Glucose-starvation is an essential signal for the vulnibactin receptor *vuuA* gene expression in *Vibrio vulnificus*

C.M. Kim[1], J.N. Park[1], Y.H. Shin[1] and S.H. Shin[1,2]*

[1] Research Center for Resistant Cells, Chosun University Medical School, Gwangju 501-759, Republic of Korea (choonmee@chosun.ac.kr)
[2] Department of Microbiology, Chosun University Medical School, Gwangju 501-759, Republic of Korea (shsin@chosun.ac.kr)
*corresponding author

Introduction

Vibrio vulnificus is a gram-negative halophilic bacterium that causes fatal septicemia and necrotizing wound infections with a high mortality rate in susceptible individuals. *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].

V. vulnificus is a ferrophilic bacterium that requires a higher level of easily-available iron for initiating growth than other pathogens [Kim *et al*., 2007]. *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 *V. vulnificus* to utilize transferrin-bound iron [Litwin *et al*., 1996; Webster and Litwin, 2000]. Nevertheless, the molecular mechanisms regulating VuuA-mediated IUS remain unknown.

Vulnibactin production or *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 *et al*., 2006; Oh *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 *Escherichia coli* [Zhang *et al*., 2005]. However, the interactions remain to be clarified in *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 *vuuA* expression.

Materials and methods

A deferrated medium was prepared as described previously [Kim *et al*., 2007; Choi *et al*., 2006]. Ferric chloride (FC) was added into the deferrated medium as an iron source. N-trismethyl-2-aminoethanesulfonic acid was added as a buffering agent to reduce pH changes resulting from glucose catabolism. We constructed *crp*- and *fur*-deletional mutant strains, as described previously [Choi *et al*., 2006; Oh *et al*., 2009]. The merozygotic P_{gene}*::lacZ* transcription reporter strains with various genetic backgrounds were constructed to compare transcriptional levels. All mutations were *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 *crp* transcription levels at less than 10 μM, but levels were not further increased by more than 10 μM FC. A *fur* mutation significantly increased *crp* expression levels (Fig. 1). A *fur* complementation normalized the increased *crp* expression levels. The *fur* mutation increased intracellular Crp levels, and the *fur* complementation normalized the increased intracellular Crp levels. These results indicate that Fur prevents *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, VuuA 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.

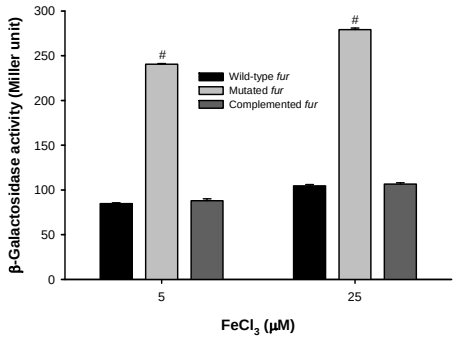

Figure 1. Effect of a *fur* mutation on *crp* expression at the transcription level. Merozygotic P_{crp}*::lacZ* transcription reporter strains with wild-type *fur*, mutated *fur* and *in trans*-complemented *fur* were cultured in media containing 5 or 25 μM ferric chloride. After culturing for 12 h,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

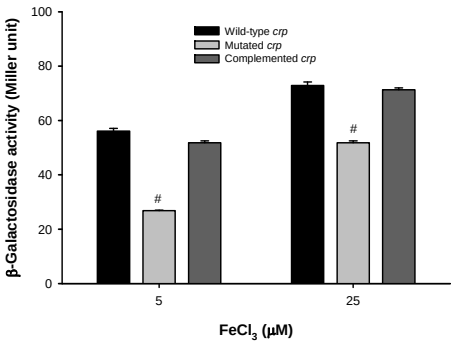

Figure 2. Effect of a *crp* mutation on *fur* expression at the transcription level. Merozygotic P_{fur}*::lacZ* transcription reporter strains with wild-type *crp*, mutated *crp* and *in trans*-complemented *crp* were cultured in media containing 5 or 25 μM ferric chloride. After culturing for 12 h,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

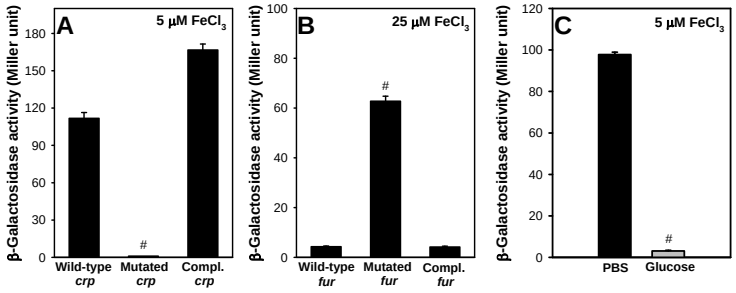

Figure 3. Effect of a *crp* mutation (A), a *fur* mutation (B) and glucose (C) on *vuuA* expression at the transcription level. A and B: P_{vuuA}*::lacZ* transcription reporter strains with the indicated genetic backgrounds were cultured media containing 5 or 25 μM ferric chloride. C: The P_{vuuA}*::lacZ* 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.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (313-2007-2-E00140) and by the National Research Foundation of Korea (NRF) grant funded by the Korea government through the Research Center for Resistant Cells (R13-2003-009).

References

[1] Choi M.H., Sun H.Y., Park R.Y., Kim C.M., Bai Y.H., Kim Y.R., Rhee J.H., Shin S.H. (2006) Effect of the *crp* mutation on the utilization of transferrin-bound iron by *Vibrio vulnificus*. FEMS Microbiol Lett 257(2), 285-292.

[2] De Lorenzo V., Herrero M., Giovannini F., Neilands J.B. (1988) Fur (ferric uptake regulator) protein and CAP (catabolite-activator protein) modulate transcription of fur gene in *Escherichia coli*. Eur J Biochem 173(3), 537-546.

[3] Gutierrez-Rios R.M., Rosenblueth D.A., Loza J.A., Huerta A.M., Glasner J.D., Blattner F.R., Collado-Vides J. (2003) Regulation network of *Escherichia coli*: consistency between literature knowledge and microarray profiles. Genome Res 13(11), 2435-2443.

[4] Jones M.K., Oliver J.D. (2009) *Vibrio vulnificus*: Disease and Pathogenesis. Infect Immun 77(5), 1723–1733.

[5] Kim C.M., Park R.Y., Choi M.H., Sun H.Y., Shin S.H. (2007) Ferrophilic characteristics of *Vibrio vulnificus* and potential usefulness of iron chelation therapy. J Infect Dis 195(1), 90-98.

[6] Kim C.M., Chung Y.Y, Shin S.H. (2009) Iron differentially regulates gene expression and extracellular secretion of *Vibrio vulnificus* cytolysin-hemolysin. J Infect Dis 200(4), 582-589.

[7] Litwin C.M., Calderwood S.B. (1993) Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of a *fur* mutant by *in vivo* marker exchange. J Bacteriol 175(3), 706-715.

[8] Litwin C.M., Rayback T.W., Skinner J. (1996) Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence, Infect Immun 64(7), 2824-2838.

[9] Miller J.H. (1992) A short course in bacterial genetics: A laboratory manual and handbook of *Escherichia coli* and related bacteria. Cold Spring Harbor Press, New York, NY, USA.

[10] Oh M.H., Lee S.M., Lee D.H., Choi S.H. (2009) Regulation of the *Vibrio vulnificus hupA* gene by temperature alteration and cyclic AMP receptor protein and evaluation of its role in virulence. Infect Immun 77(3), 1208-1215.

[11] Webster A.C.D., Litwin C.M. (2000) Cloning and characterization of *vuuA*, a gene encoding *Vibrio vulnificus* ferric vulnibactin receptor. Infect Immun 68(2), 526-534.

[12] Zhang Z., Gosset G., Barabote R., Gonzalez C.S., Cuevas W.A., Saier M.H.Jr. (2005) Function interactions between the carbon and iron utilization regulators, Crp and Fur, in *Escherichia coli*. J Bacteriol 187(3), 980-990.