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First insight into how stress exposure triggers *Vibrio harveyi* recipient successful conjugation

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Conjugation is the most common horizontal gene transfer (HGT) process that can be affected by environmental change and promote bacterial virulence and drug resistance. However, it is unknown whether environmental changes can influence the conjugation ability of the marine fish pathogen *Vibrio harveyi*, thereby affecting its pathogenicity and drug resistance. This study systematically analyzes the effect of environmental stress on the ability of *V. harveyi* to obtain shuttle plasmids from *Escherichia coli* during conjugation. The results indicate that *V. harveyi* cannot receive shuttle plasmid pMMB207 without exposure to stress. However, certain stress exposure (37–46°C, 4%–16% ethanol, 0.14–0.56 mM SDS, 0.04~0.05 M NaOH, and 0.012–0.024 M HCl for 5–60 minutes) in the log phase of *V. harveyi* before conjugation successfully induces the fertility of the *V. harveyi* recipient in intergeneric mating with *E. coli*. In particular, ethanol and heat stress showed strong induction with up to 2.5×10^5 and 5.3×10^3 transconjugants when exposed to 16% ethanol for 10 minutes and 40°C for 60 minutes, respectively. Additionally, appropriate levels of NaOH (0.05 M, 10 minutes), SDS (0.42 mM, 5 minutes), and HCl (0.024 M, 5 minutes) lead to 2.3×10^3, 4.5×10^2, and 1.8×10^2 transconjugants, respectively. These results will help establish homologous recombination gene knockout technology and greatly advance molecular theoretical research on *V. harveyi*. They will also support the establishment of disease prevention and control strategies based on the interruption of the HGT process by environmental regulation.

KEYWORDS

***Vibrio harveyi*, stress exposure, horizontal gene transfer, induced fertility, environmental regulation**

1 Introduction

About 1.6%–32.6% of genes in each microbial genome are obtained through horizontal gene transfer (HGT), which has become the most important driving force in the evolution of prokaryotes, affecting prokaryotic pathogenicity, drug resistance, metabolism, and so on (Koonin et al., 2001; Thomas and Nielsen, 2005). HGT mainly occurs in prokaryotes through three processes: transformation, transduction, and conjugation. Conjugation transfer is the

most likely HGT mode with the highest transfer efficiency in the environment due to the wide range of transferred genes, the lack of a requirement for homology and recombination, and the ability to cross species (Koonin et al., 2001).

Conjugation is a DNA exchange process that forms a "junction bridge" through the contact fusion of the cell membranes of the donor and recipient bacteria. Single-strand plasmids are transported across the cell membrane into recipient cells through the junction bridge, and complementary chains are synthesized to form new plasmid molecules (Thomas and Nielsen, 2005). As a result, conjugation requires the joint participation of the donor bacteria, recipient bacteria, and plasmid. Certain environmental stress can affect bacterial cell membrane status and immune systems, including restriction-modification (R-M) and clustered regu¬larly interspaced short palindromic repeat-associated protein (CRISPR) systems, thus affecting the conjugation efficiency and stability of foreign plasmids in recipient bacteria cells (Schäfer et al., 1994). For example, heat treatment at 50 °C for 20 minutes can inhibit the R-M system of *Salmonella typhimurium*, thus increasing its fertility in intergeneric mating with *Escherichia coli* by approximately 200 folds and enhancing the stability of hybrid recombinants (Mojica-a and Middleton, 1971). Additionally, exposure to 4%–20% ethanol for nine minutes increases the fertility of *Corynebacterium glutamicum* crosses with *E. coli* by up to 10^4 folds, probably by affecting the cytoplasmic membrane fluidity (Schäfer et al., 1994).

In recent years, *Vibrio harveyi* has become the most dominant Vibrio pathogenic bacteria of marine fish in the South China Sea, causing massive economic losses to the aquaculture industry (Deng et al., 2020a; Deng et al., 2020b). It is reported that *V. harveyi* can obtain foreign DNA through HGT, increasing its pathogenicity and drug resistance, the complexity and variability of Vibrio disease prevention and control, and posing a significant threat to breeding and food safety (Ruwandeepika et al., 2010; Deng et al., 2019; Deng et al., 2020b). In addition, the temperature rise caused by global warming and the environmental pollution caused by human activities can seriously affect the sustainable development of aquaculture (Khoshnevis Yazdi and Shakouri, 2010; Zhou et al., 2021). However, it is unknown whether the environmental changes affect the HGT of *V. harveyi*, thus impacting their pathogenicity and drug resistance. Furthermore, it was found that the laboratory conjugation transfer efficiency of *V. harveyi* is extremely low and normally unsuccessful. This precludes the use of conjugation to transfer suicide plasmids from the donor bacteria to the recipient bacteria in order to carry out in-frame gene mutations, seriously limiting research into the regulation of virulence and drug resistance in *V. harveyi*.

In this study, we investigated the effects of different environmental stresses (heat, ethanol, SDS, acid, alkali, and salinity) on the conjugation efficiency of *V. harveyi* recipient crosses with *E. coli*. This will provide new insights into the mechanisms of pathogenicity and drug resistance in *V. harveyi*, in addition to an important scientific foundation for in-frame deletions and will establish strategies for preventing and controlling outbreaks of *V. harveyi* disease based on the blocking of the conjugation transfer process.

2 Materials and methods

2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The conjugative receptor strain *V. harveyi* 345 was isolated from a diseased pearl gentian grouper with multi-drug resistance and a median lethal dose of 9.83×10^5 CFU·g^{-1} (Deng et al., 2019). It was cultured in Luria–Bertani (LB) broth with 2% additional NaCl (LBS) at 28°C. The mobilizable shuttle plasmid pMMB207 (Figure S1) is chloramphenicol (Cm) resistant and can be transferred *via* conjugation from *E. coli* to a variety of bacteria including *Aeromonas hydrophila*, *V. alginolyticus*, *V. campbellii*, *V anguillarum*, and *V. parahaemolyticus* (Zhang, 2012; Pang, 2015; Liu et al., 2017; Li et al., 2022). The conjugative donor strain, *E. coli* GEB883-pMMB207, was the GEB883 strain with pMMB207. It was cultured in LB with 20 μg/ml Cm and 0.3 mM diaminopimelate (DAP) at 37°C.

2.2 Conjugation assay

V. harveyi 345 was inoculated into an LBS liquid medium and cultured overnight at 28°C with 200 rpm shaking, then diluted 300 times and cultured under the same conditions until the early log phase (OD600nm = 0.5–1.0). The *E. coli* GEB883-pMMB207 was inoculated into LB liquid medium with 20 μg/ml Cm and 0.3 mM DAP at 37°C with 200 rpm shaking, then the overnight culture was diluted 100 times and cultured under the same conditions until the early log phase (OD600nm = 0.5–1.0).

TABLE 1 The bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Sources
Bacterial strains		
V. harveyi 345	*V. harveyi* 345: isolated from diseased grouper kidney off the South China Sea coast	(Deng et al., 2019)
E. coli GEB883	Ery^r, Tet^r,WT*E.coli* K12 Δ*dapA::erm pir RP4*-2 Δ*recAgyrA462*, *zei298::Tn10*	(Nguyen et al., 2018)
Plasmids		
pMMB207	Cm^r; Prokaryotic cell expression vector plasmid; Tac-Promoter; stable expression	(Liu et al., 2017)

The early log phase recipient bacterial cells of *V. harveyi* 345 were exposed to different stress (31–46°C for 15–60 minutes, 4%–16% ethanol for 5–30 minutes, 0.14–0.56 mM SDS for 5–30 minutes, 0.01–0.05 M NaOH for 5–20 minutes, 0.012–0.06 M HCl for 5–30 minutes), and then washed with 3% NaCl for appropriate times (temperature: did not wash, ethanol: washed three times, SDS: washed four times, NaOH: washed two times, and HCl: washed two times) according to the stress conditions. For each stress condition, 500 µL recipient bacterial cells were used to do stress exposure, and then the 500 µL stress-exposed recipient bacterial cells were mixed with 500 µL early log phase donor bacterial cells of *E. coli* GEB883-pMMB207. In addition, for salinity stress, an overnight culture of *V. harveyi* 345 was diluted 300 times in LB liquid medium with a final NaCl concentration of 0.5%, 1.0%, 1.5%, and 4.0% and cultured until the early log phase (OD600nm = 0.5–1.0). Then, 500 µL early log phase recipient bacterial cells were mixed with 500 µL early log phase donor bacterial cells. The mixture was centrifugated at 8,000 g for two minutes at room temperature. After removing the supernatant, the precipitation was suspended with 50 µL of 3% NaCl and seeded onto an LBS plate with DAP (0.3 mM). The plate was dried and conjugated for 16 hours at 28°C. The conjunctive plaques were then scraped and re-suspended in 1 mL 3% NaCl and washed once with 3% NaCl. The bacterial suspension was screened on LBS plates with Cm (34 µg/mL) at an appropriate dilution and incubated for 16 hours at 28°C. Firstly, three to four transconjugant candidates of each condition were selected to do PCR verification with the specific primers pMMB207-F (5'-ctactgagcgctgccgcaca-3') and pMMB207-R (5'-tcgttttatttgatgcctggcag-3') with a target band of 1877 bp. The PCR action was checked by 1% agarose gel electrophoresis. Then, the number of transconjugants was counted and compared under different stress conditions. The early log phase recipient *V. harveyi* 345 without exposure to stress (28°C, 3% NaCl, 0% ethanol, 0 mM SDS, 0 M NaOH, or 0 M HCl) was used as the control, and the experiment was conducted three times.

3 Results

Without exposure to stress, the shuttle plasmid pMMB207 cannot be transferred to *V. harveyi* by conjugation. However, this plasmid's intergeneric transfer could be optimized by exposing the *V. harveyi* recipient cells to stress before mating. Compared with untreated controls, fertility was obtained when bacterial cells were exposed to various treatments, including high temperatures, small alcohols (ethanol), detergents (SDS), and pH shifts (Figure 1).

Generally, ethanol has the largest effect on transconjugation, leading to as many as 2.5×10^5 transconjugants, followed by temperature (5.3×10^3), NaOH (2.3×10^3), SDS (4.5×10^2), and HCl (1.8×10^2) (Figure 2). No transconjugants grew in the absence of heat shock (28°C) or when recipient cells of *V. harveyi* 345 were heat shocked for 15–60 minutes at 31 and 34°C (Figure 2A). When the recipient cells were heat treated for 30 minutes at 37°C, pMMB207 was successfully transferred into *V. harveyi*, and 24 transconjugants grew. *V. harveyi* has a high mating ability when exposed to 40°C for 30–60 minutes, 43°C for 15–60 minutes, and 46°C for 15 minutes (Figure 2A). When exposed to 40°C, the mating ability increased with the extension of processing time (Figure 2A). However, the mating ability decreased as the processing time increased when exposed to 43 and 46°C (Figure 2A). When treated with 4% ethanol for 15 minutes, *V. harveyi* successfully incorporated the shuttle plasmid pMMB207 (Figure 2B). Relatively high fertility was induced by 4%–8% ethanol for 5–30 min and 12% ethanol for 5–10 minutes, which led to 1,970–4,500 transconjugants (Figure 2B). Additionally, optimal fertility was induced by stress conditions of 12% ethanol for 20–30 minutes and 16% ethanol for 5–10 minutes, which led to 44,770–249,770 transconjugants (Figure 2B). Figure 2C illustrates that exposure to a low concentration of 0.14 mM SDS detergent increased the fertility of *V. harveyi* as the incubation time increased. However, the opposite trend occurred when exposed to high concentrations of SDS (0.42 and 0.56 mM), and no transconjugants were obtained when exposed for 20–30 minutes. When treated with a medium concentration of 0.28 mM SDS, the transconjugants increased at first (5–20 minutes), then sharply decreased (30 minutes). Acid (HCl) and alkali (NaOH) treatments aided *V. harveyi* to receive the shuttle plasmid pMMB207 from *E. coli via* conjugation, especially when *V. harveyi* was treated with 0.04–0.05 M NaOH for 5–20 minutes, yielding up to 2,300 transconjugants, and when treated with 0.012-0.024 M HCl for 5–30 minutes, generating up to 180 transconjugants (Figures 2D, E). However, *V. harveyi* nearly failed to mate with *E. coli* when treated with 0.01–0.03 M NaOH and 0.036–0.06 M HCl (Figures 2D, E). Furthermore, when the overnight culture of *V. harveyi* 345 was diluted and cultured in LB liquid medium with different NaCl concentrations (0.5%, 1.0%, 1.5%, and 4.0%), no transconjugants were obtained (data not shown). Additionally, 252 transconjugant candidates were selected for PCR verification (Figure S2). Finally, 244 candidates met the target bands with an effective proportion of 96.83%, which confirms the effectiveness of the results.

4 Discussion

Here, we systematically studied the efficiency of foreign plasmid conjugation transfer into *V. harveyi* after exposure to different stresses and found that certain stress exposures, such as temperature, acid, alkali, SDS, and ethanol, can significantly improve the fertility of *V. harveyi* recipients in intergeneric mating with *E. coli*. This partly verifies our conjecture that warmer temperatures and antibiotic pollution could probably enhance bacterial antibiotic resistance and bacterial infection by promoting the HGT of virulence genes and antibiotic resistance genes, thus increasing the complexity and diversity of disease control (Deng et al., 2020a; Deng et al., 2020b; Deng et al., 2020c). Therefore, the results of this study have important theoretical significance for establishing disease prevention and control strategies by interrupting the HGT process with environmental regulation.

The state of the recipient cell membrane is a crucial factor affecting the formation of the junction bridge, thereby affecting conjugation transfer (Thomas and Nielsen, 2005). Genco and Clark (1988) reported that the outer membrane protein OmpA of the receptor bacteria can stabilize the junction bridge with the donor bacteria and promote the fertility of *E. coli*, *S. typhimurium*, and *Neisseria cinerea*. Sherburne and Taylor (1997) found that the deletion of lipid A synthesis genes *rfaD* and *rfaE* in *S. typhimurium* contributes to the truncation of lipid A, the reduction of porin by more than 90%, the change of cell membrane permeability, and the

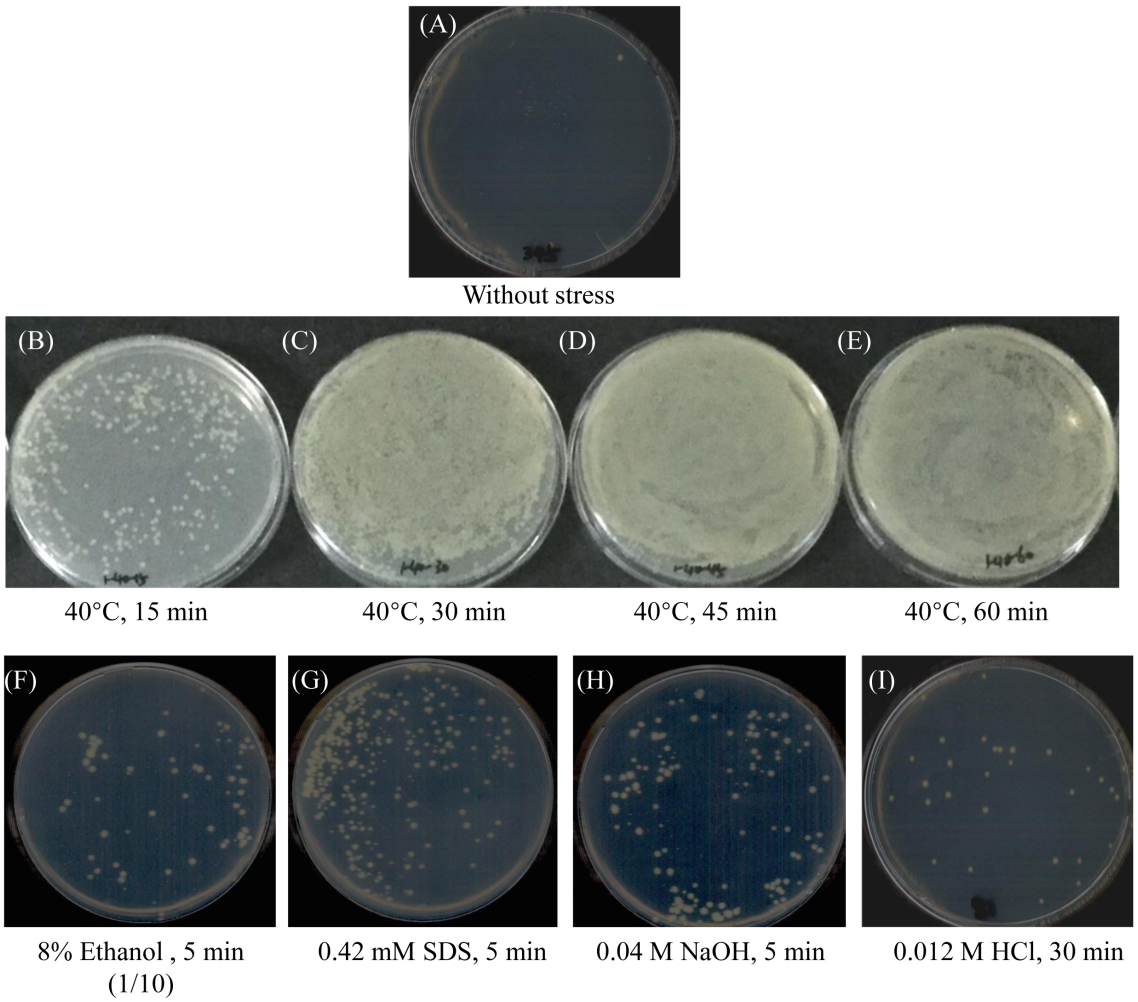

FIGURE 1
The growth of transconjugants with indicated stress on LBS plates containing 34 μg/mL Cm. **(A)** Without stress, no transconjugant growth. **(B–E)** Exposure to 40°C for 15, 30, 45, and 60 minutes, respectively. The conjunctive plaques were suspended in 100 μL of 3% NaCl and screened on LBS plates with 34 μg/mL Cm. **(F)** Exposure to 8% ethanol for 5 minutes. The conjunctive plaques were suspended in 1 mL of 3% NaCl and 100 μL of the suspension was screened on LBS plates with 34 μg/mL Cm. **(G–I)** Exposure to 0.42 mM SDS for 5 minutes, 0.04 M NaOH for 5 minutes, and 0.012 M HCl for 30 minutes, respectively. The conjunctive plaques were finally suspended in 100 μL of 3% NaCl and screened on LBS plates with 34 μg/mL Cm.

reduction of conjugation efficiency by 10–100 folds. Furthermore, successfully escaping the splicing effect of sequence-specific restriction endonucleases in the host cell immune system (R-M system, CRISPR, *etc*.) is another barricade to the success of conjugation transfer (Hickey and Hirshfield, 1990).

Heat stress can change the permeability of bacterial cell membranes (Panja et al., 2008). During DNA transformation, a heat pulse (0→42°C) can release lipids, denature membrane proteins, considerably lower cellular outer membrane fluidity, and consequently form pores on cell surfaces, allowing the DNA to cross the outer membrane barrier (Panja et al., 2008). Furthermore, Mojica-a and Middleton (1971) found that exposure to 50 °C for 20 minutes can increase the fertility of *S. typhimurium* by nearly 200 folds by inactivating its R-M system. Ethanol treatment, like heat, leads to changes in protein composition and an increased ratio of saturated to unsaturated fatty acids in the cytoplasmic membrane (Piper, 1995). Protein denaturation and fatty acid profile changes in the cell can occur as a consequence of exposure to pH shifts or detergents (Hickey and Hirshfield, 1990; Adamowicz et al., 1991; Bhuyan, 2010). Therefore, exposure to temperature, ethanol, SDS, acid, and alkali induces the fertility of *V. harveyi* recipients, probably by increasing its membrane permeability and/or inactivating the immune system. Furthermore, a lower temperature or concentration of stress cannot effectively change the cell membrane and immune system, whereas a higher temperature or concentration of stress is probably lethal to the cells and thus cannot induce the fertility of the recipients (Schäfer et al., 1994).

It should be pointed out that in the process of homologous recombination gene knockout mediated by suicide plasmid of pathogenic bacteria such as Vibrio (Val et al., 2012), conjugation transfers the recombinant suicide plasmid from the donor bacteria to the recipient bacteria and plays a key role in the success of gene knockout. However, due to the extremely low efficiency of *V. harveyi* fertility, or even *V. harveyi* that cannot be conjugated, there is no effective mutation system to study its pathogenesis and drug resistance. Based on our research, a breakthrough to improve the conjugation efficiency of *V. harveyi* has occurred, and we have already

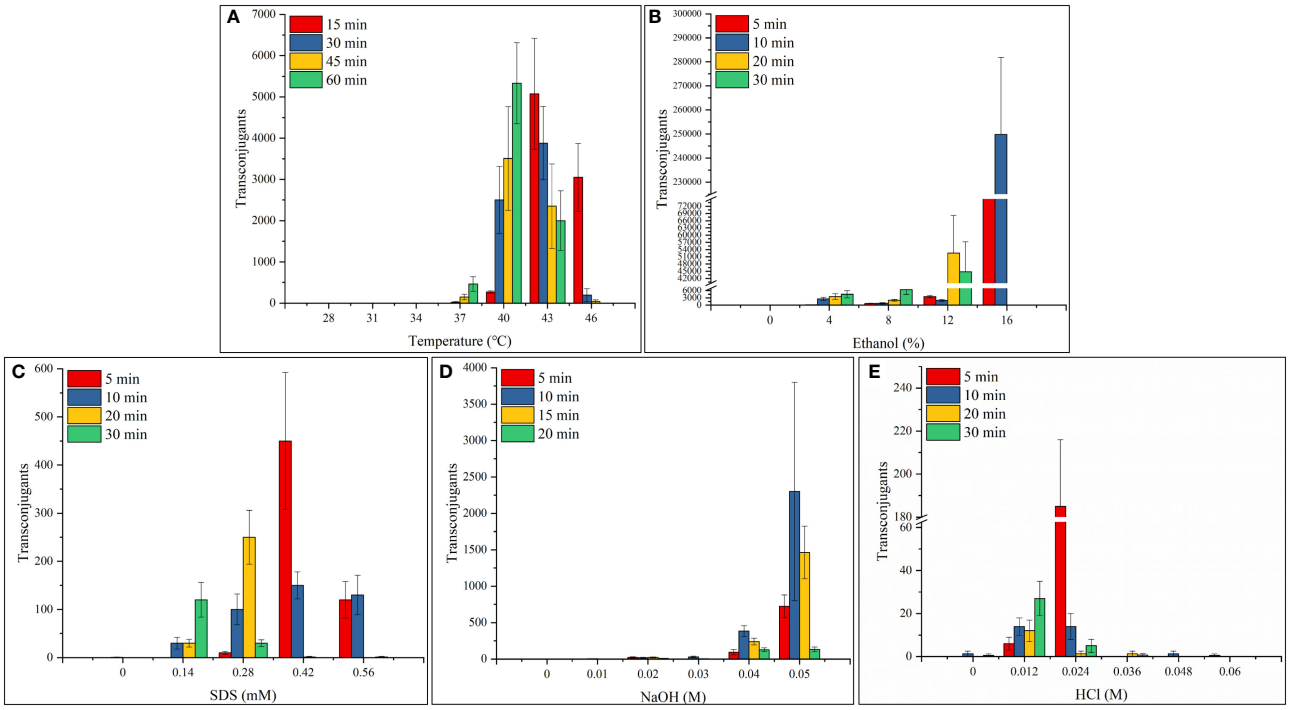

FIGURE 2
The induced interspecific mating ability of *V. harveyi* with exogenous stress. **(A)** Exposure to 31–46°C for 15–60 minutes; exposure to 28°C was used as the control. **(B)** Exposure to 4%–16% ethanol for 5–30 minutes. **(C)** Exposure to 0.14–0.56 mM SDS for 5–30 minutes. **(D)** Exposure to 0.01–0.05 M NaOH for 5–20 minutes. **(E)** Exposure to 0.012–0.06 M HCl for 5–30 minutes. Values are mean ± standard error of the mean (n = 3).

established homologous recombination gene knockout technology based on heat shock, ethanol, SDS, acid, and alkali stimulation, which has greatly promoted the research into the molecular mechanism of *V. harveyi* (Deng et al., 2017a; Deng et al., 2017b; Zhang et al., 2021).

To summarize, environmental changes affect the acquisition of foreign plasmids by *V. harveyi* recipients, thus affecting the evolution of bacterial virulence and drug resistance. Changes in the fertility of *V. harveyi* occur by affecting the status of the cell membrane and the activities of the immune system, but the specific mechanism needs to be further studied. In the future, we propose to conduct research in the following three areas: (1) how environmental stress regulates the conjugation of *V. harveyi* to obtain foreign plasmids at the cellular and molecular levels; (2) the regulation of environmental changes on the fertility of more *V. harveyi* strains and other typical aquaculture pathogens, including *Vibrio*, *Aeromonas*, and *Edwardsiella*; and (3) the regulation of environmental changes on the efficiency of other ori-plasmid conjugation transfer into typical aquaculture pathogens. In doing so, we will develop disease prevention and control strategies based on environmental regulation to block the HGT process.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

YD contributed to conception and design of the study, wrote the first draft of the manuscript. SG and HM conducted the experiment. LX performed the statistical analysis. CC wrote sections of the manuscript. JF supervised the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1083714/full#supplementary-material

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