*J. Biochem.* **128**, 371–375 (2000)

# Purification and Characterization of Cytochrome *c*-553 from *Helicobacter pylori*

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Received April 24, 2000; accepted June 8, 2000

***Helicobacter pylori*, a microaerophilic Gram-negative spiral bacterium residing in the human stomach, contains a small size soluble cytochrome *c*. This cytochrome *c* was purified from the soluble fraction of *H. pylori* by conventional chromatographies involving octyl-cellulose and CM-Toyopearl. Its reduced form gave an alpha absorption band at 553 nm, and thus the cytochrome was named *H. pylori* cytochrome *c*-553. The cytochrome, giving a band below 10,000 Da upon SDS-PAGE, was determined to have a mass of 8,998 by time of flight mass spectroscopy. Its N-terminal peptide sequence was TDVKALAKS—, indicating that the nascent polypeptide was cleaved to produce a signal peptide of 19 amino acid residues and a mature protein composed of 77 amino acid residues. The *cb*-type cytochrome *c* oxidase oxidized ferrocytochrome *c*-553 of this bacterium actively ($V_{max}$ of about 250 $s^{-1}$) with a small $K_m$ (0.9 μM). Analysis of the effect of the salt concentration on the oxidase activity indicated that oxidation of cytochrome *c*-553 is highly inhibited under high ionic conditions. The amino acid sequence of *H. pylori* cytochrome *c*-553 showed the closest similarity to that of *Desulfovibrio vulgaris* cytochrome *c*-553, and these sequences showed a weak relationship to that of the cytochrome $c_8$-group among class I cytochromes *c*.**

**Key words: cytochrome *c*-553, cytochrome *c* oxidase, cytochrome *cbb*$_3$, *Helicobacter pylori*.**

*Helicobacter pylori*, a microaerophilic Gram-negative spiral bacterium residing in the human stomach, uses a *cb*(*cbb*$_3$)-type cytochrome *c* oxidase (*1*, *2*). The purified enzyme was found to be composed of three or four subunits, and oxidized several cytochromes *c* and TMPD actively (*3*). This type of heme-Cu oxidase has been found in the alpha-subdivision of proteobacteria, such as $N_2$-fixing Rhizobiaceae, photosynthetic purple bacteria and aerobic soil bacterium *Paracoccus denitrificans*, as one of the terminal oxidases in the branched respiratory chain (*4–6*). Reading of the whole genomic DNA sequence of *H. pylori* suggested that this microaerophilic bacterium uses a simple respiratory chain composed of quinol:cytochrome *c* reductase (cytochrome $bc_1$ complex), cytochrome *c*-553 and a *cb*-type cytochrome *c* oxidase, since it contains *fbcFBC* and *fixNOQP*, but not genes encoding other terminal oxidases such as cytochrome $aa_3$ and cytochrome *bd* (*7*). This simple respiratory chain is probably relevant to the microaerophilic nature of this bacterium, since the *cb*-type oxidases are known to have a very small $K_m$ for $O_2$, as well as that complete citric acid and glyoxalate cycles are not found and NADH is oxidized only slowly (*8–10*). These results seem to be consistent with the results obtained for *H. pylori* genome (*7*), which has also been reviewed from biochemical and enzymological points of views (*11*, *12*).

Cytochrome *c*-553 seems to mediate electron transfer between the cytochrome reductase and the *cbb*$_3$-type terminal oxidase, since it is the sole small-size *c*-type cytochrome found in the genome. Other proteins having a heme C-binding motif (-CXYCH-) are fbcC of cytochrome *c* reductase, the fixO and fixP subunits of the terminal oxidase, and a peroxidase (cytochrome *c*-551). Our analyses involving heme staining after SDS-PAGE indicated that this is the sole *c*-type cytochrome in the soluble fraction, including that of periplasmic origin (*1*). The gene for cytochrome *c*-553 encodes a 96 amino acid-residue protein having one heme C motif (*7*). The present study revealed that the mature cytochrome was composed of 77 amino acids, having lost a signal peptide of 19 amino acids. The cytochrome was actively oxidized by the purified *cb*-type terminal oxidase with a small $K_m$. In this paper the purification procedure and several properties of this cytochrome are also reported.

## MATERIALS AND METHODS

*Materials*—The thermophilic *Bacillus* PS3 cytochrome *c*-

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl *p*-phenylenediamine); Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; SDS-PAGE, gel electrophoresis with sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; Da, dalton; bp, base pair.

551 was over-expressed in *Bacillus stearothermophilus* transformed with pSTEc551, and then purified as described previously (*13*). CM-Toyopearl and octyl-cellulose were products of Toso (Tokyo) and Chisso (Tokyo), respectively. PVDF membranes for protein blotting were purchased from Amersham. TMPD and *o*-tolidine were purchased from Wako Pure Chemicals (Osaka), and both equine cytochrome *c* (type VI) and cytochrome *c* (type VIII) from Sigma Chemicals (St. Louis). Other chemicals, inhibitors and detergents were obtained as described previously (*3*).

*Purification Procedure—H. pylori* NCTC11637 was cultured as described previously (*1*). The supernatant fraction (*14*) was used for the preparation of cytochrome *c*-553 as follows.

*Step 1:* Ammonium sulfate was added to the supernatant fraction to give 50% saturation, and then the mixture was centrifuged for 10 min at 16,000 $\times g$ after 20 min. The ammonium sulfate saturation of the resultant supernatant was increased to 75%, and then the mixture was centrifuged again as before.

*Step 2:* The faintly reddish supernatant fraction obtained in Step 1 was applied to a column of Octyl cellulose (1.6 × 7 cm) equilibrated with an 80% saturated ammonium sulfate solution containing 10 mM Na-$P_i$ buffer, pH 6.8. Then the upper part of the column turned red and the red band moved downward gradually, when the ammonium sulfate concentration of the elution buffer was lowered to 50% saturation. The band was finally eluted at 30% saturation.

*Step 3:* The red fraction was dialyzed against 10 mM acetate buffer, pH 5.0, for 3 h, and then against the same but fresh buffer for 12 h. The dialyzed solution, after centrifugation, was applied on a CM Toyopearl column (1 × 4 cm), and the upper part of the column turned red. The column was washed with 20 mM acetate buffer, pH 5.0, containing 20 mM NaCl. The red band moved downward slowly, when the NaCl concentration of the elution buffer was raised to 50 mM. The band was finally eluted from the column by raising the NaCl concentration to 100 mM. The red fraction was pooled (2 ml), and used as the *H. pylori* cytochrome *c*-553 preparation. When a more concentrated sample was necessary, the sample was concentrated with Centricon-10 (Amicon).

*Measurement of Enzyme Activities*—Oxygen uptake by 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, *i.e.* 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 the final electron donor, according to the following equation (*15*):

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

The net alkali formation was back-titrated with an aliquot of 10 mM HCl.

*Analytical Procedures*—Absorption spectra were measured with a Beckman DU-70 spectrophotometer. Heme contents were determined as described previously (*16*). SDS-PAGE, protein determination and heme staining were carried out as described previously (*3*). Peptide sequences were obtained by Edman degradation with an Applied Biosystem model 491A gas/liquid phase sequencer. Time of flight mass spectra were measured with a Voyager Linear Mass Spectrometer with a delay apparatus from PerSeptive Biosystems. The accelerating voltage was 25,000 V and sinapinic acid was used as the matrix.

## RESULTS AND DISCUSSION

*Purification of Cytochrome c-553*—Cytochrome *c*-553 is the sole *c*-type cytochrome in the soluble fraction, because no heme-stained band other than that of cytochrome *c*-553 was observed upon heme-staining, and its reduced form gave an alpha band peak at 553 nm. This cytochrome in the soluble fraction was effectively purified by successive hydrophobic and ion-exchange chromatographies. The heme content of the final preparation was 95 nmol/mg protein, while about 0.2 nmol/mg protein was found in the soluble fraction. From 320 ml of supernatant fraction containing 211 nmol cytochrome *c*-553, 60 nmol was recovered in the final fraction. The yield was about 28%.

*Molecular Properties of Cytochrome c-553*—The purified cytochrome gave a band corresponding to about 10 kDa (lane 3), just below the band of horse heart cytochrome *c* (lane 1) and lysozyme (lane 2) upon SDS-PAGE (Fig. 1, insertion). A very faint band at around 25 kDa observed for some preparations seems due to a contaminating protein. The precise molecular mass was determined to be 8,987.9 Da by time of flight mass spectroscopy (Fig. 1, main part). N-Terminal peptide sequencing was carried out by PVDF membrane electro-blotting with the cytochrome band in lane 3. The sequence TDVKALAKS-- is in accord with the 20th to 28th residues of the amino acid sequence deduced from DNA (*7*), indicating that the nascent polypeptide was processed so that to have 77 amino acid residues through cleavage of the signal sequence composed of 18 amino acid residues. The molecular mass, 8,987.9 Da, is very close to

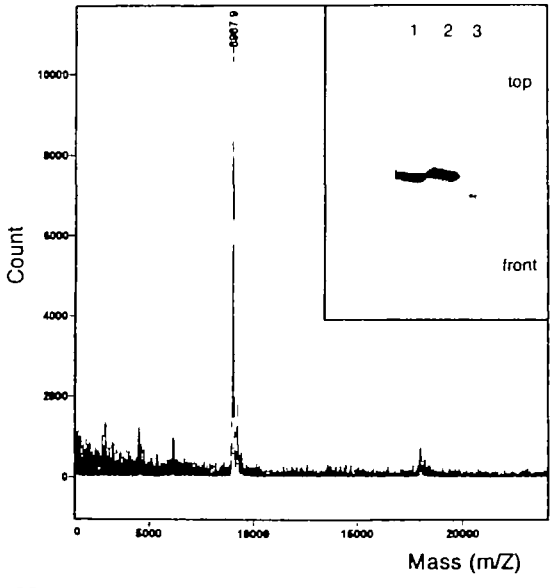


Fig. 1. **Mass spectrum and SDS-PAGE pattern (insertion) of *H. pylori* cytochrome *c*-553.** The concentration of the gel was 15%, and the gel was stained with Coomassie Brilliant Blue R-250. Horse heart cytochrome *c* (lane 1), lysozyme (lane 2), and *H. pylori* cytochrome *c*-553 (lane 3) were used.

the sum of the 77 amino acid residues (8371.8 Da) and the heme C moiety (616.5 Da), indicating that no other post-translational processing than the signal peptide cleavage takes place.

*Absorption Spectra and Redox Potential*—Absorption spectra of cytochrome *c*-553 showed peaks of the reduced form at 553 (552.7), 524 and 418 nm (solid line), and ones of the oxidized form at 526 and 410 nm (broken line), as illustrated in Fig. 2. The low optical absorption at 280 nm reflects a low content of aromatic residues; no Trp, 1 Tyr, and 2 Phe. The pyridine ferro-hemochrome gave a peak at 550 nm, indicating that the prosthetic group is heme C. Quantitative measurement gave molecular absorption coefficients of 22.3 and 135 $mM^{-1} \cdot cm^{-1}$ for the reduced *minus* oxidized difference at 553–535 nm and for the reduced form at 410 nm, respectively. The redox potential was determined to be 0.17 V, *i.e.* a little lower than that of mitochondrial cytochromes *c*.

*Cytochrome c-553 as the Substrate of the Terminal Oxidase*—Cytochrome *c*-553 from *H. pylori* was oxidized by the *cb*-type oxidase of this bacterium with a $K_m$ of 0.9 μM and a $V_{max}$ of 252 $s^{-1}$, while the $K_m$ and $V_{max}$ for yeast and equine cytochromes *c* were 15.2 and 1.1 μM, and 250 and 72.6 $s^{-1}$, respectively, indicating that cytochrome *c*-553 is the most suitable substrate of the oxidase (*3*). Cytochrome *c* oxidation by mitochondrial cytochrome *c* oxidase is known to give two kinetic constants, showing the high affinity site below 0.1 μM and the low affinity site of about several μM for cytochrome *c* (*17*). The single $K_m$ for cytochrome *c*-553 of the *cb*-type oxidase indicates that it is the low affinity site of the enzyme which cytochrome *c*-553 occupies, since the *cb*-type oxidase contained *c*-type cytochromes intrinsically as ccoO and ccoP proteins (*3*, *7*), and since TMPD, which does not interact with the high affinity site, supported the total oxidase activity with a $V_{max}$ of 247 $s^{-1}$ without cytochrome *c* addition (*3*). A very low $K_m$ for *H. pylori* cytochrome *c*-553 as the low affinity site suggests that this cytochrome may be the physiological substrate for the *cb*-type terminal oxidase.

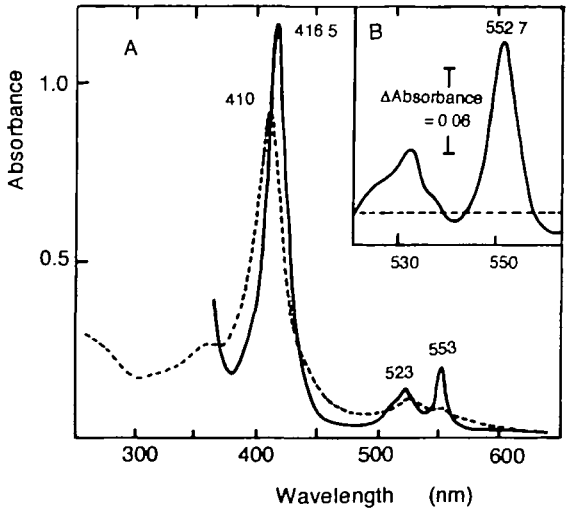


Fig. 2. **Spectra of *H. pylori* cytochrome *c*-553.** A: $Na_2S_2O_4$-reduced form spectrum (continuous line) and oxidized form spectrum as prepared (broken line). B: $Na_2S_2O_4$-reduced form giving alpha and beta bands. The cytochrome *c*-553 preparation (9.4 μg/ml) was used.

The mature cytochrome *c*-553 contains 13 Lys, 5 Glu, and 5 Asp among 77 amino acid residues, and its estimated pI based on the amino acid composition is 9.7. Interaction between acidic mitochondrial or proteobacterial cytochrome *c* oxidase and the basic substrate, cytochrome *c*, has been shown to be greatly affected by the ionic strength of the medium (*17–19*). Figure 3 shows the effect of the ionic strength on the cytochrome *c* oxidase activity of the *H. pylori cb*-type enzyme with basic yeast cytochrome *c* and acidic *Bacillus* cytochrome *c*-551 (*13*), and an artificial electron donor, TMPD, as well as cytochrome *c*-553 as substrates. The salt concentration seems to affect it in at least two different manners: one is acceleration of internal electron transfer in the oxidase, which is observed in TMPD and cytochrome *c*-551 oxidation, and the other a weakening effect of high ionic strength conditions on interaction between cytochrome *c*-553 or yeast cytochrome *c* and the *cb*-type oxidase. In fact, the 2 μM cytochrome *c*-553 as the substrate present in the experiment was far below the saturation level (not shown). These results of cytochrome *c*-553 oxidation by *cb*-type cytochrome *c* oxidase of *H. pylori* are very similar to those of mitochondrial cytochrome *c* and cytochrome $aa_3$, indicating that electrostatic interaction between the substrate and oxidase also occurs in an FixN-type (ccoNOQP) oxidase system.

*H. pylori* membranes are rich in *b*- and *c*-type cytochromes without showing any prominent absorption band around the 600–650 nm region even in the reduced state (*1*), and the genome contains no gene for an apparent cytochrome component other than *ccoNOQP* for *cb*-type cytochrome *c* oxidase, *fbcCAB* for the cytochrome $bc_1$ complex, and genes for cytochrome *c*-553 and cytochrome *c*-551 peroxidase (*7*). It is thus likely that these cytochromes form a simple microaerophilic respiratory chain oxidizing menaquinol-6 *via* cytochrome $bc_1$ complex, cytochrome *c*-553 and

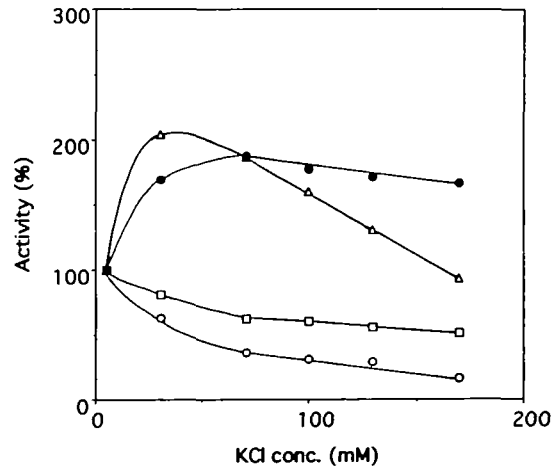


Fig. 3. **Effect of the salt concentration on oxidase activity with different substrates including cytochrome *c*-553.** The activity was followed as the change in the pH of 0.5 mM Na-$P_i$ buffer (pH 6.8–6.9) in the presence of 5 mM Na ascorbate as the final electron donor with 0.1 nmol of the *cb*-type cytochrome oxidase from *H. pylori* in a 2.5-ml glass cell. The direct electron donors added were 2.0 μM cytochrome *c*-553 (○), 3.2 μM cytochrome *c*-551 (△), 20 μM yeast cytochrome *c* (□), and 0.1 mM TMPD (●), and the activity in the reaction medium without KCl was 204 (cytochrome *c*-553), 76 (cytochrome *c*-551), 202 (yeast cytochrome *c*), or 74 $s^{-1}$ (TMPD).

*bc*-type cytochrome oxidase, which exhibits high affinity to oxygen. Cytochrome *c*-551 peroxidase may oxidize cytochrome *c*-553 using $H_2O_2$, as postulated by Kelly (*11*). Our present results directly confirmed the oxygen side of the respiratory chain. A recent report on the respiratory chain of photosynthetic bacteria showed that cytochrome $c_y$, membrane-bound *c*-type cytochrome, mediates electron transfer between cytochrome $bc_1$ and *cb*-type cytochrome *c* oxidase (*20*), but in *H. pylori* no such *c*-type cytochrome was found in the membrane (*1*), and no gene for such a cytochrome was found in the genome (*7*).

*Similarity to D. vulgaris Cytochrome c-553 and Phylogenetic Position*—Figure 4A shows alignment of *H. pylori* cytochrome *c*-553 and *Desulfovibrio vulgaris* cytochrome *c*-553. A homology search of *H. pylori* cytochrome *c*-553 by means of FASTA of the data bank revealed *D. vulgaris* cytochrome *c*-553 as the closest protein, as well as that both cytochromes give the reduced-form alpha band at around 553 nm. Homologous residues are found from the N-terminal to the C-terminal, and the percent identity is 35%, although 7 residues in the *D. vulgaris* sequence (CAA, V, and KGY) are not found in the *H. pylori* sequence. The residues such as Cys, His, and Met, necessary for heme-binding, are conserved. It is noteworthy that 7 Lys residues are conserved, probably indicating that most of these residues may play important roles in the interaction with the reductases and oxidase. Class I cytochromes *c*, a group of *c*-type cytochromes composed of 70–120 amino acid residues with heme C bound to two cysteine residues in the CXYCH-motif close to the N-terminal (*21*), are known to include subgroups such as cytochromes $c_2$, $c_4$, $c_5$, $c_6$, and $c_8$ and *Bacillus* small *c* (*22*). These groups may have been formed through gene duplication at an early stage of the history of class I cytochromes *c*, and were analyzed by constructing a phylogenetic tree using the neighbour-joining method (as well as structural and physiological aspects (*21–23*). The present phylogenetic tree analysis of *H. pylori* cytochrome *c*-553 with various class I cytochromes *c* including *D. vulgaris* cytochrome *c*-553 (*24*) showed that the two cytochromes *c*-553 form a group which separated at an early stage from members of the cytochrome $c_8$ subgroup (Fig. 4B). *D. vulgaris* cytochrome *c*-553 was shown to be present in the periplasmic space and to obtain electrons from several dehydrogenases such as formate dehydrogenase (*25*), and to donate them to the membrane-bound sulfate reducing system (*26*, *27*). In *H. pylori*, cytochrome *c*-553 in the periplasmic space may obtain electrons from some dehydrogenases such as D-lactate dehydrogenase (*2*) as well as quinol:cytochrome *c* reductase (*1*, *2*), and donate them to *cb*-type cytochrome *c* oxidase (*2*, *3*) or cytochrome *c* peroxidase to some extent (*28*). The fact that *H. pylori* cytochrome *c*-553 contains more hydrophobic amino acid residues than *D. vulgaris* cytochrome *c*-553 may be due to the fact that *H. pylori* cytochrome *c*-553 must interact with membrane proteins such as quinol:cytochrome *c* reductase and cytochrome *c* oxidase.

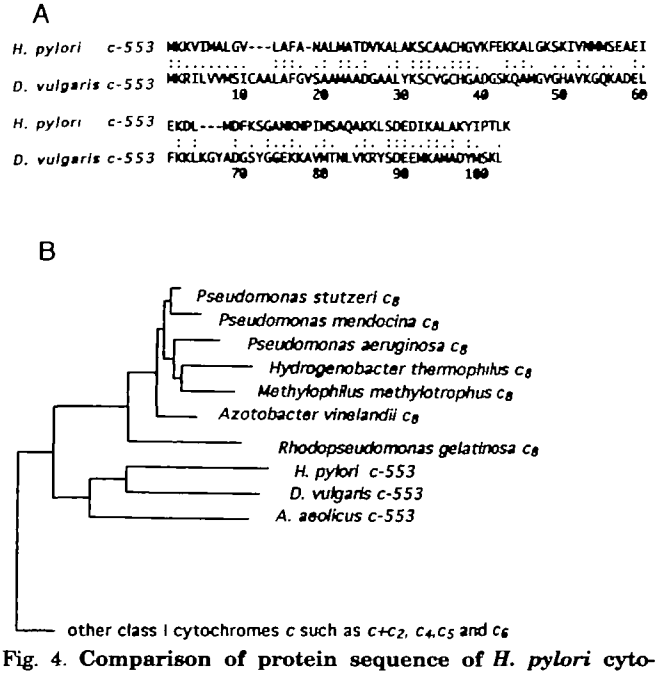


Fig. 4. **Comparison of protein sequence of *H. pylori* cytochrome *c*-553 with that of *D. vulgaris* *c*-553 (A), and a partial phylogenetic tree of class I cytochromes *c* including both cytochromes (B).** A: The results of a search (FASTA, *29*) are shown. B: The tree was drawn by adding the sequences of *H. pylori* cytochrome *c*-553 and *Aquifex aeolicus* cytochrome *c*-552 (Aq1550) to the sequences of 40 class I cytochromes *c* listed previously (*21*). Multiple alignment and phylogenetic trees were constructed with CLUSTAL W (*30*). *Rhodopseudomonas gelatinosa* is renamed *Rubrivivax gelatinosus*.

*Conclusion*—The purified cytochrome *c*-553 from *H. pylori* showed a mass of 8,988 Da with an N-terminal peptide sequence of TDVKALAKS-, indicating that the signal peptide with the first 19 residues in the precursor protein was processed. This cytochrome with an $E_m'$ of 0.17 V is oxidized rapidly by the *cb*-type cytochrome *c* oxidase of this bacterium with a relatively small $K_m$, and may mediate electron transfer between quinol:cytochrome *c* reductase and the terminal oxidase. The oxidation of this basic protein was severely affected by the ionic strength of the reaction medium, as the cytochrome *c* oxidation by mitochondrial $aa_3$-type oxidases is. It is thus likely that the oxidase activity of fixN-type terminal oxidase having monoheme protein (ccoO) instead of subunit II is also affected by the ionic strength.

*Note Added in Proof*—After this manuscript had been written, the FASTA search could access Cj1153 for the monoheme cytochrome in the whole genome (*31*) of *Campylobacter jejuni*, which belongs to the proteobacteria epsilon group including *H. pylori*. The percent identity between *H. pylori* cytochrome *c*-553 and the homologous *C. jejuni* cytochrome was 39%.

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

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