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

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## *Abstract*

*The* Yerisinia 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. entercolitica *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$^o$C) or high (37$^o$C) 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$^o$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 *vir*F gene that is localized just downstream of the *ysc*W 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$^o$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$^o$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* operon, which control expression of T3SS genes in that bacterium. Interestingly, VirF shares 56% identity to *P. aeruginosa* transcriptional activator ExsA, which can autoregulate its *exsCBA* operon by binding to promoter regions of the *exsC* gene (Allaoui *et al*. 1995; Hovey and Frank 1995).

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In this study, we investigated whether VirF might act similarly to control expression of *yscW-virF*.

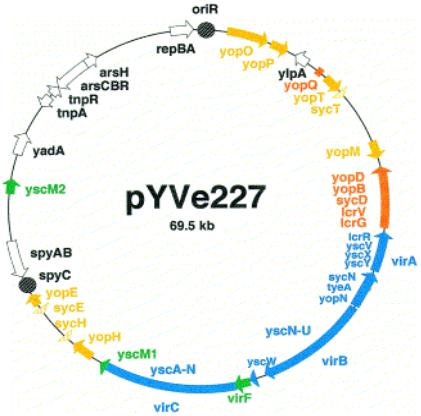


Fig. 1. The *Y. enterocolitica* virulent plasmid pYV (Bleves and Cornelis 2000).

## 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. enterocolotica* 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 $Ca^{2+}$ ion by the addition of 1.5 mg/ml $MgCl_2$ and 2.1 mg/ml $Na_2C_2O_4$ (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).

Table 1. Strains and plasmids used in this study.

| Strains or plasmids | Description | Reference |
|---|---|---|
| *Y. enterocolitica* | | |
| JB580v | Serogroup O:8, Nal, Δ*yen* ($R^-$, $M^+$) | Young Lab Collection |
| GY 6361 | $pYV^+$, pGY1060, $Nal^r$, $Tc^r$ | Young Lab Collection |
| GY 6532 | $pYV^-$, pGY1060, $Nal^r$, $Tc^r$ | This study |
| GY 6538 | $pYV^-$, pGY1060, pGY1006, $Nal^r$, $Tc^r$, $Cm^r$ | This study |
| GY6539 | $pYV^-$, pGY1060, pMMB207, $Nal^r$, $Tc^r$, $Cm^r$ | This study |
| GY6541 | $pYV^-$, pGY1060,pGY983, $Nal^r$, $Tc^r$, $Cm^r$ | This study |
| GY6542 | $pYV^-$, pGY1060,pGY984, $Nal^r$, $Tc^r$, $Cm^r$ | This study |
| *E. coli* | | |
| GY2685 | S17-1λ*pir,* pGY1006, $Cm^r$ | Young Lab Collection |
| GY2628 | S17-1λ*pir,* pMMB207, $Cm^r$ | Young Lab Collection |
| GY2631 | S17-1λ*pir,*pGY983, $Cm^r$ | Young Lab Collection |
| GY2632 | S17-1λ*pir,*pGY984, $Cm^r$ | Young Lab Collection |
| *Plasmid* | | |
| pTM100 | $mob^+$, derivative of pACYC184, $Cm^r$, $Tc^r$ | Young Lab Collection |
| pRW50 | Low copy transcriptional lacZYA | Young Lab Collection |
| pMMB207 | P*tac* expression vector | Young Lab Collection |
| pGY1006 | pTM100:: *yscW-virF* | Young Lab Collection |
| pGY1060 | pRW50 *yscW*::lacZ | Young Lab Collection |
| pGY 983 | pMMB207::*virF* | Young Lab Collection |
| pGY 984 | pMMB207::*yscW-virF* | Young Lab Collection |

### 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 $OD_{600}$ 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

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 $P_{tac}$. These plasmids were introduced into *Y. enterocolitica* strain GY6532 by conjugal mating. The process of conjugation was described as following: the culture of *Y. enterocolitica* strain GY6532 was mixed respectively with the cultures of *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). *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 secrection. 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 β-galactosidase activity as described by Miller (1972). Briefly, *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 $P_{tac}$ 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 + β-mercato-ethanol). 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-β-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 β-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. *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 *vir*F 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-o-thiogalactosidase (IPTG).

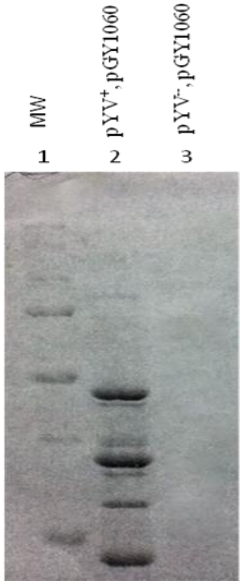


Fig. 2. Analysis of secrected Yop proteins by selected *Y. enterocolitica* strains. Lane 1: Molecular weight; Lane 2: WT GY6361 ($pYV^+$, pGY1060); and Lane 3: *virF* deletion mutant GY6532 ($pYV^{--}$, pGY1060).

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 the 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.

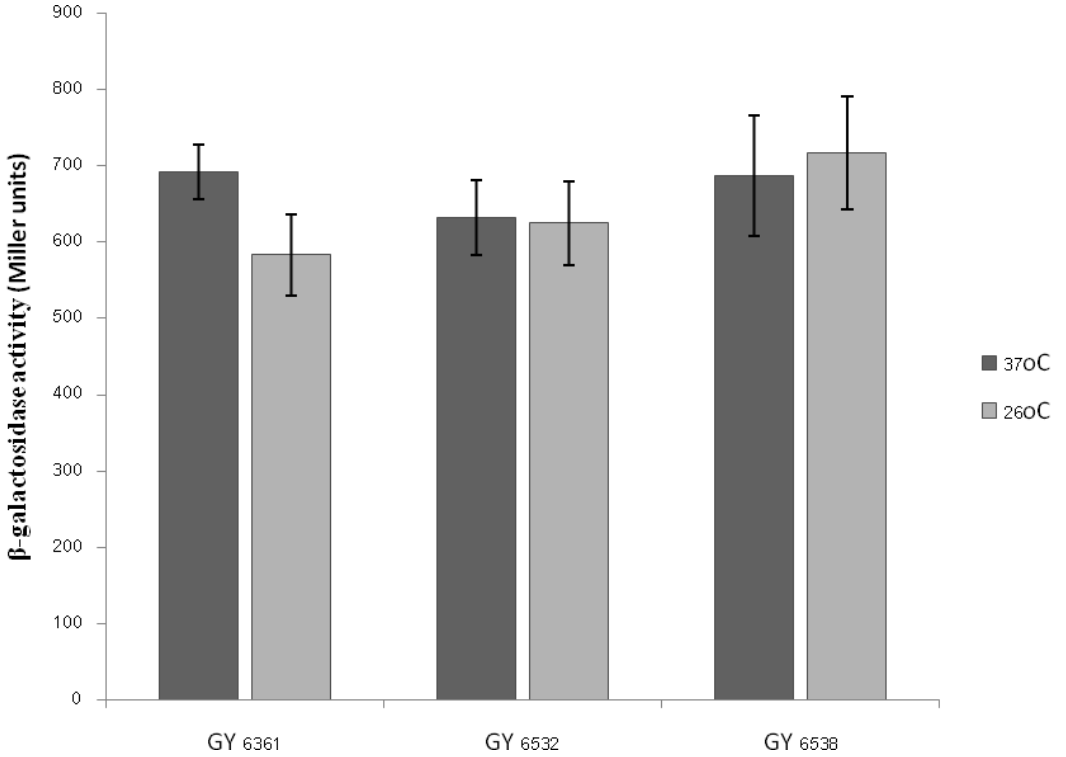


Fig. 3. Observed β-galactosidase activities at 26°c and 37°C in selected *Y. enterocolitica* strain GY6361(pYV$^{+}$, pGY1060) and *virF* deletion mutant strain GY6532(pYV$^{-}$, pGY1060) and *virF* complemented strain GY6538 where vir*F* is driven by natural promoter $P_{yscW}$ (pYV$^{-}$,pGY1060, $P_{yscW}$-*yscWvirF*). Error bars indicate standard deviation.

## 4. Discussion

*Y. enterocolitica* employs Ysc-Yop T3SS to inject Yop effectors into the cystol 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 Cà$^{2+}$ 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 ysc*W* 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 to 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*.

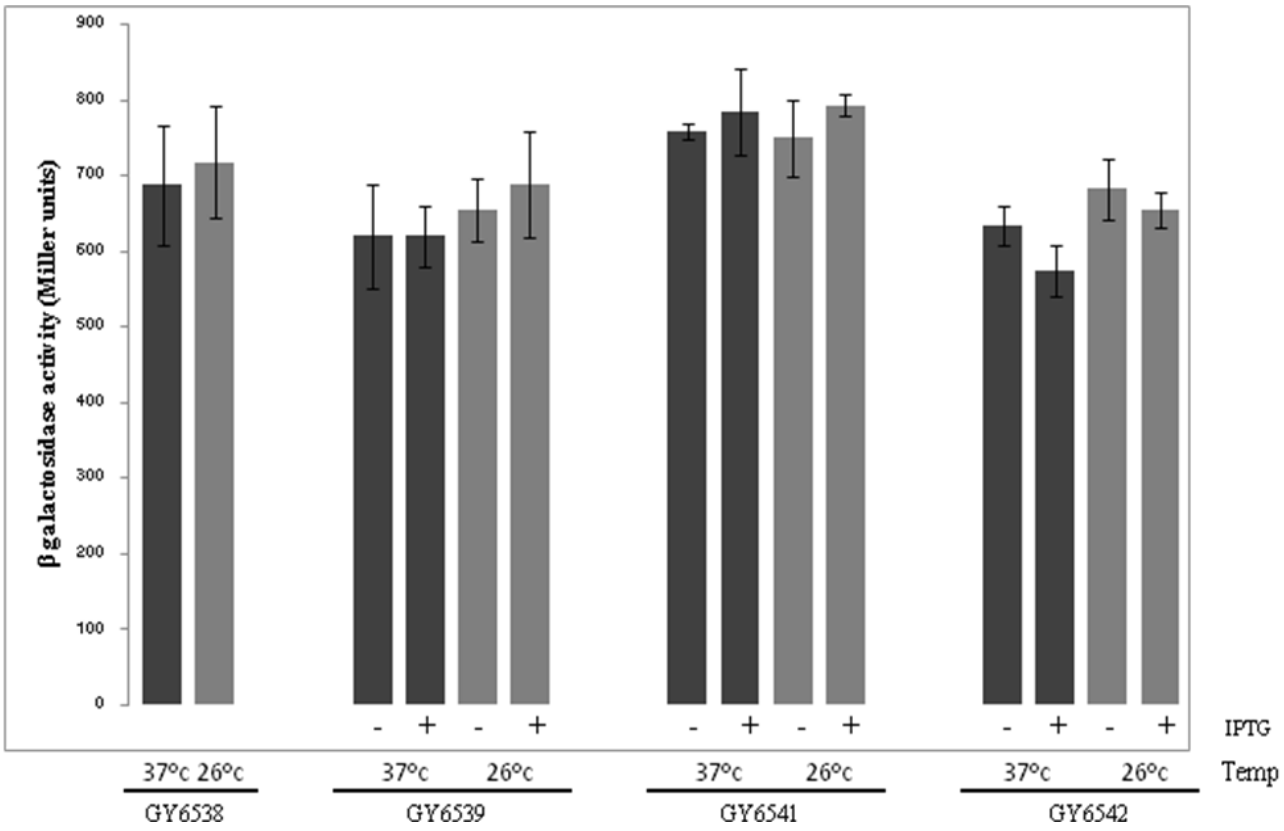


Fig. 4. Observed β-galactosidase activities in the *virF* complimented strains of *Y. enterocolitica* where the expression of *vir*F is driven by natural promoter $P_{yscW}$ or by inducible promoter $P_{tac}$. Induction of promoter $P_{tac}$ was performed by adding 1 mM IPTG in sub-cultures at 26°C and 37°C. GY6538 (pGY1060/$P_{yscW}$-*yscWvirF*), GY6539 (pGY1060/$P_{tac}$) as a control, GY6541 (pGY1060/$P_{tac}$-*virF*), GY6542 (pGY1060/$P_{tac}$-*yscWvirF*). Error bars indicate standard deviation.

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* complimented 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 *vir*F 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. Boardly, 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).

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