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The Identification and Functions of the WxxxE Motif Containing Bacterial Proteins

A thesis submitted in partial satisfaction of the requirements for the degree
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by

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The thesis of Sareh Karimilangi is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

This Thesis is dedicated to my parents for their untiring

love, support, and encouragement.

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ABSTRACT OF THE THESIS

The Identification and Functions of the WxxxE Motif
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Sareh Karimilangi

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Bacterial pathogens have evolved various strategies to aid in bacterial survival and dissemination, despite the presence of multiple host defense mechanisms. One tactic that is employed by bacterial pathogens is the secretion of effector proteins across phospholipid membranes through specialized secretion systems. Many enteric pathogens secrete effector proteins using the Type III secretion system (T3SS) to manipulate various host cellular processes

such as evading the host immune response, rearranging the host cell morphology, disrupting tight junctions, regulating cell death pathways, and modifying membrane and vesicular trafficking. Some T3SS effector proteins contain a conserved WxxxE motif with GEF-like activity that is important for their function. However, the presence of this WxxxE motif is not limited to T3SS effectors, it is also found in T2SS and T4SS virulence factors belonging to non-enteric and plant pathogens. In addition to this, in-silico data analysis revealed the presence of a conserved WxxxE motif in the TIR domains of both bacteria and eukaryotic proteins. While these virulence factors share a common WxxxE motif, their functions can vary depending on the pathogen and its mode of infection. Yet, little is known about the role of WxxxE-containing effector proteins on the bacterial pathogenesis. Hence, further functional studies will help understand the function of the WxxxE motif in these pathogenic bacteria.

Introduction

Despite many prevention efforts, foodborne illnesses continue to become a serious public health threat globally. The Center for Disease Control and Prevention (CDC) recently reported that each year approximately 48 million people become ill with foodborne infections, including hospitalizations and deaths in the United States. While there are numerous pathogens known to attribute to foodborne illnesses, *Campylobacter, Salmonella, Shigella, and Shiga toxin-producing E.coli (STEC)* persist to be the leading causes, infecting a wide range of hosts through ingestion of contaminated food or water. Eventually the infection is cleared within a few days by healthy individuals however, those with weak or compromised immune systems such as children, the elderly, or individuals with chronic disease experience severe infection. Many studies have examined the pathogenesis of enteric bacteria, but little is known about how bacteria attack different host cellular pathways to evade the host immune response. To improve the treatment and prevention of foodborne illnesses, it is crucial to examine the host immune response to enteric pathogens. By understanding the pathogenesis of enteric diseases, we could potentially identify new personalized therapeutics, as a significant amount of people do not respond to current treatment.

Chapter 1. Pathogen mimicry of host protein-protein interactions

1.1 Bacterial secretion systems

The fundamental basis for any living organism is the ability to adapt to changes in the environment. Pathogenic bacteria have evolved over time to adapt to multiple host species through the development of various sophisticated strategies. One of these tactics involves the secretion of specific virulence factors known as bacterial effector proteins into the host cell using a specialized

secretion system (Yangchun & Yu, 2021; Galan, 2009). Typically, the dedicated secretion apparatuses are divided into separate groups based on their specificity, function, and architecture (Costa et al., 2015; Green & Mecsas, 2016). Various secretion apparatuses that inject a broad range of bacterial proteins are conserved in most pathogenic bacteria, but other systems are present in a small set of bacterial species (Green & Mecsas, 2016). Often pathogenic bacteria rely on these systems to exploit the host in order to maintain a replicative niche (Green & Mecsas, 2016). They can also utilize secretion systems to take advantage of the host cellular environment by injecting bacterial proteins that aides the pathogen to compete with other microorganisms (Dörr & Blokesch, 2018; Tseng et al, 2009). Although several secretion apparatuses exist, three secretion systems will be discussed—Type II Secretion System (T2SS), Type III Secretion System (T3SS), and Type IV Secretion System (T4SS).

1.2 Enteric pathogens secrete bacterial effectors via T3SS

A large number of gram-negative bacterial pathogens such as *Salmonella, Shigella,* and *Yersenia* employ the T3SS system, which secretes a wide variety of protein substrates across both the inner and outer bacterial membrane and into the cytoplasm of the host cell (Abrusci et al., 2014), as shown in Figure 1. Because of its structure, this apparatus is often referred to as an injectisome or needle/syringe-like apparatus (Erhardt et al., 2010). During the infection process, the pathogen injects bacterial effector proteins that allow the pathogen to establish an infectious niche by disrupting various host cellular processes, including rearranging the host cell morphology, modifying membrane and vesicular trafficking, and evading the host immune response (Alto et al., 2006; Cornelis, 2006; Galán, 2009). Many of these cellular processes are carried out by low molecular weight GTPases making them essential targets for bacterial effector proteins (Mattoo et al., 2007; Wennerberg et al., 2005).

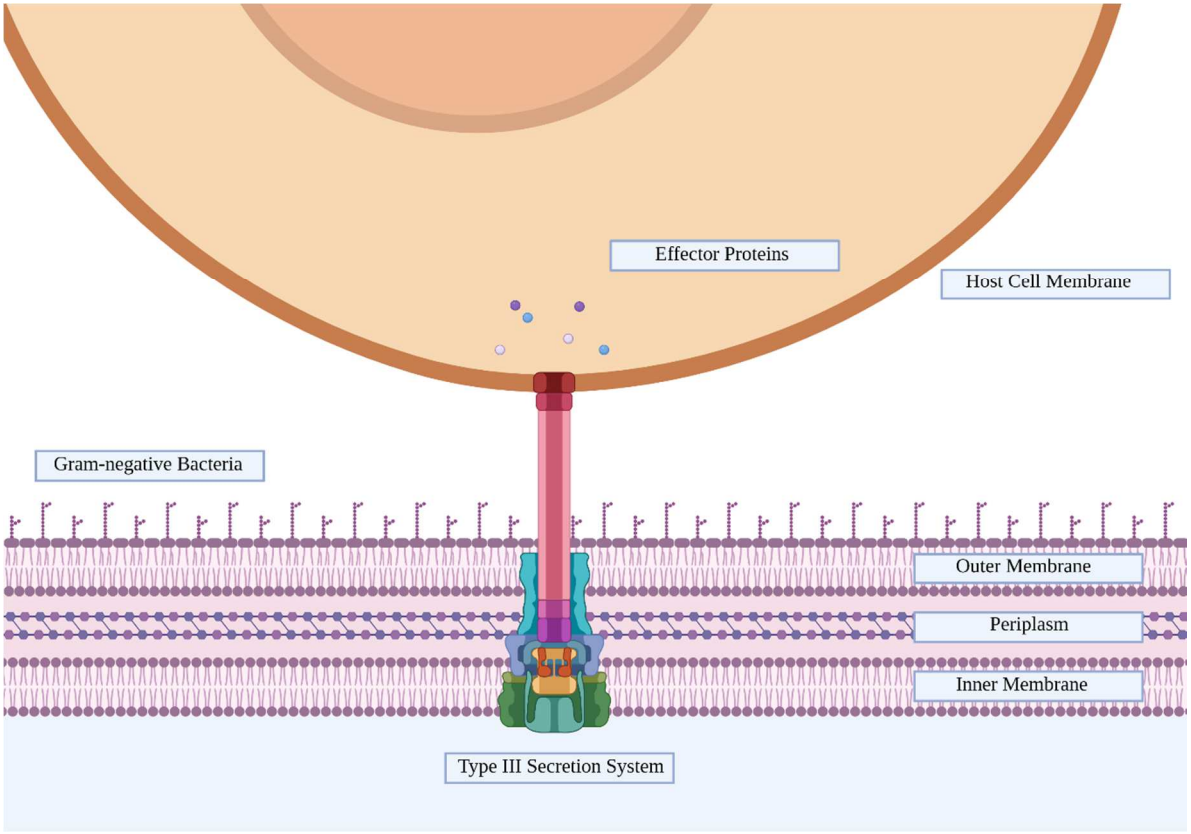

Figure 1. Type III secretion system. Complex protein secretion system employed by gram-negative bacteria to inject bacterial effector proteins across three membranes into host cytoplasm (Created with Biorender.com).

1.3 WxxxE effectors exhibit GEF mimicry to exploit host response

In the process of modulating cellular processes, small guanine nucleotide-binding (G) proteins are regulated alternately by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Once an extracellular stimulus is received, G-proteins are sequestered from the cytoplasm and localized to the membrane by the addition of a lipid moiety where they are activated by a molecular switching mechanism (Bos et al., 2007; Jaiswal et al., 2011). This involves a GTPase conversion from inactive guanosine diphosphate (GDP) bound state to active guanosine triphosphate (GTP) bound state through activation of the G-protein by GEFs as shown in Figure 2 (Bos et al., 2007; Jaiswal et al., 2011). In order to turn off the molecular switch, GAPs function in a GTPase conversion to hydrolyze GTP to an inactive GDP bound state shown in (Vetter & Wittinghofer, 2001).

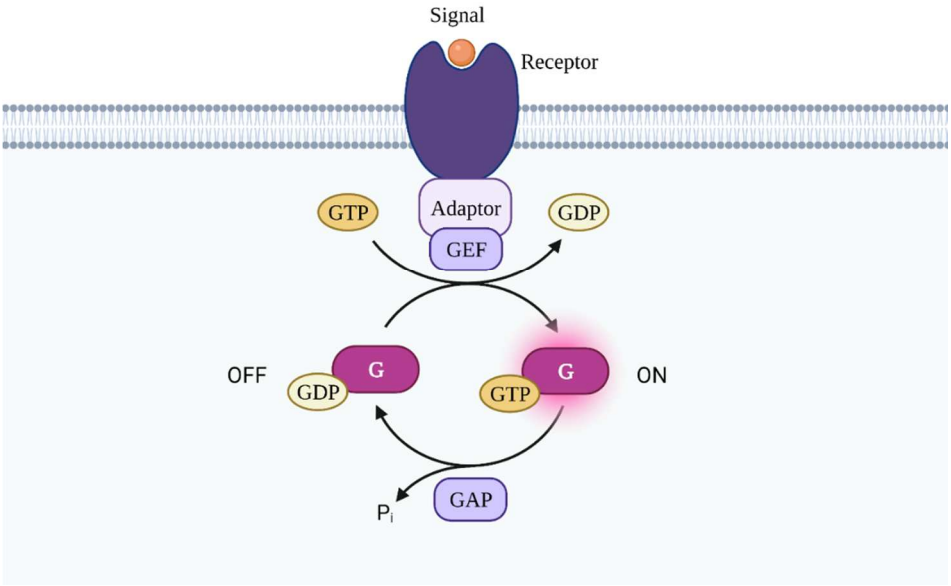

Figure 2. The Role of GAPs and GEFs in GTPase signaling pathways. G-proteins are alternately regulated by GAP and GEF proteins by a molecular switching mechanism (Adapted from Biorender.com).

Preliminary studies have shown that pathogenic bacteria manipulate the host GTPase pathways for their own benefit through the development of a unique subset of effector proteins exhibiting eukaryotic motif mimicry. This motif was first identified by Alto *et al*. (2006) to be present in the effector proteins of some pathogenic bacteria and is characterized by an invariant sequence that includes the tryptophan (W) and glutamate (E) residues hence the signature WxxxE motif. They demonstrated that substituting tryptophan or glutamic residues within the WxxxE motif with alanine resulted in a significant loss of function in the protein (Alto et al., 2006). Following this discovery, the Das lab categorized these bacterial effectors into a single family of WxxxE effector proteins that are present in pathogenic bacteria but not in commensals (Figure 3) (Sayed et al., 2021). At the time, these effectors were believed to closely mimic small GTPases to activate signaling cascades within host cells. However, recent studies examining the WxxxE protein structure questioned this notion of GTPase mimicry (Huang et al., 2009). These studies demonstrate that pathogenic bacterial effector proteins have a GEF-like fold resembling that of *Salmonella* SopE effector despite sharing no sequence homology (Huang et al., 2009). SopE had previously been documented as the first bacterial GEF mimic that induces membrane ruffling during bacterial invasion through activation of host GTP-binding proteins—Cdc42 and Rac-1 (Hardt et al., 1998). This development led to the discovery of additional bacterial GEFs that were genetically similar to SopE, which includes BopE (*Burkholderia psuedomallei*), CopE (*Chromobacterium* violaceum), and SopE2 (*Salmonella* spp.). Thus far, these bacterial effectors were the only identified Rho GEFs found in enteric pathogens. Further studies by Huang et al. reported direct evidence of the first three WxxxE effector proteins Map, IpgB1, and IpgB2 to function as GEFs for Rho-GTPases (Huang et al., 2009). Their data revealed that the bacterial GEF protein structure is a genetically inherited component that is utilized by some enteric pathogens to

trigger GTPase signaling cascades within host cells (Huang et el., 2009). For instance, crystal structure analysis confirmed that IpgB1 recognizes β2-3 residues of Cdc42 and Rac1 GTPases to stimulate the activation of these proteins through GDP-GTP exchange (Huang et al., 2009). Crystal structures and functional studies of Map showed that it directly binds to Cdc42 and has a structure like that of SopE. Despite the low sequence homology between SopE and Map, these proteins induce a similar conformational change in Cdc42, which indicates a common mechanism of nucleotide exchange (Huang et al., 2009). Figure 4 is a 3D protein structure generated by Orchard & Alto (2012), which illustrates the general similarities of guanine nucleotide exchange between SopE and WxxxE effector proteins. Numerous functional studies have investigated the WxxxE effector GEF mimics to gain a better understanding of their function. The function of these proteins can differ vastly among various pathogens, but how these effectors collectively coordinate their effects on the host cell is still being determined. Nonetheless, it is known that many of these effector proteins remodel numerous cellular processes to survive and replicate within the host.

Figure 3. Multiple sequence alignment of bacterial effector protein family with a conserved WxxxE motif. A BLAST search of amino acid sequences in enteric pathogenic bacteria that share a common amino acid sequence Trp-xxx-Glu or WxxxE motif (Retrieved from Sayed *et al.*, Gut Microbes 2021).

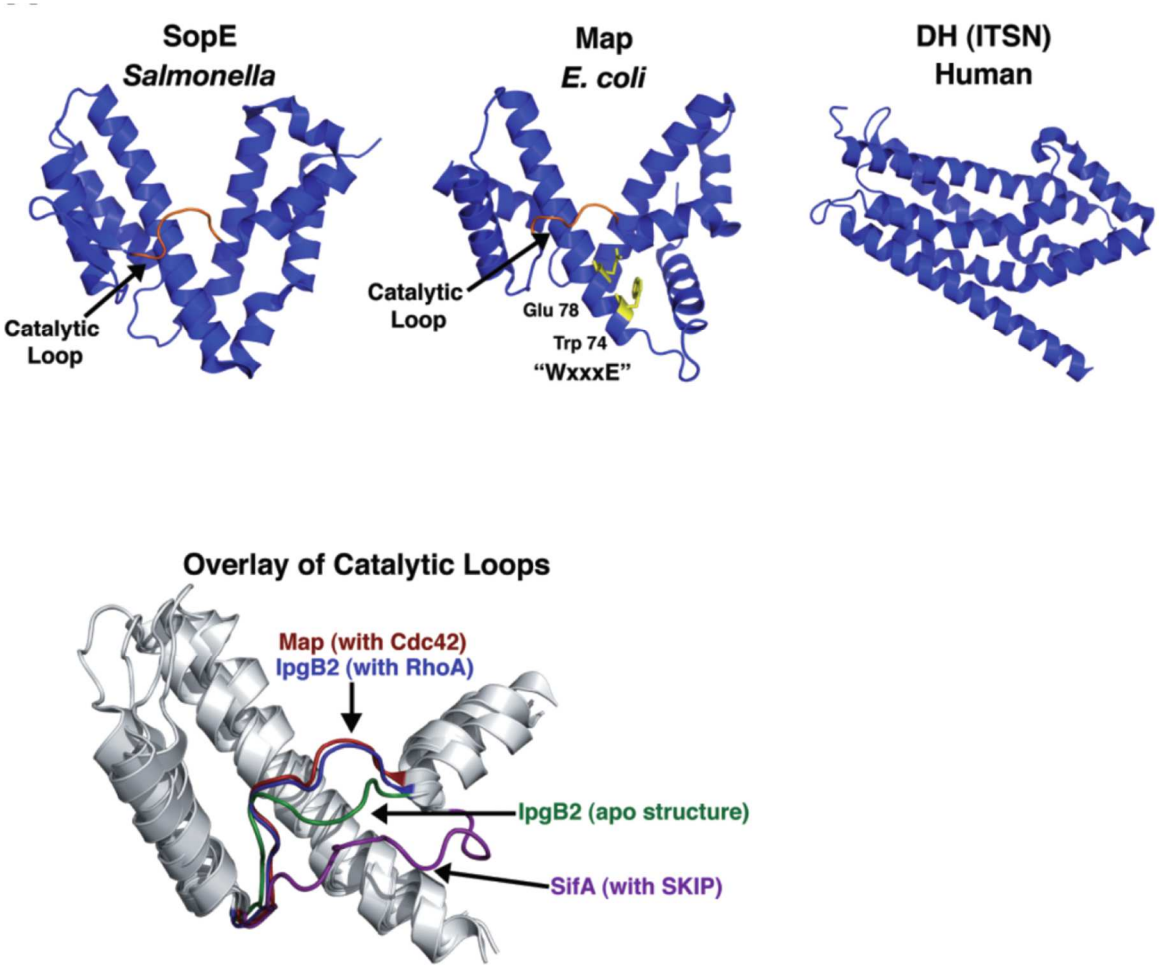

Figure 4. Comparison of GEF mimics and SopE tertiary structure. Image depicts the catalytic loops (orange) and of SopE and Map with the side chain of the Trp (W) and Glu (E) of WxxxE motif is highlighted in yellow. The GEF constructs were overlayed to depict the different loop positions in each structure—Map in complex with Cdc42 (red), IpgB2 in complex with RhoA (blue), and SifA with SKIP (purple) (Retrieved from Orchard & Alto, Cellular Microbiology 2012).

Chapter 2. Manipulation of host cell pathways by WxxxE effector Proteins

2.1 Enteric pathogens secrete effector proteins for entry and survival in the host cell

When an enteric pathogen enters the host and encounters the epithelial cells of the colon, the pathogen will depend on its T3SS to secrete bacterial effector proteins (Ehrbar et al., 2003). These proteins are expressed by genes that fall within large gene cassettes known as pathogenicity islands (PAI) (Ehrbar et al., 2003). The enteric pathogen first invades the epithelial cells by injecting one or more effector proteins to allow the bacteria to permeate the host tissue (Green & Mecsas, 2016). Once the pathogen has crossed the epithelial lining, the bacterium is then engulfed by macrophages in the lamina propria, which are thin layers of connective tissue beneath the epithelium that form part of the mucous membrane (Ribet & Cossart, 2015). As it enters the lamina propria, the pathogen encodes for another set of effector proteins that enables them to polymerize and depolymerize actin filaments (Ribet & Cossart, 2015). The secretion of the WxxxE effectors plays a key role in pathogenesis leading to numerous events including cytoskeleton rearrangement, vacuole or tubule formation, regulation of endo-lysosomal signaling, disruption of tight junctions, triggering apoptosis and the regulation of inflammation. These functions associated with WxxxE effectors depend on the bacterium and its mode of infection, illustrated in Figure 5 and Table 1 and will be further discussed in this paper.

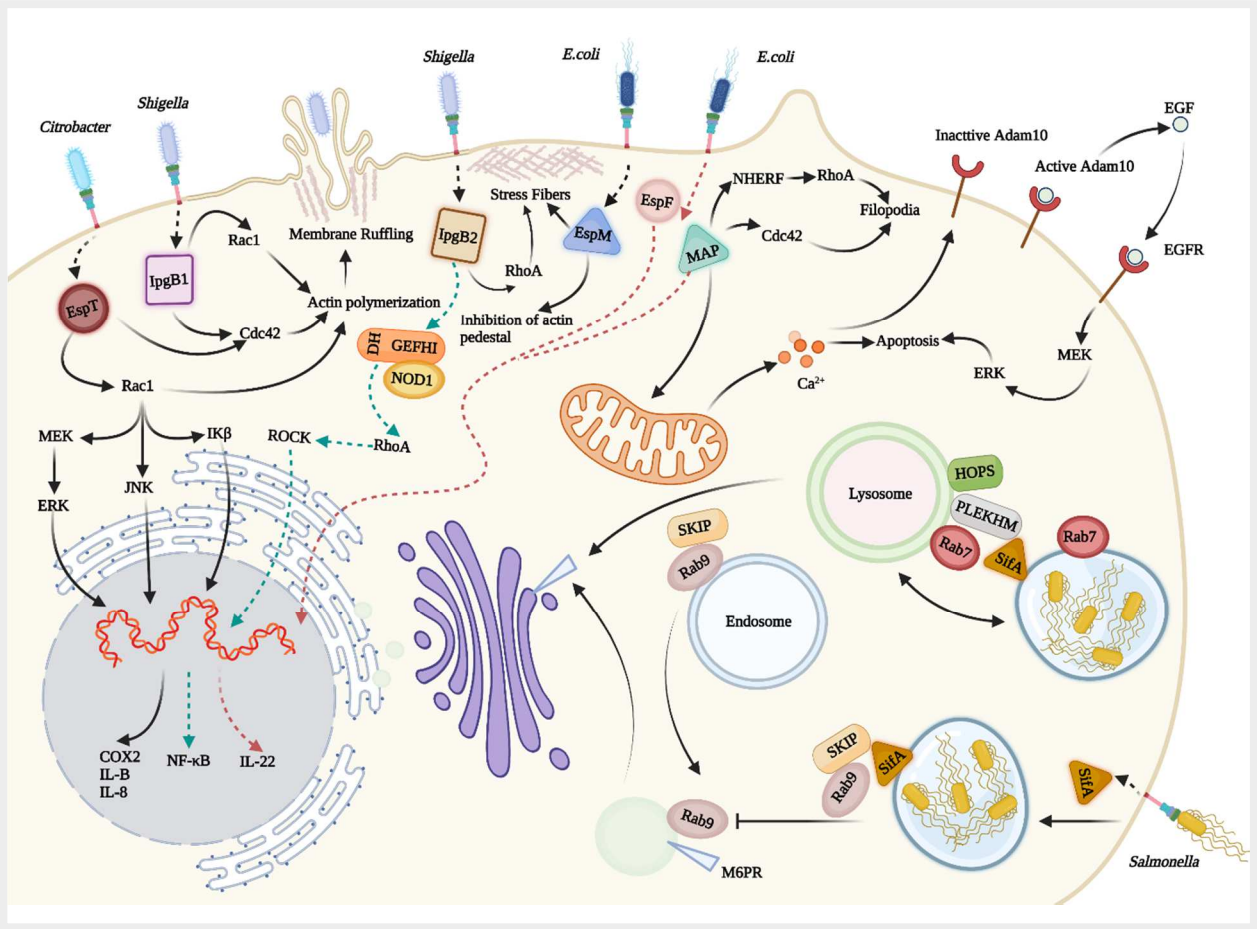

Figure 5. Manipulation of host-cell pathways by WxxxE effector proteins. An overview of enteric infection by *Shigella*, EPEC/EHEC, *Citrobacter rodentium*, and *Salmonella,* which secrete WxxxE effector proteins such as IpgB1/2, EspT/Map/EspM, and SifA respectively using the T3SS. IpgB1/2, EspT/Map/EspM activate Rho GTPases via Rac1, RhoA, and/or Cdc42, resulting in cytoskeleton rearrangement to enter the host cell. *Salmonella* enters host epithelial cells via secretion of SPI1-T3SS proteins and resides within SCV. The effector SifA is secreted by *Salmonella* to induce Sif formation. SifA interacts with host proteins—Rab7, HOPs, PLEKHM1, and LAMP1 to prevent bacterial degradation. SifA-SKIP complex interacts with Rab9 to disrupt vesicular trafficking of mannose 6 phosphate receptors (M6PRs) to the Trans-Golgi network (TGN). The WxxxE effectors induce inflammatory cytokines COX□2, IL-8, IL-1β and PGE2 through Erk, JNK and NF□κB pathways. Map stimulates IL-22 through an identified pathway (Created with Biorender.com; Adapted from Achi *et al*., Critical Reviews in Microbiology, 2021).

Table 1. The function of enteric bacterial effector proteins with a shared WxxxE motif

Bacterial Effector	Bacteria Species	Amino Acid Sequence	Function	Host Target	References
SifA	*Salmonella* Typhimurium, *Salmonella enterica*	KGHLDGWKAQLKATYLA	a) maintain the integrity of the *Salmonella*-containing vacuole (SCV), induce tubulation of the *Salmonella* phagosome forming *Salmonella*-induced filaments (SIF) b) recruits the host vesicle fusion machinery to gain access to nutrients and membranes from the late endocytic compartments and build its replicative niche inside the host cells.	Act as GEF and interact with Rho-family GTPases (Rho A, B,C) binds the mammalian kinesin-binding protein SKIP and PLEKHM2 SifA-SKIP complex interact with HOPS (HOmotypic fusion and Protein Sorting)	(Boucrot et al., 2005; Dumont et al., 2010; Garcia-del Portillo et al., 1993; McEwan et al., 2015; Ohlson et al., 2008; Sindhwani et al., 2017)
	Salmonella enterica serovar Typhi	KGNLDEWKTQLKATYLA			
	Salmonella enterica	KGILDEWKTQLKVTYLA			
	Yersinia frederiksenii	GDKFDMWKKELRTTYLS			
SifB	*Salmonella* Typhimurium, *Salmonella enterica*, *Salmonella enterica* serovar Typhi and *Salmonella enteritidis*	KGNLCDWKEQLRKAAIS	localize to the *Salmonella* containing vacuole (SCV) membrane LAMP1- positive structures. may have redundant action with other bacterial effectors	Act as GEF and interact with Rho-family GTPases	(Freeman et al., 2003; Ohlson et al., 2008)
Map	*E. coli*	TKDINGWIKDLRIVYPS	a) Map induces actin-based filopodia b) regulate the cellular process, programmed cell death (apoptosis), alter mitochondria morphology when imported into mitochondria c) Interact with human intestinal mucosa- brush border remodeling and formation of attaching and effacing lesions d) mediate uptake of EPEC into non-phagocytic cells	Act as GEF, interact with GTPases (CDC42) Interact with F-actin to control cdc42 signal interact with PSD-95/Disk-large/ZO-1 domain 1 (PDZ1) of EBP50/NHERF1 and NHERF2 interact with EbP50 of cell surface microvilli	(Alto et al., 2006; Huang et al., 2009; Jepson et al., 2003; Martinez et al., 2010; Orchard et al., 2012; Shaw et al., 2005; Simpson et al., 2006)
	E. coli EHEC O157:H7	TKDIDEWIKDLRIVYPS			
	Citrobacter rodentium	NGDTQQWFRQLQITFIS			
	E. coli EPEC	SSDTQQWFKQLQITFLS			
	EHEC	SSDTQQWFKQLQITFLS			
	E. coli	SGDTQQWFQQLQTTYIS			
	E. coli EHEC O157:H7	NGDTQQWFQQLQTTYIS			
	E. coli	SGDTQQWFKQLQITYIS			
EspT	*E. coli*	EGKMNEWMRELCICFVS	modulates the host cell cytoskeleton, triggers the formation of lamellipodia, membrane ruffles and mitochondrial localization. plays a role in production of the immune mediators (IL1β, IL8 and PGE2)	Act as GEF and interact with Rac1, cdc42 and activates signal-regulated kinases (Erk), JNK and NF-κB.	(Bulgin et al., 2009; Raymond et al., 2011)
	Citrobacter rodentium	EGKMNEWMRELCICFVS			
	E. coli	SGDTQQWFQQLQTTYIS			
EspM	*E. coli* (EHEC, EPEC) *Citrobacter rodentium*	TKDIDEWIKDLRIVYPS	Remodeling actin of mammalian cells, Delocalization of tight junction (TJ), modulate additional cellular pathways EspM2 Interact with other bacteria effector EspO1-2 to stabilize focal adhesion formation during EHEC infection	EspM2 & EspM3 are RhoA and ROCK-dependent EspM2 & EspM3 phosphorylate colifin	(Arbeloa et al., 2008; Arbeloa et al., 2010; Morita-Ishihara et al., 2013)

Table 1. The function of enteric bacterial effector proteins with a shared WxxxE motif (continued)

Bacterial Effector	Bacteria Species	Amino Acid Sequence	Function	Host Target	References
IpgB1	*Shigella flexneri*	GNQLFCWMSQERTTYVS	Stimulate actin rearrangement (lamellipodia, stress fibers and filopodia) played a role in bacteria uptake by non-phagocytic cells Target the bacteria to the host cell	Act as GEF and interact with Rac1, cdc42 Also, IpgB1 interacts with the ELMO1-Dock180 pathway (mimic RhoG activity). Interact directly with acidic phospholipids	(Alto et al., 2006; Costa and Lesser, 2014; Ohya et al., 2005; Weigele et al., 2017)
	E. coli	SGDTQQWFKQEQITYIS			
	Shigella flexneri	GNQLFCWMSQERTSYVS			
	E. coli	GDQLFCWMSQERTSYVS			
IpgB2	*E. coli*	TKDIHGWVSDERTVYPS	Stimulate actin rearrangement (lamellipodia, stress fibers and filopodia)	Act as GEF and interact with Rac1, cdc42 and Rho A activate NF-κB by a mechanism that depends on both NOD1 and GEF-H1 and requires ROCK activation	(Alto et al., 2006; Fukazawa et al., 2008)
	Shigella flexneri *E. coli*	GENITDWKNDEKKVYVS			
TrcA	*EPEC*	TKDINGWIKDERIVYPS	Remodel actin in the mammalian host cell, induce localized parallel stress fibers like EspM1 Plays a role in bacteria cytosol by binding to bfpA protein pili and outer membrane intimin		(Arbeloa et al., 2008)

2.2 WxxxE effectors manipulate the host cytoskeleton to promote bacterial invasion and movement

The cytoskeleton is a dynamic network of filamentous proteins known as the actin filaments, intermediate filaments, and microtubules that are present in the cytoplasm of all cells (Fletcher & Mullins). It helps maintain the shape and internal framework of the cell, as well as providing mechanical support that allows the cell to perform necessary functions like cell division and movement (Fletcher & Mullins). For this reason, pathogenic bacteria use bacterial effectors to exploit the host cytoskeleton to support their own survival, replication, and dissemination.

Initial studies have revealed several WxxxE effector proteins, including IpgB1, IpgB2, EspM1, EspM2, EspM3, EspT, and Map hijack host cytoskeletal components such as microtubules and actin filaments (Alto et al., 2006). For example, IpgB1 secreted by *Shigella* induce membrane ruffling for entry into epithelial cells (Ohya et al., 2005). Previous studies have demonstrated IpgB1's contribution to the formation of lamellipodia through activation of Cdc42 and Rac1 GTPases, whereas IpgB2 directly binds and activates RhoA GTPase to form actin stress fibers (Huang et al., 2009; Klink et al., 2010). IpgB1 promotes bacterial entry by translocating the ELMO1/DOCK180 complex to the membrane to activate Rac1 (Handa et al., 2007). EspT secreted by *Citrobacter rodentium* was found to have a similar phenotype to IpgB1 in which EspT triggers formation of membrane ruffling through the activation of Rac1 and cdc42 to facilitate bacterial invasion. While there are morphological similarities between IpgB1 and EspT, the mechanisms by which they induce cytoskeleton rearrangement is distinct—EspT does not form membrane ruffles and lamellipodia through the ELMO1-DOCK180 pathway as shown in IpgB1 (Bulgin et al., 2009). Moreover, the secretion of Map by Enteropathogenic E.coli (EPEC) induces the formation of filopodia in a Cdc42-dependent but Rac1-independent manner (Jepson et al., 2003). This was

further confirmed in another study, which concluded that Map does indeed activate Cdc42 (Berger et al., 2009). Furthermore, Map was shown to bind to the PDZ1 of host protein NHERF1 through its carboxy terminal PDZ ligand motif (Berger et al., 2009). This leads to filopodia formation through the recruitment of activated ezrin and in turn, the activation of the RhoA-ROCK pathway (Berger et al., 2009). Although the formation of filopodia involves the activation of Cdc42 by Map, the Map-NHERF1 complex, ezrin and the RhoA-ROCK pathway is needed for the stabilization of filopodia (Berger et al., 2009). In a similar manner to IpgB2, the translocation of EspM effectors into the host cell secreted by EPEC and *Enterohemorrhagic E.coli* (EHEC) resulted in the formation of stress fibers in a RhoA and ROCK dependent manner (Arbeloa et al., 2008). EspM1 effector protein was found to induce formation of localized parallel stress fibers while EspM2 triggered formation of global parallel stress fibers. In addition to this, EspM3 was shown to stimulate formation of localized radial stress fibers. The activity of EspM2 and EspM3 is RhoA and ROCK-dependent where both effectors activate RhoA and trigger phosphorylation of Cofilin, a downstream ROCK target (Arbeloa et al., 2008). The EspM effectors trigger the formation of actin pedestals simultaneous to the formation of stress fibers, which support the bacteria on the surface of epithelial cells (Arbeloa et al., 2008).

2.3 Regulation of the host inflammatory response by WxxxE effectors

When a bacterium enters the host cell, it is detected by macrophages (Figure 8) in the early phase of an immune response (Ribet et al., 2015; Das et al., 2015). Macrophages express pattern recognition receptors (PRRs) that sense foreign ligands known as pathogen associated molecular patterns (PAMPs) (Das et al., 2015). These foreign ligands are molecular motifs conserved within a group of microbes (Mogensen, 2009). Once a pathogen has been detected, PRRs induce the activation of downstream signaling cascades in the macrophage resulting in cytokine production

(Das et al., 2015). Even so, enteric pathogens can disrupt the host immune response through the secretion of T3SS WxxxE effectors and their mechanistic role on the host inflammatory response still remains to be elucidated.

In a recent study, Das *et al*. identified Brain Angiogenesis Receptor 1 (BAI1) as a PRR that binds to lipopolysaccharides (LPS) of gram-negative bacteria (Das et al., 2015). Upon binding, BAI1 will recruit engulfment and cell motility protein 1 (ELMO1), a cytosolic protein that interacts with the dedicator of cytokinesis 180 (DOCK180) as shown in Figure 9 (Das et al., 2015). This interaction forms the ELMO1/DOCK180 complex, which activates the small Rho GTPase Rac1 (Ras-related C3 botulinum toxin substrate 1). The activation of Rac1 ultimately leads to the reorganization of the actin cytoskeleton of the phagocyte to surround the bacteria in a membrane compartment (Das et al., 2015).

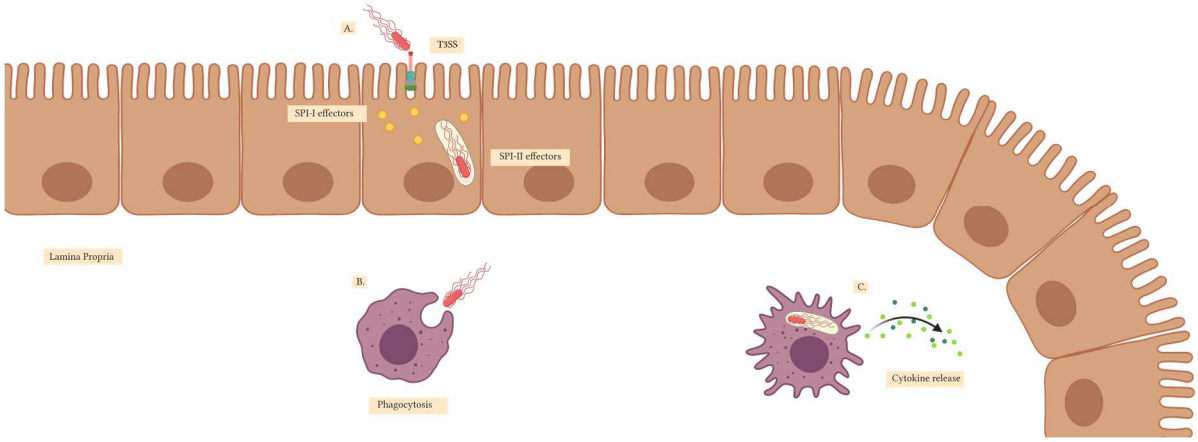

Figure 6. *Salmonella* entry into the host cell. (A) *Salmonella* employs T3SS to inject SPI-I effector proteins, promoting invasion into the host epithelial cell. Upon entry, *Salmonella* will express SPI-II effector proteins to increase bacterial survival by forming the SCV. (B) In the Lamina Propria, *Salmonella* will encounter macrophages where they will be engulfed via phagocytosis. (C) Once engulfed, the macrophage will secrete cytokines to activate the host inflammatory response. Internalized *Salmonella* will again release SPI-II effectors to evade immune response (Created with BioRender.com).

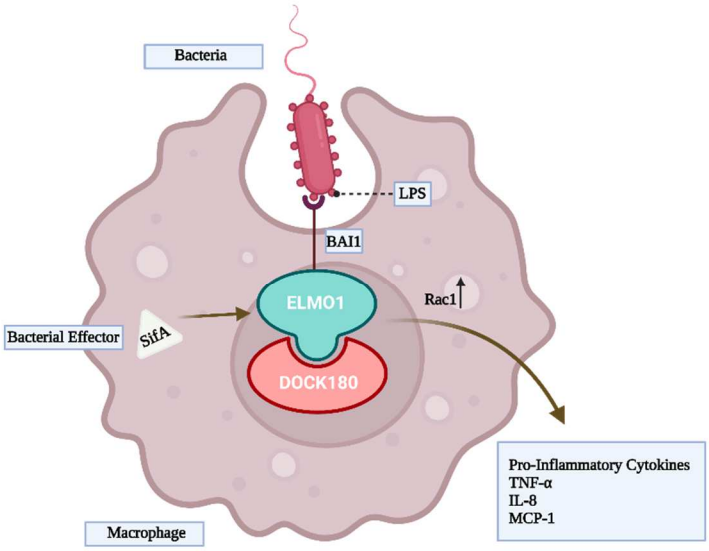

Figure 7. BAI1/ELMO1 mediated engulfment of bacteria through recognition of LPS. Detection of gram-negative bacteria by phagocytes are mediated through the interaction between BAI1 and bacterial LPS. This interaction results in the recruitment of ELMO1 and DOCK180 to the engulfment site. ELMO1 and DOCK180 form a complex in which they act as a bipartile guanine nucleotide exchange factor for Rac1 GTPase, ultimately leading to cytokine release (Created with BioRender.com).

Previous research has shown that ELMO1 assists macrophages in sensing pathogens and commensals to predict pathogenic infection through differential regulation of the host immune response (Sayed et al., 2021). For this reason, the Das lab wanted to determine whether any bacterial effector proteins can interact with ELMO1. They demonstrated that the WxxxE motif in the bacterial effector SifA interacts with the C-terminal end of the host protein ELMO1; mutations in the active site within the WxxxE motif abolish interactions with host protein ELMO1 (Figure 8). An immunopulldown with EGFP-conjugated beads confirmed the specificity of this interaction by pulldown with GFP-tagged SifA wild type or mutant and purified His- tagged ELMO1 (Sayed et al., 2021). To determine if ELMO-1 interacts with WxxxE containing effectors—IpgB1/2 and

Map, a GST pulldown assay was performed using GST-IpgB1, GST-IpgB2, and GST-MAP with purified His-ELMO1 full length (FL). Figure 9.1 confirms that the WxxxE effectors interact with host proteins ELMO1 identified by the presence of a band (Sayed et al., 2021). To further assess the overexpression of these effectors in control (C1) and ELMO1-depleted (E1) murine macrophages, they transfected C1 and E1 cells with GFP-tagged effectors including SifA, IpgB1, IpgB2, and Map. The overexpression of GFP-bacterial effectors was confirmed with immunoblotting and the interaction of the effectors with ELMO1 was specific as ELMO1 was absent in the E1 macrophages (Figure 9.2). The Das lab further investigated the relevance of this interaction in regard to the host inflammatory response. C1 and E1 cells were transfected with bacterial effector proteins and stimulated with LPS to determine host response based on cytokine production (Figure 9.3). TNF-α and IL-6 cytokine production were greater in control cells compared to ELMO1-depleted cells. Their results indicated that ELMO1-WxxxE effector protein interaction is crucial in regulating the host inflammatory response.

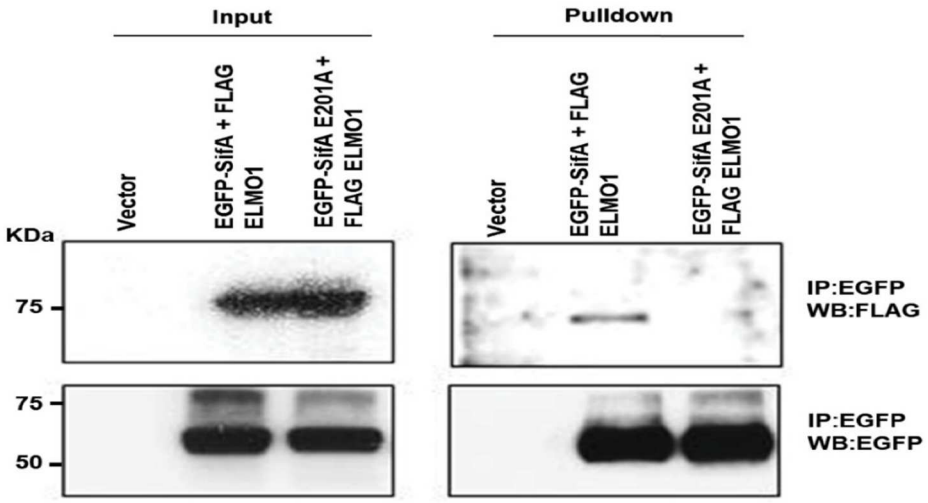

Figure 8. Mutations in the conserved WxxxE motif abolish ELMO1-SifA interaction. HEK293T cells were transfected with FLAG-ELMO1 in combination with plasmids expressing EGFP-SifA and EGFP-SifA-E201. Lysates were subjected to pulldown with EGFP antibody conjugated with A/G agarose beads. Samples were analyzed by immunoblotting with Abs against FLAG (Retrieved from Sayed *et al.*, Gut Microbes 2021).

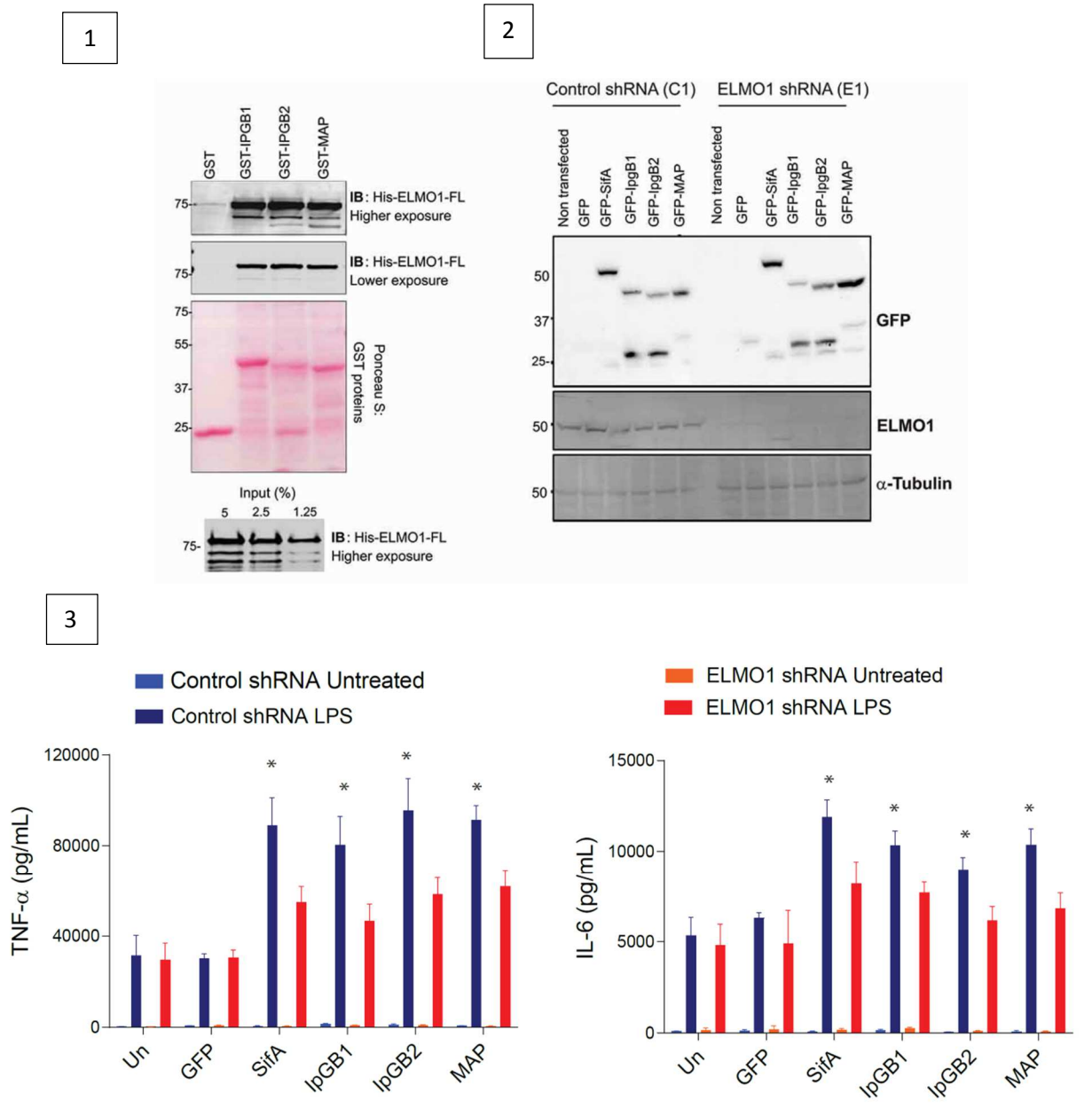

Figure 9. WxxxE effectors regulate host inflammatory response in phagocytes. (1) GST pulldown assay with GST, GST-IpgB1, GST-IpgB2, or GsT-Map was used with recombinant His-ELMO1 full length (FL). Equal loading of GST proteins was confirmed with Ponceau S staining. Lower panel indicates the input of His-ELMO1 FL. (2-3) Control (C1) or ELMO1 (E1) shRNA J774 cells were transfected with GFP vector—GFP-SifA, GFP-IpgB1, GFP-IpgB2, and GFP-Map. (3) Cells were then stimulated with bacterial LPS. After infection, supernatants were collected for cytokine production for TNF-α or IL-6 (Retrieved from Sayed *et al.*, Gut Microbes 2021).

In addition to this, Matsuda *et al.* observed Salmonella enterica serovar Typhimurium infection in the ceca of streptomycin pretreated mice (Matsuda et al., 2019). They identified five T3SS-2 effectors including SifA that are associated with T3SS-1 -independent macrophage cytotoxicity. Together, their results suggested that SifA is the most significant of the five effectors to stimulate macrophage proliferation and cytotoxicity (Matsuda et al., 2019).

In another study, the ability of *Shigella* IpgB1 to induce keratoconjunctivitis in the corneal tissue cells of the guinea pig was tested using the Sereny infection model (Hachani et al., 2008). They discovered that the intensity of infection with the IpgB1 mutant was much greater than compared to the wild type, whereas IpgB2 mutant was similar to the wild type. The differences in the degree of infection induced by IpgB1 and IpgB2 mutants indicate the variation in the functions of these effectors during pathogenesis. In the murine pulmonary model, IpgB1 and IpgB2 double mutant was significantly attenuated with a reduced inflammation score compared to the wild type. This finding is likely due to the mutant's inability to invade host cells (Hachani et al., 2008). Additionally, Fukazawa *et al*. (2008) discovered that IpgB2 activates NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells), which requires the detection of PAMPs by GEF-H1 (guanine nucleotide exchange factor H1) and NOD1 (nucleotide binding oligomerization domain containing 1) as well as the activation of the RhoA-ROCK pathway for cell invasion.

EspT secreted by *Citrobacter rodentium* was found to induce expression of pro-inflammatory mediator cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines interleukin-8 (IL-8) and IL-1β in the U937 human monocyte cell line (Raymond et al., 2011). The induction of these inflammatory molecules involves the activation of NF-κB, Jun N-terminal kinase (JNK), and extracellular-signal-regulated kinase (Erk,1/2) pathways, which control cell proliferation, inflammatory responses, and so on (Raymond et al., 2011). In order to determine if the WxxxE

motif is needed for release of inflammatory mediators by EspT, Tryptophan and Glutamine residues were substituted with Alanine and Tyrosine respectively (Raymond et al., 2011). Mutations in these putative active sites showed drastically reduced inflammatory cytokine production. Furthermore, in vivo experiments using *Citrobacter rodentium* revealed that EspT triggers expression of Keratinocyte chemoattractant (KC) and Tumor Necrosis Factor alpha (TNF-α) inflammatory mediators (Raymond et al., 2011).

A recent study by Ruano-Gallego *et al*. (2021) discovered that the T3SS WxxxE effectors form a robust intracellular network instead of operating separately. Using *C.rodentium* as a model organism, they found that the secretion of GM-CSF (granulocyte macrophage colony stimulating factor) and interleukin 6 (IL-6) was affected by the concurrence of Map with other effector proteins (Ruano-Gallego et al., 2021). GM-CSF is an immune-modulatory cytokine, that is involved in stimulating granulocyte and macrophage populations from precursor cells with a protective effect in enteric diseases, while IL-6 is a pleiotropic proinflammatory cytokine that plays a role in acute phase responses, hematopoiesis, and immune reactions (Bhattacharya et al., 2015; Tanaka et al., 2014). Further examining Maps effect on host immunity, Ruano-Gallego and his team found that the combined contribution of Map and EspF activate IL-22 responses (Ruano-Gallego et al., 2021).

2.4 The host engulfment pathway

Following the activation of Rac1 via ELMO1-DOCK180 complex, the macrophage pushes out its membrane to surround the bacterium. Once the bacteria are internalized by a macrophage, the fate of the bacterium depends on its virulence factor or its ability to infect the host (Figure 10). Non-pathogenic bacteria are internalized into a compartment known as the early phagosome, which are formed through the invagination of the plasma membrane (Uribe-Querol & Rosales, 2020). Soon this compartment undergoes swift maturation into a late phagosome through a

succession of membrane trafficking events (Uribe-Querol & Rosales, 2020). The phagosome then fuses with the lysosome that contain different hydrolytic enzymes to form the phagolysosome, where the internalized bacteria are eventually degraded (Uribe-Querol & Rosales, 2020). However, pathogenic bacteria such as *Salmonella* can avoid this fate by disrupting the trafficking mechanisms to survive and replicate inside the macrophage (Liss et al., 2017). The internalized pathogen redirects the maturation of the phagosome to protect itself within the modified phagosome known as the *Salmonella*-containing vacuole (SCV) (Liss et al., 2017). As the pathogen replicates inside the SCV, the many features of the endosome including acquiring early and late endosomal markers and maintaining an acidic pH is essential for the survival of *Salmonella* (Sarkar et al., 2017). In addition to this, *Salmonella* can block the fusion of the lysosome with the SCV through its interaction with endocytic components to prevent degradation of the bacterium (Sarkar et al., 2017).

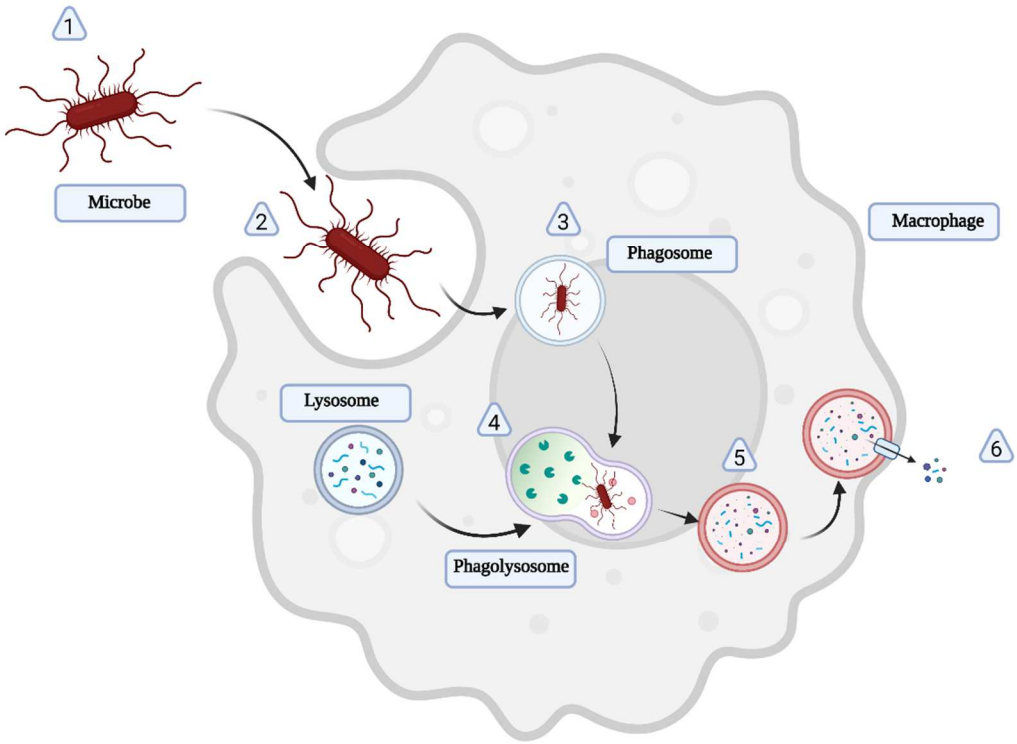

Figure 10. Phagocytosis of microbes. 1. Microbe is detected by macrophage 2. Upon detection, microbe is engulfed by phagocyte 3. The macrophage encloses the microbe in an invaginated vesicle known as the phagosome 4. The phagosome fuses with the lysosome to form the phagolysosome 5. Microbe is digested by hydrolytic enzymes 6. Digested contents are discharged through exocytosis (Created with BioRender.com).

2.5 WxxxE effectors manipulate host endocytic components to form modified vacuoles

During pathogenesis, *Salmonella* avoids degradation and replicates within the SCV by secreting bacterial effector proteins within the SPI-2 (*Salmonella* pathogenicity island 2) throughout endosomal maturation (Sarkar et al., 2017). Among the bacterial effector proteins, SifA is important for the integrity of the SCV, acquisition of the host lysosome associated membrane protein 1 (LAMP1), and the formation of *Salmonella* induced filaments (Sifs), which is a network of filaments that extend from the SCV (Knuff & Finlay, 2017; Zhao et al., 2015). At the beginning of infection, *Salmonella* converts the host endosome into the SCV and contains certain characteristics of the endosome. As the SCV matures, it loses the early endosomal markers and acquires late endosomal markers such as the lysosomal glycoprotein LAMP1 to allow Salmonella to proliferate within the SCV (Madan et al., 2012; Méresse et al., 1999; Steele□Mortimer et al., 1999). Studies have shown that among the bacterial effector proteins secreted by *Salmonella* during infection, SifA is important for the integrity of the SCV and the formation of Sifs. During phagocytosis, the normal maturation of the endosome is regulated by a family of small GTPases that are essential in the trafficking of membrane proteins known as Rab GTPases (Yeo et al., 2016). Rab GTPases are small GTPases that regulate several membrane trafficking events (Yeo et al., 2016). Inevitably, *Salmonella* will target these Rab GTPases to regulate and inhibit their function for survival (Spanò & Galán, 2017). Rab GTPases, such as Rab7, were previously shown to be targeted by SifA through PLEKHM1 (Pleckstrin homology domain-containing protein family member 1) to recruit membranes along Sifs for proper SCV formation (McEwan et al., 2015; Zhao et al., 2015).

2.6 WxxxE effectors hijack host endocytic components to ensure bacterial survival

Salmonella manipulates the host endocytic machinery by reorganizing late endosomes and lysosomes into a network of tubules or Sifs (Sindhwani et al., 2017). This allows the pathogen access to membrane components and fluid-phase cargo that is essential for the integrity of the SCV and provides intravacuolar nutrients for its survival (Sindhwani et al., 2017). Several T3SS effectors produced by *Salmonella* are involved in SCV maturation and Sif formation (Sindhwani et al., 2017). *Salmonella* mutants lacking SifA have been shown to be highly attenuated during infection and replication (Sindhwani et al., 2017). Functional analysis reveals that SifA is pivotal in SCV integrity and Sif formation. A mutational study of the putative active sites of SifA confirmed that the WxxxE motif is important for maintaining the tertiary structure and function of the effector protein (Diacovich et al., 2009). The N-terminal of SifA interacts with the pleckstrin homology domain of the host protein SKIP (SifA and kinesin-interacting protein), and in turn the SifA/SKIP complex regulates the motor protein, kinesin-1 (Diacovich et al., 2009; Sindhwani et al., 2017). Kinesin-1 is an anterograde motor protein that transports cargo along microtubules towards the cell periphery (Angerani et al., 2021).

Furthermore, SifA interacts with an endocytic protein that has a similar domain architecture as SKIP--PLEKHM1 which regulates SCV formation and proliferation of *Salmonella* (McEwan et al., 2015). SifA was also found to interact with homotypic fusion and protein sorting (HOPS), a multisubunit tethering complex that allows *Salmonella* to obtain a constant supply of nutrients and membranes to sustain the integrity of the SCV (Sindhwani et al., 2017; McEwan et al., 2015). As SifA forms a complex with host protein SKIP, it interacts with the HOPS complex to localize HOP to the SCV, enabling fusion with lysosomes (Sindhwani et al., 2017). Previous research has shown that the SifA-SKIP complex leads to the sequestering of Rab9 to increase survival of *Salmonella*.

SifA acts as an antagonist to RAB9 by binding to the pleckstrin homology domain of SKIP to prevent its interaction with Rab9 (Jackson et al., 2008). Sequestering of Rab9 leads to a decrease in lysosomal activity since Rab9 is involved in trafficking of lysosomal enzymes through mannose 6 phosphate receptors (M6PRs) from the Trans-Golgi network (TGN) to the lysosome (Jackson et al., 2008). As a result of the absence of lysosomal enzymes, bacterial clearance is significantly impaired in the phagocytes.

2.7 WxxxE effectors disrupt tight junctions to invade host cells

The TJs of the intestinal epithelium acts as a defensive barrier against invading microbes (Fukazawa et al., 2008; Zuo et al., 2020). They are multiprotein complexes consisting of transmembrane proteins, scaffolding proteins, and signaling molecules that aid in the host immune response upon disruption of the epithelial barrier (Fukazawa et al., 2008). Enteric pathogens interaction with polarized epithelial cells via secreted effector proteins, can lead to redistribution of tight junction (TJ) associated proteins. In order to colonize the intestinal epithelium and exploit host cell components, *Shigella flexneri* specifically target TJs to subdue the epithelial barrier to obtain basolateral membrane components (Sakaguchi et al., 2002, Singh et al., 2018). *S.flexneri* will consume TJ associated transmembrane proteins such as claudin1 and alter the expression of zonula occludins—ZO1 and ZO2 as well as E-cadherin and dephosphorylate occludin (Sakaguchi et al., 2002).

The pathway by which *Shigella* bacterial effectors, IpgB1 and IpgB2, disrupt TJs have yet to be identified but it is thought to involve its ability to mimic Rac and Rho respectively (Hachani et al., 2008). Other effector proteins such as Map and EspF secreted by EPEC and EHEC have been involved in the distribution of modified TJ proteins resulting in the production of diarrhea (Ugalde-Silva et al., 2016; Singh et al., 2018). Map and EspF inhibits TJ assembly by preventing

the recruitment of TJ proteins and constitutive expression of these effectors exhausted TJ protein levels (Singh et al., 2018). Map was shown to downregulate claudin-1 transcripts whereas EspF down-regulated claudin-1, occludin, and ZO1 transcript levels (Singh et al., 2018). The reduction of TJ protein by Map and EspF were caused by the lysosomal degradation of these proteins (Singh et al., 2018).

2.8 The induction of apoptosis by WxxxE effectors to subdue host response

Apoptosis is the process of programmed cell death that is controlled in the development of multicellular organisms (Alberts et al., 2002). The activation or suppression of cell death is an important factor in the aftermath of pathogenic infection (Ashida et al., 2011). Pathogen-induced regulation of apoptosis may function in the ability of the pathogen to evade host defenses that can limit the spread of disease (Ashida et al., 2011). Thereby, regulation of the host cell death pathway by bacterial effectors have shown to promote the propagation of pathogenic bacteria (Ashida et al., 2011). A recent study demonstrated Maps ability to stimulate apoptosis by targeting the host mitochondria (Ramachandran et al., 2020). This process results in the disruption of the host membrane and an efflux of Ca^{2+} ions and in turn activates ADAM10 sheddase and the release of epidermal growth factors (Ramachandran et al., 2020). The release of growth factors triggers the ERK and p38 MAPK cascade, inevitably leading to apoptosis (Ramachandran et al., 2020).

While it is known that pathogens produce WxxxE effectors during pathogenesis, the mechanisms by which these pathogens hijack the host signaling pathways and the key components that are involved in these pathways has yet to be determined. Currently, the Das lab is analyzing intracellular bacterial proteome to study the pathogen's ability to adapt to its host environment using *Salmonella* infection of murine macrophages as a model system. In this study, we have identified and analyzed unique proteins in host cells that were upregulated or downregulated upon

bacterial infection. Many of these proteins are implicated in the endolysosomal and glycolytic pathways, control membrane and vesicle transport, as well as regulation of antigen processing. In addition to this, the Das lab is using a unique method known as BioID to identify candidate host proteins that interact with the WxxxE effectors and ELMO1. Although this study is currently in progress, this approach can provide a new perspective on the host-pathogen interaction and can be applied to various microbes to further our understanding of the functional relevance of the WxxxE effector interaction with host proteins.

Chapter 3 . The conserved WxxxE motif identified in T2SS/T4SS substrates secreted by non-enteric pathogens

While the WxxxE motif was previously identified in the T3SS bacterial effector proteins belonging to a group of enteric pathogens, our lab wanted to determine if this motif is present in any non-enteric pathogens. Using the UniProt database to search for WxxxE motifs in the sequences of various bacterial proteins, we have identified additional animal pathogens that secrete the type II and type IV bacterial proteins/virulence factors that contain this signature motif (Table 2).

The T2SS (Figure 11) used by various gram-negative bacteria, transport proteins from the periplasm into the extracellular environment (Green & Mecsas, 2016). Since the T2SS apparatus is localized to the outer membrane, proteins that are secreted from this apparatus are transported to the periplasm through the Sec or Tat secretion pathway, which involves the transfer of proteins across the cytoplasmic membrane. Many pathogenic bacteria employ the T2SS to transfer virulence factors out of the bacterial cell (Green & Mecsas, 2016). Some pathogens employ the T2SS to inject protein substrates that allow the pathogen to adapt to different host environments. The type IV secretion system (T4SS) on the other hand, transfer substrates such as proteins and

DNA into a wide range of host cells, including bacteria of the same species or of a different species. The T4SS, as shown in Figure 12, can secrete substrates across several host membranes into the cytoplasm as seen with the T3SS (Green & Mecsas, 2016). The substrates transferred by these non-enteric pathogens include PltH secreted by *Bordetella pertussis* and *Bordetella bronchiseptica*, and DrrA secreted by *Legionella pneumophila*. Studies have found that the effector PulA secreted by *Klebsiella pneumoniae* employs the T2SS.

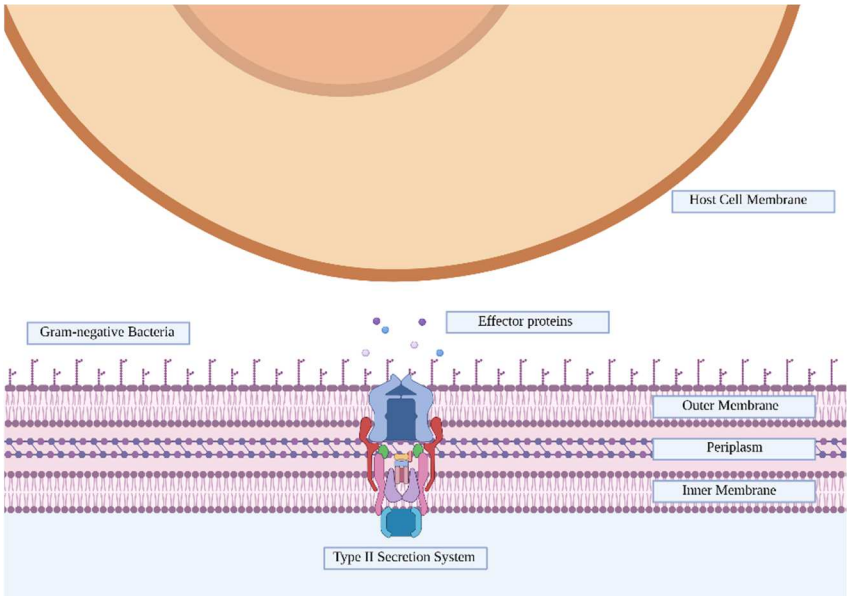

Figure 11. Type II secretion system. Complex secretion system used by gram-negative bacteria to secrete effector proteins found in the cytoplasm of the bacteria into the extracellular environment (Created with BioRender.com).

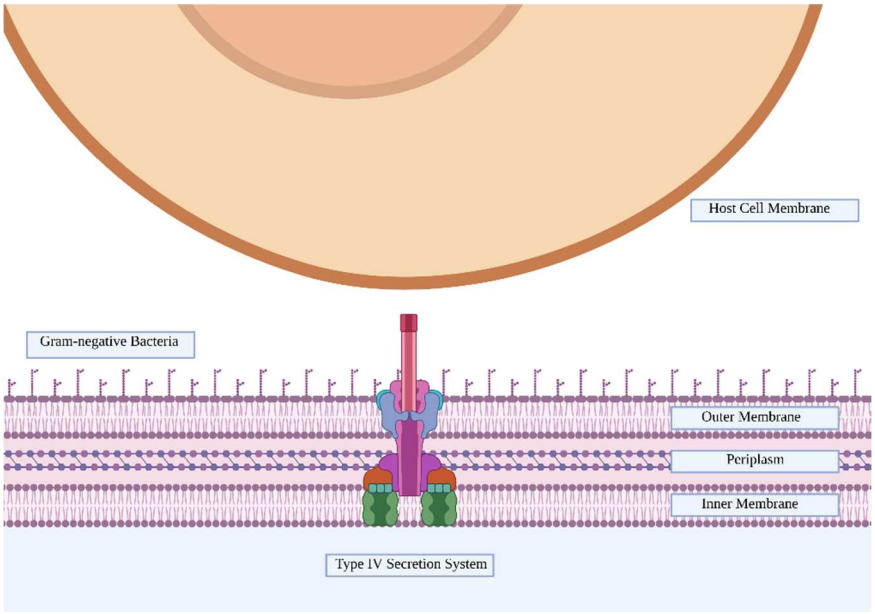

Figure 12. Type IV secretion system. A highly diverse secretion system used by both gram-negative and gram-positive bacteria to transport DNA, effector molecules, or other virulence factors from the cytoplasm to the extracellular space through pilus formation (Created with BioRender.com).

PltH is a crucial component of the Ptl system found in *Bordetella*. It's involved in forming the Ptl secretion system, a membrane spanning apparatus that is a member of the T4SS in which pertussis toxin (PT) is secreted across its outer membrane leading to whooping cough (Kotob and Burns, 1997; Verma & Burns, 2007). A previous study introduced mutations in the nucleotide binding region of PtlH plasmids and found that PT secretion was affected in wild type strains (Kotob and Burns, 1997). Additionally, PtlH localization is dependent on both the PT substrate and other Ptl proteins, therefore it is likely that PltH is a nucleotide binding protein that can interact with one or more of the Ptl proteins (Verma & Burns, 2007). It is speculated that PltH may hydrolyze ATP for PT translocation or provide energy for synthesis of the transporter (Verma et al., 2007). Conversely, PtlH might act as a signaling molecule to stimulate channel or gate opening through kinase activity (Kotab and Burns, 1997). These speculations need to be further researched to determine the exact role of PtlH in the secretion of PT.

DrrA or Sid M is secreted by *Legionella* using Dot/Icm, a T4SS effector to manipulate host vesicular transport (Du et al., 2021). This effector contains three functional domains—a C-terminal lipid phosphatidylinositol-4-phosphate binding domain (P4M) involved in membrane localization, a GEF domain for Rab1 activation, and an N-terminal adenylytransferase (NATs) (Müller et al., 2010). DrrA aids *Legionella* in the formation of its modified vacuole known as the *Legionella*-containing vacuole (LCV) derived from the endoplasmic reticulum (ER), which inhibits lysosomal fusion (Goody et al., 2011; Hardiman and Roy, 2014). DrrA effector protein contain distinct guanine nucleotide exchange activity that disrupts Rab1 mediated secretory transport to the Golgi network by acting as a switch to render Rab1 constitutively active and restrict access to endogenous exchange factors (Müller et al., 2010).

PulA (pullulanase) is a lipoprotein secreted to the cell surface by *Klebisella oxtoca* through the T2SS (East et al., 2016). In gram-negative bacteria, lipoproteins are exported to the periplasm where they are triacylated at the N-terminal cysteine residues (East et al., 2016). These mature lipoproteins can bind to periplasmic chaperones from the inner membrane and are exported to the outer membrane receptor (East et al., 2016). It was previously reported that PulA in *Klebisella pneumoniae* is involved in immune evasion by attenuating thee43 activation of the TLR4-TLR2-MyD88 pathway (Tomás et al., 2015). Their data suggests that PulA is essential for escaping the host immune response however, further research is needed to determine its exact role in pathogenesis.

Through further investigation of non-enteric bacterial proteins, we have found that many phytopathogens have been reported to contain the conserved features of the WxxxE motif in the amino acid sequences of the plant pathogen bacterial effector proteins (Ham et al., 2009). These phytopathogen effectors are known as the AvrE family of T3SS bacterial effectors, which indirectly regulate host protein phosphorylation. Effector proteins from the AvrE family of phytopathogens include WtsE, AvrE1, DspA/E, RopE secreted by *Pantoea stewartii* subsp. *Stewartii*, *Pseudomonas syringae*, *Erwinia amylovora*, and *Pseudomonas fluorescens* respectively (Table 3). As demonstrated in animal pathogens, phytopathogens secrete effector proteins to promote virulence by inhibiting PAMP triggered immunity although, the direct enzymatic activity for AvrE type effectors has yet to be clarified considering that they are toxic when expressed in plant or yeast cells (Jin et al., 2016). Despite this, researchers Jin *et al.* (2016) performed a yeast two-hybrid screen with non-lethal WtsE fragments and full length WtsE employed in a synthetic genetic array. Results from these screens suggest that WtsE interacts with protein phosphatase 2A (PP2A) regulatory subunit proteins (Jin et al., 2016). In planta experiments with AvrE1 from its

host, Pseudomonas syringae pv. tomato strain, revealed that AvrE1 interacts with several PP2A B' subunits. In addition to this, chemical inhibition of PP2A using the toxin cantharidin delayed cell death by obstructing virulence activity in WtsE and AvrE1. These results suggest that the AvrE-type effectors target PP2A by stimulating phosphatase activity in PP2A (Jin et al., 2016). Synthetic genetic arrays with WtsE and DspA/E expressed in various mutant yeast cells further supported the notion of increased stimulation of phosphatase activity in PP2A.

AvrE-type effectors, WtsE and DspA/E, both hindered sphingolipid biosynthesis in yeast cells by exhausting precursor molecules in this pathway. DspA/E interacts with the PP2A B regulatory subunit, Cdc55 in yeast which disrupt the ORM1/2 (for orsomucoid like proteins) involved in the rate-limiting step of sphingolipid biosynthesis (Jin et al., 2016). Inhibition of sphingolipid metabolism in plants affected vesicular trafficking and may lead to cell death (Markham et al, 2011; Chen et al., 2006; Dietrich et al., 2008). Moreover, their results revealed another common target of the AvrE- type effectors known as the leucine-rich repeat receptor-like kinases (LRR-RLKs). While the importance of this interaction has not been established, they speculate that LRR-RLKs role in triggering plant defense responses make them a possible target for the AvrE family effectors (Jin et al., 2016).

Lastly, we have identified a WxxxE motif in the type IV effector protein--VirB11 from the plant pathogen *Agrobacterium tumefaciens*. VirB11, which is involved in the assembly of the T4SS, is believed to be a traffic ATPase that promotes pilus polymerization through regulation of VirB4 as it disrupts pilin subunits from the inner membrane to the periplasmic region (Rozada et al., 2013). It was also reported that VirB11 is involved in nucleoprotein transfer by interacting with the VirD4 coupling protein (Rozada et al., 2013). Ultimately, VirB11 acts as a switch between substrate transport and pilus biogenesis in which VirD4, VirB11 and VirB4 interact with one

another to promote substrate transfer through an ATP-dependent and independent mechanism (Rozada et al., 2013; Atmakuri et al., 2004). The Das lab further categorized the bacterial proteins from non-enteric and plant pathogens that contain the conserved WxxxE motif (Figure 13).

Figure 13. Multiple sequence alignment of bacterial proteins with a conserved WxxxE motif from non-enteric and plant pathogens. A BLAST search of amino acid sequences in non-enteric and plant pathogenic bacteria that share a common amino acid sequence Trp-xxx-Glu or WxxxE motif (Retrieved from Sayed *et al.*, Gut Microbes 2021).

Table 2. The function of non-enteric effector proteins with a shared WxxxE motif

Bacteria Effector	Bacteria Species	Secretion System	Amino Acid Sequence	Function	Host Target	References
PtlH	*Bordetella pertussis* *Bordetella bronchiseptica*	Type IV	GQLWYEDRNGWNRQESGALTLDHLM	Potentially functions as a signaling protein for a gate/channel via kinase activity Likely provides energy for Pertussis Toxin translocation through ATO hydrolysis	a nucleotide binding protein that may interact with one or more of the Ptl proteins	(Craig-Mylius and Weiss, 1999; Farizo et al., 1996; Kotob and Burns, 1997; Verma and Burns, 2007; Williams et al., 2016)
DrrA	*Legionella pneumophila*	Type IV	RENEGNEVSPWQEWENGLRQIYKEM	contains AMPylation activity which allows it to exchange GTP for GDP on Rab1 by competing with endogenous exchange factors to recruit and constitutively activate Rab1	A Rab1 GEF that disrupts secretory transport to the Golgi	(Müller et al., 2010; Murata et al., 2006; Tan and Luo, 2011)
PulA	*Klebsiella pneumoniae*	Type II	AHWVDKTTLLWPGGENKPIVRLYYSH	Involved in attenuating inflammatory responses leading to pneumonia	Limits the activation of the TLR4-TLR2-MYD88 pathway by diminishing the activation of NF-κB	(Pugsley et al., 1990; Tomás et al., 2015)

Table 3. The function of plant pathogen effector proteins with a shared WxxxE motif

Bacteria Effector	Bacteria Species	Secretion System	Amino Acid Sequence	Function	Host Target	References
WtsE	*Pantoea stewartii subsp. Stewartii*	Type III	VHYFDQLTRG**WTEAE**AGCQQLKKGL	*disrupts sphingolipid biosynthesis via depleting sphingolipid precursor molecules*	Mimics activated G-proteins Likely targets PP2A via direct interaction with B' regulatory subunits likely interacts with LRR-RLK proteins	(Pugsley et al., 1990; Tomás et al., 2015)
AvrE1	*Pseudomonas syringae*	Type III	LYQFDPISTR**WKIPE**GLEDTAFNSL KGLMQLKAGQ**WQRFE**QRPVEENPRW	*disrupts sphingolipid biosynthesis via depleting sphingolipid precursor molecules* *promotes bacterial growth and/or suppresses callose deposition*	Mimics activated G-proteins interacts with multiple PP2A B' subunits	(Ham et al., 2009, Jin et al., 2016)
DspA	*Erwinia amylovora*	Type III	LHYFDQLTKG**WTGAE**SDCKQLKKGL	suppresses plant defense responses and promotes bacterial growth	Dephosphorylates Orm proteins by Activating PP2A Cdc55 in yeast affecting vesicular trafficking in plants and inducing cell death. -likely interacts with LRR-RLK proteins	(Ham et al., 2009, Jin et al., 2016)
DspE	*Erwinia amylovora*	Type III	KNAAYATQHG**WQGRE**GLKPLYEMQG	suppresses plant defense responses and promotes bacterial growth	Dephosphorylates Orm proteins by Activating PP2A Cdc55 in yeast affecting vesicular trafficking in plants and inducing cell death. likely interacts with LRR-RLK proteins	(Jin et al., 2016, Siamer et al., 2013, Siamer et al., 2014, Meng et al., 2006)
RopE	*Pseudomonas fluorescens*	Type III	WQGNTAIAQS**WRKVE**LPDRQPLESL	Involved in pathogenesis but direct involvement/function is unknown	unknown	(Preston et al., 2001)
VirB11	*Agrobacterium tumefaciens*	Type IV	GQVLTEGPGG**WRTYE**MPELTFEKLM	Promotes Pilus polymerization by regulating VirB4 as it disrupts pilin subunits from the inner membrane to the periplasmic space modulates VirB4 pilin dislocase activity & interacts with VirD4 by assisting TrwB to proceed with nucleoprotein substrate transport	Acts as a traffic ATPase to modulate pilus biogenesis and substrate transport by interacting with other molecular motors in T4SSs--VirB4 and VirD4.	Atmakuri et al., 2004; Ripoll-Rozada et al., 2013)

Chapter 4. Identification of prokaryotic TIR domains with a conserved WxxxE motif

The innate immune system is the first line of defense initiated against invading microbes (Jang & Park, 2014). These pathogens contain pathogen-associated molecular patterns (PAMPs), which are recognized through host pattern recognition receptors (PRRs) that ultimately lead to an intracellular signaling cascade known as a signal transduction pathway (Jang & Park, 2014). Among the subgroups of PRRs, the Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1R) play a key role in the innate immune system as they detect a wide range of pathogens from bacteria to viruses (Spear et al., 2009; Patot et al., 2017; Akira et al., 2006). Upon recognition of a pathogen, TLR signaling is triggered through the dimerization of the cytoplasmic Toll/interleukin receptor (TIR) domains to allow for protein-protein interactions between the TLRs with the TIR domains of adaptor molecules including TRAM (TRIF-related adaptor molecule), MyD88 (Myeloid differentiation primary response gene 88), TIRAP also known as Mal (TIR domain-containing adaptor protein), or TRIF (TIR domain-containing adapter-inducing interferon-β) (Chan et al., 2009; Jang and Park, 2014). Following this interaction, specific transcription factors are activated such as nuclear factor-kappaB (NF-kB) and interferon-regulatory factors (IRFs) to produce an appropriate immune response through a TLR signaling pathway (Figure 14) (Patot et al., 2017; Kawasaki & Kawai, 2014).

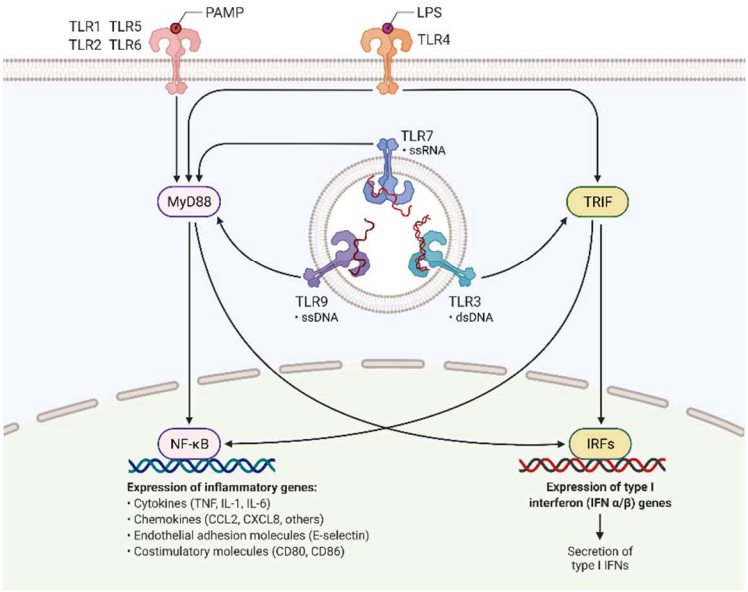

Figure 14. TLR signaling pathway. Schematic representation of the effects of pathogen recognition by host TLR proteins leading to the activation of transcription factors such as NF-kB and IRKs to trigger an immune response. TLR1/2 and TLR2/6 use MYD88 and TIRAP/MAL adapters. TLR3 uses TRIF and TLR4 uses MYD88, TIRAP/MAL, TRIF, and TRAM adapters. TLR7/8, TLR9, TLR5 and TLR11 shares MyD88 adapter. The association of these adapters with TLR proteins results in NF-kB stimulating cytokine production and IRK triggering secretion of IFNs (Adapted from Biorender.com).

Previous structural studies examining the first crystal structures of the TIR domains of TLR1 and TLR2 discovered that these TIR domains have a conserved secondary structure that spans 125-200 residues (Ve et al., 2015; Kaplan-Türköz et al., 2013). They are comprised of a five stranded parallel β-sheet (strands βA- βE) encompassed by five α helices (αA- αE) that are linked by eight surface-exposed loops labelled based on their corresponding element (loops AA-EE); in other words, loop AB connects helix αA and strand βB (Ve et al., 2015; Kaplan-Türköz et al., 2013; Xu et al., 2000). Moreover, these surface-exposed loops are necessary in homotypic protein-protein interactions in the formation and regulation of the TLR/IL-1R signaling complexes (Jang & Park, 2014).

Although TIR domains have been widely studied in human immune responses, these TIR domain proteins are also present in various multicellular and unicellular organisms including plants, mammals, and bacteria. Findings from structural analysis of the TIR-domain of bacteria compared to human TLRs, exposed a conserved core protein structure with unique conformations in loop postions among the TIR domain containing subfamilies (Jang and Park, 2014; Waldhuber et al., 2016; Xu et al., 2000). While the secondary structure of bacterial TIR domains is similar--located in either the N- or C-terminal of the protein, these TIR domains are highly variable in the remaining part of the protein structure (Xu et al., 2000). These findings from the TIR protein crystal structures and experimental data show that there are structural similarities between the TIR protein domains of humans and bacteria, which alludes to the fact that the TIR domain could have a key role in host-pathogen interactions (Zhang et al., 2011).

Since TIR domain protein-protein interactions are essential for the activation of TLR signaling pathways as a response to pathogens, there are strong indications that the bacterial TIR proteins disrupt the host signal transduction pathways through molecular mimicry (Chan et al., 2009; Rana et al., 2013). Bioinformatic analysis showed that not only are the TIR domains present in pathogenic bacteria but are also present in a significant number of non-pathogenic bacteria—nonsymbiotic and commensal bacteria (Spear et al., 2009; Zhang et al., 2011; Zhang et al., 2011). This suggests that the TIR domain containing proteins (Tdcps) of eukaryotes and non-pathogenic bacteria could play a crucial role between host and commensal interactions in the gut microbiota (Zhang et al., 2011). However, this area of Tdcps in bacteria has yet to be explored and further examination is needed to determine their role in pathogenesis.

In recent years, many studies have explored bacterial TIR proteins and their ability to cause disease through direct manipulation of the host TLR signaling pathway. Some studies determined

that Btp1/BtpA/TcpB in *Brucella* and TcpC in uropathogenic *Escherichia coli* CFT073 target MyD88 adaptor molecule to subdue downstream TLR4 and TLR2 mediated signaling (Cirl and Miethke, 2010; Salcedo et al., 2008). Another study examined TIR-like protein A (TlpA) in *Salmonella* enterica serovar Enteriditis and found that TlpA diminishes NF-kB activation by interfering with TIR-domain containing protein TLR4, IL-1 receptor, and MyD88 mediated pathways (Salcedo et al., 2008). In addition to the documented Tdcps, other protein classes have been recently identified—SEFIR and DUF1863 (TIR-like domain of unknown function) found in both pathogenic and non-pathogenic bacteria (Novatchkova et al., 2003). Results from sequence and structure-based fold predictions of the SEFIR domain, apart from the C-terminal region, proves that it is related to the TIR domain (Novatchkova et al., 2003). For this reason, the SEFIR and TIR domain belong to the STIR domain superfamily, alongside DUF1863. In a study by Patterson *et al*. demonstrated that the DUF1863 domain-containing proteins of S.aureus ST398 activate NF-kB signaling and the production of inflammatory mediators (Patterson et al., 2014; Novatchkova et al., 2003). There is still some debate about whether SEFIR, DUF1863, and TIR should be classified as single or distinct domains but despite this, recent data analysis revealed that they have distinct functions within the same signal transduction pathway (Patterson et al., 2014).

While bacterial Tdcps have been shown to downregulate inflammatory signaling pathways, some bacterial TIR proteins such as BaTcp from *Bracilius anthracis* and *Brucella* Btpa target and induce microtubule formation through a conserved Wxxxe motif (Felix et al., 2014; Cirl et al., 2008). Essentially, the conserved WxxxE motif is present in some eukaryotic TIR domain proteins and most bacterial TIR domain proteins. The WxxxE motif was also identified in human TLR proteins including TLR1, TLR2, TLR4, TLR6, TLR10, and TLR adaptor protein SARM. Moreover, multiple sequence alignment warrants that the WxxxE motif is highly conserved among

the bacterial Tdcps such as *Escherichia coli* TcpC, *Yersinia pestis* YpTdp, *Salmonella enterica* TlpA, and *Paracoccus denitrificans* PdTir (Felix et al., 2014). Using the UniProt database, we have identified a large number of enteric pathogens that contain a WxxxE motif found in either a TIR domain containing protein or TIR-like domain containing proteins shown in Table 4.

The presence of this conserved WxxxE motif in a large number of bacteria suggests that this could have a significant structural role for the TIR domain WxxxE motif as seen in the WxxxE GEF family proteins (Felix et al., 2014). The WxxxE motif in the bacterial GEF proteins are pivotal in positioning the catalytic loop involved in the interaction with specific Rho GTPase isoforms (Felix et al., 2014). However, the crystal structure of BtpA revealed that the WxxxE motif is positioned at different loop structures (Felix et al., 2014). The positioning of the WxxxE motif in these loop structures may be important for association with microtubules. While TIR proteins such as BtpA are not a part of the WxxxE GEF family despite ectopic similarities in the regulation of host actin filaments, TIR domain proteins could give us an insight into crosstalk between the TLR and GTPase signaling pathways (Felix et al., 2014).

Table 4. Identification and function of enteric bacterial TIR & TIR-like domain proteins containing the signature WxxxE motif

Bacterial Species	Protein Name	Protein Function	Domain Name	Domain	Sequence
Shigella sp. MO17	TIR domain-containing protein	Signal Transduction	TIR	118-227	185 ISKGWTNYEL
Shigella dysenteriae	TIR domain-containing protein	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	399-494	460 WPQKELRAVL
Shigella boydii	TIR domain-containing protein	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	399-494	480 WPQKELRAVL
Shigella boydii	RNA-directed DNA polymerase, msDNA specific	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	407-502	475 NKEWPQKELR
Shigella dysenteriae	RNA-directed DNA polymerase, msDNA specific	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	407-502	475 NKEWPQKELR
Shigella sp. MO17	DUF1863 domain-containing protein	unknown	DUF1863	4-111	15 DQWYKNELEI
Yersinia enterocolitica subsp. Palearctica serotype O:3	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL
Yersinia pestis bv. Antiqua (strain Nepal516)	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL
Yersinia pseudotuberculosis serotype O:1b	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL
Yersinia kristensenii	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL
Yersinia enterocolitica subsp. enterocolitica	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL
Yersinia pestis biovar Orientalis str. PEXU2	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL
Yersinia enterocolitica W22703	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL
Yersinia pseudotuberculosis	TIR domain-containing protein	Signal Transduction	TIR	4-121	120 SWELLEKSLA
Yersinia pestis	TIR domain-containing protein	Signal Transduction	TIR	139-243	205 WTCQEVGYAL

Table 4. Identification and function of enteric bacterial TIR & TIR-like domain proteins containing the signature WxxxE motif (continued)

Bacterial Species	Protein Name	Protein Function	Domain Name	Domain	Sequence
Yersinia pseudotuberculosis	Toll/interleukin-1 receptor domain-containing protein	Signal Transduction	TIR	139-243	205 **WTCQE**VGYAL
Yersinia enterocolitica subsp. Palearctica	Toll/interleukin-1 receptor domain-containing protein	Signal Transduction	TIR	139-243	205 **WTCQE**VGYAL
Yersinia enterocolitica	Lipoprotein	Signal Transduction	TIR	133-242	210 FEKN**WTQYE**L
Yersinia similis	MTH538 TIR-like domain (DUF1863)	unknown	DUF1863	6-113	75 KSK**WVKYE**IE
Yersinia frederiksenii Y225	TIR domain protein	unknown	DUF1863	6-113	75 RP**WVKYE**IEK
Campylobacter concisus UNSWCD	TIR domain-containing protein	Signal Transduction	TIR	7-102	80 **WTRHE**YRSVQ
Helicobacter pylori (Campylobacter pylori)	TIR domain-containing protein	Signal Transduction	TIR	76-219	170 YSA**WIYEE**LK
Campylobacter aviculae	TIR domain-containing protein	Signal Transduction	TIR	266-348	340 KSP**WCKYE**LN 380 **WFIEE**DFNKL
Campylobacter coli	TIR domain-containing protein	Signal Transduction	TIR	265-347	340 SR**WCKYE**LNY
Campylobacter jejuni	TIR domain-containing protein	Signal Transduction	TIR	265-347	340 SR**WCKYE**LNY 375 MDDL**WFIEE**N
Campylobacter concisus UNSW2	TIR domain-containing protein	unknown	TIR	31-138	175 KE**WDKKE**KTD
Helicobacter pylori (Campylobacter pylori)	DUF1863 domain-containing protein	unknown	DUF1863	4-107	80 K**WIRKE**VEIA
Campylobacter sp.10_1_50	DUF1863 domain-containing protein	unknown	DUF1863	2-101	70 TANRD**WVKYE**
Campylobacter geochelonis	Uncharacterized protein containing a TIR (Toll-Interleukin 1-resistance) domain	Signal Transduction	TIR	121-230	190 KG**WTNYE**LDG
Helicobacter pylori (Campylobacter pylori)	Molecular chaperone Tir	unknown	DUF1863	4-113	80 K**WIRKE**VEIA
Helicobacter pylori (Campylobacter pylori)	MTH538 TIR-like domain (DUF1863)	unknown	DUF1863	4-116	10 HS**WEYHE**HYL 80 K**WIDKE**IKIA
Salmonella gallinarum (strain 287/91/NCTC 13346)	TIR domain-containing protein	Signal Transduction	TIR	158-263	225 IKKD**WTEYE**L

Table 4. Identification and function of enteric bacterial TIR & TIR-like domain proteins containing the signature WxxxE motif (continued)

Bacterial Species	Protein Name	Protein Function	Domain Name	Domain	Sequence
Salmonella enterica subsp. enterica serovar Wilhelmsburg	TIR domain-containing protein	Signal Transduction	TIR	1-143	195 VLTL**WFLAE**R
Salmonella enterica (Salmonella choleraesuis)	TIR domain-containing protein	Signal Transduction	TIR	116-222	185 KD**WTEYE**LNG
Salmonella enterica subsp. enterica serovar Dublin str. UC16	TIR domain-containing protein	Signal Transduction	TIR	152-257	220 KKD**WTEYE**LN
Salmonella enteritidis	Molecular chaperone Tir	Signal Transduction	TIR	158-263	225 IKKD**WTEYE**L
Salmonella enteritidis (strain 2009K0958)	TIR domain-containing protein	Signal Transduction	TIR	153-285	220 KKD**WTEYE**LN
Salmonella muenster	TIR domain-containing protein	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	400-494	30 KNLK**WDNKE**L 470 H**WPKKE**LHSV
Salmonella enterica subsp. Enterica serovar Litchfield	RNA-dependent DNA polymerase	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	400-494	30 KNLK**WDNKE**L 470 H**WPKKE**LHSV
Salmonella enterica (Salmonella choleraesuis)	TIR domain-containing protein	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	401-535	30 KNLK**WDNKE**L 470 H**WPKKE**LHSV
Salmonella enterica subsp. Enterica serovar Stanley	TIR domain-containing protein	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	401-535	30 KNLK**WDNKE**L 470 H**WPKKE**LHSV
Salmonella enterica subsp. Enterica serovar Poona	RNA-dependent DNA polymerase	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	401-535	30 KNLK**WDNKE**L 470 H**WPKKE**LHSV
Salmonella enterica subsp. Enterica serovar 47:b:1,5	TIR domain-containing protein	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	400-494	30 KNLK**WDNKE**L 470 H**WPKKE**LHSV
Salmonella typhimurium (SL1344)	TIR domain-containing protein	RNA binding, RNA-directed DNA polymerase activity, Signal transduction	TIR	400-494	30 KNLK**WDNKE**L 470 H**WPKKE**LHSV
Salmonella enterica I	TIR domain-containing protein	Signal Transduction	TIR	158-263	225 IKKD**WTEYE**L
Salmonella typhimurium	TIR domain-containing protein	Signal transduction	TIR	158-263	225 IKKD**WTEYE**L

Table 4. Identification and function of enteric bacterial TIR & TIR-like domain proteins containing the signature WxxxE motif (continued)

Bacterial Species	Protein Name	Protein Function	Domain Name	Domain	Sequence
Salmonella enterica subsp. enterica serovar Agama	TIR domain-containing protein	unknown	DUF1863	7-124	85 RPWVNWEIQA
Salmonella enterica subsp. enterica serovar Kedougou	TIR domain-containing protein	unknown	DUF1863	7-124	110 VWTWKAIEDF
Salmonella enterica subsp.enterica serovar Panama	TIR domain-containing protein	unknown	DUF1863	7-124	110 VWTWKAIEDF
Salmonella senftenberg	TIR domain-containing protein	Signal Transduction	TIR	10-131	205 QWMWDSNEWN
Clostridioides difficile	TIR domain	Signal Transduction	TIR	111-278	200 LNSNWCDTEL
Clostridioides difficile CD160	TIR domain protein	Signal Transduction	TIR	4-115	10 WKNEEIADTI
Citrobacter koseri (Citrobacter diversus)	Retron-type reverse transcriptase	RNA binding, RNA directed DNA polymerase activity, Signal transduction	TIR	406-500	475 KKWPKKELNS
Citrobacter koseri (Citrobacter diversus)	TIR domain-containing protein	Signal Transduction	TIR	1-99	65 SIASTWVQRE
Citrobacter koseri (Citrobacter diversus)	TIR domain-containing protein	Signal Transduction	TIR	133-219	195 YFNSRWTSAEFG
Citrobacter koseri (Citrobacter diversus)	TIR domain protein	Signal Transduction	TIR	118-227	185 ISKGWTNYEL
Citrobacter sp. L55	Molecular chaperone Tir	Signal transduction	TIR	1-97	70 PWVVNELDVA
Citrobacter braakii	DUF1883 domain-containing protein	Signal Transduction	TIR	118-227	185 ISKGWTNYEL
Citrobacter braakii	Toll/interleukin-1 receptor domain-containing protein	Signal Transduction	TIR	118-226	255 REWCRREIIE
Citrobacter amalonaticus	RNA-dependent DNA polymerase	RNA binding, RNA directed DNA polymerase activity, Signal transduction	TIR	400-494	470 HWPKKELHSV
Klebsiella oxytoca	Molecular chaperone Tir	Signal transduction	TIR	5-104	75 KKLWTNHERK
Klebsiella variicola	Toll/interleukin-1 receptor domain-containing protein	Signal Transduction	TIR	1-143	205 VLILWFLAER
Klebsiella variicola	DUF1863 domain-containing protein	unknown	DUF1863	4-111	15 DQWYKNELEK
Klebsiella oxytoca	Molecular chaperone Tir	Signal Transduction	TIR	5-104	75 KKLWTNHERK
Klebsiella oxytoca	TIR domain-containing protein	Signal Transduction	TIR	1-143	205 VLILWFLAER
Klebsiella aerogenes (Enterobacter aerogenes)	Uncharacterized protein containing a TIR (Toll-Interleukin 1-resistance) domain	Signal Transduction	TIR	158-262	225 IKKQWTEYEL

Table 4. Identification and function of enteric bacterial TIR & TIR-like domain proteins containing the signature WxxxE motif (continued)

Bacterial Species	Protein Name	Protein Function	Domain Name	Domain	Sequence
Klebsiella quasipneumoniae	TIR domain-containing protein	Catalytic activity, Nucleoside metabolic process, Signal transduction	TIR	530-609	595 YAQSP**WCYDE**
Klebsiella michiganensis	TIR domain-containing protein	Catalytic activity, Nucleoside metabolic process, Signal transduction	TIR	530-609	595 YAQSP**WCYDE**
Escherichia coli O6:H1 (strain CFT073 / ATCC 700928 / UPEC)	NAD(+) hydrolase TcpC	Signal Transduction	TIR	169-203	105 E**WKEVE**AKKI
Escherichia coli F18+	SEFIR domain-containing protein	Signal Transduction	SEFIR	147-279	155 S**WDSPE**HKQW
Escherichia coli (strain MS 21-1)	TIR domain-containing protein	Signal Transduction	TIR	151-245	220 KK**WTQYE**LDS
Escherichia coli MS 117-3	TIR domain-containing protein	Signal Transduction	TIR	118-227	185 ISKG**WTNYE**L
Escherichia coli 541-15	TIR domain-containing protein	Signal Transduction	TIR	118-227	185 ISKG**WTNYE**L
Escherichia coli O6:K15:H31 (strain 536 / UPEC)	TIR domain-containing protein	Signal Transduction	TIR	149-243	80 HDYR**WEWKE**V
Escherichia coli	RNA-dependent DNA polymerase	RNA binding, RNA directed DNA polymerase activity, Signal transduction	TIR	399-494	465 SVNKE**WPQKE**

Methods

UniProt and Blast database search

Literature was searched to investigate published data regarding the WxxxE signature motif in enteric, non-enteric, and plant pathogens as well as bacterial TIR domains. UniProt and Blast bioinformatic databases were used to search for protein sequences that may have the WxxxE motif. Some bacterial effectors with a WxxxE motif were identified in the Blast database using *Salmonella* SifA as a query sequence to find homologous sequences in other enteric bacterial effector proteins shown in Figure 3. This was followed by literature search to determine the functions of each effector protein and then summarized in Table 1.

Using Figure 13 as reference, literature was searched to establish the function of each effector protein in non-enteric and plant pathogens. Information documented in literature on bacterial species/bacterial type that secrete these effector proteins that were different than what

was indicated in Figure 13, UniProt database was searched to verify the WxxxE motif in the protein sequences of bacterial proteins in these non-enteric and plant pathogens. The WxxxE motif were manually searched for in documented sequence entries of bacterial proteins and information from literature were summarized in Table 2 and 3.

In addition to this, using the accession number provided in UniProt-- annotations, protein sequences, and information on the TIR proteins were gathered and categorized in Table 4 as it appears in UniProt. Examining the sequence entries in UniProt, the WxxxE motif was manually searched for in documented protein sequences of bacterial proteins within the TIR domain and further organized in table 4 based on the bacterial species.

Discussion

Pathogenic bacteria have developed numerous strategies to thrive, which has been shaped by extensive co-evolution from host-pathogen interactions. The WxxxE family of bacterial GEF mimics has contributed considerable details into how pathogenic bacteria manipulate host GTPases for their own benefit. Although these bacterial GEF mimics and their eukaryotic counterparts are evolutionarily distinct, they can target and activate specific Rho GTPases by employing common biochemical strategies. Many of these effectors are known to regulate multiple host GTPase mediated cellular processes such as inflammation, endolysosomal signaling, and cytoskeleton structures. Recent studies are finding that these WxxxE GEF mimics are regulated by complex proteins and interactions with membranes as seen with eukaryotic GEFs. Future studies would need to examine the mechanisms by which these pathogens exploit the host signaling pathways and the key components that are involved in these pathways as it still remains unclear.

Furthermore, future studies will need to further examine and compare WxxxE GEF mimics to their eukaryotic counterparts to enhance our understanding of these pathways that regulate equilibrium and disruption during pathogenesis. While the presence of this WxxxE motif was initially identified in enteric bacterial proteins, it is also present in the effectors or virulence factors of non-enteric and plant pathogens. Yet, the research in this area is limited and would need to explore the functional relevance of the WxxxE motif in these pathogens. The absence of the WxxxE motif in commensal microorganisms suggests that the WxxxE effectors play a key role in the host differential recognition of pathogens.

In addition to this, our in-silica data analysis has shed light on the presence of the conserved WxxxE motif in the TIR domains of both human and bacterial proteins. However, little is known about how Tdcps enter host cells and regulate host actin dynamics (Felix et al., 2014). The presence of Tdcps in non-pathogenic bacteria indicates that these proteins also exhibit non-virulence related roles, most likely in the regulation of metabolic pathways. Further examining Tdcps could provide insight into crosstalk between the TLR and GTPase signaling pathways (Felix et al., 2014).

Overall, these WxxxE containing virulence factors are elaborate tools that aid bacteria with their ability to communicate with the host and flourish in new environments. By increasing our understanding of these bacterial systems, we can provide novel approaches to develop therapeutics that target intracellular bacterial pathogens.

Appendix

Abbreviations:

ADAM10: A Disintegrin and metalloproteinase domain-containing protein 10; ATP: Adenosine tri phosphate; AvrE: avirulence; BAI1: Brain Angiogenesis Inhibitor 1; Bep: Bartonella effector proteins; BID: Bartonella intracellular delivery; Btp: Brucella TIR domain containing protein; Cdc: Cell division control protein; COX-2: cyclooxygenase-2; CXCL: Chemokine (C-X-C motif) ligand 1; Dock: Dedicator of cytokinesis; DrrA: Defect in Rab1 recruitment A; Dsp: Disease specific; DUF: Domain of unknown function; EHEC: Enterohemorrhagic E. coli; ELMO1: Engulfment and cell motility protein 1; EPEC: Enteropathogenic E. coli; Erk,1/2: extracellular-signal-regulated kinase; Esp: EPEC secreted protein; G proteins: guanine nucleotide-binding proteins; GCC185: Golgi localized coiled-coil protein; GDP: guanosine diphosphate; GEF: guanine nucleotide exchange factors; GM-CSF: Granulocyte-macrophage colony-stimulating factor; GTP: guanosine triphosphate; HOPS: Homotypic fusion and vacuole protein sorting; Icm/Dot: intracellular multiplication/defect in organelle trafficking genes; IL: Interleukin; Ipg: Invasion plasmid gene; JNK: c-Jun N-terminal kinase; LAMP1: lysosome-associated membrane protein-1; Mal: MyD88 adaptor-like protein; Map: Mitochondrial associated protein; MAPK: mitogen-activated protein kinases; MCP: Monocyte chemoattractant protein-1; MPR: Mannose Phosphate Receptors; MyD88: Myeloid differentiation primary response gene 88; NESCA: New molecule containing an SH3 domain at the carboxyl terminus; NFkB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; NHERF: Sodium/hydrogen exchanger regulatory factor-1; NOD1: Nucleotide-binding oligomerization domain; NTP: Nucleotide tri phosphate; PGE2: Prostaglandin E2; PLEKHM: Pleckstrin homology domain-containing family M member; Ptl: Pertussis toxin liberation; PAMP: pattern-associtaed molecular pattern; PRR: pattern recognition receptor; Rab: Ras-related in brain; Rac: Ras related C3 botulinum toxin substrate; ROCK: Rho-associated protein kinase; RPIP8: Rap2-interactingprotein 8; RUN: RPIP8, UNC- 14 and NESCA; SARM: Sterile α and Armadillo motifs containing protein; SCV: Salmonella Containing Vacuoles; SEFIR: SEF/IL-17 receptor; Sid: Substrate of Icm/Dot; Sif: Salmonella-induced filament; SKIP: SifA and kinesin interacting protein; SopE: Salmonella Outer Proteins; SPI: Salmonella Pathogenicity Island; Tcp: TIR domain containing protein; Tdp: TIR domain protein; TIR: Toll/interleukin-1 receptor; TIRAP: TIR domain-containing adaptor protein; Tlp: TIR-like proteins; TLR: Toll like Receptor ; TNF: Tumor necrosis factor; TPR: Tetratricopeptide repeat; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain-containing adapter-inducing interferon-β; Vir: virulence; Wts: Water-soaking; ZO: zonula occludens

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All experimental data in this thesis is a reprint of the material as it appears in Gut Microbes, 2021, Sayed, Ibrahim M.; Ibeawuchi, Stella-Rita; Lie, Dominique; Anandachar, Mahitha Shree; Pranadinata, Rama; Raffatellu, Manuela; Das, Soumita. The interaction of enteric bacterial effectors with the host engulfment pathway control innate immune responses.

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