Vol. 110: 181–191, 2014
doi: 10.3354/dao02771

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 11

Identification and analysis of three virulence-associated TonB-dependent outer membrane receptors of *Pseudomonas fluorescens*

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ABSTRACT: *Pseudomonas fluorescens* is a Gram-negative bacterium that can infect a wide range of farmed fish. However, very little is known about the virulence mechanism of *P. fluorescens* as a fish pathogen. In this study, we identified and analyzed 3 TonB-dependent outer membrane receptors (TDRs) from a pathogenic *P. fluorescens* strain isolated from fish. *In silico* analysis revealed that all 3 proteins (named Tdr1 to 3) possess structural domains typical of TDRs. Quantitative real time RT-PCR analysis showed that *tdr1*, *tdr2*, and *tdr3* expressions were upregulated under iron-depleted conditions. Compared to the wild type, mutants defective in *tdr1*, *tdr2*, and *tdr3* were retarded in growth to different extents. Infection in a turbot *Scophthalmus maximus* model showed that all 3 mutants were impaired in their ability to desseminate into and colonize host tissues. In addition, the *tdr1* and *tdr3* mutants exhibited significantly reduced virulence. When used as subunit vaccines, purified recombinant proteins of Tdr1, Tdr2, and, in particular, Tdr3 elicited significant protection in turbot against lethal *P. fluorescens* challenge. The vaccinated fish produced specific serum antibodies, which, when incubated with *P. fluorescens*, blocked infection of *P. fluorescens* in fish cells. Together these results indicate that Tdr1, Tdr2, and Tdr3 are iron-regulated factors that participate in bacterial virulence and induce protective immunity as subunit vaccines.

KEY WORDS: *Pseudomonas fluorescens* · TonB-dependent receptor · Iron · Virulence · Vaccine

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INTRODUCTION

Pseudomonas fluorescens is an aerobic, Gram-negative bacillus found in many different environments, including soil, water, and plants. In aquaculture, *P. fluorescens* is a common pathogen to shrimp and fish, the latter including a wide range of commercially important species such as carp, tilapia, flounder, and turbot (Swain et al. 2003, 2007, Wang et al. 2009). Fish infected with *P. fluorescens* display symptoms of skin bleeding and scale loss, which can lead to heavy economic losses. To date, very little is known about the virulence mechanism of *P. fluorescens*, especially that concerning *P. fluorescens* from fish sources.

Iron is an important micronutrient for all organisms, since it is required in many basic metabolic pathways (Andrews et al. 2003). On the other hand, iron can also be toxic to the host, as free iron can lead to the generation of free oxygen radicals, which are damaging to cells. In vertebrate animals, iron is tightly sequestered by high-affinity proteins, resulting in a very low *in vivo* iron level (Schaible & Kaufmann 2004, Braun & Hantke 2011). To cope with this situation, pathogenic bacteria have developed various countermeasures to snatch iron from the host. One of these measures is the production of siderophores, which are secreted high-affinity Fe^{3+} chelators that can sequester iron from iron-containing proteins of the host (Wooldridge & Williams 1993, Neilands 1995). In Gram-negative bacteria, the Fe^{3+}-siderophore complex is too large to pass through the outer membrane, and transmembrane transport of

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the complex therefore requires outer membrane receptors such as TonB-dependent outer membrane receptors (TDRs), transperiplasmic proteins that (with partner proteins) form the ExbB-ExbD-TonB complex in the cytoplasmic membrane to obtain energy (Chimento et al. 2003, Koebnik 2005, Krewulak & Vogel 2011). The crystal structures of TDRs are highly similar, and have a 22-stranded β-barrel domain at the C-terminus and a plug domain at the N-terminus (Wiener 2005, Noinaj et al. 2010). Binding of the Fe^{3+}-siderophore complex triggers conformational change in the receptor, which induces the passage of the ligand (Braun 2006, Krewulak & Vogel 2011). As a result, TDRs are associated with bacterial pathogenicity in species such as *Flavobacterium psychrophilum*, *Escherichia coli*, *Riemerella anatipestifer*, and *Neisseria meningitides* (Alvarez et al. 2008, Hagan & Mobley 2009, Tauseef et al. 2011, Lu et al. 2013). However, the potential importance of TDRs in pathogenic *P. fluorescens* remains largely unknown.

In this study, we identified 3 TDRs (named Tdr1 to 3) from a pathogenic *P. fluorescens* strain isolated from diseased fish and examined their involvement in host infection as well as immunoprotective potential. We found that the mutation of the 3 *tdr* genes affected bacterial growth and infection and that all 3 Tdr proteins were immunogenic but induced different levels of immunoprotection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Pseudomonas fluorescens TSS is a fish pathogen that has been described previously (Wang et al. 2009). *Escherichia coli* BL21(DE3) and DH5α were purchased from Tiangen. *E. coli* S17-1λpir was purchased from Biomedal). All strains were grown in Luria-Bertani broth (LB) at 37°C (for *E. coli*) or 28°C (for *P. fluorescens*). Where indicated, the iron chelator 2,2′dipyridyl (Sigma) or $FeSO_4$ (Sangon) were added at concentrations of 600 and 100 µM, respectively. Antibiotics were supplemented as follows: chloramphenicol, 30 µg ml^{-1}; tetracycline, 5 µg ml^{-1}.

Fish

Clinically healthy turbot *Scophthalmus maximus* (average 12.9 g, ~10 cm) were purchased from a local fish farm. Fish were kept at 20°C in 325 l tanks containing ~200 l sand-filtered, activated carbon-absorbed, and aerated seawater that was changed twice daily. Fish density was maintained at less than 100 fish $tank^{-1}$. Fish were fed daily with commercial dry pellets (purchased from Shandong Sheng-suo Fish Feed Research Center). Fish were acclimatized in the laboratory for 2 wk before experimental manipulation. Before the experiment began, 5 fish were randomly sampled for bacteriological investigation of blood, liver, kidney, and spleen; no bacteria were detected from the examined tissues. In addition, ELISA analysis indicated that the randomly selected fish were negative for serum antibodies against *P. fluorescens*. Fish were euthanized with tricaine methanesulfonate (Sigma) prior to experiments involving tissue collection.

Cloning and sequence analysis of *tdr*

Selection of *tdr* genes for this study. To date the complete genomic sequences of at least 3 *P. fluorescens* strains have been reported (GenBank accession nos. CP000094.2, CP003041.1, and CP003150.1); all these strains are from soil or plants and are nonpathogenic in nature. *P. fluorescens* TSS is a pathogenic isolate from diseased fish, with some proteins exhibiting high levels (>90%) of sequence identity with the soil/plant *P. fluorescens* (e.g. AprX; Zhang et al. 2009), but others exhibiting <40% sequence identity (e.g. Pfa1; Y. Hu et al. 2009a). These observations suggest an evolutionary difference between *P. fluorescens* isolates from different sources. Nevertheless, the sequence data of soil/plant *P. fluorescens* are very useful information for the study of marine *P. fluorescens*. A search of the available sequence databank identified a number of proteins that possess TDR-typical domains and structurally belong to the TDR family, though some of these proteins are not named TDRs. Since the functions of TDRs in *P. fluorescens* are almost entirely unknown, we aimed to examine the TDRs of TSS at a functional level, in particular in relation to virulence (because TSS is a pathogenic strain). For this purpose, we designed primers based on the known sequences of TDR homologues in soil/plant *P. fluorescens*, and, with these primers, amplified the corresponding genes in TSS. In this fashion, we succeeded in obtaining the complete open reading frames (ORFs) of 4 *tdr* genes from TSS. Since we were interested in only those genes that are associated with pathogenesis, the 4 genes were each inactivated by insertional mutagenesis, and the mutants were then subjected to a preliminary study to examine their tissue dissemination capacities in

turbot. The results showed that mutation of 3 genes affected the tissue dissemination ability of TSS. These 3 genes were therefore selected for this study and named *tdr1*, *tdr2*, and *tdr3*.

Amplification and sequence analysis of *tdr* genes. The 3 *tdr* genes were amplified by PCR using the primer pairs AF1/AR1, AF2/AR2, and AF3/AR3, respectively (Table 1), which were designed based on the genes of TonB-dependent outer membrane ferric citrate receptor FecA (gene locus Pf1A506-1022) and TonB-dependent outer membrane receptors (gene loci Pf1A506-2242 and Pf1A506-5433) of *P. fluorescens* A506. The primers amplify the ORF regions of these genes. The sequences obtained for the *tdr* genes were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the NCBI conserved domain search program. The calculated molecular mass and theoretical isoelectric point were predicted using EditSeq in the DNASTAR software package. Signal peptide search was performed with SignalP 3.0. Subcellular localization prediction was performed with the PSORTb v.3.0 server.

Table 1. Primers used in this study. Restriction sites are underlined; restriction enzymes are given in parentheses

Primer	Sequence (5'–3')
705TF2	CTGCAACTTTATCCGCCTCC
705TR2	TCTGACAACGATCGGAGGAC
705TP	ACGCACTGAGAAGCCCTT
AF1	ATGCGTCGTACCCTGATTTC
AF2	GATATCATGGAGCATGACTACCAGAACTATGTAG (*Eco*RV)
AF3	ACCAGATCTTAAGCCACTGCGGACTTTGAGT (*Bgl*II)
AF4	ACCGCAATGGGTTCCCGATC
AR1	TCACAGGGTGTAGGCGATG
AR2	GATATCCAGGGTGTAGGCGATGC (*Eco*RV)
AR3	GGTAGATCTTAACTCGCCCACTACGTTCAGG (*Bgl*II)
AR4	GGCTGCTTGGACACCACATTG
BF1	ATGCCCCAGCACCCGACGC
BF2	GATATCATGTCCCAGCAGACCAACAA (*Eco*RV)
BF3	CGTAGATCTTAAGTGCTGGTGTCCTTCGGTT (*Bgl*II)
BF4	TCCTTCGGTTCGGCAGTGAC
BR1	TCAGAACGCGATAGAGGTTTGC
BR2	GATATCGAACGCGATAGAGGTTTGC (*Eco*RV)
BR3	AGCAGATCTTAA GAAGTTGACGATGCCTCCC (*Bgl*II)
BR4	GTTGCAGGCTGTAGGCGTTATC
CF1	ATGACGTACGGTCTGCAGT
CF2	GATATCATGTACCGAGACAAGGTCAAGC (*Eco*RV)
CF3	TTTAGATCTTAAGCCAATGTGATTCTGTCCTTC (*Bgl*II)
CF4	AAATGACCTTGGCCCCTACCAC
CR1	CTACCAGTTGTAAGACACCGTA
CR2	GATATCCCAGTTGTAAGACACCGTACC (*Eco*RV)
CR3	CCGAGATCTTAAGTAGGGTTCGCTGGTGGTG (*Bgl*II)
CR4	GAGTCTCGGACAGCGGTGTG
Tdr1P	GTCCAGCGGGTGGTTGTT
Tdr2P	TCTTGAAACCGTCGTGGC
Tdr3P	GTGTTGTTATCCACAGCCGTC
TSSF1	GTAAATCCGACACGCAGACC
TSSR1	CCGCCCTGGTAAGTGAAT

Quantitative real-time reverse transcription-PCR (qRT-PCR)

To examine *tdr* expression, TSS was grown in 100 ml LB medium in at 28°C to absorbance (A_{600}) = 0.5. Eighty microliters of the cells were placed into 2 new flasks (40 ml LB medium flask^{-1}), with one flask containing 600 µM 2,2'dipyridyl. Growth was continued at 28°C for 0, 10, 20, and 30 min. The cells were harvested by centrifugation (10 000 × *g*) and used for total RNA extraction with the HP Total RNA kit (Omega Bio-Tek). The RNA was treated with DNase I according to kit instructions. One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen). qRT-PCR was carried out as described by Dang & Sun (2011) in an Eppendorf Mastercycler (Eppendorf) using the SYBR ExScript qRT-PCR Kit (Takara) with the glyceraldehyde-3-phosphate dehydrogenase gene as a reference. The PCR primer pairs for *tdr1*, *tdr2*, and *tdr3* are AF4/AR4, BF4/BR4, and CF4/CR4, respectively (Table 1). The primers were designed based on the sequences of *tdr1*, *tdr2*, and *tdr3*. The PCR efficiencies were 102, 97, and 99%, respectively, for AF4/AR4, BF4/BR4, and CF4/CR4. The assay was performed 3 times (3 repeats), and each time TSS was cultured in the presence or absence of 2,2'dipyridyl as described above, and expression of *tdr* was determined by qRT-PCR. The results are shown as means ± SE.

Plasmid construction

The primers used in this study are listed in Table 1. The plasmids pEtTdr1, pEtTdr2, and pEtTdr3, which express the TonB-dependent receptor domains of Tdr1 to 3, respectively, were constructed as follows. The coding sequences of the receptor domains of Tdr1 (amino acid position 455

to 709), Tdr2 (position 510 to 778), and Tdr3 (position 539 to 796) were amplified by PCR with primer pairs AF2/AR2, BF2/BR2, and CF2/CR2, respectively (Table 1). PCR was carried out in a TaKaRa PCR Thermal Cycler™ (Takara). The reaction was performed in triplicate in a total volume of 25 µl containing 0.2 µl Ex *Taq* (Takara), 2.5µl Ex Taq Buffer (Takara), 2 µl 2.5 mM dNTP mixture, 1 µl DNA (100 ng), 0.2 µl 20 µM primers, and 18.9 µl PCR-grade water. The PCR program was 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, 57°C for 30 s, and 72°C for 45 s. The PCR products were ligated with the T-A cloning vector pBS-T (Tiangen), and *E. coli* DH5α was transformed with the ligation mixtures. Recombinant plasmids were purified from the transformants with Plasmid Mini Kit I (Omega Bio-Tek). The plasmids were digested with *Eco*RV (Takara), and the fragments containing *tdr* were retrieved and inserted into pET259 (Y. Hu et al. 2010) at the *Swa*I site (for *tdr2* and *tdr3*) or into pET32a (Novagen) at the *Eco*RV site (for *tdr1*). Plasmid p705T, which was used for insertion mutagenesis (see next subsection), was constructed as follows: the tetracycline-resistance gene of p704T (Y. Hu et al. 2009b) was cut out by *Sma*I/*Sca*I double digestion and inserted into pGP704 (Miller & Mekalanos 1988) at the *Sca*I site.

Construction of *tdr* mutants

To construct the *tdr*-defective strains TSStdr1, TSStdr2, and TSStdr3, internal fragments of *tdr1* (position 114 to 654), *tdr2* (position 168 to 753), and *tdr3* (position 138 to 681) were generated by PCR with the primer pairs AF3/AR3, BF3/BR3, and CF3/CR3, respectively. The PCR products were inserted into the suicide plasmid p705T at the compatible *Bgl*II site, resulting in p705Tdr1, p705Tdr2, and p705Tdr3. S17-1λpir was transformed separately with p705Tdr1, p705Tdr2, and p705Tdr3, and the transformants were conjugated with *P. fluorescens* TSS as described previously (Sun et al. 2009). The transconjugants were selected on LB agar plates supplemented with tetracycline and chloramphenicol, and the resistant clones were named TSStdr1, TSStdr2, and TSStdr3 respectively. Mutation of *tdr1*, *tdr2*, and *tdr3* in TSStdr1, TSStdr2, and TSStdr3 was confirmed by PCR analysis (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d110p181_supp.pdf) as follows: for examination of mutation in TSStdr1, TSStdr2, and TSStdr3, the PCR was performed with the primer 705TP (Table 1), which is specific to the common backbone plasmid p705T, paired with the primers Tdr1P, Tdr2P, and Tdr3P (Table 1), which are specific to the *tdr1*, *tdr2*, and *tdr3* genes in the chromosome, respectively. The sequences of Tdr1P, Tdr2P, and Tdr3P are not contained in the *tdr* inserts in p705Tdr1, p705Tdr2, and p705Tdr3. The PCR products were subsequently verified by sequence analysis. In addition, single-copy plasmid insertion in the mutants was further confirmed by the quantitative real-time PCR (qRT-PCR) method described by Song et al. (2002). Briefly, to obtain a standard curve that correlates the cycle threshold (Ct) values of PCR with plasmid copy number in the chromosome, PCR templates were prepared by mixing p705T DNA with TSS genomic DNA at the ratios that represent 1, 2, 4, 8, and 16 copies of plasmid per genome. qRT-PCR was then conducted with p705T-specific primers 705TF2/705TR2 (Table 1), and the results produced a standard curve (Fig. S2 in the Supplement) with the equation of $y = -3.448x + 21.948$ (y = Ct value, x = plasmid copy number). To determine the plasmid number in TSStdr1, TSStdr2, and TSStdr3, qRT-PCR was performed with the genomic DNA of each of the mutant strains as template and 705TF2/705TR2 (as above) as primers. The results showed that for all 3 mutants, the Ct values correlated with one copy of plasmid in the standard curve. Therefore, the number of plasmid insertion in TSStdr1, TSStdr2, and TSStdr3 was one.

Purification of recombinant (r) Tdr1, rTdr2, and rTdr3

Recombinant proteins were purified as follows. *E. coli* BL21(DE3) was transformed separately with pEtTdr1, pEtTdr2, and pEtTdr3; the transformants were 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 (10 000 × *g*), and His-tagged rTdr1, rTdr2, and rTrd3 were purified using Ni-NTA Agarose (Qiagen) as recommended by the manufacturer. The proteins were concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore). The concentrated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by 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.

In vivo infection assay

Experiments involving live fish were conducted in accordance with the 'Regulations for the Administration of Affairs Concerning Experimental Animals' mandated by the State Science and Technology Commission of Shandong Province. The *in vivo* infection study was approved by the ethics committee of the Institute of Oceanology, Chinese Academy of Sciences. *In vivo* infection was conducted under laboratory conditions as described in 'Materials and methods: Fish'. For tissue dissemination and colonization analysis, TSS and the mutant strains were cultured in LB medium to A_{600} = 0.8. The cells were washed in phosphate-buffered saline (PBS) and resuspended in PBS to 5×10^7 CFU ml^{-1}. Turbot were divided randomly into 5 groups of 20 fish and injected intramuscularly either with 100 µl of one of the bacterial suspensions or with 100 µl PBS (injection control). Blood, kidney, and spleen were taken from the fish at 24, 48, and 72 h post-infection (3 fish per time point). The tissues were homogenized in PBS with an OSE-20 electric tissue homogenizer (Tiangen). The homogenates and blood were diluted serially and 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 with the primers TSSF1/TSSR1 (Table 1), which are specific to TSS and the mutants, and by sequence analysis of selected PCR products. The *in vivo* infection experiment was conducted in 3 replicates, i.e. the infection trial was carried out simultaneously in 3 sets, in each set bacterial infection and recovery from the tissues of the infected fish were performed as described above. For the mortality assay, fish (15 $tank^{-1}$) were infected as above and monitored for accumulated mortality over a period of 20 d. The mortality trial was performed independently 3 times on different days (i.e. 3 repeats). The median lethal dose (LD_{50}) assay was performed as described by Wang et al. (2009).

Vaccination

rTdr1, rTdr2, and rTdr3 were resuspended in PBS to a concentration of 100 µg ml^{-1} and mixed at an equal volume with aluminum hydroxide as described by Jiao et al. (2010). As a control, PBS was mixed similarly with aluminum hydroxide without protein. Turbot (as described above) were divided randomly into 4 groups (n = 50 fish $group^{-1}$) and injected intraperitoneally with 100 µl of each of the protein mixtures or the PBS control. At 1 mo post-vaccination, 30 fish were taken from each group and challenged with TSS at the dose of 5×10^6 CFU $fish^{-1}$. The fish were monitored for mortality for a period of 3 wk. Dying fish were randomly selected for bacteriological investigation of liver, kidney, and spleen. Relative percent survival (RPS) was calculated according to the following formula: RPS = [1 − (% mortality in vaccinated fish / % mortality in control fish)] × 100. The vaccination trial was repeated once.

Enzyme-linked immunosorbent assay (ELISA)

Sera were taken from vaccinated fish and control fish (4 from each group) from one vaccination trial at 4 wk post-vaccination, and serum antibody titers were determined by ELISA as described by Sun et al. (2011). For the serum from each fish, ELISA was performed in 3 replicates.

Effect of serum antibodies on bacterial infection

The FG-9307 cell line was established from Japanese flounder gill cells (Tong et al. 1997). The cells were cultured at 22°C in 96-well cell culture plates (~10^5 cells $well^{-1}$) with Eagle's minimum essential medium (MEM) (GIBCO, Invitrogen) as described by Tong et al. (1997). *P. fluorescens* TSS was cultured in LB medium to A_{600} = 0.8 and resuspended in PBS to 1×10^8 CFU ml^{-1}. Then, 200 µl of the bacterial suspension was mixed with an equal volume of serum from control fish and fish vaccinated with rTdr1, rTdr2, and rTdr3, and the mixtures were named A, B, C, and D, respectively. The mixtures were incubated at 22°C for 30 min and then centrifuged (10 000 × *g*) at 4°C for 10 min. The bacterial cells were washed 3 times with PBS and named A1, B1, C1, and D1 (derived from A, B, C, and D mixtures respectively). A1, B1, C1, and D1 were each added to 1 well of FG cells (multiplicity of infection [MOI] 10:1) in a 96-well culture plate. The plate was incubated at 22°C for 2 h and washed 3 times with PBS. The cells were lysed with 1% Triton X-100 (200 µl $well^{-1}$), and 100 µl lysate was plated in triplicate on LB agar plates. After incubation at 28°C for 24 h, the colonies that appeared on the plates were counted. The genetic identity of the colonies was verified by PCR and sequence analysis of the PCR products. The assay was performed independently 3 times (3 repeats), and in each time the above process was conducted.

Statistical analysis

With the exception of the vaccination trial, which was repeated once, all other experiments were performed in 3 replicates/repeats, and statistical analyses were performed with the SPSS 17.0 package (SPSS Inc.). Log-rank test was used for mortality analysis, and analysis of variance (ANOVA) was used for all other analyses. In all cases, the significance level was defined as $p < 0.05$.

RESULTS

Sequence characterization of Tdr1, Tdr2, and Tdr3

Three *tdr* genes (named *tdr1*, *tdr2*, and *tdr3*) were cloned from *Pseudomonas fluorescens* TSS, a pathogenic fish isolate. Sequence analysis showed that Tdr1, Tdr2, and Tdr3 are composed of 709, 778, and 796 amino acid residues, with predicted molecular masses of 78.2, 84.8, and 87.5 kDa, respectively. *In silico* analysis indicated that all 3 proteins possess an N-terminal signal peptide, a TonB-dependent receptor plug domain, and a TonB-dependent receptor domain (Fig. 1). In addition, Tdr2 and Tdr3 contain a STN (Secretin and TonB N-terminus short domain) structure upstream of the plug domain. Subcellular localization prediction indicated that all 3 Tdr were localized to the outer membrane. Blast analysis showed that Tdr1 and Tdr3 share 98% overall sequence identity with 2 different TonB-dependent receptors of the plant isolate *P. fluorescens* A506 (GenBank accession no. AFJ57168.1 and AFJ54652.1), while Tdr2 shares 99% overall sequence identity with the TonB-dependent outer membrane ferric citrate receptor FecA of *P. fluorescens* A506 (GenBank accession no. AFJ55791.1).

tdr expression in response to iron conditions

Since some TonB-dependent receptors are known to be iron-regulated, we examined the expression profiles of *tdr1*, *tdr2*, and *tdr3* under iron-depleted conditions. For this purpose, TSS was cultured in LB medium supplemented with or without the iron chelator 2,2′dipyridyl, and *tdr* expression was determined by qRT-PCR at 10, 20, and 30 min post dipyridyl addition. The results showed that *tdr1* expression was significantly upregulated at 20 min (2-fold increase), while *tdr2* and *tdr3* expressions were significantly upregulated at all examined time points, with maximum inductions (10-fold and 6-fold increases) occurring at 30 and 20 min, respectively (Fig. 2). Hence, the presence of 2,2′dipyridyl upregulated the expression of all 3 *tdr* genes.

Mutation of *tdr* and ensuing effects

Effect on growth. To examine the functional importance of *tdr1*, *tdr2*, and *tdr3*, the genes were mutated by insertion mutagenesis, and the resulting mutant strains were named TSStdr1, TSStdr2, and TSStdr3, respectively. Single-copy plasmid insertion in the mutants was confirmed by PCR and by the qRT-PCR method described by Song et al. (2002). Growth analysis showed that when cultured in LB medium and in LB medium supplemented with iron, all 3 mutants, in particular TSStdr2 and TSStdr3, exhibited severely retarded growth profiles compared to TSS (Fig. 3A,B). When cultured in LB medium supplemented with dipyridyl, TSStdr1 exhibited a growth profile similar to that of TSS, while TSStdr2 and TSStdr3 were largely comparable to that of TSS at early logarithmic phase but reached lower cell densities at the stationary phase compared to TSS (Fig. 3C).

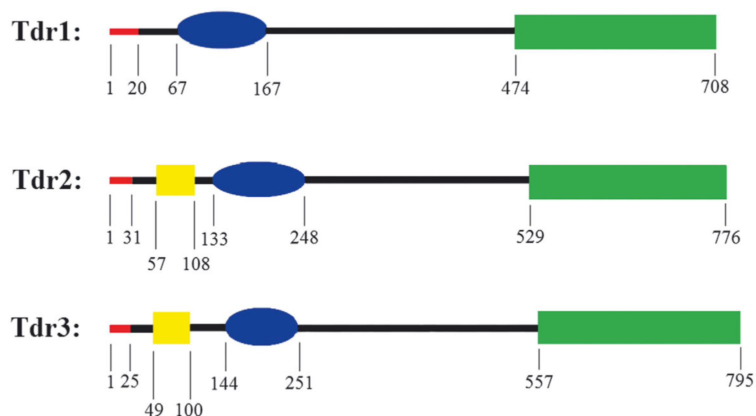

Fig. 1. Schematic representations of the structural domains of *Pseudomonas fluorescens* TonB-dependent receptor proteins Tdr1, Tdr2, and Tdr3 (red: signal peptide; yellow: secretin and TonB N-terminus short domain; blue: TonB-dependent receptor plug domain; green: TonB-dependent receptor domain). Numbers under the domains indicate the beginning and ending amino acid positions

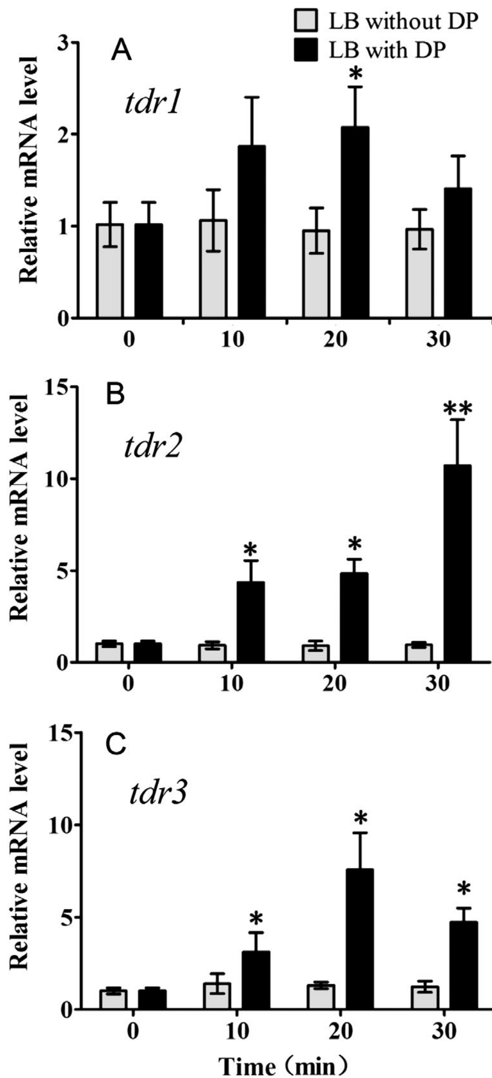

Fig. 2. Expression of *Pseudomonas fluorescens* TonB-dependent receptor gene *tdr* in response to iron conditions. *P. fluorescens* TSS was cultured in LB medium to absorbance (A_{600}) = 0.5, and the culture was added with or without the iron chelator 2,2′dipyridyl (DP). Expression of (A) *tdr1*, (B) *tdr2*, and (C) *tdr3* was determined after 10, 20, or 30 min by quantitative real time RT-PCR. For each gene, the expression level at 0 min was set as 1. Data are means ± SE from 3 independent experiments. Significant differences are indicated (*$p < 0.05$, **$p < 0.01$)

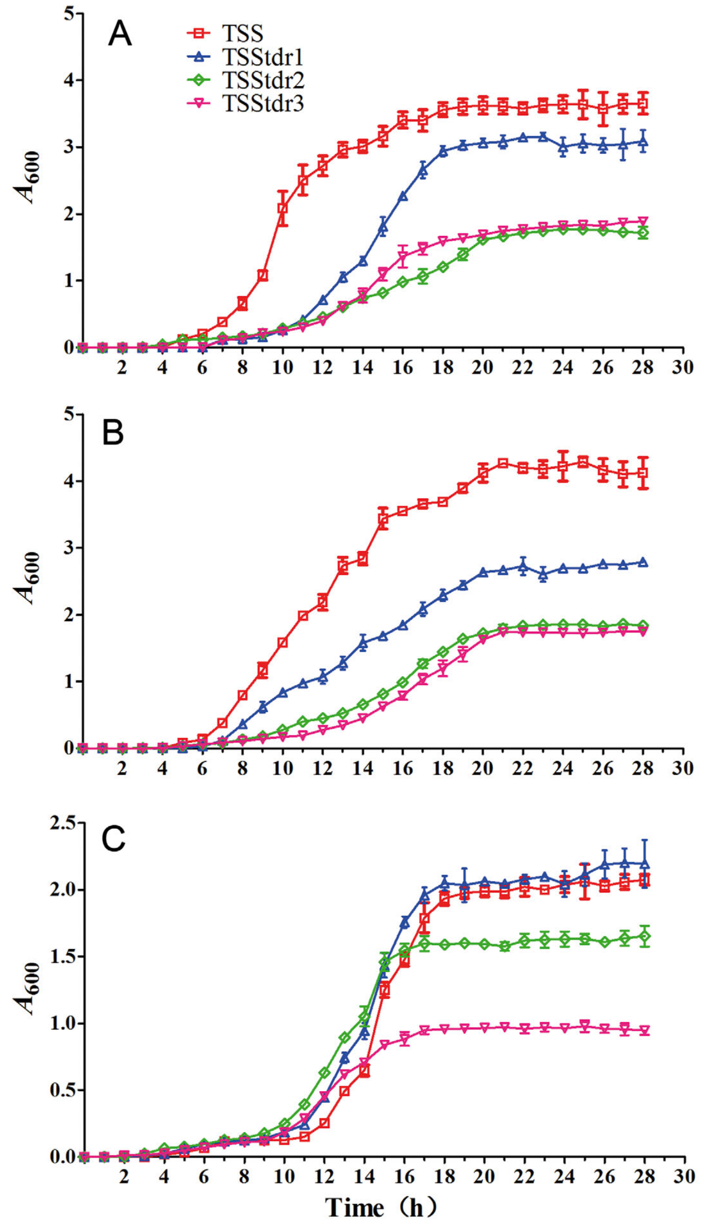

Fig. 3. Growth profiles of *Pseudomonas fluorescens* wild type (TSS) and mutant strains (TSStdr1, TSStdr2, and TSStdr3) cultured in LB medium (A) alone or supplemented with (B) iron or (C) dipyridyl. Cell density was determined by measuring absorbance at 600 nm (A_{600}). Data are means ± SE of 3 independent assays

Effect on virulence. TSStdr1, TSStdr2, and TSStdr3 exhibited LD_{50} values of 4.2×10^6, 2.7×10^6, and 5.1×10^6 CFU fish^{-1}, respectively, which were 7-, 4.5-, and 8.5-fold higher than the LD_{50} of the wild type strain TSS (6×10^5 CFU). To examine whether the *tdr* mutations had any effect on the tissue dissemination capacity of TSS, fish were infected with the same dose of TSStdr1, TSStdr2, TSStdr3, or TSS via muscle injection, and bacterial dissemination into blood, kidney, and spleen was examined at 24, 48, and 72 h post-infection by determining bacterial recovery. The results showed that for all examined tissues and at all examined time points, the recoveries of TSStdr1, TSStdr2, TSStdr3 were significantly lower than those of the wild type (Fig. 4). Mortality monitoring indicated that fish infected with TSStdr1, TSStdr2, and TSStdr3 exhibited accumulated mortalities of 60, 64.4, and 62.2%, respectively, while fish infected

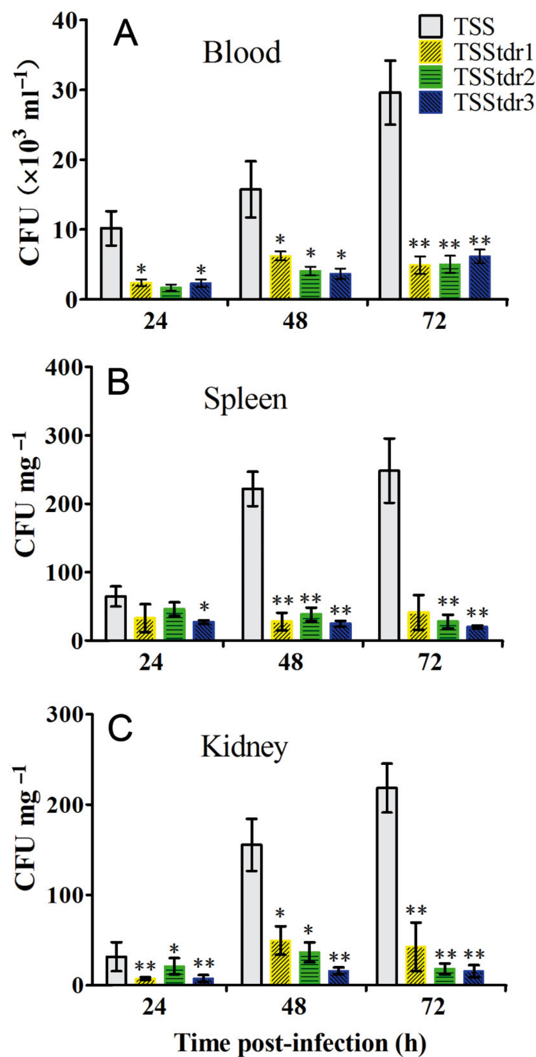

Fig. 4. *Pseudomonas fluorescens* dissemination and colonization in fish tissues. Turbot were injected intramuscularly with wild type (TSS) or mutant strains (TSStdr1, TSStdr2, and TSStdr3) and bacterial recovery from (A) blood, (B) kidney, and (C) spleen was determined at several timepoints post-infection. Data are means ± SE of 3 replicates. Significant differences are indicated (*p < 0.05, **p < 0.01)

with TSS exhibited an accumulated mortality of 86.7% (Fig. S3 in the Supplement). Statistical analysis showed that the accumulated mortalities of TSStdr1 and TSStdr3 were significantly lower than that of TSS.

Immunoprotective potential of purified rTdr

To examine the vaccine potentials of the 3 Tdr, recombinant proteins of Tdr1, Tdr2, and Tdr3 corresponding to the TonB-dependent receptor domain were purified (Fig. S4 in the Supplement) and used to vaccinate turbot. The results showed that after challenging with TSS, the accumulated mortalities of rTdr1-, rTdr2-, and rTdr3-vaccinated fish were 36.7, 30, and 26.7%, respectively, which were significantly ($p < 0.01$) lower than that of the control fish (70%). Based on these data, the protective efficacies, in terms of RPS, of rTdr1, rTdr2, and rTdr3 were 48, 57, and 62%, respectively. Microbiological examination of moribund fish indicated that TSS was the only type of bacterium recovered from kidney, liver, and spleen, suggesting that mortality was caused by TSS challenge.

Vaccine-induced serum antibodies and their effect on bacterial infection

ELISA analysis indicated that at 4 wk post-vaccination, specific serum antibodies were detected in fish vaccinated with rTdr1, rTdr2, and rTdr3 (Fig. 5). The antibody levels in rTdr1-, rTdr2-, and rTdr3-vaccinated fish were comparable. Since Tdr1, Tdr2, and Tdr3 are membrane proteins required for effective host infection, we examined whether antibody blocking of these proteins would affect TSS infectivity in FG cells. The results showed that bacterial recoveries from FG cells infected with TSS pre-treated with rTdr1, rTdr2, and rTdr3 antisera were comparable and significantly lower than that from FG cells infected with untreated TSS (Fig. 6). In contrast, pretreatment of TSS with control serum had no significant effect on bacterial recovery.

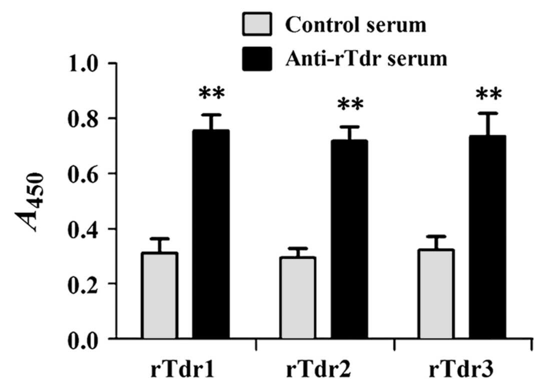

Fig. 5. Serum antibody production in fish vaccinated with recombinant TonB-dependent receptor proteins (rTdr). Sera were taken from mock-vaccinated fish (control) and fish vaccinated with rTdr1, rTdr2, and rTdr3. Serum antibodies against Tdr1, Tdr2, and Tdr3 were determined by ELISA. Data are means ± SE of 3 ELISA assays. Significant differences are indicated (**p < 0.01)

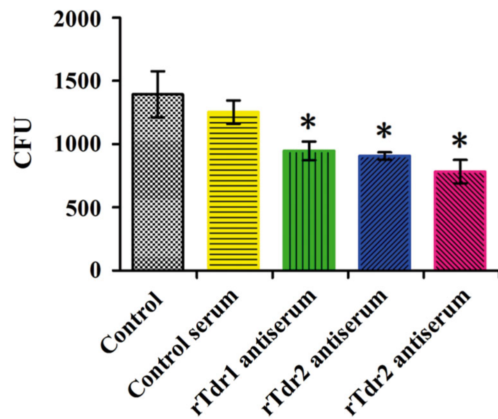

Fig. 6. Effect of of recombinant TonB-dependent receptor proteins (rTdr) antisera on bacterial infection. *Pseudomonas fluorescens* wild type TSS was pre-treated with serum from mock-vaccinated fish (control serum) or antiserum from fish vaccinated with rTdr1, rTdr2, or rTdr3, and then incubated with FG-9307 cells. Control FG cells were incubated with TSS that had not been treated with any serum. After 2 h of incubation, the number of TSS which had successfully infected FG cells was determined by plate count. Data are means ± SE of 3 independent assays. Significant differences are indicated (*$p < 0.05$)

DISCUSSION

In this study, 3 TDR homologues were cloned from a pathogenic fish isolate of *Pseudomonas fluorescens* and examined for expression under iron-variable growth conditions and potential involvement in host infection. Sequence analysis indicated that Tdr1, Tdr2, and Tdr3 possess domain structures characteristic of TDR proteins and share high levels of sequence identities with known TDR homologues. These features suggest that Tdr1, Tdr2, and Tdr3 are members of the TonB-dependent receptor family. Given their indispensable roles in iron uptake, it is not surprising to find that gene expression of TDRs is affected by iron. Y. Hu et al. (2012) found that the expression of a TDR homologue from *P. fluorescens* was induced by the iron chelator 2,2′dipyridyl. Both iron-mediated upregulation and downregulation of TonB-dependent receptors have been observed in other bacterial species. For example, a transcriptome study of *P. fluorescens* Pf-5 showed that some TonB-dependent receptors exhibited increased expression in iron-limited media while others exhibited decreased expression at the same time (Lim et al. 2012). Likewise, transcriptome analysis of *Caulobacter crescentus* and *Nitrosomonas europaea* showed that under conditions of iron deficiency, TonB-dependent receptor genes appeared in both the upregulated and the downregulated gene pools (Vajrala et al. 2012). In our study, we found that under iron-limited conditions, the expression of all 3 *tdr* genes was significantly upregulated to different extents, with *tdr2* and *tdr1* showing the highest and lowest induction levels, respectively. These results suggest that Tdr1, Tdr2, and Tdr3 may play a role in iron assimilation.

For pathogenic bacteria, iron acquisition is a vital capacity for colonization and proliferation in the host. As an important component of the iron uptake system, several TonB-dependent receptors were reported to have relevance in pathogen pathogenicity (Alvarez et al. 2008, Tauseef et al. 2011). In a comparative study of *Flavobacterium columnare* strains with high and low virulence, Li et al. (2010) identified 3 *tdr* genes in the high virulence strain but not in the low virulence strain. In a study of *Bordetella pertussis*, inactivation of a TonB-dependent receptor gene, *fauA*, was found to have a profound impact on *in vivo* growth and survival (Brickman & Armstrong 2007). In our study, potential involvement of Tdr1, Tdr2, and Tdr3 in iron utilization was suggested by 3 pieces of evidence: (1) there exist structural homologies between the 3 Tdr proteins and known TDRs participating in iron uptake, (2) the expression of *tdr1*, *tdr2*, and *tdr3* was upregulated under conditions of iron deficiency, and (3) mutation of either of the 3 *tdr* genes resulted in a retarded growth profile in LB medium with or without iron supplementation. Since LB is a rich medium, in which iron as well as other nutrients should be abundant, the growth results suggest that the 3 *tdr* mutants were unable to utilize the iron present in the medium. Taken together, these results support the hypothesis that Tdr1, Tdr2, and Tdr3 are likely to be involved in the process of iron uptake, such as that concerning iron importation into the cell, and therefore are required for the maintenance of bacterial growth. However, the exact roles played by Tdr1, Tdr2, and Tdr3 in iron utilization need to be fully demonstrated in future studies.

In vivo infection studies showed that compared to the wild type, the mutant strains TSStdr1, TSStdr2, and TSStdr3 exhibited significantly decreased tissue recoveries after experimental infection, suggesting that the 3 mutants were attenuated in their ability to disseminate and colonize tissues. Alternatively, the reduced tissue recoveries of TSStdr1, TSStdr2, and TSStdr3 may be at least in part due to the reduced growth capacities of these mutant strains. Consistent with these observations, the accumulated mortalities caused by TSStdr1, TSStdr2, and TSStdr3 were 26.7, 22.3, and 24.5%, respectively, compared with 70%

mortality caused by TSS. Hence, mortality was reduced in all of the mutants, which might correlate with lower bacterial recoveries from the mutants, though bacterial recovery was only examined on the first 3 d post-infection, while accumulating mortality was determined over a period of 20 d post-infection. These observations are in line with results using *Haemophilus ducreyi*, which showed that mutation of the TonB-dependent outer membrane receptor gene *hupA* impaired the ability of the pathogen to induce dermal lesions in an animal model (Stevens et al. 1996). However, our observations contrast with those for *Burkholderia pseudomallei*, in which mutation of the TonB-dependent receptor *fptA* caused significant increase in lethality (Kvitko et al. 2012). In our case, it is possible that mutation of the *tdr* genes upsets the uptake/homeostasis of iron as well as other nutrients that employ the Tdr, thus affecting the *in vivo* infectivity of the pathogen.

TonB-dependent receptors from several pathogens are known to be immunogenic and, in some cases, immunoprotective. For example, immunoproteomic analysis of the whole cell proteins of *Riemerella antipestifer* revealed that TonB-dependent receptor was among the immunogenic outer membrane proteins (Q. Hu et al. 2012), and a study with a Zn-uptake Tdr, ZnuD, of *Neisseria meningitidis* showed that it could induce bactericidal antibodies upon immunization of mice (Stork et al. 2010). Similarly, Tdr with vaccine potentials have been found in *Haemophilus ducreyi* and, as reported by us, *P. fluorescens* (Afonina et al. 2006, Fusco et al. 2010, Leduc et al. 2011, Y. Hu et al. 2012). In this study, we found that, when used as subunit vaccines, the recombinant proteins rTdr1, rTdr2, and rTdr3 elicited moderate but significant protections that differed in magnitude, with the highest RPS being effected by rTdr3. In line with these observations, all 3 proteins induced production of specific serum antibodies in the vaccinated fish, which may account at least in some parts for the observed protectivity of the Tdrs. These results suggest that Tdr1, Tdr2, and Tdr3 are immunogenic proteins that, especially in the case of Tdr3, have a potential as protective antigens.

In conclusion, our study indicates that Tdr1 to 3 are TonB-dependent receptor homologues that are regulated in expression by iron, participate in the virulence mechanism of *P. fluorescens*, and are required for optimal host infection. The observation that rTdr1, rTdr2, and rTdr3 induced significant immunoprotections suggests a potential for these proteins to be applied in the control of *P. fluorescens*-associated diseases in aquaculture.

Acknowledgements. This work was supported by grants from the National Natural Science Foundation of China (Nos. 41276168 and 31025030) and the Taishan Scholar Program of Shandong Province.

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Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: January 13, 2014; Accepted: May 27, 2014
Proofs received from author(s): July 12, 2014