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ORIGINAL PAPER

# Effects of temperature, growth phase and *luxO*-disruption on regulation systems of toxin production in *Vibrio vulnificus* strain L-180, a human clinical isolate

**Abdelaziz Elgaml · Kazutaka Higaki · Shin-ichi Miyoshi**

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**Abstract** *Vibrio vulnificus* is a halophilic estuarine bacterium while it causes fatal septicemia or necrotizing wound infections in humans. This pathogen secretes the metalloprotease (*V. vulnificus* protease: VVP) and the cytolysin (*V. vulnificus* hemolysin: VVH) as protein toxins; however, their production was coordinated in response to the bacterial cell density. This regulation is termed quorum sensing (QS) and is mediated by the small diffusible molecule called autoinducer 2 (AI-2). In the present study, we investigated effects of disruption of *luxO* encoding a central response regulator of the QS circuit, as well as effects of temperature and growth phase, on the toxin production by *V. vulnificus*. Disruption of *luxO* was found to increase VVP production and expression of its gene *vvpE*. The expression of *smcR*, *crp* and *rpoS*, of which products positively regulate *vvpE* expression, and *luxS* encoding the AI-2 synthetase were also significantly increased. On the other hand, the *luxO* disruption resulted in reduction of VVH production and expression of its gene *vvhA*. Expression of other two genes affecting the QS circuit, *luxT* and *rpoN*, were also significantly decreased. The regulation systems of VVP production were found to exert their action during the stationary phase of the bacterial growth and to be operated strongly at 26 °C. By contrast, those of VVH production apparently started at the log phase and were operated more effectively at 37 °C.

**Keywords** *Vibrio vulnificus* · Metalloprotease · Hemolysin · Quorum sensing · Autoinducer

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## Introduction

In pathogenic bacteria, coordinated regulation of the virulence gene expression is critical to successful colonization, invasion, in vivo growth and/or in situ toxin production. The bacterial virulence is often regulated by temperature, and this regulation occurs at both transcription and translation level (Hurme and Rhen 1998). Transfer from a natural reservoir to an infectious host offers a number of cues, which induce the signal transmission to turn on the virulence potentials (Hurme and Rhen 1998).

*Vibrio vulnificus* is a gram-negative halophilic estuarine bacterium, while it is an opportunistic human pathogen causing rapidly progressing fatal septicemia and necrotizing wound infection (Jones and Oliver 2009). The infectious diseases are preferentially in susceptible patients with hepatic diseases, hemochromatosis, heavy alcohol drinking habits, and other immunocompromised conditions (Jones and Oliver 2009). Virulence of *V. vulnificus* is multifactorial (Milton 2006), and the bacterium produces various kinds of virulent or toxic factors including capsular polysaccharides, type IV pili, hemolytic/cytolytic toxin, and proteolytic enzymes (Linkous and Oliver 1999; Strom and Paranjpye 2000). However, the metalloprotease (*V. vulnificus* protease: VVP/VvpE) and the hemolysin (*V. vulnificus* hemolysin: VVH/VvhA) are the most important toxins (Milton 2006). VVP causes serious hemorrhagic skin damage through digestion of the vascular basement membrane, especially type IV collagen forming the framework of the membrane (Miyoshi et al. 2001). It also elicits edema formation through induction of exocytotic histamine release from mast cells (Miyoshi et al. 2003) and/or activation of the factor XII-plasma kallikrein-kinin cascade (Miyoshi et al. 2004). On the other hand, VVH exhibits powerful hemolytic and cytolytic activities (Gray

and Kreger 1985), and it causes vasodilation and may play a significant role in hypotensive septic shock (Kook et al. 1996).

*Vibrio vulnificus* coordinates expression of virulence genes in response to the bacterial cell density. This regulation is termed quorum sensing (QS), which is mediated by the small diffusible signal molecule called autoinducer 2 (AI-2) (Federle and Bassler 2003; Henke and Bassler 2004). Indeed, *V. vulnificus* possesses LuxS (the AI-2 synthetase), LuxPQ (membrane bound sensor protein), LuxU-LuxO (the response regulators of QS circuit of *V. vulnificus*), and SmcR (the master transcriptional regulator for target genes controlled by the QS system) (McDougald et al. 2000, 2001; Shao and Hor 2001; Chen et al. 2003; Kim et al. 2003; Kawase et al. 2004). Moreover, five small RNAs (sRNAs) regulating SmcR were also predicted, as well as *Vibrio cholerae* and *Vibrio harveyi* (Lenz et al. 2004). Besides, Roh et al. (2006) identified LuxT as a negative regulator of SmcR. At low cell density, when the signal molecule AI-2 is absent, LuxPQ functions as kinase and it acts on LuxU and add phosphate group to the protein. The phosphorelay protein LuxU then transfers the phosphate group to LuxO. Therefore, at low cell density, LuxO remains phosphorylated which is the active form of the protein. Active LuxO, in association with sigma factor 54 RpoN, activates expression of sRNAs (small regulatory RNAs). The sRNAs along with sRNA binding protein Hfq represses the transcriptional regulator SmcR (Milton 2006). Also active LuxO activates the expression of LuxT, which is a negative regulator of SmcR (Roh et al. 2006). On the other hand at high cell density, when there is sufficient concentration of the signal molecule AI-2, it interacts with its specific sensor LuxPQ and converts its function from kinase to phosphatase. Subsequently the sensor protein dephosphorylates LuxO via LuxU. The dephosphorylated LuxO is inactive and it cannot activate the expression of sRNAs or LuxT. As a result SmcR is not inhibited any more. Therefore, at high cell density, SmcR functions actively and results in change of transcriptional status of the target genes (Milton 2006; Roh et al. 2006). In *V. vulnificus*, both AI-2 and SmcR positively regulate VVP production while negatively regulate VVH production (Shao and Hor 2001; Kim et al. 2003; Kawase et al. 2004). However, the primary target of *V. vulnificus* QS cascade may be VVP because VVP production is regulated more strongly (Milton 2006; Kim and Shin 2010, 2011). In addition to QS, two global regulators, namely RpoS (the stationary-phase sigma factor) and CRP (cAMP-receptor protein) responsible for catabolic repression, are known to control directly expression of *vvpE* encoding VVP (Kim and Shin 2011).

In the present study, we outlined effects of disruption of *luxO* on production of VVP and VVH, and on expression of their genes (*vvpE* and *vvhA*). In addition, we examined expression of genes consisting of the QS circuit (*luxS*, *luxT* and *smcR*) and those related to the QS cascade (*rpoN* that activates expression of sRNAs and *hfq* that acts together with sRNAs) (Milton 2006), *rpoS* and *crp*. The expression of *rpoD*, the house keeping sigma factor during log phase, was also examined. These experiments were carried out at two different temperatures, 26 °C (around estuarine temperature in the summer season) and 37 °C (around human intestinal temperature) and at different phases of the bacterial growth.

## Materials and methods

### Bacterial strains, plasmids and culture conditions

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

For cultivation of *V. vulnificus* strains, TYE broth (0.5 % tryptone, 0.25 % yeast extract, 2 % NaCl, 25 mM $K_2HPO_4$, pH 7.5) was used. Thiosulfate-citrate-bile-salts-

**Table 1** Bacterial strains and plasmids used

| Strain or plasmid | Relevant features[a] | References |
|---|---|---|
| *Vibrio vulnificus* | | |
| L-180 | Clinical isolate; virulent | Miyoshi et al. (1987a, b) |
| YY0507 | L-180 strain, *luxO*::Cm$^r$ | This study |
| AAER12 | Revertant strain of YY0507 | This study |
| *Escherichia coli* | | |
| SY327λ*pir* | △(*lac pro*), *argE*(Am), *rif*, *nalA*, *recA56*, *rpoB*, λ*pir*, Sm$^r$, host for π-requiring plasmids | Miller and Mekalanos (1988) |
| SM10λ*pir* | *thi-1*, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2-Tc::Mu, λ*pir*, *oriT* of RP4, Km$^r$; conjugational donor | Simon et al. (1983) |
| *Vibrio harveyi* | | |
| BB170 | Reporter strain used in auto-inducer 2 assay | Bassler et al. (1993) |
| BB152 | Positive control strain used in auto-inducer 2 assay | Bassler et al. (1993) |
| Plasmids | | |
| pKTN701 | *R6K*-ori suicide vector for gene replacement; Cm$^r$ | Nishibuchi et al. (1991) |
| pKTY0506 | pKTN701 with *luxO*; Cm$^r$ | This study |

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

sucrose (TCBS) agar plate containing chloramphenicol 10 μg/ml was used to select *luxO* mutants. In all experiments, *V. vulnificus* was cultivated in TYE broth (5 ml) at 37 °C with shaking overnight (strain YY0507, the *luxO* mutant, was cultivated in TYE broth containing chloramphenicol 10 μg/ml), and then, an aliquot of the first culture was taken and re-cultivated in fresh TYE broth at either 26 or 37 °C till reaching the desired growth phase.

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

## Construction of the *luxO* mutant and its revertant

The *luxO* mutant was constructed by the single crossover homologous recombination as described previously (Nishibuchi et al. 1991; Funahashi et al. 2002). A 781 bp region of the *luxO* was amplified by PCR using a primer set *luxO*-1, a forward primer containing the recognition sequence for *Xba*I (TCTAGA) and a reverse primer containing the recognition sequence for *EcoR*I (GAATTC) (Table 2), and digested with *Xba*I and *EcoR*I. The *Xba*I-*EcoR*I digested PCR product was inserted into the suicide vector pKTN701 (Nishibuchi et al. 1991). The hybrid plasmid obtained was transformed into *E. coli* SY327λ*pir*, then into *E. coli* SM10λ*pir*. Thereafter, it was transferred to *V. vulnificus* L-180 by conjugation, and the conjugants were cultivated on TCBS agar plates containing chloramphenicol 10 μg/ml. One suitable *luxO* mutant named strain YY0507 was selected by 48 h cultivation at 37 °C, and disruption of the *luxO* gene was confirmed by PCR. The revertant strain named AAER12 was obtained by repeated sub-culturing of strain YY0507 in LB broth at 37 °C, and reversion of the gene was confirmed by PCR.

## Measurement of bacterial growth

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

## RT-PCR

Total RNA was extracted from the bacterial cells cultivated at 26 or 37 °C at early log, late log or early stationary phase, by using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's manual. Total RNA thus obtained was added to the Ready-To-Go RT-PCR kit (GE Healthcare Bio-science, Buckinghamshire, UK) and incubated at 42 °C for 30 min for reverse transcription. Thereafter, the reverse transcriptase was inactivated by heating at 95 °C for 5 min, and PCR amplification with an appropriate primer set (Table 2) was performed as follows: 30 s denaturation at 95 °C, 30 s annealing at an appropriate temperature, and 60 s extension at 72 °C. The PCR products were electrophoresed on a 1.5 % agarose gel and visualized by staining with ethidium bromide and the intensity of bands were analyzed using imageJ program. The relative amount of each mRNA was estimated using the amount of mRNA of the house keeping gene *16s rRNA* as 1.0.

## Assay of VVP, VVH and AI-2 activity

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

The proteolytic activity of VVP was assayed with azocasein (Sigma-Aldrich, St. Louis, MO, USA) as described by Miyoshi et al. (1987a, b). Briefly, the sample was allowed to act at 30 °C for an appropriate time on 1.0 mg of azocasein in 0.6 ml of 50 mM Tris–HCl buffer (pH 8.0). The reaction was stopped by the addition of 1.4 ml of 5 % trichloroacetic acid. After centrifugation at 5,000*g* for 5 min, an aliquot of the supernatant was withdrawn and mixed with the same volume of 0.5 M NaOH. Thereafter, the absorbance at 440 nm was measured. One protease unit (PU) was defined as the amount of VVP hydrolyzing 1 μg of the substrate in 1 min. In these experiments, purified VVP was used as a positive control.

The hemolytic activity of VVH was assayed with 1 % sheep erythrocytes as described by Shinoda et al. (1985). Briefly, the sample (0.6 ml) was allowed to act on the erythrocytes (0.6 ml) at 37 °C for 2 h in 20 mM Tris–HCl buffer containing 0.9 % NaCl (pH 7.5). Thereafter, the reaction mixtures were centrifuged at 1,000*g* for 5 min, and the amount of hemoglobin released from the disrupted erythrocytes was determined by measuring the absorbance of the supernatant at 540 nm. One hemolysin unit (HU) was defined as the amount of VVH eliciting 50 % hemoglobin release. In these experiments, purified VVH was used as a positive control.

The AI-2 activity was measured using the reporter strain *V. harveyi* BB170 as described by Bassler et al. (1993).

**Table 2** Oligonucleotide primers used

| Gene | Accession number | Nucleotide sequence 5′–3′ | Position |
|---|---|---|---|
| *16s rRNA* | X76334 | | |
| | Forward | CATGATGCCTACGGGCCAAA | 171–190 |
| | Reverse | TGCCGCTATTAACGACACCAC | 447–467 |
| *luxO*-1[a] | NC_005139 | | |
| | Forward | GCTCTAGAGGTCACGGTAAACAACGCTATC | 354–375 |
| | Reverse | GCGAATTCGTTGTTAAGCACCACCACATTAC | 1,112–1,134 |
| *luxO*-2 | NC_005139 | | |
| | Forward | CCCTCTATCGCTCCTATCTCATGC | 68–91 |
| | Reverse | GTTCAAAGGGCTGGTTCAAAGGAG | 1,166–1,189 |
| *luxU* | NC_005139 | | |
| | Forward | CAGAAGAAAATTGCCTCGTTGACAG | 49–73 |
| | Reverse | TGCTACCACATCTTGCGTTTCATC | 295–318 |
| *luxT* | AE016796 | | |
| | Forward | GCCAAAGCGTAGTAAAGAAG | 3–22 |
| | Reverse | GAGACTTACCAAACAGCCAC | 423–442 |
| *smcR* | AF204737 | | |
| | Forward | GTTTCTGTGGCGACCGTCTTCAA | 389–411 |
| | Reverse | AGCGAGTAACAAATGCCGTGGAA | 743–765 |
| *luxS* | AF401230 | | |
| | Forward | CCAAAAGGCGACACCATTAC | 82–101 |
| | Reverse | CAACATCGCTTCTGGCAATG | 479–498 |
| *rpoD* | EF642870 | | |
| | Forward | ACTCAGCTTCGTAACAGCTACC | 1–22 |
| | Reverse | ATCAACCGCTTTCATCAGACCG | 567–588 |
| *rpoS* | AY163815 | | |
| | Forward | TATGCTCGACGTGCATTACG | 4–23 |
| | Reverse | TAACGCCTTCTCTCCATCTC | 452–471 |
| *rpoN* | CP002469 | | |
| | Forward | CAAGCTAGGTCAACAGTTAGCC | 21–42 |
| | Reverse | ACTGTTGGATGCGTTTGCGTAC | 568–589 |
| *hfq* | CP002469 | | |
| | Forward | ATGGCTAAGGGGCAATCTCTAC | 1–22 |
| | Reverse | TCTTCCGATTTCTCGCTTGGAC | 236–257 |
| *crp* | CP002469 | | |
| | Forward | TCAAACCGATCCAACACTAGA | 18–38 |
| | Reverse | ACCAGTTACGTCTAGGAATGC | 406–426 |
| *vvpE* | AB084580 | | |
| | Forward | CAACAGTAAAACGGGCCGTTATGAG | 618–642 |
| | Reverse | TTGAGCCGCTTTGACCACGCCGC | 1,418–1,440 |
| *vvhA* | M34670 | | |
| | Forward | AGATTAAGTGTGTGTTGCACACAAGCGGTG | 110–139 |
| | Reverse | ACCGAAAACAGCGCTGAAGGAAGAACGGTA | 894–923 |

[a] The artificial restriction enzyme site is indicated by the underline

Briefly, the reporter strain was cultured overnight in LB broth containing 3.0 % NaCl at 30 °C. The bacterial culture was diluted 1:5,000 with AB broth. An aliquot of the diluted culture (540 µl) was mixed with 60 µl of the sample, and the mixture was cultivated at 30 °C for 4 h with shaking. Thereafter, the intensity of bioluminescence (relative light unit: RLU) was measured with a luminometer K-210 (Kikkoman, Tokyo, Japan). In these

experiments, the supernatant from *V. harveyi* BB152, a mutant producing only AI-2, was used as the 100 % control.

### Western blot analysis

For Western blot analysis, proteins in the sample were precipitated by mixing with the same volume of 25 % tricloroacetic acid, and collected by centrifugation for 5 min at 15,000*g* at 4 °C. Thereafter, the protein collected was washed by 100 % ethanol, treated with 2 % sodium dodecyl sulfate (SDS) at 100 °C for 5 min and subjected to SDS-PAGE on the PhastSystem™ using a PhastGel™ Gradient 10–15 (GE Health Bio-Sciences). After SDS-PAGE, the proteins separated were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Health Bio-Sciences). The membrane with the bound proteins was then incubated with rabbit IgG antibody against VVP or VVH, and the antigen–antibody complex was visualized using the antibody against rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a chromogenic substrate 4-methoxy-1-naphthol and hydrogen peroxide. In these experiments, sterile TYE medium (the cultivation medium) was used as a negative control and purified VVP or VVH was used as a positive control.

### Statistical analysis

To evaluate the significance of difference in the results, all experiments were repeated at least three times, the data were compared by student *t* test, and the *P* values less than 0.05 were considered significantly different.

## Results

### Construction of the *luxO* mutant and its revertant

To clarify inactivation of the *luxO* gene, the RNA preparations from the *luxO* mutant (strain YY0507, *luxO*::Cm$^r$), the revertant (strain AAER12), as well as the wild type strain (strain L-180), were analyzed by RT-PCR. As shown in Fig. 1a, in the case of strain L-180 and AAER12, the significant transcription of *luxO* was observed; however, the *luxO* mRNA could not be detected in strain YY0507.

The *luxO* gene makes an operon with a downstream gene *luxU*. Therefore, the RT-PCR experiments targeting the *luxOU* mRNA also showed that null expression of the operon in the *luxO* mutant (Fig. 1b). However, a comparative amount of *luxU* mRNA was detected in all strains (Fig. 1b), indicating the *luxU* gene has own promoter and it functions normally in the *luxO* mutant.

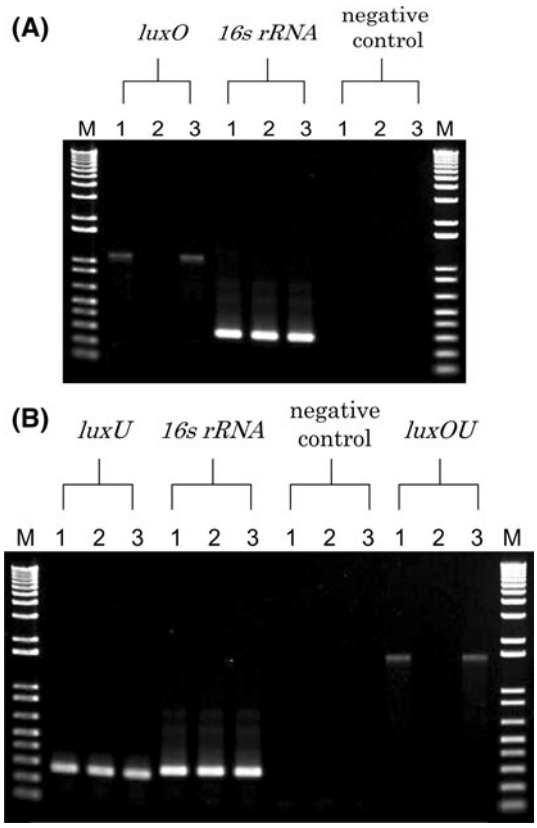


**Fig. 1** Expression of *luxO*, *luxOU* and *luxU*. In order to confirm inactivation of *luxO* in the *luxO* mutant strain YY0507 and its reversion in strain AAER12, total RNA was prepared from each strain, and RT-PCR to detect *luxO* (**a**), *luxOU* (**b**), or *luxU* mRNA (**b**) was carried out. The 16S rRNA was used as the positive control. *Lane M* 1-kb DNA ladder, *lane 1* strain L-180, *lane 2* strain YY0507 and *lane 3* strain AAER12

### Growth of the *luxO* mutant and its revertant

Strain YY0507 was found to grow slightly faster than the wild type strain at both 26 and 37 °C; however, the growth speed of strain AAER12, the revertant from strain YY0507, was the same as that of the wild type strain (Fig. 2).

### Expression of sigma factor genes (*rpoD*, *rpoS* and *rpoN*)

By disruption of *luxO* gene, the *rpoD* expression was significantly increased at log and early stationary phase, and the expression of *rpoS* was also increased at late log and early stationary phase (Fig. 3). On the other hand, disruption of *luxO* resulted in decrease in the expression of *rpoN* at late log and early stationary phase (Fig. 3). It should be noted that expression of *rpoD* and *rpoS* gene at 26 °C was higher than at 37 °C, but *rpoN* was more expressed at 37 °C (Fig. 3).

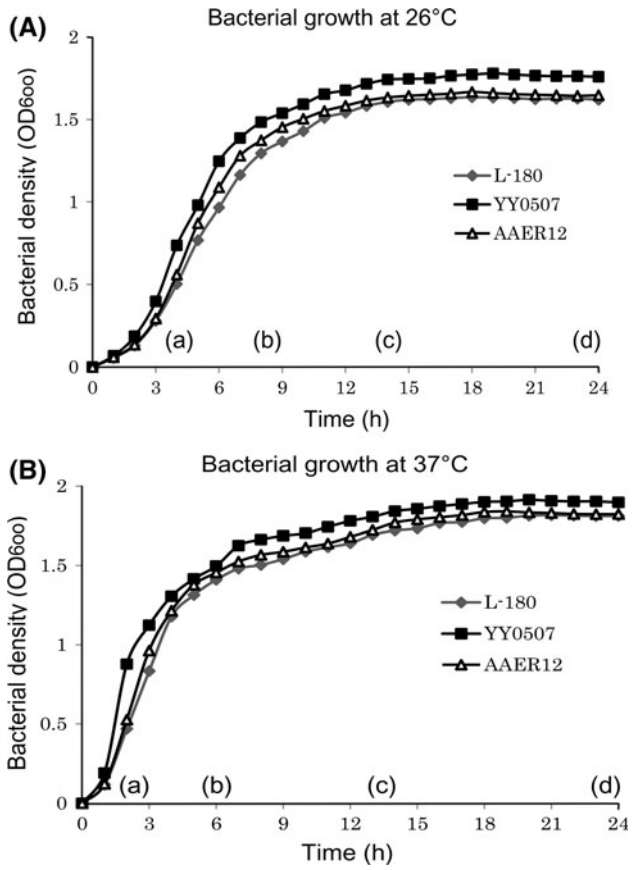


**Fig. 2** Growth of the bacterial strains at 26 °C (**a**) and 37 °C (**b**). Strain L-180 (*grey diamond*), YY0507 (*black square*) and AAER12 (*white triangle*) were cultivated in TYE broth at 26 or 37 °C, and the optical density at 600 nm ($OD_{600}$) was measured every 1 h. Thereafter, early log phase (*a*), late log phase (*b*), early stationary phase (*c*), and stationary phase (*d*) were determined. *Data* represent the mean of three experiments

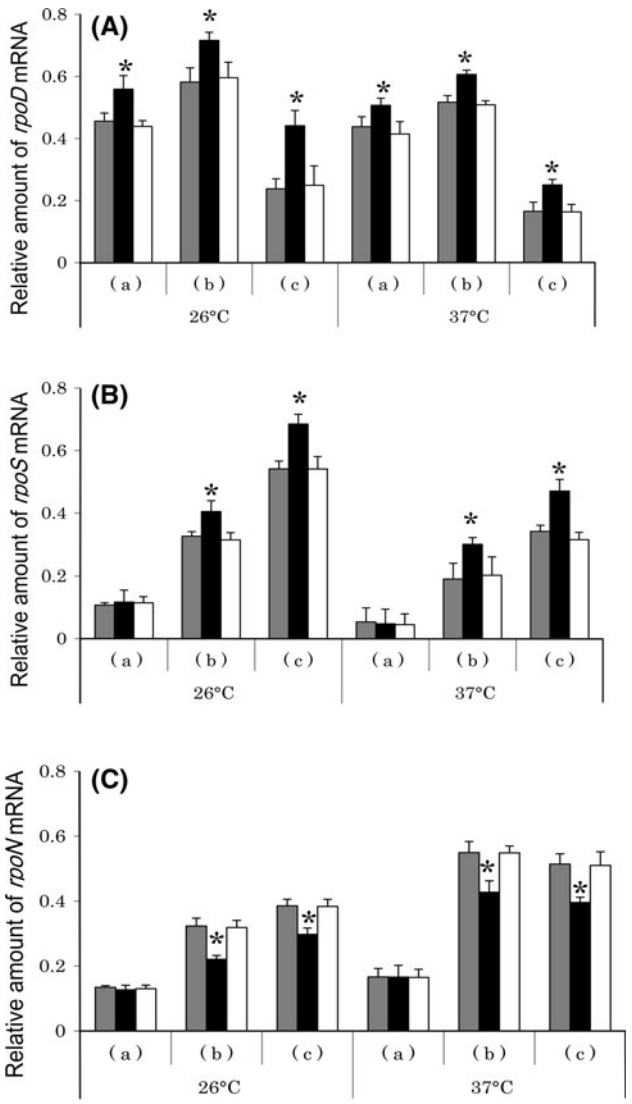


**Fig. 3** Effect of *luxO* disruption on expression of *rpoD* (**a**), *rpoS* (**b**) and *rpoN* (**c**). Strain L-180 (*grey bar*), YY0507 (*black bar*) and AAER12 (*white bar*) were cultivated in TYE broth at 26 or 37 °C, total RNA was extracted at early log phase (*a*), late log phase (*b*) and early stationary phase (*c*), and the level of mRNA was measured by RT-PCR. Thereafter, PCR products were electrophoresed on 1.5 % agarose gel, visualized by staining with ethidium bromide and analyzed by imageJ program. The amount of mRNA was represented using the amount of *16s rRNA* as 1.00. The data is the mean + SD of three experiments. The *asterisk* indicates the significant difference ($P < 0.05$) between strain YY0507 and both strain L-180 and AAER12

## Expression of the QS cascade genes (*luxS*, *luxT* and *smcR*), *crp* and *hfq*

As shown in Fig. 4, disruption of *luxO* resulted in significant increase in the expression of *luxS*, *smcR* and *crp* genes at late log and early stationary phase, and these genes were expressed more at 26 °C. By contrast, expression of *luxT* was decreased by disruption of *luxO*, and the transcription level of *luxT* was higher at 37 °C. On the other hand, no significant difference of *hfq* expression was observed by disruption of *luxO* gene (data not shown).

## Expression of *vvpE* and *vvhA* gene

Expression of *vvpE* and *vvhA* was highly dependent on the bacterial growth and cultivation temperature (Fig. 5). Namely, *vvpE* expression was maximum at early stationary phase and was distinctly higher at 26 °C. Although transcription of *vvpE* gene was started at log phase, upon the entry in the stationary phase, its level was increased about 10 times. On the other hand, *vvhA* was most expressed at log phase at 37 °C.

The disruption of *luxO* gene showed apparent increase in the *vvpE* expression. As shown in Fig. 5a, the transcription level of *vvpE* gene in the *luxO*-disrupted strain YY0507 was markedly higher than that of strain L-180 and AAER12. By contrast, the *luxO* disruption resulted in significant decrease in the expression of *vvhA* (Fig. 5b).

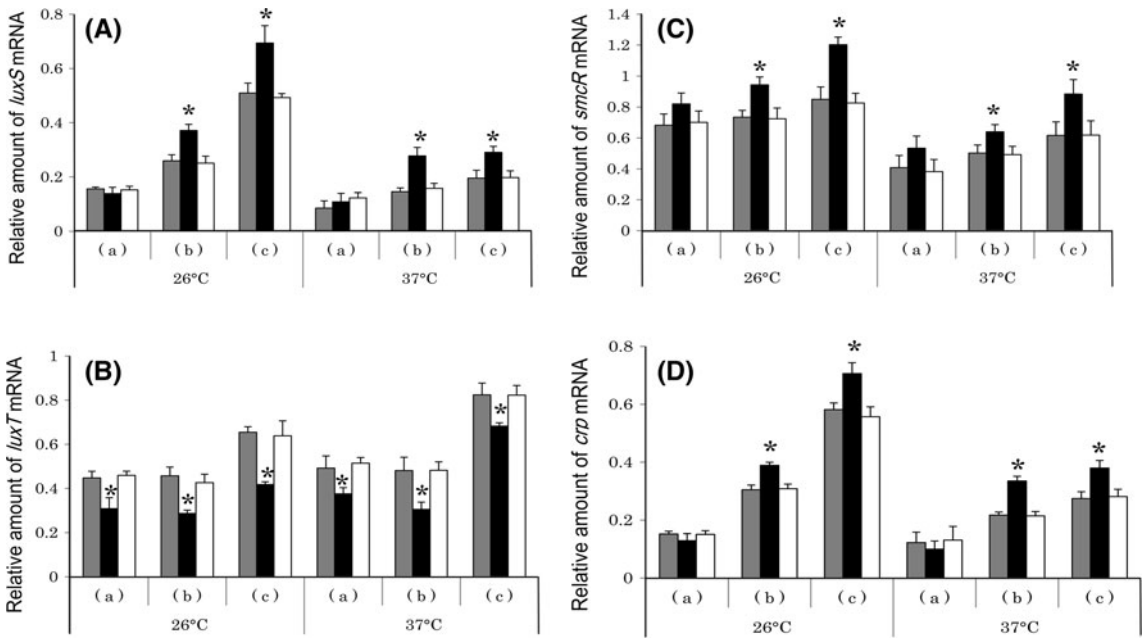


**Fig. 4** Effect of *luxO* disruption on expression of *luxS* (**a**), *luxT* (**b**) and *smcR* (**c**) and *crp* (**d**). Strain L-180 (*grey bar*), YY0507 (*black bar*) and AAER12 (*white bar*) were cultivated in TYE broth at 26 or 37 °C, total RNA was extracted at early log phase (*a*), late log phase (*b*) and early stationary phase (*c*), and the level of mRNA was measured by RT-PCR. Thereafter, PCR products were electrophoresed on 1.5 % agarose gel, visualized by staining with ethidium bromide and analyzed by imageJ program. The amount of mRNA was represented using the amount of *16s rRNA* as 1.00. The data is the mean + SD of three experiments. The *asterisk* indicates the significant difference ($P < 0.05$) between strain YY0507 and both strain L-180 and AAER12

### Production of VVP, VVH and AI-2

The VVP activity in the culture supernatant was highly dependent on the bacterial growth and cultivation temperature. Upon the entry in the stationary phase, the activity was increased about 10 times of that at log phase, and the activity was distinctly higher at 26 °C (Fig. 6a). By Western blot analysis, the VVP antigen could not be detected at early log phase; however, the antigen was steadily detected at late log and stationary phase (Fig. 6b). The disruption of *luxO* caused increase in production of VVP. The activities of VVP in the culture supernatants from strain YY0507 were significantly higher than those of strain L-180 and AAER12, and more steadily bands of the VVP antigen were detected when the culture supernatants of strain YY0507 were analyzed by western blotting (Fig. 6).

The VVH activity in the culture supernatant was also dependent on the bacterial growth and cultivation temperature. However, in contrast to the VVP activity, the VVH activity was apparently higher when cultivated at 37 °C, and the highest activity was detected in the culture supernatant at late log phase (Fig. 7). Additionally, disruption of *luxO* gene resulted in significant decrease in the activity caused by VVH at both cultivation temperatures (Fig. 7).

As well as VVP and VVH, the activity of AI-2 was highly dependent on the bacterial growth and cultivation temperature. The activity reached a maximum level at early stationary phase of cultivation at 26 °C (Fig. 8). The *luxO*-disrupted mutant YY0507 showed the higher activity than the wild type strain and the revertant strain, indicating negative regulation of *luxS* by LuxO.

## Discussion

*Vibrio vulnificus* inhabits sea water or brackish water, but it can cause infection in human. During the infection process, *V. vulnificus* must sense and sustain changes in environmental factors. The most important environmental difference in many respects is the temperature (Lee et al. 2007). Signals from the changing environmental factors are relayed to specific genes by cognate signal transduction systems, resulting in the expression of genes including specific virulence factor genes. Virulence factors required for in vivo survival and growth are produced at an appropriate place and time in a tightly regulated fashion (Heithoff et al. 1997; Lee et al. 1999; Kim et al. 2007). In *V. vulnificus*, VVP is the best-known virulence factor regulated by various environmental signals (Jeong et al. 2003; Kim et al. 2003; Roh et al. 2006; Kim and Shin 2011), and

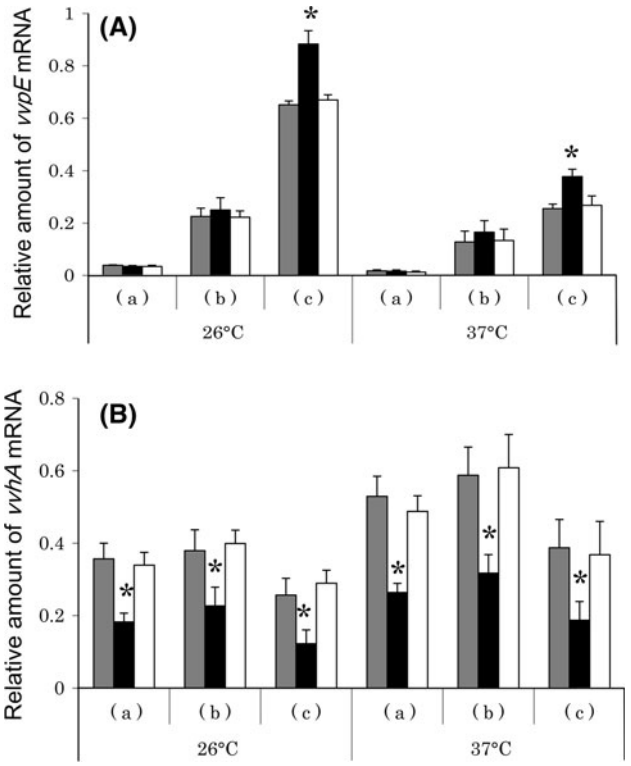


**Fig. 5** Effect of *luxO* disruption on expression of *vvpE* (**a**) and *vvhA* (**b**). Strain L-180 (*grey bar*), YY0507 (*black bar*) and AAER12 (*white bar*) were cultivated in TYE broth at 26 or 37 °C, total RNA was extracted at early log phase (*a*), late log phase (*b*) and early stationary phase (*c*), and the level of mRNA was measured by RT-PCR. Thereafter, PCR products were electrophoresed on 1.5 % agarose gel, visualized by staining with ethidium bromide and analyzed by imageJ program. The amount of mRNA was represented using the amount of *16s rRNA* as 1.00. The data is the mean SD of three experiments. The *asterisk* indicates the significant difference ($P < 0.05$) between strain YY0507 and both strain L-180 and AAER12

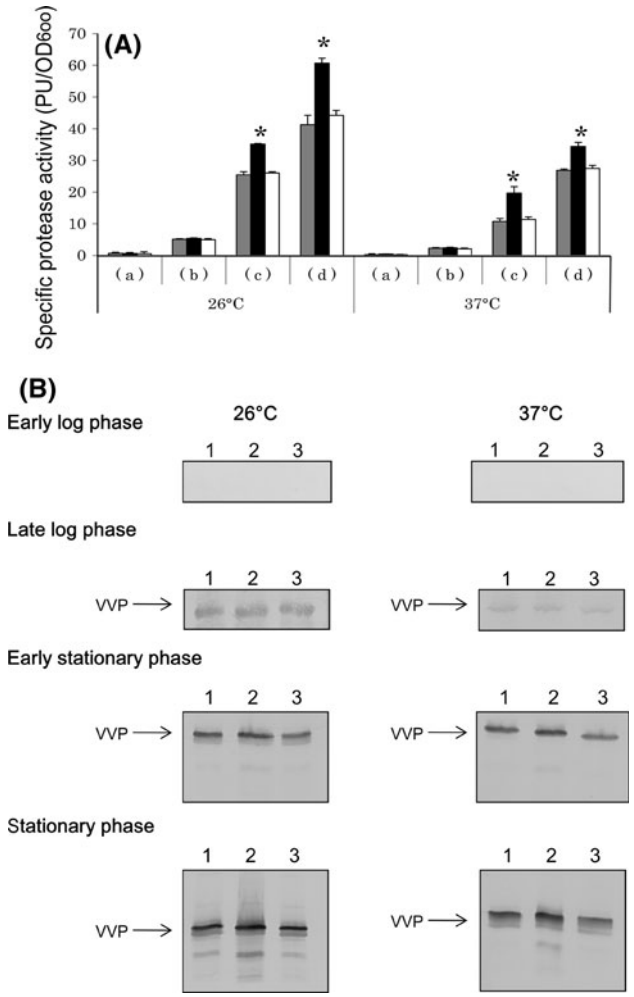


**Fig. 6** The activity of VVP in the culture supernatants (**a**) and Western blot analysis of the VVP antigen (**b**). **a** Culture supernatants were prepared from early log phase (*a*), late log phase (*b*), early stationary phase (*c*) and stationary phase (*d*) of growth of L-180 (*grey bar*), YY0507 (*black bar*) and AAER12 (*white bar*). The activities of VVP (PU/ml) in the culture supernatants were determined using azocasein as substrate, and the specific protease activity ($PU/OD_{600}$) was calculated. The data is the mean + SD of three experiments. The *asterisk* indicates the significant difference ($P < 0.05$) between YY0507 and both strain L-180 and AAER12. In these experiments, purified VVP was used as a positive control, and the cultivation media used as a negative control showed no activity. **b** Western blot analysis of the VVP antigen was performed by precipitating the proteins in the collected culture supernatants by adding the same volume of 25 % tricloroacetic acid, the protein pellets was washed by 100 % ethanol, dissolved by suspending in SDS sample buffer and boiled for 5 min, and an aliquot of each preparation was subjected to SDS-PAGE. Thereafter, the proteins were transferred to a PVDF membrane, and VVP antigens were detected with the antibody against purified VVP. Purified VVP was used as a positive control and cultivation medium used as a negative control. *Lane 1* strain L-180, *lane 2* strain YY0507 and *lane 3* strain AAER12

three global regulators, RpoS, SmcR, and CRP, have been reported to control directly production of VVP (Chiang and Chuang 2003; Kim and Shin 2010, 2011). Here we studied the effects of disruption of *luxO*, which encodes the master regulator of QS cascade, on the expression of the genes of three global regulators at different temperatures, 26 and 37 °C at different phases of the bacterial growth. Also, the effects of *luxO* disruption on the expression of sigma factor genes, the QS cascade genes, *vvpE* and *vvhA* genes, and production of VVP, VVH and AI-2 were examined.

Our results demonstrated that *V. vulnificus* produced two protein toxins, VVP and VVH, at specific times of the bacterial growth in a tightly regulated fashion. As summarized in Table 3, disruption of *luxO* showed increased expression of *rpoS*, *luxS*, *smcR*, *crp* and *vvpE* at stationary phase. Production of AI-2 and VVP was also distinctly higher in the *luxO* mutant. However, at log phase, the *vvpE* expression and VVP production was not increased. These results verified the critical roles of LuxO in the regulation of *V. vulnificus* QS system. Two promoters, promoter L (PL) and promoter S (PS), are known to direct differentially transcription of the *vvpE* gene in a growth phase-

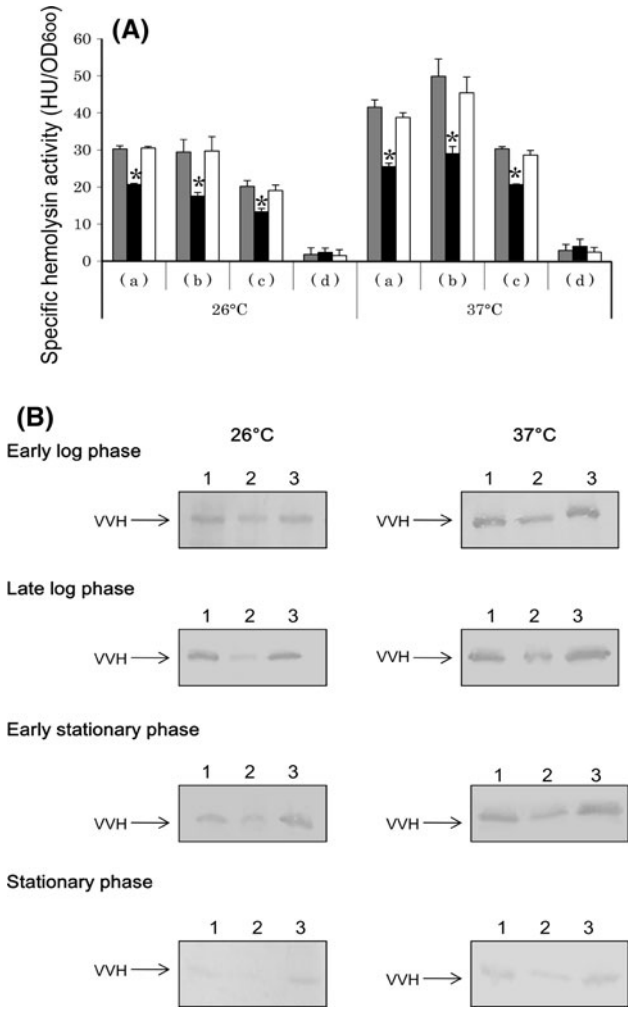


**Fig. 7** The activity of VVH in the culture supernatants (**a**) and Western blot analysis of the VVH antigen (**b**). **a** Culture supernatants were prepared from early log phase (*a*), late log phase (*b*), early stationary phase (*c*) and stationary phase (*d*) of growth of L-180 (*grey bar*), YY0507 (*black bar*) and AAER12 (*white bar*). The activities of VVH (HU/ml) in the culture supernatants were determined using 1 % sheep erythrocytes, and the specific hemolysin activity ($HU/OD_{600}$) was calculated. The data is the mean + SD of three experiments. The *asterisk* indicates the significant difference ($P < 0.05$) between YY0507 and both L-180 and AAER12. In these experiments, purified VVH was used as a positive control, and the cultivation media used as a negative control showed no activity. **b** Western blot analysis of the VVH antigen was performed by precipitating the proteins in the collected culture supernatants by adding the same volume of 25 % tricloroacetic acid, the protein pellets was washed by 100 % ethanol, dissolved by suspending in SDS sample buffer and boiled for 5 min, and an aliquot of each preparation was subjected to SDS-PAGE. Thereafter, the proteins were transferred to a PVDF membrane, and VVH antigens were detected with the antibody against purified VVH. Purified VVH was used as a positive control and cultivation media used as a negative control. *Lane 1* strain L-180, *lane 2* strain YY0507 and *lane 3* strain AAER12

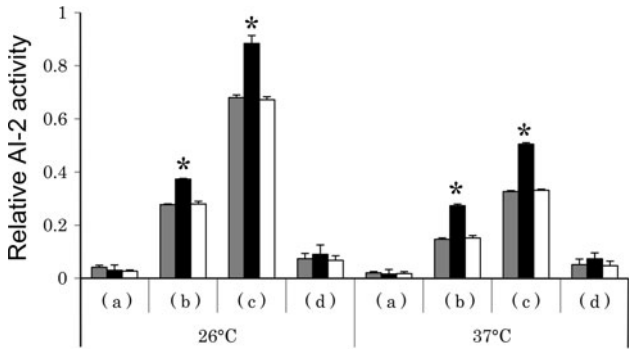


**Fig. 8** AI-2 activity in the culture supernatants. Culture supernatants were prepared from early log phase (*a*), late log phase (*b*), early stationary phase (*c*) and stationary (*d*) of growth of L-180 (*grey bar*), YY0507 (*black bar*) and AAER12 (*white bar*). Culture supernatants of test bacteria (60 µl) were added to the diluted reporter strain *V. harveyi* BB170 (540 µl) and incubated at 30 °C with shaking for 4 h. Bioluminescences of 600 µl aliqoutes of samples were measured with a luminometer, Lumitester K-210 (Kikkoman, Tokyo, Japan) that measured the amount of bioluminescence in relative luminescence unit (RLU). In these experiments, the supernatant from *V. harveyi* BB152 was used as a positive control (RLU 1.0), and the cultivation media used as a negative control showed no activity. The data is the mean + SD of three experiments. The *asterisk* indicates the significant difference ($P < 0.05$) between YY0507 and both L-180 and AAER12

dependent manner (Jeong et al. 2001, 2003, 2010). The PL activity is constitutive through the log and stationary phases, but is lower than the PS activity. The transcription from PS is induced only in the stationary phase and is dependent on RpoS, CRP and SmcR. These findings put the light on the exact time of SmcR, CRP and RpoS regulation of *vvpE* expression, namely, the regulation occurs only upon the entry to the stationary phase. Because transcription of *vvpE* from PL is starting from the log phase, RNA polymerase with RpoD, the log phase housekeeping sigma factor, would recognize the PL. However, in the present study, it was not confirmed that RpoD has significant role in VVP production during log phase. Although *rpoD* expression was significantly increased in the *luxO* mutant, *vvpE* transcription and VVP production were not significantly different from both the wild type strain and the revertant strain.

As summarized in Table 4, it is clear that both the cultivation temperature and growth phase are very critical determinants for regulation of expression *vvpE* and *vvhA* gene. The transcription level of *vvpE* and production of VVP were distinctly higher at 26 °C. It is also noteworthy that the expression of genes encoding positive regulators of *vvpE* (*smcR*, *rpoS* and *crp*) and AI-2 synthetase (*luxS*), and the AI-2 production were also higher at 26 °C. Taken together, it is concluded that the regulation systems for VVP production are operated strongly at 26 °C, but not at 37 °C. Therefore, as documented previously by Kawase et al. (2004), *V. vulnificus* produces a significant amount of VVP only in the interstitial tissue of limbs, in which temperature is lower than that in the small intestine and the

**Table 3** Effects of *luxO* disruption on the gene expression

| Growth phase | Increase | Decrease |
|---|---|---|
| 26 °C | | |
| Log phase | *rpoD* | |
| Stationary phase | *rpoS*, *luxS*, *smcR*, *crp*, *vvpE* | |
| 37 °C | | |
| Log phase | | *rpoN*, *vvhA* |
| Stationary phase | | *luxT* |

**Table 4** Growth phase and temperature dependent gene expression

| Growth phase | Cultivation temperature | |
|---|---|---|
| | 26 °C | 37 °C |
| Log phase | *rpoD* | *rpoN*, *vvhA* |
| Stationary phase | *rpoS*, *luxS*, *smcR*, *crp*, *vvpE* | *luxT* |

blood-stream, and VVP produced causes serious hemorrhagic and edematous skin damage (Miyoshi et al. 2001, 2003, 2004).

In contrast to VVP, the production of VVH started during log phase and reached a maximum level at late log phase. Disruption of *luxO* resulted in decreased expression of *vvhA* and less production of VVH. It is also noteworthy that, at 37 °C, the expression of *rpoN* was higher at log phase. This may indicate that RNA polymerase with RpoN recognizes the promoter of *vvhA* gene. Taken together, it is concluded that the regulation systems for VVH production are operated effectively at 37 °C. Therefore, *V. vulnificus* produces an enough amount of VVH in the small intestine, which results in acceleration of the bacterial invasion to the blood-stream.

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