IDENTIFICATION OF *Salmonella enterica* serovar Typhi PUTATIVE VIRULENCE FACTORS USING A YEAST GROWTH INHIBITION ASSAY

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IDENTIFICATION OF *Salmonella enterica* serovar Typhi PUTATIVE VIRULENCE FACTORS USING A YEAST GROWTH INHIBITION ASSAY

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LIST OF ABBREVIATIONS

µl	microliter
BCA	Bicinchoninic Acid Assay
bp	base pair
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
HPLC	High-performance liquid chromatography
HT	High-throughput
kbp	Kilo-base pair
kDa	kilodalton
LB	Luria Broth
M	molar
ml	milliliter
mM	millimolar
Ni-NTA	Nickel- Nitrilotriacetic acid
OD	Optical Density
PCR	Polymerase Chain Reaction
S.O.C	Super Optimal Broth
SC medium	Synthetic Complete medium
SDS	Sodium Dodecyl Sulfate
VF	Virulence factor
VFs	Virulence factors
YPD medium	Yeast extract-Peptone-Dextrose medium

PENGENALPASTIAN FAKTOR VIRULEN PUTATIF *Salmonella enterica* serovar Typhi DENGAN MENGGUNAKAN ASAI PERENCATAN PERTUMBUHAN YIS

ABSTRAK

Patogen *Salmonella enterica serovar* Typhi (*S.* Typhi) mengelak sistem imun perumah dan menyebabkan penyakit di dalam perumah dengan menghasilkan protein yang dikenali sebagai faktor virulen (VF). Apabila VF diekspresi dalam yis *Saccharomyces cerevisiae* (*S. cerevisiae*), ia didapati merosakkan sel yis (cth. menyebabkan perencatan pada pertumbuhan sel). Dalam pengertian ini, yis boleh bertindak sebagai penunjuk sensitif untuk mengenal pasti VF. Sehingga kini, masih terdapat VF yang belum dikenalpasti dan untuk yang sudah dilaporkan, peranannya dalam patogenesis masih belum difahami sepenuhnya. Kajian ini bertujuan untuk mengenalpasti VF putatif pada *S.* Typhi dengan mengekspresikannya di dalam *S. cerevisiae* dan mengkaji kesannya pada pertumbuhan yis. VF putatif dikenalpasti melalui analisis bioinformatik menggunakan pangkalan data atas talian dan semakan kajian literatur. Sejumlah 192 VF putatif yang berpotensi telah dipilih daripada proteom *S.* Typhi CT18. Kemudian, gen VF terpilih diklon dan diekspres dalam yis menggunakan format plat 96-telaga. Daripada 192 VF putatif yang dipilih, 173 VF putatif berjaya diklon dan diekspres dalam yis. Dengan menggunakan asai pertumbuhan mikroplat yang mengukur kepadatan optik kultur cair dan dengan pengesahan kebolehhidupan sel pada medium pepejal, 23 VFs putatif didapati merencat pertumbuhan yis. VFs tersebut dilakukan kajian pencirian lanjut. Dua VF putatif rekombinan ditulenkan untuk mengkaji protein perumah yang disasarkan melalui kajian interaksi VFs-protein yis dengan menggunakan asai tarik turun. Protein

perumah yang ditarik turun dikenalpasti menggunakan analisis spektrometri jisim. Dua VF putatif telah dikenalpasti iaitu - protein dirembes berkait-virulen (STY3018) dan protein yang mungkin dirembes SopD (STY3073) didapati berkemungkinan berinteraksi dan mensasarkan protein yis yang dipelihara seperti guanin nukleosida pengikat protein (protein G) yang berfungsi sebagai molekul transduksi isyarat dan protein ribosom yang diperlukan untuk proses selular yang penting. Kesimpulannya, aliran kerja ini boleh digunakan untuk mengenal pasti VF putatif pada *S*. Typhi atau protein yang terlibat dalam merencat pertumbuhan yis dan sasaran interaksinya pada sel yis.

IDENTIFICATION OF *Salmonella enterica* serovar Typhi PUTATIVE VIRULENCE FACTORS USING A YEAST GROWTH INHIBITION ASSAY

ABSTRACT

Pathogenic bacteria such as *Salmonella enterica* serovar Typhi (*S.* Typhi) evade the host immune system and cause disease in their host by producing proteins known as virulence factors (VFs). When VFs are expressed in yeast *Saccharomyces cerevisiae (S. cerevisiae)*, they have been observed to be detrimental to yeast cells (e.g. causing growth inhibition). In this sense, yeast can act as a sensitive indicator to identify VFs. There are still unidentified VFs and for those already reported, their roles in pathogenesis are still not completely understood. This study aims to identify putative *S.* Typhi VFs by expressing them in *S. cerevisiae* and examining their effects on yeast growth. First, putative VFs were identified by bioinformatics analysis using online databases and literature review. A total of 192 potential putative VFs were selected from the proteome of *S.* Typhi CT18. Then, the selected VF genes were cloned and expressed in yeast in a 96-well plate format. Of the 192 putative VFs selected, 173 putative VFs were successfully cloned and expressed in yeast. By using a microplate growth assay that measures optical density of liquid culture and by confirmation of cell viability on solid medium, 23 putative VFs were found to inhibit the growth of yeast and were subjected to further characterization studies. Two recombinant putative VFs were purified to investigate the targeted host proteins through the study of VF-yeast protein interactions using a pull-down assay. Pulled down host proteins were identified by mass spectrometry analysis. Two putative VFs were identified in this study, a virulence-associated secretory protein (STY3018) and SopD possible secreted

protein (STY3073) which were possibly interacting and targeting conserved yeast proteins such as guanine nucleotide binding proteins (G proteins) that function as signal transduction molecules and ribosomal proteins which are crucial for important cellular processes. In conclusion, this workflow can be used for the identification of *S.* Typhi putative VFs or yeast growth inhibiting proteins and their target interactions in yeast cell.

CHAPTER 1

Introduction

1.1 General Introduction

Bacterial pathogens such as *Salmonella*, *Pseudomonas aeruginosa* and *Shigella* spp. remain a major health threat worldwide despite of the advances in treatment and prevention of bacterial diseases. *Salmonella enterica* serovar Typhi (*S*. Typhi) possess a major health risk in the world according to World Health Organization (WHO), with 9.9 to 24.2 million cases reported each year resulting in approximately 208,000 deaths (Antillon et al., 2017). *S*. Typhi infection brings economic burden, especially to developing countries for the cost of surveillance, prevention and treatment of disease (Eng et al., 2015). *S*. Typhi causes typhoid fever, a systemic disease characterized by fever, haemorrhage, intestinal perforation and enlargement of the mesenteric lymph nodes, spleen and liver in humans (Wu et al., 2008).

Bacteria cause diseases in their hosts by producing proteins known as virulence factors (VFs) that act individually, cooperatively or antagonistically during infections. There are many types of VFs that possess different functions in pathogenesis, such as adherence factors that adhere to host cell surface, invasion proteins that invade host cells and toxins to kill host cells (Wu et al., 2008).

Identification of putative VFs can contribute to better understanding of bacterial pathogenesis, revealing the mechanisms of disease at the molecular level and findings of novel drugs or vaccine targets against pathogenicity (Zheng et al., 2012). Following the identification of putative VFs, the functions and roles of putative VFs in bacterial pathogenesis could be revealed through the study of the molecular mechanisms of putative VFs and their interaction with host cells (Wu et al., 2008). Therefore, putative

VFs identification serves as a first step in the fundamental study of bacterial pathogenesis that can contribute in the development of new drugs or vaccine and better treatment of diseases.

In this study, the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) was used as a model organism to study *S.* Typhi putative VFs instead of human cells which are costly to culture and sometimes hard to propagate in sufficient quantities for use in a high-throughput manner (Aleman et al., 2009; Slagowski et al., 2008). Yeast has been shown to be a good model for characterizing VFs although it cannot be used as a physiological model for human infections (Aleman et al., 2009). VFs, when expressed in yeast, have been observed to be detrimental to yeast cells and can lead to growth inhibition in yeast (Popa et al., 2016; Slagowski et al., 2008). Thus, yeast growth inhibition can be a sensitive indicator to detect the presence of VFs.

This study involves the selection of putative VF genes through bioinformatics analysis and literature review, high throughput cloning of putative VF genes into yeast expression vector for protein expression in yeast, identification of putative VFs by using a yeast growth inhibition assay and finally study of the interaction of putative VFs with host proteins through a pull-down assay. The approach used is outlined in Figure 1.1.

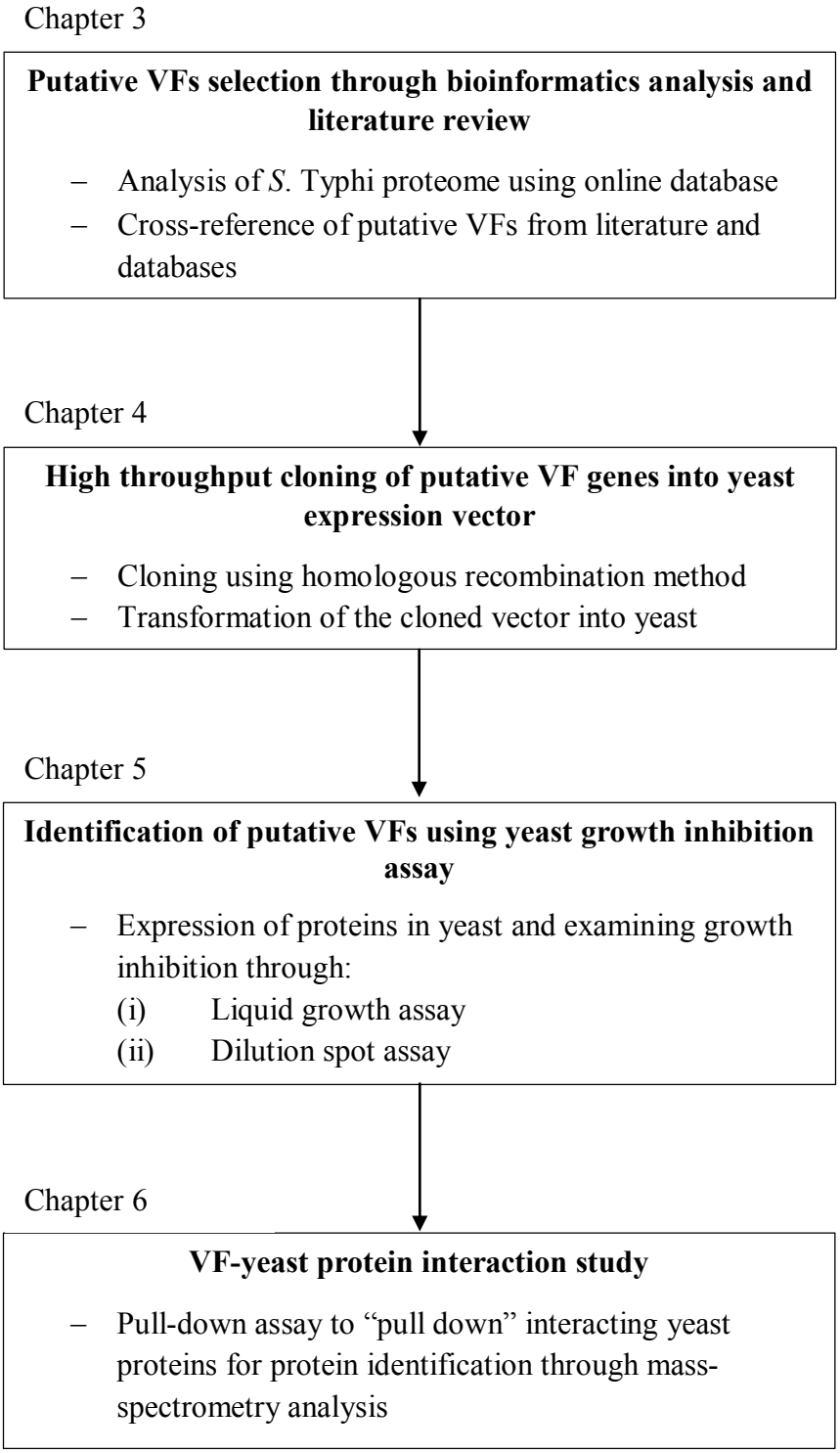

Figure 1.1 Outline and flow of study

Experimental approach taken to identify *S.* Typhi VFs and study of its interaction with host proteins

1.2 Hypothesis and Aim of Study

Many *S*. Typhi VFs remain unknown and for those that are reported, their functions are unclear. Thus, the main aim of this study is to identify *S*. Typhi putative VFs by expressing its proteins in yeast *S. cerevisiae* and using yeast growth inhibition as an indicator of the effect of putative VFs.

Specific aims were:

1. To select *S*. Typhi putative VFs from bioinformatics analysis and literature review.
2. To develop a high-throughput (HT) platform for gene cloning and expression of selected proteins in yeast *S. cerevisiae*.
3. To identify putative VFs of *S*. Typhi by using yeast growth inhibition assay.
4. To identify targeted host proteins of selected potential putative VFs through VF-yeast protein interaction study.

CHAPTER TWO

Literature Review

2.1 *Salmonella* and Typhoid fever

Salmonella is a gram negative, facultative anaerobic and rod-shaped bacterium that belongs to family of Enterobacteriaceae (Andino and Hanning, 2015). The genus of *Salmonella* contains 2600 serotypes (termed "serovar") and can be classified into two species: (i) *Salmonella enterica* and (ii) *Salmonella bongori* (Eng et al., 2015).

Salmonella enterica serovar Typhi or in short, *S*. Typhi show host specificity that only cause disease in humans. *S*. Typhi infection occurs due to the ingestion of food and water contaminated with the bacteria which usually transmitted via faecal-oral route (Wu et al., 2008). *S*. Typhi infects human gastrointestinal tract and excreted out of human body together with faeces. The bacteria may then be brought to other places by humans or animals, contaminate foods and drinking water, and infect new individuals. The infection can be spread from individual to individual and affect more people as this cycle repeats (Andino and Hanning, 2015).

S. Typhi causes typhoid fever that has incubation period of one week or more, with symptoms such as headache, abdominal pain and diarrhoea, followed by fever and other complications such as haemorrhage, hepatitis and pancreatitis (Eng et al., 2015). The severity of *S*. Typhi infection depends on dose of bacterial inoculation and health conditions of the infected patients. Doses at an approximate of 10^3 to 10^9 CFU of *S*. Typhi are sufficient to cause typhoid fever (Andino and Hanning, 2015). Clinical cases showed that children below five years old, elderly people and patients with immunosuppression are more likely to suffer from severe *S*. Typhi infection (Eng et al., 2015).

Typhoid fever remains a major health problem worldwide, with high incidence rate in many developing countries such as India, Pakistan, Indonesia and Vietnam that have more than 100 reported cases per 100, 000 population annually (Eng et al., 2015). On the other hand, USA and some European countries have low incidence rate, with less than 10 cases per 100, 000 population annually (Eng et al., 2015).

2.2 Pathogenesis of *Salmonella*

S. Typhi is a highly-adapted human pathogen that can survive in a variety of harsh conditions and defence mechanisms in the human gastrointestinal tract. *Salmonella* can survive inside host stomach in spite of the highly acidic conditions with their induced acid-tolerance response (ATR) (Wu et al., 2008).

When bacteria travel from stomach to intestine, they pass through the intestinal mucosal layer by evading host immune responses. Then, they invade intestinal epithelial cells specifically specialized epithelial cells, microfold (M) cells through bacteria-mediated endocytosis which involves the formation of membrane ruffles that uptake and engulf the bacteria (Figure 2.1) (Haraga et al., 2008).

After crossing the epithelial barrier, bacteria invade immune cells such as macrophages and induce their virulence mechanisms to survive inside macrophages. The bacteria inside macrophages can be carried to the other parts of body (e.g. liver and spleen) and cause systemic infection inside host (Figure 2.1) (Haraga et al., 2008).

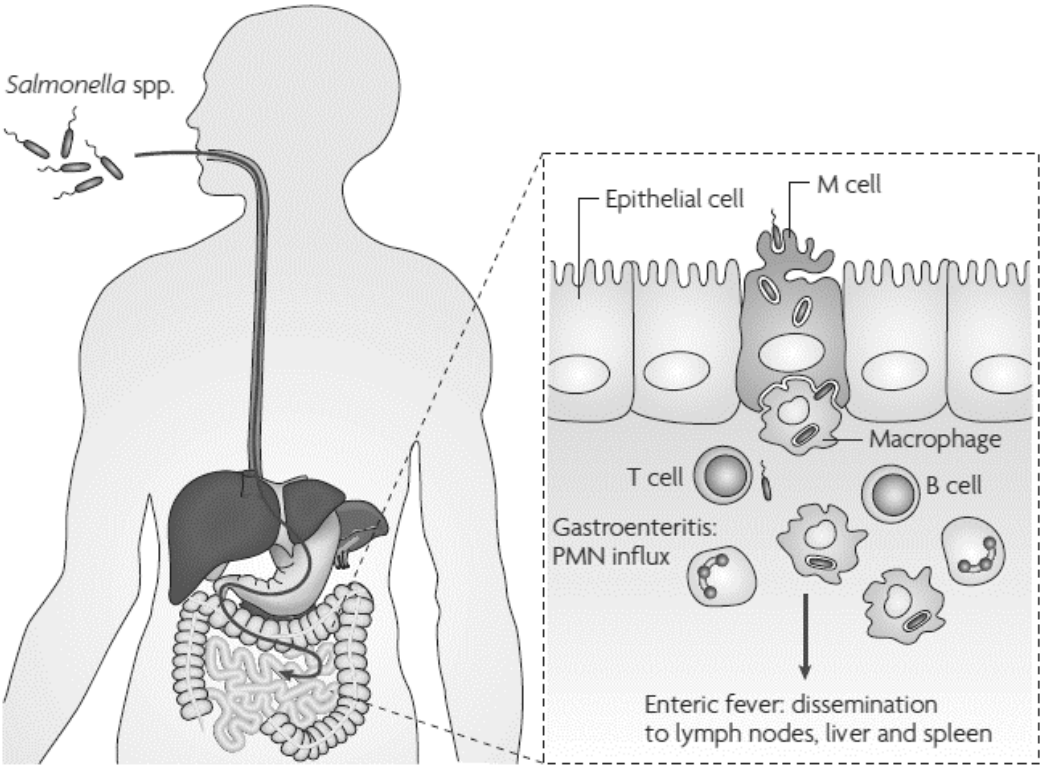

Figure 2.1 *Salmonella* infection

The ingested bacteria invade host intestinal epithelial cells specifically M cells and cross the intestinal barrier to infect macrophages and disseminate to other parts of the body such as lymph nodes, liver and spleen, causing systemic illnesses such as enteric fever (Haraga et al., 2008).

2.3 Bacterial Virulence Factors

Pathogenic bacteria depend mainly on their VFs to evade host defences and cause diseases. Gram-negative bacteria secrete and inject VFs directly into host cells via a secretion machinery known as type III Secretion System (T3SS) which are encoded in pathogenic island, a chromosome region where VF genes were located (Shames and Finlay, 2012).

Besides translocation of VFs directly into host cells via T3SS (10%), most of the VFs as such in *Salmonella* (80-90%) are secreted to the extracellular space (Srikanth et al., 2011). VFs are found to interact with host cells both intracellularly and extracellularly. As an example, the interaction of *Salmonella* invasion protein A (SipA) with host enzyme, caspase 3 for cleavage of specific motifs to functional domains which required for bacterial pathogenesis was found occurred at outer surface of epithelial cells (Srikanth et al., 2011).

A single VF can have multiple functions and host targets (Shames and Finlay, 2012). For instance, *S*. Typhi VF, SopB functions in actin cytoskeleton rearrangement (Hurley et al., 2014), stimulating fluid secretion responsible for diarrhoea (Ong et al., 2010) and cell invasion (Ibarra and Mortimer, 2009).

VFs regulate each other either cooperatively or antagonistically to promote infections. For example, VFs secreted during late stage of infection may modulate the functions of those secreted at early stage, changing the bacterial virulence strategy to promote infection. In the case of *Salmonella*, a network of VFs are cooperating in various pathogenic activities such as cell invasion (SopB, SopE, SipA, SipC and SptP), actin cytoskeleton organization (SopE and SptP), *Salmonella* Induced Filament (SIF)

formation (SifA and SseJ) and degradation of host proteins by hijacking ubiquitin system (SseL and SopA) (Shames and Finlay, 2012).

Besides, some VFs mimic host proteins to take control of important cellular processes or pathways in host. During infection, pathogenic bacteria degrade host proteins either by secreting protease or hijacking host ubiquitin proteasome pathways (UPS) which is a homeostasis system functions to regulate degradation of unwanted proteins. To subvert UPS, bacterial VFs mimic ubiquitinase enzyme and take control of ubiquitin system to promote degradation of host proteins (Shames and Finlay, 2012). As an example, *Salmonella* VFs such as SopA, SspH2 and SlrP mimic ubiquitin E3 ligase to modulate host ubiquitin pathways to their advantage (Perrett et al., 2011).

2.4 *Salmonella* Pathogenicity Islands

To date, 15 *Salmonella* Pathogenicity Islands (SPIs) (SPI-1 to SPI-15) have been identified in *S.* Typhi (Kaur and Jain, 2012). SPI-1 consists of a 40kb DNA region encoding T3SS, structural, effector and regulatory proteins whereas SPI-2 is a 40kb gene locus with two different regions, with its major region encoding major virulent factors including T3SS apparatus, regulation, chaperones and effectors (Figueira and Holden, 2012). Both SPI-1 and SPI-2 encode T3SS, a complex of proteins that is required for the transfer of VFs from bacterial cells into host cells.

Other than these two major SPIs, other SPIs (SPI-3, SPI-4, SPI-5, SPI-7, SPI-8 and SPI-9) have also been identified to play important roles in virulence and survival of the bacteria (Table 2.1) (Ong et al., 2010). However, the roles of genes in other pathogenicity islands in *S.* Typhi pathogenesis have not been investigated in depth yet.

Table 2.1 *Salmonella* Pathogenicity Islands (SPIs) and roles of their VFs in virulence adapted from (Ong et al., 2010)

SPI	Main Function	Genes	Type of Proteins	Role in Virulence
SPI-1	Invasion of epithelial cells	SopB, SopD, SopE2, SipA	Effector proteins (secreted proteins)	influence host cell functions: cytoskeletal rearrangements, neutrophil recruitment and tight-junction disruption, macrophage apoptosis
		HilA, InvF	Regulatory proteins	activate expression of effector proteins responsible for invasion of host epithelial cell
		PhoP, PhoQ	Regulatory proteins	(i) activate genes required for bacterial cells survival in macrophages (ii) activate SPI-1 encoded invasion genes
SPI-2	Survival of *S.* Typhi inside *Salmonella* Containing Vacuole (SCV)	SifA	Effector protein	(i) help bacterial cells replication in host cells (ii) maintain SCV integrity
		SseL	Effector protein	modulate host inflammatory response *in vivo*
		SseJ	Effector protein	important for intracellular bacterial survival
		SsrA/B	Regulatory protein	regulate and express SPI-2 genes
SPI-3	Survival of *S.* Typhi inside host cell	MgtA	NR	function in high affinity Mg ion uptake for *S.* Typhi survival in host cells Mg ion deficient condition
SPI-4	Adhesion to host epithelial cell surfaces	STY 4458 STY 4459	NR	encoded SiiE (non-fimbrial adhesin)

NR– Not Reported

(*Content continued on next page*)

Table 2.1. Continued

SPI	Main Function	Genes	Type of Proteins	Role in Virulence
SPI-5	Contribute to *Salmonella* enteropathogenesis	SigD/SopB	NR	(i) secreted inositol phosphatase that trigger fluid secretion responsible for diarrhoea (ii)activates mammalian protooncogene Alct, which responsible for inhibition of apoptosis in normal intestinal epithelial cells during infection
		SigE	NR	important for stabilization and secretion of SopB/Sig D
		PipD	NR	encodes a cysteine protease homolog that crucial in long term systemic infection
SPI-6	Function and regulation of SPI-6 genes still largely unknown	STY 0291	NR	predicted virulence protein
SPI-7	Encode genes for VFs	SopE	Effector protein	effect on host cell: cytoskeletal rearrangement
SPI-8	NR	STY3281, STY3283	NR	-encode colicin/pyocin that enable *S.* Typhi to kill other bacteria to compete for nutrients

NR– Not Reported

2.5 Type III secretion system

T3SS is a protein complex mainly made up of three sets of proteins: (a) base, (b) needle-like structure and (c) translocon. The main function of T3SS is to translocate VFs from bacterial cytoplasm to host cells (Kaur and Jain, 2012). The base is made up of multi-rings structures, with rings structures assembled at both bacterial inner and outer cell membranes, connected by a joint ring structure (Figure 2.2) (Kaur and Jain, 2012). On the other hand, a needle-like structure and translocon (Figure 2.2) function to deliver virulence proteins by forming pores on host cell membranes (Haraga et al., 2008).

The export of the VFs from bacterial cytoplasm to host cells via T3SS can be achieved in a well-controlled manner with the actions of several factors and proteins of the secretion system that act cooperatively in the translocation process of VFs. The ATPase at the base of the secretion system plays a crucial role as "proteins pump" that drive the export of the VFs. Besides, the VFs contain secretion signal peptides located at its amino termini that drive the transportation of proteins down the route of the secretion system. Other than secretion signals, chaperone proteins of the secretion system bind and stabilize some of the VFs, promoting the translocation of VFs (Haraga et al., 2008).

Salmonella have two distinct T3SS which encoded by SPI-1 and SPI-2 respectively. SPI1-encoded T3SS (T3SS-1) is activated upon bacteria-host cells contact and translocate VFs which are mainly required for invasion, from bacterial cell to host cytoplasm, across the host plasma membrane (Figure 2.2) (Ramos-Morales, 2012). Whilst SPI2-encoded T3SS (T3SS-2) is induced by drastic change of environmental conditions inside *Salmonella* containing vacuole (SCV) and deliver

virulence proteins which are required for bacterial intracellular survival, to host cytoplasm across the vacuolar membrane (Figure 2.2).

Despite T3SS-1 and T3SS-2 have distinct functions, they cooperate with each other for efficient functioning. There are studies reported that the mutation of T3SS-1 genes resulted in reduced expression of T3SS-2 genes (Agbor and McCormick, 2011).

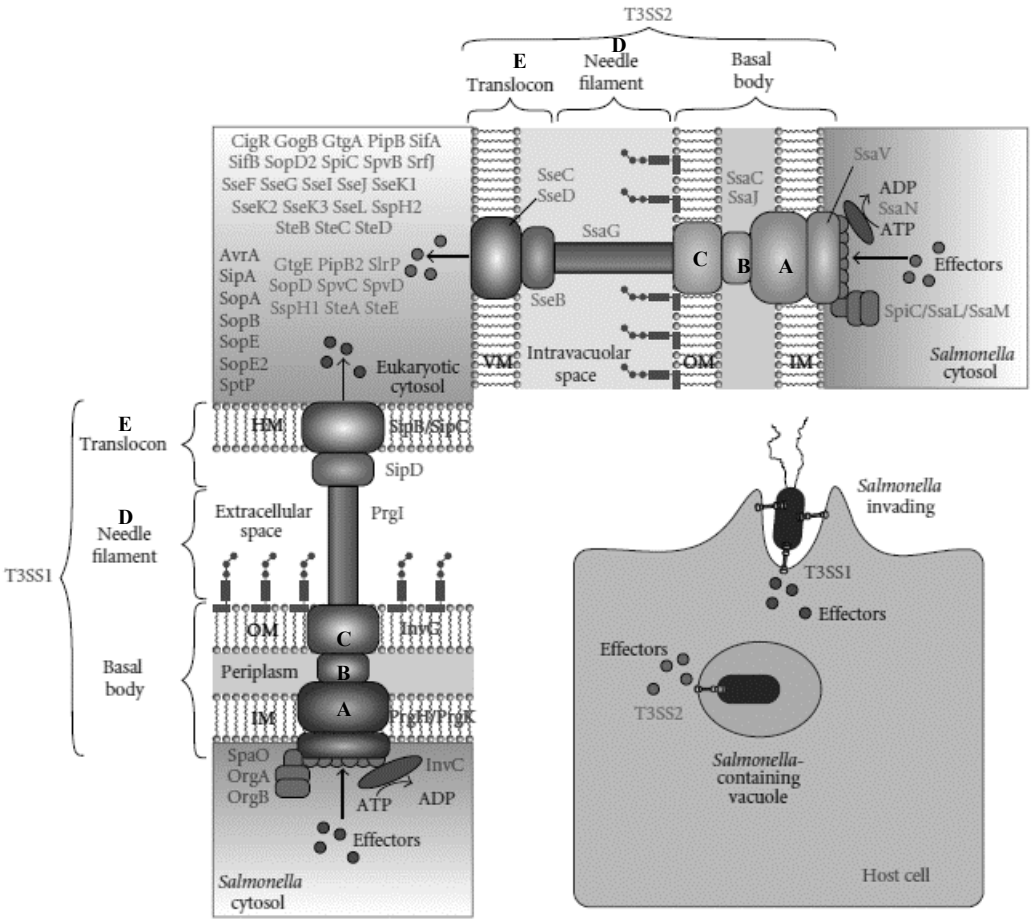

Figure 2.2 Schematic illustration of Type III secretion systems

Type III secretion systems (T3SS) comprise of three main sets of proteins: Basal body which made up of multi-ring structures, that are assembled at bacterial inner (A) and outer (C) membrane, joined by a ring-structure (B), needle filament (D) and translocon (E). SPI1-encoded T3SS (T3SS-1) translocate VFs from bacterial cytoplasm to host cells, across the host plasma membrane whereas SPI2 T3SS (T3SS-2) is expressed inside *Salmonella*-containing vacuole (SCV) and translocate VFs to host cytoplasm, across the vacuolar membrane (adapted from Ramos-Morales, 2012).

2.5.1 *Salmonella* Pathogenicity Island-1 type III secretion system

SPI-1 encodes T3SS genes which encompass of the secretion apparatus (Spa*OPQRS*), secretion system chaperone (Sic*A*), secreted virulence proteins (Avr*A*, Spt*P*, Sip*ABCD*) and secretion system transcriptional regulator (Spr*B*, Inv*F*, Hil*ACD*) (Figure 2.3) (Hurley et al., 2014).

T3SS-1 genes are expressed upon bacterial contact with host epithelial cells under regulation of important transcriptional regulatory proteins such as InvF, HilA, HilC and HilD. (Srikanth et al., 2011). The activated T3SS-1 translocate VFs from bacterial cell to host cytoplasm to induce invasion and bacterial internalization through membrane ruffling, a pinocytosis process involving remodelling of actin dynamics of host cell membrane to form a 'pocket' to engulf and uptake the bacteria (Ramos-Morales, 2012).

2.5.2 *Salmonella* Pathogenicity Island-2 type III secretion system

SPI-2 encodes genes for assembly of T3SS which comprises the secretion apparatus (Ssa*G* to Ssa*U*), secretion system chaperones (Ssc*AB*), secreted virulence proteins (Sse*BCDEFG*) and secretion system transcriptional regulators (Ssr*AB*) (Figure 2.3) (Hurley et al., 2014).

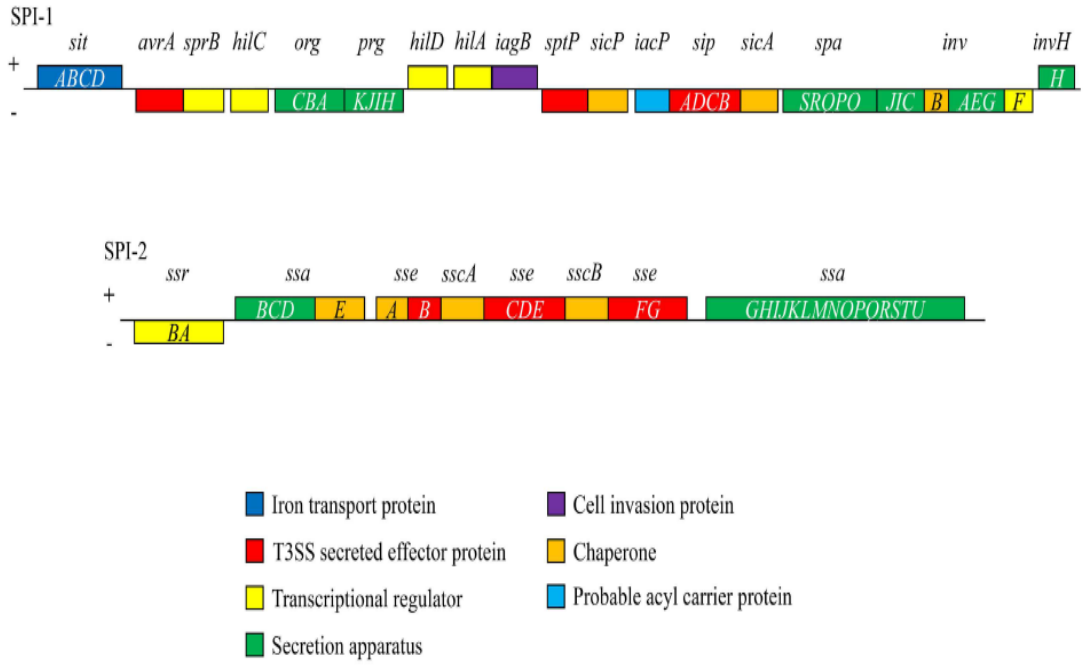

Figure 2.3 Schematic diagram showing T3SS genes encoded in *Salmonella* Pathogenicity Islands (SPI); SPI-1 and SPI-2

SPI-1 and SPI-2, each encodes for a T3SS that comprises of secretion apparatus, chaperone, secreted virulence proteins and secretion system transcriptional regulator. SPI-1 encodes T3SS-1 genes including secretion apparatus (Spa*OPQRS*), secretion system chaperone (Sic*A*), secreted virulence proteins (Avr*A*, Spt*P*, Sip*ABCD*) and secretion system transcriptional regulator (Spr*B*, Inv*F*, Hil*ACD*) while SPI-2 encodes T3SS-2 genes including secretion apparatus (Ssa*G* to Ssa*U*), secretion system chaperones (Ssc*AB*), secreted virulence proteins (Sse*BCDEFG*) and secretion system transcriptional regulators (Ssr*AB*) (Hurley et al., 2014)

The T3SS-2 genes are only expressed inside SCV, regulated by three important two-component regulatory systems including SpiR/SsrB, PhoP/PhoQ and EnvZ/OmpR which induced during harsh environmental conditions such as lacking essential nutrients, low osmolarity and pH that results in the translocation of around 30 different VFs across the vacuolar membrane to the host cells (Figueira and Holden, Kaur and Jain, 2012).

The activated T3SS-2 translocate VFs into host cytoplasm, across the vacuolar membrane to target and interact with host proteins for taking control of host cellular processes for their benefits to survive and replicate inside host cells (Kaur and Jain, 2012). For instance, SpiC virulence protein is secreted into host macrophages to affect host secretory pathways in producing and secreting bactericidal compounds and thus protect bacteria from host immune responses (Haraga et al., 2008).

2.6 Roles of VFs in Salmonella pathogenesis

2.6.1 Adherence of bacterial cells

To infect host cells, bacterial pathogens first adhere themselves to host cells membrane using their fimbriae, a hair-like structure which protrudes outside the surface of bacterial cells (de Jong et al., 2012). Fimbriae carry adhesin protein that promote adherence to different types of host cells such as epithelial, lymphoid and endothelial cells (Kisela et al., 2013).

Beside fimbriae, bacterial surface factors with adhesive properties namely adhesin recognize and bind to various host membrane molecules, promoting cells adhesion and bacterial internalization into host cells (Ribet and Cossart, 2015). Moreover, flagellar mediate motility to bring bacteria to come into contact with host cell surface membrane, promoting bacterial-host cells contact and thus facilitating

invasion and subsequent bacterial internalization (Ibarra and Mortimer, 2009; Sterzenbach et al., 2013).

2.6.2 Host cell invasion

Upon bacterial-host cells attachment, bacteria inject VFs into host cells via T3SS-1 to induce invasion and bacterial internalization. The expression of T3SS genes are modulated by important transcriptional regulatory proteins such as InvF, HilA, HilC and HilD (Srikanth et al., 2011). The expressed T3SS-1 secrete VFs including SipA, SipC, SopB, SopD, SopE, SopE2 and SptP into host cytoplasm to induce invasion of host intestinal epithelial cells through membrane ruffling by reorganization of actin cytoskeleton (Figure 2.5) (Kaur and Jain, 2012, Ramos-Morales, 2012). The VFs remodel actin dynamics of host cell membranes by activating host cell protein, Rho GTPase such as Cdc42 and Rac which function in cytoskeleton organization of mammals, promoting actin cytoskeleton rearrangement in host cells and result in the formation of membrane ruffles that can uptake and engulf the bacterial cells (Ribet and Cossart, 2015). Besides, SopB, SopE and SipC also work together to induce fusion of exocytic vesicles with the host cell membrane at the site of entry to produce source of membranes for the formation of membrane ruffles (Kaur and Jain, 2012). After invasion, SptP inhibits membrane ruffling and return host membrane to its original state (Kaur and Jain, 2012).

On the other hand, other than the invasion pathways mediated by bacterial endocytosis which governed by T3SS-1 VFs as previously discussed, *Salmonella* serotypes that cause systemic infection such as *S.* Typhi is able to pass through the epithelial barrier without interacting with epithelial cell surface (Figure 2.4) (Haraga

et al., 2008). The tight junction (Figure 2.5), serve as an epithelial barrier which seal neighbouring epithelial cells together to prevent entering of some proteins or molecules. Four T3SS-1 virulence proteins: SopB, SopE, SopE2 and SipA are found to alter the tight junction for penetrating through the epithelial barrier (Figure 2.5) (Ramos-Morales, 2012). During this stage, the transcriptional regulator TviA plays crucial roles in regulating the expression of VFs for passing through the epithelial barrier (Sterzenbach et al., 2013). TviA repress the expression of T3SS-1 and flagellar to avoid the detection of flagellin by Toll-like receptors (TLR) of epithelial cells and thus preventing recruitment of immune cells to kill the bacteria.

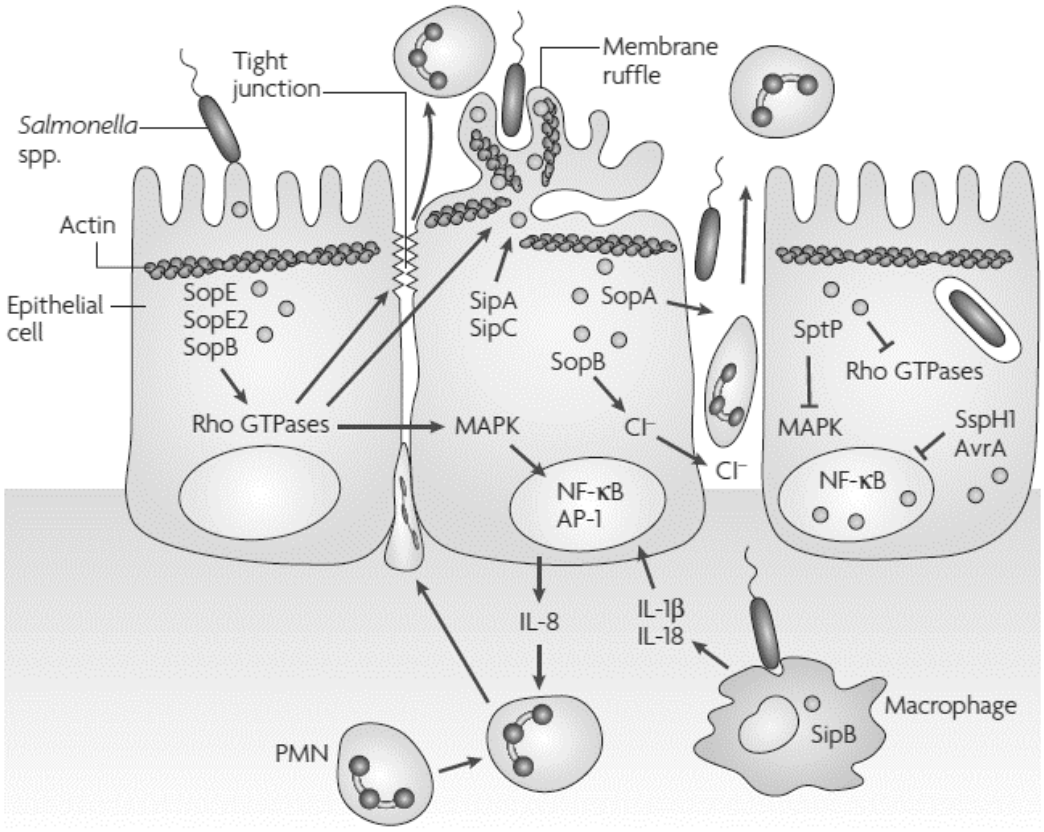

Figure 2.4 *Salmonella* induced host cells invasion

Bacteria secrete T3SS-1 virulence proteins (SopE, SopE2, SopB, SipA, SipC, SopA, SopB, SptP) to induce host cells invasion through membrane ruffling by remodelling of actin dynamics of host cell membrane. Besides, bacteria that cause systemic infection (e.g. *S.* Typhi) adopt other pathway to cross the epithelial barrier (tight junction) without interacting with the epithelial cell surface, to invade host immune cells such as macrophages to establish systemic infection (Haraga et al., 2008).

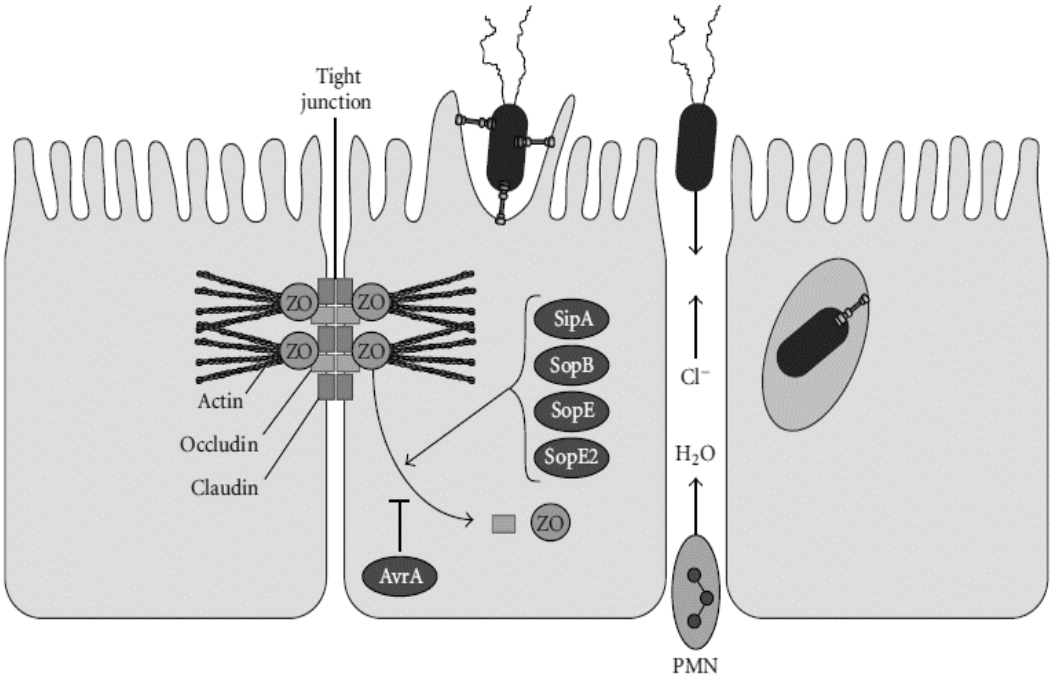

Figure 2.5 Invasion of epithelial barrier through tight junction alteration

Bacteria invade epithelial barrier, the tight junction which seal neighbouring epithelial cells together, through tight junction alteration governed by four VFs: SipA, SopB, SopE and SopE2 (Ramos-Morales, 2012).

After penetrating through the epithelial barrier, *S.* Typhi are able to invade and survive inside immune cells (e.g. macrophages) upon expression of Vi antigen (Figueira and Holden, 2012). Vi antigen is a virulence determinant that is present in *S.* Typhi but absent in other *Salmonella* serovar such as *S.* Typhimurium and *S.* Paratyphi. Vi antigen promotes infections and enhance infectivity of *S.* Typhi. It has been found to maintain bacterial survival during host immune responses by preventing recognition of host pattern recognition receptors (PRRs) (de Jong et al., 2012). Other studies have also reported that *S.* Typhi with loss of Vi antigen showed reduced virulence in mice and thus suggested the importance of Vi antigen in bacteria pathogenesis (Raffatellu et al., 2006).

The expression of Vi antigen is regulated by two-component regulatory systems RcsBC and OmpR/EnvZ which are induced due to low osmolarity condition inside host cells (Raffatellu et al., 2006). The expressed Vi antigen forms Vi capsular polysaccharide to encapsulate bacteria and protect bacteria from being phagocytized, ensuring intracellular survival inside immune cells such as macrophages and neutrophils (Raffatellu et al., 2006). The Vi capsule has the structure that prevent fusion with complement proteins and thus protecting bacteria from being phagocytized and killed by phagocytic cells (e.g. neutrophils) (Sterzenbach et al., 2013) so that bacteria can be carried inside immune cells and disseminated throughout the body organs in establishment of systemic infection inside hosts.

2.6.3 Bacterial intracellular survival and replication

Inside the host cells, bacteria reside in a vacuole that produces a microenvironment ideal for bacterial survival and replication. *Salmonella* reside in a membrane-bound vacuole known as *Salmonella*-containing vacuole (SCV) (Figure 2.6) and inject VFs via T3SS-2 to remodel the vacuole for modulation of pathogenesis preventing the recruitment of bactericidal compounds produced by host cells from killing the intracellular bacteria (Ribet and Cossart, 2015). For instance, *Salmonella* virulence protein, SopB maintain and protect the SCV by modifying membrane charge to prevent it from fusing with lysosome (Srikanth et al., 2011), and thus avoiding the killing of bacteria through lysosomal degradation (Kaur and Jain, 2012). Therefore, the SCV provides a vacuolar microenvironment necessary for bacterial survival and replication. The formation of SCV is directed by VFs such as SopB, SptP, SifA, SipA, SseF and SseG (Agbor and McCormick, 2011).

T3SS virulence proteins play important roles in each stage of SCV formation (early, intermediate and late). T3SS-1 effector proteins, SopB and SptP contribute in the early stage of SCV formation. Whilst during intermediate stage of SCV formation, T3SS-1 effector proteins, SipA and SopB function in SCV positioning to move SCV to juxtanuclear (near nucleus) position. The displacement of SCV is also driven by microtubule proteins known as kinesin which modulated by the VFs, SifA and PipB, and dynein which modulated by SseF and SseG (Figure 2.6) (Haraga et al., 2008).

Finally, in late SCV formation, a network of T3SS effectors work cooperatively and antagonistically to maintain SCV integrity and its position at perinuclear region as well as for formation of *Salmonella* Induced Filaments (SIFs). SifA and PipB2 have been found to work cooperatively in maintaining the integrity of SCV which is suggested to be important for bacterial replication (Figueira and Holden,

2012). Whilst T3SS-2 VFs such as SifA, PipB2, SopD2, SseF and SseG induce the formation of SIFs that extend from SCV throughout the cell cytoplasm (Figure 2.6) (Figueira and Holden, 2012). In the formation of SIFs that require microtubules, VFs play role in the interaction with host microtubules and associate motor proteins. Although the main function of SIFs are not completely understood, they are believed to contribute in bacterial pathogenesis and crucial for bacterial replication (Haraga et al., 2008).

Bacteria are capable of surviving inside the SCV with abilities to adapt to harsh environment (e.g. low or high pH), nutrient-deficient conditions (e.g. iron-deficient) and fight against host immune responses as well as reside microbiota that kill extracellular bacteria (Rohmer et al., 2011).