Vol. 106: 129–138, 2013
doi: 10.3354/dao02635

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published October 11

Identification of an iron acquisition machinery in *Flavobacterium columnare*

Lingyu Guan[1,2,*], Javier Santander[1,3,4,*], Melha Mellata[1], Yuangxing Zhang[2], Roy Curtiss 3rd[1,3,]**

[1]The Biodesign Institute, Arizona State University, Tempe, Arizona 85287, USA
[2]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, P R China
[3]School of Life Sciences, Arizona State University, Tempe, Arizona 85287, USA

[4]*Present address:* Center for Genomics and Bioinformatics, Nucleus for Interdisciplinary Biotechnology, Universidad Mayor, Chile

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) Δ*fur* 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 Δ*fur* 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 sequestering 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

*Contributed equally to this work.
**Corresponding author. Email: rcurtiss@asu.edu

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; Hantke 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^{2+}-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; $MgSO_4 \cdot 7\ H_2O$ 1 g; $CaCl_2 \cdot 2H_2O$ 0.74 g; NaCl 5 g; sodium citrate 1.5 g; ddH_2O 1 l) (Farmer 2004) was used for liquid growth, and tryptone yeast extract salts (TYES, tryptone 4 g; yeast extract 0.4 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $CaCl_2 \cdot 2H_2O$ 0.5 g; ddH_2O 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_2O 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}).

Table 1. Bacterial strains and plasmids. Am: ampicillin; Pm: polymyxin; Carb: carbenicillin; Gen: gentamycin; Nov: novomycin; Olean: oleandomycin; Pen: penicillin; Lin: lincomycin; Str: streptomycin. Superscript 'r' indicates antibiotic resistance

	Relevant characteristics	Source
Strain		
Escherichia coli		
Top10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 nupG recA*1 *ara*D139 Δ(*ara-leu*)7697 *galE*15 *galK*16 *rpsL*(Str^R) *endA*1 λ⁻	Invitrogen
Salmonella enterica		
χ3761	*S.* Typhimurium UK-1	Hassan & Curtiss (1990)
χ11143	*S.* Typhimurium UK-1; Δ*fur44*	Santander et al. (2012)
Flavobacterium columnare		
J201	ATCC 23463, Amp^r, $Carb^r$, Gm^r, $Olean^r$, Pen^r, Nov^r, Pm^r, Pen^r	American Type Culture Collection
Plasmids		
pEZ151	4065 bp, pSC101 *ori*, Gm GFP,	Santander et al. (2011)
pEZ161	pEZ151, Gm, pSC101 *ori*, Gm, *F. columnare* P_{fur}-*fur* gene	This study

Growth under iron-restricted conditions

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

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 $FeSO_4$ (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 ($A_{600\,nm}$). 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 $FeSO_4$ (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 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://espript.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* Typhimurium wild type (Hassan & Curtiss 1990, Luo et al. 2011) and *S.* Typhimurium Δ*fur-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_00 1296771) 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 *Adh*I restriction site in the low copy-number plasmid pEZ151 (Santander et al. 2011). The resulting plasmid pEZ161 (Table 1) was used to complement *S.* Typhimurium UK-1 Δ*fur-44* mutants. χ3761 *S.* Typhimurium UK-1 and χ11143 *S.* Typhimurium Δ*fur-44* were used as controls. The regulation function of the *F. columnare fur* gene was shown by the CAS reaction.

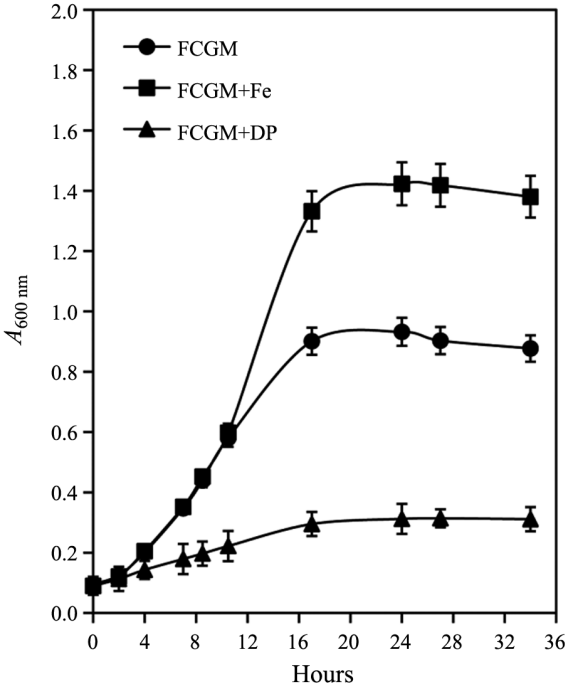


Fig. 1. *Flavobacterium columnare*. Growth in *F. columnare* growth medium (FCGM), FCGM supplemented with iron (Fe; $FeSO_4$ 40 µM), and FCGM supplemented with 2,2′-dipyridyl (DP; 100 µM), all at 28°C with aeration (200 rpm)

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 $FeSO_4$, 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 $FeSO_4$), 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).

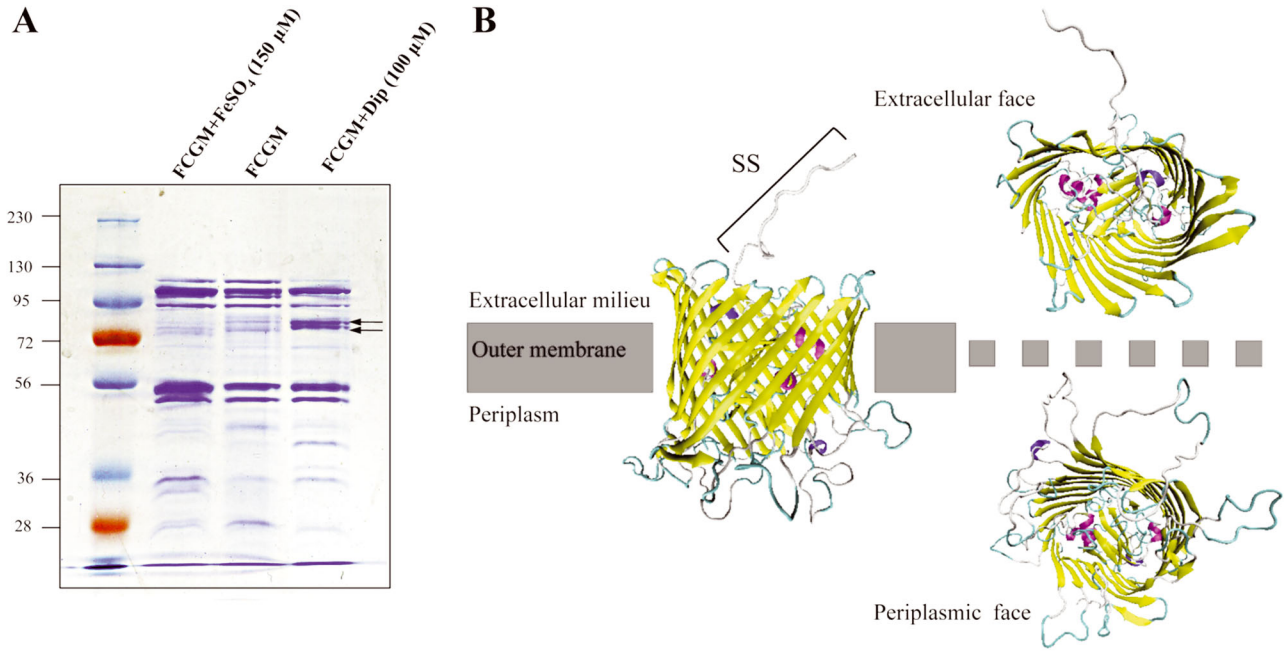


Fig. 2. *Flavobacterium columnare*. (A) SDS-PAGE profile of outer membrane proteins (OMPs) from *F. columnare* grown in FCGM with $FeSO_4$ (40 μM) (Lane 2); FCGM (Lane 3); and FCGM with 2, 2′-dipyridyl (100 μM) (Lane 4). Arrows indicate the iron-regulated OMPs. (B) 3D structure prediction of FhuA from *F. columnare*, SS: signal sequence

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%.

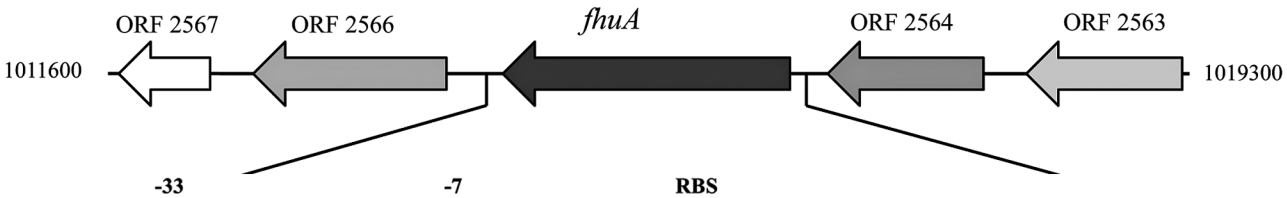
Flavobacterium columnare belongs to the *Bacteroidetes* family, which have unique promoter elements with –33/–7 consensus sequences (TTG/TAnnTTTG) separated by a spacer of variable length (generally 17 to 23 nucleotides; Pérez-Pascual et al. 2011). The predicted promoter motif TTA-N18-TATATTTG was located upstream of the *fhuA* ORF (Fig. 3), which was the most highly conserved structure as TTG/TAnnTTTG.

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 secrets 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.



TAAAAATAATGTTTAATTTTTAAAATTCTAATCTTATATTTGCAGTCCAAAAACAATTTAAAATTAGTCTAAATAAAATGAAGTTATATTGGTTAGGATGCTTATTATTC

TTGTCTGGACAAAGTTTCGGTCAAGATAGGTTTCAAGGAGAAACAGAAAATACCGTTGAAAATGATACGGCTAGAAAAAAAAGATATTCGTTAAAAGAAATAGAAATAAGAC
AACACAAACAAAAAAAGTTTGTTTCTACTGTTCGTACGGGATTAAAACCTTTTGATAATCCTCAAAGTTTACAATCTATTGGAGGAGAAGTGATTTATCAACAACAATCGGT
TCGTTTGAGCGATGTCGTAAAAAACGCAAATGGTGTTTATGTAGGCTCAGCACGTGGTGGAGCACAAGAATCCTTTTGGTCAAGAGGTTATGATATGGGTACGAATAATATT
TTTAAAAATGGTTTTCGTCAAAATGGAGGGTCAATGCCTGAGGTGATTTCATTAGAAAAAGTAGAATTTTTAAAAGGAAATAGTGCTTTGTTATACGGAAATGTAGCACCAG
GAGGAATTTTAAATTTAGTAACCAAGACCCCTTTGTTTGAAAGAGGAGGAGAGTTATCTTTTCAAACAGGAAGTTATGATTTTTACAAACCTGTAATTGATTTTTATGGGCC
TCTTAGTAAATCAATTGCTTACCGGGTAGTGAGTAGTTTTGAAAAATCAAAAAGTTTTAGAGATGTAGTAAGTAGAGAACGTTATTATATTAATCCTTCTTTTCTATTTAAG
GCAACCCAAAAAACAGATGTACTTCTTCAAATGGATTATTTGTACGATAATTGGACACCTGATTTTGGAACAGGTGCTTTTGGGAAGACTATTCTGGATGTACCTATTACTC
GTTATTTAGGTGCTAATTGGTCTAACGGACAAACACGTCAGGGATCATTATCCGCTCTTTTAAAACATCAGATTAATGATTCTTGGAAGTTTATTGCAAATATGTCATTCCA
AAATTATTCAAGACAATTTGAAGGAACAGACAGAATTCAACCTTCTAACATAACGGGTGATTTAACTAGAAAATTAGGTAAGAACAAAAATGTTGAAACACTATTAGGACAA
CAATTAGGATTACAAGGAAGTTTTAAAACTTTTGGTTTCAAACATGAATTATTTACAGGAGTAGATGGAGAAACTAGTTATACGGATAGTTATTCCTATTTGTTTTATACAA
AACAAAAAGATGGTAAATTTATTGCTATAACAGATTATGATAAAATCAATATCTTTGATCCTATTACATTTTCAAACGAAAAAGGATCACAAGAAAATTATAATTCAGTACT
AACAAAGAATCAAGGAAAACGTTTTGGCTTTTTTGCTCAAGATTTAATATCTATTCATGAAAAATTAAAAGTACTTGCTGGTTTGCGTTGGTCATGGCAAGAAGTTCAAAGT
ACTGTATATAATGCCAAAATTGATGGTGGAAATACTACAAGTCCTAAAGGACAAACTCCTTTTGTGCTTAATCATGAAGCAGATAAAGATAATGCACCAATTGAAGGCGCTA
TACAAGTAGATAAAGCATTTTCTCCTAAATTTGGTTTGGTCTTTCAACCAATGCAATCAATAGCAATTTTTGGAAGTTATTCAAATTCATTCACTACCAACACAGGAGTTGA
TATTTATAATGATCCTATAAAACCCTCTATTATAAATCAATATGAAGCAGGAGTAAAAACAGAATTTTTTAAAGGTAATTTAACAACAGGAGTAACTGTTTATAGCATTGAA
AATAATAATTTAGTCCAAATGGCAGCTACAACCTTAGATGGTAAAGCCAATTCTAATTCGAATATCAAAGAACTATCAGGTAAAACAAGAAGCAAAGGAATTGAATTAGATA
TTACCGTAACACCTATCGAAGGATTCAAGATTAATGCAGGTTATAGTTATAACGATATGCGTTTTGTGAAAACATCTGGATTAGTAGGCAGTTCATTAGAAGGGGATCGTTT
GGCGCGTACCCCTGATCAAACAGCTAATTTAAGTTTCTTTTATACAGTACAAAACGGATTTTTCAAAAATGTTTCCATAGGAGCTATAACTAACTATATAGGAAATCGTATA
GGCGGTTGGAATAACCGTTATGCAAAAGATAAAAATGGAACAGTATTTATAGAAGAACGTGAGATTCCAGTTAGTGGTTATGCAGTTGCGGATGCAACTCTAGGATACACTT
ATAAAAATATAAGTTTATTATGTAAATTGTCTAATGTGGCTAATACTTTAAATTATACAGTACATGAAAATTACAGTATTAATCCAATACCGCCAAGACAATTTATGGCAAC
ACTAAAATATCAATTTTAAAAGGTTTTGTTTTTGTGAAAGGCAAATAGTATAGTTTTAAAACT

Fig. 3. *Flavobacterium columnare fhuA* gene analysis. The putative promoter region –33/–7, and ribosomal binding site (RBS, TAAAA) are indicated. ORF: open reading frame

To evaluate the functionality of the *Flavobacterium columnare fur* gene, a complementary CAS assay was performed. As shown in Fig. 7, after 12 h growth, on the CAS plate, the *S.* Typhimurium Δ*fur*-44 mutant, which contains the *fur* gene of *F. columnare*, showed a small orange halo around the colonies which is very close to the negative control (*Salmonella* Typhimurium UK-1 wild type), but much smaller than the one produced by the *fur* mutant. This means that *S.* Typhimurium UK-1 Δ*fur*-44 was partially complemented by the *F. columnare* P_{fur}-*fur* gene, showing intermediate secretion of siderophores between the *S.* Typhimurium Δ*fur*-44 mutant and *S.* Typhimurium UK-1 wild type. These results indicate that *F. columnare* Fur is functional and may play a regulatory role in *F. columnare* iron homeostasis.

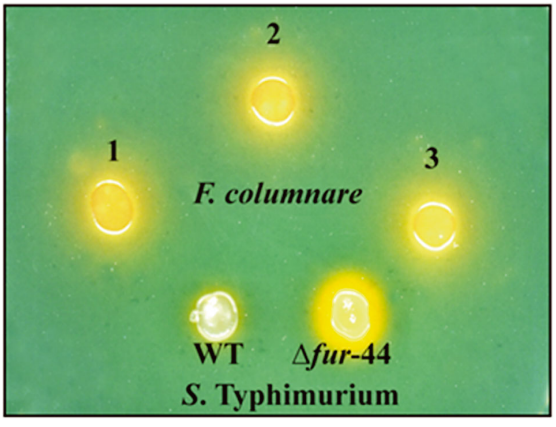


Fig. 4. Siderophore synthesis by *Flavobacterium columnare* on chrome azurol S (CAS) medium. 1, 2, 3: *F. columnare* wild-type triplicate macro-colonies. WT: *Salmonella* Typhimurium UK-1 wild type; Δ*fur*-44: *S.* Typhimurium *fur* mutant

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, *Flavobacterium columnare* can respond to iron-limited environments by upregulating the FhuA receptor protein (Fig. 2), indicating that *F. columnare* possesses at least one system for siderophore uptake.

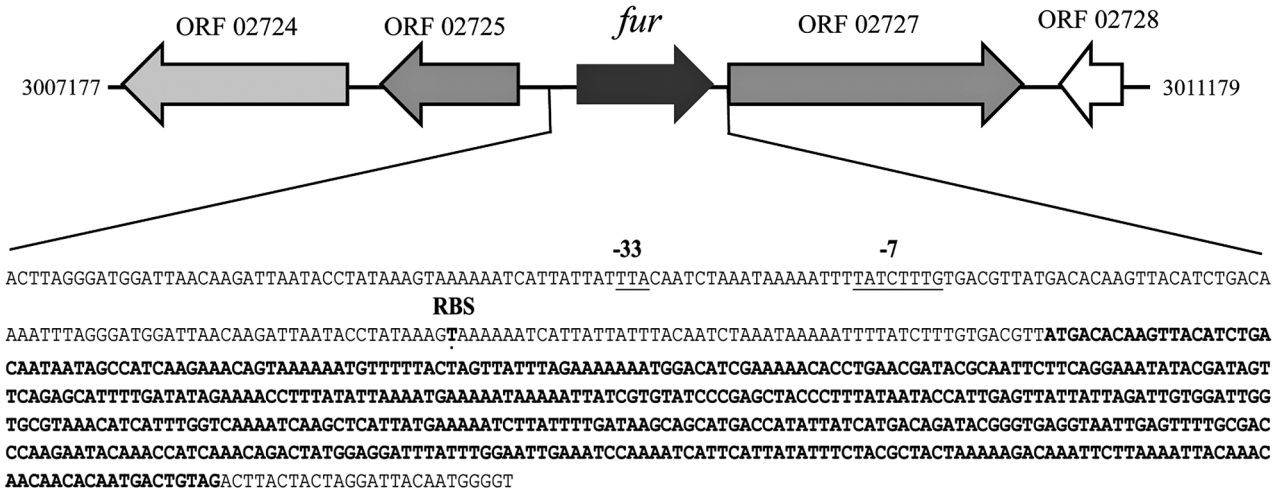


Fig. 5. *Flavobacterium columnare fur* gene analysis. The putative promoter region –33/–7, and ribosomal binding site (RBS, TAAAA) are indicated. ORF: open reading frame

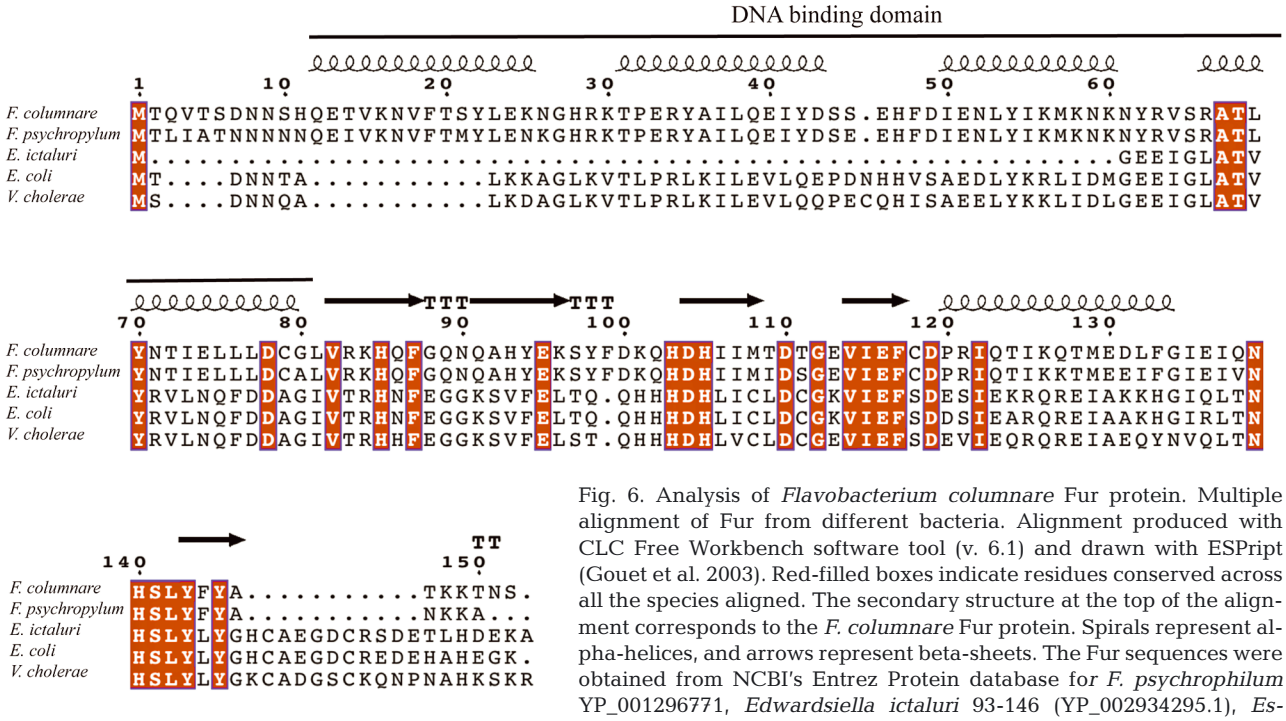


Fig. 6. Analysis of *Flavobacterium columnare* Fur protein. Multiple alignment of Fur from different bacteria. Alignment produced with CLC Free Workbench software tool (v. 6.1) and drawn with ESPript (Gouet et al. 2003). Red-filled boxes indicate residues conserved across all the species aligned. The 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

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

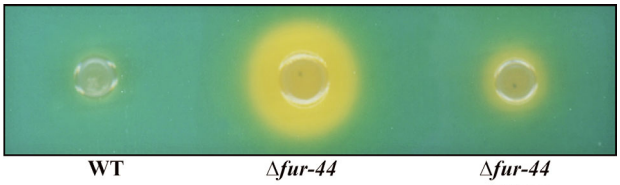


Fig. 7. CAS assay. Complementation of *Salmonella* Typhimurium Δ*fur* mutants with pEZ161 containing the *Flavobacterium columnare fur* gene. WT: wild type

(TTG/TAnnTTTG) 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 TAAAA, typically found at 2 to 12 bases from the gene start codon (Staroscik 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 Δ*fur*-44 mutant was complemented with the *F. columnare* putative *fur* gene. The *F. columnare* P_{fur}-*fur* gene partially complements *S.* Typhimurium Δ*fur*-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^{ABfr}-like, that strongly differs from the σ^{70} 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 Δ*fur*-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*.

Acknowledgements. This work was supported by USDA grant CRIS-ARZR-2009-01801, Comisión Nacional de Investigación Científica y Tecnológica (CONICYT), Gestión Propia Fellowship, Chile, and China Scholarship Council, China. We thank G. Golden for manuscript editing.

LITERATURE CITED

Ames GFL (1974) Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J Biol Chem 249:634–644

Anderson JIW, Conroy DA (1969) The pathogenic myxobacteria with special reference to fish disease. J Appl Bacteriol 32:30–39

Avendaño-Herrera R, Toranzo AE, Romalde JL, Lemos ML, Magarinos B (2005) Iron uptake mechanisms in the fish pathogen *Tenacibaculum maritimum.* Appl Environ Microbiol 71:6947–6953

Bagg A, Neilands J (1987a) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli.* Biochemistry 26:5471–5477

Bereswill S, Lichte F, Vey T, Fassbinder F, Kist M (1998) Cloning and characterization of the *fur* gene from *Helicobacter pylori.* FEMS Microbiol Lett 159:193–200

Berish SA, Subbarao S, Chen C, Trees DL, Morse SA (1993) Identification and cloning of a *fur* homolog from *Neisseria gonorrhoeae.* Infect Immun 61:4599–4606

Bertani G (1951) Studies on lysogenesis I. The mode of phage liberation by lysogenic *Escherichia coli.* J Bacteriol 62:293–300

Biegel Carson SD, Thomas CE, Elkins C (1996) Cloning and sequencing of a *Haemophilus ducreyi fur* homolog. Gene 176:125–129

Bjarnason J, Southward CM, Surette MG (2003) Genomic profiling of iron-responsive genes in *Salmonella enterica* serovar Typhimurium by high-throughput screening of a random promoter library. J Bacteriol 185:4973–4982

Brickman TJ, Armstrong SK (1995) *Bordetella pertussis fur* gene restores iron repressibility of siderophore and protein expression to deregulated *Bordetella bronchiseptica* mutants. J Bacteriol 177:268–270

Bullock G, Hsu TC, Shotts E Jr (1986) Columnaris disease of fishes. US Fish & Wildlife Publications. Paper 129, Kearneysville, VA

Cain KD, Lafrentz BR. (2007) Laboratory maintenance of *Flavobacterium psychrophilum* and *Flavobacterium columnare*. Curr Protoc Microbiol 13:1.1–1.12

Carpenter BM, Whitmire JM, Merrell DS (2009) This is not your mother's repressor: the complex role of *fur* in pathogenesis. Infect Immun 77:2590–2601

Chen S, Bagdasarian M, Kaufman MG, Bates AK, Walker ED (2007) Mutational analysis of the *ompA* promoter from *Flavobacterium johnsonia*e. J Bacteriol 189: 5108–5118

Chen S, Kaufman MG, Bagdasarian M, Bates AK, Walker ED (2010) Development of an efficient expression system for *Flavobacterium* strains. Gene 458:1–10

Crosa JH, Mey AR, Payne SM (2004) Iron transport in bacteria. ASM Press, Washington, DC

Daniel C, Haentjens S, Bissinger MC, Courcol RJ (1999) Characterization of the *Acinetobacter baumannii* Fur regulator: cloning and sequencing of the *fur* homolog gene. FEMS Microbiol Lett 170:199–209

De Lorenzo V, Herrero M, Giovannini F, Neilands J (1988) Fur (ferric uptake regulation) protein and CAP (catabolite activator protein) modulate transcription of fur gene in *Escherichia coli.* Eur J Biochem 173:537–546

Ernst JF, Bennett RL, Rothfield LI (1978) Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium.* J Bacteriol 135:928–934

Escolar L, de Lorenzo V, Pérez-Martin J (1997) Metalloregulation *in vitro* of the aerobactin promoter of *Escherichia coli* by the Fur (ferric uptake regulation) protein. Mol Microbiol 26:799–808

Escolar L, Perez-Martin J, De Lorenzo V (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein. J Bacteriol 181:6223–6229

Farmer B (2004) Improved methods for the isolation and characterization of *Flavobacterium columnare*. MS thesis, Louisiana State University, Baton Rouge, LA

Franza T, Sauvage C, Expert D (1999) Iron regulation and pathogenicity in *Erwinia chrysanthemi* 3937: role of the Fur repressor protein. Mol Plant Microbe Interact 12: 119–128

Gherna R, Woese C (1992) A partial phylogenetic analysis of the 'Flavobacter-Bacteroides' phylum: basis for taxonomic restructuring. Syst Appl Microbiol 15:513–521

Gouet P, Courcelle E, Stuart DI, Metoz F (1999) ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics 15:305–308

Gouet P, Robert X, Courcelle E (2003) ESPript/ENDscript: extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res 31: 3320–3323

Hantke K (2001) Iron and metal regulation in bacteria. Curr Opin Microbiol 4:172–177

Hassan JO, Curtiss R III (1990) Control of colonization by virulent *Salmonella typhimurium* by oral immunization of chickens with avirulent delta *cya* delta *crp S. typhimurium.* Res Microbiol 141:839–850

Hickey EK, Cianciotto NP (1994) Cloning and sequencing of the *Legionella pneumophila fur* gene. Gene 143:117–121

Litwin CM, Calderwood SB (1993) Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of a *fur* mutant by in vivo marker exchange. J Bacteriol 175:706–715

Litwin CM, Boyko SA, Calderwood SB (1992) Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. J Bacteriol 174:1897–1903

Luo Y, Kong Q, Yang J, Golden G and others (2011) Complete genome sequence of the universal killer *Salmonella enterica* Serovar Typhimurium UK-1 (ATCC 68169). J Bacteriol 193:4035–4036

Møller JD, Ellis A, Barnes A, Dalsgaard I (2005) Iron acquisition mechanisms of *Flavobacterium psychrophilum.* J Fish Dis 28:391–398

Payne SM (1994) Detection, isolation, and characterization of siderophores. Method Enzymol 235:329–344

Pérez-Pascual D, Gómez E, Álvarez B, Méndez J and others (2011) Comparative analysis and mutation effects of *fpp2–fpp1* tandem genes encoding proteolytic extracellular enzymes of *Flavobacterium psychrophilum.* Microbiology (Read) 157:1196–1204

Prince RW, Cox CD, Vasil ML (1993) Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J Bacteriol 175:2589–2598

Puente JL, Juarez D, Bobadilla M, Arias CF, Calva E (1995) The *Salmonella ompC* gene: structure and use as a carrier for heterologous sequences. Gene 156:1–9

Sambrook J, Russell W (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, NY

Santander J, Mitra A, Curtiss R III (2011) Phenotype, virulence and immunogenicity of *Edwardsiella ictaluri* cyclic adenosine 3′,5′-monophosphate receptor protein (Crp) mutants in catfish host. Fish Shellfish Immunol 31: 1142–1153

Santander J, Golden G, Wanda SY, Curtiss R III (2012) The Fur regulated iron uptake system of *Edwardsiella ictaluri* and its influence on pathogenesis and immunogenicity in the catfish host. Infect Immun 80:2689–2703

Schwyn B, Neilands J (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Söding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res 33:W244–W248

Staggs TM, Perry RD (1991) Identification and cloning of a *fur* regulatory gene in *Yersinia pestis.* J Bacteriol 173: 417–425

Staroscik AM, David H, Kate A, David N (2008) Development of methods for the genetic manipulation of *Flavobacterium columnare.* BMC Microbiol 8:115–125

Tripathi NK, Latimer MVSKS, Rakich PM (2003) Columnaris disease in freshwater fish. Compendium 25:528–536

USDA (United States Department of Agriculture) (2003) Reference of fingerling catfish health and production practices in the United States. Publ. no. N406.1103. USDA, Fort Collins, CO

Vingadassalom D, Kolb A, Mayer C, Rybkine T, Collatz E, Podglajen I (2005) An unusual primary sigma factor in the Bacteroidetes phylum. Mol Microbiol 56:888–902

Wooldridge KG, Williams PH, Ketley JM (1994) Iron-responsive genetic regulation in *Campylobacter jejuni*: cloning and characterization of a *fur* homolog. J Bacteriol 176: 5852–5856

Xie HX, Nie P, Sun BJ (2004) Characterization of two membrane associated protease genes obtained from screening out membrane protein genes of *Flavobacterium columnare* G4. J Fish Dis 27:719–729

Yamamoto S, Funahashi T, Ikai H, Shinoda S (1997) Cloning and sequencing of the *Vibrio parahaemolyticus fur* gene. Microbiol Immunol 41:737–740

Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: March 19, 2013; Accepted: June 23, 2013
Proofs received from author(s): September 30, 2013