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Original Article

Vibrio vulnificus Hemolysin Is Easily Inactivated in Spite of Being Produced at High Levels in Cirrhotic Ascites by a *fur* Mutation

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Vibrio vulnificus produces Hemolysin/cytolysin (VvhA), which is one of the most potent exotoxins capable of killing mice at submicrogram levels. However, *V. vulnificus* growth and *vvhA* expression are severely repressed and extracellular VvhA produced at low levels is easily inactivated in human body fluids. This study was conducted to obtain additional unequivocal evidence of the enigmatic characteristic of VvhA. *V. vulnificus* growth was stimulated, *vvhA* expression was de-repressed, and extracellular VvhA production was increased in cirrhotic ascites, a human *ex vivo* experimental system, by a mutation of *fur* encoding ferric uptake regulator, which acts as a transcriptional repressor. However, regardless of the presence or absence of the *fur* mutation, extracellular VvhA activity was not detected in cirrhotic ascites. These results indicate that VvhA is easily inactivated even when *vvhA* expression and extracellular VvhA production are maintained at high levels in cirrhotic ascites.

Key Words: *Vibrio vulnificus*, Hemolysin/cytolysin, Ferric uptake regulator, Ascites

INTRODUCTION

Vibrio vulnificus is a gram-negative halophilic bacterium capable of causing primary septicemia and necrotizing wound infections with a high mortality rate in susceptible individuals with underlying illnesses, especially hepatic diseases (1).

V. vulnificus produces hemolysin or cytolysin (VvhA) as an exotoxin. Purified VvhA is so potent that it can kill mice at submicrogram levels (2). However, its pathogenetic role remains enigmatic since *vvhA* mutations do not affect the virulence of *V. vulnificus* (3). Recently, VvhA was reported to exhibit only minor cytotoxicity only in the absence of the major cytotoxic factor, RtxA1 (4). From an evolutionary perspective, VvhA is currently considered to be only one of multiple factors capable of affecting the pathophysiological changes observed with *V. vulnificus* infections or the enhanced survival of *V. vulnificus* in environments or hosts (5~9).

Previous studies revealed that *vvhA* expression is severely repressed and even extracellular VvhA produced at low levels is easily inactivated in human body fluids, especially in cirrhotic ascites which is a useful human *ex vivo* experimental system (7, 8). A recent *in vitro* study revealed that *vvhA* expression is repressed by iron and de-repressed by a mutation of the *fur* gene, which encodes a transcription repressor known as ferric uptake regulator (Fur); however, extracellular VvhA secretion via the PilD-mediated type II

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Table 1. Bacterial strains used in this study

Bacterial strains	Relative characteristics and sequences	Sources and references
M06-24/O	Highly virulent clinical isolate	10
CMM2101	M06-24/O with a *lacZ* deletion	11
CMM2304	CMM2101 with a *fur* deletion	12
CMM2103	CMM2101 with a merozygotic P_{vvhA}*::lacZ* transcription fusion	11
CMM2305	CMM2304 with a merozygotic P_{vvhA}*::lacZ* transcription fusion	9
RC176	CMM2101 with a merozygotic P_{pilD}*::lacZ* transcription fusion	9
RC180	CMM2304 with a merozygotic P_{pilD}*::lacZ* transcription fusion	This study

general secretion system (PilD-mediated II-GSS) is increased by iron (9). Whether *vvhA* expression in human body fluids may also be de-repressed by a *fur* mutation remains undetermined.

Generally, to elucidate the pathogenetic roles of a gene or its product, the gene is mutated or down-regulated. In contrast, alternate methods capable of up-regulating gene expression are required to elucidate the *in vivo* pathogenetic roles of gene products, particularly in cases where the genes are expressed at very low levels in human body fluids. Accordingly, to obtain further unequivocal evidence related to the enigmatic role of VvhA, we determined extracellular VvhA activity under the condition that *vvhA* expression and extracellular VvhA production were de-repressed at high levels in an *ex vivo* experimental model of cirrhotic ascites by a *fur* mutation.

MATERIALS AND METHODS

Bacterial strains, media, cirrhotic ascites, and reagents

The bacterial strains used in this study are listed in Table 1. Luria-Bertani medium (BD, Franklin Lakes, NJ, USA) and Thiosulfate-Citrate-Bile-Sucrose medium (BD) containing 20 μg/ml ampicillin, 300 μg/ml kanamycin, 2 μg/ml tetracycline or 2 μg/ml chloramphenicol were used for selection and subculture of recombinant strains. Heart Infusion (HI; BD) agar or broth containing additional 2.0% NaCl was used as the basal medium for cultivation of *V. vulnificus* strains. HI broth was deferrated using 8-hydroxyquinoline as described by Leong and Neilands (13). The residual iron concentration of the deferrated HI broth was 1.0 μg/dl or less, which was measured as described by Stookey (14). However, *V. vulnificus* strains did not grow well in the deferrated HI broth due to the low iron concentration; therefore, 1.0 μM of ferric chloride was exogenously added to the broth. This medium could support *V. vulnificus* growth to some extent and was designated as an iron-deficient HI broth. Ascites samples were collected from five liver cirrhosis patients following their informed consent and approval of the study by the Institutional Review Board at Chosun University Hospital. Equal volumes of ascites samples were pooled to avoid individual variances and then prepared as described previously (8). Unless noted otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of transcription reporter strains

A β-galactosidase gene (*lacZ*)-deletion mutant strain (CMM2101) was constructed from M06-24/O with spontaneous streptomycin resistance as described previously (10, 11). From CMM2101, a *fur*-deletion mutant strain (CMM2304) was constructed as we previously described (12). The merozygotic P_{vvhA}*::lacZ* transcription reporter strains (CMM2103 with wild-type *fur* and CMM2305 with deleted *fur*) were constructed from CMM2101 and CMM2304, respectively, as previously described (9, 11). The merozygotic P_{pilD}*::lacZ* transcription reporter strain (RC176) was constructed from CMM2101, as described previously (9). Using the same methodology, the merozygotic P_{pilD}*::lacZ* transcription reporter strain with a *fur* mutation

(RC180) was newly constructed from CMM2304 for this study.

Culture conditions, growth and β-galactosidase activity measurements

To adapt *V. vulnificus* strains to iron-restricted conditions and to reduce intracellular iron storage, they were cultured for 12 h in HI broth containing 100 μM of the iron chelator α, α'-dipyridyl. These preconditioned strains were inoculated into iron-deficient broths or cirrhotic ascites at approximately 1×10^6 cells/ml and cultured with vigorous shaking (220 rpm) at 37℃ for 12 h. Bacterial growth was measured by the optical density of culture aliquots at a wavelength of 600 nm (OD_{600}), and β-galactosidase activity in culture aliquots was measured in triplicate by the Miller method (15).

Catechol siderophore and extracellular VvhA measurements

To measure catechol siderophore and extracellular VvhA productions, culture aliquots were centrifuged at 10,000 rpm for 5 min. In the recovered culture supernatants, catechol siderophore production was measured in triplicate without any modification by the Arnow test (16). Extracellular VvhA activity was measured in triplicate by the tube hemolysis assay using 1% human red blood cell suspensions (17). The amount of extracellular VvhA in the culture supernatants was measured by an immunoblot method. In brief, equivalent volumes (20 μl for iron-deficient HI broth and 100 μl for cirrhotic ascites) of the culture supernatants adjusted for *V. vulnificus* growth levels (OD_{600} values) were directly blotted onto nitrocellulose membranes using a Bio-Dot® SF microfiltration apparatus (Bio-Rad, Hercules, CA, USA). The membranes were allowed to react with rabbit polyclonal anti-VvhA-body, prior to reaction with goat anti-rabbit-IgG-body conjugated with horseradish peroxidase, and finally developed with diaminobenzidine and hydrogen peroxide. Additional procedures were carried out as described in our previous studies (8, 9). For quantitative analysis on a per cell basis, signal intensities were digitalized by densitometry (Molecular Imaging Software, ver. 4.0.0, KODAK, CA, USA).

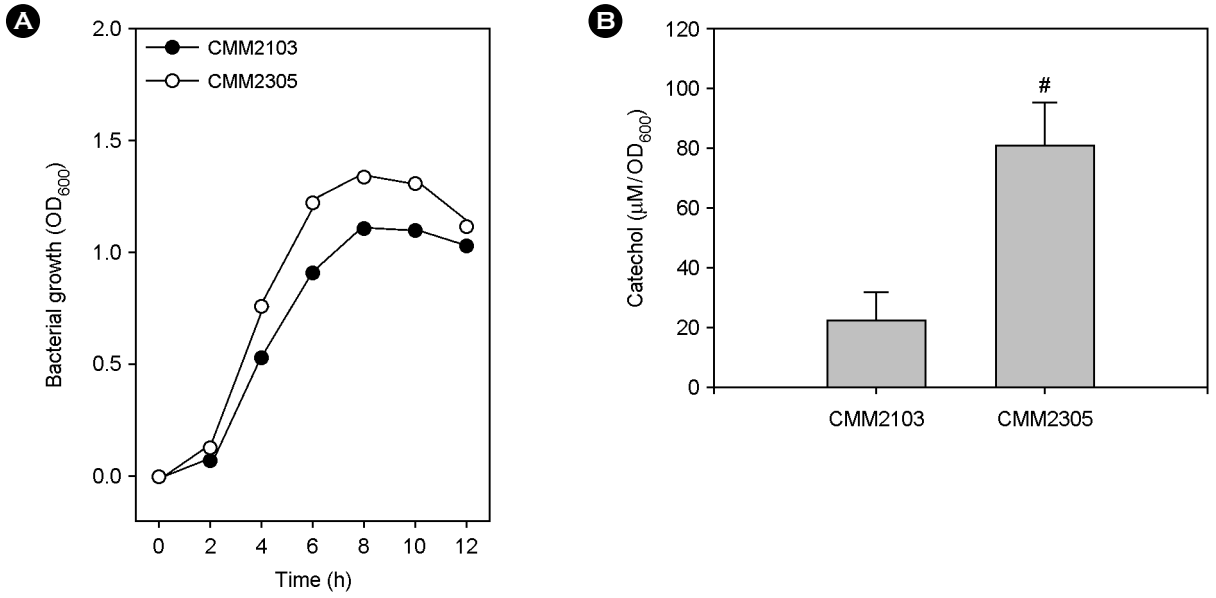


Figure 1. Effect of a *fur* mutation on the production of catechol-siderophore vulnibactin. The two P_{vhA}::*lacZ* transcription reporter strains, CMM2103 with wild-type *fur* and CMM2305 with deleted *fur*, were cultured in iron-deficient Heart Infusion broths at 37℃ for 12 h. (A) Bacterial growth was measured by the optical density of culture aliquots at 600 nm (OD_{600}). (B) Culture supernatants were obtained by centrifugation and catechol concentration in the culture supernatants was determined by the Arnow test. Numeric values are expressed as the means ± standard deviation, from triplicate measurements. The # symbol indicates a statistically significant difference between the two means ($p < 0.05$ in Student's *t*-test).

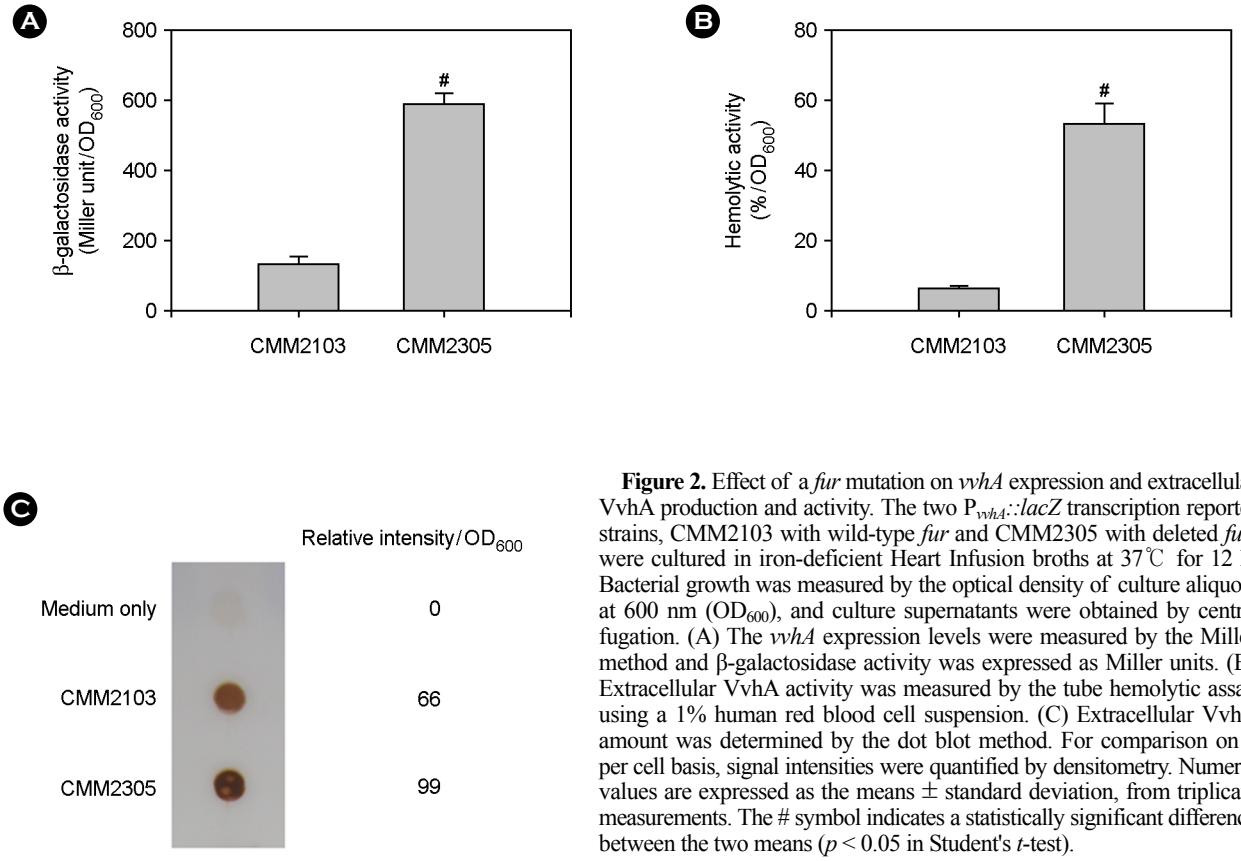


Figure 2. Effect of a *fur* mutation on *vvhA* expression and extracellular VvhA production and activity. The two P_{vvhA}*::lacZ* transcription reporter strains, CMM2103 with wild-type *fur* and CMM2305 with deleted *fur*, were cultured in iron-deficient Heart Infusion broths at 37℃ for 12 h. Bacterial growth was measured by the optical density of culture aliquots at 600 nm (OD_{600}), and culture supernatants were obtained by centrifugation. (A) The *vvhA* expression levels were measured by the Miller method and β-galactosidase activity was expressed as Miller units. (B) Extracellular VvhA activity was measured by the tube hemolytic assay using a 1% human red blood cell suspension. (C) Extracellular VvhA amount was determined by the dot blot method. For comparison on a per cell basis, signal intensities were quantified by densitometry. Numeric values are expressed as the means ± standard deviation, from triplicate measurements. The # symbol indicates a statistically significant difference between the two means ($p < 0.05$ in Student's *t*-test).

RESULTS

Effect of a *fur* mutation on vulnibactin production

The effect of a *fur* mutation on production of the catechol-siderophore vulnibactin was determined using the *V. vulnificus* CMM2103 strain with wild-type *fur* and the *V. vulnificus* CMM2305 strain with deleted *fur*. The growth level of CMM2305 was higher than that of CMM2103 (Fig. 1A). In the Arnow test, the production level of vulnibactin in CMM2103 was approximately 20 μM. In contrast, the production level of vulnibactin in CMM2305 was significantly increased compared with that in CMM2103 (Fig. 1B). Similar results were also observed when the production of vulnibactin between M06-24/O or CMM2101 and CMM2304 was compared under similar conditions, as reported in our previous study (12). These results indicate that a *fur* mutation stimulated *V. vulnificus* growth by de-repressing vulnibactin production in iron-limited *in vitro* conditions.

Effect of a *fur* mutation on *vvhA* expression and extracellular VvhA production and activity

The effects of a *fur* mutation on *vvhA* transcription and extracellular VvhA production were determined using CMM2103 with wild-type *fur* and CMM2305 with deleted *fur*. In the *lacZ*-fused transcription reporter assay, the *vvhA* expression level in CMM2305 was significantly increased compared to that of CMM2103 (Fig. 2A). In the tube hemolytic assay, extracellular VvhA activity in CMM2305 was significantly increased compared to that in CMM2103 (Fig. 2B). In the dot blot analysis, the extracellular VvhA amount in CMM2305 was also significantly increased compared to that in CMM2103 (Fig. 2C). These results indicate that a *fur* mutation increased extracellular VvhA production and activity by de-repressing *vvhA* expression under iron-limited *in vitro* conditions.

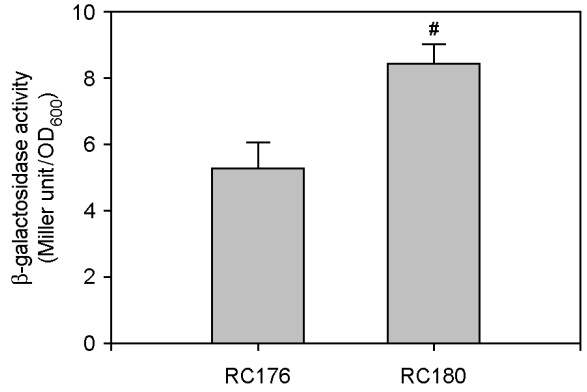


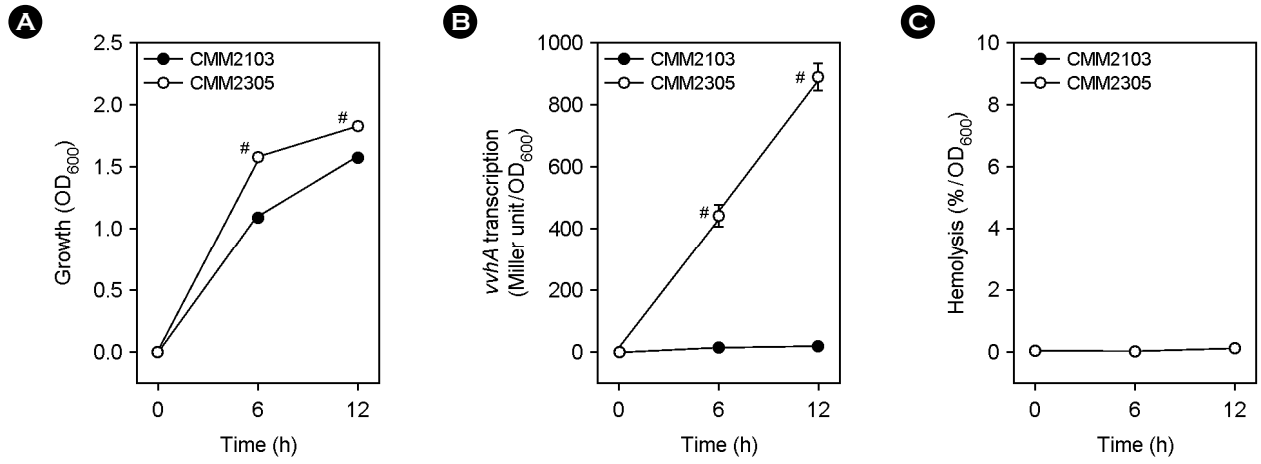
Figure 3. Effect of a *fur* mutation on *pilD* expression. The two P_{pilD}*::lacZ* transcription reporter strains, RC176 with wild-type *fur* and RC180 with deleted *fur*, were cultured in iron-deficient Heart Infusion broths at 37℃ for 12 h. Bacterial growth was measured by the optical density of culture aliquots at 600 nm (OD_{600}), and culture supernatants were obtained by centrifugation. (A) The *pilD* expression levels were measured by the Miller method and β-galactosidase activity was expressed as Miller units. Numeric values are expressed as the means ± standard deviation, from triplicate measurements. The # symbol indicates a statistically significant difference between the two means ($p < 0.05$ in Student's *t*-test).

Effect of a *fur* mutation on the activity of PilD-mediated II GSS

The effect of a *fur* mutation on the activity of PilD-mediated II-GSS responsible for extracellular VvhA secretion was determined by comparing *pilD* expression levels using the merozygotic P_{pilD}*::lacZ* transcription reporter strains, RC176 with wild-type *fur* and RC180 with mutated *fur*. The growth level of RC180 was higher than that of RC176 (data not shown). In the β-galactosidase assay, the *pilD* transcription level in RC180 was significantly increased compared to that in RC176 (Fig. 3). These results indicate that a *fur* mutation increased the PilD-mediated II-GSS activity in iron-limited *in vitro* conditions.

Easy inactivation of VvhA in cirrhotic ascites

To determine whether extracellular VvhA is easily



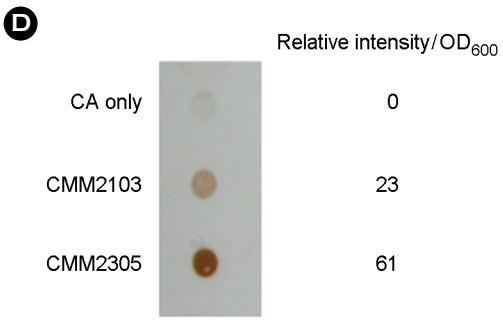


Figure 4. Effect of a *fur* mutation on *vvhA* expression and extracellular VvhA production and activity in cirrhotic ascites. The two P_{vvhA}*::lacZ* transcription reporter strains, CMM2103 with wild-type *fur* and CMM-2305 with deleted *fur*, were cultured in cirrhotic ascites at 37℃ for 12 h. (A) Bacterial growth was measured by the optical density of culture aliquots at 600 nm (OD_{600}). (B) The *vvhA* expression levels in culture aliquots were measured by the Miller method and β-galactosidase activity was expressed as Miller units. (C) Extracellular VvhA activity in culture supernatants was measured by the tube hemolytic assay using a 1% human red blood cell suspension. (D) Extracellular VvhA amount in culture supernatants was determined by the dot blot method. For comparison on a per cell basis, signal intensities were digitalized by densitometry. Numeric values are expressed as the means ± standard deviation, from triplicate measurements. The # symbol indicates a statistically significant difference between the two means ($p < 0.05$ in Student's *t*-test).

inactivated in human body fluids while *vvhA* expression is de-repressed and maintained at high levels by a *fur* mutation, the merozygotic transcriptional reporter strains CMM2103 and CMM2305 were cultured in cirrhotic ascites. The growth level of CMM2305 was higher than that of CMM2103 (Fig. 4A). In the β-galactosidase assay, the *vvhA* expression level in CMM2103 was severely repressed. In contrast, the *vvhA* expression level in CMM2305 increased significantly compared to that in CMM2103 (Fig. 4B). In the dot blot analysis, the extracellular VvhA production level in CMM2305 increased significantly compared to that in CMM2103 (Fig. 4D); however, no extracellular VvhA activity was detected in either strain (Fig. 4C). These results indicate that VvhA is easily inactivated in cirrhotic ascites while *V. vulnificus* growth is stimulated, *vvhA* expression is de-repressed, and extracellular VvhA production is increased by a *fur* mutation.

DISCUSSION

V. vulnificus often causes infections mainly in patients with elevated serum or tissue iron levels such as hemochromatosis and hepatic diseases (18~20). Elevated iron levels are considered to be one of the most reliable predisposing factors by which to determine host susceptibility to *V. vulnificus* (21). Our previous and present studies show that *V. vulnificus* growth is inhibited in cirrhotic ascites (8, 12). One of the reasons for this may be due to the fact that *V. vulnificus* is a ferrophilic bacterium which requires higher levels of available iron for growth than do other pathogenic bacteria (22); that is to say, cirrhotic ascites may be still an iron-limited condition for the ferrophilic bacterium *V. vulnificus* because it contains transferrin-bound iron rather than easily-available free iron even though it came from liver cirrhosis patients. Poor growth of *V. vulnificus* itself may repress *vvhA* expression in cirrhotic ascites. Conversely, active growth of *V. vulnificus* to some extent may be a prerequisite for efficient *vvhA* expression in cirrhotic ascites. An exogenous iron supply can stimulate *V. vulnificus* growth in cirrhotic ascites, but it can repress *vvhA* expression via Fur (9). The present study shows that a *fur* mutation can stimulate both *V. vulnificus* growth and *vvhA* expression in cirrhotic ascites by de-repressing vulnibactin production with no addition of exogenous iron. The vulnibactin-mediated iron assimilation system determines the ability of *V. vulnificus* to grow under various iron-limited conditions and also plays a significant role in the pathogenesis of *V. vulnificus* (1). Expression of the *vuuA* gene encoding the vulnibactin receptor protein is known to be under the negative control of Fur (23). In addition, our previous and present studies show that vulnibactin production is de-repressed by a *fur* mutation, indicating that the expression of genes associated with vulnibactin biosynthesis is also under the negative control of Fur (12).

Of the components of cirrhotic ascites, one of the most likely factors that can repress *vvhA* expression is glucose. Like other human body fluids, cirrhotic ascites contains a considerable level of glucose (8). It is well documented that glucose negatively regulates *vvhA* expression via the cAMP-CRP system (8, 24, 25). It seems impractical to lower glucose levels with no change of other constituents to maintain *vvhA* expression at high levels in cirrhotic ascites. Even if possible, lowering glucose levels may further decrease *vvhA* expression by inhibiting *V. vulnificus* growth in cirrhotic ascites.

Our previous study revealed that iron stimulates extracellular VvhA secretion by increasing *pilD* expression (9). The present study shows that a *fur* mutation can also facilitate extracellular VvhA production by increasing *pilD* expression. These findings suggest that a *fur* mutation may indirectly or secondarily affect *pilD* expression by increasing iron assimilation and eventually the intracellular iron level.

In cirrhotic ascites, de-repressed *vvhA* expression and facilitated extracellular VvhA secretion by a *fur* mutation can offer a simple useful means by which to measure the activity of VvhA in a setting where *V. vulnificus* growth, *vvhA* expression, and extracellular VvhA secretion are maintained at high levels. As described, *V. vulnificus* growth, *vvhA* expression, and extracellular VvhA secretion in cirrhotic ascites were all maintained at high levels by a *fur* mutation, but extracellular VvhA activity was not observed regardless of the presence or absence of a *fur* mutation. In

contrast, extracellular VvhA activity in iron-deficient HI broths was significantly increased consistently with increased *V. vulnificus* growth, *vvhA* expression and extracellular VvhA production levels by the same *fur* mutation. Our previous study revealed that *vvhA* expression is severely repressed in the presence of wild-type *fur*, even extracellular VvhA produced at very low levels is easily inactivated in cirrhotic ascites, and moreover, extracellular VvhA produced at high levels in HI broths is also inactivated in the presence of cirrhotic ascites. The present study shows more undeniable evidence that VvhA is easily inactivated in spite of being produced at high levels in cirrhotic ascites. It has been demonstrated that inactivation of VvhA in cirrhotic ascites is due to the self or cholesterol-mediated oligomerization of VvhA and the interaction of VvhA with undefined constituents of cirrhotic ascites (8, 26). In addition, the easy inactivation of VvhA provides a likely explanation of the pre-existing contradiction regarding the pathogenetic role of VvhA: *vvhA* mutations do not affect the virulence of *V. vulnificus* although purified VvhA is so potent that it can kill mice at low levels (2).

In conclusion, we were able to obtain more unequivocal evidence of the easy inactivation of VvhA in a human *ex vivo* experimental system by using a *fur* mutation which de-represses *vvhA* expression and increases extracellular VvhA production.

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