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Transcriptomic Analysis of Genes Modulated by Cyclo(L-Phenylalanine-L-Proline) in *Vibrio vulnificus* S

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Diketopiperazine is produced by various organisms, including bacteria, fungi, and animals, and has been suggested as a novel signal molecule involved in the modulation of genes with various biological functions. *Vibrio vulnificus*, which causes septicemia in humans, produces cyclo(L-phenylalanine-L-proline) (cFP). To understand the biological roles of cFP, the effect of the compound on the expression of the total mRNA in *V. vulnificus* was assessed by next-generation sequencing. Based on the transcriptomic analysis, we classified the cFP-regulated genes into functional categories and clustered them according to the expression patterns resulted from treatment with cFP. From a total of 4,673 genes, excepting the genes encoding tRNA in *V. vulnificus*, 356 genes were up-regulated and 602 genes were down-regulated with an RPKM (reads per kilobase per million) value above 3. The genes most highly induced by cFP comprised those associated with the transport and metabolism of inorganic molecules, particularly iron. The genes negatively regulated by cFP included those associated with energy production and conversion, as well as carbohydrate metabolism. Noticeably, numerous genes related with biofilm formation were modulated by cFP. We demonstrated that cFP interferes significantly with the biofilm formation of *V. vulnificus*.

Keywords: *Vibrio vulnificus*, cyclo(Phe-Pro), next-generation sequencing, transcriptome, biofilm

Introduction

Diketopiperazines (DKPs, or cyclic dipeptide molecules) are produced by various organisms, including bacteria, sponges, algae, and animals [22]. These molecules have attracted a great deal of attention owing to recent findings showing that members of this molecular family have various physiological effects, including antimicrobial [13, 18] and antitumor activities [8, 17], the promotion of plant growth [11], signaling for quorum sensing [14], and modulation of the expression of virulence factors [3, 37]. Nevertheless, the biochemical or molecular biological bases for the actions of these molecules have yet to be elucidated.

Vibrio vulnificus is a Gram-negative, halophilic bacterium, and an opportunistic pathogen causing septicemia in humans who are immunocompromised or have underlying conditions [9]. This pathogen produces cyclo(L-phenylalanine-L-proline) (cFP), which activates a quorum-sensing bioindicator [20]. The production of cFP from *V. vulnificus* reaches the highest level when cell growth enters the stationary phase. Purified cFP from culture supernatant or chemically synthesized cFP enhances the expression of *ompU* encoding a porin in *V. vulnificus* [37]. This molecule has also been identified in *V. cholerae*, and affects the expression of the virulence factor, cholera toxin [3, 37]. cFP was also identified in *Pseudomonas aeruginosa* and *P. putida*, and the compound activates several quorum-sensing bioindicators [12, 20]. Based on these findings, cFP has been suggested as a novel quorum-sensing signal molecule [27, 37].

cFP has also been reported to have additional activities. This molecule exhibits antibacterial and antifungal activities [33, 41, 45], and inhibits DNA topoisomerase [40]. The compound also inhibits cancer cell growth and induces apoptosis in colon cancer cells [4, 5]. More recently, it has

been shown that cFP produced by the human vaginal *Lactobacillus* strain represses the production of toxic shock syndrome toxin in *Staphylococcus aureus* by quenching the quorum-sensing signal [30], and that cFP produced by plant-associated bacteria mimics auxin activity, promoting plants growth [36]. Although the promiscuous biological activities of cFP are being reported, the mode of action of the compound at the molecular level remains to be investigated.

In order to obtain basic information to understand the physiological roles and working mode of cFP in *V. vulnificus*, we investigated and compared the transcriptomes of the pathogen grown in the presence or absence of exogenous cFP using the RNA sequencing (RNA-Seq) technique. Here, we report that numerous genes are modulated by cFP and suggest possible biological roles of cFP based on this analysis.

Materials and Methods

Strain and Growth Conditions

The pathogenic *V. vulnificus* strain MO6-24/O [38] was employed for this study. *V. vulnificus* MO6-24/O was grown aerobically in Luria-Bertani (LB) medium (Neogen, Baltimore, MD, USA) at 30°C. To examine the effect of cFP, *V. vulnificus* MO6-24/O cells were prepared as follows: *V. vulnificus* MO6-24/O was streaked onto a solid LB plate, and on the next day, the cells from the solid medium were inoculated in LB broth. The cells were cultured overnight, and then subcultured in the same medium. The cFP stock (Bachem, Switzerland) was dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 5 mM for the cFP-treated group. For the non-cFP-treated group, the same volume of DMSO was added to the medium. Cells in both the cFP-treated and cFP-non-treated media were further cultured at 30°C until the stationary phase (OD_{600} of ~1.5) and then used for RNA extraction.

RNA Extraction, Sequencing, and Data Analysis

Total RNA was extracted using the RNeasy midi kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Contaminated DNA was eliminated by RNase-free DNase (Invitrogen, Carlsbad, CA, USA) and the quantity and quality of the total RNA were evaluated using RNA electropherograms (Agilent 2100 Bioanalyzer) and assessing the RNA integrity number (RIN) [43]. Ten micrograms of the total RNA from each sample over a RIN value of 8.0 was used as a starting material and treated with the MICROBExpress mRNA enrichment kit (Invitrogen). The resulting mRNA samples were processed for the sequencing libraries using the Illumina mRNA-Seq sample preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's protocols.

One lane per sample was used for sequencing by the Illumina Genome Analyzer IIx (Illumina) to generate non-directional, single-ended 36-base-pair reads. Quality-filtered reads were mapped to the reference genome sequences (NCBI BioProject ID PRJNA62243) using CLC Genomics Workbench 5.5 (CLC bio, Denmark). The relative transcript abundance was computed by counting the reads per kilobase of exon model per million mapped sequence reads (RPKM) [35]. The Cluster of Orthologous Groups (COG) database was used to cluster the genes into functionally related groups [47].

Quantitative Real-Time PCR

The primers for quantitative real-time PCR are listed in Table 1. Total RNA was extracted as described above and cDNA was synthesized using a Prime Script RT reagent kit (TaKaRa Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's directions. Real-time PCR was carried out using the LightCycler 480 system with SYBR Green Master Mix (Roche Applied Sciences, Basel, Switzerland). Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.

Quantitative Assessment of Biofilm Formation

Biofilm formation assay was performed as described previously [26]. In brief, overnight culture of *V. vulnificus* in LB medium was subcultured to fresh AB broth with 1% sodium fumarate as a carbon source after washing. When the cell density reached a value of A_{600} 0.5–1.0, the culture was washed twice with AB-fumarate broth, and then subcultured to the adjusting value of A_{600} 0.05 in AB-fumarate broth containing various concentrations of cFP (0–5 mM) in a borosilicate bottle. After 48 h of incubation, the biofilm of the wall was washed twice with phosphate-buffered saline, and then stained with 1.0% crystal violet for 30 min. The stained biofilm was washed twice with distilled water and air dried, and then resuspended in 100% ethanol. The amount of crystal violet in the stained biofilm was quantified by the value of A_{550}, and normalized by dividing the planktonic cell density (A_{600}).

Results and Discussion

Next-Generation Sequencing (NGS) of Total mRNA

The concentration of cFP detected in the culture supernatant of wild-type *V. vulnificus* is approximately 0.7~1 mM [37]. We have observed that phenotypes associated with cFP are observed at 1 mM, but the phenotypes are more clearly manifested at 5 mM. For previous studies [3, 30, 36], cFP at the concentration of even higher than 10 mM was used. Therefore, we added 5 mM of cFP for cFP-treated cells to obtain clear results in this study.

Through the NGS technique, a total of 27,716,116 sequence reads were obtained from the control cFP-non-treated and cFP-treated samples. From these, we mapped 1.4 million (12%) and 2.1 million (13%) mRNA reads from total RNA. Most of the remaining reads were from rRNA. A total of

Table 1. Primers used for quantitative RT-PCR.

Genes / Function[a]	Direction	Nucleotide sequences (5′ → 3′)
MO6_01206	Forward	ATG GAA AGA GGT GTC GTT GG
Flp pilus assembly protein *cpaB*	Backward	CAA TTT CCG ATT GGC TTG TT
MO6_04451	Forward	ATGGTGTTTTGTCGCTAGGC
Isochorismatase	Backward	GTTAAATACCGGTGATGCCG
MO6_04211	Forward	GCCTATGCTCAAACCGAGAG
Ferric vulnibactin receptor *vvuA*	Backward	CTCTGTTGCACCTGGGGTAT
MO6_04202	Forward	GGCGACAACCAAAAGTTGAT
Isochorismate synthase	Backward	CGTATTGAGCAGCGATTGAA
MO6_04199	Forward	CCAAGATTTGGTGGCTCATT
NRPS biosynthase	Backward	TGTCACCAACTAACGGTGGA
MO6_02645	Forward	TCG CGC AGC ACA TAA TTT AG
LeuO homolog	Backward	GCA AAA CGC ATA TCA CAT GG
MO6_03472	Forward	TTA CTG ACG CAG TCG CAA TC
Alcohol dehydrogenase	Backward	TAG ACG CTC AGG GCA AAC TT
MO6_01966	Forward	CTC AGG ATC TCC AAC AAG CC
Cytochrome *c*-type protein *nrfB*	Backward	TGG CAA TCC ACA CAC ATC TT
MO6_04106	Forward	ACC AAG TTT AAT TGG CGT GC
TRPA-type periplasmic transporter	Backward	CCC AGA CAA CTT GGC GTA AT
MO6_01151	Forward	AAC GTA GGG CAA CTC AAT GG
Diguanylate cyclase	Backward	ATG TTC AAG CGG AAG CTG TT
MO6_02227	Forward	GTT TGG CGT GAA CAA GGT TT
Transcriptional regulator *toxR*	Backward	TCA GAA GCT GCG TCA TTC AC
MO6_00513	Forward	CGT TAG AGC ACC TGT ACG CA
AI-2 producer *luxS*	Backward	TTC ATC TAG CGA GTG CAT GG
MO6-gap[b]	Forward	TTGATTGGCCAGAATGTTAGTTTG
NADPH-dep glyceraldehyde-3-phosphate dehydrogenase	Backward	TGGTTTCAATCACGTGACCATTGA

[a]The order of these genes is as shown in Fig. 2.

[b]Used as a negative control for qRT-PCR.

4,562 genes other than the genes encoding tRNA were assigned to the COG classification [47], and were classified into 17 groups. These suggest that cFP affects the expression of numerous genes with various functions. Major COGs to which genes modulated by exogenous cFP belong are those for cellular processes, signaling, carbohydrate transport, metabolism, and energy production and conversion (Fig. 1).

The expressions of a total of 958 genes, including those with unknown functions with size ranging from 100 to 3,000 bp, were affected by exogenous cFP by at least 3-fold. This number of genes corresponds to 47.5% of the RefSeq protein-coding genes in the genome of *V. vulnificus* MO6-24/O. A total of 356 genes were positively regulated and the rest of genes were negatively regulated by cFP (Supplementary data in Tables S1 and S2). These results showed that cFP affects the regulation of a vast number of genes in the *V. vulnificus* genome.

Genes Up-Regulated by cFP

Thirty-one genes were up-regulated by cFP with an RPKM value higher than 100, excluding those annotated as "hypothetical protein" and these are summarized in Table 2. Among these, 17 genes belong to the group of inorganic ion transport and metabolism among the COG categories (Table 2). Of these genes, 15 genes are related with iron, specifically heme utilization, iron acquisition, and the regulation of these genes. It is noteworthy that two groups of genes (VVM06_01206~01212 and VVM06_04197~04211), which were closely mapped in the chromosome, were highly induced by cFP. The former gene sets are responsible for

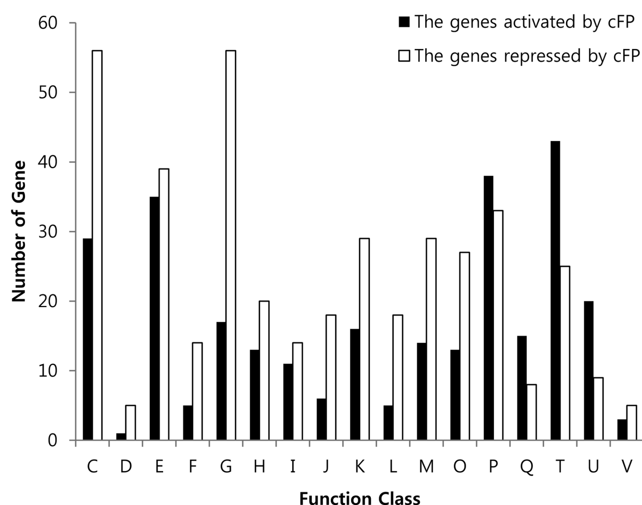

Fig. 1. The COG grouping of putative proteins encoded by genes modulated by cFP.

Each bar represents the number of genes belonging to the different functional categories. All proteins were assigned to the COG database and classified functionally into 17 molecular families. C: Energy production and conversion; D: Cell cycle control, cell division, and chromosome partitioning; E: Amino acid transport and metabolism; F: Nucleotide transport and metabolism; G: Carbohydrate transport and metabolism; H: Coenzyme transport and metabolism; I: Lipid transport and metabolism; J: Translation, ribosomal structure, and biogenesis; K: Transcription; L: Replication, recombination, and repair; M: Cell wall/membrane/envelope biogenesis; O: Post-translational modification, protein turnover, and chaperones; P: Inorganic ion transport and metabolism; Q: Secondary metabolites biosynthesis, transport, and catabolism; T: Signal transduction mechanisms; U: Intracellular trafficking, secretion, and vesicular transport; and V: Defense mechanism.

the assembly of pilus, whereas the latter are responsible for vulnibactin biosynthesis. Flp pilus mediates adhesion to surfaces and is essential for colonization and pathogenesis [42]. This type of pilus has not yet been studied in *V. vulnificus*. It is possible that, induced by cFP, this type of pilus contributes to the pathogenicity of *V. vulnificus*.

Vulnibactin is a siderophore responsible for the uptake of extracellular iron in *V. vulnificus* [44]. This protein is important for the virulence of *V. vulnificus*. We recently showed that the expression of vulnibactin is coordinately regulated by iron and the quorum-sensing pathway [48]. In an iron-rich condition, genes for vulnibactin are repressed by the Fur-iron complex. However, in an iron-depleted condition, those genes are induced only in low cell density. It is possible that cFP may exert a regulatory effect on vulnibactin expression *via* Fur-dependent or quorum-sensing regulatory pathways. Alternatively, there may be another regulatory pathway associated with the cFP-dependent regulation of these genes. The biological significance of the induction of iron-related genes by cFP has not yet been elucidated. If cFP plays a role as a quorum-sensing signal, then it supports the expression of iron-related genes at a high cell density where intracellular iron is scarce in the host environment. Future studies on cFP function need to focus on its relationship with iron metabolism and related regulatory mechanisms.

Genes Down-Regulated by cFP

In contrast to genes up-regulated by cFP, many of the genes down-regulated by cFP are for glycolysis, anaerobic energy metabolism, and fermentation (Table 3). The expression of some of these genes (alcohol dehydrogenase, pyruvate formate lyase) promotes biofilm formation in bacteria [15, 19]. The expression of the gene encoding tryptophanase is also significantly repressed by cFP. Tryptophanase is an enzyme that hydrolyzes tryptophan into pyruvate, ammonia, and indole. Indole plays various physiological roles in bacteria [21]. It is noteworthy that biofilm formation is among those roles. In *Escherichia coli*, indole controls biofilm formation by repressing motility and inducing the sensor protein for a quorum-sensing signal [21, 28]. These results suggest that cFP may inhibit biofilm formation by repressing the genes associated with biofilm.

Two groups of genes, each of which is closely mapped in the chromosome, are highly repressed by cFP; one group comprises the genes for nitrate reductase (VVM06_01962 ~01967), and the other forms an operon responsible for sialic acid biosynthesis (VVM06_04101~04107). Down-regulation of the former genes may allow cells to adapt to an oxygen limitation in the host, providing favorable circumstances for pathogens to survive in the host intestine [49]. The latter genes are associated with the acquisition, transport, and biosynthesis of sialic acid from the host's intestinal environment in *V. vulnificus* [1, 31, 23]. Sialic acid, which is rich in the epithelial surfaces of the intestine, is a good carbon and energy source for infecting pathogenic bacteria; hence, sialic acid is important for the pathogenesis of enteropathogens, including *V. vulnificus* [25]. In accordance with this study, these gene sets are only present in clinical isolates, and not in environmental isolates [34]. Recently, it was shown that the regulator NanR represses the expression of these genes in carbon-rich conditions [23]. cFP may play a role in the fine control of these genes to facilitate the survival and pathogenicity of the pathogen in sialic-acid-rich conditions in the host intestine, in accordance with the cell density of the pathogen. Taken together, these results

Table 2. List of genes of *V. vulnificus* MO6-24/O up-regulated by cFP.

GenBank Accession No.[a]	Strand	Product	RPKM[b]
VVMO6_00202	-	Manganese superoxide dismutase	242.40
VVMO6_00558	+	Iron ABC transporter substrate-binding protein	139.97
VVMO6_00638	+	Flp pilus assembly protein	616.63
VVMO6_00640	+	ABC transporter permease	151.76
VVMO6_01206	+	Flp pilus assembly protein CpaB	276.86
VVMO6_01207	+	Flp pilus assembly protein, secretin CpaC	643.90
VVMO6_01209	+	Pilus assembly protein CpaE-like protein	140.09
VVMO6_01210	+	Flp pilus assembly protein TadA	105.41
VVMO6_01212	+	Flp pilus assembly protein TadC	192.55
VVMO6_01834	+	Manganese transporter, 11 TMS	241.61
VVMO6_01907	-	Iron-regulated protein A	178.19
VVMO6_02984	-	Peptide ABC transporter ATP-binding protein	312.65
VVMO6_03382	+	Radical SAM protein	352.28
VVMO6_03384	+	Pyridoxamine 5'-phosphate oxidase-related heme iron utilization protein	122.15
VVMO6_03768	-	TonB-dependent heme and hemoglobin receptor HutA	277.16
VVMO6_04162	+	ABC transporter ATP-binding protein	386.10
VVMO6_04197	-	Phosphopantetheinyl transferase component of siderophore synthetase	133.97
VVMO6_04198	-	Non-ribosomal peptide synthetase modules siderophore biosynthesis	271.29
VVMO6_04199	-	Non-ribosomal peptide synthetase modules siderophore biosynthesis	882.65
VVMO6_04201	-	2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase	171.99
VVMO6_04202	+	Isochorismate synthase of siderophore biosynthesis	1,059.68
VVMO6_04203	+	2,3-Dihydroxybenzoate-AMP ligase	162.44
VVMO6_04205	-	Vulnibactin utilization protein VuuB	285.89
VVMO6_04206	-	Isochorismatase of siderophore biosynthesis	483.96
VVMO6_04207	-	Isochorismate pyruvate-lyase of siderophore biosynthesis	1133.43
VVMO6_04208	-	2,3-Dihydroxybenzoate-AMP ligase	370.09
VVMO6_04209	+	Aryl carrier domain	355.89
VVMO6_04210	+	Catechol siderophore ABC transporter substrate-binding protein	383.14
VVMO6_04211	-	Ferric vulnibactin receptor VuuA	250.67
VVMO6_04404	+	Ferric aerobactin ABC transporterATPase	112.27
VVMO6_04451	+	Isochorismatase	1,242.94

[a]Genes with RPKM values over 100 except for those annotated “hypothetical protein” are listed in the order of their GenBank accession numbers. Shadowed genes denote those closely mapped in the chromosome.

[b]Fold calculated as quotients of RPKM values (cFP-treated / cFP-untreated).

suggest that cFP controls the expression of genes crucial for the adaptation and pathogenicity of *V. vulnificus* in host environments.

Regulatory Genes Whose Expression Was Affected by cFP

Numerous genes possibly involved in the regulatory functions are modulated by cFP. Among these, several genes putatively encoding diguanylate cyclases that synthesize c-di-GMP have been identified (Table 4). Noticeably, one putative diguanylate cyclase (VVMO6_01151) is the most highly repressed. Diguanylate cyclase contributes to biofilm formation, as previously reported [10, 24]. As mentioned above, numerous genes possibly involved in biofilm formation are down-regulated by cFP. It is possible that the diguanylate cyclase may be related with the down-regulation of those genes. However, 10 other putative diguanylate cyclases are positively regulated by cFP, with RPKM values above 3. In the *V. vulnificus* genome, there exist more than

Table 3. List of genes of *V. vulnificus* MO6-24/O highly repressed by cFP.

GenBank Accession No.[a]	Strand	Product	RPKM[b]
VVMO6_00609	-	Protease	-33.90
VVMO6_00610	-	Protease	-64.60
VVMO6_00623	+	Thymidine phosphorylase	-33.59
VVMO6_01148	+	Transporter	-132.63
VVMO6_01149	+	Sensor histidine kinase	-44.70
VVMO6_01151	-	Diguanylate cyclase	-61.28
VVMO6_01494	+	Formate dehydrogenase subunit or accessory protein	-53.62
VVMO6_01837	-	Membrane protein	-46.26
VVMO6_01961	-	Cytochrome *c*-type heme lyase subunit NrfF nitrite reductase complex assembly	-36.08
VVMO6_01962	-	Thiol:disulfide oxidoreductase nitrite reductase complex assembly	-108.78
VVMO6_01963	-	Cytochrome *c*-type heme lyase subunit NrfE nitrite reductase complex assembly	-123.88
VVMO6_01964	-	NrfD protein	-151.68
VVMO6_01965	-	NrfC protein	-139.84
VVMO6_01966	-	Cytochrome *c*-type protein NrfB	-222.92
VVMO6_01967	+	Cytochrome *c*552	-860.72
VVMO6_02539	+	Pyruvate formate-lyase	-31.56
VVMO6_02821	-	DNA repair protein RadC	-34.09
VVMO6_03042	+	Ornithine decarboxylase	-34.36
VVMO6_03043	+	Putrescine/proton, putrescine/ornithine transporter, PotE	-42.13
VVMO6_03150	-	PTS system fructose-specific IIA IIB, IIC components	-68.94
VVMO6_03161	+	Anaerobic dehydrogenase	-52.09
VVMO6_03235	+	ABC transporter periplasmic spermidine putrescine-binding protein PotD	-34.93
VVMO6_03258	+	2-Amino-3-ketobutyrate coenzyme A ligase	-77.33
VVMO6_03259	+	L-Threonine 3-dehydrogenase	-34.43
VVMO6_03472	-	Alcohol dehydrogenase	-1,604.67
VVMO6_03498	+	Membrane-associated phospholipid phosphatase	-31.57
VVMO6_03729	-	Formate efflux transporter	-105.90
VVMO6_03816	-	Ribonucleotide reductase of class III (anaerobic) large subunit	-34.16
VVMO6_03929	+	Cobalt-zinc-cadmium resistance protein	-41.44
VVMO6_03996	-	Tripeptide aminopeptidase	-39.97
VVMO6_04101	-	Sialic-acid-induced transmembrane protein YjhT	-66.73
VVMO6_04103	-	*N*-Acetylneuraminate lyase	-52.31
VVMO6_04104	-	TRAP-type transporter large permease	-30.22
VVMO6_04105	-	TRAP-type transporter small permease	-102.65
VVMO6_04106	-	TRAP-type transporter periplasmic protein	-85.25
VVMO6_04107	+	*N*-Acetylmannosamine-6-phosphate 2-epimerase	-40.22
VVMO6_04175	+	Membrane protein	-30.79
VVMO6_04222	+	Tryptophanase	-439.05
VVMO6_04223	+	Tryptophan-specific transport protein	-52.09
VVMO6_04284	-	Acetylornithinedeacetylase	-173.53
VVMO6_04285	-	Membrane protein	-779.06
VVMO6_04524	-	Acetyltransferase	-39.07
VVMO6_t00004	+	tRNA-Arg	-82.82
VVMO6_t00007	+	tRNA-His	-37.29
VVMO6_t00046	-	tRNA-Phe	-74.58
VVMO6_t00107	-	tRNA-Cys	-57.45

[a]Genes with RPKM value below 30 except for those annotated "hypothetical protein" are listed in the order of their GenBank accession numbers. Shadowed genes denote those closely mapped in the chromosome.

[b]Fold calculated as quotients of RPKM values (cFP-treated *vs*. cFP-untreated).

Table 4. Partial list of genes associated with regulation and signal transduction, which are highly modulated by cFP.

Category	GenBank Accession No.	Product	RPKM[a]
Induced genes	VVMO6_00189	Signal-transduction protein	29.59
	VVMO6_03522	RsbR, positive regulator of sigma-B	23.28
	VVMO6_03243	Chemotactic transducer-related protein	20.03
	VVMO6_01190	Diguanylate cyclase	12.30
	VVMO6_02440	Iron-sulfur cluster regulator IscR	12.06
	VVMO6_01137	Sensor/response regulator hybrid	11.77
	VVMO6_00074	Response regulator	10.70
	VVMO6_04559	Anti-anti-sigma regulatory factor	9.57
	VVMO6_03524	Anti-sigma B factor RsbT	9.23
	VVMO6_04549	Anti-anti-sigma regulatory factor	9.18
	VVMO6_03526	Two-component system sensor protein	8.75
	VVMO6_03934	Signal transduction histidine kinase	8.66
	VVMO6_04373	CheY-like receiver	8.63
	VVMO6_03523	RsbS, negative regulator of sigma-B	8.59
	VVMO6_03285	Sensory box sensor histidine kinase/response regulator VieS	8.08
	VVMO6_03030	Diguanylate cyclase	7.95
	VVMO6_00964	Serine protein kinase (PrkA protein) P-loop containing	7.73
	VVMO6_04132	CheY-like receiver	7.18
	VVMO6_02989	Signal transduction histidine kinase	6.95
	VVMO6_03757	Diguanylate cyclase	6.78
	VVMO6_04323	Signal transduction histidine kinase	6.47
	VVMO6_01623	Sigma-54 dependent transcriptional regulator SypG	6.27
	VVMO6_00616	c-di-GMP phosphodiesterase A-related protein	6.07
	VVMO6_03638	Diguanylate cyclase	5.22
	VVMO6_02498	Autolysin sensor kinase	4.37
	VVMO6_00961	Diguanylate cyclase	4.22
	VVMO6_02521	VpsR family transcriptional regulator	4.22
	VVMO6_01119	Signal-transduction protein containing cAMP-binding and CBS domains	3.99
	VVMO6_03363	Sensor histidine kinase	3.90
	VVMO6_00881	Response regulator	3.87
	VVMO6_03073	HDIG domain-containing protein	3.80
	VVMO6_01515	Fumarate and nitrate reduction regulatory protein	3.73
	VVMO6_03284	Response regulator VieA	3.59
	VVMO6_04047	Diguanylate cyclase	3.57
	VVMO6_03411	Diguanylate cyclase	3.54
	VVMO6_01622	Signal transduction histidine kinase SypF	3.30
	VVMO6_02645	LysR-family transcriptional regulator (LeuO homolog)	3.26
	VVMO6_02887	Diguanylate cyclase	3.10
	VVMO6_03199	Diguanylate cyclase (GGDEF)/phosphodiesterase (EAL) with PAS domain	3.07
	VVMO6_01800	Diguanylate cyclase	3.06
Repressed genes	VVMO6_01151	Diguanylate cyclase	-61.28
	VVMO6_01809	Anaerobic nitric oxide reductase transcription regulator NorR	-19.46

Table 4. Continued.

Category	GenBank Accession No.	Product	RPKM[a]
	VVMO6_04512	LuxR family transcripitonal regulator	-12.51
	VVMO6_02561	LuxR family transcripitonal regulator	-11.80
	VVMO6_03151	AraC family transcripitonal regulator	-8.62
	VVMO6_03132	LacI family transcriptional regulator	-7.87
	VVMO6_01483	Sigma-54 dependent response regulator	-3.35
	VVMO6_03642	AraC family transcripitonal regulator	-3.32
	VVMO6_04012	SorC family transcripitonal regulator	-3.32
	VVMO6_02228	Transmembrane regulatory protein ToxS	-3.20
	VVMO6_02851	Two-component system response regulator OmpR	-3.05
	VVMO6_02227	Transcriptional activator ToxR	-2.66
	VVMO6_00513	Autoinducer-2 production protein LuxS	-2.65

[a]Fold calculated as quotients of RPKM values (cFP-treated/cFP-untreated); +, fold induction level; -, fold repression level. For induced genes, genes with RPKM values over 3.0 are shown, whereas for repressed genes, genes with RPKM values below -2.6 are given in this table.

40 genes possibly encoding diguanylate cyclases (data not shown). However, none of these genes' roles have been systematically studied. Among these genes, VVMO6_01151 would be the best candidate for future functional study in connection with cFP and biofilm.

The iron-sulfur cluster regulator (IscR) is positively regulated by cFP. It is responsible for the regulation of genes for the [Fe-S] protein [16], and has an unusual characteristic in that both apo- and iron-sulfur-bound holoforms regulate transcription with different DNA-binding specificities and affinities [39]. In *V. vulnificus*, this regulator appears to form an operon with six structural genes, specifically VVMO6_02434~02439 (encoding cysteine desulfurase IscS, iron-sulfur cluster assembly scaffold protein IscU, iron-binding protein IscA for iron-sulfur cluster assembly, chaperone protein HscB, and chaperone protein HscA, respectively). All of these genes are positively regulated by cFP (Supplementary data S1). It is likely that the cFP induction of these genes is mediated by IscR. As mentioned above, cFP induces numerous genes associated with iron (Table 2). In this context, the regulation of IscR by cFP would provide invaluable information on the relationship between iron and its metabolism.

Several regulators related with sigma-B and anti- and anti-anti-sigma functions are also positively regulated by cFP. The biological meaning of this result remains to be elucidated. It is noteworthy that a LeuO homolog (VVMO6_02645) is also positively regulated by cFP. We recently found that this regulator, once induced by cFP, induces the expression of a series of genes with pathogenic roles (unpublished results). We assume that this LeuO homolog is a major regulator for a cFP-dependent signal pathway(s), and we are currently studying the biological roles of this regulator.

cFP also affects the expression of *vpsR*, which encodes the positive transcriptional regulator required for the expression of *Vibrio* polysaccharides (*vps*) genes [2]. The action of VpsR is epistatic to that of the quorum-sensing master regulator HapR in *V. cholerae*. VpsR affects pathogenic phenotypes of *V. cholerae*, such as biofilm formation. cFP modulates the expression of sensor kinase *vieS* and response regulator *vieA*, which contributes to motility and biofilm formation by adjusting the cyclic diguanylate level [32]. The VieSAB three-component signal transduction system is also closely involved in cholera toxin production [46]. These findings imply that a complex regulatory interplay among cFP, VieS, VpsR, and other functions control the genes responsible for biofilm and virulence factor production in *V. vulnificus*.

The expression of ToxRS is also regulated by cFP. These membrane proteins have been well studied as sensor proteins responsible for the expression of virulence factors in *V. cholerae* [7]. In *V. vulnificus*, a mutation in *toxR* decreases the production of the virulence factor *vvhA* encoding a hemolysin [29]. These membrane proteins appear to be responsible for a cFP-mediated signal transduction [37]. The induction of the expression of *ompU* by cFP is dependent on ToxRS. In a mutant lacking in ToxR function, cFP-dependent induction of *ompU* is abolished. The relationships among cFP, *toxRS*, *ompU*, and *vvhA* still need to be investigated. The gene *luxS*, responsible for the biosynthesis of quorum-sensing autoinducer-2 signal molecule (AI-2), is

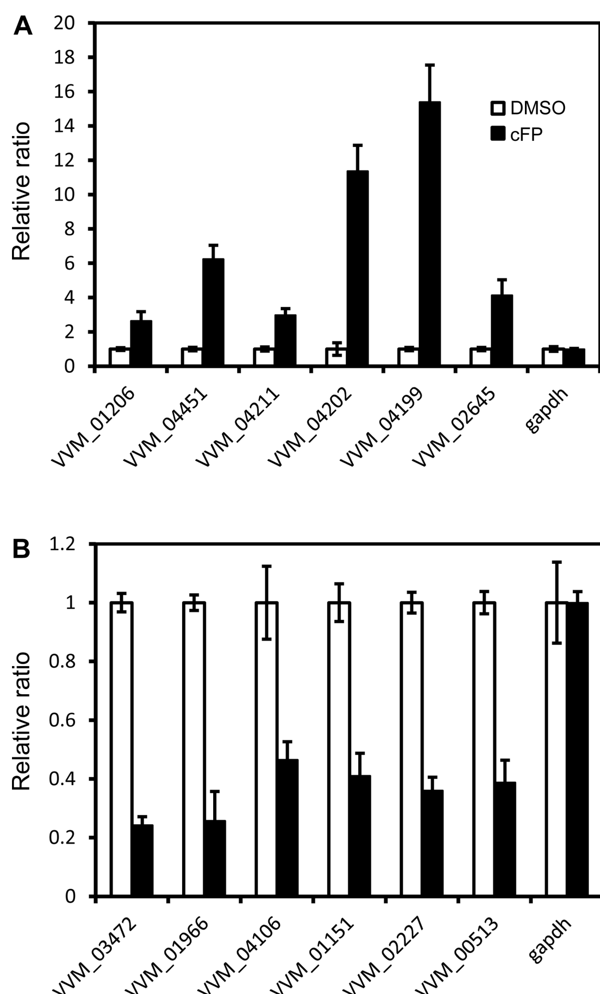

Fig. 2. Comparisons of expression levels of representative genes from *V. vulnificus* MO6/24-O grown in the presence or absence of exogenous cFP.

Transcriptional levels of selected genes from Tables 2 to 4 in cells grown in the absence or presence of cFP were compared by quantitative RT-PCR. (**A**) Genes positively regulated by cFP. (**B**) Genes negatively regulated by cFP. RNA levels were quantified using the comparative threshold cycle ($\Delta\Delta C_T$) method, and RNA fold change was normalized to the value for the expression level of the gene encoding NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase as a control. The data are the average values from three independent samples; error bars denote standard deviation.

also repressed by cFP. In well-studied *V. vulnificus* strains, such as YJ016 and MO6-24/O, whose genome sequences have been completely determined [6, 38], the effort to identify a homoserine lactone signal molecule (AI-1) compound or a gene responsible for AI-1 biosynthesis has been unsuccessful. Therefore, AI-2 appears to be a major quorum-sensing signal in this pathogen. If cFP represses the expression of *luxS*, it

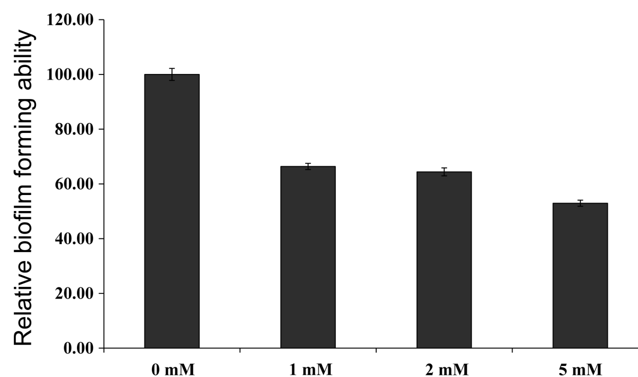

Fig. 3. Effect of cFP on biofilm formation of *V. vulnificus.*

The biofilm formation was quantitatively measured as described in Materials and Methods. The data are the average values from three independent samples; error bars denote standard deviation.

suggests that cFP antagonizes the AI-2-dependent quorum-sensing signal circuit. The production of cFP in *Vibrio* spp. is also enhanced at the early stationary phase, as is AI-2 production [3, 37]. It is possible that cFP affects not only its cognate signaling pathway, but also the AI-2-dependent quorum-sensing pathway, which interplay with each other to modulate sets of genes in accordance with complex environmental conditions, including cell density.

Confirmation Using qRT-PCR

To validate the reliability of the RNA-Seq results, 12 genes (six up-regulated by cFP and six down-regulated by cFP) were subjected to quantitative RT-PCR (qRT-PCR). These selected genes were chosen because they are of special interest in our research and are currently under investigation for their roles in connection with cFP. Even though the values of the relative ratios of transcription levels between the cFP-treated group and non-cFP-treated group are overall much smaller in qRT-PCR than those observed in RNA-seq, the results of qRT-PCR coincide well with those of RNA-Seq (Fig. 2). This suggests that the RNA-seq results are biologically significant.

cFP Inhibits Biofilm Formation in *V. vulnificus*

As mentioned above, numerous genes related with biofilm formation are modulated by cFP. Recently, several DKPs have been shown to be capable of removing the biofilm architecture [13]. However, the effect of cFP on biofilm formation has not been studied. We assessed the effect of cFP on the biofilm formation of *V. vulnificus* using the crystal violet staining method. As shown in Fig. 3, the formation of biofilm of *V. vulnificus* was reduced when

treated with cFP. Even 1 mM of cFP reduced biofilm formation more than 30%, and 5 mM of cFP was responsible for up to a 55% reduction. These results showed that cFP inhibits the biofilm formation of *V. vulnificus*. The molecular genetic basis of the effect of cFP on biofilm formation still needs to be elucidated.

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