Roles of plasmid-encoded proteins, EseH, Esel and EscD in invasion, replication and virulence of Edwardsiella ictaluri

Li Juan Zhao a,b, Jin Fang Lu b, P. Nie b,c,*, Ai Hua Li b, Bang Xi Xiong a, Hai Xia Xie b,**

a College of Fisheries, Huazhong Agricultural University, Wuhan, Hubei Province 430070, China
b State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province 430072, China
c College of Fisheries, Jimei University, 43 Yindou Road, Xiamen, Fujian Province 361021, China

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ABSTRACT

Native plasmids pEI1 and pEI2 were detected in Edwardsiella ictaluri HSN-1 isolated from diseased yellow catfish (Pelteobagrus fulvidraco). EseH encoded by pEI1 and other two proteins, Esel and EscD, encoded by pEI2, were found with homology to type III secretion system (T3SS) proteins. To investigate their roles in pathogenesis, the native plasmids were cured based on plasmid incompatibility by introducing a Kan positive and SacB negative selection marker into gene spacer of the native plasmids. Mutants with the deletion of the target genes were obtained by reverse PCR and self-ligation, and all mutants were examined for their virulence effect in yellow catfish. Compared with the HSN-1 strain, the two mutants ΔeseH and Δesel were attenuated, while mutant ΔescD had increased virulence with higher Competitive Index (CI) value. The adherence and invasion assays on fish EPC cells indicated that ΔeseH and Δesel had decreased ability in adherence. Using E. tarda as surrogate, EseH and Esel were detected in culture supernatants, but EscD was not, with the secretion of EseH depending on T3SS. In addition, EseH and Esel were found translocated into host cells, and by means of subcellular fractionation, EseH was localized in membrane fraction of ZF4 cells, and Esel in the cytosol fraction. Hence, the role of these three genes in adherence, invasion and cellular replication was revealed from the pathogenic bacterium E. ictaluri.

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1. Introduction

Edwardsiella ictaluri is a Gram-negative, facultative intracellular bacterium, and is known as the etiological agent of enteric septicemia of channel catfish (Ictalurus punctatus) (ESC) in the United States (Hawke, 1979; Hawke et al., 1981). Recently, this bacterium has been found from several species of siluriform fish in China, such as yellow catfish (Pelteobagrus fulvidraco), with the causing of heavy economic losses in the aquaculture industry (Ye et al., 2009; Liu et al., 2010).

It is reported that a set of plasmids exists constantly in E. ictaluri, of which two plasmids, named as pEI1 and pEI2 in strain 93–146 of the bacterium, were detected invariably in other strains of E. ictaluri from various sources in the US by using Southern blotting (Reid and Boyle, 1989; Lobb et al., 1993; Fernandez et al., 2001). Fernandez et al. (2001) sequenced these two plasmids in strain 93–146,
and found that some open reading frames (ORFs) in the plasmids are homologous to genes in type III secretion system (T3SS), a molecular tool for Gram-negative bacteria to inject effector proteins directly into host cells (Hueck, 1998). Thune et al. (2007) named the ORF1 encoded in pEI1 as eseH, and two ORFs encoded in pEI2, as escD and eseI, and using signature-tagged mutagenesis (STM), they found that pEI1 and pEI2 are involved in the bacterial infection in vivo in specific-pathogen-free (SPF) channel catfish. However apart from this, little is known in relation with the proteins, EseH, and EseI and EscD encoded respectively in pEI1 and pEI2 of E. ictaluri.

In consideration of the multi-copy presence of pEI1 and pEI2 in E. ictaluri, the present study was designed to cure completely each of the two plasmids in E. ictaluri for the construction of mutants. The mutants with deletion of eseH, eseI and escD, were then built up respectively, and their roles in invasion and replication were examined in host fish cells, as well as in yellow catfish. On the other hand, as T3SS in E. ictaluri is similar to that in E. tarda in gene organization (Thune et al., 2007; Rogge and Thune, 2011; Williams et al., 2012), the T3SS in E. tarda was used to analyze the secretion and translocation of EseH, EseI and EscD in fish cells. The cellular localization of these proteins was further examined in host fish cells. The present study thus provides the first report on pathogenic effects of eseH, eseI and escD, the three plasmid genes of the fish bacterial pathogen E. ictaluri.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture media and conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli was grown in Luria-Bertani (LB; BD Biosciences) broth or LB agar at 37 °C. E. ictaluri strains were grown in brain heart infusion (BHI; BD Biosciences) broth or BHI agar at 28 °C. E. tarda strains were grown in tryptic soy agar (TSA; BD Biosciences) or tryptic soy broth (TSB) at 28 °C. Stock cultures of bacteria were maintained in a suspension of broth with 25% (v/v) glycerol and stored at −80 °C. When required, antibiotics were used in the following concentrations: kanamycin (Km) at 50 μg ml⁻¹, colistin (Col) 12.5 μg ml⁻¹, gentamicin (Gn) 100 μg ml⁻¹ or 16 μg ml⁻¹, chloromycetin (Cm) 34 μg ml⁻¹, tetracycline (Tet) 15 μg ml⁻¹ and ampicillin (Amp) 100 μg ml⁻¹.

Host cells used for infection were grown at 25 °C or 35 °C in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) under a 5% (v/v) CO₂ atmosphere.

2.2. Sequencing of plasmids, pEI1 and pEI2 in strain HSN-1 of E. ictaluri

The purified plasmids were digested with Bgl I and cloned into pMD18-T (TaKaRa, China) vector for sequencing by Sangon Biotechnology Co. (Shanghai, China).

### Table 1

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td><em>Edwardsiella</em></td>
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<td>HSN-1</td>
<td><em>E. ictaluri</em> isolated from a moribund yellow catfish, Col'</td>
<td>Liu et al. (2010)</td>
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<td>HSN-GFP</td>
<td>HSN-1 carrying pFPV 25.1, Amp'</td>
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<td>pEI1</td>
<td>HSN-1 with pEI1 cured</td>
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<td>pEI2</td>
<td>HSN-1 with pEI2 cured</td>
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<td>HSN-1 cured of both pEI1 and pEI2</td>
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<td>ΔeseH::km</td>
<td>HSN-1 with eseH replaced by kanamycin resistance cassette, Kan'</td>
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<td>ΔescD::km</td>
<td>HSN-1 with escD replaced by kanamycin resistance cassette, Kan'</td>
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<td>ΔeseI::km</td>
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<td>PPD130/91</td>
<td>Wild type <em>E. tarda</em>, Col'</td>
<td>Tan et al. (2005)</td>
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<td>ΔesaN</td>
<td>PPD130/91 with in-frame deletion of esaN</td>
<td>Xie et al. (2010)</td>
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<td>DH5α</td>
<td>F', φ80lacZΔM15, ∆(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ', thi-1, gyrA96, relA1</td>
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<td>MC1061</td>
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<td>pACYC184</td>
<td>Cm', Tet'</td>
<td>Fermentas Life Sciences</td>
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<td>pACYC184 inserted by HA tagged eseI</td>
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<td>pCX341</td>
<td>pBR322 derivative, used to fuse T3SS effector to TEM-1 β-lactamase</td>
<td>Charpentier and Oswald (2004)</td>
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<td>eseH fused to TEM-1</td>
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<td>pCX-EseI</td>
<td>eseI fused to TEM-1</td>
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<td>pFPV25.1</td>
<td>Ampicillin resistant plasmid constitutively expressing Green Fluorescent Protein (GFP), Amp'</td>
<td>Valdivia and Falkow (1996)</td>
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<td>pSHK3</td>
<td>pUCori pA13ori, Kan'</td>
<td>Xiaojuan Xu, Huazhong Agricultural University</td>
</tr>
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Km, kanamycin; Col, colistin; Amp, ampicillin; Cm, chloramphenicol; Tet, tetracycline.

Superscripts: r, resistance; s, sensitivity.
2.3. Curing of pEI1, pEI2 or pEI1pEI2 and construction of deletion mutants

Plasmid pEI1 was linearized by digestion with EcoR I, before being inserted into pMD18-T to obtain pMD-EI1. SacI (1917 bp) from pRE112 was amplified and inserted into pMD-EI1 Xba I site, and Kan (909 bp) gene with its promoter was amplified from kanamycin cassette in pSHK3 and inserted into Kpn I site next to sacI in trans. All inserts were cut by EcoR I and self-ligated to achieve the incompatible plasmid and transformed directly into wild type E. ictaluri by electroporation. Positive transformants were serially inoculated onto BHI agar plates with Km, colonies were examined by PCR and electrophoretogram to confirm the loss of native pEI1. The target colonies were then screened further on 10% (w/v) sucrose to ensure the loss of incompatible plasmids for achieving the pEI1-cured E. ictaluri, i.e. pEI1’. In the same way, E. ictaluri strain cured of pEI2 (pEI2’) or cured of both pEI1 and pEI2 (pEI1‘pEI2’) were obtained (Fig. S1).

Flanking fragments of eseH, eseI and escD obtained by reverse PCR with pEI1 or pEI2 as templates were ligated to Kan cassette, before being cyclized and transformed into pEI1 or pEI2’, respectively, to obtain deletion mutants (Tables S1, Table 2).

2.4. Expression and secretion of EseH, EseD and EseI through T3SS of E. tarda

The vector pACYC184 was used to express hemagglutinating (HA) tagged EseH, EseI and EseD in the surrogate E. tarda. EseH, eseI and escD with their possible ribosomal binding site (RBS) were inserted into BamH I and SpH I sites of Tet gene, respectively. The recombinant plasmids were transformed into E. tarda PPD130/91 and its T3SS ATPase mutant ΔeseA (Table 1).

To analyze the secretion of the three proteins encoded by pEI1 and pEI2, all the strains were inoculated into T3SS inducing medium DMEM, and extracellular proteins (ECPs) and whole-cell lysates (TCPs) were harvested as described by Xie et al. (2010) for Western blotting analysis. Both TCPs and ECPs were first probed with mouse anti-DnaK monoclonal antibody (Stressgen), rabbit anti-EvpC antiserum (Zheng and Leung, 2005), and mouse anti-HA monoclonal antibody (Sigma), respectively, and then with the second antibodies, HRP-conjugated goat anti-mouse IgG (Sigma) or HRP-conjugated goat anti-rabbit IgG (Millipore).

2.5. Fluorescence microscopy of EseH and EseI translocation

Translocation usually refers to the transport of bacterial proteins across host cell membranes using T3SS (Böttner and Bonas, 2002). As EscD was found not secreted, the translocation of EseH and EseI was examined using TEM-1 β-lactamase assay (Charpentier and Oswald, 2004). Mouse macrophages (J774 cell line) (Lam et al., 2009) were seeded into wells of a 24-well plate (with coverslips at the bottom) at 3 × 10^5 cells per well in DMEM for overnight at 35 °C in a 5% CO₂ incubator. Wild-type E. tarda HSNI-GFP, J774 cells, pCX-eseH or pCX-eseI, and PPD130/91 with pCX-eseI were used to infect J774 cells at a multiplicity of infection (MOI) of 15 bacteria: 1 cell for 5 h, as described by Xie et al. (2010). The cells were incubated in CCF4-AM (with the final concentration at 1 μM; Invitrogen) for 90 min at room temperature in dark. Images were taken under a laser scanning confocal microscope (Zeiss Axiowert LSM 710 inverted microscope) with excitation at 405 nm, and emission at 460 and 530 nm, respectively.

2.6. Immunofluorescence microscopy of EseH and EseI positions in ZF4 cells

To visualize the position of translocated proteins, EseH and EseI in host cells, immunofluorescence studies were performed according to Abrahams et al. (2006). The GFP-tagged wild-type strain, HSN-1-GFP, with pACYC-eseH::HA or pACYC-eseI::HA was used to infect zebra fish fibroblast cell line, ZF4 (ATCC CRL-2050) cells at an MOI of 30:1 for 5 h. Translocated proteins were detected using mouse anti-HA monoclonal antibody, followed by AlexaFluor 594 goat anti-mouse antibody. The bacteria used in the infection were discriminated by transformed GFP vector pPF25.1 (Valdivia and Falkow, 1996), and ZF4 cell DNA was stained with DAPI (4, 6-diamidino-2-phenylindole; Molecular Probes). Cells on coverslips were visualized with a laser scanning confocal microscope.

2.7. Fractionation of host ZF4 cells to detect EseH and EseI localization

By immunofluorescence microscopy, both EseH and EseI were found out of nucleus in ZF4 cells. To determine whether EseH and EseI were localized in cytosolic or membrane fractions, ZF4 cells were infected with the E. ictaluri HSN-1 carrying pACYC-EseH::HA or pACYC-EseI::HA, respectively. Infected ZF4 cells were fractionated
as described by Xie et al. (2010) for Western blotting analysis. The membrane fraction was probed with mouse anti-HA monoclonal antibody, with rabbit anti-EvpC antisera, mouse anti-DnaK monoclonal antibody, rabbit anti-β-actin antisera (GeneTex, USA) and mouse anti-calnexin monoclonal antibody (Stressgen), respectively.

2.8. Adherence and internalization assays

These assays were performed following the procedure reported by Wang et al. (1998) with slight modifications. The epitheliohama papillosum cyprini (EPC) cell line (Fijan et al., 1983; Winton et al., 2010) was grown for 20–24 h to 100% confluence in 24-well tissue plate, and then infected respectively with wild-type E. ictaluri and its mutants in DMEM at an MOI of 10:1. The infection took place at 25 °C in a 5% CO₂ incubator for 30 min after centrifugation at 170 × g for 6 min. To measure adherence, cell monolayer was washed four times with Hank’s balanced salt solution (HBSS, Sigma), and then incubated with 1 ml 0.1% Triton X-100 (Sigma) for 10 min at room temperature. The lysates were then serially diluted and plated on BHI agar. The number of internalized bacteria was measured according to the report by Elsinghorst (1994). Briefly, the monolayer was incubated for 1 h in DMEM with Gn (100 µg ml⁻¹) to kill any remaining extracellular bacteria, and further incubated in DMEM with Gn (16 µg ml⁻¹) for 30 min, before being washed four times with HBSS; monolayers were then lysed and bacteria enumerated by serial dilution and plate-culture. The adherence and internalization or invasion to adherence were calculated respectively from four wells as the mean percentages of adhering bacteria to the total number of infected bacteria, and of internalized bacteria to the number of adhering bacteria.

2.9. Competitive index (CI) assay in yellow catfish

The competitive index (CI) of E. ictaluri strains was determined in yellow catfish obtained from Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. Mutants and wild-type E. ictaluri were grown separately. Equal amount of the wild-type strain HSN-1 and each mutants was mixed respectively with a final count of 3 ± 0.09 × 10⁶ CFU ml⁻¹ in PBS, and 0.1 ml mixed bacterial suspension was injected intraperitoneally into yellow catfish. Fish were maintained with aeration at the water temperature of 25 ± 0.5 °C. 65 h post infection (hpi), liver and spleen samples were dissected separately from 9 fish; they were homogenized, serially diluted, and drop-plated on BHI-Col separately to determine the total CFU recovered, and on BHI-Km-Col to determine the number of mutant CFU recovered. The CI values were determined by dividing the recovery ratio of (mutant CFU/wild-type CFU) by the input ratio of (mutant CFU/wild-type CFU), and expressed as the mean ± standard error, SE) from nine fish. The values, ranging from 0 to 1, indicate the level of attenuation, with values closer to 0 indicating greater attenuation, and the values of more than 1 means high virulence.

2.10. Statistical methods

The data obtained for E. ictaluri adherence, internalization, CI and replication were expressed as the mean ± SE and analyzed in one-way analysis of variance (ANOVA) using SPSS19. Differences were regarded as significant at P < 0.05.

3. Results

3.1. Sequence characters of pEI1 and pEI2

Both pEI1 and pEI2 in E. ictaluri HSN-1 were multi-copy plasmids, sharing replicons as in E. ictaluri 93–146, with the base pair numbered from the unique EcoR I site as in the strain 93–146 (Fernandez et al., 2001). The smaller plasmid, pEI1 from E. ictaluri HSN-1 was 3961 bp in size, having high level of similarity (82.2%) with pEI1 in strain 93–146 (Fernandez et al., 2001), which was of 4807 bp in size (GenBank Accession No. NC_002497). The pEI1 in HSN-1 differed from that in 93–146 mainly in the absence of 846 nt between 2436 and 3344, which was involved in transposition (Fernandez et al., 2001). The ORF of eseH was in the upstream of the transposition fragment, being close to the replicon. Hence, the eseH in the HSN-1 may function as same as eseH in other E. ictaluri strains such as strain 93–146. In addition, pEI1 in HSN-1 had a point mutation, being A rather than T in gene spacer at 4246.

EseH (YP_007447596.1), encoded by the main ORF of pEI1, was 619 aa, with homology to Sse1, SspH1 and SspH2 of Salmonella pathogenicity island-2 (SPI-2), and to IpaH of Shigella. Sse1 along with SspH1 and SspH2 are called Salmonella translocated effectors (STE)s with conserved sequence WEK(1/M)xxFF (x is any amino acid) (Miao and Miller, 2000; Miao et al., 1999; Brown et al., 2006). EseH is also similar to SspH1, SspH2 and IpaH in carboxy terminus and leucine rich repeats. Each of the homologues is of T3SS effector, suggesting that EseH may function as an effector protein in T3SS of E. ictaluri. Interestingly, EseH was found with strong homology to three ORFs (YP-002932758, YP-002932760, and YP-002933761) in the genome of E. ictaluri 93–146.

The larger plasmid, pEI2 from E. ictaluri HSN-1 was 5644 bp in size, and had almost complete nucleotide sequence with the pEI2 in E. ictaluri 93–146 (GenBank Accession No. NC_002498) (Fernandez et al., 2001), except the presence of an extra nucleotide, being G at the nt site 4230 in pEI2 of the strain HSN-1.

In pEI2, two target ORFs were found. One encoded EscD (140 aa), which shared similarity with T3SS chaperones, Spa15 in Shigella and SpaK in Salmonella. EscD is homologous to a hypothetical protein (YP-002934178) in E. ictaluri genome. Immediately downstream EscD, spaced by 3 nt, was Esel (GenBank Accession No. YP_007459743.1), which was 332 aa and had low degree of similarity with T3SS effector OspB in Shigella at the C-terminus.

Sequences of pEI1 and pEI2 from E. ictaluri HSN-1 were deposited in GenBank with the accession numbers of KC291146 and KC291147, respectively.
3.2. Curing of the plasmids, pEI1/pEI2, and deletion of eseH, eseI, and escD

To confirm the complete deletion of native plasmids, multiple PCRs were performed using plasmids isolated from the cured HSN-1 strain as templates, and the DNA gel results confirmed that no trace of native plasmid was found (Fig. S1).

For confirming the deletion of eseH, eseI and escD, primer pairs located on the flanking sequence of the three ORFs were used to determine the deletion (Tables S1 and 2), with wild-type strain HSN-1 used as positive control (expressed as ‘+’), and water template as negative control (expressed as ‘−’). Each of the primer pairs revealed the complete deletion of their target genes (Fig. S2).

3.3. Secretion of EseH and EseI, but not EscD

The 2HA fused eseH, eseI and escD were inserted into Tet gene of pACYC184, and transformed into the wild-type E. tarda PPD130/91 and the T3SS ATPase mutant esaN, respectively. Equal amount of ECPs and TCPs from E. tarda mutants and wild type were collected separately for Western blotting analyses using anti-HA, anti-EvpC and anti-DnaK antibodies. DnaK was found in TCPs but not in ECPs, indicating that ECPs was not contaminated by TCPs. Western blotting revealed that EseH, EseI and EscD were all expressed in both wild type and ΔesaN strains; EseH was detected only in the EPCs from PPD130/91, but not from esaN mutant (Fig. 1A); EseI was secreted in EPCs of both wild type and esaN mutant (Fig. 1B); However, no EscD was found in EPCs of wild type, nor ΔesaN (Fig. 1C). These results indicate that EseH is secreted and its secretion is dependent on an active T3SS, and that EseI is secreted too, but not on T3SS. However, EscD was not secreted.

3.4. Translocation of EseH and EseI

TEM-1 β-lactamase vector is a reporter to observe the translocation of T3SS effector in mammalian cells (Zlokarnik et al., 1998). Considering that E. ictaluri is of fish isolates, E. tarda was used as the surrogate. Under fluorescence microscopy, J774 cells infected with PPD130/91 strain expressing pCX341 appeared green; when infected with E. tarda expressing EseH-TEM or EseI-TEM, some cells turned blue, indicating that EseH and EseI were translocated into host cells (Fig. 2).

3.5. Localization of EseH and EseI in ZF4 cells

When EseH and EseI were translocated into host cells, their localization in host cells became important for revealing their interactions with host cells. In this study,
Fig. 3. Localizations of EseH and EseI in infected ZF4 cells using immunofluorescence (A) and Western blotting analysis (B). WT+EseH-HA+GFP and WT+EseI-HA+GFP indicating the GFP-tagged wild-type HSN-1 transformed with vector pVPF25.1 expressing HA fused EseH or EseI, respectively. Using confocal immunofluorescence analysis, bacteria (green in merged image) were confirmed by GFP fluorescence vector, and mouse anti-HA monoclonal antibody was used to detect EseH::HA or EseI::HA (red in merged image). Scale bar representing 5 μm. Cytosol and membrane were subcellular fractionations of ZF4 cells infected with bacterial strains expressing HA-tagged EseH or EseI, respectively. The host cell proteins β-actin and Calnexin were used as markers of the cytosolic and membrane fractions, respectively. EseH::HA and EseI::HA were distinguishable on the basis of their molecular masses (70 kDa and 37 kDa, respectively). EvpC and DnaK were used for indicating that the cell fractions were not contaminated.
fractionation assay and immunofluorescence microscopy were used to investigate the localization of EseH and Esel in ZF4 cells. By means of immunofluorescence, bacteria were merged with EseH or Esel in cells for visualizing the translocation of proteins. EseH and Esel were observed suffusing beyond nucleus of ZF4 cells (Fig. 3A), indicating that they were localized in cytosolic or membrane fraction of host cells.

To find out whether EseH and Esel were localized in cytosolic or membrane fractions of ZF4 cells, subcellular fractionation assays were used, with ZF4 cell proteins β-actin and Calnexin used as markers of the cytosolic and membrane fractions, respectively. EvpC and DnaK were not found in cytosolic and membrane fractions of infected ZF4 cells, indicating that these fractions were not contaminated by the bacteria. Our data showed that EseH was localized in the ZF4 cell membrane fraction, and Esel in the cytosolic fraction (Fig. 3B).

3.6. Role of EseH and Esel in adhesion to EPC cells

Adherence of bacteria to host cells is often an essential step for initiating infection, and the ability to become internalized in host cell is a determinant of virulence for pathogens. In this study, E. ictaluri mutants were found to adhere to, and to invade EPC cells (Fig. 4). In Fig. 4A, a significant decrease was observed in percentage of mutant bacteria adhering to EPC cells for mutants, ΔeseH and Δesel, being at 11.26 ± 0.29% and 12.22 ± 0.38% (P < 0.01), respectively, when compared with the wild-type strain (16.23 ± 0.52%). But, no significant difference was observed for ΔescD, despite also a decrease (13.78 ± 0.86%; P > 0.05). At 1.5 hpi, the percentage of internalization to adherence for Δesel was 40.85 ± 2.18%, which was highly significant than 33.31 ± 1.54% of wild-type (P < 0.05; Fig. 4B). EseH thus contributed to the adherence to EPC cells without affecting internalization, and Esel was involved in adherence to, and invasion into epithelial cells, and also served as to repress invasion.

3.7. Attenuation of ΔeseH and Δesel and increased virulence of ΔescD

The three proteins encoded by pEI1 or pEI2 were examined for their contribution to virulence in vivo by using CI assay. 65 hpi, CI values were determined separately from liver and spleen of each fish. The values were quite close for each mutant, despite the value in spleen was a bit lower than that in liver for ΔescD (Table 2). The mutants, ΔeseH and Δesel, had a mean CI value between zero and one, being 0.7246 and 0.7544, and 0.6373 and 0.6158 for liver and spleen respectively, indicating that the two mutants were attenuated. However, the CI of ΔescD was higher than 1, being above 1.5, implying that ΔescD may have a noticeably fast replication in catfish, when compared with wild type in vivo.

4. Discussion

Plasmids in pathogenic bacteria often play a role in their pathogenesis, and some virulent factors of enteric bacteria are even encoded in plasmids, such as pO157 in enterohaemorrhagic Escherichia coli (EHEC) (Rump et al., 2012), pYV in species of Yersinia (Hammer et al., 2012) and pWR501 in Shigella flexneri (Venkatesan et al., 2001). In the present study, the virulence impact of EseH encoded in pEI1, and EscD and Esel in pEI2 of E. ictaluri, the etiological agent of ESC, were revealed in yellow catfish and also in fish host cells through the construction of mutants.

Two native plasmids, pEI1 and pEI2 sequenced from E. ictaluri HSN-1 in this study have conserved sequences as those reported in strain 93–146 of E. ictaluri from US, despite the lack of a sequence in pEI1 of HSN-1, which corresponds to the sequence of transposon in pEI1 of the 93–146 (Fernandez et al., 2001). However, the sequence of pEI1 without transposon has not been reported previously in strains of E. ictaluri. The sequenced plasmid, pEI1 in strain HSN-1 thus provides the actual first report on this plasmid without the presence of transposon.
In order to reveal functionally unknown proteins encoded by multi-copy plasmids, it is crucial to cure the native plasmids first. Chemical or physical methods were used conventionally to remove plasmids from host bacteria, which may adversely affect the genome of host bacteria (Pickett et al., 2005). In this study, a method based on plasmid incompatibility was adopted to cure the plasmids in *E. ictaluri*, since curing plasmids in this way, according to plasmid incompatibility theory, has no side effect on other plasmids or chromosomes (Ni et al., 2008). In this study, pEI1 and pEI2 were achieved using the plasmid backbones of themselves without the risk of introducing mutation in bacterium chromosome, and virulent genes encoded in the plasmids can then be knocked out completely for examining their functions.

To obtain their mutants, the three genes, i.e. *eseH*, *eseI* and *escD*, in these two plasmids were deleted respectively after the curing of corresponding plasmids. It is confirmed that plasmids pEI1 and pEI2 were involved in virulence of the bacterium, thus playing a role in pathogenesis of the bacterium. The only functional research on the two plasmids of *E. ictaluri* was reported by Thune et al. (2007) who found that mutant No. 217UV, which had a transposon insertion in *eseH* of pEI1, and mutant No. 166ST, which had an insertion in the upstream of *escD* and *eseI*, all had a decrease in CI values in channel catfish, indicating their possible role in virulence. In the present study, Δ*eseH* and Δ*eseI* were significantly attenuated as revealed with lower CI values in yellow catfish, and decreased adherence in EPC cells, implying that they are involved in virulence of the bacterium. But, Δ*escD* had a larger CI value in yellow catfish and a slight but non-significant decrease in adherence to EPC cells, implying its fast replication in fish and its comparable virulence with the wild type. The reason why Δ*escD* had a higher CI value and thus a higher multiplication efficiency in fish is at present unknown. However, the decrease in CI value for the mutant No. 166ST, as reported by Thune et al. (2007), may be due to the regulatory influence on the expression of *eseI*, as the location in the upstream of *escD* and *eseI*, where transposon was inserted, is a region with regulatory operon which may have little effect on the expression of *escD*. But, the exact role of this regulatory operon in this plasmid may require further investigation.

Among the three proteins, EseH is the only molecule found secreted through T3SS in *E. tarda*. EseI was also secreted, as detected in cytosol fraction, but its secretion was not dependent upon the T3SS, and this protein was not affected by the regulator proteins EsrBC in the *E. ictaluri* T3SS, as reported by Rogge and Thune (2011), which is of interest for further research. The secretion of EseH and EseI may then be deployed by *E. ictaluri* to interfere with host cells. Using quantitative and Western blotting analyses, Rogge and Thune (2011) found that low pH and phosphate limitation was conducive to an active T3SS in *E. ictaluri*, whereas they failed to detect Flag-tagged EseH. The 70–99% similarity in 33 ORFs of T3SS between *E. tarda* and *E. ictaluri* may enable the functional identification of T3SS molecules in the *E. tarda* surrogate. As EscD was not even secreted, it seems possible that among the three proteins encoded in pEI1 and pEI2, EseH is the only molecule functional through T3SS, and is probably the virtual homologue to T3SS effector.

T3SS chaperones are not involved in the secretion and translocation of effectors but often interact as homo- or hetero-dimers with their cognate proteins to promote the effectors to carry out their mission (Ghosh, 2004). In *E. ictaluri*, EscD shares similarities with T3SS chaperone in low molecular weight (Mw: 15.4 kD), acidic isoelectric point (pI4.42), and predicted helical structure. It contains chaperone binding domain (CBD) homologous to PRK15336 (Lilic et al., 2006). Usually, T3SS chaperone is located next to their target genes (Jermy, 2011). In consideration of the location and protein homology of *escD* and *eseI* in pEI2T, Thune et al. (2007) proposed that EscD is a chaperone of EseI. But, the yeast two-hybrid assay failed to detect the interaction between the EscD ‘bait’ and the EseI ‘prey’ (data not shown). If EscD served as a chaperone of *eseI*, *eseI* should have defective secretion and translocation when *escD* was deleted. However, this is not the case as revealed in the present study, and it is thus considered that EscD is not a chaperone of EseI.

In addition, proteins which share certain degree of similarity to the three plasmid proteins, EseH, EseI and EscD, such as STEs in *Salmonella* (Miao et al., 1999; Brown et al., 2006), OspB (Sadzienie et al., 1993) and InvB (Porter and Curtiss, 1997) respectively, may be involved also in virulence or pathogenesis of their respective bacteria, but may be well beyond of the topic of this study.

In conclusion, two native plasmids, pEI1 and pEI2, were sequenced and successfully cured from the strain HSN-1 of *E. ictaluri*, and three genes *eseH*, *eseI* and *escD* encoded in the plasmids were deleted respectively with the construction of mutants. EseH and EseI were found secretory, with the secretion of EseH being dependent on T3SS. Mutants, Δ*eseH* and Δ*eseI* were attenuated, while Δ*escD* had increased virulence, indicating that these two plasmids are involved in virulence and pathogenesis of *E. ictaluri*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jvetmic.2013.05.023.