L-Serine, D- and L-proline and alanine as respiratory substrates of *Helicobacter pylori*: correlation between *in vitro* and *in vivo* amino acid levels

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*Helicobacter pylori* whole cells showed high rates of oxygen uptake with L-serine and L-proline as respiratory substrates, and somewhat lower rates with D-alanine and D-proline. These respiratory activities were inhibited by rotenone and antimycin A at low concentrations. Since pyruvate was produced from L-serine and D- and L-alanine in whole cells, the respiratory activities with these amino acids as substrates occurred via pyruvate. Whole cells showed 2,6-dichlorophenolindophenol (DCIP)-reducing activities with D- and L-proline and D-alanine as substrates, suggesting that hydrogen removed from these amino acids also participated in oxygen uptake by the whole cells. High amounts of L-proline, D- and L-alanine, and L-serine were present in *H. pylori* cells, and these amino acids also predominated in samples of human gastric juice. *H. pylori* seems to utilize D- and L-proline, D-alanine and L-serine as important energy sources in its habitat of the mucous layer of the stomach.

INTRODUCTION

*Helicobacter pylori* is a Gram-negative bacterium associated with gastric inflammation and peptic ulcer disease and is a risk factor for gastric cancer (Blaser, 1990; Parsonnet et al., 1991, 1994; Rabeneck & Ransohoff, 1991). The natural habitat of *H. pylori* is the mucous layer of the human gastric epithelium, where populations are considered to persist for the lifetime of the host. *H. pylori* is a microaerophilic bacterium exhibiting a strict respiratory form of metabolism and oxidizing organic acids as an energy source. The carbohydrate utilized by *H. pylori* as the energy source has been reported to be only glucose (Mendz et al., 1993). More recently the whole-genome analysis of *H. pylori* has supported these findings (Tomb et al., 1997). However, the incorporation of glucose from the culture medium was not influenced by inhibitors known to affect other bacterial glucose-related enzymes, such as glucose permease, and the $K_m$ value of its glucose transport was reported to be rather high, 4.8 mM (Burns & Mendz, 2001). In addition, glucose added to the culture medium composed of a mixture of amino acids was not utilized until the amino acids were significantly depleted (Mendz et al., 1993; Mendz & Hazell, 1995). These results suggested that glucose is not a preferred energy substrate of *H. pylori*. Candidates for the substrates of energy metabolism in this organism are thought to be organic acids such as pyruvate, D-lactate and succinate. Chang et al. (1995) reported that lower concentrations (25 μM) of pyruvate, D-lactate and succinate were rapidly oxidized, and the respiration rates were relatively high, suggesting that *H. pylori* cells may have adapted to utilizing these substrates *in vivo*. However, the oxygen uptake during lactate and pyruvate oxidation was insufficient for their complete oxidation to CO$_2$ and H$_2$O via the citric acid cycle (Chang et al., 1995; Kelly et al., 2001). Thus, instead of organic acids, amino acids are considered to be other potential respiratory substrates and energy sources in *H. pylori* cells.

Little study has been done on amino acids as respiratory substrates of *H. pylori*. *H. pylori* has genes encoding D-amino acid dehydrogenase (*dadA*), L-alanine dehydrogenase (*ald*) and L-serine deaminase (*sdaB*) (Tomb et al., 1997). These three enzymes produce pyruvate, which is the main respiratory substrate of *H. pylori* cells. In addition, *H. pylori* has a gene encoding proline dehydrogenase (*putA*), which is

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**Abbreviation:** DCPIP, 2,6-dichlorophenolindophenol.

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associated with the respiratory chain employing molecular oxygen as terminal electron acceptor in *Escherichia coli* and *Salmonella typhimurium* (Scarpulla & Soffer, 1978; Menzel & Roth, 1981). In this context, we have investigated the respiratory activity of intact whole *H. pylori* cells with alanine, serine and proline, and other amino acids of which both D- and L-isomers serve as respiratory substrates. In this report, we describe: (1) a high rate of utilization of L-serine and L-proline, followed by D-alanine and D-proline, as respiratory substrates; (2) an unusually high content of L-proline in *H. pylori* cells; and (3) the composition of free amino acids including D-isomers in gastric juice from patients infected with *H. pylori* and those from uninfected persons. These findings revealed an interesting relationship between some amino acids utilized as respiratory substrates and the composition of amino acids in gastric juice.

**METHODS**

**Materials.** Rotenone, antimycin A, D-cycloserine and DL-penicillamine were from Sigma. Other inhibitors, substrates of respiration and chemicals for enzyme assay were from Wako Pure Chemical Industries. The strain of *H. pylori* used, NCTC 11637, was cultured on Brucella agar plates (Becton Dickinson) containing 5% newborn calf serum (Sigma) under 10% CO₂ in an incubator at 37 °C for 20 h. Cultured cells were harvested by centrifugation at 10,000 g for 15 min at 4 °C, and were suspended in phosphate-buffered saline (pH 7.2) for immediate assays of respiratory and enzymic activities.

**Respiration assay of whole cells.** Respiration of whole cells (5–10 × 10⁶ cells ml⁻¹) was monitored polarographically with a Clark-type oxygen electrode (YSI Inc.) in a semi-closed vessel containing a medium of 10 mM HEPES buffer (pH 7.0) and 0.9% NaCl at 37 °C as described previously (Nagata et al., 2001). The medium was bubbled with nitrogen gas to bring the oxygen concentration to 75 µM. Various kinds of amino acids and pyruvate with a final concentration of 10 mM and inhibitors such as rotenone and antimycin A were introduced into the oxygen electrode vessel with a syringe. Respiratory activity [oxygen uptake min⁻¹ (mg protein)⁻¹] was determined based on polarographic traces of the oxygen electrode as described previously (Nagata et al., 2001).

**Enzyme assay and protein determination.** Pyruvate production from amino acids was assayed as described previously (Nagata et al., 1988). Briefly, *H. pylori* cells were added to a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM NaN₃ and 10 mM of amino acids. After 10 min incubation at 37 °C, 2,4-dinitrophenylhydrazine was added and the mixture kept at room temperature for 10 min. After addition of NaOH, hydrazone formation was measured by reading the 445 value of pure pyruvate as a standard. The amount of pyruvate was calculated based on the A₄₄₅ value of pure pyruvate as a standard.

2,6-Dichlorophenolindophenol (DCIP)-reducing activity was measured as follows. The cells were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM of an amino acid and 0.5 mM DCIP. After 10 min incubation at 37 °C, the cells were removed by centrifugation at 10,000 g for 10 min and then the A₄₄₅ of the supernatant was measured. An A₄₄₅ of 21.6 mM⁻¹ cm⁻¹ was used.

Ammonia production was measured in the same reaction mixture as used for the assay of pyruvate production except without addition of NaN₃. After incubation at 37 °C, the cells were removed by centrifugation at 10,000 g for 10 min, and the amounts of ammonia in the supernatant were measured by indophenol formation using the ammonia test reagent kit (Wako Pure Chemical Industry) as described previously (Nagata et al., 1993). The A₅₄₆ was measured. Activities of pyruvate and ammonia production and of DCIP reduction were obtained by subtracting the activities without amino acids from those with amino acids.

The amount of protein of the whole *H. pylori* cells was determined by a modification of the Lowry procedure (Markwell et al., 1981).

**Determination of free D- and L-isomers of amino acids in human gastric juice and *H. pylori* cells.** Gastric juice was collected from patients suspected of having gastroduodenal diseases. After pharyngeal anaesthesia with 1% lidocaine hydrochloride, a disinfected endoscope was inserted into the stomach and gastric juice was collected through the aspiration channel of the endoscope. The status of *H. pylori* infection was determined from the results of culture and an immunological rapid urease test described previously (Sato et al., 2000). A patient was considered to be *H. pylori*-positive if the culture was positive. *H. pylori*-negative patients were defined as those with negative results for both tests. Gastroscopy was performed to evaluate the possibility of *H. pylori* infection. Informed consent was obtained from each patient prior to inclusion in the study.

To prepare the gastric juice sample, 30% trichloroacetic acid solution was added to the juice to a final concentration of 5%, w/v. Sample preparation from *H. pylori* cells has been described previously (Nagata et al., 1998). The supernatant of the trichloroacetic-acid-treated sample was passed through a Dowex 50W × 8 (H⁺ form) column and eluted with 4 M NH₄OH after washing with distilled water to obtain purified free amino acids. The eluate was evaporated to dryness in vacuo in a centrifugal evaporator (Taidet) below 4 °C. The determination of amino acids of each enantiomer was performed as described previously (Nagata et al., 1992). The free amino acids were treated with FDAA (Marfey’s reagent, Pierce) (Marfey, 1984) to form diastereomers of amino acids. The FDAA derivatives were separated on a Silica Gel 60 plate (Merck) by two-dimensional thin-layer chromatography. FDAA amino acids recovered from the plate were analysed by HPLC for the resolution of D- and L-enantiomers, using a reversed-phase column, Nova-Pak C18 (150 × 3.9 mm i.d., Waters), and a Tosoh or Jasco gradient HPLC system. The amounts of D- and L-enantiomers of the amino acids were calculated based on the peak areas of the elution patterns as obtained by a Chromato-Integrator (D-2500, Hitachi, Tokyo). Known amounts of authentic D- and L-enantiomers of each amino acid examined were added to the samples as internal controls, and subsequently hydrolysed and analysed as described.

**RESULTS**

**Respiratory activity of whole *H. pylori* cells with amino acids as substrates**

We polarographically measured oxygen uptake of whole cells using a Clark-type oxygen electrode in a vessel containing medium bubbled with nitrogen gas to create a low concentration of oxygen. Under these conditions, the respiratory activity of endogenous substrate in cells was low [about 3 nmol oxygen min⁻¹ (mg cellular protein)⁻¹⁻¹]. Oxygen uptake of the whole *H. pylori* cells increased when pyruvate and D- and L-isomers of alanine, serine and proline were added to the cell suspension. Of the six amino acids, L-serine was found to be the most effective for increasing oxygen uptake, followed by L-proline, D-alanine and D-proline (Table 1). The respiratory activity on L-serine was comparable...
to that on pyruvate. Cells incubated with D- and L-isomers of glutamate and aspartate, L-arginine and glycine did not show significant respiratory activity (data not shown). To determine whether these respiratory activities are associated with the respiratory chain, we examined the inhibitory activity of rotenone, which is a potent inhibitor of NADH-quinone oxidoreductase, and antimycin A, which is an inhibitor of the cytochrome $bc_1$ complex. As shown in Table 1, the respiratory activities were inhibited by rotenone and antimycin A at low concentrations. The IC$_{50}$ value (concentration giving 50% inhibition) for rotenone against the respiration of D-alanine was about 8 μM, which was close to the IC$_{50}$ 3–8 μM, for rotenone against the respiration of pyruvate (data not shown). These results suggested that D- and L-isomers of alanine, serine and proline were metabolized via the respiratory chain in $H$. pylori cells.

### Table 1. Respiration of $H$. pylori cells with D- and L-isomers of alanine, serine, proline and pyruvate as substrates, and the effect of respiratory inhibitors

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Respiratory activity*</th>
<th>Activity with inhibitors (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rotenone 5 μM</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>16.32</td>
<td>53.0</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>10.21</td>
<td>46.9</td>
</tr>
<tr>
<td>D-Serine</td>
<td>5.17</td>
<td>47.7</td>
</tr>
<tr>
<td>L-Serine</td>
<td>32.72</td>
<td>18.7</td>
</tr>
<tr>
<td>D-Proline</td>
<td>16.34</td>
<td>26.3</td>
</tr>
<tr>
<td>L-Proline</td>
<td>29.00</td>
<td>61.6</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>34.22</td>
<td>58.7</td>
</tr>
</tbody>
</table>

*Respiratory activity [nmol oxygen min$^{-1}$ (mg cellular protein)$^{-1}$] determined from polarographic traces of the oxygen electrode.

Although the presence of the gene encoding D-alanine aminotransferase has not been reported for $H$. pylori, D-alanine increased time-dependently as shown in Fig. 1. Amounts of ammonia similar to those of pyruvate were produced time-dependently from D-alanine, and the rates of production of pyruvate and ammonia were the same (Fig. 1). The apparent $K_m$ value for D-alanine in the pyruvate production was 0.14 mM (data not shown).

### Pyruvate and ammonia production from D- and L-isomers of amino acids in $H$. pylori cells

$H$. pylori has the genes dadA, ald and sdaB (Tomb et al., 1997), encoding enzymes that produce pyruvate and ammonia from D- and L-alanine and from L-serine, respectively. Thus, respiratory activities with these amino acids as substrates presumably occurred via pyruvate. We assayed pyruvate- and ammonia-producing activities from these amino acids. However, whole $H$. pylori cells quickly utilized pyruvate as the respiratory substrate. To prevent this consumption of pyruvate, we added various amounts of NaN$_3$ to the reaction mixture; the maximum pyruvate production from D-alanine was obtained at 10 mM NaN$_3$ (data not shown). In the presence of 10 mM NaN$_3$, the pyruvate production from D-alanine increased time-dependently as shown in Fig. 1. Amounts of ammonia similar to those of pyruvate were produced time-dependently from D-alanine, and the rates of production of pyruvate and ammonia were the same (Fig. 1). The apparent $K_m$ value for D-alanine in the pyruvate production was 0.14 mM (data not shown).

Although the presence of the gene encoding D-alanine aminotransferase has not been reported for $H$. pylori, D-alanine increased time-dependently as shown in Fig. 1. Amounts of ammonia similar to those of pyruvate were produced time-dependently from D-alanine, and the rates of production of pyruvate and ammonia were the same (Fig. 1). The apparent $K_m$ value for D-alanine in the pyruvate production was 0.14 mM (data not shown).
an alanine aminotransferase may produce pyruvate and ammonia. The activity of D-alanine aminotransferase is dependent on pyridoxal 5'-phosphate as a cofactor and is inhibited strongly by specific inhibitors of pyridoxal 5'-phosphate, such as D-cycloserine, DL-penicillamine, NH₄OH and 3-methyl-2-benzothiazoline hydrazine hydrochloride (Yonaha et al., 1975). Since the activity of the pyruvate production from D-alanine was not affected significantly by those inhibitors (data not shown), pyruvate was probably not produced via the catalytic function of this enzyme in our experiments.

Pyruvate production from L-alanine added to whole cells of *H. pylori* was about 50% of that from D-alanine (Table 2). Ammonia production was also about half that for D-alanine, showing a specific activity of 2-46 nmol min⁻¹ (mg protein)⁻¹. These results indicated that pyruvate production from L-alanine was due to L-alanine dehydrogenase, suggesting that this enzyme activity was weak compared to the D-amino acid dehydrogenase activity on D-alanine. These facts were consistent with the results that the respiratory activity on L-alanine was lower than that on D-alanine, as shown in Table 1.

High pyruvate production from L-serine was found in whole *H. pylori* cells (Table 2). The specific activity of ammonia production from L-serine was 6-28 nmol min⁻¹ (mg protein)⁻¹, which was similar to that for pyruvate production. Since the respiration with L-serine as substrate was inhibited by rotenone and antimycin A as for D-alanine (Table 1), the oxygen uptake may have been caused by pyruvate produced by L-serine deaminase.

**DCIP-reducing activity of whole *H. pylori* cells with D- and L-isomers of alanine, serine and proline as substrate**

D-Amino acid dehydrogenase and L-alanine dehydrogenase show DCIP-reducing activity with concomitant production of pyruvate and ammonia (Olisiweski et al., 1980). We examined the DCIP-reducing activity of whole *H. pylori* cells using D- and L-isomers of alanine. Since *H. pylori* cells have strong activity of cytochrome c oxidase (Nagata et al., 1996), the reduced DCIP is considered to be oxidized immediately. To prevent DCIP oxidation, we added various amounts of NaN₃ to the reaction mixture used for the assay of DCIP reduction by D-alanine; the maximum DCIP reduction was obtained at 10 mM NaN₃ (data not shown). In the presence of 10 mM NaN₃, the specific activity of the DCIP reduction with D-alanine was close to that of pyruvate production with D-alanine (Table 2).

L-Alanine and D- and L-serine added to whole cells of *H. pylori* led to low DCIP-reducing activities (Table 2). On the other hand, high activities of DCIP reduction appeared with D- and L-proline, although the activities of pyruvate production from these amino acids were low (Table 2).

**Free amino acid composition of human gastric juice and *H. pylori* cells**

Table 3 shows the contents of free D- and L-isomers of alanine, serine, proline, aspartate and glutamate in human gastric juices from *H. pylori*-infected and uninfected subjects. The content of L-proline in infected specimens was significantly higher than that in uninfected ones. The contents of D- and L-alanine and L-serine were also high. No significant difference was observed in amino acid composition, except for L-proline, between infected and uninfected specimens. As shown in Table 4, *H. pylori* cells contained extremely large amounts of L-proline and considerable amounts of D- and L-alanine, L-serine and D-aspartate. The content of glutamate was low.

**DISCUSSION**

Although blood- or serum-containing media are commonly used for routine culture of *H. pylori*, the nature of the carbon and energy sources used in the host is unknown. *H. pylori* is very limited in its use of oxidizable carbon substrates (Doig et al., 1999). In fact, glucose does not seem to be the main energy source for *H. pylori* (Kelly et al., 2001); instead of glucose, organic acids and amino acids are considered to serve as potential respiratory substrates and energy sources. Utilization of amino acids by *H. pylori* has been investigated under various culture conditions (Mendz & Hazell, 1995; Reynolds & Penn, 1994; Stark et al., 1997). Stark et al. (1997) reported that amino acids such as alanine, arginine, asparagine, aspartate, glutamine, glutamate, proline and serine were mainly consumed from continuous culture medium by *H. pylori* cells, and suggested that the products of metabolism of these amino acids can be further metabolized as energy sources by enzymes of the TCA cycle (Stark et al., 1997). Chang et al. (1995) did not detect oxygen uptake when acetate, glycerol, L-lactate, oxaloacetate and amino acids such as aspartate and glutamate were added to *H. pylori* cells. However, they did not study the utilization of other amino acids, including the D-isomers, as respiratory substrates.
Table 3. Free D- and L-isomers of amino acids in human gastric juices from subjects infected with H. pylori and from uninfected normal subjects

Statistical analyses were carried out using Student’s t-test.

<table>
<thead>
<tr>
<th>Gastric juice*</th>
<th>Amino acid (nmol per ml gastric juice)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
</tr>
<tr>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>4.2</td>
</tr>
<tr>
<td>3</td>
<td>4.6</td>
</tr>
<tr>
<td>4</td>
<td>52.0</td>
</tr>
<tr>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td>6</td>
<td>57.8</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>21.1 ± 10.7</td>
</tr>
<tr>
<td>7</td>
<td>18.8</td>
</tr>
<tr>
<td>8</td>
<td>13.2</td>
</tr>
<tr>
<td>9</td>
<td>60.4</td>
</tr>
<tr>
<td>10</td>
<td>3.3</td>
</tr>
<tr>
<td>11</td>
<td>5.5</td>
</tr>
<tr>
<td>12</td>
<td>1.9</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>17.2 ± 9.0</td>
</tr>
</tbody>
</table>

*Gastric juices nos 1–6 were from persons infected with H. pylori and nos 7–12 from uninfected persons.

**P<0.05, specimens 1–6 compared with specimens 7–12.

In this report, we have presented evidence that H. pylori cells predominantly utilized D- and L-alanine and proline and also L-serine as respiratory substrates as well as pyruvate. The respiratory activities with these amino acids as substrate were inhibited by the respiratory inhibitors rotenone and antimycin A at low concentrations (Table 1), suggesting the respiratory activities to be coupled with ATP production. Although D-alanine as well as D-glutamate contributes to the synthesis of the cell wall of bacteria, other physiological functions and the metabolic pathways of these D-amino acids are equivocal in H. pylori cells. In the present study, we demonstrated that whole H. pylori cells utilized D-alanine but not D-glutamate as a respiratory substrate. As genome sequence analysis shows that H. pylori has the dadA gene, the reaction is considered to be the result of D-amino acid dehydrogenase function. Thus, oxygen uptake with D-alanine as substrate seems to have occurred via pyruvate, which is the main respiratory substrate in H. pylori cells (Mendz et al., 1994). Considerably high activities of oxygen uptake and pyruvate production were found when L-serine was added.

Table 4. Free D- and L-isomers of amino acids in H. pylori cells

H. pylori cells were cultured on Brucella agar plates containing 5% newborn calf serum under 10% CO₂ in an incubator at 37°C for 2 days. Harvested cells were washed once with phosphate-buffered saline (pH 7.2).

<table>
<thead>
<tr>
<th>Sample of cells</th>
<th>Amino acid (nmol per g wet weight of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>106.3</td>
</tr>
<tr>
<td>3</td>
<td>196.9</td>
</tr>
<tr>
<td>4</td>
<td>195.6</td>
</tr>
<tr>
<td>5</td>
<td>150.8</td>
</tr>
<tr>
<td>6</td>
<td>294.4</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>157.8 ± 40.1</td>
</tr>
</tbody>
</table>

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to whole cells of *H. pylori* (Tables 1 and 2). As *H. pylori* has a *sdaA* gene, this activity seems likely to be due to serine deaminase.

The presence of D-amino acid dehydrogenase has been reported in *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Escherichia coli* (Jones & Venables, 1983; Magor & Venables, 1987; Olsiewski et al., 1980; Tsukada et al., 1966; Wild et al., 1974). However, its metabolic function has not been clarified. D-Amino acid dehydrogenase and L-alanine dehydrogenase remove hydrogen from substrates of amino acids in the process of producing pyruvate and ammonia. Although the substances reduced by this hydrogen have not yet been identified in *H. pylori* cells, this hydrogen may be transferred to oxygen via the electron-transfer system leading to oxygen uptake by whole cells. However, it has not been clarified to what extent this hydrogen, besides pyruvate, participates in the oxygen uptake by D- and L-alanine in *H. pylori* cells.

In *H. pylori*, pyruvate is dehydrogenated by different dehydrogenase systems from those in the usual proteobacteria producing NADH, which is the major electron donor in the respiratory chain (Hughes et al., 1995; Kelly, 1998). Two-step systems generating NADPH seem to be present in *H. pylori*; pyruvate may be oxidized by flavodoxin, and the reduced flavodoxin reduces NADP+. NADPH-menaquinone oxidoreductase oxidized NADPH, and the menaquinone may be oxidized by the cytochrome bc complex of the respiratory chain. Thus, the reducing system in pyruvate may be different from that in the hydrogen removed from D- and L-alanine.

Moreover, *H. pylori* showed marked activities of oxygen uptake with L- and D-proline as substrate, which were also inhibited by respiratory inhibitors (Table 2). *E. coli*, *S. typhimurium* and *P. aeruginosa* have the gene putA, whose product, PutA, shows L-proline dehydrogenase activity (Ling et al., 1994; Vinod et al., 2002). Recently, Satomura et al. (2002) reported that the hyperthermophilic archaeon *Pyrobaculum islandicum* has a dye-linked D-proline dehydrogenase which is different from D-amino acid dehydrogenase. Both the L- and D-proline dehydrogenases have been reported to show DCIP reduction activity by the respective amino acids (Abrahamson et al., 1983; Satomura et al., 2002). In the present experiments, we demonstrated that *H. pylori* whole cells showed DCIP-reducing activity with D- and L-proline as substrate (Table 2). *H. pylori* has the genes *putP* and *putA*, which encode proline permease and L-proline dehydrogenase, respectively (Tomb et al., 1997). However, the gene corresponding to D-proline dehydrogenase has not been reported. From the genome sequence database, *H. pylori* *dada* encoding D-amino acid dehydrogenase shows low DNA similarity (24%) with *P. islandicum* dye-linked D-proline dehydrogenase (Satomura et al., 2002). Since the substrate specificity of D-amino acid dehydrogenase may not be strict, this dehydrogenase may metabolize D-proline as a substrate for DCIP-reducing activity as well as D-alanine. The hydrogen atoms removed from D- and L-proline by *H. pylori* whole cells may reduce a flavoprotein-like substance, and electrons produced concomitantly reduce oxygen via the electron-transport chain. Thus, different dehydrogenases and electron-transport pathways seem to participate in the oxidation of D- and L-isomers of alanine and proline. As shown in Table 2, the inhibitory action of rotenone and antimycin A against respiratory activities varied considerably among respiratory substrates, being from 10 to 48% of the control in the case of rotenone. These results may be due to the participation of different dehydrogenases and electron pathways depending on respiratory substrates.

Olson & Maier (2002) reported recently that *H. pylori* cells use molecular hydrogen as an energy source in mice. In the stomach of mice many kinds of anaerobic bacteria are present, in high density, and the molecular hydrogen derived from these anaerobic bacteria seems to be dominant. On the other hand, there are few bacteria in the human stomach, where amino acids are dominant owing to the degradation products of food, including L-serine derived from degradation of mucin. Since *H. pylori* resides in the lower layers of the gastric mucus, it is considered to use various amino acids diffused from the gastric juice as an energy source, although we cannot exclude the possibility that molecular hydrogen is also used by *H. pylori* in the human stomach as discussed by Olson & Maier (2002).

Previously, we reported the occurrence of free D-amino acids in various bacteria including *H. pylori* (Nagata et al., 1998). In the present study, we showed that of the amino acids contained in *H. pylori* cells, L-proline was present at the highest level, followed by D- and L-alanine, L-serine and D-aspartate (Table 4). All these amino acids except D-aspartate were used predominantly as respiratory substrates by *H. pylori* whole cells as described above. We also analysed the L- and D-amino acid content of human gastric juice, which to the best of our knowledge is the first report of such measurement. We found considerable amounts of L- and D-isomers of alanine and proline, and of L-serine, in the gastric juice (Table 3). Again, these amino acids coincided with the respiratory substrates used predominantly by *H. pylori*. Specimens from patients with *H. pylori* showed a significantly higher level of L-proline than those from uninfected subjects, although there was no significant difference in the contents of other free amino acids between the patients and the uninfected control group (Table 3).

There is the possibility that the free amino acids in gastric juice infected with *H. pylori* cells were derived from *H. pylori* cells since this organism contains a large amount of L-proline. *H. pylori* cells possess alanine racemase activity that converts L-alanine to D-alanine (unpublished observation). All these results, together with an apparently low *K_m* value for the pyruvate-producing reaction from D-alanine, suggest that *H. pylori* cells utilize D-alanine, L-serine, and D- and L-proline as the main energy sources in their habitat of the mucous layer.

*H. pylori* cells possess high levels of urease. The hydrolysis of
urea produces ammonia, which protects the organism from the highly acidic environment of the stomach. Not only urease but also enzymes that produce ammonia from amino acids, such as D-amino acid and L-alanine dehydrogenase, and L-serine deaminase, may contribute to protecting *H. pylori* cells from their acidic environment. Thus, the present study on the utilization of L-serine and D- and L-isomers of alanine in *H. pylori* cells offers important information on *H. pylori* metabolism, not only related to the energy source but also to protection measures against an acidic environment. These findings should be helpful for developing new anti-*H. pylori* therapies.

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