저작자표시-비영리-변경금지 2.0 대한민국

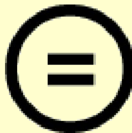
이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.



변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

[UCI]I804:24011-200000241010

2011 년 2 월

석사학위논문

Cyclic AMP 수용체 단백에 의한 패혈증 비브리오균의 편모매개 운동성의 조절

조선대학교 대학원

바 이 오 신 약 개 발 학 과

박 지 나

Cyclic AMP 수용체 단백에 의한 패혈증 비브리오균의 편모매개 운동성의 조절

Cyclic AMP-receptor Protein Regulates Flagella-mediated Motility in *Vibrio vulnificus*

2011 년 2 월 25 일

조선대학교 대학원

바 이 오 신 약 개 발 학 과

박 지 나

Cyclic AMP 수용체 단백에 의한 패혈증 비브리오균의 편모매개 운동성의 조절

지도교수 신 성 희

이 논문을 이학 석사학위 청구논문으로 제출함

2010 년 10 월

조선대학교 대학원

바 이 오 신 약 개 발 학 과

박 지 나

박지나의 석사학위 논문을 인준함

위원장 조선대학교 교수 임 성 철 (인)

위 원 조선대학교 교수 김 춘 미 (인)

위 원 조선대학교 교수 신 성 희 (인)

2010 년 11 월

조선대학교 대학원

Table of Contents

초록

I. Introduction..1

II. Materials and Methods...11

1. Bacterial strains, media, and reagents...11

2. Hemolysin assay and transferrin-bound iron utilization assay.........12

3. Mutation and complementation of the *crp* gene...............................16

4. Mutation and complementation of the *motBA* gene..........................16

5. Construction of chromosomal *motA* transcription reporters..............18

6. Observation of colony morphology and motility................................20

7. Western blot analysis for VvpE..20

8. PCR analysis...21

9. Adhesion assays..22

10. Cytotoxicity assays...23

11. Growth condition and β-galactosidase activity measurement..........24

III. Results......26

1. Bacterial characterization of *V. vulnificus* strains with reduced motility......26

2. The composition of flagella-associated genes......30

3. Relation between swarming motility and VvpE production......30

4. Adherence to HeLa P3 (S) cells......33

5. Cytotoxicity to HeLa P3 (S) cells......35

6. Correlation among motility, adherence and cytotoxicity......37

7. Analysis of non-coding regions......39

8. Effect of a *motBA* mutation on motility......43

9. Effect of a *crp* mutation on *motA* expression at the transcriptional level......47

IV. Discussion......49

V. Summary......57

VI. References......59

VII. Supplements..67

VIII. Abstract..71

IX. Acknowledgements...74

LIST OF TABLES

Table 1. Bacterial strains used in this study..13

Table 2. Plasmids used in this study..14

Table 3. Primers used in this study...15

Table 4. Characterization of *Vibrio vulnificus* strains used in this study..29

Table 5. Correlation among motility, adherence and cytotoxicity........38

Table 6. Putative CRP binding sequence in flagella associate gene....41

LIST OF FIGURES

Figure 1. Schematic view of the flagellum in a gram-negative bacterium showing the substructures and the proteins from which they are constructed……………………………………………………………………3

Figure 2. The strategy of this study……………………………………………10

Figure 3. Swarming haloes formed by *V. vulnificus* strains used in this study……………………………………………………………………………28

Figure 4. Extracellular VvpE production in *V. vulnificus* strains……………32

Figure 5. Adherence of *V. vulnificus* strains to HeLa P3 (S) cells…………34

Figure 6. Cytotoxicity of *V. vulnificus* strains to HeLa P3 (S) cells……….36

Figure 7. Putative cAMP receptor protein (CRP)-binding site in the regulatory region of the *Vibrio vulnificus motA* gene……………………….42

Figure 8. Effect of a *motBA* mutation on swarming motility of *V. vulnificus*…………………………………………………………………………44

Figure 9. Effect of a *motBA* mutation on adherence of *V. vulnificus* to HeLa P3 (S) cells..........45

Figure 10. Effect of a *motBA* mutation in *V. vulnificus* on cytotoxicity to HeLa P3 (S) cells..........46

Figure 11. Effect of a *crp* mutation on *motA* expression in *V. vulnificus*..........48

초 록

Cyclic AMP 수용체 단백에 의한 패혈증 비브리오균의 편모매개 운동성의 조절

박 지 나
지도교수: 신 성 희
바이오신약개발학과
조선대학교 대학원

패혈증 비브리오균은 그람음성 호염성 세균이며 간질환을 가지고 있는 감수성이 있는 환자들에게 괴사성 창상감염과 패혈증을 일으킨다. 패혈증 비브리오균은 극성의 편모를 가지고 활발한 운동성을 지니며, 편모매개 운동성은 중요한 독력인자 중의 하나이다. 편모매개 운동성은 패혈증 비브리오균의 세포부착능과 세포독성에 밀접하게 관련되어 있는 것으로 알려져 있으며, 운동성, 세포부착능과 세포독성은 포도당이나 에너지 이용능에 일차적으로 반응하는 만능전사조절자(global transcriptional regulator)인 cyclic AMP 수용체 단백(CRP)에 의해 양성적으로 조절을 받는다. 본 연구에서는 첫째로 임상분리주나 환경분리주에서 운동성, 세포부착능과 세포독성 사이의 관련성을 알아보았다. 운동성이 감소된

14종의 패혈증 비브리오균주를 선택하였다. Western blot으로 확인한 결과 감소된 운동성은 단백분해효소(metalloprotease) 생산성과는 관련이 없었다. 13개의 편모와 관련된 유전자들을 선택하여 PCR로 분석하였을 때 14종 균주와 M06-24/O 균주 사이에 유전자 구성은 별다른 차이를 보이지 않았다. 이들 균주의 HeLa P3 (S) 세포에 대한 세포부착능과 세포독성 실험 결과를 통계적으로 분석하였을 때 운동성과 세포부착능, 운동성과 세포독성 그리고 세포부착능과 세포독성 모두 양성의 양호한 상호관계를 나타내었다 ($0.05<r<0.75$, $p<0.05$). 두 번째로 운동성에 대한 CRP의 영향을 알아보기 위하여 편모와 관련 있는 유전자들에서 CRP결합염기서열(CRP-binding sites)을 분석하였다. CRP에 의해 조절될 가능성이 가장 높은 유전자의 하나로써 편모원동력 단백을 암호화하는 *motBA* 유전자를 선택하였다. MotBA가 결손된 돌연변이주를 제작하였고 *motBA* 유전자발현을 전사수준에서 측정할 수 있는 P_{motA}::*lacZ* transcriptional reporter 균주도 제작하였다. MotBA가 결손된 돌연변이주는 운동성, 세포부착능과 세포독성이 거의 완전히 소실되었다. 전사수준에서 *motBA*의 발현은 *crp*돌연변이에 의해 뚜렷하게 감소되었다. 감소된 *motBA* 발현은 *crp* 보충에 의해 야생균주의 수준으로 회복되었다. 이러한 결과들을 통해

운동성이 세포부착능과 세포독성에 밀접하게 관련되어 있고, CRP는 *motBA* 발현을 양성적으로 조절하여 운동성을 조절하고 더 나아가 세포부착능과 세포독성에도 영향을 주는 것임을 알 수 있었다.

중심어: 패혈증 비브리오균, 편모, 운동성, 세포부착능, 세포독성, Cyclic AMP-수용체 단백

I. Introduction

1. Bacterial flagella and motility

Flagella are helical propellers that drive the bacterial cell forward like the motor of a boat. Bacterial flagella are 10-20 μm long and between 0.01 and 0.02 μm in diameter. Three types of arrangements are known: monotrichous (single polar flagellum), lophotrichous (multiple polar flagella), peritrichous (flagella distributed over the entire cell). The schematic and typical structure of the flagellum in a gram-negative bacterium is seen in Figure 1. Each flagellum is a spiral filament of protein monomers called flagellin. The flagellum is attached to the bacterial cell body by a complex structure consisting of a hook and a basal body. The hook is a short curved structure that appears to act as the universal joint between the motor and the flagellum. The basal body bears a central rod and a set of rings (L, P and MS) in gram-negative bacteria. The flagellar motor is embedded in the layers of the cell envelope. The Mot proteins surround the MS and C rings of the motor and function to generate torque for rotation of the flagellum.

Energy for rotation comes from proton motive force. The Fli proteins act as the motor switch to trigger either clockwise or counterclockwise rotation of the flagellum and to possibly disengage the rod in order to stop motility (Slonczewski & Foster, 2009).

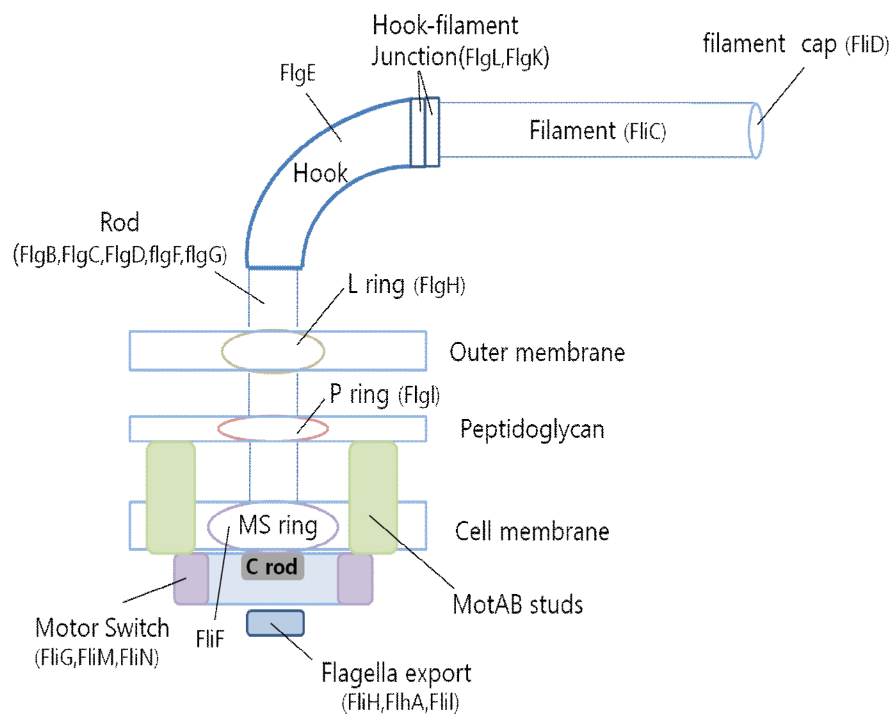


Figure 1. Schematic view of the flagellum in a gram-negative bacterium showing the substructures and the proteins from which they are constructed.

Details are in the text.

In nature, there are many chemical nutrients that bacteria need to survive. Bacteria move toward attractants by using their flagella to move up the concentration gradient. The process is called chemotaxis. In the absence of a chemical gradient, bacteria move randomly, switching back and forth between a phase called a run (swimming) and a phase called a tumble. During a run, a bacterium travels in a straight or slightly curved line. After a few seconds, the bacterium will stop and tumble (Willey, Sherwood & Woolverton, 2009). There is another type of flagella-mediated motility called swarming. Swarming is a community behavior that promotes flagellum-dependent motility on surfaces. In many bacteria, swarming is associated with the production of multiple and lateral flagella (Harshey, 1994; Fraser & Hughes, 1999).

In addition to motility, the flagella of different bacterial species presumably differ from one another in primary structure. Moreover, they are highly antigenic (called H antigen), and some of the immune responses to infection are directed against these flagellins. H antigen is useful for bacterial serotyping especially in Enterobacteriaceae (Slonczewski & Foster,

2009).

In *Pseudomonas aeruginosa*, flagella-mediated motility is involved in biofilm formation (O'Toole & Kolter, 1998). In *Vibrio cholera,* the flagellar regulatory system positively regulates transcription of a diguanylate cyclase, CdgD, which in turn regulates transcription of a novel hemagglutinin (*frhA*) that mediates adherence to chitin and epithelial cells and enhances biofilm formation and intestinal colonization in infant mice (Syed *et al*., 2009). In *Vibrio parahaemolyticus,* flagella are of particular interest because it possesses two flagellar systems. A single, sheathed polar flagellum propels the cell in liquid environments. Numerous unsheathed, lateral flagella move the cell over surfaces. Not only are flagella organelles of locomotion, but also they play important roles in attachment (kaneko *et al*., 1975; Sjoblad *et al*., 1982), biofilm formation (O'Toole *et al*., 1998; Pratt *et al*., 1998) and pathogenesis (Ottemann *et al*., 1997).

Bacterial swarming itself is regarded as a virulence factor, and constitutes a good *in vitro* model of bacterial surface adherence and colonization (Harshey, 1994; Fraser & Hughes, 1999). In *Proteus mirabilis*, a

representative gram-negative swarming bacterium, swarming differentiation is accompanied by an increase in the expression of an extracellular metalloprotease (ZapA), but ZapA is not essentially required for swarming (Walker *et al*., 1999).

2. ***Vibrio vulnificus***

V. vulnificus is a halophilic estuarine gram-negative bacterium that causes fatal septicemia and necrotizing wound infections in patients with underlying diseases, most of which are associated with elevated serum or tissue iron levels. *V. vulnificus* infections show a rapid and fulminant progression and an exceptional high mortality rate of over 50% (Paik *et al*., 1995; Tacket *et al*., 1984).

The two hallmarks of *V. vulnificus* disease are the extreme destruction of host tissues and the rapid proliferation of the bacteria in the host. Several factors including capsular polysaccharide (Simpson *et al*., 1987), RTX toxin (Kim *et al*., 2007), iron-uptake systems (Litwin *et al*., 1996), flagella (Kim *et al*.,

2003), hemolysin III (Chen *et al*., 2004), type IV pilin (Paranjpye *et al*., 1998), phospholipase A (Koo *et al*., 2007), hemolysin/cytolysin (VvhA) (Gray *et al*., 1985), and metalloprotease (VvpE) (kothary *et al*., 1987) have been known to be associated with the pathogenesis of *V. vulnificus* septicemia. The most widely accepted gold standard for molecular pathogenesis is the molecular version of Koch's postulates (Falkow, 2004). Capsular polysaccharide, RTX toxin, iron-uptake systems, flagella, hemolysin III and type IV pilin are confirmed by the postulates. In contrast, phospholipase, VvhA and VvpE have circumstantial evidence but do not satisfy requirements of the postulates. At least, each of these putative virulence factors is not a decisive factor determining mouse lethality.

V. vulnificus is a highly motile organism by virtue of a polar flagellum, like other Vibrio species. It is known that *V. vulnificus* flagella or motility is closely associated with adherence to host cells (Lee *et al*., 2004) and that it inhibits biofilm formation (Paranjpye *et al*., 2005).

Vibrio vulnificus also evidences swarming motility, which requires flagellar synthesis (Kim *et al*., 2003). Considering that the adherence and cytotoxicity

to HeLa cells and the mouse-lethality of a swarming-defective mutant significantly decreased compared with those of its wild-type *V. vulnificus* strain (Kim *et al*., 2003), *V. vulnificus* swarming is thought to play a significant role in surface adherence and colonization, as has been seen in other bacteria (Kim *et al*., 2007; Kim *et al*., 2006a,b). Moreover, metalloprotease VvpE is essentially required for the beginning of swarming unlike in *P. mirabilis* (Walker *et al*., 1999). *V. vulnificus* swarming is abolished by a *vvpE* mutation and this swarming defect is recovered by a *vvpE* complementation (Kim *et al*., 2007).

Recent studies reported that *V. vulnificus* swimming or swarming motility (or adherence or cytotoxicity) is positively regulated by the cyclic AMP (cAMP)/cAMP-receptor protein (CRP) complex (Shin, 2009; Kim *et al*., 2005). However, the related mechanisms remain totally unknown.

3. The strategy of this study

V. vulnificus infects susceptible patients via either the skin or the intestinal tract. Accordingly, *V. vulnificus* must first adhere to and colonize

the epithelial surfaces of the skin or the intestinal tract, in order to establish infections successfully.

Up to date, many studies have demonstrated the relationship among motility, adherence and cytotoxicity in the flagella-deficient background via mutations of genes that are directly associated with structure of flagella such as the *flg* or *fli* genes encoding flagella basal body or flagellin (McCarter, 2001). It is necessary to discriminate the roles of flagella or motility in adherence and cytotoxicity by determining the relationship among the three factors in the background with intact flagella but no motility.

Accordingly, we first selected *V. vulnificus* clinical or environmental isolates with reduced motility, and then attempted to determine the relationship between motility, adherence and cytotoxicity by using them. Furthermore, we attempted to elucidate the molecular mechanism connecting between motility and CRP, and to determine the relationship among motility, adherence and cytotoxicity in the background with intact flagella structure.

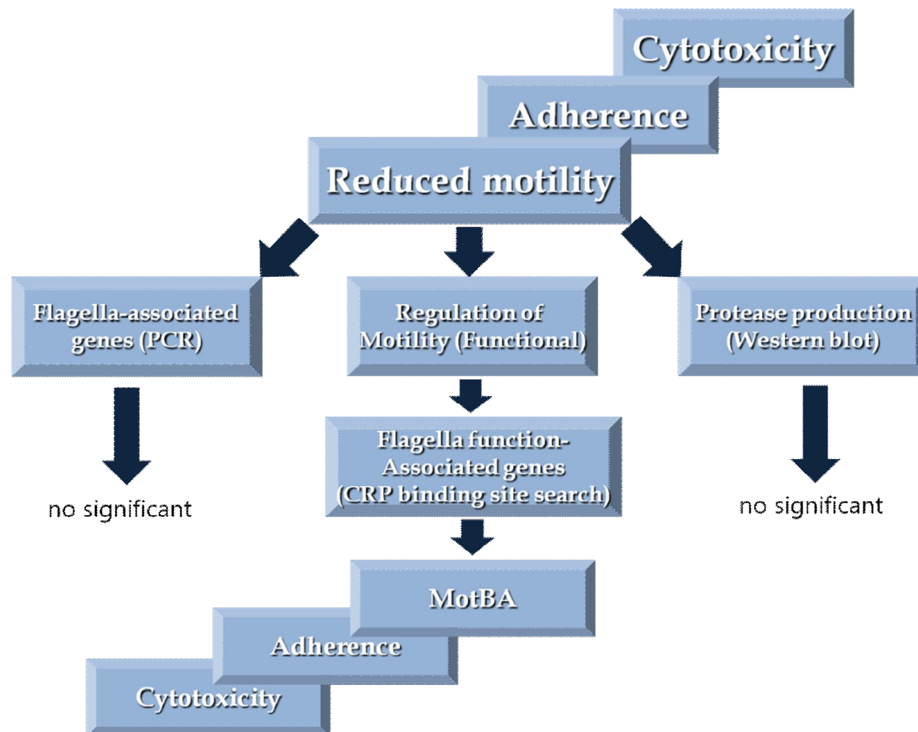


Figure 2. The strategy of this study.

Vibrio vulnificus clinical or environmental isolates with reduced motility were selected, and then the relationship between their motility, adherence and cytotoxicity was determined. Flagella-associated genes were examined by PCR and Protease production was examined by Western blot. CRP binding sites were searched in the promoters of flagella-associated genes and eventually the *motBA* gene was selected. The effect of MotBA on motility, adherence, cytotoxicity was determined by a *motBA* mutation and the effect of CRP on *motA* transcription was determined by a *lacZ*-fused *motA* transcription reporter strain.

Ⅱ. Materials and Methods

1. Bacterial strains, media and reagents

Bacterial strains, plasmids and primers used in this study are listed in Tables 1, 2 and 3. Of total 250 *V. vulnificus* clinical or environmental isolates, 14 strains showing reduced motility were selected as shown in Figure 3 and named RM (reduced motility) -1 to 14. The *V. vulnificus* M06-24/O strain showing robust motility (Reddy *et al*., 1992) and the *V. vulnificus* CMM710 strain showing reduced motility (Choi *et al*., 2006) were used as control. Heart Infusion medium (HI; BD, Franklin Lakes, NJ, USA) supplemented with 2% NaCl, Luria Bertani (LB; BD) medium supplemented with 2% NaCl, and Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS, BD) medium were used to cultivate *V. vulnificus* strains, and LB medium was used to cultivate *Escherichia coli* strains. Antibiotics (BD) were used at the following concentrations (μg/㎖). For *E. coli*, ampicillin (amp) 50, kanamycin (Km) 50, tetracycline (Tc) 12.5, and chloramphenicol (Cm) 30; for *V. vulnificus*, ampicillin 20, kanamycin 200, tetracycline 2, and chloramphenicol 2. Unless

otherwise noted, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Hemolysin assay and transferrin-bound iron utilization assay

The level of hemolysin (VvhA) production was measured as described in a previous study (Shao *et al*., 2000). The ability of *V. vulnificus* strains to utilize transferrin-bound iron (TBI) was compared as described in our previous study (Kim *et al*., 2007).

Table 1. Bacterial strains used in this study

Strains	Relative characteristics	Sources and References
Vibrio vulnificus		
M06-24/O	A highly virulent clinical isolate	Reddy *et al*., 1992
CMM2101	M06-24/O with a *lacZ* mutation	Kim *et al*., 2003
CMM710	M06-24/O with a *crp* mutation	Choi *et al*., 2006
CMM714	CMM710 with a *crp* complementation	Choi *et al*., 2006
RC100	CMM2101 with a *crp* mutation	This study
RC364	M06-24/O with a *motBA* mutation	This study
RC366	CMM2101 with a merozygotic P_{motA}::*lacZ* fusion	This study
RC368	RC366 with a *crp* mutation	This study
RC376	RC368 with a *crp* complementation	This study
RM-1 to 9	Clinical isolates	Lab collection
RM-10 to 14	Environmental isolates	Lab collection
Escherichia coli		
SY327 λ*pir*	Host for suicide vector	Miller & Mekalanos, 1988
SM10 λ*pir*	Conjugation donor	Miller & Mekalanos, 1988
DH5α	F- *recA1*; restriction negative	Lab collection

Table 2. Plasmids used in this study

Plasmids	Relative characteristics	Sources and References
pDM4	Suicide vector with R6K origin; Cm^R	McGee *et al.*, 1996
pQF52	IncP *lacZ* transcriptional fusion vector; Amp^R	Farinha & Kropinski, 1990
pLAFR3II	pLAFR3 with *bla* inserted at the cos site; Amp^R and Tc^R	Kim *et al.,* 2003
pRK2013	IncP, Km^R, Tra $Rk2^+$ *repRK2 repE1;* conjugative helper vector	Ditta *et al.*, 1980
pRC304	A *Sma*I-*Sca*I fragment containing a promoterless *lacZ* gene from pQF52 cloned into *Sma*I-cut pDM4	This study
pRC330	A 1.6-kb *Xba*I-*Spe*I fragment containing an in-frame deletion of the *V. vulnificus motBA* gene cloned into pDM4	This study
pRC336	A 2.37-kb *Hin*dIII-*Eco*RI fragment containing the *motBA* gene cloned into pLAFR3II	This study
pRC334	A 668-bp *Bgl*II-*Sma*I fragment containing the *motA* promoter region cloned into pRC304	This study

Table 3. Primers used in this study

primers	Sequences (5'→3')	Sources
motA-up-1	gc**TCTAGA**cactcaatcgctgacgcaggtg'	This study
motA-up-2	cgtacccaattataaatccacaaagcactcctcatgc	This study
motB-down-1	tttgtggatttataattgggtacgtcagttgaataaaagatg	This study
motB-down-2	gg**ACTAGT**gccacgcttgatgtgcagatagc	This study
motA-rep-up	ga**AGATCT**ccaaggtggagaaatcgctgag	This study
motA-rep-down	tcc**CCCGGG**aagaatcatcgccatcacgacgaaag	This study
motAB-comp-F	ccc**AAGCTT**ccaaggtggagaaatcgctgag	This study
motAB-comp-R	g**GAATTC**gcatttctgctcatcttttattcaactg	This study
CRP-1	tacctactggcgatgatcgatg	Choi *et al*., 2006
CRP-7	cggaatctgagagggtttagt	Choi *et al*., 2006

Capital bold letters indicate the restriction enzyme-recognition sequences: TCTAGA (*Xba*I), ACTAGT (*Spe*I), AGATCT (*Bgl*II), CCCGGG (*Sma*I), AAGCTT (*Hin*dIII), GAATTC (*Eco*RI).

3. Mutation and complementation of the *crp* gene

A deletion mutation of *crp* (CMM710) and a complementation of *crp* (CMM714) were previously described (Choi *et al*., 2006). The same *crp* mutation (RC368) or complementation (RC376) was introduced into RC366 with a P_{motA}::*lacZ* fusion. The *crp* mutation or complementation in each strain was confirmed by polymerase chain reaction (PCR) using the primers CRP-1 and -7 (data not shown).

4. Mutation and complementation of the *motBA* gene

A deletion mutant of the *V. vulnificus motBA* gene was constructed in *V. vulnificus* M06-24/O using the crossover PCR method and the suicide vector pDM4 with a R6K origin (McGee *et al*., 1996). Two pairs of PCR primers motA-up-1/motA-up-2 and motB-down-1/motB-down-2 were used for the PCR amplification of the 5′ and 3′ ends of the *motBA* gene (GeneBank Accession No. VV1_0311 and VV1_0312), respectively. The two PCR products

were used as the template for the second PCR amplification using the PCR primers, motA-up-1 with *Xba*I overhang and motB-down-2 with *Spe*I overhang. The *Xba*I-*Spe*I fragment with the deleted *motBA* gene was directly cloned into pDM4 (McGee *et al.*, 1996). The resulting plasmid (pRC330) was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (Miller & Mekalanos, 1988), and subsequently transferred to *V. vulnificus* M06-24/O by conjugation. A stable transconjugant was selected on TCBS agar containing chloramphenicol, and subsequently on HI agar containing 10% sucrose. The deletion of the *motBA* gene was confirmed by PCR using the primers motA-up-1 and motB-down-2 (data not shown). The resulting strain with the deleted *motBA* gene was named RC364.

To restore the wild type *motBA* gene in *V. vulnificus* RC364, a 2.37-kb *Hin*dIII-*Eco*RI fragment containing the full *motBA* gene was amplified using the PCR primers, motAB-comp-F with a *Hin*dIII overhang and motAB-comp-R with an *Eco*RI overhang, and the PCR product was subsequently subcloned into the broad host range vector pLAFR3II . The resulting plasmid pRC336 was transferred into *V. vulnificus* RC364 by triparental mating using

the conjugative helper plasmid pRK2013 (Ditta *et al.*, 1980). A stable transconjugant was selected on TCBS agar containing ampicillin and tetracycline. The presence of the wild type *motBA* gene was confirmed by PCR using the primers motAB-comp-F and motAB-comp-R (data not shown).

5. Construction of chromosomal *motA* transcription reporters

The merozygotic P_{motA}::*lacZ* fusion was constructed as follows. The 668-bp *Bgl*II-*Sma*I fragment containing the regulatory region of the *motA* gene was amplified using the PCR primers motA-rep-up with *Bgl*II overhang and motA-rep-down with *Sma*I overhang. The PCR product was subcloned into the vector pRC304, which had been prepared by subcloning a *Sma*I-*Sca*I fragment containing the promoterless *lacZ* gene of pQF52 (Farinha and Kropinski, 1990) into the *Sma*I site of pDM4 (McGee *et al.*, 1996). The resulting plasmid (pRC334) was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (Miller & Mekalanos, 1988), and transferred to CMM2101 by conjugation. A transconjugant was selected on TCBS agar containing chloramphenicol. The presence of the P_{motA}::*lacZ* fusion in RC366 was

confirmed by β-galactosidase assay (Miller, 1992). The resulting reporter strain with the P_{motA}::*lacZ* fusion was named RC366.

The *crp*-deleted P_{motA}::*lacZ* transcription reporter RC368 was constructed by incorporating the P_{motA}::*lacZ* fusion on pRC334 into the chromosome of RC100 by conjugation, as described above. RC100 was constructed by introducing a *lacZ* deletion mutation into CMM710 as described previously with only one exception that pDM4 was used instead of pKAS32 (Choi *et al*., 2006). The deletion of the *crp* gene was confirmed by PCR using the CRP-1 and -7 primers (data not shown).The resulting strain was named RC368

To restore the wild-type *crp* gene in the *crp*-deleted P_{motA}::*lacZ* transcription reporter RC368, the plasmid pCMM712 containing the wild-type full *crp* gene was transferred into RC368 via triparental mating using the conjugative helper plasmid pRK2013 as described previously (Choi *et al*., 2006). A stable transconjugant was selected on TCBS agar plates containing chloramphenicol and tetracycline. The presence of the wild-type *crp* gene on plasmid in RC376 was confirmed by PCR using the CRP-1 and -7 primers (data not shown). The resulting strain was named RC376.

6. Observation of colony morphology and motility

Colony morphology was observed on 2.5% NaCl HI agar. Motility was observed on the surface of 2.5% NaCl HI semisolid medium containing 0.3% agar, as described previously (Kim *et al.*, 2007; Nakhamchik *et al.*, 2008). In brief, *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 using the end of toothpick. The plates were incubated overnight at 37℃ and the diameters of the spreading halos were measured.

7. Western blot analysis for VvpE

Western blot was conducted using rabbit polyclonal anti-VvpE-body, which was prepared using recombinant GST-VvpE (Park *et al.*, 2008). Equal volumes (20 μL) of culture supernatants were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels. Thereafter, the separated proteins were transferred onto nitrocellulose membranes (PROTRAN,

Whatman GmbH, Germany). After being incubated with blocking solution [0.2% Tween 20 and 5% Skim milk in phosphate-buffered saline (PBS, pH7.2)] at 4℃ overnight, the membranes were allowed to react with the adsorbed rabbit polyclonal anti-VvpE-body (1 : 250 diluted in washing buffer consisting of 0.1% Tween 20 and 1% Skim milk in PBS) as the primary antibody, and then 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 (BCIP)-nitroblue tetrazolium (NBT) substrate solution.

8. PCR analysis

The wild type M06-24/O strain and 14 *V. vulnificus* strains with reduced motility were genetically analyzed by PCR targeting 13 flagella-associated genes (Supplement 2). The PCR primers used in this study are listed in Supplement 3. After bacteria were cultured in 2.5% NaCl HI broth overnight, genomic DNA was isolated using the bacteria genomic DNA extraction kit (G-spin^{TM}, 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°C pre-denaturation for 5 min, 95°C denaturation for 30 sec, 64°C annealing for 30 sec, 72°C extension for 30 sec, and 72°C post-extension for 10 min. The amplified PCR products were electrophoresed on 1.0% agarose gels and visualized by staining with ethidium bromide.

9. Adhesion assays

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 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 10^8 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 mℓ of DMEM. The HeLa P3 (S) cells were incubated with *V. vulnificus* cells at a multiplicity of infection (MOI) of 10 for 15 min at 37℃ in 5% CO_2 incubator. The cell monolayers were then washed six times with prewarmed PBS to remove non-adherent 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 assays

The overnight-cultured *V. vulnificus* strains were inoculated into fresh 2.5% NaCl HI broth at 5 x 10^6 cells/mℓ, 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 10^8 cells/mℓ 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/mℓ 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. Growth condition and β-galactosidase activity measurement

V. vulnificus strains were preconditioned by culturing in HI broth at 37°C overnight. Preconditioned strains were transferred to fresh HI broth to a concentration of 5 x 10^6 cells/㎖, and cultured with vigorous shaking at 37°C for 24 h. During culture, culture aliquots were withdrawn at appropriate times to measure bacterial growth and β-galactosidase activity. Bacterial growth was measured by the optical density of culture aliquots at

600 nm (OD_{600}). β-Galactosidase activity in culture aliquots was measured as previously described (Miller, 1992).

III. Results

1. Bacterial characterization of *V. vulnificus* strains with reduced motility

To determine the relationship among motility, adherence and cytotoxicity, the 14 RM strains were selected (Figure 3). Motility was compared by measuring the diameters of swarming haloes which were formed on the surface on semisolid agars after 12 h incubation at 37℃. The M06-24/O strain with wild-type *crp* exhibited swarming motility more robustly than the CMM710 strain with deleted *crp*. The diameters of swarming haloes in the M06-24/O and the CMM710 strain were about 84.6 mm and about 49.6 mm, respectively. Compared to the M06-24/O and CMM710 strains, RM strains showed reduced but various swarming motility, showing the maximum (about 39.7 mm) in the RM-7 strain and the minimum (about 3.7 mm) in the RM-9 strain. One remarkable thing was that the swarming motility of the CMM710 strain with a *crp* deletion was reduced only partially, and moreover was more active than those of RM strains.

Other bacterial characteristics of these strains are listed in Table 4. The M06-24/O strain showed opaque colonies on HI agar, grew well on 2% NaCl, formed greenish colonies on TCBS agar, produced extracellular hemolysin (VvhA) and utilized transferrin-bound iron (TBI). The CMM710 strain also greenish colonies on TCBS agar and grew well on 2% NaCl like the M06-24/O strain. In contrast, the CMM710 showed translucent colonies on HI agar, and less poorly produced extracellular hemolysin and utilized TBI than the M06-24/O strain. Ten RM strains showed opaque colonies and the other RM strains showed translucent colonies on HI agar. All RM strains grew well on 2% NaCl, formed greenish colonies on TCBS agar, produced extracellular hemolysin and utilized TBI. Overall, these results indicate that there was no significant correlation between motility and colony morphology, or the ability to grow on NaCl, to ferment sucrose, to produce hemolysin or to utilize TBI.

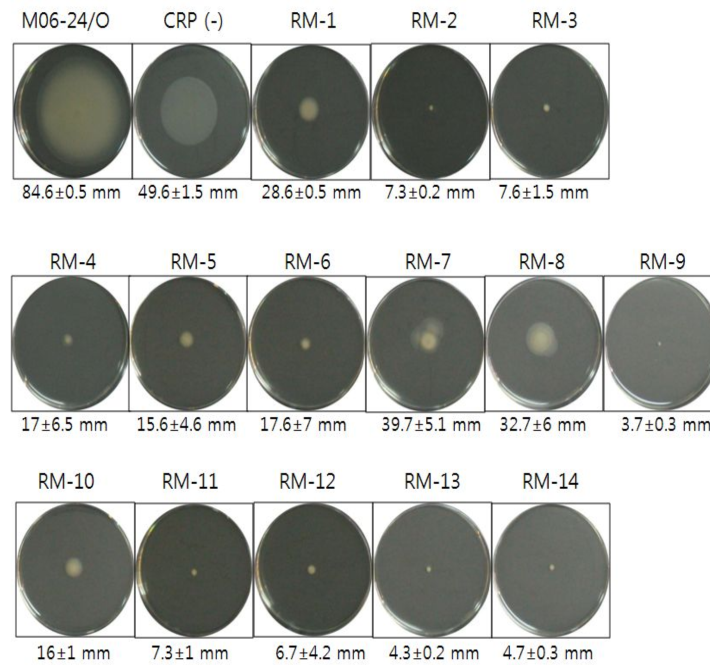


Figure 3. Swarming haloes formed by *V. vulnificus* strains used in this study. M06-24/O strain showing robust motility, CMM710 showing reduced motility, 14 *V. vulnificus* strains showing reduced swarming motility (RM-1 to 14) were used. One colony of each strain grown on 2.5% NaCl HI agar was picked 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 of the triplicate experiments with similar results is shown. Data indicate the average values and SEM obtained from the three independent experiments.

Table 4. Characterization of *Vibrio vulnificus* strains used in this study

Strains	Colony morphology	Growth on 2% NaCl	TCBS	Hemolysin	Growth on TBI
M06-24/O	Opaque	Y	G	P	Y
CMM710	Translucent	Y	G	P	Y
RM-1	Opaque	Y	G	P	Y
RM-2	Opaque	Y	G	P	Y
RM-3	Opaque	Y	G	P	Y
RM-4	Opaque	Y	G	P	Y
RM-5	Opaque	Y	G	P	Y
RM-6	Opaque	Y	G	P	Y
RM-7	Opaque	Y	G	P	Y
RM-8	Translucent	Y	G	P	Y
RM-9	Translucent	Y	G	P	Y
RM-10	Opaque	Y	G	P	Y
RM-11	Opaque	Y	G	P	Y
RM-12	Opaque	Y	G	P	Y
RM-13	Translucent	Y	G	P	Y
RM-14	Translucent	Y	G	P	Y

RM stands for 'Reduced motility'. Y: yes; G: green; P: produced; TBI: transferrin-bound iron; TCBS:

2. The composition of flagella-associated genes

Many genes are related to flagellar biosynthesis and function (Supplement 1). Of these genes, 13 genes were selected on the basis of the presence of putative promoter region (Supplement 2). We compared the composition of the 13 flagella-associated genes by using PCR. The PCR primers used for this experiment are listed in Supplement 3. No difference in the composition of the 13 flagella-associated genes was observed among the 14 RM strains and M06-24/O and CMM710 strains (Supplement 4). They possessed all 13 flagella-associated genes with only one exception that the *flaC* gene encoding for structural flagella protein missed in the RM-2 strain. Nevertheless, these results implied that there was no significant difference in the composition of flagella-associated genes among the RM strains

3. Relation between swarming motility and VvpE production

It was demonstrated that *V. vulnificus* swarming motility is affected by metalloprotease production (Kim et al., 2007). In this study, to determine the relationship between reduced motility and metalloprotease production, the

ability of the RM strains to produce metalloprotease was compared on Western blot and the intensity of positive bands were arbitrarily digitalized from 0 to 4 for statistical analysis (Figure 4). The M06-24/O produced the largest amount of metalloprotease, which was assigned to band intensity 4 and the CMM710 strain produced only a small amount of metalloprotease of band intensity 1. The metalloprotease production in the RM strains was variable. The RM-3, 6 and 9 strains produced the largest amounts of metalloprotease (band intensity 4) which were comparable to the metalloprotease production level of the M06-24/O strain. In contrast, the RM-2 and 8 strains did not produce metalloprotease (band intensity 0). These results indicated that the reduced motility of the RM strains was not related to their ability to produce metalloprotease.

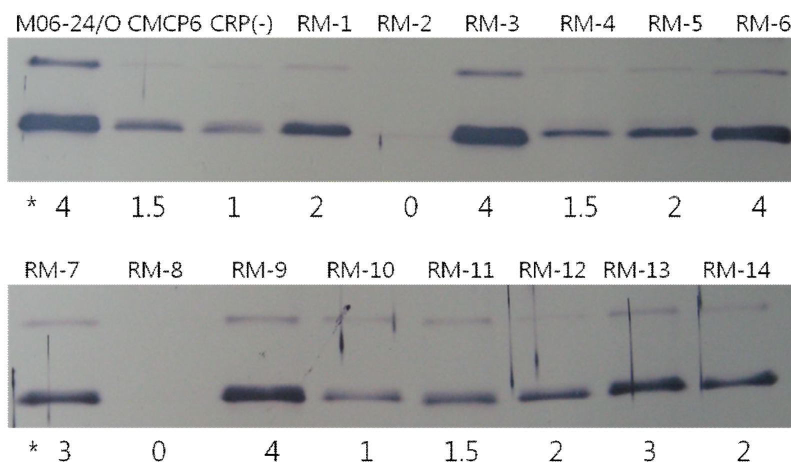


Figure 4. Extracellular VvpE production in *V. vulnificus* strains.

The *V. vulnificus* strains, wild-type M06-24/O, wild-type CMCP6, CMM710 with deleted *crp*, and 14 *V. vulnificus* strains showing reduced swarming motility (RM-1 to RM-14), were cultured in 2.5% NaCl HI broth with vigorous shaking (220 rpm) at 37℃ for 24 h. Culture supernatants were obtained by centrifugating the aliquots to observe the production profile of VvpE. Equal volumes (20 μl) of the culture supernatants were electrophoresed and Western blot was conducted using the VvpE-specific polyclonal antibody as primary antibody, as described in the Materials and Methods section. The symbol (*) indicates the band intensity.

4. Adherence to HeLa P3 (S) cells

In order to determine the relationship between motility and adherence, the adherence of the 14 RM, M06-24/O and CMM710 strains to the HeLa P3 (S) cells was compared (Figure 5). The M06-24/O strain showed adherence of about 2.94% and the CMM710 strain showed adherence of about 0.15%. All RM strains showed lower adherence with individual variance (0 to 1.14%) than the M06-24/O. The RM-4 strain only showed higher adherence than the CMM710 strain. Overall, these results indicate that the RM strains can also adhere to HeLa P3 (S) cells with individual variance but far less efficiently than the M06-24/O strain or even the CMM710 strain.

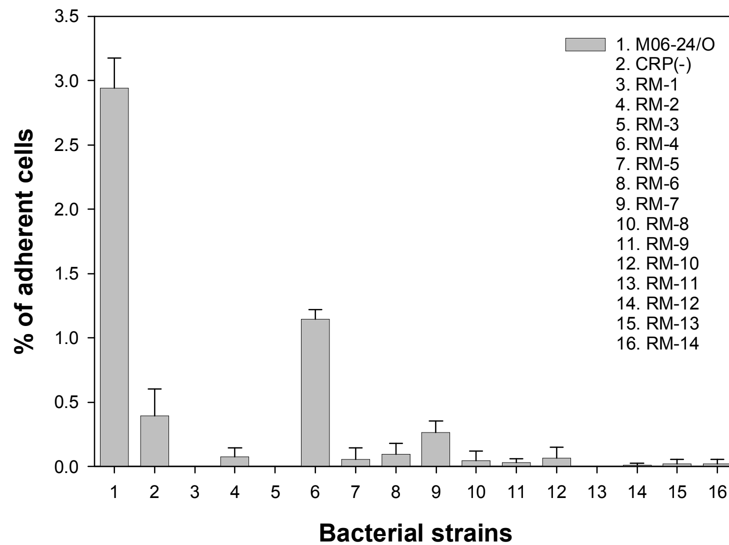


Figure 5. Adherence of *V. vulnificus* strains to HeLa P3 (S) cells.
HeLa P3 (S) cells were infected with the log-phase *V. vulnificus* strains, wild-type M06-24/O, CMM710 with deleted *crp*, and 14 *V. vulnificus* strains showing reduced swarming motility (RM-1 to RM-14), at a multiplicity of infection (MOI) of 10 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. Adherence in the CMM710 strain and all RM strains was significantly lowered compared to that of the M06-24/O strain ($p<0.05$ in Student's *t* test).

5. Cytotoxicity to HeLa P3 (S) cells

The cytotoxicity of the RM, M06-24/O and CMM710 strains to the HeLa P3 (S) cells was determined using LDH assay (Figure 6). The M06-24/O strain showed cytotoxicity of about 46.73%, and the CMM710 strain did not any cytotoxicity. All RM strains showed lower cytotoxicity with individual variance (0 to 16.39%) than the M06-24/O strain, and showed higher cytotoxicity than the CMM710 strain. Overall, the results indicate that the RM strains are also cytotoxic to HeLa P3 (S) cells with individual variance, but far less efficiently than the M06-24/O strain.

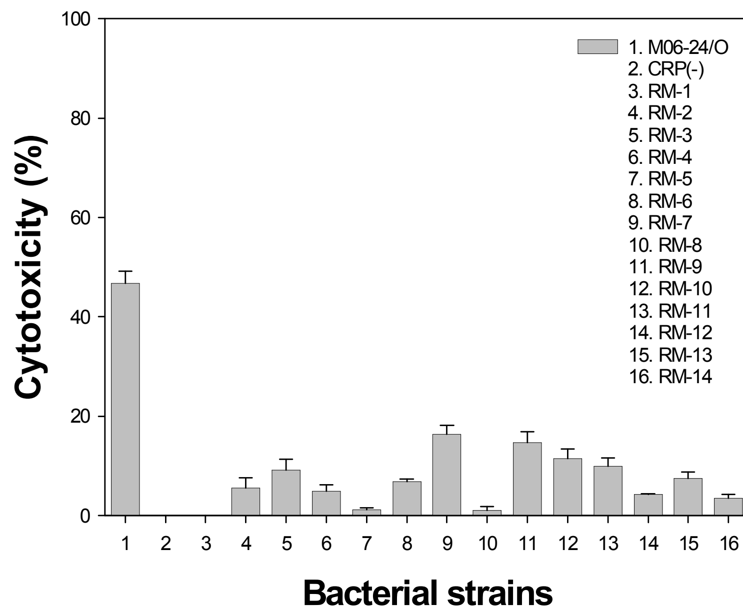


Figure 6. Cytotoxicity of *V. vulnificus* strains to HeLa P3 (S) cells.
HeLa P3 (S) cells were infected with log-phase *V. vulnificus* strains, wild-type M06-24/O, CMM710 with deleted *crp*, and 14 *V. vulnificus* strains showing reduced swarming motility (RM-1 to RM-14), at a MOI of 50 for 90 min. LDH 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. Cytotoxicity in the CMM710 strain and all RM strains was significantly lowered compared to that of the M06-24/O strain ($p<0.05$ in Student's *t* test).

6. Correlation among motility, adherence and cytotoxicity

Correlation among motility, adherence and cytotoxicity was statistically determined by correlation coefficients as shown in Supplement 5 and Table 5. There was 'moderate to good positive relationship' between motility and adherence ($R=0.599$, $p=0.0111$), between motility and cytotoxicity ($R=0.698$, $p=0.0018$) and between adherence and cytotoxicity ($R=0.724$, $p=0.010$). Overall, these results indicate that motility and adherence are positively correlated with cytotoxicity.

Table 5. Correlation among motility, adherence and cytotoxicity

Variables	Probability	Correlation coefficient	Equations
Motility & Adherence	0.0111	0.599	Adherence = 18.5 + (21.4 × Motility)
Motility & Cytotoxicity	0.0018	0.698	Cytotoxicity = 2.10+ (0.317 × Motility)
Adherence & Cytotoxicity	0.010	0.724	Cytotoxicity = 6.42 + (11.7 × Adherence)

Multiple linear regression, the statistical program SigmaStat (Jandel Corporation, Version 1.0).

7. Analysis of non-coding regions

All the fourteen flagella-associated genes examined in this study have putative CRP binding sites (Table 6). The putative CRP binding sites showed high similarities (12 to 17 of 22 bp) with the CRP binding consensus sequence of *E. coli* (Zhang *et al*., 2005). The CRP binding site of the *flaK* non-coding region was the highest similarity (17 of 22 bp). However, its location (-55 t0 -74 bp from ATG) appeared to be too near to and to be overlapped with the RNA polymerase binding site, although the transcription start site of the *flak* gene was not known. This suggested that expression of the *flak* gene might be negatively regulated by CRP. Accordingly, the *flaK* gene was excluded because this study aimed at finding out which of the flagella-associated genes are positively regulated by CRP. In contrast, the putative CRP binding site of the *motBA* regulatory region also showed a high similarity (16 of 22 bp) to the *E. coli* CRP binding consensus sequence as well as alternate sequence (Figure 7), and was located at -110 to -89 from ATG. Its location highly suggested that the *motBA* gene may be positively regulated by CRP. Accordingly, we selected

the *motBA* gene as a target gene for further experiments.

Table 6. Putative CRP binding sequences in flagella-associate genes

Promoters	Accession No	Sequences (5′→3′)	Location from translational start codon
Consensus*		**AATTGTGATCTAGATCACATTT**	
P_{flaE}	1177165	**A**GA**G**G**GAT**A**T**C**GAT**T**A**A**AT**G	17-2
P_{flaD}	1177166	**AT**C**G**CTT**T**G**T**C**GAT**G**AC**C**TTT**	303-283
P_{flaC}	1177167	**AATTG**AA**T**CTA**CTA**A**ATCA**G**A**AA**T**	145-121
P_{flgK}	1177169	**AA**A**T**CCTT**TCTA**T**ATCA**GT**CAT**G**T**	103-80
P_{flgB}	1177178	**AAT**ATC**G**G**TTA**A**A**A**CA**A**ATTT**	100-80
P_{motBA}	1177274	**AATTGTGA**CT**T**G**T**T**G**GC**CA**AG**TTT**	110-89
P_{flaF}	1178823	**AAT**AT**T**TG**T**GGC**GATCAC**CG**TT**	29-8
P_{flaB}	1178824	**TT**T**T**C**A**AAA**A**A**ATC**G**CATTT**	134-115
P_{flaA}	1178825	**AATT**C**T**CC**T**AA**AG**G**T**ATTC**TTT**	115-94
P_{flaK}	1178830	**AATTG**A**T**C**AT**T**T**T**GAT**TT**C**G**CAT**A**T**	50-74
P_{fliE}	1178833	G**A**T**TTG**AA**ATCT**T**G**G**TCA**G**ATT**	128-108
P_{fliH}	1178836	C**A**A**TG**AA**A**A**CT**CA**ATCAC**TG**A**G**TT**	54-32
P_{fliK}	1178839	C**ATTGGAT**TT**T**TA**A**C**CCATTT**	43-24
P_{flhA}	1178848	G**ATTG**CT**A**G**CTAG**CA**C**CT**A**A**TT**	106-76

-*: *Escherichia coli* CRP binding consensus sequence

-P stands for promoter

-Bold letters indicate nucleotides matched with the *E. coli* CRP binding consensus sequence

CRP

aattgtga tctagatcacattt
aattgtgattcgtttcacattt

119 cggcaaaattgtgacttgttggccaagttttttctaaaagatcgttactcattgtcgataa

agaaaataatctctttatattgcggtaaatcggaaaatagcatgaggagtgctttgtg 1

Figure 7. Putative cAMP receptor protein (CRP)-binding site in the regulatory region of the *Vibrio vulnificus motA* gene.
Sequences were analyzed using the program DNAssist (ver 2.2). Two CRP-binding site consensus/alternate sequences of *Escherichia coli* are indicated above, and the matched nucleotides are marked in red. The numbers indicate the nucleotide positions relative to the translational start site of *motA*.

8. Effect of a *motBA* mutation on motility

The *motBA* gene encodes for flagella motor protein which supplies energy required for flagella rotation. The effect of MotBA on motility, adherence and cytotoxicity was determined by the *motBA* deletional mutant RC364 strain (Figure 8). On semisolid HI agars, the M06-24/O strain formed large-sized swarming haloes with about 84.6 mm diameter and the CMM710 strain formed medium-sized swarming haloes with about 49.6 mm diameter. In contrast, the RC364 strain exhibited no swarming haloes. After 15 min incubation with HeLa P3 (S) cells (Figure 9), the M06-24/O strain showed a high adherence of about 10.6%, and the CMM710 strain showed a low adherence of about 0.5%. In contrast, the RC364 strain showed no adherence. In LDH assay (Figure 10), the M06-24/O strain showed a high cytotoxicity of about 46.72%, and the CMM710 strain showed no cytotoxicity. In contrast, the RC364 strain showed only a low cytotoxicity of 4.02%. Overall, these results indicate that MotBA positively affects flagella-mediated motility and then motility-mediated adherence and cytotoxicity by supplying energy for flagella rotation.

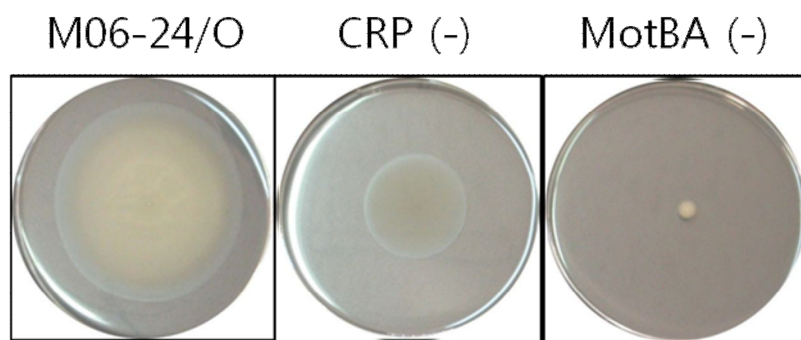


Figure 8. Effect of a *motBA* mutation on swarming motility of *V. vulnificus*.

The wild-type M06-24/O strain, CMM710 with deleted *crp* and RC364 with deleted *motBA* 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.

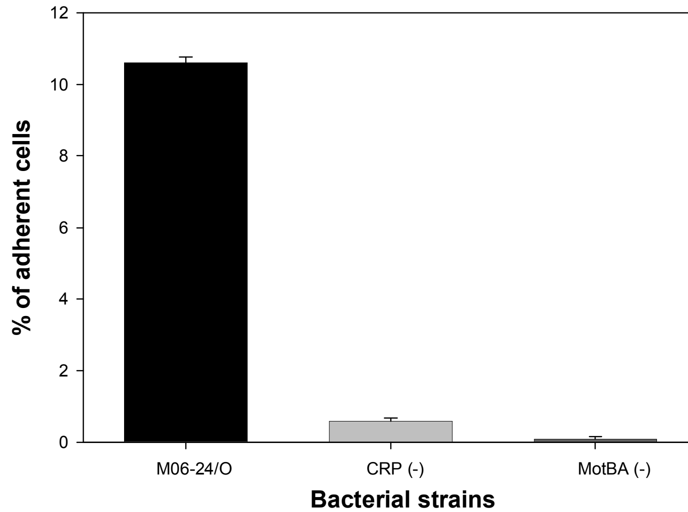


Figure 9. Effect of a *motBA* mutation on adherence of *V. vulnificus* to HeLa P3 (S) cells.

HeLa P3 (S) cells were infected with the log-phase *V. vulnificus* strains, wild-type M06-24/O, CMM710 with deleted *crp*, and RC364 with deleted *motBA*, at a multiplicity of infection (MOI) of 10 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. Adherence in the CMM710 and RC364 strains was significantly lowered compared to that of the M06-24/O strain ($p<0.05$ in Student's *t* test).

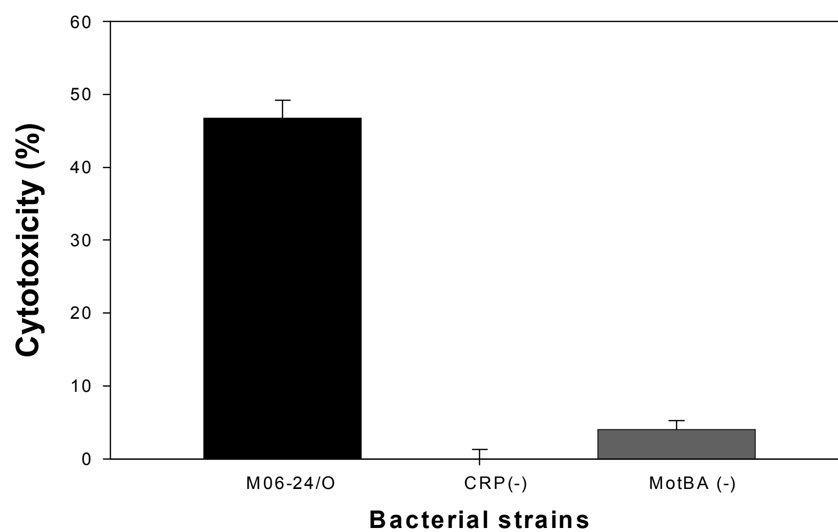


Figure 10. Effect of a *motBA* mutation in *V. vulnificus* on cytotoxicity to HeLa P3 (S) cells.

HeLa P3 (S) cells were infected with log-phase *V. vulnificus* strains, wild-type M06-24/O, CMM710 with deleted *crp*, and RC364 with deleted *motBA*, at a MOI of 50 for 90 min. 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. Cytotoxicity in the CMM710 and RC364 strains was significantly lowered compared to that of the M06-24/O strain ($p<0.05$ in Student's *t* test).

9. Effect of a *crp* mutation on *motA* expression at the transcriptional level

To determine the effect of CRP on *motA* expression at the transcriptional level, the merozygotic P_{motA}::*lacZ* transcription reporter strains, the RC366 strain with wild type *crp,* the RC368 strain with a *crp* mutation, and the RC376 strain with an *in trans crp* complementation were cultured in 2.5% NaCl HI broth for 12 h (Figure 11). The growth of RC366 strain was retarded by a *crp* mutation (RC368). The retarded growth of the RC368 strain was recovered to the wild type level by a *crp* complementation (RC376). In the β-galactosidase activity assay, the *motA* transcription levels in the RC366 strain were significantly but partially decreased by the *crp* mutation in the RC368 strain. The decreased *motA* transcription levels in the RC368 strain were recovered by the *crp* complementation in the RC376 strain. These results clearly indicate that CRP positively and partially regulates *motA* expression at the transcriptional level, and also give a plausible explanation on the result that swarming motility was not completely abolished in the CRP-deficient mutant CMM710 strain (Figure 3).

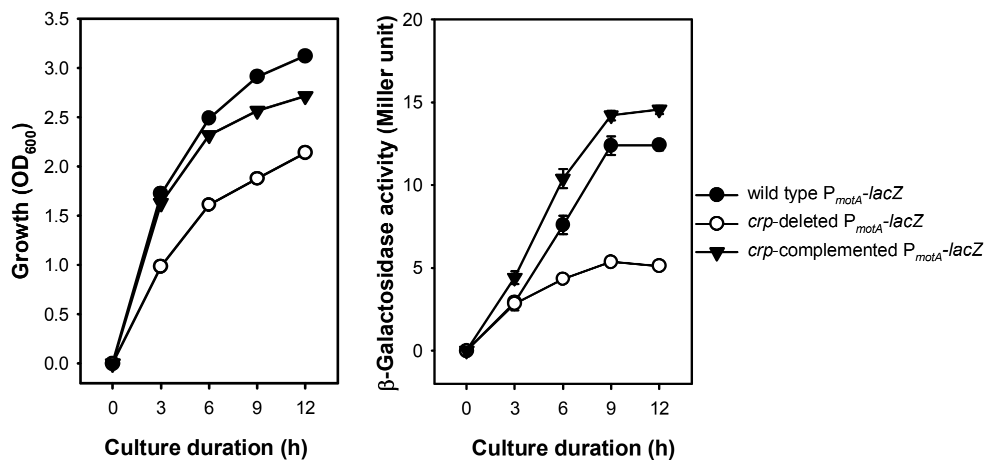


Figure 11. Effect of a *crp* mutation on *motA* expression in *V. vulnificus*. The three P_{motA}::*lacZ* transcription reporters, RC366 with wild type *crp,* RC368 with a *crp* mutation, and RC376 with *in trans crp* complementation, were inoculated at 5 x 10^6 cfu/㎖ in 2.5% NaCl HI broth, and cultured with vigorous shaking (220 rpm) at 37°C for 12 h. During culture, aliquots were removed to monitor bacterial growth and *motA* expression. Bacterial growth was measured by OD_{600}, and the level of *motA* expression was determined by measuring β-galactosidase activity with the Miller method. The growth and *motBA* transcription levels of the RC368 strain were significantly lowered compared to those of the RC366 strain or of the RC376 strain ($p<0.05$ in Student's *t* test).

Ⅳ. Discussion

Through this study, we found that flagella-mediated motility, but not the presence of flagella itself, significantly affects adherence and cytotoxicity, and that CRP controls flagella-mediated motility and then motility-mediated adherence and cytotoxicity by positively regulating *motBA* expression.

According to our results, the reduced motility of the RM strains was not related to their ability to produce metalloprotease VvpE. These findings imply that the reduced motility of the RM strains is associated with structural or functional alteration of flagella but not with metalloprotease deficiency. However, this finding is controversial. It was recently demonstrated that VvpE facilitates swarming motility (Kim *et al.*, 2007), which also fulfills the molecular version of Koch's postulates (Falkow, 2004). A hypothesis is possible that flagella may be the final and essential determinant for *V. vulnificus* swarming motility, and metalloprotease may be

an important intermediate factor that can affect *V. vulnificus* swarming. Further studies are necessary to answer this hypothesis.

In addition, our results show that there was no significant difference in the composition of 13 flagella-associated genes among the RM strains. These findings imply a possibility that the reduced motility of the RM strains might be due to the decreased expression of genes associated with flagella function (functional alteration) such as *motBA* encoding flagella motor protein, but not to the mutation of genes associated with flagella structure (structural alteration) such as the *flg* or *fli* gene encoding flagella basal body or flagellin. Further studies are necessary to determine the expression levels of the flagella-associated genes in the RM strains.

In order to establish infections successfully, *V. vulnificus* must first adhere to and colonize the epithelial surfaces of the skin or the intestinal tract at the early stage of infection. Bacterial cell to cell contact is also required for cytotoxin delivery to host cells or contact-induced cytotoxicity. This fundamental idea has led to an enormous amount of research that deals with understanding how bacterial pathogens adhere to host cells. However,

there have been a few studies that deal with *V. vulnificus* adherence to host cells. As in other pathogenic bacteria, *V. vulnificus* pili or fimbriae are involved in adherence to host cells (Paranjpye *et al.*, 2005; Paranjpye *et al.*, 1998). Mutations of the *pilA* gene which encodes a pilin structural protein, and of the *pilD* gene which encodes a prepilin peptidase, demonstrated a loss of adherence to epithelial cells and a slight increase in LD_{50} compared to the parent strains.

Interestingly, recent studies presented that flagella or flagella-mediated motility are involved in *V. vulnificus* adherence to host cells. A loss of two flagellar structural components (encoded by *flgC* and *flgE*) each resulted in significant decreases in motility, adherence and cytotoxicity compared to those of the parent strains. These mutations resulted in increased LD_{50}s, indicating that the flagellum is necessary for virulence. It has been suggested that decreases in motility, adherence, and cytotoxicity may play a concerted role in reducing virulence, because a loss of motility may lead to decreased adherence and to an inhibition of cytotoxin delivery. It has been hypothesized that decreased adherence can prevent delivery of toxic factors

to host cells or that the flagella can act as a type Ⅲ secretion system for toxins (Park *et al*., 2008; Lee *et al*., 2004). In addition, a possibility that flagella may act as adhesins cannot be excluded.

According to our results, the RM strains could also adhere and cytotoxic to HeLa P3 (S) cells with individual variance but far less efficiently than the M06-24/O strain or even the CMM710 strain. Moreover, motility and adherence were positively well correlated with cytotoxicity. These results also confirm that flagella or flagella-mediated motility are involved in adherence and further cytotoxicity in clinical or environmental isolates.
As described above, most studies have demonstrated the relationship among motility, adherence and cytotoxicity in the backgrounds deficient in one of the flagella structural components via a mutation of a specific gene encoding a flagella structure component. These studies cannot discriminate the roles of flagella from the roles of flagella-mediated motility. It is necessary to discriminate the roles of flagella or motility in adherence and cytotoxicity by determining the relationship among the three factors in the background with intact flagella but no motility, because it has been

hypothesized that reduced motility can prevent delivery of toxic factors to host cells or that the flagella can act as adhesins or a type III secretion system for toxins. In this study, the *motBA* gene encoding flagella motor protein was selected and successfully mutated for the construction of a mutant *V. vulnificus* strain with intact flagella but no motility (flagellated but paralyzed). The *motBA* mutation completely abolished flagella-mediated motility and then motility-mediated adherence and cytotoxicity by blocking energy supply for flagella rotation. These findings imply that flagella-mediated motility, but not the presence of flagella itself, is required for adherence or cytotoxicity. Accordingly flagella do not act as adhesins or a type III secretion system for toxins, but flagella-mediated motility delivers toxic factors to host cells.

The flagellar filament acts as a propeller that is turned by a reversible rotary motor, which is embedded in the membrane (McCarter, 2001; Imae & Atsumi, 1989; Yorimitsu & Homma, 2001). Energy to power flagellar rotation is derived from the transmembrane electrochemical potential of specific ions. Rotation appears to be tightly coupled to the flow of ions through the

motor. Two kinds of motors, which are dependent on different coupling ions, have been described: H^+ and Na^+ motors. In Vibrio species, the sodium motive force drives polar flagellar rotation. Sodium channel-blocking drugs, such as amiloride and phenamil, specifically can inhibit sodium-driven motility. The *Vibrio* sodium-type proteins resemble MotA and MotB of the proton type motors, which are well known in *E. coli* and *S. enterica* serovar Typhimurium. Two cytoplasmic proteins, MotA and MotB, form the force-generating unit through which the protons are channeled. Torque is transmitted from the MotA-MotB complex to the flagellar basal body. Critical electrostatic interactions between MotA and the C-ring of motor switch complex. The complex containing FliG, FliM, and FliN is essential for torque generation and the control of the direction of flagellar rotation.

Recent studies reported that *V. vulnificus* swimming or swarming motility (or adherence or cytotoxicity) is positively regulated by CRP (Shin, 2009; Choi *et al.*, 2006; Kim *et al.*, 2005). The related mechanism remains totally unknown. On the basis of these findings, we can formulate a hypothesis that CRP may regulate expression of genes encoding flagella functional

components which can affect flagella-mediated motility.

According to our results, all the fourteen flagella-associated genes examined in this study have putative CRP binding sites, indicating that expression of several flagella-associated genes may be regulated by CRP. In particular, the putative CRP binding site of the *motBA* regulatory region showed a high similarity to the *E. coli* CRP binding consensus sequence as well as alternate sequence, and was located at -110 to -89 from ATG. Its similarity and location highly suggested that the *motBA* gene may be positively regulated by CRP. Accordingly, we constructed a merozygotic P_{motA}::*lacZ* transcription reporter strains and observed the effect of a *crp* mutation on *motA* expression at the transcription level. As a result, a *crp* mutation partially inhibited a *motA* expression at the transcriptional level and partially inhibited swarming motility.

As in other pathogenic bacteria, the cAMP/CRP complex which primarily responds to carbon-availability also functions as a well-known global regulator in *V. vulnificus* (Shin, 2009). The cleavage of ATP by adenylate cyclase (encoded by *cya*) generates cAMP, which functions by binding the

CRP. The cAMP-CRP binding complex then binds DNA to influence gene expression. Several putative virulence factors of *V. vulnificus* have been shown to be under the regulation of cAMP/CRP, including hemolysin (VvhA), metalloprotease (VvpE), and the vulnibactin (VuuA) and haem receptor (HupA)-mediated iron acquisition system. Mutation of *cya* dramatically reduced hemolysin activity, with CRP binding to the *vvhA* promoter region and activating its transcription. A *crp* mutation suppressed synthesis of vulnibactin and its receptor protein, leading to a loss of transferrin-bound iron utilization. CRP works in concert with the quorum sensing regulator SmcR, and exerts its effects on *vvpE* transcription through the activity of RpoS. The cAMP-CRP system is not only necessary for regulation of individual virulence factors but also contributes to lethality, since a *cya* mutation results in an increased LD_{50}.

V. Summary

Fourteen *V. vulnificus* strains with reduced motility were selected. Their reduced motility was not associated with their metalloprotease productivity, which was measured by Western blot. When randomly-selected thirteen flagella-associated genes were analyzed by PCR, no noticeable difference in their genetic composition was detected among the fourteen strains and the reference strain M06-24/O. When their adherence and cytotoxicity to HeLa P3 (3) cells were examined and statistically analyzed, moderate to good relationship ($0.50<r<0.75$, $p<0.05$) was observed between motility and adherence, motility and cytotoxicity, and adherence and cytotoxicity. Secondly, to determine the effect of CRP on motility, we selected the *motBA* gene encoding flagella motor protein as one of the most likely target genes of CRP by analyzing the putative CRP binding sequences of the flagella-associated genes, and constructed a *motBA* deletional mutant strain and a P_{motA}::*lacZ* transcriptional reporter strain. Motility, adherence and cytotoxicity were almost completely reduced by the mutation of *motBA*. Expression of

motA at the transcription level was significantly decreased by a *crp* mutation. The decreased *motA* expression was normalized to the wild type level by a *crp* complementation. In conclusion, motility seems to be closely related to adherence and cytotoxicity and CRP affects motility and further motility-mediated adherence and cytotoxicity by positively regulating *motBA* expression.

VI. References

Chen YC, Chang MC, Chuang YC, Jeang CL (2004) Characterization and virulence of hemolysin III from *Vibrio vulnificus.* Curr Microbiol **49**: 175-9.

Choi MH, Sun HY, Park RY, Kim CM, Bai YH, Kim YR, Rhee JH, Shin SH (2006) Effect of the *crp* mutation on the utilization of transferrin-bound iron by *Vibrio vulnificus*. FEMS Microbiol Lett **257**: 285-92.

Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA **27**: 7347-7351.

Falkow S (2004) Molecular Koch's postulates applied to bacterial pathogenicity – a personal recollection 15 years later. Nature Rev **2:** 67-72.

Farinha MA, Kropinski AM (1990) Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. J Bacteriol **172**: 3496-3499.

Finlay BB, Falkow S (1997) Common Themes in Microbial Pathogenicity Revisited. Microbiol Mol Biol Rev **61**:136-169.

Fraser GM, Hughes C (1999) Swarming motility. Curr Opin Microbiol **2**:630-635.

Gray LD, Kreger AS (1985) Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus.* Infect Immun **48**: 62-72.

Harshey RM (1994) Bees aren't the only ones: swarming in Gram-negative bacteria. Mol Microbiol **13**: 389-394.

http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/flag.html.

Hülsmann A, Rosche TM, Kong IS, Hassan HM, Beam DM, Oliver JD (2003) RpoS-dependent stress response and exoenzyme production in *Vibrio vulnificus*. Appl Environ Microbiol **69**: 6114-6120.

Imae Y, Atsumi T (1989) Na^+-driven bacterial flagellar motors. J Bioenerg Biomembr **21**: 705-716.

Jones MK, Oliver JD (2009) *Vibrio vulnificus*: Disease and Pathogenesis. Infect Immun **77**: 1723-1733.

Kaneko T, Colwell RR (1975) Adsorption of *V. parahaemolyticus* onto chitin and copepods. Appl Microbiol **29**: 269-274.

Kim CM, Park RY, Choi MH, Sun HY, Shin SH (2007) Ferrophilic characteristics of *Vibrio vulnificus* and potential usefulness of iron chelation therapy. J Infect Dis **195**: 90-8.

Kim CM, Park RY, Chun HJ, Kim SY, Rhee JH, Shin SH (2007) *Vibrio vulnificus*

metalloprotease VvpE is essentially required for swarming. FEMS Microbiol Lett **269**: 170-179.

Kim CM, Park RY, Park JH, Sun HY, Bai YH, Ryu PY, Kim SY, Rhee JH, Shin SH (2006a) *Vibrio vulnificus* vulnibactin, but not metalloprotease VvpE, is essentially required for iron-uptake from human holotransferrin. Biol Pharm Bull **29**: 911-918.

Kim CM, Shin SH (2010) Regulation of the *Vibrio vulnificus vvpE* expression by cyclic AMP-receptor protein and quorum-sensing regulator SmcR. Microbial Pathogenesis **49**: 348-353.

Kim MY, Park RY, Choi MH, Sun HY, Kim CM, Kim SY, Rhee JH, Shin SH (2006b) Swarming differentiation of *Vibrio vulnificus* downregulates the expression of the *vvhBA* hemolysin gene via the LuxS quorum-sensing system. J Microbiol **44**: 226-232.

Kim SJ, Kim CM, Shin SH (2010) Virulence Characteristics of Sucrose-fermenting *Vibrio vulnificus* Strains. Korean J Lab Med **30**: 507-10.

Kim SY, Lee SE, Kim YR, Kim CM, Ryu PY, Choy HE, Chung SS, Rhee JH (2003) Regulation of *Vibrio vulnificus* virulence by LuxS quorum-sensing system. Mol Microbiol **48**: 1647-64.

Kim YK, McCarter LL (2006) ScrG, a GGDEF-EAL Protein, Participates in Regulating Swarming and Sticking in *Vibrio parahaemolyticus.* J Bacteriol

189: 4094-4107.

Kim YR, Kim SY, Kim CM, Lee SE, Rhee JH (2005) Essential role of an adenylate cyclase in regulating *Vibrio vulnificus* virulence. FEMS Microbiol Lett **243**: 497-503.

Kim YR, Lee SE, Kook H (2007) *Vibrio vulnificus* RTX toxin kills host cells only after contact of the bacteria with host cells. Cell Microbiol **10**: 848-62.

Kim YR, Rhee JH (2003) Flagella basal body *flg* operon as a virulence derminant of *Vibrio vulnificus*. Biochem Biophys Res Comm **304**: 405-10.

Koo BS, Lee JH, Kim SC (2007) Phospholipase A as a potent virulence factor of virulence factor of *Vibrio vulnificus*. Int J Mol Med **20**: 913-8.

Kothary MH, Kreger AS (1987) Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. J Gen Microbiol **133**: 1783-91.

Lee JH, Rho JB, Park KJ, Kim CB, Han YS, Choi SH, Lee KH, Park SJ (2004) Role of flagellum and motility in pathogenesis of *Vibrio vulnificus* Infect Immun **72**: 4905-4910.

Litwin CM, Calderwood SB (1993) Role of iron in regulation of virulence genes. Clin Microbiol Rev **6**: 137-149.

Litwin CM, Rayback TW, Skinner J (1996) Role of catechol siderophore

synthesis in *Vibrio vulnificus* Virulence. Infect Immun **64**: 2834-8.

McCarter LL (2001) Polar flagellar motility of the Vibrionaceae. Microbiol Mol Biol Rev **65**: 445-462.

McGee K, Horstedt P, Milton DL (1996) Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. J Bacteriol **178**: 5188-98.

Miller VL, Mekalanos JJ (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determination in *Vibrio cholerae* requires *toxR*. J Bacteriol **170**: 2575-2583.

Miller JH (1992) A short course in bacterial genetics: A laboratory manual and handbook of *Escherichia coli* and related bacteria. Cold Spring Harbor Press, New York, NY, USA.

Okunishi I, Kawagishi I, Homma M (1996) Cloning and characterization of *motY*, a gene coding for a component of the sodium-driven flagellar motor in *Vibrio alginolyticus*. J Bacteriol **178**: 2409-2451.

O'Toole PW, Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol **30**: 295-304.

Ottemann KM, Miller JF (1997) Roles for motility in bacterial-host

interactions. Mol Microbiol **24**: 1109-1117.

Paik KW, Moon B, Park CW, Kim KT, Ji MS, Choi SK, Rew JS, Yoon CM (1995) Clinical characteristics of ninety-two cases of *Vibrio vulnificus* infections. Kor J Infect Dis **27**: 355-365.

Paranjpye RN, Lara JC, Pepe JC, Pepe CM, Storm MS (1998) The type IV leader peptide/*N*-methyltransgerase of *Vibrio vulnificus* controls factors required for adherence to Hep-2 cells and virulence in iron-overloaded mice. Infect Immun **66**: 5659-68.

Paranjpye RN, Storm MS (2005) A *Vibrio vulnificus* type IV pilin contributes to biofilm formation, adherence to epithelial cells, and virulence. Infect Immun **73**: 1411-1422.

Park SD, Shon HS, Jon NJ (1991) *Vibrio vulnificus* septicemia in Korea: clinical and epidemiologic findings in seventy patients. J Am Acad Dermatol **24**: 397-403.

Park J, Ryu SY, Kim CM, Shin SH (2008) Two Forms of *Vibrio vulnificus* Metalloprotease VvpE are Secreted via the Type II General Secretion System. J Microbiol **46**: 338-343.

Pratt LA, Kolter R (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol **30**: 285-293.

Reddy GP., Hayat U, Abeygunawardana C, Fox C, Wright AC, Raneval DR jr, Bush CA, Morris JG Jr. (1992) Purification and determination of the structure of capsular polysaccharide of *Vibrio vulnificus* M06-24/O. J Bacteriol **174**: 2620-2630.

Shao CP, Hor LI (2000) Metalloprotease is not essential for *Vibrio vulnificus* virulence in mice. Infect Immun **68**: 3569-73.

Shin SH (2009) Regulation *Vibrio vulnificus* virulence by cyclic AMP-receptor protein. Med J Chosun Univ **33**: 65-74.

Simpson LM, White VK, Zane SF, Oliver JD (1987) Correlation between virulence and colony morphology in *Vibrio vulnificus*. Infect Immun **55**: 269-72.

Sjoblad RD, Doetsch RN (1982) Adsorption of polarly flagellated bacteria to surfaces. Curr Microbiol **7**: 191-194.

Slonczewski JL, Foster JW (2009) Microbiology. An evolving science. Norton & Company, New York, NY, USA.

Syed KA, Beyhan S, Correa N, Queen J, Liu J, Peng F, Satchell KJ, Yildiz F, Klose KE (2009) The *Vibrio cholera* Flagellar Regulatory Hierarchy Controls Expression of Virulence Factors. J Bacteriol **191**: 6555-6570.

Tacket CO, Brenner F, Blake NJ (1984) Clinical features and epidemiological

study of *Vibrio vulnificus* infection. J Infect Dis **149**: 559-561.

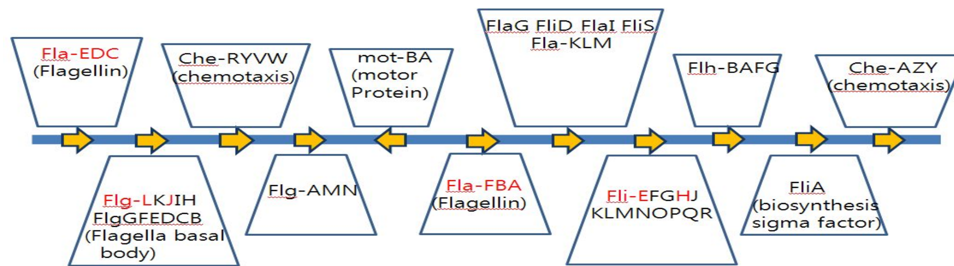
Walker KE, Moghaddame-Jafar S, Lockatell CV, Johnson D, Belas R (1999) ZapA, the IgA-degrading metalloprotease of *porteus mirabilis*, is a virulence factor expressed specifically in swarmer cells. Mol Microbiol **32**: 825-836.

Willey JM, Sherwood LM, Woolverton CJ (2009) Prescott's Principles of Microbiology. McGraw-Hill Companies, New York, NY, USA.

Yorimisu T, Homma M (2001) Na^+-driven flagellar motor of *Vibrio*. Biochim Biophys Acta **1505**: 82-93.

Zhang Z, Gosset G, Barabote R, Gonzalez CS, Cuevas WA, Saier MH Jr (2005) Functional interactions between the carbon and iron utilization regulators, Crp and Fur, in *Escherichia coli*. J Bacteriol **187**: 980–990.

VII. Supplements



Supplement 1. Flagella-associated genes.

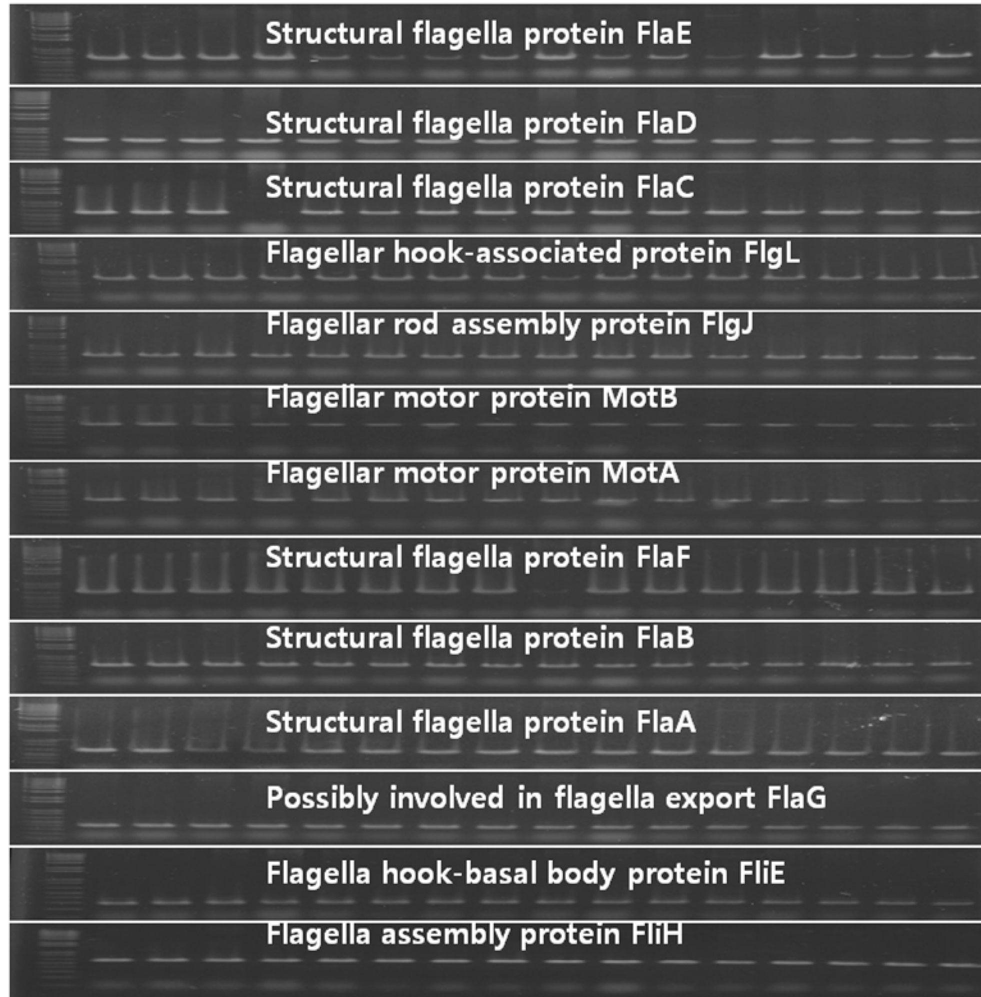
Supplement 2. The flagella-associated genes tested in this study

Genes	Characterization
flaE	Structural flagella protein FlaE (1,128 bp)
flaD	Structural flagella protein FlaD (1,134 bp)
flaC	Structural flagella protein FlaC (1,158 bp)
flgL	Flagellar hook-associated protein FlgL (1,194 bp)
flgJ	Flagellar rod assembly protein/muramidase FlgJ (921 bp)
flaF	Structural flagella protein (1,134 bp)
flaB	Structural flagella protein (1,134 bp)
flaA	Structural flagella protein (1,131 bp)
flag	Flagellar protein FlaG (429 bp)
fliE	Flagellar hook-basal body protein FliE (312 bp)
fliH	Flagellar assembly protein FliH (801bp)
motB	Flagellar motor protein MotB (801 bp)
motA	Flagellar motor protein MotA (762 bp)

Supplement 3. The primers targeting the flagella-associated genes used in

this study

Primers	**Sequences**	**Target genes**
flaE-RT-F/R	5′-aacgtgtctgcgatggtcgctc-3′/ 5′acttcttcgagactgtctccaggc-3′	*flaE*
flaD-RT-F/R	5′-cgtagcagcaatgacagcacagc-3′/ 5′ – gtcaagtcgttgtcgcctgcgg-3′	*flaD*
flaC-RT-F/R	5′-gcactaacgtatccgcgatgactg-3′/ 5′-cgatgttcagctctgcgttatccg- 3′	*flaC*
flgL-RT-F/R	5′-ccaaatgcgagctgcgaagcttg -3′/ 5′-ctaccaacgcaaaatgcgagtgtcg-3′	*flgL*
flgJ-RT-F/R	5′-gcccaactgcgatccgctttg-3′/ 5′-ctacgtcagcaagccgtgaacg-3′	*flgJ*
flaF-RT-F/R	5′-accaatgtggcagcacttgtcgc-3′/ 5′-accgatgccgtgactttgtcagtc -3′	*flaF*
flaB-RT-F/R	5′-gtagcagcaatgacagcacagcg-3′/ 5′-atcgtcaagtcgttgtcgcctgc-3′	*flaB*
flaA-RT-F/R	5′-acgtgtcagcaatgaccgcacag-3′/ 5′-tcaagatcgtcacccgctttggc-3′	*flaA*
flaG-RT-F/R	5′-cgaacatccagccttacggcttg-3′/ 5′-gtctaccagcaaaccagagctgg-3′	*flaG*
fliE-RT-F/R	5′-cggtgaaatgcgcgccatgatg-3′/ 5′-actggcatgttcataaggtctttgtatg-3′	*fliE*
fliH-RT-F/R	5′-cctgacttcgaacagccggaag-3′/ 5′-cgctcttccatccggtagctaac-3′	*fliH*
motB-RT-F/R	5′-gggtaccttcgctgacttgatgtc-3′/ 5′-cgcagagagatcccagttagaacg-3′	*motB*
motA-RT-F/R	5′-tgataggtggccttgctttcgtcg-3′/ 5′-cgcgacgaagcgccaatttatcc-3′	*motA*



Supplement 4. The composition of 13 flagella-associated genes in *V. vulnificus* strains used in this study. Result of PCR.

Supplement 5. Raw data for statistical analysis

Strains	Motility (mm, ± SE[a])	Adhesion (%, ± SD[b])	Cytotoxicity (%, ± SD)
M06-24/O	84.6 ± 0.5	2.94 ± 0.24	46.73 ± 2.48
CMM710	49.6 ± 1.5	0.15 ± 0.16	0
RM-1	28.6 ± 0.5	0.40 ± 0.21	0
RM-2	7.3 ± 2	0.08 ± 0.07	5.55 ± 2.03
RM-3	7.6 ± 1.5	0	9.18 ± 2.13
RM-4	17 ± 6.5	1.14 ± 0.08	4.83 ± 1.31
RM-5	15.6 ± 4.6	0.05 ± 0.09	1.17 ± 0.32
RM-6	17.6 ± 7	0.10 ± 0.09	6.87 ± 0.48
RM-7	39.7 ± 5.1	0.26 ± 0.09	16.39 ± 1.70
RM-8	32.7 ± 6	0.04 ± 0.08	1.06 ± 0.74
RM-9	3.7 ± 0.3	0.03 ± 0.03	14.68 ± 2.18
RM-10	16 ± 1	0.06 ± 0.09	11.46 ± 1.87
RM-11	7.3 ± 1	0	9.90 ± 1.69
RM-12	6.7 ± 4.2	0.01 ± 0.02	4.26 ± 0.18
RM-13	4.3 ± 0.2	0.02 ± 0.03	7.44 ± 1.26
RM-14	4.7 ± 0.3	0.02 ± 0.03	3.48 ± 0.77

[a]Standard error; [b]Standard deviation

VIII. Abstract

Cyclic AMP-receptor Protein Regulates Flagella-mediated Motility in *Vibrio vulnificus*

JiNa Park
(Director: Prof. Sung-Heui Shin)
Department of Bio New Drug Development
Chosun University Graduate School

Vibrio vulnificus is a gram-negative halophilic bacterium that causes necrotizing wound infections and septicemia in susceptible patients with underlying liver diseases. *V. vulnificus* is a highly motile organism by virtue of a polar flagellum and this flagella-mediated motility is considered to be one of the authentic virulence factors. It is known that the flagella-mediated motility is closely related the adherence and cytotoxicity of *V. vulnificus* to host cells, and that motility, adherence and cytotoxicity is all positively affected by cyclic AMP-receptor protein (CRP), which is a well-

known global transcriptional regulator responsive to glucose or energy-availability. In this study, we first attempted to determine the relatedness among motility, adhesion and cytotoxicity in clinical or environmental isolates. We selected fourteen *V. vulnificus* strains with reduced motility. Their reduced motility was not associated with their metalloprotease productivity, which was measured by Western blot. When randomly-selected 13 flagella-associated genes were analyzed by PCR, no noticeable difference in their genetic composition was detected among the fourteen strains and the reference strain M06-24/O. When their adherence and cytotoxicity to HeLa P3 (3) cells were examined and statistically analyzed, moderate to good relationship ($0.50<r<0.75$, $p<0.05$) was observed between motility and adherence, motility and cytotoxicity, and adherence and cytotoxicity. Secondly, to determine the effect of CRP on motility, we selected the *motBA* gene encoding flagella motor protein as one of the most likely target genes of CRP by analyzing the putative CRP binding sequences of the flagella-associated

genes, and constructed a *motBA* deletional mutant strain and a P_{motA}::*lacZ* transcriptional reporter strain. Motility, adherence and cytotoxicity were almost completely reduced by the mutation of *motBA.* Expression of *motA* at the transcription level was significantly decreased by a *crp* mutation. The decreased *motA* expression was normalized to the wild type level by a *crp* complementation. These results indicate that motility is closely related to adherence and cytotoxity and that CRP controls motility and further motility-mediated adherence and cytotoxicity by positively regulating *motBA* expression.

Key words: *Vibrio vulnificus*, Flagellum, Motility, Adherence, Cytotoxicity, Cyclic AMP-receptor protein

Ⅸ. Acknowledgements

처음 대학원에 입학할 때는 2년이란 시간이 끝나지 않을 것처럼 멀게만 느껴졌는데 벌써 시간이 흘러 학위논문을 마치게 되었습니다. 항상 밝은 미소로 가르침을 주시고 저에게 실험뿐만 아니라 학위도 할 수 있게 기회를 주시고 이끌어 주신 존경하는 신성희 교수님께 진심으로 감사 드립니다. 논문을 쓰기까지 부족한 시간이었음에도 불구하고 끝까지 저를 하나하나 세심하게 지도해 주신 마음이 따뜻하신 김춘미 교수님께도 정말 감사 드립니다. 또 따뜻하게 관심 가져주신 임성철 교수님께도 감사 드립니다. 바쁜 실험 중에도 항상 옆에서 실험도 많이 도와주고 격려 아끼지 않았던 미라와 논문 쓰는 동안에도 많은 도움을 준 영희에게도 고맙다는 말 전하고 싶습니다. 부족함 많은 큰 딸에게 묵묵히 뒷바라지 해주시고 믿어주신 아빠, 엄마께 진심으로 감사 드리고 집에서 떨어진 타지에서 혼자 열심히 일하고 있는 동생 지영이, 막내지만 언니보다도 듬직한 지민이 에게도 너무나 고맙고 사랑한다는 말 전하고 싶습니다. 또 힘들 때마다 많은 조언을 통해 용기를 내게 해준 정현씨, 사랑하는 친구들과 논문 쓰는 동안 함께 걱정해 주고 격려해 주었던 사랑하는 미.수.다 에게도 고맙다는 말 전하고 싶습니다. 논문을 쓰면서 아프기도 하고 힘든 시간도 보냈지만 그래서 인지 더욱 더 뜻 깊고 뿌듯한 마음이 앞서는 것 같습니다. 이제 끝이 아닌 또 다른 시작이라는 마음으로 한결같이 열심히 노력하도록 하겠습니다.

저작물 이용 허락서

학 과	바이오신약개발학과	학 번	20097153	과 정	석사
성 명	한글: 박지나 한문: 朴 鉣 娜 영문: JiNa Park				
주 소	광주광역시 남구 노대동 송화마을 2 단지 휴먼시아 208 동 902 호				
연락처	E-mail: nostalgia31@hanmail.net				
논문 제목	한글: Cyclic AMP 수용체 단백에 의한 패혈증 비브리오균의 편모매개 운동성의 조절 영어: Cyclic AMP-receptor Protein Regulates Flagella-mediated Motility in *Vibrio vulnificus*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

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

동의여부 : 동의(o) 반대( )

2010 년 11 월 30 일

저작자: 박 지 나 (서명 또는 인)

조선대학교 총장 귀하