J. Biochem. 125, 194-201 (1999)

Characterization of a *cb*-Type Cytochrome *c* Oxidase from *Helicobacter pylori*[1]

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Received August 24, 1998; accepted October 9, 1998

Helicobacter pylori is a microaerophilic Gram-negative spiral bacterium residing in human stomach. A cb-type cytochrome c oxidase that terminates the respiratory chain was purified to near homogeneity by solubilizing the membranes with Triton X-100 and applying anion exchange, Cu-chelating, and gel filtration chromatographies. Redox- and CO-difference spectra and pyridine ferrohaemochrome analysis showed the enzyme to contain three haems C, one low-spin protohaem, and one high-spin protohaem that probably forms a dioxygen-reducing bimetalic center with a copper atom. The enzyme actively oxidizes soluble cytochrome c from this bacterium (TNmax of about 250 s^{-1}) with a K_m of 0.9 μM. Yeast cytochrome c and N,N,N',N'-tetramethyl p-phenylenediamine (TMPD) are also oxidized at similar maximal velocities with larger K_m's. Oxygen pulse experiments on resting cells in the presence of ascorbate plus TMPD or L-lactate indicated that this sole terminal oxidase pumps H^+, although the H^+ pumping activity by proteoliposomes reconstituted from the enzyme and P-lipids was low. Two main bands with haem C at 58 and 26 kDa were observed upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and succeeding protein and haem staining. Sequencing of the operon encoding the subunits of the enzyme revealed the presence of ccoNOQP. N-terminal analysis of the 58 kDa band showed 15 or 13 amino acids coinciding with the amino acid sequences deduced from the DNA of ccoN and ccoO. CcoN, the largest subunit bearing two protohaems and copper, and ccoO, a mono-haem cytochrome subunit form a protein complex with an apparent molecular mass of 58 kDa, even in the presence of sodium dodecyl sulfate. The 26 kDa band is tentatively assumed to be ccoP with two haems C.

Key words: cytochrome c oxidase, cytochrome cb, c-type cytochrome, *Helicobacter pylori*, heme-copper oxidase.

Helicobacter pylori is a Gram-negative spiral bacterium that resides in human stomach. This bacterium is a known microaerophilic (5-7% O_2 preferable) but obligate aerobe (*1, 2*). Oxidase activity measurement of the membrane fraction has shown the presence of strong (about 0.3 μmol/min/mg protein) cytochrome *c* and *N,N,N',N'*-tetramethyl *p*-phenylenediamine (TMPD) oxidase activities (*3, 4*). Redox and CO-difference spectra indicated that the responsible terminal oxidase is not an aa_3-type but a $cb(cb_3)$-type cytochrome *c* oxidase that shows high O_2 affinity (K_m, below 0.4 μM) and was very susceptible to cyanide (K_i = 2.6 μM). A *cb*-type (or cbb_3-type) cytochrome *c* oxidase was found in *Bradirhizobium japonicum* fixing nitrogen in root nodules as a terminal oxidase (*5-7*) with a very low K_m for O_2 (*8*). This oxidase has also been found in photosynthetic bacteria such as *Rhodobacter sphaeroides* (*9*) and *Rhodobacter capusulata* (*10*), as well as in N_2-fixing Rhizobiaceae such as *Rhizobium meliloti* (*11*) and *Azorhizobium caulinodans* (*12*). All these bacteria belong to the alpha subdivision of proteobacteria. The enzymes from these bacteria have been shown to comprise three subunits with high-spin haem-Cu_B binuclear centers for dioxygen reduction to water in the largest subunit (subunit I). The genes encoding the subunits of these proteins are *fixNOQP* in Rhizobiaceae, *ccoNOQP* in photosynthetic bacteria (*13, 14*), and a closely related bacterium, *Paracoccus denitrificans* (*15*). Multiple alignment of the amino acid sequences of fixN and coxN also indicates that the genes for *cb*-type enzymes are closely homologous to the gene for subunit I of cytochrome aa_3 (*16-18*), and thus the *cb*-type enzymes also belong to the

[1] This study was supported in part by Grants-in-Aid (No. 10129225, priority area "Biometalics") from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: bp, base pair; Da, dalton; Hepes, 2-[4-(2-hyrdoxyethyl)-1-piperazinyl]ethane sulfonic acid; HOQNO, 2-heptyl-4-hydroxy-quinoline *N*-oxide; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; TMPD, *N,N,N',N'*-tetramethyl *p*-phenylenediamine.

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haem-copper oxidase super-family with well known cytochrome aa_3-type cytochrome *c* oxidases. Despite the similarity in subunit I, the other subunits of the *cb*-type oxidases are quite different from those in the usual aa_3-type cytochrome *c* oxidase; instead of Cu_A-bearing subunit II and a very hydrophobic subunit III bearing no chromophore, the known *cb*-type terminal oxidases contain monohaem and di-haem *c*-type cytochromes as the second and third subunits.

We reported that a *cb*-type cytochrome *c* oxidase functions as the solo terminal oxidase in *H. pylori* (*3*), which is far from the proteobacteria alpha subdivision (*19, 20*). Here we describe a purification procedure for *cb*-type cytochrome *c* oxidase, and show that it consists of three subunits, a catalytic subunit I, monohaem, and dihaem subunits, and catalyzes H^+-pumping cytochrome *c* oxidation. A somewhat different subunit pattern on SDS-PAGE from those of rhizobia and purple photosynthetic bacteria was interpreted in the light of DNA sequencing by us and the sequence of the entire *H. pylori* genomic DNA recently reported (*21*). This is the first enzyme-level report of a *cb*-type oxidase from a bacterium from outside the proteobacteria alpha subdivision.

MATERIALS AND METHODS

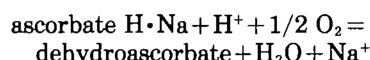
Reagents—Cytochrome *c*-553 was prepared from *H. pylori* as described elsewhere (Tsukita and Sone, to be published). T4-DNA ligase, Klenow fragment, DNA polymerase from *Thermus aquaticus* (*Taq* polymerase), restriction enzymes, the exonuclease III deletion kit, and plasmid vector pUC118 were obtained from Takara Shuzo (Kyoto). Hybond-N^+ for DNA blotting and PVDF membranes for protein blotting were purchased from Amersham. pT7Blue vector was from Stratagene (La Jolla, CA). TMPD and *o*-tolidine were purchased from Wako Pure Chemicals (Osaka), and equine cytochrome *c* (type VI), yeast cytochrome *c* (type 8), nigericin, and HOQNO were from Sigma Chemicals (St. Louis). DEAE-cellulose (DE52) was a product of Whatman (Maidstone, Kent), and Q-Sepharose FF, chelating Sepharose FF, and the Superdex gel-filtration column (HR10/30) were purchased from Pharmacia (Uppsala). Other chemicals, inhibitors, and detergents were obtained as described previously (*3, 22*).

Bacterial Strains and DNA Preparation—*H. pylori* NCTC11637 was cultured and its membrane fraction was prepared as described previously (*3*). Genomic DNA were prepared from *Campylobacter jejuni* NCTC 11951, *Wollinella succinogenes* DSM 1740, *P. denitrificans* DMS 65, and *Helicobacter felis* ATCC 49179, as well as from *H. pylori* NCTC11637.

Purification Procedure—The membrane fraction from *H. pylori* was washed once with 50 mM Hepes buffer (pH 7.0) containing 0.5% sodium cholate, 0.5 M NaCl, and 15% (w/v) glycerol and centrifuged. The resulting residue was extracted with 5 ml of a mixture of 3% Triton X-100, 50 mM Hepes buffer, 0.1 M NaCl, and 1 mM phenylmethylsulfonyl fluoride for 1 h with stirring. The soluble fraction was diluted with an equal volume of H_2O and applied to a DEAE-cellulose column (1×3 cm). The passthrough fraction was applied on a Q-Sepharose column (0.8×4 cm) equilibrated with water. The column was washed first with 20 mM, then with 40 mM, and finally with 50 mM NaCl containing 1% Triton X-100, Hepes buffer, and glycerol. This caused the red band to move slowly. The red band was eluted by increasing the NaCl concentration to 80 mM, and the fractionated eluate was measured for TMPD oxidase activity. The active fractions were pooled and applied to a chelating Sepharose column (0.4×1.5 cm) loaded with Cu(II) and 0.5 M NaCl in buffer. The column was first washed with buffer containing 1% sucrose monolaurate, 5 mM imidazol, and 0.5 M NaCl, and the oxidase was eluted by raising the concentration of imidazol to 20 mM. The reddish eluate (0.4 ml), concentrated to 0.1 ml, was fractionated through a Sepharose column (HR10-30) equilibrated with 20 mM Hepes buffer (pH 7.0) containing 0.1 M NaCl, 0.2% sucrose monolaurate, and 10% glycerol.

Proteoliposome Preparation—Proteoliposomes were prepared by freeze-thaw sonication using the partially-purified *cb*-type oxidase (step 4) and soybean P-lipids as described previously (*23*).

Measurements of Enzyme Activities—Oxygen uptake of the membrane fraction was followed with an oxygen electrode (No. 4005, Yellow Spring Instrument, Yellow Spring) in a semiclosed vessel (2.5 ml) containing the reaction medium of 50 mM sodium phosphate buffer, pH 7.1. The cytochrome *c* and TMPD oxidase activities of the purified enzyme were measured by following the pH change with ascorbate as a final electron donor according to the following equation (*24*):



$$\text{ascorbate H}\cdot\text{Na}+\text{H}^+ + 1/2\ \text{O}_2 = \text{dehydroascorbate}+\text{H}_2\text{O}+\text{Na}^+$$

The alkaline formation was back-titrated with an aliquot of 5 mM HCl. H^+ pump activity of the *cb*-type oxidase upon cytochrome *c* pulse was followed with a glass electrode (Bechman 39531) equipped in an open glass cell, while that of the resting cells was followed with a glass electrode (Horiba 3678) in a semi-closed glass cell. The contents of the vessels were all thermostatically controlled and mixed with a magnetic stirrer.

Analytical Procedures—Absorption spectra were measured on a Beckman DU-70 spectrophotometer. Haem contents were determined according to the method of Berry and Trumpower (*25*). SDS-PAGE, protein determination, and haem staining were carried out as described previously (*22*). Peptide sequences were obtained by Edman degradation with an Applied Biosystem model 491A gas/liquid phase sequencer. Time of flight mass spectra were measured in a Kratos Kompact Maldi I mass spectrometer.

DNA Manipulations—For sequencing of the *H. pylori cb*-type oxidase gene, two sets of primers were designed for PCR targeting the very conserved region in the largest subunit of the haem-copper oxidase super family, including *cb*-type enzymes; 5′-CA(A/G)TGGTGGTA(T/G)GGNG-A(T/C)AA for QWWYGHN in helix VI as a sense primer, and 5′-ATNGTCCA(A/G)TCNGT(A/C)TA(G/T)AG for HYTDWTI in helix X as an antisense primer (see Fig. 6). The PCR (94°C 2 min/45°C 1 min/72°C 2 min for 30 cycles) product obtained with *H. pylori* genomic DNA (1 μg) as a template was almost 450 bp, and cloned into pT7Blue T-vector. PCR was also carried with genomic DNA from *Helicobacter felis*, *C. jejuni*, *W. succinogenes*, and *P. denitrificans*. These PCR products were purified by agarose gel electrophoresis, and the DNA sample was sequenced with a Prism dye deoxy terminator cycle sequencing kit

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(Applied Biosystems, CA, USA) on an automated DNA sequencer (Applied Biophysics model 373), as described previously (*26*). This DNA was labeled with digoxigenin following the manufacture's protocol (Boehringer, Mannheim), and used as the probe for *H. pylori ccoNOQP* cloning. For this a cosmid library of *H. pylori*, strain HP206 (*27*) was used. The DNA sequencing was carried out by the dye primer method with a pUC118 in a Shimazu PQ100 DNA sequencer (DSQ-1000). The general gene manipulations followed those of Sambrook *et al.* (*28*). The sequence data were analyzed with a Genetix (Tokyo) software program.

RESULTS

Purification of the cb-Type Oxidase—The membrane fraction (*3*) was first washed with cholate to remove peripheral membrane proteins, and then solubilized with Triton X-100. The media for chromatography contained glycerol (10-15%) to stabilize the enzyme. A typical purification results is summarized in Table I. The last step (gel filtration) showed the enzyme to be about 200 kDa in the presence of detergent. The enzyme seems to be monomeric in buffer containing Triton X-100, since the molecular mass from the deduced amino acid composition is about 124 kDa and intrinsic membrane proteins usually bind large amounts of detergent/lipid molecules. The enzyme could be kept frozen at −80°C without severe loss of activity.

Chromophores and Subunit Structure—Figure 1A shows that only *c*-type cytochromes predominate in the reduced form with a shoulder around 561 nm indicative of the presence of *b*-type cytochromes in the enzyme. An oxidized form spectrum is shown in Fig. 1B, with the redox difference spectrum in Fig. 1C as an insert. The CO-difference spectrum (not shown) was almost the same as that of the solubilized membrane (*3*), indicating that this *cb*-type cytochrome is the sole haemprotein to react CO. The fact that no other terminal oxidase gene except *ccoNOQP* is found in the whole genomic DNA (*21*) is also noteworthy. Analysis of the haem content by pyridine haemochrome showed the content of C-haem and protohaem to be about 21 and 10 nmol/mg protein, respectively. The haem C content in the washed membranes was 0.2-0.3 nmol/mg protein, and thus about 60-90-fold concentration was attained during purification. The subunit structure was examined by gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) as shown in Fig. 2. Protein staining revealed two main (58 and 26 kDa) and one faint (32 kDa) bands, if very faint bands are neglected (lane 3). The 58 and 26 kDa bands showed peroxidase activity due to covalently-bound haems (lane 5). The 32 kDa band also showed peroxidase activity, although much weaker than the 58 or 26 kDa bands. It is noteworthy that to date no large subunit with a haem-copper binuclear center has been reported to bear haem C (*29*). Automated Edman degradation analyses of the 58 kDa band blotted to PVDF membranes gave the sequence MQENVPLSYDYSISK-. Analyses of sheets on PVDF membranes treated with 0.6 N HCl (*30*) gave a pair of amino acid residues in similar amounts for every step, indicating that one more sequence (MFSFLEKNPFFFT) preceded by fMet may be present. The former is consist with the amino acid sequence at the N-terminus of subunit I as deduced from ccoN, and the latter with that of the mono-haem protein deduced from ccoO (*21*).

In contrast, very low concentrations of amino acid derivatives were obtained with membranes blotted with the 26 kDa band, even when treated with 0.6 N HCl, indicating that the N-terminus of this protein is blocked. It

TABLE I. **A summary of the purification of cytochrome *c* oxidase.**

Step	Total protein (mg)	Total activity (yield) [μmol/min (%)]	Specific activity (μmol/min/mg)
1. Washed membrane	62.8	21.2 (100)	0.34
2. Triton X-100-solubilized	8.9	13.5 (64)	1.52
3. Q-Sepharose	1.47	7.0 (33)	4.8
4. Chelating Sepharose	0.27	1.2 (5.7)	4.4
5. Gel-filtration	0.11	0.80 (3.4)	7.3

TMPD oxidase activity was measured spectrophotometrically at 562 nm with a single-beam spectrophotometer (Beckman DU-70) using 0.1 mM TMPD at 25°C. The reaction medium comprised 0.1 ml of 1 mM EDTA and 20 mM Na-P$_i$ buffer, pH 6.0.

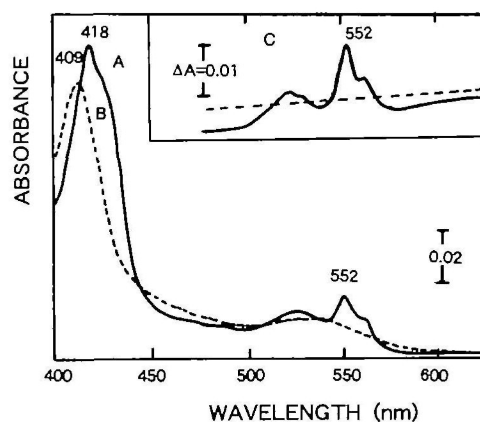

Fig. 1. **Spectra of *H. pylori* cytochrome *c* oxidase.** A, $Na_2S_2O_4$-reduced form spectrum; B, oxidized form spectrum as prepared; C (insert), reduced *minus* oxidized difference spectrum. The cytochrome *cb*-type oxidase preparation (29 μg/ml) was used.

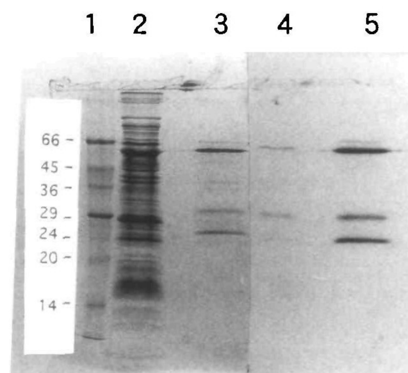

Fig. 2. **SDS-PAGE pattern of the *H. pylori* cytochrome *cb*-type oxidase preparation.** The polyacrylamide concentration in the gel was 12.5%; the gel was stained with Coomassie Brilliant Blue R-250 (lanes 1-3), or detected for heme with *o*-tolidine (lanes 4 and 5). Lane 1, marker proteins; lanes 2 and 4, membrane preparation (8.5 μg); lanes 3 and 5, the purified sample (1.1 μg).

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is thus likely that the 58 kDa band is composed of two subunits of the *cb*-type oxidase, and the 26 kDa subunit is a haem C-bearing dihaem or mono-haem subunit of the enzyme (see also "DISCUSSION"). However, the 32 kDa band seems to be due to a contaminating *c*-type cytochrome, since this band is found mainly in the more-rapidly eluted fraction from the gel filtration. It is also noteworthy that the 32 kDa band showed the strongest peroxidase activity among the membrane proteins on the SDS-PAGE gel (lane 4), although the band was relatively faint in the final preparation (lane 5).

Catalytic Properties and the Role of Cytochrome c-553—*H. pylori cb*-type cytochrome is a cytochrome *c* oxidase. Kinetic constants of the purified enzyme with cytochromes *c* from different sources and TMPD are summarized in Table II. The K_m for O_2 was 0.04 μM as reported for the membrane preparation (*3*). The *H. pylori* enzyme showed high molecular activities with these electron donors except equine cytochrome *c*. Similar substrate specificities were observed using the membrane fraction as the terminal oxidase (*3*). In other words the purified *cb*-type oxidase is just the enzyme that is working in the membrane. The pH dependency of the oxidase activity is very similar to that of membranes with an optimal pH of about 5.5. The K_i for cyanide (2.6 μM) is almost coincidental with that of the membrane also. A very low K_m for *H. pylori* cytochrome *c*-553 with an alpha band at 553 nm in the reduced form suggests that this cytochrome may be the physiological substrate for the *cb*-type terminal oxidase. Data on the respiratory chain of photosynthetic bacteria have shown that cycM, membrane-bound *c*-type cytochrome mediates electron transfer between the cytochrome bc_1-complex and aa_3-type cytochrome *c* oxidase as in the case of cytochrome *c* in mitochondoria (*31*). However, the corresponding gene is not found in the genome sequence of *H. pylori* (*21*), and in *R. leguminosarum* a soluble cytochrome *c* has been reported to be the donor for the *cb*-type oxidase (*32*).

H^+-Pumping Activity of H. pylori Cytochrome c Oxidase—Many bacterial cytochrome aa_3-type cytochrome *c* oxidases have been shown to pump H^+ (*23, 33*). Among *cb*-type oxidases, the enzyme from *P. denitrificans* is reported to pump H^+ in Hepes buffer (*15, 34*), although it has also been reported not to pump H^+ in glycylglycine buffer (*35*). In order to know whether the *cb*-type *H. pylori* oxidase pumps H^+, the partially purified enzyme was reconstituted in liposomes, and a cytochrome *c* pulse was applied. Figure 3 shows a very low H^+ release occurs outside the proteoliposomes (trace A), while H^+ uptake due to H_2O formation occurs in the presence of an uncoupler (trace B). The proteoliposomes seem not to be leaky, since they show a respiratory control ratio of about 3 when oxygen uptake is measured in the presence of 15 μM yeast cytochrome *c* (not shown). Thus, it is likely that the purified *H. pylori* oxidase pumps H^+ at a very low efficiency. We then measured the H^+/O ratio in the intact cells in which *cb*-type oxidase is working as the sole terminal oxidase, since the enzyme may lose H^+ pump activity without losing its oxidase activity during purification.

Resting *H. pylori* cells were incubated with ascorbate and TMPD in a semi-closed vessel, and an oxygen pulse was applied after anaerobiosis as shown in Fig. 4. The rate of the endogenous respiration was very low as pointed out by Chalk *et al.* (*36*). In the presence of valinomycin and KSCN and the absence of nigericin (trace A), the cells extruded H^+ at the moment of the oxygen pulse, and then gradually

TABLE II. **Oxidase activities and inhibition by KCN.**

	Substrate			
	H. pylori cyt. *c*-553	Yeast cyt. *c*	Equine cyt. *c*	TMPD
V_{max} (s^{-1})	252	250	72.6	247
K_m (μM)	0.9	15.2	1.1	108
I_{50} (μM) for KCN	–	–	–	2.6

The reaction medium (2 ml) comprised 10 mM KCl, 10 mM sodium ascorbate, and 2 mM 4-morpholinepropane sulfonic acid buffer (pH 6.5). The reaction was carried out at 30°C.

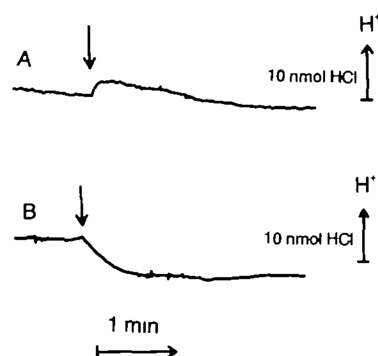

Fig. 3. **Change in pH upon cytoochrome *c* pulse by proteoliposomes reconstituted from *H. pylori cb*-type oxidase.** Proteoliposomes containing 41 μg *H. pylori cb*-type oxidase preparation and 4 mg soybean phospholipids in 0.1 ml were suspended in 2.4 ml of reaction medium composed of 25 mM K_2SO_4, 2.5 mM $MgSO_4$, and 0.1 μg/ml valinomycin at 35°C. Yeast ferrocytochrome *c* (6.8 nmol) was added at the time shown by the arrow. A, an experiment without uncoupler; B, as A but in the presence of 1 μg carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, an uncoupler.

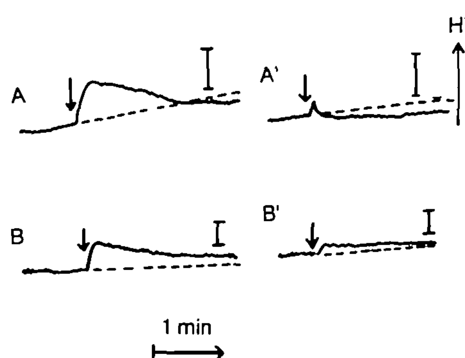

Fig. 4. **Change in pH upon oxygen pulse to an anaerobic *H. pylori* cell suspension.** Cells (1.2×10^9) were incubated at 35°C in 3.2 ml of 0.15 M KCl containing of 50 mM KSCN and 0.5 mM K-MOPS (pH 6.7-6.9). After anaerobiosis, 1 μl aliquots of 0.2 mg/ml valinomycin and 0.1 M HOQNO were added, and after about 10 min, the reaction was started by adding a 40 μl aliquot of air-saturated 0.15 M KCl. The buffer action of the medium was determined by titration with a 2 μl aliquot of 20 mM HCl. The bar indicates the pH change due to 40 nmol HCl. A, 2 mM ascorbate and 0.1 mM TMPD were added as the substrate; A′, as A but in the presence of 0.1 μg nigericin; B, 5 mM L-lactate was used as the substrate; B′, as B but in the presence of 0.1 μg nigericin.

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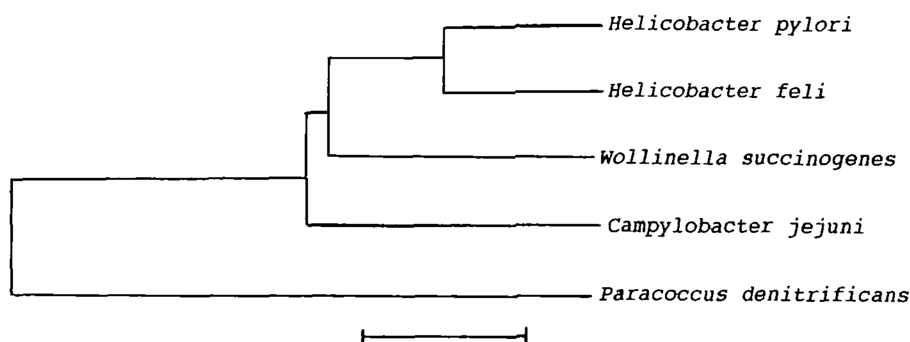

Fig. 5. A phylogenetic tree for a PCR amplified part of *ccoN/fixN* encoding subunit I. The scale bar represents a distance of 10%.

equilibrated to a new level. In the presence of nigericin, where a pH difference across the cell membrane could not be maintained, the oxygen pulse resulted in net alkalinization due to H_2O formation from $2e^- + 2H^+ + 1/2O_2$ and $1H^+$ absorption due to conversion from ascorbate to dehydroascorbate (trace A′). The apparent H^+/O ratio was about 2.5 and true H^+/O ratio is thus 1.5. A similar experiment using L-lactate as the substrate shows an apparent H^+/O ratio of 3.6 in the absence of nigericin (trace B), while no net pH change is observed in the presence of nigericin (trace B′), indicating that the true H^+/O ratio excluding scaler H^+ formation due to lactate oxidation may be 1.6. The lactate oxidation of *H. pylori* was not inhibited by HOQNO, suggesting that lactate dehydrogenases directly reduces cytochrome *c*-553 without the participation of cytochrome *c* reductase (the cytochrome bc_1 complex). On the other hand, succinate oxidation was inhibited by HOQNO (not shown). These experiments indicate that *H. pylori cb*-type cytochrome *c* oxidase has H^+ pump activity, but it is rather labile and lower in efficiency than that of aa_3-type oxidases.

Distribution of cb-Type Cytochrome c Oxidase in Proteobacteria—The proteobacterial epsilon subdivision includes several characteristic anaerobes and microaerobes including *Wollinella* and *Campylobacter* as well as *Helicobacter* (*20*). In order to examine the distribution of *cb*-type cytochrome *c* oxidases, we analyzed PCR products amplified by a set of primers targetting two conserved portions of fixN/ccoN (see "MATERIALS AND METHODS"). PCR products of about 450 bp were obtained when the genomic DNA preparations from several bacteria were used as the template. Direct sequencing revealed that these DNA to contain homologous sequences targetting helix VI to helix X in subunit I as expected. Figure 5 shows a DNA-based phylogenetic tree including *P. denitrificans* as a member of the alpha subdivision of proteobacteria. *H. pylori* forms a branch with other different species of *Helicobacter* (not shown except *H. felis*), and is different from that of the proteobacteria alpha subdivision, as represented by *P. denitrificans*. The fact that *Helicobacter*, *Wollinella*, and *Campylobacter* are far removed from other preoteobacteria, including alpha subdivision members has been pointed out (*19, 20*).

CcoNOQP Operon Encoding Subunits of cb-Type Oxidase—We cloned the whole operon encoding the subunits of the *cb*-type cytochrome *c* oxidase from *H. pylori* strain HP206 independently, since we needed the N-terminal sequences of the subunits to interprete the unexpected results of SDS-PAGE (Fig. 2), and the entire genome sequence was not then available. Cloning was carried out using the PCR product of *H. pylori* to select the cosmid library of *H. pylori* strain 206 (*27*); the *ccoNOQP* operon part found in one cosmid 4A3 was then subcloned into pUC118 for sequencing. The gene structure was the same as that reported for the whole genome (*21*), and thus the same as in proteobacteria in the alpha subdivision. The DNA sequence of *H. pylori* strain 206 shows ccoN to comprise 488 amino acid residues of 56,015 Da, ccoO, 232 residues of 26,590 Da, ccoP, 292 residues of 32,571, and ccoQ, 72 residues of 8,551. The DNA sequence around *ccoNOQP* of *H. pylori* strain HP206 has been submitted to the DDBJ/GenBank/EMBL databases under accession number, AB018105. About 96% of the bases in the coding region of *ccoNOQP* are identical to those in the corresponding region of the genome sequence reported recently (*21*).

DISCUSSION

We tried to purify a terminal oxidase from a very small amount of the membrane fraction of *H. pylori*. The enzyme, which contains haem C and protohaem, is a cytochrome *c*-oxidase that uses cytochrome *c*-553 as it substrate. The haem contents in the final preparation were 21 and 10 nmol/mg protein for haem C and protohaem, respectively. The enzyme should contain 24.6 nmol haem C and 16.4 nmol protohaem per mg protein if the enzyme contains three haems C and two protohaems in a molecular mass of 124 kDa calculated from the deduced amino acid sequences of each of the four subunits (Fig. 6). The haem C content of 21 nmol is close to the expected value, but the protohaem content is lower than expected. The present preparation may be somewhat contaminated by other proteins (lane 3 in Fig. 2), and/or the enzyme may lose protohaem during purification. In addition, the relatively high content of haem C may also be due to the presence of a 32 kDa contaminating protein containing haem C (lane 5 in Fig. 2).

The unexpected results of SDS-PAGE on this *H. pylori* oxidase, an appearance of a 58 kDa band with covalently-bound haem C (Fig. 2), was interpreted to be due to complex formation of ccoN (subunit I) and ccoO (monohaem subunit) even in the presence of SDS. The following results are in favour of the above interpretation. (i) Subunit I and the monohaem protein of the *B. japonicum* enzyme were recently reported to need another protein for their assembly (*37*). (ii) Several subunit proteins are known to exist in a complex or dimer structures upon SDS-PAGE (*38*). (iii) It seems reasonable to speculate that the complex of ccoN and O (56+26 kDa) would electrophorese at 58

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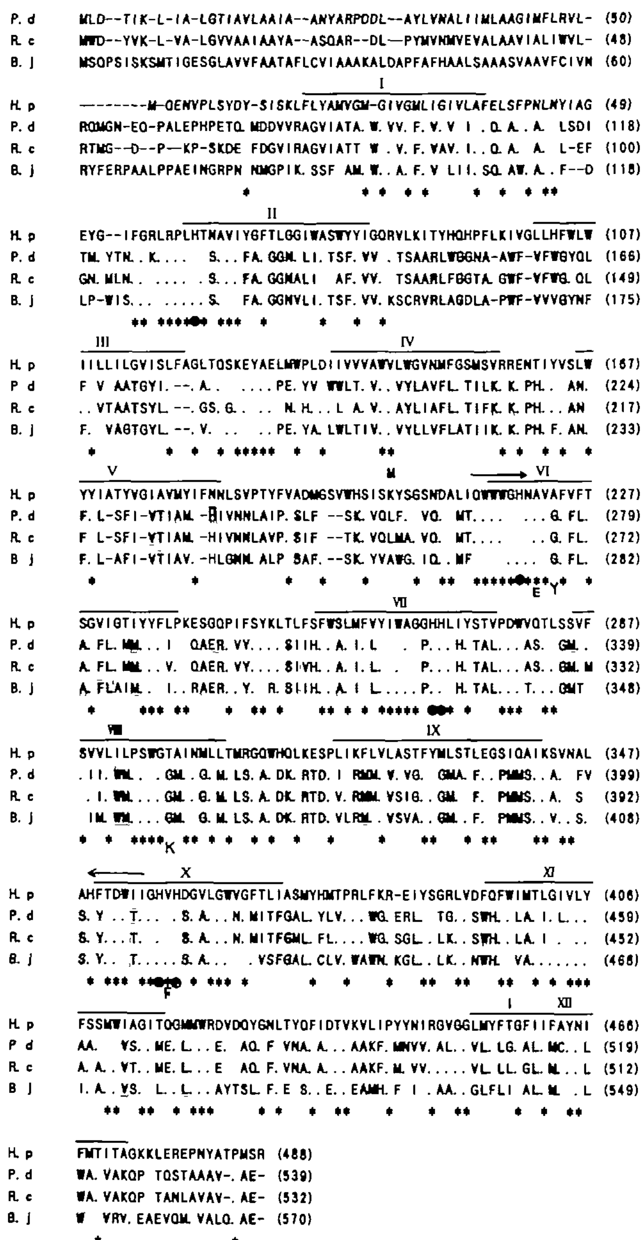

Fig. 6. **Multiple alignment of ccoN/fixN (subunit I) of *cb*-type cytochrome c oxidases.** Residues identical to those in *H. pylori* are shown by dots (·); gaps are indicated by —. The conserved residues are marked by *, while residues conserved only among the alpha group members (*i.e.* except *H. pylori*) are shadowed. Several conserved residues in the aa_3-type cytochrome *c* oxidase, which are referred to in "DISCUSSION," are indicated by one letter notations at the bottom of the alignment. The putative *trans*-membrane regions of the *H. pylori* subunits are underlined and numbered. The amino acid sequences used for a set of PCR primers are shown by the arrows on the top of the alignment. Hp, *H. pylori* strain HP206 (present work); Pd, *P. denitrificans* (*15*); Rc, *R. capusulatus* (*14*); Bj, *B. japonicum* (*7*). M and I on the top of the alignment at residues 204 and 457 as deduced from the complete genome sequence of *H. pylori* strain 26695 (*21*) are different from our present results.

kDa, since a complex containing the very hydrophobic subunit I of the haem-Cu terminal oxidase might migrate faster than proteins with the same molecular mass; *e.g.* subunit I of *Bacillus* PS3 caa_3-type oxidase (68 kDa based on the amino acid composition) electrophoreses as 46 kDa in the presence of SDS (*23*).

The 26 kDa haem C-containing band seems to be produced by the dihaem protein (cooP). It may lose the signal peptide part of the nascent 31.6 kDa protein, although we have not obtained N-terminal information because of probable N-terminal blocking. Our present preparation of *cb*-type oxidase from *H. pylori* was contaminated by a 32 kDa *c*-type cytochrome. Upon reduction, this cytochrome, found mainly in an earlier eluting fraction from gel-permeation chromatography (a higher molecular mass than the oxidase), showed a smaller alpha peak at 554 nm in addition to the main peak at 562 nm that is due to *b*-type cytochromes. These features suggest that this *c*-type cytochrome is cytochrome c_1 of cytochrome *c* reductase, which was identified in the whole genomic DNA sequence of *H. pylori* (*21*). The presence of this cytochrome may affect the absorption spectrum of *cb*-type cytochrome *c* oxidase, but its effect, if any, is not severe, since no prominent shoulder was detected at 554 nm (Fig. 1).

DNA analysis of the *ccoNOQP* locus of the bacterium indicates that the enzyme is a $cb(cbb_3)$-type haem-copper terminal oxidase composed of subunit I with a high-spin protohaem-Cu_B binuclear center and a low-spin protohaem, in addition to the monohaem (ccoO) and dihaem (ccoP) cytochromes that have been found in symbiotic nitrogen fixing and purple bacteria. Among the haem-copper oxidase super-family, *cb*-type cytochrome *c* oxidases seem to be primitive, since (i) they are expressed under microaerophilic conditions or found in microaerophilic bacteria, (ii) their smaller subunits are *c*-type cytochromes that are entirely different from the subunits II and III of the rests of the super-family, and (iii) their subunit I sequences are closely related to that of the *cb*-type NO reductase that might have evolved into the haem-copper oxidase super-family when dioxygen became available (*16–18*). *H. pylori* is a strict microaerobe living in the mucous layer of the human stomach, most frequently sited in the "grooves" at the junction of cells as the microaerophilic niche; the most suitable gaseous conditions for its growth are 5–7% oxygen and 7–10% carbon dioxide in nitrogen (*2*). The present finding that *cb*-type oxidase serves in microaerobes out of proteobacterial alpha subdivision suggests that this type of oxidase may serve as the terminal oxidase in many microaerophilic bacteria. In fact we could also amplify PCR products using DNA from *Campylobacter jejuni*, *Wollinella succinogenes*, and *H. felis*, as well as *H. pylori* as the template, although they are classified in the proteobacterial epsilone subdivision.

The *H. pylori cb*-type oxidase shows H^+-pump activity (Figs. 3 and 4), although its efficiency was lower than that of usual aa_3-type oxidases. Recent oxygen pulse experiments with mutant of *P. denitrificans* cells containing only $cb(cbb_3)$-type oxidase without the aa_3-type cytochrome *c* or bb_3-type quinol oxidases yielded a similar result, a lower H^+ pumping activity of the oxidase (*15*). A H^+-pump activity of a *cb*-type oxidase from a photosynthetic bacterium was very recently shown in aa_3-type oxidase gene-disrupted *R. sphaeroides* cells (*39*). It is thus likely that the *cb*-type oxidase is also H^+-pumping, but that its structural design differs significantly from that of aa_3-type oxidases.

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Figure 6 shows the alignment of *H. pylori* ccoN with the sequences of *cb*-type terminal oxidases from *P. denitrificans* (*15*), *R. capsulatus* (*13*), and *B. japonicum* (*7*). CcoN is homologous to subunit I of aa_3-type oxidases, which is known to be a catalytic main subunit bearing a low spin protohaem and a haem-Cu_B binuclear center, and responsible for H^+ transfer (*29*). The *H. pylori* sequence was the first one elucidated out of the alpha subdivision of proteobacteria, and is differs somewhat from the others; the identity between *H. pylori* ccoN and the other three ranges from 39.6-41.8%, while values among cco/fixNs from bacteria in the alpha subdivision are 62.9 (P.d-B.j)-79.2 (P.d-R.c). The conserved residues among all 4 sequences are dotted, while the residues conserved only among the three species of alpha division proteobacteria are shadowed. Membrane-spanning alpha helices VI-X surround the haem-copper binuclear center where dioxygen is reduced (*40, 41*). Six His residues (*) that act as ligands for three metal atoms in aa_3-type cytochrome oxidases are all conserved, and most residues in their vicinities in helices II, VI, VII, and X are also conserved in all *cb*-type oxidases. The Glu and Tyr in helix VI, which are very important for electron and proton transport in aa_3-type cytochrome oxidases (*41, 42*) are not found in the alignment of *cb*-type oxidases. Also the Lys in helix VIII, which is supposed to act in the proton pathway in aa_3-type cytochrome oxidases (*43*), is not found in the present alignment, although one or two hydroxyl residues are conserved. Two His residues in helix X are separated by Val instead of Phe, indicating that an aromatic side chain is not necessary for electron transfer from low-spin haem to the high-spin haem of the binuclear center. It is also noteworthy that there is no farnecyl side chain in protohaem.

CcoO is monohaem protein with one haem C motif. Not only this haem C motif and the distal Met, but also several charged (E, D, H, K, and R), aromatic (Y and W), and hydroxyl (S) residues spread throughout the sequence are conserved among ccoO/fixO (not shown). The sequence identity between *H. pylori* ccoO and *B. japonicum*. fixO is 39%, much higher than in the case of ccoQ and ccoP, and comparative or even higher than that of ccoN. It is thus likely that this protein interacts intimately with ccoN (subunit I) to form a 58 kDa complex even in the presence of SDS. On the other hand, the homology among the dihaem subunits is low; 80 residues are conserved among these subunits of the alpha-group proteobacteria, but only 35 residues are also conserved in the *H. pylori* sequence. If it is expressed at all, *H. pylori ccoQ* may encode a small polypeptide with 72 amino acid residues. However, to date there is no evidence that this putative polypeptide is a part of a mature enzyme, and even among three sequences of alpha sub-division proteobacteria, very few residues are conserved (not shown). It seems suspicious that these polypeptides play an important role.

We thank Drs. P.A. Chalk of the University of Sheffield and C.L. Clayton of Glaxo Research Group for information on peptide sequences deduced from the DNA of *H. pylori cb*-type oxidase and cytochrome *c*-553.

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