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Membrane Cytochromes of *Escherichia coli chl* Mutants

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The cytochromes present in the membranes of *Escherichia coli* cells having defects in the formate dehydrogenase-nitrate reductase system have been analyzed by spectroscopic, redox titration, and enzyme fractionation techniques. Four phenotypic classes differing in cytochrome composition were recognized. Class I is represented by strains with defects in the synthesis or insertion of molybdenum cofactor. Cytochromes of the formate dehydrogenase-nitrate reductase pathway are present. Class II strains map in the *chlC-chlI* region. The cytochrome associated with nitrate reductase (cytochrome b^{nr}) is absent in these strains, whereas that associated with formate dehydrogenase (cytochrome b^{fdh}) is the major cytochrome in the membranes. Class III strains lack both cytochromes b^{fdh} and b^{nr} but overproduce cytochrome *d* of the aerobic pathway even under anaerobic conditions in the presence of nitrate. Class III strains have defects in the regulation of cytochrome synthesis. An *fdhA* mutant produced cytochrome b^{nr} but lacked cytochrome b^{fdh}. These results support the view that *chlI* (*narI*) is the structural gene for cytochrome b^{nr} and that *chlC* (*narG*) and *chlI* (*narI*) are in the same operon, and they provide evidence of the complexity of the regulation of cytochrome synthesis.

Growth of *Escherichia coli* anaerobically on nitrate results in the induction of the formate dehydrogenase-nitrate reductase system. The components of this system, formate dehydrogenase and nitrate reductase, have been purified (10, 11). Cytochromes, cytochrome b^{fdh} and cytochrome b^{nr}, are present, one in each enzyme complex. The cytochromes have not been well characterized. Cytochrome b^{nr} constitutes the γ-subunit of nitrate reductase and is believed to donate electrons to the α-subunit, which is a molybdeno-iron-sulfur protein. This subunit, which may also be reduced directly by reduced benzyl viologen without the participation of cytochrome b^{nr}, has the catalytic site for reduction of nitrate. Cytochrome b^{nr} has been resolved into potentiometrically distinct components with midpoint oxidation-reduction potentials of 122 and 17 mV in the solubilized nitrate reductase (14). Little is known about cytochrome b^{fdh}, although formate dehydrogenase, like nitrate reductase, is a molybdeno-iron-sulfur protein (11).

A number of mutants in which the formate dehydrogenase-nitrate reductase system is affected have been isolated. Pleiotropic mutants at the *chlA*, *chlB*, *chlE*, and *chlG* loci lack both formate dehydrogenase and nitrate reductase activities, and these genes have been implicated in the synthesis, processing or insertion, or all three, of the molybdenum cofactor required by these enzymes (1, 21, 25, 27). The *chlC-chlI* locus is very complex and appears to contain the genes for the α-subunit of nitrate reductase and for cytochrome b^{nr}, as well as some regulatory genes (3, 7, 9, 22, 26, 27).

In the present paper we have examined the cytochrome composition of membranes from wild-type strains and mutants in the formate dehydrogenase-nitrate reductase system which have been grown anaerobically on nitrate. A variety of techniques including difference spectroscopy, redox titration, and solubilization of the cytochrome complexes were used. Four phenotypic classes differing in their cytochrome composition have been recognized in the mutants. This has enabled us to establish the absence of cytochrome b^{nr} in *chlI* mutants and to confirm the polar effect of insertions in the *chlC* gene on the synthesis of this cytochrome (3). Moreover, other factors involved in the regulation of cytochrome synthesis under anaerobic conditions have become apparent.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *E. coli* used in this investigation are listed in Table 1. The bacteria were grown anaerobically with nitrate as described in the accompanying paper (15).

Preparation of membranes. Membranes were prepared from cells disrupted in a French press as described in the accompanying paper (15).

Difference spectra and redox titration. Reduced-minus-oxidized difference spectra were recorded at 77°K. Carbon monoxide spectra were measured at

TABLE 1. Bacterial strains

Strain	Genotype	Origin
HfrH	Hfr *thi relA spoT supQ* λ^-	J. A. DeMoss
Ts9A	As PK27 but *chlC* (Ts)	J. A. DeMoss
RK4353 (=MC4100)	*lacU araD non thi rpsL gyrA*	V. Stewart
RK5206	As RK4353 but *chlG*::Mu *cts*	V. Stewart
RK5231	As RK4353 but *chlG*::Mu *cts*	V. Stewart
RK5256	As RK4353 but *chlG*::Mu *cts*	V. Stewart
RK5201	As RK4353 but *chlE*::Mu *cts*	V. Stewart
RK5218	As RK4353 but *chlE*::Mu *cts*	V. Stewart
RK5223	As RK4353 but *chlE*::Mu *cts*	V. Stewart
RK5274	As RK4353 but *narI*::Tn*10*	V. Stewart
RK5266	As RK4353 but *narK*::Tn*10*	V. Stewart
RK5270	As RK4353 but *narK*::Tn*10*	V. Stewart
RK5269	As RK4353 but *narG*::Tn*10*	V. Stewart
RK7 (KL96)	Hfr *gal thi rpsL*	C. H. MacGregor
RK7-16	As RK7 but *chlA*	C. H. MacGregor
RK7-19	As RK7 but *chlC*	C. H. MacGregor
LCB61, (=MC4100)	*lacU araD thi rpsL*	M. Chippaux
LCB68	As LCB61 but Δ*chlE68*	M. Chippaux
LCB79	As LCB61 but ϕ(*chlI-lacZ*)	M. Chippaux
LCB61-22	As LCB61 but *nirR*	M. Chippaux
LCB61-357	As LCB61 but *chlE16*	M. Chippaux
LCB79-357	As LCB79 but *chlE16*	M. Chippaux
LCB356	F^- *leu thr arg his pro purE supE lacY malA xyl ara mtl gal tonA rpsL*	M. Chippaux
LCB517	As LCB 356 but *fdhA*	M. Chippaux
LCB320 (=C600)	F^- *thi leu thr supE lacY tonA rpsL*	M. Chippaux
LCB155	As LCB320 but *ana nirC chlI*	M. Chippaux
LCB160	As LCB320 but *chlI*::Mu *cts*	M. Chippaux
LCB162	As LCB320 but *chlC*::Mu *cts*	M. Chippaux
356-15	As LCB356 but *chlA*	E. Azoulay
356-24	As LCB356 but *chlB*	E. Azoulay
CGSC 4444	*thi leuB suc bioA galT rpsL chlC*	B. Bachmann
CGSC 4459	F^+ *gal supE chlE*	B. Bachmann
NH10	As LCB68 but *narI*::Tn*10*	By P1 transduction of parent to Tet^r, using RK5274
NH30	As HfrH but *narI*::Tn*10*	
PK27	Hfr *thi*	J. A. DeMoss

room temperature. Redox titration of the membrane cytochromes was carried out at pH 7.0 in 0.1 M potassium phosphate buffer. These techniques are described in detail in the accompanying paper (15).

Fractionation of cytochromes. The cytochromes of cells grown anaerobically with nitrate were fractionated by a procedure (14) based on that of Clegg (8). All buffers were thoroughly degassed before use. Cell membranes (1,000 mg of protein) were suspended in 10 mM potassium phosphate buffer, pH 7.0, containing 0.2 mM dithiothreitol to a protein concentration of approximately 15 mg/ml. To this suspension 20% (vol/vol) Triton X-100 was slowly added to a final concentration of 2%. The mixture was stirred at room temperature for 30 min and then centrifuged at 50,000 rpm in a Beckman Ti60 rotor. The supernatant was loaded onto a column (35 by 1.5 cm) of DEAE Bio-Gel A. (DEAE-Sephacel has also been used but it gives an inferior separation.) After application, the column was washed with 200 ml of the equilibration buffer (10 mM potassium phosphate, pH 7.0, 0.2 mM dithiothreitol, 0.1% Triton X-100). Cytochromes were then eluted by a linear gradient of 0 to 0.3 M NaCl in the equilibration buffer (400-ml total volume). Fractions, 7 ml, were collected and their cytochrome content was estimated from their absorbance at 410 nm. When appropriate, fractions were also assayed for formate dehydrogenase and nitrate reductase activities.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gels were run, using the discontinuous buffer system of Laemmli (19). Either linear or exponential acrylamide gradients of 5 to 22.5% (wt/vol) were used with 3% (wt/vol) acrylamide stacking gels. Slab gels were run in a Bio-Rad 220 apparatus, using spacers of 0.75-mm thickness. They were run at 25 mA per gel until the tracking dye left the gel. Gels were stained by the procedure of Fairbanks et al. (12) and then destained and fixed in 10% (vol/vol) acetic acid. All samples were prepared by boiling for 5 min in the sample buffer of Laemmli (19).

Enzyme assays. Nitrate reductase was assayed spectrophotometrically, using reduced benzyl viologen as

electron donor, by the method of Jones and Garland (17). Formate dehydrogenase was measured as the formate-dependent reduction of 2,6-dichlorophenol indophenol.

Oxidase activities were measured with a Yellow Springs Instruments model 55 oxygen monitor coupled to an Aminco strip chart recorder. The recorder was calibrated so that a full-scale deflection represented the oxygen concentration of air-saturated buffer. This value was taken to be 0.26 μmol/ml. A concentrated membrane suspension (approximately 15 mg/ml) was diluted in 50 mM Tris-hydrochloride, pH 8.0, to a volume of 4 ml in the electrode vessel. A 0.1-ml portion of substrate was then added from a concentrated stock solution, using a Hamilton microsyringe, to give final concentrations of 1 mM for NADH and 10 mM for formate.

Measurement of the capacity of a culture to reduce nitrite. The ability to reduce nitrite was tested by growing cells overnight in a rich medium with nitrite and performing a nitrite determination on the spent medium. The medium contained, per 100 ml, 0.4 g of Bacto-Peptone (Difco Laboratories), 0.2 g of yeast extract, 0.4 g of glucose, and 1 mM nitrite. After overnight growth, 0.1 ml of medium was withdrawn and added directly to 0.9 ml of 3 M HCl. The presence or absence of nitrite in this was determined as described by Showe and DeMoss (24).

Measurement of the formate hydrogenlyase activity of growing cells, using Durham tubes. Durham tubes were used to investigate the formate hydrogenlyase activity of mutants (6). The medium used contained 0.5 g of Bacto-Peptone, 0.3 g of yeast extract, and 0.5 g of glucose per 100 ml. After growth for 24 h, the presence or absence of gas in the collector tube was noted.

Miscellaneous procedures. Protein was estimated by the method of Lowry et al. (20), using bovine serum albumin as a standard.

P1 transduction was carried out by the procedure of Pittard (23).

RESULTS

Difference spectra of chlorate-resistant mutants. Individual cytochromes can be detected by the characteristic absorption of the reduced form at wavelengths between 540 and 700 nm. Dithionite-reduced minus ferricyanide-oxidized difference spectra of membranes prepared from wild-type and chlorate-resistant mutants grown anaerobically with nitrate are shown in Fig. 1. The wild-type strain LCB61 and the *chlE* mutant CGSC 4459 showed a single α-absorption peak at 555.5 nm. (The β-absorption peaks of the cytochromes between 520 and 540 nm are of little use in characterizing cytochromes in membranes and are generally ignored.) This peak has been attributed previously to the cytochromes associated with formate dehydrogenase and nitrate reductase (15). The spectra of chlorate-resistant strains LCB68 and LCB79 were more complex. The single peak at 555 nm of LCB61 was replaced in LCB68 by a double peak with maxima at 556 and 558.5 nm. There was a pronounced shoulder on this peak at about 548 nm. LCB79 also showed a double peak in this region at 554.5 and 558 nm with a shoulder at 548 nm. By contrast with LCB61, strains LCB68 and LCB79 also showed absorption peaks which have been attributed to cytochromes a_1 (590 nm) and *d* (peak, 625 nm; trough, 646 nm). The presence of cytochromes a_1, *d*, and b_{558} in these chlorate-resistant strains is of interest since these cytochromes, which are typical of aerobically grown cells (4, 5, 16), are normally repressed by growth with nitrate under anaerobic conditions (cf. LCB61). Derepression of these cytochromes was also observed with strains CGSC 4444 (*chlC*), RK7-19 (*chlC*), LCB162 (*chlC*::Mu), LCB155 (*chlI*), LCB160 (*chlI*::Mu), RK5274 (*narI*::Tn*10*), RK5266 (*narK*::Tn*10*), RK5270 (*narK*::Tn*10*), RK5269 (*narG*::Tn*10*), NH10 (Δ*chlE68 narI*::Tn*10*), NH30 (*narI*::Tn*10*), and LCB61-22(*nirR*). With the exceptions of the last strain and LCB68 (Δ*chlE68*), all of the strains have mutations in the *chlC-chlI* locus (3, 22, 26, 27). Cytochromes b_{558}, a_1, and *d* were not derepressed in any of the *chlA*, *chlB*, *chlG*, and *fdhA* strains we examined. Their

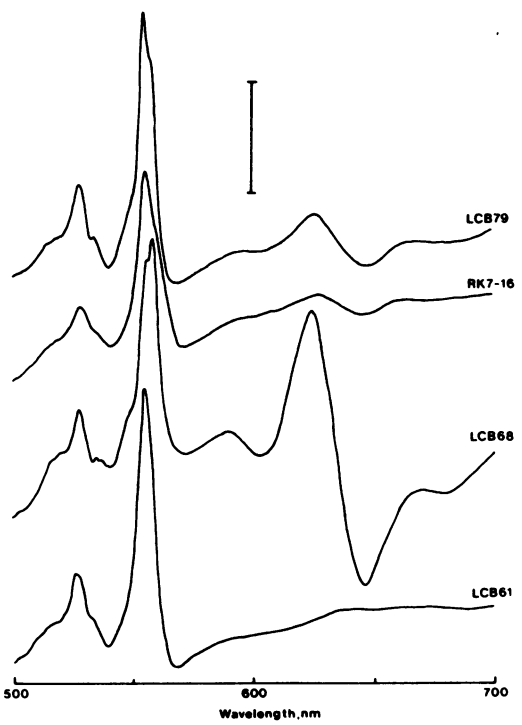

FIG. 1. Difference spectra of *chl* mutants. Dithionite-reduced minus ferricyanide-oxidized difference spectra of membranes were measured at 77°K. Bar, 0.2 absorbance unit. The concentrations of membrane protein are: LCB61, 7.7 mg/ml; RK7-16, 3.5 mg/ml; LCB79, 6.8 mg/ml; LCB68, 5.7 mg/ml.

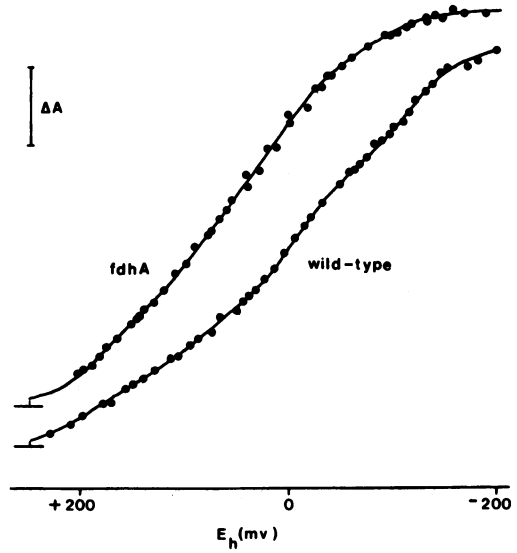

FIG. 2. Redox titration of the *b*-type cytochromes of an *fdhA* mutant compared with that of the wild type. Membranes were prepared from the *fdhA* mutant, LCB517, and the wild-type strain, HfrH, and submitted to spectrophotometric redox titration. The solid lines are four component best fits. For LCB517: ΔA (absorbance) = 0.032 U; protein, 11.8 mg/ml. For HfrH: ΔA = 0.032 U; protein, 7.4 mg/ml.

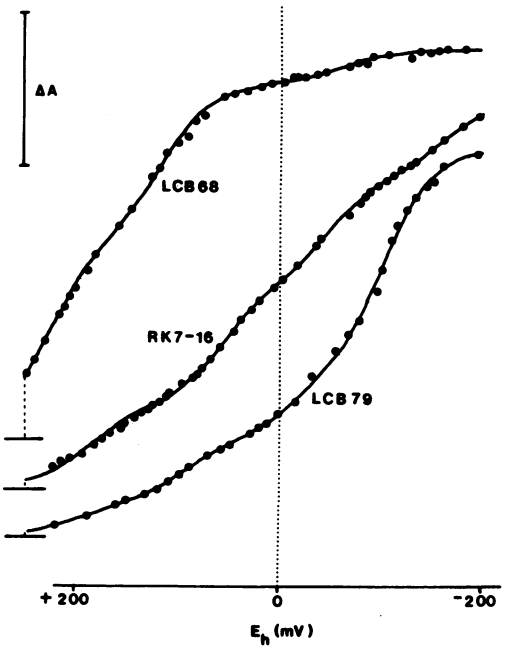

FIG. 3. Redox titrations of the *b*-cytochromes of *chl* mutants. The solid lines are best fits for LCB79 and RK7-16 (four components) and LCB68 (three components). For LCB68: ΔA = (absorbance) 0.066 U; protein, 13.4 mg/ml. For RK7-16: ΔA = 0.053 U; protein, 10.9 mg/ml. For LCB79: ΔA = 0.017 U; protein, 12.2 mg/ml.

difference spectra were like that of the wild type when grown anaerobically with nitrate. By contrast with LCB68 (*ΔchlE68*), LCB61-357 (*chlE16*) and CGSC 4459 (*chlE5*) were not derepressed for these cytochromes.

Redox titration of chlorate-resistant mutants. Figures 2 and 3 show the redox titration curves of the cytochromes in membranes from cells of the wild-type, *fdhA*, and chlorate-resistant strains of *E. coli* grown anaerobically with nitrate. The titration curves were fitted by a nonlinear least-squares program, using a function describing the theoretical behavior of the desired number of components each transferring one electron (see accompanying paper [15]). The midpoint oxidation-reduction potential (E_m) and the percent contribution of each component of this potential to the curve are summarized in Table 2. With the exception of LCB68, a minimum of four components were required to give a satisfactory fit of the titration date. Strains LCB68 and LCB79 showed striking differences when compared with the wild-type strains. The majority of the cytochromes of LCB68 titrated with a redox potential of >100 mV. This is consistent with the absence of cytochromes of the formate dehydrogenase-nitrate reductase system (see below). By contrast, most of the cytochromes of LCB79 (*chlI*) titrated with a redox potential of −106 mV. RK7-16 (*chlA*) also differed from the wild-type strain by having

TABLE 2. Midpoint oxidation-reduction potentials (E_m) and percent contribution of components resolved by redox titration of membrane cytochromes of *chl* and *fdhA* mutants[a]

Strain	Phenotypic class	Components resolved							
		E_m (mV)	%	E_m (mV)	%	E_m (mV)	%	E_m (mV)	%
HfrH	Wild type	197	15.3	99	18.5	−3	35.6	−113	30.6
RK7-16 (*chlA*)	I	188	20.5	58	31.4	−49	28.2	−165	19.8
LCB79 (*chlI*)	II	201	8.4	93	15.6	−13	17.1	−106	58.8
LCB68 (*chlE68*)	III	224	49.6	111	41.6	−67	8.8		
LCB517 (*fdhA*)		180	24.6	89	29.3	8	32.8	−83	13.2

[a] Data from Fig. 2 and 3.

TABLE 3. Classification of *chl* mutants by phenotypic class

Phenotypic class	Strain	Genotype
I	RK5200	*chlA*
	RK5201	*chlE*
	RK5206	*chlG*
	RK5208	*chlB*
	RK5218	*chlE*
	RK5223	*chlE*
	RK5231	*chlG*
	RK5256	*chlG*
	RK5266	*narK*
	RK5270	*narK*
	RK7-16	*chlA*
	356-15	*chlA*
	356-24	*chlB*
	CGSC 4459	*chlE*
	LCB61-357	*chlE*
II	TS9A (42°C)	*chlC*
	RK5269	*narG* (=*chlC*)
	RK5274	*narI* (=*chlI*)
	RK7019	*chlC*
	LCB79	*chlI*
	LCB155	*chlI*
	LCB160	*chlI*
	LCB162	*chlC*
	LCB79-357	*chlI chlE*
	NH30	*chlI*
III	CGSC 4444	*chlC* (?)
	LCB68	Δ*chlE68*
	NH10	Δ*chlE68 chlI*
	LCB61-22	*nirR* (=*fnr*)

significant amounts of cytochromes of a somewhat lower potential (<−150 mV) than found in the wild type.

The chlorate-resistant strains above are considered to be representatives of three distinct phenotypic classes (Table 2). Titration of many other *chl* mutants showed that all could be classified in one or another of these three classes (Table 3).

Formate dehydrogenase-associated cytochrome. The purified preparations of formate dehydrogenase of Enoch and Lester (10, 11) contained cytochrome. However, little information about its properties was reported. Its midpoint oxidation-reduction potential is unknown. The titration curves of Fig. 2 show that little cytochrome titrating with a potential of about −110 mV was present in membranes of the *fdhA* mutant when compared with wild-type strains. This suggests that the cytochrome of this potential detected in the membranes, particularly of anaerobically grown cells, is associated with formate dehydrogenase. Its potential is compatible with that (−420 mV) of the formate-CO_2 system for it to have a functional role in the transfer of electrons in this segment of the respiratory chain.

The absence of the formate dehydrogenase-associated cytochrome in the *fdhA* mutant was

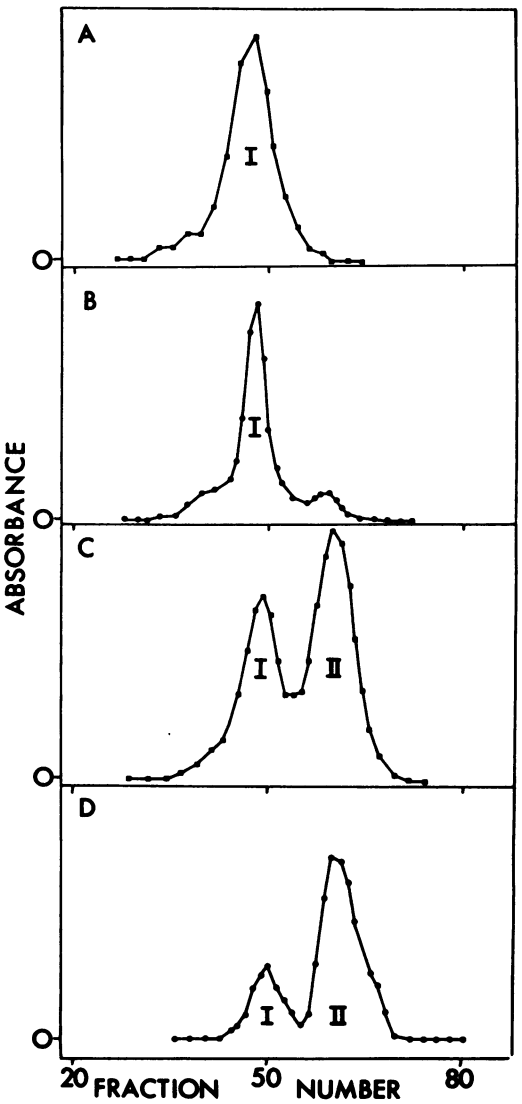

FIG. 4. Elution profiles from DEAE–Bio-Gel A of cytochromes solubilized from respiratory mutants. Cells were grown anaerobically with nitrate, and membranes were prepared, solubilized, and fractionated as described in the text. A linear gradient of 0 to 0.3 M NaCl was applied between fractions 30 and 85. The elution of cytochrome was monitored by measuring the absorbance at 410 nm of the fractions. The absorbance units for each profile are arbitrary; they have been plotted on the same axes to emphasize their similarity. The cytochromes were derived from: (A) LCB79; (B) TS9A grown at 42°C; (C) CGSC 4459; and (D) LCB517.

TABLE 4. Cytochrome content of membranes of chlorate-resistant mutants grown anaerobically with nitrate

Strain	Phenotypic class	nmol/mg of membrane protein		Cytochrome d/ cytochrome b (nmol/nmol)
		Cytochrome *o*	Cytochrome *b*	
HfrH	Wild type	0.073	0.64	0.01
LCB61	Wild type	0.064	0.60	0.04
RK7-16	I	0.250	0.80	0.06
CGSC 4459	I	0.161	0.77	0.02
LCB79	II	0.134	0.54	0.08
LCB160	II		0.44	0.10
RK7-19	II	0.182		
LCB68	III	0.024	0.82	0.55
CGSC 4444	III	0.035	0.41	0.14
LCB517	*fdhA*	0.073	0.46	0.02

confirmed by solubilization of the membrane cytochromes with Triton X-100 followed by ion-exchange chromatography on a column of DEAE-cellulose. With membranes from cells of the wild-type, or CGSC 4459, grown anaerobically on nitrate, two distinct peaks (peaks I and II) of cytochrome were eluted from the column (Fig. 4C). Formate dehydrogenase activity was associated with peak I and nitrate reductase activity was associated with peak II (14). The identification of peak II with nitrate reductase was confirmed by its absence in membrane extracts from strain TS9A grown at 42°C (Fig. 4B). This strain has a temperature-sensitive nitrate reductase, and only one-tenth of the normal level of enzyme is formed at the nonpermissive temperature (9, 14). Strain LCB79 (*chlI*) also lacked the cytochrome associated with nitrate reductase (Fig. 4A), although the cytochrome peak associated with formate dehydrogenase activity (peak I) was present. This is consistent with the large proportion of cytochrome with a midpoint redox potential of −106 mV found in this strain and with the high formate oxidase activity of the membranes (Table 4). Membranes of the *fdhA* mutant had reduced amounts of peak I. This is associated with the absence of formate dehydrogenase activity in this mutant. However, some cytochrome was still present in peak I. This is most likely due to the presence of other of the cytochromes detected in the redox titration curves of membranes of the *fdhA* mutant. These cytochromes, as well as cytochrome b^{fdh}, were present in peak I from the other strains shown in Fig. 4. This was confirmed by data of the type shown in Fig. 5. Ascorbate (in the presence of phenazine methosulfate) will reduce cytochromes with a midpoint redox potential >+10 mV. The formate dehydrogenase-associated cytochrome with a potential of about −110 mV would not be reduced by this electron donor system. By contrast, dithionite is able to reduce fully all of the cytochromes present in the membrane. Only about half of the total cytochrome of peak I from strain CGSC 4459, and of the wild type (not shown), was reducible by ascorbate. This portion would be cytochrome coeluting from the column with cytochrome b^{fdh}. Peak I from the membrane of the *fdhA* strain was completely reducible by ascorbate, as would be expected when cytochrome b^{fdh} was absent. This cytochrome had absorption maxima at 557 and 562 nm. Preliminary results indicated that it contained the cytochrome b_{555}-b_{562}-*o* complex (18).

Other properties of phenotypic classes I, II, and III. Tables 4 and 5 summarize further properties of the three classes of *chl* mutants detected on the basis of their cytochrome content. The presence of nitrate reductase and formate dehydrogenase could be detected by both activity and the characteristic mobility of their α-subunits on SDS-polyacrylamide gels. These subunits (molecular weights, 155,000 and 110,000, respectively) (10) fortuitously migrated to positions on the gels where they could usually be identified unambiguously. Although the phenotypic classes were not completely homogeneous, several features are of interest. Cytochrome *o* and *d* levels were generally elevated in classes I and II and in class III, respectively. Class III mutants lacked nitrite reductase and formate hydrogenlyase activities.

DISCUSSION

As discussed in the accompanying paper (15), growth of *E. coli* under anaerobic conditions with nitrate results in the presence in the cell membrane of several *b*-type cytochromes. Be-

TABLE 5. Respiratory activity and presence of formate dehydrogenase and nitrate reductase α-subunits in membranes of chlorate-resistant mutants grown anaerobically with nitrate

Strain	Phenotypic class	ng-atoms of oxygen reduced/min per mg of protein		Nitrate reductase (nmol of nitrate reduced/min per mg of protein)	Formate hydrogenlyase[a]	Nitrite reductase[b]	α-Subunits[c]	
		NADH oxidase	Formate oxidase				Nitrate reductase	Formate dehydrogenase
HfrH	Wild type	59	57	550	+	+	+	+
LCB61	Wild type	40	20	1140	+	+	+	+
RK7-16	I	225	<2	<10	−	+	+	−
CGSC 4459	I	209	<2	<10	−	+	+	−
LCB79	II	50	200	<10	+	+	−	+
LCB160	II	143	190	430	+	+	+	+
RK7-19	II						−	+
LCB68	III	190	<2	<10	−	−	−	−
CGSC 4444	III	209	5	70	−	−	+	±
LCB61-22	III				−	−	−	−
LCB517	*fdhA*	64	<2	610	−		+	−

[a] Estimated from gas production in Durham tubes.
[b] Estimated from the capacity to clear 1 mM nitrite from a rich medium overnight.
[c] From gel electrophoresis.

sides the cytochromes induced by the anaerobic growth conditions, there are also considerable amounts of the cytochromes formed under aerobic conditions (4, 5, 16). It is impossible to resolve the *b*-type cytochromes of these membranes by spectroscopic studies only. In the present paper we have coupled the technique of redox titration with the use of mutants in the anaerobic respiratory pathways in an attempt to resolve this complex pattern of cytochromes.

In addition to the wild type, four different phenotypes have been demonstrated by these methods. Class I is represented by mutants with defects in the *chlA*, *chlB*, *chlE*, and *chlG* loci. These *chl* mutants are all likely to be involved in the synthesis, processing, or insertion, or all three, of the molybdenum cofactor of nitrate reductase and formate dehydrogenase (1, 21, 25, 27). These strains lack formate dehydrogenase and nitrate reductase activities (see Table 5), although the nitrate reductase apoenzyme is still assembled in the membrane (Table 5) (13). The redox titration curves show appreciable amounts of cytochrome titrating in the 0- to +100-mV range, which is typical of cytochrome b^{nr}. The presence of this cytochrome was confirmed in experiments in which the membrane was solubilized with Triton X-100 and the cytochromes were fractionated on DEAE-cellulose (Fig. 4). Considerable amounts of cytochrome with midpoint potentials below 0 mV were also present in the membranes of class I mutants. The potentials of these components (−49 and −165 mV) were consistently below those (−3 and −113 mV) found in the wild-type strains. The significance of this is not clear and may be a consequence of the limitations in the titration methodology as discussed in the accompanying paper (15) or to the presence of a low-potential *c*-type cytochrome(s). This cytochrome(s) is induced under certain conditions of growth. Its potential is about −180 mV (unpublished data). The presence of a cytochrome of this potential would interfere with the resolution of cytochrome b^{fdh}, which normally shows a midpoint potential of about −110 mV. Cytochrome b^{fdh} is contributing to the redox titration curves found with class I strains since this cytochrome has been found in soluble extracts of their membranes (Fig. 4). SDS-polyacrylamide gel electrophoresis (Table 5) did not detect the α-subunit of formate dehydrogenase in class I strains. Graham et al. (13) could not detect formate dehydrogenase by immunological methods in one of the class I mutants (CGSC 4459) used in our studies. This suggests that cytochrome b^{fdh} can be incorporated into the membrane in the absence of the α-subunit of formate dehydrogenase. By contrast, Barrett and Riggs (2) with *Salmonella typhimurium* observed that this formate dehydrogenase subunit was present in cells in which cytochrome b^{fdh} could not be detected spectroscopically.

Mutants of phenotypic class II all map in the *chlC-chlI* region of the *E. coli* genome. This locus is very complex, with the genes probably

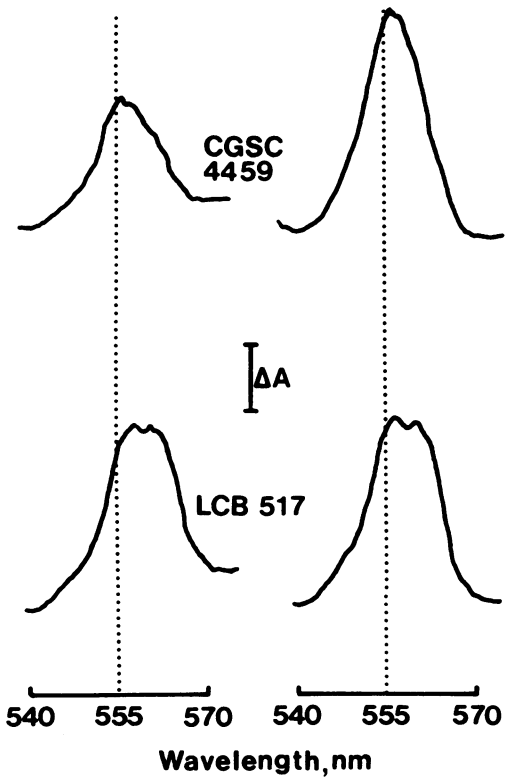

FIG. 5. Difference spectra of cytochromes of respiratory mutants on DEAE-Bio-Gel A. Peaks I from the separations shown in Fig. 4 were concentrated, and sucrose was added to a final concentration of 1 M. One-half of each sample was oxidized by the addition of ferricyanide and served as the reference sample. The other half of each sample was reduced by 2.5 mM ascorbate with 25 μM phenazine methosulfate for 15 min before freezing in liquid nitrogen and scanning against the reference sample at 77°K (left). Subsequently, it was thawed, fully reduced by dithionite, and rescanned against the reference sample at 77°K (right). For CGSC 4459, ΔA (absorbance) = 0.1 U at a protein concentation of 1.5 mg/ml. For LCB517, ΔA = 0.03 U at a protein concentration of 1.0 mg/ml.

forming an operon (3, 27). The α-subunit of the nitrate reductase appears to be the product of the *chlC* gene (equivalent to *narG* of Stewart and MacGregor [27]). It has been proposed that *chlI* (*narI*) defines the gene for cytochrome b^{nr} on the basis of our previously unpublished data (3, 27). In addition, Tn*10* insertions at the *chlC* locus produce the narK$^-$ and narL$^-$ phenotypes which reflect alterations in the control of the expression of the nitrate reductase genes and of genes for the formation of other respiratory systems (26, 27).

Our data in the present paper support these conclusions. Mutants of phenotypic class II are distinguished, when compared with the wild type, by the relatively smaller amounts of cytochrome titrating at potentials of >0 mV and by the large amount of cytochrome having a midpoint redox potential of about −110 mV. This cytochrome is cytochrome b^{fdh} as shown by both its potential and its chromatographic behavior on DEAE-cellulose (Fig. 4). The high levels of this cytochrome are reflected in the higher than normal amounts of the α-subunit of formate dehydrogenase detected by SDS gel electrophoresis and by the high formate oxidase activity (Table 5). Membranes from cells showing the class II phenotype lack cytochrome b^{nr} as shown by chromatography on DEAE-cellulose (Fig. 4) (14). The cytochrome titrating in these cells in the potential range of 0 to +100 mV, where cytochrome b^{nr} is usually found (14), is probably cytochrome of the aerobic pathway. As described in the accompanying paper (15), membranes from a mutant (NH20) lacking cytochromes b^{fdh} and b^{nr} had a component titrating with a midpoint potential of +80 mV. Our finding that cytochrome b^{nr} is absent in the *chlI* and *narI* strains of Chippaux and co-workers (3, 22) and of Stewart and McGregor (27) supports their assignment of this gene to the cytochrome. Moreover, the absence of cytochrome b^{nr} in the insertions mutants in *chlC* (*narG*) is likely due to a polar effect on the expression of the *chlI* (*narI*) gene and confirms the proposal of these workers that *chlC* (*narG*) and *chlI* (*narI*) are in the same operon.

Mutants of our phenotypic class III are distinguished by the absence (or very reduced amounts) of cytochromes with midpoint potentials more negative than +100 mV. Thus, cytochrome b^{fdh} is absent (or present in very reduced amounts), and this is reflected in the loss of formate dehydrogenase and oxidase activities (Table 5). Furthermore, cytochrome b^{nr} is absent, although benzyl viologen-dependent nitrate reductase activity may be present in some strains. These mutants are also characterized by a very high level of cytochrome *d*. Cytochrome *o* is present at one-third to one-half of the level found in the wild type. Therefore, the high NADH oxidase activity is probably mediated through the cytochrome b_{558}-cytochrome *d* pathway (4, 5, 16).

The absence of the cytochromes of the formate dehydrogenase-nitrate reductase pathway in class III mutants grown anaerobically on nitrate reflects the complex controls on the formation of the anaerobic respiratory pathways. One of the class III mutants is LCB61-22. This is a *nirR* (probably equivalent to *fnr* [7]) strain. Chippaux et al. (7) have suggested that this gene controls the synthesis of a component necessary for the expression of some of the genes involved in the synthesis of nitrate, nitrite, and fumarate reductases. LCB68 contains a small deletion in the *chlE* gene (M. Chippaux,

personal communication). Stewart and MacGregor (27) have proposed that the *chlE* gene is involved in the synthesis or insertion of the molybdenum cofactor. The absence of formate dehydrogenase and nitrate reductase activities (Table 5) in LCB68 (*ΔchlE68*) is consistent with this view. However, the absence of the α-subunits of these two enzymes, and of cytochromes b^{fdh} and b^{nr}, suggests that the product of the *chlE68* gene is also involved in the expression of the formate dehydrogenase-nitrate reductase system. Interestingly, LCB61-357 (*chlE16*) and CHSC 4459 (*chlE5*), which are also *chlE* mutants, are phenotypically class I mutants. Thus, it is probable that there is more than one gene at the *chlE* locus. Strain CGSC 4444 also falls in phenotypic class III. It is classified as a *chlC* mutant (24) but it needs further genetic and biochemical characterization.

Growth of wild-type strains and mutants of class I anaerobically on nitrate results in repression of cytochrome *d*, a component of the aerobic respiratory chain. The mechanism of the repression is unknown. The repression is relieved in mutants of classes II and III. The release from repression is particularly dramatic in LCB68, where the ratio of cytochrome *d* to cytochrome *b* is greater than is usually encountered even in wild-type cells grown aerobically to the stationary phase. These results suggest that the *chlC-narI* or *chlE68* gene products or both are involved in the repression of these aerobic cytochromes.

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LITERATURE CITED

1. **Amy, N. K.** 1981. Identification of the molybdenum cofactor in chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **148**:274–282.
2. **Barrett, E. L., and D. L. Riggs.** 1982. *Salmonella typhimurium* mutants defective in the formate dehydrogenase linked to nitrate reductase. J. Bacteriol. **149**:554–560.
3. **Bonnefoy-Orth, V., M. Lepelletier, M. C. Pascal, and M. Chippaux.** 1981. Nitrate reductase and cytochrome *b* nitrate reductase structural genes as parts of the nitrate reductase operon. Mol. Gen. Genet. **181**:535–540.
4. **Bragg, P. D.** 1979. Electron transport and energy-transducing systems of *Escherichia coli*, p. 341–449. *In* R. A. Capaldi (ed.), Membrane proteins in energy transduction. Marcel Dekker, Inc., New York.
5. **Bragg, P. D.** 1980. The respiratory system of *Escherichia coli*, p. 115–136. *In* C. J. Knowles (ed.), Diversity of bacterial respiratory systems, vol. 1. CRC Press, Inc., Boca Raton, Fla.
6. **Casse, F., M. C. Pascal, M. Chippaux, and J. Ratouchniak.** 1976. Genetic analysis of mutants from *Escherichia coli* K12 unable to grow anaerobically without exogenous acceptor. Mol. Gen. Genet. **148**:337–340.
7. **Chippaux, M., V. Bonnefoy-Orth, J. Ratouchniak, and M. C. Pascal.** 1981. Operon fusions in the nitrate reductase operon and study of control gene *nirR* in *Escherichia coli*. Mol. Gen. Genet. **182**:477–479.
8. **Clegg, R. A.** 1976. Purification and some properties of nitrate reductase from *Escherichia coli* K12. Biochem. J. **153**:533–541.
9. **DeMoss, J. A.** 1978. Role of the *chlC* gene in formation of the formate-nitrate reductase pathway in *Escherichia coli*. J. Bacteriol. **133**:626–630.
10. **Enoch, H. G., and R. L. Lester.** 1974. The role of a novel cytochrome b-containing nitrate reductase and quinone in the *in vitro* reconstruction of formate-nitrate reductase activity of *E. coli*. Biochem. Biophys. Res. Commun. **61**:1234–1241.
11. **Enoch, H. G., and R. L. Lester.** 1975. The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. J. Biol. Chem. **250**:6693–6705.
12. **Fairbanks, G., T. Steck, and D. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
13. **Graham, A., H. E. Jenkins, N. H. Smith, M. A. Mandred-Berthelot, B. A. Haddock, and D. H. Boxer.** 1980. The synthesis of formate hydrogenase and nitrate reductase proteins in various *fdh* and *chl* mutants of *Escherichia coli*. FEMS Microbiol. Lett. **7**:145–151.
14. **Hackett, N. R., and P. D. Bragg.** 1982. The association of two distinct *b* cytochromes with the respiratory nitrate reductase of *Escherichia coli*. FEMS Microbiol. Lett. **13**:213–217.
15. **Hackett, N. H., and P. D. Bragg.** 1983. Membrane cytochromes of *Escherichia coli* grown aerobically and anaerobically with nitrate. J. Bacteriol. **154**:708–718.
16. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47–99.
17. **Jones, R. W., and P. B. Garland.** 1977. Sites and specificity of the reaction of bipyridylium compounds with anaerobic respiratory enzymes of *Escherichia coli*. Biochem. J. **164**:199–211.
18. **Kita, K., and Y. Anraku.** 1981. Composition and sequence of *b* cytochromes in the respiratory chain of aerobically grown *Escherichia coli* K-12 in the early exponential phase. Biochem. Int. **2**:105–112.
19. **Laemmli, U.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **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.
21. **MacGregor, C. H., and C. A. Schnaitman.** 1973. Reconstitution of nitrate reductase activity and formation of membrane particles from cytoplasmic extracts of chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **114**:1164–1176.
22. **Orth, V., M. Chippaux, and M. C. Pascal.** 1980. A mutant defective in electron transfer to nitrate in *Escherichia coli* K12. J. Gen. Microbiol. **117**:257–262.
23. **Pittard, J.** 1965. Effect of integrated sex factor on transduction of chromosomal genes in *Escherichia coli*. J. Bacteriol. **89**:680–686.
24. **Showe, M. K., and J. A. DeMoss.** 1968. Localization and regulation of synthesis of nitrate reductase in *Escherichia coli*. J. Bacteriol. **95**:1305–1313.
25. **Sperl, G. T., and J. A. DeMoss.** 1975. *chlD* gene function in molybdate activation of nitrate reductase. J. Bacteriol. **122**:1230–1238.
26. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788–789.
27. **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.