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ORIGINAL PAPER

Role of RpoS in stress survival, synthesis of extracellular autoinducer 2, and virulence in *Vibrio alginolyticus*

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Abstract *Vibrio alginolyticus*, a marine bacterium, is an opportunistic pathogen capable of causing vibriosis with high mortality to fishes in the South China Sea. Stress resistance is very important for its survival in the natural environment and upon infection of the host. RpoS, an alternative sigma factor, is considered as an important regulator involved in stress response and virulence in many pathogens. In this study, the *rpoS* gene was cloned and characterized to evaluate the role of RpoS in *V. alginolyticus*. The predicted protein showed high identity with other reported *rpoS* gene products. The in-frame deleted mutation of *rpoS* in *V. alginolyticus* led to sensitivity of the strain to ethanol, hyperosmolarity, heat, and hydrogen peroxide challenges. Further studies showed that extracellular autoinducer 2 level, four of seven detected protease activities, and cytotoxicity of extracellular products were markedly decreased in the *rpoS* mutant compared with that in the wild-type strain. The results indicated that the global regulator RpoS was part of the regulatory networks of virulence and LuxS quorum sensing system.

Keywords RpoS · *Vibrio alginolyticus* · Stress · AI-2 synthesis · ECP

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Abbreviations

AI	Autoinducer
Ap	Ampicillin
Cm	Chloramphenicol
ECP	Extracellular product
qRT-PCR	Quantitative real-time reverse transcription PCR
QS	Quorum sensing

Introduction

The gram-negative bacterium *Vibrio alginolyticus* is one of the most invasive and devastating fish pathogens in the South China Sea. It can cause vibriosis in the large yellow croaker, sea bream, grouper, Kuruma prawn, as well as in the larvae of fish and shellfish species (Liu et al. 2004; Xie et al. 2005). Moreover, it has been reported to cause gastroenteritis, conjunctivitis, and otitis in humans (Chien et al. 2002). *V. alginolyticus* is widely distributed in marine and estuarine waters, especially in bathing areas, and has been found in many other countries, including America (Gonzalez-Escalona et al. 2006), India (Jayaprakash et al. 2006), and several European countries (Balebona et al. 1998).

Vibrio alginolyticus is always considered as an opportunistic pathogen affecting stressed fish (Austin et al. 1993). There have been extensive investigations on the morphological, biochemical, and serological characterizations of *V. alginolyticus*, its epidemiological status, and the roles of the bacterium and its extracellular products (ECPs) in the pathogenesis of vibriosis in some marine fishes (Shin et al. 1997; Balebona et al. 1998; Zanetti et al. 2000). Unfortunately, to date no key virulence mechanism has been detailed yet in *V. alginolyticus* (Xie et al. 2005).

Like the numerous bacteria living in marine environment and different host niches, adaptation to the osmotic changes, oxidative stress, temperature shifts, and famine is always the first tactics picked up by *V. alginolyticus* to survive and cause diseases in hosts following invasion and colonization. RpoS, an alternative sigma factor, also known as ^{38} or ^{s}, was originally identified in *Escherichia coli* (Lange and Hengge-Aronis 1991) and appeared to be present in many bacteria belonging to the gamma branch of the proteobacteria. RpoS is induced to express abundantly during the stationary phase of growth, which in turn governs the transcription of multiple genes to response to the stationary phase and diverse stresses. The role of RpoS in most bacteria in adapting to different environments appears to be similar to that in *E. coli* (Hengge-Aronis 2002). However, in pathogenic bacterial species, the role of RpoS in virulence is often varied. For example, in animal models, *rpoS* mutants of *Salmonella enterica* serovar Typhimurium showed significant attenuation in virulence (Fang et al. 1992), whereas an *rpoS* mutant of *Yersinia enterocolitica* appeared the same virulence as the wild type (Badger and Miller 1995). In *V. cholerae*, RpoS seemes to be required for efficient intestinal colonization (Merrell et al. 2000).

Quorum sensing (QS) is another important cell number response regulatory component, which enables bacteria to modulate their behaviors during growth in response to population density by sensing signaling molecules (autoinducers, AIs). A typical gram-negative bacteria QS system secrets two types of autoinducers: AI-1 (acylated homoserine lactones, AHL) and AI-2 (4,5-dihydroxy-2,3-pentanedione-derived molecules) (De Keersmaecker et al. 2006). QS system has been implicated in the regulation of biofilm formation, motility, ECPs, and virulence (Miller and Bassler 2001). Since both RpoS and QS are cell density related, it hints at a possible relationship between the two important regulatory systems. The view is actually supported by the facts that the two systems cross-regulate their gene expression in *Pseudomonas aeruginosa* (Whiteley et al. 2000; Schuster et al. 2004) and *P. putida* (Bertani and Venturi 2004), and RpoS positively regulates AHL level in *Ralstonia solanacearum* (Flavier et al. 1998).

Aimed at understanding the virulence mechanism of *V. alginolyticus*, our previous studies have confirmed the regulation of putative virulence factors by QS system (Wang et al. 2007; Rui et al. 2008; Ye et al. 2008). In this study, the *rpoS* gene was cloned from *V. alginolyticus*, and an in-frame deletion mutant was constructed. Furthermore, the role of RpoS in stress resistance, AI-2 level, exoenzyme activities, cytotoxicity of ECPs, and virulence was investigated to research in detail the virulence mechanism of *V. alginolyticus*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The strains and plasmids utilized in this study are listed in Table 1. *V. alginolyticus* MVP01, a pathogen isolated from vibriosis outbreak of caged-cultured *Pseudosciaena crocea* (Richardson) in the South China Sea in 1999, and its mutants were grown at 30°C in Luria-Bertani (LB) broth containing 3% NaCl (LBS) or tryptic soybean agar plus 3% NaCl (TSAS). For bioluminescence assay, *V. alginolyticus* was cultured in AI bioassay (AB) medium (Surette et al. 1999). *E. coli* strains were grown in LB broth (Oxoid, England) or on LB agar (1.5%) at 37°C. *V. harveyi* reporter strain BB170 (sensor 1^- sensor 2^+) was kindly provided by B. Bassler (Princeton University, USA) and was grown in AB medium at 30°C. Antibiotics, when necessary, were used at the following concentrations: for *V. alginolyticus*, ampicillin (Ap) at 100 □g/ml and chloramphenicol (Cm) at 7 □g/ml; for *E. coli*, Ap at 100 □g/ml and Cm at 20 □g/ml.

DNA manipulation

General recombinant DNA techniques were performed according to standard protocol (Frederick 1995). Restriction enzyme digestion, ligation, and plasmid purification were done in accordance with the manuals of the manufacturers (Takara, Dalian, China). DNA sequencing and primer synthesis were carried out by Invitrogen (Shanghai, China).

Gene cloning and construction of □*rpoS* and *rpoS*$^+$

All primers used in mutant construction are given in Table 2. A 3.0 kb PCR fragment of the *rpoS*-encoding region was generated from *V. alginolyticus* MVP01 chromosome with primers rpos-F and rpos-R.

The *rpoS* in-frame deletion mutant was constructed by allelic replacement (Fig. 1). A DNA fragment containing 90 bp of the 5′ end of *rpoS* and 321 bp upstream of the ATG initiation codon was amplified from chromosomal DNA by PCR using primers rpoSdeup-F and rpoSdeup-R. A DNA fragment containing 60 bp of the 3’ end of *rpoS* and 291 bp downstream of the stop codon was amplified using primers rpoSdedown-F and rpoSdedown-R. Both fragments were purified and fused in a subsequent PCR reaction using primers rpoSdeup-F and rpoSdedown-R. The fused segment was sequenced and ligated into suicide vector pDM4 (Wang et al. 2002). The resulting plasmid, pDM–rpoS, was mated from *E. coli* SM10 *pir* (Liang et al. 2003) into *V. alginolyticus* MVP01, and the transconjugants with the plasmid integrated into the chromosome by

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Reference or source
Vibrio alginolyticus		
MVP01	Pathogenic isolate from the aquiculture farm of South China. Ap^r	Lab collection
Δ*rpoS*	MVP01, in-frame deletion of *rpoS*	This study
rpoS$^+$	MVP01, Δ*rpoS* complemented *in trans* with intact *rpoS* gene	This study
Escherichia coli		
cc118 pir	*pir lysogen of* CC118 (Δ*(ara-leu) araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	Dennis and Zylstra (1998)
SM10 pir	*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu* Km^r *(pirR6 K)*	Liang et al. (2003)
Top10F'	F'[lacIq,Tn10(TetR)] *mcr* Δ180 *lacZ* Δm15 Δ*lac* X74 *deoR recA*	Invitrogen
Vibrio harveyi		
BB170	*luxN*::Tn5 sensor 1^-, sensor 2^+	Surette and Bassler (1998)
Plasmids		
pDM4	Cm^r; *SacBR*, suicide vector that contains an R6 K origin of replication (*pir* requiring)	Wang et al. (2002)
pMMB206	*cat*(Cm^r) IncQ *lacI*^{q} Δ*bla* $P_{tac\text{-}lac}$ *lacZa*	Morales et al. (1991)
pNQ705-1	Cm^r; suicide vector that contains an R6 K origin of replication (*pir* requiring)	Weber et al. (2008)
pDM-rpoS	Cm^r; pDM4 derivative containing *rpoS* bp 1-90 fused in-frame to bp 928–987	This study
pMMB-rpoS	Cm^r; pMMB206 derivative containing 2.3 kb fragment of *rpoS* putative promoter and ORF	This study

Table 2 Primers used for cloning and qRT-PCR

Primer	Sequence (5′–3′)[a]
rpoS-F	AATCGTTGCAATGGGAAGCAAAGCG
rpoS-R	CTTCATGGTAGAGATGACAGAAACA
rpoSdeup-F	**CTCGAG**CACAGTCGTTTATTCAGGCA
rpoSdeup-R	GTTTGATTAGGACTTTGATCTTTCGAGTTCGTTAT
rpoSdedown-F	GAACTCGAAAGATCAAAGTCCTAATCAAACAAGGT
rpoSdedown-R	**AGATCT**CGCGTAACAAGCTATCTCAG
rpoScomF	**GAATTC**AGAGATGGCGGCAAAATGGTCATTC
rpoScomR	**CTGCAG**GCGCGTAACAAGCTATCTCAGCTAG
16 s-F	AAAGCACTTTCAGTCGTGAGGAA
16 s-R	TGCGCTTTACGCCCAGTAAT
luxSF	GGTTGCCGCACAGGTTTTTA
luxSR	CCGTGCCGCATTGGTATT
pfsF	GAAGTTCGTCATCACGATGCA
pfsR	TTCTAGGAGCTCAGGCGGTATT

[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 Cm and Ap. To create the Δ*rpoS* mutant strain, a double-crossover recombination event was counter-selected on LBS agar containing 10% sucrose. The mutant without the targeted fragment in the chromosome of *V. alginolyticus* MVP01 was confirmed by PCR using primers rpoS-F and rpoS-R.

To construct complemented strain *rpoS*$^+$, an intact *rpoS* gene containing the putative promoter region was amplified with primers rpoScomF and rpoScomR, which were introduced into the *EcoR*I and *Pst*I sites of low-copy plasmid pMMB206 (Morales et al. 1991) to create pMMB-rpoS. The plasmid was introduced into the Δ*rpoS* mutant by conjugation. Cm and Ap-resistant transconjugants were selected, and the bearing of the plasmid was confirmed by PCR analysis and sequencing.

Survival assay

Overnight cultures were diluted to about 10^9 to 10^{10} cells/ml. Oxidative challenge (10 mM H_2O_2), ethanol challenge (18% ethanol), and osmotic challenge (2.5 M NaCl) were carried out in LBS medium at 30°C. Heat challenge was performed in pre-warmed (42°C) LBS medium. At the

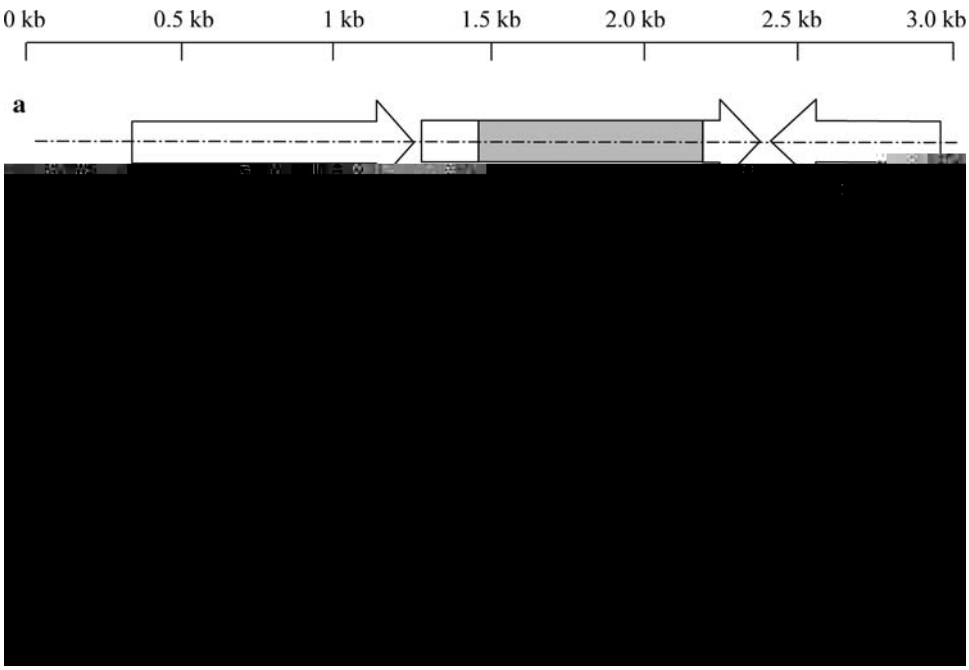

Fig. 1 **a** Genetic organization of *rpoS* and its flanking genes on the *V. alginolyticus* MVP01 chromosome. The ORF of each gene, as well as its transcriptional orientation, is indicated by a *boxed arrow*. The *gray box* represents the deleted region of *V. alginolyticus rpoS* covering bp 91-927. **b** Mapping of the targets of primers utilized in the construction of □*rpoS* allele. Primers rpoSdeup-F/R and rpoSdedown-F/R were used to amplify the DNA segments covering sequence upstream of *rpoS* and the N-terminal 90 bp as well as downstream of *rpoS* and the C-terminal 60 bp, respectively. The tails in the arrows of rpoSdeup-R and rposdedown-F indicate the overlapped sequence. The PCR products, indicated as *open box* and *boxed arrow*, are fused in a subsequent PCR using primers rpoSdeup-F and rpoSdedown-R resulting in □*rpoS* allele. **c** Diagram of the inserts used to construct the plasmid used for complementation analysis. The *boxed arrow* indicates the region amplified with the primer pair rpoScomF/R from *V. alginolyticus* chromosome. The segment is predicted to carry both the logarithmic- and stationary-phase-driven promoters of *rpoS*, and their transcript start sites are indicated by the *dashed* (P1 and P2) and *straight* (P3) *arrows*, respectively

indicated time points, an aliquot was taken to determine the survival rate by plate counts after appropriate dilution. Every experiment was repeated at least twice, and the values are the means of triplicate samples from a typical experiment. Error bars represent standard errors of the means.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Cultures of *V. alginolyticus* were grown overnight in LBS at 30°C, diluted 100-fold into fresh LBS. Cells were collected at appropriate phase and RNA was isolated with an RNA isolating kit (Tiangen, Beijing, China). The RNA was subjected to Dnase I (Promega, Madison, WI, USA) treatment to exclude the genomic DNA contaminant. Equal amount of RNA (1 □g) was used to generate cDNA (Toyobo, Tsuruga, Japan) with relevant specific primer. qRT-PCR was carried out by performing three independent experiments, each in triplicate, with a FTC-200 detector (Funglyn Biotech, Shanghai China), and transcript levels were normalized to 16*S* rRNA in each sample by the □□C_T method. The primers for qRT-PCR listed in Table 2 were designed using Primer Express software (Applied Biosystems, Foster city, CA, USA) with predicted product in the 100–200 bp size range.

AI-2 assay

AI-2 activity in the cell-free *V. alginolyticus* MVP01 culture supernatants was measured by *V. harveyi* BB170 bioluminescence reporter assay. Briefly, cell-free (CF) culture supernatants were prepared by filtration of culture broths with 0.22 □m filtration membranes. The reporter strain *V. harveyi* BB170, grown for 16 h with shaking at 30°C in AB medium, was diluted 1:10,000 in fresh AB medium, and then 90 □l of the culture was mixed with 10 □l of CF sample and incubated at 30°C. Luminescence was determined hourly using a FB12 Single Tube Luminometer (Berthold, Bad Wildbad, Germany).

Exoenzyme expression

Cultures were grown overnight in LBS, and then 5 □l of cultures was taken and spotted on the agar plates containing appropriate substrates to examine the presence of various exoenzymes. The activities of enzymes were determined

following inoculation of cultures onto LBS agar with the following substrates: 1% gelatin for gelatinase, 1% Tween 80 for lipase, 1% skim milk for caseinase, 1% egg yolk for phospholipase, 1% bovine serum albumin (Amresco, Solon, OH, USA) for albuminase, and 0.4% starch for amylase. The clearing zones were measured after 16 h of incubation at 30°C. In addition, collagenase activity was detected by spectrophotometric assay. Briefly, the bacterial cultures were harvested at the late logarithmic phase, and centrifuged at 5,000*g* for 10 min. The supernatants were obtained and filtered through 0.22 □m filters. 0.5 ml of the filtered solutions was incubated with the substrate hide powder azure (HPA; Sigma–Aldrich, St Louis, MO, USA) in 1.5 ml phosphate buffer solution (PBS) (pH 7.2) at 37°C for 2 h. After stopping the reaction by trichloracetic acid (TCA), the absorbance of the supernatants was assayed at 600 nm. All experiments were performed in duplicate and repeated at least once.

Cytotoxicity test

The toxicities of ECPs were tested by examining the amount of mitochondrial dehydrogenase released from *epothelioma papullosum cyprini* (EPC) cell line. The strains were grown on TASA agar for 24 h, and the ECPs were isolated as detailed previously (Shin et al. 1997). The cells were grown as monolayers in 24-well culture plates (Corning, Lowell, MA, USA) at 25°C by using Eagle's minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Sijiqing, Hangzhou, China). The medium was inoculated with 0.1 ml serial dilutions of ECPs samples. Cells inoculated with PBS (pH 7.2) were used as negative controls. Microtiter plates were incubated at 25°C, and the cell monolayers exposed to ECPs were observed at 1, 6, 12, and 24 h, respectively. The cytotoxicity was determined by the MTT cell viability/cytotoxicity assay kit (Beyotime, Jiangsu, China) at 7 h, with cytotoxicity calculations based on the manufacturer's instructions.

Nucleotide sequence accession number

The NCBI accession numbers for the sequence described in this study were assigned as EF382663 (*rpoS*) and EU016383 (*pfs*).

Results

Characterization and in-frame deletion of *V. alginolyticus* MVP01 *rpoS*

Based on the most conserved region of reported *Vibrio* sp. and *E. coli rpoS* gene and the flanking sequences, two primers were designed to amplify a 3.0 kb segment from *V. alginolyticus* chromosome. According to sequence analysis, two complete and one flanking partial open reading frames (ORFs) were found. One of the complete ORFs, named as *rpoS*, extends for 987 bp and codes for a protein of 328 amino acids. The predicted translation product was found to share 93% identity with *V. parahaemolyticus*, 88% with *V. vulnificus*, 83% with *V. cholerae*, as well as 75% with *V. fischeri* and *E. coli*. By searching against GenBank database, we found that the *rpoS* genes in most submitted bacterial genomes were often flanked by *nlpD* and *mutS* genes, which coded for a novel lipoprotein (NlpD) and a DNA mismatch repair protein (MutS), respectively. The same genetic arrangement was observed in the cloned gene locus of *V. alginolyticus* (Fig. 1a). A complete ORF located immediately upstream of *rpoS* gene with the same transcription direction exhibits high similarity to NlpD. The stop codon of *nlpD* is located 80 bp upstream of the predicted *rpoS* start codon. The partial ORF, downstream of the *rpoS* gene with opposite transcription orientation, is predicted to code for the carboxyl terminal region of MutS. There are 82 bases gapping the stop codons of *rpoS* and *mutS*.

In order to identify the role of RpoS in *V. alginolyticus*, an in-frame deletion mutant □*rpoS* was constructed by allelic exchange as described above (Fig. 1b). PCR and sequence determination were performed to verify that an 837 bp segment was lost in the *rpoS* ORF in □*rpoS* strain (data not shown). By analogy with the *E. coli* and *V. cholerae rpoS* genes and promoter structure (Lange et al. 1995; Yildiz and Schoolnik 1998), a 2.3 kb segment containing complete *rpoS* ORF region as well as the putative exponential-phase and stationary-phase driven promoter regions of *rpoS* was cloned and was complemented into □*rpoS* in *trans* (Table 1 and Fig. 1c).

RpoS is required for stress survival

In order to evaluate the function of RpoS in stress adaptation, the survival rates of wild-type MVP01, the in-frame deletion mutant □*rpoS*, and the complementation strain *rpoS*$^+$ were determined by plate counts after exposure to diverse stress conditions. When treated with high concentration ethanol (18%), □*rpoS* cells showed significantly less survival rate than that of wild-type cells (Fig. 2a). For osmotic challenge (2.5 M NaCl in LB), during the whole period of 120 min, though the viable cells of both the wild-type strain and the mutant strain decreased rapidly, the wild-type strain still showed more resistance than □*rpoS* did. The survival rates of wild type were 100-fold higher than that of □*rpoS* (Fig. 2b). High temperature (42°C) was another factor to which RpoS seemed to be involved in the resistance. When incubated at 42°C, cells of parental strain

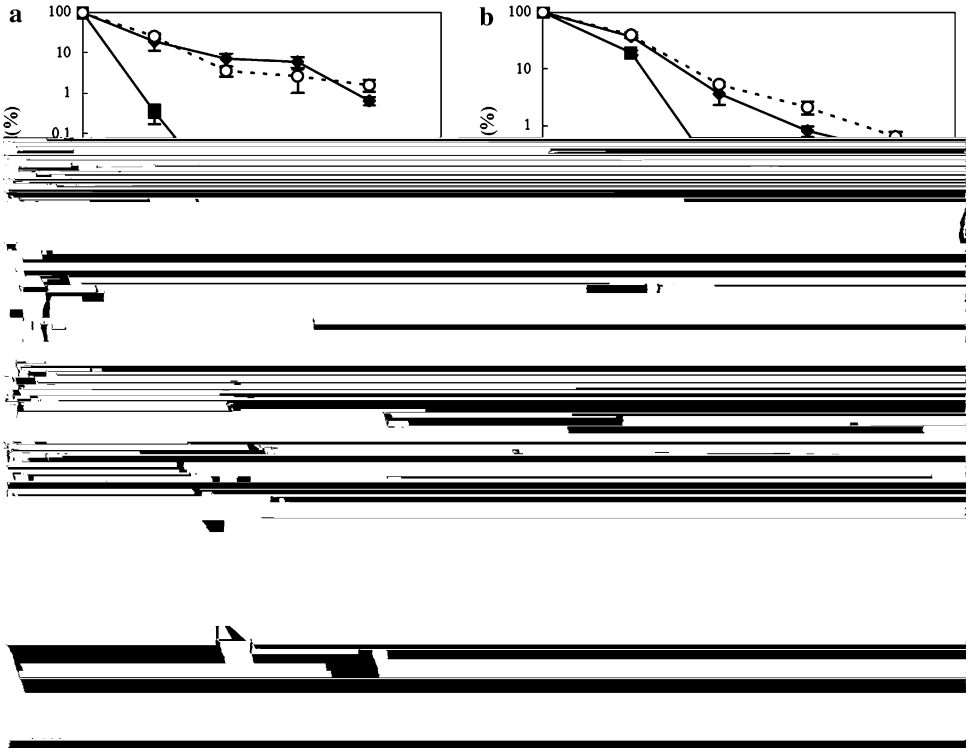

Fig. 2 Stress survival assay of stationary-phase *V. alginolyticus* cells. Bacteria of the wild-type strain MVP01 (*closed diamond*), in-frame deletion mutant □*rpoS* (*closed square*), and complemented strain *rpoS*$^+$ (*open circle*) were exposed to 18% ethanol (**a**), 2.5 M NaCl (**b**), heat (42°C) (**c**), and 10 mM H_2O_2 (**d**). The viable plate count was carried out at indicated time to determine the survival rate. 100% survival corresponds to the viable cell count determined just prior to exposure to the indicated stress

kept high survival level, while □*rpoS* cells decreased rapidly (Fig. 2c). In our experiment, it seems that the wild-type cells cannot endure high concentration H_2O_2. When challenged with 10 mM H_2O_2 for 20 min, only less than 1% parental cells survived, and at the end of the 40-min test period, survival rate decreased to 0.02%. □*rpoS* showed less survival rate than that of the wild type (Fig. 2d). The complemented strain *rpoS*$^+$ showed similar adaptation behaviors to the wild type for all the stress stimulations. Growth curve assay indicated that the low survival rate of □*rpoS* was not due to the growth deficiency (data not shown). These results indicated that RpoS indeed contributed to the stress adaptation of *V. alginolyticus* MVP01.

RpoS regulates extracellular AI-2 activity

Recently, McDougald et al. confirmed that in *Vibrio* species, the AI-2 signaling system was capable of regulating environmental adaptation (McDougald et al. 2003). Therefore, we concerned whether or not RpoS would regulate LuxS QS. The AI-2 assay with reporter stain *V. harveyi* BB170 was performed in both wild-type MVP01 and □*rpoS*. During the whole detection period, □*rpoS* synthesized 60–90% less AI-2 than the wild-type MVP01, though the AI-2 level profiles of both the wild type and □*rpoS* showed a similar trend. Moreover, the introduction of plasmid-borne *rpoS* increased the AI-2 activity to a level comparable to that of the wild type (Fig. 3a).

In order to determine whether the decreased AI-2 level induced by the *rpoS* mutation was caused by the influence of AI-2 biosynthesis, the differential expression of *luxS* and *pfs*, encoding AI-2 synthase LuxS and nucleosidase Pfs, respectively, was investigated by qRT-PCR in different growth phases in □*rpoS*. Consistently with the phenotype, expression of *luxS* and *pfs* obviously decreased in □*rpoS* compared with that in MVP01 (about two- to fivefolds) during the whole growth course (Fig. 3b). Both AI-2 production and qRT-PCR results suggested that expression of *luxS* and *pfs* was positively regulated by RpoS in *V. alginolyticus*.

RpoS affects exoenzyme activity

Vibrio alginolyticus cells produce several enzymes to allow themselves to survive, proliferate, and invade host tissue, such as collagenase, caseinase, gelatinase, phospholipase, amylase and albuminase (Balebona et al. 1998; Gomez-Leon et al. 2005). To investigate the role of RpoS in the production of exoenzymes, the ability of MVP01, □*rpoS*, and *rpoS*$^+$ to hydrolyze macromolecule was detected with the addition of substrates. As shown in Table 3, the wild-type MVP01 had the degrading ability for milk, BSA, gelatin, HPA and egg yolk, but not for starch and Tween 80. The *rpoS* mutants completely abolished the ability to produce caseinase and collagenase, and decrease the production of albuminase and gelatinase. For phospholipolytic activity, the wild-type and mutant strains displayed no

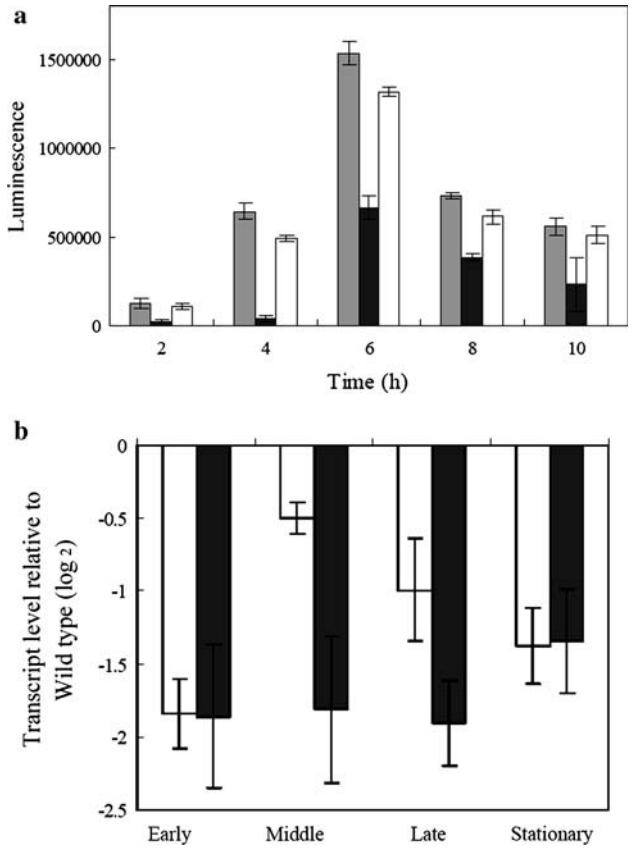

Fig. 3 Determination of RpoS effect on LuxS QS. **a** AI-2 activity assay of *V. alginolyticus* wild type MVP01 (*gray columns*), in-frame deletion mutant □*rpoS* (*black columns*), and complemented strain *rpoS*$^+$ (*white columns*) with *V. harveyi* reporter strain BB170. Overnight cultures were diluted by 100-fold in fresh LBS medium, and cell-free cultures were prepared from the culture broths of the three strains at indicated time. AI-2 activities shown are representative of three independent experiments. **b** Effect of *V. alginolyticus* RpoS on the transcription of *luxS* and *pfs*. Overnight cultures of MVP01, □*rpoS*, and *rpoS*$^+$ were diluted 100-fold in fresh LBS medium, and cells were collected in early-, middle-, late-logarithm, and stationary growth phase. Transcription levels of *luxS* and *pfs* in □*rpoS* (white columns for *luxS* and black columns for *pfs*) were detected by qRT-PCR and presented as the $\log_2$ relative to the levels in the wild type strain. The 16S rRNA was used as reference. *Error bars* indicate the standard deviation for three triplicate samples

obvious difference. In all the protease assays, the complemented stain *rpoS*$^+$ exhibited the same behavior as MVP01 did. These results indicated that RpoS was required for collagenase, caseinase, albuminase, and gelatinase activity, but not for phospholipolytic activity.

RpoS influences cytotoxicity of ECPs

The ECPs of *V. alginolyticus* wild type, the *rpoS* mutant, and the *rpoS*$^+$ strain were serially diluted by 10–50 folds to treat cell monolayers, and then the cytotoxicity was observed at different time points and was examined with

Table 3 Detection of exoenzyme activities in *V. alginolyticus* strain MVP01, □*rpoS* mutant and complemented strain *rpoS*$^+$

Exoenzyme	Width of clearing zero (cm)		
	WT	□*rpoS*	*rpoS*$^+$
Albuminase	0.33 ± 0.07	–	0.33 ± 0.03
Caseinase	0.28 ± 0.07	0.08 ± 0.02	0.43 ± 0.12
Gelatinase	0.38 ± 0.07	0.18 ± 0.02	0.42 ± 0.03
Phospholipase	0.20 ± 0.05	0.17 ± 0.03	0.28 ± 0.03
Amylase	–	–	–
Lipase	–	–	–
Protease activity/Cell density (OD_{600}/OD_{600})			
Collagenase	0.20 ± 0.03	–	0.14 ± 0.01

– not detectable

MTT method after 7 h. During the whole observation period, low concentration ECPs (ECPs diluted by 16- to 50-fold) did not lead to any morphological change in the cells until at the 6th hour of incubation, while the high concentration ECPs (ECPs diluted by 12.5- to 10-fold) showed cytotoxic activities at the first 2 h, when the cells began to shrink and round. For a long time treatment (over 12 h), cells began to detach and finally monolayer was destructed regardless of the ECPs concentration of the wild type. However, the same phenomenon was only observed on cells treated with high concentration ECPs of □*rpoS*. When diluted by 12.5- and 10-fold, the ECPs of *rpoS* mutant displayed about 70% lower cytotoxic activity than that of the wild type (Fig. 4). The ECPs of *rpoS*$^+$ strain showed comparable cytotoxicity to that of the wild-type MVP01. The observation of the decreased cytotoxicity of □*rpoS* ECPs suggested that RpoS was involved in the pathogenicity of *V. alginolyticus* due to the production of virulent component contained in ECP.

Discussion

Since the environmental adaptation plays an important role in pathogens to survive in natural environment or invade hosts, it seems that identification of key genes related to stress resistance would contribute to understand the virulence mechanism of pathogens. In the present study, the *rpoS* gene of *V. alginolyticus* MVP01 was cloned and characterized. The *rpoS* mutation led to the decreased survival rates responding to a variety of stresses, including ethanol, heat, H_2O_2, and hyperosmolarity (Fig. 2), indicating that RpoS is a key regulator in *V. alginolyticus* responsible for its adaptation to stressful conditions. Similarly, RpoS plays an important role in resisting oxidative stress and osmotic shock in *V. cholerae* and *V. vulnificus* (Yildiz and Schoolnik 1998; Hulsmann et al. 2003). In contrast, RpoS displays

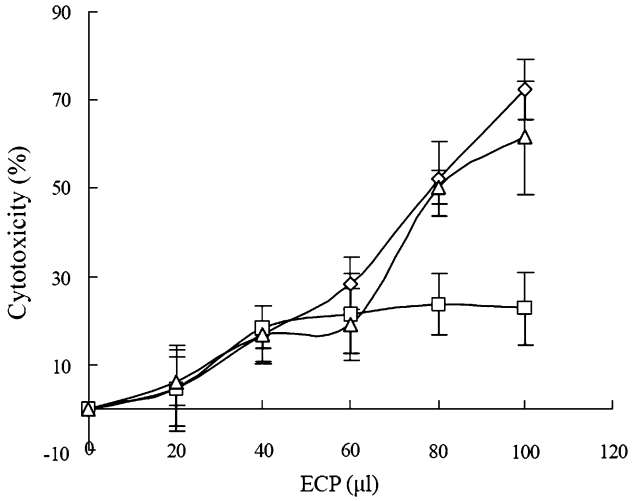

Fig. 4 Cytotoxicity effect of ECPs of *V. alginolyticus* wild type MVP01 (*diamond*), □*rpoS* (*triangle*), and *rpoS*$^{+}$ (*square*) at different dilution on the EPC cell line with MTT assay. The *X*-axis represented the various volume of ECPs added to PBS to be serially diluted. Error bars indicate the standard deviation for three triplicate samples

no benefit to oxidative and osmotic challenges in *V. harveyi* (Lin et al. 2002). Hence we postulated that in different *Vibrio* sp. context, the stress survival mechanism might be variable in different stressful conditions.

The most striking result shown here is the decreased extracellular AI-2 level in *rpoS*-deficient strain (Fig. 3a). The synthesis of AI-2 derived from *S*-adenosylmethionine (SAM) involves three enzymes: methyltransferases, Pfs and LuxS (Schauder et al. 2001). In previous studies, we found that inactive *luxS* (Ye et al. 2008) and *pfs* (Tian et al. 2008) abolished the production of AI-2. Hence, we determined the transcription of both *luxS* and *pfs* in the *rpoS* mutant and the wild type strain by qRT-PCR (Fig. 3b). The down-regulated transcription (two to fivefolds) of *luxS* and *pfs* in □*rpoS* suggests that RpoS positively affects LuxS QS by regulating AI-2 biosynthesis system. Recent observation demonstrated that *pfs* transcription induction requires RpoS in *E. coli* (Kim et al. 2006). In addition, genetic analysis indicated that *V. alginolyticus luxS* possesses an RpoS consensus promoter sequence according to a motif analysis (data not shown). Hence, we postulated that RpoS could directly transcribe *pfs* and *luxS* in *V. alginolyticus*, though it should be validated in the further study. As an important regulation system, LuxS QS was paid with more attention on how to affect other genes, other than how to be affected. Now we know that *luxS* expression can be repressed by cyclic AMP (cAMP)–CRP complex in *E. coli* (Wang et al. 2005), as well as by GppX in *Porphyromonas gingivalis* (James et al. 2006). Our result indicated a novel control pathway for *luxS* expression. To our knowledge, it is also the first report of the regulatory function of RpoS on LuxS QS in *Vibrio* sp. In other respects, the QS signals are fed to LuxO/LuxR in order to regulate the expression of virulence factors. In *V. alginolyticus*, RpoS seems to be involved in QS and virulence by modulating the regulators in QS signaling pathway as in *V. cholerae* and *V. anguillarum* (Nielsen et al. 2006; Weber et al. 2008). Our future work will disclose the putative regulatory roles of RpoS in other components of QS systems in *V. alginolyticus*.

In order to survive the environmental stress and undermine the host, pathogens adjust their protease production according to their growth phases. For example, *V. cholerae* protease production is activated by nutrient limitation (Benitez et al. 2001). Similarly, the protease was abundantly expressed at the onset of stationary phase in *V. alginolyticus* MVP01 (Rui et al. 2008). In this study, our results demonstrated that the stress adaptive regulator RpoS significantly regulated the expression of albuminase, collagenase, gelatinase and caseinase activities (Table 3). It was reported previously that alkaline serine protease (Shin et al. 1997), collagenase (Nunez et al. 2006) as well as metalloproteases (Nottage and Birkbeck 1987) contributed to the lethal factor of *V. alginolyticus*. Unfortunately, we cannot refer to the exact protein regulated by RpoS, which could induce the change of hydrolytic activity in □*rpoS*, though Rui et al. confirmed that at least one serine protease was produced in *V. alginolyticus* MVP01 (Rui et al. 2008). On the other hand, the effect of RpoS on the production of exoenzyme activities suggests that the sigma factor RpoS could be important for the pathogenesis of *V. alginolyticus*. Indeed, the virulence of □*rpoS* ECPs was decreased for EPC cells (Fig. 4). It is reasonable that RpoS influences the production of exoenzyme, which in turn affects the cytotoxicity of ECPs in *V. alginolyticus*. Furthermore, the i.p. infection results also showed that the *rpoS* mutants showed attenuated virulence, and the half lethal dose value of □*rpoS* was increased about 20-fold (data not shown) compared to the wild type, indicating that RpoS is indeed involved in the virulence of *V. alginolyticus* MVP01. Similarly, *V. cholerae* RpoS is also involved in virulence due to its positive effect on intestinal colonization in the initial invasion stages and on the hemagglutinin/protease production in *V. cholerae* cells (Yildiz and Schoolnik 1998; Merrell et al. 2000).

Taken together, our results reported here indicate that in addition to contribute to thrive in different stressful conditions, RpoS plays an important role in the regulation of extracellular AI-2 level, protease activity, and cytotoxicity of ECPs, implying that RpoS regulates the expression of potential virulence factors that are responsible for the pathogenicity of *V. alginolyticus* MVP01. These results strongly support the idea that the global regulator RpoS is part of regulatory networks of virulence and LuxS QS system in *V. alginolyticus*.

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