Mutants of *Escherichia coli* K12 Unable to use Fumarate as an Anaerobic Electron Acceptor

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SUMMARY

Mutants of *Escherichia coli* K12 strain WGAS-GF⁺/LF⁺ were selected for their inability to use fumarate as terminal electron acceptor for supporting growth on glycerol or lactate in an atmosphere of H₂ plus 5 % CO₂. Eighty-three mutants were grouped into seven different categories according to their ability to grow on different media and their ability to produce gas during glucose fermentation. Enzymological and genetic studies indicated that the major class (type I), representing nearly 70 % of the isolates, lacked fumarate reductase and corresponded to the *frdA* mutants studied previously (Spencer & Guest, 1973, 1974). Members of a second class (type II) were phenotypically similar to *men* mutants, blocked in menaquinone biosynthesis. They differed from *menA* mutants in having lesions in the 44 to 51 min region of the chromosome rather than at 87 min. It was concluded that fumarate reductase and menaquinone are essential for anaerobic growth when fumarate serves as electron acceptor but not when nitrate performs this function. Fumarate reductase and menaquinone are also essential for H₂-dependent growth on fumarate. Type III mutants, originally *frdB*, were designated *fnr* because they were defective in fumarate and nitrate reduction and impaired in their ability to produce gas. The *fnr* gene was located at 28.5 min by its cotransducibility with *pyrF* (5.7 to 9.2 %) and *trpA* (2.7 to 5.7 %) and the gene order *fnr-qmeA-pyrF-trpA* was established. It was not possible to assign specific metabolic lesions to the *fnr* mutants nor to the remaining classes, which all exhibited pleiotropic phenotypes. Nevertheless, the results demonstrate that functional or organizational relationships exist between the fumarate reductase system, nitrate reduction and hydrogen production.

INTRODUCTION

The reduction of fumarate to succinate in *Escherichia coli* K12 is catalysed by a membrane-bound flavoprotein, fumarate reductase, which is induced anaerobically and repressed aerobically (Hirsch *et al.*, 1963; Spencer & Guest, 1973, 1974). More specifically, anaerobic growth on glycerol plus fumarate induces the synthesis of an anaerobic glycerol-3-phosphate dehydrogenase and fumarate reductase as a functional membrane complex (Miki & Lin, 1973). Fumarate reductase allows fumarate to serve as a terminal acceptor permitting anaerobic growth on non-fermentable substrates. The fumarate reductase system is thus analogous in many respects to the nitrate reductase system where nitrate is the terminal electron acceptor.

Studies with a number of strict anaerobes and anaerobically grown facultative strains point to the existence of a variety of anaerobic electron transport systems involving cytochromes and/or quinones which couple the oxidation of different electron donors (e.g. H₂, NADH, formate, lactate, glycerol 3-phosphate and malate) to the reduction of fumarate. Recent

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Fig. 1. A scheme summarizing relevant metabolic pathways (---) and pathways of electron transport to nitrate and fumarate (—): MK, menaquinone; UQ, ubiquinone; cyt, specific cytochromes.

studies on *E. coli* indicate that electron transport to fumarate involves menaquinone but although cytochromes may enhance the process, their participation is not obligatory for all substrates (Singh & Bragg, 1975, 1976). Thus NADH and glycerol 3-phosphate may be oxidized by fumarate *via* cytochrome-dependent and -independent routes whereas cytochromes are essential for the oxidation of D-lactate and probably H₂ (Macy, Kulla & Gottschalk, 1976) and formate. By contrast, cytochromes and quinones appear to be obligatory components of nitrate reductase systems (Ruiz-Herrera & DeMoss, 1969; Enoch & Lester, 1975; Singh & Bragg, 1975). A current view of some relevant metabolic and electron transport pathways to fumarate and nitrate is shown in Fig. 1.

Values for molar growth yields obtained with *Propionibacterium* (de Vries, van Wyck-Kapteyn & Stouthamer, 1973), *Proteus* (Kröger, 1974) and *Bacteroides* (Macy, Probst & Gottschalk, 1975) species indicate that energy conservation is associated with fumarate reduction. Direct evidence for the phosphorylation of ADP during fumarate reduction has come from studies with cell-free preparations of *Streptococcus faecalis* plus NADH (Faust & Vandemark, 1970), *Desulphovibrio gigas* plus H₂ (Barton, LeGall & Peck, 1970) and *E. coli* plus glycerol 3-phosphate (Miki & Lin, 1975). The anaerobic transport of lactose and amino acids by *E. coli* can be coupled to glycerol 3-phosphate and formate oxidation with fumarate or nitrate as electron acceptors (Konings & Kaback, 1973; Boonstra *et al.*, 1975). Furthermore, studies of amino acid transport with a *hemA* mutant indicate that cytochromes are essential for energy conservation during electron transport to fumarate (Singh & Bragg, 1976).

In *E. coli*, fumarate also serves as electron acceptor in anaerobic pyrimidine synthesis for the oxidation of dihydroorotate with menaquinone as the electron carrier (Newton, Cox & Gibson, 1971) and in anaerobic porphyrin synthesis for the oxidation of protoporphyrinogen to protoporphyrin (Jacobs & Jacobs, 1975).

Mutants of *E. coli* K12 with defects in fumarate reduction have been isolated by an indirect selection based on their ability to grow aerobically on glycerol and inability to grow anaerobically on a glycerol plus fumarate medium (Spencer & Guest, 1973, 1974). This procedure yielded fumarate reductase mutants (*frdA*) and a variable proportion (35 to 65 %)

of uncharacterized mutants. The latter were assumed to have defects in other components of the fumarate reductase system or in functions specifically associated with anaerobic glycerol metabolism such as the anaerobic glycerol-3-phosphate dehydrogenase (*glpA*; Kistler & Lin, 1971). The purpose of the present work was to investigate biochemical and genetic characteristics of the formerly unclassified mutants. Including *frdA* mutants, seven different classes were recognized; most of these were pleiotropic, having defects in nitrate respiration or glucose fermentation as well as fumarate reduction.

METHODS

Organisms. A new spontaneous streptomycin-resistant strain of *Escherichia coli* K12, WGAS (PL2001), was isolated from strain WGA (W3110 *gal*, *trpA*, *trpR*, F⁻). From this further strains, which grew well anaerobically on GF medium (WGAS-GF⁺) and LF medium (WGAS-GF⁺/LF⁺, PL2024) but remained sensitive to chlorate, were selected sequentially according to Lambden & Guest (1976). This was necessary because the original WGAS strain was mucoid at 30 °C and its derivative, which grew well with fumarate and was used in previous isolations of fumarate reductase mutants (Spencer & Guest, 1974), proved to be chlorate-resistant. Strain WGAS-GF⁺/LF⁺ was the parental strain of the mutants described here. A collection of 21 F' donor strains containing F' plasmids in several Rec⁻ backgrounds

Table 1. *Strains of Escherichia coli* K12

Strain	Characteristics	Source or reference
W3110	F ⁻ , <i>trpR</i>	Bachmann (1972)
S729	F ⁻ , <i>met</i> , <i>hemA</i>	A. H. Stouthamer
AT3143	F ⁻ , <i>pyrF</i> , <i>pdxC3</i> , <i>ilv-277</i> , <i>met-65</i> , <i>his-53</i> , <i>purE41</i> , <i>proC24</i> , <i>cycA1</i> , <i>xyl-14</i> , <i>lacY29</i> , <i>str-97</i> , <i>tsx-63</i>	Semple & Silbert (1975)
TKG49	F ⁻ , <i>thr</i> , <i>leu</i> , <i>his</i> , <i>ilvA</i> , <i>arg</i> , <i>thi</i> , <i>pyrF</i> , <i>thyA</i> , <i>qmeA49</i> , <i>lac</i> , <i>tonA</i> , <i>tsx</i>	Wijsman (1972)
AB3303	F ⁻ , <i>thi-1</i> , <i>argE3</i> , <i>his-4</i> , <i>pabB3</i> , <i>mtl-1</i> , <i>xyl-5</i> , <i>galK2</i> , <i>lacY1</i> , <i>tsx</i> , <i>str</i> , <i>supE44</i> ?	Huang & Pittard (1967)
AT701	Hfr, <i>thi-1</i> , <i>purF59</i> , <i>tna-3</i> , <i>ptsM14</i> , <i>rel-1</i>	Taylor & Trotter (1967)
AN386	F ⁻ , <i>thi</i> , <i>menA</i> , <i>str</i>	I. G. Young

was kindly provided by Dr B. J. Bachmann for conjugation studies. The numbers of the F' were F101, F104, F111, F112, F116, F117, F123, F126, F128, F129, F133, F140, F141, F142, F143, F148, F150, F152, F196, F254 and F450. Together these represent over 95 % of the *E. coli* linkage map (Low, 1972), the only segment not represented being the 4 min region between 32 and 36 min. The donor activities of F' strains representing more than two-thirds of the chromosome (92 to 51 min plus 83 to 88 min) were confirmed with independent markers. Details of other strains which were used in linkage analyses are listed in Table 1.

Media. The basal minimal medium was that of Spencer & Guest (1973). Substrates were added to give the following final concentrations (M): glucose, 0.01; glycerol, 0.04; sodium fumarate, 0.04; potassium lactate, 0.04; sodium formate, 0.04; potassium nitrate, 0.04. Glycerol plus fumarate (GF), lactate plus fumarate (LF), formate plus fumarate (FF), fumarate (F), glycerol plus nitrate (GN), lactate plus nitrate (LN) and formate plus nitrate (FN) media also contained Casamino acids (Difco, vitamin-free; 0.5 g l⁻¹). Minimal media were supplemented with amino acids, purines, pyridines and vitamins according to individual strain requirements. Anaerobic incubations were carried out under H₂/CO₂ (95:5, v/v).

Bacterial stocks were maintained on nutrient agar (Difco nutrient broth, 0.8 %; Difco yeast extract, 0.2 %; Difco agar, 1.5 %, w/v) or stored at -15 °C with glycerol (30 %, v/v)

according to Low (1973). L-broth (Lennox, 1955) with glucose omitted was used as the complex medium for routine subculture. Media were solidified with Difco Bacto agar (15 g l⁻¹) as required. Tests for sensitivity to chlorate were done anaerobically on medium containing Oxoid nutrient broth no. 2 plus glucose (2 g l⁻¹), potassium chlorate (1 g l⁻¹), and Difco agar (15 g l⁻¹). Gas production was tested according to Guest (1969).

Isolation of mutants. Exponential-phase L-broth cultures of strain WGAS-GF⁺/LF⁺ were washed and resuspended in Tris/maleate buffer (0.1 M; pH 6.0) at 10⁹ organisms ml⁻¹. Separate suspensions were then treated with the mutagen 1-methyl-3-nitro-1-nitrosoguanidine (100 µg ml⁻¹) for 20 min at 37 °C (Adelberg, Mandel & Chen, 1965). After mutagenesis the phenotypes of mutants were expressed by aerobic growth of a washed sample of the culture for 18 h in glycerol minimal medium (GF selection) or lactate minimal medium (LF selection). These cultures were then subjected to an anaerobic penicillin (300 u. ml⁻¹) selection in GF medium or LF medium, respectively. Survivors were plated on glycerol medium (GF⁻ selection) or lactate medium (LF⁻ selection) and incubated aerobically. The desired mutants were subsequently detected by replica-plating from glycerol medium to anaerobic GF medium or from lactate medium to anaerobic LF medium. Mutants were picked and purified by streaking and finally retested anaerobically on GF or LF media.

Mutants resistant to nalidixic acid were isolated from overnight nutrient broth cultures as follows: samples of washed cultures, concentrated fivefold in saline, were plated on nutrient agar containing nalidixic acid (20 µg ml⁻¹, final concentration) and incubated for 2 to 3 days at 37 °C. Resistant colonies, presumed to be *nalA* mutants, were picked and purified by streaking on the same medium.

Growth of cultures. Cultures for enzymological studies were grown in stationary Erlenmeyer flasks filled to the neck with complex medium containing (g l⁻¹): peptone, 4; yeast extract, 4; K₂HPO₄, 6; adjusted to pH 6.8. Glucose was added, after autoclaving, to a final concentration of 10 g l⁻¹. Flasks were inoculated with an overnight L-broth culture (2 ml l⁻¹) of the desired strain and cultures were grown for 18 h at 37 °C. For manometric studies with bacterial suspensions, cultures were harvested, washed twice in cold potassium phosphate buffer (0.02 M; pH 6.9) and concentrated to give 10 mg dry wt organisms ml⁻¹. To prepare cell-free extracts, the cultures were harvested, washed twice in cold potassium phosphate buffer (0.01 M; pH 7.8), and concentrated to 0.25 g wet wt ml⁻¹. These suspensions were disrupted at 4 °C by two 90 s treatments with an ultrasonic disintegrator (MSE; 100 W) followed by sedimenting at 10000 g for 30 min. The opalescent supernatant fluids were used for enzyme assays and the protein concentrations of these ultrasonic extracts were determined by the method of Lowry *et al.* (1951).

Enzyme assays. Spectrophotometric assays were performed in a Beckman DBG T spectrophotometer at 28 °C at enzyme-limiting concentrations and the results are expressed as µmol substrate transformed (mg protein)⁻¹ h⁻¹.

(i) Fumarate reductase (BVH) was assayed by the method of Spencer & Guest (1973). The fumarate-dependent oxidation of reduced benzylviologen (BVH) was followed at 550 nm under He. The reaction mixtures (final vol. 3 ml) contained: benzylviologen, 1 µmol; sodium fumarate, 6 µmol; potassium phosphate (pH 7.8), 300 µmol; water and cell extract.

(ii) Fumarate reductase (NADH). This assay was based on the method of Kröger *et al.* (1971). The fumarate-dependent oxidation of NADH was followed at 340 nm under He. The reaction mixtures (final vol. 3 ml) contained: NADH, 0.5 µmol; sodium fumarate, 6 µmol; potassium phosphate (pH 7.8), 300 µmol; water and cell extract.

(iii) Nitrate reductase (BVH) was assayed according to Guest (1969). The nitrate-dependent oxidation of reduced benzylviologen was followed at 550 nm under He. The reaction

mixtures (final vol. 3 ml) contained: reduced benzylviologen, 1 μ mol; potassium nitrate, 50 μ mol; potassium phosphate (pH 7.4), 300 μ mol; water and cell extract.

(iv) Nitrate reductase (NADH). The nitrate-dependent oxidation of NADH was recorded at 340 nm under He. The reaction mixtures (final vol. 3 ml) contained: NADH, 0.5 μ mol; potassium nitrate, 50 μ mol; potassium phosphate (pH 7.4), 300 μ mol; water and cell extract.

(v) Nitrate reductase (HCOOH). The formate-dependent reduction of nitrate was determined by colorimetric estimations of nitrite production. The reaction mixtures (final vol. 5 ml) contained: potassium phosphate (pH 7.4), 500 μ mol; potassium nitrate, 10 μ mol; sodium formate, 10 μ mol; water and cell extract. Incubation was under He because anaerobic conditions increased the reaction rates twofold.

(vi) Formate dehydrogenase (DCPIP). This assay was based on the method of Ruiz-Herrera, Showe & DeMoss (1969). The formate-dependent reduction of 2,6-dichlorophenol-indophenol (DCPIP) was followed at 600 nm under He. The reaction mixtures (final vol. 3 ml) contained: potassium phosphate (pH 7.4), 300 μ mol; DCPIP, 0.1 mg; *N*-methylphenazonium methosulphate, 2 mg; sodium formate, 50 μ mol; water and cell extract.

The manometric methods quoted by Gray *et al.* (1966) were used to assay formate dehydrogenase (MB, methylene blue), formate dehydrogenase (BV, benzylviologen), hydrogenase and formate hydrogenlyase in washed suspensions of bacteria at 37 °C; activities are expressed as μ l gas evolved or absorbed (mg dry wt organisms)⁻¹ min⁻¹.

Genetic techniques. Conjugations were performed by cross-streak tests on solid media. The F⁻ recipient organisms were grown overnight in L-broth (without added glucose), washed in saline and streaked along the diameter of plates of selective media (GF and LF). F⁺ donor strains were grown to early exponential phase in L-broth, washed and concentrated 10-fold in sterile saline. A loopful of each donor strain was streaked at right angles across the recipient streaks. Six cross-streak tests were performed per plate of selective medium. Plates were first incubated aerobically at 37 °C for 30 min, followed by anaerobic incubation for 2 to 3 days at 37 °C. Patches of confluent growth in the areas of donor and recipient mixing were taken as a positive result that the mutation under investigation was in a region of the chromosome covered by the particular F⁺ of the donor.

Transduction with phage P1 was by the method of Guest (1969). Lysates of the donor strain were prepared in soft-agar overlays using the methods and media of Lennox (1955). Recipient organisms were infected at a multiplicity of 2 and appropriate dilutions of transduced cultures were plated on selective media. Transductants were counted after 2 to 3 days when selected aerobically, or after 3 to 5 days for anaerobic selections. Transductants were streaked on fresh selective medium and scored for the inheritance of unselected markers by replica-plating.

RESULTS

Isolation and characterization of mutants

The investigation of genetic aspects of fumarate reduction required a medium that was selective against mutants with defects in fumarate reduction, and strains that were capable of rapid growth on that medium so that single bacteria produced sizeable colonies within 2 or 3 days. Previous experience indicated that the glycerol plus fumarate (GF) medium was sufficiently selective but colony formation under anaerobic conditions was normally very slow with *E. coli* K12. However, strains (GF⁺) which grew well on this medium were readily isolated by picking larger colonies or by several successive subcultures. To distinguish between mutants with defects in fumarate reduction and those with defects in glycerol oxidation, lactate was chosen as an alternative oxidizable substrate. Tests with a lactate plus

Table 2. *Phenotypic classes of mutant and their yields from different selection procedures*

Growth phenotypes were determined by streaking washed suspensions of organisms on plates of the corresponding medium, which were incubated under H_2 plus 5 % CO_2 and scored after 48 h (or 72 h for FF and FN media). Gas production was estimated according to Guest (1969). The distribution of mutants of each type is given according to the primary selection for failure to grow on either glycerol plus fumarate (GF^-) or lactate plus fumarate (LF^-) medium, and the number of mutants of independent origin refers to the number of mutagenesis experiments which yielded mutants with the corresponding phenotypes.

	Anaerobic growth response on medium:								Gas production	Distribution of mutants		No. of mutants of independent origin
	GF	LF	FF	GN	LN	FN	Glc	GlcU		GF^-	LF^-	
WGAS- GF^+	+	—	NT	+	+	+	+	+	+++			
WGAS- GF^+/LF^+	+	+	+	+	+	+	+	+	+++			
I (<i>frdA</i>)	—	—	—	+	+	+	+	+	+++	41	22	18
II (<i>men</i>)	—	—	—	+	+	+	TR	+	+++	0	4	3
III (<i>fur</i>)	—	—	—	—	—	—	+	+	+	4	4	6
IV	—	—	—	—	—	—	—	—	—	2	1	3
V	—	—	—	+	+	+	—	—	+++/++++	0	2	2
VI	—	—	—	+	—	—	+	+	+	2	0	2
VII	—	—	—	+	+	+	+	+	—	0	1	1

NT, not tested; TR, trace growth.

Fig. 2. Effect of uracil on the anaerobic growth on glucose of type II and *menA* mutants. Cultures were shaken gently at 37 °C under an atmosphere of H₂ plus 5% CO₂ in sealed 250 ml conical flasks fitted with optically matched side-arms. The flasks contained 10 ml basal salts medium plus glucose (30 mM) and, when required, uracil (40 µg ml⁻¹). The inocula were 0.2 ml of washed suspensions of organisms (grown for 18 h as stationary cultures in glucose minimal medium) adjusted to give an initial extinction of 0.03 to 0.05 at 610 nm. Growth of representative type II (T1) and *menA* (AN386) mutants in the absence (○, □) or presence (●, ■) of uracil is shown. Growth of the parental strain WGAS-GF⁺/LF⁺ (.....) is included for comparison.

fumarate (LF) medium indicated that a second selection was necessary to obtain maximal anaerobic growth on this medium. This sequential selection yielded GF⁺/LF⁺ derivatives of strain WGAS which grew well on all test media and also remained chlorate-sensitive. Direct selection on LF medium invariably produced derivatives (LF⁺), which grew well with fumarate as electron acceptor, but failed to grow anaerobically with nitrate and were also chlorate-resistant (Lambden & Guest, 1976). The biochemical bases for these selections or 'trainings' are not understood.

Isolation of mutants. Using one GF⁺/LF⁺ derivative (PL2024) as parent, mutants which grew on glycerol or lactate in air but failed to grow anaerobically with fumarate as electron acceptor were isolated after mutagenesis with 1-methyl-3-nitro-1-nitrosoguanidine (see Methods). A total of 20 independent selections were made, 10 with glycerol (A to J) and 10 with lactate (K to T) as the oxidizable substrate. In some experiments up to 10% of the penicillin survivors were judged to be mutants of the desired phenotype. Some 93 strains were retained for further classification.

Phenotypic classification of mutants. The mutants were all tested for their ability to grow anaerobically under H₂ plus CO₂ on the following solid media: glycerol plus fumarate (GF), lactate plus fumarate (LF), formate plus fumarate (FF), glycerol plus nitrate (GN), lactate plus nitrate (LN), formate plus nitrate (FN), glucose (Glc), glucose supplemented with uracil (GlcU) and, in some cases, fumarate alone (F). In addition, they were tested for their sensitivity to chlorate and their ability to produce gas in nutrient broth plus glucose.

Like the parental strain, all the mutants grew aerobically with glycerol, lactate and glucose as substrates and they remained sensitive to chlorate. The characteristics of 83 mutants comprising seven distinct phenotypic classes are summarized in Table 2.

Table 3. *Enzymological characteristics of different mutant classes*

Organisms were grown and assayed as described in Methods. The activities of the mutants are given as percentages of the specific activities obtained with the parental strain [shown in parentheses and expressed as μmol substrate transformed $(\text{mg protein})^{-1} \text{h}^{-1}$ for the fumarate and nitrate reductases and μl gas evolved or absorbed $(\text{mg dry wt organisms})^{-1} \text{min}^{-1}$ for the other enzymes].

Enzyme	Parent (WGAS-GF ⁺ /LF ⁺)	I (KI, <i>frdA11</i>)	II (TI, <i>men</i>)	III (E9, <i>fnr-1</i>)	IV (D3)	V (L3)
Fumarate reductase (BVH)	100 (9.60)	1	11	6	77	5
Nitrate reductase (BVH)	100 (26.0)	50	15	1	5	18
Formate dehydrogenase (MB)	100 (5.00)	107	72	122	42	108
Formate dehydrogenase (BV)	100 (2.50)	87	96	27	ND	139
Hydrogenase	100 (65.0)	106	64	17	17	118
Formate hydrogenlyase	100 (3.60)	148	117	9	ND	113

ND, activity not detectable.

Mutants in the major class (type I), representing almost 70 % of the isolates, were characterized by their inability to use fumarate as an electron acceptor, although they retained the ability to use nitrate for this purpose. They also grew on glucose and fermented this substrate with the production of gas. These properties are consistent with them being similar to the fumarate reductase mutants (*frdA*) isolated previously by Spencer & Guest (1973, 1974). In the absence of an exogenous organic electron donor (e.g. glycerol, lactate or formate) the *frdA* mutants also failed to grow on fumarate medium (F) under H_2 plus CO_2 . These conditions promoted significant, though less prolific growth of the parental strain (Lambden & Guest, 1976). Growth on F medium under N_2 plus CO_2 was very poor; it was only slightly affected by the *frdA* mutation and was comparable to the growth observed with H_2 plus nitrate or N_2 plus nitrate where Casamino acids were the only source of assimilable carbon. Eleven of the type I mutants appeared to grow slightly better on glucose when uracil was added but the significance of this was not investigated further.

Four mutants were assigned to a separate class (type II) because they required uracil for best anaerobic growth on glucose (Table 2); in other respects they resembled the type I mutants. Growth tests in liquid media confirmed their requirements for uracil and representative results, obtained with mutant TI, are shown in Fig. 2. This behaviour is characteristic of mutants (*men*) blocked in menaquinone biosynthesis (Newton *et al.*, 1971) and the response of a *menA* mutant (AN386), studied with the four type II mutants under identical conditions, is included in Fig. 2. Anaerobic growth tests on solid media indicated that the *menA* mutant grew on GN and LN but not on any of the media containing fumarate (including medium F) with or without uracil. This confirmed the similarity between *men* and the type II mutants and so the type II mutants were tentatively designated *men* mutants.

Two further classes of mutant (types III and IV) used neither fumarate nor nitrate as terminal electron acceptors (Table 2). However, the type III mutants grew anaerobically on glucose medium whereas the type IV mutants would not (even when nitrate was provided). The two classes were also differentiated by their ability to produce gas; production was poor in type III mutants but undetectable in type IV mutants. The type III mutants were originally given the genetic symbol *frdB* (see Bachmann, Low & Taylor, 1976) but this was changed to *fnr* in order to denote their combined defects in fumarate and nitrate reduction (or respiration).

The fifth class of mutant (type V) was represented by two independently-isolated strains from the LF⁻ selections. They were unable to utilize fumarate but could reduce nitrate (Table 2). However, these mutants differed from the type I (*frdA*) mutants in being unable to

Table 4. *Conjugations with F' donors*

Conjugations between 21 F' donors and mutant recipients were performed by cross-streak tests (see Methods). Selection was for GF⁺ and LF⁺ conjugants with appropriate counterselections against the donors. Positive results (+) with at least one class of mutant were obtained with the four donor strains shown below; no conjugants were detected with 17 of the donor strains.

Recipient mutant	Donor strain			
	F117	F123	F126	F129
Type I (<i>frdA</i>)	+	—	—	—
Type II (<i>men</i>)	—	—	—	+
Type III (<i>fnr</i>)	—	+	+	—
Type IV	—	—	+	—
Type V	—	—	—	—

grow anaerobically on glucose unless nitrate was provided. The two type V mutants also differed from each other in producing either more (L3) or less (S4) gas than the parental strain.

Two of the mutants (type VI; A5 and D9) resembled type III (*fnr*) mutants in all respects except they were able to grow anaerobically on GN medium and one mutant (type VII; R8) had the characteristics of a type I (*frdA*) mutant but was defective in gas production and conjugation tests indicated the mutation was located outside the *frdA* region. These were not studied in the same detail as the other mutants, nor were the remaining 10 strains, which resembled strain WGAS-GF⁺ in growing poorly on LF medium. These 10 strains were only recovered from the LF⁻ selections and were assumed simply to have lost the parental facility for rapid growth on LF medium.

Enzymological studies. Cell-free extracts and washed suspensions of mutants representing the main phenotypic classes were assayed for fumarate reductase, nitrate reductase and formate hydrogenlyase systems after anaerobic growth on a rich medium: this normally derepresses the synthesis of these enzymes. Typical results, expressed as a percentage of the activities obtained for the parental strain, are summarized in Table 3. The type I (*frdA*) mutants were characteristically deficient in fumarate reductase, their nitrate reductase activities were lower than that of the parental strain, but their formate dehydrogenase, hydrogenase and formate hydrogenlyase activities were essentially unchanged. The type II (*men*) and type V mutants had reduced fumarate and nitrate reductase activities but, consistent with their ability to generate gas, the other activities were not significantly affected. The type III (*fnr*) and type IV mutants were the most pleiotropic with deficiencies in nitrate reductase, formate dehydrogenase (BV), hydrogenase and formate hydrogenlyase. Despite these deficiencies they remained sensitive to chlorate like all the other mutant classes.

Genetic studies. Attempts to locate the mutant sites on the *E. coli* linkage map were made by cross-streak conjugations using a set of 21 F' donor strains (see Methods). The ability to use fumarate as an anaerobic electron acceptor was selected on GF and LF media. Counterselection against the donor strains presented problems since most of the F' strains were streptomycin-resistant. However, depending on the F' strain, counterselection was accomplished either nutritionally (e.g. F117, Thy⁻; F123, Pyr⁻ plus Thi⁻; F126, Thi⁻) or by incorporating nalidixic acid into the selective medium and using spontaneous nalidixic-acid-resistant derivatives (*nalA*) of the various mutant types as recipients. The results (Table 4) indicated that all the type I (*frdA*) mutations are located in the F117 segment of the chromosome. This is consistent with the previous location of the *frdA* gene (Spencer & Guest, 1973).

Table 5. *Enzymic activities of selected fnr (type III) mutants*

Organisms were grown as described in Methods. The activities of the mutants (E9, F3, J2, O1) are expressed as percentages of the specific activities obtained with the parental strain [shown in parentheses as μmol substrate transformed $(\text{mg protein})^{-1} \text{h}^{-1}$ for fumarate and nitrate reductases and formate dehydrogenase (DCPIP), and μl gas evolved or absorbed $(\text{mg dry wt organisms})^{-1} \text{min}^{-1}$ for the other enzymes].

Enzyme	Parental strain (WGAS-GF ⁺ /LF ⁺)	E9 (<i>fnr-1</i>)	F3 (<i>fnr-2</i>)	J2 (<i>fnr-4</i>)	O1 (<i>fnr-8</i>)
Fumarate reductase (BVH)	100 (9.60)	6	7	7	10
Fumarate reductase (NADH)	100 (0.32)	12	12	11	9
Nitrate reductase (BVH)	100 (26.0)	1	3	2	1
Nitrate reductase (NADH)	100 (0.87)	4	2	ND	ND
Nitrate reductase (HCOOH)	100 (0.06)	ND	ND	ND	ND
Formate dehydrogenase (DCPIP)	100 (1.60)	190	260	330	300
Formate dehydrogenase (MB)	100 (5.00)	122	94	140	104
Formate dehydrogenase (BV)	100 (2.45)	27	28	20	6
Hydrogenase	100 (65.0)	17	10	8	7
Formate hydrogenlyase	100 (3.60)	9	18	0.4	ND

ND, activity not detectable.

The type II (*men*) mutations were all situated in the F129 region (Table 4). No conjugants were obtained with F111, F112 or F133, which should transfer the *menA* gene, located at 87 min on the *E. coli* linkage map (Bachmann *et al.*, 1976). The *men* mutants isolated here must therefore possess mutations in a *men* gene (or genes) located at approximately 44 to 51 min. The type III (*fnr*) mutants produced conjugants with two F' donors, F123 and F126, indicating that their mutations are located near the *trp* operon in the region common to both F'. By contrast, the type IV mutations appeared to map in the unique region of the F126 segment, i.e. somewhere in the *gal-trp* region. However, no linkage between either *gal* or *trpA* markers was detected by P1-mediated transduction using a wild-type donor. Attempts to locate the type V mutations were unsuccessful because no conjugants could be detected with any of the F' donors tested. Preliminary tests with a variety of Hfr donors suggested a location in the 86 to 14 min region. Similar results were obtained with D9, a type VI mutant; this could mean that at least one of the F' donors was inactive or deleted.

Representatives of each class of mutant (I to VII) yielded GF⁺ and LF⁺ transductants at relatively high frequencies using phage P1 lysates of the parental strain. This indicates that the mutants arose by single mutations. However, the presence of two or more closely-linked mutations was not excluded, nor was the control of fumarate and nitrate respiration by two independent mutations eliminated for the minor classes (types IV and VI).

Biochemical and genetic studies with fnr mutants

The most frequent class of mutant isolated by the selection procedures outlined above was the fumarate reductase (type I; *frdA*) mutant. Next were the mutants designated *fnr* (type III), which were recovered when either the GF⁻ or LF⁻ phenotype was selected (Table 2). The *fnr* mutants (formerly *frdB*) were chosen for further investigation.

Enzymology of fnr mutants. Preliminary analyses (Table 3) had shown the *fnr* mutants to be pleiotropic, having defects in fumarate reduction, nitrate reduction and hydrogen production. Detailed studies with four *fnr* mutants of independent origin are summarized in Table 5. All the mutants had less than 12 % of the parental fumarate reductase activities when measured with reduced benzylviologen and NADH as the reducing substrates. However, the mutants were most deficient in nitrate reductase, their activities being less than 4 %

Table 6. Linkage relationships from transductions with *fnr* mutants as recipients

The *fnr* mutants served as recipients in P1-mediated transductions with donor strains carrying markers in the *ptsM-hemA* region. *Fnr*⁺ transductants were selected anaerobically on GF medium and scored for the unselected donor markers. Similar results were obtained with four mutants (E9, F3, J2, O1) bearing independent mutations (*fnr-1*, 2, 4 and 8) and the cotransduction frequencies are based on the aggregate of the number of transductants scored for all the recipients.

Donor	Selection	Transductants per 10 ⁸ phage	Number scored	Unselected donor phenotype	Cotransduction frequency (%)
W3110	<i>Fnr</i> ⁺	76	416	TrpA ⁺	3.4
TKG49	<i>Fnr</i> ⁺	4.5	557	TrpA ⁺	2.7
				PyrF ⁻	5.7
				QmeA ⁻	5.9
AT3143	<i>Fnr</i> ⁺	4.5	310	PyrF ⁻	9.0
S729	<i>Fnr</i> ⁺	40	606	TrpA ⁺	4.5
				HemA ⁻	<0.17
AB3303	<i>Fnr</i> ⁺	68	509	TrpA ⁺	5.7
				PabB ⁻	<0.20
AT701	<i>Fnr</i> ⁺	10	461	PtsM ⁻	<0.22

of that of the parental strain when assayed with the same reducing substrates (Table 5). Furthermore, overall formate-nitrate oxidoreductase activity could not be detected in any of the mutants. Several methods were used for assaying formate dehydrogenase. The cell-free assay, using DCPIP as electron acceptor, indicated activities two- to three-fold greater than that of the parental strain. In whole cells with methylene blue as electron acceptor the formate dehydrogenase activities of the mutants were not significantly affected, but with benzylviologen the specific activities were depressed to between 6 and 28 % of the parental value (Table 5). The hydrogenase and formate hydrogenlyase activities were also low (Table 5) and consistent with the limited gas production exhibited by this mutant class (Table 2). Formate hydrogenlyase activity was extremely low in one mutant (*fnr-4*) and not detectable in another (*fnr-8*) although they both accumulated gas during the longer incubation in the gas test.

Genetic studies with fnr mutants. Conjugation studies with the F' strains indicated that the *fnr* mutations were located near the *trp* operon i.e. in the region of the *E. coli* linkage map which is common to the F123 and F126 plasmids (Table 4). This was confirmed by P1-mediated transduction using the prototroph *E. coli* W3110 as donor and selecting *Fnr*⁺ transductants anaerobically on GF medium. All the *fnr* mutations proved to be from 2.9 to 4.8 % cotransducible with the *trpA* marker of the parental strain. Furthermore, all the *Fnr*⁺ transductants regained a complete parental phenotype i.e. no segregation of the selected GF⁺ phenotype from the LF⁺, GN⁺ and LN⁺ characteristics was detected. Four mutants were chosen for more precise mapping using donor strains carrying markers in the *hemA* to *ptsM* region (26 to 40 min on the linkage map). Similar results were obtained with all four mutants and so they have been combined to give the average cotransduction frequencies recorded in Table 6. Several donor strains gave cotransduction frequencies for *fnr-trpA* of between 2.7 and 5.7 %. The transductional linkages for *fnr-pyrF* were 5.7 % and 9.0 % with TKG49 and AT3143 as donors respectively (Table 6) and with TKG49 an average cotransduction frequency of 5.9 % was obtained for *fnr-qmeA*. These results suggest that *fnr* is located on the same side of *trp* as *pyrF* and *qmeA* (the clockwise side) but is more distant from *trp*. This was confirmed by the failure to detect cotransduction between *fnr* and *hemA*, a gene which is

Table 7. *Phage P1-mediated transductions with fnr mutants as donors*

Two *fnr* mutants (eg, *fnr-1* and *fnr-8*) were used as donors in transductions with TKG49 as recipient. *QmeA*⁺ transductants were selected on appropriately-supplemented glucose minimal medium at 42 °C after an initial pre-incubation at 30 °C for 4 h and *PyrF*⁺ transductants were selected on pyrimidine-free minimal medium at 30 °C. Similar results were obtained with both donors and the number of transductants scored contains approximately equal numbers from each cross.

Recipient	Selection	Transductants per 10 ⁶ phage	Number scored	Unselected donor phenotype	Cotransduction frequency (%)
TKG49	<i>QmeA</i> ⁺	16	407	<i>TrpA</i> ⁻	22.4
				<i>Fnr</i> ⁻	9.8
				<i>PyrF</i> ⁺	82.1
	<i>PyrF</i> ⁺	26	616	<i>TrpA</i> ⁻	25.9
				<i>Fnr</i> ⁻	9.2
				<i>QmeA</i> ⁺	83.0

* Amongst the 616 *PyrF*⁺ transductants the *Fnr* and *QmeA* phenotypes were distributed as follows: *Fnr*⁺*QmeA*⁺, 464 (75 %); *Fnr*⁺*QmeA*⁻, 91 (15 %); *Fnr*⁻*QmeA*⁺, 50 (8 %) and *Fnr*⁻*QmeA*⁻, 11 (2 %).

Fig. 3. Transductional linkage map showing the position of *fnr* (formerly *frdB*) with respect to other loci. Cotransduction frequencies are given as the number of transductants inheriting the unselected marker expressed as a percentage of the number of selected transductants examined. For each cross, the appropriate cotransduction frequency is placed near the head of the arrow which points to the selected marker. Published data, presented above the map, are included for comparison: *a*, Wijsman (1972); *b*, Guest (1969); *c*, Brown & Somerville (1971); *d*, Signer, Beckwith & Brenner (1965).

situated anticlockwise to *trp* and 16 to 21 % cotransducible with *trpA* (Guest, 1969). No transductional linkage could be demonstrated between *fnr* and either *ptsM* or *pabB* markers (Table 6). In reciprocal crosses using *fnr* mutants as donors and TKG49 as the recipient, *QmeA*⁺ and *PyrF*⁺ transductants were selected (Table 7). The results indicate that the *fnr* gene is fractionally closer to *qmeA* than to *pyrF*. Assuming that the products of quadruple cross-over events will be the least frequent, a detailed analysis of the distribution of the *fnr*

and *qmeA* characteristics of 616 PyrF⁺ transductants confirmed the gene order *fnr-qmeA-pyrF* (Table 7). The cotransduction frequencies indicate that the *fnr* mutations are located between the *pabB* and *qmeA* genes and the results are summarized in Fig. 3 together with some published cotransduction data for markers in the *qmeA* to *hemA* region.

DISCUSSION

Selection for the inability to grow anaerobically on glycerol or lactate with fumarate as electron acceptor yielded a complex spectrum of mutants. It also proved difficult to assign specific metabolic lesions to most of the mutant classes, partly because of their pleiotropic phenotypes. In this respect the situation resembles that posed by the *chl* mutants which have defects in the nitrate reductase system. Originally it was anticipated that some of the mutants would possess specific lesions affecting the metabolism of the oxidizable substrate e.g. mutations affecting anaerobic glycerol-3-phosphate dehydrogenase or lactate dehydrogenase. However, all the mutants isolated by the glycerol-fumarate selection failed to grow on lactate plus fumarate (LF) and formate plus fumarate (FF) media. Likewise, most of the mutants selected with lactate media, failed to grow with GF and FF media. The reason for the absence of mutants with simple oxidation defects became apparent when it was realized that the parental strain grew on the amino acid-enriched fumarate medium with H₂ plus CO₂ (Lambden & Guest, 1976). Consequently, the presence of H₂ as an electron donor as well as glycerol or lactate in the GF⁻ and LF⁻ selections used here, automatically limited the screen to mutants with defects in the fumarate reduction system or in components essential for the anaerobic metabolism of more than one substrate. In the absence of H₂, fumarate was an extremely poor substrate for supporting anaerobic growth. Studies with one mutant (*frdA11*) and its parent (WGAS-GF⁺/LF⁺) have shown that H₂ can be used as an oxidizable substrate to support anaerobic growth of *E. coli* on L-malate or fumarate, as has also been shown by Macy *et al.* (1976).

Members of the major class of mutant (type I), representing nearly 70 % of the isolates, corresponded to the fumarate reductase mutants (*frdA*) studied previously (Spencer & Guest, 1973, 1974). Their phenotype indicates that a functional fumarate reductase is essential for the good anaerobic growth obtained with GF, LF and FF media, and also for the less prolific growth obtained with fumarate plus H₂. It is noteworthy that in selecting for the inability to reduce nitrate the major class of mutant was *chlC*, lacking nitrate reductase, despite the existence of at least seven classes of *chl* mutant (Glaser & DeMoss, 1972).

The growth phenotype of the type II mutants was similar to that observed with the reference *menA* mutant and they were tentatively classed as *men* mutants, blocked in menaquinone biosynthesis. These mutants were isolated from three of the 10 independent selections for mutants having an LF⁻ phenotype and this could constitute a useful method for isolating new classes of *men* mutant. The simplicity of this procedure and the yield (4/44 isolates) offer considerable advantages over the method previously used for isolating *men* mutants (Young, 1975). No type II mutants were recovered by primary selection for a GF⁻ phenotype. This may be significant because the GF medium was less selective than the LF medium for large inocula in genetic studies with type II mutants. It is clear from the studies with *menA* and type II mutants that menaquinone is an obligatory electron carrier for growth on all substrates (glycerol, lactate, formate and H₂) when fumarate is the electron acceptor. Under these conditions pyrimidine biosynthesis is probably blocked, but adding uracil to the GF, LF, FF or F media did not permit growth. Anaerobic growth on glucose in the absence of menaquinone is limited by pyrimidine biosynthesis. However, the oxidation of dihydro-

orotate (and protoporphyrinogen) cannot be obligatorily coupled to fumarate reduction otherwise all the *frdA* mutants would require uracil for anaerobic growth on glucose. Menaquinone is not essential for aerobic growth nor is it required anaerobically when nitrate is the terminal electron acceptor. The type V mutants closely resembled the type II mutants in their enzymology and their growth phenotypes differed in only one respect, the inability to respond to uracil for anaerobic growth on glucose. The nature and genetic location of the type V mutant lesions were not determined.

After the type I (*frdA*) mutants, the next largest class was type III, designated *fnr* because these mutants are defective in fumarate and nitrate reduction, i.e. they cannot grow anaerobically with any of the substrates (glycerol, lactate or formate) using fumarate or nitrate as electron acceptor. The enzymological studies confirmed their pleiotropy; not only did they contain reduced amounts of fumarate reductase and less than 4 % of parental nitrate reductase but they also possessed reduced amounts of formate dehydrogenase (BV), hydrogenase and formate hydrogenlyase and overall formate-nitrate oxidoreductase was not detectable. Despite their low content of nitrate reductase they were still sensitive to chlorate. The *fnr* mutations defining this phenotype were 2.7 to 5.7 % cotransducible with *trpA* and 5.7 to 9.2 % cotransducible with *pyrF*. The genetic studies also confirmed the gene order *fnr-qmeA-pyrF-trpA*, indicating that *fnr* is situated at approximately 28.5 min in the *E. coli* linkage map. The evidence suggests that these mutants have a defect in some component which is essential for electron transport from all the substrates and is common to the fumarate and nitrate reduction systems. This means that the *fnr* lesion may affect a cytochrome or electron transport to or between cytochromes. Alternatively, some regulatory or structural component may be affected.

Mutants (*nirA*) lacking NADH-dependent nitrite reductase and cytochrome c_{552} have lesions which are 6 % cotransducible with *pyrF* and have been placed at 28.5 min on the linkage map (Kavanagh & Cole, 1976). Whether *nirA* and *fnr* are related is not known. The transduction studies indicate that *qmeA* is somewhat closer to *trp* than originally proposed and they also support a *pyrF-trp* cotransduction frequency of 25 to 32 % rather than 55 to 60 % (Fig. 3). No transductional linkage could be detected between *fnr* and the clockwise markers *pabB3* and *ptsM14*. This is not surprising because the AT701 mutation, formerly *man-1*, has been redesignated *ptsM14* (B. Bachmann, personal communication) since this work was completed. Likewise *pabB* and other close markers have been moved much further from the *fnr* region during the recalibration of the *E. coli* linkage map (Bachmann *et al.*, 1976). Unfortunately there are no convenient markers in the 29 to 30 min region.

The type IV mutants had the most pleiotropic phenotype, being defective in glucose fermentation and gas production as well as unable to use fumarate and nitrate as electron acceptors. Their most striking enzymological feature was the complete lack of formate dehydrogenase (BV) and, presumably as a consequence of this, formate hydrogenlyase. It seems unlikely that this is the primary lesion because it is difficult to see how lack of formate dehydrogenase could prevent growth on glucose.

The biochemical and genetic properties of some of the mutants, particularly types III (*fnr*) and IV indicate a functional or organizational relationship between the fumarate and nitrate reductase systems. One explanation would be dependence on an energy-yielding formate to fumarate couple involving components of the formate to nitrate respiratory pathway (Fig. 1). Another indication of the interrelationship between the two systems comes from the training procedures used to obtain good growth on the fumarate-containing media. Direct selection for an LF⁺ phenotype almost invariably generated pleiotropic chlorate-resistant mutants (the WGAS-LF⁺ strains) incapable of using nitrate as an electron acceptor (Lambden & Guest,

1976). This suggests that competition from electron carriers involved in nitrate respiration can prevent efficient electron transport to fumarate.

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REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788-795.
- BACHMANN, B. J. (1972). Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriological Reviews* **36**, 525-557.
- BACHMANN, B. J., LOW, K. B. & TAYLOR, A. L. (1976). Recalibrated linkage map of *Escherichia coli* K-12. *Bacteriological Reviews* **40**, 116-167.
- BARTON, L. L., LEGALL, J. & PECK, H. D. (1970). Phosphorylation coupled to oxidation of hydrogen with fumarate in extracts of the sulphate reducing bacterium, *Desulfovibrio gigas*. *Biochemical and Biophysical Research Communications* **41**, 1036-1042.
- BOONSTRA, J., HUTTUNEN, M. T., KONINGS, W. N. & KABACK, H. R. (1975). Anaerobic transport in *Escherichia coli* membrane vesicles. *Journal of Biological Chemistry* **250**, 6792-6798.
- BROWN, K. D. & SOMERVILLE, R. L. (1971). Repression of aromatic amino acid biosynthesis in *Escherichia coli* K12. *Journal of Bacteriology* **108**, 386-399.
- ENOCH, H. G. & LESTER, R. L. (1975). The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. *Journal of Biological Chemistry* **250**, 6693-6705.
- FAUST, P. J. & VANDEMARK, P. J. (1970). Phosphorylation coupled to NADH oxidation with fumarate in *Streptococcus faecalis* 10C1. *Archives of Biochemistry and Biophysics* **137**, 392-398.
- GLASER, J. H. & DEMOSS, J. A. (1972). Comparison of nitrate reductase mutants of *Escherichia coli* selected by alternative procedures. *Molecular and General Genetics* **116**, 1-10.
- GRAY, C. T., WIMPENNY, J. W. T., HUGHES, D. E. & MOSSMAN, M. R. (1966). Regulation of metabolism of facultative bacteria. I. Structural and functional changes in *Escherichia coli* associated with shifts between the aerobic and anaerobic states. *Biochimica et biophysica acta* **117**, 22-32.
- GUEST, J. R. (1969). Biochemical and genetic studies with nitrate reductase C-gene mutants of *Escherichia coli*. *Molecular and General Genetics* **105**, 285-297.
- HIRSCH, C. A., RASMINSKY, M., DAVIS, B. D. & LIN, E. C. C. (1963). A fumarate reductase in *Escherichia coli* distinct from succinate dehydrogenase. *Journal of Biological Chemistry* **238**, 3770-3774.
- HUANG, M. & PITTARD, J. (1967). Genetic analysis of mutant strains of *Escherichia coli* requiring *p*-amino-benzoic acid for growth. *Journal of Bacteriology* **93**, 1938-1942.
- JACOBS, N. J. & JACOBS, J. M. (1975). Fumarate as alternate electron acceptor for the late steps of anaerobic heme synthesis in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **64**, 435-441.
- KAVANAGH, B. M. & COLE, J. A. (1976). Characterisation of mutants of *Escherichia coli* K12 defective in nitrite assimilation. *Proceedings of the Society for General Microbiology* **3**, 84.
- KISTLER, W. S. & LIN, E. C. C. (1971). Anaerobic L- α -glycerophosphate dehydrogenase of *Escherichia coli*: its genetic locus and its physiological role. *Journal of Bacteriology* **108**, 1224-1234.
- KONINGS, W. N. & KABACK, H. R. (1973). Anaerobic transport in *Escherichia coli* membrane vesicles. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 3376-3381.
- KRÖGER, A. (1974). Electron transport phosphorylation coupled to fumarate reduction in anaerobically grown *Proteus rettgeri*. *Biochimica et biophysica acta* **347**, 273-289.
- KRÖGER, A., DADÁK, V., KLINGENBERG, M. & DIEMER, F. (1971). On the role of quinones in bacterial electron transport: differential roles of ubiquinone and menaquinone in *Proteus rettgeri*. *European Journal of Biochemistry* **21**, 322-333.
- LAMBDEN, P. R. & GUEST, J. R. (1976). A novel method for isolating chlorate-resistant mutants of *Escherichia coli* K12 by anaerobic selection on a lactate plus fumarate medium. *Journal of General Microbiology* **93**, 173-176.
- LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190-206.
- LOW, K. B. (1972). *Escherichia coli* F-prime factors, old and new. *Bacteriological Reviews* **36**, 587-605.
- LOW, K. B. (1973). Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. *Journal of Bacteriology* **113**, 798-812.
- 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.

- MACY, J., KULLA, H. & GOTTSCHALK, G. (1976). H_2 -dependent anaerobic growth of *Escherichia coli* on L-malate: succinate formation. *Journal of Bacteriology* **125**, 423-428.
- MACY, J., PROBST, I. & GOTTSCHALK, G. (1975). Evidence for cytochrome involvement in fumarate reduction and adenosine 5'-triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. *Journal of Bacteriology* **123**, 436-442.
- MIKI, K. & LIN, E. C. C. (1973). Enzyme complex which couples glycerol-3-phosphate dehydrogenation to fumarate reduction in *Escherichia coli*. *Journal of Bacteriology* **114**, 767-771.
- MIKI, K. & LIN, E. C. C. (1975). Anaerobic energy-yielding reaction associated with transhydrogenation from glycerol 3-phosphate to fumarate by an *Escherichia coli* system. *Journal of Bacteriology* **124**, 1282-1287.
- NEWTON, N. A., COX, G. B. & GIBSON, F. (1971). The function of menaquinone (vitamin K_2) in *Escherichia coli* K-12. *Biochimica et biophysica acta* **244**, 155-166.
- RUIZ-HERRERA, J. & DEMOSS, J. A. (1969). Nitrate reductase complex of *Escherichia coli* K-12: participation of specific formate dehydrogenase and cytochrome b_1 components in nitrate reduction. *Journal of Bacteriology* **99**, 720-729.
- RUIZ-HERRERA, J., SHOWE, M. K. & DEMOSS, J. A. (1969). Isolation and characterization of mutants unable to reduce nitrate. *Journal of Bacteriology* **97**, 1291-1297.
- SEMPLE, K. S. & SILBERT, D. F. (1975). Mapping of the *fabD* locus for fatty acid biosynthesis in *Escherichia coli*. *Journal of Bacteriology* **121**, 1036-1046.
- SIGNER, E. G., BECKWITH, J. R. & BRENNER, S. (1965). Mapping of suppressor loci in *Escherichia coli*. *Journal of Molecular Biology* **14**, 153-166.
- SINGH, A. P. & BRAGG, P. D. (1975). Reduced nicotinamide adenine dinucleotide dependent reduction of fumarate coupled to membrane energization in a cytochrome deficient mutant of *Escherichia coli* K12. *Biochimica et biophysica acta* **396**, 229-241.
- SINGH, A. P. & BRAGG, P. D. (1976). Anaerobic transport of amino acids coupled to the glycerol-3-phosphate-fumarate oxidoreductase system in a cytochrome-deficient mutant of *Escherichia coli*. *Biochimica et biophysica acta* **423**, 450-461.
- 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. & GUEST, J. R. (1974). Proteins of the inner membrane of *Escherichia coli*: changes in composition associated with anaerobic growth and fumarate reductase amber mutation. *Journal of Bacteriology* **117**, 954-959.
- TAYLOR, A. L. & TROTTER, C. D. (1967). Revised linkage map of *Escherichia coli*. *Bacteriological Reviews* **31**, 332-353.
- DE VRIES, W., VAN WYCK-KAPTEYN, W. M. C. & STOUTHAMER, A. H. (1973). Generation of ATP during cytochrome-linked anaerobic electron transport in propionic acid bacteria. *Journal of General Microbiology* **76**, 31-41.
- WIJSMAN, H. J. W. (1972). Mutation affecting plasmolysis in *Escherichia coli*. *Journal of Bacteriology* **110**, 789-790.
- YOUNG, I. G. (1975). Biosynthesis of bacterial menaquinones. Menaquinone mutants of *Escherichia coli*. *Biochemistry, New York* **14**, 399-406.