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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2016.10.005

The impacts of a *fliD* mutation on the biofilm formation of *Helicobacter pylori*

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ARTICLE INFO

Article history:
Received 17 Mar 2016
Received in revised form 18 May 2016
Accepted 22 Jun 2016
Available online 17 Oct 2016

Keywords:
Helicobacter pylori
Biofilm
Flagella
fliD

ABSTRACT

Objective: To investigate the impact of the *fliD* gene on the biofilm formation of *Helicobacter pylori* (*H. pylori*).

Methods: *H. pylori fliD* mutant was constructed using inverse PCR mutagenesis. The mobility of the bacteria and its adhesion ability to human epithelial cells were assessed using a motility assay and a fluorescein isothiocyanate staining adhesion assay, respectively. The formation of biofilm was evaluated using a pellicle assay and a crystal violet staining assay. The cyto-architecture of the biofilm was documented with scanning electron microscopy.

Results: It was found that there was no significant difference in the levels of bacterial adhesion and the biofilm formation between the wild-type ATCC 43504 and the *fliD* mutant. Apart from a poor motility, the *fliD* mutant had a slightly delayed formation of its biofilm and an incomplete cyto-architecture of its biofilm. The bacterial cells residing in the biofilm of the *fliD* mutant showed a loose accumulation with less apparent cross-linking fibrils. Most of the mutant cells had truncated flagella.

Conclusions: This study provides the preliminary evidences that *fliD* potentially regulates biofilm formation and is required for the motility of *H. pylori*. Further studies need to be performed in order to develop *fliD* as a novel target for vaccine or antimicrobial agent in future.

1. Introduction

Half of the world's population, especially in developing countries, is colonized by the microaerophilic, spiral-shaped bacterium *Helicobacter pylori* (*H. pylori*) [1,2]. Without treatment, the bacterial infection can develop into various upper gastrointestinal disorders, such as chronic gastritis, peptic ulcer disease, gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer [3,4]. The bacteria were recognized as the first bacterial carcinogens and were classified by the International Agency for Research on Cancer, which is a part of World Health Organization, as a class I carcinogen in 1994 [5].

Flagella-driven movement is important for the initial colonization of the stomach mucosa and is needed for full infection [6]. Flagella also play an important role in swarming, adhesion and biofilm formation by many bacteria [7–9]. To start the process of biofilm formation, some bacteria contact surfaces using flagella, resulting in cell-to-surface adhesion involving negative electrostatic reactions. Thus, flagella are thought to be one of the initial factors used by some bacterial species for cell-to-surface adhesion and biofilm formation [10].

H. pylori has an alternative life style as a biofilm. This bacterium enables to form biofilms both *in vitro* and *in vivo*, including in the human body [9,11,12]. The biofilm formation of *H. pylori* seems to be a protective strategy for the bacteria.

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Foundation Project: Supported by the Thailand Research Fund (MRG5580161), the Research Fund Project (ASHCU57002), the Faculty of Allied Health Sciences at Chulalongkorn University and the Graduate School Thesis Grant of Chulalongkorn University.

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

For example, *H. pylori* living in biofilms can survive the host immune defense mechanisms and conquer environmental stresses, such as the highly acidic conditions in the human stomach and reactive oxygen species from phagocytic cells. Furthermore, biofilms promote bacterial survival of antimicrobial drug treatment, resulting in drug resistance, therapeutic failure and chronic infections in human [11–13].

The *fliD* gene encodes a 76 kDa flagellar capping protein called FliD or HAP2. The *fliD* operon of *H. pylori* contains the *flaG*, *fliD*, and *fliS* genes that are under the control of a 28-dependent promoter [14]. The HAP2 protein controls the polymerization of endogenous flagellin, which forms a filament [15]. Based on studies of mice infected with an *H. pylori fliD* mutant, the FliD protein is an important protein for the *in vivo* colonization and the formation of functional flagella [14,16].

Biofilm growth is most likely an important virulence factor in some bacteria and the specific molecular mechanisms controlling the formation of those bacterial biofilms have been thoroughly explored. However, in *H. pylori*, the precise molecular mechanisms of biofilm formation are still unclear. Various genes that are important for the biofilm formation by other bacteria do not seem to be involved in the biofilm formed by *H. pylori* [17]. A proteomic analysis recently reported that the flagellar protein complex, which includes FliD, is up-regulated in *H. pylori* during the mode of biofilm growth, compared with the expression of the complex during the planktonic growth mode [18]. Thus, the FliD protein may be an important factor for the formation of the biofilm of *H. pylori*. In the present study, a *H. pylori fliD* mutant was constructed to investigate the mutant's *in vitro* biofilm formation in comparison with that of the wild-type. The biofilm architectures of both bacteria were assessed using scanning electron microscopy. Flagella not only control bacterial motility but also promote surface adhesion [8], and both processes are related to the formation of biofilms by other bacteria [10,19]. Thus, our study also appraised the motility and the adhesive activity of the *fliD* mutant.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *H. pylori* strain ATCC 43504 (American Type Culture Collection, Manassas, VA, USA) was grown on brain-heart infusion (BHI) agar (Oxoid Limited, Cheshire, UK) supplemented with 7% (v/v) sheep blood at 37 °C for 3 days under microaerobic conditions (N_2: 85%, O_2: 5%, CO_2: 10%) using a gas generating kit (Mitsubishi, Japan).

2.2. PCR amplification for H. pylori fliD gene

H. pylori chromosomal DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's instructions. The chromosomal DNA of *H. pylori* was used as the template for the amplification of *fliD* by PCR using the following *fliD* gene-specific primers: *fliD*F forward primer 5′-GCTACCAATGGCGAAGTGAT-3′ and *fliD*R reverse primer 5′-CTTTTTGGATTGCGGTGTTT-3′. The PCR amplification was performed in 100 μL reaction mixtures containing 400 ng of the DNA template, 50 pmol of each specific gene primer, 200 μmol/L of each deoxynucleotide triphosphate, 1× PCR buffer, 25 μmol/L $MgCl_2$, and 1 IU of *Taq* DNA polymerase under following conditions: 35 cycles of amplification (94 °C for 15 s, 50 °C for 1 min, and 72 °C for 1 min) and a final extension at 72 °C for 7 min. Sterile distilled water was used as the negative control. The amplified PCR product was analyzed by electrophoresis using a 1.5% agarose gel containing ethidium bromide.

2.3. Construction of H. pylori isogenic fliD mutant

A defined isogenic *H. pylori* ATCC 43504 mutant was constructed as described previously with some modification [20]. Briefly, the *fliD* amplified product was introduced into a pGEM® T-Easy vector (Promega Corporation, Madison, WI, USA) and then transformed into *Escherichia coli* DH5α competent cells (New England Biolabs Inc., Ipswich, MA, USA). The transformants were grown on Luria-Bertani agar containing 100 μg/mL of ampicillin at 37 °C for 16 h. The positive colonies were distinguished by a blue/white colony screening assay. An 18-bp deletion and a unique *Bgl*II site were introduced into the cloned *fliD* gene fragment using inverse PCR mutagenesis with the following primers: In *fliD* forward 5′-GGAGATCTATCCACGCTCACTAA-3′ and In *fliD* reverse 5′-GGAGATCTTAGACTCGGTTGTCT-3′. The mutagenesis product was inserted into a 1-kb kanamycin resistant (kan^R) cassette with a *Bgl*II restriction site (kanamycinresistant cassette: Tn903) [21], synthesized from GeneArt® (Invitrogen, Grand Island, NY, USA) in the same orientation as the mutated gene. The constructed plasmid was introduced into *H. pylori* ATCC 43504 using a natural transformation. A double-crossover mutant was selected after 3–5 days of growth on complete BHI agar containing 20 μg/mL kanamycin. Successful recombination was assessed using PCR amplification with the *fliD* gene-specific primers and gene sequencing (Bioneer Sequencing Service, South Korea).

2.4. Motility assay

Three-day old colonies of wild-type *H. pylori* and the *fliD* mutant were suspended in BHI broth, and the final bacterial concentrations were adjusted equivalently for an optical density of 0.2 at 600 nm. An amount of 3 μL of the bacterial suspensions was spotted onto soft agar plates containing 0.3% (w/v) agar, 5% (v/v) heat-inactivated fetal bovine serum and 2.8% (w/v) *Brucella* broth (Oxoid Limited, Cheshire, UK) [22]. The plates were incubated at 37 °C under microaerobic condition for 5 days. The diameter of the area of outward migration was measured using vernier calipers. The results are expressed as the mean of the area over the zone of migration (in mm^2). The experiments were performed in duplicate on three separated occasions.

2.5. Adhesion assay by fluorescein isothiocyanate (FITC) staining

The ability of *H. pylori* to adhere to HEp-2 cells was assessed by FITC staining. The HEp-2 cells at 3×10^6 cells/well in complete Dulbecco's modified Eagle medium (DMEM) (Gibco, Carlsbad, CA, USA) were incubated in 6-well plates (Nunc™, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with sterile coverslips at the bottom of each well. The cells were left to adhere for 24 h at 37 °C under an atmosphere of 5% CO_2. The

HEp-2 cells were washed twice with Dulbecco's phosphate buffered saline (Gibco, Carlsbad, CA, USA). Three-day old colonies of wild-type *H. pylori* and *fliD* mutant were suspended in DMEM, and the final bacterial concentrations were adjusted equivalently to an optical density of 0.2 at 600 nm. The bacterial suspensions were then incubated with 0.1% (w/v) FITC (Sigma–Aldrich Chemical Co., St. Louis, MO, USA) and suspended in dimethyl sulfoxide for 1 h under microaerobic condition at 37 °C in the dark. The FITC-labeled bacteria were washed three times with DMEM containing 0.1% Tween 20 (Fisher Scientific, Fair Lawn, NJ, USA) using centrifugation. The HEp-2 cells were co-cultivated with 0.1% (w/v) FITC-labeled *H. pylori* at a density of 3×10^8 CFU/mL per well for 2 h at 37 °C under an atmosphere of 5% CO_2. Subsequently, the co-cultures were washed three times with Dulbecco's phosphate buffered saline. The number of *H. pylori* cells adhering to the HEp-2 cells was measured using a microplate reader (Biotek Synergy Mx, USA) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The wild-type strain values represented the control of 100% adhesion, and the mutant strain was calculated as the percent adhesion of the control. The experiments were performed in duplicate on three separate occasions.

2.6. Biofilm culture condition

The *H. pylori* biofilm was established *in vitro* using a pellicle formation assay as previously described [18,23]. Briefly, 3-day-old colonies of wild-type *H. pylori* and the *fliD* mutant were suspended in 10 mL of BHI broth supplemented with 2% (w/v) β-cyclodextrin (Sigma–Aldrich Chemical Co., St. Louis, MO, USA) in sterile glass test tubes. The final bacterial concentrations were adjusted equivalently to an optical density of 0.2 at 600 nm. The test tubes were left to stand for 7 days without shaking under microaerobic conditions at 37 °C.

2.7. Examination of biofilm formation

The biofilm characteristics were described as follows: freely floating bacterial cells that formed at an air-liquid interface were designated as a pellicle, and bacterial cells attached over the interior surface of glass test tube at an air-liquid interface were denoted as an attached biofilm. The development of the biofilm was examined as a blind test and scored daily over 7 days of incubation, as described by our previous study [23]. The levels of biofilm formation, either as a pellicle or an attached biofilm, were scored as follows: (−) neither form of biofilm was present; (+) a thin pellicle or a finely attached biofilm; (++) an accumulated pellicle or a thinly attached biofilm; or (+++) a mature pellicle covering the entire liquid surface or a dense attached biofilm. Experiments were performed in duplicate on three separate occasions.

2.8. Quantification of biofilm formation

The *H. pylori* biofilm was quantified using a crystal violet staining technique as previously described [23]. The broth suspension was removed after allowing the formation of the biofilm over 7 days. The glass-attached biofilm was rinsed twice with phosphate buffer solution (PBS) and dried at 60 °C for 30 min. The attached biofilm was stained with 0.1% (w/v) crystal violet for 5 min at room temperature, washed with PBS three times, and dried at 60 °C for 15 min. The bound crystal violet was eluted with ethanol/acetone at 80:20 (v/v) for 1 min. The solution was transferred into 96-well plates, and the absorbance at 570 nm was measured using a spectrophotometer (Biotek Synergy Mx, USA). The level of the biofilm formation was determined by subtracting the mean optical density (OD) value of the blank from the value of the test samples. The BHI broth supplemented 2% (w/v) β-cyclodextrin served as a blank control. Experiments were performed in duplicate on three separate occasions.

2.9. Scanning electron microscopic analysis

In order to observe the 3-dimensional structure among the different ages of bacterial biofilm, the bacteria were grown for 1–7 days using a pellicle formation assay as described above. After a defined incubation period, the culture broth was filtered through a Whatman® grade No. 1 filter paper to isolate the pellicle for biofilm structural analysis using scanning electron microscopy (SEM). If no apparent pellicle was observed, the planktonic cells were collected instead. The SEM was performed by the Scientific and Technological Research Equipment Center, Chulalongkorn University, Thailand. Briefly, the isolated pellicle was transferred to a vial, washed with PBS to remove the loosely attached bacteria, and fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol/L PBS at pH 7.2. The samples were successively dehydrated through a graded series of ethanol washes with a 10-min incubation per step as follows: 30%, 50%, 70%, 90% and 100% thrice (v/v). The samples were dried, coated with gold-palladium, and were examined with SEM (JEOL, Japan). Three-day old colonies of wild-type *H. pylori* and the *fliD* mutant grown on complete BHI agar were also collected for SEM analysis.

2.10. Statistical analysis

The differences in the means of the results of the motility assay, the adhesion assay, and the crystal violet staining technique between wild-type *H. pylori* and the *fliD* mutant were analyzed by student's *t*-test. The probability value of $P \leq 0.05$ was considered significantly different.

3. Results

3.1. Construction of H. pylori fliD mutant by inverse PCR mutagenesis

Our study focused on the *fliD* gene, which encodes the filament capping protein known to control the polymerization of endogenous flagellin into a filament. To investigate the possible role of the *H. pylori fliD* gene in biofilm formation, a *H. pylori fliD* mutant was constructed by allelic replacement. The kan^R cassette containing the kanamycin phosphotransferase gene that confers kanamycin resistance was cloned into the unique *Bgl*II site situated in the middle of the *fliD* gene engineered by inverse PCR mutagenesis. The constructed plasmid was transformed into the wild-type *H. pylori* by a natural transformation. PCR analysis using the primers *fliD*F and *fliD*R

confirmed that a double recombination event had successfully occurred. PCR experiments with the primers *fliD*F and *fliD*R consistently amplified a single band of 292 and 1 292 bp from the wild-type *H. pylori* and the *fliD* mutant chromosomal DNA, respectively. Sequencing analysis confirmed that a 1.29-kb fragment contained the 1-kb kan^R cassette.

3.2. In vitro characteristics of the H. pylori fliD mutant

When grown on complete BHI agar, the colony morphology and the growth rate of the *fliD* mutant showed no significant difference when compared with the wild-type strain. The impact of the *fliD* mutation on cell motility was investigated through observations of the cell movement through *Brucella* soft agar plates. The area over the zone of migration was measured after an incubation period of 5 days, and the results are presented in units of mm^2. The zone of migration of the *fliD* mutant (28.56 mm^2) was significantly smaller than the wild-type *H. pylori* strain (49.83 mm^2) ($P = 0.013$).

The adhesion of *H. pylori* to a surface seems to be the initial step for the biofilm formation of bacteria. A cell-to-cell adhesion assay was used to investigate the adhesive properties of the two *H. pylori* strains. The FITC-labeled wild-type strain and the *fliD* mutant strain were separately incubated with human HEp-2 cells. The adhesive activity of the wild-type cells was shown as 100%, and the percent adhesion of the *fliD* mutant relative to the wild-type control was 97.75%. The mutation had no significant effect on the ability of *H. pylori* to attach to the human cells.

3.3. Biofilm formation by the fliD mutant

The biofilm formation at the air-liquid interface observed in the culture tube has two distinct characteristics that were designated as a pellicle or an attached biofilm. Figure 1 shows the pellicle and the attached biofilm formed by the wild-type *H. pylori* strain and the *fliD* mutant strain after 7 days of culture. The biofilm development of the wild-type strain was first observed on Day 3 and quickly became extensive on Day 4 (Table 1). The biofilm formation of the *fliD* mutant was delayed by 1 day for both the pellicle and the attached biofilm. Both strains of *H. pylori* produced extensive biofilms at the end of the incubation period. Moreover, after 7 days of incubation, the adherent bacterial community considered as a biofilm was examined quantitatively by staining with crystal violet, and the absorbance was measured at 570 nm. The level of the formation of the biofilms was expressed as the mean of the blank OD at 570 nm subtracted from the mean of the tested ODs at 570 nm. The mutation had no significant effect on the biofilm production of *H. pylori*. The mean absorbance values obtained from the *fliD* mutant and the wild-type strain were 0.414 ± 0.051 and 0.442 ± 0.040, respectively.

The culture test tubes displayed the mature biofilms. Two biofilm characteristics were observed at the air-liquid interface, including the pellicle (white arrow) and the attached biofilm (black arrow). The score of both types of biofilms shown is (+++) extensive.

Table 1
Biofilm formation by the wild-type and *fliD* mutant *H. pylori* strains cultured as measured by the pellicle formation assay and examined over 7 days.

Days	Wild-type ATCC 43504		*fliD* Mutant	
	Pellicle	Attached biofilm	Pellicle	Attached biofilm
1	–	–	–	–
2	–	–	–	–
3	+	+	–	–
4	+++	+++	+++	+++
5	+++	+++	+++	+++
6	+++	+++	+++	+++
7	+++	+++	+++	+++

–: Neither form of biofilm was present; +: Thin pellicle or a finely attached biofilm; ++: Accumulated pellicle or a thinly attached biofilm; +++: A mature pellicle covering the entire liquid surface or a densely attached biofilm. Experiments were performed in duplicate on three separate occasions.

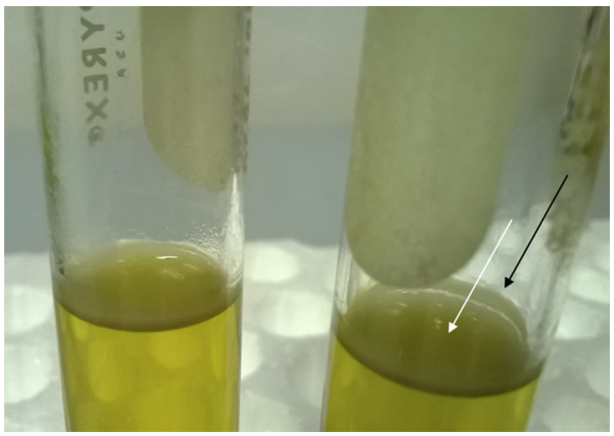

Figure 1. Photograph of Day 7 biofilm formed by wild-type and *fliD* mutant *H. pylori*.

3.4. SEM analysis

The 3-dimensional structure of the *H. pylori* biofilm was analyzed using SEM. The bacterial morphology of both wild-type (Figure 2A–G) and *fliD* mutant *H. pylori* strains (Figure 2H–N) changed from bacilli to cocci in the older biofilm cultures. There was no biofilm formation for the wild-type strain at Days 1 and 2 (Figure 2A, B) and for the *fliD* mutant strain at Days 1–3 (Figure 2H–J), which agrees with the results found in Table 1. The pellicle architectures of the wild-type strain at Days 3–7 (Figure 2C–G) were a dense accumulation of bacteria within an amorphous extracellular matrix that seemed to connect with each other through cross-linking fibrils. Although the biofilm architecture of the mutant strain appeared slightly dissimilar when compared with that of the wild-type strain, the fibril connections were lesser and had a loose accumulation (Figure 2K–N). Moreover, the wild-type cells were more rounded and more clumped (Figure 2E, F), whereas the *fliD* mutant cells were slightly dispersed and evenly distributed (Figure 2L, M). Many mature flagella were observed among the wild-type cells (Figure 3A), while fewer amounts of flagella with truncated fragments were observed among the *fliD* mutant cells (Figure 3B).

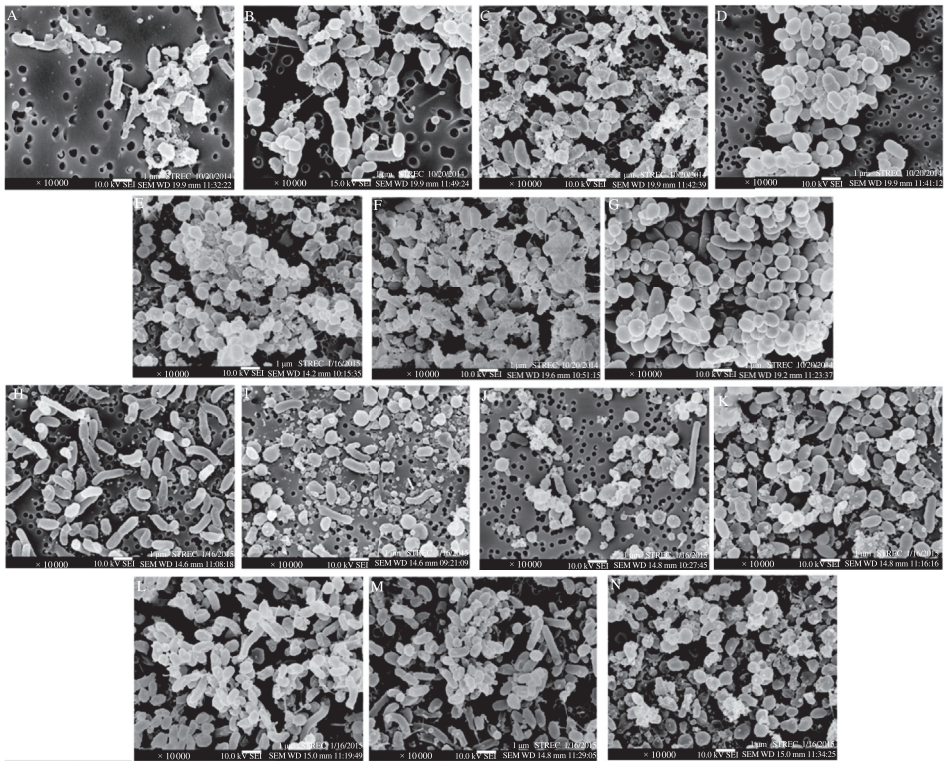

Figure 2. Scanning electron microscopic photomicrographs of the pellicle biofilm development of the wild-type and *fliD* mutant *H. pylori* strains at a magnification of 10000×.
Planktonic cells taken from the wild-type strain from cultures at (A) Day 1 and (B) Day 2; C–G: The pellicle cells of the wild-type strain taken from cultures at Days 3–7, respectively; H–J: The planktonic cells of the *fliD* mutant strain taken from cultures at Days 1–3, respectively; K–N: The pellicle cells of the *fliD* mutant strain taken from cultures at Days 4–7, respectively. Bars: 1 μm.

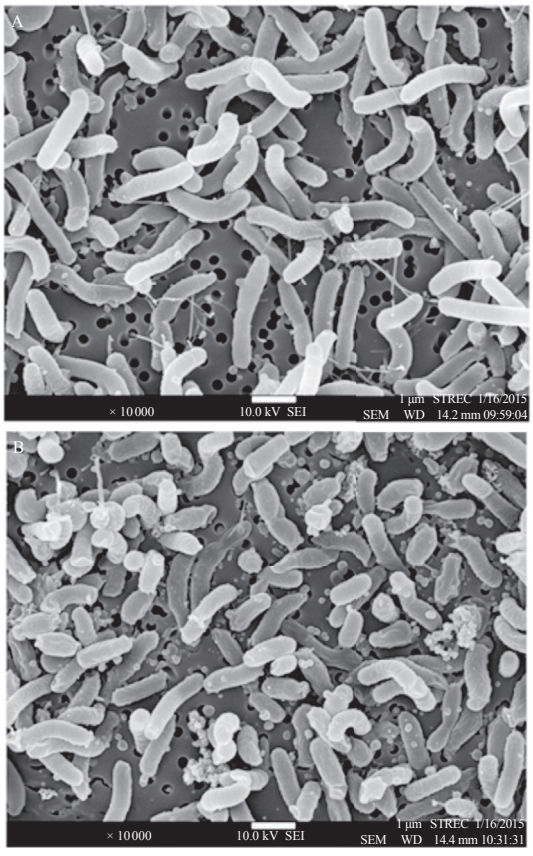

Figure 3. Scanning electron microscopic photomicrographs of wild-type and *fliD* mutant *H. pylori* cells at a magnification of 10000×.
Bacterial cells of (A) the wild-type strain and (B) the *fliD* mutant strain from 3-day-old colonies derived from complete BHI agar. Bar: 1 μm.

4. Discussion

Bacteria in the natural environment usually live in complex communities of microorganisms by attaching and proliferating on various surfaces and producing a matrix containing extracellular polymeric substances to embed themselves in, called biofilms [11]. There are similar processes of biofilm formation in many bacteria and *H. pylori*. The bacteria initially attach irreversibly or reversibly to surfaces. Then, the bacterial cells proliferate to form microcolonies within their own biofilms. The growth of biofilms can be either the proliferation of the bacteria within the biofilm or the attachment of other free living or planktonic cells, whereas the single cells can disperse from the biofilm and form new biofilms elsewhere, depending on the presence of suitable condition, including proper nutrient sources or attachment surfaces [11,12]. Thus, the adherence to a surface is always one of the initial steps in the formation of biofilms. The bacteria use their flagella to make contact with the surfaces, leading to cell-to-surface adhesion [8,10]. In many bacteria, flagellin acts as a bacterial adhesin that is required for its adhesion to epithelial cell [24]. Flagella are not restricted to only the control of bacterial motility but are also involved in the adhesion and biofilm formation of *Vibrio* spp., *Escherichia coli*, and *Aeromonas* spp. [19,25,26]. The molecular mechanisms of many bacteria that control biofilm formation have been explored. Poly-*N*-acetylglucosamine, synthesized by the *icaADBC* operon has been shown to be required for the biofilm production of *Staphylococcus aureus* and *Staphylococcus epidermidis* [27,28]. A quorum sensing system regulates the biofilm formation of *Vibrio cholerae* by directly controlling the expression of the extracellular polymeric substances biosynthesis genes, including *hapR* and *vpsR* [29].

However, the specific molecular mechanisms that control the biofilm formation of *H. pylori* remain unclear.

The *fliD* gene is important for flagellin polymerization during the flagellar biosynthesis of bacteria [30]. FliD, or HAP2, also known as a distal capping protein, is encoded by the *fliD* gene and localizes to the end of the flagellar filament. FliD plays a role in promoting the polymerization of flagellin subunits by capping the flagellin monomers at the distal end of the filament [31]. *Salmonella typhimurium* mutant strains that lack FliD were found to be non-motile and produce unassembled filament protein. The polymerization of flagellin is recovered when the FliD protein was added exogenously to the *fliD* mutant, resulting in the construction of complete flagella [32,33]. In contrast, *Vibrio parahaemolyticus fliD* mutant strains reveal different phenotypes. The *fliD* mutant strains showed slow motility on a semisolid plate [34]. In a study done by Kim *et al.*, the *fliD* gene was assessed for a regulatory role in the motility and colonization of the strain in gastric mucosa [14]. It was found that the *H. pylori* KCTC0217BP isogenic *fliD* mutant strain possessed atypical flagellar morphogenesis and incomplete flagellar elongation with an absence of the terminal bulb at the end of the flagellin, resulting in non-motility [14]. The *fliD* mutant strain had fragile, short flagella and truncated flagellar fragments [35]. In our study, the *fliD* mutant *H. pylori* strain likely had truncated flagella. Although it still retained its motility, a mutation of the *fliD* gene seemed to significantly diminish the movement of *H. pylori*.

The *fliD* gene was found to be involved in mucin adhesion by *Pseudomonas aeruginosa* (*P. aeruginosa*). The *P. aeruginosa fliD* mutant strain was not able to adhere to mucin, but when the *fliD* gene was reinserted, the adhesive phenotype is restored [36]. In addition, FliD is an important factor for the adhesion of *Clostridium difficile* [37] and the colonization of gastric mucosa of the host mice by *H. pylori* [14]. However, our data showed that when tested with an *in vitro* adherence model, the mutation of the *fliD* gene had no significant effect on the adhesion of *H. pylori* to HEp-2 cells compared with the adhesion of the wild-type strain. A previous study demonstrated that *H. pylori* strains with mutations in *flaA* and/or *flaB* did not have impaired adherence to gastric epithelial cells [38]. While the *fliD* gene encodes for the flagellar capping protein, *flaA* and *flaB* encode for the major flagellar components. The results of a previous study combined with the results of our present study suggests that the genes involved in the regulation of the *H. pylori* structural components may not play a role in promoting the adherence of *H. pylori* to epithelial cells.

The *fliD* gene may contribute to the biofilm formation of many bacteria. Some evidence shows that the *fliD* gene expression is highly up-regulated during the early stage of the biofilm formation of *P. aeruginosa* [39]. In *Cronobacter sakazakii*, a mutation of the *fliD* gene dramatically reduced the amount of biofilm, which was only 9% of the wild-type amount as detected by the crystal violet assay [35]. FliD has been found to be up-regulated in mature *H. pylori* biofilm cells in comparison with their planktonic counterparts [18]. In this present study, the *H. pylori fliD* mutant strain showed no significant reduction in the amount of biofilm as determined by the crystal violet assay compared with that of the wild-type strain. Both strains of *H. pylori* produced extensive amounts of biofilm at the end of the incubation period, when the biofilms were stained by the crystal violet. Nevertheless, the pellicle or the attached biofilm of the *fliD* mutant strain were 1 day slower in their formation than those observed for the wild-type strain. The inactivation of the flagellar genes, including *fliA*, *flaA*, *flaB*, and *flaG*, result in a delay in the pellicle formation of *Campylobacter jejuni* [40]. Because of this, we suggest that the *fliD* gene may play a critical role in promoting pellicle formation as well as in the initial attachment of *H. pylori* to a solid surface during biofilm formation. We noticed that there was a correlation between the level of pellicle and the attached biofilm during biofilm formation. Although a quantification of biofilm formation by a crystal violet staining assay was performed only with the attached biofilm compartment, this could be a representative level of biofilm in this study.

The biofilm structures of both wild-type and *fliD* mutant *H. pylori* strains were documented by SEM. The development of the biofilm formation of *H. pylori* mimics our previous report, beginning with individual bacteria adhering to the abiotic surface, its extension into microcolonies and the formation of a 3-dimensional structure [23]. SEM revealed that the wild-type biofilm consisted of a dense accumulations of cells covering with an abundant extracellular matrix and cross-linked fibrils, which is considered as a hallmark of a mature biofilm structure [17]. On the contrary, a mutation of the *fliD* gene affected the *H. pylori* biofilm at a cyto-architecture level. The *fliD* mutant biofilm had loosely aggregated cells with a small amount of extracellular matrix and fibril connections. We postulate that the *fliD* gene may play a role in the maturation of the biofilm structure in *H. pylori*.

Our study concludes that the *fliD* gene is implicated in *H. pylori* biofilm development and structure. Protection against *H. pylori* biofilm infection will provide prophylactic benefits, particularly in developing countries with high prevalence of *H. pylori* infection. FliD protein may be a candidate as a novel target for drug or vaccine development. It is known that biofilm formation of bacteria depends on the stimulation or suppression by environmental factors or gene complexes, which are involved in adhesion, quorum sensing, and stress response [41]. Further study is required in order to elucidate a precise mechanism regulating biofilm formation in *H. pylori*.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was funded by the Thailand Research Fund (MRG5580161), the Research Fund Project (ASHCU57002), the Faculty of Allied Health Sciences at Chulalongkorn University and the Graduate School Thesis Grant of Chulalongkorn University. The HEp2-cells were kindly provided by Professor Dr. Pornthep Tiensiwakul. We would like to thank Professor Dr. Kathleen L. McCoy (Department of Microbiology and Immunology, School of Medicine, Virginia Commonwealth University) for her critical reading of this manuscript.

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