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Connecting type VI secretion, quorum sensing, and c-di-GMP production in fish pathogen *Vibrio alginolyticus* through phosphatase PppA

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ABSTRACT

Vibrio alginolyticus, a Gram-negative marine bacterium, has brought about severe economic damage to the mariculture industry by causing vibriosis in various fish species. We are intrigued in the regulation of the pathogenesis in this bacterium. Here, we reported a complex regulatory connection among the newly defined type VI secretion system (T6SS), quorum sensing (QS), and 3′,5′-cyclic diguanylic acid (c-di-GMP) signal through the phosphatase PppA encoded in the T6SS gene cluster of *V. alginolyticus*. Whole-genome transcriptome analysis revealed various regulatory targets of PppA including the T6SS substrate hemolysin coregulated protein (Hcp), quorum sensing regulator LuxR, exotoxin alkaline serine protease (Asp), flagellar proteins, as well as proteins involved in polysaccharide biosynthesis and transport. Western blot analysis showed PppA served as a negative regulator of the expression and secretion of Hcp1. Mutation of *pppA* resulted in an increased level of the intracellular second messenger c-di-GMP and a decreased expression of the QS regulator LuxR as well as exotoxin Asp. Complementation of intact *pppA* gene in Δ*pppA* mutant restored the production of c-di-GMP, LuxR, and Asp to the wild-type level. Phenotypic studies suggested that PppA takes part in the modulation of biofilm formation, motility, and cell aggregation. These results demonstrated new roles of PppA in controlling virulence factors and pleiotropic phenotypes and contributed to our understanding of the regulation of pathogenesis in *V. alginolyticus*.

1. Introduction

Vibrio alginolyticus is one of the important epizootic pathogens causing high mortality outbreaks of vibriosis in sea animals and it is also an opportunistic pathogen commonly associated with ear infections (Austin, 2010; Chien et al., 2002). The abilities to adhere to the host surface, form biofilm, and produce extracellular products such as the exotoxin alkaline serine protease (Asp) and siderophore have been suggested to be critically involved in virulence in this bacterium (Cai et al., 2007; Rui et al., 2009; Wang et al., 2007a).

Pathogens mainly rely on the activity of proteins secreted by a variety of protein secretion systems for pathogenesis, niche adaptation, and utilization of nutrients. The newly defined type VI secretion system (T6SS) can secrete effector proteins lacking N-terminal hydrophobic signal sequences (Pukatzki et al., 2006). Many pathogenesis or non-pathogenesis related phenotypes are observed to be controlled by T6SS (Cascales, 2008; Jani and Cotter, 2010). T6SS are also subjected to the precise regulation by other regulatory pathways such as quorum sensing (QS) (Ishikawa et al., 2009; Sheng et al., 2012), two-component regulatory system (Schell et al., 2007), alternative sigma factors (Bernard et al., 2011), histone-like proteins (Renzi et al., 2010), and post-translational modification (Mougous et al., 2007).

In this study, we reported that the phosphatase PppA encoded in T6SS gene cluster is involved in a complex regulation network including T6SS substrate Hcp1, QS regulator LuxR, second messenger 3′,5′-cyclic diguanylic acid (c-di-GMP), and virulence-associated phenotypes in

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V. alginolyticus. Whole-genome transcriptome analysis of *pppA* deletion strain provided in depth insights into the effects of PppA on many important processes. This study advances an understanding of the complex roles of PppA in fish pathogen *V. alginolyticus* and may facilitate the study of pathogenesis of this bacterium.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Table 1. *V. alginolyticus* strains were grown at 30 °C in Luria-Bertani (LB) medium supplemented with 3% NaCl (LBS). *Escherichia coli* strains were grown at 37 °C in LB medium. Ampicillin (100 μg ml^{-1}) was used for the growth of *V. alginolyticus* wild type and mutants, and chloramphenicol (25 μg ml^{-1}) was used to maintain plasmids in *V. alginolyticus* and *E. coli*.

2.2. Cloning and sequencing of T6SS gene clusters in V. alginolyticus EPGS

The primers used for PCR amplification of putative T6SS loci of *V. alginolyticus* EPGS were based on the published sequence of T6SS gene clusters of *V. alginolyticus* 12G01 (GenBank accession Nos. AAPS01000007 and AAPS01000006) and were designed with Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). Forty primer pairs (data not shown) were used to amplify fragments that overlapped adjacent fragments by approximately 100 bp. The genomic DNA of *V. alginolyticus* EPGS was used as template for PCR amplification. Amplified PCR products were purified and subsequently sequenced. The resulting sequences were edited and assembled using GeneTool 2.0 (BioTools Incorporated, Edmonton, AB, Canada). The assembled sequences have been submitted to GenBank with the accession Nos. FJ0776 and FJ0777.

2.3. Construction of deletion mutant and complemented strain

In-frame deletion mutants were generated by the R6K origin-based allelic exchange as previously described (Milton et al., 1996). Overlap PCR was used to generate the in-frame deletion fragment from 58 to 723 bp of *pppA* gene. This fragment was cloned into the BglII/SphI sites of the R6K origin-based suicide vector pDM4, and the resulting plasmid pDM4-*pppA* was transformed into *E. coli* CC118 λ*pir*. After sequencing, pDM4-*pppA* was then transformed into *E. coli* SM10 λ*pir*. To introduce this plasmid into *V. alginolyticus*, conjugal mating was performed. Firstly, exconjugant with the plasmid integrated into the chromosome by homologs recombination was selected on LBS agar media containing Amp and Cm. The double cross-over recombination was counter selected on LBS agar containing 10% sucrose. The mutation was confirmed by PCR and sequencing.

To construct a complemented strain, a fragment containing the RBS and ORF regions of *pppA* gene was cloned into EcoRI/PstI sites of broad-host-range plasmid pMMB206, and the resulting plasmid pMMB206-*pppA* was mated from *E. coli* SM10 λ*pir* into the mutant by conjugation. Amp and Cm resistant transconjugants were selected, and the bearing of the plasmid was confirmed by PCR and sequencing. When necessary, 0.5 mM IPTG was added to induce the expression of *pppA* carried by plasmid pMMB206-*pppA*. All primers used in this study are given in Table S1.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
E. coli		
CC118 λ*pir*	λ*pir* lysogen of CC118, Δ*(ara-leu) araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	Dennis and Zylstra (1998)
SM10 λ*pir*	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km, λ*pir*R6K lysogen	Simon et al. (1983)
TOP10F'	F'{*lacI*^{q} Tn*10* (Tet^R)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ*(ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
V. alginolyticus		
EPGS	Wild type, isolated from the aquiculture farm of the South China Sea with CCTCC No. AB 209306. Amp^r	Labortaory collection
asp$^-$	EPGS, disrupted mutant in *asp*; Amp^r, Cm^r	Rui et al. (2009)
Δ*luxR*	EPGS, in-frame deletion in *luxR*, Amp^r	Rui et al. (2009)
Δ*luxO*	EPGS, in-frame deletion in *luxO*, Amp^r	Wang et al. (2007b)
Δ*pppA*	EPGS, in-frame deletion in *pppA* (VEPGS_0613), Amp^r	This study
Δ*hcp1*	EPGS, in-frame deletion of VEPGS_0021, Amp^r	Sheng et al. (2012)
Δ*pppA* Δ*luxO*	EPGS, in-frame deletion in *pppA* and *luxO*, Amp^r	This study
Δ*hcp1*/pMMB206-*hcp1*	EPGS, Δ*hcp1* complemented with plasmid pMMB206-*hcp1*	Sheng et al. (2012)
Δ*pppA*/pMMB206-*pppA*	EPGS, Δ*pppA* complemented with plasmid pMMB206-*pppA*	This study
Δ*pppA*/pMMB206	EPGS, Δ*pppA* complemented with plasmid pMMB206	This study
Δ*fliS* Δ*lafA*	EPGS, in-frame deletion in *fliS* and *lafA*, Amp^r	Sheng et al. (2012)
Plasmids		
pDM4	Suicide vector, *pir* dependent, R6K, *SacBR*, Cm^r	Milton et al. (1996)
pMMB206	IncQ *lacI*^{q} Δ*bla* $P_{tac-lac}$ *lacZα*, Cm^r	Morales et al. (1991)
pDM4-*pppA*	Cm^r, pDM4 derivative containing *ppkA1* bp 1-57 fused in-frame to bp 724-795	This study
pMMB206-*hcp1*	pMMB206 derivative *hcp1* expression plasmid	This study
pMMB206-*pppA*	pMMB206 derivative *pppA* expression plasmid	This study