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ORIGINAL ARTICLE

D-Amino acid dehydrogenase from *Helicobacter pylori* NCTC 11637

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Abstract *Helicobacter pylori* is a microaerophilic bacterium, associated with gastric inflammation and peptic ulcers. D-Amino acid dehydrogenase is a flavoenzyme that digests free neutral D-amino acids yielding corresponding 2-oxo acids and hydrogen. We sequenced the *H. pylori* NCTC 11637 D-amino acid dehydrogenase gene, *dadA*. The primary structure deduced from the gene showed low similarity with other bacterial D-amino acid dehydrogenases. We purified the enzyme to homogeneity from recombinant *Escherichia coli* cells by cloning *dadA*. The recombinant protein, DadA, with 44 kDa molecular mass, possessed FAD as cofactor, and showed the highest activity to D-proline. The enzyme mediated electron transport from D-proline to coenzyme Q_1, thus distinguishing it from D-amino acid oxidase. The apparent K_m and V_{max} values were 40.2 mM and 25.0 μmol min^{-1} mg^{-1}, respectively, for dehydrogenation of D-proline, and were 8.2 μM and 12.3 μmol min^{-1} mg^{-1}, respectively, for reduction of Q_1. The respective pH and temperature optima were 8.0 and 37°C. Enzyme activity was inhibited markedly by benzoate, and moderately by SH reagents. DadA showed more similarity with mammalian D-amino acid oxidase than other bacterial D-amino acid dehydrogenases in some enzymatic characteristics. Electron transport from D-proline to a *c*-type cytochrome was suggested spectrophotometrically.

Keywords D-Amino acid dehydrogenase · D-Proline · Gene cloning · *Helicobacter pylori* · Bacterial respiration

Abbreviations

DAD	D-Amino acid dehydrogenase
DadA	DAD purified from recombinant *Escherichia coli*
DCIP	2,6-Dichlorophenolindophenol
PMS	Phenazine methosulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Helicobacter pylori is a Gram-negative bacterium associated with gastric inflammation, peptic ulcers, and possibly gastric cancer (Dunn et al. 1997). The bacterium is microaerophilic; its natural habitat is the mucous layer of the human gastric epithelium (Dunn et al. 1997).

Database The sequence of *H. pylori* NCTC 11637 gene *dadA* reported in this paper has been submitted to DDBJ, GenBank ™ and BBI databanks under accession No. AB295062 (NCBI accession No. BAF48065).

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D-Amino acid dehydrogenase [EC 1.4.99.1] (DAD) is a membrane-bound enzyme that catalyzes dehydrogenation reactions with neutral free D-amino acids yielding 2-oxo acids, ammonia, and hydrogen, subsequently yielding protons and electrons. The electrons have been shown to be transferred to cytochromes in the *Escherichia coli* respiratory chain (Franklin and Venables 1976). In an earlier study, DAD was solubilized from the *E. coli* membrane, purified, and characterized as an iron-sulfur flavoprotein composed of two subunits with molecular masses of approximately 55 and 45 kDa (Olsiewski et al. 1980). The enzyme has been found in a few Gram-negative bacteria such as *E. coli* (Raunio et al. 1973), *Pseudomonas fluorescens* (Tsukada 1966), *P. aeruginosa* (Marshall and Sokatch 1968), and *Salmonella typhimurium* (Wild et al. 1974). The enzyme has not been studied extensively except for the one from *E. coli* (Olsiewski et al. 1980).

Because we detected D-alanine in *H. pylori* cells (Nagata et al. 2003) and in human gastric juice (Nagata et al. 2007), in this study, we demonstrate the presence of DAD in this bacterium. For that purpose, we cloned the *dadA* gene from *H. pylori* NCTC 11637, the type strain, and purified the enzyme from recombinant *E. coli* cells.

Materials and methods

Bacterial strains and culture

Helicobacter pylori NCTC 11637 was cultured on Brucella agar plates (Becton Dickinson, NJ, USA) containing Campylobacter Selective Supplement (Oxoid, Hampshire, UK) and 5% horse serum (Sigma-Aldrich, MO, USA) under 10% CO_2 at 37°C for 48 h. Cultured cells were harvested by centrifugation at 8,000×*g* for 20 min and suspended in 50 mM sodium-phosphate buffer (pH 7.0) containing 0.9% NaCl. The cells were stored at −80°C until use after being washed with the same buffer.

Escherichia coli BL21 (DE3) (Novagen, Madison, WI, USA) cells harboring pHpdadA were cultured on Luria-Bertani (LB) medium containing 25 mg/l kanamycin in continuously shaken Sakaguchi flasks at 37°C for 13 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) containing 0.9% NaCl before being stored at −80°C until use.

Partial purification of DAD from *H. pylori* cells

Harvested *H. pylori* cells (wet weight 40 g) were suspended in four volumes of 50 mM sodium-phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 10% glycerol. They were disrupted by being passed 10 times through a French press (Ohtake Works, Tokyo) at 140 MPa. All experimental procedures described hereinafter were carried out at 4°C unless stated otherwise. The supernatant of centrifugation at 18,000×*g* for 15 min (the cell-free extract) was further centrifuged at 140,000×*g* for 60 min. The resultant pellet (the membrane fraction) was suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100 (Sigma), and sonicated using an ultrasonic oscillator (Sonifier 450; Branson, CT, USA) for 20 min at 50 W (4 5-min rounds of sonication at 5-min intervals). The solubilized proteins were recovered in the supernatant after centrifugation at 140,000×*g* for 60 min. Then the supernatant was applied to a DEAE-Toyopearl column equilibrated with the same buffer. The enzyme fraction was eluted from the column with the same buffer containing a linear gradient of NaCl from 0 to 500 mM.

Isolation of DAD gene, *dad*, from *H. pylori*

The genomic DNA of *H. pylori* NCTC 11637 was isolated using a GenTLE™ yeast kit (Takara Shuzo, Kyoto, Japan). The oligonucleotide primers for PCR, (5′-GCTAAACT GCCTAAAGACGC-3′) as the sense primer and (5′-GCA CCACCGAATCAATCG-3′) as the antisense primer, were designed based on the upstream and downstream regions of the *dadA* gene of *H. pylori* 26695 (accession No. AAD07988), described as a putative gene for DAD. Amplification of nucleotides between the two primers was performed using PCR with HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) and the genomic DNA of *H. pylori* NCTC 11637 as the template. The amplified product was purified using PCR (Suprec; Takara Shuzo) followed by direct sequencing, and was analyzed using a genetic analyzer (3100; Applied Biosystems, CA, USA) equipped with a Big Dye™ Terminator ver 3.1 (Applied Biosystems). We named the gene *dadA*.

Expression of the *dad* gene in *E. coli* cells

Two primers were newly designed based on the nucleotide sequence of *H. pylori* NCTC 11637 *dadA*. The first primer was designed to contain the N-terminal region of *dadA* and the *Nde*I digestion sequence (5′-GGAATTCCATATGAAAAA AGAAGTCGTGG-3′); the second one contained the C-terminal region and the *Eco*RI digestion sequence (5′-CG GAATTCCTTAATCCCTAAAAAATGCA-3′). Amplification of the nucleotides between the two primers was performed by PCR using a PCR System (TripleMaster; Eppendorf) and the genomic DNA of *H. pylori* NCTC 11637 as a template. The amplified product was digested by *Nde*I (Takara Shuzo) and *Eco*RI (Takara Shuzo). The resulting fragment was ligated into the plasmid vector pET-41b (+)

(Novagen) that had been previously digested with the same enzymes using a DNA ligation kit (Ver. 2.1; Takara Shuzo). The constructed plasmid was named pHpdadA and transferred into *E. coli* BL21 (DE3) cells. The transformants were cultured on LB agar plates containing kanamycin (25 mg/l) at 37°C.

Purification of recombinant DAD

All experimental procedures described hereinafter were carried out at 4°C unless stated otherwise. The recombinant *E. coli* cells were suspended in 50 mM sodium-phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10% glycerol, and 50 μM FAD, and disrupted by sonication using a Sonifier 450 for 15 min (15 1-min rounds of sonication at 30-s intervals) at 28 W. Cell debris and intact cells were spun off by centrifugation at 8,000×*g* for 20 min; the resultant cell-free extract was centrifuged at 140,000×*g* for 60 min. The precipitate was used as the membrane fraction. The fraction was suspended in 20 mM Tris-HCl buffer (pH 8.0) containing 0.03% Triton X-100, 10% glycerol, and 10 μM FAD; then immediately subjected to sonication at 28 W for 1 min. The insoluble proteins from this treatment were collected by centrifugation at 140,000×*g* for 60 min, solubilized using a similar buffer containing 0.06% Triton X-100 in place of the 0.03% detergent, and subsequently subjected to sonication. Purified DAD (DadA) was obtained from the supernatant yielded by the subsequent centrifugation at 140,000×*g* for 60 min.

Fragmentation of DadA

The purified DadA (25 μg) prepared from the recombinant *E. coli* cells was dissolved in 100 μl of 70% formic acid and incubated at room temperature for 48 h. The solution was dried in a centrifugal evaporator, and the resultant residue was dissolved in a small amount of H_2O. The resultant peptide fragments were separated using SDS-PAGE, and subjected to N-terminal amino acid sequence analysis, as described below.

N-Terminal amino acid sequence determination

DadA and the internal peptide fragments from DadA separated on a SDS-PAGE gel, were transferred to a polyvinylidene fluoride membrane (Sequi-Blot™, Bio-Rad, CA, USA) using a semi-dry system (BE-320; Bio-Craft, Tokyo) and then stained with Coomassie brilliant blue R250. The bands were cut out and the N-terminal amino acid sequences of the proteins were analyzed using an automated Edman degradation protein sequencer (PPSQ-1; Shimadzu, Kyoto).

DAD enzyme assay, protein determination, electrophoresis and measurement of absorption spectra

The reaction mixture (200 μl) contained an appropriate amount of enzyme, 50 mM sodium-phosphate buffer (pH 8.0), 0.06% Triton X-100, 1.8 μM phenazine methosulfate (PMS), and 0.5 mM 2,6-dichlorophenolindophenol (DCIP), and preincubated at 37°C. Immediately after addition of 20 mM (final concentration) D-proline, reduction of DCIP was monitored by absorbance at 600 nm using a spectrophotometer (UltroSpec4300; Amersham BioSciences, NJ, USA). In an alternative measurement, 1 mM coenzyme Q_1 and 1% phosphatidylcholine were added to the above reaction mixture in place of DCIP. The initial velocity of the enzyme reaction was calculated using the extinction coefficient values for DCIP, 21.5 mM^{-1} cm^{-1} at 600 nm, and for Q_1, 14.4 mM^{-1} cm^{-1} at 275 nm. The protein concentration was determined using Bradford's method (Bradford 1976) with bovine serum albumin as a standard.

SDS-PAGE was carried out using 10% polyacrylamide gel, according to Laemmli's method (Laemmli 1970). A low-molecular weight calibration kit for SDS-PAGE (Amersham) was used for marker proteins.

Absorption spectra were measured using spectrophotometry with UltroSpec 4300.

Detection of FAD in DadA

The purified DadA was dissolved in 50 mM acetate buffer (pH 4.0) after removing the FAD-containing buffer used in DadA purification by passing through a TSKgel G-3000SW (Tosoh, Tokyo) column. The FAD liberated from DadA was separated by the TSK gel or by centricon YM-10 (Amicon, MA, USA) ultrafiltration, and identified by the thin-layer chromatography using a Silica Gel 60 plate (Merck Darmstadt, Germany) and *n*-butanol–acetic acid–water (3:1:1, v/v/v) as solvent.

Results and discussion

Purification of DAD from *H. pylori* and recombinant *E. coli* cells

DAD activity previously detected in the cell-free extract was found in the membrane fraction solubilized with 0.5% Triton X-100. The specific activity increased 65-fold from the cell-free extract by subsequent ion-exchange column chromatography. However, the protein was unstable; further purification was therefore impossible.

DadA, the *H. pylori* NCTC 11637 DAD, whose gene is *dadA*, was expressed in an active form in *E. coli* BL21 (DE3) cells. Most DadA activity detected in the recombinant

E. coli membrane fraction was then easily purified to a single band in SDS-PAGE analysis (Fig. 1) by removing impure proteins by treatment with 0.03% Triton X-100 prior to that with 0.06% one. The specific activity of DadA was 6.22 μmol min^{-1} mg^{-1} when it was measured with 20 mM D-proline as the substrate (Table 1). The apparent molecular mass of DadA was estimated as 44 kDa using SDS-PAGE analysis (Fig. 1), showing coincidence with the value of 46,040 Da, calculated based on the deduced primary structure of *dadA* (Fig. 2). Its molecular mass corresponds to that of the smaller subunit of the *E. coli* DAD, encoded by the *dadA* gene (Lobocka et al. 1994).

DNA sequence and the deduced primary structure of the dadA gene from *H. pylori*

We named the *H. pylori* NCTC 11637 gene *dadA* (DDBJ, GenBank™, BBI data bank, accession No. AB295062; NCBI accession No. BAF48065). DNA sequence analysis of *dadA* showed the predicted start codon, ATG, and an open reading frame consisting of 1,233 bases. The N-terminal amino acid sequence of the protein recovered from the band on the SDS-PAGE (Fig. 1, lane 5) gel was MKKEVVVIGG; and that from the fragmented protein was PGEVM, which coincides with the primary structure of DadA deduced from the *dadA* gene of *H. pylori* NCTC 11637 and the internal fragment of the same protein, respectively (Fig. 2). Consequently, the DadA protein obtained in the present experiment was confirmed to be the protein expressed by the *dadA* gene of *H. pylori* NCTC 11637. The primary structure deduced from the *H. pylori* NCTC 11637 *dadA* (DadA or HpDadA in Fig. 2) showed 98.5% similarity with that from *H. pylori* 26695 *dadA*. HpDadA was compared with those of other DADs (Fig. 2). Sequence similarity between HpDadA and *E. coli* DAD is only 22.3%, while the similarity among DADs from *E. coli*, *P. aeruginosa*, *Paracoccus denitrificans*, and *S. typhimurium* is 77.5%. However, the similarity of the N-terminal 33 amino acid residues among these proteins including HpDadA, and even D-proline dehydrogenase from an archaeon *Pyrobaculum islandicum* (Satomura et al, 2002) and D-amino acid oxidase [EC 1.4.3.3] from pig kidney is roughly 40%, higher than the other parts of the proteins, indicating the importance of this protein region in the evolution. Hydrophobicity patterns (plots of hydrophobicity of each amino acid residue against the amino acid position from N-terminus) of this region are also quite similar among these proteins. Therefore, FAD/NAD-binding domain (GxGxxG) and membrane-binding domain of DadA are suggested to be in the N-terminal 33 residues (Ronchi et al. 1982; Fukui et al. 1987; Lobocka et al. 1994).

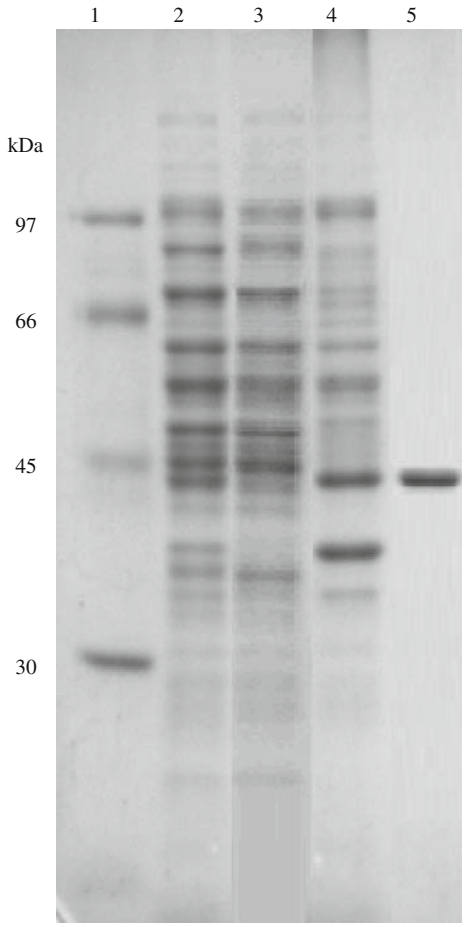

Fig. 1 SDS-PAGE analysis of *H. pylori* DadA from recombinant *E. coli* cells. *Lane 1* marker proteins, *lane 2* cell-free extract (20 μg), *lane 3* soluble fraction (20 μg), and *lane 4* membrane fraction (15 μg) of *E. coli* BL21 (DE3) cells harboring pHpdadA. *Lane 5* solubilized fraction (2 μg) of the membrane fraction shown in *lane 4*, by treatment with 0.06% Triton X-100. A representative result is shown

Table 1 Purification of *H. pylori* D-amino acid dehydrogenase from recombinant *Escherichia coli* cells

	Total protein (mg)	Total activity (μmol min^{-1})	Specific activity[a] (μmol min^{-1} mg^{-1})	Yield (%)
Cell-free extract	240	110	0.46	100
Membrane fraction	40.6	94.3	2.32	85.7
Solubilized fraction (=DadA)	13.0	81.5	6.22	74.1

[a] Assays were performed with DCIP

Characterization of DadA

All the assays were carried out under aerobic conditions because no difference was observed from those under anaerobic conditions in the enzyme activity and DadA

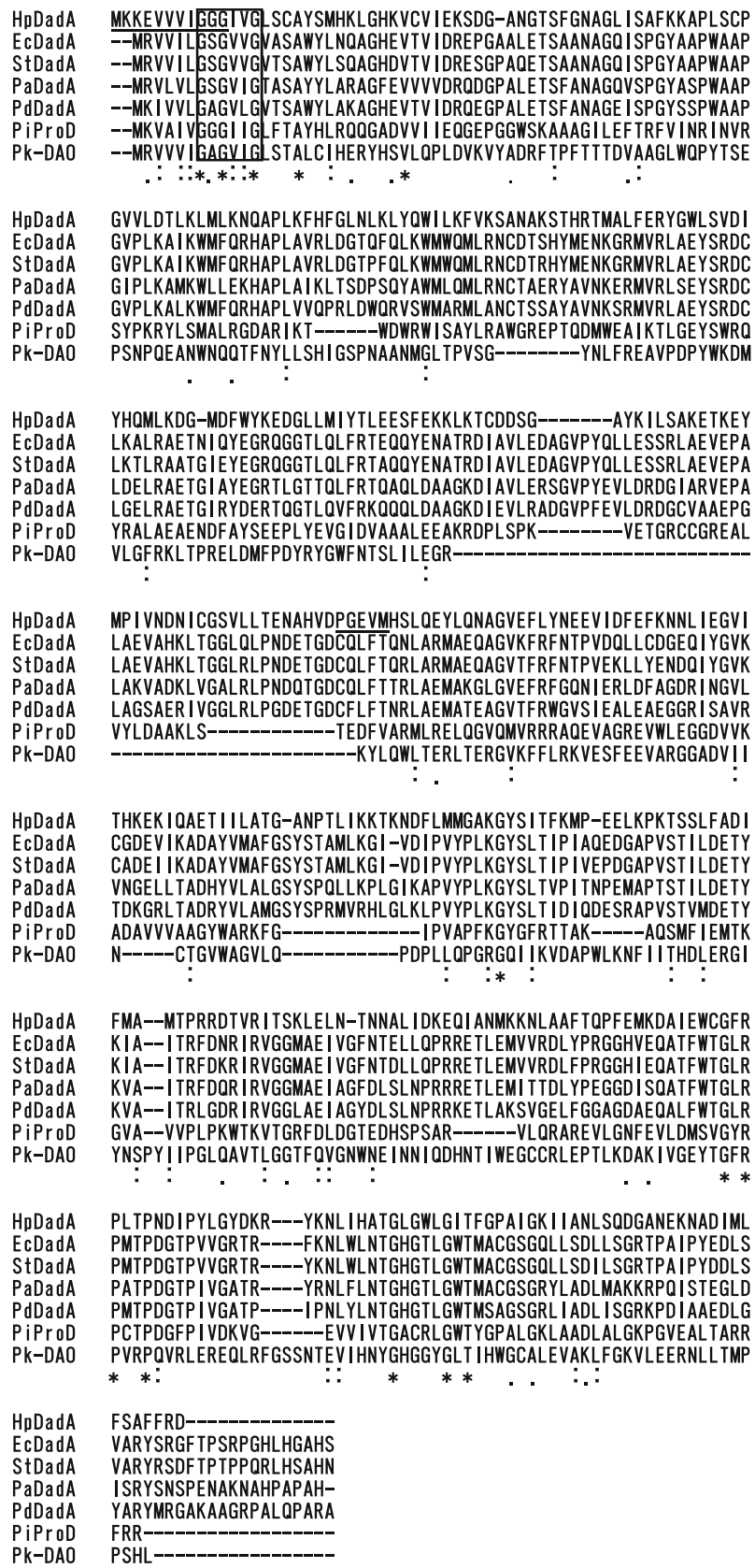

Fig. 2 Linear alignment of the amino acid sequence of *H. pylori* DadA (HpDadA) with those of other bacterial D-amino acid dehydrogenases. The DadAs are: HpDadA of *H. pylori* NCTC 11637 (NCBI accession No. BAF48065); EcDadA of *E. coli* (AAC74273); StDadA of *Salmonella typhimurium* (AAL20718); PaDadA of *Pseudomonas aeruginosa* (AAG08689); and PdDadA of *Paracoccus denitrificans* (YP_914612). PiProD, *Pyrobaculum islandicum* D-proline dehydrogenase (BAB88883), and Pk-DAO, pig kidney D-amino acid oxidase (AAA31025), are also shown. *Asterisks* indicate identical residues among the seven sequences; *double* and *single dots* respectively indicate conservative substitutions and functionally similar residues. The determined N-terminal and internal amino acid sequences of *H. pylori* DadA are shown as underscored. The nucleotide-binding motif is shown with a *rectangular frame*

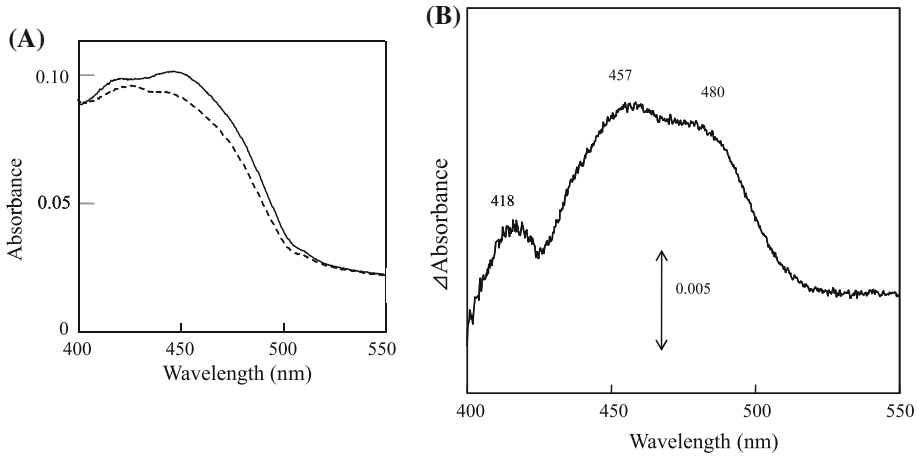

Fig. 3 Absorption spectra (**A**) and the difference spectrum (**B**) of *H. pylori* DadA. The spectrum (**B**) was obtained by subtracting the spectrum with the addition of 20 mM D-proline (*dotted lines* in **A**) from the one as prepared, without addition of D-proline (*solid lines* in **A**). The reaction mixture contained 20 μg of DadA, 50 mM sodium-phosphate buffer (pH 7.0), 0.06% Triton X-100, and ±20 mM D-proline in 200 μl. The spectra were measured 5 min after incubation at 37°C

absorption spectrum. The cofactor bound to DadA was revealed to be FAD spectrophotometrically (Fig. 3): DadA was reduced by its substrate, D-proline, and the difference absorption spectrum of oxidized (as prepared) form minus reduced form is of a typical FAD protein with a peak at 457 nm and a shoulder at 480 nm. The cofactor liberated from DadA was confirmed to be FAD by the thin-layer chromatography (Fig. 4): the cofactor moved to the same point as the authentic FAD.

DadA showed high substrate specificity to D-proline: the activity to D-proline was nearly three- and fivefold higher than that to D-alanine and D-phenylalanine, respectively, (Table 2). D-Serine was a poor substrate. The low activities observed with D-valine, D-methionine, D-aspartate, D-glutamate, L-proline, and L-alanine were insignificant. The values for L-proline and L-alanine are those after subtraction of their D-isomer contents, 0.2%, in the reagents used for the assay. The substrate specificity is the same as that obtained for the partially purified enzyme from the *H. pylori* cells (DEAE-Toyopearl eluate). The preferred substrates of the other bacterial DADs are D-alanine, D-phenylalanine, D-methionine, and D-valine (Franklin and Venables 1976; Tsukada 1966; Marshall and Sokatch 1968; Wild et al. 1974). D-Amino acid oxidase from pig kidney (Dixon and Kleppe 1965) and archaeal D-proline dehydrogenase (Satomura et al. 2002) also possess three- to fourfold higher activity to D-proline than D-alanine. The substrate specificity of other D-amino acid oxidases from 3 species of yeasts, and *Fusarium oxysporum* (Fischer et al. 1996) are different from that of DadA. Therefore, the DadA's substrate specificity is similar to those of the pig D-amino acid oxidase and the archaeal D-proline dehydrogenase rather than the bacterial DADs. Proline-utilization A flavoenzyme in PutA (Krishnan and Becker 2006) is another proline dehydrogenase of *H. pylori*, but it does not catalyze dehydrogenation of the D-enantiomer of proline (Scarpulla and Soffer 1978; Menzel and Roth 1981). Hence, DadA is distinct from PutA.

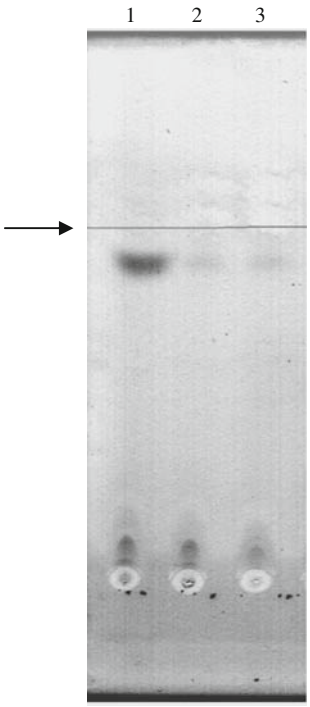

Fig. 4 Detection of FAD by thin-layer chromatography. *Lane 1* FAD, *lane 2* FAD liberated from DadA in acetate buffer (pH 4.0), and collected by gel filtration, *lane 3* FAD liberated from DadA in acetate buffer (pH 4.0), and collected by ultrafiltration. *Arrow*, solvent front

The optimum pH and temperature for DadA were 8.0 (Fig. 5A) and 37°C (Fig. 5B), respectively. These values also coincide with those shown by the enzyme partially purified from *H. pylori* cells (data not shown), suggesting the identity of the purified DadA and the protein that we intended to purify from the *H. pylori* cells. DadA mediated electron transfer from D-proline to coenzyme Q_1. In the reaction with D-proline as the electron donor and Q_1 as the electron acceptor, the apparent kinetic values for K_m and V_{max} were 40.2 mM and 25.0 μmol min^{-1} mg^{-1}, respectively, for dehydrogenation from D-proline, and were 8.2 μM and

Table 2 Substrate specificity of *H. pylori* DadA

Amino acids	Specific activity[a] (μmol min^{-1} mg^{-1})	(%)
D-Proline	6.22 ± 0.12	100
D-Alanine	2.29 ± 0.23	36.8
D-Phenylalanine	1.25 ± 0.32	19.8
D-Serine	0.42 ± 0.15	6.7
D-Valine	0.05 ± 0.02	0.8
D-Methionine	0.05 ± 0.04	0.8
D-Glutamate	0.06 ± 0.06	1.0
D-Aspartate	0.05 ± 0.10	0.8
L-Proline	0.22 ± 0.21	3.5
L-Alanine	0.12 ± 0.17	1.9

[a] Each value represents the mean ± SD of five independent assays performed with DCIP

Table 3 Effects of some reagents on DadA activity

Reagents	Specific activity[a] (μmol min^{-1} mg^{-1})	(%)
Control	6.12 ± 0.10	100
Benzoic Acid (0.1 mM)	0.12 ± 0.07	2.0
p-Hydroxybenzoic acid (0.1 mM)	1.31 ± 0.43	21.4
Iodoacetamide (0.1 mM)	2.09 ± 0.50	34.2
Iodoacetic acid (0.1 mM)	2.34 ± 0.18	38.2
$MgCl_2$ (1 mM)	5.94 ± 0.26	97.1
$CaCl_2$ (1 mM)	6.00 ± 0.34	98.0
EDTA (5 mM)	6.24 ± 0.34	102

[a] Each value represents the mean ± SD of five independent assays performed with DCIP

12.3 μmol min^{-1} mg^{-1}, respectively, for reduction of Q_1. In the reaction with DCIP substituted for Q_1, the K_m and V_{max} values were 5.3 mM and 4.5 μmol min^{-1} mg^{-1}, respectively, and that with D-alanine instead of D-proline were 60.0 mM and 18.3 μmol min^{-1} mg^{-1}, respectively. The enzyme activity was markedly inhibited by 0.1 mM benzoate (Table 3). This effect is prominent compared to other DADs, and it resembles, rather, to that of D-amino acid oxidase from pig kidney (Frisell et al. 1956). DadA was substantially inhibited also by 0.1 mM SH reagents such as p-hydroxymercuribenzoate, iodoacetamide, and iodoacetate (Table 3), indicating involvement of cysteine residues in the enzyme function. The D-amino acid oxidase is also sensitive to these reagents (Frisell and Hellerman 1956). EDTA, Mg^{2+} and Ca^{2+} had no effect on DadA activity.

Possible involvement of DAD in energy production in *H. pylori* cells

Upon addition of D-proline, the absorbance of the cell-free extract increased (Fig. 6A). The difference absorption spectrum with and without D-proline yielded a cytochrome-type spectrum showing an α-peak and a β-peak at 553 nm and 523 nm, respectively (Fig. 6B, a). A similar result was obtained with D-alanine (data not shown). Increased absorbance at α- and β-peaks of a cytochrome indicates that the cytochrome accepted electrons and was converted to its reduced form. Therefore, this result suggests that electrons liberated from D-proline were transported to the cytochrome. The liberation of electrons might have been catalyzed by DAD in the cell-free extract. In *H. pylori's* respiratory chain, electrons are suggested to be transported from an electron donor to oxygen via menaquinone 6, cytochrome bc_1, cytochrome c-553, and cytochrome cbb_3 which is the terminal oxidase, in this order (Kelly 1998).

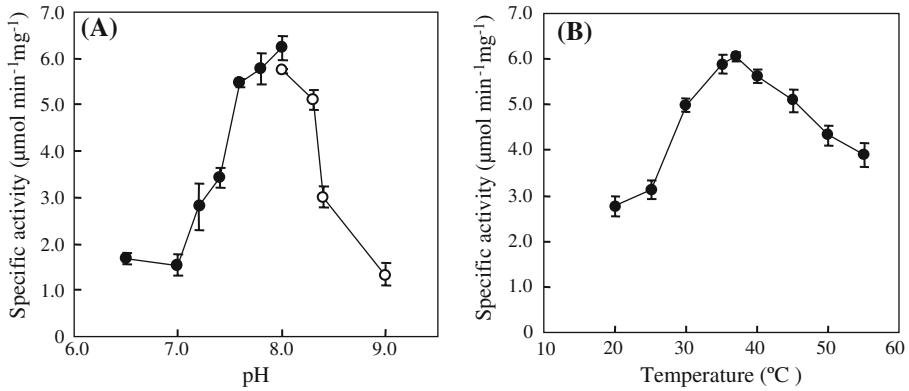

Fig. 5 pH-dependence (**A**) and temperature-dependence (**B**) of *H. pylori* DadA activity. The 200 µl assay mixture contained 2 µg of DadA, 20 mM D-proline, 0.06% Triton X-100, 1.8 µM PMS, and 0.5 mM DCIP. The buffers used in (**A**) were 50 mM sodium-phosphate buffer for pH 6.5–8.0 (*filled circle*), and 50 mM Tris–HCl buffer containing 50 mM phosphoric acid for pH 8.0–9.0 (*open circle*). The reaction was initiated by the addition of DadA and carried out at 37°C (**A**), or at various temperatures (**B**) in 50 mM sodium-phosphate buffer (pH 8.0). Each plot represents the mean ± SD of five independent assays

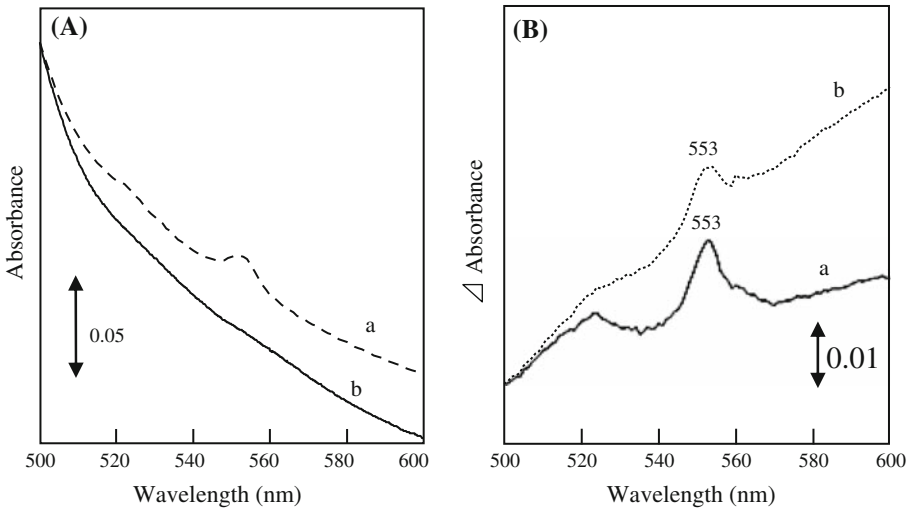

Fig. 6 Absorption spectra of *H. pylori* cell-free extract. The assay mixture contained 100 μg of cell-free extract, and 50 mM sodium-phosphate buffer (pH 7.0) in 1 ml. **A** Spectra 10 min after (*dotted line*, *a*) and before (*solid line*, *b*) the addition of 20 mM D-proline. **B** *a*, The difference spectrum with (*dotted line* in **A**) and without (*solid line* in **A**) D-proline; *b*, The difference spectrum with and without D-proline in the presence of 0.1 mM benzoate. The spectra were measured 10 min after addition of D-proline. Representative results are shown

The reduction in the α-peak height by benzoate (Fig. 6B, b) indicates that DAD is involved in the electron transport from D-proline to the *c*-type cytochrome.

H. pylori's habitat is the acidic mucous layer of the human gastric epithelium (Dunn et al. 1997), where limited oxygen is supplied. DAD requires no oxygen for its enzyme reaction. The bacterium lives in the acidic mucous layer by neutralizing the stomach acid with ammonia, which is released from urea in the reaction mediated by urease of the organism (Tsuda et al. 1994). Therefore, it might be possible that DAD whose optimum pH is 8.0 functions as the initial enzyme in *H. pylori*'s respiratory chain, like NADH dehydrogenase in the mitochondrial one. In our former study, D-proline and D-alanine were suggested to be possible energy substrates in *H. pylori* respiration because substantial oxygen uptake was measured upon the addition of these amino acids to the cells (Nagata et al. 2003). Furthermore, alanine racemase occurs in the cells (Saito et al. 2007), and an intimate relation between alanine racemase and DAD is suggested because their genes, *alr* and *dada*, are located on the same operon in *H. pylori* 26695 genome. However, the possibility that DAD functions as the initial enzyme in *H. pylori*'s respiratory chain, appears not high since the apparent K_m values of DadA to D-proline and D-alanine are much higher than the concentrations of these amino acids in *H. pylori* NCTC 11637 cells (Nagata et al. 2003) and human gastric juice (Nagata et al. 2007), although it may be possible that the concentration of these amino acids is the result of utilization by DAD.

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