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1 Short Communication

2

3 **Identification of iron regulated genes in the fish pathogen**

4 ***Aeromonas salmonicida* subsp. *salmonicida*: genetic diversity**

5 **and evidence of conserved iron uptake systems**

6

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1 **Abstract**

2 In this study we applied the Fur titration assay (FURTA) to isolate iron regulated genes
3 in *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of furunculosis in
4 fish. We have identified genes for siderophore biosynthesis and for ferri-siderophore
5 transport, some of which have been previously described in this species. A gene for a
6 hitherto uncharacterized functional hemin receptor HutE was identified, but its
7 inactivation did not affect significantly the use of hemin as sole iron source, suggesting
8 that redundant genes encoding hemin receptors exist in the *A. salmonicida* genome.
9 Additional FURTA positive clones contained genes encoding a Flavoprotein, a
10 transcriptional regulator protein, a DNA binding protein Fis, as well as genes encoding
11 putative DapD and Flp/Fap-pilin proteins. A screening of gene distribution
12 demonstrated that all the analyzed strains shared genes for siderophore biosynthesis and
13 transport and for heme utilization, indicating that these two systems of iron acquisition
14 are a conserved trait in this subspecies, whereas other genes are not common to all the
15 isolates. Thus, the Fur regulon of *A. salmonicida* subsp. *salmonicida* includes both
16 conserved and differentially-occurring genes, suggesting that the genetic diversity
17 within this fish pathogen might be higher than expected.

18

19 **Keywords:** *Aeromonas salmonicida*, iron regulation, iron uptake, Fur

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1 **1. Introduction**

2 *Aeromonas salmonicida* subsp. *salmonicida* is the etiological agent of furunculosis
3 in fish, a disease which causes significant losses in cultivated salmonids and a variety of
4 non-salmonid fish in fresh and marine waters (Toranzo et al., 2005). Several virulence
5 factors have been reported which include, among others, exotoxins (Lee and Ellis,
6 1990), surface layer protein (Chu et al., 1991), and a type III secretion system (Dacanay
7 et al., 2006).

8 The acquisition of iron is recognized as one of the key steps in the survival of
9 bacterial pathogens within their hosts, and contributes significantly to virulence (Braun,
10 2005). Bacterial pathogens can obtain iron by means of siderophores (Crosa and Walsh,
11 2002), as well as by siderophore-independent mechanisms which involve utilization of
12 host transferrins, lactoferrins, ferritins and hemoproteins. The expression of genes
13 involved in iron acquisition is tightly regulated by the ferric uptake regulator protein
14 Fur, which acts as an iron-responsive DNA binding repressor (de Lorenzo et al., 1987).
15 The Fur-binding site was demonstrated to be a 19-bp palindromic consensus sequence
16 named Fur box (de Lorenzo et al., 1988). A genetic screening known as the Fur titration
17 assay (FURTA) was devised to identify Fur-regulated genes (Stojiljkovic et al., 1994).

18 Iron-regulated *A. salmonicida* proteins have demonstrated to be protective antigens
19 for fish, and are good candidates for the improvement of vaccines (Hirst and Ellis,
20 1994). Thus, the knowledge of the Fur regulon of a bacterial pathogen is of great
21 interest. In this study we report the identification of novel Fur-regulated genes of *A.*
22 *salmonicida* subsp. *salmonicida* and show that the Fur regulon includes both conserved
23 and differentially-occurring genes.

24

25 **2. Materials and methods**

1 2.1. *Bacterial strains and culture conditions*

2 All the *A. salmonicida* subsp. *salmonicida* strains (Table 1) were isolated in our
3 laboratory from diseased fish that showed the characteristic symptoms of furunculosis.
4 Strains were subjected to taxonomical analysis by standard morphological,
5 physiological, and biochemical plate and tube tests, and all were unambiguously
6 identified as *A. salmonicida* subsp. *salmonicida*. *A. salmonicida* strains were grown at
7 22 °C in tryptic soy agar (Difco) and in Luria Bertani (LB) medium supplemented with
8 1% NaCl. Antibiotics were used at the following final concentrations: ampicillin, 50 µg
9 mL⁻¹; chloramphenicol, 20 µg mL⁻¹; tetracycline, 15 µg mL⁻¹. Bovine hemin and
10 hemoglobin (Sigma) were prepared at 5 mM in 10 mM NaOH and at 1 mM in ultrapure
11 water, respectively. 2,2'-Dipyridyl (stock 20 mM) and ethylenediamine di (*o*-hydroxy-
12 phenylacetic acid) (EDDA) (stock 138 mM) were used as iron chelating agents.

13

14 2.2. *Fur titration assay (FURTA) and DNA sequence analysis*

15 The Fur Titration Assay (FURTA) was carried out as previously described (Najimi
16 et al., 2008a). Positive clones were sequenced using the CEQ DTCS-Quick Start Kit
17 (Beckman Coulter) and a capillary DNA sequencer CEQ8000 (Beckman Coulter). The
18 European Bioinformatics Institute service and the NCBI services were used for
19 comparison of the sequence data with published sequences via the Internet.

20

21 2.3. *Analysis of hutE gene and construction of a hutE-targeted in-frame deletion mutant*

22 The *hutE* gene was PCR-amplified, cloned in pACYC184 yielding pMON37, and
23 transformed into *E. coli* 101ESD Δ (*entC-entA*) containing plasmid pSML33 (Mouriño
24 et al., 2004). This strain cannot utilize hemin unless transformed with a plasmid
25 encoding a functional outer membrane heme receptor. About 100 µl of overnight

1 cultures of the *E. coli* 101ESD transformants were added to 3 ml of molten soft CM9
2 minimal medium (Najimi et al., 2008a) and plated onto appropriate pre-poured CM9
3 plates supplemented with 110 μ M 2,2'-dipyridyl. Sterile filter-paper disks were loaded
4 with 20 μ l of either 5 mM hemin or 1 mM hemoglobin. Disks spotted with 5 mM FeSO₄
5 were included as positive controls. Results were annotated as positive or negative after
6 24 h of incubation. Deletion of an internal region of the *hutE* gene was carried out as
7 previously described (Najimi et al., 2008a), using *A. salmonicida* subsp. *salmonicida*
8 RSP74.1 strain as a host. Parental (RSP74.1) and *hutE* mutant strains (Δ *hutE*) were
9 grown under iron-sufficient (CM9 supplemented with 10 μ M hemin) and iron-deficient
10 conditions (CM9 supplemented with 100 μ M EDDA and 10 μ M hemin), and growth
11 was monitored by measuring optical densities at 600 nm (OD₆₀₀) after 12 h.

13 *2.4. DNA-probe and PCR-based screening of A. salmonicida strains for the presence of* 14 *FURTA-positive genes*

15 Suitable primers targeted to the FURTA-positive genes described in this study were
16 designed to screen a collection of *A. salmonicida* subsp. *salmonicida* strains by PCR
17 and DNA probe hybridization. Southern-blot analyses were performed using the ECL
18 Direct Nucleic Acid labelling and detection system (Amersham Biosciences).

20 **3. Results and discussion**

22 *3.1. Identification of novel Fur-regulated genes in A. salmonicida subsp. salmonicida* 23 *ACR168.1*

24 *Aeromonas salmonicida* subsp. *salmonicida* ACR168.1 (Table. 1) produces a
25 catecholate-type siderophore and utilizes heme as iron source. In a previous study we

1 applied the FURTA assay to this strain, and gene clusters involved in siderophore
2 biosynthesis (Najimi et al., 2008a) (Fig. 1A) and in heme uptake (Najimi et al., 2008b)
3 (Fig. 1B), were described. Here we analyzed 106 FURTA-positive clones not analyzed
4 previously, and eight distinct types of clones were finally selected (Fig. 1C) and
5 classified into four functional categories: siderophore biosynthesis genes, siderophore
6 transport genes, heme transport genes, and other Fur regulated genes. Table 2
7 summarizes the homologous proteins that showed high similarity to the *A. salmonicida*
8 subsp. *salmonicida* proteins.

10 3.2. Genes related to siderophore biosynthesis

11 Some FURTA clones contained the already described genes *asbB*, *asbI* and *asbD*
12 (Najimi et al. 2008a) (Fig. 1A). We did not find genes for 2,3-dihydroxybenzoic acid
13 (DHBA) synthesis, which is an essential component of catecholate siderophores (Crosa
14 and Walsh, 2002). Genes for DHBA synthesis are typically clustered with the rest of the
15 biosynthetic genes, and contain Fur boxes in their promoters. The reasons why we did
16 not isolate DHBA synthesis genes in the FURTA assay are unknown.

18 3.3. Genes related to siderophore transport

19 pFMON54 encodes two ORFs (Fig. 1C). The AsbE protein showed a 84% identity
20 to FstA, a putative outer membrane siderophore receptor identified in *A. salmonicida*
21 using a proteomic approach (Ebanks et al., 2004). Thus, the *asbE* gene is likely the
22 homologue of *fstA*. Recently it was demonstrated that *fstA* is conserved among different
23 isolates and was proposed as a useful tool for PCR-based identification of this
24 bacterium (Beaz-Hidalgo et al., 2008). AsbM shows similarity to periplasmic ferric-
25 siderophore ABC transporters. Periplasmic binding proteins (PBP) are a necessary

1 component for transport of the iron-siderophore complex into the cytoplasm. To the best
2 of our knowledge, *asbM* constitutes the first ABC-transporter gene identified in this fish
3 pathogen with a predicted function in siderophore uptake.

4

5 *3.4. Genes related to heme transport: Mutation of hutE does not affect heme iron*
6 *utilization in A. salmonicida*

7 Some FURTA positive clones harbour genes which are part of the *A. salmonicida*
8 heme uptake cluster (Fig. 1B) (Najimi et al., 2008b). We recently reported that deletion
9 of *hutA* did not completely abolish heme utilization, suggesting the existence of a yet
10 uncharacterized heme receptor. Interestingly, clone pFMON69 was found to encode a
11 novel 721 amino acid protein HutE that showed high similarity with heme receptors,
12 including HutA of *A. salmonicida* and *A. hydrophila*. When *hutE* cloned in plasmid
13 (pMON37) was transformed into *E. coli* 101ESD harbouring plasmid pSML33, the
14 resulting strain could utilize both hemin and hemoglobin as iron sources, when tested in
15 a CM9 medium plate assay (see methods above). This result clearly demonstrates that
16 *hutE* encodes a functional outer membrane heme receptor.

17 We then constructed a deletion mutant of *hutE* gene in strain RSP74.1.
18 Surprisingly, the Δ *hutE* mutant strain did not show significant differences in
19 comparison with parental strain in its ability to use hemin as sole iron source (data not
20 shown). This indicates that in this *A. salmonicida* strain, neither *hutA* nor *hutE* are
21 completely essential for the use of hemin as sole iron source. Presence of more than one
22 outer membrane heme receptor gene has been reported in other gram negative bacteria
23 such as *V. cholerae*, where at least three heme receptors contribute to heme iron
24 acquisition (Mey et al., 2001).

25

1 3.5. Other *Fur*-regulated genes

2 Plasmid pFMON10 encodes a putative DNA binding protein Fis (Fig. 1C). Fis, or
3 the factor for inversion stimulation protein, is a regulator of several bacterial functions,
4 and binds specifically to weakly related DNA sequences (Osuna et al., 1995). This
5 protein has been shown to play a role in the regulation of virulence factors in *E. coli*
6 (Goldberg et al., 2001). Plasmid pFMON20 contains two ORFs: The first encodes a
7 putative flavoprotein, and the second encodes a predicted ferritin, a protein that
8 functions as an iron storage protein in microorganisms. Although this gene does not
9 contain a *Fur* box, ferritin genes are usually isolated as FURTA-positive clones because
10 the iron-binding activity of ferritin titrates out the intracellular iron.

11 pFMON35 encodes two ORFs: PilD is homologous to Leader peptidase PilD and
12 type IV prepilin peptidase family proteins. The second is similar to Flp/Fap pilin
13 components, which are present at the bacterial cell surface and are necessary to adhere
14 to the host cell. Type IV pili was mentioned as a virulence factor in *A. salmonicida*
15 (Masada et al., 2002). Recently it was reported that FLP pili are new members of the
16 type IV class and it was also demonstrated that Flp is an iron regulated protein in *A.*
17 *salmonicida* (Boyd et al., 2008).

18 The first ORF of pFMON39 encodes a homologue of immune inhibitor A proteins
19 and proteases. Immune inhibitor A is a metallopeptidase, which specifically cleaves
20 host antibacterial proteins. A homologue of immune inhibitor A, PrtV, has been
21 identified in *V. cholerae* but a *prtV* null mutant was not attenuated in pathogenicity
22 (Ogierman et al., 1997). The second ORF encodes a hypothetical protein.

23 pFMON61 encodes a protein similar to 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate
24 N-succinyl transferases (DapD). The *E. coli* *dapD* gene is involved in diaminopimelate
25 and lysine biosynthesis (Richaud et al., 1984). Plasmid pFMON91 contained two ORFs:

1 FieF showed similarity to cation efflux proteins and cation transporter proteins, and the
2 second ORF, *cpxR*, encodes a protein similar to transcriptional regulatory proteins and
3 two component transcriptional regulators in other bacteria.

4

5 3.6. Occurrence of *Fur*-regulated genes in *A. salmonicida* subsp. *salmonicida* strains

6 It was found that only *asbD*, *asbE* and *hutA* genes are conserved in all the strains
7 (Table 1; Fig. 2). Previous studies reported that strains of *A. salmonicida* subsp.
8 *salmonicida* isolated from different sources all seemed to produce the same catechol-
9 type siderophore (Fernández et al., 1998; Najimi et al., 2008a), and a ferric siderophore
10 receptor *fstA* was found to be conserved among *A. salmonicida* strains (Beaz-Hidalgo et
11 al., 2008). The presence of *asbE* (the *fstA* homologue) in all the *A. salmonicida* strains
12 tested, reinforces the hypothesis of a siderophore-mediated iron uptake system
13 conserved in the subspecies. Distribution of *hutA* in all the isolates also suggests a
14 conservation of the heme iron utilization system (Table 1; Fig. 2B). However, *hutE* was
15 present uniquely in 3 out of 18 strains (Table 1; Fig. 2C).

16 Since all the strains analyzed were isolated from diseased fish undergoing the
17 typical symptoms of furunculosis, it is expected that all the isolates are virulent. Thus,
18 the presence of *asbD*, *asbE* and *hutA* genes shows a correlation with virulence.
19 However, the remaining genes are absent from a fraction of the strains, which suggests
20 that they are not essential for the virulence of *A. salmonicida*. A high genetic diversity
21 in presence of *Fur*-regulated genes is evidenced in this fish pathogen, since as many as
22 12 different gene patterns are observed within the 18 strains tested (Table 1). This is
23 particularly interesting because all the strains were isolate within a reduced geographic
24 area (North of Portugal, and NW Spain). Our results also suggest that siderophore
25 biosynthesis/transport and heme uptake genes *asbD*, *asbE* and *hutA* are good candidates

1 to be used as targets for DNA-based diagnosis of furunculosis, and the proteins they
2 encode could be useful targets for the design of inhibitors aimed at abolish the
3 utilization of iron sources.

4

5 3.7. Nucleotide sequence accession numbers

6 The EMBL database accession numbers of the nucleotide sequences described in
7 this study are AM712656; AM712657; AM712658; AM712659; AM712660;
8 AM712661; AM712662; AM712663; AM712664; AM712665.

9

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14

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1 **Figure Legends**

2 Fig. 1. Schematic representation of (A) the siderophore biosynthesis gene cluster of *A.*
3 *salmonicida* subsp. *salmonicida*, (B) the heme uptake gene cluster, and (C) inserts of
4 novel FURTA-positive clones of *A. salmonicida* subsp. *salmonicida* identified in this
5 study. ORFs are denoted as arrows indicating the direction of transcription. Black boxes
6 indicate the presence of a putative Fur box.

7

8 Fig. 2. Results of Southern hybridization screening for the presence of *asbD* (A), and
9 *hutA* genes (B), using digested chromosomal DNA from a collection of *A. salmonicida*
10 subsp. *salmonicida* strains, and results of PCR-based screening for presence of *hutE*
11 gene (C). Lanes 1-18, *A. salmonicida* subsp. *salmonicida* strains in the same order as in
12 Table 1. (M) Molecular mass marker. Numbers at sides indicate the size of specific
13 marker bands in kilobases.

14

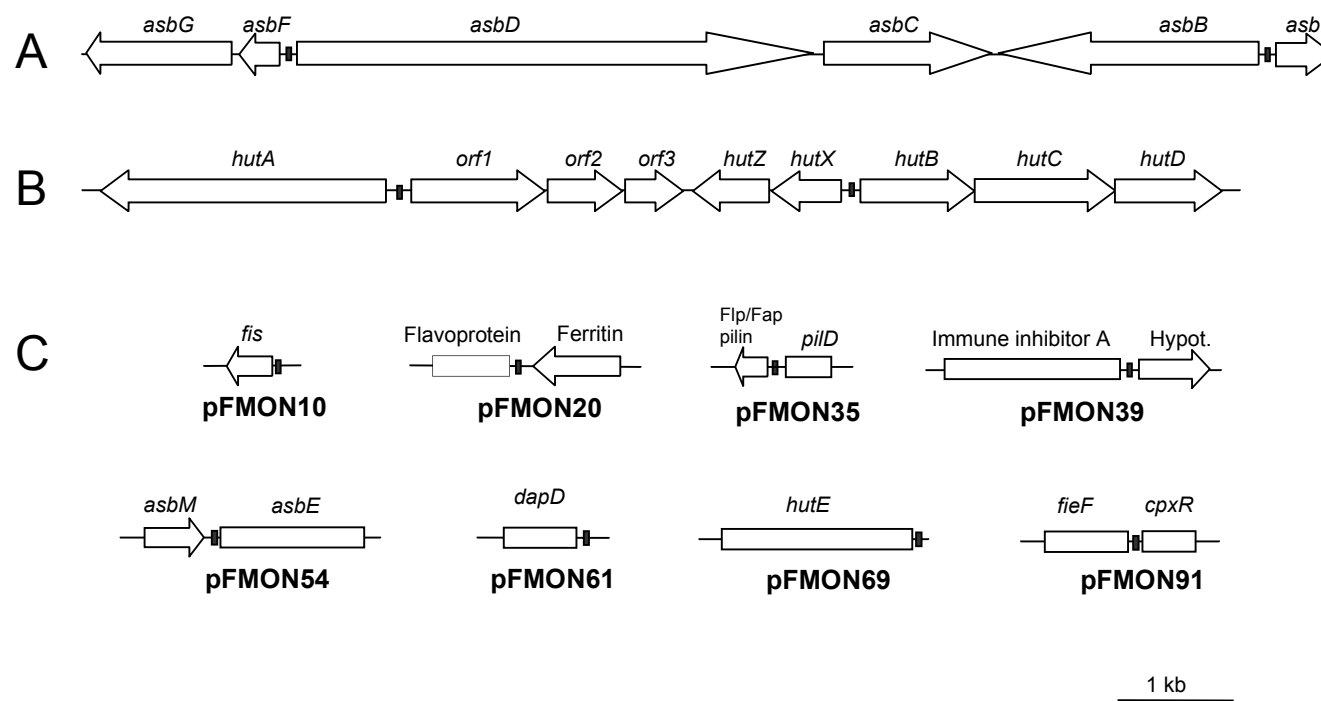


Fig. 1

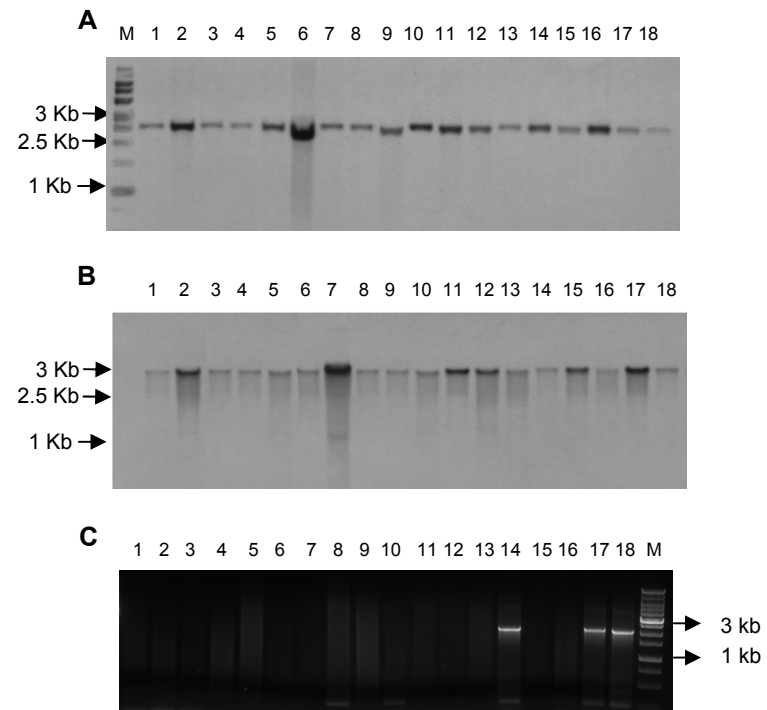


Fig. 2

1 Table 1

2 *A. salmonicida* isolates included in this study, and results of the screening for the presence of Fur regulated genes. (+) indicates presence, and (-)
 3 indicates absence of the respective gene. Results were obtained by PCR amplification using suitable primers. Presence of *asbD* and *hutA* genes
 4 was also tested by Southern hybridization with DNA probes, and results were coincident with those derived from PCR tests.

	<i>A. salmonicida</i> strain	Isolation source	<i>asbD</i>	<i>asbE</i>	<i>hutA</i>	<i>hutE</i>	<i>fis</i>	<i>Immune inhibitorA</i>	<i>Flavo protein</i>	<i>flp/ pilin</i>	<i>dapD</i>	<i>cpxR</i>
1	Santalla 8-1-03	Atlantic salmon, Spain	+	+	+	-	+	+	-	-	-	+
2	EO2103	Atlantic salmon, Spain	+	+	+	-	+	-	-	-	-	-
3	EO 0303	Atlantic salmon, Spain	+	+	+	-	+	+	+	-	-	-
4	EO1603R	Atlantic salmon, Spain	+	+	+	-	+	+	-	+	-	+
5	ULLA2002	Atlantic salmon, Spain	+	+	+	-	+	-	-	-	-	+
6	11-Louzara	Atlantic salmon, Spain	+	+	+	-	+	+	-	+	-	-
7	X-Santalla	Atlantic salmon, Spain	+	+	+	-	+	+	-	-	-	-
8	RIM37.1	Turbot, Spain	+	+	+	-	+	+	+	+	+	+
9	RSP83.1	Turbot, Portugal	+	+	+	-	+	+	+	+	+	+
10	RIM33.1	Turbot, Spain	+	+	+	-	+	+	+	+	+	+
11	PC884.1	Turbot, Spain	+	+	+	-	+	+	-	+	-	+
12	RM2 66.1	Turbot, Spain	+	+	+	-	+	-	-	+	-	-
13	ACR173.1	Turbot, Spain	+	+	+	-	-	-	-	+	-	-
14	ACR168.1	Turbot, Spain	+	+	+	+	+	+	+	+	+	+
15	SF3 1/03	Turbot, Spain	+	+	+	-	+	+	-	-	-	-
16	RO11.1	Turbot, Spain	+	+	+	-	+	+	-	+	-	+
17	RSP74.1	Turbot, Portugal	+	+	+	+	+	+	+	+	+	+
18	ACRP43.1	Turbot, Portugal	+	+	+	+	+	+	+	+	-	+

1 Table 2
 2 Proteins with homology to *A. salmonicida* subsp. *salmonicida* proteins encoded in the
 3 FURTA positive clones described in this study

FURTA clones	Accession no.	Identity (%)	Similarity (%)
<i>A. salmonicida</i> proteins			
pFMON69			
HutE			
Hemin receptor (<i>A. hydrophila</i>)	YP 856199	90	95
pFMON54			
Periplasmic ABC siderophore transporter (AsbM)			
Siderophore ABC transporter BauB (<i>P. entomophila</i>)	CAK15298	76	86
Ferric siderophore receptor (AsbE)			
Ferric siderophore receptor (<i>A. salmonicida</i>)	CAA61245.1	84	86
pFMON20			
Ferritin			
Ferritin (<i>A. hydrophila</i>)	ABK37728	91	94
Flavoprotein			
Predicted flavoprotein (<i>V. vulnificus</i>)	NP 760067	82	90
pFMON10			
DNA binding protein Fis			
DNA binding protein Fis (<i>E. coli</i>)	NP 289829	70	85
pFMON39			
Immune inhibitor A			
Immune inhibitor A (<i>A. hydrophila</i>)	ABK37971	93	97
Hypothetical protein			
Hypothetical protein (<i>A. hydrophila</i>)	YP 857946	93	94
pFMON35			
peptidase PilD			
Leader peptidase PilD (<i>A. hydrophila</i>)	ABK37795	91	93
Flp/Fap pilin component			
Flp/Fap pilin component (<i>A. hydrophila</i>)	ABK37163	100	100
pFMON61			
DapD			
Tetrahydrodipicolinate N-succinyltransferase (DapD) (<i>E. coli</i>)	AAA23667	78	87
pFMON91			
FieF			
Cation-efflux pump FieF (<i>A. hydrophila</i>)	ABK36555	94	97
CpxR			
Transcriptional regulator CpxR (<i>A. hydrophila</i>)	ABK35982	93	96

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