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Contribution of Type IV Pili to the Virulence of *Aeromonas salmonicida* subsp. *salmonicida* in Atlantic Salmon (*Salmo salar* L.)▽†

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***Aeromonas salmonicida* subsp. *salmonicida*, a bacterial pathogen of Atlantic salmon, has no visible pili, yet its genome contains genes for three type IV pilus systems. One system, Tap, is similar to the *Pseudomonas aeruginosa* Pil system, and a second, Flp, resembles the *Actinobacillus actinomycetemcomitans* Flp pilus, while the third has homology to the mannose-sensitive hemagglutinin pilus of *Vibrio cholerae*. The latter system is likely nonfunctional since eight genes, including the gene encoding the main pilin subunit, are deleted compared with the orthologous *V. cholerae* locus. The first two systems were characterized to investigate their expression and role in pathogenesis. The pili of *A. salmonicida* subsp. *salmonicida* were imaged using atomic force microscopy and Tap- and Flp-overexpressing strains. The Tap pili appeared to be polar, while the Flp pili appeared to be peritrichous. Strains deficient in *tap* and/or *flp* were used in live bacterial challenges of Atlantic salmon, which showed that the Tap pilus made a moderate contribution to virulence, while the Flp pilus made little or no contribution. Delivery of the *tap* mutant by immersion resulted in reduced cumulative morbidity compared with the cumulative morbidity observed with the wild-type strain; however, delivery by intraperitoneal injection resulted in cumulative morbidity similar to that of the wild type. Unlike the pili of other piliated bacterial pathogens, *A. salmonicida* subsp. *salmonicida* type IV pili are not absolutely required for virulence in Atlantic salmon. Significant differences in the behavior of the two mutant strains indicated that the two pilus systems are not redundant.**

Aeromonas salmonicida subsp. *salmonicida* is a gram-negative, nonmotile, rod-shaped bacterium that is the etiologic agent of an infectious bacteremia-septicemia of salmonids known as furunculosis. Furunculosis is a complex disease that exists in different forms depending on the health, age, and species of fish. Many cell-associated and secreted factors have been implicated as virulence determinants in this bacterium (for reviews, see references 5 and 7). Despite this, much of the pathogenesis of *A. salmonicida* subsp. *salmonicida* remains poorly understood, and no single characteristic or phenotype was found only in virulent strains (16, 17, 30, 42) until the recent description of an *A. salmonicida* subsp. *salmonicida* type III secretion system (6, 11).

In order to better understand the virulence strategies employed by *A. salmonicida* subsp. *salmonicida*, we have focused on the initial stages of infection: adherence and invasion. Bacteria use complex intercellular mechanisms and specific and nonspecific adhesins to achieve these aims (34). The most well-studied *A. salmonicida* subsp. *salmonicida* adhesin is the surface layer or S-layer, sometimes referred to as the A-layer (additional layer) (33). This layer is a nonspecific but important factor for adherence due to its hydrophobic nature.

Pili allow bacteria to attach to solid surfaces, including host tissues, and are considered important virulence factors in many pathogenic bacteria (36). Pili are filamentous, extracellular organelles that may be peritrichous or polar and may be present singly or in bundles. There are currently four recognized types of pili that have been found on gram-negative bacteria, and type IV pili have often been implicated in host attachment and virulence. Type IV pili are subdivided into types IVa and IVb on genetic and morphological grounds. Type IVa (non-bundle-forming) pili are important in a number of bacterial processes, including flagellum-independent "twitching" motility, DNA uptake, biofilm formation, and adherence to substrates, including host cells (27). The Tap pilus, a type IVa pilus that is encoded in part by the *tapABCD* operon, has been described for multiple aeromonad species (2, 32). It has been implicated in *A. salmonicida* subsp. *salmonicida* virulence in a rainbow trout model (*Oncorhynchus mykiss*) on the basis of an increase in the 50% lethal dose (LD_{50}) for a *tapA* mutant as assessed by intraperitoneal injection (26).

Analysis of the *A. salmonicida* subsp. *salmonicida* A449 genome sequence has revealed the presence of two type IV pilus systems in addition to the Tap system. In this study, Flp-like and mannose-sensitive hemagglutinin (MSHA)-like type IV pilus systems not previously identified in this species are described. The contribution to virulence of two of the pilus systems was assessed using a live animal model and isogenic knockout mutant strains. In addition, pili of *A. salmonicida* subsp. *salmonicida* were visualized for the first time using atomic force microscopy (AFM) and pilin-overexpressing strains.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
A. salmonicida subsp. *salmonicida* strains		
A449	Virulent, Cm^r	29
02-02	A449 *flpA*::Km, Cm^r Km^r	This study
02-06	Lab-derived mutant of A449, O side chain negative, surface layer secretor, Cm^r	43
02-10	A449 *tapA*::KO, Cm^r Ap^r	This study
03-01	A449 *flpA*::Km *tapA*::KO, Cm^r Km^r Ap^r	This study
P. aeruginosa PAK	Wild type	D. Bradley
E. coli strains		
TOP10	K-12	Invitrogen
DH10B	K-12 for large plasmids	Invitrogen
EC100D pir-116	K-12 *pir-116*	Epicenter
BW20767	K-12 pir^+, conjugation positive	28
Plasmids		
pUTkm1	Cloning vector, Ap^r Km^r	13
pCRScript-Amp	oriE1, Ap^r	Stratagene
pAH34	oriR6Kγ mobRP4, Ap^r	28
pKO	pAH34 with tmRNA tag	This study
pKO-*tapA*	Internal fragment of *tapA* in pKO, in frame with tmRNA tag	This study
pGP704	oriR6Kγ mobRP4, Ap^r	21
pJW109-pir	pGP704 with *flpA*-flanking regions surrounding kanamycin resistance cassette, Km^r Ap^r	This study
pMMB67EH	Low-copy-number, broad-host-range expression vector, mobRSF1010, Ap^r	20
pMMB67EH.Km	pMMB67EH with Ap^r replaced by Km^r	This study
ptapA.K	pMMB67EH.Km carrying complete *tapA* gene	This study
pflp1.K	pMMB67EH.Km carrying complete *flp-1* gene	This study
pBACe3.6	Large insert vector, Cm^r	19
pAs01a04	Derivative of pBACe3.6 with 71.2-kb insert carrying the *flp* locus	This study

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacteria and plasmids used in this study are listed in Table 1. The parental strain for all knockouts was *A. salmonicida* subsp. *salmonicida* strain A449, which was originally isolated from a natural furunculosis epizootic. All *Aeromonas* strains were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco) for 3 days at 17°C. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) or on LB agar at 37°C. Antibiotics were used at the following concentrations: for *E. coli*, 100 μg/ml ampicillin, 25 μg/ml kanamycin, and 20 μg/ml chloramphenicol; and for *A. salmonicida* subsp. *salmonicida*, 50 μg/ml ampicillin, 200 μg/ml kanamycin for selection, 50 μg/ml kanamycin for maintenance, and 20 μg/ml chloramphenicol. Iron concentrations were reduced by addition of 120 μM 2,2′-dipyridyl (Sigma).

DNA techniques. DNA manipulations were performed by using standard genetic and molecular techniques (1). Genomic DNA from A449 was isolated using a PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN) and was used as the PCR template for construction of all mutant strains. Oligonucleotides were prepared by Integrated DNA Technologies Inc. (Coralville, IA). PCR was performed with either *rTaq* (Amersham) or *Pfu* (MBI Fermentas) by following the manufacturer's directions. During the *A. salmonicida* subsp. *salmonicida* genomic sequencing project, a BAC library of *A. salmonicida* subsp. *salmonicida* genomic DNA was made. Briefly, *A. salmonicida* subsp. *salmonicida* A449 genomic DNA was partially digested with EcoRI to generate fragments ranging from 50 to 150 kb long; these fragments were ligated into the vector pBACe3.6 (19) digested with the same enzyme. The library was transformed into *E. coli* DH10B. BAC pAs01a04 was one of the clones obtained and contained a 71-kb insert that corresponds to the chromosomal region between bp 3126400 and 3197600 (see Fig. 2).

RT-PCR. *A. salmonicida* subsp. *salmonicida* grown under a variety of conditions was harvested in RNAprotect bacterial reagent (Qiagen). Total RNA was extracted with RNeasy mini kits (Qiagen) used according to the manufacturer's directions. DNA was removed with DNA-free (Ambion). cDNA was made from the RNA using random decamers (Ambion) and SuperScript II reverse transcriptase (RT) (Invitrogen). The primers used for amplification of *tapA* and *flpA* are shown in Table 2.

Bacterial conjugation. *E. coli* BW20767 carrying the appropriate mobilizable plasmid was grown in LB medium with the appropriate antibiotic overnight at 37°C. *A. salmonicida* subsp. *salmonicida* was grown in TSB with chloramphenicol for 3 days at 17°C. One milliliter of a culture 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. The resulting thick suspensions of the bacteria were mixed together thoroughly and spotted in the center of a TSA plate with no antibiotic. After 2 days of incubation at 17°C, the spot was removed with a sterile toothpick and resuspended in 1 ml TSB. Appropriate dilutions were plated on TSA with chloramphenicol (to select against *E. coli*) and the appropriate antibiotic to select for the transformed colonies. Large colonies were picked, streaked onto fresh plates, and passaged two more times.

Construction of mutant strains. (i) *tapA*::KO strain. The integrative knockout vector pKO was created so that the complete plasmid could be inserted into the gene of interest in such a way that the 3′ fragment of the affected gene would make an in-frame fusion to a transfer-messenger (tmRNA) tag so that any potentially translated peptide would be recognized by Clp proteases and degraded. The tmRNA tag was created using complementary oligonucleotides based on the sequence of *A. salmonicida* subsp. *salmonicida* tmRNA (45) with NotI and SstI sites at either end (Table 2). The tmRNA tag was inserted into the NotI and SstI sites of the polylinker of pCRScript-Amp (Stratagene) in such a way that the *lacZ*α fragment was not disrupted to create pCRscript-tmRNA. The new polylinker and *lacZ*α region of pCRScript-tmRNA was used to replace the polylinker of pAH34 (28) using PvuII sites to create pKO. Plasmid pAH34 and its derivative pKO are mobilizable, *pir*-dependent, ampicillin-resistant plasmids that allow blue/white selection of cloned inserts and cannot replicate in *A. salmonicida* subsp. *salmonicida.*

An internal fragment of the *tapA* gene was amplified from A449 genomic DNA using *Pfu* polymerase and primers tapA-1 and tapA-2. The fragment was blunt end ligated into the SrfI site of vector pKO to make an in-frame fusion to the tmRNA tag. The pKO-*tapA* plasmid was conjugated into A449 from *E. coli* pir^+ mating strain BW20767 as described above, and single-crossover integrants (in which the *tapA* gene was interrupted by the pKO plasmid) were selected by

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′)[a]	Description
tmRNA tag	**GGCCGCAACTAGTTGCA**GCAAACGACGAAAACTAC GCACTAGCAGCTTAATAA**CAGAGCT**	Coding strand of tmRNA tag
tmRNA tag	**CTG**TTATTAAGCTGCTAGTGCGTAGTTTTCGTCGT TTGC**TGCAACTAGTTGC**	Noncoding strand of tmRNA tag
tapA-1	ATTGCACTTCCTGCATATCAGACC	Used to amplify internal fragment for knockout
tapA-2	CGGGACCAGTATATATGTGTATGC	Used to amplify internal fragment for knockout
tapA-3	**AGGA**GAATTCACATGAAGAAGCAATCAGGC	Used for RT-PCR of *tap* mRNA and to clone complete *tapA* gene
tapA-4	**TATT**GGATCCAGAGGTCATGCGTTAGCAG	Used for RT-PCR of *tap* mRNA and to clone complete *tapA* gene
flpA-1	CGGCCTTGCATGCCAGCAAGGTTGCG	Used to amplify 5′ flanking region of *flpA* for knockout
flpA-2	**CCA**AGATCTGAGCGCTGTCACTTGCCCGCGGC	Used to amplify 5′ flanking region of *flpA* for knockout and to clone complete *flp-1* gene
flpA-3	**GCG**AGATCTCTGACCACTCCTCTATTTATGTAGTC	Used to amplify 3′ flanking region for knockout
flpA-4	GCTTGCTGGTGAGGATCACATCCACCC	Used to amplify 3′ flanking region for knockout
flpA-F	CATATTGATGGCGTGCTGGT	Used for RT-PCR of *flp* mRNA
flpA-R	CACAAAATGGCGAATCCATA	Used for RT-PCR of *flp* mRNA
flpA-5	**GGAC**GAATTCATGAAGATGCGAGTTTGGG	Used to clone complete *flp-1* gene
MMB01	CATCATAACGGTTCTGGC	Used to sequence or amplify insert of pMMB plasmids
MMB02	GAAAATCTTCTCTCATCCGC	Used to sequence or amplify insert of pMMB plasmids

[a] Restriction sites are underlined, and non-sequence-specific regions are indicated by bold type.

growth on ampicillin and passaged in fresh medium three times. Proper integration was checked by PCR amplification of genomic DNA using primers within the plasmid and flanking the insertion. As determined by immunoblotting (Fig. 1), no intact TapA subunits were produced in this strain.

(ii) *flpA*::Km strain. The *flpA* gene, encoding the putative prepilin peptidase, was interrupted by insertion of a kanamycin resistance gene cassette. Fragments flanking the *flpA* gene were amplified from genomic DNA using the primers shown in Table 2. Primers flpA-2 and flpA-3 included BglII sites that were used subsequently to introduce the 1.5-kb BamHI fragment carrying the kanamycin resistance cassette from pUTKm1. In a multistep process, the three fragments were cloned into the mobilizable, ampicillin-resistant, *pir*-dependent vector pGP704 to generate pJW109-pir. pJW109-pir was conjugated into A449 from BW20767 as described above, and single-crossover integrants were selected by growth on ampicillin. Double-crossover segregants were selected by isolation of ampicillin-sensitive, kanamycin-resistant colonies. Proper integration was checked by PCR amplification of genomic DNA using primers flanking the insertion.

(iii) *flpA*::Km/*tapA*::KO strain. The double *flpA*::Km/*tapA*::KO mutant strain was constructed by introducing the *tapA* mutation plasmid, pKO-*tapA*, into the *flpA*::Km strain.

Construction of pilin-overproducing strains. The pilin genes were amplified from A449 genomic DNA using primers flpA-2 and flpA-5 for *flp-1* and primers tapA-3 and tapA-4 for *tapA* (Table 2). The fragments were cloned into the broad-host-range expression vector pMMB67EH.Km using the EcoRI and BglII or BamHI sites provided by the amplification primers. pMMB67EH.Km was created by cloning the 1.7-kb BamHI fragment carrying the kanamycin resistance gene from plasmid pUTkm1 into the β-lactamase gene of pMMB67EH. The plasmids were conjugated into A449 and an O-side-chain-deficient, S-layer-deficient derivative of A449, IMB 02-06, as described above. Transformation was confirmed by PCR amplification of total genomic DNA with primers complementary to the vector flanking the cloned insert (MMB01 and MMB02).

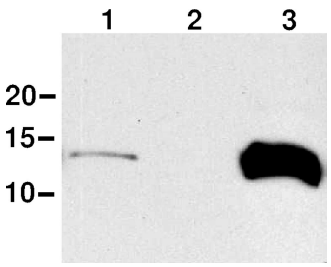

FIG. 1. Immunoblot to confirm deletion and overexpression of *tapA*. Lane 1, A449(pMMB67EH.K); lane 2, *tapA*::KO(pMMB67EH.K); lane 3, A449(ptapA.K). All strains were grown in TSB with 50 μg/ml kanamycin and 0.05 mM IPTG. The positions of molecular mass standards (in kDa) are indicated on the left.

Immunoblotting. Interruption of the *tapA* gene and overexpression of the TapA pilin in A449 (ptapA.K) were confirmed by immunoblotting with anti-TapA antibody (kindly donated by Mark Strom, National Oceanic & Atmospheric Administration, Seattle, WA) (Fig. 1). Briefly, whole-cell lysates were separated on a 15% Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (37), transferred to a polyvinylidene difluoride membrane, and visualized with anti-TapA horseradish-peroxidase conjugated secondary antibody and chemiluminescence (1). Overexpression of TapA in 02-06 and 02-10 was confirmed in the same way (data not shown). Antiserum to the Flp1 pilin is currently unavailable.

AFM. For AFM imaging, *A. salmonicida* subsp. *salmonicida* cells were grown in TSB with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), 2,2′-dipyridyl (120 μM), and isopropyl-β-D-thiogalactopyranoside (IPTG) (50 μM) for 3 days at 17°C. *E. coli* cells were grown in LB medium with chloramphenicol (20 μg/ml) and 2,2′-dipyridyl (120 μM) overnight at 37°C. After the bacteria were washed in phosphate-buffered saline (pH 7.4) and resuspended in the same buffer, 1 drop of bacterial suspension was placed on a freshly cleaved mica surface. Excess fluid was drawn off, and the bacteria were allowed to adsorb for 15 min, after which the sample was rinsed with deionized water and air dried at room temperature for 30 min. Images were recorded using the contact mode at room temperature with a Molecular Imaging AFM microscope (Tempe, AZ). Images were recorded in both height and deflection modes. While height images provided quantitative information on sample surface topography, deflection images often had higher contrast of morphological details. V-shaped cantilevers with oxide-sharpened Si_3N_4 tips were used with spring constants of 0.01 N/m. High-resolution images were recorded for dried preparations with optimized feedback parameters at a scan frequency of 4 Hz. The diameters of the pilus filaments were measured by using AFM cross sections.

Animal care. All relevant animal care committees approved the animal procedures, which were conducted under Canadian Council on Animal Care guidelines. As "death as an endpoint" is not considered acceptable under these guidelines (14; www.ccac.ca), morbid animals were euthanized when a set of previously agreed limiting clinical signs were reached. Thus, the challenge data are reported below as morbidity data rather than mortality data to reflect this.

Juvenile Saint John River or Sackville River stock Atlantic salmon were

obtained from Nova Scotia hatcheries certified under Canadian Fish Health Protection regulations. They were stocked in 100-liter fiberglass resident tanks at a stocking density of ca 2.4 kg/tank and maintained at 14 ± 2°C in flowthrough dechlorinated municipal water under subdued lighting. They were fed a maintenance ration of a commercially available extruded feed (Signature Salmon Ration; Shurgain, Truro, Nova Scotia, Canada) daily which was equivalent to 1% of the body weight. Feeding was suspended for 1 day before manipulation and for 1 day after manipulation.

In vivo culture of *A. salmonicida* subsp. *salmonicida*. In vivo culture was performed as described previously (10). Briefly, sections of dialysis tubing filled with *A. salmonicida* subsp. *salmonicida* cultures were surgically implanted into the peritoneum of Atlantic salmon. After 22 h the implants were recovered from the fish, and the bacteria were transferred to RNAprotect bacterial reagent (Qiagen) for RT-PCR analysis.

Challenge. Both intraperitoneal injection and immersion bacterial challenges of Atlantic salmon were conducted as described elsewhere (11). The parental, *tapA*::KO, and *flp*::Km strains were tested by immersion challenge in Saint John River Atlantic salmon. The *flp*::Km/*tapA*::KO strain was tested by immersion challenge in Sackville River Atlantic salmon. All three pilin mutant strains were tested by intraperitoneal injection into Sackville River Atlantic salmon.

For immersion challenges, two tanks per group with 40 fish per tank were used; the weight of the fish was ca. 60 g each. For intraperitoneal challenge, two tanks per group with ca. 25 fish per group were used; the weight of the fish was ca. 200 g each.

Morbid animals were euthanized with an overdose of tricaine methanosulfonate (Syndel Laboratories). Posterior kidney samples were placed onto TSA supplemented with 20 μg/ml chloramphenicol, as recommended for detection of *A. salmonicida* subsp. *salmonicida* in clinical infections (38). Bacteria cultivated from the posterior kidney were subsequently cultured on TSA supplemented with kanamycin or ampicillin.

For assessment of the competitive index (CI), Sackville River stock Atlantic salmon were exposed by immersion to 10^6 CFU/ml of A449 and one of the three pilus mutant strains (*tapA*::KO, *flpA*::Km, or *tapA*::KO/*flpA*::Km). The posterior kidney was sterilely dissected from moribund animals after the fish were euthanized. After the posterior kidney was weighed, it was homogenized in 1 ml of sterile phosphate-buffered saline, and the bacterial titer was determined by direct colony counting on TSA supplemented with chloramphenicol, ampicillin, or kanamycin as described above. Counts were normalized to obtain values expressed in CFU per milligram of kidney. The CI was calculated as follows: CFU of mutant mg^{-1}/CFU of parent mg^{-1}.

Statistical differences in cumulative morbidity between groups were assessed by the G test (a modified χ^2 test). Mean times to death were compared by an unpaired *t* test using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers. The nucleotide sequences for the loci described in this study have been deposited in the GenBank database under the following accession numbers: *tapABCD*, DQ396478; *tppABCD tapY1Y2 tppE*, DQ396479; *tppF*, DQ396480; *tapMNOPQ*, DQ396481; *tapTU*, DQ396482; *tapW*, DQ396483; *tapF*, DQ396484; *tapV*, DQ396485; MSHA gene, DQ396486; and *flp*, DQ396487. The accession number for the complete *A. salmonicida* subsp. *salmonicida* A449 genome is NC_009348.

RESULTS

Analysis of the genome sequence of *A. salmonicida* subsp. *salmonicida* A449 revealed the presence of genes encoding three type IV pilus complexes: Tap, Flp, and MSHA (Fig. 2). The *tapABCD* operon encoding the Tap pilin and three accessory genes were described previously for *A. salmonicida* subsp. *salmonicida* and some other *Aeromonas* species (2, 24), while the Flp pilus, which exhibits homology to pili of *Actinobacillus actinomycetemcomitans* and *Caulobacter crescentus*, has not been described previously for *Aeromonas* species. The third system is very similar to the *Vibrio cholerae* MSHA pilus. This system has been previously described for other aeromonad species (25) but not for *A. salmonicida* subsp. *salmonicida*.

Pilin genes and operons. The *tapABCD* locus of *A. salmonicida* subsp. *salmonicida* and other *Aeromonas* species (3, 32) is very similar to the *pilABCD* locus of *Pseudomonas aeruginosa*. In *P. aeruginosa*, an additional 18 genes that are scattered

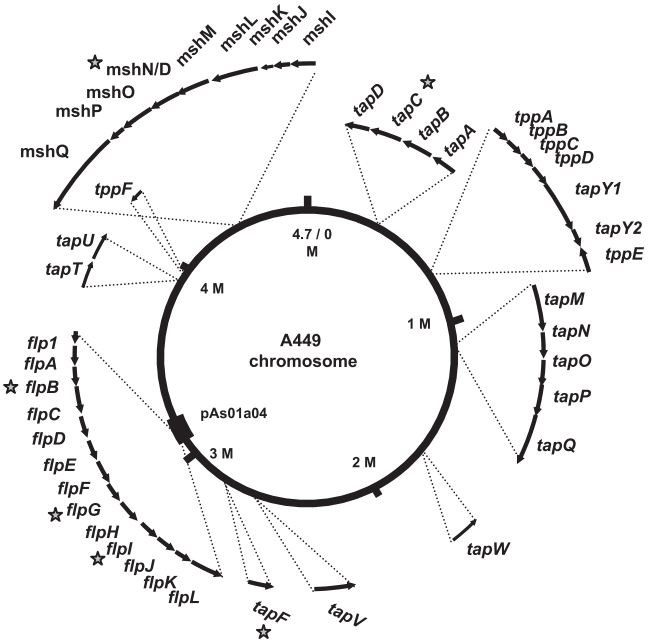

FIG. 2. Genome of *A. salmonicida* subsp. *salmonicida* showing the pilus system genes. The arrows indicate the approximate sizes and orientations of the genes. The numbering of the chromosome reflects the origin of replication. The thick line on the chromosome at 3.1 Mbp shows the extent of BAC pAs01a04 that carries the Flp locus. The stars indicate disrupted genes.

throughout the chromosome in several loci are required for pilus biogenesis (27). Homologues of these additional genes have been identified in the *A. salmonicida* subsp. *salmonicida* genome (Fig. 2). Table 3 shows the predicted gene products of the *A. salmonicida* subsp. *salmonicida* Tap system based on their similarity to the *P. aeruginosa* Pil system. When possible, the *P. aeruginosa* nomenclature is used (e.g., *tapA* is the homologue of *pilA*); the only exception is the pseudopilin genes, which are designated *tpp* (*t*ap *p*seudo*p*ilin).

An *A. salmonicida* subsp. *salmonicida* locus with similarity to *flp* gene clusters of *A. actinomycetemcomitans* and *Haemophilus ducreyi* was also identified (Fig. 2 and Table 4). This gene cluster encodes the biosynthetic proteins for a type IV pilus, and the genes are designated *flp*, *rcp*, *cpa*, or *tad* depending on the system. For clarity, the *A. salmonicida* subsp. *salmonicida* genes are designated *flpA* through *flpL*. Some of these pilus systems have more than one major pilin subunit. *A. actinomycetemcomitans* has two subunits, encoded by *flp-1* and *flp-2*, and *H. ducreyi* has three subunits, encoded by *flp-1*, *flp-2*, and *flp-3*. Even though *A. salmonicida* subsp. *salmonicida* has only one major pilin gene, in keeping with the nomenclature used for these organisms, a numerical suffix is used for the pilin subunit, Flp1.

A cluster of genes with similarity to the genes encoding MSHA pili of *V. cholerae* was also identified in A449 (Fig. 2 and Table 5). However, analysis of the *A. salmonicida* subsp. *salmonicida* A449 locus showed that 8 of 16 genes, including the gene encoding the major pilin subunit, are missing or partially deleted. The deletion creates a fused gene with the 5′ end of *mshN* and the 3′ end of *mshD*. The absence of *mshA*,

TABLE 3. Predicted gene products of the *A. salmonicida* subsp. *salmonicida* Tap pilus system based on similarity to the *P. aeruginosa* Pil system

A. salmonicida subsp. *salmonicida* gene product	Homologue in *P. aeruginosa*	% Amino acid identity[a]	Predicted function, location, and/or Gsp homologue[b]
TapA	PilA	32	Pilin subunit
TapB	PilB	56	Cytoplasm, traffic ATPase, GspE
TapC	PilC	50	Inner membrane, GspF
TapD	PilD	58	Inner membrane, prepilin peptidase
TapM	PilM	39	Inner membrane, MreB homologue
TapN	PilN	34	Inner membrane, GspL
TapO	PilO	40	Inner membrane
TapP	PilP	32	Outer membrane, pilotin, lipoprotein
TapQ	PilQ	38	Outer membrane, secretin
TapT	PilT	75	Cytoplasm, twitching ATPase
TapU	PilU	56	Cytoplasm, twitching ATPase
TapW	PilU	52	Cytoplasm, twitching ATPase
TppA	PilE	37	Pseudopilin
TppB	PilV	12	Pseudopilin
TppC	PilW	9	Pseudopilin
TppD	PilX	12	Pseudopilin
TppE	FimT	19	Pseudopilin
TppF	FimU	12	Pseudopilin
TapY1	PilY1	23	Outer membrane
TapY2	PilY2	18	Periplasm
TapV	FimV	26	Inner membrane, regulator
TapF	PilF	33	Outer membrane, tetratricopeptide repeat lipoprotein

[a] The percent amino acid identity of frameshifted gene products was calculated by reading through the frameshifts.

[b] The cellular location was predicted using the PSORTb program (http://www.psort.org/psortb/) and the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/).

encoding the major pilin subunit, as well as several critical genes involved in pilin assembly, led us to expect that the MSHA-like locus is not expressed in *A. salmonicida* subsp. *salmonicida* A449.

Six of the identified pilus genes have authentic frameshift errors compared with their closest orthologues that may affect their translation. Two genes of the Tap system show frameshifts; *tapC* has a 1-bp deletion that is predicted to cause premature termination of the protein, and *tapF* has a 7-bp duplication at the 5′ end, leading to a very short predicted protein. There are three genes in the Flp locus with frameshift errors; *flpB* and *flpG* have 1-bp changes that introduce stop codons, while *flpI* has a 4-bp duplication that moves the gene out of frame. Compared with the *V. cholerae* gene, *mshI* is either frameshifted or expressed as two separate genes. The disrupted genes are indicated in Fig. 2.

Regulation of pilus gene expression. To investigate expression of the *flp* and *tapABCD* operons, which encode the pilin subunits, RT-PCR experiments were carried out with primers specific for the *flpA* and *tapA* genes and RNA from bacteria grown under a number of different conditions (Fig. 3). *flpA* was poorly transcribed in bacteria grown in TSB, but transcription was greatly increased when the bacteria were grown in vivo in implants in Atlantic salmon. mRNA induction was seen as early as 6 h after implantation (data not shown), and mRNA was highly expressed by 22 h (Fig. 3). Since most animals, including fish, maintain a low concentration of free iron in the serum and many pathogenic bacteria respond to low iron concentrations with increased expression of virulence factors, we asked whether low iron was the signal to which *flp* transcription responded. This was indeed the case as the expression of *flp* mRNA increased dramatically when the iron-specific chelator 2,2′-dipyridyl was added to TSB (Fig. 3).

In contrast to the regulated Flp pilus, the *tapA* gene was constitutively expressed under all conditions tested, including in vivo and in media with low iron concentrations (Fig. 3). Increased temperature, variations in the salt concentration, or the growth phase had no effect on the transcription of either pilus operon (data not shown).

Challenge experiments. To investigate the role of pili in *A. salmonicida* subsp. *salmonicida* infection, isogenic mutants of strain A449 were created. Genes were inactivated using one of two methods. Plasmid pKO was introduced into the *tapA* gene in a single-crossover event to make an ampicillin-resistant, Tap-deficient strain. Alternatively, a double-crossover technique was used to introduce a kanamycin resistance cassette into *flpA*. Both methods produced stable mutants. The *flpA*::Km/*tapA*::KO double mutant was constructed as well. Reversion (the appearance of bacteria with inappropriate antibiotic

TABLE 4. Predicted gene products of the *A. salmonicida* subsp. *salmonicida* Flp pilus system based on similarity to the *A. actinomycetemcomitans* Flp system

A. salmonicida subsp. *salmonicida* gene product	Homologue in *A. actinomycetemcomitans*	% Amino acid identity	Predicted function, location, and/or Gsp homologue
Flp1	Flp1	27	Pilin subunit
FlpA	TadV	27	Inner membrane, prepilin peptidase
FlpB	RcpC	19	Periplasm, unknown function
FlpC	RcpA	27	Outer membrane, secretin
FlpD	RcpB	18	Periplasm, unknown function
FlpE	TadZ	17	Cytoplasm, ATPase, GspA
FlpF	TadA	45	Cytoplasm, traffic ATPase, GspE
FlpG	TadB	22	Inner membrane, GspF
FlpH	TadC	22	Inner membrane, GspF
FlpI	TadD	22	Outer membrane, tetratricopeptide repeat lipoprotein
FlpJ	TadE	8	Pseudopilin
FlpK	TadF	10	Pseudopilin
FlpL	TadG	7	Pseudopilin

TABLE 5. Predicted gene products of the *A. salmonicida* subsp. *salmonicida* MSHA-type cluster based on similarity to the *V. cholerae* MSHA system

A. salmonicida subsp. *salmonicida* gene product	Homologue in *V. cholerae*	% Amino acid identity	Predicted function, location, and/or Gsp homologue
MshI	MshI	25	Inner membrane, GspL
MshJ	MshJ	35	Inner membrane, GspM
MshK	MshK	33	Periplasm, unknown function
MshL	MshL	49	Outer membrane, secretin
MshM	MshM	50	Cytoplasm, ATPase, GspA
MshN/D	MshN N terminus and MshD C terminus		Fusion created by deletion
Partially deleted	MshN		Outer membrane, tetratricopeptide repeat lipoprotein
Absent	MshE		Cytoplasm, traffic ATPase, GspE
Absent	MshG		Inner membrane, GspF
Absent	MshF		Unknown function
Absent	MshB		Pseudopilin
Absent	MshA		Pilin subunit
Absent	MshC		Pseudopilin
Partially deleted	MshD		Pseudopilin
MshO	MshO	31	Pseudopilin
MshP	MshP	24	Pseudopilin
MshQ	MshQ	39	Outer membrane, similar to PilY1

sensitivities, indicating a loss of either pKO or the kanamycin cassette) was not seen. All pilus mutant strains had the same growth characteristics as the wild type in TSB at 17°C.

Two immersion challenge experiments were conducted. In the first experiment the *tapA*::KO and *flpA*::Km strains were used to challenge Saint John River Atlantic salmon (Fig. 4A to D). In the second experiment the *tapA*::KO/*flpA*::Km double mutant was used to challenge Sackville River Atlantic salmon (Fig. 4E to F). All three pilus mutant strains caused clinical disease that was visually indistinguishable from the disease caused by the parental strain. In the single-mutant challenges, the parental strain caused 40% cumulative morbidity with a mean time to morbidity (MTTM) of 20.8 days (Fig. 4A to D). In the group exposed to the *flpA*::Km strain, neither the cumulative morbidity (29%) nor the MTTM (24.3 days) was significantly different from the value obtained for the parent (Fig. 4A and B). However, the cumulative morbidity was significantly lower in the group exposed to the *tapA*::KO strain (25%) (Fig. 4C). The MTTM was also shorter in this group (16.2 days), but it was not significantly shorter (Fig. 4D). The virulence of the *tapA*::KO/*flpA*::Km double mutant, tested in a separate challenge, was similar that of the *tapA*::KO strain. In this challenge both the cumulative morbidity (76%) and the MTTM (11.8 days) were significantly different for the group exposed to the double mutant than for the group exposed to the parental strain (91% and 6.1 days) (Fig. 4E and F).

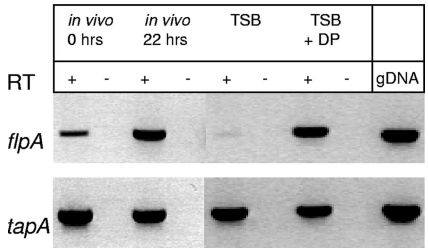

FIG. 3. RT-PCR of *A. salmonicida* subsp. *salmonicida flpA* and *tapA* genes in cultures grown under various conditions, including in vivo (bacteria from implants left in fish peritoneal cavities for 0 or 22 h), in TSB, and in TSB containing 120 μM 2,2′-dipyridyl (TSB + DP). Plus and minus signs indicate the presence and absence of the reverse transcription reaction. gDNA, genomic DNA.

The virulence of all three mutant strains and the virulence of the parental strain were also tested by intraperitoneal injection into Sackville River Atlantic salmon (Fig. 4G to L). With this route of infection there were no significant differences in cumulative morbidity between the parental strain (45%) and the *flpA*::Km, *tap*A::KO, or *tapA*::KO/*flpA*::Km strain (50, 39, and 37%, respectively). There was also no difference in MTTM between the parental strain (7.3 days) and the *flpA*::Km strain (8.1 days). However, the MTTM was significantly longer for the *tapA*::KO strain (8.7 days) and the *tapA*::KO/*flpA*::Km strain (9.8 days).

In order to more accurately assess any attenuation in virulence caused by a lack of pili, CIs of the mutants and the wild type were determined. In a separate challenge experiment, groups of fish were exposed by immersion to a mixture of equal amounts of the parental strain and one of the mutant strains. When the animals became moribund, they were euthanized, their posterior (renal) kidneys were sterilely dissected as described previously (38), and the bacteria were counted. Unlike other organs, the posterior kidney can be accessed sterilely, making it the preferred site for microbiological testing. The results are shown in Fig. 5. There were nine morbid animals following challenge with A449 and the *flpA*::Km strain, and the CI was 0.96 ± 0.38 (mean ± standard deviation), indicating that the *flpA*-deficient and wild-type strains were equally able to invade and survive within the posterior kidney. In marked contrast, however, the two *tapA* mutants had CIs of much less than 1, showing that they had decreased virulence. In the group exposed to A449 and the *tapA*::KO strain there were 12 moribund fish, and the CI was 0.07 ± 0.16 (mean ± standard deviation). In the group exposed to A449 and the double mu-

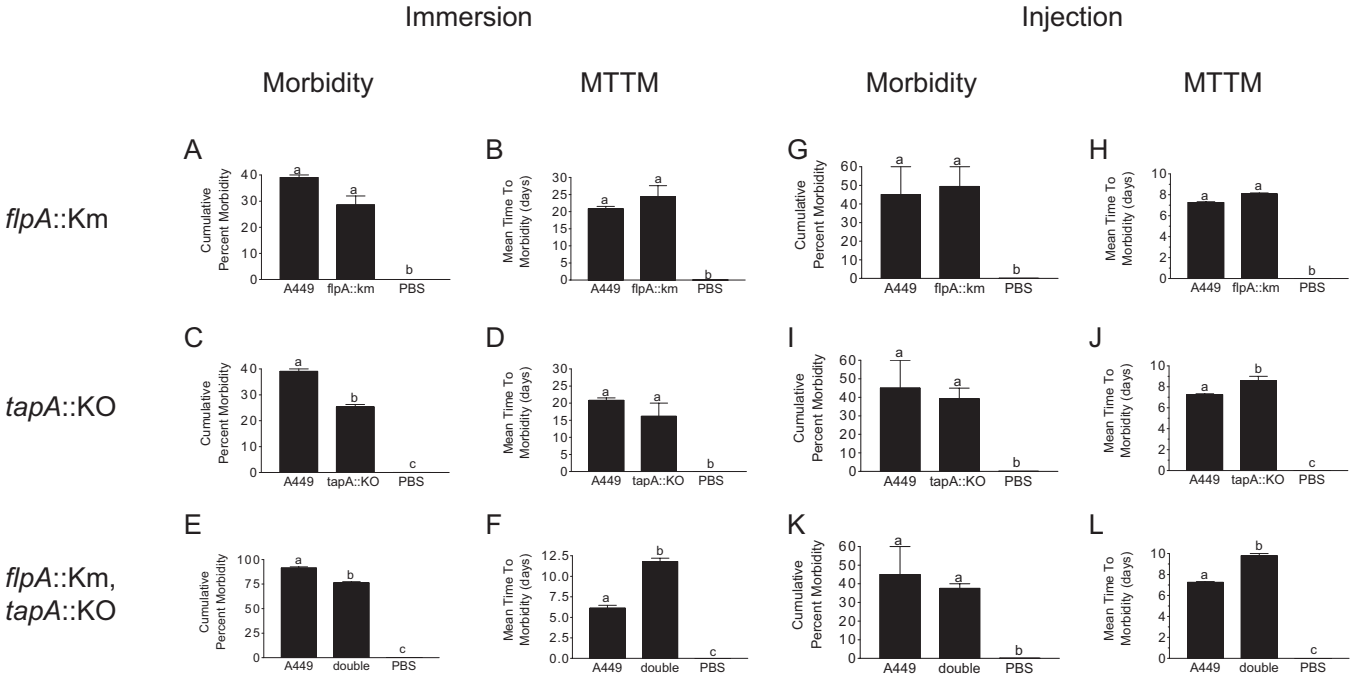

FIG. 4. Effects of single and double type IV pilus mutants used to challenge Atlantic salmon by immersion or intraperitoneal injection. The data are the cumulative morbidity and MTTM obtained using duplicate tanks. Different letters above the bars indicate statistical significance; cumulative morbidity values were compared by using the G test, and MTTM values were compared by using the *t* test. A *P* value of <0.05 was considered significant for both tests. PBS, phosphate-buffered saline.

tant there were 15 moribund fish, and the CI was 0.12 ± 0.26 (mean $\pm$ standard deviation). These results indicate that a lack of *tapA* leads to a reduced ability to invade and survive within the host.

Visualization of pili on the surface of *A. salmonicida* subsp. *salmonicida*. Despite several attempts by us and others, pili have never been visualized on the surface of *A. salmonicida* subsp. *salmonicida* using electron microscopy or immunofluorescence. We chose to use AFM to detect pili on *A. salmonicida* subsp. *salmonicida* (Fig. 6) because of our experience in detecting bacterial pili with this technique (40). The ability of AFM to visualize bacterial pili was confirmed by imaging *P. aeruginosa* strain PAK, which is known to express type IV pili. High-resolution AFM clearly demonstrated the presence of the pili on *P. aeruginosa* and also provided considerable structural detail, such as pilus width, length, and bundling characteristics (Fig. 6C). The pili in the images were between 6 and 8 nm wide and several thousand nanometers long, consistent with the size of *P. aeruginosa* pili measured by other techniques (5 to 8 nm wide and several thousand nanometers long) (9). Scans of the surface of *A. salmonicida* subsp. *salmonicida* revealed the closely packed tetragonal array of the surface layer (Fig. 6D). No pili or other extracellular structures were visible extending beyond this layer. In order to observe pili that might be hidden below the S-layer, a lipopolysaccharide O-side-chain-deficient (rough) mutant of *A. salmonicida* subsp. *salmonicida*, strain 02-06, was imaged. This strain was unable to secure the S-layer protein, VapA, to the cell surface (43), and consequently its surface was devoid of both the VapA protein and lipopolysaccharide (Fig. 6A). Pili were undetectable on this strain.

In order to visualize pili that might be too short to be seen normally, the *flp-1* and *tapA* pilin genes were overexpressed in *A. salmonicida* subsp. *salmonicida* by placing them under the control of the strong, inducible *tac* promoter and conjugating the overproducing plasmids into *A. salmonicida* subsp. *salmonicida* 02-06. Expression of the pilin subunits was induced by IPTG (50 μM). In order to induce expression of the other *flp* pilus assembly genes on the chromosome, the cultures were grown in low-iron medium using 2,2′-dipyridyl (120 μM). Under these conditions, AFM showed the appearance of long piluslike structures extending from the surface of the bacteria. Figures 6E to H show *A. salmonicida* subsp. *salmonicida* 02-06 overexpressing the *tapA* pilin gene. The pili seem to have a polar origin and have a ropelike appearance with many fibers wound together, although individual fibers were visible at the ends of the structures. The width of these bundles was between 15 and 35 nm, while the width of individual fibers varied from 3.5 to 4.6 nm, which is thinner than *P. aeruginosa* pili. Figures 6J to M show *A. salmonicida* subsp. *salmonicida* 02-06 overexpressing the *flp-1* pilin gene. The pili appear to be peritrichous, and most often a pilus from one bacterium was in close, antiparallel contact with a pilus from another bacterium. The width of these pili was approximately 15 nm. We also imaged *E. coli* DH10B carrying the BAC clone pAs01a04 (Fig. 6N to Q), which contains the entire *flp* locus in its 71-kb insert (Fig. 2). This strain also produced pili that were similar but not identical to the Flp pili. These fibers were peritrichous and approximately 4.5 nm wide but did not bundle together and did not seem to interact with pili from adjacent bacteria. In all cases, 60 to 70% of the bacterial cells imaged had pili. Figure 6B shows *E. coli* DH10B carrying the empty BAC vector

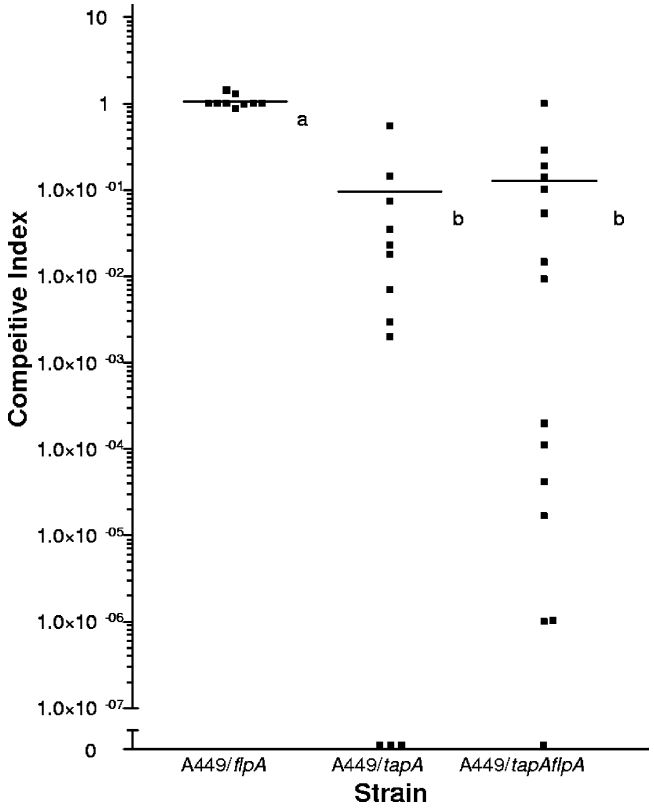

FIG. 5. CIs of single and double mutants. Atlantic salmon were challenged by immersion with equal amounts of the wild type strain and one of the mutant bacterial strains. Bacteria were isolated from the posterior kidney of moribund animals and counted. The symbols indicate the values for individual moribund fish. Different letters indicate statistical significance, as determined by the *t* test. A *P* value of <0.05 was considered significant.

pBACe3.6, and no pili or other surface structures are visible on this bacterial strain. All of the pili, as well as the surface of the bacteria and, in some places, the mica surface were covered with an unidentified thick amorphous substance that may have led to an overestimate of the fiber size.

DISCUSSION

The *A. salmonicida* subsp. *salmonicida* genome contains genes for three type IV pilus systems: Tap, Flp, and an incomplete MSHA system. Since pili are well-established virulence factors for many bacteria (22, 36), our experiments were designed to characterize the expression and function of the two presumably functional pilus systems, Tap and Flp. A previous study (26) examined the role of the Tap pilus in *A. salmonicida* subsp. *salmonicida* virulence and concluded, on the basis of an increase in the LD_{50} for rainbow trout, that the Tap pilus is a virulence factor. In this study, the contributions to virulence of Tap and Flp were assessed by both injection and immersion exposure of Atlantic salmon to pilus-deficient mutant strains.

Pilin genes and expression. The sequence, arrangement, and organization of the genes of the Tap, Flp, and MSHA pilus systems of *A. salmonicida* subsp. *salmonicida* are similar to those of the homologous systems of *P. aeruginosa*, *A. actinomycetemcomitans*, and *V. cholerae*, respectively. In addition to the previously characterized *tapABCD* cluster (26), 18 genes involved in the expression and assembly of the Tap pilus have been identified. The Tap genes are scattered widely throughout the genome, while the *flp* genes and the MSHA genes are each located in a single operon (Fig. 2). An eight-gene internal deletion in the MSHA operon encoding the pilin subunit, *mshA*, indicated that this system was inactivated and likely not expressed.

While the *A. salmonicida* subsp. *salmonicida* Tap and *P. aeruginosa* Pil systems show overall high similarity, there are differences, primarily in the regulatory genes that control pilus function and biogenesis. Most notably, *A. salmonicida* subsp. *salmonicida* contains three homologues of the *P. aeruginosa* twitching motility ATPase gene *pilT* (8): *tapT*, *tapU*, and *tapW*. While *tapT* and *tapU* are adjacent to each other, *tapW* is situated on the opposite side of the chromosome. *A. salmonicida* subsp. *salmonicida* does not appear to have orthologues of the *P. aeruginosa* genes *pilZ*, *fimX*, and *fimL*, whose products regulate twitching motility, nor are there homologues of *pilGHIJKL* and *chpABCDE*, which control *P. aeruginosa* twitching motility chemotaxis (44), suggesting that the Tap system may not provide twitching motility to *A. salmonicida* subsp. *salmonicida*. The Tap prepilin methylase/peptidase, TapD, is likely also used in assembly of the two type II secretion systems, as well as the defunct MSHA pilus system (31).

Flp pili are relatively new members of the type IVb class, the first instance having been described in 2000 (23). It is now clear that these pili are widespread among bacteria (35), where they mediate phenotypes such as biofilm formation, autoaggregation, and rough colony morphology. The Flp pilins are very small (50 to 80 amino acids) and have larger leader peptides than other type IV pilins. After processing, *A. salmonicida* subsp. *salmonicida* Flp1 is predicted to be only 49 residues long and to contain only the hydrophobic α-helix that is conserved among all members of type IV pilin group. The putative Flp prepilin leader peptidase is also different from its paralogue, TapD, in that FlpA is missing the N-terminal one-third of the protein that carries the methylation active site. The remaining portion of the molecule is an aspartyl protease and is similar to the C terminus of other type IVa prepilin peptidases (12, 39).

Gene expression analysis of the pilus subunit operons by RT-PCR indicated that the Tap pilus was constitutively expressed. On the other hand, Flp expression was iron regulated and was expressed under in vivo or iron-depleted in vitro growth conditions. In contrast to what one might expect, the apparently unregulated system, Tap, makes a greater contribution to virulence than Flp.

Pilin visualization. Since AFM easily detected the pili of *P. aeruginosa* but not those of *A. salmonicida* subsp. *salmonicida*, we reasoned that the *A. salmonicida* subsp. *salmonicida* pili are potentially too short for visualization. Consequently, strains overexpressing the TapA and Flp1 pilins were constructed. As the expression of accessory genes was not increased in these strains, only pilus length, not pilus number, was expected to increase. TapA-overexpressing strains of *A. salmonicida* subsp. *salmonicida* produced pili at their poles, the same location as the *P. aeruginosa* pili. The *A. salmonicida* subsp. *salmonicida* Tap pili appeared to be much more flexible than the pili of *P. aeruginosa* and bundled together. The overexpressed Flp pili,

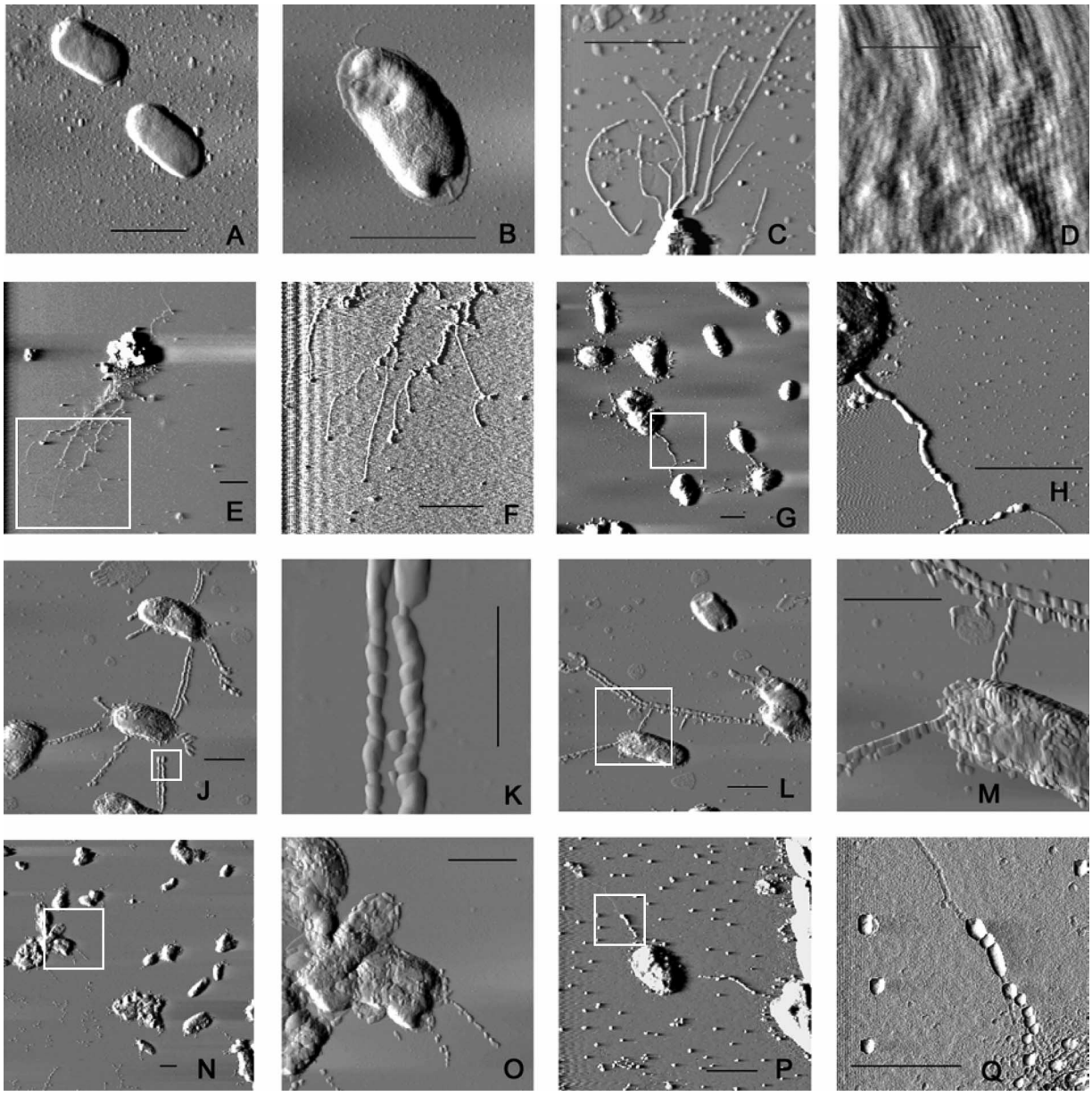

FIG. 6. AFM images of *A. salmonicida* subsp. *salmonicida* 02-06 or *E. coli* DH10B carrying pilin-overexpressing plasmids. (A) 02-06(pMMB67EH.K); (B) *E. coli* DH10B(pBACe3.6); (C) *P. aeruginosa* PAK; (D) wild-type strain A449, showing tetragonal S-layer array; (E to H) 02-06(ptapA.K); (J to M) 02-06(pflp1.K); (N to Q) *E. coli* DH10B(pAs01a04). In panels E and F, G and H, J and K, L and M, N and O, and P and Q, the second image is an enlargement of the region indicated by the box in the first image. (A, C, E to J, and L to P) Scale bars = 2 μm; (B, K, and Q) scale bars = 1 μm; (D) scale bar = 0.2 μm.

on the other hand, were peritrichous and appeared to form binary, antiparallel connections with pili from other cells. This is the first reported visualization of *A. salmonicida* subsp. *salmonicida* pili. The fact that pili were visible only under overexpressed conditions supported the hypothesis that *A. salmonicida* subsp. *salmonicida* pili are too short to be seen normally and also showed that the accessory genes responsible for pilus assembly are functional.

When the entire *flp* locus was expressed in a laboratory strain of *E. coli*, DH10B, pilus-like structures were again visible (Fig. 6N to Q). The pili were similar but not identical to the Flp pili produced by *A. salmonicida* subsp. *salmonicida*. The *E. coli* Flp pili were individual pili, while the *A. salmonicida* subsp. *salmonicida* pili were usually paired with a pilus from another bacterium. This morphological difference suggested that there may be *Aeromonas*-specific factors that are required for normal Flp pilus production that are not included on BAC pAs01a04.

Frameshifts. The genome of *A. salmonicida* subsp. *salmonicida* is littered with frameshifted genes caused by small (1- to 14-bp) insertions or deletions, as well as point mutations (M. Reith, unpublished data). There are three such lesions in the

Flp pilus system and two in Tap. It is unknown whether these lesions represent inactive pseudogenes or if the genes are somehow repaired during transcription or translation. As the data presented here demonstrate that both Flp and Tap pili can be expressed and assembled, the affected genes either are not required or are repaired during expression (4, 18). Intact genes may also be able to complement frameshifted genes as there are many sets of paralogous genes in the A449 genome, including the three type IV pilus systems, as well as two homologous type II secretion systems (one for general protein secretion and one dedicated to the S-layer). For example, the frameshifted genes *tapC* and *flpG* and the missing *mshG* gene are paralogous. However, there are intact paralogues of these genes in the Flp system (*flpH*) and in the type II secretion systems (*exeF* and *spsF*). It is possible that one or more of the uninterrupted, paralogous proteins (FlpH, ExeF, and SpsF) can substitute for the proteins with frameshifts.

Alternatively, the Flp and Tap pilins might be assembled by one of the complete type II secretion systems. This has been shown to be possible with the major pseudopilin of the *P. aeruginosa* type II secretion system, encoded by *xcpT*. Overexpression of *xcpT* generates long pilus-like structures (15, 41); however, these structures are not absolutely dependent on the Xcp secretion apparatus. When genes for this apparatus are deleted, pseudopili can still be assembled through the second type II secretion apparatus (*hxc*) or the type IV pilus (*pil*). This may happen with the Flp and Tap pilins in *A. salmonicida* subsp. *salmonicida*, but the assembly of the Flp pilin in *E. coli* (pAs01a04) cannot easily be explained by this kind of complementation.

Virulence. Immersion and intraperitoneal challenges of Atlantic salmon using strains which were deficient in *tapA* and/or *flpA* were conducted to investigate the contribution of the pili to virulence. Two TapA-deficient strains were used in live bacterial challenges of Atlantic salmon, one strain with *tapA* inactivated and one strain with both *tapA* and *flpA* inactivated. When these strains were used to challenge Atlantic salmon by immersion, the cumulative morbidity with both the *tapA*::KO and *tapA*::KO/*flpA*::Km strains was slightly, but significantly reduced. When the strains were administered by intraperitoneal injection, however, there were no significant differences in cumulative morbidity between the *tapA*::KO, *tapA*::KO/*flpA*::Km, and parental strains, although the MTTM of the mutant strains was longer. However, the CIs measured by using the immersion route for both Tap-inactivated strains were much less than one indicating decreased virulence.

The study of Masada et al. (26) showed that there was a statistically insignificant reduction in the cumulative mortality of a *tapA*-deficient strain in a rainbow trout intraperitoneal injection model. However, calculation of the LD_{50}s for the same assay showed that there was a 2.5-fold increase for the *tapA*-deficient strain compared with the wild type. These results are comparable to our results for Atlantic salmon, in that simple measurements of cumulative mortality after intraperitoneal injection did not reveal significant differences between the *tapA* mutant and the wild type; however, a closer examination of the data (mean time to death, LD_{50}) or other challenge assays (CI, immersion challenge) did show that the Tap pili play a role, albeit a small role, in virulence.

In contrast to the Tap system, Flp made little contribution to virulence when either route of infection was used. The mean CI for the *flpA*::Km strain was also about 1, indicating that the *flpA*::Km strain was not attenuated and was able to adhere to and invade Atlantic salmon as effectively as the parental strain. The Flp pilus may therefore play another role in the life of *A. salmonicida* subsp. *salmonicida*, such as adherence to other vertebrate, invertebrate, or inanimate surfaces.

Summary. *A. salmonicida* subsp. *salmonicida* has two functional type IV pilus systems, Tap and Flp, and one nonfunctional system, MSHA. The two functional systems are not redundant as they have different expression profiles, physical appearances, cellular locations, and abilities to cause clinical disease in Atlantic salmon. The Tap pili clearly contributed to virulence; however, *tapA*-deficient mutants retained much of their pathogenicity. On the other hand, the Flp pili did not contribute to virulence against Atlantic salmon. Type IV pili are well-known virulence factors for many bacterial species, including other aeromonads, but this work shows that they are not major virulence factors for *A. salmonicida* subsp. *salmonicida* infection of Atlantic salmon.

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