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Contribution of two *Aeromonas salmonicida* subsp. *salmonicida* pili to towards virulence in the Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

Aeromonas salmonicida subsp. *salmonicida* is a Gram negative, non-motile rod that is the aetiological agent of a systemic septicaemia of salmonids called furunculosis. Many steps in the pathogenesis of furunculosis are poorly understood, including the portals and mechanisms of entry into the host. Pili are filamentous, extracellular structures required by some pathogenic bacteria for the adhesion, invasion and colonization of their hosts. *Aeromonas salmonicida* has at least two apparently functional type IV pili, Flp and Tap. Both have significant nucleotide homology and similar gene order to the pili operons of *Actinobacillus actinomycetemcomitans* and *Pseudomonas aeruginosa* respectively. Isogenic knockouts of *A. salmonicida* subsp. *salmonicida* strain A449 were created in one or both of these operons. *In vitro* adherence assays using the non-phagocytic salmonid cell-line CHSE-214 or an inert borosilicate glass substrate revealed no significant difference in adherence of the mutants. Nor was their ability to form a biofilm on borosilicate glass tubes. However inactivation of the S-layer protein, VapA, significantly reduced adherence to CHSE-214 and abolished biofilm formation. When Atlantic salmon (*Salmo salar* L.) were challenged by immersion with FlpA, TapA or FlpA/TapA deficient strains, the mean time to death was extended and there was significantly reduced morbidity when compared to the group challenged with the parental strain. There was no significant difference in cumulative mortality or time to death between the pili mutants and the parental strain upon intraperitoneal challenge. These data suggest that type IV pili contribute to the initial adherence and invasion of the host but not to further steps of pathogenesis and that unlike the pili systems of some Gram negative bacteria they are not absolutely required for virulence.

INTRODUCTION

Aeromonas salmonicida subsp. *salmonicida* is a Gram negative, non-motile rod that causes a spectrum of diseases in salmonids that are collectively known as furunculosis. Acute furunculosis is characterised by rapid mortality with affected animals showing few or no clinical signs. Chronic furunculosis is characterised by focal myodermonecrotic lesions or furuncles. In the poorly understood clinically inapparent or covert form of this disease animals are outwardly healthy yet still harbour the bacterium. Under conditions of acute stress, chronic stress or immunosuppression covertly infected animals may exhibit an overt disease. Much of the pathogenesis of *A. salmonicida* subsp. *salmonicida* remain poorly elucidated including the portal of entry, mechanisms of invasion and environmental reservoirs.

Many factors have been implicated as virulence determinants. These include cellular proteins such as the surface-layer protein (VapA; Trust et al 1983), iron- or manganese-cofactored superoxide dismutase (SodA and SodB; Barnes et al 1996, Dacanay et al 2003) and iron sequestration systems (Ebanks et al 2004, Fernandez et al 1998, Hirst & Ellis 1996) in addition to extracellular proteins including cholesterol acyltransferase (GCAT, Salte et al 1992) and *AspA*, a serine protease (Nieto et al 1991). Despite the identification of multiple virulence factors, no single characteristic or phenotype is found uniquely in virulent strains (Ellis et al 1998, Olivier 1990, Vipond et al 1998, Fernandez et al 1998) with the exception perhaps of the recently described the type three secretion system of *A. salmonicida* subsp. *salmonicida* strain JF2267 (Burr et al 2003).

Early studies suggested that the posterior kidney was the site of residence for *A. salmonicida* subsp. *salmonicida* during a clinically inapparent, or covert, infection (Rose et al 1989). More recent studies agree that an exterior, rather than an interior, location is the site of

56 residence on the host. Hiney et al (1994) reported the intestine as the site of residence. Both
57 Cipriano et al (1996) and Ferguson et al (1998) reported mucous and gill as the site of reisdence
58 and, in contrast to Hiney et al (1994) neither study recovered *A. salmonicida* from the intestine.
59 Covert infections are difficult to detect with conventional microbiology but can be more
60 accurately assessed by stress-tests that utilise twin stressors: heat and exogenously applied
61 corticosteriod that immunocompromise the animal and allow progression to an overt, easily
62 recognizable disease (Cipriano et al 1997, Hiney et al 1994).

63 There are multiple strategies by which pathogenic bacteria can effect entry to the host and
64 evade, suppress or even usurp host immune effectors (reviewed by Horneff et al 2002). In order
65 to understand better the strategies employed by *A. salmonicida* subsp. *salmonicida*, we have
66 focused on the initial stages of infection by examining potential adhesins of *A. salmonicida*
67 subsp. *salmonicida* strain A449, a virulent strain. The most well studied *A. salmonicida* subsp.
68 *salmonicida* adhesin is the surface- or S-layer (Trust et al 1983). It is often implicated as an
69 important factor for invasion and adherence but may not be absolutely required as S-layer
70 deficient strains may also be virulent (Olivier 1990).

71 Pili allow bacteria to attach to solid surfaces, including host tissues and are thus
72 important virulence factors (Sauer et al 2000). They are filamentous, extracellular organelles
73 that are either peritrichous or polar and may be present singularly or in bundles. There are
74 currently four recognized types of pili found on Gram negative bacteria (Thanassa et al 2000).
75 Type IV pili are subdivided into types IVA and IVB on genetic and morphological grounds.
76 Type IVA (non-bundle forming) pili are important in a number of bacterial processes including
77 flagella-independent "twitching" motility, DNA uptake, biofilm formation, adherence to
78 substrates and, in pathogenic bacteria, adherence to host-cells. Type IV pili are important

79 virulence factors in many pathogenic gram-negative bacteria. *Aeromonas salmonicida* subsp.
80 *salmonicida* also has a number of pili systems, (this study, Masada et al 2002). A type IVA
81 pilus, encoded in part by the *tapABCD* operon, has been described in several Aeromonads
82 including *A. hydrophila* (Pepe et al 1996), *A. veronii* (Barnett et al 1999) and *A. salmonicida*
83 subsp. *salmonicida* strain A450 (Masada et al 2002). A recent study implicated this operon in
84 the virulence of *A. salmonicida* based on a 2.5 fold increase in LD_{50} for a *tapA* mutant as
85 assessed by intraperitoneal injection in rainbow trout, *Oncorhynchus mykiss* W (Masada et al
86 2002). In this study we assessed the roles of two type IV pili of *A. salmonicida* subsp.
87 *salmonicida* strain A449 in adherence to cells *in vitro*, biofilm formation and infection using a
88 live animal model using isogenic knockout mutations.
89
90

91 **MATERIALS AND METHODS**

92 **Bacterial strains and growth conditions.** Bacteria and plasmids used in this study are
93 listed in Table I. The parental strain for all knockouts was *Aeromonas salmonicida* subsp.
94 *salmonicida* strain A449 (hereon abbreviated to *A. salmonicida* A449) originally isolated from a
95 natural furunculosis epizootic in brown trout (*Salmo trutta*) at Eure, France in 1975. All
96 *Aeromonas* strains were grown in tryptic soy broth (TSB) or agar (TSA) (Difco) for 3 days at 17
97 °C with shaking. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) or agar at 37
98 °C. Antibiotics were used at the following concentrations: *E. coli*: ampicillin 100 μg mL^{-1};
99 kanamycin 25 μg mL^{-1}; *A. salmonicida*: ampicillin 50 μg mL^{-1}; kanamycin 200 μg mL^{-1} for
100 selection and 50 μg mL^{-1} for maintenance; chloramphenicol: 20 μg mL^{-1}. 2-2'-dipyridyl (120
101 μM) was added to reduce iron concentrations.

DNA techniques. DNA manipulations were performed by standard genetic and molecular techniques (Ausubel et al 1998). Genomic DNA from *Aeromonas salmonicida* strain A449 was isolated using the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN) and used for construction of all mutant strains. Oligonucleotides were prepared by Integrated DNA Technologies Inc. (Coralville, IW). PCR was performed with either rTaq (Amersham) or Pfu (MBI Fermentas) following the manufacturers' directions.

Construction of mutant strains. (i) 02-10 (*tapA*::KO). The single cross-over knock-out vector, pKO, was created to insert the complete plasmid within the gene of interest and so that the 3' fragments of the affected gene would make in-frame fusions to a tmRNA so that any translated peptide that would be recognised by Clp proteases and degraded. The tmRNA-tag was created using complementary oligonucleotides based on the sequence of *A. salmonicida* tmRNA (Williams et al 1996) with *Not*I and *Sst*I sites at either ends (Table 2). The tmRNA-tag was inserted into the *Not*I and *Sst*I sites of the polylinker of pCRScript-Amp (Invitrogen) in such a way that the *lacZα* fragment was not disrupted to create pCRscript-tmRNA. The new polylinker and *lacZα* region of pCRScript-tmRNA was used to replace the polylinker of pAH34 (Metcalf et al 1996) using *Pvu*II sites to create pKO. pAH34 is a mobilizable, pir-dependent, ampicillin resistant plasmid that allows blue/white selection of cloned inserts and that cannot replicate in *A. salmonicida* subsp. *salmonicida.*

An internal fragment of the *tapA* gene was amplified from genomic DNA using Pfu polymerase and the primers *tapA*-1 and *tapA*-2 in Table 2. The fragment was blunt-end ligated into the *Srf*I site of vector pKO to make an in-frame fusion to the tmRNA-tag. The pKO-*tapA* plasmid was conjugated into *A. salmonicida* A449 from the *E. coli* pir$^+$ mating strain BW20767 (18), single cross-over integrants were selected by growth on ampicillin, and passaged to fresh

125 media three times. Proper integration was checked by PCR of genomic DNA using primers
126 flanking the insertion.

127 **(ii) 02-02 (*flpA*::km).** The *flpA* gene was interrupted by insertion of a kanamycin
128 resistance gene cassette. Fragments flanking the *flpA* gene were amplified from genomic DNA
129 using the primers in Table 2. Primers *flpA*-2 and *flpA*-3 included *Bgl*II sites which were used
130 subsequently to introduce the 1.5 km *Bam*HI fragment carrying the kanamycin resistance
131 cassette from pUTKm1. In a multistep process, these three fragments were cloned into the
132 mobilizable, ampicillin resistant, pir-dependent vector pGP704 to generate pJW109-pir.
133 pJW109-pir was conjugated into *A. salmonicida* A449 from BW20767 and single cross-over
134 integrants selected by growth on ampicillin. Double cross-over mutants were selected by
135 isolation of ampicillin sensitive, kanamycin resistant colonies. Proper integration was checked
136 by PCR of genomic DNA using primers flanking the insertion.

137 **(iii) 03-06 (Δ*vapA*).** An in-frame, unmarked deletion of the surface layer gene, *vapA*,
138 was created using crossover PCR (Link et al 1997). Two self-complementary PCR fragments
139 were amplified from genomic DNA using the primers shown in Table 2. The internal ends of
140 each fragment included 7 identical codons, the external ends included *Xho*I and *Bam*HI
141 restriction sites. The two PCR fragments were mixed together and amplified with the two
142 external primers (VapA-No, VapA-Co) to generate a large fragment carrying *vapA* flanking
143 sequences with 254 *vapA* codons replaced with the 7 introduced codons. This fragment was
144 cloned into the pir-dependent, sucrase expressing vector pWM91 to generate pWM91-vapA.
145 pWM91-vapA was conjugated into *A. salmonicida* A449 from BW20767, single cross-over
146 integrants were isolated as described above. Double cross-over mutants were isolated by
147 selection on TSA with 15% sucrose to select against plasmid containing colonies and 0.01%

Coomassie blue to screen for *vapA*⁻ colonies. Proper integration was checked by PCR of genomic DNA using primers flanking the deletion. This strain was shown to be Surface-layer negative by SDS-PAGE (data not shown).

(iv) Double and triple mutant strains. Double and triple mutants were constructed by introducing mutated constructs into strains already containing one or more mutations.

Biofilm Assay. *Aeromonas salmonicida* A449 and isogenic mutant strains were grown in TSB with appropriate antibiotics until mid-exponential phase, cells were washed in Griffin's media and resupended in the same media to an A600 of 0.2. One mL of this culture was added to 11 mm diameter borosilicate glass tubes and incubated at 17°C with gentle rotation (150 rpm) for 7 days. Griffin's media is a defined media modified from O'Leary et al (1956) by increasing the KPO_4 concentration from 32 mM to 480 mM and reducing the NaCl from 85 mM to 43 mM to compensate for altered osmolarity. After 7 days, 200 µL of 0.1 % crystal violet was added to the cultures. After a 15-minute incubation, the media and stain were decanted, the tubes were washed gently with water and dried.

Adherence Assay. The adherence assay was modified from Merino et al. (1996) using the chinook salmon embryo (CHSE-214) cell line. Briefly, CHSE-214 cells were plated in a 96-well tissue culture plate at 1 x 10^4 cells per well and incubated at 17°C in L15 (Leibovitz) media (Sigma, St. Louis, MO) with 10% FBS and penicillin/streptomycin (both Sigma, St. Louis, MO). Bacteria were diluted in 1xHBSS to an A_{600} of 0.05. Inoculum size was later confirmed by direct colony counts on TSA. The tissue culture media was removed from the cultured cells by aspiration, the wells washed three times with L15 and filled with 200 µL of L15/5% FBS and 10 µL of the bacterial solution. Each sample was tested in triplicate. The plates were incubated for two hours at 17°C. The wells were emptied by aspiration, washed six times with L15 and the

CHSE-214 cells disrupted by hypotonic shock (0.5% Tween 20 in ddH_2O). The number of adherent bacteria was then determined by direct colony counts on TSA and expressed as a percentage of the inoculum.

Animal Care. Both the National Research Council Halifax's Local Animal Care Committee and the Dalhousie University's Committee on Laboratory Animals approved all animal procedures, which were conducted under Canadian Council on Animal Care guidelines. Juvenile St John River or Sackville River stock Atlantic salmon, ca 60 g, were obtained from Nova Scotian hatcheries certified under Canadian Fish Health Protection Regulations. They were stocked in 0.1 m^3 fibreglass resident tanks at a stocking density of ca 2.4 kg/0.1 m^3 and maintained at 14°C +/-2°C, in flow-through in dechlorinated municipal water. They were fed a maintenance ration daily of 1% bodyweight of a commercially available feed (Signature Salmon Ration, Shurgain, Truro, NS). Feeding was suspended for one-day prior to and one-day post manipulation.

Challenge. Mutant strains 02-02 and 02-10 were both tested by immersion challenge and a subsequent stress-test for clinically inapparent (covert) infections in St John River stock Atlantic salmon. Mutant strain 03-07 was tested by immersion challenge and subsequent stress-test in Sackville River stock Atlantic salmon. All three pilin mutant strains were tested by i.p. injection in Sackville River stock Atlantic salmon, the survivors of this challenge were subsequently tested for protection from re-challenge with the parental strain.

Bacteria were cultured from single colonies in tryptic soy broth (TSB, Difco, Sparks, MD) with agitation at 17°C. Absorbance was measured at 600 nm and adjusted to an OD of 0.5 with sterile, chilled 1 x phosphate buffer saline. For immersion challenges there were two tanks per group with forty fish per tank. Weight was ca. 60 g each. Fish were transferred into

commercially available plastic containers, sedated (15 mg L^{-1} Tricaine methanosulphonate, TMS, Syndel Laboratories, Vancouver, BC) and exposed for 40 minutes to ca. 10^6 cfu mL^{-1} of either the parental strain, 02-10 or 02-02. Post-exposure, the fish were returned to their designated resident tanks. Control fish experienced identical handling but were only exposed to an equal volume of PBS. For intraperitoneal (i.p.) challenge there where two tanks per group with ca. 25 fish per group. Weight was ca. 200 g each. They were anesthetised with 50 mg L^{-1} TMS prior to injection with ca 10^4 cfu of the parental strain, 02-10, 02-02 or 03-01 per animal in 100 µL PBS. Control animals were injected with an equal volume of PBS.

Animals were closely monitored post-challenge. Morbid animals were euthanised with a TMS overdose, visually surveyed for gross clinical signs and the posterior kidney was sampled onto TSA as recommended by the American Fisheries Society (Schotts 1994). If bacteria were cultivable from the posterior kidney they were subsequently cultured on TSA supplemented with either 20 µg mL^{-1} chloramphenicol, 50 µg mL^{-1}kanamycin or 50 µg mL^{-1} amplicillin. *Aeromonas salmonicida* subsp. *salmonicida* was considered the cause of death if it was cultivable from the posterior kidney and the animal displayed gross clinical signs consistent with *A. salmonicida* subsp. *salmonicida* infection such as ecchymosis of the swim bladder, punctate hemorrhaging of the perivisceral fat and focal, raised, red skin lesions (furuncules).

Clinically inapparent (covert) infection detection. Sixty days after exposure by immersion half of the surviving animals, typically between twenty and forty, from each immersion challenge group were assessed for levels of covert infection by a modified stress test based on Specker *et al* (1994). Briefly: following an i.p. injection of 100 mg kg^{-1} hydrocortisone (Sigma-Aldrich, Oakville, ON) in pasteurized vegetable oil/vegetable oil, the water temperature was raised to 18°C over ninety minutes and maintained at that level for ten days. The remaining

217 half-group was unstressed. An additional tank of twenty control fish were also subjected to a
218 stress-test. Five animals from this group were euthanised with an overdose of TMS every second
219 day for assessment of plasma cortisol and glucose using commercially available immunoassays.
220 Morbid animals were processed as for the immersion and intraperitoneal challenges. After ten
221 days, the unstressed half-group was euthanised with a TMS overdose; mucus and posterior
222 kidney were sampled onto TSA.

223 **Re-challenge**: Forty days after i.p. challenge, the surviving animals were re-challenged
224 i.p. with 10^6 cfu of the parental strain i.p. All other procedures were as for the other challenges

225 **Statistics:** Cumulative mortality between groups were assessed by G-test. Mortality
226 rates were assessed by Kaplan Meier survival analysis (GraphPad Prism version 3.0, GraphPad
227 software, San Diego, CA) to determine median survival in days and to determine if statistical
228 differences existed between mortality curves. Adherence data were compared using ANOVA
229 with Tukeys' post-test (GraphPad Prism version 3.00 for Windows, GraphPad Software, San
230 Diego CA)

231

232 **RESULTS**

233 **Knock-outs.** To investigate the role of pili in *A. salmonicida* subsp. *salmonicida*
234 infection, isogenic mutant strains were created in strain A449. Strain A449 was orginially
235 isolated from a furunculosis epizootic in brown trout (*Salmo trutta*) at Eure, France. 02-0203-06
236 Genes were inactivated using one of three different methods; introduction of the plasmid pKO
237 into the *tapA* gene in a single cross-over event to make strain 02-10; alternatively the double
238 cross-over technique was used to make an unmarked, non-polar deletion in the surface-layer of

239 strain 03-06, and to introduce the kanamycin resistance cassette into the *flpA* gene of strain 02-
240 02.
241
242 All three methods produced stable mutants. Reversion, the appearance of bacteria with
243 inappropriate antibiotic sensitivities indicting a loss of either pKO or the kanamycin cassette was
244 not seen during this study. *In vitro* 02-10, 02-02 and 03-01 grew normally in TSB and TSA at
245 17°C, 03-06 grew slightly more rapidly than wild type (data not shown). This is likely due to the
246 increased metabolic resources available to 03-06as it does not have to produce numerous copies
247 of VapA rather than an artefact of the knock-out procedure.
248 ***In vitro* assays.** The parental strain and isogenic mutant strains were assessed for their
249 ability to adhere to a solid substrate or host cells. There was no difference between the parental
250 strain and any of the single or double pili mutant strains in their ability to grow as a biofilm on
251 borosilicate glass tubes (Figure 1). Biofilm growth was abolished in single knockout S-layer
252 mutant strains as well as double or triple S-layer and pili mutant strains. Similarly, there were no
253 significant differences in the adherence of either the single or double pili mutant strains to the
254 CHSE-214 salmonid epithelial cell line when compared to the parental strain (Figure 2). In
255 contrast, there was no significant adherence to CHSE 214 by 03-06. There was no further
256 significant difference in the adherence of 03-07 (Δ *vapA/flpA*::km).
257 **Immersion Challenge.** All three pili mutant strains caused a clinical disease visually
258 indistinguishable from the disease caused by the parental strain when administered by
259 immersion. Cumulative mortality level in St. John River stock Atlantic salmon was significantly
260 lower for 02-10 (25.3%) than that for the parental strain (39.2%; G-test, $p = 0.024$) whereas there
261 was no significant difference for the 02-02 (28.7% G-test, $p = 0.64$, Table 3). In an immersion

challenge using Sackville River stock strain of Atlantic salmon and 03-01, the mortality level for 03-01 (76.3%) was significantly lower when compared to the parental strain (90.0%; G-test, p = 0.0062). There was also a delay in the median survival for all the mutants when compared to the parental strain; one day for 02-10 and five days for 02-02 and 03-01.

Intraperitoneal (i.p.) Challenge. When delivered by i.p. injection in Sackville River stock Atlantic salmon all three pili mutants again caused a clinical disease that was visually indistinguishable from the disease caused by the parental strain. There was no significant difference in cumulative mortality (G-test, p> 0.05) between the parental strain (45.0%), 02-02 (50.0 %), 02-10 (39.0%) and 03-01 (37.5%, Table 3). Median survival ranged between 6.5 days (A449) to 8 days (03-01). There was no *Aeromonas*-related mortality in any of the control groups for any immersion or i.p. challenge.

Clinically inapparent (covert) infections. After the conclusion of the immersion challenge, half the surviving animals were assessed for the presence of clinically inapparent infections (Table 4) by a modified stress test. Serum cortisol and glucose were significantly elevated in control fish by the stress-test procedure as assayed by standard immunoassays (data not shown). Ten days after administering the stress test to St. John River stock Atlantic salmon previously exposed to 02-10 or 02-02, there were no significant differences between the levels of mortality in either 02-02 (55.2%) or 02-10 (52.2%). The mortalities in both mutants were significantly higher that the parental strain (28.6%, G-test, p = 0.03). In Sackville River stock Atlantic salmon previously challenged with 03-01 there was no difference in the level of clinically inapparent infections between the parental strain (85.7%) and 03-01 (84.2%). At the conclusion of the stress-test on St John Atlantic salmon the unstressed half-group was assessed for *A. salmonicida* subsp. *salmonicida* by routine microbiology. At the

285 time of sampling, all the animals were outwardly healthy and showed no gross signs of disease.
286 *A. salmonicida* subsp. *salmonicida* was not isolated from the posterior kidney of any animal from
287 any group. It was isolated from the skin mucous of the animals exposed to A449 (8.1%, 3/37)
288 and 02-10 (10.5%, 4/38) but not from the animals exposed to 02-02 (0%, 0/38).

289 **Re-challenge**: There were no significant differences in survival when Sackville Rover
290 stock Atlantic salmon previously challenged by i.p. injection with wild-type or any of the three
291 pili mutants were re-challenged i.p. with ca 10^6 cfu/animal *A. salmonicida* A449 (Table 5).
292

293 ## DISCUSSION

294 Given their roles in adherence to host cells or inert surfaces in *Pseudomonas aeruginosa,*
295 *Neisseria gonorrhoeae*, *Vibrio cholerae* and entropathogenic *Escherichia coli*, type IV pili are
296 clearly considered virulence factors. This study investigated the role of pili, whose genes were
297 identified during *in silico* during screening of a draft *A. salmonicida* subsp. *salmonicida* strain
298 A449 genome (Singh et al unpublished data). The contribution of pili to adherence in in vitro
299 using biofilm and cellular adhesion assays was assessed in addition to their contribution
300 virulence by live bacterial challenge of Atlantic salmon..

301 The Tap pilus mutant was made by interrupting the *tapA* gene, this gene was chosen
302 because it encodes the pilin subunit and is the first gene in the *tapABCD* operon. Mutations in
303 the pilin subunit of similar pilus systems have been shown to eliminate pilus production. The
304 Flp pilus mutant was made by interrupting the *flpA*, or prepilin peptidase gene, the second gene
305 in that locus. Many Flp pilus system homologues have more than one pilin subunit gene. At the
306 time the mutation was created the number of pilus subunits was uncertain, the prepilin peptidase
307 gene, *flpA,* was interrupted to ensure inactivation of that system. It is of note that attempts to

knock out the *tapD* pre-pilin peptidase were unsuccessful, suggesting that this gene is essential. Since the insertion in the *tapA* knock-out should disrupt expression of downstream genes including *tapD*, we conclude that *tapD* has its own promoter.

***In vitro* assays.** All VapA+ strains were equally capable of forming a biofilm, regardless of the number of inactivated pili operons. Conversely VapA deficient strains were incapable of biofilm formatation, again however, regardless of the number of inactivated pili operons. Masada et al (2002) also reported that adherence to CHSE-214 of *A. salmonicida* subsp. *salmonicida* strain A450 *tapA*::ko- was not attenuated. However in this study all three pili mutants behaved equally, showing no significant difference in adherence to the parental strain. Both the *vapA* deficient strains tested in our study; 03-06 and 03-07 behaved equally; showing significantly reduced or absent adherence in both assays when compared to the parental strain. As the three pili mutants behaved as the parental strain in both adherence assays, in accordance with the observations of others (Trust et al 1983), the major *A. salmonicida* adhesin *in vitro* appeared to be the S-layer protein, VapA. Any contribution to adherence made by either Tap or Flp in these assays was apparently negligible when compared to the contribution of VapA.

***In vivo* challenges.** Due to marked differences in the susceptibility to *A. salmonicida* strain A449 of the two stocks of Atlantic salmon used during this study (St John River and Sackville River) no direct comparisons could be made between the behaviors of the single and double mutants in the immersion challenges. The varying susceptibility of individual river stocks of Atlantic salmon to bacterial pathogens has only recently been described (Lawlor et al 2004).

By immersion, the virulence of 02-10 and 03-01 as assessed by cumulative mortality was significantly reduced when compared to wild-type, whereas that of *flpA*::km was reduced but not

331 significantly. Mutant strains 02-10, 02-02 and 03-01 behaved no differently to the parental strain
332 when they were administered by intraperitoneal injection. The uniform virulence of the three pili
333 mutants when administered by injection shows that they are not required absolutely for
334 pathogenesis. As the decrease in virulence was dependent on the route of administration, pili
335 were apparently important for either adhesion to or invasion of the host but not for any
336 subsequent step(s) in pathogenesis. Masada et al (2002) also showed that the *A. salmonicida*
337 subsp. *salmonicida* strain A450 Tap pilus contributed to virulence in rainbow trout by
338 determining LD50. However unlike *P. aeruginosa*, where pili are the major adhesion, the pili of
339 *A. salmonicida* subsp. *salmonicida* do not appear to be absolutely required for adherence; all
340 three pili mutants created in this study were capable of binding host cells in vitro and a
341 significant disease could still be induced experimentally by injection or immersion of susceptible
342 hosts.

343 Another indication that pili contributed to adherence or invasion were lower mortality
344 rates leading to a slower epizootic caused by the pili deficient strains when administered by
345 immersion but not by i.p. injection. This was evident as both lengthened MTTD and an
346 decreased mortality rate when compared to the parental strain (the hazard ratio) in addition to
347 significant differences between the curves as determined by the Mantel-Haenszel test. There
348 were significant differences between the survival curves of both 02-10 and 03-01 compared to
349 the parental strain. For strain 02-10, MTTD was slightly extended by one day from twelve to
350 thirteen days for 02-10 and the hazard ratio was 1.43. However for 02-2 MTTD the hazard ratio
351 increased to 1.7 and the MTTD was increased by 40% to seventeen days. In a separate challenge
352 mortality rates were lower for 03-01 as indicated by a doubling of MTTD from five days to ten
353 and a hazard ratio of 1.45. There were no significant differences between survival curves in any

group by i.p. injection ($p > 0.05$), the hazard ratio was between 1.01 (02-2) and 1.33 (03-1) and MTTD was between one and two days longer for the mutants when compared to the parental strain.

The restoration of full virulence to all mutants when administered by injection showed that both Tap and Flp pili are evidently important for some aspect of *A. salmonicida* subsp. *salmonicida* strain A449 adherence to or invasion of Atlantic salmon. The TapA deficient strain (02-10) displayed reduced mortality, the FlpA deficient strain (02-02) a delayed epizootic whilst the double TapA/FlpA deficient strain (03-01) displayed both a reduced mortality and a delayed epizootic. It is likely that these two type IV pili are not redundant as the pili deficient strains have different effects on the outcome of challenge. This is supported by the failure to recover 02-02 from the mucus of animals that were evidently covertly infected, suggestive that perhaps FlpA is more important for adherence to the skin or mucous than TapA. They may, however act synergistically as the effect on MTTD was greater for 03-1 than for either 02-10 or 02-02.

All animals that had survived challenge with the parental strain or any of the pili-deficient mutant strains displayed a modest degree of protection from a subsequent re-challenge with the parental strain. There were, however, no significant differences between groups in this protection. The inability of the pili to confer provide protection is in contrast to previous studies of both terrestrial and aquatic vertebrates. Pili were reported as effective immunogens in cattle against *Moraxella bovis* (Lepper 1988) and in sheep against *Bacteriodes nodosus* (Stewart et al 1982, Stweart et al 1995) whilst Masada et al (2002) showed that TapA induced a degree of protection from subsequent *A. salmonicida* subsp. *salmonicida* strain A450 challenge in rainbow trout. Whereas changes in plasma profiles between naïve and exposed Atlantic salmon in response to *A. salmonicida* A449 challenge have recently been documented (Solanky et al 2005)

377 the type of immunity induced by exposure to *A. salmonicida* subsp. *salmonicida* is unknown.
378 The action of immune mechanisms other than acquired immune responses directed against TapA
379 or FlpA in surviving animals may have obscured or confounded the effect of the presence or
380 absence of either gene upon re-challenge.

381 In addition to a clinically overt disease, stress-testing and routine microbiology showed
382 that the mutants were also able to establish clinically inapparent (covert) infections in St John
383 River stock Atlantic salmon. Despite appearing outwardly healthy, many fish were found to
384 harbor *A. salmonicida* subsp. *salmonicida* with no clinical signs of disease. This phenomenon
385 was reviewed by Hiney et al (1997). In accordance with others (Cipriano et al 1997), isolation of
386 *A. salmonicida* subsp. *salmonicida* by routine microbiological techniques revealed a lower
387 incidence of clinically inapparent infection than the stress-test: for 02-10 10.5% of the animals
388 by microbiology were positive for a covert infection whilst 52.2% of the stress-tested animals
389 were positive; for the parental strain it was 8.1% vs.28.6% and finally for 02-02 0% vs. 55.2%
390 *Aeromonas salmonicida* subsp. *salmonicida* was not recovered from the posterior kidney of these
391 outwardly healthy animals despite this organ being reported as a site for clinically inapparent
392 infections by Rose et al (1990). The gastrointestinal tract, suggested as a site of colonization by
393 Hiney et al (1997), was not tested. These data support an exterior localization for *A. salmonicida*
394 A449 during a covert infection. It is of note that Cipriano et al (1997) have reported increased *A.*
395 *salmonicida* subsp. *salmonicida* titres in the mucous of furunculosis-susceptible salmonids (such
396 as *S. trutta*) when compared to furunculosis-insensitive salmonids (such as *O. mykiss*). This
397 implies that should mucous present a significant host barrier to infection, then the influence of
398 adhesins such as pili upon virulence might be different than if it were not. The data presented
399 here show that in the furunculosis susceptible Atlantic salmon, pili do play a role in virulence.

Unfortunately the challenge data for a TapA deficient strain in the furunculosis insensitive rainbow trout were obtained by Masada et al (2002) by intraperitoneal injection and cannot be directly compared with the immersion data presented here.

In summary, knockout mutants were prepared in two type IV pili systems of *A. salmonicida* subsp. *salmonicida* strain A449. When tested *in vitro* for adherence to host cells or an inert substrate the effect of the S-layer obscured any contribution to adherence made by pili. All three pili mutants caused a clinical disease indistinguishable from that of the parental strain in Atlantic salmon. When administered to Atlantic salmon by immersion there were differences between the parental strain and pili mutant strains in either cumulative mortality, mean time to death or both. Full virulence was restored when the mutants were administered by intraperitoneal injection. As the mutant strains were equally as virulence as the parental strain when the requirements for adherence to and invasion of the host were removed, these data demonstrate that whereas both Tap and Flp pili are involved in the early stages of the pathogenesis of furunculosis. Unlike other piliated Gram-negative pathogens they are not solely required for adherence, invasion or colonization of Atlantic salmon by *A. salmonicida* subsp. *salmonicida.*

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1998. Current Protocols in Molecular Biology. John Wiley & Sons, New York.
2. **Barnes, A. C., M. T. Horne, and A. E. Ellis.** 1996. Effect of iron on expression of superoxide dismutase by *Aeromonas salmonicida* and associated resistance to superoxide anion. FEMS Microbiol Lett **142:**19-26.
3. **Barnett, T. C., and S. M. Kirov.** 1999. The type IV *Aeromonas* pilus (Tap) gene cluster is widely conserved in *Aeromonas* species. Microb Pathog **26:**77-84
4. **Burr, S.E., T. Wahli, H. Segner, D Pugovkin and J Frey.** 2003. Association of Type III secretion genes with virulence of *Aeromonas salmonicida* subsp. *salmonicida.* Dis Aquat Organ **57(1-2);** 167-171
5. **Cipriano, R. C., G. L. Bullock, and A. Noble.** 1996. Nature of *Aeromonas salmonicida* carriage on asymptomatic rainbow trout maintained in a culture system with recirculating water and fluidized sand biofilters. J Aquat Anim Health **8:**47-51.
6. **Cipriano, R. C., L. A. Ford, D. R. Smith, J. H. Schachte, and C. J. Petrie.** 1997. Differences in detection of *Aeromonas salmonicida* in covertly infected salmonid fishes by the stress-inducible furunculosis test and culture-based assays. J Aquat Anim Health **9:**108-113.
7. **Dacanay, A., S. C. Johnson, R. Bjornsdottir, R. O. Ebanks, N. W. Ross, M. Reith, R. K. Singh, J. Hiu, and L. L. Brown.** 2003. Molecular characterization and quantitative analysis of superoxide dismutases in virulent and avirulent strains of *Aeromonas salmonicida* subsp. *salmonicida.* J Bacteriol **185:**4336-44.
8. **Ebanks, R. O., A. Dacanay, M. Goguen, D. M. Pinto, and N. W. Ross.** 2004. Differential proteomic analysis of Aeromonas salmonicida outer membrane proteins in response to low iron and in vivo growth conditions. Proteomics **4:**1074-85.
9. **Ellis, A. E., A. S. Burrows, and K. J. Stapleton.** 1988. Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: A-layer, extracellular proteases and extracellular haemolysins. J Fish Dis**11:**309-323.
10. **Ferguson, Y., I. R. Bricknell, L. A. Glover, D. M. MacGregor, and J. I. Prosser.** 1998. Colonisation and transmission of *lux*-marked and wild-type *Aeromonas salmonicida* strains in Atlantic salmon (*Salmo salar* L.). FEMS Microbiol. Ecol **27:**251-260.
11. **Fernandez, A. I., A. F. Fernandez, M. J. Perez, T. P. Nieto, and A. E. Ellis.** 1998. Siderophore production by *Aeromonas salmonicida* subsp. salmonicida. Lack of strain specificity. Dis Aquat Organ **33:**87-92.

12. **Grant, S. G., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc Natl Acad Sci U S A **87:**4645-9.
13. **Hiney, M. P., J. J. Kilmartin, and P. R. Smith.** 1994. Detection of *Aeromonas salmonicida* in Atlantic salmon with asymptomatic furunculosis infections. Dis Aquat Org **19:**161-167
14. **Hiney M., P. Smith and E-M. Bernoth.** 1997 Covert A. salmonicida infections. *In* Furunculosis: Multidisciplinary Fish Disease Research pp 54-97. E-M. Bernoth, A.E. Ellis, P.J. Midtlyng, G. Olivier and P. Smith (Eds). Academic Press.
15. **Hirst, I. D., and A. E. Ellis.** 1996. Utilization of transferrin and salmon serum as sources of iron by typical and atypical strains of Aeromonas salmonicida. Microbiology **142(6):**1543-50.
16. **Hornef, M. W., M. J. Wick, M. Rhen, and S. Normark.** 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. Nat Immunol **3:**1033-40
17. **Lepper A.W.** 1988 Vaccination against infectious bovine keratoconjunctivitis: protective efficacy and antibody response induced by pili of homologous and heterologous strains of *Moraxella bovis*. Aust Vet J **65(10)**:310-6.
18. **Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type Escherichia coli: application to open reading frame characterization. J Bacteriol **179:**6228-37.
19. **Masada, C. L., S. E. LaPatra, A. W. Morton, and M. S. Strom.** 2002. An *Aeromonas salmonicida* type IV pilin is required for virulence in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org **51:**13-25
20. **Merino, S., A. Aguilar, X. Rubires, D. Simon-Pujol, F. Congregado, and J. M. Tomas.** 1996. The role of the capsular polysaccharide of *Aeromonas salmonicida* in the adherence and invasion of fish cell lines. FEMS Microbiol Lett **142:**185-9.
21. **Metcalf, W. W., W. Jiang, L. L. Daniels, S. K. Kim, A. Haldimann, and B. L. Wanner.** 1996. Conditionally replicative and conjugative plasmids carrying lacZ alpha for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35:**1-13.
22. **Metcalf, W. W., W. Jiang, and B. L. Wanner.** 1994. Use of the rep technique for allele replacement to construct new *Escherichia coli* hosts for maintenance of R6K gamma origin plasmids at different copy numbers. Gene **138:**1-7.
23. **Molnos, J., R. Lange, and K. E. Amrein.** 2000. An improved vector system for insertional gene inactivation inspired by the tmRNA-tagging system of *S. pneumoniae*. J Microbiol Methods **42:**197-201.
24. **Nieto, T. P., Y. Santos, L. A. Rodriguez, and A. E. Ellis.** 1991. An extracellular acetylcholinesterase produced by *Aeromonas hydrophila* is a major lethal toxin for fish. Microb Pathog **11:**101-10.
25. **O'Leary, W. M., C. Panos, and G. E. Helz.** 1956. Studies on the nutrition of *Bacterium salmonicida*. J Bacteriol **72:**673-676.
26. **Olivier, G.** 1990. Virulence of *Aeromonas salmonicida* : Lack of relationship with phenotypic characteristics. Journal of Aquatic Animal Health **2:**119-127.
27. **Pepe, C. M., M. W. Eklund, and M. S. Strom.** 1996. Cloning of an *Aeromonas hydrophila* type IV pilus biogenesis gene cluster: complementation of pilus assembly functions and characterization of a type IV leader peptidase/N-methyltransferase required for extracellular protein secretion. Mol Microbiol **19:**857-69.

28. **Rose, A. S., A. E. Ellis, and A. Adams.** 1989. An assessment of routine *Aeromonas salmonicida* carrier detection by ELISA. Bull EFAP **9:**65-67.
29. **Salte, R., K. Norberg, J.A. Arnesen, O.R. Oedegaard and G. Eggset.** 1992. Serine protease and glycerophospholipid:cholesterol acyltransferase of Aeromonas salmonicida work in concert in thrombus formation; in vitro the process is counteracted by plasma antithrombin and alpha sub(2)-macroglobulin. J Fish Dis **15(3):** 215-227
30. **Sauer, F. G., M. A. Mulvey, J. D. Schilling, J. J. Martinez, and S. J. Hultgren.** 2000. Bacterial pili: molecular mechanisms of pathogenesis. Curr Opin Microbiol **3:**65-72.
31. **Schotts E.B.** Furunculosis. In Thoesen J.C. (eds) 1994. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4th ed., Version 1, Fish Health Section, American Fisheries Society
32. **Solanky K.S., I.W. Burton, S.L. MacKinnon, J.A.Walter and A. Dacanay.** 2005. metabolic changes in Atlantic salmon exposed to *A. salmonicida* detected by ^{1}H-nuclear magnetic resonance spectroscopy of plasma. Dis Aquat Org *in press*
33. **Specker, J.L., D.M. Portesi, S.C.Cornell and P.A. Veillette.** 1994. Methodology for implanting cortisol in Atlantic salmon and effects of chronically elevated cortisol on osmoregulatory physiology. Aquaculture, **121(1-3);** 181-193
34. **Stewart D.J., B.L. Clark, J.E. Peterson, D.A. Griffiths and E.F. Smith.** 1982 Importance of pilus-associated antigen in *Bacteroides nodosus* vaccines.Res Vet Sci **32(2):**140-147
35. **Stewart D.J., B.L. Clark, J.E. Peterson, D.L. Emery, E.F. Smith, D.A. Griffiths and I.J. O'Donnell.** 1995 The protection given by pilus and whole cell vaccines of *Bacteroides nodosus* strain 198 against ovine foot-rot induced by strains of different serogroups. Aust Vet J **62(5)**:153-9
36. **Thanassi, D. G., and S. J. Hultgren.** 2000. Multiple pathways allow protein secretion across the bacterial outer membrane. Curr Opin Cell Biol **12:**420-30.
37. **Trust T.J., E.E. Ishiguro, H. Chart and W.W. Kay.** 1983. Virulence properties of Aeromonas salmonicida. J World Maricult Soc **14;** 193-200
38. **Vipond, R., I. R. Bricknell, E. Durant, T. J. Bowden, A. E. Ellis, M. Smith, and S. MacIntyre.** 1998. Defined deletion mutants demonstrate that the major secreted toxins are not essential for the virulence of *Aeromonas salmonicida.* Infect Immun **66:**1990-8.
39. **Williams, K. P., and D. P. Bartel.** 1996. Phylogenetic analysis of tmRNA secondary structure. RNA **2:**1306-10.

541 Table 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or primer	Description, origin or sequence	Reference(s) source or description
Aeromonas salmonicida strains		
A449	A. salmonicida subsp. salmonicida isolated from a Brown trout in Eure, France, Cm	T. Trust
02-02	A449 flpA::km, Cm, Km	this work
02-10	A449 tapA::KO, Cm, Ap	this work
03-01	A449 flpA::km tapA::KO, Cm, Km, Ap	this work
03-06	A449 ΔvapA, Cm	this work
03-07	A449 ΔvapA flpA::Km, Cm, Km	this work
03-11	A449 ΔvapA tapA::KO, Cm, Ap	this work
03-12	A449 ΔvapA flpA::Km tapA::KO, Cm, Km, Ap	this work
Escherichia coli strains		
TOP10	K-12	Invitrogen, Grant 1990
EC100D pir-116	K-12, pir-116	Epicenter, Metcalf 1994
BW20767	K-12, pir+, conjugation+	Metcalf 1996
Plasmids		
pCRScript-Amp	oriE1, Ap	Clonetech
pAH34	oriR6Kγ, mobRP4, Ap	Metcalf 1996
pKO	pAH34 with tmRNA tag	this work
pKO-tapA	internal fragment of tapA in pKO, in frame with tmRNA tag	this work
pGP704	oriR6Kγ, mobRP4, Ap	Miller 1998
pUTKm1	oriR6Kγ, mobRP4, Km, Ap source of kanamycin resistance cassette	de Lorenzo 1990
pJW109-pir	pGP704 with flpA-flanking regions surrounding kanamycin resistance cassette	this work
pWM91	oriR6Kγ, sacB, mobRP4, Ap	Metcalf 1996
pWM-vapA	pWM91 with vapA-flanking regions surrounding crossover generated peptide	this work

542
543

543 Table 2. Oligonucleotides used in this study.
544

Name	Sequence (restriction sites are underlined, non-sequence specific regions in lower case)	description
tmRNA tag	ggccgcaactagttgcaGCAAACGACGAAAACTACGCACTAGCAGCTTA ATAAcagagct	coding strand of tmRNA tag
tmRNA tag	ctgTTATTAAGCTGCTAGTGCGTAGTTTTCGTCGTTTGCtgcaactagttg c	non-coding strand of tmRNA tag
tapA-1	ATTGCACTTCCTGCATATCAGACC	to amplify internal fragment for knockout
tapA-2	CGGGACCAGTATATATGTGTATGC	to amplify internal fragment for knockout
flpA-1	CGGCCTTGCATGCCAGCAAGGTTGCG	to amplify 5' flanking region for knockout
flpA-2	ccaagatctGAGCGCTGTCACTTGCCCGCGGC	to amplify 5' flanking region for knockout
flpA-3	gcgagatctCTGACCACTCCTCTATTTATGTAGTC	to amplify 3' flanking region for knockout
flpA-4	GCTTGCTGGTGAGGATCACATCCACCC	to amplify 3' flanking region for knockout
vapA-No	tgtgctcgagGCTCACAGTGCACCGAAGG	to amplify 5' flanking region for knockout
vapA-Ni	cccatccactaaacttaaacaAGTGTGAACGATGGTCAGCTGG	to amplify 5' flanking region for knockout, with crossover sequence
vapA-Co	tgtttaagtttagtggatgggTCTAACATCCTGCCTACTGAAGG	to amplify 3' flanking region for knockout, with crossover sequence
vapA-Ci	ataaggatccATGACAACTGAAATAAGGGC	to amplify 3' flanking region for knockout

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546 TABLE 3: Summary of immersion and intraperitoneal (i.p.) challenge data. Juvenile Atlantic salmon *(Salmo salar)* in fresh water
547 were challenged with wild-type *Aeromonas salmonicida* A449 or isogenic mutant strains. The flpA and tapA mutant strains were
548 tested by immersion of St John River Stock Atlantic salmon, all the remaining challenges were in Sackville River stock Atlantic
549 salmon. Data are percent (number infected/total number). Cumulative mortalities were compared by G-test. Mean time to death
550 (MTTD) was the time to 50% mortality in each group.
551

		Immersion			Intraperitoneal	
Bacterial Strain	Salmon Stock	Cumulative Percent Mortality	MTTD/days	Salmon Stock	Cumulative Percent Mortality	MTTD/days
A449	St. John	39.2% (38/97) [a]	12	Sackville	45.0% (18/40) [e]	6.5
Saline	St. John	0% (0/90) [b]	/	Sackville	0% (0/40) [f]	/
02-02	St. John	28.7% (27/94) [a]	17	Sackville	50.0% (19/38) [e]	7.5
02-10	St. John	25.3% (21/83) [b]	13	Sackville	39.0% (16/41) [e]	7.5
03-01		N/D		Sackville	37.5% (15/40) [e]	8.0
A449	Sackville	90.0% (72/80) [c]	5			
Saline	Sackville	0% (0/80) [d]	/			
03-01	Sackville	76.3% (60/80) [d]	10			

552 [a] Different letters indicate indicates statistically different values within groups (G-test; $p > 0.05$)

TABLE 4: The incidence of clinically inapparent (covert) infections in juvenile Atlantic salmon (*Salmo salar* L.) that had survived an immersion challenge with either the wild type strain or pili mutants of *Aeromonas salmonicida* subsp. *salmonicida* (Table 1). For microbiology swabs were taken from the skin mucous and the posterior kidney and cultured on TSA. For the stress test animals cortisol was administered parenterally at 100 mg kg^{-1} and the water temperature simultaneously raised to 18°C. Any animals showing signs of furunculosis within 10 days was considered to have been covertly infected. Data are percent (number infected/total number). Cumulative mortalities were compared by G-test.

		Microbiology		Stress-test
Bacterial Strain	Salmon stock	Skin mucous	Posterior kidney	Cumulative Percent Mortality
A449	St John	8.1% (3/37)	0% (0/37)	28.6% (6/21) [a]
Saline	St John	0% (0/36)	0% (0/36)	0% (0/32) [b]
02-02	St John	0% (0/38)	0% (0/38)	55.2% (16/29) [b]
02-10	St John	10.5% (4/38)	0% (0/38)	52.2% (12/23) [b]
A449	Sackville	N/D	N/D	85.7% (6/7) [c]
Saline	Sackville	N/D	N/D	0% (0/42) [c]
03-01	Sackville	N/D	N/D	84.2% (16/19) [c]

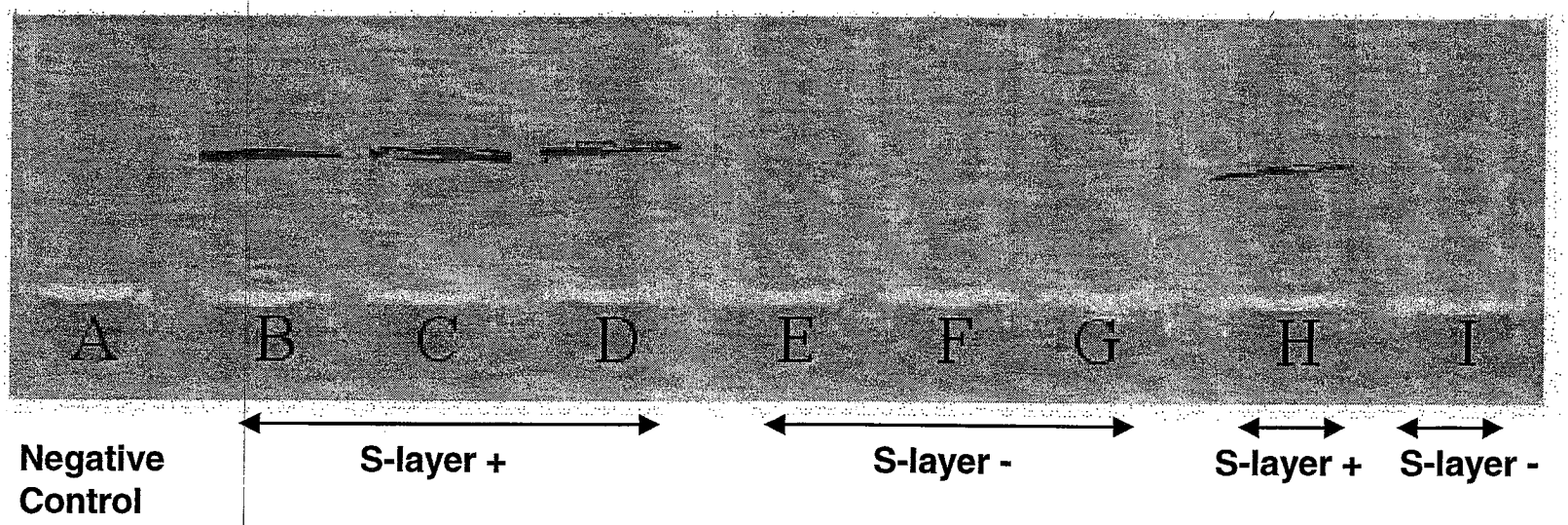
[a] Different letters indicate indicates statistically different values within groups (G-test; $p > 0.05$)

TABLE 5: Cumulative mortality in Sackville River stock Atlantic salmon in upon re-challenge with the virulent parental strain forty days after an initial i.p. challenge. Data are percent (number infected/total number). Cumulative mortalities were compared by G-test.

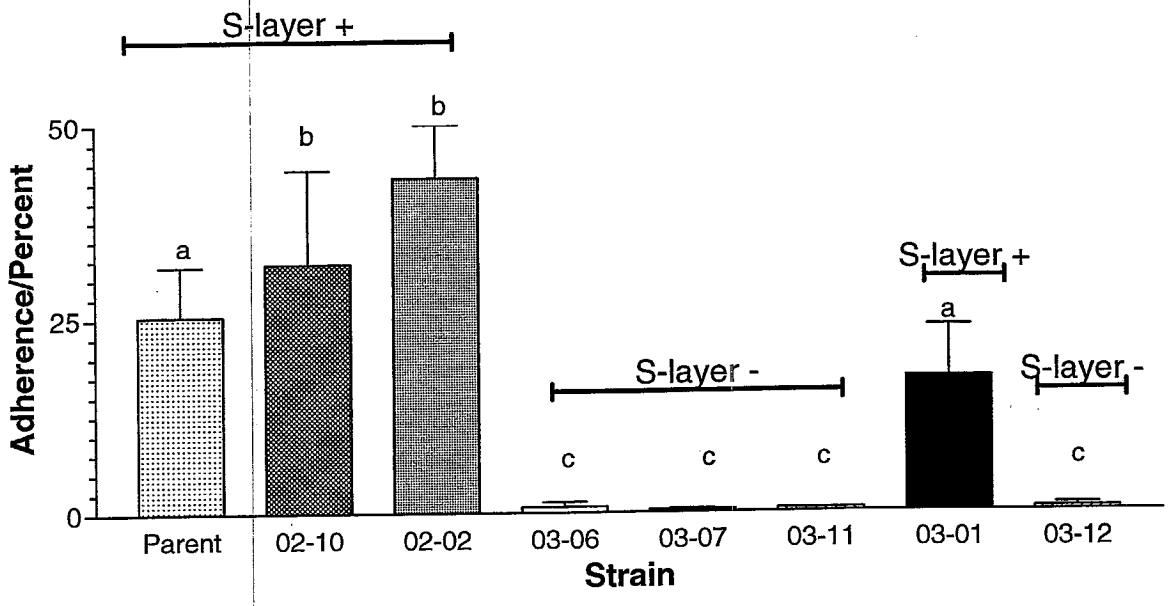
Initial i.p. challenge Strain	Re-challenge Strain	Cumulative Percent Mortality
A449	A449	22.7% (5/22) [a]
02-02	A449	26.3% (5/19) [a]
02-10	A449	24.0% (6/25) [a]
03-01	A449	36.0% (9/25) [a]
Saline	A449	48.0% (12/25) [b]
Saline	PBS	0% (0/25) [c]

[a] Different letters indicate indicates statistically different values within groups (G-test; $p > 0.05$)

FIGURE 1: Biofilm growth of parental strain and isogenic mutant strains of *Aeromonas salmonicida* A449 in borosilicate glass tubes. Bacteria were cultured in Griffin's Minimal Media for 15 days and biofilms were stained with 0.01% (w/v) crystal violet. A, media control; B, *A. salmonicida* A449; C, 02-10; D, 02-02; E, 03-06; F, 03-11; G, 03-07; H, 03-01; I, 03-12.



572 FIGURE 2: Adherence of wild-type and knock-out mutant strains of *A. salmonicida* A449 to salmonid epithelial cells (CHSE-214).
573 Data are mean +/- SE. There were no significant differences between wild-type *A. salmonicida* subsp. *salmonicida* strain A449 and
574 any of the pili mutants; *tapA*::ko *flpA*::km or *flpA*::km/*tapA*::ko. The adherence of the S-layer mutant (Δ *vapA*) was significantly
575 lower than that of the wild-type, although there was no significant difference in adherence between Δ *vapA* and Δ *vapA*/*flpA*::km.
576



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579 [a] Different letters indicate indicates sthatistically different values (G-test; $p > 0.05$)