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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 chromosome. 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*, *luxAB*, *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 initial transposon p*Mar2xT7*. The function and effectiveness of the modified transposons were verified by introducing them into *E. piscicida* EIB202. Based on the toolbox, a transposon insertion mutant library containing approximately 3.0×10^5 distinct mutants was constructed to explore the upstream regulators of *esrB*, the master regulator of the type III and type VI secretion systems (T3/T6SS) in *E. piscicida*. Following analysis by Con-ARTIST, ETAE_3474, annotated as *fabR* and involved in fatty acid metabolism, was screened out and identified as a novel regulator mediating T3SS and T6SS expression. In addition, the *fabR* mutants displayed critical virulence attenuation in turbot. Due to the broad-range host compatibility of mariner transposons, the newly built transposon toolbox can be 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 (Feschotte, 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 Matute, 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., 2013), 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 generate 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 bias 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

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mutant libraries in various bacteria with efficient, random, and unbiased insertions in the chromosome.

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* could 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) with 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). *E. piscicida* harbors 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 (Yang et al., 2012). *E. piscicida* pathogenesis involves T3/T6SS, quorum sensing, two-component systems, and exoenzymes (*e.g.*, hemolysin and chondroitinase), which make it a model organism for the study of gastrointestinal infection and secretion systems (Leung et al., 2012). The complex virulence regulatory networks and the rewiring of those networks during host invasion processes are still unclear in this bacterium.

In this study, we generated a toolbox of transposon derivatives for the study of bacterial functional genomics based on *mariner* transposon *Mar2xT7*. The newly built transposon derivatives were applied for tracking highly expressed genomic regions and essential genes in *E. piscicida*. The results demonstrated that these transposons were efficient and flexible and could be further used in any transposon-tolerant bacteria. Moreover, Tn-seq pipeline was established by using the constructed transposons and ETAE_3474 (*fabR*) was identified to be a novel T3/T6SS regulator, demonstrating the utility of the mariner plasmid toolbox in genome-wide screen of targeted genes in transposon-compatible bacteria.

2. Materials and methods

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 λ*pir* 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). 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 λ*pir*. The construct was validated by PCR and sequencing.

p*MKGR* was constructed based on p*Mmch*. The promoterless kanamycin resistance (Kan^r) cassette and *egfp* with ribosomal binding site (RBS) were amplified from pTM267 (Table 1) using the primers PKGF and PKGR. The RBS sequence used in this study was "AAGGAGG", which originated from the *E. piscicida* 16S rRNA 3′ terminal conserved sequence "CCUCCUU". An *Nhe*I restriction site and triple terminal sites "TGACTAGCTAA" were introduced into the primer PKGF, and PKGR contains an *Nhe*I site and a 48-bp T7 terminator sequence. The PCR product was digested with *Nhe*I endonuclease and ligated with the digested p*Mmch*. The constructed p*MKGR* was transformed into *E. coli* CC118 λ*pir* and validated by PCR and sequencing.

To generate p*MCGR*, a chloramphenicol resistance (Cm^r) cassette was amplified using the primers 01-P1 and 02-P2 from plasmid pTM267, and *egfp* was amplified from p*MKGR* using the primers 03-P3 and 04-P4. An *Nhe*I site, a triple terminator and an RBS site were introduced into 01-P1. The 04-P4 downstream of the T7 site contained the *Nhe*I site. The chloramphenicol and *egfp* genes were amplified and linked by overlap PCR. The following procedure was the same as for the construction of p*MKGR*. Functional validation was performed by culturing the 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 (BioTek).

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 λ*pir*, 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	Characteristics	References
Strains		
EIB202	Wild type strain, CCTCC M 208068, Col^r	Xiao et al., 2008
Δ*fabR*	EIB202, in-frame deletion of ETAE_3474 (*fabR*)	This study
fabR$^+$	Δ*fabR*, complementation of *fabR* expressed in pUTt	This study
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 lab
pMD19-T	Gene cloning vector, Amp^r	TaKaRa
pDMK	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 lab
pMar2×T7	λ*pir*-dependent *ori*, Amp^r, Gm^r	Liberati et al., 2006
p*Mmch*	pMar2×T7 derivative with *mCherry* gene	This 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* gene	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* gene	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 overnight culture of mutants was inoculated into a 96-well microplate for incubation at 30 °C for 18 h (200 μl LB in each well). The plates were centrifuged at 3000 *g* at 4 °C for 5 min. The pellets were resuspended in 200 ml of sterile PBS and centrifuged 3 times. 100 μl of resuspended bacteria was transferred to a new 96-well, black, clear-bottom plate to examine the fluorescence intensity in a fluorescence microplate reader. The excitation and emission wavelengths were set at 485 and 535 nm for GFP and 595 and 635 nm for mCherry, respectively.

2.5. Measurement of luciferase expression

For the transposon of p*MXKGR*, the luminescence of the luciferase reaction was induced by the addition of decanal to the bacterial culture. The overnight culture (2 μl) of transposon insertional mutants was inoculated into a 96-well microplate with 200 μl of LB broth in each well. Fifty microliters of 1% decanal was added to the 200 μl of culture (final concentration 0.2%, v/v). 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 arbitrary 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 arbitrary 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 30 s, (iii) 42 °C for 30 s, slope −1 °C per cycle, (iv) 72 °C for 1 min, (v) go to step ii, 6 times, (vi) 94 °C for 30 s, (vii) 58 °C for 30 s, (viii) 72 °C for 1 min, (ix) go to step vi, 25 times, (x) 72 °C for 3 min, (xi) 4 °C hold. For the second round of PCR, primers Sp2 and ABS were used. For each reaction, 1 μl of template (from first round PCR), 25 μl of 2× Taq-plus MasterMix (Venzyme) and 2.5 μl of each primer (10 μM) were mixed, and ddH_2O was added to a final volume of 50 μl. The PCR conditions were: 94 °C for 3 min, 94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min for 30 circles, 72 °C for 3 min and then 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 of DNA contamination from dead *E. piscicida* cells. The Tn-seq experiments and data analysis followed the protocols from Pritchard et al. (Pritchard et al., 2014). Briefly, bacterial 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 devices (Millipore). Proteins were separated by 12% SDS-PAGE, followed by Coomassie blue staining or Western blotting. For Western blotting, separated proteins were transferred onto PVDF membranes (Millipore) and incubated with a 1:1000 dilution of mouse anti-EseB (GL Biochem). Anti-mouse IgG (Santa Cruz Biotechnology) was used at a 1:2000

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	CTAGCTAGCTGACTAGCTAAGGAGGTATACATGAACATCAAAAAGTTTGC
14-P2	GAAAAGTTCTTCTCCTTTACTCATTTGTTAACTGTTAATTGTCC
15-P3	GGACAATTAACAGTTAACAAATGAGTAAAGGAGAAGAACTTTTC
19-SacBR	GCTCAGGCGACAACCAT
05-P1	CTAGCTAGCTGACTAGCTAAGGAGGTATACATGACCATGATTACGGATTC
08-P2	GCAATCCATCTTGTTCAATCATTTTTGACACCAGACCAAC
09-P3	GTTGGTCTGGTGTCAAAAATGATTGAACAAGATGGATTGC
21-lacZ	GTAAGTGAAGCGACCCGCATTG
16-P1	CTAGCTAGCGGTTAATTCCTCCTGTTAGC
17-P2	CTAGCTAGCCAAAAAACCCCTCAAGACC
18-PBAD	GGTTTGGTTAGCGAGAAGAG
PpMar1	AAAAGTCCGCTGGCAAAG
PpMar2	CCCTTCAAGAGCGATACAAC
AB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
AB2	GGCCACGCGTCGACTAGTACNNNNNNNNNNCCTGG
AB3	GGCCACGCGTCGACTAGTACNNNNNNNNNNCCTCG
ABS	GGCCACGCGTCGACTAGTAC
SP1	GCTCCGTAGTAAGACATTCATCGCG
SP2	GCTTACGTTCTGCCCAGGTTTGAG
pDMK-3474-P1	CCCCCCCGAGCTCAGGTTACCCGGATCTATGATCGACAAAACGGGCTTCG
pDMK-3474-P2	TCCTCCCCTAAGATCTATCTAGAAGGGCCC
pDMK-3474-P3	AGATAGATCTTAGGGGAGGAGATGCGTAAA
pDMK-3474-P4	CCCTCGAGTACGCGTCACTAGTGGGGCCCTACCATATGGAGTGCGGGGTA
pDMK-3474-in-F	GCCATTTTCGCGATGTGGAT
pDMK-3474-in-R	ACTGGTATCGTCGCGAACAG
pDMK-3474-out-F	AACTCTTCGTTGTGGCGGAT
pDMK-3474-out-R	ACGGTTTGGCTATCTCGGTG
putT-3474-F	ctcatccgccaaaacagccaTGGCAGTGATTGCGTTCATA
putT-3474-R	ttaaaaattaaggaggaattCTATGACTTTTGATGGTGTC

The underline represents digestion sites for recombination when constructing vectors; The lowercase represents overhand for homologous recombination when constructing vectors.

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*fabR* and *fabR*$^+$ 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 challenging experiments with each of the strains.

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 transposon, a gene encoding the hyperactive C9 allele of *mariner-Himar1* transposase, a λ*pir* 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_{tetA} was inserted into the

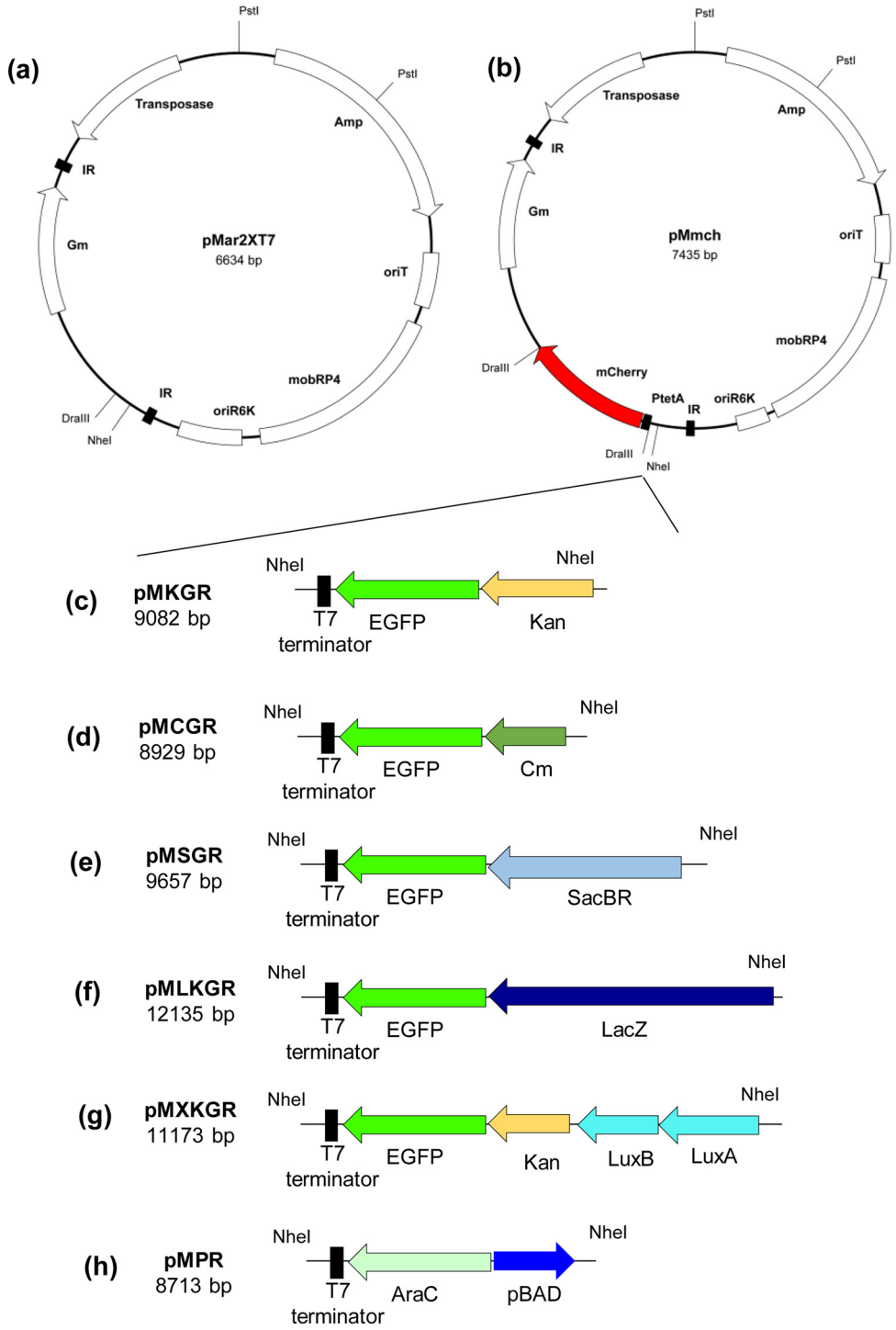


Fig. 1. The constructed p*Mar2xT7* derivative plasmids. The diagrams of the plasmids p*Mar2xT7*(a), pMmch (b), pMKGR (c), pMCGR (d), pMSGR (e), pMLKGR (f), pMXKGR (g) and pMPR (h) are shown.

enzymatic 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 1d). 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* (Espinoza-Valles et al.) 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

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

genes (*egfp* and *sacBR*) to p*Mmch*. To generate p*MSGR*, promoterless *sacBR* and *egfp* genes were inserted into the *NheI* 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 were applied to track highly expressed genomic regions. The transposon of p*MKGR* contained a promoterless kanamycin resistance gene, *egfp,* and a constitutively expressed *mCherry*. After 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, 10, 25, 50, and 100 μg/ml). The number of colony forming unit (CFU) of conjugants decreased with increasing kanamycin concentration, indicating that a portion of the transposon insertion conjugants obtained kanamycin resistance, while the decreased numbers of colonies under higher concentrations of kanamycin might be due to the low frequency of highly expressing genomic positions.

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 576 conjugants from the plate with the highest concentration of kanamycin (100 μg/ml) were picked individually and transferred to 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).

Candidate mutants with insertions in highly expressing genomic locations were further verified. Arbitrary PCR was performed with each mutant from the 96-well plates of TF001 and TF002 to identify the transposon insertion sites 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, validating that the kanamycin screening enables the localization of transposon insertions inside highly expressed regions. A total of 10 strains with the highest GFP/mCherry ratios are identified (Fig. 2b).

In addition to the fluorescent proteins, luciferase is also an efficient reporter. The transposon p*MXKGR* was built by amplifying the *luxAB* gene from *Vibrio harveyi* (Espinoza-Valles et al., 2015) 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 grown on the plate harbored transposons inside highly expressing regions. Seven mutants were examined individually for bioluminescence (Table 4). All of the seven transposon mutants showed high luciferase activity compared with that of the wild-type *E. piscicida*. The positions of the transposon insertions were determined by 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 the wild-type EIB202::Tn, in which the transposon inserted into the neutral site between *glms* and ETAE_3537 on *E. piscicida* chromosome (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 growth phase, the luminescence intensity gradually decreased to zero (Fig. 2d). Compared with the *potD* mutants, the *aroP1* mutants showed lower luminescence intensity levels on average. The luminescence intensity peaked at the early and late stages of the exponential growth phase and increased slightly from the middle of the stationary growth phase to the decline 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/T6SS 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. We have 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) (Yin et al., 2018). 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 (Input) was cultured in DMEM, a medium that induces the expression of EsrB (Srinivasa Rao et al., 2004), in the presence of either Col + Kan (output) or Col + Kan + Arab (output) (Fig. 3a). High-throughput sequencing was used to identify the sites and to analyze the insertion frequencies in the saturated 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.

We compared the transposon distribution profiles in the input and output libraries with the Con-ARTIST pipeline (Pritchard et al., 2014) to identify genes that were either under- or overrepresented ($|\log_2(FC)| > 1$ and $P < 0.05$) in the output library that likely inhibited or promoted expression of *esrB*, respectively. Based on the Con-ARTIST analysis, insertions in ETAE_3474 (*fabR*) were greatly

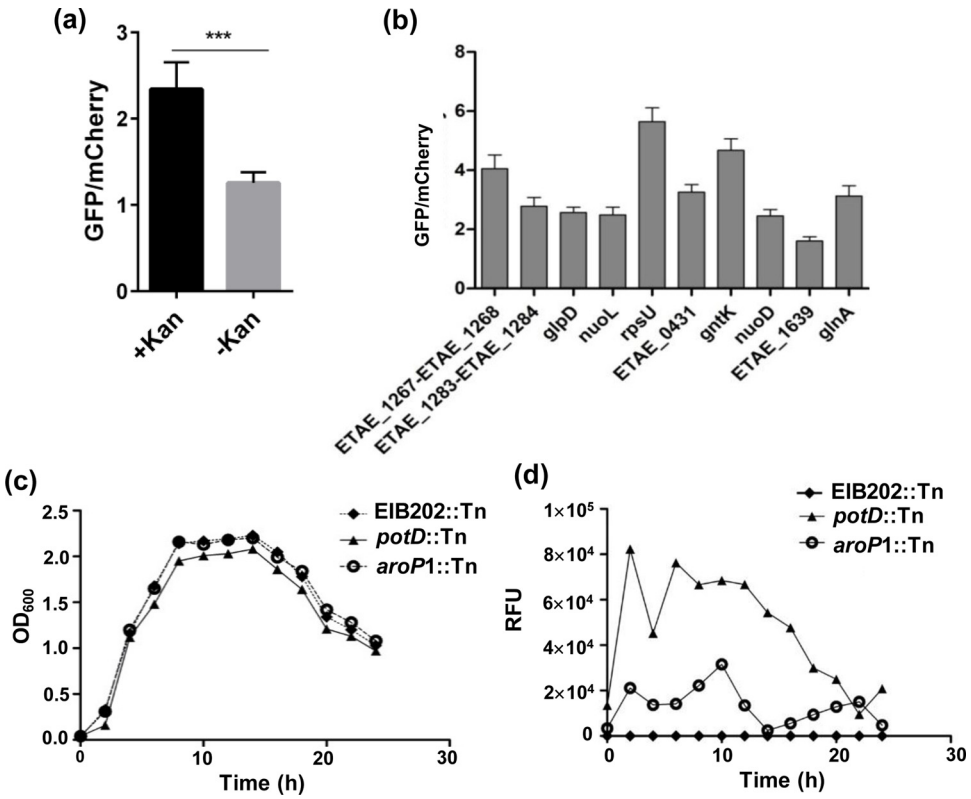


Fig. 2. Validation of the constructed p*Mar2xT7* derivative plasmid pMKGR. (a) The GFP/mCherry ratio of the Kan resistance colonies in the presence or not of Kan. (b) Transcriptional fusion mutants with relatively high GFP/mCherry ratios. (c) Growth curves of *potD*::Tn, *aroP1*::Tn, and EIB202::Tn. 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. (d) Relative luminescence units of *potD*::Tn, *aroP1*::Tn, and EIB202::Tn. More than three independent replicates were performed.

Table 4
The insertion position of the 7 strains inserted with transposon of p*MXKGR*.

Mutant ID	Genomic position	Gene ID	Insertion position within the gene (bp)	Whole gene length (bp)	Gene name
X1 (A11)	3301879	ETAE_3132	3241	3789	–
X7 (B11)	1994733	ETAE_1886	500	1059	*potD*
X8 (C11)	210899	ETAE_0188	1685	1956	*acs*
X19 (E11)	729732	ETAE_0657	343	1374	*aroP1*
X22 (F11)	2897607	ETAE_2761	310	879	–
X2 (D11)	3296391	ETAE_3126	204	1947	*yhcP*
X15 (G11)	840024	ETAE_0757	1742	2145	*cadA2*

underrepresented in the Col + Kan and Col + Kan + Arab libraries (Fig. 3b). However, insertions located upstream of *fabR* in the Col + Kan + Arab library were overrepresented compared to input and Col + Kan libraries (Fig. 3b), further suggesting that FabR activated *esrB* expression.

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

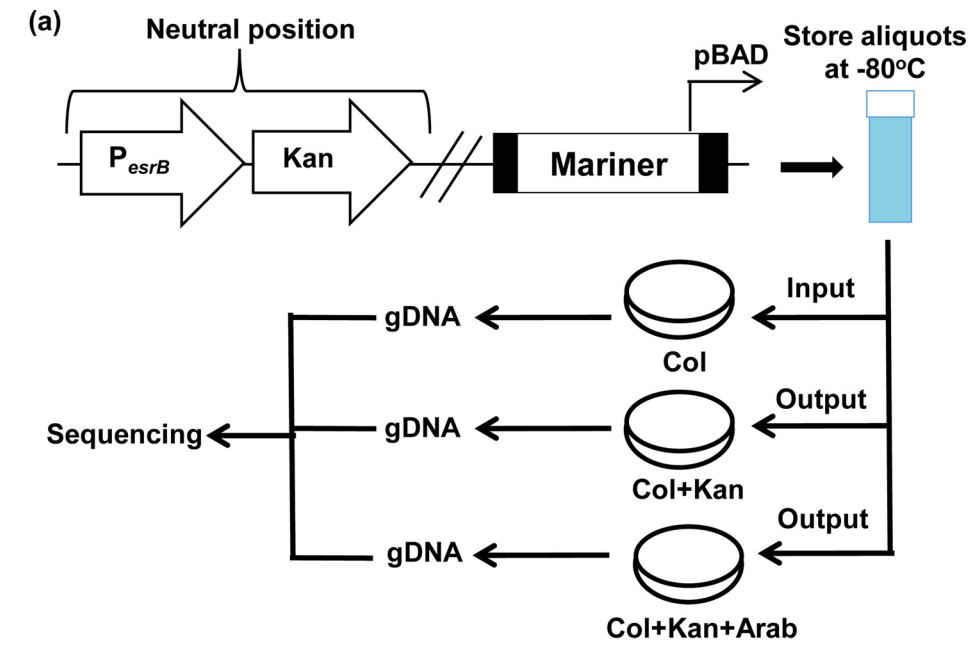
The protein encoded by *fabR*, consisting of 223 aa, is annotated as a PRK11202 superfamily protein (Fig. 4a). To validate the role of FabR in *esrB* expression, the bacterial strains including WT, Δ*fabR,* and *fabR*$^+$ were inoculated into DMEM and statically incubated at 28 °C for 24 h. The empty plasmid pUTt was introduced as control in WT and Δ*fabR*. Δ*fabR* cells showed significantly weakened auto-aggregation mediated by filamentous structures consisting of the T3SS protein EseB (Gao et al., 2015), and the reintroduction of *fabR* into Δ*fabR* restored the auto-aggregation to WT levels (Fig. 4b). The extracellular protein (ECP) profiles analyzed by SDS-PAGE indicated that Δ*fabR* showed significantly decreased production of T3/T6SS ECPs (Fig. 4c). In addition, Western blotting analysis with the anti-EseB antibody further demonstrated that FabR played an essential role in EseB expression (Fig. 4d). To further investigate the mechanism of FabR-mediated activation of T3/T6SS expression, P_{esrB}-*luxAB* reporter (Yin et al., 2018) was introduced into the WT and Δ*fabR* strains, followed by luciferase detection. Compared with the WT strain, Δ*fabR* generated greatly decreased levels of luminescence (Fig. 4e). These results collectively suggest that FabR activates T3/T6SS production through *esrB* in *E. piscicida*.

3.5. ΔfabR showed virulence attenuation in turbot

Turbot was the natural host of *E. piscicida* and was used to detect the virulence of the Δ*fabR*. WT, Δ*fabR*, and *fabR*$^+$ 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 empty plasmid pUTt was introduced as control in WT and Δ*fabR*. The mortality ratios of the fish infected with Δ*fabR* were 60% at 14 d.p.i. (Fig. 5), significantly lower than those of the fish challenged with WT and *fabR*$^+$ with 100% death at 9 d.p.i. (Fig. 5). These data revealed that FabR activates virulence expression *in vivo*.

4. Discussion

Transposable elements are widespread in eukaryotic organisms and were first discovered in maize by Barbara McClintock (1950). The transposable element *Himar1*, a member of the mariner family of transposons, was originally isolated from the horn fly (*Haematobia irritans*) by Lampe et al. (1996). Three years later, Lampe et al (1999) successfully utilized *Himar1* transposase for genetic screening in *E. coli*. Due to its characteristic of recognizing TA nucleotide sequences, *Himar1* transposable elements were always used as tools for genetic screens. Since then, several transposon insertion mutant libraries have been constructed based on the *Himar1* transposon, such as *P. aeruginosa* (Liberati et al., 2006), *V. cholerae* (Cameron et al., 2008), and *E. piscicida* (Yang et al., 2017). With the development of sequencing technologies, 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



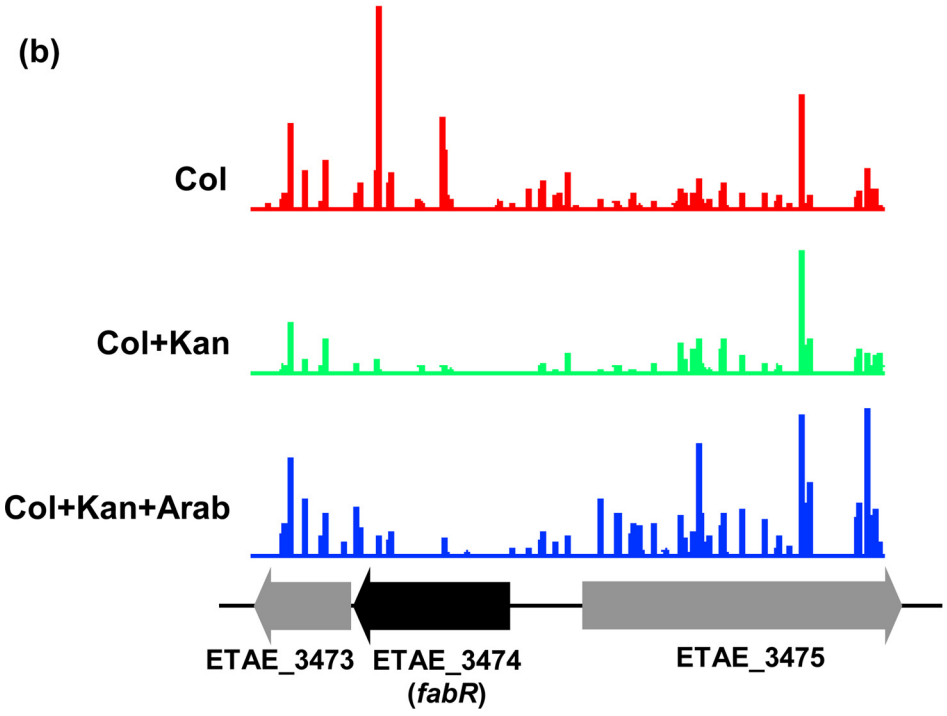


Fig. 3. The *esrB* upstream regulator screen using Tn-seq. (a) Scheme for screening of *ersB* upstream regulators. An *esrB* promoter-driven Kan^r cassette was inserted into the neutral site between *glms* and ETAE_3537 on *E. piscicida* chromosome. A transposon insertion mutant library (Input) was constructed using pMPR based on the strain carrying the P_{esrB}-Kan^r reporter, followed by exposure to Col + Kan and Col + Kan + Arab, respectively. After screening by Col + Kan and Col + Kan + Arab, the bacterial gDNA as well as that from Input libraries were extracted for next-generation sequencing. (b) Artemis screenshot of read abundance in ETAE_3474 (*fabR*) associated loci, detected in cells grown in Col (red), Col + Kan (green), Col + Kan + Arab (blue). The height of the column represents the relative read abundance of the insertion site. The read abundance represents the relative ratio or number of the mutants in library in the corresponding conditions.

facilitating researchers in performing genome-wide explorations in a wide range of bacterial species in 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 was dependent on transposon location. Interestingly, the highest GFP/mCherry ratios were observed in the strains containing insertions within the *rpsU* gene (Versalovic et al., 1993) (Fig. 2b), which encodes the protein S21 and is an essential component of the 30S rRNA. 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 the genome, the function of *rpsU* would not be necessarily deprived by the transposon insertion. In addition, the expression of *luxAB* in the strain of *potD*::Tn demonstrated that the designed *luxAB* on transposon could be facilitated 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). Besides *fabR*, various genes such as *rpoS*, *lon*, and ETAE_2184 were screened out by Tn-seq to be putatively involved in the *esrB* expression. Although ETAE_2184 was proved to be a false

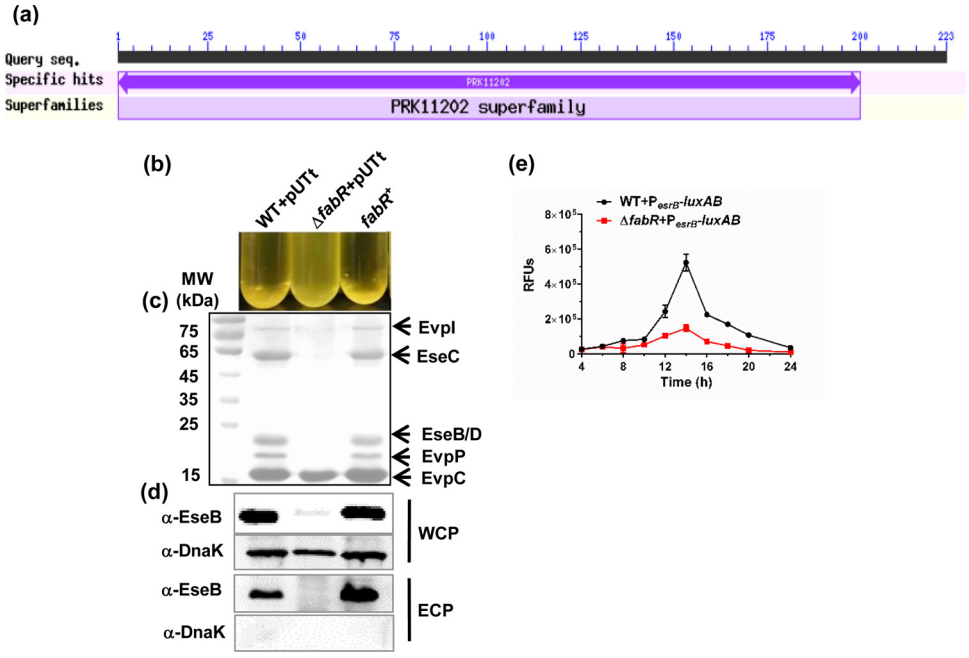


Fig. 4. Activation of T3SS expression by FabR in *E. piscicida*. (a) FabR belongs to PRK11202 superfamily. (b) Auto-aggregation phenotypes of WT, Δ*fabR*, and *fabR*$^+$, after 24 h of static growth. The empty plasmid pUTt was introduced as control in WT and Δ*fabR*. (c) The extracellular proteins (ECPs) of the indicated strains were collected from the equal volume of cells, and resolved by SDS-PAGE. More than three independent replicates were performed. (d) EseB production was detected by Western blotting with anti-EseB and anti-DnaK antibody was used as a control. More than three independent replicates were performed. (d) The P_{esrB}-*luxAB* expression plasmid was transferred into the WT and Δ*fabR* strains, followed by luciferase detection every 2 h. More than three independent replicates were performed. Mean with SD is shown.

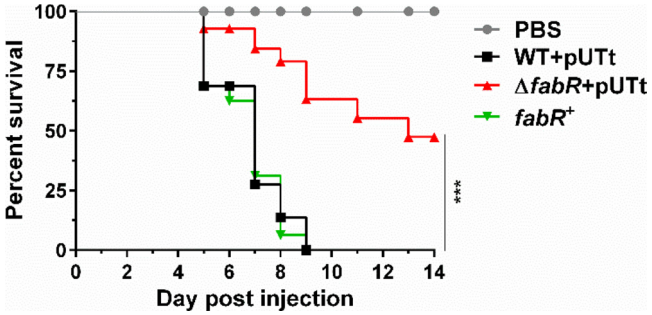


Fig. 5. Virulence assessments of WT, Δ*fabR*, and *fabR*$^+$ in turbot. The empty plasmid pUTt was introduced as control in WT and Δ*fabR*. Percent survivals of turbot were recorded for the indicated bacterial strains inoculated at a dose of 5.0×10^4 CFU/fish during a 14-day observation. ***, $P < 0.001$ based on Kaplan-Meier survival analysis with a log-rank test (Mantel-Cox).

positive (data not shown), *rpoS* and *lon* have been recently established to be essential for T3/T6SS expression by mediating the transcription of *esrB* (Yin et al., 2018). FabR was revealed to activate T3/T6SS expression through *esrB* (Fig. 4b-e), and showed virulence attenuation in its natural host turbot (Fig. 5) (Xiao et al., 2008). FabR, which is the master regulator of fatty acid metabolism, directly represses the expression of *fabA* and *fabB*, which could catalyze saturated fatty acids into unsaturated fatty acids (Zhang et al., 2002; Feng and Cronan, 2011). Therefore, FabR activating the expression of T3/T6SS may be dependent on fatty acid composition or metabolism.

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 the exploration of *esrB* upstream regulators. FabR was identified to activate T3/T6SS expression *in vivo* and *in vitro* conditions and was essential for virulence in *E. piscicida* in turbot.

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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