Effect of VirF on the Promoter Activity of yscW-virF Operon in Yersinia enterocolitica

Thuan Nghi Vu\(^1\)\(^2\) and Glenn Marvin Young\(^1\)

\(^1\) Department of Food Science and Technology, College of Agricultural and Environmental Sciences, University of California at Davis, Davis, CA, USA

\(2\) School of Biotechnology, Assumption University, Bangkok, Thailand.

Abstract

The Yersinia enterocolitica Ysc-Yop T3SS and its Yop effectors are essential for the bacteria to survive and overcome the host immune system. Expression of Ysc-Yop T3SS at the transcriptional level is thermo-controlled by the AraC transcriptional activator VirF, encoded by virF. The role of VirF in transcriptional regulation of Y. enterocolitica Ysc-Yop T3SS genes has been intensively investigated but little in known about how the virF gene is controlled. This study was aimed at understanding how virF expression is controlled. The results showed that the virF, together with the yscW located upstream of virF are transcribed as an operon from the promoter P\(_{yscW}\). We further assessed whether VirF might control transcription from P\(_{yscW}\) using a transcriptional fusion to a lacZ reporter. The analysis revealed that VirF does not have any influence to activity of promoter P\(_{yscW}\) at low (26 oC) or high (37 oC) temperature.

Keywords: Yop effectors, host immune system, transcriptional activator, T3SS gene, operon, lacZ reporter.

1. Introduction

Yersinia enterocolitica, a gastro-intestinal foodborne pathogen, harbors the Ysc-Yop T3SS for its virulence. The whole Ysc-Yop T3SS including Ysc injectisome, secreted Yop proteins and their chaperones is encoded by the virulent plasmid pYV (Cornelis et al. 2002). In the absence of Ca\(^{2+}\) ions and at 37°C, pathogenic Y. enterocolitica releases high amounts of Yop effectors that involved in pathogenesis (Lambert de Rouvroit et al. 1992).

The expression of Ysc-Yop T3SS is strongly thermo-regulated by transcriptional activator VirF (Lambert de Rouvroit et al. 1992). VirF, a 30.9 kDa protein, belongs to the AraC family of regulators and is encoded by the virF gene that is localized just downstream of the yscW gene on pYV virulent plasmid (Fig. 1) (Cornelis et al. 1989). In turn, the activation of ysc and and yop genes by VirF is controlled by YmoA.

At the temperature below 30°C, YmoA stabilizes the DNA structure and thus inhibits VirF binding to the promoter regions of ysc and yop genes (Bleves and Cornelis 2000). After a shift to 37°C, the change of DNA topology due to elevated temperature and the dislodgement of YmoA facilitates VirF binding to its recognized sites and activates the transcription of yop and ysc genes (Bleves and Cornelis 2000).

The role of VirF in transcriptional regulation of Ysc-Yop T3SS was well-studied by many authors. This study was initiated to identify mechanisms that affect transcriptional regulation of the virF gene. Data from our lab (unpublished), indicated that virF is cotranscribed with the upstream gene yscW. This yscW-virF forms an operon. This genetic organization resemble that of the Pseudomonas aeruginosa exsCBA exsC gene (Allaoui et al. 1995; Hovey and Frank 1995).
In this study, we investigated whether VirF might act similarly to control expression of yscW-virF.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

All bacteria strains and plasmids used in this study are described in Table 1. Y. enterocolitica strains were routinely grown at 26°C and E. coli strains were grown at 37°C in Luria Broth (1% tryptone, 0.5% yeast extract, 90 mM NaCl) or on Luria Agar (Difco). Media used for Y. enterocolitica Yop secretion was Luria broth which had been chelated for Ca2+ ion by the addition of 1.5 mg/ml MgCl2 and 2.1 mg/ml Na2C2O4 (Yop media). The induction of promoter P_tac was carried out by adding 1 mM IPTG to the cultures. Antibiotics were used at the following concentrations: tetracycline (15 µg/ml), nalidixic acid (20 µg/ml), chloramphenicol (25 µg/ml).

2.2 Strain construction

To construct virF deletion strain, the natural pYV virulent plasmid was cured out of Y. enterocolitica strain GY6361. The process of plasmid curing was done as follows: strain GY6361 was grown overnight in LB containing Tet and then subcultured to OD600 of 0.1 in Yop media at 37°C for 18-24 hours for two times. Subsequently, the culture was plated on LB containing Tet and incubated at 37°C for 48 hours. The loss of pYV was confirmed by SDS-PAGE analysis.

To complement the virF deletion mutants, plasmid GY1006, GY983 and GY984

Table 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td><strong>Y. enterocolitica</strong></td>
<td></td>
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<td>JB580v</td>
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<td>GY 6361</td>
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<td><strong>Plasmid</strong></td>
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<td>Low copy transcriptional lacZYA</td>
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<td>Ptac expression vector</td>
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<td>pMMB207::virF</td>
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were used. pGY1006 plasmid is a derivative of pTM100 with yscW-virF gene accompanied with its natural promoter P_{yscW} while pGY983 and pGY984 are derivatives of pBBM207 with virF gene and yscW-virF gene respectively cloned downstream of promoter P_{lac}. These plasmids were introduced into \textit{Y. enterocolitica} strain GY6532 by conjugal mating. The process of conjugation was described as following: the culture of \textit{Y. enterocolitica} strain GY6532 was mixed respectively with the cultures of \textit{E. coli} strains harbouring pGY1006, pGY983 and pGY984. Then the conjugation mixtures were centrifuged at 13,000 rpm for 2 minutes. Next, the pellets were re-suspended in LB broth and subsequently plated on LB and incubated for 6 hours. Afterward, trans-conjugants were selected on the plates that contain Cm, Nal and Tet and further confirmed by urease test.

2.3 Protein Preparation and SDS-PAGE Analysis

The detection of secreted Yops was accomplished as previously described (Petersen and Young 2002). \textit{Y. enterocolitica} were grown overnight in LB broth at 26°C and then subcultured to OD_{600} of 0.1 into Yop media for induction of Yop secretion. The sub-culture was grown at 37°C for 6 hours with shaking. To examine secreted proteins (Yops), the OD_{600} of culture was determined and bacterial cells were removed by centrifugation at 13,000 rpm for 10 minutes. Yop proteins were precipitated with 10% (wt/vol) ice-cold trichloroacetic acid (TCA) and were purified by washing with ice cold acetone. Subsequently, the protein samples were re-suspended and normalized by sample buffer containing 2-mercaptoethanol with volume that was adjusted according to the OD_{600} of culture. Normalized protein sample then was heated to 95°C for 5 minutes and exposed to SDS-PAGE on 10% acrylamide gel. The protein was visualized by staining with Coomoassie Brilliant Blue (CBB).

2.4 Promoter Activity Assay

The activity of promoter P_{yscW} was determined by measuring the \(\beta\)-galactosidase activity as described by Miller (1972). Briefly, \textit{Y. enterocolitica} strains were grown overnight at 26°C, sub-cultured to OD_{600} of 0.1 in fresh LB broth containing appropriate antibiotics and allowed to grow at 37°C and 26°C for three hours. Induction of promoter P_{lac} was carried out by adding 1 mM IPTG in sub-cultures. Following the incubation time, cells were harvested by centrifugation 13,000 rpm for 1 minute and then re-suspended in 1ml working buffer (Enzyme assay buffer + \(\beta\)-mercaptoethanol). The cell density was determined by measuring A_{600}. The 200 ul of resuspended bacterial cells was mixed with 800 ul of working buffer and then lysed by chloroforms and sodium dodecyl sulfate (SDS). Subsequently, a 200 ul amount of reaction substrate, O-nitrophenyl-\(\beta\)-D-galactopyranoside (ONPG) was added to start the assay reaction. When the yellow product became visible, the reaction was stopped by sodium carbonate and the optical densities of the samples were determined spectrophotometrically. The \(\beta\)-galactosidase activity was calculated as follows: \([((A_{420} - .175 \times A_{550}) \times 1,000) / (t \times v \times A_{600})]\), where \(t\) is the times in minutes, and \(v\) is the volume of the resuspended cells used in assay.

3. Results

3.1 Construction of virF deletion mutant and virF complimented strains

To examine the role of VirF in regulation of activity of promoter P_{yscW}, the virF deletion mutant and its complimented strains were constructed. \textit{Y. enterocolitica} strain GY6361 was constructed from the previous lab member (unpublished data from Young lab). This strain carries pYV virulence plasmid and the transcriptional fusion plasmid pGY1060 which is created by cloning promoter P_{yscW} into the upstream of a promoterless lacZYA in plasmid pRW50. This strain also harbors tetracycline resistant gene (tet). The virF deletion mutant strain (named GY6532) was constructed by removing pYV virulence plasmid. The pYV-deficient candidate strains grew faster and were recognized by large colonies on LB containing Tet. To be sure that the suspected pYV-cured candidates were the right ones, their abilities of Yop secretion were analysed by SDS-PAGE.
All yop genes and the virF gene controlling the expression of yop genes are located on the pYV, so the pYV-cured strain loses ability to produce Yop proteins (Lambert de Rouvroit et al. 1992). Figure 2 shows the ability of Yop production among selected Y. enterocolitica strains: Y. enterocolitica strain GY6361 carrying pYV (Lane 2) secreted Yop proteins in supernatant with different bands exposed in the acrylamide gel whereas Y. enterocolitica GY6532 (Lane 3) lost the ability of Yop secretion with no band found in the gel.

The complementation of virF deletion mutant was performed by introducing different plasmid pGY1006 (P_yscW-yscWvirF) or pGY983 (P_tac-virF) and pGY984 (P_tac-yscWvirF) into virF deletion mutant strain GY6532 through mating. These plasmids contain Cm resistant gene (Cm) and the virF is driven by natural promoter P_yscW or inducible promoter P_tac. The trans-conjugant candidate had ability to grow on LB that contains Cm, Nal and Tet.

The Yersinia itself is capable of producing urease that can hydrolyze urea to form carbonic acid and ammonia and results in an increase in pH medium. The trans-conjugant candidates were confirmed to be Yersinia by giving the positive result with urease test. These were named GY6538 (pGY1006), GY654 (pGY983) and GY6542 (pGY984).

### 3.2 Effect of Temperature and VirF on P_yscW Promoter Activity

In order to determine whether VirF has any influence on the promoter P_yscW, the activities of promoter P_yscW in Y. enterocolitica WT, virF mutant and virF complemented strains were determined by measuring β-galactosidase activities. All selected Y. enterocolitica strains used in this experiment harbored plasmid pGY1060 with P_yscW-lacZ gene fusion for measurement of β-galactosidase activity. The expression of virF gene by natural promoter P_yscW was controlled by temperature and its expression by the inducible promoter P_tac was controlled by isopropyl-β-D-thiogalactosidase (IPTG).

Cornelis et al. (1989) proved that VirF is maximally produced at 37°C where Ysc-Yop system is induced and VirF is poorly or not produced at lower temperature (26°C) (Lambert de Rouvroit et al. 1992). As can be seen in Fig. 3, the virF deletion mutant strain (GY6532) showed the level of β-galactosidase activity similar to those of WT strain (GY6361) at high temperature (37°C) as well as low temperature (26°C). The complementation of virF deletion mutant with plasmid pGY1006 where the virF was driven by natural promoter P_yscW also did not display any significant change in level of β-galactosidase activity at both 37°C and 26°C compared to WT and virF deletion mutant (Fig. 3).

Among the virF complemented strains where transcription of virF was driven by the inducible promoter P_tac, there was also no significant change found in the level of β-galactosidase activity in the presence or absence of IPTG at 37°C and 26°C (Fig. 4). Take together, these results indicated that VirF did not affect on activity of promoter P_yscW in the response to temperature.
Fig. 3. Observed β-galactosidase activities at 26°C and 37°C in selected *Y. enterocolitica* strain GY6361(pYV⁺, pGY1060) and virF deletion mutant strain GY6532(pYV⁻, pGY1060) and virF complemented strain GY6538 where virF is driven by natural promoter P_{yscW} (pYV⁻,pGY1060, P_{yscW}yscWvirF). Error bars indicate standard deviation.

4. Discussion

*Y. enterocolitica* employs Ysc-Yop T3SS to inject Yop effectors into the cytosol of eukaryotic cells. Among T3SSs of Gram negative pathogens, the *Y. enterocolitica* Ysc-Yop T3SS is highly similar to the *P. aeruginosa* T3SS. The expression of both T3SSs is triggered by eukaryotic cell contact in vivo or depletion of Ca²⁺ ion in medium in vitro (Hueck 1998). In addition, the T3SS genes of both the *Y. enterocolitica* and *P. aeruginosa* are regulated by the AraC transcriptional activators VirF and ExsA respectively which share 56% identity (Allaoui et al. 1995). They act as DNA-binding proteins to activate the transcription of T3SS genes by binding to the promoter regions of these T3SS genes (Hueck 1998). Moreover, both VirF and ExsA are transcribed with their upstream genes as operons. In *Y. enterocolitica*, virF and its upstream gene yscW are transcribed as an operon from the promoter of yscW (named yscW-virF operon) (unpublished data from Young lab). In *P. aeruginosa*, the exsA, together with exsB and exsC (located upstream of the exsA) are also transcribed as an operon from the promoter of exsC (named exsCBA operon) (Hovey and Frank 1995). Interestingly, the transcriptional activator ExsA can bind to the promoter P_{exsC} and auto-regulate the transcriptional level of exsCBA operon (Hovey and Frank 1995). Currently, we are underway to establish the mechanisms of transcriptional control of yscW-virF operon in *Y. enterocolitica*. Hence, based on the similarity to *P. aeruginosa* T3SS, we hypothesized that VirF may have ability to auto-regulate its yscW-virF operon by binding the promoter region of yscW.
To address this question, we investigated the effect of VirF on the activity of promoter P_{yscW} in response to temperature. The activities of promoter P_{yscW} using a transcriptional fusion to the lacZ were determined by measuring the β-galactosidase activity at mammalian body temperature (37°C) and lower temperature (26°C). The complementation analysis was used to evaluate if P_{yscW} was affected by VirF or if there were other factors involved. Based on the finding that transcription of virF itself is thermo-regulated (Cornelis et al. 1989), it was predicted that at 37°C where VirF is maximally produced, VirF binds to the promoter region of yscW and enhances the promoter activity. In this case, the WT strain (GY6361) would give higher level of β-galactosidase activity than virF deletion mutant strain (GY6532). Alternatively, at 26°C where VirF is not or poorly produced, the level of β-galactosidase activity would remain the same in both WT strain (GY6361) and virF deletion mutant strain (GY6532). However, the result revealed that there was no significant differences in the level of β-galactosidase activities among selected Y. enterocolitica strains including WT, virF deletion mutant and virF complemented strains when the production of VirF was controlled by temperature or/and by IPTG. Thus, it is concluded that Y. enterocolitica VirF does not affect the transcription of the yscW-virF operon, like its homolog P. aeruginosa ExsA.

The transcription of Y. enterocolitica yscW-virF operon is also affected by the global regulator CRP (or cAMP receptor protein). When bacteria enter host cells, CRP, together with cAMP, response to many stresses placed on bacteria in host environment and up-regulates the transcription of genes that are necessary for them to adapt and survive. Zachary W. Bent and Glenn M. Young proved that the Y. enterocolitica crp mutant shows the down-regulation of virF expression which in turn results in a decrease in Yop production (unpublished data from Young lab). They also
proved that CRP does not directly affect to the transcription of yscW-virF operon by binding to the promoter region of yscW.

5. Conclusion

It is believed that CRP indirectly controls the expression of yscW-virF operon through unknown intermediate regulator(s) (unpublished data from Young lab). Further study should be conducted to identify these intermediate regulators to give clear picture about the mechanisms of transcriptional control of yscW-virF operon. Broadly, the identification of these intermediate regulators give us more understanding about the regulation of Y. enterocolitica Ysc-Yop T3SS. In P. aeruginosa, the regulation of T3SS by global regulator Vfr (functional homolog to E. coli CRP) is also reported (Yahr and Wolfgang 2006). It is also proved that the global regulator Vfr, along with cAMP, does not directly regulated the transcription of exsCBA operon (Shen et al. 2006). Mechanisms of cAMP-Vfr complex exert the transcriptional control of P. aeruginosa T3SS genes is not clear (Yahr and Wolfgang 2006). It may indirectly affect to exsCBA operon like Y. enterocolitica or to the ExsA binding/activity (Yahr and Wolfgang 2006).

6. References


