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Role of Alkaline Serine Protease, Asp, in *Vibrio alginolyticus* Virulence and Regulation of Its Expression by LuxO-LuxR Regulatory System

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The alkaline serine protease *asp*, which was shown to be a virulence factor of *Vibrio alginolyticus* as a purified protein, was cloned from *V. alginolyticus* EPGS, a strain recently isolated from moribund *Epinephelus coioides* in an outbreak of vibriosis in a mariculture farm of Shenzhen. The *asp* null mutant was constructed by homologous recombination with suicide plasmid pNQ705-1. Compared with the wild-type strain, the *asp* null mutant exhibited a significant decrease of total extracellular protease activity, and caused a 15-fold decrease in virulence of *V. alginolyticus*. In our previous study, the *luxO* and $luxR_{val}$ genes from *V. alginolyticus* MVP01 were cloned and identified, and the *luxO*-$luxR_{val}$ regulatory couple was shown to regulate various genes expression, suggesting that it played a central role in the quorum sensing system of *V. alginolyticus*. In this study, the regulation of the *asp* gene was analyzed by using RT-PCR and quantitative real-time PCR methods; we proved that its transcription was greatly induced at the late stage of growth and was regulated by a *luxO*-$luxR_{val}$ regulatory system.

Keywords: Alkaline serine protease, *asp*, quorum sensing, *Vibrio alginolyticus*

Quorum sensing, a type of cell-cell signaling, has been studied widely in recent years [3]. A typical quorum sensing system of Gram-negative bacteria has two types of autoinducers: acylated homoserine lactones (AI-1) and furanosyl borate diester (AI-2) [4, 28]. In *Vibrio harveyi*, detection of and response to AI-1 and AI-2 occurs through two parallel two-component signal transduction circuits, and a shared response regulator called LuxO integrates the information from these two circuits and relays it to LuxR, whereupon activated LuxR regulates the expression of various genes [20]. *Vibrio* spp. could use this system to control many processes including bioluminescence, virulence, mobility, biofilm formation, and extracellular polysaccharide production [32]. Significantly, extracellular proteases, the virulence factors of *Vibrio* spp., were shown to be regulated by their respective quorum sensing systems, such as hemagglutinin protease *hap* in *V. cholerae* [13], metalloprotease *empA* in *V. anguillarum* [6], and metalloprotease *vvp* and cytolysin *vvhA* in *V. vulnificus* [25].

Vibrio alginolyticus, a ubiquitous organism in seawater, has been isolated from different organisms as part of the normal marine flora. However, it has also been suggested that this species was a pathogen of several marine animals and humans. In the South China Sea, *V. alginolyticus* was reported to be the dominant causative agent of high-mortality vibriosis in the large yellow croaker, sea bream, grouper, kuruma prawn, as well as shellfish species [16, 17].

Compared with other *Vibrio* spp., such as *V. cholerae*, *V. anguillarum*, and *V. vulnificus*, much less is known about the pathogenic mechanism of *V. alginolyticus*. Studies on the pathogenicity of various isolates of *V. alginolyticus* suggested that some extracellular enzymes with strong proteolytic activities could be important virulent factors [1, 2, 11], and alkaline serine protease from the extracellular products (ECP) of *V. alginolyticus* was reported to be lethal to fish but the detailed information about its DNA sequence and protein structure was unknown [14]. By searching the whole genome of *V. alginolyticus* 12G01, we found that two alkaline serine proteases existed in the genome, and they were totally different from each other (16% identity at the amino acid level). Blast analysis showed that one of the two alkaline serine proteases in the genome of *V. alginolyticus* 12G01 was homologous to ProA, which was only expressed in medium containing calcium and shown to be regulated by the quorum sensing system in *V. alginolyticus* MVP01 [23], and the other one was homologous to Asp, which was recently shown to be lethal to fish as purified protein with a LD_{50} of 0.25 μg protein per gram of body weight and thought to be important in the virulence mechanism of *V. alginolyticus* [5].

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In previous work, we demonstrated that *V. alginolyticus* MVP01 possessed the quorum sensing system involved in regulating extracellular proteases [23, 31]. In this study, a new *V. alginolyticus* strain termed EPGS was isolated from moribund *Epinephelus coioides* in a recent outbreak of vibriosis in a mariculture farm of Shenzhen, a southern coastal city of China. We found that the extracellular protease activity of this strain was much stronger than that of the previous *V. alginolyticus* strain MVP01, but to our surprise, it only contained the protease gene *asp* and lost gene *proA* (data not shown). Thus, a null mutant of gene *asp* was constructed by allelic exchange, and the fish infection experiments and cytotoxicity assays showed that Asp plays a role in the pathogenic mechanism of *V. alginolyticus* EPGS. Furthermore, the expression of gene *asp* was proven to be regulated by the quorum sensing system.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids are listed in Table 1. *V. alginolyticus* strains were grown at 30°C in Luria-Bertani medium supplemented with 3% NaCl (LBS). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium with 1% NaCl. Antibiotics, when needed, were added to the media at the following final concentrations: 100 μg/ml ampicillin (Amp) and 34 μg/ml chloramphenicol (Cm) for *E. coli*; 100 μg/ml Amp and 7 μg/ml Cm for *V. alginolyticus*.

DNA Manipulation

All molecular techniques were performed as previously described [24]. Enzymatic reactions and plasmid purifications were performed according to the manufacturer's instructions (TaKaRa, Dalian, China). DNA sequencing and primer synthesis were carried out by Invitrogen Co. Ltd. (Shanghai, China).

Construction of an Alkaline Serine Protease *asp* Mutant

Primers used for the mutant construction are given in Table 2. The insertion mutant of gene *asp* was constructed as previously described [31]. Briefly, an internal fragment of the gene *asp* (600 bp) was amplified by PCR from *V. alginolyticus* EPGS chromosome with primers AspmutF and AspmutR. The PCR product was treated with SacI/SpeI restriction enzymes and cloned into the corresponding restriction sites of suicide plasmid pNQ705-1 [6]. The resulting plasmid pNQ-asp was transferred from *E. coli* to *V. alginolyticus* by bacterial mating, which used *E. coli* SM10 *λpir* as the donor strain [15], and the mating was allowed to progress on an LB agar plate at 30°C for 12 h. The exconjugants with the plasmid integrated into the chromosome by homologous recombination were selected on LBS agar medium containing Cm (7 μg/ml) and Amp (100 μg/ml). The resulting mutant (NESP) was confirmed by PCR using primer pairs AmF/pNQF and AmR/pNQR, and subsequent DNA sequencing.

The quorum sensing targeted genes $luxO_{val}$ and $luxR_{val}$ of *V. alginolyticus* EPGS were amplified by PCR, and the DNA sequencing results showed that they shared 100% identity to those of *V. alginolyticus* MVP01. Thus, we used the previous suicide plasmids pDM-luxO and pDM-luxR [23, 31] to construct in-frame deletion mutants in $luxO_{val}$ and $luxR_{val}$, respectively. The plasmids were mated by conjugation from *E. coli* SM10 *λpir* to wild-type *V. alginolyticus* EPGS. After allelic exchange, the absence of targeted fragments in the mutants was confirmed by PCR amplification using primers pairs LuxO-F/LuxO-R and LuxR-F/LuxR-R, and subsequent DNA sequencing. For genetic complementation of *the* $luxR_{val}$ mutant, the plasmid pMMB-luxR [31] with intact $luxR_{val}$ gene and promoter region was introduced into the $luxR_{val}$ mutant by conjugation. The complemented strain with the presence of the plasmid was selected and confirmed by PCR analysis.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference or source
Vibrio alginolyticus		
EPGS	Amp; Pathogenic isolate from the aquiculture farm of the South China Sea	Lab collection
NESP	Amp; EPGS, null mutant in *asp*	This study
DEO	Amp; EPGS, in-frame deletion in $luxO_{val}$	This study
DER	Amp; EPGS, in-frame deletion in $luxR_{val}$	This study
CDER	Amp; EPGS, complementation *in trans* with intact $luxR_{val}$ gene	This study
Escherichia coli		
CC118 *λpir*	*λpir* lysogen of CC118 *(Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1)*	Dennis and Zylstra [10]
SM10 *λpir*	Km^r; *thi thr leu tonA lacY supE recA RP4-2-Tc::Mu λ::pir*	Liang *et al.* [15]
Top10	F' [lacIq,Tn10(TetR)] *mcrA* Φ80 *lacZ* Δm15 Δ*lac* X74 *deoR recA1*	Invitrogen
Plasmid		
pNQ705-1	Cm^r; suicide vector that contains an R6K origin (*pir* requiring)	Croxatto *et al.* [6]
pNQ-asp	Cm^r; pNQ705-1 derivative containing a 601 bp internal fragment of gene *asp*	This study
pMD-luxO	Cm^r; pDM4 derivative containing *luxO* bp 1-123 fused in-frame to bp 1281-1362	Wang *et al.* [31]
pDM-luxR	Cm^r; pDM4 derivative containing $luxR_{val}$ bp 0-56 fused in-frame to bp 474-615	Rui *et al.* [23]
pMMB-luxR	Cm^r; pMMB206 derivative containing $luxR_{val}$ bp from -782 to 1408	Rui *et al.* [23]

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

Primer	Sequence (5'-3')
AspmutF	TCA**GAGCTC**CGAACTGAGCAAAAT
AspmutR	ACG**ACTAGT**CGTTACCGAAACCATA
AmF	CTAACGTTGTGAACATGAGCC
AmR	GACCAACATACCCCAATAGCT
LuxO-F	CTACGAGCGCAGTTCATCGCA
LuxO-R	GTCTATGGACATCGCCAGTTCACAA
LuxR-F	CACGCAAACGATCACCTAA
LuxR-R	TATCCACGCTCACCCAACT
pNQF	GGTGCTCCAGTGGCTTCTGTTTCTA
pNQR	CAGCAACTTAAATAGCCTCTAAGGT
AspM-F	GGTAACAACAATGCCCACGG
AspM-R	GCCCAGATGAGTAACCCCAAC
16S-F	AAAGCACTTTCAGTCGTGAGGAA
16S-R	TGCGCTTTACGCCCAGTAAT

Measurement of Total Extracellular Protease Activity

Protease activity of culture supernatant was determined by two methods. For qualitative assay, *V. alginolyticus* strains were grown for 12 h on LBS agar plates containing 1% skimmed milk (SMLB; Oxoid, U.K.) and the clearing zones around the colonies were observed [29]. For quantitative assay, the strains were grown in LBS medium at 30°C for 9 h, and protease activity was determined by the method described elsewhere [9, 14]. Briefly, the bacterial cultures were harvested and filtered through 0.22-μm filters (Millipore, U.S.A.), and the filtered supernatants (0.5 ml) were incubated with the substrate hide powder azure (HPA; Sigma-Aldrich, U.S.A.) in 1.5 ml of PBS buffer (pH 7.2) at 37°C for 2 h. After stopping the reaction by trichlorocetic acid (TCA), the absorbance of the supernatant was assayed at 600 nm. When needed, the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to reach a final concentration of 1 mmol/l.

Gelatin-gel zymographic analysis was also used to characterize extracellular protease activity. *V. alginolyticus* strains were cultured in LBS medium for 9 h and the culture supernatants were harvested and filtered through 0.22-μm filters (Millipore, U.S.A.). Two μl of each cell-free supernatant was mixed with nonreducing buffer and loaded in a 12% denaturing polyacrylamide gel copolymerized with 0.1% gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h and incubated in 50 mmol/l Tris-HCl (pH 8.0) buffer containing 5 mmol/l $CaCl_2$ and 1 μmol/l $ZnCl_2$ for 3 h. Protein bands with gelatinase activity in the gels were visualized by Coomassie blue staining.

Fish Infection

Zebra fish (*Brachydanio rerio*) with an approximate weight of 1 g were infected with *V. alginolyticus* strains *via* route of intramuscular injection (i.m.) as described previously [33]. Four different bacterial dilutions varying by 10-fold were used and seven fish were infected for each dilution. Death due to vibriosis was determined by the observation of gross clinical signs and confirmed by isolation of *V. alginolyticus* strains from infected organs of dead fish. The mortality of the fish was recorded over a period of 2 weeks. The LD_{50} values were calculated by a method described before [22].

Cytotoxicity Assay

Cytotoxicity of *V. alginolyticus* strains was assayed with epithelioma papillosum cyprini (EPC) cells as described previously [2, 21]. The EPC cells were grown as a monolayer at 25°C in Eagle's minimum essential medium (MEM; Sigma) supplemented with 10% fetal calf serum, and harvested with trypsin ethylenediaminetetraacetic acid. A 900-μl aliquot of the cell suspension was inoculated to each well in a 24-well culture plate (Costar, U.S.A.). After incubation for 24 h, 100 μl of filtered supernatant of *V. alginolyticus* culture was added to EPC cell culture, and the EPC cells were inspected under microscopy for the morphologic damage. Cytotoxicity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Amersco, U.S.A.) after 24-h infection. Cell culture of the control group was treated with fresh LBS medium. Percent viability was calculated as follows: (optical density at 570 nm [OD_{570}] of control group-OD_{570} of treated group)/OD_{570} of control group×100%.

RNA Isolation and Quantitative Real-Time RT-PCR

Primers for quantitative real-time PCR are listed in Table 2. *V. alginolyticus* strains were grown in LBS medium at 30°C, and RNA was isolated using an RNA isolating kit (Tiangen) and then treated with RNase-free DNaseI (Promega, U.S.A.) to exclude genomic DNA contamination. Equal amounts of RNA (1 μg) were used to generate cDNA (BioToKo) with a random primer (TaKaRa). The synthesized cDNA was used as a template for PCR amplification. The primers AspM-F and AspM-R were used to amplify the internal segment of gene *asp*. 16S rRNA was selected as a control gene and amplified with primers 16S-F and 16S-R. The quantitative real-time RT-PCR (qRT-PCR) reaction system contained 0.5 μl of cDNA template, 1 μl of the gene-specific primers mentioned above, 12.5 μl of SYBR Green master mix (BioToKo), and ddH_2O up to 25 μl of total volume. qRT-PCR assays were performed in triplicate with a FTC-2000 detector (Funglyn Biotech, China) and each qRT-PCR experiment was repeated three times. Transcriptional levels were normalized to 16S rRNA in each sample by the $\Delta\Delta C_T$ method [18].

Nucleotide Sequence Accession Number

The nucleotide sequence of alkaline serine protease gene *asp* from *V. alginolyticus* EPGS has been deposited in the GenBank under Accession No. EU484572.

RESULTS

Construction of a Null Mutation Within Gene *asp*

The *asp* gene of *V. alginolyticus* EPGS was amplified by PCR and sequenced. The ORF contains 2,031 nucleotides and the deduced protein sequence shows 98%, 92%, 76%, and 72% homology to those of *V. alginolyticus* 12G01, *V. parahaemolyticus* 2210633, *V. alginolyticus* HY9901, and *V. harveyi* HY01. The *asp* deficient mutant (NESP) was generated by integrating into the chromosome a mobilizable suicide plasmid, pNQ-asp, which contained a 600-bp PCR fragment from the encoding region of the gene *asp*. Primer pairs AmF/pNQF and AmR/pNQR, located on chromosome and plasmid pNQ705-1, respectively, were chosen for PCR. Two PCR fragments were generated from the *asp* null mutant, whereas the wild-type EPGS yielded no PCR product (Fig. 1). These two PCR fragments were sequenced, and the result showed the correct integration.

The quorum sensing targeted genes $luxO_{val}$ and $luxR_{val}$ of *V. alginolyticus* EPGS were cloned and the DNA sequencing result showed that they shared 100% identity to those of *V. alginolyticus* MVP01. Thus, the recombinant plasmids pDM-luxO and pDM-luxR were used respectively for constructing in-frame deletion mutant strains DEO ($\Delta luxO_{val}$) and DER ($\Delta luxR_{val}$). For genetic complementation of strain DER, plasmid pMMB-luxR was introduced by conjunction and the transconjugant ($luxR_{val}^{+}$) was designated as strain CDER (data not shown).

Extracellular Protease Activity of Wild-Type EPGS and Null Mutant NESP

The extracellular protease activity of mutant strain NESP was examined and shown to be much less than that of wild-type strain EPGS. As seen in Fig. 2A, the clearing zone of strain NESP was smaller than that of strain EPGS. To confirm this result, the total extracellular protease activities of 9-h cultures were determined with HPA as substrate. Compared with strain NESP, the strain EPGS exhibited much higher protease activity. However, when specific serine protease inhibitor PMSF was added to the supernatants of both strains, the protease activity of strain EPGS was dramatically decreased while the protease activity of strain NESP had a little change (Fig. 2B). The similar growth profiles of both strains in LBS medium (data not shown) suggested that impairment of extracellular protease production was not caused by growth defect. Zymography revealed that wild-type *V. alginolyticus* had strong proteolytic bands at about 40 kDa, which clearly disappeared in the *asp* mutant. It also suggested that Asp was a dominant protease in the culture supernatant of *V. alginolyticus* and had more than one gelatin-degrading band (Fig. 2C). This was not unexpected,

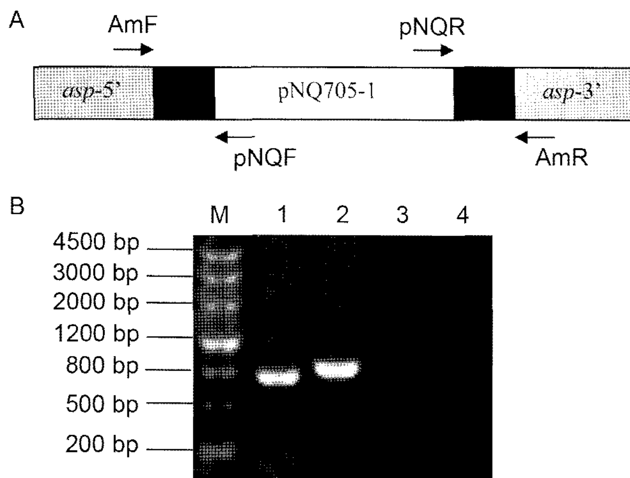

Fig. 1. Identification of the *asp* mutation.
A. A diagram of the chromosomal integration of plasmid pNQ-asp into the *asp* gene. The gray blocks represent the *asp* gene, and the black blocks represent the cloned 600-bp PCR fragment from the internal region of the *asp* gene that was used for homologous recombination. **B.** PCR screening and confirming of the *asp* null mutant. Primer pairs AmF/pNQF and AmF/pNQR were utilized to amplify the DNA regions. Lanes 1,2: chromosomal DNA of the mutant NESP; lanes 3,4: chromosomal DNA of wild-type strain; lane M: DNA molecular weight marker.

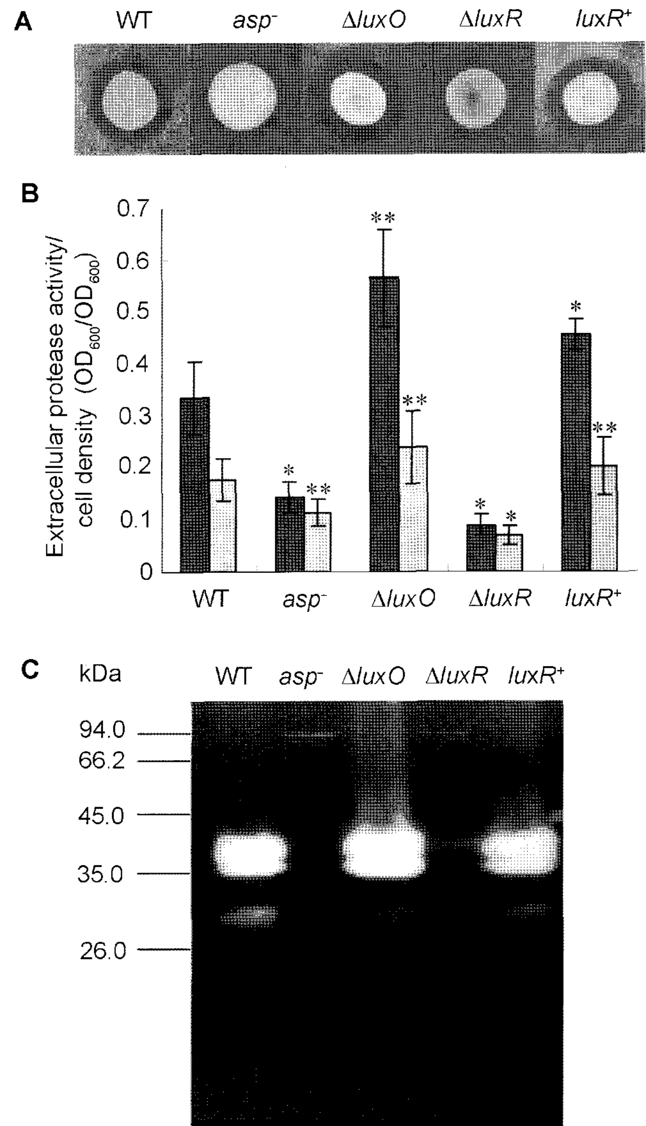

Fig. 2. Detection of extracellular protease activity.
A. Qualitative assay of extracellular protease activity on SMLB agar plates. *V. alginolyticus* strains of wild-type EPGS (WT), NESP (asp^{-}), DEO ($\Delta luxO_{val}$), DER ($\Delta luxR_{val}$), and CDER ($luxR_{val}^{+}$) were grown on SMLB agar plates for 12 h. Clearing zones around the colonies were observed. **B.** Extracellular protease activity measured by HPA digestion as described in Materials and Methods. *V. alginolyticus* strains were cultured in LBS medium for 9 h. To each sample, a solution of PMSF (gray columns) or an equal volume of ddH_2O (black columns) was added. The values shown are the means of three independent experiments, and the error bars represent the standard deviations. Data marked with one asterisk indicate p-value<0.01, and data with two asterisks indicate p-value<0.05 (Student t-test). **C.** Proteolytic activity was detected using a 12% SDS-PAGE gel copolymerized with 0.1% gelatin. Lane 1: *V. alginolyticus* wild-type; lane 2: *asp* mutant; lane 3: $\Delta luxO_{val}$ mutant; lane 4: $\Delta luxR_{val}$ mutant; lane 5: $\Delta luxR_{val}$ mutant carrying plasmid pMMB-luxR.

Table 3. Effect of the *asp* mutation on the LD_{50} and cytotoxicity of *V. alginolyticus*.

Strain	Dose/fish (CFU)	Mortality (no. dead/no. injected)	Day of death (no. dead/no. injected)	LD_{50} value (CFU/ml)	Cytotoxicity (%)
EPGS	1×10^7	7/7	1(3/7) 2(6/7) 4(7/7)	6×10^5	57
	1×10^6	6/7	1(1/7) 2(4/7) 4(6/7)		
	1×10^5	4/7	2(1/7) 3(2/7) 4(3/7) 6(4/7)		
	1×10^4	1/7	4(1/7)		
NESP	1×10^7	6/7	1(2/7) 2(6/7)	1×10^7	32
	1×10^6	3/7	2(3/7)		
	1×10^5	0/7	NA*		
	1×10^4	0/7	NA*		
Control		0/7	NA*		

*NA, not applicable: no death due to vibriosis during the 14-day experiment.

because there was evidence that the extracellular alkaline serine proteases might be degraded as they were secreted from the cytoplasm [1, 12, 14, 19].

Role of Asp as a Virulence Factor

In order to investigate the role of alkaline serine protease in the *V. alginolyticus* virulence mechanism, zebra fish (*Brachydanio rerio*) was used as the fish model to assay the virulence difference between the wild-type EPGS and the *asp* null mutant. As showed in Table 3, the intramuscular LD_{50} values were 1.0×10^7 bacteria for *asp* null mutant and 6×10^5 bacteria for wild-type EPGS, respectively. The *asp* mutation led to a 15-fold decrease in strain virulence, indicating that Asp has a role in the pathogenic mechanism of *V. alginolyticus*.

The cytotoxicity of *V. alginolyticus* strains against EPC cells was further assessed by the MTT method. After 24-h infection, the cytotoxic effects of extracellular products produced by *V. alginolyticus* strains were analyzed. As shown in Table 3, compared with the parent strain, the mutant strain NESP exhibited a 45% decrease of cytotoxicity against EPC cells. The morphological changes of cell monolayer could be observed after 8 h of infection in both strains, and the changes mainly involved cell rounding, shrinking, detachment, and finally monolayer destruction.

Growth-Dependent Expression of *asp* in *V. alginolyticus* EPGS

Since the maximum extracellular protease activity of wild-type EPGS was observed at the late stage of growth (data not shown), it was of interest to investigate whether the expression of alkaline serine protease in *V. alginolyticus* EPGS was growth-dependent. RT-PCR and quantitative real-time RT-PCR were used to measure the *asp* mRNA transcripts at different growth stages. Figs. 3A and 3B showed that *asp* transcriptional levels changed apparently during the whole growth period. A few *asp* mRNA transcripts were produced at the early stage of growth, whereas the amount of transcripts increased significantly (35-fold) at the late stage of growth. Obviously, the transcription of *asp* in *V. alginolyticus* was induced by cell density.

Positive Regulation of *asp* Expression by Quorum Sensing

The extracellular protease activities of the *V. alginolyticus* wild-type strain and quorum sensing mutants were examined. We found that the mutant $\Delta luxR_{val}$ exhibited a 70% decrease whereas the mutant $\Delta luxO_{val}$ exhibited a 2-fold increase of extracellular protease activity (Figs. 2A and 2B). This suggested that the total extracellular protease production was controlled by the quorum sensing system, which was consistent with the result previously described [23, 31].

Since the expression of *asp* in *V. alginolyticus* EPGS was induced at the late stage of growth, it was of concern whether expression of *asp* was regulated by the quorum sensing system. Zymography showed that the $\Delta luxO_{val}$ mutant had strong proteolytic bands at about 40 kDa, which clearly disappeared in the $\Delta luxR_{val}$ mutant and reappeared in $luxR^{+}$ (Fig. 2C). RT-PCR and quantitative real-time RT-PCR were then used to further examine *asp* transcription at the late stage of growth in different strains. As shown in Figs. 4A and 4B, compared with wild-type *V. alginolyticus*, the deletion mutant $\Delta luxR_{val}$ exhibited a 92% decrease of *asp* transcripts, whereas the deletion mutant $\Delta luxO_{val}$ exhibited an elevated level (3.3-fold) of *asp* transcripts, and the complemented strain $luxR_{val}^{+}$ restored the expression of *asp* to the level of wild-type strain. Meanwhile, the transcriptional levels of control gene 16S rRNA were almost the same in all strains.

DISCUSSION

Although two alkaline serine proteases have been found in the genome of *V. alginolyticus* 12G01, only the protease gene *asp* could be cloned from *V. alginolyticus* EPGS. Furthermore, we found that the deduced protein sequence of *asp* showed close homology to those of *V. alginolyticus* strain 12G01 and *V.*

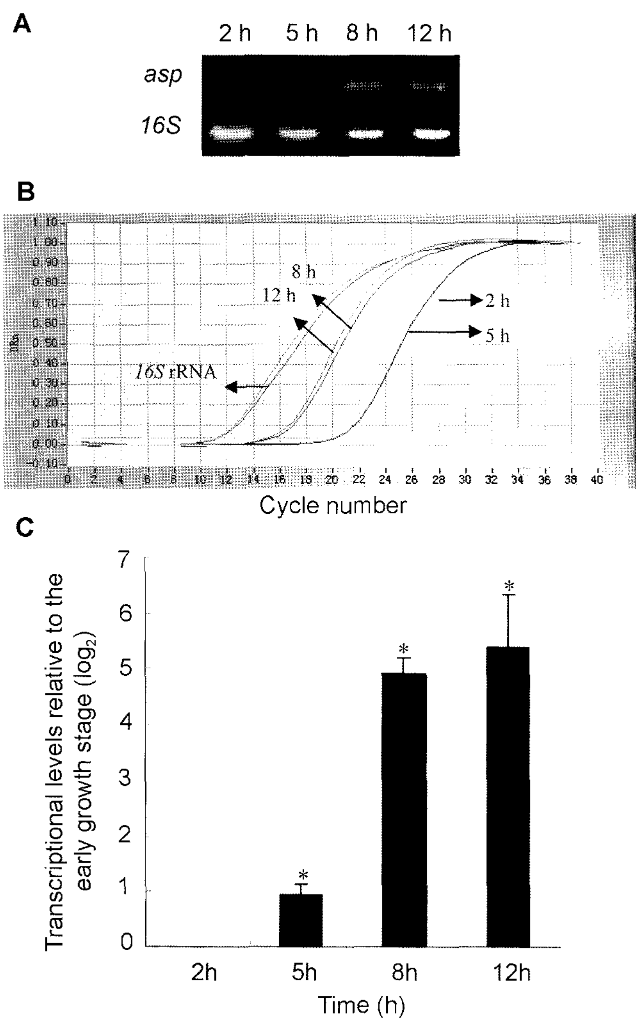

Fig. 3. The *asp* expression profile in the whole growth period.
Overnight culture of wild-type *V. alginolyticus* EPGS was diluted into fresh LBS medium and then incubated at 30°C. Transcription of *asp* at various growth stages was detected by RT-PCR (**A**) and quantitative real-time PCR (**B**). **C**. Transcriptional levels of *asp* at different growth stages are normalized to control gene 16S rRNA and are shown as log_2 difference relative to the level of early growth stage. The values shown are an average of three independent experiments with the standard deviation, indicated by the error bars. Data marked with one asterisk represent that the *asp* expression is statistically different from that of early growth stage (Student *t*-test, $p<0.01$).

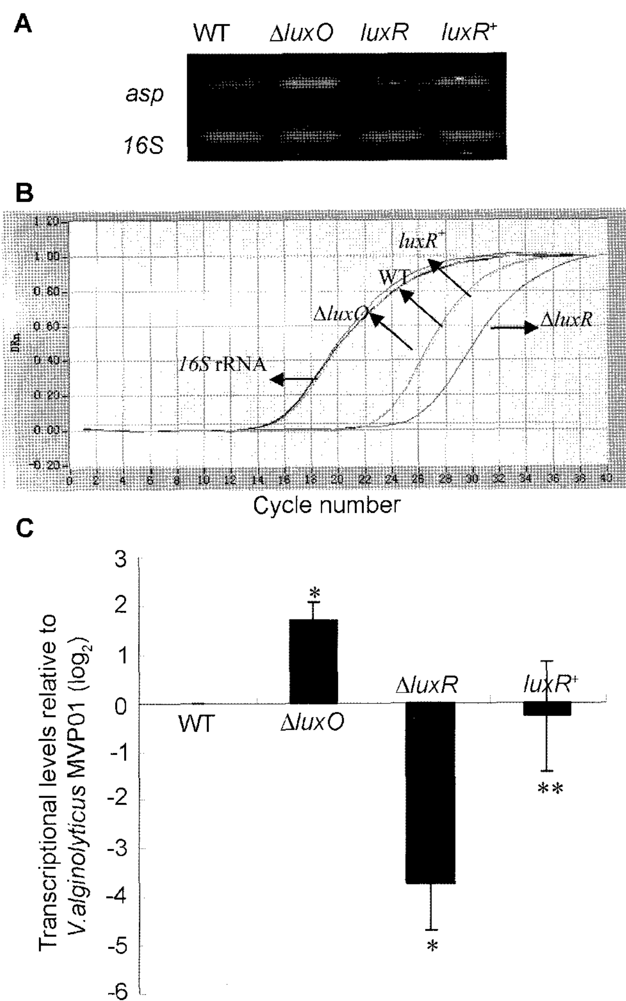

Fig. 4. Regulation of alkaline serine protease *asp* transcription by quorum sensing system.
A. *V. alginolyticus* strains EPGS (WT), DEO ($\Delta luxO_{val}$), DER ($\Delta luxR_{val}$), and CDER ($luxR_{val}^{-}$) were cultured in LBS for 9 h and mRNA transcripts of *asp* were detected by RT-PCR. 16S rRNA was selected as a control. **B**. Transcriptional levels of *asp* at the stationary growth phase (9 h) were examined by quantitative real-time PCR. **C**. The transcriptional levels of *asp* in different mutant strains are normalized to control gene 16S rRNA and are shown as log_2 difference relative to the levels of the wild-type. The values shown are an average of three independent experiments with the standard deviation, indicated by the error bars. Data marked with one asterisk represent that the *asp* expression is statistically different from that of wild-type cells (Student *t*-test, $p<0.01$), and data with two asterisks represent that the *asp* expression is not statistically different from that of wild-type cells (Student *t*-test, $p>0.05$)

parahaemolyticus strain RIMD 2210633, but not to that of *V. alginolyticus* strain HY9901. These were not unexpected results because there was evidence that the extracellular alkaline serine proteases produced by *V. alginolyticus* might differ greatly in their sequences, depending on the strain, source of the isolate, and the medium [1, 8, 12, 19, 30].

Phenylmethylsulfonyl fluoride (PMSF) is a covalently binding irreversible serine protease inhibitor. When added, the total extracellular protease activity of *V. alginolyticus* EPGS was largely inhibited, suggesting that the extracellular proteases of *V. alginolyticus* mainly consist of serine proteases. This was consistent with the result described previously [14]. Based on our data, it was also found that, when gene *asp* was knocked out, the protease activity of *V. alginolyticus* was dramatically decreased, and inhibitor PMSF had a little effect on the protease activity of strain *asp*⁻. This indicates that the Asp is a major serine protease secreted by *V. alginolyticus*.

Although alkaline serine protease was believed to be a virulence factor of *V. alginolyticus* in some reports, a direct correlation of the loss of the alkaline serine protease to a decrease in virulence of *V. alginolyticus* was not established

[5, 14]. In our experiment of fish infection, wild-type *V. alginolyticus* strain EPGS killed about 60% zebra fish when administered by i.m. injection at a dose of 1×10^5 CFU per gram of fish body, whereas the *asp* null mutant strain NESP was completely avirulent to zebra fish at the same dose. Simultaneously, an obvious decrease in cytotoxicity to epithelioma papillosum cyprini (EPC) cells was also observed in the *asp* null mutant. The results confirm that the alkaline serine protease *asp* is important for the virulence of *V. alginolyticus*.

High transcription of the alkaline serine protease gene *asp* at the late stage of growth indicates that Asp may function only when the bacterial cells reach a high population density in fish. Proteases with such an expression pattern have been demonstrated to be regulated by the quorum sensing system in many pathogenic bacteria such as *P. aeruginosa*, *V. cholerae*, *V. anguillarum*, and *V. mimicus* [7, 26, 27, 34]. Existence of a *V. harveyi*-like quorum sensing system regulatory mechanism in *V. alginolyticus* has been proposed based on the detection of AI-2 activity in the culture supernatant and the identification of a *V. harveyi*-type LuxR homolog [23, 33]. Here, we further demonstrated that *asp* transcription was regulated by a quorum sensing system, and the *asp* transcripts were greatly reduced in a $LuxR_{val}$ deficient mutant but increased in a $LuxO_{val}$ deficient mutant. Furthermore, we also found that the disruption of $luxO_{val}$ in *V. alginolyticus* resulted in a higher level of $luxR_{val}$ mRNA (data not shown). This indicates that $LuxO_{val}$ negatively regulates the expression of $luxR_{val}$, which in turn activates the expression of *asp*.

The regulation of extracellular protease by quorum sensing was found in some pathogenic bacteria, such as *A. hydrophila* and *P. aeruginosa* [26, 29], in which the production of extracellular proteases was inhibited at low cell density and rapidly induced at high cell density. The similar regulation pattern of *asp* was also found in *V. alginolyticus* in this work. This type of regulation mechanism is very important and it can be used to escape host immunity at low cell density, since if extracellular protease is expressed too early, effective host defenses will be induced and the infection probably will be terminated.

In summary, our results demonstrated that the alkaline serine protease *asp* was a virulence factor in *V. alginolyticus*. Two pivotal regulators of quorum sensing systems in *V. alginolyticus*, $LuxO_{val}$ and $LuxR_{val}$, were involved in the regulation of alkaline serine protease *asp*. Further investigation of the regulation mechanism of *asp* by the quorum sensing system in *V. alginolyticus* will be of interest.

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