Beyond the A-layer: adsorption of lipopolysaccharides and characterization of bacteriophage-insensitive mutants of *Aeromonas salmonicida* subsp. *salmonicida*

Valérie E. Paquet[1,2,3], Antony T. Vincent[4], Sylvain Moineau[2,5,6] and Steve J. Charette[1,2,3,#]

[1] Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Quebec City, QC, Canada, G1V 0A6

[2] Département de biochimie, de microbiologie et de bio-informatique, Faculté des sciences et de génie, Université Laval, Quebec City, QC, Canada, G1V 0A6

[3] Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec (IUCPQ), Quebec City, QC, Canada, G1V 4G5

[4] INRS-Institut Armand-Frappier, Laval, QC, Canada, H7V 1B7

[5] Groupe de recherche en écologie buccale (GREB), Faculté de médecine dentaire, Université Laval, Quebec City, QC, Canada, G1V 0A6

[6] Félix d'Hérelle Reference Center for Bacterial Viruses, Université Laval, Quebec City, QC, Canada, G1V 0A6

[#]To whom correspondence should be addressed: Institut de Biologie Intégrative et des Systèmes (IBIS), Pavillon Charles-Eugène-Marchand, 1030 avenue de la Médecine, Université Laval, Quebec City, QC, Canada, G1V 0A6

steve.charette@bcm.ulaval.ca; Telephone: 1-418-656-2131, ext. 406914; Fax: 1-418-656-7176

Short running title: *Phage-insensitive mutants of A. salmonicida*

Summary

Aeromonas salmonicida subsp. *salmonicida* is a fish pathogen that causes furunculosis. Antibiotherapy used to treat furunculosis in fish has led to resistance. Virulent phages are increasingly seen as alternatives or complementary treatments against furunculosis in aquaculture environments. For phage therapy to be successful, it is essential to study the natural mechanisms of phage resistance in *A. salmonicida* subsp. *salmonicida*. Here, we generated bacteriophage-insensitive mutants (BIMs) of *A. salmonicida* subsp. *salmonicida,* using a myophage with broad host range, and characterized them. Phage plaques were different depending on whether the A-layer surface array protein was expressed or not. The genome analysis of the BIMs helped to identify mutations in genes involved in the biogenesis of lipopolysaccharides (LPS) and on an uncharacterized gene (*ASA_1998*). The characterization of the LPS profile and gene complementation assays identified LPS as a phage receptor and confirmed the involvement of the uncharacterized protein ASA_1998 in phage infection. In addition, we confirmed that the presence of an A-layer at the bacterial surface could act as protection against phages. This study brings new elements into our understanding of the phage adsorption to *A. salmonicida* subsp. *salmonicida* cells.

Keywords: *Aeromonas salmonicida* subsp. *salmonicida*, bacteriophage, A-layer (S-layer), bacteriophage-insensitive mutants (BIMs), lipopolysaccharides (LPS).

Introduction

The Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* is a significant fish pathogen that causes furunculosis (Menanteau-Ledouble *et al.*, 2016), a disease prevailing in the salmonid family. Furunculosis negatively affects the aquaculture industry worldwide (Dallaire-Dufresne *et al.*, 2014a), leading to major economic losses in this fast-growing sector (FAO, 2018).

The type III secretion system (T3SS) and the A-layer are two main virulence factors of *A. salmonicida* subsp. *salmonicida* (Dallaire-Dufresne *et al.*, 2014a). The T3SS is a needle-like structure, which allows the translocation of effector proteins from the bacterial cytoplasm to host cell. These effector proteins then alter various cell functions including inhibition of phagocytosis and induction of apoptosis (Vanden Bergh & Frey, 2014). The A-layer is a major surface array protein also known as the S-layer (Doig *et al.*, 1992). Among others, it confers resistance to macrophage cytotoxicity and provides protection against specific immune defense (Dallaire-Dufresne *et al.*, 2014a).

Several plasmids that confer resistance to antibiotics have been identified in strains of this bacterial species, thereby limiting treatment options for furunculosis outbreaks (Vincent *et al.*, 2014, Vincent *et al.*, 2015, Tanaka *et al.*, 2016, Trudel *et al.*, 2016). Moreover, prophylactic immunization for this pathology suffers from logistic constraints, side effects, or, in some cases, a lack of efficiency (Larsen & Pedersen, 1997, Dallaire-Dufresne *et al.*, 2014a). Therefore, phage therapy is now being explored as a possible alternative for treatment of furunculosis (Imbault, 2006, Pereira *et al.*, 2011, Kim *et al.*, 2015).

If properly selected, virulent phages can rapidly penetrate into their target bacteria and lyse them promptly (Jamal *et al.*, 2018). The initial step of the phage lytic cycle is its adsorption

to the host receptor (Labrie *et al.*, 2010). Lipid A of the lipopolysaccharide (LPS) was previously identified as a receptor for the *A. salmonicida* subsp. *salmonicida* myophage 55R-1 (Ishiguro *et al.*, 1983). The A-layer was also reported as a receptor for temperate myophage TP446 of *A. salmonicida* (Ishiguro, 1984), indicating a diversity of phage receptors in this species.

Over a decade ago, a study reported the testing of the virulent myophage HER110 to treat trouts with furunculosis (Imbault, 2006). After 45 days, only 10% of the fish showed mortality or serious illness while 100% of the untreated fish were severely sick or died (Imbault, 2006). The potential efficacy of phage therapy on *A. salmonicida* subsp. *salmonicida* was also evaluated with infected rainbow trout. The myophage PAS-1 showed notable protective effects, revealed by the increased survival rates of infected rainbow trouts (Kim *et al.*, 2015).

However, treatments with bacteriophages also present complications and some disparate effects (Torres-Barcelo, 2018). Bacteria have a wide range of phage defense mechanisms (Labrie *et al.*, 2010). Understanding the mechanisms that drive the emergence of phage-resistant strains is crucial to provide a successful path to phage therapy as an alternative treatment method in aquaculture (Samson *et al.*, 2013, Moreirinha *et al.*, 2018). Usually, all mechanisms rely on protein interactions or functions, but defence against phage infections based on bacterial secondary metabolites was also recently reported in *Streptomyces* (Kronheim *et al.*, 2018).

In the case of *A. salmonicida* subsp. *salmonicida*, mechanisms of resistance to phages by the bacterium are still elusive. Recently, we characterized several phages infecting *A. salmonicida* subsp. *salmonicida* including phages with broad host range such as the myophage SW69-9 that contains no lysogeny module (Vincent *et al.*, 2017). A good way to study these mechanisms of resistance is to isolate bacteriophage-insensitive mutants (BIMs) for these phages and compare their genomes with the sensitive parental wild type strain (Millen & Romero, 2016).

In *Lactobacillus delbrueckii* subsp. *bulgaricus*, analysis of BIMs showed that the attachment and the adsorption of the phage to the bacterial receptor was a critical step, and that it was hindered by a change in the polysaccharide structure on their S-layer (Deng *et al.*, 2018). Other studies pointed to the presence of quorum-sensing-regulated phage defense mechanisms or changes in the gene sequence of the receptor (Hossain *et al.*, 2012, Tan *et al.*, 2015).

In this study, we generated *A. salmonicida* subsp. *salmonicida* BIMs following infection with the lytic phage SW69-9 and characterized their phenotypes and genotypes with the goal to improve our knowledge on mechanisms of resistance to phages for this bacterium. This helped to identify a potential new gene involved in the adsorption of *A. salmonicida*' bacteriophages while presenting new clues about the role of LPS and the A-layer in the regulation of bacteriophage-bacterium interaction.

Results

Altered lytic plaque phenotype

A. salmonicida subsp. *salmonicida* M15879-11 was used as a bacterial host to amplify the newly characterized *A. salmonicida* lytic phage SW69-9 (Vincent *et al.*, 2017). A spot test of SW69-9 on the M15879-11 strain shows a turbid phenotype of the lytic plaque (**Figure 1A**). In order to create mutants showing new phenotypes in regard of bacteriophage's interaction, M15879-11 strain was grown at 25ºC, a temperature known to promote genomic rearrangements that include the loss of the T3SS locus (Daher *et al.*, 2011, Tanaka *et al.*, 2017). We generated the M15879-11-R3 (AS-R3) and M15879-11-R5 (AS-R5) mutant strains, and noticed that those had lost their T3SS, confirming that genomic alterations occurred (**Figure S.1**). We noticed that AS-R5 showed a clear lytic plaque phenotype (**Figure 1B**) in spot test assay against SW69-9, contrary to the wild type strain and AS-R3. In addition to the lost T3SS, AS-R5 also lost its A-layer, as was revealed by growth on brilliant blue Coomassie agar (**Figure 1C**). The wild-type bacteria had a functional A-layer that absorbed the brilliant blue colorant pigment, while AS-R5 remained non-pigmented. PCR amplification was done to confirm the presence of the *vapA* gene that encodes the A-layer protein (**Figure 1D**). Wild type strains (01-B526 and M15879-11) and natural mutants (AS-R3 and AS-R5) had positive amplification product of 1900 pb for *vapA*. Only strain HER1110, which is the negative control and is known to lack a functional A-layer (Daher *et al.*, 2011) on brilliant blue Coomassie agar, did not possess the *vapA* gene.

In light of these results, we sequenced the genome of AS-R5 by Illumina MiSeq. A mutation in the *vapA* gene produced a premature stop codon, leading to an ORF that encodes 87 amino acid proteins instead of the 502-residue polypeptide found in the wild-type strain. This

explained the absence of the A-layer while the *vapA* gene is still present in AS-R5. Therefore, the absence of the A-layer at the surface of the cell could contribute to the clear lytic plaque observed for this strain.

To confirm the hypothesis that turbid lytic plaque is associated with the presence of a functional A-layer, 24 strains of *A. salmonicida* subsp *salmonicida* were screened for the presence of the *vapA* gene, the presence of an A-layer and, finally the appearance of lytic plaque on a phage spot test assay (**Table 1**). Three different profiles were observed: presence of *vapA* and functional A-layer leading to the turbid lytic plaque phenotype with phage SW69-9 on spot test (11/24, e.g.: M15879-11), presence of *vapA* and non-functional A-layer leading to clear lytic plaque with phage SW69-9 on spot test (8/24, e.g.: AS-R5) and finally absence of *vapA* and non-functional A-layer leading to clear lytic plaque with phage SW69-9 on spot test (5/24, e.g.: HER1110). The presence of a functional A-layer was linked to the turbid lytic plaque phenotype against phage SW69-9. Eleven other *A. salmonicida* subsp. *salmonicida* specific bacteriophages from a previous study (Vincent *et al.*, 2017) were tested and confirmed that this phenomenon is bacterial host strain dependent (**Table S.1**), i.e. linked to the absence of a functional A-layer at the surface of the bacterial strain and not linked to an effect due to the phage.

Phage adsorption assay

Lipid A, one of the major structures of the LPS, is known to be required for binding of *A. salmonicida* subsp. *salmonicida* bacteriophages to bacterial cells (Ishiguro *et al.*, 1983). However, it was described in the same bacterium that more than 60% of the LPS is covered by the A-layer (Ebanks *et al.*, 2005). Knowing that the A-layer could act as a protection against bacteriophages, we compared the adsorption activity of phage SW69-9 on host M15879-11 with

a functional A-layer, compared to AS-R5 with a non-functional A-layer (**Figure 2**). After 5 minutes, less than 15% of the phage's population was adsorbed to the M15879-11 cells comparatively to more than 70% with strain AS-R5. Even after 20 minutes, the adsorption of SW69-9 to M15879-11 was limited at 43% compared to more than 90% to AS-R5. After 30 minutes, the adsorption rate of the mutated strain remained constant comparatively of the low and variable adsorption rate for the wild type strain.

BIM generation and genomic analysis

To investigate phage-bacteria interactions in *A. salmonicida* subsp. *salmonicida*, we isolated a set of BIMs (**Table S.2**) following the prolonged incubation of the mutated strain AS-R5 with phage SW69-9. Characterisation of 9 BIMs revealed a strong level of phage resistance when compared with the parental strain even after repeated growth on plates and freezing/thawing cycles. One of these BIMs was randomly selected (BIM93) and its genome sequenced to shed light on the genetic determinant involved in the phage resistance phenotype. Only one non-synonymous mutation was found in the genome of BIM93 as compared to AS-R5. This mutation occurred in a gene, named *ASA_1998* in the reference strain *A. salmonicida* subsp. *salmonicida* A449 (Reith *et al.*, 2008) and codes for a 544 amino acid hypothetical protein of unknown function. The mutation resulted in the modification of cysteine at position 219 to a phenylalanine. Because LPS are known to act as receptor for *A. salmonicida* subsp. *salmonicida* bacteriophages (Ishiguro *et al.*, 1983), and because no mutation was detected on LPS-biogenesis genes on BIM93, this result suggests that the adsorption of the phage was blocked or that the resistance mechanism used by the bacteria is not at the adsorption level.

The genomic DNA of 8 other BIMs were pooled and sequenced to analyse and to compare their mutation profiles (**Table 2).** This second round of sequencing allowed detecting and confirming mutations on genes involved in LPS synthesis: i.e., two heptosyltransferase family proteins with mutations at different positions on these genes for all other BIMs.

Complementation assays

To confirm the various phage phenotypes observed above, we cloned the wild-type *vapA* and *ASA_1998* genes in plasmid pAsa7 and transformed the recombinant plasmids into BIM93. The expression of a functional A-layer (*vapA*) in BIM93 was confirmed on Coomassie brilliant blue agar (**Figure 3A** and **C**) but it did not restore the phage sensitivity phenotype. On the other hand, BIM93 expressing *ASA_1998* was totally lysed by phage SW69-9, confirming its role in phage infectivity (**Figure 3B**). Of note, the complemented strain BIM93-ASA_1998 stayed non-pigmented on Coomassie brilliant blue agar (**Figure 3C)**, suggesting no link between ASA_1998 and the A-layer biogenesis.

LPS profile on SDS-PAGE

To verify if the *ASA_1998* gene was linked to LPS production, the LPS profile of various strains were analyzed on 12% SDS-PAGE and revealed by silver staining (**Figure 4**). Wild-type isolates M15879-11, AS-R5 and HER1110 had no mutation in the LPS gene and had very similar LPS profiles with several well-distributed LPS-moieties at various molecular weights. On the other hand, BIM93, which has a point mutation in the *ASA_1998* gene, showed an altered LPS profile. The LPS profiles of all other BIMs, possessing mutations required in the LPS biogenesis (**Table 2),** were very similar to the one of BIM93 (**Figure S.2**), suggesting that the

193 uncharacterized *ASA_1998* gene is directly or indirectly involved in LPS transportation,
194 structure, synthesis or anchoring. Finally, the rescue of the *vapA* gene leading to an over
195 expression of A-layer protein at the surface of the positive electro-transformant BIM93 did not
196 allow a return of the LPS biosynthesis. The complementation of *ASA_1998* in BIM93 led to a
197 partial return of LPS biosynthesis, further supporting the hypothesis that ASA_1998 plays a role
198 in the biosynthesis of LPS.

Discussion

This study has shed light on the role of the A-layer during the phage-bacteria interactions in *A. salmonicida* subsp. *salmonicida*. Deprived of the A-layer, the strain AS-R5 showed a clear phage plaque phenotype and was used to generate stable BIMs. The characterization of these BIMs led to the identification of a new gene (*ASA_1998*) involved in phage adsorption. The function of the uncharacterized protein encoded by this gene is also associated with LPS biosynthesis.

Outer proteins and LPS

At the cellular membrane, the expression of a functional A-layer anchored to LPS potentially hides the sugar residues and makes the accessibility to the receptor, the lipid A of LPS (Ishiguro *et al.*, 1983), difficult for phages. Bacterial outer membrane proteins play a significant role in virulence because they cover the major part of the cell wall and they are the first bacterial defense line against the molecules and the cells of the immune system (Rollauer *et al.*, 2015). The A-layer complex is the major outer surface protein in *A. salmonicida* subsp. *salmonicida* (Ebanks *et al.*, 2005) and it is attached to LPS on the cell surface (Chart *et al.*, 1984). The A-layer can impart various functions to its host. It physically protects the cell against bacteriophages, as well as proteases and molecules (complement) of the fish immune system (Kay & Trust, 1991). Belland and Trust demonstrated in 1987 that the cultivation of *Aeromonas* at 30°C may lead to genetic rearrangements which can result in the loss of the expression of the A-layer (Belland & Trust, 1987).

For a long time, the LPS was identified as the receptor for phage infections (Paterson, 1969). In the 80s, Ishiguro's team clarified the portion of the LPS involved in phage interaction: lytic phage 55R-1 linked to lipid A (Ishiguro *et al.*, 1983). Subsequently, the same team indicated that the A-layer is a component of the receptor for temperate phage TP446, specific to *A. salmonicida* strain A446 (Ishiguro, 1984).

Attached to the outer membrane, the inner core region of the LPS is composed of lipid A, a hydrophobic membrane anchor. The structure of lipid A is strongly conserved among different isolates of *A. salmonicida* subsp. *salmonicida* (Wang *et al.*, 2006). Next, the hydrophilic core-region of the LPS, bound to lipid A, is composed of an O-polysaccharide chain followed by the highly variable polysaccharide known as the O-antigen region in the outer region of the bacterial cell envelope. In several Gram-negative bacteria, the LPS O-antigen is one of the essential components of the bacterial survival toolkit in hostile environments (Kalynych *et al.*, 2014). In *A. salmonicida*, the core region or the O-polysaccharide chain composition of the smooth LPS purified is very similar and homogeneous (Chart *et al.*, 1984) and evolutionary conserved (Forn-Cuni *et al.*, 2017). Only *A. salmonicida* subsp. *pectinolytica* presents a different outer LPS core compared to the other *A. salmonicida* species (Merino & Tomas, 2016). The O-polysaccharide chain is also strongly immunogenic and a part of this chain crosses the A-layer surface and is exposed to the cell surface (Chart *et al.*, 1984). Thereby, LPS is a key target for phages with a broad host range in *A. salmonicida*.

Discovery of A. salmonicida genes regulating phages adsorption

Since the complementation of BIM93 with *vapA* did not restore sensitivity to phages, we confirmed that the A-layer is not the receptor for *A. salmonicida* phage SW69-9. Conversely, the LPS and the protein expressed by gene *ASA_1998* are involved in phage binding to bacterial cells. It is possible that the LPS represent only a part of the receptor for myophage SW69-9, as was demonstrated for several coliphages including T4 of the *Myoviridae* family (Mutoh *et al.*, 1978). It is possible that phage SW69-9 uses a combination of LPS and outer membrane protein receptors, with the ASA_1998 protein being the potentially second element of a composite receptor. However, the LPS profile of BIM93 is clearly identical to LPS migration profiles of the other BIMs (Figures 4 and S.2). Since these BIMs have mutations in genes involved in enzymes of the LPS biosynthesis pathway, this suggests that the *ASA_1998* gene found mutated in BIM93 may also be associated with the biosynthesis or the attachment of LPS to the cell outer-membrane.

The mutation in the *ASA_1998* gene of BIM93 is the unique non-synonymous mutation comparatively to sensitive AS-R5 strain. This gene encodes a hypothetical protein predicted to have a signal peptide and a transmembrane domain (**Figure S.3**), which let suppose that a region of the protein may be exposed outside the cell. The mutation resulted in the modification of cysteine at position 219 to a phenylalanine. Cysteines are known to be sometime implicated in the formation of disulfide bridges permitting the stabilisation of the native conformation of a protein. The impact of the replacement of a cysteine by a phenylalanine (hydrophobic amino acid) in *ASA_1998* remains to be elucidated. Nevertheless, since it is the only mutation found in BIM93, this mutation clearly has an impact that explains the inability of the SW69-9 phage to infect BIM93.

267

268 *Phage adsorption and lytic plaque phenotype*

269 It was previously reported that the adsorption rate of the myophage 55R-1 was slower on
270 the A-layer positive strain compared to A-layer negative strain (Chart *et al.*, 1984). Because the
271 LPS is not completely decorated by A-layer proteins on the cell surface, the adsorption rate is
272 weak but remains (Ebanks *et al.*, 2005). Therefore, the synthesis of A-layer protein and the
273 synthesis of LPS may be involved in coordinate regulation (Belland & Trust, 1985) for phage
274 receptor availability. We showed interest in the AS-R5 strain because phage SW69-9 formed
275 clear plaques compared to all those observed in the previous study which were turbid (Vincent
276 *et al.*, 2017). The observation of such phage plaque differences has been made also by others in
277 *A. salmonicida* (Easwaran *et al.*, 2017). By comparing the lytic phenotype of several strains with
278 or without functional A-layers, the role of the A-layer in turbid plaque seems evident.

279

280 *LPS profile and BIM mechanisms*

281 The LPS profiles on SDS-PAGE are very similar between *A. salmonicida* subsp.
282 *salmonicida* strain M15879-11 with a functional A-layer or not (AS-R5 and HER1110). Having
283 an A-layer anchored on the LPS did not seem to modify the gel migration pattern (Shaw *et al.*,
284 1986). A BIM may lose its ability to resist a phage if it is no longer exposed to it, or if the
285 bacterium is subcultured several times (Deng *et al.*, 2018). As previously noted (Imbault, 2006),
286 BIMs generated from the A-layer positive strain did not retain the resistance phenotype over
287 time. Some standard assays to generate BIMs required a clear plaque phenotype (Hynes *et al.*,
288 2017). Using the AS-R5 strain helped to obtain stable BIM that kept the resistance phenotype
289 over time. The hypotheses that have been proposed to explain this phenomenon are an increased

expression of *vapA* leading to an increase in the cell surface covered by the A-layer (Ebanks *et al.*, 2005) thus rendering the receptor totally inaccessible, or, a reversible epigenetic mutation by colony phase variation (Van den Broek *et al.*, 2005).

The BIM study and the identification of the underlying mechanisms of resistance are essential in the context of phagotherapy. Among the mechanisms studied on *A. salmonicida* subsp. *salmonicida,* the alteration of the intracellular protein expression levels of phage-resistant mutants was observed. These proteins are associated with phage transcription or stress regulation (Moreirinha *et al.*, 2018). Here, after sequencing several BIMs and sensitive strains, we revealed a role for genes involved in the biogenesis of LPS, such as the heptosyltransferase family enzymes (**Table 2**). Indeed, this enzyme family catalyzes the addition of multiple heptose sugars to form the core region of LPS. Cells deficient in HepI, the most studied heptosyltransferase in Gram-negative bacteria, display a truncated LPS on their cell surface (Cote & Taylor, 2017). In the present case, the mutation clearly causes a truncated LPS (**Figure 4**), as demonstrated in another study (Jimenez *et al.*, 2009). By the absence of sugar residues on the LPS, the phage receptor thus becomes non-functional to initiate the adsorption step. This also seems to be the case when phages infect the catfish pathogen *Edwardsiella ictaluri*, because major modification on outer membrane protein, receptor for adsorption, is a principal molecular determinant of phage susceptibility in this pathogen (Hossain *et al.*, 2012).

Taken altogether, virulent phages with broad host range will likely be part of phage therapy including in aquaculture environments. Understanding how targeted bacteria will evolve toward phage resistance is crucial for the long-term option of this alternative treatment. Genome analysis of BIMs identified a novel mutation in an uncharacterized protein (ASA_1998), thereby linking this host protein to phage biology.

Experimental procedures

Bacteriophage amplification

A. salmonicida subsp. *salmonicida* M15879-11 was used as a bacterial host to amplify phage SW69-9 as previously described (Vincent *et al.*, 2017).

Strains and homologous recombination

A. salmonicida subsp. *salmonicida* M15879-11 was cultured at 25°C twice over 3 days to initiate the homologous recombination of the plasmid pAsa5 (Daher *et al.*, 2011) under stressful conditions. After a week, isolated colonies were analysed by PCR to detect clones that lost genes encoding the T3SS (Tanaka *et al.*, 2017). Two rearranged strains derived from *A. salmonicida* subsp. *salmonicida* M15879-11 were used in this study, namely M15879-11-R3 (AS-R3) and M15879-11-R5 (AS-R5). Information about other strains used in this study is available in **Table S.3**.

Spot test assay, Coomassie brilliant blue agar and PCR genotyping

The phage spot test assay was previously described (Vincent *et al.*, 2017). The phenotype characterization of the A-layer membrane protein was done by Coomassie brilliant blue agar using the standard protocol (Daher *et al.*, 2011). The virulent reference strain *A. salmonicida* subsp *salmonicida* 01-B526 (Charette *et al.*, 2012) was used as positive control for the functional A-layer and PCR amplification. The strain *A. salmonicida* subsp *salmonicida* HER1110 was used as the non-functional A-layer and negative PCR control (Daher *et al.*, 2011).

Primers used to amplify *vapA* were 5'-CAGGACATGAGCATCAGTAGTTTCC-3' and 5'-CGACTAGATTCGCTCTTACAGAGTG-3'. DNA lysates and PCR mixtures were prepared as previously described (Trudel *et al.*, 2013). The PCR program was 5 min at 95ºC, then denaturation at 95ºC for 30 s, annealing at 57ºC for 30 s and extension for 2 minutes at 68ºC repeated 30 times with a five minutes final extension at 68ºC. The PCR products were made visible on 1% agarose gel containing 0.5 $\mu g.mL^{-1}$ ethidium bromide. All PCR reactions were performed at least twice with appropriate controls. The quick load 2-log DNA ladder (NEB) was used as molecular markers.

Adsorption assay

Adsorption assay of phage SW69-9 was performed on the wild-type and As-R5 strains in triplicate as reported (Duplessis & Moineau, 2001). Strains were grown in 10 mL TSB (EMD, Millipore, Canada) until an OD_{600} between 0.6 and 0.8. The phage lysate was diluted up to 0.0001 and 100 µL of this dilution were added to 900 µL of the host strain in a 1.5 mL tube. In parallel, a negative control, which was used to determine the initial phage titer, was done with 100 µL of the phage dilution in 900 µL of TSB. Six tubes were incubated simultaneously at 18°C with agitation at 200 rpm. Every 5 minutes for 30 minutes, a test tube and a control tube were centrifuged and 100 µL of the supernatant were plated on soft-top agar in technical triplicate. The percentage of phage adsorption was calculated as described (Duplessis & Moineau, 2001).

Generation of BIMs

The BIMs were generated based on Mills' procedure (Mills *et al.*, 2007). In summary, the rearranged sensitive host AS-R5 was completely lysed by soft-top agar plaque assay with a

SW69-9 phage at MOI ≥ 1. After overnight incubation at 25°C or 18°C, some phage-resistant colonies appeared. All of the phage-resistant colonies were harvested and suspended in 10 mL of TSB, then incubated overnight at 25°C or 18°C at 200 rpm. The next day, in fresh 10 mL aliquots of TSB, 10% of the BIM preculture was inoculated with SW69-9 phage at a MOI of 10 and incubated overnight at 25°C or 18°C at 200 rpm. The BIM and phage culture was replicated four times, doubling the phage MOI each time. The fourth day, serial dilutions of the BIM culture were plated on tryptic soy agar (TSA) (EMD, Millipore, Canada) and incubated overnight at 25°C. Twenty isolated colonies were streaked on TSA and incubated overnight at 25°C, then frozen in TSA-15% glycerol stock at -80°C. The stability of the phage resistance phenotype was measured by phage spot test assay against an undiluted solution of SW69-9 phage, by 4 passes on TSA in the absence of the phage, and finally, by phage spot test assay post-freezing. Only BIMs that kept the resistance phenotype were used for the study, i.e. 9 mutants (**Table S.2**).

Bacterial DNA extraction, sequencing and de novo assembly

The DNA of the *A. salmonicida* subsp *salmonicida* strains M15879-11, AS-R5 and BIMs were extracted with a DNeasy Blood and Tissue Kit (Qiagen, Canada). Sequencing libraries were prepared with a Nextera DNA Library Preparation Kit and sequenced on an Illumina MiSeq apparatus at the IBIS sequencing platform (Université Laval, Canada). The sequencing reads for M15879-11 were *de novo* assembled using an A5-miseq version 20160825 (Coil *et al.*, 2015). The mutations in strain AS-R5 were found using snippy version 3.2-dev (https://github.com/tseemann/snippy) while those from the mixed BIMs were found using a combination of bwa version 0.7.17-r1188 (Li *et al.*, 2009, Li, 2013), samtools version 1.6 (Li *et*

380 *al.*, 2009), freebayes version v1.1.0-54-g49413aa (Garrison, 2016) and SnpEff version 4.3p
381 (Cingolani *et al.*, 2012).
382
383 *Cloning of vapA and ASA_1998*
384 The plasmid pAsa7 (Vincent *et al.*, 2016) is a small ColE1-type replicon plasmid (~5 270
385 bp) that bears a functional resistance to chloramphenicol and is naturally found in *A. salmonicida*
386 subsp. *salmonicida.* The plasmid pAsa7 was modified by the insertion of the lac promoter and
387 terminator from the vector pGFPuv (Clontech Lab, CA, USA). Then, each gene of interest (*vapA*
388 or *ASA_1998*) **(Figure S.4)** was cloned between the lac promoter and terminator before
389 transformation into *E. coli* MC1061 (Michelsen, 1995) competent cells and selection on
390 chloramphenicol-containing LB plates. Clones with the sequenced-confirmed right insert were
391 electroporated in *A. salmonicida* subsp. *salmonicida* BIM93 cells (Dallaire-Dufresne *et al.*,
392 2014b). Electro-transformants were tested for sensitivity to phage SW69-9 (spot test assay) and
393 a functional A-layer (Coomassie brilliant blue agar).
394
395 *LPS extraction*
396 The LPS extraction was done with a kit from Boca Scientific (USA). Briefly, 2 mL of a
397 growing bacterial culture (OD_{600} between 0.8 and 1.2) were collected by centrifugation before
398 continuing with the rest of manufacturer's protocol. Then, the purified LPS was suspended in
399 buffer (10 mM Tris-HCl pH 8) and treated for 30 minutes at 37°C with 20 µg mL^{-1} of RNase A
400 (Ambion, Can) and then 30 minutes at 50°C with 2.5 µg of proteinase K (Sigma-Aldrich, Can)
401 for each 1 µg of LPS. The LPS extraction was performed at least in duplicate for each strain and
402 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue

staining was done to ensure the absence of contaminating proteins. The absence of DNA and RNA was also confirmed on agarose gel.

LPS characterization

The SDS-PAGE was done by Laemmli's method in denaturizing conditions (5% ß-mercaptoethanol) (Laemmli, 1970). A mini-PROTEAN Tetra cell system (Bio-Rad, USA) was used to migrate all LPS trials. Samples of LPS were diluted 2/3 in TEX 3X (188 mM Tris-HCl pH 6.8, 3% SDS, 30% glycerol, 0.01% bromophenol blue) to a final concentration of TEX 1X and boiled 5 min before it was loaded in 12% acrylamide separating gel. Gels were run for 10 min at 100 V then 45 min at 200 V. To visualize LPS, the gels were fixed for 30 min, washed 3 times during 10 min and stained over 15 min using the Silver Staining Plus Kit (Bio-Rad, USA).

Acknowledgements

We are grateful to Michel Frenette for critical reading of the manuscript. We acknowledge funding from the Ministère de l'agriculture, des pêcheries et de l'alimentation du Québec (INNOVAMER Program), the Natural Sciences and Engineering Research Council of Canada and Ressources Aquatiques Québec. ATV received an Alexander Graham Bell Canada Graduate Scholarship from NSERC. SJC is a research scholar of the Fonds de Recherche du Québec en Santé. SM holds a Tier 1 Canada Research Chair in Bacteriophages.

Author contributions

VEP, ATV, SM, and SJC contributed to the concepts and design of the study. VEP, ATV and SJC participated in the acquisition, analysis, and interpretation of the data. VEP drafted the manuscript. All authors reviewed the manuscript.

Competing Interests: The authors declare that they have no competing interests.

Data Availability Statement: Data available on request from the authors.

References

Ahmad S, Khan H, Shahab U*, et al.* (2017) Protein oxidation: an overview of metabolism of sulphur containing amino acid, cysteine. *Front Biosci (Schol Ed)* **9**: 71-87.

Belland RJ & Trust TJ (1985) Synthesis, export, and assembly of *Aeromonas salmonicida* A-layer analyzed by transposon mutagenesis. *J Bacteriol* **163**: 877-881.

Belland RJ & Trust TJ (1987) Cloning of the gene for the surface array protein of *Aeromonas salmonicida* and evidence linking loss of expression with genetic deletion. *J Bacteriol* **169**: 4086-4091.

Charette SJ, Brochu F, Boyle B, Filion G, Tanaka KH & Derome N (2012) Draft genome sequence of the virulent strain 01-B526 of the fish pathogen *Aeromonas salmonicida*. *J Bacteriol* **194**: 722-723.

Chart H, Shaw DH, Ishiguro EE & Trust TJ (1984) Structural and immunochemical homogeneity of *Aeromonas salmonicida* lipopolysaccharide. *J Bacteriol* **158**: 16-22.

Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X & Ruden DM (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly (Austin)* **6**: 80-92.

Coil D, Jospin G & Darling AE (2015) A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics* **31**: 587-589.

453 Cote JM & Taylor EA (2017) The Glycosyltransferases of LPS Core: A Review of Four
454 Heptosyltransferase Enzymes in Context. *Int J Mol Sci* **18**.

455 Daher RK, Filion G, Tan SG, Dallaire-Dufresne S, Paquet VE & Charette SJ (2011) Alteration
456 of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas*
457 *salmonicida* in stressful conditions. *Vet Microbiol* **152**: 353-360.

458 Dallaire-Dufresne S, Tanaka KH, Trudel MV, Lafaille A & Charette SJ (2014a) Virulence,
459 genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida,* the causative
460 agent of fish furunculosis. *Vet Microbiol* **169**: 1-7.

461 Dallaire-Dufresne S, Emond-Rheault JG, Attere SA, Tanaka KH, Trudel MV, Frenette M &
462 Charette SJ (2014b) Optimization of a plasmid electroporation protocol for *Aeromonas*
463 *salmonicida* subsp. *salmonicida*. *J Microbiol Methods* **98**: 44-49.

464 Deng K, Fang W, Zheng B, Miao S & Huo G (2018) Phenotypic, fermentation characterization,
465 and resistance mechanism analysis of bacteriophage-resistant mutants of *Lactobacillus*
466 *delbrueckii* ssp. *bulgaricus* isolated from traditional Chinese dairy products. *J Dairy Sci* **101**:
467 1901-1914.

468 Doig P, Emody L & Trust TJ (1992) Binding of laminin and fibronectin by the trypsin-resistant
469 major structural domain of the crystalline virulence surface array protein of *Aeromonas*
470 *salmonicida*. *J Biol Chem* **267**: 43-49.

471 Duplessis M & Moineau S (2001) Identification of a genetic determinant responsible for host
472 specificity in *Streptococcus thermophilus* bacteriophages. *Mol Microbiol* **41**: 325-336.

473 Easwaran M, Dananjaya SHS, Park SC, Lee J, Shin HJ & De Zoysa M (2017) Characterization
474 of bacteriophage pAh-1 and its protective effects on experimental infection of *Aeromonas*
475 *hydrophila* in Zebrafish (*Danio rerio)*. *J Fish Dis* **40**: 841-846.

476 Ebanks RO, Goguen M, McKinnon S, Pinto DM & Ross NW (2005) Identification of the major
477 outer membrane proteins of *Aeromonas salmonicida*. *Dis Aquat Organ* **68**: 29-38.

478 FAO (2018) The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable
479 development goals. p.^pp. Rome.

480 Forn-Cuni G, Merino S & Tomas JM (2017) Comparative Genomics of the Aeromonadaceae
481 Core Oligosaccharide Biosynthetic Regions. *Int J Mol Sci* **18**.

482 Garrison GMaE (2016) Haplotype-based variant detection from short-read sequencing. p.^pp.

483 Hossain MJ, Rahman Kh S, Terhune JS & Liles MR (2012) An outer membrane porin protein
484 modulates phage susceptibility in *Edwardsiella ictaluri*. *Microbiology* **158**: 474-487.

485 Hynes AP, Lemay ML, Trudel L, Deveau H, Frenette M, Tremblay DM & Moineau S (2017)
486 Detecting natural adaptation of the *Streptococcus thermophilus* CRISPR-Cas systems in research
487 and classroom settings. *Nat Protoc* **12**: 547-565.

488 Imbault S, Parent, S., Lagacé M., Uhland C.F. and Blais JF. (2006) Using Bacteriophages to
489 Prevent Furunculosis Caused by *Aeromonas salmonicida* in Farmed Brook Trout. *Journal of*
490 *Aquatic Animal Health* **18:3**: 203-214.

491 Ishiguro EE, Ainsworth T, Shaw DH, Kay WW & Trust TJ (1983) A lipopolysaccharide-specific
492 bacteriophage for *Aeromonas salmonicida*. *Can J Microbiol* **29**: 1458-1461.

493 Ishiguro EE, Ainsworth, T., Harkness, R.E. et al. (1984) A temperate bacteriophage specific for
494 strains of *Aeromonas salmonicida* possessing A-layer, a cell surface virulence factor. *Current*
495 *Microbiology* **10**: 199.

496 Jamal M, Bukhari S, Andleeb S, Ali M, Raza S, Nawaz MA, Hussain T, Rahman SU & Shah
497 SSA (2018) Bacteriophages: an overview of the control strategies against multiple bacterial
498 infections in different fields. *J Basic Microbiol*.

499 Jimenez N, Lacasta A, Vilches S, Reyes M, Vazquez J, Aquillini E, Merino S, Regue M & Tomas
500 JM (2009) Genetics and proteomics of *Aeromonas salmonicida* lipopolysaccharide core
501 biosynthesis. *J Bacteriol* **191**: 2228-2236.

502 Kalynych S, Morona R & Cygler M (2014) Progress in understanding the assembly process of
503 bacterial O-antigen. *FEMS Microbiol Rev* **38**: 1048-1065.

504 Kay WW & Trust TJ (1991) Form and functions of the regular surface array (S-layer) of
505 *Aeromonas salmonicida*. *Experientia* **47**: 412-414.

506 Kim JH, Choresca CH, Shin SP, Han JE, Jun JW & Park SC (2015) Biological control of
507 *Aeromonas salmonicida* subsp. *salmonicida* infection in rainbow trout (*Oncorhynchus mykiss)*
508 using *Aeromonas* phage PAS-1. *Transbound Emerg Dis* **62**: 81-86.

509 Kronheim S, Daniel-Ivad M, Duan Z, Hwang S, Wong AI, Mantel I, Nodwell JR & Maxwell
510 KL (2018) A chemical defence against phage infection. *Nature* **564**: 283-286.

511 Labrie SJ, Samson JE & Moineau S (2010) Bacteriophage resistance mechanisms. *Nat Rev*
512 *Microbiol* **8**: 317-327.

513 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of
514 bacteriophage T4. *Nature* **227**: 680-685.

515 Larsen JL & Pedersen K (1997) Vaccination strategies in freshwater salmonid aquaculture. *Dev*
516 *Biol Stand* **90**: 391-400.

517 Li H (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM.
518 p.^pp.

519 Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R
520 & Genome Project Data Processing S (2009) The Sequence Alignment/Map format and
521 SAMtools. *Bioinformatics* **25**: 2078-2079.

522 Menanteau-Ledouble S, Kumar G, Saleh M & El-Matbouli M (2016) *Aeromonas salmonicida*:
523 updates on an old acquaintance. *Dis Aquat Organ* **120**: 49-68.

524 Merino S & Tomas JM (2016) The *Aeromonas salmonicida* Lipopolysaccharide Core from
525 Different Subspecies: The Unusual subsp. *pectinolytica*. *Front Microbiol* **7**: 125.

526 Michelsen BK (1995) Transformation of *Escherichia coli* increases 260-fold upon inactivation
527 of T4 DNA ligase. *Anal Biochem* **225**: 172-174.

528 Millen AM & Romero DA (2016) Genetic determinants of lactococcal C2viruses for host
529 infection and their role in phage evolution. *J Gen Virol* **97**: 1998-2007.

530 Mills S, Coffey A, McAuliffe OE, Meijer WC, Hafkamp B & Ross RP (2007) Efficient method
531 for generation of bacteriophage insensitive mutants of *Streptococcus thermophilus* yoghurt and
532 mozzarella strains. *J Microbiol Methods* **70**: 159-164.

533 Moreirinha C, Osorio N, Pereira C, Simoes S, Delgadillo I & Almeida A (2018) Protein
534 Expression Modifications in Phage-Resistant Mutants of *Aeromonas salmonicida* after AS-A
535 Phage Treatment. *Antibiotics (Basel)* **7**: 1-21.

536 Mutoh N, Furukawa H & Mizushima S (1978) Role of lipopolysaccharide and outer membrane
537 protein of *Escherichia coli* K-12 in the receptor activity for bacteriophage T4. *J Bacteriol* **136**:
538 693-699.

539 Paterson WD, R. J. Douglas, , I. Grinyer, and , L. A. McDermott (1969) Isolation and Preliminary
540 Characterization of Some *Aeromonas salmonicida* Bacteriophages. *Journal de l'office des*
541 *recherches sur les pêcheries du Canada,* **26(3)**: 629-632.

542 Pereira C, Salvador S, Arrojado C, Silva Y, Santos AL, Cunha A, Gomes NC & Almeida A
543 (2011) Evaluating seasonal dynamics of bacterial communities in marine fish aquaculture: a
544 preliminary study before applying phage therapy. *J Environ Monit* **13**: 1053-1058.

545 Reith ME, Singh RK, Curtis B*, et al.* (2008) The genome of *Aeromonas salmonicida* subsp.
546 *salmonicida* A449: insights into the evolution of a fish pathogen. *BMC Genomics* **9**: 427.

547 Rollauer SE, Sooreshjani MA, Noinaj N & Buchanan SK (2015) Outer membrane protein
548 biogenesis in Gram-negative bacteria. *Philos Trans R Soc Lond B Biol Sci* **370**.

549 Samson JE, Magadan AH, Sabri M & Moineau S (2013) Revenge of the phages: defeating
550 bacterial defences. *Nat Rev Microbiol* **11**: 675-687.

Shaw DH, Squires MJ, Ishiguro EE & Trust TJ (1986) The structure of the heptose-3-deoxy-D-mannooctulosonic-acid region in a mutant form of *Aeromonas salmonicida* lipopolysaccharide. *Eur J Biochem* **161**: 309-313.

Tan D, Svenningsen SL & Middelboe M (2015) Quorum Sensing Determines the Choice of Antiphage Defense Strategy in *Vibrio anguillarum*. *MBio* **6**: e00627.

Tanaka KH, Vincent AT, Trudel MV, Paquet VE, Frenette M & Charette SJ (2016) The mosaic architecture of *Aeromonas salmonicida* subsp. *salmonicida* pAsa4 plasmid and its consequences on antibiotic resistance. *PeerJ* **4**: e2595.

Tanaka KH, Vincent AT, Emond-Rheault JG, Adamczuk M, Frenette M & Charette SJ (2017) Plasmid composition in *Aeromonas salmonicida* subsp. *salmonicida* 01-B526 unravels unsuspected type three secretion system loss patterns. *BMC Genomics* **18**: 528.

Torres-Barcelo C (2018) The disparate effects of bacteriophages on antibiotic-resistant bacteria. *Emerg Microbes Infect* **7**: 168.

Trudel MV, Tanaka KH, Filion G, Daher RK, Frenette M & Charette SJ (2013) Insertion sequence AS5 (IS AS5) is involved in the genomic plasticity of *Aeromonas salmonicida*. *Mob Genet Elements* **3**: e25640.

Trudel MV, Vincent AT, Attere SA, Labbe M, Derome N, Culley AI & Charette SJ (2016) Diversity of antibiotic-resistance genes in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida:* dominance of pSN254b and discovery of pAsa8. *Sci Rep* **6**: 35617.

570 Van den Broek D, Bloemberg GV & Lugtenberg B (2005) The role of phenotypic variation in
571 rhizosphere *Pseudomonas* bacteria. *Environ Microbiol* **7**: 1686-1697.

572 Vanden Bergh P & Frey J (2014) *Aeromonas salmonicida* subsp. *salmonicida* in the light of its
573 type-three secretion system. *Microb Biotechnol* **7**: 381-400.

574 Vincent AT, Tanaka KH, Trudel MV, Frenette M, Derome N & Charette SJ (2015) Draft genome
575 sequences of two *Aeromonas salmonicida* subsp. *salmonicida* isolates harboring plasmids
576 conferring antibiotic resistance. *FEMS Microbiol Lett* **362**.

577 Vincent AT, Paquet VE, Bernatchez A, Tremblay DM, Moineau S & Charette SJ (2017)
578 Characterization and diversity of phages infecting *Aeromonas salmonicida* subsp. *salmonicida*.
579 *Sci Rep* **7**: 7054.

580 Vincent AT, Emond-Rheault JG, Barbeau X, Attere SA, Frenette M, Lague P & Charette SJ
581 (2016) Antibiotic resistance due to an unusual ColE1-type replicon plasmid in *Aeromonas*
582 *salmonicida*. *Microbiology* **162**: 942-953.

583 Vincent AT, Trudel MV, Paquet VE, Boyle B, Tanaka KH, Dallaire-Dufresne S, Daher RK,
584 Frenette M, Derome N & Charette SJ (2014) Detection of variants of the pRAS3, pAB5S9, and
585 pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug resistance,
586 interspecies exchanges, and plasmid reshaping. *Antimicrob Agents Chemother* **58**: 7367-7374.

587 Wang Z, Li J & Altman E (2006) Structural characterization of the lipid A region of *Aeromonas*
588 *salmonicida* subsp. *salmonicida* lipopolysaccharide. *Carbohydr Res* **341**: 2816-2825.

589

590 **Table 1:** A-layer phenotype, *vapA* genotype and response to phage infection of 24
591 *A. salmonicida* subsp *salmonicida* strains.

Strains	*VapA* genotype[a]	A-layer phenotype[b]	Lytic capacity[c]	Lytic plaque[d]
A449	(+)	(+)	(++)	Turbid
01-B522	(+)	(+)	(+)	Turbid
01-B526	(+)	(+)	(+)	Turbid
HER1084	(+)	(-)	(+)	Clear
HER1085	(-)	(-)	(++)	Clear
HER1098	(-)	(-)	(++)	Clear
HER1104	(+)	(-)	(++)	Clear
HER1107	(+)	(+)	(++)	Turbid
HER1108	(+)	(-)	(++)	Clear
HER1110	(-)	(-)	(++)	Clear
09-0167	(+)	(+)	(++)	Turbid
5704-5	(-)	(-)	(++)	Clear
5704-3	(+)	(-)	(++)	Clear
M23067-09	(+)	(+)	(++)	Turbid
2009-144 K3	(+)	(+)	(+)	Turbid
M15879-11	(+)	(+)	(++)	Turbid
AS-R3	(+)	(+)	(++)	Turbid
AS-R5	(+)	(-)	(++)	Clear
JF2267	(+)	(+)	(+)	Turbid
RS 530	(+)	(-)	(++)	Clear

RS 595	(+)	(-)	(++)	Clear
JF3224	(+)	(+)	(++)	Turbid
JF3507	(+)	(-)	(++)	Clear
A527	(-)	(-)	(++)	Clear

592 [a]*vapA* genotype: (+) the *vapA* gene is present on the chromosome; (-) the *vapA* gene is absent
593 on the chromosome confirmed by PCR analysis (see Figure 1D).
594 [b]A-layer-phenotype: (+) the A-layer protein is functionnal (-) the A-layer protein is non-
595 functionnal (see Figure 1C).
596 [c]Lytic capacity: (++) the strain is easily lysed by a weak proportion of phage SW69-9; (+) the
597 strain is lysed by a medium proportion of phage, (see Material and methods section).
598 [d]Lytic plaque: Clear= the background on agar is clear, transparent and clean; Turbid= the
599 background on agar is trouble, indefinite and dim (see Figure 1A and B).
600
601

602 **Table 2**: Detection of mutations in gene of BIMs of *A. salmonicida* subsp *salmonicida*.

Locus tag[a]	Position	Protein function	BIM93	BIM95	BIM96	BIM97	BIM98	BIM99	BIM100	BIM101	BIM102
ASA_1998	656	Hypothetical protein/sorting domain-containing protein	**yes**	no	no	no	no	no	no	no	no
ASA_4222	149	LPS heptosyltransferase family protein	no	no	no	no	no	**yes**	no	no	no
ASA_4222	404	LPS heptosyltransferase family protein	no	no	no	no	**yes**	no	**yes**	**yes**	**yes**
ASA_4220	331	LPS heptosyltransferase II	no	no	no	**yes**	no	no	no	no	no
ASA_4220	587	LPS heptosyltransferase II	no	**yes**	**yes**	no	no	no	no	no	no

603

604 a: The locus tags and annotations are given according to the curated genome sequence of *A. salmonicida* subsp. *salmonicida* A449
605 (GenBank CP000644.1). A more complete description of the different mutations is available in Table S.4.

606 **Figure legends**

607

608 **Figure 1: Spot test assay, Coomassie brilliant blue agar and PCR genotyping.** Serial
609 dilutions of a SW69-9 phage lysate were spotted on M15879-11 and AS-R5 bacterial lawn. The
610 dilution 10^5 was zoomed to show the turbid lytic plaque of M15879-11 host comparatively to
611 the clear plaque of AS-R5 host (**A** and **B**). The Coomassie brilliant blue agar revealed that the
612 strain AS-R5 did not have functional A-layer outer proteins, because the bacteria did not absorb
613 the brilliant blue pigment as the negative control HER1110. However, M15879-11 as the positive
614 control 01-B526, absorbed the colorant, which indicates the presence of a functional A-layer
615 protein on the cell surface (**C**). PCR detection of *vapA* gene amplicon (1900 bp) from the
616 chromosome of *A. salmonicida* subsp. *salmonicida* strains revealed that the wild type strain
617 M15879-11 and rearranged strains AS-R3 and AS-R5 had the gene on chromosome as the
618 positive control strain 01-B526. Only the strain HER1110 did not have the *vapA* gene (**D**).

619

620 **Figure 2: Adsorption of phage SW69-9 on wild type *A. salmonicida* subsp. *salmonicida* or**
621 **rearranged strains.** The percent of adsorption of SW69-9 on M15879-11 and AS-R5 cells was
622 determined by calculating the initial titer and the adsorption titer each 5 minutes up to 30 minutes
623 on TSA top plate agar. All experimentations were done at least in biological and technical
624 triplicate.

625

626 **Figure 3: BIM-complementation.** Positive electro-transformants of BIM93 were confronted to
627 phage SW69-9 to confirm the role of VapA and ASA_1998 as potential phage receptors (**A** and
628 **B**). The totality of the plaque was lysed for BIM93+ASA_1998 in comparison of BIM93 and

BIM93+VapA that were still resistant. The Coomassie brilliant blue agar (**C**) confirmed that the complementation of VapA was effective as shown by the absorption of colorant by the bacterial growth.

Figure 4: LPS profile on SDS-PAGE. The LPS extractions were migrated on 12% SDS-PAGE and were revealed by silver staining. The experimentation was done in biological triplicate. One representative result is shown.

637 **Supporting information**

638 Figure S.1: Loss of T3SS in AS-R3 and AS-R5.

639 Table S.1: Lytic phenotype of 12 specific *A. salmonicida* bacteriophages against AS-R5.

640 Table S.2: Characteristics of BIMs of *A. salmonicida* subsp *salmonicida.*

641 Figure S.2: LPS extraction and profile on 12% SDS-PAGE of BIMs.

642 Figure S.3: Map prediction of gene *ASA_1998*.

643 Table S.3: Information on *A. salmonicida* subsp *salmonicida* strains used in this study.

644 Figure S.4: Gene sequence of *vapA* and *ASA_1998* for cloning.

645 Table S.4: A complete description of the different mutations found in BIMs.

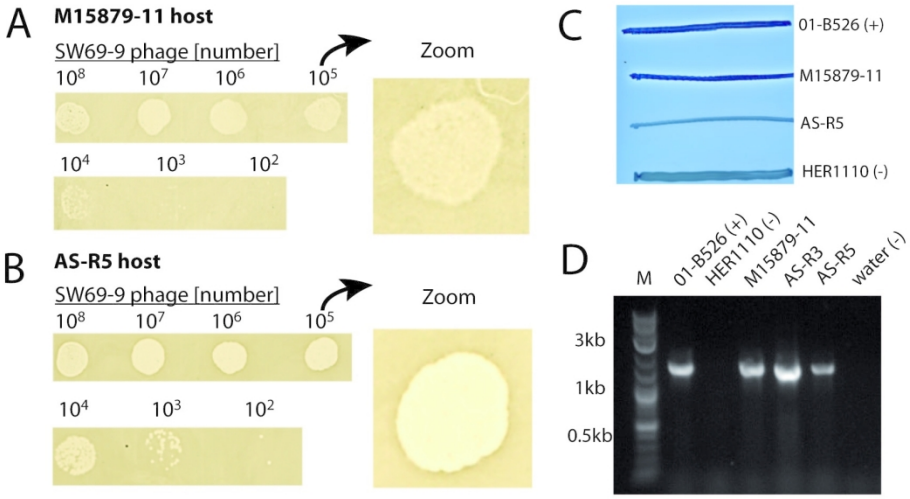

Figure 1: Spot test assay, Coomassie brilliant blue agar and PCR genotyping. Serial dilutions of a SW69-9 phage lysate were spotted on M15879-11 and AS-R5 bacterial lawn. The dilution 10^5 was zoomed to show the turbid lytic plaque of M15879-11 host comparatively to the clear plaque of AS-R5 host (A and B). The Coomassie brilliant blue agar revealed that the strain AS-R5 did not have functional A-layer outer proteins, because the bacteria did not absorb the brilliant blue pigment as the negative control HER1110. However, M15879-11 as the positive control 01-B526, absorbed the colorant, which indicates the presence of a functional A-layer protein on the cell surface (C). PCR detection of *vapA* gene amplicon (1900 bp) from the chromosome of *A. salmonicida* subsp. *salmonicida* strains revealed that the wild type strain M15879-11 and rearranged strains AS-R3 and AS-R5 had the gene on chromosome as the positive control strain 01-B526. Only the strain HER1110 did not have the *vapA* gene (D).

159x107mm (300 x 300 DPI)

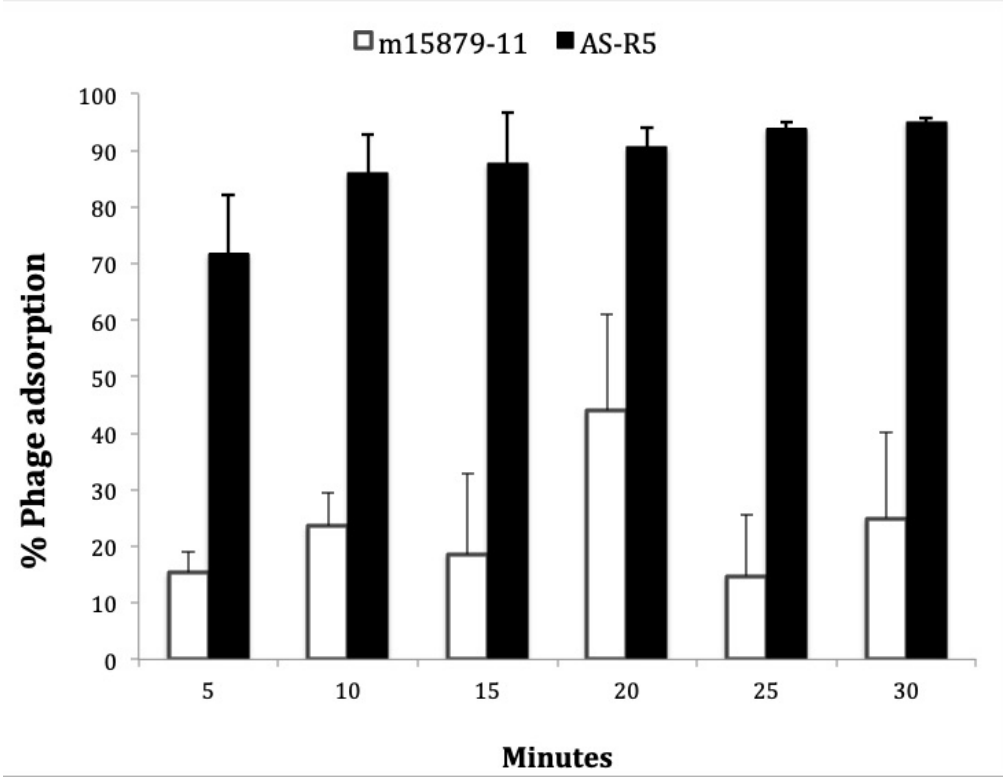

Figure 2: Adsorption of phage SW69-9 on wild type *A. salmonicida* subsp. *salmonicida* or rearranged strains. The percent of adsorption of SW69-9 on M15879-11 and AS-R5 cells was determined by calculating the initial titer and the adsorption titer each 5 minutes up to 30 minutes on TSA top plate agar. All experimentations were done at least in biological and technical triplicate.

256x199mm (72 x 72 DPI)

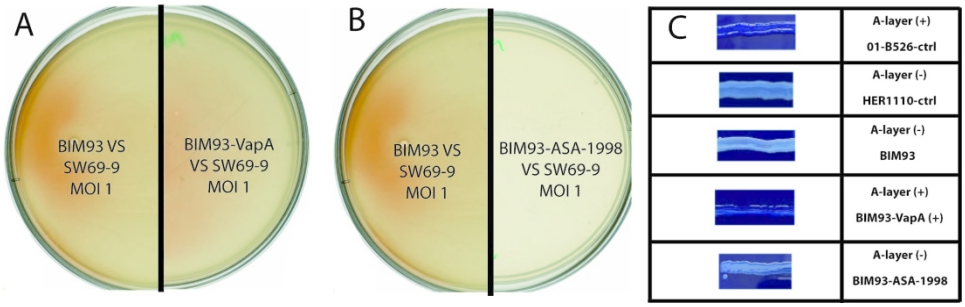

Figure 3: BIM-complementation. Positive electro-transformants of BIM93 were confronted to phage SW69-9 to confirm the role of VapA and ASA_1998 as potential phage receptors (A and B). The totality of the plaque was lysed for BIM93+ASA_1998 in comparison of BIM93 and BIM93+VapA that were still resistant. The Coomassie brilliant blue agar (C) confirmed that the complementation of VapA was effective as shown by the absorption of colorant by the bacterial growth.

179x53mm (300 x 300 DPI)

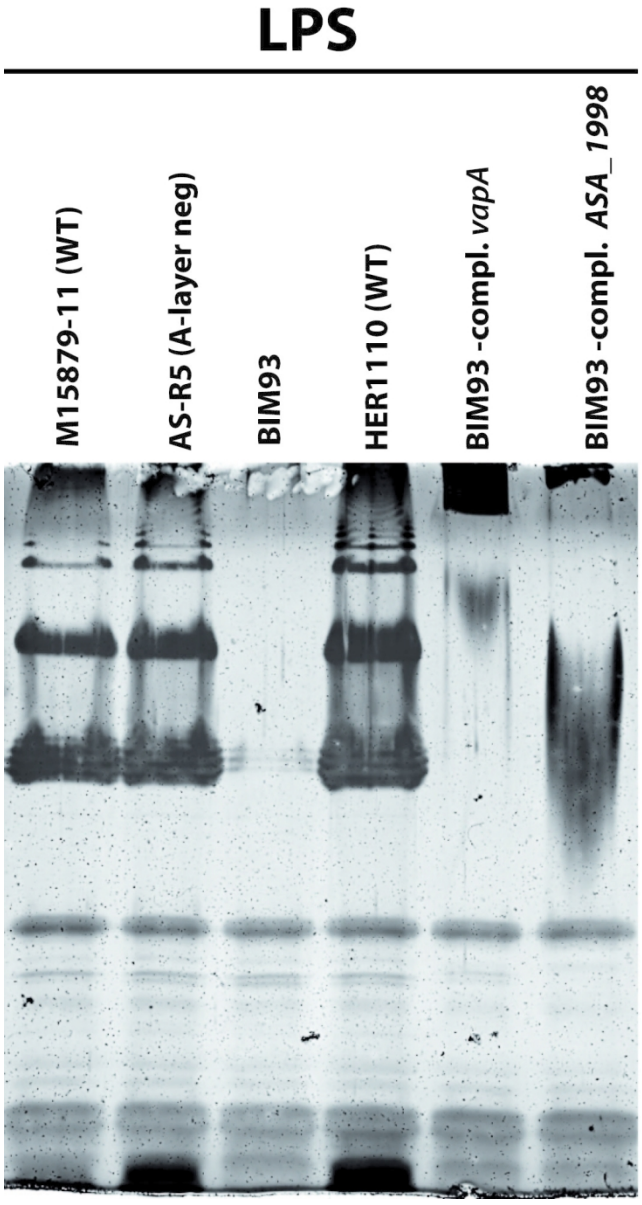

Figure 4: LPS profile on SDS-PAGE. The LPS extractions were migrated on 12% SDS-PAGE and were revealed by silver staining. The experimentation was done in biological triplicate. One representative result is shown.

98x168mm (300 x 300 DPI)

Supporting information

Beyond the A-layer: adsorption of lipopolysaccharides and characterization of bacteriophage-insensitive mutants of *Aeromonas salmonicida* subsp. *salmonicida*

Valérie E. Paquet[1,2,3], Antony T. Vincent[4], Sylvain Moineau[2,5,6] and Steve J. Charette[1,2,3,#]

1. Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Quebec City, QC, Canada, G1V 0A6

2. Département de biochimie, de microbiologie et de bio-informatique, Faculté des sciences et de génie, Université Laval, Quebec City, QC, Canada, G1V 0A6

3. Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec (IUCPQ), Quebec City, QC, Canada, G1V 4G5

4. INRS-Institut Armand-Frappier, Laval, QC, Canada, H7V 1B7

5. Groupe de Recherche en Écologie Buccale (GREB), Faculté de médecine dentaire, Université Laval, Quebec City, QC, Canada, G1V 0A6

6. Félix d'Hérelle Reference Center for Bacterial Viruses, Université Laval, Quebec City, QC, Canada, G1V 0A6

[#]To whom correspondence should be addressed: Institut de Biologie Intégrative et des Systèmes (IBIS), Pavillon Charles-Eugène-Marchand, 1030 avenue de la Médecine, Université Laval, Quebec City, QC, Canada, G1V 0A6

steve.charette@bcm.ulaval.ca; Telephone: 1-418-656-2131, ext. 406914; Fax: 1-418-656-7176

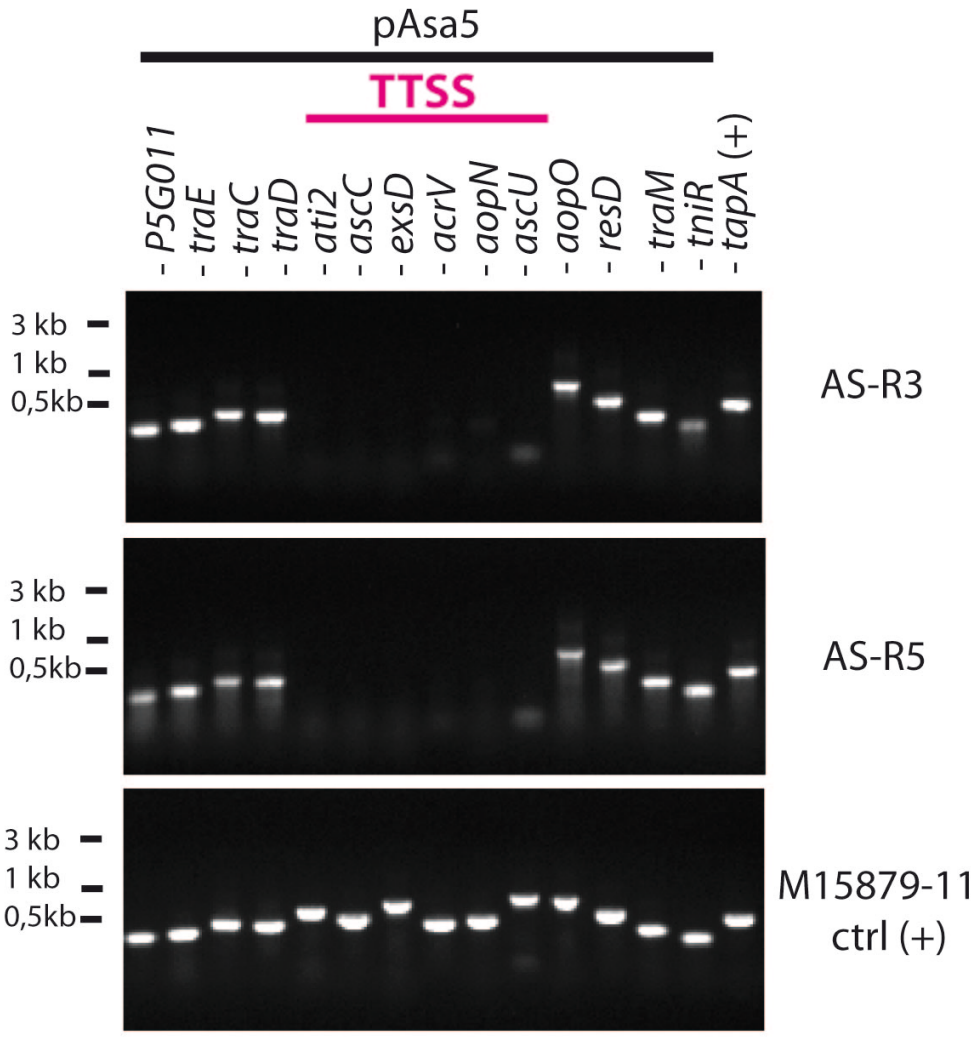

Figure S.1: Fourteen genes associated with the pAsa5 plasmid (including 6 genes in the TTSS locus) were amplified by PCR on AS-R3, AS-R5 and M15879-11 strains to detect the presence of rearrangements in the plasmid. The gene *tapA* on the chromosome was used as a positive control.

Table S.1: Lytic phenotype of 12 specific *A. salmonicida* bacteriophages against AS-R5.

Bacterophage	Lytic capacity[a]	Lytic plaque[b]
3 (HER84)	Sensitive (++)	Clear
31.2 (HER105)	Sensitive (++)	Clear
32 (HER106)	Sensitive (++)	Clear
44RR2.8t.2 (HER98)	Sensitive (+)	Clear
51 (HER108)	Sensitive (++)	Clear
56 (HER109)	Resistant	N/A
59.1 (HER100)	Resistant	N/A
65.2 (HER110)	Sensitive (+)	Clear
Asp37 (HER99)	Sensitive (+)	Clear
L9-6	Sensitive (++)	Clear
Riv-10	Sensitive (++)	Clear
SW69-9	Sensitive (++)	Clear

[a]Lytic capacity: Sensitive (++) the strain is easily lysed by a weak proportion of phage; Sensitive (+) the strain is lysed by a medium proportion of phage, Resistant, the strain cannot be lysed by the phage (see Material and methods section).

[b]Lytic plaque: Clear= the background on agar is clear, transparent and clean; Hazy= the background on agar is trouble, indefinite and hazy; N/A= the strain is resistant, no lytic plaque was visible (see Figure 1A and B).

Table S.2: Characteristics of BIMs of *A. salmonicida* subsp *salmonicida* analyzed in this study.

# collection BIM	Full name	Temp. of incubation	Phage resistant	Confirmation of phenotype
BIM93	*A. salmonicida* AS-R5-BIM-1	25ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM95	*A. salmonicida* AS-R5-BIM-3	25ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM96	*A. salmonicida* AS-R5-BIM-4	25ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM97	*A. salmonicida* AS-R5-BIM-5	25ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM98	*A. salmonicida* AS-R5-BIM-V2-1	18ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM99	*A. salmonicida* AS-R5-BIM-V2-2	18ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM100	*A. salmonicida* AS-R5-BIM-V2-3	18ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM101	*A. salmonicida* AS-R5-BIM-V2-4	18ºC	SW69-9	spot test, serial plating, freezing and pcr
BIM102	*A. salmonicida* AS-R5-BIM-V2-5	18ºC	SW69-9	spot test, serial plating, freezing and pcr

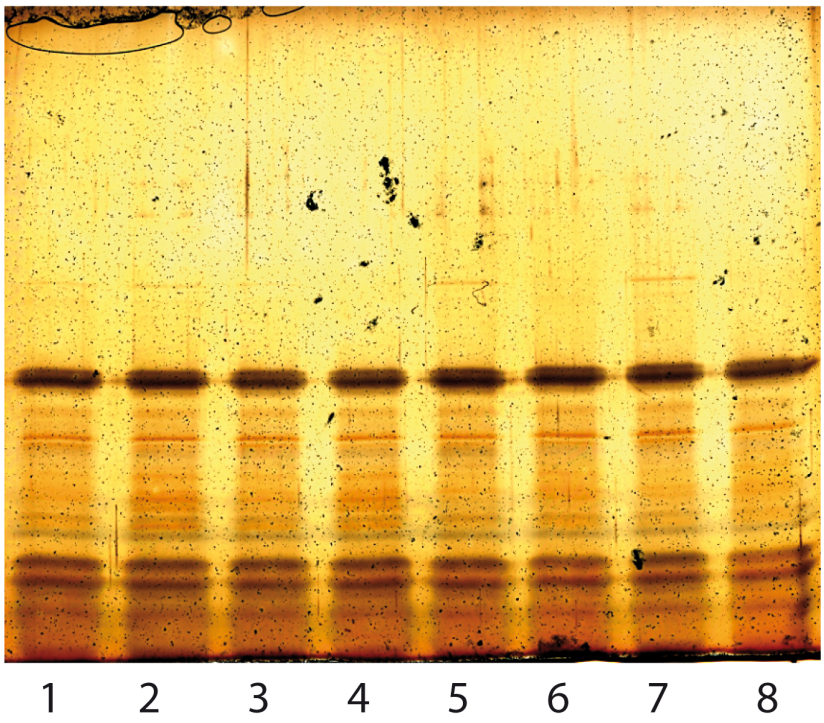

Figure S.2: LPS profile of BIMs.The LPS extractions were migrated on 12% SDS-PAGE and were revealed by silver staining. The experimentation was done in biological triplicate. One representative result is shown. All profiles are very similar to BIM93. Line 1: BIM95, line 2: BIM96, line 3: BIM97, line 4: BIM98, line 5: BIM99, line 6: BIM100, line 7: BIM101, and line 8: BIM102.

Figure S.3: Map prediction of the gene *ASA_1998*. The gene possesses a signal peptide and a membrane region. The unique non-synonymous mutation occurred at position 219, modifying a cysteine residue into a phenylalanine residue (black arrow).

Table S.3: Information on *A. salmonicida* subsp. *salmonicida* strains used in this study.

# collection	Bacteria full name	Origin
1	*Aeromonas salmonicida* subsp *salmonicida* 01-B522	Quebec, Canada
2	*Aeromonas salmonicida* subsp *salmonicida* 01-B526	Quebec, Canada
4	*Aeromonas salmonicida* subsp *salmonicida* HER1098	Félix D'Hérelle reference center
5	*Aeromonas salmonicida* subsp *salmonicida* HER1110	Félix D'Hérelle reference center
6	*Aeromonas salmonicida* subsp *salmonicida* HER1108	Félix D'Hérelle reference center
7	*Aeromonas salmonicida* subsp *salmonicida* HER1104	Félix D'Hérelle reference center
8	*Aeromonas salmonicida* subsp *salmonicida* HER1085	Félix D'Hérelle reference center
9	*Aeromonas salmonicida* subsp *salmonicida* HER1084	Félix D'Hérelle reference center
10	*Aeromonas salmonicida* subsp *salmonicida* HER1107	Félix D'Hérelle reference center
11	*Aeromonas salmonicida* subsp *salmonicida* A449	Michael Reith, Canada
17	*Aeromonas salmonicida* subsp *salmonicida* 09-0167	Quebec, Canada
31	*Aeromonas salmonicida* subsp *salmonicida* 5704-5	Quebec, Canada
33	*Aeromonas salmonicida* subsp *salmonicida* 5704-3	Quebec, Canada
37	*Aeromonas salmonicida* subsp *salmonicida* m23067-09	Quebec, Canada
57	*Aeromonas salmonicida* subsp *salmonicida 2009-144 K3*	New-Brunswick, Canada
69	*Aeromonas salmonicida* subsp *salmonicida* M15879-11	Quebec, Canada
82	*Aeromonas salmonicida* subsp *salmonicida* JF2267	Joachim Frey, Suisse
86	*Aeromonas salmonicida* subsp *salmonicida* RS 530	Ontario, Canada
87	*Aeromonas salmonicida* subsp *salmonicida* RS 595	France
88	*Aeromonas salmonicida* subsp *salmonicida* JF3224	Europe
89	*Aeromonas salmonicida* subsp *salmonicida* JF3507	Europe
193	*Aeromonas salmonicida* A527	India
AsR-60	*Aeromonas salmonicida* subsp *salmonicida* M15879-11-(#69)-25-R3	Rearranged from M15879-11
AsR-62	*Aeromonas salmonicida* subsp *salmonicida* M15879-11-(#69)-25-R5	Rearranged from M15879-11

Figure S.4 : Gene sequence of *vapA* and *ASA_1998* for cloning

>*vapA* (1549 pb)
CTCGAGCTCGAG
ATGTTTAAGAAGACTTTGATTGCAGCTGCCATTGTGGTCGGTTCCGCTGCACCTGCGTTT
GCTGATGTCGTGATTAGCCCGAACGACAACACTTTCGTTACTACCTCCCTCGCATCTGTA
ACTAAGCAGCCGGTACTGGACTTCTCCACTGCTCAACAAAACCTGACCCTTAACTTCAGT
GAAGTTGGTGACCTTAAGAACAACGGTTTCATTGTGTTGGAAATCCAAGGTGAAGGCCAA
TTCAACGACGCGGAAATCCGTCAGTGGCTGTCCAACGGTTTCTGGCGTAGGCCGTTTACC
GGTCTGCTGGTTAACCCGAATGATCATGGTAATTTTGCCAATAGCGGTGAAGTTAATGAC
GTTCGGAAGTTCTTTAAGATTATTTCCGACGGTACCCAGCTGACCATCGTTCACACTATC
GACAGCAATGGCAAGCGTCTGCGTCTTGCTCTTGCTTCTGATGTAGAAGAGACAATCAAT
TTTGCTGATGCAGAGGTTGAGCTGAAGCTGAACTTAGCTAACCAAGCCTTTAAGCTGACC
TCCGGTTCTCAAGGTACAGTAGCTCTGACCGCAGGAGCTCTGTGGAACGCTTCTTACACT
GCTGATCCGGTTGCTACCAAGCCGCTGTTCAAACTGGGTAAGCTGTTCCAGTTGAGTTTG
ACTAACGCTGGTAAAGCTACCGCTCTGGTTTCCGAAGGTTTCTTGAAACTTAATATCGGT
GATGCGAATATTTCTGCTACTGATTTCGCGATTACCAACGTTACTACTAACCAGACCATC
CAACGTGACAAGGTTAACCTGACCCTGACTGGTGATGTTTCTGCCTTCAAGAAAGATGCC
AACGGTAACTTGGTAAACAAAGCTGGTGCTAGCATCGGTTGGAAAGCTGCTGCTGATGGT
CAATCTGCTACAGCTGTCTTGGGTGCTGGCAACATGGCCGGTGGGGTTCAAAATGCTCTG
GCTGCTTTTGGTACACTGTACGTTGCTGCAGATAACACTGTTCCGGTTCCTGCTGTTAAC
TTCAATGTTAAGGCTGAAATCCAAGGTGATAGCCAAGCTACCTATAACTACTTCAAGGAC
GAGCTGGCTGATCTCTTCATCCTCACCCGTGATGGTATGAAGTTTGACACAATTACTACT
GGTACCACTTCTGCCAACCTCATCCACATTCGTGATGTATCTAACATCCTGCCTACTGAA
GGTGGCAAGATCTTCGTAACTATCACTGAATATGCAGATCATGCTGCCAATGGTCGTGGT
GAAGGTACTGTATTGGTTACCCGTAAAGCACTGTCTGTTACCCTGCCAAGCGGTGGTGCA
GTGACTCTGAAGCCTGCTGATGTTGCTGCTGACGTTGGTGCTTCTATCACTGCTGGCCGT
CAGGCTCGCTTCCTGTTTGAAGTTGAAACCAATCAGGGTGAAGTAGCTGTTAAGAAATCC
AATGCTGAAGGCGTGGATATTCAGAATGGTACCCGCGGCACAGCACCGCTG**GTAGATTTC**
ACTCTG**TAA**TCTAGATCTAGA

ATG start codon
TAA stop codon

XhoI sites: CTCGAGCTCGAG
XbaI sites: TCTAGATCTAGA

VapA PRIMERS
F1:CTCGAGCTCGAG**ATG**TTTAAGAAGACTTTG

R1:TCTAGATCTAGA**TTA**CAGAGTGAAATCTAC

Amplicon: 1533 pb

>*ASA_1998* (1635 pb)
CTCGAGCTCGAG**ATG**AAAAAGCAATTATCCAT
GCTGGCCATTCTGGTCAGCGCCAGCCTGCACGCCCAGGCGGCACAAAGCCCCTTTTTCAC
CATTGTTGACGGGTCTGACAAGGGATTCGCCAGCGCGATCAGTGCCGATGGCAACGGGCT
CGTCGGGATCAACACCAAAAACAACATGGCCGATCACTTCTCGACCGTACGTTTCGCCGA
CTTCCTGGTCGATCGTTTCCGTTTTGAGCAGGGCTGCATGCTCTCCAACTCGGTTTGCAA
CGCCTTCTGGAACGACAAGAGCAACTTTGCGTATCAGTGGCGTGTCGACTTTCTGGCCAA
AACCGATCAGCGCAGCAATGTGGGTGACCTAGTCAGCAACGAAACTGATGGTTTGGTTAC
CGCTTTGGGTGACGTAGCAGGCACTCGTGTGGGCTACAAGATGGATGGCAAACTGCGCAC
CGCATTTGCCATCGTCGAGGAAGGCAACGCCGTCAACCTCAAGGATGGCGCCAGCACCCA
CAGCCTGAGCGTGCCGACCAGCATCAAGAAACTGGACAACGGCCAGTACCTGGTGACCGG
TACTGCCGCCACCGGCAAGACCATTGCCGTCAATTCCGAGACCTACAACTGGTGCTTCGC
CGGTAACGACGGCCAGTATGGTGACTACCGCTACTGCCCGGGCCTCAACACCCAGGCCAG
CTTCTGGTTGCTCAACAGCACAGGCACCTTCAACAAGTTGATCCAGGCCAATGAGTATTC
CCGTGGCCGTAACGAAGTGATCCAGACTGCGTCTGCACTCGGGGTTGGCAACGTCAATGG
CAGCTTGTTCGGTGTGGGCTACTCCTCTACCGGTGAAATTGGCACCAACTACCTGGATGG
CCGCAACCTGGCGGCATACTGGACGCTGGATCTGGCCGGTGGCAAGGTCGGTACCACCCA
GACCATACCGCTGGCCGAAGGCGAGCCGGGCAAGGATGACGCCAAGCTGCAACACAGCTG
GGCTGTGGCGGTCAACGACAACGGTTATGTGATTGGCAACCAGCTCTATCGCATCAACAA
GGGGCAGAATCGCCCGGTCGAGATGTTCGTGTTCAACTTGAACACCAAGCAGACGGCGAG
TGTGCCGTTGCAAGACAGCCCAATCAGCGGTGCAGGCTCGGAAGCTGCCGCCATCAACAA
CCACAACATGGTCGTGGGCTGGCGTGACTCTCGTCACCAGACCCAGCCAGTGGTCAATGG
CACCAATCGCATGCAGGAAGGTTTCCTGCTCAATGCTGCAACCAGCAAAAATTGGTATCT
GAACGACCTCATCTGCGGGCTGGATGATGCCGGTGCCAAGCAGTGTGCCCAGAACGGCTA
CTACTACCACATCGCGTATGCCAGCGGCATCAGCACGGATGGTACTGTCGCTGCGACGGC
TTTTCGCTATAACAGCGAGAGCGATCTCAACAATCGTACCAATTCCACCATCGTTAGCGT
GAAGCTGACGCCAGCCGTTGCCGATTACAAGAACAACGAGCCTGCAAGCTATGTGGTTGC
GAATGCGCCGGTCAATAACCAGACTGGTCAGGATGGTGGCAGTGGTGGTGGCAGCCTGTT
CTGGTTGACTCTGCTGGCCCTGCCGTTTACCTGGCTGCGCCGTTACCAACGA**TAA**TCTAGA
TCTAGA

ATG start codon
TAA stop codon

XhoI sites: CTCGAGCTCGAG
XbaI sites: TCTAGATCTAGA

Primers NR
F1: CTCGAGCTCGAG**ATG**AAAAAGCAATTATCC

R1: TCTAGATCTAGA**TTA**TCGTTGGTAACGGCG

Amplicon (1659 pb)

Table S.4: A complete description of the different mutations found in BIMs.

TYPE	REF	ALT	EVIDENCE	FTYPE	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE	PRODUCT
snp	C	A	A:161 C:0	CDS	656/1635	219/544	missense_variant c.656G>T p.Cys219Phe	ASA_1998		hypothetical protein
del	TGGATCGACGAGATTATCCTGGA	TA	TA:17 TGGATCGACGAGATTATCCTGGA:78	CDS	149/1044	50/347	disruptive_inframe_deletion c.149_169delGGATCGA CGAGATTATCCTGG p.Trp50_Asp57delinsTyr	ASA_4222		LPS heptosyltransferase family protein
del	CTGGATCTGGTGCGTCGCTTTCTC	CC	CC:38 CTGGATCTGGTGCGTCGCTTTCTC:94	CDS	404/1044	135/347	frameshift_variant c.404_425delTGGATCTG GTGCGTCGCTTTCT p.Leu135fs	ASA_4222		LPS heptosyltransferase family protein
del	AGGGGGA	AGGGGA	AGGGGA:23 AGGGGGA:112	CDS	331/1116	111/371	frameshift_variant c.331delG p.Glu111fs	ASA_4220	*rfaF*	LPS heptosyltransferase II
del	CGCTGGCCGGAAGGGCACTACG CCGTGGTGGCGCAGAAGTATCTG GATGAGGG	CG	CG:22 CGCTGGCCGGAAGGGCACTACG CCGTGGTGGCGCAGAAGTATCTGGATG AGGG:62	CDS	587/1116	196/371	disruptive_inframe_deletion c.587_637delCGGAAGGG CACTACGCCGTGGTGGCGCAGAAGTATCTGGATGA GGGCTGGC p.Pro196_Trp212del	ASA_4220	*rfaF*	LPS heptosyltransferase II