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Phosphorylation of PppA at threonine 253 controls T6SS2 expression and bacterial killing capacity in the marine pathogen *Vibrio alginolyticus*

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ABSTRACT

Type VI secretion systems (T6SSs) are multi-protein secretory nano-machines that mediate inter-bacterial competition. *Vibrio alginolyticus* is an abundant gram-negative marine bacterium that efficiently kills other bacteria with its T6SS2. The *V. alginolyticus* T6SS2 gene cluster encodes a phosphatase, PppA, and a type II membrane-spanning Hanks-type threonine kinase, PpkA2, which have been implicated in the activation of T6S. Meanwhile, T6SS2 gene expression is under the control of quorum sensing. However, the role of PppA in T6SS2 activity is unclear. Here, our phosphoproteomic screen identified PppA as a novel PpkA2 substrate. Phosphorylation at threonine 253 (T253) of PppA is not conserved in other bacteria, suggesting that PppA may play a unique role in T6SS2 activation in *V. alginolyticus*. Interestingly, PppA phosphatase activity was modulated by the cognate kinase PpkA2, which implied that a homeostasis is required for optimal T6S activity. PppA and phosphorylation of PppA at T253 are important for T6S activity and T6SS2-mediated bacterial killing. Moreover, PppA and the phosphorylation of PppA are also essential for the expression of LuxR, the master regulator of quorum sensing, thus augmenting T6SS2 expression. Collectively, our data demonstrated that phosphorylation of PppA at T253 controls the activity of T6SS2, thereby enhancing the competitive fitness of *V. alginolyticus*.

1. Introduction

Bacteria often depend on special secretion machineries, including type VI secretion systems (T6SSs), to adapt to their environmental niches. T6SSs are widespread in gram-negative bacteria (Mougous et al., 2006; Pukatzki et al., 2006). Some bacteria harbour more than one T6SS, and these T6SSs can often have different functions (Cascales, 2008). Ordinarily, T6SSs consist of 13 core subunits and several additional components (Boyer et al., 2009), which form three main modules: a baseplate, a contractile sheath-tube, and an anchor attached to the membrane (Böck et al., 2017). The Hcp (TssD) assembly constitutes the secretory tube, which is surrounded by the VipA/VipB sheath (Basler and Mekalanos, 2012; Mougous et al., 2006). ClpV (TssH) interacts with and can disassemble the sheath after contraction, and the VipA/VipB subunits are then recycled (Kapitein et al., 2013). VgrG (TssI) and some PAAR proteins form the spike at the tip of the Hcp tube, which can be decorated with various effector proteins (Shneider et al., 2013), targeting neighbouring prokaryotic as well as eukaryotic cells (Böck et al., 2017; Silverman et al., 2012). During bacterial killing, bacteria deliver several kinds of effectors and use specific immunity proteins to protect themselves (Benz and Meinhart, 2014).

As complex interbacterial 'nanoweapons', T6SSs are tightly controlled by various systems acting at multiple levels. Various cues, such as surface contact, iron limitation, c-di-GMP, and chitin (Joshi et al., 2017; Lazzaro et al., 2017; Silverman et al., 2012) are sensed by many kinds of regulators to coordinate the activation of T6S at the transcriptional, post-transcriptional, and post-translational levels. A subset of T6SS gene clusters harbour orthologues of PpkA, a Ser/Thr protein kinase, PppA, a phosphatase, and Fha, a forkhead-associated-domain-containing protein, which constitute the Ser/Thr phosphorylation cascades that control T6S activity (Cascales, 2008; Fritsch et al., 2013; Silverman et al., 2011). Moreover, other non-T6SS genes and phenotypes, i.e., oxidative-stress resistance, quorum sensing and virulence, have been observed to be associated with the PppA-PpkA pair, suggesting much more complex roles for this pair than T6SS activation (Goldová et al., 2011).

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PppA is a PP2C-family protein phosphatase in the T6SS gene cluster. PP2C phosphatases belong to the PPM family, members of which are dependent on Mn^{2+}/Mg^{2+}. PP2C represents a large family of conserved protein phosphatases (Lammers and Lavi, 2007; Xue et al., 2008). The primary function of PP2C-family proteins is the regulation of stress signalling; however, these proteins also play a role in growth, survival, cell differentiation, metabolism, and apoptosis (Lu and Wang, 2008). Genomic analysis of the bacterial kingdom, particularly cyanobacteria, revealed a large number of PPM homologs (Shi, 2004; Zhang and Shi, 2004; Zhang et al., 2005). However, the function of PppA during T6SS activation is not clear.

Vibrio alginolyticus, a gram-negative, mesophilic and moderately halophilic bacterium, is abundant in marine environments (Ruwandeepika et al., 2012). This bacterium is also an important pathogen, causing vibriosis in various marine animals, including coral, and resulting in significant damage to marine ecological systems and to the aquaculture industry worldwide (Bhotra and Singh, 2016; Xie et al., 2013). The pathogenesis of this bacterium relies mainly on quorum sensing (QS), the cell-to-cell communication system mediating collective gene expression by a population (Wang et al., 2007). *V. alginolyticus* encodes two T6SS gene clusters (T6SS1 and T6SS2), each of which encode homologues of the 13 characteristic core T6SS proteins (Hcp, VgrG, IcmF, ClpV, DotU, VipA, VipB, COG3515, and COG3518-3522) (Sheng et al., 2013). The organism principally relies on PpkA2 activated T6SS2 for interbacterial competition and T6SS2 can be positively regulated by QS key regulator LuxR (Yang et al., 2018). Here, we found that PppA could be the phosphorylation substrate of PpkA2 at threonine 253 and PppA phosphatase activity was also modulated by the cognate kinase PpkA2. Moreover, PppA and its phosphorylation site mediated T6SS2 expression and activity. Moreover, our data indicated that QS was modulated by PppA and the related phosphorylation cascade. Our data facilitate the understanding of T6SS regulation and the coordination of T6SS regulation with QS, thereby enhancing the competitive fitness in marine environments and the pathogenesis of *V. alginolyticus*.

2. Materials and methods

2.1. Bacterial stains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 1. The *V. alginolyticus* strains were grown in Luria-Bertani (LB) broth containing 3% sodium chloride (LBS) broth at 30 °C. *E. coli* MC4100, *E. coli* DH5α (*λpir*), *E. coli* SM10 (*λpir*), and *E. coli* BL21 (DE3) were grown in LB broth at 37 °C. The following antibiotics were added at the concentrations indicated: carbenicillin (100 μg ml^{-1}), tetracycline (12.5 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or L-arabinose (0.2 mg ml^{-1}).

2.2. Deletion mutant and complement strain construction

In-frame deletion mutants were generated as described in a prior study (Sheng et al., 2013). The fragment was cloned into the XbaI sites of the R6K-origin-based suicide vector pDM4 using the Gibson-assembly protocol (Gibson et al., 2009), and the resulting plasmid, pDM4, was transformed into *E. coli* DH5α *λpir*. After sequencing, pDM4 was then transformed into *E. coli* SM10 *λpir*. This plasmid was introduced into *V. alginolyticus* by conjugation. The double-crossover recombinant was selected for on LBS agar containing 15% sucrose. The mutation was confirmed by PCR and sequencing. A fragment containing the ORF region was cloned into the plasmid pBAD33 to construct a complementation strain (Gu et al., 2016).

The site-directed mutagenesis in the genome, i.e. *pppA*^{T253A}, *pppA*^{T253D}, and *pppA*^{T253E}, were generated by creation of the point mutation by specific primers followed by subsequent procedures of double cross-over recombination and PCR selection of the point mutation in the mutants as above-mentioned (Sheng et al., 2013).

2.3. Purification of PppA and PpkA2

The PppA or PpkA2 open reading frame (ORF) sequence was amplified by PCR and inserted into pET28a with a His6 tag at the carboxyl terminal. Then, the recombinant plasmid was transformed into *E. coli* BL21 (DE3). When the cells grew to OD_{600} = 0.6 at 37 °C, expression was induced with 1 mM IPTG, and the cells were grown for 12–16 h at 16 °C to allow PppA or PpkA2 expression. Finally, the protein was purified using nickel affinity chromatography (Best chrom, Shanghai, China).

The point mutation variants such as *ppkA2*^{D224AN229A} were generated by creation of point mutation with specific primers and then cloned and expressed in pET28a after transformed into *E. coli* BL21 (DE3) strain.

2.4. Mass spectrometric analysis of the phosphoproteome

Phosphoproteomic assays were carried out by the Beijing Genomics Institute (BGI Shenzhen, Guangdong, China) (Shen et al., 2016). Bacterial cells were harvested from LBS plates and washed with LBS. The cell suspensions were then washed with lysis buffer containing 1 mM Phenylmethanesulfonyl fluoride (PMSF), 2 mM Ethylenediaminetetraacetic acid (EDTA) and 10 mM DL-Dithiothreitol (DTT). The bacterial cells were sonicated for 15 min on ice, and the lysates were cleared by centrifugation at 25,000*g* at 4 °C for 20 min. The lysates were treated with 10 mM DTT at 56 °C for 60 min. The lysates were alkylated by addition of 55 mM Iodoacetamide (IAM) for 45 min and then precipitated by cold acetone. The precipitates were dissolved in Tetraethylammonium bromide (TEAB) for 15 min. The digest was desalted using a Strata × C_{18} column and then vacuum dried. One milligram of dry peptide was dissolved in a 2% Trifluoroacetic acid (TFA) and 65% Acetonitrile (ACN) solution, and saturated with glutamic acid (20 mg ml^{-1}, pH 2.0–2.5). Phosphopeptides were enriched using TiO_2 chromatography (GL Science, Saitama, Japan). The phosphopeptides were analysed on an LC-20AD (Shimadzu, Kyoto, Japan) followed by a Triple TOF 5600 mass spectrometer (AB SCIEX, Concord, ON) equipped with an ion source (AB SCIEX, Concord, ON) and radiator (New Objectives, Woburn, MA). High-confidence hits were those that had a localization probability > 0.75 and the peptide ion scores of which were greater than 30 (the threshold score suggested by MASCOT for high-confidence matching of a single peptide sequence at $P < 0.001$).

2.5. Bacterial killing assay

The bacterial killing assay was performed as previously described for *V. parahaemolyticus* (Salomon et al., 2013). The bacteria used in the killing assay were grown overnight in LBS (*V. alginolyticus*) or LB (*E. coli*). The bacterial cultures were diluted to an optical density of 0.5 and then mixed at a ratio of 4:1 (predator:prey). Twenty-five microlitres of the mixtures were spotted on LBS plates and then incubated at 30 °C for 4 h. The CFUs of the mixtures at t = 0 were determined by plating 10-fold serial dilutions on different antibiotic plates. After 4 h, bacterial spots were harvested from LBS plates, and the CFUs of the surviving predator and prey were determined. An empty pBAD33 or pRK415 plasmid was used to provide *E. coli* and *V. alginolyticus* with resistance to chloramphenicol and tetracycline, respectively.

2.6. Quantitative real-time reverse transcription PCR (qRT-PCR)

alginolyticus strains were harvested as described for the killing assay, and the total RNA was treated with DNaseI (Promega, Madison, WI, USA). Total RNA (1 μg) was used to generate cDNA using reverse transcriptase (Toyobo, Tsuruga, Japan) and 6-mer random primers. The qRT-PCR assay was performed for at least three independent experiments, each in triplicate, by an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The transcript