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Identification and characterisation of the *fur* genes in *Photobacterium damselae* ssp. *piscicida* and ssp. *damselae*

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ABSTRACT: The gene encoding the ferric uptake regulator protein (*fur* gene) of the fish pathogenic bacterium *Photobacterium damselae* ssp. *piscicida* Strain DI21 was partially amplified using degenerate oligonucleotides. Complete sequencing of the *fur* gene and neighbouring DNA was accomplished by primer walking. An open reading frame of 447 bp, coding for a protein of 148 amino acids, and with high homology to previously described Fur proteins, was identified. The *fur* gene of *P. damselae* ssp. *damselae* ATCC 35083 was subsequently amplified by PCR with specific primers and its sequence determined, showing a 99.3% similarity to the *P. damselae* ssp. *piscicida fur* gene. The *P. damselae fur* gene was able to complement the *fur* mutation of *Escherichia coli* Strain H1681 in an iron-dependent fashion.

KEY WORDS: *Photobacterium damselae* · Pasteurellosis · *fur* gene · Fur protein · Iron uptake

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INTRODUCTION

The species *Photobacterium damselae* includes Gram-negative marine bacteria belonging to 2 different subspecies, namely *P. damselae* ssp. *damselae* and ssp. *piscicida* (Gauthier et al. 1995). *P. damselae* ssp. *piscicida* (formerly *Pasteurella piscicida*) is the causative agent of fish pasteurellosis, a serious disease which results in considerable economic losses in marine aquaculture worldwide (Magariños et al. 1996a). *P. damselae* ssp. *damselae* (formerly *Vibrio damsela*), has been reported to cause wound infections and fatal disease in a variety of marine animals and humans (Clarridge & Zighelboim-Daum 1985).

The lethality of *Photobacterium damselae* ssp. *piscicida* to fish is increased when haemin or haemoglobin are injected intraperitoneally (Magariños et al. 1994). Iron availability also regulates the amount of capsular polysaccharide and the production of an extracellular protease in this subspecies (Magariños et al. 1996a,b). Production of siderophores and several iron-regulated outer membrane proteins has also been reported in *P. damselae* ssp. *piscicida* strains (Magariños et al. 1994). Similarly, utilisation of haemoglobin and ferric ammonium citrate as sole iron sources *in vitro* has been reported in *P. damselae* ssp. *damselae* (Fouz et al. 1994), and virulence of this subspecies is significantly increased in iron-overloaded animals. However, despite the evidence that iron uptake mechanisms play a role in *P. damselae* virulence, little is known about the genetic basis of iron-sequestering systems in this species.

Despite the importance of iron in bacterial physiology, concentrations of this metal over physiological levels is toxic for cells, as it catalyses the Fenton reaction that leads to formation of hydroxyl radicals (OH) (Halliwell & Gutteridge 1984). Thus, iron homeostasis must be strictly controlled. Expression of genes involved in iron uptake systems is regulated at the level of transcription by the Fur (ferric uptake regulator) repressor protein (Escolar et al. 1999). Homologues of Fur protein have been characterised in many bacterial genera, where it has been found to control expression of iron-responsive genes (Hantke

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2001). This protein requires ferrous iron as a cofactor for dimerisation and DNA binding. When Fur is associated with ferrous iron, it is able to bind operator sites (called Fur boxes) within the promoter region of iron uptake genes, so that transcription of these genes is shut off in iron-replete cells. In iron-depleted conditions, Fur cannot bind operator sites, thus allowing transcription of iron uptake-related genes. In this study, the *fur* gene has been cloned and sequenced in the 2 *P. damselae* subspecies, and its ability to function as an iron-dependent co-repressor has been tested.

MATERIALS AND METHODS

Strains used are listed in Table 1. *Photobacterium damselae* strains were routinely grown at 25°C in Tryptic Soy Agar (Difco) supplemented with 1% NaCl (TSA-1). *Escherichia coli* strains were routinely grown at 37°C in Luria Bertani (LB) medium. MacConkey Agar (Cultimed) was supplemented either with 200 µM 2,2′-dipyridil (iron-restricted conditions) or with 0.1 mM $FeCl_3$ (iron-sufficient conditions). All strains were stored frozen at –80°C in LB broth with 20% glycerol. Ampicillin sodium salt stock solutions (100 mg ml^{-1} in water) were filter sterilized and stored at –20°C.

Standard methods for DNA manipulations were carried out as described by Sambrook & Russell (2001). Total genomic DNA from *Photobacterium damselae* was prepared with the Easy-DNA kit (Invitrogen). Plasmid DNA purification and extraction of DNA from agarose gels were done with kits from Qiagen. DNA-probe labeling and Southern blot analysis were carried out with the ECL DNA labeling and detection system (Amersham Biosciences), following the manufacturer's instructions. Plasmids used in cloning experiments and those derived from this study are summarised in Table 1. Degenerate PCR primers for partial amplification of the *P. damselae fur* gene were designed on the basis of conserved amino-acid sequences evidenced by comparative analysis of Fur proteins of *Vibrio* spp. and *Escherichia coli*. PCR reactions were carried out in a T-Gradient thermal Cycler (Biometra), with *Taq* polymerase BioTaq (Bioline). Oligonucleotide primers used are listed in Table 1.

DNA sequences were determined by the dideoxy chain termination method on either plasmid or PCR products using the Big Dye Terminator v3.0 DNA

Table 1. Strains, plasmids and oligonucleotide primers used in this study. Added restriction sites are shown in italics. Positions are with respect to the translational starting point of *Photobacterium damselae* ssp. *piscicida fur* gene

	Relevant features/ sequence	Source/isolated from/position
Bacteria		
Escherichia coli		
DH5α	*supE4* Δ*lacU169* (Φ80 *lac*ZΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
H1681	*thr ser fhuA lacy rpsL galK hsdR mcrA fhuF:*λ*pLacMu fur-31 zbf::Tn10*	Heidreich et al. (1996)
Photobacterium damselae ssp. *piscicida* DI21		*Sparus aurata*
Photobacterium damselae ssp. *damselae* ATCC 35083		*Carcharinus plumbeus*
Plasmids		
pGEMT-Easy	General TA cloning vector, Amp^R	Promega
pWKS30	Low copy cloning vector, Amp^R	Wang & Kushner (1991)
pCAR106	*Hind*III-*Bgl*II fragment of DI21, containing partial *fur* and *glnS* ORFs, cloned in pGEM-T	This study
pCAR113	*Hind*III-*Bgl*II fragment of DI21, containing *orf1* and partial *fur* and *fld* genes, cloned in pGEM-T	This study
pSJR21	DI21 complete *fur* gene in pWKS30	This study
Oligonucleotides		
Fur-5′	5′-AACRGGAAAGTRTATGTCAG-3′	*fur* (-13-7)
Fur-3′	5′-CCRTAHARRTADAGGCTGTG-3′	*fur* (303-322)
IP-DI21-fur-2	5′-GAAGATCTTCAAACGAGGTAGTGTAAT-3′	*fur* (43-67)
Fur-pisc-5′-1	5′-ACAACCAGAGTGTCAACACA-3′	*fur* (81-100)
Delfur-DI21-3′-1	5′-GC*AAGCTT*TACGTGATACTTGGGCTAAAA-3′	*glnS* (600-580)
Delfur-DI21-5′-1	5′-GC*GGATCC*CATGATACATCCTGATAACAA-3′	*orf1* (-260-240)
M13-fw	5′-GTTTTCCCAGTCACGAC-3′	pWKS30 polylinker
M13-rev	5′-CAGGAAACAGCTATGAC-3′	pWKS30 polylinker

Sequencing Kit (Applied Biosystems) and an automated sequencer ABI 377 (Applied Biosystems). Both strands of DNA were completely sequenced. The European Bioinformatics Institute services were used to consult the EMBL (European Molecular Biology Laboratory) database with the FASTA3 and BLAST algorithms. Sequences were aligned with DNATools software (Version 6.0). Phylogenetic trees were constructed according to the neighbour-joining method (Saitou & Nei 1987) using the ClustalX software. The stability of the groupings was evaluated by performing a bootstrap analysis (1000 replicates). Additional DNA and peptide sequence analysis was performed using BioEdit software (Version 5.0.6).

Escherichia coli H1681 is a *fur*-defective strain that harbours a β-galactosidase reporter gene placed under control of the *fur*-regulated *fhuF* gene promoter (Heidreich et al. 1996). Thus, this strain is ideal for testing the function of a putative *fur* gene as an iron-regulated transcriptional repressor. The complete *P. damselae fur* gene was PCR-amplified with primers delfur-DI21-5′-1 and delfur-DI21-3′-1, cloned in pWKS30 to create pSJR21, and transformed into H1681. The resultant colonies were tested on MacConkey Agar plates under both iron-rich and iron-restricted conditions for fl-galactosidase activity, and recorded as Lac^+ (red colonies) or Lac^- (white colonies).

RESULTS

Using degenerate primers fur-5′ and fur-3′, a fragment of 392 bp was obtained by PCR using DNA of *Photobacterium damselae* ssp. *piscicida* Strain DI-21 as a template. This fragment was excised from the agarose gel, cloned in pGEMT-Easy vector, and sequenced in full. A partial open reading frame was deduced from the nucleotide sequence, and showed 91% similarity to the Fur protein of *Vibrio fischeri.* In order to clone the complete *fur* gene, the PCR-amplified partial *fur* was used as a probe in Southern blots of restriction-digested *P. damselae* ssp. *piscicida* DI21 chromosomal DNA. This probe hybridised to a *Hind*III fragment of ca. 3 kb. *Hind*III-*Bgl*II-digested chromosomal DNA was ligated into plasmid pWKS30 which had been similarly digested, and ligation products were used as DNA template in 2 different PCR reactions, one with primers Fur-pisc-5′-1 and M13-fw and the second with primers IP-DI21-fur2 and M13-rev. PCR products of ca. 1.5 and 1.2 kb were amplified and cloned in pGEMT-easy to create pCAR106 and pCAR113, respectively (Fig. 1). This strategy allows the amplification of unknown DNA sequences upstream or downstream of a known DNA fragment, without the need of screening gene libraries.

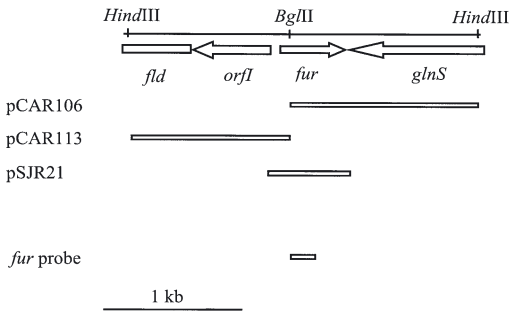

Fig. 1. *Photobacterium damselae*. Restriction map and schematic representation of the *fur* region. Open arrows indicate open reading frames (ORFs; arrows point in the direction of transcription). Relevant plasmids and the partial *fur* gene used as DNA probe are also indicated. Only relevant restriction sites are shown

After sequencing the DNA insert in pCAR106, containing the 3′-end of the *fur* gene, the complete *Photobacterium damselae fur* gene was established as an open reading frame coding for a 148-amino acid protein, which showed high percentage similarity with Fur proteins of *Vibrio fischeri* (86%), *V. logei* (86%) and *V. anguillarum* (84%). The 3′-end of pCAR106 contained a partial new open reading frame (ORF), which was interrupted by a *Hind*III site (Fig 1). The closest database matches for this new ORF were the *glnS* genes of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae*. Sequence analysis of pCAR113 revealed 2 ORFs that were tentatively identified according to homology with published sequences. These were: a partial flavin mononucleotide-containing electron transferase (*fld*) gene showing significant homology to *fldA* genes of *V. fischeri*, *V. salmonicida* and *V. cholerae*, and an ORF (ORF1) with homology to hypothetical proteins of *Vibrio* spp. (Fig. 1). The *fur* gene was PCR-amplified from *P. damselae* ssp. *damselae* strain ATCC 35083 with primers delfur-DI21-5′-1 and delfur-DI21-3′-1, and its sequence determined. The percentage of *fur* gene sequence similarity between the 2 subspecies was 99.3%, and 2 nucleotide substitutions were found between the 2 subspecies, one of which leads to a single amino acid substitution at Position 60 (N in DI21, S in ATCC 35083). Nucleotide sequences determined in this study have been assigned the following EMBL accession numbers: AJ440780 (*P. damselae* ssp. *piscicida* partial *fld*, *glnS* genes and complete *orf1* and *fur* genes) and AJ564701 (*P. damselae* ssp. *damselae* complete *fur* gene).

The predicted sequences of *Photobacterium damselae* ssp. *piscicida* and ssp. *damselae* Fur proteins were aligned with their closest homologues from *Vibrio* species, as well as with that of *Escherichia coli* (Fig. 2). A high degree of sequence conservation was observed in most of the protein regions, but significant sequence

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subsp. piscicida   MSDNNHALKQAGLKITLPRLKILEVLQQPECQHISAEDLYKKLIDIGEEIGLATVYRVLN 60
subsp. damselae    ............................................................S 60
V. parahaemolyt    .....Q...D....V..............D..............L.............. 60
V. vulnificus      .....Q...D....V..............D..............L.............. 60
V. anguillarum     .....Q...D....V......................E.......L.............. 60
V. cholerae        .....Q...D....V......................E.......L.............. 60
V. salmonicida     .....Q...K....V.........I.....S.............................. 60
V. logei           .....Q...K....V.........I.....S.............................. 60
V. fischeri        .....Q...K....V............E.............................. 60
A. hydrophila      .A...Q...K....I....V....I....D...T.......R...Q.............. 60
E. coli            .T...T...K....V............E.DNH.V.......R...M.............. 60

subsp. piscicida   QFDDAGIVTRHHFEGGKSVFELATQHHHDHLVCLDCGKVIEFSDELIERRQKEIAAQYNV 120
subsp. damselae    ............................................................ 120
V. parahaemolyt    ......................S..............E......DI..E..R....K... 120
V. vulnificus      ......................S..............E......DI..E.......A... 120
V. anguillarum     ......................S..............E.......V..Q..R...E.... 120
V. cholerae        ......................S..............E......DV..Q.......K... 120
V. salmonicida     ..............................................D...E...D..NK... 120
V. logei           ..............................................D...E...D..NK... 120
V. fischeri        ..............................................T..Q...QV.EK... 120
A. hydrophila      ........................E...................V..Q..T...KKH.I 120
E. coli            ...........N..........TQ.......I............DS..A..R....KHGI 120

subsp. piscicida   DLTNHSLYLYGHC-ANGDCCKDESLHDQK  148
subsp. damselae    ............-.............  148
V. parahaemolyt    T..........K.-SD.G.KENPDA.KPAK 149
V. vulnificus      Q..........K.-GD.S.KGNPDA.KR.S 149
V. anguillarum     Q..........K.-.D.S.KQNPNA.KS.R 149
V. cholerae        Q..........K.GSD.S.KDNPNA.KP.K 150
V. salmonicida     R............-IT...IENTTA.NG   147
V. logei           R............-IT...IENTTA.NG   147
V. fischeri        R............-IT...ADNDDA.NA   147
A. hydrophila      R............-.A...KH.D        142
E. coli            R............-.E...RE..HA.EG.  148
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Fig. 2. Amino-acid sequence alignment of Fur (ferric uptake regulator) proteins in *Photobacterium damselae* ssp. *piscicida* and *damselae*, *Aeromonas hydrophila*, *Escherichia coli* and *Vibrio* spp. Identical residues are denoted with dots, and deletions are denoted with hyphens

divergence was encountered at the C-terminal end. Phylogenetic trees were constructed with *fur* gene and Fur protein sequences (Fig. 3a,b). For comparative purposes, a phylogenetic tree was constructed with the 16S rRNA gene sequences of the same bacterial species (Fig. 3c). Tree topologies produced with the 3 sequences revealed congruous phylogenetic relationships, and were reinforced by the bootstrap analysis.

The *Photobacterium damselae* ssp. *piscicida fur* gene contained in plasmid pSJR21 was able to functionally complement *Escherichia coli* H1681, and this complementation was dependent on the iron concentration of the culture medium (Table 2).

Table 2. Results of complementation of *Escherichia coli* H1681 with plasmid pSJR21 harbouring *Photobacterium damselae* ssp. *piscicida* complete *fur* gene. Lac phenotype (fl-galactosidase activity) was recorded after 24 h incubation at 37°C

Strain	MacConkey agar + Fe (iron-rich)	MacConkey agar + dipyridil (iron-restricted)
H1681	Red colonies (Lac^+)	Red colonies (Lac^+)
H1681 + pSJR21	White colonies (Lac^-)	Red colonies (Lac^+)

DISCUSSION

The arrangement of the sequenced ORFs in *Photobacterium damselae* ssp. *piscicida* was *fld-orf1-fur-glnS*, which is the same pattern encountered in *Vibrio salmonicida* and *V. logei* (Colquhoun & Sorum 2002). Database searches conducted on the finished genome projects of *V. vulnificus* and *V. parahaemolyticus* also showed this same gene organisation. The existence of a conserved gene order in the vicinity of the *fur* locus is thus evident in the family *Vibrionaceae*. In addition, this gene order is similar to that encountered in *Escherichia coli* and *Aeromonas hydrophila*. The existence of *fldA* homolog upstream of *fur* gene has also been reported in *Alteromonas* sp. (Tsujibo et al. 2000).

The sequence of the *fur* gene has been determined in several members of the family *Vibrionaceae*, including *Vibrio cholerae* (Litwin et al. 1992), *V. parahaemolyticus* (Yamamoto et al. 1997), *V. logei* and *V. salmonicida* (Colquhoun & Sorum 2002), *V. vulnificus* (Litwin & Calderwood 1993)

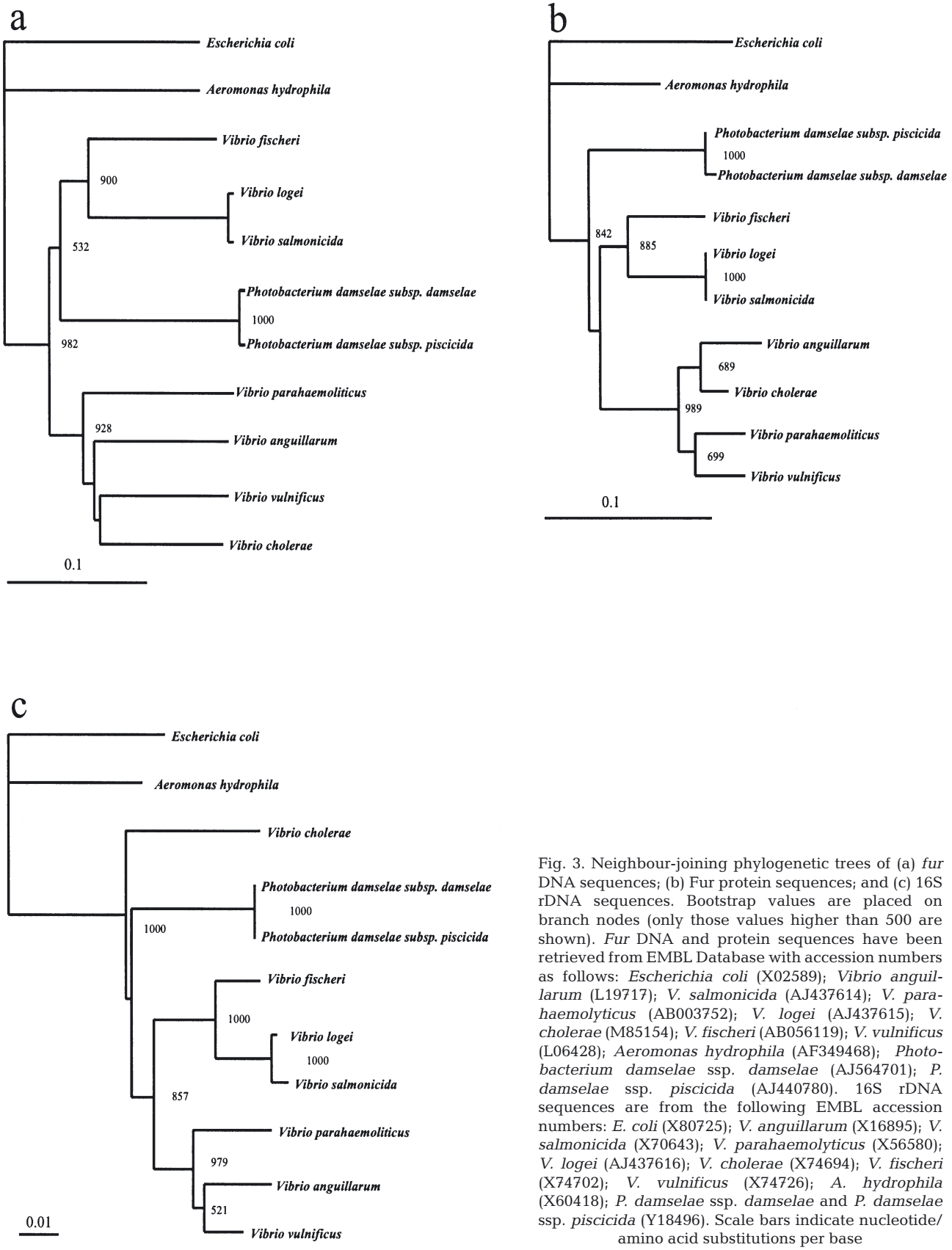

Fig. 3. Neighbour-joining phylogenetic trees of (a) *fur* DNA sequences; (b) Fur protein sequences; and (c) 16S rDNA sequences. Bootstrap values are placed on branch nodes (only those values higher than 500 are shown). *Fur* DNA and protein sequences have been retrieved from EMBL Database with accession numbers as follows: *Escherichia coli* (X02589); *Vibrio anguillarum* (L19717); *V. salmonicida* (AJ437614); *V. parahaemolyticus* (AB003752); *V. logei* (AJ437615); *V. cholerae* (M85154); *V. fischeri* (AB056119); *V. vulnificus* (L06428); *Aeromonas hydrophila* (AF349468); *Photobacterium damselae* ssp. *damselae* (AJ564701); *P. damselae* ssp. *piscicida* (AJ440780). 16S rDNA sequences are from the following EMBL accession numbers: *E. coli* (X80725); *V. anguillarum* (X16895); *V. salmonicida* (X70643); *V. parahaemolyticus* (X56580); *V. logei* (AJ437616); *V. cholerae* (X74694); *V. fischeri* (X74702); *V. vulnificus* (X74726); *A. hydrophila* (X60418); *P. damselae* ssp. *damselae* and *P. damselae* ssp. *piscicida* (Y18496). Scale bars indicate nucleotide/amino acid substitutions per base

and *V. anguillarum* (Tomalsky et al. 1994). Conservation of the relative positions of *Vibrio* species in the phylogenetic trees, with regard to *Photobacterium damselae* subspecies and to the species included as outgroup (*E. coli*), indicate the suitability of the *fur* gene as a phylogenetic marker. Despite its small size (ca. 450 bp), phylogenies deduced with *fur* gene sequences are in accordance with those deduced from the 16S rRNA gene (ca. 1.5 kb), indicating that the *fur* gene is not an erratic evolutionary clock, and can be employed as a phylogenetic marker, as suggested by other authors (Achenbach & Yang 1997, Colquhoun & Sorum 2002).

The ability of *Photobacterium damselae* Fur protein to recognise the Fur-regulated promoters in an *Escherichia coli* background suggests that a similar regulatory mechanism (via the Fur protein) of iron-regulated genes is expected to occur in *P. damselae*, with a Fur protein which is able to sense the iron availability in the cell. This opens the door for future genetic studies aimed at identifying Fur-regulated promoters in *P. damselae* strains using a Fur titration assay (FURTA), a technique which has proved very useful for identification of iron-regulated genes (Stojiljkovic et al. 1994). Fur-regulated outer-membrane proteins are candidates that elicit a strong immune response in the host, as reported in *Aeromonas salmonicida* (Hirst & Ellis 1994). Therefore, characterisation of the *fur* gene in *P. damselae* will allow the construction of *fur* mutant strains for further identification and characterisation of iron-regulated proteins.

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