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Construction and characterization of the *angR* mutant of *Vibrio anguillarum* via insertional inactivation

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Abstract

Vibrio anguillarum strain M3 was isolated from cultured diseased flounder *Paralichthys olivaceus*. In order to determine whether chromosomal *angR* gene plays an important role in infecting flounder host, part fragment of a virulence regulatory gene *angR* from *V. anguillarum* M3 genome was cloned and ligated with suicide plasmids pNQ705 in the present study. The conserved fragment of *angR* gene was amplified from M3 genome and inserted into suicide plasmid pNQ705, a kind of conditional replicon. The recombinant plasmid was transferred into *Vibrio anguillarum* M3 strain genomic DNA through bacterial conjugation and homologous recombination. A mutant *V. anguillarum* strain with *angR* gene mutation was constructed and screened successfully using TCBS medium containing chloramphenicol. PCR identification and sequencing showed that the recombinant plasmid inserted into *Vibrio anguillarum* genome DNA, as expected design, resulted in insertional inactivation of angR gene. Results of artificial infection experiment showed that virulence of mutant *Vibrio anguillarum* strain reduced dramatically as compared with the wild strain M3.

Key words

angR gene, Iron uptake system, Insertional inactivation, *Vibrio anguillarum*

Introduction

Vibrio species are important bacterial pathogens of fish and shellfish in aquaculture, causing a disease known as vibriosis (Austin *et al*., 1995; Beaz-Hidalgo *et al.* 2010). *Vibrios* are also known to cause diseases in humans, most often following the consumption of contaminated seafood. Among the potentially pathogenic Vibrios, *Vibrio anguillarum* is one of the most widespread causative agents of hemorrhagic septicemia among fish and shellfish (Mo *et al*., 2001; Frans *et al*., 2011). *V. anguillarum* is a common member of microbial community in marine and estuarine environments and is an important opportunistic pathogen for different species of wild and cultured fish and shellfish. It has been identified as the main causative agent of fatal skin ulcer and haemorrhage that affects marine fish aquaculture and leads to severe economic loss (Chen *et al*., 2002; Tolmasky and Crosa, 1991). In recent years, there have been extensive studies on the pathogenesis of *Vibrio anguillarum*. Some important virulence factors have been identified that play an important role in the etiology and pathogenesis of flounder hemorrhagic and septic diseases, including protease, hemolysin, outer membrane protein and plasmid-mediated iron uptake system (Farrell and Crosa, 1991; Conchas *et al*., 1991; Rui *et al*., 2009). The iron uptake system allows bacteria to grow at low concentrations of available iron imposed by high-affinity iron-binding proteins (Stork *et al*., 2002; Tang *et al*. 2014). A regulatory gene of this system, *angR*, appears to be essential for virulence of this pathogen and play a crucial role in regulation of expression of iron transport genes *fatDCBA* and production of siderophore anguibactin (Wertheimer *et al*., 1999; Chen *et al*., 1996; López and Crosa, 2007).

In the present study, a virulent strain *V. anguillarum* M3 was isolated from diseased left-eyed flounder, *Paralichthys olivaceus* and plasmid in M3 strain was absent. It suggested that the iron uptake system of the strain must be chromosomally encoded. To analyze whether chromosomal *angR* gene plays an important role in infecting flounder host, a mutant strain was

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constructed by insertional inactivation of *angR* gene in *V. anguillarum* M3 chromosome and its properties were evaluated as well in the present study.

Materials and Methods

Bacterial strains, plasmids and media : Pathogenic *V. anguillarum* strain M3, with no virulent plasmid, was isolated from diseased cultured flounder *Paralichthys olivaceous*. M3 strain was cultured in trypticase soy agar or broth (TSA or TSB) medium supplemented with 1% NaCl at 28ºC. M3 strain genome DNA extraction was performed using *TaKaRa* DNA extraction kit. The suicide plasmid pNQ705 was reproduced in *E. coli* SY327 that was inoculated in LB medium containing chloramphenicol at 37ºC. *E. coli* SY327, S17-1 and JM109 strains were cultured in LB medium at 37ºC. Thiosulfate citrate bile salts sucrose (TCBS) agar was used as Vibrio species selective medium. The bacterial strains and plasmids, used in this study, were all stored in laboratory and are listed in Table 1.

Cloning of angR gene fragment : *V. anguillarum* M3 genomic DNA was prepared using bacterial DNA extraction kit (Huashun, China). A set of primers was designed and synthesized to amplified angR gene conserved region. The forward primer was: 5'-TCGAGCTCTAGGGTCGTTGCTTAGTGTTG-3' (*Sac* I site and stop codon TAG were added to 5' end) and the reverse primer was: 5'-CTCGTCGACGGCTGTCCCATTGTAAGA-3' (*Sal* I site was added to 5' end). The expected amplified fragments were approximately 620 base pair (bp). The PCR products were purified using Huashun PCR products purification kits after agarose gel electrophoresis and sequenced by Shanghai Sangon Biotech Company.

Construction and screen of mutant strain : The purified PCR products were cloned into pUCm-T vector and then transformed into *E. coli* JM109 competent cells. Both the recombinant plasmid and suicide plasmid pNQ705 were extracted and digested with *Sac* I and *Sal* I restriction enzymes. The products of ligation were transformed into *E. coli* SY327 competent cells and inoculated in LB media supplemented with chloramphenicol at 37ºC. The recombinant suicide plasmids were extracted and transformed into donor bacteria *E. coli* S17-1 competent cells. The recipient bacteria *V. anguillarum* M3 were cultured in TSA media at 28ºC. Both fresh donor and recipient bacteria were inoculated into respective liquid medium until OD_{600} reached 0.5. The donor and recipient bacteria were mixed 1:1 and centrifuged at 4000 rpm for 5 min. The mixtures were suspended with fresh TSB medium and spotted onto TSA plate to perform conjugation at 28ºC for 24 hr. Bacteria were resuspended with fresh TSB medium and inoculated onto TCBS agar supplemented with chloramphenicol at 28ºC for 24 hr to screen mutant strain.

Identification of insertional inactivation mutant strain : Mutant strains were subcultured on TCBS agar supplemented with chloramphenicol repeatedly to confirm whether antibiotic resistant of the mutant had been inherited steadily. A set of primers was designed to amplify insertional regions of *angR* gene in M3 genome to identify whether recombinant suicide plasmids have been integrated into exact region of M3 genome. The sequence of forward primer was: 5'-CTTCGCTTTCCCTGAGAC-3' (corresponding to positions 381-398 of M3 *angR* gene) and reverse primer was: 5'-CCAGTGGCTTCTGTTTCTATCA-3' (located at suicide plasmid). The expected amplified fragment was approximately 1200 bp. The PCR products were purified using Huashun PCR products purification kits after agarose gel electrophoresis and sequenced by Sangon Biotech Company.

Characteristics of mutant strain : Morphological characters of the mutant strain cultured on TSA and TCBS agar were observed. To investigate the change of virulence of mutant strain, 50 % lethal dose (LD_{50}) was tested and calculated with developed Karber's method by challenging healthy cultured flounder. Different concentrations of bacterial suspensions ($3.5 \times 10^4 \sim 3.5 \times 10^8$ CFU ml^{-1}), used for artificial challenge, were diluted with phosphate buffered saline (PBS, pH 7.4). For each challenge concentration 18 individuals were distributed into three aquariums and were intramuscularly injected with 0.2 ml bacterial suspensions per fish and kept at 18°C for 14 days to calculate cumulative mortality. SPSS 19.0 was used for statistical analyses. LD_{50} Calculations were based on the following formula:

$$\lg LD_{50} = X_m - d(\Sigma P - 0.5)$$

Where, $\lg LD_{50}$, X_m and d; logarithm of LD_{50}, of highest challenge

Table 1 : Strains and plasmids used

Strains or plasmids	Relevant characteristics	Source
Vibrio anguillarum		
M3	Wild type	Laboratory collection
Escherichia coli		
SY327	Δ (*lac pro*) *argE*(Am) *recA56rpoB*λ*pir*, host for π-requiring plasmids	Invitrogen
S17-1	Tpr Smr*recAthipro* rK- mK-*RP4:2-Tc:*MuKm Tn7λ*pir*	Invitrogen
JM109	K-12 *recA1 supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*)	Laboratory collection
Plasmid		
pNQ705	Cmr, 4.5 kb, R6Kγ*ori*, *oriT* of RP4, suicide vector	Laboratory collection
pUCm-T vector	Ampr, 2.7 kb, high-copy-number cloning vector	Sangon, China

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concentration and the common ratio of concentration gradient. ΣP : sum of mortalities.

Results and Discussion

Electrophoresis results showed that the amplified *angR* fragments were approximately 620 bp (Fig. 1). Homology search found that homogeneity of amplified partial sequences was 98% with *Vibrio anguillarum* angR gene fragment between 918 ~ 1537 bp.

Recombinant plasmid was constructed successfully and transformed into *E. coli* S17-1, which was used as donor bacteria of the conjugation reaction and cultured in LB medium supplemented with chloramphenicol at 37ºC. Recombinant plasmid was transferred into *Vibrio anguillarum* M3 strain genomic DNA through bacterial conjugation and homologous recombination. Mutant strain with angR gene mutations was successfully screened using TCBS medium containing chloramphenicol.

A set of primers (corresponding to *Vibrio anguillarum* M3 *angR* gene and the recombinant plasmid respectively) were designed to amplify insertional regions of *angR* gene in mutant strain genome to identify whether recombinant plasmids were integrated into M3 genome steadily. 1200 bp amplified fragment from the mutant strain was in line with expectations, while no PCR product was found from wild *Vibrio anguillarum* M3 (Fig. 2). Sequencing results showed that part of PCR product sequenced was from the plasmid and part of PCR product sequenced was from *Vibrio anguillarum* angR gene. The results proved that recombinant plasmid were inserted into *Vibrio anguillarum* genome and resulted in insertional inactivation of angR gene.

The mutant *Vibrio anguillarum* strain could grow on TCBS agar medium containing chloramphenicol and still retained capacity of sucrose decomposition which could change the color of selective medium from green to yellow (Fig. 3).

The LD_{50} of *Vibrio anguillarum* wild strain was about 3.5×10^7 cfu ml^{-1}, while that of mutant strain was more than 3.5×10^8 cfu ml^{-1} (Table 2). The statistical result of artificial infection experiment to healthy flounders showed that the virulence of *Vibrio anguillarum* mutant strain dropped off dramatically (at least decreased 10 fold) as compared to wild strain. The challenge results proved that intact angR gene played an important role in *Vibrio anguillarum* virulence system and the virulent effects of *Vibrio anguillarum* were highly influenced by insertional inactivation of angR gene.

Vibrio anguillarum iron intake system is an important virulence factor and angR gene, as an indispensable element, plays an important role in biosynthesis and regulation of the system in the process of pathogenesis (López and Crosa, 2007; Frans *et al*., 2011; Busschaert *et al*., 2014). Studies have shown that in angR defect strains, expression of iron transport gene fatB and fatA were greatly reduced under iron deficiency condition (Wertheimer *et al*., 1999; López and Crosa, 2007). To extend the knowledge of virulence gene regulation during infection, many genetic techniques have been developed (Lee *et al*., 2001;

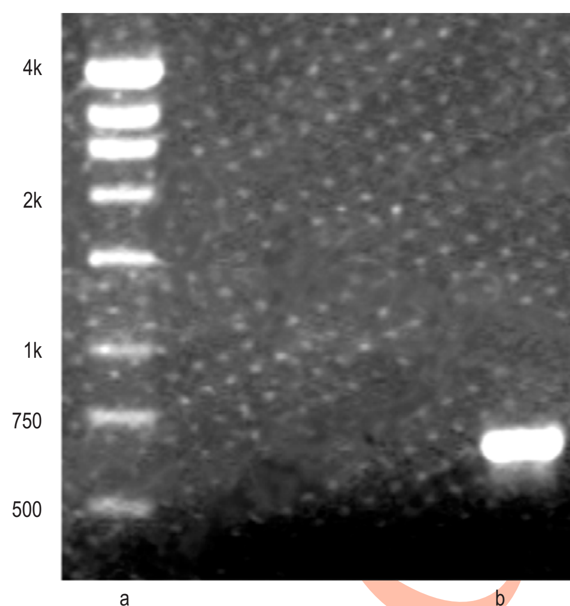

Fig. 1 : PCR fragment of angR gene in *Vibrio anguillarum* M3 strain: a lane-DL4000 Maker; b lane-PCR fragment of angR gene

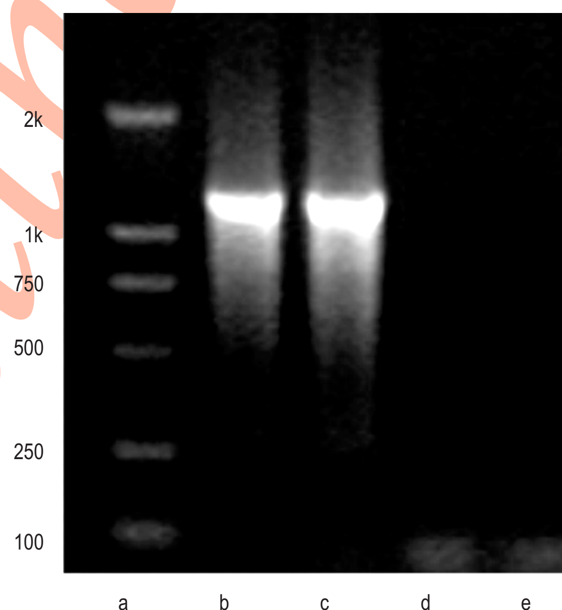

Fig. 2 : PCR identification of mutant strain: a lane-DL2000 Maker b ~ c lane-mutant strain d ~ e lane-wild strain

Table 2 : Results of artificial challenge experiment by mutant and wild strain

Challenge concentration (CFU ml^{-1})	Fish number in each group	Wild strain group		Mutant strain group	
		Average dead fish	Mortality	Average dead fish	Mortality
3.5×10^8	18	18 ± 0	(100 ± 0.0)%	0	0
3.5×10^7	18	9 ± 2.46	(50 ± 13.6)%	0	0
3.5×10^6	18	0	0	0	0
3.5×10^5	18	0	0	0	0
3.5×10^4	18	0	0	0	0
Control	18	0	0	0	0
		$LD_{50} = 3.5\times10^7$	$LD_{50} > 3.5\times10^8$		

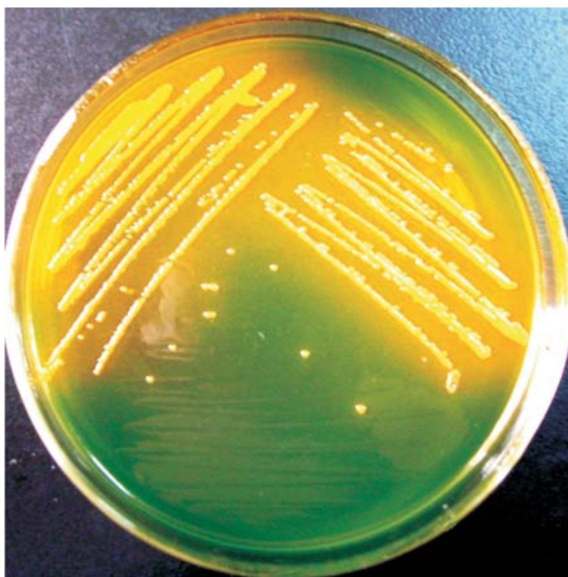

Fig. 3 : Colony morphology of mutant strain on selective TCBS medium containing chloramphenicol

Chang *et al.*, 2014). One of the most effective pathways to study bacterial gene function is to construct gene mutant strain via homologous recombination (Singer *et al.*, 1996; Yildiz and Schoolnik, 1998). Subcloning a DNA fragment into an active gene will disrupt the function of that gene and result in inactivation of gene. In order to study the virulence effect of angR genes of *Vibrio anguillarum* M3 strain, angR gene mutant strain was constructed in the present study. Part of angR gene fragments was cloned into suicide plasmid pNQ705 and the recombinant plasmid suicide was transferred into M3 strain genomic DNA via bacterial conjugation and homologous recombination which resulted in inactivation of angR gene. The results of artificial infection experiment showed that the virulence of mutant strain declined greatly which proved that intact angR gene played an important role in *Vibrio anguillarum* M3 strain virulence system.

One of the most creative methods in the present study was to screen mutant strains with combination of Vibrios TCBS selective medium and chloramphenicol resistance, which significantly had higher screening efficiency as compared to traditional double antibiotic resistance screening methods (Borrell *et al.*, 2013; Zhao and Drlica, 2002). As compared to mutant strains, the donor bacteria and recipient bacteria were unable to live on chloramphenicol TCBS medium.

A mutant *V. anguillarum* strain with *angR* gene mutation was successfully constructed through homologous recombination technique using a suicide plasmid to determine whether its virulence could be influenced. Results of artificial infection experiment showed that virulence of mutant *Vibrio anguillarum* strain reduced dramatically as compared with wild strain. It was proved that intact angR gene played an important role in the course of infecting flounder host.

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