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Potentiometric Analysis of the Cytochromes of an *Escherichia coli* Mutant Strain Lacking the Cytochrome *d* Terminal Oxidase Complex

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A combination of potentiometric analysis and electrochemically poised low-temperature difference spectroscopy was used to examine a mutant strain of *Escherichia coli* that was previously shown by immunological criteria to be lacking the cytochrome *d* terminal oxidase. It was shown that this strain is missing cytochromes *d*, a_1, and b_{558} and that the cytochrome composition of the mutant is similar to that of the wild-type strain grown under conditions of high aeration. The data indicate that the high-aeration branch of the respiratory chain contains two cytochrome components, b_{556} (midpoint potential [E_m] = +35 mV) and cytochrome *o* (E_m = +165 mV). The latter component binds to CO and apparently has a reduced-minus-oxidized split-α band with peaks at 555 and 562 nm. When the wild-type strain was grown under conditions of low aeration, the components of the cytochrome *d* terminal oxidase complex were observed: cytochrome *d* (E_m = +260 mV), cytochrome a_1 (E_m = +150 mV) and cytochrome b_{558} (E_m = +180 mV). All cytochromes appeared to undergo simple one-electron oxidation-reduction reactions. In the absence of CO, cytochromes b_{558} and *o* have nearly the same E_m values. In the presence of CO, the E_m of cytochrome *o* is raised, thus allowing cytochromes b_{558} and *o* to be individually quantitated by potentiometric analysis when they are both present.

The membrane-bound cytochromes of *Escherichia coli* which participate in the aerobic respiratory chain have been subject to considerable biophysical characterization. Fourth-order finite difference spectroscopy resolves a total of seven cytochromes (18, 19), including five *b*-type cytochromes and cytochromes a_1 and *d*. Potentiometry has been utilized by several laboratories to resolve the various *b* cytochromes further (5–7, 15–17, 21). The results from different laboratories are often difficult to compare, probably in large part because of differences in cell growth conditions. When the cells are grown under conditions of oxygen limitation, i.e., harvested in the late logarithmic or stationary phase, cytochromes b_{558}, a_1, and *d* are present (15, 18). It has recently been demonstrated that all three of these cytochromes are part of the cytochrome *d* terminal oxidase complex (13). When cells are grown with high aeration, i.e., harvested in the early log phase, cytochrome *o* is present. Cytochromes *d* and *o* bind carbon monoxide (1, 17). Cytochrome b_{556} appears to be present under both growth conditions.

The goal of this paper is to use potentiometry to identify each cytochrome present in aerobically grown *E. coli*. The strains employed for this work include wild-type strains, MR43L and GR17N, grown under low and high aeration; an F-prime plasmid-carrying strain, MR43L(F152), which overproduces the cytochrome *d* complex by two- to threefold (21); and a mutant strain, GR19N, which does not produce the cytochrome *d* complex under any growth conditions (4). Strain MR43L(F152) offers the additional advantage of having very low levels of cytochrome *o* when grown under low aeration (11).

The cytochromes in these strains have been studied by potentiometry and electrochemically poised difference spectroscopy in both the absence and presence of carbon monoxide. The results obtained by potentiometry are compared with those from other laboratories and are discussed in terms of recent biochemical and immunological characterizations of the respiratory chain components (4, 10–12).

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MATERIALS AND METHODS

Materials. Aldrich Chemical Co. supplied duroquinone, ferrocene, benzoquinone, 2-hydroxy-1,4-naphthoquinone, and 5-hydroxy-1,4-naphthoquinone. Quinhydrone, 1,2-naphthoquinone, anthraquinone-2-sulfonate, and sodium dithionite were obtained from Eastman Kodak Co. Benzyl viologen, phenazine methosulfate, phenazine ethosulfate, and dimethylphenylenediamine were purchased from Sigma Chemical Co. Potassium ferricyanide was supplied by Mallinckrodt, Inc.

Bacterial strains. All strains were derived from *E. coli* K-12. The strains MR43L (20), MR43L(F152) (20), GR19N (4), and GR17N (4) have been fully described.

Growth conditions. Cells were grown at 37°C on the basal medium of Cohen and Rickenberg (2) supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1.5 g of Casamino Acids (Difco Laboratories) per liter, and 5 g of DL-sodium lactate per liter.

Two types of growth conditions were used. Cells grown under conditions of high aeration in a 38-liter New Brunswick fermentor were harvested in the early exponential growth phase. The air flow rate was 25 liters per min, and the agitation was 350 rpm. Cells were grown under conditions of low aeration by using an air flow rate of 5 liters per min and an agitation of 200 rpm and by harvesting 5 h into the stationary phase. The inoculum was a 100-ml culture grown overnight in Penassay broth.

Preparation of membranes, protein determination, and room-temperature difference spectroscopy. Procedures for membrane preparation, protein determination, and room-temperature difference spectroscopy have been described previously (4).

Potentiometric titrations. For chemical titrations, the method of Dutton (3) was used with minor modifications.

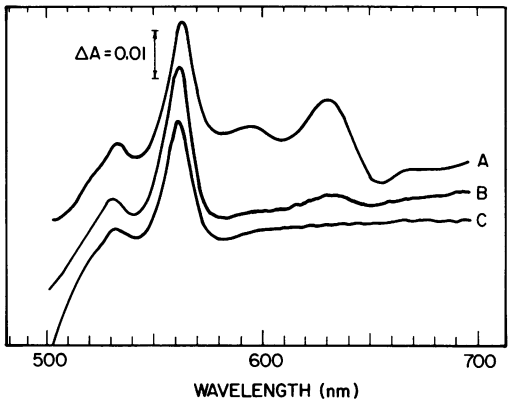

FIG. 1. Dithionite-reduced-minus-ferricyanide-oxidized difference spectra of *E. coli* membrane vesicles recorded at room temperature. (A) Strain MR43L(F152) grown with low aeration and harvested in the stationary phase (0.7 mg of protein per ml); (B) strain MR43L grown with high aeration and harvested in the early exponential phase (3.4 mg of protein per ml); (C) cytochrome *d* mutant GR19N grown with low aeration and harvested in the stationary phase (2.0 mg of protein per ml).

Membrane vesicles were suspended in 0.1 M potassium phoshate buffer, pH 7.0, and contained approximately 10 μM *b*-type cytochromes quantitated by using $\Delta\varepsilon_{red\text{-}ox}$ = 17.5 per mM per cm for the wavelength pair 560 and 575 nm (4). Titrations of 7.0-ml samples of this solution were done at 22°C in a cuvette (path length, 1 cm) equipped with argon or carbon monoxide input and output lines and with side arms for a platinum wire electrode, a mechanical stirrer, and a salt bridge (diameter, 1 mm) leading to a calomel reference electrode. The redox mediators used at a final concentration of 4 μM were quinhydrone (midpoint potential [E_m] = +280 mV), 1,2-naphthoquinone-4-sulfonate (E_m = +215 mV), 1,2-naphthoquinone (E_m = +143 mV), phenazine methosulfate (E_m = +10 mV), 2-hydroxy-1,4-naphthoquinone (E_m = −145 mV), and anthraquinone-2-sulfonate (E_m = −225 mV). To reach potentials above +330 mV, 100 μM potassium ferricyanide (E_m = +430 mV) was required. Oxygen was removed by passing argon or carbon monoxide (Linde, Union Carbide Corp.) over the solution while stirring for 50 min. All chemical titrations were done in the reductive direction. Small amounts of sodium dithionite (10 mM) were added to the cuvette with a Hamilton syringe through a side arm, which was also used for extracting samples for poised low-temperature spectra. Room-temperature spectra were scanned from +400 or +300 mV to −200 mV every 15 to 20 mV by using the split-beam mode of an Aminco DW2 spectrophotometer with a 1.5-nm slit width.

Electrodic titrations in both the oxidative and reductive directions were performed with an electrodic potentiometric system (J. G. Koland, Ph.D. thesis, University of Illinois, Urbana, 1983). KCl (0.2 M) was included in the titration samples, as were the following mediators at a concentration of 5 μM each: dimethylphenylenediamine (E_m = +371 mV), benzoquinone (E_m = +270 mV), 1,2-naphthoquinone (E_m = +143 mV), phenazine methosulphate (E_m = +80 mV), phenazine ethosulphate (E_m = +55 mV), 5-hydroxy-1,4-naphthoquinone (E_m = −5 mV), and 2-hydroxy-1,4-naphthoquinone (E_m = −139 mV). Benzyl viologen (E_m = −350 mV) and ferrocene (E_m = +420 mV) were included at a final concentration of 50 μM.

Data analysis. By the use of an LSI-11 minicomputer (Digital Equipment Co.), each spectrum from the titration was recorded with an Aminco DW2 spectrophotometer and then was stored, along with the cell potential, on a magnetic disk. Fractional reduction of cytochromes *d*, a_1, and *b* were found by measuring A_{628} minus A_{603}, A_{595} minus A_{603}, and A_{560} minus A_{579}, respectively. Plots of fractional reduction versus cell potential were fitted to various numbers of components, assuming Wernstian behavior with n = 1.0.

Poised low-temperature difference spectroscopy. To achieve the better signal-to-noise ratios required for low-temperature spectroscopy, both the cytochrome and media-

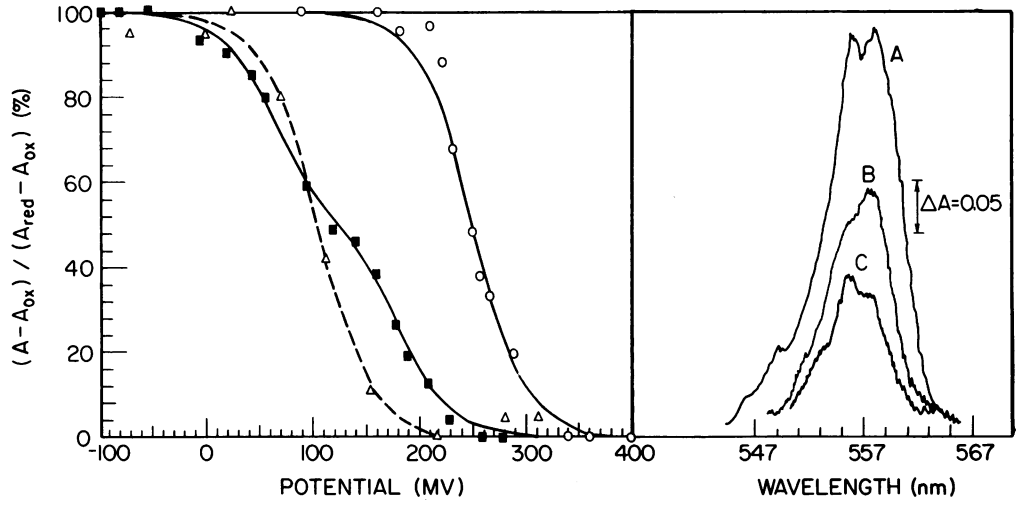

FIG. 2. Characterization by potentiometric analysis and by poised low-temperature (77 K) difference spectroscopy, each performed in the absence of carbon monoxide, of the membrane-bound cytochromes of strain MR43L(F152) grown to the stationary phase. In the chemical titration shown here, one-component fits of cytochrome *d* (○) and cytochrome a_1 (△) generated E_m values of +252 and +110 mV, respectively. A two-component fit for the *b*-type cytochromes (■) generated E_m values of +180 and +63 mV. Fractional reduction of the *d*, a_1, and *b*-type cytochromes was determined by measurement of A_{628} minus A_{603}, A_{595} minus A_{603}, and A_{560} minus A_{579}, respectively. The α peak of the *b*-type cytochromes was analyzed by poised low-temperature difference spectra: (A) fully reduced minus oxidized; (B) +140 mV minus oxidized; (C) fully reduced minus +80 mV.

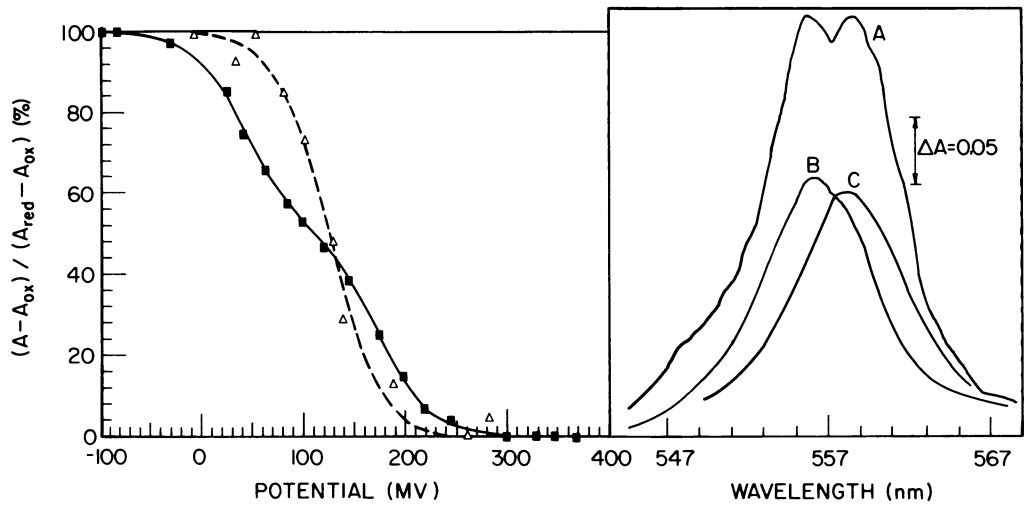

FIG. 3. Characterization by potentiometric analysis and by poised low-temperature (77 K) difference spectroscopy, each performed in the presence of carbon monoxide, of the membrane-bound cytochromes of strain MR43L(F152) grown to the stationary phase. In a typical titration shown above, cytochrome *d* was found to be fully reduced at potentials in excess of 400 mV. With a one-component fit, cytochrome a_1 (△) was titrated with an E_m of +125 mV. E_m values of +174 and +44 MV were generated by a two-component fit of the *b*-type cytochromes (■). Fractional reduction of the cytochromes was determined as stated in the legend to Fig. 2. The α peak of the *b*-type cytochromes was analyzed by poised difference spectra at 77 K: (A) fully reduced minus oxidized; (B) fully reduced minus +100 mV; (C) +120 mV reduced minus oxidized.

tor concentrations were increased fivefold over the concentrations used for the titration experiments described above. The Amino DW2 low-temperature accessory was used with minor modifications. At various potentials along the titration, samples (0.5 ml) were removed with a 1-ml gas-tight Hamilton syringe and were placed in an argon-flushed sample compartment fitted with a rubber stopper so as to maintain the anaerobic state. In the other compartment was placed a titration sample fully reduced with dithionite or oxidized with ferricyanide. Samples were then frozen in liquid nitrogen (77 K) and analyzed by difference spectroscopy in the region of 560 nm with the DW2 spectrophotometer.

RESULTS

Room-temperature difference spectroscopy. Figure 1 shows the dithionite-reduced-minus-ferricyanide-oxidized spectra of membranes from the three strains used in this work. Strain MR43L(F152) had high quantities of cytochromes *d* (peak at 628 nm) and a_1 (peak at 595 nm) when grown to the

TABLE 1. Summary of the potentiometric analysis of the membrane-bound cytochromes of the *E. coli* cytochrome *d* mutant GR19N and the wild-type strains MR43L, MR43L(F152), and GR17N.

Strain	Growth phase	Type of titration	CO present	No. of trials	Cytochrome E_m (mV ± SD)[a]				
					b_{558}	b_{556}	*o*	a_1	*d*
MR43L(F152)	Stationary	Chemical	No	6	182 ± 8 (60)	50 ± 15 (40)		150 ± 8	247 ± 8
		Electrodic	No	3	184 ± 9 (63)	30 ± 8 (37)		160 ± 20	260 ± 10
		Chemical	Yes	4	185 ± 10 (60)	20 ± 15 (40)		125 ± 14	>400
MR43L	Stationary	Chemical	No	8	185 ± 5 (?)	32 ± 5 (40)	185 ± 5 (?)	140 ± 6	247 ± 8
			Yes	3	190 ± 10 (41)	57 ± 10 (40)	>400[b] (15)	175 ± 15	>400
GR17N	Stationary	Chemical	No	3	205 ± 15 (?)	60 ± 12 (50)	205 ± 15 (?)	180 ± 14	235 ± 12
			Yes	2	190 ± 14 (45)	70 ± 14 (40)	>400[b] (15)	180 ± 15	>400
MR43L	Early log	Chemical	No	4		17 ± 12 (67)	150 ± 14 (33)		
		Electrodic	No	3		51 ± 10 (65)	181 ± 10 (35)		
		Chemical	Yes	5		10 ± 27 (72)	335 ± 16 (28)		
Mixture of MR43L(F152) and MR43L[c]		Chemical	No	4	~200 (?)	42 ± 10 (60)	~200 (?)	122 ± 15	271 ± 8
			Yes	3	167 ± 8 (35)	20 ± 15 (50)	350 ± 12 (15)	120 ± 9	>400
GR19N	Stationary	Chemical	No	5		20 ± 18 (67)	170 ± 14 (33)		
			Yes	4		21 ± 11 (74)	310 ± 18 (26)		

[a] Value in parentheses shows percentage of total α peak.
[b] The anaerobic cuvette was flushed with a high carbon monoxide flow rate.
[c] A 1:1 mixture of stationary-phase MR43L(F152) and early-log-phase MR43L was formed on the basis of the α peak of the *b*-type cytochromes.

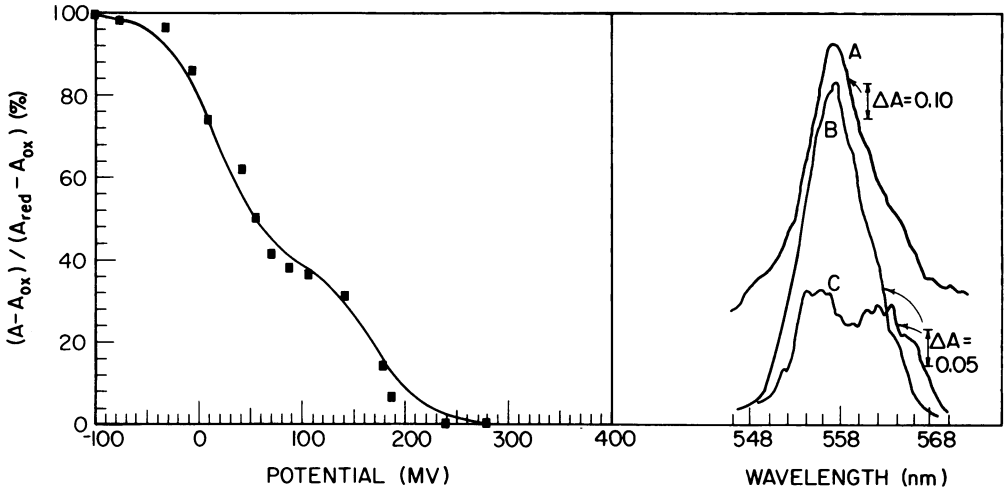

FIG. 4. Characterization by potentiometric analysis and by poised low-temperature difference spectroscopy, each performed in the absence of carbon monoxide, of the membrane-bound *b*-type cytochromes of strain MR43L grown to the early log phase. A two-component fit of the above titration yielded E_m values of +160 and +17 mV. Fractional reduction of the *b*-type cytochromes was found by measuring A_{560} to A_{579}. The α peak was further characterized by poised spectra at 77 K: (A) fully reduced minus oxidized; (B) fully reduced minus +50 mV; (C) +180 mV minus oxidized.

stationary phase. Also shown is the spectrum from strain MR43L grown to early exponential phase so as to yield low levels of cytochromes *d* and a_1. Spectrum C in Fig. 1 shows that mutant strain GR19N lacked both cytochromes *d* and a_1. Further analysis of the *b*-type cytochromes required the application of potentiometry as detailed below.

Potentiometric analysis of stationary-phase strain MR43L(F152). Identical results for the potentiometric analysis of the a_1 and *b*-type cytochromes in strain MR43L(152) were observed for titrations in the absence (Fig. 2) and in the presence (Fig. 3) of carbon monoxide. In each case, cytochrome a_1 gave a one-component fit with an E_m of approximately +150 mV. Two components were found for the *b*-type cytochromes and were further characterized on the basis of poised low-temperature difference spectroscopy. The high-potential component (E_m = +180 mV) and the low-potential component (E_m = +45 mV) were assigned to cytochromes b_{558} and b_{556} since they exhibited peak maxima at 77 K of 558 and 556 nm, respectively. In the absence of carbon monoxide, cytochrome *d* was titrated as a single component with an E_m of +250 mV (Fig. 2), whereas in the presence of CO, its E_m shifted to above +400 mV. Electrodic and chemical titrations gave identical results (Table 1).

Potentiometric analysis of early-exponential-phase strain MR43L. The *b*-type cytochromes of strain MR43L grown to early log phase were resolved into two species by potentiometric analysis. In both the absence and presence of carbon monoxide, the high-potential *b*-type cytochrome could be assigned to $b_{555,562}$ with a split-α spectrum (Fig. 4, curve C, and 5, curve C), and the low-potential component had an α

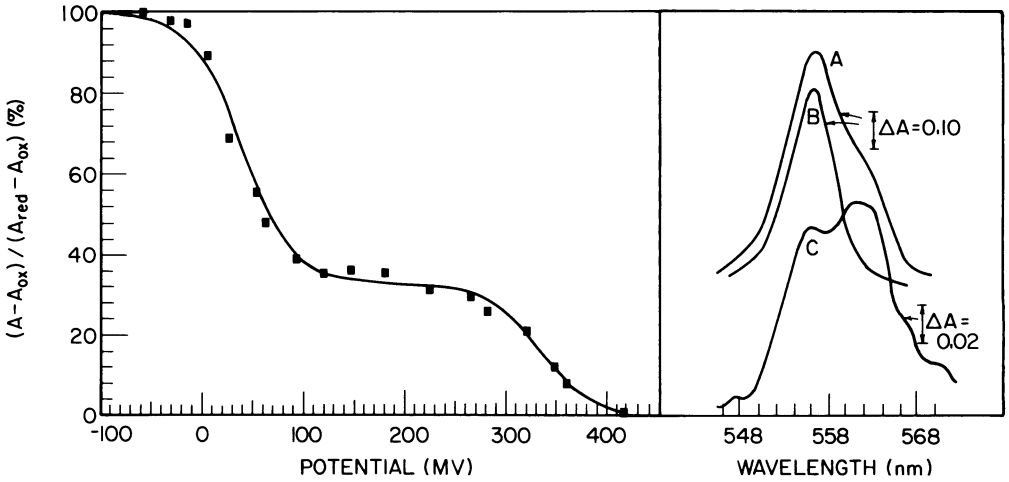

FIG. 5. Characterization by potentiometric analysis and by poised low-temperature difference spectroscopy, each performed in the presence of carbon monoxide, of the membrane-bound *b*-type cytochromes of strain MR43L grown to the early exponential phase. A two-component fit of the above titration yielded E_m values of +334 and +40 mV. Fractional reduction was found as described in the legend to Fig. 4. The α peak was characterized by poised spectra at 77 K: (A) fully reduced minus oxidized; (B) fully reduced minus +120 mV; and (C) +180 mV minus oxidized.

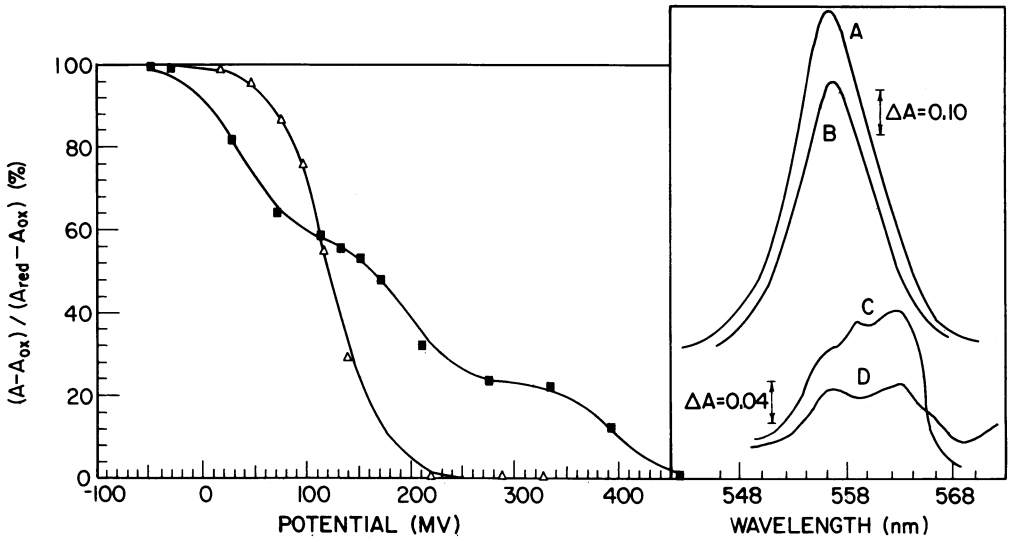

FIG. 6. Characterization of the cytochromes in a mixture (1:1 on the basis of α-peak absorbance in the reduced-minus-oxidized spectra) of strains MR43L(F152) grown to stationary phase and MR43L grown to early exponential phase. Both the potentiometric analysis and poised low-temperature difference spectroscopy were performed in the presence of carbon monoxide. Cytochrome *d* was found to be fully reduced at potentials in excess of +400 mV. By using a one-component fit, cytochrome a_1 (△) was found to be titrated with an E_m of +125 mV. E_m values of +396, +195, and +36 mV were generated by a three-component fit for the *b*-type cytochromes (■). Fractional reduction by the cytochromes was found as described in the legend to Fig. 2. The α peak of the *b*-type cytochromes was analyzed by poised difference spectra at 77 K: (A) fully reduced minus oxidized; (B) fully reduced minus +100 mV; (C) +150 mV minus oxidized; and (D) +240 mV minus oxidized.

peak at 556 nm (Fig. 4, curve B and Fig. 5, curve B). Carbon monoxide caused the $b_{555,562}$ component to be titrated at much higher potentials, whereas the b_{556} component remained unaffected by carbon monoxide. Electrodic and chemical titrations again gave identical results (Table 1).

Potentiometric analysis of a mixture of stationary-phase strain MR43L(F152) and early-exponential-phase strain MR43L in the presence of CO. To test the ability to differentiate the three *b*-type cytochromes, a mixture of stationary-phase strain MR43L(F152) and early-exponential-phase strain MR43L was titrated in the presence of carbon monoxide (Fig. 6). Titrations of cytochromes a_1 and *d* yielded results identical to those found for MR43L(F152) grown to stationary phase. Clear resolution of all three *b*-type cytochromes was obtainable with these titrations in the presence of carbon monoxide (Fig. 6). The cytochrome $b_{555,562}$ component was titrated at higher potentials, whereas the cytochrome b_{556} and b_{558} components were found to be unaffected by carbon monoxide.

Potentiometric analysis of strain GR19N. Potentiometric and poised spectral analyses of strain GR19N grown to the stationary phase (Fig. 7 and 8) indicated that the *b*-type cytochromes are nearly identical to those found in strain MR43L grown to the early exponential phase. The absence of cytochrome b_{558} was indicated both by the spectroscopic assignment of the high-potential component to only $b_{555,562}$

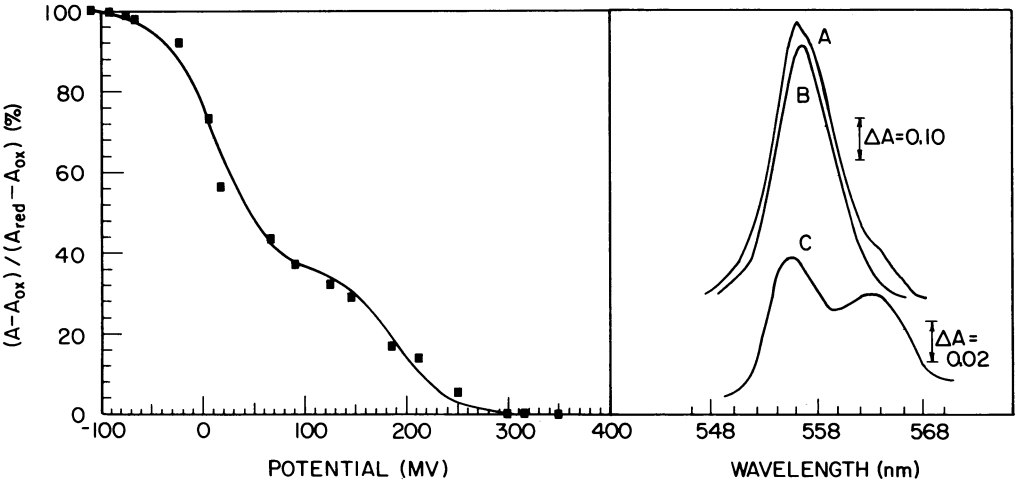

FIG. 7. Characterization by potentiometric analysis and by poised low-temperature difference spectroscopy, each performed in the absence of carbon monoxide, of the membrane-bound *b*-type cytochromes of strain GR19N grown to the stationary phase. A two-component fit of the above titration yielded E_m values of +190 and +12 mV. Fractional reduction of the *b*-type cytochromes was found by measuring A_{560} minus A_{579}. The α peak was further characterized by poised spectra at 77 K: (A) fully reduced minus oxidized; (B) fully reduced minus +40 mV; and (C) +180 mV minus oxidized.

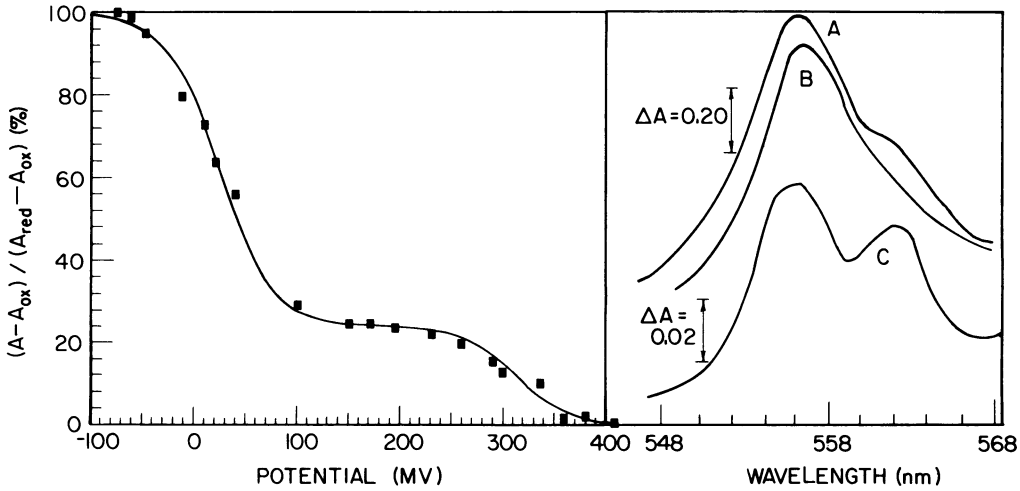

FIG. 8. Characterization by potentiometric analysis and by poised low-temperature difference spectroscopy, each performed in the presence of carbon monoxide, of the membrane-bound *b*-type cytochromes of GR19N grown to the stationary phase. A two-component fit of the above titration yielded E_m values of +314 and +25 mV. Fractional reduction was found as described in the legend to Fig. 7. The α peak was characterized by poised spectra at 77 K: (A) fully reduced minus oxidized; (B) fully reduced minus +100 mV; and (C) +100 mV minus oxidized.

(Fig. 7, curve C, and 8, curve C) and by the absence of a third cytochrome *b* component in the range typical of cytochrome b_{558} in the titration with carbon monoxide (Fig. 8).

Potentiometric analysis of strains MR43L and GR17N grown to stationary phase. The wild-type strain MR43L is not isogenic with mutant strain GR19N. To eliminate possible strain differences as a contributing factor in these experiments, strain GR17N, which is the isogenic parent of GR19N, was examined. Both MR43L and GR17N were grown to the stationary phase under conditions identical to those used for GR19N. Potentiometric titrations of both the wild-type strains yielded identical results (Table 1). In addition to cytochromes b_{556}, b_{558}, a_1, and *d*, some cytochrome *o* was also present.

Reduced-minus-carbon monoxide-reduced difference spectrum of early-exponential-phase strain MR43L. The split-α peak of this difference spectrum at 77 K (Fig. 9) reveals that both the 555- and 562-nm peaks can be assigned to cytochrome *o*.

DISCUSSION

Analysis of the potentiometric data, supported by poised low-temperature difference spectroscopy, lends itself to rather straightforward interpretation concerning the number and kind of *b* cytochromes found in the membranes. The low-potential cytochrome (E_m = +35 mV) was assigned on the basis of difference spectroscopy to cytochrome b_{556}. This component was present in wild-type cells grown with both high and low aeration and was also present in the mutant strain GR19N. Most likely, it represents a single biochemical species, corresponding to the cytochrome purified and characterized by Kita et al. (9). In strain GR19N, which lacks the cytochrome *d* complex, there was present a high-potential *b* cytochrome (E_m = +165 mV) which had absorption peaks in the reduced-minus-oxidized difference spectrum at 555 and 562 nm. In the presence of carbon monoxide, the E_m of this component shifted to higher values, i.e., the reduced form was stabilized by binding to carbon monoxide. Both cytochromes b_{555} and b_{562} were titrated as a single component in the presence or absence of carbon monoxide. For this reason, it is likely that this represents a single cytochrome with a split-α band, $b_{555\text{-}562}$, although one cannot rule out the possibility that this component represents two distinct cytochromes that are thermodynamically coupled. This component is identical to cytochrome *o* (8, 11, 12). The presence of carbon monoxide raised the E_m of cytochrome *o* to above 300 mV. Higher carbon monoxide flow rates apparently increased the concentration of carbon monoxide and further raised the midpoint of cytochrome *o* to above 400 mV. Hackett and Bragg (5) concluded that the b_{562} and b_{555} components were titrated with distinctly different E_m values, but the soluble preparation used was not pure, and their poised low-temperature spectra did not offer clear resolution of these cytochromes. Other workers (14) have concluded that only the b_{555} component binds to carbon monoxide, based on the spectral perturbation accompanying the binding of the ligand to the membranes. However, the data presented in Fig. 9 suggest that both the b_{555} and b_{562} components are affected by carbon monoxide binding. The question whether the b_{555} and b_{562} components represent a single cytochrome with a split-α absorbance band will be easily answered by a careful examination of the purified cytochrome *o* complex (8, 12).

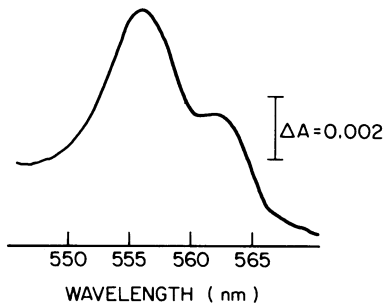

FIG. 9. Reduced-minus-carbon monoxide-reduced difference spectrum at 77 K of membranes from strain MR43L grown to the early exponential phase.

Cytochrome b_{558} is a component of the cytochrome *d* terminal oxidase complex (13) and has an E_m (+180 mV) quite close to that of cytochrome *o*. Since cytochrome b_{558} does not bind to carbon monoxide, it can be resolved from cytochrome *o* potentiometrically in the presence of carbon monoxide. It is clearly absent from both mutant strain GR19N and wild-type strains grown with high aeration and harvested in the early exponential phase of growth. The other components of the cytochrome *d* complex are cytochromes *d* (E_m = +260 mV) and a_1 (E_m = +150 mV). Carbon monoxide caused the E_m of cytochrome *d* to increase beyond +400 mV so that it was not oxidized under the conditions of the experiments. The cytochrome a_1 component was not affected owing to the presence of carbon monoxide, as was also observed by Reid and Ingledew (17). It should be noted that cytochrome a_1 is titrated as a single Nernstian component, and previous interpretations of two components (17) were probably due to complications owing to the titration of cytochrome *d*, whose spectrum overlaps that of cytochrome a_1 (J. G. Koland, M. J. Miller, and R. B. Gennis, Biochemistry, in press).

Other laboratories have in some cases reported the presence of low-potential cytochromes ($E_m < -50$ mV) in the membranes of aerobically grown *E. coli* (5, 6, 15, 17, 21). These were absent from the membrane preparations utilized in this work, which have been shown to have normal electron transport activity (4). Reid and Ingeldew have made a similar observation (17). The *b* cytochrome associated with formate dehydrogenase ($E_m \simeq -100$ mV) is not associated with the aerobic electron transport system but appears to be present in different amounts, depending on the strain utilized and the growth conditions (5).

In summary, the results in this work demonstrate that all the cytochromes of aerobically grown *E. coli* can be resolved by potentiometry in the presence of carbon monoxide and that cytochrome b_{558} is not present in the mutant strain *E. coli* GR19N. In addition, it has been shown that cytochrome *o* probably contains a single *b* cytochrome with a split-α absorption band in the reduced-minus-oxidized spectrum. It is evident that these methods will be useful in the analysis of those mutant strains of *E. coli* with defective respiratory chain components.

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