

Full Paper

Bacterial virulence analysis using brine shrimp as an infection model in relation to the importance of quorum sensing and proteases

(Received January 27, 2014; Accepted June 28, 2014)

Mi-Nan Lee, Soo-Kyoung Kim, Xi-Hui Li, and Joon-Hee Lee*

Department of Pharmacy, College of Pharmacy, Pusan National University, Busan, 609–735, South Korea

Brine shrimp are aquatic crustaceans belonging to a genus of *Artemia*. This organism is widely used for testing the toxicity of chemicals. In this study, brine shrimp were evaluated as an infection model organism to study bacterial virulence. *Artemia* nauplii were infected with various pathogenic bacteria, such as *Vibrio vulnificus*, *Pseudomonas aeruginosa*, *Burkholderia vietnamiensis*, *Staphylococcus aureus*, and *Escherichia coli*, and the susceptibility to these bacteria was investigated by counting the survival of the infected nauplii. While all of the tested bacteria have significant virulence to brine shrimp, killing the nauplii in a few days, *V. vulnificus* showed the strongest virulence. *P. aeruginosa* also showed a dose-dependent virulence to brine shrimp, but the virulence was weaker than that of *V. vulnificus*. The virulence tests using the virulence-attenuated mutants of *V. vulnificus* and *P. aeruginosa*, such as quorum sensing (QS) mutants or protease-deficient mutants showed a significant attenuation of virulence, demonstrating that the QS mechanism is important in the virulence of these bacteria to brine shrimp. *B. vietnamiensis*, *S. aureus*, and *E. coli* were also virulent to brine shrimp and the virulence was correlated with dosage within 24 h under our conditions. *Salmonella enterica* Typhimurium and *Bacillus subtilis* were also virulent to brine shrimp, but the virulence was weak and slowly exerted compared with that of other bacteria. Taken together, we suggest that brine shrimp are a good infection model to assay bacterial virulence, especially for *V. vulnificus* and *P. aeruginosa*, and QS is important in the bacterial virulence to brine shrimp.

Key words: brine shrimp; infection model; protease; *Pseudomonas aeruginosa*; quorum sensing; *Vibrio vulnificus*

Introduction

The brine shrimp is an aquatic crustacean that is frequently used as a standard organism for testing the toxicity of chemicals and as live food in the larviculture of economically important fishes and crustaceans (Sorgeloos et al., 1978; Peroone and Wells, 1987; Orozco-Medina et al., 2002; Marques et al., 2006). A genus of brine shrimp, *Artemia*, is known to exist worldwide in inland saltwater lakes and is able to live in waters of high salinity (up to 25%), which feature makes the brine shrimp a good host model organism for the virulence study of marine pathogenic bacteria (Marques et al., 2006; Gajardo and Beardmore, 2012). Moreover, *Artemia* produces dormant eggs, known as cysts, that are stably stored for long periods and hatched as needed (Gajardo and Beardmore, 2012). This characteristic also provides a convenient way to use this organism for scientific applications, as well as for practical uses. Brine shrimp may have additional advantages like their short life-span and good resilience when used as a model organism (Sorgeloos et al., 1978; Peroone and Wells, 1987; Marques et al., 2006; Gajardo and Beardmore, 2012).

In this study, we intended to use brine shrimp for testing the virulence of pathogenic bacteria, instead of toxic chemicals. Currently, there are many host model organisms used for the virulence study of bacteria but most organisms are usually difficult to use in high salinity, being not eligible for marine pathogens like *Vibrio vulnificus*. Some human pathogens like *Pseudomonas aeruginosa* and *Staphylococcus aureus* are salt-tolerant, which is a habitation-determi-

*Corresponding author: Joon-Hee Lee, Laboratory of Microbiology, Department of Pharmacy, College of Pharmacy, Pusan National University, Research building 537, San 30, Jangjun-Dong, Geumjung-Gu, Busan, 609–735, South Korea.
Tel: 82–051–510–2821 Fax: 82–051–513–6754 E-mail: joonhee@pusan.ac.kr

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

nant feature in the human body and important for their physiology. Therefore, in order to obtain more appropriate host model organism for marine or salt-tolerant pathogens, brine shrimp were evaluated for bacterial virulence analysis in this study. In addition, the importance of quorum sensing (QS) in the bacterial virulence to brine shrimp was addressed as well.

We tested five representative pathogenic bacteria, *V. vulnificus*, *P. aeruginosa*, *Burkholderia vietnamiensis*, *S. aureus*, and *Escherichia coli*, for the evaluation of brine shrimp. *V. vulnificus* and *P. aeruginosa* were further studied to determine whether brine shrimp could reflect a difference in virulence depending on the mutation of virulence genes like QS and exoprotease genes. Both *V. vulnificus* and *P. aeruginosa* are Gram-negative human pathogens causing serious infections with a high mortality rate (Hardalo and Edberg, 1997; Strom and Paranjpye, 2000), and dependent on the QS system for the expression of their virulence genes.

QS refers to the mechanism in which microbes communicate with each other by producing and responding to diffusible small molecules as signals. The virulence factor production and pathogenicity are closely related to the QS mechanism in *V. vulnificus* and *P. aeruginosa* (Henke and Bassler, 2004; Antunes et al., 2011). In *V. vulnificus*, a major QS regulator, SmcR, has been reported to regulate the expression of virulence factors including exoproteases (Roh et al., 2006). One of them, an elastase that is a 45 kDa metalloprotease encoded by the *vvpE* gene, is important in the pathogenicity (Jeong et al., 2001; Gulig et al., 2005; Roh et al., 2006). In *P. aeruginosa*, the QS signal receptors LasR, RhlR, and QscR express many virulence factors (Fuqua and Greenberg, 2002; Lee et al., 2006). Among them, Protease IV (encoded by *piv*), a lysine-specific endoprotease, was suggested to be involved in the *Pseudomonas* virulence for

corneal infection (Engel et al., 1998). Here, we show that QS is important in the virulence of *V. vulnificus* and *P. aeruginosa* to brine shrimp, using QS and protease mutants.

Materials and Methods

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were basically grown in Luria-Bertani medium (LB; 5 g/L yeast extract, 10 g/L bacto-tryptone, 5 g/L NaCl), but the NaCl was increased to 2.0% (wt/vol) in the experiments using *V. vulnificus* strains (this medium is indicated as LBS). Bacterial cells were cultivated with vigorous shaking at 37°C. Cell growth was measured by optical density at 600 nm (OD₆₀₀).

Culture of brine shrimp. *Artemia*, a brine shrimp, was purchased as dormant eggs, also known as cysts (Artemio® Mix, JBL, Neuhofen/Pfalz, Germany). The cysts may be stored for long periods and hatched on demand. Half a spoonful (about 3.2 g) of the cysts was suspended in 166 ml of distilled water (the cysts were mixed with sea salts) and incubated with air bubbling at 28–30°C for 24–36 h. Then the cysts hatched and grew to be nauplii, which were used for bacterial infection experiments. The nauplii of *Artemia* were further cultivated in artificial seawater that was prepared by dissolving 40 g of sea salts (S9883, Sigma, St. Louis, MO) in 1 L of distilled water.

Virulence assay with brine shrimp. Virulence tests with the nauplii of *Artemia* were performed as described previously (Brackman et al., 2008), but with minor modifications. Briefly, after hatching, twenty nauplii of brine shrimp were transferred into a petri dish (35 × 10 mm) containing 5 ml of autoclaved artificial seawater. The bacterial cells were prepared by being grown overnight and sub-cultivated

Table 1. Organisms used in this study.

Organisms	Genotype	References
Bacteria		
<i>Vibrio vulnificus</i>		
M06-24/O	Wild-type, highly virulent clinical isolate	(Roh et al., 2006)
HS031	M06-24/O, <i>smcR::nptII</i> , Km ^R	(Jeong et al., 2003)
CMM111	M06-24/O <i>vvpE::pKC9844</i> ; elastase deficient	(Jeong et al., 2000)
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild type of <i>P. aeruginosa</i>	(Pearson et al., 1997)
MW1	<i>lasI</i> [−] , <i>rhlI</i> [−] double mutant of PAO1	(Whiteley et al., 1999)
DH0001	<i>piv</i> [−] mutant of PAO1, Tc ^R	(Yeom, 2013)
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169 (Ø80lacZΔM15) hsd17 recA1 gyrA96 thi-1 relA1</i>	Lab. collection
<i>Burkholderia vietnamiensis</i>		
G4	A type of <i>B. vietnamiensis</i>	(Ha et al., 2012)
<i>Staphylococcus aureus</i>		
RN4220	Wild type of <i>S. aureus</i>	(Nair et al., 2011)
<i>Salmonella enterica</i> Typhimurium		
SL1344	A type of <i>Salmonella enterica</i> Typhimurium	Lab. collection
<i>Bacillus subtilis</i>		
ATCC6051	A type of <i>Bacillus subtilis</i>	Lab. collection
Animal		
<i>Artemia salina</i>	A species of brine shrimp	Artemio® Mix, JBL, Neuhofen/Pfalz, Germany

*Km, kanamycin; Tc, tetracycline.

in 5 ml of fresh LB (or LBS for *V. vulnificus*) broth up to $OD_{600} \approx 0.4$. Various CFU (colony forming unit) of bacterial cells were then added to the seawater to infect the brine shrimp, and incubated at 28°C for several days. As a control, 1×10^5 CFU of bacterial cells killed by autoclave were added to shrimp in seawater and incubated in the same manner. The survival of the shrimp was scored every day after the addition of bacteria. For the comparison of virulence between wild type and QS- or protease-mutant strains, the same number of bacterial cells (1×10^5 CFU) was infected to the shrimp nauplii and the survival was counted daily after the infection. The experiment with 20 nauplii was repeated at least three times and the data were statistically analyzed using the *t*-test (two-sample assuming equal variances) of MS Office Excel (Microsoft, Redmond, WA). If the *p*-value was lower than 0.05, it was considered significant.

Results

The brine shrimp is a good host model for bacterial virulence analysis

For the evaluation of brine shrimp as a host model for the bacterial virulence study, various pathogenic bacteria were infected to the brine shrimp by feeding. As a control, bacterial cells killed by autoclave were infected in the same manner. First, *V. vulnificus* cells were infected and survival of the brine shrimp nauplii was daily measured. When different CFUs (colony forming units) of *V. vulnificus* cells were infected, the shrimp were killed in dose-dependent manner (Fig. 1A). Even the smallest amount of *V. vulnificus* cells tried in this experiment (10^3 CFU) significantly induced shrimp death (Fig. 1A). This result indicated that the brine shrimp is susceptible to *V. vulnificus* and the virulence of *V. vulnificus* can be sensitively assayed with brine shrimp.

Next, *P. aeruginosa* cells were infected and the survival of brine shrimp was investigated. When different CFUs of *P. aeruginosa* cells were infected in the same manner, the shrimp were similarly killed in a dose-dependent manner, but less sensitively, compared with the result for *V. vulnificus* (Fig. 1B). The significant killing effect occurred at an infectious dose of 3×10^4 CFUs (Fig. 1B). This result showed that the brine shrimp is less susceptible to *P. aeruginosa* than *V. vulnificus*, but nonetheless, the brine shrimp is apparently suitable for measuring *P. aeruginosa* virulence.

Other pathogenic bacteria, *B. vietnamiensis*, *S. aureus* and *E. coli*, were tested for their virulence in the same manner. These bacteria also showed strong virulence to brine shrimp even at a low dosage and the virulence was correlated with the dosage within 24 h (Fig. 2A, B, C). After the 2nd day, even a small dose significantly killed the shrimp nauplii and the difference between dosages became small (Fig. 2A, B, C). So, brine shrimp can be used for the virulence assay of these bacteria, but the experiment should be very carefully performed and is not recommended for long-time analysis. Otherwise, the experimental conditions should be improved. *Salmonella enterica* Typhimurium and *Bacillus subtilis* were also virulent to brine shrimp, but the virulence was relatively weak and slowly exerted compared with that of other bacteria (Fig. 3A, B).

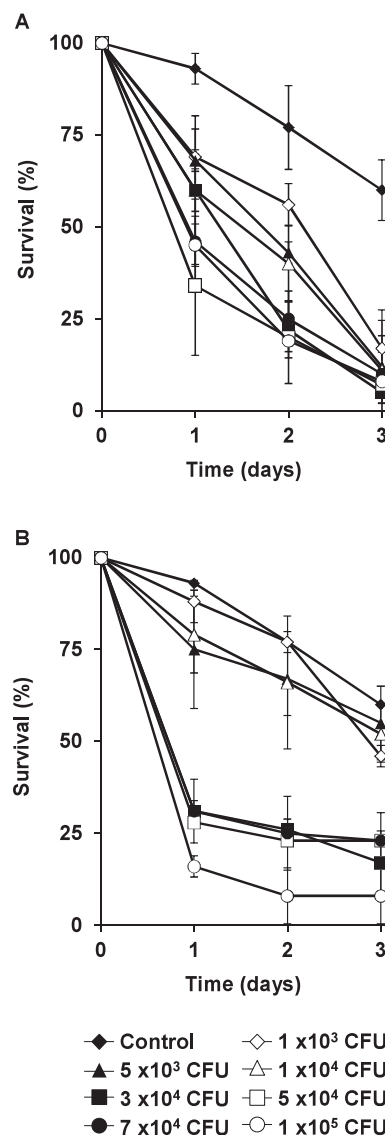


Fig. 1. Virulence of *V. vulnificus* (A) and *P. aeruginosa* (B) to brine shrimp.

Different CFUs of wild type *V. vulnificus* and *P. aeruginosa* cells were fed to the nauplii of brine shrimp and as a control, 1×10^5 CFUs of bacterial cells were killed by autoclave and fed to brine shrimp nauplii. The survival of nauplii was counted every day. CFUs, colony forming units.

QS is important in the bacterial virulence to brine shrimp

To better understand whether the assay using the brine shrimp well reflects the *V. vulnificus* virulence, and to learn whether the bacterial QS system is important in the virulence to brine shrimp, two mutant strains of *V. vulnificus*, *smcR*[−] and *vvpE*[−] mutants were tested for virulence. Recently, it was reported that SmcR, a major QS regulator of *V. vulnificus*, is a key virulence factor in *V. vulnificus* in experiments using mouse and human cell line models (Shao et al., 2011; Kim et al., 2013). The *vvpE* encoding a metalloprotease was also reported to cause dermal necrosis and edema in a mouse model, when the purified protein was injected (Kothary and Kreger, 1987; Molla et al., 1989; Gulig et al., 2005; Roh et al., 2006). When the virulence of the *smcR*[−] and *vvpE*[−] mutants was tested with brine shrimp, the *vvpE*[−] mutant showed a fairly clear attenuation of virulence, while the *smcR*[−] mutant showed a slight reduction of virulence (Fig. 4). The reduction of virulence in both mutants was statistically significant (*p*-value, <0.05).

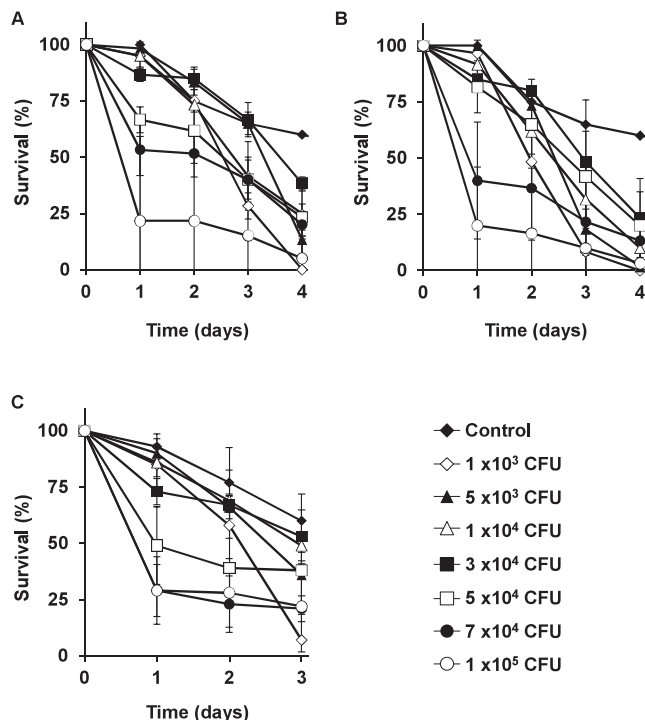


Fig. 2. Virulence of *B. vietnamiensis* (A), *S. aureus* (B), and *E. coli* (C) to brine shrimp.

Different CFUs of the bacterial cells were fed to brine shrimp nauplii and the survival was counted daily. 1×10^5 CFUs of bacterial cells were killed by autoclave and fed to brine shrimp nauplii as a control.

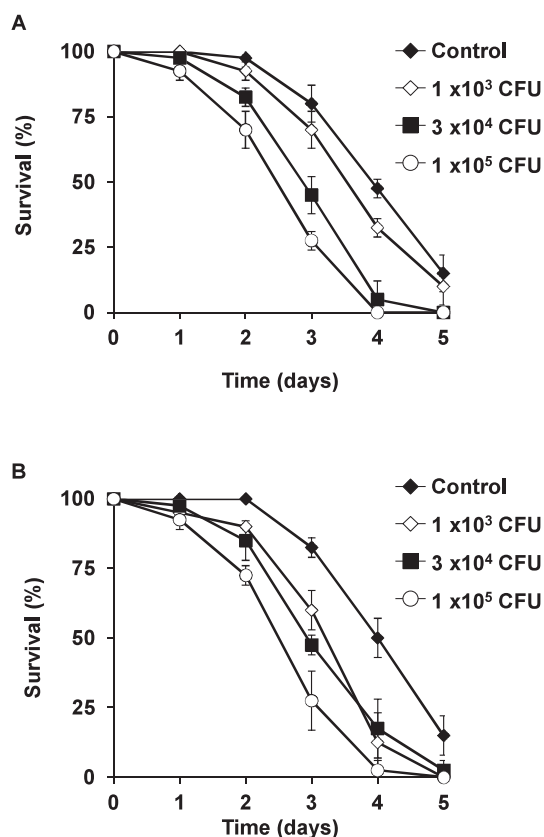


Fig. 3. Virulence of *S. enterica* Typhimurium (A) and *B. subtilis* (B) to brine shrimp.

Various CFUs of the bacterial cells were fed to brine shrimp nauplii and the survival was counted daily. 1×10^5 CFUs of bacterial cells were killed by autoclave and fed to brine shrimp nauplii as a control.

As in the experiment with *V. vulnificus*, two mutant strains of *P. aeruginosa* were tested for the virulence assay. The QS system has been well documented to be crucial for the *P. aeruginosa* virulence to many hosts including mice, insects, and nematodes (Rumbaugh et al., 2000; Chugani et al., 2001; Hentzer et al., 2003), and Protease IV encoded by the *piv* gene is positively regulated by the QS system and was also reported to be crucial in corneal infection (Engel et al., 1998). So, the QS mutant MW1 (*lasI*⁻, *rhlI*⁻ double mutant) and the *piv*⁻ mutant were tested for their virulence with brine shrimp. Both the MW1 and *piv*⁻ mutants were dramatically attenuated in virulence (Fig. 5).

Taken together, our results demonstrate that the brine shrimp is a good infection model to assay bacterial virulence and the QS system and proteases are crucial for the bacterial virulence to brine shrimp. In particular, *V. vulnificus* and *P. aeruginosa* are well assayed for their virulence with brine shrimp.

Discussion

The success of culturing brine shrimp depends on the establishment of a favorable microbial environment, which may be a reason why brine shrimp can be used for bacterial virulence study. Based on our results, we suggest that brine shrimp are suitable for the virulence analysis of marine or salt-tolerant pathogens like *V. vulnificus* and *P. aeruginosa*. So far, brine shrimp have been used for the virulence analysis of some *Vibrio* spp., such as *Vibrio harveyi* and *Vibrio parahaemolyticus* (Ricomora and Voltolina, 1995; Roque and Gomez-Gil, 2003). This study also showed that other disease-causing pathogens can be assayed using brine shrimp. Under our experimental conditions, the virulence assay for *B. vietnamiensis*, *S. aureus*, and *E. coli* was well correlated with infectious doses only in a short-time range (within 24 h). However, since these bacteria also showed strong virulence to brine shrimp, this limitation may be resolved by optimizing the experimental conditions via the

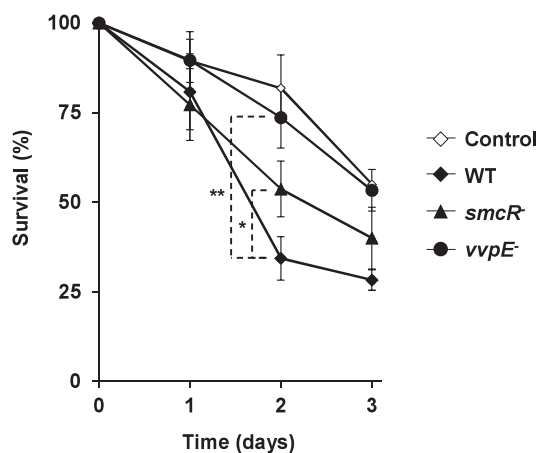


Fig. 4. Attenuation of the *V. vulnificus* virulence to brine shrimp by mutations on the QS system and metalloprotease.

The QS mutant, *smcR*⁻ and metalloprotease mutant, *vvpE*⁻ of *V. vulnificus* were grown to $OD_{600} \approx 0.4$ and 1×10^5 CFUs of the cells were fed to brine shrimp nauplii and the survival was counted every day. As a control, the same amount of bacterial cells was killed by autoclave and fed to brine shrimp nauplii. WT, wild type. *p*-value; * < 0.05, ** < 0.01.

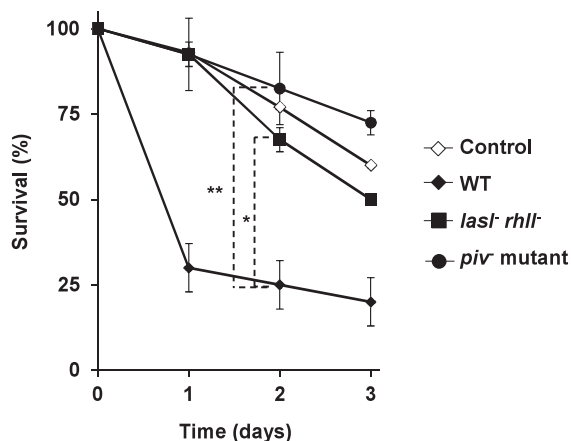


Fig. 5. Attenuation of the *P. aeruginosa* virulence to brine shrimp by mutations on the QS system and Protease IV.

The QS mutant, MW1 (*lasI⁻ rhII⁻*) and Protease IV mutant, *piv⁻* of *P. aeruginosa* were grown to $OD_{600} \approx 0.4$ and 1×10^5 CFUs of the cells were fed to brine shrimp nauplii and the survival was counted daily. The same amount of the killed bacterial cells was used for the control. *p*-value; * < 0.05 , ** < 0.01 .

modification of the culture medium, temperature, or size of inoculum. We think that this organism has an advantage over other model organisms for bacterial virulence analysis, because: 1) it does not require the maintenance of stock cultures, since its eggs are extremely stable under most environmental conditions, 2) it can be cultivated in massive numbers in a limited volume of water, which facilitates replication for statistical analyses, 3) it has a very short life-span, enabling many experiments to be conducted in a short time, 4) as it is transparent, microscopic examinations of live specimens are possible, 5) it has good resilience, which is ideal for running biological toxicity assays, and 6) it can be gnotobiotically grown under laboratory conditions (Sorgeloos et al., 1978; Peroone and Wells, 1987; Marques et al., 2006; Gajardo and Beardmore, 2012).

In this study, we also showed that the QS mechanism is important in the bacterial virulence to brine shrimp. All bacteria used in this study belong to very important pathogenic groups and are known to be dependent on the QS system for their virulence. While the importance of QS and proteases in the virulence to brine shrimp is well consistent with previous virulence studies using other host models, there are some noticeable points in our results. The first point is that although both VvpE and Protease IV have similar effect on the bacterial virulence to brine shrimp, they are not homologous with each other. Actually, the homologue of the *V. vulnificus* VvpE is LasB (elastase) in *P. aeruginosa*. The second point is that although SmcR was found to have a key role in the virulence of *V. vulnificus* to mice (Shao et al., 2011; Kim et al., 2013), the mutation effect of *smcR* was relatively small in brine shrimp infection (Fig. 4). Instead, the mutation on *vvpE* had a more dramatic effect on the virulence in our study. This is interesting in that the mouse experiments have given controversial results about the role of *vvpE* in *V. vulnificus* virulence. While the direct injection of the purified VvpE caused dermal necrosis and edema in a mouse model (Kothary and Kregger, 1987; Molla et al., 1989; Gulig et al., 2005; Roh et al., 2006), the mutation of *vvpE* showed virulence comparable

to or higher than did the wild type strain in mouse models (Jeong et al., 2000; Shao and Hor, 2000; Gulig et al., 2005). This may be due to differences in the host system. Actually, when we investigated the role of QS and *vvpE* using an insect model, the *Tenebrio molitor* larva (a mealworm) that is a host similar to the brine shrimp, both SmcR and VvpE were crucial in the *V. vulnificus* virulence to the insect (Ha et al., 2014). Consistently, QS and Protease IV of *P. aeruginosa* were also crucial for the virulence of *P. aeruginosa* to the insect, suggesting that QS and proteases may be generally important in the bacterial virulence to small invertebrate animals. However, the host-dependent difference in the role of VvpE in the pathogenesis of *V. vulnificus* infection remains to be clarified.

Acknowledgments

We are grateful to Dr. Sang Ho Choi, a professor of the Department of Agricultural Biotechnology, Center for Food Safety and Toxicology, and Center for Food and Bioconvergence, Seoul National University, for providing the mutant strains. This work was supported by a National Research Foundation of Korea Grant funded by the Korean Government (2010-0015901). This research was also supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2013R1A1A2012220)

References

- Antunes, L. C., Ferreira, R. B., Buckner, M. M., and Finlay, B. B. (2011) Quorum sensing in bacterial virulence. *Microbiology*, **156**, 2271–2282.
- Brackman, G., Defoirdt, T., Miyamoto, C., Bossier, P., Van Calenberg, S. et al. (2008) Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. *BMC Microbiol.*, **8**, 149.
- Chugani, S. A., Whiteley, M., Lee, K. M., D'Argenio, D., Manoil, C., and Greenberg, E. P. (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, **98**, 2752–2757.
- Engel, L. S., Hill, J. M., Moreau, J. M., Green, L. C., Hobden, J. A., and O'Callaghan, R. J. (1998) *Pseudomonas aeruginosa* protease IV produces corneal damage and contributes to bacterial virulence. *Invest. Ophthalmol. Vis. Sci.*, **39**, 662–665.
- Fuqua, C. and Greenberg, E. P. (2002) Listening in on bacteria: Acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell. Biol.*, **3**, 685–695.
- Gajardo, G. M. and Beardmore, J. A. (2012) The brine shrimp artemia: Adapted to critical life conditions. *Frontiers Physiol.*, **3**, 185.
- Gulig, P. A., Bourdage, K. L., and Starks, A. M. (2005) Molecular pathogenesis of *Vibrio vulnificus*. *J. Microbiol.*, **43 Spec No**, 118–131.
- Ha, C., Kim, S. K., Lee, M. N., and Lee, J. H. (2014) Quorum sensing-dependent metalloprotease VvpE is important in the virulence of *Vibrio vulnificus* to invertebrates. *Microb. Pathog.*, **71–72C**, 8–14.
- Ha, C., Park, S. J., Im, S. J., and Lee, J. H. (2012) Interspecies signaling through QscR, a quorum receptor of *Pseudomonas aeruginosa*. *Mol. Cells*, **33**, 53–59.
- Hardalo, C. and Edberg, S. C. (1997) *Pseudomonas aeruginosa*: Assessment of risk from drinking water. *Crit. Rev. Microbiol.*, **23**, 47–75.
- Henke, J. M. and Bassler, B. L. (2004) Bacterial social engagements. *Trends Cell Biol.*, **14**, 648–656.
- Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B. et al. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.*, **22**, 3803–3815.
- Jeong, H. S., Jeong, K. C., Choi, H. K., Park, K. J., Lee, K. H. et al. (2001) Differential expression of *Vibrio vulnificus* elastase gene

- in a growth phase-dependent manner by two different types of promoters. *J. Biol. Chem.*, **276**, 13875–13880.
- Jeong, H. S., Lee, M. H., Lee, K. H., Park, S. J., and Choi, S. H. (2003) SmcR and cyclic AMP receptor protein coactivate *Vibrio vulnificus* vvpE encoding elastase through the RpoS-dependent promoter in a synergistic manner. *J. Biol. Chem.*, **278**, 45072–45081.
- Jeong, K. C., Jeong, H. S., Rhee, J. H., Lee, S. E., Chung, S. S. et al. (2000) Construction and phenotypic evaluation of a *Vibrio vulnificus* vvpE mutant for elastolytic protease. *Infect. Immun.*, **68**, 5096–5106.
- Kim, S. M., Park, J. H., Lee, H. S., Kim, W. B., Ryu, J. M. et al. (2013) LuxR homologue SmcR is essential for *Vibrio vulnificus* pathogenesis and biofilm detachment, and its expression is induced by host cells. *Infect. Immun.*, **81**, 3721–3730.
- Kothary, M. H. and Kreger, A. S. (1987) Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. *J. Gen. Microbiol.*, **133**, 1783–1791.
- Lee, J. H., Lequette, Y., and Greenberg, E. P. (2006) Activity of purified QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Mol. Microbiol.*, **59**, 602–609.
- Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P., and Bossier, P. (2006) Gnotobiotically grown aquatic animals: Opportunities to investigate host-microbe interactions. *J. Appl. Microbiol.*, **100**, 903–918.
- Molla, A., Yamamoto, T., Akaike, T., Miyoshi, S., and Maeda, H. (1989) Activation of hageman factor and prekallikrein and generation of kinin by various microbial proteinases. *J. Biol. Chem.*, **264**, 10589–10594.
- Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M. et al. (2011) Whole-genome sequencing of *Staphylococcus aureus* strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. *J. Bacteriol.*, **193**, 2332–2335.
- Orozco-Medina, C., Maeda-Martinez, A. M., and Lopez-Cortes, A. (2002) Effect of aerobic Gram-positive heterotrophic bacteria associated with *Artemia franciscana* cysts on the survival and development of its larvae. *Aquaculture*, **213**, 15–29.
- Pearson, J. P., Pesci, E. C., and Igilewski, B. H. (1997) Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.*, **179**, 5756–5767.
- Peroone, G. and Wells, P. G. (1987) *Artemia* in aquatic toxicology: A review. *Artemia Res. Its Appl.*, **1**, 259–275.
- Ricomora, R. and Voltolina, D. (1995) Effects of bacterial isolates from *Skeletonema*-Costatum cultures on the survival of *Artemia*-*Franciscana* Nauplii. *J. Invertebr. Pathol.*, **66**, 203–204.
- Roh, J. B., Lee, M. A., Lee, H. J., Kim, S. M., Cho, Y. et al. (2006) Transcriptional regulatory cascade for elastase production in *Vibrio vulnificus*: LuxO activates luxT expression and LuxT represses smcR expression. *J. Biol. Chem.*, **281**, 34775–34784.
- Roque, A. and Gomez-Gil, B. (2003) Therapeutic effects of enrofloxacin in an experimental infection with a luminescent *Vibrio harveyi* in *Artemia franciscana* Kellogg 1906. *Aquaculture*, **220**, 37–42.
- Rumbaugh, K. P., Griswold, J. A., and Hamood, A. N. (2000) The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microbes Infect.*, **2**, 1721–1731.
- Shao, C. P. and Hor, L. I. (2000) Metalloprotease is not essential for *Vibrio vulnificus* virulence in mice. *Infect. Immun.*, **68**, 3569–3573.
- Shao, C. P., Lo, H. R., Lin, J. H., and Hor, L. I. (2011) Regulation of cytotoxicity by quorum-sensing signaling in *Vibrio vulnificus* is mediated by SmcR, a repressor of hlyU. *J. Bacteriol.*, **193**, 2557–2565.
- Sorgeloos, P., Remiche-Van Der Wielen, C., and Peroone, G. (1978) The use of *Artemia nauplii* for toxicity test—A critical analysis. *Ecotoxicol. Environ. Safety*, **2**, 249–255.
- Strom, M. S. and Paranjpye, R. N. (2000) Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes Infect.*, **2**, 177–188.
- Whiteley, M., Lee, K. M., and Greenberg, E. P. (1999) Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, **96**, 13904–13909.
- Yeom, D. H. (2013) Effects of Protease IV knock-out, acyltransferase overexpression, and growth restriction on virulence of *Pseudomonas aeruginosa*. Master thesis, College of Pharmacy, Pusan National University.