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Vibrio alginolyticus MviN is a LuxO-regulated Protein and Affects Cytotoxicity Towards Epithelioma Papulosum Cyprini (EPC) Cells

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***Vibrio alginolyticus*, a Gram-negative marine bacterium, is one of the causative agents of fish vibriosis. Its virulence factors and pathogenesis mechanism are barely known, except for some extracellular products (ECPs) that are known to be regulated by quorum sensing system. Therefore, the present study used a microarray to analyze the transcription profiles of the wild-type *V. alginolyticus* and a deletion mutant of *luxO*, the pivotal regulator in *Vibrio* quorum sensing systems, which resulted in the identification of a putative virulence factor, MviN. Quantitative real-time reverse transcription PCR confirmed that the transcription of *mviN* was upregulated in the *luxO* mutant when compared with wild-type, and downregulated in a *luxO-con* complemented strain. Furthermore, Western blotting indicated that MviN was greatly induced during the late-exponential and stationary phases of growth, indicating that the expression of MviN was cell-density dependent and quorum sensing regulated in *V. alginolyticus*. Meanwhile, the *mviN* null mutant displayed a much slower growth rate than the wild type, signifying the essential role of MviN in *V. alginolyticus*. Western blotting also revealed that MviN was present as an extracellular protein in *V. alginolyticus*. When epithelioma papulosum cyprini (EPC) cells were treated with the ECPs of the *mviN* mutant, no cytotoxicity was observed, whereas EPC cells treated with the wild type exhibited pathological changes, which increased with the ECPs concentration and treatment time. Therefore, the results demonstrated that MviN is a LuxO-regulated ECPs component and involved in the pathogenicity of *V. alginolyticus*.**

Keywords: *mviN*, *Vibrio alginolyticus*, quorum sensing, LuxO, virulence

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The Gram-negative bacterium *Vibrio alginolyticus* is one of the causative agents of vibriosis, a well-known fish disease in many countries such as China [20], America [14], India [16], and several European countries [2]. The organism can be isolated from marine and estuarine waters, diseased large yellow croaker, sea bream, grouper, and Kuruma prawns, as well as the larvae of certain fish and shellfish species [23, 39]. Moreover, *V. alginolyticus* has also been reported to cause diarrhea, extra-intestinal infections such as otitis, and wound infections in humans [7]. However, little is known about the virulence mechanisms of *V. alginolyticus* as a pathogen for fish.

V. alginolyticus produces a number of virulent extracellular products (ECPs), which mainly include proteases, hemolysins, and siderophores, and these ECPs would appear to be associated with the virulence of the bacterium [1, 2, 13, 19, 38]. For example, the lethal attributes of the extracellular serine proteases ProA and Asp, and thermostable direct hemolysin (TDH) secreted by *V. alginolyticus* strains have already been confirmed in relation to Kuruma prawns *Penaeus japonicas*, *Lutjanus erythopterus*, and mice, respectively [3, 4, 6]. In *V. alginolyticus*, the production of ECPs, including extracellular proteases, hemolysins, and siderophores, is known to be regulated by a *V. harveyi*-like LuxO/LuxR quorum sensing (QS) system, which is a type of cell–cell signaling that monitors the population density and results in the coordinated expression of multiple genes [9, 15, 38]. Further studies have also revealed that the LuxO/LuxR QS system regulates two types of serine protease, ProA and Asp, in *V. alginolyticus* strains to mediate vibriosis in a maritime aquaculture [31, 32].

Accordingly, building on the knowledge that ECPs are important virulence factors in *V. alginolyticus*, and that LuxO regulates the production of ECPs, as shown in a previous study by the current authors [38], the present study further examines the pathogenicity and role of LuxO in the QS system of *V. alginolyticus* by comparing the transcription profiles of the wild type and a *luxO* mutant

using a whole-genome microarray to unveil the putative extracellular virulence genes regulated by LuxO. The results identified many upregulated or downregulated genes in the *luxO* mutant when compared with the wild type, including some unknown transcriptional regulators (LysR, GnyR, GntR, and AraC/XylS family), metabolism-related enzymes, membrane proteins, and proteins involved in motility. In addition, MviN is characterized as another protein, in addition to the alkaline serine protease (Asp), that is negatively regulated by LuxO and a virulence-associated extracellular product in *V. alginolyticus*.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids are all listed in Table 1. The *V. alginolyticus* strains were grown at 30°C in Luria–Bertani (LB) medium (Oxford, England) supplemented with 3% NaCl (LBS), or M9 medium supplemented with 3% NaCl and 0.4% glucose (M9S). Meanwhile, the *E. coli* strains were grown at 37°C in LB medium or on LB agar (1.5%). When needed, antibiotics were added to the media at the following final concentrations: 100 μg/ml ampicillin (Amp) and 25 μg/ml chloramphenicol (Cm) for *E. coli*; 100 μg/ml Amp and 7 μg/ml Cm for *V. alginolyticus*.

DNA Manipulation

All the molecular techniques were performed as described previously [34], the enzymatic reactions and plasmid purifications performed according to the manufacturer's instructions (TaKaRa, Dalian, China), and the DNA sequencing and primer synthesis carried out by Invitrogen Co. (Shanghai, China).

Microarray Experiments

The microarray experiments were performed using NimbleGen DNA microarrays (NimbleGen, Madison, WI, U.S.A.) according to the manufacturer's instructions. Spotted microarrays containing full-length ORFs derived from *V. alginolyticus* strain 12G01 were purchased from NimbleGen. The RNA was extracted from cells grown in the M9S medium using the TRIzol reagent (Invitrogen, Shanghai, China), and purified using an RNeasy kit (Qiagen, Chatsworth, CA, U.S.A.). Fluorescently labeled cDNA was prepared by direct incorporation of fluorescent nucleotide analogs (Cy3-dCTP) during a first-strand randomly primed reverse transcription reaction. The differentially labeled cDNAs were then applied to different array surfaces under conditions that favor hybridization. The microarray slides were scanned using a GenePix 4000B (Molecular Devices, Union City, CA, U.S.A.). For every ORF-specific spot, the resulting fluorescence intensity of each label was measured and compared using NimbleScan software.

Construction of *luxO-con* Complemented Strain and Null Mutant of *mviN* Gene

The primers used for the mutant construction are given in Table 2. To construct a complemented strain *luxO-con* (*luxO* D47E), the primers LuxOCF/LuxOMR and LuxOCR/LuxOMF were used to amplify two fragments, which were then fused by overlap PCR using the primers LuxOCF/LuxOCR targeted for the entire *luxO* gene. The primers LuxOMF/LuxOMR harbored a mutation of the Asp-47 GAT codon to GAG. The intact *luxO* gene containing the putative promoter region and desired site mutation was then introduced to the *Bam*HI and *Pst*I sites of the low-copy plasmid pMMB206 [26] to create pMMB-luxO. Thereafter, the plasmid was introduced to the *luxO* mutant by conjugation, and the presence of the plasmid confirmed by PCR analysis and sequencing on automated ABI 3730 capillary sequencers (Applied Biosystems, Foster City, CA, U.S.A.).

An internal fragment of the *mviN* gene (1,300 bp) was PCR-amplified from the *V. alginolyticus* EPGS chromosome using the primers MviNinF and MviNinR (Table 2). The PCR product was then treated with *Bgl*II/*Spe*I restriction enzymes and cloned into the corresponding restriction sites of the suicide plasmid pNQ705-1 [8], generating pNQ-MviN. The resulting plasmid pNQ-MviN was mated from *E. coli* SM10 λ*pir* [21] into *V. alginolyticus* EPGS, and exconjugants with the plasmid integrated into the chromosome by

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference or source
Vibrio alginolyticus		
EPGS	Amp; pathogenic isolate from aquiculture farm of South China Sea	Lab. collection
mviN⁻	Amp; EPGS, insertional mutant in *mviN*	This study
Δ*luxO*	Amp; EPGS, in-frame deletion in *luxO*	Rui *et al.* [31]
luxO-con	Amp; EPGS, complementation *in trans* with intact *luxO* gene containing point mutation of D47E	This study
Escherichia coli		
CC118 λ*pir*	λ*pir* lysogen of CC118 [Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*]	Dennis and Zylstra [10]
SM10 λ*pir*	Km^r; *thi thr leu tonA lacY supE recA RP4-2-Tc*::*Mu* λ::*pir*	Liang *et al.* [21]
Top10	F' [lacIq,Tn10(TetR)] *mcrA* Φ80 *lacZ* Δm15 Δ*lac* X74 *deoR recA1*	Invitrogen
Plasmid		
pNQ705-1	Cm^r; suicide vector containing R6K origin (*pir* requiring)	Croxatto *et al.* [8]
pNQ-MviN	Cm^r; pNQ705-1 derivative containing 1,300 bp internal fragment of gene *mviN*	This study
pMMB-luxO	Cm^r; pMMB206 derivative containing *luxO* bp 84–2333	This study

Table 2. Primers used for cloning and qRT–PCR.

Primer	Sequence (5'–3')[a]
LuxOCF	CG**GGATCC**CGAATCACCAACTC
LuxOCR	AA**CTGCAG**CCGCACTGCTTTTC
LuxOMF	TATTTTACTCGAGCTCCGTCTGCCAGATAT-GAC
LuxOMR	GCAGACGGAGCTCGAGTAAAATAA-GATCTGGAA
MviNF	G**GAATTC**CCGTTAGGTCTGTTTGGTATTG
MviNR	CCG**CTCGAG**TCAGTCTGTCCCAGCTTTTA
MviNinF	T**AGATCT**AATGGGGGCAGGAGCAAG
MviNinR	C**GCATGC**TCCAGTTGCCATAAGATT
pNQF	GGTGCTCCAGTGGCTTCTGTTTCTA
pNQR	CAGCAACTTAAATAGCCTCTAAGGT
CMviN-F	GCTCCCGATAGGGCAACAA
CMviN-R	CGGAGTGGTGACTGCACTGT
16S-F	AAAGCACTTTCAGTCGTGAGGAA
16S-R	TGCGCTTTACGCCCAGTAAT

[a]Nucleotides in bold represent restriction enzyme sites added to the 5' region of the primer.

homologous recombination were selected on LBS agar medium containing 100 μg/ml Amp and 7 μg/ml Cm. The resulting mutant (*mviN*$^-$) was confirmed by PCR using the primer pairs MviNinF/pNQF and MviNinR/pNQR, as previously described [25], followed by DNA sequencing.

Quantitative Real-Time Reverse Transcription PCR (qRT–PCR)

Cultures of *V. alginolyticus* were grown overnight in LB at 30°C and diluted 100-fold into a fresh M9S medium. The cells were then collected at the appropriate phase and the RNA was isolated using an RNA isolating kit (Tiangen, Beijing, China). Next, the RNA was subjected to DNase I (Promega, Madison, WI, U.S.A.) treatment to exclude any genomic DNA contaminant. Equal amounts of the RNA and a random primer were used to generate cDNA (ToYoBo, Tsuruga, Japan). qRT–PCR was then carried out by performing three independent experiments, each in triplicate, using a FTC-200 detector (Funglyn Biotech, Shanghai, China), and the transcript levels were normalized to 16S rRNA in each sample using the $\Delta\Delta C_T$ method [18]. The primers for the qRT–PCR, listed in Table 2, were designed using Primer Express software (Applied Biosystems) with the predicted product in the 100 to 200 bp size range.

Expression of Recombinant MviN Protein and Preparation of Anti-MviN Serum

A 714-bp sequence of *mviN* from its carboxy-terminal was amplified using the primers MviNF and MviNR (Table 2). The amplified fragment was ligated into the *Eco*RI and *Xho*I sites of the vector pIGH1 (ImmunoGen, Shanghai, China), and then transformed into *E. coli* BL21 (DE3) (Novagen, Madison, WI, U.S.A.). A single positive colony was incubated in an LB broth with 100 μg/ml of Amp. Isopropyl-β-D-thiogalactoside (IPTG) was then added to the culture at a final concentration of 0.5 mM and the culture was incubated at 30°C overnight. Thereafter, the cells were harvested, resuspended in a buffer (50 mmol/l Tris-HCl, pH 7.0), lysed by ultrasonication, and centrifuged. The supernatant was then applied to a Ni-NTA spin column (Qiagen, Valencia, CA, U.S.A.). An antibody for the purified MviN was prepared by immunizing two New Zealand white rabbits, which were injected with the purified MviN protein. After three successive booster injections, blood was collected from the rabbits and the specific antibody was purified by affinity chromatography on an Ag column.

Western Blotting Analysis

The *V. alginolyticus* wild-type EPGS was cultured in M9S, and the bacterial cells were then harvested in different growth phases and centrifuged at 5,000 ×*g* for 10 min at 4°C. The supernatants were filtered through 0.22-mm filters (Millipore, U.S.A.) and concentrated using an ultrafiltration device (Millipore) after adding the protease inhibitor PMSF (Meijing, Shanghai, China). The cell pellets were then suspended in PBS. Equivalent amounts of the cell pellets and supernatants were examined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were then transferred to a PVDF membrane (Millipore) using a semi-dry electroblotter (Bio-Rad, Hercules, CA, U.S.A.). The membrane was then incubated with the rabbit anti-MviN serum (1:1,000). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (H+L) (Langdun, Shanghai, China) diluted at 1:3,000 was used as secondary antibody.

Growth Curve Assays

The *V. alginolyticus* wild type and *mviN* insertional mutant were separately grown in LB broth and LB broth with Cm at 30°C. They were then diluted at 1:50 and inoculated into a fresh medium. Samples were taken every hour and the optical density was measured at 600 nm (OD_{600}).

Cytotoxicity Test

The toxicities of the ECPs were tested by examining the amount of mitochondrial dehydrogenase released from the epithelioma papulosum cyprini (EPC) cell line. The cells were grown as a monolayer in 24-well culture plates (Corning, Lowell, MA, U.S.A.) at 25°C using Eagle's minimum essential medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China). The medium was inoculated with 0.1-ml serial dilutions of the ECPs samples. Cells inoculated with PBS (pH 7.2) were used as the negative controls. The microtiter plates were incubated at 25°C, and the cell monolayers exposed to the ECPs were observed after 1, 6, 12, and 24 h. The cytotoxicity was determined using an MTT cell viability/cytotoxicity assay kit (Beyotime, Jiangsu, China) after 7 h, and the cytotoxicity calculations were made based on the manufacturer's instructions.

Fish Infections

The *V. alginolyticus* strains were cultured in LBS at 30°C. After harvesting the cells by centrifugation at 2,000 ×*g* for 10 min, the cell pellets were washed and suspended in a 2.5% NaCl solution, and diluted to the appropriate inoculation concentrations. Zebra fish (*Brachydanio rerio*) were then infected with the wild type or mutant strains *via* the route of intramuscular injection. In each case, five different bacterial dilutions were used with 6-fold variations, and 10 fish were infected with each dilution. Death due to vibriosis was determined by the observation of gross clinical signs and confirmed by the isolation of *V. alginolyticus* strains that were resistant to the appropriate antibiotics from infected organs of the dead fish. The mortality of the fish was recorded over a period of 7 days. The LD_{50} values were calculated using the method described previously [29].

Nucleotide Sequence Accession Number
The NCBI accession number for *mviN* described in this study is FJ548628.

RESULTS

Transcriptional Analysis of *luxO* Deletion Mutant of *V. alginolyticus*

The whole-genome DNA microarray (NimbleGen) designed against *V. alginolyticus* 12G01 was used to screen novel virulence factors regulated by *luxO* in *V. alginolyticus* EPGS. Dozens of genes, including transcriptional regulators, membrane proteins, cellular processing and metabolism enzymes, and unknown function proteins, were selected and confirmed to be regulated by *luxO* using qRT–PCR, indicating that LuxO plays a versatile role in *V. alginolyticus* (Table 3). Interestingly, several iron metabolism-related genes (V12G01_06671, V12G01_03497, V12G01_03596, and V12G01_09412) were identified to be LuxO-regulated, which was consistent with the previous finding that LuxO controls the siderophore production in *V. alginolyticus* [38]. However, the present study focused on V12G01_13344, which was 1.61-fold upregulated in the *luxO* mutant when compared with the wild type in the microarray data and annotated as an "uncharacterized membrane protein, putative virulence factor." When searching against the GenBank, V12G01_13344 exhibited a high identity to homologs in other *Vibrio* species annotated as the MviN protein. Therefore, V12G01_13344 was named MviN, which would seem to exist as a putative virulence factor in *V. alginolyticus*.

Genetic Characteristics of *mviN* in *V. alginolyticus*

PCR amplification was performed to obtain the 1,563 bp *mviN* gene encoding 520 amino acids in *V. alginolyticus* EPGS. BlastP search against the GenBank indicated that MviN is highly conserved in *Vibrio* species with identity ratios of 95%, 96%, 88%, 81%, and 80% with other homologs from *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. cholerae*, and *V. fischeri*, respectively. Moreover, the similarity ratios to MviN homologs from species *Burkholderia pseudomallei*, *Neisseria meningitidis*, *Rhizobium leguminosarum*, *Rhizobium tropici*, *Sinorhizobium meliloti*, and *Salmonella typhimurium* were 33%, 9%, 24%, 31%, 33%, and 20% (Fig. 1). A putative signal peptide of 23 amino acids was found at the N-terminus of the predicted protein (http://www.cbs.dtu.dk/services/SignalP/), and 12 transmembrane helixes were predicted when mapping in the protein (http://imgweb.jgi-psf.org/cgi-bin/w/main.cgi) (Fig. 1), suggesting that MviN may be an integral membrane or extracellular protein in *V. alginolyticus*.

When searching the genome of *V. alginolyticus* 12G01, MviN was found to be flanked by sequences encoding a 30S ribosomal protein S20 (*rpsT*) with an opposite transcription orientation and a hypothetical protein 70 bp downstream (Fig. 2A). Additionally, sequencing results indicated that *V. alginolyticus* EPGS, MVP01, and 33787 all harbor the *mviN* locus and share the same genetic arrangement (data not shown).

To perform a functional characterization of MviN in *V. alginolyticus*, a site-directed null mutant was constructed using the suicide plasmid pNQ705-1 [25]. A gene fragment of approximately 1,300 bp inside the *mviN* gene was cloned into the corresponding restriction sites of pNQ705-1. The resulting plasmid was then conjugated into *V. alginolyticus* for PCR screening and to obtain an *mviN* null mutant (Fig. 2B and 2C).

mviN is Negatively Regulated by *luxO* in *V. alginolyticus*

qRT–PCR indicated that the expression of *mviN* was more than 4-fold higher in *luxO* when compared with the wild type (Fig. 3). To further characterize the role of LuxO in the regulation of MviN, a constitutively active allele of

Table 3. Parts of genes differentially expressed in *luxO* mutant.

Gene ID	Functional category	Fold change	
		Microarray	qRT–PCR
V12G01_06411	Putative tyrosine-specific transport protein	−1.94	−2.72
V12G01_12850	Putative outer membrane protein A	−1.57	−2.06
V12G01_06671	DPS family protein	−1.74	−2.17
V12G01_03315	Immunogenic protein	1.96	8.43
V12G01_10848	Universal stress protein UspB	1.53	3.56
V12G01_07978	Putative transcriptional regulator (LysR)	1.51	2.27
V12G01_01190	Putative transcriptional regulator (GnyR, BltR)	1.56	4.83
V12G01_13344	MviN protein	1.61	5.11
V12G01_03497	TonB system transport protein ExbB2	1.67	3.40
V12G01_03596	ABC transporter permease protein	1.51	4.05
V12G01_09412	Iron(III) ABC transporter	1.52	2.47

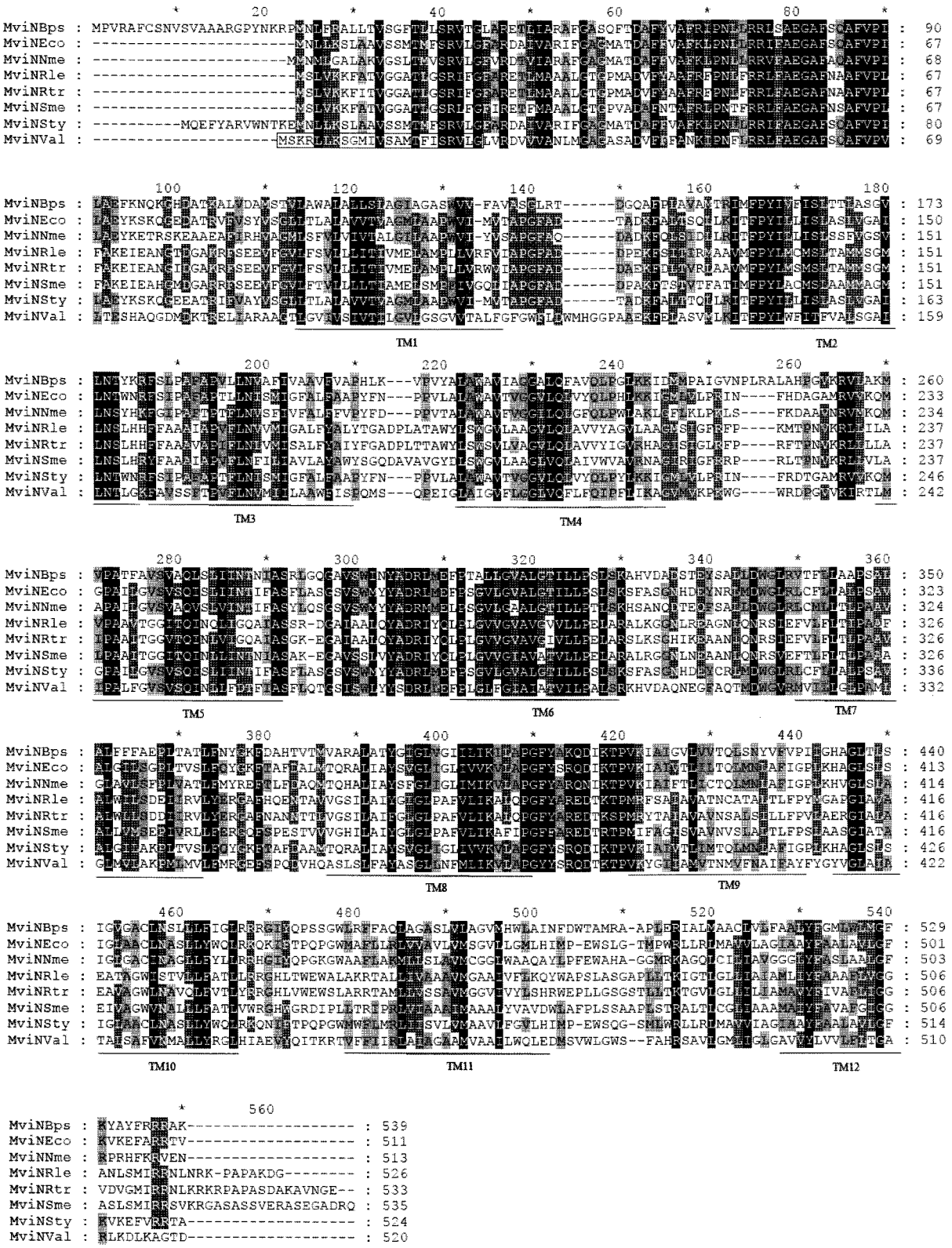

Fig. 1. Amino acid sequence alignment of predicted *V. alginolyticus* MviN protein (MviNVal) with selected homologs of *B. pseudomallei* (MviNBps), *E. coli* (MviNEco), *N. meningitidis* (MviNNme), *R. leguminosarum* (MviNRle), *R. tropici* (MviNRtr), *S. meliloti* (MviNSme), and *S. typhimurium* (MviNSty).

The identical and highly conserved amino acids in the sequences are highlighted in black and gray. The 12 underlined stretches of amino acids termed from TM1 to TM12 are predicted transmembrane helices, whereas the possible signal peptide in *V. alginolyticus* is boxed.

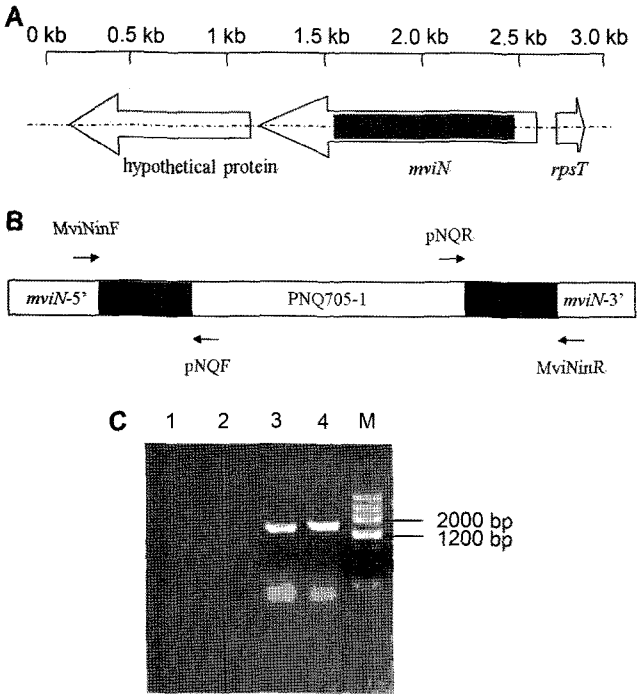

Fig. 2. A. Organization of ORFs corresponding to *mviN* and its flanking genes in *V. alginolyticus* EPGS chromosome.
The ORFs are indicated by the three boxed arrows. The black box represents the internal region of *mviN* that was used for the homologous recombination. **B.** Mapping of primer targets utilized for constructing plasmids and validating mutants. The primer pair MviNinF/MviNinR was used to amplify the internal region of *mviN*, whereas the screening for mutants utilized two primer pairs, MviNinF/pNQF and MviNinR/pNQR. **C.** PCR screening and confirming of *mviN* insertional mutant. Amplifications with the chromosomal DNA of the wild type as the template (Lanes 1 and 2), and with the chromosomal DNA of the *mviN* insertional mutant as the template (Lanes 3 and 4) are shown. Lane M, DNA molecular weight marker.

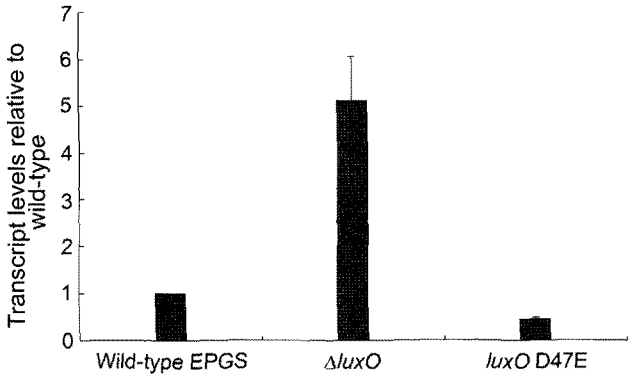

Fig. 3. qRT-PCR analysis of MviN expression regulated by LuxO.
The *V. alginolyticus* wild-type EPGS, Δ*luxO* mutant, and *luxO-con* (*luxO* D47E) were grown in M9S for 8 h at 30°C. The transcriptional levels of *mviN* were then examined by qRT-PCR. The results are normalized to the control gene 16S rRNA using the $\Delta\Delta C_T$ method and show the difference relative to the levels of the wild-type. The error bars indicate the standard deviation for three triplicate samples.

luxO (*luxO-con*) was constructed by site-directed mutation of the site at which LuxO is phosphorylated (*e.g.*, D47E), as previously described [36]. As LuxO negatively regulates extracellular protease production in *V. alginolyticus* [31, 38], Hide Powder Azure (HPA) assay was carried out to detect the minimum extracellular protease activity in *V. alginolyticus luxO-con* (data not shown), confirming that LuxO D47E was present as a constitutively active LuxO protein. qRT–PCR also showed that MviN was drastically repressed in the *luxO-con* strain, demonstrating that *mviN* was negatively regulated by *luxO* in *V. alginolyticus* (Fig. 3).

mviN Expression is Cell-Density Dependent

Based on the finding that MviN was regulated by the QS regulator LuxO, the question of *mviN* expression being cell-density dependent was also explored. Thus, qRT–PCR and Western blotting were used to detect the *mviN* mRNA transcript and protein expression at different growth phases. As a result, the *mviN* mRNA transcription levels in the late-exponential and stationary phases were found to be 20-fold higher than those in the early and mid-exponential phases (Fig. 4A). During the late stationary phase, the *mviN* transcription activity decreased quickly to a level comparable to that in the early exponential phase. These results were consistent with those shown in the Western blotting (Fig. 4B), confirming that *mviN* was synthesized in a cell-density dependent manner in *V. alginolyticus*.

mviN Plays an Essential Role for Growth of *V. alginolyticus*

During the mutant construction and selection process, it was intriguing to find that the *mviN* insertional mutant grew very slowly on the agar plate. When compared with the wild type, the mutant displayed an irregular growth with long lagging growth phases (*e.g.*, approximately 10 h for the lag phase and 20 h for the stationary phase) (Fig. 5). Moreover, the overall cell density of the *mviN* mutant was much lower than that of the wild type during the stationary phase (Fig. 5), emphasizing the important roles of *mviN* in *V. alginolyticus*.

MviN is Secreted into Extracellular Medium

To determine the localization of MviN, part of MviN was expressed. The purified protein was then injected into New Zealand white rabbits to generate anti-MviN serum. The cell pellets and supernatants were concentrated and equal volumes subjected to SDS–PAGE and probed with the antibody (Fig. 6, lanes 1 and 2). A 56-kDa band corresponding to MviN was detected in the supernatant, indicating that the MviN had been secreted into the extracellular medium in *V. alginolyticus*.

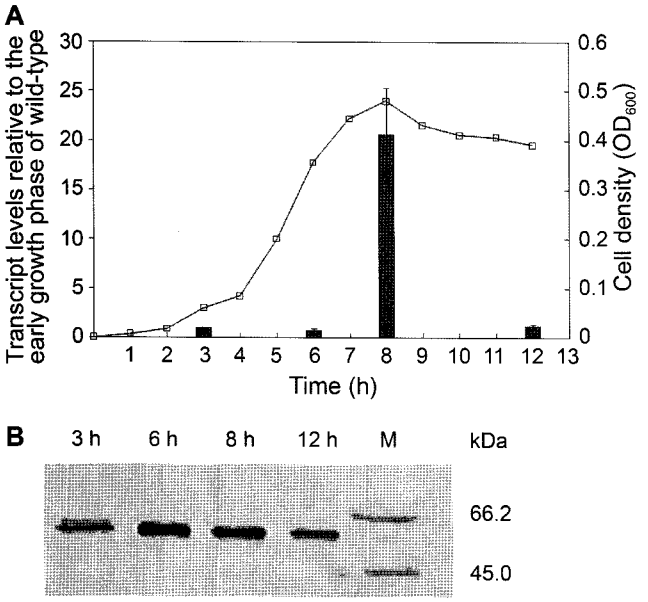

Fig. 4. *mviN* expression profile of *V. alginolyticus* for the whole growth period.

An overnight culture of *V. alginolyticus* EPGS was diluted into a fresh M9S medium and then incubated at 30°C. **A.** Samples were taken at various time points and analyzed for growth (OD_{600}, open squares). The transcriptional levels of *mviN* in different growth phases were determined by qRT-PCR and normalized to the 16S rRNA. The results are shown relative to the levels of the early phase (3 h) of growth (black columns). The error bars indicated the standard deviation for three triplicate samples. **B.** Western blotting analysis of MviN expression. The wild-type strain was grown in 50 ml M9S medium. The culture supernatants were collected in different growth phases and concentrated. The proteins in the supernatants were then resolved by SDS-PAGE and probed with anti-MviN serum. Lane M, protein molecular mass marker.

Role of MviN as Virulence Factor

Zebra fish (*Brachydanio rerio*) was used as the fish model to test the virulence of the wild-type EPGS and *mviN* insertional mutant. The LD_{50} value for the *mviN* insertional mutant was 1.8×10^6 CFU/ml, whereas that for the wild-type EPGS was 4.9×10^5 CFU/ml (Table 4), suggesting a marginal attenuation in the fish model when disrupting the *mviN* in *V. alginolyticus*.

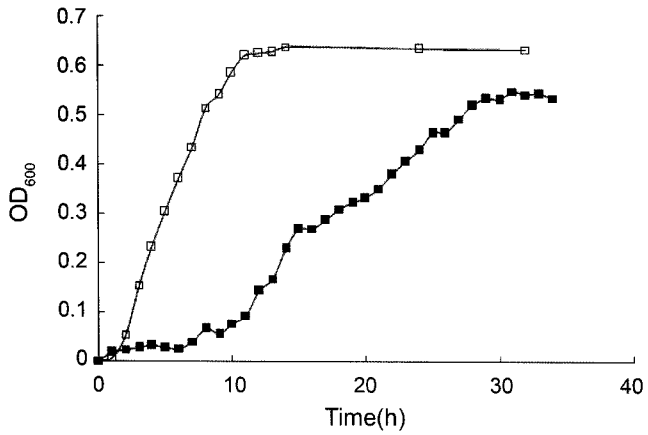

Fig. 5. Growth of *V. alginolyticus* wild-type EPGS (open squares) and *mviN* insertional mutant (filled squares).

An overnight culture of each strain was subcultured at a dilution of 1:50 into fresh LB medium.

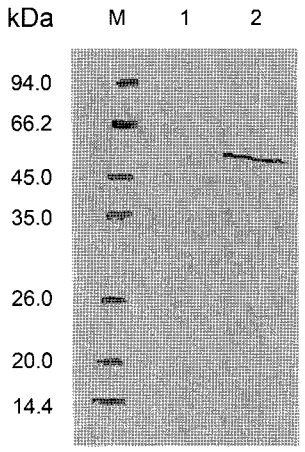

Fig. 6. Western blotting analysis of cell pellet and supernatant.

V. alginolyticus EPGS was cultured in M9S medium at 30°C. The cell pellet and supernatant were then separated and concentrated. Equal amounts of the cell pellet (lane 1) and supernatant (lane 2) were subjected to SDS-PAGE and probed with anti-MviN serum. Lane M, protein molecular mass marker.

The ECPs from the wild type and the *mviN* insertional mutant of *V. alginolyticus* were serially diluted to treat the EPC cell monolayer, and the cell morphology was observed at different time points and assessed using the MTT method. When treated with a low concentration of ECPs from the wild type, no change in cell shape was observed until 6 h post incubation. In contrast, with a high concentration of ECPs from the wild type, the cells began to shrink within the first 2 h (Fig. 7D). When the cells were incubated over a period of 12 h, the cell monolayer was destroyed, regardless of the ECPs concentration (Fig. 7E). Meanwhile, the ECPs from the *mviN* insertional mutant showed no effect on the cells, regardless of the ECPs concentration and incubation time (Fig. 7C), indicating that the ECPs from the *mviN* insertional mutant exhibited no cytotoxic activity against the EPC cells. Thus, the results of the fish and cell experiments indicated that MviN was involved in the pathogenicity of *V. alginolyticus*.

DISCUSSION

Extracellular products (ECPs), such as alkaline serine proteases, siderophores, and hemolysins, were established to be important virulence factors in *V. alginolyticus* and to be controlled by central regulator LuxO in QS system. To identify more virulence factors regulated by the QS system, a DNA microarray against the *V. alginolyticus* genome sequence was used in this study. Zhu *et al.* [40]

Table 4. Virulence of *Vibrio alginolyticus* wild-type EPGS and *mviN* insertional mutant in zebra fish after i.p. injection.

Strain	Dose/fish (CFU)	Mortality (no. dead/no. injected)	LD_{50} value (CFU/ml)	Day of death (no. dead/no. injected)
Wild type	3.66×10^7	10/10	4.9×10^5	1(8/10), 2(9/10), 4(10/10)
	6.10×10^6	8/10		2(7/10), 3(8/10)
	1.02×10^6	9/10		2(1/10), 3(5/10), 4(9/10)
	1.69×10^5	1/10		5(1/10)
	2.82×10^4	1/10		5(1/10)
mviN mutant	2.22×10^7	10/10	1.8×10^6	1(4/10), 2(10/10)
	3.70×10^6	7/10		2(1/10), 3(7/10)
	6.17×10^5	1/10		5(1/10)
	1.03×10^5	1/10		5(1/10)
	1.71×10^4	0/10		NA
Control (2.5% NaCl)		0/10	NA	NA

NA, not applicable: no death due to vibriosis during 7-day experiment.

previously found a lot of virulence-related genes regulated by LuxO using a DNA microarray of *V. cholerae*. In the present study, the results suggested that LuxO plays specific regulatory roles in the *V. alginolyticus* scenario. Among the 11 genes identified to be regulated by LuxO in *V. alginolyticus*, the putative virulence factor *mviN* was identified and subjected to further characterization (Table 3).

BlastP analysis indicated a high identity between the *V. alginolyticus* MviN and homologs in other *Vibrio* species, and suggested that MviN may be an integral membrane or secreted protein. Although MviN was localized extracellularly in *V. alginolyticus* (Fig. 6), it is still possible that MviN may be an integral membrane protein, as the extracellular localization of an outer membrane protein has already been observed in several bacteria, including *Helicobacter pylori*, *Brucella melitensis*, and *Aeromonas* spp. [12, 17, 24, 37]. In *H. pylori*, membrane fragments are released as vesicles that may be involved in the delivery of virulence factors, along with specific secretion pathways and autolysis [11].

The role of MviN (or MviS) was first described as a factor required by *S. typhimurium* for virulence in mice [5], and deletion of *mviN* greatly reduced the virulence in a mouse model. The involvement of MviN in virulence was also revealed in *B. pseudomallei* and *R. tropici* [22, 28]. In this study, the ECPs from the *mviN* insertional mutant exhibited no cytotoxicity towards the EPC cells (Fig. 7), suggesting that MviN may one of the ECPs and involved in the pathogenicity of *V. alginolyticus*. Notwithstanding, the decreased toxicity towards the ECP cells by the *mviN* insertional mutant could also be ascribed to the fact that MviN may be a membrane protein and the mutation in *mviN* may have caused a defective delivery pathway for the virulence factors.

In addition to virulence, MviN has been essentially associated with growth in other bacteria. In the nitrogen-fixing symbiont *S. meliloti*, when *mviN* was cotranscribed with *glnD*, encoding uridylyl transferase, isolating an in-frame deletion mutant of either gene was impossible unless a complementing cosmid was provided, suggesting that *glnD* and *mviN* are essential genes in *S. meliloti* [30]. The significant role of MviN in bacterial growth has also been observed in *N. meningitidis* and *B. pseudomallei* [22, 27]. In the case of *V. alginolyticus* EPGS, the *mviN* insertional mutant was found to grow much more slowly than the wild-type strain (Fig. 5). Moreover, all attempts to construct an *mviN* in-frame deletion mutant failed, further suggesting that *mviN* may be an essential gene in *V. alginolyticus*. A recent study identified MviN (renamed MurJ) as a peptidoglycan flippase, an essential inner membrane protein in *E. coli* [33]. However, MviN in *V. alginolyticus* showed no homolog to MurJ (data not shown). Therefore, the growth defect exhibited by the *mviN* insertional mutant may not have been due to the repression of peptidoglycan biogenesis, which causes cell lysis mediated by MurJ, as established in *E. coli* [33].

In this study, MviN was characterized to be regulated by LuxO and the cell density (Fig. 3 and 4). Previous work on *V. alginolyticus* already indentified a *V. harveyi*-like QS system that included active players, such as LuxO and LuxR, and regulated the multiple phenotypes in the organism [31, 32, 38]. Therefore, it is reasonable to speculate that MviN could play a part in the consequences of the QS regulatory cascade in *V. alginolyticus*. A recent study revealed that the global regulator RpoS is involved in the LuxS/AI-2 QS system by positively affecting the expression of *luxS* and regulating the cytotoxicity of the ECPs in *V. alginolyticus* [35], hinting at the regulation of MviN by RpoS in this bacterium.

In summary, a microarray analysis enabled the identification of the putative virulence factor MviN regulated by LuxO, the pivotal regulator in the QS system of *V. alginolyticus*. In addition, the results of qRT–PCR with Western blotting

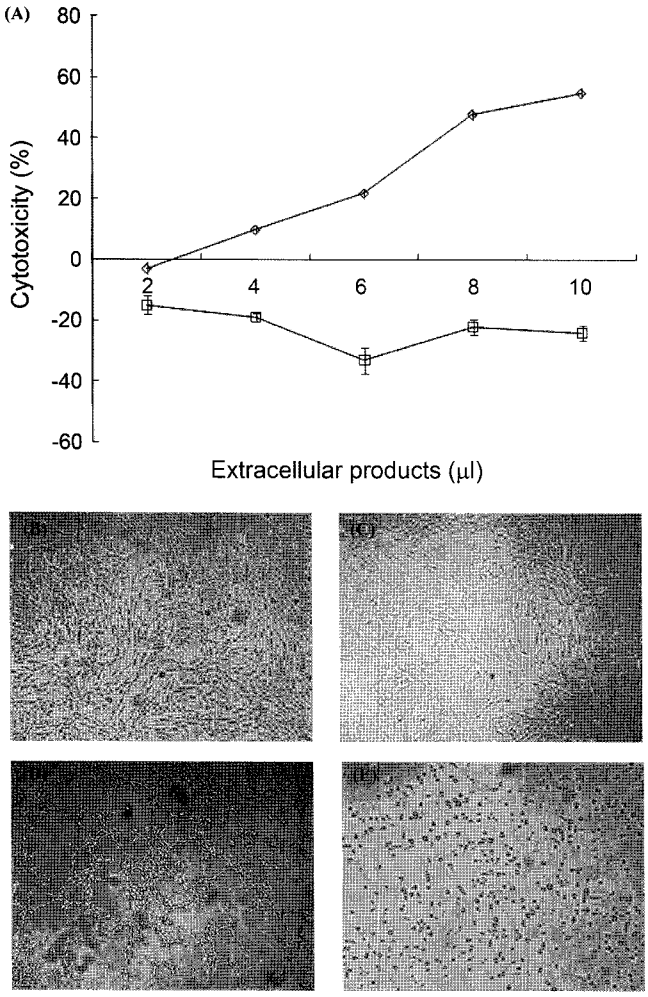

Fig. 7. A. Cytotoxicity effect of ECPs from *V. alginolyticus* wild-type EPGS (diamonds) and *mviN* insertional mutant (squares) at different dilutions on EPC cells using the MTT assay.
The error bars indicate the standard deviation for three triplicate samples. **B–E.** Micrographs of EPC cells when treated with ECPs from different *V. alginolyticus* strains: uninfected EPC cells as control (**B**) and EPC cells infected with the highest concentration of ECPs from *mviN* insertional mutant for 12 h (**C**), ECPs from wild type for 7 h (**D**), and ECPs from wild type for 12 h (**E**).

confirmed the microarray data and revealed the cell-density dependent expression pattern of MviN. Finally, MviN was also shown to be secreted into the extracellular medium and to be involved in the growth and pathogenicity of *V. alginolyticus*.

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