Involvement of CO\textsubscript{2} generated by urease in multiplication of \textit{Helicobacter pylori}

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Abstract

\textit{Helicobacter pylori} (\textit{H. pylori}) urease generates both ammonia (NH\textsubscript{3}) and carbon dioxide (CO\textsubscript{2}) from urea. NH\textsubscript{3} helps \textit{H. pylori} to survive in the stomach in part by neutralizing gastric acid. However, the relationship between CO\textsubscript{2} and \textit{H. pylori} is not completely cleared. We examined the effect of CO\textsubscript{2} generated by urease on multiplication of \textit{H. pylori} by using isogenic \textit{ureB} mutant and \textit{ureB} complemented strain from \textit{H. pylori} strain JP26. Wild-type strain survived in the medium supplement with 1mM urea in room air, however, the urease negative strain did not. To discern whether CO\textsubscript{2} was incorporated into \textit{H. pylori}, \textsuperscript{14}C in bacillus was counted after 6 hours incubation with \textsuperscript{14}C urea in both acidic and neutral medium. Significant more \textsuperscript{14}C uptake was detected in wild-type strain compared to \textit{ureB} mutant strain and this uptake in the wild-type strain was more under acidic condition compared to under neutral condition, but no difference was identified in the mutant strain. These results suggest that CO\textsubscript{2} generated by urease plays a role in multiplication of \textit{H. pylori}.

Keywords: \textit{Helicobacter pylori}; Urease; CO\textsubscript{2}; \textit{ureB}

1. Introduction

\textit{Helicobacter pylori} (\textit{H. pylori}) is a pathogen which leads to the development of peptic ulcer [1, 2], MALT lymphoma [3] and distal gastric cancer [4, 5]. Several \textit{H. pylori} constitutions have been proposed to be pathogenic factors, among which is a remarkably high level of urease activity. Involvement of urease in colonization was first proposed by Eaton et al [6], and several subsequent investigations have suggested a role of \textit{H. pylori} urease in multiplication and pathogenesis [7, 8, 9, 10]. Urease catalyzes the hydrolysis of urea to form carbon dioxide (CO\textsubscript{2}) and ammonia (NH\textsubscript{3}). It has been reported that \textit{H. pylori} urease functions to neutralize gastric acid by producing NH\textsubscript{3}, and enhanced production of NH\textsubscript{3} may also facilitate the formation of NH\textsubscript{3}-derived compounds, such as monochloramine, which exert cytotoxic effects on host cells [10]. Enhancement of bacterial motility [9] and inhibition of phagocytic clearance of bacteria [7] have also been reported to be dependent upon urease activity. The potential of urease to be a pathogenic factor has been attributed to NH\textsubscript{3}. In contrast, little attention has been paid to the role of CO\textsubscript{2} that is produced in the same reaction [11]. Therefore, in the present study, we attempted to clarify the role of urease-generated CO\textsubscript{2} on multiplication of \textit{H. pylori}.  

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2. Material and methods

2.1. Bacterial strains, culture conditions

**Table 1** Bacterial strains and plasmids used in this study

| Strain (alternate name) or plasmid | Genotype or characteristics | Reference or source |
|-----------------------------------|-----------------------------|---------------------|
| JP26                             | *H. pylori* wild-type        | [17]                |
| JP26ureB                         | *H. pylori* JP26/HP0072: *aphA* | This study         |
| JP26ureBcomp                     | *H. pylori* JP26ureB containing HP0072 | This study         |
| pGEM-T Easy                      | ColE1, AmpR, PCR cloning vector | Promega            |
| pUK4K                            | ColE1 (AmpR, KanR)          | GE Healthcare Bio-Sciences |
| pGEMTureB                        | pGEM-T Easy containing HP0072 | This study         |
| pGEMTureBKm                      | pGEMTureB, HP0072: *aphA*    | This study         |
| pHel2                            | shuttle vector              | [15]                |
| pHel2ureB                        | pHel12 containing HP0072     | This study         |

*H. pylori* strains used in this study and their origins are listed in Table 1. *H. pylori* strains were cultured at 37 °C under microaerophilic condition in brucella broth (BB, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) medium supplemented with 7% fetal calf serum (FCS, Thermo fisher scientific corporation, Waltham, MA, USA) or agar medium (1.5% agar). For long term storage, *H. pylori* strains were suspended in BB containing 15% (wt. / vol) glycerol and kept at -80 °C. Stock cultures of *H. pylori* were grown for 4 days on BB agar plates supplemented with 7% heat-inactivated FCS at 37 °C in a microaerophilic atmosphere. Broth cultures of *H. pylori* were prepared by subculturing colonies from agar plates into BB supplemented with 7% FCS and grown for 48 hours at 37 °C in a microaerophilic atmosphere. The identification of *H. pylori* was confirmed by characteristic colony morphology, Gram’s stain, and positive reactions for urease, catalase, and oxidase.

**Table 2** Primers used for PCRs

| Primer | Sequence                              |
|--------|---------------------------------------|
| ureBF1 | ACGCACTATGACACACTTTCC                  |
| ureBR1 | TGCCCACTTTCTACAGAACCT                  |
| ureLF1 | CCAAGTGATCCTAAAAGCAC                  |
| ureLR1 | AAAGGGTAAGCCACAAAACAC                 |
| 27F    | CGACTTGTCGAGCGCAAA                     |
| 1492R1 | TTATCAGTTTCAACACGTCAAA                 |

2.2. Rapid urease activity

Bacteria from agar plates were transferred with a sterile loop into 100 μl of urea solution containing 2% (wt / vol) urea (Fuji film wako pure chemical, Osaka, Japan) and 0.001% (wt / vol) phenol red (Fuji film wako pure chemical) in 0.01 M phosphate-buffered saline (PBS) (pH 6.8). A positive reaction was indicated by a change in color from yellow to pink within 5 min [12].

2.3. Construction of *ureB* mutant suicide vector and *ureB* complement vector

The HP0072 ORF of strain 26695 [13] was amplified with 100 pmol of primers ureBF1 and ureBR1 (Table 2) in a 50-μl reaction mixture containing 0.25 μl of Ex Taq polymerase (Takara biomedicals, Ohtsu, Japan) for 25 cycles of 1 min at
94 °C, 1 min at 55 °C, and 1 min at 72 °C in a DNA thermal cycler (GeneAmp PCR System 9700, Thermo fisher scientific co). PCR product was purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The product was ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into E. coli DH5α. Plasmid pUC4K (GE healthcare bio-sciences co, NJ, USA) was digested with BamHI, and the 1.27 kb kanamycin resistance (Km; aphⅡ) cassette was isolated by agarose gel electrophoresis, and this cassette was inserted into the BamHI site located in the HP0072 ORF in the pGEMTureB to disrupt the ureB gene, creating pGEMTureBkm. No nucleotide error by PCR in plasmid was confirmed by sequencing (CEQ2000XL, Beckman Coulter. Inc. Brea, CA, USA) [14]. Plasmid pHel2 is E. coli-H. pylori shuttle vector containing chloramphenicol cassette [15]. Plasmid pHel2ureB is pHel2 derivative carrying the ureB gene of H. pylori strain 26695. The HP0072 ORF of strain 26695 was amplified with 100 pmol of primers ureBF1 and ureBR1 in a 50-μl reaction mixture containing 0.25 μl of Pyrobest (Takara biomedicals) for 25 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C in a DNA thermal cycler. This product was ligated into pHel2 digested by SmaI and transformed into E. coli DH5α, creating pHel2ureB.

### 2.4. Construction of an isogenic ureB mutant strain

The resulting plasmid, designated pGEMTureBkm was used as donor DNA. H. pylori strain JP26 was used as the transformation recipient [16]. Recipient H. pylori cells harvested for 48 hours were suspended into 1 ml of PBS and centrifuged at 8,500 g for 5 min. The pellet was then resuspended in 300 μl of PBS. Each transformation mixture, consisting of 25 μl of recipient cells and 1 μg of donor DNA, was spotted onto a BB agar plate (approximately 600 ng of DNA /25 μl of cells). Plates were incubated overnight at 37 °C in a microaerophilic atmosphere. The transformation mixture was spread onto BB agar plates containing 25 μg / mL Km. All plates were incubated for 5 days at 37 °C in a microaerophilic atmosphere to select transformants [17]. All transformants examined were urease negative as determined by rapid urease assay. One such transformant, designated JP26ureB, was chosen for the further study.

### 2.5. Construction of an isogenic ureB complemented strain

The resulting plasmid, designated pHel2ureB was used as donor DNA. H. pylori strain JP26ureB was used as the transformation recipient. Transformation method was almost same as described above except using BB agar plates containing 25 μg / mL Km and 10 μg / mL chloramphenicol and we created JP26ureBcomp as ureB complemented strain.

### 2.6. Detection of H. pylori UreB protein

H. pylori strain JP26, JP26ureB and JP26ureBcomp were spread onto blood agar plates and incubated at 37 °C in a microaerophilic atmosphere for 3 days. Bacteria grown on the agar plates were transferred to BB medium with 0.5% cycloheximide (Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for an additional 3 days. The bacterial suspensions were then diluted in 20 μl of fresh broth and incubated up to 48 hours. Before each experiment, H. pylori viability was confirmed by observing active motility using phase-contrast microscopy. To extract cytoplasmic proteins from H. pylori, bacteria cultures were centrifuged at 5000 g for 20 min and pellets were suspended in distilled water and disrupted by sonication. Supernatants were then centrifuged (10,000 g, at 4 °C for 20 min). The supernatants were filtered through a 0.2-μm pore filter to remove cellular debris. Soluble proteins present in the culture supernatant were precipitated by mixing with a 0.1 volume of trichloroacetic acid (TCA) and was kept on ice for 15 min. Subsequently, the mixture was centrifuged (10,000 g at 4 °C for 20 min) and pellets were washed with cold acetone to remove residual TCA. After this procedure was repeated, the pellets were air-dried and used for analysis of H. pylori cytoplasmic proteins. The electrophoresis of proteins was carried out on a 12% polyacrylamide gel. Gel was then electrotransferred onto polyvinylidene difluoride (PVDF) membrane (ProBlott; Applied Biosystems). After that, western blotting was performed for detection of UreB protein with anti-Helicobacter pylori urease B monoclonal antibody (Institute of Immunology, Tokyo, Japan).

### 2.7. Bacterial multiplication in various pH conditions

Bacteria were harvested from culture plates and suspended in PBS to yield a final suspension of approximately 10^10 colony-forming units (CFU) / mL. To evaluate the multiplication of H. pylori under a variety of pH conditions, cell suspensions were diluted 1:10 in BB, BB containing with 100mM MES(2-[N-Morpholino] ethanesultonic acid (Fuji film wako pure chemical) (BB MES) (pH 5.4), with or without urea, and incubated for 6 hours at 37°C with a microaerophilic condition. To count viable bacteria after 6 hours incubation, serial dilutions of cell suspensions were made in PBS, inoculated onto BB agar plates, and incubated for 3 days at 37°C in a microaerophilic condition. The number of CFU/mL was then determined. The initial and final pH of each suspension was determined using a pH electrode (ISFET pH meter KS723; Shindengen, Tokyo, Japan) that was calibrated before each set of measurement.
2.8. Assessment of mRNA levels by semi quantitative RT-PCR

For reverse transcription-PCR (RT-PCR), bacterial cells were cultured in 5 ml of BB for 3 days. Total RNA was prepared from 1ml of culture with an RNA-protected Bacteria Reagent (Qiagen), followed by treatment with RNase-free DNase (Qiagen). A total of 100 ng of total RNA was reverse transcribed in the presence of ureI and 16S rRNA-specific primers with the Omniscript RT Kit (Qiagen), according to the instructions of the manufacturer in a 10 μl reaction volume. The primers used for amplification of the ureI gene and 16S rRNA gene were described in Table 2. The 0.4 μl of the 10 μl reverse transcriptase reaction mixture was used for the next PCR (reaction volume, 10 μl). Amplification and detection of ureI and 16S rRNA were performed with the following cycle profile: 25 cycles annealing at 56°C and extension at 72°C for 90 s. The amount of contaminating chromosomal DNA in each sample was determined in control reactions without reverse transcriptase. The quantity of cDNA used in the experiments for each gene was normalized to the quantity of 16S rRNA cDNA in each sample. Triplicate assays were performed with RNA from at least three independent cultures.

2.9. Measurement of carbon—14 CO₂ uptake

14C-labeled urea (GE healthcare bio-sciences co) with specific activity of 1 μCi / M was added in incubation medium. We used BB and BB MES as incubation medium. Incubation was carried out in 96-well-plate for 6 hours. After incubation, bacterial cells were washed three times by PBS, and the 14C count in cells was measured as disintegrations per minute (dpm), using a liquid scintillation counter.

2.10. Statistical method

Results are expressed as the mean ± SD. The pH change and bacteria multiplication under different conditions were compared using a paired t test. The p < 0.05 was considered significant.

3. Results

3.1. Construction of an isogenic ureB mutant and ureB complemented mutant strain

We first constructed an isogenic ureB mutant that was derived from H. pylori strain JP26. We checked the genotype of the isogenic urease mutant and confirmed that aphA was inserted within the ureB gene by sequencing analysis.

![Western blot analysis of UreB in H. pylori strain JP26, disruption of ureB disrupted strain JP26ureB and ureB complemented strain JP26ureBcomp. Arrows shows UreB protein by western blotting analysis. A: JP26, B: JP26ureB, C: JP26ureBcomp. (B): Confirmation of expression of ureI in H. pylori strain (a) JP26, (b) JP26ureB, (c) JP26ureBcomp by RT-PCR analysis. The RT-PCR products of ureI and 16sRNA were shown in upper and lower panel, respectively. M represented molecular marker. We also checked the phenotype of the isogenic urease mutant by using urease activity test. Wild-type strain JP26 caused an immediate color change from yellow to pink. In contrast, the isogenic ureB mutant strain did not cause a color change when inoculated into rapid urease assay solution, demonstrating that this strain does not possess a functional urease enzyme. We next constructed ureB complemented strain from ureB mutant strain. In ureB complemented strain JP26ureBcomp, we saw a color change under urease assay. Thus, we verified that complementation was successful.](image-url)
also ascertained successful disruption of ureB in JP26ureB and complementation of ureB in JP26ureBcomp by western blotting (Figure 1(A)).

### 3.2. Multiplication of \textit{H. pylori} in the absence or presence of urea

To assess the role of urea utilization in bacterial multiplication, \textit{H. pylori} wild-type and mutant strains were incubated at 37°C in BB (pH 7.0) and BB MES (pH 5.4) in the presence of 1mM urea.

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\caption{Comparison of CFU at 0, 6, 24 hours in \textit{H. pylori} strain JP26 (A), JP26ureB (B), and JP26ureBcomp (C). The values for the log number of CFU/mL at 0, 6, 24 hours were shown as a bar graph. Representative results from 5 independent experiments are shown. *: \( p < 0.05 \).}
\end{figure}
Wild-type strain JP26 and ureB complement strain grew in both BB and BB MES after 6 hours (Figure 2(A), 2(C)). But there was no significance of bacterial growth between BB and BB MES. On the contrary, there was significant difference in multiplication of the urease mutant between BB and BB MES after 6 hours ($p < 0.05$) (Figure 2(B)). Although wild-type and ureB complement strains survived after 24 hours, the ureB mutant strain did not (Figure 2). The results indicate that multiplication of wild-type and ureB complement strain was urea-dependent and pH dependent. We then tested if medium pH was changed by addition of urea. There was an increase in medium pH containing the wild-type $H.\ pylori$ strain following the addition of urea (pH 5.4→pH 6), but no change of pH occurred without urea (pH 5.4→pH 5.4).

3.3. Comparison of 14C internalization between wild-type and urease-negative $H.\ pylori$ strains

To assess if CO$_2$ derived from urea play a role in multiplication of $H.\ pylori$, $^{14}$C uptake experiments were performed (Figure 3A). The value of dpm represents the rate of $^{14}$C uptake. This study was performed at room air (0.03% CO$_2$) to decrease the effect of external CO$_2$. The uptake of $^{14}$C in mutant strain was significantly reduced under both pH conditions (pH 7 and 5.4) compared to that in wild-type strain ($p < 0.05$). In wild-type strain, the uptake of $^{14}$C was higher in BB MES (pH 5.4) than in BB (pH 7) ($p < 0.01$). But in the mutant strain, there was no difference of $^{14}$C intake between BB and BB MES. The results of complemented strain were like those of wild-type strain.

**Figure 3** Comparison of 14C internalization in the absence of exogenous CO2 between $H.\ pylori$ strain JP26, JP26ureB, and JP26ureBcomp. The dpm values were shown as a bar graph. Representative results from 5 independent experiments are shown. *: $p < 0.05$.

**Figure 4** Comparison of $^{14}$C internalization in $H.\ pylori$ strain JP26 with or without 5% CO$_2$. The dpm values were shown as a bar graph. Representative results from 5 independent experiments are shown. *: $p < 0.05$. 


We next investigated whether exogenous CO$_2$ affect $^{14}$C uptake (Figure 4). The uptake of $^{14}$C in the absence of CO$_2$ was higher than that with 5% CO$_2$ at both pH 7 and 5.4 conditions. In pH 5.4, this difference was statistically significant ($p < 0.05$). The uptake of $^{14}$C in the mutant strain was essentially the same at both pH 7 and 5.4 under 5% CO$_2$.

4. Discussion

*H. pylori* produces a large amount of urease, which amounts to 5% of the total protein of the bacterium [18]. The urease of *H. pylori* catalyzes the degradation of urea to NH$_3$ to protect the bacterium from the harmful effects of acid. Urease has an optimum pH between 7.5 and pH 8.0 and is irreversibly inactivated below pH 4.0 [19, 20, 21]. Cytoplasmic urease has been proposed to protect *H. pylori* from acid because it may increase the periplasmic pH and membrane potential in combination with UreI, a proton-gated urea channel. The urease activity in *H. pylori* increases in acidic media since UreI increases the availability of urea to intra bacterial urease [22]. That is, after acidity opens a urea channel in the inner membrane of the bacterium, urea is taken up passively and catalyzed by urease in the cytoplasm [23].

*H. pylori* survives between pH 4.0 and 8.0, grows at neutral pH, and grows poorly at pH <6.0 or > 8.0 [21]. At neutral pH, in the absence of external buffer, urea is bactericidal to *H. pylori in vitro* because of the elevation of pH by NH$_3$. The toxic effect correlates with an irreversible loss of potential difference. If urea and NH$_3$ are transported into the cytoplasm in large amounts, they may cause degeneration of cytoplasmic proteins and alkalinization of the bacterial cytoplasm. To overcome toxicity, *H. pylori* may have evolved a protective mechanism which regulates urease production, and these bacteria may escape toxicity related to excess NH$_3$ concentration [23].

In this study, we clarified the utilization of urease-generated CO$_2$ by *H. pylori* at room air. Although wild-type strain survived in the medium supplement with 1mM urea in room air, the urease mutant strain did not. Significant decrease of $^{14}$C uptake was detected in urease mutant strain. The uptake of $^{14}$C was detected in urease mutant strain. The difference of $^{14}$C uptake between wild-type strain and mutant strain was essentially the same at both pH 7 and 5.4. This result suggests that the uptake of CO$_2$ generated by urease may increase to compensate the lack of CO$_2$.

From these results, we hypothesis that CO$_2$ generated by urease as a carbon source is important for multiplication of *H. pylori* under low exogenous CO$_2$ condition. Carbonate is essential for bacterial multiplication, as this compound is used to carboxylate and synthesis of carbamoyl phosphate, the first metabolite for the nucleotide synthetic pathway. Inhibition of de novo synthesis of purine nucleotides induces *H. pylori* cell death [19]. The level of carbonate is extremely low in acidic solutions, since carbonate is protonated at low pH and protonated carbonate converts to carbon dioxide and H$_2$O in the presence of carbonic anhydrase, an enzyme encoding DNA is present in the genome of *H. pylori* [24]. The CO$_2$ content in solution is low at any pH without a supply of CO$_2$ gas because air contains only 0.036% CO$_2$ [25]. A supply of carbonate is particularly important in acidic media. In fact, amino acid decarboxylases are synthesized at low pH under anaerobic multiplication conditions in E. coli [26]. From this hypothesis, decarboxylases may supply carbonate under anaerobic multiplication conditions for this organism [27]. Glutamate decarboxylase has been reported to enhance multiplication at acidic pH for E. coli [28], and it is possible that carbonate is also required to permit *H. pylori* to survive under acidic condition. As *H. pylori* has an acetyl-CoA carboxylase and malic enzyme [29], these enzyme activities may also play a part in metabolism of internal urease generated CO$_2$ from urea.

Recently the role of the periplasmic α-carboxylic anhydrase (α-CA) (HP1186) in acid acclimation of *H. pylori* was reported [30]. The α-CA catalyzes the conversion of CO$_2$ to HCO$_3^-$ . Hence, buffering of the periplasm to a pH consistent with viability depends not only on NH$_3$ efflux from the cytoplasm but also on the conversion of CO$_2$ generated by urease, to HCO$_3^-$ by the periplasmic α-CA. NH$_3$ production neutralizes acid entering into the periplasm. CO$_2$ produced by urea hydrolysis diffuses into the periplasm and is converted to HCO$_3^-$ by the periplasmic α-CA. HCO$_3^-$ then acts as an essential periplasmic buffer, maintaining periplasmic pH.

Compared to this study, our multiplication study was designed under low CO$_2$ condition for long time and the result suspected that CO$_2$ generated by urease is not only periplasmic buffering factor but also another multiplication factor.
5. Conclusion

In this study, we describe the relationship between H. pylori and CO₂ generated from urea in the context of bacterial multiplication. A complete understanding of the mechanism of utilizing CO₂ by H. pylori therefore awaits further investigation.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declare no conflict of interest regarding the publication of this paper.

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