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HU-induced polymorphous filamentation in fish pathogen *Edwardsiella tarda* leading to reduced invasion and virulence in zebrafish

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ABSTRACT

Edwardsiella tarda is a rod-shaped Gram-negative pathogenic bacterium that causes hemorrhagic septicemia in fish. Nucleoid-associated protein HU is a basic DNA-binding protein with structural specificity in regulating genes expression. In wild-type *E. tarda* EIB202, HU is composed of two subunits HUα (*hupA*) and HUβ (*hupB*), and exists in homodimer or heterodimer forms. Different from the wild-type and Δ*hupB* mutant, Δ*hupA* mutant was found to be defective in cell growth, H_2S production, acid adaptation, and exhibited abnormal cell division resulting in a filamentous phenotype in log phase bacteria. The qRT-PCR result showed that deletion of *hupA* significantly up-regulated the transcription levels of *recA* and *sulA*, which in turn stimulated RecA-dependent pathway to prevent cell division, resulting in filamentous morphology in *E. tarda*. Furthermore, the elongated Δ*hupA* cells showed a striking defect in EPC cell invasion, and the adhesion and internalization rates were reduced to 25% and 27% of the wild-type in log phase cultures. Confocal laser scanning microscopy revealed that filamentous bacteria failed to adhere to and could not be internalized into EPC. When some of the bacteria regained the rod-shape morphology in stationary cultures, the Δ*hupA* mutants showed increased adhesion and internalization rates into EPC. Moreover, Δ*hupA* mutant exhibited delayed mortalities (for two days) in zebrafish but the LD_{50} increased 17 folds. Immunohistochemical analysis showed that Δ*hupA* mutant reduced proliferation abilities in the muscle, liver and intestine of zebrafish. This study indicates that HU protein and strains morphology play essential roles in the virulence network of *E. tarda*.

1. Introduction

Edwardsiella tarda is a versatile rod-shaped Gram-negative bacterium that causes systemic hemorrhagic septicemia in a wide range of animals and leads to severe financial losses in the aquaculture industry (Mohanty and Sahoo, 2007). Several activities, such as the production of hemolysin, ability to invade epithelial cells, and resistance to phagocyte-mediated killing, have been suggested to contribute to the pathogenesis of *E. tarda*. Furthermore, type III and type VI secretion systems (T3SS and T6SS) are required for intracellular replication of the bacterium in murine and fish phagocytes, but the exact mechanism remains. unknown (Leung et al., 2012; Tan et al., 2005).

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The bacterial nucleoid-associated protein (NAP) HU was first identified in *Escherichia coli* strain U93 (Rouviere-Yaniv and Gros, 1975). Different from the other members of NAPs, HU binds to the DNA with structural specificity to regulate cellular behaviors that include replication, transcription, transposition, and recombination (Dillon and Dorman, 2010). Inactivation of the HU protein in pathogens like *Salmonella enterica* serovar Typhimurium and *Porphyromonas gingivalis* showed reduced virulence in hosts. Mangan et al. and Schechter et al. reported that mutation of the HU in *S.* Typhimurium resulted in down-regulation of SPI-1 and SPI-2 virulence genes, which significantly impaired the capacity to invade and proliferate in epithelial cells (Mangan et al., 2011; Schechter et al., 2003). The expression of surface polysaccharide, a crucial virulence determinant of the oral anaerobe *P. gingivalis*, was also modulated by HU protein (Alberti-Segui et al., 2010). HU is composed of two subunits, HUα and HUβ, encoded by *hupA* and *hupB* separately. It exists in three different forms, the HUαβ heterodimer and the $HU\alpha_2$ and $HU\beta_2$ homodimers (Claret and Rouviere-Yaniv, 1997). The abundance of different forms varies with the growth phase of the bacteria (Claret and Rouviere-Yaniv, 1997). Generally, expression of *hupA* varies with growth phase while *hupB* expression increases to compensate for the lacking of α-subunit (Bonnefoy et al., 1989). The HUαβ heterodimer is the dominant form at most growth phases, except during early log phase when the $HU\alpha_2$ form is predominant. Accumulation of the $HU\beta_2$ homodimer is rarely observed and is usually in low abundant (Claret and Rouviere-Yaniv, 1997).

Although HU was shown to play significant role in many pathogenic bacteria, its role in virulence gene regulation has not yet been determined in the fish pathogen *E. tarda*. Based on the annotated genome of *E. tarda* EIB202, distinct genetic loci ETAE-0183 (*hupA*) and ETAE-0995 (*hupB*) were found to encode the subunits HUα and HUβ, respectively (Wang et al., 2009a,b). Our previous work showed that the deletion of *hupA* led to the significant attenuation in virulence to zebrafish (*Danio rerio*) (Xiao et al., 2011a,b), which suggested that there were correlations between HU and virulence in *E. tarda*. In the present study, the distinct function of HU in the pathogenesis of *E. tarda* was investigated. Moreover, invasion assays *in vitro* with epithelial papilloma of carp (EPC) cell as well as *in vivo* in zebrafish were carried out to reveal the roles of HU protein in pathogenesis.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid preparations were performed with *E. coli* Top10F' and *E. coli* CC118 λ*pir*. Plasmids were introduced into Ca^{2+} treated *E. coli* strains by transformation, and into *E. tarda* strains through conjugal mating with *E. coli* SM10 λ*pir* strain or transformed by electroporation.

E. coli was grown at 37 °C in Luria-Bertani broth (LB; Oxoid, UK) and agar plates, while *E. tarda* was grown at 28 °C in LB or deoxycholate hydrogen sulphide lactose agar (DHL; Nissui, Japan). Stock cultures were maintained at −86 °C in a suspension of LB containing 20% (v/v) glycerol. When required, appropriate antibiotics (Sigma, St. Louis, MO, USA) were added at the following concentrations: ampicillin (Amp, 100 μg ml^{-1}), colistin (Col, 12.5 μg ml^{-1}), kanamycin (Km, 50 μg ml^{-1}).

2.2. Construction of in-frame deletion mutants, complemented strains, overexpression strains, and GFP-labelled strains

In-frame deletion mutants of *hupA* gene and *hupB* gene were generated by the sacB-based allelic exchange as previously described (Xiao et al., 2009). The targeted in-frame deletion mutants were confirmed by PCR and sequencing separately using various primers (Supplemental Table 1S). The EIB202-derived mutants harboring the targeted deletions in *hupA* gene and *hupB* gene were designated as Δ*hupA* and Δ*hupB*, respectively.

To generate complemented strains, a 563 bp for *hupA* and a 586 bp for *hupB* intact genes were amplified from *E. tarda* EIB202 chromosome with primers *hupA*-S1/S2 and *hupB*-S1/S2 (Supplemental Table 1S). Products were cloned into the HindIII/SalI sites of the stable plasmid pUTat (Xiao et al., 2011a,b). The derivative plasmids were transformed into *E. tarda* EIB202 by electroporation. Colonies harboring plasmid pUTat-*hupA* or pUTat-*hupB* was isolated on LB plate containing both Amp and Col, and the presence of the plasmids were confirmed by PCR analysis with primer pair M14-F/M14-R (Supplemental Table 1S) and sequencing.

SulA and RecA overexpression strains were also constructed. Briefly, a 753 bp and a 1140 bp intact genes containing the putative promoter region and open reading frame (ORF) were amplified from *E. tarda* EIB202 chromosome with primer pairs *sulA*-OE1/OE2 and *recA*-OE1/OE2 (Supplemental Table 1S). The constructs were introduced into the HindIII/EcoRI sites of the broad-host-range plasmid pAK*gfp* (Karsi and Lawrence, 2007). The resulting plasmids were mated from *E. coli* SM10 λ*pir* into *E. tarda* EIB202. Finally, the EIB202-derived constructs harboring the putative promoter regions and ORFs of *sulA* or *recA* gene were designated as EIB202/pAK-SulA and EIB202/pAK-RecA, respectively. A negative control with the empty vector of pAK*gfp* was also introduced into *E. tarda* EIB202 and named as EIB202/pAK.

2.3. Bacterial two-hybrid assay

The bacterial two-hybrid system was used to determine protein–protein interactions between HUα and HUβ subunits in *E. tarda* EIB202 as previously described (Yang et al., 2010). Bacterial culture medium and plates were prepared according to the Agilent Technologies (USA) instruction manual (http://www.genomics.agilent.com). *hupA* and *hupB* genes were amplified by PCR using specific primer pairs (Supplemental Table 1S) from genomic DNA of *E. tarda* EIB202. After digesting with EcoRI/XhoI, gene fragments were cloned into the modified pBT and pTRG to produce recombinant vectors (pBT-*hupA*, pBT-*hupB*, pTRG-*hupA* and pTRG-*hupB*) (Table 1). A pair of pBT/pTRG plasmids was co-transformed into the reporter strain and spotted onto M9 screening medium containing