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Identification of the *Vibrio vulnificus fexA* Gene and Evaluation of its Influence on Virulence

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Abstract *Vibrio vulnificus* is the causative agent of foodborne diseases such as gastroenteritis and life-threatening septicemia. Microbial pathogenicity is a complex phenomenon in which expression of numerous virulence factors is frequently controlled by a common regulatory system. In the present study, a mutant exhibiting decreased cytotoxic activity toward intestinal epithelial cells was screened from a library of *V. vulnificus* mutants constructed by a random transposon mutagenesis. By a transposon-tagging method, an open reading frame, *fexA*, a homologue of *Escherichia coli arcA*, was identified and cloned. The nucleotide and deduced amino acid sequences of the *fexA* were analyzed, and the amino acid sequence of FexA from *V. vulnificus* was 84% to 97% similar to those of ArcA, an aerobic respiration control global regulator, from other Enterobacteriaceae. Functions of the FexA were assessed by the construction of an isogenic mutant, whose *fexA* gene was inactivated by allelic exchanges, and by evaluating its phenotype changes *in vitro* and in mice. The disruption of *fexA* resulted in a significant alteration in growth rate under aerobic as well as anaerobic conditions. When compared to the wild-type, the *fexA* mutant exhibited a substantial decrease in motility and cytotoxicity toward intestinal epithelial cell lines *in vitro*. Furthermore, the intraperitoneal LD_{50} of the *fexA* mutant was approximately 10^1–10^2 times higher than that of parental wild-type. Therefore, it appears that FexA is a novel global regulator controlling numerous genes and contributing to the pathogenesis as well as growth of *V. vulnificus*.

Key words: *V. vulnificus*, *fexA*, aerobic respiration regulator

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The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of foodborne diseases such as life-threatening septicemia and possibly gastroenteritis in individuals with underlying predisposing conditions such as liver damage, excess levels of iron, and immunocompromised conditions [for recent reviews, see 19, 32]. Wound infections can result from exposure to seawater or from the handling of shellfish contaminated with *V. vulnificus*. Mortality from septicemia is very high (>50%), and death may occur within one to two days after the first sign of illness [for recent reviews, see 19, 32]. Several potential virulence factors, including an endotoxin, polysaccharide capsule, iron sequestering systems, cytolytic hemolysin, elastase, phospholipase A2, and other exotoxins, have been identified in *V. vulnificus* [19, 27, 32].

Microbial pathogenicity is multifactorial and a complex phenomenon that involves the products of many genes. For development of a disease, survival and multiplication are clearly the priorities of the infecting microorganisms. Therefore, it has been generally accepted that virulence factors include all those factors contributing to survival and multiplication within the host [21]. Most of these virulence factors act cooperatively to obtain maximum effectiveness in the pathogenesis, and their expression is coordinately controlled by a common global regulatory system in response to environmental signals [22]. This coordinated regulation by global regulators would facilitate cooperation of the virulence factors, and would be crucial for the overall success of the infectious microorganisms during pathogenesis [13]. Indeed, many global regulatory proteins that control expression of genes required for adaptation and optimal survival in response to environmental stresses also modulate the expression of numerous virulence factors in bacterial pathogens [22, 13].

V. vulnificus has to survive adverse conditions both inside and outside of its hosts to ensure developing illness. Similar to other physiological sites of infection of many enteropathogenic bacteria, a characteristic feature of the environmental conditions of the *in vivo* infection site of *V. vulnificus* is a low level of oxygen concentration. It has been reported that anaerobiosis affects the production of virulence factors, and enzymes involved in anaerobic respiration appear to play a role in virulence in many pathogenic bacteria [2, 12]. Bacterial adaptation to availability of oxygen is coordinated by a group of global regulatory systems, which include FNR (fumarate, nitrate reduction) protein and the two-component Arc (aerobic respiration control) signal transduction system [7, 20]. The Arc system consists of ArcB, a transmembrane sensor kinase, and its cognate response regulator, ArcA [10, 11]. In response to oxygen deficiency, ArcB is autophosphorylated, and the phosphoryl group is then transferred to ArcA [6]. The ArcA regulator has a pleiotropic effect on a number of cellular functions [10]. Recent DNA microarray analysis revealed that expression of a total of 110 genes in *E. coli* is altered by ArcA [25].

Although ArcA has been shown to positively modulate the expression of *toxT* of *V. cholerae* [29], no studies have yet been reported on the role of ArcA in virulence regulation of bacterial pathogens other than *V. cholerae*. Accordingly, in an effort to examine the effects of ArcA on virulence of *V. vulnificus*, an open reading frame, *fexA*, a homologue of *Escherichia coli arcA*, was identified and cloned from *V. vulnificus* in the present study. The function of the ArcA during an infectious process was accessed by constructing an isogenic *arcA* mutant of *V. vulnificus* and applying the molecular version of Koch's postulates [5, 9]. The possible roles of ArcA in virulence of *V. vulnificus* have been demonstrated by comparing the virulence of the mutant with that of its parental wild-type in *in vitro* cell culture and in mice.

Materials and Methods

Strains, Plasmids, and Culture Media

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (w/v) agar. Unless otherwise noted, the *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). When required, appropriate antibiotics were added to the media as follows: ampicillin 100 μg/ml, kanamycin 50 μg/ml, and tetracycline 10 μg/ml. All the media components were purchased from Difco (Detroit, MI, U.S.A.), and the chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

General Genetic Procedures

Procedures for the isolation of plasmid DNA, genomic DNA, and transformation were carried out as described by Sambrook and Russell [28]. Restriction and DNA-modifying

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

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
V. vulnificus		
M06-24/O	Clinical isolate; virulent	Laboratory collection
HM03	M06-24/O, *fexA*::*nptI*	This study
E. coli		
DH5α	*supE44 Δ lacU169 (ϕ80 lacZ* ΔM15*) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pir*; Km^r; host for π-requiring plasmids; conjugal donor	[24]
Plasmids		
pBR322	ColEI *oriV*; cloning vector; Tc^r, Ap^r	Laboratory collection
pUC19	Cloning vector; Ap^r	Laboratory collection
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	[24]
pRK415	Broad-host-range vector, IncP *ori*, *oriT* of RK2; Tc^r	[15]
pCVD442	R6K *ori*, *sacB*, *oriT* of RP4; Ap^r	[4]
pLAFR3	IncP *ori*; cosmid vector; Tc^r	[31]
pHM043	Cosmid containing *fexA*	This study
pHM0431	9-kb *Bam*HI fragment containing *fexA*; cloned into pBR322; Tc^r, Ap^r	This study
pHM044	0.9-kb *Kpn*I/*Sac*I fragment containing part of *fexA*; cloned into pUC19; Ap^r	This study
pHM045	pHM044 with *fexA*::*nptI*; Ap^r, Km^r	This study
pHM0452	pCVD442 with *fexA*::*nptI*; Ap^r, Km^r	This study
pHM046	pRK415 with *fexA*; Tc^r	This study

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

enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, MA, U.S.A.). The DNA fragments were purified from the agarose gels using a High Pure PCR product kit (Roche, Mannheim, Germany). Primary DNA cloning and manipulation were conducted in *E. coli* DH5α, and restriction mapping was used to confirm that the transformants contained appropriate plasmids. PCR amplification of DNA was performed using a Mastercycler gradient (Eppendorf, Hamburg, Germany) and standard protocol [28].

Cloning of the *V. vulnificus fexA*

Previously, the current authors had generated a library of *V. vulnificus* mutants by a random transposon mutagenesis using a mini-Tn5 *lacZ*1 [3, 27]. A mutant that exhibited decreased cytotoxic activity toward INT-407 intestinal epithelial cells was screened from the mutant library. A DNA segment flanking the transposon insertion was amplified from genomic DNA of the mutant by PCR as a template, as described previously [16, 27]. Since the nucleotide sequence of the resulting PCR product, a 663-bp DNA fragment, revealed 89% identity with that of *V. vulnificus fexA* [*V. vulnificus* CMCP6 genome sequence database, http://www.ncbi.nlm.nih.gov], the DNA was labeled with [α-^{32}P]dCTP and named FexAP. To clone the full gene of the *V. vulnificus fexA*, a cosmid library of *V. vulnificus* ATCC29307 was constructed using pLAFR3 [16, 27] and screened using FexAP as a probe. A colony showing a positive signal was isolated, and the cosmid DNA was purified and named pHM043. A 9.0-kb band from the cosmid DNA digested with *Bam*HI was purified and ligated into pBR322 (NEB) to result in pHM0431, as shown in Fig. 1.

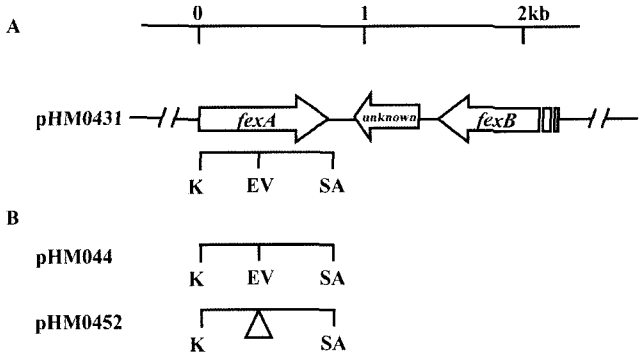

Fig. 1. Physical map of the *fexA* gene on the *V. vulnificus* chromosome and plasmids used in this study.
(A) Plasmid pHM0431 was used to determine the nucleotide sequence of *fexA*. Open boxes and thick lines represent the coding regions of *fexA*, unknown, and part of *fexB* genes and chromosomal DNA, respectively. (B) Depicted are regions cloned in each of the plasmids used for the construction of the *fexA*::*nptI* mutants. The insertion positions *nptI* cassette are indicated by open triangles. Abbreviations: K, *Kpn*I; EV, *Eco*RV; SA, *Sac*I.

Construction of the *fexA*::*nptI* Cartridge

The *fexA* gene in pHM044 that was constructed by ligation of a 0.9-kb PCR fragment carrying the open reading frame (ORF) of *fexA* with pUC19 was inactivated *in vitro*. The 1.2-kb DNA fragment carrying *nptI*, encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin [24], was isolated from pUC4K (Pharmacia, Piscataway, NJ, U.S.A.) and inserted into a unique *Eco*RV site present within the ORF of *fexA* in pHM044. The 2.1-kb *fexA*::*nptI* cartridge from the resulting construct (pHM045) was liberated and ligated with *Sma*I-digested pCVD442 [4], forming pHM0452 (Fig. 1B). The resulting construct, pHM0452, is a derivative of pCVD442 and maintained in *E. coli* SM10 λ *pir* [23].

Generation of the *fexA*::*nptI* Mutant by Allelic Exchange

To generate the *fexA*::*nptI* mutant in *V. vulnificus* by homologous recombination (Fig. 3A), *E. coli* SM10 λ *pir*, *tra* [4, 23] was transformed with pHM0452 and used as a conjugal donor to *V. vulnificus* M06-24/O. Conjugation was conducted according to methods previously described [13, 14]. The desired transconjugants that showed a green colony formation on TCBS (thiosulfate citrate bile salts) agar, supplemented with ampicillin and sucrose (6%, w/v), were selected. The transconjugants that were ampicillin-sensitive because of the absence of pHM0452 were confirmed for the presence of *nptI* in the *fexA* gene by PCR using a pair of primers, FexA043 (5'-GTATTTTTGAAGC-AGAGGGA-3') and FexA044 (5'-ACGCTTCTAAATCAC-CACAG-3') (Fig. 3B).

Complementation of the *fexA*::*nptI* Mutant

The ORF and upstream region of the *fexA* were amplified by PCR using the following pairs of primers: FEXACF (5'-AAACTGCAGCCCAGTAGAATAATCCAGTA-3') and FEXACR (CCGGAATTCTAAAGTGTTTTAAGCCAAAC-

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V.vulnificus        MQTPQILIVEDEQVTRNTLKSIFEAEGYAVFEASNGDEMHHMLSENSINLVIMDINLPGK
V.parahaemolyticus  MQTPQILIVEDEQVTRNTLKSIFEAEGYAVFEASDGEEMHQVLSDNSINLVIMDINLPGK
V.cholerae          MQTPQILIVEDEQVTRNTLKSIFEAEGYAVFEASNGEEMHQVLSDYPINLVIMDINLPGK
E.coli              MQTPHILIVEDELVTRNTLKSIFEAEGYDVFEATDGAEMHQILSEYDINLVIMDINLPGK
                    **** ******* ********** **** * *** ** ***********

V.vulnificus        NGLLLARELREQADVALMFLTGRDNEVDKILGLEIGADDYITKPFNPRELTIRARNLLSR
V.parahaemolyticus  NGLLLARELREQANIALMFLTGRDNEVDKILGLEIGADDYITKPFNPRELTIRARNLLSR
V.cholerae          NGLLLARELREQADVALMFLTGRDNEVDKILGLEIGADDYITKPFNPRELTIRARNLLSR
E.coli              NGLLLARELREQANVALMFLTGRDNEVDKILGLEIGADDYITKPFNPRELTIRARNLLSR
                    ********** ****************************************

V.vulnificus        SMSSSVMPEEKRSVEKYEFNGWVLDINSRSLISPSGDGYKLPRSEFRALLHFCENPGKIQ
V.parahaemolyticus  SMSTNAVQEEKRSVEKYEFNGWVLDINSRSLVSPAGDSYKLPRSEFRALLHFCENPGKIQ
V.cholerae          SMHAGTTQEEKRSVEKYVFNGWELDINSRSLVSPDGDSYKLPRSEFRALLHFCENPGKIQ
E.coli              TMNLGTVSEERRSVESYKFNGWELDINSRSLIGPDGEQYKLPRSEFRAMLHFCENPGKIQ
                     *     ** **** * **** ******** * *  ********** **********

V.vulnificus        TRADLLKKMTGRELKPHDRTVDVTIRRIRKHFESVSGTPEIIATIHGEGYRFCGDLEA
V.parahaemolyticus  TRADLLKKMTGGELKPHDRTVDVTIRRIRKHFESVSGTPEIIATIHGEGYRFCGDLED
V.cholerae          TRADLLKKMTGRELKPHDRTVDVTIRRIRKHFESVSGTPEIIATIHGEGYRFCGDLED
E.coli              SRAELLKKMTGRELKPHDRTVDVTIRRIRKHFESTPDTPEIIATIHGEGYRFCGDLED
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Fig. 2. Sequence relatedness of FexA of *V. vulnificus* and ArcA homologues of other enterobacteriaceae.
Identical sequences are indicated with asterisks, and blanks represent missing sequences. Alignment was based on the amino acid sequences in the GenBank (NCBI) database and derived by the CLUSTRALW alignment program (http://www.ch.embnet.org/software/ ClustalW.html).

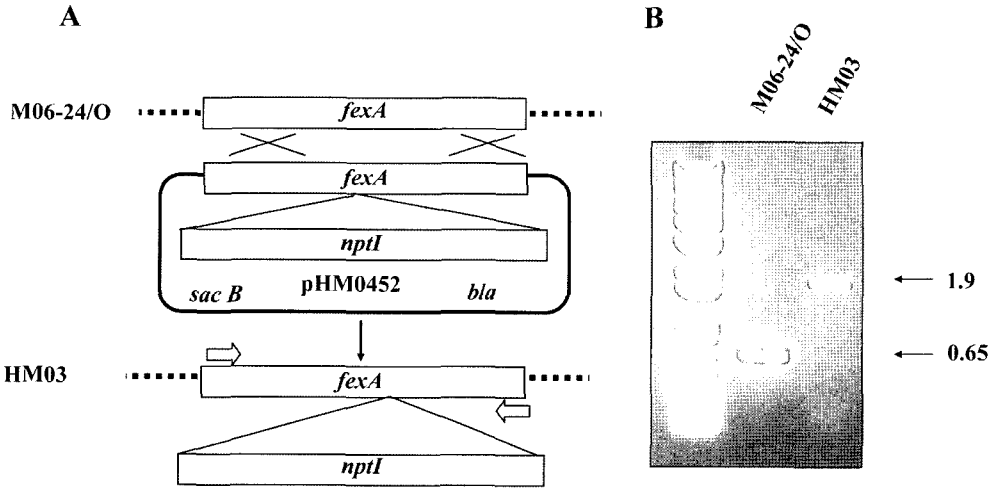

Fig. 3. Allelic exchange procedure and construction of the *fexA::nptI* isogenic mutant.
(A) Double homologous recombinations between strain M06-24/O and plasmid pHM0452 led to interruption of the *fexA* gene and resulted in construction of the mutant HM03. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open box, the target *fexA* gene; shaded box, the *nptI* gene; open arrows, locations of the oligonucleotide primers used for confirmation of the *nptI* insert; large X's represent genetic crossing over. Abbreviations: *sacB*, levansucrase gene; *bla*, β-lactamase gene. (B) PCR analysis of M06-24/O and isogenic mutant HM03 generated by allelic exchange. Molecular size markers (1 kb plus DNA ladder, Invitrogen, Carlsbad, CA, U.S.A.) and PCR products are indicated.

3'). The amplified *fexA* was digested with *Eco*RI and *Pst*I and then ligated with pRK415 [15] digested with the same enzymes, resulting in pHM046. Since the broad host range vector pRK415 has an IncP1 origin and RP4 *oriT*, the resulting plasmids were mobilizable into *V. vulnificus* by conjugation.

Measurement of Cell Growth and Motility Test

For comparison of the growth rates, 50 ml of each culture of parental, wild-type *V. vulnificus* M06-24/O, and its isogenic *fexA* mutant inoculated with an initial cell density of approximately 0.005 (OD_{600}), were grown at 30°C under aeration in 250-ml Erlenmeyer flasks. For anaerobic growth, the test tubes (10-mm diameter and 13-cm long, Fisher, New York, NY, U.S.A.) filled with cultures to the brim were incubated without shaking, as described previously [29]. The inocula were from late exponential phase cultures in LB. Bacterial growth was monitored by measuring OD_{600} of cultures.

For motility test, wild-type and *fexA* mutant were grown overnight, and subsequently an equal amount of the strains were stabbed into LBS semi-solid media solidified with 0.3% agar (Bactoagar, Difco). The plates were incubated at 30°C, and migration through the agar was monitored over a period of 24 h and photographed by the same procedure as described previously [17].

Cytotoxicity Assay

To examine the effects of the *fexA* mutation on the ability of *V. vulnificus* to damage epithelial cells, we performed cytotoxicity assays by using INT-407 human intestinal epithelial cells. M06-24/O and HM03 were grown in LBS broth overnight at 30°C. The following day, 0.1 ml of the culture was inoculated into 100 ml of LBS broth and shaken at 30°C. After 4 h of cultivation, bacterial cells were harvested by centrifugation and suspended in cell culture medium, MEM [minimum essential medium containing 1% (v/v) fetal bovine serum (GIBCO-BRL, Gaithersburg, MD, U.S.A.)], to appropriate concentrations. Preparation of INT-407 cells and infection with bacterial cultures were performed in 96-well tissue culture plates (Nunc, Roskilde, Denmark), as described previously [14]. The cytotoxicity was determined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant using the Cytotoxicity Detection Kit (Roche, Mannheim, Germany), and was expressed by the total LDH activity of cells completely lysed by 1% Triton-X 100 as 100%.

Mouse Model of Infection

The 50% lethal doses (LD_{50}s) of wild-type and the *fexA* mutant were compared using ICR mice (Specific Pathogen-Free, Institute of Laboratory Animal Resources Seoul National University, Seoul, Korea), as described elsewhere [14]. For the determination of LD_{50}, bacteria grown in LBS broth overnight at 30°C were harvested and suspended in PBS to appropriate concentrations, ranging from 10^1 to 10^8 CFU in 10-fold increments.

A group of (n=6) 7-weeks-old normal female mice were injected intraperitoneally with 0.1 ml of serial dilutions of bacterial suspensions. The infected mice were observed for 24 h, and the LD_{50}s were calculated by the method of Reed and Muench [26]. Mice were injected intraperitoneally with 250 μg of iron dextran per g of body weight immediately before injection with bacterial cells.

Data Analysis

Averages and standard errors of the mean (SEM) were calculated from at least three independent determinations. The statistical significance of difference among *V. vulnificus* strains was evaluated using Student's unpaired *t* test (SAS software, SAS Institute Inc., Cary, NC, U.S.A.). Significance was accepted at $p<0.05$.

Nucleotide Sequence Accession Number

The nucleotide sequence of *fexA* gene of *V. vulnificus* M06-24/O was deposited into the GenBank under accession number AY968673.

RESULTS

Cloning and Sequencing Analysis of *V. vulnificus fexA*

The nucleotide sequences of the DNA fragment in pHM0431 were determined by primer walking (National Instrumentation Center for Environmental Management, Seoul, Korea), and the sequence data were submitted to the GenBank [Accession number AY968673]. A coding region consisting of 717 nucleotides was identified (data not shown). Comparison of the nucleotide sequence of the coding region with that of *V. vulnificus* CMCP6 genome sequence revealed that the coding region is the *fexA* gene of *V. vulnificus* [http//www.ncbi.nml.nih.gov]. There are only 2 mismatches between the nucleotide sequences of *fexA* genes in M06-24/O and CMCP6, both of which are located in chromosome 1. The *arcA* from *E. coli* was 81% identical in nucleotide sequences with the *fexA* of *V. vulnificus* (data not shown). This information indicates that the coding region *fexA* is a homolog of the *arcA* gene reported in *E. coli*.

The amino acid sequence deduced from the *fexA* coding sequence revealed a protein, FexA, composed of 238 amino acids with a theoretical molecular mass of 27,005.7 Da and PI of 5.81. The amino acid composition and molecular weight of this FexA are quite similar to those of ArcA homologues from Enterobacteriaceae. The amino acid sequence of the *V. vulnificus* FexA was 84% to 97% identical to those of ArcA homologues from *E. coli*, *V. cholerae*, and *V. parahaemolyticus*, and their identity appeared evenly throughout the whole proteins (Fig. 2, http://www.ch.embnet.org/software/ClustalW.html). The ArcA is a response regulator of the ArcA/ArcB two-component signal transduction system, and the ArcB is a transmembrane sensor kinase. These two components together regulate anoxia-responsive genes. In the course of our sequencing analysis, the part of *arcB* homology (*fexB*), located downstream of *fexA*, was found (Fig. 1). The *arcA* and *arcB* genes of *V. cholerae*, a species closely related to *V. vulnificus*, are organized in the same orientation as in *V. vulnificus arcA* and *arcB* (data not shown). All of this information suggested that the *fexA* gene also encodes the protein required for the response and adaptation of *V. vulnificus* to availability of oxygen.

Construction and Confirmation of the *V. vulnificus fexA* Mutant

A double crossover, in which the wild-type *fexA* gene was replaced with the *fexA*::*nptI* allele, was confirmed by PCR using a pair of primers; FexA043 (5'-GTATTTTTGAAG-CAGAGGGA-3') and FexA044 (5'-ACGCTTCTAAATC-ACCACAG-3'). The PCR analysis of the genomic DNA from M06-24/O with the primers produced a 0.65-kb fragment (Fig. 3B), whereas the genomic DNA from the *fexA*::*nptI* mutant resulted in an amplified DNA fragment of approximately 1.9-kb in length. The 1.9-kb fragment was in agreement with the projected size of the DNA fragment containing the wild-type *fexA* (0.65-kb) and the *nptI* gene (1.25-kb). To determine the stability of the insertional mutation, strain HM03 was grown overnight without kanamycin selection. The inserted *nptI* DNA was stably maintained, as determined by the maintenance of kanamycin resistance and by the generation of appropriate-sized DNA fragment by PCR (data not shown). The *V. vulnificus fexA* mutant chosen for further analysis was named HM03, as shown in Fig. 3B.

Effects of *fexA* Mutations on the Growth of *V. vulnificus* Under Aerobic and Anaerobic Conditions

The growth of the *fexA* mutant was compared with that of parent strain. Based on the cell densities, the maximum specific growth rate, (μ_{max}; defined as a specific growth rate at an exponential phase) was determined as described elsewhere [31]. When LBS was used and cultured at 30°C with aeration, the wild-type showed μ_{max} of 3.680 (Table 2). Growth rate of the HM03, the *fexA* mutant, was significantly reduced with μ_{max} of 1.510, suggesting that FexA was responsible for optimum growth in the conditions used.

Similarly, the growth rate of the wild-type was much higher than that of the *fexA* mutant, when the strains were cultured with LBS at 30°C under anaerobic condition (Table 2). However, as expected, the growth rates of both

Table 2. Comparison of specific growth rate (μ_{max}) of *V. vulnificus* M06-24/O and its isogenic *fexA* mutant, grown at different conditions[a].

Growth condition	Strain[b]	μ_{max} (h^{-1})
Aerobic	WT	3.680
	HM03	1.510
	HM03(pHM046)	2.860
Anaerobic	WT	2.01
	HM03	1.18
	HM03(pHM046)	1.845

[a]: Details are in Materials and Methods.
[b]: WT, M06-24/O; HM03, *fexA* mutant; HM03 (pHM046), complemented strain.

strains of *V. vulnificus* decreased under anaerobic condition, when compared with those under aerobic condition: The maximum specific growth rate of the wild-type was 2.010, compared with 1.180 of the *fexA* mutant. The magnitude of decrease of growth rate of the mutant was similar to the decrease of growth rate that was observed in the wild-type (Table 2).

It seems quite unlikely that the decrease of growth rate was due to polar effects of the *fexA* insertional mutation on downstream genes; however, this possibility cannot be ruled out *a priori*. Therefore, we examined whether reintroduction of recombinant *fexA* could complement the decrease of growth rate of HM03. In both aerobic and anaerobic conditions, HM03 (pHM046), in which the *fexA* mutation is complemented by introducing recombinant *fexA*, revealed the growth rates restored to substantial levels, but still did not reach those of the wild-type. One plausible explanation for this incomplete recovery of growth rates of HM03, carrying pHM046, is that *fexA* on the pHM046 was either not stably maintained or not efficiently expressed in the cell culture conditions used. Nonetheless, the results indicate that FexA is a physiologically important regulator for optimum metabolism and growth in *V. vulnificus*.

Effect of *fexA* Mutation on Motility

It has been demonstrated that motility is one of the mechanisms by which bacteria can get appropriate niche inside the host after infection. Several studies have also demonstrated that motility of pathogenic bacteria facilitates biofilm formation and colonization on host epithelial cells. To determine if the *fexA* in *V. vulnificus* was required for motility, the *fexA* mutant HM03 was tested for its ability to migrate on a semi-solid plate surface. As shown in Fig. 4, the growth of *fexA* mutant away from the inoculation point decreased, compared with the wild-type, and the swimming diameter of the mutant was consistently reduced by about 50% of that of the wild-type, indicating that *fexA* is necessary for optimum motility of *V. vulnificus*.

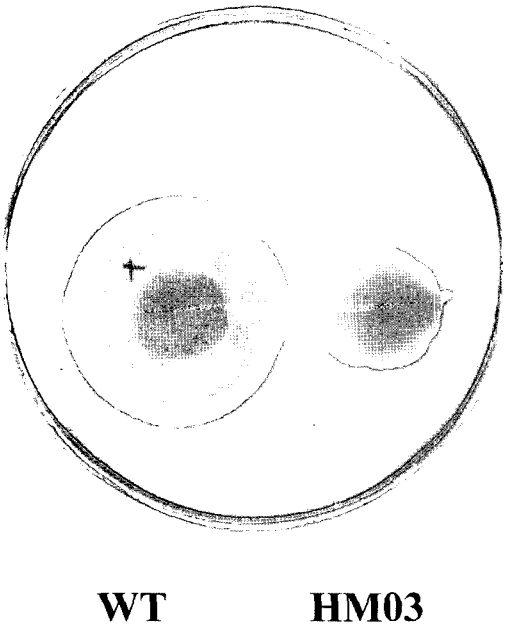

Fig. 4. Motility of *V. vulnificus* strains in a semi-solid medium. Wild-type strain (M06-24/O) displays motility in LBS containing 0.3% agar, which is reduced by mutation in *fexA* (HM03).

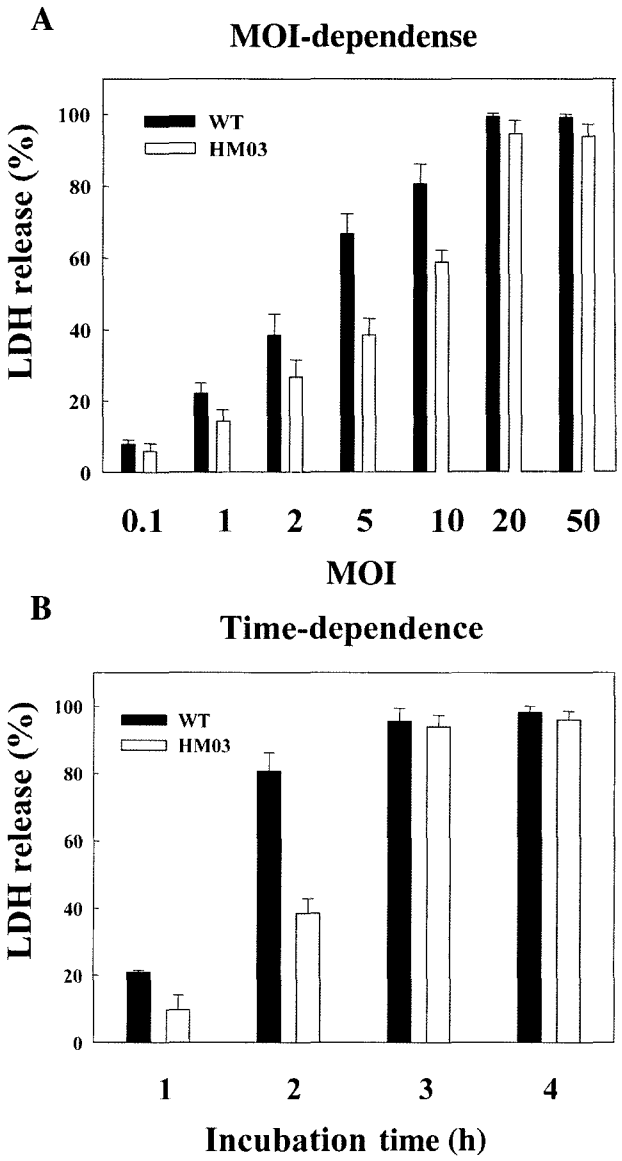

Fig. 5. Effect of *fexA* mutation on the virulence of *V. vulnificus* to INT-407 cells.
INT-407 cells were infected with M06-24/O (WT) or HM03 (*fexA* mutant) of *V. vulnificus* at various MOIs for 2 h (A), or at MOI of 5 for varying incubation time (B). Afterward, cell cytotoxicity was determined by LDH release assay. The data represent mean±SEM from three independent experiments. *$P<0.01$, **$P<0.05$, relative to groups infected with wild-type of *V. vulnificus* at each MOI or each incubation time.

Table 3. Effects of *fexA* mutation on the lethality of *V. vulnificus* to mice[a].

Strain	Intraperitoneal LD_{50} (CFU)
M06-24/O (n=6)	9.5×10^{1}–9.5×10^{0}
HM03 (n=6)	1.5×10^{3}–1.5×10^{2}

[a]: n, number of iron-treated mice for each inoculation group, ranging from 1 to 10^{8} CFU in 10-fold increments.

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

In order to examine the effect of the *fexA* mutation on the virulence of *V. vulnificus*, the LDH activity from monolayers of INT-407 cells, which were infected with 100 μl of suspension of either M06-24/O on HM03 strains at a different multiplicity of infection (MOI) and incubated for 2 h, were determined (Fig. 5A). The *fexA* mutant HM03 exhibited much less LDH activity as long as the MOI was not higher than 10. The level of LDH activity from INT-407 cells, infected with HM03 at a MOI of 5, was almost 2-fold less than that from the cells infected with the wild-type. Similarly, INT-407 cells were infected at an MOI of 5, and the LDH activity from the cells was compared at different incubation times, as indicated in Fig. 5B. The cells infected with HM03 released a lower level of LDH activity than that of the cells infected with the wild-type for as long as 2.5 h while the cells were incubated with bacterial suspension. Therefore, an optimal incubation period of 2 h with a constant MOI of 5 was chosen for further experiments.

Virulence in Mice is Dependent on *fexA*

The role of the *V. vulnificus fexA* gene in virulence was further examined using a mouse model. The LD_{50}s in iron-overloaded mice after intraperitoneal infection of *V. vulnificus* strain are shown in Table 3. The LD_{50} of HM03 was greater than 10^{2} CFU, compared with an LD_{50} lower than 10^{1} CFU for the wild-type. Therefore, in the mouse model of intraperitoneal infection, in which the *fexA* mutant showed an approximately 1-log increase in LD_{50} over the wild-type, the *fexA* mutant appeared to be much less virulent than its parental wild-type. Taking these results together, it is reasonable to conclude that the *fexA* is essential for virulence of *V. vulnificus* in mice as well as in *in vitro* cell culture.

Discussion

Bacterial pathogenesis is a complex phenomenon in which multiple virulence factors participate. Genes and operons encoding these virulence factors are often coordinately regulated by a common regulator in response to environmental conditions [22]. The diseases resulting from infection with *V. vulnificus* are remarkable in regards their invasive nature, ensuing severe tissue damage, and rapidly fulminating course. This multifaceted nature of pathology of the diseases indicates that numerous virulence factors are typically involved in pathogenesis of the organism. Like many other pathogenic bacteria, *V. vulnificus* has to alter its physiology and virulence by regulatory circuits that respond to environmental conditions. Understanding the changes in virulence in response to environmental conditions, typically resembling the *in vivo* sites of infection, has long been a goal of investigators interested in the pathogenesis of *V. vulnificus*. Indeed, relative to *in vitro* conditions, the intestinal environment is featured by its substantially lower level of oxygen [8]. The change of oxygen availability could serve as a signal to stimulate (and/or repress) global changes in expression of genes including many virulence factors.

ArcA is a transcriptional regulator that controls the expression of a large number of genes in response to a shift from aerobiosis to anaerobiosis [20]. The gene product of *E. coli arcA* is a biochemically as well as genetically well-characterized global regulator [10]. The ArcA, previously known as Dye, SrfA, FexA, MspA, or SegA, is a regulator component of the ArcAB two-component system. When phosphorylated in response to oxygen deficiency, the ArcA has been shown to repress the expression of oxygen-requiring catabolic genes, such as the genes of the TCA cycle and cytochrome oxydase. ArcA is also required for proper expression of certain catabolic genes for utilization of pyruvate and for fermentation of sugar [18, 20, 33]. It has been demonstrated that the major role of ArcA is to adjust catabolism under oxygen-restricted (microaerobic) growth conditions, rather than to adjust catabolism under fully aerobic or anaerobic conditions [1]. Recent transcriptome analysis using DNA microarrays revealed that mutations in the *arcAB* system in *E. coli* altered the expression level of more than 100 genes, including genes for energy metabolism.

From the fact that a feature of the adaptation strategy of pathogens for optimal survival in their host is the integration of virulence gene expression with a regulatory system responding to environmental conditions [8], it is most likely that ArcA, a global regulator responding to anaerobiosis, participates in the regulation of virulence genes of bacterial pathogens. However, compared with the substantial body of literature concerning the function of ArcA of *E. coli*, only a few studies on the role of ArcA or ArcA homologues in the regulation of expression of virulence factors of pathogenic bacteria have been reported [29]. It has been reported that the ArcA positively modulates the expression of *toxT* of *V. cholerae*, the transcriptional activator of the genes encoding cholera toxin (CT) and toxin-coregulated pilus (TCP), and increases *V. cholerae* virulence in the infant cholera mouse model [29]. To investigate whether ArcA has a role in the regulation of virulence, *fexA*, an

arcA homologue, was identified from *V. vulnificus*, and a *V. vulnificus fexA* mutant was then constructed. Compared with wild-type, the *fexA* mutant was less toxic to intestinal epithelial cells *in vitro*, and also showed significantly diminished virulence in mice as measured by their abilities to cause death. These results suggest that FexA is an important regulator for optimal expression of virulence factors in the pathogenesis of *V. vulnificus*. However, additional works are needed to identify the specific genes encoding the virulence factors that are modulated by FexA of *V. vulnificus*.

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