The Bacterial Pathogen Yersinia Creates a Complex Structure on the Surface of Macrophages

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THE BACTERIAL PATHOGEN YERSINIA CREATES A COMPLEX STRUCTURE ON THE SURFACE OF MACROPHAGES

By

Wael Bahnan

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2014
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

THE BACTERIAL PATHOGEN *YERSINIA* CREATES A UNIQUE STRUCTURE
ON THE SURFACE OF MACROPHAGES

Wael Bahnan

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During an infectious process, bacterial pathogens interact with host innate immune cells. These innate cells (primarily macrophages) play a major role in the antimicrobial defense and bacterial clearance. In this work, we identify the niche which *Yersinia pseudotuberculosis* creates during macrophage contact. We show that the pYV virulence plasmid is essential for the ability of the bacterium to create an acidified, partially enclosed, and durable niche along the macrophage surface. These compartments are uncoupled from the normal host cell endocytic trafficking pathway and are exposed to the extracellular medium. We present data which shows that the formation of the *Yersinia* Acidified Compartment (YAC) is independent of type III secretion system mediated translocation of effectors into the host cell. However, the maintenance of the YAC requires a fully functional type III secretion system, especially the effector YopJ which seems to play an important role in YAC maintenance. We provide evidence that the loss of YAC acidity by exogenous neutralizing agents or through deletion of YopJ is detrimental to *Yersinia* viability during macrophage contact. We also show that when expressed in fission yeast, the GFP-YopJ fusion is able to undergo a stress dependent subcellular redistribution which requires the first 70 amino acids of
YopJ. We also show that YopJ is able to impose an endosomal trafficking defect in fission yeast cells. Taken together, our data sheds light on the unique structure which *Yersinia* creates along the macrophage border, its importance as well as bacterial factors which are involved in its creation.
Acknowledgments

I would like to express my thanks and gratitude for my mentor Dr. Kurt Schesser. He has been a source of inspiration and motivation through difficult times. The tough times which I went through gave me an excellent perspective on how to deal with problems. Those were lessons which I will carry into my scientific career and life in general. Working in his lab has been a wonderful learning experience.

In addition to Dr. Kurt Schesser, I would like to express my thanks to Dr. Gregory Plano, Dr. Antonio Barrientos, Dr. Wasif Khan and Dr. Robert Levy for serving as my dissertation committee members. Special thanks go to Dr. Ralf Landgraf for serving as my external examiner. I would like to thank Dr. Douglas Boettner for his help and support. Also, I want to thank my previous mentor and friend Dr. Roy Khalaf for his support and guidance. Special thanks go to Dr. George Abdel Nour who was the reason behind my love for microbiology.

My greatest source of energy and strength is my family; my father, mother and brother. Had it not been for their support, this achievement would not have been possible. I am eternally indebted to you and will always carry your love in my heart. I also want to express my heartfelt thanks to my cousin Mike Bahnan as well as my best friend Maria Chiara Di Luca for their never ending love and support.
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<td>EEA1</td>
<td>Early Endosome Antigen 1</td>
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<tr>
<td>LAMP-1/2</td>
<td>Lysosomal-Associated Membrane Protein-1/2</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein 1A/1B-light chain 3 (LC3)</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Patterns</td>
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<tr>
<td>PMG</td>
<td>Pombe Minimal Glutamate</td>
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<td>RNI</td>
<td>Reactive Nitrogen Intermediates</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>YAC</td>
<td>Yersinia Acidic Compartment</td>
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<td>Yop</td>
<td>Yersinia Outer Protein</td>
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CHAPTER 1: INTRODUCTION

The initial events which occur during the host-pathogen interaction usually dictate the outcome of an infection, from the cell to the animal level. For example, whether or not a bacterial pathogen will be able to establish a niche for itself despite the presence of patrolling host innate immune cells is the first obstacle a pathogen faces in an infection. Macrophages are usually the first line of innate immune defense in mammalian systems. These cells are involved in bacterial detection, phagocytosis, killing and in the induction of the adaptive immune response. Bacterial pathogens, however, have evolved alongside their hosts so that they can disrupt or even subvert immune defense mechanisms. One major way through which bacteria disturb the proper immune response is through the use of a Type III Secretion System which delivers (translocates) its toxins (also called effectors) directly into the host cell cytoplasm [1]. Different pathogens have diverse effectors which they utilize to allow them to establish their replicative niche. In many cases, the mammalian host cells recognize these effectors and pathogenic molecules and use them to initiate an anti-bacterial immune response. Two common examples of bacteria which utilize Type III Secretion Systems are the human pathogens, *Shigella* and *Salmonella*. In these bacteria, the Type III Secretion Systems allow the invasion and replication inside their target host cells. Whereas in the case of the extracellular pathogens *Yersinia* and *E. coli* the Type III Secretion System serves an anti-phagocytic role, allowing the
bacteria to remain extracellular [2]. Regardless of the role which the Type III Secretion System plays in bacterial pathogenesis, it is clear that it allows bacteria to modulate the intimate interaction between themselves and host cells.

1.1 The role of macrophages in immune defense

Mammalian hosts are protected from invading pathogens by tissue resident and circulating immune cells. These immune cells recognize pathogen-associated molecules, and then initiate innate and adaptive immune responses. The innate immune response is mediated by cells such as macrophages, dendritic cells, monocytes and neutrophils. These cells are either professional killers of invading microbes (such as neutrophils), or are professional antigen presenting cells whose role is to digest invading bodies and present their antigens to adaptive immune cells such as B and T cells. Macrophages and dendritic cells are at the heart of the innate immune response. They are typically resident in tissues and are activated by pathogen activated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) or peptidoglycan. Upon sensing PAMPs, quiescent macrophages differentiate into a pro-inflammatory subtype called M1 macrophages [3]. These macrophages are responsible for initiating and maintaining the inflammatory process. Macrophages can also differentiate into an anti-inflammatory/immunosuppressive subtype called M2 macrophages upon exposure to cell associated danger signals or bacterium-antibody complexes. The M2 macrophages usually induce tissue repair and remodeling while promoting and maintaining an anti-inflammatory state [4].
Macrophages play an important role in initiating an adaptive immune response against invading bacterial pathogens. They express Toll like receptors and C-type lectin receptors on their surface as well as RIG-like receptors and NOD-like receptors in their cytoplasm. Upon interaction of these receptors with their respective ligands, macrophages undergo signaling events which lead to the production of pro-inflammatory cytokines such as TNF-α, Interleukin (IL)-1β, IL-12 and others. The production of these cytokines at the interface between macrophages and T-cells leads the T-cells to produce Interferon (IFN)-γ. The production of IFN-γ further activates the macrophages and drives them towards the M1 phenotype [4].

1.2 The phagocytic process and phagosomal maturation

The role of macrophages is not only sensing invading bacterial pathogens, but also their uptake through phagocytosis. Following internalization, the pathogens are degraded and a fraction of its peptides is presented to T-cells through the Major Histocompatibility Complex II (MHCII)- T-Cell receptor interaction. Phagocytosis of invading bacteria starts at the level of the macrophage membrane where the bacterium gets in contact with host cells either directly with a membrane receptor, or indirectly through opsonizing antibodies (or complement factors deposited on the bacterial surface) interacting with Fc and complement receptors [5].
1.2.1 Initiation of phagocytosis

After ligation of the appropriate receptors, membrane ruffling occurs and the cells identify the geometric shape and size of the particle on its surface. If the cell estimates the size of the particle to be >0.5µm, phagocytosis starts (in contrast to endocytosis for smaller particles) [6]. As the bacteria which we deal with are typically >0.5µm in size, and macrophages are professional phagocytes, we will be discussing the details of phagocytosis and not general endocytosis or macropinocytosis. It is worth noting that particle size, geometry/topography [7] and rigidity [8] have been identified as factors that determine the efficiency of phagocytosis and the outcome in terms of cytokine production. The immediate next step after particle recognition is particle uptake. The best studied example of phagocytic uptake is the Fc receptor (FcR)-mediated phagocytosis of antibody-opsonized particles. Once FcR binds the IgG coated bacterium, the cytoplasmic domain of the FcR gets modified leading to the recruitment/activation of actin-mediated cellular motility machinery in order to spread the phagocytic membrane around the opsonized surface, creating a “half-eaten” state of the phagocytosed particle [9, 10]. The actual phagocytic process is highly dependent on lipid mediators which modulate the internalization process. Upon ligation of the FcR, the phosphatidyl inositol PI(4,5)P₂ is recruited and mediates the nucleation of actin to allow membrane motion and encompassment of the foreign particle. During this phase of large particle uptake, host membranes originating from endosomes, lysosomes, endoplasmic reticulum as well as other membranous organelles fuse with the cup of the forming phagosome to allow remodeling and
encasement of the particle [11-13]. Once this occurs, PI3-kinase phosphorylates PI(4,5)P₂, changing it into PI(3,4,5)P₃. This burst of PI(3,4,5)P₃ is crucial for progression of the phagocytic process, ending finally with membrane fusion around the foreign particle and scission of the compartment followed by detachment of the host cell plasma membrane [14]. While the mechanism of cup closure is not exactly known, fusion and scission events dependent on Myosin X and dynamin proteins [15] need to occur in order to dislocate the phagosome from the plasma membrane. Once detached, the phagosome undergoes a maturation process which consists of a series of fusion events with normal endosomal compartments. The first maturation step is the fusion with early endosomes, which allows the phagosome to mature into early endosome-like compartments by acquiring markers such as Rab5. The function or Rab5 is to mediate the further fusion of phagosomes with sorting endosomes, allowing the trafficking of other early endosomal markers such as EEA1. It is in this stage that the phagosome cargo undergoes the majority of its sorting events, where receptors are recycled to the cell membrane whereas the bacteria are destined for the lysosome [16]. After the progression through the early endosomal phase, the phagosome starts to lose early endosomal markers and acquire late endosomal markers through fusion events with late endosomes. An excellent example is the loss of Rab5 and the acquisition of Rab7 through the aforementioned process. Experiments in which blocking the activation of Rab7 was attempted showed a disruption of both phagosomal maturation and fusion with lysosomes, highlighting the importance of Rab7 for normal phagosomal
maturation and acidification [17]. In addition to Rab7, other major components of the late phagosomal membrane are the Lysosome-Associated Membrane Proteins 1 and 2 (LAMP1- and 2). These proteins are heavily glycosylated and are important for maintaining the late endosomal/lysosomal integrity. LAMP1 and 2 proteins trafficked directly from the trans-Golgi network into the late endosomes and are retained throughout the phagosome-lysosome fusion [18]. The formation of the phagolysosome gives rise to the most acidic and restrictive cellular compartment to bacterial growth.

1.2.2 Late endosome and lysosome acidification

The late endosome is more acidic (pH 5.5-6.5) than the early endosome (pH 6.5-7) as it has less passive proton leakage and acquires the V-ATPase proton pump. The V-ATPase is a large multi-meric complex composed of two main functional complexes, $V_0$ and $V_1$. This proton pump mediates the shuttling of proton ions from the cytoplasm into the vacuolar space, creating a highly acidified environment [19, 20]. The acidic environment mediated by the proton pumps in late endosomes is necessary for the next step in phagosome maturation, which is fusion with the lysosomes [21]. The late phagosome fusion with lysosomes occurs through the calcium dependent activity of a SNARE protein complex containing Syntaxin 7 and VAMP7, giving rise to the phagolysosome [22, 23]. The phagolysosome then matures into a lysosome by loss of late endosomal markers such as M6PR, and gain of lytic enzymes. The lysosome is the final destination and the last step in the maturation process of
the incoming phagosome. It is the main site of degradation of the phagosome contents.

### 1.2.3 Antimicrobial properties of phagolysosomes

The antimicrobial effects of the phagolysosome are due to the numerous bactericidal components which reside in this compartment. Phagolysosomes are the most acidic compartments inside the cell with a pH ranging between 4.5 and 5. This low pH is due to a strong enrichment of the V-ATPase proton pump in the vacuolar membrane. The phagolysosomal acidification serves two main purposes. The first is to create a restrictive environment for bacterial growth. The second is to create an optimal environment for the activity of the antimicrobial components of the phagolysosome. In fact, the acidic pH is important for the proper functioning of the antimicrobial proteases Cathepsin D and L [24, 25]. In addition to Cathepsin function, the increased concentration of protons inside the vacuolar lumen is important for the synthesis of reactive oxygen species [26]. Reactive oxygen species (ROS) and reactive Nitrogen intermediates (RNI) are concentrated inside the lysosome and play a major role in bacterial killing. The production of ROS is due to the activity of the NOX2 oxidase enzyme. NOX2 assembly is increased under pro-inflammatory conditions. Once assembled, NOX2 transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to molecular Oxygen, generating the superoxide anion $O_2^-$. While $O_2^-$ is cytotoxic, it can be dismutated inside the phagolysosome to generate the highly bactericidal compound $H_2O_2$. Through a Fenton chemistry reaction, $H_2O_2$ can
react with other phagolysosomal components and generate unstable hydroxyl radicals. In addition to this, the lysosomal enzyme myeloperoxidase utilizes \( \text{H}_2\text{O}_2 \) to covert Chloride ions into the highly toxic hypochlorous acid [14]. The effectiveness of ROS in combatting microbial pathogens is pronounced to the extent that it has affected the co-evolution of several pathogens with their hosts. Several pathogens such as *Salmonella* and *Mycobacterium tuberculosis* try to ameliorate the toxicity of ROS by producing superoxide dismutase or catalase enzymes that render ROS harmless [27]. Other pathogens such as *Ehrlichia* target host proteins which are important for NOX2 function thus block the host cell ability to generate ROS [28]. Similar to ROS, RNI are also bactericidal molecules made inside the phagolysosome. They also nonspecifically damage bacterial proteins, lipids and nucleic acids [29]. The synthesis of RNI however, is different from ROS in that it is under the control of the inducible Nitric Oxide Synthetase 2 (iNOS) enzyme. iNOS is expressed in phagocytic cells under stress signals from the p38 MAPK or the Janus kinase-signal transducer and activator of transcription-interferon regulatory factor (JAK-STAT-IRF) signaling pathways. Signals which activate iNOS production range from pro-inflammatory cytokines to conserved bacterial molecules such a LPS or lipoteichoic acid [30-32]. Once expressed, iNOS (which acts as a homodimer) mediates electron transport between NADPH and L-Arginine, creating NO\(^{\cdot}\) and L-citrulline [33]. After NO\(^{\cdot}\) is synthesized on the cytosolic surface of the phagosomal membrane, it diffuses into the phagolysosomal lumen where it reacts with ROS to form the highly reactive peroxynitrite ONOO\(^{\cdot}\). It is important to note here that ROS and RNI work
in concert to create a hostile environment for bacterial growth through destabilizing the bacterial genome, disturbing electron transport or other crucial bacterial processes. As with ROS, several pathogens have developed ways to counteract the toxicity of RNI. One example is the highly successful *Mycobacterium tuberculosis* which blocks the recruitment of the scaffolding protein EBP-50, and by that blocks the delivery of iNOS into the phagolysosome [34].

In addition to ROS and RNI, phagocytic cell phagosomes contain a variety of antimicrobial proteins and peptides which play major roles in inhibiting vacuolar bacterial growth. One major class of proteins serves to chelate ions which are important for bacterial growth. Of particular importance is Lactoferrin which is delivered to phagolysosomes and binds Iron ions, and thus inhibits bacterial growth due to metabolic starvation [35]. Another important example of nutritional limitation in phagolysosomes is through the activity of the macrophage protein NRAMP1. NRAMP1 is localized to the lysosomes of phagocytes, and it mediates the exclusion of Zinc and Manganese ions from the vacuolar lumen, thus limiting bacterial growth due to nutritional stress [36]. Another major class of antibacterial host proteins is the directly lytic enzymes. Of major importance is lysozyme, which cleaves β1-4 glycosidic bonds which hold together peptidoglycan molecules in bacterial cell walls [37]. Cationic antimicrobial peptides bind strongly and permeabilize the Gram negative outer membrane, leading to an ionic imbalance along the bacterial surface and subsequently, bacterial lysis [38]. In addition to the previous two classes of antimicrobial effectors, host cells make
more than 50 hydrolytic enzymes which are activated under acidic conditions. These lysosomal acid hydrolases include phosphatases, proteases, lipases and glycosidases which aid in degrading vacuolar bacteria. The best studied of these enzymes is the Cathepsin family of proteases. It includes Cathepsins A and G which are serine proteases, Cathepsins D and E which are aspartate proteases and Cathepsins B, C, F, H, K, L, O, S, V, X and W which are cysteine proteases. All of these proteases degrade complex macromolecules in the acidic conditions of the phagolysosome [14].

1.3 Bacterial strategies to resist host bactericidal effects

Different microorganisms have developed methods to disrupt their phagocytosis, killing and activation of an adaptive immune response [39]. In order to simplify the study of how bacterial pathogens create hospitable niches for themselves inside the phagocytic cells, we will attempt to classify the bacteria into three categories. The first category is comprised of the bacteria which derail proper phagosome maturation, and by that mediate their survival in an intracellular permissive vacuole. Of importance in this class is the Gram negative intracellular bacterium Legionella pneumophila, which creates a specialized phagosome that is associated with rough endoplasmic reticulum and mitochondrial markers, and is studded with ribosomes. This niche does not acquire LAMP1, does not fuse with lysosomes and does not acidify, allowing the bacteria to replicate [40]. Coxiella burnetti on the other hand, creates a large phagolysosome-like structure that acquires the endosomal markers Rab5 and Rab7 as well as the autophagy
marker LC3. This compartment however does not fuse with lysosomes and by that supports the growth of the bacteria [41, 42]. The Gram positive bacterium *Rhodococcus equi* creates an early endosome-like compartment, acquiring markers such as EEA1, Rab5 and PI3P, but does not progress to Cathepsin or V-ATPase positive compartments. These compartments have been shown to support growth of the bacterium for up to 48 hours post infection [43]. The common theme for the bacterial intracellular replicative compartments discussed previously is the inhibition of maturation and acidification of the phagocytic compartment. Given the potency and effectiveness of the phagolysosome in bacterial killing, the diversity of strategies by which bacteria try to avoid phagolysosomes is not surprising.

The second class of bacteria lyses the phagocytic vacuoles and escapes into the cytoplasm of the host cell where they undergo replication. The best studied example of this class of bacteria is the food pathogen *Listeria monocytogenes*. Once internalized, *Listeria*, permeabilizes the phagocytic membrane and destroys its vacuole through the activity of its phospholipases and cholesterol dependent cytolysin Listerolysin O (LLO). This is followed by release of the bacteria into the cytosol [44]. While other Gram negative bacteria such as *Shigella* and *Rickettsia* have similar mechanisms of intracellular growth as *Listeria*, *Francisella tularensis* is clearly different. Once internalized into phagosomes, *Francisella* containing compartments acquire early and late endosomal markers such as EEA1, LAMP1 and LAMP2 but not Cathepsin proteins. This means that the compartment matures but does not fuse with
lysosomes. The bacteria then escape the vacuole through an unidentified mechanism, enter the endosomal-autophagic pathway and begin to replicate. This replication activates the phagocyte inflammasome and leads to host cell pyroptosis, releasing the bacteria to repeat their infectious cycle [39].

The methods utilized by the bacteria mentioned above aim to avoid the phagosome fusion with the lysosome by escaping into the cytosol. While this is possible in bacteria which possess the proper set of toxins/effectors that mediate such vacuolar escape, other bacteria have evolved mechanisms that allow them to cope with the acidic conditions of the phagolysosome. The best studied example of bacteria which inhabit an acidic "restrictive" vacuole is *Helicobacter pylori*. After internalization, *H. pylori* creates a compartment which retains EEA1, Rab7 and usually proceeds to acquire LAMP1 and LAMP2. While the progression of phagosome maturation is not completely normal, the aforementioned markers have been documented to co-localize with the bacteria containing vacuole [45]. The bacteria express a multitude of proteins and enzymes such as urease [46], superoxide dismutase and catalase to counteract the toxicity of ROS and RNI [47]. In addition to *H. pylori*, *Streptococcus agalactiae* (commonly referred to as Group B Streptococcus) is internalized by phagocytes, and resides in an acidic, LAMP1 positive compartment. The acidity of the compartment is essential for proper *Streptococcus agalactiae* intracellular growth, as when the cells are treated with a V-ATPase inhibitor, the bacterium loses the ability to replicate properly. It is thought that the acidified environment
signals the proper bacterial stress response pathway, mainly the CovR/S two component system, to be able to respond the phagolysosome toxicity [48, 49].

1.4 *Yersinia* pathogenesis

Of the genus *Yersinia*, three species are pathogenic to humans. *Yersinia pestis* is a vector borne bacterium which is the causative agent of the pneumonic and bubonic plague, while *Yersinia pseudotuberculosis* and *enterocolitica* are causative agents of gastro-enteritis.

Of interest to us is *Yersinia pseudotuberculosis* which causes self-limiting gastrointestinal symptoms in immuno-competent patients. In an experimental mouse model of oral *Y. pseudotuberculosis* infection, the bacteria traverse the intestinal lining and enter the Peyer’s patches. After uptake by resident lymphoid cells (macrophages and dendritic cells), the bacteria progress to infect the spleen and lymph nodes. Through neutrophil mediated clearance, a low dose infection is typically contained within 5-8 days [50].

1.4.1 The *Yersinia* Type III Secretion System

The diseases caused by the three *Yersinia* species are different in clinical presentation, severity and outcome. However, all three species share a common set of virulence factors to help them deal with host immune cells. The three *Yersinia* species harbor a 70 Kilo-base extra-chromosomal plasmid, named pYV. This plasmid encodes the apparatus and the effectors that make up the Type III Secretion System which is crucial for *Yersinia* virulence [51].
After the virulence plasmid was discovered in \textit{Yersinia} species, its regulation by extracellular calcium was described. \textit{Yersinia} incubated in media at 37°C in the presence of millimolar concentrations of calcium did not secrete effectors into the extracellular space. However, when calcium was chelated, \textit{Yersinia} Outer Proteins (Yops) secretion was initiated [52, 53]. Afterwards, the thermo-regulated property became apparent. It was shown that a protein which was harbored on the virulence plasmid was only expressed at 37°C but not at 26°C. This protein also mediated pathogenesis and virulence in Swiss albino mice [54]. These two characteristics of the Type III Secretion System (calcium and thermo-regulation) made its study and analysis it in the lab settings.

The \textit{Yersinia} Type III Secretion System apparatus is composed of 20 proteins which are assembled on the bacterial surface, spanning the inner membrane, peri-plasmic space, and outer membrane. The Type III Secretion System is required to deliver several \textit{Yersinia} protein substrates (Yops) from the bacterial intracellular cytoplasmic space onto the extracellular surface of the bacterium [55]. The needle like structure which the Type III Secretion System encodes is required for Yop translocation into host cells. The needle has a tip complex formed of three proteins, YopB, YopD, and LcrV. YopB and YopD are pore-forming proteins [56] which are thought to maintain contact and create a pore in the host cell membrane, indeed, that the YopB/D complex has been isolated from infected red blood cell membranes [57].
Historically, the scientific community had accepted the model by which the Type III Secretion System needle served as a conduit which translocated the bacterial effectors directly from the bacterial cytoplasm into the host cell. This model had been recently challenged by a report [58] which showed that when bacterial effectors are coated onto the extracellular surface of the bacteria, they are translocated into the host cell through a Type III Secretion dependent process. In the aforementioned paper, bacteria (which have a deletion of the yopH locus) coated with exogenously added YopH (YopH is known to inhibit calcium spikes after infection) were able to inhibit the infection-induced calcium spikes in infected neutrophils. This indicated that there might be an alternate route for Yop delivery which does not require the Type III Secretion System needle to function as a conduit. Regardless of the method of Yop delivery, some components of the Type III Secretion System remained crucial for proper Yop translocation.

As mentioned earlier, the tip complex of the Type III Secretion System is composed of YopB/D and LcrV. Of interest to us in this work is the YopB protein. YopB showed resemblance to leukotoxins and hemolysins [56]. A further study [59] showed that the proper translocation of YopE is dependent on the presence of YopB. This termed YopB as a “translocator” protein. The deletion of YopB makes Yersinia unable to translocate the Yop effectors into the host cell. However, the mutant bacteria retain their ability to secrete the Yop effectors into the extracellular milieu upon calcium chelation [60]. This shows that there can be an uncoupling between Yop secretion and translocation into mammalian cells.
The Type III Secretion System in *Yersinia* translocates six known effectors into target host cells. The effectors YopE, YopH, YpkA and YopT all target the host cell actin cytoskeleton. YopE acts as a Rho GTPase activating protein (GAP) which deactivates Rho, Rac and CDC42 proteins. YopH is a tyrosine phosphatase which deactivates focal adhesion kinases such as FAK and the adaptor protein p130\textsuperscript{cas}. YopT is a cysteine protease which cleaves RhoA which is attached to the membrane and disrupts its subcellular localization, leading to its deactivation. YpkA is a eukaryotic serine/threonine-like kinase which phosphorylates G\text{q} proteins and also serves as a Guanosine Dissociation Inhibitor (GDI) for Rho and Rac proteins. The function of these four effectors is to disrupt the host cell cytoskeleton and prevent the endocytosis/phagocytosis of cell-associated *Yersinia*. While YopM seems to be involved in Caspase-1 activation, it has no detectable role in anti-phagocytosis [51].

The first study to identify the involvement of YopJ in bacterial pathogenesis [61] showed that this effector mediates macrophage apoptosis upon *Yersinia* infection. Subsequent studies [62, 63] showed that YopJ has a major role in inhibiting host immune response activation after *Yersinia* infection, specifically NF-kB activation and TNF-\(\alpha\) induction. Furthermore, YopJ was shown to bind Mitogen Activated Protein Kinase Kinase (MAPKK) and IKK\(\beta\) proteins [64] leading to inhibition of their phosphorylation and activation. This led to a disruption of the MAPK and NF-kB signaling pathways. The same group later showed that YopJ possesses a de-ubiquitinating-like protease activity [65]. In this report, YopJ was shown to cleave SUMO proteins which mediate signal
transduction in a manner similar to ubiquitin. The molecular mechanism of how YopJ blocks NF-κB and MAPK pathways remained unknown until 2006, when two reports came out from different groups which showed that YopJ possesses an acetyltransferase activity. Through this acetyltransferase activity, YopJ was proposed to acetylate its target proteins on specific activation residues and thus inhibit their activation [66, 67]. Recently, several reports have studied the role of YopJ in the context of a murine model of *Yersinia* infection. The reports show that TAK1 is also a target of YopJ and that by de-activating TAK1, YopJ promotes intestinal barrier dysfunction and infection progression [68, 69].

A highly simplified model of the *Yersinia* Type III Secretion System function is shown in Fig. 1-1. The Type III secretion translocation machinery is utilized to deliver the Yop effectors directly from the bacterium into the host cell. This translocation process is important might have an intermediate step at which the effectors are bound at the surface of the bacterial cell, but not yet translocated into the host cell. The translocator YopB is known to mediate the final step which is formation of a pore in the host cell membrane and mediation of Yop delivery into the infected cell. Once translocation is complete, the Yops are able to bind to their respective host targets and exert their functions.

### 1.4.2 The *Yersinia* replicative niche

As with many other pathogens, upon host cell contact and pathogen internalization, *Yersinia pseudotuberculosis* creates a permissive intracellular niche which sustains its replication. The earliest report about the intracellular
T3SS
YopB
Yop
Active Yop capable of binding host target

Figure 1-1. The Yersinia Type III secretion System delivers effectors into host cells to mediate pathogenesis. Type III Secretion effectors are delivered from the bacterial cytosol onto the bacterial surface, and later into the host cell cytoplasm through the type III secretion system and the pore-forming protein YopB. Once the Yops have been translocated into the host cell, they bind their respective targets and exert their biological activity which is necessary for the proper Yersinia infection.
Yersinia containing compartment came out in 1999 [70]. This report showed that when Yersinia pseudotuberculosis with an un-induced Type III Secretion System was used (grown at 27°C) to infect host cells, it created an intracellular compartment inside macrophages. (This is not surprising as the major role of the Type III Secretion System is to resist phagocytosis). This Yersinia intracellular compartment was shown to have a neutral pH due to a block imposed by the bacteria on the V-ATPase function (shown by decreased ATP hydrolysis). While the mechanism of the proton pump inhibition remained unstudied, it was shown to occur when un-induced Yersinia either harboring or cured of the virulence plasmid were used for infections. After this study, another report [71] showed that un-induced Yersinia pestis creates an intracellular compartment which has markers of late endosomes/lysosomes such as LAMP-1 and Cathepsin D. The intracellular compartments were shown to be accessible to the endocytic pathway components (able to fuse to lysosomes). Also, the bacterial two component regulator phoP was shown to be important for modifying the bacterial response in a way that allows formation and maintenance of this intracellular niche. Further studies [72] showed that un-induced Yersinia pestis which has been internalized into host cells reside in regular endosomes or autophagosome-like structures. These structures which contain viable bacteria acquire the lapidated form of the autophagic marker LC3-II and have a characteristic double membrane which is consistent with autophagosomes. These compartments eventually fuse with lysosomes and change into single membrane enclosed autophagolysosomes which have a neutral pH. These autophagosome-like
structures had no apparent effect on bacterial viability, as the inhibition of autophagy had no detrimental effect on *Yersinia pestis* survival inside the infected macrophages. This report also showed that when the *Yersinia pseudotuberculosis* strain 32777 was used (un-induced) to infect J774A.1 macrophages, it created an intracellular compartment which had a neutral pH. The most detailed study of the *Yersinia pseudotuberculosis* intracellular niche [73] showed that in bone marrow derived macrophages; un-induced *Yersinia* gets internalized and through interactions with the host cell autophagic pathway, creates an LC3 positive compartment. This phenotype went “hand-in-hand” with the activation of the host cell autophagic response. Even when the Type III Secretion System was induced, this compartment was still created; hence it is independent of Type III Secretion System function. (It is crucial to note here that the authors of the report added gentamicin to their infected cells to kill extracellular bacteria, and that allowed them to focus on internalized *Yersinia* only regardless of the Type III Secretion System status. While this makes their data analysis easier, it could lead to a misinterpretation of results because *Yersinia* which has a functional Type III Secretion System would resist phagocytosis. This means that the internalized portion of Type III secretion expressing bacteria might have been partially defective for anti-phagocytosis and other aspects of Type III secretion function). The formation of these autophagic LC3 positive compartments was important for intracellular *Yersinia* replication because *Yersinia* was unable to grow inside ATG5 knockout cells. This report also showed that the autophagic *Yersinia* containing compartment was not
acidified. Also, it was shown that a disruption of the host cell autophagic machinery (mainly ATG5 deletion) as well as the use of heat killed *Yersinia* led to the formation of an acidic intracellular compartment, consistent with a normal phagolysosome. Hence, viable bacteria required functional host autophagic machinery in order to create the proper intracellular *Yersinia* containing compartment.

It was recently shown by the Grinstein group that when *Yersinia* which is cured of the pYV virulence plasmid was used to infect epithelial like cells, a transient delay was present during the phagocytic process. The plasmid-cured *Yersinia* created an open "pre-vacuole" structure which was open to the extracellular environment and incompletely limited by host cell membrane. This structure was open for up to 10 minutes before it started to seal, while normal phagocytosis usually is completed in 1-3 minutes [74]. The closure of the open compartment occurs through the recruitment of the PI(4,5)P$_2$ phosphatases OCRL and Inpp5b through a Rab5 dependent process. These phosphatases dephosphorylate PI(4,5)P$_2$ and mediate *Yersinia* uptake. This study established that the interaction between *Yersinia* (lacking its Type III Secretion System) and epithelial cells is initiated by the formation of a unique cellular structure along the interface between the bacterium and the host cell.

In this work, we are studying the interaction between virulent *Yersinia pseudotuberculosis* and host macrophages. We describe the formation of a stable, open niche along the host cell surface which is limited by host cell
membrane. We also show that this niche is dissociated from normal host endocytic maturation and that it is important for proper bacterial viability during the infection process.
CHAPTER 2: YERSINIA PSEUDOTUBERCULOSIS CREATES A UNIQUE COMPARTMENT ALONG THE MACROPHAGE SURFACE

2.1 Materials and Methods

2.1.1 Cell culture and reagents

RAW 264.7 macrophage-like cells were cultured in DMEM complete media without antibiotics (supplemented with 10% fetal calf serum (FCS)). The day before infection, $10^5$ cells were plated directly onto 12mm glass coverslips (for microscopy experiments) which were placed in wells of a 24 well plate. For bacterial growth and other experiments, the RAW cells were seeded directly into 24 well tissue culture plates. Bone marrow-derived macrophages (BMMs) and Peritoneal macrophages (PEMs) were prepared as previously described [75, 76] and were seeded either directly into 24 well tissue culture plates or onto 12mm coverslips. PEMs were allowed to attach to the glass or plastic surfaces for 2 hours before the experiments were started. In experiments where Dynasore, ammonium chloride or Tris buffer were used, the cells were pre-treated with 80μM Dynasore, 30mM Tris pH 7.45 or 30mM ammonium chloride for 60 minutes and were kept in the same conditions during the infection.

2.1.2 Bacterial culture and infection conditions

Yersinia pseudotuberculosis strain YIII/pYV+ (pIB102 [54]) and its derivatives: the plasmid-cured YIII/pYV-, yopB mutant YIII/pYV+ΔyopB (pIB604 [60]) and yopJ mutant YIII/pYV+ΔyopJ (pIB232, [77]) were used in this study. We generated the GFP expressing versions of the previous strains by transforming
the constitutively expressing pGFP plasmid (Clonetech) into these Y. 
*pseudotuberculosis* strains to allow their analysis in microscopy based 
experiments. For the Type III Secretion System translocation experiments, the 
*yopE_{1-120-ELK}* [78] expressing plasmid was transformed into the YPIII/pYV+ and 
YPIII/pYV- strains. For infections, bacteria were grown to saturation overnight in 
DMEM+10%FCS medium at 27 °C in an aerated shaking incubator. 50µl of the 
overnight culture was diluted into 2 ml of the complete media and allowed to 
grow at 27°C for 2 hours. The cultures were then shifted to a 37°C incubator to 
activate the expression of the Type III Secretion System genes. For bacterial 
viability assays, macrophages infected with bacteria at a 1:40 ratio before being 
washed three times with sterile PBS. The cells were then lysed with sterile 
distilled water. The lysates were vortexed vigorously and serial dilutions were 
plated onto LB plates. The plates were incubated at 30°C for 2 days before the 
colonies were counted. In experiments which required a gentamicin treatment 
step, 2µg/ml of the antibiotic was added to the infected cells 30 minutes before it 
was washed off and the cells were lysed with water.

**2.1.3 Labeling conditions and microscopy**

To detect the acidified conditions inside the *Yersinia* containing compartments, 
we used the acid-sensitive pHrodo dye (10,000MW red dextran, Life 
Technologies). pHrodo emits fluorescent signal under acidified conditions. The 
emission starts at a pH of 6.8 and plateaus out at a pH of 5. For macrophage 
labeling experiments, the cells were washed off their media and then incubated
with PBS supplemented with 10% FCS and 10µg/ml pHrodo for 40 minutes. The cells were then fixed with paraformaldehyde for 12 minutes. For the simultaneous infection and co-labelling experiments, bacteria were washed off their tissue culture-growth media with pre-warmed PBS three times, and then resuspended in PBS+10% FCS. The titer of the bacteria was determined by OD$_{595}$, and the bacteria were added directly with the pHrodo to the macrophage at a ratio of 40:1. After infection, the cells were fixed with 3.7% paraformaldehyde for 12 minutes. The coverslips were then mounted onto pre-cleaned glass slides using pro-long Gold (contains DAPI) anti-fade (Invitrogen). An Olympus fluorescence BX61 upright microscope was used for the microscopic analysis of the experiments. The microscope was equipped with a Uplan S Apo 100x objective (NA 1.4), Nomarski differential interference contrast (DIC) optics, a Roper CoolSnap HQ camera, Sutter Lambda 10-2 excitation and emission filter wheels, and a 175 watt Xenon remote source with liquid light guide. Image capture was automated using Intelligent Imaging Innovations Slidebook 4.01 for the Mac. For all the cells analyzed, a series of optical Z-sections (0.35 µm) was captured. Prior to analysis, the stacks were deconvolved using the nearest neighbor algorithm. Representative projected images were chosen to be included in the figures. Image J software was used to quantify the fluorescent signal (in arbitrary units) per cell or per bacterium. To generate the signal/cell ration, the total cellular fluorescence was divided by the cellular area. To generate the signal/bacterium ration, the signal per bacterium was divided by the bacterial area. The
percentage of bacteria co-localized with pHrodo* (i.e., fluorescent pHrodo) was calculated by counting the bacteria associated with >30 cells in at least two independent experiments and dividing the number of bacteria in a red environment by the total number of bacteria associated with the macrophages. To calculate the percentage of internalized bacteria, each Z-stack image of the infected and CD11b stained cells was analyzed for the subcellular localization of the macrophage associated bacteria. Only clearly extracellular bacteria or bacteria still within the membrane but not dislodged into the cell were termed “extracellular”. The bacteria in which the phagocytic process was completed were termed “internalized”. For EEA1, LAMP1 or CD11b staining, the cells were fixed, permeabilized with PBS+0.3% Triton X-100, then blocked with PBS+3% Bovine serum albumin. CD11b (eBioscience 14-0112-82), EEA1 (Abcam 2900) or LAMP1 (Abcam 24170) specific antibodies were used at a 1:250 dilution to stain the cells for 60 minutes at room temperature. Appropriate secondary antibodies with the AlexaFluor 555 fluorophore were used at a 1:500 dilution to stain the cells for one hour at room temperature before the coverslips were washed and mounted on slides. Live cell imaging was performed as previously described [75]. Briefly, BMMs were infected and imaged by differential interference contrast (DIC) using a Nikon Eclipse Ti-E microscope using an Andor iXon + EMCCD camera. Images were acquired with 5 second intervals for 30 minutes. The collected data were then transformed into a video with a display rate of 9 frames/second.
2.2 Results

2.2.1 Pathogenic Yersinia establishes an extracellular niche on the macrophage surface

We used DIC time-lapse microscopy to analyze the kinetics by which bone marrow derived macrophages phagocytose and traffic Yersinia pseudotuberculosis. We first experimented with the plasmid-cured strain YPIII/pYV-, which was readily taken up into the macrophages and trafficked into the peri-nuclear region (Fig. 2-1 and video files http://youtu.be/YcA-eXEdfUA). This is consistent with our previous findings of a zipper-like phagocytosis mechanism of Yersinia uptake [75]. The wild-type strain (YPIII/pYV+), however, was able to resist phagocytosis by the macrophages (Fig. 2-1 and video files http://youtu.be/6PIOS_hc2ww). These data are consistent with previous published work which proved that Yersinia utilizes its type III secretion system (harbored on the pYV plasmid) to counteract phagocytosis ([79, 80]).

2.2.2 An acidified compartment is created at the Yersinia-macrophage interface

In order to study the environment inside which Yersinia resides along the macrophage border, we used the pH sensitive dye pHrodo to study the Yersinia occupied niche. pHrodo is a dextran which is conjugated to a rhodamine fluorophore. The fluorophore is excited under acidic conditions starting at a pH of 6.8 and reaches a maximum at a pH of 5.
Figure 2-1. The *Yersinia pseudotuberculosis* virulence plasmid pYV impacts its interaction with bone marrow-derived macrophages. *Yersinia* pseudotuberculosis YPIII/pYV+ or YPIII/pYV- strains (harboring or missing the virulence plasmid) were used to infect BMDM. The infections were recorded for 30 minutes, and two still shot images are shown above (video files in *supporting information*). *(top)* we show a highlighted YPIII/pYV- bacterium that is internalized by the macrophage in a movie time of 24 seconds (the process in real time takes the equivalent to 11.25 minutes). *(bottom)* we show a highlighted YPIII/pYV+ bacterium which remains surface bound on the macrophage without being phagocytosed for a similar amount of time.
In steady state macrophages, pHrodo is endocytosed as a bulk-phase dye and is trafficked into the lysosomal area of the cells. Since the lysosome is the most acidic environment of mammalian cells, pHrodo reflects the strong increase in acidification with increased fluorescence (Fig. 2-2A; red-fluorescent pHrodo will be referred to as ‘pHrodo*’). Macrophages infected with YPIII/pYV+ Yersinia and simultaneously labeled with pHrodo* showed in addition to the endocytic staining, a pHrodo* based encasing of the Yersinia bacteria. Eighty four percent of the macrophage associated bacteria showed this phenotype of encasement with pHrodo* signal (Fig. 2-2B). This intimate interaction between pHrodo* and the pYV+ bacteria was not seen when macrophages were infected with the plasmid-cured YPIII/pYV- Yersinia. In fact, only 9% of the pYV- bacteria were seen to be encased with the pHrodo* signal. Experiments using the murine macrophage-like cell like RAW264.7 showed similar results to peritoneal macrophages where 90% of the wild-type bacteria associated with pHrodo* while only 10% of the plasmid-cured bacteria were localized in pHrodo* staining compartments ($P < 0.05$, student t-test; data not shown). This led us to hypothesize the presence of a Yersinia Acidified Compartment (YAC) in which the wild-type resides but not the plasmid-cured strain. While macrophage associated wild-type bacteria were seen to be encased in pHrodo* signal, unattached bacteria did not show this phenotype. This indicated the requirement of macrophage contact for the formation of YACs (Fig. 2-2A, bottom row). In fact, we analyzed the signal of
Figure 2-2. The pYV virulence plasmid allows *Yersinia pseudotuberculosis* to create an acidified compartment upon macrophage contact. (A) Phrodo was used to label peritoneal macrophages which were simultaneously being infected with GFP-expressing wild-type (pYV+) or plasmid-cured (pYV-) *Yersinia* for 40 minutes. The cells were then analyzed by fluorescent microscopy. (B) The percentage of *Yersinia* co-localized with fluorescent phrodo* was calculated by counting the bacteria associated with more than 40 infected cells in multiple independent experiments. (C) The phrodo* signal per macrophage-associated or detached bacteria were studied and plotted. (*P < 0.05 using student t-test*)
pHrodo* per bacterium for both attached and unattached bacteria. The signal to bacteria ratio was much higher in the attached bacteria compared to the unattached (Fig. 2-2C). In contrast to the strong pHrodo* signal seen associated with wild-type bacteria in infected macrophages, when epithelial like cell lines were infected (Cos7 and HeLa), the pHrodo* signal was barely visible and was hardly distinguishable from background (data not shown).

We then studied the temporal stability of the YAC. For that reason, we modified our infection protocol by infecting macrophages for 60 minutes, then after washing, adding pHrodo for an extra 40 minutes. This gave us an infection regimen which lasted up to 100 minutes. This modification of our infection procedure showed that the YPIII/pYV+ bacteria were still situated inside the YAC as seen by the intense pHrodo* staining. Hence, the architecture and acidification of the YAC are characteristics which last at least up to 100 minutes post infection (Fig. 2-3A). Interestingly, the pHrodo* signal was much more pronounced in the 100 minute infection protocol than in Fig. 2-2A, suggesting that YACs are increasingly acidified during the infection process. Collectively, these data indicate that YPIII/pYV+ Yersinia are able to create a durable and acidified compartment along the macrophage border which is dependent on the presence of the pYV virulence plasmid.

As mentioned earlier, pHrodo normally stains the lysosomes of steady state cells. We were clearly able to visualize this distinct staining even in infected cells. To test this, we quantified the total macrophage fluorescence in uninfected as well
Figure 2-3. *Yersinia* acid-containing compartments (YACs) are temporally stable. (A) GFP-expressing wild-type or plasmid-cured *Yersinia* were used to infect RAW cells for 60 minutes. The cells were then washed and labeled with pHrodo for an additional 40 minutes before microscopic analysis. (B) The fluorescent intensity per macrophage was determined from macrophages which were infected as in A. (C) The total fluorescent signal per cell (from B) was plotted as a function of the number of bacteria per infected cell. Then, the linear regression was calculated. The data shown are representative of more than 3 experiments done, with more than 30 cells analyzed per experiment. (* P < 0.05 using student t-test)
as in YPIII/pYV+ and YPIII/pYV- infected macrophages. We saw that the increase in acidified pHrodo*signal seen in YPIII/pYV+ infected macrophages was attributed solely to the YAC, as this was not seen in uninfected or pYV-infected cells (Fig.2-3B).

Knowing that these YACs are stable for up to 100 minutes, we studied the interaction between the YAC and the host cell endocytic maturation pathway. We first tested whether YACs co-localize with early endosomes by staining YPIII/pYV+ infected cells for EEA1 which is a common early endosome marker. Only 1% of the bacteria were associated with EEA1. Therefore, YACs do not appear to co-localize with this early endosomal marker. We then tested whether the YACs co-localize with LAM P1, which is a late endosomal/lysosomal marker. LAMP1 is normally delivered into the late endosomes and then traffics to the lysosome of cells. Upon infecting the macrophages and staining for LAMP1, it was evident that only a minor population of the bacteria (9.5%) was associated with this marker (Fig. 2-4). Additionally, infected cells were treated with an inhibitor of dynamin (Dynasore) to impair host endosomal trafficking. Dynamin is a host cell protein which is involved in scission of incoming endosomes. It has been also implicated in vesicular fission from the Golgi and ER. The inhibition of dynamin proteins using the dynamin inhibitor Dynasore has been shown to lead to a defect in lysosomal acidification and endosome/lysosome fusion [81]. When macrophages were treated with dynasore for 60 minutes, there was
Figure 2-4. YACs are uncoupled from normal endocytic maturation. RAW macrophages were infected with wild-type Yersinia for 100 minutes. The cells were then fixed and stained for EEA1 or LAMP1. The same results were obtained in infections of RAW cells or peritoneal macrophages. The images shown are representative of two independent experiments.
complete loss of lysosomal acidification and there was no detectable pHrodo* signal in the cells. However, cells infected with YPIII/pYV+ and pretreated with dynasore still showed the formation of YACs which had a much higher fluorescent pHrodo* signal than the cellular background (data not shown). This data indicates that YAC formation and acidification is uncoupled from normal cellular endocytic acidification machinery.

2.2.3 YAC formation does not require delivery of the type III effectors into the host cell cytosol

It has been previously shown that Yersinia can deliver their effectors into host cells via a two-step process. The first step is the export of the effectors onto the extracellular surface of its cell wall. The second step would then follow by translocating the exported effectors into the host cell [58]. The ΔyopB Yersinia mutant is fully capable of exporting the effectors onto the bacterial cell wall, but is missing the YopB translocator protein which mediates pore formation in the host cell. This is necessary for effector translocation from the bacterial extracellular surface into the infected cell [60]. To assess whether the creation of YACs requires effector translocation into host cells, we infected peritoneal macrophages with YPIII/pYV+ or YPIII/pYV+/ΔyopB bacteria. Unexpectedly, the ΔyopB strain was able to induce the formation of the YACs to a similar extent as the wild-type (Fig. 2-5A). To study if there is an involvement of the YopB translocator protein in the stability of the YACs, we infected macrophages with either the YPIII/pYV+ or YPIII/pYV+/ΔyopB strains according to the 100 minute
infection protocol described earlier (see Fig. 2-3). We discovered that while the wild-type strain was better able to maintain the YACs for up to 100 minutes (89% of the bacteria were co-localized with pHrodo*), than the yopB mutant strain. Only 46.3% of the YPIII/pYV+/ΔyopB bacteria were able to maintain their co-localization with pHrodo* (Fig. 2-5B and C). These data suggest that the formation of the YACs is not dependent on effector translocation into the host cells, but the maintenance of the YACs along the host cell membrane requires a fully functional Type III Secretion System.

YopB proteins are conserved between Yersinia species and other Gram negative organisms which utilize a Type III Secretion System. However, to the best of our knowledge, there has not been a function attributed to this protein other than effector translocation into the host cell. Given that the most important role of the Type III Secretion System is to resist phagocytosis, we tested whether the YPIII/pYV+/ΔyopB strain differed in anti-phagocytic capability from the wild-type or the plasmid-cured strain (see Fig. 2-1; [51]). We infected peritoneal macrophages with YPIII/pYV+ or YPIII/pYV+/ΔyopB or YPIII/pYV- bacteria for 40 or 100 minutes. After infection, we stained the fixed cells for the CD11b macrophage surface marker. This allowed us to analyze the localization of the three strains using the host cell membrane as a reference. Forty minutes after infection, a minor fraction of the YPIII/pYV+ (8%) and YPIII/pYV+/ΔyopB (27%) bacteria were internalized which significantly differed from that of YPIII/pYV- strain where 59% of the bacteria were internalized (Fig. 2-6). One hundred
Figure 2-5. Creation of YACs is independent of effector translocation. (A) Peritoneal macrophages were infected with GFP-expressing wild-type (YPIII/pYV+) or a type III translocation defective mutant (YPIII/pYV+/ΔyopB) and simultaneously labeled with pHrodo for 40 minutes as previously described in Fig. 2-2. (B) Peritoneal macrophages were infected with the GFP-expressing wild-type (YPIII/pYV+) or the yopB mutant (YPIII/pYV+/ΔyopB) strains for 60 minutes before being washed and labeled with pHrodo for 40 minutes as described in Fig. 2-3. (C) The percentage of the bacteria co-localized with the pHrodo fluorescence from (A) and (B) is calculated from counting the bacteria associated with more than 30 macrophages from three independent experiments. (* P < 0.05 using student t-test)
minutes after infection, the percentage of internalized YPIII/pYV+ bacteria remained steady at 8%. On the contrary to the phenotype seen at 40 minutes, the percentage of internalized YPIII/pYV+/ΔyopB bacteria did not significantly differ from that observed for YPIII/pYV- bacteria (62% and 58%, respectively).

### 2.2.4 YACs are exposed to the extracellular milieu

To examine a different angle on the internalization question, we utilized the impermeability of mammalian membranes to the antibiotic gentamicin to test the localization of the two bacterial strains (YPIII/pYV+ or YPIII/pYV+/ΔyopB) with respect to the host cell membrane. This assay relies on the fact that if bacteria are exposed to the extracellular environment, they will be exposed to gentamicin in the medium and will lose viability. Only internalized bacteria will be protected from gentamicin and will be able to form colonies upon plating on bacteriological medium. After a 40 minute infection of peritoneal macrophages, gentamicin was added to the wells and then the cells were lysed. The lysates were plated onto agar plates and CFU enumeration showed that around 80% of both with YPIII/pYV+ and YPIII/pYV+/ΔyopB strains were sensitive to gentamicin. This indicated that at this time, both strains were exposed to the extracellular environment (Fig. 2-7). After a prolonged infection of 100 minutes, gentamicin treatment was able to kill around 90% of the YPIII/pYV+ strain while only 20% of the YPIII/pYV+/ΔyopB strain remained sensitive to the antibiotic. These data are consistent with our previous findings (from microscopy experiments) showing that there is a transient, YopB independent anti-phagocytic activity which arises from
Figure 2-6. Secreted type III effectors confer transient anti-phagocytic potential. (A-C) Wild-type (YPIII/pYV*), YopB mutant (YPIII/pYV+/ΔyopB) or plasmid-cured (YPIII/pYV-) Yersinia were used to infect peritoneal macrophages for 40 or 100 minutes. The cells were then fixed and stained for CD11b before being analyzed by fluorescence microscopy. (D) Localization of the bacteria in A-C with respect to the cell membrane was analyzed for each bacterium by examining individual Z-stacks of each image. The shown images are representative of three independent experiments in which more than 40 cells were analyzed. (* P < 0.05 using student t-test)
Figure 2-7. Wild-type and translocation defective bacteria are equally exposed to extracellular medium early in, but not after prolonged infection. Wild-type (YPIII/pYV+) or YopB mutant (YPIII/pYV+/ΔyopB) strains were used to infect peritoneal macrophages for 40 or 100 minutes. Thirty minutes prior to cell lysis for the CFU assay (as described in the materials and methods), gentamicin was added to the infected cells to kill all the extracellular bacteria. The percentage of gentamicin sensitive bacteria was calculated by dividing the number of CFUs recovered between untreated or gentamicin treated wells. The data shown is representative of one experiment which was repeated multiple times, yielding similar results.
an unknown factor harbored on the pYV virulence plasmid. This data also indicates that the majority of the YPIII/pYV+ *Yersinia* seen in Fig. 2-6 associated with the host membrane are sensitive to gentamicin. This is evidence that macrophage associated *Yersinia* are found inside an acidified, membrane bound compartment which is sensitive to gentamicin, and thus, exposed to the extracellular medium. The data presented previously in the pHrodo experiments (Fig 2-5) as well as in both the membrane localization assay and the gentamicin protection assay indicate that the both YPIII/pYV+ or YPIII/pYV+/ΔyopB start off in YACs and are sensitive to gentamicin at early stages of infection, but then the YPIII/pYV+/ΔyopB gets internalized and loses its YAC acidification.

### 2.2.5 The acidified environment enhances *Yersinia* survival vis-à-vis the macrophage

In order to identify the importance of the acidic environment of the YACs for the establishment of a productive infection, we attempted to neutralize the YAC using exogenously added NH₄Cl or Tris buffer. The addition of either of these two compounds led to a strong decrease in pHrodo* signal inside the YACs. Tris only neutralized the extracellular YAC, further supporting the hypothesis that YACs are open to the external milieu. NH₄Cl, however, neutralized the entire cell including the lysosomes (Fig. 2-8). When combined with infection conditions, neutralization of the YACs did not correlate to any change in the localization of the bacteria at 40 and 100 minutes. Surprisingly, we noticed that significantly more YPIII/pYV+ bacteria were associated with the macrophages after 100
Figure 2-8. Exogenous buffering agents disturb the acidification in the *Yersinia* containing compartment. (A) YPIII/pYV+ *Yersinia* was used to infect RAW macrophages which had been treated with ammonium chloride (NH₄Cl) or Tris for 60 minutes prior to being infected. After allowing the infection to proceed for 60 minutes, the macrophages were washed and labeled for an additional 40 minutes with pHrodo. (B) The fluorescent intensity of pHrodo per bacterium (of cells infected as in A) was quantified and plotted. The data shown is representative of a single experiment which was repeated twice yielding similar results. (*P < 0.05 using student t-test)
Figure 2-9. Disrupting the YAC acidity affects the *Yersinia*-macrophage interaction. (A) Ammonium chloride (NH₄Cl) or untreated peritoneal macrophages were infected with GFP-expressing wild-type *Yersinia* for 40 or 100 minutes. After infection, the macrophages were stained for CD11b and analyzed by fluorescence microscopy. (B) The number of bacteria associated per cell was enumerated through microscopic analysis and the data were plotted. The data shown are representative of three experiments with similar results. (* P < 0.05 using student t-test)
minutes of infection. This was seen only when the cells were treated with NH₄Cl or Tris (Fig. 2-9). This increase in bacterial association, however, was inversely related to *Yersinia* viability. When we infected macrophages for 40 and 100 minutes, then osmotically lysed the cells and plated the lysates. CFU analysis revealed that at the 100 minute mark, even though there were more YPIII/pYV+ associated with the cells treated with NH₄Cl or Tris, the majority of the attached bacteria were not viable and did not form colonies (Fig. 2-10). This supports the idea that the acidic conditions inside the YACs are important for bacterial viability and their resistance to macrophage attack. It is important to note here that YPIII/pYV+ bacteria which were cultivated in bacteriological medium in the presence of NH₄Cl or Tris were not affected in terms of their growth (Fig. 2-11).

The Type III Secretion System, specifically the translocator protein YopB has been shown to play an important role in the viability of *Yersinia* when under macrophage attack [79]. For this reason, we decided to test whether the presence of NH₄Cl or Tris was affecting the functioning of the Type III Secretion System. This is measured by the YopE₁₋₁₂₀-ELK translocation assay in which the YPIII/pYV+ strain used to infect macrophages expresses the YopE₁₋₁₂₀-ELK fusion protein. Once this protein is translocated into the host cell, it gets phosphorylated by host kinases. Therefore, the phosphorylated signal of YopE₁₋₁₂₀-ELK is a direct measure of Type III Secretion System translocation of its effectors [78]. We were able to readily detect phosphorylated YopE₁₋₁₂₀-ELK in untreated macrophages infected with the YopE₁₋₁₂₀-ELK-expressing *Yersinia*
Figure 2-10. Disrupting the acidic environment negatively affects *Yersinia* survival and Type III Secretion System activity. (A) Wild-type *Yersinia* was used to infect ammonium chloride treated or untreated cells for 40 or 100 minutes. The infected cells were then washed, lysed, and the lysates were plated for CFU enumeration. The data shown is representative of a single experiment which was repeated several times yielding similar results (*P* < 0.05 using student t-test). (B) Peritoneal macrophages were pretreated with ammonium chloride or left untreated and then infected with wild-type *Yersinia* which expresses the YopE_{1-120}ELK for 90 minutes. The cells were then lysed in sample buffer and samples were run on SDS-PAGE gels. After western blotting, the membranes were probed for phosphorylated ELK, total ELK and Actin (as a loading control). The total ELK signal is indicative of the fusion protein synthesized inside the bacterium, while the phosphorylated ELK is a direct measure of translocated ELK which was phosphorylated by host cell kinases.
In contrast to this result, *Yersinia* infecting NH$_4$Cl treated macrophages was unable to translocate the YopE$_{1-120}$-ELK fusion. This was seen as the loss of phosphorylated fusion protein under NH$_4$Cl treated conditions. The YPIII/pYV- strain serves as a negative control for our experiment as it is neither able to make the YopE$_{1-120}$-ELK fusion, nor translocate effectors into host cells. However, in the context of the reduced bacterial viability data, we attribute the loss of phosphorylated YopE$_{1-120}$-ELK signal to bacterial death rather than Type III Secretion System malfunctioning.

2.2.6 YAC maintenance is dependent on the type III effector YopJ

Based on our findings that the *Yersinia* Type III Secretion System is involved in YAC formation and maintenance, we infected macrophages with several Type III Secretion System mutants, each deleted for a specific effector protein. Among the mutants which we assayed, there was a clear indication that the $\Delta$yopJ mutant (YPIII/ pYV+/ΔyopJ) strain was unable to maintain the YACs upon macrophage infection, compared to the YPIII/pYV+ strain. In the 100 minute infection protocol, 90% of the YPIII/pYV+ strain were inside YACs whereas only 28% of the YPIII/ pYV+/ΔyopJ strain was associated with pHrodo* signal (Fig. 2-12A, B). This reduction in the ability of the YPIII/ pYV+/ΔyopJ strain to maintain YACs was correlated with a reduced bacterial viability when compared to YPIII/pYV+ strain (Fig. 2-12C). The yopB and YPIII/pYV+/ΔyopBΔyopJ double mutant were equally unable to maintain YACs as the YPIII/pYV+/ΔyopB strain (data not shown). This indicates that the involvement of YopJ in YAC
Figure 2-11. Ammonium Chloride or Tris do not affect the growth of YPIII/pYV+ *Yersinia in vitro*. Overnight cultures of wild-type *Yersinia* (YPIII/pYV+) were grown at 27 °C. The cultures were then diluted to an optical density (OD) of 0.05 in tissue culture media and allowed to grow for 2 more hours. The cultures were then shifted to a 37 °C incubator and allowed to grow for one hour to allow the expression of the Type III Secretion System genes. The cultures were then diluted to an OD of 0.05 in tissue culture media which was either supplemented with either ammonium chloride or Tris (at a concentration of 30mM). The diluted cultures were allowed to grow for 2 hours at 37 °C to mimic the infection conditions. The OD of the cultures was recorded at T=0 and after 2 hours. The data shown is representative of two independent experiments with similar results.
Figure 2-12. YopJ is required for *Yersinia*-containing acidic compartment (YACs) stability. (A) GFP-expressing wild-type (YPIII/pYV+) or YopJ-deleted mutant (YPIII/pYV+/ΔyopJ) bacteria were used to infect RAW macrophages for 60 minutes. The cells were then washed and labeled with and additional pHrodo for 40 minutes as described for Fig. 3. (B) The percentage of bacteria co-localized with pHrodo was calculated by counting the bacteria associated with more than 40 infected cells in three independent experiments. (C) RAW macrophages were infected with the wild-type of YopJ mutant strains for 90 minutes. The number of viable bacteria associated with the macrophages was enumerated by plating the lysates and enumerating the CFUs as described before. The data shown is representative data set of a single experiment which was repeated multiple times yielding similar results. (* P < 0.05 using student t-test)
maintenance depends on the presence of YopB. Collectively, our data suggests that the presence of YopJ is necessary to maintain YACs along the macrophage border and the loss of YopJ is detrimental to the infection processes and negatively affects bacterial viability.

2.3 Discussion

The Type III Secretion System comprises a set of virulence factors which *Yersinia* uses to combat immune cells; mainly macrophages [51]. However, in any given *Yersinia* infection, 10-15% of the bacteria are internalized by the macrophages and are able to replicate in non-acidified intracellular compartments ([71-73]). The intracellular replicative niche of *Yersinia* has been studied by several groups. However, the extracellular replicative niche has been mysterious. Knowing that *Yersinia* is mainly an extracellular pathogen, this gap in the literature is troubling. Recently, Sarantis and colleagues [74] showed that the plasmid-cured *Yersinia pseudotuberculosis* strain (equivalent to YPIII/pYV- used in this study) creates an interesting compartment during its internalization process by host epithelial-like cells. The internalized bacteria formed what was term a ‘pre-vacuole’ along the cell surface. This pre-vacuole was shown to be transient (it starts to close in 10 minutes) and was partially open to the extracellular medium. The transient nature of this pre-vacuole was shown through the ability of the cell to seal the compartment and internalize it through a Rab5 dependent process.
In this report, we shed light on some fundamental aspects of the macrophage-
*Yersinia* (pYV+) interaction by showing the formation of the unique YAC structure. These YAC compartments are partially open to the extracellular environment. This unique YAC structure is uncoupled from the typical mammalian cell endocytic maturation pathway as it does not show co-localization with either early or late endosomal markers (EEA1 or LAMP1, respectively). The absence of the LAMP1 marker also indicates that the acidification inside the YACs is not due to fusion events between lysosomes and the bacterium containing structure. While the acidification requires the presence of the pYV virulence plasmid and its Type III Secretion System, translocation of effectors from the bacterium into the host cell is not required to form these YACs. To the best of our knowledge, this is the first report showing a function which is associated with secreted, but not translocated Type III Secretion System effectors. The role of secreted effectors in creating the YACs seems to be correlated to the transient anti-phagocytic ability of the YIII/pYV+/ΔyopB mutant strain. While the wild-type strain was able to maintain acidity in the YACs and functionally preserve its anti-phagocytic potential, the YIII/pYV+/ΔyopB mutant lost the acidity in its compartments in the 100 minute infections and this correlated with an increase in its internalization by the host cells. These results support the idea that the acidification of the YACs correlate with anti-phagocytic potential.
The Type III Secretion System effector YopJ also seems to be important for YAC maintenance. Our data shows that YPIII/pYV+/ΔyopJ mutant bacteria were unable to maintain the acidification inside the YACs in the 100 minute infections. This previously undescribed role of YopJ in the infection process might (or might not) be related to its known effect of blocking pro-inflammatory cytokine production upon macrophage and animal infection [67, 82]. However, the role of YopJ in a Yersinia infection should not be limited to its known effects. We have previously shown that YopJ from Y. pseudotuberculosis inhibits the maturation of dendritic cells [75] while others have shown similar effects with YopJ from the related pathogens Y. enterocolitica and Y. pestis [83, 84]. Whether or not the role of YopJ in the stability of YACs is involved with the dendritic cell maturation defect remains to be determined. In addition to the effects on YopJ in mammalian cells described by our lab and others, we had previously shown that YopJ is active in fission yeast and is involved in protecting yeast cells from the toxicity of the type III system effector YpkA [85]. While our findings show that YopJ is a highly complex protein with possibly multiple conserved targets between mammalian cells and yeasts, the molecular mechanism behind the involvement of YopJ in YAC maintenance remains to be described. It also remains to be determined whether the acidic environment that is created by Yersinia is a cause or an effect of its various Type III Secretion System dependent activities.

The YACs containing YPIII/pYV+/ΔyopB- or YPIII/pYV+/ΔyopJ bacteria as well as the exogenously neutralized (YPIII/pYV+ containing) YACs share the
commonality that they have lost their acidification. This loss of acidity seems to correlate with loss of anti-phagocytic ability (yopB mutant versus wild-type) and a loss of bacterial viability (ammonium chloride or Tris treated cells). Both of these outcomes can be attributed to the defect of Type III Secretion System function and ability to translocate effectors into host macrophages.

YACs might also serve a role in modulation of the Type III Secretion System and effector delivery into host cells. It has previously been shown [86] that when Yersinia is grown under acidic conditions, this creates a negative feedback effect onto effector secretion. The acidic environment inside the YACs might be an added level of control which Yersinia uses to modulate the amount of effectors it delivers into host cells. As explained by [87] there is a strong similarity between the mechanism which type III secretion effectors enter host cells, and the AB toxin mode of entry. The study of diphtheria toxin (an AB type toxin) has shown clearly that pH is crucial for proper uptake and delivery of AB toxins into host cells [88]. In fact, the low endosomal pH seems to partially unfold the diphtheria toxin, allowing exposure of hydrophobic residues. These residues embed into the endosomal membrane allowing the toxin to leave the endosome and enter the cytosol [89]. Understanding how Yersinia utilizes the acidic environments to achieve a productive infection will allow us to develop new antimicrobial strategies to target pathogenic processes which would render a virulent bacterium harmless.
3.1 Materials and Methods

3.1.1 Yeast culture and growth conditions

The *Schizosaccharomyces pombe* strain h⁻ ade6-704 leu1-32 ura4-D18 was used as a recipient strain for all the GFP or GFP-YopJ constructs. Unless indicated otherwise, the pREP3X plasmid (harbors a leucine nutritional selection marker) was used for expression of GFP, GFP-YopJ or GFP-YopJ mutant proteins in this study. Genes cloned into pREP3x are expressed of the nmt3x high strength thiamine repressible promoter. Before performing experiments, yeast cultures were grown in PMG media supplemented with 225mg/ml Adenine, Uridine and Thiamine (PAUT) at 32°C. The cultures were maintained in logarithmic growth (OD₅₉₅<0.2) for at least 36 hours. The cultures were then washed with sterile distilled water three times, and were inoculated into PAU media (lacking thiamine) to induce the expression of the nmt1 promoter. After 16-18 hours of induction at 32°C, the cultures were ready for experiments as the expression of the nmt1 promoter would be at its peak level.

For heat shock experiments, cultures in logarithmic growth were either left untreated or placed directly in a 42°C shaking incubator for 30 minutes. The cultures were then either concentrated for microscopy of processed for western blotting (see below). The yeast strains utilized in this study are shown in Table 1.
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Table 3.1 The yeast strains which were used in this study.
3.1.2 Generation of GFP-YopJ mutants

The primers used to create the truncated mutants are shown below in Table 3-2. The pREP3x GFP-YopJ (p345W) plasmid was used as a template to generate the mutants used in this study. We developed a whole plasmid PCR protocol using the Q5 high fidelity polymerase (New England Biolabs) to make the mutants. Briefly, 100 ng of plasmid DNA was used as PCR template and the PCR was run under the following conditions: 200µM dNTP, 0.5 µM forward and reverse primers, 0.02U/µl Q5 polymerase and 1X PCR enhancer. The reactions were ran using the following cycling conditions in an Eppendorf Mastercycler epgradient PCR machine: 98°C for 30 seconds for initial denaturation, 30 cycles of 98°C for 10 seconds (denaturation), 52°C for 30 seconds (annealing), 72°C for 6 minutes (extension), followed by a final extension at 72°C for 7 minutes. The samples were then run on 1% agarose gels. The bands were extracted from the gels using the Gel/PCR extraction kit (IBI Scientific) according to the manufacturer’s instructions. Following extraction, the DNA was treated with the restriction enzyme DpnI for 3 hours at 37°C (New England Biolabs), to digest contaminating methylated template DNA. The samples were then purified and concentrated using pellet paint (Novagen), according to the manufacturer’s instructions. The concentrated samples were kinase treated using Optikanse DNA kinase (Affymetrix) in T4 ligase buffer (New England Biolabs) for one hour at 37°C. After kinase treatment, the DNA was ligated using the ligase 2X
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Table 3-2 The primer sets which were used in this study to generate the GFP-YopJ mutants.
mastermix (New England Biolabs) and transformed into chemically competent *Escherichia coli*. The chemically competent *E. coli* was prepared in house by growing 10mls of culture to logarithmic phase. The cultures were then centrifuged at 3,000RPM for 10 minutes in a cold (4°C) centrifuge. The bacteria were kept cold, and washed 3 times with sterile cold distilled water. The bacteria were finally suspended in 10% polyethylene glycol and kept on ice until transformation. After transformation, the plasmids were verified by sequencing, and then transformed into the yeast wild-type strain 831W.

### 3.1.3 Western blotting and protein analysis

For yeast cell lysis, exponentially growing cultures which were stressed or unstressed were centrifuged and resuspended in lysis buffer (40mM HEPES, pH7.5, 50mM KCl, 1% Triton X-100, 2mM EDTA and 5mM PMSF). 200µl of glass beads were added to the cells which were broken in a bead beater at maximum speed for 35 seconds. The lysis was repeated 3 times with 1 minute incubation on ice between each round of bead beating. Complete lysis (>80%) was checked by mixing the lysates with 2% SDS and assessing the formation of ghosts and broken cells by light microscopy. The lysates were then harvested and allowed to settle for 60 minutes on ice. This allowed the settling of intact cells and nuclei. After the initial settling period, the supernatant was collected and centrifuged at 3,500RPM for 12 minutes in a cold (4°C) centrifuge. The resultant supernatants and pellets were resuspended in 2X sample buffer and ran on SDS-PAGE gels.
The proteins were transferred to nitrocellulose membranes and probed with an anti-GFP antibody.

3.1.4 **Microscopy and Immunofluorescence**

For yeast microscopy, stressed or unstressed exponentially growing cultures were collected by centrifugation at 5000RPM for 5 minutes. The cells were then directly visualized by fluorescent microscopy. Cells with 1 or more granules in them were considered as “granulated signal” cells when the data were analyzed. A 1.5X intermediate magnification plus a 60X apochromat objective on a TE2000U inverted Nikon microscope (Nikon, Melville, NY) were used to acquire the images. The microscope was equipped with a Retiga EXi 1394 12-bit CCD camera (QImaging, Surrey, BC, Canada). MetaMorph imaging software v. 6.3r2 was utilized to process the images after acquisition. In the case of the pellet microscopy experiments, yeast cells were lysed as stated before (see western blotting lysis protocol) with a modified lysis buffer which lacks Triton X-100. Also, the samples were centrifuged at 12,000 RPM rather than 3,500 RPM for 12 minutes. The pellets were then directly loaded onto slides and analyzed by fluorescent or phase contrast microscopy. For pHrodo and FM4-64 labeling of fission yeast: yeast cells were grown in logarithmic phase for more than 36 hours with GFP-YopJ expression being induced or un-induced (as explained previously). The cells were concentrated into a pellet then incubated on ice for 10 minutes. The cells were pulsed with 10µg/ml pHrodo or with 8µM of the membrane binding dye FM4-64 (Life Technologies) for 5 minutes before being
washed with media. After washing, fresh media was added and the cells were allowed to grow at 32°C for 45 minutes. Living pHrodo stained cells were directly imaged using fluorescent microscopy. Cells pulsed with FM4-64 were mixed with molten agar and loaded onto glass slides before microscopic examination.

3.2 Results

3.2.1 YopJ undergoes a rapid subcellular redistribution under heat stress

Previously in the lab [85], a fission yeast model was developed to study the effects of YopJ on eukaryotic cells. We extended the findings of the previous report by discovering that the hybrid protein GFP-YopJ, when expressed in fission yeast, undergoes a rapid subcellular redistribution under heat stress conditions. GFP-YopJ expressed off a high strength promoter (pREP3X) under unstressed conditions (32°C) showed a homogeneous distribution throughout the yeast cell. However, when the cultures were shifted to a 42°C incubator, GFP-YopJ underwent a rapid redistribution, from the homogenous diffused signal, into a sharp punctate signal, whereas GFP only did not (Fig. 3-1A). This subcellular redistribution of signal started to occur after 8 minutes of heat shock (data not shown). This change in signal was seen in the majority of stressed cells by 15 minutes, and was present in 99% of the analyzed cells by 30 minutes. This is in contrast to that only 19% of unstressed cells showed granulated GFP-YopJ signal (Fig. 3-1B).
Figure 3-1. YopJ-GFP undergoes a rapid subcellular redistribution upon heat stress. 

A) Yeast cells expressing GFP or GFP-YopJ were grown in exponential phase for more than 36 hours. The cells were then left untreated at 32°C or shifted to a 42°C water bath for the indicated periods of time. The cells were then fixed with 4% paraformaldehyde and analyzed by fluorescent microscopy. 

B) The percentage of cells with granulated GFP-YopJ signal was calculated by counting the number of cells with granulated signal and dividing the number over the total counted cells. The data shown is representative of multiple independent experiments with similar results. (* P < 0.05 using student t-test)
Our lab had previously generated and characterized several mutants of YopJ. These mutants were resultant of a genetic screen which inserted 5 amino acid “scar sequence” in the yopJ gene leading to a small mutation. Since the canonical function of YopJ is to inhibit inductive TNF-α expression, these mutant forms of YopJ were then expressed in Yersinia and assayed for TNF-α inhibition potential [85]. The inactive mutant yopJ genes had insertions in the amino acid sequence sites 52, 170 and 231. We characterized whether those mutants underwent the heat stress dependent subcellular re-localization of GFP-YopJ. Surprisingly, all of the tested mutants had the ability to undergo the localization with similar kinetics to the wild-type protein (Fig. 3-2A and data not shown).

3.2.2 The first 70 amino acids of YopJ are important for the stress dependent redistribution

We then took a different approach to study the domains of YopJ which were responsible for this stress dependent subcellular redistribution. We made deletion mutations in pREP3x plasmid encoded GFP-yopJ in which we progressively truncated the C-terminus of the YopJ protein (Fig. 3-3C). We assayed the truncated GFP-YopJ mutants for their ability to undergo the stress responsive redistribution. We found that the first 70 amino acid fragment of the protein (GFP-YopJ^{1-70}) was required to maintain the stress dependent redistribution. The GFP-YopJ^{1-70} mutant underwent a comparable subcellular redistribution as the full length protein under heat shock conditions. A further
Figure 3-2. "Scar" mutations in YopJ do not disrupt the ability of GFP-YopJ to undergo the stress dependent subcellular redistribution. Yeast strains expressing wild-type GFP-YopJ or mutant variants were grown exponentially for more than 36 hours. The cells were then subjected to heat shock, fixed and analyzed as in Fig. 3-1.
truncation of GFP-YopJ, leaving only the first 40 amino acids of YopJ was not enough to mediate the stress dependent subcellular redistribution of GFP-YopJ\textsuperscript{1-40} (**Fig. 3-3A**).

The percentage of cells in which GFP-YopJ underwent the subcellular redistribution under unstressed conditions was 11%, 25% and 13% for GFP-YopJ\textsuperscript{FL}, GFP-YopJ\textsuperscript{1-70} and GFP-YopJ\textsuperscript{1-40} mutants respectively. Under heat shock conditions, the percentage of cells with granulated GFP-YopJ\textsuperscript{FL} and GFP-YopJ\textsuperscript{1-70} increased to 99% and 83% respectively while it remained at 16% for the GFP-YopJ\textsuperscript{1-40} mutant. While the change in the percentage of cells with granulated signal for the GFP-YopJ\textsuperscript{FL} and GFP-YopJ\textsuperscript{1-70} between the unstressed and stressed conditions is statistically significant, this is not the case for GFP-YopJ\textsuperscript{1-40} (**Fig. 3-3B**).

### 3.2.3 YopJ creates an endosomal trafficking defect in fission yeast

Knowing that the redistribution is stress dependent, we studied whether or not the punctate “granulated” GFP-YopJ signal co-localized with common stress markers inside the yeast cells. Using fluorescent microscopy, we saw that the “granulated” form of GFP-YopJ did not co-localize with endogenous RFP-tagged stress granules, RFP-tagged P-bodies or mitotracker stained mitochondria (*data not shown*). We then created a mCherry-YopJ construct expressed off a pREP3X plasmid. We saw that while the mCherry-YopJ still underwent the stress dependent redistribution, it did not co-localize with the endogenous GFP-tagged
yeast stress response mitogen activated protein kinase Sty1, nor with the endogenous GFP-tagged stress response mitogen activated protein kinase kinase Wis1 (*data not shown*). We analyzed whether GFP-YopJ re-localized into granules situated in the plasma membrane by analyzing Z-planes of heat-stressed GFP-YopJ expressing cells, however GFP-YopJ did not seem to localize with the membranes in our experiments (*data not shown*). We then took a biochemical approach to study the stress dependent redistribution of the GFP-YopJ. GFP and GFP-YopJ expressing cells were left unstressed or subjected to heat shock. The cells were lysed in non-denaturing yeast lysis buffer using a bead beater. The cell extracts were separated into supernatants and pellets after centrifugation. Western blotting of the supernatants and pellets using an anti-GFP antibody showed that the heat shock dependent re-localization of GFP-YopJ which we observed through fluorescent microscopy was associated with a differential enrichment of the protein in the pellet fraction while GFP did not show such enrichment (*Fig. 3-4A*). We then took an “unorthodox” approach to study the compartment with which GFP-YopJ was associating under heat stress conditions. Cells expressing GFP or GFP-YopJ were lysed in detergent free, non-denaturing buffer and the lysates were separated into supernatants and pellets. Phase contrast microscopy showed that the pellets were mostly composed of large refractile membranous structures. Fluorescent microscopy showed that the GFP-YopJ signal associated with these membrane structures in
Figure 3-3. The first 70 amino acids of YopJ are required for the stress dependent subcellular redistribution. A) Yeast cells expressing the full length or the truncated versions of GFP-YopJ were grown for 36 hours in logarithmic phase and then subjected to heat shock. The cells were then fixed and analyzed by fluorescent microscopy. B) The percentage of cells expressing the YopJ variants in A was calculated and plotted. C) Summary of the deletion mutants which we generated in this work. (* $P < 0.05$ using student $t$-test)
Figure 3-4. YopJ undergoes a heat stress dependent enrichment in yeast membranous compartments. A) Yeast cells expressing GFP or GFP-YopJ were grown exponentially and then subjected to heat shock in a 42°C shaking water bath for 30 minutes or left unstressed. The cells were then lysed using bead beating in non-denaturing protein extraction buffer. Equal portions of the supernatants and pellets were loaded on an 8% SDS-PAGE gel and probed with a α-GFP antibody. B) Yeast cells expressing GFP or GFP-YopJ were subjected to heat shock as in (A) and were lysed using bead beating in detergent-free, non-denaturing extraction buffer lacking (Triton X-100). The pellets were mounted onto slides and analyzed by fluorescent microscopy. C) GFP-YopJ expressing yeast cells were pulsed with pHrodo for 5 minutes on ice, washed, and chased for 45 minutes in a 32°C incubator. The live cells were directly visualized using fluorescent microscopy. D) Yeast cells with GFP-YopJ being induced or un-induced were pulsed with FM4-64 for 5 minutes on ice before being washed and chased for 45 minutes at 32°C. The live cells were mixed with molten agar and loaded onto glass slides before being analyzed by fluorescent microscopy.
unstressed cells, and was enriched under stress conditions. Also, the enrichment consisted mainly of the “granulated” form of GFP-YopJ (Fig. 3-4B).

We then suspected that GFP-YopJ was associated with endosomal structures. We used a GFP-YopJ expressing strain in which the expression was driven by a weaker promoter (pREP41X). Fluorescent microscopy on this strain showed that YopJ was localizing to vacuole-like compartments under normal growth conditions. We utilized the pHrodo acid-sensitive fluorescent bulk-phase dye to study the localization of GFP-YopJ within the yeast cells. We found that when GFP-YopJ was expressed at low levels co-localizes with pHrodo*. Thus, GFP-YopJ traffics into acidified endosomal compartments (Fig. 3-4C). To test the functional significance of the endosomal localization of GFP-YopJ, we grew cells (expressing GFP-YopJ off a chromosomally integrated pJK plasmid) under GFP-YopJ inducing or non-inducing conditions. The cells were then pulsed with the membrane binding fluorescent dye FM4-64 for 5 minutes on ice. The cells were then washed and chased in dye-free media for 45 minutes to study the progression of endosomal trafficking. Our results show that the expression of GFP-YopJ causes the accumulation of immature endosomes with an abnormal appearance. This is consistent with a delay in the maturation of endosomes, as seen in Fig. 3-4D.

Collectively, our data indicates that YopJ undergoes a (heat) stress-dependent subcellular re-localization into membranous compartments. This stress dependency of the redistribution is dependent of the first 70 amino acids of YopJ.
Also, this redistribution is independent of the known catalytic and biological function of YopJ. Our results also indicate that YopJ trafficks into eukaryotic endosomal compartments and causes a delay in endosomal maturation when expressed in fission yeast.

3.3 Discussion

The pathogenesis of the *Yersinia* effector YopJ has been a mystery ever since its discovery. Early on, contradictory reports had showed that the YopJ mutant *Yersinia* was either fully virulent in a mouse model [77], or severely attenuated [90]. Even several years after identification of the trans-acetylase activity of YopJ (which was built on solid biochemical evidence) [67], this biochemical mechanism has never been shown to occur in the context of a cell-based infection without the need for over expressing YopJ.

Our yeast based experiments have shown that YopJ can undergo a rapid subcellular redistribution upon heat stress. This redistribution of the protein from a homogeneous to a punctate form occurred under heat shock. In the context of fission yeast, heat shock is a rapid variation of temperature between the permissive 32°C and the non-permissive 37°C or higher temperatures. The fact that this phenotype occurs at close to human body temperature (37-42°C) leads us to believe that this subcellular re-localization is relevant for mammalian infection conditions. We have also identified that the first 70 amino acids of YopJ are very important for this stress responsiveness. This is a very important finding
especially because YopJ is a Type III Secretion System effector. Typically, the type III secretion signal in other effectors (mainly YopE) had been mapped to a region within the first 70 amino acids of the protein [91]. This leads us to believe that there are multiple roles for the Type III Secretion System translocation signal in pathogenesis.

Our findings concerning the membrane localization of YopJ are exciting; however, they were not surprising. Taking into consideration that type III secretion effectors get translocated from the bacterial cell straight into the host cell, implies that the effectors will be in intimate contact with the host cell membrane at a certain point. In addition to this discovery, we found that this membrane localization was associated to a disruption of normal endocytic trafficking in the yeast cell expressing YopJ. We saw that while endocytosis actually occurs in yeast cells expressing YopJ, endocytic maturation is disrupted. Rather than the formation of normal mature late endosomes which fuse with lysosomes in the case of cells expressing GFP only, the cells expressing GFP-YopJ had endosomes which were abnormal in shape, size and localization after a 45 minute chase with FM4-64.

These yeast based findings however, should be approached with caution due to the nature of the model in which they were discovered. YopJ is a type III secretion substrate which is translocated directly into host cells. This translocation step is missing in our yeast models in which the cells express YopJ off a plasmid. The levels of expression in the yeast model are also much higher
than those translocated from *Yersinia* into the host cell upon infection. Also, it is important to remember that the fission yeast is not a normal host of *Yersinia* infection, so we should approach these data with caution until the results of our yeast based experiments have been evaluated in a mammalian cell infection system.

Collectively, our data suggest that YopJ has the ability to undergo a rapid subcellular re-localization which is dependent on the first 70 amino acids of its sequence. YopJ is also able to create a blockage on endocytic maturation in yeast cells. While the phenotypic analysis of this trafficking defect is clear, the molecular mechanisms underlying it remain unknown and are exciting new ventures for future research. Also, it will be interesting to try and link the observations made in the yeast concerning the YopJ mediated trafficking defect with the observations made in mammalian cells concerning maintenance of the *Yersinia* containing compartment.
Chapter 4: Conclusions

4.1 Summary

Our work has shown that the pathogen *Yersinia* creates a unique compartment along the macrophage border. This compartment is acidic and is limited by host cell membrane, while remaining exposed to the extracellular environment. While effector secretion is sufficient for creating this compartment, translocation is crucial for its maintenance. In fact, the translocated effector YopJ plays an important role in the maintenance of this compartment. This compartment gives the bacteria a permissive niche in which they can replicate along the macrophage border. Any disruption of this compartment be it through loss of a bacterial factor (YopJ) or through the addition of exogenous neutralizing agents led to a decrease in bacterial viability.

Our findings concerning the role of YopJ in the formation of the *Yersinia* containing compartment led us to study the biology of this effector using fission yeast as a model. Our studies have shown that YopJ is able to undergo a rapid subcellular redistribution upon heat stress. This stress dependent re-distribution is dependent on the first 70 amino acids of YopJ. We also found that YopJ associates with a yeast membranous structure. YopJ is also able to cause an endocytic trafficking defect in the yeast cells. Whether or not the yeast based YopJ data coincides with its role in YAC maintenance remains to be established.
4.2 Future perspectives and directions

Our work opens several doors for upcoming research topics. The nature of the membrane surrounding the bacterium along the macrophage border is worth looking into. Along those lines, it is common knowledge that *Yersinia* expresses 4 Type III Secretion System effectors that disrupt the actin cytoskeleton. This raises a major question as to how the *Yersinia* containing compartment is supported along the membrane. Thus, studying the host cell cytoskeletal scaffold which underlies the compartment is an interesting topic to pursue.

It is interesting to hypothesize that the interference of YopJ in normal endocytic trafficking in fission yeast and its involvement in the stabilization of the acidified *Yersinia* containing compartment are related. It will be exciting to pursue the yeast based findings with more in-depth analysis of the endocytic defect, specifically looking at the early-late endosome transition events. Also, these findings should be translated into the mammalian host to see whether or not YopJ disrupts endocytosis underneath the *Yersinia* containing compartment. Knowing that endocytosis is highly important for propagation of cytokine signaling in *Yersinia* infection (*data not shown*), it is interesting to study whether the YopJ endocytic defect is related to the canonical YopJ function of inhibiting pro-inflammatory cytokine signaling in infected cells.
References


