JOURNAL OF BACTERIOLOGY, May, 1973, p. 563-570
Vol. 114, No. 2

Printed in U.S.A.

Isolation and Properties of Fumarate Reductase Mutants of *Escherichia coli*

MARGARET E. SPENCER AND J. R. GUEST
Department of Microbiology, Sheffield University, Sheffield, S10 2TN, England

Received for publication 27 December 1972

Escherichia coli produces two enzymes which interconvert succinate and fumarate: succinate dehydrogenase, which is adapted to an oxidative role in the tricarboxylic acid cycle, and fumarate reductase, which catalyzes the reductive reaction more effectively and allows fumarate to function as an electron acceptor in anaerobic growth. A glycerol plus fumarate medium was devised for the selection of mutants (*frd*) lacking a functional fumarate reductase by virtue of their inability to use fumarate as an anaerobic electron acceptor. Most of the mutants isolated contained less than 1% of the parental fumarate reduction activity. Measurements of the fumarate reduction and succinate oxidation activities of parental strains and *frd* mutants after aerobic and anaerobic growth indicated that succinate dehydrogenase was completely repressed under anaerobic conditions, the assayable succinate oxidation activity being due to fumarate reductase acting reversibly. Fumarate reductase was almost completely repressed under aerobic conditions, although glucose relieved this repression to some extent. The mutations, presumably in the structural gene (*frd*) for fumarate reductase, were located at approximately 82 min on the *E. coli* chromosome by conjugation and transduction with phage P1. *frd* is very close to the *ampA* locus, and the order of markers in this region was established as *ampA-frd-purA*.

The properties of the succinate dehydrogenases found in aerobic and anaerobic cells differ considerably. The aerobic enzyme has a predominantly oxidative function, as a participant in the tricarboxylic acid cycle. It is a membrane-bound flavoprotein and, unique among the tricarboxylic acid cycle enzymes, it serves as a direct electron donor to the cytochrome electron transport chain leading to oxygen. By contrast, the succinate dehydrogenase characteristic of obligate anaerobes such as *Micrococcus lactilyticus* serves a predominantly reductive role, leading to succinate as a fermentation product. This enzyme might, more appropriately, be termed "fumarate reductase" (30). Facultative anaerobes such as *Escherichia coli* should have a physiological requirement for both types of enzyme. Hirsch et al. (16) found that anaerobic fumarate reduction in *E. coli* was unimpaired in a mutant lacking succinate dehydrogenase and suggested the existence of a distinct fumarate reductase; this was later confirmed by the isolation of a mutant lacking the reductase (C. A. Hirsch, Fed. Proc. 24:228, 1965). Fumarate reductase has subsequently been found in yeast and in various anaerobically-grown bacteria (15, 25).

The fumarate reductase in *E. coli* has a lower K_m for fumarate than for succinate and is adapted to catalyze fumarate reduction faster than succinate oxidation. Like succinate dehydrogenase, fumarate reductase is a membrane-bound flavoprotein. The enzyme is induced under anaerobic conditions, where it permits fumarate to be used as an electron acceptor, with the formation of succinate as a fermentation product (6, 9, 10, 20, 24, 28). Nitrate, which can also function as an anaerobic electron acceptor in *E. coli*, is known to serve as the terminal electron acceptor for a cytochrome-dependent electron transport chain in which nitrate reductase is a membrane-bound component (27). An analogous electron transport function for fumarate reductase has been proposed in *Streptococcus faecalis* (9) and *Bacillus megaterium* (20) and may also occur in *E. coli*. Fumarate reductase also serves to provide succinate for biosynthetic use during anaerobic growth when the tricarboxylic acid cycle enzymes are repressed (2, 12; C. A. Hirsch, Fed.

Proc. 24:288, 1965). Because this anaerobic route from oxaloacetate to succinate can function in the absence of malate dehydrogenase, Courtright and Henning (5) have proposed a pathway that includes aspartate which is converted to fumarate by the anaerobically inducible enzyme aspartase.

In the present study a glycerol-fumarate medium was devised which was suitable for genetic studies with mutants lacking fumarate reductase. With this medium, mutants of *E. coli* unable to utilize fumarate as an electron acceptor were isolated, and aspects of their biochemistry and genetics were investigated.

MATERIALS AND METHODS

Organisms. Mutants were selected in the following strains of *E. coli* K-12: R4 (Hfr,*met*) from R.H. Pritchard, and W945*sdh* (F^-, *thr*, *thi*, *ara*, *lac*, *gal*, *mal*, *xyl*, *mtl*, *sdh*, *tsx*, *str*) from U. Henning. Strain P678 (F^-, *thr*, *leu*, *thi*, *lac*, *gal*, *mal*, *xyl*, *mtl*, *tonA*, *str*) and a derivative containing a mutation in fumarate reductase, P678*frd*, were obtained from C. A. Hirsch. A series of F' strains, KLF17 ($pyrB^+$), KLF18 ($pyrB^+$), KLF19 ($pyrB^+$), and KLF21 ($pryB^+$, thr^+) in the KL132 background (F^-, *thr*, *leu*, *proA*, *his*, *thy*, *thi*, *pyrB*, *lac*, *mal*, *xyl*, *mtl*, *recA*), were obtained from B. Low. Other strains used in mapping studies are shown in Table 1.

Abbreviations. The genetic nomenclature conforms to the recommendations of Demerec et al. (7). The abbreviations are according to the current *E. coli* linkage map (29). The structural genes for the enzymes fumarate reductase and succinate dehydrogenase are abbreviated to *frd* and *sdh*, respectively.

Media. The basal minimal medium contained (per liter): KH_2PO_4, 5.44 g; K_2HPO_4, 10.49 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $MnSO_4 \cdot 4H_2O$, 5 mg; $FeSO_4 \cdot 7H_2O$, 0.125 mg; $CaCl_2$, 0.5 mg. Substrate additions where indicated were made to give the following concentrations: glucose 0.01 M; glycerol, 0.04 M; sodium fumarate, 0.04 M. The glycerol plus fumarate medium (GF medium) used for genetic studies contained, in addition, Casamino Acids (0.5 g/liter). Anaerobic incubation of solid media was carried out under H_2/CO_2 (95:5, vol/vol). All minimal media were supplemented with vitamins and amino acids according to the particular requirements of the bacteria to be grown.

Bacterial stocks were maintained on L agar (21), and L broth was used as the complex medium for routine subculture. Media were solidified with 1.5% agar (Difco) where required.

Selection of mutants. Derivatives of the parental strains, R4 and W945*sdh*, which grew well anaerobically on GF medium, were first isolated. Cultures were treated with mutagen according to the method of Adelberg et al. (1). Log-phase L-broth cultures were washed and suspended in tris(hydroxymethyl)-aminomethane-maleate buffer (0.1 M pH 6.0) at a concentration of 10^9 cells/ml and were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml) for 20 min at 37 C. After being washed, a sample of each treated culture was given an aerobic expression period in glycerol minimal medium, followed by an aerobic penicillin selection (300 U/ml) on GF medium. Survivors were plated on glycerol minimal medium, and mutants were detected by replica plating to GF medium and incubating anaerobically. Mutants with the desired phenotype were purified, and cell-free extracts were examined for fumarate reductase and succinate dehydrogenase activities.

Growth of cultures. Aerobic cultures were grown in 2-liter Erlenmeyer flasks containing 500 ml of medium (peptone, 0.4%; yeast extract, 0.4%; K_2HPO_4, 0.6%; pH 6.8) inoculated with 1 ml of overnight L-broth culture. The cultures were shaken at 37 C and harvested in late log phase. Anaerobic cultures were grown from the same inoculum in stationary 1-liter Erlenmeyer flasks filled to the neck with the same complex medium plus glucose (1%, added after autoclaving); the cultures were harvested after 18 h at 37 C.

Preparation of extracts. Harvested cells were washed twice in cold potassium phosphate buffer (0.01 M, pH 7.8) and resuspended at a concentration of 0.25 g (wet wt) per ml. The suspensions were disrupted at 0 C by two periods of 2 min in an ultrasonic disintegrator (M.S.E., 100 W) and sedimented at 15,000 × *g* for 20 min, and the supernatnat fluids were used for enzymology. Protein concentra-

TABLE 1. *Characteristics of bacterial strains used in linkage analysis*

Strain	Characteristics	Source
PA256	F^-, *pro*, *his*, *argH*, *thi*, *purA*, *str*	K. G. Eriksson-Grennberg
G11a1	Hfr, *ilv*, *metB*, *ampA*	K. G. Eriksson-Grennberg
Q11	Hfr, *fdp*	K. G. Eriksson-Grennberg
KG20	F^+, *pro*, *his*, *argH*, *thi*, *purA*, *ampA*, *str*	K. G. Eriksson-Grennberg
PA2004	F^-, *thr*, *leu*, *his*, *pyrB*, *str*	K. G. Eriksson-Grennberg
RM1003	F^-, *purA*	W. T. Drabble
AB2500	F^-, *thr*, *leu*, *proA*, *his*, *thy*, *argE*, *thi*, *ara*, *lac*, *gal*, *xyl*, *mtl*, *drn*, *uvrA*, *tsx*, *sup37*	P. Howard-Flanders
P10	Hfr, *thr*, *leu*, *thi*, *lac*, *malA*, *ton*	F. Jacob
Ra-2	Hfr	B. Low
H	Hfr, *thi*	W. Hayes

tions were determined by the method of Lowry et al. (22).

Enzyme assays. All assays were carried out in a Unicam SP800 spectrophotometer in 1-cm light path cuvettes at 30 C. Specific activities were expressed in micromoles of substrate transformed per milligram of protein per hour.

(i) Fumarate reductase (frd) was assayed by following the fumarate-dependent oxidation of reduced benzyl viologen at 550 nm under He (17). The extract (containing 0.1 to 2.0 mg of protein) was added to 3.0 ml of potassium phosphate buffer (0.01 M, pH 7.8), and the cuvette was flushed out with He. A sample (about 0.1 ml) of reduced benzyl viologen, prepared by gassing with H_2 in the presence of palladized asbestos, was transferred anaerobically into the cuvette to give an initial extinction of 0.8 to 1.0 at 550 nm. The extinction was measured for 1 to 2 min to determine any nonspecific dye oxidation, and the reaction was started by adding sodium fumarate (0.1 ml, 0.06 M). The molar extinction coefficient of benzyl viologen was estimated as $7.78 \times 10^3\ cm^{-1}$, by reducing a standard amount of dye with successive small amounts of solid sodium dithionite. Two molecules of benzyl viologen are required to reduce one molecule of fumarate.

(ii) Succinate dehydrogenase (sdh) was measured by the method of Arrigoni and Singer (3). The reaction mixture (3.0 ml) contained: potassium phosphate buffer (0.1 M, pH 7.4, KCN (0.65 mg, freshly prepared and neutralized with HCl), 2,6-dichlorophenol indophenol (DCPIP, 0.2 mg) *N*-methylphenazonium methosulphate (2 mg), sodium succinate (20 μmol), and protein (up to 2 mg). Sample and reference cuvettes, containing all reagents except succinate, were compared for 1 to 2 min, and the reaction was started by the addition of succinate. The reduction of DCPIP (molar extinction coefficient $21 \times 10^3\ cm^{-1}$) was followed at 600 nm. One molecule of DCPIP is reduced by one molecule of succinate.

Genetic techniques. Conjugations were performed by incubating log-phase cultures of F^- recipient bacteria (2.5×10^8 cells/ml) with Hfr donors (2.5×10^7 cells/ml) for 60 min, or with F′ donors (2.5×10^7 cells/ml) for 30 min in L broth at 37 C. Conjugating bacteria were separated by shaking, and serial dilutions were plated out on selective media. The frequency of recombinant formation was expressed as a percentage of the number of donor bacteria in the conjugation mixture. Transduction with phage P1kc was by the method of Guest (14). Lysates of donor strains were prepared by confluent lysis using the media and method of Lennox (21). Recipient bacteria were infected with a multiplicity of 2, and appropriate dilutions of transduced cultures plated on selective medium enriched with nutrient broth (Difco; 0.2% vol/vol). Recombinant colonies were counted after 2 to 4 days, with the exception of the Frd^+ colonies selected on GF medium, which were counted after 5 to 15 days, according to the strain used. Transductants were purified on selective medium before scoring the inheritance of nonselective markers.

RESULTS

Isolation and characterization of mutants. *E. coli* cannot grow anaerobically on glycerol without an exogenous electron acceptor, and fumarate can serve this function in the presence of an active fumarate reductase. On this basis, the inability to grow anaerobically in a medium containing glycerol plus fumarate may be used to select mutants deficient in fumarate reductase. Aerobic growth on glycerol, followed by anaerobic penicillin selection in GF medium, proved an effective method for enriching mutants since approximately 3% of the surviving colonies did not grow anaerobically on GF medium after a single treatment.

A total of 14 mutants with the desired phenotype, derived from two parental strains, R4 and W945*sdh*, were investigated enzymologically. Cell-free extracts of these were tested for their ability to reduce fumarate and oxidize succinate, after anaerobic growth on complex medium (Table 2). Results obtained with the strains P678 and P678*frd* are included for comparison.

Clearly there are several classes of mutant, but the predominant type (type *a*), which included 10 of the 14 mutants, contained less than 1% of the parental activity for fumarate reduction. One mutant (type *d*) contained 17% of the parental activity, and two others (type *c*) were classified as Frd^+ on the basis of their

TABLE 2. *Fumarate reduction and succinate oxidation specific activities in parental and mutant strains after anaerobic growth in complex medium with glucose (0.1%)*

Strain	No. of mutants	Fumarate reduction		Succinate oxidation	
		Specific activity[a]	Parental activity (%)	Specific activity[a]	Parental activity (%)
R4		29.00		3.46	
R4 *a* mutants (*frd*-1)	5	0.21	<1	0.19	5
R4 *b* mutants	1	0.77	2	1.01	30
R4 *c* mutants	2	13.1	45	1.98	57
W945*sdh*		26.52		2.07	
W945*sdh a* mutants (*frd*-2)	5	0.15	<1	0.02	<1
W945*sdh d* mutants	1	4.40	17	0.07	3
P678		12.70		1.66	
P678*frd*	1	1.02	8	1.99	>100

[a] Expressed as micromoles of substrate transformed per milligram of protein per hour.

enzymology, although they did not grow on GF medium anaerobically.

Two mutants of type *a*, named R4*frd*-1 and W945*sdh frd*-2, were selected for further biochemical and genetic studies. Anaerobic growth curves in GF medium were measured for these mutants together with their respective parental strains (Fig. 1). Compared with their parental strains, the growth of both mutants is greatly impaired although strain R4*frd*-1 shows a low, but definite, growth. None of the strains grew when either glycerol or fumarate were omitted.

A complication of estimating fumarate reductase in cell-free extracts is that succinate dehydrogenase, which may also be present, is a reversible enzyme and can reduce fumarate to some extent. For this reason the enzyme activities measured are recorded as the rates of reduction of fumarate and oxidation of succinate, rather than as the specific activities of the distinct enzymes. Because both fumarate reductase and succinate dehydrogenase are reversible and, as mutants in each may contain a residual amount of activity, there is no direct means of determining the absolute activities of each enzyme. However, reasonable estimates may be made by comparing the activities of parental and mutant strains, in conditions where the enzymes are induced.

Most of the Frd$^-$ mutants of strain R4, compared with the parental strain, were deficient in their ability to oxidize succinate as well as reduce fumarate after anaerobic growth (Table 2). This suggested that the mutants may be (i) double mutants with lesions in both *frd* and *sdh* genes, (ii) pleiotropic mutants with lesions affecting a membrane-binding site common to both enzymes or (iii) single *frd* mutants, if the succinate oxidation activity of the anaerobically grown parental strain is due to fumarate reductase acting in reverse. Evidence supporting the latter view comes from several observations. First, the Frd$^-$ mutants of strain W945*sdh* also showed similar deficiencies in both succinate oxidation and fumarate reduction activities (Table 2), indicating that the succinate oxidation activity of the parent was due to fumarate reductase. Furthermore, because the succinate oxidation activities of anaerobically grown strain R4 and P678 were comparable to that of strain W945*sdh* (Table 2), it can be concluded that succinate dehydrogenase synthesis was virtually completely repressed under anaerobic conditions. Finally, when strain R4*frd*-1 was grown under conditions which should induce succinate dehydrogenase (aerobically in the absence of glucose; 12), the rate of succinate oxidation was as high as that for the parental strain (Table 3), confirming the presence of a wild-type *sdh* gene. By contrast, the rate of succinate oxidation by strain W945*sdh* was very much less after aerobic growth, further confirming that the anaerobic acitivity was due to fumarate reductase. The results are therefore consistent with the view that type *a* mutants have lesions in the *frd* structural gene only.

After aerobic growth in the absence of glucose, the rates of succinate oxidation for all Sdh$^+$ strains were increased and the rates of fumarate reduction of Frd$^+$ strains were much

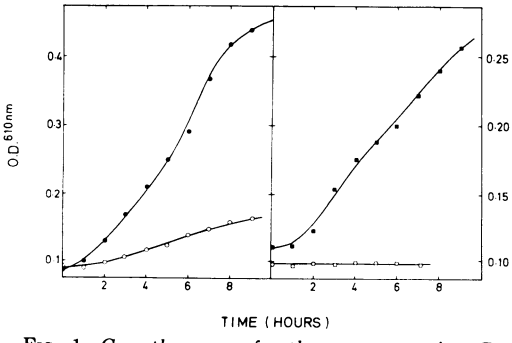

FIG. 1. *Growth curves for the parent strains, R4 (●) and W945sdh (■), and the mutants, R4frd-1 (○) and W945sdh frd-2 (□), on GF medium (anaerobic growth). The cells were grown in side-arm flasks inoculated with a washed overnight L-broth culture and gassed with H_2/CO_2 (95:5; vol/vol). One optical density (O.D.) unit at 610 nm is equivalent to 10^9 cells/ml.*

TABLE 3. *Specific activities for fumarate reduction and succinate oxidation for parental and mutant strains grown aerobically in complex medium with or without glucose (0.1%)*

Strain	Specific activity[a]			
	Without glucose		With glucose	
	Fumarate reduction	Succinate oxidation	Fumarate reduction	Succinate oxidation
R4	2.91	5.90	8.01	9.24
R4*frd*-1	0.76	7.64	0.95	6.64
W945*sdh*	1.45	0.35	4.53	0.97
W945*sdh frd*-2	0.11	0.14	0.18	0.05
P678	3.06	6.11	11.82	3.90
P678*frd*	0.62	4.63	0.66	2.77

[a] Expressed as micromoles of substrate transformed per milligram of protein per hour.

lower (Table 3), when compared with the corresponding anaerobic rates. Furthermore, a comparison of the rates of fumarate reduction for strain W945*sdh* and other strains, particularly W945*sdh frd*-2, indicated that fumarate reductase was highly, but not completely, repressed during aerobic growth. It was also possible to estimate that, although the overall rates of succinate oxidation and fumarate reduction increased two- to threefold or decreased four- to tenfold, respectively, during transition from anaerobic to aerobic conditions with Sdh$^+$Frd$^+$ strains (R4 and P678), these changes probably corresponded to an increase in specific activity for succinate dehydrogenase approaching 40-fold and a 20-fold decrease in fumarate reductase activity. This aerobic repression of fumarate reductase and induction of succinate dehydrogenase was greatest in the absence of glucose; when glucose (0.1%) was added aerobically, intermediate levels of fumarate reductase were obtained and succinate dehydrogenase was less fully induced (Table 3). Parental strains were also grown on the GF medium anaerobically, but the fumarate reducing activities obtained were no higher than after growth on complex medium.

Strain P678*frd* was atypical because it maintained a high rate of succinate oxidation after anaerobic growth (Table 2); only one of the mutants isolated here (R4 type *b*) exhibited a somewhat similar phenotype. A possible explanation is that, unlike the *frd*-1 and *frd*-2 lesions, the P678*frd* and type *b* lesions affect only the fumarate reducing activity of fumarate reductase and not the reverse reaction.

Genetic studies with frd mutants. In preliminary conjugations between several Hfr strains and strain P678*frd*, the frequency of Frd$^+$Str^R recombinant formation was relatively high with R4 and Ra-2 as donors, but very low with the H and P10 (Table 4). This indicates that the *frd* marker lies in the 79- to 88-min region of the *E. coli* chromosome (Fig. 2). Similar studies with F' donors carrying different segments within this region further limited the *frd* marker to that segment in the 79- to 84-min region which is common to strains KLF17 and KLF18 (Table 4, Fig. 2).

The mutants R4*frd*-1 and W945*sdh frd*-2 were transduced to Frd$^+$ with parental donors at normal frequencies; both complete and abortive transductants were seen. Selected Frd$^+$ transductants regained both fumarate reduction and succinate oxidation activities after anaerobic growth, suggesting that the observed phenotype results from a single lesion. Transductions were performed between *frd* mutants and strains containing various genetic markers in the region between 79 and 84 min (Table 5). The *frd* mutations were cotransducible with *fdp*, *purA*, and *ampA*, but not with *pyrB* or *uvrA*. In most cases the results with different *frd* mutants agreed well. An exception was the failure to find co-transduction between the lesion in strain P678*frd* and *ampA* when the other mutations (*frd*-1 and *frd*-2) were closely-linked to *ampA* (as would be expected on the basis of other linkage relationships). This anomaly appeared to be a property of the P678 background; the same *frd* lesion transduced into other backgrounds (PA256 and Q11) showed, in most cases, close linkage with *ampA*. In view of this, the genetic mapping of strain P678*frd* was not investigated further. The co-transduction frequencies obtained using *frd*-1 and *frd*-2 are summarized in Fig. 3, together with the cotransduction frequencies between the other markers which have been determined by previous workers.

The reported co-transduction frequencies between *ampA* and *purA* are much higher when Amp^R is selected first (8). Similar results were

TABLE 4. *Frequency of Frd$^+$ recombinants in conjugations between P678frd and several Hfr and F' donors*

Donor strain	Type	Frd$^+$ recombinants (% donor input)
R4	Hfr	4.8
P10	Hfr	0.0036
H	Hfr	0.0068
Ra-2	Hfr	0.34
KLF17	F'	15.4
KLF18	F'	2.5
KLF19	F'	<0.0001
KLF21	F'	<0.0001

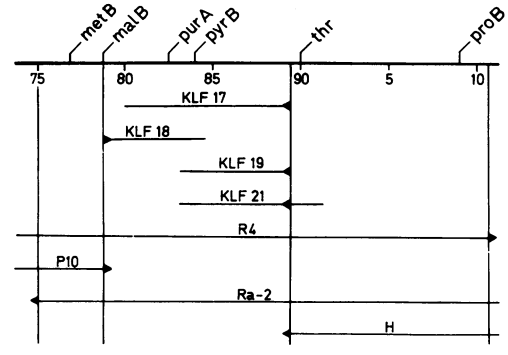

FIG. 2. *Linkage map of Escherichia coli showing origin and polarity of Hfr and F' donor strains, and the approximate limits of the F' episomes used in conjugation.*

TABLE 5. *Phage P1-mediated co-transduction between genetic markers in the uvrA to pyrB region*[a]

Donor	Recipient	Selection	Transductants per 10^8 P1	Inheritance of unselected donor marker		
				No. scored	Marker	Cotransduction (%)
P678*frd*	PA256	$PurA^+$	18	151	Frd^-	18.5
R4*frd*-1	PA256	$PurA^+$	61	99	Frd^-	37
W945*sdh frd*-2	PA256	$PurA^+$	30	80	Frd^-	41
R4*frd*-1	RM1003	$PurA^+$	243	129	Frd^-	38
P678*frd*	Q11	Fdp^+	15	87	Frd^-	5
R4*frd*-1	Q11	Fdp^+	226	104	Frd^-	6
W945*sdh frd*-2	Q11	Fdp^+	106	109	Frd^-	3
P678*frd*	PA2004	$PyrB^+$	30	100	Frd^-	<1
G11a1	P678*frd*	Amp^R	3	100	Frd^+	<1
		Frd^+	3	100	Amp^R	<1
G11a1	R4*frd*-1	Amp^R	78	100	Frd^+	97
		Frd^+	204	101	Amp^R	87
G11a1	W945*sdh frd*-2	Amp^R	71	106	Frd^+	98
		Frd^+	30	104	Amp^R	100
AB2500	R4*frd*-1	Frd^+	3	100	$UvrA^S$	<1
RM1003	R4*frd*-1	Frd^+	66	81	$PurA^-$	<1
G11a1	PA256	Amp^R	86	92	$PurA^+$	74
		$PurA^+$	106	106	Amp^R	38

[a] $PurA^+$ and $PurB^+$ transductants were selected on glucose minimal medium, Fdp^+ on glycerol minimal medium, and Amp^R on L agar containing ampicillin (10 μg/ml). Transductants were scored as Uvr^S if they failed to grow after irradiation for 60 s on L agar, 75 cm from a 15-W ultraviolet light source.

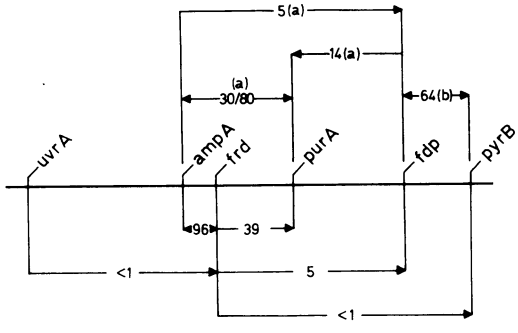

FIG. 3. *Linkage map of Escherichia coli from 79 to 84 min showing the position of markers in the region and their cotransduction frequencies. Data above the map was obtained by previous authors (a) Eriksson-Grennberg (8), (b) Fraenkel and Horecker (11). Data below the map summarizes the results of Table 5.*

obtained here (Table 5), and it is possible that selection against $PurA^-$ transductants occurs during the period of 4 h allowed for Amp^R expression. The strain PA256 was not used as a donor, because no lysate of reasonable titer could be made. Instead a *purA* strain RM1003 was used, but no $PurA^-$ recombinants were found. This finding is in agreement with the unexplained observation of Eriksson-Grennberg (8) that *purA* mutant markers are not found in recombinants obtained by transduction with P1·*purA* lysates.

The above results show that *frd* is very close to *ampA*, but the relative order of the *frd*, *ampA*, and *purA* genes cannot be specified. The order of these markers was determined by a three-factor cross, with KG20 (*purA*,*ampA*) as a recipient and a P1 lysate of strain R4*frd*-1, the donor strain. $PurA^+$ transductants were selected, and the distribution of the nonselective Amp^S and Frd^- markers was examined (Table 6). The Amp^SFrd^+ class is clearly the least frequent and, if this is presumed to derive from a quadruple cross-over, it may be concluded that the order of the genes is *ampA-frd-purA*.

DISCUSSION

The glycerol-fumarate medium devised for the selection of *frd* mutants proved effective, and most of the mutants isolated appeared to have lesions only in the presumed structural gene for fumarate reductase (*frd*). However some of the mutants (type *c*) which failed to grow on glycerol plus fumarate possessed an active fumarate reductase: the nature of their lesions is currently under investigation. If fumarate reductase does participate in an electron transport system, it is possible that these

mutants are deficient in other components of the system. Alternatively, they may lack the soluble, flavin-linked α-glycerophosphate dehydrogenase which is used for anaerobic catabolism of glycerol and is distinct from the particulate dehydrogenase used aerobically (18).

Compared with their parental strains, most of the newly isolated mutants were deficient in both fumarate reduction and succinate oxidation activities after anaerobic growth. In this respect they differed from the P678*frd* mutant which retained parental succinate oxidation activity. The mutants appeared, however, to have lesions only in the *frd* gene: they could be readily transduced to Frd$^+$ with the simultaneous recovery of both activities, and the *frd* mutants of strain R4 clearly possessed a wild-type *sdh* gene as they showed parental activities for succinate oxidation after aerobic growth. No mutants of the phenotype of strain P678*frd* were isolated, but one R4 mutant (type *b*) was found to retain 30% of its parental succinate oxidation activity although it had only 2% fumarate reduction activity.

The isolation of single *frd* mutants which had also lost all their anaerobic succinate oxidation activity led to the conclusion that succinate dehydrogenase is completely repressed in anaerobic growth, the succinate oxidation activity measured in parental strains resulting from fumarate reductase acting reversibly. This is supported by the observation that strain W945*sdh* showed almost wild-type anaerobic succinate oxidation activities, but after aerobic growth, when fumarate reductase was highly repressed, the succinate oxidation activity declined in parallel. It seems likely that the succinate oxidation activity reported for anaerobically grown *E. coli* (12, 13, 17) is mainly due to fumarate reductase and that succinate dehydrogenase is completely repressed anaerobically, as is α-ketoglutarate dehydrogenase (2). Fumarate reductase was induced anaerobically, and highly, but not completely repressed after anaerobic growth in the absence of glucose. In the presence of glucose, a small induction of fumarate reductase was observed, at the same time as some catabolite repression of the tricarboxylic acid cycle enzymes, including succinate dehydrogenase (13, 26). This shows that the regulation of fumarate reductase is correlated with the regulation of the tricarboxylic acid cycle, such that fumarate reductase is induced when the TCA cycle enzymes are repressed. Unlike nitrate reductase (27), fumarate reductase is apparently not substrate induced, and anaerobic growth on glycerol plus fumarate did not result in higher activities than anaerobic growth on complex glucose medium. However this situation may reflect the difference between an exogenous electron acceptor (nitrate) and one which is normally generated endogenously (fumarate). It is possible that anaerobic growth on complex glucose medium generated sufficient fumarate to induce fumarate reductase fully.

TABLE 6. *Three-factor cross between frd, purA, and ampA markers*

Donor	Recipient	Selection	Transductants per 10^8 phage	Distribution of unselected markers	
				Type	No.
R4*frd*-1	KG20 (*amp purA*)	PurA$^+$	110	Amp^{R}Frd$^+$	131
				Amp^{S}Frd$^-$	62
				Amp^{S}Frd$^+$	0
				Amp^{R}Frd$^-$	20

The ratios of succinate oxidation to fumarate reduction activity for succinate dehydrogenase and fumarate reductase are best obtained when only one enzyme is operating. This is the case for succinate dehydrogenase in aerobically grown *frd* mutants and for fumarate reductase in any anaerobically grown Frd$^+$ strain. The ratios observed here are 7 to 13 for succinate dehydrogenase and 0.08 to 0.13 for fumarate reductase. Thus fumarate reductase is found to catalyze the reduction of fumarate 7 to 13 times faster than the oxidation of succinate. Similar ratios of activity have been reported for fumarate reductase from yeast, *E. coli*, and *Micrococcus lactilyticus* (19, 24, 30). The results presented here differ from those of Hirsch et al. (16) who deduced succinate oxidation to fumarate reduction ratios of 25 for succinate dehydrogenase and 1.5 for fumarate reductase. However, different methods were used for assaying both activities, so the results are not strictly comparable.

An analysis of the cotransduction results shows that *frd* lies at about 82 min on the *E. coli* chromosome. This is about 25 min away from the *sdh* gene (4), but it is interesting to note that the structural gene for aspartase, *aspA*, has been located in this region of the chromosome (23). The possibility of the two genes comprising part of a regulatory unit is being investigated.

ACKNOWLEDGMENT

One of the authors (M.E.S.) was a recipient of a Medical Research Council Junior Research Fellowship.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965.

Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. Amarasingham, C. R., and B. D. Davis. 1965. Regulation of α-ketoglutarate dehydrogenase formation in *Escherichia coli*. J. Biol. Chem. **240**:3554–3668.
3. Arrigoni, C., and T. P. Singer. 1962. Limitation of the phenazine methosulphate assay for succinic and related dehydrogenases. Nature (London) **193**:1256–1258.
4. Creaghan, I. T., and J. R. Guest. 1972. *Amber* mutants of the α-ketoglutarate dehydrogenase gene of *Escherichia coli* K12. J. Gen. Microbiol. **71**:207–220.
5. Courtright, J. B., and U. Henning. 1970. Malate dehydrogenase mutants in *Escherichia coli* K-12. J. Bacteriol. **102**:722–728.
6. Deibel, R. H., and M. J. Kvethas. 1964. Fumarate reduction and its role in the diversion of glucose fermentation by *Streptococcus faecalis*. J. Bacteriol. **88**:858–864.
7. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1968. A proposal for a uniform nomenclature in bacterial genetics. J. Gen. Microbiol. **50**:1–14.
8. Eriksson-Grennberg, K. G. 1968. Resistance of *Escherichia coli* to penicillins. II. An improved mapping of the *ampA* gene. Genetics **12**:147–156.
9. Faust, P. J., and P. J. Vandemark. 1970. Phosphorylation coupled to NADH oxidation with fumarate in *Streptococcus faecalis* loci. Arch. Biochem. Biophys. **137**:392–398.
10. Forget, P., and P. Pichinoty. 1964. Influence de la respiration anaérobic du nitrate et du fumarate sur le métabolisme fermentaire d-*Aerobacter aerogenes*. Biochem. Biophys. Acta **82**:441–444.
11. Fraenkel, D. G., and B. L. Horecker. 1965. Fructose-1,6-diphosphatase and acid hexose phosphatase of *Escherichia coli*. J. Bacteriol. **90**:837–842.
12. Gray, C. T., J. W. T. Wimpenny, D. E. Hughes, and M. R. Mossman. 1966. Regulation of metabolism in facultative bacteria. I. Structural and functional changes in *Escherichia coli* associated with shifts between the aerobic and anaerobic states. Biochem. Biophys. Acta **117**:22–32.
13. Gray, C. T., J. W. T. Wimpenny, and M. R. Mossman. 1966. Regulation of metabolism in facultative bacteria. II. Effects of aerobiosis, anaerobiosis and nutrition on the formation of Krebs cycle enzymes in *Escherichia coli*. Biochem. Biophys. Acta **117**:33–41.
14. Guest, J. R. 1969. Biochemical and genetic studies with nitrate reductase *C*-gene mutants of *Escherichia coli*. Mol. Gen. Genet. **105**:285–297.
15. Hauber, J., and T. P. Singer. 1967. Studies on succinate dehydrogenase. 14. Intracellular distribution, catalytic properties and regulation of fumarate reductases in yeast. Eur. J. Biochem. **3**:107–116.
16. Hirsch, C. A., M. Rasminsky, B. D. Davis, and E. C. C. Lin. 1963. A fumarate reductase in *Escherichia coli* distinct from succinate dehydrogenase. J. Biol. Chem. **238**:3770–3774.
17. Jones, R. G. W., and J. Lascelles. 1967. The relationship of 4-hydroxybenzoic acid to lysine and methionine formation in *Escherichia coli*. Biochem. J. **103**:709–713.
18. Kistler, W. S., and E. C. C. Lin. 1971. Anaerobic L-α-glycerophosphate dehydrogenase of *Escherichia coli*: its genetic locus and its physiological role. J. Bacteriol. **108**:1224–1234.
19. Kovac, L. 1960. Enzymic reduction of fumarate in yeast. Enzymologia **22**:27–36.
20. Kröger, A., and V. Dadak. 1969. On the role of quinone in bacterial electron transport. Eur. J. Biochem. **11**:328–340.
21. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein Measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. Marcus, M., and Y. S. Halpern. 1969. Mapping of the aspartase gene in *Escherichia coli* K-12. Israel J. Med. Sci. **5**:413–415.
24. Peck, H. D., O. H. Smith, and H. Gest. 1957. Comparative biochemistry of the biological reduction of fumaric acid. Biochem. Biophys. Acta **25**:142–147.
25. Pichinoty, F., L. LeMinor, and H. H. Mollaret. 1965. An investigation of fumarate reductase in facultative anaerobes. Can. J. Microbiol. **11**:287–290.
26. Ruiz-Herrera, J., and L. G. Garcia. 1972. Regulation of succinate dehydrogenase in *Escherichia coli*. J. Gen. Microbiol. **72**:29–35.
27. Showe, M. K., and J. A. DeMoss. 1968. Localization and regulation of synthesis of nitrate reductase in *Escherichia coli*. J. Bacteriol. **95**:1305–1313.
28. Sinclair, P. R., and D. C. White. 1970. Effect of nitrate, fumarate and oxygen on the formation of the membrane-bound electron transport system of *Haemophilus influenzae*. J. Bacteriol. **101**:365–372.
29. Taylor, A. L. 1970. The current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155–175.
30. Warringa, M. G. J., O. H. Smith, A. Giuditta, and T. P. Singer. 1958. Studies on succinic dehydrogenase. VIII. Isolation of a succinic dehydrogenase-fumaric reductase from an obligate anaerobe. J. Biol. Chem. **230**:97–109.