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Novel Na+/H+ antiporter (NapA) regulates the motility in *Helicobacter pylori*

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Abstract

Na+/H+ antiporter plays an important role in maintaining cellular homeostasis by regulating osmotic pressure and intracellular pH. It plays an important role in maintaining cellular homeostasis. In *Helicobacter pylori*, whole genome sequencing has revealed the presence of two types of Na+/H+ antiporter. A gene (*nhaA*) homologous to the Na+/H+ antiporter of *Escherichia coli* has been investigated and its function has been analyzed. However, another gene homologous to the Na+/H+ antiporter of *Enterococcus hirae* (*napA*) is not yet known in detail. In this study, we investigated the function of this gene (*napA* in *H. pylori*). First, to confirm the genetic presence of *napA* in 20 *H. pylori* clinical isolates, PCR analysis was performed, and the *napA* gene was confirmed in all strains. The amount of Na+ extrusion was measured by atomic absorption spectroscopy. The results showed that the Na+ concentration was decreased in the wild-type strain compared to the *napA* mutant strain. In addition, there was a significant dose-dependent difference in CFU of Na+ concentration in the *napA* mutant strain compared to the wild-type strain. We examined whether the *napA* gene is related to motility using both wild-type and *napA* mutant strains. As a result, in the motility agar test, the bacterial motility observed in the wild-type strain was not observed in the *napA* mutant strain. However, no difference in flagellar proteins was observed by SDS-PAGE analysis. These results suggest that the *napA* gene of *H. pylori* may regulate homeostasis by extruding Na+ and may also regulate motility.

Keywords: *Helicobacter pylori*; NapA; Na+/H+ antiporter; Motility; Flagella

1. Introduction

Na^+/H^+ antiporter is ubiquitous membrane protein found in cytoplasmic and organelle membranes of many organisms from bacteria to humans [1]. It plays a primary role in the regulation of intracellular pH and cellular Na^+ concentrations by exchanging Na^+ for H^+ [1]. In *Escherichia coli*, three Na^+/H^+ antiporters (EC NhaA, NhaB, and ChaA) are known, and their functional characteristics have been well described [2]. Among the three antiporters, EC NhaA plays a major role in regulating intracellular Na^+ concentrations [3].

In *Helicobacter pylori*, two Na^+/H^+ antiporters (NhaA, and NapA) are known by TIGR (The Institute for Genomic Research) sequence [4]. The *nhaA* gene of *H. pylori* is homologue of EC *nhaA* gene and this function was well studied [5, 6]. Another putative Na^+/H^+ antiporter gene, *napA* gene (HP1183) is annotated the homologue of *Enterococcus hirae napA* gene [4, 7]. The similarity and identity between *napA* gene of *H. pylori* and Na^+ / H^+ antiporter of *Enterococcus hirae* were 53.5 % and 26.6 %, respectively [7]. But the function of this gene has been uncleared yet.

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Many motile bacteria move by rotating their flagella, the filamentous organelles that extend from the cell body [8]. Flagellar rotation is carried out by a reversible rotary motor embedded in the cytoplasmic membrane at the base of each flagellar filament [9]. These motors are powered by an electrochemical gradient of specific ions across the cytoplasmic membrane and are classified into two types of coupling ions: an H^+-driven motor (in *Escherichia coli*, *Salmonella typhimurium*, and Bacillus spp.) and a Na^+-driven motor (in alkalophilic Bacillus and marine Vibrio spp.) [9]. At neutral pH, the Na^+ motive force is secondarily generated by a Na^+ / H^+ antiporter from the H^+ motive force [9]. But the relationship between *napA* and motility has been unclear in *H. pylori*.

Now we try to clarify the prevalence of *napA* in *H. pylori*, the Na^+ transportation ability of *napA*, the Na^+ sensitivity of *napA*, and the motility associated with *napA*.

2. Material and methods

2.1. Bacterial strains, culture condition

To confirm the presence of the *napA* gene in clinical isolates, we analyzed 20 *H. pylori*

Table 1 Bacterial strains and plasmids used in this study

Strain (alternate name) or plasmid	Genotype or characteristics	Reference or source
H. pylori		
JP26	Wild type	[11]
JP26nap	JP26/HP1183: *aphA*	This study
E.coli DH5α	*endA1 hsdR17($r_K^- m_K^+$) supE44 thi-1 recA*	
Plasmids		
pGEM-T Easy	ColE1, Amp^r, PCR cloning vector	Promega
pUK4K	ColE1(Amp^r,Kan^r)	[12]
pGEMTnapA	pGEM-T Easy containing HP1183	This study
pGEMTnapA:km	pGEMT/HP1183: *aphA*	This study

Strains in this study. Those *H. pylori* strains including 26695, J99, JP26 and 17 clinical isolates from Nagoya university hospital were used [4, 10, 11]. Stock cultures of *H. pylori* were grown for 4 days on Brucella broth agar plates (Becton Dickinson and Company, MD, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL, MD, USA) (BB agar) at 37 °C in a microaerophilic atmosphere.

Broth cultures of *H. pylori* were prepared by subculturing colonies from freshly cultured agar plates into Brucella broth (Becton Dickinson and Company) supplemented with 10% FCS (BB) 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 in urease, catalase, and oxidase tests.

Table 2 Oligonucleotide primers used in this study

Primer	Sequence
napA1	aatcatgtccgaaattggct
napA2	gtatttgcgccacaataagg
napAinv1	cataggatcccacctaccccacataagc
napAinv2	gataggatccgcctaaaagcagtcagtt

2.2. DNA techniques and sequence analysis.

Standard molecular techniques were used. *H. pylori* chromosomal DNA was prepared from cells of each strain after 48 hours of growth on two BB agar plates as described previously [12]. Plasmid DNA was prepared from *H. pylori* after 48 hours of growth or from *E. coli* after overnight cultures, using a midi-prep protocol (Qiagen Inc., Valencia, CA, and USA) according to the manufacturer's instructions. Strains and plasmids used in this study are listed in Table 1. *E. coli* was routinely grown at 37°C in Luria-Bertani broth or agar supplemented with ampicillin (Fuji film wako pure chemical Co., Osaka, Japan) (100 μg/ml), kanamycin (Fuji film wako pure chemical Co.) (25 μg/ml), when appropriate. *H. pylori* strains were grown on BB agar with 10% FCS plates at 37°C in a 5% CO_2 atmosphere. Antibiotic-resistant *H. pylori* transformants were selected with kanamycin (25 μg/ml). Primers were listed in Table 2. The HP1183 ORF of strain 26695 was amplified by PCR using primers napA1 and napA2. The presence of a single 800 bp band was verified on a 1% agarose gel (Fuji film wako pure chemical Co.). The PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN). This product was ligated into pGEM-T Easy (Promega Co, WI, and USA) and transformed into *E. coli* DH5α according to manufacture protocols. A unique BamHI site was created by inverse PCR with primers napAinv1 and napAinv2. Plasmid pUC4K was digested with BamHI [12]. After that, the kanamycin resistance (Kan^r; *aphA*) cassette was isolated by agarose gel electrophoresis and ligated into the inverse PCR product to disrupt the HP1183 ORF, creating pGEMTnapA:km. *H. pylori* JP26 were transformed to Kan^r with pGEMTnapA to create JP26/1183::*aphA* (JP26napA). The procedure of natural transformation was performed as previous method [13]. Briefly, recipient *H. pylori* cells were harvested from 48-hours growth on one BB agar plate into 1 ml of phosphate-buffered saline (PBS) and then centrifuged at 8,500 × g for 5 min. The pellet was resuspended in 300 μl of PBS. Each transformation mixture, consisting of 25 μl of recipient cells and 30 ng of donor DNA (pGEMTnapA:Km), was spotted onto a BB agar plate. Plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. After 18 hours of incubation, the transformation mixture was harvested into 1 ml of PBS, and 100-μl aliquots of appropriate serial dilutions were plated on BB agar with kanamycin plates. All plates were incubated for 4 days at 37°C in a 5% CO_2 atmosphere, after which transformants were collected. Chromosomal DNA was isolated from strains JP26, JP26napA, and the insertion of the *aphA* cassette within HP1183 in the transformants was confirmed by PCR using primer pair's napA1 and napA2.

2.3. Measurement of Na^+ sensitivity to *H. pylori*

Bacteria were harvested from culture plates and suspended in PBS to yield a final suspension of approximately 10^8 colony-forming units (CFU) / mL. To evaluate the survival of *H. pylori* under a variety of Na^+ concentration conditions, cell suspensions were diluted in BB, BB with 50mM NaCl (Fuji film wako pure chemical Co.), BB with 100mM NaCl, and incubated for 2 days at 37°C under microaerophilic conditions. To enumerate viable bacteria after 2 days, serial dilutions of cell suspensions were made PBS, inoculated onto BB agar plates, and incubated for 4 days at 37°C under microaerophilic conditions. The number of CFU was then determined. For acid and alkali stress, BB with 100mM MESS (Fuji film wako pure chemical Co.) and BB with 100mM MOPS (Fuji film wako pure chemical Co.) were used.

2.4. The measurement of Na^+ extrusion by atomic absorbance experiment.

H. pylori were cultured for 4 days in BB agar plate with adequate antibiotics. *H. pylori* were collected from plate and mixed with 1mM Tris-HCl (pH6.8) (Fuji film wako pure chemical Co.) and centrifuged 8000 × g for 5 minutes. Supernatant was removed and remixed with 1mM Tris-HCl (pH6.8) 7mL. 5M NaCl 210mL was added to mixed solution. After incubation on ice for 1 hour, aliquot of bacterial solution was added to 1mM Tris-HCl (pH6.8) 4mL. Then mixed solution was added to 0.35mL Lauryl Bromide (Sigma Aldrich Co., MO, and USA) and centrifuged 8000 × g for 5 minutes after 0, 1, 3, 5 minutes of adding. After removal of water layer, adding water and washed and removed. 500mL milliQ and 50 mL trichloroacetic acid (TCA) (Fuji film wako pure chemical Co.) were added and mixed. After 5 minutes of incubation at 90°C, centrifuged 6000 × g for 5 minutes. Supernatant was collected and was investigated to measure Na^+ concentrations by Polarized Zeeman atomic absorption spectrometer (Z-5710, Hitachi High-Tech Co., and Tokyo, Japan). Pellet was measured for the quantities of protein.

2.5. Motility test

Bacterial motility experiments were performed by modifying previous research methods [9, 14]. Bacterial cells grown micro aerobically at 37°C for 4 days on BB agar were stabbed with toothpicks into BB 0.4 % agar as motility agar plate. The diameters of the growth zone were measured in the mortality agar plate and expressed as degree of bacteria motility.

2.6. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) of Flagellar protein

Flagellar protein experiments were performed by modifying previous research methods [15]. Bacteria were harvested into distilled water and homogenized, and deflagellated cells removed by centrifugation according to the previous

protocol. The bacteria - free supernatant was centrifuged at 100,000 × g for 1 hour, and the pellet of flagella was retained. This pellet was suspended in distilled water, adjusted to pH 2.0 with HCl, and held on ice for 15 minutes. Material insoluble at pH 2.0 was removed by centrifugation at 100,000 × g for 1hour. The supernatant was then adjusted to pH 7.0 with NaOH and left on ice for 30 minutes to allow for flagellar reassociation.

After that, sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the previous method. Protein solubilized in sample buffer was stacked in a 4.5% (wt/vol) acrylamide (Fuji film wako pure chemical Co.) and separated with 7.5 % (wt / vol) acrylamide. Protein was stained with Coomassie blue R-250 (Fuji film wako pure chemical Co.).

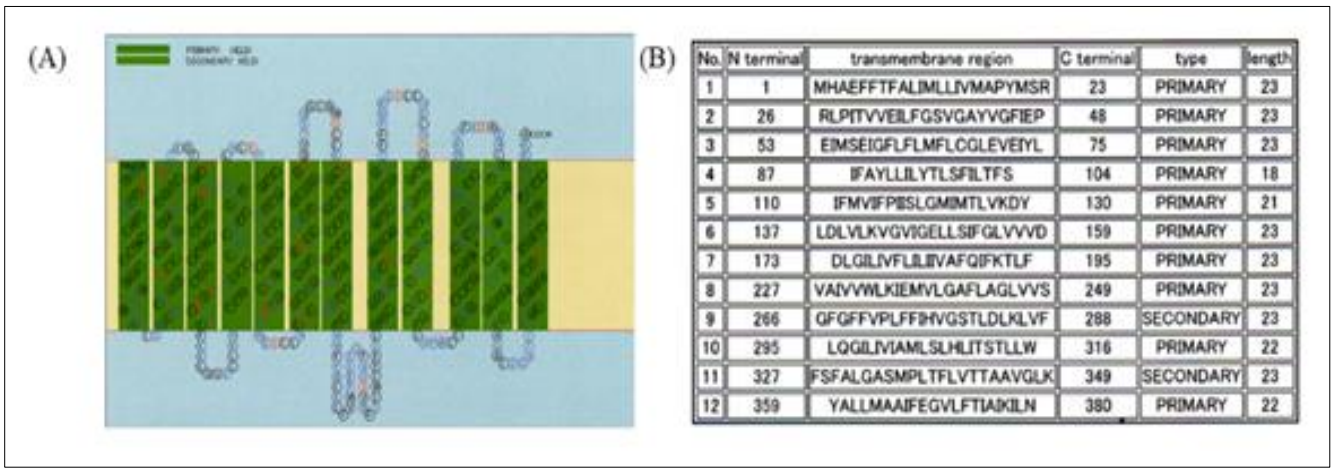
2.7. Statistical analysis

All the experiments were repeated at least five times, and the results were expressed as the mean values ± standard deviations and compared using a paired *t* test.

3. Results

3.1. The confirmation of *napA* gene in *H. pylori* strains

At first, the amino acid structure of NapA was analyzed by SOSUI engine (https://harrier.nagahama-i-bio.ac.jp/sosui/) (Figure 1). This analysis revealed that NapA antiporter was a 12-transmembrane protein. Moreover, we checked whether *napA* gene is widely conserved in *H. pylori* clinical strain. By PCR method, approximately 800 bp bands were represented in all 20 *H. pylori* strains (Figure 2).



No.	N terminal	transmembrane region	C terminal	type	length
1	1	MHAEFFTFALIMLLIVMAPYMSR	23	PRIMARY	23
2	26	RLPITVVEILFGSVGAYVGFIEP	48	PRIMARY	23
3	53	EIMSEIGFLFLMFLCGLEVEIYL	75	PRIMARY	23
4	87	IFAYLLILYTLSFILTFS	104	PRIMARY	18
5	110	IFMVIFPIISLGMIMTLVKDY	130	PRIMARY	21
6	137	LDLVLKVGVIGELLSIFGLVVVD	159	PRIMARY	23
7	173	DLGILIVFLILIIVAFQIFKTLF	195	PRIMARY	23
8	227	VAIVVWLKIEMVLGAFLAGLVVS	249	PRIMARY	23
9	266	GFGFFVPLFFIHVGSTLDLKLVF	288	SECONDARY	23
10	295	LQGILIVIAMLSLHLITSTLLW	316	PRIMARY	22
11	327	FSFALGASMPLTFLVTTAAVGLK	349	SECONDARY	23
12	359	YALLMAAIFEGVLFTIAIKILN	380	PRIMARY	22

Figure 1 Characteristic of NapA structure and amino acid sequence (A) Two-dimensional structure, (B) amino acid sequence

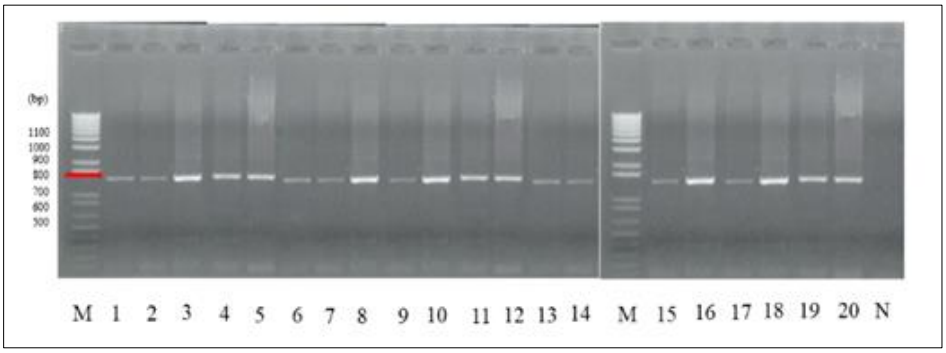


Figure 2 Prevalence of *napA* gene in 20 *H. pylori*

M: marker 1:26695 2:J99 3:JP26 4-20:HP1-HP17 N: negative control. Red Arrow indicates the band of the ***napA*** gene

3.2. Na^+ extrusion measurement

Next, we checked the Na^+ extrusion test in both wild-type (JP26) and isogenic *napA* mutant (JP26napA) strain. The value of Na^+ concentration in measured was decreasing according to time in both wild-type strain and mutant strain. But the rate of decreasing in wild-type strain is more rapid than that in *napA* mutant strain. (Figure 3)

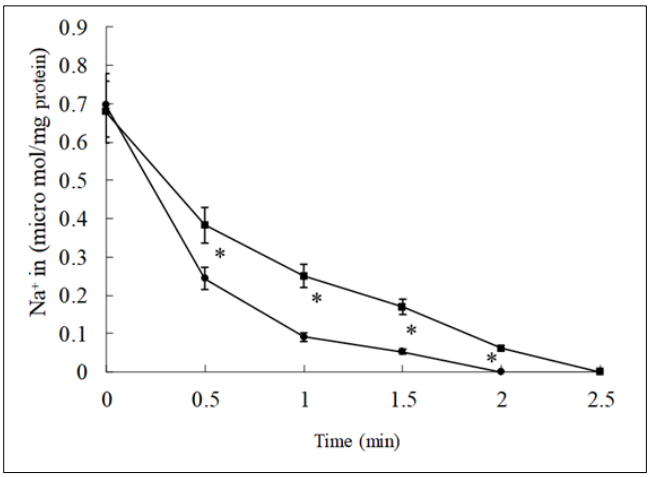


Figure 3 Comparison of Na^+ extrusion between JP26 and JP26napA

Closed circle: JP26, Closed box: JP26napA. *: $p < 0.05$

3.3. Na^+ sensitivity assay

We investigated whether the *napA* gene is involved in the effect of Na^+ concentration on bacteria. At first, we found no significant changes of the CFU in wild-type strain by 200mM Na^+ at neutral condition (pH 7.4). But the CFU dropped down under over 200mM Na^+ concentration.

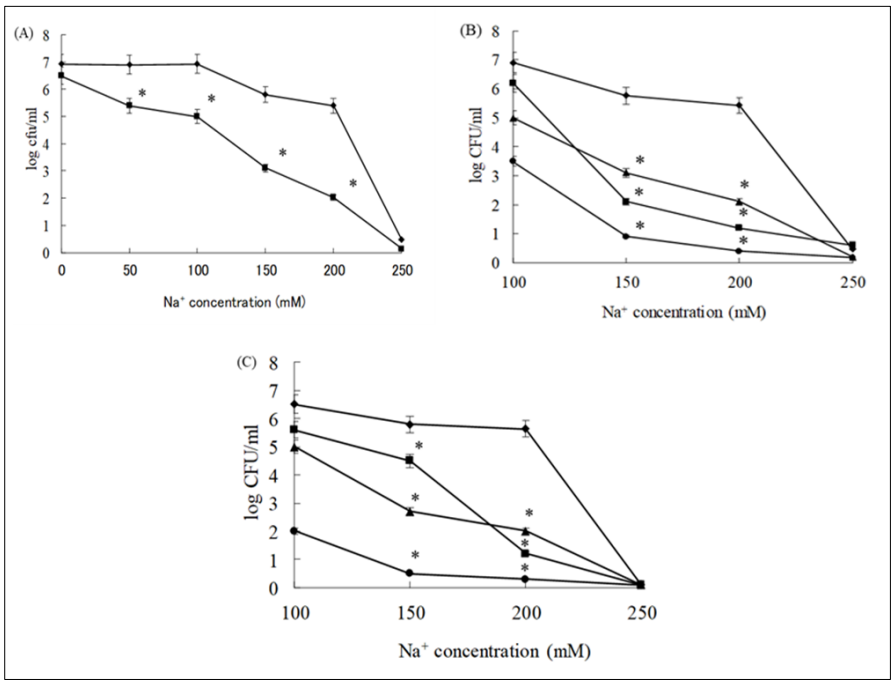


Figure 4 Comparison of CFU by Na^+ stress in between JP26 and JP26napA

(A) At neutral condition (pH 7.4): closed diamond: JP26, closed box: JPnapA. (B) At acidic condition (pH 5.4), closed diamond: JP26, closed box: JPnapA. Closed triangle: JP26 (pH 5.4). Closed circle JP26napA (pH5.4). (C) At basic condition (pH 8.0): closed diamond: JP26, closed box: JP26napA. Closed triangle: JP26 (pH 8.0). Closed circle JP26napA (pH8.0). *: $p < 0.05$

But the colony of *napA* mutant strain was reduced even if under 50 mM Na^+ concentration significantly ($p < 0.05$) (Figure 4(A)). And as the Na^+ concentration increased, the difference in CFU between the wild strain and the *napA* mutant strain increased significantly ($p < 0.05$). At a Na^+ concentration of 250 mM, none of the bacteria were detected. Next, the involvement of the *napA* gene in the Na^+ concentration under acidic conditions was examined. In the wild-type strain, bacterial CFU decreased significantly as the Na^+ concentration increased under acidic condition (pH 5.4). However, more than that, the *napA* mutant strain showed a significant decrease in CFU under acidic condition (pH 5.4). These results were suggested the loss of Na^+/H^+ antiporter activity. At a Na concentration of 250 mM, none of the bacteria were detected. We also investigated the involvement of the *napA* gene when the Na^+ concentration increased under basic conditions. In the wild-type strain, no significant decrease in CFU was observed at a Na^+ concentration of 150 mM under basic condition (pH 8.0), but a significant decrease in CFU was confirmed at a Na^+ concentration of 200 mM under basic condition (pH 8.0) ($p < 0.05$). Even if the Na^+ concentration under basic conditions was 100 mM, the CFU of the mutant was 1/1000 or less of that of the wild-type strain, and at a Na^+ concentration of 150 mM or more under basic condition (pH 8.0), the bacteria could hardly be confirmed. At a Na^+ concentration of 250 mM, none of the bacteria were detected.

3.4. Motility assay

We investigated whether Na^+/H^+ antiporter was associated with motility ability. Single-colony motilities of wild-type and mutant strains were measured in motility of BB 0.4% agar plate. The wild-type strain formed diffuse colonies with large swarming halos (Figure 5(A)). However, as the *napA* mutant formed dense colonies, the suppression effects on bacterial motility in *napA* mutant strains were found (Figure 5(B)). Compared with wild-type strain, *napA* mutant strain had less than 1/10 the motility diameter of bacteria ($p < 0.01$) (Figure 5(C))

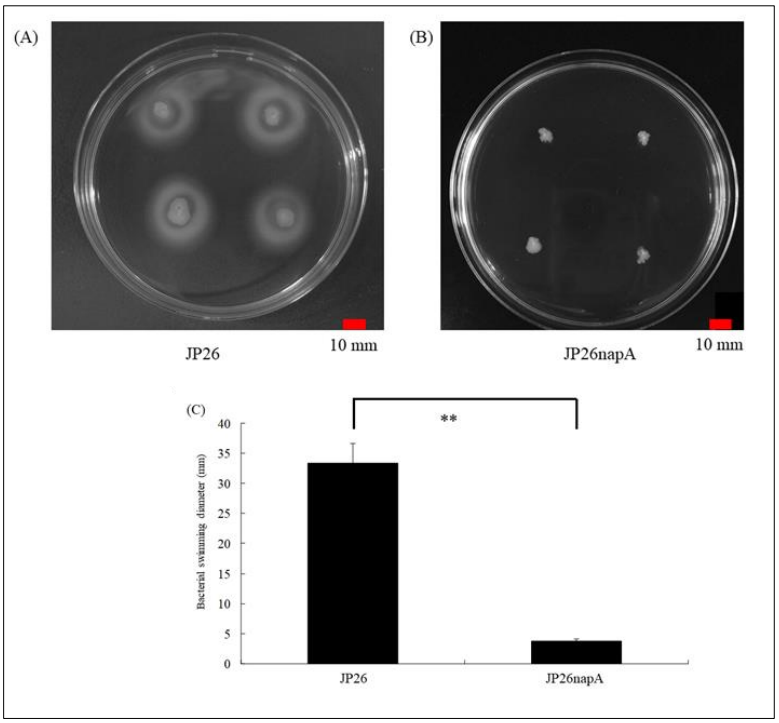


Figure 5 Comparison of bacterial motility between JP26 and JP26napA

(A) Motility photo of JP26, (B) Motility photo of JP26napA, (C) Bacterial motility diameter shows movement distance of bacteria. **: $p < 0.01$

3.5. Confirmation of flagellin proteins.

We checked the existence of flagellin proteins both wild-type and mutant strain. The flagella of wild-type and *napA* mutant strains were analyzed by SDS-PAGE to determine whether the difference in bacterial motility was due to changes in flagella. However, there was no difference in the protein bands of the flagella (Figure 6).

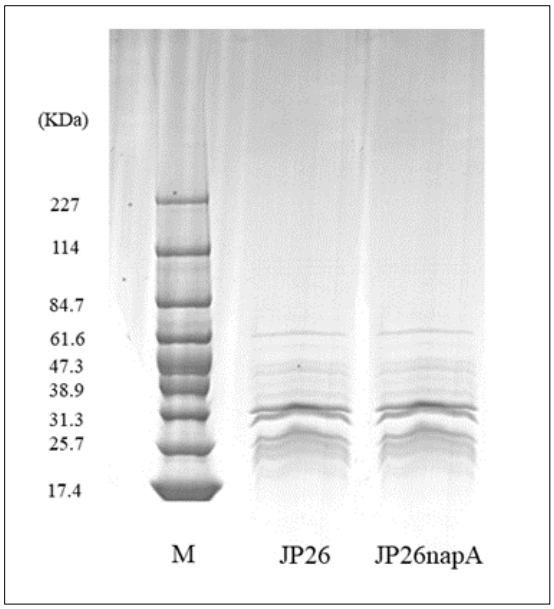


Figure 6 Comparison of flagellin proteins between JP26 and JP26napA

M: protein marker

4. Discussion

In this study, we investigated the *napA* gene in *H. pylori*. The *napA*-disrupted mutants were more sensitive to Na^+ concentration than wild-type strains. This effect was most pronounced in the neutral pH range. In addition, the *napA* mutant showed little movement on motility agar, despite having apparently normal flagella. These results indicate that the *napA* gene is closely related to the Na^+/H^+ exchanger and likely encodes a Na^+/H^+ antiporter.

Na^+ and H^+ are the most common ions and play a major role in the physiological functions of cells [16]. Both are most important for the bioenergetics of the cell, and too high or too low a concentration of these ions turns them into powerful stressors for all cells [16]. Therefore, all cells have a very efficient homeostasis mechanism for these ions [16]. A protein that plays a major role in this homeostasis mechanism is the Na^+/H^+ antiporter [17]. Na^+/H^+ antiporters are membrane proteins that exchange Na^+ and H^+ [17]. In addition to their ability to exchange ions on the cell membrane, they have the unique property of being involved in homeostasis [18]. In most bacterial and plant cells, Na^+/H^+ antiporters are involved in maintaining cytoplasmic pH homeostasis and in the elimination of Na^+, which is toxic to the cytoplasm at high concentrations [18]. In bacteria, Na^+ excretion is also essential for maintaining the inward Na^+ gradient that drives many transport systems [19]. Since Na^+/H^+ antiporters are involved in bacterial motility, it is possible that the flagellum of *H. pylori*, like the flagellum of *E. coli*, moves not only by Na^+ motive force but also by H^+ motive force [20]. Our results suggest that NapA may be one of the motility-related proteins.

Limitations of this study are discussed. Wild-type *H. pylori* cells have at least two Na^+ / H^+ exchanger systems, NhaA and NapA [4, 10]. The *napA* gene does not show significant homology to any of the *E. coli nhaA*, *nhaB*, or *chaA* genes [4]. NhaA expresses antiporter function under general pH conditions, whereas NapA expresses antiporter function only under neutral conditions [5]. Thus, NapA is a member of a new class of membrane proteins. The *napA* gene is widely conserved among clinical *H. pylori* isolates and is thought to regulate cellular homeostasis. Considering the importance of another Na^+/H^+ antiporter, NhaA, Repeated attempts to inactivate *nhaA* gene on *H. pylori* chromosomes by allelic exchange with a copy of the gene interrupted by a kanamycin resistance cassette have failed, indicating that *nhaA* is an essential gene in *H. pylori*. However, little is known about the basic mechanisms and factors underlying the Na^+/H^+ antiporter in *H. pylori*, and future studies, including the relationship between NhaA and NapA in *H. pylori*, are awaited.

5. Conclusion

The NapA is the novel Na^+ / H^+ antiporter associated with the regulation of motility of *H. pylori*. Further studies on the Na^+/H^+ antiporter of *H. pylori* may provide more detailed information on the mechanism of the bacterial Na^+/H^+ antiporter.

Compliance with ethical standards

Acknowledgments

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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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