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NOTE

Transferable green fluorescence-tagged pEI2 in *Edwardsiella ictaluri* and preliminary investigation of its effects on virulence

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ABSTRACT: *Edwardsiella ictaluri* is the etiologic agent of enteric septicemia of catfish, which causes substantial losses in catfish aquaculture. To determine pathogen–host interactions, previous studies have used the green fluorescence protein (GFP) gene. Here, the pEI2 plasmid of *E. ictaluri* isolate I49 was tagged using a Tn10-*GFP-kan* cassette to create the green fluorescence-expressing derivative I49-*gfp*. The Tn10-*GFP-kan* insertion site was mapped by plasmid sequencing to 663 bp upstream of open reading frame 2 and appeared to be at a neutral site in the plasmid. Purification of the pEI2::*GFPKan* plasmid and mobilization into *E. coli* resulted in GFP expression. The isolated pEI2::*GFPkan* plasmid was used to retransform the wild type I49 isolate (ensuring a single Tn10-*GFP-kan* insertion) and an independent *E. ictaluri* isolate, S97-73-3. The wild type and the green fluorescent-tagged strains were compared for modulation of pathogenicity in channel catfish *Ictalurus punctatus* by immersion challenge. A significant reduction in mortalities occurred for the I49*GFPkan* strain as compared to its isogenic parent, but no difference was observed between the S97-73-3*GFPkan* strain and the S97-73-3 wild type. This GFP-tagged plasmid will be useful for determining the effects that the pEI2::*GFPkan* plasmid has on virulence and host–pathogen interactions between *E. ictaluri* isolates.

KEY WORDS: Edwardsiellosis · Plasmid tag · Catfish · *Ictalurus punctatus* · Host–pathogen interaction

INTRODUCTION

Edwardsiella ictaluri, the etiologic agent of enteric septicemia of catfish (ESC), is a Gram-negative bacterium that was originally isolated from diseased channel catfish *Ictalurus punctatus* (Hawke 1979). ESC causes substantial losses in catfish aquaculture and remains one of the largest problems in this industry (Freund et al. 1990, Shoemaker et al. 2007, Thune et al. 2007). Detection and tracking of pathogens using the green fluorescence protein (GFP) gene from *Aequorea victoria* is a useful tool to determine pathogen–host interactions (Valdivia et al. 1996), and this approach has been applied to several fish pathogens (Ling et al. 2000, 2001, Welch & Wiens 2005), including *E. ictaluri* (Karsi et al. 2009).

Two plasmids exist in clones isolated from channel catfish: pEI1 and pEI2 (Newton et al. 1988) (also referred to as pCL1 and pCL2; Lobb & Rhoades 1987). Both plasmids contain genes with sequence similarity to functional proteins in other bacteria, but little is known about the functions of either plasmid

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(Fernandez et al. 2001). A study using high-throughput mutation and screening revealed that mutation of a hypothetical protein in the pEI1 plasmid attenuated pathogenicity (Karsi et al. 2009), suggesting that the pEI1 plasmid is important for virulence in catfish. A study by Rogge & Thune (2011) suggested that a pEI2 associated gene, *eseI*, is expressed under low-pH and low-phosphate conditions. This expression was not associated with the type III expression system and suggests that the pEI2 plasmid is functional in multiple pathogenic pathways.

In this study we report the tagging of the pEI2 plasmid with a Tn10-*GFP-kan* cassette and transformation of the tagged pEI2 to *Edwardsiella ictaluri*. The tag is retained without selection with kanamycin and after isolation from challenged fish. We report the effects of this transformation on the virulence of 2 different *E. ictaluri* isolates. This tagged plasmid will be a useful tool for comparing and contrasting differences in isolates of *E. ictaluri* by determining effects that the pEI2::*GFPkan* plasmid has on virulence in different *E. ictaluri* isolates and changes to host–pathogen interactions.

MATERIALS AND METHODS

Bacteria and culture conditions

Two isolates of *Edwardsiella ictaluri*, I49 and S97-73-3, were taken from moribund channel catfish during ESC pond outbreaks in Mississippi (USA) and stored at the Catfish Genetics Research Unit (CGRU), Jamie Whitten Delta States Research Center. The isolates were classified as *E. ictaluri* by 16S sequencing, Gram staining, and colony morphology. TOP10 *Escherichia coli* cells were purchased from Invitrogen. All *E. ictaluri* isolates were maintained as frozen stocks and grown on tryptic soy agar (TSA) or tryptic soy broth (TSB). The *E. coli* strains were grown from frozen stocks on Luria Bertani (LB) agar or broth. Kanamycin was added to growth media when appropriate at a concentration of 100 µg ml^{-1}.

Transposition of Tn10-*GFP-kan* cassette

Transposition of the mini-Tn10-*GFP-kan* transposon (Stretton et al. 1998) into *Edwardsiella ictaluri* I49 was accomplished as reported elsewhere (de Lorenzo et al. 1990, Welch & Wiens 2005). Briefly, conjugation mixes were incubated at 24°C for 12 h on TSA media, resuspended and diluted in TSB, and plated on TSA containing 100 µg ml^{-1} kanamycin. Plates were incubated at 14°C to counter select the *E. coli* donor.

Transformation

Electroporation of *Edwardsiella ictaluri* was performed using the protocol established by Russo et al. (2009). Briefly, mid-log-phase *E. ictaluri* cultures were chilled 10 min on ice and centrifuged at 3400 × *g* (4°C, 15 min). Supernatants were discarded and cells were washed once with ice-cold distilled water and twice with ice-cold 10% glycerol, to produce electrocompetent cells. Then, 0.2 µg of plasmid DNA was added to electrocompetent *E. ictaluri* cells (ca. 2×10^9 CFU, confirmed by plating); cells and plasmid were transferred to a chilled 0.1 cm electroporation cuvette (BioRad Laboratories) and electroporated at 200 Ω, capacitance 25 µF and 12.5 kV cm^{-1} for 4 ms. Transformation of *E. coli* TOP10 cells was performed as recommended by the manufacturer (Invitrogen). Plasmid transference confirmation was determined by kanamycin resistance on LB plates for *E. coli*, and TSA for *E. ictaluri*, containing 100 µg ml^{-1} kanamycin and by visualization of green fluorescence.

Bacterial challenge

Thirteen flow-through aquaria (75 l capacity) were stocked with 25 fish from a single family of CGRU channel catfish, with apparently healthy fish weighing an average of 7.4 ± 0.7 g. Following 1 wk of acclimation, the water flow was stopped and water volumes were reduced to 14 l while maintaining aeration. Water temperature averaged 27.0 ± 0.3°C. Three tanks each were challenged with approximately 1×10^6 CFU ml^{-1} final concentrations of an overnight culture of the 4 *Edwardsiella ictaluri* strains: I49, I49*GFPkan*, S97-73-3, and S97-73-3*GFP-kan*. CFUs were confirmed by serial dilution plate counts and OD600 reading. One tank treated with the same protocol but lacking bacteria served as a negative control. The immersion challenge was allowed to proceed for 30 min, after which water flow was resumed. Fish were fed daily to satiation before and during the challenge and were observed for mortality for 21 d. All mortalities were examined for the classic signs of enteric septicemia (bloody ascites, hemorrhage and necrosis in multiple organs, skin lesions, and lesions of the cranial foramen). Greater than 10% of random mortalities were collected and sent to the Mississippi State University Veterinary Diagnostic

Laboratory for pathological analysis. Confirmation of death by *E. ictaluri* was determined by Gram staining of liver tissue, ESC pathological signs, and isolation of *E. ictaluri*. All mortalities submitted for diagnostic evaluation were confirmed to have died from ESC.

Statistical analysis

Survival curves were generated by Kaplan-Meier analysis, and statistical differences were determined using both the log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests performed on GraphPad version 5 software. A p-value of <0.05 was deemed significant.

RESULTS

Generation of GFP-expressing bacteria

A single clone demonstrating high green fluorescence following the insertion of a Tn10-*GFP-kan* transposon was selected for further study. The transposon cassette had randomly inserted into the pEI2 plasmid of *Edwardsiella ictaluri* I49. The *GFP-kan* cassette insertion was confirmed by kanamycin resistance, sequencing (Fig. 1), and plasmid isolation (Fig. 2). The size of the native pEI2 plasmid is 5643 bp, but the pEI2::*GFPkan* plasmid increased it to 8309 bp. The open reading frame 2 (Orf2), a putative replication initiation site, occurred 663 bp downstream of the transposon insertion site (Fig. 1); this insertion site has no known associated function and no obvious open reading frames, thus making it a neutral site. The transformed pEI2::*GFPkan* plasmid from *E. ictaluri* I49 was isolated and transformed into TOP10 *E. coli* cells for selection and propagation. Plasmids were isolated from the transformed TOP10 *E. coli*, and colonies either contained both pEI1 and pEI2::*GFPkan* or just the pEI2::*GFPkan* plasmids (Fig. 2). Although the pEI2::*GFPkan* plasmid was propagated in the TOP10 *E. coli*, it was also positive for green fluorescence as compared to the sham-transformed colonies (Fig. 3). One *E. coli* isolate, TOPpEI2*GFPkan*, containing only the pEI2::*GFPkan* plasmid, was selected and used for further propagation and isolation. The isolated pEI2::*GFPkan* plasmid was then used to transform the wild type (wt) I49 isolate and a different *E. ictaluri* isolate, S97-73-3. This step ensured that a single *GFP-kan* cassette is sufficient to confer a green fluorescent phenotype. In colonies positive for green fluorescence, the plasmid DNA was isolated and a single band was visualized by gel electrophoresis, confirming that only the pEI2::*GFPkan* plasmid was present (Fig. 2). The transformed *E. ictaluri* strains were sub-cultured (>10 times) on TSA plates that did not contain kanamycin, and no loss of green fluorescence was observed (Fig. 3).

Fig. 1. Schematic of the Tn10-*GFP-kan* insertion site into pEI2 plasmid of *Edwardsiella ictaluri* between Orf2/Repeat II and Repeat III. Insertion site is 663 bp downstream of the Orf2 site

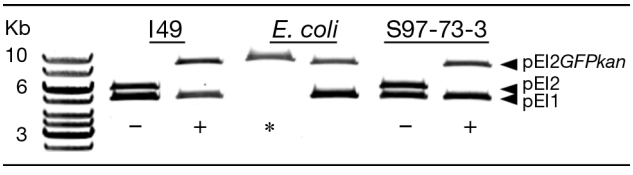

Fig. 2. Plasmid isolation from parent *Edwardsiella ictaluri* I49 and S97-73-3 (–) isolates and the I49*GFPkan* and S97-73-3*GFPkan* mutants (+), and TOP10 *E. coli* bacteria, one containing only the pEI2::*GFPkan* plasmid and a separate isolate containing both pEI1 and pEI2::*GFPkan* plasmids. *indicates the isolate used for propagation of the pEI2::*GFP-kan* construct. The native pEI2 plasmid is 5643 bp and pEI1 is 4807 bp, but the pEI2::*GFPkan* plasmid is 8309 bp. Corresponding bands are indicated with (◄) and are labeled

Bacterial challenge

To determine whether pathogenicity was altered, immersion challenges were performed using the wt isolates I49 and S97-73-3, and comparing them to the GFP-expressing I49*GFPkan* and S97-73-3*GFPkan*

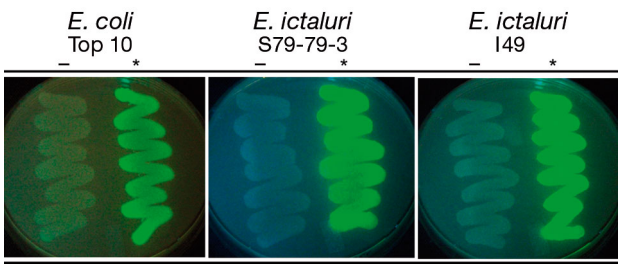

Fig. 3. Detection of green fluorescent protein on tryptic soy agar plates using a blue light-emitting diode (LED), with wild type isolates of *E. coli* and *Edwardsiella ictaluri* (–), and the strains containing pEI2::*GFPkan* (*). All forms were grown on TSA plates without kanamycin

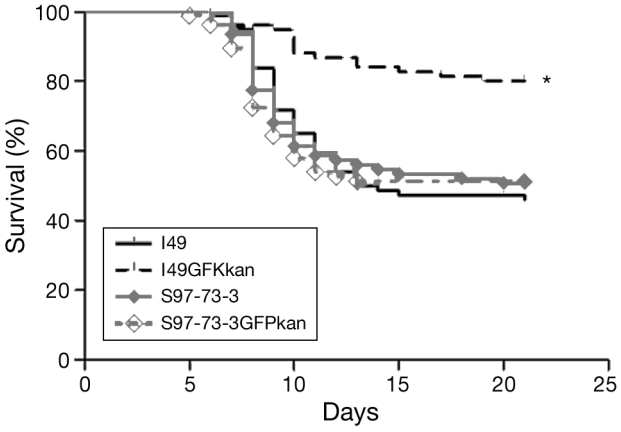

Fig. 4. *Ictalurus punctatus.* Survival curves of channel catfish following challenge with pEI2::*GFPkan* containing *Edwardsiella ictaluri* strains I49 and S97-73-3, and their isogenic parents. Fish were challenged by immersion for 30 min in a bacterial concentration of approximately 10^6 CFU ml^{-1}. Mortalities were recorded daily for 21 d. *Significant difference ($p < 0.0001$) in mortality between strain I49 and pEI2::*GFPkan* containing I49

strains. No significant difference in mortality was observed between the S97-93-3 wt and the S97-73-3*GFPkan* derivative, both strains causing approximately 50% mortality. Conversely, there was a significant ($p < 0.0001$) attenuation of pathogenicity from the I49*GFPkan* stain as compared to the wt I49 isolate, which resulted in 20 versus 51% mortality, respectively (Fig. 4).

DISCUSSION

Collectively, the current study and previous reports (Thune et al. 2007, Karsi et al. 2009, Rogge & Thune 2011) suggest that the conserved plasmids from catfish-derived *Edwardsiella ictaluri* are associated with pathogenicity in relation to a type III secretion system and possibly multiple pathways. In the current study, the insertion site of the Tn10-*GFP-kan* transposon into what was perceived as a neutral site was based on the sequence analysis and an apparent lack of an open reading frame (Orf). This insertion was considered advantageous (Fig. 1) as this site would have minimal interference with normal bacterial function. However, a previously reported mutant, 166ST, with an insertion into the Repeat 1 region of the pEI2 plasmid, a region also without an obvious ORF, demonstrated attenuated pathogenicity (Thune et al. 2007). This attenuation was thought to occur due to a disruption in the regulatory region upstream to the start codon of Orf1. Another study (Rogge & Thune 2011) showed that during periods of low pH and phosphate limitation, conditions mimicking a phagosomal environment, the pEI2 encoded gene *eseI* was modestly upregulated at the RNA level and increased expression at the protein level. Interestingly, this increased expression was not associated with several type III secretion system genes, suggesting that the genes encoded on the pEI2 plasmid are associated with multiple pathways.

The *GFP-kan* cassette insertion into the pEI2 plasmid allows for relatively easy isolation and transfection in multiple *Edwardsiella ictaluri* isolates, which is useful for functional comparisons between isolates. We determined that the *GFP-kan* cassette insertion site would elicit minimal interference in normal bacterial function, but as the bacterial challenges demonstrate, this is not always the case (Fig. 4). The variation in pathogenicity between the wt I49 strain and the I49*GFPkan* derivative suggests that an insertion into regions even with no suggestive genetic function can modulate pathogenicity. Interestingly with this particular insertion site, the effect is not universal between all *E. ictaluri* isolates. The I49*GFPkan* had an attenuated pathogenicity as compared to the I49 strain, but no attenuation was observed between the S97-93-3 wt and the S97-73-3*GFPkan* strains. These results suggest variation between *E. ictaluri* isolates and the effect the pEI2*GFPkan* plasmid has on pathogenicity.

The electroporation protocol established by Russo et al. (2009) proved effective in transfecting the pEI2::*GFPkan* plasmid into multiple *Edwardsiella ictaluri* isolates, but electroporation was unnecessary for transference into the TOP10 *E. coli* strain. The TOP10 *E. coli* propagation elicited unexpected results, specifically with regard to GFP expression in *E. coli* and maintaining the pEI1 plasmid when co-transfected (Figs. 2 & 3). For example, the insertion of the Tn10-*GFP-kan* transposon into pEI2 did not inhibit the production of GFP in the *E. coli* intermediate, indicating that this plasmid construct may be useful for generating multiple species of GFP-tagged bacteria. The presence of the pEI1 plasmid in some colonies could be maintained for several reasons, including (1) a higher stability in *E. coli* even in the presence of selective media, or (2) a relationship between pEI1 and the pEI2::*GFPkan* plasmid which allows the pEI1 plasmid to be replicated and not eliminated. Once the pEI2::*GFPkan* plasmid was transfected into wt *E. ictaluri* strains I49 and S97-93-3, the native pEI2 plasmid was rapidly lost (Fig. 3). The native pEI2 plasmid was, presumably, selected against due to the absence of the kanamycin gene,

while the native pEI1 plasmid should not have been affected due to the transference of only the pEI2::*GFPkan* plasmid. When the I49*GFPkan* and S97-73-3*GFPkan* strains were isolated out of infected fish, or passed several times without the presence of a selective agent, green fluorescence was not diminished, suggesting that the plasmid was stable and not eliminated over time.

This study, along with previous studies, suggests that the conserved plasmids from catfish-derived *Edwardsiella ictaluri* strains are important in pathogenicity, but not all pEI2 mutations will affect each strain in a similar manner. The GFP-tagged plasmid generated in this study is a useful tool for generating green fluorescent strains of *E. ictaluri* with an identical GFP tag and allowing studies between these various isolates. In one isolate, the insertion had no effect on pathogenicity whereas in a second we observed modulation of pathogenicity, indicating a variation in function for the pEI2::*GFPkan* plasmid between isolates. The pEI2::*GFPkan* plasmid may alter host versus pathogen interactions, indicating more pEI2-related functions, which can be determined in future studies.

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