Regulation of iron-uptake systems in *Vibrio vulnificus*, a ferrophilic bacterium

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Vibrio vulnificus is a gram-negative ferrophilic bacterium that causes necrotizing wound infections and fatal septicemia, which mainly occur in patients with elevated levels of iron in serum or tissue, despite the presence of well-developed bacterial multiple iron-uptake systems (IUSs). These IUSs play important roles in the pathogenesis of *V. vulnificus* infections and are primarily regulated at the transcriptional level by a ferric uptake regulator called Fur responding to iron availability and their own specific regulators. Recent studies have shown that the IUSs are also controlled by other global regulators, including cyclic AMP-receptor protein responding to carbon availability and SmcR, a master regulator of the quorum-sensing system responding to bacterial density. This review presents an update on this sophisticated regulation of IUSs in *V. vulnificus*.

Keywords: *Vibrio vulnificus*; Iron; Quorum sensing; Ferric uptake regulator; cAMP-receptor protein

INTRODUCTION

Iron (Fe) is the fourth most abundant metal on Earth and is an essential ingredient for all living things, including bacteria. However, bacteria must be able to acquire Fe to settle, proliferate, and cause infections in their hosts, and therefore, many pathogenic bacteria have their own well-developed iron-uptake systems (IUSs). The activity of IUSs has long been recognized as being closely related to the virulence of pathogenic bacteria. Therefore, IUSs have been considered a target in the development of vaccines or new therapeutic modalities [1–3].

Fe is quickly oxidized to an insoluble form in an environment where oxygen is present at neutral pH. Therefore, the concentration of soluble Fe that is easily available to most

living things is very low. The human body contains 3–5 g of Fe, most of which is intracellular. The majority of Fe in the body is present as hemoglobin in erythrocytes. Extracellular Fe is rapidly removed by high-affinity Fe-binding proteins, such as transferrin, ferritin, and lactoferrin [1–3]. Thus, a normal human body contains a very low concentration of free Fe and is an Fe-deficient environment. This low Fe-availability is considered an innate defense mechanism called “innate nutritional immunity”, which helps in suppressing the growth of pathogenic bacteria [2]. However, the concentration of easily available Fe as well as the degree of Fe saturation of transferrin or lactoferrin can be increased, thereby allowing pathogenic bacteria to grow in many pathological conditions, such as liver diseases, hemosiderosis, as well as during transfusions and chemotherapy [1–3].

Bacteria have adapted to Fe-deficient environments, developing their own unique IUSs that can efficiently absorb environmental Fe present at low concentrations. Generally, bacterial IUSs can be divided into three types. First, several bacterial species can obtain Fe by expressing specific receptors that directly bind to Fe-withholding proteins such as hemoglobin, transferrin, and lactoferrin [4]. For instance, *Neisseria* and *Haemophilus* species express receptors that are unique only to human transferrin and lactoferrin. Other bacterial species can acquire Fe by directly binding to heme proteins such as hemin and hemoglobin by expressing receptors specific to heme [5-7]. Second, most bacteria, except *Neisseria* or *Haemophilus*, can produce small molecules (600-1,500 kDa), called siderophores, with a high affinity for Fe. Most siderophores have a higher affinity for Fe than Fe-withholding proteins such as transferrin or lactoferrin so that they can deprive these proteins of Fe. The siderophore-Fe complexes are again absorbed through specific receptors. Many bacteria produce one or more siderophores [8,9]. Third, some bacteria can also use xenosiderophores (also called heterologous or exogenous siderophores) produced by other bacteria or fungi [10-15] through the phenomenon called “siderophore piracy”. This siderophore piracy may play an important role in the survival and proliferation of pathogenic bacteria especially in mixed bacterial environments.

Fe is an essential substance for efficient energy production in bacterial cells. Therefore, the amount of Fe required by bacteria is closely related to the metabolic activity in bacterial cells. The more active the metabolism, the more Fe is required to support efficient energy production. However, excessive Fe can be toxic because this can promote free radical formation within bacterial cells. Therefore, the expression of IUSs in bacterial cells must be thoroughly and sensitively controlled [16].

A FERROPHILIC BACTERIUM *Vibrio vulnificus*

Vibrio vulnificus is an opportunistic pathogen that can cause necrotizing fasciitis or fatal sepsis mainly in patients with underlying diseases such as alcoholic hepatitis, cirrhosis, and hemosiderosis [17-20]. Most of these patients have elevated serum Fe concentrations [21,22]. Fe has long been

known to play an important role in the pathogenesis of *V. vulnificus* infections [23]. First, an elevated Fe concentration is directly related to host sensitivity to *V. vulnificus* [21,22]. In a mouse experimental model, the 50% lethal dose was significantly reduced by exogenous Fe administration [24, 25]. These Fe-overloaded mice are used as the most susceptible experimental animal for *V. vulnificus* infection. Second, an elevated Fe concentration promotes the production of exotoxins from *V. vulnificus*, and the production of hemolysin and metalloproteinase is increased in response to iron concentration [26,27]. Third, *V. vulnificus* is a ferrophilic or Fe-sensitive bacterium that requires higher levels of soluble Fe for growth than other pathogenic bacteria do [28,29]. The reason for this ferrophilic characteristic is the low activity of *V. vulnificus* well-equipped and versatile IUSs [30]. For example, although *V. vulnificus* produces several siderophores, the amount produced is very low compared to those produced by other pathogenic bacteria.

IUSs IN *V. vulnificus*

Vulnibactin-mediated IUS

V. vulnificus produces a siderophore called vulnibactin that has a very high affinity for Fe and is structurally classified as catechol or phenolate siderophore [31,32]. Virulent *V. vulnificus* strains generally produce vulnibactin and use Fe bound to transferrin, but less virulent or avirulent *V. vulnificus* strains do not produce vulnibactin and are unable to use transferrin-bound Fe [33]. Besides vulnibactin, other siderophores are known to be produced, but vulnibactin has the greatest effect on the growth of *V. vulnificus* in Fe-deficient environments [34].

Genes involved in the production of vulnibactin were first discovered in the *ven* operon [35]. The *venB* gene shows 41% homology at the amino acid level with and the *Escherichia coli entB* gene, which encodes isochorismatase, and this enzyme is required for the synthesis of a precursor (2,3-dihydroxybenzoic acid) of enterobactin, a catechol siderophore produced by *E. coli*. *V. vulnificus* containing a mutation in *venB* can neither produce vulnibactin nor use Fe bound to transferrin, resulting in the weakened virulence. The *vis* gene encoding vulnibactin-specific isochorismate synthase is also involved in the synthesis of vulnibactin as a mutation in this gene prevents the production of vulnibactin

and the use of Fe bound to transferrin [36,37]. In addition, *vvsAB*, which encodes a member of the nonribosomal peptide synthase, is also required for the biosynthesis of vulnibactin, and a mutation in either *vvsA* or *vvsB* decreases the production of vulnibactin in *V. vulnificus* [38]. These show that various enzymes are required for the synthesis of vulnibactin and that vulnibactin has a higher affinity for Fe than transferrin. Thus, *V. vulnificus* can use Fe bound to transferrin through vulnibactin.

Extracellular vulnibactin binds Fe to form the Fe-vulnibactin complex, which is then absorbed via its cognate specific receptor, VuuA, located on the outer membrane of *V. vulnificus*. The 72 kDa VuuA receptor is encoded by *vuuA*, and a mutation in *vuuA* prevents the internalization of Fe-vulnibactin into the cytoplasm and inhibits the ability of *V. vulnificus* to use Fe bound to transferrin to grow [39].

Hydroxamate siderophore-mediated IUS

Virulent *V. vulnificus* strains produce both vulnibactin and a hydroxamate siderophore, but avirulent *V. vulnificus* strains only produce the hydroxamate siderophore [31]. The hydroxamate siderophore can also promote the proliferation of *V. vulnificus* in Fe-deficient environments [31,34]. However, neither the structure of this siderophore nor the genes related to hydroxamate siderophore-mediated IUS have been described.

Xenosiderophore-mediated IUSs

Desferrioxamine is a hydroxylate siderophore produced by *Streptomyces pilosus*, and it has long been used as an Fe-chelating agent for the treatment of Fe overload [40]. Several pathogenic bacteria, including *V. vulnificus*, can absorb Fe using this xenosiderophore [10-15], and this has been a major limitation in the use of desferrioxamine as a therapeutic Fe-chelating agent. As a result, new Fe-chelating agents that inhibit the growth of pathogenic bacteria capable of utilizing desferrioxamine, including *V. vulnificus*, have been developed [28,41-44]. Accordingly, these Fe-chelating agents have the potential to prevent and treat fatal infections caused by such pathogenic bacteria [45].

In *V. vulnificus*, desferrioxamine is utilized via its cognate receptor called DesA, which is an Fe-responsive outer membrane protein of 78 kDa [11,12]. A mutation in *desA*

abolishes the ability of *V. vulnificus* to use desferrioxamine and inhibits the growth of *V. vulnificus* in the presence of desferrioxamine. This DesA-mediated IUS is widespread in clinical or environmental isolates of *V. vulnificus* [15].

V. vulnificus can also use another xenosiderophore called aerobactin, which is produced by *E. coli*, via the cognate receptor IutA, an outer membrane protein of 76 kDa [13]. A mutation in *iutA* abolishes the ability of *V. vulnificus* to utilize aerobactin for Fe acquisition and can inhibit the growth of *V. vulnificus* in the presence of aerobactin.

Fe-aerobactin or Fe-desferrioxamine is transported into the cytoplasm by an ATP-binding cassette (ABC) transport system containing VatB, VatD, and VatB. The *vatC*, *vatD*, and *vatB* genes encode an ATP-binding protein, a periplasmic binding protein, and an inner membrane permease, respectively. A mutation in these genes also results in the loss of the ability of *V. vulnificus* to utilize aerobactin as well as desferrioxamine [13]. This indicates that this ABC transport system is shared between the IutA-mediated and DesA-mediated IUSs.

Heme receptor-mediated IUS

The first identified heme receptor was a 77kDa outer membrane protein called HupA, which responds to Fe availability [6]. A mutation in *hupA* encoding this receptor protein prevents *V. vulnificus* from using hemin or hemoglobin as an Fe source and reduces virulence in mice and in tissue culture [46,47]. This implies that HupA plays a significant role in the pathogenesis of *V. vulnificus* infections. Recently, a new heme receptor called HvtA (79 kDa) was identified, and this receptor acts with HupA to enable *V. vulnificus* to make more effective use of hemin or hemoglobin [48]. The growth of *V. vulnificus* is not solely impaired by a mutation in *hvtA*. Accordingly, HvtA, in conjunction with HupA, is needed for optimal hemin utilization, and HupA and HvtA are considered major and minor heme receptors, respectively. In addition, the HupBCD components of the ABC transport system are shared by HupA and HvtA. Therefore, a mutation in the *hupBCD* genes also abolishes the ability of *V. vulnificus* to utilize hemin or hemoglobin in the absence of vulnibactin [49].

REGULATION OF IUSs BY Fur AND THEIR SPECIFIC REGULATORS

Regulation of the vulnibactin-mediated IUS by Fur (Figs. 1 and 2)

The expression of bacterial IUSs is controlled by a global regulator called ferric uptake regulator (Fur), which is primarily responsive to Fe availability [50]. In Fe-sufficient environments, the Fe-Fur complex binds to a specific sequence called the “Fur box” in the regulatory region of its target genes [51]. This prevents the binding of RNA polymerase and represses the transcription of target genes. By contrast, under Fe-deficient environments, apo-Fur dissoci-

Fig. 1. Schematic illustration of the regulation of target genes by Fe and ferric uptake regulator (Fur) in *Vibrio vulnificus*. The thickness of line indicates the main function of Fur in *V. vulnificus*.

ates from the Fur box so that the transcription of the target genes is derepressed.

The expression of the vulnibactin-mediated IUS is primarily controlled by Fur. A Fur box is not present in the upstream region of *venB* which is transcribed in a polycistronic operon [35]. The presence of a Fur box in the regulatory region of *vis* remains to be experimentally determined. However, the expression of both *venB* and *vis* is negatively regulated by Fe or derepressed by a mutation in *fur* [35,52]. A Fur box is present at the regulatory regions of *vvsAB* and *vuua*, and the expression of these genes is also negatively regulated by Fe and derepressed by a mutation in *fur* [38,39, 52-55]. Thus, the transcription of genes involved in the vulnibactin-mediated IUS is primarily controlled by Fur, a transcription repressor responding to Fe availability.

Regulation of the heme receptor-mediated IUS by Fur and HupR (Figs. 1 and 3)

The expression of *hupA*, *hvtA*, and *hupBCD* is negatively regulated by Fe. A Fur box is present in the regulatory region of these genes, and a mutation in *fur* de-represses the expression of these genes [46-49]. This indicates that Fe is a primary essential signal for the expression of the heme receptor-mediated IUS. In addition, HupR, a LysR-like transcription activator, acts as a transcription activator for the expression of *hupA* only when hemin or hemoglobin is supplied as an Fe source in Fe-deficient conditions [56]. The expression of *hupA* occurs at relatively low levels even in the

Fig. 2. Schematic illustration of the regulation of vulnibactin (A) and vulnibactin receptor protein (B) production in *Vibrio vulnificus*. Several enzymes such as *vis*, *venB*, and *vvsAB* are known to be involved in vulnibactin synthesis. Crp: cyclic AMP-receptor protein, SmcR: a master regulator of the autoinducer-2-mediated quorum sensing system, LuxS: an enzyme required for autoinducer-2 synthesis, Fur: ferric uptake regulator.

absence of heme or hemoglobin or the absence of HupR. A mutation in *hupR* decreases the expression of *hupA* only in the presence of heme or hemoglobin as an Fe source. HupR upregulates the expression of *hupA* but not that of *hvtA* in the presence of heme or hemoglobin. It is hypothesized that a small amount of heme can be transported to the cytoplasm via HvtA, resulting in the upregulation of *hupA*

Fig. 3. Schematic illustration of the regulation of heme receptor protein production in *Vibrio vulnificus*. HupR acts as a transcription activator only in the presence of heme. Other abbreviations are the same as those in Fig. 2.

Fig. 4. Schematic illustration of the regulation of desferrioxamine receptor protein production in *Vibrio vulnificus*. DesR acts as a transcription activator only in the presence of desferrioxamine. Other abbreviations are the same as those in Fig. 2.

transcription by HupR [49]. Whether HupR itself is Fe-responsive remains unproven. Thus, HupR is a local or specific, but not essential, regulator that controls the expression of *hupA* in response to the presence of heme.

Regulation of the DesA-mediated IUS by Fur and DesR (Figs. 1 and 4)

In Fe-deficient environments, *desA* is expressed only in the presence of desferrioxamine. DesR, an AraC-like transcription activator, is essentially required for the expression of *desA* [11,12]. Putative Fur boxes are present in the regulatory regions of both *desA* and *desR*, indicating that Fe is a primary signal for their expression. A mutation in *desR* results in a loss of production of DesA even in Fe-deficient environments or in the presence of desferrioxamine, which indicates that Fe-responsive DesR is a local, specific, and essential activator responding to the presence of desferrioxamine.

Regulation of the IutA-mediated IUS by Fur and IutR (Figs. 1 and 5)

The expression of *iutA* is also under the control of Fur [13,57], which indicates that Fe is a primary signal for the expression of *iutA*. IutR, a GntR-like transcription repressor, is also involved in the expression of *iutA* [13]. IutR itself is

Fig. 5. Schematic illustration of the regulation of aerobactin receptor protein production in *Vibrio vulnificus*. IutR acts as a transcription repressor in the absence of exogenous aerobactin, whereas in the presence of aerobactin, the IutR-aerobactin complex (probable but not proved) derepresses IutA production. Other abbreviations are the same as those in Fig. 2.

not regulated by Fe. The expression of *iutA* occurs at low levels even in the absence of exogenous aerobactin [13,57]. When aerobactin is supplied exogenously, *iutA* is fully expressed. A mutation in *iutR* derepresses the expression of *iutA* regardless of the presence of aerobactin. IutR binds directly to the regulatory region of *iutA* in the absence of aerobactin, thereby repressing the expression of *iutA*, whereas in the presence of aerobactin, *iutA* expression is derepressed by the interaction of IutR with an inducer (probably Fe-aerobactin), leading to a loss of the DNA binding affinity [13]. Accordingly, IutR acts as a local and specific repressor responding to the presence of aerobactin in iron-deficient environments. The expression of *vatC*, *vatD*, and *vatB* encoding components of the ABC transport system is also under the negative control of Fe-Fur [13].

REGULATION OF IUSs BY cAMP OR Crp

The expression of virulence factors is under the control of several global regulators in pathogenic bacteria including *V. vulnificus* [58]. Of these global regulators, cyclic AMP and its receptor protein (Crp) complex are primarily responsive to carbon availability and regulate the expression of a variety of genes, including those related to iron metabolism in *E. coli* [51]. In *V. vulnificus*, cAMP or Crp also regulates the expression of a variety of virulence factors [59,60].

Fig. 6. Schematic illustration of the regulation of target genes by the cyclic AMP (cAMP)-cAMP receptor protein (Crp) complex in *Vibrio vulnificus*. The thickness of line indicates the main function of Crp in *V. vulnificus*.

The expression of *vis* and *vuuA* is positively regulated by cAMP or Crp (Figs. 2 and 6) [52]. A mutation in *cyaA*, which encodes adenylate cyclase required for the synthesis of cAMP, or *crp*, which encodes Crp protein, downregulates the expression of *vis* and *vuuA* and attenuates the ability of *V. vulnificus* to use transferrin-bound Fe. The cAMP-Crp complex is thought to promote the progression of RNA polymerase by binding to a specific base sequence called the Crp-binding site in the regulatory region of *vis* or *vuuA* although direct binding remains to be experimentally determined. The effect of cAMP or Crp on the expression of *venB* or *vvsAB* also remains to be determined.

The expression of *hupA* is also under the positive control of Crp (Figs. 3 and 6) [46,47]. The cAMP-Crp complex promotes the progression of RNA polymerase by binding to a specific base sequence in the regulatory region of *hupA* [46]. A mutation in *cyaA* or *crp* decreases the expression of *hupA*. Moreover, the addition of exogenous cAMP increases the expression of *hupA* in the *cyaA*-mutated background, whereas the addition of glucose dose-dependently decreases the expression of *hupA* [47].

In addition, the expression of *iutA* is under the positive control of Crp (Figs. 5 and 6) [57]. A mutation in *cyaA* or *crp* also decreases the expression of *iutA*. As with *hupA*, the expression of *iutA* in the *cyaA*-mutated background was increased by the addition of exogenous cAMP and is dose-dependently decreased by the addition of glucose.

The regulation of IUSs by cAMP or Crp indicates that the amount of iron required by *V. vulnificus* is controlled in accordance with the activity of carbon metabolism via the cAMP-Crp complex.

REGULATION OF IUSs BY QUORUM SENSING

In many pathogenic bacteria including *V. vulnificus*, harmonious organization of virulence gene expression is a key factor in appropriate colonization, invasion, *in vivo* growth, and *in situ* toxin production [61]. The cell-to-cell signaling system known as quorum sensing (QS) modulates the expression of virulence factors and synchronizes group behavior for improved survival [62]. In *V. vulnificus*, the autoinducer-2 (AI-2)-mediated QS system has been well studied [63,64] despite the presence of other QS signal mol-

Fig. 7. Schematic illustration of the regulation of target genes by the autoinducer-2 (AI-2)-mediated quorum sensing system in *Vibrio vulnificus*. HlyU is a small regulatory protein, whose expression is directly and negatively regulated by SmcR, a master regulator of the AI-2-mediated quorum sensing system. Details are described in the text.

ecules [65]. *V. vulnificus* possesses a well-organized QS circuit (Fig. 7), consisting of LuxS (AI-2 synthetase), LuxP-LuxQ (membrane bound sensor proteins), LuxU-LuxO (response regulators), and SmcR (the master transcriptional regulator), all of which are involved in the regulation of virulence factors, such as metalloprotease and hemolysin [65]. Moreover, small RNAs are involved in the repression of SmcR [66]. In addition, LuxT acts as a negative transcriptional regulator of SmcR [66–68].

At low cell densities, the concentration of AI-2 is insufficient to enable QS, LuxQ acts as a kinase and adds a phosphate group to LuxU, which, in turn, transfers the phosphate group to LuxO. LuxO is activated by phosphorylation, and together with RpoN (a nitrogen-limitation sigma factor), sRNAs, and Hfq (a sRNA binding protein), it acts to suppress the production of SmcR. The activated LuxO can also stimulate LuxT, which inhibits the expression of *smcR* [68]. By contrast, at high cell densities, AI-2 is present at sufficient concentration to interact with LuxP-LuxQ and modulate their function to a phosphatase, which dephosphorylates LuxO. The dephosphorylated LuxO is inactive and can no longer inhibit SmcR. SmcR can therefore activate or repress the expression of target genes, such as *vvpE* which encodes a metalloprotease and *vvhA* which encodes a hemolysin [68].

A previous study [53] reported that SmcR acts as a tran-

scriptional repressor of the expression of *vvsAB* by directly binding to the regulatory region of *vvsAB*, especially in Fe-deficient conditions (Fig. 2). A mutation in *smcR* derepresses vulnibactin production. The binding site of SmcR overlaps with that of Fe-Fur in the regulatory region of *vvsAB*. However, Fe-Fur binds to the regulatory region of *vvsAB* with higher affinity than SmcR. Therefore, Fur is the key repressor that prevents the overexpression of *vvsAB* in response to Fe in Fe-sufficient environments, whereas SmcR is another key repressor that prevents the overexpression of *vvsAB* in response to bacterial density in Fe-deficient environments. In another study [69], a mutation in *luxS* slightly decreased the expression of *vuuA* but slightly increased the expression of *iutA* and *hupA* in *V. vulnificus* (Figs. 2, 3 and 5). There is a controversy on the effect of AI-2-QS on the expression of IUSs between the two studies; that is to say, a mutation in *smcR* derepresses vulnibactin production, whereas a mutation in *luxS* decreases vulnibactin receptor production. This controversy remains unexplainable to date. However, it is undeniable that Fe-Fur and QS cooperate to regulate the expression of IUSs in response to Fe availability for the fine-tuning of the intracellular iron level and improved survival of *V. vulnificus*. Further coherent studies need to reveal details on the interaction between IUSs and QS.

REGULATION OF IUSs BY TEMPERATURE

The effect of other environmental factors affecting the expression of IUSs in addition to Fe availability has not been extensively studied. *V. vulnificus* resides in estuarine environments and can infect humans, and thus, *V. vulnificus* is capable of sensing and responding to diverse environmental changes for either survival or successful establishment of infections in the human body [70,71]. Upon entering the human body, *V. vulnificus* must withstand a temperature change from 25°C to 37°C. The change of temperature modulates the expression of several virulence factors that are required for the bacterial survival and successful infection [67,72]. *V. vulnificus* modulates the expression of *hupA* by sensing and responding to the change of temperature especially in clinical isolates [46,48,49]. In addition, the change of temperature resulted in increased expression of *vuuA* and *crp*, and Crp can then act as a transcriptional

activator in the expression of *vuuA* [73]. These indicate that a change of temperature is an environmental signal for the regulation of IUSs. Details on the temperature-mediated regulation of IUSs remain to be determined.

EXOTOXINS AFFECTING Fe AVAILABILITY AND THEIR REGULATION

Regulation of *V. vulnificus* hemolysin and proteases by Fe-Fur (Fig. 8)

Most of Fe in the human body exists in the form of hemoglobin in erythrocytes. *V. vulnificus* produces several cytotoxins [19,20,58], especially hemolysin, which have a great impact on Fe availability because they can destroy host cells (especially erythrocytes) to release Fe [74]. There is still much doubt concerning the pathogenic role of hemolysin in infections of *V. vulnificus* [75]. However, even if only a very small amount of active hemolysin is produced, which subsequently destroys only a very small amount of erythrocyte, sufficient Fe is made available for use by *V. vulnificus*.

In addition, *V. vulnificus* is hypothesized to utilize the metalloprotease VvpE activity to assimilate Fe by degrading heme proteins, transferrin and lactoferrin, thereby releasing Fe and increasing its availability [76]. However, this hypothesis has not been supported by further studies [36,37,77]. A mutation in *vvpE* did not affect the ability of *V. vulnificus* to produce vulnibactin or to acquire Fe from transferrin or

hemoglobin in Fe-deficient media or in ascitic fluids obtained from liver cirrhosis patients, which is considered an *ex vivo* experimental system for *V. vulnificus*. By contrast, a mutation in *fur* derepressed the production of vulnibactin, and facilitated the assimilation of Fe from transferrin, and a mutation in *vis* abolished the production of vulnibactin and the assimilation of Fe from transferrin under the same conditions. These results show that vulnibactin is essentially required for the assimilation of Fe from transferrin or hemoglobin, and VvpE has no direct effect on the assimilation of Fe from transferrin or hemoglobin via the vulnibactin-mediated IUS under *in vitro* or *ex vivo* conditions. Discrepancies among these studies on the roles of VvpE in the assimilation of Fe from transferrin or hemoglobin may be due to the use of different experimental conditions and methods.

A Fur box exists in the regulatory region of *vvhA* in *V. vulnificus* [78]. The expression of *vvhA* is repressed by Fe and derepressed by a mutation in *fur*. However, the extracellular secretion of VvhA hemolysin is conversely increased by Fe [26,79]. VvhA is secreted extracellularly via the type II general secretion system. The extracellular secretion of VvhA is reduced by a mutation in *pilD*, which encodes PilD, a component of the type II general secretion system. Fe increases the expression of *pilD*. Thus, Fe represses the expression of *vvhA* via Fur at the transcriptional level but increases the extracellular secretion of VvhA via the type II general secretion system. In addition, extracellular VvhA is digested by

Fig. 8. Schematic illustration of the regulation of hemolysin and metalloprotease production in *Vibrio vulnificus*. ToxR: a regulator of undefined signal transduction system, which is a homolog of the ToxRS system in *Vibrio cholerae*. H-NS is a histone-like nucleoid protein, which is involved in the temperature-mediated regulation of gene expression. HlyU is a small regulatory protein, whose expression is directly and negatively regulated by SmcR, a master regulator of the autoinducer-2-mediated quorum sensing system. RpoS is a sigma factor (σ^{38}) for RNA polymerase, which is involved in various stationary stress responses. Other abbreviations are the same as those in Fig. 2.

VvpE and VvpM, which are produced by *V. vulnificus*. These results indicate that Fur regulates the production of VvhA at the transcriptional level by directly binding to the regulatory region of *vvhA* and at the post-translational level by regulating the expression of two VvhA-degrading exoproteases, namely, VvpE and VvpM.

The expression of *vvpE*, but not *vvpM*, is also negatively regulated by direct binding of Fur to its regulatory region [78], indicating that VvpE production is repressed by Fe. However, in other studies [27,36,37,77], Fe increases the expression of *vvpE* in *V. vulnificus*. Fe stimulated the expression of *vvpE* and the extracellular production of VvpE even in a *crp* and/or *smcR* mutated background. These indicate that Fe increases the expression of *vvpE* through factor(s) other than Crp and SmcR. This controversy among these studies may also be due to the use of different experimental conditions and methods.

Regulation of hemolysin and protease by cAMP or Crp (Fig. 8)

The role of the global regulator cAMP-Crp complex in both virulence and general metabolism in *V. vulnificus* has been extensively investigated. cAMP is generated through cleavage of ATP by adenylate cyclase, which is encoded by *cyaA* in *V. vulnificus* [58]. cAMP acts as a regulator by binding to Crp, which then binds to the regulatory regions of target genes, modulating their expressions. In *V. vulnificus*, several virulence or toxic factors, including VvpE and VvhA, have been shown to be regulated by the cAMP-Crp system. A mutation in *cyaA* or *crp* dramatically decreases VvhA production [59,60]. The cAMP-Crp complex directly binds to the regulatory region of *vvhA* activating its expression [80,81]. Thus, the cAMP-Crp complex is the primary positive regulator for the expression of *vvhA*.

The cAMP-Crp complex also regulates the expression of *vvpE*. The complex works in concert with QS via SmcR and exerts its effects on the RpoS-mediated expression of *vvpE* [82,83]. Two different promoters, namely L and S, are involved in the expression of *vvpE* [31,82,83]. The activity of the L promoter is constitutive throughout bacterial growth and is lower than that of the S promoter. The expression of *vvpE* via the S promoter is induced only in the stationary growth phase, and is dependent on RpoS and positively regulated by both the cAMP-Crp complex and SmcR. The

cAMP-Crp complex and SmcR bind to the different sites of the S promoter. They then act synergistically to activate the expression of *vvpE*. In other studies [84-86], a mutation in *crp* decreases the expression of *vvpE* in the early growth stage, whereas a mutation in *smcR* decreases the expression of *vvpE* only at the late growth stage. A double mutation in *crp* and *smcR* can severely inhibit the expression of *vvpE* from the early growth stage, and the inhibited expression of *vvpE* is restored only at the early growth stage by a single complementation of *crp*, but not *smcR*. Taken together, the cAMP-Crp complex is the primary positive regulator, and SmcR synergistically cooperates with the cAMP-Crp complex for the RpoS-dependent expression of *vvpE*.

In addition, a study reported that *crp* and *rpoS* are expressed more strongly at 26°C than at 37°C [67]. Other studies [73,85,86] controversially showed that *V. vulnificus* growth is stimulated and *crp* or *smcR* or *vvpE* is expressed more strongly by a temperature change from 25°C to 37°C. This inconsistency remains to be clarified via further coherent studies.

Regulation of hemolysin and protease by QS (Fig. 8)

The QS system has a key role in the coordinate expression of *vvhA* and *vvpE* in *V. vulnificus*. The expression of *vvpE* is decreased by a mutation in *luxS* or *smcR* but increased by a mutation in *luxO*, whereas the expression of *vvhA* is affected in the opposite manner and increased by a mutation in *luxS* and *smcR* but decreased by a mutation of *luxO* [63,64,66,67]. Thus, the QS system in *V. vulnificus* appears to positively regulate the expression of *vvpE*, but negatively regulate the expression of *vvhA*.

Regulation of hemolysin and protease by HlyU or HNS (Fig. 8)

The small regulatory protein HlyU also regulates the expression of *vvhA* in *V. vulnificus*. The importance of HlyU in the virulence of this pathogen was first recognized by detection of antibodies against HlyU in the serum of patients infected by *V. vulnificus* [70]. A mutation in *hlyU* reduces the cytotoxicity and mouse lethality of *V. vulnificus* and decreases the expression of *vvhA* [70,87]. The expression of *vvhA* regulated by HlyU is also dependent on the QS system [88]. SmcR directly binds to a regulatory region of *hlyU*, re-

pressing the expression of *hlyU*. A double mutation in *smcR* and *hlyU* restores the production of VvhA in the absence of H-NS, a histone-like nucleoid structuring protein that is known to be involved in the temperature-mediated regulation of gene expression [89]. Collectively, SmcR regulates the expression of *vvhA* by repressing *hlyU*.

In addition, a mutation in *hns* increases the expression of *vvhA* but decreases the expression of *vvpE* [89]. H-NS directly binds and competes with HlyU for binding to the regulatory region of *vvhA* but not *vvpE*. H-NS positively affects the mRNA level of *rpoS*, and then RpoS positively regulates the expression of *vvpE*. These indicate that the role of H-NS is dependent on the QS in the regulation of *vvhA*, but not in the regulation of *vvpE*.

Regulation of hemolysin and protease by ToxRS (Fig. 8)

V. vulnificus is known to possess another signal transduction system, which is mediated by ToxRS. The ToxRS system of *V. vulnificus* is functionally similar to that of *V. cholerae*. In *V. cholerae*, ToxR regulates the expression of multiple virulence factors including cholera toxin, toxin-coregulated pilus, and accessory colonization factor genes [90]. A mutation in *toxR* decreases the production of VvhA in *V. vulnificus* [91]. However, there have been no further studies on the ToxRS system in *V. vulnificus*.

INTERACTION AMONG Crp, Fur, QS AND/OR RpoS

In addition to the functional cooperation among Crp, Fur, QS and/or RpoS, the direct interaction among these global regulators has been investigated (Fig. 8). Crp primarily responding to carbon availability is involved in the expression of *smcR*, which encodes the QS master regulator SmcR primarily responding to bacterial density [92]. A mutation in *crp* impaired *V. vulnificus* growth, decreased AI-2 production, and repressed the expression of *smcR*. Furthermore, *smcR* expression was repressed by glucose in the presence of Crp but not in its absence. A mutation in *cyaA* also impaired *V. vulnificus* growth and repressed *smcR* expression. These results indicate that cAMP or CRP modulates the AI-2-QS system in response to glucose availability in *V. vulnificus*, demonstrating the presence of a connection between

catabolite repression and QS in *V. vulnificus*.

It has not been determined yet whether Crp is involved in the expression of Fur, the global regulator responding to Fe availability, in *V. vulnificus*. However, it has long been known that Crp positively regulates the expression of Fur in *E. coli* [93]. This suggests that this direct interaction between Crp and Fur may also exist in *V. vulnificus*. Further studies on this interaction are necessary. Interestingly, Fur positively autoregulates the expression of Fur in *V. vulnificus* [94] as well as in *E. coli* [93]. In *V. vulnificus*, a mutation in *fur* downregulates the expression of *fur* in a low-Fe condition, and Fur can directly bind to the regulatory region of *fur* [94,95].

Fe or Fur also directly or indirectly affects the activity of AI-2-QS system in *V. vulnificus* [66,96].

The expression of *vvpE*, a member of the QS regulon, is repressed under iron-rich conditions, and Fe-Fur represses the expression of *smcR* by directly and reversibly binding to the regulatory region of *smcR*. Apo-Fur does not bind to the regulatory region of *smcR*. Furthermore, Fe-Fur represses the expressions of *qrr1-5*, which are quorum-regulatory small RNAs that inhibit the expression of *smcR* at the post-transcriptional level, by directly binding to their regulatory regions. The expression of *luxO* and *qrr* RNAs is also repressed by Fe independently of Fur. All of these findings indicate that Fe and QS are linked together in the coordinated expression of virulence factors.

Crp also regulates the expression of *rpoS* [97]. RpoS is an alternative sigma factor (σ^{38}) for RNA polymerase, which is involved in various stationary stress responses. Cellular cAMP levels are inversely correlated with RpoS levels, and a mutation in *cyaA* or *crp* derepresses the expression of *rpoS*. The cAMP-Crp complex represses the expression of *rpoS* by directly binding to its regulatory region.

CONCLUSION

V. vulnificus is a Gram-negative ferrophilic bacterium that contains well-developed multiple IUSs. *V. vulnificus* causes necrotizing wound infections and fatal septicemia that mainly occur in patients with elevated levels of Fe in serum or tissues. These IUSs play important roles in the pathogenesis of *V. vulnificus* infections, and are sophisticatedly controlled at the transcriptional level by global regulators as well

as their local or specific regulators. Moreover, functional and direct interactions of global regulators are also present. Accordingly, this sophisticated and delicate regulation of the IUSs allows *V. vulnificus* to sensitively or appropriately respond to a variety of intrinsic or environmental signals to enable bacterial survival and regulation of virulence factors, thereby causing fatal infections. It is expected that a continued understanding of IUSs will contribute to the development of new treatments and preventive measures for *V. vulnificus* infections.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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