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Purification of *Helicobacter pylori* NCTC 11637 Cytochrome bc_1 and Respiration with D-Proline as a Substrate▽§

Minoru Tanigawa,[1] Tomomitsu Shinohara,[1] Katsushi Nishimura,[1,2] Kumiko Nagata,[3]† Morio Ishizuka,[4] and Yoko Nagata[1]*

Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, Tokyo 101-8308, Japan[1]; Department of Applied Chemistry, Junior College, Nihon University, Chiba 274-8501, Japan[2]; Department of Bacteriology, Hyogo College of Medicine, Hyogo 663-8501, Japan[3]; and Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, Tokyo 112-0003, Japan[4]

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Helicobacter pylori **is a microaerophilic bacterium associated with gastric inflammation and peptic ulcers. Knowledge of how pathogenic organisms produce energy is important from a therapeutic point of view. We found D-amino acid dehydrogenase-mediated electron transport from D-proline or D-alanine to oxygen via the respiratory chain in *H. pylori*. Coupling of the electron transport to ATP synthesis was confirmed by using uncoupler reagents. We reconstituted the electron transport chain to demonstrate the electron flow from the D-amino acids to oxygen using the recombinant cytochrome bc_1 complex, cytochrome *c*-553, and the terminal oxidase cytochrome cbb_3 complex. Upon addition of the recombinant D-amino acid dehydrogenase and D-proline or D-alanine to the reconstituted electron transport system, reduction of cytochrome cbb_3 and oxygen consumption was revealed spectrophotometrically and polarographically, respectively. Among the constituents of *H. pylori*'s electron transport chain, only the cytochrome bc_1 complex had been remained unpurified. Therefore, we cloned and sequenced the *H. pylori* NCTC 11637 cytochrome bc_1 gene clusters encoding Rieske Fe-S protein, cytochrome *b*, and cytochrome c_1, with calculated molecular masses of 18 kDa, 47 kDa, and 32 kDa, respectively, and purified the recombinant monomeric protein complex with a molecular mass of 110 kDa by gel filtration. The absorption spectrum of the recombinant cytochrome bc_1 complex showed an α peak at 561 nm with a shoulder at 552 nm.**

Helicobacter pylori is a Gram-negative bacterium associated with duodenal and gastric ulcers and, possibly, gastric cancer (3). The bacterium is an obligate microaerophile that prefers 5 to 7% oxygen and colonizes the mucus layer of the human gastric epithelium (3). It requires oxygen as a terminal acceptor but cannot grow under high oxygen tension. Oxygen is both necessary for and deleterious to the growth of *H. pylori* cells. In contrast to the mitochondrial respiratory chain, complex I appears to be absent in *H. pylori* (2). Although knowledge of the electron transport coupled with ATP synthesis in *H. pylori* is likely to contribute to therapeutic strategies, a fundamental understanding of the bacterial physiology and metabolism is lacking, and potential energy substrates remain unknown (1). The constituents of the respiratory electron transport chain of a microaerobe must play a major role in adaptation to environmental changes such as oxygen tension (16). Therefore, a constituent different from those in aerobic respiration appears to be involved in microaerobic respiration.

D-Amino acid dehydrogenase (DAD) is a flavoenzyme that catalyzes the deamination of free neutral D-amino acids yielding the corresponding 2-oxo acids, ammonium, protons, and electrons without using oxygen (14). DAD has been known to occur in Gram-negative *Gammaproteobacteria*, such as facultatively anaerobic *Escherichia coli* (15) and *Salmonella typhimurium* (20), and bacteria such as *Pseudomonas fluorescens* (18) and *P. aeruginosa* (11) that are able to grow using nitrate as a final electron acceptor in anaerobic respiration. We detected DAD activity in an obligate anaerobe, *Pyrobaculum islandicum* (13), and purified *H. pylori* DAD from the recombinant *E. coli* cells (17). The enzyme has not been reported in obligatory aerobic organisms such as vertebrates and higher plants. The distribution of DAD among the organisms above led us to speculate that DAD functions in respiration under microaerobic or anaerobic conditions, since DAD-mediated electron transport from D-alanine to cytochromes was suggested in *E. coli* membranes (5).

To test this hypothesis, in the present study, we examined whether electrons from D-amino acids are transferred to the terminal component of *H. pylori*'s respiratory chain, cytochrome cbb_3, and oxygen in the presence of DAD, by using an electron transport system consisting of each purified component from *H. pylori* cells or recombinant *E. coli* cells. For that purpose, we purified the cytochrome bc_1 complex, the only component that had not been purified, from recombinant *E. coli* cells and then reconstituted the electron transport chain.

MATERIALS AND METHODS

Cell culture. *Helicobacter pylori* NCTC 11637, the type strain, was cultured on brucella agar plates (Becton Dickinson, NJ) containing campylobacter selective supplement (Oxoid, Hampshire, United Kingdom) and 5% horse serum (Sigma-Aldrich, St. Louis, MO) under 10% CO_2 at 37°C for 48 h. Cultured cells were harvested by centrifugation at 8,000 × *g* for 20 min and washed once with 50 mM

* Corresponding author. Mailing address: Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, 1-8-14 Kanda-Surugadai, Chiyoda Ward, Tokyo 101-8308, Japan. Phone: 81-3-3259-0861. Fax: 81-3-3293-7572. E-mail: nagata@chem.cst.nihon-u.ac.jp.

† Present address: Department of Food and Nutrition, Faculty of Human Life Sciences, Senri Kinran University, Osaka 565-0873, Japan.

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sodium phosphate buffer (pH 7.0) containing 0.9% NaCl before being stored at −80°C until being used.

Escherichia coli Rosetta 2 (Merck, Darmstadt, Germany) cells harboring plasmid pHpcytbc1 were cultured in Luria-Bertani (LB) medium containing 25 μg/ml of kanamycin and 25 μg/ml of chloramphenicol in Erlenmeyer flasks at 37°C with continuous shaking. After a 4-h culture, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the culture was continued for another 4 h. The cells were collected by centrifugation at 8,000 × *g* for 10 min and washed once with 50 mM sodium phosphate buffer (pH 7.0) before being stored at −80°C.

Purification of cytochrome bc_1 from *H. pylori* cells. The *H. pylori* NCTC 11637 cell pellet (wet weight, 100 g) was suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, and 1 mM EDTA. The cells were broken by being passed through a French press (Ohtake, Tokyo, Japan) at 140 MPa. The entire experimental procedure was carried out at 4°C. Unbroken cells and cell debris were removed by centrifugation at 18,000 × *g* for 20 min. The resulting supernatant, the cell extract, was centrifuged at 140,000 × *g* for 60 min. The pellet was suspended in 50 mM HEPES (pH 7.2) containing 0.5% sodium cholate and stirred for 30 min. After removal of peripheral membrane proteins by centrifuging at 140,000 × *g* for 60 min, the pellet was solubilized with 1.0% *n*-octyl-β-D-thioglucoside (Dojin, Kumamoto, Japan) in 50 mM HEPES buffer (pH 7.2) by being stirred for 60 min. The solubilized proteins were applied to a DEAE-cellulose column equilibrated with 50 mM HEPES buffer (pH 7.2) containing 1.0% Triton X-100 and eluted from the column by increasing the NaCl concentration with a linear gradient from 0 to 500 mM in the equilibration buffer. The cytochrome bc_1 fraction was eluted with 70 mM NaCl to measure its absorption spectra.

Purification of cytochrome *c*-553 and cytochrome cbb_3. The entire experimental procedure was carried out at 4°C. The soluble fraction of the cell extract of *H. pylori* cells, the supernatant of the centrifugation at 140,000 × *g* for 60 min (described above), was dialyzed against 50 mM acetate buffer (pH 5.0) before being applied to a CM-Toyopearl column (φ1.5 × 3 cm; Tosoh, Tokyo, Japan) equilibrated with the dialysis buffer, and was eluted with a linear gradient of 0 to 500 mM NaCl in the same buffer (8). The fraction containing cytochrome *c*-553 was applied to a Sephacryl S-200 column (φ1.5 × 45 cm; Amersham Biosciences, United Kingdom) equilibrated with 50 mM acetate buffer (pH 5.0) containing 200 mM NaCl. The purified cytochrome *c*-553 was stored at −80°C until use.

The membrane fraction of the cell extract was used for the preparation of the cytochrome cbb_3 complex. The fraction was suspended in 50 mM HEPES buffer (pH 7.0) containing 100 mM NaCl and centrifuged at 140,000 × *g* for 60 min. The precipitated membranes were solubilized with 3.0% Triton X-100 for 3 h and dialyzed against 50 mM HEPES buffer containing 3.0% Triton X-100 (pH 7.2) (19). The dialysate was applied onto a Q-Sepharose column equilibrated with the dialysis buffer and was eluted by increasing the concentration of NaCl with a linear gradient of 0 to 500 mM in the buffer. The active fractions were pooled and applied onto a Sephacryl S-200 column equilibrated with 50 mM HEPES buffer (pH 7.2) containing 200 mM NaCl beforehand. The purified enzyme fraction was stored at −80°C until use.

Purification of D-amino acid dehydrogenase (DAD). DAD was purified from recombinant *E. coli* cells harboring the *H. pylori* NCTC 11637 *dadA* gene (accession number AB295062) according to a previously reported method (17).

Purification of recombinant cytochrome bc_1. Harvested cells of recombinant *E. coli* harboring pHpcytbc1 (wet weight, 2.5 g) were suspended in 10 ml of 50 mM sodium phosphate buffer (pH 7.0) and were disrupted by being passed 15 times through a French press at 140 MPa. All the experimental procedures described hereinafter were carried out at 4°C unless otherwise stated. After the removal of unbroken cells and cell debris by centrifugation at 6,000 × *g* for 20 min, the supernatant solution was centrifuged at 140,000 × *g* for 60 min. The pellet, the membrane fraction, was suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 1% *n*-octyl-β-D-thioglucoside and stirred for 14 h. The solubilized proteins, the supernatant yielded by the centrifugation at 140,000 × *g* for 60 min, were applied onto a HisTrapHP column (GE Healthcare, United Kingdom) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1% Tween 20, 5 mM imidazole, and 500 mM NaCl. The cytochrome bc_1 fraction was eluted with the same buffer containing a linear gradient of imidazole from 5 to 500 mM, and subjected to further purification with a TSKgel G3000 column (φ7.5 × 600 mm; Tosoh), equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1% Tween 20 and 200 mM NaCl.

Analyses of K_m values for cytochrome bc_1 and DAD-mediated reactions. The reactions were monitored spectrophotometrically using an Ultrospec 4300Pro spectrophotometer (Amersham Biosciences). The reaction to obtain the K_m value for the DAD-mediated dehydrogenation of D-proline was carried out in a mixture containing various amounts of D-proline, 1 μg of DAD, 50 μM quinone (Q_0 or Q_1, Sigma), 1.8 μM phenazine methosulfate, 0.06% Triton X-100, and 50 mM sodium phosphate buffer (pH 8.0) in a total volume of 200 μl at 37°C for 10 min. The velocity of D-proline's dehydrogenation was calculated based on the rate of reduction of quinone using a molar extinction coefficient of 14.4 mM^{-1} cm^{-1} for Q_0 and Q_1 at 275 nm, and the K_m value was calculated using a Lineweaver-Burk plot. The reaction to obtain the K_m value for the reduction of quinone was monitored at 275 nm in a mixture similar to the one above except that it contained various amounts of quinone, 100 mM D-proline, and 1% phosphatidylcholine (wt/vol) instead of 50 μM quinone and various amounts of D-proline. The reduction of cytochrome bc_1 was measured by monitoring the A_{275} upon the addition of 1 μg of the recombinant cytochrome bc_1 to the reaction mixture containing various amounts of quinone, 100 μM cytochrome *c*-553, 0.1% Tween 20, 1% phosphatidylcholine, and 50 mM sodium phosphate buffer (pH 7.0) in a total volume of 200 μl at 37°C. The oxidation of cytochrome bc_1 was analyzed in a reaction medium similar to the one above except that it contained various amounts of cytochrome *c*-553 and 50 μM quinone instead of various amounts of quinone and 100 μM cytochrome *c*-553. Upon the addition of 1 μg of the recombinant cytochrome bc_1, the increase in A_{553} was monitored, and the molar extinction coefficient value at 553 nm, 22.3 mM^{-1} cm^{-1}, was used for calculation of the K_m value.

Spectrophotometric detection of electron transport in the reconstituted system. Upon the acceptance of electrons, the absorption spectrum of a cytochrome is converted from that of an oxidized form to a reduced form. Electron transport from D-proline to the terminal oxidase, cytochrome cbb_3, was investigated by monitoring the cytochrome's spectral change. The reaction mixture consisted of 30 μg of DAD, 50 μM Q_0, 10 μg of the recombinant cytochrome bc_1, 20 μg of cytochrome *c*-553, and 80 μg of cytochrome cbb_3 in 50 mM sodium phosphate buffer (pH 7.0) containing 1% phosphatidylcholine and 25%-saturated oxygen. After a 5-min incubation at 37°C, D-proline was added to make a concentration of 100 mM.

Polarometric assay of respiration. Respiration was monitored polarographically with a Clark-type oxygen electrode (YSI Model 5300, Yellow Springs Instruments, OH) in a semiclosed vessel containing 600 μl of a reaction mixture composed of 10 μg of DAD, 20 μM Q_0, 5 μg of the recombinant cytochrome bc_1, 5 μg of cytochrome *c*-553, 5 μg of cytochrome cbb_3, 1% phosphatidylcholine, and 25%-saturated oxygen in 50 mM sodium phosphate buffer (pH 7.0). The 25%-saturated oxygen concentration was obtained by mixing the 50 mM sodium phosphate buffer saturated with oxygen (100%) and the buffer solution containing no oxygen. After a 5-min incubation at 37°C, the reaction was started by the addition of 100 mM (final concentration) D-proline.

The respiration of whole cells was assayed in a manner similar to that described above in 10 mM HEPES buffer (pH 7.0) containing 100 mM NaCl and 25%-saturated oxygen (12). The various respiratory substrates were introduced into the oxygen electrode vessel with a syringe. The ATP synthesis uncouplers used were 2,4-dinitrophenol (Wako), valinomycin (Sigma), and nigericin (Sigma).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 12.5% polyacrylamide gel according to the method of Laemmli (10). After the electrophoresis, the proteins were stained with Coomassie brilliant blue R250 or *o*-toluidine to visualize proteins or hemes (4), respectively.

Isolation, cloning, and expression of the cytochrome bc_1 gene cluster. The genomic DNA of *H. pylori* NCTC 11637 was isolated using a Genomic DNA kit (Qiagen, Hilden, Germany). A pair of oligonucleotide primers was designed for PCR based on the upstream region of the *H. pylori* HPAG1 cytochrome bc_1 gene (DDBJ accession number NC008086), 5′-GCCTTTAGTCGTTAAAAGGTAA T-3′ (sense primer), and the downstream region of the *H. pylori* J99 cytochrome bc_1 gene (DDBJ accession number NC 000921), 5′-GAATTTTCTAACCACC ACCAG-3′ (antisense primer). Amplification of nucleotides between the two primers was performed by PCR using Ready-To-Go PCR beads (Amersham Biosciences) and *H. pylori* NCTC 11637 genomic DNA as a template. The amplified product was purified by ultrafiltration with Suprec PCR (Takara Shuzo, Kyoto, Japan) and directly sequenced by a 3100 genetic analyzer (Applied Biosystems, CA). Six pairs of primers were designed (Table 1) to sequence the amplified product. To insert the gene cluster into plasmid pET-41b (Novagen, Madison, WI), we used two primers: the first designed to contain the N-terminal region of the cytochrome bc_1 complex of *H. pylori* NCTC 11637 and an EcoRI recognition sequence (5′-GGAATTCCATATGGCAGATATTCAAAGGC-3′), and the second designed to contain the C-terminal region of cytochrome bc_1 and a XhoI recognition sequence (5′-CCGCTCGAGATGCACTTCGCTCCAAAC TT-3′). The histidine tag sequence was inserted into the C-terminal region. Amplification of nucleotides between the two primers was performed by PCR using a Triple Master PCR system (Eppendorf, Hamburg, Germany) and the genomic DNA of *H. pylori* NCTC 11637 as the template. The amplified product

TABLE 1. Primers employed to sequence *H. pylori* NCTC 11637 cytochrome bc_1 complex

Reverse primer	Forward primer
5′-GCCTTTAGTCGTTAAAAGGTAAT-3′	5′-TTAGTGCCTTCAATTTTAAA-3′
5′-ACTTTTGGTGAAGCCGGGGC-3′	5′-AGTGATAACCGCTGCGGCCCA-3′
5′-TTTGGAGGCATTCCTTTCAT-3′	5′-GGAGCGACGACTGGGCTTCT-3′
5′-GGAGCGACGACTGGGCTTCT-3′	5′-TTCAAAACCCCCGCCACATG-3′
5′-TTTCTTAGCCCACTTCATCA-3′	5′-ATGCACTTCGCTCCAAAC-3′
5′-AAAAATCATGATTTTCTTTGCGGTGC-3′	5′-GAATTTTCTAACCACCACCAG-3′

was digested with the restriction enzymes EcoRI (Takara Shuzo) and XhoI (Takara Shuzo), and the resulting fragment was ligated into pET-41b that had been digested with the same enzymes, using a DNA ligation kit (version 2.1; Takara Shuzo). The construction was confirmed by sequencing. The resultant plasmid was introduced into *E. coli* Rosetta 2, and the cytochrome bc_1 gene was overexpressed in the *E. coli* cells.

Nucleotide sequence accession number. The nucleotide sequence of the *H. pylori* NCTC 11637 cytochrome bc_1 complex has been deposited in the DDBJ/GenBank/BBI databanks under accession number AB281426.

Protein structure accession number. The amino acid sequence of the *H. pylori* NCTC 11637 cytochrome bc_1 complex can be accessed through the NCBI Protein Database under accession numbers BAF36623.1 for the 2Fe-2S subunit, BAF36624.1 for the cytochrome *b* subunit, and BAF36625.1 for the cytochrome c_1 subunit.

RESULTS

Cloning of the *H. pylori* NCTC 11637 cytochrome bc_1 gene clusters. Sequencing of the gene showed a predicted start codon, ATG, at three points, resulting in a deduced primary structure composed of three subunits, Rieske Fe-S protein, cytochrome *b*, and cytochrome c_1, each with methionine as the N-terminal amino acid (see Fig. S1 in the supplemental material). ORFs coding the former two proteins are separated by gaps of 14 nucleotides (nucleotide positions 16 to 506 for the Fe-S protein, 520 to 1765 for cytochrome *b*). Nucleotides 1763 to 1768, AGATGA, are for arginine and the termination codon in cytochrome *b*, while nucleotides 1765 to 1770, ATGAAA, are for the start codon and lysine in the cytochrome c_1 subunit (nucleotide position 1765 to 2619); i.e., nucleotides 1765 to 1768 overlapped. The calculated molecular masses of these proteins are 18 kDa, 47 kDa, and 32 kDa, respectively.

The Fe-S protein and cytochrome *b* subunits, respectively, had high similarity to those of aerobic microbes in sequence alignments (Fig. S1). The alignments of Fe-S protein subunits from *H. pylori*, *Rhodobacter capsulatus*, *Paracoccus denitrificans*, and *Saccharomyces cerevisiae* showed 49.4% sequence identity and two conserved ion-sulfur centers (Fig. S1). Two pairs of heme-ligating histidine residues were also conserved in the alignment of cytochrome *b* subunits among *H. pylori*, *R. capsulatus*, *P. denitrificans*, *Thiobacillus novellus*, and *Rickettsia typhi* (Fig. S1), with 60.8% identity. In contrast, the sequence identity of the cytochrome c_1 subunit among *H. pylori*, *R. capsulatus*, *R. shaeroides*, *P. denitrificans*, *Rhizobium legminosarum*, and *T. denitrificans* was rather lower, 33.1% (Fig. S1). One heme *c*-binding site was conserved, and another heme *c*-attachment motif was seen in only the *H. pylori* subunit.

Purification of *H. pylori* cytochrome bc_1 from recombinant *E. coli* cells. A pure form of *H. pylori*'s cytochrome bc_1 complex was obtained for the first time, as far as we know, by overexpressing the cytochrome bc_1 gene in an active form in *E. coli* Rosetta 2 cells. The absorption spectra of the purified cytochrome bc_1 (Fig. 1A) were similar to those of the partially

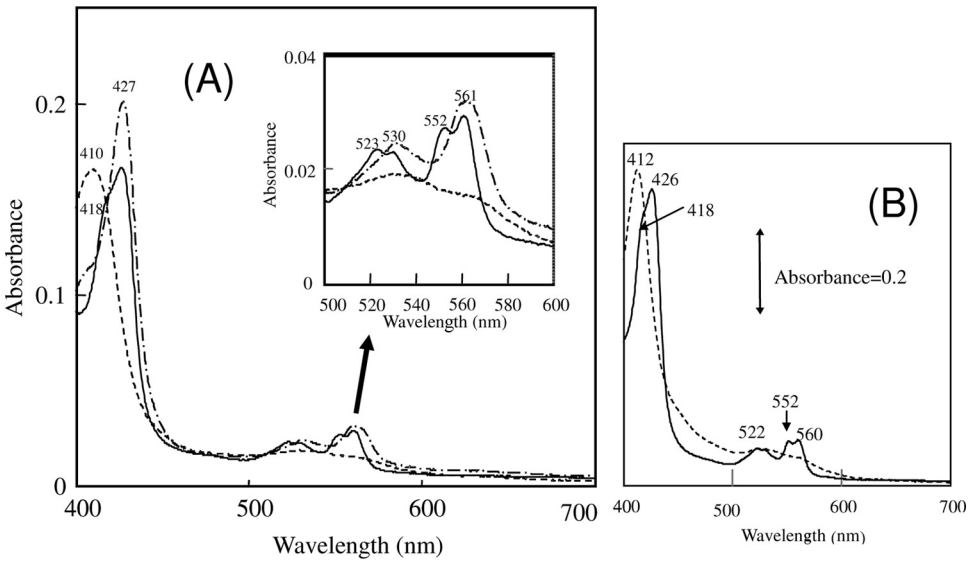

FIG. 1. Absorption spectra of the *H. pylori* NCTC 11637 cytochrome bc_1 complex prepared from recombinant *E. coli* cells (A) and *H. pylori* NCTC 11637 cells (B). Fifty micrograms of the recombinant cytochrome bc_1 or an appropriate amount of the cytochrome bc_1 fraction prepared from *H. pylori* cells was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 7.0). Absorption spectra were measured at room temperature. ——, reduced with 0.1 mM TMPD together with 1.0 mM ascorbate; —·—, reduced with a small grain of hydrosulfite; – – – –, as prepared.

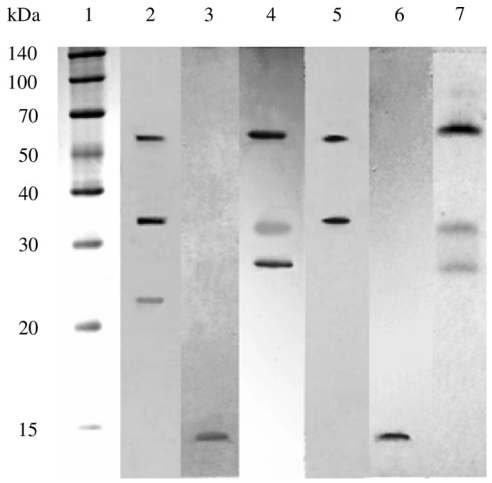

FIG. 2. SDS-PAGE pattern of *H. pylori* NCTC 11637 cytochromes used in the reconstitution experiment. Lane 1, marker proteins for molecular masses, 140 kDa, 100 kDa, 70 kDa, 50 kDa, 44 kDa, 30 kDa, and 20 kDa; lanes 2 and 5, recombinant cytochrome bc_1 (20 μg); lanes 3 and 6, cytochrome *c*-553 (15 μg); lanes 4 and 7, cytochrome cbb_3 (35 μg). Lanes 1, 2, 3, and 4, stained for protein with Coomassie brilliant blue R250; lanes 5, 6, and 7, stained for heme with *o*-tolidine.

purified cytochrome bc_1 prepared from *H. pylori* NCTC 11637 cells (Fig. 1B), suggesting that the cytochrome bc_1 from recombinant *E. coli* cells is the same protein complex as that produced in the *H. pylori* NCTC 11637 cells. The recombinant cytochrome bc_1 complex showed distinct α, β, and γ peaks at 561 nm, 523 nm, and 427 nm with shoulders at 552 nm, 530 nm, and 418 nm in the *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD)-ascorbate-reduced form, and peaks at 561 nm, 530 nm, and 427 nm without shoulders in the hydrosulfite-reduced form. The oxidized, native form of the cytochrome showed a prominent absorption maximum at 410 nm.

The apparent molecular masses of the Fe-S protein, cytochrome *b*, and cytochrome c_1 were estimated to be 21 kDa, 55 kDa, and 33 kDa using SDS-PAGE (Fig. 2), corresponding to the values of 18 kDa, 47 kDa, and 32 kDa, respectively, calculated based on the deduced primary structure. The discrepancy in values between the two proteins may be due to the detergents combined to the purified proteins. The results of an analysis of cytochrome bc_1 complex carried out on a TSKgel G3000 gel filtration column showed a molecular mass of 110 kDa (data not shown) based on marker proteins (Oriental Yeast, Tokyo, Japan) with molecular masses of 290 kDa, 142 kDa, 67 kDa, 32 kDa, and 12.4 kDa in the presence of 50 mM sodium phosphate buffer (pH 7.2) containing 200 mM NaCl and 0.1% Tween 20. Since this value well coincides with the value of 109 kDa (the sum of 21, 55, and 33), cytochrome bc_1 was indicated to be a monomeric complex. Since the 55-kDa and 33-kDa bands were stained for heme, it was confirmed that the two bands were of cytochromes.

Purification of cytochrome *c*-553 and cytochrome cbb_3. These cytochromes were purified to homogeneity from *H. pylori* NCTC 11637 cells as judged by SDS-PAGE analyses (Fig. 2). The purified cytochrome *c*-553 (Fig. 3A) and cytochrome cbb_3 (Fig. 3B) showed absorption spectra almost identical to those reported by Koyanagi et al. (8), and Tsukita et al. (19), respectively. The 32-kDa and 29-kDa bands of cytochrome cbb_3 were stained for heme faintly because of loose binding of protohemes to the protein moiety.

Respiration with the reconstituted system. In the *H. pylori* respiratory chain, electrons are suggested to be transported from an electron donor to the electron acceptor, oxygen, via menaquinone-6, cytochrome bc_1, cytochrome *c*-553, and cytochrome cbb_3, in this order (7). We prepared a reconstituted respiratory chain, the electron transport system, by adding each purified component, i.e., DAD, coenzyme Q_0, cytochrome bc_1, cytochrome *c*-553, and cytochrome cbb_3 to sodium phosphate buffer (pH 7.0) containing 1% phosphatidylcholine. Since the intrinsic quinone of *H. pylori*, menaquinone-6 was not commercially available, we used Q_0. Upon the addition of D-proline, the most active substrate of DAD (17), to the reconstituted respiratory system, a reduced type of absorption spectrum of cytochrome cbb_3 appeared (Fig. 4A), although the height of the α peak was lower than that reduced with hydrosulfite (Fig. 3B). The absorption spectrum of the DAD-free

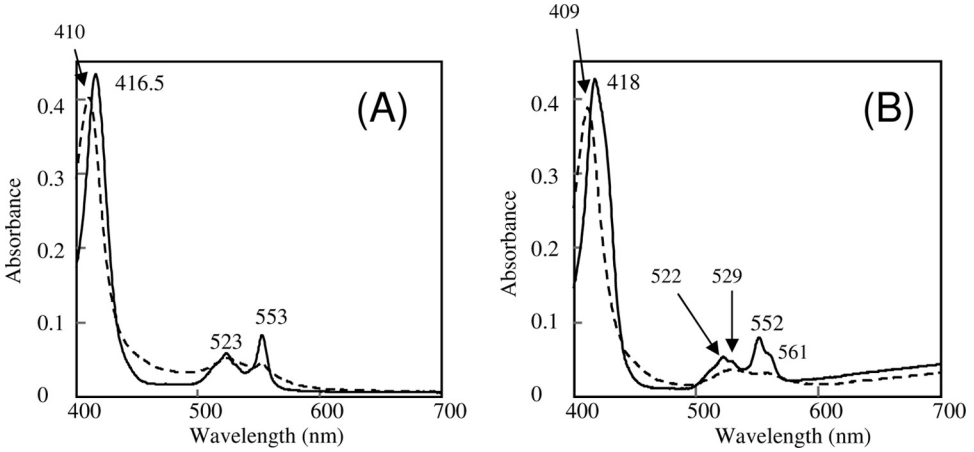

FIG. 3. Absorption spectra of purified cytochrome *c*-553 (A) and cytochrome cbb_3 (B). The cytochromes were prepared as described under Materials and Methods. Four micrograms of cytochrome *c*-553 or 100 μg of cytochrome cbb_3 was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 7.0), and the absorption spectra were measured at room temperature. ——, reduced with hydrosulfite; – – –, oxidized (as prepared).

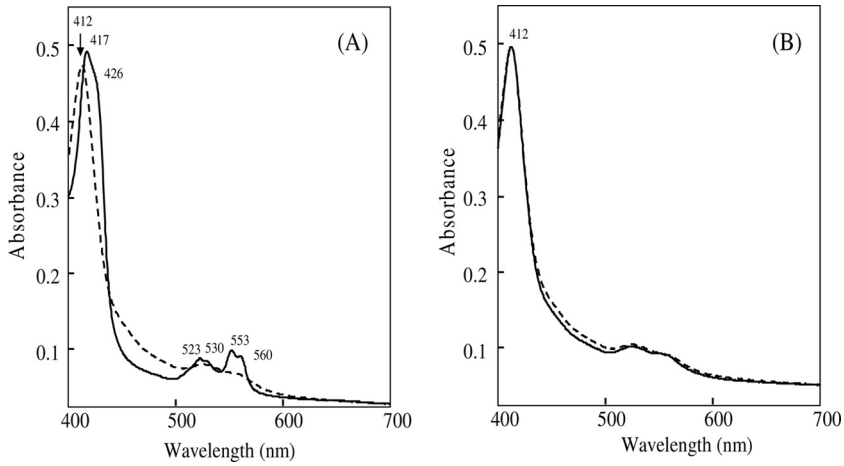

FIG. 4. Absorption spectra of the *H. pylori* NCTC 11637 reconstituted electron transport system. The reaction medium contained 30 μg of DAD, 50 μM Q_0, 10 μg of the recombinant cytochrome bc_1, 20 μg of cytochrome *c*-553, and 80 μg of cytochrome cbb_3 in 50 mM sodium phosphate buffer (pH 7.0) containing 1% phosphatidylcholine and 25%-saturated oxygen. After a 5-min incubation at 37°C, 100 mM (final concentration) D-proline was added. (A) Absorption spectra of the complete reaction mixture; (B) spectra of an incomplete reaction mixture without DAD. ——, after addition of D-proline; – – –, before addition of D-proline.

reconstitution mixture did not change to the reduced form upon the addition of D-proline (Fig. 4B). All other components were also demonstrated to be indispensable to respiration in the same manner as above (data not shown). These results clearly showed that electrons liberated from hydrogen molecules yielded from D-proline by the dehydrogenation reaction mediated by DAD were transported to cytochrome cbb_3 via Q_0, cytochrome bc_1, and cytochrome *c*-553. A similar result was obtained with D-alanine as the electron donor (data not shown).

The K_m value for the dehydrogenation reaction of D-proline mediated by DAD was 13.0 mM or 44.5 mM when ubiquinone Q_0 or Q_1 was used, respectively, as the electron acceptor. Under anaerobic conditions in a Thunberg-type tube, the K_m values for the reactions were similar. The K_m value for the reduction of Q_0 or Q_1 by the electrons released from D-proline was much smaller than the above values, i.e., 10.2 μM or 8.2 μM, respectively. The K_m values for the reduction and oxidation of cytochrome bc_1, were, respectively, 2.31 μM and 1.37 μM when Q_0 was used as the electron donor, and 1.37 μM and 1.52 μM, respectively, when Q_1 was used. The last process of the electron transport, the oxidation of cytochrome *c*-553 by cytochrome cbb_3 is reported to have a K_m value of 0.9 μM (19).

We also examined the oxygen-consuming activity of the reconstituted electron transport chain. Oxygen dissolved in the reconstituted mixture was quickly used up on the addition of D-proline (Fig. 5). The oxygen consumption was completely inhibited by 40 μM sodium cyanide (data not shown). It was revealed that all the components of the mixture are necessary for respiration because a mixture lacking any of the components showed no oxygen consumption (data not shown).

Respiration by *H. pylori* whole cells. The rate of oxygen consumption by whole cells with 100 μM D-proline and D-alanine was 1.4 ± 0.6 and 1.3 ± 0.8 nmol oxygen min^{-1} mg^{-1} cells (wet weight) (mean ± standard deviation of three independent experiments), respectively, while the values with 100 μM pyruvate and D-lactate were 25.4 ± 2.2 and 21.4 ± 4.2 nmol oxygen min^{-1} mg^{-1} cells, respectively. The activity was increased 10-fold with 10 mM D-proline and D-alanine to 14.9 ± 1.4 and 15.3 ± 0.6 nmol oxygen min^{-1} mg^{-1} cells, respectively.

Effect of ATP synthesis uncouplers. As indicated in Table 2, the respiratory activity was increased approximately 3-fold by the addition of valinomycin plus nigericin or dinitrophenol when D-proline, D-alanine, or pyruvate was used as the respiratory substrate.

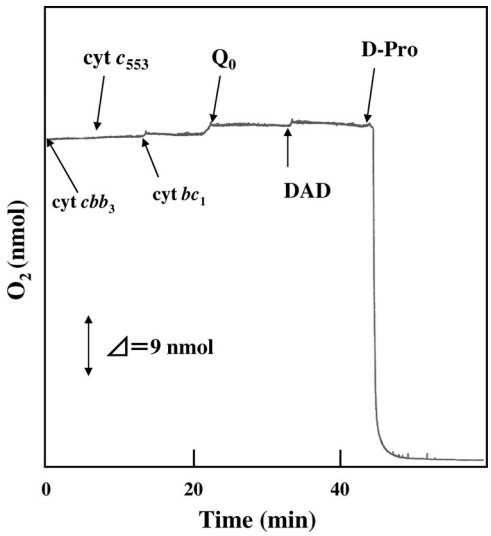

FIG. 5. Oxygen consumption by the *H. pylori* NCTC 11637 reconstituted electron transport system. After incubation of an electrode vessel containing 1% phosphatidylcholine and 25%-saturated oxygen in 50 mM sodium phosphate buffer (pH 7.0), at 37°C for 5 min, 5 μg of the recombinant cytochrome bc_1, 5 μg of cytochrome *c*-553, 5 μg of cytochrome cbb_3, 20 μM Q_0, 10 μg of DAD, and 20 mM D-proline were added in this order to make 600 μl. Oxygen consumption was monitored with a Clark-type oxygen electrode.

TABLE 2. Effect of ATP-synthesis uncoupler reagents on the respiratory activity of *H. pylori* cells

Substrate	Oxygen consumption activity, nmol oxygen min^{-1} mg^{-1} cells[a]		
	Control	Valinomycin + nigericin (10 μM)	DNP (100 μM)
D-Proline (10 mM)	14.9 ± 1.9	50.5 ± 3.0	48.3 ± 4.5
D-Alanine (10 mM)	15.3 ± 1.2	48.7 ± 3.2	50.2 ± 2.2
Pyruvate (100 μM)	25.4 ± 0.8	70.2 ± 4.3	75.2 ± 1.2

[a] Fresh *H. pylori* cells (1 mg, wet weight) were added to an oxygen electrode vessel containing 25% saturated oxygen, either D-alanine, D-proline, or pyruvate as a respiratory substrate, either valinomycin together with nigericin or 2,4-dinitrophenol (DNP) as the uncoupler, 100 mM NaCl, and 10 mM HEPES buffer (pH 7.0) in a total volume of 600 μl and incubated at 37°C for 5 min. Values are the means ± standard deviations for results of three independent experiments.

DISCUSSION

In the present study, the possibility that D-proline or D-alanine is utilized by the organisms that live in the microaerobic milieu as an electron donor in DAD-mediated electron transport has been shown. Electron transport from D-alanine to the cytochrome *bd* complex in *E. coli* membrane was suggested spectrophotometrically (5). However, a precise transport route of the substrate-derived electrons has not been revealed in any organisms. We have shown each component involved in the above electron transport, for the first time as far as we know, by using a reconstituted electron transport system composed of pure constituents, including the recombinant cytochrome bc_1 complex and DAD. Previously (12), we suggested the involvement of a *b*-type cytochrome in the D-proline- or D-alanine-stimulated oxygen uptake because the stimulation was repressed by antimycin A. In the present study, the involvement of cytochrome bc_1, cytochrome *c*-553, and cytochrome cbb_3 in D-proline or D-alanine respiration has been demonstrated. In addition, the respiration was indicated to be coupled with ATP synthesis based on an increase in oxygen consumption activity with the uncoupler reagents.

The level of oxygen consumption by the whole *H. pylori* cells was considerably higher with NADPH, pyruvate, or D-lactate (1) as the respiratory substrate than with D-proline or D-alanine (Table 2). The moderate activity with these amino acids may be ascribed to the large K_m value of the DAD-mediated reaction with the best substrate, D-proline (17), although the K_m value is comparable to that of *E. coli* enzyme (14). The reaction was carried out at pH 7.0, while the optimum pH of DAD is 8.0 (17) because almost no oxygen uptake was observed at pH 8.0, which may be due to other relevant reactions repressed at pH 8.0. Since *H. pylori* cells reside in the mucus layer of the gastric epithelium, where the occurrence of D-alanine was confirmed (12), the environment of the colonized cells is different from the one in the present experiments, and *H. pylori* cells may use D-alanine or D-proline as the respiratory substrate in the human stomach.

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