Measurement of Haemolysin Activities in *Vibrio vulnificus*

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[Abstract] VvhA produced by *Vibrio vulnificus* exhibits cytolytic activity to human cells including erythrocytes. Since haemolysis by VvhA may provide iron for bacterial growth and pathogenicity, we investigated the expression of VvhA to elucidate the regulatory roles of Fur, a major transcription factor controlling iron-homeostasis. Fur repressed the transcription of *vvhBA* operon via binding to the promoter region. However, haemolysin content and haemolytic activity were lowered in cell-free supernatant of *fur* mutant. This discrepancy between the levels of *vvhA* transcript and VvhA protein in *fur* mutant was caused by exoproteolytic activities of the elastase VvpE and another metalloprotease VvpM, which were also regulated by Fur. *vvpE* gene expression was repressed by Fur via binding to the Fur-box homologous region. Regulation of VvpM expression by Fur did not occur at the level of *vvpM* transcription. *In vitro* proteolysis assays showed that both proteases efficiently degraded VvhA. In addition, the extracellular levels of VvhA were higher in culture supernatants of *vvpE* or *vvpM* mutants than in the wild type. Thus this study demonstrates that Fur regulates haemolysin production at the transcription level of the *vvhBA* operon and at the post-translation level by regulating the expressions of two VvhA-degrading exoproteases, VvpE and VvpM.

This protocol can be applied to other Vibrio strains with haemolysin activities, such as *Vibrio parahaemolyticus* (*V. parahaemolyticus*) or other human pathogen strains with similar heamolysin activities.

Materials and Reagents

1. Bacterial strains
   a. Wild type (*Vibrio vulnificus MO6-24/O*, Clinical isolate: biosafety level 2) with pRK415 plasmid (vector control)
   b. *fur* mutant with pRK415
   c. *fur* mutant with pRK415-*fur*

2. 1% human red blood cells (RBCs) (from healthy person who is a volunteer and diluted with PBS buffer to make a 1% RBC solution)
3. Tetracycline (3 mg/ml) (Sigma-Aldrich)
4. PBS buffer (Sigma-Aldrich, catalog number: P5368)
5. 0.02% Triton X-100 (Triton™ X-100 for molecular biology) (Sigma-Aldrich, catalog number: T8787)
6. LB broth (see Recipes)

Equipment

1. Shaking incubator
2. Centrifuge (14,000 rpm or 21,000 *x g*)
3. Spectrophotometry (540 and 600 nm)
4. 0.22 micron filter

Procedure

1. Grow 5 ml of bacterial cells at 30 °C shaking incubator, in modified Luria–Bertani (LB) [addition of NaCl to LB at a final concentration of 2.5% (w/v): *Vibrio vulnificus* is a marine bacterium, so 2.5% NaCl makes cell happy to grow] medium supplemented with antibiotics (tetracycline 3 mg/ml).
2. Take bacterial samples and measure cell density by spectrophotometry at 600 nm.
3. Collect the supernatants of cultures by centrifugation (14,000 rpm, 5 min, RT) and filtration (use with syringe filter: pore size 0.22 μm).
4. Prepare cell-free supernatants from each culture and serial dilute them with PBS by 1/2, 1/4 and 1/8 (final volume: 1 ml).
5. Incubate 1% RBC solution with the equal volume of the diluted supernatants at 37 °C for 1 h (no agitation).
6. After 1-h incubation, centrifuge of each samples to remove unlysed RBCs (14,000 rpm, 5 min, RT).
7. To make the fully lysis of 1% RBC solution, use equal volume of 0.02% Triton X-100 at 37 °C for 1 h (no agitation).
8. After centrifuge of each sample, take the upper part of samples. Measure the lysed RBCs samples by spectrophotometry at 540 nm, as described by Shinoda *et al*., 1985 (for the blank at 540 nm, you can use culture broth without bacteria cells).
9. Calculate Haemolytic activity (HU) (HU was expressed as the reciprocal of the dilution factor showing 50% haemolysis) (Shinoda *et al*., 1985).

Representative data

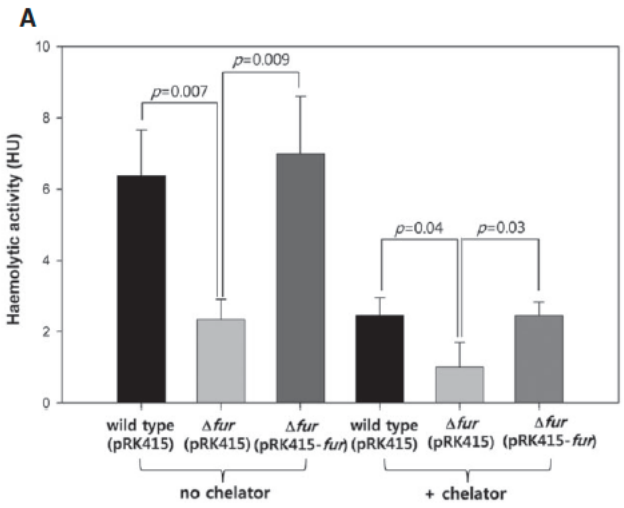

Figure 1. Haemolytic activities are decreased by *fur* mutation or iron deprivation. Haemolytic activities in the supernatants of wild type (pRK415), *Δfur* strain (pRK415) and *Δfur* strain (pRK415-fur) cultures. *V. vulnificus* strains were grown in LBS supplemented with 3 μg/ml tetracycline for 2 h, and each culture was treated for 4 h with 2,2′-dipyridyl at a concentration of 0.1 mM (+ chelator) or 0 mM (no chelator). OD600 of the cultures of wild type (pRK415) and *Δfur* strain (pRK415-fur) were 2.0–2.5, and OD600 of the *Δfur* (pRK415) cultures were 1.0–1.5. Serial dilutions of cell-free supernatants were added to 1% RBC solution, and lysed RBCs were measured by spectrophotometry. Activity was expressed as haemolytic units (HU), the reciprocal of the dilution factor showing 50% haemolysis (Lee *et al.*, 2013).

Recipes

1. LB broth
   10 g of Peptone
   10 g of Yeast extract
   5 g Sodium Cloride per one liter

Acknowledgments

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