Comparative analysis and mutation effects of *fpp2–fpp1* tandem genes encoding proteolytic extracellular enzymes of *Flavobacterium psychrophilum*

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Flavobacterium psychrophilum is a very significant fish pathogen that secretes two biochemically characterized extracellular proteolytic enzymes, Fpp1 and Fpp2. The genes encoding these enzymes are organized as an *fpp2–fpp1* tandem in the genome of strain *F. psychrophilum* THC02/90. Analysis of the corresponding encoded proteins showed that they belong to two different protease families. For gene function analysis, new genetic tools were developed in *F. psychrophilum* by constructing stable isogenic *fpp1* and *fpp2* mutants via single-crossover homologous recombination. RT-PCR analysis of wild-type and mutant strains suggested that both genes are transcribed as a single mRNA from the promoter located upstream of the *fpp2* gene. Phenotypic characterization of the *fpp2* mutant showed lack of caseinolytic activity and higher colony spreading compared with the wild-type strain. Both characteristics were recovered in the complemented strain. One objective of this work was to assess the contribution to virulence of these proteolytic enzymes. LD₅₀ experiments using the wild-type strain and mutants showed no significant differences in virulence in a rainbow trout challenge model, suggesting instead a possible nutritional role. The gene disruption procedure developed in this work, together with the knowledge of the complete genome sequence of *F. psychrophilum*, open new perspectives for the study of gene function in this bacterium.

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INTRODUCTION

Flavobacterium psychrophilum is the aetiological agent of ‘cold water disease’ and ‘rainbow trout fry syndrome’ in salmonid fish. The disease mainly affects fingerlings, and is currently considered one of the most significant bacterial infections in the salmonid industry, causing high mortality and serious economic losses worldwide. Although *F. psychrophilum* is considered a rather fastidious bacterium because it is difficult to isolate and handle (Michel *et al.*, 1999), significant advances have been made over the last few years in culture (Michel *et al.*, 1999; Cepeda *et al.*, 2004; Álvarez & Guijarro, 2007), typing (Izumi *et al.*, 2003; Soule *et al.*, 2005; Arai *et al.*, 2007; Nicolas *et al.*, 2008), molecular diagnosis (Urdaci *et al.*, 1998; Cepeda & Santos, 2000; del Cerro *et al.*, 2002; Fujiwara-Nagata & Eguchi, 2009) and genomics (Duchaud *et al.*, 2007). Moreover, a genetic manipulation system based on conjugation, transformation and transposon mutagenesis has been

developed (Álvarez *et al.*, 2004). Nevertheless, the very low frequency of conjugation and the very low number of *F. psychrophilum* strains that can be genetically manipulated (Álvarez *et al.*, 2004) have hampered the development of mutagenesis techniques and tools. Furthermore, genetic systems for targeted gene mutation have not been reported.

Several molecules secreted by *F. psychrophilum* and involved in adherence (Møller *et al.*, 2003), haemagglutination and haemolysis (Lorenzen & Olesen, 1997; Møller *et al.*, 2003) and iron acquisition (Álvarez *et al.*, 2008) have been suggested to play a role in virulence. In addition, different authors have proposed extracellular proteolytic activities as putative virulence factors (Bertolini *et al.*, 1994; Madsen & Dalsgaard, 1998). Among them, the extracellular proteases Fpp1 and Fpp2 have been purified and enzymically characterized as metalloproteases, and the N-terminal sequences of both proteins have been determined (Secades *et al.*, 2001, 2003). In addition, 13 genes, encoding

putative extracellular proteolytic activities, including Fpp1 and Fpp2, have been identified in the genome of strains JIP02/86 (Duchaud *et al.*, 2007) and THC02/90 (E. Duchaud, unpublished data). There is not conclusive evidence, however, of their involvement in disease development. Although extracellular proteolytic enzymes have been considered as virulence factors in many bacterial fish pathogens, comparative analyses of wild-type strains and isogenic mutants devoid of proteolytic activity have seldom been performed. In particular, proteases from *Vibrio anguillarum* (Yang *et al.*, 2007), *Yersinia ruckeri* (Fernández *et al.*, 2003), *Pseudomonas fluorescens* (Zhang *et al.*, 2009) and *Moritella viscosa* (Bjornsdottir *et al.*, 2009) have been clearly proved to be involved in bacterial virulence in fish.

The aim of this study was to develop a disruption system for specific gene inactivation of *F. psychrophilum*. For this task, the *fpp1* and *fpp2* genes, encoding extracellular proteolytic activities, were chosen. The results showed that insertional mutation of both genes was stable *in vivo*. Additionally, *fpp1*[−] and *fpp2*[−] mutants were used to study the roles of the corresponding proteases in the physiology and virulence of the bacterium.

METHODS

Bacterial strains, plasmids, growth conditions and proteolytic activity. *Escherichia coli* strain S17-1 λ pir (Simon *et al.*, 1983) was grown at 37 °C in 2 $\times$ TY medium (per litre: 10 g tryptone, 10 g yeast extract, 5 g NaCl) with 20 g agar l^{−1} added for solid medium. This strain was used to transfer DNA into *F. psychrophilum*. The wild-type strain *F. psychrophilum* THC02/90 was grown at 12 or 18 °C in nutrient broth (NB; Pronadisa) or nutrient broth containing 10 mM CaCl₂ (NBF) (Secades *et al.*, 2001). Nutrient agar (NA; NB containing 15 g agar l^{−1}) or nutrient agar charcoal [NAC; NA supplemented with activated charcoal (Sigma)] were used for solid cultures, as previously described (Álvarez & Guijarro, 2007). For selective growth of *E. coli* S17-1 λ pir, 50 μ g streptomycin ml^{−1} was used and transformants were selected with 100 μ g ampicillin ml^{−1}. Selection of *F. psychrophilum* transconjugants was carried out with 10 μ g erythromycin ml^{−1} and 10 μ g tetracycline ml^{−1}. The plasmids and primers used are listed in Table 1.

Colony spreading and biomass production were analysed according to Pérez-Pascual *et al.* (2009). Briefly, plates containing one-sixth NA were inoculated in the centre with 6 μ l liquid culture in mid-exponential growth phase. The plates were then incubated at 20 °C, and spreading and biomass production were evaluated at 72, 96 and 120 h by measuring the colony diameter (cm) and biomass production (OD₅₂₅) of 1 ml aqueous suspensions of colonies.

Extracellular proteolytic activity on solid medium was visualized by using NBF containing 15 g agar l^{−1} and supplemented with 0.75 % (w/v) gelatin or 1 % (w/v) casein. In these media, proteolytic activity was visualized as the formation of clear haloes around the colonies. Azocasein assays using supernatants of liquid cultures were carried out according to Secades *et al.* (2001). One unit of enzyme activity (enzymic units; EU) was defined as the amount of enzyme which yielded an increase in the A₄₂₀ of 0.01 in 2 h at 30 °C.

Generation of *fpp1*[−] and *fpp2*[−] mutants by single-crossover homologous recombination and complementation of the *fpp2*[−]

mutant. Fpp1 and Fpp2 had previously been purified and biochemically characterized (Secades *et al.*, 2001, 2003). The amino acid N-terminal sequences for Fpp1 (SSTGQLKTMRLAQ(S/C/W)NGQYA; our unpublished results) and Fpp2 (Secades *et al.*, 2003) had previously been determined and their sequences used to locate the *fpp1*–*fpp2* tandem genes in the genome of the THC02/90 strain.

Internal fragments of 2481 and 1967 bp of the predicted *fpp1* and *fpp2* genes, respectively, were amplified by PCR with the following primers: Fpp1-F (nucleotides 315–323) and Fpp1-R (nucleotides 2785–2764) for *fpp1* (both with a *Sall* restriction site), and Fpp2-F (nucleotides 452–470) and Fpp2-R (nucleotides 2426–2406) for *fpp2* (with *XbaI* and *PstI* restriction sites) (Table 1). All PCRs were performed in a Perkin-Elmer 9700 GeneAmp thermocycler with an initial denaturation cycle at 94 °C for 5 min, followed by 25 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 3 min), and a final 7 min elongation step at 72 °C. The generated amplicons were digested with the corresponding restriction enzymes and ligated into the pLYL03 suicide plasmid, previously digested with the same enzymes and dephosphorylated when necessary. Each ligation mixture of target genes was used to transform electrocompetent cells of *E. coli* S17-1 λ pir. Clones carrying pLYL03 containing inserts (pLYL03Fpp1 and pLY03Fpp2) were used to conjugate with *F. psychrophilum* THC02/90, as previously described (Álvarez *et al.*, 2004), by selecting the transconjugants using NAC plates supplemented with erythromycin. Erythromycin-resistant colonies of *F. psychrophilum* appeared after 7–10 days of incubation at 20 °C. Selected transconjugant colonies were analysed by Southern blotting using a DIG DNA detection kit (Roche) following the protocol described by the manufacturer. Total DNA from transconjugants and the wild-type strain was isolated and digested with *HindIII* and *XbaI* restriction enzymes. Then, DNA fragments were subjected to 0.8 % agarose gel electrophoresis at 100 V for 2 h, transferred to a nylon membrane (Amersham Biosciences) and fixed with UV irradiation. The labelled internal regions of *fpp1* (2.5 kb) and *fpp2* (1.9 kb) genes were used as probes to perform hybridization for each mutant.

To complement the *fpp2*[−] mutant strain, the *fpp2* gene was amplified from the wild-type strain by PCR using an Expand Long Template PCR system (Roche) and primers Fpp2C-F (nucleotides −384 to −366) and Fpp2C-R (nucleotides 3128 to 3146), obtaining a fragment of 3530 bp (Table 1). The *KpnI* and *SphI* restriction sites were introduced into the sequences of Fpp2C-F and Fpp2C-R, respectively, to clone the PCR product digested with *KpnI* and *SphI* into pCP23. The resulting plasmid was designated pCP23-Fpp2 (Table 1). Transfer of pCP23-Fpp2 to *F. psychrophilum* was carried out by conjugation, as described by Álvarez *et al.* (2004), and pCP23-Fpp2 was recovered from the transconjugants, digested with *KpnI* and *SphI*, and analysed by agarose gel electrophoresis to confirm the presence of the insert.

RT-PCR. Total RNA was obtained from 2 ml of early stationary phase cultures of wild-type strain THC02/90 and mutant strains *fpp1*[−] and *fpp2*[−] grown in NBF at 12 °C. RNA was isolated using a High Pure RNA Isolation kit (Roche) and treated with DNase I (RNase-free) (Ambion) to eliminate traces of DNA. Reverse transcription (RT-PCR) was performed using Superscript One-Step with Platinum *Taq* (Invitrogen Life Technologies). Ten nanograms of RNA were used in each reaction. In order to determine whether RNA was free of contaminant DNA, PCRs using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies) were performed and also reactions omitting the reverse-transcription step were included in each run as negative controls. The primers used for RT-PCR amplifications were 1F (nucleotides 2109–2032) and 1R (nucleotides 2526–2544) for the intragenic region of *fpp2*, 2F (nucleotides 2674–2695) and 2R

Table 1. Strains, plasmids and primers used in this study

Plasmid, strain or primer	Description or sequence*	Source or reference
Plasmids		
pCP23	ColE1 ori, (pCP1 ori), Ap ^r (Tc ^r), <i>E. coli</i> - <i>F. psychrophilum</i> shuttle plasmid	Agarwal <i>et al.</i> (1997)
pCP23-Fpp2	<i>fpp2</i> gene, derived from pCP23	
pLYL03	ColE1; Ap ^r (Emr ^b); suicide plasmid	Li <i>et al.</i> (1995)
pLYL03Fpp1	<i>fpp1</i> internal fragment, derived from pLYL03	This study
pLYL03Fpp2	<i>fpp2</i> internal fragment, derived from pLYL03	This study
<i>F. psychrophilum</i> strains		
THC02-90	Wild-type	Chakroun <i>et al.</i> (1998)
fpp1 ⁻	<i>fpp1</i> mutant created by specific homology recombination	This study
fpp2 ⁻	<i>fpp2</i> mutant created by specific homology recombination	This study
fpp2 ⁻ pCP23-Fpp2 complemented	Mutant fpp2 ⁻ carrying pCP23-Fpp2, complemented strain	This study
<i>E. coli</i> strain		
S17-1 λ pir	λ pir <i>hsdR pro thi</i> ; RP4-2 Tc::Mu Km::Tn7	Simon <i>et al.</i> (1983)
Primers†		
Fpp1-F	5'-ATTTCGTCGACGATTGCAAGCG-3'	This study
Fpp1-R	5'-TTATTGTCGACGTGACTGTGT-3'	This study
Fpp2-F	5'-AGACCCTGCAGGAAATCC-3'	This study
Fpp2-R	5'-GCTGTTCTAGAATTCCTGT-3'	This study
Fpp2C-F	5'-ATCGGGTACCTTATTTGGGTGTTATTT-3'	This study
Fpp2C-R	5'-ATCGGCATGCTTTTGTGCGAGAATAATG-3'	This study
3F	5'-CATATACTATTCTACGATGGC-3'	This study
3R	5'-AATGCTGATAAAGGTGCTGAAG-3'	This study
2F	5'-GCGATATTAGCGGGCGTGAAG-3'	This study
2R	5'-CCGTCTTTGCTAGTGGTCCA-3'	This study
1F	5'-TACCAAGCTTCAGTAAACATTGA-3'	This study
1R	5'-GTTCCAAATAATAAACCCACACA-3'	This study

*Antibiotic-resistance phenotypes: Ap^r, ampicillin; Tc^r, tetracycline; Emr^b, erythromycin. Antibiotic-resistance phenotypes and other features listed in parentheses are those expressed by *F. psychrophilum* but not by *E. coli*.

†Restriction sites for cloning are in bold type.

(nucleotides 95–75) for the *fpp2*–*fpp1* intergenic region, and finally 3F (nucleotides 1898–1920) and 3R (nucleotides 3013–3035) for the intragenic region of *fpp1* (Table 1).

Animal experiments. Animal experiments were performed in accordance with the European legislation governing animal welfare, and they were authorized and supervised by the Animal Experimentation Ethics Committee of Universidad de Oviedo.

Rainbow trout (*Oncorhynchus mykiss*) fingerlings weighing between 5 and 7 g used for the animal experiments were obtained from a commercial fish farm. Fish were kept in 60 l tanks at 14±1 °C in continually flowing dechlorinated water.

To determine the stability of *fpp1* and *fpp2* mutations *in vivo*, two groups of 10 rainbow trout were injected intramuscularly with 50 µl of a cell suspension in PBS containing 10⁶ c.f.u. from an exponential-phase culture of each mutant grown in NB. After 24, 48 and 72 h, fish were euthanized, and muscle, spleen, liver and intestine from each infected fish were homogenized together in NB. Aliquots of the suspensions were plated onto NAC and incubated for 5 days at 20 °C. Randomly taken *F. psychrophilum* colonies were used to inoculate flasks containing NB, and cultures were incubated at 12 °C for 24 h. Total DNA from each culture was obtained and analysed by Southern

blotting as described above. In total, eight different colonies (two per time point) from each mutant were analysed. As a control for the recovery of the mutants, erythromycin was used in all the steps, except during the fish infections. Control fish injected with 10⁶ wild-type strain cells were also analysed.

For LD₅₀ determinations, *F. psychrophilum* cultures were grown to exponential phase, harvested by centrifugation and washed with PBS. Cells were resuspended in PBS and serial dilutions were prepared. Groups of 10 fish were challenged by intramuscular injection of 50 µl of dilutions containing 10³–10⁹ c.f.u., and LD₅₀ was calculated according to the method of Reed & Muench (1938). LD₅₀ experiments were performed twice.

Genetic analysis. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was used to compare protein sequences and Simple Modular Architecture Research Tools (SMART) to detect conserved domains. MotifScan software from MyHits was used to identify the motifs present in each sequence. The MEROPS peptidase database was used to classify each protease. The ProtParam program (ExPASy) was used for molecular mass computation and SignalP3.0 (Center for Biological Sequence Analysis; CBS) to predict the location of a signal peptide cleavage site. The alignment of the protein sequences of both Fpp1 and Fpp2 was

performed with CLUSTAL_X software. *fpp1* and *fpp2* gene sequences were obtained from the ongoing genome sequence of *F. psychrophilum* THC02/90 (E. Duchaud, unpublished data) deposited in EMBL under accession number FR667216.

RESULTS

fpp1 and *fpp2* bioinformatic analysis

The genes encoding Fpp1 and Fpp2 proteases have been definitely localized as an *fpp2*–*fpp1* tandem in the *F. psychrophilum* JIP02/86 genome (Duchaud *et al.*, 2007) and also in the ongoing genome of strain THC02/90 (Fig. 1a), where these genes are separated by an intergenic space of 109 bp. Putative *Flavobacterium* –7 (TAnnTTTG) and –33 (TTG) promoter sequences, and a ribosome-binding site (TAAAA) (Chen *et al.*, 2007), were identified upstream of the respective ORFs, and stem–loop palindromic sequences corresponding to rho-independent terminators were located at the 3' ends of both genes.

Additionally, Fpp2 and Fpp1 putative prosequences of 82 and 194 aa, respectively, could be deduced based on the comparison between the encoding sequence and the N-terminal amino acid sequence of both proteins.

The *fpp2* gene encoded a predicted 942 aa protein with a theoretical molecular mass of 100 240 Da. The deduced

Fpp2 protein (Fig. 2) displays a modular structure encompassing (i) an N-terminal signal peptide of 26 aa; (ii) a region similar to other metalloproteases (MEROPS M43, cytophagalyisin family), with a typical HEXXH-XXGXXH motif (amino acids 284–294); (iii) a 'cleaved adhesin domain' (amino acids 526–686) of 160 aa characteristic of a group of adhesins/peptidases represented by the extracellular virulence complex RgpA, RgpB and Kgp of *Porphyromonas gingivalis* (Pike *et al.*, 1994; Bhogal *et al.*, 1997; Rangarajan *et al.*, 1997), a species belonging to the same phylum as *F. psychrophilum*; and (iv) a highly conserved C-terminal region of 95 aa (amino acids 847–942) (Duchaud *et al.*, 2007). This module is likely involved in cell-anchoring of proteins to the outer membrane (Karlsson *et al.*, 2004).

The *fpp1* gene encoded a protein of 1138 aa with a predicted molecular mass of 119 587 Da. The putative Fpp1 (Fig. 2) also displays a modular structure encompassing (i) an N-terminal signal peptide of 25 aa; (ii) a region similar to other metalloproteases (MEROPS M12, adamalysin family), with a typical HEXXHXXGXXH zinc metalloprotease motif (amino acids 364–374); (iii) a Drmip–Hesp domain, characteristic of a mitogen-activated protein kinase involved in growth and development in many organisms (Szeto *et al.*, 2007) in the central region (amino acids 600–675), and a regulatory P domain (amino

Fig. 1. Gene organization of the *fpp2*–*fpp1* locus in the *F. psychrophilum* genome (a). The position of each gene and the direction of transcription are shown by arrows. The *fpp2*–*fpp1* promoter (P), the rho-independent transcriptional terminator (hairpin loop), and the flanking genes *ribF* (riboflavin kinase; FP0233) and *pth* (peptidyl-tRNA hydrolase; FP0230) are indicated. (b) RT-PCR analysis of the *fpp2*–*fpp1* locus in *F. psychrophilum*. Each PCR was performed with 10 ng *F. psychrophilum* RNA. As controls for DNA contamination, PCRs were also carried out with the primers used for RT-PCR analysis by omitting the reverse-transcription step (lanes 2, 6 and 9). Lanes: 1, 100 bp molecular marker (Biotools); 6–8, RT-PCRs performed with the RNA from the *fpp2*[–] mutant strain; 9–11, RT-PCRs performed with the RNA from the *fpp1*[–] mutant strain. The following primers were used: 3R and 3F for lanes 5, 8 and 11; 2R and 2F for lane 4; 1F and 1R for lanes 3, 7 and 10. The localization of the primers used for RT-PCR analysis and the lengths of the amplicons generated are also shown.

Fig. 2. Structural organization and domains of the Fpp1 and Fpp2 proteolytic enzymes from *F. psychrophilum* strain THC02/90. Domain regions are labelled, motifs are shown and the initial and final residues of each domain are indicated above each protein.

acids 938–1043) containing an AGDL motif (amino acids 952–955) similar to the RGDL motif conserved in most eukaryotic proprotein convertases belonging to the S8 family (Fig. 2); and (iv) a highly conserved C-terminal region of 95 aa (amino acids 1043–1138) likely involved in cell-anchoring (Karlsson *et al.*, 2004).

Generation of *fpp1*[−] and *fpp2*[−] mutants by single-crossover homologous recombination

Internal fragments from the *fpp1* and *fpp2* genes were used to generate the suicide pLYL03Fpp1 and pLYL03Fpp2 plasmids, respectively. These vectors were introduced into *E. coli* S17-1λpir and mobilized into *F. psychrophilum* THC02/90 by conjugation, and transconjugants were selected on NAC plates supplemented with erythromycin as described in Methods. Southern blot analysis of the selected transconjugants showed that the pLYL03Fpp1 and pLYL03Fpp2 plasmids were integrated into the *fpp1* and *fpp2* genes of the chromosome of *F. psychrophilum* THC02/90, generating the corresponding *fpp1*[−] and *fpp2*[−] mutants (Fig. 3a, b). Two new *Xba*I fragments of 7.2 and 0.5 kb appeared from *fpp1*[−] DNA digestion in addition to the 4, 2 and 0.7 kb fragments originating from the parental wild-type strain (Fig. 3a, b). In the same way, a new *Xba*I fragment of 7.9 kb was found from the *fpp2*[−] DNA digestion in addition to the 2.7 kb fragment from the parental wild-type strain (Fig. 3a, b). Similar results were obtained when the *Hind*III enzyme was used for DNA digestion (data not shown). Furthermore, and according to the patterns of labelled DNA bands after Southern blot hybridization, one can conclude that the integration of pLYL03Fpp1 and pLYL03Fpp2 into the chromosome had occurred by a single-crossover event between the *fpp1* and *fpp2* internal DNA fragments harboured in the plasmids and the respective homologous sequences from the bacterial chromosome (Fig. 3). The resulting mutants were stable during *in vivo* infections. Southern blot hybridization showed the presence of the corresponding labelled

*Xba*I fragments resulting from *fpp1* and *fpp2* gene disruption in the genome of all the colonies analysed that were recovered from fish (Fig. 3a, b). Therefore, no reversion was observed in the absence of selective antibiotic pressure *in vivo*. Identical results were obtained when erythromycin was used to recover the mutants from the fish.

RT-PCR

RT-PCR assays were carried out to elucidate the transcriptional structure of the *fpp1* and *fpp2* genes. According to sequence analysis, two putative promoter regions may be present at the 5' ends of both the *fpp2* gene and the *fpp1* gene. When mRNA from the wild-type strain was used as template for RT-PCR analysis, *fpp2* and *fpp1*, as well as the intergenic region, were amplified (Fig. 1b, lanes 3–5). However, when the *fpp1* gene was disrupted the *fpp2* upstream gene was amplified (Fig. 1b, lane 10), whereas no amplification was detected when the *fpp2* gene was mutated (Fig. 1b, lanes 7 and 8). These results indicate that under the conditions used, there is a unique promoter activity located upstream of the *fpp2* gene that drives the transcription of both genes, generating a single polycistronic mRNA. Therefore, genes *fpp2* and *fpp1* constitute an operon. The resulting transcriptional structure is presented in Fig. 1(a).

Phenotypic characterization and LD₅₀ determination of *fpp1*[−] and *fpp2*[−] mutants

To further characterize the *F. psychrophilum* *fpp1*[−] and *fpp2*[−] mutants, some of their phenotypic traits were studied. The growth of *fpp1*[−] (data not shown) and *fpp2*[−] mutants in NBF at 12 °C was similar to that of the wild-type strain (Fig. 4a). The levels of proteolytic activity in the culture supernatants were similar in the wild-type strain and the *fpp1*[−] mutant (data not shown). However, the *fpp2*[−] mutant exhibited a caseinolytic activity significantly lower than that of the wild-type strain (Fig. 4a).

Fig. 3. Construction of *fpp1*[–] and *fpp2*[–] isogenic mutants by insertional mutagenesis. (a) *Xba*I restriction map of the area surrounding the *fpp2*–*fpp1* locus of the wild-type THC02/90 strain and isogenic *fpp1*[–] and *fpp2*[–] mutants. The labelled DNA fragments of *fpp1* and *fpp2* used as probes and the size of the fragments revealed by Southern blot hybridization are indicated in kb. X, *Xba*I; (i) THC02/90 strain, (ii) *fpp1*[–] mutant, (iii) *fpp2*[–] mutant. (b) Southern blot analysis of the wild-type *F. psychrophilum* strain and of the *fpp1*[–] and *fpp2*[–] mutants. Genomic DNA from the wild-type *F. psychrophilum* strain (lanes 1 and 3) and the mutant strains *fpp1*[–] (lane 2) and *fpp2*[–] (lane 4) was digested with *Xba*I and hybridized with the 2.5 kb *fpp1* and 1.9 kb *fpp2* fragments. The sizes of a molecular mass ladder are indicated in the centre in kb.

Analysis of colony spreading on diluted NA medium showed that when *fpp1*[–] and *fpp2*[–] mutants were grown on half-strength NA the diameters of the colonies were similar to those of the wild-type strain (data not shown). By contrast, when they were grown on one-sixth NA, the colonies of the *fpp2*[–] mutant presented a significantly larger diameter than those of the wild-type strain (Fig. 4b, c). This difference was correlated with an increase in biomass production (Fig. 4b).

Plasmid pCP23-Fpp2 was constructed as described in Methods to complement the *fpp2*[–] mutant. Introduction of this plasmid into the *fpp2*[–] mutant restored the extracellular caseinolytic activity, determined both qualitatively on NBF plates supplemented with casein (data not shown), and quantitatively, showing an approximately twofold higher azocasein activity of the complemented strain compared with the wild-type after 120 h of incubation (Fig. 4a). Additionally, colony spreading of the complemented *fpp2*[–] mutant was considerably decreased (Fig. 4c).

In order to evaluate the effects of the *fpp1* and *fpp2* mutations on virulence, LD₅₀ experiments were carried out on rainbow trout. The LD₅₀ 10 days post-injection was calculated to be 2.53×10^6 c.f.u. for the wild-type strain. Under the same conditions, strains *fpp1*[–] and *fpp2*[–] showed LD₅₀ values of 2.12×10^6 and 1.73×10^6 c.f.u., respectively. These results indicate that the disruption of the *fpp1* and *fpp2* genes had no significant effect on the virulence of the bacteria in rainbow trout under the

conditions used. Additionally, visual examination of skin lesions showed that the wild-type strain and the two mutants caused the same tissue damage around the injection site (data not shown).

DISCUSSION

The *fpp1* and *fpp2* genes have been unambiguously identified in the genomes of *F. psychrophilum* strains JIP02/86 (Duchaud *et al.*, 2007) and THC02/90 (this study). The deduced Fpp1 and Fpp2 proteins display modular structures encompassing an unusual mixture of well-established domains.

The Fpp2 protein is composed of four domains, including a typical ‘cleaved adhesin domain’ of 160 aa. This domain is characteristic of the RgpA–Kgp protease–adhesin complexes of *P. gingivalis* (Curtis *et al.*, 2001; Pathirana *et al.*, 2006). Both Rgp and Kgp belong to the cysteine proteinase family, but also display some characteristics of serine proteases and metalloproteases (Kadowaki *et al.*, 1994; Abe *et al.*, 1998). Nevertheless, the Fpp2 protein sequence showed the highest similarity with the M43 family of metalloproteases, in concordance with the biochemical and enzymic properties of the purified Fpp2 enzyme (Secades *et al.*, 2003).

The Fpp1 protein is also composed of four domains and contains a P domain (amino acids 938–1043). This domain is found in subtilisin proprotein convertases, and seems to be necessary to fold and maintain the endopeptidase

Fig. 4. Growth, extracellular proteolytic activity and spreading of *F. psychrophilum* THC02/90, the *fpp2*⁻ mutant and the pCP23-Fpp2-complemented *fpp2*⁻ mutant. (a) Bacterial growth at 12 °C in NBF was monitored by determining OD₅₂₅. Solid lines, growth (●) and proteolytic activity (○) of strain THC02/90; dotted lines, growth (▲) and proteolytic activity (△) of the *fpp2*⁻ mutant; dashed and dotted lines, growth (◆) and proteolytic activity (◇) of the pCP23-Fpp2-complemented *fpp2*⁻ mutant. The growth curve and proteolytic activity corresponding to the *fpp1*⁻ mutant were similar to those of strain THC02/90 (not shown). The extracellular proteolytic activity in cell-free supernatants was determined by azocasein assay (Secades *et al.*, 2001); EU, enzymic units. (b) Spreading of and relative biomass production by colonies of *F. psychrophilum* THC02/90 and the *fpp2*⁻ mutant. The histogram represents the diameters of colonies of strain THC02/90 (white bars) and the *fpp2*⁻ mutant (grey bars) at different incubation times. Lines represent the relative biomass production of strain

THC02/90 (●, solid line) and of the *fpp2*⁻ mutant (▲, dashed line) at different incubation times. (c) Colony spreading of (i) *F. psychrophilum* THC02/90, (ii) the *fpp2*⁻ mutant and (iii) the pCP23-Fpp2-complemented *fpp2*⁻ mutant. The strains were grown on one-sixth NA for 96 h at 20 °C according to Pérez-Pascual *et al.* (2009). Bar, 1 cm.

catalytic domain and to regulate the calcium- and acidic pH-dependence of this type of protease. This P domain is therefore likely responsible for the calcium-dependent activity of the purified Fpp1 protein. Nevertheless, Fpp1 has metalloprotease characteristics, as previously determined (Secades *et al.*, 2001), and belongs to the M12 family (MEROPS database).

Initially, the tandem organization of the *fpp2* and *fpp1* genes with putative regulatory sequences for each gene suggested that they might be transcribed as two units. However, RT-PCR analysis of the transcripts in the *fpp1*⁻ and *fpp2*⁻ mutants showed that only one active promoter was detected upstream of the *fpp2* gene and that it was responsible for the generation of an *fpp2*–*fpp1* polycistronic mRNA. The Fpp1 putative promoter seems not to be active under the assayed conditions.

Proteolytic activity quantification during *in vitro* growth indicated that *fpp2* is likely the main gene driving caseinolytic activity in the bacterium. Intriguingly, the *fpp2* mutation caused a conditional hypergliding phenotype and consequently a biomass increase when growing on diluted NA. Therefore, colony spreading and Fpp2 extracellular proteolytic activity are negatively correlated in this bacterium, suggesting that these two cellular processes may be in some way related in *F. psychrophilum*. It should be emphasized that a low nutrient concentration induces colony spreading in *F. psychrophilum* (Pérez-Pascual *et al.*, 2009). There was no clear decrease of growth of the *fpp1*⁻ and *fpp2*⁻ mutants compared with the wild-type strain in both standard and diluted broth cultures. Growth conditions are considerably different between solid and liquid media, especially regarding the factors that affect motility, such as nutrient availability, cell density, surface characteristics, and diffusion of extracellular factors related to nutrient utilization. Therefore, the high-spreading phenotype of the *fpp2*⁻ mutant may result from the alteration of some of these characteristics, which are reverted in the complemented mutant. For instance, the absence of *fpp2* may decrease nutrient availability, stimulating colony spreading to colonize unexploited areas of the agar plate. The alteration of bacterial motility by the mutation of genes encoding proteolytic activity that has been reported in different bacteria is usually related to regulatory enzymes with a proteolytic function, such as the ClpP protease from *Bacillus subtilis* (Gupta & Rao, 2009) and the Lon protease from *Proteus mirabilis* (Clemmer & Rather, 2008), rather than to extracellular proteases. Alteration in motility phenotypes may also result from changes in expression of virulence factors, including

proteolytic enzymes, as reported in *Vibrio cholerae*, in which production is increased in hyperswarmer mutants (Gardel & Mekalanos, 1996).

Extracellular proteolytic activities have been reported to be involved in the virulence of different pathogens, including a number of fish pathogenic bacteria (Fernández *et al.*, 2003; Yang *et al.*, 2007; Zhang *et al.*, 2009). However, the implication of proteases in pathogenesis is not a general rule, as shown in this study in a rainbow trout model. Although purified Fpp1 and Fpp2 proteases have been shown to degrade tissue proteins *in vitro* (Secades *et al.*, 2001, 2003), the inactivation of the *fpp1* and *fpp2* loci did not have any effect on bacterial virulence. This, together with the fact that similar necrotic lesions appeared around the injection site of the wild-type strain and the two mutants in this study, suggests that these proteins are not directly involved in virulence, at least in rainbow trout challenged by intramuscular injection. Nevertheless, it cannot be ruled out that they may play a role in virulence in a natural infection process or in another fish host. Because the genome of *F. psychrophilum* encodes 11 other extracellular proteases, this genetic redundancy may conceal the loss of Fpp1 and Fpp2.

This paper describes for the first time a method for specific gene inactivation in *F. psychrophilum* by using homologous recombination. The resulting mutants were stable, and no reversion was observed in the absence of selective antibiotic pressure during *in vivo* growth.

This technique, together with the knowledge of the complete genome sequence, opens promising perspectives for the study of this bacterium.

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