A pGpG-specific phosphodiesterase regulates cyclic di-GMP signaling in Vibrio cholerae

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The bacterial second messenger bis-(3′-5′)-cyclic diguanylate monophosphate (c-di-GMP) controls various cellular processes, including motility, toxin production, and biofilm formation. c-di-GMP is enzymatically synthesized by GGDEF domain–containing diguanylate cyclases and degraded by HD-GYP domain–containing phosphodiesterases (PDEs) to 2 GMP or by EAL domain–containing PDE-As to 5′-phosphoguananyl-(3′,5′)-guanosine (pGpG). Since excess pGpG feedback inhibits PDE-A activity and thereby can lead to the uncontrolled accumulation of c-di-GMP, a PDE that degrades pGpG to 2 GMP (PDE-B) has been presumed to exist. To date, the only enzyme known to hydrolyze pGpG is oligoribonuclease Orn, which degrades all kinds of oligoribonucleotides. Here, we identified a pGpG-specific PDE, which we named PggH, using biochemical approaches in the gram-negative bacteria Vibrio cholerae. Biochemical experiments revealed that PggH exhibited specific PDE activity only toward pGpG, thus differing from the previously reported Orn. Furthermore, the high-resolution structure of PggH revealed the basis for its PDE activity and narrow substrate specificity. Finally, we propose that PggH could modulate the activities of PDE-As and the intracellular concentration of c-di-GMP, resulting in phenotypic changes including in biofilm formation.

Bis-(3′-5′)-cyclic diguanylate monophosphate (c-di-GMP) is a widely conserved bacterial second messenger found in all major bacterial phyla (1). It was first discovered in 1987 as an activator of the cellulose synthase from Komagataeibacter xylinus (formerly known as Gluconacetobacter xylinus) by Ross et al. (2). c-di-GMP is associated with diverse cellular processes, such as bacterial growth, motility, virulence, biofilm formation, and cell cycle progression (3). Low levels of cellular c-di-GMP upregulate motility by inducing flagellar expression, assembly, or motor function (4) and are also required for the expression of acute virulence genes (5). In contrast, high c-di-GMP levels stimulate the biosynthesis of fimbriae, adhesins, various matrix exopolysaccharides, and biofilm formation (4, 6, 7). Moreover, c-di-GMP–dependent spatiotemporal control of protein degradation is crucial in cell cycle progression in Caulobacter crescentus (8).

c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases harboring the GGDEF domain, named after its conserved motif Gly-Gly-Asp-Glu-Phe (3). The degradation of c-di-GMP is mediated by two types of phosphodiesterases (PDEs) containing either the HD-GYP or EAL domain (9). HD-GYP domain proteins hydrolyze c-di-GMP to two molecules of GMP in a one-step reaction (10). In contrast, EAL domain-containing PDEs (PDE-As) degrade c-di-GMP into the linear product 5′-phosphoguananyl-(3′,5′)-guanosine (pGpG) (11, 12), requiring a second PDE enzyme (PDE-B), which hydrolyzes pGpG into two GMP molecules, to complete the signaling process (13). In the two-step degradation mechanism via pGpG, the intermediate pGpG has been reported to competitively inhibit c-di-GMP-specific PDE-As catalyzing the first reaction by a feedback inhibition mechanism. The PDE activity of Escherichia coli YfgF, an EAL-containing PDE-A, has been found to be inhibited by high concentrations of pGpG (14). Furthermore, pGpG acts as a competitive inhibitor of the EAL-containing response regulator RocR and inhibits c-di-GMP turnover in Pseudomonas aeruginosa (15). These findings suggest that the appropriate degradation of pGpG by PDE-B is crucial for sustaining the c-di-GMP signaling pathway.

Recently, a high-throughput DRaCALA-based screen on a Vibrio cholerae El Tor N16961 complete genome ORF library has revealed several pGpG-binding proteins including oligoribonuclease VC0341 (hereafter VcOrn), as well as EAL and HD-GYP domain proteins, and some proteins of unknown functions. Of these, VcOrn, which possesses an RNase–T domain (Fig. 1A), was then determined to exhibit pGpG PDE activity, similar to the previous findings in P. aeruginosa (16–18). However, unlike its ortholog in P. aeruginosa, VcOrn has been shown to be an essential exonuclease (19), similar to that in E. coli, which is responsible for the degradation of nanoRNAs, from di-ribonucleotides to penta-ribonucleotides (20). Thus, the pGpG-hydrolyzing function of this protein appeared to be a part of RNA turnover (18). In most bacteria, the intracellular concentration of c-di-GMP is acutely...
modulated by the specific activities of its metabolic enzymes responding to the environmental signals (4). Therefore, we speculated whether VcOrn could degrade pGpG rapidly enough to meet diverse metabolic needs and thus fulfill the role of a PDE-B which is responsible for the c-di-GMP signal decay in V. cholerae, even in the presence of nanoRNAs. These considerations prompted us to search for a PDE-B that is primarily responsible for the degradation of pGpG in the c-di-GMP signaling network.

Here, we report that V. cholerae VCA0593 is a pGpG-specific phosphodiesterase (Fig. 1B; Table 1). Similar to its ortholog in E. coli, VcOrn could hydrolyze nanoRNAs including pentaribonucleotides 5’-GGAAA-3’ and 5’-AAAAA-3’, albeit at a considerably slower rate than the diribonucleotides, pApG, pApA, and pGpG, which is consistent with a previous study (18). We then tested whether the pGpG-hydrolyzing activity of VcOrn would be affected by other nanoRNAs. We found that the pGpG-hydrolyzing activity of VcOrn was inhibited in the presence of the indicated ribonucleotides (20 μM pGpG; 100 μM pApG and pApA). These results were calculated based on the Michaelis–Menten equation (dotted line). Statistical significance was assessed using Student’s t test (***p value < 0.005). Shown are means and SDs (n = 3, independent measurements).

### Table 1

| Substrate | Specific activity (μmol/mg/min) |
|-----------|-------------------------------|
| pAp       | PggH: 0; Orn: 0                |
| c-di-GMP   | PggH: 0; Orn: 0                |
| c-di-AMP   | PggH: 0; Orn: 0                |
| AAAA      | PggH: 0.25 ± 0.03; Orn: 0.26 ± 0.05 |
| GAAAA     | PggH: 0.26 ± 0.05; Orn: n.d.   |
| ssDNA     | PggH: n.d.; Orn: n.d.          |
| pApA      | PggH: 0.34 ± 0.01; Orn: n.d.   |
| pApG      | PggH: 0.32 ± 0.02; Orn: n.d.   |
| pGpG      | PggH: 0.20 ± 0.01; Orn: 0.35 ± 0.01 |

PggH, phosphoguanylyl guanylate hydrolase; n.d., not determined; Orn, oligoribonuclease.

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**Results**

### Oligoribonuclease activity of V. cholerae Orn

First, we determined the exonuclease activity of purified VcOrn using several nanoRNAs, including pGpG as a substrate (Fig. 1B; Table 1). Similar to its ortholog in E. coli, VcOrn could hydrolyze nanoRNAs including pentaribonucleotides 5’-GGAAA-3’ and 5’-AAAAA-3’, albeit at a considerably slower rate than the diribonucleotides, pApG, pApA, and pGpG, which is consistent with a previous study (18). We then tested whether the pGpG-hydrolyzing activity of VcOrn would be affected by other nanoRNAs. We found that the pGpG-hydrolyzing activity of VcOrn was inhibited in the...
presence of excess amounts of pApA, pApG, and 5′-GGAAA-3′ (Figs. 1C and S1). This inhibition by other nanoRNAs was also observed when we measured the pGpG-degrading activity of WT *V. cholerae* N16961 cell crude extract in the absence and presence of excess amounts of pApG (Fig. 1D). Interestingly, we found that the inhibitory effect of pApG on the pGpG-hydrolyzing activity of the crude extract was significantly lower than that simulated based on the result in Figure 1C. These results led us to speculate the existence of another pGpG-binding protein(s) responsible for the specific degradation of pGpG in *V. cholerae*.

**pGpG-specific PDE activity of PggH**

VCA0593 has also been reported to bind pGpG in *V. cholerae* El Tor N16961 (15). An analysis of the primary structure of VCA0593 revealed that this protein possesses DHH (residues 11–101) and DHHA1 (residues 264–303) domains (Fig. 1A). Therefore, it can be classified as a DHH/DHHA1 family enzyme. The DHH/DHHA1 family enzymes are involved in the hydrolysis of oligonucleotides in various bacterial species (21), such as the *Thermotoga maritima* PDE TM1595, which hydrolyzes various linear dinucleotides (22); PDE YybT, which hydrolyzes cyclic dinucleotides (c-di-GMP and c-di-AMP) (23); the 3′-phosphoadenosine 5′-phosphate (pAp) phosphatase YtqI (24) in *Bacillus subtilis*; and the ssDNA exonuclease RecJ, which is highly conserved in most bacteria and archaea (25). Thus, we assumed that VCA0593 might exhibit nucleotide-hydrolyzing activity. First, we assessed the PDE activity of VCA0593 toward several known substrates of DHH/DHHA1 family proteins; c-di-GMP, c-di-AMP, pApA, and ssDNA (Table 1). However, these substrates were not hydrolyzed by VCA0593. We then examined the oligoribonuclease activity of VCA0593 toward the pentaribonucleotides 5′-GGAAA-3′ and 5′-AAAAA-3′ and the diribonucleotides pApA, pApG, and pApG. Although VCA0593 exhibited PDE activity toward pApG, neither the pentaribonucleotides nor the diribonucleotides nor the diribonucleotides pApA and pApG were degraded by VCA0593 (Fig. 1B; Table 1), suggesting that, unlike VcOrn and TM1595 that hydrolyze other linear dinucleotides as well as pGpG, VCA0593 is a PDE that specifically hydrolyzes pGpG. Therefore, we named it PggH (phosphoguananyl guanylate hydrolase). PggH is highly conserved among species of the genus *Vibrio* (Fig. S2). A protein BLAST search against the nonredundant protein sequences database at NCBI showed that the amino acid sequence identity of PggH was >77% among species of the genus *Vibrio* (26).

Most DHH/DHHA1 domain proteins function as homodimers, except the monomeric DNase RecJ (27). Gel filtration analysis revealed that PggH forms a homodimer (Fig. 2, A and B). PggH showed the highest PDE-B activity in the presence of Mn2+ among the divalent cations tested in this study (Fig. 2C). Thus, further biochemical studies were performed in the presence of Mn2+. The Michaelis–Menten constant (K_m) and turnover number (k_cat) of PggH for pGpG were determined by analyzing the initial rate of pGpG hydrolysis at different substrate concentrations. A double-reciprocal plot of the pGpG-hydrolyzing activity of PggH showed K_m and k_cat values of 12.11 μM and 0.14 s⁻¹, respectively (Fig. 2, D and E). Since it has been previously shown that increasing the concentrations of pGpG over a range of 2 to 20 μM causes a progressive inhibition of c-di-GMP degradation by PDEs (13), the kinetic properties of PggH suggest that this enzyme might play a significant role in the c-di-GMP signal decay in vivo. Unlike that in VcOrn, the pGpG-degrading activity of PggH was not substantially affected by nanoRNAs (Figs. 2F and S1), but interestingly, it showed a slight decrease in the presence of an excess amount of pApG.

**Structural basis for pGpG PDE activity of PggH**

To investigate the structural basis for the pGpG-specific PDE activity of PggH, we determined the crystal structure of the protein in the presence of Mn2+ at 1.9 Å resolution (Fig. 3). The asymmetric unit contained two protein molecules that closely interact with each other, consistently with the dimeric size in a gel filtration chromatography column (Fig. 2B). The two DHHA1 domains in the dimer formed a tight interaction, whereas no direct interaction between the DHH domains was found in the dimer (Fig. 3A). Each subunit contains a putative active site between the DHH and DHHA1 domains. The subunit structure further showed that Mn2+ is coordinated by Asp15, Asn55, His68, His69, and Asp121 at the active site, where His68 and His69 are from the conserved DHH motif (Fig. 3B). In addition, Asp13 and Asp67 are indirectly involved in the binding of Mn2+, in a hydrogen bond network with Asn55 and His69, respectively. All these residues are strictly conserved among PggH homologs in *Vibrio* species (Fig. S2). Mutations at His68 and Asp121 completely abolished enzymatic activity, indicating that the coordination with divalent cation is essential for its activity as other DHH/DHHA1 family PDEs (Fig. S3) (21). Mutations at the two aspartate residues, Asp13 and Asp67, also abolished the enzymatic activity, implicating their importance in stabilizing the positions of the Mn2+-coordinating residues (Fig. S3).

The DHHA1 domain of PggH contains a highly conserved GGGH motif, which is known to be responsible for the binding of nucleic acid in several enzymes of the DHH/DHHA1 family (22, 28). To examine the role of the GGGH motif in PggH, we mutated His282 to alanine. The mutation abolished pGpG hydrolysis activity of PggH, confirming the role of the GGGH motif in binding the nucleotide (Fig. S3).

By conducting structural comparisons with other DHH/DHHA1 domain–containing proteins, we found several unique features of PggH. While all other structure-annotated DHH/DHHA1 proteins including TM1595 form side-by-side butterfly-like dimers without any interference with an induced-fit model by the other subunit (22, 27–29), PggH forms a tightly intertwined dimer, apparently providing increased structural rigidity between the two subunits. The DHH and DHHA1 domains in one subunit are intervened by...
the DHHA1 domain of the other subunit in the dimer (Fig. 3C). This domain arrangement reduces the space between the two subunits, which also appears to limit the mobility of the domains in response to the substrate binding. Structural rigidity was further found within the subunit. The DHH and DHHA1 domains in PggH are connected via a long rigid α-helix, which is also different from the canonical DHH/DHHA1 family phosphoesterases in which a flexible loop connects the two domains (Fig. 3D) (22, 28).

The molecular structures of TM1595 were determined both in the ligand-free form and in complex with the substrate pApA (22). A closing movement between DHH and DHHA1, which was induced by pApA binding was observed, demonstrating a typical example of the induced-fit model (30). However, compared to the flexible domain arrangement of other DHH/DHHA1 family proteins, the tightly intertwined domain arrangement of PggH would allow a very limited conformational change upon the binding of a substrate. (Fig. S4) Thus, our findings suggest that PggH could possess distinct mechanisms of substrate binding and its catalysis, which could explain the molecular basis underlying the narrow substrate specificity.

Mechanism of substrate selectivity of PggH

We then measured the binding affinity of PggH for linear diribonucleotides (pGpG, pApG, and pApA) using isothermal titration calorimetry (ITC) (Fig. S5) to further explore the mechanism of substrate specificity in more detail. Thermodynamic parameters indicated the enthalpy-driven interaction of PggH with pGpG (KD = 14.13 ± 7.35 nM) and pApG (KD = 104.67 ± 20.13 nM), whereas pApA did not bind to PggH (Figs. 4A and S5). These data suggested that the 3′-guanine in the substrate is crucial for the binding to PggH, whereas the 5′-guanine is essential for the formation of the active enzyme–substrate complex for catalysis. However, tetraribonucleotides with guanine at either 5′- or 3′-end did not bind PggH (Fig. S5), supporting that PggH has a narrow substrate specificity. Unfortunately, we were not able to test binding of trinucleotides to PggH, since we could not find any supplier.
To identify the crucial residues required for substrate binding, we generated five PggH variants in which the hydrophilic residues in the putative active site were mutated and measured binding affinities of these mutants for pGpG and pApG, respectively (Fig. 4A). Interestingly, mutation of His282 to alanine led to a significantly decreased binding affinity (by >10-fold) for pGpG, but it showed a similar affinity for pApG, compared to that shown by the WT protein. These data explain the reason why the PggH(H282A) mutant completely lost pGpG hydrolase activity (Fig. S3), suggesting that His282 is critical for the recognition of 5′-guanine for the catalysis. Although Asp121 coordinates Mn^{2+} and thus is essential for the catalytic activity, mutation in this residue did not significantly affect the binding affinity for pGpG and pApG, implying that Asp121 is not involved in the substrate binding.

Based on these mutation analyses, we simulated the binding modes of the PggH crystal structure to pGpG and pApG using GalaxyDock3 software, a protein-ligand docking program (Fig. 4, B and C) (31). In these models, 3′-guanosine of both pGpG and pApG are bound in a narrow cleft surrounded by Arg228, Arg146, and Asn227. The guanidinium group of Arg228 forms hydrogen bonds with N3 and the amide group of Asn146 with N7 of 3′-guanine. Meanwhile, according to the docking models, the side chain carbonyl oxygen of Asn227 forms hydrogen bonds with the N1 imino proton and the C2 amine group of 3′-guanine. Because N1 is not protonated and there is no amino group at C2 in adenine, dinucleotides with

Figure 3. Crystal structure of PggH. A, overall structure of a PggH homodimer. Ribbon representations of the dimer structure of PggH. The two molecules are presented in different colors as follows: subunit A (DHH domain, light cyan; DHHA1 domain, cyan; α11 helix, gray) and subunit B (DHH domain, pink; DHHA1 domain, magenta; α11 helix, gray). B, the active site and the metal binding site. The active site is presented in a blue rectangle (left). Detailed views of the active site and metal binding site are presented right. Metal coordinating residues are colored green, two sulfates are colored yellow, and a Mn^{2+} ion is presented as a purple sphere in (A) and (B). C, comparison of the dimeric structures between TM1595 and PggH. Surface representations of the homodimeric structures of TM1595 and PggH. The DHH domains are colored light cyan and pink. The DHHA1 domains are colored cyan and magenta. The active sites between the DHH and DHHA1 domains are presented as dotted black circles. D, ribbon representations of the monomeric structures of TM1595 and PggH. The DHH domains are colored light cyan and pink. The linker helix of PggH is colored gray. Metal ions are represented as purple spheres. The linker loop of TM1595 and the linker helix of PggH are highlighted as dotted black rectangles. PggH, phosphoguanylyl guanylate hydrolase.
**Figure 4. PggH distinguish its substrate at two stages.** A, binding affinities of PggH and its variants for the pGpG and pApG were measured using isothermal titration calorimetry (ITC). Titration experiments consisted of a single initial injection of 1 μl followed by 13 injections of 2 μl nucleotides (100 μM pGpG and pApG) in 25 mM Hepes–NaOH (pH 7.6), 100 mM NaCl, and 1 mM EDTA into the reaction cell containing 10 μM His-PggH or the same amount of its variant (H282A, R11A, R146A, R228A, D121A, and N227W) in the same buffer. The dissociation constants were obtained using MicroCal PEAK-ITC Analysis Software with a single-site binding model. Data shown are means and SDs (n = 3, independent measurements). Statistical significance was assessed using Student’s t test versus WT (p values > 0.05 were presented, ***p values < 0.005 for pGpG group; **p values < 0.005 for pApG group). B and C, the binding pose of pGpG (B) and pApG (C) in the binding pocket of PggH was simulated using GalaxyDock3 software. Overall simulated models are presented in small rectangles (top). Active sites of PggH are presented in red and blue rectangles in pGpG- and pApG-bound forms, respectively, and detailed views of the active sites are presented below. The black dotted lines indicated the hydrogen bonds between nucleotides and amino acid residues. The distance between the phosphorus atom of the phosphodiester bond (orange) and Mn^{2+} ion (purple) is indicated by the red dotted line along with the value. pGpG, 5'-phosphoguanylyl-(3′,5')-guanosine; PggH, phosphoguanylyl guanylate hydrolase.

3′-adename, such as pApA, may not be recognized by Asn227, at least partly accounting for the substrate selectivity of PggH at the 3′-base of the diribonucleotide. When we substituted Asn227 with tryptophan, we were unable to detect any binding of pGpG and pApG to PggH, supporting the simulated docking model. The substrate binding is further stabilized by several hydrogen bonds between ribose of 3′-guanosine with the guanidinium group of Arg146 and backbone carboxyl groups of Gly120 and Asp121. This was supported by the ITC data, which showed that the mutation at Arg146 reduced the binding affinity for both pGpG and pApG.

Recognition of the base at the 5′-position appears to be accomplished by His282 of the GGGH motif. The formation of hydrogen bonds between Nε2 of His282 and the N1 and C2 amine groups of 5′-guaine introduced a bend in the pGpG molecule, rendering the phosphodiester bond more accessible to Mn^{2+} ion (~2.6 Å). However, because 5′-adename could not form hydrogen bonds with His282, pApG remained in a straight configuration. In this pose, the distance between phosphate and Mn^{2+} was not adequately close to allow a nucleophilic attack by a Mn^{2+}-activated water molecule (~7.8 Å). This structural model indicated that binding with His282 is indispensable for the appropriate positioning of the substrate (Fig. 4B).

**PggH is involved in the regulation of c-di-GMP signaling**

Because our results indicated that PggH is a pGpG-specific PDE with its intrinsic structural nature, we compared pGpG hydrolysis activities between the cell lysates from WT and a pggH-deficient mutant (ΔpggH) strains to evaluate the pGpG degradation by cellular PggH (Fig. 5A). Lysates of the pggH mutant still degraded pGpG into GMP, suggesting that there exist additional proteins possessing pGpG-hydrolase activity, including VcOrn and some RNases (32). However, the deletion of pggH significantly decreased the pGpG-hydrolizing activity of cell lysates, indicating that PggH plays a substantial role in hydrolyzing pGpG in *V. cholerae* cells.

Previous biochemical studies revealed that the accumulation of pGpG could inhibit the c-di-GMP PDE activity of PDE-As (13) and suggested that appropriate degradation of pGpG is required for the maintenance of the c-di-GMP signaling. Thus, we speculated whether PggH could regulate the c-di-GMP signaling in *V. cholerae*. To assess the effect of PggH on the regulation of the c-di-GMP concentration, we compared intracellular concentrations of pGpG and c-di-GMP between the WT and a pggH-deficient mutant (Fig. 5B). Quantification using LC-MS/MS revealed that ΔpggH cells had an approximately 2.6-fold higher concentration of pGpG than WT cells, indicating that PggH possesses the pGpG PDE activity in *V. cholerae* cells. We also found a higher level of c-di-GMP in ΔpggH cells compared to that in WT cells, leading to the conclusion that PggH is involved in regulating the c-di-GMP signaling.

To examine the role of PggH in this signaling pathway, we measured biofilm formation, which requires a high level of c-di-GMP (33, 34). We observed that ΔpggH cells exhibited a higher biofilm-forming activity than WT cells, indicating that PggH negatively regulates the c-di-GMP signaling in the cells (Fig. 5C). The pggH-complemented strain, constructed by transforming the PggH expression plasmid to the *V. cholerae* pggH mutant, exhibited a similar level of biofilm formation.
with that of the WT strain, whereas the pggH mutant strain expressing the biochemically inactive PggH(D13N) did not complement this phenotypic change. These results confirmed the regulatory role of PggH in c-di-GMP signaling through its pGpG-PDE activity (Fig. S6A). However, this modulatory effect was less significant in the pggH mutant carrying the VcOrn expression plasmid (Fig. S6B). Therefore, it could be assumed that PggH can hydrolyze pGpG more efficiently than VcOrn to relieve the feedback inhibition of c-di-GMP degradation.

**PggH regulates c-di-GMP signaling in cooperation with PDE-As**

It has been shown that c-di-GMP turnover by PDE-As is inhibited by pGpG (14, 15). To verify that the increased biofilm formation of the pggH mutant is associated with the inhibition of PDE-As by the accumulation of pGpG and thus the increased concentration of c-di-GMP, the c-di-GMP PDE activity of PDE-A was determined in the absence or presence of PggH in vitro. Recently, we identified a novel sugar-responsive PDE-A, named PdeS in *V. cholerae* (35). As the expression of PdeS increased in the stationary phase, that of PggH also increased (Fig. S7), and accordingly, we assumed that PdeS was suitable to assess the effects of PggH on the activity of PDE-As and the c-di-GMP signaling. As expected, the PDE activity of PdeS was stimulated by the addition of PggH (Fig. 5D), suggesting that the product pGpG inhibits the activity of PDE-A by a negative feedback mechanism and that PggH functions as a modulator of PDE-A by controlling the pGpG level. We then constructed a pggH mutant expressing the c-di-GMP PDE PdeS and measured biofilm formation, using pJK1113 plasmid which possesses an arabinose-inducible PBAD promoter of arakBAD operon upstream of a multiple cloning site (Fig. 5E). Compared to the pggH mutant carrying pJK1113, cells carrying pJK-PdeS exhibited an apparent decrease in biofilm formation to a level similar to that of cells carrying pJK-PggH. However, the pggH mutant harboring pJK-PdeS(E450A), which expresses an inactive form of PdeS (35), did not exhibit the rescued phenotype, indicating that the pggH mutant accumulates c-di-GMP. The decreased biofilm formation of the WT strain, whereas the pggH mutant strain expressing the biochemically inactive PggH(D13N) did not complement this phenotypic change. These results confirmed the regulatory role of PggH in c-di-GMP signaling through its pGpG-PDE activity (Fig. S6A). However, this modulatory effect was less significant in the pggH mutant carrying the VcOrn expression plasmid (Fig. S6B). Therefore, it could be assumed that PggH can hydrolyze pGpG more efficiently than VcOrn to relieve the feedback inhibition of c-di-GMP degradation.

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formation of the pggH mutant was also observed when we transformed the mutant with an expression vector for VieA, a well-studied PDE (Fig. S6B) (12). Based on these findings, we concluded that PggH regulates c-di-GMP signaling by modulating the overall activities of PDE-As in V. cholerae.

**Discussion**

The degradation of oligoribonucleotides by nanoRNases is a critical process for cellular physiology in terms of RNA recycling and the completion of cyclic nucleotide signaling. In Gammaproteobacteria, the only protein previously identified to degrade diribonucleotides is the oligoribonuclease Orn, and its regulatory roles in c-di-GMP signaling have been well studied in P. aeruginosa (15, 18). However, in bacterial species in which Orn is essential for cell growth, such as E. coli and V. cholerae, whether Orn is involved in cyclic nucleotide signaling has not been clearly determined.

In this study, we investigated two proteins that have been reported to bind pGpG in the V. cholerae ORFeome from the DraCALA-based screen (15). Biochemical analysis suggests that Orn and PggH cooperate to reduce the cellular pGpG level and ensure the timely decay of the c-di-GMP signal, as a high level of pGpG inhibits the c-di-GMP PDE activity of PDE-As in V. cholerae (13–15). PggH exhibits a lower $K_{m}$ value than Orn (PggH, 12.11 μM; Orn, 41.15 μM), in addition to a considerably lower $K_{cat}$ value (PggH, 0.14 s$^{-1}$; Orn, 23.33 s$^{-1}$) (Figs. 2, D and E and S8). Considering that Orn has a variety of substrates from diribonucleotides to pentaribonucleotides present at similar levels in the cell, whereas PggG specifically digests pGpG and its activity is little affected in the presence of other nanoRNAs, it can be assumed that optimal pGpG hydrolysis activity of the two proteins would be exerted in the cell under different physiological contexts. At higher PggG concentrations, Orn might play the major role in the hydrolysis of pGpG as well as other nanoRNAs, whereas PggG likely plays a role in the degradation of pGpG under physiological PggG concentrations to allow for a finer tuning of the c-di-GMP signaling network. This notion is consistent with our result that the ectopic expression of either PggG or Orn in the ΔpggH mutant reduced biofilm formation by ~29% and ~13%, respectively (Fig. S6B). These data suggest that PggG could play a pivotal role in the degradation of pGpG and the completion of c-di-GMP signaling in their specific conditions in V. cholerae.

We determined the crystal structure of PggH at high resolution to gain molecular insights into the PggG-specific activity of PggH. Most DHH/DHHA1 family PDEs show broad substrate specificity to various oligonucleotides, which could be explained by an induced-fit model accompanied by a large conformational change upon substrate binding (22, 28, 29). Compared to the open active site of these enzymes in the absence of substrate (apo state), PggH has a small and narrow active site. When we calculated the volume of active site of PggH and TM1595 using CASTp software with a probe radius of 2.5 Å, we found that PggH has an ~2-fold smaller active site (383.796 Å$^3$) than TM1595 (748.513 Å$^3$). In addition, a rigid linker between the DHH and DHHA1 domains and the unique intervening dimeric arrangement of PggH further restrict the interdomain motion (Fig. 3). These distinct structural features lead to an unusually narrow substrate specificity of PggG compared to that shown by other DHH/DHHA1 family enzymes (Table 1, Figs. 2F and S1).

Linear dinucleotide intermediates can exhibit a regulatory effect on cyclic dinucleotide signaling. Here, we found that the degradation of pGpG by PggH is slightly inhibited in the presence of excess amounts of pApG (Fig. 2F) that can be produced following the degradation of 3′, 3′-cGAMP (36). Several enzymes have been reported to exhibit bifunctional activity that produces or degrades both c-di-GMP and cGAMP (37–39), suggesting that the concentration of two cyclic dinucleotides are interactively regulated. In this context, it is probable that PggH could act as a mediator in the cross talk between the c-di-GMP and cGAMP signaling pathway. Moreover, to the best of our knowledge, PggH is the first identified enzyme that specifically degrades pGpG. We therefore expect that further studies on the regulation of the pGpG hydrolysis activity of PggH might help to better understand the overall c-di-GMP signaling network in V. cholerae.

**Experimental procedures**

**Bacterial strains, plasmids, and culture conditions**

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S1 and S2. Bacterial cells were grown at 37 °C in LB medium. All plasmids were constructed using the standard polymerase chain reaction (PCR)-based cloning procedures and verified by sequencing.

To construct a pggH deletion mutant of the V. cholerae N16961 strain, the upstream and downstream regions of the pggH gene were amplified by PCR using the primer pairs PB421/PB422 and PB423/PB424, respectively. After digestion of the two PCR products, the Xmal-SphI and SphI-XbaI fragments, respectively, were cloned into the corresponding sites of the suicide vector pDM4 to generate the plasmid pDM4-PggH. The E. coli SM10 λpir strain carrying pDM4-PggH was conjugated with V. cholerae N16961, and the transconjugants were selected. Integration was confirmed by PCR, and the exoconjugants were selected in LB containing 10% sucrose. Deletion of pggH in the selected colonies was confirmed by PCR (40, 41).

To construct pJK-PggH-lacZ, pJK1113 and the pggH promoter region was amplified by PCR using the primer pairs PB425/PB426 and PB427/PB428, respectively. And then, these PCR products were combined using the EZ-Fusion Cloning kit. Then, the lacZ ORF amplified by PCR from E. coli chromosome using the primers PB429 and PB430 was digested with NcoI and SalI and inserted into the corresponding sites of the suicide vector pDM4 to generate the plasmid pDM4-PggH. The E. coli SM10 λpir strain carrying pDM4-PggH was conjugated with V. cholerae N16961, and the transconjugants were selected. Integration was confirmed by PCR, and the exoconjugants were selected in LB containing 10% sucrose. Deletion of pggH in the selected colonies was confirmed by PCR (40, 41).

To construct pJK1113-VieA, pJK1113 and the vieA (VC1652) ORF region was amplified by PCR using the primer pairs PB437/PB438 and PB439/PB440, respectively. And then, these PCR products were combined using the EZ-Fusion Cloning kit.
Purification of proteins

His-tagged proteins (His-PggH and His-Orn) were purified in the E. coli ER2566 strain by adding 1 mM IPTG and incubating at 30 °C for 4 h. The proteins were purified using TALON metal affinity resin according to the manufacturer’s instructions (Takara Bio). After elution with buffer A (20 mM Hepes–NaOH (pH 7.6), 100 mM NaCl, 5 mM β-mercaptoethanol, and 5% glycerol) containing 150 mM imidazole, His-tagged proteins were concentrated using Amicon Ultracel-3K centrifugal filters (Merck Millipore). To increase the purity of proteins (>98% pure) and remove imidazole, the concentrated fractions were subjected to gel filtration chromatography using a HiLoad 16/600 Superdex 200 200 column (GE Healthcare) equilibrated with buffer A (44, 45).

In vitro assay for pGpG-hydrolyzing activity

pGpG-hydrolyzing activity was assayed in a 100 μl reaction volume containing 50 mM Tris–HCl (pH 8.0), 10 mM MnCl2, 250 mM NaCl, 5 mM β-mercaptoethanol, and indicated concentrations of pGpG and purified proteins. After incubation at 37 °C for the indicated time, the reaction was terminated by the addition of 20 mM EDTA, and then trifluoroacetic acid was added to a final concentration of 10%. The reaction product generated from pGpG was then analyzed by HPLC (15).

Crystallization of PggH for structural determination

The crystals of the native protein were obtained as described previously (47). Briefly, the crystals were obtained by mixing the protein solution (1 μl, 10 mg/ml) and the reservoir solution (1 μl) containing 0.2 M magnesium chloride, Tris–HCl (pH 8.0), 12% PEG 4000, and 2 mM tris(2-carboxyethyl)phosphine hydrochloride at 14 °C by the vapor diffusion hanging-drop method.

Structural determination and refinement

The crystals were transferred to cryoprotection buffer (2 μl, the reservoir solution supplemented with 30% glycerol) for 1 min and then were flash-cooled in liquid nitrogen for data collection under cryogenic conditions. To solve the phasing problem, 10 mM zinc chloride was added in cryoprotection buffer of several crystals. The datasets of Zn-soaked crystals were collected on a direct X-ray detector Pilatus 6M (Dectris), equipped in beamline 5C of Pohang Accelerator Laboratory, Republic of Korea, at a wavelength of 1.2822 Å and Mn2+ bound crystals at 0.9795 Å. The crystal of PggH belonged to the space group P212121 with unit cell dimensions of a = 68.4 Å, b = 148.3 Å, and c = 58.8 Å. The program HKL-2000 was employed to process, merge, and scale the diffraction datasets (48). Data collection statistics are provided in Table S3. Anomalous signals from four Zn sites were found in each subunit, and the resulting electron density map was sufficiently clear to build an initial model using the programs PHENIX and COOT (49, 50). The Mn2+-bound structure was determined by direct refinement using the Zn2+-soaked structure.

Isothermal titration calorimetry

All the ITC experiments were carried out at 25 °C using MicroCal PEAQ-ITC calorimeter (Malvern Panalytical). Each titration experiment consisted of a single initial injection of 1 μl, followed by 33 injections of 2 μl nucleotides (100 μM pGpG, pApG, and pApA) in 25 mM Hepes–NaOH (pH 7.6), 100 mM NaCl, and 1 mM EDTA into the reaction cell containing 10 μM His-PggH in the same buffer. The experimental data were analyzed using MicroCal PEAQ-ITC Analysis Software with a single-site binding model.

Measurement of biofilm formation

Overnight-cultured cells were 1:100 inoculated in buffered LB medium with potassium phosphate (pH 7.0) and incubated under static conditions in a 96-well polystyrene microtiter plate at 37 °C for 24 h. Planktonic cells were removed and washed away with PBS, and the biofilm-associated cells were stained using 0.1% crystal violet solution. After several rinses using PBS, the stained biofilm was eluted with 100% ethanol and measured at 550 nm (35).

Computational simulation of substrate binding

A protein–ligand docking program GalaxyDock3 (31) was used to predict the binding poses of pGpG and pApG to the PggH structure. To account for protein conformational
changes induced by substrate binding, alternative structures for the loops surrounding the binding site were generated using a loop modeling program GalaxyLoop (51). Multiple ensemble docking simulations of pGpG and pApG were performed on the protein structures generated by loop modeling, and the docking poses compatible with mutagenesis analyses were selected.

Data availability
Coordinates and structure factors have been deposited in the Protein Data Bank (PDB) under accession codes 7D62.

Supporting information—This article contains supporting information (35, 52–54).

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: c-di-GMP, bis-(3′-5′)-cyclic diguanylate monophosphate; ITC, isothermal titration calorimetry; PDE, phosphodiesterase; pGpG, 5′-phosphoguananyl-(3′,5′)-guanosine; PgGHy, phosphoguananyl guanylate hydrolase.

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