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Original Article

Change of *Vibrio vulnificus* Metalloprotease VvpE Production by Temperature and Salinity

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Vibrio vulnificus, a gram-negative halophilic marine bacterium and opportunistic pathogen, must withstand various environmental changes, especially the simultaneous change of temperature and salinity (SCTS) from 25℃/2.5% to 37℃/0.9% upon entering the human body. Previous studies have suggested that temperature and salinity may affect the production of metalloprotease VvpE via the LuxS-mediated autoinducer-2 quorum sensing system (AI-2-QSS). However, this hypothesis remains to be verified through coherent experiments. In this study, SCTS stimulated *V. vulnificus* growth with no increase in total growth levels. The SCTS-mediated prolongation of the stationary growth phase resulted in a significant increase in growth phase-dependent *luxS* and *vvpE* transcriptions; however, SCTS did not affect *luxS* or *vvpE* transcription levels during the exponential growth phase. SCTS also advanced extracellular VvpE production, which was consistent with *vvpE* transcription and *V. vulnificus* growth. SCTS-mediated modulation of *vvpE* expression was slightly attenuated but still observed in the background of a *luxS* mutation which seriously repressed *vvpE* expression. These results indicate that SCTS stimulates *luxS* and *vvpE* expression by stimulating *V. vulnificus* growth; however, the LuxS-mediated AI-2-QSS plays only a minor role, if any, in the SCTS-mediated modulation of *vvpE* expression.

Key Words: *Vibrio vulnificus*, Temperature, Salinity, Regulation of metalloprotease VvpE, Autoinducer-2, Quorum sensing

INTRODUCTION

Vibrio vulnificus is a gram-negative halophilic marine bacterium and an opportunistic pathogen that causes rapidly progressing fatal septicemia and necrotizing wound infections with a high mortality rate, preferentially in susceptible patients with hepatic diseases, hemochromatosis, heavy alcohol drinking habits, and other immunocompromised conditions (1).

V. vulnificus inhabits sea water, but can also infect the human body. The two environments differ in many respects. As a result, *V. vulnificus* must overcome and survive diverse environmental changes to establish a successful human infection. Virulence expression is a survival strategy of pathogenic bacteria (2). *V. vulnificus* is capable of sensing and responding to diverse environmental changes by modulating the expression of virulence factors for survival (3, 4).

V. vulnificus produces a metalloprotease known as VvpE. Purified VvpE induces hemorrhagic damage and dermonecrosis, enhances vascular permeability and edema, and is lethal to mice (1). Although the pathogenetic role of VvpE is enigmatic (5), *vvpE* expression or VvpE production is a useful tool for studying how *V. vulnificus* senses and responds to diverse environmental signals since it is the

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best known phenotype directly controlled by the three global regulators, RpoS (the stationary-phase sigma factor responsible for general stress response), SmcR (the master regulator of the LuxS-mediated autoinducer-2 quorum sensing system [AI-2-QSS] responsible for bacterial density-dependent gene expression), and Crp (cAMP-receptor protein responsible for catabolite repression) (6). In addition, extracellular VvpE production occurs via the type II general secretion system (7).

V. vulnificus encounters simultaneous changes in a variety of environmental factors, including temperature, osmolarity, iron and oxygen levels, upon entering the human body. Most of these environmental factors are known to affect *vvpE* expression (8). In particular, temperature of 25℃ and salinity of 2.5% can mimic estuarine condition in summer seasons, as 37℃ and 0.9% does the human body condition. Previous studies suggested that *V. vulnificus* might sense and respond to temperature changes by modulating *vvpE* expression via AI-2-QSS, which functions more efficiently at 26℃ than at 37℃ (8, 9). However, these findings are somewhat controversial. AI-2-QSS modulates *vvpE* expression primarily in response to bacterial density (6, 10). Empirically, we know that the growing ability of *V. vulnificus* is higher at 37℃ than at 25℃. Furthermore, our previous study revealed that the simultaneous change of temperature and salinity (SCTS) from 25℃/2.5% to 37℃/0.9% stimulated *V. vulnificus* growth with increased hemolysin (VvhA) production (11). Accordingly, this controversy needs to be solved by defined experimentation using mutants and transcriptional reporters of suitable genes. In this study,

Table 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid and primer	Relative characteristic and sequence	Source or Reference
V. vulnificus		
M06-24/O	Highly virulent clinical isolate	12
CMM2201	M06-24/O with a *luxS* mutation	13
CMM2211	CMM2201 with an *in trans luxS* complementation	13
CMM2101	M06-24/O with a $lacZ_{Vv}$ mutation	13
RC138	CMM2101 with a merozygotic P_{luxS}::$lacZ_{Ec}$ transcriptional fusion	This study
CMM2106	CMM2101 with a P_{vvpE}::$lacZ_{Ec}$ transcriptional fusion	13
CMM2207	CMM2106 with a *luxS* mutation	13
E. coli		
SY327 λ*pir*	Host for suicide vector	14
SM10 λ*pir*	Conjugation donor	14
Plasmid		
pQF52	IncP *lacZ* transcriptional fusion vector; Amp^R	15
pDM4	Suicide vector with R6K origin; Cm^R	16
pRC130	A 1075-bp *Bam*HI-*Hin*dIII fragment containing the *luxS* promoter region cloned into pQF52	This study
pRC136	A *Bam*HI-*Sca*I fragment containing a P_{luxS}::*lacZ* fragment from pRC130 cloned into *Bgl*II-*Sma*I-cut pDM4	This study
Primers		
luxS-rep-F	5'-cg**GGATCC**gctcatcgtgtgtttgcagagc-3'	This study
luxS-rep-R	5'-ccc**AAGCTT**cggtaaaactatctaataatggc-3'	This study

Vv and *Ec* stand for *V. vulnificus* and *E. coli*, respectively.
Cm^R and Amp^R stand for chloramphenicol-resistance and ampicillin-resistance, respectively.
Capital bold letters indicate the restriction enzyme-recognition sequences: GGATCC for *Bam*HI and AAGCTT for *Hin*dIII.

therefore, we first determined the effect of SCTS on *luxS* expression using P_{luxS}::*lacZ* transcription reporter, and then compared the effect of SCTS on *vvpE* expression and extracellular VvpE production between the backgrounds of wild-type *luxS* and mutated *luxS* using P_{vvpE}::*lacZ* transcription reporters and Western blotting.

MATERIALS AND METHODS

Bacterial strains, media and reagents

Bacterial strains, plasmids and primers used in this study are listed in Table 1. Heart Infusion (HI; Becton-Dickinson (BD), Franklin Lakes, NJ, USA) broth and agar with 2.5% sodium chloride were used to cultivate *V. vulnificus* strains. Luria-Bertani medium (BD) and Thiosulfate-Citrate-Bile-Sucrose medium (BD) with or without appropriate antibiotics were used for selection and subculture of recombinant strains. Antibiotics (BD) were used at the following concentrations; for *Escherichia coli*, ampicillin 50 μg/ml, kanamycin 50 μg/ml, tetracycline 12.5 μg/ml, and chloramphenicol 30 μg/ml; for *V. vulnificus*, ampicillin 20 μg/ml, kanamycin 200 μg/ml, tetracycline 2 μg/ml, and chloramphenicol 2 μg/ml. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Culture conditions, bacterial growth measurement, and β-galactosidase assay

V. vulnificus strains were preconditioned by culturing in HI broth containing 2.5% NaCl at 25℃ overnight to adapt them to a condition mimicking their natural habitat. The preconditioned strains were inoculated into HI broth containing 0.9% NaCl or 2.5% NaCl at 1 × 10^6 cells/mL and cultured with vigorous shaking (220 rpm) at 37℃ or 25℃ for 24 h. Culture aliquots were withdrawn at appropriate times to measure bacterial growth and β-galactosidase activity. To measure extracellular VvpE production, culture supernatants were obtained from culture aliquots by centrifugation (10,000 × *g* for 5 min.). Bacterial growth was determined by measuring the optical density of culture aliquots at a wavelength of 600 nm (OD_{600}). β-Galactosidase activity in culture aliquots was measured by the Miller method (17).

Western blotting for VvpE

Rabbit polyclonal anti-VvpE antibody was prepared as described in our previous studies (7). VvpE production was measured using Western blots as described previously (18). In brief, equal volumes of culture supernatants were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were incubated with rabbit polyclonal anti-VvpE antibody and subsequently with anti-rabbit-IgG antibody conjugated with alkaline phosphatase. Finally, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium solution was used for visualization.

Construction of the *lacZ*-fused *luxS* transcription reporter

The merozygotic P_{luxS}::*lacZ* transcription reporter was constructed as follows. The 1,075-bp *Bam*HI-*Hin*dIII fragment containing the regulatory region of *luxS* was amplified using the polymerase chain reaction (PCR) primers luxS-rep-F with a *Bam*HI overhang and luxS-rep-R with a *Hin*dIII overhang. The PCR product was then subcloned into pQF52 (15). From the resulting plasmid pRC130, the *Bam*HI-*Sca*I fragment containing the P_{luxS}::*lacZ* fragment was isolated and subcloned into pDM4 (18). The resulting plasmid pRC136 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (14), and transferred to CMM2101 by conjugation. Eventually, a transconjugant (RC138) was selected on TCBS agar containing chloramphenicol. The presence of the P_{luxS}::*lacZ* fusion in RC138 was confirmed by β-galactosidase assay (17).

RESULTS

To determine the effect of SCTS on AI-2-QSS activity, we measured *luxS* transcription levels using RC138 with the P_{luxS}::*lacZ* transcriptional fusion. SCTS stimulated and advanced the growth of RC138 with no increase in the total growth level, and subsequently prolonged the duration of the stationary growth phase (Fig. 1A). The *luxS* expression

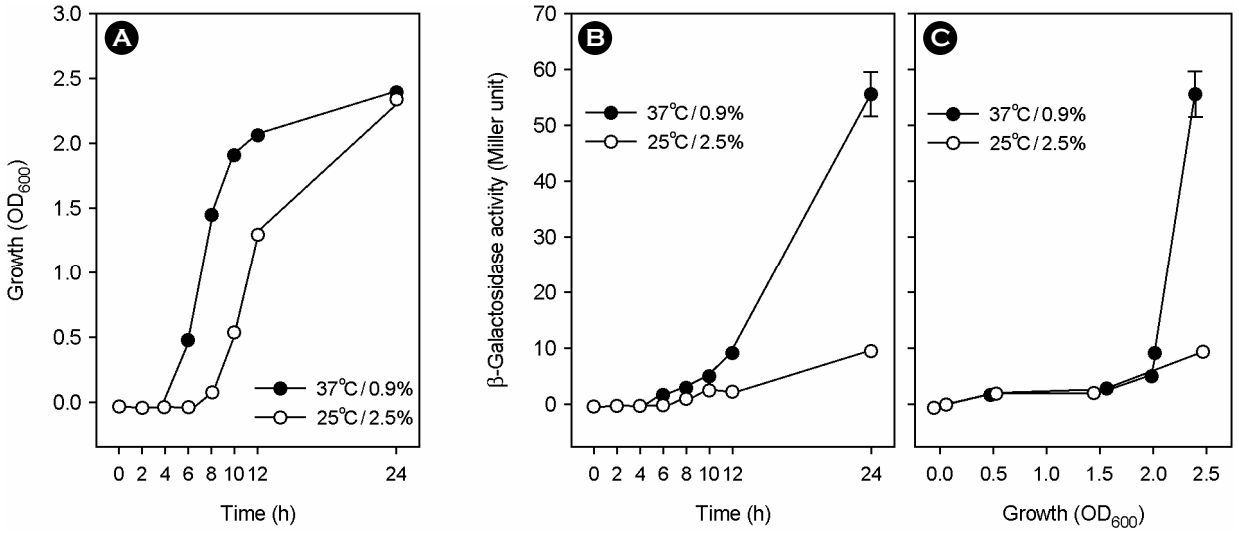

Figure 1. Effect of the simultaneous change of temperature and salinity from 25℃/2.5% to 37℃/0.9% on *luxS* transcription. After preconditioning by culturing at 25℃/2.5% overnight, the *V. vulnificus* RC138 strain with the P_{luxS}::*lacZ* transcriptional fusion was transferred into fresh broths and cultured with vigorous shaking at 25℃/2.5% or 37℃/0.9%. Bacterial growth (A) was expressed as the optical density of culture aliquots at a wavelength of 600 nm. Accumulated β-galactosidase activity in culture aliquots was expressed as the Miller unit, and plotted against culture time (B) and bacterial growth (C). Means and standard deviations were from triplicate measurements.

pattern by SCTS was growth phase- or bacterial density-dependent (Fig. 1B and 1C): *luxS* expression was initiated at low bacterial densities, gradually increased during the exponential growth phase, and thereafter steeply increased during the stationary growth phase or at high bacterial densities. SCTS did not affect *luxS* transcription levels on a per-cell basis during the exponential growth phase. However, SCTS significantly increased *luxS* transcription levels on a per-cell basis once the growth of RC138 arrived at the stationary growth phase. Accordingly, SCTS significantly increased total *luxS* expression levels, prolonging the duration of the stationary growth phase in the 24-h batch culture. Overall, these results indicate that SCTS primarily stimulates *V. vulnificus* growth and can increase total AI-2-QSS activity by facilitating growth phase-dependent *luxS* transcription.

The effect of SCTS on *vvpE* transcription in the background of wild-type or mutated *luxS* was determined using the two P_{vvpE}::*lacZ* transcriptional reporter strains, CMM-2106 with wild-type *luxS* and CMM2207 with mutated *luxS*. SCTS stimulated and advanced the growth of the two strains, prolonging the duration of the stationary growth phase (Fig. 2A and 2D). In CMM2106, the *vvpE* expression pattern was growth phase- or bacterial density-dependent (Fig. 2B and 2C): *vvpE* expression was initiated at low bacterial densities, gradually increased during the exponential growth phase, and thereafter steeply increased during the stationary growth phase or at high bacterial densities. SCTS did not increase *vvpE* expression levels on a per cell basis during the exponential growth phase. However, SCTS significantly increased *vvpE* transcription levels once the growth of CMM2106 arrived at the stationary growth phase. Accordingly, SCTS increased total *vvpE* expression levels, prolonging the duration of the stationary growth phase in the 24-h batch culture. Similarly, SCTS also increased *vvpE* transcription levels in CMM2207 in which *vvpE* transcription was severely repressed (Fig. 2E and 2F). However, when quantitatively compared, the SCTS-mediated increases in *vvpE* transcription were approximately 4-fold in CMM-2106, and approximately 2-fold in CMM2207. These results indicate that SCTS primarily stimulates *V. vulnificus* growth and can secondarily facilitate growth phase-dependent *vvpE* transcription, and AI-2-QSS only partially affects the SCTS-mediated modulation of *vvp*E expression, if at all.

The effect of SCTS on extracellular VvpE production in the background of wild-type *luxS*, mutated *luxS* or *in*

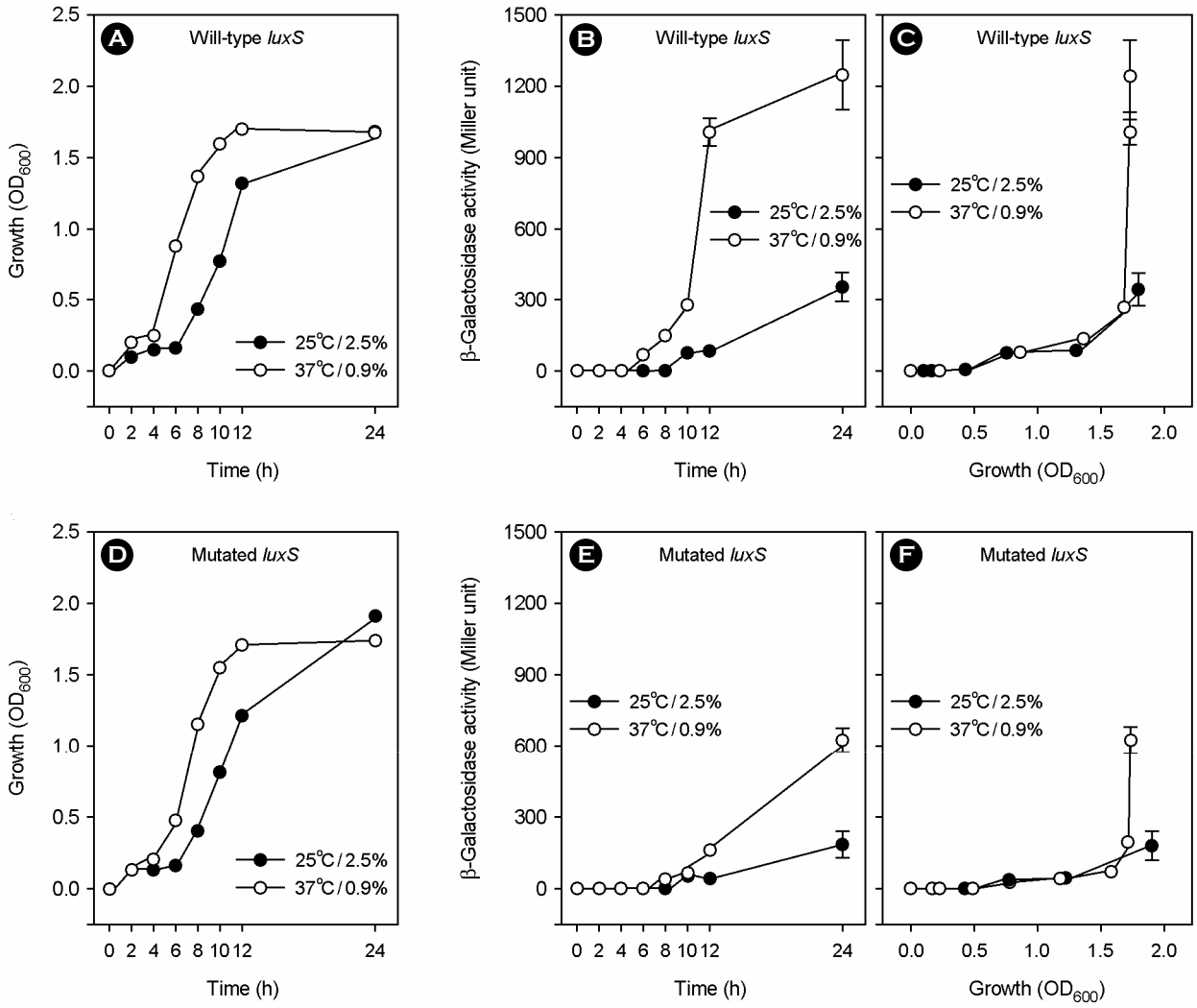

Figure 2. Effect of a *luxS* mutation on the regulation of *vvpE* transcription by the simultaneous change of temperature and salinity from 25℃/2.5% to 37℃/0.9%. After preconditioning by culturing at 25℃/2.5% overnight, the two *V. vulnificus* strains with P_{vvpE}::*lacZ* transcriptional fusion, CMM2106 with wild-type *luxS* (A to C) and CMM2207 with mutated *luxS* (D to F) strains, were transferred to fresh Heart Infusion broths and cultured with vigorous shaking at 25℃/2.5% or 37℃/0.9%. Bacterial growth (A and D) was expressed as the optical density of culture aliquots at a wavelength of 600 nm (OD_{600}). Accumulated β-galactosidase activity in culture aliquots was expressed as the Miller unit, and plotted against culture time (B and E) and bacterial growth (C and F). Means and standard deviations were from triplicate measurements.

trans-complemented *luxS* was determined using the three strains, M06-24/O with wild-type *luxS*, CMM2201 with mutated *luxS* and CMM2211 with *in trans* complemented *luxS*. SCTS stimulated and advanced the growth of all three strains, prolonging the duration of the stationary growth phase (Fig. 3A). A *luxS* mutation or *in trans* complementation did not affect *V. vulnificus* growth. In M06-24/O, SCTS also advanced and stimulated extracellular VvpE production, which was consistent with advanced and stimulated growth levels. At 25℃/2.5% (Fig. 3B), extracellular VvpE production was observed at 10~11 h after culture initiation. At 37℃/0.9%, extracellular VvpE production began to be observed at 4 or 5 h after culture initiation. Extracellular VvpE production in CMM2201 was lowered and delayed by a *luxS* mutation, and these changes were completely recovered by an *in trans luxS* complementation in CMM2211. However, SCTS also facilitated extracellular VvpE production even in CMM2201. These results indicate

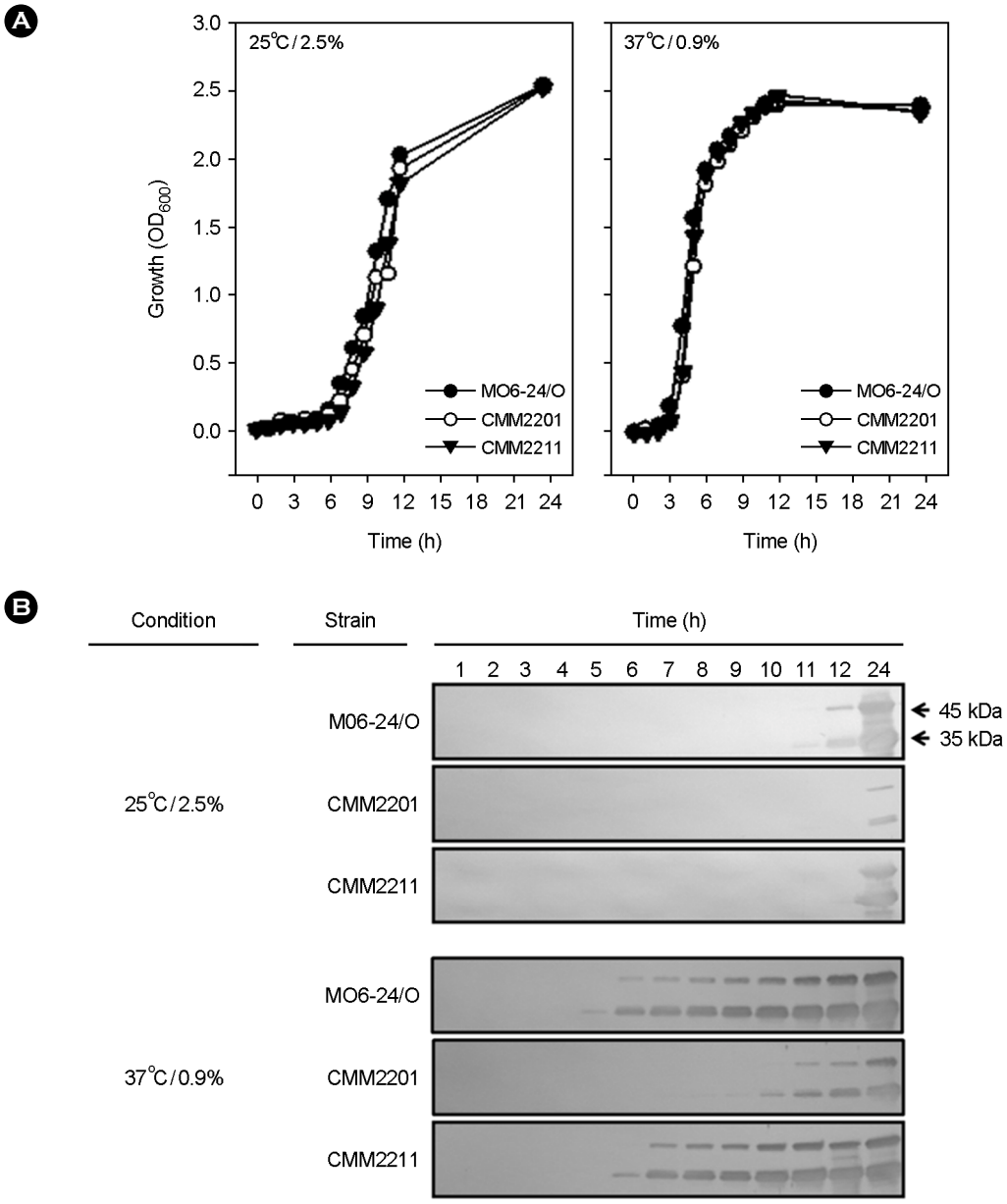

Figure 3. Effect of a *luxS* mutation on the regulation of extracellular VvpE production by the simultaneous change of temperature and salinity from 25℃/2.5% to 37℃/0.9%. After preconditioning by culturing at 25℃/2.5% overnight, the *V. vulnificus* M06-24/O, CMM2201 (with the mutated *luxS* gene) and CMM2211 (with the *in trans* complemented *luxS* gene) strains were transferred to fresh Heart Infusion broths and cultured with vigorous shaking at 25℃/2.5% or 37℃/0.9%. (A) Bacterial growth was expressed as the optical density of culture aliquots at a wavelength of 600 nm (OD_{600}). (B) Culture supernatants were obtained by centrifugation of culture aliquots to measure extracellular VvpE production by Western blotting. A representative one of the twice repeated experiments is shown. Arrows indicate the two forms of VvpE.

that SCTS can stimulate extracellular VvpE production with stimulated *V. vulnificus* growth and AI-2-QSS may only partially affect the SCTS-mediated modulation of extracellular VvpE production.

DISCUSSION

In this study, SCTS stimulated *V. vulnificus* growth and facilitated *vvpE* expression, suggesting that VvpE may

function as a virulence factor required for the better survival of *V. vulnificus* upon entering the human body. Virulence expression is a survival strategy of pathogenic bacteria (2). *V. vulnificus* inhabits sea water and can also infect the human body. The two environments differ in many respects including temperature and salinity. During the infection process, *V. vulnificus* must be able to sense and sustain changes in environmental factors (19). Signals from the changing environmental factors are relayed to specific genes by cognate signal transduction systems, resulting in the expression of genes associated with specific virulence factors. Virulence factors required for *in vivo* survival and growth of *V. vulnificus* are produced at the appropriate place and time in a tightly regulated fashion, as reported for other pathogens (18, 20, 21).

According to our results, *luxS* expression is dependent on bacterial density. In general, AI-2 production increases in proportion to increasing bacterial density (10). In *E. coli*, *luxS* expression is also increased in proportion to increasing bacterial density (22). This study also revealed another interesting finding that *luxS* expression is growth phase-dependent. This finding suggests that metabolic status may affect *luxS* expression via the stationary-phase sigma factor RpoS in *V. vulnificus*. We are now investigating this possibility. RpoS is known to positively affect AI-2 production and *luxS* expression in *V. alginolyticus* (23).

According to our results, SCTS stimulates *V. vulnificus* growth with no increase in the total growth level, and the consequent prolongation of the stationary growth phase results in a significant increase in growth phase-dependent *luxS* transcription. These findings indicate that AI-2-QSS functions more efficiently at 37℃/0.9% than at 25℃/2.5%. In contrast, previous studies reported contradictory results, in which both AI-2 production measured by the AI-2 bioassay and the *luxS* mRNA level determined by the reverse transcriptase-polymerase chain reaction (RT-PCR) method were higher at 26℃ than at 37℃, which suggested that AI-2-QSS might function more effectively at 26℃ than at 37℃ (8, 9). This significant difference may be explained by the following research results. In *V. vulnificus*, AI-2 is known to be produced at the exponential growth phase and thereafter abruptly declines (13). Accordingly, only measuring AI-2 production at one or two time points may not represent actual AI-2 production or AI-2-QSS activity. Therefore, it may be more suitable to serially measure and compare transcriptional levels with culture time, as in this study. In addition, the AI-2 bioassay is known to be too vulnerable to accurately measure the activity of AI-2-QSS (24). Similarly, the *luxS* expression level alone may not represent actual AI-2-QSS activity because AI-2 production is mediated by several spontaneous non-enzymatic reactions, as well as the enzymatic action of LuxS (10). LuxS produces the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) as a metabolic by-product, during the conversion of S-ribosylhomocysteine to homocysteine in the activated methyl cycle, and DPD is spontaneously converted to the AI-2 signal molecule furanosyl borate diester via several intermediates. Accordingly, LuxS can function as a metabolic enzyme as well as an AI-2 synthase although the presence or absence of LuxS unequivocally determines the presence or absence of AI-2 production. All of these findings warrant that the expression of multiple genes associated with AI-2 signaling, as well as AI-2 production, should be simultaneously measured to quantitatively assess actual AI-2-QSS activity.

In *V. vulnificus*, AI-2 production is mediated by the enzymatic action of LuxS (13, 18), and AI-2 signaling is transferred to the master regulator SmcR (a LuxR homolog) via LuxO and LuxT (25). It has been well documented that *vvpE* transcription is positively or negatively regulated by *luxS*, *luxO*, or *smcR* mutations, respectively (6, 13). Indeed, the best known phenotype directly regulated by *V. vulnificus* AI-2-QSS is the production of VvpE. This study re-confirms that *vvpE* transcription and extracellular VvpE production are under the control of AI-2-QSS.

According to our results, SCTS stimulated *V. vulnificus* growth and facilitated *vvpE* transcription. The *vvpE* transcription level was determined by the *lacZ*-fused *vvpE* transcriptional reporter assay, which is a very stable method capable of measuring gene transcription. Similar findings were also observed in extracellular VvpE production, which was determined by the Western blot method, one of the

most sensitive methods capable of measuring the simultaneous production of the two forms of VvpE (7, 26). The SCTS-mediated modulation of *vvpE* transcription and extracellular VvpE production appeared to be slight attenuated but still clearly observed in the background of a *luxS* mutation. These findings indicate that *V. vulnificus* senses SCTS to increase *vvpE* expression and that AI-2-QSS only partially affects the SCTS-mediated modulation of *vvpE* expression, if at all. In contrast, previous studies reported that *V. vulnificus* could sense and respond to temperature changes by modulating *vvpE* transcription and extracellular VvpE production via AI-2-QSS, and that AI-2 production or *luxS* and *vvpE* expression levels were higher at 26℃ than at 37℃ (8, 9). There are plausible explanations for the significant differences in our research results compared to others. One persuasive factor of this study is that *vvpE* expression was serially measured with culture time, and analyzed on a per cell basis through coherent experiments using transcriptional reporters, suitable mutants, and Western blotting. In contrast, the other researchers used the RT-PCR method to measure *vvpE* transcription levels, and the conventional function assay method which detects caseinolytic activity to measure extracellular VvpE activity. In addition, in this study, *V. vulnificus* strains were preconditioned by culturing at 25℃/2.5% while the other researchers preconditioned *V. vulnificus* strains by culturing at 37℃/2.5%. This difference in preconditioning may also affect *luxS* or *vvpE* expression levels. *V. vulnificus* does not mutually circulate between natural habitats and the human body, but moves only one way from its natural habitat to the human body. In this context, the shifting from 25℃/2.5% to 37℃/0.9% salinity applied in this study may be more rational than the opposite shifting used by others.

According to our results, SCTS prolongs the duration of the stationary growth phase by stimulating *V. vulnificus* growth, and significantly increases the *vvpE* transcription level only during the prolonged stationary growth phase in 24-h batch culture. These findings indicate that the duration of the stationary growth phase plays an important role in stimulating growth phase-dependent *vvpE* expression. The SCTS-mediated growth stimulation and the resulting prolongation of the stationary growth phase may increase metabolic or oxidative stress with increased Crp and/or RpoS, which may increase *vvpE* expression. It is well documented that Crp and SmcR synergistically affect the RpoS-dependent *vvpE* promoter [6]. Our recent research also revealed that Crp functions as an essential activator, and SmcR functions as a modulator for full *vvpE* expression only in the presence of Crp (27).

Generally, bacterial growth is not synchronous but asynchronous even *in vitro*. Accordingly, different growth phases of *V. vulnificus* can exist simultaneously *in vivo*; some could already arrive at the stationary growth phase, but others could begin to grow at the same time. The VvpE produced from *V. vulnificus* cells that already arrived at the stationary growth phase can affect the growth or survival of *V. vulnificus* cells which begin to grow by destroying various host proteins. Our previous study revealed that VvpE is essentially required for *V. vulnificus* swarming and can destroy various host proteins lactoferrin and immunoglobulin A (18). Bacterial swarming is defined as a rapid and coordinated population migration of flagellated bacteria across solid surfaces, and is considered as a good model for studying adherence, colonization and invasiveness to/on/into mucosa, which are essential steps for the early stage of infection (28). Moreover, VvpE may affect the growth and survival of *V. vulnificus* at the early stage of infection by destroying the iron-withholding protein lactoferrin and immunoglobulin A, both of which are important components for mucosal immunity (29). Accordingly, VvpE is likely to play significant roles in the adherence, colonization and invasiveness to/on/into mucosa of *V. vulnificus* at the early stage of infection, although it is mainly produced at the stationary growth phase.

The fact that *V. vulnificus* can sense and respond to SCTS by stimulating *vvpE* expression implies that *V. vulnificus* can also sense SCTS to up-regulate the expression of other virulence factors. Our previous study revealed that SCTS increases the production of VvhA, another exotoxin produced by *V. vulnificus* (11). Moreover, the elevation of temperature up-regulates the expression of *hupA* encoding haem receptor protein, by which *V. vulnificus* acquires iron

from various haem proteins (30). However, the SCTS-mediated up-regulation of *vvpE* expression does not directly indicate that *vvpE* expression is increased more within the human body than under environmental conditions. Several factors including the availability of iron and glucose can also directly or indirectly affect *in vivo vvpE* expression (6, 31) in a similar mode as in the case of *in vivo vvhA* expression (11, 32, 33).

In summary, SCTS facilitated both *luxS* expression and *vvpE* expression, stimulating *V. vulnificus* growth. However, the SCTS-mediated up-regulation of *vvpE* expression was slightly attenuated but still observed in the background of a *luxS* mutation. Accordingly, SCTS facilitates *luxS* and *vvpE* expression with stimulated *V. vulnificus* growth, but the LuxS-mediated AI-2-QSS is likely to play only a minor role, if any, in the SCTS-mediated modulation of *vvpE* expression.

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