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Regulation system of serine protease production in *Vibrio vulnificus* strain NCIMB 2137, a metalloprotease-gene negative strain isolated from a diseased eel

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ABSTRACT

Vibrio vulnificus is a ubiquitous estuarine microorganism but causes fatal systemic infections in cultured eels or shrimps, as well as in immunocompromised humans. An extracellular metalloprotease has been reported to be a potential virulence factor of the bacterium; however, a few strains isolated from a diseased eel or shrimp were recently found to produce a serine protease termed VvsA. In the present study, we first clarified the regulatory characteristics of the VvsA production in *V. vulnificus* strain NCIMB 2137, a metalloprotease-gene negative strain isolated from a diseased eel. *V. vulnificus* coordinates expression of virulence genes in response to the bacterial cell density, which is termed quorum sensing (QS) and is mediated by the small diffusible molecule called autoinducer 2 (AI-2). When cultivated at 26 °C, the *vvsA* expression was closely related with expression of the *luxS* gene encoding the synthase of the AI-2 precursor LuxS. Both VvsA and AI-2 were sufficiently secreted at early stationary phase of the bacterial growth. In contrast, when cultivated at 37 °C, far less amounts of the AI-2 and VvsA were produced even at the stationary phase. Disruption of the *luxS* gene was found to decrease significantly the *vvsA* expression and VvsA production. Disruption of *luxO* encoding the central response regulator of the QS circuit caused an increase in the *vvsA* expression and VvsA production at the logarithmic growth phase. These findings indicate that VvsA production is positively regulated by the *V. vulnificus* LuxS-dependent QS system, which operated more effectively at 26 °C than at 37 °C.

1. Introduction

Vibrio vulnificus is a pathogenic bacterial species that occurs in warm aquatic environments with intermediate levels of salinity. This species is commonly isolated in temperate, subtropical, and tropical areas, and it is able to infect fish including eel, shellfish and humans (Valiente et al., 2008a).

V. vulnificus is heterogeneous and has been divided into three biotypes (BTs), two human pathogenic biotypes (BT1 and BT3) and one fish–shellfish pathogenic biotype (BT2) (Amaro and Biosca, 1996; Marco-Noales et al., 2001; Tison et al., 1982). BT2 is also subdivided into several serovars (Biosca et al., 1997; Fouz et al., 2007); however, serovar E is epidemiologically the most relevant because it causes outbreaks of vibriosis with high mortality in eel farms (Biosca et al., 1997; Fouz et al., 2007; Tison et al., 1982) and sporadic cases of human septicemia (Valiente et al., 2008b).

Vibriosis by BT2 affects captured eels (*Anguilla anguilla* L.) maintained in farms, occasionally resulting in economic losses to fish farmers. The bacterium enters the blood through the intact gill epithelium and spread to the internal organs, causing death (Marco-Noales et al., 2001). Diseased eels present external and internal hemorrhages and, sometimes, superficial ulcers close to the head or anus. These symptoms are similar to those displayed by humans affected by the bacterium, thus, eel diseases could be considered a model to study human diseases (Valiente et al., 2008c).

V. vulnificus excretes a number of toxic factors to the extracellular milieu including a heat-labile metalloprotease named *V. vulnificus* protease (Milton, 2006; Miyoshi et al., 2012). It was reported that, based on the metalloprotease gene, *V. vulnificus* might be classified into the genotype A or B. Namely, by the PCR (polymerase chain reaction) method, most strains isolated from human cases have the type A gene, whereas most strains from diseased eels or shrimps carry type B gene. However, a few strains from diseased eel or shrimp could not be categorized because no PCR product was obtained, which suggested the absence of the metalloprotease gene (Miyoshi et al., 2012; Wang et al., 2008). Interestingly, theses strains were found to produce a serine protease termed VvsA (Miyoshi et al., 2012; Wang et al., 2008). The serine protease VvsA is an ortholog of an extracellular protease produced by *Vibrio parahaemolyticus*, a causative agent of human wound infection as well as gastroenteritis (Chakraborty et al., 1997; Janda et al., 1988). *V. parahaemolyticus* protease has been reported to possess various toxic activities including the collagenolytic, cytotoxic, hemolytic and edema-forming activity (Ishihara et al., 2002; Lee et al., 2002).

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Therefore, VvsA serine protease may be also a virulent factor in eel vibriosis, which is characterized by external and internal hemorrhages affecting the major organs (Miyoshi et al., 2012; Wang et al., 2008).

V. vulnificus coordinate the expression of virulence genes in response to the bacterial cell density, and this regulation is termed as quorum sensing (QS). As well as other *Vibrio* species, the small diffusible molecule called autoinducer-2 (AI-2) may mediate the QS system of *V. vulnificus* (Federle and Bassler, 2003; Henke and Bassler, 2004; Milton, 2006). In addition to the bacterial cell density, the cultivation temperature also affects significantly the expression of virulence genes (Lee et al., 2007; Watanabe et al., 2004).

In the present study, in order to clarify whether VvsA production is regulated in response to the bacterial cell density and cultivation temperature, the *vvsA* expression and VvsA activity were compared among a wild type strain NCIMB 2137, a metalloprotease-gene negative strain isolated from a diseased eel, and its mutants of genes consisting of the QS circuit at 26 °C (the temperature of the estuarine water at the warm season, which is the most optimum for eel growth) and 37 °C (human body temperature). Herein, it is reported that the VvsA production is regulated by the QS system which operated more strongly at 26 °C than at 37 °C.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown on Luria–Bertani (LB) agar plate or in LB broth containing 0.5% NaCl, and when required an appropriate antibiotic was added to the media as follows: chloramphenicol 10 μg/ml, streptomycin 50 μg/ml and kanamycin 50 μg/ml.

For cultivation of *V. vulnificus* strains, TYE broth (0.5% tryptone, 0.25% yeast extract, 2% NaCl, 25 mM K_2HPO_4, pH 7.5) was used. Thiosulfate–citrate–bile–salts–sucrose (TCBS) agar plate containing chloramphenicol 10 μg/ml was used to select *luxS*, *luxO* and *smcR* mutants. In all experiments, *V. vulnificus* was cultivated in TYE broth (5 ml) at 37 °C with shaking overnight (strains LXSD0713 (*luxS* disruptant), AA0507 (*luxO* disruptant) and SMRD113 (*smcR* disruptant) were cultivated in TYE broth containing chloramphenicol 10 μg/ml), and then, an aliquot of the first culture was taken and re-cultivated in fresh TYE broth at either 26 °C or 37 °C till reaching the desired growth phase.

For the AI-2 assay, autoinducer bioassay (AB) broth (1 mM L-arginine, 2% glycerol, 10 ng/ml riboflavin, 1 μg/ml thiamin, 300 mM NaCl, 10 mM K_2HPO_4, 50 mM $MgSO_4$, 0.2% casamino acids; pH 7.5) was used.

Table 1
Bacterial strains and plasmids used.

Strain or plasmid	Relevant features[a]	Reference
Vibrio vulnificus		
NCIMB 2137	Eel clinical isolate; virulent.	Amaro and Biosca (1996)
LXSD0713	NCIMB 2137 strain, *luxS*::Cm^r.	This study
LXSR0813	Revertant strain of LXSD0713.	This study
AA0507	NCIMB 2137 strain, *luxO*::Cm^r.	This study
AAER22	Revertant strain of AA0507.	This study
SMRD113	NCIMB 2137 strain, *smcR*::Cm^r.	This study
SMRR213	Revertant strain of SMRD113.	This study
Escherichia coli		
SY327λ*pir*	Δ(*lac pro*), *argE*(Am), *rif*, *nalA*, *recA56*, *rpoB*, λ*pir*, Sm^r, host for π-requiring plasmids.	Miller and Mekalanos (1988)
SM10λ*pir*	*thi-1*, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2-Tc::Mu, λ*pir*, *oriT* of RP4, Km^r; conjugational donor.	Simon et al. (1983)
Vibrio harveyi		
BB170	Reporter strain used in auto-inducer 2 assay.	Bassler et al. (1993)
BB152	Positive control strain used in auto-inducer 2 assay.	Bassler et al. (1993)
Plasmids		
pKTN701	*R6K*-ori suicide vector for gene replacement; Cm^r.	Nishibuchi et al. (1991)
pKTA0613	pKTN701 with *luxS*; Cm^r.	This study
pKTA0506	pKTN701 with *luxO*; Cm^r.	This study
pKTA1112	pKTN701 with *smcR*; Cm^r.	This study

[a] Cm^r, chloramphenicol-resistant; Sm^r, streptomycin-resistant; Km^r, kanamycin-resistant.

2.2. Measurement of bacterial growth

V. vulnificus strains were grown at 37 °C under aeration in TYE broth (5 ml) overnight with shaking. Then, an aliquot of the first culture was taken and re-cultivated in fresh TYE broth at 26 °C or 37 °C, and the growth monitored by measuring the optical density at 600 nm (OD_{600}) of the cultures every 1 h. Then, the growth curves were drawn. Thereafter, early log phase, late log phase and early stationary phase were determined.

2.3. Real time RT-PCR

Cultures of *V. vulnificus* strains were treated with RNA protect bacterial reagent (Qiagen), and total RNA was extracted from the bacterial cells cultivated at 26 °C or 37 °C at early log, late log or early stationary phase, by using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's manual.

Quantitative RT-PCR (qRT-PCR) was conducted using an iScript™ one-step RT-PCR kit with SYBR® green (Bio-Rad Laboratories). The reaction mixtures composed of 12.5 μl of SYBR® Green PCR Master Mix (Bio-Rad Laboratories), 0.75 μl of each of forward and reverse primers (the final concentration of each primer is 200 nM) (Table 2), 0.5 μl of iScript™ one-step RT-PCR reverse transcriptase, 0.5 μl of RNA (100 ng/μl), and nuclease free water was added to adjust the final volume to 25 μl. The reactions were performed in MiniOpticon™ real time PCR detection system (Bio-Rad Laboratories) as follows: an initial incubation at 50 °C for 10 min for reverse transcription. Thereafter, the reverse transcriptase was inactivated by heating at 95 °C for 5 min, and PCR amplification of target gene was performed for 40 cycles of denaturation at 95 °C for 10 s and annealing at an appropriate temperature for 30 s. DNA polymerization was conducted in a range of temperatures from 55 °C to 95 °C within 20 min to obtain the melting curve for determining the PCR amplification specificity. The RT reaction without the reverse transcriptase was used as a negative control for each gene. The house keeping gene *16s rRNA* was used as an internal control. The amount of each mRNA was detected by comparing C_t value of each sample to a standard curve. The relative amount of mRNA of tested genes was normalized and estimated using the amount of mRNA of the house keeping gene *16s rRNA* as 1.0.

2.4. Assay of the AI-2 activity

Quantitative assays of the AI-2 activity of the culture supernatants were performed. Cultures of *V. vulnificus* strains were grown in TYE medium until early log phase, late log phase and early stationary phase. Cell free culture supernatants were prepared from these cultures by centrifugation at 12,000 *g* for 5 min at 4 °C and filtration through a 0.2 μm Millipore filter.

The AI-2 activity was measured using the reporter strain *Vibrio harveyi* BB170 as described by Bassler et al. (1993). Briefly, the reporter strain was cultured overnight in LB broth containing 3.0% NaCl at 30 °C. The bacterial culture was diluted 1:5000 with AB broth. An aliquot of the diluted culture (540 μl) was mixed with 60 μl of the sample, and the mixture was cultivated at 30 °C for 4 h with shaking. Thereafter, the intensity of bioluminescence (relative light unit: RLU) was measured with