



FlgN plays important roles in the adhesion of *Aeromonas hydrophila* to host mucus

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ABSTRACT. Adhesion to the host mucus is a crucial step in the early infection stage of pathogenic bacteria. To investigate the mechanisms of the adhesion of *Aeromonas hydrophila* to its host mucus, a mutant library was constructed using the mini-Tn10 transposon mutagenesis system. Of 276 individual colonies, the mutant strain with the most attenuated adhesion ability in this study was screened out and designated A77. Molecular analysis showed that a 414-bp sequence flanking mini-Tn10 in A77 had the highest identity (97%) with the bacterial flagellar protein gene *flgN*. A complemented strain *flgN*⁺ was constructed and the biological characteristics of the wild-type, mutant A77, and complemented *flgN*⁺ strains were investigated. The results showed that the decreased abilities of motility, adhesion to mucus, and biofilm formation in the mutant strain were partially recovered in the complemented *flgN*⁺ strain, which suggested that *flgN* plays an important role in the adhesion of *A. hydrophila* to its host.

Key words: Adhesion; *Aeromonas hydrophila*; *flgN*; Mini-Tn10Km; ELISA

INTRODUCTION

Pathogen adhesion to the cells and mucosal surfaces of the host is a critical step in the early infection process (Acord et al., 2005). For many pathogenic bacteria, it is only when they adhere to sensitive cells through adhesins they produce toxins in the focus of infection and eventually penetrate the tissues of the host. For this reason, bacterial adhesion has been regarded as a virulence factor of pathogenic bacteria (Chhatwal, 2002). In recent decades, the mechanisms of adhesion of many clinical pathogens to their hosts have been elucidated. Studies on *Burkholderia pseudomallei* demonstrated that the *pilA* deletion mutant strain JAB16 showed lower adhesion to human epithelial cell lines than the wild type strain *B. pseudomallei* K96243, and that *Caenorhabditis elegans* infected with JAB16 survived significantly longer than those infected with K96243 (Essex-Lopresti et al., 2005). Kelly et al. (2004) studied the adhesion of *Candida albicans* and found that a *CaACE2* gene knock-out caused a two-fold decrease in adhesion to 96-well polystyrene plates, and that female mice infected with the wild type strain survived only for 5-7 days, while those infected with the *CaACE2* gene knock-out mutant strain remained alive after 28 days.

Although adhesion is an important virulence factor for pathogens, most studies on bacterial adhesion have focused on clinical pathogenic bacteria; hardly any fish pathogens have been studied to uncover the mechanisms of their adhesion.

Aeromonas hydrophila is a widespread representative of *Aeromonas* and is found in water, water inhabitants, and in domestic animals and food. It is not only responsible for several kinds of diseases of farmed and feral fish including hemorrhagic ulceration and hemorrhagic septicemia, but is also responsible for gastrointestinal and extra-intestinal infections of humans; for example, acute diarrheal disease, bacteremia, and meningitis, among others (Daskalov, 2006). Several structures or molecules have previously been demonstrated to be related to the pathogenicity of *A. hydrophila*, such as toxins, proteases, outer membrane proteins, lipopolysaccharide, and motility proteins, among others (Merino et al., 1996; Gavín et al., 2003; Khajanchi et al., 2009; McCoy et al., 2010; Khushiramani et al., 2012). However, the adhesion of *A. hydrophila* is not well understood. Pianetti et al. (2012) once evaluated the adhesion properties of a *A. hydrophila* strains isolated from fish following prolonged storage at refrigerated and room temperatures in the presence of different NaCl concentrations, and using microscopic analysis, determined that adhesive capacity decreased over time with an increase NaCl concentrations. However, there have been no reports on the mechanisms of adhesion of *A. hydrophila*. In this study, we explore the role of genes related to *A. hydrophila* adhesion through construction of mutant and complemented strains, which will contribute to a better understanding of the adhesion mechanisms of *A. hydrophila*.

MATERIAL AND METHODS

Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Table 1. *A. hydrophila* strains were routinely grown overnight in Luria-Bertani (LB) broth at 28°C, unless otherwise indicated. *Escherichia coli* strains were grown in LB at 37°C. The semi-soft medium used for motility assays was LB plus 0.3% agar. The medium was supplemented with the appropriate antibiotics

at the following concentrations when required: 100 µg/mL kanamycin (Km); 50 µg/mL ampicillin and 50 µg/mL streptomycin (Sm); or 25 µg/mL chloromycetin.

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Characteristic(s)	Source or reference
Strains		
<i>A. hydrophila</i>		
W	Wild-type strain (Sm ^R), isolated from diseased eel	This study
A77	Mini-Tn10Km insertion mutant deficient in adhesion (Sm ^R Km ^R)	This study
flgN+	A77 complementation with intact <i>flgN</i> gene (Km ^R Cm ^R)	This study
<i>E. coli</i>		
Sm10	<i>thi thr leu tonA lacY supE recA</i> RP4-2-Tc::Mu::Km (λpir)	Herrero et al. (1990)
DH _α	F' <i>endA hdsR17</i> (r _k ⁺ m _k ⁺) <i>supE44 thi-l recA1 gyr-A96 80lacZ</i>	Hanahan (1983)
Plasmids		
pLOFKm	Ap ^R ; Tn10-based delivery plasmid with Km ^R	Herrero et al. (1990)
pACYC184	Cloning vector, Tc ^R Cm ^R	Altarriba et al. (2003)
pACYC184-flgN	pACYC184 with complete <i>flgN</i> gene, Cm ^R	This study

General DNA methods

Plasmid DNA was isolated using a plasmid DNA extraction kit (Omega Bio-Tek, Norcross, GA, USA). *A. hydrophila* chromosomal DNA isolation was carried out according to the instructions of the bacterial DNA kit (TaKaRa Bio, Otsu, Shiga, Japan). DNA restriction digestions and T4 ligations were carried out according to manufacturer's instructions. DNA samples were separated on 1% agarose gels; when required, DNA extraction from gels was carried out using a Gel Extraction Kit (Omega Bio-Tek).

Insertional mutagenesis of *A. hydrophila*

The mini-Tn10Km transposon was introduced into *A. hydrophila* on the suicide vector pLOFKm carried by *E. coli* Sm10 through a filter mating technique developed by Herrero et al. (1990) with minor modifications. Briefly, 0.22 µm filters with a 1:4 mixture of the donor strain, *E. coli* Sm10 (pLOFKm) carrying the mini-Tn10Km, and the recipient strain, *A. hydrophila*, were incubated for 4 h at 28°C on LB plates supplemented with 3 mM IPTG. The filters were transferred into Eppendorf tubes containing 1 mL LB and vortexed. A 100-µL suspension aliquot was spread onto LB plates supplemented with 600 µg ampicillin and 100 µg streptomycin per mL to select for *A. hydrophila* carrying the mini-Tn10Km transposon. Following incubation at 28°C for 24 h, single colonies were selected for analysis in the adhesion assay. All colonies were further investigated with an indirect ELISA assay.

Adhesion assay

The adhesion assay was performed by the method of indirect ELISA using 96-well microtiter polystyrene plates, as previously described with some modifications (Collado et al., 2005). Briefly, 100 µL sterilized eel skin mucus was immobilized into each well by incubation overnight at 4°C. The negative control was sterile phosphate buffer saline (PBS). After incubation, the plates were washed twice with PBS to remove unbound mucus, and 100-µL bacterial suspension was added into the wells and immobilized by incubation for 2 h before

being washed three times by PBS plus Tween 20 and dried at 60°C. The plates were blocked with 200- μ L fetal calf serum for 1 h at 37°C and washed in PBS-T. Subsequently, 100 μ L sera raised in rabbits against the antigen *A. hydrophila* was diluted to 1:100 with PBS and added to homologously antigen-coated wells, incubated at 37°C for 1 h, and then washed three times in PBS-T. Rabbit anti-goat horseradish peroxidase (HRP) conjugate at a dilution of 1:1000 was added to each well and incubated at 37°C for 1 h; the wells were then thoroughly washed and 50 μ L substrate solution [4 mg O-phenylenediamine tetrahydrochloride and 20 μ L H₂O₂ (30%, v/v) in 10 mL acetate buffer, pH 5.0] was added. The plates were incubated at 37°C for 30 min in a dark chamber and finally the optical density (OD) was recorded at 492 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Molecular analysis of the mutant

The DNA sequence flanking the transposon mutants was determined using thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). The arbitrary primer was supplied by the Genomic Walking Kit (TaKaRa Bio), and the nested primers specific to the transposon are listed in Table 2. Among the nested primers, LSP4-6 were used to amplify upstream sequences flanking the transposon, and RSP4-6 were used to amplify the downstream sequences flanking the transposon. TAIL-PCR products were purified using a Gel Extraction Kit (Omega Bio-Tek), after which they were cloned into pMD18-T (TaKaRa Bio) and sequenced. DNA sequences were analyzed by BLASTn (National Center for Biotechnology Information, Bethesda, MD, USA) and other softwares such as ClustalW and MegAlign (DNASStar).

Table 2. Specific primers used in TAIL-PCR.

Primer	Sequence
LSP4	5' ATGCTTGATGGTCGGAAGAGGC 3'
LSP5	5' CATCGGGCTTCCCATAACAATCG 3'
LSP6	5' ATTATCGCGAGCCCATTTATAACC 3'
RSP4	5' CCTGTTGAACAAGTCTGGAAAGAAATG 3'
RSP5	5' GATCTTGCCATCCTATGGAAGCTG 3'
RSP6	5' TTACGCTGACTTGACGGGACGG 3'

Construction of the complemented strain (*flgN*+))

The mutant *flgN* gene was amplified using the primers NF: 5'-GGA TCC GAC CTC GTC CTC CGA CAA CC-3' and NR: 5'-GCA TGC CTT ACT AGA GGC TAG CAT AAT CAG GAA CAT CAT ACG GAA GGC CTT GAT GCC GAT GGA GC-3', where enzyme restriction sites are underlined, and the hemagglutinin (HA)-tag sequence is italicized. The PCR product was ligated to the pMD18-T vector (TaKaRa Bio) and introduced into *E. coli* DH5 α . Single bacterial colonies were inoculated into 5 mL LB medium containing 50 mg/mL ampicillin with shaking overnight at 37°C. Recombinant plasmid was extracted and digested with *Bam*HI and *Sph*I. The digested products were subcloned into the expression vector pACYC184 previously digested with the same endonuclease, and the final construct was named pACYC184-*flgN*. Plasmid pACYC184-*flgN* was independently introduced into the mutant strain A77 by electroporation. Transconjugants were selected on chloromycetin plates. The expression of FlgN was detected by western blot according to supplier's instructions.

Western blot

Whole extracts of the wild-type and complemented strains were resuspended in 0.5 mL loading buffer. Samples were heated for 10 min at 95°C and subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins resolved by SDS-PAGE were transferred to polyvinylidene fluoride membranes (GE Healthcare, Little Chalfont, UK) and blocked overnight with PBS with 1% milk powder (w/v) at 4°C with gentle agitation. Blots were probed with the anti-HA tag (1:3000) and a goat HRP-conjugated secondary anti-mouse antibody (Sigma) (1:5000), and then developed using ECL Plus (Ruisai, Shanghai, China).

Motility assay

Freshly grown bacterial colonies were stabbed into the center of test tubes filled with semi-soft medium (LB base, 0.3% agar) and incubated at 28°C for 16-24 h to assess motility.

Biofilm formation

Quantitative biofilm formation was performed in a microtiter assay as described previously with minor modifications (Pratt and Kolter, 1998). Briefly, overnight cultures were diluted 1:100 into fresh broth. Then 100 µL aliquots were added on a 96-well microtiter plate (polystyrene) and incubated in humidified conditions for 48 h at 30°C without shaking. To remove any unattached bacteria, the microtiter plates were rinsed thoroughly with sterile PBS and surface-attached bacteria were dried for 30 min at 60°C, and subsequently stained with 1.0% crystal violet for 10 min. After solubilization with 33% acetic acid, the OD was recorded at 590 nm using a microplate reader (Bio-Rad).

Statistical analysis

All data were statistically analyzed using analysis of variance tests in SPSS17.0 (SPSS, Chicago, IL, USA).

RESULTS

Screening of adhesion-defective mutants

A collection of 276 random insertion mutants of *A. hydrophila* was obtained using mini-Tn10Km mutagenesis. Each mutant in the library was subjected to the adhesion assay to identify adhesion. The mutant strain with the most attenuated adhesion ability in this study was designated A77. The adhesion assay showed that the adhesion ability of A77 reduced approximately 50% compared to the wild-type strain (Figure 1).

Sequence analysis of the *A. hydrophila* mini-Tn10 insertion site

Sequence analysis showed that the 414-bp sequence flanking mini-Tn10 in A77 shared the highest identity (97%) with a flagellar protein *flgN* gene of *A. hydrophila* ATCC 7966 (accession No. CP000462.1) and with the FlgK/FlgL-specific chaperone (*flgN*) gene

of *A. hydrophila* AL09-73 (accession No. Q216196.1). Analysis of the *flgN* gene of the *A. hydrophila* wild-type strain showed that this gene contains a 414-bp open reading frame and encodes a FlgN protein which is predicted to have 137 amino acids of approximately 15 kDa molecular weight.

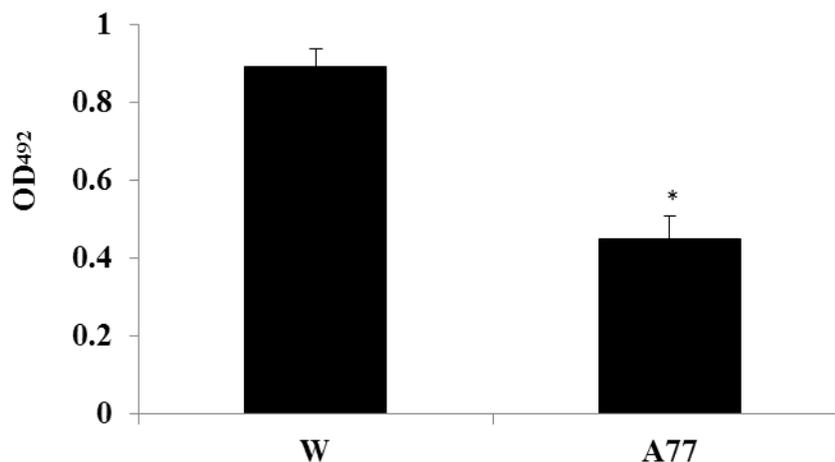


Figure 1. Adhesion of wild-type and mutant strain of *Aeromonas hydrophila* to fish mucus. Results are reported as the optical density (OD) recorded at 492 nm in a microplate reader (mean and SD). *Significant difference at $P < 0.05$ among adhesion difference from wild-type strain. All values are the average of three independent experiments.

Western blot

To detect the expression of the pACYC184-*flgN* vector in the complemented strain *flgN*⁺, western blot analysis was performed. The results demonstrated that the FlgN protein was detectable in the strain *flgN*⁺, which confirmed that the FlgN protein can be expressed in the complemented strain (Figure 2).

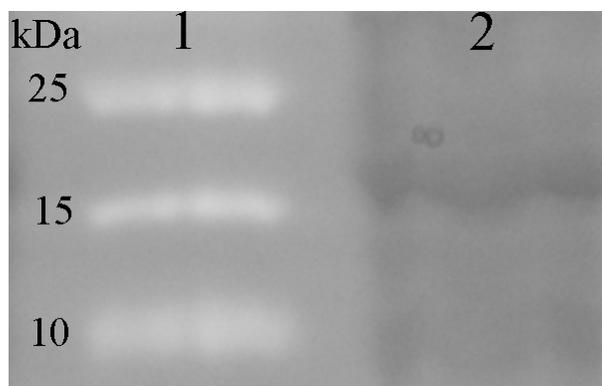


Figure 2. Western blot analysis of the complementation strain. Lane 1 = protein markers; lane 2 = purified protein.

Bacterial motility

The bacterial motility assay showed that all the strains migrated from the line of inoculation causing turbidity in the semi-soft medium. However, the mutant strain A77 displayed defective motility when compared with the wild type and complementation strains (Figure 3). This result suggests that mutation in *flgN* can markedly attenuate bacterial motility.



Figure 3. Motility of the wild-type, the mutant and the complementation in semi-soft medium.

Adhesion assay

The wild-type strain, the mutant strain A77, and the complemented strain *flgN+* all demonstrated adhesion properties. The results showed that the number of adhesive bacteria of the mutant strain exhibited an approximate 75% reduction compared to the wild type strain, and that the numbers of adhesive bacteria of the complemented strain had recovered over 80% those of the wild-type strain (Figure 4). These data suggested that mutation in *flgN* significantly affected bacterial adhesion.

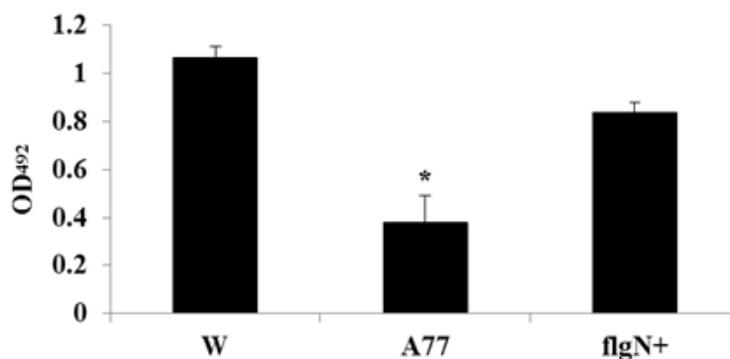


Figure 4. Adhesion of *Aeromonas hydrophila* wild-type, mutant and complementation strains to skin mucus of eel. Results are reported as the optical density (OD) recorded at 492 nm in a microplate reader (means and SD). *Significant difference from wild-type strain ($P < 0.05$). All values are the average of three independent experiments.

Biofilm formation

Solid surface-associated biofilm formation of these bacteria was also evaluated. The results revealed that the biofilm formed by the mutant strain was only about 35% of that formed by the wild-type strain as measured by optical density, while the biofilm formed by the complemented strain was restored to nearly 90% of that formed by the wild-type strain. These data suggested that mutation in *flgN* can attenuate bacterial biofilm formation (Figure 5).

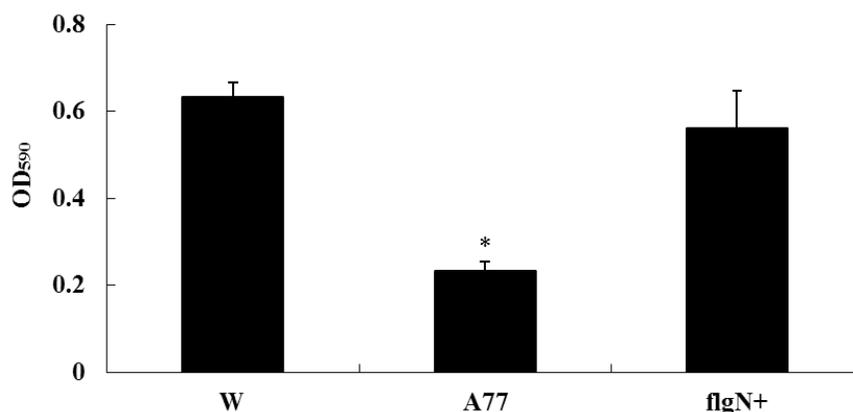


Figure 5. Biofilm formation of the *Aeromonas hydrophila* wild-type, mutant and complementation strains. OD₅₉₀ quantifies the amount of crystal violet retained by the biofilm on the microtitre plates after staining. The average of three independent experiments (each experiment performed in duplicate) is shown. Error bars represent standard deviations. *Significant difference from wild-type strain ($P < 0.05$).

DISCUSSION

Previous studies have shown that adhesion is one of the critical factors necessary for pathogenic bacteria to invade their host. In this study on *A. hydrophila* adhesion, we found that mutation in *flgN* led to defective motility, adhesion, and biofilm formation.

It is known that *A. hydrophila* expresses two flagellar systems, a single polar flagellum for swimming in all culture conditions and multiple lateral flagella for swarming on solid or semisolid media (Gavin et al., 2002; Canals et al., 2006a,b). Studies on *A. hydrophila* flagellar gene systems has revealed that 55 genes are involved in the polar flagellum and 38 genes are involved in the lateral flagella (Merino et al., 2006), and that over 20 genes are shared by these two flagellar systems, which include the *flgN* gene (Fraser et al., 1999). Further studies revealed that FlgN is an approximately 16-kDa protein composed of 140 amino acid residues, and acts as a flagellar type III export chaperone specific for the hook-filament junction proteins, FlgK and FlgL (Yeh and Klesius, 2011). FlgN is also a translational regulator of the anti-sigma factor FlgM, and thus is predicted to play an important role in fine-tuning the flagellar assembly process (Minamino et al., 2012). Loss of FlgN reduces the initiation of filament assembly and the number of flagellar filaments, but not the stability of mature filaments, resulting in a motile but non-swarming mutant of *Proteus mirabilis* (Gygi et al., 1997). Disruption of the *flgN* gene of *Salmonella* significantly abolished the motility of the pathogen (Minamino

et al., 2012). These results suggest that FlgN plays different roles in the motility of different bacteria. In this study, FlgN mutation led to significantly defective motility in *A. hydrophila*, which suggested that FlgN plays an important role in its motility.

The data in our study also revealed that defective motility directly affected the adhesion and biofilm formation of *A. hydrophila*. This might be due to the close relationship between motility and adhesion and biofilm formation.

It is well known that there are different kinds of adhesins that exist on the bacterial surface, which permit the bacteria to recognize many different elements of host surfaces (Pizarro-Cerdá and Cossart, 2006). It is only when bacteria find the adaptors on the host surfaces that they can adhere to their host. Flagellar motility is necessary for bacteria adhesion and colonization, as has been confirmed by previous studies (Gavín et al., 2002). Rabaan et al. (2001) found that single mutations of *flaA* and *flaB* resulted in decreased adhesion of approximately 50%, and that mutation of *flaH*, *flaJ*, or both flagellin genes resulted in the complete loss of adhesion. Mutation in the *flgN* gene of *A. hydrophila* AH-3 showed an approximately 80% reduction in adhesion to HEp-2 cells compared to their wild type strain (Canals et al., 2006a). In our study, mutation in *flgN* led to an approximately 50% reduction in adhesion to host skin mucus. We interpreted this to mean that the bacteria utilize flagellar motility to search for the adaptors on host surfaces; if their motility is impaired, it becomes difficult for the bacteria to find the appropriate binding sites for adhesion.

It has also been well understood that the first step of biofilm formation is the initial adhesion to the solid surface (O'Toole et al., 2000). Therefore, flagellar motility and adhesion would also play an important role in the biofilm formation of many bacteria. Lemon et al. (2007) demonstrated that flagellum-mediated motility is critical for *Listeria monocytogenes* adhesion and biofilm formation. Mutation of *A. hydrophila* AH-3 FlgN also resulted in a 62% reduction in the ability to form biofilm compared with their wild type strains (Canals et al., 2006a). These data are similar to the result in our study that mutation in FlgN led to an approximately 65% reduction in bacterial biofilm formation.

Adhesion and biofilm formation are both involved in bacterial virulence, which has been demonstrated in *Gardnerella vaginalis*, *Staphylococcus epidermidis*, *E. coli*, and other bacteria (Cerca et al., 2005; Saldaña et al., 2009). Therefore, we believe that FlgN mutation might also attenuate the virulence of *A. hydrophila* through reduction of bacterial motility, adhesion, and biofilm formation.

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