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Swarming Differentiation of *Vibrio vulnificus* Downregulates the Expression of the *vvhBA* Hemolysin Gene via the LuxS Quorum-Sensing System

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Swarming has proven to be a good *in vitro* model for bacterial surface adherence and colonization, and the swarming differentiation of a bacterium has been shown to be coupled with changes in the expression of virulence factors associated with its invasiveness, particularly in the early stages of infection. In this study, we attempted to determine whether the expression of *vvhA*, which encodes for hemolysin/cytolysin (VvhA), is either upregulated or downregulated during the swarming differentiation of *V. vulnificus*. The insertional inactivation of *vvhA* itself exerted no detectable effect on the expression of *V. vulnificus* swarming motility. However, in our *lacZ*-fused *vvhA* transcriptional reporter assay, *vvhA* expression decreased in swarming *V. vulnificus* as compared to non-swarming or planktonic *V. vulnificus*. The reduced expression of *vvhA* in swarming *V. vulnificus* increased as a result of the deletional inactivation of *luxS*, a gene associated with quorum sensing. These results show that *vvhA* expression in swarming *V. vulnificus* is downregulated via the activity of the LuxS quorum-sensing system, suggesting that VvhA performs no essential role in the invasiveness of *V. vulnificus* via the adherence to and colonization on the body surfaces required in the early stages of the infection. However, VvhA may play a significant role in the pathophysiological deterioration occurring after swarming *V. vulnificus* is differentiated into planktonic *V. vulnificus*.

Keywords: hemolysin, quorum-sensing, swarming, *Vibrio vulnificus*

Vibrio vulnificus is a halophilic estuarine bacterium, which is capable of inducing fatal septicemias and necrotizing wound infections. A number of factors have been implicated in the virulence and pathogenesis of *V. vulnificus*, including capsular polysaccharide, lipopolysaccharide, metalloprotease, hemolysin or cytolysin, phospholipase, motility, and iron-assimilation systems (Strom and Parangpye, 2000; Gulig *et al.*, 2005). Among these virulent factors, hemolysin or cytolysin (VvhA) is an extremely potent exotoxin, which is able to kill mice and exhibit a variety of biological activities at very low concentrations (Lee *et al.*, 2004). However, the pathogenetic roles of this exotoxin remain somewhat controversial, as the specific mutation of the *vvhA* gene does not appear to alter the virulence of *V. vulnificus* in mouse models (Wright and Morris, 1991; Fan *et al.*, 2001).

Swarming is defined as the 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. Swarming itself is considered a virulence factor, and is associated with the expression of invasiveness -associated virulence factors in *Proteus mirabilis*, *Serratia liquefaciens*, *Yersinia enterocolitica*, *Salmonella typhimurium* and *Bacillus subtilis* (Allison *et al.*, 1992; Fraser and Hughes, 1999; Fraser *et al.*, 2002; Connelly *et al.*, 2004; Wang *et al.*, 2004). Studies of *P. mirabilis*, a representative swarming bacterium (Allison *et al.*, 1992; Fraser *et al.*, 2002), have revealed that the ability to invade human urothelial cells *in vitro* is a specific property of swarmer cells,

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and that mutations affecting swarming motility also tend to significantly reduce invasiveness. In addition, the swarming differentiation of *P. mirabilis* is accompanied by an increase in the activities of virulence factors, including the extracellular hemolysin HpmBA. Swarming is considered a virulence factor for intestinal-tract pathogens, including *Salmonella typhimurium* as well, and is accompanied by an increase in the expression of a variety of genes related to pathogenicity (Connelly *et al.*, 2004; Wang *et al.*, 2004).

V. vulnificus infects susceptible patients via the skin and intestinal tract (Strom and Parangpye, 2000; Gulig *et al.*, 2005). *V. vulnificus* must first, accordingly, adhere to and colonize the surfaces of the skin and intestinal tract, in order to establish infections effectively. *V. vulnificus* manifests swarming motility, which essentially requires flagellar synthesis (Kim and Rhee, 2003) and is controlled by global regulatory systems, including RpoS and the cAMP-cAMP receptor protein complex (Hulsmann *et al.*, 2003; Kim *et al.*, 2005). This swarming motility in *V. vulnificus* has also been theorized to play an important role in the surface adherence and colonization of the bacterium, as well as the switching on or off the expression of invasiveness-associated virulence factors. However, no reports have yet been filed regarding the expression of virulence factors in swarming *V. vulnificus*. Therefore, in this study, we initially attempted to determine the levels of expression of *vvhA,* which encodes for the hemolysin/ cytolysin VvhA, the most potent exotoxin produced by the bacterium, in swarming *V. vulnificus*. Our results revealed that *vvhA* expression was downregulated via the activity of the LuxS quorum-sensing system in swarming *V. vulnificus,* versus non-swarming or planktonic *V. vulnificus*, and thus we briefly report the findings here.

Materials and Methods

Bacterial strains, media and reagents

V. vulnificus MO6-24/O wild-type strain (Wright *et al.*, 1990), a *vvhA*-insertional mutant (CVD707) (Wright and Morris, 1991), a P*vvhA::lacZ* transcription reporter strain (CMM2105) and its *luxS*-deletional mutant (CMM2206) (Kim *et al.*, 2003), and a *luxS* -deletion mutant and its *luxS-in trans*-complemented strain (Kim *et al.*, 2003) were used in this study. Heart infusion media (BD) containing additional 2% NaCl (NaCl-HI) were used as the basal media for the cultivation of the *V. vulnificus* strains. X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) was purchased from Bioneer. Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. (USA).

Measurement of bacterial growth, β-galactosidase activity, and hemolytic activity during broth culture

V. vulnificus strains were inoculated into HI broth to concentrations of 1×10^6 cfu/ml, then cultured with vigorous shaking (220 rpm) at 37°C for 12 h. During the culture period, culture aliquots were removed at appropriate times to monitor bacterial growth and β-galactosidase activity. Bacterial growth was monitored by measuring the OD_{600} of the culture aliquots. β-Galactosidase activity in the culture aliquots was measured by the method described by Miller (1992). Culture supernatants were obtained at the same times via the centrifugation of the culture aliquots at 10,000 rpm for 5 min. Hemolytic activity in the culture supernatants was measured using 1% human RBS suspensions by the method described by Shao and Hor (2001).

Observation of swarming of V. vulnificus

V. vulnificus strains grown overnight on HI agar were inoculated onto the surfaces of semisolid HI agars, which contained various concentrations of Bacto agar (BD) using the end of a toothpick, and the plates were incubated at 37°C for 24 h. During incubation, the sizes of the swarming haloes were checked at the appropriate times.

Measurement of β-galactosidase activity on semisolid agars

β-Galactosidase activity on semisolid HI agars was measured using the two methods described by Walker *et al.* (1999). (1) The CMM2105 or CMM2206 strains were inoculated onto the surfaces of semisolid HI agars containing various concentrations of Bacto agar and 96 μg/ml of X-gal, and incubated at 37°C for 12 h. During incubation, the color change of the X-gal from colorless to greenish-blue was monitored at the appropriate times. (2) The CMM2105 or CMM2206 strain was inoculated onto the surfaces of semisolid HI agars containing various concentrations of Bacto agar, but not containing X-gal, then incubated at 37°C for 12 h. After incubation, the *V. vulnificus* cells were harvested, and β-galactosidase activity was measured by the Miller method (Miller, 1992). In addition, *V. vulnificus* cells grown on the surfaces of semisolid agars containing 0.3% Bacto agar were harvested separately from the central and peripheral areas of swarming haloes, and the β-galactosidase activity of each was measured by the Miller method.

In our previous report (Kim *et al.*, in press), X-gal itself was determined to exert an inhibitory effect on the swarming motility of *Vibrio* species, including *V. vulnificus*. However, this effect of X-gal could be partially overcome via increases in the bacterial inoculum as well as a prolonging of culture duration.

Results and Discussion

vvhA expression in planktonic V. vulnificus cells

To first observe the expression of *vvhA* and its corresponding VvhA production in planktonic *V. vulnificus,* the three *V. vulnificus* strains, MO6-24/O (wild type), CVD707 (*vvhA*-insertional mutant), and CMM2105 (P*vvhA::lacZ* transcriptional reporter), were cultured with vigorous shaking (220 rpm) in HI broth (Fig. 1). No noticeable differences were observed with regard to the growths of the three strains (Fig. 1A). The expression of *vvhA* in the CMM2105 strain increased rapidly throughout the logarithmic growth phase, exhibiting a peak level in excess of 200 Miller units (Fig. 1B). In the MO6-24/O strain, the total hemolytic activity in the culture supernatants also increased rapidly, but declined abruptly during the late growth phase (Fig. 1C). No hemolytic activity was observed in the CVD707 strain. This pattern of *vvhA* expression or extracellular VvhA production has been well-documented, and VvhA in the late growth phase has been shown to be inactivated rapidly via self-oligomerization in our previous studies (Kim *et al.*, 2003; Shin *et al.*, 2005). Therefore, our results indicate that *vvhA* is expressed at a substantial level in planktonic *V. vulnificus*.

V. vulnificus expresses swarming motility on semisolid agars with low viscosity

V. vulnificus evidenced no swarming motility on the surfaces of semisolid HI agars containing more than 0.5% agar, but were also shown to exhibit remarkable swarming motility on the surfaces of semisolid agars containing 0.3% agar. *V. vulnificus* manifested an obvious bull's eye-patterned cyclic swarming motility on the surfaces of semisolid HI agars containing 0.3% agar (Fig. 2, 3, 4 and 5). Moreover, upon light microscopic visualization, the *V. vulnificus* cells obtained from the periphery of the swarm haloes appeared to be elongated to a greater degree than were those obtained from the liquid cultures, and the mutations of the *cya* gene, which encodes for adenylate cyclase. and the *crp* gene, which encodes for the cAMP receptor protein (CRP), completely lost the ability to swarm on the surfaces of semisolid HI agars containing 0.3% agar (Kim *et al.*, 2005) although the individual cells in this case retained the ability to actively swim when resuspended in fresh HI broth and observed under dark-field microscopy (data not shown). Flagella synthesis is known to be a prerequisite for the swarming motility of *V. vulnificus* (Kim *et al.*, 2003). In general, bacterial swarming has been shown to occur on the surface of semisolid agars containing more than 0.5% agar, and this phenomenon has been associated with both cell elongation and hyperflagellation (Fraser and Hughes, 1999). In addition, the most striking macroscopic characteristic of a swarm colony is the 'bull's eye' appearance generated by cyclic waves of swarming (Walker *et al.*, 1999; Harshey, 1994; Allison *et al.*, 1992). Overall, our results show that *V. vulnificus* exhibits flagella-mediated swarming motility only on

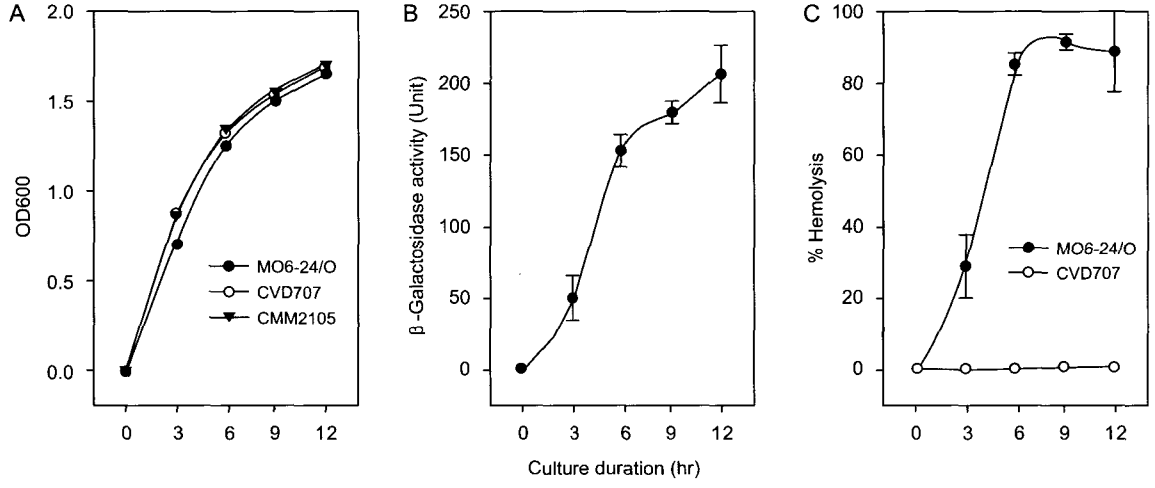

Fig. 1. The expression of *vvhA* in planktonic *V. vulnificus*. The three *V. vulnificus* strains, MO6-24/O wild type, CVD707 (*vvhA*-insertional mutant) CVD707, and CMM2105 (P*vvhA::lacZ* transcriptional reporter), were cultured with vigorous shaking (220 rpm) at 37°C for 24 h in HI broth. Culture aliquots were removed in order to measure bacterial growth and β-galactosidase activity at the indicated times, and then the culture supernatants were obtained by centrifuging the culture aliquots at 10,000 rpm for 5 min, and used to measure hemolytic activity. (A) Bacterial growth was measured by the OD_{600} of the culture aliquots, (B) β-galactosidase activity in the CMM2105 strain was measured via the Miller method, and (C) hemolytic activity in the MO6-24/O and CVD707 strains was measured using a 1% suspension of human RBC.

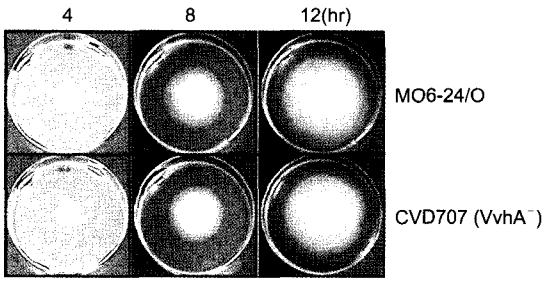

Fig. 2. *vvhA* mutation has no effect on the expression of swarming motility in *V. vulnificus*. The two *V. vulnificus* strains, MO6-24/O wild type and CVD707 (*vvhA*-insertional mutant), grown on HI agar overnight, were inoculated onto the surfaces of semisolid HI agar containing 0.3% Bacto agar, then incubated at 37°C. During incubation, photographs were obtained at the indicated times.

the surfaces of semisolid agars with exceptionally low viscosity.

vvhA mutation exerts no effects on the expression of the swarming phenotype of *V. vulnificus*

We also observed the effects of *vvhA* mutation on the expression of *V. vulnificus* swarming motility (Fig. 2). No noticeable differences were observed in the size of swarming haloes between the wild type MO6-24/O strain and the *vvhA*-insertional mutant strain, CVD 707. These results indicate that *vvhA* mutation exerts no effects on the expression of swarming motility in *V. vulnificus*. *V. vulnificus* swarming has been shown to be either reduced or abolished by mutations of the *rpoS* gene, which is associated with general stress responses (Hulsmann *et al.*, 2003), the *cya* gene, which encodes for adenyl cyclase in synthesis of cAMP (Kim *et al.*, 2005), and the *crp* gene, which encodes for the cAMP-receptor protein (CRP) (our unpublished observation). All these mutations have also been associated with a downregulation of VvhA production. However, our results show that the swarming defects induced by these mutations are not directly associated with deficiencies in VvhA.

vvhA expression is downregulated in swarming *V. vulnificus*

In order to determine the levels of *vvhA* expression in swarming *V. vulnificus*, we cultured the CMM2105 strain (*PvvhA::lacZ*) on semisolid HI agars containing 96 μg/ml of X-gal (Fig. 3A). The levels of *vvhA* expression (blue coloration by β-galactosidase) in swarming *V. vulnificus* were found to be far lower than those detected in non-swarming *V. vulnificus*, and were less pronounced in the peripheral area than in the central area of the swarming haloes. In order to reconfirm these findings, we cultured the CMM2105

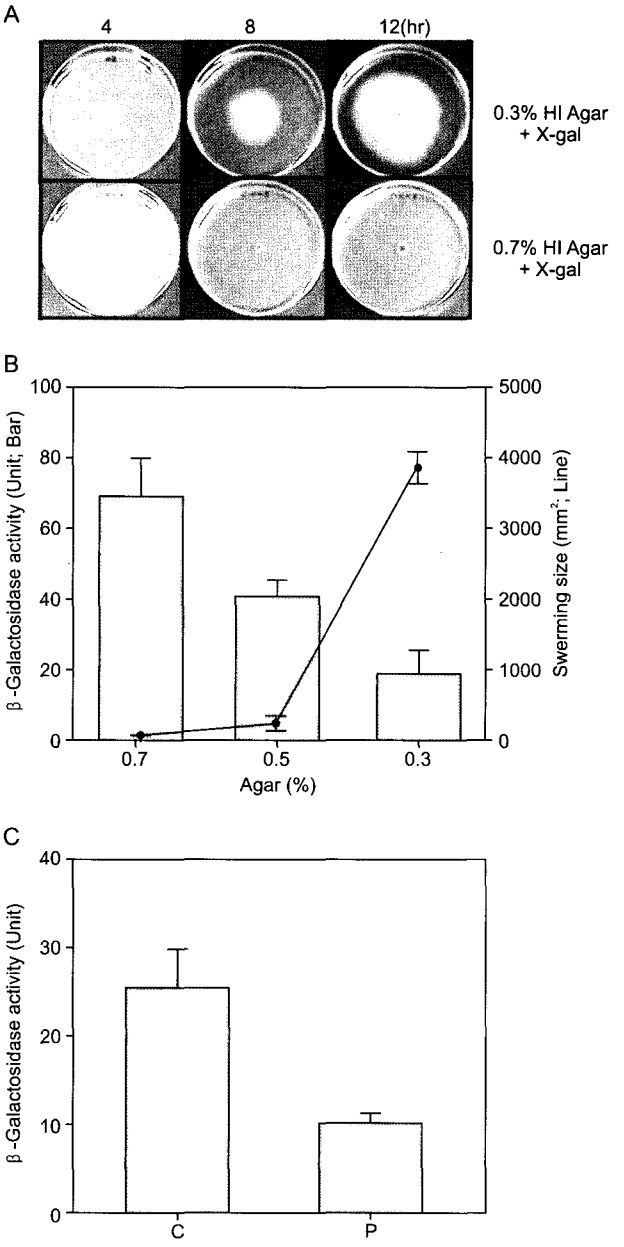

Fig. 3. *vvhA* expression is downregulated in swarming *V. vulnificus*. (A) The CMM2105 (P*vvhA::lacZ* transcriptional reporter) strain, grown on HI agar overnight, was inoculated onto the surface of semisolid HI agar containing 0.3 or 0.7% Bacto agar and 96 μg/ml of X-gal, then incubated at 37°C. During incubation, photographs were taken at the indicated times. (B) The CMM2105 strain was inoculated onto the surface of semisolid HI agar containing 0.3, 0.5 or 0.7% Bacto agar, but not containing X-gal, and then incubated at 37°C for 12 h. After incubation, the sizes of the swarming haloes were measured, after which the bacterial cells were harvested from the whole surfaces of the semisolid agars, and the β-galactosidase activity was measured by the Miller method. (C) Under the same conditions as in (B), bacterial cells were harvested separately from the central (the first cycle: C) area and the peripheral (the second or third cycle: P) area of the swarming haloes, after which the levels of β-galactosidase activity were measured by the Miller method.

strain on semisolid HI agars that did not contain X-gal, harvested the cells from the agar surfaces, and then measured β-galactosidase activity using the Miller method (Miller, 1992), which is a widely used and standard method to measure β-galactosidase activity. The level of *vvhA* expression measured by the Miller method was down-regulated in swarming *V. vulnificus* versus non-swarming *V. vulnificus*. The levels of *vvhA* expression were inversely related to the viscosity of the media and to the size of swarming haloes (Fig. 3B), and were also found to be lower in the peripheral area (i.e., the second or third cycle of swarming) than in the central area (i.e., the first cycle of swarming) of the swarming haloes (Fig. 3C). Moreover, the levels of *vvhA* expression in swarming *V. vulnificus* did not increase above 20 Miller units even under prolonged culture conditions, and the measured levels were far lower than those (~more than 200 Miller units) in the planktonic *V. vulnificus*, as was shown in Fig. 1B. These results show that *vvhA* expression is severely downregulated in swarming *V. vulnificus* cells versus non-swarming or planktonic *V. vulnificus*.

vvhA expression in swarming V. vulnificus is increased by the mutation of luxS

It has been well-documented that *vvhA* expression in planktonic *V. vulnificus* cells is down-regulated by the LuxS-quorum sensing system, which is considered to be a coordinator that is responsible for the cell density-dependent expression of virulence factors (Kim *et al.*, 2003). The *luxS* mutation upregulates *vvhA* expression, and the *in trans luxS* complementation downregulates *vvhA* expression in planktonic *V. vulnificus*. However, the role of the LuxS-quorum sensing system in regulating *vvhA* expression has yet to be observed in swarming *V. vulnificus*, which is a cell density-dependent behavior. Thus, in order to determine whether *vvhA* expression in swarming *V. vulnificus* is also regulated by the activity of the LuxS quorum-sensing system, we cultured the CMM2105 strain (P*vvhA::lacZ* reporter) and the CMM2206 strain (*luxS*-deleted P*vvhA::lacZ* reporter) on 0.3% semisolid agar containing X-gal. The *luxS* mutation enhanced the *vvhA* expression in swarming *V. vulnificus* to a fairly substantial degree (Fig. 5A). Similar results were also observed in cases in which the same strains were cultured on semisolid HI agars not containing X-gal and harvested the cells from the agar surfaces, and the β-galactosidase activity was measured via the Miller method (Fig. 5B). The *luxS* mutation and complementation appeared not to have any effect on *V. vulnificus* swarming (Fig. 4). The significantly reduced swarming behavior exhibited by the CMM2206 strain as what was observed in the CMM2105 strain

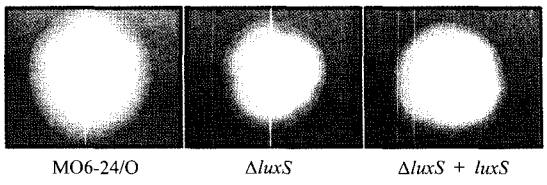

Fig. 4. Effect of the *luxS* mutation on the swarming motility of *V. vulnificus*. The *V. vulnificus* strains, MO6-24/O, *luxS*-deleted mutant (Δ*luxS*) and *luxS*-complemented strain (Δ*luxS* + *luxS*), grown on HI agar overnight were inoculated onto the surfaces of semisolid HI agar containing 0.3% Bacto agar and 96 μg/ml of X-gal, then incubated at 37°C. During incubation, photographs were taken at the indicated times.

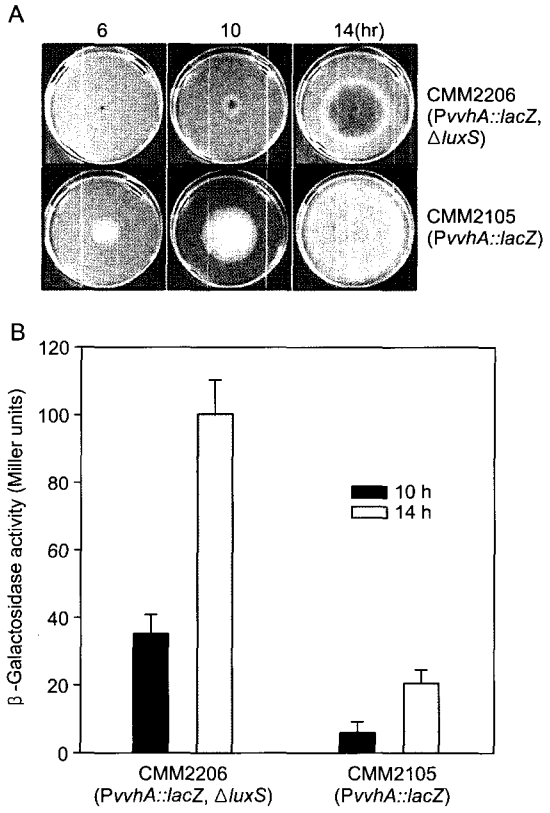

Fig. 5. Effect of the *luxS* mutation on *vvhA* expression in swarming *V. vulnificus*. (A) The *V. vulnificus* strains, CMM2105 (P*vvhA::lacZ* transcriptional reporter) and CMM2206 (*luxS*-deleted P*vvhA::lacZ* transcriptional reporter), grown on HI agar overnight were inoculated onto the surfaces of semisolid HI agar containing 0.3% Bacto agar and 96 μg/ml of X-gal, then incubated at 37°C. During incubation, photographs were taken at the indicated times. (B) The two strains were inoculated onto the surface of semisolid HI agar containing 0.3% Bacto agar, but not containing X-gal, then incubated at 37°C for 14 h. During incubation, bacterial cells were harvested from the whole surfaces of semisolid agars, and the β-galactosidase activity was measured by the Miller method.

(Fig. 5A) was attributed to the effect of X-gal, but not to the possible effect of the *luxS* mutation. In our

recent study, X-gal was shown to inhibit the growth and swarming of the functional *lacZ*-harboring *V. vulnificus* (Kim *et al.*, in press). Taken together, our results indicate that *vvhA* expression in swarming *V. vulnificus* cells is down-regulated via the activity of the LuxS-quorum sensing system, as is also the case in planktonic *V. vulnificus* cells. However, a significant level of *vvhA* (in excess of 200 Miller units) was expressed in planktonic *V. vulnificus* (Fig. 1B), whereas an insignificant level of *vvhA* (approximately 20 Miller units) was expressed in swarming *V. vulnificus* (Fig. 3B), despite the presence of the functional LuxS-quorum sensing system. Considering these results, *vvhA* expression appears to be downregulated via the LuxS quorum-sensing system to a more profound degree in swarming *V. vulnificus* than in planktonic *V. vulnificus*.

Bacteria not only live freely, but also adhere to the solid surfaces of a variety of materials in diverse environments, including that of the human body (Harshey, 1994; Fraser and Hughes, 1999). In particular, bacterial adherence to and colonization on the surfaces of the human body constitute a prerequisite for the successful establishment of infections, particularly in the early stages of infection. Swarming is an excellent example of bacterial surface adherence and colonization, especially in the context of *in vitro* studies (Harshey, 1994), and is itself considered a virulence factor for intestinal and urogenital pathogens (Allison *et al.*, 1992; Fraser and Hughes, 1999; Fraser *et al.*, 2002; Connelly *et al.*, 2004; Wang *et al.*, 2004). In the case of the urogenital pathogen *P. mirabilis* (Allison *et al.*, 1992; Fraser *et al.*, 2002), it has been fairly well-documented that mutations affecting swarming motility also significantly reduce its invasiveness via adherence to and colonization on urothelial cells. Moreover, the swarming differentiation of *P. mirabilis* is accompanied by increases in the expression of virulence factors, including the intracellular urease and extracellular hemolysin, HpmBA, and the metalloprotease, ZapA, all of which have also been thought to play central roles with regard to its invasiveness, particularly in the early stages of infection. In the case of the intestinal pathogen, *S. typhimurium*, swarming differentiation is also accompanied by increases in the expression of a variety of pathogenicity genes, some of which are thought to play important roles in its invasiveness (Wang *et al.*, 2004). These would appear to be true in the case of *V. vulnificus*. The motility of *V. vulnificus* has been fairly associated with adherence to cultured cells (Kim and Rhee, 2003; Kim *et al.*, 2005). Accordingly, the evidence collected in this study suggests that VvhA is not required for the invasiveness of *V. vulnificus* via adherence to and colonization on the intestinal epithelium or the skin, particularly in the early stages of *V. vulnificus* infection. However, it can rather play a significant role in the deterioration of the pathophysiological changes observed in the late stages of infection, by which time swarming *V. vulnificus* has already differentiated into planktonic *V. vulnificus*.

In conclusion, the results of this study indicated that *vvhA* expression is down-regulated via the activity of the LuxS quorum-sensing system in swarming *V. vulnificus*. This report may constitute a starting point for studies concerning the up- or downregulation of virulence factors in swarming *V. vulnificus*. Further studies will be necessary in order to further characterize the behavior of the upregulated virulence factors observed in swarming *V. vulnificus*.

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