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Original article

Colonization of an acid resistant *Kingella denitrificans* in the stomach may contribute to gastric dysbiosis by *Helicobacter pylori*

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ABSTRACT

In the stomach of a gastric ulcer patient who had been administered an anti-acid, a gram-negative and urease-negative bacillus similar in size to *Helicobacter pylori* was infected together with *H. pylori*. According to biochemical test and 16S rRNA gene analysis, the urease-negative bacterium was identified as *Kingella denitrificans*, a human nasopharyngeal commensal. In contrast to the standard strain of *K. denitrificans*, the isolate showed catalase activity, did not produce acid from glucose, and exhibited acid tolerance. Acid tolerance of *H. pylori* was increased by cocultivation with the *K. denitrificans* isolate, but not with other isolates of *K. denitrificans*. Disruption of physiological and immunological niche by dysbiotic colonization of bacterium may provide pathological attributes to human stomach. Collectively, a careful administration of anti-acids to the elderly, especially those with atrophic gastritis, is necessary to avoid repression of the gastric barrier to bacteria.

1. Introduction

Helicobacter pylori (*H. pylori*) colonizes approximately half of the world's population and causes chronic gastritis, peptic ulcers, and gastric adenocarcinoma [1]. Eradication of this bacterium improves the symptoms of patients with peptic ulcer and gastric lymphoma of mucosa-associated lymphoid tissue [2,3]. Isolation of *H. pylori* from endoscopic gastric biopsy specimens is the most reliable method for detecting *H. pylori* infection and essential for drug susceptibility testing [4].

The gastric acid determines bacterial susceptibility to the stomach and inhibits infectious agents from reaching the intestine [5]. Urease activity is crucial for *H. pylori* to colonize the stomach through neutralizing the acidic environment and providing chemotactic motility [6]. However, colonization of urease-negative *H. pylori* and *Campylobacter jejuni* is reported in patients receiving acid-reducing compounds [7,8]. Moreover, predisposed decrease of acid secretion, due to therapy, disease, or age, increased bacterial population in gastric juice [9,10]. Disproportional use of proton pump inhibitors is considered to promote small intestinal bacterial overgrowth, which is prevalent in patients with irritable bowel disease (IBD) [11]. The gastrointestinal microbiota clearly contributes to development of IBD both in mouse models and patients [12].

A gram-negative bacillus, *Kingella denitrificans* (*K. denitrificans*), is a component of the normal upper respiratory and genitourinary tract flora and sometimes causes severe infection [13–15]. *Kingella* species are plump gram-negative bacilli and positive for cytochrome *c* oxidase [16]. Unlike the related species, such as *Neisseriae* and *Moraxellae*, *Kingella* species are catalase-negative similar to *Cardiobacterium hominis* and *Eikenella corrodens*. However, strain UB-75 of *Kingella oralis* and strain UB-204 of *E. corrodens* were catalase positive [17]. The type-strain of *K. denitrificans* characteristically produces acid from glucose and is positive for prolyl-aminopeptidase. Different from other species in the genus, *K. denitrificans* reduces nitrate to nitrite [16].

Necessity for careful identification of urease-negative bacteria in the gastric mucosa is highlighted in this paper. Of particular

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interest, disruption of integrated immunological niche by dysbiotic colonization of commensal bacteria is discussed.

2. Materials and methods

2.1. Patient

A 78-year-old man suffering from gastric ulcer had been administered 40 mg of histamine receptor 2 (H2) antagonist, ranitidine, per day for two years. Endoscopic observation revealed multiple gastric ulcer scars with severe atrophic gastritis. Gastric mucosal biopsy from the antrum and the body was performed to determine histological findings and detect *H. pylori*. The biopsy specimen was positive for the CLO-test (Kimbarly-Clark, Roswell, GA).

The study was approved by the Yamaguchi University Hospital Ethics Committee. Informed consent was obtained from the patient. The research was carried out in accordance with the Declaration of Helsinki.

2.2. Bacterial isolation and culture conditions

The gastric biopsy specimen was homogenized and a loopful of inoculum was streaked onto a plate of an HP selective medium (Eiken Chemical Inc., Tokyo, Japan) containing amphotericin B (2 μg/ml), trimethoprim (5 μg/ml), polymyxin B (2.5 IU/ml), and vancomycin (10 μg/ml). The plate was grown in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) at 37 °C for 5 days. The type-strain of *K. denitrificans* ATCC33394 was obtained from the American Type Culture Collection. *K. denitrificans* KDY1 was isolated from a nasopharyngeal swab of a leukemic patient and *H. pylori* CPY3401 was from gastric biopsy specimen [18] in Yamaguchi University Hospital, respectively. And HPT73 is an isogenic *ureB*-disrupted mutant of CPY3401. The culture condition of *K. denitrificans* was exactly the same as that of *H. pylori*.

2.3. Morphology

Hematoxylin-eosin, Giemsa, and Gram stainings were performed by a standard method. For electron microscopy, bacteria were grown in brucella broth containing 3% horse serum for 24 h, washed once with 5 volumes of 10% glycerol MOPS buffer and suspended in 5 volumes of saline. Samples were dried onto a collodion-carbon-coated grid. Shadowing was performed and samples were observed with a JEM-200CX (JEOL) transmission electron microscope as described [18].

2.4. Detection of a H. pylori-specific gene in paraffin-embedded biopsy samples

DNA was extracted from paraffin-embedded gastric biopsy tissues using DEXPAD (Takara BioCo. Shiga, Japan) and subjected to PCR. The primers ureF1 (ATA TTA TGG AAG AAG CGA GAG C) and ureR (ATG GAA GTG TGA GCC GAT TTG), corresponding to bases 2783–2804 and 3076–3096, respectively, of the ureA gene of *H. pylori* amplified 314-bp fragments. For the second round of PCR amplification, primers ureR and ureF2 (CAT GAA GTG GGT ATT GAA GC; +2893–2912) were used.

2.5. Biochemical characterization

Catalase production was tested by placing bacteria from the plates into a drop of 3% hydrogen peroxide on a slide glass. Cytochrome *c* oxidase activity was tested on an oxidase strip (Eiken Chemical Inc.). Hydrolysis of urea was detected with Christensen urea agar (Eiken Chemical Inc.).

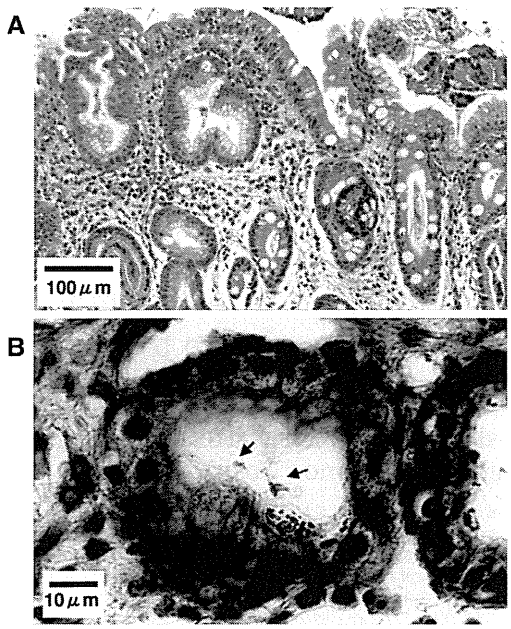

Fig. 1. Histologic section of the biopsy sample. A. The specimen from the antral lesions of chronic active gastritis was stained with hematoxylin and eosin. Infiltration by mononuclear cells and grandular atrophy and intestinal metaplasia could be observed. Magnification, ×140. B. H. pylori-like bacteria were stained with Giemsa in the gastric pit of the same antral biopsy specimen as in panel A. Arrows indicate curved bacilli. Magnification, ×1000.

The ID test HN-20 rapid NISSUI (Nissui Pharmaceutical Co., Tokyo, Japan) was used for identifying *Haemophilus* and *Neisseria* species. This system assays activities for alanine aminopeptidase, alkaline phosphatase, nitrate and nitrite reduction, urease, ornithine decarboxylase, indole production, proline aminopeptidase, glucosidase, γ-glutamyl transpeptidase, and β-galactosidase. This system also examines acid production from glucose, maltose, fructose, mannose, mannitol, trehalose, sucrose, lactose, and xylose.

2.6. 16S ribosomal RNA genome sequencing and data analysis

Bacterial 16S rRNA genes were amplified using universal primers for eubacterial 16S rRNA genes [19]. 16S rRNA sequences were compared by using the Clustal W suite of program [20]. The sequences were between 1467 and 1473 bp long, and the 5′ end was located at position 9 and the 3′ end was position 1482 in the *Escherichia coli* numbering system. A rooted phylogenetic tree [21] has been created.

2.7. Acid sensitivity

The survival of the isolate under different pH conditions [22] was evaluated. Cell suspensions from 48 h cultures were incubated at 37 °C for 1 h with glycine-HCl buffer (pH 2.0), McIlvain's buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, [pH 4.0]), and 0.1 M phosphate buffer (pH 7.0). After incubation, serial 10-fold dilutions of the cell suspensions in 150 mM NaCl were plated onto brucella agar plates containing 3% horse serum and incubated for 72 h at 37 °C to determine CFU.

2.8. Urease assay

Urease activity in bacterium [18] was determined and expressed in micromoles of urea hydrolyzed per minute per milligram of protein in the crude extract.

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Table 1
Properties of the isolate, *K. denitrificans*, and *H. pylori*.

	H. pylori CPY3401 Urease (+)	*H. pylori* HPT73 Urease (−)	*K. denitrificans* NHP1	*K. denitrificans* ATCC3394	*K. denitrificans* KDY1
Catalase production[a]	+	+	+	−	−
Oxidase production[a]	+	+	+	+	+
Alanine aminopeptidase	+	+	+	+	+
Phosphatase	−	−	−	−	−
Nitrate reduction	−	−	+	+	+
Nitrite reduction	−	−	+	+	+
Urease activity[a]	+	−	−	−	−
Indole production	−	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−
Glucosidase	−	−	−	−	−
Proline aminopeptidase	−	−	+	+	+
γ-Glutamyl aminopeptidase	+	+	−	−	−
Acid production from					
Glucose	−	−	−	+[b]	+
Maltose	−	−	−	−	−
Fructose	−	−	−	−	−
Mannose	−	−	−	−	−
Mannitol	−	−	−	−	−
Trehalose	−	−	−	−	−
Sucrose	−	−	−	−	−
Lactose	−	−	−	−	−
Xylose	−	−	−	−	−
Growth at 42°	−	−	+	+	+

[a] Items for routine assay to identify *H. pylori*.
[b] Weakly positive.

3. Results

3.1. Isolation of a gram-negative and urease-negative bacterium

The histology of the gastric biopsy specimens indicated granular atrophy and intestinal metaplasia accompanied by infiltration of mononuclear cells to the lamina propria, a typical observation in gastric mucosa infected with *H. pylori* (Fig. 1A). Though it is not specific, a few bacteria-like organisms could be seen in the gastric lumen (Fig. 1B). *H. pylori ureA* gene was amplified in the paraffin-embedded gastric tissue (not shown).

A bacterium isolated from the culture of biopsy specimen was named NHP1. The bacterial colonies corroded the agar surface and had no hemolytic activity on sheep blood agar. Growth was obtained at 37 and 42 °C under the microaerobic conditions (Table 1). A gram-negative bacillus, quite similar in size and morphology with *H. pylori* was observed (Fig. 2). However, NHP1 lacked the urease activity.

3.2. Morphological and genetical analysis

The electron microscopy showed that *H. pylori* CPY3401 had a curved body with a bundle of sheathed flagella at one pole (Fig. 3A), whereas strain NHP1 was rod-shaped with no flagella and sometimes appeared in pairs (Fig. 3B).

The basic local alignment search tool showed that the 16S rRNA sequence of NHP1 had the highest similarity with the gene of *K.*

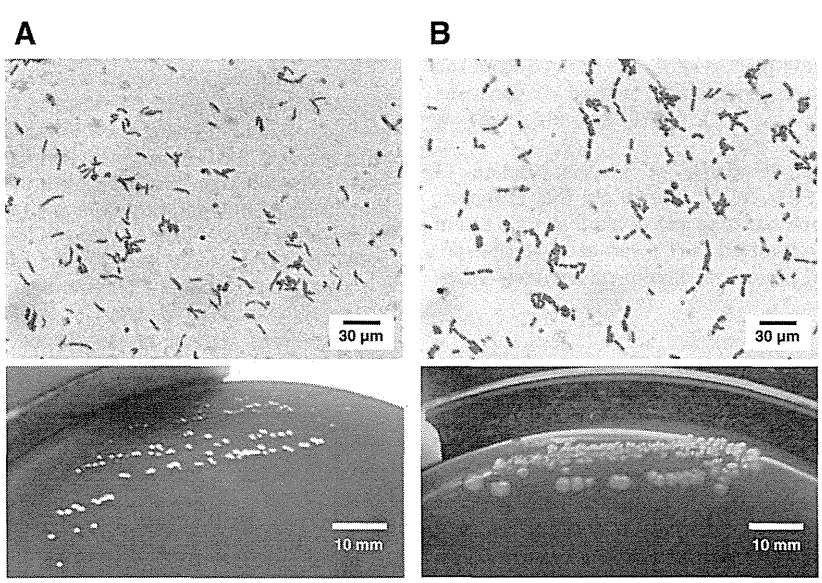

Fig. 2. Gram stain and colonies of bacteria. Bacteria were cultured for 2 days with HP selective plate. The grown bacterial colonies (lower pictures) were smeared on the glass and Gram-stained (upper pictures). A. *H. pylori* CPY3401, B. NHP1, Magnification of Gram Stain, ×1000.

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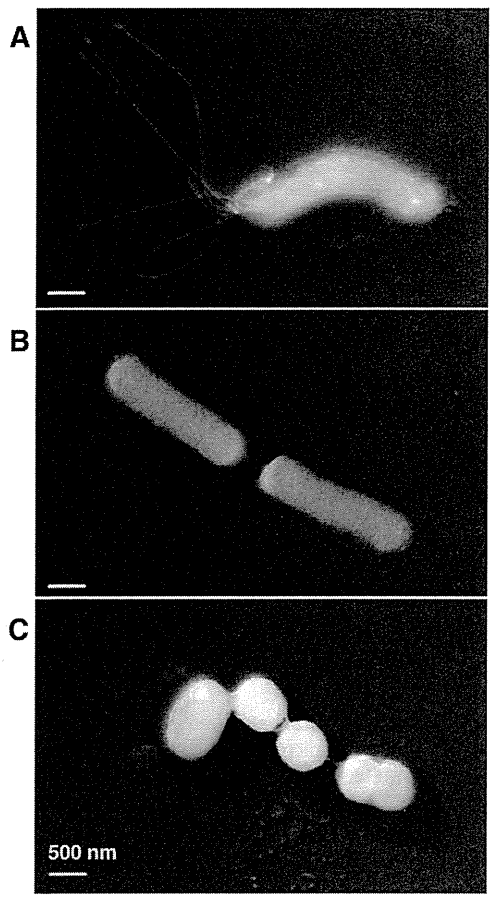

Fig. 3. Electron micrographs. A. *H. pylori* CPY3401, B. NHP1 C. *K. denitrificans* KDY1, Bar = 500 nm.

denitrificans (Fig. 4A). The 1468 sequence alignment of NHP1 and *K. denitrificans* ATCC33394 (type-strain) had 99% identity (not shown). NHP1 differs in its sequence by only 3 bases from UB-294, an oral isolate of *K. denitrificans* [23] and by 5 bases from ATCC33394 (Fig. 4B). Since a phylogenetic tree derived from the distant matrix by using the neighbor joining method [28] formed a tight cluster on the tree (Fig. 4A), NHP1 was diagnosed as *K. denitrificans*.

However, the electronmicrograph of another isolate of *K. denitrificans*, KDY1, was 0.5–0.6 by 0.5–1.0 μm that is shorter than NHP1 and CPY3401 and had long and thin pili about 5 nm in diameter (Fig. 3C). NHP1 resembled to *H. pylori* at a glance especially in size, but *H. pylori* possessed slightly curved body when observed carefully (Fig. 2).

3.3. Biochemical identification

A scoring-based test system for *Haemophilus* and *Neisseria* species showed NHP1 as *K. denitrificans* at the highest probability (12% in Table 1). Since other species name having the similar score was not presented by the ID test, the isolate was identified as *K. denitrificans*. Biochemical features of NHP1 were almost identical to those of *K. denitrificans* strains, ATCC33394 and KDY1, except for catalase activity and acid production from glucose. The scoring system diagnosed the NHP1 isolate as *K. denitrificans*, consistent with the sequencing result.

3.4. Acid sensitivity

The acid sensitivity of *H. pylori* CPY3401 was compared with *K. denitrificans* NHP1, KDY1, and ATCC33394 (Table 2). Colony forming units per ml (CFU/ml) of the initial inoculum of NHP1 numbered $10^{7.9}$, declining to $10^{6.2}$ after a 1 h exposure to pH 4.0 at 37 °C. On the other hand, CFU/ml of the initial inoculum of *H. pylori* CPY3401 numbered $10^{8.2}$, decreasing to $10^{4.1}$ after 1 h at pH 4.0. Thus, the survival ratio of bacteria after 1 h at pH 4.0 was 1 in $10^{1.7}$ in *K. denitrificans* NHP1, compared to 1 in $10^{4.1}$ in *H. pylori*. *K. denitrificans* NHP1 was indicated $10^{2.4}$-fold more tolerant to acid (pH 4.0) than *H. pylori* CPY3401. In contrast, the type-strain (ATCC33394) and a clinical isolate (KDY1) were vulnerable to acid. Their initial inocula numbered $10^{8.3}$ and $10^{7.8}$, respectively, declining to less than $10^{1.0}$ after exposure to pH 4.0.

Each isolate of *K. denitrificans* was mixed one-on-one with *H. pylori* CPY3401 and exposed to buffers with different pH. The initial CFU/ml of *H. pylori* and NHP1 mixture numbered $10^{7.9}$, declining to $10^{6.5}$ after a 1 h exposure to pH 4.0. Five hundred clones were picked up from the colonies exposed to pH 4.0, then subjected to urease assay. The urease activity of *H. pylori* CPY3401 and NHP1 was 353.5 mmol/min/mg and 15.4 mmol/min/mg, respectively. None of the pH 4 plates either from *H. pylori* and ATCC33394 or *H. pylori* and KDY1 showed more than 100 mmol/min/mg of urease activity. On the other hand, number of colonies which showed more than 100 mmol/min/mg of urease activity in the pH 4 plate of the *H. pylori* and NHP1 was 116 (23.2%), indicating CFU/ml of survived *H. pylori* was $10^{6.15}$. When *H. pylori* was mixed with other isolates of *K. denitrificans* (ATCC33394 and KDY1), all of the survived bacteria after exposure to pH 4.0 showed urease activity and CFU/ml did not exceeded 10^4. Acid tolerance of *H. pylori* was increased up to 160-fold by cocultivation with acid tolerant *K. denitrificans* NHP1.

We have repeated the experiment by changing the time length (20, 40, and 60 min) for bacterial exposure to acidic conditions (pH 2, 4, and 7). In the acidic condition (pH 4), single culture of *H. pylori* could not survive. However, mixture of *H. pylori* with *K. denitrificans* showed survival of *H. pylori* after 20 min in pH 4, which was better when mixed with acid tolerant NHP1 (65.4%) than with ATCC33394 (32.1%) (Fig. 5).

4. Discussion

The gastric juice represents a barrier to microbes in saliva and ingested food, mainly by the bactericidal activity of hydrochloric acid [23]. A study in patients with hypochlorhydria being treated with anti-acid and histamine receptor 2 (H2) antagonists identified bacteria originating from the mouth in the gastric contents [24]. Moreover, acid-inhibiting proton pump inhibitors caused gastric colonization by oral-type bacteria in healthy volunteers [10]. The gastric barrier to infection has more significant meaning to hosts having a weakened immunological defense [25].

We isolated a rod-shaped isolate of *K. denitrificans*, which is different from general plump-shaped isolate not only by the morphology. However, the 16S rRNA sequence of the isolate showed 99% identity with the type-strain ATCC33394. The novel *K. denitrificans* isolate, NHP1, was better able to survive in acidic conditions than the type-strain of *K. denitrificans* (Table 2). Though acid exposure of *H. pylori* alone did not show survival of the bacterium, mixture of *H. pylori* with *K. denitrificans* showed survival of *H. pylori* (Fig. 5). We assume that *K. denitrificans* may bind with *H. pylori*, thus, *K. denitrificans* enables *H. pylori* more acid resistant by coating the bacterial body. This coating may be more effective in acid tolerant NHP1 than acid sensitive ATCC43349. Such a difference in acid tolerance between isolates could also be observed in

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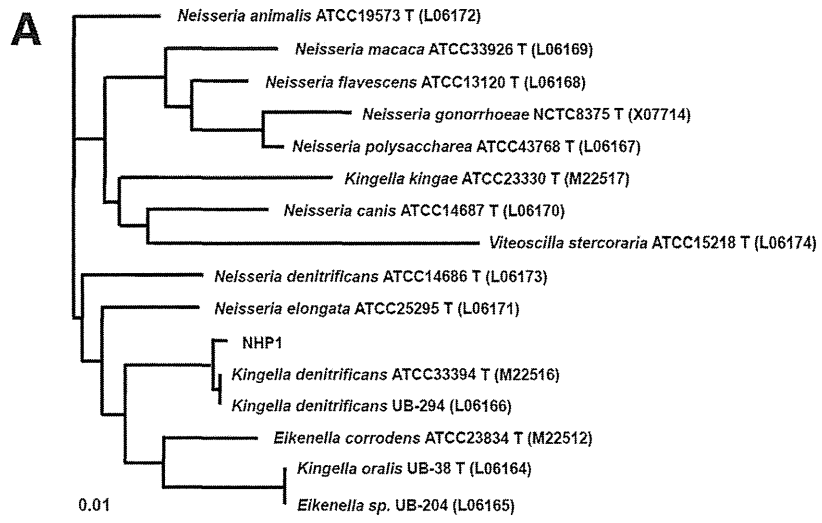

Fig. 4. Genetic analyses of 16S rRNA sequences of *K. denitrificans*. A. Rooted phylogenetic tree based on 16S rRNA sequence comparisons. Bar indicates 0.01% differences in nucleotide sequences. Horizontal distances are equivalent to genetic distances. Type strains are indicated by adding T at the last of each name. 16S rRNA sequences are available for electronic retrieval from GenBank under the accession numbers indicated in each parenthesis. B. Variations of DNA sequences of the 16S rRNA gene of *K. denitrificans*. Numbers are corresponding to positions in the *Escherichia coli* 16S rRNA numbering system. Bases deviated from the sequencing result of type-strain ATCC33394 were underlined.

Neisseria gonorrhoeae [26]. Thus, a specific strain of *K. denitrificans* might be able to survive in the human stomach. Furthermore, *Kingella kingae*, a commensal of the human respiratory tract [27], also causes acute gastroenteritis before the onset of systemic symptoms [28]. Though an association of *K. denitrificans* with gastrointestinal disease has not yet been described, our experimental results showed *K. denitrificans* NHP1 isolate help survive *H. pylori* in the acidic condition.

Human alimentary tract harbors hundreds of commensal microbes that interact with the host and provide genetic, metabolic, and immunological attributes [29]. On the other hand, infection with dysbiotic microbes or environmental stresses such as exposure to xenobiotics could alter compositional or functional properties of gut microbes and disrupt immune homeostasis by specific members of this community [30,31]. Since *K. dentrificans* could be colonized into atrophic gastric epithelium where the mucosal barrier systems are perturbed due to chronic inflammation, it might profoundly affect pathology and clinical prognosis of chronic gastritis caused by *H. pylori* infection. Moreover, the commensal may interact with *H. pylori* to stimulate inflammatory signals that have a great impact on the tumor development and progression [32]. Consistent with this idea, the commensal microbes switch their contribution from gastrointestinal homeostasis to pathogenic inflammation, once they communicate with dysbiotic pathogens such as *Salmonella typhimurium* [33]. Furthermore, it is of great interest to evaluate whether infections with *K. dentrificans* or other commensals are associated with the susceptibility to gastric

Table 2
Survival of bacteria after incubation in solutions of different pH.

	Survival after incubation in buffers[a]		
	pH 2	pH 4	pH 7
Strain			
H. pylori CPY3401	<1.0	4.1	8.2
K. denitrificans NHP1	<1.0	6.2	7.9
K. denitrificans ATCC33394	<1.0	<1.0	8.3
K. denitrificans KDY1	<1.0	<1.0	7.8
Strains			
NHP1 + *H. pylori* CPY3401	<1.0	6.5(6.15)[b]	7.9
ATCC33394 + *H. pylori* CPY3401	<1.0	3.9[c]	7.8
KDY1 + *H. pylori* CPY3401	<1.0	4.0[c]	7.9

[a] Numbers are expressed as log10 CFU/ml of the mean results of more than two experiments.
[b] Since 23.2% of the colonies were positive for urease. log10 CFU/ml of *H. pylori* is 6.15, which is shown in the parenthesis.
[c] All the colonies are urease positive.

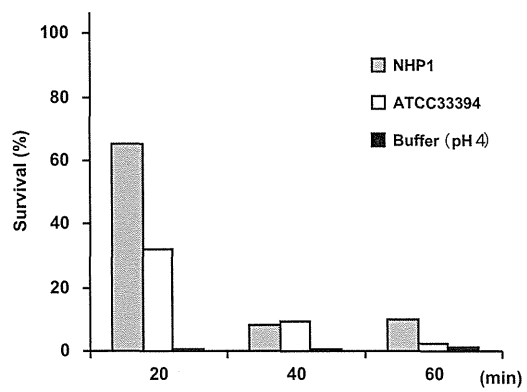

Fig. 5. Acid tolerance of *H. pylori*. Acid tolerance of *H. pylori* at 20, 40, and 60 min in the pH 4 buffer was assayed by mixing *H. pylori* CPY3401 with *K. denitrificans* NHP1, *K. denitrificans* ATCC3394, and buffer alone. Percent survival of *H. pylori* CPY3401 at pH 4 condition in contrast to pH 7 condition was calculated by comparing the numbers of colonies.

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inflammation and tumorigenicity in patients with *H. pylori* infection. Conclusively, a careful administration of anti-acids to the elderly, especially those with atrophic gastritis, is required to maintain the gastric barrier to other bacteria.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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