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Aeromonas salmonicida Type I pilus system contributes to host colonization but not invasion

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ABSTRACT: The host-adherence strategies employed by *Aeromonas salmonicida* subsp. *salmonicida*, the etiological agent of an infectious bacteremia of salmonids, are poorly understood. In addition to the outer protein coat or S-layer, *A. salmonicida* has both Type I and Type IV pili loci. The *A. salmonicida* Type I or Fim pilus is encoded by an operon with genes for a chaperone, an usher, and 3 pilus subunits and is predicted to be similar to the Pap fimbriae of uropathogenic *Escherichia coli*, which are considered significant virulence factors. A Fim-deficient strain of *A. salmonicida* strain A449, Δ*fim*, was created by deleting this operon. Virulence of Δ*fim* was unchanged in direct live challenges of Atlantic salmon *Salmo salar* L., a natural host for *A. salmonicida*. A measure of clinically inapparent (covert) infections suggested Fim was required to establish or maintain a covert infection. This was confirmed by an ex vivo adherence and invasion assay using freshly excised salmon gastrointestinal (GI) tract, which showed that, compared to the parental strain, the ability of the isogenic Δ*fim* mutant strain to adhere to the salmon GI tract was reduced but, once adhered, its ability to invade was unchanged. Thus the Fim pilus functions as an adhesin in *A. salmonicida* and the presence of a functional Fim improved the efficiency of *A. salmonicida* infection of Atlantic salmon.

KEY WORDS: Pilin · Adhesin · Furunculosis · Virulence

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INTRODUCTION

Aeromonas salmonicida subsp. *salmonicida* (hereinafter referred to as *A. salmonicida*) is the etiological agent of an infectious bateriemia of Atlantic salmon called furunculosis. While *A. salmonicida* is transmitted horizontally through the water column, little is understood about the strategies it uses to colonize the host during infection. Furunculosis presents as a spectrum of diseases depending on the age and health status of the salmon. Acute and per-acute infections are characterized by high mortality and morbidity rates and affected animals show few or no clinical signs except for ecchymosis of the swim bladder. Low morbidity rates are seen in sub-acute and chronic infections where affected animals display the characteristic, focal dermomyonecrotic lesions or furuncles that are considered pathognomonic for the disease. The clinically inapparent, or covert, infection state is characterized by no morbidity or mortality and an outwardly healthy appearance. A disease state is manifested when the salmon is placed under acute or chronic stress. Such conditions temporarily immunosuppress the salmon, allowing the covert infection to become overt.

Recent work (Jutfelt et al. 2008) indicates that live *Aeromonas salmonicida* can cross the salmon intestinal

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epithelium by a trans-cellular route and that this translocation requires bacteria with intact and functional surface structures. Our continued interest in *A. salmonicida* adhesins leads us to ask if we could identify factors important for adherence to salmon intestinal tissue.

The most well studied *Aeromonas salmonicida* adhesin is its outer protein coat, the S-layer, the major protein constituent of which is VapA. While the S-layer has long been demonstrated to be both an important virulence factor and an adhesin (Ishiguro et al. 1981, Trust et al. 1983, Garduño et al. 1992, Kay & Trust 1997, Garduño et al. 2000), *VapA*-deficient strains may still be infectious (Ellis et al. 1988, Olivier 1990). In addition to the S-layer, *A. salmonicida* strain A449 also has genes for at least 4 specific adhesion systems: Tap, Flp, MSHA, and Fim (Reith et al. 2008). The first 3 are Type IV pili and have been characterized elsewhere (Masada et al. 2002, Boyd et al. 2008). The latter system, Fim, is predicted to encode a Type I pilus, similar to the fimbriae of pathogenic *Escherichia coli* that are considered to be important virulence factors in that organism. The present study reports on the contribution of Fim to *A. salmonicida* strain A449 virulence as assessed by a Fim-deficient isogenic laboratory strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacteria and plasmids used in the present study are listed in Table 1. The parental strain for all knockouts was *Aeromonas salmonicida* subsp. *salmonicida* strain A449 (hereinafter A449) originally isolated from a natural furunculosis epizootic in brown trout *Salmo trutta* (Michel 1979). All *A. salmonicida* strains were grown in tryptic soy broth (TSB) or agar (TSA) (Difco) for 3 d at 17°C with shaking. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) or agar at 37°C. Antibiotics were used at the following concentrations: *E. coli*: ampicillin, 100 µg ml^{-1} and kanamycin, 25 µg ml^{-1}; *A. salmonicida*: ampicillin, 50 µg ml^{-1} and chloramphenicol, 20 µg ml^{-1}.

Mutant construction. An in-frame, unmarked deletion of the entire *fim* locus, hereinafter referred to as Δ*fim,* was created using crossover PCR (Link et al. 1997). Briefly, 2 self-complementary PCR fragments of ca. 500 bp flanking the *fim* locus were amplified from A449 chromosomal DNA using the primers shown in Table 2. The 2 PCR fragments were mixed together and amplified with the 2 external (Fim-No and Fim-Co) primers to generate a ca. 1 kbp fragment carrying DNA flanking the *fimA* and *fimF* genes. The joined fragments were cloned into the mobilizable, pir-dependent, sucrase-expressing vector pWM91 to generate pWM-fim, which was conjugated into A449 from *Escherichia coli* BW20767. Single-crossover integrants were selected by plating on TSA supplemented with ampicillin to select for integrants and chloramphenicol to select against *E. coli*. Double-crossover segregants were isolated by selection on TSA with 15% sucrose to select against plasmid-containing colonies. Correct deletion of the *fim* locus was determined by PCR with primers complementary to the chromosome outside of the flanking regions and with primers complementary to the *fimC* gene. This resulted in the creation of a small open reading frame encoding the first 8 amino acids (aa) of FimA and the last 17 aa of FimF connected by the 7 aa of the crossover tag.

Bacterial conjugation. *Escherichia coli* BW20767 carrying pWM-fim was grown in LB with ampicillin overnight at 37°C. A449 was grown in TSB with chloramphenicol for 3 d at 17°C. One ml of each bacterial species was harvested and washed twice in fresh TSB without antibiotics. After the last wash, the bacterial pellet was resuspended in a small volume (15 to 30 µl) of TSB. These thick suspensions of each bacterium were mixed together thoroughly and spotted in the center of a TSA plate without antibiotics. After 2 d of incubation at 17°C, the spot was removed with a sterile

Table 1. Bacterial strains and plasmids used. Cm^R: chloramphenicol resistant; Ap^R: ampicillin resistant

Strain or plasmid	Description	Source
Aeromonas salmonicida		
A449	*A. salmonicida* subsp. *salmonicida* isolate, Cm^R	Michel (1979)
Δ*fim*	A449 Δ*fim*, Cm^R	Present study
Escherichia coli **strains**		
TOP10	K-12	Invitrogen
EC100D pir-116	K-12, pir-116	Epicenter
BW20767	K-12, pir+, conjugation+	Metcalf et al. (1996)
Plasmids		
pWM91	*oriR6Kγ*, *sacB*, *mobRP4*, Ap^R	Metcalf et al. (1996)
pWM-fim	pWM91 with fim flanking regions, Ap^R	Present study

Table 2. Oligonucleotides used. Restriction sites are underlined, non-sequence-specific regions in lower case

Primer name	Sequence	Purpose
Fim-No	tgtgctcgagCGTGCTCAATGTTGGTTTTT	To amplify 5′ flanking region for knockout, carries XhoI site
Fim-Ni	cccatccactaaacttaaacaAACCAAAATTTTATTAGCGTTCATTC	To amplify 5′ flanking region for knockout, carries crossover sequence
Fim-Ci	tgtttaagtttagtggatgggATCAAACCTGGCGATTATACCG	To amplify 3′ flanking region for knockout, carries crossover sequence
Fim-Co	atatcacgatgcggccgcGGGAGAGAATGTTTCCCACA	To amplify 3′ flanking region for knockout, carries *Not*I site
Fim-UF1	AATCCTGATTGCCTGATTGC	To verify deletion, flanks deletion on left side
Fim-DR1	CCATTCTCATCGGAAAGGAA	To verify deletion, flanks deletion on right side
Fim-F1	GCTGACTTGACCCAATCCAT	To verify deletion, within deletion
Fim-R1	TATCAAGATTACAGCCGGCC	To verify deletion, within deletion

toothpick and resuspended in 1 ml TSB, diluted, and plated on TSA with the appropriate antibiotics. Large colonies were picked, streaked to fresh plates, and passaged 2 more times.

Animal care. Both the National Research Council Halifax's Local Animal Care Committee and Dalhousie University's Committee on Laboratory Animals approved all animal procedures, which were conducted under Canadian Council on Animal Care guidelines. Under these guidelines death is considered an unacceptable endpoint (Demers et al. 2006; www.ccac.ca). Moribund animals were euthanized when a previously agreed set of limiting clinical signs were reached. Data are thus reported as cumulative morbidity and mean time to morbidity (MTTM) to better reflect this reality.

Juvenile St. John River stock Atlantic salmon (*Salmo salar* L. 1758), ca. 120 g, were obtained from a Nova Scotia hatchery certified under Canadian Fish Health Protection Regulations. They were stocked in 100 l fiberglass resident tanks at a stocking density of ca. 2.4 kg 100 l^{-1} and maintained at 14 ± 2°C, in flow-through in dechlorinated municipal (fresh) water. They were fed a maintenance ration daily of 1% bodyweight of a commercially available feed (Signature Salmon Ration, Shurgain). Feeding was suspended for 1 d prior to and 1 d post-manipulation.

Challenge. The virulence of the mutant strain Δ*fim* and its parental strain A449 were tested by intraperitoneal (i.p.) and immersion challenges conducted as per Dacanay et al. (2006). Briefly, for the immersion challenge there were 2 tanks $group^{-1}$ with ca. 40 fish $tank^{-1}$. The fish were transferred into commercially available plastic containers, sedated (15 mg l^{-1} tricaine methanosulphonate [TMS]; Syndel Laboratories), and exposed for 40 min to ca. 10^6 cfu ml^{-1} of the appropriate strain before being returned to their designated resident tanks. Control fish experienced identical handling but were only exposed to an equal volume of phosphate-buffered saline (PBS). For i.p. challenge, there were 2 tanks per group with ca. 30 fish per tank. They were anesthetized with 50 mg l^{-1} TMS prior to injection with ca. 10^4 cfu $animal^{-1}$ of the appropriate strain in 100 µl PBS. Control animals were injected with an equal volume of PBS. Doses were confirmed retrospectively by direct colony counts on TSA supplemented with 20 µg ml^{-1} chloramphenicol.

Animals were closely monitored post-challenge. Morbid animals were euthanized with a TMS overdose once they had met the minimum criteria for euthanasia. The posterior kidney was sampled aseptically onto TSA as recommended by the American Fisheries Society (Schotts 1994). The TSA was supplemented with 20 µg ml^{-1} chloramphenicol.

Statistical significance in cumulative morbidity between groups was assessed by the *G*-test, a modified χ^2, which was calculated manually. Statistical significance in MTTM between groups was assessed by *t*-test and was calculated by GraphPad Prism 5.0. Significance was set as $p < 0.05$ for both tests.

Stress test. It has been long known that *Aeromonas salmonicida* can induce a clinically inapparent or covert infection state (Hiney et al. 1997) and that adhesins such as Fim may play an important role in establishing such a state. Sixty days after exposure by immersion, covert infection levels in the remaining animals were assessed using a modified stress test (Specker et al. 1994). Briefly, the animals were anesthetized as before (see 'Challenge' above) and 100 mg kg^{-1} cortisol (hydrocortisone; Sigma-Aldrich) emulsion in 1:1 vegetable oil:vegetable fat was administered by i.p. injection. The animals were returned to their resident tank and monitored for 10 d, after which they were euthanized. To ensure sufficient numbers of animals for statistics, animals from both A449 and Δ*fim* tanks were combined to give a total number of 40 animals for both. Statistical significance in cumulative morbidity and MTTM between groups was assessed as for the challenges.

Ex vivo adherence. An ex vivo assay was performed to determine if the Δ*fim* strain had reduced host-adherence abilities. The gastrointestinal (GI) tract was chosen as the substrate against which bacterial adherence would be tested as it could be readily collected and manipulated in a sterile manner. Hindgut was sterilely excised from large (>2 kg) Atlantic salmon following euthanasia with an overdose of anesthetic (>100 mg l^{-1} TMS). Gut was prepared by first cutting the tube of gut open sagittally, followed by cutting it axially to produce rectangles of ca. 100 mm^2. Feces were removed with sterile forceps and by washing in sterile PBS (pH 7.4). The pieces of gut were placed on sterile TSA and 10 µl of an OD_{600} 0.001 solution of either Δ*fim* or the parental strain A449 was added to the lumenal side of the gut square. The bacteria and gut were incubated for 2 h at room temperature. After this time non-adherent bacteria were removed by gently washing the gut square with sterile PBS. Adherent bacteria were removed by vigorous vortexing in PBS and subsequently enumerated by direct colony counts on TSA supplemented with 20 µg ml^{-1} chloramphenicol. After removal of the adherent bacteria, gut squares were placed in Liebowitz-15 (L-15) medium (Sigma-Aldrich) supplemented with 21.5 µg ml^{-1} gentamicin (Gibco) and incubated for a further 2 h at room temperature to kill extracellular bacteria. Invasive bacteria were enumerated following hypotonic shock: gut squares were transferred into a lysis solution (sterile 0.1% Tween 20 in ddH_2O) and vortexed vigorously. Bacterial titer was determined by direct colony counts as before.

The area of the gut squares was measured and the bacterial titer normalized per mm^2. Bacteria adherent after 2 h are reported as a percentage of the original innoculum. Invasive bacteria are reported as a percentage of the adherent bacteria. Statistical differences in adherence or invasion were determined by *t*-test (GraphPad Prism 5.00); significance was set at $p < 0.05$.

RESULTS

Gene identification

A region of the *Aeromonas salmonicida* strain A499 genome (Reith et al. 2008) was identified that contained 6 genes predicted to encode a Type I or chaperone/usher type pilus (Table 3). The proteins encoded by these genes are predicted to be: a major pilus subunit (*fimA*), a protein of unknown function (*fimB*), an usher (*fimC*), a chaperone (*fimD*), and 2 minor subunit proteins (*fimEF*). The 6 genes are located between bases 4 016 890 and 4 022 860 of the A449 genome (GenBank accession no. NC_009348). The amino acid (aa) sequences have been assigned GenBank accession numbers YP_001143434 to YP_001143439. Table 3 also shows the relatedness of the *A. salmonicida* Fim proteins to the homologous proteins of another aeromonad, *A. hydrophila*, and to the *Proteus mirabilis* Pmf proteins. While *Proteus* spp. are not closely related to aeromonads, the number and order of the *fim* and *pmf* genes are almost identical in these 2 species and the aa sequences are among the most similar in GenBank (Massad & Mobley 1994).

To investigate the role of Type I pili in *Aeromonas salmonicida* pathogenesis, an unmarked deletion mutant strain of A449 was successfully created in which the entire locus *fimABCDEF* was deleted. There were no differences in *in vitro* growth characteristics between the parental A449 strain and the Δ*fim* strain (data not shown).

Challenge

Challenge data are summarized in Table 4. When administered by injection there were no significant differences in overall cumulative morbidity between the Δ*fim* mutant strain (50.0%) and the parental strain A449 (42.4%; *G*-test, $p > 0.05$). Similarly there were no significant differences when Δ*fim* (45.1%) and A449

Table 3. Predicted gene products of the *Aeromonas salmonicida* subsp. *salmonicida* strain A449 Fim pilus system

A. salmonicida gene name and GenBank accession no.	Homologue GenBank accession no. (% identity)		
	A. hydrophila ATCC 7966	*Proteus mirabilis*	Predicted function
fimA, YP_001143434	YP_855052 (93)	*pmfA* Q04681 (27)	Major pilus subunit
fimB, YP_001143435	YP_855053 (61)	Not present	Unknown
fimC, YP_001143436	YP_855054 (86)	*pmfC* P53514 (44)	Periplasmic chaperone
fimD, YP_001143437	YP_855055 (86)	*pmfD* P53520 (44)	Outer membrane usher
fimE, YP_001143438	YP_855056 (63)	*pmfE* P53522 (28)	Minor subunit, putative tip adhesin
fimF, YP_001143439	YP_855057 (75)	*pmfF* P53521 (24)	Minor subunit

(44.1%) were administered by immersion (*G*-test, $p > 0.05$). The MTTM of A449 (14.3 d) was significantly shorter than the MTTM for the Δ*fim* strain (20.7 d) when administered by immersion, but MTTM was equal at 9.9 d in both strains when administered by injection.

Stress test

Six days after the initiation of the stress test, *Aeromonas salmonicida*-related cumulative morbidity was 75% in the A449-exposed group and 66.7% in the Δ*fim* group (Table 5). This difference was not significant (*G*-test, $p > 0.05$). MTTM was significantly longer in the Δ*fim* group (5.0 d) than in the A449-exposed group (4.2 d; *t*-test, $p < 0.001$).

Table 4. Summary of live bacterial challenges. Cumulative morbidity is the number of morbid animals/total number of animals; statistical significance was assessed by a *G*-test with significance set at $p < 0.05$. Mean time to morbidity (MTTM) is the arithmetic mean of time of morbidity in days; statistical significance was assessed by a *t*-test with significance set at $p < 0.05$. Different superscripted letters denote statistical significance. nd = not determined

Strain	Intraperitoneal		Immersion	
	Morbidity (%)	MTTM (d)	Morbidity (%)	MTTM (d)
A449	42.37[a]	9.9[c]	44.05[d]	14.3[f]
Δ*fim*	50.0[a]	9.9[c]	45.12[d]	20.7[g]
PBS	0[b]	nd	0[e]	nd

Table 5. Stress test results. See Table 4 for statistics and abbreviations

Strain	Morbidity (%)	MTTM (d)
A449	75 (30/40)[a]	4.2[c]
Δ*fim*	66.7 (26/39)[a]	5.0[d]
PBS	0 (0/40)[b]	nd

Ex vivo adherence

Significantly more A449 (192%) adhered to the gut squares after 2 h at room temperature (ca. 20°C) than Δ*fim* (12.7%; *t*-test, $p > 0.05$) (Fig. 1). The doubling time of A449 at room temperature is ca. 2 h, accounting for the nearly 100% increase in titer from the original inoculum over the incubation period. However, the number of adherent bacteria that invaded the gut squares was approximately the same in both strains (A449, 35.4% and Δ*fim*, 50.7%; *t*-test, $p < 0.05$).

DISCUSSION

Host colonization, the adherence of a pathogenic bacterium to the host, is a crucial first step in pathogenesis. The S-layer of *Aeromonas salmonicida* has been repeatedly demonstrated to be its most important adhesin (Ishiguro et al. 1981, Trust et al. 1983, Garduño et al. 1992, 2000, Kay & Trust 1997). S-layer-mediated adherence is reportedly against basement membrane components such as Type IV collagen and laminin (Noonan & Trust 1997). The genome of *A. salmonicida* also carries genes for at least 4 additional

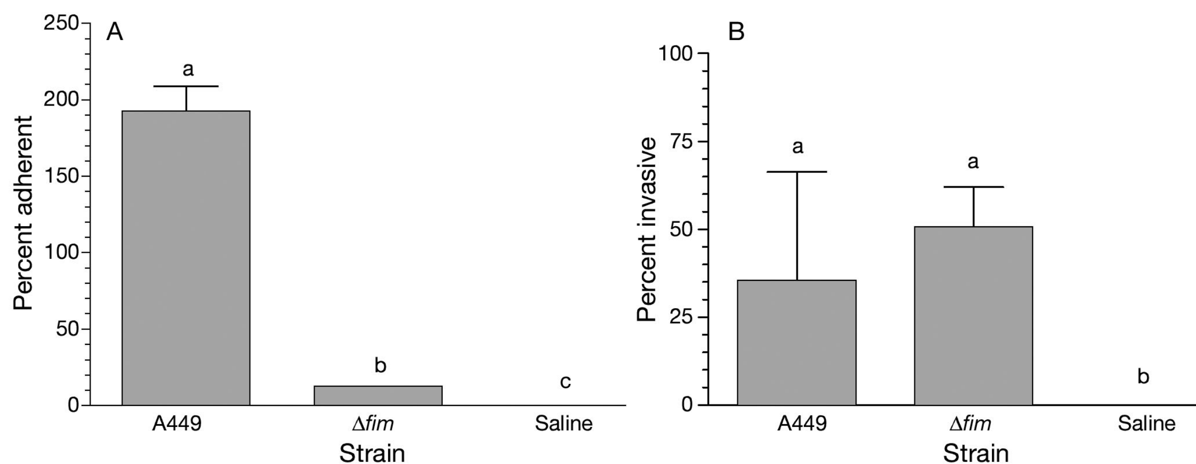

Fig. 1. Contribution of the Fim pilus system to host-cell adherence and invasion. (A) *Aeromonas salmonicida* strain A449 and the isogenic mutant strain Δ*fim* were allowed to adhere to freshly excised squares of Atlantic salmon gut and counted after 2 h incubation. (B) After a further 2 h incubation in media supplemented with gentamicin, the epithelial layer was lysed by hypotonic shock and invasive bacteria were counted. Different superscripted letters indicate statistical significance (*t*-test, $p < 0.05$). Data are means (+SD) from 3 gut squares

adhesins: 3 type IV pili systems, Tap (Masada et al. 2002, Boyd et al. 2008), Flp, and MSHA, of which the latter is expected to be non-functional (Boyd et al. 2008); and 1 Type I or chaperone/usher type pilus, Fim (the present study).

The Fim operon contains 6 genes, which are predicted to encode the major pilin subunit protein (*fimA*), a gene of unknown function (*fimB*), an outer membrane usher (*fimC*), a periplasmic chaperone (*fimD*), and 2 minor pilus subunits (*fimEF*). Based on the aa sequence of the outer membrane usher protein (FimC) and gene order, the *Aeromonas salmonicida* pilus is in the same group (π) as the pyelonephritis-associated (P) pili that allow uropathogenic *Escherichia coli* to adhere to kidney epithelial cells (Nuccio & Bäumler 2007). P fimbriae are composed of a rigid helical shaft that ends in a thinner flexible tip fibrillum, and the single adhesive subunit is carried at the distal end of this fibrillum. The *A. salmonicida* Fim locus does not have a homologue of the tip fibrillum subunit (PapE) and is therefore not expected to have this structure. The adhesive subunits of P pili (PapG) are larger than the structural subunits and have distinctive N-termini that carry the binding site. Because of its size and sequence, the *A. salmonicida fimE* gene likely encodes this function.

Despite the presence of 3 presumably functional pili loci, Tap and Flp (Boyd et al. 2008) and Fim (the present study), pili have never been visualized directly on the surface of *Aeromonas salmonicida*. Seroconversion and the expression of an anti-Tap antibody in rainbow trout experimentally infected with *A. salmonicida* strain A450 has been reported (Masada et al. 2002), suggesting that Type IV pili are expressed under certain *in vivo* conditions. The surface of strain A449 was extensively examined by atomic force microscopy following growth in high-iron and low-iron media and yet neither peritrichous or polar fimbrial structures were seen (Boyd et al. 2008). It may be that pili and fimbriae in *A. salmonicida* are either short enough that they do not protrude past the crystalline S-layer, or they are only assembled on contact with the host.

The Type 1 or chaperone/usher pili of Gram-negative bacteria contribute to virulence by allowing the bacterium to adhere to host tissues (reviewed in Pizarro-Cerdá & Cossart 2006). In the present study the contribution of Fim to the virulence of *Aeromonas salmonicida* was assessed using an isogenic mutant knockout strain with *fimABCDEF* deleted (Δ*fim*) in live animal challenges and ex vivo adherence and invasion assays. The entire *fimABCDEF* operon was deleted as the function of all the genes in the *fim* operon were not fully understood (for example *fimB*), and deletion of the whole operon ensured that all adhesive elements were absent in the Δ*fim* strain.

Atlantic salmon were challenged with bacteria by either immersion or i.p. injection (Table 4). The former route is considered a more authentic route of exposure than the latter, which bypasses the requirement for adherence and invasion. Perhaps unsurprisingly, given that *fim* purportedly encodes an adhesin, there were no differences in virulence between Δ*fim* and the parental strain, as measured by both cumulative morbidity and MTTM when administered by i.p. injection. Surprisingly, when the Δ*fim* strain was administered by immersion, cumulative morbidity was also unchanged with respect to the parental strain. There was, however, a significant increase in MTTM from 14.3 d (A449) to 20.7 d in the Δ*fim* strain. These results suggest that the Δ*fim* strain is equally as virulent as the parental strain, but is impaired in its ability to invade the host.

The stress test data also showed no significant difference between the covert infection levels between the parental strain and Δ*fim* (Table 5). However, MTTM was again significantly longer for Δ*fim* than it was for the isogenic parental strain. Changes in MTTM for Δ*fim* could be due to either a decrease in bacteria resident in the covertly infected fish or the requirement for attachment for the resident bacteria. The latter situation might arise if the covert bacteria are resident in the gut and must adhere and invade to cause infection.

The ex vivo assay confirmed the attenuated colonization properties of the Δ*fim* mutant. The gastrointestinal tract (GI) tract was chosen as it could be easily dissected and prepared in a sterile manner. Unlike the resident GI flora of the Atlantic salmon, A449 is chloramphenicol-resistant, allowing it to be discriminated easily (data not shown). It was not possible to use skin as, in contrast, many bacterial species present in the resident tank water are chloramphenicol-resistant, making the discrimination of otherwise unmarked *Aeromonas salmonicida* strains from the environmental background difficult, and it is impossible to adequately sterilize fish skin without destroying its structural integrity. Ex vivo *A. salmonicida* adherence and invasion in the salmonid GI has been reported (Ringø et al. 2004, Jutfelt et al. 2008) and most aeromonad species (e.g. *A. hydrophilia*, *A. caviae*, *A. bestiarum*) and the related taxon *Pleisomonas* are GI pathogens. In the adherence assay, 192% of the added A449 bacteria were adherent after 2 h at ca. 20°C. High adherence data are common in *A. salmonicida* studies, as the hydrophobic S-layer tends to drive bacteria onto surfaces. Furthermore the generation time of *A. salmonicida* at these temperatures is on the order of 2 h (data not shown), accounting for the increase in bacterial titer. The adherence of the Δ*fim* mutant strain was significantly lower than that for the parental strain (Fig. 1), indicating that the Fim pili are the major *A. salmonicida* adhesin for this tissue.

However, when invasion into the gut epithelium was determined, there was no significant difference between the 2 strains, with invasion rates between 35 and 50% of the adherent bacteria. Thus while the adherent properties of the Δ*fim* strain were significantly attenuated, once adhered to the host (presumably using another adhesin or gravity), it was equally as capable of invasion as the parental strain. Invasion of the epithelial layer by Gram-negative bacteria is often mediated by Type III secretion systems (T3SS). *Aeromonas salmonicida* has a functioning T3SS (Burr et al. 2002, 2003) and we have shown it to be critical for virulence in Atlantic salmon (Dacanay et al. 2006). The results of the present study suggest that the Fim pili allow *A. salmonicida* to come into close contact with the gut epithelium. This in turn would allow the T3SS to activate cell invasion mechanisms and internalize the bacterium, thereby protecting it from the added gentamicin. However, the data presented here only show that the invasive *A. salmonicida* were protected from the antibiotic and do not allow us to determine if the invasive bacteria were internalized within the GI epithelial cells or were resident in the intracellular space. The results of Jutfelt et al. (2008), however, suggest that once internalized within the epithelial cell, *A. salmonicida* can translocate across the epithelium to the serosal side of the intestinal wall, from where it can spread systemically.

In summary, the *fim* pili are major adhesive factors that allow *Aeromonas salmonicida* to adhere to salmon intestinal epithelial cells. Lack of *fim* causes only slight reduction in overall virulence however, reinforcing the notion that *A. salmonicida* virulence is complex and multifactorial.

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