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IVET-based Identification of Virulence Factors in *Vibrio vulnificus* MO6-24/O

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Abstract *Vibrio vulnificus* is an opportunistic pathogen that causes septicemia in humans. To identify the genes associated with its pathogenicity, *in vivo* expression technology (IVET) was used to select genes specifically expressed in a host, yet not significantly *in vitro*. Random *lacZ*-fusions in the genome of *V. vulnificus* strain MO6-24/O were constructed using an IVET vector, pSG3, which is a suicide vector containing promoterless-*aph* and -*lacZ* as reporter genes. A total of ~18,000 resulting library clones were then intraperitoneally injected into BALB/c mice using a colony forming unit (CFU) of 1.6×10^6. Two hours after infection, kanamycin was administered at 200 μg per gram of mouse weight. After two selection cycles, 11 genes were eventually isolated, which were expressed only in the host. Among these genes, VV20781 and VV21007 exhibiting a homology to a hemagglutinin gene and *tolC*, respectively, were selected based on having the highest frequency. When compared to wild-type cells, mutants with lesions in these genes showed no difference in the rate of growth rate, yet a significant decrease in cytotoxicity and the capability to form a biofilm.

Key words: *Vibrio vulnificus*, IVET, virulence factor, TolC

Vibrio vulnificus is a gram-negative halophilic bacterium that causes fatal septicemia in humans. Several virulence factors have already been identified in *V. vulnificus*, including hemolysin/cytolysin [9], polysaccharide capsules [30], siderophores [18], phospholipase A2 [32], and metalloprotease [22]. However, the determinants responsible for the virulence of the pathogen are still poorly understood despite various efforts to elucidate the virulence mechanisms.

The virulence of pathogens is closely related to a series of complex processes required for the coordinated expression of numerous genes. Thus, various *in vitro* systems have been developed to understand the infectious processes that cause damage to a host, such as the use of specific culture conditions to mimic the host environment [7]. It has already been established that *V. vulnificus* contains several genes that allow it to survive and adjust to environmental changes, such as *cadC*, *lrp*, and *rpoS* [13, 24, 27]. However, efforts to identify the virulence factors have been limited, partly due to difficulties reproducing the complex environment of a host under laboratory conditions. A pathogen may encounter several radically different environments in a host and have different requirements during infection, particularly in the context of developing an immune response. For these reasons, *in vivo* experimental models are highly advantageous, and several methods, such as *in vivo* expression technology (IVET) [20], have recently been developed that greatly simplify *in vivo* analysis. IVET is a promoter trap strategy for identifying genes induced in a specific environment, and is based on the assumption that genes specifically induced in a host are important in the pathogenic processes. Since the first IVET system was utilized to search for virulence factors in *Salmonella typhimurium* [20], various IVET systems have been designed and used in a number of different organisms, such as *Pseudomonas aeruginosa* [34], *Yersinia enterocolitica* [35], *Vibrio cholerae* [4], and *Staphylococcus aureus* [19]. As a result, IVET systems have enabled the identification of a number of known virulence factors, metabolic and biosynthetic genes, and unknown genes. For example, *sitABCD*, an iron transport operon in *S. typhimurium* [12], *motAB*, and *motY* encoding the flagella motor proteins in *V.*

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cholerae [16], and *fur*, a transcriptional regulator in *P. aeruginosa* [34], have all been identified by IVET. As such, IVET systems provide an insight into the genes, which are required for survival and multiplication *in vivo*, and the identified gene products can represent new targets for attenuating mutation, antimicrobial agents, or recombination vaccines. In many cases, the inactivation of genes identified by IVET systems has resulted in the attenuation of virulence, indicating the importance of the role of genes in pathogenesis [11].

Accordingly, to extend current knowledge of the genes responsible for the pathogenesis of *V. vulnificus*, the present study utilized IVET to select bacterial genes expressed in host tissue, while minimally expressed *in vitro*. As a result, several potential virulence factors were identified, along with their relationship to the pathogenicity of *V. vulnificus*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strains were grown in an LB (Sigma, St. Louis, U.S.A.) broth with aeration at 37°C, while the *V. vulnificus* strains were grown in either LB supplemented with 2% NaCl (LBS) or TCBS (Difco Laboratories, Detroit, U.S.A.) media at 28°C. Antibiotics were used at the following concentrations: 100 μg/ml ampicillin; 3 μg/ml chloramphenicol for *V. vulnificus*; 50 μg/ml ampicillin; 25 μg/ml chloramphenicol; 10 μg/ml tetracycline for *E. coli*. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a concentration of 40 μg/ml.

DNA Manipulations and Transformation

The isolation of the plasmid DNA was performed using an alkaline lysis procedure [28]. When required, the DNA samples were purified using Wizard Plus SV Minipreps (Promega, Madison, U.S.A.). The ligations and digestions were performed with enzymes supplied by New England Biolab (Beverly, U.S.A.) or MBI fermentas (Amherst, U.S.A.) according to the manufacturer's instructions. The plasmid DNA was introduced to the *E. coli* by transformation, electroporation using a Bio-Rad Gene Pulser II apparatus, or biparental mating using *E. coli* strain S17-1 (λ-*pir*) [28].

Isolation of *Vibrio vulnificus* Total DNA

The *V. vulnificus* was grown in LBS medium overnight, and the cells in the stationary phase recovered by centrifugation.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmid	Relevent genotype and Charateristics	Source/reference
Strains		
Escherichia coli		
DH5α	*SupE44 ΔlacU169(ϕ80lacZΔM15) hsd17 secA1 gyrA9 Thi-1 relA1*, Nal^r	[28]
S17-1	*recA, thi, pro, hsdR$^-$M* [RP4-2Tc::Mu::Kmr::Tn7], Tp^r, Str^r	[29]
S17-1 λ-*pir*	*recA, thi, pro, hsdR$^-$M* [RP4-2Tc::Mu::Kmr::Tn7], (λ-*pir*), Tp^r, Str^r	[8].
Vibrio vulnificus		
MO6-24/O	Clinical isolate	[26]
MO6-24/ΔZ	*lacZ*-null mutation of MO6-24/O	[2]
20781KO	Derivative of MO6-24/O containing Cm^r cassette insertion in ORF of VV20781	This study
21007KO	Derivative of MO6-24/O with in-frame deletion in ORF VV21007	This study
20781-415	20781KO harboring pRK415, Tc^r	This study
20781-Com	20781KO harboring derivative of pRK415 cloned with VV20781.	This study
21007-415	21007KO harboring pRK415, Tc^r	This study
21007-Com	21007KO harboring derivative of pRK415 cloned with VV21007	This study
Plasmids		
pSG3	IVET vector, ColE1 derivative with *oriT* pl-*aph* pl-*lacZ*, Ap^r Gm^r	[2]
pKNG101	Suicide vector for allelic exchange, R6K, Sm^r	[14]
pDM4	Suicide vector for allelic exchange, R6K, Cm^r	[23]
pSUP202	Derivative of ColE1 plasmid *mob*$^+$, Ap^r Tet^r Cm^r	[29]
pRK415	Broad-host-range IncP cloning vector, Tc^r	[15]
pDM4::d21007	1.4-kb SalI-SphI fragment containing in-frame deletion in VV21007 cloned into pDM4	This study
pKNG::d20781	2.7-kb fragment containing Cm^r cassette insertion in VV20781 cloned into pKNG101	This study
pRK21007	1.9-kb DNA fragment containing VV21007 cloned into pRK415	This study
pRK20781	1.8-kb fragment containing VV20781 cloned into pRK415	This study

Abbreviations: Ap^r, ampicillin-resistant; Gm^r, gentamycin-resistant; Nal^r, naladixic acid-resistant; Sm^r, streptomycin-resistant; Tc^r, tetracycline-resistant; Tp^r, trimethoprim-resistant; pl-*aph*, promoterless kanamycin-resistant gene; pl-*lacZ*, promoterless *lacZ*.

These cells were then resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) with 5% sarkosyl and added with protease K (5 mg/ml), then, incubated at 37°C until complete cell lysis was obtained. The lysate was extracted three times with 3% NaCl-saturated phenol and subsequently with chloroform: isoamyl alcohol (24:1) and diethyl ether. The total DNA of *V. vulnificus* was finally precipitated by adding 95% ethanol.

Construction of *V. vulnificus* MO6-24/O Libraries in pSG3

The plasmid pSG3 is a promoter-trapping vector containing promoterless *aph-* and *lacZ-* as the reporter genes, and was specially designed for IVET (Fig. 1) [2]. As such, the genomic library of *V. vulnificus* was constructed in this IVET vector. The total DNA of MO6-24/O was partially digested with *Sau3A*I, and the digested DNA fragments size-fractionated in a 10–40% (w/v) sucrose gradient by centrifugation at 26,000 rpm at 15°C for 16 h in a Beckman SW41 rotor. The DNA fragments containing 3–5 kb DNA fragments were pooled and precipitated by adding cold 95% ethanol. The purified DNA fragments were then ligated into the BamHI site of pSG3, and the ligated sample introduced into *E. coli* strain S17-1 λ-*pir* by electroporation [5].

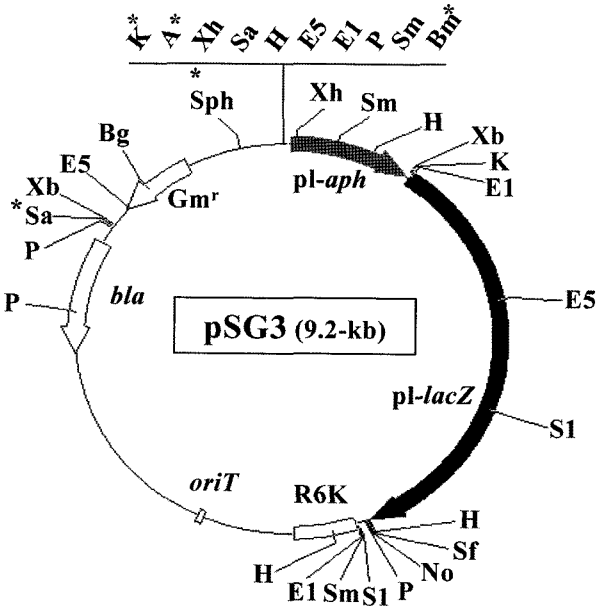

Fig. 1. IVET vector pSG3.
The IVET vector pSG3 contains a promoterless kanamycin resistance gene and *lacZ*, denoted as pl-*aph* and pl-*lacZ*, respectively, in the region just downstream of the multi-cloning site (MCS). In addition, the vector also contains *oriR*6K, which allows autonomous replications of the plasmid upon the *trans*-supplement of the Pi replication protein, *oriT* that is a conjugal transfer origin, and gentamycin and ampicillin-resistance genes (Gm^r and *bla*, respectively) as selection markers. Abbreviations for restriction sites: A, ApaI; Bm, BamHI; Bg, BglII; E1, EcoRI; E5, EcoRV; H, HindIII; K, KpnI; P, PstI; S1, SacI; Sa, SalI; Sf, SfiI; Sm, SmaI; Sph, SphI; Xb, XbaI; Xh, XhoI. Asterisks (*) denote restriction sites useful for retrieval of inserts from genome [2].

Construction of Transcriptional *aph-lacZ* Fusions in *V. vulnificus*

Clones of the *V. vulnificus* genomic library in pSG3 were introduced by biparental mating into *V. vulnificus* strain MO6-24/Δ*Z* that includes a deletion mutation in *lacZ* [2]. Briefly, twenty-microliter cultures of *E. coli* strain S17-1 λ-*pir* containing the library clones and *V. vulnificus* strain MO6-24/Δ*Z* were grown on an LB agar in the presence of appropriate antibiotics to the exponential phase. Both cultures were then suspended in an LB medium, washed three times, and resuspended in an LB broth. Thereafter, the two resuspensions were mixed, spotted on an LB agar, and incubated at 37°C for 6 h. Finally, the cells were collected and spread on a TCBS agar containing ampicillin to select the *V. vulnificus* cells containing pSG3 fused into the genome by homologous recombination.

IVET Selection

One hundred microliters of the *V. vulnificus aph-lacZ* fusion pool was incubated in LBS at 28°C with vigorous aeration. After two hours, the cells were washed two times with phosphate-buffered saline (PBS) (pH 7.2) [28] and resuspended in PBS. Female 6-week-old BALB/c mice pretreated with kanamycin at a concentration of 200 μg per gram of mouse weight were intraperitoneally injected with the PBS-washed *V. vulnificus aph-lacZ* fusion clone cells based on a colony forming unit (CFU) value of approximately 1.6×10^6. After two hours, an equal concentration of kanamycin was administered again and the infection allowed to progress for a minimum of six hours. After visible signs of illness, livers and spleens were removed from the treated mice and homogenized by adding 1 ml of PBS. The bacterial cells within the homogenate were grown overnight on a TCBS agar containing ampicillin, and the finally selected clones subjected to further studies.

DNA Nucleotide Sequence Analysis

To determine the DNA sequences of the inserts in the *aph-lacZ* fusion clones recovered from the mice, a synthetic oligonucleotide, pl-Km (5'-TAAATCAGCATCCATGTTGG-3'), which is complementary to the nucleotide sequence of the 5' end of the promotorless-*aph* gene in pSG3, was used as the primer. The plasmid DNA was isolated by Wizard Plus SV Minipreps (Promega) and the DNA nucleotide sequences determined using an ABI-373A automated DNA sequencer (Perkin Elmer Corp., Foster City, U.S.A.) or DNA sequencing service (Core Bio System Co., Ltd., Seoul, Korea). The search for sequence similarities was performed using the BLAST program [1], while multiple alignment of the deduced amino sequences was carried out using ClustalW [33].

Construction of Mutations in ORFs VV20781 and VV21007

The regions of ORF VV20781 and VV21007 in *V. vulnificus* MO6-24/O were amplified by a PCR using purified genomic DNA as the template and the following oligonucleotide PCR primers: for VV20781, 20781F-Bam (5'-ACGGATCCGTAGTACAATATGCCGC-3') and 20781R-Spe (5'-ATACATGTCGAGTGATGCTTTCAGG-3'); for VV21007, 21007F (5'-GAGAGCGATCAGCACCACCA-3') and 21007R (5'-ATGGCAGTTGGGGCTCTC-3'). The resulting PCR products were purified and cloned into a pGEM-T easy vector (Promega), and the DNA nucleotide sequences of the cloned fragments determined. To construct an insertion mutation in ORF VV20781, a Cm^r cassette from pSUP202 [29] was inserted into the *Xba*I site in the coding region. Meanwhile, an in-frame deletion mutation of VV21007 was achieved by removing a 516-bp BclI fragment from the coding region of VV21007. The two mutated fragments were then cloned into the suicide shuttle vectors pKNG101 and pDM4, to construct pKNG::d20781 and pDM4::d21007, respectively. *E. coli* S17-1 λ-*pir* carrying the resulting constructions was mated with *V. vulnificus* MO6-24/O to generate each mutant by homologous recombination. The conjugation and isolation of the transconjugants were performed by methods previously described [17]. Each mutation was confirmed by a PCR of the chromosomal DNA from the respective mutant and wild-type strains using appropriate primer sets (for VV20781, 20781F, 5'-ATGTCCAAATCAGATTTG-3' and 20781R, 5'-TTATTCTTGTGGACGCCA-3'; for VV21007, 21007F, and 21007R) and Southern hybridization. To complement the VV20781 or VV21007 mutant strain with the wild-type allele *in trans*, DNA fragments containing the VV20781 or VV21007 gene amplified with the primer sets 20781F-Bam/20781R-Spe or 21007F/21007R were cloned into the *Eco*RI site of pRK415 under the P_{lac} promoter. The resulting plasmids were then transferred into the respective mutant strains by biparental mating [28].

Biofilm Formation Assay

Biofilm formation was determined using polyethylene tubes as an abiotic surface for bacterial attachment. Overnight cultures of the examined *V. vulnificus* strain were inoculated into an LB broth in a polyethylene tube, incubated with gente agitation at 28°C for 72 h, and the OD_{600} value of the cell culture measured. When the value reached approximately 0.3, the cell cultures were discarded, then the tubes were gently washed with distilled water and filled with a 0.1% crystal violet solution. After 15 min, the tubes were gently washed four times with distilled water and dried at room temperature. The crystal violet associated with biofilms was suspended in dimethyl sulfoxide (DMSO) and the OD_{570} of the suspension measured. The formation of a biofilm was then quantitatively expressed as the ratio between the A_{570} value for the crystal violet elute and the A_{600} value for cell growth.

Cytotoxicity Assay

The cytotoxicity was determined by measuring the activity of lactate dehydrogenase (LDH) released from the HeLa cell line using a CytoTox96 Non-Radioactive Cytotoxic Assay Kit (Promega). 6×10^4 HeLa cells grown for 3 or 4 days in a Dulbecco modified Eagle medium (DMEM) at 37°C in a 5% CO_2 incubator were placed in 96-well plates and incubated overnight. The bacterial cells were then added to each well at different multiplicities of infection and incubated for 90 min at 37°C. The activity level of released LDH was measured as recommended by the manufacturer.

RESULTS

Construction of *V. vulnificus* MO6-24/O Genomic Libraries in pSG3

The total DNA of *V. vulnificus* strain MO6-24/O was partially digested with *Sau*3AI and fractionated according to size. The 3–5 kb-sized fractionated DNA fragments were ligated to the *Bam*HI site of pSG3 (Fig. 1; reference 2) and the ligated plasmids transformed into *E. coli* S17-1 λ-*pir*. A total of ~15,000 library clones containing *V. vulnificus* MO6-24/O genomic DNA were obtained and pooled. The heterogenecity of the resulting library was confirmed by examining the restriction enzyme profiles of 21 randomly selected clones.

Construction of Random *aph-lacZ* Fusions in Genome of *Vibrio vulnificus* MO6-24/O

The pooled MO6-24/O genomic libraries in *E. coli* S17-1 λ-*pir* were conjugated with MO6-24/ΔZ, a *lacZ*-null mutant derived from MO6-24/O [2]. By selection on TCBS containing ampicillin at 100 μg/ml, a total of 18,800 transconjugants were obtained and pooled. In the resulting transconjugants, pSG3 was randomly integrated into the genome by a single cross-over to generate transcriptional *aph-lacZ* gene fusions.

Selection of Fusion Clones Using IVET

The pool of *aph-lacZ* fusion clones was injected intraperitoneally based on a 1.6×10^6 CFU into kanamycin-treated BALB/c mice, then surviving clones were recovered from the livers and spleens. After the first selection round, about 8,200 surviving clones were recovered from the livers of the treated mice. These clones were pooled and re-injected into kanamycin-treated BALB/c mice, and a total of 1,647 clones finally obtained from the livers and spleens of those mice (895 clones from the liver and 792

Table 2. *V. vulnificus* genes selected by IVET.

Classification	Gene designation[1]	Predicted product (Species, identity%/similarity%)[1]	Number of identified clones	Organ originated
Biosynthesis and metabolism	VV10342	Cytosine/Adenosine deaminase, YftC (*V. cholerae*, 77/89)	1	Spleen
	VV11423	Mehionine synthase I (*V. parahaemolyticus*, 91/95)	2	Liver
	VV13165	1-acyl-sn-glycerol-3-phosphate-acyltransferase (*V. parahaemolyticus*, 80/91)	6	Liver & Spleen
	VV21348	Mannose-6-phosphate isomerase (*V. cholerae*, 76/83)	3	Liver & Spleen
Regulation	VV11166	SOS response regulator LexA (*V. parahaemolyticus*, 86/91)	1	Spleen
Membrane protein and transport	VV11759	Chromate transport protein ChrA (*V. parahaemolyticus*, 60/71)	1	Liver
	VV21007	Outer membrane protein TolC (*V. choleare*, 50/68)	14	Liver & Spleen
Proteolysis	VV11605	Zn-dependent peptidase (*V. parahaemolyticus*, 73/86)	1	Liver
Hypothetical protein	VV20781	Unkown	33	Liver & Spleen
	VV13143	Unkown	1	Liver
	VV21194	Unkown	1	Liver

[1]Proteins predicted from complete genome sequence of *V. vulnificus* CMCP6 in GenBank database.

clones from the spleen). The level of β-galactosidase expression by these clones was monitored by examining the color of the colonies on an LBS agar containing X-Gal at 40 μg/ml and ampicillin at 100 μg/ml. Among the clones, 184 clones (approximately 12% of all the examined clones) showed low β-galactosidase activities on the medium, as determined by visual examination of the color development.

Retrieval of Chromosomal Genes Fused with pSG3 from Selected Clones

The 184 clones finally selected by IVET were assumed to contain the *aph-lacZ* reporter genes fused with the genes in the genome of *V. vulnificus*, which were only expressed in the mouse host, and not *in vitro*. The DNA fragments fused to the pSG3 derivatives were retrieved from the chromosome of the isolated clones. For this, the genomic DNA was isolated from the clones and digested with ApaI or SalI, which cuts a single site of pSG3 (Fig. 1). The digested DNA fragments were self-ligated, and introduced into *E. coli* S17-1 λ-*pir*, then, selected by ampicillin.

Nucleotide Sequence Analyses of Inserts in Retrieved pSG3 Derivatives

The DNA sequences of the inserted DNA fragments in the pSG3 derivatives from the selected clones were determined using the oligomer pl-Km as the primer, as described in Materials and Methods, that is complementary to the 5' end part of the promotorless-*aph* gene in pSG3. The analysis of the nucleotide sequences of the individual inserts and comparison with the annotated genes of *V. vulnificus* CMCP6 in the GenBank database identified 11 genes. The list of the genes identified in this study is shown in Table 2. The 11 putative genes included genes for biosynthesis and metabolism (cytosine/adenosine deaminase, methionine synthase I and phosphomannose isomerase), transcriptional regulation (SOS response transcriptional repressor), protease (Zn-dependent peptidase), lipid metabolism (1-acyl-sn-glycerol-3-phosphate-acyltransferase), and transport (ChrA and TolC homolog). The remaining three genes did not show any significant homologies with any known genes in the GenBank database. Among the 11 identified genes, genes VV20781 and VV21007 exhibited the highest frequencies (33 clones and 14 clones, respectively). Hence, these two genes were chosen for further studies.

```
         10 ⬇      20        30        40        50        60
MSKSDLVKQQPELIIKTDGDFWGLGQEGTFDLAGLYNGQYSRHASNSSWFDTISTSKGEM

         70        80        90        100       110       120
AANITNTQNNQMWTMSCSGGGTNVNYMGVQFGGNKPYQCTIFQAGEQVGQFIMQEKSAVI

         130       140       150       160       170       180
DLGLEKKETGYIEVGHTRFELETVHTGEGLLMPLESPLGYSFKHNGREIAAVQTNGMLTV

         190       200
QWLPELTSHEKDVLAIGAIAGALSWRPQE
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Fig. 2. Deduced amino acid sequences of VV20781 gene.
VV20781 possibly encodes 209 amino acids. The underlined amino acid sequences represent a prokaryotic membrane lipoprotein lipid attachment site [31]. The arrow indicates the site at which the gene was inserted by the reporter genes in pSG3 in the selected clone.

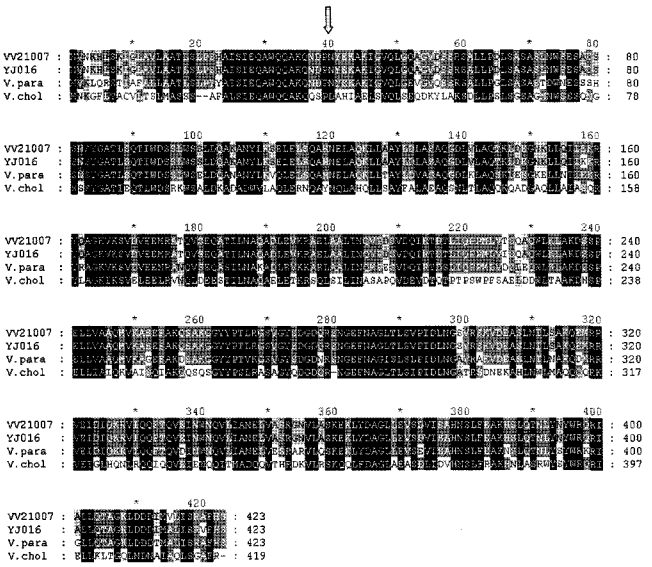

Fig. 3. Alignment of VV21007 with TolC proteins.
The deduced amino acids for VV21007 showed high similarities with the TolC proteins from *V. vulnificus* strain YJ016 [6], *V. parahaemolyticus* [21], and *V. cholerae* [10]. The black boxes and shadowed boxes represent identical and highly conserved regions among the compared proteins, respectively. The alignment was performed using the multiple alignment program ClustalW. The arrow indicates the site at which the gene was inserted by the reporter genes in pSG3 in the selected clone.

Analysis of Deduced Amino Acid Sequences of VV20781 and VV21007

The function of the protein encoded by the VV20781 gene was difficult to deduce from information in the GenBank database due to the lack of significantly homologous genes. However, since it contained a prokaryotic membrane lipoprotein lipid attachment site: TQNNQMWTMSC (Fig. 2) [31], this suggests a membrane protein. Meanwhile, the VV21007 gene encoded the outer membrane protein and showed 99%, 91%, and 71% similarities with the TolC proteins from *V. vulnificus* YJ016 [6], *V. parahaemolyticus* [21] and *V. cholerae* [10], respectively (Fig. 3).

Construction of Mutations in VV20781 and VV21007

Mutations of the VV20781 and VV21007 genes were constructed in *V. vulnificus* MO6-24/O by allelic exchange. A mutant of the VV21007 gene, 21007KO, was constructed by deleting the BclI fragment in the gene (Fig. 4A). The size of the deleted BclI fragment was 516-bp, therefore, the deletion of this fragment generated an in-frame deletion mutation in the VV21007 gene. The generation of the deletion was confirmed by both a PCR (data not shown) and Southern hybridization (Fig. 4A). To generate a mutation in the VV20781 gene, the Cm^r cassette from pSUP202 was inserted into the XbaI site within the VV20781 coding region (Fig. 4B), and confirmed by a PCR (data not shown) and Southern hybridization (Fig. 4B). The resulting two mutants grew well on an LB medium

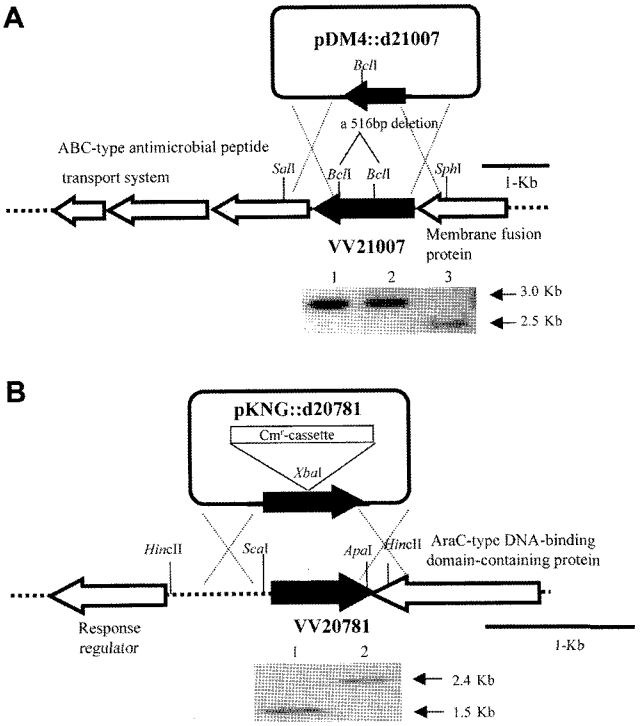

Fig. 4. Construction of mutations in VV21007 and VV20781 by allelic exchange.
A. Homologous recombination between chromosomal gene VV21007 from strain MO6 24/O and pDM4::d21007, and confirmation of deletion mutant by Southern hybridization using VV21007-coding DNA as a probe. Lane: 1, MO6-24/O wild-type; 2, pDM4 single-crossover; 3, VV21007 mutant. **B.** Homologous recombination between chromosomal gene VV20781 from strain MO6 24/O and pKNG::d20781, and confirmation of insertion mutant by Southern hybridization using VV20781-coding DNA as a probe. Lane: 1, MO6-24/O wild-type; 2, VV20781 mutants. The dashed and solid lines indicate the chromosomal DNA and plasmid DNA, respectively. The expected band sizes are indicated on the right side.

and TCBS agar plate, and the growth rates for both mutants in an LB liquid medium were not significantly different from that for the parental wild-type cells (data not shown).

Cytotoxicity

The fact that VV20781 and VV21007 were selected by IVET suggests that these two genes are involved in the virulence of *V. vulnificus*. Furthermore, the deduced amino acid sequences for these genes were found to be homologous to genes in the GenBank database, which are closely related to the pathogenicities of other bacteria. Since wild-type *V. vulnificus* cells are highly cytotoxic to cultured animal cell lines, the effect of the mutations in VV20781 and VV21007 on the cytotoxicity towards HeLa cells was examined. The cytotoxicity was determined by measuring the level of LDH released from the HeLa cells with different multiplicities of infection (MOIs). For the MOIs examined, the cytotoxicity of both mutants towards the HeLa cells was significantly attenuated when compared

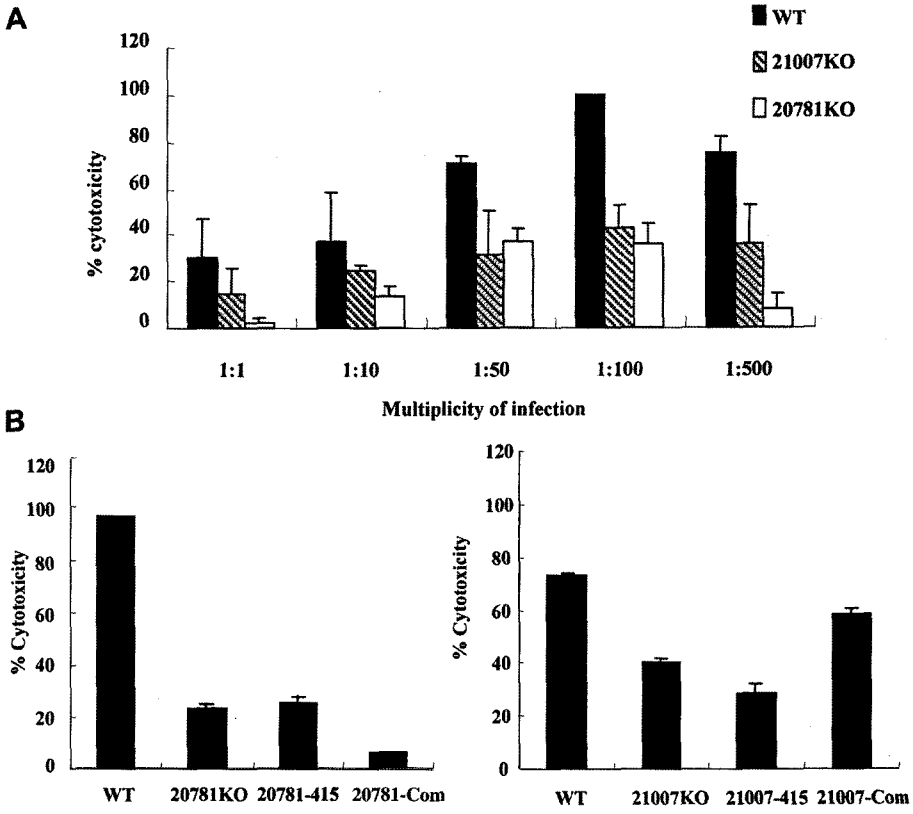

Fig. 5. Effect of mutations of VV20781 and VV21007 on cytotoxicity towards HeLa cells.
VV29781KO and VV21007KO cells were incubated with HeLa cells for 90 min and the cytotoxicity measured. **A.** Cytotoxicity towards HeLa cells with different multiplicities of infection (MOI), where MOI means ratio of number of HeLa cells to number of infected bacterial cells; WT, wild-type cells. **B.** Complementation of wild-type copies of VV20781 and VV21007 at the MOI value of 100.

to that of the wild-type strain (Fig. 5A). At the MOI value of 100, the wild-type cells killed 100% of the HeLa cells, however, 21007KO and 20781KO only killed about 45% and 40% of the HeLa cells, respectively, suggesting that VV21007 and VV20781 play an important role in the cytotoxicity of *V. vulnificus*. The cyctotoxic deficiency of the mutant 21007KO was almost completely restored by the introduction of a wild-type copy of the VV21007 gene (in strain 21007-Com) (Fig. 5B). However, the cytotoxic deficiency of 20781KO was not restored by the *in-trans*

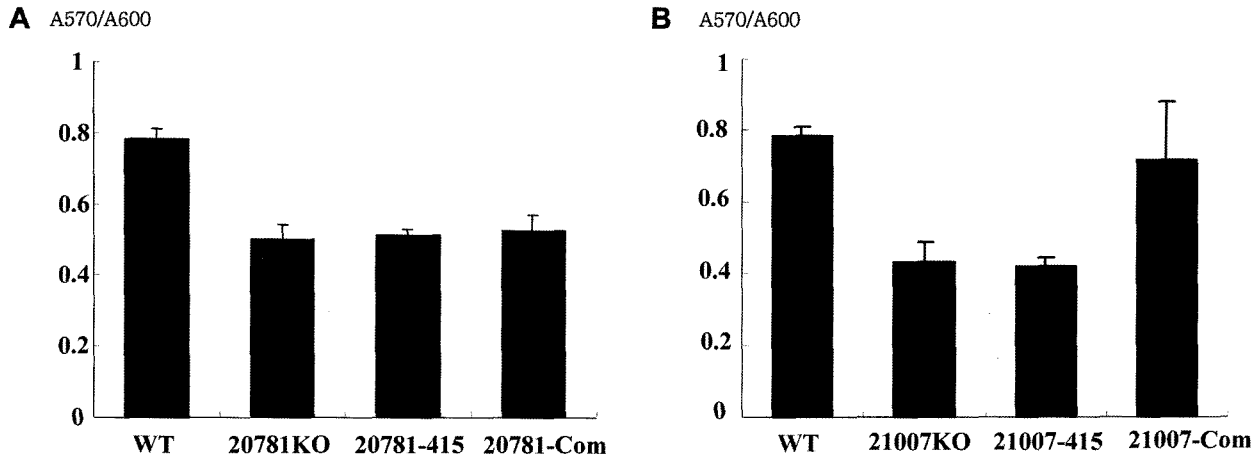

Fig. 6. Effect of mutations of VV20781 and VV21007 on biofilm formation.
The biofilm formation was measured using polyethylene tubes as the surface. Effect of mutation in (**A**) VV20781 and (**B**) VV21007. WT denotes wild-type strain.

introduction of a wild-type copy of the VV20781 gene (20781-Com) (Fig. 5B).

Biofilm Formation

Based on the deduced amino acid sequence analysis, VV20781 and VV21007 genes were both assumed to encode membrane proteins, leading to the possibility that the genes may be involved in attachment to other cells or biofilm formation. Therefore, a quantitative comparison of the ability to form a biofilm was made between the wild-type and two mutants, 20781KO and 21007KO. The cells were grown in polyethylene tubes for 72 h with gentle agitation, and the biofilm formations quantitatively measured, as described in Materials and Methods. The amount of biofilm produced by the mutant strains was about 37% (20781KO) and 43% (21007KO) lower than the amount of biofilm produced by the wild-type strain (Fig. 6). The reduced biofilm formation in 20781KO was not even recovered after a wild-type copy of the VV20781 gene was introduced *in-trans* (Fig. 6A). In contrast, the reduced biofilm formation of 21007KO was almost fully recovered by the introduction of a wild-type copy of VV21007 (21007-Com in Fig. 6B).

DISCUSSION

Identifying the genes *V. vulnificus* specifically induces *in vivo* is important to understand the mechanisms by which this pathogen causes disease. Thus, an IVET strategy, involving an original IVET vector pSG3 with a promoterless *aph* and *lacZ* gene [2], was employed to determine novel virulence factors in *V. vulnificus*. As such, the vector allowed convenient monitoring of the transcriptional activities of the genes fused by the *lacZ* and selection *in vivo* based on antibiotic resistance.

Using the IVET system, 184 *V. vulnificus* genomic library clones were selected, in which the randomly inserted reporters were expressed in the host yet not *in vitro*. The percentage of the selected clones showing kanamycin resistance *in vivo*, yet low β-galactosidase activities *in vitro*, differed according to the organ: 16% of the clones recovered from the liver and 9% of those recovered from the spleen. Some fusion clones were only isolated from one of the organs: clones with reporter genes fused to genes VV11423, VV11605, VV11759, VV13143, and VV21194 were only isolated only from the liver, while clones with reporter genes fused to genes VV10342 and VV11166 were only isolated from the spleen (Table 2). Thus, the different levels of gene expression were likely due to differences in the physiological environments of the different organs. Yet, the biological implications of this observation remain to be clarified.

In this study, the nucleotide sequences of the selected clones were determined and 11 different genes identified (Table 2). Among the 11 genes, four genes, VV10342, VV11423, VV13165, and VV21348, were assumed to be involved in biosynthetic and metabolic functions. Growth *in vivo* is essential for bacterial pathogenesis, and numerous genes already detected by IVET in *S. typhimurium* have been associated with metabolic functions [20]. Meanwhile, two genes, VV11759 and VV21007, were identified as involved in secretion mechanisms, plus a transcriptional regulator gene (VV11166) associated with an SOS response and Zn-dependent peptidase (VV11605) assumed to act as cytolysin and hemolysin were also identified. However, their exact roles in pathogenecity require further study. Nonetheless, despite the identification of several putative virulence genes, previously reported genes as virulence determinants in *V. vulnificus*, such as cytolysin/hemolysin [9], metalloprotease [22], and capsular polysaccharide [30], were not identified using the method employed in this study. It is possible that these factors were also expressed at relatively high levels *in vitro*, thereby excluding them from the IVET *in vitro* screening.

Among the 11 finally selected genes, two genes, VV20781 and VV21007, were of interest due to their high frequency. The deduced amino acid sequence for VV20781 was found to contain a membrane lipoprotein lipid attachment motif (Fig. 2). Thus, since the production of bacterial lipoproteins has been reported to be associated with resistance to bactericidal effects in complemented strains [25], it is possible that this gene may be associated with colonization or survival within a host based on protecting the bacterial cell membrane structure. Meanwhile, the translated product of VV21007 possessed amino acid sequence similarities with the outer membrane protein TolC. In a previous study, it was suggested that TolC plays a role in the colonization *in vivo* of *V. cholerae* [3]. Therefore, to investigate the relatedness of these genes to virulence, mutations were constructed in each of the two genes in the *V. vulnificus* MO6-24/O strain. Two ORFs neighboring VV20781 were transcribed in the opposite direction to VV20781, while an in-frame deleted mutation was included in 21007KO. As such, the resulting genetic organization in 20781KO and mutation in 21007KO eliminated any possibility of a polar effect on the expression of the adjacent genes.

To elucidate the roles of the VV20781 and VV21007 genes, the cytotoxicity assay and the biofilm formation assay were performed for the null-mutants. The cytotoxicity and biofilm production by 20781KO and 21007KO were both decreased compared to those by the wild-type strain (Fig. 5A and 6A), suggesting that VV20781 and VV21007 are associated with the virulence of *V. vulnificus*. However, while the reduced cytotoxicity and biofilm formation

in 21007KO were almost completely restored in the complemented strain 21007-Com (Fig. 5B and 6B), the same reductions in 20781KO were not complemented by the *in trans* supplement of a wild-type copy (Fig. 5B and 6B). Although the precise reasons for this result are not yet understood, the mutation in VV20781 may somehow have interfered with the function of the wild-type product supplemented *in trans*, while another possibility is that the excess amount of the wild-type product encoded by the multi-copy plasmid may have kept the membrane protein from forming a natural structure.

The purpose of this study was to investigate the virulence mechanisms in *V. vulnificus*. Although the genes selected by the current IVET system were shown to have a relationship with the bacterial virulence, IVET systems have an inherent drawback in that they discriminate too strongly against genes expressed *in vitro*. It is reasonable to expect that genes expressed *in vitro* are also important for survival in a host and will cause damage to the host [7], but some genes associated with virulence may still be excluded from the pool at certain points during IVET. Plus, genes down-regulated during infection may also be excluded from the screening. Therefore, for a complete understanding of the virulence mechanisms, other analyses are required that supplement the disadvantages of IVET.

In summary, the use of IVET in the current study facilitated the identification of a TolC-homolog as a new virulence factor in *V. vulnificus*, yet the mechanism underlying its function requires for further study.

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