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Identification and Characterization of the *wbpO* Gene Essential for Lipopolysaccharide Synthesis in *Vibrio vulnificus*

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Abstract A *wbpO* gene encoding a putative UDP-*N*-acetyl-D-galactosamine dehydrogenase was identified and cloned from *Vibrio vulnificus*. LPS production was altered by disruption of the *wbpO* gene through allelic exchanges. The function of the *wbpO* gene in virulence assessed *in vitro* and in mice revealed that WbpO is important in both the pathogenesis of *V. vulnificus* and the biosynthesis of LPS.

Key words: *V. vulnificus wbpO*, LPS, pathogenesis

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, excess levels of iron, and immunocompromised conditions [9, 18, 33]. Wound infections can also result from exposure to seawater or handling of shellfish contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death can occur within one to two days after the first signs of illness [9, 18, 33].

The symptoms accompanied with *V. vulnificus* septicemia as well as the inflammatory response observed in wound infections are associated with the endotoxic activity of lipopolysaccharides (LPS) molecules, making them a prime candidate as a major virulence factor. So far, very little is known about the biosynthetic pathway for the LPS of *V. vulnificus*, and the genes encoding enzymes involved in the production of *V. vulnificus* LPS have not yet been identified. However, it is generally believed that a similar biosynthetic pathway operates in Gram-negative bacteria. As such, the biosynthetic pathways and molecular genetics of LPS production in *Pseudomonas aeruginosa* have been widely studied [1]. The common glycolytic metabolite Glc-1-P (glucose-1-phosphate) is first converted to UDP-GlcNAc (UDP-*N*-acetyl-D-glucosamine), the main activated precursor of surface associated carbohydrate synthesis [1, 32]. UDP-GalNAc (UDP-*N*-acetyl-D-galactosamine) is then formed by C_4 epimerization of UDP-GlcNAc [3]. UDP-GalNAcA (UDP-*N*-acetyl-D-galactosaminuronic acid), the product of further dehydrogenation of UDP-GalNAc, is an important intermediate used for the biosynthesis of different uronic acid sugars of LPS and CPS (capsular polysaccharides) that contain GalNacA or derivatives, not only in *P. aeruginosa* but also in other organisms [36]. Furthermore, it has recently been reported that the epimerization and dehydrogenation are carried out by the gene products of *wbpP* and *wbpO*, respectively [1].

The characteristics of *V. vulnificus* LPS as a potential virulence factor have been studied primarily using the purified LPS in animal models [21]; intravenous injections of *V. vulnificus* LPS into mice caused mean arterial pressure to decrease within 10 min, with further decline and subsequent death in 30 to 60 min. One of the leading factors believed to contribute to fatality in Gram-negative septicemia is an overproduction of tumor necrosis factor (TNF) in response to LPS, leading to overstimulation of nitric oxide (NO) synthase [6]. In fact, increased mortality in cirrhotic mice due to *V. vulnificus* infection was found to depend on the serum TNF-α level [6]. In a recent study, purified LPS caused an increase of TNF-α expression in primary human peripheral blood mononuclear cells *in vitro* [24]. Therefore, these data suggest that the stimulation of TNF in response to *V. vulnificus* LPS is followed by nitric oxide production, and leads to endotoxic shock.

However, despite the reported effects of administering *V. vulnificus* LPS to experimental animals, a definitive

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Table 1. Plasmids and bacterial strains used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial Strains		
V. vulnificus		
ATCC29307	Clinical isolate; virulent	Laboratory collection
NY012	ATCC29307 with *wbpO*::*nptI*; Km^r	This study
E. coli		
DH5α	*supE44 Δ lacU169 (ϕ80 lacZ* ΔM15*) hsdR17 recA1 endA1 gyrA96 thi-1 relAI*	Laboratory collection
SM10 λ *pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*; Km^r; host for π-requiring plasmids; conjugal donor	[22]
Plasmids		
Mini-Tn5 *lacZ*1	R6K γ *ori*; suicide vector; *oriT* of RP4; Ap^r	[4]
pCVD442	R6K γ *ori*; *sacB*; suicide vector; *oriT* of RP4; Ap^r	[5]
pLAFR3	IncP *ori*; cosmid vector; Tc^r	[32]
pUC18	Cloning vector; Ap^r	Laboratory collection
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	[23]
pRK415	IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc^r	[13]
pNY0200	Cosmid library containing *wbpO*; Tc^r	This study
pNY0202	2.8-kb SphI-BglI fragment containing *wbpO* cloned into pUC18; Ap^r	This study
pNY0305	1.3-kb SacI-SacI fragment containing *wbpO* cloned into pUC18; Ap^r	This study
pNY0307	pNY0305 with *wbpO*::*nptI*; Ap^r, Km^r	This study
pNY0309	pCVD442 with *wbpO*::*nptI*; Ap^r, Km^r	This study
pNY0311	pRK415 with *wbpO*; Tc^r	This study

[a]: Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

analysis of the role of *V. vulnificus* LPS, through construction of a defined mutation, has not yet been reported. We, therefore, undertook this study to identify the function of *V. vulnificus* LPS during the infectious process, by constructing an isogenic *wbpO* mutant of *V. vulnificus* and applying the molecular version of Koch's postulates [7, 8]. The possible role of WbpO in virulence was demonstrated by comparing the virulence of the mutant with that of its parental wild-type in *in vitro* cell cultures and in mice.

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used for the plasmid DNA replication or conjugational transfer of plasmids were grown in a Luria-Bertani (LB) broth or LB broth containing 1.5% (w/v) agar. Unless otherwise noted, the *V. vulnificus* strains were grown in an LB medium supplemented with 2.0% (w/v) NaCl (LBS). When required, appropriate antibiotics were added to the media as follows: ampicillin 100 μg/ml, kanamycin 50 μg/ml, and tetracycline 10 μg/ml. Media components were all purchased from Difco (Detroit, MI, U.S.A.), and the chemicals from Sigma (St. Louis, MO, U.S.A.). For data analysis, the averages and standard errors of the mean (SEM) were calculated from at least three independent determinations. The statistical significance of the difference among the *V. vulnificus* strains was evaluated using Student's unpaired *t* test (SAS software, SAS Institute Inc., Cary, NC, U.S.A.). Significance was accepted at $p<.05$.

Cloning and Sequence Analysis of *wbpO* Gene

Previously, we generated a library of *V. vulnificus* mutants by random transposon mutagenesis using a mini-Tn5 *lacZ*1 [4, 28]. In the present study, therefore, a mutant exhibiting decreased cytotoxic activity toward INT-407 intestinal epithelial cells was screened from this mutant library. A DNA segment flanking the transposon insertion was amplified from the genomic DNA of the mutant by PCR, as described previously [14, 28]. Since the nucleotide sequence of the resulting PCR product, a 663-bp DNA fragment, revealed 49% identity with that of *P. aeruginosa wbpO*, the DNA was labeled with [α-^{32}P]dCTP and named WbpOP. To clone the full gene of the *V. vulnificus wbpO*, a cosmid library of *V. vulnificus* ATCC29307 constructed using pLAFR3 [14, 28] was screened using WbpOP as a probe. A colony showing a positive signal was isolated, and the cosmid DNA was purified and named pNY0200 (Fig. 1A). A 2.8-kb band from the cosmid DNA digested with SphI and BglI was then purified and ligated into pUC18 to result in pNY0202, as shown in Fig. 1.

The nucleotide sequence of the DNA fragment in pNY0202 was determined by primer walking (Korea Basic Science Research Center, Kwang-Ju, Korea), and the sequence data were submitted to the GenBank [Accession number AY350749]. The nucleotide sequence revealed a coding region consisting of 1,284 nucleotides (Fig. 1A). The nucleotide sequence *wbpO* from *P. aeruginosa* was

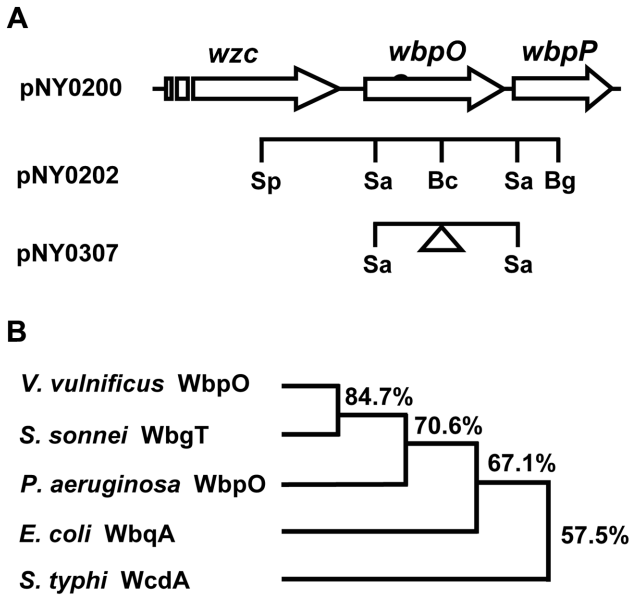

Fig. 1. *V. vulnificus wbpO* gene locus, and relatedness of *wbpO* and polysaccharide biosynthesis genes reported from other Enterobacteriaceae.

A. Plasmid pNY0202 was used to determine the nucleotide sequence of *wbpO*. The open boxes represent the locations of a complete ORF (*wbpO*) and two less characterized ORFs (*wzc* and *wbpP*) and the directions of their transcription. Plasmid pNY0307 was used for the construction of the *wbpO*::*nptI* mutants. The insertion position of the *nptI* cassette is indicated by an open triangle. Abbreviations; Bc, BclI; Bg, BglI; Sa, SacI; Sp, SphI. **B**. Dendrogram showing the amino acid sequence relatedness of *V. vulnificus* WbpO, and gene products of putative polysaccharide biosynthesis genes derived using the DNASIS alignment program (version 2.6, Hitachi Software Engineering Co., Ltd., Japan), and based on the amino acid sequences in the GenBank databases (NCBI).

75% identical with the coding region in pNY0202 (data not shown). This information suggested that the coding region is a homolog of the *wbpO* gene reported from *P. aeruginosa*, and therefore, we named it the *wbpO* of *V. vulnificus*.

The amino acid sequence deduced from the *wbpO* nucleotide sequence revealed a protein, WbpO, composed of 427 amino acids with theoretical molecular mass of 47,423 Da and pI of 5.40. A database search for amino acid sequences similar to those deduced from the *wbpO* coding region revealed three other proteins with high levels of identity, which are involved in surface carbohydrate (polysaccharide) biosynthesis in *Shigella sonnei*, *E. coli*, and *Salmonella enterica* serovar Typhi (Fig. 1B, http//www.ncbi.nml.nih.gov). The amino acid sequence of the *V. vulnificus* WbpO was 64–87% identical to those of the proteins, and their identity appears evenly throughout the whole proteins (data not shown). The amino acid sequences for the WbpO from *V. vulnificus* and WbgT from *S. sonnei* were significantly similar (87%, 371 out of 427 amino acids). However, 10 extra N-terminal amino acid residues are present within 437 amino acids of the WbgT from *S. sonnei*.

Among these, the gene product of *P. aeruginosa wbpO* has been biochemically and genetically well characterized [1, 36] Furthermore, the enzymatic characteristics of the WbpO have recently been confirmed [36]. The WbpO is an $NAD(P)^+$-dependent UDP-GalNAc dehydrogenase that produces UDP-GalNAcA, which is an important intermediate for LPS biosynthesis in *P. aeruginosa* [1]. The functions of the WbgT of *S. sonnei*, WbqA of *E. coli*, and WcdA of *S. enterica* serovar Typhi are less well characterized, and therefore, these proteins have different names. However, recent genetic studies support that they are all homologues with similar, if not identical, functions, and that they all contribute to surface carbohydrate biosynthesis [30, 35]. All of this information suggests that the *wbpO* gene also encodes the protein required for surface carbohydrate biosynthesis by *V. vulnificus*.

Generation and Confirmation of *wbpO*::*nptI* Mutant

The role of the *wbpO* gene product in virulence was examined by constructing a *wbpO* mutant of *V. vulnificus*. For this purpose, the *wbpO* gene in pNY0305 (Table 1), that was constructed by ligation of a 1.3-kb SacI-SacI fragment of pNY0202 with pUC18, was inactivated *in vitro* by insertion of *nptI* encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin [23]. The 1.2-kb DNA fragment carrying *nptI* was isolated from pUC4K (Pharmacia, Piscataway, NJ, U.S.A.) and inserted into a unique BclI site present within the open reading frame (ORF) of *wbpO*. The 2.5-kb *wbpO*::*nptI*

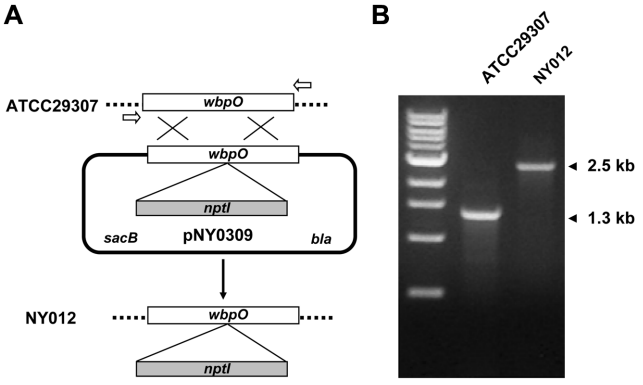

Fig. 2. Allelic exchange procedure and construction of *wbpO*::*nptI* isogenic mutant.

A. Double homologous recombinations between strain ATCC29307 and plasmid pNY0309 led to an interruption of the *wbpO* gene and resulted in the construction of mutant NY012. The dashed lines represent the bacterial chromosome; the full line, the plasmid DNA; the open box, the target *wbpO* gene; the shaded box, the *nptI* gene; the open arrows, the locations of the oligonucleotide primers used to confirm the *nptI* insert; and the large X's represent genetic crossing over. Abbreviations; *sacB*, levansucrase gene; *bla*, β-lactamase gene. **B**. PCR analysis of ATCC29307 and isogenic mutant NY012 generated by allelic exchange. Molecular size markers (1-kb-plus DNA ladder, New England Biolabs) and PCR products are indicated.

cartridge from the resulting construct (pNY0307, Fig. 1A) was liberated and ligated with SmaI-digested pCVD442 [5], forming pNY0309 (Table 1).

E. coli SM10λ*pir* (containing pNY0309) was used as a conjugal donor to generate the *wbpO::nptI* mutant of *V. vulnificus* ATCC29307 by homologous recombination (Fig. 2A). The *V. vulnificus wbpO* mutant chosen for further analysis was named NY012. The conjugation and isolation of the transconjugants were conducted using previously described methods [17, 27], and a double crossover, in which each wild-type *wbpO* gene was replaced with the *wbpO::nptI* allele, was confirmed by PCR, as shown in Fig. 2B. The PCR analysis of the genomic DNA from ATCC29307 with primers WbpO005F (5'-GCTCTGCAG-TAGAACCTATTTTTATGTATTAAAG-3') and WbpO006R (5'-TTAGAATTCATTTATCTCTATCCTCCCAGGAC-3') produced a 1.3-kb fragment (Fig. 2B), whereas the genomic DNA from NY012 resulted in an amplified DNA fragment of approximately 2.5-kb in length. This 2.5-kb fragment is in agreement with the projected size of the DNA fragment containing the wild-type *wbpO* (1.3-kb) and *nptI* gene (1.2-kb). The inserted *nptI* DNAs were stably maintained in the mutant, as determined by the maintenance of kanamycin resistance (all of more than 500 colonies tested) and by generation of the appropriate-sized DNA fragment by PCR (data not shown).

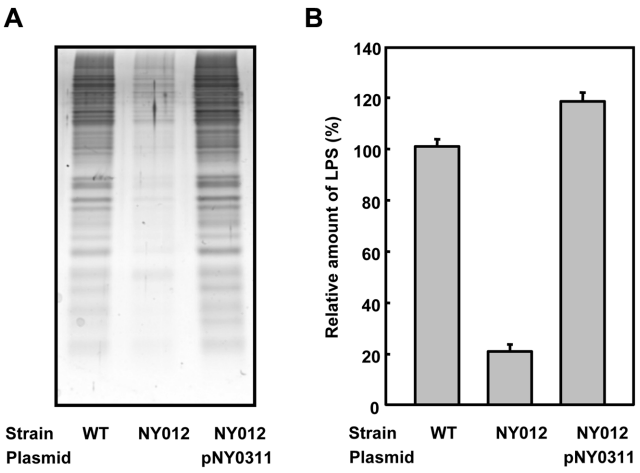

Fig. 3. Analysis of LPS.
A. The method described by Slauch *et al.* [31] was used to isolate the LPS from the wild-type (ATCC29307), *wbpO* mutant (NY012), and complemented strain NY012 (pNY0311). After separation on 12% polyacrylamide gels, LPS were visualized by silver staining as described by Hitchcock and Brown [10]. **B**. The relative amounts of LPS from each strain are presented using the amount of LPS of the wild-type as 100%.

Effects of *wbpO* Gene Mutation on Biosynthesis of LPS

To date, the WbpO of *P. aeruginosa* is the first UDP-GalNAc dehydrogenase that has been characterized at the molecular and biochemical levels. It has been suggested that the product of the dehydrogenation of UDP-GalNAc, UDP-GalNAcA, is an important intermediate for LPS biosynthesis in Gram-negative bacteria. Thus, in order to examine whether WbpO is indeed involved in the LPS biosynthesis of *V. vulnificus*, the LPS of the parental wild-type and isogenic *wbpO* mutant were compared.

For this purpose, LPS was prepared from whole cells as described by Slauch *et al.* [31]. Briefly, cells were harvested from 100 ml of LBS cultures grown to an OD_{525} of 0.6, washed with phosphate-buffered saline (PBS, pH 7.4), and then resuspended in 20 ml of LPS buffer [150 mM NaCl-20 mM MOPS (3-[*N*-morpholino] propanesulfonic acid), pH 6.9]. The cells were lysed by mixing with an equal volume of phenol previously saturated with the LPS buffer, and then the top aqueous phase was recovered. LPS in the aqueous phase was precipitated with ethanol, and resuspended in 2.5 ml of the LPS buffer. The bulk RNA and DNA collected with LPS were removed by treatment with RNase (0.25 Kunitz U, Sigma) and DNase I (100 U, Sigma), as described previously [31]. The LPS was then purified by centrifugation, and the LPS pellet was redissolved in 0.5 ml of the LPS buffer. The LPS preparations were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the LPS profiles were visualized by silver staining using the method of Hitchcock and Brown [10].

Figure 3 shows LPS from each strain after separation on an SDS-gel. For ATCC29307, LPS migrated throughout the gel and revealed distinct bands for each LPS ladder with different lengths of polysaccharide chains. When the overall migration profiles of the LPS ladders in ATCC29307 and NY012 were compared, it was apparent that the disruption of *wbpO* resulted in changes in the pattern and amount of LPS production (Figs. 3A and 3B). Quantitative measurement of LPS was carried out by measuring the intensities of each band using a UMAX digital imaging system (UTA-1100, UMAX Technologies, Inc. Fremont, CA, U.S.A.) and Kodak 1D Image Analysis software (Eastman Kodak Co. Rochester, NY, U.S.A.). As shown in Fig. 3B, The LPS synthesis was reduced in NY012 and the residual level of LPS corresponded to approximately one-third of that found in the wild-type. Moreover, it is apparent that the reduction in the LPS synthesis was not uniform throughout the LPS ladders; the lower bands of the polyacrylamide gel that represent mostly small molecular weight LPS preferentially disappeared, whereas the others were still evident in the *wbpO* mutant. A possible explanation for this observation is that the *wbpO* mutant synthesizes a structurally altered LPS complex of which a certain O side chain(s) or core carbohydrate(s) are different from those of the wild-type LPS complex. Therefore, it is obvious that the mutation in the *wbpO* of *V. vulnificus* affected both the structure and the amount of LPS.

To rule out the possibility that the alteration in the amount and pattern of LPS production resulted from polar effects of the *wbpO* insertional mutation on downstream genes, we examined whether reintroduction of pNY0311 carrying recombinant *wbpO* could reverse the alteration in the LPS production of NY012 cells. Thus, pNY0311 was constructed by subcloning the *wbpO*, which was amplified by PCR using primers WbpO005F and WbpO006R and then digested with PstI and EcoRI into the broad host-range vector pRK415 [13] linearized with the same enzymes. The resulting plasmid was mobilized into *V. vulnificus* by conjugation. The LPS production of NY012 (pNY0311) was restored to a level comparable to the level of wild-type ATCC29307 (Fig. 3). Furthermore, the overall profile for LPS ladders of the complemented strain was indistinguishable from that for ATCC29307. Therefore, the alteration in the LPS production of NY012 resulted from the inactivation of functional *wbpO* rather than any polar effects on the genes downstream of *wbpO*.

WbpO is Required for Cytotoxicity to Epithelial Cells *In Vitro*

To examine the effects of the *wbpO* mutation on the ability of *V. vulnificus* to damage epithelial cells, two different kinds of assays were performed using INT-407 (ATCC CCL-6) human intestinal epithelial cells. ATCC29307 and NY012 were grown overnight in LBS broth at 30°C. On the following day, 0.1 ml of the culture was inoculated into 100 ml of LBS broth and shaken at 30°C. After 4 h of cultivation, the bacterial cells were harvested by centrifugation and suspended at appropriate concentrations in a cell culture medium, MEM (minimum essential medium) containing 1% (v/v) fetal bovine serum (GIBCO-BRL, Gaithersburg, MD, U.S.A.). Preparation of the INT-407 cells and infection with bacterial cultures were performed in 96-well tissue culture plates (Nunc, Roskilde, Denmark), as described previously [11]. The cytotoxicity was then determined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant using a Cytotoxicity

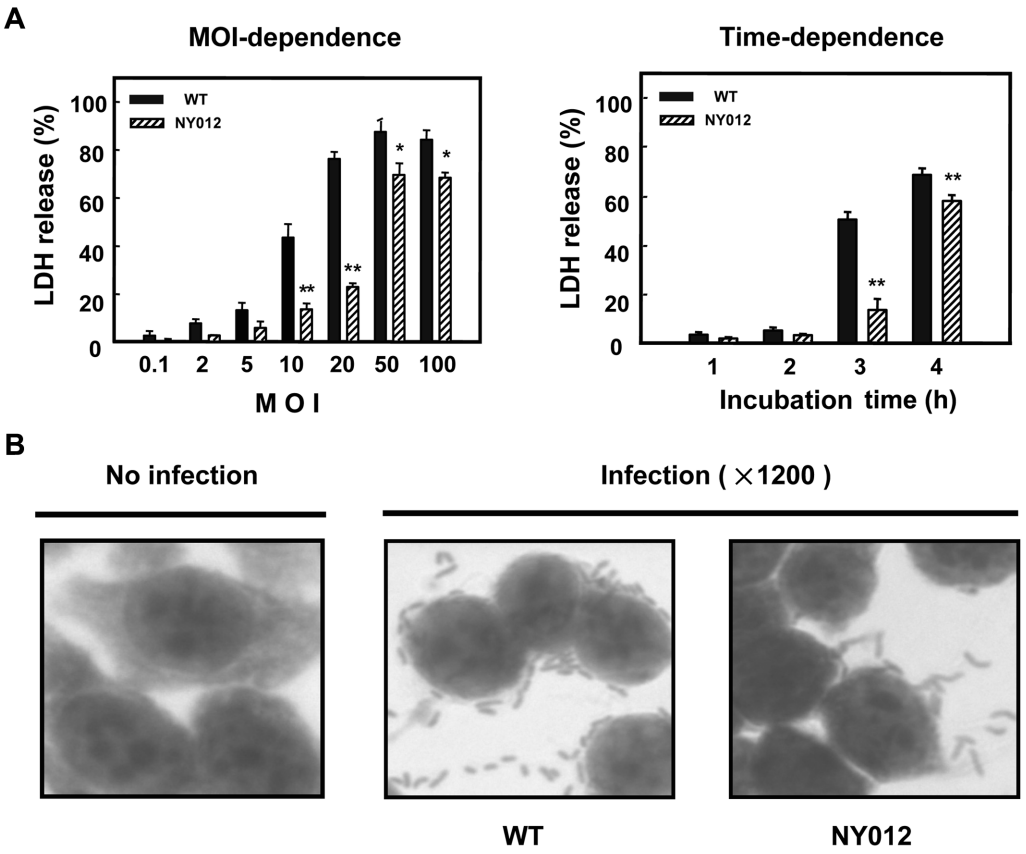

Fig. 4. Effect of *wbpO* mutation on virulence of *V. vulnificus* to INT-407 cells.
A. INT-407 cells were infected with wild-type (ATCC29307) or NY012 (*wbpO* mutant) of *V. vulnificus* at various MOIs for 3 h (left), or at an MOI of 10 for various incubation times (right). Thereafter, the cell cytotoxicity was determined by an LDH release assay. The data represent mean±SEM from three independent experiments. **P*<0.01, ***P*<0.05, relative to groups infected with the wild-type of *V. vulnificus* at each MOI or each incubation time. **B**. Microscopic observation of INT-407 cells infected with the wild-type or *wbpO* mutant of *V. vulnificus*. INT-407 cells were cultured on 18-mm-diameter glass covers in a culture dish for 24 h. Afterward, the cells were infected with the wild-type or *wbpO* mutant of *V. vulnificus* at an MOI of 10 for 3 h or not infected. The cells were observed using a light microscope (×1,200) after Giemsa staining. Left, uninfected control; middle, infected with wild-type; right, infected with *wbpO* mutant.

Detection Kit (Roche, Mannheim, Germany), and expressed by the total LDH activity of the cells completely lysed by 1% Triton-X 100 as 100%.

LDH activities from monolayers of INT-407 cells that were infected with 20 ml of a suspension of ATCC29307 and NY012 strains at different multiplicities of infection (MOI) and incubated for 3 h were determined (Fig. 4A). The *wbpO* mutant NY012 exhibited much less LDH activity when the MOI was not higher than 20. The level of LDH activity in the INT-407 cells infected with NY012 was almost 3-fold less than that in the cells infected with the wild-type. Similarly, INT-407 cells were infected at an MOI of 10, and LDH activity was assayed at different incubation times as indicated (Fig. 4A). The cells infected with NY012 exhibited lower LDH activity than the cells infected with wild-type, when the cells were incubated with the bacterial suspension as long as 3 h. Therefore, an optimal period of 3 h incubation with a constant MOI of 10 was chosen for further experiments.

To further investigate the cytotoxicity of the *wbpO* mutant NY012 and its parental wild-type, morphologic studies were also carried out using INT-407 cells infected with *V. vulnificus* strains (Fig. 4B). Microscopic observation of the INT-407 cells infected with ATCC29307 at an MOI of 10 for 3 h was carried out as previously described [15], and the stained cells were assessed for size, regularity of the cell margin, and the morphological characteristics of the nuclei. As shown in Fig. 4B, many Giemsa-stained INT-407 cells exhibited marked cellular damage after infection with the wild-type. Cytoplasmic loss and nuclear material condensation, typical phenotypes of cell death, were observed in the intestinal cells infected with the wild-type, and many *V. vulnificus* cells were observed in the disrupted cytoplasmic region of the infected cells. In contrast, fewer dead cells were observed after incubation with NY012: The cells infected with NY012 exhibited less damaged surface and cytoplasmic loss. Accordingly, these results suggest that WbpO is important with regard to the ability of *V. vulnificus* to infect and injure host cells.

WbpO is Required for Adhesion to Epithelial Cells *In Vitro*

In order to further characterize the role of WbpO in the interaction of *V. vulnificus* and intestinal epithelial cells, adhesion of ATCC29307 and NY012 to INT-407 cells was assayed. For the adhesion assay, INT-407 monolayers were prepared and infected as described above at an MOI of 10, except that the INT-407 cells were seeded onto glass coverslips placed at the bottom of the tissue culture plate (Corning, Inc., Corning, NY, U.S.A.). The bacteria were then allowed to adhere for different lengths of time. After thorough washing with PBS (pH 7.4), the cells were then fixed in methanol, stained with 0.4% Giemsa, and examined under a light microscope (Axioskop 50; Carl Zeiss Inc., Jena, Germany). The mean number of attached bacteria per cell, calculated by examining 100 cells, was used to represent the adhesion index of the strains.

When the adhesion assay was performed with the wild-type *V. vulnificus*, observation under light microscopy revealed the formation of small clusters of aggregated bacteria on the INT-407 cell surface (Fig. 5A). The number and size of the clusters increased as the incubation time increased (data not shown), and each cluster contained about 20 bacteria after 3 h of incubation (Fig. 5A). The wild-type strain adhered to the INT-407 cells and reached an adhesion index of 17.4 bacteria per cell after 3 h of infection (Fig. 5B). In contrast, with NY012, a much smaller area of the intestinal cell surface was covered with bacteria, and no clusters of aggregated bacteria were observed (Fig. 5A). At all time points studied, NY012 was consistently and significantly

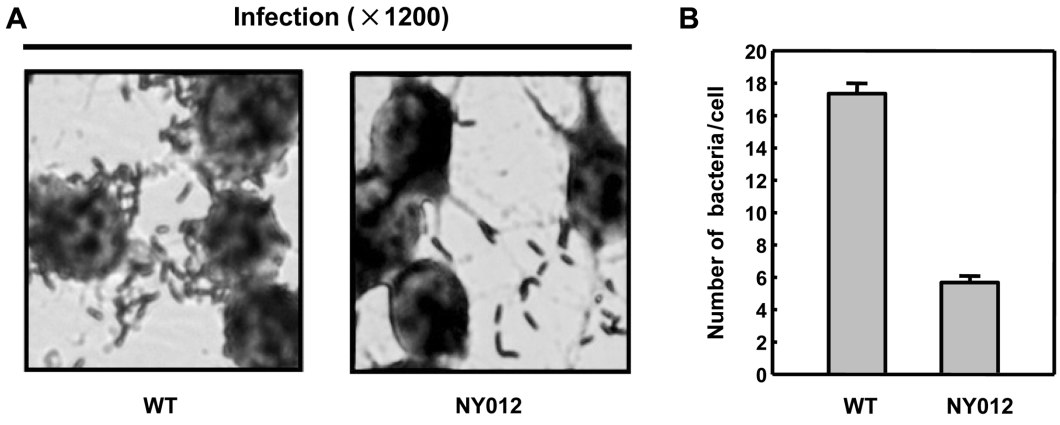

Fig. 5. Adhesion of *V. vulnificus* wild-type and *wbpO* mutant to INT-407 cells.
For both panels, INT-407 cells were cultured on glass coverslips and infected at an MOI of 10 for 3 h, using the same methods as described in the legend of Fig. 4B. **A**. After incubation with the bacteria for 3 h, the INT-407 monolayers were rinsed to remove any nonadhering bacteria. Light micrographs show the adhesion of wild-type (ATCC29307) and NY012 (*wbpO* mutant) to the INT-407 cells. **B**. The adherent bacteria were quantified and expressed as the number of bacteria per cell in the coverslip tissue culture.

less adherent than the wild-type parent strain (data not shown). When infected for 3 h, the number of the *wbpO* mutant per cell of INT-407 monolayers was about 3-fold less than that of the wild-type (Fig. 5B). It was impossible to perform adhesion assays when the incubation period was longer than 4 h, since most of the INT-407 cells were lysed, as observed in Fig. 4. Nonetheless, the results clearly revealed that the *wbpO* mutant had significantly impaired ability to attach to the epithelial cells.

Several studies have demonstrated that motility of pathogenic bacteria facilitates attachment to host epithelial cells. Thus, to explore this possibility, the motility of NY012 and the wild-type was compared. Thus, 3-μl aliquots of cultures were inoculated onto the surface of motility plates using a pipette, as described elsewhere [2]. The ability to migrate away from the inoculation point did not differ significantly (data not shown), indicating that the mutant is as motile as the wild-type. Therefore, although other explanations are possible, it is most likely that the impaired adherence exhibited by the *wbpO* mutant is due to its altered LPS production, as observed in Fig. 3.

Virulence in Mice is Dependent on *wbpO*

The role of the *V. vulnificus wbpO* gene in virulence was further examined using a mouse model. The 50% lethal doses (LD_{50}s) of the wild-type and the *wbpO* mutant were compared using ICR mice (Specific Pathogen-Free; Daehan Animal Co., Taejon, Korea), as described elsewhere [11, 12, 16]. To determine LD_{50}, the bacteria grown overnight in LBS broth at 30°C were harvested and suspended to appropriate concentrations in PBS, ranging from 10^2 to 10^8 CFU in 10-fold increments. Group of 7-week-old normal female mice (n=6) were injected intraperitoneally with 0.1 ml of serial dilutions of the bacterial suspensions. The infected mice were then observed for 24 h, and the LD_{50}s were calculated using the method of Reed and Muench [26]. The mice were also injected intraperitoneally with 250 μg of iron dextran per g of body weight immediately before injection of bacterial cells.

The LD_{50}s in the iron-overloaded mice after intraperitoneal infection with *V. vulnificus* strains are shown in Table 2. The LD_{50} for NY012 was greater than 10^7 CFU, compared with an LD_{50} lower than 10^4 CFU for the wild-type. Therefore, in the mouse model of intraperitoneal infection, in which the *wbpO* mutant exhibited more than 3-log increase in the LD_{50} over the wild-type, the *wbpO* mutant appeared to be virtually less virulent than its parental wild-type. This result indicated that the WbpO required for the synthesis of the complete LPS complex of *V. vulnificus* is apparently important for the pathogenesis of the bacteria. Taken together, it is reasonable to conclude that the *wbpO* is essential for the virulence of *V. vulnificus* in mice as well as in tissue cultures.

Table 2. Effects of *wbpO* mutation on the lethality of *V. vulnificus* to mice.[a]

Strain	Intraperitoneal LD_{50} (CFU)
ATCC29307 (n=6)	5.9×10^3
NY012 (n=6)	4.2×10^7

[a]n, number of iron-treated mice for each inoculation group, ranging from 10^2 to 10^8 CFU in 10-fold increments.

The diseases resulting from infection with *V. vulnificus* are remarkable as regards their invasive nature, ensuing severe tissue damage, and rapidly fulminating course. This multifaceted nature of the pathology of the diseases typically indicates that numerous virulence factors are involved in the pathogenesis of the organism. Therefore, understanding the molecular pathogenesis of the virulence factors is critical in developing improved treatment and prevention, as well as elucidation of how the bacteria can circumvent host defenses, multiply in the host, and cause such extensive damage. A variety of endotoxins and exotoxins, including polysaccharide capsules, iron-sequestering systems, an elastolytic protease, cytolytic hemolysin, and phospholipase A2, have been implicated as putative virulence factors for *V. vulnificus* [9, 18, 33]. Another putative virulence factor is LPS. However, most of the data on *V. vulnificus* virulence factors have been derived from animal models in which the putative virulence factors are purified and used to inject to animals.

Therefore, the major problem that needs to be addressed is discrepancy between the infection experiments using *V. vulnificus* mutant strains lacking presumed virulence factors and those studies that relied primarily on the injection of the virulence factors into animals. Notably, a null mutation either in the cytolytic hemolysin or in the elastolytic protease had no effect on virulence in mice [11, 34]. In the present study, the function of LPS during an infectious process was examined by constructing an isogenic *wbpO* mutant of *V. vulnificus* and comparing the virulence of the mutant with that of the parental wild-type. When compared with the wild-type, the *wbpO* mutant, which is an LPS mutant, was less adherent and less toxic to intestinal epithelial cells *in vitro*, and also exhibited significantly diminished virulence in mice as measured by its ability to cause death. These results led us to confirm that LPS is an important virulence factor in the pathogenesis of *V. vulnificus*. Besides LPS, to date, only two other virulence factors, the capsule and iron acquisition systems, have been confirmed to be essential for the virulence of *V. vulnificus*, according to the molecular version of Koch's postulates [19, 34].

The interaction of LPS with specific receptors of cells could generate a wide range of effects on various cell systems; LPS elicits the release of a different set of cytokines, which can trigger the release of bioactive lipids, cascades of other cytokines, and reactive oxygen species from a wide variety of cells. These mediators cause increased vascular permeability, decreased cardiac contractility, vasodilation,

pulmonary hypertension, and disseminated intravascular coagulopathy, ultimately leading to shock and death [25]. Although a definitive role of LPS has not yet been established for *V. vulnificus*, it probably plays an important role in modulating the host cytokine response *in vivo,* similar to other enterobacterial LPS.

It has been noted that LPS and CPS are partially composed of the same sugar subunits, meaning that certain precursors are shared in the synthesis of both compounds [29]. Nonetheless, the gene(s) responsible for the biosynthesis of LPS has not yet been identified and characterized. To our knowledge, the data in this work represent the first description of an LPS gene in *V. vulnificus*. In the course of current sequencing analysis, parts of the *wzc* and *wbpP* homologues flanking the *wbpO* were found. Although it has been previously reported that these genes are also clustered and required for the biosynthesis of surface carbohydrates in Enterobacteriaceae [1, 30, 35], it is still unclear whether these genes are also involved in the synthesis of surface carbohydrates in *V. vulnificus*. However, based on our observation that the organization of the genes is similar, and that the sequence homology is present, it is most likely that the roles of *wzc* and *wbpP* would be analogous to those observed in other Enterobacteriaceae.

Adhesion to intestinal epithelial cells is an important step in the disease process of pathogenic bacteria; however, the contribution of LPS molecule in bacterial attachment to epithelial cells is still not well understood. It has been reported that the presence or absence of LPS polymers and differences in the structural and/or chemical nature of LPS molecules influence the physicochemical characteristics of the cell surface and hence the ability of bacteria to attach [29]. Furthermore, altering the surface hydrophobicity and surface charge of LPS has been postulated to modify the relative adhesive properties of bacteria [20]. In the current study, we have shown that *wbpO* is a potential virulence factor required for the synthesis of a complete LPS complex, and therefore, is involved in the adhesion of *V. vulnificus*. Although other explanations could be made, it is also reasonable to suggest that LPS plays a role in the virulence of *V. vulnificus* by aiding adhesion to epithelial cells. Therefore, it would be of interest to determine in future studies the nature of chemical and structural alterations in LPS polymers of the *V. vulnificus wbpO* mutant.

The nucleotide sequence of the *wbpO* gene of *V. vulnificus* ATCC29307 was deposited in the GenBank under accession number AY350749.

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