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Pattern analysis of conditional essentiality (PACE)-based heuristic identification of an *in vivo* colonization determinant as a novel target for the construction of a live attenuated vaccine against *Edwardsiella piscicida*

Ruiqing Ma[a], Guanhua Yang[a], Rongjing Xu[d], Xiaohong Liu[a,c], Yuanxing Zhang[a,c], Yue Ma[a,c,**], Qiyao Wang[a,b,c,*]

[a] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China
[c] Shanghai Engineering Research Center of Maricultured Animal Vaccines, Shanghai, 200237, China
[d] Yantai Tianyuan Aquatic Co., Ltd., Yantai, Shandong, China

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ABSTRACT

Edwardsiella piscicida is the aetiological agent of fish edwardsiellosis, causing huge economic losses in aquaculture industries. The use of a live attenuated vaccine (LAV) will be an effective strategy to control the disease in farmed fish. Thus, methods facilitating exploration of targets used for construction of an LAV will be of great significance. Previously, we devised an algorithm termed pattern analysis of conditional essentiality (PACE) to perform genome-wide analysis of the temporal dynamic behaviour of *E. piscicida* mutants colonizing turbot. Here, we correlated the conditional essentiality patterns of the PACE-derived colonization determinants with that of the *aroC* gene encoding chorismate synthase, the established target for LAV construction in *E. piscicida,* and identified ETAE_0023 as a novel valuable LAV target. ETAE_0023 encodes an uncharacterized DcrB family protein. Deletion of ETAE_0023 dramatically impaired *E. piscicida* invasion capability in ZF4 cells as well as colonization in fish and resulted in *in vivo* clearance at ~30 days post-infection. Δ*ETAE_0023* showed an ~2500-fold higher 50% lethal dose (LD_{50}) than that of the wild type strain. Vaccination with Δ*ETAE_*0023 by intraperitoneal (i.p.) injection upregulated expression of immune factors, i.e., IL-1β, IgM, MHC-I and MHC-II, and produced significantly high levels of *E. piscicida*-specific IgM as well as serum bactericidal capacities in turbot. Moreover, a single i.p. inoculation with Δ*ETAE_0023* generated significant protection comparable to the established WED LAV strain in turbot against challenge with the wild type strain after 5 weeks of vaccination. Taken together, we demonstrated a PACE-based method for heuristic identification of targets for LAV construction and presented Δ*ETAE_0023* as a new LAV candidate against edwardsiellosis.

1. Introduction

Edwardsiella piscicida (formerly included in *Edwardsiella tarda*) is a Gram-negative, rod-shaped bacterium leading to edwardsiellosis for over 20 species of piscine hosts, including both freshwater and marine fish [1,2]. In recent years, *E. piscicida* infection has resulted in great economic losses for cultured flatfish, including turbot and flounder, worldwide [3]. Since antibiotic abuse represents a problematic method of treating bacterial infections in the aquaculture industry and has led to the evolution of multi-drug resistance strains, it is urgent to develop vaccines for the prevention and treatment of this destructive pathogen.

Live attenuated vaccines (LAVs) are an effective way to elicit protective immune responses and thus provide more persistent memory responses, a higher survival rate of fish, and more robust humoral and cell-mediated immune responses [4]. Until now, a number of potential virulence factors associated with the pathogenicity of *Edwardsiella piscicida* have been used as targets to produce LAVs, including haemolysis-associated protein EthA, the twin-arginine translocation system TatABCD, and chorismic acid synthetase AroC [5–7]. Moreover, similar to other enteropathogenic bacteria, the type III secretion system (T3SS) also plays important roles in the process of infection by *E. piscicida* [8,9], and the T3SS has also been used as a target in the construction of the LAV strain WED [10]. All these targets are identified by the trial-and-error method by following the literature related to investigations in

* Corresponding author. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China.
** Corresponding author. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China.
E-mail addresses: mymarine@ecust.edu.cn (Y. Ma), oaiwqiyao@ecust.edu.cn (Q. Wang).

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other enteropathogenic bacteria. It will be of interest to identify other targets for highly efficient LAV development, especially from the pool of ~28% hypothetical or uncharacterized genes [11].

Transposon insertion sequencing (TIS) is a genome-wide screening method coupling transposon mutagenesis and high-throughput sequencing [12], which has been employed for gene function research in a wide range of bacterial species [13]. TIS is also an efficient way to identify putative gene function, greatly facilitating gene annotations with unprecedented depth [14]. In TIS analysis of high-density transposon insertion libraries, insertion frequency at each locus or the relative abundance of corresponding mutants is generally inversely correlated with the locus's contribution to fitness following the imposition of a selective pressure, such as a host, antibiotics and so on [13]. Recently, we established an algorithm termed pattern analysis of conditional essentiality (PACE) to perform time series analysis of TIS data to characterize the fitness profiles and dynamic behaviour of *E. piscicida* mutants colonizing turbot [15]. In this study, we further carried out a heuristic search for new LAV targets based on the previously established virulence attenuation gene *aroC*, encoding chorismate synthase. Our investigation indicated that ETAE_0023 encodes an uncharacterized DcrB family protein in *E. piscicida* that could be exploited as a novel LAV target. This work advances a new LAV design method and presents Δ*ETAE_0023* as a new LAV candidate against edwardsiellosis.

2. Materials and methods

2.1. Bacterial and cell strains, media, and culture conditions

The bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2. *Escherichia coli* strains and *E. piscicida* were cultured in Luria Bertani (LB) broth at 37 °C and 30 °C, respectively. *E. coli* CC118 λ*pir* [16] was used for plasmid preparation. Plasmids were introduced into *E. coli* strains by transformation and into *E. piscicida* strains through conjugation with *E. coli* SM10 λ*pir* [17]. When required, ampicillin (Amp), colistin (Col), chloramphenicol (Cm) and kanamycin (Kan) were added at final concentrations of 50, 12.5, 34 and 50 μg/ml, respectively. The *E. piscicida* FKC suspensions were produced as described [18]. ZF4 zebrafish fibroblast cells (ATCC CRL-2050) were cultured in DMEM-Ham's F-12 medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco) in a 5% CO_2 atmosphere.

2.2. Construction of in-frame deletion mutants

The in-frame deletion mutants were generated by *sacB*-based allelic exchange as previously described [19]. The upstream and downstream fragments were amplified by PCR with primer pairs P1/P2 and P3/P4 (Table 2), respectively. The product was cloned into the suicide vector pDM4 with BglII/SphI sites and transformed into CC118 λ*pir*. The resulting plasmid was validated by sequencing with the primer pair pDM4-PF/PR and then transformed into SM10 λ*pir*. The correct plasmid was mated into WT(Δp) by conjugation and integrated into the chromosome by homologous recombination. Single crossover recombination events were selected on LB agar medium containing Cm and Col, while double crossover recombination events were selected on LB agar medium containing 12% sucrose. The mutant was confirmed by PCR and sequencing using the primers OUT-F/R.

2.3. Turbot colonization and survival assays

The 3-month-old turbot from a commercial farm (Tianyuan Corp., Yantai, China) weighing approx. 25 ± 3 g were maintained in aerated tanks supplied with a continuous flow of sand-filtered seawater at 15 ± 1 °C. Fish were fed 48 h before and after the inoculation. In addition, whether the fish were infected with *E. piscicida* was first analysed by plating tissues including liver, kidney and spleen on DHL selective plates (Difco, Detroit, USA). For sacrifice, fish were anaesthetized (10 min) in sea water supplemented with MS-222 (0.02% v/v) and aseptically dissected to harvest tissues.

For colonization assays, bacteria resuspended in phosphate-buffered saline (PBS) were injected into fish by the intraperitoneal (i.p.) route with 3×10^5 CFU/fish, and tissues dissected from fish ($n = 5$) were harvested at different days post-infection (DPI). The cell-forming units (CFU) per gram of tissue were enumerated by plating homogenized tissue on DHL agar containing appropriate antibiotics. For fish survival assays, bacteria resuspended in PBS were injected into fish by the i.p. route with four concentration gradients ($3 \times 10^5 \sim 10^8$ CFU/fish) and fish survival was monitored over the following 30 days. The LD_{50} values were calculated as previously described [10].

2.4. Cell invasion assays

ZF4 cells were used for the cell infection assays, in which cells were seeded in 24-well plates at 10^5 cells per well. *E. piscicida* was washed twice with PBS and resuspended in DMEM. Then, bacteria were added to ZF4 cells at a multiplicity of infection (MOI) of 50:1. The plates were then centrifuged at 600 g for 10 min at 28 °C. After a 2-h incubation, every well was washed with PBS 3 times, and the cells were lysed with 1% Triton-X 100 for 30 min. The bacterial load was enumerated by plating the lysate on DHL agar medium. The invasion rate was the ratio of bacterial CFU to ZF4 cell number.

2.5. Serum bactericidal ability

After 28 days post-vaccination, peripheral blood was collected from the caudal vein of each vaccinated fish [15]. After clotting at 4 °C for 1 h, serum was separated by centrifugation at 3,000 rpm at 4 °C for 10 min. The serum of five fish inoculated with the same LAV candidate was pooled and then stored at −80 °C for serum bactericidal and ELISA tests. To test bacterial survival in serum, 30 μl of a wild type (WT) strain

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Characteristics	Reference or source
Edwardsiella piscicida		
EIB202	Wild type strain (formerly known as *E. tarda*), CCTCC M 208068, Col^r, Cm^r, Kan^s	[10]
EIB202Δp	Wild type strain, pEIB202-cured, Col^r, Cm^s, Kan^s	[18]
Δ*aroC*	Δ*aroC*Δp, in-frame deletion of *aroC*, Col^r, Cm^s, Kan^s	[18]
WED	EIB202Δp, in-frame deletion of *eseB*, *eseC*, *eseD*, *escA* and *aroC*, CCTCC M 2010278, Col^r, Cm^s, Kan^s	[18]
Δ*ETAE_0023*	EIB202Δp, in-frame deletion of *ETAE_0023*	This study
Escherichia coli		
SM10 λpir	*thi thr leu tonA lacy supE recA*::*RP4-2-Tc*::*Mu*, *pirR6K*, Kan^r	[16]
CC118 λ*pir*	λ*pir* lysogen of CC118, Δ (*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA1*	[16]
Plasmid		
pMar2xT7	Mariner Himar1 transposon encoding a gentamycin resistance gene. Suicide plasmid, *pir* dependent, R6K, Amp^r, Gm^r	Lab collection
pDM4	Suicide plasmid, *pir* dependent, R6K, SacBR, Cm^r	[26]

Table 2
Primers used in this study.

Primers	Sequence (5′–3′)
P1	GAGCTCAGGTTACCCGGATCTATTGCCCGTCGCACAGGGTATTTTTTC
P2	CGACGCTTACATGGAAATTCCTTATGTTGTCCGA
P3	ATTTCCATGTAAGCGTCGCGGGGGGCCGACAGAC
P4	CCCTCGAGTACGCGTCACTAGTGGGGCCCTTGCAGTTCAATGCCAGCGAGGCTAC
OUT-F	CAGTGGAATGGATCGCGCCGGAGCC
OUT-R	TATTAACCCCGACTTTCCATTTCAG
IN-F	GGGCTGTTGGTTACCGGACT
IN-R	GCGATATTGGCCGCTTCTTG
IL-1-F	GGAGTGCAACATGAGCGAGAT
IL-1-R	TCATGGGATGGTGGGAGATC
MHC-I-F	CATCGCTGCCATTGGAGTCT
MHC-I-R	CCCTGCGTTGTTTACAGGAGAT
MHC-II-F	TGTCCTCAGTGTCTCTGCTGAAG
MHC-II-R	GTATGTCTCCTCTCCACCAGTGTCT
IgM-F	GTGTTCCCAGTCCTAGACCTGACT
IgM-R	CGAGTACACTAGAAATGCAGTGATACAG
β-actin-F	TGAACCCCAAAGCCAACAGG
β-actin-R	AGAGGCATACAGGGACAGCAC

suspension with 10^6 CFU was mixed with 270 μl of serum and incubated at 30 °C. Bacterial survival was assessed 8 h after inoculation ($n = 3$). The assay was performed in triplicate three independent times.

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA assays were performed to measure the serum antibodies against *E. piscicida* [18]. Microtiter plate wells were overnight coated with 100 μl of formalin-killed cells (FKC) of the WT strain at 4 °C. Excess cells were discarded, and wells were blocked with 100 μl of PBS containing 2% bovine serum albumin (BSA) for 3 h at 22 °C. The blocking solution was then removed, and every well was washed three times with PBST (PBS + 0.05% Tween-20). The wells were then incubated for 3 h at 22 °C with 100 μl turbot serum diluted 20-fold with PBS and washed with PBST three times. The wells were then incubated with 100 μl of mouse anti-turbot IgM monoclonal antibody (Aquatic Diagnostic, Stirling, UK) for 1 h, washed with PBS 3 times, and further incubated with 100 μl of goat anti-mouse IgG-HRP (Tiangen, Beijing, China) (1:1000) for 1 h at 22 °C. Finally, the wells were washed three times, followed by the addition of TMB solution (Tiangen, Beijing, China) as a colour-developing substrate. Reactions were terminated by the addition of 5 μl of 2 M H_2SO_4, and the absorbance at 450 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA).

2.7. RNA extraction and quantitative real-time reverse transcriptase-PCR (qRT-PCR)

Total RNA extraction and qRT-PCR detection of bacteria and head-kieney were performed as described previously [18]. Primers used for qRT-PCR were listed in Table 2. All the analysis of relative gene expression were determined by $\Delta\Delta C_T$ method with β-actin as reference control.

2.8. Immunization and challenge

Each vaccine strain was intraperitoneally injected at a dose of $\sim 3 \times 10^5$ CFU/fish into a 3-month-old turbot (25 ± 3 g; $\sim 1.2 \times 10^4$ CFU/g) with formalin-killed bacteria and PBS as negative controls. Fish were maintained in 1,000 L tanks at 15 ± 1 °C supplied with sand-filtered sea water as previously described for 5 weeks. For challenge experiments, fish were challenged by the intramuscular (i.m.) route with 2×10^3 CFU/fish (approx. $2 \times LD_{50}$ for i.m. injection) of WT cells. All challenge tests were performed in triplicate with 30 fish for each group. The mortality of challenged fish was recorded daily for 28 days after inoculation, and the relative protection ratio (RPS) of the vaccinated group was calculated as follows [18]:

$$RPS = 100\% \times \left(1 - \frac{mortality\ of\ vaccinated\ fish}{mortality\ of\ control\ fish}\right)$$

Additionally, WT colonization in the kidney (CFU/g) was determined at 5, 10, 15, 20, and 28 DPI ($n = 5$ fish per timepoint).

2.9. Ethics statement

All animal protocols used in this study were approved by the animal care committee of the East China University of Science and Technology (2006272). The Experimental Animal Care and Use Guidelines from the Ministry of Science and Technology of China (MOST-2011-02) were strictly followed.

2.10. Statistical analysis

Statistical significance was determined with GraphPad Prism 6.0 for Windows as indicated in the experiments. Significant differences were considered at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

3. Results

3.1. In vivo PACE discovered ETAE_0023 as a potential target for the construction of an LAV

Previously, we devised an algorithm termed pattern analysis of conditional essentiality (PACE) to perform genome-wide analysis of the temporal dynamic behaviour of *E. piscicida* mutants colonizing turbot [15]. From a temporal series of TIS data, PACE derived a quantitative evaluation of each mutant's fitness over the course of the infection and identified 364 *in vivo* decreasing (IVD) genes and assigned these genes into 4 clusters. We have shown that cluster 2 genes could be used as targets for LAV development because they exhibit similar *in vivo* fitness dynamic patterns to those of T3SS and T6SS genes. Here, we used a heuristics approach to identify more novel targets for LAV construction. We hypothesize that the clearance of the mutant by the host immune system is essential for the safety of a live vaccine strain. As the cluster 2, 3, and 4 genes have mean $\log_2$(FC) values that converge by day 14, we set the average value of $\log_2$(FC) at 8, 11, 14 days post-injection as the outbreak index to identify genes with similar fitness in the late stages of infection. On the other hand, we correlated the genes' dynamic PACE patterns to that of an established LAV target, *rpoS*. We focused on the 156 *in vivo* conditionally essential genes (including 103 IVD genes) and plotted their corresponding correlation to the RpoS pattern against the

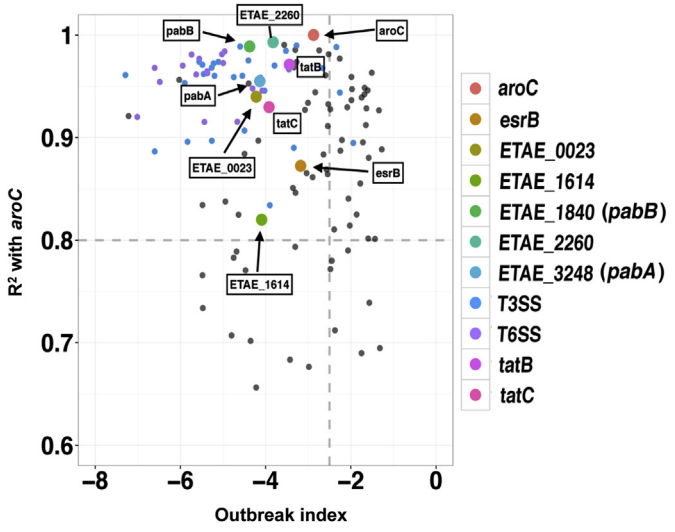

Fig. 1. Heuristic clustering analysis of fitness dynamics of *in vivo* conditionally essential genes based on PACE analysis. The fitness of the gene during the late infection phase (outbreak index) was plotted against the correlation (R^2) to relative abundance time series curves of the known *in vivo* conditional essential gene *aroC*. PACE analysis of previous transposon insertion sequencing (TIS) data established 156 *in vivo* conditional essential gens in *E. piscicida* [15]. The outbreak index is the average value of log_2(FC) at 8, 11, and 14 days post-injection (DPI) relative to the initial abundance of the insertions in the gene before injection (input). The known virulence factors or genes controlling *in vivo* colonization in *E. piscicida* are highlighted.

outbreak index (Fig. 1). Using a cut-off outbreak index < -2.5 and R^2 with the *aroC* mutant > 0.8, our analysis included ~75 genes that fit into the category of most likely to be an LAV target, including almost all the established LAV target genes for T3SS and T6SS, *esrB*, *tatBC* as well as *phbAB* genes [15], thus validating our screen analysis. We chose ETAE_0023 (outbreak index, −4.05; R^2, 0.94) as the potential target for the construction of an LAV candidate to further assess our PACE-facilitated vaccine design strategy. Lower colonization ability (outbreak index) of the ETAE_0023 insertion mutant than the *aroC* mutant will be a benefit as an LAV in terms of biosafety. Compared with the master T3SS virulence factor mutant strain [8], the transposon-inserted *ETAE_0023* mutant strain exhibited a similar defective colonization dynamic curve in turbot (Fig. 2A). Analysis of the survival curves in turbot with PACE revealed that the mutant of *ETAE_0023* in cluster 2 has a high correlation with the mutant of the reported vaccine target *aroC,* which controls the biosynthesis of chorismic acid [7], as well as other reported targets, including *pabA*, *pabB*, *tatB* and *tatC* [5,15]. There should be little or no polar effects for the insertion in ETAE_0023 on neighbouring genes, as the disruption in ETAE_0022 and 0024 did not show significant *in vivo* growth deficiency (Fig. 2A and B). *ETAE_0023* was annotated as a conserved hypothetical protein belonging to the DcrB family in *E. piscicida* and may be involved in membrane homeostasis and phage absorption. We then constructed an in-frame deletion mutant of *ETAE_0023,* and the deletion had no influence on the growth of *E. piscicida* in LB (Fig. 2C) as well as the production of T3SS and T6SS proteins (Fig. 2D). Moreover, Taken together, we identified a conserved hypothetical gene, ETAE_0023, as a potential virulence attenuation target in *E. piscicida.*

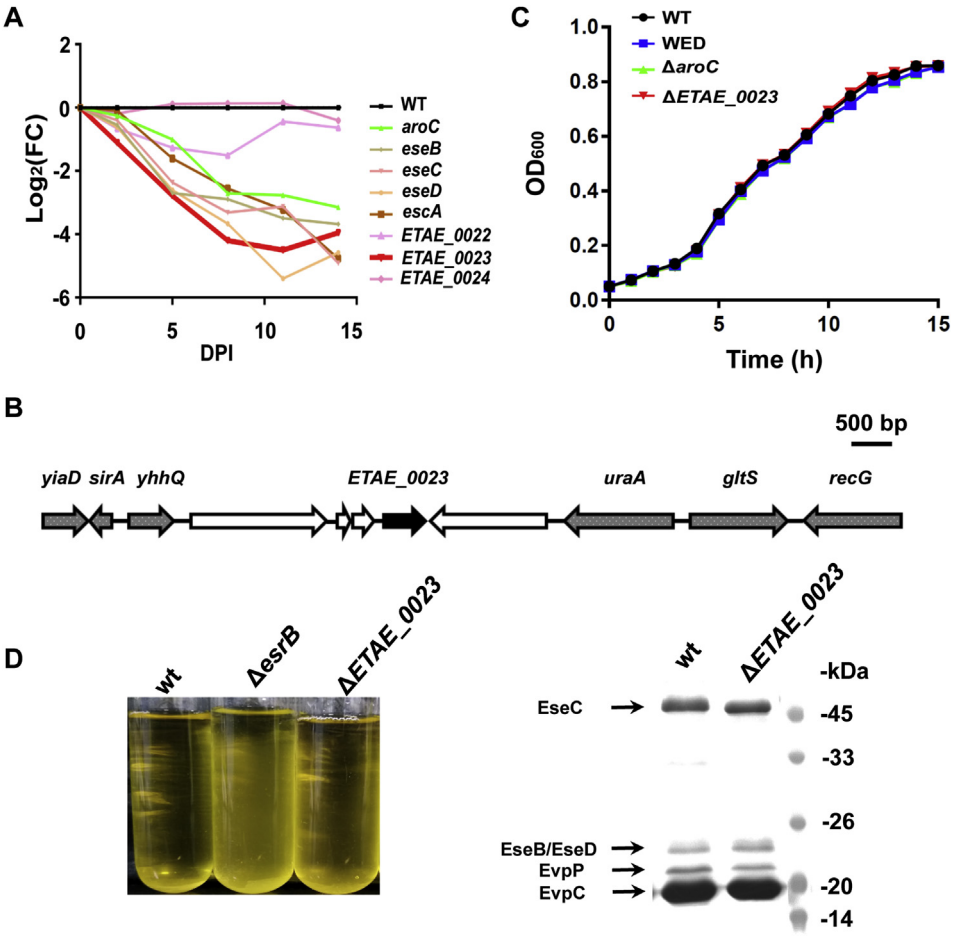

Fig. 2. PACE identifies *ETAE_0023* as an *in vivo* colonization-associated determinant in *E. piscicida*. **A.** *In vivo* time series TIS analysis of dynamic colonization by specific mutants. The PACE-derived dynamic fold change (FC) data [15] for each transposon insertion mutant strain recovered from liver relative to the WT strain ere plotted against the days post-injection (DPI) by intraperitoneal (i.p.) injection in turbot. Relative abundance time series curves of WT and mutant strains with insertions in typical virulence factors (T3SS genes), aromatic amino acid biosynthesis (*aroC*), *ETAE_0023*, and the neighbouring genes (0022 and 0024) are shown. **B.** The location of *ETAE_0023* in the *E. piscicida* genome. *ETAE_0023* is marked with a black box, and white boxes are annotated as hypothetical genes. **C.** Growth curve of the WT, WED, Δ*aroC*, and Δ*ETAE_0023* strains in LB at 30 °C. **D.** Auto-aggregation and production of T3SS and T6SS proteins in extracellular proteins shown in SDS-PAGE analysis. The T3/T6SS regulator *esrB* mutant was used a negative control.

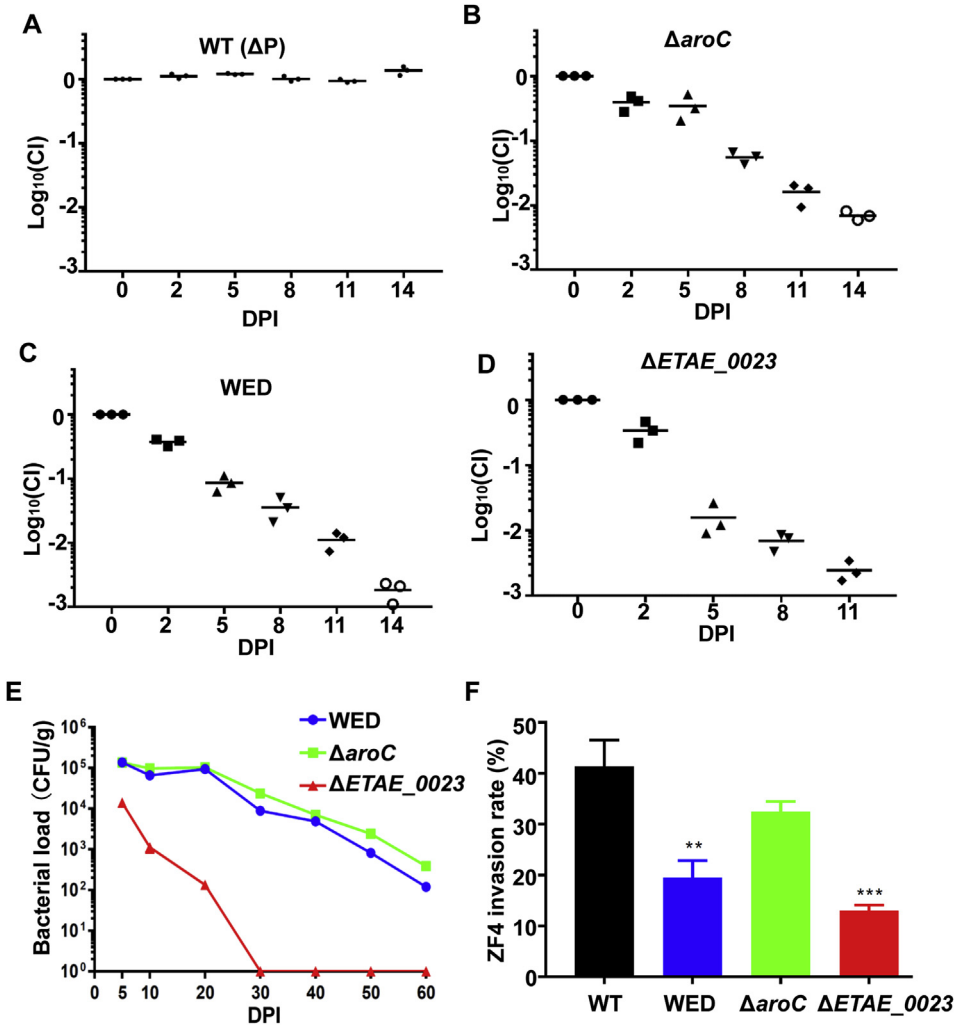

Fig. 3. Validation of the roles of *ETAE_0023* in *in vivo* colonization in *E. piscicida*. A-D. Competitive index (CI) assays for the indicated strains (WED, Δ*aroC*, and Δ*ETAE_0023*) compared with the WT strain carrying Cm-resistant pEIB202 at the indicated time points. The CIs of WT vs. WT(ΔP), WT strain cured of pEIB202, are shown. **E.** Bacterial loads recovered from kidneys of fish i.p. infected with a dose of 3×10^5 CFU/fish during 60 DPI. **F.** ZF4 invasion capabilities of the indicated strains with an MOI = 5 0. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, based on one-way ANOVA and Fisher's LSD multiple comparison post-test.

3.2. ΔETAE_0023 displayed reduced viability in vivo

To verify the abovementioned TIS results and the virulence attenuation resulting from mutagenesis in *ETAE_0023*, we first determined the competition index (CI) for Δ*ETAE_0023* vs. the WT strain. The previously established control strain WT(ΔP), WT with the endogenous R plasmid pEIB202 cured, showed no *in vivo* colonization defect (Fig. 3A). The reported LAV strains Δ*aroC* and WED with T3SS and *aroC* knockout and pEIB202 cured [18] exhibited gradually reduced colonization and were eventually outcompeted by WT (Fig. 3B and C). Similarly, the CI results for equally mixed Δ*ETAE_0023* and WT also indicated that Δ*ETAE_0023* was gradually cleared away in turbot during the 2-week observation period (Fig. 3D). Meanwhile, the LD_{50} values of these strains were also determined with doses from 10^5 to 10^9 CFU/fish. The LD_{50} value for all the mutant strains was higher than that for WT, which was 4.8×10^3 CFU/g after 4 weeks (Table 3). Notably, the LD_{50} value of Δ*ETAE_0023* was 1.2×10^7 CFU/g, ~2,600-fold higher than that of WT. Previous reports showed *E. piscicida* colonized the turbot kidney more robustly than the liver [18]. We further inoculated the LAV candidates Δ*aroC*, WED and Δ*ETAE_0023* in the fish and recovered these bacteria from kidney which at different days two months post-inoculation to monitor their dynamic survival in turbot. As shown in the kidney, Δ*ETAE_0023* was cleared away by the host after 30 days, which is mostly earlier than Δ*aroC* and WED (Fig. 3E and data not shown). In addition, bacterial invasion into ZF4 cells by Δ*ETAE_0023* was significantly lower than by WT (Fig. 3F). Taken together, these data demonstrated an essential role for ETAE_0023 in *in vivo* colonization.

Table 3
LD_{50} value of the mutant strains and their attenuation folds compared with the WT strain.

LAV strain	Genotype	Annotation	LD_{50} (CFU/g)	Attenuation fold[a]
WED	ΔT3SS; Δ*aroC*	T3SS components; *aroC*	1.17×10^7	2603
Δ*aroC*	Δ*aroC*	chorismate synthase	5.44×10^6	1125
Δ*ETAE_0023*	Δ*ETAE_0023*	conserved hypothetical protein	1.16×10^7	2589

[a] The LD_{50} of the WT strain was 4.48×10^3 CFU/g.

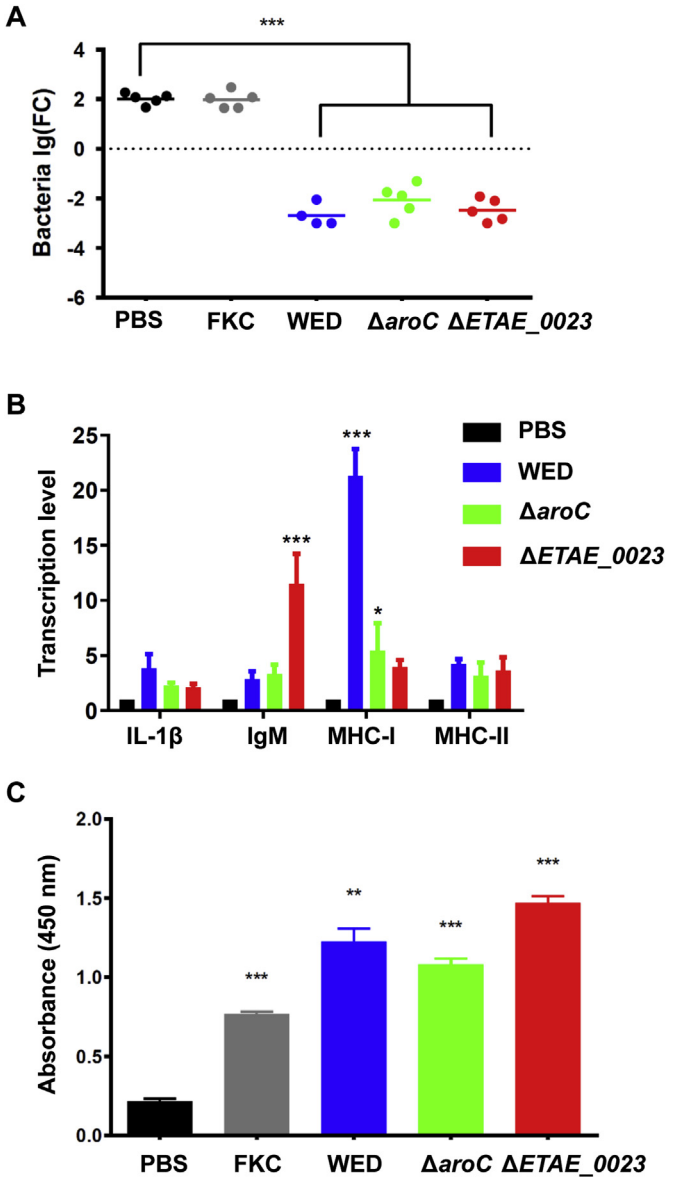

Fig. 4. Protective immune responses in naïve turbot induced by inoculation with Δ*ETAE_0023*. **A.** Bactericidal capacities of turbot serum after inoculation with PBS or the indicated vaccines. *E. piscicida* EIB202 was incubated with serum extracted from vaccinated turbot for 8 h, and the CFU was counted. FC is the fold change of the final CFU relative to the initial CFU for each serum sample ($n = 5$). **B.** Expression of immune-related genes in head kidneys induced by vaccination 30 DPI. The mRNA level of each gene was normalized to β-actin utilizing the $\Delta\Delta C_T$ method. Triplicate assays were performed with the head kidneys from at least three independent fish 30 DPI. **C.** Levels of *E. piscicida*-specific serum IgM in turbot 30 DPI were assayed by ELISA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ based on one-way ANOVA and Fisher's LSD multiple comparison post-test.

3.3. Immune response of turbot elicited by ΔETAE_0023

Serum bactericidal activity induced by Δ*ETAE_0023* was tested 30 days post-vaccination. PBS- and formalin-killed WT bacteria (FKC)-treated fish were also included in the analysis. FKC could not trigger bactericidal activities in turbot serum [15]. Similar to WED and Δ*aroC*, Δ*ETAE_0023* could also generate significant bactericidal activity in the serum of turbot (Fig. 4A). To monitor immune responses stimulated by Δ*ETAE_0023,* transcription levels of immune-associated genes in head kidneys after inoculation with Δ*ETAE_0023* were then analysed by qRT-PCR. The results showed that Δ*ETAE_0023* upregulated the expression

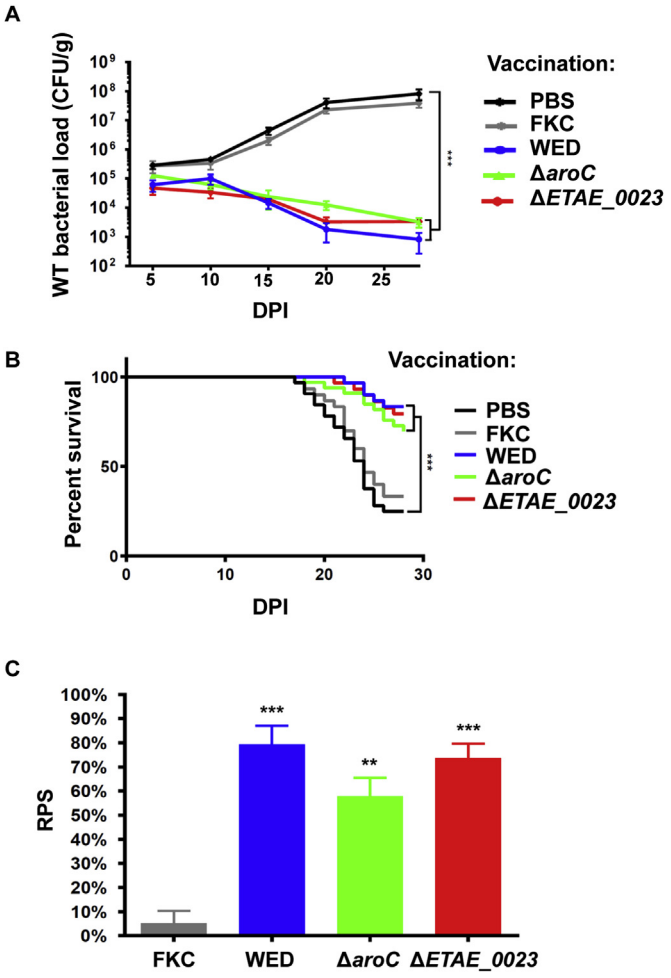

Fig. 5. Δ*ETAE_*0023 is a live attenuated vaccine that induces protective immunity in turbot. **A.** Bacterial loads in kidneys of vaccinated fish after challenge with WT (Cm^r) *E. piscicida* EIB202. The mean and SEM CFU per gram of tissue are shown ($n = 5$ fish per time point). **B.** Survival of vaccinated turbot challenged with WT *E. piscicida* EIB202. A total of 90 fish were challenged for each group to monitor their survival 30 days after vaccination. ***, $P < 0.001$ comparing the LAV vaccine strain group with the PBS group using Kaplan-Meier survival analysis with a log rank test (Mantel-Cox). **C.** Relative protection index (RPS ± SEM) of each vaccine candidate, based on mortality at 28 DPI for $n = 3$ groups of 30 challenged fish.

of all the tested immune-related genes, i.e., IL-1β, IgM, MHC-1, and MHC-II, which have been revealed to associate the protective immune responses triggered by WED in head kidneys [18]. In particular, a significantly higher level of IgM transcript was generated by Δ*ETAE_0023* than by WED and Δ*aroC* (Fig. 4B). Moreover, Δ*ETAE_0023* was confirmed to generate a significantly higher titre of *E. piscicida*-specific IgM in serum in fish than in the PBS-treated group of fish as analysed by ELISA (Fig. 4C). These results indicated that Δ*ETAE_0023* can elicit a strong immune response in turbot as an LAV candidate.

3.4. Immune protection capacity of turbot vaccinated with ΔETAE_0023

Fish vaccinated with the LAV candidates, i.e., Δ*ETAE_0023*, Δ*aroC*, and WED, as well as FKC were then challenged with WT *E. piscicida* 5 weeks after inoculation. During 28 days of observation, WT bacterial load declined over time in Δ*ETAE_0023*-inoculated fish in a similar to WED- and Δ*aroC*-immunized fish (Fig. 5A). This result indicated that Δ*ETAE_0023* can trigger significant protection against an *E. piscicida* challenge as an LAV candidate. In addition, compared with the negative control fish treated with FKC or PBS, the fish vaccinated with

Δ*ETAE_0023* showed largely delayed death (21 dpi) and significantly lower mortality (RPS = 73.7%), which is parallel to WED (22 dpi, RPS = 79.4%) and better than Δ*aroC* vaccination (~20 dpi, RPS = 57.8%) (Fig. 5B and C). Consequently, a markedly higher immune protection capacity was observed for the LAV candidate Δ*ETAE_0023* group than for the FKC-immunized group. Collectively, these assays demonstrated that the new LAV candidate strain Δ*ETAE_0023* can produce superior immune efficacy against *E. piscicida* for turbot and merits consideration as a potential vaccine candidate use by the aquaculture industry.

4. Discussion

Vaccination is a boon strategy to combat bacterial diseases in aquaculture industries. To date, several types of vaccines have been reported and used in aquaculture industries. LAV use for aquaculture has been paid much more attention for many years because it can stimulate cell-mediated immunity better than whole-cell killed vaccine administration [20]. In terms of *Edwardsiella* bacteria, LAVs for fish, such as the avirulent strains ATCC 15947 [21] and E22 [22] as well as a rifampicin-resistant mutant strain TX5RM [23], have been established as ideal candidates administered through oral, immersion and injection routes for various species of fish. However, their residual virulence, virulence recovery and reactogenicity might be major concerns for developing vaccine products. Thus, exploration of specific gene targets to generate virulence-attenuated vaccine candidates is essential for the development of an LAV. Previously, we performed systematic screens, and multiple genes in *E. piscicida* have been reported to be potential LAV candidates [5–7]. The genome sequence-based trials identified *aroC*-, T3SS-, and T6SS-related genes as well as the Tat translocation system as suitable loci for construction of an LAV; however, some of the genes, e.g., LPS, severely affect the fitness of the strain when disrupted, do not seem to be ideal LAV targets, and give rise to low protection against challenge by the WT strain in fish. All these so-called rational designs and constructions of LAVs are based on knowledge of the virulence and metabolism network in the context of *Salmonella*, *E. coli* or other phylogenetically related pathogens. The trial-and-error strategy is time consuming and sacrifices many experimental animals [24]. Moreover, all the LAV targets identified by this method are annotated genes, which exclude approximately 30% of the uncharacterized genes in the genome. Although the Vaccine Investigation and Online Information Network (VIOLIN) [25] provides a web-based central resource that integrates vaccine literature data mining, vaccine research data curation and storage, and curated vaccine data analysis for vaccines and vaccine candidates developed against various pathogens, including *E. piscicida*, species- or strain-specific LAV design will require functional genomics-based systematic screens for suitable gene target identification. In the present study, with the assistance of TIS and PACE, we were able to identify a hypothetical gene, *ETAE_0023*, for which the mutant strain had a similar survival pattern with authenticated targets, such as *aroC*. We thus demonstrated that this is a novel heuristic strategy for LAV target identification, which will facilitate the identification of more *Edwardsiella* bacterial-specific LAV targets. With the rapid development of functional genomics research technologies such as TIS technology, we believe that this strategy would also be adaptable to other pathogens for rapid LAV development.

In this study, we identified ETAE_0023 as a new LAV target that was essential for the colonization ability of *E. piscicida* (Figs. 2A and 3), thus endowing the deletion strain with a high level of biosafety. We were intrigued by which mechanisms Δ*ETAE_0023* was severely impaired in its virulence and infectivity towards host or fish cells. The expression of the major T3SS and T6SS virulence factors in Δ*ETAE_0023* was not different from that in WT in DMEM cultures (Fig. 2D). The cell invasion capacities of Δ*aroC*, which had intact T3SS expression, showed no significant difference with WT but significantly higher invasion than that of Δ*ETAE_0023* (Fig. 3F), further suggesting that ETAE_0023 might not be related to T3SS expression or function during infection. Δ*ETAE_0023* exhibited exceptionally weak colonization ability in turbot, which may be due to the loss of cell invasion ability, as shown in Fig. 3F. These features indicate that Δ*ETAE_0023* possesses high safety as an LAV. However, as bioinformatics analysis reveals a potential interaction of ETAE_0023 family proteins with phages, these proteins may be involved in prophage stability and cell lysis. Further investigation will be required for the illumination of its biological function.

Meanwhile, Δ*ETAE_0023* could also provide efficient immune protection against challenge by a highly pathogenic *E. piscicida* strain in turbot (Fig. 4), and the protection is comparable to or higher than that elicited by the established vaccine strains candidates WED and Δ*aroC* (Fig. 5). Interestingly, Δ*ETAE_0023* showed the highest level of IgM expression as well as *E. piscicida*-specific IgM titre. Moreover, the MHC-I expression patterns largely differed from those of other vaccines tested (Fig. 4B). These data suggest that even though they shared equal or comparable RPS levels against WT challenge, they may protect the fish through totally different immune mechanisms, therefore warranting further exploration of gene targets for LAV design as well as rational construction of LAVs. In terms of development of an ideal LAV strain, one must balance high biosafety and high virulence attenuation to enable longer *in vivo* clearance by the host to trigger high protective immune responses as well as immune memory in the host. Our heuristics searching strategy based on the *in vivo* colonization dynamics of a mutant strain will facilitate the identification of novel gene targets that meet the requirements of the abovementioned balance.

In conclusion, our study took advantage of high-throughput *in vivo* TIS data, discovered a conserved virulence-associated factor of *E. piscicida* and identified *ETAE_0023* as a new LAV target. This work is significant as it deepens our understanding of the virulence mechanism of *Edwardsiella* and gives new perspective for the exploitation of novel LAV targets for pathogenic bacteria.

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