

## Modulation of Quorum-Regulatory sRNA on Various Physiological Functions and Virulence of *Vibrio alginolyticus*

Huan Liu<sup>1</sup>, Jinfang Yang<sup>2</sup>, Shanshan Zhang<sup>3</sup>, Wang Liu<sup>4</sup>, Xuefeng Chen<sup>5</sup>

<sup>1,2,3,4,5</sup>School of Food and Biological Engineering, Shaanxi University of Science & Technology, Xi'an Shaanxi, 710021, China

**Key words:** Qrr1, *Vibrio alginolyticus*, Physiological function, Virulence.

**Abstract.** *Vibrio alginolyticus* is one of the important fish pathogen in China, bringing about serious economic loss to aquaculture. sRNA (Small non-coding RNA, sRNA) modulate the target genes on the post-transcriptional level with the aid of the chaperon protein, Hfq and play essential roles in the regulation of various physiological functions, like virulence. In this paper, a quorum regulatory sRNA, Qrr1, was screened and cloned. Besides, An in-frame deletion was made in *qrr1* by allelic exchange, using the suicide plasmid. Meanwhile, the plasmid, pMMB206 was utilized to carry the contact *qrr1* gene into the mutant strain  $\Delta qrr1$  to generate the complemented strain, *qrr1*<sup>+</sup>. The roles of this gene in different physiological functions of *Vibrio alginolyticus* were characterized. The results demonstrated that Qrr1 obviously promoted the growth of *Vibrio alginolyticus* during exponential and stationary stage. Moreover, the ability of mobility was almost absent, the biofilm formation and extracellular protease production were all impaired in the  $\Delta qrr1$  mutant strain, contrast to the wild-type strain, which means Qrr1 participates in the virulence regulation as an important regulator in *Vibrio alginolyticus*.

### Introduction

*Vibrio alginolyticus* is a kind of important aquatic pathogenic bacteria, widespread around the world, can infect a variety of fish and shellfish, bacterial septicemia caused by its infection brings great economic loss for aquaculture industry.

Toxic factors of *vibrio alginolyticus* include extracellular proteases, bacterial biofilm, moveability and so on, it is mainly regulated and controlled by quorum-sensing system and sRNA molecular chaperone protein Hfq(Liu et al. 2011; Gu et al. 2016). Hfq is initially identified as endogenous host protein that necessary for bacteriophage to replicate in the *escherichia coli*. Deletion analysis of Hfq reveals that this protein participates in the physiological regulation like growth of *escherichia coli*, cell division, response of the osmotic pressure(Tsui et al. 1994). Later, Hfq is founded in many bacteria, and it display regulation and control on the physiological function of bacteria(Chao and Vogel 2010). Hfq displays its regulation and control as a kind of uncoded sRNA chaperone proteins. sRNA is a kind of uncoded RNA with regulation and control functions in the bacteria, with size of 60~300 basic groups. sRNA can be rapid synthesis under the cooperated function of Hfq chaperone proteins, combine with different target mRNA through complementary base pairing, affecting its stability and translation activity to realize the rapid regulation and control on genetic expression. Hfq forms a six dimer structure of donut shape, with two independent bonding sites surfaces, one is in the far end and used for the combination of Poly (A tail), the other one is in the close end and is used for the combination of AU enrichment region. Therefore, Hfq provides RNA molecule with moored platform to promote the interaction of uncoded sRNA and target mRNA molecule (Mikulecky et al. 2004; Schu et al. 2015). Currently, more and more researches founded that Hfq and sRNAs has an essential effect on various physiological functions of many bacteria, such

as growth features, environmental stress and virulence, when the Hfq is damaged, vibrio cholera, haemophilus bleeding and yersinia enterocolitica will be significantly attenuated (Lenz et al. 2004; Hempei et al. 2013; Kakoschke et al. 2014; Feliciano et al. 2016).

This research filtrates quorum sensing regulation sRNA molecule -- Qrr1 in the splendidus vibrio genome through bioinformatics method, then to gain nullmutant for biological characteristics research through unmarked box method to gain qrr1 gene from the genome.

## Materials and methods

### Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Growth in Luria-Bertani (LB) broth or on LB plates containing 3% (w/v) NaCl at 30°C was used for *Vibrio alginolyticus* culture in the whole study. *Escherichia coli* strains used for plasmid DNA replication and conjugation were grown in LB broth or medium containing 1% (w/v) NaCl supplemented with appropriate antibiotics at 37°C. And the antibiotics were used at the following concentrations: 100 µg/ml ampicillin (Amp) and 7 µg/ml chloramphenicol (Cm) for *V. alginolyticus* while 100 µg/ml Amp and 25 µg/ml Cm for *E. Coli*.

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Reference or source
Vibrio alginolyticus		
EPGS	Pathogenic isolate from aquaculture farm. Ampr	Lab collected
asp-	EPGS carrying the null mutation in asp gene	Lab collected
ΔluxR	EPGS carrying in-frame deletion in luxR gene	Lab collected
ΔaphA	EPGS carrying in-frame deletion in aphA gene	Lab collected
Δqrr1	EPGS carrying in-frame deletion in qrr1 gene	This study
qrr1+	EPGS, Δhfq complemented with intact hfq gene	This study
Plasmids		
pDM4-Δqrr1	Cmr, pDM4 derivative containing the truncated qrr1 from bp 7-121	This study
pMMB-qrr1	Cmr, pMMB206 derivative containing the intact qrr	This study

### DNA manipulation

All DNA manipulations were carried out as previously described. Enzymatic reactions and plasmid purifications were performed according to the manufacturer's instruction (TaKaRa, Dalian, China), and DNA sequencing and primer synthesis were finished in Generay Co. (Shanghai, China).

#### *Clone and analysis of qrr1 gene*

According to the published vibrio alginolyticus 12G01 genome, vibrio cholera and qrr1 gene order in the vibrio harveyi, make compare analysis by using bioinformatics method, choose quorum sensing regulation sRNA and Qrr1 in the vibrio alginolyticus to design specific primers to clone qrr1 gene and use Mfold to make secondary structure prediction analysis.

#### *The construction of Δqrr1 mutant strain*

Set vibrio alginolyticus EPGS genome as template, respectively amplify primers, qrr1upF and qrr1upR, qrr1doF and qrr1doR to get upstream and downstream homologous segments -- qrr1up and qrr1do. After recycling, PCR products connect upstream and downstream homologous segments qrr1up and qrr1do through overlap PCR by using qrr1upF and qrr1doR as primers to get Δqrr1 fragment. Use suicide plasmid pDM4 to combine vibrio alginolyticus EPGS to get PCR and correctly verified mutant strain Δqrr1 through two rounds of homologous recombination.

#### *The construction of complementary strain qrr1+*

Set EPGS genome as template, use primers *qrr1comF* and *qrr1comR* to carry out PCR reaction, enlarge to get DNA fragment with complete *qrr1* gene ORF region, use complementary plasmid pMMB206 to integrate it into the recipient bacterium  $\Delta qrr1$  to get complementation strain *qrr1*<sup>+</sup>.

#### *The measurement of growth curve*

Inoculate bacterial strain that keeping in the -80°C glycerin tube into LBS fluid medium, 200 r/min resuscitation culture overnight in the 30°C table concentrator. Use fresh LBS fluid medium to dilute OD<sub>600nm</sub> to 1.0, inoculate it into 50 ml LBS fluid medium according to 1% (v/v), culture with 200 r/min in the 30°C table concentrator, sampling every 1h and measure the OD<sub>600nm</sub> to draw the growth curve of each bacterial strain.

#### *Detection of biofilm*

Use fresh LBS fluid medium to dilute the overnight cultured bacterial strain to same OD<sub>600nm</sub> value. Then inoculate each bacterial strain to sterile tubes with 10ml LBS fluid medium, stationary culture for 48h under 30°C. Each tube added with 2% (w/v) crystal violet solution, dyeing for 5 minutes, pour out the culture and wash with tap water, each tube added with 33% (v/v) glacial acetic acid solution, detect its light absorption value at 570 nm.

#### *Dynamic analysis*

Respectively dot same amount activated vibrio alginolyticus EPGS,  $\Delta qrr1$  mutant strain and *qrr1*<sup>+</sup> complementary strains on the LBS slab with 0.3% (w/v) (soft slab) and 1.5% (w/v) (hard slab) agar powder, culture under 30°C until bacterial colony grows to reasonable size, take out for photo and measure the diameter.

#### *Activity analysis of extracellular proteases*

Qualitative determination use method of skin milk slab, quantitative analysis is to measure the amount of soluble sky blue dye released by the enzymolysis of Hide powder azure, HPA. Measuring method refers to Liu et al. 2011.

## **Results**

### **Qrr1 order analysis and the construction of $\Delta qrr1$ mutant strain**

Set vibrio alginolyticus EPGS genome as template to enlarge *qrr1* order to 121 bp and keep high homology with vibrio harveyi, vibrio parahaemolyticus, *qrr1* order in the vibrio cholera. Use Mfold online analytical tools to make secondary structure prediction for the transcription RNA, we found that Qrr1 has typical stem loop structure, its 3' normally has U tail, and there is AU-rich region in the assumed Hfq binding site in the stem loop structure.

Further to get the 7-121, 115bp mutant strain  $\Delta qrr1$  of deficiency *qrr1*. PCR verification and order measurement result showed that 7-121 basic groups totally 115bp internal nucleotide of mutant strain  $\Delta qrr1$  was missing.

### **The construction of *qrr1*<sup>+</sup> complementary strains**

Connect complementary fragments into pMMB206 plasmid and combine into  $\Delta qrr1$  mutant strain on the basis of mutant strain. Use primers *qrr1comF* and *qrr1comR* to make PCR verification of bacterial strain. The result showed that the *qrr1*<sup>+</sup> complementary strains can enlarge to get two fragments differ 115 bp, that is the frame missing fragment in the  $\Delta qrr1$  mutant strain and the complete complementary fragment of pMMB206 plasmid that can start subdomain, which proves that covering plasmid pMMB-*qrr1* with target fragment is successfully transited to the  $\Delta qrr1$  mutant strain.

### **Influence on the growth of vibrio alginolyticus**

In order to investigate the function of Qrr1 on the growth of vibrio alginolyticus, we respectively measure the growth curve of vibrio alginolyticus wild type strain and complementary strain. The result showed that all of them get into logarithmic phase after being inoculated into the fresh nutrient

medium; wild type strain grows fast in the logarithmic phase, in the stable phase, thallus intensity reaches  $OD_{600nm}=6.0$ . During the logarithmic phase, compared with wild type strain, the growth of mutant strain and complementary strain declines obviously; after getting into the stable phase, the growth of mutant strain reaches the growth of complementary phase, wild type strain keeps higher germ concentration. It is visible that the missing of *Qrr1* gene has influence on the whole growth period of *vibrio alginolyticus*, has biggest influence on the logarithmic phase.

### **The regulation and control of *Qrr1* on *vibrio alginolyticus* moveability**

Moveability detection result showed that wild type strain has strong moveability on hard slab and soft slab, on the hard slab, it creep diffuses to surrounding from the sampling dot, it almost covers the whole soft slab. While the moveability of  $\Delta qrr1$  mutant strain almost lost. It is visible that the positive regulation function of Hgq on *vibrio alginolyticus* is mainly realized from the function of *Qrr1*.

### **Influence of *Qrr1* on the biofilm formation of *vibrio alginolyticus***

Through detection on the biofilm of different bacterial strain we found that *vibrio alginolyticus* wild type can form visible and thick biofilm on the surface of test tube wall and LBS fluid nutrient medium. Under the same culture condition, the biofilm of  $\Delta qrr1$  mutant strain is reduced, while though the biofilm formation of  $qrr1^+$  complementary strain did not reach that of the wild type, but has obvious increase. Through crystal violet staining analysis we found that the biofilm formation of  $\Delta qrr1$  mutant strain is reduced by 50%, compared with the wild type. It is visible that *Qrr1* has positive regulation function on the biofilm formation of *vibrio alginolyticus*.

### **Influence of *Qrr1* on the extracellular proteases formation of *vibrio alginolyticus***

Wild type bacterial strain can form obvious transparent zone around the bacterial colony on the skim milk slab, and the diameter and transparency of the transparent zone are biggest. While the diameter and transparency of the transparent zone of the  $\Delta qrr1$  mutant strain are decreased; when there is complete gene open reading order complementary to the mutant strain, its extracellular proteases activity will be improved on a certain extent. The HPA chromogenic reaction results conform with milk slab, the yield of extracellular proteases of the wild type is highest, when *Qrr1* is damaged, its acellular proteases production is lowest, and the production of complementary strain is close to the wild type. It is visible that *Qrr1* has some promotion on the production of extracellular proteases of *vibrio alginolyticus*.

## **Discussion**

The main virulence factors of *vibrio alginolyticus* are extracellular proteases, biofilm and moveability. Former researches found that there is sRNA chaperone protein – Hfq in the *vibrio alginolyticus*, this protein has important regulations on the environmental stress ability, moveability, biofilm, extracellular protease activity, survival in the host and virulence of the *vibrio alginolyticus* (Liu et al. 2011). As an important transcription regulatory factor, Hfq mainly assists the interaction of different sRNA molecule and target mRNA molecule. In the *Escherichia coli*, Hfq displays regulation on nutrition metabolism through two sRNA molecules, SgrS and CsrA (Papenfert et al. 2013; Wang et al. 2005); displays oxidative stress through the transition of *fh1A* and *rpoS* gene (Updegrave and Wartell. 2011). In the *vibrio cholera*, Hfq mainly realizes its regulation on virulence through the influence of *Orr* molecule on the quorum sensing key components LuxR and AphA (Shao and Bassler. 2012).

This paper screened quorum sensing regulation sRNA molecule—*Qrr1* from the 12G01 genome of *vibrio alginolyticus* by using bioinformatics method. Establish a mutant strain missing 7-121 basic group, through comparing its different physiological property with wild type bacterial strain and complementary strain we found that *Qrr1* has some promotion on the synthesis of extracellular proteases of *vibrio alginolyticus*, while former researches found that when Hfq is missing, its extracellular proteases production is obviously improved, it is visible that in the *vibrio alginolyticus*,

Hfq mainly affect extracellular proteases through one or many sRNA but not Qrr1. When Qrr1 is missing, the motility of *Vibrio alginolyticus* is totally lost, which is the same as the Hfq missing strain, which showed that the regulation of Hfq on the motility of *Vibrio alginolyticus* is mainly realized by Qrr1. Besides, when the Qrr1 is damaged during transition, only the cell concentration of *Vibrio alginolyticus* in the logarithmic phase and stable phase is obviously lower than wild type, while when the Hfq is damaged, only the cell concentration of *Vibrio alginolyticus* in the logarithmic phase is decreased a little by comparing with the wild type, when in the stable phase, it reaches the level of the wild type, which showed that Hfq displays regulation on the growth and metabolism of *Vibrio alginolyticus* through Qrr1 and other unknown sRNA molecule.

Through research in this paper, we identified the sRNA molecule - Qrr1, which is related with the growth and virulence of *Vibrio alginolyticus*, provided the regulation of Hfq on the virulence and other physiological functions with reference. Meanwhile, research results also showed that the complexity of Hfq's virulence regulation system on *Vibrio alginolyticus*, there are still a large number of unknown sRNA molecules which take part in the different physiological function of *Vibrio alginolyticus* need further identification.

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