Involvement of *tolQ* and *tolR* genes in *Edwardsiella ictaluri* virulence

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ABSTRACT

Edwardsiella ictaluri is a Gram-negative intracellular facultative pathogen causing enteric septicemia of channel catfish (ESC). The Tol system, consisting of four envelope proteins TolQ, TolR, TolA, and TolB, are required for colicin import and contributes to bacterial virulence in several pathogenic bacteria. However, the Tol system and its importance in *E. ictaluri* virulence have not been investigated. Here we present construction and evaluation of the *E. ictaluri* TolQ, TolR and TolQR mutants (*EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR*). The Tol mutants were developed using in-frame gene deletion and their attenuation and vaccine efficacy were determined in catfish fingerlings. The *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* mutants showed reduced virulence in catfish (28.93%, 19.70%, and 39.82% mortality, respectively) compared to wild type (46.91% mortality). Further, vaccination with mutants protected catfish against subsequent wild-type infection. This study suggests that the Tol system contributes to *E. ictaluri* virulence in catfish.

Keywords: *Edwardsiella ictaluri*; Tol system; TolQ; TolR; Virulence

1. Introduction

Edwardsiella ictaluri is a Gram-negative facultative intracellular pathogen. It causes enteric septicemia of catfish (ESC), which manifests as acute septicemia or chronic meningoencephalitis [1, 2]. It is estimated that ESC is responsible for 25-30% production loss accounting for \$60 million annually [3].

In Gram-negative bacteria, the Tol system is highly conserved. It consists of four proteins: TolQ, TolR, TolA, and TolB. These proteins are involved in maintaining the stability of the outer membrane using energy derived from proton motive force [4, 5]. The Tol system is also used by filamentous bacteriophage to import DNA into bacteria, and it enables uptake of group A colicins [6, 7]. Disruption or deletion of Tol system proteins caused defects in outer membrane integrity, which resulted in the release of periplasmic content into the extracellular medium, formation of outer membrane vesicles, and hypersensitivity to bile salts [8-10]. Besides structural functions, *tol* genes also appear to play a significant role in the pathogenesis of several Gram-negative bacteria such as *E. coli* [11], *Haemophilus ducreyi* [12], *Salmonella enterica* [13], and *Vibrio cholerae* [14].

TolQ and TolR proteins are the two most widely disseminated proteins of the Tol system, sharing structural and functional homology with ExbB/ExbD and the proton motive force (PMF)-responsive flagellar motor proteins MotA/MotB. There is a high degree of DNA and amino acid sequence homology between TolQ/R and ExbB/D systems, and evidence now suggests the two systems can partially replace one another [15, 16]. The *tolQ* and *tolR* genes are often annotated in most bacterial genomes as *exbB* and *exbD* homologs. Moreover, overexpressed TolQ can substitute for missing ExbB, and overproduced TolR can complement

missing ExbD [17]. ExbB and ExbD mediate group A colicin uptake via TonB; similarly, TolQ/R mediate group A colicin uptake via TolA [16]. .

In the current work, we report organization of the *tol* genes in the *E. ictaluri* genome, construction of three *E. ictaluri tol* mutants (*EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR*), and determination of *tolQ* and *tolR* roles in *E. ictaluri* virulence.

2. Material and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *E. ictaluri* strain 93-146 was cultured in brain heart infusion (BHI) agar or broth (Difco Laboratories, Detroit, MI) and incubated at 30°C throughout the study. *E. coli* strains were cultured using Luria–Bertani (LB) agar or broth (Difco) and incubated at 37°C throughout the study. When required, the following antibiotics and reagents (Sigma-Aldrich, St. Louis, MO) were added to culture medium: ampicillin (Amp: 100 µg/ml), colistin (Col: 12.5 µg/ml), sucrose (5%), and mannitol (0.35%). Suicide vector pMEG-375 (*sacRB mobRP4* R6K *ori* Cm^r Amp^r) was used to construct in-frame gene deletion by allelic exchange.

2.2. Sequence analysis

Nucleotide sequences of the *E. ictaluri tol* genes (GenBank accession: CP001600) [18] were compared to *tol* genes from *E. tarda* FL6-60 (GenBank accession: CP002154) and *E. coli* K-12 MG165 (GenBank accession: U00096) using Clustal V in Megalign (DNASTAR Inc, Madison, WI).

2.3. Construction of *Ei* Δ *tolQ*, *Ei* Δ *tolR*, and *Ei* Δ *tolQR* in-frame deletion mutants

E. ictaluri 93-146 *Ei* Δ *tolQ*, *Ei* Δ *tolR*, and *Ei* Δ *tolQR* were constructed as described previously [19]. Briefly, approximately 1 kb upstream and downstream regions of the *tolQ* and *tolR* genes were amplified from *E. ictaluri* 93-146 genomic DNA using A/B and C/D primer pairs, respectively. The positions and sequences of PCR primers are listed in Table 2. Upstream and downstream amplicons were diluted and used as template for a second PCR [20] with the A/D primers to generate Δ *tolQ* and Δ *tolR* deletion fragments. The upstream fragment of the *tolQ* gene and the downstream fragment of the *tolR* gene were used for producing Δ *tolQR* deletion fragment. The three fusion fragments were digested with *Sac*I and *Sma*I and ligated separately into pMEG-375 digested with the same restriction enzymes. Screening for plasmid with inserts was conducted in *E. coli* CC118 λ *pir* using colony PCR, and positive clones were confirmed by restriction digestion and sequencing.

Plasmids were transferred into SM10 λ *pir*, which was used as a donor to transfer plasmids into *E. ictaluri* strain 93-146 by conjugation. Transformants were selected on BHI agar containing Amp and Col at 30°C for two days. To allow the second homologous recombination, a single Amp resistant merodiploid colony was streaked on BHI agar and incubated at 30°C for two days. Loss of the suicide vector by the second homologous recombination was selected by spreading overnight cultures onto LB agar with 5% sucrose and 0.35% mannitol, followed by incubation at 28°C for three days. Amp-sensitive colonies were screened by colony PCR using the A/D primers, which was confirmed by sequencing.

2.4. Growth characteristics of the *E. ictaluri* *Ei* Δ *tolQ*, *Ei* Δ *tolR* and *Ei* Δ *tolQR* mutants

Growth kinetics of the three *E. ictaluri* mutants in BHI broth was compared to parent strain 93-146. Briefly, overnight cultures of *E. ictaluri* strain 93-146, *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* were adjusted to OD₆₀₀=1.0, diluted 1:100 with fresh BHI, and cultivated at 30°C. The optical absorbance at 600 nm was measured at 6, 12, 18, and 24 h. Four replicates were performed, and the mean optical absorbance at each time-point were used to determine the growth rate.

2.5. Determination of the virulence of *EiΔtolQ*, *EiΔtolR* and *EiΔtolQR* mutants

All fish experiments were conducted using a protocol approved by the Institutional Animal Care and Use Committee at Mississippi State University. Virulence of the three Tol mutants were determined as described [19]. Briefly, 400 specific-pathogen-free (SPF) channel catfish fingerlings (13.88 ± 0.27 cm, 27.77 ± 1.04 g) were stocked into 20 40-liter tanks supplied with flow-through dechlorinated water. The tanks were divided into five groups of four replicates. The first treatment group was challenged with *EiΔtolQ*, the second group was challenged with *EiΔtolR*, the third group was challenged with *EiΔtolQR*, the fourth group was challenged with *E. ictaluri* strain 93-146, and the last group was negative (BHI) control. Channel catfish were acclimated for one week; then the fish were challenged by immersion in water containing approximately 3.32 x 10⁷ CFU/ml water for one h. Water flow was resumed for gradual removal of the bacteria. Mortalities were recorded daily for 21 days

The ability of each mutant to generate protection against ESC was determined by re-challenging surviving fish with *E. ictaluri* strain 93-146 by immersion (3.83 x 10⁷ CFU/ml). Fish mortalities were recorded daily for 14 days, and the percent survival was calculated.

2.6 Statistical analyzes

Mean percent mortalities for each tank were calculated for each treatment and subjected to arcsine transformation. For the virulence experiment, the mean transformed percent mortality for the fish challenged with *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* mutant strains were compared with *E. ictaluri* WT group by Student's t-test. For the protection trial, transformed mean percent survival data for fish vaccinated with *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* were compared with the sham vaccination treatment using Student's t-test. Differences between growth of *E. ictaluri* mutants and *E. ictaluri* 93-146 was conducted using Student's t-test ($p < 0.05$).

3. Results

3.1. Genomic analysis of the *E. ictaluri* Tol system

Analysis of the *E. ictaluri* genome revealed the presence of four consecutive *tol* genes in the following order: *tolB*, *tolA*, *tolR*, and *tolQ* from NT01EI_2857 to NT01EI_2860 (Fig. 1). The whole operon size is 3,860 bp. The distance between the stop codon of *tolB* and the beginning of *tolA* is 144 bp. Similarly, the distance between the stop codon of *tolA* and the start codon of *tolR* is 135 bp, and the distance between the stop codon of *tolR* and the start codon of *tolQ* is 12 bp.

The identity between the TolB, TolA, TolR, and TolQ proteins encoded by *E. ictaluri* and *E. tarda* FL6-60 were 95%, 96.9%, 89%, and 95, respectively. The identity between TolB, TolA, TolR, and TolQ proteins encoded by *E. ictaluri* and *E. coli* K-12 MG165 were 73.5%, 69%, 72%, and 77%, respectively (Fig. 1).

3.2. Construction of the *E. ictaluri* *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* mutants

The *EiΔtolQ* strain had an in-frame deletion of 194 aa from the 227 aa protein (85.23%), and *EiΔtolR* strain had a deletion of 114 aa from the 140 aa protein (81.42%). The *EiΔtolQR* strain had a deletion of 308 aa out of 367 aa (83.92%) from the *tolQR* region.

3.3. Growth of the *E. ictaluri* *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* mutants

No significant differences in growth kinetics were detected between *E. ictaluri* strain 93-146 and the three mutant strains in BHI broth ($p < 0.05$).

3.4. Virulence and efficacy of the *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* mutants

Percent mortalities in fish experimentally infected with *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR* was 28.9%, 19.7%, and 39.8%, respectively, compared to 46.9% for parent strain 93-146. *EiΔtolQ* and *EiΔtolR* were significantly attenuated compared to parent strain ($p < 0.05$), but there was no significant difference between fish challenged with *EiΔtolQR* compared with *E. ictaluri* strain 93-146 (Fig. 2A). Fish surviving infection from *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQ* had 100%, 98.61%, and 96.55% survival, respectively, when they were re-challenged with wild type strain 93-146. This was significantly higher than percent survival of the sham-vaccinated group (40.69%) (Fig. 2B).

4. Discussion

This study was undertaken to explore the role of the *tolQ* and *tolR* genes in *E. ictaluri* virulence. The organization of the *tol* operon in the *E. ictaluri* chromosome is similar to the *tol* operon in other Gram-negative pathogens, including *Pseudomonas putida* [21], *Actinobacillus actinomycetemcomitans* [22], *Erwinia chrysanthemi* [23], *Brucella abortus* [24], and

Haemophilus influenza [25, 26]. As expected, there is high amino acid identity between Tol system proteins encoded by *E. ictaluri*, *E. tarda*, and *E. coli*. In fact, the Tol system has been identified in most sequenced genomes of Gram-negative bacteria. Therefore, *E. ictaluri* is similar to other Gram-negative bacteria in having a well conserved Tol system gene cluster.

Analysis of TolQ/TolR homologs from many Gram-negative bacteria showed that TolQ/TolR are structurally and functionally similar to ExbB/D of the TonB system and also with MotA and MotB [27]. In fact, *tolQ* and *tolR* genes are often annotated in most bacterial genomes as *exbB* and *exbD* homologs. The *E. ictaluri* genome does not have *exbB* and *exbD* genes, suggesting that *E. ictaluri* does not utilize the ExbB and ExbD proteins of TonB-ExbB-ExbD complex. However, the *E. ictaluri* genome contains the MotA/MotB system and a complete Tol system. This supports the idea that ExbB and ExbD function can be partially replaced by activities of the homologous proteins TolQ and TolR, and these two systems TolQ-TolR-TolA and TonB-ExbB-ExbD may derive from a common ancestor[16]. Thus, it is possible that TolQ and TolR in *E. ictaluri* are involved not only in energy transduction, but also in iron uptake by contributing to the ExbB and ExbD complex.

The Tol system is uncharacterized in *E. ictaluri*. However, it is likely to play a critical part in the virulence of this pathogen given the roles of this complex in other Gram-negative bacteria. Because the TolQ-TolR pair are the two most widely disseminated proteins of the Tol system, and because they share structural and functional homology with ExbB/ExbD and MotA/MotB [27, 28], we hypothesized that deletion of *tolQ* and *tolR* may affect virulence of *E. ictaluri*. Therefore, we constructed three in-frame deletion mutants: *EiΔtolQ*, *EiΔtolR*, and *EiΔtolQR*. We were able to delete 85.23% of *tolQ*, 81.42% of *tolR*, and 83.92% *tolQR* operon.

Our virulence assessment demonstrated that deletion of *tolQ* and *tolR* caused a significant reduction in virulence (greater than two-fold) when compared to *E. ictaluri* parent strain 93-146. Currently, no information is available on the role of *tolQ* and *tolR* in *E. ictaluri* pathogenesis, but our results are similar to findings from other Gram-negative pathogens. In *Salmonella enterica* serovar Typhimurium strain SL1344, a *tolA* mutant was highly attenuated in mouse infections via the oral and intravenous routes [29]. A *S. enterica* Typhimurium *tolB* mutant was unable to survive within macrophages, resist the bactericidal activity of non-immune serum, or cause fatal infection in mice [13]. In the plant pathogen *Erwinia chrysanthemi*, *tolQ*, *tolA*, *tolB* or *pal* mutants displayed reduced virulence, reduced bacterial motility, altered morphology, and increased susceptibility to various antimicrobial agents [23]. In *V. cholerae*, *tolQRA* mutants showed defects in the uptake of prophage CTXphi, which encodes cholera toxin [14].

In *S. enterica* Typhimurium, immunization with the *tolA* mutant provided significant protection against subsequent challenge with wild-type strain SL1344. Therefore, *tolA* was considered a potential target for the development of attenuated live vaccines [29]. In *E. ictaluri*, *tolQ* and *tolR* mutants were not as attenuated as the *S. enterica tolA* mutant, which will limit their ability to be used as live attenuated vaccines. However, results of our protection challenge were similar to *S. enterica* and indicated that the three mutants provided significant protection of channel catfish against subsequent infection with wild type *E. ictaluri*.

Interestingly, deletion of both *tolQ* and *tolR* did not result in a significant reduction in virulence in channel catfish compared to parent strain 93-146.

In conclusion, our study shows that the *E. ictaluri* Tol system is involved in pathogenesis of infection in channel catfish. Further research is warranted to delineate function of these proteins and their specific roles in pathogenesis of ESC. Our results also indicate that these

217 genes could be included as components of a strategy for development of live attenuated vaccines
218 for *E. ictaluri*.

219

220 **Conflict of interest**

221 The authors declare that they have no competing interests.

222

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References

- [1] Miyazaki T, Plumb JA. Histopathology of *Edwardsiella ictaluri* in channel catfish, *Ictalurus punctatus* (Rafinesque)*. *Journal of fish diseases*. 1985;8:389-92.
- [2] Shotts EB, Blazer VS, Waltman WD. Pathogenesis of Experimental *Edwardsiella ictaluri* Infections in Channel Catfish (*Ictalurus punctatus*). *Canadian Journal of Fisheries and Aquatic Sciences*. 1986;43:36-42.
- [3] USDA. DEVELOPMENT OF APPROACHES TO PREVENT AND AMELIORATE DISEASES OF CATFISH. Agricultural Research Service Annual Report. 2014.
- [4] Goemaere EL, Cascales E, Lloubes R. Mutational analyses define helix organization and key residues of a bacterial membrane energy-transducing complex. *Journal of molecular biology*. 2007;366:1424-36.
- [5] Vankemmelbeke M, Zhang Y, Moore GR, Kleanthous C, Penfold CN, James R. Energy-dependent immunity protein release during tol-dependent nuclease colicin translocation. *The Journal of biological chemistry*. 2009;284:18932-41.
- [6] James R, Kleanthous C, Moore GR. The biology of E colicins: paradigms and paradoxes. *Microbiology*. 1996;142 (Pt 7):1569-80.
- [7] Lazdunski CJ. Colicin import and pore formation: a system for studying protein transport across membranes? *Molecular microbiology*. 1995;16:1059-66.
- [8] Bernadac A, Gavioli M, Lazzaroni JC, Raina S, Lloubes R. *Escherichia coli* tol-pal mutants form outer membrane vesicles. *Journal of bacteriology*. 1998;180:4872-8.
- [9] Lazzaroni JC, Portalier R. The excC gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL). *Molecular microbiology*. 1992;6:735-42.
- [10] Vianney A, Lewin TM, Beyer WF, Jr., Lazzaroni JC, Portalier R, Webster RE. Membrane topology and mutational analysis of the TolQ protein of *Escherichia coli* required for the uptake of macromolecules and cell envelope integrity. *Journal of bacteriology*. 1994;176:822-9.

- [11] Hellman J, Roberts JD, Jr., Tehan MM, Allaire JE, Warren HS. Bacterial peptidoglycan-associated lipoprotein is released into the bloodstream in gram-negative sepsis and causes inflammation and death in mice. *The Journal of biological chemistry*. 2002;277:14274-80.
- [12] Fortney KR, Young RS, Bauer ME, Katz BP, Hood AF, Munson RS, Jr., et al. Expression of peptidoglycan-associated lipoprotein is required for virulence in the human model of *Haemophilus ducreyi* infection. *Infection and immunity*. 2000;68:6441-8.
- [13] Bowe F, Lipps CJ, Tsois RM, Groisman E, Heffron F, Kusters JG. At least four percent of the *Salmonella typhimurium* genome is required for fatal infection of mice. *Infection and immunity*. 1998;66:3372-7.
- [14] Heilpern AJ, Waldor MK. CTXphi infection of *Vibrio cholerae* requires the tolQRA gene products. *Journal of bacteriology*. 2000;182:1739-47.
- [15] Koebnik R. The molecular interaction between components of the TonB–ExbBD–dependent and of the TolQRA-dependent bacterial uptake systems. *Molecular microbiology*. 1993;9:219-.
- [16] Braun V, Herrmann C. Evolutionary relationship of uptake systems for biopolymers in *Escherichia coli*: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. *Molecular microbiology*. 1993;8:261-8.
- [17] Braun V. The structurally related exbB and tolQ genes are interchangeable in conferring tonB-dependent colicin, bacteriophage, and albomycin sensitivity. *Journal of bacteriology*. 1989;171:6387-90.
- [18] Williams ML, Gillaspay AF, Dyer DW, Thune RL, Waldbieser GC, Schuster SC, et al. Genome sequence of *Edwardsiella ictaluri* 93-146, a strain associated with a natural channel catfish outbreak of enteric septicemia of catfish. *Journal of bacteriology*. 2012;194:740-1.
- [19] Abdelhamed H, Lu J, Shaheen A, Abbass A, Lawrence ML, Karsi A. Construction and evaluation of an *Edwardsiella ictaluri* fhuC mutant. *Veterinary microbiology*. 2013;162:858-65.
- [20] Horton RM, Cai ZL, Ho SN, Pease LR. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *BioTechniques*. 1990;8:528-35.

279 [21] Llamas MA, Rodriguez-Herva JJ, Hancock RE, Bitter W, Tommassen J, Ramos JL. Role of
280 *Pseudomonas putida* tol-oprL gene products in uptake of solutes through the cytoplasmic membrane.
281 *Journal of bacteriology*. 2003;185:4707-16.

282 [22] Paul-Satyaseela M, Karched M, Bian Z, Ihalin R, Boren T, Arnqvist A, et al. Immunoproteomics of
283 *Actinobacillus actinomycetemcomitans* outer-membrane proteins reveal a highly immunoreactive
284 peptidoglycan-associated lipoprotein. *Journal of medical microbiology*. 2006;55:931-42.

285 [23] Dubuisson JF, Vianney A, Hugouvieux-Cotte-Pattat N, Lazzaroni JC. Tol-Pal proteins are critical
286 cell envelope components of *Erwinia chrysanthemi* affecting cell morphology and virulence.
287 *Microbiology*. 2005;151:3337-47.

288 [24] Tibor A, Weynants V, Denoel P, Lichtfouse B, De Bolle X, Saman E, et al. Molecular cloning,
289 nucleotide sequence, and occurrence of a 16.5-kilodalton outer membrane protein of *Brucella*
290 *abortus* with similarity to pal lipoproteins. *Infection and immunity*. 1994;62:3633-9.

291 [25] Deich RA, Metcalf BJ, Finn CW, Farley JE, Green BA. Cloning of genes encoding a 15,000-dalton
292 peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton
293 protein from *Haemophilus influenzae*. *Journal of bacteriology*. 1988;170:489-98.

294 [26] Sen K, Sikkema DJ, Murphy TF. Isolation and characterization of the *Haemophilus influenzae* tolQ,
295 tolR, tolA and tolB genes. *Gene*. 1996;178:75-81.

296 [27] Cascales E, Lloubes R, Sturgis JN. The TolQ-TolR proteins energize TolA and share homologies
297 with the flagellar motor proteins MotA-MotB. *Molecular microbiology*. 2001;42:795-807.

298 [28] Eick-Helmerich K, Braun V. Import of biopolymers into *Escherichia coli*: nucleotide sequences of
299 the exbB and exbD genes are homologous to those of the tolQ and tolR genes, respectively. *Journal*
300 *of bacteriology*. 1989;171:5117-26.

301 [29] Paterson GK, Northen H, Cone DB, Willers C, Peters SE, Maskell DJ. Deletion of tolA in
302 *Salmonella Typhimurium* generates an attenuated strain with vaccine potential. *Microbiology*.
303 2009;155:220-8.

304 [30] Lawrence ML, Cooper RK, Thune RL. Attenuation, persistence, and vaccine potential of an
305 *Edwardsiella ictaluri* purA mutant. Infection and immunity. 1997;65:4642-51.

306 [31] Herrero M, de Lorenzo V, Timmis KN. Transposon vectors containing non-antibiotic resistance
307 selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative
308 bacteria. Journal of bacteriology. 1990;172:6557-67.

309 [32] Miller VL, Mekalanos JJ. A novel suicide vector and its use in construction of insertion mutations:
310 osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires
311 toxR. Journal of bacteriology. 1988;170:2575-83.

312 [33] Dozois CM, Daigle F, Curtiss R, 3rd. Identification of pathogen-specific and conserved genes
313 expressed in vivo by an avian pathogenic *Escherichia coli* strain. Proceedings of the National
314 Academy of Sciences of the United States of America. 2003;100:247-52.

315

Figure legends

Fig. 1. Organization of the Tol operon in the *E. ictaluri* genome. Arrows indicate the direction of transcription, and numbers at the beginning and the end indicate genomic coordinates of the *tol* operon.

Fig. 2. Cumulative percent mortalities of channel catfish fingerlings experimentally infected with the *Ei* Δ *tolQ*, *Ei* Δ *tolR*, and *Ei* Δ *tolQR* mutants (A). Percent survival of channel catfish fingerlings experimentally infected with *Ei* Δ *tolQ*, *Ei* Δ *tolR*, and *Ei* Δ *tolQR* mutants and subsequently challenged with wild type *E. ictaluri* strain 93-146 (B).

326 **Table 1**

327 Bacterial strains and plasmids used in the present study.

Bacterial strain	Strains	Reference
<i>Edwardsiella ictaluri</i>		
93–146	Wild type; pEI1 ⁺ ; pEI2 ⁺ ; Col ^r	[30]
<i>EiΔtolQ</i>	93–146 derivative; pEI1 ⁺ ; pEI2 ⁺ ; Col ^r	This study
<i>EiΔtolR</i>	93–146 derivative; pEI1 ⁺ ; pEI2 ⁺ ; Col ^r <i>ΔtolR</i>	This study
<i>EiΔtolQR</i>	93–146 derivative; pEI1 ⁺ ; pEI2 ⁺ ; Col ^r : <i>ΔtolQR</i>	This study
<i>Escherichia coli</i>		
CC118λpir	<i>Δ(ara-leu); araD; ΔlacX74; galE; galK; phoA20; thi-1; rpsE; rpoB; argE(Am); recA; λpirR6K</i>	[31]
SM10λpir	<i>thi; thr; leu; tonA; lacY; supE; recA; ::RP4-2-Tc::Mu; Kmr; λpirR6K</i>	[32]
Plasmids		
pMEG-375	8142 bp, Amp ^r , Cm ^r , <i>lacZ</i> , R6K <i>ori</i> , <i>mob incP</i> , <i>sacR sacB</i>	[33]
p <i>EiΔtolQ</i>	10354 bp, pMEG-375, <i>ΔtolQ</i>	This study
p <i>EiΔtolR</i>	10300 bp, pMEG-375, <i>ΔtolR</i>	This study
p <i>EiΔtolQR</i>	10313 bp, pMEG-375, <i>ΔtolQR</i>	This study

328

329 **Table 2**

330 Primers used to generate and verify in-frame deletion of the *E. ictaluri tolQ*, *tolR*, and *tolQR*
 331 genes.

Primers		Sequence (5'→3') ^b	RE ^a
EitolQF01	A	AAG AGCT CCCTGGATTTTAGGAACGCTTA	<i>SacI</i>
EitolQR48	B	CAGCAGACTTGCTTTCAGGA	
EitolQF630	C	<u>TCCTGAAAGCAAGTCTGCTGGAAGAGTTCATCGCCATTCTG</u>	
EitolQR01	D	AACCC GGG CTCGGCCTGTTTCTTAGCTTC	<i>SmaI</i>
EitolQF01S		CTGAAGGCTTGGTTCTCAGTC	
EitolRF01	A	AAG AGCT CACGCTACATCGCCTTTTATGA	<i>SacI</i>
EitolRR48	B	GATGTTGATTTCGACTTGAGA	
EitolRF390	C	<u>TCTCAAGTCGGAAATCAACATCACCTCCGTGGGACTGATGAC</u>	
EitolRR01	D	AACCC GGG CATAGCCGCTAATTCTGCAC	<i>SmaI</i>
EitolRF01S		GGGTGAATAAGCTGGAGCAGA	

332 ^aRE stands for restriction enzyme added to the 5' end of the primer sequence.

333 ^bBold letters at the 5' end of the primer sequence represent RE site added. AA nucleotides were
 334 added to the end of each primer containing a RE site. Underlined bases in primer C indicate
 335 reverse complemented primer B sequences.

336 Fig. 1.

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338