Effect of VirF on Promoter activity of *yscW-virF* operon in *Yersinia enterocolitica* and Evaluation of *Yersinia enterocolitica tol-excC* (*pal*) gene cluster in maintenance of outer membrane integrity

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&

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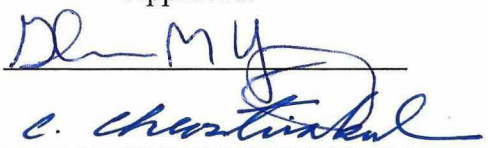

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CHAPTER 1

Introduction

Y. enterocolitica, a worldwide foodborn pathogen

Yersinia enterocolitica is gram-negative bacillus which belongs to the family of *Enterobacteriaceae.* It is a foodborne pathogen with worldwide distribution although *Y. enterocolitica* infections are sporadic and its foodborne outbreaks are rare (18). *Y. enterocolitica* itself confers properties that allow it to grow in food as well as in the harsh environment. The optimum growth temperature of *Y. enterocolitica* is 28°C (40). However, *Y. enterocolitica* is a psychrotropic organism that (allow it) can grow properly in refrigerated temperature (40). Some strains of *Y. enterocolitica* even can grow slowly at temperature as low as -5°C (21, 36). *Y. enterocolitica* is also capable of growing under modified atmosphere due to its facultative anaerobic properties (21). In addition, it can grow in the wide range of pH 4-10 and can tolerate in NaCl concentration up to 5% (21).

Based on biochemical properties, *Y. enterocolitica* is divided into six biotypes (1A, 1B, 2, 3, 4 and 5) (18). Biotype 1A is ubiquitous in the environment and considered to be non-pathogenic strain (43). Biotypes 2 to 5 consist of low pathogenic *Y. enterocolitica* strains which cause mild gastrointestinal illness in human, and are not mouse lethal at low doses (7). They are frequently found in Europe and Japan (18, 21). Biotype 1B includes highly pathogenic *Y. enterocolitica* strains which cause severe systemic disease in human, and are mouse lethal at low doses (7). They predominate in North America and until now only strain 8081 whose genome has been sequenced (18, 44).

All pathogenic *Y. enterocolitica* strains (Biotypes 1B, 2-5) contain a conserved 70kb virulent plasmid (pYV) to express their virulence. The pYV virulent plasmid codes for Ysc-Yop type III serection system (T3SS) and its effectors which enhance survival of *Y.enterocolitica* in host cells and help bacteria overcome host immune system (12). High

pathogenic strains of *Y. enterocolitica* (Biotype 1B) are differentiated from low pathogenic strains of *Y. enterocolitica* (Biotype 2-5) by the presence of specific chromosomal virulent genes. Chromosomal virulent genes of *Y. enterocolitica* consist of *inv, ail, rovA*, *yst, ure*, *myf*, high pathogenic island (HPI) and plasticity zone (PZ) (21, 44). However, HPI and PZ are only found in highly pathogenic *Y. enterocolitica* Biotype 1B strains (7, 44). The specific chromosomal virulent genes allow highly pathogenic *Y. enterocolitica* Biotype 1B strains spread systemically in host cells and more deadly than other pathogenic *Y. enterocolitica* strains. The PZ is localized on the *Y. enterocolitica*'s chromosome and accounts for 16% unique feature of Biotype 1B (44). It contains the *Yersinia* adhesion pathogenicity island (YAPI) which harbors a *pil* operon encoding for Type IV pilus. Type IV pilus facilitates for bacterial cells to colonize in the intestinal mucosa (44). Another T3S system, named Ysa is also found in PZ. The Ysa T3S system injects the Ysp effector proteins into the cytosol of eukaryotic cells to interfere the host's innate immune system (44). Moreover, PZ also carries T2S system, named Yst1 which is responsible for the colonization in GI tract. Finally, PZ harbors several clusters of genes that involve in the adaptation of *Y. enterocolitica* in gut or wider environment (44).

High pathogenic island (HPI) consists of a cluster of chromosomal genes that encodes the biosynthesis, transport and regulation of yersiniabactin (Ybt) (7). The role of HPI is involved in iron acquisition of *Y. enterocolitica* in mammalian cells (7). Iron plays a key role for bacterial growth and systemic dissemination inside mammalian cells. Highly pathogenic *Y. enterocolitica* strains (Biotype 1B) carry HPI that allow them secrete a siderophore, namely Yersiniabactin (Ybt), which is capable of chelating iron bound to eukaryotic proteins and bring it back to the cytosol of bacteria (7). By contrast, low

pathogenic *Y. enterocolitica* strains (Biotype 2-5) do not produce yersiniabactin due to the absence of HPI. Therefore, these low pathogenic strains usually cause local intestinal infection. The systemic infection occurs when low pathogenic strains utilize the exogenous siderphores like ferrioxamine to obtain iron (7).

Y. enterocolitica infections are commonly acquired through consumption of contaminated food or water, especially in raw pork or pork products such as chitterling (18). In addition, *Y. enterocolitica* infection mostly occurs in infant and young children although all ages are at risk (18). After ingestion, bacterial cells begin to migrate through intestinal tract to the terminal end of ileum. Microfold or M cells are specialized epithelium cells which are located on follicle-associated epithelium and have ability to take up bacterial cells from the lumen of small intestine via phagocytosis (6). M cells can be distinguished with surrounding epithelial cells by the presence of villi on their apical surface. Attachment and invasion of M cells is mediated by Inv, Ail and YadA protein (18). The internalized bacteria attach on M cells to cross intestinal epithelium barrier and colonize on specialized lymphoid follicle or Peyer's Patch (6).

Unlike *Y. pseudotuberculosis*, the spreading of *Y. enterocolitica* from Peyer's patch to mesenteric lymph nodes or spleen or liver is rarely occurred. Most *Y. enterocolitica* infections are usually limited to Peyer's patch with symptoms including self-limited diarrhea, fever, nausea and abdominal pain (6, 18). Sometimes, the additional symptoms lead to misdiagnosis as appendicitis.

Ysc-Yop Type III serection system of *Y. enterocolitica*

The whole Ysc –Yop T3S system is encoded by the virulent plasmid pYV (12). It consists of Ysc injectisome, Yop translocators, Yop effectors and their chaperones (13). The *ysc* genes encoding for Ysc injectisome are contained in four contiguous loci of pYV plasmid: *virA*, *virB* , *virG/yscW* and *virC* whereas the *yop* genes encoding for Yop translocators and Yop effectors scatter throughout the plasmid (figure 1.1) (4). The *virF* encoding for the primary regulator of VirF is located between the *virB* and *virC* operon.

Some Yop proteins require specific cognate chaperones, named Syc (specific Yop chaperones) for their proper secretion. Generally, Syc chaperone is the small acidic protein (less than 20kDa) encoded by the *syc* gene which is located next to the gene encoding for its cognate protein (25). In the absence of the chaperone, its cognate protein is not stable or can not be secreted (25).

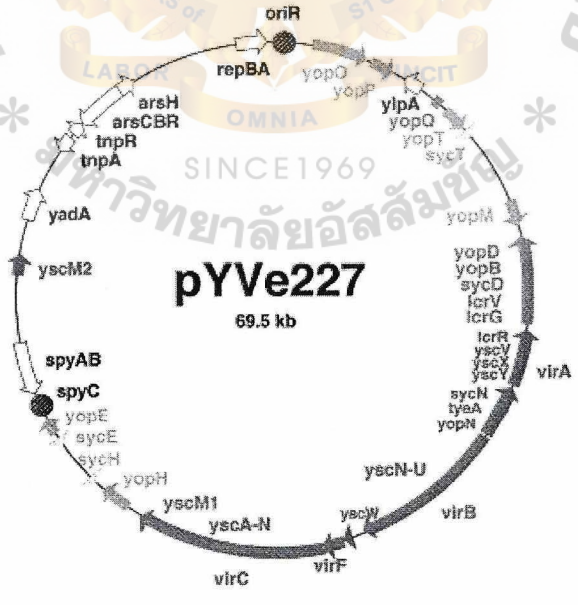

Figure 1.1 : The *Y. enterocolitica* Virulent Plasmid pYV (5)

The Ysc injectisome is a needle complex that consists of two parts: the internal part is basal body which spans peptidoglycan layer and two bacterial membranes, and the external part is a hollowed needle protruded from the bacterial surface. In the absence of eukaryotic cells at 37°C, Ysc injectisome is built but stays in resting state and no Yop is secreted (13). Upon Ca^{2+} depletion or contact with eukaryotic target cells, *Y. enterocolitica* injects six of Yop effectors into the cytosol of target cells. Yop translocators, are required to support bacteria to translocate Yop effectors across the eukaroytic cell membranes (13). In vitro, the expression of Yop serection is induced after culture of *Y. enterocolitica* at 37°C and their secretion only starts when Ca^{2+} is depleted from medium, conditions that are mimic host environment (13).

Three Yop translocators including YopB, YopD and LcrV are encoded by large *lcrGV-sycD-yopBD* operon (11). This operon also encodes for the chaperone SycD that is the partner of YopB and YopD. LcrV is a hydrophilic protein whereas YopB and YopD contain central hydrophobic domains which act as transmembrane helices. In the resting state of Ysc Injectisome, Lcrv is believed to form the tip complex on the YscF needle tip of Ysc injectisome (35). The LcrV needle tip is thought to mediate pore formation by YopB and YopD on eukaryotic cell membrane (35). Due to the presence of transmembrane helices, secreted YopB and YopD insert together into eukaryotic cell membrane and form the translocation pore (12, 35). Yop effectors are delivered through this pore to enter the cytosol of eukaryotic cells. YopQ (YopK in *Y. pseudotuberculosis*) is encoded by the gene located outside the *lcrGV-sycD-yopBD* operon and is thought to regulate the size of translocation pore (11).

Six Yop effectors delivered into the cytosol of eukaryotic cells include YopH, YopO (YpkA), YopE, YopT, YopP (YopJ) and YopM (12). YopE, YopH and YopT require their cognate chaperones, namely SycE, SycH, SycT respectively for their proper secretion and stability in bacterial cytoplasm (13). The host innate immune system is the first line of defense against infection. The roles of Yop effectors inside eukaryotic cells are to inhibit host innate immune response. YopE ,YopH, YpkA/YopO and YopT prevent rearrangement of actin cytoskeleton which is essential for the phagocytosis of bacteria and hence, play a key role in resistance to phagocytosis by macrophages and polymorphonuclear leukocytes (PMNs) (12). YopP has ability to suppress inflammatory response and induces apotosis in macrophage (12). The pathogenic function of YopM is still mysterious although YopM is an important virulent factor in *Yersinia* infection in mice (12).

The expression of Ysc-Yop system is thermo-regulated by transcriptional activator VirF at transcriptional level (14). 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 the virulent plasmid pYV (figure 1.1) (10). The activity of VirF on the transcription control of *ysc* as well as *yop* genes is modulated by YmoA (14). YmoA chromosomally encoded by the *ymoA* gene is a histone-like protein and involves in chromosomal structure and DNA compaction. 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 (4). After a shift to 37°C, the change of DNA's topology due to elevated temperature and the dislodgement of YmoA facilitates VirF bind to its recognized site and activates the transcription of *yop* and *ysc* genes (4). Under this condition, the released

YmoA is degraded by ClpXP and Lon protease (17). The expression of YmoA is further controlled by the nucleoid-associated protein, H-NS.

Tol-Pal system of Gram negative bacteria

The *tol-pal* genes cluster is widely distributed and its gene organization is highly conserved in Gram negative bacteria (42). The Tol-Pal system has been originally identified in *Escherichia coli*. The *E. coli tol-pal* genes are contained in a continuous region at 17.3 min in genomic map and organized in the following order: *ybgC-tolQ-tolR-tolA-tolB-pal-ybgF* (45). These genes are transcribed from two promoters located upstream of 5' end *ybgC* gene and *tolB* gene (20). Later on, a similar organization of *tol-pal* genes has been found in other related species such as *Pseudomonas putida* (named *tol-oprL* in this organism) (32), *Erwinia chrysamthemi* (16), *Caulobacter* (48).

The Tol-Pal system of Gram negative bacteria composes of five core envelope proteins: three inner membrane proteins (TolR, TolQ and TolA), one periplasmic protein (TolB) and the peptidoglycan associated lipoprotein (Pal) (33). TolR and TolA have one transmembrane (TM) domain (TolR1 and TolA1 respectively) and two remaining domains in each protein protrude into periplasm while TolQ has three TM domains (33). Three these proteins interact each other via their transmembrane domains at the inner membrane to form the TolQRA complex (33). The peptidoglycan associated lipoprotein (Pal) interacts with TolB to form the second complex TolBPal near the outer membrane (30). The TolBPal complex can link to other structural proteins such as Braun lipoprotein (Lpp) and OmpA (33). The Braun lipoprotein is abundant in outer membrane and makes the connection between outer membrane and peptidoglycan layer by covalent bond (38).

OmpA is also the outer membrane protein but links to peptidoglycan by non-covalent bond (38). Two Tol-Pal complexes link each other via C-terminal domain of TolA and N-terminal domain of TolB (20). The arrangement of Tol-Pal system is showed in figure 1.2.

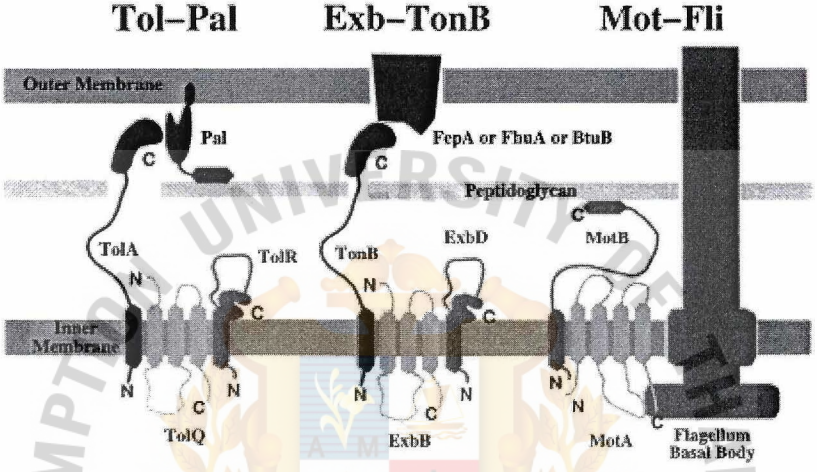

Figure 1.2 : Schematic representation of *E. coli* Tol-Pal system (left), TonB system (center) and flagella motor (right) (8).

In *E. coli*, the TolQRA complex shows similarity to ExsB-ExsD-TonB complex (or Exs/TonB system) which is also arranged and located in the inner membrane (figure 1.2) (28). The Exs/TonB system involves in the uptake of iron-siderophore complexes and vitamin B12 through the specific receptors of outer membrane such as FhuA, FepA or BtuB (33). TolQ and TolR are the homologs of ExbB and ExbD respectively while TolA is homologous to TonB (30). In addition, like TonB, TolA of Tol-Pal system is proved to be an energized protein. The energization of TolA induced by its transmembrane domain causes a change in conformation of C-terminal domain that makes it easily interact with

Pal (33). TolQ and TolR are also found to share homologies to MotA and MotB respectively which are torque generating elements of flagella motor (8).

The Tol-Pal system plays an essential role in maintenance of outer membrane stability. It is originally proved from *E. coli* that mutation on any *tol* and *pal* genes causes disruption of outer membrane integrity resulting in phenotypic changes such as hypersensitive to some drugs and detergents, release of periplasmic proteins into extracellular medium and formation of outer membrane vesicles (20). The first gene *ybgC* and the last gene *ybgF* in *E. coli tol-pal* gene cluster do not contribute to the maintenance of outer membrane stability since the inactivation of two these genes does not show any phenotypic changes (20). The *ybgC* encodes for a cytoplasmic protein with the thoesterase activity while the *ybgF* encodes for the uncharacterized periplasmic protein (20). Recently, works by Krachler et al demonstrated that YbgF is the highly elongated protein which interacts with TolA to form its trimer structure. This maybe a explanation why *ygbF* is retained in *tol-pal* operon (26). However, mutation on *ybgF* in *Er. chrysanthemi* shows a phenotypic change with the sensitivity to sodium cholate (16). Thus, whether the first gene *ybgC* and last gene *ybgF* contributing to stabilize the outer membrane are still unclear. The exact function of each Tol- Pal proteins in maintenance of outer membrane protein integrity is largely unknown. The Tol-Pal proteins may involve in the assembly and/or translocation of (an) outer membrane components crucial for its stability such as porin which serves as a channel for importing and exporting molecules across the cell membrane (38), lipopolysarcharide (LPS) which acts a protective barrier to against the harmful compounds such as antibiotics, drugs, chemical compounds, etc (20). In *E. coli*, the *tolA* and *pal* mutants elicit sustained extracytoplasmic stress responses that in turn reduce O-

antigen polymerization (46). Another possible function of Tol-Pal proteins may associate with the formation of cell envelope in daughter cell such as the role of Tol-Pal in OM invagination during cell constriction or in connecting the OM with IM and peptidoglycan layer during septum formation (19).

In addition to maintenance of outer membrane integrity, the Tol-Pal proteins also play a role in uptake /transport of group A colicins (A, E1 to E9, K,L,N bacteriocin 28b and cloacin DF13) (22, 33). To enter cells, groups A colicins bind to outer membrane receptors such as the vitamin B12 receptors (BtuB), nucleoside receptors (Tsx) and the OmpF porin and utilize Tol proteins for translocation across the membrane (27). N-terminal domain of Colicin A interacts with Tol proteins which help maintain the unfolded conformation of colicin A and facilitate them pass through periplasm and reach to the target (28). The Tol-Pal proteins also involve in the uptake of filamentous phage. In *E.coli*, infection by the filamentous phage F1 or M13 is initiated by binding to the tip of the F conjugative pilus via pIII protein (22). The TolA binds to pIII protein and serves as a co-receptor for phage entry into bacterium (22). Similarly, CTXΦ phage utilizes pilus and TolQRA in order to enter into *V. cholera* (22).

CHAPTER 2

Effect of VirF on the promoter activity of *yscW-virF* operon in *Y. enterocolitica*

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Abstract

The *Y. enterocolitica* Ysc- Yop T3SS and its effectors are essential for bacteria to survive and overcome host immune system. Its expression at the transcriptional level is thermo-controlled by the AraC transcriptional activator VirF. The role of VirF in transcriptional regulation of *Y. enterocolitica* Ysc- Yop T3SS genes has been well documented by many authors. Currently, our lab has been underway to study about transcriptional regulation of *virF*. We has proved that the *virF* together with the *yscW* located upstream of *virF* are transcribed as an operon from the promoter P_{yscW} like the manner of their homologs, *exsA* and *exsB* in *P. aeruginosa*. The *P. aeruginosa exsA* encodes for its T3SS transcriptional activator ExsA which can auto-regulate transcription of its *exsCBA* operon. In this study, we investigated the effect of VirF on the activity of promoter P_{yscW} using a transcriptional fusion to the lacZ in response to temperature. The complementation analysis revealed that VirF does not have any influence to activity of promoter P_{yscW} at low (26°C) or high (37°C) temperature in the manner of its homolog ExsA.

Introduction

Type III secretion system (T3SS) is essential for the virulence of many gram negative pathogenic bacteria by enabling bacteria inject toxin proteins into cytosol of eukaryotic cells (24). It enhances the survival of bacterial pathogens in host cells and helps bacteria overcome the host immune system.

The *Y. enterocolitica* Ysc-Yop system is one of the best understood systems among T3SSs of bacteria and plays an important role in the pathogenesis of *Y. enterocolitica*. The whole Ysc–Yop T3S system including Ysc injectisome, secreted Yop proteins and their chaperones is encoded by the virulent plasmid pYV (13). Upon Ca^{2+} depletion or contact with eukaryotic target cells, pathogenic *Y. enterocolitica* utilizes Ysc injectisome to inject six of Yop effectors into the cytosol of target cells (13). These Yop effectors can interfere with a number of cellular functions such as rearrangement of the actin cytoskeletion, pathogen phagocytosis and pro-inflammotory immune responses (12).

In vitro, the expression of Ysc-Yop system is induced after culture of *Y. enterocolitica* at 37°C and Yop secretion only starts when Ca^{2+} is depleted from medium, conditions that are mimic host environment (13).

The expression of Ysc-Yop system is strongly thermo-regulated by transcriptional activator VirF at the transcriptional level (14). In turn, the activity of VirF is controlled by YmoA. At the elevated temperature (37°C), the chromatin structure is changed and the modulator YmoA is dislodged out of pYV, so VirF can bind to promoter regions of *ysc* as well as *yop* genes to activate their transcription (14).

The role of VirF in transcriptional regulation of Ysc-Yop T3SS was well-studied by many authors. Currently, our lab has been underway to find out mechanisms on transcriptional regulation of *virF* gene. From the unpublished data in our lab, it is found *virF* is transcribed with its upstream gene *yscW* as an operon from the promoter of the *yscW*. 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 (1, 23). Thus, we hypothesized that VirF may has auto-regulation function on its *yscW-virF* operon. Since it was previous known that the transcription of *virF* is thermo-regulated (10), the purpose of this study is to examine the role of VirF and temperature in regulation of activity of promoter P_{yscW}.

Materials and methods

Bacterial Strains and Growth conditions

All bacteria strains and plasmids used in this study are described in Table 2.1. *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, 90mM NaCl) or on Luria Agar (Difco). Media used for *Y. enterocolitica*'s Yop secretion was Luria broth which had been chelated for Ca^{2+} ion by the addition of 1.5mg/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 1mM IPTG to the cultures. Antibiotics were used at the following concentrations: tetracycline (15µg/ml), nalidixic acid (20µg/ml), chloramphenicol (25µg/ml).

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 described as following: strain GY6361 was grown overnight in LB containing Tc 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 13000 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.

Protein preparation and SDS-PAGE analysis

The detection of secreted Yops was accomplished as previously described (37). *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).

Promoter activity assay

The activity of promoter P_{yscW} was determined by measuring the β-galactosidase activity as described by Miller (34). 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 1mM IPTG in sub-cultures. Following the incubation time, cells were harvested by centrifugation 13000 rpm for 1 minute and then re-suspended in 1ml working buffer (Enzyme assay buffer + β-mercatoethanol). The cell density was determined by measuring A_{600}. The 200ul of resuspended bacterial cells was mixed with 800ul of working buffer and then lysed by chloroforms and sodium dodecyl sulfate (SDS). Subsequently, a 200ul 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 follow: $[(A_{420} - .175 \times A_{550}) \times 1{,}000]/[t \times v \times A_{600}]$, where t is the times in minutes, and v is the volume of the resuspended cells used in assay.

Result

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 (Bent and Young 2010, unpublished data). 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 (Tc^R). 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 Tc. 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 (14). Figure 2.1 showed the ability of Yop production among selected *Y. enterocolitica* strains: *Y. enterocolotica* strain GY6361 carrying pYV (lane1) secreted Yop proteins in supernatant with different bands exposed in the acrylamide gel whereas *Y. enterocolitica* GY6532 (lane2) 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^R) 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 Tc. 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).

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). Corniel at el 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) (14). As can be seen in figure 2.2, 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 (figure 2.2). 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 (figure 2.3). Take together, these results indicated that VirF did not affect on activity of promoter P_{yscW} in the response to temperature.

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 Ca^{2+} ion in medium in vitro (24). 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 (1). They act as DNA-binding proteins to activate the transcription of T3SS genes by binding to the promoter regions of these T3SS genes (24). 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) (3). 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) (23). Interestingly, the transcriptional activator ExsA can bind to the promoter P_{exsC} and auto-regulate the transcriptional level of *exsCBA* operon

(23). 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*.

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 (10), 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. Bent and Young proved that the *Y. enterocolitica crp* mutant shows the downregulation of *vir*F expression which in turn results in a decrease in Yop production (Bent and Young, unpublished data). They also proved that CRP does not directly affect to the transcription of *yscW-virF* operon by binding to the promoter region of *yscW*. Thus, it is believed that CRP indirectly controls the expression of *yscW-virF* operon through unknown intermediate regulator(s) (Bent and Young, unpublished data). 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 (47). It is also proved that the global regulator Vfr, along with cAMP, does not directly regulated the transcription of *exsCBA* regulon (41). Mechanisms of cAMP-Vfr complex exert the transcriptional control of *P. aeruginosa* T3SS genes is not clear (47). It may indirectly affect to *exsCBA* operon like *Y. enterocolitica* or to the ExsA binding/activity (47).

Figures and tables

Figure 2.1 Analysis of secrected Yop proteins by selected *Y. enterocolitica* strains. Lane 1 : Molecular weight; Lane 2: WT GY6361 (pYV^+, pGY1060); Lane 3 : *virF* deletion mutant GY6532 (pYV^{--}, pGY1060).

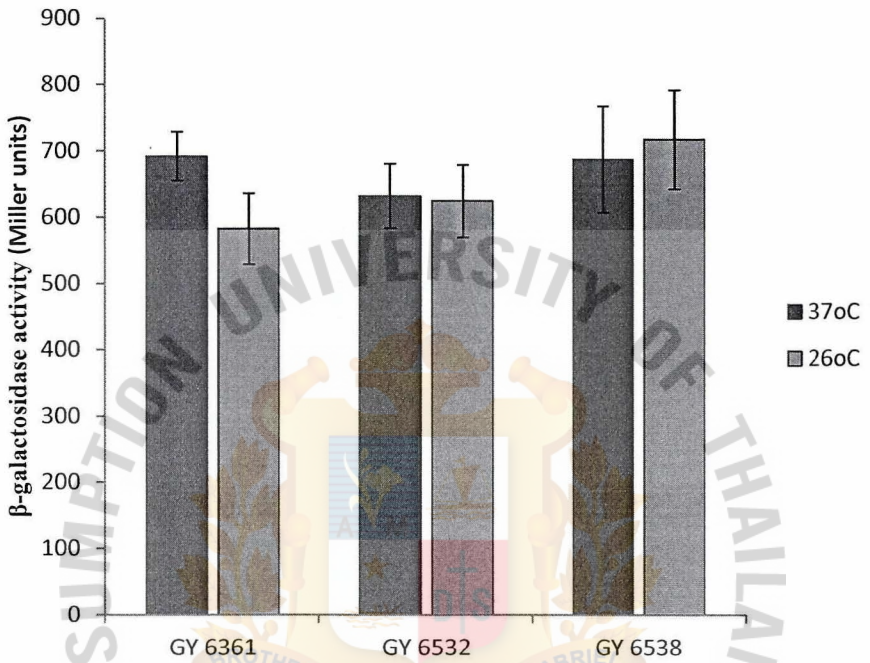

Figure 2.2 : β-galactosidase activities at 26°c and 37°C in selected *Y. enterocolitica* strain GY6361(pYV^{+}, pGY1060) and *virF* deletion mutant strain GY6532(pYV^{-}, pGY1060) and *virF* complemented strain GY6538 where vir*F* is driven by natural promoter P_{yscW} (pYV^{-},pGY1060, P_{yscW}-*yscWvirF*). Error bars indicated standard deviation.

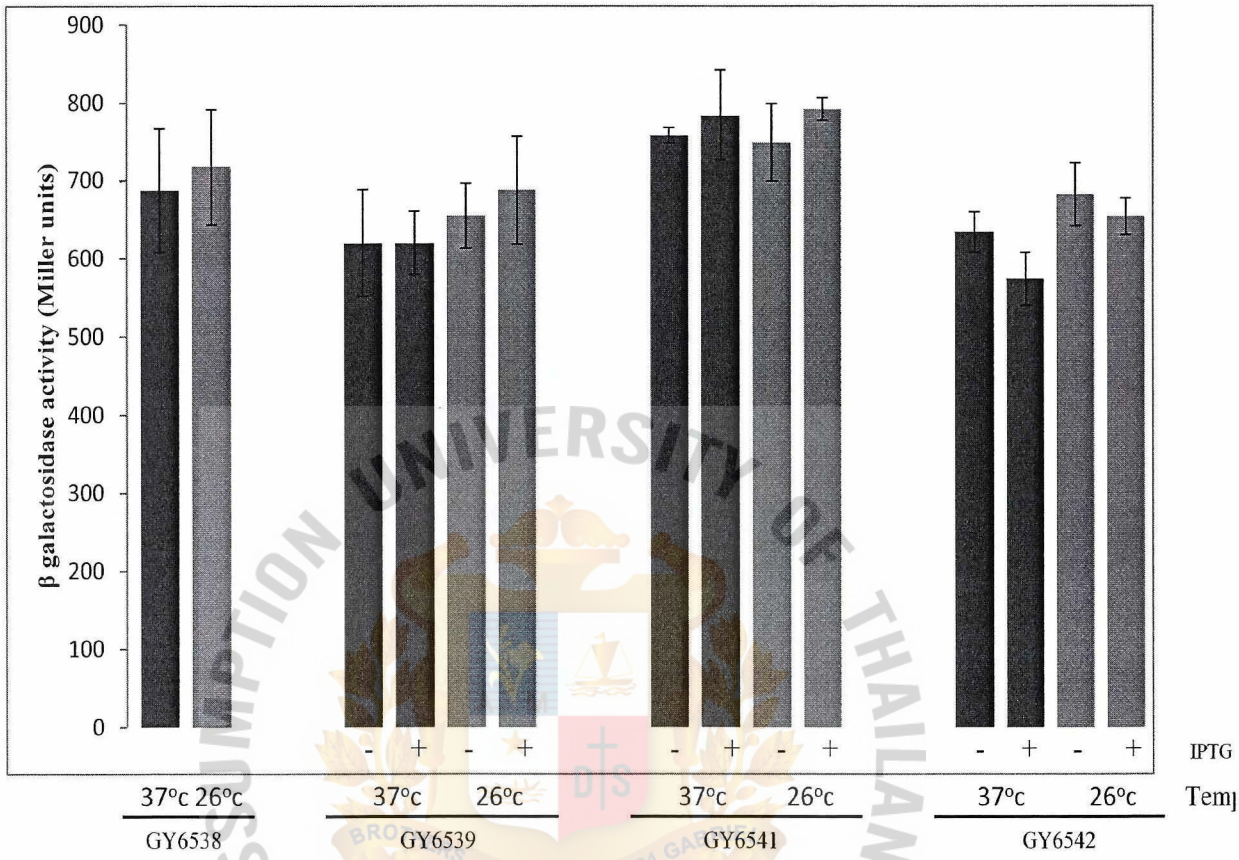

Figure 2.3: β-galactosidase activities in the *virF* complimented strains of *Y. enterocolitica* where the expression of *vir*F is driven by natural promoter P_{yscW} or by inducible promoter P_{tac}. Induction of promoter P_{tac} was performed by adding 1mM IPTG in sub-cultures at 26°C and 37°. GY6538 (pGY1060/P_{yscW}-*yscWvirF*), GY6539 (pGY1060/P_{tac}) as a control, GY6541 (pGY1060/P_{tac}-*virF*), GY6542 (pGY1060/P_{tac}-*yscWvirF*). C. Error bars indicated standard deviation

Table 2.1: 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

CHAPTER 3

Evaluation of *Y. enterocolitica tol-excC* (*pal*) gene cluster in maintenance of outer membrane integrity

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Abstract

Tol-Pal system is well conserved in Gram negative bacteria and plays an important role in maintenance of outer membrane integrity. This role originally is identified in *E. coli* and later on in *P. putida*, *Er. chrysanthemi* and *Caulobacter*. In *Y. enterocolitica*, the organization of *tol-excC (pal)* gene cluster resembles as observed in *E. coli tol-pal* gene cluster. The predicted *Y. enterocolitica* Tol-ExcC (Pal) proteins are also highly similar to those of *E. coli*. The investigation of the function of *Y. enterocolitica tol-excC* (*pal*) gene cluster in maintenance of outer membrane integrity was first conducted by using a transposon mutant. The *Y. enterocolitica tolQ, tolR, tolB* mutants displayed slow-growth phenotypes when they were cultured overnight for tri-parent mating. In addition, these *Y. enterocolitica tol* mutants showed the sensitivity to SDS with 0.1% concentration. However, *Y. enterocolitica* complemented *tol* strains could not be successfully constructed in this study, so we were unable to evaluate the role of the Tol-ExcC (Pal) in maintenance of outer membrane integrity further.

Introduction

The outer membrane (OM) of Gram negative bacteria protects the cells from the external environment. The outer membrane is linked to peptidoglycan layer as well as inner membrane via envelope's proteins such as Braun's lipoprotein (Lpp), OmpA, Tol-Pal system (28). Mutation on *lpp*, *ompA* and *tol-pal* gene causes disruption of outer membrane integrity resulting in phenotypic changes such as hypersensitive to some drugs and detergents, release of periplasmic proteins into extracellular medium and formation of outer membrane vesicles (20).

The Tol-Pal system of Gram negative bacteria composes of five core envelope proteins: three inner membrane proteins (TolR, TolQ and TolA), one periplasmic protein (TolB) and the peptidoglycan associated lipoprotein (Pal) (33). The Tol-Pal proteins form two complexes (TolQRA and TolBPal) to bring inner and outer membrane in close proximity. Two Tol-Pal complexes links each other via C-terminal domain of TolA and N-terminal domain of TolB (20). These two complexes also can link to other structural outer membrane proteins such as Lpp and OmpA (33).

The Tol-Pal proteins in *E. coli* are encoded by a cluster of genes transcribed under two promoters. The large transcript is from *ybgC-tolQ-tolR-tolA-tolB-pal-ybgF* and the small one is from *tolB-pal-ybgF* (33). Neither cytoplasmic protein YbgC nor periplasmic protein YbgF contributes to maintenance of outer membrane integrity because the inactivation of *ybgC* and *ybgF* does not show any phenotypic changes in cells (20). The exact function of Tol- Pal proteins in maintenance of outer membrane protein integrity is

still largely unknown. Two possible functions have emerged: involvement in the assembly and/or translocation of (an) outer membrane components crucial for its stability such as porin, LPS (20) and/or in the OM invagination during cell constriction (19). In addition to maintenance of outer membrane integrity, the Tol-Pal proteins also play a role in uptake /transport filamentous phage DNA and group A colicin (33).

The *tol-pal* gene cluster is widely distributed and its gene organization is highly conserved in Gram negative bacteria (42). However, the role in maintenance of outer membrane integrity has been identified more in several related species; *P. putida* (31), *Er. chrysanthemi* (16), *C. crescents* (48). *Y. enterocolitica* is a Gram negative foodborn pathogen which causes gastrointestinal illness in human. Since no study related to Tol-Pal system in *Y. enterocolitica* has been conducted, the organization of *tol-pal* gene cluster in *Y. enterocolitica* and its role in maintenance of cell envelope integrity was analysed in this study.

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 3.1. *Y. enterocolitica* strains were routinely grown at 26°C and *E. coli* strains were grown at 37°C in TYE media (10g/L tryptone and 5g/L yeast extract). Antibiotics were used at the following concentrations: kannamycin (50µg/ml), tetracycline (15µg/ml) and nalidixic acid (20µg/ml).

Construction of *tol-pal* complementation plasmid

To construct the *tol-pal* complementation plasmid, the *tol-pal* gene fragment was amplified by PCR with *Pfu* polymerase (Stratagene) using 2 primers (Primer Tol1, 5'-GTGTACCGGTGTGATTCATC-3', Primer Tol2, 5-CGTTACCAACTGAGCTAACG-3'). Primer Tol1 was located 248 bp upstream of gene *ybgC* (YE2936) and Primer Tol2 was located 197 bp downstream of gene *ybgF* (YE2930). Genomic DNA from *Y. enterocolitica* GY6495 was served as a template DNA for PCR reactions. The resulting PCR product was examined by agarose gel electrophotoresis. Subsequently, the resulting PCR product was purified by Qiaex® Gel extraction Kit (150) (Qiagen) and then cloned into plasmid pCR-Blunt II-TOPO following the manufacturer's instruction to create pTOPO/*tolpal*. Next, the pTOPO/*tol-pal* was transformed into One Shot competent *E. coli* cells by heat shock. The *E. coli* carrying pTOPO/*tol-pal* was selected on LB containing kannamycin.

The *tol-pal* fragment was subcloned from pTOPO to pTM100. The process was described as following: pTOPO/*tol-pal* from One Shot competent *E. coli* was purified by using QIA® Spin Miniprep (Qiagen). Afterwards, both pTOPO/*tol-pal* and pTM100 were digested by enzyme EcoRI for 2hours at 37°C. The resulting fragments were confirmed by agarose gel electrophotoresis.

SDS sensitivity test of *Y. enterocolitica tol* mutants

Y. enterocolotica tol mutants were screened from our lab strain library. SDS sensitivity test was performed by streaking *Y. enterocolitica* WT and *Y. enterocolitica tolB, tolQ, tolR* mutants on TYE plates containing different concentrations of sodium dodecyl sulfate (SDS): 0.1%, 0.01%, 0.001%, 0.0001% and 0.00001%.

Construction of *tol* complemented strains by triparent mating

To construct *tol* complemented strains, the *E. coli* donor strain GY3257 carrying the cosmid pLAFR library of *Y. enterocolitica* WT; and two *E. coli* strains , GY2118 and GY2121, carrying helper plasmid pRK290 and pRK2930 respectively were used. Both helper plasmids contain *tra* gene but carry different marker genes: tetracycline resistance gene (Tc^R) on pRK290 and kanamycine resistance gene (Kn^R) on pRK2930. All *Y. eneteroclitica tol* mutants harbor tetracycline resistance gene (Tc^R) and nalidixic acid resistance gene (Nal^R) and the *E. coli* donor strain carries tetracycline resistance gene (Tc^R).

Six sets of triparental mating were established (Table 3.2): three of them with the helper strain GY2118 and three of them with helper strain GY2121. All *Y. enterocolitica* and *E. coli* strains were cultured overnight before performing the conjugation. The process of conjugation was described as following: the overnight culture of *Yerisnia tol* mutant was mixed with the overnight of *E. coli* donor strain and the *E. coli* helper strain. Then the conjugation mixture was centrifuged at 13000 rpm for 2 minutes. Next, the pellet was re-suspended in TYE broth and subsequently plated on TYE and incubated for 6 hours. Afterward, the trans-conjugants were plated on different TYE based selective medium and later confirmed by urease test. The selective medium used for each sets of triparent mating listed in Table 3.2.

Computer analysis

Y. enterocolitica tol-excC (*pal*) gene cluster analysis was done by using Artemis program (39). Amino acid sequence similarities were detected by using BLAST program (2) available at the National Center for Biotechnology Information network server.

Result

The organization of *Y. enterocolitica tol-excC (pal)* gene cluster

Based on *Y. enterocolitica* strain 8081 genome sequence recently released by Thomson et al, the genes which encode for *Y.enterocolitica* Tol-Pal system are similar to those in *E. coli.* They are contained in a compacted region in the order *ybgC* (YE2936) *tolQ* (YE2935) *tolR* (YE2934) *tolA* (YE2933) *tolB* (YE2932) *excC* (or *pal*) (YE2931) and *ybgF* (YE2930) (figure 3.1A). In the upstream region of the gene *ybgC* (YE2936), the homolog of gene *ybgE* of *E. coli* is also identified. The downstream of the gene YE2930 is a gene tRNA-*lys* (YEt061) that is highly similar to tRNA-*lysT* in *E. coli.* Overall, the organization of *Y. enterocolitca tol-excC*(*pal*) cluster is highly similar to the organization of *E. coli tol-pal* gene cluster.

The amino acid (aa) length of predicted *Y. enterocolitica* Tol-Pal proteins and their percentage identities to *E. coli* Tol-Pal proteins are displayed in Figure 3.1B. The gene YE2936 potentially encodes for a polypeptide of 133 amino acid residues that share 74% identity with *E. coli* YbgC. Similarly, the gene YE2930 probably encodes a polypeptide of 296 amino acid residues that share 70% identity with *E. coli* YbgF. The predicted TolQ protein is the most identical (89%) to *E. coli* TolQ , followed by the predicted TolB and Pal (86% identity to *E. coli*) and TolR (82 % identity to *E. coli*). The predicted TolA protein shows the lowest percentage of identity to *E. coli* (63%).

Construction of *tol-pal* complementation plasmid

As described in Materials and methods, PCR was used to amplify the *tol-pal* gene fragment from genomic DNA and the resulting PCR product was confirmed by the agarose gel electrophoresis. Based on the genomic sequence of *Y. enterocolitica* WT strain 8081, it is expected that *tol-excC* (*pal*) fragment is 6054bp in length. The resulting PCR product amplified from genomic DNA had the correct size (figure 3.2). Following PCR amplification, the resulting PCR product was cloned into 3519 bp of pTOPO to create the recombinant plasmid pTOPO/PCRproduct. To subclone into plasmid pTM100, the recombinant plasmid pTOPO/PCR product and pTM100 were digested by the EcoRI enzyme. The digested plasmids were then analyzed by the agarose gel electrophoresis. The uncut pTM100 and uncut pTOPO/PCRproduct were used as control. Theoretically, EcoRI enzyme cut only single EcoRI site on pTM100 to produce the opened pTM100 with two sticky ends and its expected digested plasmid was 5050 bp. With plasmid pTOPO/*tolexcC(pal),* there are three EcoRI sites : one is located on the *ygbC* gene and two remaining ones are localized outside the *tol-excC (pal)* fragment (figure 3.1). Hence, after digesting with EcoRI enzyme, 3 expected different DNA fragments are generated: ~3519 bp of TOPO vector, ~556 bp of *tol-pal* fragment and ~5500 bp of *tol excC* (*pal)* fragment. As seen in figure 3.3, digested plasmid pTM100 (lane 2) had correct size whereas the digested plasmid pTOPO/PCRproduct released a ~3500 bp of fragment implied is TOPO vector and 2 unexpected fragments (~3900 bp and ~2200 bp) (lane4) (figure 3.3). This indicated that there is also one EcoRI site inside PCR product fragment but its location differs from expected one. Thus, it could be concluded that PCR product was not *tol-excC* (*pal*) fragment. Since *tol-excC* (*pal*) fragment has unsuccessfully created, the *tol-pal* complementation plasmid was not constructed.

SDS sensitivity of *Y. enterocolitica tol* mutants

Y. enterocolitica WT and *Y. enterocolitica tolQ, tolR, tolB* mutants were streaked on TYE containing different SDS concentrations from 0.1% to 0.00001%. The result (table 3.3) revealed that *Y. enterocolitica* WT could tolerate with all concentrations of SDS used this study while all of *Y. enterocolitica tol* mutants showed sensitivity to SDS with 0.1 % concentration.

Construction of *tol* complemented stains by triparental mating

To complement the *Y. enterocolitica tol* mutants with the pLaFR cosmid library containing the *Y. enterocolitica* WT inserts, the tri-parental conjugation was used. The pLaFR cosmid contains the origin of transfer (oriT) and *mob* genes that involve in the mobilization of DNA during conjugative process. The *tra* genes on helper plasmid (SY327/pRK290 or DH5α/pRK2013) in *E. coli* facilitate the conjugation by forming the pilin complex and nicking the oriT of pLaFR which initiates the transfer of single DNA strand of cosmid pLaFR to recipient *Y. enterocolitica tol* mutants.

The result revealed that the *Y. enterocolitica tol* mutants showed slow-growth phenotypes when they cultured overnight for tri-parental conjugation mating. Since *Y. enterocolitica tol* mutants were sensitive to 0.1% SDS, the complemented *tol* strains were expected to grow on the TYE containing 0.1% SDS. After conjugation, the trans-conjugants were selected by plating on TYE containing 0.1% SDS and different types of antibiotics (TYE based selective media) depending on the sets of conjugation mixture. The results showed that trans-conjugants grew on all TYE based selective medium that did not contain Kn (Table 3.4). However, all of these trans-conjugants growing on these medium gave

negative urease test. This indicated the tri-parental conjugation mating did not occur and somehow *E. coli* strains could resist to antibiotics that they did not carry and grew on TYE based selective medium.

Discussion

The *Y. enterocolitica* genome sequence reveals that the *Y. enterocolitica tol-excC*(*pal*) region is well conserved. The gene order of *Y. enterocolitica tol-exc*C(*pal*) gene cluster (*ygbCtolQtolRtolAtolBpalygbF)* is identical to *E. coli, P. aeruginosa, P. putida and Er. Chrysamthemi* (15, 16, 32, 45). However, transcriptional organization of *tol-pal* gene cluster is different in these species. Both *tol-pal* genes of *E. coli* and *P. putida* are transcribed under two operons but the gene content in each operon is different: for *E. coli* (*orf1tolQtolRtolAtolB and tolBpalorf2*) (45) and for *P.putida* (*orf1tolQtolRtolAtolB and oprLorf2*) (32). The *tol-pal* genes of *P. aeruginosa* are arranged into 3 operons (*orf1tolQtolRtolA*, *tolB* and *oprLorf2*) (15) . Further study should be conducted to analyze the transcriptional organization of *Y. enterocolitica tol-excC* (*pal*) gene cluster.

Among *Y. enterocolitica* predicted Tol-Pal proteins, TolQ is the most conserved protein whereas TolA is the least conserved protein. This is also observed in other species such as *P. putida* (31), *Er. chrysamthemi* (16) , *V. Cholera* (22) and *Caulobacter* (48). TolQ contains three transmembrane domain located all in inner membrane and links to both TolA and TolR. The role of TolQ in assembly of TolQRA complex and localization of this complex in inner membrane probably support the high level of TolQ conservation (20, 31). TolA involves in many interactions with other cell envelope's proteins such as

TolB and Pal to bring two membranes in close proximity (20). In addition, it acts as a co-receptor for entry of phages into bacteria, e.g F1 phage into *E. coli*; CTXΦ phage into *V. cholera* (9, 22). The reason for poor conservation of *tolA* gene among Gram negative species is not still clearly known. It is possible that there are selective advantages in the mutations in *tolA* gene among species such as selection of phage uptake, providing immunity to cocilins as well as phages (42).

The Tol-Pal system of Gram negative bacteria plays an important role in maintenance of outer membrane integrity. Mutation on *tol* or *pal* genes exposes phenotypic changes in bacteria. The hypersensitivity to variety of antibiotics and chemical compounds is one of phenotyphic changes found in strains having mutations on the *tol-pal* gene cluster and has been proved in several species. In *E. coli*, all *tol* and *pal* mutants showed the sensitivity to deoxycholate and EDTA (29) . *P. putida orf*1 (or *ybgC* in *E. coli*), *tolR, tolA* and *tolB* mutants displayed the sensitivity to variety of chemical agents like SDS, DOC, and EDTA and some antibiotics (31). In *Er. chrysanthemi,* it was proved that all *tol* and *pal* mutants were sensitive to sodium cholate, SDS and CCCP (16).

To investigate the role of *Y. enterocolitica* Tol-ExcC (Pal) system in maintenance of outer membrane integrity, the transposon *tolQ, tolR, tolB* mutants were observed to have a decreased growth rate and be sensitive to salt and SDS. However, since the *tol* complemented strains would not be constructed, the complementation analysis could not be used to evaluate *Y. enterocolitica* further. In this study, two different approaches were used to complement *tol-excC* (*pal*) genes into the *tol* mutants. In the first method, the *tol* complemented strains would be constructed by introducing plasmid containing *tol-excC* (*pal*) fragment into *Y. enterocolitica tol* mutants. To construct the *tol* complemented

plasmid, *tol-excC* (*pal*) fragment from *Y. enterocolitica* genomic DNA was amplified, cloned into plasmid pTOPO and then subcloned into pTM100. Nevertheless, *tol-excC* (*pal*) fragment has not successfully created in this study. Tol Primers are designed approximately 250bp far away from the *tol-excC* (*pal*) region were not specific for only *tol-pal* gene cluster. They can hybridize with other regions of *Y. enterocolitica* genomic DNA or pYV plasmid and amplify them. Hence, although length of PCR product was right size, it is believed that PCR product was not the *tol-excC* (*pal*) gene fragment because pTOPO/PCR product generated 2 unexpected fragments (~3900 bp and ~2200 bp) after restriction digestion (figure 3.3).

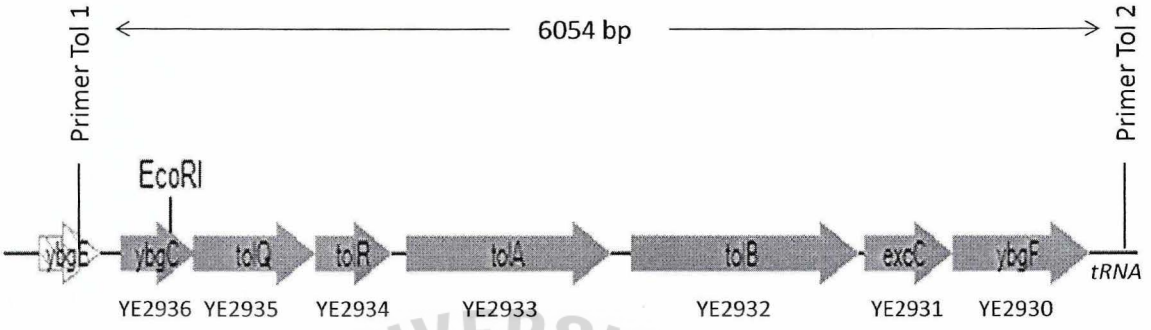
Since getting troubles with primer design and determination of restriction enzymes, another approach was used to construct the *tol-pal* complemcnted strain. In the second method, *Y. enterocolitica tol* mutants were complemented with the pLAFR cosmid that carries the *Y. enterocolitica* WT inserts by tri-parental mating. However, attempts to conjugate three different *Y.enterocolitica tolQ, tolB and tolR* mutant strains, the *E. coli* carrying pLaFR library of *Y. enterocolitica* with *E. coli* helper strain resulted in isolation of only *E. coli* for unknown reasons.

The unsuccessful construction of *tol-pal* complemented strains limited the evaluation of Tol-Pal system in maintenance of cell envelope integrity in *Y. enterocolitica*. However, a some information of *Y. enterocolitica* Tol-ExcC(Pal) system have been revealed. *Y. enterocolitica tol* mutants show better growth in non-salt environment (TYE broth: tryptone and yeast extract) than salt-containing environment (LB broth: tryptone, yeast extract, sodium chloride). For this reason, TYE based medium were used throughout the experiment. However, *Y. enterocolitica tol* mutants still display slow-growth phenotypes

compared to *Y. enterocolitica* WT when they are grown in TYE based media. Finally, *Y. enterocolitica tol* mutants are sensitive to 0.1% SDS while *Y. enterocolitica* WT show well resistance at this level of SDS concentration. All these information reveals that *Y. enterocolitica tol* mutants expose changes in phenogtype, compared to *Y. enterocolitica* WT.

Figures and tables

A.



B..

	YbgC	TolQ	TolR	TolA	TolB	ExcC (or Pal)	YbgF
Y. enterocolitica	133	228	142	386	430	168	269
E. coli	134	230	142	421	430	173	263
% identity with *E. coli*	74%	89%	82%	63%	86%	86%	70%

Figure 3.1. Organization of the *tol-excC* (*pal*) gene cluster in *Y. enterocolitica* strain 8081. (A) Localization of *tol-excC* (*pal*) genes in *Y. enterocolitica* genomic DNA and positions of primers used for *tol-excC* (*pal*) amplification. (B) Predicted length of the different proteins and homology between *Y. enterocolitica* and *E. coli*.

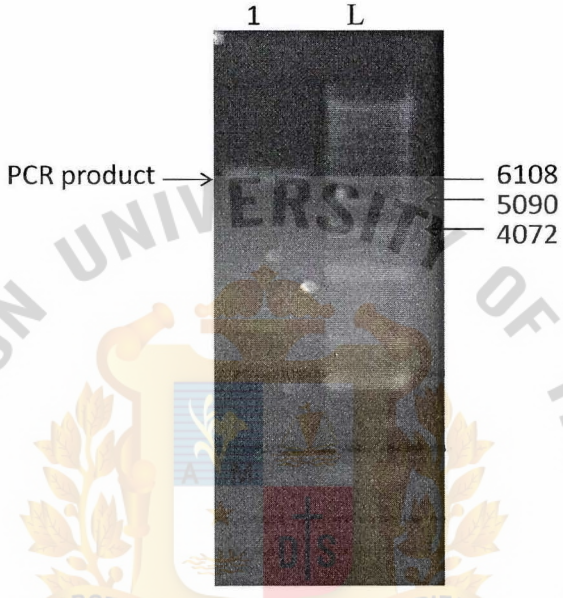


Figure 3.2.Confirmation of PCR product on 0.8% (w/v) agarose gel stained with SYBR Safe®. Lane 1 : PCR product; L:1kb DNA ladder

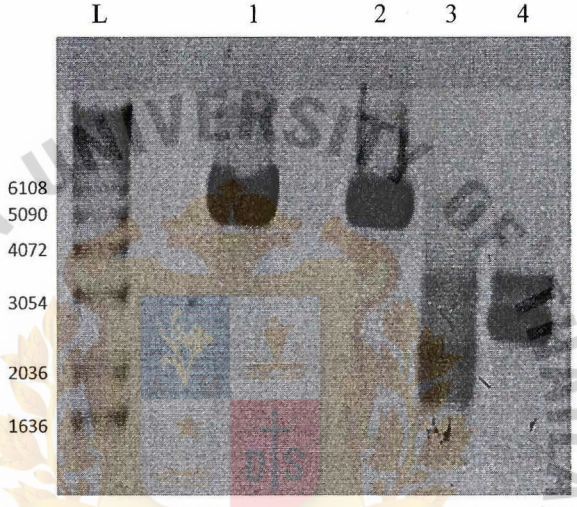


Figure 3.3. Restriction digest analysis of pTM100 and pTOPO/PCR product. Both plasmids were digested by EcoRI enzyme for 2 hours at 37°C and the resulting digested plasmids were analyzed on 0.8% (w/v) agarose gel stained with SYBR Safe®. L: 1kb DNA ladder; Lane 1: uncut pTM100; Lane 2: cut pTM100; Lane 3: Uncut pTOPO/PCR product; Lane 4: Cut pTOPO/PCR product

Table 3.1 : Bacterial strains used in this study

Strains	Description	Reference
Y. enterocolitica		
JB580v	Serogroup O:8, Nal^R, Δ*yen*R (R-M+)	Young Lab Collection
GY6495	ΔyspK/K(K53A), Nal^R	Young Lab Collection
GY695	*tol*B::TnMod-R-Kn' 93,Nal^R,Kn^R,yplA-	Young Lab Collection
GY573	*tol*Q::TnMod-R-Kn' 135, Nal^R,Kn^R,yplA-	Young Lab Collection
GY694	*tol*R::TnMod-R-Kn' 97, Nal^R,Kn^R,yplA-	Young Lab Collection
E. coli		
GY2118	SY327/pRK290 (derivative of pRK2), *mob*$^+$, *tra*$^+$, Tc^R	Young Lab Collection
GY2121	DH5α/pRK2013, (derivative of pRK2), *mob*$^+$, *tra*$^+$, Kn^R	Young Lab Collection
GY 3257	LE392/pLAFR*, Tc^R, *Y.enterocolitica* YVM159 cosmid library	Young Lab Collection

Table 3.2: Sets of triparental conjugation mixture and the TYE based selective media used for transconjugant selection.

No.of set	Conjugation mixture	TYE based selective media
1	*E. coli* helper GY2118+ *E. coli* donor GY3257 + *Y. enterocolitica tolR*⁻ 694	Tet15 + Kn50 + 0.1%SDS Tet15 + Nal20 + 0.1%SDS Kan50 + 0.1SDS
2	*E. coli* helper GY2118+ *E. coli* donor GY3257 + *Y. enterocolitica tolB*⁻ 695	Tet15 + Kn50 + 0.1%SDS Tet15 + Nal20 + 0.1%SDS Kan50 + 0.1SDS
3	*E. coli* helper GY2118+ *E. coli* donor GY3257 + *Y. enterocolitica tolQ*⁻ 573	Tet15 + Kn50 + 0.1%SDS Tet15 + Nal20 + 0.1%SDS Kan50 + 0.1SDS
4	*E. coli* helper GY2121+ *E. coli* donor GY3257 + *Y. enterocolitica tolR*⁻ 694	Tet15 + Nal20 + 0.1%SDS Tet15 + Nal20 Nal20 + 0.1%SDS
5	*E. coli* helper GY2121+ *E. coli* donor GY3257 + *Y. enterocolitica tolB*⁻ 695	Tet15 + Nal20 + 0.1%SDS Tet15 + Nal20 Nal20 + 0.1%SDS
6	*E. coli* helper GY2121+ *E. coli* donor GY3257 + *Y. enterocolitica tolQ*⁻ 573	Tet15 + Nal20 + 0.1%SDS Tet15 + Nal20 Nal20 + 0.1%SDS

Table 3.3 : SDS sensitivity of *Y. enterocolitica tol* mutants

SDS concentration (w/v)	***Y.enterocolitica* strains.**			
	WT	*tolB* mutant	*tolQ* mutant	*tolR* mutant
0.1%	+	--	--	--
0.01%	+	+	+	+
0.001%	+	+	+	+
0.0001%	+	+	+	+

+ resistance; -- sensitive.

Table 3.4 : The growth of trans-conjugants on TYE based selective media.

No. of set	**TYE based selective media.**		
	Tet15 + Kn50 + 0.1%SDS	Tet15 + Nal20 + 0.1%SDS	Kan50 + 0.1%SDS
1	--	+	--
2	--	+	--
3	--	+	--
	Tet15 + Nal20 + 0.1%SDS	Tet15 + Nal20	Nal20 + 0.1%SDS
4	++	+	++
5	++	+	++
6	++	+	++

The number of set corresponding to the conjugation mixture described in Table 3.3. + growth ; -- no growth.

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