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Membrane Cytochromes of *Escherichia coli* Grown Aerobically and Anaerobically with Nitrate

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Redox titration has been coupled to spectroscopic techniques, enzyme fractionation, and the use of mutants to examine the cytochrome composition of the membranes from cells grown aerobically and anaerobically with nitrate. A combination of techniques was found to be necessary to resolve the cytochromes. At least six *b*-type cytochromes were present. Besides cytochromes b^{fdh} and b^{nr}, components of the formate dehydrogenase-nitrate reductase pathway, cytochromes b_{556}, b_{555}, b_{562}, and *o*, characteristic of aerobic respiratory pathways, were present. The midpoint oxidation-reduction potentials of the aerobic *b*-type cytochromes suggested that the sequence of electron transfer is: cytochrome $b_{556} \rightarrow b_{555} \rightarrow b_{562} \rightarrow O_2$.

Escherichia coli, a facultative anaerobe, can respond to changes in its environment by synthesizing different types of respiratory pathways. It is generally held that growth under conditions where oxygen is not limiting results in the formation of predominantly cytochromes b_{556}, b_{562}, and *o*. These constitute the "high aeration" pathway. When the oxygen tension is low, the cytochromes of the "low aeration" pathway, cytochromes b_{558} and *d*, are induced in addition to the others. Cytochromes *o* and *d* are the oxidases of the two pathways (1, 2, 18). In addition, several anaerobic respiratory pathways are known. The best-defined one is the formate dehydrogenase-nitrate reductase system, which is induced by anaerobic growth with nitrate as terminal electron acceptor (26). These conditions give cells containing cytochromes with an α-absorption maximum at about 555 nm (12, 22) in the reduced-minus-oxidized difference spectrum. This is in contrast to the cytochromes absorbing at 556, 558, and 562 nm detected in aerobically grown cells. The peak at 555 nm has been attributed to the presence of two cytochromes, cytochrome b^{fdh} and b^{nr}, which can also be found in purified preparations of formate dehydrogenase and nitrate reductase, respectively (8, 9). These two cytochromes were once suggested to be the only species present in cells grown anaerobically with nitrate (22). However, the complex kinetics of cytochrome reduction in the membranes of these cells have been interpreted to show the presence of both of the aerobic respiratory pathways in addition to cytochromes b^{fdh} and b^{nr} (23).

The resolution and characterization of bacterial cytochromes have relied greatly on the use of the absorption spectra of the reduced cytochromes. This technique cannot resolve cytochromes with identical or nearly identical spectra. In practice, it is found that the presence of several cytochromes results in the formation of a single fused peak sometimes showing some resolution into minor peaks or shoulders. Various mathematical procedures such as curve simulation (27), or taking fourth-order finite difference spectra (24, 25), have been applied to resolve such spectra. The results are unsatisfactory in the absence of other criteria (27).

One such criterion is the midpoint oxidation-reduction potential of the cytochrome. The midpoint potentials of the cytochromes of *E. coli* have been examined by a number of workers (14, 19, 20, 27). However, with the exception of the recent work of Hendler and Schrager (14) and van Wielink et al. (27), the method of approach has been too simplistic. These workers improved the resolving ability of the spectroscopic and potentiometric titration techniques by linking the two in their analyses.

In this, and the accompanying paper (11), we have examined the composition of the *b*-type cytochromes in membranes of *E. coli* grown aerobically and anaerobically with nitrate. To improve our ability to resolve these cytochromes, we have coupled spectroscopic techniques with redox titration, enzyme fractionation, and the use of mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following strains of *E. coli* were used in this investigation (the name of the donor is given in brackets): HfrH (*thi relA spoT supQ* λ⁻) [J. A. DeMoss]; RK7 (KL96) (Hfr

thi gal rpsL) [C. H. MacGregor]; MR43L (F^- *thi lac gal recA*) [R. B. Gennis]; LCB517 (F^- *thi leu thr arg his pro purE supE lacY malA xyl ara mtl, gal tonA rpsL fdhA*) [M. Chippaux]; RK5274 (*thi lacU araD non rpsL gyrA narI*::Tn*10*) [V. Stewart]; NH20 (as LCB517, but *narI*::Tn*10*) (by P1 transduction of LCB517 to tetracycline resistance, using RK5274).

The basic minimal medium consisted of salts (4) supplemented with thiamine (1 μg/ml), 1 μM ammonium molybdate, 1 μM selenous acid, and 12 μM ferric citrate. When necessary, essential nutrients or tetracycline was added from sterile stock solutions to a level of 20 μg/ml. For aerobic growth, glucose (or glycerol, where indicated) was present at a concentration of 0.4%. A 500-ml amount of medium in a 2-liter Erlenmeyer flask was shaken at 250 rpm in a New Brunswick rotatory incubator at 37°C. The medium for anaerobic growth with nitrate was supplemented with 0.5% Bacto-Peptone (Difco Laboratories) and 0.5% potassium nitrate. After autoclaving, sterile glucose (1%) and sodium bicarbonate (50 mM) were added. The cells were grown at 37°C as nonstirred standing cultures in 2-, 4-, or 6-liter flasks completely filled with medium.

E. coli MR43L was grown aerobically with vigorous shaking in Penassay broth.

Preparation of membranes. Cells were harvested by centrifugation at 5,000 rpm for 20 min in a JA10 rotor of a Beckman J-21 centrifuge. The cells were suspended in 25 mM Tris-hydrochloride, pH 8.0, containing 0.9% NaCl and resedimented by centrifugation at 10,000 rpm in a JA20 rotor for 15 min. They were resuspended to a concentration of approximately 1 g (wet weight)/5 ml in 50 mM Tris-H_2SO_4, pH 7.8, containing 10 mM $MgCl_2$ and disrupted in a French press at 20,000 lb/in^2. Whole cells were removed by centrifugation in a JA20 rotor as described above. The resulting crude extract was separated into a membrane fraction and a soluble fraction by ultracentrifugation for 2 h at either 40,000 rpm in a Beckman 42.1 rotor or 45,000 rpm in a Beckman Ti60 rotor. The membrane pellet was then suspended in the Tris-magnesium buffer and resedimented as before. The membranes were suspended in an appropriate buffer immediately before use.

Low-temperature difference spectra. Spectra were taken at the temperature of liquid nitrogen (77°K) in a Perkin-Elmer 356 spectrophotometer operated in its split beam mode, using its cryogenic unit. A 1-ml portion of a membrane suspension was diluted in an equal volume of 2.0 M sucrose, and the sample was divided into two portions. These two samples were reduced and oxidized as appropriate before being transferred to the cuvette compartments of the cryogenic cell holder. The whole-cell holder was then immersed in liquid nitrogen for about 2 min. When reductants other than dithionite were used, freezing was delayed by 5 min after addition of the sample to the cuvette to allow the depletion of any oxygen introduced in the transfer process. The frozen samples were then devitrified by illumination from a distance of 5 cm with a 150-W incandescent bulb for 3 min. This step improved the sensitivity and was necessary for obtaining reproducible spectra. The samples were then refrozen in the cryogenic unit and scanned. Typically, a rapid scan of the wavelength region 700 to 500 nm was performed at 60 nm/min, with a slit width of 3 nm and the pen response switch at the "medium" position. This was then followed by a slow scan at 10 nm/min of the α-peak region of the *c*- and *b*-type cytochromes, with a 1-nm slit width and "slow" response setting. This allowed an accurate determination of the absorption maximum of a particular sample.

The height of the α-peak of cytochrome *b* was measured above a base line drawn between the two adjacent troughs of the spectrum. It was found that the procedure described for measuring spectra at 77°K increased the absorbance for a particular sample by a factor of 23 over that found at room temperature. Extinction coefficients for typical *b*-type cytochromes at room temperature are about 17 mM^{-1} cm^{-1} (5, 12). Therefore, an extinction coefficient of 390 mM^{-1} cm^{-1} has been taken for total *b*-type cytochrome measured at 77°K. Although this value may be in error, it was found useful in comparing the cytochrome content of various preparations. An extinction coefficient of 8.5 mM^{-1} cm^{-1} at 628 nm, using 650 nm as the reference wavelength, was taken for cytochrome *d* (12).

Carbon monoxide difference spectra. Carbon monoxide difference spectra were recorded at room temperature. A membrane suspension in each of two cuvettes was reduced by the addition of a few grains of dithionite. One was used as reference and the other was bubbled with carbon monoxide for 2 min. The difference spectrum was then scanned at 60 nm/min, with a 3-nm slit width at medium pen response. No change was seen in the spectrum after longer periods of bubbling with carbon monoxide. No effort was made to keep samples in the dark during this procedure. An extinction coefficient of 170 mM^{-1} cm^{-1} between the peak at 417 nm and the trough at 432 nm was taken for cytochrome *o* (3).

Spectrophotometric redox titration. Potentiometric titrations were performed by the method of Dutton (7). A sample of membranes was suspended in 15 ml of degassed 0.1 M potassium phosphate buffer, pH 7.0, to a protein concentration of approximately 10 mg/ml. A 12.5-ml portion of this was placed in a cuvette of the design shown by Dutton but with just one sidearm. The vessel was sealed with a serum stopper in the sidearm and a rubber stopper in the top containing ports for the electrode and for gassing. The electrode was a Fisher combination platinum electrode (13-639-82) which had an internal reference electrode. This was standardized periodically against a series of cross-checked standard calomel electrodes. The atmosphere in the cuvette was replaced by nitrogen (Linde U.S.P.), and the cuvette was intermittently flushed with nitrogen throughout the titration. The redox mediators used were as follows: sodium anthraquinone-2-sulfonate, phenazine methosulfate, phenazine ethosulfate, 2,6-dichlorophenol indophenol, menadione, 1,2-naphthoquinone, and 2-hydroxy-1,4-naphthoquinone. These were added from concentrated stock solutions to final concentrations of 10 to 100 μM. To reduce a sample, small additions of stock solutions of NADH or dithionite were added. To oxidize the sample, atmospheric oxygen was introduced or a small volume of a phenazine methosulfate stock solution was added. Reductant stock solutions (25 mM) were always made up in 0.1 M Tris-hydrochloride, pH 8.0. The remaining 2.5 ml of membrane suspension was oxidized by the addition of ferricyanide and used as reference. The material being titrated was scanned

against this at a series of measured potentials between +250 and −200 mV. The wavelength range covered was 590 to 530 nm scanned at a speed of 60 nm/min with a 3-nm slit width. At the end of all titrations the redox potential was lowered to about −300 mV and the difference spectrum was scanned. This ensured that no cytochrome of very low potential had been overlooked and that cytochrome had not become degraded during the lengthy titration procedure.

The amount of cytochrome reduced at a given potential was estimated by measuring the height of the α-peak above a line drawn tangentially to the troughs on either side of the peak in the spectrum. This method is equivalent to the "three-point fit" of Hendler and Shrager (14). The data were fitted by a nonlinear least-squares program (6), using a function describing the theoretical behavior of the desired number of components each transferring one electron.

Preparation of soluble cytochrome b_{555}-b_{562} complex (16). The membrane fraction from *E. coli* MR43L was suspended at a concentration of 10 mg of protein per ml in 10 mM Tris-hydrochloride buffer, pH 8.0, containing 10 mM $MgCl_2$, and 20% Triton X-100 was added to give a final concentration of 5%. The mixture was stirred at room temperature for 30 min before being centrifuged at 150,000 × *g* for 2 h in a Beckman 42.1 rotor. The supernatant (150 ml) was applied to a column (35 by 2 cm) of DEAE-Sepharose CL-6B equilibrated with 10 mM Tris-hydrochloride buffer, pH 8.0, containing 1 mM EDTA and 1% Triton X-100. The column was then washed with 200 ml of the equilibration buffer before a linear gradient from 0 to 0.4 M NaCl in equilibration buffer (500-ml total volume) was applied. The cytochrome-containing peak which was eluted when the conductivity of the buffer was 7 mmho (0.007 S) was used for the experiments.

Chemicals. All chemicals were obtained from commercial sources and were of reagent grade. Special chemicals were obtained as follows: NADH, phenazine methosulfate, phenazine ethosulfate, 2,6-dichlorophenol indophenol, horse heart cytochrome *c* (type IV), and tetracycline from Sigma Chemical Co.; 1,2-naphthoquinone and 2-hydroxy-1,4-naphthoquinone from Eastman; menadione from Nutritional Biochemicals Corp.; anthraquinone-2-sulfonate from Fisher Scientific Co.; ruthenium hexamine from Alfa Division; and 3,6-diaminodurene from Aldrich Chemical Co.

RESULTS

Difference spectra of cytochromes from cells grown aerobically and anaerobically with nitrate. Dithionite-reduced minus ferricyanide-oxidized difference spectra of membranes of *E. coli* HfrH grown aerobically to the stationary phase showed maxima at 556, 558, 590, and 625 nm which have been attributed to cytochromes b_{556}, b_{558}, a_1, and *d*, respectively (Fig. 1) (1, 2, 13). The trough at 646 nm is part of the cytochrome *d* spectrum. The shoulder at 548 nm on the cytochrome *b* α-peak is due to a *c*-type cytochrome, whereas that at 562 nm is due to cytochrome b_{562}. This species is much more prominent in the membranes of cells grown aerobically in a Casamino Acids-supplemented medium to the exponential phase of growth (Fig. 1b). These membranes have an absorption maximum at 555 nm and a lower content of cytochrome *d* than in stationary-phase cells. The difference spectrum of membranes from cells grown anaerobically with nitrate was simpler (Fig. 1c). Cytochromes a_1 and *d* were barely detectable. The *b*-type cytochrome showed a single peak at 555.5 nm on which a shoulder at 547 nm, due to a *c*-type cytochrome, was discernible. Cytochromes b_{558} and b_{562} were not clearly present in the difference spectrum.

In both the membrane and purified preparations, cytochrome b_{562} is always found associated with cytochrome *o*, whereas cytochrome b_{558} is associated with cytochrome *d* (13, 16, 21; M. J. Miller and R. B. Gennis, Fed. Proc. **41**:3653, 1982). Although the difference spectrum suggested that cytochromes b_{562} and b_{558} might be absent from the membranes of cells grown anaerobically with nitrate, this would be inconsistent with the suggestion of Sanchez-Crispin et al. (23) that these cells contain aerobic cytochromes. It was thought possible that low levels of b_{558} and b_{562} might be masked by the high levels of cytochromes b^{fdh} and b^{nr}. However, their presence might be inferred if cytochromes *o* and *d* could be detected by carbon monoxide difference spectroscopy. This technique was applied to the membranes of cells grown anaerobically with nitrate with the result shown in Fig. 2. For comparison, the carbon monoxide difference spectra of aerobically grown cells from both exponential and stationary phases are presented. The spectrum of the aerobic exponential-phase cells was typical of cytochrome *o*, with maxima at 415, 534 and 567 nm and a pronounced minimum at 428 nm. The spectrum of the stationary-phase cells showed cytochrome *o* but with addition of the spectrum of the cytochrome *d*-carbon monoxide complex (minimum at 442 nm; maximum at 641 nm). By contrast, the carbon monoxide difference spectrum of membranes from cells grown anaerobically with nitrate showed some features of cytochrome *o* (maxima at 417, 537, and 569 nm), although the prominent minimum at 428 nm was absent. The presence of cytochrome *o* suggests that cytochrome b_{562} was present even though it was not evident in the difference spectrum. As might be predicted from the difference spectrum, no trace of the cytochrome *d*-carbon monoxide complex was detected. Thus, cytochrome b_{558} is unlikely to be present in these cells.

Potentiometric titration of membrane cytochromes. Difference spectroscopy was clearly insufficient to define the total *b*-type cytochrome content of the membranes of cells grown anaerobically with nitrate. Spectrophotometric redox

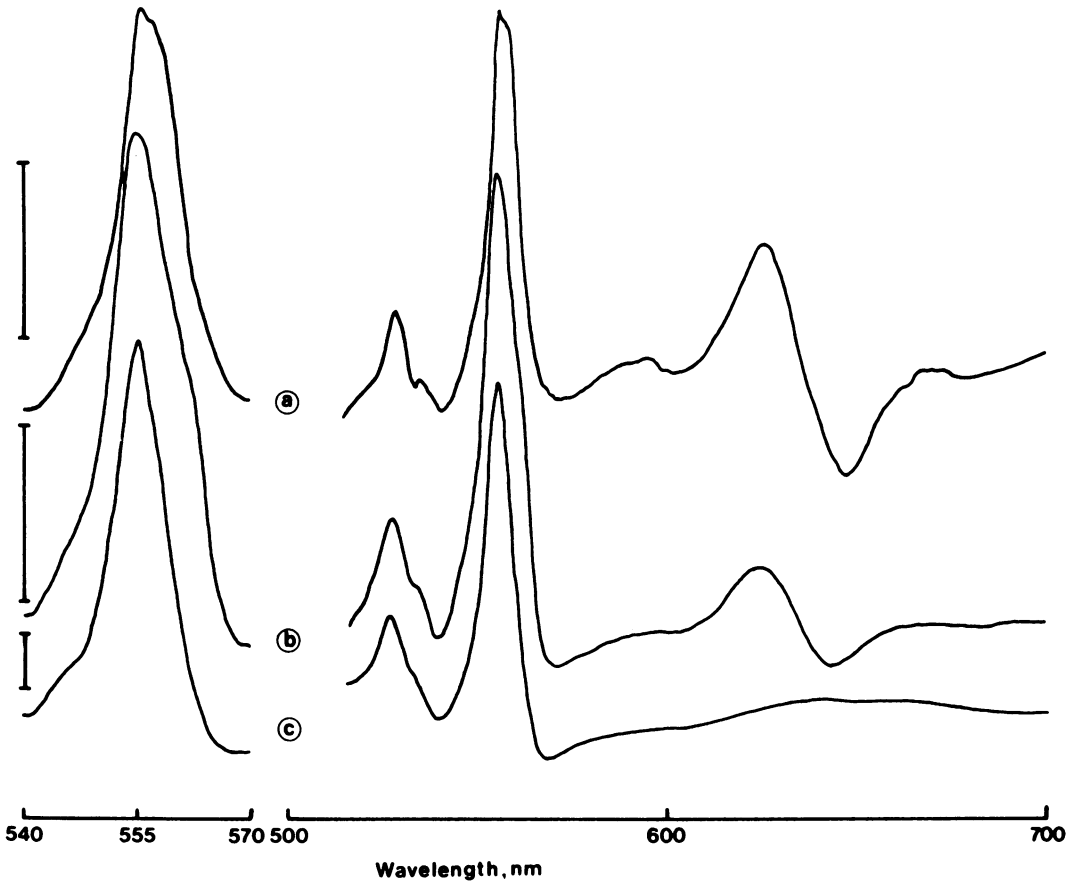

FIG. 1. Effect of growth conditions on the cytochrome content of membranes of strain HfrH. Dithionite-reduced minus ferricyanide-oxidized difference spectra were measured at 77°K. Bar, Absorbance difference of 0.06 U. a, Cells grown aerobically to the stationary phase on minimal medium with glucose (protein, 2.5 mg/ml); b, cells grown aerobically to the exponential phase on minimal medium supplemented with 0.5% glycerol and 0.5% Casamino Acids (protein, 5.8 mg/ml); c, cells grown anaerobically with nitrate (protein, 6.1 mg/ml).

titration was considered to be a potentially useful technique. Previous applications of this method have emphasized the membranes of cells grown under aerobic conditions (14, 19, 20, 27). Therefore, these were studied also to verify the data obtained from anaerobically grown cells. The method was first applied to the membrane cytochromes of the wild-type strain after either anaerobic growth with nitrate or aerobic growth to the stationary phase. Direct examination of the data indicated that more than two components were present. Figure 3 shows the results analyzed by a nonlinear least-squares program for the presence of three and of four components. It is clear that the lines drawn for four components fit the data points most closely. This value indicates the minimum number of cytochromes likely to be present in the membranes. Analysis of the data for the presence of more than four components did not noticeably improve the closeness of the fit of the calculated line to the data points. For this reason, we have generally analyzed our potentiometric data in terms of the minimum number of components required to give acceptable agreement between theoretical and experimental values. The extent of agreement was determined from the sum of the squared differences between the predicted and experimental values. For example, with the data for the aerobically grown cells plotted in Fig. 3 the sum of the squared differences for a four-component fit (sum = 5) is markedly smaller than for a three-component fit (sum = 30), indicating that the former is a better fit than the latter.

The reproducibility of the redox titration data is shown in Table 1. The results from titration of three separate preparations of membranes of

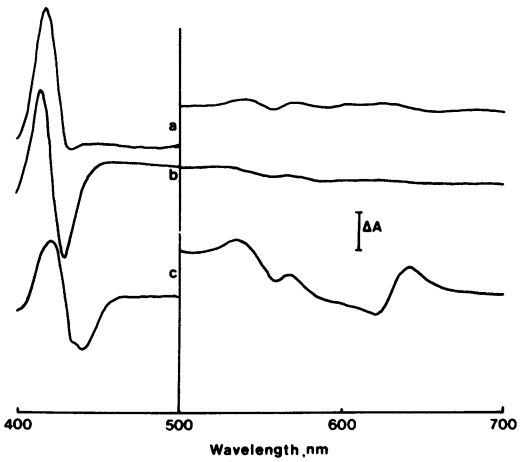

FIG. 2. Effect of growth conditions on the carbon monoxide difference spectrum of membrane preparations of strain HfrH. When necessary the absorbance (A) scale was changed between the α-band region (700 to 500 nm) and the Soret band region (500 to 400 nm). Cells were grown: a, anaerobically with nitrate (ΔA = 0.03 in the α-band and Soret band regions; protein concentration, 9.0 mg/ml); b, aerobically with 0.5% glycerol–0.5% Casamino Acids to the exponential phase (ΔA = 0.03 in α-band and Soret band regions; protein, 7.1 mg/ml); and c, aerobically with glucose to the stationary phase (ΔA = 0.03 in α-band region, 0.1 in Soret band region; protein, 10.7 mg/ml).

strain HfrH and of three different titrations of the same preparations of membranes of strain RK7 are compared. Analysis of the cytochromes in terms of a three-component fit showed that for both strains the maximum standard deviation of the mean of the midpoint potential was ±5 mV.

The effect of pH on the midpoint potentials of the cytochromes in cytoplasmic membranes from cells grown anaerobically on nitrate is shown in Table 2. The potential of all of the cytochromes in the membrane fell with increasing pH. However, the change was much smaller than the 60 mV per pH unit predicted by the chemiosmotic hypothesis for cytochromes involved in proton translocation (18).

Effect of growth conditions on the potentiometrically detectable cytochromes of *E. coli*. The spectra in Fig. 1 and 2 showed alterations in the pattern of cytochromes synthesized by strain HfrH under different conditions of growth. In Fig. 3 are shown the redox titration curves resolved for four components for membranes grown aerobically to the stationary phase or anaerobically on nitrate. The midpoint potentials of these components, together with their percent contributions to the titration curves, as well as data for exponential-phase aerobically grown cells, are summarized in Table 3. The values in Table 3 are given merely to facilitate the comparison of the *b*-type cytochrome content under various growth conditions. Since the number of cytochromes in the membrane is not established, the quoted values for midpoint potential cannot be interpreted to mean that a cytochrome of that potential is present.

A comparison of aerobic cells from different phases of growth showed that larger amounts of high-potential cytochrome (E_m, +200 mV) are produced when the cells enter the stationary phase. For cells grown anaerobically with nitrate the pattern of cytochrome formation was independent of the growth phase. In comparison with aerobically grown cells, the membranes from nitrate-grown cells were enriched in the lower-potential cytochromes. This is consistent with the presence of the formate dehydrogenase-nitrate reductase system. The cytochromes associated with nitrate reductase have potentials near 0 and +100 mV (10), whereas that of formate dehydrogenase has a potential of about −100 mV (11).

TABLE 1. Reproducibility of redox titration data

Expt[a]	Strain	Components resolved[b]					
		E_m (mV)	%	E_m (mV)	%	E_m (mV)	%
A	HfrH	180	48	48	28	−127	23
B	HfrH	169	44	39	30	−128	26
C	HfrH	173	51	42	31	−130	18
D1	RK7	184	42	51	35	−119	23
D2	RK7	178	43	47	36	−118	21
D3	RK7	173	44	48	36	−119	20
Mean (±SD)		176 (±5.0)	45 (±3.1)	46 (±4.1)	32 (±3.1)	−124 (±4.9)	22 (±2.5)

[a] The strains indicated were grown aerobically on minimal medium to the stationary phase. Membranes were prepared and titrated as described in the text. A, B, C are titrations of different preparations of membranes. D1, D2, D3 are three titrations of the same preparations of membranes.

[b] The titration curves were resolved as three-component fits. E_m, Midpoint oxidation-reduction potential; %, percent contribution of the component to the titration curve.

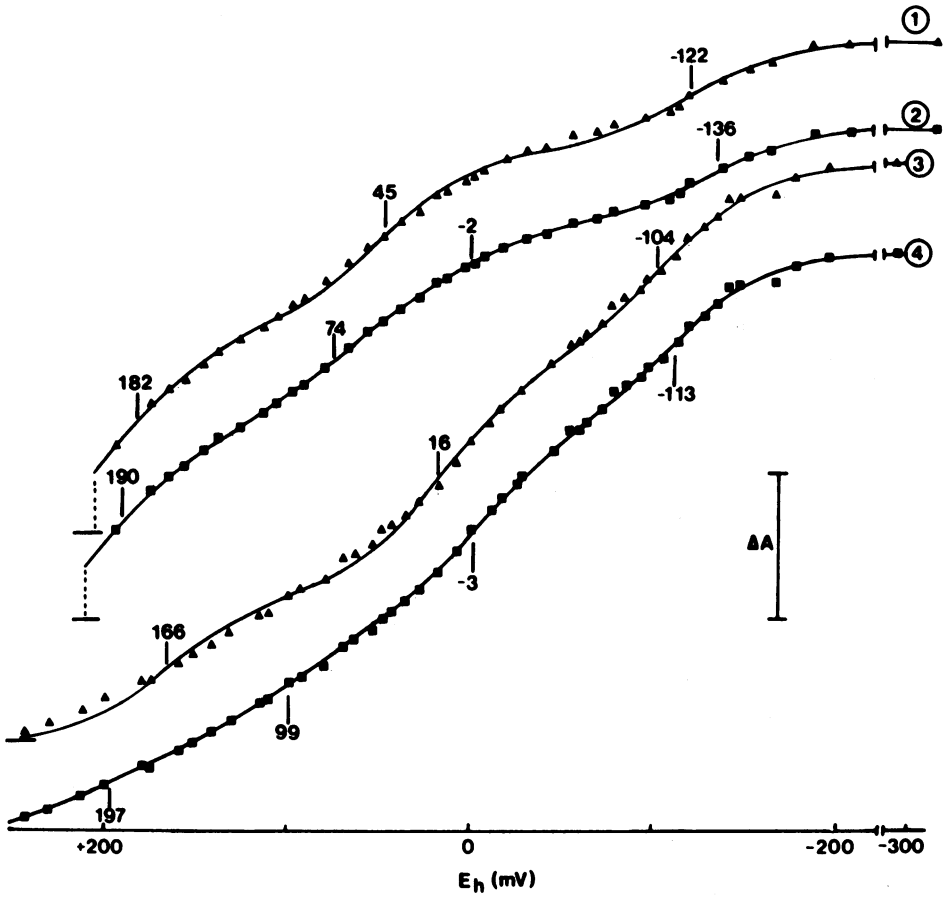

FIG. 3. Comparison of three (curves 1 and 3)- and four (curves 2 and 4)-component best fits of redox titration data of cytochromes. The solid lines are the theoretical curves describing the behavior of the components whose midpoint oxidation-reduction potentials are indicated on the curves. Curves 1 and 2, Membranes (16.4 mg of protein per ml) from cells of RK7 grown aerobically on a minimal medium with glucose to the stationary phase (ΔA, 0.036 absorbance unit); curves 3 and 4, membranes (7.4 mg of protein per ml) of HfrH grown anaerobically with nitrate (ΔA, 0.063 absorbance unit).

Potentiometric titration of solubilized cytochromes of *E. coli*. The redox titrations of whole membranes led to the conclusion that at least four components were present irrespective of the growth conditions. Therefore, simplified systems were sought to study by this method.

Anraku and co-workers (15–17) have described the solubilization, fractionation, and

TABLE 2. pH dependence of midpoint potentials of cytochromes in membranes of cells grown anaerobically with nitrate

pH of titration	Buffer	Cytochromes resolved[a]					
		E_m (mV)	%	E_m (mV)	%	E_m (mV)	%
5.6	MES[b]	201	28	55	43	−64	29
7.0	Potassium phosphate	166	26	16	40	−104	34
8.4	Tris-hydrochloride	123	33	−43	40	−171	27

[a] The titration curves were resolved as three-component fits. E_m, Midpoint oxidation-reduction potential; %, percent contribution of the component to the titration curve.

[b] MES, 2-(*N*-Morpholino)ethanesulfonic acid.

TABLE 3. Midpoint oxidation-reduction potentials (E_m) and percent contribution of components resolved by redox titration of membrane cytochromes of strain HfrH

Growth conditions	Components resolved							
	E_m (mV)	%	E_m (mV)	%	E_m (mV)	%	E_m (mV)	%
Aerobic, exponential	206	26	96	28	5	35	−141	10
Aerobic, stationary	190	39	74	27	−2	16	−136	19
Anaerobic with nitrate	197	15	99	19	−3	36	−113	30

partial purification of the cytochromes of *E. coli* membranes. Two fractions were characterized from the membranes of exponential-phase cells. A cytochrome with an α-absorption maximum at 556 nm in the reduced form and a midpoint potential of −45 mV was obtained as a homogeneous preparation. A complex containing cytochromes b_{555} and b_{562} was also isolated. This complex contained cytochrome *o*, and it was suggested that cytochromes *o* and b_{555} were identical. Using the procedure of Kita et al. (16), the cytochrome b_{555}-b_{562} complex was isolated from aerobically grown wild-type strain MR43L by ion-exchange chromatography on DEAE-Sepharose as described in Materials and Methods (it was found that this procedure worked much less satisfactorily with other strains). The second chromatography step of Kita et al. was omitted so that we should have sufficient material for redox titration. The dithionite-reduced minus ferricyanide-oxidized difference spectrum of the complex is shown in Fig. 4a. Well-separated maxima at 555.5 and 562 nm were observed in the α-band region. Cytochromes b_{558}, a_1, and *d* were absent from the preparation. The carbon monoxide difference spectrum (Fig. 4b) showed maxima characteristic of cytochrome *o* at 416, 535, and 567 nm. Minima at 433 and 476 nm were present. The minimum at 476 nm was not observed in the spectrum of the membrane-bound cytochrome *o* (Fig. 2). Using published extinction coefficients, it was calculated that approximately one-third of the *b*-type cytochrome in the complex was cytochrome *o*.

Potentiometric titration of the cytochrome b_{555}-b_{562} complex revealed three components of midpoint potentials of +196, +129, and −43 mV, contributing 43.7, 38.6, and 17.7%, respectively, to the absorbance (Fig. 5). At certain electrode potentials samples were removed under nitrogen from the titration mixture and frozen at 77°K, and their spectra were measured with a fully oxidized sample (without mediators) as the reference (Fig. 6). The difference spectra showed that cytochrome b_{562} was reduced to a

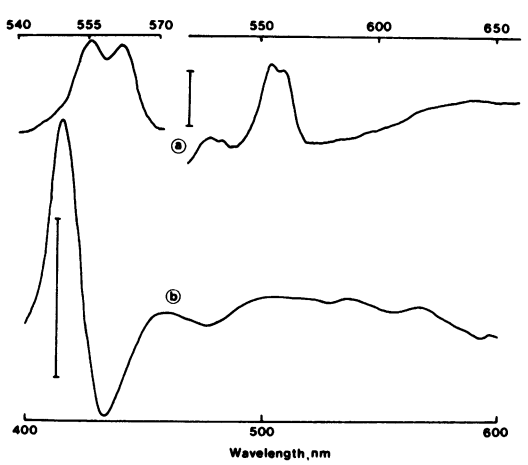

FIG. 4. Cytochromes of cytochrome b_{555}-b_{562} complex. a, Dithionite-reduced minus ferricyanide-oxidized difference spectrum measured at 77°K (protein, 0.5 mg/ml); b, carbon monoxide difference spectrum (protein, 1.1 mg/ml). Bar, Absorbance difference of 0.03 unit.

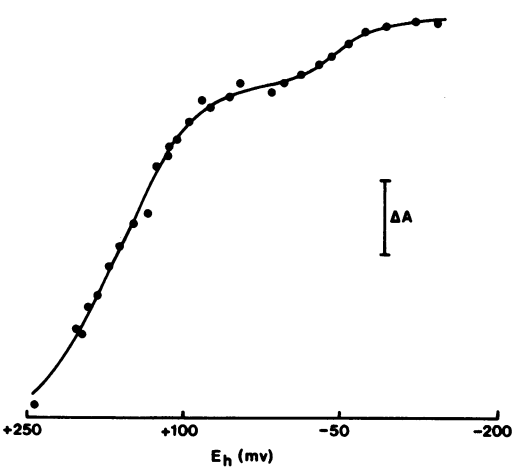

FIG. 5. Redox titration of cytochromes of cytochrome b_{555}-b_{562} complex. The complex (3.0 mg of protein per ml) in 0.1 M potassium phosphate buffer, pH 7.0, was titrated in the presence of 30 μM diaminodurene and 5 μM ruthenium hexamine as mediators. The solid line is the theoretical line for a three-component fit for components of midpoint potentials +196, +129, and −43 mV, contributing 43.7, 38.6, and 17.7%, respectively, to the absorbance (A).

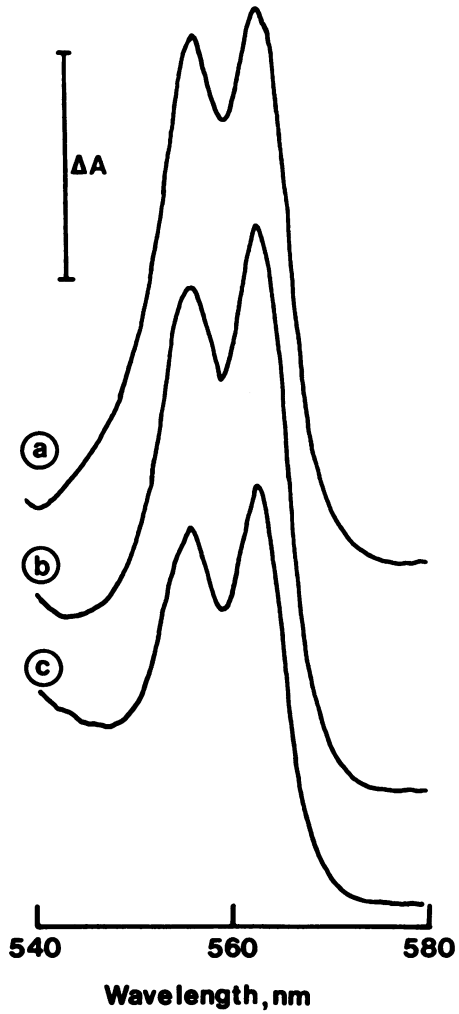

FIG. 6. Difference spectra at 77°K of cytochromes of the cytochrome b_{555}-b_{562} complex poised at different electrode potentials. Samples were removed under nitrogen at the indicated potentials during the course of a redox titration of the complex and frozen at 77°K, and the spectra were measured against a fully oxidized sample (without mediators) as the reference. The redox titration was carried out with the mediator mixture described in the text. a, Dithionite-reduced minus oxidized difference spectrum; b, +160 mV minus oxidized difference spectrum; c, +183 mV minus oxidized difference spectrum. ΔA is 0.06 absorbance unit.

greater extent than was cytochrome b_{555} at the higher potentials. This suggested that cytochrome b_{562} was responsible for the midpoint potential of +196 mV and cytochrome b_{555} was responsible for that of +129 mV. The preparation used for titration was incompletely separated from some cytochrome b_{556}. This gave the component with a midpoint potential of −43 mV (17). In all of our preparations of this complex, the apparent ratio of the two major *b*-type cytochromes was approximately unity.

Properties of a nitrate reductase-formate dehydrogenase double mutant. It is a concern that fractionated cytochromes may be modified during their preparation in such a way that their midpoint potential is altered from its value in the membrane. Therefore, we wished to obtain a membrane preparation in which the cytochrome *o* pathway was the major respiratory pathway in order to compare this with the soluble complex. To achieve this, *fdhA chlI*::Tn*10* strain NH20, in which cytochromes b^{nr} and b^{fdh} were lacking, was constructed by P1 transduction of LCB517 (*fdhA*) to tetracycline resistance, using RK5274 (*chlI*::Tn*10*) as donor (see accompanying paper [11]). One of the transductants was shown to retain nitrate reductase activity with benzyl viologen as donor, but not with ascorbate and phenazine methosulfate, while lacking a formate oxidase activity. This strain, when grown anaerobically with nitrate, produced primarily the cytochromes of the cytochrome *o* pathway. The dithionite-reduced minus ferricyanide-oxidized difference spectrum showed a major α-band maximum at 555 nm, with a pronounced shoulder on the peak at 562 nm (Fig. 7a). A small amount of cytochrome *d* (maximum, 625 nm; minimum, 646 nm) was also present. This spectrum is similar to that of aerobically grown exponential-phase wild-type cells. Cytochrome

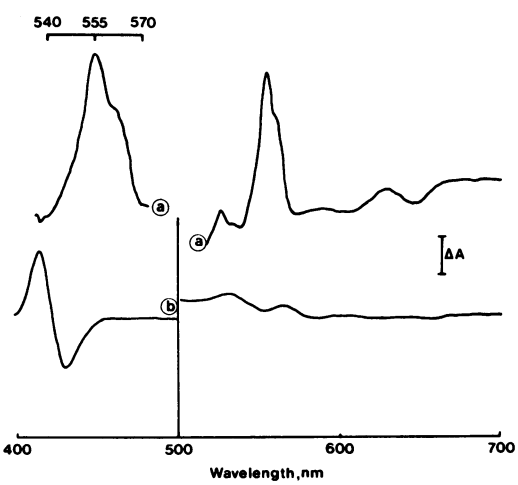

FIG. 7. Cytochromes of membranes of NH20 grown anaerobically with nitrate. a, Dithionite-reduced minus ferricyanide-oxidized difference spectrum measured at 77°K (protein, 3.9 mg/ml; Δ*A*, 0.03 U); b, carbon monoxide difference spectrum (protein, 6.8 mg/ml; Δ*A*, 0.03 U in α-band region and 0.1 U in Soret band region).

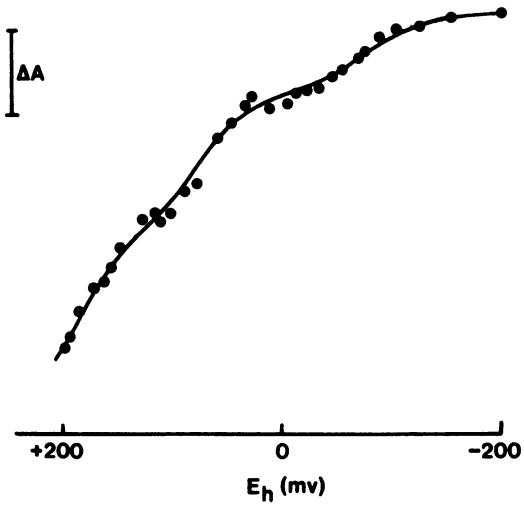

FIG. 8. Redox titration of cytochromes in membranes of NH20 grown anaerobically with nitrate. The membranes (6.9 mg of protein per ml) were titrated as described in the text. The solid line is the theoretical line for a three-component fit for components of midpoint potentials +194, +80, and −69 mV, contributing 47.5, 33.3, and 19.2%, respectively, to the absorbance (*A*). Bar, Absorbance difference of 0.009 U.

o was also present (Fig. 7b). The stoichiometry of cytochrome *o* to total *b*-type cytochrome was calculated to be about 1:2.

A redox titration of membranes from NH20 grown anaerobically with nitrate (Fig. 8) was resolved into three components with midpoint potentials of +194, +80, and +69 mV, contributing 47.5, 33.3, and 19.2% of the total absorbance, respectively. These are likely due to cytochromes b_{562}, b_{555}, and b_{556}, respectively, although these values differ to some extent from those found with the soluble complex. These results indicate that cells grown anaerobically with nitrate also form cytochromes characteristic of the aerobic respiratory chains.

DISCUSSION

In the present paper the technique of redox titration has been coupled to spectroscopic techniques, enzyme fractionation, and the use of mutants to examine the cytochrome composition of membranes from cells grown aerobically or anaerobically with nitrate. A combination of techniques was found to be necessary to resolve the cytochromes since no individual method gave complete or unambiguous results. Difference spectroscopy was an unsatisfactory method by which to resolve multiple *b*-type cytochromes. For example, in the membranes of cells grown anaerobically with nitrate it failed to demonstrate the presence of the cytochromes of the aerobic pathway. Therefore, spectrophotometric redox titration was used as an alternative approach.

Although previous applications (14, 19, 20, 27) of this technique to the cytochromes of *E. coli* have not led to a consensus on the number and properties of the cytochromes present, there is reason to believe that the data obtained in the present study are valid. In a three-component analysis of the cytochromes of cells grown aerobically to the exponential phase, we found cytochromes of midpoint potentials +195, +69, and −100 mV. These correspond well, in terms of both potential and relative amount, to the species of midpoint potentials +186, +57, and −105 mV reported by Hendler and Schrager (14). Since their values were obtained with a rather different system from that reported here, the agreement of the results supports the validity of both approaches. However, the present data differ slightly from those of van Wielink et al. (27). In our four-component analyses of the cytochromes in the cell membrane of aerobically grown cells, species of potentials +200, +80, 0, and −140 mV were found. These differ from the values of van Wielink et al. (+194, +122, +27, and −76 mV), particularly in the low-potential region. This is partly due to the different compositions of the membranes used in the two studies. The cytochromes in the experiments of van Wielink et al. were fully reduced at −100 mV, whereas about 15% of the cytochrome was still oxidized at this potential in our experiments. The amount of cytochrome of potential $<+100$ mV was dependent on the strain used and on the growth conditions. For example, the usually high activity of formate dehydrogenase in our preparation is likely due to the inclusion of ammonium molybdate and selenous acid in our growth media. Formate dehydrogenase is accompanied by a low-potential cytochrome (see accompanying paper [11]).

The redox titration results showed that irrespective of the growth conditions at least four cytochromes were present in the membrane. Thus, a four-component analysis compared with that for one, two, or three components in most cases gave a much better fit of the results. The closeness of fit was not markedly improved by analyzing for greater than four components. The value for the midpoint potentials and the relative amounts of each were sensitive to the value assigned to the number of components present. For example, the three-component analysis of the cytochromes of cells grown anaerobically with nitrate suggested the existence of species of potentials +166, +16, and −104 mV, whereas the four-component analysis indicated species of potentials +197, +99, −3, and −113 mV.

van Wielink et al. (27) have attempted to

improve the resolution of the redox potential technique by linking it to measurements of the spectra obtained at selected potentials. This required the use of a curve-fitting program to resolve the components contributing to the peak observed in the spectrum. Unfortunately, this technique also depends on which value is chosen for the number of components expected to be present. With this procedure, four major components were detected with absorption maxima at 77°K of 555.7, 556.7, 558. 6, and 563.5 nm and with midpoint potentials of +46, +174, −75, and +187 mV, respectively.

Our approach to improving the resolution of the redox titration method has been to use subfractions of the respiratory chain. Reid and Ingledew (21), Miller and Gennis (Fed. Proc. **41**:3653, 1982), and Kita et al. (15–17), using detergents, have separated the respiratory chain of aerobically grown *E. coli* into three fractions which have been characterized by the absorption maxima of the cytochromes measured in reduced-minus-oxidized difference spectra at 77°K. These fractions are (i) cytochrome b_{556}, with a midpoint potential of −45 mV; (ii) the cytochrome b_{555}-cytochrome b_{562} complex in which cytochrome b_{555} may be cytochrome *o*; and (iii) the cytochrome b_{558}-cytochrome *d* complex. Redox titration of the cytochrome b_{555}-cytochrome b_{562} complex gave midpoint potentials of +129 and +196 mV for these two cytochromes. Since our complex was contaminated by a small amount of cytochrome b_{556}, we were also able to confirm its potential of −45 mV.

Our data are consistent with the suggestion of Kita and Anraku (15) that cytochromes b_{556}, b_{555}, and b_{562} constitute the high-aeration quinol oxidase pathway. However, the finding that cytochrome b_{562} (E_m, +196 mV) has a higher midpoint potential than cytochrome b_{555} (E_m, +129 mV) suggests that the order of electron transfer should be from cytochrome b_{556} to b_{555} to b_{562} and not that suggested by these authors. Neither do we feel that either of the species b_{562} or b_{555} can be equated to cytochrome *o*. With our preparation the hydrogen peroxide-oxidized minus dithionite-reduced difference spectrum was not significantly altered when the reduced sample was bubbled with carbon monoxide. This confirms the suggestion of Scott and Poole (24) that cytochrome *o* may have a broad α-band which is exceptionally small in relation to its Soret band. If this is correct, the sequence of electron transfer would be: cytochrome $b_{556} \rightarrow b_{555} \rightarrow b_{562} \rightarrow o \rightarrow O_2$.

Use of the double mutant NH20 (*fdhA chlI*::Tn*10*) allowed us to examine the other cytochromes present in the membranes of cells grown anaerobically with nitrate without interference from the major cytochromes b^{fdh} and b^{nr}. This strain produced cytochromes with properties similar to those of the purified cytochrome b_{555}-b_{562} complex. The slight differences are attributed to the presence of cytochrome b_{558} in the membranes of NH20. This cytochrome is normally repressed by anaerobic growth with nitrate except in certain *chl* mutants such as *chlI* (see 11). The occurrence of this cytochrome is deduced from the presence of cytochrome *d* and from the indistinct separation of cytochromes b_{555} and b_{562} in the difference spectrum. In a previous study (19), we found that cytochrome b_{558} had a midpoint potential of +196 mV. Thus, it would not be separated from cytochrome b_{562} (E_m, +196 mV) in the redox titrations of the membranes of NH20. The apparent shift in the potential of cytochrome b_{555} from +80 mV, as found in the membranes of NH20, to +129 mV in the purified complex may be due to environmental perturbation of the heme caused by solubilization or to the presence of an additional, unrecognized *b*-type cytochrome in the membranes of NH20.

The data on NH20 confirm the suggestion of Sanchez-Crispin et al. (23) that both aerobic and anaerobic respiratory pathways occur together in the membranes of cells grown anaerobically with nitrate. In fact, our analysis is consistent with the presence in these membranes of at least six *b*-type cytochromes. These are cytochromes b^{nr} (E_m, +120 mV and +20 mV; 10), b^{fdh} (E_m, −110 mV; see 11), b_{556} (E_m, −45 mV; 17), b_{555} (E_m, +130 mV), and b_{562} (E_m, +196 mV). In addition, cytochrome *o* is present and may not be identical to any of these cytochromes. Cytochrome b_{558} (and cytochrome *d*) is usually repressed by the presence of nitrate and is not normally found except in certain *chl* mutants (see accompanying paper [11]).

We are presently unable to relate our data to the conclusions of van Wielink et al. (27), although it is likely that cytochrome b_{562} (E_m, +196 mV) is identical to their cytochrome $b_{563.5}$ (E_m, +187 mV).

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LITERATURE CITED

1. **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.
2. **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.

3. **Daniel, R. M.** 1970. The electron transport system of *Acetobacter suboxydans* with particular reference to cytochrome *o*. Biochim. Biophys. Acta **216**:328–341.
4. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
5. **Deeb, S. S., and L. P. Hager.** 1964. Crystalline cytochrome b_1 from *Escherichia coli*. J. Biol. Chem. **239**:1024–1031.
6. **Dixon, W. J.** 1973. BMD biomedical computer programs. University of California Press, Los Angeles.
7. **Dutton, P. L.** 1978. Redox potentiometry: determination of midpoint potentials of oxidation-reduction components of biological electron-transfer systems. Methods Enzymol. **54**:411–435.
8. **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.
9. **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.
10. **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.
11. **Hackett, N. R., and P. D. Bragg.** 1983. Membrane cytochromes of *Escherichia coli chl* mutants. J. Bacteriol. **154**:719–727.
12. **Haddock, B. A., J. A. Downie, and P. B. Garland.** 1976. Kinetic characterization of the membrane-bound cytochromes of *Escherichia coli* grown under a variety of conditions by using a stopped-flow dual-wavelength spectrophotometer. Biochem. J. **154**:285–294.
13. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47–99.
14. **Hendler, R. W., and R. I. Shrager.** 1979. Potentiometric analysis of *Escherichia coli* cytochromes in the optical absorbance range of 500 nm to 700 nm. J. Biol. Chem. **254**:11288–11299.
15. **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.
16. **Kita, K., M. Kasahara, and Y. Anraku.** 1982. Formation of a membrane potential by reconstituted liposomes made with cytochrome b_{562}-*o* complex, a terminal oxidase of *Escherichia coli* K 12. J. Biol. Chem. **257**:7933–7935.
17. **Kita, K., I. Yamato, and Y. Anraku.** 1978. Purification and properties of cytochrome b_{556} in the respiratory chain of aerobically grown *Escherichia coli* K12. J. Biol. Chem. **253**:8910–8915.
18. **Mitchell, P.** 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. **41**:445–502.
19. **Pudek, M. R., and P. D. Bragg.** 1976. Redox potentials of the cytochromes of the respiratory chain of aerobically grown *Escherichia coli*. Arch. Biochem. Biophys. **174**:546–552.
20. **Reid, G. A., and W. J. Ingledew.** 1979. Characterization and phenotypic control of the cytochrome content of *Escherichia coli*. Biochem. J. **182**:465–472.
21. **Reid, G. A., and W. J. Ingledew.** 1980. The purification of a respiratory oxidase complex from *Escherichia coli*. FEBS Lett. **109**:1–4.
22. **Ruiz-Herrera, J., and J. A. De Moss.** 1969. Nitrate reductase complex of *Escherichia coli* K-12: participation of specific formate dehydrogenase and cytochrome b_1 components in nitrate reduction. J. Bacteriol. **99**:720–729.
23. **Sanchez-Crispin, J. A., M. Dubourdieu, and M. Chippaux.** 1979. Localization and characterization of cytochromes from membrane vesicles of *Escherichia coli* K-12 grown in anaerobiosis with nitrate. Biochim. Biophys. Acta **547**:198–210.
24. **Scott, R. I., and R. K. Poole.** 1982. A re-examination of the cytochromes of *Escherichia coli* using fourth-order finite difference analysis: their characterization under different growth conditions and accumulation during the cell cycles. J. Gen. Microbiol. **128**:1685–1696.
25. **Shipp, W. S.** 1972. Cytochromes of *Escherichia coli*. Arch. Biochem. Biophys. **150**:459–472.
26. **Stouthamer, A. H., J. Van't Riet, and L. F. Oltmann.** 1980. Respiration with nitrate as acceptor, p. 19–48. *In* C. J. Knowles (ed.), Diversity of bacterial respiratory systems, vol. 2. CRC Press, Inc., Boca Raton, Fla.
27. **van Wielink, J. E., L. F. Oltmann, F. J. Leeuwerik, J. A. De Hollander, and A. H. Stouthamer.** 1982. A method for in situ characterization of *b*- and *c*-type cytochromes in *Escherichia coli* and in Complex III from beef heart mitochondria by combined spectrum deconvolution and potentiometric analysis. Biochim. Biophys. Acta **681**:177–190.