Role of the *toxR* Gene from Fish Pathogen *Vibrio alginolyticus* in the Physiology and Virulence

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Received: 28 September 2017 / Accepted: 21 October 2017 / Published online: 28 October 2017
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Abstract A mutant strain of *Vibrio alginolyticus* with an in-frame deletion of the *toxR* gene was constructed to reveal the role of ToxR in the physiology and virulence of *V. alginolyticus*. The statistical analysis showed no significant difference in the growth ability, swarming motility, activity of extracellular protease and the virulence by injection (the value of LD₅₀) between the wild-type and the *toxR* mutant. However, the deletion of *toxR* could decrease the level of biofilm formation. The comparative proteomic analysis demonstrated the deletion mutation of *toxR* could up-regulate the expression of glutamine synthetase and levansucrase, and down-regulate the expression of 10 proteins such as OmpU, DnaK, etc. These results suggest that ToxR may be involved in the early stages of infection by influencing colonization of the bacteria on the surface of the intestine through enhancing the biofilm information of *V. alginolyticus* via modulating the expression of glutamine synthetase, levansucrase and OmpU.

Keywords *Vibrio alginolyticus* · *toxR* mutant · Comparative proteomic analysis · Physiology · Virulence

Introduction

Vibrio alginolyticus is a halophilic (salt-tolerant) Gram-negative bacterium found naturally in temperate marine and estuarine environments [1]. This bacterium is one of the important epizootic pathogens causing vibriosis outbreak and high mortality in sea animals with clinical symptoms of bacterial septicaemia and skin ulcer. It is also an opportunistic pathogen commonly associated with ear infections and gastrointestinal diseases in humans [2]. In addition, clinical isolates usually have high lethality in fish and high cytotoxicity to various cultured cells compared with environmental isolates [3]. Several putative virulence factors of *V. alginolyticus*, such as metalloprotease, alkaline serine protease and iron acquisition factors, have been reported in vivo and in vitro [4–6].

ToxR was first identified in *V. cholerae* in which it regulates several virulence factors in response to changes in environmental parameters like pH, temperature, amino acid composition and osmolarity of the growth medium [7]. ToxR has also been identified and characterized from a variety of *Vibrio* species and strains isolated from marine organisms and environments. The regulatory protein ToxR of *V. cholerae* contains a cytoplasmic DNA-binding-transcriptional activation domain, a trans-membrane domain and a periplasmic domain [8]. ToxR and TcpP of *V. cholerae* could stimulate the expression of some virulence genes by combining to activate the *toxT* promoter, and then ToxT activates various virulence genes including cholera toxin (CT) and the toxin co-regulated pilus (TCP) directly [9]. Lee et al. [10] reported ToxR could stimulate the expression of the hemolysin gene *vhA* of *V. vulnificus*. ToxR of *V. parahaemolyticus* could regulate acid, bile salts, and sodium dodecyl sulfate stresses and affect intestinal colonization in an orogastric adult murine model

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[11]. Similarly, Chen et al. [12] found ToxR of *V. alginolyticus* might have enhanced the bile resistance and biofilm formation.

Biofilm is microbial communities that are embedded in a matrix comprising polysaccharides, nucleic acids and extracellular proteins. The matrix enables the biofilm to adhere to the surfaces of planktonic cells, forms the scaffold for the construction of the biofilm, and provides mechanical stability once the biofilm is established. When grown in biofilm, bacteria have access to nutrients accumulating at interfaces [13]. *Vibrio* can survive as free-living or in association with zooplankton and form biofilm on biotic and abiotic surfaces in order to protect themselves with the exopolymer barrier [14]. The biofilm can also provide protection from toxic compounds, such as antibiotics, thermal stress, and predation [15]. Although biofilm formation has been studied extensively in *V. cholera* and *V. harveyi* [14, 16], much of the focus has been directed toward the survival of *Vibrio* in aquatic environment and its role in pathogenicity, and little is known about the related genes that can regulate biofilm formation.

In the current study, a mutant strain of *V. alginolyticus* with an in-frame deletion of the *toxR* gene was constructed, and then physiology and virulence of the mutant were observed. Furthermore, we compared the proteomes of the wild-type and *toxR* mutant to identify the proteins possibly regulated by *toxR* gene, and to determine whether some of the proteins may be involved in regulating biofilm formation.

Materials and Methods

Bacterial Strains, Plasmids and Media

The bacterial strains and plasmids used in this study are shown in Table 1. *V. alginolyticus* strain HY9901 was isolated from the diseased *Epinephelus coioides* and maintained in our laboratory. *V. alginolyticus* was grown in tryptic soy broth (TSB, Huankai, Guangzhou, China) supplemented with 2% NaCl at 25 °C. *Escherichia coli* strains were cultured in Luria broth (LB, Huankai, Guangzhou, China) at 37 °C. When required, appropriate antibiotics such as ampicillin (Amp, 100 µg ml⁻¹) or chloramphenicol (Cm, 30 µg ml⁻¹) were added.

Construction of a *toxR* Mutant

PCR-amplified DNA fragments used for constructing the in-frame deletion mutation of *toxR* were generated by overlap PCR [17]. Briefly, two PCR fragments were obtained from *V. alginolyticus* strain HY9901 genomic DNA with the primer pairs of ToxR-for/ToxR-int-rev and ToxR-int-for/ToxR-rev (Table 2) using the *pfu* Taq polymerase (Promega, Madison, WI). The resulting products generated a 216-bp fragment containing the DNA upstream of *toxR* and a 230-bp fragment containing the DNA downstream of the *toxR*. A 15 bp overlap in the sequences (Italic) permitted amplification of a 442-bp product containing a deletion from nucleotides 206–812 of *toxR* during

Table 1 Plasmids and bacterial strains used in this study

Strains and plasmids	Phenotype	Source or references
<i>E. coli</i> strains		
DH5α	<i>SupE44ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1</i>	Sangon
MC1061 <i>λpir</i>	<i>lacY1 galK2 ara-14 xyl-5 supE44 λpir</i>	Rubires et al. [31]
MC1061-pRE112- <i>toxR</i>	MC1061 containing plasmid of pRE112- <i>toxR</i> , Cmr	This study
S17-1 <i>λpir</i>	Tp ^r Sm ^r <i>recA thi pro hsdR</i> ⁻ M ⁺ RP4:2-Tc:Mu:Km Tn7 <i>λpir</i>	
S17-1-pRE112- <i>toxR</i>	S17-1 containing plasmid of pRE112- <i>toxR</i> , Cmr	This study
<i>Vibrio alginolyticus</i> strain		
HY9901	Wild-type, isolated from the diseased fish	Cai et al. [5]
HY9901-pRE112- <i>toxR</i>	HY9901 containing plasmid of pRE112- <i>toxR</i> , Cm ^r	This study
HY9901Δ <i>toxR</i>	HY9901 carrying an in-frame deletion of <i>toxR</i> ₂₀₆₋₈₁₂	This study
C-HY9901Δ <i>toxR</i>	HY9901Δ <i>toxR</i> containing plasmid of pACYC184- <i>toxR</i> , Cm ^r	This study
Plasmid		
pMD18-T	Cloning vector; Amp ^r	TaKaRa
pRE112	pGP704 suicide plasmid, <i>pir</i> dependent, <i>oriT</i> , <i>oriV</i> , <i>sacB</i> , Cm ^r	Simon et al. [32]
pRE112- <i>toxR</i>	pRE112 containing <i>toxR</i> gene in-frame deletion of <i>toxR</i> ₂₀₆₋₈₁₂ , Cm ^r	This study
pACYC184	Cm ^r , Tc ^r	Amersham
pACYC184- <i>toxR</i>	pACYC184 containing <i>toxR</i> gene, Cm ^r	This study

Table 2 Sequences of primers used in this study

Name	Sequence (5'—3')	Targeting sequence	Product size (bp)
ToxR-T1	AAGAACTAAATGACTAACAT	Flanking region and ORF sequence of <i>toxR</i>	1106
ToxR-T2	GAGATATTTCACGTTTGA		
ToxR-for	CGGGGTACCATGACTAACATTGGCACC (<i>Kpn</i> I)	Upstream of <i>toxR</i>	216
ToxR-int-rev	AGTCATCCACCTCAAAACCTTG		
ToxR-int-for	TTGAGGTGGATGACTACGTAACACTGCGTAT	Downstream of <i>toxR</i>	230
ToxR-rev	CGGAGCTCAGTATCGTCAGGCAAGCT (<i>Sac</i> I)		
ToxR-F1	CGCGGATCCTTATTTCAGATGTCTGT (<i>Bam</i> HI)	ORF sequence of <i>toxR</i>	879
ToxR-F2	ACATGCATGCATGACTAACATTGGCAC (<i>Sph</i> I)		

a second PCR with primers of ToxR-for and ToxR-rev, which were introduced at a *Kpn* I or *Sac* I restriction site (underlined), respectively. The resulting PCR product was digested with *Kpn* I or *Sac* I, then inserted into the same sites of the suicide plasmid pRE112 which carried a *sacB* sucrose-sensitivity gene and conferred chloramphenicol resistance, generating the recombinant plasmid pRE112-*toxR* transformed into *E. coli* MC1061 λ pir and subsequently S17-1 λ pir. The plasmid pRE112-*toxR* was sequenced to confirm the correct construction. *E. coli* S17-1 λ pir containing the plasmid pRE112-*toxR* was conjugated with wild-type *V. alginolyticus* strain HY9901. Recipient cells were plated on TSA containing the antibiotics chloramphenicol (30 μ g ml⁻¹) to select the clone pRE112-*toxR* that had integrated the vector by a single crossover of allelic exchange. The conjugants were plated on counter-selective TSA containing 10% (w/v) sucrose. Colonies that grew on this medium were tested for chloramphenicol sensitivity to ensure the loss of plasmid by using TSA supplemented with chloramphenicol. The selected strains were confirmed by PCR analysis and sequencing of the chromosome. One of the mutants was named HY9901 Δ *toxR* and subjected to further study.

To create a complementary plasmid for the *toxR* mutant, the *toxR* gene was amplified using primers of ToxR-F1 and ToxR-F2 (Table 2), and then the amplified fragment was cloned into *Bam*H I and *Sph* I sites of the pACYC184 to construct pACYC184-*toxR*. The plasmid pACYC184-*toxR* was first transformed into *E. coli* DH5 α and plasmid DNA was isolated, and transformed into the HY9901 Δ *toxR* mutant to produce the complementary strain C-HY9901 Δ *toxR*. The presence of the plasmid was confirmed by PCR analysis and sequencing.

Characterization of *V. alginolyticus* Strain HY9901 Δ *toxR*

The phenotypes of the mutant were characterized including growth ability, biofilm formation, extracellular protease activities and swarming motility changes as previously

described [14]. Briefly, the wild-type strain HY9901, the mutant strain HY9901 Δ *toxR* and the complementary strain C-HY9901 Δ *toxR* were each cultured in TSA at 28 °C for 24 h. To measure the growth level of bacteria in TSB, overnight cultures of wild-type, the mutant and the complementary strain were inoculated into TSB with an initial OD₆₀₀ of 0.01, respectively. Samples were removed every 1 h and OD₆₀₀ was measured. Biofilm formation, extracellular protease activities, swarming motility were assayed and measured [18–20]. All experiments were performed in triplicate.

The medium lethal dose (LD₅₀) determination was conducted to assess the virulence of wild-type, the mutant and the complementary strain. The grouper fish (*E. coioides*) approximately weighing 50 g were obtained from a commercial farm in Zhanjiang city of China, cultured in tanks with circulating sea water at 28 °C and fed to satiation daily with commercial dry pellets. After 7 days of acclimatizing in the tank, the LD₅₀ was determined to assess the mortality titration response of the wild-type, the mutant and the complementary strain. Briefly, the different strains of *V. alginolyticus* were grown to an OD₆₀₀ of 0.4 in TSB, pelleted by centrifugation, and resuspended in sterilized phosphate-buffered saline (PBS, pH 7.4) to densities of 1 $\times$ 10⁸, 1 $\times$ 10⁷, 1 $\times$ 10⁶, 1 $\times$ 10⁵ and 1 $\times$ 10⁴ cfu ml⁻¹. For each bacterial density, a group of 20 grouper fish was injected intraperitoneally with 0.1 ml of bacterial suspension, and the control group was injected intraperitoneally with 0.1 ml of PBS. All of the experiments repeated in triplicate. The fish were monitored for mortality for 7 days and the values of LD₅₀ were calculated.

Two-Dimensional Gel Electrophoresis (2-DE) Profiles of Whole-Cell Lysates and the Identification of Protein Spots

Vibrio alginolyticus strain HY9901 Δ *toxR* and HY9901 were grown in TSB supplemented with 2% NaCl with gentle agitation at 28 °C for 18 h, respectively. Preparation of whole-cell protein extract and 2-DE was carried out

essentially [21]. The first-dimensional isoelectric focusing (IEF) and second-dimensional SDS-gel electrophoresis were performed according to the Amersham Biosciences manual. IEF was carried out with IPG strips (Immobiline DryStrip™, pH 4–7, 7 cm, GE Healthcare) by the IPG-phor TM system (GE Healthcare, Uppsala, Sweden). After IEF, the IPG strips were equilibrated for two intervals of 15 min in the equilibration solution. Equilibrated strips were placed onto 12.5% SDS–polyacrylamide gels, with a Bio-Rad Cell system (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed with an initial constant current of 80 V for 30 min, followed by 180 V until the tracking dye reached the gel bottom. The 2-DE experiments were repeated in triplicate.

Gel analysis was processed, and then the detection and calculation of standardized abundance of protein spots were carried out [21]. The volume ratio was calculated as the ratio of logarithmic values of the standardized volumes. Student's *t* test was used to determine the differential expression of protein spots. The differential spots reported had *p* values of ≤ 0.01 and volume ratio of ≥ 2.0 . The differential protein spots were excised, digested with trypsin and then analyzed by Liquid Chromatography Mass Spectrometry analysis (LC–MS/MS) between wild-type and the mutant [22]. MS/MS fragmentation patterns for each protein spot were searched against the NCBI database using a MASCOT search engine (www.matrixscience.com) and NMPDR database (<http://www.nmpdr.org/FIG/wiki/view.cgi>). Protein identification was considered significant if at least 2 different peptides were matched to the database. Mixture hits were ignored.

Results

Construction of the *toxR* Mutant

To understand the possible role of *toxR* in *V. alginolyticus*, an unmarked *toxR* deletion mutant was constructed by allelic exchange. Using the double selection strategy of allelic exchange mutagenesis by means of suicide vector pRE112, we deleted 607-bp (residues 206–812) of the *toxR* gene in *V. alginolyticus* strain HY9901, and obtained the *toxR* mutant. The mutant was confirmed by the mutant's inability to grow on TSA supplemented with chloramphenicol ($30 \mu\text{g ml}^{-1}$), and also verified by generating a 442-bp fragment after PCR analysis. Chromosomal sequencing showed that the mutation did not alter the reading frame of the downstream genes of *toxR*.

Characterization of the *toxR* Mutant

Several physiological phenotypes were compared among the mutant, wild-type and the complementary strain (Fig. 1). The mutant showed no discernible morphological difference from wild-type and the complementary strain when cultured in TSB. The mutant also showed a similar growth level with wild-type and the complementary strain. No significant difference of the activity of ECPase and swarming diameter was found between the mutant and wild-type when cultured in TSA, or TSB medium ($p > 0.05$). The *toxR* mutant of *V. alginolyticus* had suppressed biofilm formation, which was confirmed by the statistical analysis ($p < 0.05$).

Fig. 1 The physiological phenotypes of the mutant, wild-type and the complementary strain. **a** The growth curve; **b** the biofilm formation; **c** the swarming motility; **d** the activity of ECPase; **e** the value of LD₅₀. * $p < 0.05$; ** $p < 0.01$

To investigate the virulence of *ToxR* in vivo, the grouper fish were intraperitoneally injected with the mutant, wild-type and the complementary strain, and the value of LD₅₀ was then determined. The results showed the values of LD₅₀ for the mutant, wild-type and the complementary strain were 6.82×10^6 , 2.42×10^6 and 3.01×10^6 cfu per fish, respectively, indicating no significant difference in the virulence among the mutant, wild-type and the complementary strain under the experimental conditions ($p > 0.05$). The pathologic signs of vibriosis were observed by ulcers on the skin, hemorrhagic and swelling in the liver and kidney in the experimental group. None of fish died or became diseased in the control group.

2-DE Profiles of Whole-Cell Lysates and Protein Identification

The whole-cell lysates from wild-type and the mutant were resolved by 2-DE, in triplicates for each of the two independent biological samples. A total number of 427 protein

spots were detected, out of which 2 (0.05%) proteins increased and 12 (2.81%) decreased in expression levels significantly ($p < 0.01$) after Coomassie R-250 staining (Fig. 2). Most of the spots were found to be in the pH 4–7 range and had molecular masses of 10–130 kDa. Of the 427 protein spots detected, 14 protein spots of interest were excised to detect by LC–MS/MS analysis (Table 3). The LC–MS/MS results showed only 12 protein spots corresponding to 12 proteins could be identified among 14 different protein spots, and the LC–MS/MS results of spot 2 and 3 were negative due to the very low abundance of the proteins. In the identified 12 proteins, the expression levels of 2 proteins including glutamine synthetase and levansucrase were up-regulated, and the expression levels of 10 proteins including metabolism-related proteins, transport-related proteins, molecular chaperone DnaK and outer membrane protein U (OmpU) were down-regulated in the *toxR* mutant compared with that in wild-type (Table 3).

Table 3 Identified protein spots with changed expression levels by LC-MALDI

Protein no.	Protein name	R	Accession no.	Function	Subcellular localization
T1	Pterin-4- α -carbinolamine dehydratase	– 1.76	gil91227037	Biosynthesis of cofactors, prosthetic groups, and carriers	Cell inner membrane
T4	Electron transfer flavoprotein, α -subunit	– 1.57	gil91224794	Iron-binding	Cell inner membrane, cytoplasm
T5	Amino acid ABC transporter, periplasmic amino acid-binding protein	– 1.42	gil91227965	Transport and binding proteins	Periplasm
T6	Putative pyruvate dehydrogenase E1 component, α subunit	– 1.29	gil91225779	Unknown	Cytoplasm
T7	Dihydrolipoamide acetyltransferase	– 1.54	gil91225777	Energy metabolism	Cytoplasm
T8	Pyruvate kinase	– 1.27	gil91227129	Energy metabolism	Cell inner membrane, cytoplasm
T9	D-fructose-6-phosphate amidotransferase	– 1.42	gil91227131	Energy metabolism	Cytoplasm
T10	Molecular chaperone DnaK	– 1.31	gil91225094	Promote protein folding, interaction, and translocation, both constitutively and in response to stress,	Cytoplasm
T11	Glutamine synthetase	+ 1.36	gil91224957	Amino acid biosynthesis	Cytoplasm
T12	Outer membrane protein U	– 1.88	gil237846493	Cell envelope	Cell outer membrane
T13	Chain A, crystal structure of levansucrase	+ 1.53	gil38492844	Hydrolysis of sucrose	Extracell
T14	Hypothetical protein V12G01_14960	– 1.23	gil91227427	Unknown	Cytoplasm

R = + (the mutant strain/wild-type strain) for up-regulated proteins, and R = – (wild-type strain/the mutant strain) for down-regulated

Fig. 2 2D map (pH 4–7) of a whole-cell lysate of *Vibrio alginolyticus* strain HY9901 and HY9901 Δ *toxR*. The positions of molecular weight standards are indicated on the right. **a** The typical 2D gel of

Vibrio alginolyticus strain HY9901. **b** The typical 2D gel of *Vibrio alginolyticus* strain HY9901 Δ *toxR*

Discussion

Vibrio alginolyticus can adopt several survival strategies in aquatic environments. The bacterium can survive as free-living or in association with zooplankton, and penetrate the surface barriers of the host and disseminate effectively in the host tissue as a major cause of fish bacterial septicaemia [12]. Biofilm formation is a multicellular behavior by which bacteria can colonize a surface of the host tissue, leading to resistance to antibiotics and host immune-killing [23]. It was reported that *V. alginolyticus* invaded the intestine of fish where some adverse factors such as antimicrobial peptides could easily be overcome for successful colonization and initiation of infection with the assistance of biofilm formation [24]. In this study, a mutant strain of *V. alginolyticus* with an in-frame deletion of *toxR* gene was constructed to reveal the role of ToxR in the physiology and virulence of *V. alginolyticus*. ToxR was shown to enhance biofilm formation, which should assist bacterial survival and colonization in the fish intestine. However, we found the deletion mutation of *toxR* didn't clearly affect the growth ability, swarming motility, activity of ECPase and the virulence (the value of LD₅₀). These indicate that ToxR may not be needed for the development of disseminated disease. Therefore, ToxR may be only involved in the early stages of infection by influencing colonization of the bacteria on the surface of the intestine through enhancing the biofilm information. The results were in accordance with that of Chen et al. [12] in *V. alginolyticus* strain ZJ51-O.

Biofilm formation of *Vibrio* depends on specific structural genes (flagella, pili and exopolysaccharide biosynthesis) and complex regulatory processes (two-component regulators, quorum sensing and c-di-GMP signaling) [25].

In the study, the deletion mutation of *toxR* could up-regulate the expression of glutamine synthetase and levansucrase, and down-regulate the expression of 10 proteins such as OmpU. It was proved that biofilm information was suppressed in case of glutamine synthetase mutant in *Mycobacterium bovis* [26]. The glutamine synthetase also overexpressed during biofilm growth in exponential and stationary phases of bacterial growth by DNA array analysis of an *Escherichia coli* biofilm formation [27]. It was reported that levansucrase could contribute to biofilm formation in *Pseudomonas syringae*, and its expression occurred mainly in the early stages of bacterial growth and biofilm development [28]. Levansucrase is also involved in tolerance to NaCl, sucrose and desiccation, and in biofilm formation in *Gluconacetobacter diazotrophicus* [29]. In the investigation of biofilm formation, the ToxR of *V. alginolyticus* had significantly enhanced biofilm formation. Therefore, there are good theoretical reasons to believe that the expression of glutamine synthetase and levansucrase might have related to biofilm formation of *V. alginolyticus*.

However, an evidence showed that the expression of OmpU was positively regulated by ToxR, and a *toxR* mutant showed a thicker biofilm with better surface area coverage compared to that of wild-type in *V. anguillarum* [30]. Interestingly, our results showed that the expression of OmpU was suppressed and biofilm formation was decreased in the *toxR* mutant, and were inconsistent with that in *V. anguillarum*. This indicated OmpU could negatively regulate biofilm formation in *V. alginolyticus*. Furthermore, it should be further studied that how OmpU negatively regulates biofilm formation of *V. alginolyticus*.

In conclusion, the results of our study showed that ToxR didn't clearly affect the growth ability, swarming motility,

activity of ECPase and the virulence, and could only enhance biofilm formation of *V. alginolyticus* probably via modulating the expression of glutamine synthetase, levansucrase and OmpU. The suggests ToxR may be involved in the early stages of infection by influencing colonization of the bacteria on the surface of the intestine through enhancing the biofilm information, and cannot affect directly the later stage of disseminated infection as indicated by the failure of *toxR* mutation to attenuate the virulence of *V. alginolyticus*.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 31572656), Fishing Port Construction and Fishery Development Funds of Guangdong Province (Prevention of Fish Disease), Natural Science Foundation of Guangdong Province (No. 2015A030308020, 2017A030307033 and 2017A030313174), and High-level Talents Project of Sailing Plan in Guangdong Province (No. GDOU2017030501).

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