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The mutagenesis of a type IV secretion system locus of *Piscirickettsia salmonis* leads to the attenuation of the pathogen in Atlantic salmon, *Salmo salar*

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Abstract

Piscirickettsiosis is a threatening infectious disease for the salmon industry, due to it being responsible for significant economic losses. The control of outbreaks also poses considerable environmental challenges. Despite *Piscirickettsia salmonis* having been discovered as the aetiological agent of the disease more than 25 years ago, its pathogenicity remains poorly understood. Among virulence factors identified so far, type four secretion systems (T4SS) seem to play a key role during the infection caused by the bacterium. We report here the genetic manipulation of *P. salmonis* by means of the transference of plasmid DNA in mating assays. An insertion cassette was engineered for targeting the *icmB* gene, which encodes a putative T4SS-ATPase and is carried by one of the chromosomal T4SS clusters found within the genome of *P. salmonis* PM15972A1, a virulent representative of the EM-90-like strain. The molecular characterization of the resulting mutant strain demonstrated that the insertion interrupted the target gene. Further in vitro testing of the *icmB* mutant showed a dramatic drop in infectivity as tested in CHSE-214 cells, which is in agreement with its attenuated behaviour observed in vivo. Altogether, our results demonstrate that, similar to other facultative intracellular pathogens, *P. salmonis*' virulence relies on an intact T4SS.

KEYWORDS
EM-90, mutant, *Piscirickettsia salmonis*, strain, T4SS

1 | INTRODUCTION

Piscirickettsiosis is an infectious disease affecting reared salmonids worldwide. *Piscirickettsia salmonis*, a Gram-negative bacterium and causative pathogen, is responsible for an acute, septicaemic disease and subsequent negative economic and environmental impacts especially in Chile. Considerable amounts of antibiotics are needed each year to control outbreaks as the existing vaccines, formulated mainly with inactivated antigens, have yielded poor efficacy in the field (Jakob et al., 2014). Owing to the increased risk of antibiotic resistance and the substantial economic losses implicated, piscirickettsiosis is a serious threat for the sustainability of the salmon industry. Therefore, new, more effective vaccines are required. In this regard, the first live attenuated vaccine was introduced to the Chilean market in 2016 (Maisey, Montero, & Christodoulides, 2016). At present and despite initially high expectations towards an improved field efficacy, information concerning the true performance of this pioneering product is not yet available (Evensen, 2016).

Type four secretion systems (T4SS) are bacterial multiprotein complexes that form a gate for the exportation of diverse molecules

such as DNA and proteins. Phylogenetically, they are related to the conjugation machinery, and four subgroups can be distinguished as follows: F-T4SS (Tra/Trb), archetypal P-T4SS (VirB/D4), I-T4SS (Dot/Icm) discovered in *Legionella pneumophila* and a genomic island-associated type (GI-T4SS) (Souza et al., 2012). T4SS encompass a protein transmembrane complex, which creates a pore-like opening in the membrane of the host cell and allows specific molecules or effectors to trespass this barrier. These effectors can interfere with the physiology of host cells, thereby subverting key cellular processes during the infection (Cascales & Christie, 2003). It has been estimated that the minimal genetic set needed for the functionality of T4SS in Gram-negative bacteria is about 12 genes (Waksman & Orlova, 2014), although the Dot/Icm-related group seems to be more complex and may require up to 20 genes (Nagai & Kubori, 2011). Paradigmatic descriptions of T4SS are based on results obtained in the plant symbiont *Agrobacterium tumefaciens*: here, T4SS are required for the establishment of a long-lasting ecological relationship, which can be achieved by the introduction of the oncogenic plasmid *T* into the host cell (Christie, 1997). Furthermore, the T4SS of *Brucellae*, which is also called *virB*, is absolutely necessary for those pathogens to display full virulence in brucellosis, a zoonotic disease also known as Malta fever (O'callaghan et al., 1999). The Gram-negative pathogens *Coxiella* and *Legionella* that are related to *Piscirickettsia* possess a Dot/Icm (defect in organelle trafficking/intracelullar multiplication) system whose name is derived from the phenotypes observed in mutagenesis studies and that is clasified into the I-T4SS group. This system has been shown to be critical in the pathogenesis of the respective diseases (Segal & Shuman, 1997; Zamboni, Mcgrath, Rabinovitch, & Roy, 2003). In this context, genes closely related to the Dot/Icm system found in *Legionella* were previously identified in the genome of the *P. salmonis* LF-89 strain (Eppinger et al., 2013). Consistent with their putative pathogenic role, the transcripts for genes encoding several T4SS components in *P. salmonis*-infected cell cultures have been detected, and evidence of their protein translation was also recently added (Gomez et al., 2013; Cortes et al., 2017). Moreover, some putative T4SS effectors have been identified bioinformatically (Labra, Arredondo-Zelada, Flores-Herrera, Marshall, & Gomez, 2016). But even though in silico and expression studies have proven to yield valuable data for the identification of virulence factors, there is still a need for the functional characterization to confirm these hypotheses.

Thus far, data on the pathogenicity of *P. salmonis* have been obtained using a single organism: the LF-89^T strain, also known as VR-1361 (Fryer, Lannan, Giovannoni, & Wood, 1992). However, recent studies suggest that *P. salmonis* isolates can be splitted into at least two genogroups (Otterlei et al., 2016; Bravo & Martinez, 2016). Although disease presentation and gross pathology are similar, a difference in the number of T4SS gene clusters was described at genomic level: in comparison with its EM-90-like counterpart, the genome of the LF-89-like isolate is enriched in this kind of genes (Bohle et al., 2014). Interestingly, the EM-90-like strain, which tolerates a warmer and less nutrient-rich environment, was responsible for the majority of piscirickettsiosis cases recorded in Chile in 2015, infecting exclusively *Salmo salar* (Saavedra et al., 2017). However, and despite their relevance in the current epidemiological situation of piscirickettsiosis, data concerning the experimental behaviour of EM-90-like isolates have only recently been presented (Rozas-Serri et al., 2017).

An important step towards the elucidation of pathogenicity and thus the development of vaccine strains with improved efficacy is the establishment of a protocol for genetic manipulation. To this end, several methods have been reported, but due to its specificity, site-specific mutagenesis using linear DNA or plasmid constructs introduced by gene transfer technologies is most broadly used. Nevertheless, little is known about the molecular genetics of the *Piscirickettsia* genus and, to the best of our knowledge, no protocol for the mutagenesis of *P. salmonis* has been reported to date. Considering the gaps of knowledge mentioned above, this study aims at gaining insights into the virulence patterns of EM-90-like isolates by means of controlled in vivo testing and unravelling the role of the T4SS in the pathogenicity of *P. salmonis* by adapting a well-known mutagenesis tool.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains, growth conditions and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Culture of *P. salmonis* on liquid or solid medium was performed on ADL-PSB or PSA, respectively (Henriquez, Kaiser, Bohle, Bustos, & Mancilla, 2016), at 18°C, while *Escherichia coli* strains were cultured on tryptic soy broth, TSB (Becton-Dickinson, Franklin Lakes, NJ, USA) or agar (TSA) at 37°C. All media were supplemented with 10 μg/ml of polymyxin B, 50 μg/ml of kanamycin, or both, depending on the purpose of the experiment. *P. salmonis* growth kinetics were analysed using a microtiter method described elsewhere (Saavedra et al., 2017). Briefly, frozen bacteria were inoculated into 3 ml of ADL-PSB, and the medium was maintained statically for 4 days at 18°C. After centrifugation, bacteria were washed with saline-buffered solution (SBS; 0.15 M NaCl, 7.3 mM KH_2PO_4, 11.5 mM K_2HPO_4, pH 6.0) supplemented with 4% lactose, and the suspension was subsequently adjusted to an optical density (OD) = 1.0 as measured at a wavelength of 600 nm. 30 μl of a 1:10 dilution of this cell suspension were used to inoculate wells of flat-bottom 96-well microtiter plates prefilled with 120 μl of ADL-PSB, thereby reaching a final concentration of ~3×10^5 colony-forming units (CFU) per ml. The microplates were incubated at 18 ± 0.1°C for 48 hr, with 1 min of shaking before reading OD_{600} each hour in an EPOCH2 microplate spectrophotometer (Biotek, Winooski, VT, USA). Minimal inhibitory concentrations for polymyxin B and kanamycin (both purchased from Sigma-Aldrich, St Louis, O, USA) were determined applying a conventional microdilution method but using PSB (Yanez et al., 2014). Plasmid pJQK was kindly donated by Prof Ignacio Moriyón, Department of Microbiology and Parasitology, University of Navarra, Pamplona, Spain. The plasmid was propagated and maintained in *E. coli* S17-1λ*pir* (see Table 1).

TABLE 1 Bacteria and plasmids used

Strain/plasmid	Characteristic	Source or Reference
P. salmonis PM15972A1 (PM15972A1)	Parental *P. salmonis* field isolate recovered from a diseased Atlantic salmon in 2010	Bohle et al. (2014)
P. salmonis icmB::Km (*icmB*)	*P. salmonis* insertional mutant containing a pJQK interrupting the *icmB* gene of the parental strain	This study
E. coli TOP10	Cloning strain	Invitrogen
E. coli S17-1λ*pir*	Mating strain with plasmid RP4 inserted into the chromosome. Supports the replication of R6K plasmids	Simon et al. (1983)
E. coli pCR*icmB*	Recombinant clone, TOP10 background, containing the plasmid pCR*icmB*	This study
E. coli pJQ*icmB*	Recombinant clone, S17-1λ*pir* background containing the plasmid pCR*icmB*	This study
pCR2.1-TOPO	Cloning plasmid. Kanamycin and ampicillin resistances	Invitrogen
pCR*icmB*	pCR2.1-TOPO derivative containing a 882-bp fragment resulting from the amplification with primers icmB_F and R	This study
pJQK	Mobilizable plasmid with RP4 origin of transfer. Kanamycin resistance	Quandt and Hynes (1993)
pJQ*icmB*	pJQK derivative containing the *Bam*HI-*Xba*I fragment of pCR*icmB*	This study

2.2 | Sequence analysis

The chromosome sequence of *P. salmonis* PM15972A1 was retrieved from GenBank (accession no CP012413). Similarity searches were accomplished applying conventional algorithms (BLAST). Secretion system components were predicted with T346Hunter (Martinez-Garcia, Ramos, & Rodriguez-Palenzuela, 2015). Primers were designed using the Primer3 web tool (Untergasser et al., 2012). Promoter analysis was accomplished with the BPROM web tool (Solovyev & Salamov, 2011).

2.3 | DNA manipulation

Genomic DNA (gDNA) was extracted from bacterial colonies and purified using the GeneJET gDNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Primers used are listed in Table 2. A fragment of 882 bp was amplified using primers icmB_F and icmB_R, and 5 ng of gDNA from *P. salmonis* PM15972A1 as template. The fragment was cloned into pCR2.1TOPO and *E. coli* TOP10 served as host (Invitrogen, Carlsbad, CA, USA). Recombinant clones were recovered from selective plates and then screened under identical PCR conditions. A miniprep of pCRicmB was used for restriction digestion by *Xba*I-*Bam*HI (Thermo Fisher Scientific). The resulting fragment was purified using the Qiaquik kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The fragment was subcloned into the pJQK vector (Quandt & Hynes, 1993) using *E. coli* S17-1λ*pir* as host (Simon, Priefer, & Pehle, 1983), a procedure that yielded the plasmid pJQicmB. *E. coli* carrying pJQicmB were used for a mating assay with *P. salmonis* PM15972A1. Aliquots of fresh cultures of both bacteria were centrifuged at 6,030 *g* for 3 min, washed twice with SBS-lactose, and mixed in a proportion of 1:10. Bacteria were suspended in 100 μl of saline and placed onto a non-selective PSA plate. Mating was permitted for 24 hr at 18°C on ADL-PSA plates, before the cells were transferred onto selective plates containing 50 μg/ml of kanamycin and 10 μg/ml of polymyxin B. Insertional mutants were confirmed by PCR using a combination of *icmB*-flanking (icmB_Fb and Rb) and plasmid backbone primers (M13F and R). To study homologous insertion sites, PCR-RFLP using the icmB_T4SS2 primer pair and digestion with *Xba*I and *Pst*I were performed. An estimation of the stability of plasmid integration was realized by culturing bacteria in antibiotic-free media for around 100 generations (duplication time ~10 hr). Subsequently, a 10 μl aliquot was plated on PSA plates with or without kanamycin. The stability was based on the ratio between kanamycin-resistant and total colonies.

2.4 | Cell infection assay

To test infectivity, an end-point dilution assay was performed. Chinook salmon embryo cells, CHSE-214, were propagated in MEM (Gibco, Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (FBS; HyClone GE Healthcare, Little Chalfont, UK), using 25-cm^2 polystyrene flasks (Nunc; Thermo Fisher Scientific) at 18°C. For tissue culture infection dose titration (TCID), 96-well microplates were prepared so that each well contained ~2.4×10^4 cells in a volume of 100 μl. The cells were maintained in MEM supplemented with 2% FBS to achieve ~90% of confluence in 48 hr. Tenfold serial dilutions of fresh bacterial culture suspensions, starting at DO_{600} = 1.0 diluted 1:100 in saline, were used to infect cell monolayers. The $TCID_{50}$/ml was defined as the maximum dilution of pathogen still able to yield cytopathic effect (CPE) in 50% of inoculated wells. Titres were assessed 14 days post-infection in triplicate. Calculations were based on Reed & Muench method (Reed & Muench, 1938). For T4SS relative gene expression kinetics, *S. salar* head kidney cells, SHK-1, were cultured in Leibovitz's L-15 medium (Gibco, Thermo Fisher Scientific) plus 10% FBS at 18°C. We followed the protocol reported by Salazar and colleagues with modifications (Salazar, Haussmann, Kausel, & Figueroa, 2016). 24-well plates were seeded with 7.2×10^4 cells/well, left for 48 hr, and then infected at a multiplicity of infection (MOI) of 10. Plates were

TABLE 2 Sequences of primers used in the study

Name	Sequence (5′-3′)	Reference or Source
OspA_F	GCTGTGCCCAGAACTTTAG	This study
OspA_R	GACCACTRCCTTTACCAAAC	This study
OspA_P	FAM-CAGCAACACCGCCAACAACAGCC-IBFQ	This study
icmB_F	ATTACCCGCGATCTGAAATG	This study
icmB_R	AGTCGCCATGTTTTGGTGA	This study
icmB_Fb	TCACCACGCTCATCGAGATA	This study
icmB_Rb	GGCATTAGTTGACGGGCTAT	This study
M13F	GTAAAACGACGGCCAG	Invitrogen
M13R	CAGGAAACAGCTATGAC	Invitrogen
16S_F	CTTATCGCGTTAGCTACGCCAC	This study
16S_R	CGACTTTCTGGATCAATACTGACG	This study
ADL_DotA_F	CACGGCGGTTGTTGTTGTC	This study
ADL_DotA_R	AGCATTTCAGATAGCCCCAAAGA	This study
ADL_1828_F	GTGCCTGCACGGCTTTTAAT	This study
ADL_1828_R	CTGGAGCATCACCTCAGGC	This study
ADL_icmP_F	CATGGATTGCCCGATCTACCATC	This study
ADL_icmP_R	AGGCCGTTTTTGTTGAGGCT	This study
ADL_sdbC_F	AGTTCCTATGGTGATGAGTGTATCA	This study
ADL_sdbC_R	CTACCCTAATGTCACTCCGCTAT	This study
T4SS2_icmE_F	ATTCAGACGCTGCCTGTTGG	This study
T4SS2_icmE_R	AAACAAGTGCAGCAACCGAG	This study
T4SS2_icmK_F	CTTGTGCGGAACCTCCTGTT	This study
T4SS2_icmK_R	GCACTTGCAGGAACAACACC	This study
T4SS2_2375_F	AGAACTCTGTTCTGACTGGC	This study
T4SS2_2375_R	GGCTGGTCTGGTCAAAGTAA	This study
icmB_T4SS2_F	TACGAACAGCAACTGTAGTT	This study
icmB_T4SS2_R	CAAGATGATGATCGCAGATT	This study

centrifuged for 10 min at 4°C and 200 *g* to facilitate the contact between bacteria and cells. After an additional incubation of 1 hr at 18°C, extracellular bacteria were killed by adding 400 μl of culture medium plus gentamicin (50 μl/ml). Subsequently, the cells were softly rinsed and left at 18°C in Leibovitz's L-15 medium plus 2% FBS (time 0). Nucleic acid extraction and purification were performed from infected cells at 0, 24, 48 and 72 hr post-infection using the E.Z.N.A. Total RNA kit (Omega Biotek, Norcross, GA, USA). RNA was prepared by removing DNA through an exhaustive RQ1 DNAse treatment (Promega, Madison, WI, USA) achieved with 2 U of enzyme per sample for 30 min at 37°C. cDNA synthesis was facilitated by mixing enzyme buffer, 2 μl random hexamers, 1 μg of RNA and 2 μl of RevertAid reverse transcriptase (Thermo Fisher Scientific). RT reaction was accomplished by incubating the mix for 10 min at 25°C, 60 min at 42°C and 10 min at 70°C.

2.5 | Real-time PCR

Real-time PCR, qPCR, for *P. salmonis* detection was performed in a final volume of 20 μl. The reaction mix contained 10 μl of 2× TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 1.0 μl of forward and reverse primers (OspA_F and R) and probe (OspA_P) at a final concentration of 500 and 250 nM, respectively, 7.0 μl of ultrapure water and 2 μl of template obtained as described above. The same conditions were used when gene expression experiments were carried out, but using only the corresponding primers and SYBR Green Expression Master Mix (Applied Biosystems). qPCR were conducted in 48-well microplates (MicroAmp fast optical reaction plate) in a StepOne Real-Time PCR machine (Applied Biosystems). The thermal profile included 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers for several components of the T4SS were specifically designed to distinguish gene expression between T4SS loci and to assess a possible polar effect on genes located downstream of the mutation (Figure 1a, Table 2). To this end, genes located upstream (*sdbC* and *icmP*) and downstream (KW89_1828 and *dotA*) from the predicted insertion site were selected. Transcription of components of the homologous T4SS was also studied using *icmK*, *icmE* and KW89_2375 sequences. For a relative quantification, fold changes were calculated according to the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the 16S rRNA gene as an endogenous control of expression (Livak & Schmittgen, 2001).

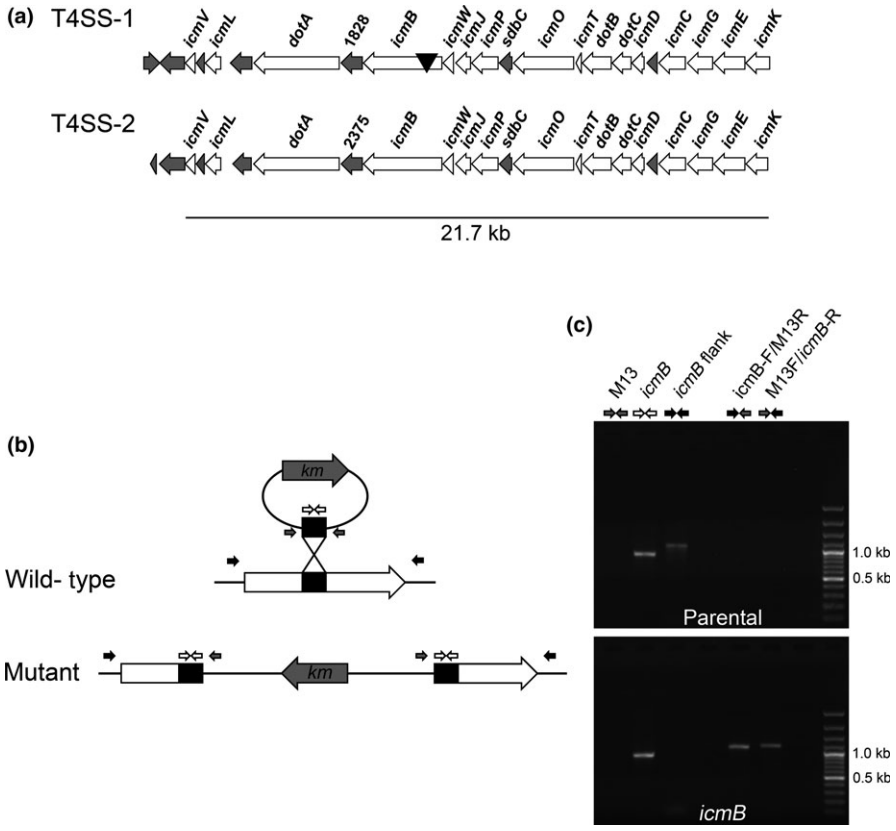

FIGURE 1 (a) Genetic organization of *Legionella*-like T4SS gene clusters in *Piscirickettsia salmonis* PM15972A1. Genes coding for T4SS structural components appears as white arrows. The site of insertion is shown by the black triangle. (b) Mutagenesis strategy. Insertion of the engineered plasmid encoding kanamycin resistance will give rise a distinctive mutant genotype, which can be distinguished by PCR. Position of primers used is indicated by arrows: Black, flanking *icmB* primers; white, *icmB* primers; grey, plasmid backbone primers. (c) PCR characterization of the insertional mutant *icmB*. Specific PCR amplicons resulting from the combination of primers are shown in the corresponding lanes

2.6 | In vivo virulence assessment

Animal testing was conducted in the experimental hatchery of Aquadvise S.A., located in Quillaipe, Puerto Montt, Chile (ID registration number 104161, Resolución Exenta #1255, Subpesca). Accommodation, handling and killing were strictly performed in accordance with good animal practices as defined by "Ley 20.380, Sobre Protección de Animales" of República de Chile and the "Manual de Aspectos Bioéticos de la Experimentación Animal" from Comisión Nacional de Investigación Científica y Tecnológica, CONICYT, Chile. Animal work was approved by the corresponding ethics and animal welfare committee at Aquadvise S.A.

A cohort of juvenile Atlantic salmons was reared to smoltification and then transferred to sea water. Sanitary conditions were exhaustively verified by means of molecular, microbiological and veterinary assessments. Six tanks each containing 50 naïve fish (*S. salar*, starting average weight 100.6 g) were maintained at 13.5 ± 0.4°C for 17 days at a density of 17.2 kg/m^3 of sea water. Subsequently, fish from two tanks were intraperitoneally injected with 0.1 ml of a cell suspension containing 4.3 × 10^8 CFU/ml of the *icmB* strain. Positive control groups were inoculated with identical volumes of a cell suspension of 1.2 × 10^8 (two tanks) or 2.5 × 10^7 CFU/ml (one tank) of the parental PM15972A1 strain, while the negative control group was injected with saline only. Fish pertaining to distinct experimental groups were maintained in separate tanks for 30 days at 15.0 ± 0.3°C. Random sampling of three fish from one of the high-dose positive controls and one of the *icmB* groups was performed for qPCR and histological studies at 7, 14 and 28 days post-inoculation (dpi). Accumulated mortality, along with abiotic and feed parameters, was recorded on a daily basis. At the end of the assay, fish were killed with a lethal dose of benzocaine. All animals were tested for the presence of *P. salmonis* by qPCR run on kidney samples. DNA samples were extracted and purified as outlined above.

2.7 | Histopathological analysis

Tissue samples from liver and kidney were fixed in buffered formaldehyde 10% at room temperature. Tissue samples were dehydrated with Histo-Clear (National Diagnostics, Atlanta, GA, USA) in an automatic Leica TP1020 tissue processor (Leica Microsystems, Wetzlar, Germany). Samples were embedded in paraffin (Sigma-Aldrich). Sections of 4 μm were cut with a Sakura Accu-Cut SRM-200 rotary microtome (Sakura Finetek Europe B.V, Alphen aan den Rijn, the Netherlands) and mounted on glass slides. Samples were stained with haematoxylin and eosin. Tissues were examined at different levels of magnification using optical microscope Leica DM1000 LED equipped with a Leica ICC50HD digital camera to capture histopathological alterations. All analyses were performed on several tissue sections per animal to ensure consistent reproducibility.

3 | RESULTS

A previous study using drafted genomes suggested that *P. salmonis* PM15972A1 lacks one of the T4SS loci detected in the LF-89 strain (Bohle et al., 2014). We confirmed this fact in a T346Hunter analysis

using the closed version of the sequence. Indeed, *P. salmonis* PM15972A1 bears two chromosomal T4SS loci, each of 21.7 kb in length, which exhibit a high similarity in the number and genetic organization of components as well as their amino acid sequences. They are arbitrarily designated here as T4SS-1 and T4SS-2 (Figure 1a, Table S1). Additionally, a more classic, *virB*-like T4SS of about 9 kb comprising 10 genes was also detected in the PM15972A1 strain (Figure S1). Chromosomal T4SS loci were found to each encode 16 proteins, likely representing the core components of the respective multiprotein structures. BLASTX searches identified highly conserved components between those two T4SS loci: *icmB* (*dotO*) and *icmO*. The *icmB* gene of T4SS-1 (protein ID WP_016209956), which encodes a T4SS-ATPase of unknown function and 996 amino acids in length, is conserved not only among members of *Piscirickettsia* but also in their phylogenetic relatives *Legionella* and *Coxiella* and was selected for further characterization.

To determine the role of one of the T4SS carried by PM15972A1, a mutant on what we predicted to be a structural protein not forming the pore needle was constructed. A suicide plasmid containing an R6K replication origin and a homologous fragment of the *icmB* gene was engineered. The plasmid, a derivative of pJQ200sk, also contained an *oriT* region that allowed its mobilization (Quandt & Hynes, 1993). The plasmid was successfully transferred in mating assays, thereby producing polymyxin/kanamycin-resistant transconjugants, albeit in low numbers. The outlined mutagenesis process can be seen in Figure 1b. The corresponding colonies showed the wild-type grey, rounded morphology in PSA plates. Microscopic examination confirmed the presence of pleomorphic Gram-negative bacteria, which were then PCR-confirmed as *P. salmonis*. Plasmid insertion was verified by PCR using several primer pairs (Table 2). As expected for recombinant clones, homologous recombination occurred only in the target site, interrupting the *icmB*

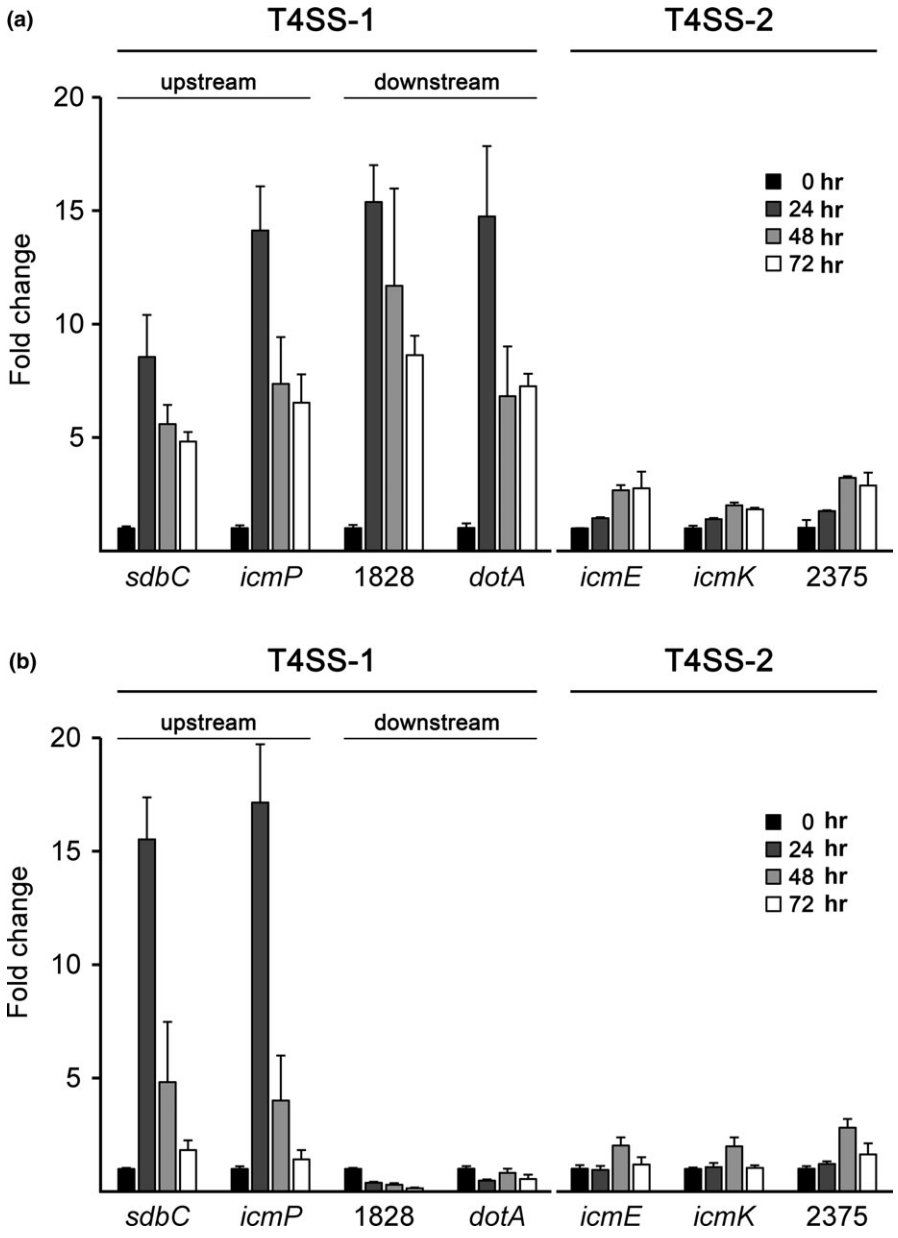

FIGURE 2 In vitro relative gene expression of T4SS genes. SHK-1 cells were infected at a MOI = 10 with (a) Parental PM15972 strain or (b) *icmB* mutant strain. Terms upstream and downstream refer to the position of genes relative to the insertion site in T4SS-1 (see Figure 1a). Similar positions are those for *icmE* and *icmK* (upstream), and KW89_2375 (downstream) relative to the putative homologous insertion site in T4SS-2

gene (Figure 1c). Despite the high similarity at sequence level displayed by both chromosomal T4SS loci, all assayed clones harboured the plasmid at this specific point, that is, at *icmB* of T4SS-1. This finding was confirmed by a PCR-RFLP study (Figure S2). Notably, the resistant phenotype was maintained for at least 100 generations in a proportion of 10^6–10^7 resistant colonies over ~10^8 in total, indicating that the mutant strain is stable. A kinetics study disclosed that the growth of both mutant and parental strains was similar in PSB medium (not shown).

The in vitro behaviour of the *icmB* mutant was tested in two cell lines. A selected colony of the *icmB* mutant strain was further characterized. Colonies from plate cultures were used to seed PSB broth, which served as inoculum to infect CHSE-214 monolayers. A CPE was evident after 5 days in wells infected with the parental strain, leading to the destruction of the monolayer. Strikingly, cells infected with the *icmB* mutant maintained an apparently healthy monolayer until the end of the assay. In fact, in three independent experiments, null CPE was obtained for the *icmB* mutant, in contrast to titres recorded for the parental strain were in average $10^{5.8}$ $TCID_{50}$/ml. In infected SHK-1 cells, parental T4SS-1 genes turned out to be highly expressed at all tested points in time in comparison with those genes related to T4SS-2 (Figure 2a). In contrast, a polar effect on genes located downstream of the insertion in T4SS-1 was evident in the *icmB* mutant (Figure 2b). In that strain, the expression of T4SS-1 genes upstream of the insertion was not disturbed. Genes belonging to T4SS-2 showed a lower expression patterns similar to that registered for the same locus in the parental strain suggesting that it remained unchanged.

To confirm the reduced infectivity of the *icmB* mutant as observed in vitro, groups of fish were inoculated with equivalent doses of mutant or parental strains. The comparison of accumulated mortality curves revealed a dose-effect relationship for fish inoculated with different amounts of parental strain (Figure 3). Remarkably, even though the *icmB* strain was administered in concentrations as high as 10^8 CFU/ml, fish experienced no mortality. This result confirms the attenuation of this mutant. On the other hand, dark pigmentation and lethargy were observed in moribund fish exposed to the parental strain. In this group, gross pathology indicated abdominal distension, ascites and widespread haemorrhages in internal organs (Figure S3). Upon microscopic examination although, an extensive inflammatory response with macrophage and neutrophil infiltration in livers and kidneys of all samples derived from PM15972A1-infected fish became visible, and these constitute findings typical of a productive bacterial infection (Figure S4). Interestingly, in this group, *P. salmonis* could not be detected by means of PCR in hepatic tissue after 7 days, while the pathogen was identified in kidney samples up to 14 dpi (Table 3). On the contrary, histological alterations compatible with a milder inflammatory process were detected at the end of the assay in only a fraction of fish belonging to the *icmB*-group and were mostly limited to hepatic tissues. However, pathogen DNA was barely or not detectable in those samples. Kidney samples obtained from this group tested positive for *P. salmonis* only at the beginning of sampling (Table 3). Furthermore, as expected for fish suffering from septicaemia, Ct values for *P. salmonis* in internal organs of dead fish in the positive control groups amounted to an average of 28.08 (24.75–35.76).

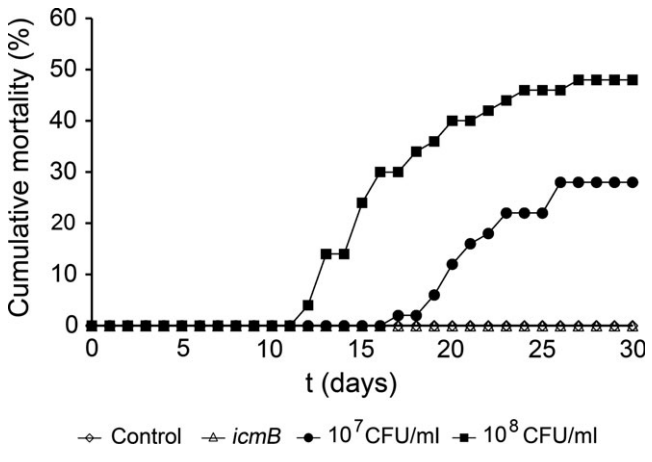

FIGURE 3 Cumulative mortality of challenged fish (*Salmo salar*). Two dilutions of the parental strain were used (~10^7 and ~10^8 CFU/ml, 0.1 ml). Mutant *icmB* was inoculated at ~10^8 CFU/ml using an identical volume. Negative control (Control) was inoculated with 0.1 ml saline

4 | DISCUSSION

This study shows for the first time that the genetic manipulation of *P. salmonis* is feasible. We took advantage of a well-known genetic tool used for *Brucella* mutagenesis, another facultative intracellular pathogen (Scupham & Triplett, 1997). This genetic tool resulted suitable for *P. salmonis* mutagenesis, yielding a stable insertional mutant. Although the function of the *icmB* gene remains elusive, it appears to be essential for *P. salmonis* replication in CHSE-214 cells and to cause disease in Atlantic salmon. This hypothesis is in agreement with other bacterial models where the disruption of a similar T4SS component led to a drastic reduction in their respective virulence (Purcell & Shuman, 1998; Beare et al., 2011). We cannot discard the possibility that the phenotype exhibited by the *icmB* mutant may also be due to the interference with the expression of additional T4SS-1 genes. In this context, it is worth commenting the null complementation observed for the T4SS-1 *icmB* mutant, despite the high similarity shared with the T4SS-2 locus. Sequence homology and

TABLE 3 Average Ct values for hepatic and renal tissues of challenged fish

Strain	T_7	T_{14}	T_{28}
icmB	ND / 35.37 (1/3)	ND / ND	ND / ND
PM15972A1	ND / 35.65 (3/3)	ND / 36.47 (2/3)	ND / ND

Fifty fish were maintained in separate tanks and were inoculated with the mutant or parental strain, respectively. Three fish were chosen randomly for sampling at 7, 14 and 28 dpi. Left values obtained using hepatic samples, right values derived from renal tissue. ND, Non-detectable. Fraction within braces represents the number of PCR-positive samples.

synteny allow to hypothesize that a duplication event could have given rise to the T4SS-2 in *P. salmonis*. Even though a paralogous *icmB* copy was found in the T4SS-2 (85% amino acid identity), the T4SS-1 mutant was not functionally rescued. To explain this, it seems plausible to infer that genes and gene products from both T4SS are not interchangeable, possibly because of a certain genetic drift or divergence between them. Indeed, a *Legionella icmB* mutant was not rescued by complementation with the orthologous *Coxiella* gene, indicating that some interactions between T4SS proteins are highly specific (Zusman, Yerushalmi, & Segal, 2003). Additionally, when looking at bioinformatic findings regarding what we think is the promoter region of the T4SS operons, the expression of T4SS-2 genes might be influenced by a more complex regulatory circuit (Table S2). Taking this into account, we might also speculate that the T4SS-2 plays a role in a different stage of the infection. This hypothesis is supported by the low expression level detected for T4SS-2 genes, which are apparently switched on more than 24 hr post-infection. On the other hand, gene decay has not yet driven the inactivation of components of this locus, suggesting that the T4SS-2 is likely important for *P. salmonis*' intracellular lifestyle. Further research will aid to elucidate whether only one or both T4SS loci harboured in the genome of PM15972A1 are required for the full virulence of this *P. salmonis* EM-90-like strain.

The strain dichotomy hypothesis for *P. salmonis* has recently been proposed, suggesting that the EM-90-like isolates should be grouped as a *S. salar*-specific variant (Saavedra et al., 2017). Thus, it would be reasonable to expect that low doses of pathogen suffice to provoke high mortality rates. However, the observed outcome of an experimental infection of Atlantic salmon with high doses of PM15972A1 is similar to that obtained after the injection of coho salmons with low doses of LF-89-like isolates (House, Bartholomew, Wynton, & Fryer, 1999). This apparent disagreement with an expected low dose requirement for a host-specific strain should be interpreted carefully, as a reliable comparison with results obtained using LF-89-like isolates in other studies cannot be made. In this regard, Rozas-Serri and colleagues reported higher acute mortality in fish intraperitoneally injected with EM-90-like isolates than we observed in those treated with PM15972A1, but clinical findings were similar in both studies. Therefore, the difference in mortality curves can be ascribed to the bacterial isolate employed.

Notwithstanding the level of accumulated mortality obtained (roughly 50%), the attenuation of the *icmB* strain could be shown clearly both in vitro and in vivo. In fact, the mutant did not replicate in CHSE-214 cells and was unable to persist in fish tissues. The clearance of pathogens from liver and kidneys was confirmed by qPCR in different stages of the infection (Table 3). In contrast, all samples obtained from fish that died of the infection with the parental strain contained bacterial loads similar to those detected in animals that succumbed to an infection with the LF-89 strain (Dettleff, Bravo, Patel, & Martinez, 2015). Moreover, a less severe inflammatory response was caused by the mutant strain when compared with the parental PM15972A1. This finding is of particular interest for screenings for potential candidates for a live attenuated vaccine, as appropriate strains should trigger a strong, but limited cellular immune response to elicit long-term protection (Titball, 2008). In a scenario where the inactivated-antigen-based vaccines show a low efficacy to prevent the disease, live vaccine candidates arise as promising alternatives. Upcoming research on *icmB* and additional mutants will allow their prophylactic capabilities to be defined, but also to outline the pathogenic determinants of *P. salmonis*.

In conclusion, we have set up a protocol for the introduction and integration of DNA constructs into *P. salmonis* cells and obtained the first mutant on a T4SS component, thereby demonstrating that the genetic manipulation of this pathogen is feasible. This study provides a reliable methodology to carry out mutagenesis of this fastidious, slow-growing pathogen. Our current research is focused on improving the adapted mutagenesis method to remove selective markers from the mutants, enhance the stability of the mutation and avoid undesirable polar effects. Altogether, our results confirm the essential role of one of the chromosomal T4SS for the virulence of a representative isolate of the EM-90-like strain. Mutations on other hypothetical virulence markers are underway and will help us to get a deeper understanding of the pathogenic arsenal used by this bacterium.

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