODS

Bacterial strains. The characteristics and sources of the strains of *Escherichia coli* are listed in Table 1. Strain KI and its derivatives, KI-1 and KI-ILR8-16, were originally defined as Hfr and *thi* but these characteristics had been lost. Strain KI-ILR8-16 was one of the Ace^{c} strains which synthesize the pyruvate dehydrogenase complex constitutively (Flatgaard *et al.*, 1971). The parent for strains A2T3, A6 and A10 is probably Ymel (Henning, Herz & Szolyvay, 1964). Deletion mutants were isolated in three parental strains, H, KI-1 and KI-ILR8-16, and designated by the prefixes HΔ, KΔ and CΔ (C denoting the constitutive parent), respectively. Each deletion was given a different number and when deletions isolated in strain H were transferred to the KI-1 background by conjugation, these strains were designated by the prefix KHΔ but retained the original number.

Media. Minimal medium E of Vogel & Bonner (1956) was used in all experiments. Carbon sources were D-glucose (10 mM), potassium acetate (50 mM) or potassium succinate (50 mM). Supplements were added as required: acetate (2 mM with glucose as substrate, or 4 mM with succinate), succinate (2 mM), L-lysine (40 μg ml^{-1}), L-methionine (20 μg ml^{-1}), other amino acids (30 μg ml^{-1}), vitamins (10 μg ml^{-1}) and streptomycin sulphate (200 μg ml^{-1}). The complete medium used for routine growth and maintenance was L broth (Lennox, 1955). Media were solidified with Bacto-agar (15 g l^{-1}) when required. With Thy^{-} strains, thymine (50 μg ml^{-1}) was added to complete and defined media to ensure optimal growth.

Isolation of spontaneous aroP mutants. Independent stationary-phase cultures of the parental strains were prepared by inoculating single colonies into 5 ml L broth and shaking for 16 h at 37 °C. The bacteria were washed twice and concentrated 10-fold (to about 5×10^{10} organisms ml^{-1}) in sterile saline (9 g l^{-1}). Samples (0·05 and 0·1 ml) were plated on a selective glucose minimal medium (Gnaslm-aro) containing supplements of nicotinate, acetate, succinate, lysine, methionine and the analogues DL-5-methyltryptophan (20 μg ml^{-1}) and β-2-thienyl-DL-alanine (20 μg ml^{-1}), plus other specific supplements required by the parental strains. After 48 h at 37 °C, 50 to 200 resistant colonies appeared on each plate. Some 50 to 150 colonies from each starting culture were picked, diluted in saline and grown as small patches on the same medium (25 to 50 patches per plate). These plates served as master plates for replica-plating to sub-master plates (G-aro), which contained the analogues but not the supplements, and to control plates (Gnaslm-aro). The sub-master plates were also replica-plated immediately to plates (G) which contained neither analogues nor

supplements, and to control plates (Gnaslm) which lacked the analogues. Examination of all the plates after incubation confirmed the presence of *aroP* mutants and permitted the detection of auxotrophic *aroP* strains. The relatively large inoculum provided by the first replica plates was essential for demonstrating the AroP$^-$ phenotype. Conversely, the second set of replica plates improved the sensitivity of the method for detecting auxotrophs by testing growth from small inocula in the absence of the amino-acid analogues. Potential auxotrophic *aroP* mutants were purified on the selective medium (Gnaslm-aro) and details of their growth requirements were elucidated by further tests. Only one mutant of a particular phenotypic class was selected from each independent parental culture.

Reversion tests. Cultures (16 h at 37 °C in L broth) of mutant organisms were washed twice with saline and duplicate samples containing 10^8 and 10^9 bacteria were plated on appropriate media. The plates were incubated at 37 °C, examined at regular intervals for 5 days and, when present, the number of spontaneous revertant colonies was scored.

Conjugation. Deletions isolated in strain H were transferred by conjugation to the KI-1 (Pps$^-$, Met$^-$) background in order to investigate the regulatory state of the *ace* operon by lactate-sensitivity tests and to provide a complete set of deletions in one genetic background containing a reference marker (*metB*$^-$) for subsequent fine-structure genetic analyses. Overnight L broth cultures of the recipient, KΔ18S, a spontaneous streptomycin-resistant derivative of mutant KΔ18 (Δ*aroP–lpd, metB, thy, pps*; see Results), were diluted 10-fold in fresh L broth and grown for 90 min at 37 °C with shaking. The donor strains (nine stable HΔ strains, see Results) were grown as stationary slanted 5 ml cultures and likewise were subcultured into fresh L broth. Equal portions (1 ml) of the exponential-phase cultures containing approximately 2×10^9 donor and 2×10^8 recipient bacteria per ml were mixed gently and incubated at 37 °C without shaking for 20 min. Mating pairs were separated by vortex mixing for 1 min and Lpd$^+$ Str^R recombinants were selected immediately by plating dilutions in saline on acetate minimal medium plus streptomycin (200 μg ml^{-1}) and appropriate supplements, and enriched with nutrient broth (Difco; 2 ml l^{-1}). The recombinants from each cross were purified and tested and a series of strains possessing the characteristics of the donor deletion and the recipient (Met$^-$, Thy$^-$, Pps$^-$, Str^R) were readily isolated. These strains were designated by the prefix KHΔ but retained the number of the original H deletion strain.

Preparation of bacteria-free extracts. Organisms were grown in 500 ml batches of salts medium E, with glucose (10 mM) and relevant supplements, in 2 l Erlenmeyer flasks shaken at 37 °C. Bacteria were harvested in the late-exponential phase by centrifuging, washed twice, and resuspended in potassium phosphate buffer (0·04 M, pH 7·0) to obtain 250 mg wet wt organisms ml^{-1}. Suspensions were disrupted with an ultrasonic cell disintegrator (M.S.E., 150 W) for 4 min at 0 °C (two 2 min treatments with an interval for cooling). The supernatant ultrasonic extracts were obtained after centrifuging (24000 *g* for 30 min), and protein was determined according to Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Samples of each mutant culture were tested to ensure that reversion had not occurred.

Enzyme assays. Specific activities [μmol substrate transformed (mg protein)$^{-1}$ h^{-1}] were determined in the region of proportionality between initial reaction velocity and protein concentration. All spectrophotometric assays were carried out in 10 mm light-path silica cuvettes at 25 °C with a Beckman DB GT double-beam spectrophotometer coupled to a Servoscribe Is potentiometric recorder.

Pyruvate dehydrogenase (pdh) complex and α-ketoglutarate dehydrogenase (kgdh) complex were assayed spectrophotometrically at 366 nm and pH 8·5 by following the α-keto acid-

dependent reduction of 3-acetylNAD according to Guest and Creaghan (1973); the only modification was the addition of $MgCl_2$ (5 μmol) to obtain maximum activity when assaying the pyruvate dehydrogenase complex.

Pyruvate dehydrogenase (E1p; EC 1.2.4.1) and α-ketoglutarate dehydrogenase (E1 kg; EC 1.2.4.2) were assayed spectrophotometrically at 420 nm and pH 6·3 with ferricyanide as the electron acceptor, according to Hager & Kornberg (1961). As discussed by Langley & Guest (1974), the assay is not specific for E1p as the flavoprotein pyruvate oxidase (EC 1.2.2.2), which is present in extracts in varying amounts, is also active in this assay.

Lipoamide dehydrogenase (E3; EC 1.6.4.3) was assayed spectrophotometrically at 366 nm and pH 7·8 by recording the dihydrolipoate-dependent reduction of 3-acetylNAD according to Creaghan & Guest (1972).

Dihydrolipoamide acetyltransferase (E2p; EC 2.3.1.12) was assayed at 30 °C and pH 7·0 by the method of Willms *et al.* (1967) which measures the production of the heat-stable thioester *S*-acetyldihydrolipoamide from dihydrolipoate and acetyl-CoA (generated from acetylphosphate and CoA with phosphotransacetylase). Reaction mixtures containing 1 to 2 mg protein were incubated for 5 to 10 min and the product was determined by the hydroxamate procedure of Lipmann & Tuttle (1945). Wild-type and constitutive rates were readily detected but the method lacked the sensitivity to detect with any degree of certainty specific activities of less than 1 (i.e. less than 15 % of the wild type).

More sensitive tests for the presence of acetyltransferase (E2p) and positive identifications of which component(s) of the dehydrogenase complexes were missing in specific mutants were achieved by complementation assays. Overall α-keto acid dehydrogenase complex activities could be reconstituted by mixing extracts of mutants which lack specific components or by adding purified lipoamide dehydrogenase to extracts of *lpd* mutants. Details of the complementation assays are described by Guest & Creaghan (1973).

Materials. Coenzyme A, acetylphosphate (potassium-lithium salt), 3-acetylNAD and phosphotransacetylase were obtained from Boehringer; DL-lactate (lithium salt), α-ketoglutarate (monosodium salt), pyruvate (sodium salt), thiamin pyrophosphate and β-2-thienyl-DL-alanine were from Sigma; and α-lipoic acid (DL-6,8-thioctic acid) and DL-5-methyltryptophan were from Koch–Light. DL-Dihydro-α-lipoic acid was prepared by the method of Gunsalus & Razzell (1957). Purified pig heart lipoamide dehydrogenase was from Miles-Seravac, Maidenhead, Berkshire, and a sample of the same enzyme from *E. coli* B was kindly provided by Dr C. H. Williams, Jr (Department of Biochemistry, University of Michigan, Ann Arbor, Michigan, U.S.A.).

RESULTS

Isolation of potential deletion strains amongst spontaneous aroP mutants

The close proximity of the general amino-acid permease gene (*aroP*) to the genes determining the pyruvate dehydrogenase complex (*aceE*, *aceF* and *lpd*) in *Escherichia coli* K12 (Guest, 1974), and the existence of two strains of *Salmonella typhimurium* LT2 deleted for these genes (Langley & Guest, 1974), suggested that comparable strains with deletions extending into neighbouring genes may be found among spontaneous *aroP* mutants of *E. coli*. Mutants (*aroP*) defective in the general aromatic amino-acid permease are characterized by their resistance to inhibitory analogues such as *p*-fluorophenylalanine, β-2-thienylalanine and 5-methyltryptophan. Different combinations of these analogues were tested over a range of concentrations to determine optimum selective conditions. The combination of at least two analogues ensured that only *aroP* mutants survived, because resistance to single

Table 2. *Isolation of spontaneous aroP mutants and the detection of potential deletion strains as auxotrophs*

Spontaneous mutants resistant to DL-5-methyltryptophan and β-2-thienyl-DL-alanine were isolated and screened for auxotrophy, as described in Methods. The results include four independent *aroP* auxotrophs of strain H which were later found not to be deletion mutants.

Strain	Independent cultures	Colonies tested	Confirmed *aroP* mutants	Auxotrophic *aroP* mutants	Independent isolates
H	15	1037	969	25	13
KI-I	12	940	612	27	9
KI-ILR8-16	14	915	741	13	10
Totals	41	2892	2322	65	32

analogues could be due to mutations affecting the regulation of amino-acid biosynthesis or specific transport systems. Spontaneous *aroP* mutants were accordingly selected on appropriate glucose minimal media supplemented with 5-methyltryptophan plus β-2-thienylalanine and nicotinate, acetate, succinate, lysine and methionine, which may be required as a consequence of deleting genes adjacent to *aroP* (see Methods). Three parental strains, H, KI-I (Pps$^-$), KI-ILR8-16 (Pps$^-$, Ace^c), were chosen as the most suitable for studying the effects of deletion on the expression of the *ace* operon and the regulation of the *lpd* gene.

Potential *aroP* mutants appeared at a frequency of approximately 1 per 3×10^7 bacteria plated. However, the actual frequency may be lower because it is likely that, after plating, several generations occur before growth is inhibited. The results of 41 independent experiments are summarized in Table 2. Of the 2892 potential AroP$^-$ colonies tested, 80 % retained their resistance to the inhibitory analogues. The remaining 20 % failed to grow when transferred to the same medium on which they had been selected. Growth of these isolates during the initial selection may have been due to neighbouring AroP$^-$ colonies excreting aromatic amino acids which neutralized the effects of one or both analogues. Of the confirmed *aroP* mutants, 65 (i.e. 2·8 %) were auxotrophs, which could have arisen by deletion of neighbouring genes (Table 2), and these were recovered from 28 of the 41 starting cultures.

Nutritional classification of the auxotrophic aroP mutants

Using a variety of different media, tests with the 65 auxotrophic *aroP* mutants indicated the presence of six nutritionally-distinct classes (Table 3). Only one representative of a particular phenotypic class was retained from each starting culture so that a total of 32 independently-derived auxotrophic *aroP* mutants were chosen for further study (Table 2). The number of independent isolates in each case is included in Table 3. Eight independent isolates were Nad$^-$, requiring a single supplement of nicotinate for growth on glucose, succinate and acetate media (Table 3). The strains exhibited a typical Ace$^-$ phenotype by requiring acetate for growth on glucose and succinate media and being able to grow on acetate minimal medium (Table 3). A further mutant required acetate for growth on succinate but the acetate supplement was not essential for growth on glucose although it did improve the rate and extent of growth. This appeared to represent a distinct class of Ace$^-$ lesion which was designated 'Ace$^-$' to signify the leaky or partial nature of the phenotype (Table 3). Eight independent isolates with a composite Nad$^-$ Ace$^-$ phenotype required nicotinate and acetate for growth on glucose and succinate media and needed nicotinate for growth on acetate. The acetate requirement of a further three nicotinate-dependent strains was less apparent on succinate as well as glucose media and these were placed in a separate

Table 3. *Nutritional classes of auxotrophic aroP mutants*

The nutritional requirements of 65 auxotrophic *aroP* mutants were investigated by streaking dilute suspensions in saline on the test media shown below. The six nutritional classes are based on the corresponding growth responses. The number of independent isolates representing each class is also recorded. Growth was scored after 48 h at 37 °C as: ++, very good; +, good; ±, poor; −, none. Abbreviations for media (substrates in capital letters, supplements in lower case letters) are: G, glucose; A/a, acetate; S/s, succinate; n, nicotinate; aro, 5-methyltryptophan plus β-2-thienylalanine. All media included relevant supplements for the parental strains.

	Nutritional class					
Test medium	Nad⁻	Nad⁻'Ace⁻'	Nad⁻Ace⁻	'Ace⁻'	Ace⁻	Lpd⁻
G	−	−	−	+	−	−
Gn	++	+	−	+	−	−
Ga	−	−	−	++	++	−
Gna	++	++	++	++	++	−
Gs	−	−	−	+	−	−
Gas	−	−	−	++	++	++
Gnas	++	++	++	++	++	++
Gnas-aro	+	+	+	+	+	+
S	−	−	−	±	−	−
Sn	−	±	−	±	−	−
Sa	−	−	−	++	++	−
Sna	++	++	++	++	++	−
A	−	−	−	+	+	−
An	+	+	+	+	+	−
No. of independent isolates	8	3	8	1	10	2

class designated Nad⁻ 'Ace⁻' (Table 3). Only two strains exhibited an Lpd⁻ phenotype, requiring acetate plus succinate for growth on glucose and failing to grow on acetate and succinate media (Table 3). These two strains could combine Ace⁻ and Lpd⁻ phenotypes but the Ace⁻ nutritional phenotype is included in, and would be masked by, the Lpd⁻ nutritional phenotype.

Further properties of the auxotrophic aroP mutants

Stability. An important characteristic of most deletion mutants is their stability; failure to detect reversion is often considered a useful criterion for classifying mutations as deletions and for discriminating between deletions and pleiotropic or multiple mutations. Most point mutations revert spontaneously at frequencies similar to a spontaneous mutation rate of approximately 1 in 10^7 to 10^9 whilst deletions exhibit reversion frequencies of less than 1 in 10^{10} (Demerec, 1960).

The 32 auxotrophic *aroP* strains were tested for spontaneous reversion to prototrophy on the relevant media (listed in Table 3). Where an auxotroph had more than one nutritional phenotype, the stabilities of the characteristics were tested singly and together, e.g. the Nad⁻ Ace⁻ strains were tested for reversion to Nad⁺ Ace⁻, Nad⁻ Ace⁺ and Nad⁺ Ace⁺ phenotypes. Allowing for four generations of bacterial growth after plating, the tests could detect reversion frequencies of 1 in 10^{10} or lower. These tests showed that the Ace⁻ phenotypes of three strains (HΔ7, HΔ8 and HΔ9) and the Lpd⁻ phenotype of one strain (HΔ12) had estimated reversion frequencies greater than 1 in 3×10^7, which were higher than the rates obtained with representative *ace* (A2T3, A10) and *lpd* (T3A58*lpd*1) mutants. These strains were presumed to combine *aroP* mutations (including deletions) with mutations in the *ace* (HΔ7, HΔ9) or *lpd* (HΔ12) genes and a deletion (*nadC–aroP*) with an *ace* point mutation (HΔ8). One

Table 4. *The auxotrophic aroP deletion mutants*

The 28 independently-isolated *aroP* mutants exhibiting stable auxotrophy are listed in the six phenotypic classes. Each strain is prefixed to denote its parent strain, H, K (KI-1) or C (KI-ILR8-16, the constitutive strain), followed by Δ designating the deletion and an individual number given to each potential deletion strain. The dotted line in the *aroP–ace* region indicates that the deletion may not extend into the *ace* genes but their expression is affected.

Phenotypic class*	Strain designations	Extent of deletion *nadC–aroP–ace–lpd*	No. of deletion strains
Nad⁻	HΔ1, HΔ2, HΔ4, HΔ5, HΔ6, HΔ14, KΔ27, CΔ44	————	8
Nad⁻ 'Ace⁻'	HΔ3, CΔ35, CΔ39	————·····	3
Nad⁻Ace⁻	HΔ10, KΔ17, KΔ22†, KΔ32, KΔ34, CΔ42†, CΔ46	————————	7
'Ace⁻'	CΔ47	——·····	1
Ace⁻	HΔ13, HΔ15, KΔ21, KΔ33, CΔ37, CΔ40, CΔ41†, CΔ45	————	8
Lpd⁻	KΔ18	————————	1

* All strains possess the AroP⁻ phenotype.

† Ace⁻ deletions which contain detectable E2p, the *aceF* gene product; the other 12 Ace⁻ deletions lack E1p and E2p, products of both the *aceE* and *aceF* genes.

other unstable strain, found among the auxotrophic *aroP* mutants but not included in the foregoing results, required succinate for growth on glucose, grew poorly on succinate media and failed to grow on acetate. This strain (HΔ11) was probably a spontaneous double mutant (*aroP*, *suc*). The remaining 28 strains were stable and it was tentatively concluded that they represent spontaneous *aroP* deletion mutants and that their auxotrophy is due to extension of the deletions into neighbouring genes. The presumptive deletion mutants are listed in Table 4 according to their phenotypic classes. The range of nutritional phenotypes and the relative abundance of the fundamental classes (Nad⁻, Nad⁻ Ace⁻, Ace⁻, Lpd⁻) confirms the gene order *nadC–aroP–ace–lpd*, and the extents of the individual classes of deletion can be approximated as shown in Table 4. If strain KΔ18 is assumed to be a deletion strain, its Lpd⁻ nutritional phenotype must be masking an Ace⁻ phenotype in order to form a series consistent with the other classes of deletion.

Streptomycin resistance. One of the characteristics of the *S. typhimurium* deletion strains (SM16 and SM51) was resistance to low concentrations (20 μg ml^{-1}) of streptomycin (Carrillo-Castaneda & Ortega, 1970; Langley & Guest, 1974). None of the auxotrophic *aroP* strains of *E. coli* were resistant to this concentration of streptomycin. The phenomenon appears to be a special feature of the *S. typhimurium* deletion strains and it could be due to the deletion of a specific gene in this region of the *S. typhimurium* chromosome or the combined effects of the deletions and some other mutation present in SM16 and SM51.

Regulatory state of the ace operon. Growth on acetate of mutants (*pps*) lacking phosphoenolpyruvate synthetase is inhibited by low concentrations of pyruvate or lactate (Brice & Kornberg, 1967). This is because pyruvate represses the glyoxylate cycle and, whereas wild-type strains can remove the pyruvate with phosphoenolypyruvate synthetase, this is not possible in the mutants. However, the *pps* mutants can become resistant to pyruvate (or lactate) by further mutations which cause (i) constitutive synthesis of the glyoxylate cycle enzymes (*iclR*; Kornberg, 1966), (ii) defects in pyruvate or lactate transport (e.g. *usp*; Kornberg & Smith, 1967) or (iii) constitutive synthesis of the pyruvate dehydrogenase complex (*ace*^{c}; Flatgaard *et al.*, 1971). To determine whether the regulation of pyruvate dehydro-

genase complex synthesis was altered by the *aroP* deletions, the deletion strains were tested for lactate-resistance on acetate (except the Lpd$^-$ strain, which was unable to grow on acetate, and the unstable strains presumed not to be deletion strains). Direct tests were possible with the derivatives of strains KI-1 and KI-ILR8-16 because they already possessed the *pps* mutation. The deletions in strain H (Pps$^+$) were all transferred to the KI-1 (Pps$^-$) background by conjugation with a streptomycin-resistant derivative of strain KΔ18 (KΔ18s) to yield a series of streptomycin-resistant deletion strains designated KHΔ. Preliminary tests with the strain H derivatives (Pps$^+$) indicated that they were all lactate-resistant despite the fact that several lacked the pyruvate dehydrogenase complex and could be deficient in the ability to remove the inhibitor.

All the Pps$^-$ deletion and parental (KI-1 and KI-ILR8-16) strains plus the Pps$^+$ strain KI, were tested at 37 °C by streaking dilute suspensions on acetate minimal medium supplemented with lithium DL-lactate at concentrations of 0, 5, 10, 15, 30 and 50 μM. If a strain was sensitive to lactate then the formation of single colonies was prevented. Strain KI (Pps$^+$) was completely unaffected by 50 μM-lactate, whereas KI-1 (Pps$^-$) tolerated 15 μM but not 30 μM-lactate and strain KI-ILR8-16 (Pps$^-$, Ace^c), constitutive for the pyruvate dehydrogenase complex, grew with 30 μM but not 50 μM-lactate. None of the deletion strains (Pps$^-$, KΔ, KHΔ and CΔ) were resistant like the constitutive strain (KI-ILR8-16) and seven of the eight Nad$^-$ Ace$^+$ deletion strains exhibited the same sensitivity as KI-1. The other Nad$^+$Ace$^+$ deletion strain (KΔ27) was more sensitive than its parent, being inhibited by 15 μM-lactate; a possible explanation for this could be a lower expression of the *ace* operon. Strain CΔ44, the only Nad$^-$ Ace$^+$ derivative of KI-ILR8-16, was also interesting because by resembling KI-1 it had lost the ability to tolerate the higher concentrations of lactate characteristic of its parent. The 'Ace$^-$' strains were also more sensitive than the parental strains, being inhibited by 15 μM-lactate, and this is consistent with impaired expression of the *ace* operon. Most sensitive were the deletion strains possessing an Ace$^-$ phenotype. They were inhibited by 10 μM-lactate and their sensitivity is presumably due to the absence of both *pps*- and *ace*-mediated routes of pyruvate metabolism. Clearly none of the deletions produced lactate-resistance corresponding to an Ace^c phenotype and direct attempts to select such strains on acetate minimal medium plus nicotinate, the aromatic amino-acid analogues and DL-lactate (30 μM) were unsuccessful. Higher concentrations of lactate were required for inhibition than those reported by Flatgaard *et al.* (1971). The discrepancy may be due to the fact that the earlier tests were performed at 42 °C because the *pps* mutation is probably leaky at 37 °C. Unfortunately, the strain of KI-1 used in the present studies grew very poorly on acetate at 42 °C and the tests had to be performed at 37 °C.

Enzymological studies

Ultrasonic extracts of all the auxotrophic *aroP* mutants were assayed for the pyruvate dehydrogenase (pdh) and α-ketoglutarate dehydrogenase (kgdh) complexes and their components E1p, E2p, E3 and E1kg in order to investigate the enzymological phenotype and the effects of the deletions on expression of the *ace* and *lpd* genes. The results are summarized in Table 5.

Parent strains. The parental strains H and KI-1 had very similar activities for all the enzymes tested. Furthermore, the results obtained with strain KI, the Pps$^+$ parent of KI-1, were also similar indicating that the *pps* mutation had no effect on these enzymes under the conditions used. The third parental strain (KI-ILR8-16) had approximately twice as much pdh complex activity as strain KI-1 (Table 5) and this is in good agreement with the ratio 60:25 reported by Flatgaard *et al.* (1971) for the pdh complex activities of the same strains grown on glucose.

Table 5. *Specific activities of the α-keto acid dehydrogenase complexes and components in parental and auxotrophic aroP deletion strains*

Ultrasonic extracts of cultures grown on glucose medium (supplemented as required) were prepared and the enzymes were assayed as described in Methods. The average activities for determinations with at least two different extracts are quoted. The results quoted for some strains of each phenotypic class are representative of all the other strains not listed but of the same category (see text). Specific activities are expressed as μmol substrate transformed (mg protein)$^{-1}$ h^{-1}. Abbreviations: pdh complex, overall pyruvate dehydrogenase complex; E1p, pyruvate dehydrogenase plus oxidase; E2p, dihydrolipoamide acetyltransferase; E3, lipoamide dehydrogenase; kgdh complex, α-ketoglutarate dehydrogenase complex; E1kg, α-ketoglutarate dehydrogenase.

Strain	Relevant phenotype	Specific activity					
		pdh complex	E1p	E2p	E3	kgdh complex	E1kg
H	Ace$^+$	5·98	1·71	5·90	2·07	2·50	4·85
K1	Ace$^+$	4·60	1·80	6·20	2·25	3·10	5·40
K1-1	Ace$^+$ Pps$^-$	5·00	2·01	6·10	2·49	2·09	4·30
K1-ILR8-16	Ace^c Pps$^-$	10·22	3·33	13·80	3·90	3·00	3·04
HΔ4	Nad$^-$ Ace$^+$	4·20	2·02	3·70	2·08	3·07	4·51
KΔ27	Nad$^-$ Ace$^+$	1·52	0·92	2·30	1·63	1·66	3·31
CΔ44	Nad$^-$ Ace$^+$	4·13	2·27	6·10	3·80	2·14	2·12
CΔ39	Nad$^-$ 'Ace$^-$'	0·36	0·37	1·60	1·98	2·32	5·14
CΔ47	'Ace$^-$'	0·33	0·45	1·60	1·47	1·55	3·65
KΔ33	Ace$^-$	< 0·01	< 0·01	< 1·00	0·85	1·84	2·40
CΔ42	Nad$^-$ Ace$^-$	< 0·01	< 0·01	< 1·00	1·56	1·78	2·88
KΔ18	Lpd$^-$	< 0·01	< 0·01	< 1·00	< 0·01	< 0·01	5·04

The activities for E1p, E2p and E3 were also correspondingly higher in the constitutive strains.

Ace$^+$ *deletion strains.* Of the eight deletion mutants which were nutritionally Ace$^+$ (i.e. all the members of the Nad$^-$ class in Table 4), the enzyme activities of six, represented by strain HΔ4 in Table 5, were almost unaffected. One strain KΔ27 had only 30 % of the parental pdh complex activity, approximately 50 % of the E1p and E2 components and a reduced E3 activity (Table 5). Although this strain was acetate-independent, the deletion clearly affected expression of the *ace* operon and this is consistent with the increased lactate-sensitivity of strain KΔ27. The remaining Nad$^-$ deletion strain, CΔ44, no longer expressed the *ace* operon at the constitutive level (Table 5) and this again had been indicated by its lactate-sensitivity. Presumably the deletion in strain CΔ44 removes a regulatory element or fuses the *ace* operon to another, less efficient, promoter. The E3 activities of the Ace$^+$ strains were between 67 % and 100 % of the corresponding parental strains.

'Ace$^-$' *deletion strains.* The four strains possessing the leaky 'Ace$^-$' phenotype, represented by strains CΔ39 and CΔ47 in Table 5, had only about 7 % of the pdh complex activity of the inducible strain K1-1. The E1p and E2p components were also considerably reduced. The E3 activities were affected but less dramatically; between 38 % and 56 % of the parental activity was retained. Again, these deletions appear to have removed one or more control elements or fused the *ace* structural genes to a promoter with very low efficiency. However, it is interesting that despite the low pdh complex activities, these strains grow on glucose without added acetate but the activities fail to support good growth on unsupplemented succinate medium.

Ace$^-$ *deletion strains.* None of the 15 stable deletions which were nutritionally Ace$^-$ (eight Ace$^-$ and seven Nad$^-$ Ace$^-$), represented by strains KΔ33 and CΔ42 in Table 5, possessed detectable pdh complex activity. Seven of the strains gave small positive results in

Table 6. *Complementation between extracts of* Ace⁻ *and* Lpd⁻ *deletion strains and an aceF mutant*

The pyruvate dehydrogenase (pdh) complex activities of mixtures of ultrasonic extracts containing 1 mg protein of the test strain and an equal amount of the *aceF* mutant (strain A10) in 1 ml reaction mixtures were assayed. Activities are expressed as total activity in the samples (in μmol h^{-1}) after subtracting the very small activities of both participating extracts tested separately.

Test strain	Phenotype	Activity of pdh complex (μmol h^{-1})
A10	AceF⁻	< 0·01
A2T3	AceE⁻	2·26
A6	AceE⁻	0·42
W1485A1	AceE⁻	2·42
HΔ13	Ace⁻	< 0·01
KΔ33	Ace⁻	< 0·01
CΔ46	Nad⁻ Ace⁻	< 0·01
KΔ22	Nad⁻ Ace⁻	0·53
CΔ41	Ace⁻	0·76
CΔ42	Nad⁻ Ace⁻	0·44
KΔ18	Lpd⁻	< 0·01

the E1p assay which could be due to pyruvate oxidase but none possessed E2p activity when assayed by the direct method. However, it was difficult to discriminate between the low E2p activities of the leaky 'Ace⁻' strains (which exhibit weak pdh complex activities) and would be expected to possess E2p) and zero activity which would be predicted for a few Ace⁻ strains. The complementation assay proved more sensitive for detecting the presence of the active E2p component. Extracts of each Ace⁻ deletion strain were mixed with an extract of strain A10 (an *aceF* mutant which contains E1p and E3 components but no E2p) and active E2p was detected by its ability to reconstitute overall pdh complex activity. By this criterion, three deletion strains with Ace⁻ phenotypes (KΔ22, CΔ41 and CΔ42) complemented strain A10 (Table 6) and it was concluded that they retain intact and functional *aceF* genes. No complementation was detected with the 12 remaining Ace⁻ strains represented by strains HΔ13, KΔ33 or CΔ46 in Table 6. All the Ace⁻ strains contained lipoamide dehydrogenase (E3) at between 30 % and 95 % of the parental level and they all possessed the activities of the α-ketoglutarate dehydrogenase complex and its dehydrogenase component E1kg.

Lpd⁻ *deletion strain.* The one stable strain designated Lpd⁻ by nutritional criteria (KΔ18) had no detectable E3, pdh or kgdh complex activities, although E1kg was present (Table 5). Furthermore, strain KΔ18 lacked E1p (Table 5) and no E2p activity could be detected either by direct or complementation tests (Tables 5 and 6). This confirms that the Lpd⁻ nutritional phenotype is masking the Ace⁻ phenotype. Complementation studies were also performed by adding graded amounts of purified lipoamide dehydrogenase from *E. coli* B and pig heart to extracts of strain KΔ18 in studies similar to those with *lpd* mutants of *E. coli* and the deletion strains of *S. typhimurium* (Guest & Creaghan, 1973; Langley & Guest, 1974). Overall kgdh complex activity was restored, but no pdh complex activity could be recovered with either source of the E3 component. It may thus be concluded that the deletion in strain KΔ18 extends from the *aroP* gene through the *ace* operon and probably into the *lpd* gene (Table 4) unless it prevents *lpd* expression by some other mechanism. Immunological studies with antiserum raised against purified *E. coli* lipoamide dehydrogenase were used to test for the presence or absence of cross-reacting material (CRM) by immunodiffusion according to the methods described by Guest & Creaghan (1974). No cross-reacting

material could be detected in ultrasonic extracts of strain KΔ18. This CRM$^-$ phenotype is also consistent with a deletion extending into the *lpd* gene or one which prevents *lpd* gene expression.

Other mutants. The unstable auxotrophic *aroP* mutants were also examined enzymologically by direct and complementation assays to elucidate further details of their phenotypes. It was concluded that strain HΔ8 combines an *nadC–aroP* deletion with an *aceF* mutation and strains HΔ7, HΔ9, HΔ11 and HΔ12 have *aroP* mutations plus mutations in the *aceE*, *aceF*, *sucB* and *lpd* genes, respectively (the *lpd* mutation being CRM$^-$). The data also suggested that all these strains, except HΔ7, may have deletions in the *aroP* region which affect *ace* operon expression.

DISCUSSION

The results validated the prediction, based on the properties of two *S. typhimurium* strains (SM16 and SM51), that strains of *E. coli* deleted in *aroP* and adjacent genes may be found among spontaneous *aroP* mutants. Strains deleted for the *nadC*, *aceE*, *aceF* and *lpd* genes were isolated. The frequency of independent auxotrophic deletions amounted to 1·2 % of the spontaneous *aroP* mutants examined. This fell within the expected range of 1 % to 5 % for deletions among spontaneous mutants; a higher frequency would have been expected if non-auxotrophic *aroP* deletion mutants could have been detected and included. The use of a combination of aromatic amino-acid analogues presented a direct and fairly specific selection for *aroP* mutants (only 20 % of the mutants from the first screening failed to exhibit the AroP$^-$ phenotype in subsequent tests). Since the specific aromatic amino-acid transport systems have higher Michaelis constants than the general aromatic amino-acid permease (Brown, 1970; Thorne & Corwin, 1975), it is probably essential to have the correct balance of analogues and concentrations sufficient to be inhibitory via the general (*aroP*) systems but not too high to afford entry via the specific permeases. The concentrations were critical and this may explain why the *aroP* mutants could still be retarded or inhibited when grown from small inocula.

The nutritionally-distinct classes within the group of 28 stable deletion strains, Nad$^-$, Nad$^-$ Ace$^-$, Ace$^-$, Lpd$^-$ (including leaky 'Ace$^-$' types), and further subdivisions based on the complement of functional *aceE* and *aceF* gene products, confirmed the gene order *nadC–aroP–aceE–aceF–lpd* (Guest, 1974). The frequency with which different types were recovered did not appear to be strictly related to the length of genome deleted because the basic (Nad$^-$, Ace$^-$ and Nad$^-$ Ace$^-$) types arose with almost equal frequency (8, 10 and 9 respectively). However, only one strain (KΔ18) with an *aroP–lpd* deletion, analogous to the deletion in *S. typhimurium* SM16, was isolated and no strain with a deletion of the complete *nadC–lpd* region, corresponding to strain SM51, was recovered. A further characteristic of the *S. typhimurium* strains, resistance to low concentrations of streptomycin, was not exhibited by any of the *E. coli* deletion strains, including strain KΔ18. The *S. typhimurium* mutants also differed in that they did not require succinate for growth on glucose (Langley & Guest, 1974). However, a succinate-independent partial revertant of strain KΔ18, which was phenotypically more like strain SM16, was still streptomycin-sensitive. The existence of viable deletions in the *nadC–lpd* region indicates that it is unlikely to contain other 'essential' genes.

Of the 15 Ace$^-$ deletions having no pyruvate dehydrogenase complex or E1p, only three had detectable E2p activity. Although this confirms the gene order *aroP–aceE–aceF* it cannot be concluded that all but three of these deletions end in the *aceF* gene. The polarity of the *ace* operon is such that two-thirds of random deletions in the *aceE* gene may be expected to generate reading-frame shifts affecting expression of the *aceF* gene.

Conclusions regarding the effects of the deletions on the control of *ace* and *lpd* gene expression must await a thorough genetic analysis. Nevertheless, the direct relationship between the lactate-resistance of *pps* mutants growing on acetate and the activity of the pyruvate dehydrogenase complex proved a useful criterion for evaluating the degree of expression of the *ace* operon. Constitutive synthesis of the complex produces resistance (Flatgaard *et al.*, 1971) but none of the deletions generated resistance, e.g. by removing a gene specifying a negatively-acting repressor or by fusing the *ace* operon to a more efficient promoter. On the contrary, decreased resistance generated by the deletions in several Nad$^-$ strains and strains with 'Ace$^-$' phenotypes corresponded to a lowering of the *ace* gene expression. Control elements between *aroP* and *aceE* could again be affected by deletion or the *ace* operon could have been brought under the control of other, less effective, regulatory mechanisms. The leaky or 'Ace$^-$' phenotype of several deletion mutants with decreased *ace* gene expression is noteworthy. The ability (albeit impaired) to grow on unsupplemented glucose and, to a lesser extent, succinate media, indicates that growth-limiting amounts of acetate are synthesized and that there is a greater demand for acetate during growth on succinate or that other sources of acetate (pyruvate oxidase or the phosphoroclastic reaction) are induced or derepressed to a greater extent on glucose. Point mutants with this 'Ace$^-$' phenotype have not been reported. They may have been overlooked but could prove useful for studying the control of *ace* expression. The deletions generating the greatest lactate-sensitivity on acetate were totally deficient in pyruvate dehydrogenase complex activity as well as lacking phosphoenolpyruvate synthetase activity. In a Pps$^+$ background strains lacking pyruvate dehydrogenase complex activity, as well as Ace$^+$ strains, were unaffected by lactate. This suggests that phosphoenolpyruvate synthetase is primarily responsible for removing inhibitory pyruvate (formed from lactate) during growth on acetate plus lactate.

Only one strain, KΔ18, lacked lipoamide dehydrogenase activity and this could be due to a deletion affecting either the *lpd* structural gene or its expression. All the deletion strains with Ace$^-$ or 'Ace$^-$' phenotype retained E3 activity at between 30 % and 95 % of the parental level. Expression of the *lpd* gene can thus be independent of the *ace* operon. The lower E3 activities of some deletion strains indicate that the *ace* operon may influence lipoamide dehydrogenase synthesis, e.g. by exerting polar or pseudo-polar effects on *lpd* gene expression at the level of transcription or translation. Alternatively, the deletions may have brought the *lpd* gene under the control of new regulatory elements which promote the observed levels of lipoamide dehydrogenase synthesis. However, similar effects on E3 synthesis have been observed with *ace* polar mutants; *lpd* expression was never less than 30 % (Henning *et al.*, 1966; Creaghan & Guest, 1972) and this is consistent with some form of *ace*-independent *lpd* gene expression.

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