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Pseudomonas fluorescens: identification of Fur-regulated proteins and evaluation of their contribution to pathogenesis

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ABSTRACT: *Pseudomonas fluorescens* is a Gram-negative bacterium and a common pathogen to a wide range of farmed fish. In a previous study, we found that the ferric uptake regulator gene (*fur*) is essential to the infectivity of a pathogenic fish isolate of *P. fluorescens* (wild-type strain TSS). In the present work, we conducted comparative proteomic analysis to examine the global protein profiles of TSS and the *P. fluorescens fur* knockout mutant TFM. Twenty-eight differentially produced proteins were identified, which belong to different functional categories. Four of these proteins, viz. TssP (a type VI secretion protein), PspA (a serine protease), OprF (an outer membrane porin), and ClpP (the proteolytic subunit of an ATP-dependent Clp protease), were assessed for virulence participation in a model of turbot *Scophthalmus maximus*. The results showed that the *oprF* and *clpP* knockouts exhibited significantly reduced capacities in (1) resistance against the bactericidal effect of host serum, (2) dissemination into and colonization of host tissues, and (3) inducing host mortality. In contrast, mutation of *tssP* and *pspA* had no apparent effect on the pathogenicity of TSS. Purified recombinant OprF, when used as a subunit vaccine, induced production of specific serum antibodies in immunized fish and elicited significant protection against lethal TSS challenge. Antibody blocking of the OprF in TSS significantly impaired the ability of the bacteria to invade host tissues. Taken together, these results indicate for the first time that in pathogenic *P. fluorescens*, Fur regulates the expression of diverse proteins, some of which are required for optimal infection.

KEY WORDS: *Pseudomonas fluorescens* · Ferric uptake regulator · Proteomic · Infection · Virulence · Vaccine

INTRODUCTION

Ferric uptake regulator (Fur) is a transcriptional regulator that controls the expression of target genes by recognizing and binding to specific DNA sequences (Bagg & Neilands 1987, de Lorenzo et al. 1987). Fur was first discovered in *Escherichia coli*, in which mutation of the *fur* site caused constitutive expression of the genes involved in iron uptake (Hantke 1981, 1984, Bagg & Neilands 1985). Subsequently, Fur was found to exist almost ubiquitously in Gram-negative bacteria and is a master regulator of iron homeostasis (Crosa 1997, Hantke 2001, Andrews et al. 2003). The activity of Fur requires Fe^{2+}, which serves as a co-repressor. When iron is abundant, Fur associates with Fe^{2+}, and the Fur-Fe^{2+} complex binds to the operator site in the promoter of the target gene, whereby it blocks binding of RNA polymerase and

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inhibits transcription of the target gene (Escolar et al. 1999, 2000, Lee & Helmann 2007). For pathogenic bacteria, iron acquisition is essential to host infection, and thus Fur is closely linked to pathogenicity (Ellermeier & Slauch 2008, Johnson et al. 2011, Troxell et al. 2011). In addition to acting as a repressor, Fur can also function as an activator and can regulate genes other than those involved in iron homeostasis (Troxell & Hassan 2013, Fillat 2014).

Pseudomonas fluorescens is a Gram-negative, psychrotrophic bacterium that grows optimally at 25–30°C. It is present widely in soil, water, plants, and animals. *P. fluorescens* is a common aquaculture pathogen, infecting both invertebrate and vertebrate animals, notably shrimp and fish (Swain et al. 2007, Wang et al. 2009). In China, diseases due to *P. fluorescens* have been observed in various species of farmed fish, including grass carp, common carp, Japanese flounder, and turbot (Wang et al. 2009, Shen et al. 2014). *P. fluorescens* can also infect humans and cause outbreaks of bacteremia (Gershmanet al. 2008). To date, documented research on the pathogenicity of *P. fluorescens* is very limited for both human clinical isolates and fish isolates (Hu et al. 2009a, 2012, W. Zhang et al. 2009, Madi et al. 2010, Dagorn et al. 2013, S. Zhang et al. 2014). As a result, very little is known about the virulence factors that participate in the infection process of *P. fluorescens*.

In a previous study with a fish model, Wang et al. (2009) observed that deletion of the *fur* gene of the pathogenic *P. fluorescens* wild-type strain TSS dramatically attenuated the overall virulence of the bacterium. Here we compared the proteomic profiles of the *fur*-defective mutant TFM and the wild-type strain TSS. Of the differentially produced proteins thus identified in TFM, we further examined their potential involvement in bacterial infection as well as the immunoprotective effect of one of the proteins as a subunit vaccine.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Pseudomonas fluorescens TSS and TFM have been decribed previously (Wang et al. 2009). *Escherichia coli* BL21(DE3) was purchased from Tiangen. All strains were grown in Luria-Bertani broth (LB) at 28°C (for *P. fluorescens*) or 37°C (for *E. coli*). Where indicated, 2,2′-dipyridyl (Sigma), tetracycline, and chloramphenicol were added at concentrations of 600 µM, 20 µg ml^{-1}, and 50 µg ml^{-1}, respectively.

Fish

Clinically healthy turbot *Scophthalmus maximus* were purchased from a local fish farm. Fish were maintained at 20°C in aerated seawater that was changed twice daily, and they were fed daily with commercial dry pellets (purchased from Shandong Sheng-suo Fish Feed Research Center, Shandong, China). Before the experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen by plate counts as reported previously (Hu et al. 2009b), and no bacteria were detected from the sampled fish. An enzyme-linked immunosorbent assay (ELISA) detected no serum antibodies against *P. fluorescens*. Fish were euthanized with tricaine methanesulfonate (Sigma) as reported previously (Wang et al. 2009) prior to tissue collection.

Two-dimensional gel electrophoresis (2-DE)

TFM and TSS were cultured in LB medium at 28°C to OD_{600} 0.8, and the cells were collected by centrifugation at 4000 × *g* (15 min at 4°C). The cells were washed 3 times with phosphate-buffered saline (PBS) and resuspended in extraction solution (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 2% IPG buffer). The cells were disrupted by intermittent sonic oscillation for a total of 15 min on ice with intervals of 30 s. Unbroken cells and cellular debris were removed by centrifugation at 20 000 × *g* for 60 min. The proteins in the supernatant were purified with a 2D-Clean-Up Kit (GE Healthcare) and resuspended in IEF sample loading solution (7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.5% IPG buffer, 0.002% bromophenol blue). Protein concentration was determined using the BCA Protein Assay Kit (Sangon). Two-DE was performed as reported previously (Zhang et al. 2013). The gel images were acquired using ImageScanner III (GE Healthcare) and analyzed with ImageMaster 2D Platinum 6.0 (GE Healthcare). Triplicate runs were made for each sample to ensure gel reproducibility. For comparative analysis, the percentage intensity volume (%vol) of each spot was used for comparison of matched spots between TFM and TSS. To reduce potential errors, a ratio of ≥2 (or ≤0.5) and analysis of variance (ANOVA) $p < 0.05$ were taken as a threshold for differential expression.

Extraction and proteolysis

Differentially expressed protein spots were subjected to in-gel enzymatic digestion and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry as reported previously (Zhang et al. 2013). MALDI-TOF mass spectrometry (MS) analysis was performed with ultrafleXtreme (Bruker) as follows. One microliter peptide solution was dripped onto the Anchorchip target plate and allowed to dry at room temperature. Matrix solution (CHCA) was added to the plate, and the plate was loaded into the spectrometer. The mass range was from 500 to 3500 Da, and the scan resolution was 50 000. After the scan, the 5 most abundant MS peaks were selected for MS/MS scanning. Protein identification was performed as described previously (Zhang et al. 2013).

Quantitative real-time reverse transcription-PCR

TFM and TSS were cultured in LB medium to an OD_{600} of 0.8. Total RNA was extracted with an EZNA Total RNA Kit (Omega Bio-tek). The RNA was treated with RNase-free DNaseI (TaKaRa). One microgram of RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen). qRT-PCR was carried out in an Eppendorf Mastercycler using SYBR ExScript qRT-PCR Kit (TaKaRa) as described previously (Zheng & Sun 2011) with primers listed in Table S1 in the Supplement, available at www.int-res.com/articles/suppl/d115p067_supp.pdf. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only 1 PCR product was amplified and detected. The expression level of the target genes was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Gene cloning and sequence analysis

The open reading frames of *clpP*, *oprF*, *pspA*, and *tssP* were cloned by PCR using the primer pairs ClpPF1/ClpPR1, OprFF1/OprFR1, PspAF1/PspAR1, and TssPF1/TssPR1, respectively (Table 1), which were designed based on the sequences of *clpP*, *oprF*, *pspA*, and *tssP* homologues in *P. fluorescens* A506 (GenBank accession nos. AFJ59834.1, AFJ59305.1, AFJ58776.1, and AFJ56203.1, respectively). The PCR products were ligated into the T-A cloning vector T-Simple (TransGen Biotech), and the recombinant plasmids were subjected to sequence analysis. The amino acid sequences encoded by the 4 genes were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. A domain search was performed with the conserved domain search program of NCBI.

Gene knockout and complementation

The primers used in gene knockout are listed in Table 1. To construct the *oprF* knockout TSSΔ*oprF*, in-frame deletion of an 855 bp segment (positions 82 to 936) of *oprF* was performed by overlap extension PCR as follows: the first overlap PCR was performed with the primer pair OprFF2/OprFR2, the second overlap PCR was performed with the primer pair OprFF3/OprFR3, and the fusion PCR was performed with the primer pair OprFF2/OprFR3. The PCR

Table 1. Primers used in this study. Underlined nucleotides are restriction sites of the enzymes indicated in parentheses

Primer	Sequence (5′→ 3′)
ClpPF1	CTAAGGTGACCGATAAAGCG
ClpPF2	GTACCCGGGGAATGAGCAGCAGATGAACG (*Sma*I)
ClpPF3	TGACTGCGCCCGCATAATATAAGCCG
ClpPR1	GCCACAGAAGGAGCAATAGA
ClpPR2	TATGCGGGCGCAGTCACCGAACCAC
ClpPR3	GGACCCGGGGTTGTCCGACTTGCGC (*Sma*I)
OprFF1	ATGAAACTGAAAAACACCTTGG
OprFF2	GTTCCCGGGGACATTGACCTGACTGTCGG (*Sma*I)
OprFF3	AAGGCCAAGTTAACCGTCGCGTAGAAGC
OprFF4	GATATCATGAAACTGAAAAACACCTTGG (*Eco*RV)
OprFR1	TTACTGAGCGGTAGCTTCAAC
OprFR2	CGGTTAACTTGGCCTTGTGCCAGAAC
OprFR3	CGCCCCGGGCCGCAGGAGTTTATTCACC (*Sma*I)
OprFR4	GATATCCTGAGCGGTA GCTTCAACC (*Eco*RV)
PspAF1	ATGATCACCGATTCACCAC
PspAF2	ACCGATATCCGCTGAACCAGAGCTTTGC (*Eco*RV)
PspAF3	CACGTTTCCGCGCGAAAACCCAGGGCG
PspAR1	GACTGCAGCAGGCTTTCATA
PspAR2	TTCGCGCGGAAACGTGGTGAATCGGT
PspAR3	TGTGATATCTGGCGAACTCCGGGTGCT (*Eco*RV)
TssPF1	ATGACGGACAATACCGCCC
TssPF2	GCGCCCGGGAGGCCGTCAGTGAAGACAA (*Sma*I)
TssPF3	TAATCATGGCCTAATTAACCGGACCG
TssPR1	TTATGCGCTTTTTGCCGA
TssPR2	AATTAGGCCATGATTACTGCTCCCCTGC
TssPR3	ACGCCCGGGGGAAGCGATTTCAGTTACCAC (*Sma*I)

products were inserted into the suicide plasmid p7TS (Wang et al. 2009) at the *Sma*I site, resulting in plasmid p7TSoprF. p7TSoprF was introduced into *E. coli* S17-1λpir (Biomedal) by transformation. The transformant S17-1λpir/p7TSoprF was conjugated with TSS. The transconjugants were selected first on LB plates supplemented with tetracycline and chloramphenicol and then on LB plates supplemented with 12% sucrose and chloramphenicol. The colonies that appeared on the plates were analyzed by PCR; the PCR products were subsequently subjected to DNA sequencing to confirm the deletion in *oprF*.

The *clpP*, *pspA*, and *tssP* knockouts TSSΔ*clpP*, TSSΔ*pspA*, and TSSΔ*tssP* respectively, were constructed in the same fashion as TSSΔ*oprF*. To construct TSSΔ*clpP*, in-frame deletion of a 360 bp segment (positions 268 to 627) of *clpP* was performed by overlap extension PCR as follows: the first overlap PCR was performed with the primer pair ClpPF2/ClpPR2, the second with the primer pair ClpPF3/ClpPR3, and the fusion PCR with the primer pair ClpPF2/ClpPR3. To construct TSSΔ*pspA*, in-frame deletion of a 2886 bp segment (positions 25 to 2910) of *pspA* was performed by overlap extension PCR as follows: the first overlap PCR was performed with the primer pair PspAF2/PspAR2, the second with the primer pair PspAF3/PspAR3, and the fusion PCR with the primer pair PspAF2/PspAR3. To construct TSSΔ*tssP*, in-frame deletion of a 1488 bp segment (positions 4 to 1491) of *tssP* was performed by overlap extension PCR as follows: the first overlap PCR was performed with the primer pair TssPF2/TssPR2, the second with the primer pair TssPF3/TssPR3, and the fusion PCR with the primer pair TssPF2/TssPR3. In all cases, the fusion PCR products were inserted into p7TS, and the recombinant plasmid was introduced into *E. coli* S17-1λpir by transformation as described above for the construction of TSSΔ*oprF*. The transformants were conjugated with TSS, and the transconjugants were selected as described above for the construction of TSSΔ*oprF*. Deletion of *clpP*, *pspA*, and *tssP* in TSSΔ*clpP*, TSSΔ*pspA*, and TSSΔ*tssP* respectively, was confirmed by PCR as above and sequence analysis of the PCR products.

The *oprF* complement strain TSSΔ*oprF*C was created as described previously (Hu et al. 2009b) by introducing into TSSΔ*oprF* a low-copy plasmid expressing *oprF*. Briefly, the coding sequence of *oprF* was amplified by PCR as above, and the PCR product was inserted into pBT (Hu et al. 2009b) at the *Sma*I site; the recombinant plasmid was digested with *Swa*I, and the fragment carrying *oprF* was inserted into pJT (Hu et al. 2009b) at the *Swa*I site, resulting in pJToprF. pJToprF was introduced into S17-1λpir by transformation. The transformant S17-1λpir/pJToprF was conjugated with TSSΔ*oprF*. The transconjugants were selected on LB plates supplemented with tetracycline (selection marker of pJToprF). The colonies that appeared on the plates were analyzed by PCR, and the PCR product was subsequently subjected to DNA sequencing. One of the colonies was named TSSΔ*oprF*C. Growths of the knockouts and the complement strain TSSΔ*oprF*C were conducted as described above for the wild type, except that tetracycline was added during the growth of TSSΔ*oprF*C.

Serum survival assay and *in vivo* infection

Serum survival analysis was performed as reported previously (Wang et al. 2013).

Experiments involving live fish were conducted in accordance with the 'Regulations for the Administration of Affairs Concerning Experimental Animals' promulgated by the State Science and Technology Commission of Shandong Province. To investigate tissue dissemination and colonization ability, TSS and the mutants (TSSΔ*tssP*, TSSΔ*pspA*, TSSΔ*oprF*, and TSSΔ*clpP*) were cultured in LB medium to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS to 2×10^7 CFU ml^{-1}. Turbot (average 11.8 g) were divided randomly into 6 groups (N = 15 fish $group^{-1}$) and infected by intramuscular (i.m.) injection with 50 µl of TSS, TSSΔ*tssP*, TSSΔ*pspA*, TSSΔ*oprF*, TSSΔ*clpP*, or PBS. Fish were maintained as described above in 52 l tanks containing 40 l of seawater. Kidney and spleen were taken from the fish at 12, 24, and 48 h post-infection (5 fish at each time point). The tissues were weighed, and equal weights of tissues from each fish were homogenized in PBS with an OSE-20 electric tissue homogenizer (Tiangen). One hundred microliters of the homogenates were diluted serially in PBS, and 100 µl of the dilutes were plated in triplicate on LB agar plates. The plates were incubated at 28°C for 48 h, and the colonies that appeared on the plates were enumerated. The genetic identity of the colonies was verified by PCR amplification of the *fur* gene with primers FurF1 (5′-ATG GTT GAA AAT AGC GAA CT-3′) and FurR1 (5′-CTT TTT CTT ACG GAT GTA CA-3′); the PCR product was ligated into the T-A cloning vector T-Simple (TransGen Biotech), and *E. coli* Trans 5α (TransGen Biotech) was transformed with the ligation mixture and

plated on LB agar plate containing 50 µg ml^{-1} kanamycin. The plate was incubated at 37°C overnight, and 5 of the colonies that emerged on the plate were selected for sequence analysis of the inserted *fur* gene. For the mortality assay, fish (20 $group^{-1}$) were infected as above with TSS, TSSΔ*oprF*, and TSSΔ*clpP* at a dose of 1×10^7 CFU $fish^{-1}$. The fish were maintained as described above in 52 l tanks containing 40 l seawater. The fish were monitored for accumulated mortality over a period of 20 d. The experiments were performed independently 3 times.

Expression and purification of recombinant protein

The plasmid pEtOprF, which expresses recombinant OprF (rOprF), was constructed as follows. The coding sequence of OprF was amplified by PCR with the primer pair OprFF4/OprFR4 (Table 1). The PCR products were ligated with the T-A cloning vector pBS-T (Tiangen); the recombinant plasmid was digested with *Eco*RV, and the fragment containing *oprF* was inserted into pET259 (Hu et al. 2010) at the *Swa*I site. Recombinant protein was purified as follows. *E. coli* BL21(DE3) was transformed with pEtOprF; the transformant was cultured in LB medium at 37°C to mid-logarithmic phase, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growing at 37°C for an additional 4 h, the cells were harvested by centrifugation, and His-tagged rOprF was purified using Ni-NTA Agarose (Qiagen) as recommended by the manufacturer. The protein was treated with Triton X-114 to remove endotoxin as reported previously (Chen et al. 2013). The protein was then concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore). The concentrated protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of the protein was determined using the Bradford method with bovine serum albumin as the standard.

Vaccination

rOprF was resuspended in PBS to a concentration of 200 µg ml^{-1} and mixed at an equal volume with aluminum hydroxide as described previously (Jiao et al. 2010). As a control, PBS was mixed similarly with aluminum hydroxide without protein. Turbot (average 18.4 g) were divided randomly into 2 groups (N = 60 fish $group^{-1}$) and injected intraperitoneally with 50 µl of the protein mixture or the PBS control. The fish were maintained as described above in 52 l tanks containing 40 l of seawater. At 1 mo post-vaccination, 40 fish were taken from each group and challenged with TSS at a dose of 1×10^7 CFU $fish^{-1}$. The fish were monitored for mortality for a period of 20 d. Dying fish were randomly selected for the examination of bacterial recovery from liver, kidney, and spleen. Relative percent of survival (RPS) was calculated according to the following formula: RPS = [1 – (% mortality in immunized fish/% mortality in control fish)] × 100. The vaccination experiment was performed 2 times.

ELISA

Sera were taken from vaccinated fish and control fish (8 each) at 1 mo post-immunization, and serum antibody titers were determined by ELISA as reported previously (Sun et al. 2011).

Effect of serum antibody blocking on bacterial infection

Rat antiserum against rOprF was prepared as reported previously (Jiao et al. 2010). The serum was diluted 10 times in PBS. TSS was cultured in LB medium to an OD_{600} of 0.8. The cells were washed in PBS and resuspended in PBS to 2×10^7 CFU ml^{-1}. One milliliter of bacterial cells was mixed with 10 µl rOprF antiserum or pre-immune serum and incubated at 28°C for 1 h. After incubation, 50 µl of the mixture were inoculated into turbot via i.m. injection. At 12, 24, and 48 h post-infection, bacterial recovery from kidney and spleen was determined as described above.

Statistical analysis

All experiments were performed 3 times, and statistical analyses were carried out with the SPSS 17.0 package. Except for the mortality analysis in the *in vivo* infection experiment and the survival analysis in the vaccination experiment, in which the statistical methods are indicated in the relevant figure legends, ANOVA was used for all other analyses. In all cases, the significance level was defined as $p < 0.05$.

RESULTS

Identification of differentially expressed proteins between TFM and TSS

Two-DE analysis showed that compared to TSS, TFM exhibited 33 proteins which were differentially produced (Fig. 1). Of these proteins, 16 were significantly upregulated (ratio of TFM:TSS ≥ 2, $p \leq 0.05$), and 17 were significantly downregulated (ratio of TSS:TFM ≥ 2, $p \leq 0.05$). MALDI-TOF/TOF analysis successfully identified 28 of the 33 protein spots (Table 2). Of these 28 proteins, 14 were putatively involved in cellular metabolism. These proteins were glutamine synthetase, ATP-dependent protease ATP-binding subunit, arginine deiminase, isocitrate lyase, purine nucleoside permease, ketol-acid reductoisomerase, acetyl-CoA carboxylase subunit alpha, serine protease A (PspA), dipeptidase, urocanate hydratase, isocitrate dehydrogenase, exodeoxyribonuclease III, ATP-dependent Clp protease proteolytic subunit (ClpP), and superoxide dismutase. Four proteins (EvpB family type VI secretion protein [TssP], sugar ABC transporter substrate-binding protein, ABC transporter, and ABC transporter substrate-binding protein) were associated with transport, 4 proteins (carbohydrate-selective porin OprB, outer membrane porin OprE3, flagellin, and outer membrane porin OprF) were membrane proteins, 2 proteins (glucan biosynthesis protein G and 50S ribosomal protein L25) were associated with biosynthesis, 1 protein was involved in chemotaxis, and the other 2 proteins were unknown in function (Table 2).

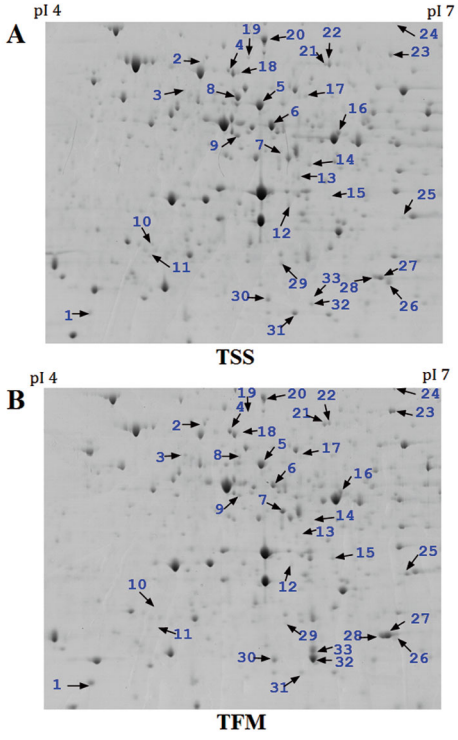

Fig. 1. Representative 2-dimensional gel electrophoresis (2-DE) maps of the protein profiles of *Pseudomonas fluorescens* (A) wild-type strain TSS and (B) the *fur* knockout mutant TFM. Whole-cell proteins prepared from *P. fluorescens* TSS and TFM were subjected to 2-DE analysis. Numbers indicate protein spots with differential expression

Validation of differential expression at the mRNA level

To examine whether the above-observed differential productions in protein level correlated with the expressions of mRNA, qRT-PCR was conducted to determine the transcription of 20 randomly selected genes encoding 12 proteins upregulated and 8 proteins downregulated in TFM. The results showed that of the 12 genes encoding the upregulated proteins, all were significantly upregulated at the mRNA level (Table 3). Of the 8 genes encoding downregulated proteins, 5 were significantly downregulated at the mRNA level and 3 were significantly upregulated at the mRNA level (Table 3).

Construction of TSS genetic variants with deletions of differentially expressed genes

Since Fur is known to be a key regulator of genes involved in pathogenicity, we wished to examine the virulence potentials of some differentially produced proteins in TFM. For this purpose, we cloned 4 genes, viz. *tssP*, *pspA*, *oprF*, and *clpP*, which encode 4 proteins identified above, i.e. type VI secretion protein, serine protease A, outer membrane porin OprF, and ATP-dependent Clp protease proteolytic subunit, respectively (Table 2). Sequence analysis showed that the amino acid sequence of PspA is 99.1% identical to that of *P. fluorescens* A506 (GenBank accession no. AFJ58776.1), while the amino acid sequences of ClpP, TssP, and OprF are 100% identical to their counterparts in *P. fluorescens* A506 (GenBank accession nos. AFJ59834.1, AFJ56203.1, and AFJ59305.1, respectively). To examine their functional importance, *tssP*, *pspA*, *oprF*, and *clpP* knockouts were created and named TSSΔ*tssP*, TSSΔ*pspA*, TSSΔ*oprF*, and TSSΔ*clpP* respectively. In addition, an *oprF* complement strain, TSSΔ*oprF*C, was also created.

Table 2. Summary of differentially expressed proteins in *Pseudomonas fluorescens* wild type (TSS) and *fur* knockout mutant (TFM). Spot ID represents the number on the 2-dimensional gel electrophoresis (2-DE) gels in Fig. 1. The MASCOT score is −10 log (p), where p is the probability that the observed match is a random event (based on the NCBInr database using the MASCOT searching program as MS/MS data). Scores greater than 65 are significant ($p < 0.05$). Coverage indicates the number of amino acids spanned by the assigned peptides divided by the protein sequence length. For the fold ratio, the mean is the average protein abundance ratio for 3 paired samples, and SD is the standard deviation of protein abundance ratios of 1 certain spot of 3 paired samples. NA: not analyzed; NUP: purine nucleoside permease; NCBI: National Center for Biotechnology Information; pI: theoretical isoelectric point; M_r: relative molecular weight; TFM: *P. fluorescens fur* knockout mutant; TSS: *P. fluorescens* wild-type strain

Spot no.	NCBI no.	Protein name [source species or genus]	Abbrev.	MASCOT score	pI	M_r (kDa)	Coverage (%)	Fold ratio TFM:TSS (mean ± SD)
Signal transduction								
1	gil489314654	Chemotaxis protein [*Pseudomonas fluorescens*]		69	4.96	58.73	42.33	3.12 ± 1.06
Metabolism								
2	gil489280166	Glutamine synthetase [*Pseudomonas*]		307	5.16	52.00	86.20	2.21 ± 0.73
3	gil213521160	ATP-dependent protease ATP-binding subunit [*Pseudomonas fluorescens*]	ClpX	184	5.18	48.14	54.12	2.24 ± 0.78
5	gil489286234	Arginine deiminase [*Pseudomonas fluorescens*]		297	5.36	46.75	67.85	0.48 ± 0.06
8	gil489266921	Isocitrate lyase [*Pseudomonas*]		132	5.31	48.79	60.73	−∞
13	gil504531739	Purine nucleoside permease, NUP family / hypothetical protein	NUP	159	5.54	38.20	58.77	0.36 ± 0.13
14	gil489268327	Ketol-acid reductoisomerase [*Pseudomonas*]		215	5.48	36.42	70.18	0.42 ± 0.17
15	gil489281554	Acetyl-CoA carboxylase subunit alpha [*Pseudomonas*]		255	5.65	35.02	62.36	2.84 ± 0.58
17	gil387999487	Serine protease A [*Pseudomonas fluorescens* SS101]	PspA	151	5.58	102.74	57.29	0.44 ± 0.16
21	gil387893092	Dipeptidase [*Pseudomonas fluorescens* A506]		181	5.85	62.42	51.78	2.17 ± 0.55
22	gil387891588	Urocanate hydratase [*Pseudomonas fluorescens* A506]		310	5.66	60.70	67.18	0.41 ± 0.11
24	gil504531801	Isocitrate dehydrogenase [*Pseudomonas fluorescens*]		141	5.84	80.28	52.03	∞
25	gil388000088	Exodeoxyribonuclease III [*Pseudomonas fluorescens*]	Xth	457	5.59	30.91	85.78	2.98 ± 1.05
30	gil213521159	ATP-dependent Clp protease proteolytic subunit	ClpP	232	5.42	23.41	49.53	2.19 ± 0.76
32	gil387892089	Superoxide dismutase [*Pseudomonas fluorescens*]	SodA1	192	5.59	22.33	57.49	4.57 ± 1.22
Transport								
4	gil 489287996	EvpB family type VI secretion protein [*Pseudomonas*]	TssP	256	5.27	56.05	71.43	2.20 ± 0.65
7	gil504529570	Sugar ABC transporter substrate-binding protein/ putative iron-binding periplasmic protein [*Pseudomonas*]	AfuA	180	6.14	37.07	54.79	3.51 ± 1.04
9	gil489280456	ABC transporter [*Pseudomonas fluorescens*]		82	5.62	40.20	35.23	0.44 ± 0.10
29	gil515541981	ABC transporter substrate-binding protein		154	6.19	27.44	82.63	0.46 ± 0.15
Chaperones								
12	gil489286359	Molecular chaperone GroEL [*Pseudomonas fluorescens*]	GroEL	106	4.93	56.90	52.66	0.35 ± 0.04
Membrane proteins								
6	gil489286177	Carbohydrate-selective porin OprB	OprB	162	5.88	49.93	58.42	−∞
16	gil387891539	Outer membrane porin OprE3 [*Pseudomonas fluorescens* A506]	OprE3	552	6.99	47.64	91.94	2.88 ± 0.74
18	gil516097028	Flagellin [*Pseudomonas fluorescens*]		81	5.26	55.93	48.99	0.44 ± 0.04
20	gil387895056	Outer membrane porin OprF [*Pseudomonas fluorescens*]	OprF	242	5.73	34.51	87.62	0.33 ± 0.09
Biosynthesis								
23	gil489280211	Glucan biosynthesis protein G [*Pseudomonas fluorescens*]		588	6.17	65.01	91.41	2.93 ± 0.82
27	gil387891951	50S ribosomal protein L25 [*Pseudomonas fluorescens*]		577	5.73	21.38	68.34	2.48 ± 0.66

(Table 2 continued on next page)

Table 2 (continued)

Spot no.	NCBI no.	Protein name	Abbrev.	MASCOT score	pI	M_r (kDa)	Coverage (%)	Fold ratio TFM:TSS (mean ± SD)
Unknown function and hypothetical proteins								
19	gil387892131	Hypothetical protein PflA506_0906 [*Pseudomonas fluorescens* A506]		170	5.62	66.67	55.52	0.31 ± 0.08
26	gil489281996	Hypothetical protein [*Pseudomonas fluorescens*]		314	5.79	26.53	88.93	0.40 ± 0.07
10	NA	NA		NA	NA	NA	NA	0.42 ± 0.47
11	NA	NA		NA	NA	NA	NA	0.48 ± 0.05
28	NA	NA		NA	NA	NA	NA	2.16 ± 0.81
31	NA	NA		NA	NA	NA	NA	0.45 ± 0.22
33	NA	NA		NA	NA	NA	NA	3.45 ± 0.96

Growth profiles of the mutants

Growth analysis showed that when cultured in LB medium, TSSΔ*tssP* was comparable to TSS in growth profile, while TSSΔ*pspA* and TSSΔ*clpP* exhibited apparently lower growth rates than TSS (Fig. 2A). The growth rate of TSSΔ*oprF* was largely similar to that of TSS at the early logarithmic phase but reached much lower cell densities at the stationary phase. When cultured in LB medium supplemented with the iron chelator 2,2′-dipyridyl, TSSΔ*tssP* was comparable to TSS in growth profile, while TSSΔ*pspA* and TSSΔ*clpP* grew faster than TSS at the logarithmic phase and were similar to TSS at the stationary phase (Fig. 2B). TSSΔ*oprF* grew much faster than TSS at the logarithmic phase and reached higher cell densities at the stationary phase (Fig. 2B). Compared to TSSΔ*oprF*, the *oprF* complement strain TSSΔ*oprF*C exhibited faster growth, albeit still slower than that of TSS (Fig. S1 in the Supplement).

Table 3. Summary of mRNA expression in the *Pseudomonas fluorescens fur* knockout mutant TFM (in comparison with that in the wild-type strain TSS) as determined by qRT-PCR. 2-DE: 2-dimensional gel electrophoresis; NUP: purine nucleoside permease

Spot no.	Description [source species or genus]	Protein level (by 2-DE)	mRNA level (by qRT-PCR)
21	Dipeptidase [*Pseudomonas fluorescens* A506]	Up (2.17 ± 0.55)	Up (3.64 ± 1.24)
30	ATP-dependent Clp protease proteolytic subunit [*Pseudomonas fluorescens* SS101]	Up (2.19 ± 0.76)	Up (4.18 ± 0.21)
4	EvpB family type VI secretion protein [*Pseudomonas*]	Up (2.20 ± 0.65)	Up (9.00 ± 0.29)
2	Glutamine synthetase [*Pseudomonas*]	Up (2.21 ± 0.73)	Up (1.71 ± 0.28)
27	50S ribosomal protein L25 [*Pseudomonas fluorescens*]	Up (2.48 ± 0.66)	Up (2.08 ± 0.08)
15	Acetyl-CoA carboxylase subunit alpha [*Pseudomonas*]	Up (2.84 ± 0.58)	Up (2.42 ± 0.23)
16	Outer membrane porin OprE3 [*Pseudomonas fluorescens* A506]	Up (2.88 ± 0.74)	Up (36.80 ± 17.07)
23	Glucan biosynthesis protein G [*Pseudomonas fluorescens*]	Up (2.93 ± 0.82)	Up (2.78 ± 0.20)
25	Exodeoxyribonuclease III [*Pseudomonas fluorescens*]	Up (2.98 ± 1.05)	Up (1.90 ± 0.20)
7	Sugar ABC transporter substrate-binding protein/ putative iron-binding periplasmic protein [*Pseudomonas*]	Up (3.51 ± 1.04)	Up (15.80 ± 7.10)
32	Superoxide dismutase [*Pseudomonas fluorescens*]	Up (4.57 ± 1.22)	Up (88.85 ± 17.02)
24	Isocitrate dehydrogenase [*Pseudomonas fluorescens*]	Up (∞)	Up (1.74 ± 0.25)
8	Isocitrate lyase [*Pseudomonas*]	Down (−∞)	Down (0.10 ± 0.06)
13	Purine nucleoside permease, NUP family/hypothetical protein [*Pseudomonas*]	Down (0.36 ± 0.13)	Down (0.05 ± 0.02)
14	Ketol-acid reductoisomerase [*Pseudomonas*]	Down (0.42 ± 0.17)	Down (0.26 ± 0.06)
18	Flagellin [*Pseudomonas fluorescens*]	Down (0.44 ± 0.04)	Down (0.06 ± 0.01)
9	ABC transporter [*Pseudomonas fluorescens*]	Down (0.44 ± 0.10)	Down (0.65 ± 0.12)
22	Urocanate hydratase [*Pseudomonas fluorescens* A506]	Down (0.41 ± 0.11)	Up (1.82 ± 0.17)
5	Arginine deiminase [*Pseudomonas fluorescens*]	Down (0.48 ± 0.06)	Up (2.01 ± 0.18)
26	Hypothetical protein [*Pseudomonas fluorescens*]	Down (0.40 ± 0.07)	Up (7.34 ± 3.32)

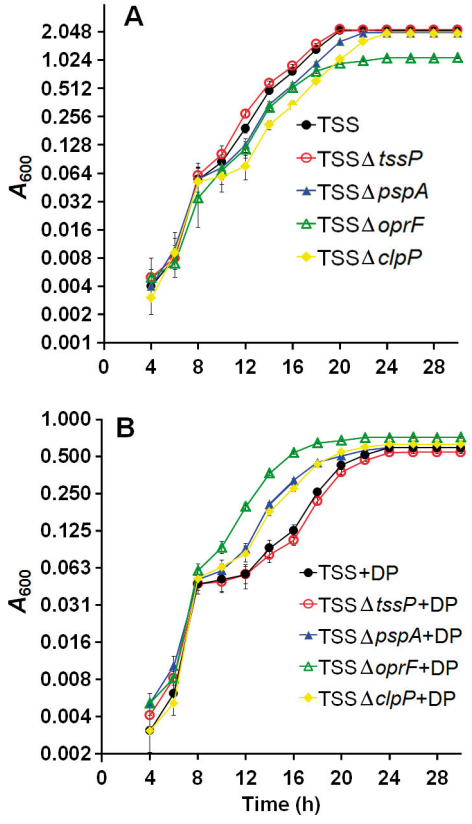

Fig. 2. Growth profiles of *Pseudomonas fluorescens* wild-type strain TSS and its genetic variants. TSSΔ*tssP*, TSSΔ*pspA*, TSSΔ*oprF*, and TSSΔ*clpP* cultured in Luria-Bertani medium supplemented (A) without or (B) with 2,2′-dipyridyl (DP). Cell density was determined at various time points by measuring absorbance at 600 nm (A_{600}). Data are means ± SEM of 3 independent assays

Virulence potentials of the mutants

Survival in host serum

Serum survival analysis showed that when incubated with turbot serum, TSSΔ*oprF* exhibited a survival rate that was 34-fold lower than that of TSS (Fig. 3). The survival rate of TSSΔ*clpP* was also significantly reduced compared to TSS. The survival rates of TSSΔ*tssP* and TSSΔ*pspA* were comparable to that of TSS.

Invasion of host tissues

To examine their ability to disseminate into and colonize host tissues, the wild-type and mutant TSS were inoculated into turbot via muscle injection, and bacterial loads in kidney and spleen were determined at 12, 24, and 48 h post-infection. The results showed that the bacterial recoveries of TSSΔ*tssP* and TSSΔ*pspA* were comparable to those of TSS at all examined time points (data not shown). In contrast, the bacterial recoveries of TSSΔ*oprF* in both tissues and at all examined time points were significantly lower (4- to 14-fold) than those of TSS (Fig. 4). Similarly, TSSΔ*clpP* also exhibited significantly reduced bacterial recoveries, but the reduction magnitudes (2- to 7-fold) were in general lower than those of TSSΔ*oprF* (Fig. 4).

Ability to cause host mortality

To examine the ability of the mutants to cause mortality in the host, turbot were inoculated with the same dose of the wild-type and mutant TSS, and the mortality of the fish was monitored. In fish infected with TSS, TSSΔ*clpP*, and TSSΔ*oprF*, mortality began to occur at 3 d post-infection, and the mortality rates were 40, 60, and 10%, respectively (Fig. 5). At 5 d post-infection, the accumulated mortalities of TSS-, TSSΔ*clpP*-, and TSSΔ*oprF*-infected fish were 100, 90, and 50%, respectively. At 6 d post-infection, TSSΔ*clpP*-infected fish reached 100% mortality. In contrast, by the end of the monitored period, there was still 20% survival of TSSΔ*oprF*-infected fish, which was significantly higher than that of TSS-infected fish.

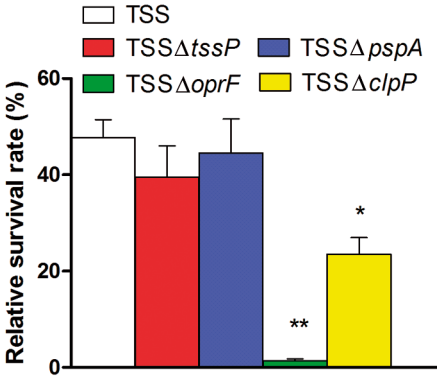

Fig. 3. Survival of *P. fluorescens* wild-type strain TSS and its genetic variants TSSΔ*tssP*, TSSΔ*pspA*, TSSΔ*oprF*, and TSSΔ*clpP* incubated with turbot (*Scophthalmus maximus*) serum for 1 h. Numbers of surviving bacterial cells were determined after incubation. Data are means ± SEM of 3 independent experiments. Significant difference between the survival rates of TSS and mutants are indicated by asterisks: *$p < 0.05$, **$p < 0.01$

Immunoprotective effect of rOprF

Since OprF is an outer membrane protein, we investigated whether it would elicit any immunoprotection in fish against TSS infection. To answer this question, rOprF was purified from *E. coli* as a His-tagged protein (Fig. S2) and used to vaccinate turbot. Fish were challenged with TSS at 1 mo post-vaccination and monitored for mortality and survival. The results showed that there was no survival in the control fish by 8 d post-challenge, whereas by the end of the monitored period, the survival rate of rOprF-vaccinated fish was 58%, which was significantly higher than that of the control fish (Fig. 6). The protection rate, in terms of RPS, of rOprF was also 58% (because the mortality of the control fish was 100%). Comparable protection (48%) was observed in the repeat vaccination trial. ELISA analysis showed that at 1 mo post-vaccination, specific serum antibodies were detected in fish vaccinated with rOprF (titer: 2^7).

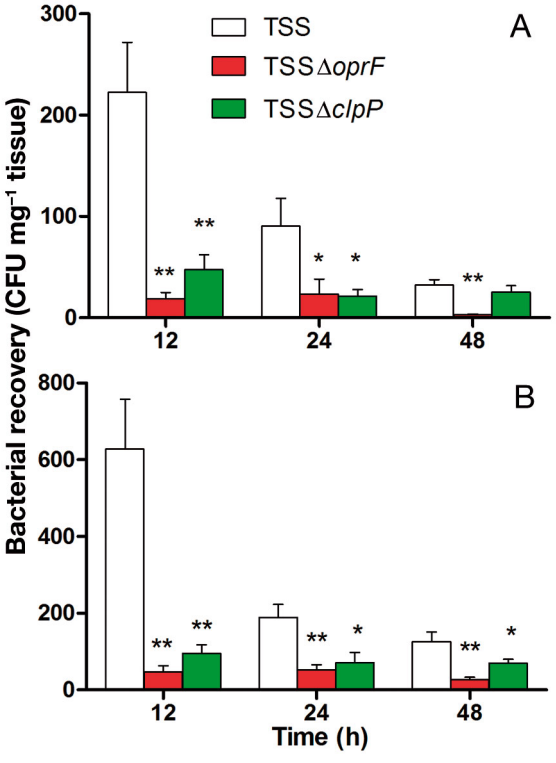

Fig. 4. Bacterial dissemination and colonization in tissues of turbot *Scophthalmus maximus* infected with *Pseudomonas fluorescens* wild-type strain TSS or its genetic variants TSSΔ*oprF* or TSSΔ*clpP* via intramuscular injection. Bacterial recovery (as colony-forming units, CFU) from (A) kidney and (B) spleen was determined at different times post-infection. Results are means ± SEM of 3 independent experiments. Significant differences between TSS and mutants indicated by asterisks: *p < 0.05; **p < 0.01

Antibody blocking of OprF and its effect on bacterial infection

Since, as shown above, OprF is required for effective host infection, we examined whether antibody blocking of this protein would affect TSS infection. For this purpose, turbot were infected with TSS that had been treated with rOprF antiserum or control serum, and bacterial dissemination in and colonization of kidney and spleen were determined at 12, 24, and 48 h post-infection. The results showed that for

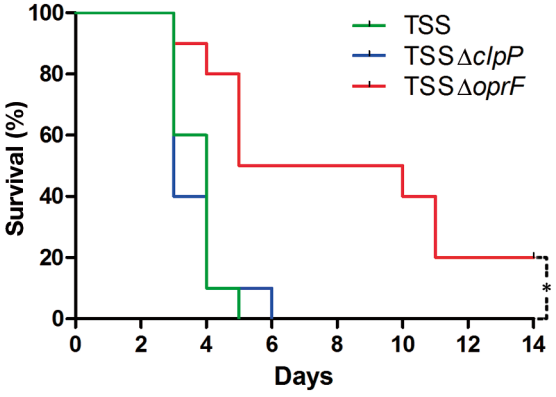

Fig. 5. Survival of turbot *Scophthalmus maximus* infected with *P. fluorescens* wild-type strain TSS or its genetic variants TSSΔ*oprF* or TSSΔ*clpP* via intramuscular injection. Fish were monitored daily for mortality and survival for 20 d (only 14 d are shown, since no further mortalities occurred thereafter). Results are means of 3 independent experiments. Significant difference between survival of TSS- and TSSΔ*oprF*-infected fish was determined with a logrank test (*p < 0.05)

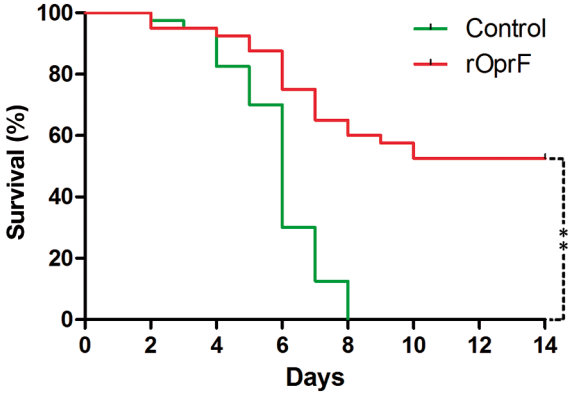

Fig. 6. Survival of turbot *Scophthalmus maximus* vaccinated with or without (control) recombinant OprF (rOprF) challenged with *Pseudomonas fluorescens* wild-type strain TSS and monitored daily. The graph shows 1 representative vaccination trial. Significant difference between survival of vaccinated fish and control fish was determined with a logrank test (**p < 0.01)

both tissues and at all examined time points, bacterial recoveries from the fish infected with TSS pretreated with rOprF antiserum were significantly lower than those from the fish infected with TSS pretreated with the control serum (Fig. 7).

DISCUSSION

In this study, we employed a proteomic approach and systematically examined the proteins that are affected in production by Fur. In Gram-negative bacteria such as *Pseudomonas aeruginosa* and *E. coli*, Fur is known to be a master regulator that controls iron homeostasis as well as other functions (Hantke 2001, Vasil 2007, Cornelis et al. 2009). Global transcriptome and proteomic studies have indicated that in *Neisseria meningitidis*, *Campylobacter jejuni*, and *Shewanella oneidensis*, *fur* mutation alters the expression of a large number of genes involved in diverse aspects of bacterial life (Thompson et al. 2002, Grifantini et al. 2003, Holmes et al. 2005). However, the regulatory role of Fur in pathogenic *P. fluorescens* is essentially unknown. In this study, proteomic analysis identified 28 proteins that were differentially produced between TFM and TSS, with 26 proteins having known functional homologues in other bacteria. Of the 26 functional proteins, 16 are putatively involved in metabolism and biosynthesis, which is in line with previous reports that *fur* regulons are associated with various metabolic processes in many bacterial species (Sebastian et al. 2002, Grifantini et al. 2003, Frangipani et al. 2014). The other 10 differentially expressed proteins identified in our study are categorized as ABC transporters, membrane proteins, and molecular chaperones. These results indicate that in TSS, Fur regulates the expression of a broad array of proteins participating in various aspects of biological processes. qRT-PCR analysis showed that of the 20 randomly selected genes, 17 exhibited mRNA profiles consistent with the 2-DE results, suggesting that Fur probably exerts its regulation on these genes at the transcription level. The other 3 genes exhibited mRNA profiles different from the 2-DE results. It is likely that Fur may regulate these genes indirectly at the posttranscriptional level as reported previously (Massé & Gottesman 2002, Wilderman et al. 2004).

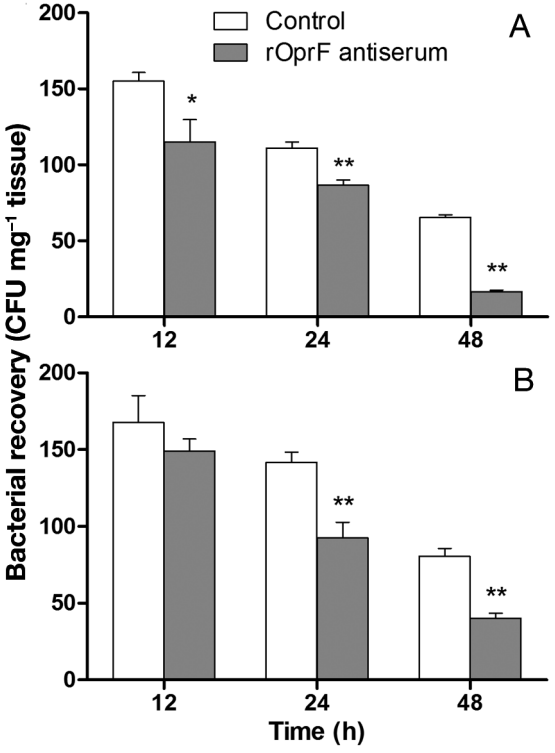

Fig. 7. Effect of recombinant OprF (rOprF) antibodies on bacterial infection in turbot *Scophthalmus maximus* infected with *Pseudomonas fluorescens* wild-type strain TSS that had been treated with rOprF antiserum or preimmune serum (control). Bacterial recovery (as colony-forming units, CFU) from (A) kidney and (B) spleen was determined at different times post-infection. Results are means ± SEM of 3 independent experiments. Significant differences between (anti) sera indicated by asterisks: $^{*}p < 0.05$, $^{**}p < 0.01$

Previous reports have shown that many Fur-regulated proteins are virulence factors involved in host infection. For example, in *P. aeruginosa*, *Haemophilus influenzae*, *Shigella*, *Vibrio vulnificus*, *Aeromonas salmonicida*, and *Edwardsiella tarda*, Fur modulates the expression of genes such as those involved in siderophore production and heme utilization, outer membrane proteases, hemolysin, and the PhoB-PhoR 2-component system, all of which participate in bacterial pathogenicity (Chandu & Nandi 2004, Africa et al. 2011, Chakraborty et al. 2011, Ebanks et al. 2013, Harrison et al. 2013, Lee et al. 2013). In our study, virulence analysis showed that compared to the wild type, TSSΔ*clpP* exhibited significantly reduced survival in host serum and lower bacterial loads in fish tissues following experimental infection. These results suggest that *clpP* is required for effective serum resistance and tissue dissemination, which is in line with the observations that Clp proteases are essential enzymes participating in the virulence of various bacteria including *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (Gaillot et al. 2000, Yamamoto et al. 2001, Kwon et al. 2004, Farrand et al. 2013). OprF plays multiple roles in bacterial pathogenesis and

immunity (Azghani et al. 2002, Krishnan & Prasadarao 2012). In *P. aeruginosa*, OprF is involved in adhesion to eukaryotic cells (Yoon et al. 2002), biofilm formation (Yoon et al. 2002), recognition of host immune factors (Fito-Boncompte et al. 2011), modulation of the quorum-sensing network (Funken et al. 2012), and association with lectin (Sharma et al. 2013). In our study, the *oprF* knockout (TSSΔ*oprF*) was drastically impaired in its ability to resist the killing effect of host serum and to disseminate in host tissues. Consistently, fish infected with TSSΔ*oprF* exhibited significantly reduced mortality. These results suggest that, as in *P. aeruginosa*, in *P. fluorescens* OprF is required for optimal bacterial infection. Compared to TSS, TSSΔ*oprF* exhibited retarded growth; introducing a low-copy plasmid expressing *oprF* into TSSΔ*oprF* partially compensated for the growth defect, but the growth of the complement strain TSSΔ*oprF*C was still slower than that of TSS, probably because, unlike the chromosomal *oprF*, the *oprF* in TSSΔ*oprF*C exists in multiple copies and is expressed from an alien promoter. In the presence of 2,2′-dipyridyl, TSSΔ*oprF* grew faster than TSS. Since 2,2′-dipyridyl creates an iron-deplete condition mimicking that of the environment *in vivo* in a host, the attenuated infectivity of TSSΔ*oprF* is not likely due to a growth defect during infection. Likewise, since TSSΔ*clpP* grew better than TSS under iron-deplete conditions, the reduced *in vivo* infection of this mutant is not likely the result of a growth defect.

As a major outer membrane protein, OprF has been considered an ideal target for vaccine development. In *P. aeruginosa*, OprF can induce strong protective immunity in mammalian systems (Price et al. 2001, Worgall et al. 2007, Weimer et al. 2009, Krause et al. 2011). Likewise, in our study, fish vaccinated with rOprF displayed significantly increased survival against lethal *P. fluorescens* challenge, and that the vaccinated fish produced specific serum antibodies against rOprF. These results indicate that OprF of *P. fluorescens* is a protective immunogen with vaccine potential. Consistent with the results of virulence analysis, administration of turbot with TSS plus rOprF antiserum inhibited tissue dissemination and colonization of TSS, which suggests that blocking the OprF in TSS probably inhibits the normal functioning of the protein and thus impairs the infectivity of the pathogen.

In conclusion, the results of this study provide for the first time a large-scale picture of Fur-regulated proteins in pathogenic *P. fluorescens*. As observed in many human pathogens, in *P. fluorescens* the Fur mutation affects the expression of a large number of proteins involved in diverse biological functions, including those associated with bacterial pathogenicity. The observation that rOprF elicited protective immunity in fish suggests a potential application of this protein in the control of *P. fluorescens* infection.

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