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Purification, crystallization and preliminary X-ray crystallographic analysis of VCA0593 with a c-di-GMP binding activity in *Vibrio cholerae*

Yongdae Jang[1,†], Young-Ha Park[2,†], Jae-Woo Lee[3], Inseong Jo[1], Chaewoon Park[1], Yeong-Jae Seok[2,*] and Nam-Chul Ha[1,*]

[1]Department of Agricultural Biotechnology, Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 08826, Republic of Korea, [2]Department of Biological Sciences, Seoul National University, Seoul 08826, Republic of Korea, [3]Department of Biophysics and Chemical Biology, Seoul National University, Seoul 08826, Republic of Korea.

*Correspondence: hanc210@snu.ac.kr, yjseok@snu.ac.kr

[†]These authors equally contributed to this work.

Many kinds of nucleotides are differentially synthesized and degraded in response to external and internal signaling in bacterial cells. Cyclic di-GMP (c-di-GMP) is ubiquitous bacterial nucleotide secondary signaling molecule, and is involved in virulence, antibiotic resistance, biofilm formation and cell division. VCA0593 was predicted or screened as a c-di-GMP or 5'-phosphguanylyl-(3',5')-guanosine binding protein in *Vibrio cholerae* genome by genome-wide system approaches, suggesting that VCA0593 might be involved in a c-di-GMP-mediated signaling pathway. In this study, the full-length VCA0593 was overexpressed in the *E. coli* expression system, and successfully crystallized. X-rays were diffracted by the crystals to 1.60 Å resolution, revealing that the crystals belong to space group $P2_12_12$, with unit cell parameters a = 68.9, b = 149.0, and c = 58.9 Å. According to cell content analysis, the asymmetric unit was expected to contain two molecules of VCA0593. The molecular replacement was not available due to lack of the homologous models. We are now under way to solve the crystal structure using the anomalous signals from Zn-soaked crystals. The crystal structure would help reveal the function of the protein and its mechanism of action, which could be associated with the pathogenesis of *V. cholerae.*

INTRODUCTION

Bacteria incessantly change their cellular physiology in response to diverse environmental stimuli (D'Argenio and Miller, 2004). For example, changes in cell surface can induce the dispersion of the cells to the new sites or the adhesion of the cells. Cyclic di-GMP (or c-di-GMP) are ubiquitously used as a secondary messenger in many cellular signal pathways, involved in virulence, antibiotic resistance, biofilm formation and cell division (Corrigan et al., 2011). The cellular level of c-di-GMP is finely tuned by its controlled synthesis and degradation. C-di-GMP is synthesized by dinucleonylate cyclases and degraded by phosphodiesterase. The cyclase in synthesizing c-di-GMP commonly contains GGDEF domain, and the enzyme produces one molecule of c-di-GMP from two GTP molecules. C-di-GMP is degraded by one or two steps. C-di-GMP is degraded to 5'-phosphguanylyl-(3',5')-guanosine (pGpG) and then finally to two molecules of GMP by two different enzymes in case of two step mechanisms, whereas it can be degraded into two GMP molecules in a single enzyme in the one step cases (Corrigan et al., 2011).

Vibrio cholerae, which live in aquatic environment, is a representative human pathogen (Conner et al., 2017). *V. cholerae* copes with the dramatic environmental change during transition to human hosts. To adapt to these environmental change, c-di-GMP is used as a key regulator in *V. cholerae* between motile and sessile states. High cellular level of c-di-GMP is a hallmark of sessile state of *V. cholerae*, while the low level of c-di-GMP renders the bacteria highly motile. Many diguanylate cyclases were identified in *V. cholerae* genome by systematic analysis, and lack of some of the genes is associated with a phenotype of an increased motility (Beyhan and Yildiz, 2007; Conner et al., 2017). The phosphodiesterase containing EAL domain catalyzes the hydrolysis of c-di-GMP to pGpG, and pGpG is then hydrolyzed by oligoribonuclease Orn. Without the activity of Orn, pGpG can be accumulated and is responsible for inhibition of the break-down of c-di-GMP by the EAL-containing phosphodiesterases via a product inhibition mode in *Pseudomonas aeruginosa* (Cohen et al., 2015).

VCA0593 was screened as a c-di-GMP and pGpG binding protein in *V. cholerae* genome by a genome-wide system approach (Orr et al., 2015; Roelofs et al., 2015), suggesting that VCA0593 might be involved in a c-di-GMP-mediated signaling pathway (Roelofs et al., 2015). However, the function of this

gene in regard with c-di-GMP or pGpG are not known yet. VCA0593 belongs to NrnA superfamily, and is annotated as exopolyphosphatase, DHHA1 containing phosphodiesterase, or nanoRNase in GENBANK. However, any crystal structure of the protein including its homologous proteins has not been determined yet, and the function of this gene in regard with c-di-GMP are not investigated, either. To gain structural and functional information of VCA0593, we undertook to determine the crystal structure of VCA0593. In this study, we successfully overexpressed the protein in the *E. coli* expression system, and obtained the single crystals diffracting X-rays up to 1.60 Å resolution.

RESULTS AND DISCUSSION

To overexpress the full-length VCA0593 protein (residues 1-310), the *E. coli* expression system was employed. Two subsequent chromatography was conducted to purify the protein to homogeneity: affinity chromatography (Ni-NTA), and gel filtration chromatography (HiLoad 16/600 Superdex 200 pg). The final protein sample was concentrated to 10 mg/ml, as judged by Bradford assay. The purity of the protein was examined on SDS-PAGE, indicating >98% (Figure 1). The protein band appeared in a molecular weight of 36.5 kDa on the gel.

The crystals grew under several conditions from initial screening trials in an automated protein crystallization screening trial. The finally optimized crystals were obtained from 0.2 M magnesium chloride, Tris:HCl (pH 8.0), 12% PEG 4000, 2 mM TCEP by hanging drop vapour diffusion method at 14°C (Figure 2). The VCA0593 protein crystals were flash-cooled in liquid nitrogen after being soaked in reservoir solution containing additional 30% MPD. A highly brilliant undulator X-ray beam (beamline 5C in Pohang Accelerator Laboratory, Pohang, Republic of Korea) was utilized at a wavelength of 0.97940 Å to collect the diffraction dataset. The dataset with 99.3% completeness and 1.60 Å resolution was finally collected. The crystal lattice belonged to the primitive orthogonal space group, and analysis of the diffraction along the h, k and l axes further revealed a space group of $P2_12_12$ with unit cell parameters of a = 68.9, b = 149.0, and c = 58.9 Å. Data collection statistics are given in Table 2.

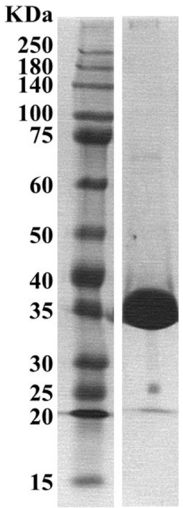

FIGURE 1 | The protein band on 12%-20% gradient SDS-polyacrylamide gel. The size marker is presented in the left line.

Cell content analysis suggested that two molecules in the asymmetric unit were suggested by with Matthews coefficient of 2.06 Å^3/Da and a solvent content of 40.4% (Matthews, 1968)(Table 2). Since one of NrnA superfamily protein was a dimeric in solution, VCA0593 is also suggested to be dimeric in the functional state (Uemura et al., 2013). Thus it is most likely that one dimeric unit is contained in the asymmetric unit. PSI-BLAST search results indicated that any structure with a substantial homology are not available (Li et al., 2011). In order to obtain the experimental phases by the single-wavelength anomalous diffraction (SAD) approach, we are now underway to collect anomalous signals from the crystal soaked in Zn-contained solution.

TABLE 1 | VCA0593 production information

	VCA0593
Source organism	*Vibrio cholerae* O1 biovar El Tor str. N16961
DNA source	*Vibrio cholerae* O1 biovar El Tor str. N16961 genomic DNA
Forward primer	GGATCCCATGTCATCACTCAAGTATCG
Reverse primer	GTCGACCTATTTGCGCTGAATCAAATTTAACCATCCGCA
Expression vector	pET-DUET
Expression hose	*E. coli* BL21 (DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHHHSQDPMSSLKYRLVTRSDFDGLVCAVLLKS IELIDDIQFVHPKDMQDGKVPITERDIITNLPYVANAHLVF DHHHSETLRNKGERPNHIINPNAPSAARVVWEHYGGTKTF PFEWVEMMEAVDKGDSAQFTRDEVLDSTGWNLLNFLMD ARTGLGRFHNFRISNYNLMMALIDHCTHASIDEILQLPDVK ERVELYRKHETLFKEQIQRCGKVYQNLVLLDLTEEETIYA GNRFIIYALYPQCNISIHKMWGFQKQNIVFATGKSIFDRSSR TNIGELMLKYGGGGHAAAGTCQIAIEDADRVEKALITQINADG

CONCLUDING REMARKS

The crystal structure would help elucidate the function and action mechanism of VCA0593, expanding the structural diversity of NrnA superfamily, which could be associated with the pathogenesis of *V. cholerae*.

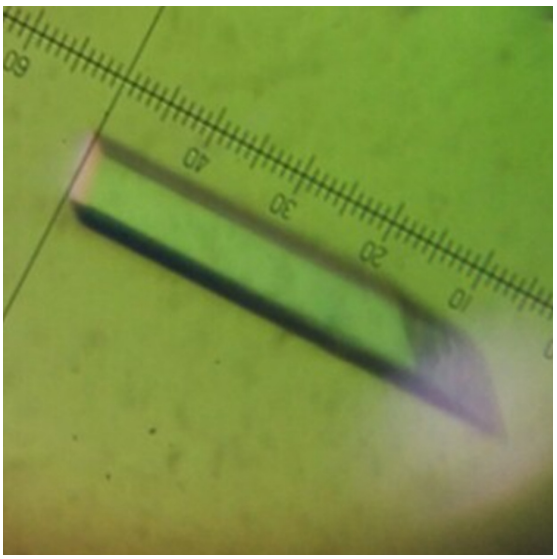

FIGURE 2 | A crystal of VCA0593. The approximate dimension of the crystal is 0.45 x 0.45 x 0.05 mm.

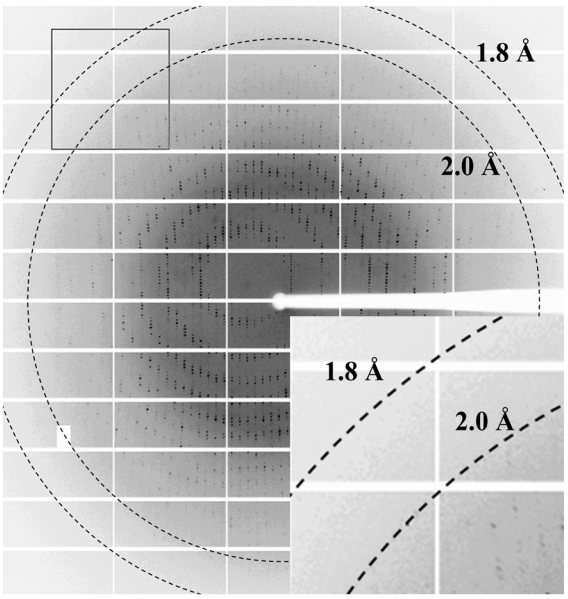

FIGURE 3 | A representative diffraction image. The resolution circles are shown in dotted lines. A rectangular area is enlarged in the left bottom corner.

METHODS

Construction for the protein expression of VCA0593

The gene coding for the VCA0593 was amplified from the V. cholerae O1 biovar El Tor str. N16961 genome by PCR (NCBI Reference Sequence: NP_232983.1). The primer sequences are described in Table 1. The PCR products were digested with BamHI and SalI and cloned into pETDuet vector (EMD Biosciences), resulting in pETDuet-VCA0593 that contains the hexahistidine tag at the N-terminus of the protein for purification (Table 1).

Protein expression and purification of the VCA0593 protein

The recombinant plasmid pETDuet-VCA0593 was transformed into *Escherichia coli* BL21 (DE3) cells. The cells were cultured in 2.0 L LB medium supplemented with 100 μg/mL ampicillin at 37°C until OD_{600} reached 0.8. The VCA0593 was induced by 0.5 mM IPTG, and further cultured for 7~8 h at 30°C. The cells were harvested by centrifugation at 1400xg for 10 min at 4°C. The cell pellet was resuspended with 50 ml of lysis buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl and 2 mM β-mercaptoethanol. A continuous cell disruptor (Constant Systems, England) was employed to disrupt the cells, and centrifugation was conducted by at 20000xg for 30 min at 4°C to remove the cell debris. The cell lysate (50 ml) was incubated with 2 ml of Ni–NTA agarose resin (Qiagen, Germany) and incubated in a column for 30 min at 4°C. After the resin was washed with 300 ml of a buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 20 mM imidazole (pH 8.0), and 2 mM β-mercaptoethanol. The protein was subsequently eluted with 30 ml of a buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 250 mM imidazole (pH 8.0), and 2 mM β-mercaptoethanol. The fractions containing the protein were pooled and concentrated to 5 ml using a Vivaspin centrifugal concentrator (30 kDa molecular-weight cutoff; Millipore, USA). Then the resulting protein sample was further purified using a gel filtration chromatographic column (HiLoad 16/60 Superdex 200; GE Healthcare), which was pre-equilibrated with 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and 2 mM β-mercaptoethanol. The pooled fractions were finally concentrated to 10 mg/ml using the same concentrator, and stored frozen at -80°C until use.

TABLE 2 | X-ray diffraction and cell content analysis

	VCA0593
Data collection	
Beam line	PAL 5C
Wavelength (Å)	0.97940
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	$P2_12_12$
Cell dimensions	
a, *b*, *c* (Å)	68.9, 149.0, 58.9
α, β, γ (°)	90, 90, 90
Resolution (Å)	50.0-1.60 (1.63-1.60)
Total No. of reflections	2490936
No. of unique reflections	80072
R_{merge}	0.068 (0.515)
High resolution shell CC1/2	0.341
$I/\sigma I$	27.67 (2.09)
Completeness (%)	99.3 (96.0)
Redundancy	8.5 (4.4)
No. of molecules in asymmetric unit	2
V_M (Å^3/Da)	2.06
Solvent contents (%)	40.4

*Values in parentheses are for the highest resolution shell.

Crystallization

Initial crystallization of VCA0593 was performed using automated crystal screening device MOSQUITO by sitting-drop vapour-diffusion method. Commercially available sparse-matrix screening solutions (MCSG, Anatrace) was selected for the initial crystallization screening trials at 14°C. The protein solution (0.2 µl, 10 mg/ml) was mixed with a reservoir solution (0.2 µl) and equilibrated against 60 µl of the reservoir solution in a 96-well crystallization plate. Small cuboid crystals were obtained using the condition containing 0.2 M magnesium chloride, Tris:HCl (pH 8.5), 16% PEG 4000, 2 mM Tris(2-carboxyethyl) phosphine (TCEP). The crystallization conditions were further optimized using hanging-drop diffusion method under a reservoir solution containing 0.2 M magnesium chloride, Tris:HCl (pH 8.0), 12% PEG 4000, 2 mM TCEP at 14°C. In the final optimization experiments, 1 µl of the protein solution (10 mg/ml) was mixed with 1 µl of the reservoir solution, and equilibrated against 500 µl of the reservoir solution in a 15-well plate. Thick plate-shaped crystals for data collection appeared in 3-4 days (Figure 2).

Data collection and processing

The crystals were transferred to 2 µl of cryo-protection buffer containing the reservoir solution and additional 30% (±)-2-methyl-2,4-pentanediol (MPD) for 1 min, and then were flash-cooled in liquid nitrogen for data collection under cryogenic conditions. The datasets were collected on a direct X-ray detector Pilatus 6M (Dectris, Switzerland), equipped in beamline 5C of Pohang Accelerator Laboratory, Republic of Korea, at a wavelength of 0.97940 Å. The program HKL-2000 was employed to process, merge, and scale the diffraction datasets (Otwinowski and Minor, 1997). The data-collection statistics are given in Table 2.

CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

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