Expression of flagellin and key regulatory flagellar genes in the non-motile bacterium *Piscirickettsia salmonis*

Gabriela P. Carril1, Fernando A. Gómez1, Sergio H. Marshall1,2,3,*

1Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso 2340000, Chile
2Núcleo de Biotecnología Curuama, Pontificia Universidad Católica de Valparaíso, Valparaíso 2340000, Chile
3Fraunhofer Chile Research, Foundation on System Biotechnology, Santiago 7550296, Chile

ABSTRACT: The *Piscirickettsia salmonis* genome was screened to evaluate potential flagella-related open reading frames, as well as their genomic organization and eventual expression. A complete and organized set of flagellar genes was found for *P. salmonis*, although no structural flagellum has ever been reported for this bacterium. To gain further understanding, the hierarchical flagellar cascade described for *Legionella pneumophila* was used as a reference model for putative analysis in *P. salmonis*. Specifically, 5 of the most relevant genes from this cascade were chosen, including 3 regulatory genes (*fleQ*, triggers the cascade; *fliA*, regulates the σ28-coding gene; and *rpoN*, an RNA polymerase-dependent gene) and 2 terminal structural genes (*flaA* and *flaB*, flagellin and a flagellin-like protein, respectively). Kinetic experiments evaluated gene expressions over time, with *P. salmonis* assessed in 2 liquid, cell-free media and during infection of the SHK-1 fish cell line. Under all conditions, the 5 target genes were primarily expressed during early growth/infection and were differentially expressed when bacteria encountered environmental stress (i.e., a high-salt concentration). Intriguingly, the flagellin monomer was fully expressed under all growth conditions and was located near the bacterial membrane. While no structural flagellum was detected under any condition, the recombinant flagellin monomer induced a proinflammatory response in SHK-1 cells, suggesting a possible immunomodulatory function. The potential implications of these observations are discussed in the context of *P. salmonis* biology and pathogenic potential.

KEY WORDS: Flagella · Gram-negative bacteria · *fleQ* · *fliA* · *flaB* · *flaA* · *rpoN* · Immunomodulatory · *Legionella pneumophila*

INTRODUCTION

*Piscirickettsia salmonis* is the etiological agent of piscirickettsiosis, an infectious disease that seriously affects salmonid fish farming. Although reported at other latitudes, *P. salmonis* has had a particular impact in Chile, where it constitutes a permanent threat to the sustainability of this important economic industry (Marshall et al. 2011, Wilhelm et al. 2006, Gómez et al. 2011). The first case of piscirickettsiosis in Chile was reported in 1989 in Coho salmon *Oncorhynchus kisutch*, with certain farms recording stock mortalities of up to 90% (Fryer et al. 1992, Toblar et al. 2011). Since then, this disease has also been described in Canada (Brocklebank et al. 1992), Ireland (Rodger & Drinan 1993), and Norway (Olsen et al. 1997). Additionally, *P. salmonis* has been reported in other salmonids, as well as in related fish species such as European seabass *Dicentrarchus labrax* in Greece and white seabass *Atractoscion nobilis* in...

*P. salmonis* is a non-encapsulated, pleomorphic, generally coccolid bacterium with a diameter ranging from 0.5 to 1.5 µm (Fryer et al. 1992). This bacterium is classified as a facultative intracellular member of the *Gammaproteobacteria* that is able to grow in cell-free media (Mauel et al. 2008, Mikalsen et al. 2008, Gómez et al. 2009, Henriquez et al. 2013), belongs to the *Piscirickettsiaceae* family, and is closely related to the genera *Coxiella*, *Francisella*, and *Legionella* (Fryer & Hedrick 2003). Indeed, a recent report described the presence of a fully functional Dot/Icm-like type IV secretion system in *P. salmonis* that is highly similar to that of *L. pneumophila*, a species in which this system is directly associated with pathogenicity (Zusman et al. 2008). The Dot/Icm-like type IV secretion system may be responsible for the intracellular survival of *P. salmonis* in fish macrophages (Gómez et al. 2013).

Regarding survival, *P. salmonis* is also able to persist for extended periods of time in seawater (Oliveares & Marshall 2010), and it was recently confirmed that stress is fully responsible for triggering biofilm formation in this bacterium. Biofilm formation is a known persistence and survival strategy used by pathogenic bacteria under harsh metabolic conditions, such as in the marine environment where *P. salmonis* must live, survive, and evolve (Marshall et al. 2012). Two genetic features have been described in *P. salmonis* that could play key roles in pathogen survival. Firstly, at least 2 different insertion sequences are constituent parts of the bacterial genome, acting as functional structures that regulate gene expression (GenBank: AF184152; Marshall et al. 2011). Secondly, 2 different functional chromosomal toxin-antitoxin modules are particularly upregulated under stress, with distinctive features contributing to the bacterium’s adaptation to environmental cues (Gómez et al. 2011, Marshall et al. 2012). The search for new pathogenic features has been facilitated by the recent genome sequencing of *P. salmonis* isolates (Eppinger et al. 2013).

To gain a better understanding of the pathogenic potential of this bacterium, we screened the *P. salmonis* genome for genes associated with virulence factors, particularly in relation to the flagellum, a motility structure. An array of flagella-related genes was detected, despite the non-motile classification of *P. salmonis*. Considering this finding, further research characterized pivotal genes associated with flagellar expression, many of which have been extensively described in flagella-positive bacteria. Due to the similarities between *P. salmonis* and *L. pneumophila*, genes described in *L. pneumophila* were used as references to evaluate putative flagellar expression in *P. salmonis*.

*L. pneumophila* flagella result from the expression of a well-characterized hierarchical transcriptional cascade, which is comprised of genes categorized into 4 classes (Albert-Weissenberger et al. 2010). Five of the most relevant genes from this cascade were evaluated in *P. salmonis* under different growth conditions. The selected genes were (1) *fleQ*, a principal regulator and potential initiator of the cascade; (2) *flia* (sigma 28 factor ([σ28]), a gene with a regulatory role in cascade progression; (3) *flaA* and *flaB*, flagellin and flagellin-like genes, respectively, key components of the flagellin monomer; and (4) *rpoN*, which codes for the sigma 54 factor ([σ54], a key component of bacterial RNA polymerase.

*P. salmonis* presented a similar genomic organization of cascade genes and a similar expression pattern to that of *L. pneumophila*. Additionally, evidence was found of flagellin monomer presence in *P. salmonis*. Despite these findings, this bacterium did not assemble a flagellum under any of the assayed growth conditions. The significance and potential of these results are discussed.

**MATERIALS AND METHODS**

**Screening of the *Piscirickettsia salmonis* genome**

The genomic sequence of the *P. salmonis* LF-89 reference strain (NCBI Genome project No.: PRJNA 174046) was annotated by the RAST Server (Aziz et al. 2008). After RAST analysis, all *P. salmonis* genomic regions containing flagella-related genes were analyzed with the Softberry online software (http://linux1.softberry.com/berry.phtml), using the FGENESB algorithm to confirm the predicted open reading frames (ORFs). All flagella-related ORFs were then analyzed with BLASTP to confirm the RAST results. Once the genes were confirmed, the CLC Main Workbench 5 software (www.clcbio.com) was used to complement sequence analysis, specifically for accurate alignments, and to determine the putative operon organization of flagellar genes in *P. salmonis*. Finally, flagellar gene organization was compared between different *P. salmonis* strains using recently reported genomes (Eppinger et al. 2013, Bohle et al. 2014, Yañez et al. 2014, Pulgar et al. 2015).
In silico analyses of putative regulatory and structural flagellar proteins

Based on the L. pneumophila regulation cascade for flagellar expression, 4 selected putative equivalents were matched in P. salmonis (i.e. the σ54 activator protein FleQ; the motility-related σ28 protein FliA; the flagellin protein FlaA; and the flagellin-like protein FlaB) by amino acid sequence alignments with homologue proteins. These proteins were obtained via BLASTP analysis using the ClustalW tool (Larkin et al. 2007) and by image processing using the Jalview software (Clamp et al. 2004). Additionally, the protein sequences were submitted to the NCBI Conserved Domain Search Tool (Marchler-Bauer et al. 2011) to determine the presence of key domains in corresponding homologues, and the flagellin amino acid sequence was analyzed with the MOTIF Search Server (www.genome.jp/tools/motif/) to predict the presence of signal peptides.

Expression kinetics of P. salmonis flagellar genes in liquid cultures

To determine the expression of the selected putative flagellar genes, 2 different media were used, a nutrient-rich broth (BM3) (Henríquez et al. 2013) and a marine broth (MB) (Difco) that is stressful for P. salmonis due to its higher-than-seawater salt and mineral concentrations. For each broth, 2 growth conditions were assessed, i.e. static growth with no shaking and dynamic growth with shaking at 100 rpm.

To obtain sufficient P. salmonis for the evaluations, a single colony from a master plate was inoculated in 3 ml of BM3 broth and incubated overnight at 23°C with shaking at 100 rpm. The next morning, the culture was inoculated in 50 ml of BM3 broth and incubated at 23°C with shaking at 100 rpm for 24 h, overnight. After this, identical 3 ml aliquots of P. salmonis were taken from the stock colony and inoculated in 50 ml of either BM3 or MB media for both the static and dynamic growth conditions. Triplicate cultures were used for each medium/condition at each evaluated time point (3, 6, 12, 24, and 48 h). Both media for the respective growth conditions were grown in parallel at 23°C. Identical 10 ml aliquots were sampled from each culture at 3, 6, 12, 24, and 48 h for RNA extraction.

For kinetic evaluations in liquid cultures, bacterial viability was measured at each time point using the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) according to the manufacturer’s instructions.

Expression kinetics of P. salmonis flagellar genes during infection in the salmon head kidney macrophage cell line (SHK-1)

To determine the expression profile of the putative flagellar genes during the infection process, an in vitro infection kinetics assay was designed using the Salmo salar macrophage-derived SHK-1 cell line (Dannevig et al. 1995). The SHK-1 cells (passage 42) were grown at 18°C in Leibovitz’s L-15 medium (Invitrogen) supplemented with 15% fetal bovine serum (Gibco) in 25 cm² culture flasks. For infection assays, cells were grown in 6-well plates for 5 d or until they reached a 90% confluence. Then, 50 µl of P. salmonis, grown in BM3 broth and close to an OD600 of 0.3, was used to infect individual wells at a multiplicity of infection of 50. Infected cells were incubated for 2 h at 20°C and then thoroughly washed 5 times with sterile 1x phosphate-buffered saline (PBS) to eliminate excess bacteria. Finally, fresh L-15 medium was added. Infection kinetic analysis for the expression of the selected flagellar gene cascade was performed using the whole contents of individual duplicate wells at 3, 6, 12, 24, and 48 h. Kinetic assays began from the moment when cells were exposed to the bacterial inoculum. Uninfected wells for each time point were used as controls. Samples were immediately processed for RNA extraction.

RNA extraction and cDNA synthesis

RNA purification was carried out using the TRIzol LS reagent (Invitrogen) in accordance with manufacturer’s instructions. From the liquid cultures, 10 ml aliquots were centrifuged at 4600 × g (20 min at 4°C), and RNA was extracted from the resulting pellet. From infected SHK-1 cells, individual wells were harvested with a cell scraper; the whole mixture was recovered and centrifuged at 300 × g (10 min), washed with 1× PBS, and centrifuged again. The supernatant was discarded, and the cellular pellet was processed for RNA purification. All obtained RNA was stored at −80°C until further analysis.

Prior to cDNA synthesis, 2 µg of RNA were pre-treated with DNase RQ1 (Promega) to eliminate putative DNA contamination, and reverse transcription
Table 1. Specific primers designed for qRT-PCR analysis of *Piscirickettsia salmonis* flaQ, rpoN, fliA, flaA, and flaB. *Salmo salar* innate immune response genes *il1*-α, membrane toll-like receptor 5 (*tlr5M*), and soluble *tlr5* (*tlr5S*), and the housekeeping gene *ef-1α*. *T*<sub>m</sub>: melting temperature.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ → 3’</th>
<th><em>T</em>&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlaQ-F</td>
<td>GCCCCCCATGGTTAAGCAAGAGATGG</td>
<td>60</td>
<td>This study</td>
</tr>
<tr>
<td>FlaQ-R</td>
<td>GACTTAAACATCGTGCCGCCACAC</td>
<td>60</td>
<td>This study</td>
</tr>
<tr>
<td>RpoN-F</td>
<td>GCACTGAACAGCTCCTCATCACTCGG</td>
<td>60</td>
<td>This study</td>
</tr>
<tr>
<td>RpoN-R</td>
<td>GTGAAGCCGGGTGAAGAATG</td>
<td>60</td>
<td>This study</td>
</tr>
<tr>
<td>FliA-F</td>
<td>ATCACCATGCGTCACATTGC</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>FliA-R</td>
<td>GACTTAAATCGGCGGTAT</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>EfliA-F</td>
<td>GCTTCAATCAGTCCAATCATGCGGCC</td>
<td>60</td>
<td>This study</td>
</tr>
<tr>
<td>EfliA-R</td>
<td>CTTGACGGACACGTTCTTGA</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>IL1-α-Ftp1</td>
<td>ATCACCAAGCCTCATACATTC</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>IL1-α-Rtp1</td>
<td>GTCCTTGAACTCGGTTCCCA</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>TLR5M Ftp2</td>
<td>TTAGGCCGCAAGTTCTGAT</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>TLR5M Rtp2</td>
<td>AACAGGCGGTTCTAACC</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>TLR5S Ftp</td>
<td>GCTGCTGGAGCTAAGGAACA</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>TLR5S Rtp</td>
<td>GACTTAAAGCATCGTGCGGT</td>
<td>60</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>EF-1α F</td>
<td>GCTTAAATCGGCGGTAT</td>
<td>62</td>
<td>Salazar et al. (2016)</td>
</tr>
<tr>
<td>EF-1α R</td>
<td>CTGGACGGACACGTTCTTGA</td>
<td>62</td>
<td>Salazar et al. (2016)</td>
</tr>
</tbody>
</table>

was performed using M-MLV Reverse Transcriptase (Promega) and random primers (Promega) according to the manufacturer’s instructions.

### Western blot analysis to determine the presence of flagellin (FlaA) protein

*P. salmonis* protein extracts were obtained from 48 h old cultures in BM3 and MB media under dynamic and static growth conditions. Additionally, bacterial protein lysates of *Escherichia coli* TOP10 (Invitrogen) were used as positive controls, and total protein lysates from uninfected SHK-1 cells and *Micrococcus luteus* were used as negative controls. All protein samples were quantified using the BCA Protein Assay Kit (Thermo Fisher), and 20 μg of each extract was separated on a 12% polyacrylamide SDS-PAGE and transferred onto a 0.45 μm pore nitrocellulose membrane (Bio-Rad), in accordance with standardized conditions. Briefly, a polyclonal monospecific antibody against a conserved region of flagellin in *Vibrio anguillarum* (Fig. A1 in the Appendix) was prepared in the laboratory according to published methodology (González-Stegmaier et al. 2015) and was used for specific detection. The membrane was blocked with 3% bovine serum albumin (BSA) for 1 h at 37°C and incubated with the first anti-flagellin antibody in a 1:200 dilution for 1 h at 37°C with gentle agitation. After extensive washing with 1× PBS and 1× PBS + 0.05% Tween 20 (PBST), an
anti-IgG mouse horseradish peroxidase secondary antibody was added at a 1:10,000 dilution and incubated at 37°C for 1 h with gentle agitation. After extensive washing, the membrane was revealed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Confocal laser scanning microscopy (CLSM) analysis of flagellin localization**

SHK-1 cells were grown on glass cover slips and infected with the *P. salmonis* LF-89 reference strain at a multiplicity of infection of 50. At 33 h post-infection (hpi), the cells were washed with 1× PBS and then fixed for 15 min with 3% paraformaldehyde. After washing with 1× PBS, the fixed cells were permeabilized with 0.1% triton X-100 (Invitrogen) for 10 min. The polyclonal monospecific anti-flagellin antibody was added in a 1:500 dilution to the permeabilized cells, which were then incubated for 1 h at 37°C and washed once with PBST and twice with 1× PBS. Following this, an anti-mouse FITC-conjugated antibody was added in a 1:10,000 dilution to detect the bound primary antibody, for which the cells were incubated for 1 h at 37°C and then washed with PBST and with 1× PBS.

An anti-ChaPs (HSP60 of *P. salmonis*) antibody was prepared in the laboratory and was used as a control to recognize the bacterium (Marshall et al. 2007). Detection was performed under the same conditions as described above, although an anti-mouse Alexa Fluor 647-conjugated antibody was used for specific detection. Nuclear staining was carried out with 10 µg ml⁻¹ of propidium iodide for 5 min at room temperature, and cells were washed 3 times with 1× PBS and mounted with Dako Mounting Medium (Invitrogen). Finally, the samples were analyzed using a Leica TCS SP5 II Spectral Confocal Microscope (Leica Microsystems). Images were obtained with a Leica HCX PL APO 40×/1.25 OIL, CS objective (Leica Microsystems) at a resolution of λ488 xy = 132 nm.

**Immunogold assay for transmission electronic microscopy (TEM) of flagellin localization**

SHK-1 cells were grown in 25 cm² flasks under the previously described conditions until reaching 80% confluence. Cells were infected with *P. salmonis*. At 33 hpi, the infected cells were collected in 15 ml Falcon tubes, and the medium (supernatant) and cells were separately processed. The tubes were centrifuged at 300 × *g* (5 min) to collect a pellet sample for the cells or to eliminate cellular debris in the case of the supernatant. Both samples were passed to 1.5 ml Eppendorf tubes and fixed overnight with 4% paraformaldehyde (Sigma-Aldrich). Thin sections of the samples (60–70 nm) were obtained with a Sorvall Ultramicrotome 139 (MT-5000). Following this, the sections were layered over copper grills and stained with 4% uranyl acetate in methanol for 2 min and lead citrate for 5 min. The samples were then washed with 1× PBS and blocked with 3% BSA containing 0.5% Tween-20 for 30 min at room temperature. A polyclonal monospecific anti-flagellin antibody was then added in a 1:100 dilution, and the grids were incubated for 1 h at room temperature and washed 3 times with PBST. An anti-mouse IgG conjugated to gold particles (Sigma-Aldrich) in a 1:200 dilution was used as a secondary antibody, with a 1 h incubation period at room temperature followed by washing 3 times with 1× PBST. Finally, the grids were washed, filtered with distilled water, and left to dry. The samples were contrasted with uranyl 4% and observed under a Philips Tecnai 141 TEM at a range of 12 to 80 kV.

**Immune response of SHK-1 cells to recombinant *P. salmonis* flagellin**

To produce recombinant flagellin, the full flaA ORF of the *P. salmonis* genome was amplified with the primers FlaA-NdeI-F: 5’-GGC ATA TGG GAA TTT CTA TTA ATA CCA AC-3’ (*Tm*: 60°C); and FlaA-BamHI-R2: 5’-GGG GAT CCT TAT TGC AGC AAC TGC AAA GCG GT-3’ (*Tm*: 60°C). The resulting PCR products were first cloned into the pCR 2.1 TOPO-TA (Thermo) and then subcloned into the pET28a expression vector (Novagen) between the NdeI and BamHI restriction sites, generating a His-tagged flaA (His-FlaA) gene (pET28a/FlaA vector). The *E. coli* Rosetta BL21 (DE3) pLysS strain was then transformed with the pET28a/FlaA vector to produce the recombinant FlaA protein. The recombinant protein was produced in 100 ml *E. coli* Rosetta BL21 (DE3) pLysS cultures using 1 mM of isopropyl β-D-1-thiogalactopyranoside (Winkler). Finally the His-FlaA protein was purified from the solubilized *E. coli* cells using the TALON His-Tag Purification Resin (Clontech Laboratories) according to the manufacturer’s instructions. To eliminate the potential contamination of bacterial endotoxins, such as lipopolysaccharide, during protein prepara-
tion, the purified recombinant protein was loaded onto a Polymyxin B column (Sigma-Aldrich). Purified His-FlaA was confirmed by SDS-PAGE and Western blotting.

To evaluate the inflammatory response, SHK-1 cells (n = 3 cultures) were incubated either with recombinant flagellin (0.01 or 0.1 µg ml⁻¹ concentration) or a negative control without FlaA (cells only treated with 1x PBS) for 6, 12, and 24 h. Total RNA extraction, DNase treatment, and cDNA synthesis were performed for each time point as previously described. Expression levels of the innate immune response genes *il1-β*, membrane toll-like receptor 5 (*tlr5M*), and soluble TLR5 (*tlr5S*) were analyzed by qRT-PCR and normalized to the expression of the housekeeping gene *ef-1α*. The expression levels were calculated as described above using the Livak method (2⁻ΔΔCt). The qRT-PCR was performed with specific primers (Table 1) in the CFX96 qPCR System (Bio-Rad) using the following parameters: 95°C for 3 min, 40 cycles of 95°C for 15 s, 60°C for 10 s (or 62°C for the housekeeping gene), and 60°C for 25 s. For melting curve analysis, samples were heated from 65 to 95°C in 0.5°C increments with a dwell time of 5 s at each temperature and with continuous fluorescence monitoring. For all assays, primer efficiencies were determined as previously described.

**Statistical analysis**

All qRT-PCR data were analyzed using 1-way ANOVA to determine significant differences in gene expression. The comparisons were performed between the selected gene at different times and the calibrator individually for each different culture medium or kinetic infection in an independent manner. All analyses were done using a confidence level of 95%. The analyses were performed using Star Plus Mac Software, and all graphics were made in Prisma Software.

**RESULTS**

**Genomic organization of putative *Piscirickettsia salmonis* flagellar genes**

Using the gene organization and expression pattern of *Legionella pneumophila*, the genomic organization of all structural and regulatory genes involved in *P. salmonis* flagellar biosynthesis were defined and compared against available *P. salmonis* genomes. The principal flagellar genes were clustered in 4 different operons in all of the analyzed *P. salmonis* strains (Fig. 1). Additionally, the organization of flagellar genes was conserved between different *P. salmonis* strains, except for *fliI*, which was absent in the Austral-005 strain, and for *flit*, which was absent in the genome published by Pulgar et al. (2015).

*fleQ*, the cascade initiator, was located in an operon with other regulatory genes, including *fleS*, *fleR*, and the *fliE-F-G-H-I-J-K* family of coding genes (Fig. 1A). *fliA* (σ28) was located in a second operon with other regulatory genes, including *flhF*, *flhA*, *fleN*, and *fliQ* (Fig. 1B). *flaA* and *flaB* were located together in a third operon with other structural flagella genes, including *flag* and *flID-S-T* (Fig. 1C). *flgM*, a putative anti-σ28 gene, was located in a fourth operon (Fig. 1D). Finally, *rpoN* (σ54) was also located in the fourth identified operon with genes unrelated to flagellar biosynthesis.

**P. salmonis** flagellar genes conserve sequences with homologues

The 4 specific *P. salmonis* genes coding for flagellum-related functions in the putative transcriptional cascade for flagellar biosynthesis were comparatively analyzed in silico against homologous sequence alignments in *L. pneumophila* and with other Gram-negative bacteria. All 4 proteins displayed a high degree of conservation, especially for the active sites of FleQ and FliA (σ28) and for the C- and N-terminal domains of FlaA and FlaB, 2 flagellum-filament structural proteins (Figs. 2 & 3).

**Detection of flagellin expressed as a protein monomer**

Cultures grown for 48 h in BM3 or MB media under static and dynamic conditions were used to obtain crude protein extracts. These extracts were assessed via Western blotting and were exposed to a monospecific polyclonal antibody generated against a conserved epitope of the flagellin protein monomer (González-Stegmaier et al. 2015). There was an unequivocal presence of the flagellin monomer in all *P. salmonis* extracts at the expected 53.34 kDa, as well as in the positive *E. coli* control (Fig. 4). The protein was absent from the SHK-1 cell line and the non-flagellated bacterium *Micrococcus luteus*. 
Fig. 1. Operon organization of the putative *Piscirickettsia salmonis* flagellar genes selected for analyses. Images were processed using the CLC Main Workbench Software. (A) Operon containing the *fleQ* gene. (B) Operon containing the *fliA* gene (σ28). (C) Operon containing the *flaA* (flagellin) and *flaB* genes. (D) Operon containing the anti-FliA (σ28), *flgM* gene.
Cellular localization of the flagellin monomer

The location of the flagellin monomer in bacteria in infected cells was determined using CLSM and TEM. At 33 hpi of the SHK-1 cell line, flagellin appeared circumscribed to the periphery of the bacteria, as observed through CLSM (Fig. 5). As a control for the identification of *P. salmonis* in CLSM observations, an anti-ChaPs antibody (HSP60 of *P. salmonis*) was used, as ChaPs is an abundant protein in *P. salmonis* (Marshall et al. 2007). This approach was further supported with TEM analysis, where FlaA was only found located on the periphery and in the cytosol of the host cell-released bacteria (Fig. 6).

Expression of the 5 flagellar genes under different growth conditions

In both the optimal (BM3) and sub-optimal (MB) cell-free media growth conditions and in infected SHK-1 fish cells (see Fig. 8), all flagellar genes were transcribed at 3, 6, 12, 24 and 48 h, but these were differentially expressed depending on the culture/
Fig. 3. Sequence alignment of 2 flagellar-filament structural proteins in *Piscirickettsia salmonis* compared to phylogenetically related homologues. (A) FlaA alignment. (B) FlaB alignment. In both cases, black boxes show flagellin C- and N-terminal domains, present in both proteins. The image was processed using Jalview Software, and color shows the degree of amino acid conservation between each protein and the respective homologue.
A high fold change of flagellar gene expression was observed when *P. salmonis* was grown in MB compared to BM3 (Fig. 7). Moreover, the static growth condition seemed to act in synergy with nutritional stress, favoring flagellar gene expression (Fig. 7A vs. 7B). These observed differences were statistically significant (*p* < 0.05), and cell viability was not a limiting factor in the overall evaluation (Table 2). The transcription profile of *P. salmonis* flagellar genes during infection of the macrophage cell line SHK-1 tended to be preferentially expressed during early infection stages (Fig. 8), with a peak at 6 h of *fliA* (σ28), a gene with an important regulatory role in cascade progression, specifically activating expression of class IV genes, under which flagellin is classified. It is also worth noting that in the *in vitro* infected SHK-1 cells, the expression of all target genes tended to show diminished transcription profiles over infection progression.

**Immunomodulatory effect of *P. salmonis* FlaA protein**

All 3 immune genes (*il-1β*, *tlr5M*, and *tlr5S*) were highly expressed *in vitro* in SHK-1 cells after challenge with recombinant FlaA protein (Fig. 9). Interestingly, expression of *il-1β* peaked 3 hpi and averaged 200-fold higher than in non-stimulated cells. This expression decreased at 12 h before increasing again at 24 hpi.
DISCUSSION

The expression of flagellar genes in Gram-negative bacteria constitutes a prime example of how numerous and complex molecular mechanisms need to be coordinated to generate a fully functional structure, such as a flagellum. In general, bacterial flagellar expression is highly regulated by different environmental conditions and is usually driven by a transcriptional and hierarchal cascade of genes in which full translation of the first component of the cascade is required to activate the expression of subsequent genes (Macnab 2003, Moisi et al. 2009, Martinez et al. 2010). Additionally, since the organization of the flagellar system appears to be evolutionarily conserved in Gram-negative bacteria (Tsang & Hoover 2014), a similar cascade, if one exists in Piscirickettsia salmonis, would likely share common features.

Table 2. Piscirickettsia salmonis viability (%) in cell-free media. BM3: nutrient-rich medium; MB: marine broth medium with high salt concentration; dynamic growth: with shaking at 100 rpm; static growth: no shaking

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>BM3 Dynamic growth</th>
<th>BM3 Static growth</th>
<th>MB Dynamic growth</th>
<th>MB Static growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>76.1</td>
<td>75.7</td>
<td>80.2</td>
<td>81.5</td>
</tr>
<tr>
<td>6</td>
<td>93.3</td>
<td>95.5</td>
<td>90.6</td>
<td>91.7</td>
</tr>
<tr>
<td>12</td>
<td>85.2</td>
<td>93.1</td>
<td>85.8</td>
<td>81.6</td>
</tr>
<tr>
<td>24</td>
<td>97.1</td>
<td>97.2</td>
<td>84.1</td>
<td>88.1</td>
</tr>
<tr>
<td>48</td>
<td>84.9</td>
<td>99.1</td>
<td>93.3</td>
<td>90.2</td>
</tr>
</tbody>
</table>

Fig. 6. Transmission electron microscope image of Piscirickettsia salmonis FlaA in infected SHK-1 cells. Location of FlaA on bacterial surface and in cytosol.

Fig. 7. Expression profiles of Piscirickettsia salmonis flagellar genes during growth kinetic experiments in cell-free media. Gene expression was determined by qRT-PCR, using relative quantification, and was calculated relative to the growth in the nutrient-rich broth (BM3), at equivalent time points. Expression in marine broth (MB) with (A) dynamic growth and (B) static growth. Data were validated by ANOVA (p < 0.05). Error bars show SD; fold change is presented on a logarithmic scale. *Significant difference between growth in nutrient-rich broth (BM3) and marine broth (MB).

Fig. 8. Expression profiles of Piscirickettsia salmonis flagellar genes during growth kinetic experiments in an infected fish cell line (SHK-1). Gene expression was determined by qRT-PCR, using relative quantification, and using 3 h post-infection as a calibrator. Data were validated by ANOVA (p < 0.05). Error bars show SD; fold change is presented on a logarithmic scale. *Significant difference between growth in nutrient-rich broth (BM3) and marine broth (MB).
From the Class III genes, reported organizations in a strikingly similar flagellar cascade organization to genome, resulting in the identification of *P. salmonis* while *cally, required to complete the flagellum structure. Specifically, *fliA* genes encode for 2 proteins responsible for assembly. Finally, 2 genes were selected from Class IV. These pivotal genes encode for 2 proteins required to complete the flagellum structure. Specifically, *fliA* encodes for the master regulator protein FleQ, the expression of which is likely controlled by the σ70 factor (Jacobi et al. 2004). Since FleQ together with factor σ54 (RpoN) positively regulate the expression of Class II genes, the second selected gene was *rpoN*. From the Class III genes, *fliA* (σ28), which is also FleQ-dependent, was selected. The expression of *fliA* controls genes involved in motor and flagellum assembly. Finally, 2 genes were selected from Class IV. These pivotal genes encode for 2 proteins required to complete the flagellum structure. Specifically, *fliA* is responsible for the flagellin monomer while *fliB* codes for a flagellin-like protein.

This information was used to thoroughly screen the *P. salmonis* genome, resulting in the identification of a strikingly similar flagellar cascade organization to reported organizations in *Legionella* and *Pseudoomonas* species (Jacobi et al. 2004), with the structural and regulatory genes assembled into 4 different operons (Fig. 1). Moreover, we have demonstrated that all characterized *P. salmonis* strains have flagellar genes and share the same genetic organization (Eppinger et al. 2013, Bohle et al. 2014, Yañez et al. 2014, Pulgar et al. 2015). Our results suggest that *P. salmonis* has the potential to develop a flagellum controlled by this transcriptional cascade.

In *silico* comparative analysis (Figs. 2 & 3) demonstrated a high degree of conservation for the protein sequence of FlaA (Fig. 3A), and *P. salmonis* FlaA contained the specific flagellin-conserved regions reported for most Gram-negative bacteria. These observations strengthened the possibility of the *P. salmonis* FlaA variant acting as an active protein, and this was confirmed through detection of a protein in *P. salmonis* extracts using an anti-flagellin antibody (Fig. 4). Indeed, synthesis of the flagellin monomer, the end product of the cascade, was translated as a mature 53.34 kDa monomer, as expected from the FlaA sequence. This study represents the first detection of a *P. salmonis* flagellin protein, although no flagellum structure has been observed in the bacteria.

Using CLSM (Fig. 5) and TEM (Fig. 6) flagellin was observed, under the conditions assayed, on the bacteria periphery during infection of SHK-1 cells, despite the flagellin protein sequence not possessing a translocation signal (data not shown).

The presence of flagellin protein is a clear indicator that an active flagellar cascade exists in *P. salmonis*. In addition to the complex flagellar cascade, the generation of a functional flagellum also requires a highly regulated assembly process (Macnab 2003). Since *P. salmonis* lacks a functional flagellum, the flagellin monomer might have additional functions. Notwithstanding these observations, flagellar gene expression and assembly are not necessarily perfectly coupled processes. Indeed, some bacteria have lost the capacity to assemble a functional flagellum despite expressing all of the flagella-related genes (Akerley et al. 1992, Tominaga et al. 1994, Parkhill et al. 2001). A similar situation might explain the absence of functional flagella in *P. salmonis*. Indeed, flagella development is normally regulated by a Type III secretion system (McCarver 2006), which is absent in *P. salmonis* (Gómez et al. 2013). In order to demonstrate activity of the *P. salmonis* flagellar cascade, the expression of the selected genes (*fleQ, fliA, rpoN, flaB, and flaA*) was evaluated under different culture and growth conditions. Our results show that all of the evaluated genes were transcribed, although the conditions and time post initiation of growth/infection resulted in different expression levels. In cell-free media, all genes showed higher expression in the nutrient-poor medium (MB), particularly under the static condition (i.e. without shaking; Fig. 7B). These results agree with those reported for flagellar expression in *L. pneumophila*, where stressors, such as limited nutrition and high osmolality, upregulate expression of genes involved in the flagellar cascade, and flagellum proteins, while abundant nutrients...
have a contrary effect (Heuner et al. 1999). Additionally, the regulatory genes of the *P. salmonis* flagellar cascade share similar expression profiles to those described for *L. pneumophila*, where *rpoN* (σ54) and *flIA* (σ28) are induced, triggering a high expression of the final genes in the cascade, such as *flaA* and *flaB* (Prouty et al. 2001, McCarter 2006). Notably, even in the nutrient-rich medium (BM3), all of the flagellar genes were transcribed, and, independent of the growth conditions, all flagellar genes followed the same induction profile (data not shown). Also worth highlighting, *P. salmonis* grown on the MB medium did evidence biofilm formation by the bacteria (Marshall et al. 2012). Future studies should determine the role of flagellar genes, if any, in *P. salmonis* biofilm formation.

In the SHK-1 cells infected *in vitro* (Fig. 8), all of the flagella-related genes were preferentially expressed during early infection, with high expression 6 hpi. This would support the idea that flagellin might trigger the activation of immune responses in the host to attract macrophages and initiate productive infection (Steiner et al. 2000, McCarthy et al. 2008, Rojas et al. 2009, Duan et al. 2013, Rozas & Enriquez 2014).

Knowing that flagellin is a pathogen-associated molecular pattern that directly interacts with TLR5 and considering the descriptions in *Salmo salar* of TLR5 and TLR5M (Tsujita et al. 2004, Tsoi et al. 2006), the impact of *P. salmonis* flagellin on TLR5 expression was evaluated. Specifically, a lab-produced recombinant His-tagged FlaA protein was used to stimulate SHK-1 cells. The results indicated an up-regulated expression of *tlr5M, tlr5S*, and *il-1β* (Fig. 9), strongly suggesting that *P. salmonis* flagellin monomer could be a pro-inflammatory molecule for the immune system of the host, as has been previously suggested (Salazar et al. 2016).

Our results strongly indicate that a regulatory flagellar hierarchy promotes flagellin expression in *P. salmonis*. However, the question remains as to what is missing from *P. salmonis* for flagellar assembly to occur. Based on the present results, there is an array of possible explanations, of which the following 3 are the most reasonable: (1) the flagellum assembly machinery is either impaired in *P. salmonis*, independent of the bacteria’s growth conditions, or is highly regulated by an alternative, yet unidentified molecular system (Toft & Fares 2008); (2) the cascade itself and/or some of its components are key structural/functional components but do not necessarily lead to the formation of a structural flagellum; and (3) monomeric flagellin plays a still unknown functional role associated with the bacteria’s cell membrane, providing alternative functions. Furthermore, the evidence presented in this work strengthens the idea that the flagellin monomer may act as a proinflammatory molecule (Steiner et al. 2000), modulating the salmonid immune system and initiating the systemic infection characteristic of *P. salmonis* (Fig. 9).

In conclusion, in the complex context of flagellar gene regulation, we found evidence that *P. salmonis* contains and expresses key genes necessary for generating a flagellum. Moreover, *P. salmonis* synthesized flagellin monomers, which were observed near the cell membrane, although final assembly into a functional structure was not detected under any of the assayed conditions. Additionally, the genetic organization of the assessed genes was highly conserved, corresponding to a highly regulated hierarchical cascade characteristic of flagellar biosynthesis in most Gram-negative bacteria. This finding suggests that the flagellar genes are not required for flagella assembly, highlighting the fact that the flagellin monomer of *P. salmonis* evidenced proinflammatory effects in *S. salar* macrophage-like cells, stimulating the expression of 3 key innate immunity components, and therefore representing an alternative function for the flagellar cascade in facilitating *P. salmonis* infection of macrophages.

**Acknowledgements.** This study was supported by the Comisión Nacional de Investigación Científica y Tecnológica de Chile (CONICYT) through grant FONDECYT 11130407 and by Fundación COPEC-UC through grant 2014.J.116. We also acknowledge the support of the Fraunhofer Chile Research given to S.H.M. and to the Vice Rectoría de Investigación y Estudios Avanzados at the Pontificia Universidad Católica de Valparaíso, Chile. We thank Dr. Luis Mercado for providing the specific antibody against a conserved epitope of the flagellin monomer, and we acknowledge Ramón Ramírez and Fabián Henríquez for technical support.

**LITERATURE CITED**


rickettsiosiis in cultured sea bass (Dicentarchus labrax L.) from Greece. J Appl Ichthyol 20:525−529


González-Stegmaier R, Romero A, Estapa A, Montero J, Mulero V, Mercado L (2015) Effects of recombinant flagellin B and its ND1 domain from Vibrio anguillarum on macrophages from gillhead seabream (Sparus aurata L.) and rainbow trout (Oncorhynchus mykiss, W.). Fish Shellfish Immunol 42:144−152


Marshall SH, Henríquez V, Gómez FA, Cárdenas C (2011) ISPsa2, the first mobile genetic element to be described and characterized in the bacterial facultative intracellular pathogen Piscirickettsia salmonis. FEMS Microbiol Lett 314:18−24


Appendix

Table A1. Primer efficiencies for qPCR analyses

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FleQ</td>
<td>109.0</td>
</tr>
<tr>
<td>RpoN</td>
<td>109.7</td>
</tr>
<tr>
<td>FliA</td>
<td>98.2</td>
</tr>
<tr>
<td>FlaB</td>
<td>101.9</td>
</tr>
<tr>
<td>FlaA</td>
<td>101.9</td>
</tr>
<tr>
<td>IL1-β</td>
<td>100.4</td>
</tr>
<tr>
<td>TLR5M</td>
<td>103.2</td>
</tr>
<tr>
<td>TLR5S</td>
<td>94.6</td>
</tr>
<tr>
<td>EF-1α</td>
<td>102.0</td>
</tr>
</tbody>
</table>

Fig. A1. Sequence alignment of *Piscirickettsia salmonis* and *Vibrio anguillarum* FlaA proteins. Red box indicates the conserved epitope used for *V. anguillarum* anti-flagellin antibody production (González-Stegmaier et al. 2015). The alignment was made using ClustalΩ and the image was processed with Jalview software.