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Identification of Fur regulated genes in the bacterial fish pathogen *Photobacterium damselae* ssp. *piscicida* using the Fur titration assay

Carlos R. Osorio[1,2,*], Manuel L. Lemos[1] & Volkmar Braun[2]
[1]*Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, 15782, Santiago de Compostela, Galicia, Spain.* [2]*Mikrobiologie/Membranphysiologie, Universität Tübingen, Auf der Morgenstelle 28, D-72076, Tübingen, Germany*

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Abstract

Bacteria have developed a series of iron-scavenging and transport systems. The expression of many of the iron utilization genes is tightly regulated by the Fe^{2+} loaded Fur repressor protein. In this study, the Fur titration assay (FURTA) was used to screen for DNA fragments from a genomic DNA library of *Photobacterium damselae* ssp. *piscicida* containing potential Fe^{2+}Fur binding sites or iron binding-proteins which withdraw iron from Fur. One of the clones encoded a *tonB* gene and adjacent a functionally related *exbB* gene. An additional and complete *tonB exbB exbD* gene cluster was identified and sequenced. A gene homologous to the ferritin gene was found whose FURTA-positive phenotype may be explained by its iron-binding ability. Genes encoding a putative complete iron-regulated outer membrane transport protein and a pseudogene of a transport protein were found. The FURTA assay also revealed iron regulation of the AraC type transcriptional regulation.

Introduction

Iron is an essential element for growth and survival of most bacteria. Although iron is the fourth most abundant element on earth, free iron is limited and not readily available since the ferrous iron undergoes rapid oxidation to form iron (III) which is practically insoluble. Bacteria respond to iron limitation by multiple mechanisms. One of these involves the synthesis and secretion of low-molecular-weight Fe(III) chelators named siderophores (Neilands, 1995). Bacterial pathogens are also capable of acquiring iron by means of siderophore-independent mechanisms through utilization of host iron-binding compounds such as transferrins, lactoferrins, ferritins and hemoproteins.

The expression of genes involved in iron acquisition is strictly regulated in response to the iron concentration in the environment. The ferric uptake regulator (Fur) protein acts as an iron-responsive DNA binding repressor protein (Hantke 1981, Braun & Hantke 1991, Neilands, 1990), and requires ferrous iron as a cofactor for dimerization and DNA binding. When Fur is loaded with ferrous iron, it is able to bind operator sites (called Fur boxes) within the promoter region of iron-regulated genes so that their transcription is shut-off in iron-replete conditions. In iron-depleted conditions, unloaded Fur cannot bind operator sites, thus allowing transcription of iron-regulated genes.

The marine Gram-negative bacterium *Photobacterium damselae* ssp. *piscicida* is the causative agent of fish pasteurellosis, a disease affecting wild and cultured marine fish and which causes important economical losses in marine aquaculture worldwide (Magariños *et al.* 1996b). This species acquires iron via siderophores and heme and its virulence for fish is increased when fish are previously inoculated with hemin or hemoglobin (Magariños *et al.* 1994). Strains of *P. damselae* ssp. *piscicida* produce siderophores under iron-limiting conditions, and chemical tests and

*Corresponding author: Carlos Rodríguez Osorio, Departamento de Microbioloxía e Parasitoloxía, Instituto de Acuicultura, Universidade de Santiago de Compostela, 15782-Santiago de Compostela, Galicia, Spain (Tel.: 34-981563100 (Ext. 16062); E-mail: crosorio@usc.es)

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristic(s)	Isolation source/ Reference
Strains		
P. damselae subsp. piscicida		
DI21		Seabream, Spain
554.2		Sole, Spain
B51		Seabass, Spain
IT-2		Seabream, Italy
ATLIT-2		*Morone sp.* Israel
10831		Seabass, France
EPOY-8803-II		Red grouper, Japan
ATCC 29690		Yellowtail, Japan
ATCC 17911		White perch, USA
Escherichia coli		
DH5α	Cloning strain	Laboratory stock
H1717	*araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF*::λ placMu	Hantke 1987
Plasmids		
PT7-7	Cloning vector, Ap^r,	Tabor and Richardson 1985
pGEMT-Easy	PCR cloning vector, Ap^r	Promega
pCAR149	Inverse-PCR product containing DNA downstream of clone F-30	This study

cross-feeding assays showed that the siderophore is neither a phenolate nor a hydroxamate (Magariños *et al.* 1994). In addition, iron availability regulates the amount of capsular polysaccharide and the production of an extracellular protease (Do Vale *et al.* 2001; Magariños *et al.* 1996a,b). Iron-regulated outer membrane proteins have been described in *P. damselae* ssp. *piscicida* (Magariños *et al.* 1994) but genes encoding these proteins have not been identified. Recently, we reported on the cloning and sequencing of a Fur homologue in *P. damselae* ssp. *piscicida*, and demonstrated that it acts as an iron-responsive transcriptional regulator (Juíz-Río *et al.* 2004). In the present study, we report the identification of novel Fur-regulated genes in *P. damselae* ssp. *piscicida* using the Fur titration assay (FURTA).

Methods

Bacterial strains and culture conditions. Strains used are listed in Table 1. *P. damselae* ssp. *piscicida* was routinely grown at 25^{o}C in Tryptic Soy Agar (Difco Laboratories, Augsburg, Germany) supplemented with 1% NaCl (TSA-1). *Escherichia coli* strains were grown at 37 °C in Luria Bertani (LB) medium. McConkey agar base (Difco) was supplemented with 1% lactose and 0.04 mM $FeSO_4$ (M+Fe). If required ampicillin was added at a concentration of 0.1 mg ml^{-1} All strains were stored frozen at -80 °C in LB broth with 20% glycerol.

DNA techniques. Total genomic DNA was extracted from *Photobacterium damselae* ssp. *piscicida* with the Easy-DNA kit (Invitrogen, Karlsruhe, Germany). DNA extraction from agarose gels and plasmid DNA purification were carried out with kits from Qiagen, Hilden, Germany. Standard methods for restriction endonuclease analyses, ligations and other DNA cloning techniques were carried out as described by Sambrook and Rusell (2001). Plasmids used in cloning experiments and those derived from this study are summarized in Table 1.

For inverse PCR, chromosomal DNA was cut with a single restriction enzyme and self-ligated. Ligation products were used as template in a PCR reaction

with suitable primers, using the Expand Long Template Kit (Roche Diagnostics, Mannheim, Germany). DNA sequences were determined by the dideoxy chain termination method on either plasmid or PCR product templates by using the Big Dye Terminator v3.0 DNA Sequencing Kit (Applied Biosystems) and an automated sequencer ABI 377 (Applied Biosystems). The European Bioinformatics Institute services were used to consult the EMBL database with the FASTA3 and BLAST algorithms. Additional DNA and peptide analysis were performed using BioEdit Software (Version 5.0.6). For Southern hybridization, chromosomal DNA (3 μg) was cut with appropriate enzymes, gel-electrophoresed and transferred onto nylon membranes (0.45 μm pore size, Boehringer Mannheim). Labeling of DNA probes, hybridization and detection were performed with the ECL Direct Nucleic Acid labeling and detection system (Amersham Biosciences, Braunschweig, Germany).

FURTA. Fur-regulated promoters and iron-binding proteins carried on a multicopy plasmid can be identified by transformation into *E. coli* strain H1717. This strain carries a Fur-regulated *fhuF*::*lac*Z gene fusion which is particularly sensitive to changes in the concentration of the Fur repressor (Stojiljkovic *et al.* 1994). Iron-binding proteins cloned on a plasmid and expressed in *E. coli* H1717 will compete with Fur proteins for iron, thus lowering the amount of Fur molecules which can dimerize and repress the *fhu*F::*lac*Z gene fusion. Similarly, Fur boxes introduced on a multicopy plasmid can cause derepression of the fusion by titrating the Fur protein, thus leading to transcription of the *lacZ* gene and the expression of a *lac*$^+$ phenotype. This can be used as a selectable marker for cloning Fur-regulated promoters and iron-binding proteins, since *lac*$^+$ colonies are red on McConkey plates supplemented with Fe, while *lac*$^-$ colonies remain white.

Fur titration assay was performed as previously described (Stojiljkovic *et al.* 1994). In brief, chromosomal DNA of *P. damselae* ssp. *piscicida* DI21 was partially digested with the restriction enzyme *Sau*3A. Fragments between 0.5 and 5 kb were cloned into the *Bam*HI site of vector PT7-7 and transformed into *E. coli* DH5-α. Approximately 20.000 colonies were pooled into four groups. Plasmid DNA isolated from these pools was used to transform *E. coli* H1717. Red transformants on M+Fe were reisolated twice, and plasmid DNA was isolated.

Nucleotide sequence accession number. The EMBL accession number for the sequences described in this article are AJ548760, AJ567909, AJ699417, AJ699306, AJ699365.

Results and discussion

Sequence analysis of FURTA positive DNA regions.

Using the FURTA assay we isolated 72 Furta-positive clones which formed red colonies on iron containing M+Fe medium. Duplicate clones were eliminated on the basis of identical *Eco*RI-*Hind*III restriction patterns. Since several small clones were contained in the collection of larger ones, five distinct groups of clones were finally inferred (Figure 1).

Plasmid F-15 contained five ORFs (Figure 1). ORF1 and ORF2 showed homology with AraC-type transcriptional activators (Table 2). AraC regulates the *E. coli* arabinose operon by binding through a helix-turn-helix motif to promoter DNA (Gallegos *et al.* 1993). Members of this family act in *Pseudomonas aeruginosa* (PchR) (Heinrichs and Poole, 1993) and *Yersinia pestis* (YbtA) (Fetherston *et al.* 1996) as positive regulators of genes coding for siderophore receptors and for enzymes involved in siderophore biosynthesis. In fact, a low similarity was found between ORF2 and YbtA in a BLAST similarity search (data not shown). Thus, the *P. damselae* ssp. *piscicida* ORF2 may constitute a positive regulator of gene functions related to iron acquisition. The potential Fur-box upstream of ORF2 matched the *E. coli* consensus sequence in 16 of 19 bases (Figures 1, 2). ORF3 encoded a protein with 660 amino acids which was homologous to siderophore receptors of various species. It displayed the highest similarity to FyuA, a putative siderophore receptor from *Photorhabdus luminescens* (Table 2), while the closest homologue with well-known function was the quinolobactin receptor of *Pseudomonas fluorescens* (Matthijs *et al.* 2004) (Table 2). Since *P. damselae* is a member of the family *Vibrionaceae*, a similarity search was conducted among the siderophore receptors in *Vibrio* species. ORF3 showed 25% identity to the *V. vulnificus* ferric vulnibactin receptor (Figure 3). ORF3 may constitute the receptor for the yet uncharacterized *P. damselae* ssp. *piscicida* siderophore. Although *P. damselae* is closely related to *Vibrio* species, ORF3 has no close homologue in Vibrios. It is concluded that ORF3 may transport a novel ferric siderophore. In fact, strains of

Table 2. Proteins with homology to *Photobacterium damselae* ssp. *piscicida* ORFs described in this study.

Photobacterium damselae protein	Homologue	EMBL Acc No	Amino acid Identity (%)	Amino acid Similarity (%)
ORF1 (F-15)	*R. solanacearum* AraC family transcriptional regulator	Q8XYE5	30	48
ORF2 (F-15)	*Anabaena sp*. AraC family transcriptional regulator	Q8YTY8	44	59
ORF3 (F-15)	*P. luminescens* putative ferrisiderophore receptor FyuA	Q7N4L5	40	61
	P. fluorescens quinolobactin receptor	Q84HG2	25	42
ORF4 (F-15)	*Y. pestis* YbtX protein	Q9ZC28	35	52
ORF5 (F-15)	*Y. enterocolitica* HMWP2 non-ribosomal peptide synthetase (Irp2)	P48633	47	62
	V. anguillarum AngR protein	P19828	31	50
ORF6 (F-30)	*V. parahaemolyticus* probable ferrisiderophore receptor	Q87JU8	75*	86*
	E. coli FhuE receptor	P16869	31*	48*
ORF7 (F-30)	*V. cholerae* hypothetical protein VC1548	Q9ZHW2	55	76
ORF8 (F-30)	*V. cholerae* ExbB-related protein	Q9ZHW1	59	72
ORF9 (F-57)	*V. parahaemolyticus* ferritin	Q87TJ2	88	95
ORF10 (F-64)	*V. parahaemolyticus* PhuW protein	Q87J26	59	73
ORF11 (F-64)	*V. cholerae* TonB1 protein	O52042	43	59
ORF12 (F-64)	*V. parahaemolyticus* ExbB protein	Q9XCY5	66	83
ORF13 (F-19)	*V. vulnificus* Glutathione-S-transferase	Q8D4M6	84	91
ORF14 (F-19)	*V. parahaemolyticus* uncharacterized iron-regulated membrane protein	Q87JU1	52	73
ExbB2	*V. cholerae* ExbB2	Q9ZHWO	70	84
ExbD2	*V. cholerae* ExbD2	Q9ZHV9	84	93
TonB2	*V. cholerae* TonB2	Q9ZHV8	50	70

*Data obtained by comparing the first 245 amino acids of the pseudogene.

P. damselae ssp. *piscicida* produce under iron-limiting conditions siderophores which are not of the phenolate or hydroxamate type (Magariños *et al.* 1994).

PCR and Southern blot hybridization were used to screen a collection of *P. damselae* ssp. *piscicida* strains for presence of the putative ferrisiderophore receptor coded by ORF3. For PCR detection, primers F15-in-5′ (GCAGATAAAGGAGCACCACG) and F-44-in-3′ (CCACCATATAGCGTAGCTTG) targeted to internal regions of ORF3 gene were designed. The ca. 1 kb fragment including part of the putative ferrisiderophore receptor was amplified in only four *P. damselae* ssp. *piscicida* strains (Table 3). In addition, the complete ORF3 of clone F-15 was excised with *Bam*HI and *Sal*I and used as a probe for hybridization. The results coincided with those obtained by PCR screening, indicating a diverse ORF3 content in *P. damselae* ssp. *piscicida* strains.

The genetic makeup can vary considerably between strains of the same species. This is particularly frequent for genes coding for putative virulence genes such as those which encode iron-sequestering systems. In this study we have shown that the occurrence of the putative ferric siderophore receptor ORF3 is variable among *P. damselae* strains and subspecies. The two avirulent *P. damselae* ssp. *piscicida* strains ATCC 29690 and EPOY 8803-II tested proved negative for ORF3. However, since other ORF3 negative strains (Table 3) have been described as virulent (Magariños *et al.* 1992), we cannot relate ORF3 to virulence.

The F-15 plasmid was completely sequenced and revealed two additional Orfs, ORF4 and ORF5 (Figure 1). ORF4 showed homology to the YbtX protein of *Yersinia pestis* (Table 2). Though this protein was initially believed to be involved in siderophore yersin-

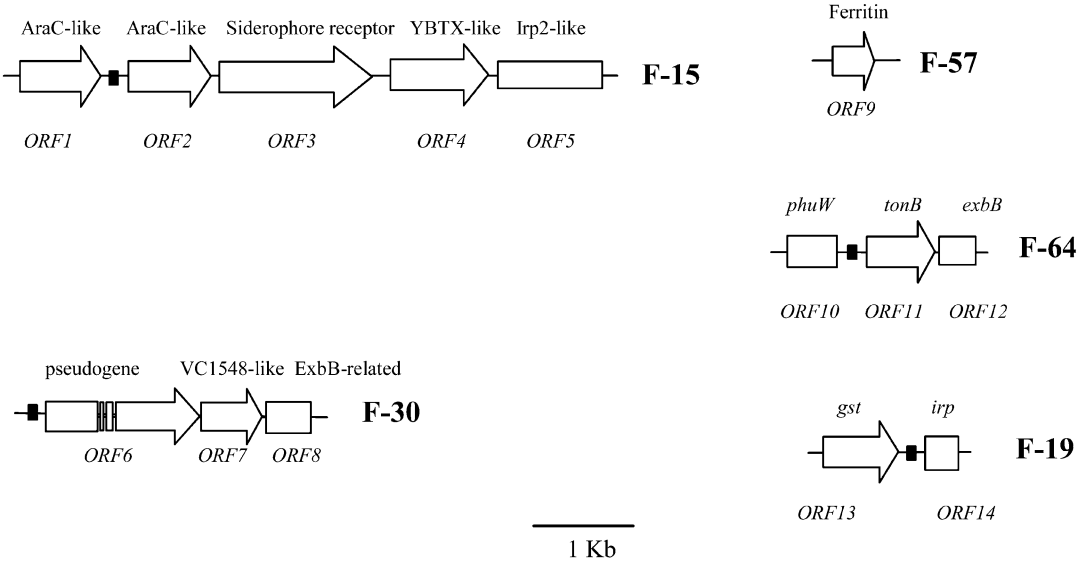

Figure 1. Schematic representation of inserts of FURTA-positive clones of *P. damselae* ssp. *piscicida*. Open arrows indicate complete open reading frames and direction of transcription. Squares indicate incomplete open reading frames. Black boxes indicate the presence of a putative Fur box.

iabactin biosynthesis recent studies have shown that YbtX is a highly hydrophobic cytoplasmic membrane protein of uncertain function, whose mutation does not have any significant effect in yersiniabactin synthesis or utilization (Fetherston *et al.* 1999). ORF5 showed homology to the IRP2 protein of *Yersinia enterocolitica*, an enzyme with non-ribosomal peptide synthase domains involved in the initial cyclization and condensation reactions involving salicylate and two cysteine molecules, implicated in the biosynthetic pathway of the siderophore yersiniabactin (Bobrov *et al.* 2002). ORF5 showed also homology to AngR, an iron regulated *Vibrio anguillarum* protein involved in the biosynthesis of the siderophore anguibactin (Crosa, 1997; Wertheimer *et al.* 1999). Since no Fur box was apparent upstream of the ORF3, ORF4 and ORF5 genes they may be regulated through ORF2 (AraC) which itself is iron regulated. Clone F-15 apparently encodes part of a ferric siderophore transport and synthesis gene cluster.

ORF6 of the FURTA positive clone F-30 coded for a 291 amino acid protein and contained a Fur box sequence which matched 14 of 19 nucleotides with the *E. coli* consensus Fur-box (Figure 2) It showed the highest similarity to the N-proximal half of a putative ferrichrome-iron receptor of *Vibrio parahaemolyticus* (Makino *et al.* 2003) (Table 2). The closest homologue with well-known function was the *Escherichia coli* FhuE receptor for coprogen, ferrioxamine B and

Consensus:	GATAATGAT	A	ATCATTATC
F-64	GATAATGAT	A	AcgATTcTt
F-30	GAaAATaAg	A	ATggTTATC
F-44	GATAATGtT	t	cTCATTATC
F-19	acTAATacg	A	ActATTATC

Figure 2. Comparison of potential Fur binding sites in FURTA positive clones with the *E. coli* Fur-box consensus sequence. Uppercase letters indicate residues which are identical to the consensus, and lowercase letters indicate residues differing from the consensus.

rhodotorulic acid (Sauer *et al.* 1990) (Table 2). The size of ORF6 was much shorter than the sequences encoding outer membrane iron receptors which are in the range of 80 kDa. Analysis of the downstream sequence indicated that ORF6 may constitute a pseudogene. Approximately 50 nucleotides downstream of the stop codon of ORF6 started a sequence of 377 amino acids which showed homology to the second half of the *V. parahaemolyticus* siderophore receptor. To see whether the two pseudogenes are only contained in the studied strain, the region comprising the frame-shift mutation was PCR-amplified in the *P. damselae* ssp. *piscicida* strains B51, IT-2, EPOY 8803-II, and ATCC 29690 and sequenced. All four strains harboured similar frame-shift mutations indicating that this pseudogene may be an ancestral feature of *P. damselae* ssp. *piscicida* and not the result of a recent genetic event in a single strain.

Table 3. Occurrence of ORF3 coding for a putative ferrisiderophore receptor in different *Photobacterium damselae* ssp. *piscicida* strains.

Strain	Origin/Source	Virulence (LD_{50}) for fish[a]	PCR detection of ORF3	DNA probe detection of ORF3
DI21	Seabream, Spain	virulent (10^3–10^5)	+	+
554.2	Sole, Spain	ND	+	+
B51	Seabass, Spain	ND	+	+
IT-2	Seabream, Italy	virulent (10^4–10^5)	−	−
ATLIT-2	*Morone sp.* Israel	ND	+	+
10831	Seabass, France	virulent (10^3–10^4)	−	−
EPOY-8803-II	Red grouper, Japan	Nonvirulent ($>10^8$)	−	−
ATCC 29690	Yellowtail, Japan	Nonvirulent ($>10^8$)	−	−
ATCC 17911	White perch, USA	virulent (10^5–10^6)	−	−

[a]Data obtained from Magariños *et al.* 1992. (LD50) is the number of cells which killed half of the fish. ND, not determined.

Clone F-57 contained an ORF coding for a 175-amino acid protein with homology to ferritin proteins (Table 2). Since no Fur box was evidenced in the region upstream of the ferritin gene, it is likely that the FURTA-positive phenotype is caused by withdrawal of iron from Fur by the iron-binding activity of ferritin.

The insert of F-64 contained the promoter region and the sequence of a *tonB* (ORF11) gene, coding for a putative protein which showed the highest similarity to the TonB1 protein of *Vibrio cholerae* (Table 2). F-64 also contained the 5′ region of a putative oxygen-independent coproporphyrinogen oxidase homologue (*phuW*) (ORF10) and an *exbB* gene (ORF12) (Figure 1). The TonB, ExbB (and ExbD) proteins are key components in the transport of ferric siderophore complexes and heme through the outer membrane of gram negative bacteria. A consensus Fur-box sequence which matched 15 of 19 nucleotides with *E. coli* consensus Fur-box (Fig. 2) is contained in the intergenic region between the *tonB* and *phuW* homologues. The PhuW oxidase may degrade heme and release iron. *V. cholerae* contains two sets of *tonB exbB exbD* genes of which *tonB1 exbB1 exbD1* are linked to heme transport genes (Occhino *et al.* 1998).

The insert of clone F-19 contained two partial ORFs. ORF13 showed homology to the carboxy-terminal region of glutathione-S-transferases. Domains of ORF14 showed homology to FptA, the pyochelin receptor of *Pseudomonas aeruginosa* (Ankenbauer and Quan, 1994). A putative Fur-box matching 12 of 19 nucleotides with the *E. coli* consensus sequence was evidenced upstream of the ORF14 start codon (Figure 2).

Cloning and sequence analysis of the complete TonB2 system. F-30 harboured a complete ORF7 and a partial ORF8 with homology to *V. cholerae* hypothetical protein VC1548, and a protein which on the basis of C-terminal homologies was tentatively related to ExbB in several genome sequences of Vibrionaceae although the proteins have double the size of ExbB proteins with defined functions. These two genes are part of a cluster of *V. cholerae* genes which includes the TonB2 system (Occhino *et al.* 1998). In order to determine whether *P. damselae* ssp. *piscicida* harboured a second set of *tonB* genes, chromosome walking downstream of F-30 was carried out by inverse PCR. Chromosomal DNA was cut with *Bgl*II, self-ligated and used as a template for inverse-PCR with suitable primers. A band of ca. 7 kb was amplified, cloned in pGEMT-Easy to yield pCAR149, and partially sequenced. Four ORFs were found downstream of the VC1548 homologue which showed homology to the ExbB-related protein, and the ExbB2, ExbD2 and TonB2 proteins (TonB2 system) of *V. cholerae,* respectively (Figure 4, Table 2).

Like *V. cholerae, P. damselae* ssp. *piscicida* apparently contains two sets of *tonB exbB exbD* genes. To examine iron regulation, plasmid pCAR149 was transformed into the *E. coli* H1717 indicator strain and showed a *lacZ*$^-$ phenotype, suggesting that none of the genes of the second TonB system is under Fur regulation. The same gene arrangement is encountered in the recently annotated genome of *V. parahaemolyticus* (Makino *et al.* 2003). In contrast, *V. cholerae* (Heidelberg *et al.* 2000) and *Vibrio vulnificus* (Chen *et al.*

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                    *         20         *         40         *         60
P.damselae      M-----------YRNSFSLSPLSLAILAATLSPVVSADEHNP---EVTVMETMIVEATKQ :  46
V.vulnificus    MAALRPARTSVAEKKIFKLHALSAVVMGLCASGQAYAQTENTNSNKKEEMPVVVVIGEKT :  60

                    *         80         *        100         *        120
P.damselae      NISLQQVDSSVLIKTGKELEKAG-IHEVKDLEKAFPGLMIQTRGNRTYAN-------TTI :  98
V.vulnificus    ERTIYDTSSSVQVFDQETIDNTPGATEIDDLLQLIPNMVDSGQGNSMPTVRGIDGSGPSI : 120

                    *        140         *        160         *        180
P.damselae      RGVSSPDYYSPTVSIYVDG---VLQDSAFLTQQLLNVEQVELLRGPQATLYGGNSQGGII : 155
V.vulnificus    GGLASFAGTSPRLNMSIDGRSLTYSEIAFGPRSLWDMQQVEVYLGPQSYIQGRNASAGAI : 180

                    *        200         *        220         *        240
P.damselae      NIITKKATD--QNLASGGVTYSDRSKQLDASAAVAISDSTYADIAIRSLKDEGNIRHIPS : 213
V.vulnificus    VMKTNDPTHHFESAVKAGVGERNYSQTAAMISAPIIQDELAFRLSFDQQKRDSFVDLASY : 240

                    *        260         *        280         *        300
P.damselae      NTDDANDSKDFSGTARFHYLPQQSPLAVTFSVSSAHLDSHEEWYLTQQEYDKRATSQDIP : 273
V.vulnificus    EPAGDAKKIEMNSVRGKLLYEPSALAGFKTTLGVSHMDSRGPQSESTNVVGN-EAFRPVY : 299

                    *        320         *        340         *        360
P.damselae      ELKRVVNSYALHIDYDLGATQLTSITAYQ---NRNVDREYIGGKWVEDQNTFSQEVRANT : 330
V.vulnificus    ETKSLSTAWDISWQLNEVLTFENNLVYSKFAFERYTNPLQKGDYTAEGKEFHVEPLLRYL : 359

                    *        380         *        400         *        420
P.damselae      QFSDDLSALVGG-YYEHRN----FDVSAGANNDIETQTYALFGQMTYAMTDSIDLTGGLR : 385
V.vulnificus    SLGGRVNALVGARYYKSSQDDEYVDATSANPMSGSTKTQSAFAELTYALTQSIDVTVAGR : 419

                    *        440         *        460         *        480
P.damselae      ASRFFSESDFGGNSAWRIDSYQSDKSENVISPKAAIGWQINPDSRVFASLASGYRPG--G : 443
V.vulnificus    YEKERVKRKVS-DPRFKLD---HDDTLSVFLPKFDIAFKPDMAQTFGFKVAKGYNSGGAG : 475

                    *        500         *        520         *        540
P.damselae      FSPAPRSSGDKNGYSPEKSFNGELGWRT-NLAENTVRFSGALYWIDTKDIQLYTGNP-GS : 501
V.vulnificus    LAFNPILGGGFSPYQFEEEYIWNYEFYTRHRLGNSVELMTNTFYNDFDNMQMTQTLSNGD : 535

                    *        560         *        580         *        600
P.damselae      QVLRNMGDAQSRGIELDLAYYPTDDLTLTLGATFGQSTFESGNDGIEGKTLPYAPDTTVV : 561
V.vulnificus    VLIANLDNAKTYGAEIGTRWYATDSLELFANLGLLKTEYKEVNG--TSKELERAPNMTGN : 593

                    *        620         *        640         *        660
P.damselae      AGIEYYLPQSWLDGELSIATNARYTSKIEFDESN--TLAQDGYTLIDLAVQYAMNDNISF : 619
V.vulnificus    LGGQY----SFFDG-FELSANAAYTGDYESDRSNTEIVKIDAYWVANAQLAYVFENGRAA : 648

                    *        680         *        700
P.damselae      RVFSNNLTDKEYTTYAYSFGNTRFNNYGSEREVGVNMKLEW : 660
V.vulnificus    LFATNLFDSDKTTLYARGSLNEPLK--QQPRMIGASLQLNF : 687
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Figure 3. Sequence alignment of the putative *P. damselae* ssp. *piscicida* ferrisiderophore receptor of clone F-15 with the *V. vulnificus* ferrisiderophore receptor. Shaded residues denote identical positions in the two proteins.

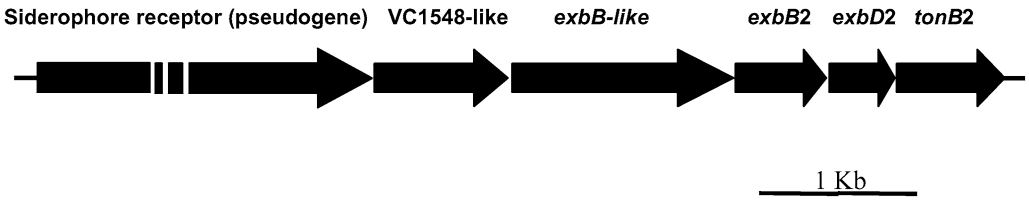

Figure 4. Structure of the *P. damselae* ssp. *piscicida* second TonB system and upstream genes.

2003) do not harbour a putative siderophore receptor linked to the TonB2 System.

Our study has shown that *P. damselae* ssp. *piscicida* harbours at least two sets of genes encoding TonB, ExbB and ExbD proteins. In Gram-negative bacteria, these three proteins are part of a cytoplasmic membrane complex involved in the active transport of a series of iron-containing ligands across the outer membrane. The arrangement of genes in the two TonB systems of *P. damselae* ssp. *piscicida* is the same as described for *V. cholerae*, the first species in which two sets of TonB genes were discovered (Occhino *et al.* 1998). Thereafter, other Gram-negative bacteria were shown to contain two sets of TonB genes, including several representatives of the genus *Vibrio* (O'Malley *et al.* 1999). This study represents the first description of two sets of TonB genes in a *Photobacterium* species, adding a new genus to the increasing list of bacterial genera which harbour two TonB systems.

In a previous study (Magariños *et al.* 1994) it was found that three outer membrane proteins of 105, 118 and 145 kDa were overproduced in *P. damselae* ssp. *piscicida* DI21 under iron-restricted conditions. None of these proteins were identified in the present study which indicates that the number of Fur-regulated genes in *P. damselae* ssp. *piscicida* may be higher than those isolated in this study. Fur boxes which differ substantially from the *E. coli* consensus Fur boxes may not bring about sufficient Fur titration as to allow derepression of the *fhu*F::*lac*Z gene fusion. This would cause an underestimation of the iron-responsive genes as has been reported for example in *Helicobacter pylori* (Fassbinder *et al.* 2000), and in *Campylobacter jejuni* (van Vliet *et al.* 1998). Similarly, in *Salmonella typhimurium* eight Fur-regulated promoters were isolated by FURTA, while the number of Fur-regulated genes may be between 80 to 100 (Panina *et al.* 2001).

In conclusion, we have identified four iron-regulated genes and/or operons and a putative iron-binding protein gene in *Photobacterium damselae* ssp. *piscicida*. These results represent the first survey for iron-regulated genes in this fish pathogenic bacterium, setting the basis for future genetic analyses aimed at understanding the role played by iron-sequestering systems in the virulence of *P. damselae* ssp. *piscicida*.

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References

Ankenbauer RG, Quan HN. 1994 FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. *J Bacteriol* **176**, 307–319.

Bobrov AG, Geoffroy VA, and Perry RD. 2002 Yersiniabactin production requires the thioesterase domain of HMWP2 and YbtD, a putative phosphopantetheinylate transferase. *Infect Immun* **70**, 4204–4214.

Braun V, Hantke K. 1991 Genetics of bacterial iron transport. In: Winkelmann G, ed. *CRC Handbook of microbial iron chelates*. Boca Raton: CRC Press: 107–138.

Chen CY, Wu KM, Chang YC, *et al*. 2003 Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. *Genome Res* **13**, 2577–2587.

Crosa JH. 1997 Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. *Microbiol Mol Biol Rev* **61**, 319–336.

Do Vale A, Ellis AE, Silva MT. 2001 Electron microscopic evidence that expression of capsular polysaccharide by *Photobacterium damselae* subsp. *piscicida* is dependent on iron availability and growth phase. *Dis Aquat Org* **44**, 237–240.

Fassbinder F, van Vliet AHM, Gimmel V, Kusters JG, Kist M, Bereswill S. 2000 Identification of iron-regulated genes of *Helicobacter pylori* by a modified Fur titration assay (FURTA-Hp). *FEMS Microbiol Lett* **184**, 225–229.

Fetherston JD, Bearden SW, and Perry RD. 1996 YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. *Mol Microbiol* **22**, 315–325.

Fetherston JD, Bertolino VJ, and Perry RD. 1999 YbtP and YbtQ: two ABC transporters required for iron uptake in *Yersinia pestis*. *Mol Microbiol* **32**, 289–299.

Gallegos MT, Michán C, and Ramos JL. 1993 The XylS/AraC family of regulators. *Nucl Acids Res* **21**, 807–810.

Hantke K. 1981 Regulation of ferric iron transport in *Escherichia coli* K-12: isolation of a constitutive mutant. *Mol Gen Genet* **182**, 288–292.

Hantke K. 1987 Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12: *fur* not only affects iron metabolism. *Mol Gen Genet* **210**, 135–139.

Heidelberg JF, Eisen JA, Nelson WC, *et al*. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**, 477–483, 2000.

Heinrichs DE, Poole K. 1993 Cloning and sequence analysis of a gene (*pchR*) encoding an AraC family activator of pyochelin and ferripyochelin receptor synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **175**, 5882–5889.

Juíz-Río S, Osorio CR, Lemos ML. 2004 Identification and characterisation of the *fur* genes in *Photobacterium damselae* ssp. *piscicida* and ssp. *damselae*. *Dis Aquat Org* **58**, 157–164.

Magariños B, Santos Y, Romalde JL, Rivas C, Barja JL, Toranzo AE. 1992 Pathogenic activities of the live cells and extracellular products of the fish pathogen *Pasteurella piscicida*. *J Gen Microbiol* **138**, 2491–2498.

Magariños B, Romalde JL, Lemos ML, Barja JL, Toranzo AE. 1994 Iron uptake by *Pasteurella piscicida* and its role in pathogenicity for fish. *Appl Environ Microbiol* **60**, 2990–2998.

Magariños B, Bonet R, Romalde JL, Martínez MJ, Congregado F, Toranzo AE. 1996a Influence of the capsular layer on the virulence of *Pasteurella piscicida* for fish. *Microb Pathog* **21**, 289–297.

Magariños B, Toranzo AE, Romalde JL. 1996b Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. *Annu Rev Fish Dis* **6**, 41–64.

Makino K, Oshima K, Kurokawa K, *et al*. 2003 Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet* **361**, 743–749.

Matthijs S, Baysse C, Koedam N, Tehrani KA, Verheyden L, Budzikiewicz H, Schafer M, Hoorelbeke B, Meyer JM, De Greve H, and Cornelis P. 2004 The *Pseudomonas* siderophore quinolobactin is synthesized from xanthurenic acid, an intermediate of the kynurenine pathway. *Mol Microbiol* **52**, 371–384.

Neilands JB. 1990 Molecular aspects of regulation of high affinity iron absortion in microorganisms. *Adv Inorg Biochem* **8**, 63–90.

Neilands JB. 1995 Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* **270**, 26723–26726.

Occhino DA, Wyckoff EE, Henderson DP, Wrona TJ, Payne SM. 1998 *Vibrio cholerae* iron transport: haem transport genes are linked to one of two sets of *tonB*, *exbB*, *exbD* genes. *Mol Microbiol* **29**, 1493–1507.

O'Malley SM, Mouton SL, Occhino DA, *et al*. 1999 Comparison of the heme iron utilization systems of pathogenic vibrios. *J Bacteriol* **181**, 3594–3598.

Panina EM, Mironov AA, and Gelfand MS. 2001 Comparative analysis of Fur regulons in gamma-proteobacteria. *Nuc Acids Res* **29**, 5195–5206.

Sambrook J, Rusell DW. 2001 *Molecular cloning: a laboratory manual.* Cold Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press.

Sauer U, Hantke K, and Braun V. 1990 Sequence of the FhuE outermembrane receptor gene of *Escherichia coli* K-12 and properties of mutants. *Mol Microbiol* **4**, 427–437.

Stojiljkovic I, Bäumler AJ, Hantke K. 1994 Fur regulon in Gram-negative Bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. *J Mol Biol* **236**, 531–545.

Tabor S, Richardson CC. 1985 A bacteriophage T7 RNA polymerase-promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA*. **82**, 1074–1078.

van Vliet AHM, Wooldridge KG, Ketley JM. 1998 Iron-responsive gene regulation in a *Campylobacter jejuni fur* mutant. *J Bacteriol* **180**, 5291–5298.

Wertheimer AM, Verweij W, Chen Q, *et al*. 1999 Characterization of the *angR* gene of *Vibrio anguillarum*: essential role in virulence. *Infect Immun* **67**, 6496–6509.