

Proteomic Identification and Characterization of *Vibrio vulnificus* Proteins Induced upon Exposure to INT-407 Intestinal Epithelial Cells

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Proteomic analysis led to identification of the proteins of *Vibrio vulnificus* that were induced upon exposure to INT-407 cells, and 7 of which belong to the functional categories such as amino acid transport/metabolism, nucleotide transport/metabolism, posttranslational modification/protein turnover/chaperones, and translation. Among the genes encoding the host-induced proteins, disruption of *purH*, *trpD*, *tsaA*, and *groEL2* resulted in reduced cytotoxicity. The *purH*, *trpD*, and *tsaA* mutants showed impaired growth in the INT-407 lysate; however, the growth rate of the *groEL2* mutant was not significantly changed, indicating that the possible roles of the host-induced proteins in the virulence of *V. vulnificus* are rather versatile.

Keywords: Proteomics, *V. vulnificus*, virulence

A successful infection of pathogenic bacteria depends primarily on their ability to survive and multiply within the harsh environments of the host. Many novel genes that are not expressed during *in vitro* growth are differentially expressed in order to evade antimicrobial defense systems and to proliferate within the host [3]. Therefore, it has been generally accepted that bacterial genes that are preferentially expressed within the environment of the host are likely important for pathogenesis [20]. So far, several experimental approaches, such as *in vivo* expression technology (IVET) [23] and signature-tagged mutagenesis (STM) [8], have been used for the extensive screening of bacterial genes that are specifically induced upon exposure to the host. These screenings and subsequent characterization have led to the identification of many bacterial genes encoding potential virulence factors [20].

The pathogenic marine bacterium *Vibrio vulnificus* is a causative agent of foodborne diseases such as life-threatening

septicemia and possibly gastroenteritis in individuals with underlying predisposed conditions, including liver damage and excess levels of iron [7, 22, 33]. Disease caused by infection with *V. vulnificus* is remarkable for the invasive nature of the infection, ensuing severe tissue damage, and rapidly fulminating course, indicating that the pathogenicity of the bacteria involves the products of many genes. Similar to many other pathogenic bacteria, *V. vulnificus* must survive adverse environments within the host by preferentially expressing numerous genes to ensure developing illness. However, until now, there have been few studies on the identification and characterization of the genes that are highly expressed in the host [13]. As such, extensive identification of the virulence factors of *V. vulnificus* has been limited owing to the inability to mimic host environments in the laboratory.

Recently, cell culture model systems have been used to mimic the interaction between bacterial pathogens and host, and thus protein or transcript profiles of the bacteria exposed to host cells have been analyzed [1, 2, 4, 5, 14, 34]. Many virulence factors have been identified on a global scale by screening and subsequent characterization of proteins or genes induced upon exposure to host cells [1, 2, 5]. The present study used the INT-407 (ATCC CCL-6) cell culture model combined with proteomic analysis to screen and identify proteins of the *V. vulnificus* induced in host tissue. Functions of the host-induced proteins were assessed by constructing isogenic mutants, in which genes encoding the proteins were separately inactivated by allelic exchanges and by evaluating their phenotype changes in virulence and growth rate.

The strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, the *V. vulnificus* strains were grown in Luria-Bertani (LB) medium supplemented with 2.0% (w/v) NaCl (LBS). Total cellular proteins were prepared from *V. vulnificus* M06-24/O exposed to INT-407 human epithelial cells. For this purpose, monolayers of INT-407 cells were prepared and maintained with 40 ml of

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

Strain or plasmid	Relevant characteristics ^a	Reference or source
Bacterial strain		
<i>V. vulnificus</i>		
M06-24/O	Clinical isolate; virulent	Laboratory collection
OH0501	M06-24/O with <i>tig::nptI</i> , Km ^r	This study
OH0502	M06-24/O with <i>fbpA::nptI</i>	This study
OH0503	M06-24/O with <i>purH::nptI</i>	This study
OH0504	M06-24/O with <i>trpD::nptI</i>	This study
OH0505	M06-24/O with <i>tsaA::nptI</i>	This study
OH0506	M06-24/O with <i>groEL2::nptI</i>	This study
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 (ϕ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Laboratory collection
SM10 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu λ pir</i> ; conjugal donor	[25]
Plasmids		
pGEM-T easy	PCR product cloning vector; Ap ^r	Promega
pUC4K	pUC4 with <i>nptI</i> ; Ap ^r , Km ^r	Pharmacia
pDM4	Suicide vector; <i>oriR6K</i> ; Cm ^r	[26]
pJH0311	0.3-kb NruI fragment containing multicloning site of pUC19 cloned into pCOS5; Ap ^r , Cm ^r	[6]
pOH0514	pDM4 with <i>tig::nptI</i> ; Cm ^r , Km ^r	This study
pOH0515	pDM4 with <i>fbpA::nptI</i> ; Cm ^r , Km ^r	This study
pOH0518	pDM4 with <i>purH::nptI</i> ; Cm ^r , Km ^r	This study
pOH0520	pDM4 with <i>trpD::nptI</i> ; Cm ^r , Km ^r	This study
pOH0522	pDM4 with <i>tsaA::nptI</i> ; Cm ^r , Km ^r	This study
pOH0526	pDM4 with <i>groEL2::nptI</i> ; Cm ^r , Km ^r	This study
pOH031	pJH0311 with <i>purH</i> ; Ap ^r , Cm ^r	This study
pOH032	pJH0311 with <i>trpD</i> ; Ap ^r , Cm ^r	This study
pOH033	pJH0311 with <i>tsaA</i> ; Ap ^r , Cm ^r	This study
pOH034	pJH0311 with <i>groEL2</i> ; Ap ^r , Cm ^r	This study

^aAp^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

the culture medium [minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin] in 300-cm² culture flasks as described previously [10]. M06-24/O was incubated with INT-407 cells at an MOI of 20 for 2 h. The mixture of the INT-407 and *V. vulnificus* cells was centrifuged at 250 \times g for 10 min to precipitate INT-407 cells, and the bacterial cells were then harvested from the supernatant by centrifugation at 2,430 \times g for 20 min.

Proteomic Analysis

Proteins from the bacterial cells exposed to either INT-407 cells or the culture medium alone were prepared as described elsewhere [35], and then resolved by two-dimensional gel electrophoresis (2-DE). For the first dimension, pH 4–7 IPG (immobilized pH gradient) strips (13 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) were rehydrated overnight in a rehydration buffer [15] containing 100 μ g of protein sample and then IEF (isoelectric focusing) was performed at 20°C for 12 h [30]. The IPG strips were equilibrated with SDS equilibration solution [15] and embedded on top of the 12% SDS-polyacrylamide gels. The SDS-PAGE was run at 20 mA for 12 h and the gels

were fixed and stained with silver according to the manufacturer's procedure (Amersham Pharmacia Biotech). The gels were scanned and the intensity of each spot, from two gels of each culture, was measured with the Image Master 2D Elite software (Version 3.10; Amersham Pharmacia Biotech). The spots more abundant in bacterial cells exposed to INT-407 cells were excised, digested with trypsin (Promega, Madison, WI, U.S.A.), and used for MS analysis with a MALDI-TOF mass spectrometer (matrix-assisted laser desorption ionization mass spectrometer; Voyager-DE STR Biospectrometry Workstation, Germany).

Identification of the *V. vulnificus* Proteins Induced upon Exposure to Host Cells

2-DE gels for analysis of proteins from *V. vulnificus* incubated with and without INT-407 cells were compared, and 8 spots that were more abundant in the proteins from *V. vulnificus* exposed to INT-407 cells were selected for MS analysis (Figs. 1A and 1B). For reliable identification of the *V. vulnificus* proteins, MS-fit (<http://prospector.usuf.edu/ucsfhtml4.0/msfit.htm>) was used as the main searching program that provides an option to specify the target proteins using the tryptic peptide masses. Among the spots

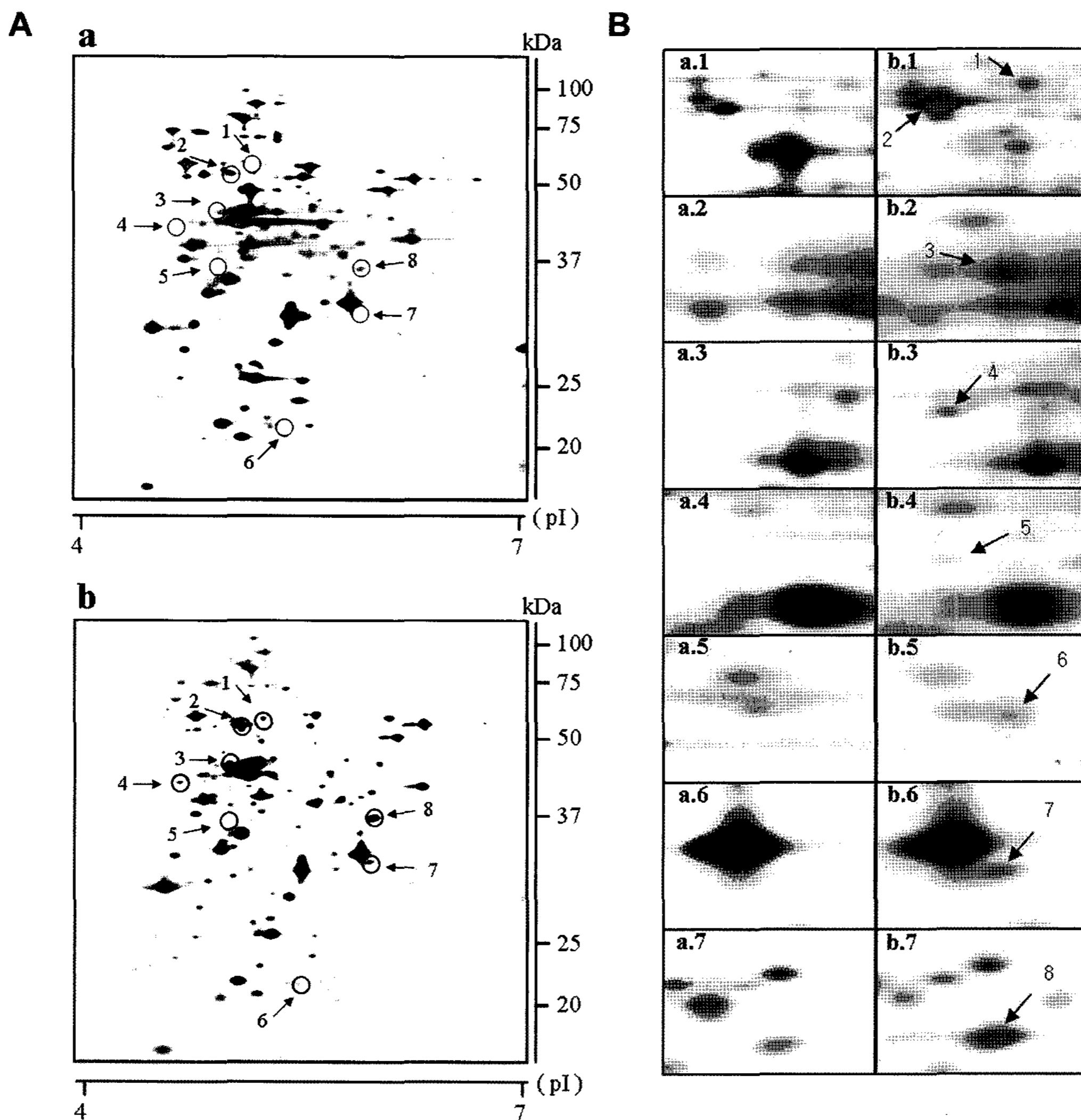


Fig. 1. 2-DE protein profiles of *V. vulnificus* M06-24/O.

A. Proteins were isolated from the bacteria incubated without (a) or with (b) INT-407 epithelial cells. Proteins of which expression was upregulated in the presence of INT-407 cells are indicated with a circle and were subjected to MALDI-TOF MS analysis. B. Part view of the selected protein spots whose abundance increased following exposure of *V. vulnificus* to INT-407 cells. Numbers represent the spot identification numbers listed in Table 2.

analyzed, 7 protein species were identified and encoded by 5 genes on chromosome 1 and 2 genes on chromosome 2, respectively (Table 2). Although one protein was of unknown function, most belonged to functional categories (clusters of orthologous group, COG) on the basis of the databases of the *V. vulnificus* CMCP6 and YJ016 genomes, which were retrieved from GenBank (AE016795; AE016796; BA000038) (Table 2). Three proteins involved in the COG posttranslational modification/protein turnover/chaperones such as GroEL2, Tig, and TsaA were induced upon exposure to INT-407 cells (Table 2, Fig. 1). Proteins such as TrpD (COG amino acid transport/metabolism), FbpA (COG inorganic ion transport/metabolism), PurH (COG nucleotide transport/metabolism), and TufB (COG translation) were also found to be more abundant in the *V. vulnificus* exposed to INT-407 cells (Table 2, Fig. 1). Consequently, it is apparent

that exposure to host cells induces expression of numerous proteins in *V. vulnificus*.

Generation and Confirmation of the Mutants that Lack the Host-induced Proteins

To evaluate the roles of the host-induced proteins in virulence, the 6 genes (*tig*, *fbpA*, *purH*, *trpD*, *tsaA*, and *groEL2*) encoding the proteins were inactivated separately by allelic exchanges. For inactivation *in vitro*, each gene was amplified by PCR using a pair of primers as indicated in Table 3. The resulting PCR products were cloned and disrupted by insertion of the 1.2-kb *nptI* DNA conferring resistance to kanamycin into the unique restriction enzyme sites within the ORF of each gene, as described previously [9, 27]. The disrupted genes were liberated and cloned into pDM4 [26] to result in pOH0514 (*tig::nptI*), pOH0515

Table 2. Protein species more abundant in the *V. vulnificus* exposed to the INT-407 epithelial cells.^a

Functional category	Protein	Gene	Locus tag	Sequence coverage (%) ^b	M _r /pI (observed)	Ratio ^c	Spot No.
Amino acid transport and metabolism	Anthranilate phosphoribosyl transferase	<i>trpD</i>	VV1_3066	38	35.4/4.9	2.8	5
Inorganic ion transport and metabolism	ABC-type Fe ³⁺ transport system, periplasmic component	<i>fbpA</i>	VV2746	37	37/5.9	2.1	8
Nucleotide transport and metabolism	AICAR transformylase/IMP cyclohydrolase	<i>purH</i>	VV1_1227	55	57.4/5	1.7	1
Posttranslational modification, protein turnover, chaperones	Chaperonin GroEL2 (HSP60 family)	<i>groEL2</i>	VV2_1134	47	55.9/4.9	2.1	2
	FKBP-type peptidyl-prolyl <i>cis-trans</i> isomerase	<i>tig</i>	VV1_0024	49.0	48.2/4.8	7.3	4
	Peroxiredoxin (AhpC/Tsa family)	<i>tsaA</i>	VV1_0453	59	22.2/5.2	2.6	6
Translation	GTPase-translation elongation factor	<i>tufB</i>	VV1_1339	29	43.1/4.9	2	3
	Uncharacterized protein			53	34.8/5.8	3.8	7

^aFunctional categories, protein names, gene names, and locus tag numbers are based on the database of the *V. vulnificus* CMCP6 (GenBank: AE016795, AE016796), except *fbpA* that is identified based on the YJ016 genome (GenBank: BA000038). Mass fingerprints of the proteins were obtained by MALDI-TOF MS and analyzed using MS-fit (<http://prospector.usuf.edu/ucshtml4.0/msfit.htm>).

^bBased on complete protein sequences.

^cRatio of the protein spot intensities of M06-24/O cultured with versus without INT-407 cells.

(*fbpA::nptI*), pOH0518 (*purH::nptI*), pOH0520 (*trpD::nptI*), pOH0522 (*tsaA::nptI*), and pOH0526 (*groEL2::nptI*) (Fig. 2A, Table 1). *E. coli* SM10 λ *pir*, *tra* containing each of the resulting plasmids was used as a conjugal donor to generate isogenic mutants of *V. vulnificus* M06-24/O by homologous recombination [25].

The conjugation and isolation of the transconjugants were conducted according to methods previously described [16, 17, 19]. As shown in Fig. 2B, double crossovers, in which wild-type gene was replaced with the disrupted allele, were confirmed by PCR. For example, PCR analysis

of the genomic DNA from the wild-type M06-24/O using the primers TIG001 Forward and Reverse produced a 0.8-kb fragment (Fig. 2B), whereas the genomic DNA from the *tig* mutant OH0501 resulted in an amplified DNA fragment approximately 2.0 kb in length. This 2.0-kb fragment was in good agreement with the projected size of the DNA fragment containing the wild-type *tig* (0.8-kb) and *nptI* gene (1.2-kb). In a similar way, the mutants in which *fbpA*, *purH*, *trpD*, *tsaA*, and *groEL2* were inactivated were confirmed by PCR using a pair of primers as indicated in Table 3 (Fig. 2B), and named OH0502,

Table 3. Oligonucleotides used for PCR in this study.

Oligonucleotide	Oligonucleotide sequence, 5'→3' ^a	Target genes ^b	
For construction of mutants			
	Forward	Reverse	
TIG001	CCGTCGTTTCGATGGTTTCC	CGTCGATCGCTTGCTCTTTG	<i>tig</i>
FBPA001	GGCTGCTGAAGAGGTGAATG	ATGTGCTGTGCTTTGTCCG	<i>fbpA</i>
PURH001	AGGCATCGCAGTCACTGAAG	TCGGATCGGTTTGGTAAGCG	<i>purH</i>
TRPD001	GGAAACACCCGATGAAATTG	TTACGTCGATCGCTTGTTC	<i>trpD</i>
TSAA001	AACGTCGATTGGCTCACAAA	ACTTCGCCGTGCTTCTGGTG	<i>tsaA</i>
GROEL2001	GCTCTAGAGCTCGCTGGCAATCTTAGTC	GCTCTAGAGCGAGGAGAATATCATGGCTG	<i>groEL2</i>
For complementation of mutants			
	Forward	Reverse	
PURH002	<u>GAGCTCAGGAAATTGGAAGCATGAAC</u>	<u>GGGTACCCATAAAAACTAGTGGCGGAAGT</u>	<i>purH</i>
TRPD002	<u>GAGCTCGGGAGAAAAGATTATGG</u>	<u>GGTACCATCTTGGCTAGGACTTCC</u>	<i>trpD</i>
TSAA002	<u>GAGCTCGAGGAGCAAAAAAATGGTACTA</u>	<u>GGGGTACCCCTTATTTTTTAAGGTCAGCT</u>	<i>tsaA</i>
GROEL2002	<u>GGGTACCCTCCTTATAGTACCGAC</u>	<u>GGGGATCTAAAGTCTGCTACACA</u>	<i>groEL2</i>

^aRegions of oligonucleotides not complementary to corresponding target genes are underlined.

^bTo where the oligonucleotides are hybridized.

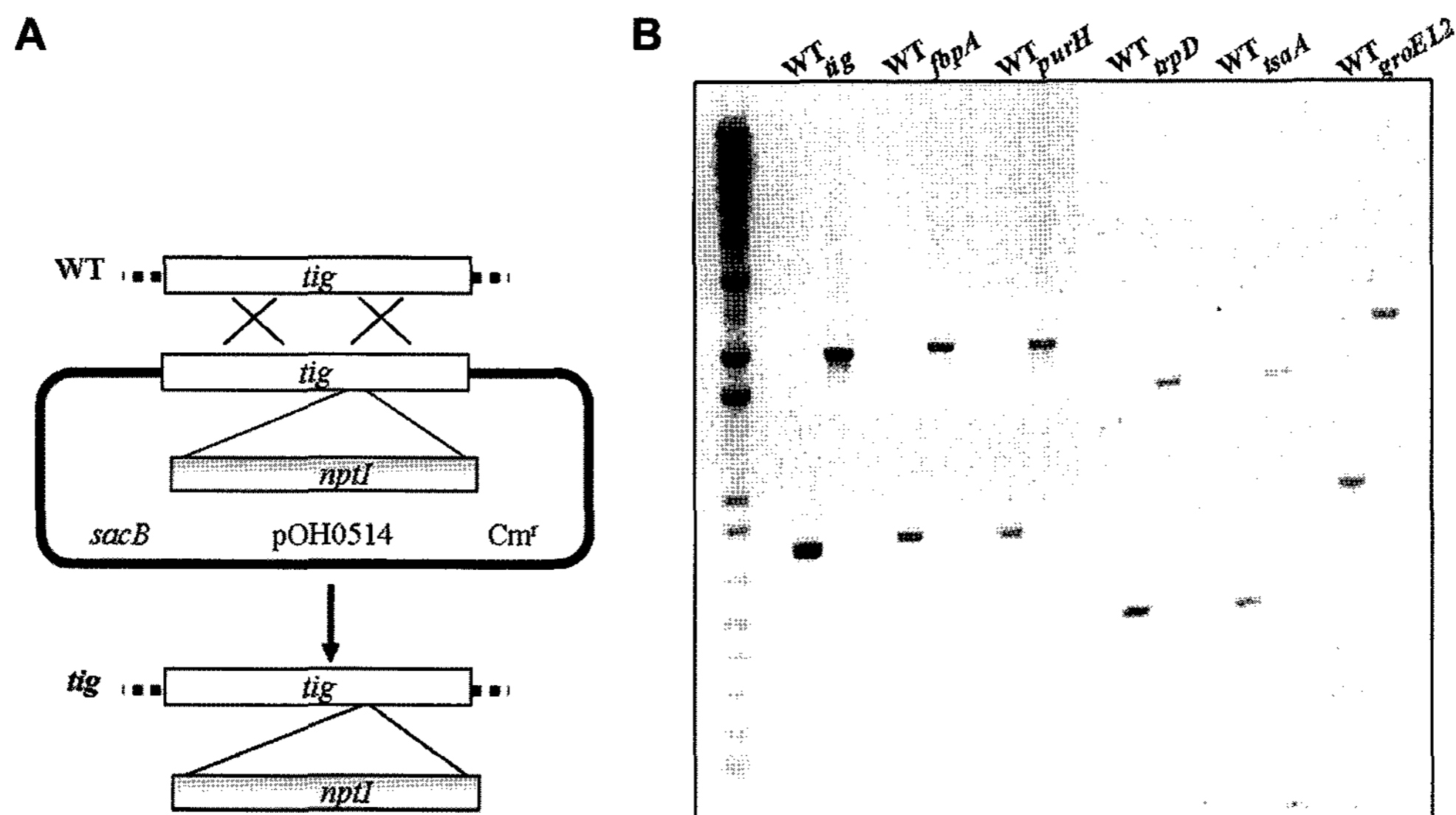


Fig. 2. Allelic exchange procedure and construction of the *tig*, *fbpA*, *purH*, *trpD*, *tsaA*, and *groEL2* mutants.

A. Schematic representation of construction of the *tig* mutant as an example. Double homologous recombination between the wild-type gene on the chromosome and mutant allele on the plasmid pOH0514 (Table 1) led to interruption of the *tig* gene and resulted in construction of the *tig* mutant. The *fbpA*, *purH*, *trpD*, *tsaA*, and *groEL2* mutants were constructed using similar procedures (details in text). Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open boxes, the target *tig* gene; shaded boxes, the *nptI* gene; large X's represent genetic crossing over; *sacB*, levansucrase gene. **B.** PCR analysis of the wild type and the mutants generated by allelic exchanges. The sizes of the PCR products from the mutants are approximately 1.2 kb bigger than those from wild type, indicating that the target genes were successfully disrupted by insertion of the *nptI* cassette (1.2 kb) (details in text). Molecular size markers (1-kb plus ladder; Invitrogen, Carlsbad, CA, U.S.A.) are on the left.

OH0503, OH0504, OH0505, and OH0506, respectively (Table 1).

Effects of the Lack of the Host-induced Proteins on the Cytotoxicity of *V. vulnificus*

Effects of the *tig*, *fbpA*, *purH*, *trpD*, *tsaA*, and *groEL2* mutations on the cytotoxicity to INT-407 cells were examined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant. The preparation of the INT-407 cells and infection with the bacterial cultures were performed in a 96-well tissue culture plate (Nunc, Roskilde, Denmark), as described previously [10, 28, 29]. Among the mutants, the *purH* mutant OH0503, *trpD* mutant OH0504, *tsaA* mutant OH0505, and *groEL2* mutant OH0506 exhibited significantly less LDH activity at an MOI of 10 for 90 min (Fig. 3). The levels of LDH activities from the INT-407 cells infected with OH0503, OH0504, OH0505, and OH0506 were from 2-fold to 10-fold less than that obtained with wild type (Fig. 3).

It was examined whether the re-introduction of the *purH*, *trpD*, *tsaA*, and *groEL2* genes could complement the decrease of cytotoxic activity of OH0503, OH0504, OH0505, and OH0506 respectively. For this purpose, the *purH*, *trpD*, *tsaA*, and *groEL2* amplified by PCR with the primers (Table 3) were subcloned into the broad host-range vector pJH0311 [6] to result in pOH031, pOH032, pOH033, and pOH034, respectively (Table 1). The lower LDH activities of each mutant were restored to the levels comparable to those obtained from the wild type, when INT-407 cells

were infected with the respective complemented strains (Fig. 3). Therefore, the decreased cytotoxic activities of OH0503, OH0504, OH0505, and OH0506 were confirmed as results from the inactivation of functional *purH*, *trpD*, *tsaA*, and *groEL2*, rather than any polar effects on genes downstream of each. These results suggest that PurH,

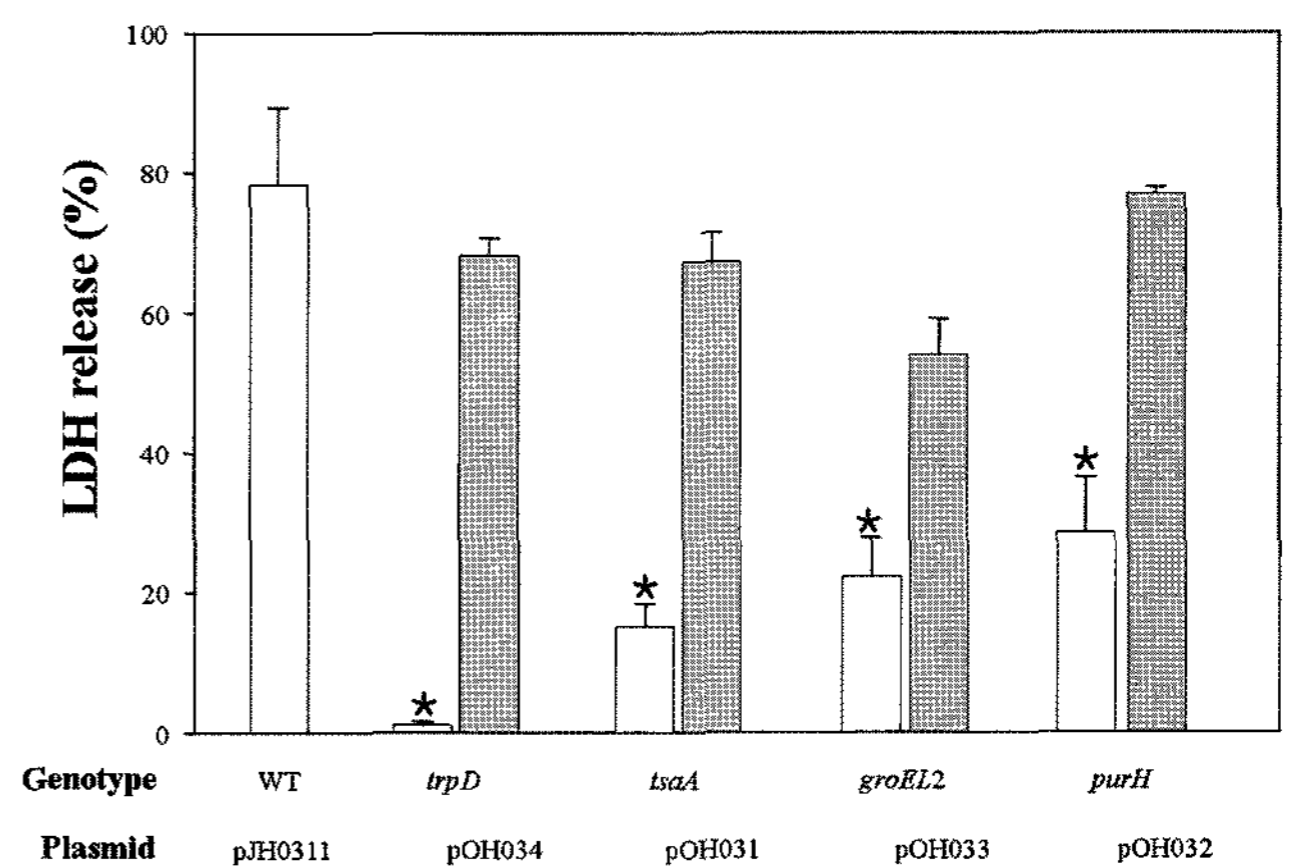


Fig. 3. Effects of the *purH*, *trpD*, *tsaA*, and *groEL2* mutations on the cytotoxicity to INT-407 cells.

INT-407 cells were infected with the wild-type and mutant strains containing pJH0311 (control, white bar) or respective plasmids for complementation (gray bar) as indicated at an MOI of 10 for 90 min. The cell cytotoxicity was determined by an LDH release assay. The data represent mean \pm SEM from three independent experiments. *, $P < 0.01$ relative to groups infected with the wild type.

Table 4. Comparison of specific growth rate of the wild type and the *purH*, *trpD*, *tsaA*, and *groEL2* mutants in various media.

Medium ^a	Specific growth rate (μ_{\max}) ^b (h ⁻¹)				
	WT	<i>purH</i>	<i>trpD</i>	<i>tsaA</i>	<i>groEL2</i>
LBS	1.764	1.972	1.746	1.609	1.786
INT-407 lysate	1.792	0.170	0.51	0.459	1.656

^aDetails are in the text.^bSpecific growth rate at an exponential growing stage.

TrpD, TsaA, and GroEL2 are essential for virulence of the *V. vulnificus* in tissue cultures.

Comparison of Growth Rate of the *V. vulnificus* Strains

To examine whether reduced cytotoxicity of the *purH*, *trpD*, *tsaA*, and *groEL2* mutants resulted from defects in their growth, we compared growth rates of the *purH*, *trpD*, *tsaA*, and *groEL2* mutants with that of the wild type (Table 4). To prepare the INT-407 lysate, INT-407 cells cultured as described above were scraped and sonicated to complete lysis. Wild-type and mutant strains were inoculated into the LBS broth, and INT-407 lysate and bacterial growth in a respective growth medium was monitored by counting colony forming units (CFU) on LBS agar plates. Based on these values of CFU, maximum specific growth rates, μ_{\max} , defined as a specific growth rate at an exponential phase, were calculated by methods described elsewhere [18].

The growth rates of the mutants in LBS were not significantly different from that of the wild type. However, the growth rates of the *purH*, *trpD*, and *tsaA* mutants in the INT-407 lysate were much lower than that of the wild type. These results suggest that the decreased virulence of the *purH*, *trpD*, and *tsaA* mutants likely resulted from their growth defect when infected to the INT-407 cells. In contrast, the growth rate of the *groEL2* mutant showed no difference in the INT-407 lysate when compared with that of the wild type. This indicated that GroEL2 could play a role in the pathogenesis of *V. vulnificus* by contributing to production of a potential virulence factor(s) rather than assuring growth of the pathogen in the host.

In the present study, eight proteins of *V. vulnificus* were induced by exposure to INT-407 cells, and among these, PurH, TrpD, TsaA, and GroEL2 appeared to be essential for virulence of the pathogen, as measured by its ability of cytolytic activity. However, it is apparent that these proteins are not directly involved in damaging or injuring host cells. Rather, these proteins seem to be primarily involved in the metabolism and utilization of nutrients in the host environments, or are required for maintaining the protein conformation for survival under stresses such as oxidative stresses. Undoubtedly, the abilities to outwit the host, acquire essential nutrients, and multiply in the host are often important for bacterial pathogenesis. Consistent with this, the *pur* mutant of *S. typhimurium* is defective for intracellular growth and is avirulent [21]. The

mutation of *trpD* also resulted in an auxotrophic mutant of *Mycobacterium tuberculosis* with reduced intracellular growth and survival in macrophages [32]. Therefore, it is not difficult to imagine that the growth defect in the INT-407 cells is the major, if not sole, reason for the attenuated virulence of the *purH* and *trpD* mutants of *V. vulnificus* (Table 4, Fig. 3).

Pathogenic bacteria are highly adapted microorganisms with a survival strategy that requires multiplication on or within another living organism [24]. TsaA, a member of the AhpC/Tsa family, is a thiol-specific antioxidant enzyme. Proteins of the AhpC/Tsa family are predicted to control the cellular level of reactive oxygen intermediates, which are detrimental for the optimal growth by damaging the cellular molecules [11, 31]. Consistent with this, the growth rate of the *tsaA* mutant in the INT-407 lysate was lower than that of the wild type (Table 4). These results suggest that TsaA aids the growth and adaptation in host environments, potentially contributing to the virulence of *V. vulnificus*.

Additionally, GroEL2, a chaperonin of the HSP60 family, was also induced upon exposure to host cells (Fig. 1, Table 2) [12]. GroEL2 is induced by a variety of stresses and plays an important biological role in protein folding and translocation across the membrane [36]. It is noted that the growth rate of the *groEL2* mutant was not significantly different from that of wild type in the INT-407 lysate, indicating that the decreased virulence of the *groEL2* mutant is not correlated with the growth defect as observed in the *tsaA* mutant (Table 4). Although other explanations are possible, this indicated that the folding and translocation of many virulence factors involved in injuring host cells may be regulated by GroEL2. However, the exact role of GroEL2 in the virulence of *V. vulnificus* needs additional study.

Acknowledgments

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