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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* Δ*fur* 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

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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_{fur} 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 CHSE-214 monolayer cell culture, inoculated in CHAB agar plates (brain heart infusion supplemented with L-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 ~10^8 cells/ml (OD_{600} ~1.0). *Escherichia coli* DH5α and *Salmonella enterica* serovar Typhimurium χ11143 Δ*fur*-44 strain were grown at 37 °C in LB broth (tryptone 10 g/l; yeast extract 5 g/l and NaCl 5 g/l) or LB agar (tryptone 10 g/l; yeast extract 5 g/l; NaCl 5 g/l and 1.5% agar). Nutrient media were supplemented with ampicillin (Amp, 100 μg/ml), kanamycin (Km, 50 μg/ml) and/or chloramphenicol (Cm, 25 μg/ml) as indicated in Table 1.

***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 predicted by PSI-BLAST alignment and HHpred [31]. *P. salmonis fur* genetic context was analyzed with Softberry web-base software, using the Bprom algorithm [32].

The evolutionary history of *P. salmonis* Fur was inferred by using the maximum likelihood method based on the JTT matrix-based model [15]. An initial tree for the heuristic search was obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [33].

A total of 24 Fur proteins were analyzed, comprising those of *Aeromonas salmonicida* subsp. *salmonicida* A449 (ABO90840.1), *Bacillus cereus* (WP_002010356.1), *Coxiella burnetii* RSA 493 (NC_002971.3), *Cycloclasticus* sp. PY97M (EPD14371.1), *Edwardsiella ictaluri* 93-146 (NC_012779.2), *Edwardsiella tarda* strain PPD 130/91 (AEO72442.1), *Escherichia coli* str. K-12 substr. MG1655 (NP_415209.1), *Flavobacterium columnare* ATCC 49512 (AEW85616.1), *Flavobacterium psychrophilum* JIP02/86 (CAL43969.1), *Francisella tularensis* subsp. *tularensis* SCHU S4 (YP_169106.1), *Haemophilus influenzae* Rd KW20 (NP_438359.1), *Hydrogenovibrio marinus* (WP_029909427.1), *Legionella pneumophila* (NZ_CCZV01000002.1), *Methylophaga nitratireducenticrescens* (AFI85361.1), *Piscirickettsia salmonis* LF-89 = ATCC VR-1361 (KJ804204.1), *Pseudomonas aeruginosa* PAO1 (NP_253452.1), *Pseudomonas putida* (WP_003249922.1), *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (NP_459678.1), *Staphylococcus aureus* subsp. *aureus* NCTC 8325 (YP_500494.1), *Streptococcus mutans* UA159 (NP_721026.1), *Thioalkalimicrobium cyclicum* ALM1 (AEG31828.1), *Thiomicrospira chilensis* (WP_028486797.1), *Vibrio cholerae* (AAA27519.1), and *Yersinia pestis* strain KIM5 (WP_002210357.1).

Cloning procedures. Genomic DNA extraction was performed using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA). PCR amplifications of *P. salmonis* P_{fur}-*fur* and P_{lac}-*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_{fur}-*fur* fragment contains 200 bp upstream of the *fur* starting codon and PCR amplified using the forward primer 5′-GACTTGTGTTTGTGTCATGCC-3′. The Plac-fur sequence was PCR amplified using the forward primer5′-TTTACACTTTATGCTTCC GGCTCGTATGTTTAAAGGAGAGGTAACCTATGTCTCAACAAG-3′. The reverse primer utilized was 5′-CAGCCATGCCAATCACAACG-3′. The PCR fragments were cloned into TOPO-TA (Invitrogene). The obtained plasmids were named pEZ287 (P_{fur}) and pEZ283 (P_{lac}) (Table 1).

Heterologous *fur* complementation in χ11143 *Salmonella enterica* subsp. *enterica* serovar Typhimurium Δ*fur*-44. The Δ*fur*-44 strain was complemented with *P. salmonis fur* expressed under its promoter and P_{lac}, independently. *Edwardsiella ictaluri fur* gene was used as positive control (pEZ116) [27]. Complemented *S. enterica* subsp. *enterica* serovar Typhimurium Δ*fur*-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 37ºC with aeration (180 rpm). Bacterial growth was monitored by optical density (A_{600nm}). Samples were measured in triplicate and identical experiments were performed twice.

SDS-PAGE and western blotting. Synthesis of *P. salmonis* Fur in χ11143 *S. enterica* subsp. *enterica* serovar Typhimurium Δ*fur*-44 harboring pEZ283, 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 μl 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-

Table 1. Strains and plasmids used in this study

	Relevant characteristics	Reference
Strains		
Piscirickettsia salmonis LF-89	ATCC VR 1361 Type culture strain	ATCC; [13]
Escherichia coli DH5α	F–Φ80*lacZ*ΔM15 Δ*(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44* λ– *thi-1 gyrA96 relA1*	Invitrogen
Salmonella enterica serovar Typhimurium c3761	UK-1; wild-type	[27]
Salmonella enterica serovar Typhimurium c11143	UK-1; Δ*fur44*	[27]
Plasmids		
TOPO vector	3.9 kb, pUC *ori*, Km^r, Amp^r	Promega
pEZ283	4.3 kb, pUC *ori*, Km^r, Amp^r, P_{lac}–*fur (P. salmonis fur)*, TOPO	This study
pEZ287	4.3 kb, pUC *ori*, Km^r, Amp^r, P_{fur}–*fur (P. salmonis fur),* TOPO	This study
pEZ116	4.6 kb, P_{fur}-*fur (E. ictaluri fur),* Cm^r, pSC101*ori*, pACYC184	[27]

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 chromoazurol S (CAS) agar plates [6,28]. One ml of an overnight culture of *S. enterica* subsp. *enterica* serovar Typhimurium χ11143 Δ*fur*-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 (OD_{600} of 1.0) supplemented with $FeSO_4$ (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 µl of 10 N HCl and extracted twice with 4 ml of ethyl-acetate to obtain catechol siderophores [17]. The aqueous phase was dried with gaseous N_2 and resuspended in 80 µl of methanol. Twenty µl were spotted onto 225-µm-layer (20 × 20 cm) TLC Silica gel 60 F_{254} aluminum sheets and the chromatography was developed with benzene-glacial acetic acid-water (125:72:3, vol./vol./vol.) in a closed chamber. The aluminum sheets were removed, dried and immersed in 0.1% $FeCl_3$ to visualize the sideropheres.

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 Δ*fur*-44 grown in iron-replete conditions (LB supplemented with 150 µM $FeSO_4$) and iron-regulated outer membrane proteins (IROMPs) were isolated from *Salmonella enterica* subsp. *enterica* serovar Typhimurium Δ*fur*-44 grown in LB supplemented with 2′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 Fe^{2+} and Zn^{2+} binding pockets are perfectly aligned in all the compared sequences (Fig. 2A). The differences in the amino acid residues between *P. salmonis* Fur and

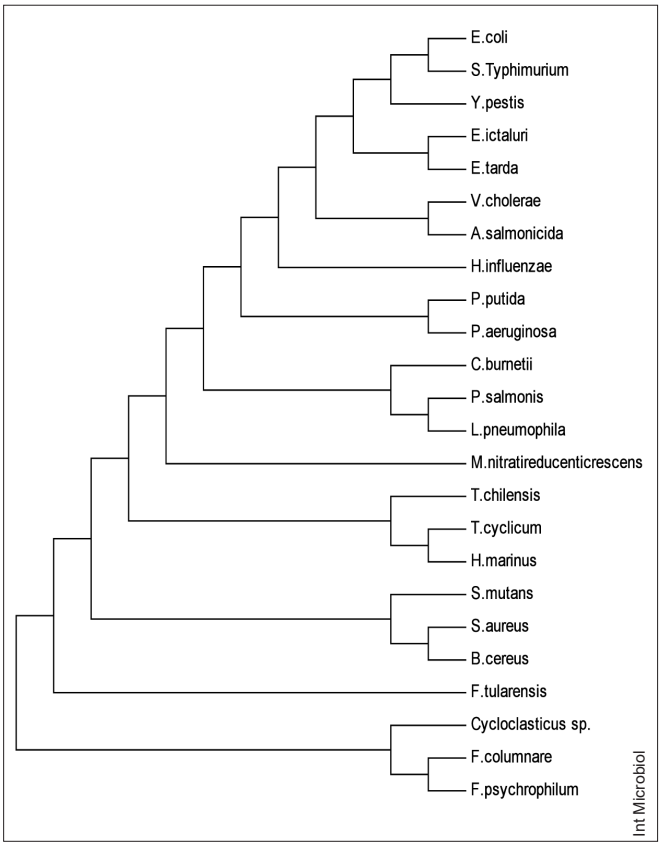

Fig. 1. Evolutionary relationships based on Fur protein sequence.

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 Δ*fur*-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 Δ*fur*-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 Δ*fur*-44 transformed with pEZ283 (*P. salmonis*, P_{lac}-*fur*) or pEZ287 (*P. salmonis*, P_{fur}-*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 Δ*fur*-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 ($FeSO_4$, 150 μM) the wild type and the complemented Δ*fur*-44 strains did not synthesize siderophores (Fig. 3C). In contrast, the non-complemented Δ*fur*-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, Δ*fur*-44 strain showed a constitutive IROMPs expression (Fig. 3D). IROMPs regulation was restored in *S.* Typhimurium Δ*fur*-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 $FeSO_4$ 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 Δ*fur*-44 complemented with *P. salmonis* P_{fur}-*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 Zn^{2+} and Fe^{2+} 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).

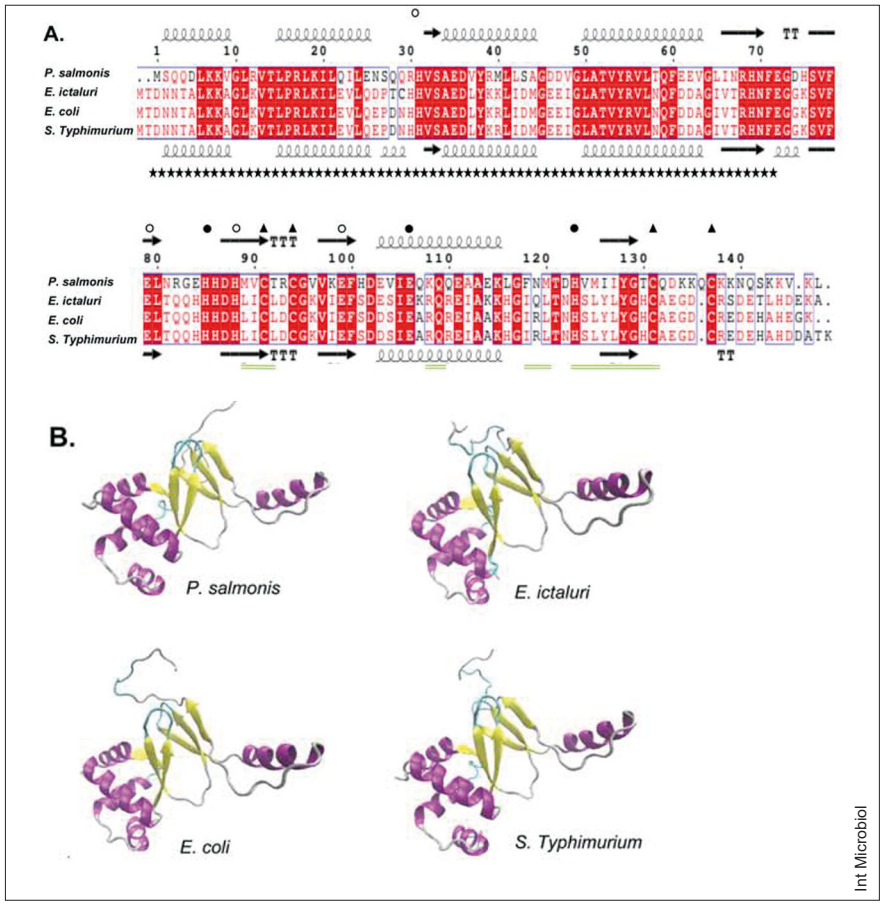

Fig. 2. *Piscirickettsia salmonis* Fur alignment and structure. (**A**) Fur alignment and secondary structure. The secondary structure is displayed as spirals (representing α-helix) and arrows (representing β-sheets). The structure on the top of the alignment is *P. salmonis* Fur protein and the structure 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 Zn^{2+} or Fe^{2+} binding pocket respectively. Black triangles indicate the cysteine residues related to *Escherichia coli* Fe^{2+} and Zn^{2+} binding pockets. (**B**) Three dimensional structure predictions of Fur proteins.

Piscirickettsia salmonis fur functionality was confirmed in *S.* Typhimurium Δ*fur*-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 Δ*fur*-44. However, the expression of *P. salmonis fur* in *S.* Typhimurium Δ*fur*-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.

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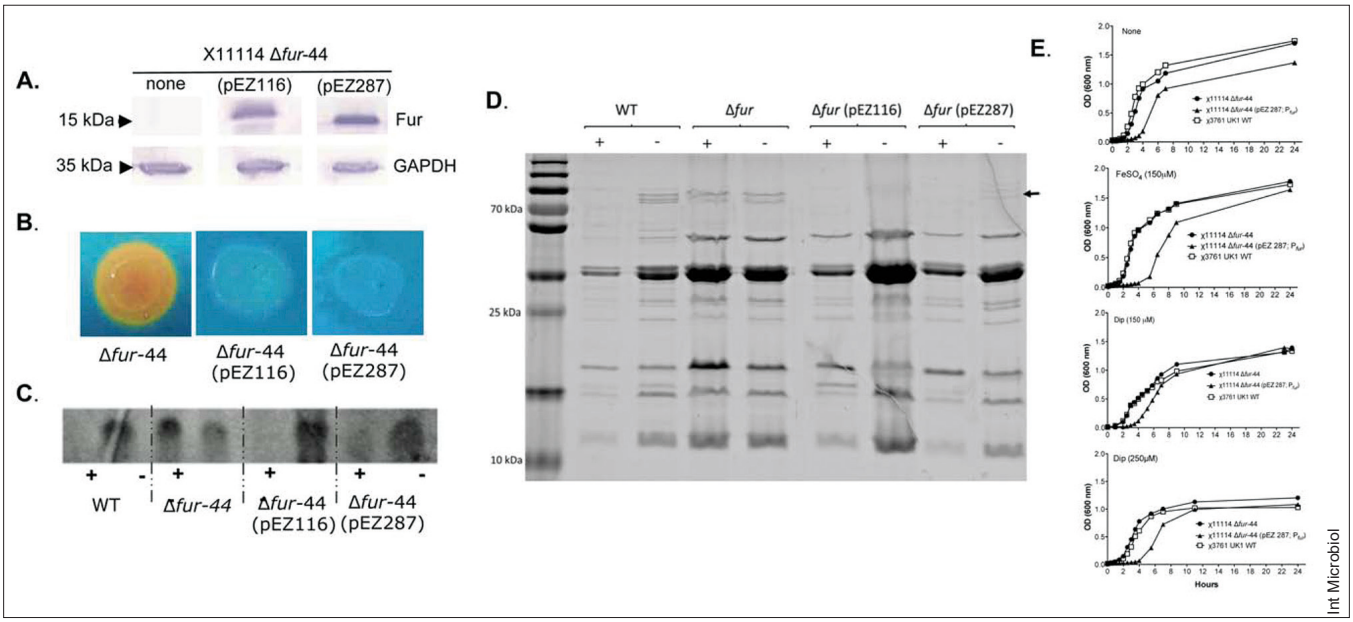

Fig. 3. Fur protein expression and complementation assays. (**A**) Synthesis of Fur in *Salmonella enterica* subsp. *enterica* serovar Typhimurium Δ*fur*-44 verified by western blot analysis. GAPDH was used as control; pEZ116 (*Escherichia ictaluri* Fur); pEZ287 (*Piscirickettsia salmonis* Fur). (**B**) 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 arrowhead indicate Fur-regulated IROMP proteins. (**E**) Growth of *S. enterica* subsp. *enterica* serovar Typhimurium Δ*fur*-44 growth at 37 °C with aireation (180 rpm).

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