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

By D. LANGLEY* AND J. R. GUEST

Department of Microbiology, University of Sheffield, Sheffield S10 2TN

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Twenty-eight spontaneous auxotrophic *aroP* mutants with deletions in the *azi–nadC–aroP–aceE–aceF–lpd* region of the *Escherichia coli* K12 chromosome were characterized genetically with respect to various *azi*, *nadC*, *ace* and *lpd* markers by P1-mediated transduction. One mutant (KΔ18; *aroP–lpd*$^{\Delta}$) had a deletion which extended through the *aceE* and *aceF* genes to end within the *lpd* gene. The polarity of the *ace* operon (*aceE* to *aceF*) was confirmed. It was concluded that 10 out of 15 deletions generating a strict requirement for acetate terminated in the *aceE* gene. Of the ten, three mutants (KΔ22, CΔ41 and CΔ42) synthesized detectable dihydrolipoamide acetyltransferase (the *aceF* gene product) and seven were assumed to possess deletions generating polar effects on *aceF* gene expression. Five deletions appeared to extend into the *aceF* gene. A further five deletions, which limited the expression of the *ace* operon without generating an Ace$^-$ phenotype or a complete Ace$^-$ phenotype, ended closest to the *aroP*-proximal *aceE* markers. The opposite ends of all these deletions appeared to terminate before (10), within (2) or extend beyond (9) the *nadC* gene. There was no obvious correlation between the deletion end-points and the corresponding lipoamide dehydrogenase activities, which ranged from 30 to 95 % of parental levels in different deletion strains. The remaining seven deletions simply extended between the *aroP* and *nadC* genes (*nad–aroP*$^{\Delta}$) without affecting expression of the *ace* operon.

Regulation of the synthesis of the pyruvate and α-ketoglutarate dehydrogenase complexes was investigated in some of the parental and deletion strains under different physiological conditions including thiamin-deprivation. The results indicate that the syntheses of the two dehydrogenase complexes are independently regulated. Expression of the *lpd* gene appears to be coupled to complex synthesis but can be dissociated under some conditions. Mechanisms for regulating *lpd* gene expression are discussed and an autogenous mechanism involving uncomplexed lipoamide dehydrogenase functioning as a negatively acting repressor at the operator site of an independent *lpd* gene is proposed as the simplest mechanism which is consistent with all available information.

INTRODUCTION

Mutants of *Escherichia coli* K12 requiring acetate for aerobic growth on glucose and lacking overall pyruvate dehydrogenase complex (pdh complex) activity have been characterized as: *aceE* mutants, lacking activity of the dehydrogenase (E1p) component; *aceF* mutants, lacking dihydrolipoamide acetyltransferase (E2p) activity; and polar *aceE* mutants (mainly of the nonsense type), lacking both E1p and E2p activities (Henning *et al.*, 1966, 1968, 1969). The mutations are clustered at approximately 2 min in the *E. coli* linkage map and those of the third class are confined to a relatively large segment at one

* Present address: Beecham Pharmaceuticals, Clarendon Road, Worthing, West Sussex, BN14 8QH.

Table 1. *Gene–protein relationships of the enzymic components of the α-keto acid dehydrogenase complexes of Escherichia coli* K12

The genes determining specific and shared components of the two dehydrogenase complexes are shown with the corresponding abbreviations. The polarities of the *ace* and *suc* operons are indicated by the arrows.

Gene	Component	
aceE ↓	Pyruvate dehydrogenase E1p	pdh complex
aceF ↓	Dihydrolipoamide acetyltransferase E2p	pdh complex
lpd	Lipoamide dehydrogenase E3	
sucA ↓	α-Ketoglutarate dehydrogenase E1kg	kgdh complex
sucB ↓	Dihydrolipoamide succinyltransferase E2kg	kgdh complex

end of the *aceE* gene, thus defining the polarity of expression of the *ace* operon as *aceE* to *aceF*. The *ace* operon is inducible by pyruvate (Dietrich & Henning, 1970) and the *aceE* gene product is believed to function autogenously as a regulatory protein (repressor) as well as an enzyme (Flatgaard, Hoehn & Henning, 1971; Henning *et al.*, 1972; Goldberger, 1974). The gene–protein relationships of the pyruvate and α-ketoglutarate dehydrogenase complexes are summarized in Table 1.

Mutants requiring succinate for aerobic growth on glucose and lacking overall α-ketoglutarate dehydrogenase complex (kgdh complex) activity have likewise been characterized as: *sucA* mutants, lacking α-ketoglutarate dehydrogenase (E1kg) activity; *sucB* mutants, lacking dihydrolipoamide succinyltransferase (E2kg) activity; and polar *sucA* amber mutants, lacking both E1kg and E2kg activities (Herbert & Guest, 1969; Creaghan & Guest, 1972). The corresponding mutations are clustered at approximately 16 min in the *E. coli* linkage map constituting an analogous *suc* operon with the same polarity (*sucA* to *sucB*) as the *ace* operon (Table 1).

Studies with mutants requiring acetate plus succinate for best aerobic growth on glucose and lacking lipoamide dehydrogenase activity indicate that a single gene (*lpd*), located very close to the *aceF* gene at the distal end of the *ace* operon (Table 1), specifies the E3 components of both α-keto acid dehydrogenase complexes (Guest & Creaghan, 1972, 1973, 1974; Guest, 1974; Alwine, Russell & Murray, 1973).

The existence of a single *lpd* gene and its close association with the *ace* operon raised the problem of how its expression is controlled relative to the syntheses of the two enzyme complexes. Some form of regulatory coupling was indicated by the observation that polar *ace* and *suc* mutants contain less lipoamide dehydrogenase (E3) activity and that this deficiency was more pronounced in *ace* polar *suc* polar double mutants, although E3 synthesis was not abolished by combining the most polar mutations (Henning *et al.*, 1966; Creaghan & Guest, 1972). Further insight into this and related problems has been sought by studying several classes of spontaneous auxotrophic *aroP* mutants which have been isolated recently (Langley & Guest, 1977). These mutants were characterized as lacking the general aromatic amino-acid permease, by their resistance to aromatic amino-acid analogues, and they were presumed to possess deletions extending in one or both directions from the *aroP* gene into the *nadC–aroP–aceE–aceF–lpd* region, by virtue of their stability and simultaneous acquisition of requirements for one or more essential nutrients: nicotinate (Nad^-), acetate (Ace^-) and acetate plus succinate (Lpd^-). This paper describes a genetic characterization of the deletion mutants for the purpose of correlating the genetic lesions with the corresponding phenotypes. Attempts to define the regulatory defects generated by some of the deletions and to investigate the basic mechanisms controlling expression of the *ace* operon and *lpd* gene are also described. In particular, the response of the *lpd* gene in strains lacking part or most of the *ace* operon was studied to evaluate the independence of the *lpd* gene.

Table 2. *Strains of Escherichia coli* K12

Strain	Characteristics	Source
H	Hfr; *thi-1, azi-7, relA1*?	W. Hayes
K1-1	F⁻; *metB, relA1, thyA56, tsx-87, pps, azi, ton*?	H. L. Kornberg
K1-1LR8-16	F⁻; *metB, relA1, thyA56, tsx-87, pps, azi, ton*?, *ace*^{c}*	U. Henning
W3110	F⁻; *trpR, iclR*	C. Yanofsky
WGAaceE64	F⁻; *gal-25, trpA9761, iclR, aceE64*	J. R. Guest
WGAaceE131	F⁻; *gal-25, trpA9761, iclR, aceE131*	J. R. Guest
A2T3	*aceE2, trpE61* (λ)	U. Henning
A6	*aceE6* (λ)?	U. Henning
A10	*aceF10* (λ)	U. Henning
W1485A1	*aceE1, supE42*	J. R. Guest
T3A58lpd1	F⁻; *trpE61, trpA58, iclR, lpd1*	J. R. Guest
Hlpd6	Hfr; *thi-1, azi-7, relA1*?, *lpd6*	J. R. Guest
Hlpd9	Hfr; *thi-1, azi-7, relA1*?, *lpd9*	J. R. Guest
E126	F⁺; *nadC13*	R. K. Gholson
KL14	Hfr; *thi-1, relA1*	B. J. Bachmann

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

Table 3. *Nutritional classes of aroP deletion mutants*

The 28 independent *aroP* deletion strains were isolated as aromatic amino-acid analogue-resistant mutants exhibiting stable auxotrophy. The phenotypic symbols are based on the nutritional requirements for aerobic growth on glucose: Nad⁻, nicotinate; Ace,⁻ acetate; Lpd⁻, acetate plus succinate (characteristic of a lipoamide dehydrogenase deficiency). 'Ace⁻' denotes the leaky or partial Ace⁻ phenotype of mutants which require acetate for growth on succinate but not glucose (Langley & Guest, 1977).

Phenotype	Strains
Nad⁻	HΔ1, HΔ2, HΔ4, HΔ5, HΔ6, HΔ14, KΔ27, CΔ44
Nad⁻'Ace⁻'	HΔ3, CΔ35, CΔ39
'Ace⁻'	CΔ47
Nad⁻Ace⁻	HΔ10, KΔ17, KΔ22*, KΔ32, KΔ34, CΔ42*, CΔ46
Ace⁻	HΔ13, KΔ15, KΔ21, KΔ33, CΔ37, CΔ40, CΔ41*, CΔ45
Ace⁻Lpd⁻	KΔ18

* Ace⁻ deletion strains which contain detectable E2p, the *aceF* gene product; the remaining 12 Ace⁻ deletion strains lack E1p and E2p, the products of the *aceE* and *aceF* genes.

METHODS

Bacterial strains. The characteristics and sources of the basic parental strains and the *nadC*, *ace* and *lpd* mutants are listed in Table 2. Further details of several of these strains have been described by Langley & Guest (1977). The nutritional and enzymological properties of the deletion mutants have also been described previously (Langley & Guest, 1977). The 28 deletion mutants were isolated as independent spontaneous *aroP* mutants of strains H, K1-1 and K1-1LR8-16, and designated by the prefixes HΔ, KΔ and CΔ (C denoting the constitutive, *ace*^{c}, parent), respectively. The mutants fell into the six nutritional categories shown in Table 3. Only three of the mutants possessing Ace⁻ phenotypes (KΔ22, CΔ41 and CΔ42) retained detectable dihydrolipoamide acetyltransferase (E2p) activity. Deletions isolated in strain H were transferred to the K1-1 background by conjugation with a spontaneous streptomycin-resistant derivative of mutant KΔ18 in order to provide a complete set of deletions in a single genetic background containing a *metB* mutation as a reference marker for fine structure genetic analysis. The corresponding products were designated by the prefix KHΔ but retained the numbers of the original H deletion strains. Unfortunately, it was subsequently discovered that strain KΔ18 and all the KHΔ derivatives except KHΔ10 could not be transduced to Met⁺. Nutritional studies indicated that KΔ18 had acquired a second *met* mutation which was not cotransducible with *metB*. Furthermore, one of the two *met* mutations must have reverted in KHΔ10.

Media. Minimal medium E of Vogel & Bonner (1956) was used in all experiments. Carbon sources were: D-glucose (10 mM, unless stated otherwise); 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-amino acids (30 μg ml^{-1}), thymine (50 μg ml^{-1}), nicotinic acid (10 μg ml^{-1}), thiamin hydrochloride (10 μg ml^{-1}, unless stated otherwise), Casamino acids (Difco, vitamin-free; 0·05 %, w/v).

Selective minimal media were sometimes enriched with Difco Bacto Nutrient Broth (0·2 %, v/v). The complete medium for routine growth and maintenance was L broth (Lennox, 1955) with additional thymine (50 mg l^{-1}), required for optimal growth of Thy$^-$ strains. Media were solidified with Difco Bacto agar (15 g l^{-1} or 6 g l^{-1}) as required.

Transduction with phage P1. Lysates of donor strains were prepared from a stock of the temperate phage P1 by the overlayer technique. Confluent lysis was obtained by incubating 0·1 ml of a stationary phase culture of the donor (approx. 5×10^8 bacteria) and 10^6 P1 in a 2·5 ml soft agar overlayer for 6 h at 37 °C. The phages were extracted with L broth and assayed with *Shigella dysenteriae* 16. The lysates routinely contained 5×10^9 to 5×10^{11} plaque-forming units (p.f.u.) ml^{-1} and, for fine structure mapping, at least two successive cycles of lysis were used in their preparation to eliminate any transducing phages derived from previous hosts.

For transductions, 0·2 ml of a stationary phase L broth culture (containing 10^9 recipient organisms) was incubated with 0·1 ml of $CaCl_2$ (50 mM) for 30 min at room temperature after which 1 ml of donor lysate was added to give a multiplicity of infection of 2. The transduction mixture was incubated at 37 °C for 20 min, then 4 ml of cold saline (0·9 %, w/v) containing $CaCl_2$ (2·5 mM) was added, and the bacteria were sedimented by centrifugation. They were washed once with saline plus $CaCl_2$ and resuspended in an appropriate volume of saline plus $CaCl_2$ for plating on selective media. Samples containing bacteria exposed to up to 5×10^8 P1 were plated in duplicate, thus setting the limit for detecting transduction at 0·001 transductants per 10^6 P1 phages. Control experiments were always performed: (i) with no phage, to check for reversion in the recipient cultures; (ii) with no bacteria, to check the sterility of the lysates; and (iii) 'selfing mixtures' were tested to determine whether the lysates of specific mutant donor strains possessed any ability to transduce to prototrophy the identical mutant recipient.

Most of the P1-mediated transductions involved detecting recombination between donors with point mutations in the *azi*, *nadC*, *aceE*, *aceF* and *lpd* genes and recipients with deletion mutations. Failure to observe significant recombination indicated that the sites corresponding to the point mutations were deleted in the recipient. Wherever possible, recombination frequencies were obtained by expressing transduction frequencies as a percentage of the frequency of transduction of a standard marker (*metB*) in the same cross. Closely related media were used for different selections to ensure the highest degree of reproducibility and comparability of the results. Thus, most types of Ace$^+$ or Ace$^+$Lpd$^+$ transduction frequencies were determined with enriched succinate minimal media plus methionine, thymine, thiamin and nicotinate (as required), and the corresponding Met$^+$ transduction frequencies were obtained with the same media lacking methionine but including acetate, as required. In crosses involving the *nadC* marker, similar succinate- or glucose-based media were used without enrichment. To score the inheritance of the non-selective *azi*$^+$ marker, transductants were grown as small patches on nutrient agar and replica-plated to fresh plates of nutrient agar (Bacto) with and without sodium azide (3 mM).

Enzymology. Cultures were grown aerobically in minimal salts media and harvested in late-exponential phase (almost stationary phase) or mid-exponential phase (two to three divisions before entering stationary phase) according to Langley & Guest (1977). Anaerobic growth was obtained in stationary Erlenmeyer flasks (1 litre) filled to the neck with medium. The preparation of bacteria-free extracts and the methods for assaying overall pyruvate dehydrogenase complex (pdh complex), pyruvate dehydrogenase (E1p; EC 1.2.4.1), α-ketoglutarate dehydrogenase complex (kgdh complex), α-ketoglutarate dehydrogenase (E1kg; EC 1.2.4.2) and lipoamide dehydrogenase (E3; EC 1.6.4.3) have been described by Langley & Guest (1977). Enzyme activities in ultrasonic extracts are expressed in the same units [μmol substrate transformed (mg protein)$^{-1}$ h^{-1}] but the values obtained for different assays can only be compared in relative and not absolute terms.

RESULTS

Genetic analysis of auxotrophic aroP deletion mutants

The 28 auxotrophic *aroP* deletion mutants were grouped into six nutritionally distinct categories (Table 3). The ends of the corresponding deletions were determined by P1-mediated transductional crosses using a variety of donors (*azi*$^+$, *nadC*, *ace* and *lpd* strains, Table 2) and the deletion mutants as recipients. The results are most conveniently presented by combining the different groups of deletion mutants into three basic classes: Ace$^-$ (Nad$^-$Ace$^-$, Ace$^-$ and Ace$^-$Lpd$^-$); 'Ace$^-$' (Nad$^-$'Ace$^-$' and 'Ace$^-$'); and Ace$^+$ (Nad$^-$).

Mapping the Ace$^-$ *deletion mutants.* The positions of the right-hand termini of these deletions were determined using six *ace* mutants and one *lpd* mutant as fixed points and an *ace*$^+$ control. The results (Table 4) are summarized in Fig. 1. The relative orientation of the

Table 4. *Mapping studies with* Ace⁻ *deletion mutants*

Depending on the recipients, selections were made for Nad⁺Ace⁺ or Nad⁺Ace⁺Lpd⁺ transductants with the *nadC* donor (using unenriched succinate minimal medium) and for Nad⁺Ace⁺ or Ace⁺Lpd⁺ transductants with all the other donors listed (using enriched succinate minimal medium). In general, recombination frequencies representing the relative distances between the deletion and the corresponding point mutation were derived by expressing the numbers of transductants selected as a percentage of the number of Met⁺ transductants selected on comparable media from the same cross. The actual transduction frequencies for Met⁺ selections were in the range 1 to 123 per 10^6 P1 using the different recipients with mutant donors, and between 6 and 148 per 10^6 P1 with w3110, the wild-type donor. The frequencies of different types of Nad⁺ and Ace⁺ transduction ranged between < 0·001 and 8·0 per 10^6 P1 with mutant donors, and 11 to 123 per 10^6 P1 with the wild-type donor. Recombination frequencies normalized with respect to *metB* were not obtained with KHΔ13 and KΔ18 because these strains yielded no Met⁺ transductants. The values quoted for these strains are consequently the numbers of Ace⁺ or Ace⁺Lpd⁺ transductants per 10^6 P1 (in italics) or recombination frequencies normalized to the reference marker (*metB*) by determining the ratios Nad⁺Ace⁺:Ace⁺ (KHΔ13) or Nad⁺Ace⁺Lpd⁺:Ace⁺Lpd⁺ (KΔ18) and dividing by the average value for the ratio of the Ace⁺:Met⁺ transduction frequencies (1·19), obtained from all the other crosses.

The relevant mutations of the donors are arranged in the sequence obtained by independent mapping. The results obtained with an *ace*⁺ donor (w3110) as an external control are also included.

Recipient	Phenotype	Recombination frequency (%) with donor markers (or, in italics, transductants per 10^6 P1)								
		nadC13	*aceE64*	*aceE131*	*aceE2*	*aceE6*	*aceE1*	*aceF10*	*lpd1*	*ace*⁺
KHΔ10	Nad⁻Ace⁻	0·02	0·140	0·015	< 0·003	< 0·004	0·008	< 0·006	1·55	60
KHΔ13	Ace⁻	16·65	*0·274*	*0·146*	*0·066*	*0·560*	*1·089*	*0·304*	*0·423*	*58*
KΔ15	Ace⁻	10·12	0·695	0·085	< 0·005	< 0·008	0·022	< 0·003	2·47	195
KΔ17	Nad⁻Ace⁻	< 0·02	0·508	0·040	< 0·003	< 0·007	0·009	2·57	9·70	87
KΔ18	Ace⁻Lpd⁻	9·47	*0·100*	*0·002*	*< 0·001*	*< 0·001*	*< 0·001*	*< 0·001*	*< 0·001*	*48*
KΔ21	Ace⁻	8·48	0·672	0·226	< 0·008	0·012	0·022	1·32	11·06	172
KΔ22*	Nad⁻Ace⁻	0·04	0·241	0·015	< 0·003	0·252	0·484	3·06	21·17	304
KΔ32	Nad⁻Ace⁻	< 0·02	6·050	2·200	0·420	1·580	2·510	4·65	30·14	219
KΔ33	Ace⁻	17·92	0·447	0·246	< 0·007	< 0·007	0·042	0·716	4·90	103
KΔ34	Nad⁻Ace⁻	1·30	0·478	0·055	< 0·004	0·683	0·808	5·35	17·82	128
CΔ37	Ace⁻	10·61	0·678	0·183	< 0·002	< 0·002	0·050	< 0·002	4·20	52
CΔ40	Ace⁻	8·89	1·259	0·244	< 0·002	0·002	0·113	3·85	21·18	236
CΔ41*	Ace⁻	12·32	0·649	0·061	< 0·002	< 0·002	0·048	6·25	12·19	78
CΔ42*	Nad⁻Ace⁻	< 0·01	0·542	0·040	< 0·003	< 0·003	0·021	1·61	6·05	332
CΔ45	Ace⁻	17·39	0·576	0·159	< 0·002	< 0·002	0·137	8·05	15·85	153
CΔ46	Nad⁻Ace⁻	3·06	0·570	0·250	< 0·001	< 0·002	0·048	5·34	28·57	148

* Nad⁻Ace⁻ and Ace⁻ deletion mutants possessing detectable E2p, the *aceF* gene product.

aceE mutants *64*, *131*, *2* and *6* (from left to right) has been reported by Henning *et al.* (1968). The order and positions of *aceE2* and *aceF10* with respect to the *lpd1* marker were established by three-point crosses with *leu* and *nadC* as outside markers (Guest, 1974). Similar experiments with *nadC* as outside marker established that the *aceE6* and *aceE1* sites are between *aceE2* and *aceF10* and the recombination frequencies indicated that *aceE6* is nearer than *aceE1* to the *aceE2* site (J. R. Guest, unpublished observations). The *aceF10* mutation is reported at the most leftward (*aceE* proximal) of the *aceF* mutations (Henning & Hertz, 1964; Bisswanger & Henning, 1973). Other *aceF* mutations (*16* and *19*) originally included in this analysis proved to be either too leaky (*aceF19*) or had to be reclassified as *aceE* (*aceF16*) by failing to recombine with any of the 16 Ace⁻ deletion mutants and by exhibiting an AceE⁻F⁺ enzymological phenotype. The overall order of the *ace* mutations was confirmed in the present deletion analysis (Table 4, Fig. 1).

Two problems were encountered in the deletion analysis. First, the two *aceE* amber mutations (*aceE64* and *aceE131*) invariably gave rather high recombination frequencies with all the deletions (Table 4), even when no recombination could be detected with at least one donor mapping to the right of *aceE131*. Nevertheless, the end-points could be defined

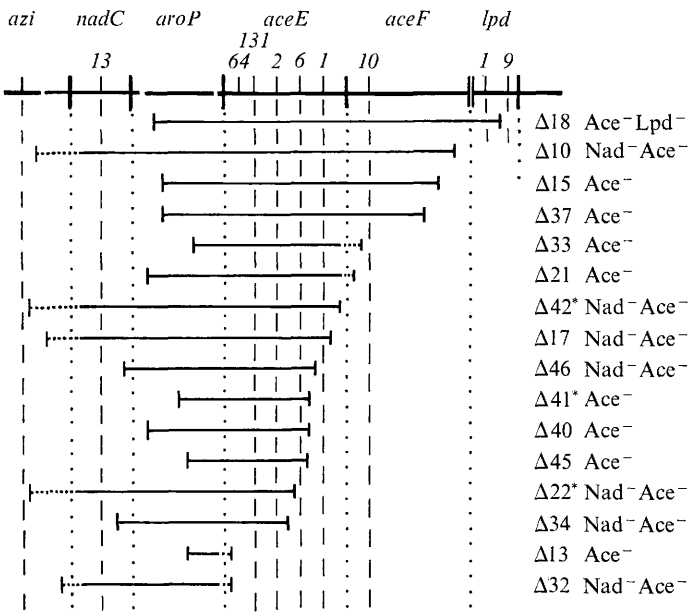

Fig. 1. Map illustrating the approximate positions and extents of the deletion mutations of spontaneous auxotrophic *aroP* mutants with Ace⁻ (Nad⁻Ace⁻, Ace⁻ and Ace⁻Lpd⁻) phenotypes. The extremities of the deleted regions relative to *azi*, *nad*, *ace* and *lpd* markers are based on the mapping data summarized in Table 4. The vertical dotted lines denote possible limits to the corresponding structural genes and broken horizontal lines indicate regions of uncertainty with respect to the deletion end-points. The asterisks indicate the three deletion mutations which generate Ace⁻ phenotypes without preventing the synthesis of detectable dihydrolipoamide acetyltransferase (E2p), the *aceF* gene product.

with some confidence using other information and it appeared that the recombination with *aceE64* was significant for only two deletions (Δ13 and Δ32). The second problem concerned two of the deletion strains (KHΔ13 and KΔ18) because Met⁺ transduction frequencies could not be obtained for the purpose of normalizing the other transduction frequencies (see Methods). However, it was possible to locate their end-points from the overall pattern of transduction frequencies (*ace* or *lpd* ends) or by reference to the Ace⁺Lpd⁺ transduction frequencies (*nadC* ends).

Six of the deletions definitely end in the *aceE* gene (between *aceE2* and *aceE1*) but the two showing significant recombination with *aceE64* (Δ13 and Δ32) may either end in the *aceE* gene or generate the Ace⁻phenotype by removing or inactivating an essential regulatory region outside the *aceE* gene. In this connexion it should be remembered that the position of the *aceE64* mutation (an extremely polar *aceE* mutation) relative to the end of the *aceE* structural gene is unknown. Two other deletions (in KΔ17 and CΔ42) probably terminate between *aceE1* and the right-hand end of the *aceE* gene (Fig. 1). This is because CΔ42 has a functional *aceF* gene product (Langley & Guest, 1977) and because the KΔ17 deletion is presumed to fall within the CΔ42 deletion by virtue of its greater recombination frequency with *aceF10* (Table 4).

The right-hand termini of the deletions in two strains (KΔ21 and KΔ33) were not assigned with certainty (Fig. 1). They could enter the *aceF* gene because they produce an AceF⁻ phenotype and recombine with *aceF10* at lower frequencies than KΔ17 and CΔ42. Alternatively, they could terminate in the *aceE* gene, beyond the *aceE1* site, and generate a polar effect on *aceF* expression (as proposed for KΔ17). Three strains (KHΔ10, KΔ15 and CΔ37) failed to recombine with *aceF10* (Table 4), but because they have functional *lpd* genes (Langley & Guest, 1977) it was assumed that they end in the *aceF* gene as shown. With

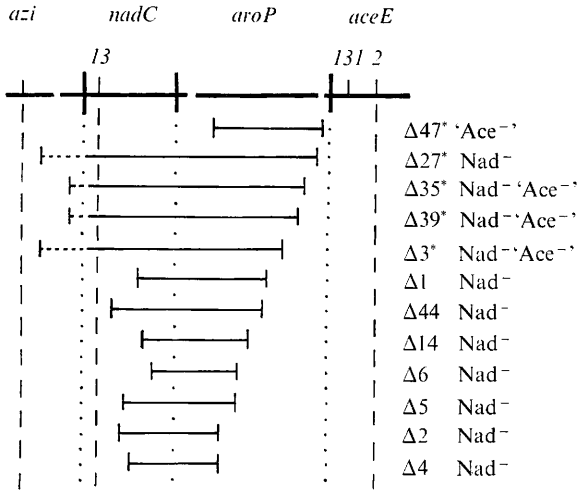

Fig. 2. Map showing the approximate positions and extents of the deletion mutations of spontaneous auxotrophic *aroP* mutants with Ace⁺ and 'Ace⁻' (Nad⁻, Nad⁻'Ace⁻' and 'Ace⁻') phenotypes. The end-points are based on the mapping data summarized in Tables 5 and 6; the precise location of the end of the *aceE* structural gene is uncertain. Deletions marked with asterisks affect expression of the *ace* operon in such a way as to generate a leaky Ace⁻ phenotype ('Ace⁻') or limit enzyme synthesis without generating any nutritional requirement for acetate.

KΔ18, the Ace⁻Lpd⁻ deletion strain, no recombination was apparent with any of the *ace* donors (except *aceE64*; see above) nor with *lpd1* (the extreme left-hand *lpd* mutation; Guest & Creaghan, 1974). This deletion appeared to terminate in the *lpd* gene between *lpd1* and *lpd9* because Ace⁺Lpd⁺ transductants (frequency 0·13 per 10^6 p.f.u.) were obtained with Hlpd9 as donor. A higher transduction frequency was obtained with Hlpd6 (0·32 per 10^6 p.f.u.) indicating a marker order *lpd1–9–6*, rather than the order *lpd1–6–9* originally reported by Guest & Creaghan (1974); unfortunately standardization of these frequencies by reference to *metB* was not possible.

The left-hand extremities of deletions in the Nad⁻Ace⁻, Ace⁻ and Ace⁻Lpd⁻ mutants were obtained from recombination frequencies with *nadC13* (Table 4) and cotransduction frequencies with an *azi* marker (see below). The deletions in the Nad⁺ strains (Ace⁻ and Ace⁻Lpd⁻) all enter and disrupt the *aroP* gene but terminate at different points to right of the *nadC* gene (Table 4; Fig. 1). Two of the seven Nad⁻Ace⁻ strains (KΔ34 and CΔ46) recombined with the *nadC* mutant indicating that their deletions terminate within the *nadC* gene but to the right of the *nadC13* site (Fig. 1). The approximate positions of the end-points of the deletions extending through the *nadC13* marker (Fig. 1) are based on cotransductional linkage with *azi* obtained by selecting Nad⁺Ace⁺ transductants with a wild-type donor (W3110) and scoring for the inheritance of the unselected *azi*⁺ marker (KHΔ10, 95 %; KΔ17, 86 %; KΔ22, 98 %; KΔ32, 72 %; CΔ42, 98 %). This assumes that there is no marked interference stemming from the different sizes of the deletions.

Mapping the 'Ace⁻' *deletion mutants.* The right-hand termini of the corresponding deletions were mapped by transduction relative to the *aceE131* and *aceE2* markers (Table 5). The distances obtained with *aceE2* as donor were used to construct the deletion map (Fig. 2) because these were considered more reliable than the rather high recombination frequencies obtained with *aceE131*. The deletion of strain CΔ47 appeared to extend closest towards (or even into) the *aceE* gene. The left-hand terminus of the CΔ47 deletion was determined relative to *nadC13* (Table 5). None of the three Nad⁻'Ace⁻' deletion mutants gave significant recombination with *nadC13* indicating that their deletions may extend beyond this marker (Fig. 2). Further evidence concerning their left-hand termini came from measurements of the cotransductional linkage with *azi* (KHΔ3, 95 %; CΔ35, 78 %; CΔ39, 78 %).

Table 5. *Mapping studies with* 'Ace$^-$' *deletion mutants*

With the Nad$^-$'Ace$^-$' recipients, selections were made for Nad$^+$ transductants on glucose minimal medium (*nad* donor) and for Nad$^+$Ace$^+$ transductants on succinate medium (*ace* donors). Transduction frequencies are expressed as the number of transductants per 10^6 P1 phage and, where possible, they are also expressed as a percentage of the number of Met$^+$ or total Nad$^+$ transductants selected on corresponding glucose or succinate media from the same cross (recombination frequencies).

With the 'Ace$^-$' recipient, Nad$^+$Ace$^+$ transductants (*nad* donor) and Ace$^+$ transductants (*ace* donors) were selected on unenriched and enriched succinate media, respectively; the transduction frequencies are related to Met$^+$ and Nad$^+$ transduction as above.

		Transduction frequencies with donor markers:								
		nadC13			*aceE131*			*aceE2*		
Recipient	Phenotype	Selection	No. per 10^6 P1	(% Met$^+$)	Selection	No. per 10^6 P1	(% Nad$^+$; % Met$^+$)	Selection	No. per 10^6 P1	(% Nad$^+$; % Met$^+$)
KHΔ3	Nad$^-$'Ace$^-$'	Nad$^+$	0·013	(—)	Nad$^+$Ace$^+$	1·04	(12·8 ; —)	Nad$^+$Ace$^+$	0·29	(6·4 ; —)
cΔ35	Nad$^-$'Ace$^-$'	Nad$^+$	< 0·001	(< 0·002)	Nad$^+$Ace$^+$	2·20	(8·0 ; —)	Nad$^+$Ace$^+$	0·72	(2·5 ; 13·1)
cΔ39	Nad$^-$'Ace$^-$'	Nad$^+$	0·010	(0·015)	Nad$^+$Ace$^+$	1·68	(5·2 ; —)	Nad$^+$Ace$^+$	1·04	(3·9 ; 15·5)
cΔ47	'Ace$^-$'	Nad$^+$Ace$^+$	10·0	(15·8)	Ace$^+$	4·06	(— ; 12·8)	Ace$^+$	3·30	(— ; 8·1)

Table 6. *Mapping studies with* Ace$^+$ *deletion mutants*

Selections were made for Nad$^+$ transductants with *nadC* (E126) and *nad*$^+$ (W3110) donors and for Nad$^+$Ace$^+$ transductants with *aceE* donors; the results are expressed as transductants per 10^6 P1 phage. Met$^+$ transductants could be selected with very few of the recipients but recombination frequencies for Nad$^+$Ace$^+$ transduction with *aceE* donors are expressed as a percentage of the Nad$^+$ transduction observed in the same cross.

	Transduction frequencies with donor markers:				
	nadC13	*aceE131*		*aceE2*	
Recipient	Nad$^+$ per 10^6 P1	Nad$^+$Ace$^+$ per 10^6 P1	(% Nad$^+$)	Nad$^+$Ace$^+$ per 10^6 P1	(% Nad$^+$)
KHΔ1	0·16	1·17	(7·9)	0·44	(7·1)
KHΔ2	0·10	2·18	(19·1)	0·68	(17·0)
KHΔ4	0·14	1·86	(25·5)	0·70	(17·1)
KHΔ5	0·11	1·64	(18·2)	0·60	(12·0)
KHΔ6	0·20	1·78	(18·2)	0·72	(10·0)
KHΔ14	0·19	1·30	(11·2)	0·54	(8·7)
KΔ27	< 0·001	1·26	(5·5)	0·36	(1·7)
CΔ44	0·08	5·20	(11·0)	2·60	(7·7)

Mapping the Ace$^+$ *deletion mutants.* The *ace*-proximal termini of the deletions of the Nad$^-$ mutants were evaluated from recombination frequencies with the *aceE131* and *aceE2* markers (Table 6, Fig. 2). Greater reliance was placed on the results obtained with *aceE2* because *aceE131* again gave characteristically high recombination frequencies. At the other end, all the deletions except one (in KΔ27) yielded Nad$^+$ transductants with *nadC13* at frequencies consistent with termini in the *nadC* gene (Table 6; Fig. 2). The deletion in KΔ27 appeared to extend beyond the *nadC* gene and this was supported by the high cotransductional linkage observed with *azi* (95 %).

It should be noted that although KΔ27 is completely acetate-independent, enzymological studies and lactate-sensitivity tests (in a *pps* background) indicated that expression of the *ace* operon was depressed in this strain (Langley & Guest, 1977). It may be significant that most of the deletion mutants exhibiting reduced expression of their *ace* operons (KHΔ3, CΔ39, CΔ35 and KΔ27) have deletions terminating very close to *aceE* at one end and extending through the *nadC13* site at the other (Fig. 2). However, CΔ47 is an exception because the *nadC* gene is not deleted. Consequently, events at either or both extremities of these deletions may be responsible for limiting the expression of the *ace* operon.

Regulatory studies on ace and lpd gene expression

The regulation of the *ace* operon and *lpd* gene were investigated using some of the deletion mutants. The response of the *lpd* gene in mutants lacking part or most of the *ace* operon was particularly relevant to investigating whether the *lpd* gene is independently regulated.

Effect of growth conditions on enzyme specific activities. Preliminary studies with one parental strain, H, showed that synthesis of the pyruvate dehydrogenase complex was induced approximately 2-fold by growth on pyruvate and repressed approximately 3·5-fold by growth on acetate, relative to growth on 10 mM-glucose (Table 7*a*). Synthesis of the complex was also repressed by growth on α-ketoglutarate and succinate, and during mid-exponential phase with excess glucose (50 mM); although relatively little repression was observed in the same phase of growth with an initial glucose concentration of 20 mM (Table 7*a*). During anaerobic growth, when the complex probably cannot function (Hansen & Henning, 1966), its synthesis was repressed but not abolished. Many of these observations confirm the findings of Dietrich & Henning (1970).

The highest specific activities of the α-ketoglutarate dehydrogenase complex were

Table 7. *Specific activities of α-keto acid dehydrogenase complexes and their components in a parental strain* (H) *and the nadC–aceF$^{\Delta}$ mutant* (HΔ10) *after different conditions of growth*

All cultures were harvested in late-exponential phase except the cultures (*) grown aerobically with glucose at 20 and 50 mM, which were harvested in mid-exponential phase. For the mutant, HΔ10, the media were supplemented with nicotinate and also acetate (2 or 4 mM) except during anaerobic growth when acetate was omitted. Enzyme specific activities were assayed in ultrasonic extracts and are expressed as μmol substrate transformed (mg protein)$^{-1}$ h^{-1}.

	Enzyme specific activity				
Growth substrate (concentration)	pdh complex	E1p	kgdh complex	E1kg	E3
(*a*) Parental strain H					
Glucose (10 mM)	5·20	1·69	2·42	4·70	3·47
Glucose (20 mM)*	4·60	0·77	2·34	3·55	2·48
Glucose (50 mM)*	2·23	0·46	0·75	2·05	0·73
Glucose (50 mM; anaerobic)	2·68	0·42	0·09	0·31	1·70
Pyruvate (50 mM)	9·88	2·25	4·28	5·10	4·78
Acetate (50 mM)	1·48	0·33	4·03	7·08	3·93
α-Ketoglutarate (50 mM)	2·37	0·61	3·63	7·12	1·56
Succinate (50 mM)	2·26	0·76	1·08	4·25	0·49
(*b*) Deletion strain HΔ10 (*nadC–aceF$^{\Delta}$*)					
Glucose (10 mM)	ND	ND	1·50	3·83	1·89
Glucose (50 mM)*	ND	ND	2·44	5·10	2·31
Glucose (50 mM; anaerobic)	ND	ND	0·22	0·41	1·30
Acetate (50 mM)	ND	ND	4·51	8·63	2·59
α-Ketoglutarate (50 mM)	ND	ND	2·74	8·60	0·94
Succinate (50 mM)	ND	ND	1·59	5·56	0·64

ND, Not detectable.

observed with pyruvate and acetate as substrates (Table 7*a*); the values with α-ketoglutarate were slightly lower. This indicates that acetate (or a derivative) induces kgdh complex synthesis, and, if α-ketoglutarate is the primary inducer, then inducing concentrations would appear to be formed more effectively from pyruvate and acetate than from exogenous α-ketoglutarate. The synthesis of the kgdh complex was repressed anaerobically and by growth with excess glucose, as has been noted by Amarasingham & Davis (1965). In further studies, diauxic growth was observed with glucose (the first substrate) plus α-ketoglutarate. However, this diauxie was not affected by concentrations of cyclic adenosine 3′:5′-monophosphate (cyclic AMP, 7·5 mM) which affected the glucose plus lactose diauxie. Engel-Rae & Henning (1973) have also shown that synthesis of the pdh complex is insensitive to cyclic AMP.

The relative ratios of pdh complex to kgdh complex varied over an 8-fold range for aerobic growth on different substrates [e.g. glucose (50 mM) relative to acetate] and over a greater range (88-fold) when anaerobic growth on glucose is considered (Table 7*a*). This indicates that the syntheses of the two complexes are independently regulated. The lipoamide dehydrogenase (E3) activities of strain H varied over a 10-fold range in response to the same changes in growth substrate, glucose concentration and aeration (Table 7*a*). No strict correlation between E3 activity and the corresponding amount of either one or the sum of both complex activities was apparent. However, the E3 activity was generally high when one or both complexes were high (e.g. with pyruvate as the substrate), indicating that some form of coupling may exist between E3 synthesis and the synthesis of the two complexes. A noteworthy exception occurs during anaerobic growth where the E3 activity appears greater than might be expected from the relative amounts of the two complexes. The E3 activity of succinate-grown organisms was also low but this was not investigated further.

With HΔ10 (*nadC–aceF$^{\Delta}$*), the mutant deleted for most of the *ace* operon, growth on acetate again induced higher activities for the kgdh complex than α-ketoglutarate (Table 7*b*).

Table 8. *Effect of thiamin-starvation on the synthesis of the α-keto acid dehydrogenase complexes and components during growth on glucose*

Cultures were grown in pairs in minimal medium containing glucose (20 mM), vitamin-free Casamino acids (0·05 %, w/v) and relevant supplements, with and without thiamin (1 μg ml^{-1}). The cultures were inoculated with bacteria, grown with thiamin, to an initial concentration of 2×10^6 organisms ml^{-1}, and shaken at 37 °C before harvesting at densities of between 3×10^8 and 5×10^8 organisms ml^{-1}. Enzyme specific activities were assayed in ultrasonic extracts in the presence or absence of thiamin pyrophosphate (+TPP, −TPP) and are expressed as μmol substrate transformed (mg protein)$^{-1}$ h^{-1}.

		Enzyme specific activity					
		pdh complex			kgdh complex		
Strain (phenotype)	Thiamin during growth	+TPP	−TPP	E1p +TPP	+TPP	E1kg +TPP	E3
H (Nad$^+$Ace$^+$)	−	6·68	< 0·01	1·96	0·34	1·43	3·37
	+	2·61	0·33	0·84	1·32	3·17	1·92
HΔ1 (Nad$^-$)	−	8·05	< 0·01	2·56	1·33	2·44	3·27
	+	4·38	0·62	1·45	1·45	2·13	2·12
HΔ2 (Nad$^-$)	−	10·70	0·10	3·20	0·79	2·21	4·48
	+	2·72	0·50	1·15	0·97	2·69	1·60
HΔ3 (Nad$^-$'Ace$^-$')	−	1·54	< 0·01	0·66	1·28	2·40	1·65
	+	0·67	0·07	0·32	2·24	2·79	1·72
HΔ4 (Nad$^-$)	−	10·16	0·04	2·40	0·72	2·10	5·04
	+	4·88	0·73	1·67	1·30	2·71	3·14
HΔ6 (Nad$^-$)	−	8·57	0·10	2·14	0·18	0·73	4·03
	+	2·92	0·40	1·25	1·05	2·92	1·78
KL14 (Nad$^+$Ace$^+$)	−	5·00	< 0·01	0·54	0·30	0·68	2·89
	+	1·13	0·21	0·18	0·18	0·70	0·48
H-LR8-16 (Nad$^+$Ace^c)	−	16·66	< 0·01	4·72	0·74	2·02	7·03
	+	10·98	0·90	2·73	2·15	2·61	5·49

Synthesis of the kgdh complex was also repressed anaerobically but, in contrast to the parental strain, it was not repressed aerobically with 50 mM-glucose. This difference is probably due to the presence of acetate (2 mM) as an essential nutrient for the deletion strain.

The lipoamide dehydrogenase (E3) activities of HΔ10 were high after growth on acetate (Table 7*b*) indicating that, in the absence of the *ace* operon, the *lpd* gene still responds to conditions inducing the kgdh complex. The presence of some form of independent expression for the *lpd* gene is also indicated because anaerobically grown HΔ10 contained considerable E3 activity despite the lack of pdh complex and the severely repressed kgdh complex (Table 7*b*). Consequently, the results obtained with strains H and HΔ10 point to the existence of a regulatory system for the *lpd* gene which reacts to couple lipoamide dehydrogenase synthesis with the expression of the *ace* and *suc* operons but also permits some independent synthesis of the E3 component.

Induction of pyruvate dehydrogenase complex synthesis by thiamin-deprivation in glucose medium. Dietrich & Henning (1970) reported that thiamin-starvation of thiamin-requiring strains induces the synthesis of the pyruvate dehydrogenase complex. This is an indirect effect which stems from the fact that thiamin (as thiamin pyrophosphate, TPP) is essential for the formation of an active complex. Consequently, thiamin-deprivation leads to the accumulation of high concentrations of pyruvate (up to 10 mM), which cannot be metabolized but continues to induce the synthesis of an inactive 'apo-complex'. This 'apo-complex' is readily assayed by adding TPP (normally present in the reaction mixture for the pdh complex).

This technique was used to investigate whether the regulation of pdh complex synthesis

Table 9. *Effect of thiamin-starvation on the synthesis of the α-keto acid dehydrogenase complexes and components during growth on acetate*

Cultures were grown in pairs in minimal medium containing acetate (50 mM), vitamin-free Casamino acids (0·05 %, w/v) and relevant supplements, with and without thiamin (1 μg ml^{-1}). The cultures were inoculated with bacteria, grown with thiamin, to an initial concentration of 2×10^6 organisms ml^{-1}, shaken at 37 °C and harvested at densities of between 3×10^8 and 5×10^8 organisms ml^{-1}. Enzyme specific activities [μmol substrate transformed (mg protein)$^{-1}$ h^{-1}] were assayed in ultrasonic extracts with TPP added to all reaction mixtures except for the E3 assay.

Strain (phenotype)	Thiamin during growth	Enzyme specific activity: pdh complex	Elp	kgdh complex	Elkg	E3
H (Nad$^+$Ace$^+$)	−	1·79	0·31	3·68	8·38	2·91
	+	1·06	0·27	1·65	6·43	1·29
HΔ10 (Nad$^-$Ace$^-$)	−	ND	ND	4·39	8·00	3·29
	+	ND	ND	2·39	7·24	1·42
HΔ13 (Ace$^-$)	−	ND	ND	3·90	8·15	2·93
	+	ND	ND	1·43	5·50	0·99

ND, Not detectable.

had been affected in several of the deletion mutants of strain H having Nad$^-$ and Nad$^-$'Ace$^-$' phenotypes (Table 8). The activities for pdh complex, Elp and E3 were increased some 2- to 2·5-fold by thiamin-starvation of the parental strain. The efficacy of the technique is apparent from the fact that no pdh complex could be detected in the absence of TPP after thiamin-starvation. Similar results were obtained with the four Nad$^-$ mutants (HΔ1, HΔ2, HΔ4 and HΔ6), confirming that these deletions have no effect on expression of the *ace* operon. It is also clear that the limited expression of the *ace* operon of HΔ3 (Nad$^-$'Ace$^-$'), characteristic of mutants with 'Ace$^-$' phenotypes (Langley & Guest, 1977), is still inducible by thiamin-starvation (Table 8). Evidently, the deletion of HΔ3 lowers the degree of expression of the *ace* operon but the control elements which respond to inducing conditions appear to be retained. A similar approach was attempted with the other deletion mutants (KΔ and CΔ; Ace$^+$ and 'Ace$^-$') and also *aroP* derivatives of KH (Ace$^+$) and K1-1LR8-16 (Ace^c). The *thi-1* mutation of KL14 was introduced by conjugation, but due to some factor residing in the K1-1 background, none of the Thi$^-$ derivatives could be satisfactorily starved of thiamin in liquid medium. Thus, in order to determine whether the constitutive expression of K1-1LR8-16 could be further induced, its *ace^c* mutation was transferred to the H background. The *nadC–aceF*$^\Delta$ mutant, HΔ10, was transduced to Nad$^+$Ace$^+$ with K1-1LR8-16 as donor and when a representative transductant, designated H-LR8-16, was subjected to thiamin-deprivation the pdh complex, Elp and E3 activities were all increased (Table 8). Thus the degree of expression dictated by the *ace^c* mutation can be modified. Some substrate-dependent variations in pdh complex activity have also been observed with *ace^c* mutants (Flatgaard *et al.*, 1971).

Thiamin-starvation tended to decrease rather than increase the activity of the kgdh complex (Table 8). This probably stems from the deficiency in active pdh complex limiting the formation of acetyl-CoA and hence lowering the overall activity of the tricarboxylic acid cycle. Despite these reciprocal changes in pdh and kgdh complex activities, there was a net increase in the sum of both complex activities during thiamin-starvation in nearly every case and this was accompanied by an increase in lipoamide dehydrogenase (E3) activity (Table 8).

Effect of thiamin-deprivation in acetate medium. If it is assumed that α-ketoglutarate (or a precursor) induces the synthesis of the kgdh complex, it should accumulate during thiamin-deprivation and induce the synthesis of an inactive kgdh 'apo-complex'. Further-

more, the use of acetate medium and mutants deleted in the *ace* operon should permit a study of the regulation of kgdh complex and lipoamide dehydrogenase synthesis in the absence of pdh complex.

The specific activities for the kgdh complex and lipoamide dehydrogenase were increased over 2-fold in the parental strain, H, during thiamin-starvation in acetate medium (Table 9). Similar changes were observed with two mutants, HΔ10 and HΔ13, deleted for the *aceE* plus *aceF* and *aceE* genes, respectively. The E1kg activities were also increased by thiamin-starvation. Consequently, the results obtained with all three strains confirm that synthesis of the kgdh complex is increased during thiamin-starvation in acetate medium, possibly by the accumulation of an inducer. Furthermore, the results confirm that in the absence of a functional *ace* operon, the *lpd* gene is not only expressed but this expression responds to environmental stimuli.

DISCUSSION

The detailed genetic characterization of spontaneous auxotrophic *aroP* mutants with deletions extending towards and through the *aceE*, *aceF* and *lpd* genes offered the possibility of correlating the nature and extent of the deletions with the corresponding phenotypes, and gaining some insight into the mechanisms controlling expression of the single *lpd* gene relative to the synthesis of the two multienzyme complexes which it serves. The results of the genetic analysis were entirely consistent with the nutritional and biochemical phenotypes of the deletion mutants. They confirmed the gene order: *azi–nadC–aroP–aceE–aceF–lpd* and also the *aceE* to *aceF* polarity of the *ace* operon. The regulatory experiments with parental and some of the deletion strains indicated that the syntheses of the two dehydrogenase complexes are independently regulated and that expression of the *lpd* gene is normally coupled to complex synthesis (*ace* and *suc* expression) but a limited amount of independent expression is also possible.

The 15 mutants with Ace$^-$ phenotypes appeared to be deleted into the *aceE* (10) or *aceE* plus *aceF* (5) structural genes. Since only three of these (KΔ22, CΔ41 and CΔ42) had detectable dihydrolipoamide acetyltransferase (E2p) activity, it must be assumed that seven of the ten deletions terminating in the *aceE* gene generate polar effects which prevent expression of the *aceF* gene. This proportion supports the prediction that two-thirds of random deletions should generate reading-frame shifts and only one-third should involve the excision of an exact multiple of three base-pairs from a given gene.

The group of deletions that limited expression of the *ace* operon to 30 % or to less than 10 % of the parental level, often generating the 'Ace$^-$' or partial mutant phenotype, were particularly interesting. Their deletions terminated closest to the standard set of *ace* point mutations. Their phenotypes could be due to fusing the *ace* genes to another, less efficient, promoter. Deletion of the *ace* promoter plus operator would lead to 'constitutive' expression of the *ace* genes at the level dictated by the new promoter, whereas expression of the *ace* operon could remain subject to normal regulatory stimuli if the operator region remained intact. The low but inducible pdh complex of one 'deletion' strain (HΔ3; Nad$^-$'Ace$^-$') is consistent with the latter situation. Recently, mutants possessing the 'Ace$^-$' phenotype have been recovered after selecting for mutagen-induced *ace* mutants (J. R. Guest, unpublished observations). They appear to have been overlooked previously but could represent *ace* promoter mutants as well as 'leaky' *ace* structural gene mutants.

The *ace* (and *lpd*) genes of some deletion mutants could be fused to regulatory elements controlling the *nadC* gene (see Fig. 1), but this possibility was not investigated because expression of the *nadC* gene is unaffected by exogenous nicotinate (Saxton *et al.*, 1968). Also, where tested, expression of the *ace* operon in Nad$^-$ deletion mutants (Ace$^+$ and 'Ace$^-$') was still subject to normal regulatory stimuli. The 'constitutive' expression of the *ace*^{c} strain (H-LR8-16) could be induced to higher levels by thiamin-starvation, but the extension

of this approach to Nad$^-$ deletion mutants of the *ace*^{c} strain (Ace$^+$ and 'Ace$^-$') proved impossible in the K1-1 background.

The inability to interpret and correlate the phenotypes and genotypes more precisely stems not only from the problem of fusion with unknown genes or genetic elements but also the present inability to define, by genetic markers, the limits of structural genes and regulatory regions in the *ace* operon. The same problems apply when considering expression of the *lpd* gene. All the deletion mutants synthesized between 30 % and 95 % of parental lipoamide dehydrogenase. However, there was no obvious correlation between deletion end-point and degree of *lpd* gene expression. Thus lipoamide dehydrogenase synthesis may be influenced by the *ace* operon, and deletions into the *ace* operon may exert polar effects on expression of the *lpd* gene.

Many different mechanisms could be proposed for the regulation of *lpd* expression. These depend to some extent on the uncertain sub-unit stoicheiometries in the multienzyme complexes and whether they are fixed or variable under different physiological conditions. The *aceF* and *lpd* genes are probably separated by less than one 'average' gene (Guest & Creaghan, 1974) and although it may have been assumed from studies with *ace* polar mutants that the *lpd* gene is expressed with the same polarity as the *ace* operon, this has not been proved. It seems unlikely that the *lpd* gene is merely the distal gene of the *ace* operon unless the *aceF–lpd* region contains a secondary transcriptional promoter and a transcribable operator, responding to regulatory factors controlling the *suc* operon. Some such modifications would be essential for explaining the apparent inducibility of E3 synthesis under conditions which induce the kgdh complex and repress the pdh complex, in the presence and absence of an intact *ace* operon.

A simpler view is to consider the *lpd* gene as a separate operon and regard the apparent polar effects on *lpd* expression of *ace* and *suc* polar mutations and some *ace* deletions as indirect effects reflecting changes in the synthesis of the E1– E2 sub-complexes (pseudo-polarity). Then, the lipoamide dehydrogenase gene could be regulated by a simple autogenous mechanism in which uncomplexed lipoamide dehydrogenase molecules serve as negatively acting repressors at an *lpd* operator region. Coupling to the *ace* and *suc* operons would be mediated by the association of uncomplexed E3 components to the E1–E2 sub-complexes. Also, the lipoamide dehydrogenase in *ace suc* double polar mutants and in HΔ10 growing anaerobically may represent the basal level of the enzymic repressor.

The evolution of the single *lpd* gene is worth considering, particularly with respect to its dual metabolic function and its close physical association with the *ace* operon. The *ace* and *suc* operons could have arisen by duplication of a common ancestral operon containing an *lpd* gene followed by deletion of the gene associated with the *suc* operon. Other evolutionary routes could be postulated and it would be interesting to investigate the organization of the corresponding genes in other bacteria and in eukaryotes.

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