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MarTrack: A versatile toolbox of *mariner* transposon derivatives used for functional genetic analysis of bacterial genomes

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ABSTRACT

The mariner transposon family of *Himar1* has been widely used for the random mutagenesis of bacteria to generate single insertions into the chromosomes. Here, a versatile toolbox of mariner transposon derivatives was generated and applied to the functional genomics investigation of fish pathogen *Edwardsiella piscicida*. In this study, we combined the merits of the random mutagenesis of mariner transposon and common efficient reporter marker genes or regulatory elements, *mcherry*, *gfp*, *cAB*, *lacZ*, *sacBR*, and P_{BAD} and antibiotic resistance cassettes to construct a series of derivative transposon vectors, p*Mmch*, p*MKGR*, p*MCGR*, p*MXKGR*, p*MLKGR*, p*MSGR*, and p*MPR*, based on the improved transposon p*Mar2xT7*. The function and effectiveness of the modified transposons were verified by introduction into *E. piscicida* EIB202. Based on the toolbox, a transposon insertion mutant library containing approximately 3.0×10^5 separated mutants was constructed to explore the upstream regulators of *rB*, the master regulator of the type III and type VI secretion systems (T3/T6SS) in *E. piscicida*. Following analysis by arbitrary PCR, ETAE_2184 (renamed as EsrR) was screened out and identified as a novel regulator mediating T3SS expression. In addition, the *esrR* mutants displayed critical virulence attenuation. Due to the broad-range host compatibility of mariner transposons, the newly built transposon toolbox can be broadly applied for functional genomics studies in various bacteria.

1. Introduction

Transposon-mediated mutagenesis is a powerful tool for functional genomics studies in the post-genomic era (Fescheet al., 2008). During the process of transposition, the chromosome is excised by a transposase, followed by the integration of the transposon into the cutting site (Serrato-Capuchina and Matic, 2018). Transposons are categorized into two families depending on transposition behavior: (1) replicate and paste; (2) cut and paste. The 'cut and paste' transposition mode only produces one insertion and is useful for mutagenesis applications. The random insertion transposons, including the mariner, Mu and Tn families, are widely applied in generating mutant libraries (Jacobs et al., 2003; Liberati et al., 2006; Gallagher et al., 2007; Cameron et al., 2008; Gallagher et al., 2011), genetic footprinting (Smith et al., 1995), transcriptional fusion (Pozsgai et al., 2012), and signature-tagged mutagenesis (Grant et al., 2005). Based on the traditional transposons, mini-transposons are created in which the naturally occurring transposase genes have been rearranged artificially outside of the transposons to originate shorter mobile elements. The modified transposons carrying reporter genes and promoter elements are also useful for the random generation of transcriptional or translational fusions, which has been verified as an efficient approach to determine gene expression levels and essentiality under different conditions (Pozsgai et al., 2012).

Himar1, a mariner family element isolated from the horn fly *Haematobia irritans* (Lampe et al., 1996; Lampe et al., 1998), can efficiently mediate *in vivo* and *in vitro* transposition in bacteria (Picardeau, 2010). The *Himar1* transposon has been demonstrated to have little host or targeted DNA site specificity beyond the known requirement for the dinucleotide TA (Liu et al., 2007). *Himar1*-derived mini-transposons, such as the typical and widely used Mar2xT7 (Liberati et al., 2006; Menanteau-Ledouble and Lawrence, 2013), made up of short inverted repeats flanking antibiotic resistance markers, delivered by suicide vector plasmid, have been constructed for random mutagenesis in various bacteria and have been successfully used in creating large mutant libraries in various bacteria with efficient, random, and unbiased insertions in the chromosome.

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Whereas the *Himar1* transposon Mar2xT7 is an excellent transposon for random insertional mutagenesis, it is not without limitations. First, the Mar2xT7 is limited to generating gentamicin resistance-marked insertions (Liberati et al., 2006). Second, the Mar2xT7 does not allow monitoring of bacterial mutant viability in real time; although bioluminescence marker plasmids carrying *luxCDABE* or *gfp* should be co-transferred into the receptor bacterium to facilitate monitoring. Third, the Mar2xT7 is limited primarily to loss-of-function mutations, as random insertions commonly abolish gene activity while not allowing quantitative tracking of the expression levels of genes and promoters. Fourth, the transposon displays limited compatibility for next-generation sequencing-based transposon sequencing (Tn-seq) (Fu et al., 2013; Chao et al., 2016) or essential gene investigation in various bacterial contexts.

Edwardsiella piscicida is a broad host bacterial pathogen (Xiao et al., 2008) that mainly inhabits freshwater and marine animals, causing edwardsiellosis in over 20 piscine species, such as flatfish, eel and tilapia, resulting in large economic losses in the aquaculture industry worldwide (Leotta et al., 2009; Green, 2010; Park et al., 2012). *E. piscicida* is a facultative intracellular pathogen (Wang et al., 2011) and develops the capacity to resist killing by professional phagocytes and to colonize and replicate in macrophages (Okuda et al., 2009; Hou et al., 2016; Zhang et al., 2016). In our former study, sequencing of the complete genome of a highly virulent *E. piscicida* strain EIB202 was accomplished (Wang et al., 2009). The genomics study revealed that *E. piscicida* harbors the type III and type VI secretion systems (T3SS and T6SS) (Tan et al., 2002; Srinivasa Rao et al., 2003; Zheng et al., 2005; Leung et al., 2012; Lv et al., 2012), which comprise the core ammunition for *E. piscicida* to invade host cells (Okuda et al., 2009). *E. piscicida* is phylogenetically close to other model enteric pathogens, such as pathogenic *Escherichia coli*, *Shigella* and *Salmonella* species et al., 2012). *E. piscicida* pathogenesis involves T3/T6SS, quorum sing, two-component systems, and exoenzymes (*e.g.*, hemolysin chondroitinase), which make *E. piscicida* a model organism for the stu of gastrointestinal infection and secretion systems (2012). The complex virulence regulatory networks and rewiri of those networks during host invasion processes are s nclear bacterium.

In this study, we aimed to combine nsposons efficient reporter genes. To generate a toolbox fo udy of bacte functional genomics, a series of transposon de ative e constructed based on *mariner* transposon *Mar2xT7*. T newly bu nsposon derivatives were applied for tracking h y expressed ge regions and essential genes in *E. piscicida* 202. T results demonstrated that these transposons are efficie d flex and can be further used in any transposon-tolerant bacte g the c ructed transposons, some attenuated mutan e dis red, i uding ETAE_2184 (renamed EsrR), demons ing utility plasmid toolbox in any mariner transposon patible cteria.

2. Materials an thods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are detailed in Table 1. *Escherichia coli* SM10 *λpir* was used as the donor strain in conjugations for transferring the transposon vectors into the host strain, *E. piscicida* EIB202. *E. coli* and *E. piscicida* were grown in Luria-Bertani broth (LB, Oxoid, Hampshire, England) for routine incubation. Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) was used to induce T3SS expression. When required, antibiotics were supplemented at the following concentrations: gentamicin (Gm; 15 μg/ml), colistin (Col; 20 μg/ml), ampicillin (Amp; 50 μg/ml), kanamycin (Kan; 50 μg/ml), and chloramphenicol (Cm; 25 μg/ml).

2.2. Construction of pMar2xT7 derivatives

The transposon derivatives were constructed based on p*Mar2xT7* containing *mariner* transposase outside the *mariner* transposon. The functional genes were amplified from various plasmids (Table 1). The first derivative, p*Mmch,* was constructed as follows. The *mcherry* gene with constitutive promoter P_{tetA} was PCR-amplified from pTM267 (Table 2) using the primers PTT1 and PTT2 (Table 2), with a *Dra*III restriction site and protection nucleotides. The PCR products were digested with *Dra*III restriction endonuclease (NEB, England), followed by ligation into the *Dra*III site in the p*Mar2xT7* transposon. The constructed vector was transformed into *E. coli* CC118 *λpir*. The construct was validated by PCR and sequencing.

p*MKGR* was constructed based on *mch*. The moterless kanamycin resistance (Kan^r) cassette an with ribos al binding site (RBS) were amplified from pTM267 (1) using primers PKGF and PKGR. The RBS sequen sed in t study as "AAGGAGG", which originated from the *piscicida* 16S r terminal conserved sequence "CCUCCUU". *Nhe*I re tion site and triple terminal sites "TGACTAGCTAA" w e i d into t primer PKGF, and PKGR contains an *Nhe*I and a p T7 minator sequence. The PCR product was di d with *Nhe* lease and ligated with the digested p*Mm* 1 nstructed p*MKGR* was transformed into *E. coli* CC118 *λpir* and vali d by PCR and sequencing.

To ate p*MCGR* hloramphenicol resistance (Cm^r) cassette wa mplified using the primers 01-P1 and 02-P2 from plasmid p 267, and *egfp* was amplified from p*MKGR* using the primers 03-P3 a 04-P4. An N site, a triple terminator and an RBS site were in- ed into 0 1. The 04-P4 downstream of the T7 site contained the e chloramphenicol and *egfp* genes were amplified and linked by verlap PCR. The following procedure was same as for the truction of p*MKGR*. Functional validation was performed by cul- e conjugants on LB agar plates containing Col, Gm and Cm. Colonies were picked onto 96-well plates, and GFP and mCherry expression levels were confirmed using a fluorescence microplate reader.

The construction of p*MXKGR* was similar to that of p*MKGR*. The *luxAB* gene was cloned from *V. harveyi* using the primers 10-P1 and 11-P2. The promoterless *luxAB*, Kan^r cassette, and *egfp* genes with individual RBSs were inserted into the *Nhe*I site of p*Mmch*.

For p*MSGR*, *sacBR* was PCR-amplified from pDM4 using the primers 13-P1 and 14-P2, and *egfp* was PCR-amplified from p*MKGR*. The 13-P1 contains an *Nhe*I site, a triple terminator and an RBS sequence. The *sacBR* and *egfp* genes were linked by overlap PCR, and a combination start and stop codon ("ATGA" sequence) was added between them to enhance the transcription of *egfp*.

For p*MLKGR*, the promoterless *lacZ*, Kan^r cassette and *egfp* genes with individual RBSs were inserted into the *Nhe*I site of p*Mmch*. The *lacZ* gene was PCR-amplified from pDM5 using the primers 05-P1 and 08-P2. The Kan^R and *egfp* genes were PCR-amplified from p*MKGR* using the primers for Kan^R and *egfp*. For p*MPR*, the *araC* and promoter P_{BAD} were PCR-amplified from the vector pBAD-myc-His and inserted into the *Nhe*I site of p*Mmch*.

2.3. Mutagenesis by in vivo transposition

The plasmids of transposon derivatives were transformed into SM10 *λpir*, which served as the donor. The introduction of plasmids into *E. piscicida* was performed by conjugation. Briefly, 2 ml of recipient (OD_{600} = 0.6) and 1 ml of donor (OD_{600} = 0.6) were mixed with 15 μl of 10 mM $MgCl_2$ and dropped onto the surface of a filter film (0.22 μm diameter) on LB agar plates followed by incubation at 37 °C for 3 h. The matings were resuspended in LB broth and plated on selective medium with colistin (Col) and gentamicin (Gm) supplementation. For screening, different reagents were added according to each transposon. For p*MKGR* and *MXKGR*, different Kan concentrations of 0, 10, 25,

Table 1
Strains and plasmids used in the study.

Strains or plasmids	Genotype	References
Strains		
EIB202	Wild type strain, CCTCC M 208068, Col^r	Xiao et al. (2008)
Δ2184	EIB202, in-frame deletion of 2184	This study
Δ2184$^+$	EIB202, complementation of 2184	This study
2184::Tn	EIB202, transposon insertion mutant	This study
Δ*esrB*	EIB202, in-frame deletion of *esrB*	Lv et al. (2012)
Top10	Tn10 (TetR) *mcr* Φ180 *lacZ* Δm15Δ*lac* X74 *deoR recA*	Invitrogen
CC118 λ*pir*	λ*pir* lysogen Δ(*ara-leu*) *araD* Δ(*lacX74*) *phoA20 thi-1 rpoB argE* (*am*) *recA1*	Our lab
SM10 λ*pir*	*thi thr leu tonA lacy supE recA::RP4-2-Tc::Mu, pirR6K*, Kan^r	Our lab
Vibrio harveyi	Wild type strain, VIB647	Espinoza-Valles et al. (2015)
Plasmids		
pTM267	Promoterless-*egfp* reporter plasmid	Our l
pMD19-T	Gene cloning vector, Amp^r	TaKaRa
pDM4	Suicide plasmid, *pir* dependent, R6K, *sacBR*, Cm^r	Our lab
pDM5	promoterless *lacZ* gene, Cm^r Tc^r	Our lab
pBAD-myc-His	Protein expression vector harboring P_{BAD} promoter	Our la
pMar2×T7	λ*pir*-dependent ori, Amp^r, Gm^r	et al. (2006)
p*Mmch*	pMar2×T7 derivative with *mcherry* gene	study
p*MKGR*	p*Mmch* derivative harboring promoterless *Kan* and *egfp* gene	This study
p*MCGR*	p*Mmch* derivative harboring promoterless *Cm* and *egfp* gene	This study
p*MXKGR*	p*Mmch* derivative harboring promoterless *luxAB*, *kan* and *egfp* ge	This study
p*MSGR*	p*Mmch* derivative harboring promoterless *sacBR* and *egfp* gene	This study
p*MLKGR*	p*Mmch* derivative harboring promoterless *lacZ*, *kan* and *egfp* e	This study
p*MPR*	p*Mmch* derivative harboring outward promoter P_{BAD}	This study
P_{esrB}-*luxAB*	*esrB* promoter driven *luxAB* expression, Amp^r	Yin et al. (2018)

50 μg/ml were added. For p*MCGR*, 25 μg/ml of chloramphenicol was added. For p*MSGR*, p*MLKGR* and p*MPR*, selective medium with dual antibiotics was supplemented with sucrose (10%), X-gal (20 μg/ml) and arabinose (1% v/v).

2.4. Examination of fluorescence intensity

The GFP and mCherry fluorescence intensities were ied usi a fluorescence microplate reader (BioTek). The might ture o mutants was inoculated into a 96-well micro for in ation at 30 °C for 18 h. (200 μl LB in each well). The plate re t 3000 *g* at 4 °C for 5 min. The pellets we esuspe in 200 ml of sterile PBS and centrifuged 3 times. microliters esuspended bacteria was transferred to a new 9 k, clear-bo plate to examine the fluorescence intensity a fluor ce microplate reader. The excitation and emission w lengths were at 485 and 535 nm for GFP and 595 and 635 r mCherry, respec ely.

2.5. Measurement of lucif ession

For the tra p*MX* luminescence of the luciferase reaction wa uced b e additi of decanal to the bacterial culture. The overni cultur f transposon insertional mutants was inoculated into l micropate with 200 μl of LB broth in each well. Fifty microliters % decanal was added to the 200 μl of culture (final concentration 0.2%). At this concentration, the luminescence intensity rapidly rose to its peak and maintained the peak level for 10 min. Light emission was monitored in real time by microplate luminometry (Orion II, Titertek-Berthold).

2.6. Transposon insertion site mapping by two-round semiarbitrary PCR

Transposon insertion sites were identified by two-round arbitrary PCR (Liu and Chen, 2007). Primers are listed in Table 2. For the first round of semiarbitrary PCR, primers SP1 and a mixture of AB1, AB2 and AB3 were used. For each reaction, 1 μl of template (overnight cultured cells), 10 μl of 2× Taq-plus MasterMix (Venzyme) and 1 μl of each primer (10 μM) were mixed, and ddH_2O was added to a final volume of 20 μl. The PCR conditions were: (i) 94 °C for 3 min, (ii) 94 °C for 3 (iii) 42 °C f 30 s, slope −1 °C per cycle, (iv) 72 °C for 1 min, (v) g step ii, 6 ti s, (vi) 94 °C for 30 s, (vii) 58 °C for 30 s, (viii) 72 °C fo in, (ix) s o step vi, 25 times, (x) 72 °C for 3 min, (xi) 4 °C hold. For t ound of PCR, primers Sp2 and ABS were used. For each eaction, 1 μl of template (from first round PCR), 25 μl of 2× Taq-plus Mix (Venzyme) and 2.5 μl of each primer (10 μM) were mixed, and dH_2O was added to a final volume of 50 μl. The PCR conditions were: (i) 94 °C for 3 min, (ii) 94 °C for 30 s, (iii) 64 °C for 30 s, (iv) 72 °C for 1 min, (v) go to step ii, 30 times, (vi) 72 °C for 3 min, (vii) 4 °C hold. The PCR products were gel-purified and sequenced using primer Mar-seq2. The sequence adjacent to the transposon was mapped to the *E. piscicida* chromosome by BLAST (Altschul et al., 1997).

2.7. Transposon insertion sequencing (Tn-seq)

After the input libraries were subjected to a selective condition, the surviving population was outgrown for a limited number of generations in LB to achieve high cell density to eliminate the potential for DNA contamination from dead *E. piscicida* cells. The Tn-seq experiments and data analysis followed the protocols from (Pritchard et al. (2014). Briefly, the genomic DNA was extracted and fragmented by sonication. Then, the DNA fragments were subjected to end repair and A-tailing and were supplemented with adapters and P5, P7 sequences by two-round PCR to generate the sequencing libraries. The sequencing libraries of one input and two replicated outputs were applied to high-throughput sequencing on Illumina's MiSeq platform, and ~ 2 million reads were generated for each library. The sequencing results were processed with adapter trimming, mapping to the genome, and tallying to each locus of *E. piscicida*.

2.8. SDS-PAGE and Western blotting analysis

Whole cell proteins (WCPs) and extracellular proteins (ECPs) were extracted and concentrated as previously described (Lv et al., 2012). For both preparations, overnight cultures were subcultured into 50 ml of fresh DMEM and incubated for 24 h at 28 °C without shaking; bacteria were then harvested by centrifugation at 5000 × *g* for 10 min at 4 °C for WCPs. The supernatants were filtered with 0.22-μm filters (Millipore) and concentrated using 10-kDa cutoff centrifugal filter

Table 2
Primers used in this study.

Primers	Sequence (5'–3')
PTT1	GTACCACACCGTGCGGTACCGTTGACACTCTATC
PTT2	GTACCACGGTGTGCCGGTACCTTACTTGTACAGCTCGTCCATGCCGCCGGTCGAGTG
M13F	GTTTTCCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC
PMar2	TGGAACAACACTCAACCCT
PMar1	ATCGTCACCGTAATCTGCT
PtongF	GATTTTGGTCATCGGTGCG
PKGF	CTAGCTAGCTGACTAGCTAATCAAGAGACAGGATGAGGATCG
PKGR	CTAGCTAGCCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGGCGGCCAATTCATTATTTGTA
PGI	AGGTGATGCTACATACGGAAAG
01-P1	CTAGCTAGCTGACTAGCTAAGGAGGTATACATGGAGA
02-P2	CCTTCTTAAATCTAGAACGCTTACGCCCCGCCCTGCCACT
03-P3	AGTGGCAGGGCGGGGCGTAAGCGTTCTAGATTTAAGAAGG
04-P4	CTAGCTAGCCAAAAAACCCCTCAAGAC
P-Cm-IFPC	CTTGCCCGCCTGATGAATG
PmhI	AGCGCATGAACTCCTTGATG
10-P1	CTAGCTAGCTGACTAGCTAATACCAACAAATAAGGAAATG
11-P2	GATCCTCATCCTGTCTCTTGATTACGAGTGGTATTTGACGAT
12-P3	ATCGTCAAATACCACTCGTAATCAAGAGACAGGATGAGGATC
04-P4	CTAGCTAGCCAAAAAACCCCTCAAGAC
20-lux	GTAGATGGTGAAGCAGCAAGG
13-P1	CTAGCTAGCTGACTAGCTAAGGAGGTATACATGAACA[illegible]AAGTTTGC
14-P2	GAAAAGTTCTTCTCCTTTACTCATTTGTTAACTGTT[illegible]
15-P3	GGACAATTAACAGTTAACAAATGAGTAAAGGAGA[illegible]AACTT
19-SacBR	GCTCAGGCGACAACCAT
05-P1	CTAGCTAGCTGACTAGCTAAGGAGGTATA[illegible]CATGATTACGC
08-P2	GCAATCCATCTTGTTCAATCATTTTTGA[illegible]CAGACCAAC
09-P3	GTTGGTCTGGTGTCAAAAATGATTGA[illegible]AGATGGATTGC
21-lacZ	GTAAGTGAAGCGACCCGCATTG
16-P1	CTAGCTAGCGGTTAATTCCTCCTGTTA[illegible]
17-P2	CTAGCTAGCCAAAAAACCCCTCAAGAC[illegible]
18-PBAD	GGTTTGGTTAGCGAGA[illegible]
PpMar1	AAAAGTCCGCTGGCA[illegible]
PpMar2	CCCTTCAAGAGCGATA[illegible]C
AB1	GGCCACGCGTCGACTAG[illegible]CGCC
AB2	GGCCACGCGTCGACTAGT[illegible]NNNNNNCCTGG
AB3	GGCCAC[illegible]CTAGTA[illegible]NNNNNNNCCTCG
ABS	GGCC[illegible]TAGTAC
SP1	GC[illegible]TAGTAA[illegible]CATTCAT[illegible]G
SP2	[illegible]ACGTTC[illegible]GGTTTGAG
pDM4-2184-P1	ccc[illegible]atGCAGCAGGGCGGGAATAAAC
pDM4-2184-P2	CGCC[illegible]CATTTTTCACCCTGCGTATCG
pDM4-2184-P3	GTGAAA[illegible]TAAGCGGGCGATGGCGTCAAC
pDM4-2184-P4	gagtacgcgtc[illegible]gggcccttctagAGGCTGAGGGCATCCACACC
pDM4-2184-in-F	[illegible]CCACAGAAG[illegible]TGAGCGAGAA
pDM4-2184-in-R	[illegible]ACAAACTGGCGGCTAATGT
pDM4-2184-out-F	[illegible]CTTTGCTGGCTGACGC
pDM4-2184-out-R	CA[illegible]AGGTCACCGTAGAAGAGC
pUTt-2184-F	ctcatccgccaaaacagccaGGGCGGGAATAAACAGTAAAG
pUTt-2184-R	ttaaaaattaaggaggaattTTAGCGCGTCAGCATCTGCAC

The underlin[illegible]represen[illegible]restion[illegible]s for recombination when constructing vectors; The lowercase represents overhand for homologous recombinat[illegible]onstru[illegible]tors.

devices (Mi[illegible]e) [illegible]rote[illegible]ere separated by 12% SDS-PAGE, followed by Coo[illegible]e blue staining or Western blotting. For Western blotting, separate[illegible]oteins were transferred onto PVDF membranes (Millipore) and incu[illegible]ed with a 1:1000 dilution of mouse anti-EseB (GL Biochem). Anti-mouse IgG (Santa Cruz Biotechnology) was used at a 1:2000 dilution as a secondary antibody. The bands were visualized with TMB substrate (Amresco). Anti-DnaK (Santa Cruz Biotechnology) was used as a cytoplasmic protein control.

2.9. Turbot fish maintenance and virulence assay

Healthy turbot weighing 50.0 ± 5.0 g were selected and acclimatized for 2 weeks with a continuous flow of seawater at 14–18 °C in clean, aerated tanks. The virulence in turbot was measured with the *E. piscicida* WT, Δ*esrR* and *esrR*::Tn strains inoculated *via* intraperitoneal (I.P.) injection at a dose of 5.0×10^4 CFU. The turbot mortalities were recorded after injection for up to 14 days. A total of 15 turbot were grouped for one strain.

2.10. Statistics

Statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software).

3. Results

3.1. Construction of mariner transposon derivatives harboring multifunctional reporter genes or elements

The vector p*Mar2xT7* (pMFLGM.GB-2xT7) is an engineered derivative of the *Himar1* transposon, which contains transposon *Mar2xT7* (Tn*Mar2xT7*), with a gentamicin resistance cassette internal to the

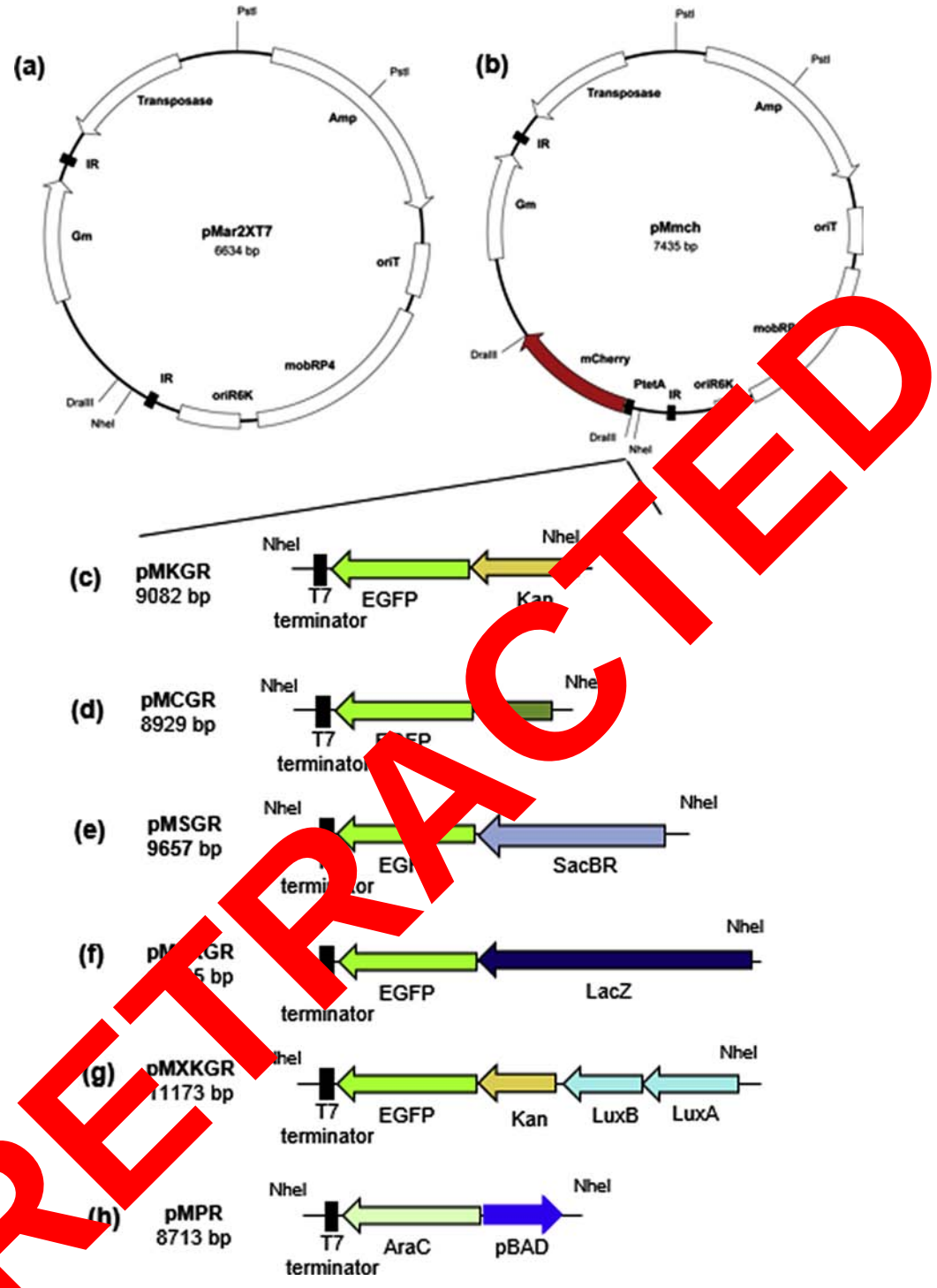

Fig. 1. The constructe ar2xT7 derivative plasmids. The diagrams of the plasmids pMmch (a), pMKGR (b), pMCGR (c), pMXKGR (d), pMSGR (e), pMLKGR (f), and pMPR (g) are shown.

transposon, a gene encoding the hyperactive C9 allele of *mariner-Himar1* transposase, a *λpir* dependent replicon (oriR6K), and an ampicillin (Amp) resistance cassette external to the transposon (Liberati et al., 2006). p*Mmch* is the first constructed derivative of p*Mar2xT7*. The *Dra*III endonuclease recognition site inside Tn*Mar2xT7* was digested using the corresponding restriction endonuclease. The reporter gene *mcherry* with constitutive promoter P_{Tet} was inserted into the enzyme site (Fig. 1a). The constitutively expressed mCherry fluorescence protein from Tn*Mmch* was proportional to the amount of host bacteria, which could be treated as a background reference. Tn*Mmch* was utilized as an initial vector to generate other transposon derivatives.

Six derivative vectors of transposons harboring different functional reporter genes were generated based on p*Mmch*, *i.e.*, p*MKGR*, p*MCGR*, p*MXKGR*, p*MSGR*, p*MLKGR*, and p*MPR* (Fig. 1b-h, Table 1). The transposons of p*MKGR* and p*MCGR* both contain the promoterless fluorescent protein-coding gene *egfp* from vector pTM267 inserted upstream of the P_{tetA} promoter and a promoterless antibiotic resistance cassette (either kanamycin or chloramphenicol) inserted upstream of the *egfp* gene (Fig. 1c and d). Both the *egfp* gene and the antibiotic

resistance cassette were promoterless but had ribosomal binding sites (RBSs) upstream of the coding sequence to ensure gene translation. The p*MKGR* was used to generate p*MXKGR*, from which the promoterless luciferase gene *luxAB* from *Vibrio harveyi* with an RBS sequence was inserted into the *Nhe*I endonuclease site upstream of the kanamycin resistance cassette (Fig. 1g).

The transposon of p*MSGR* was constructed by adding two functional genes (*egfp* and *sacBR*) to p*Mmch*. To generate p*MSGR*, promoterless *sacBR* and *egfp* genes were inserted into the *Nhe*I site of the p*Mmch* transposon (Fig. 1e). The *sacBR* terminal codon was coupled with the start codon of the *egfp* gene *via* an "ATGA" sequence between them, which ensured the efficiency of the *egfp* protein translation. For the transposon of p*MLKGR*, the promoterless *lacZ* gene with an RBS sequence was inserted upstream of the kanamycin resistance gene of the p*MKGR* transposon (Fig. 1f). The *lacZ*, the kanamycin resistance gene and the *egfp* sequence were linked together to form an expressional fusion and could be translated into individual proteins. The transposon of p*MPR* contained two functional genes in addition to the *mcherry* inside the p*Mmch*, the regulator gene (*araC*) and the promoter (P_{BAD}) of the L-arabinose operon. A promoterless *araC* and an outward P_{BAD} (facing the adjacent IR) were designed (Fig. 1h).

The transposition efficiencies of the six newly constructed transposons were examined by introducing them into the *E. piscicida* strain EIB202. The relative transposition frequency of each transposon was normalized to the frequency of the original plasmid p*Mar2xT7* (Table 3), and the transposition frequencies decreased with increasing transposon size (Table 3).

3.2. Screening for highly expressed genes by antibiotics and bioluminescence

The versatile reporter markers in the transposon derivatives [illegible] applied to track highly expressed genomic regions. The transposon of p*MKGR* contained a promoterless kanamycin resistance gene, *egfp*, a constitutively expressed *mcherry*. After the conjugation between *E. piscicida* EIB202 and *E. coli* SM10 λ*pir* (p*MKGR*), the same amount of bacterial mixture was plated on LB agar supplemented with colistin, gentamicin and different concentrations of kanamycin (0, [illegible], and 100 μg/ml). The number of colony forming units (CFU) of conjugants decreased with increasing kanamycin concentration, indicating that a portion of the transposon insertion conjugants obtained kanamycin resistance, while the decreased number of colonies under higher concentrations of kanamycin might be due to the low frequency of highly expressing genomic locations.

To confirm the effectiveness of GFP protein expression, the ratio of GFP compared with mCherry fluorescence intensity, which reflected the relative expression level of the *egfp* gene, was used as the assessment index. A total of [illegible] conjugants on the plate with the highest concentration of kanamycin (100 μg/ml) were picked individually and transferred into six 96-well plates (TF001-TF006), and the protein fluorescence intensities of GFP and mCherry were examined. The ratios of GFP to mCherry fluorescence from the six 96-well plates were much higher than the average, verifying that the expression of the *egfp* gene increased along with the increasing expression of the kanamycin resistance gene (Fig. 2a).

Table 3
Transposition frequencies of different transposon derivatives.

Plasmids	Transposon size (bp)	Absolute frequency	Relative frequency
p*Mar2×T7*	994	1.22×10^{-2}	1.0
p*MPR*	3073	6.07×10^{-3}	0.50
p*MCGR*	3289	3.38×10^{-3}	0.28
p*MKGR*	3442	3.01×10^{-3}	0.25
p*MSGR*	4017	1.52×10^{-3}	0.12
p*MXKGR*	5533	8.38×10^{-4}	0.069
p*MLKGR*	6495	9.46×10^{-5}	0.0078

Candidate mutants with insertions in highly expressing genomic locations were further verified. Each mutant from the 96-well plates of TF001 and TF002 underwent semi-arbitrary PCR to locate the transposon inside the genome (Liu and Chen, 2007). The sequencing results from 147 strains were mapped to the genome of *E. piscicida* EIB202, and 71 out of 147 (48.3%) transposons were inserted inside the rRNA operon, a naturally highly expressing genetic element. Therefore, the high probability of insertion further validated that the kanamycin screening method forces the transposon to insert inside highly expressing regions. A total of 10 strains with the highest GFP/mCherry ratios are listed (Fig. 2b).

In addition to the fluorescent proteins, luciferase is also an efficient bioreporter. The transposon p*MXKGR* was [illegible] excising the *luxAB* gene from *Vibrio harveyi* (Espinoza-Valdez et al., [illegible]) and cloning it into the p*MKGR* transposon. To demonstrate the functionality of p*MXKGR*, the conjugants from *E. piscicida* and SM10 λ*pir* (p*MXKGR*) were screened on LB agar plates supplemented with high concentrations of kanamycin to ensure the colonies grew. The plate harbored transposons inside high expression regions. Seven mutants were examined individually for bioluminescence (Table 4). All of the seven transposon mutants showed high luciferase activity compared with wild-type *E. piscicida*. The positions of the transposon insertions were determined by [illegible] arbitrary PCR and sequencing (Table 4). To further confirm the screening results, mutants with the highest luminescence (B11 inside *potD*) and lower luminescence levels (E11 inside *aroP1*) were evaluated for growth rate and long-term luciferase activity. The results showed that the growth rates of the *potD* and *arop1* mutants were similar to that of wild-type EIB202: Tn, in which the transposon inserted into the neutral site between *glms* and ETAE_3537 on *E. piscicida* [illegible] (Fig. 2c). Nevertheless, the luminescence intensities of the three strains behaved distinctly during the 24-h period (Fig. 2D). For the *potD* mutants, the intensity reached its peak during the early stage of the exponential phase (2 h) and decreased slightly in the middle of the exponential phase (4 h), but soon recovered (Fig. 2d). After entering the stationary phase, the luminescence intensity decreased gradually (Fig. 2d). Compared with the *potD* mutant, the *aroP1* mutant showed lower luminescence intensity levels on average. The luminescence intensity peaked at the early and late stages of the exponential phase and increased slightly from the middle of the stationary phase to the late of stationary phase (Fig. 2d). Therefore, we confirmed that the expression levels of the *potD* and *aroP1* genes behave differently over the course of the bacterial growth phases, and p*MXKGR* is a powerful tool to not only screen highly expressing genes but also to monitor gene expression *in situ* throughout the bacterial growth phases.

3.3. Tracking upstream regulators of T3/SS with tn-seq

Since EsrB is a master regulator of T3/T6SS in *E. piscicida* (Leung et al., 2012), a Tn-seq-based screen was designed to identify genes that mediate its expression (Yin et al., 2018). Initially, we created a reporter of the *esrB* promoter by fusing the 500-bp segment located upstream of the *esrB* start codon to a kanamycin (Kan) resistance gene (yielding P_{esrB}-kan). This reporter was introduced into a neutral site (between *glms* and ETAE_3537) on the *E. piscicida* EIB202 chromosome (Fig. 3a). Previous studies have shown that introduction of DNA into this site does not alter *E. piscicida* growth (Yin et al., 2018). Then, a high-density transposon insertion library was created in this strain (WT: P_{esrB}-kan) based on the designed plasmid pMPR. The constructed library was cultured in DMEM, a medium that induces the expression of EsrB (Srinivasa Rao et al., 2004), in the presence of either Gm + Kan (input) or Gm + Kan + Arab (output) (Fig. 3a). High-throughput sequencing was used to identify the sites and to analyze the insertion frequencies in the input and output libraries. Due to the designed arabinose promoter (Arab) on the out-flanking of the transposon (pMPR), the abundance of insertions located upstream of the genes that would activate the expression of *esrB*, would be overrepresented in the output library.

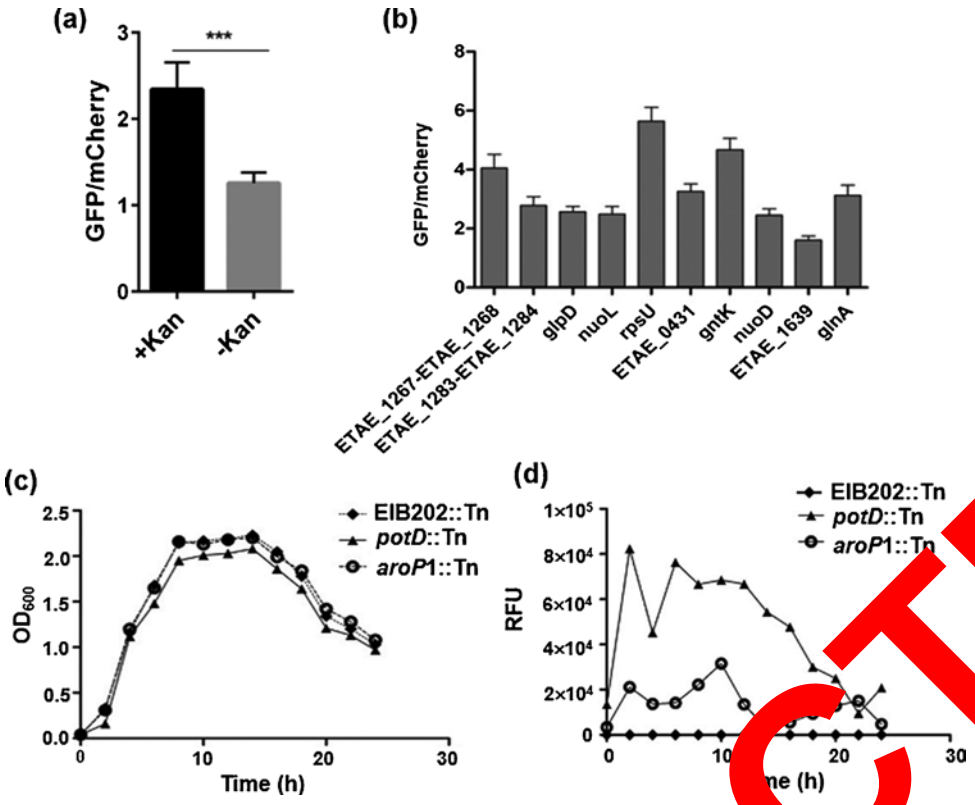

Fig. 2. Valid ation of the constructed pMar2xT7 derivative plasmids. (a) Transcriptional fusion mutants with relatively high GFP/mCherry ratios. The transposon was inserted near the 3′ end of the *rpsU* ORF (121/126). (b) Growth curves of *potD*::Tn, *aroP1*::Tn, and EIB202::Tn. The transposon inserted into the neutral site between ETAE_3551 and ETAE_3552 on the *E. piscicida* chromosome, which appeared not to significantly affect the strain's growth *in vivo* and *in vitro* and was designated as EIB202::Tn. (c) Relative luminescence units of *potD*::Tn, *aroP1*::Tn, and EIB202::Tn. More than three independent replicates were per[illegible]

Table 4
The insertion position of the 7 strains inserted with transposon of p*MXK*[illegible]

Mutant ID	Genomic position	Active gene ID	Insertion p[illegible]hin "Active" gene (bp)	"Active" gene length (bp)	"Active" gene name
X1 (A11)	3301879	ETAE_3132		3789	–
X7 (B11)	1994733	ETAE_1886	5[illegible]	1059	*potD*
X8 (C11)	210899	ETAE_018[illegible]	[illegible]	1956	*acs*
X19 (E11)	729732	ETAE_0657	[illegible]	1374	*aroP1*
X22 (F11)	2897607	ETA[illegible]61	310	879	–
X2 (D11)	3296391	E[illegible]3126	204	1947	*yhcP*
X15 (G11)	840024	[illegible]0757	[illegible]42	2145	*cadA2*

We compared the transpo[illegible] distribution p[illegible]s in the input and output libraries with the Co[illegible]RTIST pipeline (Prit[illegible]ard et al., 2014) to identify genes that [illegible]re eit[illegible] under- or overrepresented ($|\log_2(FC)| > 1$ and $P <$ [illegible]05) [illegible] the o[illegible]ut library that likely inhibited or promoted [illegible]xpress[illegible] of *esrB*, [illegible]pectively. Based on the CoARTIST analysi[illegible] hetica[illegible]amed ETAE_2184, was greatly underrepres[illegible]d in th[illegible]nput and [illegible]tput libraries (Fig. 3b). However, the insert[illegible]cated [illegible] of ETAE_2184 in the output library was overrepresen[illegible]3b), fu[illegible]er suggesting that ETAE_2184 activated *esrB* expression.

3.4. Identification of ETAE_2184 as a novel T3/T6SS regulator

The protein encoded by ETAE_2184, consisting of 203 aa, is annotated as an AcrR/TetR family protein (Fig. 4a). Because ETAE_2184 associates with EsrB expression or activity, we rename ETAE_2184 as EsrR (EsrB regulator). To investigate the relationship between EsrR and *esrB* expression, the bacterial strains, including WT, Δ*esrB*, Δ*esrR*, Δ*esrR*$^+$, *esrR*::Tn, were inoculated into DMEM and statically incubated at 28 °C for 24 h. The strains Δ*esrR*, and *esrR*::Tn, like Δ*esrB*, significantly weakened auto-aggregation mediated by filamentous structures consisting of the T3SS protein EseB (Gao et al., 2015), and the reintroduction of EsrR into Δ*esrR* restored the auto-aggregation to WT levels (Fig. 4b). The extracellular protein (ECP) profiles were analyzed by SDS-PAGE, which indicated that Δ*esrB* completely abolished all T3/T6SS ECPs, and *esrR* mutants showed significantly decreased production of T3/T6SS ECPs (Fig. 4c). In addition, Western blotting analysis to determine EseB expression with the anti-EseB antibody was consistent with the results of those auto-aggregation and SDS-PAGE (Fig. 4d), suggesting that EsrR activates T3/T6SS expression. To further investigate the mechanism of EsrR-mediated activation of T3/T6SS expression, P_{esrB}-*luxAB* (Yin et al., 2018) was introduced into the WT, Δ*esrB*, Δ*esrR*, Δ*esrR*$^+$, and *esrR*::Tn strains, followed by luciferase detection. Compared with the WT strain, the expression levels of *luxAB* in Δ*esrB*, Δ*esrR*, and *esrR*::Tn were greatly decreased (Fig. 4e). Complementation of Δ*esrR* restored the phenotype to WT (Fig. 4b-e). These results suggest that EsrR activates T3/T6SS.

3.5. esrR mutants showed virulence attenuation in turbot

Turbot was the natural host of *E. piscicida* and was used to detect the virulence of the *esrR* mutants. *E. piscicida* WT, Δ*esrR*, and *esrR*::Tn were I.P. injected into turbot at a dose of 5.0×10^4 CFU per fish, and fish mortalities were then recorded for up to 14 days. The mortality ratios of the fish infected with Δ*esrR* and *esrR*::Tn were 13.5% and 22.5% at 14 d.p.i., respectively (Fig. 5), significantly lower than those of the fish challenged with WT, with 100% death at 9 d.p.i. (Fig. 5). The mortality ratios of Δ*esrR* and *esrR*::Tn were similar to those of *esrB* or T3SS

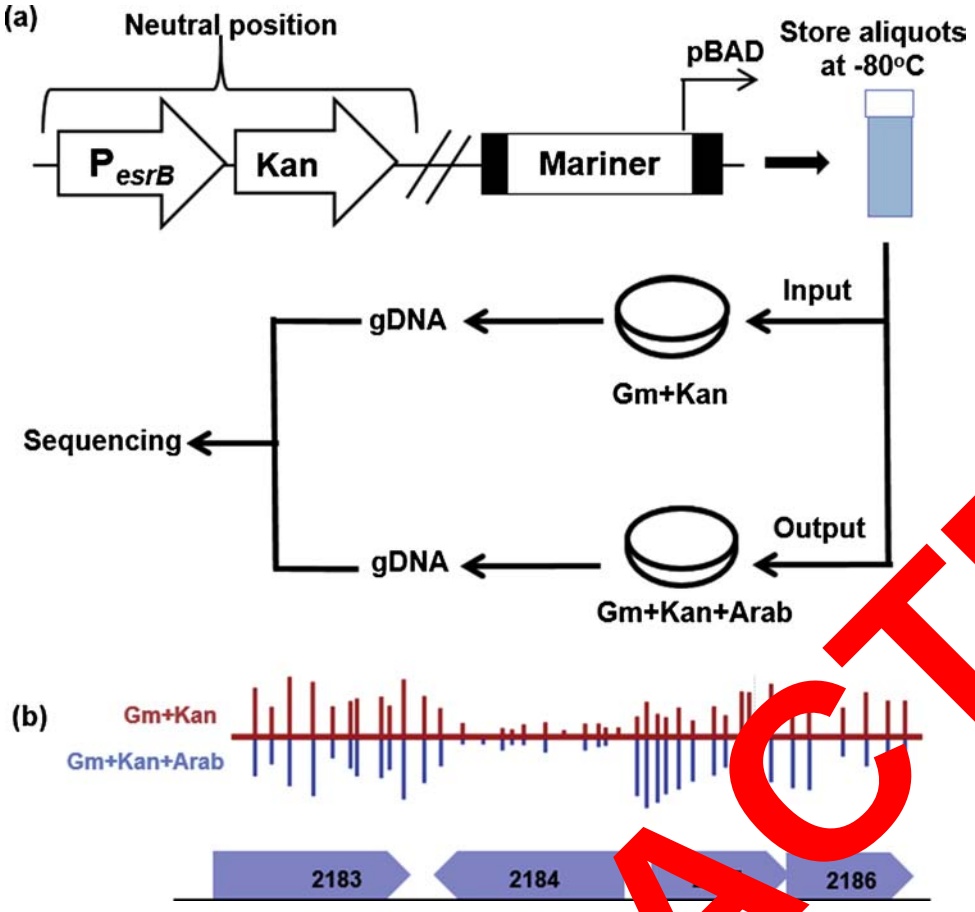

Fig. 3. The *esrB* upstream regulator screen using Tn-seq. (a) Screening model for *ersB* upstream regulators. An *esrB* promoter-driven Kan^r cassette was inserted into the neutral site between *glms* and ETAE_3537 on *E. piscicida* chromosome. A transposon insertion mutant library was constructed using pMPR based on the strain carrying the P_{esrB}-Kan^r reporter, followed exposure to Gm + Kan and Gm + Kan + Arab, respectively. After screening by Gm + Kan and Gm + Kan + Arab, the bacterial gDNA was extracted for next-generation sequencing. (b) Artemis screenshot of read abundance in ETAE_2184, detected in Gm + Kan (*versus* Gm + Kan + Arab (blue). The re[illegible] blue[illegible]ns represent screen conditions[illegible]Gm + Kan, [illegible] + Kan + Arab, respectively[illegible] height of th[illegible]olumn represents the [illegible]d abu[illegible]e of the in[illegible]ion site. The read [illegible]dance repr[illegible] the [illegible]ve ratio or number [illegible] the mu[illegible]nts in li[illegible]or interpretation of the referen[illegible]to colour [illegible] this figure legend, the [illegible]s referre[illegible] the web version of this ar-

mutants (Srinivasa Rao et al., 2004; Wei et al., 2018)[illegible]gly, th[illegible] *in vivo* Tn-seq screen in turbot revealed that the Es[illegible]utant [illegible]ibited a critical colonization defect as well (Yang et al[illegible]17). Th[illegible]re-vealed that EsrR activates virulence expressi[illegible] *in*

4. Discussion

Transposable elements are w[illegible]espread in [illegible]ryotic organisms and were first discovered in mai[illegible]y MCCLINTOCK[illegible]50). The transposable element *Himar1*, a m[illegible]ber of t[illegible] mariner family of transposons, was originally isolated f[illegible] the he[illegible]ly (*Haematobia irritans*) by Lampe (1996). Three years later[illegible] et al[illegible]99) successfully utilized *Himar1* transposa[illegible]n *E.* c[illegible]ssay f[illegible] genetic screening. Due to its characteristic[illegible]ecog[illegible]ing T[illegible]otide sequences, *Himar1* transposable ele[illegible]nts were[illegible]lways use[illegible] as tools for genetic screens and explorations[illegible]nce t[illegible] transposon insertion mutant libraries have been co[illegible]ed based on the *Himar1* transposon, such as *P. aeruginosa* (Liber[illegible]al., 2006), *V. cholerae* (Cameron et al., 2008), and *E. piscicida* (Yang [illegible], 2017). As the development of sequencing technology, transposon mutant libraries coupled with next-generation sequencing (Tn-seq) greatly promote the exploration of genetics (Chao et al., 2016). Tn-seq is revolutionizing microbiological studies by facilitating researchers in performing genome-wide explorations in a wide range of bacterial species with unprecedented depth and under a multitude of conditions.

Here, based on the *Himar1* transposon pMAR2×T7, six transposons were constructed, which carried different reporters for screens. To validate the designed transposons, we detected the expression levels of GFP, mCherry, and *luxAB* in various environments. As shown in Fig. 2b, all of the transposon mutants were able to express mCherry, but GFP expression depended on transposon location. Interestingly, there were the highest GFP/mCherry ratios in the strains containing insertions within the *rpsU* gene (Versalovic et al., 1993) (Fig. 2b), which codes the protein S21 and is an essential component of the 30S rRNA, have the highest GFP/mCherry ratios. Furthermore, the transposon insertion position located at 121 bp of the right C-terminal of the 126 bp *rpsU* gene, Due to the essentiality of *rpsU* to *E. piscicida* and only single-copy on genome, the function of *rpsU* would not necessarily be deprived by the transposon insertion. In addition, the expression of *luxAB* in the strain of *potD*::Tn demonstrated that the designed *luxAB* on transposon was able to be promoted by promoters from *E. piscicida* genome (Fig. 2d).

To discover novel T3/T6SS regulators, the genome of *E. piscicida* was modified with P_{esrB}-kan inserting on the neutral site (Yin et al., 2018). Based on the modified strain, a transposon insertion library, containing approximately 3.0×10^5 different mutants, was constructed using pMPR (Fig. 1h). ETAE_2184 (EsrR), annotated as a TetR/AcrR family protein (Fig. 4a), was screened by the Tn-seq and revealed to activate T3/T6SS expression through EsrB (Fig. 4b-e). Although the phenotype of *esrR* deletion was similar to that of the transposon mutant, slight differences in auto-aggregation (Fig. 4b), SDS-PAGE (Fig. 4c), Western blotting (Fig. 4d), and P_{esrB}-*luxAB* expression (Fig. 4e) were observed. The transposon mutant of *esrR* displayed a more critical T3/T6SS defect than the *esrR* deletion. As we know, transposon insertion mutants cause polar effects (Jacobs et al., 2003), which could greatly influence the downstream gene expression profile and lead to differences in phenotype. Excitingly, both the *esrR* deletion and the transposon mutant showed high levels of virulence attenuation in its natural host turbot (Fig. 5) (Xiao et al., 2008), consistent with the *in vivo* screen data from our previous investigation (Yang et al., 2017), suggesting that this mutant could be potential as a live-attenuated vaccine.

In conclusion, based on the *Himar1* transposon p*Mar2×T7*, a transposon toolbox was successfully built for functional genomics studies. A transposon mutant library was constructed using the toolbox for

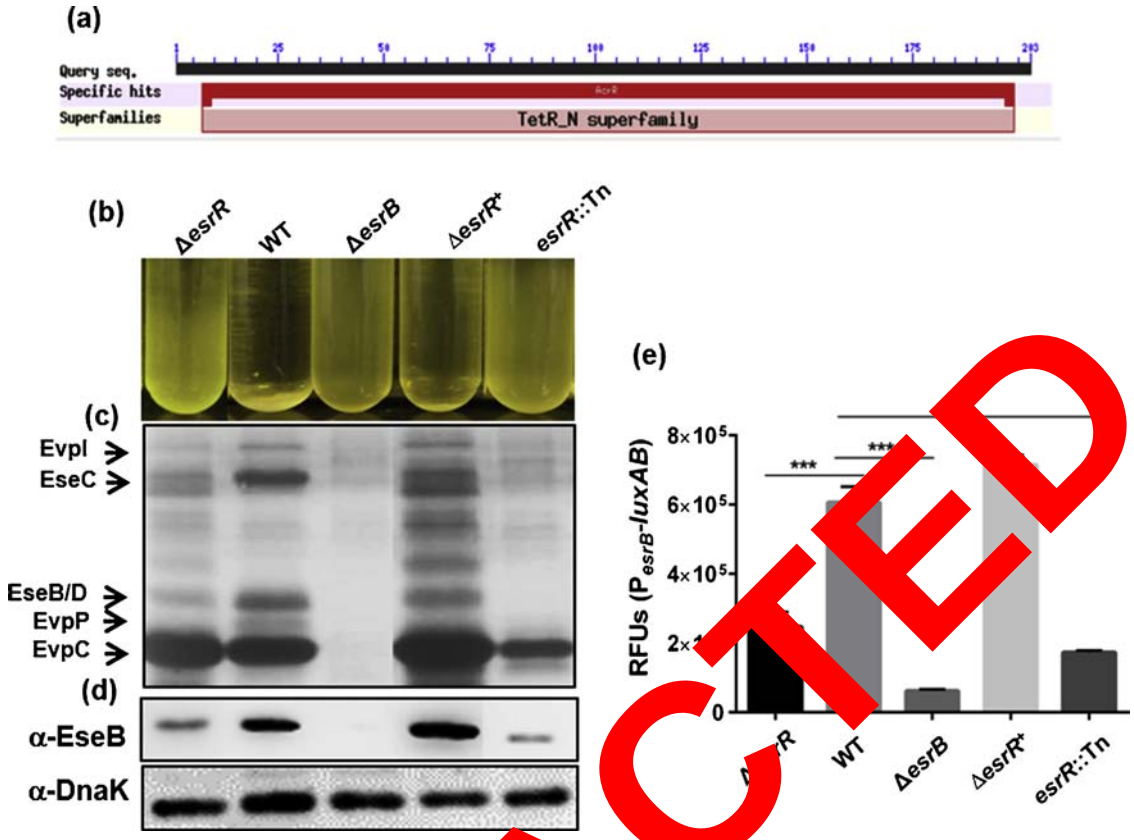

Fig. 4. Activation of T3SS expression by EsrR in *E. piscicida*. (a) Conserve[d] [a]nalysis of EsrR. EsrR obtains AcrR family domains and TetR. (b) Auto-aggregation phenotypes of WT, Δ*esrB*, *esrR*::Tn, Δ*esrR*, and *esrR*$^+$, after 24 h of static gr[owth]. (c) [E]xtracellular proteins (ECPs) of the indicated strains were collected from the same number of cells, and aliquots of the same volume were resolved by SDS-PAGE. [T]hree independent replicates were performed. (d) EseB production was detected by Western blotting with anti-EseB and anti-Dnak antibodies was us[ed as] control. More than three independent replicates were performed. (d) Expression of *esrB* in the WT, Δ*esrB*, *esrR*::Tn, Δ*esrR*, and *esrR*$^+$ strains. T[he] *PesrB-luxAB* exp[ression] plasmid was transferred into the above strains, followed by luciferase detection at 12 h post-inoculation. More than three independent r[eplic]ates w[ere] perform[ed]. ***, $P < 0.001$; *, $P < 0.05$ based on Student *t*-test.

Fig. 5. Virul[ence] assessm[ent] of the Es[rR] mutants in turbot. Percent survivals of turbot were [re]corded f[ollow]ed bacterial strains inoculated at a dose of 5.0×10^4 CFU[/fish] [d]uring a 14-day observation. ***, $P < 0.001$; *, $P < 0.05$ based on Kaplan-[Meier] survival analysis with a log-rank test (Mantel-Cox).

the exploration of *esrB* upstream regulators. EsrR was identified to activate T3/T6SS expression *in vivo* and *in vitro* conditions and was attenuated in turbot, meriting as a candidate of live-attenuated vaccine.

5. Ethics statement

All animal experiments presented in this study were approved by the Animal Care Committee of the East China University of Science and Technology (2,006,272). The Experimental Animal Care and Use Guidelines from Ministry of Science and Technology of China (MOST-2011-02) were strictly followed.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Cameron, D.E., Urbach, J.M., Mekalanos, J.J., 2008. A defined transposon mutant library and its use in identifying motility genes in Vibrio cholerae. Proc. Natl. Acad. Sci. U. S. A. 105, 8736–8741.

Chao, M.C., Abel, S., Davis, B.M., Waldor, M.K., 2016. The design and analysis of transposon insertion sequencing experiments. Nat. Rev. Microbiol. 14, 119–128.

Espinoza-Valles, I., Vora, G.J., Lin, B., Leekitcharoenphon, P., González-Castillo, A., Ussery, D., Høj, L., Gomez-Gil, B., 2015. Unique and conserved genome regions in *Vibrio harveyi* and related species in comparison with the shrimp pathogen *Vibrio harveyi* CAIM 1792. Microbiology 161, 1762–1779.

Feschotte, C., 2008. Transposable elements and the evolution of regulatory networks. Nat. Rev. Genet. 9, 397–405.

Fu, Y., Waldor, M.K., Mekalanos, J.J., 2013. Tn-Seq analysis of *Vibrio cholerae* intestinal colonization reveals a role for T6SS-mediated antibacterial activity in the host. Cell Host Microbe 14, 652–663.

Gallagher, L.A., Ramage, E., Jacobs, M.A., Kaul, R., Brittnacher, M., Manoil, C., 2007. A comprehensive transposon mutant library of *Francisella novicida*, a bioweapon surrogate. Proc. Natl. Acad. Sci. U. S. A. 104, 1009–1014.

Gallagher, L.A., Ramage, E., Patrapuvich, R., Weiss, E., Brittnacher, M., Manoil, C., 2013. Sequence-defined transposon mutant library of Burkholderia thailandensis. mBio 4, e604–e613.

Gao, Z.P., Nie, P., Lu, J.F., Liu, L.Y., Xiao, T.Y., Liu, W., Liu, J.S., Xie, H.X., 2015. Type III secretion system translocon component EseB forms filaments and mediates auto-aggregation and biofilm formation by Edwardsiella tarda. Appl. Environ. Microbiol. 81, 6078–6087.

Grant, A.J., Coward, C., Jones, M.A., Woodall, C.A., Barrow, P.A., Maskell, D.J., 2005. Signature-tagged transposon mutagenesis studies demonstrate the dynamic nature of cecal colonization of 2-week-old chickens by Campylobacter jejuni. Appl. Environ. Microbiol. 71, 8031–8041.
Green, D.M., 2010. A strategic model for epidemic control in aquaculture. Prev. Vet. Med. 94, 119–127.
Hou, M., Chen, R., Yang, D., Núñez, G., Wang, Z., Wang, Q., Zhang, Y., Liu, Q., 2016. Identification and functional characterization of EseH, a new effector of the type III secretion system of Edwardsiella piscicida. Cell. Microbiol. 19, e12638.
Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenthner, D., Bovee, D., Olson, M.V., Manoil, C., 2003. Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A 100, 14339–14344.
Lampe, D.J., Churchill, M.E., Robertson, H.M., 1996. A purified mariner transposase is sufficient to mediate transposition in vitro. EMBO J. 15, 5470–5479.
Lampe, D.J., Grant, T.E., Robertson, H.M., 1998. Factors affecting transposition of the *Himar1* mariner transposon *in vitro*. Genetics 149, 179–187.
Lampe, D.J., Akerley, B.J., Rubin, E.J., Mekalanos, J.J., Robertson, H.M., 1999. Hyperactive transposase mutants of the *Himar1* mariner transposon. Proc. Natl. Acad. Sci. U. S. A. 96, 11428–11433.
Leotta, G.A., Piñeyro, P., Serena, S., Vigo, G.B., 2009. Prevalence of *Edwardsiella tarda* in Antarctic wildlife. Polar Biol. 32, 809–812.
Leung, K.Y., Siame, B.A., Tenkink, B.J., Noort, R.J., Mok, Y., 2012. *Edwardsiella tarda* - virulence mechanisms of an emerging gastroenteritis pathogen. Microbes Infect. 14, 26–34.
Liberati, N.T., Urbach, J.M., Miyata, S., Lee, D.G., Drenkard, E., Wu, G., Villanueva, J., Wei, T., Ausubel, F.M., 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. U. S. A. 103, 2833–2838.
Liu, Y.G., Chen, Y., 2007. High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. BioTechniques 43, 649–656.
Liu, Z.M., Tucker, A.M., Driskell, L.O., Wood, D.O., 2007. Mariner-based transposon mutagenesis of Rickettsia prowazekii. Appl. Environ. Microbiol. 73, 6644–6649.
Lv, Y., Xiao, J., Liu, Q., Wu, H., Zhang, Y., Wang, Q., 2012. Systematic mutation analysis of two-component signal transduction systems reveals EsrA-EsrB and PhoP-PhoQ as the major virulence regulators in Edwardsiella tarda. Vet. Microbiol. 157, 190–199.
Mcclintock, B., 1950. The origin and behavior of mutable loci in maize. Proc. Natl. Acad. Sci. U. S. A 6, 344–355.
Menanteau-Ledouble, S., Lawrence, M.L., 2013. Use of bioluminescence mutant screening for identification of *Edwardsiella ictaluri* genes involved in channel catfish (Ictalurus punctatus) skin colonization. Vet. Microbiol. 162, 724–730.
Okuda, J., Kiriyama, M., Suzaki, E., Kataoka, K., Nishibuchi, M., Nakai, T., 2009. Characterization of proteins secreted from a type III secretion system of *Edwardsiella tarda* and their roles in macrophage infection. Dis. Aquat. Organ. 84, 115–121.
Park, S.B., Aoki, T., Jung, T.S., 2012. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. Vet. Res. 43, 67.
Picardeau, M., 2010. Transposition of fly mariner elements in bacteria as a genetic tool for mutagenesis. Genetica 138, 551–558.
Pozsgai, E.R., Blair, K.M., Kearns, D.B., 2012. Modified mariner transposons for inducible-expression insertions and transcriptional reporter fusion insertions in *Bacillus subtilis*. Appl. Environ. Microbiol. 78, 778–785.
Pritchard, J.R., Chao, M.C., Abel, S., Davis, B.M., Baranowski, C., Zhang, Y.J., Rubin, E.J., Waldor, M.K., 2014. ARTIST: high-resolution genome-wide assessment of fitness using transposon-insertion sequencing. PLoS Genet. 10, e1004782.
Serrato-Capuchina, A., Matute, D., 2018. The role of transposable elements in speciation. Genes 9, e254.
Smith, V., Botstein, D., Brown, P.O., 1995. Genetic footprinting: a genomic strategy for determining a gene's function given its sequence. Proc. Natl. Acad. Sci. U. S. A 92, 6479–6483.
Srinivasa Rao, P.S., Lim, T.M., Leung, K.Y., 2003. Functional genomics approach to the identification of virulence genes involved in *Edwardsiella tarda* pathogenesis. Infect. Immun. 71, 1343–1351.
Srinivasa Rao, P.S., Yamada, Y., Tan, Y.P., Leung, K.Y., 2004. Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. Mol. Microbiol. 53, 573–586.
Tan, Y.P., Lin, Q., Wang, X.H., Joshi, S., Hew, C.L., Leung, K.Y., 2002. Comparative proteomic analysis of extracellular proteins of *Edwardsiella tarda*. Infect. Immun. 70, 6475–6480.
Versalovic, J., Koeuth, T., Britton, R., Geszvain, K., Lupski, J.R., [illegible] Conservation and evolution of the *rpsU-dnaG-rpoD* macromolecular synthesis operon in bacteria. Mol. Microbiol. 8, 343–355.
Wang, Q., Yang, M., Xiao, J., Wu, H., Wang, X., Lv, Y., Li, L., Zhang, H., Wang, S., Zhao, G., Liu, Q., Zhang, Y., 2009. Genome sequence of the fish pathogen *Edwardsiella tarda* provides insights into adaptation to broad host ranges and intracellular niches. PLoS One 4, e7646.
Wang, X., Wang, Q., Yang, M., Zhang, Q., Wu, H., Zhang, Y., 2011. QseBC controls flagellar motility, [illegible]al hemolysination and intracellular virulence in fish pathogen Edwardsiella tarda. Fish Shellfish Immunol. 30, 944–953.
Wei, L., Wu, Y., Gu, Xu, W., Zhang, X., Wang, Q., 2018. YebC controls virulence [illegible]T3SS gene expression in the pathogen. FEMS Microbiol. Lett. 1, 365.
Xiao, J., Wang, Q., Liu, Q., Wu, Y., Liu, H., Zhang, Y., 2008. Isolation and identification of pathogen *Edwardsiella* [illegible] from mariculture in China. Aquac. Res. 40, 13–17.
Yang, [illegible], Billings, G., Hubbard, T.P., Park, J.S., Leung, K., Liu, Q., Davis, B.M., Zhang, Y., Wang, Q., Waldor, M.K., 2017. Time-resolved transposon insertion sequencing reveals genome-wide fitness dynamics during infection. mBio 8, e01581–17.
Yang, M., Lv, Y., Xiao, J., Wu, H., Zheng, H., Liu, Q., Zhang, Y., Wang, Q., 2012. *Edwardsiella* comparative phylogenomics reveal the new intra/inter-species taxonomy [illegible]ps, virulence evolution and niche adaptation mechanisms. PLoS One 7, e[illegible].
[illegible]a, K., Guan, Y., Ma, R., Wei, L., Liu, B., Liu, X., Zhou, X., Ma, Y., Zhang, Y., Waldor, [illegible] Wang, Q., 2018. Critical role for a promoter discriminator in RpoS control of [illegible]nce in Edwardsiella piscicida. PLoS Pathog. 14, e1007272.
Zhang, L., Ni, C., Xu, W., Dai, T., Yang, D., Wang, Q., Zhang, Y., Liu, Q., 2016. Intramacrophage infection reinforces the virulence of Edwardsiella tarda. J. Bacteriol. 198, 1534–1542.
Zheng, J., Tung, S.L., Leung, K.Y., 2005. Regulation of a type III and a putative secretion system in *Edwardsiella tarda* by EsrC is under the control of a two-component system, EsrA-EsrB. Infect. Immun. 73, 4127–4137.