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.
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 (Hardoal and Edberg, 1997; Strom and Paranjpye, 2000), and dependent on the QS system for the expression of their virulence.

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 system (Hardalo and Edberg, 1997). *V. vulnificus* and *P. aeruginosa* are Gram-negative human pathogens causing serious infections with a high mortality rate (Hardoal and Edberg, 1997; Strom and Paranjpye, 2000), and dependent on the QS system for the expression of their 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, 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). 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>
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<th>Table 1. Organisms used in this study.</th>
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<tr>
<td><strong>Organisms</strong></td>
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<tr>
<td><em>Vibrio vulnificus</em></td>
</tr>
<tr>
<td>M06-24/O</td>
</tr>
<tr>
<td>HS031</td>
</tr>
<tr>
<td>CMM111</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
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<tr>
<td>PAO1</td>
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<tr>
<td>MW1</td>
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<tr>
<td>DH0001</td>
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<tr>
<td><em>Escherichia coli</em></td>
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<tr>
<td>DH5α</td>
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<tr>
<td><em>Burkholderia vietnamiensis</em></td>
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<td>G4</td>
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<tr>
<td><em>Salmonella enterica</em> Typhimurium</td>
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<tr>
<td>SL1344</td>
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<tr>
<td><em>Bacillus subtilis</em></td>
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<td>ATCC6051</td>
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*Km*, kanamycin; *Tc*, tetracycline.
in 5 ml of fresh LB (or LBS for V. vulnificus) broth up to OD<sub>600</sub> = 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<sup>5</sup> 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<sup>5</sup> 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<sup>5</sup> 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<sup>4</sup> 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).

**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, smc<sup>R</sup>− and vvpE<sup>E</sup>− 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 smc<sup>R</sup>− and vvpE<sup>E</sup>− mutants was tested with brine shrimp, the vvpE<sup>E</sup>− mutant showed a fairly clear attenuation of virulence, while the smc<sup>R</sup>− mutant showed a slight reduction of virulence (Fig. 4). The reduction of virulence in both mutants was statistically significant (p-value, <0.05).
and the survival was counted daily. 10^5 CFUs of bacterial cells were fed to brine shrimp nauplii and the survival was counted daily. Different CFUs of the bacterial cells were fed to brine shrimp nauplii and the survival was counted daily. As a control, the same amount of bacterial cells were killed by autoclave and fed to brine shrimp nauplii.

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 Vololima, 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

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 (las^-rhl^- 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

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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 gnotobiologically 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 Aeruginosa (2006), the mutation of 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

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