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RESEARCH ARTICLE

Effects of Elevated Intracellular Cyclic di-GMP Levels on Biofilm Formation and Transcription Profiles of *Vibrio vulnificus*

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Abstract Effects of elevated intracellular 3',5'-cyclic diguanylic acid (c-di-GMP) levels on biofilm formation and transcription profiles were evaluated to assess the functions of c-di-GMP in *Vibrio vulnificus*. Elevated c-di-GMP levels promoted biofilm formation and rugose colony development. Microarray analysis revealed that c-di-GMP influenced expression of genes belonging to different functional categories and more than 5% of the *V. vulnificus* genome. Among these, 10 genes potentially involved in biofilm formation were experimentally verified as subject to c-di-GMP regulation. c-di-GMP contributes to biofilm formation based on modulation of diverse cellular processes in *V. vulnificus.*

Keywords: 3',5'-cyclic diguanylic acid (c-di-GMP), *Vibrio vulnificus*, transcriptome analysis, biofilm, rugose colony

Introduction

Many bacteria live within multicellular structures known as biofilms, which are embedded in a matrix of extracellular polymeric substances, including polysaccharides, proteins, nucleic acids, and lipids (1). Biofilm formation facilitates survival of bacteria against stress, including starvation, temperature change, antimicrobial agent challenge, and host immune defense systems (2,3). Previous studies have reported that the pathogenic marine bacterium *Vibrio vulnicus* forms biofilms on different biotic surfaces, such as eel body surfaces and crab and oyster shells, to enhance survival in natural environments (4-6). Recent studies have provided several lines of evidence supporting the hypothesis that biofilm formation is essential for pathogenesis and survival of *V. vulnificus* in a host (7-9).

The bacterial global second messenger 3',5'-cyclic diguanylic acid (c-di-GMP) is known to be a key molecular factor in biofilm formation, adhesion, motility, virulence, and bacterial cell morphogenesis (10). c-di-GMP is synthesized by diguanylate cyclases (DGCs) and degraded by c-di-GMP-specific phosphodiesterases (PDEs). DGCs and PDEs are ubiquitous in bacteria, and c-di-GMP networks consisting of these enzymes have been identified in many bacteria (11). *V. vulnificus* diguanylate cyclase protein A (DcpA) has been reported to influence exopoly-saccharide (EPS) production, biofilm formation, and rugose colony development (12).

c-di-GMP-influenced biofilm development and colony morphology of *V. vulnificus* were examined in this study. Whole-genome microarray analysis revealed that more than 5% of *V. vulnificus* genes were differentially expressed depending on levels of intracellular c-di-GMP.

Materials and Methods

Strains, plasmids, and culture conditions Bacterial strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, *V. vulnificus* strains were grown in Luria-Bertani medium supplemented with 2.0% (wt/vol) NaCl (LBS) at 30°C. *Vibrio fischeri* minimal medium containing 32.6 mM glycerol (VFMG) was used for biofilm formation (9). All the chemicals were puchased from Sigma (St. Louis, MO, USA).

Manipulation of intracellular c-di-GMP levels The *dcpA* region encoding diguanylate cyclase (12) was amplified

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
V. vulnificus		
CMCP6	Clinical isolate; virulent	Laboratory collection
E. coli		
DH5α	*supE44* Δ*lacU169* (80*lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*; plasmid replication	Laboratory collection
S 17-1λ*pir*	λ-*pir* lysogen; *thi pro hsdR hsdM*$^+$ *recA* RP4-2 Tc::Mu-Km::Tn7;Tp^r Sm^r; host for π-requiring plasmids; conjugal donor	26
Plasmids		
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pJH0311	0.3-kb NruI fragment containing multi-cloning site of pUC19 cloned into pCOS5; Ap^r, Cm^r	13
pJN1002	pJH0311 with *dcpA*	This study

[a]Tp^r , trimethoprim resistant; Sm^r, streptomycin resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant

using PCR with *V. vulnificus* genomic DNA and the primers dcpA_F (5'-GAGCTCTCATCCCATCCTAAACT TTGT-3') and dcpA_R (5'-CCCGGGTGGGTTTTCTGTT G-3') for manipulation of intracellular levels of c-di-GMP. The amplified *dcpA* coding region was cloned into the broad host-range vector pJH0311 (13) under an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, resulting in pJN1002 (Table 1). The plasmid pJN1002 was delivered into *V. vulnificus* CMCP6 via conjugation (9), and intracellular c-di-GMP levels of *V. vulnificus* strains were measured using LC-MS, as previously described (14).

Biofilm formation and colony morphology assays The biofilm formation activity of *V. vulnificus* strains in static and shaking cultures was assessed as previously described (9,15). Static biofilms were formed by standing polystyrene microtiter plates (Nunc, Roskilde, Denmark) containing 200 μL of VFMG culture supplemented with 1 mM IPTG for 24 h at 30°C. Once planktonic cells were removed, biofilm cells on walls were washed with phosphate-buffered saline (PBS, pH 7.4), then stained with a 1% (wt/vol) crystal violet (CV) solution for 15 min at room temperature. Biofilms were quantified based on elution of CV using 100% ethanol and measurement of absorbance values at 570 nm (A_{570}) (16). For biofilms in shaking cultures, 5 mL of VFMG with 1 mM IPTG in a glass tube was inoculated with *V. vulnificus* strains and incubated in shaking incubator (SW-90R; Sang-woo, Seoul, Korea) for 12 h at 30°C with shaking at 220 rpm. Biofilm rings at the air-liquid interface of shaking cultures were photographed using a digital camera (PowerShot SX220 HS; Canon, Tokyo, Japan). For analysis of colony morphology, 2 μL of culture grown to A_{600}=0.8 were spotted onto LBS agar plates supplemented with 1 mM IPTG. Colonies grown at 30°C for 24 h were photographed (PowerShot SX220 HS; Canon).

Microarray analysis For transcriptome analysis, *V. vulnificus* strains containing either pJH0311 or pJN1002 were grown to A_{600}=0.7 using AB medium (300 mM NaCl, 50 mM $MgSO_4$, 0.2% vitamin-free casamino acids, 10 mM potassium phosphate, 1 mM L-arginine, pH 7.5; 17) in the presence of 1 mM IPTG, and total cellular RNAs were isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Aminoallyl-cDNAs were synthesized using an Amino Allyl cDNA Labeling Kit (Ambion, Austin, TX, USA) and were labeled using Cy3 or Cy5 (Amersham Pharmacia, Uppsala, Sweden). Equal amounts of labeled cDNAs were combined to hybridize with the *V. vulnificus* Whole-Genome Twin-Chip (9) at 42°C for 16 h and the arrays were washed, dried, scanned, and analyzed using GenePix Pro 3.0 software (Axon Instruments, Union City, CA, USA). ORF spots that showed a 2× or greater difference in expression with a p value of 0.05 were considered to be regulated by c-di-GMP. The M value in Table S1 represents the $\log_2$ ratio of DNA hybridization intensity of each gene in *V. vulnificus* containing pJN1002 versus *V. vulnificus* containing pJH0311. The database for the *V. vulnicus* CMCP6 genome was retrieved from GenBank (accession numbers AE016795 and AE016796).

Quantitative real-time PCR (qRT-PCR) cDNA was synthesized for qRT-PCR using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real-time PCR amplification of cDNA was performed using a Chromo 4 real-time PCR detection system (Bio-Rad) with a pair of specific primers (Table S2) as described previously (9). Relative expression levels of specic transcripts were calculated using the 16S rRNA expression level as an internal reference for normalization.

Data analysis Mean values and standard errors of the mean (SEM) were calculated based on at least 3 independent experiments. Data were analyzed using Student's t test with

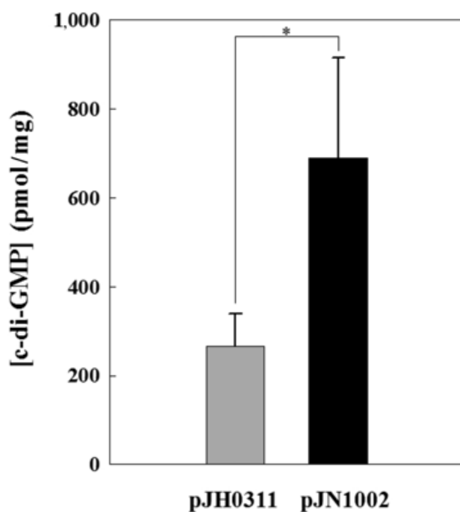

Fig. 1. Intracellular c-di-GMP levels of *V. vulnificus* strains. *V. vulnificus* containing either pJH0311 or pJN1002 was grown in VFMG supplemented with 1 mM IPTG; Intracellular levels of c-di-GMP were measured using LC-MS and normalized to mg of total protein; Error bars represent the SEM; *p<0.05 relative to *V. vulnificus* containing pJH0311.

SAS software (SAS Institute Inc., Cary, NC, USA). Significance of differences between experimental groups was accepted at a p value of <0.05.

Microarray data accession number All primary microarray data were deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo/; 18) under accession number GSE51459.

Results and Discussion

Effects of c-di-GMP on biofilm formation and colony morphology In order to examine the effects of c-di-GMP on biofilm formation and colony morphology, the plasmid pJN1002 containing *dcpA* under control of the IPTG-inducible promoter was constructed and transferred to *V. vulnificus*. After induction using 1 mM IPTG, the intracellular c-di-GMP levels in *V. vulnificus* containing pJN1002, determined using LC-MS, were approximately 3× greater than for the pathogen containing the control vector pJH0311 (Fig. 1). Thus, pJN1002 was an appropriate plasmid for manipulation of intracellular c-di-GMP levels.

After induction of *dcpA* expression, the biofilm formation activity of *V. vulnificus* containing pJN1002 was 1.6× higher than for the pathogen containing pJH0311 (negative control) under static conditions (Fig. 2A). Under shaking conditions, *V. vulnificus* containing pJN1002 formed a thick biofilm, while the pathogen containing pJH0311 did not produce a biofilm at the air-liquid interface (Fig. 2B). *V. vulnificus* containing pJN1002 formed rugose colonies while the pathogen containing pJH0311 exhibited smooth colony morphology (Fig. 2C). Elevated c-di-GMP levels in *V. vulnificus* cells promoted biofilm formation, particularly at the air-liquid interface, and colony rugosity of the pathogen.

Effects of c-di-GMP on transcription profiles Comparison of transcripts from *V. vulnificus* carrying pJN1002 with transcripts from the pathogen carrying pJH0311 resulted in prediction of 233 genes potentially regulated by c-di-GMP. Among them, 109 genes were up-

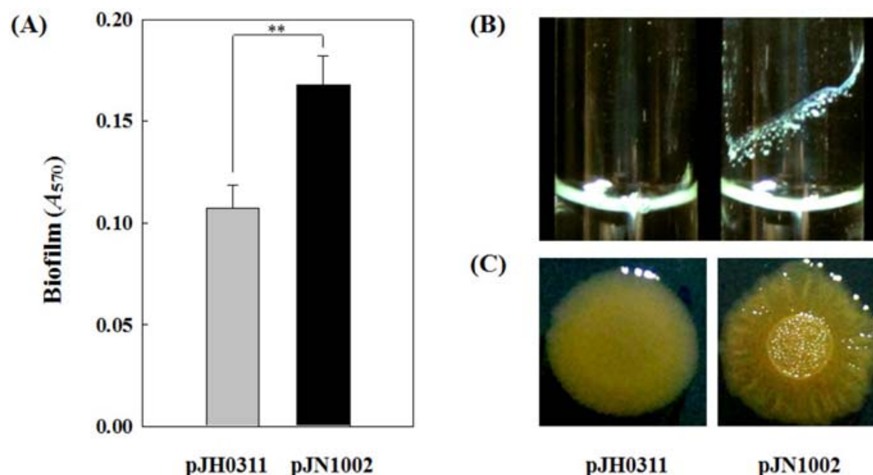

Fig. 2. Effects of c-di-GMP on biofilm formation and colony morphology. (A and B) Biofilms of *V. vulnificus* containing either pJH0311 or pJN1002 were grown in VFMG supplemented with 1mM IPTG under static (microtiter plates, A) and shaking (test tubes, B) culture conditions; Static biofilms were quantified using the CV staining method; (C) Colonies of *V. vulnificus* containing either pJH0311 or pJN1002 grown on LBS agar plates supplemented with 1 mM IPTG were photographed; **p<0.005 relative to *V. vulnificus* containing pJH0311.

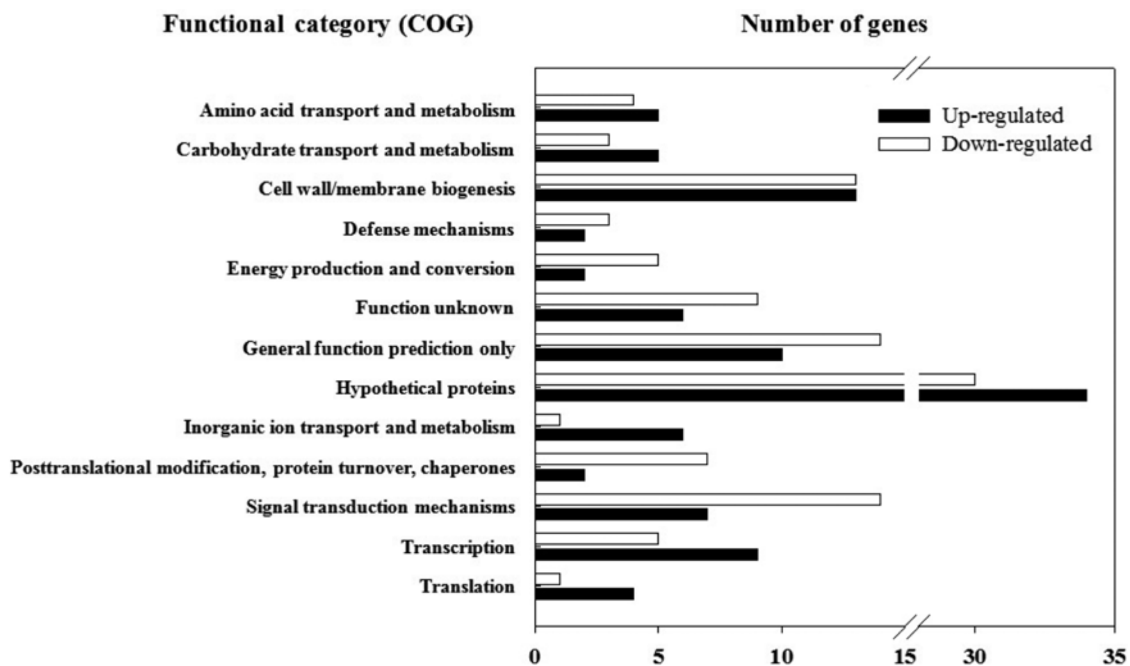

Fig. 3. Number of genes regulated by c-di-GMP. Genes with expression ratios of 2 on the basis of microarray analytical results were considered to be regulated by c-di-GMP; Functional categories (COG) corresponding to at least 5 genes are presented and are based on the database for the *V. vulnicus* CMCP6 genome; Closed and open bars represent genes up-regulated and down-regulated by c-di-GMP, respectively.

regulated and 124 genes were down-regulated. The predicted genes of the c-di-GMP regulon were distributed throughout the 2 chromosomes of *V. vulnicus*. A complete list of locus tags of the 233 genes is shown as supplementary material (Table S1). Although many of the predicted genes were of hypothetical or unknown function, some belonged to functional categories (Fig. 3), of which the 10 categories of amino acid transport and metabolism, carbohydrate transport and metabolism, cell wall/membrane biogenesis, defense mechanisms, energy production and conversion, inorganic-ion transport and metabolism, post-translational modification/protein turnover/chaperones, signal transduction mechanisms, transcription, and translation contained at least 5 genes (Fig. 3).

c-di-GMP-regulated genes involved in biofilm formation Among the genes regulated by c-di-GMP, 10 genes that probably participated in biofilm formation of *V. vulnificus* were up-regulated by c-di-GMP (Fig. 4). The products of the 10 genes were BrpA, BrpT (5), DGC-family proteins (11), an OmpA protein (19,20), components of type 1 secretion systems (T1SS) (21-23), and putative calcium binding proteins (24). Since many of the genes that the microarray analysis predicted were not previously reported to be c-di-GMP-regulated, c-di-GMP regulation of the selected 10 genes was experimentally verified using qRT-PCR. qRT-PCR analysis revealed that c-di-GMP regulated transcription of all of the 10 genes predicted, of which VV1_3061 and VV2_1571 encoding putative calcium binding proteins were the most up-regulated (approximately 100x) by c-di-GMP (Fig. 4).

The *V. vulnificus brp* gene cluster and *brpT* were homologues to the *V. cholerae vps*-II gene cluster and *vpsT*, respectively, which were responsible for VPS (*Vibrio* polysaccharide) production (25). BrpA, the product of the first gene of the *brp* gene cluster, and BrpT are known to participate in biofilm formation in *V. vulnificus* (5). DGC-family proteins (gene products of VV1_1829 and VV2_0264) are known to maintain appropriate levels of c-di-GMP in bacterial cells (11). OmpA has been suggested to function as an adhesin in many bacteria (20) and to promote biofilm formation (19). Gene products of VV1_2716, VV2_1572, and VV2_1573 are components of T1SS, which is required for pellicle formation (21-23). Putative calcium binding proteins harbor calcium-binding motifs and the acidic glycine-rich nonapeptides (data not shown), and presumably function as adhesins required for bacteria-to-bacteria interactions during biolm formation (24).

c-di-GMP probably regulates many cellular processes, including EPS synthesis, c-di-GMP production, adhesin biogenesis, protein secretion, and cell-to-cell interaction, in which the combined effects of regulation appear to enhance *V. vulnificus* biofilm formation.

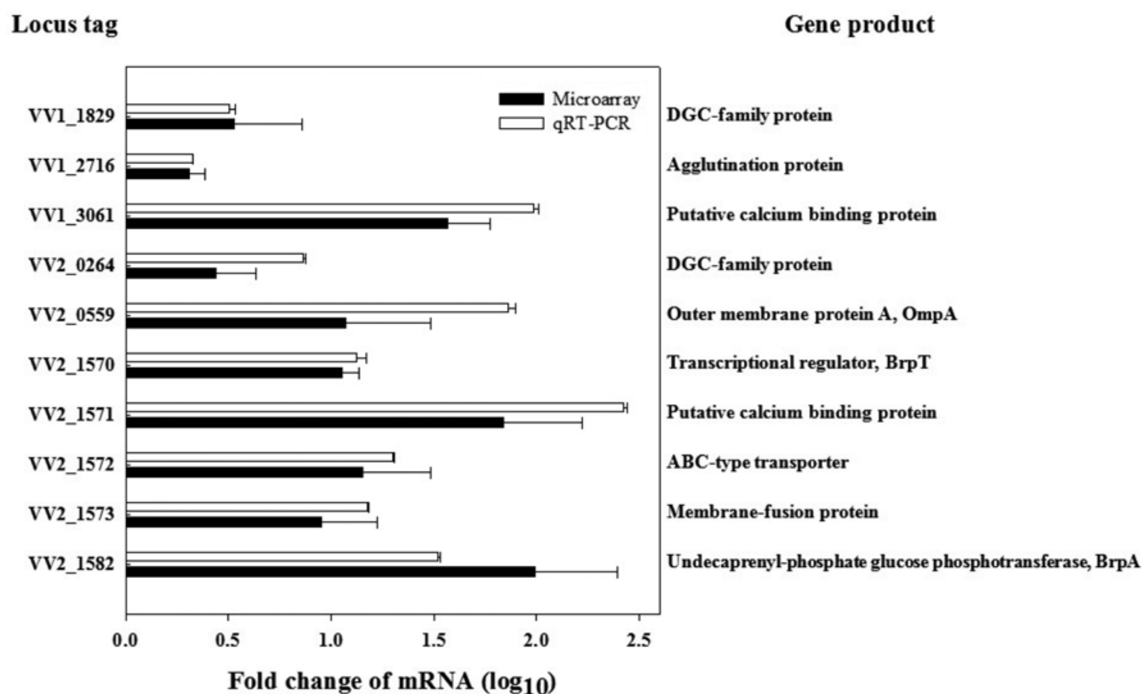

Fig. 4. Verification of newly predicted genes as part of the c-di-GMP regulon. Ten genes from the pool of c-di-GMP-regulated gene members newly predicted on the basis of microarray analytical results were analyzed using qRT-PCR. Each column represents the mRNA expression level in *V. vulnificus* containing pJN1002 relative to the level in *V. vulnificus* containing pJH0311. Mean values and SEM were calculated from results of at least 3 independent experiments. Locus tags are based on the database of the *V. vulnicus* CMCP6 genome, and the products of the 10 genes are listed on the right. ABC transporter, ATP-binding cassette transporter.

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Disclosure The authors declare no conflict of interest.

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