Vol. 123: 29–43, 2017
doi: 10.3354/dao03079

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published February 8

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

Gabriela P. Carril[1], Fernando A. Gómez[1], Sergio H. Marshall[1,2,3,*]

[1]Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso 2340000, Chile
[2]Núcleo de Biotecnología Curauma, Pontificia Universidad Católica de Valparaíso, Valparaíso 2340000, Chile
[3]Fraunhofer 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

*Corresponding author: sergio.marshall@pucv.cl

southern California, USA (Athanassopoulou et al. 2004, Arkush et al. 2005). Therefore, *P. salmonis* constitutes a potential danger to the fish farming industry worldwide.

P. salmonis is a non-encapsulated, pleomorphic, generally coccoid 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, Henríquez 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 (Olivares & 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 cm^2 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 1× 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 pretreated with DNase RQ1 (Promega) to eliminate putative DNA contamination, and reverse transcription

Table 1. Specific primers designed for qRT-PCR analysis of *Piscirickettsia salmonis fleQ*, *rpoN*, *fliA*, *flaA*, and *flaB*; *Salmo salar* innate immune response genes *il1-β*, membrane toll-like receptor 5 (*tlr5M*), and soluble TLR5 (*tlr5S*); and the housekeeping gene *ef-1α*. T_m: melting temperature

Primer	Sequence 5′→ 3′	T_m (°C)	Reference
FleQ-F	GCCCCCATGGTTAAGCAGAGAGTGG	60	This study
FleQ-R	GACTTAAAGCATCGTGCGCCACAC	60	This study
RpoN-F	GCATGAAACAGTCCCTTCAACTGCG	60	This study
RpoN-R	GGTATTGCCACTATTATCTGAGTCGC	60	This study
FliA-F	GTGAAGCGGGCTGAAAAGTATGCT	60	This study
FliA-R	GCTTCAATCAGTCCAATCATGCCCGC	60	This study
FlaB-F	GCGATGGGAATTTCTATTAATACCAAC	60	This study
FlaB-R	CGTAATGATCGCATAGCCAGCCGC	60	This study
FlaA-F	GCATGGCACTTTCAGTTGTAACCAAC	60	This study
FlaA-R	CAAGGCCAATACTCGCATCCGCTG	60	This study
IL1-β Ftp1	ATCACCATGCGTCACATTGC	60	Salazar et al. (2016)
IL1-β Rtp1	GTCCTTGAACTCGGTTCCCA	60	Salazar et al. (2016)
TLR5M Ftp2	TTGACCGCCAGGATCCTTGA	60	Salazar et al. (2016)
TLR5M Rtp2	AACAGGGCGGTTCTACCCA	60	Salazar et al. (2016)
TLR5S Ftp	GCTGCTGGAGCTAAGGAACA	60	Salazar et al. (2016)
TLR5S Rtp	GAGCCCTCAGCGAGTTAAGC	60	Salazar et al. (2016)
EF-1α F	GCTTACAAAATCGGCGGTAT	62	Salazar et al. (2016)
EF-1α R	CTTGACGGACACGTTCTTGA	62	Salazar et al. (2016)

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

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of expression kinetics

Expression of the selected putative *P. salmonis* flagellar genes from all growth conditions were measured by qRT-PCR using relative quantification. qRT-PCR assays were performed in triplicate using a total reaction volume of 20 µl for each sample, which contained 1× Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix (Agilent), 300 nM of gene-specific primers (Table 1), and 1 µl of template (1:10 dilution of cDNA). PCR assays were performed in a CFX96 Real-Time qPCR System (Bio-Rad) using the following parameters: 95°C for 3 min, 40 cycles at 95°C for 15 s, and 60°C for 30 s. For melting curve analysis, samples were heated from 65 to 95°C in 0.5°C increments, with a dwell time at each temperature of 5 s and with continuous fluorescence monitoring. The *P. salmonis* ribosomal operon internal transcribed spacer (*its*) was used as the housekeeping gene together with primers RTS1 and RTS4, as previously described (Marshall et al. 1998, Gómez et al. 2013) and using the following PCR conditions: 95°C for 3 min, 40 cycles at 95°C for 15 s, 51°C for 10 s, and 60°C for 20 s. For all assays, primer efficiencies were established with the equation E = 10(−1/slope) (Table A1 in the Appendix). Relative gene expression were estimated using threshold cycle (Ct) values and the Livak $2^{-\Delta\Delta Ct}$ method without modification (Livak & Schmittgen 2001). For growth kinetics in MB, the Ct value obtained for each sample at each time point for ITS was used as a normalization factor (Marshall et al. 2012, Gómez et al. 2013). The growth kinetics in BM3 were used as a calibrator: static for static and mixing for mixing, at each time point for all genes to measure relative fold differences in expression under the other media conditions. For infection kinetics in SHK-1 cells, the ITS Ct values were used as a normalization factor and the initial *P. salmonis* flagellar gene expression in the inoculum at time 0 as the calibrator.

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 cm^2 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′ (T_m: 60°C); and FlaA-BamHI-R2: 5′-GGG GAT CCT TAT TGC AGC AAC TGC AAA GCG GT-3′ (T_m: 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 *Nde*I and *Bam*HI 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 1× 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 ($2^{-\Delta\Delta Ct}$). 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*.

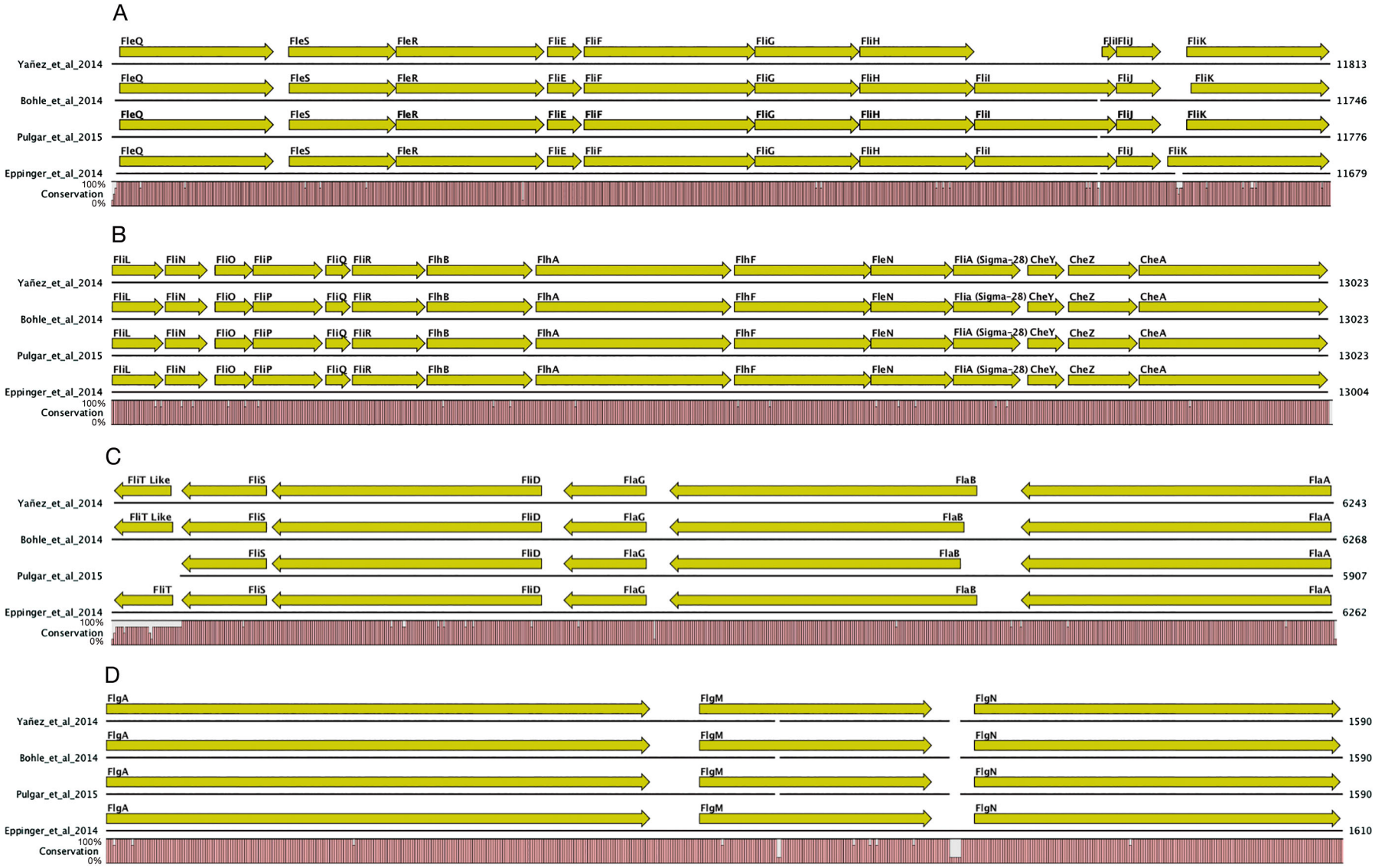

Fig. 1. Operon organization of the putative *Piscirickettsia salmonis* flagellar genes selected for analyses. Images were processed using the CLC Main Workbench Software. (A) Operon containing the *fleQ* gene. (B) Operon containing the *fliA* gene (σ28). (C) Operon containing the *flaA* (flagellin) and *flaB* genes. (D) Operon containing the anti-FliA (σ28), *flgM* gene

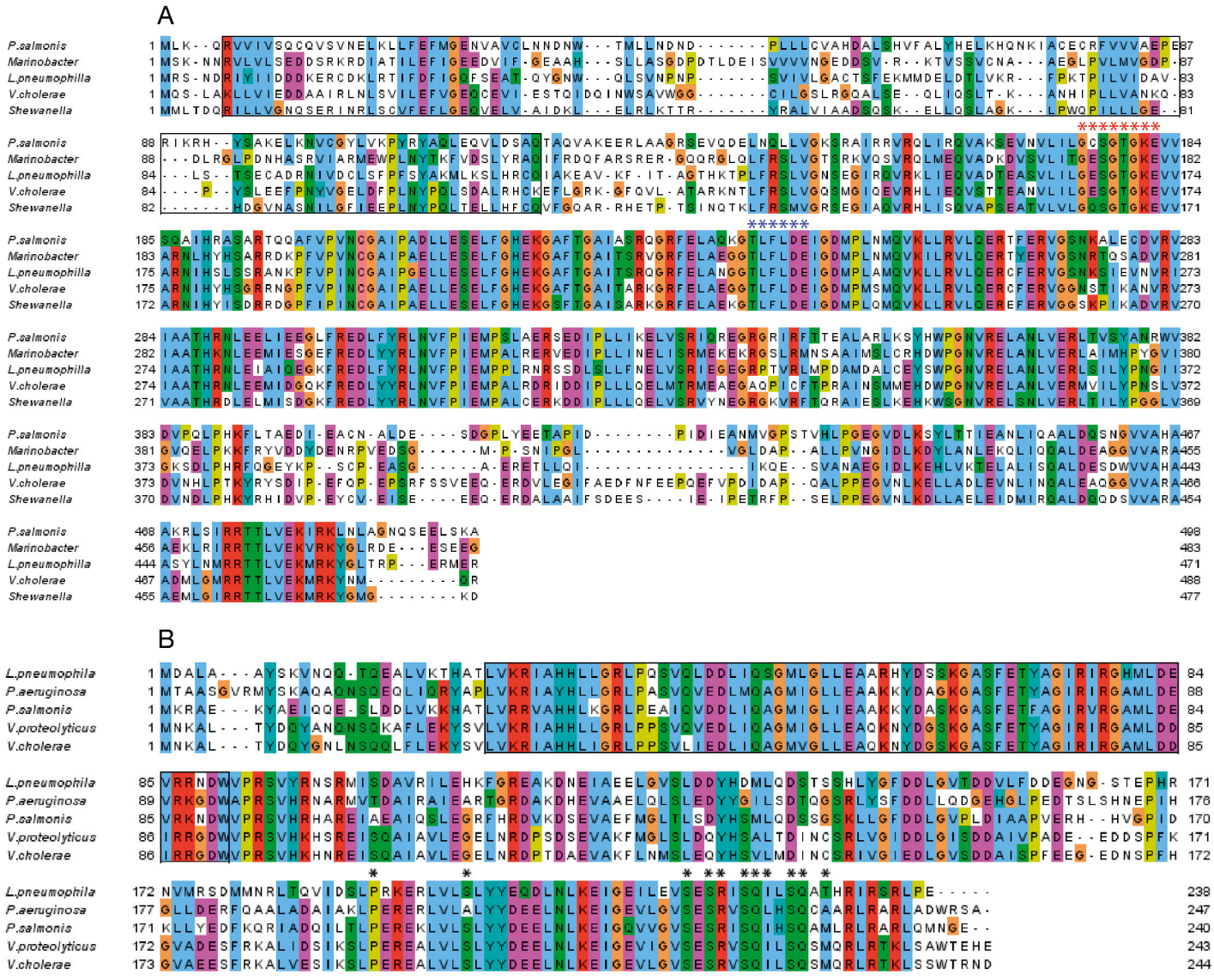

Fig. 2. Sequence alignments of putative *Piscirickettsia salmonis* flagellar-regulatory proteins compared to phylogenetically related homologues. (A) FleQ alignment. Red and blue asterisks show the Walker A and Walker B motifs, respectively (ATP-binding site). (B) FliA (σ28) alignment. Black asterisks indicate the amino acids implicated in the putative DNA-binding domain. In both panels, black boxes show the conserved domains of each protein family. The images were processed using Jalview Software, and color shows the degree of amino acid conservation between each protein and the respective homologue

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/

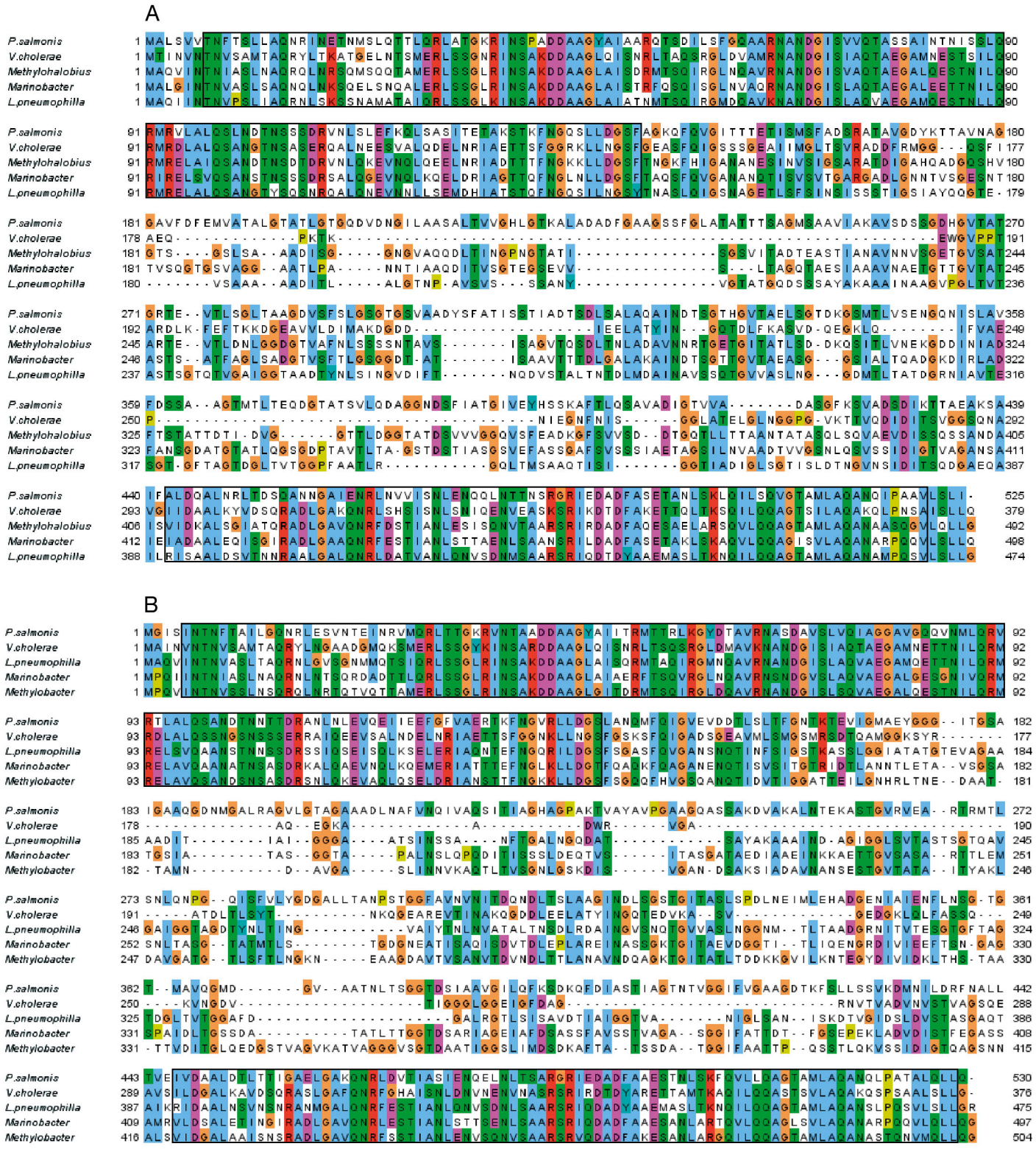

Fig. 3. Sequence alignment of 2 flagellar-filament structural proteins in *Piscirickettsia salmonis* compared to phylogenetically related homologues. (A) FlaA alignment. (B) FlaB alignment. In both cases, black boxes show flagellin C- and N-terminal domains, present in both proteins. The image was processed using Jalview Software, and color shows the degree of amino acid conservation between each protein and the respective homologue

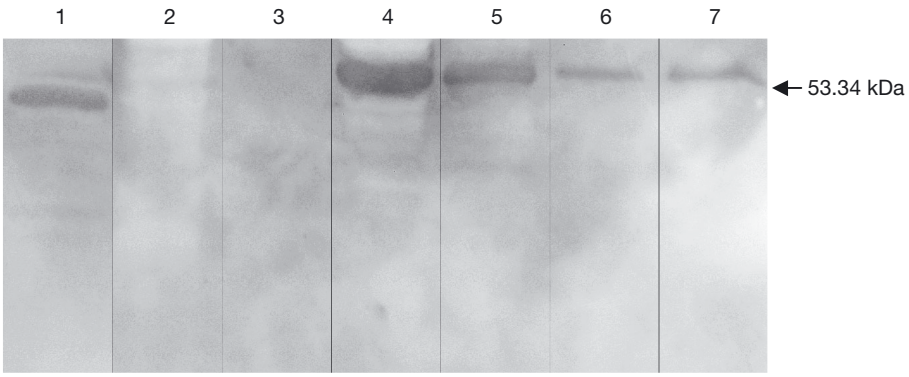

Fig. 4. Detection of *Piscirickettsia salmonis* flagellin by Western blot analysis using a monospecific polyclonal anti-flagellin antibody. Similar crude extracts from related bacteria were used as controls. Lanes: 1, *Escherichia coli*; 2, SHK-1 cell line; 3, *Micrococcus luteus*; 4, *P. salmonis* grown in a nutrient-rich broth (BM3) with dynamic growth; 5, *P. salmonis* grown in BM3 with static growth; 6, *P. salmonis* grown in marine broth (MB) with dynamic growth; 7, *P. salmonis* grown in MB with static growth

growth conditions and the time of evaluation. 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 tlr*5S*) 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.

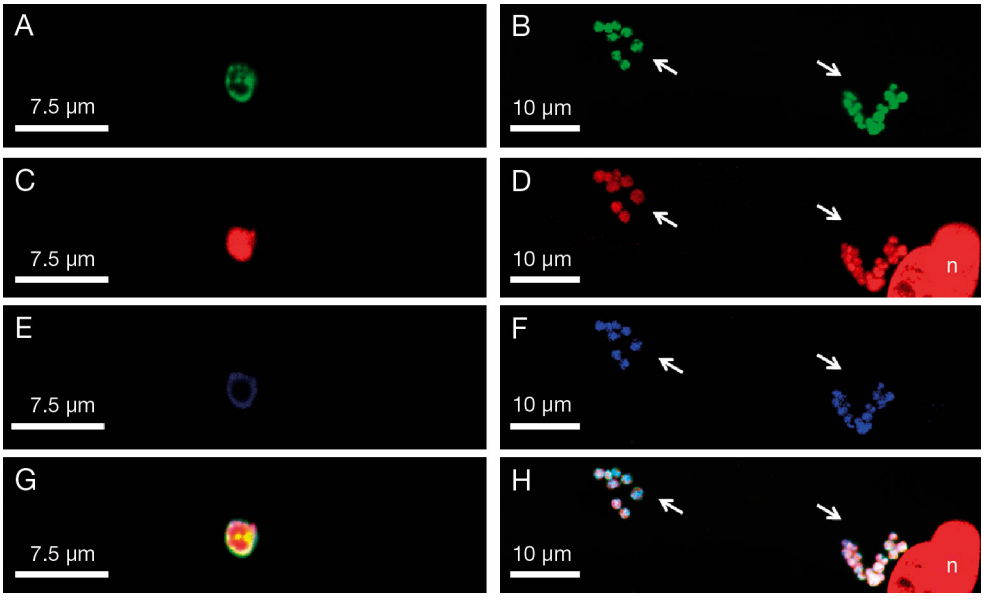

Fig. 5. Cellular localization of *Piscirickettsia salmonis* flagellin in infected SHK-1 cells. Infected cells were screened for bacterial flagellin using confocal laser scanning microscopy and fluorescent markers as tracers. All images were obtained under a 100× objective. (A, B) Flagellin identification with an FITC-conjugated secondary antibody. (C, D) Bacterial and host cell DNA stained with propidium iodide. (E, F) Localization of the *P. salmonis* transmembrane ChaPs protein using an Alexa Fluor 647-conjugated antibody. (G) Merged image of panels A, C, and E. (H) Merged image of panels B, D, and F. White arrows show *P. salmonis* aggregations near a host cell; n: nucleus of an infected SHK-1 cell

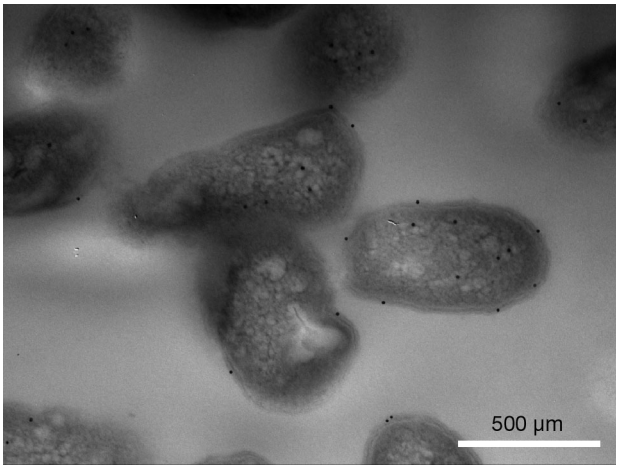

Fig. 6. Transmission electron microscope image of *Piscirickettsia salmonis* FlaA in infected SHK-1 cells. Location of FlaA on bacterial surface and in cytosol

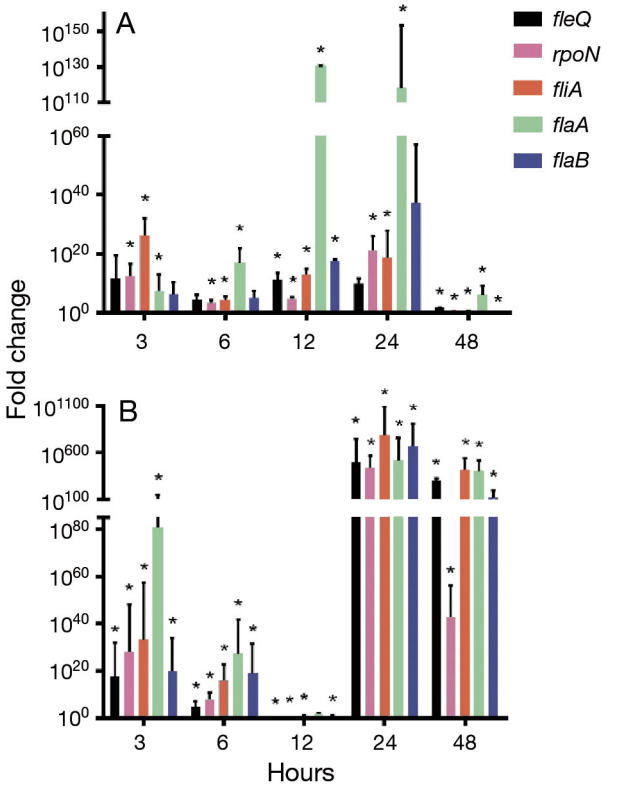

Fig. 7. Expression profiles of *Piscirickettsia salmonis* flagellar genes during growth kinetic experiments in cell-free media. Gene expression was determined by qRT-PCR, using relative quantification, and was calculated relative to the growth in the nutrient-rich broth (BM3), at equivalent time points. 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)

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

Time (h)	BM3		MB	
	Dynamic growth	Static growth	Dynamic growth	Static growth
3	76.1	75.7	80.2	81.5
6	93.3	95.5	90.6	91.7
12	85.2	93.1	85.8	81.6
24	97.1	97.2	84.1	88.1
48	84.9	99.1	93.3	90.2

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.

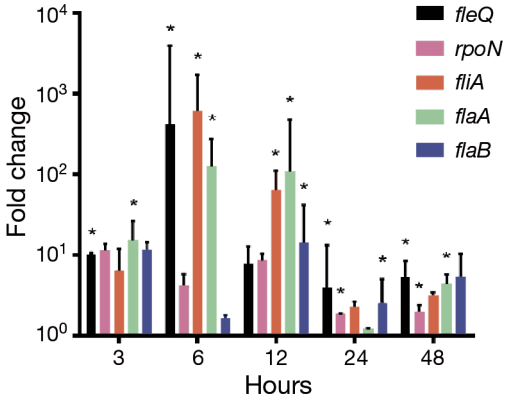

Fig. 8. Expression profiles of *Piscirickettsia salmonis* flagellar genes during growth kinetic experiments in an infected fish cell line (SHK-1). Gene expression was determined by qRT-PCR, using relative quantification, and using 3 h post-infection as a calibrator. Data were validated by ANOVA ($p < 0.05$). 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)

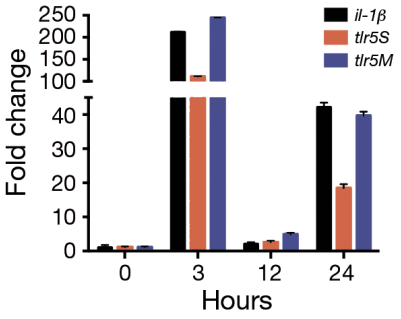

Fig. 9. Expressions of *il-1β*, *tlr5S*, and *tlr5M* in SHK-1 cells challenged with a recombinant *Piscirickettsia salmonis* FlaA (flagellin). Expressions were determined by qRT-PCR using relative quantification. In all cases, *ef-1α* was used as the housekeeping gene while time 0 (no exposed cells) was used as the calibrator. The SHK-1 cell line was exposed to 0.1 µg ml^{-1} of recombinant *P. salmonis* FlaA for 3, 12, and 24 h. Data were validated by ANOVA ($p < 0.05$). Error bars show SD. *Significant difference between the calibrator and the time post-infection

Considering this information and the phylogenetically close relationship between *P.* salmonis and *Legionella pneumophila,* the flagellar cascade organization present in *L. pneumophila,* which has 4 gene classes, was used as a basis for selecting putative homologues in a potential cascade in *P. salmonis*. The *fleQ* gene was selected from Class I. This gene 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, *flaA* is responsible for the flagellin monomer while *flaB* 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 *Pseudomonas* 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 (McCarter 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 TLR5S 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 the *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

Akerley BJ, Monack DM, Falkow S, Miller JF (1992) The bvgAS locus negatively controls motility and synthesis of flagella in *Bordetella bronchiseptica*. J Bacteriol 174: 980–990

Albert-Weissenberger C, Sahr T, Sismeiro O, Hacker J, Heuner K, Buchrieser C (2010) Control of flagellar gene regulation in *Legionella pneumophila* and its relation to growth phase. J Bacteriol 192:446–455

Arkush KD, McBride AM, Mendonca HL, Okihiro MS and others (2005) Genetic characterization and experimental pathogenesis of *Piscirickettsia salmonis* isolated from white seabass *Atractoscion nobilis*. Dis Aquat Org 63: 139–149

Athanassopoulou F, Groman D, Prapas T, Sabatakou O (2004) Pathological and epidemiological observations on

rickettsiosis in cultured sea bass (*Dicentrarchus labrax* L.) from Greece. J Appl Ichthyol 20:525–529

Aziz RK, Bartels D, Best AA, DeJongh M and others (2008) The RAST server: rapid annotations using subsystems technology. BMC Genomics 9:75

Bohle H, Henríquez P, Grothusen H, Navas E and others (2014) Comparative genome analysis of two isolates of the fish pathogen *Piscirickettsia salmonis* from different hosts reveals major differences in virulence-associated secretion systems. Genome Announc 2:e01219-14

Brocklebank JR, Speare DJ, Armstrong RD, Evelyn T (1992) Septicemia suspected to be caused by a rickettsia-like agent in farmed Atlantic salmon. Can Vet J 33:407–408

Clamp M, Cuff J, Searle SM, Barton GJ (2004) The Jalview Java alignment editor. Bioinformatics 20:426–427

Dannevig BH, Falk K, Namork E (1995) Isolation of the causal virus of infectious salmon anemia (ISA) in a long-term cell-line from Atlantic salmon head kidney. J Gen Virol 76:1353–1359

Duan Q, Zhou M, Zhu L, Zhu G (2013) Flagella and bacterial pathogenicity. J Basic Microbiol 53:1–8

Eppinger M, McNair K, Zogaj X, Dinsdale EA, Edwards RA, Klose KE (2013) Draft genome sequence of the fish pathogen *Piscirickettsia salmonis*. Genome Announc 1: e00926-13

Fryer JL, Hedrick RP (2003) *Piscirickettsia salmonis*: a Gram-negative intracellular bacterial pathogen of fish. J Fish Dis 26:251–262

Fryer JL, Lannan CN, Giovannoni SJ, Wood ND (1992) *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. Int J Syst Bacteriol 42:120–126

Gómez F, Henríquez V, Marshall SH (2009) Additional evidence of the facultative intracellular nature of the fish bacterial pathogen *Piscirickettsia salmonis*. Arch Med Vet 41:261–267

Gómez FA, Cardenas C, Henríquez V, Marshall SH (2011) Characterization of a functional toxin-antitoxin module in the genome of the fish pathogen *Piscirickettsia salmonis*. FEMS Microbiol Lett 317:83–92

Gómez FA, Tobar JA, Henríquez V, Sola M, Altamirano C, Marshall SH (2013) Evidence of the presence of a functional Dot/Icm type IV-B secretion system in the fish bacterial pathogen *Piscirickettsia salmonis*. PLOS ONE 8:e54934

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

Henríquez M, González E, Marshall SH, Henríquez V, Gómez FA, Martínez I, Altamirano C (2013) A novel liquid medium for the efficient growth of the salmonid pathogen *Piscirickettsia salmonis* and optimization of culture conditions. PLOS ONE 8:e71830

Heuner K, Brand BC, Hacker J (1999) The expression of the flagellum of *Legionella pneumophila* is modulated by different environmental factors. FEMS Microbiol Lett 175:69–77

Jacobi S, Schade R, Heuner K (2004) Characterization of the alternative sigma factor σ54 and the transcriptional regulator *fleQ* of *Legionella pneumophila*, which are both involved in the regulation cascade of flagellar gene expression. J Bacteriol 186:2540–2547

Larkin MA, Blackshields G, Brown NP, Chenna R and others (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Macnab RM (2003) How bacteria assemble flagella. Annu Rev Microbiol 57:77–100

Marchler-Bauer A, Lu SN, Anderson JB, Chitsaz F and others (2011) CDD: a conserved domain database for the functional annotation of proteins. Nucleic Acids Res 39:D225–D229

Marshall S, Heath S, Henríquez V, Orrego C (1998) Minimally invasive detection of *Piscirickettsia salmonis* in cultivated salmonids via the PCR. Appl Environ Microbiol 64:3066–3069

Marshall SH, Conejeros P, Zahr M, Olivares J, Gómez F, Cataldo P, Henríquez V (2007) Immunological characterization of a bacterial protein isolated from salmonid fish naturally infected with *Piscirickettsia salmonis*. Vaccine 25:2095–2102

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

Marshall SH, Gómez FA, Ramírez R, Nilo L, Henríquez V (2012) Biofilm generation by *Piscirickettsia salmonis* under growth stress conditions: a putative *in vivo* survival/persistence strategy in marine environments. Res Microbiol 163:557–566

Martinez RM, Jude BA, Kirn TJ, Skorupski K, Taylor RK (2010) Role of FlgT in anchoring the flagellum of *Vibrio cholerae*. J Bacteriol 192:2085–2092

Mauel MJ, Ware C, Smith PA (2008) Culture of *Piscirickettsia salmonis* on enriched blood agar. J Vet Diagn Invest 20:213–214

McCarter LL (2006) Regulation of flagella. Curr Opin Microbiol 9:180–186

McCarthy UM, Bron JE, Brown L, Pourahmad F and others (2008) Survival and replication of *Piscirickettsia salmonis* in rainbow trout head kidney macrophages. Fish Shellfish Immunol 25:477–484

Mikalsen J, Skjaervik O, Wiik-Nielsen J, Wasmuth MA, Colquhoun DJ (2008) Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. FEMS Microbiol Lett 278:43–47

Moisi M, Jenul C, Butler SM, New A and others (2009) A novel regulatory protein involved in motility of *Vibrio cholerae*. J Bacteriol 191:7027–7038

Olivares J, Marshall SH (2010) Determination of minimal concentration of *Piscirickettsia salmonis* in water columns to establish a fallowing period in salmon farms. J Fish Dis 33:261–266

Olsen AB, Melby HP, Speilberg L, Evensen Ø, Håstein T (1997) *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway — epidemiological, pathological and microbiological findings. Dis Aquat Org 31: 35–48

Parkhill J, Wren BW, Thomson NR, Titball RW and others (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature 413:523–527

Prouty MG, Correa NE, Klose KE (2001) The novel σ^{54}- and σ^{28}-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. Mol Microbiol 39:1595–1609

Pulgar R, Travisany D, Zuniga A, Maass A, Cambiazo V (2015) Complete genome sequence of *Piscirickettsia salmonis* LF-89 (ATCC VR-1361) a major pathogen of farmed salmonid fish. J Biotechnol 212:30–31

Rodger HD, Drinan EM (1993) Observation of a rickettsia-like organism in Atlantic salmon, *Salmo salar* L., in Ireland. J Fish Dis 16:361–369

Rojas V, Galanti N, Bols NC, Marshall SH (2009) Productive infection of *Piscirickettsia salmonis* in macrophages and monocyte-like cells from rainbow trout, a possible survival strategy. J Cell Biochem 108:631–637

Rozas M, Enriquez R (2014) Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review. J Fish Dis 37:163–188

Salazar C, Haussmann D, Kausel G, Figueroa J (2016) Molecular cloning of *Salmo salar* Toll-like receptors (TLR1, TLR22, TLR5m and TLR5s) and expression analysis in SHK-1 cells during *Piscirickettsia salmonis* infection. J Fish Dis 39:239–248

Steiner TS, Nataro JP, Poteet-Smith CE, Smith JA, Guerrant RL (2000) Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. J Clin Invest 105:1769–1777

Tobar JA, Jerez S, Caruffo M, Bravo C, Contreras F, Bucarey SA, Harel M (2011) Oral vaccination of Atlantic salmon (*Salmo salar*) against salmonid rickettsial septicaemia. Vaccine 29:2336–2340

Toft C, Fares MA (2008) The evolution of the flagellar assembly pathway in endosymbiotic bacterial genomes. Mol Biol Evol 25:2069–2076

Tominaga A, Mahmoud MAH, Mukaihara T, Enomoto M (1994) Molecular characterization of intact, but cryptic, flagellin genes in the genus *Shigella*. Mol Microbiol 12: 277–285

Tsang J, Hoover TR (2014) Themes and variations: regulation of RpoN-dependent flagellar genes across diverse bacterial species. Scientifica (Cairo) 2014:681754

Tsoi S, Park KC, Kay HH, O'Brien TJ and others (2006) Identification of a transcript encoding a soluble form of toll-like receptor 5 (TLR5) in Atlantic salmon during *Aeromonas salmonicida* infection. Vet Immunol Immunopathol 109:183–187

Tsujita T, Tsukada H, Nakao M, Oshiumi H, Matsumoto M, Seya T (2004) Sensing bacterial flagellin by membrane and soluble orthologs of toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*). J Biol Chem 279:48588–48597

Wilhelm V, Miquel A, Burzio LO, Rosemblatt M and others (2006) A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based on recombinant proteins. Vaccine 24:5083–5091

Yañez AJ, Molina C, Haro RE, Sanchez P and others (2014) Draft genome sequence of virulent strain AUSTRAL-005 of *Piscirickettsia salmonis*, the etiological agent of piscirickettsiosis. Genome Announc 2:e00990-14

Zusman T, Degtyar E, Segal G (2008) Identification of a hypervariable region containing new *Legionella pneumophila* Icm/Dot translocated substrates by using the conserved *icmQ* regulatory signature. Infect Immun 76: 4581–4591

Appendix

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

Table A1. Primer efficiencies for qPCR analyses

Primer pair	Efficiency (%)	Primer pair	Efficiency (%)
FleQ	109.0	IL1-β	100.4
RpoN	109.7	TLR5M	103.2
FliA	98.2	TLR5S	94.6
FlaB	101.9	EF-1α	102.0
FlaA	101.9		

Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: March 16, 2015; Accepted: November 7, 2016
Proofs received from author(s): January 25, 2017