Glucose-starvation is an essential signal for the vulnibactin receptor\textit{vuuA} gene expression in \textit{Vibrio vulnificus}

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Introduction

\textit{Vibrio vulnificus} is a gram-negative halophilic bacterium that causes fatal septicemia and necrotizing wound infections with a high mortality rate in susceptible individuals. \textit{V. vulnificus} possesses a variety of virulence factors, including acid neutralization, capsular polysaccharide expression, iron acquisition, cytotoxicity, motility, and expression of proteins involved in attachment and adhesion [Jones and Oliver, 2009]. \textit{V. vulnificus} is a ferrophilic bacterium that requires a higher level of easily-available iron for initiating growth than other pathogens [Kim \textit{et al.}, 2007]. \textit{V. vulnificus} possesses multiple iron-uptake systems (IUSs) that can utilize various iron sources. In particular, the vulnibactin receptor (VuuA)-mediated IUS determines the ability of \textit{V. vulnificus} to utilize transferrin-bound iron [Litwin \textit{et al.}, 1996; Webster and Litwin, 2000]. Nevertheless, the molecular mechanisms regulating VuuA-mediated IUS remain unknown. Vulnibactin production or \textit{vuuA} expression is negatively regulated by the ferric uptake regulator (Fur) [Litwin and Calderwood, 1993]. Recently, VuuA and heme receptor (HupA) expressions were demonstrated to be under the positive control of cyclic AMP-receptor protein (Crp), which primarily responds to carbon availability [Choi \textit{et al.}, 2006; Oh \textit{et al.}, 2009]. These findings imply the presence of interactions between carbon and iron metabolism or interactions between the carbon utilization regulator Crp and the iron utilization regulator Fur. Diverse interactions between Crp and Fur have been identified in \textit{Escherichia coli} [Zhang \textit{et al.}, 2005]. However, the interactions remain to be clarified in \textit{V. vulnificus}. Accordingly, this study was designed to identify the interactions between Crp and Fur and to determine that the two global regulators cooperatively regulate \textit{vuuA} expression.

Materials and methods

A deferrated medium was prepared as described previously [Kim \textit{et al.}, 2007; Choi \textit{et al.}, 2006]. Ferric chloride (FC) was added into the deferrated medium as an iron source. N-tris(methyl-2-aminoethanesulfonic acid was added as a buffering agent to reduce pH changes resulting from glucose catabolism. We constructed \textit{crp-} and \textit{fur-} deletional mutant strains, as described previously [Choi \textit{et al.}, 2006; Oh \textit{et al.}, 2009]. The merozygotic \textit{P}\textsubscript{gus}::\textit{lacZ} transcription reporter strains with various genetic backgrounds were constructed to compare transcriptional levels. All mutations were \textit{in trans} complemented by plasmids harboring wild-type genes. Transcription levels were indicated as β-galactosidase activity [Miller, 1992]. We conducted Western blot using rabbit polyclonal antibodies specific for each protein to compare protein levels (details will be reported elsewhere).

Results and discussion

Effect of iron and fur mutation on crp expression.

FC dose-dependently increased growth and \textit{crp} transcription levels at less than 10 μM, but levels were not further increased by more than 10 μM FC. A \textit{fur} mutation significantly increased \textit{crp} expression levels (Fig. 1). A \textit{fur} complementation normalized the increased \textit{crp} expression levels. The \textit{fur} mutation increased intracellular Crp levels, and the \textit{fur} complementation normalized the increased intracellular Crp levels. These results indicate that Fur prevents \textit{crp} over-expression in response to increasing iron level.
Effect of iron and crp mutation on fur expression.

FC dose-dependently increased fur expression levels at less than 10 μM, but levels were not further increased by more than 10 μM FC. A crp mutation partially but significantly decreased fur transcription levels with impaired growth, and a crp complementation normalized the decreased fur transcription and growth levels (Fig. 2). The crp mutation also decreased intracellular Fur levels and the crp complementation also normalized the decreased intracellular Fur levels. These results indicate that Crp modulates fur expression.

Effect of iron, glucose, crp mutation and fur mutation on vuuA expression.

FC repressed vuuA expression; namely, vuuA expression was highly induced at 5 μM FC, but severely repressed at 25 μM FC. Adding glucose significantly decreased the induced vuuA expression levels at 5 μM FC. The crp mutation also significantly decreased the induced vuuA expression levels at 5 μM FC, and the crp complementation normalized the decreased vuuA expression levels. In contrast, the fur mutation de-repressed the repressed vuuA expression levels at 25 μM FC, whereas the fur complementation normalized the de-repressed vuuA expression levels (Fig. 3). In Western blot, VuA production levels were observed with the same trends as vuuA transcription levels, indicating that glucose-starvation as well as iron-starvation is essential for vuuA expression, and that Crp is essential for inducing vuuA expression in response to glucose starvation, whereas Fur prevents vuuA over-expression in response to increasing iron concentration.

![Figure 1](image1.png)  
Figure 1. Effect of a fur mutation on crp expression at the transcription level. Merozygotic P<sub>crp</sub>:lacZ transcription reporter strains with wild-type fur, mutated fur and in trans-complemented fur were cultured in media containing 5 or 25 μM ferric chloride. After culturing for 12 h, β-galactosidase activity was measured by the Miller method [Miller, 1992]. The # symbol indicates a statistically significant difference (p<0.05).

![Figure 2](image2.png)  
Figure 2. Effect of a crp mutation on fur expression at the transcription level. Merozygotic P<sub>fur</sub>:lacZ transcription reporter strains with wild-type crp, mutated crp and in trans-complemented crp were cultured in media containing 5 or 25 μM ferric chloride. After culturing for 12 h, β-galactosidase activity was measured by the Miller method [Miller, 1992]. The # symbol indicates a statistically significant difference (p<0.05).

![Figure 3](image3.png)  
Figure 3. Effect of a crp mutation (A), a fur mutation (B) and glucose (C) on vuuA expression at the transcription level. A and B: P<sub>vuuA</sub>:lacZ transcription reporter strains with the indicated genetic backgrounds were cultured media containing 5 or 25 μM ferric chloride. C: The P<sub>vuuA</sub>:lacZ transcription reporter strain with wild-type crp and fur was cultured in media containing 5 μM ferric chloride plus PBS or 0.25% glucose. After culturing for 12 h, β-galactosidase activity was measured by the Miller method [Miller, 1992]. The # symbol indicates a statistically significant difference (p<0.05).
Diverse interactions among global regulators are believed to coordinate the activities of the different metabolons, so that the supply of one type of nutrient matches the supply of other essential types of nutrients. These interactions serve as the bacterial “nervous system,” coordinating the various activities of bacterial cells [Zhang et al., 2005; Gutierrez-Rios et al., 2003]. Diverse functional interactions between Fur and Crp have been identified in *E. coli* [Zhang et al., 2005]. Moreover, Crp modulates *fur* expression in *E. coli* [De Lorenzo et al., 1988]. This study also showed that there was a functional interaction between Crp and Fur in regulating *vuuA* expression, and that Crp modulated *fur* expression in *V. vulnificus*. Moreover, this study presented a new finding that Fur repressed *crp* expression in *V. vulnificus*. To our knowledge, the regulation of *crp* expression by Fur remains unknown even in *E. coli*. A putative Crp binding site was found in the regulatory region of the *V. vulnificus fur* gene and a putative Fur-binding site was found in the regulatory region of the *V. vulnificus crp* gene although binding assays were not performed in this study. Accordingly, a mutual or horizontal interaction, rather than a hierarchical or unidirectional interaction, is likely to be present between Crp and Fur in *V. vulnificus*.

Iron is essential for activating many catabolic enzymes, especially those involved in the electron transport system. Glucose is the most preferable energy source in most bacteria. Based on this study, glucose-starvation or energy-depletion is likely to be an essential signal for *vuuA* expression. This implies that iron uptake should be increased to stimulate catabolism or to efficiently produce energy under glucose-poor stressful conditions. In summary, glucose-starvation as well as iron-starvation was essential for *vuuA* expression, and Crp was required for *fur* and *vuuA* expression in response to glucose-starvation, whereas Fur prevented *crp* and *vuuA* over-expression in response to increasing iron level.

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**References**


