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Pseudomonas fluorescens: Iron-responsive proteins and their involvement in host infection

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ABSTRACT

For pathogenic bacteria, the ability to acquire iron is vital to survival in the host. In consequence, many genes involved in iron acquisition are associated with bacterial virulence. *Pseudomonas fluorescens* is a bacterial pathogen to a variety of farmed fish. However, the global regulatory function of iron in pathogenic *P. fluorescens* is essentially unknown. In this study, in order to identify proteins affected by iron condition at the expression level, we performed proteomic analysis to compare the global protein profiles of *P. fluorescens* strain TSS, a fish pathogen, cultured under iron-replete and iron-deplete conditions. Twenty-two differentially expressed proteins were identified, most of which were confirmed to be regulated by iron at the mRNA level. To investigate their potential involvement in virulence, the genes encoding four of the 22 proteins, *i.e.* HemO (heme oxygenase), PspB (serine protease), Sod (superoxide dismutase), and TfeR (TonB-dependent outermembrane ferric enterobactin receptor), were knocked out, and the pathogenicity of the mutants was examined in a model of turbot (*Scophthalmus maximus*). The results showed that compared to the wild type, the *hemO*, *pspB*, and *tfeR* knockouts were significantly impaired in the ability to survive in host serum, to invade host tissues, and to cause host mortality. Immunization of turbot with recombinant TfeR (rTfeR) and PspB induced production of specific serum antibodies and significant protections against lethal TSS challenge. Further analysis showed that rTfeR antibodies recognized and bound to TSS, and that treatment of TSS with rTfeR antibodies significantly impaired the infectivity of TSS to fish cells. Taken together, these results indicate for the first time that in pathogenic *P. fluorescens*, iron affects the expression of a large number of proteins including those that are involved in host infection.

1. Introduction

Pseudomonas fluorescens is a Gram-negative bacterium existing in diverse ecological niches. It is a pathogen to a variety of farmed fish, including grass carp, Indian major carp, Japanese flounder, tilapia, and turbot (Wang et al., 2009). Fish infected with *P. fluorescens* develop 'Red Skin' disease that can lead to heavy mortality. In addition to fish, *P. fluorescens* can also infect birds and humans. Unlike non-pathogenic *P. fluorescens*, such as those from plant and soil, very limited studies on pathogenic human and fish *P. fluorescens* have been reported (Dagorn et al., 2013; Hu et al., 2009; Madi et al., 2010; Picot et al., 2004; Zhang et al., 2014), and consequently the infection mechanism of this bacterium is poorly understood.

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Iron is an essential nutrient for most organisms, because it is a cofactor for many enzymes involved in fundamental metabolic pathways. Although iron is the most common element on Earth, it is difficult to utilize by living organisms on the account of its low solubility under aerobic conditions (Litwin and Calderwood, 1993). In vertebrate animals including human and fish, iron is sequestered by iron binding proteins such as transferrin, lactoferrin, and ferritin, which create an *in vivo* condition of extremely low iron availability for invading microorganisms (Braun and Hantke, 2011). As a result, microbes have evolved various strategies to acquire iron. For pathogenic bacteria, overcoming the iron restraint in the host is a basic virulence mechanism that enables *in vivo* infection and survival (Hantke, 2001). On the other hand, since iron can generate highly reactive oxygen species that are detrimental to the cells, iron uptake has to be tightly regulated (Troxell and Hassan, 2013). Reports have shown that in well-studied pathogens such as *Escherichia coli* and *Pseudomonas aeruginosa*, many genes involved in iron homeostasis are also virulence factors associated with bacterial infection (Schaible and Kaufmann, 2004).

Like all bacteria that prefer an aerobic metabolism, *P. fluorescens* depends on iron for the normal functioning of the iron-containing enzymes in the respiratory electron-transport chain. However, how the iron conditions affect gene expression in pathogenic *P. fluorescens* is unknown. In this study, we conducted proteomic analysis to examine the global protein profiles of TSS, a pathogenic fish isolate of *P. fluorescens*, cultured under iron-replete and iron-deplete conditions. Furthermore, we selected several differentially expressed proteins and investigated their virulence potentials.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. fluorescens TSS is a pathogenic fish isolate that has been reported previously (Wang et al., 2009). The 16S rDNA sequence of TSS has been deposited into GenBank database (Accession number KP663368). *E. coli* BL21 (DE3) was purchased from Tiangen (Beijing, China). Both 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, St. Louis, USA), tetracycline, and chloramphenicol were added at the concentrations of 600 μM, 20 μg/ml, and 50 μg/ml respectively.

2.2. Fish

Clinically healthy turbot (*Scophthalmus maximus*) were purchased from a local fish farm. Fish were maintained at 20 °C in aerated seawater and fed daily with commercial dry pellets. Before experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen, and no bacteria were detected from the sampled fish. Enzyme-linked immunosorbent assay (ELISA) detected no serum antibodies against *P. fluorescens*. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA), followed by severing the spinal cord of the fish with a scalpel.

2.3. Two-dimensional gel electrophoresis (2-DE)

TSS was inoculated into a 1000-ml flask obtaining 200 ml LB medium supplemented with or without 600 μM 2,2′-dipyridyl (DP) (Sigma, USA). The cells were cultured under aerobic condition at 28 °C with shaking (180 rpm). At OD_{600} 0.8, the cells were collected by centrifugation at 4000 × *g* for 15 min at 4 °C. The cells were washed three times with 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 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 soluble proteins in the supernatant were purified with 2D-Clean-Up Kit (GE Healthcare, Piscataway, NJ, USA) and resuspended in IEF sample loading solution (7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.5% IPG buffer, and 0.002% bromophenol blue). Protein concentration was determined using the BCA Protein Assay Kit (Sangon, Shanghai, China). Two-DE was performed as reported previously (Zhang et al., 2013). The gel images were acquired using ImageScanner III (GE healthcare, Piscataway, NJ, USA) and analyzed with ImageMaster 2D Platinum 6.0 (GE healthcare, Piscataway, NJ, USA). 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 TSS cultured under different conditions. 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. The 2-DE experiment was performed independently for three times, each time with three replicates.

2.4. In-gel enzymatic digestion and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry

Extraction and proteolysis of differentially expressed protein spots were performed as reported previously (Zhang et al., 2013). MALDI-TOF mass spectrometry (MS) analysis was performed with ultrafleXtreme (Bruker, Germany) 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, five most abundant MS peaks were selected for MS/MS scan. Protein identification was as described previously (Zhang et al., 2013).

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

TSS was cultured in LB medium supplemented with or without DP to OD_{600} 0.8 as described above. Total RNA was