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Hemolysin EthA in *Edwardsiella tarda* is essential for fish invasion *in vivo* and *in vitro* and regulated by two-component system EsrA–EsrB and nucleoid protein Hha_{Et}

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ABSTRACT

Edwardsiella tarda is a Gram-negative pathogen for hemorrhagic septicemia in fish. Recently, two-component system (TCS) EsrA–EsrB in *E. tarda* has been found to play key roles in regulating type III secretion system (TTSS) and type VI secretion system (T6SS). In this study, a markedly attenuated Δ*esrB* mutant was investigated to exhibit enhanced cell-invasion capability, as well as the increased cytotoxicity of its extracellular products (ECPs). Compared with the parental strain, the Δ*esrB* mutant unexpectedly displayed the significantly increased hemolytic activity, and the restoration of hemolysin production was observed in the complemented strain $esrB^{+}$. A hemolysis-associated 147 kDa protein, EthA, was found to be up-regulated in the ECPs of Δ*esrB*. The deletion of *ethA* gene in *E. tarda* wild type and Δ*esrB* strains drastically decreased their capacities in internalization of epithelial papilloma of carp (EPC) cells. These results indicated that the increased production of EthA was responsible for the enhanced cell-invasion related capabilities in Δ*esrB*. Furthermore, the expression of EthA in Δ*esrB* exhibited a temperature-induced manner, and a nucleoid protein Hha_{Et} was identified to mediate *ethA* expression by directly binding to its promoter. These results demonstrated that the virulence determinant EthA was fully required for invasion abilities of *E. tarda* and was subjected to the control of a complicated and precisely regulated network primed for its invasion, colonization and infection process in fish.

1. Introduction

Edwardsiella tarda is a versatile Gram-negative pathogen that generally causes systemic hemorrhagic septicemia in marine and freshwater aquaculture, accounting for severe economical losses worldwide [1]. During the infection to fish, *E. tarda* colonizes in various organs, such as intestine, liver, spleen, kidney and even heart in both extracellular and intracellular locations [2–4]. The capacities to produce extracellular pathogenicity factors such as hemolysin [5], chondroitinase [6], and effector proteins delivered via type III or VI secretion systems (TTSS or T6SS) [7,8] are implicated to be essential for its pathogenesis. The recently completed genome sequencing of *E. tarda* unravels the genetic basis for habitat adaptation, invasive nature, and virulence determinants of the bacterium [9].

Hemolytic activities of *E. tarda* have been suggested to contribute to its symptoms in systemic hemorrhagic septicemia [10]. The 34 kDa hole-forming β-hemolysin HlyA has been described by Chen et al. [11] in several *E. tarda* strains. The 4782 bp gene *ethA* for a second hemolysin of 165.3 kDa has been cloned and sequenced by Hirono et al. [12] and investigated to be predominant in most pathogenic isolates of *E. tarda*. The deduced amino acid sequence of EthA shares homology (47%) with the ShlA hemolysin of *Serratia marcescens* [5]. Just upstream of *ethA* resides the gene *ethB*, encoding an activation/secretion protein of 61.9 kDa. The *ethA* and *ethB* genes are transcribed independently [12]. For the present, EthA as a large protein has not yet been identified in the secreted components of this bacterium. With the exception of *hlyA*, hemolysin genes *ethA* and *ethB* are present in the genome of completely sequenced *E. tarda* EIB202, probably accounting for the usually observed hemorrhage symptoms in affected fish [9].

Several virulence regulators have been addressed in *E. tarda* with the purpose to control the infection by the bacterium. RpoS, the alternative sigma factor in *E. tarda*, is established to regulate stress response, biofilm formation, autoinducer synthesis and invasion capability, but not significantly affect lethality toward fish [6]. LuxS/AI-2 quorum sensing (QS) system is believed to be involved in regulating of the production of virulence-associated

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elements such as TTSS genes and biofilm production [13], and inhibition of the system by two small peptides attenuates the virulence of *E. tarda* TX1 and blocks its infection [14]. EthR, a transcription regulator of the GntR family, represses in combination with ferric uptake regulator (Fur) the expression of hemolysin activator *ethB* by direct interaction with the *ethB* promoter region [15]. EsrB is identified as a response regulator of bacterial two-component signal transduction system (TCS) EsrA–EsrB, a homolog of SsrA–SsrB in *Salmonella* spp. [7,16]. In *E. tarda*, EsrA–EsrB system effectively regulates TTSS and T6SS via EsrC, a regulatory protein of the AraC family [7], and is considered as the potential target for live-attenuated vaccine against edwardsiellosis [8,17]. On the other hand, it has been appreciated that the nucleoid protein directly binds to the curved sequence upstream of target promoter and affects the transcription of a very wide variety of genes in Gram-negative pathogens [18–20]. As a nucleoid protein, Hha has been identified as a hemolysin expression-modulating protein in the thermoregulation of the expression of invasion genes in *Salmonella* spp. [21] and virulence genes in the locus of enterocyte effacement in enterohemorrhagic *Escherichia coli* [22]. Unfortunately, the identifications of EsrA–EsrB and Hha_{Et} associated virulent determinants as well as their regulatory network in this bacterium have not been detailed yet.

In this present study, we have attempted to dissect the attenuated manner of *esrB* mutant, and intriguingly detect the significantly enhanced cell-invasion capability, as well as the increased cytotoxicity of its extracellular products (ECPs) as a consequence of *esrB* deletion in *E. tarda* EIB202. EthA, the major hemolysin in *E. tarda* EIB202, was identified to be regulated by EsrB and essential for fish invasion. Moreover, the expression of EthA was also mediated by temperature and nucleoid protein Hha_{Et}, underlying its complex regulation network during invasion process of *E. tarda*.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains used in this study and their sources are listed in Table 1. *E. tarda* strains were routinely grown in tryptic soy broth (TSB) (Difco, Detroit, MI, USA) or tryptic soy agar (TSA) (Difco, Detroit, MI, USA) at 28 °C, while *E. coli* strains were cultured in Luria broth (LB) (Difco, Detroit, MI, USA) at 37 °C. Plasmid preparation was performed with *E. coli* Top10F′ or *E. coli* cc118 *λpir*. Stock cultures were maintained at −80 °C in a suspension of TSB containing 20% (v/v) glycerol. When required, ampicillin (Amp), chloramphenicol (Cm), colistin (Col), kanamycin (Km), IPTG and X-Gal were supplemented at final concentrations of 100, 50, 12.5, 25, 100 and 50 μg ml^{-1}, respectively.

2.2. Construction of site-directed mutants

For construction of an *esrB* site-directed deletion mutant, PCR amplifications were performed to generate the upstream fragment of *esrB* with primer pair MBU-for/MBU-rev and the downstream part of *esrB* with primer pair MBD-for/MBD-rev (Table 2) [8]. The PCR product containing in-frame deletion fragment from bp 154 to 642 in *esrB* was obtained with next run of overlap PCR with primer pair MBU-for/MBD-rev and then sequenced and cloned into the *Bgl*II/*Sph*I sites of the suicide vector pDMK (Table 1), which carried R6K *ori*, *sacB* sucrose-sensitivity gene [23], Cm resistance gene and Km resistance gene.

The resulting plasmid pDMDesrB (Table 1) was mated from *E. coli* SM10 *λpir* into *E. tarda* EIB202 by conjugation. The unmarked in-frame deletion mutants were selected in two sequential homologous recombination steps on LB agar medium containing Km, Cm and Col and then on LB agar with 10% (w/v) sucrose, respectively [8]. The targeted in-frame deletion mutants were confirmed by sequencing of the deleted region on chromosome. The EIB202 derived mutants with the targeted deletion of *esrB* gene were designated as Δ*esrB*. Following the above-mentioned procedures, the deletion mutants Δ*ethA* and Δ*esrB*/Δ*ethA* and insertion mutant *hha*$^{-}$ were isolated (Table 1). The Δ*esrB*/*hha*$^{-}$ double mutation strain was also isolated by allelic integration of the plasmid pDMDhha (Table 1) into the chromosome of Δ*esrB*.

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Description	Reference of source
Escherichia coli		
Top10F′	General cloning strain	Invitrogen
cc118 *λpir*	Host for π requiring plasmids	[39]
SM10 *λpir*	Host for π requiring plasmids, conjugal donor	[40]
BL21(DE3)	F^{-} *ompT hsdS gal*	Tiangen
Edwardsiella tarda		
EIB202	Wild type isolated from turbot, Yantai, China, Col^{r}, Cm^{r}	[41]
EIB202G	EIB202, containing plasmid pUCPG, Col^{r}, Cm^{r}, Amp^{r}	[8]
Δ*esrB*	EIB202, in-frame deletion of *esrB* from aa 51 to 214, Col^{r}, Cm^{r}	[8]
esrB^{+}	complementation of Δ*esrB*, Col^{r}, Cm^{r}, Amp^{r}	This study
Δ*ethA*	EIB202, in-frame deletion of *ethA* from aa 85 to 41, Col^{r}, Cm^{r}	This study
Δ*esrB*/Δ*ethA*	EIB202, in-frame deletions of *esrB* from aa 51 to 214 and *ethA* from aa 85 to 411, Col^{r}, Cm^{r}	This study
hha^{-}	EIB202, insertion mutant of hha_{Et}, Col^{r}, Cm^{r}, Km^{r}	This study
Δ*esrB*/*hha*$^{-}$	ΔesrB, insertion mutant of hha_{Et}, Col^{r}, Cm^{r}, Km^{r}	This study
HhaOEx	EIB202, over-expression of hha_{Et}, Col^{r}, Cm^{r}, Amp^{r}	This study
Plasmids		
pDM4	Suicide vector that contains an R6K origin of replication, Cm^{r}, SacB	[42]
pDMK	pDM4 derivative with Km fragment inserted in SalI site, Km^{r}, Cm^{r}	[8]
pDMDesrB	pDMK derivative containing *esrB* in-frame deletion of codons 153–642, Cm^{r}, Km^{r}	[8]
pDMDethA	pDMK derivative containing *ethA* in-frame deletion of codons 253–1234, Cm^{r}, Km^{r}	This study
pDMDhha	pDMK derivative containing hha_{Et} fragment mapping codons 10–204, Cm^{r}, Km^{r}	This study
pMD19-T Simple	Cloning vector, Amp^{r}	Takara
pUC18	Cloning vector, Amp^{r}	Takara
pET28a	Expressing vector, Km^{r}	Novagen
pUEB	pUC18 derivative with *esrB* fragment, Amp^{r}	This study
pUEH	pUC18 derivative with hha_{Et} fragment, Amp^{r}	This study
pHhaE	pET28 expressing Hha_{Et}, Km^{r}	This study
pUCPG	pUC18 containing promoter of *evpP* fused promoterless *gfp*, Amp^{r}	[8]

2.3. ECPs preparation and identification

Extracellular proteins were isolated by trichloroacetic acid (TCA) (Sigma, MO, USA) methanol precipitation [24]. Briefly, whole cells were removed from the culture by centrifugation (6000 × *g*, 10 min at 4 °C). The supernatant was then filtered through a 0.22 μm low protein-binding Millex filter (Millipore, Bedford, MA, USA). TCA (10%, w/v) was added and the acidified mixture was left on ice for 30 min to precipitate the proteins. The mixture was centrifuged (20000 × *g*, 20 min at 4 °C) to isolate the proteins, and the pellets were washed three times with an equal volume of ice-cold