The respiratory chain of *Helicobacter pylori*: identification of cytochromes and the effects of oxygen on cytochrome and menaquinone levels

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Abstract

The quinone and cytochrome components of the respiratory chain of the microaerophilic bacterium *Helicobacter pylori* have been investigated. The major isoprenoid quinone was menaquinone-6, with traces of menaquinone-4; no methyl-substituted or unusual menaquinone species were found. Cell yield was highest after growth at 10% (v/v) oxygen and menaquinone levels (per dry cell mass) were maximal at 5–10% (v/v) oxygen. *Helicobacter pylori* cells and membranes contained b- and c-type cytochromes, but not terminal oxidases of the a- or d-types, as judged by reduced minus oxidised difference spectra. Spectra consistent with the presence of a CO-binding terminal oxidase of the cytochrome b- or o-type were obtained. The soluble fraction from disrupted cells also contained cytochrome c. There were no significant qualitative differences in the cytochrome complements of cells grown at oxygen concentrations in the range 2–15% (v/v) but putative oxidases were highest in cells grown at 5–10% (v/v) oxygen.

Keywords: Cytochrome; *Helicobacter pylori*; Menaquinone; Microaerophilia; Oxygen metabolism; Respiration; Oxidases

1. Introduction

*Helicobacter pylori* is found in the stomach and upper intestine of man and is the major aetiological agent of active chronic gastritis. It is also associated with peptic ulcer formation and has been linked in epidemiological studies to gastric cancer and heart disease. However, understanding of these relationships and the development of selective antimicrobial agents is limited by a lack of fundamental knowledge of the metabolism and physiology of the organism. *Helicobacter pylori* is Gram-negative, motile, catalase-positive and ‘oxidase-positive’, but cannot apparently metabolise nitrate [1,2]. It is also microaerophilic, with optimal growth in atmospheres of 5–10% (v/v) oxygen [1] supplemented, typically, with 10% (v/v) carbon dioxide. Nevertheless, the presence of fumarate reductase suggests that anaerobic respiration may occur [3,4].

Glucose may act as an energy source in defined media, although reports on the metabolic products...
conflict [5]. Cells from broth cultures readily oxidise exogenous ethanol, fumarate, glucose, β-lactate, pyruvate and succinate, and there is evidence for a tricarboxylic acid cycle [6]. Cell membranes oxidise fumarate, β-lactate, NADH, NADPH and succinate, but only the quinone components of the oxygen-terminated electron transport chains responsible for substrate oxidation have been studied. Menaquinone appears to be the sole isoprenoid quinone [7] but possible changes in quinone with growth conditions have not been studied [8]. Here we give the first description of the cytochrome composition of *H. pylori* and the effects of dissolved oxygen tension on cytochrome and quinone levels.

2. Materials and methods

2.1. Organisms, culture conditions and cell fractionation

The strains used were: *Helicobacter pylori* NCTC 11637 and two clinical isolates (Glaxo Wellcome strains 8091 and 4187E); *Helicobacter mustelae* (ferret isolate, Glaxo Wellcome strain 8093569E); *Helicobacter acinonyx* (ATCC 51101); and *Helicobacter nemestrinae* (ATCC 49396). Stock cultures were maintained on 5% chocolate Columbia agar (Oxoid) [6], supplemented with polymyxin B, amphotericin B and vancomycin (each at 10 mg l⁻¹) and sub-cultured twice weekly. Plates were incubated in a VAIN cabinet (Don Whitley) with 5% (v/v) oxygen and 10% (v/v) carbon dioxide. For growth at various oxygen concentrations, bacterial lawns were prepared on chocolate blood agar plates and incubated for 2 days at 37°C in gas mixtures containing variable amounts of oxygen (2–15%, v/v) and N₂ with 10% (v/v) CO₂ prepared using a Mart® Anoxomat. For quinone determinations, cells were harvested into ice-cold phosphate-buffered saline (PBS), washed twice and then freeze-dried. Biomass was determined by dry weight measurements. For spectroscopic studies, cells were suspended in 200 mM MES-Tris buffer (pH 7) containing 100 μM phenyl methyl sulfonyl fluoride (PMSF) and stored at −20°C. Cell fractions were obtained by sonication [6]; the whole sonicate was centrifuged at 700 × g for 10 min to remove whole cells and then at 100 000 × g for 60 min to obtain a pellet (‘membranes’) and a supernatant ‘cytoplasm’. Membranes were washed thrice and resuspended in quarter-strength Ringer’s solution (Sigma) containing 50 mM PIPES buffer (pH 6.5) and stored as aliquots at −70°C. Dilutions for spectral studies were made in 100 mM K phosphate buffer (pH 7.0). Spectra (see Fig. 1) were also obtained for cells grown in a 45 liter batch of BHI medium (Oxoid) containing 0.5% (v/v) foetal calf serum. A Biolafitte 75 liter stirred tank fermenter, fitted with three Rushton turbine impellors and baffles, was used. The fermenter was gassed with CO₂, N₂ and air and balanced on the off gas line by mass spectrometry to achieve 12% (v/v) CO₂ and 5% (v/v) O₂. Cells were harvested at 24 h and cell fractions prepared as described above.
2.2. Extraction and determination of isoprenoid quinones

Procedures were essentially as described by Collins [9]. Lipid from samples of 30 mg dry weight of cells was extracted into 30 ml of chloroform/methanol (2:1 v/v) over 2 h with stirring. After filtration, the solvent was rotary evaporated under vacuum at 40°C. The extract was resuspended in 1 ml of chloroform/methanol (2:1 v/v) and applied to a TLC plate (Merck Art 5735 Kieselgel 60) with menaquinone and ubiquinone standards. The plate was developed in hexane-diethyl ether (85:15 v/v) and visualised under UV light (254 nm). The dark band of menaquinone had an Rf of 0.7–0.8. The quinone was eluted from the silica in 1 ml chloroform through a sintered glass filter. The sample was evaporated to dryness under nitrogen and stored under nitrogen at −20°C if necessary. Concentrations of menaquinones were estimated spectrophotometrically [10]. The change in absorption at 245 nm after reduction with borohydride was compared to that for menaquinone-4 (MK-4; Sigma) to calculate menaquinone content. The identity of menaquinones was confirmed by electron impact mass spectroscopy (MS) and after further purification using reverse phase HPLC by spectral analysis and thermospray MS [11].

2.3. Cytochrome and other assays

Visible electronic absorption spectra were recorded in a Johnson Foundation SDB3 dual-wavelength scanning spectrophotometer. Spectra were scanned at room temperature from 400 to 800 nm (reference 575 nm), at a scan rate of 2.9 nm s⁻¹ using a spectral band width of 4 nm. Absorption coefficients used were: cytochrome c, 552–538 nm, 18.3 mM⁻¹ cm⁻¹ [12]; cytochrome b, 558–575 nm, 17.5 mM⁻¹ cm⁻¹ [13]; high-spin CO-binding haemoprotein, 419–436 nm, 215 mM⁻¹ cm⁻¹ [14]; low-spin CO-binding haemoprotein, 569–556 nm, 25 mM⁻¹ cm⁻¹ [14]. Protein determinations were as described before [6].

3. Results

3.1. Identification and quantification of quinones

Separation of lipid extracts from H. pylori NCTC 11637 by TLC showed a single menaquinone band. Electron impact MS revealed M⁺ of 580 (menaquinone-6, MK-6, molecular mass 580 Da), with m/z ions of 511, 443, 375, 307 and 239 (fragmentation ions as isoprene units are successively cleaved off), 225 and 187 (fragmentation ions derived from

Table 1

Cytochrome and menaquinone levels in H. pylori NCTC 11637 grown at various oxygen concentrations

<table>
<thead>
<tr>
<th>[O₂] (%) v/v</th>
<th>Menaquinone content (nmol g cells⁻¹) ¹</th>
<th>Haemoprotein content (nmol (mg protein)⁻¹)</th>
<th>'high-spin CO-binding pigment' b</th>
<th>'low-spin CO-binding pigment' c</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>38</td>
<td>0.16</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>0.38</td>
<td>0.36</td>
<td>0.27</td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>0.29</td>
<td>0.22</td>
<td>0.13</td>
</tr>
<tr>
<td>15</td>
<td>14</td>
<td>0.38</td>
<td>0.38</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The cytochrome data are from one experiment but similar patterns of change were seen in a replicate experiment. The quinone data are also from a single experiment but similar patterns of change were observed in two further experiments.

¹ Cell yield was determined as dry weight harvested from the plates.

² 'High-spin CO-binding pigment' is defined as the component(s) observed in CO difference spectra with an absorption maximum in the Soret region near 418 nm and a trough near 440 nm [14].

³ 'Low-spin CO-binding pigment' is defined as the component(s) observed in CO difference spectra with an absorption minimum in the α/β region near 552 nm and measured relative to the neighbouring peak at about 576 nm [14].
the naphthoquinone nucleus) and 69 (isoprene unit) confirming the identity of MK-6. A M+ of 444 suggested a lower (approx. 10%) level of MK-4. Reverse phase HPLC confirmed a major peak of MK-6 eluting after a minor peak of MK-4 by observation of the expected UV absorption profiles and, following thermospray MS, detection of pseudomolecular ions of 581 and 445 Da, equivalent to MK-6 and MK-4, respectively. H. pylori strains 8091 and 4187E, H. mustelae and H. acinonyx gave similar results, although H. nemestrinae lacked MK-4. No ubiquinone was detected.

Yields of cells after 2 days of growth on plates were markedly dependent on oxygen concentration. Growth (1.7 mg dry wt per plate) was poorest at 2% (v/v) oxygen, consistent with the absolute requirement of H. pylori for oxygen [1], and increased to reach a maximum yield (7.1 mg dry wt per plate) at 10% (v/v) oxygen. At 15% (v/v) oxygen, growth (4.0 mg dry wt per plate) was inhibited, consistent with the well-established view that H. pylori is a microaerophile [1]. No evidence was obtained for a qualitative change in the quinones extracted from cells grown at various oxygen concentrations. However, there were marked changes in the menaquinone content (Table 1), which was maximal at 5–10% (v/v) oxygen.

### 3.2. Haem proteins in whole cells, membranes and cytoplasmic fractions

In reduced minus oxidised difference spectra (Fig. 1a) of whole cells, the peak at 552 nm is attributed to cytochrome(s) c and the shoulder at about 560 nm to cytochrome(s) b. The β-band (524 nm) and the Soret (γ) band (430 nm) are each attributed to the fused bands of cytochromes b and c. Notable was the absence of signals in the red region of the spectrum that would indicate the presence of cytochromes aa₃ (about 600 nm), cytochrome d (630 nm) or cytochrome b₅₆₃ (595 nm).

In the CO difference spectrum of cells (Fig. 1d), the 416 nm peak is typical of the CO-ligated form of cytochrome o. However, the 438 nm trough is at too long a wavelength to be typical of the unligated, reduced form of cytochrome o and is probably due to a CO-binding cytochrome b. The breadth and asymmetry of both the γ-bands and the α-trough (with a minimum at 553 nm) reveal more than one CO-binding component. The 628 nm band suggests the presence of a component that reacts with CO to form an adduct with an absorption maximum at this wavelength. The band position is similar to that of the CO adduct of cytochrome d (about 636 nm [15]) despite the lack of a 630 nm peak in Fig. 1a (see Section 4).

The reduced minus oxidised difference spectrum (Fig. 1b) of the soluble fraction reveals cytochrome c. The α- (552), β- (524 nm) and γ-bands (420 nm) are all characteristic of this cytochrome. The CO spectrum (Fig. 1e) reveals that only a small fraction of cytochrome c binds CO, a property described for numerous bacterial c-type cytochromes [14] and not in itself evidence of an oxidase function.

In the reduced minus oxidised spectrum of membranes (Fig. 1c), the twin peaks at 552 and 560 nm are probably the contributions of cytochromes c and b respectively. The dominant γ-band (430 nm) is from cytochrome b, and the split β-band (peak at 522 nm) is due to c- and b-type cytochromes. The absence of bands near and above 600 nm confirm the absence of cytochrome aa₃ or d-type terminal oxidases. However, the CO spectrum (Fig. 1f) retained the 628 nm band of intact cells. The undulations between 500 and 600 nm indicate the presence of CO-binding c- and/or b-type haemoproteins. The γ-bands are broad and have contributions from more than one component. The 418 nm peak is consistent with, but not proof of, the presence of cytochrome o or a CO-binding cytochrome b.

The spectra of cells grown under various oxygen concentrations were qualitatively similar (not shown) but, in cells grown at 2% (v/v) and 5% (v/v) oxygen, the α-bands were superimposed on a broad featureless signal. At no oxygen concentration were signals attributable to cytochromes 4₃₃ or d observed. The CO difference spectra showed no striking differences at the various oxygen concentrations. The γ-peaks were at 418–419 nm and troughs at 436 nm at all oxygen concentrations except 15% (v/v). Here, the band was broader, shifted to 438 nm and the γ- and α-signals of much lower amplitude (not shown). The levels of all haem proteins assayed (cytochromes c and b, and the CO-binding components) were lowest at sub-optimal oxygen concentrations (2% (v/v)) (Table 1). Both CO bind...
ing haem proteins, particularly the high-spin type which is likely to represent the terminal oxidase, were highest at the oxygen concentrations (5–10% O₂) that were also optimal for growth and menaquinone content (Table 1).

4. Discussion

In agreement with earlier findings [16], MK-6 was the dominant menaquinone of *Helicobacter* and ubiquinones were not detected. The 5-methyl-substituted MK-6, present in certain *Campylobacter* species [7], and the unusual, unidentified MK-6 species of *H. pylori* reported by Moss et al. [16], were not seen. There was no evidence that quinone composition varied with oxygen supply and the reduced menaquinone concentration of *H. pylori* cells grown in 2 and 15% (v/v) oxygen (Table 1) may reflect diminished biosynthetic capabilities under sub-optimal growth conditions. In facultatively aerobic bacteria such as *E. coli*, oxygen supply influences quinone composition [8]. The relatively narrow range of oxygen concentrations in which *H. pylori* is thought to grow in vivo may require no drastic changes in respiratory chain composition or gene expression (c.f. [15]). This suggestion is supported by cytochrome assays, which showed no substantial changes in cytochrome levels other than slight decreases in total cytochrome content at sub-optimal oxygen concentrations. A puzzling and perhaps novel feature of spectra reported here is the absorbance in membranes and whole cells at about 628 nm in CO difference spectra. This band is close to the position (about 636 nm) for the CO complex of cytochrome *d*, yet the corresponding band of the ferrous form is not seen when the ferrous form is trapped as the CO adduct. It is perhaps pertinent to microaerophily that cytochrome *d* confers on *Azotobacter vinelandii* the fastest respiration rates observed for any bacterium and the ability to fix nitrogen in aerobic conditions by respiratory protection [17].

The terminal oxidase supporting aerobic respiration of *H. pylori* probably contains haems O and/or B [15]. In the CO difference spectrum of membranes (Fig. 1f), the γ-band at 418 nm is consistent with this. The trough at about 430–432 nm [18] that would be expected to accompany this signal for a cytochrome *o* is not clear, but may be obscured in the broad trough centred at 442 nm. Similarly the α/β bands at 544 and 576 suggest the possible presence of cytochrome *o*, although the trough centred at 562 is not typical of a high-spin ligand-binding haem (and has been quantified in Table 1 on the assumption that the signal arises largely from a CO-binding pigment that is low-spin in the ferrous state [14]). Recently, evidence has been obtained from random genome sequencing of *H. pylori* [19] for a cytochrome *cb*-type oxidase homologous to FixNOQP found in *Bradyrhizobium japonicum* and other bacteria [20]. The FixN (= CcoN) cytochrome *b* may be the CO-binding component seen in our spectra. Other components that might contribute to the absorption spectra are hydroperoxidases and haemoglobin-like proteins.

Cytochrome *c* is clearly present in cytoplasmic fractions of *H. pylori* (Fig. 1b) and is probably also responsible for the 552 nm component in membranes (Fig. 1c). Previously, cytochrome *c* was tentatively identified (without spectral data) as the soluble 14 kDa ascorbic acid-oxidising component of *H. pylori* [21]. It is probable that the oxidase-positive character of *Helicobacter* is due to the presence of a membrane-bound terminal oxidase, such as FixN (= CcoN), capable of accepting electrons from cytochrome *c*.

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