Identification of an iron acquisition machinery in *Flavobacterium columnare*

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ABSTRACT: *Flavobacterium columnare*, a fastidious Gram-negative pathogen and the causative agent of columnaris disease, is one of the most harmful pathogens in the freshwater fish-farming industry. Nevertheless the virulence mechanisms of *F. columnare* are not well understood. Bacterial iron uptake from the host during infection is an important mechanism of virulence. Here we identified and analyzed part of the iron uptake machinery of *F. columnare*. Under iron-limited conditions during *in vitro* growth, synthesis of an outer membrane protein of ~86 kDa was upregulated. This protein was identified as a TonB-dependent ferrichrome-iron receptor precursor (FhuA). Synthesis of siderophores in *F. columnare* was corroborated by chrome azurol S assays. A putative ferric uptake regulator (Fur) protein was also identified in the *F. columnare* genome. Structural analysis of the *F. columnare* Fur protein revealed that it was similar to Fur proteins involved in iron uptake regulation of other bacteria. Furthermore, *Salmonella enterica* serovar Typhimurium (S. Typhimurium) Δfur mutants were partially complemented by the *F. columnare* fur gene. We conclude that a siderophore-mediated iron uptake system exists in *F. columnare*, and fur from *F. columnare* could partially complement S. Typhimurium Δfur mutant.

KEY WORDS: *Flavobacterium columnare* · Siderophore · FhuA · Fur · Iron uptake

INTRODUCTION

*Flavobacterium columnare* is a rod-shape, yellow-pigmented Gram-negative bacterium that causes columnaris disease in a wide variety of freshwater fish globally (Bullock et al. 1986). Outbreaks of columnaris disease have been reported worldwide in the fish aquaculture industry; affected species include channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, goldfish *C. auratus*, rainbow trout *Oncorhynchus mykiss*, Japanese eel *Anguilla japonica*, brown trout *Salmo trutta*, and tilapia *Oreochromis* spp.) (Anderson & Conroy 1969). Currently, *F. columnare* is the second most prevalent bacterial disease in channel catfish, which is the dominant aquaculture species in the US (USDA 2003). However, despite the importance of this pathogen, *F. columnare* virulence mechanisms are still poorly understood.

Iron is an essential element in a variety of metabolic cellular pathways. The bioavailability of iron in the biological fluids within the vertebrate host is extremely limited because of sequestration by high-affinity iron-binding proteins, such as transferrin in serum and extracellular fluids, lactoferrin on mucosal surfaces, and ovotransferrin in eggs (Payne 1994). Consequently, the ability to obtain iron *in vivo* is considered an important determinant in bacterial pathogenicity and virulence; in fact, low levels of iron are a signal for the expression of several virulence genes (Møller et al. 2005). To obtain this bound iron, most pathogenic bacteria have developed iron uptake systems that...
usually involve 2 components, viz. low-molecular-weight siderophores that are synthesized and secreted by bacteria to chelate ferric iron and subsequently transfer it to the pathogen, and iron-regulated outer membrane proteins (iROMPs), which are high-affinity receptors of the iron-siderophore complexes (Crosa et al. 2004, Avendaño-Herrera et al. 2005).

In Gram-negative bacteria, gene regulation of siderophore utilization, and iron homeostasis in general, is mediated at the transcriptional level by the ferric uptake repressor (Fur; Han ties 2001). As Escherichia coli Fur is among the best studied, numerous studies have detailed Fur regulation of iron uptake systems in this model organism, including the ferrichrome-iron receptor (FhuA; Carpenter et al. 2009). The Fur protein of E. coli is a 17 kDa polypeptide (Bagg & Neilands 1987a), which acts as a transcriptional repressor of iron-regulated promoters by virtue of its Fe$^{3+}$-dependent DNA binding activity (Escolar et al. 1997). Homologues of the fur gene have been described in numerous Gram-negative bacteria, including several important human pathogens like Yersinia (Staggs & Perry 1991), Salmonella (Ernst et al. 1978), Vibrio (Litwin et al. 1992, Litwin & Calderwood 1993, Yamamoto et al. 1997), Pseudomonas (Prince et al. 1993), Helicobacter pylori (Bereswill et al. 1998), Bordetella (Brickman & Armstrong 1995), Campylobacter (Wooldridge et al. 1994), Acinetobacter baumannii (Daniel et al. 1999), Legionella (Hickey & Cianciotto 1994), Neisseria (Berish et al. 1993), and Haemophilus (Biegel Carson et al. 1996), and even in plant pathogens like Erwinia chrysanthemi (Franza et al. 1999). Most of these homologues are able to complement E. coli fur mutants, suggesting that the molecular mechanisms that control transcriptional regulation by iron are shared by many microorganisms (Escolar et al. 1999).

Here, we describe the iron uptake machinery in Flavobacterium columnare under iron-limited conditions, which induce the upregulation of the TonB-dependent protein FhuA (ferrichrome-iron receptor precursor), and the presence of siderophores and the Fur protein. The objective of this study was to obtain a first insight into F. columnare iron assimilation mechanisms to benefit understanding virulence in this bacterial species.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and reagents**

The bacterial strains and plasmids are listed in Table 1. Bacteriological media and components were acquired from Difco, and antibiotics and reagents were obtained from Sigma. *Flavobacterium columnare* growth medium (FCGM; tryptone 8 g; yeast extract 0.8 g; MgSO$_4$·7 H$_2$O 1 g; CaCl$_2$·2H$_2$O 0.74 g; NaCl 5 g; sodium citrate 1.5 g; ddH$_2$O 1 l) (Farmer 2004) was used for liquid growth, and tryptone yeast extract salts (TYES, tryptone 4 g; yeast extract 0.4 g; MgSO$_4$·7H$_2$O 0.5 g; CaCl$_2$·2H$_2$O 0.5 g; ddH$_2$O 1 l) (Cain & Lafrentz 2007) supplemented with 1% agar were used for plate growth of *F. columnare* ATCC 23463 at 28°C. *Salmonella Typhimurium* and *E. coli* strains were routinely grown in LB broth (tryptone, 10 g; yeast extract 5 g; NaCl, 10 g; glucose 1 g; ddH$_2$O, 1 l) (Bertani 1951) at 37°C. When required, the media were supplemented with 1.5% agar, ampicillin (Amp; 100 µg ml$^{-1}$), gentamycin (Gm; 20 µg ml$^{-1}$), or kanamycin (Km; 50 µg ml$^{-1}$).

**Growth under iron-restricted conditions**

The minimum inhibitory concentration of 2, 2-dipyridyl (Sigma-Aldrich) was determined in FCGM broth sup-

### Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tr>
<td><em>Escherichia coli</em></td>
<td></td>
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<tr>
<td>Top10</td>
<td>F$^{-}$, mcrA Δ(mrr-hsdRMS-mcrBC)</td>
<td>Invitrogen</td>
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<td></td>
<td>ε80lacZΔM15 ΔlacX74 nupG, recA1 araD139 Δ ara-leu7697, galE15 galK16 rpsL (Str$^R$) endA1 λ$^−$</td>
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<tr>
<td><em>Salmonella enterica</em></td>
<td></td>
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<tr>
<td>χ3761</td>
<td>S. Typhimurium UK-1</td>
<td>Hassan &amp; Curtiss (1990)</td>
</tr>
<tr>
<td>χ11143</td>
<td>S. Typhimurium UK-1, Δfur44</td>
<td>Santander et al. (2012)</td>
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<tr>
<td><em>Flavobacterium columnare</em></td>
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<tr>
<td><em>Plasmids</em></td>
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<tr>
<td>pEZ151</td>
<td>4065 bp, pSC101 ori, Gm GFP, pEZ151, Gm, pSC101 ori, Gm, F. columnare p$_{fur}$-fur gene</td>
<td>Santander et al. (2011)</td>
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<td>pEZ161</td>
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<td>This study</td>
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plemented with different concentrations of 2,2'-dipyridyl (150, 100, 75, 50, and 25 µM). We determined that the minimal inhibitory concentration of 2,2'-dipyridyl was 100 µM. FCGM, FCGM supplemented with 2, 2-dipyridyl (100 µM), and FCGM supplemented with FeSO₄ (40 µM; 195 ml) were inoculated with 5 ml of early log-phase culture. The cultures were incubated at 28°C with aeration (200 rpm). Bacterial growth was monitored by measuring the optical density (A₆₀₀ nm). Samples were measured from triplicate cultures per medium, and identical experiments were performed twice. Data from one experiment were used to describe the results.

Outer membrane protein (OMP) analysis

The OMPs of *Flavobacterium columnare* were prepared according to the method of Xie et al. (2004) with modifications (Santander et al. 2011). Briefly, 25 ml of FCGM broth, non-supplemented or supplemented with 2,2'-dipyridyl (100 µM) or FeSO₄ (40 µM), were inoculated with 5 ml of late log phase *F. columnare* culture (36 h). The liquid cultures were grown at 28°C with aeration (200 rpm) until they reached the stationary phase. The cells were collected by centrifugation at 10 000 × g (10 min at 4°C). *F. columnare* cells resuspended in Tris-OH/EDTA buffer pH 7.4 (20 mM Tris-OH; 1 mM EDTA; 1 mM PMSF) were lysed by passing the culture twice through a French press (Thermo Electron Corporation) at 10 000 psi (6.9 MPa; 40K cell). The lysed cell preparation was centrifuged at 7000 × g (10 min at 4°C) to remove cell debris and unlysed cells. OMPs were prepared as described previously (Puente et al. 1995), except 0.5 % (w/v) Sarkosyl was used instead of Triton X-100. The pellet was resuspended in 10 ml of 0.5 % (w/v) Sarkosyl and incubated on ice for 1 h. The suspension was then centrifuged at 16 000 × g (1 h at 4°C) to obtain the stationary phase. The cells were collected by centrifugation at 10 000 × g (10 min at 4°C). *F. columnare* cells resuspended in Tris-OH/EDTA buffer pH 7.4 (20 mM Tris-OH; 1 mM EDTA; 1 mM PMSF) were lysed by passing the culture twice through a French press (Thermo Electron Corporation) at 10 000 psi (6.9 MPa; 40K cell). The lysed cell preparation was centrifuged at 7000 × g (10 min at 4°C) to remove cell debris and unlysed cells. OMPs were prepared as described previously (Puente et al. 1995), except 0.5 % (w/v) Sarkosyl was used instead of Triton X-100. The pellet was resuspended in 10 ml of 0.5 % (w/v) Sarkosyl and incubated on ice for 1 h. The suspension was then centrifuged at 16 000 × g (1 h at 4°C) to obtain the OMPs. The total proteins were normalized to 25 µg µl⁻¹ by using the nanodrop spectrophotometer (ND-1000, NanoDrop) and separated by 8% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Coomassie blue staining was performed to visualize proteins (Ames 1974).

OMP identification

Two protein bands of ~86 kDa synthesized in the iron-limited condition were excised from the SDS-PAGE gel separately for peptide sequencing analysis (performed by ProtTech Inc, Norristown, PA, USA) by using the NanoLC-MS/MS peptide sequencing technology. The protein gel band was destained, cleaned, and digested in-gel with sequencing grade modified trypsin (Promega). The resulting peptide mixture was analyzed by an LC-MS/MS system, using high performance liquid chromatography (HPLC) with a 75 µm inner diameter reverse phase C18 column coupled to an ion trap mass spectrometer (Thermo). The mass spectrometric data were used to search the non-redundant protein database in the *Flavobacterium columnare* open reading frame (ORF) database (www.miangel.msstate.edu/databases/FcProteinDatabase.fasta).

In silico analysis

Amino acid sequence alignments for FhuA and, Fur proteins were performed using the CLC Free Workbench software tool (v. 6.1, CLC bio). Protein structural-based alignments for the Fur protein were performed using the web-based interface for ESPript v.2.2 (http://escript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi; Gouet et al. 1999). The 3D protein structure of *Flavobacterium columnare* FhuA protein was predicted using position specific iterative (PSI)-BLAST alignment and HHpred (Söding et al. 2005). The putative promoter regions of *F. columnare fhuA* and *fur* genes were analyzed by the Discriminating Word Enumerator (DWE; http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=analysisMotifDWEForm).

Detection of siderophores

Siderophore synthesis was evaluated by the universal method of the Chrome Azurol S (CAS) assay (Schwyn & Neilands 1987) with modifications to support *Flavobacterium* growth (Møller et al. 2005) and buffered with 10 mM of MOPS (TYES-CAS). A yellow-orange halo around the colony after 3 d of incubation at 28°C was considered positive for siderophore production. *Salmonella* Typhi murium wild type (Hassan & Curtiss 1990, Luo et al. 2011) and *S. Typhi murium Δfur-44* (Santander et al. 2012) were used as controls for siderophore synthesis.

Cloning of *Flavobacterium columnare fur* gene and complementation of *fur* mutants

Methods for DNA isolation, restriction enzyme digestion, DNA cloning, and use of PCR for construction
and verification of vectors followed standard protocols (Sambrook & Russell 2001). In order to identify the flavobacterium columnare fur gene and study its function, the putative fur gene and its promoter were determined by blasting F. psychrophilum fur (YP_001296771) with the F. columnare genome database from GenBank with accession no. NC016510. Based on the putative sequence from the blast results, the upstream primer Fur-for: 5’-TCA TAC TAG ACA AGA CTA TAA AAA T-3’ and the downstream primer Fur-rev: 5’-TAT CAT ATT TAG AGG TTA ATA CAT C-3’ were designed, and the fur gene with its promoter sequence from F. columnare ATCC 23463 was verified by sequencing the amplified DNA fragment.

To evaluate the functionality of the flavobacterium columnare fur gene, the P_fur-fur fragment (859 bp) was cloned into the Adhl restriction site in the low copy-number plasmid pEZ151 (Santander et al. 2011). The resulting plasmid pEZ161 (Table 1) was used to complement S. Typhi murium UK-1 Δfur-44 mutants. χ3761 S. Typhimurium UK-1 and χ11143 S. Typhimurium Δfur-44 were used as controls. The regulation function of the F. columnare fur gene was shown by the CAS reaction.

RESULTS

Growth under iron-restricted conditions

Growth curves of Flavobacterium columnare ATCC 23463 were determined at 28°C, by growing in FCGM, FCGM supplemented with 40 µM FeSO₄, and FCGM supplemented with 100 µM 2,2’-dipyridyl, respectively. As shown in Fig. 1, F. columnare generation time was dependent on the iron concentration. Under iron-rich conditions (FCGM with 40 µM FeSO₄), the generation time was shorter than for bacteria grown under standard conditions with FCGM medium. F. columnare grown under iron-restricted conditions had the longest generation time among these 3 conditions (Fig. 1). Thus, we conclude that iron has an important role in F. columnare physiology.

IROMP analysis

Flavobacterium columnare ATCC 23463 grown in iron-limited conditions upregulated 2 IROMPs of ~86 kDa, indicating that the secretion of these proteins was iron-regulated and suggests a possible relationship to iron acquisition (Fig. 2A). We also observed that these IROMPs were upregulated in standard growth conditions, indicating that FCGM is an iron-limited medium for F. columnare (Fig. 2A). Protein identification indicated that these 2 IROMPs correspond to the same protein, the TonB-dependent outer membrane ferrichrome-iron receptor precursor protein (FhuA) of F. psychrophilum. The amino acid sequences of F. columnare FhuA were obtained through blasting the FhuA sequence of F. psychrophilum with the non-redundant F. columnare protein database. Structural analysis showed that F. columnare FhuA presented a signal secretion peptide at the N-terminal region, indicating that the FhuA protein with the lower molecular weight observed in the IROMPs profile corresponded to the FhuA protein without the secretion signal peptide, which was predicted to be cleaved between the 18th and 19th amino acid residues (data are not shown). The predicted 763 amino acids of the F. columnare FhuA protein sequence shared homologies with a variety of siderophore receptors from many bacteria, such as the similarity to F. psychrophilum FhuA (55% identity; 70% similarity), Escherichia coli FhuA (23% identity; 42% similarity), Vibrio cholerae FhuA (21% identity; 39% similarity), Neisseria meningitidis FhuA (24% identity; 42% similarity), and Salmonella Typhimurium FhuA (23% identity; 43% similarity).
The 3D structure prediction also shows that *F. columnare* FhuA has similar domain architecture, with a 22-stranded transmembrane β-barrel that encloses a globular plug domain (Fig. 2B). Ligand-binding sites are formed from residues on the extracellular side of the plug domain, as well as from residues on the walls and extracellular loops of the β-barrel. The TonB box is found at the N-terminus of the plug domain, and in some structures it protrudes into the periplasm (Fig. 2B). The *F. columnare* fhuA gene (GeneID: 11477636) is highly homologous with *F. psychrophilum* fhuA (GeneID: 5300370), with a similarity of 96.9%.

*Flavobacterium columnare* belongs to the Bacteroidetes family, which have unique promoter elements with −33/−7 consensus sequences (TTG/TAnnTTTG) separated by a spacer of variable length (generally 17 to 23 nucleotides; Pérez-Pascual et al. 2011). The predicted promoter motif TTA-N18-TATCTTTG was located upstream of the *fur* ORF (Fig. 5). The 152 amino acids, which shared similar size with common Fur from other bacteria such as *E. coli* (148 aa), *Salmonella* Typhimurium (150 aa), and *Vibrio cholerae* (150 aa) (de Lorenzo et al. 1988, Litwin et al. 1992, Bjarnason et al. 2003). Sequence and structural alignment between functional representative bacterial Fur proteins revealed that 26 amino acid residues (−17%) are strictly conserved out of 153 residues in *F. columnare* Fur (Fig. 6). *F. columnare* Fur has 43, 43, 32, 42, and 90% amino acid similarity to the Fur of *S. Typhimurium*, *E. coli*, *Edwardsiella tarda*, *V. cholerae*, and *F. psychrophilum*, respectively, which means that *F. columnare* Fur may be functionally similar to Fur from other bacteria.

**Siderophore synthesis**

The CAS assay was applied to test whether *Flavobacterium columnare* could produce siderophores. After 3 d of incubation on CAS agar, *F. columnare* produced a yellow halo around the colonies (Fig. 4), and a similar yellow halo also appeared around the positive control, *Salmonella* Typhimurium *fur* mutant, but not around the negative control, *S. Typhimurium* wild type. These results indicate that *F. columnare* synthesizes and secretes siderophores on CAS medium modified for *F. columnare* growth.

**Identification of the fur gene of *Flavobacterium columnare***

The predicted promoter motif TTA-N18-TATCTTTG was located upstream of the *fur* ORF (Fig. 5). The *F. columnare* Fur protein had 152 amino acids, which shared similar size with common Fur from other bacteria such as *E. coli* (148 aa), *Salmonella* Typhimurium (150 aa), and *Vibrio cholerae* (150 aa) (de Lorenzo et al. 1988, Litwin et al. 1992, Bjarnason et al. 2003). Sequence and structural alignment between functional representative bacterial Fur proteins revealed that 26 amino acid residues (−17%) are strictly conserved out of 153 residues in *F. columnare* Fur (Fig. 6). *F. columnare* Fur has 43, 43, 32, 42, and 90% amino acid similarity to the Fur of *S. Typhimurium*, *E. coli*, *Edwardsiella tarda*, *V. cholerae*, and *F. psychrophilum*, respectively, which means that *F. columnare* Fur may be functionally similar to Fur from other bacteria.
To evaluate the functionality of the \textit{Flavobacterium columnare} \textit{fur} gene, a complementary CAS assay was performed. As shown in Fig. 7, after 12 h growth, on the CAS plate, the \textit{S. Typhimurium} \textit{Δfur-44} mutant, which contains the \textit{fur} gene of \textit{F. columnare}, showed a small orange halo around the colonies which is very close to the negative control (\textit{Salmonella Typhi murium} UK-1 wild type), but much smaller than the one produced by the \textit{fur} mutant. This means that \textit{S. Typhi murium} UK-1 \textit{Δfur-44} was partially complemented by the \textit{F. columnare Pfur} gene, showing intermediate secretion of siderophores between the \textit{S. Typhi murium} \textit{Δfur-44} mutant and \textit{S. Typhi murium} UK-1 wild type. These results indicate that \textit{F. columnare} Fur is functional and may play a regulatory role in \textit{F. columnare} iron homeostasis.

\section*{DISCUSSION}

Vertebrates sequester iron from invading pathogens as a means of nutritional immunity, using high-affinity iron-binding proteins to limit levels of free iron in biological fluids and tissues in order to deprive pathogens of this key nutritional component. Invading bacterial pathogens sense this iron depletion as a signal that they are within a host and induce the expression of genes that allow iron uptake in order to overcome the host defenses. Similar to other pathogens, \textit{Flavobacterium columnare} can respond to iron-limited environments by upregulating the FlhA receptor protein (Fig. 2), indicating that \textit{F. columnare} possesses at least one system for siderophore uptake.
Flavobacterium columnare belongs to the Bacteroidetes family (also known as the Cytophaga-Flavobacterium-Bacteroides group; Gherna & Woese 1992). Bacteroidetes have strong differences in their mechanisms of gene regulation at either transcription or translation levels compared to proteobacteria (Vingadassalom et al. 2005). As mentioned previously, Bacteroidetes have unique promoter elements with −33/−7 consensus sequences.

Fig. 5. Flavobacterium columnare fur gene analysis. The putative promoter region −33/−7, and ribosomal binding site (RBS, TAAA) are indicated. ORF: open reading frame

Fig. 6. Analysis of Flavobacterium columnare Fur protein. Multiple alignment of Fur from different bacteria. Alignment produced with CLC Free Workbench software tool (v. 6.1) and drawn with ESPript (Gouet et al. 2003). Red-filled boxes indicate residues conserved across all the species aligned. The secondary structure at the top of the alignment corresponds to the F. columnare Fur protein. Spirals represent alpha-helices, and arrows represent beta-sheets. The Fur sequences were obtained from NCBI’s Entrez Protein database for F. psychrophilum YP_001296771, Edwardsiella ictaluri 93-146 (YP_002934295.1), Escherichia coli NP_286398, and Vibrio cholerae NP_231738.

Fig. 7. CAS assay. Complementation of Salmonella Typhimurium Δfur mutants with pEZ161 containing the Flavobacterium columnare fur gene. WT: wild type
(TTG/TAnTGG) separated by spacer or variable length nucleotides (generally 17 to 23 bases; Chen et al. 2010). The putative ribosomal binding site (RBS) consensus sequence has been described as TAAA, typically found at 2 to 12 bases from the gene start codon (Starosick et al. 2008). The F. columnare FhuA gene presents all of these elements at its promoter region as predicted (Fig. 3). However, the F. columnare fhuA gene is not part of an organized operon, and the surrounding genes have no similarities with the fhuBCD genes, which are required for siderophore transport. The rest of the machinery for siderophore transport across the F. columnare cell membrane still needs to be identified.

As mentioned above, Flavobacterium columnare FhuA belongs to a siderophore receptor precursor protein family. Thus, the presence of siderophores was evaluated. Siderophores of F. columnare were detected using CAS plates (Fig. 4). However, further studies are required to determine the gene regulation of siderophore synthesis and secretion for F. columnare.

Usually in bacteria, iron acquisition is tightly controlled by the Fur protein (Crosa et al. 2004). A Fur-like regulation pattern may therefore exist during iron uptake in Flavobacterium columnare. We identified a putative fur gene in the chromosome of F. columnare. To evaluate its activity, Salmonella Typhimurium Δfur-44 mutant was complemented with the F. columnare putative fur gene. The F. columnare P_fur-fur gene partially complements S. Typhimurium Δfur-44 mutant (Fig. 7). This partial complementation could be due to differences in the promoter regions between S. Typhimurium and Flavobacterium. Although the F. columnare Fur protein possesses a predicted functional 3D structure (data not shown), its amino acid sequence differed from the enteric Fur protein, which could lead to a partial repression activity. As mentioned, the promoter regions of the fur gene in S. Typhimurium are different from those in Flavobacterium, indicating that the F. columnare Fur binding box is also different. On the other hand, Bacteroides possesses an unusual primary sigma factor, called σ^Allo-like, that strongly differs from the σ^20 factor of proteobacteria (Chen et al. 2007). Thus, the genes in Bacteroides are not usually expressed when transferred into proteobacteria, and proteobacteria genetic elements do not function well in Bacteroidetes (Chen et al. 2010). This correlates with the partial complementation of S. Typhimurium Δfur-44 by the F. columnare P_fur-fur gene.

Pathogenesis of Flavobacterium columnare is not clear, but it is known that the columnaris disease process involves bacterial invasion and external tissue damage, with no occurrence of systemic infection found (Tripathi et al. 2003). This may indicate that the extracellular capsule or proteases are more critical than iron acquisition in establishing F. columnare infection. Therefore, more experiments are needed to identify whether iron acquisition of F. columnare can influence its virulence. In addition, only one strain, F. columnare ATCC 23463, was used in all of the experiments described here; thus our conclusions are not all-inclusive. The lack of feasible genetic means and techniques for mutant construction in F. columnare ATCC 23463 is also a major limitation in advancing in-depth investigation of the iron uptake mechanisms of F. columnare. In summary, we identified part of the principal iron uptake machinery of F. columnare, which contains a putative siderophore iron uptake system, FhuA, that is regulated in an iron-dependent fashion and likely regulated in a Fur-dependent fashion as well. Siderophores were detected in F. columnare. Further studies should be conducted at the genetic level to determine the regulatory relationship of these components of the iron uptake system, as well as their potential role in the pathogenesis of F. columnare.

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