Two Mutations Affecting Utilization of C₄-Dicarboxylic Acids by *Escherichia coli*

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SUMMARY

Studies with a mutant of *Escherichia coli* reported to be deficient in phosphoenolpyruvate carboxykinase activity (AB257^{mut-}) indicate that it possesses two mutations. One affects growth on succinate (*S*⁻), and the other primarily affects growth on fumarate and malate and affects growth on succinate to a lesser extent (*FM*⁻). Neither of these mutations has a direct effect on carboxykinase activity. Presence of the *S*⁻ mutation also prevents growth on acetate. It delays growth on other substrates such as lactate, glycerol and malate and it specifically inhibits the oxidation of succinate by mutant suspensions. The only biochemical lesion which could be detected was a deficiency in succinate dehydrogenase and it is thought that the *S*⁻ locus may be situated in the structural gene for this enzyme. Transduction with phage P1 indicates linkages between *S*⁻ and *gltA* and *sucA* of 97 and 90%, respectively and the gene order *S*...*sucA*...*gal* was established. The *FM*⁻ mutation was also found in the parental strain AB257. It affects the oxidation of succinate, fumarate and malate by mutant suspensions. The uptake of the dicarboxylic acids is also impaired but no other biochemical lesion could be detected. Genetic studies indicate that the *FM*⁻ mutation is located near *xyl*.

INTRODUCTION

Several mutations specifically affecting the ability of *Escherichia coli* to utilize C₄-dicarboxylic acids such as succinate, fumarate or malate as sole carbon and energy sources have been described. A mutant lacking succinate dehydrogenase is capable of growth on fumarate but not on succinate (Hirsch, Rasminsky, Davis & Lin, 1963). Several mutants blocked in the biosynthesis of ubiquinone (*ubi*⁻) are unable to grow on malate or succinate (Cox, Gibson & Pittard, 1968; Cox, Young, McCann & Gibson, 1969). Mutants with a defective C₄-dicarboxylic acid transport system (*dct*⁻) have been isolated by virtue of their resistance to 3-fluoromalate. These are unable to grow on malate or fumarate and their ability to grow on succinate is markedly impaired (Kay & Kornberg, 1969). Mutants which are virtually devoid of phosphoenolpyruvate carboxykinase (Kornberg, 1965) or possess abnormally low carboxykinase activity (Hsie & Rickenberg, 1966) are also unable to grow on intermediates of the tricarboxylic acid cycle, presumably due to inadequate provision of phosphoenolpyruvate for biosynthetic purposes.

Carrillo-Castaneda & Ortega (1967) have reported the isolation of a phosphoenolpyruvate carboxykinase-less strain of *Salmonella typhimurium*, apparently the result of a deletion which also imposes requirements for nicotinic acid and lysine + methionine.

This indicated that the *pck* and *lys* + *met* loci are close. In view of the high degree of homology between the linkage maps of *Escherichia coli* and *S. typhimurium* (Sanderson, 1967), evidence for the same proximity in *E. coli* was sought during genetic studies with two types of lysine + methionine-requiring mutant: *lip*⁻, blocked in lipoate biosynthesis, and *suc*⁻, lacking α -ketoglutarate dehydrogenase (Herbert & Guest, 1968). The phosphoenolpyruvate carboxykinase-deficient mutant of Hsie & Rickenberg, AB257^{suc}⁻, was therefore examined. This strain was selected for its ability to utilize glycerol but not succinate after treatment of the parent strain AB257 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It grew on a variety of hexoses and trioses but not on intermediates of the tricarboxylic acid cycle; however, in its uptake of succinate the mutant was comparable with the parental strain. Our genetic studies indicate that AB257^{suc}⁻ possesses at least two mutations, one affecting succinate utilization (*S*⁻) and another which primarily affects growth on fumarate and malate (*FM*⁻). The latter mutation is unstable and it is also present in the parental strain AB257.

METHODS

Organisms. *Escherichia coli* strain AB257 and its derivative AB257^{suc}⁻, which is unable to grow on succinate, were kindly provided by Dr H. V. Rickenberg. Both strains were *metB*⁻ and although Hfr activity could not be detected, a weak polarized donor activity remained. To avoid confusion in the use of *suc*⁻ to describe the requirement for succinate (e.g. in mutants lacking α -ketoglutarate dehydrogenase) and inability to utilize succinate, and because the parent strain proved to be unable to grow on fumarate and malate, these strains will be designated AB257 *FM*⁻ and AB257 *FM*⁻, *S*⁻. A revertant of the former which grows on fumarate and malate will be called AB257 *FM*⁺. The characteristics of other strains of *E. coli* K12 used are as follows: AB1325 (*F*⁻, *proA*⁻, *lacY*⁻, *gal*⁻, *purB*⁻, *his*⁻, *str*⁻, *mtl*⁻, *xyl*⁻, *thi*⁻); XG3 *lip*32 (*F*⁻, *leu*⁻, *ara*⁻, *lac*⁻, *proC*⁻, *tsx*⁻, *purE*⁻, *lip*⁻, *trp*⁻, *str*⁻, *xyl*⁻, *thi*⁻); W620 (*F*⁻, *gltA*⁻, *galK*⁻, *ura*⁻, *str*⁻); W1485 (*F*⁺); HfrH (*Hfr*: *o-thr-pro-gal*...*F*); P10 (*Hfr*, *thr*⁻, *leu*⁻, *lacY*⁻, *thi*⁻, *malA*⁻, λ^r , *T*_{1.5}^r: *o-thi-metA-ilv*...*F*) and P13 (*Hfr*, *his*⁻, *cys*⁻, *thi*⁻, *gal*⁻, *T*_{1.3}^r: *o-ilv-met-thi*...*F*). The origins of several derivatives of these strains, AB1325 *lip*13 (*lip*⁻), AB1325 *suc*1 (*sucA*⁻) and W1485 *suc*1 (*sucA*⁻) have been described previously (Herbert & Guest, 1968). In addition, W1485 *S*⁻ was isolated by P1 transduction between AB257 *FM*⁻, *S*⁻ and W1485 *gal*⁻ and selection for *gal*⁺; the isolation of *FM*⁻ and *S*⁻ derivatives of AB1325 is described in the Results section. Phage P1 *kc* was originally obtained from Dr C. Yanofsky and T₆ was kindly provided by Dr B. A. Fry.

Media. The minimal medium used throughout contained (/l.): NaH₂PO₄·2H₂O, 6.08 g.; K₂HPO₄, 10.6 g.; (NH₄)₂SO₄, 2 g. and 5 ml. of a salts solution which in turn contained (g./l.): MgSO₄·7H₂O, 10; MnCl₂·4H₂O, 1; FeSO₄·7H₂O, 0.05; CaCl₂, 0.1 and a trace of conc. HCl to clarify. Carbon sources were added to a final concentration of 50 mM for acetate, succinate, DL-malate, fumarate, lactate, glycerol, 0.5 % for galactose, mannitol, xylose and 0.2 % for glucose. Media were supplemented with vitamins and amino acids according to the particular requirements of the bacteria to be grown or selected. The complex medium was L-broth (Lennox, 1955) and bacterial stocks were maintained on plates of L-agar. All incubations were at 37°.

Genetic techniques. Mixtures of log phase cultures containing 2×10^7 donor and 2 to 4×10^8 recipient bacteria/ml. were used in interrupted mating experiments. The

methods of Pittard & Wallace (1966) and Herbert & Guest (1968) were used when exconjugant donor organisms were to be killed with phage T₆ or streptomycin respectively. Transduction with phage P1*kc* was according to Guest (1969). For estimating linkages, recombinant colonies were picked, diluted and purified by single colony isolation on the medium used for their selection, prior to scoring the inheritance of non-selective markers.

Growth tests. Growth was followed in L-tubes rocked in a water bath at 37° according to Herbert & Guest (1968). Turbidities were measured with an EEL colorimeter fitted with a neutral density filter; an EEL reading of ten was equivalent to 0.3 mg. dry wt/ml. All cultures which grew were tested for reversion.

Oxygen consumption. The oxidation of several substrates was recorded polarographically using a simple oxygen electrode in a reaction vessel of 1 ml. capacity (Estabrook, 1967). Organisms were grown in glucose minimal medium, harvested late in the log phase, washed twice and resuspended at 1 mg. dry wt/ml. in potassium phosphate buffer (0.04 M, pH 7.0). Substrate oxidation is expressed as μ l. O₂ consumed/mg. dry wt/h. at 25° after subtracting the endogenous rate.

Enzyme assays. Unless otherwise stated, cell-free extracts were prepared from organisms harvested from minimal medium in late log phase, washed twice and resuspended in potassium phosphate buffer (0.04 M; pH 7.0) at 50 mg. dry wt/ml. These suspensions were disrupted with an ultrasonic cell disintegrator (M.S.E., 100 W) for 4 min. at 0°. Cell debris was removed by centrifuging at 23,000g for 10 min. Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard. All spectrophotometric assays were carried out in 1 cm. light-path cuvettes at 25° with a Beckman DB spectrophotometer. The results are expressed as μ moles substrate transformed/mg. protein/h.

Succinate dehydrogenase (EC.1.3.99.1) was measured according to Arrigoni & Singer (1962). The following reactants were contained in 3.0 ml.: potassium phosphate buffer, pH 7.4, 300 μ moles; KCN (freshly neutralized with HCl), 10 μ moles; 2,6-dichlorophenol indophenol (DCPIP), 0.2 mg.; *N*-methylphenazonium methosulphate, 2 mg.; sodium succinate, 20 μ moles and protein up to 1.5 mg. The reaction was started with succinate and DCPIP reduction was followed at 600 nm. using a control which lacked succinate. The oxidation of succinate involves the reduction of two molecules of DCPIP and millimolar extinction coefficient of 21 was used for DCPIP.

Fumarase. (EC.4.2.1.2) was measured by the method of Racker (1950). For this assay extracts were centrifuged at 100,000g for 1 h. and the reaction mixture (3 ml.) contained: potassium phosphate buffer pH 7.3, 100 μ moles, and protein 0.5 mg. The reaction was started with sodium L-malate, 50 μ moles and fumarate formation was assessed at 240 nm. using a millimolar extinction coefficient of 2.44 for fumarate.

Malate dehydrogenase (EC.1.1.1.37) was assayed according to Ochoa (1955). The reaction mixture contained in 3.0 ml.: potassium phosphate buffer, pH 7.4, 75 μ moles; NADH, 0.15 μ moles; protein, 10 μ g. and reaction was started with 0.76 μ mole of freshly prepared oxalacetate. The decrease in extinction at 340 nm. was compared with that of a control lacking oxalacetate.

Malic enzymes. The NAD- and NADP-linked malic enzymes (EC.1.1.1.38 and EC.1.1.1.40) were assayed in extracts prepared in 0.05 M-tris-HCl buffer, pH 7.9, containing 10 mM-MgSO₄ and centrifuged for 20 min. at 24,000g and 90 min. at 100,000g by the methods of Takeo (1969).

Phosphoenolpyruvate carboxykinase was assayed by a modification of the method of Bridgeland (1968) using organisms grown with succinate or fumarate as substrate. The reaction mixture contained (μ mole in 1 ml.): tris-maleate-NaOH buffer (equimolar tris and maleic acid; pH 7.5), 5; phosphoenolpyruvate, 5; ADP, 5; MnCl_2 , 5; potassium L-glutamate, 10; $\text{NaH}^{14}\text{CO}_3$ ($0.19 \mu\text{Ci}/\mu\text{mole}$), 15; glutamate-oxalacetate transaminase, 3.6 units and extract containing 0.5 mg. protein. Incubation was at 30° and at 3 min. intervals, samples (0.1 ml.) were pipetted into 0.9 ml. formic acid (0.1 M) in scintillation vials and left overnight at room temperature to remove dissolved $^{14}\text{CO}_2$. Then 12 ml. of dioxan-based liquid scintillator NE220 (Nuclear Enterprises Ltd) was added and the radioactivity measured in a Nuclear-Chicago scintillation counter (Model 6801), at an efficiency of approximately 72 %. Controls lacking phosphoenolpyruvate or ADP were run with all experiments; CO_2 -fixation in the absence of ADP never exceeded 14 % of the total activity and was deducted from activities quoted.

Malate oxidation by particulate fractions. The oxidation of L-malate by subcellular fractions was measured according to Cox, Snoswell & Gibson (1968). The results quoted for the small particulate fraction are expressed in $\mu\text{mole O}_2$ uptake/mg. protein/h.

Quinone content. The presence of ubiquinone and vitamin K was detected using the methods for extraction and thin-layer chromatography described by Cox, Gibson & Pittard (1968).

Uptake of fumarate and succinate. The uptake of ^{14}C -labelled fumarate and succinate was measured at 37° with log phase bacteria washed and suspended in phosphate buffer (0.1 M, pH 7.4) at $70 \mu\text{g. dry wt/ml.}$ by the method of Kay & Kornberg (1969). The radioactive acids were added at 0.1 mM (1 Ci/mole), samples (1 ml.) were withdrawn at intervals over 20 min. and the bacteria were collected on Millipore filters ($0.45 \mu\text{m.}$ pore size; presoaked in 0.1 mM-fumarate or succinate), washed three times with 3 ml. of buffer and transferred to vials containing 4 ml. of NE220 scintillation fluid. Radioactivity was assayed by scintillation counting, as above, and the initial rates of C_4 -dicarboxylic acid uptake are expressed in nmoles/mg. dry wt organisms/h.

Materials. Phosphoenolpyruvate and glutamate-oxalacetate transaminase were from C. F. Boehringer & Soehne, Mannheim, Germany. $[2,3\text{-}^{14}\text{C}]$ -Fumaric and succinic acids were purchased from The Radiochemical Centre, Amersham, and 3-fluoromalate was kindly provided by Dr P. W. Kent.

RESULTS

Genetic Studies with AB257 and AB257^{suc-}

Conjugation

The first indication that AB257^{suc-} contained more than one mutation affecting C_4 -dicarboxylic acid metabolism came from preliminary conjugation studies aimed at mapping its *pck*⁻ lesion. With AB257^{suc-} as donor and AB1325*lip*13 as recipient, a high proportion of *gal*⁺ and *lip*⁺ recombinants inherited the donor strain's inability to use succinate as substrate (Table 1). However, despite their inability to use succinate, the majority grew on fumarate and malate. Derivatives lacking the ability to use fumarate and malate were found in significant numbers only amongst the *xyl*⁺ recombinants. The presence of at least two mutations was confirmed by reversion

studies. Revertants of AB257^{suc-} selected for ability to grow on either fumarate or malate invariably grew on both these substrates, but the majority would not grow on succinate. Likewise, most of the revertants selected on succinate failed to use fumarate and malate although some revertants capable of using all three substrates were again

Fig. 1. Kinetics of marker transfer from HfrH to AB1325^{S-}, *proA*⁻, *gal*⁻, *str*⁻ (a) and from P10 to AB1325^{FM-}, *metB*⁻, *xyl*⁻, *str*⁻, *tsx*⁻ (b). In (a) counter-selection against the donor was with streptomycin and *S*⁺ recombinants were selected on media containing succinate as the carbon source. In (b) counter-selection against the donor was with phage T₆ and *FM*⁺, *metB*⁺ recombinants were selected on fumarate medium to avoid undue interference from reversion of the *FM*⁻ marker.

Table 1. Conjugation with AB257^{suc-} and AB257 as donors

Overnight cultures were diluted into fresh L-broth to 5×10^8 bacteria/ml. for each strain and incubated at 37° for 90 min. Recombinants were selected on appropriate media containing methionine and streptomycin; 50 or 100 were purified by single colony isolation and scored for their inability to grow on succinate (*S*⁻) or on fumarate and malate (*FM*⁻).

Selected marker	AB257 ^{suc-} × AB1325 <i>lip</i> 13		AB257 × AB1325	
	Recombinants/ml.	<i>S</i> ⁻ (%)	Recombinants/ml.	<i>FM</i> ⁻ (%)
<i>pro</i> ⁺	76,000	< 1	186,000	< 1
<i>mtl</i> ⁺	—	—	7,300	33
<i>xyl</i> ⁺	3,400	< 2	9,500	57
<i>his</i> ⁺	—	—	1,500	< 1
<i>gal</i> ⁺	260	62	430	< 2
<i>lip</i> ⁺	150	65	—	—

recovered. The parental strain, AB257, grew on succinate but, in contrast to published results (Hsie & Rickenberg, 1966), it failed to grow on fumarate and malate. A high frequency of reversion occurred on fumarate and particularly on malate. All the revertants selected on fumarate grew on malate and most of the revertants selected on malate grew on fumarate, but some grew less well than others on this substrate. On succinate the revertants grew better than AB257 itself. The simplest interpretation of these results is that AB257 and AB257^{suc-} possess a mutation (*FM*⁻) which primarily

affects growth on fumarate and malate and to a lesser extent on succinate, and that, in addition, AB257^{suc⁻} possesses a mutation which primarily affects growth on succinate. The strains may therefore be designated AB257^{FM⁻} and AB257^{FM⁻, S⁻}. The revertants of AB257^{FM⁻, S⁻} which grow on all three carbon sources are thought to arise by double events which would be favoured by the instability of the mutations and the fact that each mutation seems to impair growth on any of the three selective media. The presence of a mutation affecting fumarate and malate utilization in AB257 was confirmed by transfer to AB1325 during conjugation (Table 1). Some 57 % of *xyl*⁺ recombinants simultaneously inherited the inability to grow on both fumarate and malate.

For more precise mapping of *S* and *FM*, recombinants from the above conjugations were used as recipients in interrupted mating experiments. With HfrH as donor and a *lip*⁺ recombinant AB1325^{S⁻}, *proA*⁻, *gal*⁻, *str*⁻ as recipient, the *S*⁺ marker entered 8 min. after *proA*⁺ and 3 min. before *gal*⁺ (Fig. 1*a*). Attempts to map the *FM* locus proved difficult due to its instability. However, with a *tsx*⁻ derivative of a *mtl*⁺ recombinant AB1325^{FM⁻}, *metB*⁻, *xyl*⁻, *str*⁻, *tsx*⁻ as recipient and by selecting for joint transfer of *metB*⁺ and *FM*⁺, the *FM*⁺ marker appeared to enter 12.5 min. after *metB*⁺ and 2.5 min. after *xyl*⁺ (Fig. 1*b*). Linked transfer of *FM*⁺ and *metB*⁺ was not obtained with the Hfr strain P13 which transfers its genome with the order *o...ilv...met*.

Transduction

The position of the *S*⁻ site was further investigated in transduction studies using phage P1kc and recipients possessing markers in the *purE* to *gal* region of the *Escherichia coli* chromosome. The results indicated that *S* was not cotransduced with either *purE* or *lip*, but cotransduction of *S* with *gal* (40 to 50 %), *sucA* (90 %) and *gltA* (97 %) was observed (Table 2). These linkages suggest that the *S* locus is very close to

Table 2. Tests for cotransduction of *S* and *FM* with other markers

P1kc lysates of donor strains were used to infect stationary phase recipient cultures at a multiplicity of 2. *Gal*⁺ and *xyl*⁺ transductants were selected on galactose and xylose minimal medium respectively and other transductants were selected on appropriate glucose media. Transductants were purified by single colony isolation and the distribution of non-selective markers was determined by replica plating.

Donor	Recipient	Selective marker	Transductants per ml. × 10 ³	Distribution of non-selective donor marker		
				No. scored	Marker	Cotransduction (%)
AB257 <i>FM</i> ⁻ , <i>S</i> ⁻	XG3 <i>lip</i> 32	<i>lip</i> ⁺	5.1	100	<i>S</i> ⁻	< 1
		<i>purE</i> ⁺	8.5	100	<i>S</i> ⁻	< 1
AB257 <i>FM</i> ⁻ , <i>S</i> ⁻	AB1325 <i>sucI</i>	<i>sucA</i> ⁺	4.3	100	<i>S</i> ⁻	90
		<i>gal</i> ⁺	3.7	100	<i>S</i> ⁻	50
AB257 <i>FM</i> ⁻ , <i>S</i> ⁻	W620	<i>gltA</i> ⁺	31.7	100	<i>S</i> ⁻	97
		<i>gal</i> ⁺	28.0	100	<i>S</i> ⁻	40
W1485	AB1325 <i>FM</i> ⁻ , <i>met</i> ⁻ , <i>mtl</i> ⁺	<i>xyl</i> ⁺	98.2	123	<i>FM</i> ⁺	5
AB257 <i>FM</i> ⁻ , <i>S</i> ⁻	AB1325	<i>xyl</i> ⁺	12.4	126	<i>FM</i> ⁻	0.8

gltA and closer to *sucA* than to *gal*. To determine the order of *S* and *sucA* relative to *gal*, the reciprocal crosses shown in Table 3 were performed and the distribution of the non-selective *gal* marker in *S*⁺, *sucA*⁺ transductants scored. If the marker order is *S*...*sucA*...*gal*, then the linkage between *S*⁺, *sucA*⁺ and the donor *gal*⁺ marker would be normal (40 to 50 %) in the direction I cross but much reduced for *S*⁺, *sucA*⁺ transductants obtained in the direction II cross. Opposite predictions would be for the order *sucA*...*S*...*gal*. Difficulty was experienced in finding a medium suitable for direct selection of *S*⁺, *sucA*⁺ recombinants because the *S*⁻ mutants grew slowly on plates of acetate medium, and *sucA*⁻ mutants usually grew on succinate, albeit after a considerable lag. Nevertheless, *S*⁺, *sucA*⁺ transductants could be isolated satisfactorily as the large colonies which appeared early on succinate medium with the *S*⁻ recipient and on acetate

Table 3. Order of *sucA* and *S* markers relative to *gal*

Transduction with P 1 kc was as described in the Methods section, *S*⁺, *sucA*⁺ recombinants were selected on acetate minimal medium (I) or succinate minimal medium (II) and purified by single-colony isolation before testing the distribution of the donor *gal*⁺ marker by replica plating

Direction of cross	Donor	Recipient	Distribution of donor <i>gal</i> ⁺ in <i>S</i> ⁺ , <i>sucA</i> ⁺ transductants	
			No. scored	Per cent <i>gal</i> ⁺
I	AB 257 <i>FM</i> ⁻ , <i>S</i> ⁻ (<i>S</i> ⁻ , <i>sucA</i> ⁺ , <i>gal</i> ⁺)	AB 1325 <i>suc</i> I (<i>S</i> ⁺ , <i>sucA</i> ⁻ , <i>gal</i> ⁻)	52	59.6
II	w 1485 <i>suc</i> I (<i>S</i> ⁺ , <i>sucA</i> ⁻ , <i>gal</i> ⁺)	AB 1325 <i>S</i> ⁻ (<i>S</i> ⁻ , <i>sucA</i> ⁺ , <i>gal</i> ⁻)	75	1.3

medium with the *sucA*⁻ recipient. The results indicate that the order is *S*...*sucA*...*gal*. Similar crosses were attempted to order *S* and *gltA* relative to *gal*, and similar difficulties were experienced in finding suitable media for direct selection of *S*⁺, *gltA*⁺ transductants. Some success was obtained using acetate medium in a cross between AB 257 *FM*⁻, *S*⁻ (donor) and w 620 *gltA*⁻, *gal*⁻ (recipient) where the distribution of the donor *gal*⁺ in *S*⁺, *gltA*⁻ recombinants was low (5/34) and suggested the order *gltA*...*S*...*gal*. However, no satisfactory selection procedure could be found for the reciprocal cross because *S*⁺, *gltA*⁻ recombinants grew well enough on succinate medium to make them indistinguishable from *S*⁺, *gltA*⁺ recombinants. Consequently, the relative order of *S* and *gltA* could not be deduced with certainty but a tentative order *gltA*...*S*...*gal* may be indicated.

The *FM*⁻ mutation was too unstable to permit direct selection of *FM*⁺ in transduction crosses but attempts were made to use it as a non-selective marker. With AB 257 *FM*⁻ and AB 257 *FM*⁻, *S*⁻ as donors, no linkage between *FM*⁻ and any of the genes in the *purE* to *gal* region was observed. A very weak linkage between *FM* and *xyl* was observed (Table 2) but its significance is difficult to assess in view of the instability of this mutation.

Physiological and biochemical studies with S⁻ and FM⁻ mutants

In order to investigate the metabolic lesions associated with the *S*⁻ and *FM*⁻ mutations, two pairs of mutant and wild-type strains were chosen for detailed study.

These were AB257 *FM*⁻ and a revertant AB257 *FM*⁺ (selected on fumarate medium), and w1485 *S*⁻ and w1485. In all these studies cultures of the mutant organisms were tested to ensure that significant reversion had not occurred during growth.

Growth tests. The growth of the test organisms in liquid media with several different carbon sources is shown in Fig. 2. It can be seen that the *S*⁻ mutation prevented growth on succinate and acetate and it delayed growth on glucose, glycerol, lactate and malate

Fig. 2. Growth of *Escherichia coli* *FM*⁻ and *S*⁻ mutants and the corresponding wild-type strains on various substrates: O, glucose; ●, glycerol; x, lactate; Δ, malate; ▲, fumarate; □, succinate; ■, acetate.

Table 4. Respiratory activities of washed suspensions of mutant and wild-type strains grown in glucose medium

Strain	Respiration rates (μl. O ₂ consumed/mg. dry wt/h.) with substrate		
	Succinate	Fumarate	Malate
AB257 <i>FM</i> ⁺	114	53	55
AB257 <i>FM</i> ⁻	55	20	35
w1485	63	32	28
w1485 <i>S</i> ⁻	32	31	35

but not fumarate. Supplementing the succinate medium with lactate and glycerol (2 mM) permitted very slow growth of w1485 *S*⁻ and full growth was reached only after 70 h. The *FM*⁻ mutation prevented growth on malate and fumarate and growth on succinate was impaired in such a way that it paralleled the response with acetate. Reversion usually occurred on malate after about 45 h. but rarely occurred on fumarate up to 80 h. Supplementing malate and fumarate media with lactate and glycerol (2 mM) promoted rapid growth to a submaximal extent. Neither mutant responded to glutamate, 4-hydroxybenzoate or 2,3-dihydroxybenzoate when added to non-permissive media, but both grew anaerobically with glycerol plus fumarate. The results

obtained with solid media were not as well defined because after prolonged incubation some growth of *S*⁻ strains occurred on acetate and succinate. *FM*⁻ strains invariably reverted after prolonged incubation and the mutant organisms were fed by the revertant colonies.

Respiratory activities. A study of the oxidation of succinate, fumarate and malate by washed suspensions of the test strains showed that whereas the *FM*⁻ mutation affected the oxidation of all three dicarboxylic acids, the *S*⁻ mutation only affected the oxidation of succinate (Table 4).

Table 5. *Phosphoenolpyruvate carboxykinase in FM*⁻ and *S*⁻ mutants

Organisms were grown with succinate or fumarate as carbon sources and the enzyme was assayed in cell-free extracts as described in the Methods section.

Strain	Phosphoenolpyruvate carboxykinase (μ mole/mg. protein/h.) after growth on	
	Succinate	Fumarate
W 1485	0.48	0.38
W 1485 <i>S</i> ⁻	—	0.37
AB 257 <i>FM</i> ⁺	0.49	0.39
AB 257 <i>FM</i> ⁻	0.45	—

Table 6. *Enzymic activities and uptake of C₄-dicarboxylic acids*

Organisms were grown in minimal medium with glucose or acetate as carbon sources. Enzymes were assayed in cell-free preparations as described in the Methods section and the results are expressed as μ moles substrate transformed/mg. protein/h. or as μ moles O₂ taken up/mg. protein/h. for particulate malate oxidation. The initial rates of dicarboxylic acid uptake by whole cell suspensions are expressed as nmoles/mg. dry wt organisms/h.

Organism	W 1485	W 1485 <i>S</i> ⁻	AB 257 <i>FM</i> ⁺	AB 257 <i>FM</i> ⁻	AB 257 <i>FM</i> ⁺	AB 257 <i>FM</i> ⁻
Growth substrate	Glucose	Glucose	Glucose	Glucose	Acetate	Acetate
Succinate dehydrogenase	4.5	0.9	4.3	3.9	—	—
Fumarase	33	28	32	31	—	—
Malate dehydrogenase	290	282	250	242	564	620
Malic enzyme (NAD)	6.4	3.6	6.3	6.8	6.9	9.5
Malic enzyme (NADP)	6.7	3.9	5.3	2.8	18.1	25.4
Particulate malate oxidation	0.8	0.6	0.7	0.6	—	—
Succinate uptake	119	116	104	86	1460	360
Fumarate uptake	111	124	146	60	1460	380

Enzymic activities. The AB 257 *FM*⁻, *S*⁻ was originally characterized by a deficiency in phosphoenolpyruvate carboxykinase activity, so the effects of the individual mutations on this enzyme were examined. Since strains carrying the *FM*⁻ or the *S*⁻ lesion can grow on at least one C₄-dicarboxylic acid, it could be argued that if the carboxykinase is essential for growth on these substrates both types of mutant must possess adequate amounts of the enzyme. This was confirmed by showing that neither the *FM*⁻ nor *S*⁻ mutations affected the carboxykinase activity of organisms grown on succinate and fumarate respectively (Table 5).

Several other enzymes concerned with the oxidation and metabolism of C₄-dicarboxylic acids were assayed in an attempt to define the *FM*⁻ and *S*⁻ lesions; typical results are shown in Table 6. The most striking deficiency in W 1485 *S*⁻ was the low

succinate dehydrogenase activity. The presence of only 20 % of S^+ activity was confirmed with other S^- derivatives (e.g. AB1325 S^- and AB257 FM^- , S^-). The specific activities of the malic enzymes were also lower in the S^- mutant, but this could be a secondary effect. The FM^- mutation had no significant effect on the enzymes tested. Several of the enzymes were also assayed in organisms grown on acetate in case a regulatory defect could be detected. However, no differences between FM^+ and FM^- strains were found although the specific activities of malate dehydrogenase and the NADP-linked malic enzyme were considerably higher after growth on acetate (Table 6). The behaviour of this malic enzyme contrasts with the *Pseudomonas putida* enzyme which is repressed by acetate (Jacobson, Bartholomaeus & Gunsalus, 1966). The *Escherichia coli* enzyme is also induced by malate (Katsuki, Takeo, Kameda & Tanaka, 1967) and it is thought to supply NADPH₂ and acetyl CoA (via pyruvate). Presumably, the provision of NADPH₂ is its main function during growth on acetate.

Uptake of C₄-dicarboxylic acids. The S^- mutation had no effect on the initial rate of succinate or fumarate uptake by organisms grown on glucose (Table 6). By contrast, the FM^- mutation decreased the rate of uptake of both substrates and this was particularly apparent with organisms grown on acetate. However, growth of all four test strains on several substrates was inhibited by 3-fluoromalate (0.1 mM), and more precise tests with AB257 FM^+ and AB257 FM^- growing on acetate indicated that the FM^- strain was only slightly less sensitive over a range of analogue concentrations.

Quinone content. No differences were found between mutant and wild-type strains in tests for the presence of ubiquinone and vitamin K.

DISCUSSION

The genetic studies with AB257^{suc-} and its derivatives clearly indicated the presence of at least two mutations, FM^- and S^- , which primarily affect growth on fumarate and malate and on succinate, respectively. The FM^- mutation mapped in the *xyl* region of the *Escherichia coli* chromosome and it was unstable. The biochemical lesion associated with this mutation is obscure; it had no significant effect on the key enzymes of C₄-dicarboxylic acid metabolism, nor did it appear to affect ubiquinone or vitamin K synthesis. The only deficiency which could be detected was an impaired capacity for dicarboxylic acid transport. Mutants (*dct*⁻), which apart from being resistant to 3-fluoromalate have the same phenotype, also map in the *xyl* region (Kay & Kornberg, 1969). However, the greatest inhibition of fumarate uptake observed with an FM^- mutant was 74 % and this alone seems unlikely to account for the failure to grow on this substrate. The effect on transport may be secondary to a more fundamental defect such as a regulatory defect or a defect in the provision of either energy or essential metabolites required specifically for growth on malate and fumarate. This is supported by the fact that supplements of lactate and glycerol permit growth on these substrates. Another uncharacterized mutation which affects growth on malate but not on succinate has also been discovered in AB3283, which also carried two mutations affecting ubiquinone biosynthesis (Cox *et al.* 1968). The origin of the FM^- mutation in AB257 is unknown but it is not present in the similar strain HfrC.

The S^- mutation was closely linked to the *gltA* and *sucA* loci and it may be situated between them. It produces a deficiency in succinate dehydrogenase activity, and al-

though the deficiency is not as complete as was found in the succinate dehydrogenase-less mutant of Hirsch *et al.* (1963), both types have many properties in common. The structural gene for succinate dehydrogenase maps in this region of the chromosome (U. Henning, personal communication) and it seems likely that this gene is the site of the *S*⁻ mutation. It is interesting to note that all the structural genes for enzymes of the tricarboxylic acid cycle which have been mapped (citrate synthase, α -ketoglutarate and succinate dehydrogenase) are clustered in the *lip* to *gal* region of the chromosome.

To account in part for the results observed by Hsie & Rickenberg (1966) it is necessary to propose that AB257^{suc-} (*FM*⁻, *S*⁻) was prepared by single-step mutation of AB257*FM*⁻ but was subsequently compared with an *FM*⁺ revertant of the parent strain. Since neither of the two mutations affects phosphoenolpyruvate carboxykinase activity, the deficiency observed in AB257^{suc-} may be a secondary consequence of combining the two mutations, or alternatively it may be due to a third mutation which was not detected during the present work. No evidence concerning either the location of the carboxykinase (*pck*) gene or the question of whether this enzyme is essential for growth on intermediates of the tricarboxylic acid cycle was obtained.

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