Biochimica et Biophysica Acta 933 (1988) 114–123
Elsevier

BBA 42738

The microaerophilic respiration of *Campylobacter mucosalis*

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(Received 25 May 1987)
(Revised manuscript received 26 October 1987)

Key words: Microaerophilic respiration; Cytochrome *c*; Peroxidase; Oxidase; (*Campylobacter*)

A model is proposed for the respiratory adaptation to falling oxygen concentration during growth of the microaerophilic bacterium *Campylobacter mucosalis*. During the early stages of growth, the oxidation of formate is a two-stage branched process involving the production of H_2O_2 followed by its peroxidatic removal. In later stages of growth, at lower oxygen concentrations, the predominant electron flow is linear to a membrane-bound cytochrome-*c* oxidase which reduces O_2 directly to H_2O. Several components of this model have been investigated. H_2O_2 was produced during formate oxidation and accumulated when electron transfer to the cytochrome-*c* peroxidase was inhibited. A cytochrome *c*-553, of the Class 1 type, was purified and shown to be the specific electron donor to both the peroxidase and the membrane-bound oxidase. The levels of this cytochrome *c* and of the peroxidase were higher in cells harvested early in growth. In later stages of growth, the activity of the membrane-bound oxidase increased. Proton pumping across the membrane was detected with either H_2O_2 or oxygen as terminal electron acceptor. The novel energy-conserving role of H_2O_2 in this catalase-negative bacterium is discussed in relation to its microaerophilic nature.

Introduction

Microaerophilic bacteria utilise oxygen as a terminal electron acceptor in respiration, but are killed by high oxygen concentrations. This toxicity is probably due to the generation of partially reduced species, such as superoxide and hydrogen peroxide. The optimum oxygen tension for a particular microaerophile will be influenced by a number of factors, which include the rate of production of such toxic species, the susceptibility of enzyme systems to damage, and the presence and efficiency of detoxifying enzymes such as superoxide dismutase, catalase and peroxidase [1].

The genus *Campylobacter* includes gram-negative, non-fermenting microaerophilic organisms [2]. Some, such as *C. sputorum* subspecies *bubulus* and *C. mucosalis,* are catalase-negative. In *C. sputorum* ss *bubulus,* Stouthamer and coworkers have proposed that hydrogen peroxide production during formate oxidation was a major cause of oxygen toxicity. The oxidation of formate occurred via two pathways. One, which was of low oxygen affinity, was inhibited by CN^- but not by quinol analogues, and produced hydrogen peroxide. The other, which was of high oxygen affinity, was inhibited by quinol analogues but not by CN^- and produced water as an end-product [3–5]. When the high affinity route was inhibited by glutaraldehyde treatment, hydrogen peroxide accumulated

Abbreviations: TMPD, *N,N,N′N′*-tetramethyl-*p*-phenyldiamine dihydrochloride; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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[6]. These authors propose that in uninhibited cells, hydrogen peroxide is decomposed by a cytochrome-*c* peroxidase supplied by the electron transport system. However the components of this electron transport system to the peroxidase were not investigated.

We studied the related catalase-negative organism *Campylobacter mucosalis* [7] originally isolated from porcine intestinal adenomatosis [8]. We found that this organism, like *C. sputorum* subspecies *bubulus,* produces substantial amounts of hydrogen peroxide during formate oxidation.

In this paper, we report a model for the respiration of *C. mucosalis* which involves an adaptation to changing oxygen concentrations during growth. Central to this model are the interactions of a soluble cytochrome *c*-553 with a soluble peroxidase and a membrane-bound cytochrome oxidase. In addition, we show that removal of hydrogen peroxide involves electron flow through an energy-conserving electron transport system.

Materials and Methods

Growth of cells

Campylobacter mucosalis f. *C. sputorum* subspecies *mucosalis* (Strain 1248, NCTC 253/72, [7]) was a gift from Dr. G.H.K. Lawson, [8] and was grown microaerobically on plates of Columbia agar base (Oxoid, Basingstoke, U.K.) supplemented with 5% horse serum or whole horse blood (Gibco, Paisley, U.K.) in an atmosphere initially comprising 4.5% O_2/5% CO_2/15.5% N_2/75% H_2. After growth at 37° C for 24 or 72 h, the cells were harvested by scraping from the agar surface and were washed in 10 mM sodium phosphate (pH 7).

Preparation of cell extracts and membranes

All operations were conducted at 4° C. Washed cells were homogenised in 3–5 vols. of 10 mM sodium phosphate (pH 7), and were disrupted either by freezing and thawing twice, or by passage through a French pressure cell at $82.7 \cdot 10^6$ Pascals (12000 psi).

Soluble and membrane fractions were prepared by centrifugation at $23000 \times g$ for 30 min in the case of freeze-thawing or $100000 \times g$ for 60 min in the case of French pressing. Membranes were resuspended in 3–5 vols. of 10 mM sodium phosphate (pH 7).

Cytochromes c

Horse heart cytochrome *c* (Type VI) was from Sigma (Poole, U.K.). Cytochrome *c*-551 was prepared from *Pseudomonas stutzeri* (strain 224, ATCC 17591) by conventional methods.

Assay of peroxidase activity

Cytochrome-*c* peroxidase activity was measured by the decline in absorbance at the α-band of ferrocytochrome *c* (concentrations in Table legends) on addition of hydrogen peroxide (17 μM) and a cell extract. The cytochrome *c* was reduced in situ in a stirred cuvette under argon by titration with 10 mM sodium dithionite (prepared anaerobically in 20 mM sodium phosphate (pH 7)). Anaerobic conditions were required to prevent interference by oxidase activity of small membrane vesicles, and also to minimise production of H_2O_2 from the reaction of dithionite with O_2. Such peroxide production led to variable re-oxidation of cytochrome *c* after addition of the cell supernatant, but prior to addition of H_2O_2.

The oxidation of the ferrocytochrome by peroxidase and hydrogen peroxide followed an exponential (pseudo-first-order) progress curve. Rate constants were derived from plots of log $A_t - A_\infty$ against time (where A_t is the absorbance of the α-peak at time t and A_∞ is the absorbance after addition of ferricyanide). Velocities were derived from the initial reaction rate or were calculated from $v = k \times [C_r]$ where k = pseudo-first-order rate constant and C_r = concentration of ferrocytochrome *c*. The two methods gave similar results.

Assays of oxygen utilisation

Oxygen utilisation was measured in an oxygen electrode (Rank Brothers, Cambridge, U.K.) at 25° C in 10 mM sodium phosphate (pH 7)/1 mM EDTA, in a final volume of 2 ml. Substrates used were 1 mM ascorbate with 0.3 mM TMPD (Sigma) or 10 mM sodium formate. Experiments were also performed with pulses of 250 nmol formate.

The presence of hydrogen peroxide formed during these experiments could be detected by addition of 1400 Sigma units of catalase to release O_2 by dismutation.

HOQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide, Sigma), when present, was preincubated for 2 min with membranes at a concentration of 50 μM.

Assay of cytochrome-c oxidase activity

Cytochrome-*c* oxidase activity was measured by the decline in absorbance at the α-band of ferrocytochrome *c* on addition of a cell membrane fraction. The cytochrome *c* (9 μM) was reduced in situ in a cuvette by the addition of sodium ascorbate (pH 7). Different final concentrations of ascorbate were required to produce comparable levels of reduction of the different cytochromes studied. 67 μM ascorbate gave 96%, and 91% reduction in horse cytochrome *c*, and *Ps. stutzeri* cytochrome *c*-551, respectively, while 400 μM ascorbate was required to produce 52% reduction in *C. mucosalis* cytochrome *c*-553. After a steady level of reduction had been achieved, addition of a cell membrane fraction resulted in oxidation of the cytochrome *c*, which followed an exponential progress curve to a new steady-state level of partial reduction. Velocities were derived from the initial rate of oxidation, monitored at the α-peak.

Proton translocation

The method used was that of Dawson and Jones [9]. The chamber contained 3 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma) (pH 7.2) 140 mM KCl, 200 μg carbonic anhydrase, 20 μg valinomycin (both from Sigma) and 0.1 ml fresh cell suspension (4 mg protein) in a final volume of 3.5 ml. The chamber was stirred, maintained at 27 °C, and flushed with oxygen-free nitrogen.

Either formate (1 mM) or H_2 (0.78 mM) were present as substrates, the latter by flushing with H_2 gas in place of N_2. Pulses of O_2 (39 nmol) were added by injection of 0.15 ml of air-saturated buffer. Pulses of hydrogen peroxide (88 nmol) were injected in 10 μl of O_2-free solution. 5 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone, Sigma) was present as required.

Acidification of the medium was recorded with a pH electrode connected to a chart recorder. The pH change was calibrated by addition of 0.01 M HCl or 0.01 M KOH and was found to be linear between pH 6.8 and 7.2.

Purification and properties of cytochrome c-553

All procedures were carried out at 4 °C. The soluble cell extract from *C. mucosalis* (3 g wet weight cells) was passed through Sephadex G-25 (fine) equilibrated in 2 mM sodium phosphate (pH 7) and the coloured eluate was applied to a column (10 × 2 cm) of Whatman DEAE-52 cellulose equilibrated in the same buffer. The unadsorbed eluate was applied to a column (8 × 1 cm) of Whatman CM-52 cellulose and the adsorbed proteins were eluted with a linear gradient of 2–100 mM sodium phosphate (pH 7) (400 ml). Cytochrome *c*-553 was the only coloured protein eluted from this column, and red fractions were combined, diluted 6-fold readsorbed to a small CM-52 cellulose column (1 × 2 cm), and eluted as a sharp band in 100 mM sodium phosphate (pH 7). This solution was passed through a column of Sephadex G75 superfine equilibrated in 20 mM Tris-HCl (pH 8)/100 mM NaCl and the red fractions were diluted 10-fold, and concentrated on CM-52 as described above.

Amino-acid analysis of the purified cytochrome was carried out on a Locarte analyser after hydrolysis in 6 M HCl in vacuo for 70 h at 105 °C. Cysteine was measured as cysteic acid after removal of the haem group [10], performic acid oxidation [11] and acid hydrolysis for 20 h. The sample for analysis contained 5.9 nmol haem, as determined by the pyridine haemochrome method. Tryptophan was not determined by amino-acid analysis. An estimate of the tryptophan content was obtained by subtracting the tyrosine and haem contributions at 280 nm from the absorbance of the holoprotein. This calculation assumes

$$A_{280\,nm}^{tyrosine} = 1.1\ mM^{-1}\cdot cm^{-1} \text{ and } A_{280\,nm}^{haem} = 13.8\ mM^{-1}\cdot cm^{-1}$$

the latter being calculated from cytochromes *c* of known tryptophan content.

Redox titration of cytochrome *c*-553 was carried out in 10 mM sodium phosphate (pH 7) in a stirred anaerobic cuvette fitted with a Pt : Ag | AgCl combination electrode (Russell pH, Auchtermuchty, U.K.). The redox buffer-mediator was 20 μM ferric ammonium sulphate/400 μM EDTA. Oxidative titration was performed with a potassium ferricyanide solution; reductive titration was performed with a ferrous ammonium sulphate solution.

Results

Respiratory models for cells after 1 day and 3 days growth

The relative importance of different pathways of oxygen utilisation by *C. mucosalis* changes during growth in a culture jar. The features of these different pathways are shown in Fig. 1 and the characterisation of the individual components of the model is described below.

Hydrogen peroxide production by C. mucosalis

Addition of small pulses of the respiratory substrate, formate, to whole cells resulted in O_2 uptake (Fig. 2). After exhaustion of formate, the presence of hydrogen peroxide can be detected by the addition of catalase, which releases O_2 by dismutation. More H_2O_2 was found in 'older' cells (Fig. 2a). However 'younger' cells accumulate H_2O_2 if the quinone analogue and respiratory inhibitor HOQNO is present (Fig. 2c). Similar results (not shown) were obtained under conditions of formate excess when catalase was added during the process of oxygen uptake.

Because H_2O_2 accumulation is enhanced by HOQNO, we propose that its production occurs on the substrate side of the block. We have accordingly placed it in Fig. 1 as part of the formate-oxidising system.

After dismutation by catalase of any H_2O_2 present, the final level of oxygen uptake in Figs. 2a, b and c should be quantitatively related to the amount of formate added by the equation:

$$2HCOO^- + O_2 \rightarrow 2HCO_3^- \quad (1)$$

The measured stoichiometries ($HCOO^-/O_2$) for

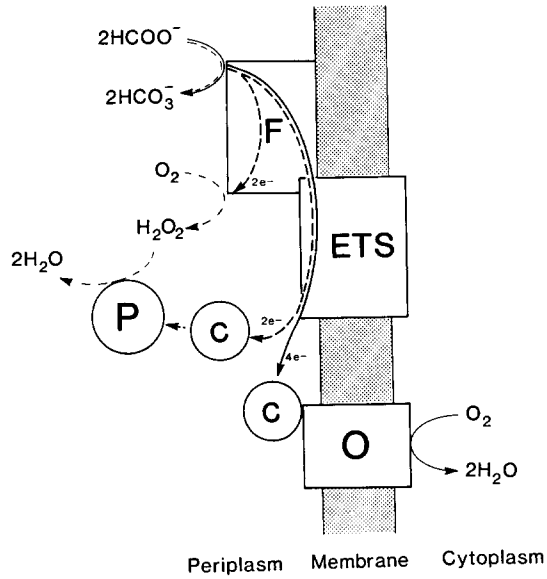

Fig. 1. Patterns of electron flow in the respiration of *C. mucosalis*. Two different pathways for the oxidation of formate by *C. mucosalis* are shown. In cells harvested early in growth, we propose that the broken line shows the dominant flow of electrons. This involves direct reduction of O_2 to hydrogen peroxide by a formate-oxidising enzyme (F) followed by reduction of hydrogen peroxide to H_2O by a cytochrome-*c* peroxidase (P) which receives electrons from the electron transport system (ETS) via a cytochrome *c*-553 (C). In cells harvested later in growth, the solid line shows the proposed dominant flow of electrons leading to a conventional reduction of O_2 to H_2O by a membrane-bound terminal oxidase (O).

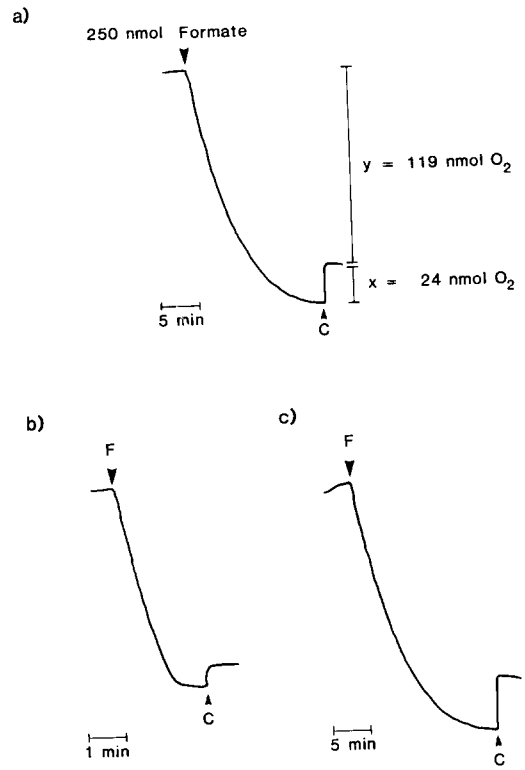

Fig. 2. The oxidation of formate by *C. mucosalis*. Formate pulses (250 nmol) were added to *C. mucosalis* grown for 3 days (a) or 1 day (b, c). HOQNO (50 μM) was preincubated with the cells in (c). O_2 uptake was measured in an O_2 electrode (see Materials and Methods) and, after uptake was complete, catalase (C) was added.

Figs. 2a, b and c are 2.11, 2.22 and 2.06, respectively, in reasonable agreement with the expected figure of 2.

Prior to dismutation by catalase of any H_2O_2 present, the traces should enable calculation of the relative importance of partial reduction to H_2O_2 and complete reduction to H_2O [6]. For example, the value x/y in Fig. 2a gives the proportion of the flow of electrons from formate to give H_2O_2 (= 20%). However, this is a useful estimate only if there is stable accumulation of H_2O_2. In this organism, catalase is absent but removal of H_2O_2 may occur via the cytochrome-*c* peroxidase (see below). We propose that the enhanced accumulation of H_2O_2 in 'young' cells treated with HOQNO is due to blockage of the electron supply to the cytochrome-*c* peroxidase (Fig. 2c).

The properties of cytochrome c-553 and variation in amounts with age of cells

A soluble cytochrome *c*-type component with an asymmetric α-peak and a mid-point potential near +100 mV was identified by analysis of complex potentiometric titrations of soluble cell extracts from *C. mucosalis* [12]. This cytochrome has been purified by chromatography on CM-cellulose (see Materials and Methods) and its basic nature was confirmed by electrophoresis in the native state (results not shown).

On the basis of molecular exclusion chromatography on Sephadex G-75 and polyacrylamide gel electrophoresis in SDS (results not shown), cytochrome *c*-553 has a similar molecular weight to cytochrome *c*-551 of *Pseudomonas aeruginosa* (Molecular weight from amino-acid sequence, 9329 [13]). Parallel determinations of amino-acid composition and haem content showed that cytochrome *c*-553 contains 68–72 amino acids per haem group (Table I). In conjunction with the estimates of molecular weight, this indicates that the molecule has a single haem.

Spectroscopic properties of cytochrome *c*-553 are summarised in Table II with absorption coefficients based on pyridine haemochrome determination. The presence of a 695 nm band in the ferricytochrome suggests methionine coordination of the iron [14]. Potentiometric titration in the region of the α-band (Fig. 3a) was used to derive a mid-point potential ($E_{m,7}$) of +99 mV for a single

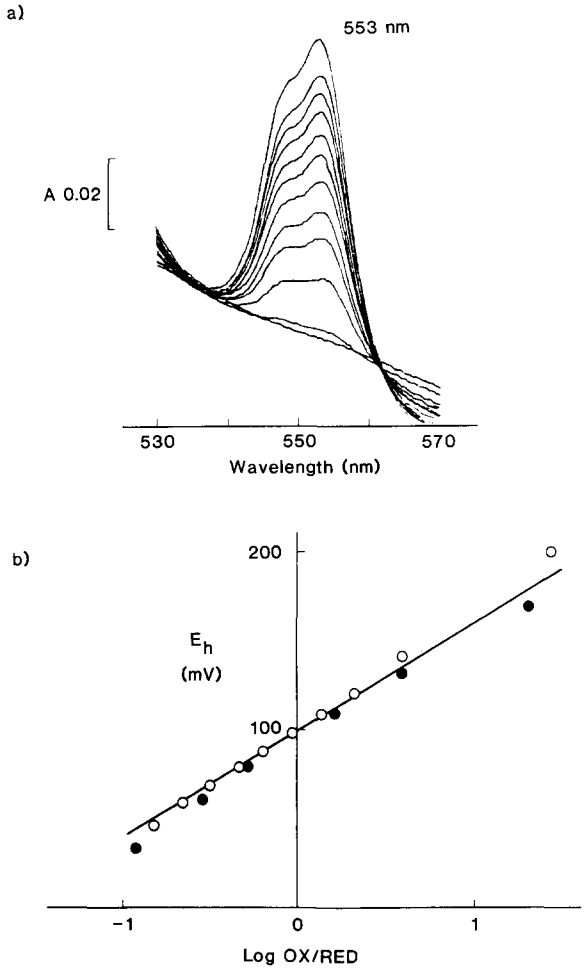

Fig. 3. Potentiometric redox titration of *C. mucosalis* cytochrome *c*-553. (a) Spectra were recorded during a reductive titration of cytochrome *c*-553 as described in materials and Methods. Each spectrum corresponds to a measured redox potential and was used to derive the plot shown in (b). (b) Points were calculated from the reductive titration (○) shown in (a), and from an oxidative titration (●, not shown).

redox species (Fig. 3b) in good agreement with that predicted from titration of the cell extract [12].

2–5-times more cytochrome *c*-553 was present in cells grown for 1 day compared with those grown for 3 days. This was shown by molecular exclusion chromatography of the soluble extract, by quantitative analysis of haem-stained SDS electrophoretic gels and by the degree to which the α-band of a soluble extract was reducible with

TABLE I

AMINO-ACID COMPOSITION OF CYTOCHROME *c*-553

	nmol	mol/mol haem	Integer
Asp	70.2	11.9	12
Thr	19.5	3.3	3
Ser	7.1	1.2	1
Glu	10.2	1.7	2
Pro	22.5	3.8	4
Gly	31.8	5.4	5/6
Ala	65.2	11.1	11
Val	28.4	4.8	5
Met	14	2.4	2/3
Ile	24.3	4.1	4
Leu	20.2	3.4	3/4
Tyr	19.8	3.4	3/4
Phe	5.1	0.9	1
His	5.3	0.9	1
Lys	54.1	9.2	9
Arg	0	0	0
Cys		1.7	2
Trp		0	0
		No of residues	68/72
		M_r	7795–8259

ascorbate and phenazine methosulphate, since cytochrome *c*-553 is the only high potential cytochrome *c* present (results not shown).

The reactivity of cytochrome *c*-553 with cytochrome *c* peroxidase and cytochrome *c* oxidase of *C. mucosalis* is described below.

Cytochrome c peroxidase

Soluble extracts of *C. mucosalis* contain a cytochrome *c* peroxidase activity assayed using horse ferrocytochrome *c* as substrate. This activity was nearly 4-times greater in cells grown for 1 day compared to 3-day cells (Table III). The peroxidase showed a preference for basic cytochromes (Table IV) and the cytochrome *c*-553 of *C. mucosalis* was the best donor tested under these particular conditions. Not only was the rate at 5 μM 3-times higher than that for horse cytochrome *c*, but the cytochrome *c*-553 approached saturation at this concentration, while horse cytochrome *c* was well below saturation. However, because of the small quantities of cytochrome *c*-553 available, routine assays were performed using horse ferrocytochrome *c*.

TABLE II

SPECTROSCOPIC PROPERTIES OF CYTOCHROME *c*-553

Figures in parentheses are absorption coefficients ($mM^{-1} \cdot cm^{-1}$) based on pyridine haemochrome determination.

Ferricytochrome		Ferrocytochrome	
Spectral maxima			
280	(18.2)	Soret 416	(151.8)
Soret 408	(110.2)	β 522	(15.6)
524	(8.3)	α 553	(20)
695	(0.6)		
Spectral ratios			
α/β	1.28		
α/280	1.1		

TABLE III

THE EFFECT OF CULTURE AGE ON OXIDASE AND PEROXIDASE ACTIVITIES

Age (days)	Cytochrome-*c* peroxidase [a] (nmol per min per mg cellular protein)	Ascorbate-TMPD oxidase [b] (nmol O_2 per min per mg cellular protein)
1	230	119
3	60	210

[a] The assay was as described in Materials and Methods, using horse ferrocytochrome *c* (8.5 μM) in 10 mM phosphate (pH 7)/1 mM EDTA. Initial rates were calculated from $k \times [C_r]$, where k is the pseudo-first-order rate constant, and $[C_r]$ is the concentration of ferrocytochrome *c*.

[b] Rates were determined using the membrane fraction. The soluble fraction typically contained less than 10% of the oxidase activity.

Cytochrome-c oxidase

Almost all the ascorbate-TMPD oxidase activity of *C. mucosalis* was found in the membranes (Table III). The small amount of apparently soluble activity may be due to the presence of vesicles. Membranes from cells grown for 3 days contained twice the activity found in membranes from younger cells (Table III).

Ascorbate-TMPD oxidase activity is considered to reflect the presence of a cytochrome *c* donor to an oxidase [15]. Cytochrome-*c* oxidase activity itself was detected using ferrocytochromes *c* as electron donors (Table IV). The assay is com-

TABLE IV

PEROXIDASE AND OXIDASE REACTIVITY WITH CYTOCHROME *c*

The oxidation of each cytochrome was monitored at the α-peak maximum.

	Net charge at pH 7	Cytochrome-*c* peroxidase [a]		Cytochrome-*c* oxidase [d]	
		v ($\mu M \cdot min^{-1}$)	%	*v* ($\mu M \cdot min^{-1}$)	%
C. mucosalis *c*-553	+ve	20.7 [b]	100	11.3 [e]	100
C. mucosalis *c*-553	+ve	16.3 [c]	79		
Horse cytochrome *c*	+ve	7.0 [b]	34	7.0 [f]	62
Horse cytochrome *c*	+ve	3.3 [c]	16		
Ps. stutzeri *c*-551	−ve	0.3 [b]	1	0.8 [g]	7

[a] The assay was as described in Materials and Methods using: [b] 5.0 μM or [c] 3.3 μM ferrocytochrome *c* in 10 mM sodium phosphate (pH 7)/1 mM EDTA. The reaction was initiated using 25 μl soluble cell extract (derived from a broken cell suspension containing 20 mg protein per ml) Rates are expressed relative to 5 μM *C. mucosalis* *c*-553 (100%).

[d] The assay was as described in Materials and Methods with 9 μM cytochrome *c* in 10 mM phosphate/1 mM EDTA. Cytochromes *c* were reduced with ascorbate. Concentration of ferrocytochrome *c* attained was: [e] 4.7 μM, [f] 8.6 μM or [g] 8.2 μM and the oxidation was initiated by 5 μl of a membrane fraction derived from a broken cell suspension containing 20 mg protein per ml. Rates are expressed relative to *C. mucosalis* *c*-553 (100%).

plicated by the tendency for cytochrome *c*-553 to oxidise in air. Also, unlike the peroxidase assay, the cytochrome cannot be reduced in the cuvette by dithionite because the latter reacts with the oxygen required for the oxidase reaction. Thus, the assay involves a reduction of the cytochrome in the cuvette using a low concentration of ascorbate. However, this, in itself, gives rise to two problems. One is that the subsequent oxidase rate is influenced by the excess ascorbate, which tends to re-reduce the cytochrome *c*. The contribution of this re-reduction rate increases as more oxidised cytochrome is formed until a steady-state level is reached. The second problem is that reduction is incomplete for some cytochromes (52% for cytochrome *c*-553), making it difficult to compare cytochromes under identical conditions. For these reasons, no attempt has been made to study the cytochrome concentration dependence of the reaction.

Nevertheless, the results of Table IV show the same clear qualitative preference for basic cytochromes as was seen for the peroxidase activity. Cytochrome *c*-553 was again more active than horse cytochrome *c*.

Topography of electron transport, energy conservation and hydrogen peroxide production

Our proposal in Fig. 1 incorporates a periplasmic location for both H_2O_2 production by formate oxidation and H_2O_2 removal by cytochrome-*c* peroxidase action. Formation of spheroplasts and examination of the periplasmic and cytoplasmic fractions is an important method for establishing topography of bacterial respiratory processes. However, in spite of numerous attempts, we have been unable to form spheroplasts from *C. mucosalis*. In all cases, extensive damage to the cell membrane and release of isocitrate dehydrogenase or malate dehydrogenase as cytoplasmic marker enzymes was observed. However, although we have no such direct evidence for the topography outlined in Fig. 1, persuasive arguments can be offered in its favour.

Thus, it is probable that the cytochrome-*c* peroxidase is periplasmic. Wood [16] has proposed what may be a general rule, that *c*-type cytochromes are either periplasmic or located at the periplasmic side of the cell membrane. We have proposed that the substrate of the peroxidase is cytochrome *c*-553, suggesting a periplasmic location for both.

We propose that hydrogen peroxide production is also periplasmic. Thus, if formate oxidase is facing the periplasmic side of the cell membrane, the pattern of H^+ release and uptake during formate oxidation with H_2O_2 as an intermediate is shown in Fig. 4. In this diagram, there is no net

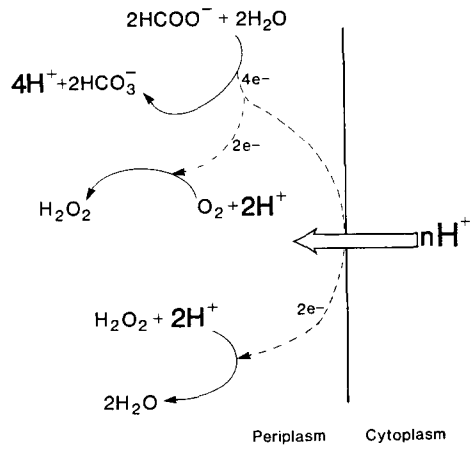

Fig. 4. A model for energy conservation during oxidation of formate by *C. mucosalis*. The diagram is a modified form of that shown in Fig. 1, in which only the electron flow predominating after 1 day of growth is considered. The membrane separating the periplasm and cytoplasm is shown as a single vertical line. Proton release and uptake in the periplasmic space are emphasised, and balance. Protons translocated across the membrane to acidify the periplasm are shown by the open arrow. Under anaerobic conditions, with a pulse of H_2O_2 as electron acceptor (as in Fig. 5), there is no reduction of oxygen and all four electrons from the two formate molecules will pass via the electron transport system to reduce two molecules of H_2O_2. This again results in a balance of proton release and uptake in the periplasmic space.

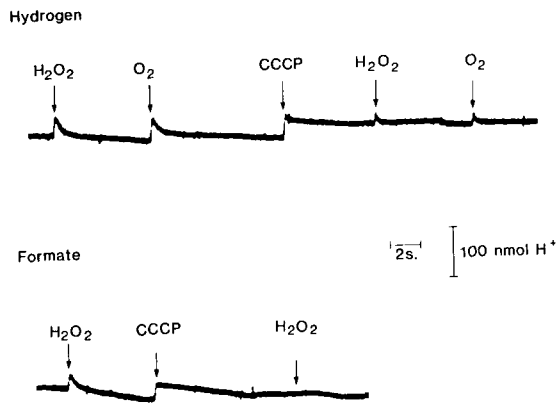

Fig. 5. Proton effects during respiration of *C. mucosalis*. The acidification of the medium after addition of pulses of O_2 or hydrogen peroxide to suspensions of *C. mucosalis* was measured as described in Materials and Methods. Acidification was studied in the presence of two substrates, hydrogen (top trace) and formate (lower trace).

change in the H^+ concentration in the periplasmic space due to the reactions themselves, while if formate oxidase were cytoplasmic, there would be an acidification inside the cell and an alkalinisation outside. Thus, the only possible contribution to a bioenergetically useful acidification of the periplasm would have to come from the protons pumped by the electron transport system (shown as the transmembrane arrow in Fig. 4).

Whole cells generated a proton gradient, as measured by acidification of the external medium with either formate or hydrogen as substrates and pulses of oxygen as electron acceptor. Similar acidification was observed with H_2O_2 as oxidant. In both cases, the effects were abolished by the protonophore CCCP (Fig. 5).

The stoichiometry of H^+ pumping is very low for both oxygen (approx. 0.7 H^+/O) and H_2O_2 (approx. 0.6 H^+/H_2O_2). It seems probable that this reflects the fragility of the cell membrane of *C. mucosalis* already seen in attempts to form spheroplasts. Thus, in the proton extrusion experiment, only a small proportion of cells may have an intact membrane and therefore be capable of producing a vectorial H^+ effect.

From these results, it is probable that the mechanism for removal of H_2O_2 may act not only to detoxify this compound, but may also contribute to energy conservation via a proton-pumping electron transport system. In that case, the bacteriological status of *C. mucosalis* as catalase-negative is interesting because the presence of catalase would remove this possibility of energy conservation, and reduce growth. We have tested this directly by comparing the growth of *C. mucosalis* on plates containing blood (and therefore erythrocyte catalase) with plates containing serum. The qualitative observation was that cells grew faster on plates lacking catalase.

Discussion

The sealed culture jars used for the growth of *C. mucosalis* initially contain oxygen at 20% of atmospheric level, which then decreases due to cellular respiration. We propose that this change in oxygen concentration is accompanied by a change in the nature of the respiration. When the oxygen concentration is relatively high in the early stages of growth, the oxidation of formate involves the production of hydrogen peroxide. This is then

reduced to water by a cytochrome-*c* peroxidase which receives electrons from the membrane electron transport system via a cytochrome *c*-553. Thus, this pattern of respiration involves a two-stage reduction of oxygen to water and a branched flow of electrons (Fig. 1). As the oxygen concentration falls in the culture jar, less hydrogen peroxide is formed and the electron flow occurs predominantly by a conventional linear pathway to a terminal cytochrome-*c* oxidase.

We examined the changes that occur in individual components of this model during adaptation to lower oxygen concentrations. Although little accumulation of hydrogen peroxide by cells harvested early in growth could be detected, due to its rapid removal by the peroxidase pathway, the capacity for large-scale production of hydrogen peroxide could be seen in cells inhibited by HOQNO (Fig. 2c). The rapid removal of the hydrogen peroxide in early growth was achieved by elevated levels of cytochrome-*c* peroxidase and its specific electron donor, cytochrome *c*-553. In later stages of growth, lower levels of these components, but higher levels of cytochrome oxidase, were observed. Although the oxidase also utilises cytochrome *c*-553, less of this may be required to efficiently mediate electron transfer between membrane complexes than between a membrane complex and a soluble peroxidase.

This adaptive response of *C. mucosalis* to changing oxygen concentrations is relevant to our understanding of microaerophilic bacteria. One possible reason for the toxicity of oxygen to such bacteria is the presence of autoxidisable dehydrogenases which generate hydrogen peroxide by direct interaction with oxygen [1]. Formate dehydrogenase may be one such enzyme in *C. sputorum* [4,5], *C. jejuni* [17] and *C. mucosalis*. If such enzymes have a relatively high K_m for oxygen [4], the rate of production of the toxic product will increase as the oxygen concentration is raised. With *C. mucosalis*, we propose that induction of the peroxidase system allows some adaptation, but a point will be reached where the removal of the hydrogen peroxide cannot keep pace with its production, and cell death occurs.

The status of catalase in this scheme is of interest. Both *C. mucosalis* and *C. sputorum* are catalase-negative but, while the addition of catalase to the medium can enhance the aerotolerance of some microaerophiles, this is not the case for these two organisms. A possible explanation for this is that, although catalase will allow removal of the toxic hydrogen peroxide, it will also eliminate the possibility of energy conservation by a proton-translocating electron transport chain that supplies electrons to a peroxidase. We have demonstrated that such proton translocation can occur in response to pulses of hydrogen peroxide in *C. mucosalis*. Thus, although production of toxic hydrogen peroxide seems unavoidable in this organism, once formed, it appears to play a positive role in energy conservation. This novel method of energy conservation may not be available to catalase-positive organisms, or to parasitic organisms that are exposed to the peroxide-scavenging mechanisms of their hosts.

Another possible reason for the failure of catalase to enhance oxygen tolerance in *C. mucosalis* is one of access. If hydrogen peroxide causes damage close to its site of production in the cell, the presence of added catalase in the medium may be ineffective. In this connection, it is of interest to note that some bacteria may possess both catalase and peroxidase. This apparent duplication of function might be explained if catalase were cytoplasmic and dealt with hydrogen peroxide generated in that compartment, while cytochrome-*c* peroxidase removed periplasmic hydrogen peroxide. Such locations for catalase and cytochrome-*c* peroxidase have been demonstrated in *Ps. stutzeri* (Wilson, Goodhew, Hunter and Pettigrew, unpublished data).

A common pattern in biological electron transport is for a small, soluble, high potential cytochrome *c* (Class 1 of Ambler [18]) to act as donor to both a membrane oxidase and soluble enzymes [19]. In size, mid-point potential and spectrum, the cytochrome *c*-553 of *C. mucosalis* clearly falls within Class 1, although it is in a small minority of basic cytochromes. Other *Campylobacter* species probably contain related high-potential cytochromes *c* [5,20,21]. Of the Class 1 cytochromes, cytochrome *c*-553 most closely resembles cytochrome *c*-555 from *Chlorobium limicola* f. *thiosulphatophilum* in spectrum, basic nature and mid-point redox potential. Indeed, we have shown that this cytochrome *c*-555 was reactive with both the per-

oxidase and oxidase of *C. mucosalis* (results not shown), although this apparent similarity is somewhat surprising in view of the disparate lifestyles of the two organisms. However, precedents exist for the occurrence of similar cytochromes in apparently divergent bacterial genera [18].

In conclusion, we have presented evidence of the production of H_2O_2 by *C. mucosalis,* and the utilisation of H_2O_2 and O_2 as terminal acceptors for energy-conserving electron transfer through the respiratory chain. The reactivity of *c*-553 with the peroxidase and oxidase, and the levels of these proteins during growth, was consistent with the proposal that cytochrome *c*-553 donates electrons predominantly to the peroxidase in the early stages of growth, and subsequently to the oxidase in later growth.

Acknowledgements

Preliminary experiments were carried out by Callum Livingstone. We wish to thank John Leaver and John Phillips for their helpful criticism of the manuscript. This work was generously supported by a Wellcome Trust project grant and a Wellcome Trust Research Leave Fellowship to G.W.P.

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