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Involvement of CO_2 generated by urease in multiplication of *Helicobacter pylori*

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Abstract

Helicobacter pylori (*H. pylori*) urease generates both ammonia (NH_3) and carbon dioxide (CO_2) from urea. NH_3 helps *H. pylori* to survive in the stomach in part by neutralizing gastric acid. However, the relationship between CO_2 and *H. pylori* is not completed cleared. We examined the effect of CO_2 generated by urease on multiplication of *H. pylori* by using isogenic *ureB* mutant and *ureB* complemented strain from *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_2 was incorporated into *H. pylori*, ^{14}C in bacillus was counted after 6 hours incubation with ^{14}C urea in both acidic and neutral medium. Significant more ^{14}C uptake was detected in wild-type strain compared to *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_2 generated by urease plays a role in multiplication of *H. pylori.*

Keywords: *Helicobacter pylori*; Urease; CO_2; *ureB*

1. Introduction

Helicobacter pylori (*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 *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 *H. pylori* urease in multiplication and pathogenesis [7, 8, 9, 10]. Urease catalyzes the hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3). It has been reported that *H. pylori* urease functions to neutralize gastric acid by producing NH_3, and enhanced production of NH_3 may also facilitate the formation of NH_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_3. In contrast, little attention has been paid to the role of CO_2 that is produced in the same reaction [11]. Therefore, in the present study, we attempted to clarify the role of urease-generated CO_2 on multiplication of *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	ACGCACTATGCACACTTTCC
ureBR1	TGCCCACTTCTACAGAACCT
ureIF1	CCAAAGTCGATCCTAAAAGCAC
ureIR1	AAAGCGGTAAGCCACAAAACAC
27F	CGACTTGTCTGAACGCCAAA
1492R1	TTATCACGTTCCAAACCAGTCAA

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 *Bam*HI, and the 1.27kb kanamycin resistance (*Km*^{r}; *aphA*) cassette was isolated by agarose gel electrophoresis, and this cassette was inserted into the *Bam*HI 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 *Sma*I 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%-cyclodextrin (Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for an additional 3 days. The bacterial suspensions were then diluted in 20 mL 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. Sonicates 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 16sRNA 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 CO_2 uptake

^{14}C-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 ^{14}C count in cells was measured as disintegrations per minute (dpm), using a liquid scintillation counter.

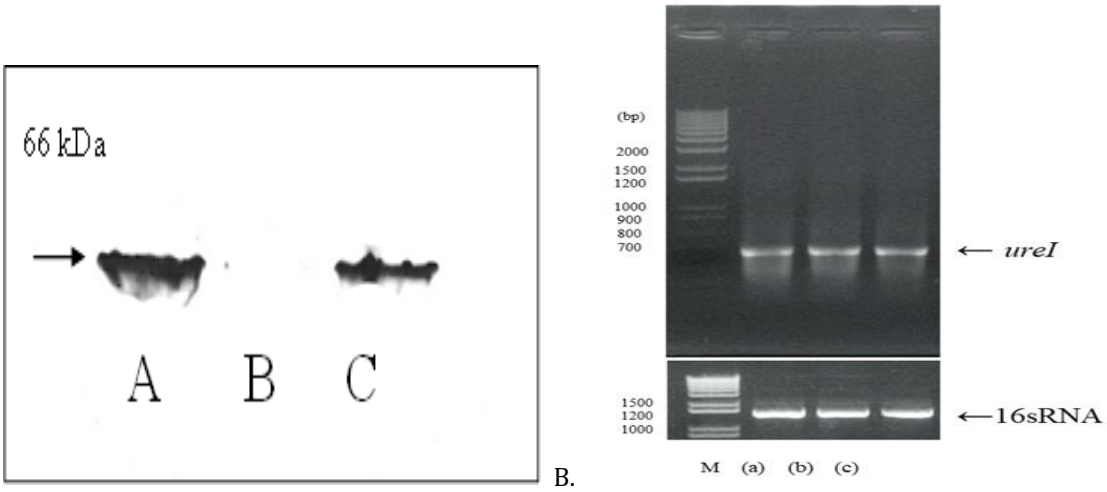
2.10. Statistical method

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 *ure*B gene by sequencing analysis.



A. B.

Figure 1 (A): 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 *ure*B 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. We

also ascertained successful disruption of *ureB* in JP26ureB and complementation of *ureB* in JP26ureBcomp by western blotting (Figure 1(A)).

3.2. Multiplication of *H. pylori* in the absence or presence of urea

To assess the role of urea utilization in bacterial multiplication, *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.

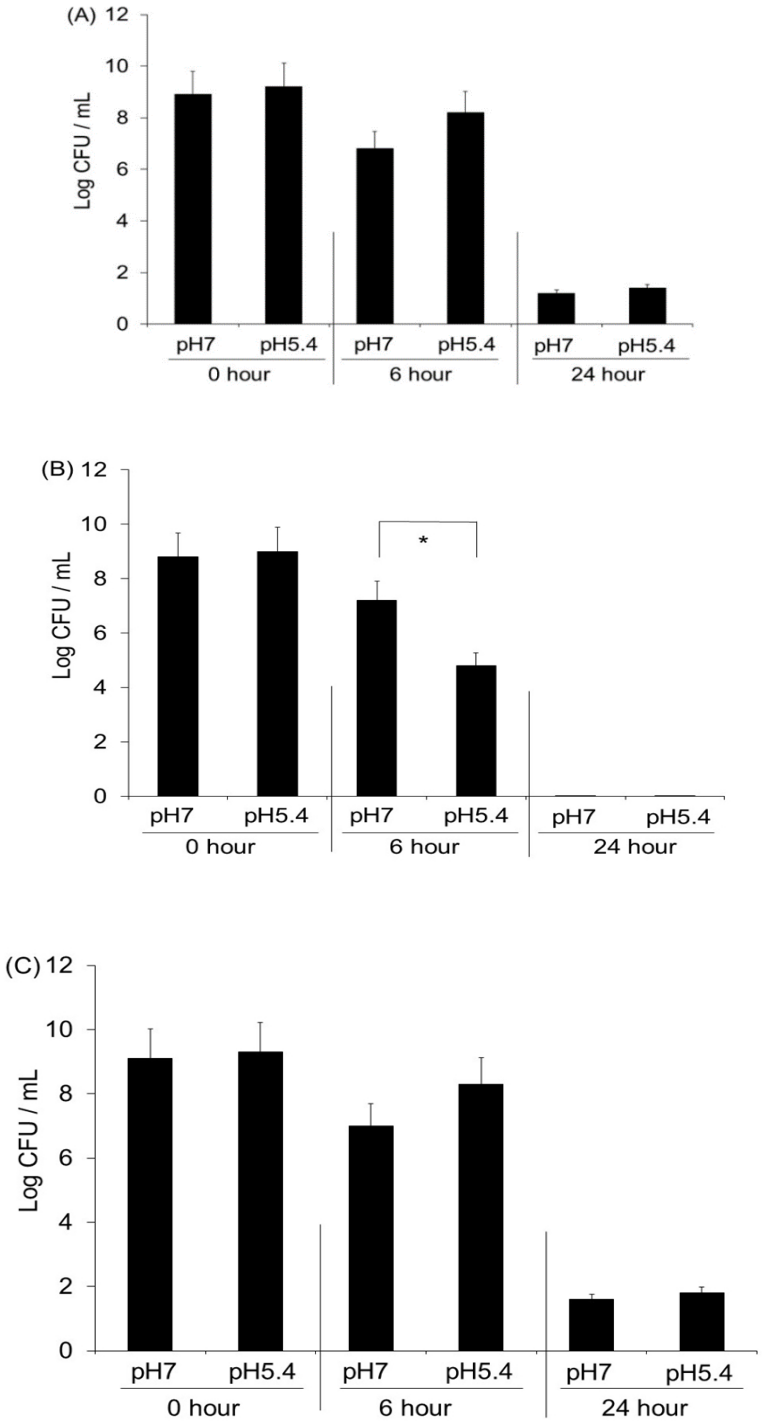


Figure 2 Comparison of CFU at 0, 6, 24 hours in *H. pylori* strain JP26 (A), JP26ureB (B), and JP26ureBcomp (C). The values for the log number of CFU/mL at 0, 6, 24 hours were shown as a bar graph. Representative results from 5 independent experiments are shown. *: $p < 0.05$.

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→pH5.4).

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 14C 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 14C -intake between BB and BB MES. The results of complemented strain were like those of wild-type strain.

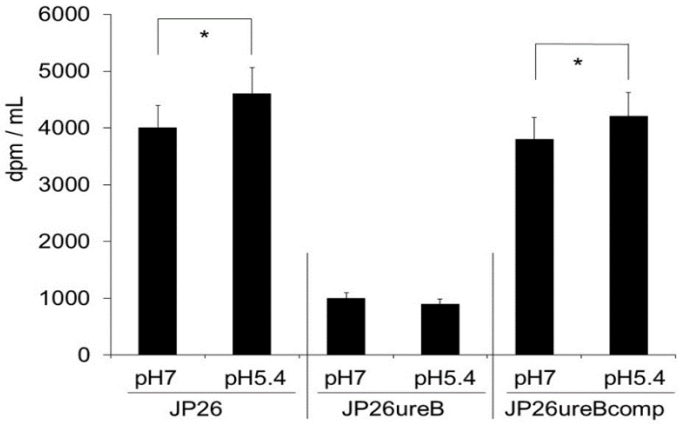


Figure 3 Comparison of 14C internalization in the absence of exogenous CO2 between H. pylori strain JP26, JP26ureB, and JP26ureBcomp. The dpm values were shown as a bar graph. Representative results from 5 independent experiments are shown. *: $p < 0.05$.

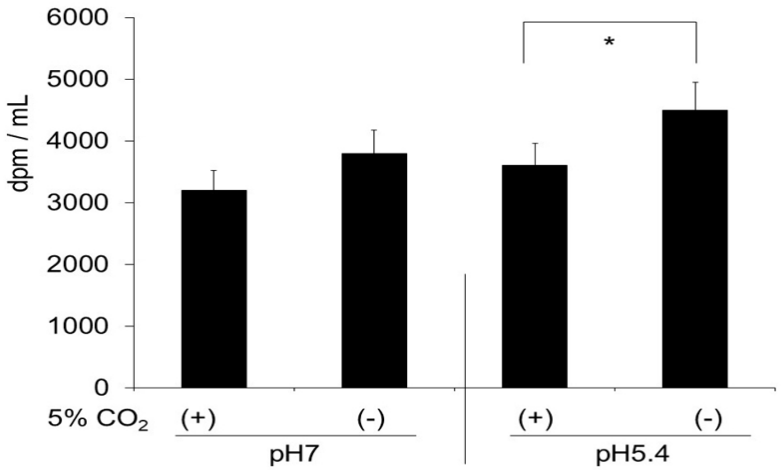


Figure 4 Comparison of ^{14}C internalization in *H. pylori* strain JP26 with or without 5% CO_2. The dpm values were shown as a bar graph. Representative results from 5 independent experiments are shown. *: $p < 0.05$.

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 correlates the count of ^{14}C -urea transported only from Ure I channel in mutant strain, because urease-generated CO_2 do not exist, and Ure I channel has transport activity in even urease mutant strain. The differences of ^{14}C uptake between wild-type strain and mutant strain represent the uptake of CO_2 generated by urease. In addition, more ^{14}C uptake was detected in the wild-type strain under acidic condition compared to that under neutral condition, but no differences were identified in the mutant strain. In the absence of exogenous CO_2, ^{14}C count increases in 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_2O 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-carbonic 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 NH3 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 CO_2 generated from urea in the context of bacterial multiplication. A complete understanding of the mechanism of utilizing CO_2 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 of regarding the publication of this paper.

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