**Pseudomonas fluorescens**
Filamentous Hemagglutinin, an Iron-Regulated Protein, Is an Important Virulence Factor that Modulates Bacterial Pathogenicity

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**INTRODUCTION**

For many bacterial pathogens, attachment to host tissues by various adhesins is the critical step in the process of infection (Pizarro-Cerda and Cossart, 2006). Adhesins are a large family of proteins that includes fimbrins (Romantschuk, 1992; Ojanen-Reuhs et al., 1997; Shakhnovich et al., 2007), type IV pili (Roine et al., 1998; Poole et al., 2007), flagella (Roy et al., 2009), trimeric autotransporter adhesins (Mil-Homens and Fialho, 2012; Mikula et al., 2013), and filamentous hemagglutinin (FHA).

Filamentous hemagglutinin is an important adhesin present both in a secreted and surface-associated form in *Bordetella* (Weiss and Hewlett, 1986; Locht et al., 1993; Jacob-Dubuisson et al., 2000). One FHA that has been extensively characterized is from *Bordetella pertussis*. It is secreted in a Sec-dependent manner by the two-partner secretion (TPS) system, which includes an
outer membrane-associated accessory protein interacting with FHA N-proximal secretion domain. Functionally, it has proapoptotic activity and promotes bacterial aggregation (Locht et al., 1993, 2001; Romero et al., 2014). In Xanthomonas axonopodis pv. citri, a FHA-like protein is required for tissue colonization, surface attachment, cell-to-cell aggregation and biofilm formation (Gottig et al., 2009). Another FHA-like protein in Acinetobacter baumannii plays a role in virulence in a mouse lethal model of infection, promoting biofilm formation and mediating the adhesion of A. baumannii to epithelial cells (Astaneh et al., 2014). Apart from its role as an adhesin, FHA of B. pertussis and Bordetella bronchiseptica also possesses immunomodulatory properties which may contribute to subversion of host innate and adaptive immunity (Abramson et al., 2001; Braat et al., 2007; Julio et al., 2009; Henderson et al., 2012; Romero et al., 2014).

Pseudomonas fluorescens is a Gram-negative bacterium existing widely in soil, water, plant, and animals. In aquaculture, it is a common pathogen for shrimp and a wide range of fish species (Swain et al., 2007; Wang et al., 2009). In addition, P. fluorescens can also infect humans and is known to cause outbreaks of bacteremia (Gershman et al., 2008). Unlike environmental P. fluorescens from water and soil, pathogenic P. fluorescens from fish have been studied on a very limited base. In this study, with an aim to gain new insight into the infection mechanism of P. fluorescens, we investigated the function of P. fluorescens FHA in an infection model of turbot (Scophthalmus maximus).

MATERIALS AND METHODS

Ethics Statement

Experiments involving live animals were conducted in accordance with the “Regulations for the Administration of Affairs Concerning Experimental Animals” promulgated by the State Science and Technology Commission of Shandong Province. The study was approved by the ethics committee of Institute of Oceanology – Chinese Academy of Sciences.

Bacterial Strains and Growth Conditions

Pseudomonas fluorescens TSS is a pathogenic fish isolate that has been reported previously (Wang et al., 2009). Escherichia coli BL21(DE3) and DH5α were purchased from TransGen Biotech (Beijing, China). E. coli S17-1 λpir was purchased from Biomedal. All strains were grown in Luria-Bertani broth (LB) at 37°C (for E. coli) or 28°C (for TSS). Where indicated, 2,2′-dipyridyl (Sigma, St. Louis, MO, USA), tetracycline, and chloramphenicol were added at the concentrations of 600 μM, 20 and 50 μg/ml, respectively.

Two-Dimensional Gel Electrophoresis (2-DE), MALDI-TOF/TOF-MS and Protein Identification

Two-dimensional gel electrophoresis, MALDI-TOF/TOF-MS, and protein identification were performed as reported previously (Liu et al., 2015).

Sequence Analysis

The sequence of P. fluorescens fha has been reported previously (GenBank accession no. WP_014719704.1). The amino acid sequence was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the conserved domain search program of NCBI. Subcellular localization prediction was performed with the PSORTb v.3.0 server.

Construction of TSSfha and TSSΔfha

In order to create TSSfha, we first created the plasmid p705T, which was used for gene mutagenesis, as follows: the tetracycline-resistance gene of p704T (Hu et al., 2009) was cut out by SmaI/Scal double digestion and inserted into pGP704 (Miller and Mekalanos, 1988) at the Scal site. To construct the Pffha-defective strain TSSfha, the internal fragment of Pf fha (positions 241–408) was amplified by PCR with the primer pairs F (5′-AGATCTGTGTGTTGAAACAAGGCC-3′, underlined sequence, BglII site) and R (5′-AGATCTATCGGGCGGCTGGC GAA-3′, underlined sequence, BglII site). The PCR product was inserted into the suicide plasmid p705T at the compatible BglII site, resulting in p705Fha. S17-1 λpir was transformed with p705Fha, and the transformant was conjugated with P. fluorescens TSS as described previously (Sun et al., 2009). The transconjugant was selected on LB agar plates supplemented with tetracycline and chloramphenicol, and one of the resistant clones was named TSSfha. Mutation of Ffha in TSSfha was confirmed by PCR in addition, single-copy plasmid insertion in TSSfha was further confirmed by the quantitative real-time PCR (qRT-PCR) method described previously (Zhang et al., 2014).

To construct TSSΔfha, in-frame deletion of a 582 bp segment (positions 40–621) of Ffha was performed by overlap extension PCR as follows: the first overlap PCR was performed with the primers F2 (5′-CCCCGGGAACGTGCTACAAAGACGT-3′, underlined sequence, Smal site) and R2 (5′-CGACCTCTCCTGG GGTGAAAGGTGGA-3′), the second overlap PCR was performed with the primers F3 (5′-CACCCAAGAAGGTGG CCTAGTCTGGC-3′) and R3 (5′-CGCGGGGTTGATGCTG CTTGGTTCG-3′, underlined sequence, Smal site), and the fusion PCR was performed with the primer pair F2/R3. The PCR products were inserted into the suicide plasmid p7TS (Wang et al., 2009) at the Smal site, resulting in p7TSFha. p7TSFha was introduced into S17-1 λpir (Biomedal, Spain) by transformation. The transformant S17-1 λpir/p7TSFha was conjugated with TSS. The transconjugants were selected first on LB plates supplemented with tetracycline and chloramphenicol and then on LB plates supplemented with 12% sucrose and chloramphenicol. The colonies that appeared on the plates were analyzed by PCR, and the PCR products were subjected to sequence analysis to confirm deletion of Ffha.

Adhesion to FG Cells

FG-9307 cells, a cell line established from Japanese flounder gill cells (Tong et al., 1997), were cultured at 22°C in 96-well cell culture plates (∼10^5 cell/well) with L-15 medium (GIBCO,
Invitrogen, Carlsbad, CA, USA) as described previously (Tong et al., 1997). TSS and TSSfha were cultured in LB medium to an OD$_{600}$ of 0.8 and were resuspended in L-15 medium to $1 \times 10^7$ CFU/ml. One hundred microliter of bacterial suspension was added to FG cells cultured as above. The plates were incubated at 28°C for 1, 2, and 4 h, followed by washing three times with PBS. FG cells were then lysed with 1% Triton X-100, and 50 µl lysate was plated in triplicate on LB agar plates. The plates were incubated at 28°C for 48 h, and the colonies that appeared on the plates were enumerated. The genetic identity of the colonies was verified by PCR and sequence analysis of selected PCR products. The experiment was performed three times.

Autoaggregation and Production of Extracellular Matrix

For autoaggregation analysis, TSS and TSSfha were cultured at 28°C in test tubes containing LB broth in a shaking incubator to an OD$_{600}$ of 0.8. The tubes were removed from the shaker, and 100 microliters of cell suspension were added into 96-well microplates and incubated overnight. Meanwhile, the static cultures in the test tubes were monitored for sedimentation for 48 h. Bacterial autoaggregation in microplates and glass tubes were then examined. For microscopy, TSS and TSSfha were cultured as above, 100 microliters of cell suspension were added into a 12-well plate with embedded coverslips for 8 h at 28°C. Autoaggregation of cells on coverslips were photographed with a scanning electron microscope (S-3400N, Hitachi, Japan; Wang and Sun, 2015). For extracellular matrix analysis, TSS and TSSfha were cultured as above, 100 microliters of cell suspension were added into a 12-well culture plate with embedded coverslips for 20 h at 28°C. The cells were then observed with a scanning electron microscope. All experiments were performed three times.

Motility Assay and Flagella Formation

To measure motility, TSS, TSSfha, and TSSAfha were cultured in LB medium to an OD$_{600}$ of 1.0, and 5 µl cell suspension were spotted onto the center of fresh swimming plates containing LB medium plus 0.3% (w/v) agar. The plates were then incubated at 28°C. Two days later, the motility of the bacteria was assessed by examining the diameter of the motility halo on the soft agar. To measure flagella formation, the bacteria were cultured in LB agar plates at 28°C for 20 h and examined with a transmission electron microscope (JEM-1200, Jeol, Japan) as reported previously (Sun and Sun, 2016). The assays were performed three times.

Biofilm Formation Assay

Quantitative biofilm formation on polystyrene surfaces (96-well microtiter plates) was investigated as reported previously (Wang et al., 2013a). Briefly, $10^7$ CFU of TSS, TSSfha, and TSSAfha were placed into a sterile 96-well flat-bottomed tissue culture plate and incubated at 28°C for 12 h. After incubation, the plates were washed to remove unbound cells, and the bound cells were stained with crystal violet. Quantification of the bound cells was performed by measuring the dissolved crystal violet at OD$_{570}$ after the addition of 100 µl 30% acetic acid. To quantify biofilm formation in glass tubes, the bacteria were cultured as above and the cultures in tubes were incubated under static conditions for 48 h at 28°C. Then the culture mediums were removed and the glass tubes were washed three times with PBS. Subsequently, 5 ml of 1% crystal violet dye was added to glass tube for 15 min at room temperature. Following, the tubes were washed three times with phosphate buffered saline (PBS). After addition of 5 ml of 30% acetic acid for 15 min at room temperature to solubilize the crystal violet, the glass tubes were allowed to dry and then photographed. The experiment was performed three times.

Hemagglutination Assay

TSS and TSSfha cultured as above were resuspended in PBS to $2 \times 10^9$, $2 \times 10^8$, $2 \times 10^7$, and $2 \times 10^6$ CFU/ml respectively. Turbot red blood cells were collected, washed three times in PBS, and resuspended to a final concentration of 2% (v/v). Fifty microliters of bacterial suspension or PBS (control) was mixed with 50 µl of turbot erythrocytes in a 96-well microtiter V-bottom plate. The plate was incubated for 1 h at room temperature. Hemagglutination was detected by visual inspection as reported previously (Astaneh et al., 2014). A small pellet of erythrocytes at the bottom of the well after incubation was considered negative as against positive reactions exhibiting an even sheet of erythrocytes across the wells. The experiment was performed three times.

Serum Survival Assay

Serum survival analysis was performed as reported previously (Wang et al., 2013b).

Tissue Infection and Mortality Assay

Clinically healthy turbot (average 13.6 g) were purchased from a local fish farm. The fish were maintained as reported previously (Sun and Sun, 2015). For tissue dissemination and colonization analysis, TSS, TSSfha, and TSSAfha were cultured in LB medium to an OD$_{600}$ of 0.8. The cells were washed with PBS and resuspended in PBS to $10^8$ CFU/ml. Turbot were divided randomly into four groups ($N=15$) and infected by intramuscular (i.m.) injection with 50 µl of TSS, TSSfha, TSSAfha, or PBS. Kidney and spleen were taken from the fish at 12, 24, and 48 h post-infection (5 fish/time point). The tissues were homogenized in PBS with an OSE-20 electrotissue homogenizer (Tiangen, Beijing, China). The homogenates were diluted serially in PBS and plated in triplicate on LB agar plates. After incubation at 28°C for 48 h, the colonies that appeared on the plates were counted. The genetic identity of the colonies was verified as above. For mortality assay, turbot were infected with TSS, TSSfha, and PBS as above, and the fish were monitored for mortality over a period of 20 days. All experiments were performed three times.

Purification of Recombinant Proteins and Antibody Preparation

The plasmids pETFha, which expresses recombinant Pf$_{fha}$ (rFha), was constructed as follows. Pf$_{fha}$ containing the hemagglutination activity domain (positions 1–684) was amplified by PCR with the primer pairs F (5′-GATATCGATGCGACTATGCCGACT-3′) and R (5′-GATATCGATGCGACTATGCCGACT-3′) and subcloned into the pET28a(+) vector. The resulting plasmids, pETFha, were then purified from E. coli BL21 DE3 cells using a plasmid purification kit (Qiagen, USA). The recombinant protein was expressed in E. coli BL21 DE3 cells by inducing the expression with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 4 h. The cells were harvested by centrifugation and lysed with 1% Triton X-100. The resulting lysate was then applied to a nickel affinity column (Qiagen, USA) and eluted with a gradient of imidazole. The purified recombinant protein was then dialyzed against PBS and stored at -80°C.

The purified recombinant protein was used to purify antibodies. Turbot sera were collected from fish that were infected with TSS, TSSfha, or PBS, and the sera were incubated with recombinant protein at 4°C overnight. The precipitated antibody was collected by centrifugation and dialyzed against PBS. The antibody was then affinity purified on a nickel affinity column and eluted with a gradient of imidazole. The purified antibody was then dialyzed against PBS and stored at -80°C.
The PCR product was ligated with the T-A cloning vector pEASY-T1 Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the PfFha-containing fragment, which was inserted into pET259 (Hu et al., 2010) at the SwaI site, resulting in pETFha. To purify rFha and the control protein rTrx, E. coli BL21(DE3; TransGen Biotech, Beijing, China) was transformed with pETFha and pET32a (Novagen, San Diego, CA, USA), the latter plasmid expresses Trx; the transformants were cultured in LB medium at 37°C to mid-logarithmic phase, and isopropyl-α-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growing at 16°C for an additional 16 h, the cells were harvested by centrifugation, and His-tagged rFha and rTrx were purified using Ni-NTA Agarose (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. The purified proteins were reconstituted, removed of endotoxin, and concentrated as described previously (Wang and Sun, 2015). Mouse antibody against rFha was prepared as reported previously (Sun and Sun, 2015). Control antibody from pre-immune mouse was also prepared. The antibody was purified using rProtein G Beads (Solarbio, Beijing, China). The specificity of the rFha antibody was determined by Western immunoblot as reported previously (Wang and Sun, 2015).

**Immunofluorescence Microscopy**

To detect PfFha on TSS, the bacteria were cultured in LB medium supplemented with 2,2′-dipyridyl as indicated above to OD_{600} 0.9; the cells were collected by centrifugation, washed with PBS, and resuspended in PBS to 1 × 10^8 CFU/ml. One hundred microliters of bacterial suspension (∼1 × 10^7 CFU) were seeded on a glass slide pre-treated with 0.001% polylysine (Sigma, St. Louis, MO, USA). For fluorescence microscopy, the samples were blocked with 1% bovine serum albumin for 2 h at 30°C and incubated with mouse anti-rFha antibody or control antibody from pre-immune mouse (1/500 dilution) for 1 h at 30°C. The cells were washed three times with PBST (0.05% Tween 20 in PBS) and incubated with goat anti-mouse IgG (1/1000 dilution; Bioss, Beijing, China) coupled to fluorescein isothiocyanate (FITC) for 1 h at 30°C. The cells were washed with PBST and stained with 4′,6-diamidino-2-phenylindole (DAPI) according to the instructions of the manufacturer (Bioss, Beijing, China). The cells were then analyzed with a Zeiss fluorescence microscope (Carl Zeiss Imager A2, Jena, Germany).

To examine interaction between rFha and FG cells, the cells were seeded on 0.001% polylysine-treated glass coverslips in 12-well cell culture plates; the cells were incubated with rFha or rTrx (100 μg/ml) at 22°C for 2 h and treated with paraformaldehyde (Sigma, St. Louis, MO, USA) at 22°C for 30 min. The cells were then treated with 1% bovine serum albumin at 4°C for overnight. The cells were incubated with mouse-anti His antibody (1/1000 dilution; Bioss, Beijing, China) for 1 h at 37°C. Subsequently, cells were washed three times with PBST and incubated with FITC-coupled goat-anti mouse IgG (1/1000 dilution; Bioss, Beijing, China) for 1 h at 37°C. After washing three times with PBST, the cells were stained with DAPI and subjected to microscopy as described above.

**Effect of Antibody Blocking on Bacterial Infection**

Turbot (as described above) were divided randomly into three groups (N = 15). TSS was cultured in LB medium to an OD_{600} of 0.8. The cells were washed and resuspended in PBS to 10^8 CFU/ml. One milliliter bacterial cells were mixed with 5 μl rFha antibody, control antibody, or PBS (control) and incubated at 28°C for 1 h. After incubation, 50 μl of the mixture was inoculated into turbot via i.m. injection. Kidney and spleen were taken from the fish (five at each time point) at 12, 24, and 48 h post-bacterial infection, and bacterial recovery from the tissues was determined as above.

**Immunization and Enzyme-Linked Immunosorbent Assay (ELISA)**

rFha was resuspended in PBS to a concentration of 200 μg/ml and mixed at an equal volume with aluminum hydroxide as described previously (Jiao et al., 2010). As a control, PBS was mixed similarly with aluminum hydroxide without protein. Turbot (as described above) were divided randomly into two groups (N = 55) and injected intraperitoneally with 50 μl of the protein mixture or the PBS control. At 1 month post-immunization, the fish were challenged with TSS at the dose of 5 × 10^6 CFU/fish. The fish were monitored for mortality for a period of 20 days. Dying fish were randomly selected for the examination of bacterial recovery from liver, kidney, and spleen. Relative percent of survival (RPS) was calculated according to the following formula: RPS = [1 – (% mortality in immunized fish/% mortality in control fish)] × 100. The immunization experiment was performed two times.

**Statistical Analysis**

With the exception of the immunization trial which was performed twice, all other experiments were performed three times, and statistical analyses were carried out with the SPSS 17.0 package (SPSS, Inc., Chicago, IL, USA). Except for the survival analysis in the immunization experiment and the in vivo infection experiment, for which logrank test was used, analysis of variance (ANOVA) was used for all other analyses. In all cases, the significance level was defined as P < 0.05.

**RESULTS**

**Identification and Characterization of PfFha**

In an effort to identify secreted proteins of TSS regulated by iron, TSS was cultured in the presence and absence of the iron chelator 2,2′-dipyridyl, and the extracellular proteins were examined by two-DE analysis. A total of 15 differentially expressed proteins were identified, of which six were significantly upregulated (ratio ≥ 2, P ≤ 0.05) in the presence of 2,2′-dipyridyl (Figure 1; Supplementary Table S1). PfFha was one of
FIGURE 1 | Representative 2-DE maps of the extracellular protein profiles of *Pseudomonas fluorescens* TSS cultured under different conditions. Extracellular proteins of *P. fluorescens* TSS cultured in the presence (B) and absence of 2,2'-dipyridyl (DP) (A) were subjected to 2-DE analysis. Numbers indicate protein spots with differential expression. The spot of PfFha (number 6) is indicated.

the upregulated proteins (Figure 1). Sequence analysis showed that PfFha contains an N-terminal signal peptide (residues 1–32) and a hemagglutination activity domain (residues 50–170; Supplementary Figure S1).

**In vitro Effect of PfFha Mutation**

**Cellular Motility, Flagella Formation, and Autoaggregation**

To examine the function of PfFha, a TSS mutant defective in PfFha was created based on insertion mutagenesis and named TSSfha. In addition, another PfFha-defective mutant, TSSAfha, was also created by markerless in-frame deletion. Compared to TSS, the motility of TSSfha in soft LB agar plate was almost abolished (Figure 2A). Similar observation was made with TSSAfha (Supplementary Figure S2A). Electron microscopy showed that while polar flagella were observed with the cells of TSS, no apparent flagella were observed with the cells of TSSfha (Figure 2B). Similar observation was made with TSSAfha (Supplementary Figure S2B). Cultured TSS cells were able to aggregate when left standing in the culture tube or being placed into a microplate, whereas, TSSfha failed to do so (Figure 3A). Consistently, scanning electron microscopy detected apparent aggregation of TSS cells but not TSSfha cells (Figure 3B).

**Biofilm and Extracellular Matrix Production**

Vegetative growth analysis showed that when cultured in LB medium, TSSfha was comparable to TSS in growth profile, while when cultured in LB medium supplemented with 2,2'-dipyridyl, TSSfha grew slightly slower than TSS at the logarithmic phase and reached similar cell densities to TSS at the stationary phase (Supplementary Figure S3). The growth profile of TSSAfha was very similar to that of TSSfha (Supplementary Figure S3). Biofilm growth showed that TSSfha exhibited significantly reduced capacity to form biofilm (Figure 4). Similar observation was made with TSSAfha (Supplementary Figure S4). Scanning electron microscopy revealed that when cultured TSS cells were left on a coverslip for 20 h, a matrix of networks formed by fiber-like structures was observed abundantly among the cells, whereas very little such structure was formed by TSSfha (Figure 5).

**Host Cell Adhesion, Hemagglutination, and Survival in Host Serum**

Immunofluorescence microscopy showed that PfFha was localized on the surface of the bacteria (Supplementary Figure S5). With this observation, we examined the potential involvement of PfFha in host cell adhesion. The results showed that when incubated with flounder FG cells, the numbers of host cell-bound TSS increased with time; in contrast, the numbers of
host cell-bound TSS/ha barely changed and were 10.8-, 17.2-, and 60.8-fold lower than those of host cell-bound TSS at 1, 2, and 4 h of incubation (Supplementary Figure S6). These results suggested a requirement of PfFha for host interaction. With this result, we further investigated whether PfFha could bind directly to host cells. For this purpose, FG cells were incubated with recombinant PfFha (rFha), which was purified as a His-tagged protein (Supplementary Figure S7), and cell-bound rFha was detected with immunofluorescence microscopy. The results showed that rFha was detected on FG cells (Figure 6). In contrast, when FG cells were similarly incubated with rTrx, the control protein purified under the same condition as rFha, no cell-bound protein was detected (Figure 6).

Hemagglutination analysis indicated that incubation of TSS with turbot red blood cells induced agglutination of the blood cells in a manner that depended on the concentration of TSS; in contrast, no apparent hemagglutination was caused by TSS/ha even at high concentrations (Figure 7). Serum survival analysis showed that following incubation with turbot serum for 1 h, TSS and TSS/ha exhibited survival rates of 53.8 ± 10.3 and 30.1 ± 3.9%, respectively, the latter being significantly (P < 0.05) lower than that of the former. Similar to TSS/ha, the survival rate of TSS/ha in turbot serum (28.3 ± 2.8%) was significantly (P < 0.05) lower than that of TSS.

**FIGURE 3** | Autoaggregation of *Pseudomonas fluorescens* TSS and TSS/ha. (A) TSS (left) and TSS/ha (right) were cultured in LB medium at 28°C to an OD<sub>600</sub> of 0.8; 100 microliters of cell suspensions were added into 96-well microplates and incubated overnight (Top), or the cell cultures were left standing for 2 days at room temperature without shaking (Bottom). (B) Autoaggregation observed by scanning electron microscope (SEM). TSS and TSS/ha were cultured in LB medium at 28°C to an OD<sub>600</sub> of 0.8; cell suspensions were added onto coverslips and observed by SEM after incubating for 8 h at room temperature. The pictures represent one of three independent experiments.

**FIGURE 4** | Biofilm formation of *Pseudomonas fluorescens* TSS and TSS/ha. (A) Image of crystal violet (CV)-stained biofilms formed inside glass tubes. Cells were grown in LB medium overnight at 28°C and then stood for 2 days at room temperature before staining with CV. Image shown is from one representative experiment of three independent experiments. (B) Quantitation of biofilm. Stained biofilms were dissolved in 30% acetic acid, and the optical density at OD<sub>570</sub> was recorded. Data are the means of three independent assays and presented as mean ± SEM. **P < 0.01.

**FIGURE 5** | Production of extracellular matrix structure by *Pseudomonas fluorescens* TSS and TSS/ha. TSS (A,B) and TSS/ha (C,D) were cultured in LB medium at 28°C to an OD<sub>600</sub> of 0.8; cell suspensions were added onto coverslips and observed by a scanning electron microscope after incubation for 20 h at room temperature. The results represent one of three independent experiments.

**FIGURE 6** | Binding of rFha to FG-9307 cells. FG cells were incubated with rFha (A,B) or the control protein rTrx (D,E), and the cell-bound protein was detected with FITC-labeled antibody. The cells were stained with DAPI and observed with a fluorescence microscope. (C) A merge of (A) and (B); (F) A merge of (D) and (E). Magnifications: 20 × 10, scale bars: 50 µm.
**In vivo Effect of PfFha Mutation**

In vivo infection assay showed that following inoculation into turbot, TSS disseminated into and multiplied in kidney and spleen, in which the numbers of TSS increased rapidly with time (Figure 8A). In contrast, in turbot inoculated with TSS/fha, the bacterial numbers recovered from kidney and spleen were significantly lower than that from TSS-infected fish at 12, 24, and 48 h post-infection. Similar results were obtained in repeated infection analyses with TSS, TSS/fha, and TSSA/fha in parallel, which showed that at 12, 24, and 48 h, the amounts of TSSA/fha in kidney and spleen were comparable to those of TSS/fha and were significantly lower than those of TSS (Supplementary Figure S8). Consistent with these observations, the survival rate of TSS/fha-infected fish (66.7%) was significantly higher than that of TSS-infected fish (6.7%; Figure 8B). To further investigate the importance of PfFha to host infection, turbot were infected with TSS in the presence of anti-rFha antibody, and bacterial dissemination in and colonization of kidney and spleen were subsequently determined. The results showed that at 12, 24, and 48 h post-infection, bacterial recoveries from the fish infected with TSS plus rFha antibody were significantly lower than those from the fish infected with TSS alone or with TSS plus control antibody (Figure 9).

**Immunoprotection Induced by rFha**

Since, as shown above, PfFha was a secreted protein essential to host infection, we examined the immunoprotective potential of rFha in a model of turbot. For this purpose, turbot were vaccinated with rFha and challenged with TSS at 1 month post-vaccination. The fish were subsequently monitored for mortality and survival. The results showed that the survival rate of the vaccinated fish was 45.7%, which was significantly higher than that of the control fish (14.3%; Figure 10). The RPS of the vaccinated fish was 36.7%.

**DISCUSSION**

Our previous study has shown that in TSS, iron limitation altered the expression of a large amount of cytoplasmic and membrane associated proteins involved in diverse functions (Sun and Sun, 2015). In the present study, we found that PfFha was a secreted protein whose expression was upregulated during iron starvation. The presence of PfFha in the extracellular milieu is in agreement with the previous reports that FHA is both surface associated and secreted (Weiss and Hewlett, 1986; Locht et al., 1993). Consistently, PfFha contains an N-terminal putative signal peptide and is predicted to be an extracellular protein. PfFha also contains a hemagglutination activity domain, which is usually found near the N terminus of FHA and heme/hemopexin-binding proteins (Ruer et al., 2008).

Reports have indicated that in Xanthomonas campestris pv. vesicatoria, mutation of fha significantly reduced swarming motility (Choi et al., 2013), whereas in X. axonopodis pv. citri, mutation of fha resulted in faster swarming (Gottig et al., 2009); but neither of these phenotypes related to flagella. In our study, TSS/fha displayed very little motility compared to the wild type and, surprisingly and interestingly, exhibited no apparent flagellum. To our knowledge, this is the first evidence that suggests a link between FHA and flagella. These observations imply that PfFha is possibly involved in the normal synthesis/transport of flagella. The lack of flagella may
also to some degrees account for the reduced self-aggregation observed with TSSfha, since it has been reported that in E. coli and Xylella fastidiosa, autoaggregation is influenced by surface structures such as type I fimbriae and type IV bundle-forming pili (Ulett et al., 2006; De La Fuente et al., 2008). For many pathogens, motility is often intimately linked to pathogenicity by complex regulatory networks (Josenhans and Suerbaum, 2002). In Burkholderia pseudomallei, flagella are necessary virulence determinants during intranasal and intraperitoneal infection of mice (Chua et al., 2003). In the coral pathogen Vibrio corallilyticus, flagellar mutation disables coral attachment, chemotaxis, and host infection (Meron et al., 2009). Considering these observations, it is likely that the motility/flagella-defectiveness of TSSfha may contribute at least in part to the attenuated virulence of this mutant.

Many studies have indicated a close relationship between biofilm and bacterial pathogenicity. In Pseudomonas aeruginosa, biofilm production significantly facilitates bacterial infection (Bjarnsholt et al., 2010); in Xanthomonas campestris, biofilm is required for full virulence to plants (Dow et al., 2003); in methicillin-resistant Staphylococcus aureus, the biofilm-forming capacity is crucial for intracellular persistence and chronic infections (Oyama et al., 2016). In X. axonopodis pv. citri, B. pertussis, and A. baumannii, it is known that fha mutation reduced biofilm formation (Gottig et al., 2009; Serra et al., 2011; Astaneh et al., 2014). Likewise, we observed a significant reduction in biofilm production by TSSfha. Accumulating evidences indicate that the motility of bacteria plays an important role in biofilm development (Wood et al., 2006; Meshcheryakov et al., 2013). For example, E. coli lacking flagella or possessing paralyzed flagella is defective in biofilm growth (Wood et al., 2006; Houry et al., 2010), and Edwardsiella tarda with impaired flagella exhibited reduced biofilm production (Xu et al., 2014). Biofilms are multicellular aggregates of bacteria bound by a matrix of extracellular polymers (O’Toole et al., 2000; Branda et al., 2006; Monds and O’Toole, 2009). The matrix of biofilm not only allows the bacteria to cohere to one another but also adhere to solid surfaces (Flemming and Wingender, 2010). In our study, we found that while the wild type TSS was able to form a matrix of fiber-like structures on the solid surface of a cover slip, TSSfha completely lost this capacity. It is possible that the matrix formed by the fiber structures connects the bacterial cells into a network and assists cellular adhesion to the attached surface, thus promoting biofilm growth.

Filamentous hemagglutinin plays a crucial role in host interaction by involvement in the adherence to host surface and hemagglutination (Rojas et al., 2002; Guilhabert and Kirkpatrick, 2005; Gottig et al., 2009; Serra et al., 2011). A previous study showed that in Erwinia chrysanthemi, mutation of hecA, the hemagglutinin homolog of fha, decreased the ability to attach to leaves and form aggregates on them (Rojas et al., 2002). In our study, TSSfha was markedly impaired in attachment to FG cells, suggesting that PfFha was vital to the binding of the bacteria to

FIGURE 9 | Effect of rFha antibody on Pseudomonas fluorescens infection. Turbot were infected with TSS in the presence or absence of rFha antibody, control antibody, or PBS (control). Bacterial recovery from kidney (A) and spleen (B) was determined at different hours post-infection. The results are the means of three independent experiments and presented as mean ± SEM. **P < 0.01.

FIGURE 10 | Protective effect of rFha as a vaccine. Turbot were vaccinated with or without (control) rFha and challenged with P. fluorescens TSS at 1 month post-vaccination. The fish were monitored daily for survival. Significances between the survivals of the vaccinated fish and the control fish were determined with logrank test. **P < 0.01.
host cells. Consistent with its structural prediction, TSS exhibited apparent hemagglutination activity, which, however, was absent in TSS/fha, suggesting that as observed previously in bacteria such as B. pertussis (Colombi et al., 2004), Pf_fha functions as a hemagglutinin.

Filamentous hemagglutinin as a key virulence factor has been reported in both plant and animal pathogens including E. chrysanthemi, B. pertussis, and A. baumannii (Rojas et al., 2002; Astaneh et al., 2014; Romero et al., 2014). In our study, we found that compared to the wild type, TSS/fha exhibited a distinct reduction in serum survival, which is the first observation of an association between bacterial FHA and serum resistance. Given the fact that Pf_fha is a surface protein of the bacteria, which enables it to be in direct contact with host factors, it is possible that, as observed previously with other bacterial factors (Hallström et al., 2013; Bernhard et al., 2014), Pf_fha may be able to interact with, and consequently sequester or inactivate, serum components essential to the functioning of the complement system. In vivo study showed that the tissue dissemination and mortality-inducing capacities of TSS/fha were markedly weakened. Consistently, the presence of rFha antibody, which most likely interacted with and thus blocked the function of the natural Pf_fha, significantly reduced the dissemination and colonization of TSS in turbot tissues. Together these results indicate that Pf_fha is required for the full virulence of TSS during the process of host infection.

In B. pertussis, FHA is considered one of the most important immunogens, because it is the major secreted protein and mediates host-pathogen interaction (van den Berg et al., 1999; Balder et al., 2007). Similarly, purified FHA protein confers protection against Bordetella bronchiseptica in mice (Goebel et al., 2009). In our study, we found that when used as a subunit vaccine, rFha was able to induce a significant protection on immunized turbot, suggesting a potential of rFha as a vaccine candidate for the control of P. fluorescens in agriculture. These results are also in line with the secreted nature of Pf_fha and with the involvement of Pf_fha in host interaction.

In summary, we in this study provide a systematic analysis of the biological properties of P. fluorescens FHA associated with pathogenicity. We found for the first time that mutation of bacterial FHA affects flagella formation, extracellular matrix production, and serum survival. Like the FHA of other bacteria, P. fluorescens Pf_fha is required for adhesion to host cells, hemagglutination, biofilm formation, mortality, and pathogenicity. In addition, P. fluorescens Pf_fha in the form of recombinant protein can induce protective immunity in fish. These results add new insights into the function of bacterial FHA.

AUTHOR CONTRIBUTIONS
LS and Y-YS conceived and designed the experiments; Y-YS performed the experiments; Y-YS and HC analyzed the data; Y-YS and LS wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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