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Aerobic and Anaerobic Respiratory Systems in *Campylobacter fetus* subsp. *jejuni* Grown in Atmospheres Containing Hydrogen

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Maximum growth of *Campylobacter fetus* subsp. *jejuni*, strain C-61, occurred when the cultures were incubated with shaking in atmospheres containing approximately 30% hydrogen, 5% oxygen, and 10% CO_2. Suspensions of cells grown under these conditions consumed oxygen with formate as the substrate in the presence of 0.33 mM cyanide, which completely inhibited respiration with ascorbate-*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine and with lactate. Spectroscopic evidence with intact cells suggested that a form of cytochrome *c*, reducible with formate but not with lactate or ascorbate-*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, can be reoxidized by a cyanide-insensitive system. Analysis of membranes from the cells showed high- and low-potential forms of cytochrome *c*, cytochrome *b*, and various enzymes, including hydrogenase, formate dehydrogenase, and fumarate reductase. The predominant carbon monoxide-binding pigment appeared to be a form of cytochrome *c*, but the spectra also showed evidence of cytochrome *o*. The membrane cytochromes were reduced by hydrogen in the presence of 2-heptyl-4-hydroxyquinoline-*N*-oxide at concentrations which prevented the reduction of cytochrome *c* with succinate as the electron donor. Reoxidation of the substrate-reduced cytochromes by oxygen was apparently mediated by cyanide-sensitive and cyanide-insensitive systems. The membranes also had hydrogen-fumarate oxidoreductase activity mediated by cytochrome *b*. We conclude that *C. fetus jejuni* has high- and low-potential forms of cytochrome which are associated with a complex terminal oxidase system.

Campylobacter fetus is an important agent of disease in humans and cattle (15). It is readily isolable from clinical specimens such as feces by exploitation of its microaerophilic characteristics and its resistance to antibiotics. The organisms grow in complex media in atmospheres enriched with CO_2 and containing 5% O_2 with hydrogen or nitrogen; suitable atmospheres for incubation of plate cultures are provided by the GasPak system (BBL Microbiology Systems, Cockeysville, Md.) without catalyst (7, 13).

We have previously worked with *C. fetus* subsp. *intestinalis* grown microaerophilically (3). This organism was found to be rich in cytochrome *c*, consisting of high- and low-potential forms distinguishable with ascorbate–*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (ascorbate-TMPD) and dithionite as the electron donors. A major proportion of the cytochrome *c* formed a complex with carbon monoxide, but other carbon monoxide-binding pigments were not detected. A branched terminal oxidase system was suggested by observations of the effects of cyanide upon respiration with formate and other substrates.

These studies have now been extended to *C. fetus* subsp. *jejuni* grown microaerophilically in a hydrogen-enriched atmosphere, and this paper concerns the respiratory systems linked with oxygen and with fumarate in intact cells and in membrane preparations.

MATERIALS AND METHODS

Organism and culture conditions. *C. fetus jejuni* strain C-61 was from the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va. Stock cultures were in fluid thioglycolate (BBL Microbiology Systems) and were subcultured weekly; working cultures were derived from 72-h thioglycolate cultures with 0.5% inocula. All cultures were incubated at 37°C.

Cells for experiments were grown in brucella broth (Difco Laboratories, Detroit, Mich.) in 100- to 200-ml volumes in 500-ml flasks, shaken in an atmosphere containing approximately 5% O_2, 10% CO_2, and 30% H_2. The inoculated flasks (0.5% inoculum) were placed in GasPak jars with the GasPak generating

system without a catalyst to provide this atmosphere (BBL Microbiology Systems), and the jars were shaken on a gyratory shaker at 200 rpm.

The growth experiments, shown in Table 1, were done under similar conditions in 50-ml flasks containing 20 ml of brucella broth. Six flasks were in each GasPak jar; the atmosphere was generated by the GasPak system without a catalyst or by evacuating the jars and refilling them with appropriate gas mixtures.

Preparation of cell suspensions and extracts. Cultures were harvested after 36 to 40 h at the end of the logarithmic phase; the absorbance, measured with the Zeiss PMQ II spectrophotometer (540 nm cm^{-1}), was approximately 1.2, equivalent to 0.36 mg of protein ml^{-1}. The cells were washed in 0.2 of the original volume with 40 mM Tris-hydrochloride buffer (pH 7.5) and suspended in 2 to 4 ml of this buffer per 100 ml of culture. Extracts were prepared with the French pressure cell and were divided into the cytoplasmic and membrane fractions as before (3).

Respiration and enzyme activities. Oxygen consumption was measured at 37°C by the Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, England); the vessel contained 3 ml of air-saturated 40 mM Tris-hydrochloride buffer (pH 7.5) with cells or membranes containing 0.5 to 1 mg of protein.

Ascorbate-TMPD oxidase activity was measured with cells or membranes in the oxygen electrode as above with final concentrations of 3.3 mM sodium ascorbate and 0.5 mM TMPD. Enzyme activities in the membranes were measured spectrophotometrically at room temperature. Succinate and lactate dehydrogenase were assayed as before with phenazine methosulfate (*N*-methylphenazonium methosulfate) and dichlorophenol-indophenol or with ferricyanide as the electron acceptor (3). Formate dehydrogenase was assayed with benzyl viologen as the electron acceptor in anaerobic cuvettes gassed with argon. The cuvettes contained membranes (0.5 mg of protein) in 2.5 ml of 40 mM Tris-hydrochloride buffer (pH 7.5)–0.8 mM benzyl viologen. Formate (2 μmol) was added to the experimental cuvette, and the increase in absorbance was measured at 578 nm (8.6 mm^{-1} cm^{-1}). For the assay of hydrogenase a similar system was used, except that the reaction was begun by the addition of benzyl viologen to the experimental and reference cuvettes, and gassed with hydrogen and argon, respectively. Fumarate reductase was assayed as before with reduced methyl viologen as electron donor (3).

Spectroscopic measurement of cytochromes. All spectroscopic measurements were made with the Aminco DW-2a spectrophotometer in the split- or dual-beam mode with a band pass of 1 nm. The concentration of cytochromes was determined by difference spectroscopy with dithionite or ascorbate-TMPD as the electron donor. The Δε values of 17.3 and 17.5 nM^{-1} cm^{-1} were applied for cytochromes *c* and *b*, respectively (16). The wavelength pairs were 548 to 537 nm for cytochrome *c* and 558 to 571 nm for cytochrome *b*. Total cytochrome *c* was calculated from the dithionite-reduced minus ferricyanide-oxidized spectrum, and low-potential cytochromes were estimated from the dithionite-reduced minus ascorbate-TMPD-reduced spectrum.

Carbon monoxide-binding pigments were determined with dithionite-reduced material by observation of the dithionite-reduced plus CO minus dithionite-reduced spectrum. The experimental cuvette was bubbled gently for 30 s with CO, and the spectra were measured after maximum absorbance was reached (5 to 10 min). The amount of CO-binding cytochrome *c* was calculated by assuming an extinction (Δε) of 55 mM^{-1} cm^{-1} for the peak to trough of the Soret band. This value is derived from data for soluble forms of CO-binding cytochrome *c* and has been applied to methylotrophic bacteria (1, 14).

Analytical determinations. Volatile and nonvolatile acids in the growth media were analyzed by gas-liquid chromatography by the methods of Holdeman et al. (5). Protein was determined with bovine serum albumin as the standard by a modification of the Lowry method (2).

Materials. Gases were from Matheson Scientific, Inc. (Newark, Calif.). Hydrogen and argon were passed through heated copper filings to remove traces of oxygen. Electron donor and acceptor dyes and enzyme inhibitors were from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions (10 mM) of 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) were in ethanol.

RESULTS

Growth conditions. Preliminary experiments showed that maximum growth of *C. fetus jejuni* occurred in brucella broth incubated in atmospheres of H_2 with 5% O_2 and 10% CO_2. A suitable atmosphere was generated in the GasPak system without a catalyst, and this was used routinely. Cultures grown under these conditions attained maximum cell density within 40 h.

Anaerobic growth in brucella broth was also observed, provided that the cultures were incubated in an atmosphere containing both H_2 and CO_2 (Table 1). Growth in this atmosphere was significantly improved by the addition of fuma-

TABLE 1. Effect of culture conditions on growth and succinate accumulation[a]

Atmosphere	Addition to medium (μmol/ml)		Growth[b]	Succinate in culture (μmol/ml)
	Fumarate	Formate		
GasPak			1.02	1.2
GasPak	10		1.12	11.6
GasPak	10	10	1.23	11.0
H_2-Ar-CO_2			0.23	2.2
H_2-Ar-CO_2	10		0.36	13.0
H_2-Ar-CO_2	10	10	0.54	13.1
Ar-CO_2			0.02	1.3
H_2-Ar			0.02	2.2

[a] Cultures were in 50-ml flasks containing 20 ml of brucella broth with fumarate and formate added as shown. Flasks were incubated with shaking at 37°C in GasPak jars filled with the appropriate atmosphere. The atmospheres contained: H_2, with O_2 and CO_2 as provided by the GasPak system without a catalyst; H_2-Ar-CO_2, 35:55:10; Ar-CO_2, 90:10; H_2-Ar, 35:65.

[b] Growth was measured after 72 h of incubation as absorbance at 540 nm; the absorbance given by the uninoculated medium (0.02) has been subtracted.

rate with or without formate, and analysis of the spent medium showed that fumarate had been reduced to succinate (Table 1). The yield of cells attained anaerobically was less than half that attained with oxygen at the concentration provided by the GasPak system (Table 1). Under these conditions, fumarate with formate slightly improved the yield, and succinate was accumulated as observed in the anaerobic systems (Table 1).

Respiration of cell suspensions. Cells harvested after growth in the GasPak system consumed oxygen with ascorbate-TMPD, formate, and DL-lactate at rates of, respectively, 347, 209, and 211 nmol min^{-1} mg^{-1} of protein. Other substrates, including succinate, malate, fumarate, aspartate, and glutamate, only slightly increased the rate above the endogenous level of 10 to 20 nmol min^{-1} mg^{-1} of protein.

The respiratory activity with lactate was stable for at least 48 h in cells stored at 0°C, in contrast to our observations with *C. fetus intestinalis*, which lost this activity within 12 h (3).

Cyanide (0.33 mM) completely inhibited oxygen consumption with ascorbate-TMPD and with lactate (Fig. 1). Oxygen consumption with formate continued after the addition of cyanide, though at a declining rate (Fig. 2a). The declining rate with formate in the presence of cyanide was not due to inactivation of the enzyme system; oxygen consumption resumed upon reaeration of the system, and this cycle could be repeated several times (Fig. 2b). It is possible that the oxidase concerned with formate oxidation in the presence of cyanide has a lower affinity for oxygen than does the cyanide-sensitive system concerned with the oxidation of ascorbate-TMPD and lactate.

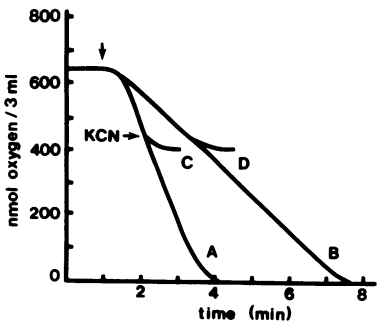

FIG. 1. Effect of cyanide on ascorbate-TMPD and lactate oxidase activity of cells. The oxygen electrode vessel contained cells (0.7 mg of protein) in 3 ml of air-saturated 40 mM Tris-hydrochloride buffer (pH 7.5) with the following substrates added after 1 min (arrow): ascorbate-TMPD (A, C); DL-lactate (B, D). Cyanide (1 μmol) was added to (C) and (D) 1 min after the substrate.

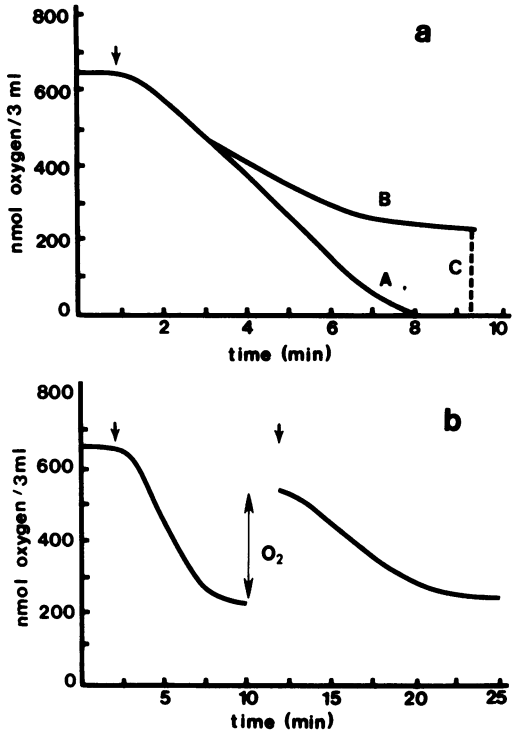

FIG. 2. Effect of cyanide on formate oxidase activity of cells. The oxygen electrode vessel contained cells in buffer as described in the legend to Fig. 1. (a) Formate (20 μmol) was added without cyanide (A) and with 1 μmol of cyanide (B) at the time shown by the arrow; dithionite was added at the time shown by the dashed line C. (b) Cyanide (1 μmol) was added at zero time, with additions of formate (20 μmol) as shown by the arrows. The reaction mixture was aerated to restore the oxygen concentration as indicated.

Enzyme activities in cell fractions. Assays for various activities were made with the cytoplasmic and membrane fractions derived from the crude cell extracts (Table 2). The membranes had formate dehydrogenase and hydrogenase activities, measured with benzyl viologen as the electron acceptor; neither enzyme was detectable in the cytoplasm. Succinate and lactate dehydrogenases were also located in the membrane, and the latter was found to be specific for the D-isomer.

It is likely that the succinate dehydrogenase activity was attributable to fumarate reductase. This enzyme was present in high activity in the membrane fraction (Table 2), and it was completely inhibited by 20 μM *p*-chloromercuriphenylsulfonate. The inhibitor also completely abolished succinate dehydrogenase activity measured with ferricyanide as the electron acceptor (data not shown). Sensitivity to this inhibitor has been attributed to an iron-sulfur

TABLE 2. Enzyme activities in membrane fraction[a]

Enzyme	Sp act
Hydrogenase	68
Formate dehydrogenase	43
L-Lactate dehydrogenase	<10
D-Lactate dehydrogenase	84
Succinate dehydrogenase	221
Fumarate reductase	1,515
Ascorbate-TMPD oxidase	54

[a] Enzymes were assayed as described in the text; activities are shown as nanomoles per minute per milligram of membrane protein.

protein associated with fumarate reductase (8–10).

Ascorbate-TMPD oxidase activity was found in the membrane fraction only (Table 2); it was completely inhibited by 0.08 mM cyanide.

Cytochromes in cell fractions. The difference spectra of intact cells and crude extracts showed cytochrome *c*, with a maximum at 548 nm, to predominate. No evidence was found of cytochromes *a* or *d* in scans extending to 650 nm.

The distribution and amounts of the various cytochromes were determined in the cytoplasmic and membrane fractions derived from the crude extracts (Table 3, Fig. 3). Approximately 50% of the cytochrome *c* in the crude extract was recovered in the cytoplasmic fraction, and most of this soluble pigment was a high-potential form, reducible by ascorbate-TMPD (Fig. 3a, trace 3). Only a small amount (approximately 11%) of the cytoplasmic cytochrome *c* formed a complex with CO (Table 3, Fig. 3b).

Cytochrome *c* also predominated in the membranes, as shown by the dithionite-reduced minus air-oxidized spectrum (Fig. 3c, trace 2).

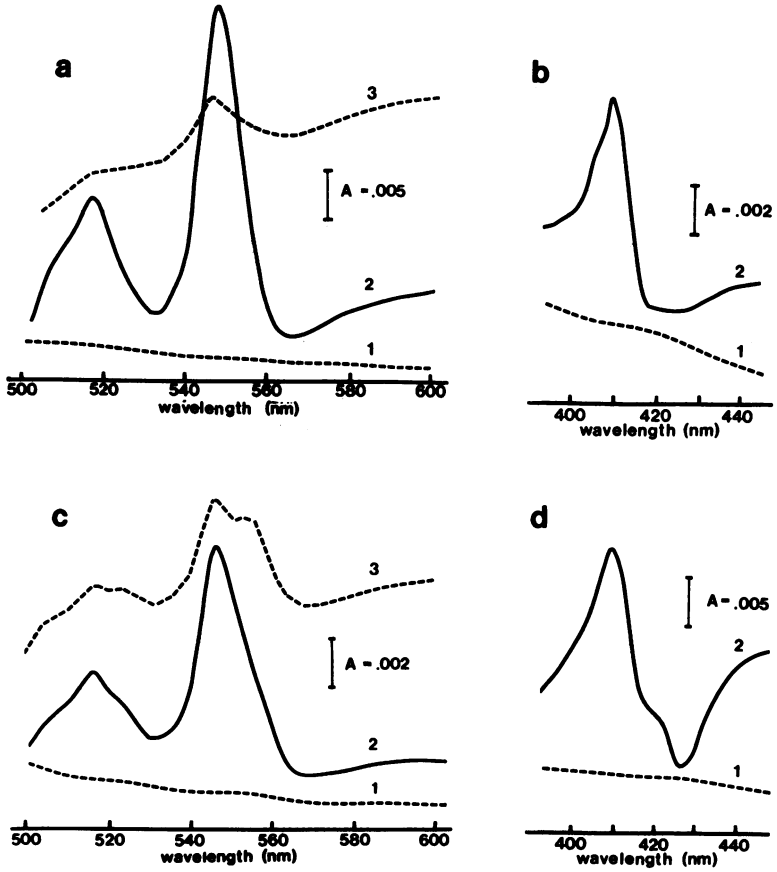

FIG. 3. Cytochrome difference spectra of cytoplasmic and membrane fractions. (a) Cytoplasm (1.13 mg of protein per ml): trace 1, base line; trace 2, dithionite reduced minus ferricyanide oxidized; trace 3, dithionite reduced minus ascorbate-TMPD reduced. (b) Cytoplasm (1.1 mg of protein per ml): trace 1, base line; trace 2, dithionite reduced plus CO minus dithionite reduced. (c) Membranes (0.6 mg of protein per ml): trace 1, base line; trace 2, dithionite reduced minus air oxidized; trace 3, dithionite reduced minus ascorbate-TMPD reduced. (d) Membranes (0.6 mg of protein per ml): trace 1, base line; trace 2, dithionite reduced plus CO minus dithionite reduced. A, Absorbance.

TABLE 3. Forms of cytochrome in cytoplasmic and membrane fractions[a]

Fraction	Type of cytochrome	Concn (nmol per mg of protein) in prepn: I	II
Cytoplasm	Total cytochrome c[b]	1.42	1.52
	Low-potential cytochrome c	Trace	Trace
	CO-binding cytochrome c	0.15	0.14
Membranes	Total cytochrome c[b]	1.54	1.92
	Low-potential cytochrome c	0.87	1.00
	CO-binding cytochrome c	0.54	0.67
	Cytochrome b	0.70	0.87

[a] Determinations were made in fractions from two batches of cells (I and II). Calculation of the cytochromes is explained in the text. The proportion of total cytochrome c in the crude extracts which was recovered in the cytoplasmic fraction was 53 and 47% in preparations I and II, respectively.

[b] Total cytochrome, high- and low-potential forms.

However, cytochrome b was revealed as a maximum at 558 nm in the dithionite-reduced minus ascorbate-TMP-reduced spectrum (Fig. 3c, trace 3). This spectrum also showed a maximum at 548 nm, deemed to represent a low-potential form of cytochrome c which was reducible by dithionite but not by ascorbate-TMPD. From these spectra it was calculated that 50 to 60% of the total cytochrome c in the membrane was of the low-potential form (Table 3).

The CO difference spectra of the membranes suggested the presence of two pigments capable of combining with CO (Fig. 3d). The maximum at 408 to 410 nm indicated a complex with cytochrome c, and the shoulder at 420 nm could be due to a form of cytochrome o. The amount of CO-binding cytochrome c was calculated to be approximately 35% of the total cytochrome c in the membrane (Table 3).

Effect of cyanide on the reduction and reoxidation of cytochromes in cells. Intact cells in air-saturated buffer were examined by dual wavelength spectroscopy to determine the reduction levels attained by the cytochromes with various substrates (Fig. 4). Little or no reduction of cytochrome c was observed for periods of at least 1 min after the addition of succinate, lactate, or formate. However, the addition of cyanide resulted in rapid reductions to different levels with the different substrates. Reduction of cytochrome c with formate as the electron source occurred in several phases; the initial rapid reduction upon the addition of cyanide was followed by a slower phase which continued until the level of reduced cytochrome reached approximately 86% of that with dithionite (Fig. 4a, b). The level of reduction attained with succinate or lactate was approximately 62% of that with dithionite (Fig. 4c, d).

We interpret the initial reduction upon the addition of cyanide to be due to the high-potential form of cytochrome c which is linked to the cyanide-sensitive terminal oxidase. The second phase, observed with formate, could be due to reduction of the low-potential cytochrome c as oxygen is depleted by the cyanide-insensitive oxidase.

This interpretation of the spectral changes with the various substrates was supported by further spectroscopic observations. First, the cuvettes were vigorously aerated upon attainment of the final reduction level in the presence of cyanide. Partial reoxidation of the formate-reduced cytochrome occurred (Fig. 4b), but no reoxidation of the succinate- or lactate-reduced cytochrome was observed (Fig. 4c, d). Second, difference spectra measurements were made of the substrate-reduced cytochromes (cyanide present) by split-beam spectroscopy. With air-oxidized cells as the reference, the characteristic reduced cytochrome c maximum at 548 nm predominated with all substrates (Fig. 4, solid lines). The same preparations were then scanned against a reference of ascorbate-TMPD-reduced cells (Fig. 4, dashed lines). These scans blanked out the absorbance due to the reduced high-potential cytochrome c and revealed the reduced low-potential cytochrome. The formate-reduced cells showed two maxima attributable to low-potential cytochrome c and cytochrome b (Fig. 4b). In contrast, no maximum was shown in the succinate- or lactate-reduced cells; the slight trough in the difference spectra at 548 nm indicates that the high-potential cytochrome c was almost, but not completely, in the reduced state in these cells (Fig. 4c, d).

Reduction and reoxidation of cytochromes in membrane preparations. The reduction of membrane cytochromes and their reoxidation by oxygen was monitored by difference spectroscopy against a reference of air-oxidized membranes or of preparations in an argon atmosphere. Under both conditions, the membranes remained in the oxidized state unless a substrate was added.

The membrane cytochromes were reduced by hydrogen, and approximately 82% of the total became reduced with this substrate (Fig. 5a, trace 1). Reduction of both cytochrome c and cytochrome b also occurred in the presence of 40 μM HQNO (Fig. 5a, trace 2). Upon aeration, the hydrogen-reduced cytochromes were completely reoxidized, even in the presence of 0.8

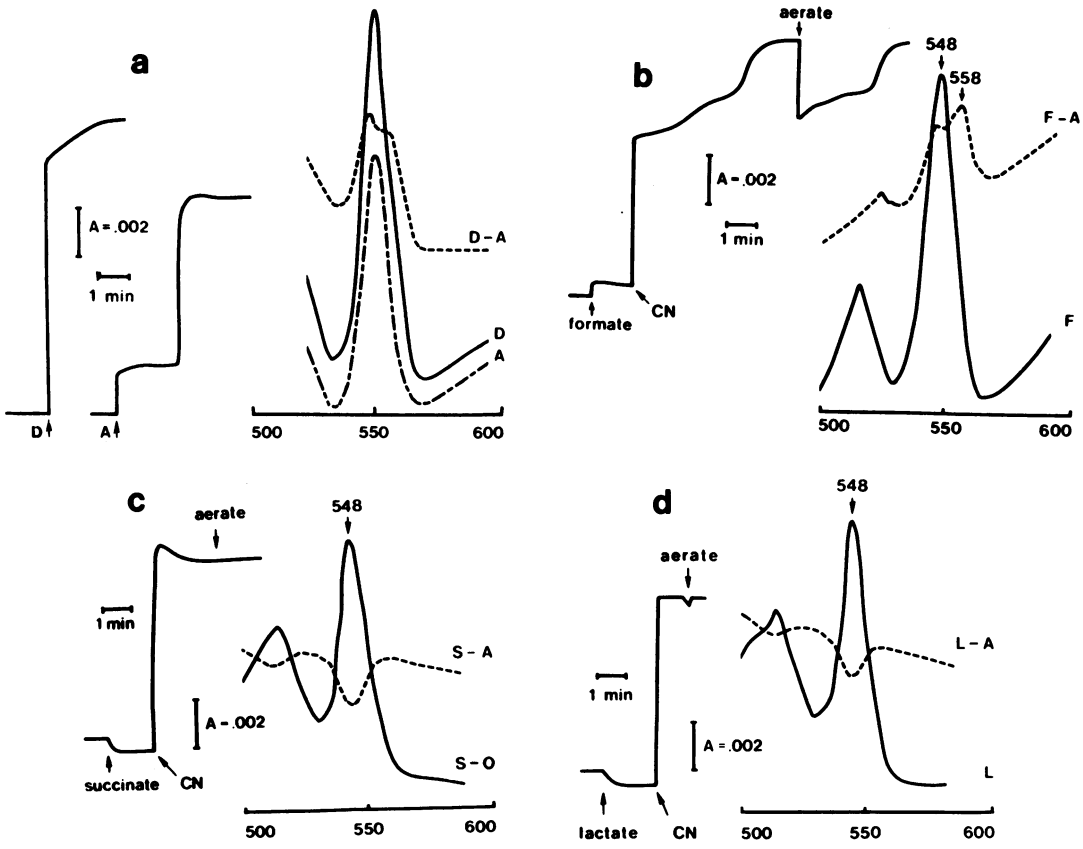

FIG. 4. Reduction and reoxidation of cytochromes observed by dual wavelength and difference spectroscopy of whole cells. The experimental cuvette contained cells suspended in air-saturated 40 mM Tris-hydrochloride buffer (pH 7.5), and the samples were monitored by dual beam (548 to 537 nm) with additions as indicated in the left-hand traces in each panel. These reduced preparations were then monitored by difference spectroscopy against the appropriate reference to give the scans shown to the right in each panel. (a) Reduction with dithionite (D) or with ascorbate-TMPD (A); difference spectra against a reference of air-oxidized cells (A, D) or dithionite-reduced minus ascorbate-TMPD-reduced cells (D-A). (b) Formate (F; 4 mM) as the reductant, with the addition of cyanide (CN; 0.38 mM) and with aeration as indicated. The difference spectra measured after completion of the experiment were against air-oxidized cells (F) or against ascorbate-TMPD-reduced cells (F-A). (c) As in (b) but with 4 mM succinate (S) as the substrate. (d) As in (b) but with 4 mM DL-lactate as the substrate.

mM cyanide (Fig. 5a, trace 3). Similar observations were made with formate as the electron donor (data not shown).

With succinate as the substrate, the reduction level of cytochrome *c* reached 43% of the total (Fig. 5b, trace 1). Reduction of cytochrome *c* by succinate was prevented by 40 μM HQNO, and reduced cytochrome *b* became evident in the spectrum (Fig. 5b, trace 2). Reoxidation of cytochrome *b* with HQNO present was not observed upon aeration of the cuvettes either with or without cyanide (Fig. 5b, trace 3). Reoxidation of succinate-reduced cytochrome *c* was examined in the presence of HQNO added after the pigment had become reduced (Fig. 5c). Upon aeration, complete reoxidation occurred within 1.5 min (Fig. 5c, trace 2). Such reoxidation was severely inhibited by the addition of 0.8 mM cyanide and was not complete even after 5 min (Fig. 5c, trace 3).

We suggest that the succinate-reduced cytochrome *c* represents the high-potential form and that an HQNO-sensitive step between cytochrome *b* and cytochrome *c* participates in the electron transfer. It seems that the cyanide-sensitive terminal oxidase is primarily involved in the reoxidation of the succinate-reduced cytochrome *c*; however, the slow reoxidation observed in the presence of 0.8 mM cyanide could be due to the operation of an alternative oxidase. We assume that hydrogen reduces both high- and low-potential forms of cytochrome *c* and

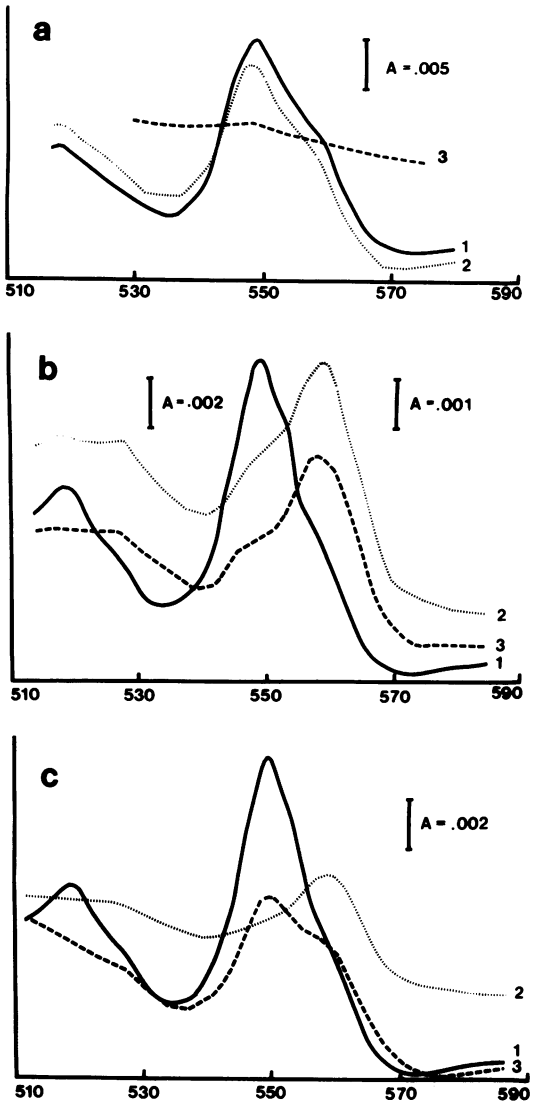

FIG. 5. Effect of HQNO and cyanide on reduction and oxidation of cytochromes observed by difference spectroscopy of membranes. Anaerobic cuvettes contained membranes (0.7 mg of protein per ml) in 40 mM Tris-hydrochloride buffer (pH 7.5) saturated with argon; the base line was established in an atmosphere of argon. Additions were made to the experimental cuvettes, and the spectra were recorded against the reference of membranes in an argon atmosphere. (a) Hydrogen as the substrate, without addition (trace 1), and with 40 μM HQNO added before gassing with hydrogen (trace 2). Cyanide (0.8 mM) was added to the latter after maximum absorbance was reached and the cuvette was aerated; trace 3 shows the spectrum recorded 1.5 min after aeration. (b) Succinate (4 mM) as the reductant in an atmosphere of argon, without addition (trace 1) and with 40 μM HQNO added before succinate (trace 2). Cyanide (0.8 mM) was added to the latter after maximum absorbance was reached, and the cuvette was aerated; trace 3 was recorded 5 min after aeration. (c) Succinate (4 mM) as the reductant; 40 μM HQNO was added after maximum absorbance was reached (trace 1). The cuvette was then aerated, and trace 2 was recorded after 5 min. In trace 3, 0.8 mM cyanide was added before aeration, and the spectrum was recorded after 5 min.

that the HQNO-sensitive step may not be obligatory. It might be expected from the observations with succinate-reduced cytochrome *c* that cyanide would inhibit the reoxidation of a proportion of the hydrogen-reduced pigment (representing the high-potential form). We did not detect such inhibition under the conditions of these experiments, and we are unable to account for this discrepancy.

Reoxidation of cytochromes by fumarate. The coupling of hydrogenase with fumarate reductase via cytochrome *b* was explored by difference spectroscopy of membrane preparations under anaerobic conditions. In these experiments, the absorbance due to the high-potential cytochrome *c* was canceled out by the addition of ascorbate-TMPD to reference and experimental cuvettes in an argon atmosphere (Fig. 6; compare traces a and d). The experimental cuvette was then flushed with hydrogen to give the spectrum shown in Fig. 6, trace b. This revealed a predominance of cytochrome *b* at 558 nm with the shoulder at 548 to 550 nm, attributed to low-potential cytochrome *c*. Upon the addition of fumarate (Fig. 6, trace c), the reduced cytochrome *b* peak vanished, and considerable reoxidation of the low-potential cytochrome *c* also occurred. The reoxidation of the hydrogen-reduced cytochromes by fumarate was completely inhibited by 40 μM *p*-chloromercuriphenylsulfonate (data not shown).

DISCUSSION

The pattern of cytochromes found in *C. fetus jejuni* resembles qualitatively that found in *C. fetus intestinalis* (3). Cytochrome *c* predominates in both organisms and includes high- and low-potential forms. In *C. fetus jejuni*, approximately 35% of the membrane cytochrome *c* appeared to be capable of forming a complex with carbon monoxide; by the same calculation, all of the membrane cytochrome *c* in *C. fetus intestinalis* formed a complex with carbon monoxide. *C. fetus jejuni* also showed evidence of cytochrome *o*, but this was not detected in *C. fetus intestinalis*. Thorough identification of the various forms of cytochrome *c* requires their purification and characterization, and progress in this direction has begun. Hoffmann and Goodman (4) have partially purified high- and low-potential forms from solubilized membranes of *C. fetus jejuni* H48 and have found that the latter forms a complex with carbon monoxide.

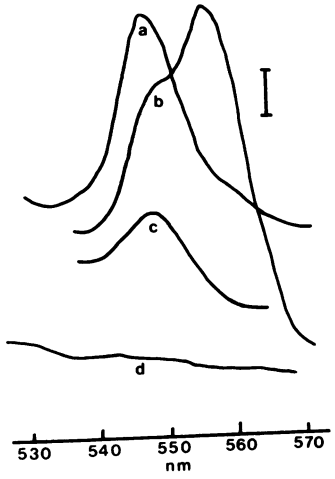

FIG. 6. Oxidation of reduced membrane cytochromes observed by difference spectroscopy. The anaerobic cuvettes contained membranes in argon-saturated 40 mM Tris-hydrochloride buffer (pH 7.5) with ascorbate-TMPD added to the experimental cuvette only (a) or to both cuvettes (b, c, d). In (b) and (c), the argon atmosphere in the experimental cuvette was replaced by hydrogen, and the spectrum was recorded before (b) and after (d) the addition of 1 mM fumarate (c). The base line (d) was with ascorbate-TMPD in both cuvettes in an atmosphere of argon. The protein concentration was 0.7 mg per ml; the bars represent absorbances of 0.002 for (a) and 0.001 for (b), (c), and (d).

C. fetus jejuni, like *C. fetus intestinalis*, apparently has several terminal oxidases distinguished on the basis of cyanide sensitivity with various substrates. Formate can be oxidized via a cyanide-insensitive system, as shown by our work with intact cells and by Hoffman and Goodman (4) with membrane preparations. We have made similar observations with hydrogen as the substrate (data not shown). The kinetics of oxygen consumption with formate suggest that the cyanide-insensitive oxidase may have a relatively low affinity for oxygen (Fig. 2b). The rate declined progressively as the reaction mixture became depleted of oxygen but was restored by aeration. The latter observation argues against the inactivation of formate dehydrogenase; in *Campylobacter sputorum* (lacking catalase), the oxidation of formate in the presence of cyanide produces hydrogen peroxide, which irreversibly inactivates the dehydrogenase (12, 13). We conclude that the cyanide-insensitive oxidase in *C. fetus jejuni* becomes progressively less effective as the concentration of oxygen decreases, but confirmation requires more exact kinetic experiments with varying oxygen concentrations.

The spectroscopic observations with intact cells (Fig. 4) suggest that the cyanide-insensitive oxidase may be particularly concerned with the low-potential cytochrome *c*, whereas the cyanide-sensitive oxidase may be more concerned with the high-potential cytochrome. Thus, formate-reduced cytochrome *c*, representing low- and high-potential forms, was partially reoxidized by aeration in the presence of cyanide; the same concentration of inhibitor completely prevented the reoxidation of high-potential cytochrome *c* reduced with succinate or lactate. However, in membrane preparations, succinate-reduced cytochrome *c* was slowly reoxidized in the presence of cyanide, and we were unable to show an effect of cyanide on reoxidation of the hydrogen-reduced cytochrome. We propose as a working hypothesis the pathways of electron transfer in the membranes outlined in Fig. 7. The preferred route of electron transfer from high-potential cytochrome *c* to oxygen is via the cyanide-sensitive oxidase, but slow reoxidation (Fig. 7, dashed arrow) may also occur by the insensitive system, available also for reoxidation of the low-potential cytochrome *c*. Reduced cytochrome *b* is presumed to be reoxidized by the fumarate reductase system under anaerobic conditions; aerobically it is reoxidized via an HQNO-sensitive site before the high-potential cytochrome *c* oxidase system. Electron transfer may also occur between the low- and high-potential forms of cytochrome *c*, and this route may circumvent cytochrome *b* in the presence of HQNO (Fig. 7, dotted arrow). The nature of the terminal oxidases is quite unknown. Prime candidates include the cytochrome *c* and the cytochrome *o* indicated by the carbon monoxide difference spectra.

The physiological functions of the various cytochromes should be considered in relation to the microaerophilic peculiarities of *C. fetus jejuni* and related organisms. They have a respiratory metabolism based on the Krebs cycle, but they also possess membrane-associated enzyme systems found in some strict anaerobes. In anaerobes such as *Vibrio succinogenes*, the coupling of hydrogen or formate oxidation with fumarate reductase is a major source of ATP (8), and the growth experiments (Table 1) indicate

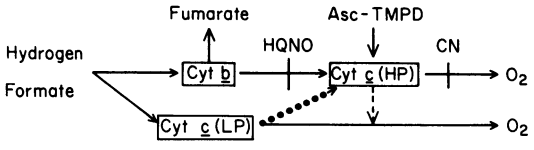

FIG. 7. Scheme for electron transfer in membranes of *C. fetus jejuni*. Cyt, Cytochrome; HP, high potential; LP, low potential; Asc, ascorbate.

that *C. fetus jejuni* derives some benefit from such systems. However, anaerobic growth is poor relative to that with oxygen present at concentrations below normal atmospheric pressure. It seems likely that the high-potential cytochrome *c* oxidase system in the membranes provides *C. fetus jejuni* with an additional site for energy coupling, as apparently occurs in strict aerobes with similar terminal oxidase systems (6). This possibility is supported by preliminary observations of the active transport of glutamate, which is inhibited by cyanide with lactate but not with hydrogen as the energy source (G. M. Carlone and J. Lascelles, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K25, p. 140).

The role of the low-potential cytochrome *c* is unclear. Low-potential forms of the pigment are found in many strict anaerobes, and they seem to be in the cytoplasmic or periplasmic fraction (9–11). However, in *C. fetus jejuni* the low-potential cytochrome was in the membranes. It may possibly provide an oxygen buffer which maintains an appropriate redox environment for electronegative dehydrogenase systems. The function of the cytoplasmic cytochrome *c* is also open to speculation. It may mediate electron transfer between NADP-linked cytoplasmic dehydrogenases and the high-potential cytochrome *c* oxidase system in the membranes. We have made preliminary observations in support of this with unfractionated extracts. Isocitrate and NADP reduce all the cytochrome *c* in the extracts, and complete reoxidation occurs upon aeration; in this context, the high level of NADP-linked isocitrate dehydrogenase found in the cytoplasm of *C. fetus jejuni* and in *C. fetus intestinalis* may be relevant.

Finally, how can the problem of oxygen sensitivity in the microaerophile be reconciled with the presence of a cytochrome *c* terminal oxidase system characteristic of some highly aerobic bacteria? We suggest that a major locus of oxygen toxicity is at the strongly electronegative primary dehydrogenase systems. Such systems are evidently vital since the organisms are sensitive to metronidazole (7, 15). The electronegative dehydrogenase systems could include hydrogenase, formate dehydrogenase, and pyruvate dehydrogenase, all likely to involve iron-sulfur proteins (including ferredoxin) and susceptible to auto-oxidation by oxygen (17). Dependence of *C. fetus jejuni* upon such primary dehydrogenase systems would require that the concentration of dissolved oxygen be maintained by the terminal oxidase systems below the critical inactivating level.

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