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Suppression of the Succinate Requirement of Lipoamide Dehydrogenase Mutants of *Escherichia coli* by Mutations Affecting Succinate Dehydrogenase Activity

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Reversion studies with a variety of different lipoamide dehydrogenase mutants of *Escherichia coli* K12 led to the discovery of a class of partial revertant which no longer required succinate for aerobic growth on glucose but retained all the other nutritional characteristics of the Lpd$^-$ phenotype. These revertants were given the phenotypic designation Lpd$^-$Sin$^+$ to denote their succinate independence, and mutations in the gene(s) designated *sin* were considered responsible for generating the succinate-independent phenotype of *lpd* mutants. Enzymological and genetic studies confirmed that the revertants retained their *lpd* mutations. Moreover, all of the 24 independent revertants tested possessed *sin* mutations located in the *gal* region of the chromosome, presumably suppressing by an intergenic and indirect mechanism. The *sin* mutations exhibited no allele specificity for suppression and they were recessive to the wild-type *sin*$^+$ allele in merodiploid strains.

A common feature of the Lpd$^-$Sin$^+$ revertants (succinate-independent derivatives of *lpd* mutants) was a deficiency in succinate dehydrogenase. On their own, the *sin* mutations produced a phenotype analogous to that of *sdh* mutants. Furthermore, the probable identity of *sin* and *sdh* mutations was confirmed by reconstruction studies in which an *lpd sdh* double mutant was shown to exhibit an Lpd$^-$Sin$^+$ phenotype. It was concluded that the requirement for exogenous succinate by mutants lacking lipoamide dehydrogenase (and hence overall 2-oxoglutarate dehydrogenase complex activity) can be eliminated by inactivating succinate dehydrogenase because the latter enzyme depletes the intracellular succinate and succinylCoA pools by removing succinate faster than it can be supplied by other endogenous mechanisms. In fact, it would appear that the presence of active succinate dehydrogenase is responsible for the nutritional requirement for succinate of mutants lacking overall 2-oxoglutarate dehydrogenase complex activity during aerobic growth on glucose. A gene-dosage effect on the activities of the 2-oxoglutarate dehydrogenase complex was also demonstrated with corresponding merodiploid strains.

INTRODUCTION

The lipoamide dehydrogenase components (E3) of the pyruvate and 2-oxoglutarate dehydrogenase complexes of *Escherichia coli* K12 are specified by a single gene designated *lpd* (Guest & Creaghan, 1973, 1974; Guest, 1974). This gene is situated at 2·3 min in the *E. coli* linkage map, very close to the *aceE* and *aceF* genes which specify the dehydrogenase (E1) and dihydrolipoamide acyltransferase (E2) components of the pyruvate dehydrogenase complex, but approximately 14 min from the *sucA* and *sucB* genes specifying the corresponding components of the 2-oxoglutarate dehydrogenase complex. Lipoamide dehydrogenase-deficient mutants (*lpd*) require exogenous acetate and succinate for aerobic growth

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on glucose minimal medium, but not for anaerobic growth on glucose. The *lpd* mutants also fail to utilize either acetate or succinate as sole carbon and energy sources.

Reversion studies, which indicated that a wild-type phenotype could be restored by a single reversion event, were consistent with the existence of just one *lpd* gene (Guest & Creaghan, 1973). During the course of further studies with the *lpd* mutants, different classes of revertant were detected. Derivatives of several *lpd* mutants which still require acetate but no longer require succinate for aerobic growth on glucose are described here. The results indicate that suppression of this succinate-dependent component of the Lpd$^-$ phenotype is due to failure to synthesize an active succinate dehydrogenase.

METHODS

Bacterial strains. The properties of the *lpd* mutants and their parental strains have been described previously (Guest & Creaghan, 1973, 1974; Langley & Guest, 1977) but new designations have been allocated to many of the mutants previously referred to by abbreviated genotypic or phenotypic symbols. Some of the corresponding designations and genotypes are as follows. Strain JRG74: *trpE61 trpA58 iclR* (formerly T3A58), the parent strain of JRG301 (*lpd1*) and the Lpd$^+$ revertant JRG325 (formerly T3A58*lpd1RA1*). Strain JRG160: *gal trpA9761 trpR iclR lpd5* (formerly WGA*lpd5*). Strains JRG355 and 359: Hfr *thiA azi lpd8* and *9* (formerly H*lpd8* and H*lpd9* respectively). Strain JRG599: *metB thy azi ton pps aroP–lpdΔ18* (formerly KΔ18). A leucine-requiring derivative (JRG384) of a spontaneous streptomycin-resistant strain (JRG419: *gal trpA9761 trpR iclR str*, formerly WGAS, originating in W3110) was used to prepare P1-lysogenic *lpd1* (JRG367), *lpd8* (JRG374) and *lpd9* (JRG375) derivatives by selecting Leu$^+$ transductants with the corresponding *lpd* mutant donors. The *aroP–lpdΔ18* deletion of JRG599 was transferred to a similar background to provide the P1-sensitive strain JRG721 (*gal trpR iclR str aroP–lpdΔ18*) by first selecting a Trp$^+$ transductant of JRG384 and then transducing the product (JRG719) to Leu$^+$ with JRG599 as donor and testing for simultaneous inheritance of AroP$^-$ and Lpd$^-$ phenotypes. Twelve spontaneous succinate-independent revertants containing *sin* mutations (*sin-1* to *sin-12*) were isolated from several *lpd* parents (see Results) for detailed enzymological and genetic studies. A further 12 independent *sin* mutations (*sin-13* to *sin-24*) were obtained by spontaneous reversion of the *lpdΔ* strains, JRG721 and JRG721R.

Three strains possessing two independent succinate dehydrogenase mutations were used: W945*sdhgal*$^+$ (*sdh thr thi*; Creaghan & Guest, 1972); JRG660 (*gal trpA trpR iclR str sdh-18*; isolated by Dr P. Lambden); and a Gal$^+$ derivative, JRG691, obtained by conjugation between F151-1(*gal*$^+$)/JC1553 and JRG660.

Several F′ donor strains containing F′ plasmids in different Rec$^-$ backgrounds were used for strain construction and mapping. These included: KLF4 carrying the F104 (*leu*$^+$*lpd*$^+$) plasmid in AB2463 from K. B. Low and a series of F-*gal*$^+$ strains derived by transferring the F152-1, F8, F450 (F100-1), F1s (F100-2) and F152 plasmids from strain N23–53 (E. Ohtsubo) to JC1553.

To construct recombination-deficient derivatives of JRG721 and JRG730, spontaneous *thy* mutants were first isolated by plating these cultures (range 10^7 to 10^{10} organisms per plate) on appropriately supplemented glucose minimal medium containing trimethoprim (10 μg ml^{-1}) and thymine (50 μg ml^{-1}) according to Stacey & Simson (1965). Some 98 % of the surviving colonies were found to be Thy$^-$ and representatives were conjugated with KL16–99 (Hfr, *recA1 thi thy*$^+$). A proportion of the selected Thy$^+$Str^R conjugants were recognized as Rec$^-$ by their high sensitivity to ultraviolet radiation. The products used in dominance tests were: JRG721R (*aroP–lpdΔ18 gal trpR iclR str recA1*) and JRG730R (*sin-8 gal trpA trpR? iclR str recA1*).

Abbreviations. Most of the genetic symbols (and corresponding phenotypic abbreviations) are those used in the recalibrated *E. coli* linkage map (Bachmann, Low & Taylor, 1976). Mutation in the gene(s) designated *sin* is responsible for the succinate independence (Sin$^+$ phenotype) of *lpd* mutants during aerobic growth on glucose. The Lpd$^-$ phenotype is characterized by the requirement for succinate (lack of succinate independence, Sin$^-$) and the requirement for acetate (lack of acetate independence, Ain$^-$) for aerobic growth on glucose, as well as the inability to utilize succinate (Sut$^-$) and the inability to utilize acetate (Aut$^-$) as sole carbon and energy sources, i.e. Lpd$^-$ = Sin$^-$Ain$^-$Sut$^-$Aut$^-$. Consequently, revertants possessing *sin* mutations have the Lpd$^-$Sin$^+$, phenotype i.e. the Sin$^+$Ain$^-$Sut$^-$Aut$^-$ nutritional phenotype and the *lpd*$^-$*sin*$^-$ genotype. On their own, all the *sin* mutations studied in the present work generated the same nutritional phenotype as *sdh* mutations, i.e. Sin$^+$ and Sdh$^-$ = Sin$^+$Ain$^+$Sut$^-$Aut$^-$.

Media. The basal minimal medium E of Vogel & Bonner (1956) was used in all experiments except for the growth tests in liquid medium where the citrate-free medium of Spencer & Guest (1973) was used. Independent tests showed that the presence of citrate had no effect on the nutritional phenotypes described. Substrates were (mM): D-glucose, 11; D-galactose, 28; potassium succinate, 40; and potassium acetate, 40. Supplements of acetate (2 mM), succinate (2 mM), L-lysine (40 μg ml^{-1}), L-methionine (20 μg ml^{-1}) and other

appropriate supplements were added to minimal media where necessary. Thymine (50 μg ml^{-1}) was added to media when required. The complex medium used for growth and maintenance of organisms was L broth and L agar (Lennox, 1955), except for merodiploids, which were grown on minimal media designed to maintain their plasmids. All media were solidified with Difco Bacto agar (15 g l^{-1}). Some of the selective media containing acetate or succinate as substrates were enriched with Difco Bacto nutrient broth (0·2 %, v/v).

Preparation of extracts for enzymology. Organisms were grown in 500 ml of glucose minimal medium plus relevant supplements, in 2 l Erlenmeyer flasks shaken at 37 °C. Cultures were harvested in late exponential phase, washed twice with potassium phosphate buffer (0·04 M; pH 7·8) and finally resuspended in the same buffer at a concentration of 0·2 g wet wt ml^{-1}. The suspensions were disrupted at 0 °C for two periods of 2 min with an ultrasonic cell disintegrator (M.S.E., 150 W) and debris was removed by centrifuging at 15000 *g* for 20 min. The supernatant ultrasonic extracts were assayed for protein according to Lowry *et al.* (1951) using bovine serum albumin as standard. Samples of each culture were tested to ensure that reversion had not occurred.

Enzyme assays. Enzyme activities of the ultrasonic extracts were all measured spectrophotometrically in the region of proportionality between initial reaction velocity and protein concentration at 25 °C (unless stated otherwise) and are quoted as μmol substrate transformed (mg protein)$^{-1}$ h^{-1}.

Pyruvate dehydrogenase complex and 2-oxoglutarate dehydrogenase complex were assayed at 366 nm and pH 8·5 by recording the 2-oxo acid-dependent reduction of 3-acetylNAD by the method of Guest & Creaghan (1973) except that $MgCl_2$ (5 μmol) was added to obtain maximum specific activity when assaying the pyruvate dehydrogenase complex.

2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was assayed at 420 nm and pH 6·3 with ferricyanide as the electron acceptor by a spectrophotometric method based on the manometric procedure of Hager & Kornberg (1961).

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

Succinate dehydrogenase (EC 1.3.99.1) was assayed with 2,6-dichlorophenolindophenol as electron acceptor at 600 nm by the method of Spencer & Guest (1973).

Fumarate reductase (EC 1.3.99.1) was assayed anaerobically (under He) at 550 nm by recording the fumarate-dependent oxidation of reduced benzylviologen by the method of Spencer & Guest (1973).

Succinate-semialdehyde dehydrogenase (EC 1.2.1.16) was assayed by recording the substrate-dependent reduction of NADP at 340 nm, pH 9·2 and 40 °C by the method of Dover & Halpern (1972) with substrate synthesized according to Hendler & Anfinson (1954).

Isocitrate lyase (EC 4.1.3.1) was assayed at 324 nm and pH 6·8 according to the method of Dixon & Kornberg (1959), except that ethylenediaminetetraacetate was substituted for cysteine as suggested by Kennedy & Dilworth (1963).

Genetic methods. Conjugations were performed by cross-streak tests on plates of selective medium at 37 °C. Washed suspensions of 10-fold concentrated early-exponential phase cultures of donor strains were streaked at right-angles across washed suspensions of stationary phase recipient cultures. Conjugants were picked and purified from the tail of the donor streak and then tested for the inheritance of non-selective markers.

Transductions with phage P1 were performed by the method of Spencer, Lebeter & Guest (1976) using a multiplicity of infection of 2 (or 0·1 for constructing non-lysogenic transductants). Transductants were purified on the selective medium before scoring the inheritance of non-selective markers.

RESULTS

Reversion studies with lipoamide dehydrogenase mutants

The *lpd* mutants of *E. coli* may be regarded as having a composite nutritional phenotype, Sin$^-$Ain$^-$Sut$^-$Aut$^-$, denoting failure to grow on glucose without succinate and acetate (lack of succinate and acetate independence, Sin$^-$Ain$^-$) and the inability to utilize succinate (Sut$^-$) and acetate (Aut$^-$) as sole carbon and energy sources. In a detailed investigation of the reversion of three representative *lpd* mutants, different classes of revertant were obtained depending on the primary nutritional phenotype selected (Table 1). The mutants were chosen for their different immunological properties (Guest & Creaghan, 1974): JRG301 (*lpd1*, poor CRM-positive), JRG355 (*lpd8*, CRM-negative) and JRG359 (*lpd9*, CRM-positive), but each gave similar reversion patterns. Complete reversion to a wild-type phenotype (Lpd$^+$) was observed in each of the primary selections; the back-mutant was indeed the sole

Table 1. *Reversion of lpd mutants*

Spontaneous revertants of three *lpd* mutants were selected by spreading washed saline suspensions containing 5×10^7 bacteria derived from several independent L broth cultures on different media: unsupplemented glucose medium (Sin^+Ain^+ selection), acetate- or succinate-supplemented glucose medium (Sin^+ or Ain^+ selections) and enriched acetate medium (Aut^+ selection). After incubating at 37 °C for 2 to 5 days, revertant colonies were picked and purified on the primary selective medium before investigating the complete nutritional phenotype. The Lpd^-Sin^+ revertants arising from the Sin^+Ain^+ selections were also recognized by their slow growth during purification in the absence of added acetate. Aggregate results for the three mutants are quoted because the reversion patterns of the individuals were similar.

Primary selection	Number tested	Phenotype	Number (%)	Reversion frequency (no. per 10^6 plated)
Sin^+Ain^+	344	$Sin^+Ain^+Sut^+Aut^+$ (Lpd^+)	203 (59)	1·52
		$Sin^+Ain^-Sut^-Aut^-$ (Lpd^-Sin^+)	141 (41)	1·10
Sin^+	418	$Sin^+Ain^+Sut^+Aut^+$ (Lpd^+)	23 (6)	0·17
		$Sin^+Ain^-Sut^-Aut^-$ (Lpd^-Sin^+)	395 (94)	2·74
Ain^+	215	$Sin^+Ain^+Sut^+Aut^+$ (Lpd^+)	157 (73)	0·25
		$Sin^-Ain^+Sut^-Aut^-$ (Lpd^-Ain^+)	58 (27)	0·10
Aut^+	80	$Sin^+Ain^+Sut^+Aut^+$ (Lpd^+)	80 (100)	0·30

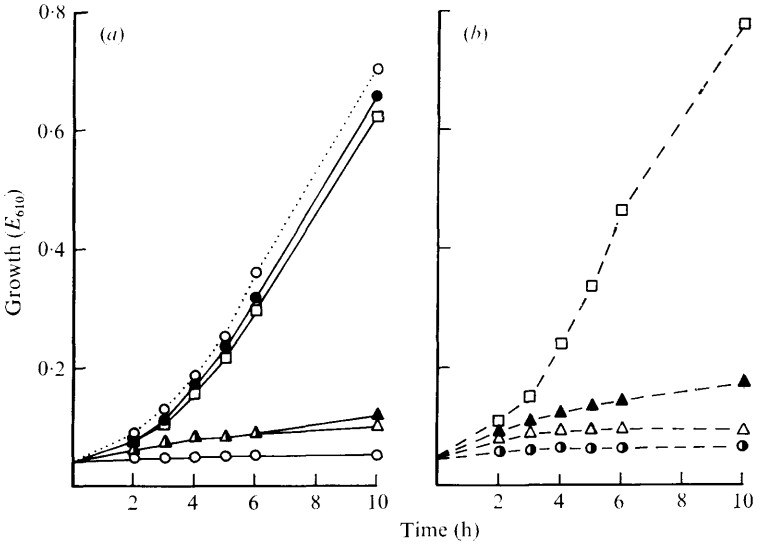


Fig. 1. Growth of a lipoamide dehydrogenase mutant, Lpd^- (JRG301, *lpd1*), and its revertant derivatives, Lpd^+ (JRG325, *lpd*$^+$) and Lpd^-Sin^+ (JRG326, *lpd1 sin-1*), in glucose minimal medium. Cultures were shaken at 37 °C in 250 ml Erlenmeyer flasks fitted with optically-matched side arms. Each flask contained 10 ml of citrate-free basal medium plus glucose (0·4 %, w/v), L-tryptophan (30 μg ml^{-1}) and supplements: ○, none; ●, acetate; △, succinate; ▲, lysine plus methionine; □, acetate plus succinate. The inocula were prepared from washed suspensions of 16 h cultures of the organisms grown in appropriately supplemented glucose minimal medium and added to give an initial extinction of 0·05 (5×10^7 bacteria ml^{-1}). Growth was measured as extinction at 610 nm for (*a*) the revertants: ·····, Lpd^+; ——, Lpd^-Sin^+; and (*b*) the parental mutant: – – –, Lpd^-. No significant reversion was detected in any of the cultures which grew.

class recovered by selecting for the ability to utilize acetate. However, relatively large numbers of revertants, designated Lpd^-Sin^+ due to their succinate independence but retention of all the other components of the Lpd^- phenotype, were recovered by primary selection on glucose minimal medium with or without an acetate supplement. The appearance of these succinate-independent but acetate-dependent revertants on acetate-free medium is probably permitted

by acetate excretion from adjacent Lpd$^+$ revertants. Acetate-independent but otherwise Lpd$^-$ revertants (Lpd$^-$Ain$^+$) arose only on succinate-supplemented glucose minimal medium and represented a third class of revertant. The same revertant classes were obtained with all of the 10 independent *lpd* mutants isolated by Guest & Creaghan (1973) and with derivatives obtained by transferring their *lpd* mutations into other genetic backgrounds. Furthermore, Lpd$^-$Sin$^+$ and Lpd$^-$Ain$^+$ (but not Lpd$^+$) revertants of a mutant (KΔ18; JRG599) deleted for the *aroP–lpd* region were also readily isolated (Langley & Guest, 1977), and this seemed to rule out explanations of the revertant phenotypes based on secondary mutations in the *lpd* gene generating complex-specific lipoamide dehydrogenase in sufficient amount to promote growth on glucose.

Growth tests in liquid media were performed in order to define the revertant phenotypes under more stringent conditions (Fig. 1). The results confirmed that the Lpd$^+$ revertants grew on unsupplemented glucose minimal medium and that the Lpd$^-$Sin$^+$ revertants required acetate (Fig. 1*a*) whereas the original Lpd$^-$ mutants required both acetate and succinate (Fig. 1*b*). However, the revertants designated Lpd$^-$Ain$^+$ by growth tests on solid media, proved to be acetate-dependent and indistinguishable from the Lpd$^-$ mutants in liquid media. The reason for this discrepancy is not clear. Presumably these revertants retain some requirement for acetate which may be spared by exogenous nutrients present in the agar or by endogenously generated acetate providing a permissive concentration of this nutrient within and around a developing colony but insufficient when diluted in liquid medium. This class of revertant was not investigated further.

A total of 12 independent Lpd$^-$Sin$^+$ revertants were retained for parallel enzymological and genetic studies. They all needed acetate (Ain$^-$) but not succinate (Sin$^+$) for aerobic growth on glucose and used neither succinate nor acetate as sole carbon and energy sources (Sut$^-$Aut$^-$). Independent spontaneous mutations generating the Sin$^+$ phenotype were given individual *sin* allele numbers and these were first isolated in the following mutant strains. Strain JRG301 (*lpd1*): *sin-1* (JRG326), *sin-2* (JRG629) and *sin-3* (JRG630). Strain JRG160 (*lpd5*): *sin-4* (JRG631). Strain JRG355 (*lpd8*): *sin-5* (JRG632). Strain JRG359 (*lpd9*): *sin-6* (JRG633) and *sin-7* (JRG634). Strain JRG367 (*lpd1*): *sin-8* (JRG709). Strain JRG374 (*lpd8*): *sin-9* (JRG710). Strain JRG375 (*lpd9*): *sin-10* (JRG711). Strain JRG599 (*aroP–lpd*$^{\Delta}$*18*): *sin-11* (JRG682) and *sin-12* (JRG683).

Enzymological studies with Lpd$^-$Sin$^+$ *revertants*

Ultrasonic extracts of the Lpd$^-$Sin$^+$ revertants were assayed for relevant enzyme activities and compared with the corresponding Lpd$^-$ mutant strains as well as typical Lpd$^+$ parental (JRG74) and Lpd$^+$ revertant (JRG325) strains. Representative results (Table 2) show that the activities of both the pyruvate and 2-oxoglutarate dehydrogenase complexes and lipoamide dehydrogenase were restored in the Lpd$^+$ revertant but not in the Lpd$^-$Sin$^+$ revertants. Similar results were obtained for all 12 Lpd$^-$Sin$^+$ derivatives examined, regardless of the nature of the original *lpd* mutation or the genetic backgrounds into which different mutations had been placed before reversion.

These results posed the problem of how the requirement for succinate could be suppressed in the absence of lipoamide dehydrogenase. Several possibilities were considered. One involved the derepression or feedback resistance of enzymes potentially capable of generating succinate or succinylCoA but normally repressed or inhibited during aerobic growth on glucose. For example, fumarate reductase, which is repressed aerobically (Peck, Smith & Gest, 1957; Spencer & Guest, 1973), could become derepressed to generate succinate from fumarate. Such a possibility would be expected to increase the rate of interconversion of succinate and fumarate as measured in the assays for fumarate reductase or succinate dehydrogenase. However, no such effect was detected when Lpd$^-$ strains and the corresponding Lpd$^-$Sin$^+$ revertants were compared (Table 2). Isocitrate lyase is also normally repressed and inhibited during aerobic growth on glucose (Kornberg, 1966). However, the

Table 2. *Enzyme activities of representative succinate-independent revertants* (Lpd$^-$Sin$^+$) *and related strains*

Ultrasonic extracts of cultures grown in glucose minimal medium supplemented with acetate and succinate (2 mM) were assayed as described in the Methods. The specific activities are expressed as μmol substrate transformed (mg protein)$^{-1}$ h^{-1} and represent averages of several determinations. Abbrevations: pdh complex, overall pyruvate dehydrogenase complex; ogdh complex, overall 2-oxoglutarate dehydrogenase complex; lpdh, lipoamide dehydrogenase; ogdh, 2-oxoglutarate dehydrogenase; ssdh, succinate-semialdehyde dehydrogenase; icl, isocitrate lyase; frd, fumarate reductase; sdh, succinate dehydrogenase.

		Enzyme specific activity							
Strain	Relevant genotype	pdh complex	ogdh complex	lpdh	ogdh	ssdh	icl	frd	sdh
JRG74	*lpd*$^+$ (wild-type)	2·40	1·60	2·61	1·26	4·44	10·2	1·2	9·81
JRG301	*lpd1*	< 0·01	< 0·01	< 0·01	0·63	4·78	5·0	1·1	3·57
JRG325	*lpd*$^+$ (back mutant)	3·02	1·84	2·21	7·00	3·42	14·2	1·1	9·80
JRG326	*lpd1 sin-1*	< 0·01	< 0·01	< 0·01	0·87	1·93	2·9	1·1	0·31
JRG355	*lpd8*	< 0·01	< 0·01	< 0·01	3·49	2·14	0·8	1·0	2·23
JRG632	*lpd8 sin-5*	< 0·01	< 0·01	< 0·01	1·02	1·92	0·8	1·0	0·06
JRG359	*lpd9*	< 0·01	< 0·01	0·04	3·20	2·52	1·3	1·0	4·19
JRG634	*lpd9 sin-7*	< 0·01	< 0·01	0·15	4·02	2·46	1·4	1·0	0·06
JRG367	*lpd1*	< 0·01	< 0·01	< 0·01	0·27	< 0·02	3·6	1·0	2·10
JRG709	*lpd1 sin-8*	< 0·01	< 0·01	0·07	0·39	< 0·02	4·3	1·0	0·06
JRG599	*lpd*$^{\Delta}$*18*	< 0·01	< 0·01	< 0·01	3·08	0·94	0·4	1·0	2·55
JRG683	*lpd*$^{\Delta}$*18 sin-12*	< 0·01	< 0·01	< 0·01	2·95	0·83	0·5	1·0	0·11

Lpd$^-$Sin$^+$ phenotype was not correlated with an increase in the specific activity for this enzyme (Table 2). Considerable variation in isocitrate lyase activity was observed between different sets of strains. This was traced to differences in their genetic backgrounds rather than being a consequence of the *lpd* and other mutations. Thus, isocitrate lyase was found to be synthesized constitutively in strains W3110, W1485E and their derivatives, but it was inducible in HfrH and its derivatives.

Another possibility was the activation of other routes leading to succinate, succinylCoA or compounds which require succinylCoA for their synthesis (e.g. 5-aminolaevulinate, porphyrins, methionine, lysine and diaminopimelate). For example, a route involving the decarboxylation of 2-oxoglutarate and the oxidation of succinate semialdehyde could yield succinate. Succinate-semialdehyde dehydrogenase was present in many of the mutants (Table 2) and its substrate could be generated from 2-oxoglutarate [via the thiamin pyrophosphate (TPP) adduct] by 2-oxoglutarate dehydrogenase, which is present in all the strains. However, succinate independence was not correlated with an increase in succinate-semialdehyde dehydrogenase activity. Furthermore, because this enzyme was not detectable in any of the mutants and revertants isolated in the W3110 background (e.g. JRG367 and JRG709) it could not be responsible for the Sin$^+$ phenotype in these strains. No other mechanism for the production of succinate or succinylCoA from 2-oxoglutarate which by-passes the 2-oxoglutarate dehydrogenase complex could be found.

Finally, the possibility of blocking metabolic reactions which might normally deplete the intracellular succinate pool, e.g. succinate oxidation or reduction and succinate transport, was considered. No mechanism involving the reduction of succinate, including the reversal of the succinate-semialdehyde dehydrogenase reaction, could be detected. However, a common feature of the Lpd$^-$Sin$^+$ revertants was a deficiency in succinate dehydrogenase (Table 2). This was true for all of the 12 Lpd$^-$Sin$^+$ revertants examined. Thus it was concluded that succinate independence stems from a deficiency in succinate dehydrogenase. Conversely, the presence of an active succinate dehydrogenase may be presumed responsible

Table 3. *Linkage relationships between sin, sdh and gal mutations*

Phage P1-mediated transduction was performed by the technique described in the Methods. Gal+ transductants were selected on galactose minimal medium supplemented with acetate and succinate, purified on the same medium and tested for the Lpd−Sin+ and Lpd− phenotypes on appropriately supplemented glucose media as well as succinate and acetate minimal media. In crosses 1 and 2, Sin+ (Lpd−Sin+) transductants were selected on glucose medium supplemented with acetate. As controls the recipients were plated on the same media to ensure that reversion was insignificant compared with the numbers of transductants isolated after exposure to P1 lysates.

Cross	Donor	Recipient	Selection	Number scored	Unselected donor phenotype	Cotransduction frequency (%)
1	JRG326 (*lpd1 sin-1*)	JRG367 (*gal lpd1*)	Gal+	54	Sin+	81
			Sin+	102	Gal+	84
2	W945*sdhgal*+	JRG367 (*gal lpd1*)	Gal+	95	Sin+ (*sdh*−)	89
			Sin+ (*sdh*−)	104	Gal+	67
3	JRG725 (*sin-3*)	JRG721 (*gal lpd*Δ)	Gal+	72	Sin+	79
4	JRG727 (*sin-5*)	JRG721 (*gal lpd*Δ)	Gal+	35	Sin+	68
5	JRG729 (*sin-7*)	JRG721 (*gal lpd*Δ)	Gal+	74	Sin+	53
6	JRG691 (*sdh-18*)	JRG721 (*gal lpd*Δ)	Gal+	74	Sin+ (*sdh*−)	68

for the succinate requirement of *lpd* mutants. By depleting the endogenously generated intracellular succinate pool, an exogenous supply of succinate becomes necessary to compensate for the loss.

Genetic studies with Lpd−Sin+ *revertants*

Chromosomal location of the sin mutations. Genetic methods were originally adopted to determine whether the Lpd−Sin+ phenotype was due to intragenic suppression by secondary mutations (*sin*) in the *lpd* gene. The *lpd* and *nadC* genes are 70 % cotransducible (Guest, 1974) but no *nadC–sin* linkage (< 0·5 %) could be detected when Nad+ transductants from crosses between *lpd*−*sin*− revertant donors and *lpd*−*nadC*− and *nadC*− recipients were examined. Retention by the revertants of the *lpd* mutation was confirmed and it was concluded that the *lpd* and *sin* mutations are not closely linked. Also the isolation of Lpd−Sin+ revertants of an *lpd* deletion mutant ruled out intragenic suppression as the basis of this partial reversion. Consequently, the *sin* gene(s) in its mutated form, *sin*−, was defined as generating succinate independence in *lpd* mutants.

Because the enzymological studies had implicated succinate dehydrogenase and because the succinate dehydrogenase gene (*sdh*) is known to be cotransducible with *gal* (Creaghan & Guest, 1972), further attempts to locate the *sin* mutations were made by testing for cotransducibility with *gal*. Transduction between *gal*+*lpd*−*sin*− revertant donors and *gal*−*lpd*− mutant recipients enabled primary selections for both Gal+ and Sin+ (Lpd−Sin+) transductants. In every case tested, cotransducibility of *gal* and *sin* markers was clearly demonstrated (see Table 3, cross 1, for representative results). Using an independent *gal*+*sdh*− donor in analogous experiments (Table 3, cross 2), similar frequencies of cotransduction between *gal* and *sdh* markers were observed. Furthermore, transductants possessing the characteristic Lpd−Sin+ phenotype (but presumably *lpd sdh* double mutants) were recovered from these crosses either as a class of Gal+ transductants or by direct selection for the Sin+ phenotype. In related transductional crosses using wild-type donors and *lpd*− *sin*− revertant recipients, selection for acetate-independent growth on glucose yielded transductants (Ain+) which were all incapable of growth on succinate and acetate (Sut− Aut−). These transductants had presumably inherited the *lpd*+ allele from the donor to gain the Ain+ phenotype but failed to express the complete Lpd+ phenotype because they retained the unlinked *sin* mutation. Furthermore, no succinate-utilizing (Sut+) or acetate-utilizing (Aut+) transductants were ever recovered from these crosses by direct selection. On their own the *sin* mutations must therefore be responsible for the inability to utilize succinate and

Table 4. *Conjugation with F′ donors*

The chromosomal markers carried by the plasmids are delineated by the horizontal lines. Conjugations were performed by cross-streak tests (see Methods) using appropriate nutritional counterselection against the donors. Selection was for succinate-utilizing conjugants (Sut$^+$: *sin*$^+$ or *sdh*$^+$) and the results obtained with different donors are recorded as: +, positive; or −, negative. All the donors produced Gal$^+$ conjugants with this group of recipients.

	Chromosomal markers carried by plasmid	Recipient				
Donor	*fep lip supE ubiF sdh sucAB tol nadA aroG gal pgl attλ uvrB*	JRG726 (*sin-4*)	JRG730 (*sin-8*)	JRG731 (*sin-9*)	JRG732 (*sin-10*)	JRG660 (*sdh-18*)
F152-1		−	−	−	−	−
F8		−	−	−	−	−
F450 (F100-1)		−	−	−	−	−
F1s (F100-2)		+	+	+	−	+
F152		+	+	+	−	+

acetate, the Sut$^-$Aut$^-$ phenotype, which is identical to the nutritional phenotype of *sdh* mutants. Consequently, these genetic studies confirmed the relationship between succinate dehydrogenase and the succinate independence of *lpd* mutants, i.e. *sdh* and *sin* mutations are analogous and *lpd sdh* and *lpd sin* double mutants possess the Lpd$^-$Sin$^+$ phenotype.

Further genetic studies were designed to test 10 independent Lpd$^-$Sin$^+$ revertants for similar *gal*-linked *sin* mutations and to test the suppressor-specificity of individual *sin* mutations against *lpd* mutations other than that resident in the strain in which they were originally selected. In one group of experiments the wild-type *lpd*$^+$ allele was introduced into the Lpd$^-$Sin$^+$ revertants (*lpd*$^-$*sin*$^-$) by conjugation with the F′ donor, KLF4, followed by selection for acetate-independent aerobic growth on glucose. The *lpd*$^+$*sin*$^-$ products containing the *sin-1* to *sin-10* mutations were given the designations JRG723 to 732 and they all resembled *sdh* mutants by being unable to utilize acetate or succinate as sole carbon and energy sources. However, the *sin* designation was retained to emphasize their origin in the Lpd$^-$Sin$^+$ revertants, despite the apparent identity of the phenotype (Sut$^-$Aut$^-$) generated by the *sin* and *sdh* mutations. The *lpd*$^+$*sin*$^-$ derivatives were more suitable for use as donors in P1-mediated crosses because they gave lysates of high titre relative to the very poor lysates obtained with *lpd*$^-$*sin*$^-$ strains. The *sin* mutants and another representative *sdh* mutant were each crossed with the *lpd* deletion mutant, JRG721 (*gal aroP–lpd*$^{\Delta}$*18*; see Methods for construction). Gal$^+$ transductants were selected and the frequencies for *sin–gal* cotransduction ranged between 50 and 80 % (see Table 3, crosses 3 to 5 for representative results); *sdh* and *gal* were 68 % cotransducible in the analogous cross (Table 3, cross 6). These experiments confirmed the presence of *gal*-linked *sin* mutations in the Lpd$^-$Sin$^+$ revertants. They also demonstrated that the mechanism of *sin*-mediated suppression is indirect and not allele-specific because *sin* mutations isolated with a variety of different *lpd* mutants (and also an independent *sdh* mutation) were all capable of suppressing the *lpd* deletion mutation.

The location of the *sin* mutations was also investigated by a series of cross-streak conjugations using a group of five F′ donors carrying well characterized F-*gal*$^+$ plasmids (Sharp *et al.*, 1972; Ohtsubo & Hsu, personal communication) in a Rec$^-$ background (Table 4). The series of *lpd*$^+$*sin*$^-$ strains (*sin-1* to *sin-10*) derived from the Lpd$^-$Sin$^+$ revertants by conjugation with KLF4 (see above) plus JRG660 (*gal sdh-18*) were used as recipients and nutritional counterselection was employed against the donors. All the F′ donors gave Gal$^+$ conjugants but only two, F1s (F100-2) and F152, produced succinate-utilizing (Sut$^+$; *sin*$^+$ or *sdh*$^+$) conjugants (see Table 4 for representative results). These observations limit the location of the *sin* mutations to the segment of genome between *supE* and *tol* which contains the *sdh* gene. Related conjugations indicated that a further 14 independent *sin* mutations (*sin-11* to *sin-24*, all isolated in strains containing the *aroP–lpd*$^{\Delta}$*18* deletion) were located in the same region.

Dominance tests. The interaction of *sin*$^+$ and *sin*$^-$ alleles was studied by constructing merodiploids in *recA*$^-$ backgrounds. In one experiment a *recA*$^-$ derivative of JRG730 (*gal trpA*

Table 5. *Enzymology of representative merodiploid strains*

Extracts of organisms grown on glucose plus acetate and succinate were prepared and assayed as described in Methods except for the 2-oxoglutarate dehydrogenase complex of the *lpd* mutants which was assayed by complementation with pig heart lipoamide dehydrogenase by the method of Guest & Creaghan (1973). Specific activities are expressed as μmol substrate transformed (mg protein)$^{-1}$ h^{-1}. Abbreviations as in Table 2. —, Not tested.

Strain	Relevant genotype	Enzyme specific activity			
		lpd	sdh	ogdh	ogdh complex
JRG719	*lpd*$^+$	2·32	6·54	—	—
JRG721	*lpd*$^\Delta$	< 0·01	2·01	2·63	0·72
JRG721R	*lpd*$^\Delta$ *recA*$^-$	< 0·01	1·69	—	
JRG721RS24	*lpd*$^\Delta$ *sin-24 recA*$^-$	< 0·01	0·02	2·57	1·88
FIs/JRG721RS24	*sin*$^+$/*lpd*$^\Delta$ *sin-24 recA*$^-$	< 0·01	3·14	6·80	2·01
FIs/JRG721RS24-S	*sin*$^-$/*lpd*$^\Delta$ *sin-24 recA*$^-$	< 0·01	0·08	6·00	1·94

str sin-8) which has a Sut$^-$Aut$^-$ phenotype by virtue of the *sin-8* mutation (originally isolated as a partial suppressor of *lpd*1 in JRG367) was selected and designated JRG730R (see Methods). Corresponding merodiploids were then prepared by conjugation with FIs/JC1553 and F152/JC1553 which transfer the relevant segment of the chromosome (see Table 4). The constructed strains, designated FIs/JRG730R and F152/JRG730R were checked for their ultraviolet sensitivity and ability to transfer *gal*$^+$ and *sdh*$^+$ markers by cross-streak conjugations in order to confirm their Rec$^-$ phenotype and the presence of the F plasmids. Growth tests and enzymological studies with these F *sin*$^+$*gal*$^+$/*sin-8 gal*$^-$ *recA*$^-$ merodiploids established the dominance of the wild-type allele because they used both succinate and acetate as sole carbon and energy sources; they also synthesized active succinate dehydrogenase.

Further experiments were performed with a strain deleted for the *lpd* gene, JRG721 (*aroP–lpd*$^\Delta$*18 gal str*), in order to study the effects of partial diploidy on the Lpd$^-$Sin$^+$ phenotype. A *recA*$^-$ derivative was isolated (JRG721R) and a series of six Lpd$^-$Sin$^+$ revertants were selected on acetate-supplemented glucose minimal medium (e.g. JRG720RS24 carrying the *sin-24* mutation). When FIs and F152 were inserted into these revertants by Gal$^+$ selection, the merodiploids became dependent on both acetate and succinate for aerobic growth on glucose and they were unable to grow on succinate or acetate even though substantial succinate dehydrogenase activities could be detected. The merodiploid state was confirmed by tests for plasmid maintenance (as above) so it was concluded that the Lpd$^-$Sin$^+$ phenotype of the revertants is restored to Lpd$^-$ in the merodiploids (F *sin*$^+$*gal*$^+$/*lpd*$^\Delta$*sin*$^-$*gal*$^-$*recA*$^-$). This confirms that the wild-type (*sin*$^+$) allele is dominant to the suppressor (*sin*$^-$) mutations.

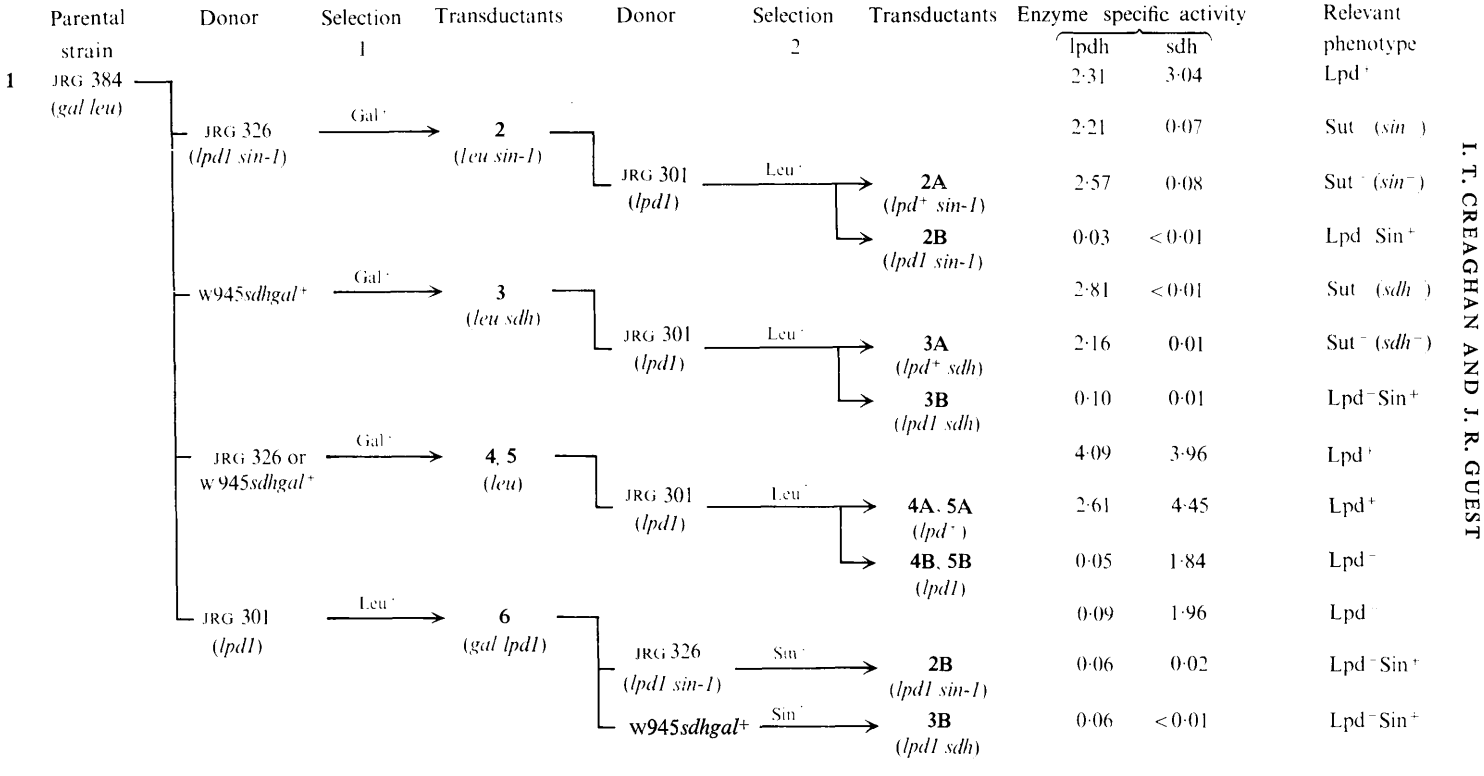
The merodiploids FIs/JRG720RS24 and F152/JRG720RS24 were subjected to a further selection for Lpd$^-$Sin$^+$ revertants using acetate-supplemented galactose medium (galactose was used in order to maintain the plasmids). These revertants had properties consistent with the genotype F *sin*$^-$*gal*$^+$/*lpd*$^\Delta$*sin-24 gal*$^-$*recA*$^-$, in which the *sin*$^-$ character is analogous to *sdh*$^-$. They retained the Rec$^-$ phenotype (ultraviolet sensitivity) and although they transferred *gal*$^+$ by conjugation, no *sdh*$^+$ transfer could be detected. Rather, the plasmids transferred an *sdh* lesion (presumably due to the *sin* mutation) which was 66 % cotransferable with the selective *gal*$^+$ marker. This observation shows that restoration of the Lpd$^-$Sin$^+$ phenotype following the second reversion step is due to *sin* mutation in the plasmid and not to removal of the *sdh* gene by plasmid shortening.

Enzymological investigations with representative merodiploids and their parental strains are summarized in Table 5. The simplest interpretation is that the *sin* mutations affect the *sdh* structural gene. It is also interesting to note the presence of a gene-dosage effect on the 2-oxoglutarate dehydrogenase activities in the merodiploids; the corresponding *suc* genes are also carried by the plasmids.

Reconstruction studies. To confirm that suppression of the succinate requirement of *lpd*

Table 6. *Reconstruction of strains possessing the* Lpd$^-$Sin$^+$ *phenotype using lpd, sin and sdh mutations*

The scheme outlines the reconstructions of strains analogous to the Lpd$^-$Sin$^+$ revertants starting with an Lpd$^+$ parental strain (**1**) by two alternative routes. Mutations were introduced by P1 cotransduction using Gal$^+$ selection (for *sin*$^-$ and *sdh*$^-$), Leu$^+$ selection (for *lpd*$^-$) or direct Sin$^+$ selection (for introducing *sin* and *sdh* mutations into *lpd*$^-$ strains). The ultimate products, **2B** and **3B**, were derived by two routes; corresponding *gal*$^-$ derivatives were also obtained from intermediate strain **6** but these are not shown. Other derivatives are included for comparison.



mutants in the Lpd$^-$Sin$^+$ revertants is a consequence of inactivating the *sdh* gene or its expression, reconstruction of the Lpd$^-$Sin$^+$ phenotype was attempted starting with an Lpd$^+$ parental strain. The strategy is outlined in Table 6. Representative *sin*, *sdh* and *lpd* mutations were introduced by appropriate transductional selections and two basic routes were used. The first involved introducing the *sin* or *sdh* lesions followed by the *lpd* mutation. The reverse sequence was adopted in the second route: *lpd* first and then *sin* or *sdh*. Some of the other derivatives synthesized in these constructions are included as controls. A comparison of the growth phenotype and the enzymology of the *lpd1 sin-1* (**2B**) and *lpd1 sdh* (**3B**) products constructed by either route shows them to be indistinguishable. Thus both sets of constructions confirm that the *sin* and *sdh* mutations are analogous in affecting succinate dehydrogenase and suppressing the succinate requirement of *lpd* mutants.

DISCUSSION

The common features of all the lipoamide dehydrogenase mutants after reversion to succinate independence were the presence of *gal*-linked mutations (*sin*) and deficiency in succinate dehydrogenase. None of the *sin* mutations were allele-specific; they all suppressed the succinate requirement of a lipoamide dehydrogenase deletion mutant. The *sin* mutations were recessive to the wild-type (*sin*$^+$) allele and their ability to generate the Lpd$^-$Sin$^+$ phenotype in *lpd* mutants was paralleled by two independent succinate dehydrogenase mutations (*sdh*). Furthermore, the reconstruction of Lpd$^-$Sin$^+$ strains from different combinations of *lpd*, *sin* and *sdh* mutations by different routes, makes it unlikely that the phenotype is dependent on any other mutation. The simplest interpretation of the results is that the *sin* and *sdh* mutations affect the same gene. If this is correct then the isolation of succinate-independent revertants of *lpd* mutants provides an extremely simple and direct method for selecting *sdh* mutants. The existence of other mechanisms for suppressing the succinate requirement of *lpd* mutants was not excluded but no evidence for alternative mechanisms was detected.

The results indicate that the suppression of the succinate requirement of *lpd* mutants is due to eliminating the depletion of intracellular succinate, caused by its conversion to fumarate, rather than to inducing or derepressing alternative mechanisms for the synthesis of succinate. This mechanism for suppression may explain why two mutants of *Salmonella typhimurium* LT2 deleted for the *lpd* gene did not require succinate for aerobic growth on glucose minimal medium (Langley & Guest, 1974). The succinate dehydrogenase activities of these mutants were less than 20 % of the wild-type (Langley & Guest, unpublished observations) and may be sufficiently low to suppress the requirement for exogenous succinate.The findings may also explain repeated failures in this laboratory to isolate double mutants deficient in both succinate dehydrogenase and 2-oxoglutarate dehydrogenase complex activities by starting with an *sdh* mutant and selecting for mutants with a Suc$^-$ (Sin$^-$) phenotype. It would appear that the desired mutants may not require exogenous succinate for growth on glucose. It will be interesting to determine whether the succinate requirement of *lip* (lipoate biosynthesis) and *sucA,B* (2-oxoglutarate dehydrogenase, dihydrolipoamide succinyltransferase) mutants (Herbert & Guest, 1968, 1969) can be suppressed by a similar mechanism.

The present interpretation of the results suggests that the requirement for exogenous succinate shown by *lpd* mutants growing aerobically on glucose stems as much from the presence of an active succinate dehydrogenase as the absence of a functional 2-oxoglutarate dehydrogenase complex. However, this does not imply that the Lpd$^-$Sin$^+$ revertants (*lpd*$^-$*sin*$^-$ or *lpd*$^-$*sdh*$^-$), deficient in overall 2-oxoglutarate dehydrogenase complex and succinate dehydrogenase activities, have no metabolic requirement for succinate or succinylCoA, rather that their demand is small and this can be met from other endogenous sources. One endogenous source depends on the E1 component of the 2-oxoglutarate dehydrogenase complex generating succinate semialdehyde-TPP which could then be oxidized to succinate.

Alternatively, the E1 plus E2 components of this complex could be responsible for the production of a limited amount of succinylCoA in the absence of lipoamide dehydrogenase if the enzyme-bound dihydrolipoamide can be re-oxidized non-enzymically, albeit at a low rate. Other sources include isocitrate and fumarate which could supply limited amounts of succinate by the action of isocitrate lyase or fumarate reductase, if these enzymes are not completely repressed or inhibited in the strains or conditions investigated.

REFERENCES

BACHMANN, B. J., LOW, K. B. & TAYLOR, A. L. (1976). Recalibrated linkage map of *Escherichia coli* K-12. *Bacteriological Reviews* **40**, 116–167.

CREAGHAN, I. T. & GUEST, J. R. (1972). *Amber* mutants of the α-ketoglutarate dehydrogenase of *Escherichia coli* K12. *Journal of General Microbiology* **71**, 207–220.

DIXON, G. H. & KORNBERG, H. L. (1959). Assay methods for key enzymes of the glyoxylate cycle. *Biochemical Journal* **72**, 3P.

DOVER, S. & HALPERN, Y. S. (1972). Utilization of γ-aminobutyric acid as the sole carbon and nitrogen source by *Escherichia coli* K-12 mutants. *Journal of Bacteriology* **109**, 835–843.

GUEST, J. R. (1974). Gene–protein relationships of the α-keto acid dehydrogenase complexes of *Escherichia coli* K12: chromosomal location of the lipoamide dehydrogenase gene. *Journal of General Microbiology* **80**, 523–532.

GUEST, J. R. & CREAGHAN, I. T. (1973). Gene–protein relationships of the α-keto acid dehydrogenase complexes of *Escherichia coli* K12: isolation and characterization of lipoamide dehydrogenase mutants. *Journal of General Microbiology* **75**, 197–210.

GUEST, J. R. & CREAGHAN, I. T. (1974). Further studies with lipoamide dehydrogenase mutants of *Escherichia coli* K12. *Journal of General Microbiology* **81**, 237–245.

HAGER, L. P. & KORNBERG, H. L. (1961). On the mechanism of α-oxoglutarate oxidation in *Escherichia coli*. *Biochemical Journal* **78**, 194–198.

HENDLER, R. W. & ANFINSON, C. B. (1954). The incorporation of carbon dioxide into both carboxyl groups of glutamic acid. *Journal of Biological Chemistry* **209**, 55–62.

HERBERT, A. A. & GUEST, J. R. (1968). Biochemical and genetic studies with lysine + methionine mutants of *Escherichia coli*: lipoic and α-ketoglutarate dehydrogenase-less mutants. *Journal of General Microbiology* **53**, 363–381.

HERBERT, A. A. & GUEST, J. R. (1969). Studies on α-keto acid dehydrogenase mutants of *Escherichia coli*. *Molecular and General Genetics* **105**, 182–190.

KENNEDY, I. R. & DILWORTH, M. J. (1963). Activation of isocitrate lyase and triosephosphate dehydrogenase in *Azotobacter vinelandii* extracts. *Biochimica et biophysica acta* **67**, 226–239.

KORNBERG, H. L. (1966). The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochemical Journal* **99**, 1–11.

LANGLEY, D. & GUEST, J. R. (1974). Biochemical and genetic characteristics of deletion and other mutant strains of *Salmonella typhimurium* LT2 lacking α-keto acid dehydrogenase complex activities. *Journal of General Microbiology* **82**, 318–335.

LANGLEY, D. & GUEST, J. R. (1977). Biochemical genetics of the α-keto acid dehydrogenase complexes of *Escherichia coli* K12: isolation and biochemical properties of deletion mutants. *Journal of General Microbiology* **99**, 263–276.

LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

PECK, H. D., SMITH, O. H. & GEST, H. (1957). Comparative biochemistry of the biological reduction of fumaric acid. *Biochimica et biophysica acta* **25**, 142–147.

SHARP, P. A., HSU, M. T., OHTSUBO, E. & DAVIDSON, N. (1972). Electron microscopic heteroduplex studies of sequence relations among plasmids of *E. coli*: I. Structure of F-prime factors. *Journal of Molecular Biology* **71**, 471–497.

SPENCER, M. E. & GUEST, J. R. (1973). Isolation and properties of fumarate reductase mutants of *Escherichia coli*. *Journal of Bacteriology* **114**, 563–570.

SPENCER, M. E., LEBETER, V. M. & GUEST, J. R. (1976). Location of the aspartase gene (*aspA*) on the linkage map of *Escherichia coli* K12. *Journal of General Microbiology* **97**, 73–82.

STACEY, K. A. & SIMSON, E. (1965). Improved method for the isolation of thymine-requiring mutants of *Escherichia coli*. *Journal of Bacteriology* **90**, 554–555.

VOGEL, H. & BONNER, D. M. (1956). A convenient growth medium for *Escherichia coli* and some other micro-organisms. *Microbial Genetics Bulletin* **13**, 43–44.