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Identification and Characterization of the Replication Region of Virulence Plasmid pEIB202 in *Edwardsiella piscicida*

Xinyue Chang[1], Chengli Teng[1], Haizhen Wu[1,2]*, Jiang Ye[1], Qiyao Wang[1], and Huizhan Zhang[1,2]

[1]*State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, P.R. China*
[2]*Department of Applied Biology, East China University of Science and Technology, Shanghai, P.R. China*

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**Corresponding author*
Phone: +86-21-64252507;
Fax: +86-21-64252515;
E-mail: wuhzh@ecust.edu.cn

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Edwardsiella piscicida is the causative agent of edwardsiellosis, which has caused enormous economic losses worldwide. In our previous research, an attenuated live vaccine known as WED and based on the virulent strain *E. piscicida* EIB202 can effectively protect turbots against edwardsiellosis via intraperitoneal injection, while vaccination by immersion exhibits a weaker effect. During the development of the immersion vaccine, we surprisingly found the counts of ΔpEIB202/ EIB202 colonized on zebrafish were 100 times lower than those of EIB202. However, pEIB202 carries 53 predicted ORFs and has several copies in *E. piscicida* EIB202, impeding the study of its function. Thus, the replication region is located at a 1,980 bp fragment (from 18,837 to 20,816 bp), containing a transcriptional repressor and a replication protein. Moreover, the minimal replication plasmid, named pRep-q77, has low copies in both *E. coli* and *E. piscicida*, but is more stable in *E. piscicida* than in *E. coli*. This work lays a foundation for further examination of the function of the virulence plasmid pEIB202.

Keywords: *Edwardsialla piscicida*, pEIB202, replication region, location, characterization

Introduction

Edwardsiella piscicida, a Gram-negative, short rod-shaped, motile, facultatively anaerobic bacterium [1], can cause edwarsiellosis characterized by symptoms of petechial hemorrhage, bloody ascites, exophthalmia, rectal hernia, and congested organs [2]. It has infected an enormous range of hosts, including fish, amphibians, reptiles, birds, and mammals [3]. Moreover, *E. piscicida* has been recognized to induce systematic disease in over 20 valuable farmed fish species, resulting in tremendous economic losses worldwide [4].

With the significant growth of aquaculture during the past five decades, the application of antibiotics has also increased dramatically, which induces drug resistance in bacteria or suppresses the immune system of host [5]. In addition, *E. piscicida* is insensitive to a majority of antibiotics since it is an intracellular pathogen. In this case, developing vaccines is an effective way to protect fish against edwarsiellosis. Several vaccines against various virulence factors including flagella [6], invasin [7], catalase [8], and two-component systems [9, 10] have been developed. Previously, we developed WED, a live attenuated vaccine, with deletion of *aroC*, TTSS, and pEIB202 of *E. piscicida* EIB202. It was found that the immunoprotective effect via immersion was lower than that of injection vaccination [11]. But since immersion is a labor-and-time-efficient method, improving the effectiveness of immersion vaccination is of significance to commercial application of this vaccine. Much work has been done on the development of immersion vaccines. And we surprisingly found the plasmid pEIB202 (43,703 bp) in *E. piscicida* EIB202 could play an important role in its colonization on zebrafish.

Plasmid pEIB202 is an endogenous multi-drug resistance plasmid (GenBank Accession No. CP001136) in *E. piscicida* EIB202 [12]. The plasmid carries 53 ORFs, including antibiotic resistance genes, conjugation genes, and an incomplete set of a type IV secretion system. It also has more than one copy in EIB202 (not shown by data), hindering the deletion of genes on plasmid and examination of the function of this virulence plasmid. Even though the replication initiator protein was predicted [12], the exact locus of the replicon

Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Characteristics	Source
Strains		
E. coli JM83	F⁻, *ara*, Δ(*lac-pro*AB), *rps*L,(Str^r), ϕ80, Δ(*lacZ*), M15	Our lab
E. piscicida EIB202	Col^r, Cm^r, wild type	Our lab [13]
ΔpEIB202/EIB202	Col^r, pEIB202 curing derivative of EIB202	Our lab
WED	A live attenuated vaccine with deletion of *aroC*, TTSS, and pEIB202 of *E. piscicida* EIB202	Our lab [11]
Plasmids		
pEIB202	Endogenous plasmid in *E. piscicida* EIB202	Our lab [12]
pRep 33	*Sau3A* I partially digested fragment, located at 18,249~21,335 bp in pEIB202, ligated with an 892 bp DNA fragment (KCm^r) containing Cm resistance gene.	This work
pRep 23	*Sau3A* I partially digested fragment, located at 17,961~21,335 bp in pEIB202, ligated with an 892 bp DNA fragment (KCm^r) containing Cm resistance gene.	This work
pRep 19	*Sau3A* I partially digested fragment, located at 18,134~21,602 bp in pEIB202, ligated with an 892 bp DNA fragment (KCm^r) containing Cm resistance gene.	This work
pRep 13	*Sau3A* I partially digested fragment, located at 17,123~21,602 bp in pEIB202, ligated with an 892 bp DNA fragment (KCm^r) containing Cm resistance gene.	This work
pRep-q77	Plasmid with minimal replicon, located at 18,837~20,816 bp in pEIB202, ligated with an 892 bp DNA fragment (KCm^r) containing Cm resistance gene.	This work
pDMK	Km^r, Cm^r, pDM4 derivative, suicide vector, R6K, *pir* requiring	Our lab [14]

has not been identified. The understanding of pEIB202 would be facilitated by adding specific genes to the replicon instead of deleting genes on plasmid. To this end, we successfully located and characterized the replication region of pEIB202, which lays a foundation for studying the virulent function of pEIB202.

Materials and Methods

Strains, Plasmids and Growth Conditions

Strains, plasmids, and primers used in this study are listed in Tables 1 and 2. *E. piscicida* strains were cultivated in Luria broth (LB) or LB with 1.8% agar at 28°C. *E. coli* strains were cultivated in LB or LB with 1.8% agar at 37°C. When required, chloramphenicol (Cm) was added at 30 μg/ml right after inoculation.

Plasmid DNA Isolation

The isolation of plasmid pEIB202 from *E. piscicida* EIB202 was performed as described by Xi X *et al.* with some adjustments [15]. Strains were grown overnight and then collected by centrifugation and lysed in Buffer I (50 mM glucose, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Then lysozyme (final concentration: 5 mg/ml) was added, after which the mixture was incubated at 37°C for 30 min. Buffer II was then added and incubated at 37°C until the solution was clear, followed by addition of Buffer III and an ice-bath for 30 min. A plasmid extraction kit (GK2002-200; Generay, China) was used to isolate plasmid DNA from *E. coli*. DNA products were stored at −20°C.

E. piscicida Electroporation

To transform plasmid to *E. piscidida* ΔpEIB202/EIB202, electroporation was carried out with glass cuvettes. Briefly, the electrocompetent bacteria were ice-bathed till glycerol melted and plasmid DNA was added and mixed evenly. Then the bacteria-DNA mixture was transferred to glass cuvette and the outside was kept dry. The cuvette was placed in an electroporator (MicroPulser, Bio-Rad, USA) and a 3 kV, 6 ms pulse was performed. Afterwards, 1 ml of pre-warmed LB media was pipetted into the cuvette immediately and mixed gently. Next, the bacteria were cultured at

Table 2. Primers used in this study.

Primer name	Nucleotide sequence (5′-3′)
Rep q77-F	CCCAAGCTTAATCAAAACACCCCATCTT
Rep q77-R	CGCGGATCCTGAGGCTTCGCCATTAGTAATAGGG
KCm-F	CGCGGATCCCGCACTTTGCGCCG
KCm-R	CGCGGATCCTTACGCCCCGCCCTGCCAC
Cm30	ATTTATTCTGCGAAGTGATCTTCCG
Cm840	TTCCATGTCGGCAGAATGCTTAATG
gap-F	AACATCATCCCGTCCTCTAC
gap-R	CCATACCAGTCAGTTTGCCA
hlyB-F	GCCTATTTCGTCTTTCCTAA
hlyB-R	ACATCGCTATCCATCCGTCT
rep-F	AGCTGCCACCGCTGTACAC
rep-R	CTTCCTGTCACGCTCCCTA

28°C with 190 rpm shaking for 2 h, followed by plating the transformed bacteria to LB agar containing proper antibiotics.

DNA Sequencing and Analysis

Partially digested pEIB202 by *Sau3A* I were ligated to an 892 bp fragment encoding chloramphenicol resistance, which was amplified by KCm-F/R primers on pDMK. The ligation products were then transformed into *E. coli* JM83. The locations of subclones were determined by aligning the sequence of each subclone with pEIB202. The sequencing was performed with Cm30/Cm480 primers on KCm^r fragment. Sequence analysis was performed by using the NCBI database or the DNASTAR (Lasergene.v 7.1, DNASTAR Inc) software. The phylogenetic NJ tree of RepA protein from pEIB202 was formed by clustering the top 15 hits after BLASTP similarity searches at Genbank. Then, the relationships were constructed by MEGA 7.

Determination of Plasmids Copy Number with qPCR Assays

The copy number of the derivates of pEIB202 in *E. coli* and *E. piscicida* was measured by qPCR, as described previously [16]. A Bio-Rad CFX96 Real-time PCR System (Bio-Rad) was used for qPCR amplification and detection. The qPCR assay was performed in a 20 µl reaction system in triplicate, with 10 µl SYBR Green Real-Time PCR Master Mix (TOYOBO, Japan), 2 µl Plus solution (TOYOBO), 1 µl forward primer, 1 µl reverse primer, 1 µl template and 5 µl ddH_2O. Cultured *E. coli* and *E. piscicida* were added as template. PCR primers were designed by Primer Premier 5.0 software. The glyceraldehyde-3-phosphate dehydrogenase gene *gap*, a single-copy gene in the genome of *E. coli*, and hemolysin activator HlyB domain gene *hlyB* [17], single-copy in *E. piscicida*, were used as the reference genes. The *rep*-F/R were designed for detection of pEIB202 derivates. The copy counts of plasmids were calculated as follows: $N = 2^{-\Delta Ct}$. ΔCt represented the difference between the threshold cycle counts (Ct) of the reference gene and the *rep* reaction.

Assessment of Plasmid Stability

The stability of plasmid pRep-q77 containing the proposed minimal replication region in *E. coli* and *E. piscicida* were assessed as follows: *E. coli* and *E. piscicida* with pRep-q77 were inoculated in LB (Cm^+) and grown for 12 h and 24 h respectively. Then, the bacterial suspension was taken to antibiotic-free LB at 1% (v/v) ratio. The *E. coli* cells were collected at 0, 3, 6, 9, 12, 20, 24 h, and *E. piscicida* cells were collected at 0, 8, 16, 24 h after inoculation. Harvested *E. coli* and *E. piscicida* were then serially diluted and inoculated on plates with and without Cm. The curves describing the plasmid loss were drawn as the ratio of counts of colonies on antibiotic-containing plates to antibiotic-free plates.

Immersion Infection of Zebrafish, Sampling and qPCR

E. piscicida EIB202, ΔpEIB202/EIB202 and WED were processed as described previously with modifications [18]. The bacteria were harvested, washed and then suspended with sterile PBS at 10^8 CFU/ml. *E. piscicida*-free zebrafish were bath-infected with bacterial suspension for 30 min, then washed with clean water, and returned to original tanks. Intestines, skin, and gills of ten zebrafish were pooled and weighed at 0, 3, 6, 12, 24, 48, 72, 120, 168 h post infection. Total DNA from zebrafish tissue was extracted by using a TIANamp Marine Animals DNA Kit (DP324; TIANGEN, China). Quantitative PCR assays were performed as described previously [19]. Colonization on tissues was determined by bacterial load per gram tissue.

Results

Role of pEIB202 in Zebrafish Colonization

We previously developed a live attenuated vaccine called WED, which can effectively protect turbots against edwardsiellosis via intraperitoneal injection. However, the relative percent survival (RPS) in the turbot bath vaccinated with WED was lower than that of vaccination via intraperitoneal injection, which exerted an adverse impact on the wide use of the vaccine. So much work has been done on the development of immersion vaccines, including analysis of the counts of bacteria colonized on

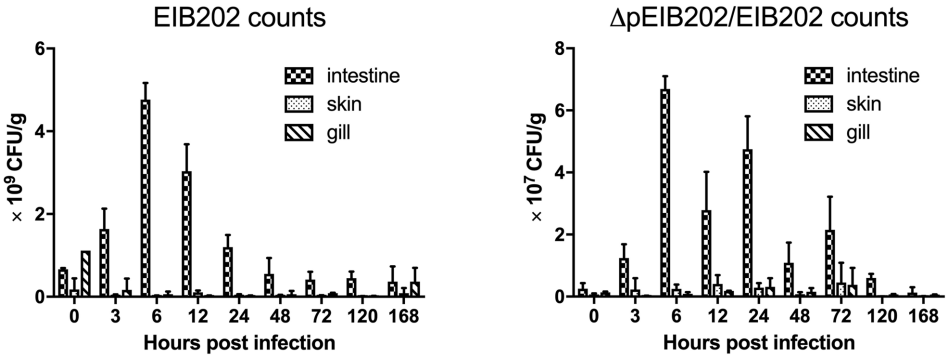


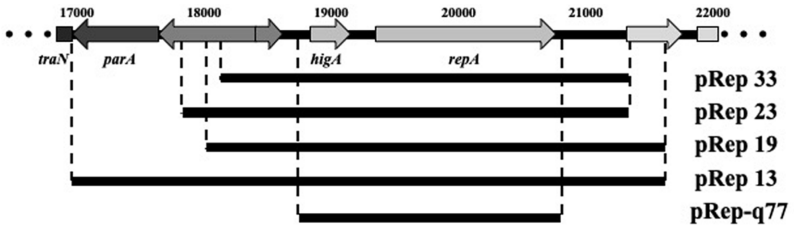
Fig. 1. The counts of *Edwardsiella piscicida* colonized on intestine, skin, and gills of zebrafish after exposure to 10^8 CFU/ml bacteria. Total DNA of each tissue from ten zebrafish was extracted for qPCR analysis. Bars represented the mean counts of three biological replicates and the error bars represented standard deviations.

zebrafish by qPCR. Mucosal tissues were obtained after zebrafish bath-infection or bath-vaccination with 10^8 CFU/ml bacteria. qPCR was performed using *E. piscicida* specific primers. The result shows that *E. piscicida* mainly colonize on intestines, which suggests intestine is the main route for *E. piscicida* infection. The counts of *E. piscicida* on the intestine peaked at 6 h post infection, and then decreased to the level at initial infection. The counts using WED are too low to be detected (data not shown). Then we tested the ΔpEIB202 strain, which colonizes on the intestine at a level 100 times lower than that of *E. piscicida* EIB202 during a week post infection (Fig. 1). These results indicate that pEIB202 may play an important role in zebrafish colonization.

Location of the Replicon of pEIB202

Based on the whole sequence of pEIB202 submitted to GenBank (Accession No. CP001136) [12], the plasmid carries 53 predicted ORFs, which confers the multiple functions, of the bacteria such as antibiotic resistance, conjugation, and transposon. Furthermore, pEIB202 encodes an incomplete type IV secretion system, which is involved in horizontal DNA transfer. There is more than one copy of pEIB202 in *E. piscicida* EIB202, but the origin of replication has not been elucidated. It is necessary to determine the replicon of pEIB202 for characterizing the virulent plasmid. To this end, *Sau3A* I partial digestion products were screened: 43 transformants were obtained and sequenced, among which the No. 33 transformant contained the minimal *Sau3A* I DNA fragment of pRep33 (from 18,249 to 21,335 bp). Subsequently, PCR was performed as shown in Fig. 2 and 5 fragments were succeeded. A shorter fragment has been though it unfortunately failed to replicate in *E. coli* JM83. Therefore, we located the minimal replication origin from 18,837 to 20,816 bp, and named this subclone pRep-q77/JM83.

Copy counts of these subclones were measured through qPCR (Fig. 2). Results showed that these plasmids have low copy counts in ΔpEIB202/EIB202 and JM83. Copy counts in JM83 are lower than those in ΔpEIB202/EIB202, which may be attributed to the uncoordinated operation of the replication apparatus between pEIB202 and *E. coli*. The relative copy counts of pRep33 (36) and pRep19 (4.7) are the highest among five subclones in ΔpEIB202/EIB202 and JM83 respectively. Interestingly, pRep13, pRep19, and pRep23 contain the same fragment as pRep33, while having lower copy numbers than pRep33. The differences among them have been carefully checked and a fragment ranging from 18,134 to 18,249 has been found missing in pRep33. In this 116 bp fragment, there are 2 different direct repeats shown in a supplementary Fig., which may regulate the copy number of the subclone. Direct repeats participate in plasmid copy number controlling, by means of cooperation with initiation protein. Moreover the mechanisms of direct repeats to negatively regulate plasmid copy number have been elucidated, one of which is a titration model [20]. Briefly, the higher concentration of direct repeats leads to fewer plasmid copies, due to the competition for a finite amount of initiation protein between parent and daughter plasmid copies. Here, we propose that the high copy number of pRep33 is due to the absence of those two direct repeats, which present in pRep23, pRep19, and pRep13. In conclusion, there are several differences between replication mechanisms involved in ΔpEIB202/EIB202 and JM83.



Subclone	Location in pEIB202	Size (bp)	Copy number in ΔpEIB202/EIB202	Copy number in JM83
pRep 33	18249~21335	3086	36.03	4.48
pRep 23	17961~21335	3374	6.86	3.60
pRep 19	18134~20602	3469	7.56	4.74
pRep 13	17123~21602	4479	8.50	2.14
pRep-q77	18837~20816	1980	7.86	1.55

Fig. 2. Relative locations, sizes and copy numbers both in ΔpEIB202/ *E. piscicida* EIB202 and *E. coli* JM83 of the 5 subclones.

Fig. 3. Sequence of the origin of replication of pEIB202.

The numbers refer to the plasmid position. Inverted repeats (IR) are underlined and shadowed and palindromes are underlined with curved lines. Direct repeats (DR) are double underlined. Also the AT-rich region is boxed. The putative ORFs are shown in gray color, and sequences between 18918 and 19229 bp are predicted to be the transcriptional regulator, while sequences after 19414 encode the putative replication protein.

Sequence Features of Minimal Replication Origin

The minimal replication origin in plasmid pRep-q77 is located between 18,837 and 20,816 bp, containing a transcriptional repressor encoding gene and *repA* gene. Inverted repeats, direct repeats, and palindrome sequences in this region were screened by DNASTAR (Fig. 3), and an AT-rich region was predicted by mEMBOSS. The direct repeats are viewed as the binding sites for replication protein [21] and play a part in plasmid incompatibility [22], while the inverted repeats are regarded as the interaction region with the HTH motif [23] of replication protein dimer. The AT-rich region is agreed to be an initial part of replication due to poor stability [24].

The transcriptional repressor, consisting of 103 aa, shares

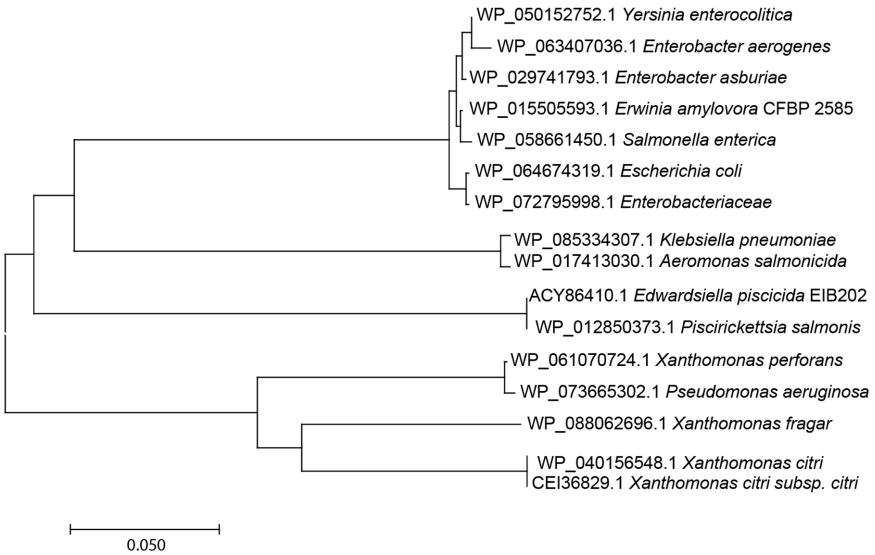


Fig. 4. The phylogenetic tree is constructed with the MEGA 7 using proteins obtained from the Genbank protein database.

Top 15 hits for RepA protein sequences from pEIB202 after BLASTP similarity searches are clustered, and the relationships among the proteins are determined. The bar indicates the number of amino acid substitutions per site.

high amino acid sequence similarity to the putative antidote protein of the plasmid maintenance system (ZP_00107635.1) from *Nostoc punctiforme* PCC 73102 (identity: 39%, coverage: 6 ~ 98 aa). It belongs to the antidote protein HigA family, which forms a distinct group of helix-turn-helix proteins. The HigB/HigA toxin-antitoxin system of *E. coli* CFT073 participates in many physiological activities, including cell growth and maintenance of plasmid DNA [25]. HigB is a sequence-specific endoribonuclease, which contacts to the 50S ribosomal subunit. The antitoxin HigA shows an interrelated format of shuffling with altered antitoxin folds to neutralize toxins. Thus, we believe that the transcriptional repressor is likely to contribute to plasmid maintenance.

RepA constitutes 454 aa, among which there is a RepB primase domain between 130 and 220 aa. The conserved motif between 361 and 432 aa shows 31% identity with the HTH in the C termini of ColE2-P9 Rep. Notably, ColE2-P9 Rep protein is reported as a single protein for DNA unwinding [26], and the HTH module exclusively binds to *Ori*, which is critical for Rep to anchor on the DNA [27]. Hence, the RepA of pEIB202 is able to unwind the plasmid duplex DNA independently. Besides, the amino acid sequence shows 100% identity with replication proteins of *Piscirickettsia salmonis* AY6532B, AY3800B, AY6297, and AY3864B (100% coverage). Furthermore, RepA presents high similarity with the replication proteins of *Yersinia enterocolitica*, *Erwinia amylovora* CFBP, *Salmonella enterica*, and *E. coli* (identity: 73%, coverage: 98%). The phylogenetic NJ tree based on RepA is shown in Fig. 4. RepA exhibits a close evolution relationship with hypothetical replication proteins of the pathogenic bacteria, indicating the transfer/evolution traces of pEIB202.

Stability of pRep-q77

The stability of pRep-q77 in *E. coli* JM83 and ΔpEIB202 EIB202 is assessed. After 24-hour antibiotics-free culturing, 73% ΔpEIB202 EIB202 carried the plasmid pRep-q77 and only 25% *E. coli* JM83 carried plasmid pRep-q77 (Fig. 5). The result proves that plasmid pRep-q77 is more stable in *E. piscicida* than in *E. coli*.

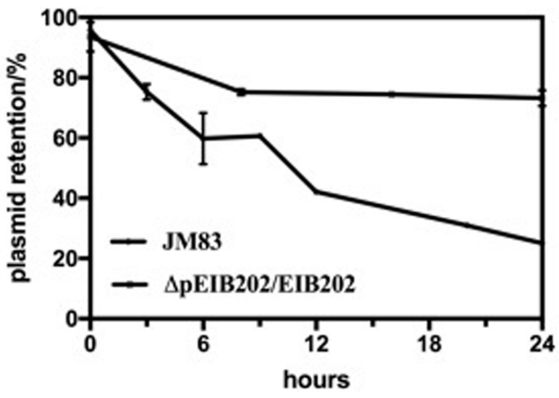


Fig. 5. The plasmid loss rate of pRep-q77 in ΔpEIB202/*E. piscicida* EIB202 and *E. coli* JM83 during 24 h culturing.

Discussion

In this study, the minimal replication region of the virulence plasmid pEIB202 is located at a 1,980 bp DNA fragment ranged from 18,837 bp to 20,816 bp and characterized in *E. piscicida*.

Mucosal tissues (intestines, skin and gills) of fish are possible accesses for pathogen invasion [28]. Previous study reported that the effectiveness of bath vaccination is related to the amount of antigen uptake [29]. To elucidate genetic factors in *E. piscicida* EIB202 contributing to the immunoprotection of WED via immersion, we measured the bacteria loads on main mucosal tissues of zebrafish after bath vaccination via qPCR. According to the results, pathogen colonized on the intestine exhibit a significant proliferation at 6 h post infection, after which bacteria are cleared by the host. The wild-type *E. piscicida* EIB202 is proved to colonize on the intestine at a level 100 times higher than ΔpEIB202/EIB202, suggesting the plasmid may play a vital role in colonization. However, the plasmid has several copies in *E. piscicida* EIB202, impeding further study about its function due to the difficulty of deleting specific genes on pEIB202. Thus, it is necessary to figure out the replication region of pEIB202, although the replicon of pEIB202 was predicted by bioinformatics [12]. Here, the replication region is found to be located in a 1,980 bp region, containing the replication protein RepA and a transcriptional repressor encoding gene. Generally, the replication by the theta type mechanism is more common in gram-negative bacteria. Furthermore, the plasmid-encoded replication protein Rep is required [21], implying that pEIB202 exerts the theta mechanism. In addition to Rep protein, general features like an adjacent AT-rich region containing sequence repeats, the *dnaA* boxes for host DnaA initiator protein binding [30], and the Dam methylation sequences [31] are found in numerous theta-replicating plasmids' original region. Methylation may help to improve the efficiency of replication initiation both in vivo and in vitro. Though it is unnecessary for

replication, it primarily matters in post-replication [32]. Furthermore, it was reported that methylation sites in the replicon of plasmid P1 are required to sequester hemi-methylated *oriC* into the membrane [33]. The stimulation can result from the increased twist or unwinding of the DNA or accumulated recycling of initiators [34]. This may explain the differences of the copy counts between *E. coli* and *E. piscicida*. As the sequence exhibits subtypical features, such as no typical *E. coli dnaA* boxes [35] and methylation sequences or few inverted repeats, the novel replicon is difficult to predict. Also, *E. piscicida* specific replication features should be noted.

The RepA shows a close relationship with replication protein of various pathogens including aquatic gamma-proteobacteria pathogen *P. salmonis*. In addition, the plasmid carries genes encoding DNA transfer proteins, implying that the plasmid is able to transfer among different species and confer them multidrug resistance. Maintenance and replication of plasmid may induce a "metabolic burden" in *E. coli*. It is clarified as the amount of resources (raw material and energy) that are obtained from the host cell metabolism [36]. In *E. coli* DH5α, gene *gapA* encoding glyceraldehyde-phosphate dehydrogenase (GAPDH) in the glycolytic pathway is observed to be up-regulated in plasmid-bearing cells [37]. The expression of *fba* gene (encoding fructose bisphophate aldolase) in *E. coli* BL21(DE3) is increased as well [38]. Those demonstrate that house keeping genes for energy metabolism are likely to up-regulate due to the plasmid introduction, which can be attributed to "metabolic burden" brought from plasmid. In addition, GAPDH and FBA are found at the non-cytoplasmic parts, and present protection against several pathogens in turbots [39, 40], suggesting these house keeping genes act as virulent factors when secreted. This suggests that pEIB202 may confer up-regulation of some genes, such as GAPDH and FBA in *E. piscicida* EIB202, hence its potential virulent-related effects on *E. piscicida* EIB202.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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