Protective Efficacy of Four Recombinant Fimbrial Proteins of Virulent *Aeromonas hydrophila* Strain ML09-119 in Channel Catfish

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ABSTRACT

Aeromonas hydrophila is a reemerging pathogen of channel catfish (*Ictalurus punctatus*); recent outbreaks from 2009 to 2014 have caused the loss of more than 12 million pounds of market size catfish in Alabama and Mississippi. Genome sequencing revealed a clonal group of *A. hydrophila* isolates with unique genetic and phenotypic features that is highly pathogenic in channel catfish. Comparison of the genome sequence of a representative catfish isolate (ML09-119) from this virulent clonal group with lower virulence *A. hydrophila* isolates revealed four fimbrial proteins unique to strain ML09-119. In this work, we expressed and purified four *A. hydrophila* fimbrial proteins (FimA, Fim, MrfG, and FimOM) and assessed their ability to protect and stimulate protective immunity in channel catfish fingerlings against *A. hydrophila* ML09-119 infection for vaccine development. Our results showed catfish immunized with FimA, Fim, FimMrfG, and FimOM exhibited 59.83%, 95.41%, 85.72%, and 75.01% relative percent survival, respectively, after challenge with *A. hydrophila* strain ML09-119. Bacterial concentrations in liver, spleen, and anterior kidney were significantly ($p < 0.05$) lower in vaccinated fish compared to the non-vaccinated sham groups at 48 h post-infection. However, only the Fim immunized group showed a significantly higher antibody titer in comparison to the non-vaccinated treatment group ($p < 0.05$) at 21 days post-vaccination. Altogether, Fim and FimMrfG recombinant proteins have potential for vaccine development against virulent *A. hydrophila* infection.

Keywords: Fimbrial proteins, *Aeromonas hydrophila*, Channel catfish.

1. Introduction

Aeromonas hydrophila is an important Gram-negative bacterial pathogen associated with disease outbreaks in farmed fish with estimated losses of millions of dollars per annum (Fang et al., 2004). *A. hydrophila* is ubiquitous in aquatic environments and is responsible for causing a number of different diseases including “Motile Aeromonad Septicemia” (MAS), “Hemorrhagic Septicemia”, “Ulcer Disease,” and “Red-Sore Disease” (Esteve et al., 1995) in carp, tilapia, perch, salmon, catfish, and other fish species (Janda and Abbott, 2010).

A. hydrophila was not historically considered a pathogen of major concern in channel catfish aquaculture. However, since April of 2009, a clonal population of highly virulent *A. hydrophila* strains have been isolated from disease outbreaks on commercial catfish farms in western Alabama (Hemstreet, 2010). Since then, disease outbreaks have spread to Mississippi and Arkansas (Pridgeon and Klesius, 2011). Until 2014, this outbreak has been responsible for an estimated loss of more than \$12 million in commercially raised catfish operations in the Southeastern United States (Hossain et al., 2014). The signs of *A. hydrophila* infection caused by these virulent *A. hydrophila* (VAh) in catfish include acute onset of anorexia, hemorrhage in muscles and visceral organs, and bloody ascites. Outbreaks are associated primarily with marketable size catfish with mortality rates up to 50-60%.

MAS infection can be difficult to treat in aquaculture systems due to antibiotic resistance (Shariff, 1998). Therefore, vaccination would be a more efficient method to control and prevent *A. hydrophila* infection. During recent years, numerous studies investigated the use of several recombinant surface and extracellular proteins as vaccines against *A. hydrophila* infection, including Omp38 (Wang et al., 2013b), Aha1 and OmpW (Maiti et al., 2012), extracellular protease (Wu et al., 2012), Omp48 (Khushiramani et al., 2012), flagellar protein FlgK (Yeh and

Klesius, 2012), and Omp-G (Guan et al., 2011). Although these different preparations have provided varying degrees of protection in fish, a commercial vaccine for protection of farmed fish against *A. hydrophila* infection does not exist.

A. hydrophila strain ML09-119 was isolated from an outbreak of MAS in a commercial catfish operation, and it is our representative strain of the clonal group of VAh strains. Using comparative genomics, we determined that some fimbrial proteins are unique to VAh strains compared to historical *A. hydrophila* strains isolated from catfish aquaculture. The goal of the current study was to amplify, express, and purify four of these VAh-unique fimbrial proteins from *A. hydrophila* strain ML09-119 [(P pilus assembly protein, pilin FimA (AGM42215.1), fimbrial protein (AGM42222.1), fimbrial protein MrfG (AGM42218.1), and fimbrial biogenesis outer membrane usher protein (AGM42220.1)]. Further, we determined whether these proteins have potential to protect and stimulate antibody response in catfish against *A. hydrophila* ML09-119 infection.

2. Material and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table 1. *A. hydrophila* strain ML09-119 (Griffin et al., 2013) was isolated in 2009 from a large-scale disease outbreak in a commercial catfish farm in Alabama. It is a representative strain of the clonal group of virulent *A. hydrophila* that have impacted U.S. channel catfish aquaculture. The strain was grown on brain heart infusion (BHI) agar or broth (Difco, Sparks, MD, USA) and incubated at 37°C. *Escherichia coli* strains NovaBlue (Novagen, Madison, WI, USA) and BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) were used for cloning and expression, respectively. *E. coli* strains were cultured on Luria–

Bertani (LB) agar or broth (Difco) and incubated at 37°C throughout the study. The expression vector pET-28a (Novagen) was used for expression of fimbrial proteins. Whenever required, isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin (Kan, 50 µg/ml), ampicillin (Ap, 100 µg/ml), and colistin (Col, 2.5 µg/ml) (Sigma–Aldrich, Saint Louis, MN, USA) were added to culture medium.

2.2. Construction of the four fimbrial protein expression plasmids

The coding regions of selected genes were amplified by PCR using primers synthesized by Sigma–Aldrich. Two different restriction sites were incorporated into primer ends for cloning. The primers and restriction sites used in this study are listed in Table 2. Product sizes were 588 bp, 438 bp, 570 bp, and 2566 bp for *fimA*, *fim*, *fimMrfG*, and *fimOm*, respectively. The amplified coding regions were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), digested, and ligated into the corresponding sites in linearized pET-28a. Ligation product was transformed into NovaBlue competent cells by heat shock at 42°C. Positive clones were selected on LB Kan plates and verified by colony PCR using T3 and T7 terminator primers to confirm the in-frame insertion. Four recombinant plasmids (pETfimA, pETfim, pETmrfG, and pETom) were isolated from *E. coli* NovaBlue, then transformed into competent BL21 (DE3).

2.3. Expression of four recombinant fimbrial proteins in BL21 (DE3)

E. coli BL21 (DE3) carrying recombinant plasmids was grown in LB broth containing Kan with constant shaking at 37°C until cultures reached an optical density (600 nm) of 0.5–0.6; subsequently, bacteria were induced with 100 mM IPTG for 8 h at 37°C. Whole bacterial proteins were isolated using Bug Buster protein extraction reagent (Novagen) and solubilized in

tricine sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) for 5 min at 80°C. Protein separation was conducted using 12% SDS-PAGE (Laemmli, 1970) to check expression of each fimbrial protein. Non-recombinant *E. coli* BL21 (DE3) and uninduced recombinant clone were used as controls. After positive clones were identified, bacteria were stored at -80°C in 20% glycerol.

2.4. Purification of recombinant fimbrial proteins

Fimbrial proteins were purified by His-Bind (Novagen) resin column. Briefly, *E. coli* BL21 (DE3) was grown in 500 ml of LB broth and induced by IPTG. Bacteria were harvested by centrifugation (12,000 x g for 20 min at 4 °C), and pellets were lysed using Bug Buster protein extraction reagent with benzonase nuclease and protease inhibitory cocktail set III (Sigma). Soluble fractions were removed by centrifugation, and recombinant proteins were purified from inclusion body pellets by suspending in lysis buffer (Tris-HCl buffer pH 8.0, 6 M urea) with gentle sonication (4 cycles, 10 s each) on ice. After centrifugation (12,000 xg for 20 min at 4°C), recombinant protein was bound to resin column, washed (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9), and eluted in 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. Protein yield was determined on a spectrophotometer at 280 nm, and purity was assessed by SDS-PAGE. The identity of the recombinant proteins was confirmed by MALDI-TOF mass spectrometry.

2.5. Establishment of an immersion challenge model for *A. hydrophila* ML09-119 in catfish

To determine the exposure time and temperature that caused 50% mortalities (LD50) in channel catfish by bath immersion with *A. hydrophila* ML09-119, fish were divided into four groups of four replicates with 40 fish in each group (10 fish/tank). Immersion challenge dose was

1.7x10¹⁰ colony forming unit (CFU)/ml in water. The first group was challenged by immersion for 3 h at 25°C, the second group by immersion for 6 h at 25°C, the third group by immersion for 3 h at 30°C, and the last group by immersion for 6 h at 30°C. Fish were monitored daily for two weeks, and the cause of mortality was confirmed by isolating bacteria from liver, spleen, and anterior kidney. Control fish in each group were given an equal volume of BHI broth. The experiment was performed twice.

2.6. Vaccine efficacy of recombinant proteins

The vaccine efficacy trial was performed at Mississippi State University according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC). On the day of immunization, the four fimbrial proteins FimA, Fim, FimMrfG, and FimOM were mixed with the non-mineral oil adjuvant Montanide ISA 763 AVG (Seppic, Paris, France) at a ratio of 30:70 fimbrial protein to adjuvant with final concentration of 250 µg/ml of each recombinant protein. Specific pathogen free channel catfish (mean weight: 68.77g) were stocked in 40-L tanks supplied with flow-through dechlorinated municipal water with constant aeration and fed twice daily. Water temperature was maintained at 30°C throughout the experiments. Fish were divided into seven treatments with five replicate tanks and 20 fish/tank: non-vaccinated control; PBS-adjuvant injected treatment; heat killed *A. hydrophila*-injected treatment (0.1 ml of 1 x 10⁶ killed bacteria/ml); and four treatments injected intraperitoneally with 0.1 mL of recombinant fimbrial protein. Fish were anesthetized using MS-222 for injections.

Three weeks post-vaccination, fish were experimentally infected by immersion with *A. hydrophila* ML09-119 (approximately 2.3x10¹⁰ CFU/ml) for 6 h at 30°C. At 48 h post-infection, a total of five fish (one fish per tank) from each treatment were euthanized, and liver, spleen, and

anterior kidney tissues were collected aseptically. Tissues were homogenized in 1 ml PBS each. Tissue suspensions were diluted serially and spread in triplicate on BHI agar plates containing Ap and Col. Plates were incubated at 37°C for 48 h, and viable bacterial colonies were enumerated.

The remaining fish were monitored for mortalities daily for two weeks, and relative percent survival (RPS) was calculated based on the formula $RPS = [1 - (\% \text{ mortality in vaccinated group} / \% \text{ mortality in control group})] \times 100$ (Amend, 1981).

2.7. Serum antibody response

Three weeks post-vaccination and prior to experimental infection with strain ML09-119, ten fish per group (two fish per tank) were removed for blood collection. Blood was collected from the caudal vein, and after blood clotted overnight at 4 °C, serum was obtained by centrifugation at 3500x g for 10 min.

Antibody titers were determined from the immunized fish serum by enzyme-linked immunosorbent assay (ELISA) (Waterstrat et al., 1991). Immulon™ plates (Bloomington, MN, U.S.A.) were coated overnight at 4°C with 50 µl per well of heat killed whole bacteria (10^8 CFU/ml). Wells were washed and blocked with 100 µl/well of 5% nonfat dry milk (Bio-Rad) in PBS overnight at 4°C. Wells were washed three times in PBS containing 0.05% Tween-20 (PBS-T). Fifty microliters of fish serum diluted 1:100 was added to each well. Plates were incubated for 1 h at 37°C and washed. Fifty microliters of a 1:4 dilution of monoclonal antibody 9E1 (anti-catfish Ig) (Lobb and Clem, 1982; Miller et al., 1987) were added to each well, plates were incubated for 1 h at 37°C, and then they were washed.

Goat anti-mouse antibody conjugate (Fisher Scientific) was added, plates were incubated at room temperature for 1 h, and washed. Finally, 100 µl of *p*-nitrophenyl phosphate substrate (Sigma 104 phosphatase substrate) dissolved in 10% diethanolamine buffer was added to each well, and plates were incubated for 45 min at room temperature. Optical density of each well was measured at 405 nm in an ELISA Microplate Reader (CA, USA). Controls included wells with PBS buffer (in place of serum) and wells with known positive *A. hydrophila* immune sera. To standardize, average background absorbance for each plate was subtracted from the measured absorbance.

2.8. Statistical analysis

The effect of vaccination with the different proteins on fish survival after challenging with *A. hydrophila* was assessed with mixed model logistic regression using PROC GLIMMIX in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC, USA). The number of live fish in a tank at the end of the trial was the outcome assessed using an events/trials syntax. Protein was the fixed effect assessed in the model. Tank within protein group was included as a random effect in the model. The wild type was the referent for comparisons of the effect of protein.

The effect of the different proteins on the number of CFU in tissue samples and on ELISA titers was assessed by analysis of variance using PROC GLM in SAS for Windows 9.4. Separate models were used to assess CFU/g in liver, spleen, and anterior kidney samples as well as the ELISA results. The CFU/g data was transformed by first adding 1 to each CFU/g value and then taking the base 10 logarithm. The ELISA data was transformed by simply taking the base 10 logarithm of each ELISA value. The distribution of the residuals was evaluated for each model to determine the appropriateness of the statistical model for the data. If the effect of

protein was found to be statistically significant, the least squares means were compared using the Dunnett adjustment for multiple comparisons with wild type as the referent. A significance level of 0.05 was used for all analyses.

3. Results

3.1. Production of four recombinant fimbrial proteins of *A. hydrophila* ML09-119

Recombinant proteins migrated to the expected size on coomassie blue stained SDS-PAGE gels. No expression was observed with non-recombinant *E. coli* and uninduced recombinant clones, and optimal induction time with IPTG was determined to be 6-8 h (Fig. 1). The four fimbrial recombinant proteins (20.59 kDa, 20.11 kDa, 19.56 kDa, and 93.52 kDa for FimA, Fim, FimMrfG, and FimOM, respectively) were found in the insoluble fraction (Fig. 2). To further confirm these proteins, MALDI-TOF mass spectrometry amino acid sequencing was conducted, resulting in 100% identity with the published sequences (accession numbers AGM42215.1, AGM42222.1, AGM42218.1 and AGM42220.1) (data not shown).

3.2. Establishment of an immersion challenge model of *A. hydrophila* ML09-119 in catfish

No mortalities occurred in the two treatments where fish were experimentally infected at 25°C. In addition, no mortalities occurred when fish were experimentally infected at 30°C for 3 h. However, when fish were immersed for 6 h at 30°C, mortalities were 46.53% in the first trial and 52.33% in the second trial. In each trial, all fish mortalities occurred within 48 h of experimental infection. Therefore, this method was used as the *A. hydrophila* immersion challenge protocol for the vaccine study.

3.3. Vaccine efficacy of recombinant fimbrial proteins

Significantly higher mortalities occurred in the non-vaccinated control treatment (43.57%) compared to mortalities in fish vaccinated with Fim (2.00%; $p=0.0069$), FimMrfG (6.22%; $p=0.0058$), and FimOM usher protein (10.89%; $p=0.0171$). There was no significant difference in mortalities between non-vaccinated control treatment and fish vaccinated with FimA (17.83%; $p=0.0621$), as well as fish injected with PBS-adjuvant (29.54%; $p=0.3588$) (Fig. 3). RPS for the four recombinant fimbrial proteins (FimA, Fim, FimMrfG, and FimOM) was 59.83%, 95.41%, 85.72%, and 75.01%, respectively. RPS for the PBS-adjuvant treatment was 32.19 %.

Mean bacterial concentrations in liver were significantly lower ($p<0.0003$) in fish vaccinated with FimA, Fim, FimMrfG, and FimOM than both non-vaccinated fish and fish injected with PBS-adjuvant (Fig. 4). Furthermore, the mean spleen and anterior kidney bacterial loads were significantly lower ($p<0.0001$ and $p<0.03$, respectively) in fish vaccinated with the four recombinant fimbrial proteins compared to the non-vaccinated control treatment and the PBS-adjuvant treatment.

3.4. Serum antibody response

Fish vaccinated with Fim protein had significantly higher ($p=0.02$) antibody production than non-vaccinated fish and fish injected with PBS-adjuvant (Fig. 5). Fish injected with killed *A. hydrophila* also had significantly ($p<0.05$) higher antibody titers than non-vaccinated treatment. However, antibody response in fish vaccinated with FimA, FimMrfG, and FimOM did not significantly differ ($p=1.00$, 0.61, and 0.16, respectively) from non-vaccinated fish or the

PBS-adjuvant treatment. Fish vaccinated with adjuvant only also did not have significantly higher antibody concentrations than non-vaccinated control treatment.

4. Discussion

A. hydrophila strain ML09-119 represents a clonal group of *A. hydrophila* isolates causing outbreaks of bacterial septicemia in channel catfish since 2009. This isolate is significantly more virulent to channel catfish than historical *A. hydrophila* strains isolated from sporadic cases of MAS in fish and a Chinese carp isolate (Hossain et al., 2014). We compared the genome sequence of strain ML09-119 (GenBank accession no: CP005966.1) (Tekedar et al., 2013) with the *A. hydrophila* ATCC 7966T genome sequence (NC_008570.1) to identify four fimbrial proteins unique to ML09-119 (AGM42215.1, AGM42222.1, AGM42218.1, and AGM42220.1). We hypothesized these proteins will be effective in stimulating significant protective immunity in catfish against strain ML09-119. Our aim is to identify protective protein antigens from virulent *A. hydrophila* for potential use as recombinant antigens that can be expressed in a live attenuated vaccine carrier.

Fimbriae are adhesive organelles that are often important virulence factors in a wide range of pathogenic bacteria (Sauer et al., 2004). They typically facilitate invasion of host tissues and are involved in other diverse functions such as phage binding, DNA transfer, biofilm formation, cell aggregation, and twitching motility (Doughty et al., 2000; Kline et al., 2009; Proft and Baker, 2009). Fimbriae are divided into four classes based on their assembly pathways, of which the type I fimbriae are encoded by the *fim* gene cluster (Jones et al., 1995; Knight and Bouckaert, 2009). Type I fimbriae are the most prevalent type and are found on most uropathogenic *Escherichia coli* (UPEC), where they contribute significantly to urinary tract infection. Type 1 fimbriae are also responsible for invasion and persistence in target cells

(Baorto et al., 1997). They are found in several species, including *Salmonella enterica*,
Pseudomonas putida, *Klebsiella pneumoniae*, and *Yersinia* (Sauer et al., 2004).

Due to their extracellular location and their role in colonizing host tissue, fimbrial
proteins have been considered important targets for vaccine development against several
bacterial diseases. For instance, purified recombinant FimA induces protective immunity against
Edwardsiella tarda in turbot fish, suggesting that rFimA is an effective subunit vaccine (Wang et
al., 2013a). In *Proteus mirabilis*, a common cause of urinary tract infection, structural fimbrial
protein (MrpA) protects mice from infection when used as a purified recombinant protein
(Pellegrino et al., 2003). Immunization of pigs with the Type IV fimbrial recombinant protein of
Actinobacillus pleuropneumoniae induces high levels of protection that could be a valuable
component of an efficient subunit vaccine for the prevention of porcine pleuropneumonia
(Sadilkova et al., 2012).

The pET plasmids are very effective for expression of recombinant proteins in *E. coli*
based on the T7 promoter (Rosenberg et al., 1987), and we found it to be effective for expression
of the four fimbrial *A. hydrophila* genes *fimA*, *fim*, *fimMrfG*, and *fimOm* encoding structural
fimbrial subunits. The four recombinant proteins were found in the insoluble fraction during
purification, which is consistent with what was observed for *P. mirabilis* fimbrial proteins (Sauer
et al., 2004). It is possible that the expressed protein formed inclusion bodies due to
overexpression.

The level of protection provided by the four proteins in catfish against experimental
infection with *A. hydrophila* strain ML09-119 varied. Fish vaccinated with Fim and FimMrfG
proteins had strong protection against *A. hydrophila* infection (RPS: 95.41% and 85.72%), while
fish immunized with FimA and FimOM had less protection (RPS: 59.83% and 75.01%). Not

surprisingly, the two most effective recombinant proteins as vaccines are structural fimbriae proteins.

Consistent with these findings, the mean bacterial counts recovered from liver, spleen, and anterior kidney of catfish vaccinated with FimA, Fim, FimMrfG, and FimOM were significantly lower than bacterial counts of non-vaccinated control group within 48 h post-infection. This indicates that all four proteins are effective at reducing infection caused by VAh, even though some are better at protecting. Mean bacterial concentrations from anterior kidney were more consistent and higher than spleen and liver, indicating that anterior kidney may be the preferred tissue for recovery and quantification of *A. hydrophila* during infection. However, posterior kidney was not tested in this study.

The antibody titers in non-vaccinated catfish serum may be due to environmental exposure to *A. hydrophila*, which is ubiquitous in aquatic environments (Janda and Abbott, 2010). Another factor likely contributing to the background antibody concentrations detected in non-vaccinated fish is that our plates were coated with whole cell lysate of *A. hydrophila* ML09-119. An alternative method that would yield more specific detection of antibody is to use respective recombinant proteins to coat ELISA plates.

In the current study, only Fim immunized catfish had a statistically significant increase in antibody concentration compared to the control non-vaccinated group. Fim vaccinated catfish also had the best protection against subsequent challenge with strain ML09-119, suggesting that humoral immunity contributes to protection against VAh infection. However, antibodies do not account for all of the protection, because fish vaccinated with FimA, FimMrfG, and FimOM all had significant protection but no significant increase in antibody concentration.

For some pathogens, anti-fimbrial antibodies show significant contributions to protection against infection. For example, vaccination of turbot fish with recombinant FimA as a subunit vaccine induced production of specific serum antibodies that bound live *E. tarda* via interaction with FimA. This antibody-*E. tarda* interaction effectively blocked infection (Wang et al., 2013a). Fimbrial protein of *Pasteurella multocida* is also antigenic and stimulated antibody production (IgG and IgA) in goats that provided good protection against high dose challenge (Ina Salwany, 2009; Mohd Yasin et al., 2011). In contrast, *Salmonella* fimbrial vaccines did not induce high antibody titers in poultry; however, they were very effective for control of experimental infection (Menão et al., 2013). Further studies are necessary to delineate the roles of humoral and cell-mediated immunity in fimbrial protein-mediated protection against VAh infection in catfish.

In conclusion, our results confirm that Fim is a promising vaccine candidate against VAh infection in channel catfish. FimMrfG also provided good protection. Both of these proteins have potential for integration into a live attenuated vaccine carrier. The mechanism of this protective effect remains unknown, and future studies are needed to characterize the immune responses elicited by these *A. hydrophila* fimbrial proteins.

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445

446 **Table 1**

447 Bacterial strains and plasmids used

Strain or plasmid	Description	Source
<i>A. hydrophila</i> ML09-119	Isolate from a disease outbreak on a commercial catfish farm	(Griffin et al., 2013)
<i>E. coli</i>		
NovaBlue	endA1 hsdR17(rK12– mK12+) supE44 thi-1recA1 gyrA96 relA1 lac F'[proA+B+ lacIqZΔM15 ::Tn10(TcR)]	Novagen
BL21(DE3)	F ⁻ <i>ompT hsdS gal</i> ; expression host, vaccine delivery vector	Invitrogen
Plasmid		
pET-28a	Expression vector; Km ^r	Novagen
pET <i>fimA</i>	pET-28a,:: <i>fimA</i>	This study
pET <i>fim</i>	pET-28a,:: <i>fim</i>	This study
pET <i>mrfG</i>	pET-28a,:: <i>fimMrfG</i>	This study
pET <i>Tom</i>	pET-28a,:: <i>fimOm</i>	This study

448

449 **Table 2**

450 Properties of *A. hydrophila* ML09-119 fimbrial proteins and oligonucleotide primers used for
451 PCR amplification

Protein	Locus	Number of tag nucleotides/ amino acids	M.W. (kDa)	Isoelec- tric point	Primer	Sequence	Restriction enzyme
P pilus assembly protein, pilin FimA	AHML_ 02150	591/197	20.59	4.45	FIM1-F	AAA A GCTTACTGGTAGGTC ATGATAAAGTCG	HindIII
					FIM1-R	AAG G ATCCTATGAAACCC ATGATGAAACC	BamHI
Fimbrial protein	AHML_ 02185	441/147	20.11	4.982	FIM2-F	AAG G ATCCTTGGAAAATG AGGTTTGCAGT	BamHI
					FIM2-R	AAA A GCTTCTGATAATTCA TGACAAAGTCTGC	HindIII
Fimbrial protein	AHML_ 02165	561/186	19.56	9.362	MrfG-F	AAA A GCTTATAGGTCAGCT TGAGGGTTGAC	HindIII
MrfG					MrfG-R	AAG G ATCCCTGAAGGAGG TAACGATGAACC	BamHI
Fimbrial biogenesis outer membrane usher protein	AHML_ 02175	2538/846	93.52	4.999	OM-F	AAG A GCTCAACGGGTCTC AGTGACAGCTC	SacI
					OM-R	AAG A ATTCCCCCTTACAGA CAGTGACGAT	EcoRI

452 ^aBold letters at the 5' end of the primer sequence represent RE site added. AA nucleotides were
453 added to the end of each primer containing a RE site to increase the efficiency of enzyme cut.

Figure legends

Fig. 1. SDS-PAGE with Coomassie blue stain showing expression of recombinant *A. hydrophila* ML09-119 proteins FimA (A), Fim (B), FimMrfG (C), and FimOM (D) in *E. coli* BL21 (DE3) after induction with 100 mM IPTG. Lanes: (1) standard protein marker, (2) uninduced 2 h, (3) uninduced 4 h, (4) uninduced 6 h, (5) uninduced 8 h, (6) induced 2 h, (7) induced 4 h, (8) induced 6 h, and (9) induced 8 h. Arrows indicate recombinant proteins.

Fig. 2. SDS-PAGE with Coomassie blue stain showing purified recombinant FimA, Fim, FimMrfG, and FimOM. Molecular weights in kilodaltons are shown for the standard protein marker in the right column.

Fig. 3. Percent mortalities in catfish vaccinated with FimA, Fim, FimMrfG, and FimOM recombinant proteins following experimental infection with *A. hydrophila* ML09-119 at three weeks post-vaccination. Data are the mean $\pm$ SE of five replicate tanks (each containing 20 fish). Significant differences between vaccinated and non-vaccinated treatments are indicated with asterisks ($p < 0.05$). Significant differences between vaccinated treatments and PBS-adjuvant treatment are indicated with two asterisks (**) ($p < 0.05$).

Fig. 4. Mean bacterial concentrations (CFU/g) in liver, spleen, and anterior kidney of catfish vaccinated with recombinant FimA, Fim, FimMrfG, and FimOM fimbrial proteins at 48 h post-infection with *A. hydrophila* ML09-119. Data represents the mean $\pm$ SE of five fish per treatment. Significant differences between vaccinated and non-vaccinated treatments are indicated with asterisks ($p < 0.05$). Significant differences between vaccinated treatments and

PBS-adjuvant treatment are indicated with two asterisks ($\ast\ast$) ($p < 0.05$). $\dagger$ indicates CFU/g was below the detectable limit for this treatment.

Fig. 5. Antibody response determined by ELISA in channel catfish serum at 21 days post-vaccination with FimA, Fim, FimMrfG, and FimOM proteins. Optical densities at 405 nm are means of ten fish. Vertical bars denote standard errors of the mean. Asterisks (*) indicate statistically significant differences between non-vaccinated treatment and vaccinated treatments ($p < 0.05$). Two asterisks ($\ast\ast$) indicate significant differences between vaccinated treatments and PBS-adjuvant ($p < 0.05$).

Fig. 1

Fig. 2

Fig. 3.

Fig. 4.

Fig. 5