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Identification of Virulence Factors in *Vibrio vulnificus* by Comparative Transcriptomic Analyses between Clinical and Environmental Isolates Using cDNA Microarray

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We compared the gene expression among four clinical and five environmental *V. vulnificus* isolates, using a cDNA microarray containing 131 genes possibly associated with pathogenicity, transport, signal transduction, and gene regulations in the pathogen. cDNAs from total RNAs of these isolates were hybridized into the cDNA microarray using the cDNA of the wild-type strain MO6-24/O as a reference. We focused on selecting differentially expressed (DE) genes between clinical and environmental isolates using a modified t-statistic. We could detect two statistically significant DE genes between virulent isolates and less-virulent isolates with a marginal statistical significance (p-value of 0.008). These were genes putatively encoding pilin and adenlyate cylase. Real time-PCR confirmed that these two selected genes transcribed in significantly higher levels in virulent isolates than in less-virulent isolates. Mutants with lesions in the gene encoding pilin showed significantly higher LD_{50} values than that of wild type.

Keywords: *Vibrio vulnificus*, virulence factors, cDNA microarray, pilin

Vibrio vulnificus is a motile, curved, rod-shaped with a single polar flagellum, Gram-negative marine bacterium, causing infectious diseases in humans, especially in people who are immunocompromised or have an underlying condition such as hemochromatosis, liver cirrhosis, or alcoholism [1, 12]. Roles of several factors such as cytolysin-hemolysin, sideropores, and metalloproteases as virulence determinants for *V. vulnificus* have been reported [8, 10, 13, 26]. In addition, there are some cases of a correlation between pathogenicity and characteristics such as the presence of a polysaccharide capsule, the resistance to the bactericidal components and phagocytosis of sera, and the ability to acquire iron from transferrin in *V. vulnificus* [7, 9, 24, 29]. There have also been many attempts to distinguish virulent and avirulent strains isolated from a variety of sources by comparison of phenotypic characteristics in order to correlate virulence with expression of a particular factor [19, 22]. Studies accumulated to date indicate that the disease elicited by *V. vulnificus* is onset and developed not by a single or a few factors but rather by complex activities of numerous factors including those unidentified so far. Therefore, the screening and identification of virulence factors in the pathogen is important for understanding the pathogenic mechanism of the disease elicited by *V. vulnificus*.

In this study, employing a cDNA microarray, we compared the expression of genes possibly associated with pathogenicity among *V. vulnificus* isolates that have different potentials to elicit disease. Microarray technology represents a powerful high-throughput tool that provides useful large-scale quantitative information about important cellular pathways and processes [2, 17]. Co-hybridization assays for differential microarray can compare the relative gene expression between paired queries and reference samples, allowing the identification of informative patterns of gene expression across multiple experiments. This study showed that employing a microarray for the measurement of the differential expression of genes between virulent and less-virulent isolates of pathogen, and between sources of isolates as well, provided valuable information to identify virulence factors of pathogenic microbes.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The *V. vulnificus* isolates employed in this study are listed in Table 1. Cells were grown in Luria–Bertani (LB) medium at 30°C. Some

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Table 1. *Vibrio vulnificus* isolates used in this study.

Strains	Isolation source/ Serum resistance[a]	Genotype[b]	LD_{50} value
MO6 24/O	Clinical/ND	C	3.48×10^5
LSU1866	Clinical/3	C	1.97×10^6
C71840	Clinical/3	C	6.86×10^4
YJ016	Clinical/3	C	1.29×10^6
SPRC10143	Clinical/3	C	5.06×10^5
SPRC10215	Oyster/2	E	5.53×10^5
ENV1	Oyster/0	E	7.84×10^6
JY1701	Oyster/0	E	2.06×10^7
SS108A3A	Seawater/ND	C	2.68×10^7
3001C1	Seawater/1	E	1.14×10^7

[a]Resistance to human serum; 0, not resistant; 1, weak; 2, moderate; 3, strong resistance [16].
[b]Genotype as determined by PCR analyses; C, correlates with clinical origin; E, correlates with environmental origin [16].
ND = Not determined.

characteristics of these isolates have been previously determined [16], and are summarized in Table 1. All media and antibiotics were purchased from Difco (Detroit, MI, USA) and Sigma-Aldrich (St. Louis, MO, USA).

Measurement of the LD_{50} (Lethal Dose 50) Value

To measure LD_{50} values, tested cells cultured overnight in LB medium were diluted to OD_{600}=0.1 and grown until the OD_{600} value reached approximately 1.0 (exponential growth phase). The cells were harvested by centrifugation at 7,000 rpm for 2 min. Cells were suspended in phosphate-buffered saline (PBS) to approximately 10^8 CFU/ml and diluted to the concentration to be used. Four-weeks-old female ICR mice (Samtako Bio, Korea) were injected hypodermically in their back with 0.1 ml of serially diluted bacteria suspensions. The injected mice were observed for 48 h and the LD_{50} value was quantified.

Isolation of *V. vulnificus* Genomic DNA

Preparation of genomic DNA was carried out as previously reported with some modifications [5]. In brief, *V. vulnificus* cells were grown in LB medium for overnight, and cells at the stationary phase were harvested by centrifugation. These cells were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with 5% sarkosyl and protease K (5 mg/ml) and incubated at 37°C until complete lysis of cells was obtained. The lysate was extracted three times with 3% NaCl–saturated phenol and subsequently with chloroform:isoamyl alcohol (24:1) and diethyl ether. Then, total DNA of *V. vulnificus* was precipitated by adding 95% ethanol and dissolved in TE buffer (pH 8.0) containing RNase A (40 μg/ml).

Preparation of cDNA Microarray of *V. vulnificus* Genes

A total of 131 genes known to be associated with virulence, quorum sensing, metabolite transport, gene regulation, and signal transduction in *Vibrio* species were contained in the cDNA microarray (Table 2). DNA fragments of all genes were prepared by PCR amplification using the genomic DNA of *V. vulnificus* MO6 24/O as a template and 18-bases oligonucleotides as primers. Each PCR primer set was

Table 2. List of genes printed in the cDNA microarray.

Gene ID[a]	Size (bp)	Putative product
VV1_0019	293	Bacterial nucleoid DNA-binding protein
VV1_0023	626	ATP-dependent Clp protease
VV1_0041	3,017	RTX toxin
VV1_0126	668	Response regulator VC0826 (TcpP)
VV1_0175	449	Fe^{2+}/Zn^{2+} uptake regulation proteins (Fur)
VV1_0189	1,904	HtpG
VV1_0212	509	Phosphotransferase system IIA component
VV1_0213	1,127	Flagellin FlaE
VV1_0214	1,133	Flagellin FlaD
VV1_0215	1,124	Flagellin FlaC
VV1_0218	920	Peptidoglycan hydrolase
VV1_0219	1,049	FlgI
VV1_0221	788	FlgG
VV1_0222	749	FlgF
VV1_0223	1,304	FlgE
VV1_0224	707	FlgD
VV1_0225	416	FlgC
VV1_0226	395	FlgB
VV1_0306	924	Transcriptional regulator, LysR family
VV1_0310	1,448	Thiamine biosynthesis protein, ThiI
VV1_0353	386	PilE
VV1_0357	1,910	DnaK
VV1_0365	551	GrpE
VV1_0447	1,160	Serine protease
VV1_0464	2,102	Polyphosphate kinase
VV1_0465	1,430	Exopolyphosphatase
VV1_0493	986	Membrane protein
VV1_0525	740	Sigma-54-dependent transcriptional regulator
VV1_0608	824	Cyclic AMP phosphodiesterase
VV1_0636	1,757	Putative hemolysin
VV1_0852	2,120	Guanosine-pyrophosphohydrolase
VV1_0858	719	OmpR
VV1_0867	761	EpsN
VV1_0868	473	EpsM
VV1_0870	1,022	EpsK
VV1_0871	701	EpsJ
VV1_0875	1,217	EpsF
VV1_0877	2,021	EpsD
VV1_1028	1,529	Threonine dehydratase
VV1_1043	644	Hemolysin
VV1_1123	2,534	Adenylate cyclase
VV1_1132	2,489	FOG: EAL domain
VV1_1215	485	Unknown
VV1_1224	272	Bacterial nucleoid DNA-binding protein
VV1_1252	608	Superoxide dismutase
VV1_1362	317	Transcriptional regulator of *met* regulon
VV1_1364	1,166	Cystathionine gamma-lyase
VV1_1365	2,411	Aspartate kinase II/Homoserine dehydrogenase

Table 2. Continued.

Gene ID[a]	Size (bp)	Putative product
VV1_1375	899	Transcriptional regulator
VV1_1377	1,007	Tfp pilus assembly protein
VV1_1378	590	PilN
VV1_1379	590	PilO
VV1_1380	512	PilP
VV1_1390	449	Ribosomal protein L9
VV1_1423	3,680	B12-dependent methionine synthase
VV1_1469	776	Beta-galactosidase/beta-glucuronidase
VV1_1573	2,786	Signal transduction histidine kinase
VV1_1575	1,700	Guanosine polyphosphate pyrophosphohydrolase
VV1_1588	992	DNA-directed RNA polymerase
VV1_1605	2,858	Predicted Zn-dependent peptidases
VV1_1608	518	LuxS
VV1_1609	1,177	CorB
VV1_1623	869	Type 4 prepilin leader peptide processing enzyme
VV1_1624	1,226	Type IV pilin biogenesis protein
VV1_1625	1,688	PilB
VV1_1626	446	Type IV pilin PilA
VV1_1634	554	SmcR
VV1_1686	1,022	OmpU
VV1_1759	1,151	Chromate transport protein ChrA
VV1_1777	332	Putative tRNA synthetase
VV1_1855	737	Pseudouridylate synthase
VV1_1937	800	Polar flagellar assembly protein
VV1_1959	494	Chemotaxis signal transduction protein
VV1_1966	1,982	Cytochrome c-type biogenesis protein CcmF
VV1_1967	515	Thiol-disulfide interchange protein CcmG
VV1_2059	1,337	Cadaverine/lysine antiporter
VV1_2060	2,108	Lysine decarboxylase
VV1_2066	1,967	Methyl-accepting chemotaxis protein
VV1_2219	2,336	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
VV1_2237	1,058	Extracellular solute-binding protein
VV1_2241	638	Transcriptional regulator
VV1_2255	1,625	Methyl-accepting chemotaxis protein
VV1_2324	359	Conserved hypothetical protein
VV1_2604	1,133	Putrescine transport system, ATPase component
VV1_2703	1,028	ABC-type amino acid transport, signal transduction systems, periplasmic component/domain
VV1_2755	2,171	Catalase
VV1_2764	761	ABC-type amino acid transport, periplasmic component/domain
VV1_2768	1,073	4-Hydroxyphenylpyruvate dioxygenase (Hemolysin VllY)
VV1_2771	1,784	5'-Nucleotidase/2',3'-cyclic phosphodiesterase

Table 2. Continued.

Gene ID[a]	Size (bp)	Putative product
VV1_2805	1,370	Di- and tricarboxylate transporter
VV1_2908	1,310	Cysteinyl-tRNA synthetase
VV1_2923	407	DNA-binding protein H-NS
VV1_2942	1,157	CqsA homolog
VV1_3054	644	Response regulator
VV1_3114	485	V10 pilin
VV1_3134	419	Periplasmic serine proteases
VV1_3143	1,688	Conserved hypothetical protein
VV1_3145	1,751	Predicted metal-dependent hydrolase
VV1_3165	1,874	1-Acyl-sn-glycerol-3-phosphate acytransferase
VV2_0032	1,787	Zinc metalloprotease
VV2_0077	3,218	FOG: CheY-like receiver
VV2_0180	452	*S*-Adenosylhomocysteine hydrolase
VV2_0191	1,031	Secreted trypsin-like serine protease
VV2_0361	404	Biopolymer transport protein
VV2_0403	455	Membrane-fusion protein
VV2_0404	1,406	Cytolysin VCA0219 (HlyA)
VV2_0552	359	Transaldolase
VV2_0580	977	Outer membrane receptor protein
VV2_0713	551	Outer membrane receptor protein
VV2_0741	1,145	Acetyl-CoA acetyltransferase
VV2_0753	1,100	ABC-type transport system, periplasmic component
VV2_0781	629	Unknown
VV2_0831	4,562	Nonribosomal peptide synthetase modules
VV2_0833	1,070	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase
VV2_0838	887	Aryl carrier domain
VV2_1007	1,271	Outer membrane protein
VV2_1118	3,131	Delta 1-pyrroline-5-carboxylate dehydrogenase
VV2_1143	1,058	Outer membrane protein
VV2_1146	2,423	Conserved hypothetical protein
VV2_1169	359	FOG: CheY-like receiver
VV2_1250	2,453	Glucan phosphorylase
VV2_1270	2,738	Predicted Zn-dependent peptidase
VV2_1322	656	Transcriptional regulator (AcrR/TetR family)
VV2_1325	1,388	ABC-type transport system, ATPase component
VV2_1330	2,120	Alpha-galactosidase
VV2_1337	2,165	Outer membrane receptor protein
VV2_1348	1,217	Phosphomannose isomerase
VV2_1467	2,879	Chemotactic transducer-related protein
VV2_1575	1,313	Membrane protein involved in the export of *O*-antigen and teichoic acid
VV2_1607	461	Transcriptional regulator LuxT
VV2_1638	473	Signal transduction histidine kinase

[a]GenBank accession number denoted for *V. vulnificus* strain CMCP6.

designed for the amplification of approximately 700 base-pair fragments for each ORF (data not shown). If the size of a gene is shorter than 700 bp, the full length of the ORF was amplified. PCR was performed in a 100 μl total reaction volume, containing autoclaved deionized water, 60 pmol each of forward and reverse primers, 1× PCR buffer with $MgCl_2$ (Genenmed, Seoul, Korea), dNTP (7.5 mM each), 2.5 U of Top-pfu DNA Polymerase (CoreBioSystem, Seoul, Korea), and 100 ng of the genomic DNA template. PCR was executed under the following condition: an initial denaturation step at 95°C for 5 min, and 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 5 min. At the same condition, the 16S rRNA gene was employed as a positive control. The resulting PCR products were confirmed by electrophoresis in 0.8% agarose/TBE gels. Subsequently, the amplified products were purified with the PCR clean-up kit (CoreBioSystem, Seoul, Korea). Eluents were evaporated on Modulspin40 (BioTron, Seoul, Korea) and dissolved in 15 μl of spotting buffer (50% DMSO). Microarrays were spotted on Corning GAPS II coated slides (Corning, NY, USA) using an Affymetrix 427 arrayer (Affymetrix, Massachusetts, USA).

Preparation of cDNA

All experiments were performed following the same protocol with the same batch of microarrays. Total RNA was isolated from cells grown in LB medium at 37°C [14] by using TRI REAGENT (Molecular Research Center, Cincinnati, USA) according to the manufacturer's protocol and further purified using RNeasy mini kit columns (Qiagen, Valencia, USA). The purified RNA was precipitated with ethanol and dissolved in 20 μl of distilled deionized water. About 200 μg of the total RNA sample was used to prepare cDNA. The total pool of messenger RNA from each cell population is used to prepare fluorescent-labeled cDNA by reverse transcription in the presence of aminoallyl-labeled nucleotide precursors, and then cDNA coupling with Cy3 or Cy5.

Labeling of cDNA with Cy3 and Cy5

5-Aminoallyl-dUTP (Sigma, USA) at 200 μM was incorporated in the first strand by randomly primed reverse transcription reaction using 400U Superscript II (Stratagene, USA). *N*-Hydroxysuccinimide (NHS) ester (4.5 μl) of Cy3 or Cy5 dye (Amersham Pharmacia, Sweden) in DMSO was added for coupling. The labeled cDNAs were purified with the QIAquick PCR purification kit (Qiagen, CA, USA) and dried in a speed vacuum. The slide with microarray was placed in a conical tube with the prehybridization buffer (5× SSC, 0.1% SDS, 1% BSA) at 42°C for 45 min.

Hybridization, Washing, and Scanning

The labeled cDNAs were denatured at 95°C for 2–3 min, cooled at 20°C, and then combined and added to 15 μl of hybridization buffer (50% formamide, 10× SSC, 0.2% SDS). The mixture was applied to a microarray covered with a spaced cover glass L (Takara, Japan). Hybridization was carried out for 16–18 h at 42°C in a humidified hybridization chamber (Corning, CA, USA). The slides were washed in 1× SSC, 0.1% SDS at 42°C by agitation for 4 min, then 0.1×SSC, 0.1% SDS at 42°C for 4 min, and rinsed in 0.1×SSC for 4 min 3–5 times and dried by centrifugation at 500 rpm for 5 min. Microarray slides were scanned by using a GenePix 4000B dual-color confocal laser scanner (Axon Instruments, USA). For every spot, the resulting fluorescence intensity of each of the labels was measured and compared by using the GenePix Pro 5.0 software system (Axon Instruments, USA).

Design of cDNA Microarray

Each array had 131 bacterial genes duplicated 10 times on it using eight print-tips. For the hybridization of two samples, we employed the reference design in which the cDNA of *V. vulnificus* MO6-24/O was used as the common reference. Each cDNA sample of *V. vulnificus* isolate was hybridized together with the reference cDNA on the same array. Each bacterial strain has four technical replicates, where two were labeled forward and the other two labeled backward to minimize the dye bias. Therefore, the total number of arrays for the experiment is 36 (9 strains × 4 replicates/stain).

We have to note, here, that three levels of replications (*i.e.*, biological and technical replicates and duplications within an array), were imbedded in the design [2], where nine bacterial strains correspond to biological replicates, four arrays per strain represent technical replicates, and multiple spotting in an array is the duplication.

Statistical Analysis of cDNA Microarray Data

We filtered out poor quality spots on an array by excluding spots whose background intensities were lower than 1.3 times of the background intensities at either channel. The number "1.3" was determined by trial and error and was based on how one could efficiently eliminate the low intensity signals often represented by a "banana shape" [4]. The number of genes having at least one measurement out of 10 duplicate spots for 36 arrays ranged from 68 to 126 (mean=105.1, sd=15.50, median=104, first quartile=99, third quartile=119.2). We took a median of k duplicate spots (k=1, …, 10) for a gene, and this median represented the log intensity ratio $y_{grs} = \log_2\left(\frac{Cy5_{grs}}{Cy3_{grs}}\right)$, where the subscript "grs" corresponds to the g-th gene in the r-th replicate array for the s-th strain, g=1, … G_{rs}, r=1, …, 4, s=1, … 9.

We performed within print-tip group location-scale normalization using loess [28]. That is, we set the median of each print-tip group to be zero and the variance being equal for each print-tip group within an array. Then multiple slide scale normalization was followed. We used 0.65 for the cut point of no-missing proportion (NMP) to delete spots containing missing values for more than 35% of the total number of arrays. This filtering process yielded 102 genes with 36 arrays, which is denoted by a 102 × 36 matrix. Missing values in the 102 × 36 matrix were imputed by the k-nearest neighbor (KNN) method using an R package "impute" [23]. The imputed 102 × 36 matrix is now the data set for the statistical analysis.

Identifying a set of DE genes between VG and LVG can be formulated as a problem of multiple hypothesis testing as follows:

$$H_0^{(g)}: \mu_{VG}^{(g)} = \mu_{LVG}^{(g)} \; vs \; H_a^{(g)}: \mu_{VG}^{(g)} \neq \mu_{LVG}^{(g)}, \; g=1, \ldots, 102$$

or

$$H_0^{(g)}: \mu_{C}^{(g)} = \mu_{E}^{(g)} \; vs \; H_a^{(g)}: \mu_{C}^{(g)} \neq \mu_{E}^{(g)}, \; g=1, \ldots, 102,$$

where $\mu_{VG}^{(g)}$, $\mu_{LVG}^{(g)}$, $\mu_{C}^{(g)}$, and $\mu_{E}^{(g)}$ represent means of y_{grs} for $s \in VG$, $s \in LVG$, $s \in C$, and $s \in E$, respectively, for g-th gene, g=1, …, 102.

There are several approaches to multiple hypotheses testing in the context of the cDNA microarray experiment [3]. We used two R packages, "Limma" [18] and "Multtest" (http://www.bioconductor.org/packages/release/bioc/html/multtest.html), which output false discovery

Table 3. DNA nucleotide sequences of primers used for RT-PCR.

Gene ID	Directions	Nucleotide sequence of primers
VV1_1123	Forward	5'-TATCACCAGTTTAGCGGC-3'
	Reverse	5'-CTATACGTTGACCGCTTT-3'
VV1_1364	Forward	5'-GGTGTCGAAAGCTTGGTT-3'
	Reverse	5'-TTAACAGCTCTCCTTCGC-3'
VV1_1605	Forward	5'-ATAAAGCGCCGCTTAACG-3'
	Reverse	5'-CTATCGGATGATTTCAAG-3'
VV1_3114	Forward	5'-TCCCGATTGGTGGATCAT-3'
	Reverse	5'-TCAACATCCGTCTGTGAT-3'
16s rDNA	Forward	5'-TGGAGGTTGTGGCCTTGA-3'
	Reverse	5'-TAAGGAGGTGATCCAGCG-3'

rate (FDR) and family-wise error rate (FWER), respectively, as a means of controlling the type I error rate. We used the maxT procedure in the Multtest R package.

Comparative Analysis of the Gene Expression Using Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA from each tested *V. vulnificus* isolate (200 μg per strain) was prepared to synthesize cDNA. Primers for a real-time PCR were designed using ABI PRISM Primer Express software (PE Applied Biosystems, Foster, USA). The primer sets used in this study are shown in Table 3. The RT-PCR was carried out using a SYBR Green PCR master mix with Applied Biosystems' 7500 Real-Time PCR System (PE Applied Biosystems, Foster, USA). The PCR was carried out for 40 cycles as follows; hybridization for 2 min at 50°C, activation for 10 min at 95°C, denaturation for 15 s at 95°C, and annealing/extension for 1 min at 56°C. The comparative cycle threshold value (Ct) was measured for quantitative comparisons. For relative normalizing, the housekeeping gene encoding 16S rDNA was employed as an internal control. The amount of gene expression relative to that in the internal reference MO6 24/O was expressed by the fold difference ($2^{-\Delta\Delta}$CT) [30].

Construction of a Deletion in VV1_3114

We constructed a null mutant with a deletion in VV1_3114 putatively encoding a pilin. A DNA fragment spanning the 3' end of VV1_3115, which locates in the upstream of VV1_3114, and the 5' end of VV1_3114 was PCR-amplified using primers 3114F5 (5'-GGGCCC AATCGATGGGTACTTGT-3') and 3114B7 (5'-CTGCAGACTACG ATCAATTCTATTAG-3'). The resulting fragment F5B contains *Apa*I and *Pst*I sites at each of both ends. In the same manner, the fragment spanning the 3' end of VV1_3114 and a half part of VV1_3113, which locates in the downstream of VV1_3114, was amplified using primers 3114F8 (5'-CTGCAGGGGGGCGGACTTTGCCGTG-3') and 3114B6 (5'-AGATCTACTGGGCCATTGTGA-3'). The resulting fragment F8B6 contains *Pst*I and *Bgl*II sites at each of both ends. The F5B and F8B6 fragments were cloned into pGEMT-Easy (Promega) to construct pGEM-F5B and pGEM-F8B6, respectively. These constructs were digested with *Pst*I and ligated to make a concatamer. From this construct, the *Apa*I-*Bgl*II fragment, which has a deletion in VVI_3114, was cut and cloned into pBluescriptII (Strategene). The 1.2-kb Km^r-cassette from pUC4K [21] was cloned at the center of the *Apa*I-*Bgl*II fragment using the *Pst*I site. The resulting *Apa*I-*Bgl*II fragment containing the Km^r-cassette at the center was cloned into pDM4 [11], and the resulting plasmid was introduced into MO6-24/O. By selecting Km- and sucrose-resistant and Cm-sensitive transconjugants, a derivative of MO6-24/O, which has a Km-resistant cassette inserted in VV1-3114 by an allelic exchange, was isolated and named MO6-3114K. The construct was confirmed by PCR and Southern hybridization (data not shown).

RESULTS

LD_{50} Values and Grouping of Ten *V. vulnificus* Isolates

Isolation sources and serum resistance of ten *V. vulnificus* strains employed in this study were described in a previous report [16], and are summarized in Table 1. To assess the pathogenicity of ten *V. vulnificus* isolates, we measured the LD_{50} values toward mice. The result indicated that the group classified upon the basis of LD_{50} values of ten strains is well consistent with the group categorized by the sources of those strains, with one exception of SPRC10215 (Table 1). Generally, if not all, clinical isolates are more virulent than environmental isolates. We obtained 3.21 (p-

Table 4. The list of genes expressing in a higher level in the virulent group than in the less-virulent group.

FWER-rank	Gene ID	Products	Teststat	Adj-p	p-Value
1	VV1_3114	V10 pilin	-3.81762	0.4047619	0.00209
2	VV1_1123	Adenylate cyclase	-3.38884	0.5634921	0.01025
3	VV1_1364	Cystathionine gamma-synthase	-3.14451	0.6666667	0.01418
4	VV1_1634	SmcR	-3.03225	0.7063492	0.01322
5	VV1_1605	Predicted Zn-dependent peptidase	-2.98543	0.7142857	0.01211
6	VV2_1638	Signal transduction histidine kinase	-2.58213	0.8730159	0.04011
7	VV1_3054	Response regulator	-2.52598	0.8809524	0.0229
8	VV1_1959	Chemotaxis signal transduction protein	-2.39963	0.9047619	0.0323
9	VV1_1224	Bacterial nucleoid DNA-binding protein	-2.38798	0.9047619	0.02148
10	VV2_1250	Glucan phosphorylase	2.321339	0.9285714	0.06974

The list of genes with statistical significance was obtained after source-dependent grouping. This ranking is based on the FWER value (for strong control of the family-wise Type I error rate) [3]. In the teststat, the values of the minus signs mean that expression of the genes in the less virulence group is lower than that in the virulence group. The Adj-p and p-value indicate adjusted p-value and p-value of the empirical Bayes statistic, respectively.

value=0.008) for the two sample t-statistic for comparing two groups based on the LD_{50} values in Table 1. According to these results, those ten isolates could be divided into two groups; one is the more virulent group (VG) including MO6 24/O, LSU1866, C71840, YJ016, SPRC10143, and SPRC10215, and the other is the less-virulent group (LVG) including ENV1, JY1701, SS108A3A, and 300-1C1.

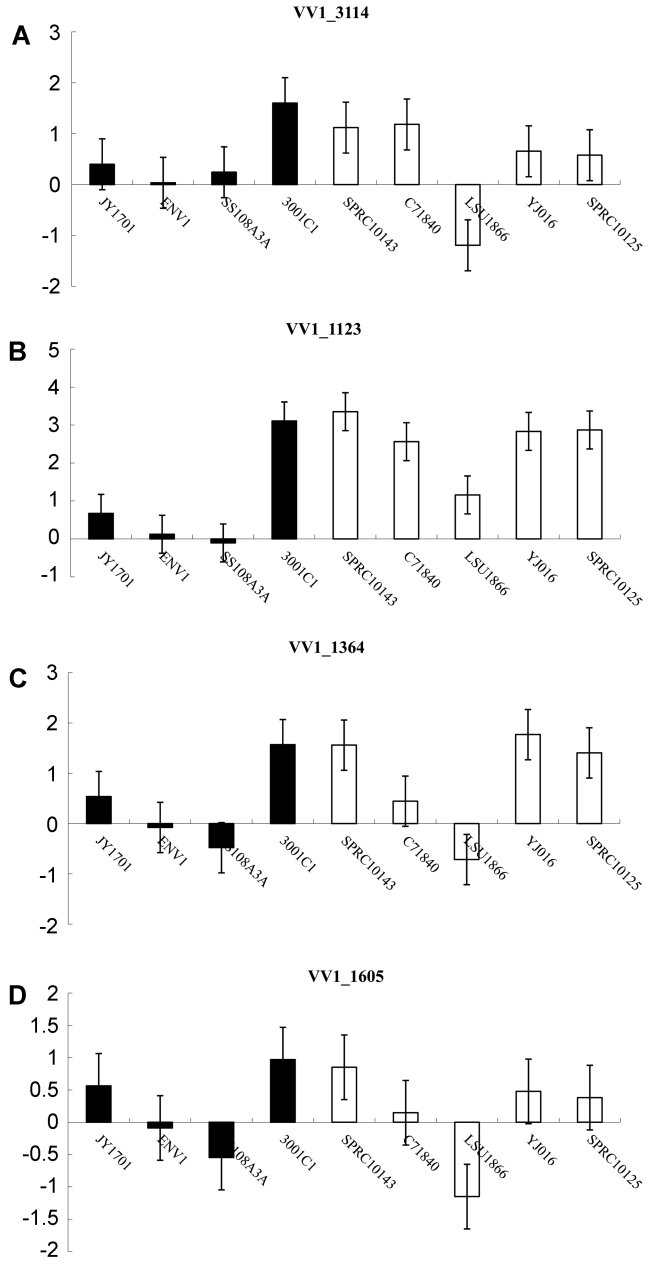

Fig. 1. Transcription level of genes as determined by RT-PCR.
(**A**) VV1_3114 (pilin); (**B**) VV1_1123 (adenylate cyclase); (**C**) VV1_1364 (cystathionine gamma-synthase); (**D**) VV1_1605 (predicted Zn-dependent peptidase). These results are average values from experiments performed five times, with the error bars showing standard deviations. The solid bars and the empty bars denote LVG and VG, respectively. The Y axis represents the log10 value of the relative quantification as described in Materials and Methods.

Genes Expressing Differently Between VG and LVG

We compared the differences in the level of transcriptions between virulence and less virulence groups through microarray analysis. As summarized in Table 4, we determined the ranking of gene expression profiling according to statistical significance as described above. We obtained ten genes that expressed highly specific to virulence groups. The putative gene VV1_3114, possibly encoding a pilin, showed the highest expression level in the virulence group comparing with the less virulence group. In addition, the putative genes encoding adenylate cyclase, cystathionine gamma-synthase, *smcR*, and Zn-dependent peptidase were ranked highly within the FWER values.

RT-PCR Analysis for Two Genes Selected by the Statistic Analysis

To validate the cDNA microarray results, we quantitatively measured the amount of transcripts of four genes that showed the best scores in the FWER rank by RT-PCR. For this, we did not include the gene VV1_1634 encoding SmcR, since this major quorum-sensing regulator has been well documented to be involved in the pathogenicity of *V. vulnificus* [6]. As shown in Fig. 1, the levels of transcripts of these genes were generally higher in VG than in LVG, with the exception of the strains LSU1866 and 3001C1. The strain LSU1866 is a clinical isolate and showed relatively

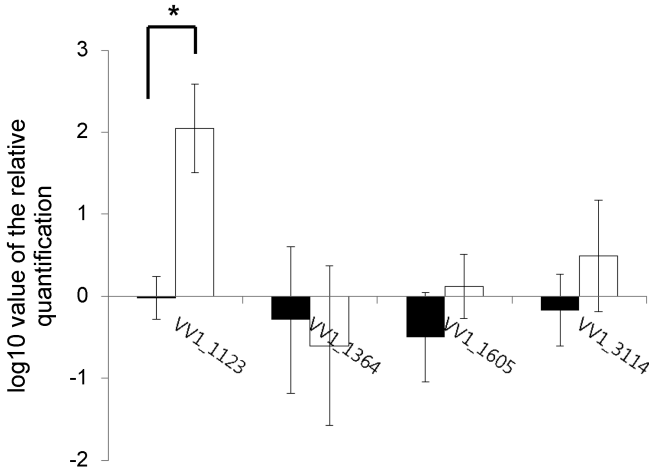

Fig. 2. Relative quantifications of statistically significant genes using RT-PCR.
Comparison of gene expression levels of four genes, which were detected on microarray experiment for the VG and LVG of *Vibrio vulnificus* isolates, is estimated by RT-PCR. These results are from a single representative experiment from experiments performed five times, with the error bars showing standard deviations. The solid bars and the empty bars denote LVG and VG, respectively. The Y axis represents the log10 value of the relative quantification, as described in Materials and Methods. The asterisk denotes a significant difference ($p<0.05$, t-test) between LVG and VG.

low LD50, but the expression levels of VV1_3114, VV1_1605, and VV1_1364 were relatively low among the ten strains. Meanwhile, the strain 3001C1 belonging to LVG showed the relative expression levels of all the four genes in a much higher level than other strains in LVG did, and the level was even higher than those of strains belonging to VG.

Fig. 2 shows the comparisons of average values of the relative expression levels of those four genes between strains in VG and in LVG. In accordance with the cDNA microarray results, genes VV1_1123 and VV1_3114 clearly showed expression levels significantly higher in VG than in LVG. We also compared the LD_{50} value of MO6 24/O with MO6-3114K carrying a deletion mutation in VV1_3114 upon 4-week-old female ICR mice. The value of the mutant was about 100-folds higher than wild type, showing that the putative pilin-encoding gene is associated with the pathogenicity of *V. vulnificus.*

DISCUSSION

The pathogenesis of *V. vulnificus* shows extreme and rapid invasiveness and concomitant tissue destruction in susceptible human hosts [20]. The symptom of the infection appears so swiftly that the development of a rapid diagnostic technique is critical for the proper treatment of patients. To develop effective diagnostic kits or therapeutic agents for pathogenic *V. vulnificus*, the genes related with bacterial virulence have to be continuously searched and the virulence mechanism regarding those genes need to be scrutinized.

In this study, genes differentially expressed between virulent strains and less-virulent strains of *V. vulnificus* were searched by employing the cDNA microarray technique. This study was based upon the assumption that there are more actively expressed genes in virulent isolates compared with less-virulent isolates, and that those genes are associated with active pathogenicity. LD_{50} values of ten *V. vulnificus* isolates were measured to categorize those into two groups: virulent group (VG) and less-virulent group (LVG). According to the LD_{50} value, MO6 24/O, LSU1866, C71840, YJ016, SPRC10143, and SPRC10215 strains were categorized as VG. ENV1, JY1701, SS108A3A, and 300-1C1 strains were classified as LVG. This categorization coincided with results of the study in the James D. Oliver group, except for the ENV1 and SPRC10215 strains [16].

For the cDNA microarray analysis, we selected 131 representative genes that are possibly associated with virulence of the pathogen. These genes had been chosen by two criteria. First, we chose genes previously showed to be associated with the virulence in *V. vulnificus* (*e.g.*, s*mcR*, *rtx*, genes encoding hemolysin, proteases, and sigma factors), or in related pathogenic *Vibrio* spp. (*e.g.*, genes encoding ABC-type transporters, pilins, quorum-sensing components). Secondly, we additionally chose genes that showed a higher expressed level in a cDNA microarray experiment by the addition of cyclic(L-Phe-L-Pro) (unpublished data), which is a diffusible molecule possibly acting as quorum-sensing signal molecules in pathogenic *Vibrio* spp. [15]. We were interested in finding virulence factors among these genes.

The cDNA samples of ten *V. vulnificus* strains including the reference MO6 24/O strain were used for hybridization of the cDNA microarray. The microarray profiling showed that the expression of VV1_3114 (V10 pilin), VV1_1123 (adenylate cyclase), VV1_1364 (cystathionine gamma-synthase), VV1_1634 (*SmcR*), and VV1_1605 (predicted Zn-dependent peptidase), and genes associated with signal transduction, response regulator, DNA-binding protein, and glucan phosphorylase were significantly different between virulence and less virulence groups. Among them, the genes VV1_3114 (pilin) and VV1_1123 (adenylate cyclase) showed the most differentially expressed level between VG and LVG with a statistical significance. As we confirmed this result using real-time PCR, those two genes showed a significantly higher level of expression in the virulent group compared with the less-virulent group.

Adenylate cyclase catalyzes the cyclization of the ATP molecule to produce cyclic AMP. This compound has been well characterized to be involved in numerous cellular controlling processes in response to various intra- and extracellular signals [25]. cAMP also has been implicated in pathogenicity of *V. vulnificus* [6]. V10 pilus has been less well characterized in terms of pathogenicity. This protein was first identified in *V. cholerae* [27]. Cells carrying this protein hemagglutinated human, rabbit, and sheep erythrocytes, although the purified protein did not. The implications of the protein in virulence of the pathogen remain obscure. We have observed that the mutation in the gene encoding the pilus significantly attenuated the virulence of *V. vulnificus*, implying the relatedness of the protein with pathogenicity. Further study needs to be carried out to elucidate the precise role of the protein in pathogenity of *V. vulnificus*.

Discovery of new markers associated with the pathogenicity of pathogenic bacteria will be helpful in understanding of virulence mechanism as well as worthy to being applied as a detection probe. DNA microarray is a useful high-throughput technical tool and has been widely employed to compare the expression levels of genes in different conditions in a given organism. In this study, we developed an analytical method to compare groups of data to extract genes expressed differentially depending upon *V. vulnificus* isolates with different pathogenic potentials. We obtained a list of genes that showed different levels of expression according to the virulence. RT-PCR confirmed that this list is valid significantly. This study showed that the transcriptomic differences between pathogenic isolates and

less-pathogenic isolates provide useful information for the identification of virulence factors in pathogenic microorganisms. Further extension of this application to the whole genes in the genome could provide results useful to identifying novel virulent factors.

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