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온도와 염분농도 변화에 의한 패혈증 비브리오균 단백분해효소 생산 조절

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Regulatory Mechanism of *Vibrio vulnificus* Metalloprotease Production by Temperaure and Salinity

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송 준 섭

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조선대학교 대학원 의학과

패혈증 비브리오균은 해양미생물 중 하나로서 주요 자연서식처는 민물이 섞이는 연안 바닷물이다. 이 균이 인체감염을 일으키기 위해서는 온도와 염분농도의 변화, 영양분의 차이 등 다양한 환경인자들의 변화를 극복할 수 있어야 한다. 환경인자들 중 온도와 염분농도의 변화는 패혈증 비브리오균이 생산하는 독력인자 중 하나인 단백분해효소의 생산을 조절하는 신호 중 하나로서 알려져 왔으나 그 조절기전은 아직까지 규명되지 못하였다. 본 연구에서는 여름철 바닷물의 온도인 25°C에서 체온인 37°C로 온도변화를 주고 이와 더불어 바닷물의 염분농도인 2.5%에서 인체의 염분농도인 0.9%로 염분농도의 변화를 주었을 때 패혈증 비브리오균에 의한 단백분해효소의 생산이 어떻게 조절되는지를 관찰하였다. 또한, 조절기전을 밝히기 위하여 동일한 온도와 염분농도의 변화가 세균집단의 밀도에 반응하여 단백분해효소 등 독력인자들의 발현을 조화롭게 조절하는 것으로 알려진 autoinducer-2 (AI-2)에 의해 매개되는 quorum-sensing system (QSS)의 활성에 어떠한 영향을 미치는가, 그리고 단백분해효소 등 여러 가지 단백들의 세포외 분비를 담당하고 있는 PilD에 의해 매개되는 type II general secretion system (II-GSS)의 활성에 어떠한 영향을 미치는 가를 관찰하였다. 연구결과를 보면,

온도와 염분농도의 변화에 의해 세균성장이 촉진되었다. 이와 함께 QSS의 신호전달물질인 AI-2의 생합성 효소 LuxS를 암호화하는 유전자 *luxS*의 전사와 AI-2-QSS의 주요 전사조절인자 SmcR를 암호화하는 유전자 *smcR*의 전사도 촉진되었다. 단백분해효소를 암호화하는 유전자 *vvpE*의 전사도 동일한 온도와 염분농도의 변화에 의해 촉진되었다. 단백분해효소 유전자 *vvpE*의 이러한 변화는 QSS에 관여하는 유전자 *luxS*와 유전자 *smcR*를 돌연변이시켰을 때 약화되었고, 유전자 *luxS*를 돌연변이시켰을 때 보다는 유전자 *smcR*를 돌연변이시켰을 때 더욱 약화되었다. 또한, 온도와 염분농도의 변화는 II-GSS에 반드시 필요한 효소인 PilD를 암호화하는 유전자 *pilD*의 발현을 촉진하였다. 유전자 *pilD*의 이러한 변화는 QSS에 관여하는 유전자 *luxS* 또는 유전자 *smcR*를 돌연변이시킨 경우에도 큰 변화 없이 관찰되었다. 단백분해효소의 세포외 생산 또한 온도와 염분농도의 변화에 의해 촉진되었다. 단백분해효소 세포외 생산의 이러한 변화는 QSS에 관여하는 유전자 *luxS*와 유전자 *smcR*를 돌연변이시켰을 때 약화되었고 두 유전자의 야생형을 이용하여 돌연변이를 보완시켜 주었을 때에는 원래대로 회복되었다. 따라서, 이러한 결과는 패혈증 비브리오균이 인체감염을 일으킬 때 온도와 염분농도의 변화를 감지하고 유전자발현 수준에서는 AI-2-QSS을 통해 그리고 단백질 세포외 분비 수준에서는 PilD-II-GSS를 통해 단백분해효소의 생산을 조절함으로써 균의 독력을 변화시킬 수 있음을 분명하게 보여준다.

핵심용어: 패혈증 비브리오균, 온도, 염분농도, 단백분해효소, Autoinducer-2, Quorum-sensing system, Type II general secretion system

Introduction

1. General review

Vibrio vulnificus is a gram-negative halophilic estuarine bacterium that inhabits sea water and an opportunistic pathogen that can reside in the human body. *V. vulnificus* causes a rapidly progressing fatal septicemia and a necrotizing wound infection with a high mortality rate, preferentially in susceptible patients with hepatic diseases, hemochromatosis, heavy alcohol drinking habits, and compromised immune functions. *V. vulnificus* infections occur as the consequence of ingesting raw seafood contaminated with the bacterium, or of wound infections by sea water contaminated with the bacterium. In particular, *V. vulnificus* infections are characterized by formation of hemorrhagic and edematous lesions and extensive dermonecrosis on the limbs [1-4].

Korean people, especially residents of the west-southern Korea, have been often exposed to *V. vulnificus* through seafood and occupational exposure. The number of reported cases of *V. vulnificus* septicemia has increased since the first case was reported in 1980. The increasing number of cases may be caused by greater disease activity or improved recognition by clinicians or laboratory workers. However, it appears that many factors are associated with increased vulnerability of Korean people to *V. vulnificus* infection. These include the high prevalence of hepatitis B virus infection-related or alcohol-related liver diseases such as liver cirrhosis and hepatoma, the environment, and the popularity of preparing and eating raw or undercooked seafood. Most cases of reported *V. vulnificus* septicemia occur in summer season from June to November when the temperature of estuarine seawater reaches approximately 25^{o}C [1, 2, 5, 6]. Further continuous education, measures, and studies

are needed to prevent this emerging fatal disease. Currently, *V. vulnificus* septicemia is being thoroughly controlled as one of the group III communicable diseases designated as reportable by Korean law [http://dis.cdc.go.kr/].

1) Quorum sensing: bacterial cell-to-cell communication

In recent years there has been a paradigm shift in our understanding of the unicellular bacterial world from the perspective that bacterial cells are non-co-operative to one which incorporates social interactions and multicellular behavior. Many bacteria monitor their cell-population densities through the exchange of chemical signaling molecules called autoinducers (or called pheromones) that accumulate extracellularly and trigger alterations in behavior at high population densities. This phenomenon is referred to as quorum sensing (QS). QS enables a bacterial population to mount a co-operative response that improves access to nutrients or specific environmental niches, promotes collective defense against other competitor prokaryotes or eukaryotic defense mechanisms and facilitates survival through differentiation into morphological forms better able to combat environmental threats [7-9].

As the bacterial population density increases, the synthesis of autoinducers increases more and more, and eventually their concentration continues to rise in the external environment. Once a threshold concentration has been attained, activation of a signal transduction cascade leads to the induction or repression of QS target genes. QS can also be considered in the context of ‘diffusion sensing’ or ‘compartment sensing’ or even ‘efficiency sensing’, where the signal molecule supplies information with respect to the local environment and spatial distribution of the cells rather than, or as well as, cell population density [10-12]. In addition, QS continues to attract significant interest from the

perspective of social evolution, fitness and the benefits associated with costly co-operative behaviors [13,14].

Bacterial quorum sensing system (QSS) was first identified and described in the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi* [15, 16]. Since then, several QSS have been described and they are categorized into the follows: (1) the LuxR/I-type systems, primarily used for intraspecies communication by gram-negative bacteria, in which the signaling molecule is an acyl-homoserine lactone (also called autoinducer-1; AI-1), (2) the peptide signaling systems used primarily by gram-positive bacteria, (3) the LuxS or autoinducer-2 (AI-2) signaling systems used for interspecies communication, and (4) the epinephrine or norepinephrine (AI-3) interkingdom signaling system [9].

2) QSS in *V. vulnificus*

V. vulnificus possesses only AI-2-mediated QSS (AI-2-QSS), as shown in Figure 1. AI-2 is a furanosyl borate diester, which is synthesized by the LuxS enzyme. LuxS converts S-ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione called DPD. DPD is a very unstable compound that reacts with water and cyclizes to form several furanones, one of which is thought to be the precursor of AI-2 [17,18]. However, some researchers have raised the question whether AI-2 is a true signal molecule, or whether it is released as a waste product or used as a metabolite rather than a signal molecule [11,19]. In addition, LuxS is an enzyme involved in the biochemical pathway for the detoxification of S-adenosyl-methionine. Accordingly, altered gene expression by *luxS* mutation will comprise both genes affected by QS *per se* and genes differentially expressed by the interruption of the metabolic pathway. To address which genes are truly regulated through AI-2 signaling, it is impor-

tant to use pure AI-2 signal. Hence, the only phenotype demonstrated to be AI-2 dependent is the production of metalloprotease called VvpE in *V. vulnificus* [20-26].

The receptor for AI-2 is a periplasmic protein called LuxP, which complexes with LuxQ controlling whether LuxQ behaves as a sensor kinase or phosphatase according to the concentration of AI-2 present [27]. In the absence of AI-2 signal or in the presence of low levels of AI-2 signal, LuxQ as a hybrid enzyme acts as an intrinsic kinase and phosphorylates a complex phophorelay system, with LuxU and LuxO as intermediaries [27, 28]. Phosphorylated LuxO then activates *luxT* transcription by direct binding to the regulatory region, and LuxT represses *smcR* transcription by direct binding to the regulatory region [25]. SmcR as the master QS regulator acts by direct binding to the regulatory region of *vvpE* and is essentially required for full expression of *vvpE* [21,23]. Accordingly, down-regulated SmcR can no longer induce *vvpE* transcription. Conversely, as the bacterial population density increases and AI-2 reaches a critical threshold, LuxQ acts as a phosphatase and de-phosphorylates LuxU and LuxO. De-phosphorylated LuxO de-represses *smcR* transcription because it can no longer activate LuxT. Eventually, up-regulated SmcR activates *vvpE* transcription. In addition, it has been known in *V. harveyi* and *V. cholerae* that phosphorylated LuxO in conjunction with σ54 can activate transcription of small regulatory RNAs, which destabilize the messenger RNA of *smcR*, which in turn can no longer activate *vvpE* transcription [29]. However, it has not yet determined whether *V. vulnificus* QSS can also regulate *smcR* expression at the post-transcriptional level by using small RNAs.

AI-2 QS also affects a variety of *V. vulnificus* phenotypes other than *vvpE* expression [30]. The mutation of *smcR* results in a significant alteration in bio-

film formation, in type of colony morphology, in motility, in reduced survival under adverse conditions, such as acidic pH and hyperosmotic stress, and in decreased cytotoxic activity toward INT 407 cells *in vitro*. Furthermore, intraperitoneal LD_{50} against mouse becomes higher by *smcR* mutation. Therefore, it appears that SmcR is a novel global regulator, controlling numerous genes contributing to the pathogenesis as well as survival of *V. vulnificus*

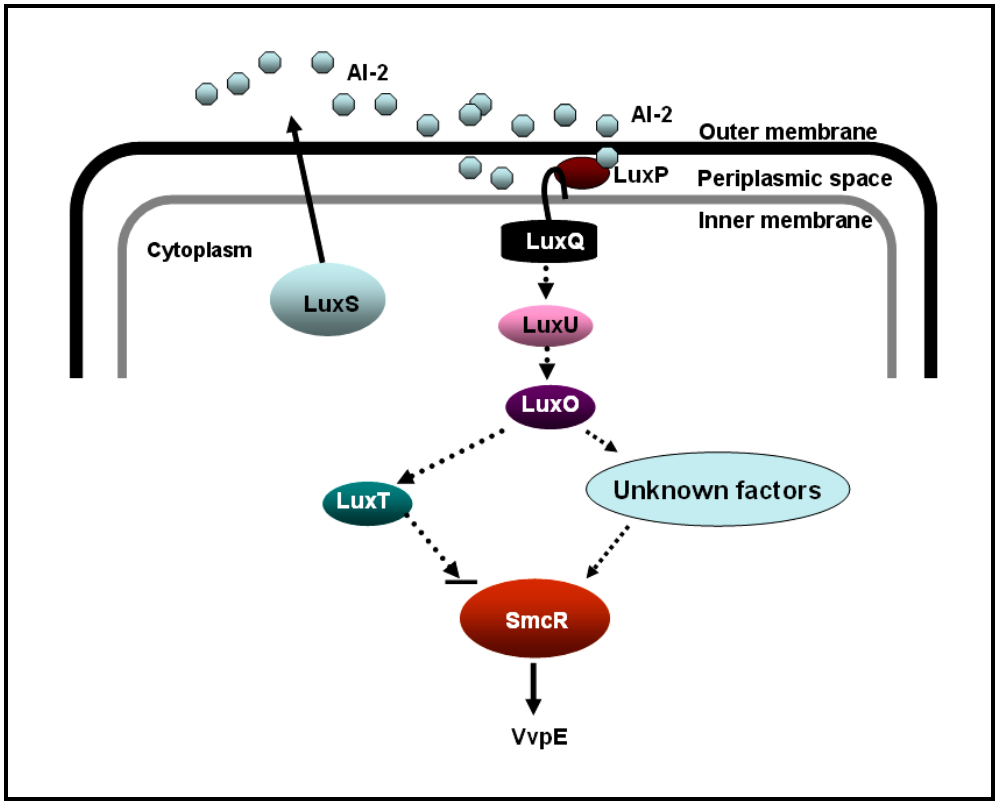


Figure 1. Schematic illustration for AI-2-QSS in *Vibrio vulnificus*. At high population densities, LuxS synthesizes and secretes AI-2. Accumulated AI-2 binds to a membrane receptor called LuxP and transduces signals to a sensor kinase called LuxQ, which dephosphorylates from LuxO. De-phosphorylated LuxO no longer activates LuxT, which acts as a transcriptional repressor, and activates transcription of the *smcR* gene. SmcR acts as a transcriptional activator on the promoter of the *vvpE* gene. In addition, LuxO is known to be able to regulate SmcR at the posttranscriptional level via yet-undefined factors.

3) Pathogenetic roles of metalloprotease VvpE

Several potential virulence factors have been reported to be associated with *V. vulnificus* infections, including capsule [31], lipopolysaccharide [32], metalloprotease VvpE [33], hemolysin or cytolysin [34], phospholipase [35], motility [36], iron-assimilation systems [37], and RTX toxin [38-40].

V. vulnificus produces the thermolysin-like VvpE, which has been considered as an important virulence factor to evoke the typical skin lesions, so has been extensively studied. Purified VvpE induces hemorrhagic damage and dermonecrosis, enhances vascular permeability and edema, and has also proven lethal to mice [41-51]. Furthermore, VvpE functions as a prothrombin activator and fibrionolytic enzyme, suggesting that it interferes with blood homeostasis and thereby facilitate the development of systemic *V. vulnificus* infections [52]. *V. vulnificus* produces the two forms of VvpE with different activities via the type II general secretion system (II-GSS) [53]. VvpE of 34 kDa has sufficient and similar proteolytic activity toward soluble proteins such as azocasein and plasminogen but displays reduced activity toward insoluble proteins such as fibrin and elastin [46, 52]. In contrast, VvpE of 45 KDa has sufficient proteolytic activity toward insoluble proteins as well as soluble proteins, and agglutinates rabbit erythrocytes, binds to the erythrocyte ghosts, and digests the ghost membrane proteins [41-45].

In spite of these extensive studies, some serious doubts have been raised with regard to the roles of VvpE in the pathogenesis of *V. vulnificus* infections. VvpE-deficient mutants evidenced comparable virulence than were seen in the wild-type strains in studies employing mouse experimental models [54-56]. In addition, our recent studies show that VvpE exerts no direct effects on iron-assimilation from human transferrin [57-59] or on the inactivation of the hemo-

lysin from *V. vulnificus* [60]. Nevertheless, from an evolutionary perspective, it is believed that VvpE is generated because it is essential for the survival of *V. vulnificus* in external environments as well as within human hosts. Accordingly, new paradigms will be required in order to elucidate the currently obscure pathogenetic roles of VvpE.

More recently, it was reported that VvpE is essentially required for the swarming motility of *V. vulnificus*, which is thought to be a good model for bacterial surface adherence and colonization, and that VvpE destroys IgA and lactoferrin, which are responsible for mucosal immunity [26]. Bacterial swarming is defined as a rapid and coordinated population migration of flagellated bacteria across solid surfaces, and has proven to be a good *in vitro* model for bacterial surface adherence and colonization [61, 62]. Swarming itself is generally regarded to be a virulence factor in urogenital- and intestinal-tract pathogens; moreover, swarming differentiation is linked to the expression of virulence factors associated with invasiveness in many bacterial species [63-66]. In particular, swarming differentiations in *P. mirabilis* and *B. subtilis* has been closely related with the expression of metalloproteases [64, 66].

V. vulnificus infects susceptible patients via either the skin or the intestinal tract. Accordingly, *V. vulnificus* must first adhere to and colonize the epithelial surfaces of the skin or the intestinal tract, in order to successfully establish infections. *V. vulnificus* also evidences swarming motility, which requires flagellar synthesis [36], and is controlled via global regulatory systems, such as RpoS and the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex [67, 68]. As has been seen in other bacteria, *V. vulnificus* swarming is believed to play a significant role in surface adherence and colonization, as well as in the expression of invasiveness-related virulence factors, because the adhesion and cyto-

toxicity to cultured cells of a swarming-defective mutant was attenuated [36]. These findings strongly suggest that VvpE may play important roles in the surface adherence and colonization of *V. vulnificus* by facilitating swarming, and in the invasiveness of *V. vulnificus* by facilitating the proteolytic cleavage of IgA and lactoferrin.

4) Regulation of VvpE production

Expression of the *vvpE* gene encoding VvpE is affected by a variety of growth conditions, including temperature, osmolarity, and iron and oxygen levels [20, 24, 57, 58, 69], and is controlled by the stationary sigma factor RpoS [23, 67], AI-2-QSS [20-26], and the cAMP-CRP complex [23, 68], as shown in Figure 2.

Expression of the *vvpE* gene begins constitutively during the early logarithmic growth phase, and it is profoundly induced during the late logarithmic or early stationary growth phase [21, 23, 59]. In addition, it was recently documented that *vvpE* expression is increased during *V. vulnificus* swarming and *luxS* mutation attenuates *vvpE* expression in swarming *V. vulnificus* [26]. Expression of the *vvpE* gene is directed by two different promoters, promoter L and promoter S. The activity of promoter L is constitutive and lower than the activity of promoter S. The activity of promoter S is induced only in the stationary growth phase and is dependent on RpoS, a sigma factor of RNA polymerase that is required for transcription of many stationary-phase-responsive genes, and is also dependent on AI-2-QSS which coordinately regulates transcription of many genes in responsive to bacterial cell density. The activity of promoter S is also under the positive control of CRP, and the influence of CRP on the promoter S is mediated by SmcR, a master transcription regulator of AI-

2-QSS. The binding sites for CRP and SmcR were juxtapositioned and centered 220 and 198-bp upstream of the transcription start site of the RpoS-dependent promoter S, respectively. These findings indicate that CRP and SmcR function synergistically to co-activate *vvpE* expression via promoter S and that the two activators exert their effect by directly binding to the promoter S.

Exotoxins including VvpE are secreted into media through II-GSS, which essentially requires PilD, the type IV leader peptidase/N-methyltransferase [53, 70]. It was recently demonstrated that two forms of VvpE are produced and secreted from the early growth phase through the PilD-mediated II-GSS (PilD-II-GSS) by using more reliable or confirmative methods [53, 59].

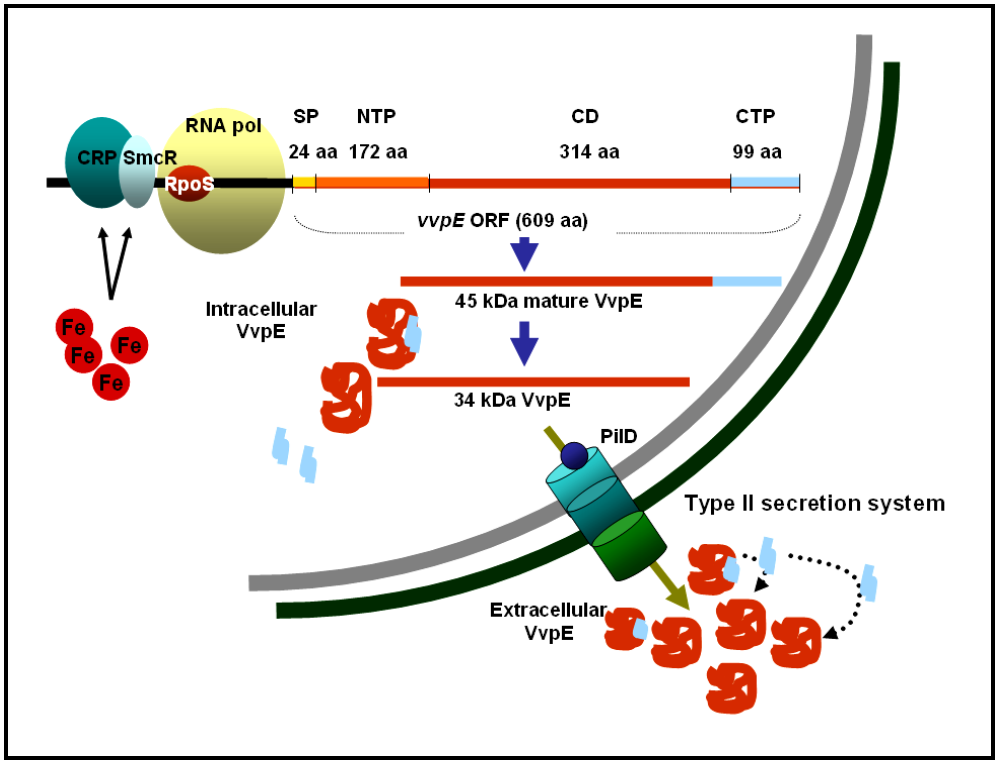


Figure 2. Schematic illustration for the production of metalloprotease VvpE in *Vibrio vulnificus*. RpoS (sigma factor S), SmcR (master regulator of AI-2-QSS) and CRP (cAMP-receptor protein) positively or directly regulate *vvpE* transcription. The ORF of *vvpE* consists of the four regions with 609 amino acids: a region for the signal peptide (SP) with 24 amino acids, a region for the N-terminal propeptide (NTP) with 172 amino acids, a region for the catalytic domain (CD) with 314 amino acids and a region for the C-terminal propeptide (CTP) with 99 amino acids. The mature VvpE with the molecular size of 45 and 34 kDa are secreted via the type II general secretion system, which is mediated by PilD, an enzyme essential for post-transcriptional protein modification.

2. The aim of this study

Again, *V. vulnificus* is a halophilic bacterium that inhabits sea water and an opportunistic human pathogen that can reside in the human body. The two environments of *V. vulnificus* inhabits differ in many respects including temperature, salinity, and the type and concentration of nutrients. Establishment of a successful infection requires *V. vulnificus* to overcome and to survive the diverse environmental conditions [71]. Virulence expression is a survival strategy for pathogenic bacteria. Like other Vibrio species [72-74], *V. vulnificus* also appears to be able to sense environmental changes and correspondingly regulate virulence expression for survival [20, 24, 71]. Previously, it was demonstrated that the change of environmental factors, especially salinity and temperature, modulate the production of hemolysin, the most potent exotoxin produced by *V. vulnificus* [75, 76].

Recently, it was reported that *V. vulnificus* can sense environmental changes to modulate the production of VvpE via AI-2-QSS [24, 55]. It was well documented that AI-2-QSS positively regulates VvpE production [20-26]. However, it has not been determined yet whether or not the changes of environmental factors affect the activity of AI-2-QSS to modulate VvpE production.

V. vulnificus encounters numerous stresses upon entry into the human host. In this study, we particularly focused on the change of temperature and salinity. Temperature 25^{o}C and salinity of 2.5% are mimicry estuarine conditions in summer seasons [77-79]; Temperature 37^{o}C and salinity of 0.9% reflect human body conditions. In this study, we attempted to determine whether the shifting of temperature (25 to 37^{o}C) and salinity (2.5 to 0.9%) modulates VvpE production via AI-2-QSS.

Moreover, the activity of AI-2-QSS has been commonly measured by an

AI-2 bioassay [22]. The assay is based on the ability of *Vibrio harveyi* BB170 to specifically bioluminate in response to AI-2 [80]. However, this assay is seriously affected by the presence of glucose in media. Nearly all bacterial culture media contain glucose at various levels [81]. Currently, this bioassay is being considered as a qualitative but not quantitative method because it is too vulnerable to accurately measure the activity of AI-2-QSS [81]. The direct measurement of *luxS* expression is thought to be more accurate and rational than the indirect measurement of AI-2 using the bioassay. Accordingly, in this study, the activity of AI-2-QSS was monitored by directly measuring the *luxS* and *smcR* expression levels using the P_{luxS}*::lacZ* or P_{smcR}*::lacZ* transcription reporter strains.

VvpE is secreted through II-GSS, which essentially requires PilD [53, 70]. However, it has not been determined whether the activity of II-GSS may be affected by the changes of temperature and salinity, and by the change of AI-2-QSS activity. Accordingly, in this study, the activity of II-GSS was monitored by directly measuring the *pilD* expression levels using the P_{pilD}*::lacZ* transcription reporter strains.

Materials and Methods

1. Bacterial strains, plasmids, primers, media and reagents

Bacterial strains, plasmids and primers used in this study are listed in Table 1, 2 and 3. Heart Infusion (HI; BD, Flanklin Lakes, NJ, USA), Luria Bertani (LB; BD) media with additional 2% NaCl and Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS; BD) medium were used to cultivate *V. vulnificus* strains, and HI and LB media not containing additional NaCl were used to cultivate *Escherichia coli* strains. Antibiotics were used at the following concentrations. For *E. coli*, ampicillin 100 μg/ml, kanamycin 100 μg/ml, tetracycline 12.5 μg/ml, chloramphenicol 30 μg/ml; for *V. vulnificus*, ampicillin 20 μg/ml, kanamycin 200 μg/ml, tetracycline 2 μg/ml, chloramphenicol 2 μg/ml. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Bacteria strains used in this study

Strains	Relative characteristics	Sources
MO6-24/O	Wild-type, highly virulent clinical isolate	#
CMM2100	spontaneous streptomycin resistance	[22]
CMM2101	*lacZ* mutation	[22]
RC138	merozygotic P_{luxS}*::lacZ* fusion	#
RC196	merozygotic P_{smcR}*::lacZ* fusion	#
CMM2201	*luxS* mutation	[22]
CMM2202	*lacZ* and *luxS* mutation	[22]
CMM2210	*in trans luxS*-complementation	[22]
RC174	*smcR mutation*	#
RC162	*lacZ and smcR* mutation	
RC186	*in trans smcR*-complementation	#
CMM2106	P_{vvpE}*::lacZ* transcription fusion	[22]
CMM2207	*luxS*-mutated P_{vvpE}*::lacZ* transcription fusion	[22]
RC164	*smcR*-mutated P_{vvpE}*::lacZ* transcription fusion	#
RC104	*pilD* mutation	#
RC108	*in trans pilD*-complementation	#
RC176	merozygotic P_{pilD}::*lacZ* fusion	#
RC182	*luxS*-mutated merozygotic P_{pilD}::*lacZ* fusion	#
RC184	*smcR*-mutated merozygotic P_{pilD}::*lacZ* fusion	#
SY327 λ*pir*	*Escherichia coli*; Host for suicide vector	[72]
SM10 λ*pir*	*Escherichia coli*; Conjugation donor	[72]
DH5α	*Escherichia coli*; Host for pRC108	#
HB101	*Escherichia coli*; Host for pRK2013	[82]

#: laboratory stock strains or strains constructed in this study.

Table 2. Plasmids used in this study

Plasmids	Relative characteristics	Sources
pCR®2.1-TOPO®	PCR cloning vector	Invitrogen
pDM4	Suicide vector with R6K origin; Cm^R	[83]
pLAF3	IncP cosmid vector; Tc^R	[84]
pLAF3II	pLAFR3 with *bla* inserted at the cos site; $Amp^R Tc^R$	#
pRK2013	IncP, Km^R, Tra $Rk2^+$ *repRK2 repE1*	[82]
pUTKm1	Tn5-based insertion delivery plasmid, Amp^R	[85]
pQF52	IncP *lacZ* transcriptional fusion vector; Amp^R	[86]
pRC152	2.05-kb *Sma*I-*Spe*I fragment containing an in-frame deletion of the *V. vulnificus smcR* gene cloned into pDM4	#
pRC154	934-bp *Bgl*II-*Kpn*I fragment containing the *pilD* promoter region cloned into pQF52	#
pRC156	*Bgl*II-*Sca*I fragment containing P_{pilD}::*lacZ* fragment from pRC154 cloned into *Bgl*II-*Sma*I cut pDM4	#
pRC158	1.39-kb *Hin*dIII-*Eco*RI fragment containing the *smcR* gene cloned into pLAFR3II	#
pCMM237	pDM4 with *Sac*I-*Xba*I fragment of pCMM236	#
pRC104	pDM4 with 1.94-kb *Bam*HI-*Spe*I fragment containing an *in-frame* deletion of *V. vulnificus pilD* gene	#
pRC108	pLAFR3II with 1.81-kb *Bam*HI-*Hin*dIII fragment containing *pilD* gene	#
pRC130	A 1075-bp *Bam*HI-*Hin*dIII fragment containing the *luxS* promoter region cloned into pQF52	#
pRC136	A *Bam*HI-*Sca*I fragment containing P_{luxS}::*lacZ* fragment from pRC130 cloned into *Bgl*II-*Sma*I-cut pDM4	#
pRC162	926-bp *Bam*HI-*Hin*dIII fragment containing the *smcR* promoter region cloned into pQF52	#
pRC164	*Bam*HI-*Sca*I fragment containing P_{smcR}::*lacZ* fragment from pRC162 cloned into *Bgl*II-*Sma*I cut pDM4	#

#: Plasmids constructed in this study.

Table 3. Primers used in this study

Primers	Sequences	Sources
luxS-rep-F	5'-cg**GGATCC**gctcatcgtgtgtttgcagagc-3'	#
luxS-rep-R	5'-ccc**AAGCTT**cggtaaaactatctaataatggc-3'	#
smcR-rep-F	5'- cg**GGATCC**caaagccaatccacttcactgg-3'	#
smcR-rep-R	5'- ccc**AAGCTT**gccacgacgagcaaacacttcc-3'	#
pili-rep-F	5'- ga**AGATCT**ggcaaagtgaccattgaatggc -3'	#
pili-rep-R	5'- gg**GGTACC**ccaccactatcattaactcaatc -3'	#
Cont-1	5'-cgggatccgccatatccccaaatgccgatg-3'	#
Cont-2	5'-cggaattctcaatattgtagctttaacgtcac-3'	#
smcR-up-1	5'- tcc**CCCGGG**ttatttatctcaacctttgcccac -3'	#
smcR-up-2	5'- ttactggtgttacatgagctgctgtttacgttttag -3'	#
smcR-down-1	5'-cagcagctcatgtaacaccagtaacctcatatcaag-3'	#
smcR-down-2	5'- gactagtgaaagctgctgatcatgggtgg -3'	#
smcR-comp-1	5'- ccc**AAGCTT**gttcaatggcacgagcaagatcc -3'	#
smcR-comp-2	5'- g**GAATTC**tgttggtctagcggcggaagtg -3'	#
PilD-1	5'-cg**GGATCC**gcatttatgttgacttagtcgcc-3'	#
PilD-2	5'-aaagccatattacatataatcgtcgttctctttg-3'	#
PilD-3	5'-gacgattatatgtaatatggctttagtgattggattg-3'	#
PilD-4	5'-g**ACTAGT**atcgctgacgaattgagctgag-3'	#
PilD-5	5'-ccc**AAGCTT**atccaatcactaaagccatattacgc-3'	#
PilD-6	5'-tgaatacaacatcacaccaccgac-3'	#
PilD-7	5'-ccttctttccctgtcagcaatctg-3'	#

#: Primer sequences first introduced in this study.
Capital bold letters indicate the restriction enzyme-recognition sequences: GGATCC (*Bam*HI), AAGCTT(*Hin*dIII), ACTAGT (*Spe*I), AGATCT(*Bgl*II), and GGTACC(*Kpn*I).

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

The *V. vulnificus* strains were preconditioned by culturing them in HI broth containing 2.5% NaCl at 25^{o}C overnight to adapt them to the condition reflecting their natural habitat. Portions of the resulting cultures of the adapted strains were transferred to HI broth containing 0.9% NaCl or 2.5% NaCl to concentrations of 1 x 10^{6} cells/ml and cultured with vigorous shaking (220 rpm) at 37^{o}C or 25^{o}C for 12 h. During culture, aliquots were withdrawn at appropriate times to measure bacterial growth and β-galactosidase activity, and to obtain culture supernatants for the measurement of VvpE production. Bacterial growth was measured by 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 [87].

3. Western blot for the detection of VvpE

Recombinant VvpE was prepared using the GST Gene Fusion System [53]. Using the resulting GST-VvpE fusion protein, rabbit polyclonal anti-VvpE-body was prepared, and the specificity of the polyclonal anti-VvpE-body was tested by Western blot in our previous studies [26, 53, 59]. VvpE production was measured using Western blot. In brief, equal volumes (10 μl) of culture supernatants were electrophoresed on 10% SDS-PAGE gels after denaturation by heating and β-mercaptoethanol. After electrophoresis, proteins were transferred to PROTRAN nitrocellulose membranes (Whatman GmbH, Germany). The membranes were incubated with blocking solution (0.2% Tween-20 and 5% Skim milk in PBS) at 4^{o}C overnight, allowed to react with rabbit polyclonal anti-VvpE-body (1000:1 diluted in washing buffer consisting of 0.1% Tween-20

and 1% Skim milk in PBS) and subsequently with anti-rabbit-IgG-body conjugated with alkaline phosphatase (15,000:1 diluted in washing buffer), and finally visualized with BCIP-NBT substrate solution.

4. Construction of the merozygotic P_{luxS}*::lacZ* transcription reporter strain

The merozygotic P_{luxS}::*lacZ* transcriptional fusion was constructed by using the broad-host-range vector pQF52 containing promoterless *lacZ* [86] and R6K origin suicide vector pDM4 [83] (Figure 3). The 1075-bp *Bam*HI-*Hin*dIII fragment containing the promoter region of *luxS* was amplified using the PCR primers; luxS-rep-F with *Bam*HI overhang and luxS-rep-R with *Hin*dIII overhang, and was subcloned into pQF52. The resulting plasmid was designated pRC130. The *Bam*HI-*Sca*I fragment containing the P_{luxS}::*lacZ* fragment from pRC130 was subsequently subcloned into pDM4, which is cut with *Bgl*II-*Sma*I. The resulting plasmid was designated pRC136, transformed into *Escherichia coli* SY327 λ*pir* and SM10 λ*pir* [72], and transferred to CMM2101 by conjugation. Eventually, a transconjugant was selected on TCBS agar containing chloramphenicol and named RC138. The presence of the P_{luxS}::*lacZ* transcriptional fusion was confirmed by a β-galactosidase assay [87].

5. Construction of the merozygotic P_{smcR}*::lacZ* transcription reporter strain

The merozygotic P_{smcR}::*lacZ* transcriptional fusion was also constructed by using the broad-host-range vector pQF52 containing promoterless *lacZ* [86] and R6K origin suicide vector pDM4 [83] (Figure 3). The 926-bp *Bam*HI-*Hin*dIII fragment containing the promoter region of *smcR* was amplified using the PCR primers; smcR-rep-F with *Bam*HI overhang and smcR-rep-R with *Hin*dIII over-

hang, and was subcloned into pQF52. The resulting plasmid was named pRC162. The *Bam*HI-*Sca*I fragment containing the P_{smcR}::*lacZ* fragment from pRC162 was subsequently subcloned into pDM4, which was cut with *Bgl*II-*Sma*I. The resulting plasmid was named pRC164, transformed into *Escherichia coli* SY327 λ*pir* and SM10 λ*pir* [72], and transferred to CMM2101 by conjugation. Eventually, a transconjugant was selected on TCBS agar containing chloramphenicol and named RC196. The presence of the P_{smcR}::*lacZ* transcriptional fusion was confirmed by β-galactosidase assay [87].

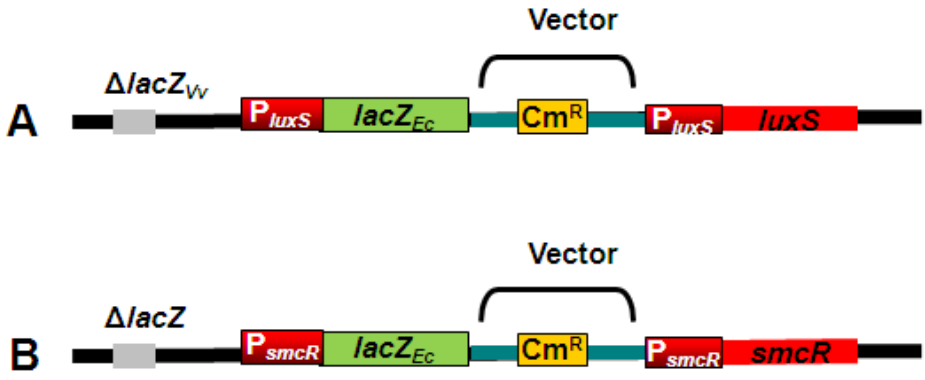


Figure 3. Schematic illustration for the gene structure of RC139 with the merozygotic P_{luxS}*::lacZ* transcriptional fusion (A) and RC196 with the merozygotic P_{smcR}*::lacZ* transcriptional fusion (B). Δ*gene*: deletional mutation of the gene, Vv: *Vibrio vulnificus*, P_{gene}: promoter of the gene, Ec: *Escherichia coli*, Cm^R: chloramphenicol resistance gene.

6. Construction of a *smcR* deletion mutant and its *in trans smcR*-complemented strain

A deletion mutant of *smcR* was constructed using the suicide vector pDM4 with R6K origin [83] (Figure 4). The 5' and 3' ends of *smcR* were amplified by PCR using the primers; smcR-up-1/2 and smcR-down-1/2. The resulting PCR products were used as the DNA template for the second crossover PCR using the primers; smcR-up-1 with a *Sma*I overhang and smcR-down-2 with a *Spe*I overhang. The resulting *Sma*I-*Spe*I fragment with deleted *smcR* was cloned into pDM4. The resulting plasmid was named pRC152, transformed into *Escherichia coli* SY327 λ*pir* and SM10 λ*pir* [72], and subsequently transferred to M06-24/O by conjugation. Finally, a stable transconjugant was selected on TCBS agar containing chloramphenicol and on HI agar containing 10% sucrose, and was named RC174. The deletional mutation of *smcR* was confirmed by PCR using the primers smcR-up-1 and smcR-down-2.

To restore wild-type *smcR* in RC174 with mutated *smcR* (Figure 5), the *Hin*dIII-*Eco*RI fragment containing the promoter region and open reading frame of *smcR* was amplified using the PCR primers; smcR-comp-1 with a *Hin*dIII overhang and smcR-comp-2 with a *Eco*RI overhang, and was subsequently subcloned into the broad host range vector pLAFR3II, which had been prepared by subcloning the *Bam*HI-*Bgl*II fragment containing the *bla* gene of pUTKm1 into a *Bgl*II site of pLAFR3 [84, 85]. The resulting plasmid was named pRC158, transferred into RC174 by triparental mating using the conjugative helper plasmid pRK2013 [82]. Finally, a stable transconjugant was selected on TCBS agar containing ampicillin and tetracycline and named RC186. The presence of wild-type *smcR* on plasmid DNA was confirmed by PCR using the primers smcR-comp-1 and 2.

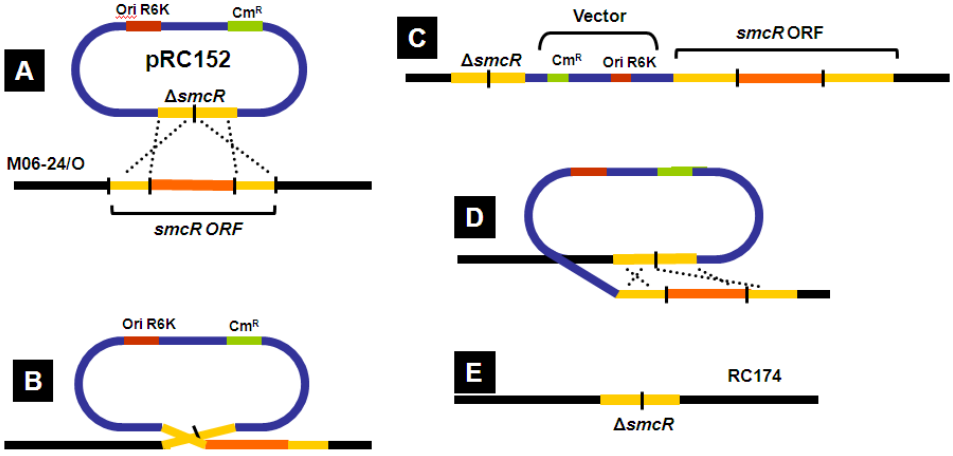


Figure 4. Schematic diagram for the construction of *Vibrio vulnificus* RC174 with deleted *smcR*. (A and B) The first homogenous recombination (dot line) occurs between the plasmid pRC152 and the chromosome of M06-24/O. **(B and C)** The intermediate form after the first homogenous recombination has transiently both mutated *smcR* and wild-type *smcR,* as well as the vector DNA. **(D)** The second homogenous recombination occurs within the chromosome and the vector DNA is expelled. **(E)** Finally, RC174 has only mutated *smcR*. Δ*gene*: deletional mutation of the gene, Cm^R: chloramphenicol resistance gene.

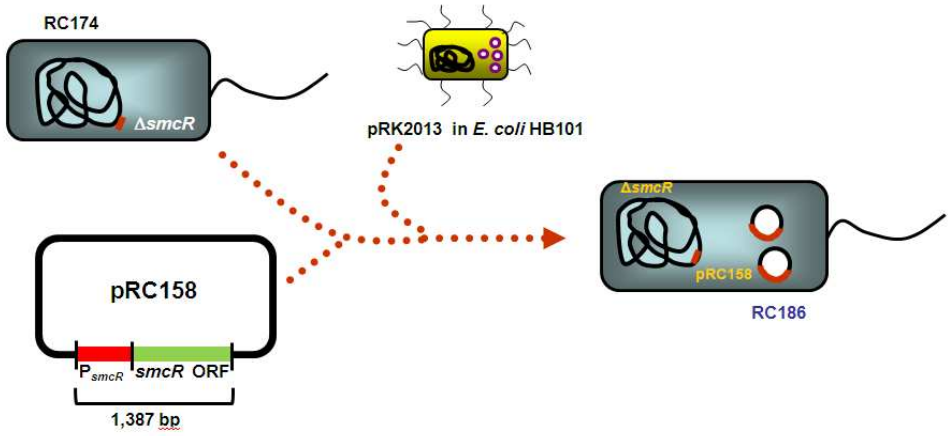


Figure 5. Schematic diagram for the construction of *Vibrio vulnificus* RC186 with *in trans* complemented *smcR*. The 1,387-kb fragment containing the promoter region and ORF of *smcR* was cloned into the plasmid pRC158. The resulting plasmid pRC158 was transferred into RC174 with mutated *smcR* by tri-parental mating using the conjugative helper plasmid pRK2013. Eventually, RC186 has wild-type *smcR* on plasmid DNA and mutated *smcR* on the chromosome. Δ*gene*: deletional mutation of the gene, P_{gene}: promoter of the gene.

7. Construction of the P_{vvpE}::*lacZ* transcriptional reporter strains

CMM2106 with the P_{vvpE}::*lacZ* transcriptional fusion and CMM2207 with the *luxS*-mutated P_{vvpE}::*lacZ* transcriptional fusion were constructed as previously described [22]. In this study, a deletion mutation of *smcR* was newly introduced into CMM2106 as described above, and the deletion of *smcR* was confirmed by PCR using the primers smcR-up-1 and smcR-down-2. The resulting strain with the *smcR*-mutated P_{vvpE}::*lacZ* transcriptional fusion was designated RC164. The schematic gene structures of the P_{vvpE}::*lacZ* transcriptional reporter strains are shown in Figure 6.

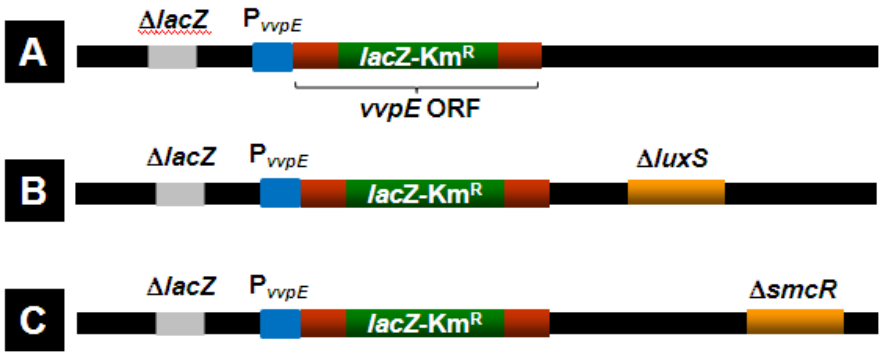


Figure 7. Schematic illustration for the gene structure of CMM2106 with the P_{vvpE}*::lacZ* transcriptional fusion (A), CMM2207 with the *luxS*-deleted P_{vvpE}*::lacZ* transcriptional fusion (B) and RC164 with the *smcR*-deleted P_{vvpE}*::lacZ* transcriptional fusion (C). Δ*gene*: deletional mutation of the gene, Km^R: kanamycin resistance gene.

8. Construction of a *pilD*-deleted mutant and its *in trans pilD*-complemented strain

A *pilD* deletion mutant was constructed in M06-24/O by crossover PCR. Two pairs of PCR primers PilD-1/PilD-2 and PilD-3/PilD-4 were used for the PCR amplification of the 5'- and 3'-ends of *pilD,* respectively. The resulting two PCR products were used as the template for the second PCR amplification using the PCR primers; PilD-1 with *Bam*HI overhang and PilD-4 with *Spe*I overhang. The *Bam*HI-*Spe*I fragment with deleted *pilD* was cloned into the suicide vector pDM4 [83]. The resulting plasmid was named pRC104, transformed into *Escherichia coli* SY327 λ*pir* and SM10 λ*pir* by electroporation [72], and subsequently transferred to M06-24/O by conjugation. Finally, a stable transconjugant was selected on TCBS agar containing chloramphenicol, subsequently on HI agar containing 10% sucrose, and named RC104. The deletion mutation of *pilD* was confirmed by PCR using the primers PilD-1 and PilD-4 (refer to Figure 4).

To restore wild-type *pilD* in RC104 with mutated *pilD*, the 1.81-kb *Bam*HI-*Hin*dIII fragment containing 870-bp *pilD* was amplified using the PCR primers; PilD-1 with a *Bam*HI overhang and PilD-5 with a *Hin*dIII overhang, and was subsequently subcloned into the broad host range vector pLAFR3II, which had been prepared by subcloning the *Bam*HI-*Bgl*II fragment containing the *bla* gene of pUTKm1 into the *Bgl*II site of pLAFR3 [84, 85]. The resulting plasmid was named pRC108, transferred into RC104 with mutated *pilD* by triparental mating using the conjugative helper plasmid pRK2013 [82]. Finally, a transconjugant was selected on TCBS agar containing ampicillin and tetracycline and named RC108. The presence of wild type *pilD* on plasmid DNA was confirmed by PCR using the primers PilD-1 and PilD-5 (refer to Figure 5).

7. Construction of merozygotic P_{pilD}::*lacZ* transcriptional reporter strains

The merozygotic P_{pilD}::*lacZ* transcriptional fusion was constructed by using the broad-host-range vector containing promoterless *lacZ* pQF52 [86] and R6K origin suicide vector pDM4 [83]. The 934-bp *Bgl*II-*Kpn*I fragment containing the promoter region of *pilD* was amplified using PCR primers; pili-rep-F with *Bgl*II overhang and pili-rep-R with *Kpn*I overhang, and was subcloned into pQF52. The resulting plasmid was named pRC154. The *Bgl*II-*Sca*I fragment containing the P_{pilD}::*lacZ* fragment from pRC154 was subsequently subcloned into pDM4 which was cut with *Bgl*II-*Sma*I. The resulting plasmid pRC156 was transformed into *Escherichia coli* SY327 λ*pir* and SM10 λ*pir* [72], and transferred to CMM2101 by conjugation. Finally, a transconjugant was selected on TCBS agar containing chloramphenicol and named RC176. The presence of the P_{pilD}::*lacZ* transcriptional fusion was confirmed by β-galactosidase assay [87]. Using the same method as above, RC182 with the *luxS*-mutated P_{pilD}::*lacZ* transcriptional fusion and RC184 with the *smcR*-mutated P_{pilD}::*lacZ* transcriptional fusion were constructed from CMM2202 and RC162, respectively (Table 1).

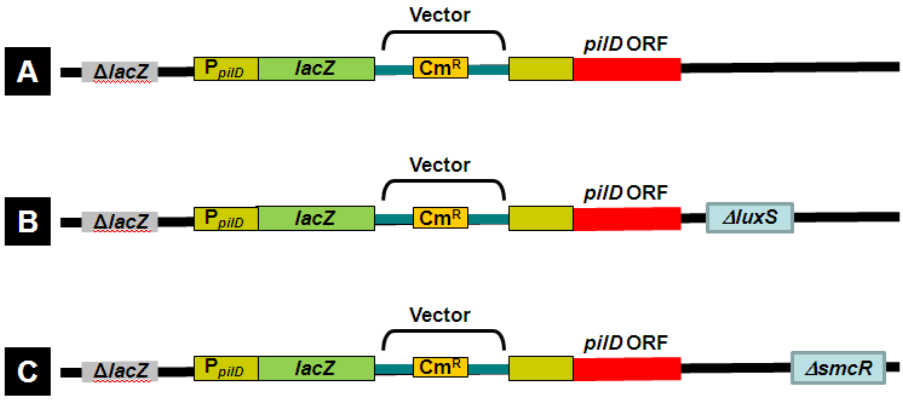


Figure 8. Schematic illustration for the gene structure of RC176 with the merozygotic P_{pilD}*::lacZ* transcriptional fusion (A), RC182 with the *luxS*-deleted merozygotic P_{pilD}*::lacZ* transcriptional fusion (B) and RC184 with the smcR-deleted merozygotic P_{pilD}*::lacZ* transcriptional fusion (C). ORF: open reading frame, Δ*gene*: deletional mutation of the gene, P_{gene}: promoter of the gene, Cm^R: chloramphenicol resistance gene.

Results

1. Regulation of VvpE production by AI-2-QSS

To observe the effect of *luxS* mutation on VvpE production, the three *V. vulnificus* strains were used; M06-24/O with wild-type *luxS*, CMM2201 with a mutated *luxS*, CMM2210 with *in trans* complemented *luxS* (Table 1). No growth difference was observed among the three strains (Figure 8A). VvpE production was observed by Western blot (Figure 8B). Two forms of VvpE with molecular sizes of 35 and 45 kDa began to be produced from the early growth phase and continued to increase throughout culture. VvpE with 35 kDa was the major form throughout culture. VvpE production was decreased and delayed in CMM2201 with mutated *luxS*. The decreased and delayed VvpE production was recovered to the wild-type level in CMM2210 with *in trans* complemented *luxS*. The production profile of VvpE appeared not to be changed by *luxS* mutation.

To observe the effect of *smcR* mutation on VvpE production, the three *V. vulnificus* strains were used; M06-24/O with wild-type *smcR*, RC174 with mutated *smcR*, RC186 with *in trans* complemented *smcR* (Table 1). No growth difference was observed among the three strains (Figure 8A). VvpE production was decreased and delayed in RC174 with mutated *smcR* (Figure 8B). The decreased and delayed VvpE production was recovered to the wild-type level in RC186 with *in trans* complemented *smcR*. VvpE production appeared to be more seriously decreased in RC174 with mutated *smcR* than in CMM2201 with mutated *luxS*.

Overall, these results clearly indicate that VvpE production is positively regulated by AI-2-QSS.

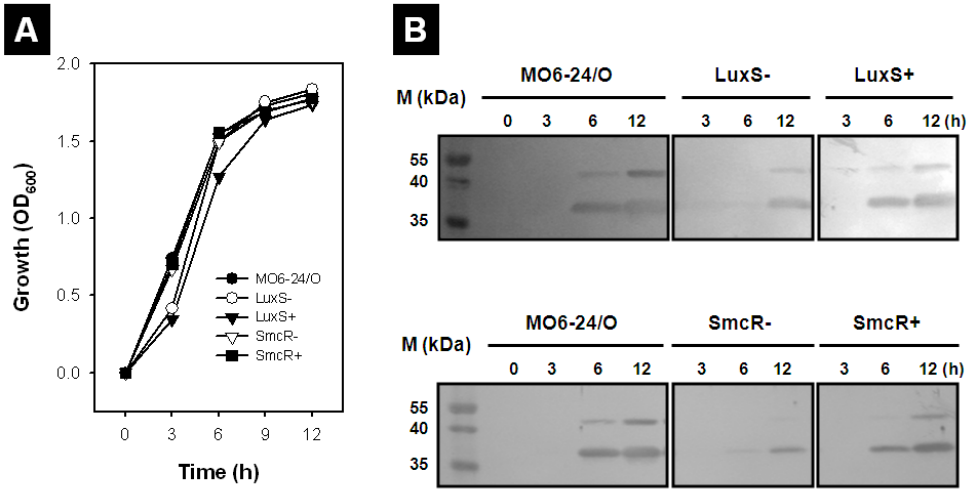


Figure 8. Regulation of VvpE production by the AI-2-mediated quorum sensing system. The five *V. vulnificus* strains were used; M06-24/O with wild type *luxS* or *smcR*, CMM2201 with mutated *luxS* (LuxS-), CMM2210 with *in trans* complemented *luxS* (LuxS+), RC174 with mutated *smcR* (SmcR-) and RC186 with *in trans* complemented *smcR* (SmcR+). The strains were inoculated into fresh HI broth containing 2.5% NaCl at 1×10^6 cells/ml, and cultured with vigorous shaking (220 rpm) at 37°C for 12 h. During culture, aliquots were obtained to monitor bacterial growth, and culture supernatants were obtained by centrifugating the aliquots to observe VvpE production. **(A)** Bacterial growth was monitored by measuring the optical densities of the culture aliquots at 600 nm (OD_{600}). **(B)** VvpE production was determined by Western blot, which was conducted as described in the Materials and Methods section. M: molecular size marker.

2. Regulation of VvpE production by PilD-II-GSS

To determine that VvpE is produced through PilD-II-GSS, the three strains were used; M06-24/O with wild-type *pilD*, RC104 with mutated *pilD* and RC108 with *in trans* complemented *pilD* (Table 1). No growth difference was observed among the three strains (Figure 9A). On Western blot, M06-24/O with wild-type *pilD* and RC108 with *in trans* complemented *pilD* began to produce VvpE 3 h after culture initiation (Figure 9B). VvpE with molecular size of 34 kDa was produced to higher levels throughout the culture period than VvpE with molecular size of 45 kDa. In contrast, RC104 with mutated *pilD* began to produce only a small amount of VvpE 6 h after culture initiation. The reason why a small amount of VvpE was detected in RC104 with mutated *pilD* only the late exponential or stationary growth phase might be because accumulated intracellular VvpE was released from autolyzed cells. Overall, these results clearly indicate that VvpE is produced via PilD-II-GSS.

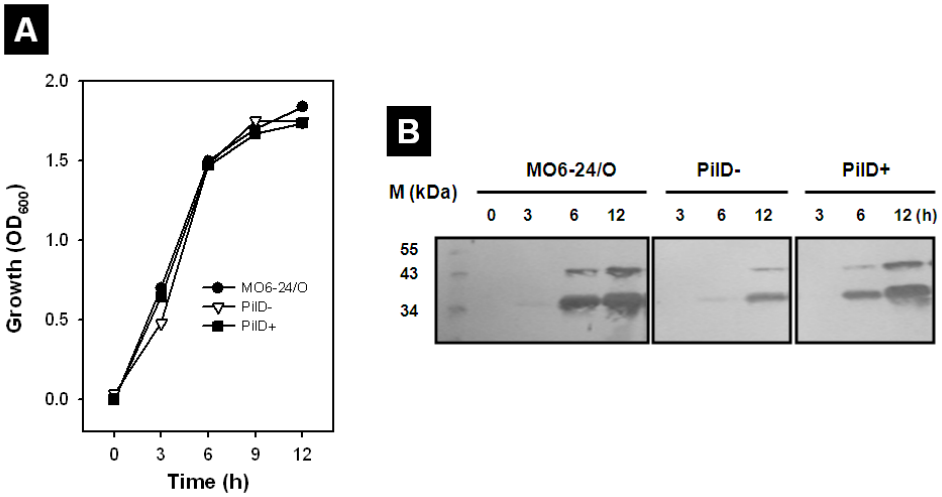


Figure 9. Regulation of VvpE production by the PilD-mediated type II general secretion system. The three *V. vulnificus* strains, M06-24/O with wild-type *pilD*, RC104 with mutated *pilD* (PilD-) and RC108 with *in trans* complemented *pilD* (PilD+), were cultured with vigorous shaking (220 rpm) in HI broth at 37^{o}C for 12 h. During culture, culture aliquots were obtained at the indicated times to monitor bacterial growth, and culture supernatants were obtained by centrifugation of the culture aliquots at 10,000 rpm for 5 min to observe VvpE production. **(A)** Bacterial growth was monitored by the measurement of the optical density at 600 nm (OD_{600}) of the culture aliquots. **(B)** VvpE production was determined by Western blot, which was carried out as described in the Materials and Methods section. M: molecular size marker.

3. Modulation of the AI-2-QSS activity by the shifting of temperature and salinity

To determine that the activity of AI-2-QSS was affected by the shifting of temperature and salinity, the transcriptional reporter strains were used; RC138 with the P_{luxS}*::lacZ* transcriptional fusion and RC196 with the P_{smcR}*::lacZ* transcriptional fusion (Table 1). Bacterial growth was more active at 37^{o}C than at 25^{o}C, and more affected by the shifting of temperature (25 to 37^{o}C) than by the shifting of salinity (2.5 to 0.9%) (Figures 10A and 10B). The transcriptional levels of *luxS* and *smcR* were determined by measuring β-galactosidase activity. The transcriptional level of *luxS* was far higher at 37^{o}C and in 0.9% salinity than at 25^{o}C and in 2.5% salinity (Figure 10C). The transcriptional level of *smcR* was far higher at 37^{o}C than at 25^{o}C (Figure 10D). However, the transcription level of *smcR* was the highest at 37^{o}C and in 2.5% salinity, which was different from the transcriptional level of *luxS*. Overall, these results clearly indicate that the total activity of AI-2-QSS is higher at 37^{o}C and in 0.9% salinity than at 25 ^{o}C and in 2.5% salinity.

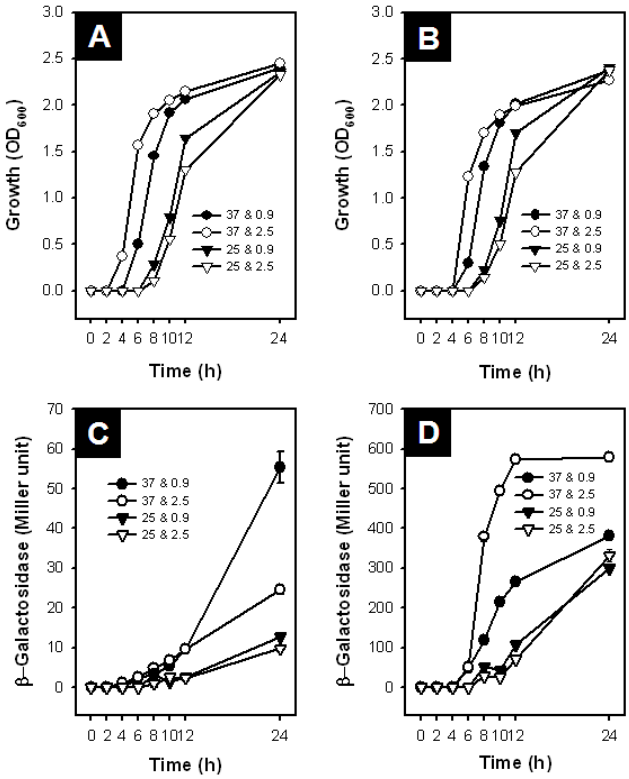


Figure 10. Modulation of the AI-2-QSS activity by the shifting of temperature and salinity. The two *V. vulnificus* strains, RC138 with the P_{luxS}*::lacZ* transcriptional fusion **(A and C)** and RC196 with the P_{smcR}*::lacZ* transcriptional fusion **(B and D)**, were cultured with vigorous shaking (220 rpm) in HI broth at 25/37^{o}C and in 2.5/0.9% salinity for 24 h. During culture, culture aliquots were obtained at the indicated times in order to monitor bacterial growth and to measure β-galactosidase activity. **(A and B)** Bacterial growth was monitored by the measurement of the optical density at 600 nm (OD_{600}) of the culture aliquots. **(C and D)** β-Galactosidase activity (Miller unit) was measured as previously described [87] and expressed as the means and standard errors of values measured in triplicate.

4. Modulation of *vvpE* expression by the shifting of temperature and salinity

To determine that the shifting of temperature and salinity modulates *vvpE* expression via AI-2-QSS, the three P_{vvpE}*::lacZ* transcription reporter strains were used; CMM2106 with wild-type *luxS* and *smcR*, CMM2207 with mutated *luxS*, and RC164 with mutated *smcR* (Table 1). Bacterial growth was more active at 37^{o}C than at 25^{o}C, and more affected by the shifting of temperature (25 to 37^{o}C) than by the shifting of salinity (2.5 to 0.9%). However, no growth difference was observed among the three strains (Figures 11A, 11B and 11C). The transcription level of *vvpE* was determined by measuring β-galactosidase activity. In CMM2106 with wild-type *luxS* and *smcR*, *vvpE* transcription began from the early growth phase and reached peak at the stationary growth phase, and began at low levels at low bacterial densities ($OD_{600} \leq 1.5$) and increased steeply at high bacterial densities ($OD_{600} \geq 1.5$) (Figure 11D). The transcription level of *vvpE* was severely decreased from the early growth phase in CMM2207 with mutated *luxS* (Figure 11E) and RC164 with mutated *smcR* (Figure 11F), and the decrease was more severe in RC164 with mutated *smcR* than in CMM2207 with mutated *luxS*. The transcription level of *vvpE* in CMM2106 with wild-type *luxS* and *smcR* was markedly increased by the shifting of temperature (25 to 37^{o}C) and salinity (2.5 to 0.9%). In contrast, the transcription level of *vvpE* in CMM2207 with mutated *luxS* and RC164 with mutated *smcR* was only slightly increased or not affected by the shifting. These results clearly indicate that *V. vulnificus* senses the shifting of temperature and salinity to modulate *vvpE* expression via AI-2-QSS.

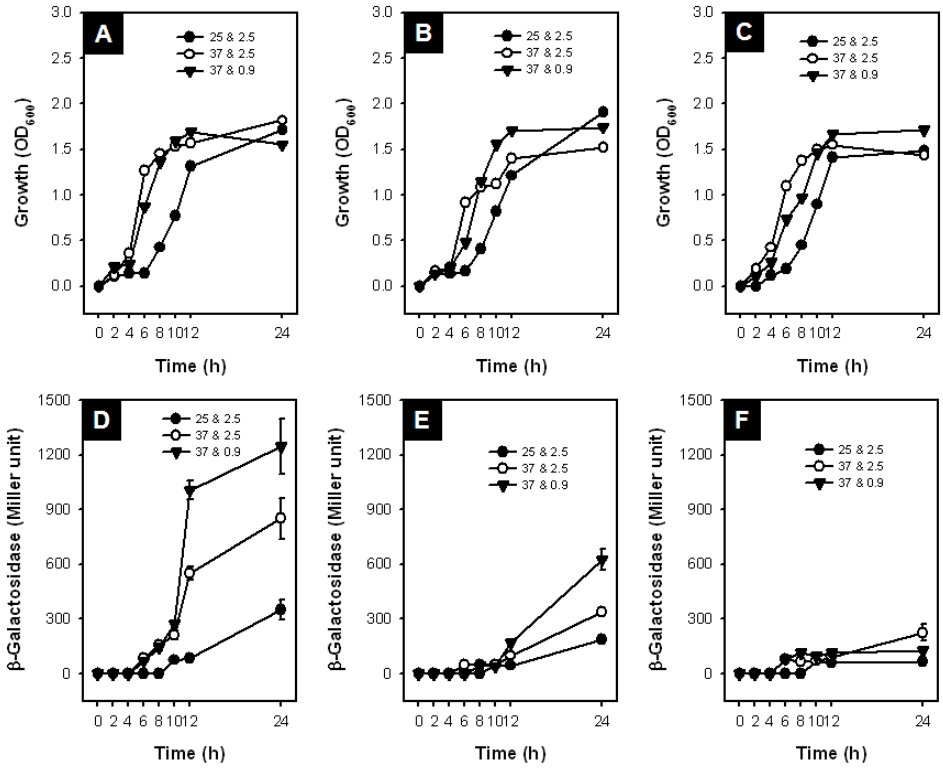
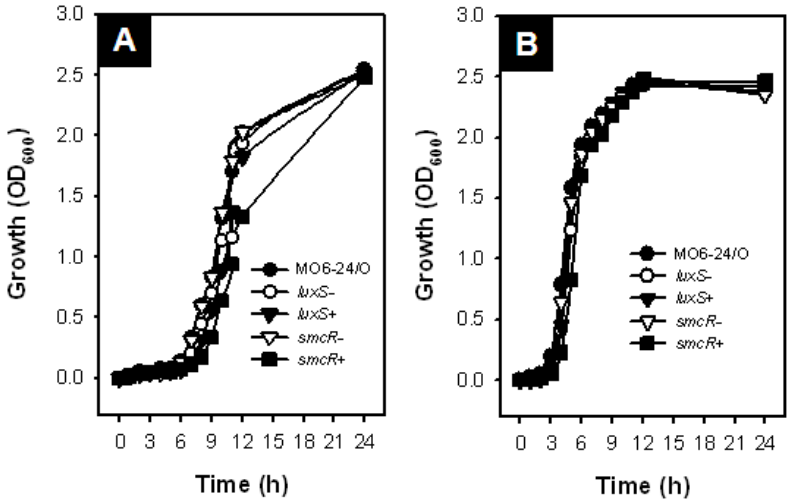


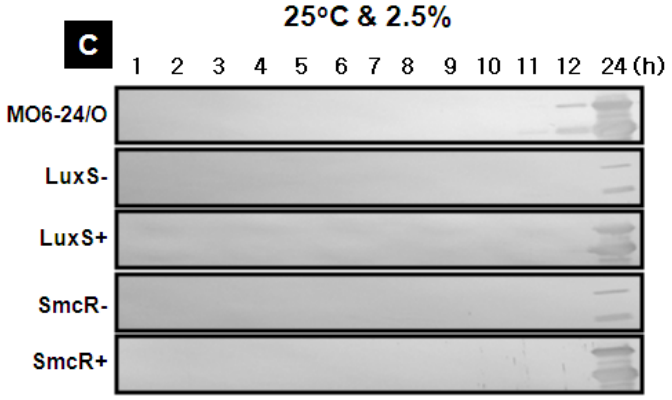
Figure 11. Modulation of *vvpE* expression by the shifting of temperature and salinity. The three P_{vvpE}*::lacZ* transcription reporter strains, CMM2106 with wild-type *luxS* or *smcR* **(A and D),** CMM2207 with mutated *luxS* **(B and E)** and RC164 with mutated *smcR* **(C and F)**, were cultured with vigorous shaking (220 rpm) at 25/37°C and in 2.5/0.9% salinity for 24 h. During culture, aliquots were obtained to monitor bacterial growth and β-galactosidase activity. **(A, B and C)** Bacterial growth was monitored by measuring the optical density of the culture aliquots at 600 nm (OD_{600}). **(D, E and F)** β-Galactosidase activity (Miller unit) in the culture aliquots was measured as previously described [87], and expressed as the means and standard errors of values measured in triplicate.

5. Modulation of the extracellular VvpE production by the shifting of temperature and salinity

To determine that the shifting of temperature and salinity modulates extracellular VvpE production via AI-2-QSS, the five strains were used; M06-24/O with wild-type *luxS* and *smcR*, CMM2201 with mutated *luxS*, and CMM2210 with *in trans* complemented *luxS*, RC174 with mutated *smcR*, and RC186 with an *in trans* complemented *smcR* (Table 1). Bacterial growth was more active at 37^{o}C and in 0.9% salinity than at 25^{o}C and in 2.5% salinity. However, no growth difference was observed among the five strains (Figures 12A and 12B). Extracellular VvpE production was determined by Western blot. When cultured at 25^{o}C and in 2.5% salinity (Figure 12C), extracellular VvpE production in all the five strains was observed only 12 or 24 h after culture initiation. When cultured at 37^{o}C and in 0.9% salinity (Figure 12D), extracellular VvpE production in M06-24/O began from the early growth phase, at low levels at low bacterial densities ($OD_{600} \leq 1.5$), and increased gradually even at high bacterial densities ($OD_{600} \geq 1.5$). Extracellular VvpE production was delayed and severely decreased from the early growth phase in CMM2201 with mutated *luxS* and RC174 with mutated *smcR*. The decrease was completely or partially recovered in CMM2210 with *in trans* complemented *luxS* and RC186 with *in trans* complemented *smcR*. Extracellular VvpE production was accelerated and increased by the shifting of temperature (25 to 37^{o}C) and salinity (2.5 to 0.9%), but the extent was far less in CMM2201 with mutated *luxS* and RC174 with a mutated *smcR* than in M06-24/O with wild-type *luxS* and *smcR*, CMM2210 with *in trans* complemented *luxS* or RC186 with *in trans* complemented *smcR*. The reason that extracellular VvpE production was facilitated even in CMM2201 with mutated *luxS* or RC174 with mutated *smcR* by the shifting of temperature

and salinity appeared to be because the PilD-II-GSS activity was also increased to produce more VvpE by the shifting, as described below. Overall, these results clearly indicate that *V. vulnificus* senses the shifting of temperature and salinity to modulate extracellular VvpE production via AI-2-QSS.


A
B
Growth (OD_{600})
Time (h)
MO6-24/O
luxS-
luxS+
smcR-
smcR+


C
25°C & 2.5%
1 2 3 4 5 6 7 8 9 10 11 12 24 (h)
MO6-24/O
LuxS-
LuxS+
SmcR-
SmcR+

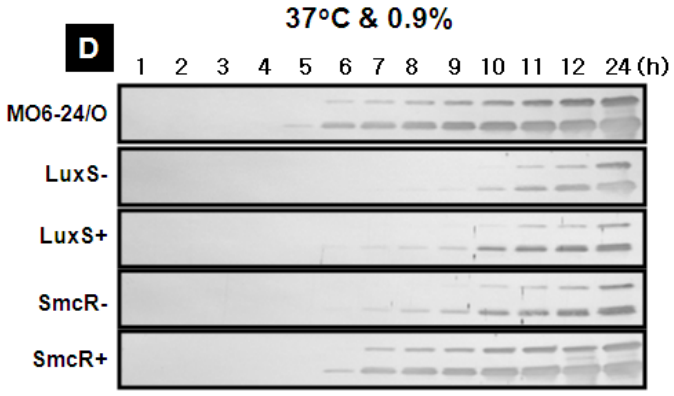

D
37°C & 0.9%
1 2 3 4 5 6 7 8 9 10 11 12 24 (h)
MO6-24/O
LuxS-
LuxS+
SmcR-
SmcR+

Figure 12. AI-2-QSS-mediated modulation of extracellular VvpE production by the shifting of temperature and salinity. The five *V. vulnificus* strains were used; M06-24/O with wild type *luxS* or *smcR*, CMM2201 with mutated *luxS* (LuxS-), CMM2210 with *in trans* complemented *luxS* (LuxS+), RC174 with mutated *smcR* (SmcR-) and RC186 with *in trans* complemented *smcR* (SmcR+). The strains were cultured with vigorous shaking (220 rpm) at 25/37^{o}C and in 2.5/0.9% salinity for 24 h. During culture, aliquots were obtained to monitor bacterial growth, and culture supernatants were obtained by centrifugating the aliquots to observe VvpE production. **(A and B)** Bacterial growth was monitored by measuring the optical densities of the culture aliquots at 600 nm (OD_{600}). **(C and D)** VvpE production was determined by Western blot, which was conducted as described in the Materials and Methods section.

6. Modulation of the PilD-II-GSS activity by the shifting of temperature and salinity

To determine that the PilD-II-GSS activity is modulated by the shifting of temperature and salinity, the three P_{pilD}*::lacZ* transcription reporter strains were used; RC176 with wild-type *luxS* and *smcR*, RC182 with mutated *luxS*, and RC184 with mutated *smcR* (Table 1). No noticeable growth difference was observed among the three strains (Figures 13A, 13B and 13C). The transcription level of *pilD* was determined by measuring β-galactosidase activity. In RC176 with wild-type *luxS* and *smcR* (Figure 13D), *pilD* transcription began at the early growth phase and reached peak at the stationary growth phase, and began at low levels at low bacterial densities ($OD_{600} \leq 1.5$) and increased gradually at high bacterial densities ($OD_{600} \geq 1.5$). The transcription level of *pilD* appeared to be slightly increased in RC182 with mutated *luxS* (Figure 13E) and RC184 with mutated *smcR* (Figure 13F) especially at 37^{o}C and in 0.9% salinity. No noticeable difference was observed between RC182 with mutated *luxS* and RC184 with mutated *smcR*. Regardless of the presence of *luxS* or *smcR* mutation, the transcription level of *pilD* was markedly increased by the shifting of temperature (25 to 37^{o}C) and salinity (2.5 to 0.9%), showing the highest level at 37^{o}C and in 0.9% salinity. These results clearly indicate that the shifting of temperature and salinity modulates the PilD-II-GSS activity.

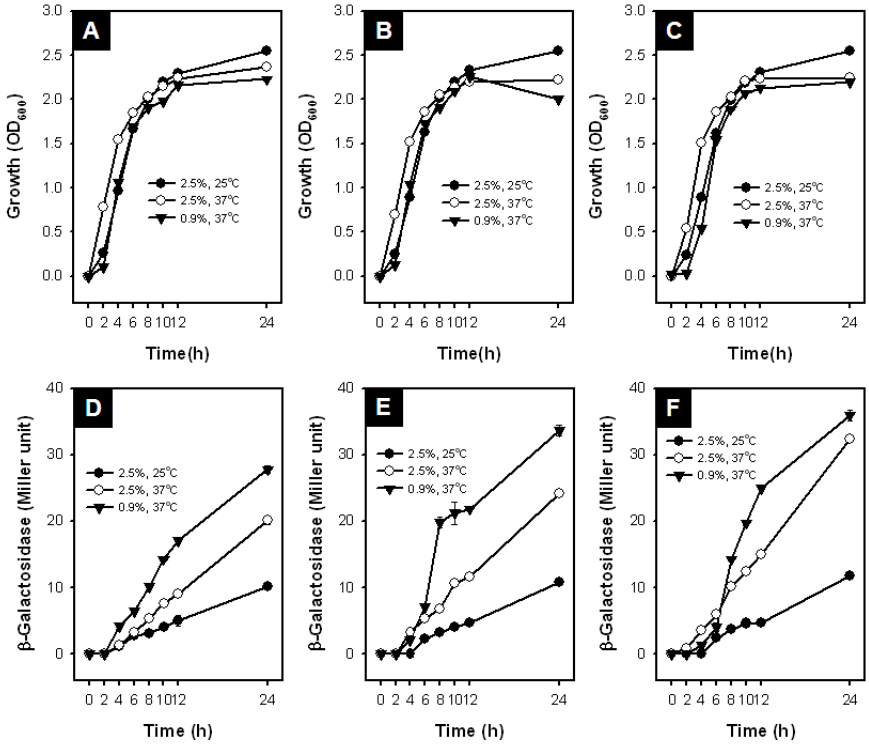


Figure 13. Modulation of the PilD-II-GSS activity by the shifting of temperature and salinity. The three P_{pilD}*::lacZ* transcription reporter strains, RC176 with wild-type *luxS* or *smcR* **(A and D)**, RC182 with mutated *luxS* **(B and E)** and RC164 with mutated *smcR* **(C and F)**, were cultured with vigorous shaking (220 rpm) at 25/37°C and in 2.5/0.9% salinity for 24 h. During culture, aliquots were obtained to monitor bacterial growth and β-galactosidase activity. **(A, B and C)** Bacterial growth was monitored by measuring the optical densities of the culture aliquots at 600 nm (OD_{600}). **(D, E and F)** β-Galactosidase activity (Miller unit) in the culture aliquots was measured as previously described [87], and expressed as the means and standard errors of values measured in triplicate.

Discussion

The present study has demonstrated that *V. vulnificus* senses the dual shifting of temperature and salinity to regulate VvpE production by up-regulating the activity of AI-2-QSS and PilD-II-GSS. Other environmental factors such as the change of iron concentration or oxygen tension are also known to affect *vvpE* expression or VvpE production, but details on these factors will be discussed elsewhere. In this study, only the relation between temperature/salinity and VvpE will be discussed.

As previously known [20-26], VvpE production was positively regulated by AI-2-QSS (Figure 8). The production level of VvpE was decreased and delayed by *luxS* or *smcR* mutation. Moreover, the present study revealed that VvpE production was more seriously affected by *smcR* mutation than by *luxS* mutation. The reason may be because SmcR regulates *vvpE* expression by directly binding to the promoter of *vvpE*, but LuxS is only one of the enzymes required for the AI-2 synthesis. The production profile of VvpE was not affected by *smcR* or *luxS* mutation; VvpE with 35 kDa was the major secreted form throughout culture, as previously described [53]. In addition, VvpE was produced from the early growth phase, as reported in our previous study [59]. SmcR functions as a transcriptional activator cooperatively with CRP mainly on the RpoS-dependent promoter S of *vvpE* [23], which means that AI-2-QSS generally operated mainly during the late growth phase or at high bacterial densities. However, our results clearly show that *luxS* or *smcR* mutation delays the beginning of VvpE production, as well as decreases the level of VvpE production. Accordingly, AI-2-QSS appears to function albeit at low levels from the early growth phase or at lower bacterial densities. However, this opinion can be proved only by directly observing *luxS* or *smcR* expression.

As previously known [53, 59, 70], VvpE was secreted through PilD-II-GSS (Figure 9). MO6-24/O and RC108 with wild-type *pilD* began to produce VvpE 3 h after culture initiation. In contrast, RC104 with mutated *pilD* began to produce only a small amount of VvpE beginning 6 h after culture initiation. A small amount of VvpE was detected in RC104 with mutated *pilD* only after the late exponential or stationary growth phase. Similar finding was also observed in our previous studies [53, 59]. Expression of *vvpE* was not affected by *pilD* mutation (data not shown). Accordingly, these results may be because intracellular VvpE, which had been being accumulated due to the blockage of II-GSS, was released from autolyzed bacterial cells which naturally occur and increase during the late growth phases.

PilD is a type IV leader peptidase/N-methyltransferase, a bi-functional enzyme that proteolytically cleaves the specialized leader sequence of type IV pilin precursors and induces *N*-methylation of the newly exposed N-terminal amino acid before assembly into the pilus structure [70]. However, the actual role of PilD in *V. vulnificus* is not known except that it is required for the secretion of VvpE via the type II general secretion system.

The present study clearly shows that the AI-2-QSS activity is modulated by the shifting of temperature and salinity. To directly determine the AI-2-QSS activity, the $P_{luxS::lacZ}$ or $P_{smcR::lacZ}$ transcriptional reporter strains were used. The *luxS* or *smcR* expression level was higher at 37^{o}C and in 0.9% salinity than at 25^{o}C and in 2.5% salinity (Figure 10). These results clearly indicate that the total activity of AI-2-QSS is higher at 37^{o}C and in 0.9% salinity than at 25 ^{o}C and in 2.5% salinity. In contrast, another research group reported that the activity of AI-2 measured by a bioassay and the level of *luxS* mRNA determined by RT-PCR were higher at 26^{o}C than at 37^{o}C, and so AI-2-QSS could function

effectively at 26°C than at 37°C [24]. We think that the direct measurement of *luxS* or *smcR* expression is more accurate and rational than the indirect measurement of AI-2 using a bioassay. RT-PCR is also too vulnerable to determine and compare transcriptional levels. Moreover, it may be inaccurate to determine transcriptional levels only at a time point. Accordingly, it is more desirable to serially measure and compare transcriptional levels with culture time, as in this study. This opinion was supported by a previous report, in which the indirect measurement using a bioassay was too vulnerable to accurately measure the activity of AI-2-QSS, and was recommended as a qualitative but not quantitative method [81]. However, another explanation may be possible. In this study, *V. vulnificus* strains were pre-conditioned by culturing at 25°C and in 2.5% salinity. In contrast, another research group [24] preconditioned *V. vulnificus* strains by culturing at 37°C. This difference of preconditioning may sufficiently affect *luxS or smcR* expression. However, *V. vulnificus* does not mutually circulate between natural habitats and the human body, but move only in one way from natural habitats to the human body. Therefore, the shifting from 25°C/2.5% to 37°C/0.9% salinity is thought to be more rational than the opposite shifting. Another noticeable finding was that *smcR* expression was the highest at 37°C and in 2.5% salinity, but *luxS* expression was the highest at 37°C and in 0.9% salinity. However, the condition of 37°C and 2.5% salinity is only an experimental condition, which cannot mimic the human body.

The finding that *V. vulnificus* senses the shifting of temperature and salinity to modulate the AI-2-QSS activity suggests that QSS may not recognize only bacterial population density, as described by other researchers. They proposed that QSS can be considered in the context of ‘diffusion sensing’ or ‘compartment sensing’ or even ‘efficiency sensing’, where the signal molecule

supplies information with respect to the local environment and spatial distribution of the cells rather than, or as well as, cell population density [10-14].

This study clearly shows that *vvpE* expression is regulated by AI-2-QSS. In transcription reporter assay, *vvpE* expression was down-regulated by *luxS* or *smcR* mutation, and more seriously affected by *smcR* mutation than by *luxS* mutation (Figure 11). The down-regulation of *vvpE* expression by either *luxS* or *smcR* mutation has also been observed by other researchers [20-26]. In this study, we compared the effect of *luxS* or *smcR* mutation on *vvpE* expression simultaneously under the same conditions. SmcR is the master regulator of AI-2-QSS. Without SmcR, AI-2-QSS cannot be operated by LuxS or AI-2 only. However, AI-2-QSS can be operated by only SmcR because LuxS is only one of the enzymes required for the synthesis of AI-2. Thus, the effect of *smcR* mutation on *vvpE* expression appears to be more serious than the effect of *luxS* mutation.

One of the most important findings in this study is that *vvpE* expression was up-regulated by the shifting of temperature and salinity from 25^{o}C and 2.5% to 37^{o}C and 0.9%, and this modulation was seriously inhibited by *luxS* or *smcR* mutation (Figure 11). This finding clearly indicates that *V. vulnificus* senses the shifting of temperature and salinity to modulate *vvpE* expression via AI-2-QSS. Like *vvpE* expression, extracellular VvpE production was up-regulated by the shifting of temperature and salinity from 25^{o}C and 2.5% to 37^{o}C and 0.9%, and this modulation was seriously inhibited by *luxS* or *smcR* mutation (Figure 12). The reason that extracellular VvpE production was facilitated by the shifting of temperature and salinity even in a *luxS* or *smcR*-mutated background appeared to be because the PilD-II-GSS activity was also increased to produce more VvpE by the shifting (Figure 13). In contrast,

another research group reported that *vvpE* expression measured by RT-PCR and extracellular VvpE activity were higher at 26^{o}C than at 37^{o}C [24]. As described above, RT-PCR is also too vulnerable to determine and compare transcriptional levels. Moreover, it may be inaccurate to determine transcriptional levels only at a time point. Accordingly, it is more desirable to serially measure and compare transcriptional levels with culture time, as in this study. A functional assay which measures proteolytic activity is also too vulnerable to compare VvpE production, especially when VvpE is produced at low levels, and gelatin-zymography or Western blot is known to be a more sensitive or useful method to measure VvpE production than the functional assay [59]. However, another explanation may be possible. In this study, *V. vulnificus* strains were pre-conditioned by culturing at 25^{o}C and in 2.5% salinity. In contrast, another research group [24] preconditioned *V. vulnificus* strains by culturing at 37^{o}C. This difference of preconditioning may sufficiently affect *vvpE* expression. However, *V. vulnificus* does not mutually circulate between natural habitats and the human body, but move only in one way from natural habitats to the human body. Therefore, the shifting from 25^{o}C and 2.5% salinity to 37^{o}C and 0.9% salinity is thought to be more rational than the opposite shifting.

The present study clearly shows that the shifting of temperature and salinity modulates the PilD-II-GSS activity. Regardless of the presence of *luxS* or *smcR* mutation, the transcription level of *pilD* was markedly increased by the shifting of temperature (25 to 37^{o}C) and salinity (2.5 to 0.9%), showing the highest level at 37^{o}C and in 0.9% salinity. This finding clearly indicates that *V. vulnificus* senses the shifting of temperature and salinity to modulate extracellular VvpE production via PilD-II-GSS. In addition, this finding answers to the

reason that extracellular VvpE production was facilitated by the shifting of temperature and salinity even in a *luxS* or *smcR*-mutated background.

II-GSS also controls the extracellular secretion of other several exoproteins including pilin, VvpE and cytolysin/hemolysin, all of which are putative virulence factors. Moreover, Loss of PilD results in significant decreases in CHO cell cytotoxicity, adherence to HEp-2 cells, and virulence in a mouse model [24]. More recently, it has been demonstrated that type IV pilin, which is exported via II-GSS, contributes to biofilm formation, adherence to epithelial cells, and virulence [88]. Accordingly, our results show that the shifting of temperature and salinity can stimulate the production of several virulence factors as mentioned above, and eventually increase the virulence of *V. vulnificus* by increasing the activity of II-GSS.

Virulence expression is a survival strategy for pathogenic bacteria. *Vibrio vulnificus* is an estuarine bacterium that inhabits sea water and an opportunistic human pathogen that can survive the human body. The two environments differ in many respects including temperature, salinity, and the type and concentration of nutrients. *V. vulnificus* should be able to overcome the changes of a variety of environmental factors and survive the human body for establishment of a successful infection [71]. Like other Vibrio species [72-74], *V. vulnificus* also appears to be able to sense environmental changes and correspondingly regulate virulence expression for survival [20, 24, 71]. Previously, it was demonstrated that the change of environmental factors, especially salinity and temperature, modulate the production of hemolysin, the most potent exotoxin produced by *V. vulnificus* [75, 76].

In addition, *V. vulnificus*, after entering the susceptible hosts, passes through gastric acidity, experiences an abrupt pH increase in the duodenum,

encounters bile secretion, invades into intestinal mucosa, and eventually enters bloodstream where the pathogen multiplies. During this complicated infection process, *V. vulnificus* should be able to sense changes in the environmental factors in the host milieu. The changing signals are likely relays to specific genes by cognate signal transduction systems, resulting in the expression of specific virulence factors [88]. Virulence factors required for *in vivo* survival and growth of *V. vulnificus* are expected to be produced at the right place and time in a tightly regulated fashion, as reported for other pathogens [89, 90]. Recently, a few *in vivo*-preferentially-expressed *V. vulnificus* antigens are identified [91]. Among them, *pyrH* encoding UMP kinase catalyzing phosphorylation of UMP to UDP is demonstrated to be an essential *in vivo* survival factor [92]. UMP kinase is known to sense the environmental pyrimidine pool and directly regulate pyrimidine-specific CarP1 promoter of carbamoylphosphate synthetase of *Escherichia coli* responsible for the early stage *de novo* synthesis of pyrimidines [93]. However, the mechanism is not identified in *Vibrio vulnificus*.

QSS is currently considered as a therapeutic target for novel antimicrobials. As widespread resistance to traditional antibacterial agents continues to pose a major threat, the demand for novel therapeutic approaches to the treatment of infection is increasing. The ability to switch off virulence gene expression exogenously may therefore offer a novel strategy for the treatment or prevention of infection. The discovery that diverse bacterial pathogens employ QSS to co-ordinate the control of virulence gene expression offers one such opportunity [94]. In particular, interference with transmission of the molecular message by an antagonist which competes for the AI-1 binding site of the transcriptional activator protein (a LuxR homologue) thereby switching off virulence gene expression so attenuating the pathogen, is an attractive strategy. In this

context, the ability of various AI-1 analogues to inhibit the action of the cognate AHL *in vivo* has been demonstrated [95]. Furthermore, furanone compounds produced by the Australian macroalgae, *Delisea pulchra*, is known to be able to competitively inhibit AHL-regulated processes including swarming in *Serratia liquefacuens* and bioluminescence in *Vibrio fisheri* [96]. To date, it is known that QSS is identified in most pathogenic bacteria as well as nonpathogenic bacteria. This ubiquity of QSS may offer a generic target for controlling bacterial infection.

Through this study, we found that *V. vulnificus* can sense the changes of temperature and salinity and can up-regulate VvpE production when it enters the human body. However, this finding does not directly indicate that VvpE production is more stimulated within the human body than under environmental conditions. Recently it was reported that *V. vulnificus* produces only small amount of VvhA *in vivo*, and such small amount of VvhA can produce other pathological manifestations such as hypotension other than mouse lethality [97]. One of the possible mechanisms that can suppress the *in vivo*-VvhA-expression is the catabolic repression by glucose contained in human and animal blood. It is well documented through *in vitro* experiments that glucose can suppress VvhA expression via the cAMP-CRP regulating system [98-100]. In A recent study, the transcription of *vvhA* in cirrhotic ascites, a human *ex vivo* system was more suppressed in the presence of glucose than in its absence [101]. Therefore, it is likely that the *in vivo*-expression of VvhA remains suppressed because of the absence of a potent transcriptional activator, CRP. Like VvhA production, VvpE production is also regulated by the cAMP-CRP regulating system [23, 68]. In addition, VvpE production is up-regulated by iron [24]. However, *in vivo*-iron-availability is very low. Accordingly, only a small amount of

VvpE would be produced *in vivo*, especially with the human body. Some studies reported that only a low level of *vvpE* transcription and a trace amount of VvpE is observed in human body fluids or under iron-limited conditions [57,58, 69].

Summary

This study shows that AI-2-QSS is involved in the modulation of *vvpE* expression by temperature and salinity. Along with bacterial growth, transcription of *luxS* encoding an enzyme for AI-2 synthesis and of *smcR* encoding a master regulator of AI-2-QSS was stimulated by the dual shifting of temperature (25^{o}C to 37^{o}C) and salinity (2.5% to 0.9%). Transcription of *vvpE* was stimulated by the shifting; this regulation was attenuated by *luxS* or *smcR* mutation, and was more greatly affected by *smcR* mutation than by *luxS* mutation. Extracellular VvpE production was stimulated by the shifting; this regulation was also attenuated by *luxS* or *smcR* mutation, and this attenuation was partially recovered by *in trans luxS* or *smcR* complementation. In addition, the shifting stimulated the expression of *pilD* encoding an essential component of II-GSS, which is responsible for extracellular VvpE secretion, even in the *luxS*- or *luxR*-mutated background. These results indicate that the shifting regulates VvpE production via AI-2-QSS and PilD-II-GSS in *V. vulnificus.*

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Abstract

Regulatory Mechanism of *Vibrio vulnificus* Metalloprotease Production by Temperaure and Salinity

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Vibrio vulnificus can survive in the two distinct environments, sea water and the human body, which differ in many respects including temperature, salinity, and the type and concentration of nutrients. Of these environmental factors, the change of temperature and salinity has been known as a signal that modulates the expression of *vvpE* encoding a metalloprotease VvpE in *Vibrio vulnificus,* but the mechanisms remains to be clarified. This study shows that the autoinducer-2-mediated quorum-sensing system (AI-2-QSS) involves the temperature and salinity-mediated modulation of *vvpE* expression, and that the type II general secretion system (II-GSS) involves the temperature and salinity-mediated modulation of extracellular VvpE production. Along with bacterial growth, transcription of *luxS* encoding an enzyme for AI-2 synthesis and of *smcR* encoding a master regulator of AI-2-QSS was stimulated by the dual shifting of temperature (25^{o}C to 37^{o}C) and salinity (2.5% to 0.9%). Transcription of *vvpE* was stimulated by the shifting; this regulation was attenuated by *luxS* or *smcR* mutation, and was more greatly affected by *smcR* mutation than by *luxS* mutation. Extracellular production of VvpE was stimulated by the shifting; this regulation was attenuated by *luxS* or *smcR* mutation, and this attenuation was partially

recovered by *in trans* complementation of wild type *luxS* or *smcR*. In addition, the shifting stimulated the expression of *pilD* encoding an essential component of II-GSS, which is responsible for extracellular VvpE secretion, even in the *luxS*- or *luxR*-mutated background. These results indicate that *V. vulnificus* senses the shifting of temperature and salinity to regulate VvpE production by up-regulating the AI-2-QSS activity at the gene expression level, and by up-regulating the PilD-mediated II-GSS activity at the protein secretion level, and can eventually upgrade its virulence for better survival within the human body.

Key words: *Vibrio vulnificus*, Temperature, Salinity, Metalloprotease, Autoinducer, Quorum-sensing system, Type II general secretion system

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	영문: Regulatory Mechanism of *Vibrio vulnificus* Metalloprotease Production by Temperaure and Salinity				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

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