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Cytochrome Spectrum of an Obligate Anaerobe, *Eubacterium lentum*

JAY F. SPERRY* AND TRACY D. WILKINS

Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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An obligately anaerobic bacterium, *Eubacterium lentum*, was shown to contain cytochromes *a*, *b*, and *c* and a carbon monoxide-binding pigment. Extracts of cells grown with hemin gave a typical absorption spectrum for cytochrome *c* with maxima at 424, 525, and 553 nm. Extracts from cells grown in the absence of hemin also had an absorption peak corresponding to cytochrome *b* (562 nm) in their reduced versus oxidized spectrum. Extraction of hemes and formation of pyridine hemochromes allowed quantitation of protoheme IX and heme *c*. Large amounts of cytochrome *c* masked the presence of cytochrome *b* in cells grown in medium containing hemin. When cells were grown in the presence of 50 mM nitrate, cytochrome *a* (605 nm) was detected. In anaerobic extracts of cells grown either with or without nitrate, cytochromes *b* and *c* were reduced by formate and oxidized by NO_3. Cytochrome *a* appeared to be partially oxidized by NO_3 and completely oxidized by air.

Cytochromes have been described from a number of anaerobic bacteria (3, 4, 10–12, 18, 19, 21), and electron transport-associated phosphorylation (adenosine 5'-triphosphate generation) has been demonstrated in the obligate anaerobe *Desulfovibrio gigas* (17). However, cytochromes have not been found in many anaerobic bacteria. The clostridia have long been regarded as not containing cytochromes until a recent report of cytochrome *b* in *Clostridium formicoaceticum* and *Clostridium thermoaceticum* (6).

In this paper we report the occurrence of cytochromes in *Eubacterium lentum* and believe that this is the first demonstration of cytochromes occurring in bacteria of the genus *Eubacterium*. *E. lentum* was of interest to us because it comprises 5 to 10% of anaerobic isolates in hospitals (2), yet almost nothing is known about the organism. *E. lentum* also is unusual in dehydrogenating all three hydroxyl groups of cholic acid (15), and such reactions have been suggested to be of possible importance in colon cancer (7).

MATERIALS AND METHODS

Bacterial strain. The organism used in this study was the neotype strain of *E. lentum* (ATCC 25559, VPI 0255).

Cultural conditions. Stock cultures in chopped meat broth medium (9) were transferred under oxygen-free nitrogen every 2 months. The growth medium (TYG) contained (per liter): Trypticase (BBL), 10 g; glucose, 6 g; yeast extract (Difco), 2.5 g; cysteine-hydrochloride, 0.3 g; sodium formaldehyde sulfoxalate, 0.3 g; hemin (Sigma), 5 mg; menadione (Sigma), 0.5 mg; and sodium hydroxide to pH 7.0. Medium was also prepared with the above formula minus hemin and menadione (these flasks were washed in HCl to remove residual hemin). The medium was dispensed in 2.5-liter amounts per 3-liter Erlenmeyer flask, autoclaved (121 C, 15 min), flushed, and sealed under oxygen-free nitrogen. Other medium components were added as autoclave-sterilized (121 C, 15 min) concentrates immediately prior to inoculation. Initially, tubes containing 20 ml of medium were each inoculated with 4 drops of the stock culture and incubated at 37 C for 24 h. One 20-ml culture was added to each 2.5 liters of medium, and the large flasks were incubated for 24 h at 37 C. The cells were harvested aerobically by centrifugation at 6,000 × *g* for 10 min and washed once by resuspending the cells in 40 ml of 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4) and again centrifuging. The supernatant fluid was discarded, and the centrifuge tube containing the cell pellet was flushed with oxygen-free nitrogen, sealed, and stored at −10 C until used.

Cell disruption. Cell pellets (0.3 to 0.6 g, wet weight) were resuspended in 10 to 20 ml of 0.05 M Tris-hydrochloride buffer (pH 7.4) containing 10 mM $MgSO_4$. The cell suspension was either passed through a French pressure cell once at 16,000 lb/in^2 (aerobically) or the cell extracts were prepared anaerobically. The cells were resuspended in 20 ml of deoxygenated (boiled 2 to 3 min and cooled under oxygen-free nitrogen) Tris-hydrochloride buffer, pH 7.4, containing 10 mM $MgSO_4$ and added to a precooled (5 C) 50-ml Bronwill bottle containing 10 g of 0.11-mm glass beads (B. Braun Melsungen Aparate-

bau) inside an anaerobic chamber (1). The glass bottle was sealed with a glass stopper having a fine coat of high-vacuum grease (Dow Corning). The bottle was then removed from the anaerobe chamber and shaken twice at 4,000 rpm for 1-min periods in a Bronwill MSK cell homogenizer. The glass bottle was returned to the anaerobe chamber, and the cell extract was removed from the bottle with a 1-ml Eppendorf pipette.

Difference spectra. Difference spectra were performed with a Gilford model 240 scanning spectrophotometer with a split cuvette. The crude cell extract was centrifuged for 1 min in a Brinkman Eppendorf 3200 centrifuge and 1 ml was added to each side of the cuvette, either aerobically or anaerobically (in the anaerobic chamber). All components used in the anaerobic experiments were degassed by boiling for 2 to 3 min, cooled under oxygen-free nitrogen, and added to the cuvette in the anaerobic chamber. The top of the cuvette was sealed with Plasticine. The protein content of the cell extracts was measured by the Lowry assay (14).

Extraction of hemes and formation of pyridine ferrohemochromes. The method of Rizza et al. (19) was employed. Whole cells of *E. lentum* were extracted with 20 volumes of 4% (vol/vol) HCl-acetone at 0 to 4 C over a period of 1 to 2 h with the aid of a glass homogenizer. This suspension was centrifuged at 12,100 × *g* for 10 min, and the supernatant fluid was removed and saved. The pellet was resuspended in 2 to 4 ml of 50% (vol/vol) aqueous pyridine, transferred to a glass homogenizer, and homogenized for 2 min. An equal volume of 0.15 N sodium hydroxide was added to the homogenate, followed by another minute of homogenization, and the dithionite-reduced versus oxidized spectrum for the protein-bound pyridine hemochrome was determined immediately. The hemes from the supernatant fluid from the HCl-acetone extraction were extracted into 2 volumes of diethyl ether. The ether layer was extracted with an equal volume of 0.27 N HCl. The aqueous layer was extracted with an equal volume of ether, and the ether fractions were combined and evaporated to dryness. The residue was dissolved in 2 to 4 ml of 50% (vol/vol) aqueous pyridine, an equal volume of 0.15 N sodium hydroxide was added, and the dithionite-reduced versus oxidized spectrum of the soluble pyridine hemochromes was determined.

Carbon monoxide-binding pigment. Equal volumes of dithionite-reduced cell extract were added to each side of the cuvette, carbon monoxide was bubbled through the sample side for 2 min, and the spectrum was determined.

RESULTS

Presence of cytochromes c and b. The reduced (dithionite) versus oxidized spectrum of a crude cell lysate of the neotype strain of *E. lentum* was similar to that of cytochrome *c* with maxima at 424, 525, and 553 nm (Fig. 1). Cytochrome *c* is the only cytochrome for which the heme portion cannot be extracted from cells with acid-acetone, so we determined the reduced versus oxidized spectrum of the residue of such extracted cells. The spectrum of the pyridine hemochrome (Fig. 2) confirmed that cytochrome *c* (heme *c*) was present (maxima at 414, 522, and 551 nm). The supernatant of the extracted cells was also examined; the pyridine hemochrome of the extract had a spectrum identical to protoheme IX (Fig. 3), indicating that cytochrome *b* was also present. The concentration of cytochrome *c* was 10-fold higher than that of cytochrome *b* (Table 1), so the presence of the latter was obscured in the crude

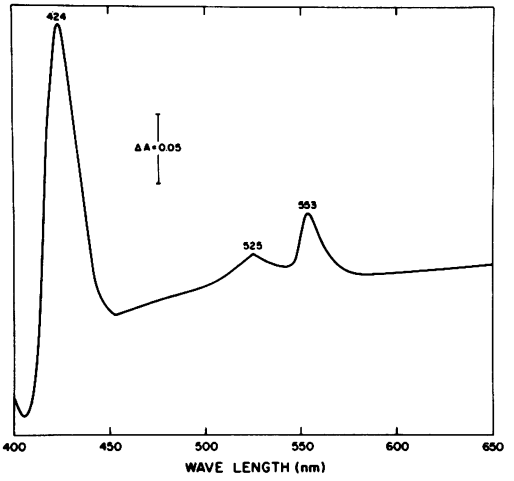

FIG. 1. *Dithionite-reduced versus air-oxidized difference spectrum of a crude cell extract (2.2 mg of protein per ml) of E. lentum (ATCC 25559).*

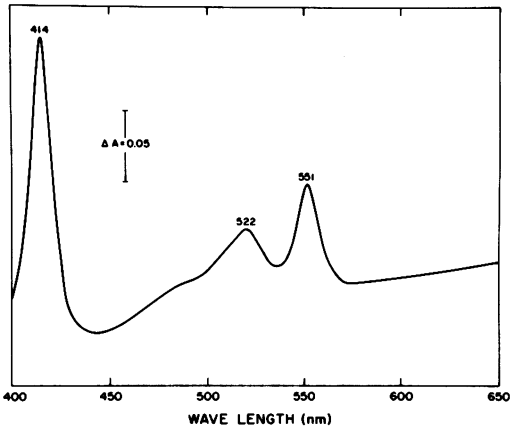

FIG. 2. *Dithionite-reduced versus air-oxidized difference spectrum of pyridine hemochrome from the residue of the acid-acetone extract of E. lentum. The residue of 27 mg of HCl-acetone-extracted cell protein was resuspended in 6 ml of pyridine-NaOH.*

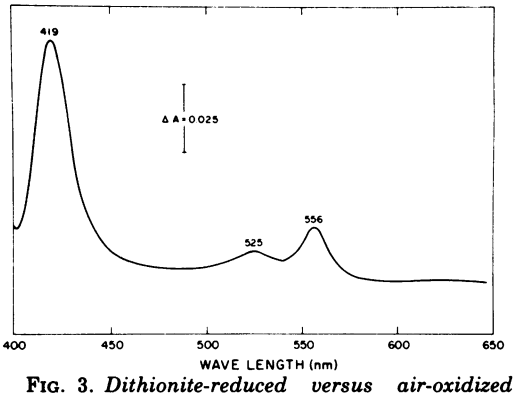

FIG. 3. *Dithionite-reduced versus air-oxidized difference spectrum of pyridine hemochrome from acid-acetone extract of E. lentum. The HCl-acetone extract from 27 mg of cell protein was resuspended in 6 ml of pyridine-NaOH.*

TABLE 1. *Heme concentration of E. lentum grown in different media*

Growth medium	Concn (μmol/g of protein)		Ratio[b]
	Heme c[a]	Protoheme IX[a]	
TYG	0.954	0.077	12.4
TYG + KNO_3	0.421	0.081	5.2
TYG − hemin, − menadione	0.174	0.226	0.77
TYG + KNO_3 − hemin, − menadione	0.184	0.406	0.45

[a] Heme concentrations are calculated from the $\Delta A_{551-536}$ for heme c and $\Delta A_{556-540}$ for protoheme IX using $mM\epsilon = 20$.

[b] This is the ratio of heme c to protoheme IX.

cell lysates. When *E. lentum* was grown in medium without added hemin and menadione, cytochrome *b* could be detected in the crude cell lysates (Fig. 4, peak at 562 nm). This was due to an increase in the concentration of cytochrome *b* (protoheme IX) as well as a decrease in the concentration of cytochrome *c* (heme *c*) (Table 1), which resulted in almost equal amounts of each cytochrome in the cells. The omission of hemin and menadione from the growth media had no effect on either growth rate or total cell yield.

Induction of cytochrome a by nitrate. Most strains of *E. lentum* reduce nitrate (16). Since nitrate is an electron acceptor, we examined the spectrum of cells grown in heme-free medium containing 50 mM nitrate. The portion of the reduced (formate) versus oxidized (air) spectrum from 400 to 575 nm was identical to that of cells grown without nitrate or hemin, but an additional large peak was present at 605 nm (Fig. 5). These results suggested that the presence of nitrate had induced the synthesis of cytochrome *a*. However, when we examined the pyridine hemochrome of the acid acetone extracts of cells grown in the presence of nitrate, we could only detect cytochrome *b*; no peak was present in the region of 580 to 590 nm where heme *a* has maximal absorbance. Because cytochrome *a* is reportedly difficult to work with (13) and might be degraded by the solvents we used, a milder solvent (ethyl acetate-acetic acid) for extraction (5) was tried, but without success. However, when the whole cells were homogenized directly with pyridine-

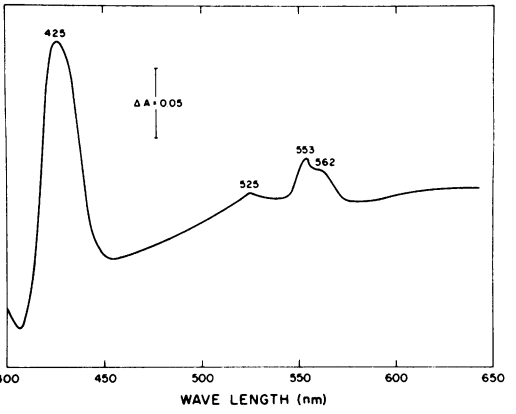

FIG. 4. *Formate-reduced versus nitrate-oxidized difference spectrum of a crude cell extract (1.9 mg of protein per ml) of E. lentum grown in TYG media (minus hemin) containing 50 mM nitrate.*

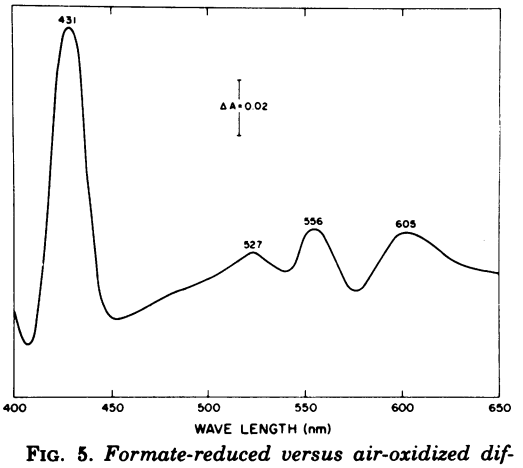

FIG. 5. *Formate-reduced versus air-oxidized difference spectrum of a crude cell extract (1.9 mg of protein per ml) of E. lentum grown in TYG media (minus hemin) containing 50 mM nitrate.*

KOH, the pyridine hemochrome of cytochrome *a* (maxima at 587 nm) was formed. Even under these mild conditions, this peak vanished within several minutes.

Carbon monoxide-binding pigment. Several anaerobes that contain cytochromes have a carbon monoxide-binding pigment; therefore, we examined the reduced plus carbon monoxide versus reduced spectrum of *E. lentum* extracts. The observed spectra of cell extracts of *E. lentum* grown on TYG media (Fig. 6) or TYG media minus hemin were almost identical, and they are consistent with the presence of a cytochrome *o*-like pigment.

Substrate reduction. We were unable to obtain reduction of the cytochromes in cell lysates either aerobically or anaerobically with the following compounds: glucose, L-alpha-glycerophosphate; *d,l*-lactate; hydrogen; reduced nicotinamide adenine dinucleotide phosphate; pyruvate; succinate; or cholate. Reduction could be obtained with reduced nicotinamide adenine dinucleotide in some preparations but not in others. Dithionite reduced the cytochromes, but ascorbate and reduced glutathione did not. Formate reduced the cytochromes, but consistent reduction was only obtained when cell disruption, substrate addition, and determination of the spectrum were all performed anaerobically. Although nitrate induced synthesis of cytochrome *a*, cytochrome *a* was not oxidized by nitrate when formate was the electron donor but it was oxidized when dithionite was used to reduce the cytochromes. Nitrate and nitrite oxidized cytochromes *b* and *c* under anaerobic conditions, even in extracts of cells that had not been grown in the presence of nitrate. Fumarate, which is a common electron acceptor for anaerobes, did not oxidize the cytochromes.

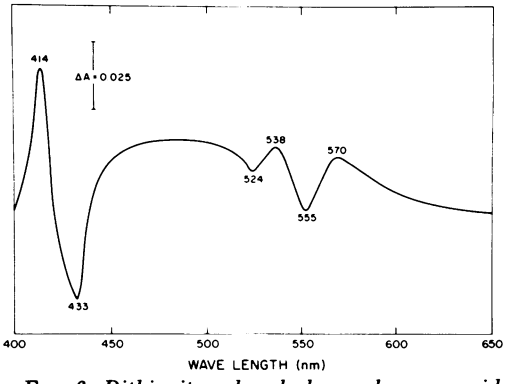

FIG. 6. *Dithionite-reduced plus carbon monoxide versus dithionite-reduced difference spectrum of a crude cell extract (4.3 mg of protein per ml) of E. lentum grown on TYG media.*

Cell yield. Because *E. lentum* only grows to a very low turbidity in the usual bacteriological culture media (optical density of 0.1 at 650 nm), we attempted to increase growth by addition of formate (20 mM) and nitrate (20 mM) to TYG medium. There was no increase in turbidity when this electron donor-acceptor pair was added.

DISCUSSION

E. lentum is unusual in containing cytochromes *a*, *b*, and *c*; no other anaerobe has been reported to contain all three. However, cytochrome *b* and the carbon monoxide-binding pigment may be identical, since both share the same prosthetic group (protoheme IX). Carbon monoxide-binding pigments have been found in most cytochrome-containing anaerobes (3, 4, 19, 21), but no function has been ascribed to them. The induction of cytochrome *a* by nitrate has not been reported previously for anaerobic bacteria, but *Haemophilus parainfluenzae* possesses a branched electron transport system in which increased production of cytochrome a_1 was stimulated by anaerobic growth (in nitrate-containing media) and the cytochrome a_1 was oxidized by nitrate (20).

Some component of the electron transport system for *E. lentum* appeared to be oxygen sensitive, since the system only functioned reproducibly under anaerobic conditions. However, under aerobic conditions, the cytochromes of *E. lentum* were rapidly reduced chemically by dithionite, but not by ascorbate or reduced glutathione. This could result if the oxidation-reduction potentials of the cytochromes were below that of the latter two compounds or if the electron transport chain was so tightly coupled that reduction only occurred when the initial component of the system was reduced. Under anaerobic conditions, the cytochromes were rapidly reduced by formate but only slowly, if at all, by reduced nicotinamide adenine dinucleotide, which further indicates that the electron transport system is tightly coupled. A tightly coupled formate dehydrogenase system has been shown for *Escherichia coli*, where cytochrome b_1 was reduced by formate but not by succinate or reduced nicotinamide adenine dinucleotide (22).

We had hoped that the investigation of the electron transport system of *E. lentum* would result in identification of electron donor-acceptor pairs that could be added to the media to increase the growth of the organism. Although we did identify compounds that functioned in cell extracts, addition of these to the media did not increase growth. This suggested to us that

either growth stopped due to lack of some growth factor rather than an energy source, or the electron donors were not transported into the cells in appreciable quantities. We have recently found that *E. lentum* can be grown to a high turbidity by addition of substrate amounts of arginine to the medium (unpublished data). We are currently determining whether *E. lentum* has an obligate arginine dihydrolase pathway (8) for addenosine 5'-triphosphate formation. If it does, the presence of the cytochromes would be even more of an enigma, since cytochromes are not needed in the arginine dihydrolase pathway.

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