Biochemistry and Biophysics Reports 31 (2022) 101282

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

VfqI-VfqR quorum sensing circuit modulates type VI secretion system VflT6SS2 in *Vibrio fluvialis*

Yu Han, Jingjing Pan, Yuanming Huang, Qian Cheng, Ping Liu, Baowei Diao, Jie Li, Biao Kan **, Weili Liang *

State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changbai Road 155, Changping District, Beijing, China

ARTICLE INFO

Keywords:
T6SS
QS
VfqI
VfqR
V. fluvialis

ABSTRACT

V. fluvialis is an emerging foodborne pathogen and could cause cholera-like gastroenteritis syndrome and poses a potential threat to public health. VflT6SS2 is a functionally active type VI secretion system (T6SS) in *V. fluvialis* which confers bactericidal activity. VflT6SS2 is composed of one major cluster and three *hcp-vgrG* orphan clusters. Previously, we identified two quorum sensing (QS) systems CqsA/LuxS-HapR and VfqI-VfqR in *V. fluvialis* and demonstrated that the former regulates VflT6SS2. However, whether VfqI-VfqR QS regulates VflT6SS2 is unknown. In this study, we showed that the mRNA abundances of VflT6SS2 *tssD*2 (*hcp*), *tssI*2 (*vgrG*) and *tssB*2 (*vipA*) were all significantly decreased in VfqI or/and VfqR deletion mutant(s). Consistently, Hcp expression/secretion was reduced too in these mutants. Complementation assay with VfqR mutant further confirmed that the reduced Hcp expression/secretion and impaired antibacterial virulence are restored by introducing VfqR-expressing plasmid. Reporter fusion analyses revealed that VfqR modulates the promoter activities of VflT6SS2. Bioinformatical prediction and further reporter fusion assay in *E. coli* supported that VfqR acts as a transcriptional factor to bind and regulate the gene expression of the VflT6SS2 major cluster. However, VfqR seems to promote transcription of *hcp* (*tssD2*) in the orphan clusters through elevating the expression of *vasH* which is encoded by the VflT6SS2 major cluster. Additionally, we found that the regulation intensity of VfqR on VflT6SS2 is weaker than that of HapR. In conclusion, our current study disclosed that in *V. fluvialis*, VfqI-VfqR circuit upregulates the expression and function of VflT6SS2 by directly or indirectly activating its transcription. These findings will enhance our understanding of the complicated regulatory network between QS and T6SS in *V. fluvialis*.

1. Introduction

Vibrio fluvialis is a gram-negative, polarly-flagellated, halophilic, oxidase-producing bacterium and is considered as an emerging foodborne pathogen with epidemic potential [1]. It was first isolated from a patient with severe diarrhea in Bahrain in 1975 [2] and is globally distributed in the aquatic milieu, mostly in the seas, estuaries, and brackish water [1]. People get infected by eating untreated seafood and suffer acute gastroenteritis and cholera-like diarrhea [3,4]. The isolation rates of *V. fluvialis* from patients with cholera-like diarrhea, extra-intestinal infections and the appearance of multidrug-resistant strains are gradually increasing [5,6]. So, *V. fluvialis* is becoming a great threat to food safety and public health.

Type VI secretion system (T6SS) is a contact-dependent bacterial weapon that allows for direct killing of competitors through the translocation of proteinaceous toxins. T6SS was first found in *Vibrio cholerae*, it can mediate *V. cholerae* non-O1/non-O139 infection in humans [7]. Since its discovery, T6SS-mediated pathogenic effect was reported in many microbes such as *Pseudomonas aeruginosa* [8], *Escherichia coli* [9], *Edwardsiella piscicida* [10], etc. Therefore, originally T6SS was considered as another way of bacterial pathogenesis [7–9], later on, continuously increasing evidence showed that T6SS is involved in a variety of physiological processes, such as bacterial competitors [11], environmental survival [12], metal ion uptake [13–15]. In *V. fluvialis* 85003, we identified two T6SS clusters, namely VflT6SS1 and VflT6SS2. VflT6SS2 is functionally expressed and mediates antibacterial virulence, and its

* Corresponding author. Changbai Road 155, Changping District, Beijing, China.
** Corresponding author. Changbai Road 155, Changping District, Beijing, China.
E-mail addresses: kanbiao@icdc.cn (B. Kan), liangweili@icdc.cn (W. Liang).

https://doi.org/10.1016/j.bbrep.2022.101282
Received 2 March 2022; Received in revised form 20 April 2022; Accepted 16 May 2022
2405-5808/© 2022 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

activity is regulated by environmental signals such as temperature, osmotic pressure, and global regulators such as HapR and IHF [16–18]. VflT6SS2 is composed of one major cluster and three *hcp-vgrG* orphan clusters, *tssD*2_a-*tssI*2_a, *tssD*2_b-*tssI*2_b and *tssD*2_c-*tssI*2_c [18]. The secretion activity of VflT6SS2 depends on host growth phase and it works when optical density (OD_{600}) reached between 1.0 and 2.0 [18]. The underlying mechanism of this phenomenon remains unclear and might be involved in the regulation of quorum sensing (QS).

QS is a process of bacterial cell-to-cell chemical communication that relies on the production, detection and response to extracellular signaling molecules called autoinducers. QS controls group behaviors in complex and dynamically changing environments [19] and many physiological activities are subject to QS regulation such as bioluminescence [20], virulence factor production [21], secondary metabolite production [22], biofilm formation [23], and host-microbe interaction [24,25]. Due to its broad-spectrum regulatory effect, QS has been considered as an attractive target for antimicrobial therapy and a means of biological control in agriculture [21,24,26]. Previously, we identified two QS systems in *V. fluvialis*: one is CqsA/LuxS-HapR with cholera autoinducer 1 (CAI-1) and autoinducer 2 (AI-2) as signal molecules, and the other is VfqI-VfqR with acyl-homoserine lactone (acyl-HSL or AHL) as the autoinducer [27]. We have shown that CqsA/LuxS-HapR system modulates VflT6SS2 by directly activating the transcription of its major cluster and orphan clusters [17]. However, whether VfqI-VfqR system participates in the regulation of VflT6SS2 is unknown and thus is the focus of this study.

AHL-dependent QS was firstly discovered in a marine bioluminescence bacterium *Vibrio fisheri* [28]. It contains two main components, AHL synthase LuxI and transcriptional regulator LuxR. LuxI catalyzes the synthesis of AHLs from acylated-acyl carrier protein and S-adenosylmethionine [29]. LuxR then binds AHL and the resultant LuxR-AHL complex promotes transcription of target genes, such as *lux* operon responsible for bioluminescence [30]. In addition, *luxR* homolog without a linked *luxI* homolog termed an orphan or solo, has been found and *luxI* solo also exists [31]. In *V. fluvialis,* LuxI homolog VfqI produces three AHL molecules with 3-oxo-C_{10}-HSL as the major component and they are sensed by LuxR homolog VfqR. VfqR-AHL is required to activate *vfqI* expression and auto-repress *vfqR* expression. The other regulatory targets of VfqI-VfqR remain to be investigated in addition to activating hemolysin expression and therefore showing toxicity to tissue culture cells [27]. In this study, we proved that VfqI-VfqR QS regulates the expression and function of VflT6SS2 thus contributing to environmental fitness of *V. fluvialis*.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are shown in Supplementary Table S1. Δ*vfqI* and Δ*vfqR* mutants were constructed previously [27]. Δ*vfqI-vfqR* was generated by introducing pWM91-*vfqI* into Δ*vfqR*. Exconjugants and sucrose-resistant mutants were selected as described [16]. *V. fluvialis* were grown in Luria-Bertani (LB) broth with 2% NaCl at 30 °C. *E. coli* was cultured in LB with 1% NaCl at 37 °C. Culture media were supplemented with antibiotics as required: ampicillin (Amp, 100 μg/mL), streptomycin (Sm, 100 μg/mL), chloramphenicol (*Cm*, 10 μg/mL for *E. coli*, 2.5 μg/mL for *V. fluvialis*), tetracycline (Tc, 10 μg/mL for *E. coli*, 2.5 μg/mL for *V. fluvialis*).

Complementation plasmid pSR*vfqR* was constructed by amplifying and cloning *vfqR* coding sequence into pSRKTc. VfqR was expressed from the *lac* promoter with the induction of isopropyl-b-D-thiogalactopyranoside (IPTG).

2.2. RNA extraction and quantitative real-time PCR (qPCR)

V. fluvialis strains were cultured to OD_{600} 1.5. Total RNA extraction, chromosomal DNA removal, cDNA synthesis, qPCR and relative expression values (R) were performed or calculated as previously described [16]. The related primers were listed in Supplementary Table S2. A control reaction using DNase-treated RNA as a template was performed for each sample to exclude chromosomal DNA contamination.

2.3. Analyses of VflT6SS2 Hcp expression and secretion

Overnight cultures were diluted 1:100 into 20 mL LB medium and incubated to OD_{600} 1.5 at 30 °C. As previously described, protein samples were prepared from cell pellets and cell-free supernatants [16,18]. Western blot analysis was performed with polyclonal rabbit anti-Hcp antibody and anti-*E. coli* cyclic AMP receptor protein (CRP) antibody. CRP was used as a control for Hcp secretion to exclude the cell lysis.

For VfqR complementation, pSR*vfqR*/Δ*vfqR* or pSRKTc/Δ*vfqR* were cultured to OD_{600} 0.5. Then, each sample was divided into two parts. One was induced with 0.5 mM IPTG, and the other was not induced as a control. The cultures were continually incubated to OD_{600} 1.5 for protein samples preparation.

2.4. Bacterial killing assay

The assay was performed as described previously [16,17]. The predator strains pSRKTc/WT, pSRKTc/Δ*vfqR*, pSR*vfqR*/Δ*vfqR*, and pSRKTc/Δ*vasK* were mixed with *E. coli* prey MG1655 at 9:1 ratio in triplicates. Then, 10 μL of the 10-fold concentrated mixtures were spotted on filter membrane placed on 2% NaCl LB agar plate containing 1 mM IPTG and incubated at 30 °C for 12 h. The colony-forming units (CFU) of the prey MG1655 at the beginning (T0) and after 12 h incubation with predators (T12) were calculated by plating 10-fold serial dilutions on Rfp^R (50 μg/mL) plates.

2.5. Luminescence activity assay

The overnight culture of *V. fluvialis* containing *lux* reporter fusion plasmid was diluted at 1:100 in fresh LB and incubated at 30 °C with shaking. Luminescence activity and OD_{600} were measured and calculated as previously described [16]. The culture of *E. coli* SM10*λpir* containing pVflT6SS2-*lux* or p*tssD*2*a*-*lux* together with pSR*vfqR* or pSRKTc was complemented with 0.5 mM IPTG and various concentrations of exogenous 3-oxo-C10-HSL as indicated.

2.6. Statistical analysis

GraphPad Prism 9 software was used for data statistics and analysis. Statistical significance was determined by unpaired two-tailed Student's *t*-test.

3. Result

3.1. VfqI-VfqR QS regulates VflT6SS2 expression

The products of *tssD*2 (*hcp*) and *tssI*2 (*vgrG*) function not only as the structural components but also the secreted effectors of VflT6SS2. *tssB*2 (*vipA*) is the first gene of VflT6SS2 major cluster and encodes the sheath component. To determine whether VfqI-VfqR QS regulates VflT6SS2, we detected the mRNA levels of *tssD*2 (*hcp*), *tssI*2 (*vgrG*) and *tssB*2 (*vipA*) in mutants Δ*vfqI*, Δ*vfqR* and Δ*vfqI-vfqR*. Compared with WT, the mRNA abundances of *tssD*2 (*hcp*), *tssI*2 (*vgrG*) and *tssB*2 (*vipA*) were greatly reduced in Vfql-VfqR mutants, implying that VfqI-VfqR QS was involved in the regulation of VflT6SS2 (Fig. 1A–C). To further confirm, we detected Hcp levels in WT and above mutants. As expected, both the expression and secretion levels of Hcp in Δ*vfqI*, Δ*vfqR* and Δ*vfqI-vfqR* were obviously less than WT (Fig. 1D). These results proved that VfqI-VfqR QS circuit positively regulates VflT6SS2.

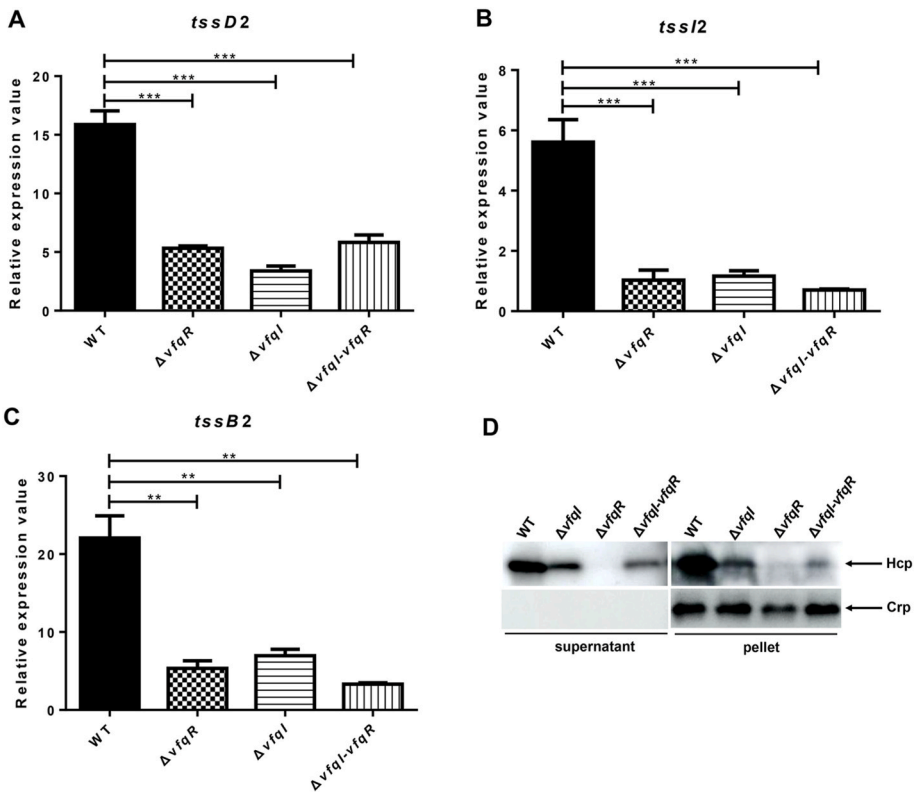

Fig. 1. VfqI-VfqR QS modulates VflT6SS2 expression. (A–C) Comparison of the mRNA levels of *tssD*2 (A), *tssI*2 (B) and *tssB*2 (C) in WT, Δ*vfqR*, Δ*vfqI* and Δ*vfqI-vfqR* mutants. The mRNA abundances around OD600 of 1.5 were determined using qPCR. **P < 0.01, ***P < 0.001. (D) Expression and secretion of Hcp in WT, Δ*vfqI*, Δ*vfqR*, and Δ*vfqI-vfqR*. Western blot analysis was performed to detect target protein in cell pellets or culture supernatants using anti-Hcp and anti-Crp antibodies. The arrows show the immunoblot bands to Hcp or Crp.

3.2. *VfqR is the key player of VfqI-VfqR circuit on VflT6SS2*

We have shown that in *V. fluvialis* VfqI functions as an AHL synthase and VfqR is the AHL-dependent LuxR homolog [27]. *vfqR* and *vfqI* are convergently-transcribed with an intergenic region of 1 bp and a potential *lux* box (5′-AACTGTTCGATCGGACAGGT-3′) was found upstream of *vfqI* which is centered at −68.5 from its start codon. The *lux* box functions as the LuxR binding site to activate target loci [32]. The existence of *lux* box in *vfqI* promoter correlates well with our previous results, i. e. VfqR-AHL is required to activate *vfqI* expression [27]. VfqR-AHL binding to the *lux* box results in a remarkable increase of AHL production and creates a positive-feedback loop. We did find that the level of AHL was increased in a cell density-dependent pattern [33]. Based on the facts that homologs of LuxR are widely considered to be transcriptional regulators [34] and the regulation of *vfqR* on *vfqI* [27], and consistently reduced expression of VflT6SS2 coding genes in Vfql-VfqR mutants, we supposed that VfqR is the master regulator of VfqI-VfqR QS on VflT6SS2.

Domain analysis (Pfam) revealed two principal conserved domains in VfqR: N-terminal AHL-binding domain (residues 16–163) and C-terminal DNA binding HTH domain (residues 174–230). Sequence alignments of VfqR with canonical LuxR homologs revealed the presence of strongly conserved residues both in the AHL-binding domain (W61, Y65, D74, P75, W89, G114) and the DNA-binding domain (E180, W186, G190) which are present in at least 95% of LuxR-type proteins [35,36] (Fig. 2A). The highly conserved residues in the AHL-binding domain are involved in the formation of intermolecular H-bonds with AHL molecules, reflecting the common activation mechanism by AHLs with identical HSL headgroups [37]. Phylogenetic analysis of VfqR and other LuxR-type proteins (Fig. 2B) indicated that VfqR shared the highest amino acid similarity with VanR of *Vibrio anguillarum*, followed by LuxR of *V. fischeri*, LasR from *Pseudomonas aeruginosa* and AhyR from *Aeromonas hydrophilia* (Pairwise alignment: VfqR = 76%, 38%, 36% and 31%, respectively).

To further confirm the regulation of VfqR on VflT6SS2, we performed complementation assay. As shown in Fig. 2C, inclusion of pSR*vfqR* in Δ*vfqR* restored Hcp expression and secretion with or without IPTG induction, indicating that VfqR regulate VflT6SS2, and the amount of VfqR needed seems very small considering the uninduced (leaky) expression of VfqR recovered Hcp expression/secretion. Consistently, the bactericidal ability of Δ*vfqR* was greatly impaired in contrast to WT but restored after *vfqR* complementation (Fig. 2D). Though it did not recover to WT level, the bactericidal ability of pSR*vfqR*/Δ*vfqR* was still significantly higher than those of pSRKTc/Δ*vfqR* and pSRKTc/Δ*vasK*, the latter was used as a T6SS negative control. These data demonstrated that VfqR is the key regulator of VfqI-VfqR QS on VflT6SS2 function.

3.3. *VfqR is required for the promoter activities of VflT6SS2 clusters*

VflT6SS2 consists of one major cluster and three orphan clusters, *tssD*2_a-*tssI*2_a, *tssD*2_b-*tssI*2_b and *tssD*2_c-*tssI*2_c (Fig. S1) [18]. Since VfqR works as a transcriptional factor to regulate target gene expression, we checked the luminescence activities of *lux* reporter plasmids containing the promoter regions of VflT6SS2 major cluster (pVflT6SS2-*lux*) and three orphan clusters (p*tssD*2a-*lux*、p*tssD*2b-*lux* and p*tssD*2c-*lux*) in WT and Δ*vfqR*, which we constructed in previous study [16]. As shown in Fig. 3, the promoter activities of pVflT6SS2-*lux*、p*tssD*2a-*lux*、p*tssD*2b-*lux* and p*tssD*2c-*lux* in Δ*vfqR* were all significantly lower than in WT. To compare with the CqsA/LuxS-HapR circuit, we also monitored the promoter activities of the above reporter constructs in deletion mutant of *hapR* which is the master regulator of CAI-1/AI-2 based QS. By contrast, in Δ*hapR*, the promoter activities diminished to a much lower level (Fig. 3). Therefore, these results demonstrated that VfqR regulates the major and three orphan clusters of VflT6SS2 at the promoter level, and VfqI-VfqR circuit is the secondary QS regulatory system of VflT6SS2 when comparing to CqsA/LuxS-HapR circuit. However, whether VfqR directly works on the promoter regions of VflT6SS2 still needs to be determined.

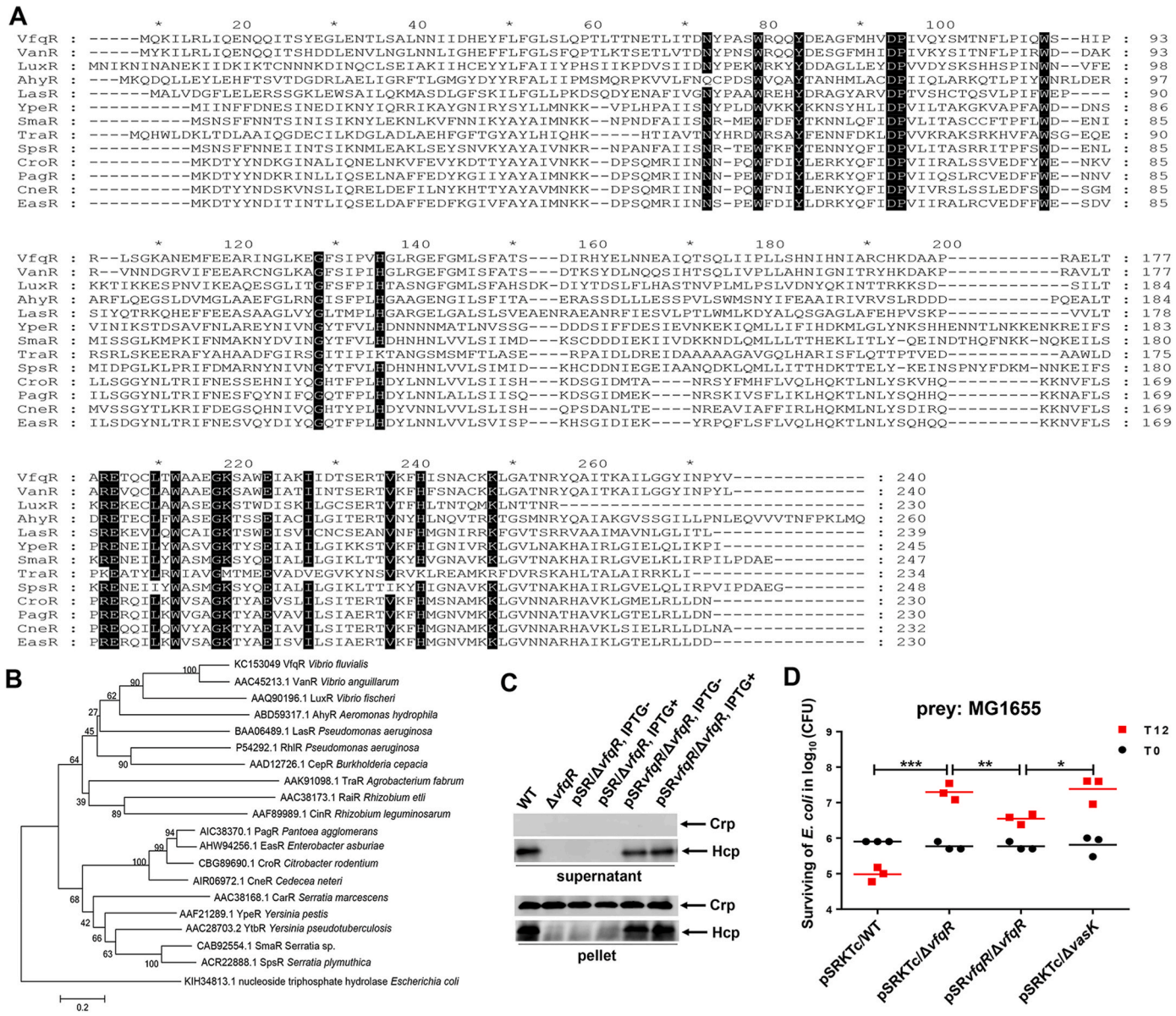

Fig. 2. VfqR is the key modulator of VfqI-VfqR QS on VflT6SS2 expression. (A) Mega 7 multiple sequence alignment of *V. fluvialis* VfqR protein with canonical LuxR-type proteins. Their GenBank accession numbers are listed in Supplementary Table S3. Highly conserved residues are given a black background. (B) Phylogenetic tree showing the evolutionary distances between VfqR and other canonical QS LuxR-type proteins. The tree was generated using the Neighbor-Joining method and was drawn to scale, with branch lengths showing the evolutionary distances. The bootstrap values are given as numbers at the nodes (percentage of 1000 replicates). *E. coli* KIH34813.1 was used as an outgroup. The horizontal bar indicates evolutionary distance as 0.2 changes per nucleotide position. (C) The effect of *vfqR* on Hcp expression and secretion. Protein extracts from cell pellets and culture supernatants of WT and Δ*vfqR* with pSR*vfqR* or pSRKTc were subjected to Western blot analysis with the anti-Hcp or anti-CRP antibody. (D) Bacterial killing assay. The CFU of the prey MG1655 was determined at the start point (T0) and after 12 h (T12) incubation with *V. fluvialis* predators. *P < 0.05, **P < 0.01, ***P < 0.001.

3.4. VfqR directly activates the promoter of VflT6SS2 major cluster

To clarify whether VfqR directly activates VflT6SS2 major cluster and orphan clusters, we screened and identified a partially conserved VfqR binding element (5′-ATATGTAAGTTTAACTTGTA-3′) based on a relaxed *lux* box-like sequence [32] in the promoter region of VflT6SS2 major cluster which is positioned at nucleotides −196 to −177 relative to the *tssB*2 (*vipA*) start codon, closely downstream of previously identified HapR binding site 1 (Fig. 4A).

To functionally prove the direct regulation, we measured the target promoter activity in *E. coli* SM10*λpir* strain as literature reported [30]. *E. coli* has a heterogenous genetic background where possible additional regulation existed under circumstances within the native host, *V. fluvialis* could be excluded. pT6SS2-*lux* displayed very low luminescence activity in SM10*λpir* with control plasmid pSRKTc (Fig. 4B). However, induction of VfqR from pSR*vfqR* greatly increased the luminescence activity of pT6SS2-*lux*, suggesting that VfqR could directly activate the major cluster promoter of VflT6SS2. Beyond expectation, the activation of pT6SS2-*lux* by VfqR in SM10*λpir* seems independent of 3-oxo-C10-HSL, as without addition of exogenous 3-oxo-C10-HSL, pSR*vfqR* still activated pT6SS2-*lux* in SM10*λpir* (Fig. 4B). Consistent with the absence of potential *lux* box-like element in the *hcp* promoters, p*tssD*2a-*lux* (used as a representative) could not be activated by pSR*vfqR*, but was obviously activated by pSR*hapR* via the direct regulation of HapR on *hcp* promoter which we reported before [17] (Fig. 4C). Therefore, we reasoned that the decreased promoter activities of orphan

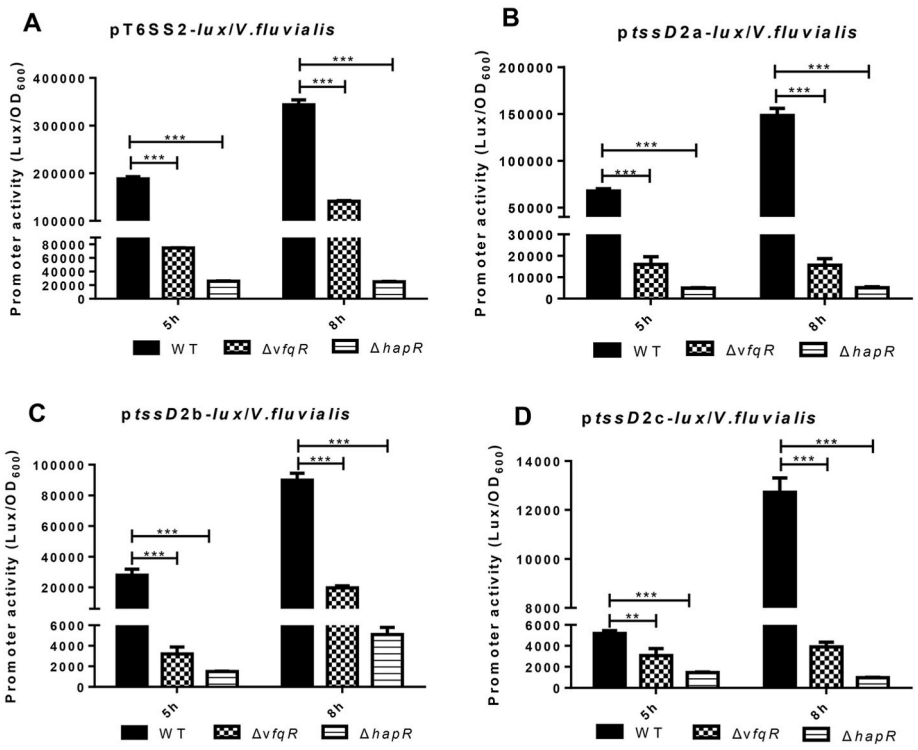

Fig. 3. Luminescence activities of *lux* reporter fusion plasmids in *V. fluvialis* WT, Δ*vfqR* and Δ*hapR*. Overnight cultures of WT and mutants containing pVflT6SS2-*lux* (A), p*tssD*2a-*lux* (B), p*tssD*2b-*lux* (C) or p*tssD*2c-*lux* (D) were 1:100 diluted in fresh 2% NaCl LB with *Cm* and incubated at 30 °C with shaking. The luminescent activities were reported as luminescence/OD_{600} at the designated time points. **P < 0.01, ***P < 0.001.

clusters in Δ*vfqR* were due to the indirect effect caused by the reduced VasH expression which is encoded within the major cluster and required to activate *hcp* [16,18]. As demonstrated, the *vasH* mRNA level was indeed down-regulated in VfqI-VfqR circuit deletion mutants (Fig. 4D). For further validation we introduced the VasH expressing construct pSR*vasH* or pSRKTc into WT or Δ*vfqR* containing p*tssD*2a-*lux*. As shown in Fig. 4E, the luminescence activity of p*tssD*2a-*lux* in pSR*vasH*/Δ*vfqR* was raised to the same level as in pSRKTc/WT. Though the average signal intensity in pSR*vasH*/WT was higher than that in pSR*vasH*/Δ*vfqR,* statistical analysis indicated no significant difference between them. Together, these results showed that VfqR modulates VflT6SS2 by directly or indirectly activating their promoters.

4. Discussion

As an energy-consuming secretion system, T6SS is under tight control to benefit bacterial fitness and pathogenicity [39]. Disecting the regulation network of T6SS is an important way to understand the modulation and function of T6SS.

V. fluvialis has relatively complicated QS by producing and detecting at least three autoinducers [27]. CAI-1 and AI-2 signals of CqsA/LuxS-HapR circuit are transmitted via the shared phosphorylation–dephosphorylation cascade through LuxU and LuxO, and finally converged to the master regulator HapR [40]. Previously, we showed CqsA/LuxS-HapR circuit activates VflT6SS2 through the master regulator HapR [17]. Here, we clarified that AHL-based VfqI-VfqR circuit also manipulates the function of VflT6SS2 and further proved that the AHL-responsive receptor, VfqR, is the key player which works at the transcriptional level. Though both QS circuits are involved in regulating VflT6SS2, differences exist between each other. First, the intensity of activation mediated by CqsA/LuxS-HapR QS circuit is much stronger than that of VfqI-VfqR. Mutation of HapR almost fully abolished the VflT6SS2 promoter activities, while deletion of VfqR still maintained partial promoter activities. So, we proposed that VfqR is the secondary QS regulator of VflT6SS2 while HapR is the predominant one. Second, HapR directly activates the promoters of both the major cluster and orphan clusters [17], while VfqR only directly targets the promoter of the major cluster. Furthermore, there are two highly conserved HapR binding sites [17] but only one less conserved VfqR binding element in the major cluster promoter, which may account for the difference of regulation efficiency between HapR and VfqR. The specific biological significance of VfqR and HapR regulation on VflT6SS2 is still unknown. Previously, we found that at similar cell density, *V. fluvialis* produces higher levels of CAI-1 and AI-2 at 37 °C, while AHL production peaks at 30 °C [33], so we reasoned that high levels of CAI-1 and AI-2 at 37 °C might benefit the fitness of *V. fluvialis* in infected host intestine, whereas AHL probably helps the survival in environment.

In addition, we found that in SM10*λpir* the activation of VflT6SS2 major cluster promoter by VfqR was independent of exogenous complemented 3-oxo-C_{10}-HSL, which is the most abundant one in three AHL molecules identified in *V. fluvialis*: 3-oxo-C10-HSL, C10-HSL and 3-oxo-C12-HSL [27]. A possible interpretation of this phenomenon is that VfqR might function as a luxR solo (orphan LuxR) regulator homolog in *E. coli*, which could sense more signals than just cognate AHLs [41]. Some LuxR solos were reported to be implicated in intraspecies, interspecies, and interkingdom signaling [42]. On the other hand, this result suggested that although 3-oxo-C10-HSL is the main component of AHLs in *V. fluvialis*, it might not be the real AHL molecule sensed and bound by VfqR to activate the AHL-VfqR controlled promoters. It was recently reported that LuxR-homolog YasR in *Pseudoalteromonas* sp. R3 only responds to C8-HSL but not 3-OH-C8-HSL, which are synthesized by LuxI homolog YasI [43]. Similarly, YasR could promote the expression of itself in an AHL-independent manner [43]. Previously, we used arabinose-induced P_{BAD}-*vfqI* construct or concentrated spent medium from WT *V. fluvialis* as the sources of AHL which theoretically produces or contains above three AHLs instead of only 3-oxo-C10-HSL [27]. We are interested in determining the true VfqR-responsive AHL in *V. flivialis* in future study.

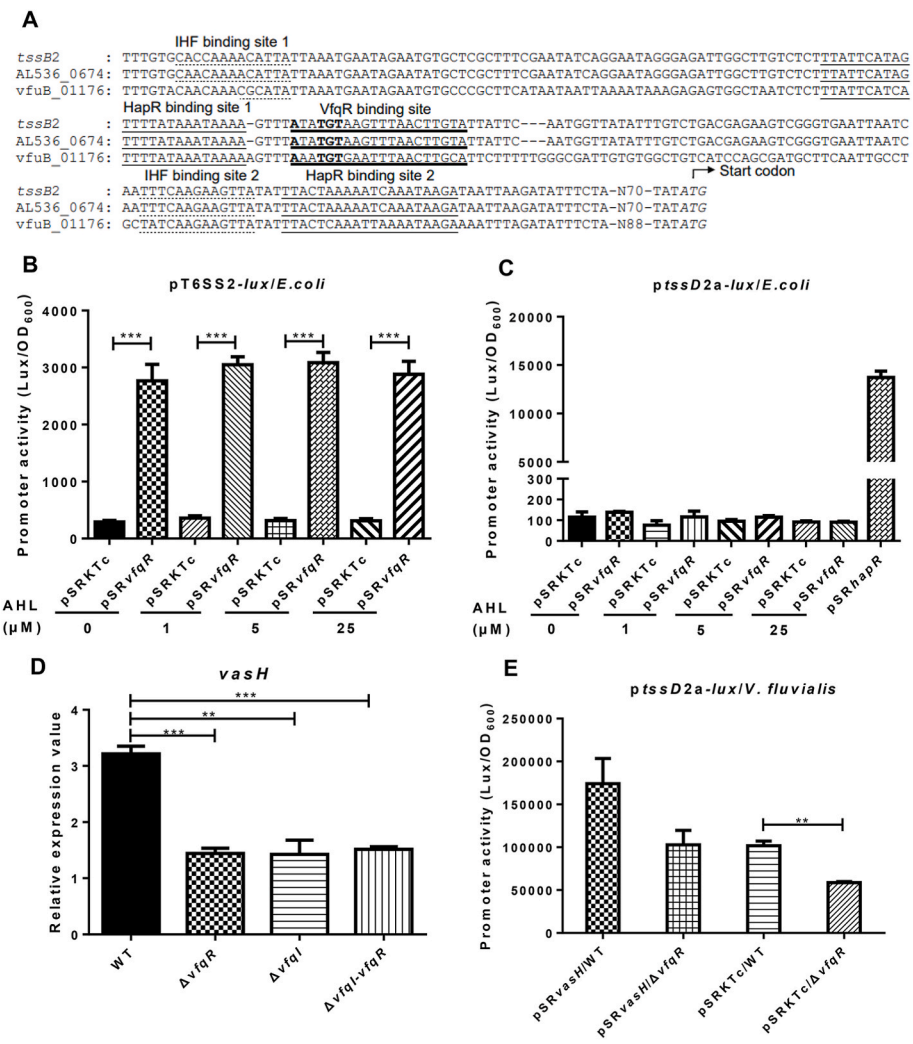

Fig. 4. VfqR directly activates VflT6SS2 major cluster and indirectly activates *tssD*2 (*hcp*). (A) Characteristics of the promoter region of VflT6SS2 major cluster. Nucleotide sequences of the promoter region of *tssB*2 (*V. fluvialis* 85003), AL536_06745 (*V. fluvialis* 33809) and vfuB_01176 (*V. furnissii* NCTC11218) were aligned. The predicted VfqR binding sites are underlined and the conserved bases according to literature [38] were highlighted in boldface type. Previously characterized IHF and HapR binding sites are also shown. (B and C) Luminescence activities of pT6SS2-*lux* and p*tssD*2a-*lux* with pSR*vfqR* or pSRKTc in SM10*λpir*. Overnight cultures of the strains were 1:100 diluted in fresh LB with 0.5 mM IPTG induction at 30 °C. Different concentrations of 3-oxo-C10-HSL (0, 1, 5, 25 μM) were added to the corresponding cultures as indicated. pSR*hapR* was used as a positive control. ***P < 0.001. (D) mRNA level of *vasH* in WT, Δ*vfqR*, Δ*vfqI* and Δ*vfqI-vfqR* mutants. **P < 0.01, ***P < 0.001. (E) The activity of p*tssD*2a-*lux* in WT and Δ*vfqR* containing either pSR*vasH* or pSRKTc control vector. The cultures were induced with 0.5 mM IPTG at 30 °C. **P < 0.01.

Although we identified a potential *lux*-like box in the promoter region of VflT6SS2 major cluster and demonstrated the VfqR-dependent activation of the promoter activity in a heterogeneous genetic background, we did not establish the direct binding of VfqR to the VflT6SS2 major promoter *in vitro* due to unsolved VfqR expression problem. Specifically, we could not get recombinant VfqR protein no matter with pET or pTXB1 vector under various inducing conditions in *E. coli* host. The underlying issue needs more exploration in future.

In summary, we clarified the regulation of VfqI-VfqR QS on VflT6SS2 in this study. These findings further broadened our understanding of biological functions of VfqI-VfqR circuit and regulation complexity of VflT6SS2.

Declaration of competing interest

We declare that we have no conflicts of interest.

Acknowledgments

This study is supported by the National Key R&D Program of China (2021YFC2300302 and 2017YFC1601503), and the National Natural Science Foundation of China (81772242).

Abbreviations

qPCR, quantitative real-time PCR; T6SS, type VI secretion system; WT, wild type; AHL, acyl-homoserine lactone; CFU, colony-forming units.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101282.

References

[1] E.O. Igbinosa, A.I. Okoh, *Vibrio fluvialis*: an unusual enteric pathogen of increasing public health concern, Int. J. Environ. Res. Publ. Health 7 (2010) 3628–3643.
[2] A.L. Furniss, J.V. Lee, T.J. Donovan, Group F, a new Vibrio? Lancet 2 (1977) 565–566.
[3] B.D. Tall, S. Fall, M.R. Pereira, et al., Characterization of *Vibrio fluvialis*-like strains implicated in limp lobster disease, Appl. Environ. Microbiol. 69 (2003) 7435–7446.
[4] S. Kitaura, K. Okamoto, Y. Wakabayashi, et al., *Vibrio fluvialis* liver abscess and bacteremia in a sashimi lover: a case report and review of the literature, Open Forum Infect. Dis. 7 (2020) 212.
[5] G. Chowdhury, T. Ramamurthy, A. Ghosh, et al., Emergence of azithromycin resistance mediated by phosphotransferase-encoding *mph*(A) in diarrheagenic *Vibrio fluvialis*, mSphere 4 (2019).
[6] T. Ramamurthy, G. Chowdhury, G.P. Pazhani, et al., *Vibrio fluvialis*: an emerging human pathogen, Front. Microbiol. 5 (2014) 91.
[7] S. Pukatzki, A.T. Ma, D. Sturtevant, et al., Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 1528–1533.
[8] J.D. Mougous, C.A. Gifford, T.L. Ramsdell, et al., Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*, Nat. Cell Biol. 9 (2007) 797–803.

[9] L. Journet, E. Cascales, S.T. Lovett, et al., The type VI secretion system in *Escherichia coli* and related species, EcoSal Plus 7 (2016).
[10] K. Yin, J. Zhang, J. Ma, et al., MviN mediates the regulation of environmental osmotic pressure on esrB to control the virulence in the marine fish pathogen *Edwardsiella piscicida*, Microbiol. Res. 239 (2020), 126528.
[11] S.L. Murdoch, K. Trunk, G. English, et al., The opportunistic pathogen Serratia marcescens utilizes type VI secretion to target bacterial competitors, J. Bacteriol. 193 (2011) 6057–6069.
[12] K. Yu, P. Xue, Y. Fu, et al., T6SS mediated stress responses for bacterial environmental survival and host adaptation, Int. J. Mol. Sci. 22 (2021) 478.
[13] J. Lin, W. Zhang, J. Cheng, et al., A Pseudomonas T6SS effector recruits PQS-containing outer membrane vesicles for iron acquisition, Nat. Commun. 8 (2017), 14888.
[14] T. Wang, M. Si, Y. Song, et al., Type VI secretion system transports Zn^{2+} to combat multiple stresses and host immunity, PLoS Pathog. 11 (2015), e1005020.
[15] L. Song, J. Pan, Y. Yang, et al., Contact-independent killing mediated by a T6SS effector with intrinsic cell-entry properties, Nat. Commun. 12 (2021).
[16] J. Pan, M. Zhao, Y. Huang, et al., Integration host factor modulates the expression and function of T6SS2 in *Vibrio fluvialis*, Front. Microbiol. 9 (2018) 962.
[17] X. Liu, J. Pan, H. Gao, et al., CqsA/LuxS-HapR Quorum sensing circuit modulates type VI secretion system VflT6SS2 in *Vibrio fluvialis*, Emerg. Microb. Infect. 10 (2021) 589–601.
[18] Y. Huang, P. Du, M. Zhao, et al., Functional characterization and conditional regulation of the type VI secretion system in *Vibrio fluvialis*, Front. Microbiol. 8 (2017) 528.
[19] S. Mukherjee, B.L. Bassler, Bacterial quorum sensing in complex and dynamically changing environments, Nat. Rev. Microbiol. 17 (2019) 371–382.
[20] J. Engebrecht, K. Nealson, M. Silverman, Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*, Cell 32 (1983) 773–781.
[21] T.R. de Kievit, B.H. Iglewski, Bacterial quorum sensing in pathogenic relationships, Infect. Immun. 68 (2000) 4839–4849.
[22] A.M. Barnard, S.D. Bowden, T. Burr, et al., Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria, Philos. Trans. R. Soc. Lond. B Biol. Sci. 362 (2007) 1165–1183.
[23] R. Ruhal, R. Kataria, Biofilm patterns in gram-positive and gram-negative bacteria, Microbiol. Res. 251 (2021), 126829.
[24] M. Phour, A. Sehrawat, S.S. Sindhu, et al., Interkingdom signaling in plant-rhizomicrobiome interactions for sustainable agriculture, Microbiol. Res. 241 (2020), 126589.
[25] J. Gosai, S. Anandhan, A. Bhattacharjee, et al., Elucidation of quorum sensing components and their role in regulation of symbiotically important traits in Ensifer nodulating pigeon pea, Microbiol. Res. 231 (2020), 126354.
[26] A. Shukla, P. Parmar, B. Patel, et al., Breaking bad: better call gingerol for improving antibiotic susceptibility of *Pseudomonas aeruginosa* by inhibiting multiple quorum sensing pathways, Microbiol. Res. 252 (2021), 126863.
[27] Y. Wang, H. Wang, W. Liang, et al., Quorum sensing regulatory cascades control *Vibrio fluvialis* pathogenesis, J. Bacteriol. 195 (2013) 3583–3589.
[28] W.C. Fuqua, S.C. Winans, E.P. Greenberg, Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators, J. Bacteriol. 176 (1994) 269–275.
[29] M.E. Churchill, L. Chen, Structural basis of acyl-homoserine lactone-dependent signaling, Chem. Rev. 111 (2011) 68–85.
[30] N. Qin, S.M. Callahan, P.V. Dunlap, et al., Analysis of LuxR regulon gene expression during quorum sensing in *Vibrio fischeri*, J. Bacteriol. 189 (2007) 4127–4134.
[31] H.M. Gan, H.Y. Gan, N.H. Ahmad, et al., Whole genome sequencing and analysis reveal insights into the genetic structure, diversity and evolutionary relatedness of *luxI* and *luxR* homologs in bacteria belonging to the Sphingomonadaceae family, Front. Cell. Infect. Microbiol. 4 (2015).
[32] L.C. Antunes, R.B. Ferreira, C.P. Lostroh, et al., A mutational analysis defines *Vibrio fischeri* LuxR binding sites, J. Bacteriol. 190 (2008) 4392–4397.
[33] X. Hu, B. Diao, J. Li, et al., Prevalence of quorum sensing (QS) systems and its signal molecules production in *Vibrio fluvialis*, J. Nanjing Agric. Univ. (2019) 682–688.
[34] N.A. Whitehead, A.M. Barnard, H. Slater, et al., Quorum-sensing in gram-negative bacteria, FEMS Microbiol. Rev. 25 (2001) 365–404.
[35] A.V. Patankar, J.E. González, Orphan LuxR regulators of quorum sensing, FEMS Microbiol. Rev. 33 (2009) 739–756.
[36] Y.Y. Lau, K.Y. How, W.F. Yin, et al., Functional characterization of quorum sensing LuxR-type transcriptional regulator, EasR in *Enterobacter asburiae* strain L1, PeerJ 8 (2020), e10068.
[37] M.J. Bottomley, E. Muraglia, R. Bazzo, et al., Molecular insights into quorum sensing in the human pathogen *Pseudomonas aeruginosa* from the structure of the virulence regulator LasR bound to its autoinducer, J. Biol. Chem. 282 (2007) 13592–13600.
[38] L.C.M. Antunes, R.B.R. Ferreira, C.P. Lostroh, et al., A mutational analysis defines *Vibrio fischeri* LuxR binding sites, J. Bacteriol. 190 (2008) 4392–4397.
[39] A. Joshi, B. Kostiuk, A. Rogers, et al., Rules of engagement: the type VI secretion system in *Vibrio cholerae*, Trends Microbiol. 25 (2017) 267–279.
[40] M.B. Miller, K. Skorupski, D.H. Lenz, et al., Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*, Cell 110 (2002) 303–314, 10.
[41] G. Xu, Evolution of LuxR solos in bacterial communication: receptors and signals, Biotechnol. Lett. 42 (2020) 181–186, 10.
[42] C. Bez, S. Covaceuszach, I. Bertani, et al., LuxR solos from environmental fluorescent pseudomonads, mSphere 6 (2021).
[43] S. Li, S. Wu, Y. Ren, et al., Characterization of differentiated autoregulation of LuxI/LuxR-type quorum sensing system in *Pseudoalteromonas*, Biochem. Biophys. Res. Commun. 590 (2022) 177–183.