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Use of Φ(*glp-lac*) in Studies of Respiratory Regulation of the *Escherichia coli* Anaerobic *sn*-Glycerol-3-Phosphate Dehydrogenase Genes (*glpAB*)

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Expression of the *glpA* operon encoding the extrinsic membrane anaerobic *sn*-glycerol-3-phosphate dehydrogenase complex of *Escherichia coli* K-12 was studied in five strains carrying independent *glpA-lac* operon fusions. The location of the fusions was confirmed by transduction. Two of the strains produced an enzymatically active anaerobic *sn*-glycerol-3-phosphate dehydrogenase that accumulated in the cytoplasmic fraction of the cells. This suggests the loss of a specific membrane anchor subunit encoded by a distal gene, *glpB*, which was disrupted by the insertion. β-Galactosidase in all five strains carrying Φ(*glpA-lac*) was highly inducible by glycerol only anaerobically. A mutation in *fnr*, a pleiotropic activator gene, prevented full induction of the Φ(*glpA-lac*), demonstrating that the Fnr protein is a positive regulator of the primary dehydrogenase as well as of the terminal reductases of anaerobic respiratory chains. Low concentrations of the respiratory poison KCN had a permissive effect on aerobic expression of Φ(*glpA-lac*). Aerobic expression of the hybrid operon was also enhanced in isogenic derivatives of the fusion strains deficient in protoporphyrin biosynthesis (*hemA*). Thus, heme proteins may play a role in mediating aerobic repression of the anaerobic respiratory chain.

In *Escherichia coli* K-12, the dissimilation of glycerol and *sn*-glycerol-3-phosphate (G3P) with fumarate as the sole exogenous hydrogen acceptor requires the anaerobic G3P dehydrogenase-fumarate reductase system (19). The transfer of electrons through this system energizes the membrane, thus providing the proton-motive force for ATP synthesis and amino acid transport (22, 37, 38). Anaerobic G3P dehydrogenase and fumarate reductase are membrane-bound flavoproteins, inducible anaerobically by G3P and fumarate, respectively (14, 16, 20, 44, 45, 54). Both are subject to respiratory controls: induction of these enzymes is antagonized by nitrate or molecular oxygen, which are electron acceptors with higher redox potentials than fumarate (16, 19, 48). Respiratory repression of anaerobic G3P dehydrogenase by molecular oxygen and nitrate is retained in a mutant with a deletion in the specific repressor of the *glp* regulon ($glpR^{del}$), and such an effect is not counteracted by the addition of cyclic AMP in the growth medium (15). Menaquinone and certain *b* cytochromes were shown to be intermediary electron carriers linking the energy-generating process (5, 23, 41, 47). An iron-sulfur protein also plays a role in both anaerobic G3P dehydrogenase and the fumarate reductase complex (11).

Mutants affected in the anaerobic G3P dehydrogenase (*glpA*) (19), menaquinone biosynthesis (*menA* and *menCEBD*), porphyrin biosynthesis (*hemA*) (46), fumarate reductase (*frdAB*) (24), and the positive regulatory protein (*fnr*) (24), required for production of several anaerobic terminal electron transport proteins (including fumarate reductase), have been isolated. As expected, defects in these unlinked genes all impair anaerobic growth on glycerol and G3P as the carbon sources and fumarate as the exogenous hydrogen acceptor. As yet, little is known about how the expression of most of these genes is controlled by respiratory conditions. The operon fusion technique of Casadaban and Cohen (7) was used to construct *glpA-lac* fusions for the purpose of studying the respiratory control of the *glpA* operon. Some of the regulatory properties of these fusions are reported here.

MATERIALS AND METHODS

Materials. Phenazine methosulfate, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), flavin adenine dinucleotide, flavin mononucleotide (grade I), benzyl viologen, *o*-nitrophenyl-β-D-galactopyranoside, fusaric acid, chlorotetracycline, 5-aminolevulinic acid (ALA), EDTA, and DL-dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo.; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XG) was from Bachem, Inc., Torrance, Calif. Nutrient broth, tryptone, yeast extract, and MacConkey medium were obtained from Difco Laboratories, Detroit, Mich. Vitamin-free casein acid hydrolysate was obtained from ICN Nutritional Biochemicals, Cleveland, Ohio. All other reagents used were commercial products of the highest grade available.

Bacterial and phage strains. The genotypes of the *E. coli* K-12 strains and bacteriophages used are given in Table 1.

Growth conditions. Bacteria were cultivated and phages were propagated by generally used methods (3, 39). Growth was carried out at 30°C in experiments with cells bearing phage Mu dl; otherwise, incubations were carried out at 37°C. LB medium (1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl) was used for routine liquid cultures. The glycerol content of rich media is sufficient to cause the accumulation of growth-inhibiting concentrations of G3P in *glpD* strains. Therefore, glucose was added to a concentration of 10 mM for growth of these strains in LB medium to prevent the emergence of mutants in glycerol kinase (13). A standard

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TABLE 1. *E. coli* and bacteriophage strains

Strain	Derived from strain	Genotype or phenotype	Source and reference
Bacteria			
DL-2		F⁻ *thi zei-723*::Tn*10 gyrA glpR araD139 ΔlacU169 rpsL relA fldB ptsF25*	(30)
JRG861a		F⁻ *fnr-1 trpA9761 gal-25 rpsL195*	B. Bachmann
JP2144		F⁻ *tryR366 trpA9605 his-85 ilv-632 tsx-84*	B. Bachmann
JW380		F⁻ *zch-506*::Tn*10*; Tn*10* near *tyrT*	B. Bachmann
MAL103		F⁻ Mu cts d1 (Ap^r *lac*) Mu cts Δ(*pro-lac*) XIII *rpsL*	(7)
MC4100		F⁻ *thi araD139 ΔlacU169 rpsL relA fldB ptsF25*	(6)
NK6970	W3110	F⁻ *nadA*::Tn*10*	N. Kleckner
SASX41B		HfrC *hemA41 metB1 relA spoT1*	B. Bachmann
W945 *sdh*		F⁻ *thi thr sdh-9 gal rpsL*	B. Bachmann
ECL1	E15	HfrC *phoA8 relA1 tonA22* T2^r λ$^+$	(27)
ECL95	ECL1	HfrC *glpD phoA8 relA1 tonA22* T2^r λ$^+$	(27)
ECL317	MC4100	F⁻ *thi araD139 ΔlacU169 ΔglpD102 rpsL relA fldB ptsF25*	This work
ECL321	ECL317	F⁻ *thi araD139 ΔlacU169 ΔglpD102 nadA*::Tn*10 recA rpsL relA fldB ptsF25*	This work
ECL323	MC4100	F⁻ *thi araD139 ΔlacU169 fnr zci*::Tn*10 rpsL relA fldB ptsF25*	This work
ECL371	ECL321	F⁻ *thi sdh-9 araD139 ΔglpD102 ΔlacU169 rpsL relA fldB ptsF25*	This work
ECL372	ECL371	F⁻ *thi sdh-9 araD139 ΔglpD102 ΔlacU169 hemA41 zch-506*::Tn*10 rpsL relA fldB ptsF25*	This work
ECL381	ECL371	F⁻ *glpA101*::Mu d1	This work
ECL382	ECL371	F⁻ *glpA102*::Mu d1	This work
ECL383	ECL371	F⁻ *glpA103*::Mu d1	This work
ECL384	ECL371	F⁻ *glpA104*::Mu d1	This work
ECL385	ECL371	F⁻ *glpA105*::Mu d1	This work
ECL390	ECL381	F⁻ *glpA101*::Mu d1 *fnr-1 zci*::Tn*10*	This work
ECL391	ECL381	F⁻ *glpA101*::Mu d1 *hemA41 zch-506*::Tn*10*	This work
ECL394	ECL384	F⁻ *glpA104*::Mu d1 *fnr-1 zci*::Tn*10*	This work
ECL395	ECL384	F⁻ *glpA104*::Mu d1 *hemA41 zch-506*::Tn*10*	This work
ECL398	ECL382	F⁻ *glpA102*::Mu d1 *fnr-1 zci*::Tn*10*	This work
ECL399	ECL383	F⁻ *glpA103*::Mu d1 *fnr-1 zci*::Tn*10*	This work
ECL454	SASX41B	HfrC *hemA41 zch-506*::Tn*10 metB1 relA1 spoT1*	This work
ECL501	ECL385	F⁻ *glpA104*::Mu d1 *fnr-1 zci*::Tn*10*	This work
Phages			
P1 *vir*			(31)
λNK55		*c*I1857 *b*221 *O*am *c*III::Tn*10*	(21)
Mu d1		cts *trp'CBA' lac'OZYA'* Tn*3* Ap^r	(7)

minimal medium (SM) buffered at pH 7.0 by 0.1 M phosphate was used when growing cultures for enzyme assays (52). Where indicated, glycerol and lactate were added to 20 mM, pyruvate was added to 30 mM, glucose, maltose, and xylose were added to 10 mM, and lactose was added to 5 mM as carbon and energy sources. When added as hydrogen acceptors for anaerobic growth, fumarate was present at 20 mM, and potassium nitrate was present at 10 mM. Casein hydrolysate was added to 0.03% in liquid SM or minimal agar medium to stimulate anaerobic growth. When added as the carbon and energy source, casein hydrolysate was present at 1.0%. Media were supplemented with vitamins and amino acids where needed.

LB agar plates containing XG (40 μg/liter) and the appropriate inducer were used to screen for the presence of β-galactosidase activity in isolated colonies. When present, ampicillin was added to 50 μg/ml, tetracycline was added to 20 μg/ml (10 μg/ml in liquid medium), and nalidixic acid was added to 50 μg/ml. Anaerobic incubation was carried out in sealed jars made anaerobic by an H_2-CO_2 generator (GasPak Anaerobic Systems; BBL Microbiology Systems, Cockeysville, Md.). GasPak anaerobic indicators (BBL) were used to document anaerobiosis.

The blue color imparted to Lac$^+$ colonies on XG agar results from hydrolysis of the indolyl-β-galactoside and subsequent condensation of two molecules of the halogenated indoxyl ring to form the indigo dye which requires an oxidant (2). Therefore, anaerobically incubated plates were exposed to air at 4°C for 2 to 3 h to allow Lac$^+$ colonies to become blue.

For assays of anaerobic G3P dehydrogenase and fumarate reductase activities in aerobically grown cells, 250-ml culture volumes were vigorously agitated in 2.5-liter flasks. Anaerobic cultures for determination of these activities were grown undisturbed in 500-ml screw-capped flasks filled to the top. Cells for the assays of β-galactosidase activity were grown aerobically to the mid-exponential phase in 5 ml of medium, vigorously agitated in 55-ml tubes or grown anaerobically overnight in undisturbed screw-capped tubes filled to capacity (15 ml). When added as inducers, fumarate was present at 20 mM but glycerol was present at 0.2 mM to avoid growth inhibition of *glpD* strains (13). Growth of liquid cultures was monitored turbidimetrically with a Klett-Summerson colorimeter (no. 42 filter).

Strains were tested for growth on single carbon sources by being streaked on appropriate agar plates. For definitive analysis of growth phenotypes, 10 μl of 10^{-5} (for comparison of colony size) and 10^{-1} (for scoring reversion frequency) dilutions of an overnight culture were spread in adjacent sectors of a plate along with strains serving as positive and negative controls (where available). A stamp with metal prongs that fit one half of a microtiter plate with 96 wells (Falcon Plastics, Oxnard, Calif.) was used for replica plating.

Preparation of cell extracts. Cells harvested for the fumarate reductase assay were washed in 10 mM potassium

phosphate buffer (pH 7.0) plus 1 mM DL-dithiothreitol and suspended in the same solution at 0.5 g of fresh cells per ml of buffer. The suspended cells were disrupted with cooling in a 60-W sonic disintegrator (Measuring and Scientific Equipment , Ltd., London), allowing 1 min of treatment per ml of suspension. Cellular debris was removed by centrifugation at 10,000 × *g* for 30 min. Cells for anaerobic G3P dehydrogenase assay were treated identically except that 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol and 1 mM EDTA was used. EDTA was omitted from this buffer, and 1 mM $MgCl_2$ was added instead when extracts were prepared for subcellular fractionation.

Subcellular fractionation. Crude extracts were fractionated into cytoplasmic and membrane components by centrifugation at 200,000 × *g* for 2 h in a Beckman SW50.1 rotor. After removal of the supernatant fluid (referred to as the cytoplasmic fraction), the pellet (referred to as the membrane fraction) was gently suspended in 2 ml of buffer with a hand-operated Teflon homogenizer.

Enzyme assays. Anaerobic G3P dehydrogenase activity was assayed at 30°C by the phenazine methosulfate-mediated reduction of MTT monitored at 570 nm (19). Since crude extracts contained substantial endogenous substrates to give nonlinear rates of MTT reduction even in the absence of added G3P, the reaction mixture was aerated by shaking and incubated for 3 min before the addition of MTT. Endogenous reducing power was thus channeled to phenazine methosulfate which was autoxidized by the dissolved oxygen.

The reduction of fumarate was assayed at 30°C in an anaerobic cuvette (no. 195; Hellma Cells, Jamaica, N.Y.) containing cell extract (0.3 to 1.0 mg of protein), 800 nmol of benzyl viologen, 0.5 mM DL-dithiothreitol, and 10 mM potassium phosphate buffer at pH 7.0 in a final volume of 2.0 ml (modified from [14]). After the cuvette and its content were made anaerobic by flushing them with N_2, an amount of a freshly prepared solution of sodium dithionite (2 mg/ml, kept under N_2) sufficient to reduce one-half of the benzyl viologen was introduced with a Hamilton syringe through the gas outlet. The cuvette was stoppered and observed for 1 to 2 min to check for complete anaerobiosis and endogenous activity. The reaction was then started by the addition of 20 μmol of fumarate (kept under N_2). The reoxidation of reduced benzyl viologen was monitored at 500 nm (48), and the initial rate was used to calculate the enzyme activity. Controls in which either the fumarate or the enzyme extract were omitted had no activity. This assay gave an activity about 15 to 20 times higher than a previously employed assay based on the reduction of fumarate at the expense of the oxidation of reduced flavin mononucleotide (36).

Specific activities of anaerobic G3P dehydrogenase and fumarate reductase are expressed in nanomoles per minute per milligram of protein. Protein concentrations in cell extracts were estimated (37) with bovine serum albumin as the standard (29).

β-Galactosidase activity was assayed in whole cells by following the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside at 420 nm, and the specific activity was expressed in units by the method of Miller (39).

P1 transduction. P1 *vir* was used for transductions (3). All transductants were purified on the same selective medium and replica plated to screen for unselected markers. Antibiotic-resistant transductants were selected on LB agar with the appropriate drug. For screening *hemA*$^+$ transductants, LB agar was used without ALA supplement.

Isolation of a Δ*lac* Δ*glpD sdh* strain. For the isolation of *glpA-lac* fusions, a Δ*lac* Δ*glpD sdh* mutant was constructed as the parental strain. The absence of aerobic G3P dehydrogenase (*glpD*) and succinate dehydrogenase (*sdh*) facilitated accurate measurement of anaerobic G3P dehydrogenase and fumarate reductase activities. First, a *glpD*::Tn*10* insertion mutant was isolated from strain MC4100 by a modified (3) method of Kleckner et al. (21). A spontaneous tetracycline-sensitive derivative (4, 34) of this mutant, strain ECL317, was shown to have a deletion in *glpD* by its failure to give GlpD$^+$ revertants and its inability to yield GlpD$^+$ transductants when crossed with strain ECL95, which carries a point mutation in *glpD*. The inducibility of glycerol kinase was tested to insure that neighboring *glpR* (12) was not included in the deletion. Strain ECL317 was transduced to Tc^r with P1 grown on strain NK6970 (*nadA*::Tn*10*), and the *nadA*::Tn*10* transductant (strain ECL321) was subsequently transduced to *nadA*$^+$ *sdh* with P1 grown on strain W945 (*sdh*) to give strain ECL371 (Δ*lac* Δ*glpD sdh*).

Isolation of Mu d1(Ap^r*lac*) fusions in *glpA*. Fusions of *lacZYA* to the *glpA* promoter were isolated by the method of Casadaban and Cohen (7). Cells of strain ECL371 were distributed into 10 tubes and infected at a multiplicity of infection of 0.2 with a lysate of Mu d1(Ap^r*lac*) phage prepared by heat induction of strain MAL103. The infected cells were grown about fourfold in SM-glucose medium supplemented with ampicillin, after which they were plated on MacConkey-ampicillin agar supplemented with glycerol plus fumarate and incubated anaerobically. Wild-type colonies utilizing glycerol anaerobically in the presence of fumarate were pink to red; *glpA* mutants appeared pale.

Construction of *fnr* Φ(*glpA-lac*) strains. To obtain a cell line containing a Tn*10* insertion closely linked to *fnr*, strain JRG861a (*fnr*) was transduced with a P1 lysate of a population of MC4100 harboring a pool of about 15,000 random Tn*10* insertions (resulting from infection with λNK55). Transductants were selected anaerobically for growth on glycerol-nitrate agar supplemented with tetracycline. The linked *fnr*$^+$ and Tn*10* were transduced to a fresh genetic background (strain MC4100) to eliminate possible extraneous copies of Tn*10*. The resulting transductant was used as a Tn*10* donor to strain JRG861a, and an *fnr zci*::Tn*10* transductant was identified by its inability to grow anaerobically on glycerol-fumarate and glycerol-nitrate agar and its resistance to tetracycline. This pair of markers was introduced into strain MC4100 by P1 transduction to produce strain ECL323. The *zci*::Tn*10* was ca. 10% cotransduced with *fnr* and 30% cotransduced with *tyrR* (28.8 min). Strain ECL323 was used as the standard transduction donor of *fnr* to *glpA-lac* fusion strains by selecting for Tc^r and screening for the loss of ability to grow anaerobicially on lactate-nitrate agar.

Construction of *hemA* Φ(*glpA-lac*) strains. The Tn*10* insertion of strain JW380 (*zch-506*::Tn*10*), closely linked to the *hemA* locus, was first transduced into strain SASX41B (*hemA*). Strain ECL454, a Tc^r transductant which retained *hemA*, was in turn used as the transduction donor of *hemA* to strains ECL381 and ECL384 by selecting for Tc^r on LB-tetracycline-ALA agar and screening for the requirement of ALA. HemA$^-$ strains were unable to grow on either glycerol or lactate with nitrate as terminal hydrogen acceptor. The *zch-506*::Tn*10* insertion was ca. 30% linked to *hemA* (strain ECL372).

RESULTS

Mu d1(Ap^r *lac*) fusions to *glpA* promoters. From each of the 10 Mu d1-infected populations of strain ECL371, 5,000 colonies were screened on MacConkey-ampicillin agar sup-

TABLE 2. β-Galactosidase activity of *glpA-lac* fusion strains[a]

Strain	β-Galactosidase activity (U)				
	Aerobic	Anaerobic			
	+GF	NA	+G	+GF	+GN
ECL381	35	24	430	480	200
ECL382	160	140	420	1,000	500
ECL383	74	26	—	770	—
ECL384	99	110	380	740	400
ECL385	78	81	1,100	700	420

[a] Cells were grown anaerobically on SM-xylose medium on SM-xylose-casein hydrolysate. Abbreviations: NA, no additions; +G, glycerol added; +GF, glycerol and fumarate added; and GN, glycerol and nitrate added. —, Not assayed.

plemented with glycerol and fumarate. A total of 48 pale, small, translucent colonies from each of the 10 infected lines was purified on LB-glucose-ampicillin agar for replica plating. A total of 61 Lac^+ colonies (blue on LB-XG agar containing glycerol plus fumarate) was found, repurified on LB-glucose-ampicillin agar, and transferred to new master plates for further replica plating. Minimal agar containing glycerol or lactate as the carbon source with fumarate or nitrate as the hydrogen acceptor was used for this screening. As controls, colonies were also replica plated on standard minimal medium-glucose agar. Thirteen mutants were found to be specifically impaired in anaerobic growth on glycerol supplemented with either fumarate or nitrate; these were considered possible Φ(*glpA-lac*) mutants.

Genetic purification of *glpA-lac* fusions by P1 transduction. Five independent mutants of 13 had patterns of *lac* gene induction that suggested fusion to *glpA*. To eliminate possible extraneous copies of Mu dl, these fusions were reintroduced into strain ECL371 by P1 transduction, selection for Ap^r, and screening for anaerobic growth on glycerol-fumarate and glycerol-nitrate medium. The data reported below represent experiments performed with these genetically purified fusion strains (ECL381 to ECL385).

Characterization of *glpA-lac* fusions. All five fusion-bearing strains failed to grow anaerobically on glycerol with either fumarate or nitrate as the hydrogen acceptor. Growth on lactate-nitrate agar and simple glucose agar was unimpaired. Thus, the insertion of Mu dl in these strains specifically disrupted the anaerobic pathway for glycerol dissimilation.

The pattern of β-galactosidase activity of these strains is given in Table 2. The *lac* genes were inducible by glycerol only under anaerobic conditions. Nitrate or fumarate had no consistent effect on anaerobic induction by glycerol. Strain ECL381 was studied further. There was no significant difference in the basal level of β-galactosidase activity in cells grown aerobically or anaerobically, and the addition of glycerol to an aerobic culture resulted in less than twofold induction of Φ(*glpA-lac*) (data not shown).

Mapping of Mu dl insertions in strains ECL381 to ECL385. Mu dl insertions in strains ECL381 to ECL385 were mapped by P1 transductions in which strain DL-2 served as the donor. This strain carries a Tn*10* inserted between *glpA* (48.7 min) and the closely linked *gyrA* (48.3 min) locus (*zei-723*::Tn*10* [30]). Transductants were selected for Tc^r and scored for anaerobic growth on glycerol-fumarate or glycerol-nitrate agar, as well as for Ap^r and resistance to nalidixic acid (*gyrA*). In strain ECL381, $GlpA^+$ was 81% cotransducible with *zei-723*::Tn*10* (Table 3). $GlpA^+$ was similarly linked to Tc^r when transduced to the other $GlpA^-$ strains. A small fraction of $glpA^+$ transductants were found to be Ap^r, perhaps because of additional Mu dl copies elsewhere on the recipient chromosome.

TABLE 3. Mapping of *glpA-lac* fusions by transduction with strain DL-2 as the donor[a]

Recipient strain	No. of Tc^r transductants	Unselected marker		% Cotransduction of $GlpA^+$ with Tc^r
		$GlpA^+$	Ap^s	
ECL381	96	78	73	81
ECL382	48	36	32	76
ECL383	50	41	30	82
ECL384	48	34	31	70
ECL385	48	38	27	79

[a] Transductants were selected for Tc^r and scored for $GlpA^+$ and Ap^s as described in the text.

Levels of anaerobic G3P dehydrogenase activity in *glpA-lac* fusion strains. Anaerobic G3P dehydrogenase activity was absent from crude extracts of strains ECL381, ECL383, and ECL384 grown under inducing conditions (Table 4). In contrast, the anaerobic G3P dehydrogenase activities of strains ECL382 and ECL385 were not significantly different from that of the parental strain ECL371 bearing the wild-type $glpA^+$ operon. Induced levels of fumarate reductase in all five strains were normal as expected. These results, together with the data from mapping experiments and β-galactosidase assays, show that the *lac* genes are inserted in the *glpA* operon and that in strains ECL381, ECL383, and ECL384 the *lac* genes disrupted the structural genes of the catalytic subunits of anaerobic G3P dehydrogenase (44).

Loss of membrane attachment of anaerobic G3P dehydrogenase in two fusion strains. The presence of wild-type levels of anaerobic G3P dehydrogenase activity distinguished strains ECL382 and ECL385 from the other *glpA* fusion strains. The preservation of in vitro but not in vivo activity of the anaerobic G3P dehydrogenase suggested that Mu dl was inserted in a distal gene of the *glpA* operon, which will be referred to as the *glpB* gene. [For convenience, fusions of either *glpA* or *glpB* to the *lac* genes will still simply be referred to as Φ(*glpA-lac*) in this work.] Since the dehydrogenase purified as a soluble dimer of two different kinds of subunits, a flavoprotein and an Fe-S protein (44), and the coupling of this enzyme to fumarate reductase requires formation of a membrane-associated complex (36), the possibility exists that a third protein component is necessary for attachment of the enzyme as an extrinsic membrane enzyme

TABLE 4. Anaerobic G3P dehydrogenase and fumarate reductase activities of *glpA-lac* fusion strains[a]

Strain	Anaerobic G3P dehydrogenase activity (nmol min^{-1} mg of $protein^{-1}$)		Fumarate reductase activity (nmol min^{-1} mg of $protein^{-1}$)
	With flavins	Without flavins	
ECL371	93	14	1,300
ECL381	2	0	1,100
ECL382	72	2	1,200
ECL383	1	0	850
ECL384	0	0	1,100
ECL385	80	7	1,100

[a] Cells were grown anaerobically in SM-xylose-casein hydrolysate supplemented with glycerol plus fumarate. Flavins were added as a combination of flavin adenine dinucleotide and flavin mononucleotide.

TABLE 5. Subcellular distribution of specific anaerobic G3P dehydrogenase activity[a]

Strain	Activity (nmol min^{-1} mg of $protein^{-1}$) with (+) and without (−) flavins						Ratio of C/M[b]
	Crude extract		Cytoplasmic fraction		Membrane fraction		
	+	−	+	−	+	−	
ECL371	41	6	74	12	66	9	1.1
ECL382	25	0	67	7	5	0	13
ECL385	30	7	38	5	3	0	13

[a] Cell extracts were fractionated as described in the text.
[b] Ratio of flavin-stimulated activities of the cytoplasmic and membrane fractions.

and that strains ECL382 and ECL385 lack this component. The possibility was tested by fractionating crude cell extracts into cytoplasmic and membrane components.

Membrane fractions of crude extracts were sedimented by centrifugation at 200,000 × *g* for 2 h. The specific activity of anaerobic G3P dehydrogenase was approximately equal in the cytoplasmic and membrane fractions of strain ECL371 (Table 5). In contrast, the specific activity of the enzyme in the cytoplasmic fraction was more than 10-fold that in the membrane fraction of strains ECL382 and ECL385. These results suggest the loss of an anchor subunit by the two fusion strains, since it seems unlikely that Mu dl insertion in the gene encoding the flavoprotein or the Fe-S subunit would have resulted in loss of membrane attachment without concomitant loss of enzyme activity.

Effect of the *fnr* mutant allele on anaerobic Φ(*glpA-lac*) expression. The product of the *fnr*$^+$ gene (29.4 min) is required for expression of β-galactosidase in strains carrying operon fusions between the promoter of the nitrate reductase genes (*chlC* or *nar*) and the *lac* structural genes (8, 50). Introduction of the *fnr* mutant allele into *glpA-lac* fusion strains impeded induction of the fusion operon by about threefold under anaerobic conditions but did not affect the basal levels of enzyme activity (Table 6).

Permissive effect of KCN and heme deficiency on aerobic Φ (*glpA-lac*) expression. Stimulation of aerobic synthesis of anaerobic electron carriers by low concentrations of KCN (1) raised the question of whether or not aerobic expression of β-galactosidase in *glpA-lac* fusion strains grown in the presence of glycerol and fumarate could also be lifted by cyanide. Indeed, strains ECL381 and ECL384 poisoned with 150 μM KCN had more than threefold-higher levels of β-galactosidase activity than unpoisoned cells (Table 7).

The effect of KCN on respiratory repression suggested a role for iron porphyrins or heme proteins in the operation of respiratory control. This hypothesis was tested by the use of *hemA* derivatives of strains ECL381 and ECL384 that could not synthesize functional cytochromes and other heme proteins. As positive controls, heme synthesis in these strains was restored by ALA supplementation.

Aerobic levels of β-galactosidase of strains ECL381 and ECL384 were compared with those of the respective isogenic *hemA* derivatives, strains ECL391 and ECL395, after growth in a medium supplemented with glycerol and fumarate. The levels of Φ(*glpA-lac*) operon expression were about fourfold greater in *hemA* mutants than in *hemA*$^+$ strains. The levels of β-galactosidase in *hemA* strains were reduced to those of the wild-type strains when ALA was provided in the growth medium. Therefore, full aerobic repression of the *glpA* operon appears to require the presence of functional heme proteins.

If the effect of KCN is mediated through the cytochromes of the respiratory chain or other heme proteins, then the aerobic β-galactosidase level in *hemA* strains should not be further increased by cyanide. In fact, aerobically grown cells of *hemA* strains poisoned with KCN had lower enzyme levels than unpoisoned cells. Provision of *hemA* cells with ALA made them behave like wild-type cells in the presence or absence of KCN. Thus, KCN and the *hemA* mutant allele are nonsynergistic in their effect and appear to act by a similar mechanism.

Data from the above experiments might be distorted by the fact that overnight cultures grown to the stationary phase on LB medium become partially anaerobic, thereby allowing partial derepression of *glpA-lac* fusion operons. When these stationary-phase cells were inoculated into the inducing medium (xylose-casein hydrolysate-glycerol-fumarate) and harvested at a uniform time, differences in β-galactosidase levels of control and experimental cultures might therefore reflect differences in the rate of dilution of the enzyme as a result of the slower growth in this medium of *hemA* cells or of cells poisoned with KCN, as compared with controls. However, the results of these experiments were unchanged when cells were harvested at the same culture density. Indeed, cells from *hemA* strains poisoned with KCN, which had the slowest growth rate, also had the lowest levels of β-galactosidase. Another possible source of error was that poor growth conditions might result in alterations in cell morphology which could affect the light-scattering properties. Since calculation of β-galactosidase activity was based on the optical density at 600 nm of the cell suspension (39), changes in cell size could spuriously alter calculations of enzyme levels. However, when β-galactosidase activities were calculated as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein, the results were unchanged.

TABLE 6. β-Galactosidase activity of *fnr* derivatives of *glpA-lac* fusion strains[a]

Strain	β-Galactosidase activity (U)		
	Aerobic (+GF)	Anaerobic	
		NA	+GF
ECL381	39	14	400
ECL390 (*fnr*)	40	10	130
ECL382	71	38	790
ECL398 (*fnr*)	70	29	270
ECL383	74	26	770
ECL399 (*fnr*)	58	21	230
ECL384	57	8	670
ECL394 (*fnr*)	50	8	210
ECL385	63	24	730
ECL501 (*fnr*)	58	25	200

[a] Cells were grown anaerobically on SM-xylose medium or aerobically on SM-xylose-casein hydrolysate. Glycerol plus fumarate were added where indicated (+GF). NA, No additions.

TABLE 7. Permissive effects of KCN and *hemA* mutation on induced β-galactosidase levels in aerobically grown cells of *glpA-lac* fusion strains[a]

Strain	*hemA* allele	Addition to inducing growth medium	β-Galactosidase activity (U)
ECL381	+	None	46
		KCN	120
ECL391	−	None	160
		KCN	63
		ALA	46
		KCN + ALA	100
ECL384	+	None	85
		KCN	280
ECL395	−	None	320
		KCN	180
		ALA	82
		KCN + ALA	240

[a] Strain ECL391 carried the same *glpA-lac* fusion as strain ECL381; strain ECL395 carried the same *glpA-lac* fusion as strain ECL384. Cells were grown aerobically in SM-xylose-casein hydrolysate medium in the presence of glycerol plus fumarate, harvested in the mid-exponential growth phase, and assayed for β-galactosidase. Where indicated, KCN was added to 150 μM, and ALA was added to 50 μg/ml.

DISCUSSION

In an initial report, it has been suggested that the anaerobic G3P dehydrogenase is a soluble enzyme, in contrast to the membrane-bound aerobic dehydrogenase (18). A subsequent study revealed that the enzyme is, in fact, associated with the membrane and that transhydrogenation from G3P to fumarate requires the formation of a particulate complex with fumarate reductase (36). The ready dissociation of this enzyme from the membrane under nondenaturing conditions suggests that it is an extrinsic protein of the inner membrane (44). This conclusion is reinforced by the likely existence of a specific anchor protein whose gene is disrupted by Mu dl insertions in strains ECL382 and ECL385. The similarity of the control patterns of β-galactosidase activity in these two strains to those of strains ECL381, ECL383, and ECL384 and the close map positions of all five fusions suggest that the catalytic subunit (*glpA* product) and the anchor subunit (*glpB* product) of the anaerobic G3P dehydrogenase complex are encoded in a single operon. The *glpB* locus is most likely at the distal end, since Mu dl insertions in that gene have no polar effect on the synthesis of the catalytic subunit of the dehydrogenase.

No membrane anchor subunit was noticed during the purification of anaerobic G3P dehydrogenase. It is possible that the protein remained embedded in the membrane or bound to the phenyl Sepharose column used in the study (44). The hydrophobic properties of the fumarate reductase anchor proteins (FrdC and FrdD subunits) caused them to be overlooked in the original purification of that enzyme (25, 26, 28). Attachment to the membrane by specific hydrophobic subunits appears to be a common feature of enzymes of the respiratory chain. Deletions or mutations in these genes result in the accumulation of catalytic subunits in the cytoplasmic fraction of induced cells (17, 26, 32, 51). Evolutionary precursors of respiratory enzymes were likely to be soluble oxidoreductases that catalyzed the reoxidation of NADH anaerobically at the expense of fumarate or nitrate without concomitant proton translocation. A contemporary example of such an enzyme responsible for fumarate fermentation (rather than fumarate respiration) might be the soluble fumarate reductase of *Veillonella alcalescens* (*Micrococcus lactilyticus*) (53). The location of catalytic and membrane-binding domains on different subunits of some present-day respiratory enzyme complexes would suggest that the anchoring subunits were later acquisitions during the evolution of electron transport chains.

The 7- to 29-fold induction of β-galactosidase in the fusion strains are somewhat lower than the ca. 30-fold induction reported for the wild-type *glpAB* operon (19). Although subtle regulatory features might be obscured by the intruding bases in the junction between the *glp* promoter and the *lac* structural genes or local changes in topology (see, for example, reference 49), valid qualitative statements regarding the regulatory behavior of the intact wild-type operon can probably still be made, especially when several independent hybrid operons behave in consistent ways.

Synthesis of a number of proteins for terminal anaerobic respiratory chains has been shown to be under positive regulation of the fnr^+ gene product (9, 10, 24, 40). The present study showed that the *glpAB* operon, encoding a dehydrogenase complex initiating an electron transport chain, is also under the control of the Fnr protein. However, the induction of these *glpA-lac* fusion operons is not totally dependent on this activator protein, as are the *chlC-lac* (*narG-lac*) fusion operons (8, 50). The *fnr-1* allele used in the present study does not appear to be leaky, since the mutation lowered the induced fumarate reductase activity to only 6% of the wild-type level (24).

Two different mechanisms of hierarchal control of respiratory and fermentation enzymes have been proposed. The first invokes intracellular redox potential, E_h, as the key signal (see, for example, references 1, 35, 55). According to this model, terminal hydrogen acceptors having relatively positive standard redox potentials repress the synthesis of proteins that function with hydrogen acceptors of more negative standard redox potentials not by direct interaction with specific regulator proteins but via influencing the relative concentrations of an oxidized and reduced effector, as a redox coenzyme does. Through this mechanism, molecular oxygen would repress all the anaerobic respiratory enzymes, nitrate would repress enzymes such as fumarate reductase, and fumarate would repress enzymes such as ethanol dehydrogenase.

An alternative model invokes a series of regulatory proteins that assures the preferential utilization of the exogenous hydrogen acceptor which has the most positive redox potential (42, 43, 50, 51). For instance, an oxygen-sensitive regulator (e.g., a heme protein) would control transcription of the genes encoding not only enzymes of the aerobic respiratory chain, but also a pleiotropic repressor molecule that prevents expression of genes encoding all anaerobic respiratory proteins: in the presence of O_2, proteins of the aerobic respiratory chain and the pleiotropic repressor would be synthesized, and consequently anaerobic pathways would not be expressed. In the absence of O_2, the nitrate reductase system would become inducible. When this system is induced, another repressor would be synthesized. This repressor would prevent the induction of systems of lower redox potential, such as the fumarate reductase complex. Similarly, when the fumarate system is expressed, the genes for formate-hydrogenlyase and ethanol dehydrogenase would not be inducible. Such a hierarchal mechanism would dispense with a control system that has to sense the intracellular redox potential. Support for this model comes primarily from the observations that mutations in *narK* prevent repression by nitrate of tertiary amine *N*-oxide reductase expres-

sion. It thus appears that respiratory repression by nitrate is mediated by the *narK*$^+$ gene product, which is induced together with the *narGHI* genes encoding the nitrate reductase complex (51). (The model would be strengthened if the *nar* mutation also allows the induction of fumarate reductase in the presence of nitrate.) Results of experiments with KCN and with *hemA* derivatives of the strains bearing *glpA-lac* fusions are consistent with the second (multitiered) model. If the putative regulator of the aerobic respiratory chain is indeed a heme protein, then mutations in *hemA* or the addition of cyanide might prevent the full induction of genes encoding proteins for aerobic electron transport along with the aerobic repressor. This would create a permissive condition for the induction of other respiratory chains. On the other hand, repression of other systems by nitrate should be unaffected by the *hemA* mutation, since the repressor of the *nar* genes appears to require molybdenum rather than heme as corepressor (42). Indeed, respiratory repression of *glpA* and *frd* by nitrate is not relieved in the *hemA* strains (unpublished data). However, it is difficult to explain why normal aerobic repression of nitrate reductase still occurs in *hemA* strains (33). Thus, decisive evidence to support either model is still lacking. The isolation of pleiotropic regulatory mutants that allow the expression of Φ(*glpA-lac*) under nonpermissive conditions might help to resolve the problem.

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LITERATURE CITED

1. **Ashcroft, J. R., and B. A. Haddock.** 1975. Synthesis of alternative membrane-bound redox carriers during aerobic growth of *Escherichia coli* in the presence of potassium cyanide. Biochem. J. **148**:349–352.
2. **Barka, T., and P. J. Anderson.** 1962. Histochemistry: theory, practice, and bibliography, p. 265–269. Harper & Row, New York.
3. **Berman, M. L., L. W. Enquist, and T. J. Silhavy.** 1981. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Bochner, B. R., H.-C. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
5. **Boonstra, J., J. A. Downie, and W. N. Konings.** 1978. Energy supply for active transport in anaerobically grown *Escherichia coli*. J. Bacteriol. **136**:844–853.
6. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
7. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. U.S.A. **76**:4530–4533.
8. **Chippaux, M., V. Bonnefoy-Orth, J. Ratouchniak, and M.-C. Pascal.** 1981. Operon fusions in the nitrate reductase operon and study of the control gene *nirR* in *Escherichia coli*. Mol. Gen. Genet. **182**:477–479.
9. **Chippaux, M., D. Giudici, A. Abou-Jaoude, F. Casse, and M.-C. Pascal.** 1978. A mutation leading to the total lack of nitrite reductase activity in *Escherichia coli* K12. Mol. Gen. Genet. **160**:225–229.
10. **Cole, J. A., and F. B. Ward.** 1973. Nitrite reductase-deficient mutants of *Escherichia coli* K-12. J. Gen. Microbiol. **76**:21–29.
11. **Cole, T., T. Grundstrom, B. Jaurin, J. J. Robinson, and J. H. Weiner.** 1982. Location and nucleotide sequence of *frdB*, the gene coding for the iron-sulphur protein subunit of the fumarate reductase of *Escherichia coli*. Eur. J. Biochem. **126**:211–216.
12. **Cozzarelli, N. R., W. B. Freedberg, and E. C. C. Lin.** 1968. Genetic control of the L-α-glycerophosphate system in *Escherichia coli*. J. Mol. Biol. **31**:371–387.
13. **Cozzarelli, N. R., J. P. Koch, S. Hayashi, and E. C. C. Lin.** 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. **90**:1325–1329.
14. **Dickie, P., and J. H. Weiner.** 1979. Purification and characterization of membrane-bound fumarate reductase from anaerobically grown *Escherichia coli*. Can. J. Biochem. **57**:813–821.
15. **Freedberg, W. B., and E. C. C. Lin.** 1973. Three kinds of controls affecting the expression of the *glp* regulon in *Escherichia coli*. J. Bacteriol. **115**:816–823.
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. **Holmgren, E., L. Hederstedt, and L. Rutberg.** 1979. Role of heme in synthesis and membrane binding of succinic dehydrogenase in *Bacillus subtilis*. J. Bacteriol. **138**:377–382.
18. **Kistler, W. S., C. A. Hirsch, N. R. Cozzarelli, and E. C. C. Lin.** 1969. Second pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase in *Escherichia coli* K-12. J. Bacteriol. **100**:1133–1135.
19. **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.
20. **Kistler, W. S., and E. C. C. Lin.** 1972. Purification and properties of the flavine-stimulated anaerobic L-α-glycerophosphate dehydrogenase of *Escherichia coli*. J. Bacteriol. **112**:539–547.
21. **Kleckner, N., D. Baker, D. G. Ross, D. Botstein, I. A. Swan, and M. Zaben.** 1978. Properties of the tetracycline-resistance element Tn*10* in *Escherichia coli* and bacteriophage lambda. Genetics **90**:427–461.
22. **Konings, W. N., and H. R. Kaback.** 1973. Anaerobic transport in *Escherichia coli* membrane vesicles. Proc. Natl. Acad. Sci. U.S.A. **70**:3376–3381.
23. **Kröger, A.** 1978. Fumarate as terminal acceptor of phosphorylative electron transport. Biochim. Biophys. Acta **505**:129–145.
24. **Lambden, P. R., and J. R. Guest.** 1976. Mutants of *Escherichia coli* unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. **97**:145–160.
25. **Lemire, B. D., J. J. Robinson, R. D. Bradley, D. G. Scraba, and J. H. Weiner.** 1983. Structure of fumarate reductase on the cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **155**:391–397.
26. **Lemire, B. D., J. J. Robinson, and J. H. Weiner.** 1982. Identification of membrane anchor polypeptides of *Escherichia coli* fumarate reductase. J. Bacteriol. **152**:1126–1131.
27. **Lin, E. C. C.** 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. **30**:535–578.
28. **Lohmeier, E., D. S. Hagen, P. Dickie, and J. H. Weiner.** 1980. Cloning and expression of the fumarate reductase gene of *Escherichia coli*. Can. J. Biochem. **58**:158–164.
29. **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.
30. **Ludtke, D., T. J. Larson, C. Beck, and W. Boos.** 1982. Only one gene is required for the *glpT*-dependent transport of *sn*-glycerol-3-phosphate in *Escherichia coli*. Mol. Gen. Genet. **186**:540–547.
31. **Luria, S. E., J. N. Adams, and R. C. Ting.** 1960. Transduction of lactose-utilizing ability among strains of *E. coli* and *S. dysenteriae* and the properties of the transducing phage particles. Virology **12**:348–390.
32. **MacGregor, C. H.** 1976. Biosynthesis of membrane-bound nitrate reductase in *Escherichia coli*: evidence for a soluble precursor. J. Bacteriol. **126**:122–131.
33. **MacGregor, C. H., and C. W. Bishop.** 1977. Do cytochromes function as oxygen sensors in the regulation of nitrate reductase biosynthesis? J. Bacteriol. **131**:372–373.
34. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of

tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145**:1110–1112.
35. **McPhedran, P., B. Sommer, and E. C. C. Lin.** 1961. Control of ethanol dehydrogenase levels in *Aerobacter aerogenes*. J. Bacteriol. **81**:852–857.
36. **Miki, K., and E. C. C. Lin.** 1973. Enzyme complex which couples glycerol-3-phosphate dehydrogenation to fumarate reduction in *Escherichia coli*. J. Bacteriol. **114**:767–771.
37. **Miki, K., and E. C. C. Lin.** 1975. Anaerobic energy-yielding reaction associated with transhydrogenation from glycerol-3-phosphate to fumarate by an *Escherichia coli* system. J. Bacteriol. **124**:1282–1287.
38. **Miki, K., and T. H. Wilson.** 1978. Proton translocation associated with anaerobic transhydrogenation from glycerol 3-phosphate to fumarate in *Escherichia coli*. Biochem. Biophys. Res. Commun. **83**:1570–1575.
39. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Newman, B. M., and J. A. Cole.** 1978. The chromosomal location and pleiotropic effects of mutations of the *nirA*$^+$ gene of *Escherichia coli* K12: the essential role of *nirA*$^+$ in nitrite reduction and in other anaerobic redox reactions. J. Gen. Microbiol. **106**:1–12.
41. **Newton, N. A., G. B. Cox, and F. Gibson.** 1971. The function of menaquinone (vitamin K_2) in *Escherichia coli* K-12. Biochim. Biophys. Acta **244**:155–166.
42. **Pascal, M.-C., J.-F. Burini, J. Ratouchniak, and M. Chippaux.** 1982. Regulation of the nitrate reductase operon: effect of mutations in *chlA*, *B*, *D*, and *E* genes. Mol. Gen. Genet. **188**:103–106.
43. **Pascal, M.-C., and M. Chippaux.** 1982. Involvement of a gene of the *chlE* locus in the regulation of the nitrate reductase operon. Mol. Gen. Genet. **185**:334–338.
44. **Schryvers, A., and J. H. Weiner.** 1981. The anaerobic *sn*-glycerol-3-phosphate dehydrogenase of *Escherichia coli*. Purification and characterization. J. Biol. Chem. **256**:9959–9965.
45. **Schryvers, A., and J. H. Weiner.** 1982. The anaerobic *sn*-glycerol-3-phosphate dehydrogenase: cloning and expression of the *glpA* gene of *Escherichia coli* and identification of the *glpA* products. Can. J. Biochem. **60**:224–231.
46. **Singh, A. P., and P. D. Bragg.** 1975. Reduced nicotinamide adenine dinucleotide dependent reduction of fumarate coupled to membrane energization in a cytochrome deficient mutant of *Escherichia coli* K12. Biochim. Biophys. Acta **396**:229–241.
47. **Singh, A. P., and P. D. Bragg.** 1976. Anaerobic transport of amino acids coupled to the glycerol-3-phosphate-fumarate oxidoreductase system in a cytochrome-deficient mutant of *Escherichia coli*. Biochim. Biophys. Acta **423**:450–461.
48. **Spencer, M. E., and J. R. Guest.** 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. **114**:563–570.
49. **Sternglanz, R., S. DiNardo, K. A. Voelkel, Y. Nishimura, Y. Hirota, K. Becherer, L. Zumstein, and J. C. Wang.** 1981. Mutations in the gene coding for *Escherichia coli* DNA topoisomerase I affect transcription and transposition. Proc. Natl. Acad. Sci. U.S.A. **78**:2747–2751.
50. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1320–1325.
51. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788–799.
52. **Tanaka, S., S. A. Lerner, and E. C. C. Lin.** 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J. Bacteriol. **93**:642–648.
53. **Warringa, M. G. P. J., O. H. Smith, A. Guiditta, 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.
54. **Weiner, J. H., and P. Dickie.** 1979. Fumarate reductase of *Escherichia coli*. Elucidation of the covalent-flavin component. J. Biol. Chem. **254**:8590–8593.
55. **Wimpenny, J. W. T.** 1969. The effect of E_h on regulatory processes in facultative anaerobes. Biotechnol. Bioeng. **11**:623–629.