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석사학위논문

Translucent Colony-type *Vibrio vulnificus* 균주에서 Group 1 CPS Operon의 유전자 구성과 잠재적인 독력인자의 발현

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The Composition of Group 1 CPS Operon in Translucent Colony-type *Vibrio vulnificus* Strains and Their Potential Virulence Factors

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신영희의 석사학위 논문을 인준함

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Vibrio vulnificus 패혈증은 기회감염을 유발하며 높은 치사율을 가진 병원균이다. 협막(capsular polysaccharide: CPS)을 포함하여 철획득능, 세포독성, 운동성 그리고 부착이나 유착과 관련된 단백질 발현 등이 이 병원균의 독력인자로써 발병에 중요한 역할을 한다. 협막은 가장 중요한 독력인자 중 하나로 알려져 있다. Translucent colony-type (T-type) 균주는 협막 형성이 감소하였거나 없는 균으로 다른 독력인자들의 변화를 동반할 수 있는 것으로 알려져 있다.

본 연구에서는 15가지의 T-type 균주를 선택하여 group 1 CPS operon의 구성유전자들의 조성을 조사하였다. 또한 협막 이외의 다른 독력인자의 발현을 조사하였고 이들 독력인자들 사이에 상호연관성을 분석하였다. Southern blot 분석에서 T-type 균주들은 group 1 CPS operon을 가지고 있지만 관련된 몇 몇의 유전자들이 결손되어 있었으며 이와 같이 결손된 유전자의 종류에 있어서는 개체 간에 차이를 보였다.

PCR과 Western blot 분석에서 대부분의 T-type 균주는 철획득능과 관련된 몇 몇의 유전자와 이들 유전자 산물들이 발현하였으나 몇 몇 균주들은 이러한 유전자와 유전자 산물에 결손이 있었다. Urea-gel electrophoresis에서 T-type 균주들은 transferrin-bound iron을 이용할 수 있었으며 그 정도는 개체 간에 차이를 보였다. 반고체배지에서 T-type 균주들은 유주 운동성을 보였으며 그 정도는 개체 간에 차이를 보였다.

T-type 균주들은 개체 간의 차이가 있으나 HeLa P3 (S) cells에 효율적으로 부착하였다. LDH assay에서 그들은 HeLa P3 (S)에 세포독성을 나타내었고 그 정도는 개체 간에 차이를 보였다. Multi-well plate에서 T-type 균주들은 생물막을 형성하였고 그 정도는 개체 차이를 보였다. 이들 독력인자들 사이의 상호관계를 통계적 분석을 하였을 때 유주운동성은 세포부착능, 세포독성과 양성의 상호관계를 나타내었고 이러한 인자들은 생물막 형성과 음성의 상호관계를 나타내었다. 한 균주를 제외한 모든 T-type 균주들의 마우스에 대한 치사율이 협막을 가진 M06-24/O 균주의 마우스 치사율에 비해 현저히 감소되었다.

이상의 결과를 종합하여 볼 때, 협막의 존재유무가 마우스 치사율을 결정하는 가장 중요한 독력인자인 것으로 보이며 T-type 균주들은 다른 독력인자들의 변화를 동반하지 않으며 유주운동성은 세포부착과 세포독성에서 중요한 역할을 하지만 생물막 형성은 세포부착과 세포독성을 저해하는 것으로 여겨진다. 예외적으로 협막 외에 마우스 치사율을 결정할 가능성이 있는 아직 알려지지 않은 다른 독력인자가 있을 수 있음을 알 수 있었다.

중심어: *Vibrio vulnificus*, 협막, 철흡수체계, 유주 운동성, 세포부착성, 세포독성, 생물막 형성

I. Introduction

Vibrio vulnificus is an opportunistic but highly lethal human pathogen. Human infections by *V. vulnificus* occur via wound contamination or ingestion of raw oysters in susceptible patients (Grau *et al*., 2005; Strom *et al*., 2000). The fatality rate of patients with primary septicemia is greater than 50%, and death often occurs within hours of hospital admission (Kim *et al*., 2003; Nakhamchik *et al*., 2007).

A wide array of virulence factors, including capsular polysaccharide (CPS) expression, iron acquisition, cytotoxicity, motility, and expression of proteins involved in attachment or adhesion, play significant roles in the pathogenesis of the organism.

CPS is one of the few known virulence factors that are absolutely required for pathogenicity. The CPS mediates resistance of bacteria to complement-mediated bacteriolysis and phagocytosis (Nakhamchik *et al*., 2007; Park *et al*., 2005). Both CPS expression and virulence are associated with opaque colony morphology, but opaque colonies can spontaneously revert to T-type colonies in a process termed phase variation. It has been known that strains with T-type colonies (T-type) are less virulent, serum-sensitive, and cannot grow in iron-limited media, even in the presence of transferrin that is fully iron saturated (Wright *et al*., 1990), suggesting that other virulence factors may be accompanied with reduced CPS expression.

Nevertheless, the genetic variation of the group 1 CPS operon in T-type strains has not been determined. In addition, there are no comprehensive studies on which virulence factors are accompanied with reduced CPS expression, or on the relationship between virulence factors.

Therefore, in this study, we selected T-type *V. vulnificus* strains

exhibiting reduced CPS expression, investigated their genetic variation in group 1-CPS operon genes and the expression of their virulence factors, and statistically analyzed the correlation between virulence factors.

II. Materials and Methods

1. Bacterial strains, media, and culture conditions

The T-type *V. vulnificus* strains used in this study are described in Table 1. Heart Infusion (HI; BD, Flanklin Lakes, NJ, USA) medium containing 2.5% NaCl was used as a basal medium for the cultivation of *V. vulnificus* strains. Iron-limited HI broth was prepared by adding 200 μM dipyridyl into HI broth. In addition, deferrated (DF) HI broth was prepared by the method described by Leong and Neilands (Leong *et al*., 1982) to a residual iron concentration of less than 1 μM. When necessary, holotransferrin (HT; 1,200~1,600 ㎍ iron per 1 g protein) was added to DF-HI broth as an iron-source.

Of 250 *V. vulnificus* strains, 15 strains showing stable T-type colonies on 2.5% NaCl HI agar were selected. The *V. vulnificus* M06-24/O (Reddy *et al*., 1992) and CMCP6 strains showing opaque colony, and CMM710 strain with deleted *crp* showing translucent colony were used as control (Table 1).

Colony morphology was observed on 2.5% NaCl HI agar, and swarming motility was observed on the surface of 2.5% NaCl HI semisolid medium containing 0.3% agar (Kim *et al*., 2007). Unless otherwise noted, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Southern blot analysis

The wild-type M06-24/O strain and 15 T-type *V. vulnificus* strains were inoculated into 2.5% NaCl HI broths at 5 × 10^6 cells/㎖, and cultured with vigorous shaking at 37℃ for 6 h. Genomic DNAs from the *V. vulnificus* strains were isolated using the Bacteria Genomic DNA Extraction kit (G-spin™, iNtRON Biotechnology, Korea). For Southern blot analysis

(Zuppardo *et al.*,1998), the genomic DNAs were digested completely with various restriction enzymes (TaKaRa). The *Pst*I-digested genomic DNAs were used to hybridize with the probes of *wza* and *wzb*; the *Bgl*II-digested genomic DNAs with the probes of *wzc, rfaG, wbfT, wbpP,* and *wbfU*; the *Cla*I-digested genomic DNAs with the probes of *wecC* and *wbfY*; the *Hin*dIII-digested genomic DNAs with the probes of *wzx, wbjB, rmlD, wbjD,* and *wbuB*; the *Eco*R*V*-digested genomic DNAs with the *wbjV* probe. Approximately 15 ㎍ of the digested genomic DNAs were separated by 1% agarose gel electrophoresis, and transferred overnight to positively charged nylon membranes (Roche Applied Science, Germany) by capillary action. The blotted membranes were then fixed using a UV crosslinker.

Each digoxigenin (DIG)-labeled DNA probe specific for the group 1 CPS operon genes was hybridized to DNA on the blots and detected using DIG luminescent detection kit (Roche) according to the DIG system user's guide. Prehybridization and hybridization were performed in DIG Easy Hyb solution (Roche) at 37℃.

The wza-ORF-F/R, wzb-ORF-F/R, wzc-F/R, wecC-F/R, wbpP-F/R, wzx-F/R, rfaG-F/R, wbjB-F/R, rmlD-F/R, wbjD-F/R, wbuB-F/R, wbfT-F/R, wbfU-F/R, wbfY-F/R, and wbfV-F/R primers specific for the group 1 CPS operon genes were designed and yielded the correctly sized PCR fragments of 1,233-bp, 441-bp, 509-bp, 576-bp, 621-bp, 618-bp, 539-bp, 541-bp, 685-bp, 561-bp, 584-bp, 509-bp, 432-bp, 597-bp, and 621-bp respectively. Each amplified PCR product was subcloned into pCR2.1-TOPO by TA cloning, and was named pRC254, pRC256, pRC264, pRC284, pRC286, pRC266, pRC268, pRC270, pRC288, pRC290, pRC292, pRC272, pRC294, pRC280, and pRC274, respectively. The *Eco*RI-digested DNA fragments from the resulting plasmids were used as probe with only one exception that the *Spe*I-*Xho*I-digested DNA fragment was used for

rfaG probe, and each probe was labeled by using a DIG DNA labeling kit (Roche).

3. Lethality of T-type *V. vulnificus* strains to mice

Overnight-cultured *V. vulnificus* strains were inoculated into fresh 2.5% NaCl HI broth at 5 x 10^6 cells/㎖ and cultured with vigorous shaking at 37℃ for 4.5 h. Bacterial cells were harvested and washed three times, and suspended with PBS. Groups of eight-week-old specific pathogen-free female mice (n=5) were injected intraperitoneally with each bacterial strains of 1 x 10^7 cells, and the control group was given PBS (0.2 ㎖) alone. The mice were observed for 24 h.

4. PCR analysis

The wild-type M06-24/O strain and 15 T-type *V. vulnificus* strains were genetically identified by PCR targeting genes related the group 1 CPS operon or genes related to iron-uptake. The PCR primers used in this study are listed in Table 3 and Table 5. After bacteria were cultured in 2.5% NaCl HI broth overnight, genomic DNA was isolated using the bacteria genomic DNA extraction kit (G-spin™, iNtRON Biotechnology, Korea). The *V. vulnificus* genomic DNAs were mixed with PCR premix (Bioneer) containing the primers. A total 30 cycles of PCR were performed in a thermal cycler (Exicycler A2060, Bioneer, Korea) as follows: 95℃ pre-denaturation for 5 min, 95℃ denaturation for 30 sec, 55-60℃ annealing for 30 sec, 72℃ extension for 30 sec, and 72℃ post-extension for 10 min. The amplified PCR products were electrophoresed on 1.0% agarose gels and visualized by staining with ethidium bromide.

5. Western blot analysis

Western blot was conducted using the adsorbed rabbit polyclonal anti-VuuA, -IutA, and -HupA antibodies (details will be reported elsewhere). The wild-type M06-24/O strain and 15 T-type *V. vulnificus* strains were inoculated into fresh 2.5% NaCl HI broth containing 200 μM dipyridyl at 5 x 10^6 cells/㎖ and cultured with vigorous shaking at 37℃ for 12 h. The bacterial pellets containing approximately 1 x 10^8 cells were boiled for 10 min, and were used as cell lysates. The cell lysates were electrophoresed on 10% SDS-PAGE gels. Thereafter, separated proteins were transferred to nitrocellulose transfer membrane (PROTRAN, Whatman GmbH, Germany). The membranes were incubated with blocking solution (0.2% Tween 20 and 5% Skim milk in PBS) at 4℃ overnight, allowed to react with the adsorbed rabbit polyclonal anti-VuuA, -IutA, or -HupA antibody (1:250 diluted in washing buffer consisting of 0.1% Tween 20 and 1% Skim milk in PBS) as the primary antibody and with anti-rabbit-IgG-body conjugated with alkaline phosphatase (1:10,000 diluted in washing buffer) as the secondary antibody, and finally visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium substrate solution (Sigma).

6. Urea-gel electrophoresis

To observe iron-removal from transferrin during culture, 6 M urea-gel electrophoresis was used. The wild-type M06-24/O strain, the CMM710 strain with deleted *crp*, 15 T-type *V. vulnificus* strains were cultured in 2.5% NaCl HI broth containing 100 μM dipyridyl at 37℃ overnight to adapt to iron-limited conditions. These preconditioned bacteria were inoculated into DF-HI broth containing 0.5 ㎎/㎖ of HT at 1 x 10^6 cells/㎖,

and cultured with vigorous shaking at 37℃ for 24 h. Culture aliquots were with drawn at 24 h, and culture supernatants were obtained by centrifuging at 13,000 rpm for 3 min. Total 20 ㎕ of culture supernatants mixed with urea-gel sample buffer were electrophoresed on a 5% stacking and 6% running urea-gel (Kim *et al*., 2007; Makey *et al*., 1976). Proteins were visualized by Coomassie blue staining. Transferrin molecules can harbor two ferric ions per one transferrin molecule and are separated into the four forms according to iron saturation states on a 6 M urea-gel: apoferric, N-terminal monoferric, C-terminal monoferric and diferric forms (Makey *et al*., 1976).

7. Arnow test

Arnow test was used to measure the concentration of phenolate type siderophore (Arnow, 1937). The wild-type M06-24/O strain, the CMM710 strain with deleted *crp*, and 15 T-typet *V. vulnificus* strains cultured in 2.5% NaCl HI broth containing 100 μM dipyridyl were inoculated into DF-HI broth containing 1 μM ferric chloride to a concentration of 5 x 10^6 cells/㎖, and cultured with vigorous shaking at 37℃ for 24 h. Culture supernatants were obtained by centrifuging at 13,000 rpm for 3 min, and 1 ㎖ of culture supernatants were added into test tube. A 1 ㎖ of standard catechol solution was also placed in another test tube. The following reagents were added sequentially and mixed well after each addition; 1 ㎖ of 0.5 N HCl, 1 ㎖ of of nitrite-molybdate reagent (a yellow color results at this point), 1 ㎖ of 1 N NaOH (a red color results), and 1 ㎖ of distilled water. The OD_{510} was then measured. Data indicate average values and standard deviations from three experiments.

8. Swarming motility test

The wild-type M06-24/O strain, the CMM710 strain with deleted *crp*, and 15 T-type *V. vulnificus* strains grown on 2.5% NaCl HI agars at 37℃ overnight were inoculated onto the surfaces of 2.5% NaCl HI semisolid agar (0.3% Bacto-agar) using the end of toothpick. The plates were incubated overnight at 37℃ and the diameters of the spreading halos were measured (Kim *et al*., 2007; Nakhamchik *et al*., 2008).

9. Adhesion assay

Adherence assay (Lee *et al*., 2004) was performed with HeLa P3 (S) cells derived from human uterine cancer cell (Kataoka *et al.,* 1992). The overnight-cultured *V. vulnificus* strains, wild-type M06-24/O and CMCP6, CMM710 with deleted *crp* and 15 T-type *V. vulnificus* strains, were inoculated into fresh 2.5% NaCl HI broth at 5 x 10^6 cells/㎖ and cultured with vigorous shaking at 37℃ for 4.5 h. The *V. vulnificus* cells were harvested and washed three times, and resuspended to 5 x 10^7 cells/㎖ with PBS. HeLa P3 (S) cells were seeded in 24-well plates at 1 x 10^5 cells/㎖ and cultured in the presence of 5% CO_2 at 37℃. After 24 h, the cells were washed twice with 1 ㎖ of pre-warmed serum-free DMEM (PAA Laboratories GmbH, Austria), and then added with 1 ㎖ of DMEM. The HeLa P3 (S) cells were incubated with *V. vulnificus* cells at a multiplicity of infection (MOI) of 5 for 15 min at 37℃ in 5% CO_2 incubator. The cell monolayers were then washed six times with prewarmed PBS to remove nonadherent bacteria. Following the last wash, the HeLa P3 (S) cells were broken with 0.1% Triton X-100 solution for 15 min. The bacteria were recovered and pour plated onto 2.5% NaCl HI agar. The number of input

bacteria was also determined by pour plating diluted bacterial cultures onto 2.5% NaCl HI agar.

10. Cytotoxicity assay

The overnight-cultured *V. vulnificus* strains, the wild-type M06-24/O strain and the CMCP6 strain, the CMM710 strain with deleted *crp* and 15 T-type *V. vulnificus* strains, were inoculated into fresh 2.5% NaCl HI broth at 5 x 10^6 cells/㎖ and cultured with vigorous shaking at 37℃ for 4.5 h. The *V. vulnificus* cells were harvested and washed three times, and resuspended to 1 x 10^9 cells/㎖ with PBS. The CytoTox non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) was used to measure the activity of lactate dehydrogenase (LDH). HeLa P3 (S) cells were seeded in 24-well plates at 1 x 10^5 cells/㎖ and cultured in the presence of 5% CO_2 at 37℃. After 24 h, the cells were washed twice with 1 ㎖ of pre-warmed serum-free DMEM (PAA Laboratories GmbH, Austria). The HeLa P3 (S) cells were incubated with *V. vulnificus* cells at a multiplicity of infection (MOI) of 50 for 2 h. At appropriate times, supernatants were obtained and centrifuged at 13,000 rpm for 5 min at 4℃. The supernatants (50 ㎕) were transferred to a 96-well plate and mixed with the same volume of reconstituted LDH substrate mixture. After incubation at room temperature in the dark for 30 min, the reaction was stopped and OD_{495} (Park *et al*., 2005).

11. Biofilms assay

Biofilm assay was adapted from the original protocol of O'Toole and Kolter (Nakhamchik *et al*., 2008; O'Toole *et al*., 1998; O'Toole *et al*., 1998). The overnight-cultured *V. vulnificus* strains, the wild-type M06-24/O strain, the CMM710 strain with deleted *crp* and 15 T-type *V. vulnificus*

strains, were adjusted to 1 x 10^6 cells/㎖ in 2.5% NaCl HI broth, and 150 ㎕ of cell suspension was added into 96-well microtiter plates (3 wells per strain). Microtiter plates were statically incubated for 24 h at 30℃. Following measurement of the planktonic cell density at OD_{600} (OD_{Cells}) with a Epoch™ microplate spectrophotometer (Biotek, USA), medium was carefully removed and the wells were stained with 150 ㎕ of 0.1% crystal violet (CV) solution for 20 min. The stained wells were then washed three times with PBS and biofilms were resolubilized in 150 ㎕ of isopropanol-acetone (4:1). The OD_{595} of each well (OD_{CV}) was then measured. The OD_{CV}/OD_{Cells} ratio was calculated to determine the relative level of biofilm formation.

12. Correlation among virulence factors

The relationship among virulence factors in the T-type *V. vulnificus* strains was investigated through statistical analysis using the program, SigmaStat (Jandel Corporation, Version 1.0). The relationship is expressed as correlation coefficient (r): $0.00 \leq r \leq 0.25$ indicates 'little or no relationship', $0.25 \leq r \leq 0.50$ indicates 'fair relationship', $0.50 \leq r \leq 0.75$ indicates 'moderate to good relationship', and $0.75 \leq r \leq 1.00$ indicates 'very good to excellent relationship'.

III. Results

1. The genetic variation of the group 1 CPS operon

1) Southern blot analysis

The genetic organization of the typical group 1 CPS genes is illustrated in Figure 1 and the functions of the genes are summarized in Table 2. In order to determine the genetic variation of the group 1 CPS operon in T-type strains, southern blot was conducted using the specific probes for each gene (Figure 2). Of the tested 15 genes, more-than-one genes were deleted in all the 15 T-type strains. All the genes were deleted in 7 T-type strains (T-1, T-3, T-4, T-5, T-6, T-8 and T-9), 13 genes in one T-type strain (T-10), 11 genes in two T-type strains (T-2 and T-11), 9 genes in two T-type strains (T-12 and T-15), and 8 genes in three strains (T-7, T-13 and T-14). Conversely, the six genes, *wbpP*, *wzx*, *rfaG*, *wbjB*, *rmlD*, and *wbjD,* were commonly deleted in all the 15 T-type strains, which suggested that the gene products may be essential for the CPS synthesis.

The other genes were deleted with individual variance among strains, which suggested that the gene products may be unnecessary for the CPS synthesis. In addition, the same results were obtained in PCR using the specific primers for each gene (data not shown). Overall, these results indicate that T-type strains harbor group 1 CPS operon, with the more-than-one related genes being deleted with individual variance.

2) Mouse lethality

In order to determine the mouse lethality of T-type strains, the time required for killing mouse was measured after 1 x 10^7 cells of T-type strains were injected intraperitoneally. All the T-type strains did not kill

mice (n=5) within 24 h except the T-15 strain (Figure 3). The T-15 and M06-24/O strains killed all mice within 5 h. These results indicate that CPS is one of the major virulence factors determining mouse lethality.

The mouse lethality of the T-15 strain with reduced CPS expression and deleted group 1 CPS operon genes was comparable to that of M06-24/O strain with sufficient CPS expression. This suggests that yet-unknown virulence factor(s) determining mouse lethality might be discovered through the T-15 strain.

2. Iron-uptake systems

1) Expression of genes related to iron-utilization

The composition of iron-uptake systems in T-type strains was determined by PCR using the specific primers for each *vuuA, iutA,* and *hupA* (Figure 4) and by the Western blot method using the specific polyclonal antibody for each gene product (Figure 5).

All the T-type strains had the *vuuA* gene encoding the vulnibactin-receptor protein (VuuA) and the *iutA* gene encoding a receptor protein (IutA). However, the T-10, T-12, and T-13 strains did not have the *hupA* gene encoding haem receptor protein (HupA). Overall, these results indicate that T-type strains possess several iron-uptake systems with individual variance, and that the absence of CPS is unlikely to be associated with the composition of iron-uptake systems.

2) Utilization of transferrin-bound iron

The ability of T-type strains to utilize transferrin-bound iron was compared on 6 M urea-gel electrophoresis (Figure 6). The T-1, T-2 and T-3 strains utilized transferrin-bound iron more efficiently than the M06-24/O strain. The T-4, T-5 and T-6 strains utilized transferrin-bound

iron less efficiently than the M06-24/O strain. No significant difference in the ability of the T-10, T-11 and T-12 strains to utilize transferrin-bound iron was observed as compared to that of the M06-24/O strain. Overall, these results indicate that T-type strains can utilize transferrin-bound iron with individual variance, and that the absence of CPS is unlikely to be associated with the ability to utilize transferrin-bound iron.

3) Production of vulnibactin

The ability of T-type strains to produce vulnibactin was determined using the arnow test (Figure 7). The T-1, T-5 and T-12 strains produced vulnibactin more than the M06-24/O strain. The T-2, T-3, T-7, T-8 and T-9 strains produced vulnibactin less than the M06-24/O strain. No significant difference to produce vulnibactin was observed in the ability of the T-4, T-6, T-10, T-11, T-13, T-14 and T-15 strains as compared to that of the M06-24/O strain. Overall, these results indicate that T-type strains can production vulnibactin with individual variance, and that the absence of CPS is unlikely to be associated with the ability to produce vulnibactin.

3. Swarming motility

The swarming motility of T-type strains was determined on the surface of semisolid agars (Figure 8). The T-1, T-4, T-5, T-6, T-7, T-8, and T-9 strains exhibited swarming motility more robustly than the M06-24/O strain. The T-3, T-13 and T-14 strains exhibited swarming motility less robustly than the M06-24/O strain. No significant difference was observed in the ability of the T-2, T-10, T-11, T-12 and T-15 strains to express swarming motility as compared to that of the M06-24/O strain. Overall, these results indicate that T-type strains can exhibit swarming motility with individual

variance, and that the absence of CPS is unlikely to be associated with the ability to express swarming motility.

4. Adherence to HeLa P3 (S) cells

In order to determine the relationship between the presence of capsule and adherence, the adherence of T-type strains to the HeLa P3 (S) cells was compared (Figure 9). The T-1, T-4, T-5, T-6, T-7, T-8, T-9, T-10, T-11 and T-15 strains adhered more efficiently than the M06-24/O strain. The T-3, T-12, T-13 and T-14 strains adhered less efficiently than the M06-24/O strain. No significant difference was observed in the adhering ability of the T-2 strain compared to that of the M06-24/O strain. Overall, these results indicate that T-type strains can adhere to HeLa P3 (S) cells with individual variance, and that the absence of CPS is unlikely to be associated with the ability to adhere to cell surfaces.

5. Cytotoxicity to HeLa P3 (S) cells

The cytotoxicity of T-type strains to the HeLa P3 (S) cells was determined using LDH assay (Figure 10). The T-4, T-7, and T-10 strain showed stronger or comparable cytotoxicity than the M06-24/O strain. The T-1, T-2, T-3, T-5, T-6, T-8, T-9, T-11, T-12, T-13, T-14 and T-15 strains showed weaker cytotoxicity than the M06-24/O strain. Overall, the results indicate that T-type strains are cytotoxic to HeLa P3 (S) cells with individual variance, and that the absence of CPS is unlikely to be associated with cytotoxicity.

6. Biofilm formation

The ability of T-type strains to form biofilm was determined on microtiter plates (Figure 11). The T-1, T-2, T-10, T-11, T-13, T-14 and T-15 strains formed biofilm more efficiently than M06-24/O strain.

The T-8 and T-9 strains formed biofilm less efficiently than the M06-24/O strain. No significant difference was observed in the ability of the T-3, T-4, T-5, T-6, T-7 and T-12 strains to form biofilm as compared to that of the M06-24/O strain. Overall, these results indicate that T-type strains can form biofilm with individual variance, and that the absence of CPS is unlikely to be associated with the ability to form biofilm.

7. Correlation among virulence factors

No significant correlation was observed between CPS and the other virulence factors. Correlation between the virulence factors other than CPS was also statistically determined by correlation coefficient. As shown in Table 6 and Table 7, there was ‘moderate to good positive relationship’ between motility and cytotoxicity (R=0.718, p=0.0012), between adherence and cytotoxicity (R=0.604, p=0.0103), and between adherence and motility (R=0.506, p=0.0383). No significant correlation was observed between the ability to utilize transferrin-bound iron and cytotoxicity (R=0.155, p=0.5533).

In contrast, there was ‘moderate to good negative relationship’ between biofilm formation and swarming motility (R= -0.590, p=0.0126). No significant correlation was observed between biofilm formation and cytotoxicity (R= -0.478, p=0.0525), between adherence and biofilm formation (R= -0.214, p=0.4098), and between the ability to produce vulnibactinand cytotoxicity (R= -0.0290, p=0.9122). Overall, these results indicate that swarming motility and adherence are positively associated

with cytotoxicity, but swarming motility is negatively associated with biofilm formation, and that the absence of CPS is unlikely to be associated with the expression of the other virulence factors.

IV. Discussion

The presence of CPS is related to the colony morphology, with encapsulated strains being opaque and unencapsulated strains being T-type (Grau *et al.,* 2008; Jones *et al.,* 2009; Simpson *et al*., 1987; Wright *et al.*, 2001). In *V. vulnificus*, immune evasion is primarily associated with CPS expression, and the presence of CPS provides resistance to opsonization by complement and thus avoidance of phagocytosis by macrophages (Starks *et al*., 2000). CPS expression also confers resistance to the bactericidal effects of serum (Chatzidaki-Livanis *et al.*, 2005), suggesting that CPS may mask immunologic structures that normally would activate the nonspecific host responses. In addition, the virulence of unencapsulated mutants was attenuated in mouse models (Stelma *et al.*, 1992). Accordingly, CPS has been considered to be one of the few known virulence factors that are required for *V. vulnificus* pathogenicity.

Antibodies to the *V. vulnificus* capsule were protective in animal models (Stelma *et al.*, 1992). In this study, the time required for killing mice was prolonged in the T-type strains than in the M06-24/O strain, and the T-type strains could not kill mice within 24 h except one strain. These results confirm that CPS is one of the major virulence factors determining mouse lethality.

Recently, genes involved with CPS biosynthesis have been identified after the elucidation of the genome sequences of two *V. vulnificus* strains (Jeong *et al.*, 2001; Jeong *et al.*, 2003; Jeong *et al.*, 2008). The group 1 CPS operon is composed of highly conserved transport genes followed by more-variable biosynthetic genes (Chatzidaki-Livanis *et al.*, 2005). Multiple genes outside of the group 1 CPS operon are considered to be essential CPS expression (Chatzidaki-Livanis *et al.*, 2005). Overall, a variety of

genes involved in CPS production underscores the complexity and importance of this surface structure for *V. vulnificus*. In this study, more-than-one genes in the group 1 CPS operon were deleted with individual variance in all the 15 T-type strains. This confirms a variety of genes are involved in CPS production in *V. vulnificus*. The heterogeneity at the biochemical level makes the development of a capsule-based vaccine nearly impossible (Chatzidaki-Livanis *et al.*, 2005). *V. vulnificus* can also undergo phase-variable expression of CPS, and CPS expression is not constitutive but could be regulated by environmental factors (Starks *et al*., 2000). In addition, we recently found that colony morphology is changed from opaque to T-type by a mutation of *crp* encoding cyclic AMP receptor protein (Shin, 2009). However, the molecular mechanism remains to be clarified.

Iron-uptake systems are authentic virulence factors in *V. vulnificus* (Alice *et al.*, 2008). It has been known that reduced CPS expression is accompanied with the decreased activities of iron-uptake systems. It is reported that T-type strains cannot grow in iron-limited media containing transferrin-bound iron (Simpson *et al.*, 1987), and is also reported that T-type strains cannot grow in iron-limited media, even in the presence of transferrin that is fully iron saturated (Wright *et al.*, 1981; Wright *et al.*, 1990).

However, in this study, T-type strains utilized transferrin-bound iron with individual variance, harbored several genes and receptor proteins related to iron-uptake with individual variance, and produced vulnibactin with individual variance. Accordingly, these results indicate that expression of iron-uptake systems or utilization of transferrin-bound iron is independent of the presence of CPS. However, it was recently demonstrated that expression of the vulnibactin or VuuA-mediated iron-uptake system and of

the HupA-mediated iron-uptake system is inhibited by a mutation of *crp* (Choi *et al.,* 2006; Oh *et al.,* 2009; Shin, 2009). As described above, CPS expression is also inhibited by a mutation of *crp*. These facts indicate that expressions of CPS and of iron-uptake systems are regulated by a common regulator, and suggest that change in CPS expression may be accompanied with change in expression of iron-uptake systems in the particular conditions that can affect cyclic AMP and CRP levels.

Motility or the presence of flagella is one of the decisive virulence factors (O'Toole *et al.*, 1998), which has been demonstrated by the molecular version of Koch's postulates (Falkow, 1988). Both swimming and swarming motility rely on the presence of flagella (Kim *et al*., 2003; Lee *et al*., 2004). The Tn Himar1 insertion mutation results in a significant decrease in motility, adhesion, cytotoxicity, indicating that *flg* genes, which are components of the flagellum biogenesis gene cluster, might play an important role in the virulence of *V. vulnificus* (Kim *et al*., 2003; Srivastava *et al.,* 2009). A loss of each flagella structural components results in significant decreases in motility, cellular adhesion and cytotoxicity compared to those of the parent strains, and increased LD_{50} against mice (Kim *et al*., 2003; Lee *et al*., 2007). Host cell contact by motility and/or adherence may be a prerequisite for *V. vulnificus* cytotoxin secretion. As reported (Lee *et al*., 2004), the motility of *V. vulnificus* may be required for localization to the sites of infection and that the flagellar apparatus may serve as an adhesin or as a structure for invasion into the host cells. As reported (Kim *et al*., 2008), RTX toxin is the major cytotoxic factor and the virulence factor that affects mouse lethality, which was demonstrated by the molecular version of Koch's postulates (Falkow, 1988). In this study, T-type strains could exhibit swarming motility with individual variance, adhere to HeLa P3 (S) cells with individual variance, and show cytotoxicity

to HeLa P3 (S) cells with individual variance. In addition, swarming motility and adherence were positively correlated with cytotoxicity. These results indicate that the closely-related three factors, motility or the presence of flagella, adherence and cytotoxicity, are independent of the presence of CPS.

In this study, T-type strains formed biofilm with individual variance, indicating that the absence of CPS is unlikely to be associated with the ability to form biofilm. Moreover, a previous study reported that CPS expression inhibits attachment and biofilm formation (Joseph *et al.,* 2003). In this study, swarming motility was also negatively correlated with biofilm formation. These noticeable findings are contrasted with those in other studies, in which polysaccharides are involved in biofilm formation in related species (Paranjpye *et al.*, 2005).

In conclusion, the genetic compositions of the group 1 CPS operon in T-type strains are determined to be varied. CPS seems to be the most reliable virulence factor, which determines the lethality of *V. vulnificus* against mouse. Expression of iron-uptake systems or utilization of transferrin-bound iron, swarming motility, adherence, cytotoxicity, and biofilm formation are likely to be independent of the presence of CPS. Motility seems to be positively associated with adherence or cytotoxicity to HeLa P3 (S) cells, and the three factors seems to be negatively associated with biofilm formation.

Table 1. Bacterial strains used in this study

Strains	Morphology	Source
M06-24/O	Opaque	Clinical isolate
CMCP6	Opaque	Clinical isolate
CRP[a](-)	Translucent	*crp* deletion mutant
T-1	Translucent	Environment isolate
T-2	Translucent	Environment isolate
T-3	Translucent	Environment isolate
T-4	Translucent	Environment isolate
T-5	Translucent	Environment isolate
T-6	Translucent	From USA
T-7	Translucent	From USA
T-8	Translucent	From USA
T-9	Translucent	From USA
T-10	Translucent	From USA
T-11	Translucent	Clinical isolate
T-12	Translucent	Clinical isolate
T-13	Translucent	Clinical isolate
T-14	Translucent	Clinical isolate
T-15	Translucent	Clinical isolate

[a]cyclic-AMP receptor protein

Table 2. The group 1 CPS operon genes tested in this study

Gene	Characterization
ORF 1[a]	lacks start codon (252- bp)
wza	Outer membrane capsular polysaccharide (1233- bp)
wzb	Cytoplasmic phosphatase Wzb (441- bp)
wzc	Putative tyrosine protein kinase (2184- bp)
wecC	Polysaccharide biosynthesis protein (1281- bp)
wbpP	Polysaccharide biosynthesis protein (1032- bp)
wzx	Polysaccharide biosynthesis protein Wzx (1284- bp)
rfaG	Glycosyltransferase RfaG (1104- bp)
wbjB	Epimerase/dehydratase WbjB (1038- bp)
rmlD	dTDP-4-dehydrorhamnose reductase (873- bp)
wbjD	UDP-N-acetylglucosamine 2-epimerase (1131- bp)
wbuB	Putative L- fucosamine transferase (1149- bp)
wbfT	Probable UDP-galactose 4-epimerase (963- bp)
wbfU	Probable galactosyl transferase (555- bp)
wbfY	Predicted nucleoside-diphosphate sugar (1953- bp)
wbfV	Predicted UDP-glucose 6-dehydrogenase (1167- bp)

[a]Open reading frame

Table 3. The primers targeting the group 1 CPS operon genes used in this study

Primers	Sequences	Target genes
wza-ORF-F/R	5'-atgctttcaaaagcgttagaaagaaatgg-3' / 5'-ttacggccacgttttaacgcgtag-3'	*wza*
wzb-ORF-F/R	5'-atgtttaacaaaatcttagtcgtttgtgtc-3' / 5'-ttacaatttctttgcccaggcttgtg-3'	*wzb*
wzc-F/R	5'-caacgtattgatgatctcaggccc-3' / 5'-ttagaatcacgccttttacttcgatg-3'	*wzc*
wecC-F/R	5‘-aaggtgatagaaaatacacagcgtgatg-3’ / 5'-aatagcttctgcgcccatttcacg-3'	*wecC*
wbpP-F/R	5'-gaagcccaatggcaacgatttacc-3' / 5'-gtttaaggttgttctgtctcctactgc-3'	*wbpP*
wzx-F/R	5'-ggtcaccggattatccaggatgc-3' / 5'-ggtaattgaggccatgagaaatcc-3'	*wzx*
rfaG-F/R	5'-gcattgtcagcaaagttaggtgg-3' / 5'-tcaggtatatgatgagcaatcggag-3'	*rfaG*
wbjB-F/R	5'-gttcttttggtaatgcggttcttcg-3' / 5'-cttggatcggtaatggtgatagg-3'	*wbjB*
rmlD-F/R	5'-tattaatcttaggtgcaacaggcatgc-3' / 5'-aaggtgtacttatcaataggatcaaccg-3'	*rmlD*
wbjD-F/R	5'-agtcggtacacgtccagaaattattcg-3' / 5'-ttagataaagaaaggcggtcaagtacatc-3'	*wbjD*
wbuB-F/R	5'-agcgcttggttaaaggatattatgatgg-3' / 5'-agccaagcgcaataaattagccatatc-3'	*wbuB*
wbfT-F/R	5'-aggtcgtgacaatcctaaagggc-3' / 5'-tgttgttcttgatgcatccaaacgg-3'	*wbfT*
wbfU-F/R	5'-gcattctttggtttgctattcttatggc-3' / 5'-tcggtcggtcttagcgagtaac-3'	*wbfU*
wbfY-F/R	5'-atttgagttatctgaatacggcttgtacg-3' / 5'-acatcgccacctttgcccattg-3'	*wbfY*
wbfV-F/R	5'-gtgcccattttgctgactaacc-3' / 5'-tcagtcactaccaaacaaatcgcg-3'	*wbfV*

Table 4. Southern blot result

Strains / Genes	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10	T-11	T-12	T-13	T-14	T-15
wza	N[a]	Ⓟ[b]	N	N	N	N	Ⓟ	N	N	N	Ⓟ	Ⓟ	Ⓟ	Ⓟ	Ⓟ
wzb	N	Ⓟ	N	N	N	N	Ⓟ	N	N	N	Ⓟ	Ⓟ	Ⓟ	Ⓟ	Ⓟ
wzc	N	Ⓟ	N	N	N	N	Ⓟ	N	N	N	Ⓟ	Ⓟ	Ⓟ	Ⓟ	Ⓟ
wecC	N	N	N	N	N	N	N	N	N	N	N	Ⓟ	Ⓟ	Ⓟ	Ⓟ
wbpP	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
wzx	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
rfaG	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
wbjB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
rmlD	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
wbjD	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
wbuB	N	N	N	N	N	N	Ⓟ	N	N	Ⓟ	N	N	N	N	N
wbfT	N	N	N	N	N	N	Ⓟ	N	N	N	N	N	N	N	N
wbfU	N	N	N	N	N	N	N	N	N	N	N	N	Ⓟ	Ⓟ	N
wbfY	N	N	N	N	N	N	Ⓟ	N	N	N	N	Ⓟ	Ⓟ	Ⓟ	Ⓟ
wbfV	N	Ⓟ	N	N	N	N	Ⓟ	N	N	Ⓟ	Ⓟ	Ⓟ	Ⓟ	Ⓟ	Ⓟ

[a]N: negative, [b]Ⓟ: positive

Table 5. The primers targeting genes related to iron-uptake used in this study

Primers	Sequences	Target genes	Sources
vuuA-1/3	5'-gcctatgctcaaaccgagag-3' / 5'-gcagcggtttgtgaatagtttcg-3'	*vuuA*	This study
iutA-1/2	5'-gcttttgctcaagtcgattacg-3' / 5'-tcgacgctgttggtcttaatcc-3'	*iutA*	This study
hupA-1/2	5'-aaattgcaatctcagctgagcg-3' / 5'-ctaccttgtaagtggcacctag-3'	*hupA*	This study

Table 6. Relationship among virulence factors

Strains	6 M- Urea (rank)	Arnow test (μM ± SD[c])	Motility (cm ± SE[d])	Adhesion (% ± SD)	Cytotoxicity (% ± SD)	Biofilm formation (OD_{CV}/OD_{Cells}[e] ± SD)	HupA (rank)	IutA (rank)	VuuA (rank)
MO[a]	3	10.10 ± 0.23	4.07 ± 0.06	0.21 ± 0.03	91.97 ± 2.17	2.04 ± 0.16	1	1	1
CRP(-)[b]	1	8.20 ± 0.47	2.33 ± 0.12	0.10 ± 0.01	13.16 ± 0.41	3.19 ± 0.25			
T-1	3	13.07 ± 0.60	4.9 ± 0.17	1.33 ± 0.07	81.55 ± 8.64	3.62 ± 0.08	1	2	1
T-2	3	9.21 ± 0.72	4.47 ± 0.55	0.38 ± 0.05	34.12 ± 1.22	4.74 ± 0.19	1	2	0.5
T-3	2	8.38 ± 0.26	0.37 ± 0.06	0.08 ± 0.02	26.96 ± 2.71	2.25 ± 0.12	1	2	1
T-4	3	11.06 ± 1.63	6.23 ± 0.25	1.40 ± 0.31	86.30 ± 3.67	2.20 ± 0.19	1	1	1
T-5	3	12.17 ± 0.13	4.87 ± 0.06	1.64 ± 0.39	81.27 ± 8.21	2.51 ± 0.23	1	1	1
T-6	1	10.78 ± 0.13	5.23 ± 0.15	3.17 ± 0.24	83.30 ± 6.23	2.24 ± 0.11	1	1	3
T-7	1	8.97 ± 0.26	5.13 ± 0.21	6.86 ± 0.39	88.66 ± 6.04	2.65 ± 0.17	1	2	2
T-8	3	9.59 ± 0.23	5.57 ± 0.21	1.07 ± 0.05	60.21 ± 0.66	1.61 ± 0.10	1	1	1
T-9	3	9.19 ± 0.22	6.2 ± 0.27	2.77 ± 0.44	67.68 ± 1.66	1.57 ± 0.06	1	1	1
T-10	2	9.95 ± 0.46	4.3 ± 0.31	4.87 ± 0.37	91.39 ± 3.15	3.64 ± 0.19	0	2	1
T-11	2	10.35 ± 0.56	4.07 ± 0.12	2.13 ± 0.26	33.31 ± 4.02	4.07 ± 0.31	1	1	1
T-12	3	15.62 ± 0.68	3.43 ± 0.06	0	17.20 ± 0.99	2.58 ± 0.13	0	1	3
T-13	2	11.04 ± 0.29	1.47 ± 0.12	1.33e-3 ± 8.11e-4	13.24 ± 0.38	4.76 ± 0.02	0	2	1
T-14	2	11.30 ± 0.51	0.22 ± 0.03	4.00e-4 ± 4.00e-4	21.89 ± 0.76	7.65 ± 0.71	1	1	1
T-15	3	10.93 ± 0.52	4.4 ± 0.1	3.38 ± 0.13	60.42 ± 1.55	3.70 ± 0.03	1	1	1

[a]M06-24/O; [b]CMM710 with deleted *crp*; [c]Standard deviations

[d]Standard errors; [e]See the Materials and Methods section for details

Table 7. Correlation among virulence factors

Variables	p[a]	ɣ[b]	Equations
Cytotoxicity & Motility	0.0012	0.718	Cytotoxicity = 8.51 + (12.1 × Motility)
Cytotoxicity & Adherence	0.0103	0.604	Cytotoxicity = 39.9 + (9.54 × Adherence)
Motility & Adherence	0.0383	0.506	Motility = 3.14 + (0.475 × Adherence)
Motility & Biofilm	0.0126	-0.590	Motility = 6.28 - (0.719 × Biofilm)
Cytotoxicity & Biofilm	0.0525	-0.478	Cytotoxicity = 88.1 - (9.80 × Biofilm)
Biofilm & Adherence	0.4098	-0.214	Biofilm = 3.52 - (0.165 × Adherence)
Cytotoxicity & 6 M Urea	0.5533	0.155	Cytotoxicity = 41.9 + (6.01 × 6 M Urea)
Cytotoxicity & Arnow	0.9122	-0.0290	Cytotoxicity = 61.1 - (0.482 × Arnow)

[a]The probability of obtaining a value of the test statistic

[b]Correlation coefficient

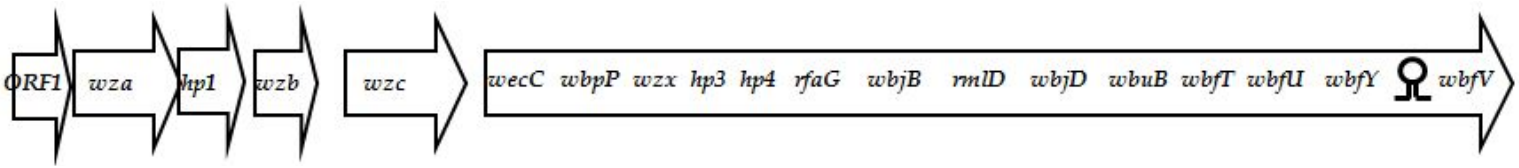

Figure 1. Genetic organization of group 1 CPS operon of *V. vulnificus* M06-24/O.

The arrows indicates the direction of transcription of the genes, and the predicted stem-loop transcriptional terminator (Ω) is shown for *V. vulnificus* operon.

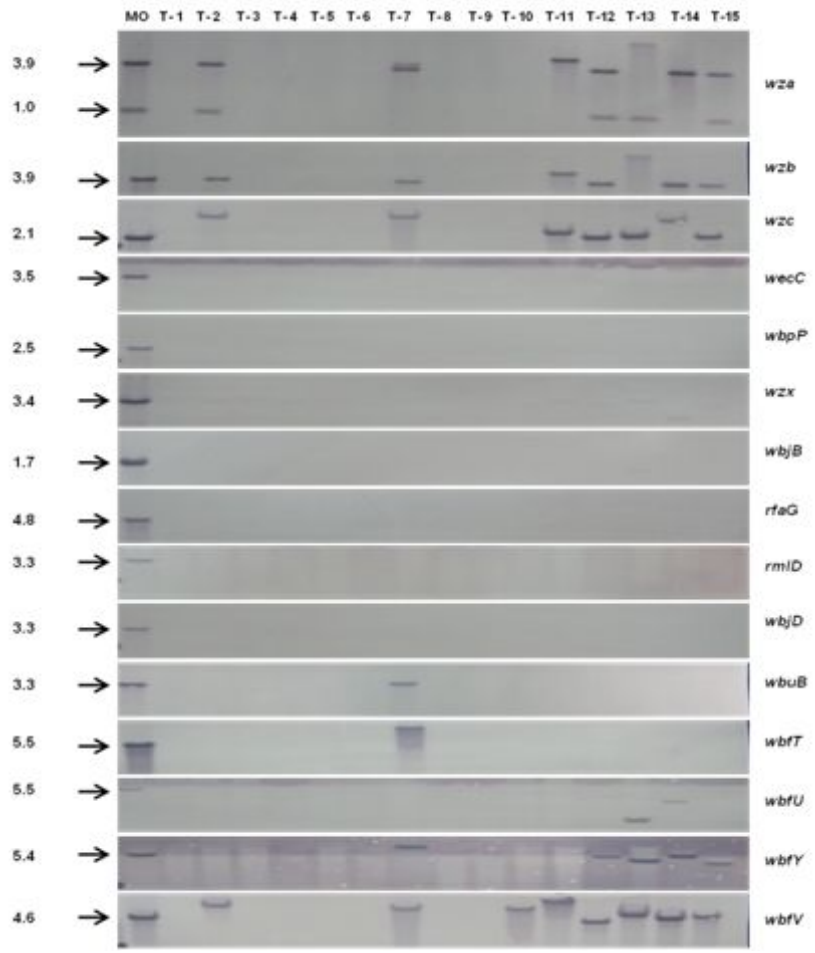

Figure 2. Southern blot analysis of the group 1 CPS operon genes in T-type *V. vulnificus* strains

The genomic DNAs were digested completely with various restriction enzymes and hybridized to each DIG-labeled DNA probe specific for the group 1 CPS operon genes, as described in the Materials and Methods section. The sizes of hybridization bands specific for the group 1 CPS operon genes are indicated in kilobases at the left, and derived from the sequence analysis of group 1 CPS operon of wild-type M06-24/O. The lane MO indicates the wild-type M06-24/O strain.

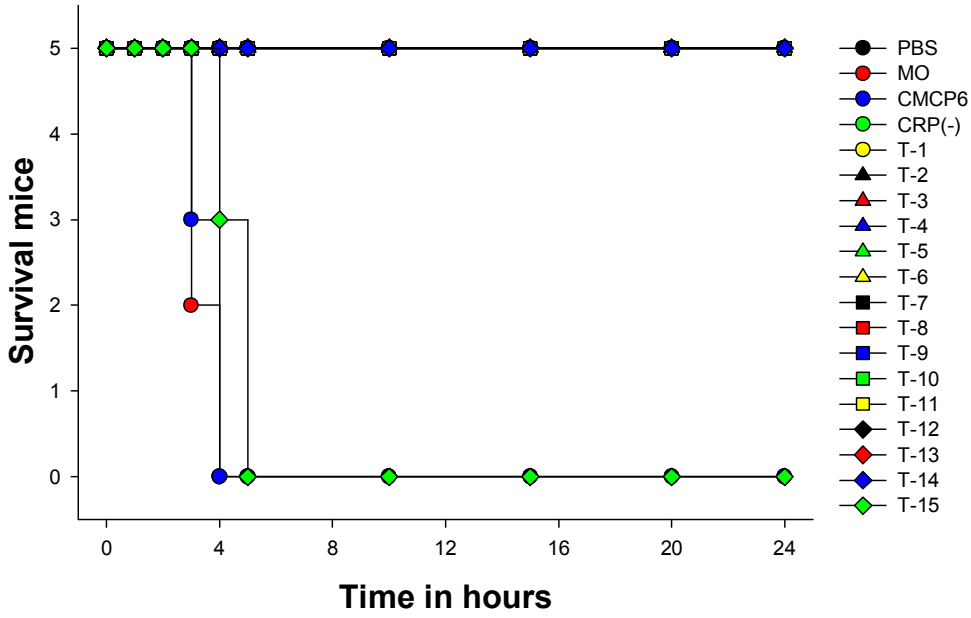

Figure 3. Survival curve of mice infected intraperitoneally with T-type *V. vulnificus* strains.

The overnight-cultured *V. vulnificus* strains, wild-type M06-24/O (MO) and CMCP6, CMM710 with deleted *crp*, and 15 T-type *V. vulnificus* strains (T-1 to T-15) were inoculated into fresh 2.5% NaCl HI broth to a concentration of 5 x 10^6 cells/㎖ and cultured with vigorous shaking at 37℃ for 4.5 h. The bacterial suspension of 1 x 10^7 cells was injected intraperitoneally to eight-week-old specific pathogen-free female mice (n=5), and the mice were observed for 24 h.

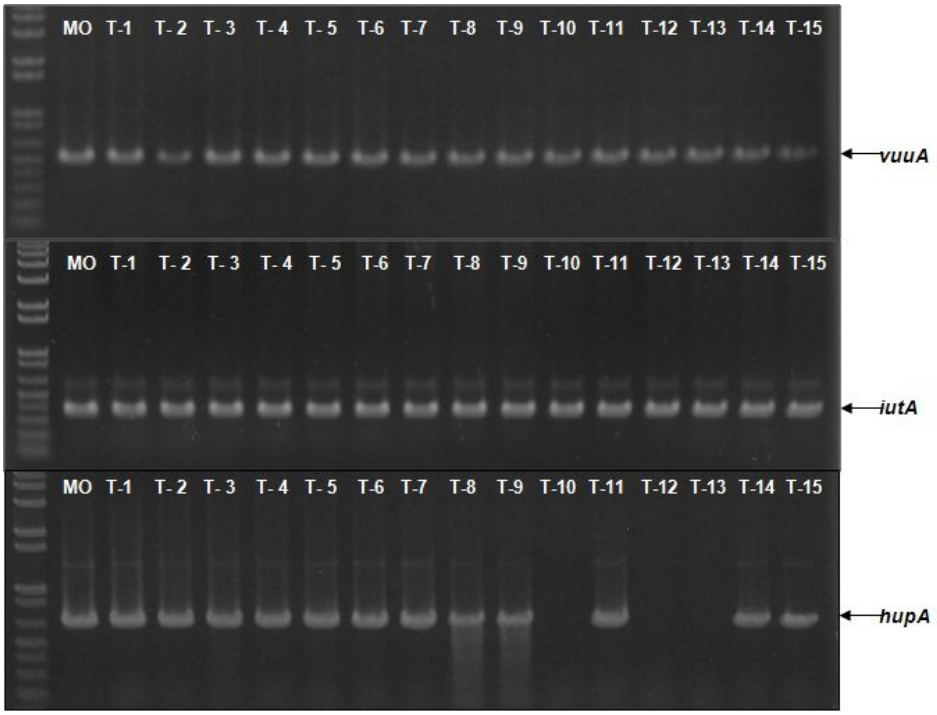

Figure 4. PCR analysis of *vuuA, iutA,* and *hupA* genes in T-type *V. vulnificus* strains.

Genomic DNA was extracted from the overnight culture of *V. vulnificus* strains, and PCR was performed using the primers: *vuuA*-specific primers, vuuA-1/3; *iutA*-specific primers, iutA-1/2; *hupA*-specific primers, hupA-1/2. The lane MO indicates the wild-type M06-24/O strain.

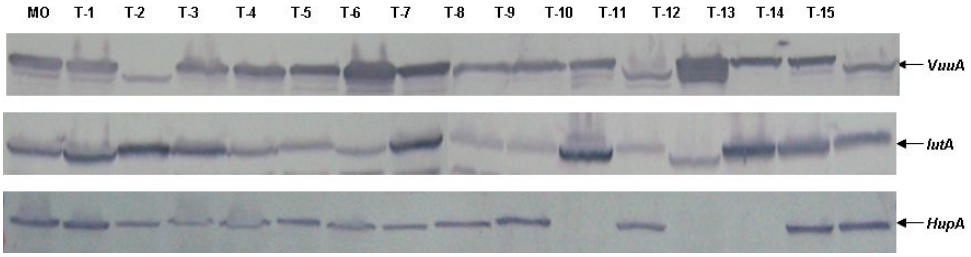

Figure 5. Expression of VuuA, IutA, and HupA in T-type *V. vulnificus* strains.

Overnight-cultured *V. vulnificus* strains were inoculated into fresh 2.5% NaCl HI broth containing 200 μM dipyridyl at 5 x 10^6 cells/㎖ and cultured with vigorous shaking at 37℃ for 12 h. The final bacterial densities were then adjusted to 1 x 10^8 cells/㎖, and the cell lysates were obtained from the bacterial pellets. After electrophoresis of the cell lysates, Western blot was conducted using the adsorbed rabbit polyclonal anti-VuuA, -IutA, and -HupA antibodies as primary antibody, as described in the Materials and Methods section. The lane MO indicates the wild-type M06-24/O strain.

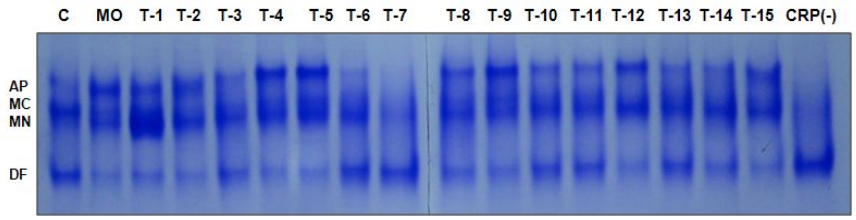

Figure 6. Iron-removal from transferrin in T-type *V. vulnificus* strains.

The overnight-cultured *V. vulnificus* strains, wild-type M06-24/O (MO), CMM710 with deleted *crp* and 15 T-type *V. vulnificus* strains (T-1 to T-15), were inoculated into deferrated HI broth containing 0.5 ㎎/㎖ of human holotransferrin at 1 x 10^6 cells/㎖, and cultured with vigorous shaking at 37℃ for 24 h. Equal volumes (20 ㎕) of culture supernatants were electrophoresed on a 6 M urea-gel and stained with Coomassie blue. Transferrin molecules are separated into four forms according to iron-saturation level: apoferric (AP), C-terminal monoferric (MC), N-terminial monoferric (MN), and diferric (DF) form. Uninoculated deferrated HI broth containing holotransferrin was used as the control (C). The lane MO indicates the wild-type M06-24/O strain.

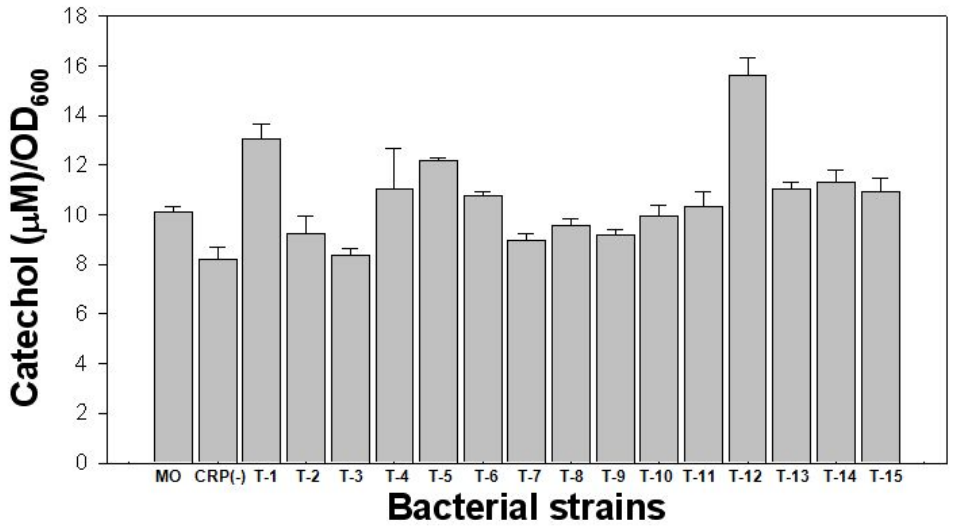

Figure 7. Phenolate siderophore production of T-type *V. vulnificus* strains.

The culture supernatants of the *V. vulnificus* strains, wild-type M06-24/O, CMM710 with deleted *crp*, 15 T-type *V. vulnificus* strains (T-1 to T-15), were obtained after 24 h and phenolate siderophore was detected by Arnow test. Data indicate mean and standard deviations from the three independent experiments.

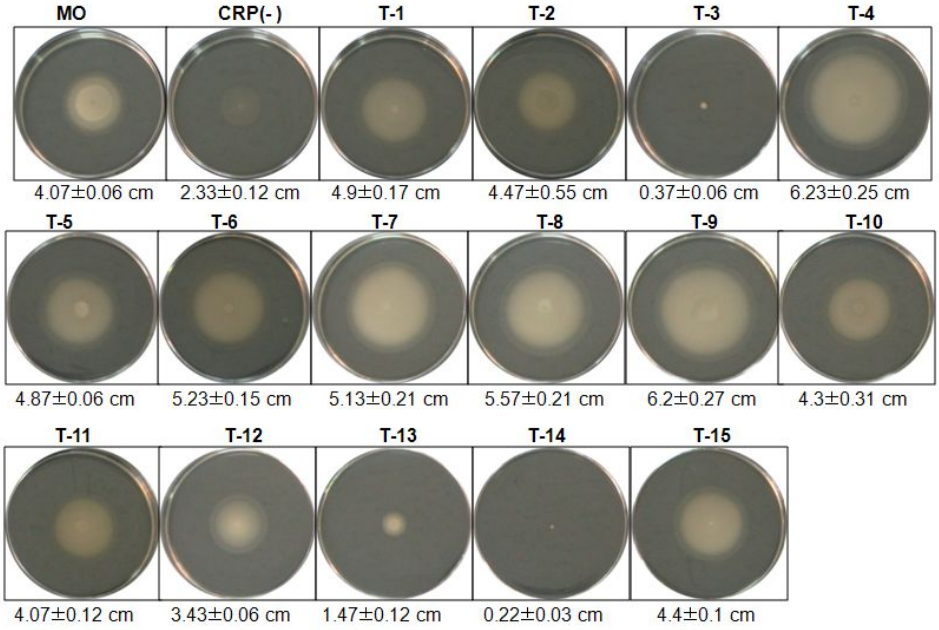

Figure 8. Swarming haloes formed by T-type *V. vulnificus* strains.

The wild-type M06-24/O strain, CMM710 with deleted *crp*, 15 T-type *V. vulnificus* strains (T-1 to T-15) were used. One colony of each strain grown on 2.5% NaCl HI agar was picked up to be inoculated onto the surface of semisolid HI agar containing 0.3% Bacto-agar using the end of toothpick, and incubated at 37℃ for 12 h. A representative one observed in three times-repeated experiments of similar results is shown. Data indicate the average values and SEM from the three independent experiments.

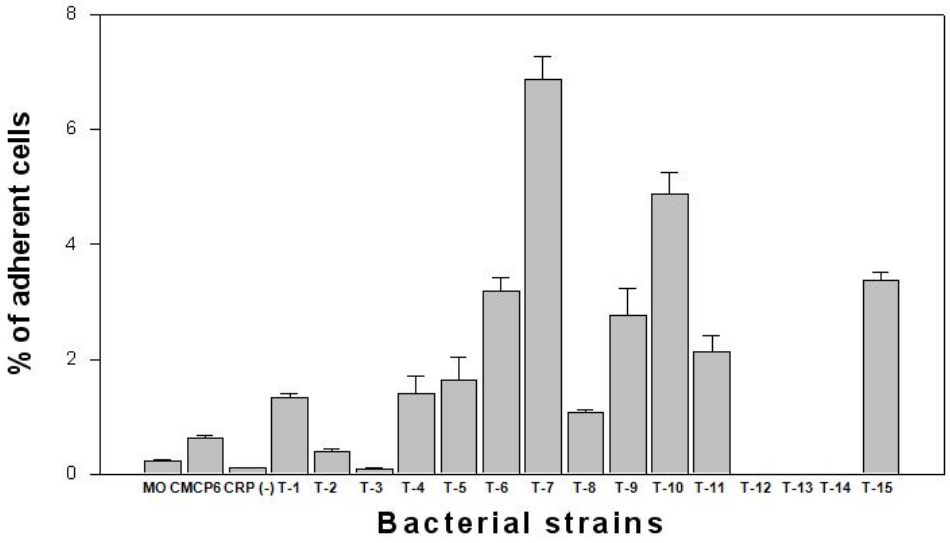

Figure 9. Adherence of T-type *V. vulnificus* strains to HeLa P3 (S) cells.

HeLa P3 (S) cells were infected with the log-phase *V. vulnificus* strains, wild-type M06-24/O (MO) and CMCP6, CMM710 with deleted *crp*, and 15 T-type *V. vulnificus* strains (T-1 to T-15), at a multiplicity of infection (MOI) of 5 for 15 min. The HeLa P3 (S) cells were then washed six times and were broken with 0.1% Triton X-100 solution for 15 min. The bacteria were recovered and pour plated onto 2.5% NaCl HI agar. Adherence values are indicated as the percentage of the numbers of adhered bacteria to those of bacteria initially added to HeLa P3 (S) cells. Data indicate the average values and standard deviations from the three independent experiments.

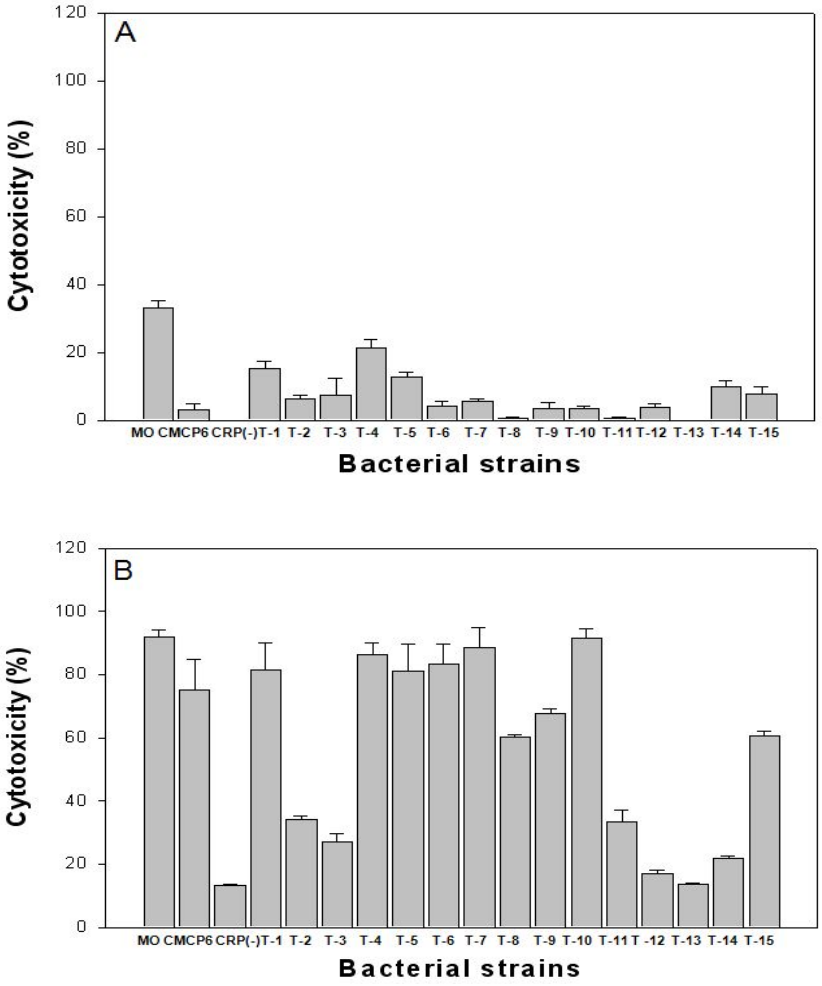

Figure 10. Cytotoxicity of T-type *V. vulnificus* strains to HeLa P3 (S) cells.

HeLa P3 (S) cells were infected with log-phase *V. vulnificus* strains, wild-type M06-24/O and CMCP6, CMM710 with deleted *crp*, and 15 T-type *V. vulnificus* strains (T-1 to T-15), at a MOI of 50 for 90min (A) and 120min (B). LDH released in the supernatant was measured as a marker of the cytotoxicity and data indicate the average values and standard deviations from the three independent experiments.

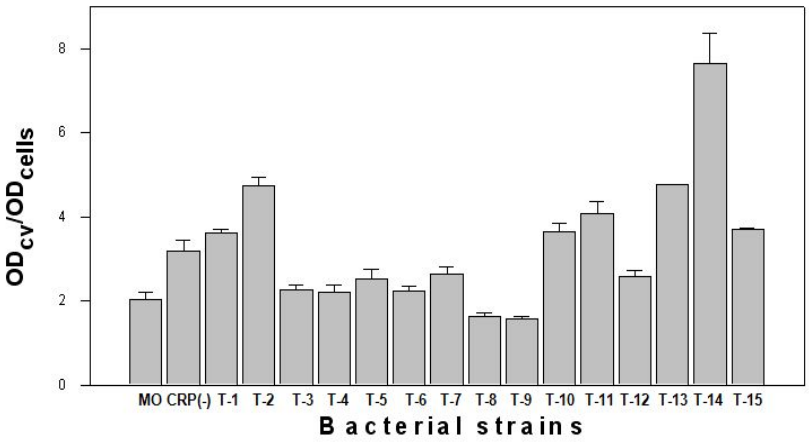

Figure 11. Biofilm formation of T-type *V. vulnificus* strains.

Microtiter plates were stained with 0.1% crystal violet (CV) solution after static incubation of *V. vulnificus* strains for 24 h at 30℃. Biofilm formation was quantified by measuring absorbance at OD_{595} (OD_{CV}) and is presented as values normalized by planktonic cell density at OD_{600} (OD_{Cells}). Data indicate the average values and standard deviations from the three independent experiments.

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VII. Abstract

The Composition of Group 1 CPS Operon in Translucent Colony-type *Vibrio vulnificus* Strains and Their Potential Virulence Factors

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*Vibrio vulnificus*is an opportunistic but highly lethal human pathogen. A wide array of virulence factors, including capsular polysaccharide (CPS) expression, iron acquisition, cytotoxicity, motility, and expression of proteins involved in attachment or adhesion, play significant roles in the pathogenesis of the organism. CPS is one of the few known virulence factors that are absolutely required for pathogenicity. It has been known that translucent colony-type (T-type) strains with reduced or no capsule can accompany the changes of the other virulence traits.

In this study, 15 T-type strains were selected to inspect group 1 CPS operon and virulence factors. On PCR and Southern blot, they harbored group 1 CPS operon with the related several genes being deleted with individual variance. On PCR and Western blot, they harbored several genes and proteins related to iron-uptake with individual variance. On 6 M urea-gel electrophoresis, they could utilize transferrin-bound iron with individual variance. On semisolid agar, they exhibited swarming motility

with individual variance. In an adhesion assay, they adhered to HeLa P3 (S) cells with individual variance. In LDH assay, they exhibited cytotoxicity to HeLa P3 (S) cells with individual variance. On multi-well plates, they formed biofilm with individual variance. When relationship among these virulence traits was statistically analyzed, swarming motility was positively associated with adherence or cytotoxicity, and the three traits were negatively associated with biofilm formation.

These results imply that T-type strains do not accompany the change of the other virulence traits and motility seems to play an important role in adhesion and cytotoxicity but inhibits biofilm formation in *V. vulnificus*.

Key words: *Vibrio vulnificus*, Capsular polysaccharide, Iron-uptake system, Motility, Adhesion, Cytotoxicity, Biofilm

VIII. 감사의 말씀

저작물 이용 허락서

학 과	바이오 신약개발학과	학 번	20087521	과 정	석사・박사
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연락처	e-mail : chunsaring@hanmail.net				
논문제목	한글 : Translucent Colony-type *Vibrio vulnificus* 균주에서 Group 1 CPS Operon의 유전자 구성과 잠재적인 독력인자의 발현				
	영문 : The Composition of Group 1 CPS Operon in Translucent Colony-type *Vibrio vulnificus* Strains and Their Potential Virulence Factors				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 - 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함(다만, 저작물의 내용변경은 금지함)
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함

동의여부 : 동의(○) 반대( )

2010 년 8 월

저작자: 신 영 희 (인)

조선대학교 총장 귀하