A functional ferric uptake regulator (Fur) protein in the fish pathogen *Piscirickettsia salmonis*

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**Summary.** *Piscirickettsia salmonis*, a Gram-negative fastidious facultative intracellular pathogen, is the causative agent of the salmonid rickettsial septicemia (SRS). The *P. salmonis* iron acquisition mechanisms and its molecular regulation are unknown. Iron is an essential element for bacterial pathogenesis. Typically, genes that encode for the iron acquisition machinery are regulated by the ferric uptake regulator (Fur) protein. *P. salmonis fur* sequence database reveals a diversity of fur genes without functional verification. Due to the fastidious nature of this bacterium, we evaluated the functionality of *P. salmonis* fur in the *Salmonella* Δfur heterologous system. Although *P. salmonis* fur gene strongly differed from the common Fur sequences, it restored the regulatory mechanisms of iron acquisition in *Salmonella*. We concluded that *P. salmonis* LF-89 has a conserved functional Fur protein, which reinforces the importance of iron during fish infection. [Int Microbiol 2016; 49-55]

**Keywords:** *Piscirickettsia salmonis* · ferric uptake regulator protein (Fur) · transcriptional regulatory element · iron acquisition · fish pathogens

**Introduction**

*Piscirickettsia salmonis* is the bacterial pathogen with the most significant impact in the Chilean aquaculture industry and the causative agent of the salmonid rickettsial septicemia (SRS) or piscirickettsiosis [25]. Since its first description [4,7], several SRS outbreaks have occurred. Currently, 60% of the Chilean salmon farming centers are positive for *P. salmonis* detection [29]. *P. salmonis* is the only member of the *Piscirickettsia* genus, having the virulent strain LF-89 (ATCC VR-1361) as type strain [11,12]. This Gram-negative intracellular facultative bacteria replicates in infected fish cells, specifically in *P. salmonis* containing vesicles [13,24]. Despite the significant advancement in *P. salmonis* in vitro culture [14,16,36], there is a lack of knowledge about its pathogenic regulatory mechanisms due to its fastidious nature.

Salmon susceptibility to *P. salmonis* infection correlates with the expression of iron withholding genes [20]. For instance, salmon macrophages infected with *P. salmonis* trigger the expression of ferritin and transferrin [21,22]. This indicates that salmonid fish sequester iron from invading pathogens, and *P. salmonis* has unknown mechanisms to get this essential nutrient from the host. Iron is both a nutritional and regulatory element, determining the adaptation of bacteria to host by adjusting the expression of functional genes as well as virulence factors [27]. Bacteria capture iron from host tissues...
but also it must prevent toxic iron overloads [19]. Typically, the ferric uptake regulator (Fur) protein regulates the iron acquisition and also several virulence factors in Gram-negative bacteria [3,8,9,10]. Fur protein is a homodimer of 17-kDa subunits, originally described as a repressor upon interaction with its co-repressor Fe^{2+} [1,34].

Sequence analysis of the *P. salmonis* genome has led to the finding of several putative genes encoding for proteins related to iron metabolism (HemH, TonB, TbpB), siderophore utilization, and the ferric uptake regulator protein Fur [20,35]. Despite the relevance of this bacterial pathogen, and the well-known relation between iron homeostasis and virulence related genes, there is no experimental verification of the functionality of *P. salmonis* fur gene. In addition, there are several different sequences of *P. salmonis* fur gene at the genome databases without functional verification. This implies no information regarding the consensus sequence necessary for DNA-binding and set of genes regulated through this interaction.

The aim of this study was to verify the functionality of *P. salmonis* LF-89 Fur as an iron-dependent regulator and validate the P_m and fur sequence of the *P. salmonis* LF-89 type strain as a conserved regulatory mechanism.

### Materials and methods

**Bacterial strains, media and reagents.** Bacterial strains and plasmids used in this study are described in Table 1. *Piscirickettsia salmonis* LF-89 (ATCC VR-1361) was grown on CHAB monolayer cell culture, inoculated in CHAB agar plates (brain heart infusion supplemented with cysteine 1 g/l and 5% ovine blood) and incubated at 15 °C for 20 days. A single colony was inoculated onto Austral-SRS broth [35,16] and incubated at 15 °C for another 10 days with gentle shaking (100 rpm) until ~10^6 CFU/ml sample buffer [18].

**In silico sequence analysis.** Gene sequences were obtained from the National Center for Biotechnology Information (NCBI). Aligned amino acid sequences of Fur were obtained with EMBL-EBI Clustal Omega [29] and visualized using ESPript v.3.0 [23]. Fur three-dimensional structures were visualized using ESPript v.3.0 [23]. Fur three-dimensional structures were visualized using ESPript v.3.0 [23].

**Cloning procedures.** Genomic DNA extraction was performed using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA). PCR amplifications of *P. salmonis* P_m-fur and P_m-fur were performed using Vent DNA polymerase (New England, Biolabs). The products were purified with a purification kit from Qiagen (Valencia, CA, USA) and submitted for sequencing at Macrogen (Seoul, South Korea). The P_m-fur fragment contains 200 bp upstream of the fur starting codon and PCR amplified using the forward primer 5'-GACTTGTGTTGTGTAGCATGCC-3'. The Plac-fur sequence was PCR amplified using the forward primer 5'-TTTACACTTATGCTTCCGGCTCGTATGTTTAAAGGAGAGGTAACCTATGTCTCAACAAG-3'. The reverse primer utilized was 5'-CAGCCATGCCAATCACAACG-3'. The PCR fragments were cloned into TOPO-TA (Invitrogen). The obtained plasmids were named pEZ287 (P_m) and pEZ283 (P_m) (Table 1).

**Heterologous fur complementation in *Salmonella enterica* subsp. enterica serovar Typhimurium Δfur-44.** The Δfur-44 strain was complemented with *P. salmonis* fur expressed under its promoter and P_m, independently. *Escherichia coli* fur was used as a positive control (pEZ116) [27]. Complemented *S. enterica* subsp. enterica serovar Typhimurium Δfur-44 cells were transformed with each plasmid using the CaCl_2 method [26]. Transformant colonies were used to evaluate *P. salmonis* Fur synthesis and functionality.

**Growth under iron-restricted conditions.** Fifty ml of LB supplemented with iron (FeSO_4 μM) and LB supplemented with 2, 2-dipyridyl (150 μM and 250 μM) were inoculated with 50 μl of early log-phase culture. The cultures were incubated at 37°C with aeration (180 rpm). Bacterial growth was monitored by optical density (A_600). Samples were measured in triplicate and identical experiments were performed twice.

**SDS-PAGE and western blotting.** Synthesis of *P. salmonis* Fur in *Salmonella enterica* subsp. enterica serovar Typhimurium Δfur-44 harboring pE2283, pEZ287 or pEZ116 was evaluated by Western blot. One ml of middle log culture, grown at 37°C with aeration (180 rpm), were harvested. After centrifugation, bacterial cells were resuspended in 100 ml sample buffer [18] and heat denatured for 10 min. Total protein were separated in 12% SDS-PAGE transferred onto a nitrocellulose membrane and blocked using a fat-free milk solution (5% wt/vol) in phosphate-buffered saline with 0.05% Tween 20 (PBS-T). The membranes were incubated overnight at 4°C with 1:10,000 dilution of anti-Fur primary rabbit polyclonal antibody [19]. After washing with PBS-T, the membranes were incubated with a 1:10,000 dilution of the secondary antibody (alkaline phosphatase-conjugated anti-rabbit im-

munoglobulin G, Sigma). Immune reactive proteins were detected using a chromogenic substrate for alkaline phosphatase (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium). A primary rabbit serum against GAPDH was used as control.

### Detection of secreted siderophores.
Siderophore synthesis was evaluated in chromozaurol S (CAS) agar plates [6,28]. One ml of an overnight culture of *S. enterica* subsp. *enterica* serovar Typhimurium χ11143 Δfur-44 complemented strain was pelleted, washed two times and resuspended in 50 μl of PBS. A 10-μl aliquot of each bacterial culture was spotted on CAS agar plates, and incubated overnight at 37 °C. Siderophore production was visualized as a yellow-orange halo around the bacterial growth.

### Detection of iron-free siderophores by thin layer chromatography (TLC).
*Salmonella enterica* iron-free siderophores were obtained by the following method. Bacterial cultures grown overnight in 10 ml of LB broth for 18–24 h at 37 °C (OD₅₄₀ of 1.0) supplemented with FeSO₄ (150 μM) or with 2, 2′-Dipyridyl (150 μM) were harvested by centrifugation at 5,000 ×g for 10 min. The supernatants were passed through 0.2 µm pore-size membrane filter to remove residual bacteria, acidified with 50 N HCl and extracted twice with 4 ml of ethyl-acetate to obtain catechol siderophores [17]. The aqueous phase was dried with gaseous N₂ and extracted twice with 4 ml of ethyl-acetate to obtain catechol siderophores [17]. The aqueous phase was dried with gaseous N₂ and extracted twice with 4 ml of ethyl-acetate to obtain catechol sideropheres [17].

### Outer membrane protein analysis.
Sarkosyl-insoluble outer membrane proteins (OMPs) were obtained as previously described [5]. OMP proteins were isolated from *S. enterica* subsp. *enterica* serovar Typhimurium Δfur-44 grown in iron-replete conditions (LB supplemented with 150 μM FeSO₄) and iron-regulated outer membrane proteins (IROMPs) were isolated from *Salmonella enterica* subsp. *enterica* serovar Typhimurium Δfur-44 grown in LB supplemented with 2′′-dipyridyl (150 μM) (iron-limited conditions). The total proteins were normalized to 25 μg/μl by using the nano Genova spectrophotometer (Jenway) and separated by 10% (wt/vol) SDS-PAGE. Coomassie blue staining was performed to visualize proteins.

## Results

### *Piscirickettsia salmonis* fur sequence analysis.
*Piscirickettsia salmonis* fur gene has a 444 bp length, similar to *S. Typhimurium* (453 bp), *E. coli* (447 bp), *E. ictaluri* (450 bp) and *A. salmonicida* (429 bp). However, the G+C content of *P. salmonis* fur (40.7%) is lower compared to *S. Typhimurium* (47.2%), *E. coli* (47.9%), *E. ictaluri* (53.8%) and *A. salmonicida* (54.1%). Also, *P. salmonis* fur gene has a low percentage of identity compared to *E. coli* and *Salmonella fur* genes, with 29% and 52% respectively. The low G+C content of *P. salmonis* fur is shared with phylogenetic relatives from the genera *Coxiella* and *Legionella* (Fig. 1). *Piscirickettsia salmonis* Fur contains 147 amino acid residues, which 71 residues are identical to *E. ictaluri* (149 aa) and *S. enterica* subsp. *enterica* serovar Typhimurium (150 aa) (Fig. 2A). In terms of structure, we found that *P. salmonis* Fur has two putative functional domains: a DNA-binding domain in the N-terminal region, and a C-terminal domain involved in dimerization (Fig. 2B). According to its metalloprotein nature, the amino acid residues related to Fe²⁺ and Zn²⁺ binding pockets are perfectly aligned in all the compared sequences (Fig. 2A). The differences in the amino acid residues between *P. salmonis* Fur and

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<th>Table 1. Strains and plasmids used in this study</th>
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<td><strong>Strains</strong></td>
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<td><em>Piscirickettsia salmonis</em> LF-89</td>
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<tr>
<td><em>Escherichia coli</em> DH5α</td>
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<tr>
<td><em>Salmonella enterica</em> serovar Typhimurium c3761</td>
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<td><em>Salmonella enterica</em> serovar Typhimurium c11143</td>
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| **Plasmids** | Relevant characteristics | Reference |
|-------------|
| TOPO vector | 3.9 kb, pUC ori, Km, Amp′ | This study |
| pEZ283 | 4.3 kb, pUC ori, Km, Amp′, P₅ fur (P. salmonis fur), TOPO | Promega |
| pEZ287 | 4.3 kb, pUC ori, Km, Amp′, P₅ fur (P. salmonis fur), TOPO | Promega |
| pEZ116 | 4.6 kb, P₅ fur (E. ictaluri fur), Cm, pSC101 ori, pACYC184 | This study |

other Fur proteins have no evident impact on the secondary and tertiary structure (Figs. 2A-2B).

**Piscirickettsia salmonis fur gene cloning and protein expression.** *Piscirickettsia salmonis fur* was expressed in χ11143 Δfur-44 under control of its promoter (Fig. 3A) and under control of P lac promoter. *P. salmonis* Fur showed a molecular weight of 16 kDa, similar to the 16.9 kDa predicted *in silico* (Fig. 3A).

**Detection of siderophores synthesis regulated by Piscirickettsia salmonis Fur.** *Salmonella* Typhimurium Δfur-44 displays a constitutive secretion of siderophores, forming an orange-yellow halo around the bacterial colony on CAS agar plates [28]. Complementation with a functional Fur protein restores the iron-mediated gene repression, precluding the constitutive synthesis of siderophores, as observed in the strain transformed with pEZ116 (positive control). *S. Typhimurium* Δfur-44 transformed with pEZ283 (*P. salmonis*, P _ω-fur_) or pEZ287 (*P. salmonis*, P _α-fur_) restores the iron-mediated gene repression, evidencing that *P. salmonis fur* gene encodes a functional Fur (Fig. 3B).

Iron-free catechol siderophore synthesis in *S. Typhimurium* Δfur-44 complemented with *P. salmonis* Fur was evaluated by TLC analysis. All bacterial cultures grown under iron-deprived conditions were able to synthesize siderophores. In contrast, when the bacterial cultures were supplemented with iron (FeSO₄, 150 μM) the wild type and the complemented Δfur-44 strains did not synthesize siderophores (Fig. 3C). In contrast, the non-complemented Δfur-44 strain synthesized siderophores constitutively (Fig. 3C).

**Outer membrane protein profile.** Wild-type *S. Typhimurium* expressed IROMPs (iron regulated outer membrane proteins) only under iron-deprived condition. In contrast, Δfur-44 strain showed a constitutive IROMPs expression (Fig. 3D). IROMPs regulation was restored in *S. Typhimurium* Δfur-44 complemented with *P. salmonis fur* gene (Fig. 3D).

**Growth under iron-restricted conditions.** Growth curves were determined in LB, LB supplemented with 150 μM FeSO₄ and LB supplemented with 150 μM and 250 μM of 2,2′-dipyridyl at 37 °C. As shown in Fig. 3E, *S. enterica subsp. enterica* serovar Typhimurium Δfur-44 complemented with *P. salmonis P _fur_* (pEZ287) showed a longer generation time (Fig. 3E).

**Discussion**

The NCBI and UNIPROT databases have a diversity of *P. salmonis fur* genes without functional validation. Here, we analyzed the fur genes of the virulent *P. salmonis* LF-89 type strain. The phylogenetic analysis showed that *P. salmonis* Fur grouped with *Francisella*, *Coxiella* and *Legionella*, which are closely related genera (Fig. 1). Despite the nucleotide differences of *P. salmonis fur* genes, the structure of *P. salmonis* Fur protein was very similar to other Gram-negative Fur proteins (Fig. 2). The structural identity and amino acid similarity at the active sites of Fur, supported the *P. salmonis* Fur functionality. For instance, the amino acids related to Zn²⁺ and Fe²⁺ binding were conserved, in agreement with the iron-dependent regulation found in the complementation assays. Sequence analysis and comparisons showed that *P. salmonis* Fur was prone to dimerization since residues required for dimerization and meta binding were present in *P. salmonis* Fur (Fig. 2B). Cysteines in positions 92 and 95 are essential for the activity of Fur proteins family [6]. These cysteine residues are found in a CXYCG motif [2], which is also present in *P. salmonis* Fur (Fig. 2A).
**Piscirickettsia salmonis** fur functionality was confirmed in *S. Typhimurium Δfur-44*, revealing that, despite the differences in G+C content of *P. salmonis* genome, the predicted *fur* promoter (with standard -10 and -35 sequences) was functional when tested in *S. Typhimurium*. However, the expression of *P. salmonis* fur in *S. Typhimurium Δfur-44* under control of its promoter caused a longer generation time, in contrast to *P. salmonis* fur under *P* _lac_ control (Fig. 3E). These results suggest that *P. salmonis* Fur might recognize similar consensus sequences than *Salmonella* and *E. coli* Fur. However, the promotor of *P. salmonis* fur gene seems to have a pleiotropic effect on *S. Typhimurium* growth (Fig. 3E).

The fastidious nature of *P. salmonis* and its facultative intracellular life cycle precluded the study of basic aspects of its biology, however utilization of heterologous systems, like *S. Typhimurium*, can be utilized to understand virulent mechanisms of *P. salmonis*.

In summary, we identified the *fur* gene of *P. salmonis* and demonstrated the functionality of the encoded protein in a heterologous complementation system. The analysis also showed that *P. salmonis* Fur transcriptional regulation was iron-dependent. This study provided a useful starting point for the analysis of the Fur-regulated genes in *P. salmonis*, the elucidation of its Fur-box sequence and the unveiling of novel mechanisms of bacterial infection in fish.

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**Competing interests:** None declared.

**References**


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**Fig. 2. Piscirickettsia salmonis** Fur alignment and structure. (A) Fur alignment and secondary structure. The secondary structure is displayed as spirals (representing α-helix) and arrows (representing β-sheets). The structure on the top of the alignment is *P. salmonis* Fur protein and the structure at the bottom is from *Salmonella enterica* subsp. *enterica* serovar Typhimurium Fur protein. DNA-binding protein domain is marked with black stars, and the dimerization domains are double underlined. The white and black circles indicate the amino acid residues related with Zn²⁺ or Fe²⁺ binding pocket respectively. Black triangles indicate the cysteine residues related to *Escherichia coli* Fe²⁺ and Zn²⁺ binding pockets. (B) Three-dimensional structure predictions of Fur proteins.
Fig. 3. Fur protein expression and complementation assays. (A) Synthesis of Fur in Salmonella enterica subsp. enterica serovar Typhimurium Δfur-44 verified by western blot analysis. GAPDH was used as control; pEZ116 (Escherichia ictaluri Fur); pEZ287 (Piscirickettsia salmonis Fur). (B) Detection of siderophore in CAS agar plates. (C) Detection of secreted siderophores in S. enterica subsp. enterica serovar Typhimurium grown under iron-rich (+) and iron-limited (–) conditions by TLC. (D) Outer membrane protein profile of S. enterica subsp. enterica serovar Typhimurium grown under iron supplemented (+) or iron deprived (–) conditions. The arrowheads indicate Fur-regulated IROMP proteins. (E) Growth of S. enterica subsp. enterica serovar Typhimurium Δfur-44 growth at 37 °C with aeration (180 rpm).


