Discovery of chromosomal factors contributing to the virulence of *Yersinia pseudotuberculosis,* including Fis, a regulator of resistance to reactive oxygen species

A thesis

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ABSTRACT

All three pathogenic *Yersinia* species share a conserved virulence plasmid that encodes a Type 3 Secretion System (T3SS) and its effectors. This system contributes to growth and virulence in mammalian infection by injecting effector proteins into innate immune cells to dismantle their bactericidal functions. However, in the absence of this plasmid, the enteric pathogen *Yersinia pseudotuberculosis* (*Yptb*) retains the ability to colonize and grow within host organs. To uncover chromosomal factors that contribute to pathogenicity of *Yptb* in the absence of the virulence plasmid, we utilized a high-throughput, transposon screen (TnSeq). More than 30 genes, including many that were previously uncharacterized, were identified. Next, I developed a deep sequencing-based approach to validate and further characterize the phenotype of 18 of those genes in both WT and plasmid-deficient *Yptb* strains by infecting mixed pools of in-frame knockouts into immunocompetent mice. Fourteen mutants in the plasmid-deficient strain and 13 in the WT strain were attenuated for growth in livers, indicating that most of these factors were essential, even in the presence of the virulence plasmid. The growth of these mutants was assessed in immunocompromised mice to determine which of these factors disarm host defenses produced by innate immune cells. Mutants containing deletions of the *dusB-fis* operon, which encodes the nucleoid-associated protein Fis, were restored for growth in mice lacking neutrophils and inflammatory monocytes, two of the major cell types responsible for controlling *Yersinia* infection. This operon was not important for secretion or translocation of T3SS effectors, but protected *Yptb* from oxidative stress by regulating the transcription of several ROS-detoxifying genes. Strikingly, this protection was critical for virulence, as mice unable to produce ROS were more sensitive to

infection by *ΔdusB-fis* than WT mice. Notably, this is the first report of the requirement for Fis during *Yersinia* infection and also highlights a novel mechanism by which *Yptb* defends against ROS in mammalian tissues.

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cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
DNA	Deoxyribonucleic acid
GALT	Gut associated lymphoid tissue
H_2O_2	Hydrogen peroxide
Kan	Kanamycin
LPS	Lipopolysaccharide
MLN	Mesenteric lymph nodes
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
$O_2^{\bullet -}$	Superoxide
T3SS	Type 3 Secretion System
Yptb	*Yersinia pseudotuberculosis*

CHAPTER 1: INTRODUCTION

1.1 *YERSINIA* AND *YERSINIA PSEUDOTUBERCULOSIS*

1.1.1 Evolution, transmission, clinical presentation, and epidemiology

The gram-negative *Yersinia* genus contains three species that are pathogenic to humans: *Yersinia pestis,* the causative agent of the bubonic plague, as well as the enteropathogenic *Yersinia pseudotuberculosis* (*Yptb*) and enteropathogenic *Yersinia enterocolitica*. Whereas *Yptb* and *Y. enterocolitica* are predicted to have diverged from a common, non-pathogenic ancestor 41-186 million years ago, *Y. pestis* evolved more recently, diverging from a common ancestor of *Yptb* 1,500-20,000 years ago (Wren 2003, Zimbler, Schroeder et al. 2015). Because these three organisms share a close evolutionary history, a tropism for lymphoid tissue, and similar modes of cellular pathogenesis, *Y. enterocolitica* and *Yptb* have served as useful paradigms for studying some shared aspects of the pathogenesis of this genus, due to their safety and ease of manipulation in the lab. Because *Yptb* shares 97% of its genome with *Y. pestis*, it thus serves as a particularly useful model organism for studying genetic factors that may contribute to the pathogenesis of plague (Wren 2003). However, there are also a number of pronounced differences between these organisms, such as the ability of *Y. pestis* to modify its LPS at 37° C, and its expression of capsule and PLA protease (Montminy, Khan et al. 2006, Derbise, Chenal-Francisque et al. 2010, Zimbler, Schroeder et al. 2015). My thesis work has focused on *Yptb*, and thus information presented below will describe factors expressed by this organism.

Yptb is an enteric pathogen transmitted by the fecal-oral route of infection. Reservoirs of *Yptb* include swine, unpasteurized or improperly pasteurized dairy products, and water (Galindo, Rosenzweig et al. 2011). However, in rare cases, person-

to-person transmission of *Yptb* can occur (Benenson 1995). Clinical presentations of disease can range from acute gastroenteritis and mesenteric lymphadenitis, which are often accompanied by fever, abdominal pain, and bloody or watery diarrhea to, in individuals lacking healthy immune function, a more serious systemic disease (Long, Jones et al. 2010). In immunocompetent individuals, the GI manifestations of the disease are usually self-limiting; however, systemic infection with *Yptb* is characterized by surprisingly high mortality rates (Dube 2009). Between the years of 1997-2006, 10 U.S. states reported 18 cases of *Yptb* infection, 2 of which resulted in death of the patient (Long, Jones et al. 2010). However, many case of *Yptb* go unreported because they are mild or simply not cultured, or cases are initially misdiagnosed, as symptoms of the disease are often confused with those of acute appendicitis (Dube 2009).

1.1.2 Sites of host colonization

Following ingestion from a contaminated food or water source, *Yptb* is delivered to the GI tract, where it first colonizes the mucosal epithelium of the small intestine. From there, the bacteria can invade the Peyer's patches by way of M-cells, specialized microfold epithelial cells that normally function to sample invading microbes (Isberg and Barnes 2001). *Yptb* replicates extracellularly in the Peyer's patches, from which it may disseminate into other structures in the gut associated lymphoid tissue (GALT) network, specifically the mesenteric lymph nodes (MLN). The GALT is composed of lymphoid tissues containing several types of immune cells that are required for sampling, presenting and destroying invading pathogens (Pearson, Uhlig et al. 2012). GALT structures are lymph nodes, with distinct B and T cell zones, and circulating antigen-presenting cells (Brandtzaeg 2002). However, most GALT structures, ie the Peyer's

patches and cecal lymph tissues, do not contain afferent lymphatic vessels, which normally supply other types of lymphoid tissues with antigens for immunological stimulation. Rather, M cells serve as entry points for exogenous stimuli, by sampling microbes in the intestinal epithelium and delivering them to the Peyer's patches (Brandtzaeg 2002). Peyer's patches are aggregated lymphoid follicles having dome-like structures (Murphy, Travers et al. 2012). The Peyer's patches reside on the intestinal wall, between the lamina propria of the submucosa and the epithelium. Sampling by the Peyer's patches is believed to be critical for both the induction of a local immune response and for the development of adaptive mucosal immunity (Brandtzaeg, Baekkevold et al. 1999, Neutra, Mantis et al. 2001). Because of its lymphoid tissue tropism, *Yptb* has exploited the sampling properties of M cells and Peyer's patches to gain access to the GALT network.

From the Peyer's patches, *Yptb* can disseminate into the MLN, which are connected to the Peyer's patches by draining lymphatics (Murphy, Travers et al. 2012). The MLN are located in the connective tissue that tethers the intestine to the rear wall of the abdomen (Murphy, Travers et al. 2012). Like the Peyer's patches, MLN are part of the GALT tissue network and contain organized B and T cell zones and circulating antigen presenting cells (Brandtzaeg 2002). These cells are divided into three anatomical regions: the cortex, paracortex and the medulla. Interaction between B and T cells with antigen presenting cells in the MLN is crucial for establishing adaptive mucosal immunity.

Contrary to other models of enteropathogenic dissemination, the GALT does not serve as a primary reservoir for systemic dissemination of *Yptb* (Barnes, Bergman et al.

2006). Rather, the bacteria can disseminate directly from the small intestine to the bloodstream, which carries them to the more distal organ sites, such as the spleen and liver (Barnes, Bergman et al. 2006, Dube 2009). The spleen is a vascular, lymphocyte-rich organ that serves two major physiological purposes: production of immunological responses against blood-borne pathogens, and removal of aged or defective red blood cells from the circulation (Burkitt, Young et al. 1993). Like the Peyer's patches and MLN, the spleen possesses a highly organized architecture. It contains two major tissue regions, the red and the white pulp. The red pulp consists of a mixture of blood cells, including platelets, granulocytes, erythrocytes and plasma (Murphy, Travers et al. 2012). Infection can also lead to the recruitment of innate immune cells to the red pulp region of the spleen. The white pulp, which is separated from the red pulp by barriers referred to as marginal zones, consists almost exclusively of B and T cell lymphocytes, with specialized regions that function to promote lymphocyte-antigen presenting cell interactions (Murphy, Travers et al. 2012).

Blood-borne pathogens, including *Yptb*, also frequently colonize the liver. The major functions of the liver include storage and synthesis of important metabolic products, detoxification of metabolic waste products, destruction of spent red blood cells and the synthesis of critical components of plasma (Burkitt, Young et al. 1993). Unlike the spleen, which plays a direct role in alerting our immune system to blood-borne disease, the liver does not serve as a reservoir of circulating immune cells. However, infection of the liver can result in a pronounced immune response, usually originating from the interaction of the invading microorganism with sinusoidal endothelial cells, which have some antigen presenting functions (Knolle and Gerken 2000). Other resident

cells in the liver, including dendritic cells, Kupffer cells, liver-associated lymphocytes and perhaps even hepatocytes, can also contribute to immune clearance of invading pathogens (Knolle and Gerken 2000).

Yptb grows within tissue sites in extracellular aggregates, where bacteria come into close contact with responding immune cells (Simonet, Richard et al. 1990, Davis, Mohammadi et al. 2015). *Yptb* is also capable of growing intracellularly, and it has been proposed that intracellular growth within phagocytes may be important during early stages of infection (Pujol and Bliska 2005). Indeed, *Yptb* invades M cells to gain access to the Peyer's patches (Marra and Isberg 1997); however, there is no clear evidence to date that dissemination to other tissues or growth within these tissues requires an intracellular phase.

1.1.3 The Yersinia T3SS and translocation

1.1.3.i Genetic organization and conservation

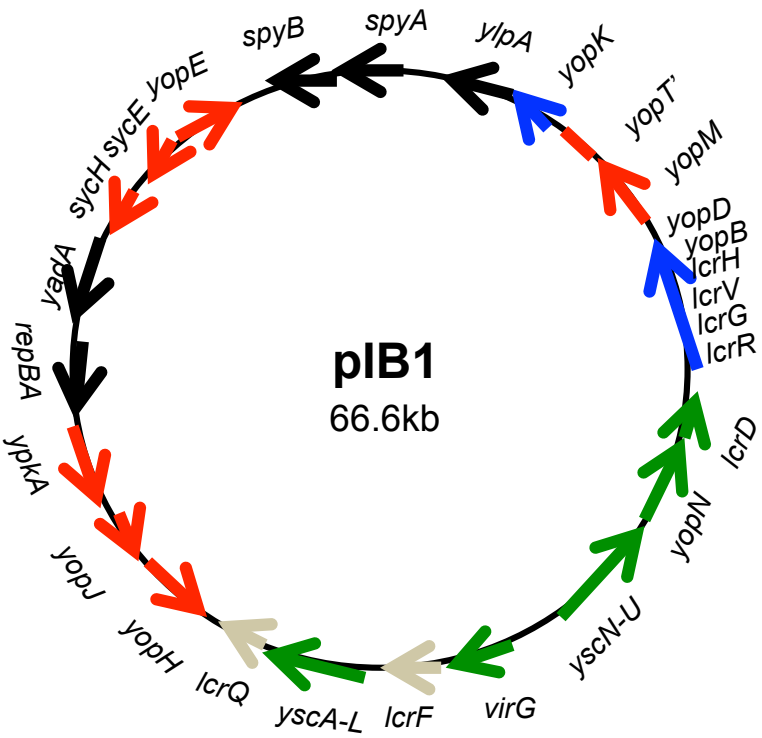
In order to colonize and grow in the sites of infection presented above, *Yptb* utilizes sophisticated tools to rapidly adapt to the mammalian host, attach to and invade M cells and to subvert the host immune response. Chief among these tools is a ~70-kb virulence plasmid (Fig 1-1). This plasmid, which is conserved across all three species of pathogenic *Yersinia*, is called pIB1 in *Yptb,* pYV in *Ye*, and pCD1 in *Yersinia pestis* (Gemski, Lazere et al. 1980) (Portnoy and Falkow 1981). The pIB1 and pCD1 plasmids share very similar genomes and replicative properties, whereas the pYV plasmid differs somewhat in the region coding its origin of replication (Portnoy and Falkow 1981). In all three species, the virulence plasmid encodes a Type 3 Secretion System (T3SS) and its associated effector proteins.

T3SSs are found in a large number of Gram-negative bacterial pathogens and symbionts, including pathogenic species of *Shigella, Salmonella, E. coli, Chlamydia* and *Pseudomonas* (Buttner 2012). T3SSs have been described as "injectisomes" and "needle and syringe"-like apparatuses because of their structure (Fig 1-2). They secrete a wide variety of proteinaceous substrates across both the inner and outer bacterial membranes. In addition, most T3SSs also transport substrates into a target eukaryotic cell membrane in the same step and, therefore actually transport proteins across three membranes. Secretion of T3SS substrates is generally thought to be a one-step process, although recently this notion has been challenged in *Yersinia* (discussed in the next section).

All known T3SSs share a conserved core of 9 proteins that are highly conserved among all known systems (Abrusci, McDowell et al. 2014, Burkinshaw and Strynadka 2014). They share 8 of these proteins with the flagellar apparatus found in many bacteria and are evolutionarily related to flagellin (Troisfontaines and Cornelis 2005). Two operons on pIB1, *ysaA-L* and *yscN-U* (Fig 1-1), encode the structural genes of the *Yptb* sorting platform, base, and needle components. A third operon, *lcrGVHyopBD,* encodes the translocon, while the effector proteins are scattered in either mono-cistronic or bi-cistronic operons.

Figure 1-1. The pIB1 virulence plasmid of *Yptb*

Yptb carries a conserved 66.6kb virulence plasmid. This plasmid encodes a T3SS, as well as its 5 associated effector proteins, and the adhesin *yadA*. Highly homologous plasmids, pYV and pCD1, are carried by *Y. enterocolitica* and *Y. pestis,* respectively.



Adapted from (Cornelis, Boland et al. 1998)

1.1.3.i T3SS machinery

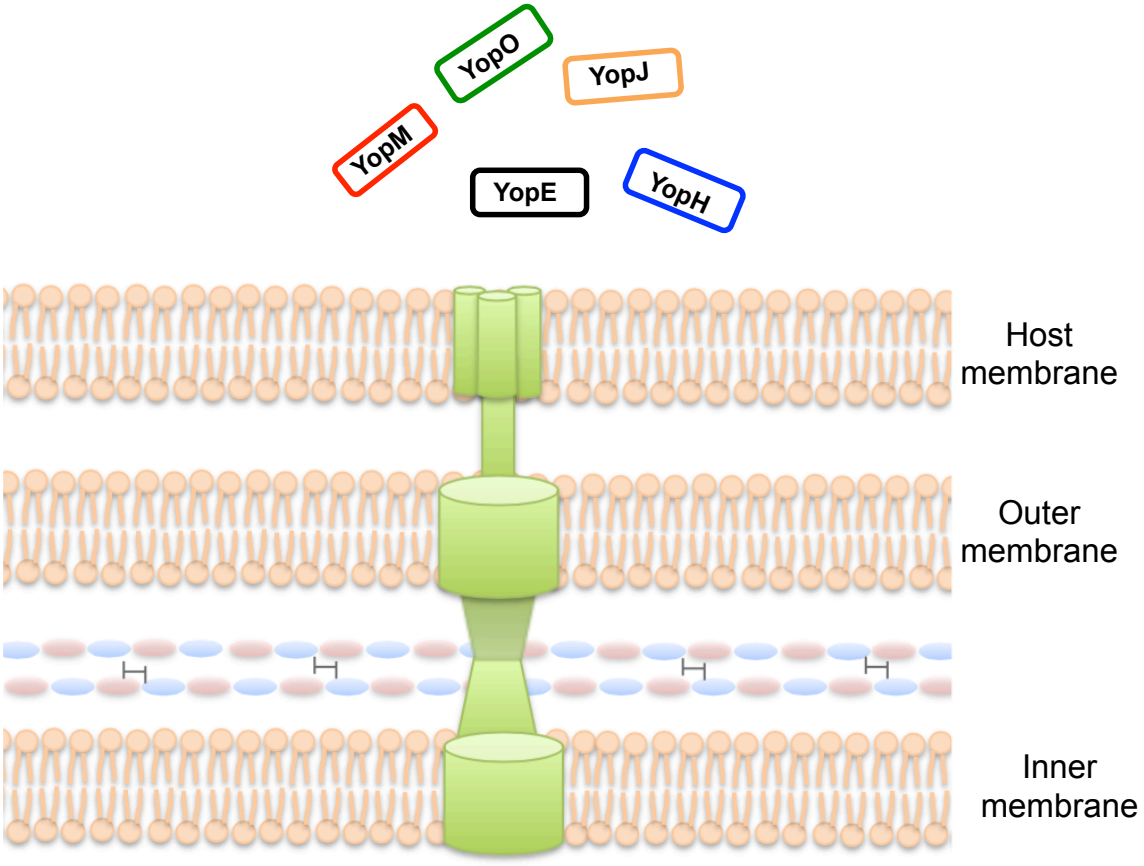
The T3SS can be broken down into three main components: a base complex or basal body, the needle component, and the translocon (Abrusci, McDowell et al. 2014). The base complex contains cytoplasmic components and spans the inner and outer membrane, forming a socket-like structure consisting of several rings with a center rod (Kubori, Matsushima et al. 1998). In most systems it is comprised of at least 15 proteins (Abrusci, McDowell et al. 2014, Burkinshaw and Strynadka 2014). Encased by and emanating from this socket and rod-like structure is a filament called the needle, which extends through the secretin and into the extracellular space (Kubori, Matsushima et al. 1998). The T3SS needle has an inner hollow core that is wide enough to permit an unfolded effector to traverse (Deane, Cordes et al. 2006, Demers, Habenstein et al. 2014). Excitingly, recent studies of the T3SSs of *Shigella* and *Salmonella* have visualized "trapped" effector proteins by cryo-EM and single particle analysis, supporting the model that substrates can traverse through the needle (Dohlich, Zumsteg et al. 2014, Radics, Konigsmaier et al. 2014).

While assembly of the T3SS needle-like structure is sufficient for the secretion of proteins into the extracellular space, upon contact with a eukaryotic cell, a translocon is assembled at the needle end. This assembly is dependent upon the "tip" protein LcrV, which is a pentamer that caps the needle and plays a role in regulating secretion of effectors (Price, Cowan et al. 1991, Harmon, Murphy et al. 2013). The translocon consists of two other proteins YopB, and YopD, which are directly inserted into the cytoplasmic membrane of the host cell to form a small pore. Following pore insertion, effector proteins, or Yops (*Yersinia* outer proteins) can be transported from the bacterial

cytosol through the base, needle, and translocon pore to be delivered directly to the host cell cytosol. Recently, however, a two-step model of translocation of Type 3 effectors has been proposed. In this model, the needle and/or tip sense contact with host cells and then effectors and translocon components, which have been sequestered in a protected niche on the outer surface (perhaps in lipid vesicles), are released from the outer surface, rapidly traverse the intercellular space, and form a pore through which effectors pass (Akopyan, Edgren et al. 2011, Edgren, Forsberg et al. 2012). However, additional experiments are needed to determine the mechanism by which translocation occurs.

Figure 1-2. The *Yersinia* T3SS

Yptb expresses a T3SS that translocates a number of effector proteins, called Yops, directly into the cytosol of a recipient eukaryotic cell. The T3SS spans the bacterial inner and outer membranes, as well as the host cell membrane and delivers effectors to the cell in a 1- or 2-step process.



1.1.3.iii Functions of T3SS Yop effector proteins in *Yersinia*

Following translocation into host cells, Yops inhibit host cell defenses, such as phagocytosis, ROS production, proinflammatory cytokine production and, in some cell types, act to promote cell death (Fallman, Andersson et al. 1995, Monack, Mecsas et al. 1997, Palmer, Hobbie et al. 1998, Songsungthong, Higgins et al. 2010) (Bliska and Black 1995). The catalytic functions of individual Yop proteins and their roles in promoting *Yersinia* survival and growth within a mammalian host have been well studied and are summarized briefly below, with a particular emphasis on functions that are important to consider for this work.

YopE

YopE is a GTPase activating protein (GAP). Upon injection into the infected host cell, YopE contributes to resistance to phagocytosis by disrupting the host's actin microfilament structure (Rosqvist, Forsberg et al. 1991). This is not a consequence of direct interaction between YopE with the actin network, but instead, results from YopE's ability to catalyze GTP hydrolysis by the Rho-family GTPases RhoA and Rac1, which serve as master regulators of actin cytoskeleton (Black and Bliska 2000, Von Pawel-Rammingen, Telepnev et al. 2000, Aepfelbacher 2004). Additionally, inactivation of the Rac2 GTPase by YopE inhibits oxidative burst in neutrophil-like cells infected with *Yptb* (Songsungthong, Higgins et al. 2010). *Yptb* strains containing deletions of *yopE* are attenuated for virulence in animal models of infection, however the functions and contributions of this protein to virulence vary by tissue site (Logsdon and Mecsas 2003, Songsungthong, Higgins et al. 2010). In *Yptb*, a number of point mutants have been constructed that separate YopE targets; using these point mutants, it was determined that

having only partial anti-phagocytic and ROS-inhibitory activity is sufficient for growth in the Peyer's patches, but not in the spleen, suggesting that different thresholds of YopE activities are required for growth in different tissue sites (Songsungthong, Higgins et al. 2010). Interestingly, the YopE of *Yptb* and *Y. pestis* contains an epitope recognized by $CD8^+$ T cells and, accordingly, infection by both pathogens induces a large YopE-specific $CD8^+$ T cell response (Lin, Szaba et al. 2011, Szaba, Kummer et al. 2014, Zhang, Mena et al. 2014).

YopH

YopH is a powerful phosphotyrosine phosphatase containing a eukaryotic phosphatase domain at its carboxy terminus and a phosphotyrosylpeptide-binding domain at its amino terminus (Cornelis 2002). A number of proteins involved in focal adhesion have been identified as targets of YopH in cell culture models (Cornelis 2002); other targets identified in cell culture models include paxillin, Lck, Fyb and SKAP-HOM (Cornelis 2002). Recently, it was demonstrated that YopH primarily targets the PRAM-1/SKAP-HOM and the SLP-76/Vav/PLCγ2 signal transduction axes of neutrophils during tissue infection; blocking this pathway resulted in abrogation of calcium flux and IL-10 induction in isolated neutrophils (Rolan, Durand et al. 2013). In addition to blocking calcium flux and IL-10 release, translocation of YopH into immune cells exerts a number of other physiological effects, including the suppression of phagocytosis and oxidative burst (Bliska and Black 1995, Andersson, Carballeira et al. 1996, Andersson, Magnusson et al. 1999). As a consequence, Δ*yopH* mutants are significantly attenuated for virulence in animal infection models (Logsdon and Mecsas 2003). Notably, the virulence of a Δ*yopH* mutant is restored in neutropenic mice, suggesting that the primary

role of this effector in infection by *Yptb* is to inhibit the actions of neutrophils (Rolan, Durand et al. 2013).

YopM

The YopM protein is a member of the family of leucine-rich-repeat (LRR) effector proteins. Deletions of *yopM* in pathogenic *Yersinia spp.* result in dramatic loss of virulence in mouse models of *Yersinia* infection (Leung, Reisner et al. 1990, McPhee, Mena et al. 2010); however its direct mode of action is still largely unknown. Once inside the cytoplasm of host cells, YopM at least partly traffics to the nucleus. This trafficking may depend on interaction between YopM and two mammalian serine-threonine protein kinases, RSK1 and PRK2 (McDonald, Vacratsis et al. 2003, McCoy, Marre et al. 2010). Additionally, during infection of LPS-primed murine macrophages, YopM inhibits activation of caspase-1 through an unknown mechanism (LaRock and Cookson 2012). This inhibition is at least partially responsible for the virulence of *Yptb*, as infection of caspase-1/11$^{-/-}$ mice rescues the virulence of a Δ*yopM* strain (LaRock and Cookson 2012, Chung, Philip et al. 2014). Recent studies have shown that another translocated effector, YopJ, may collaborate with YopM to inhibit caspase-1 dependent inflammatory responses during infection of *Y. pestis* and *Yptb* and that, accordingly, strains lacking both effectors are significantly more attenuated than single deletion mutants during murine infection (Ratner, Orning et al. 2016, Schoberle, Chung et al. 2016).

YopO

YopO, also referred to as YpkA, is a multifunctional protein with guanine nucleotide dissociation inhibitor (GDI) and serine-threonine kinase domains that modulates the actin cytoskeleton dynamics of infected host cells (Cornelis 2002). Though

the mechanism of YopO action remains unclear, it has been shown to target actin, Rho, Rac, otubain 1, Gαq-dependent signaling pathways, and vasodilator-stimulated phosphoprotein (VASP) (Barz, Abahji et al. 2000, Aepfelbacher 2004, Navarro, Koller et al. 2007, Trasak, Zenner et al. 2007, Groves, Rittinger et al. 2010, Ke, Tan et al. 2015, Lee, Grimes et al. 2015). These actions function to prevent phagocytic uptake, and are important for virulence, as strains of *Yptb* lacking *yopO* are attenuated in mouse models of infection (Logsdon and Mecsas 2003).

YopJ

While the enzymatic activity of YopJ remained largely unclear for some time, recent work suggests that this protein functions as a serine-threonine actetyltransferase (Paquette, Conlon et al. 2012). This activity allows YopJ to acetylate various mitogen activated protein kinase kinases (MAP2Ks), which blocks their activities (Orth 2002, Paquette, Conlon et al. 2012). However, in macrophages, inhibition of NF-κB and MAPK pathways by YopJ also drives caspase-8 and receptor-interacting protein (RIP) kinase-mediated activation of caspase-1, leading to cell death (Philip, Dillon et al. 2014, Weng, Marty-Roix et al. 2014). Interestingly, mice lacking caspase-8 and RIPK3 are more susceptible to *Yersinia* infection, suggesting that the effects of YopJ on this pathway may actually induce a more potent immune response. There is also evidence that YopJ acetylates critical sites in the RICK and TAK1 kinases, which serve as mediators of Nod1 signaling, in order to induce intestinal barrier dysfunction (Meinzer, Barreau et al. 2012). Additionally, a recent report demonstrated that YopJ of *Yptb* blocks TRIF signaling in macrophages and dendritic cells, resulting in decreased production of type I IFN and other pro-inflammatory cytokines (Rosadini, Zanoni et al. 2015). Likely as a result of

YopJ's ability to both suppress and activate different arms of the innate immune response, Δ*yopJ* mutants are mostly virulent in *Yersinia* infection models (Galyov, Hakansson et al. 1994, Lemaitre, Sebbane et al. 2006, Rosadini, Zanoni et al. 2015); however, mild defects in the virulence of a Δ*yopJ* mutant have been observed at late time points in systemic tissues sites following oral-gastric infection (Philip, Dillon et al. 2014).

In summary, the Yops have many important activities in phagocytic and epithelial cells that can alter mammalian immune responses to infection. Of particular relevance to my studies, however, are the findings that YopE and YopH can block ROS production in neutrophils and macrophages.

1.1.3.iii Bacterial factors that influence effector translocation: The Adhesins

In order to achieve high levels of effector translocation, *Yersinia* must establish close contact with recipient cells. This binding is largely mediated by specialized proteins called adhesins. Adhesins play important roles in mediating the binding of both commensal and pathogenic bacteria to eukaryotic host cells (Kline, Falker et al. 2009). This binding can occur via direct contact between adhesins and host cell receptors or through the interactions of adhesins with soluble macromolecules or ligands that bridge bacteria to eukaryotic cells (Kline, Falker et al. 2009). These adhesive surface structures are found in both gram-negative and gram-positive bacteria and can range in size from monomeric surface-bound proteins to multi-component apparatuses like the Type IV pili of *E. coli* (Kline, Falker et al. 2009). While it was originally believed that adhesion to host cells was only important for establishing colonization and avoiding clearance by peristalsis and mucosal secretions, recent work has highlighted the important roles

adhesins play in modulating innate immune responses, either directly, via their interactions with immune receptors, or indirectly, by facilitating interactions with host cells that promote delivery of effector proteins and toxins that modulate immune responses (Kline, Falker et al. 2009).

Yptb encodes a number of adhesins, the best-studied being Invasin, pH 6 antigen, Ail, and YadA (Leo and Skurnik 2011). Importantly, clones containing mutations in genes encoding Invasin and pH 6 antigen were highly attenuated in the transposon mutant screen performed in Chapter 2 of this work (Crimmins, Mohammadi et al. 2012); these findings indicate that these adhesins are important for virulence even in the absence of Type 3 effector translocation. The known functions of these proteins and their roles in promoting colonization and survival of *Yptb* during infection are summarized briefly below.

Invasin

Invasin is an outer membrane protein consisting of an N-terminal signal peptide, followed by a transmembrane β-barrel domain and a C-terminal (extracellular) rod-like structure containing 3-4 all β-barrel domains (Hamburger, Brown et al. 1999). Invasin binds β1-integrin receptors on eukaryotic cells to facilitate adhesion and invasion (Isberg, Voorhis et al. 1987, Isberg and Leong 1988, Leong, Fournier et al. 1990, Isberg and Van Nhieu 1995). *Yptb* expresses Invasin initially during infection, in order to mediate passage through M cells that line the small intestine (Marra and Isberg 1997, Clark, Hirst et al. 1998). In addition to M cells, Invasin confers binding to cultured epithelial cells, and also induces aggregation of platelets *in vitro* (Simonet and Falkow 1992, Simonet, Triadou et al. 1992). Binding of Invasin to β1-integrin receptors normally results in

bacterial internalization. This process has been well studied and occurs as follows: binding of invasin to β1-integrin receptors results in clustering of the receptors, which, in turn, induces a cascade of signaling that ultimately leads to formation of adhesion foci containing activated Fac and Src proteins (Alrutz and Isberg 1998, Isberg, Hamburger et al. 2000, Weidow, Black et al. 2000). Formation of these foci induces actin cytoskeleton rearrangements, which allow for uptake of the Invasin-expressing bacteria (Isberg, Hamburger et al. 2000).

Invasin is optimally expressed at 26°C, and thus the bacteria are primed for adhesion upon reaching the small intestine (Isberg, Swain et al. 1988, Ellison, Young et al. 2003, Grassl, Bohn et al. 2003, Ellison and Miller 2006). Expression is inhibited at 37°C by RovA, and as a consequence, it is believed that Invasin's major role in infection is to promote survival in the GI tract at early stages of infection, as well as to allow bacteria to breach the intestinal epithelium and reach the Peyer's patches (Pepe and Miller 1993, Marra and Isberg 1996, Mecsas, Bilis et al. 2001, Handley, Newberry et al. 2005, Hudson and Bouton 2006). Additionally, Invasin has been shown to collaborate with other adhesins to promote effector translocation by *Yptb* both *in vitro* and *in vivo*, suggesting that its expression may be sufficiently high at later stages of *Yptb* infection to promote this phenotype (Maldonado-Arocho, Green et al. 2013). Notably, *inv* is not expressed by *Y. pestis*, due to a frameshift mutation in the gene's coding region (Simonet, Riot et al. 1996).

pH 6 antigen

pH 6 antigen is a multi-component outer-membrane pilus-like structure that is most highly expressed at 37°C under low pH conditions (Price, Freeman et al. 1995). In

Y. pestis, pH 6 antigen can bind to glycosphingolipids and phosphotidylcholine on host cells (Payne, Tatham et al. 1998, Galvan, Chen et al. 2007) and is important for Yop delivery and virulence (Felek, Tsang et al. 2010) (Cathelyn, Crosby et al. 2006). Studies in *Y. pestis* have also demonstrated that pH 6 antigen binds apolipoprotein B (a low density lipoprotein present host serum) (Makoveichuk, Cherepanov et al. 2003). One proposed model for the significance of this interaction is that *Y. pestis* may use pH 6 antigen-mediated binding of host low-density lipoproteins to avoid phagocytic recognition (Huang and Lindler 2004).

In *Yptb*, the pH 6 antigen complex is expressed from the *psaABCEF* locus, where PsaE and PsaF positively regulate expression of the three structural components PsaA-C, and loss of either of these positive regulators abrogates pH 6 antigen activity (Yang, Merriam et al. 1996). Expression of pH 6 antigen by *Yptb* promotes agglutination of erythrocytes and facilitates thermoinducible cell binding *in vitro* (Yang, Merriam et al. 1996); however, prior to work presented in Chapter 3 of this thesis and in our 2012 publication (Crimmins, Mohammadi et al. 2012), no further evaluations of the role of pH 6 antigen in *Yptb* virulence had been reported.

Ail

Ail is an OmpX family outer membrane protein with an 8-strand β-barrel fold, containing 4 extra-cellular loops and 8 membrane-spanning domains (Vogt and Schulz 1999, Fernandez, Hilty et al. 2001, Yamashita, Lukacik et al. 2011). Ail is homologous to OmpX of *Escherichia coli* and Rck and PagC of *Salmonella enterica* (Pulkkinen and Miller 1991, Heffernan, Wu et al. 1994, Mecsas, Welch et al. 1995). Studies of Ail in the all three pathogenic *Yersinia* species have revealed a role for Ail in host cell invasion and

binding, which is believed to mediate delivery of translocated effector proteins into the host cell (Miller, Beer et al. 2001, Bartra, Styer et al. 2008, Tsang, Felek et al. 2010), (Felek and Krukonis 2009, Maldonado-Arocho, Green et al. 2013) (Paczosa, Fisher et al. 2014). Studies of Ail in *Y. enterocolitica* and *Yptb* have demonstrated a role for Ail in blocking membrane attack complex (MAC) formation by complement proteins, and it is believed that this may occur through the binding of Ail to Factor H ad C4 binding protein (Biedzka-Sarek, Salmenlinna et al. 2008, Kirjavainen, Jarva et al. 2008, Paczosa, Fisher et al. 2014). In addition to blocking MAC formation by complement, inhibition of complement signaling by Ail may also prevent opsonophagocytosis *in vivo* (Bartra, Styer et al. 2008, Hinnebusch, Jarrett et al. 2011). Additionally, a recent report found that Ail of *Y. pestis* binds the host extracellular matrix protein vitronectin; this binding allows the Pla protease to cleave vitronectin, which may enhance adherence to eukaryotic cells and/or evasion of complement-dependent killing (Bartra, Ding et al. 2015).

YadA

YadA is a homotrimeric protein belonging to the trimeric autotransporter adhesin family. The protein has a lollipop-like structure, with a globular head that extends from a coiled-coiled stalk(Koretke, Szczesny et al. 2006). YadA, unlike the other adhesins described above, is encoded by the *Yersinia* virulence plasmid (Cornelis, Boland et al. 1998). Like Invasin, this protein is not expressed by *Y. pestis,* which contains a mutation in the *yadA* coding region (Skurnik and Wolf-Watz 1989). YadA has several predicted functions. As an adhesin, it confers binding to extracellular matrix (ECM), collagen, fibronectin and laminin, with the preferred ligand of *Yptb* YadA being fibronectin (Hudson, Bliska et al. 2005, Heise and Dersch 2006). *Yptb* YadA promotes adhesion to

and invasion of cultured epithelial cells, and it is thought that this occurs through the bridging of YadA to β1-integrin receptors through its binding partners (Eitel and Dersch 2002). YadA also has affinity for itself and can therefore induce autoagglutination of *Yptb in vitro* (Balligand, Laroche et al. 1985, Skurnik and Wolf-Watz 1989).

In *Y. enterocolitica,* YadA plays an important role in conferring resistance to complement-mediated killing; however, this function has not been demonstrated in *Yptb* (Balligand, Laroche et al. 1985, Biedzka-Sarek, Salmenlinna et al. 2008). Additionally YadA plays a key role in promoting translocation of Yops into target cells via its adhesive properties, as mutants lacking YadA and one or more additional adhesins are less efficient at translocating Yops into cultured epithelial cells and *in vivo* (Boyd, Grosdent et al. 2000, Mejia, Bliska et al. 2008, Maldonado-Arocho, Green et al. 2013, Paczosa, Fisher et al. 2014).

1.1.4 Virulence of T3SS and Yop mutants

As stated above, expression and translocation of Yops is critical for successful infection of *Yptb.* In fact, strains of *Yptb* that lack one or more effector proteins are highly attenuated for virulence by the oral, intravenous, and intraperitoneal routes of infection, with many of these mutants defective for colonizing and growing within intestinal and lymphoid tissue sites (Logsdon and Mecsas 2003). Accordingly, strains of *Yptb* lacking multiple adhesins, which are impaired for Yop translocation, are also highly attenuated for virulence (Maldonado-Arocho, Green et al. 2013).

However, strikingly, strains of *Yptb* lacking one or more Yop proteins are frequently more attenuated for infection in certain tissue sites than strains lacking the T3SS or the virulence plasmid completely (Logsdon and Mecsas 2003, Balada-Llasat and

Mecsas 2006). Plasmid-deficient *Yptb* colonizes the Peyer's patches and MLN at comparable levels to WT, but is defective in colonizing the GI tract, spleen, and liver following oral infection (Balada-Llasat and Mecsas 2006). By contrast, a *Yptb* strain lacking all 5 Yop effector proteins (*ΔyopHEMOJ*) colonizes and persists in the Peyer's patches and MLN at significantly lower levels than WT (Logsdon and Mecsas 2003). One reason that multiple Yop mutants are highly attenuated for infection may be that expression of the T3SS and insertion of pore into host cell membranes induces high levels of inflammation and immune cell recruitment (Balada-Llasat and Mecsas 2006, Auerbuch, Golenbock et al. 2009, Crimmins, Mohammadi et al. 2012, Davis, Mohammadi et al. 2015). While *Yptb* containing a full complement of Yops may be equipped to deal with this influx of immune cells, Yop mutants are unable to subvert the bactericidal actions of these cells. By contrast, pIB1$^-$ *Yptb* induces a much weaker immune response than WT during growth within the MLN (Balada-Llasat and Mecsas 2006, Crimmins, Mohammadi et al. 2012); therefore, this strain does not depend on Yop proteins to withstand host restriction.

Additional studies have demonstrated that pIB1$^-$ *Yptb* can also successfully colonize and persist in systemic tissue sites when plasmid-deficient bacteria are infected by the intravenous route (Simonet, Mazigh et al. 1984, Une and Brubaker 1984). Notably, this route of infection bypasses colonization of the small intestine (a major reservoir for bacterial dissemination to the bloodstream), where the plasmid-deficient bacteria cannot survive (Barnes, Bergman et al. 2006). These data suggest that persistence of virulence plasmid-deficient *Yptb* is not limited to the GALT, and may imply a broader mechanism whereby virulence plasmid-deficient bacteria are capable of persisting, or "hiding out" in

protective or non-inflammatory tissue sites in the infected animal. In fact, plasmid-deficient mutants are defective at colonizing lymphoid tissue following oral infection of mice lacking B and T cells, suggesting that the plasmid-deficient strain of *Yptb* may have a tropism for the B and T cell zones of the MLN (and potentially of other tissue sites), where it replicates extracellularly; however these mice also lacked Peyer's patches, so the defects observed could also be attributed to a failure in reaching the MLN (Balada-Llasat and Mecsas 2006).

This mechanism of T3SS-independent persistence may be indicative of the evolutionary history of *Yersinia* pathogenesis. It is widely believed that a common, non- or mildly pathogenic ancestor of *Yptb* and *Ye* acquired the pYV virulence plasmid prior to their evolutionary divergence (Wren 2003). This ancestor may have served as a commensal organism, colonizing tissue sites at low levels. Acquisition of the T3SS-containing virulence plasmid may have enabled the bacteria to spread to and survive in additional tissue sites with phagocytic cells, including the mammalian small intestine. Whether *Yersinia* ever established a niche in lymphocyte-rich tissue sites in its evolutionary history is speculation; nonetheless, possession of an intact *Yptb* chromosome appears to be sufficient for colonization of and persistence in lymphocyte-rich tissue sites. In addition to the experimental data outlined above, several patients have been hospitalized with infections caused by plasmid-deficient *Yersinia spp* (Fukushima, Sato et al. 1991, Grant, Bennett-Wood et al. 1998). While the majority of these cases resulted from pYV$^-$ *Y. enterocolitica* infection, at least one case was the result of infection with a plasmid-deficient *Yptb* strain (Fukushima, Sato et al. 1991). Taken

together, these data suggest that chromosomal factors are critical contributors to the colonization and growth of *Yptb* in a mammalian host.

1.1.4 Contributions of chromosomal factors to Yersinia pathogenesis

A number of transposon mutagenesis screens have been performed to identify chromosome-encoded virulence factors in the pathogenic *Yersinia spp.* (Darwin and Miller 1999, Karlyshev, Oyston et al. 2001, Mecsas, Bilis et al. 2001, Flashner, Mamroud et al. 2003, Palace, Proulx et al. 2014). Examples of known chromosomal virulence factors of *Yersinia* include the adhesins Invasin, Ail, and pH 6 antigen, the PhoP/PhoQ regulatory system, transcriptional regulators RovA and IscR, the nitric oxide detoxifying protein Hmp, and the cytotoxic necrotizing factor CNFY (Ben-Efraim and Bichowsky-Slomnichi 1964, Isberg, Voorhis et al. 1987, Wren, Olsen et al. 1995, Cathelyn, Crosby et al. 2006, Schweer, Kulkarni et al. 2013, Miller, Kwuan et al. 2014, Davis, Mohammadi et al. 2015). However, prior to our 2012 publication (Crimmins, Mohammadi et al. 2012), no *Yersinia* screens had been performed using a transposon library with a complexity of more than 2,000 insertion mutants, suggesting the potential for discovery of many novel chromosome-encoded virulence factors. Therefore, the work presented in Chapter 3 of this thesis specifically sought to identify chromosomal virulence factors by exploiting the observation that strains lacking the T3SS can colonize and grow within systemic tissue sites.

1.2 HOST RESPONSE TO *YERSINIA* INFECTION

1.2.1 Innate immune recognition of Yersinia

The mammalian immune system has evolved specialized mechanisms for recognizing and responding to invading microbes at early stages of infection. These sensing mechanisms generally involve the detection of distinct structures on the surface of microorganisms, often referred to as pathogen associated molecular patterns (PAMPS), by host encoded receptors present on a variety of innate immune cells. Bacterial structures recognized by these pattern recognition receptors (PRRs) include the lipid A component of bacterial lipopolysaccharide (LPS), flagellin, and peptidoglycan. Recognition of one or more of these structures by a PRR activates a cascade of signaling that ultimately induces the transcription of inflammatory cytokines, which, upon secretion, recruit immune cells to the site of infection. The pathogenic *Yersinia* are recognized by a number of PRRs, including TLR4, TLR1, TLR6 and TLR2 (Zhang and Bliska 2003, Montminy, Khan et al. 2006, DePaolo, Kamdar et al. 2012, Vladimer, Weng et al. 2012).

However, because aberrant or unnecessary activation of TLR-dependent pathways by non-pathogenic, commensal bacteria can be damaging to the mammalian host, additional receptors, which directly sense infection by pathogenic bacteria, may act in concert with PRRs to recruit a robust immune response capable of clearing infection of a pathogen. For example, certain cytosolic receptors and signaling proteins recognize products secreted by bacterial pathogens or the actions of effector proteins themselves (Broz, Newton et al. 2010, Burdette, Monroe et al. 2011, Zhao, Yang et al. 2011, Philip, Dillon et al. 2014, Weng, Marty-Roix et al. 2014); activation of these pathways leads to assembly of the inflammasome complex. Further, remnants of necrotic cells (called

damage associated molecular patterns, or DAMPs) can also stimulate immune responses (Kono and Rock 2008).

Additionally, there is evidence that the pore formed by the *Yersinia* translocon induces a potent immune response during tissue infection. Initiation of translocation by *Yptb* induces the expression of NF-kB and Type I interferon-regulated genes in the host cell(Auerbuch, Golenbock et al. 2009). This induction is dependent on pore formation, and not simply T3SS expression, as a pore-forming mutant (*ΔyopB* or *ΔyopD*) fails to induce expression of these genes (Auerbuch, Golenbock et al. 2009). Further, whereas strains of *Yptb* lacking the T3SS induce very little inflammation or necrosis in infected lymphoid tissue sites, infection of these tissue sites with WT *Yptb* results in a pronounced increase in inflamed, necrotic foci (Balada-Llasat and Mecsas 2006). While activation of NF-kB and Type I interferon by T3SS-dependent pore formation is TLR-independent (Auerbuch, Golenbock et al. 2009), activation of other immune signaling pathways by pathogenic *Yersinia* sometimes requires collaboration between TLR and these cytosolic, "infection-sensing" receptors. For example, NLRP12 activation by *Yptb,* which results in inflammasome assembly and pro-inflammatory cytokine production, is dependent on both TLR4 activation and Yop translocation (Vladimer, Weng et al. 2012). Likewise, detection of YopJ activity by a RIP1-caspase-8 or RIP3-dependent caspase-1 activation pathway is also TLR4 dependent (Philip, Dillon et al. 2014, Weng, Marty-Roix et al. 2014).

1.2.2 Phagocyte restriction of Yersinia infection

Yersinia encounter a number of phagocytic cells subsets, including neutrophils, inflammatory monocytes, macrophages, and dendritic cells, during mammalian tissue

infection (Balada-Llasat and Mecsas 2006, Crimmins, Mohammadi et al. 2012, Westermark, Fahlgren et al. 2014, Davis, Mohammadi et al. 2015). These cells serve as the primary targets of Yop translocation in both mammalian infection models and "*ex vivo"* tissue analyses (Marketon, DePaolo et al. 2005, Koberle, Klein-Gunther et al. 2009, Durand, Maldonado-Arocho et al. 2010), and also act as important mediators of *Yersinia* clearance. The functions of these cells and their interactions with pathogenic *Yersinia* and *Yptb,* in particular, are summarized below.

1.2.2.i Neutrophils

Neutrophils, also called polymorphonuclear cells (PMNs), are the most abundant human immune cells and serve as the first line of defense against invading pathogens. These cells are short-lived and normally develop in the bone marrow from haematopoietic stem cells in a process called granulopoiesis (Bugl, Wirths et al. 2012). Mature neutrophils are characterized by their segmented nucleus and granules, which are filled with more than 700 proteins (Rorvig, Ostergaard et al. 2013). During microbial infection, these cells migrate from the bloodstream into infected tissue sites, through a process called extravasation (Kolaczkowska and Kubes 2013). Neutrophils utilize a number of factors to facilitate bacterial clearance, including phagocytosis of bacteria, release of extracellular traps (NETs), chelation of iron, release of the bactericidal contents of granules, and production of toxic reactive oxygen and nitrogen species (Kruger, Saffarzadeh et al. 2015).

The interactions between *Yptb* and neutrophils have been well characterized. Histological studies have shown that neutrophils are the primary cells in contact with *Yptb* during infection of lymphoid tissue sites (Crimmins, Mohammadi et al. 2012, Davis,

Mohammadi et al. 2015) (Fig 1-3). Accordingly, these cells are the most abundant targets of *Yptb* translocated effectors (Durand, Maldonado-Arocho et al. 2010). In fact, depletion of neutrophils from mice with the Ly6G-specific antibody 1A8 restores growth of mutants lacking YopH, which targets the PRAM-1/SKAP-HOM and SLP-76/ Vav/PLCg2 signal transduction pathways in these cells (Rolan, Durand et al. 2013). Interestingly, while the attenuation of specific mutant strains is restored in neutropenic mice, these mice are no more susceptible to WT *Yptb* infection (Rolan, Durand et al. 2013), suggesting that other phagocytic cells must collaborate with neutrophils to restrict infection by this bacterium.

1.2.2.ii The mononuclear phagocytes: macrophages, monocytes, and dendritic cells

Macrophages, monocytes, and dendritic cells are members of the mononuclear phagocyte system (MPS); these cells are found throughout the body and exhibit many diverse functions depending on their developmental origin and tissue tropisms, as detailed below:

Macrophages

In general, macrophages are distinguished as large vacuolar cells that specialize in clearing debris, apoptotic cells, and microbial pathogens. In mice, macrophages are often phenotypically defined by the presence of specific markers, including F4/80 and Cd68 (Martinez-Pomares, Platt et al. 1996). Generally, these cells derive from embryonic progenitors; once circulation is established they spread into the blood and reach peripheral tissues sites, where they differentiate and are predominantly maintained through self-renewal (Yona, Kim et al. 2013). Additionally, monocytes recruited to tissue

sites are capable of differentiating into macrophages in response to inflammation; however, it is unclear whether these "new" macrophages possess functions similar to resident macrophages (Jakubzick, Gautier et al. 2013, Epelman, Lavine et al. 2014). Examples of resident tissue macrophages include liver Kupffer cells, lung alveolar macrophages, splenic macrophages, and peritoneal macrophages (Yona, Kim et al. 2013).

The interactions between pathogenic *Yersinia* and macrophages have been well defined in a variety of *in vivo* and tissue culture system models. The effectors YopJ and YopM prevent activation of caspase-1 by *Yptb* in cultured macrophages, and these activities are critical for infection, as mutants lacking both effectors are attenuated in murine infection (Chung and Bliska 2016) (LaRock and Cookson 2012, Ratner, Orning et al. 2016). Interestingly, YopJ can also indirectly trigger caspase-1 activation in naïve macrophages, where acetylation of MAPK kinases by this protein ultimately drives caspase-8 and RIP3 kinase-mediated caspase-1 activation (Philip, Dillon et al. 2014, Weng, Marty-Roix et al. 2014). Mice lacking both caspase-8 and RIP3-kinase are more susceptible to infection by *Yersinia*, indicating that macrophage-mediated detection of *Yersinia* infection is critical for controlling infections (Philip, Dillon et al. 2014, Weng, Marty-Roix et al. 2014).

During infection of red pulp regions in the spleen, extracellular microcolonies of *Yptb* are surrounded by and in direct contact with neutrophils; however $Cd68^{pos}$ cells are also observed at sites of infection, usually outside of the neutrophil populations (Fig 1-3); these $Cd68^{pos}$ cells are iNOS-positive and were shown to restrict *Yptb* growth through the release of nitric oxide (Davis, Mohammadi et al. 2015) (Fig 1-3). Because Cd68 can be

found on monocytes and macrophages (Iqbal, McNeill et al. 2014), this marker could potentially represent either or both populations (Davis, Mohammadi et al. 2015).

Monocytes

Unlike some tissue-resident macrophages, all monocytes arise from precursor cells in the bone marrow, called common monocyte progenitors, which are committed to the monocyte lineage (Yona, Kim et al. 2013). Monocytes possess several markers that are common across most subsets, including CD11b, F4/80, and Cd68 (Rose, Misharin et al. 2012, Iqbal, McNeill et al. 2014). Once released into the bloodstream as non-dividing cells, monocytes can traffic to different tissue sites. In general, there are two subsets of monocytes present in the bloodstream of mice: a short-lived, $CX_3CR1^{lo}CCR2^{pos}Gr1^{pos}$ "inflammatory monocyte" subset, which is recruited by CCL2 to inflamed tissue sites, and a longer-lived $CX_3CR1^{hi}CCR2^{neg}Gr1^{neg}$ "resident monocyte" subset, which is recruited to non-inflamed sites in a CX_3CR1-dependent manner (Geissmann, Jung et al. 2003). Upon reaching tissue sites, these cells either maintain their phenotype or further differentiate depending on the tissue microenvironment in which they reside. For example, inflammatory monocytes can differentiate into inflammatory dendritic cells within 18 hours of reaching inflamed tissues sites (Geissmann, Jung et al. 2003); monocytes are also capable of differentiating into macrophages (Jakubzick, Gautier et al. 2013).

Several studies suggest that $CX_3CR1^{lo}CCR2^{pos}Gr1^{pos}$ "inflammatory monocytes" are important for controlling *Yersinia* infection. First, mice depleted of all cells containing the Gr1 epitope, which include inflammatory monocytes, neutrophils, activated T cells and endothelial cell subsets (Daley, Thomay et al. 2008), are more

highly colonized with *Yptb* than those lacking just $Ly6G^{pos}$ neutrophils, indicating that a $Gr1^{pos}Ly6G^{neg}$ cell type collaborates with neutrophils to control *Yersinia* infection (Keith 2016). Additionally, $Gr1^{pos}$ cells surround *Yptb* microcolonies in the red pulp of spleens, suggesting that inflammatory monocytes may also be present alongside neutrophils and macrophages in these areas of inflammation (Keith 2016) (Fig 1-3). Furthermore, a study with *Y. pestis* demonstrated that the virulence of a Δ*yopM* mutant was restored in CCR2-/- mice, suggesting that inflammatory monocytes are targeted directly or indirectly by this effector during tissue infection (Ye, Uittenbogaard et al. 2011).

Dendritic cells

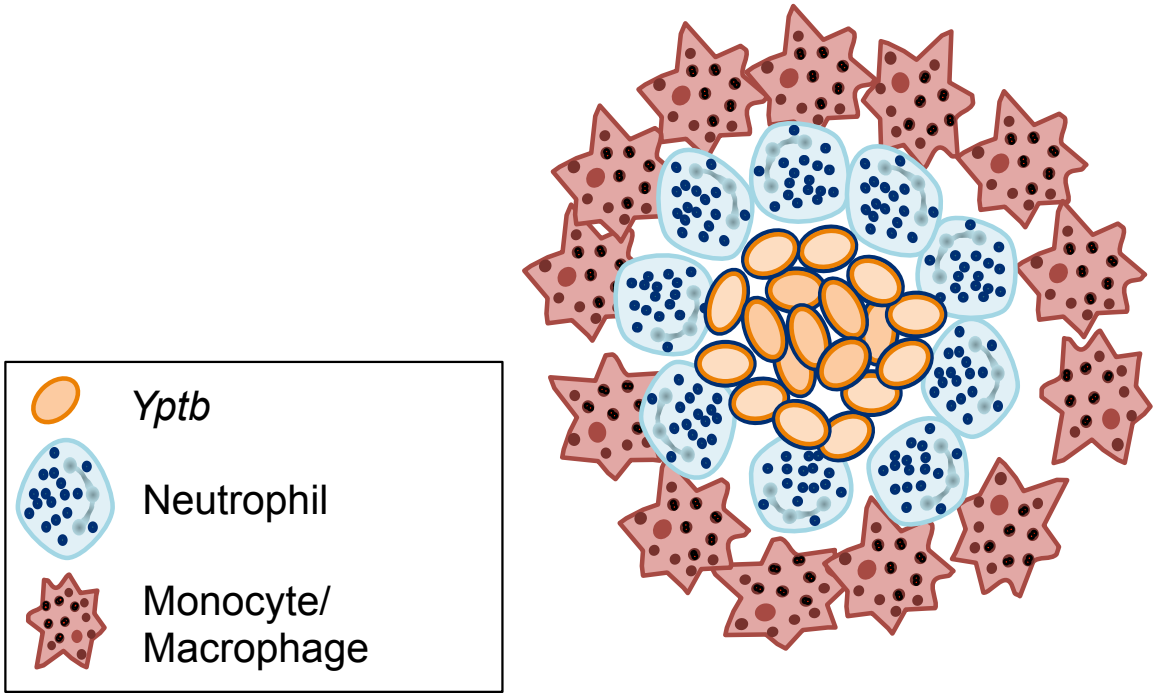
Dendritic cells (DCs) primarily function as antigen presenting cells, and efficiently present antigens on MHC molecules to activate naïve T cells. In mice, these cells are defined as $Cd11c^{hi}MHCclassII^{pos}$. DCs have been divided into a number of different subsets that depend on the presence of additional markers, which are often specific to certain tissue sites. In mice, these cells arise from common DC progenitor (CDP) cells in the bone marrow. Once they reach tissue sites, DCs can differentiate into several subsets, including both $CD8^+$ and $CD8^-$ DCs in lymphoid tissues and $CD103^+$ $CD11b^-$ DCs in peripheral non-lymphoid tissues (Chow, Brown et al. 2011).

Some interactions between *Yersinia* and DCs have been characterized. First, like the other phagocytic cells described, DCs are primary targets of translocated effectors during *Yersinia* infection (Marketon, DePaolo et al. 2005, Koberle, Klein-Gunther et al. 2009, Durand, Maldonado-Arocho et al. 2010). Effector proteins have been shown to inhibit several antibacterial functions in DCs, including phagocytosis, and the capacity of some DC subsets to stimulate $CD8^+$ T cells (Fahlgren, Westermark et al. 2009,

Autenrieth, Linzer et al. 2010). However, the specific role(s) of DCs in promoting innate immune clearance of *Yersinia* is unclear, as depletion of these cells in CD11c-diptheria toxin mice actually results in enhanced innate immune clearance of *Y. enterocolitica* in infected spleens (Autenrieth, Warnke et al. 2012). Furthermore, no DCs were detected surrounding microcolonies of *Yptb* during growth in spleens, suggesting that these cells may not interact with *Yersinia* during growth in this tissue site (Davis, Mohammadi et al. 2015). Instead, DCs may play a more important role in controlling *Yptb* infection at early time points in the GI tissues, as interactions between *Yptb* and resident DC populations have been observed in the Peyer's patches and cecal lymphoid follicles at early time points (Westermark, Fahlgren et al. 2014).

Figure 1-3. *Yptb* is surrounded by phagocytic cells during extracellular growth in spleens

Below is a cross-sectional depiction of the host response to *Yptb* infection in the spleen, based largely off of previously published histological analyses (Durand, Maldonado-Arocho et al. 2010, McCoy, Marre et al. 2010, Crimmins, Mohammadi et al. 2012, Davis, Mohammadi et al. 2015, Keith 2016). *Yptb* grows within the red pulp of spleens in extracellular aggregates referred to as microcolonies. These aggregates are in direct contact with a shell of $Ly6G^{pos}$ neutrophils. Surrounding the neutrophils is a shell of $Cd68^{pos}$ cells, which may be macrophages, inflammatory monocytes, or potentially both populations.



1.2.3 Reactive oxygen species as an effector function of phagocytic cells

1.2.3.i The NADPH oxidase complex

One effector function shared between most subsets of phagocytic cells is the production of reactive oxygen species (ROS) in response to fungal and bacterial infections (Adams, Dinauer et al. 1997, Mastroeni, Vazquez-Torres et al. 2000, Rokutan, Kawahara et al. 2006). In phagocytic cells, oxidative burst occurs via the activation and assembly of the NADPH oxidase (also called NOX2) complex on either the plasma or phagosomal membrane, usually in response to microbial contact, phagocytosis, PRR activation, or cytokine activation (Panday, Sahoo et al. 2015).

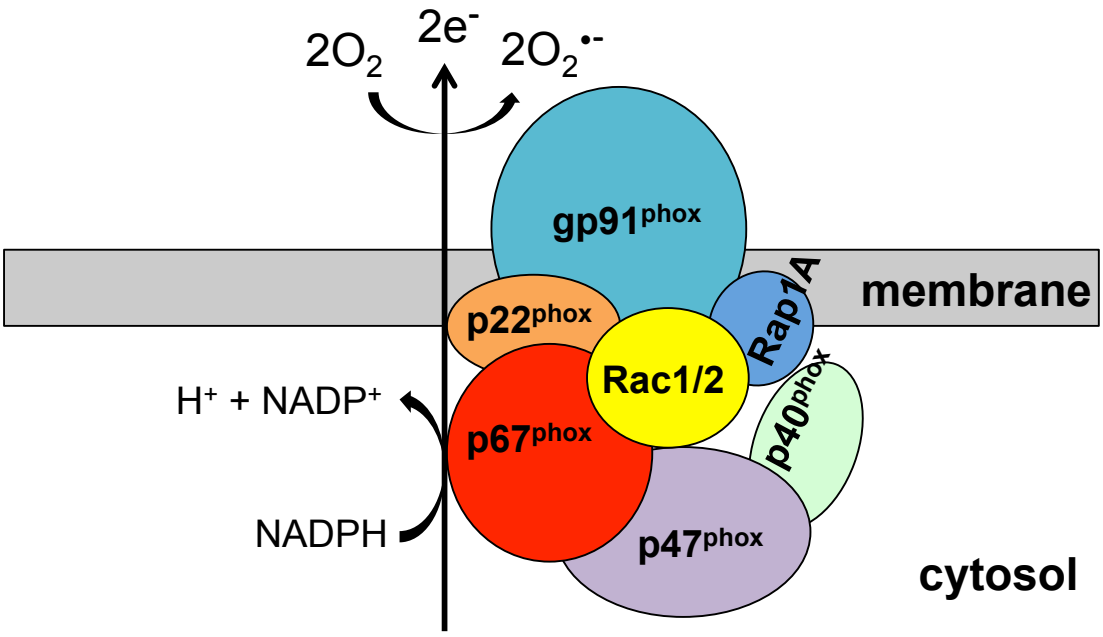
The NOX2 complex consists of several subunits that interact to produce superoxide (Fig 1-4). Two of these subunits, $gp91^{phox}$ and $p22^{phox}$, are integral membrane proteins and together form the heterodimeric subunit flavocytochrome b_{558} ($cytb_{558}$) (Panday, Sahoo et al. 2015). Additionally, three NOX2 subunits, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, reside in the cytosol as a complex, where they serve as regulatory factors for oxidase activation (Touyz, Chen et al. 2002). Specifically, upon stimulation, $p47^{phox}$ undergoes phosphorylation, which causes the entire cytosolic complex to translocate to the membrane and associate with $cytb_{558}$ to form the active oxidase (Panday, Sahoo et al. 2015). Then, in a series of electron transfer reactions, this activated complex ultimately transfers electrons from an NADPH donor to oxygen in order to generate $O_2^{\cdot -}$ (superoxide). Activation of the NOX2 complex also relies on the guanine nucleotide binding protein Rac. Rac is localized to the cytosol, in a dimeric complex with the guanine nucleotide dissociation inhibitor Rho-GDI. Upon activation, Rac binds guanosine triphosphate (GTP) and translocates to the membrane with the

$p40^{phox}$/$p47^{phox}$/$p67^{phox}$ cytosolic complex (Diebold and Bokoch 2001, Grizot, Faure et al. 2001). Two different isoforms of Rac contribute to NADPH oxidase activation, depending on the cell type. Rac1 has been shown to be important for activation of this complex in mononuclear phagocytes, while Rac2 appears to be more important for NOX2 activation in neutrophils (Diebold and Bokoch 2001, Grizot, Faure et al. 2001). Another guanine nucleotide binding protein, Rap1A, is also believed to promote activation of the NOX2 complex. This protein is localized to the membrane and associates with $cytb_{558}$; however, its role in NOX2 activation is currently poorly defined (Takahashi, Dillon et al. 2013).

Once generated, superoxide often undergoes spontaneous or enzymatic dismutation to hydrogen peroxide (H_2O_2). H_2O_2 diffuses freely across membranes and thus may exert a bactericidal effect on nearby extracellular bacteria, as well as on internalized bacteria present in the phagosome. Additionally, in neutrophils, H_2O_2 can be converted into hypochlorous acid (HOCl) by the enzyme myeloperoxidase (MPO) (Rosen and Klebanoff 1979, Foote, Goyne et al. 1983). Though HOCl is highly bactericidal (Klebanoff 1967), MPO does not appear to be essential for host defenses against bacteria, as genetic deficiencies in this enzyme do not generally result in increased susceptibility to microbial infections (Parry, Root et al. 1981), with the exception of infections by pathogenic fungus *Candida albicans* (Aratani, Koyama et al. 1999).

Figure 1-4. NADPH oxidase complex activation in phagocytic cells

The NADPH oxidase complex consists of multiple subunits: 2 of these subunits, $gp91^{phox}$, and $p22^{phox}$ are found in the membrane and together form the $cytb_{558}$ complex. Four proteins, $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and Rac1 or Rac2, are found in the cytosol and play important regulatory roles in stimulating activation of this complex. Additionally, another membrane protein, Rap1A, also contributes to NOX2 activation, though its mechanism of action is currently unknown. Following activation of $p47^{phox}$ by phosphorylation, the cytosolic complex translocates to the membrane and associates with $cytb_{558}$. Next, a series of electron transport reactions occur, which ultimately result in the transfer of an electron from NADPH to oxygen, generating superoxide ($O_2^{\bullet-}$).



Adapted from (Gardiner, Deffit et al. 2013)

1.2.3.ii Mechanisms of ROS-induced damage within bacterial cells

A major cytotoxic consequence of H_2O_2 in the bacterial cell is its ability to oxidize ferrous iron (II) to ferric iron (III), in a reaction that is referred to as Fenton chemistry, which results in formation of highly reactive hydroxyl (OH^-) radicals (Imlay 2008). Superoxide itself is also damaging to bacteria and can act synergistically with H_2O_2 in promoting the Fenton reaction; this is thought to occur through a mechanism in which superoxide mobilizes iron from iron-sulfur clusters, thus increasing the amount of free iron available for Fenton chemistry (Keyer, Gort et al. 1995, Keyer and Imlay 1996). The Fenton reaction can be particularly damaging to proteins containing iron-sulfur clusters; these proteins often participate in metabolic pathways and, thus, their damage can have impacts on bacterial survival in nutrient-limited environments (Imlay 2006). Additionally, generation of $OH^{\bullet}$ by the Fenton reaction can result in DNA damage as a consequence of strand breaks and base oxidation; this damage is thought to be the main mechanism of ROS-dependent antibacterial activity (Imlay and Linn 1988, Imlay 2008). Many cellular proteins are also vulnerable to oxidative attack, particularly at cysteine, methionine, tyrosine, phenylalanine, and tryptophan residues (Tamarit, Cabiscol et al. 1998). Finally, the peroxidation of lipids by ROS can result in membrane damage, which may have bactericidal effects (Shohet, Pitt et al. 1974).

1.2.3.iii Mechanisms of bacterial resistance to ROS

Bacteria use a variety of strategies to resist the actions of ROS. First, a number of bacterial enzymes are utilized to transform ROS into less toxic products; these include superoxide dismutases (SODs), catalases, and peroxiredoxins (Imlay 2008). Catalases and peroxiredoxins function as H_2O_2 scavengers. Examples of these proteins in *E. coli*

include the peroxiredoxin AhpC and the catalase KatG (Imlay 2008). While these scavengers exhibit some functional redundancy, they typically contribute to detoxification at different H_2O_2 concentrations; at low concentrations, AhpC serves as the primary scavenger, whereas KatG becomes the primary scavenging enzyme at high concentrations (Hillar, Peters et al. 2000, Seaver and Imlay 2001). In *E. coli* and many organisms, the transcriptional response to H_2O_2 is dependent on the global regulator OxyR (Imlay 2008). Oxidation of this protein results in a conformational change that allows for the binding of a large number of DNA promoter sequences. The OxyR regulon includes peroxiredoxins and catalases, as well as several other factors important for responding to oxidative damage, including the reducing agents glutathione reductase (*gor*), glutaredoxin 1 (*grxA)*, and thioredoxin 2 (*trxC*), which function to minimize the frequency of aberrant disulfide bond formation occurring as a result of exposure to ROS (Zheng, Aslund et al. 1998) (Zheng, Wang et al. 2001, Imlay 2008).

In contrast to the peroxiredoxins and catalases, which scavenge H_2O_2, SODs scavenge superoxide. *E. coli* encodes two cytoplasmic SOD izosymes, one (MnSOD or SodA), which uses the co-factor manganese, and another (FeSOD or SodB), which uses the co-factor iron (Imlay 2008). Additionally, because superoxide does not easily cross membranes at a neutral pH, *E. coli* also secretes another, copper- and zinc-co-factored SOD (CuZnSOD or SodC) into the periplasm (Korshunov and Imlay 2002). Interestingly, SODs may also reduce overall H_2O_2 levels, by preventing further H_2O_2 production through the interaction of superoxide with other reductants in the cell (Liochev and Fridovich 1994). While baseline expression of SODs is usually high (Imlay and Fridovich 1991), activation of the SoxRS regulatory system further enhances expression

of these enzymes in response to superoxide stress (Liochev, Benov et al. 1999). Much like OxyR, SoxR undergoes a conformational change upon detection of redox stress (Hassan and Fridovich 1977). In this case, SoxR contains an iron sulfur cluster, which, upon oxidation, induces a structural change in that protein; oxidized SoxR then promotes increased transcription of the DNA-binding protein SoxS (Hidalgo, Ding et al. 1997). In *E. coli*, SoxS positively regulates about a dozen genes, including those encoding SODs and also several other genes involved in detoxification, iron-sulfur cluster repair, and drug efflux (Imlay 2008). In some species of bacteria, such as *Pseudomonas,* which lack a SoxS homolog, SoxR serves as both the redox sensor and direct inducer of the regulon (Kobayashi and Tagawa 2004) (Eiamphungporn, Charoenlap et al. 2006).

Because free iron is susceptible to Fenton chemistry, bacteria utilize a number of mechanisms to sequester iron or control its uptake in response to encountering ROS in the environment. For example, a family of proteins known as ferritins acts to maintain iron homeostasis; the ferritin Dps, in particular, is critical for withstanding oxidative stress, as it both sequesters iron and binds DNA to protect it from damage (Halsey, Vazquez-Torres et al. 2004, Velayudhan, Castor et al. 2007). Furthermore, because oxygen radicals can release iron from iron-sulfur clusters, factors that promote regeneration of iron-sulfur clusters may also play a role in recovering from oxidative damage. In *E. coli,* the Suf iron-cluster repair machinery, in particular, plays an important role in recovery from H_2O_2-mediated damage (Imlay 2008, Jang and Imlay 2010).

DNA damage is another major antibacterial consequence of ROS. Therefore, bacteria encode numerous DNA repair enzymes that are crucial for recovering from ROS attack (Imlay 2008). These include proteins involved in base excision and

recombinational repair pathways. Many DNA repair factors are essential for growth and/or survival under aerobic conditions; however, the expression of some of these proteins is enhanced under oxidative stress conditions in an OxyR and/or SoxRS-dependent manner (Zheng, Wang et al. 2001) (Imlay 2008), suggesting that increased levels of some DNA repair factors may be required for resistance to ROS.

Bacterial pathogens also employ a number of measures to prevent exposure to increased levels of ROS at sites of infection. One mechanism of ROS evasion is the suppression of oxidative burst in phagocytes, usually through the actions of secreted effector proteins or toxins. For example, *Bordetella pertussis* can inhibit respiratory burst by secreting an adenylate cyclase toxin into host phagocytes. This toxin increases cAMP levels in host cells, which inhibits oxidative responses to a variety of stimuli (Pearson, Uhlig et al. 2012). Another example is the pathogen *Salmonella typhimurium,* which utilizes its SPI2 pathogenicity island to at least partially suppress activation of the NOX2 complex in macrophages (Vazquez-Torres, Xu et al. 2000, Gallois, Klein et al. 2001, Vazquez-Torres and Fang 2001, Rosenberger and Finlay 2002, Felmy, Songhet et al. 2013). And as discussed earlier in this chapter, *Yersinia* are also capable of directly suppressing the oxidative burst of phagocytic cells, due to the actions of the T3SS effectors YopH and YopE (Bliska and Black 1995, Songsungthong, Higgins et al. 2010). These and other mechanisms by which *Yersinia* responds to oxidative stress *in vitro* and *in vivo* will be discussed in further detail below.

1.2.3.ii *Yersinia* and ROS

While the specific factors required by *Yptb* or other *Yersinia* to resist *in vitro* sources of oxidative stress have not been well characterized, BLAST analysis of the YPIII *Yptb* sequence indicates that this organism encodes many homologous genes to those characterized in *E. coli,* including *oxyR*, *sodA, sodB, sodC*, *katG*, *ahpC*, *dps,* the *suf* operon, and numerous DNA repair and recombination factors. Additionally, a study performed in *Y. pestis* found that the catalase KatA was important for protection against exogenous H_2O_2 *in vitro* (Han, Geng et al. 2008). Interestingly, a BLAST search revealed no putative SoxRS homologs in *Yptb*, which is consistent with studies finding no such homology in *Y. pestis* (Deng, Burland et al. 2002). However, PHYRE analysis uncovered some structural homology between the putative, redox-sensitive transcriptional regulator encoded by YPK_1908 and SoxR, suggesting that, similar to *Pseudomonas* (Kobayashi and Tagawa 2004), *Yptb* may utilize just SoxR to activate the regulon.

As mentioned earlier in this chapter, two *Yersinia* T3SS effectors, YopE and YopH, can prevent oxidative burst in phagocytic cells. YopE has been shown to inhibit oxidative burst in the neutrophil-like HL-60 cell line; this inhibition occurs through the GAP-mediated inactivation of Rac2 (Songsungthong, Higgins et al. 2010). Interestingly, point mutants of *yopE* lacking Rac2 inhibitory activity are unable to colonize the spleens of WT mice but can grow within spleens of mice lacking the $gp91^{phox}$ component of the NOX2 complex, suggesting that the ROS-inhibitory activity of this protein is critical for splenic infection (Songsungthong, Higgins et al. 2010). YopH prevents Fc-receptor mediated oxidative burst in macrophages, though the mechanism of how this occurs is not known (Bliska and Black 1995). Nonetheless, Δ*yopH* mutants are significantly

attenuated for virulence in several murine tissue sites, particularly in a Δ*yopE* background (Logsdon and Mecsas 2003, Rolan, Durand et al. 2013), suggesting that ROS inhibition by this protein may be critical for tissue infection.

Because *Yersinia* can suppress oxidative burst in Yop-intoxicated immune cells, there is some debate as to how much oxidative stress these pathogens experience during tissue infection. A study performed in *Y. enterocolitica* found that mutants lacking a functional *sodA* gene were significantly impaired for survival and growth within the spleen and liver following intravenous infection (Roggenkamp, Bittner et al. 1997). By contrast, this mutant had no defect in growth in the gut and Peyer's patches, suggesting that this bacterium may encounter oxidative stress during growth in systemic tissue sites, but not in GI tissues (Roggenkamp, Bittner et al. 1997). In possible contrast to this finding, a study analyzing the transcriptional induction of *sodA* in *Yptb* during splenic infection showed little difference in the expression of this gene relative to *in vitro* growth, and also found that a *katG* transcriptional reporter was not uniformly induced across microcolonies (Davis, Mohammadi et al. 2015). However, in the case of *sodA* in *Yptb,* RNA levels were comparable to stationary phase growth, where bacteria may encounter higher endogenous levels of oxidative stress than they do in exponential phase (Benov and Fridovich 1995, Imlay 2008). In regard to the *katG* reporter result, activation of the reporter was detected in a few individual bacteria in each microcolony. However, because *katG* requires high concentrations of H_2O_2 to be induced (Hillar, Peters et al. 2000), it is possible that bacteria did encounter some oxidative stress, but concentrations of ROS in the microcolony were not uniformly high enough to induce transcription of *katG* in the entire population.

Additionally, a microarray analysis performed in *Y. pestis* isolated from infected buboes determined that oxidative stress response genes were not highly induced in this tissue site (Sebbane, Lemaitre et al. 2006). However, this study measured bacterial gene expression in the bubo at later time-points, where tissues were colonized with 10^7-10^9 CFU of *Y. pestis* growing in extensive masses (Sebbane, Lemaitre et al. 2006). At this stage of infection, these tissues are highly necrotic (Sebbane, Lemaitre et al. 2006), and therefore immune mechanisms of bacterial clearance could potentially differ from those utilized against *Yptb* in the spleen.

Altogether, these studies suggest that *Yersinia* may rely on multiple mechanisms to prevent clearance by ROS during infection of certain tissue sites, and that levels of ROS encountered may differ between tissue sites and species of *Yersinia*. However, the contributions of ROS resistance mechanisms to *Yersinia* survival in different tissue sites and at different time points needs further clarification.

1.3 The nucleoid associated protein Fis

A major focus of this work is the *dusB-fis* operon, which we found to be essential for the virulence of *Yptb*. In particular, we determined that possession of this operon was critical for resisting killing by H_2O_2 *in vitro*, and that mutants lacking *dusB-fis* failed to up-regulate a number of ROS-responsive genes. Consistent with this result, the virulence of this mutant was restored in mice lacking a functional NADPH oxidase complex, suggesting that the primary role of this operon during *Yptb* infection is to protect against ROS.

1.3.1 The dusB-fis operon

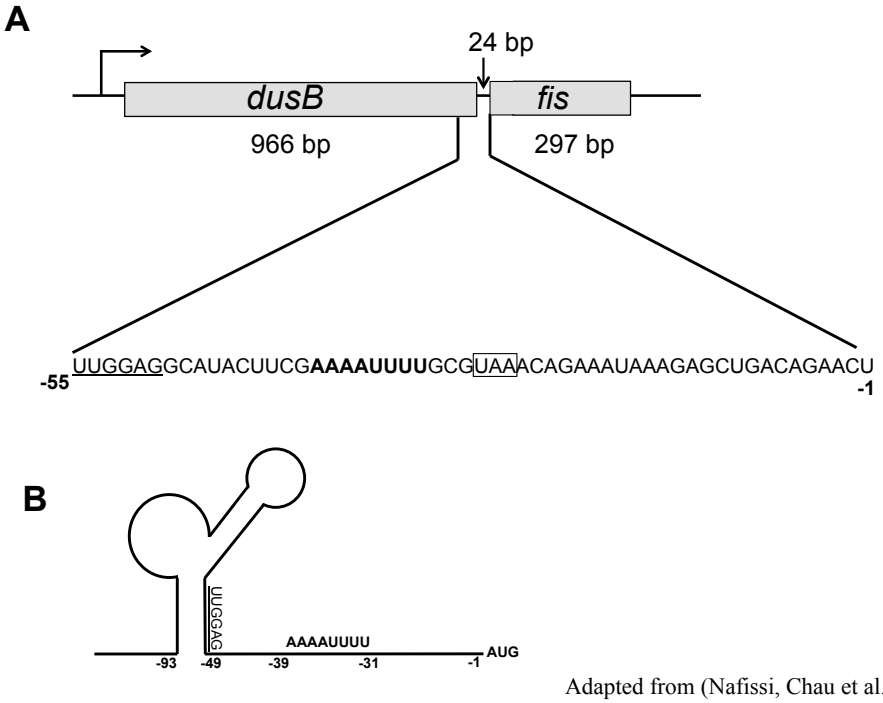
This *dusB-fis* operon is conserved in *Enterobacteriaceae* family members of the *Gammaproteobacteria* (Nafissi, Chau et al. 2012), where the two genes are co-regulated and transcribed from a single promoter upstream of *dusB* (Fig 1-5) (Ball, Osuna et al. 1992, Beach and Osuna 1998). The *fis* gene encodes the small, abundant nucleoid-associated protein (NAP) Fis, which is known to play roles in promoting recombination, replication and transcription (Duprey, Reverchon et al. 2014). The *dusB* gene encodes a tRNA dihydrouridine synthase; this family of proteins modifies tRNAs by converting uridine to 5,6-dihydrouridine within D loops (Bishop, Xu et al. 2002). Importantly BLAST analysis indicates that the *dusB* and *fis* sequences in *Yptb* are highly homologous to those in *E. coli* and *Salmonella,* where much work has been performed to determine the regulation and functions of this operon.

Interestingly, in *E.coli,* transcripts from two genes are highly abundant at different growth stages, however, translation of DusB occurs at very low leels relative Fis, likely due to the fact that the 5' untranslated region upstream of *dusB* contains no canonical Shine-Dalgarno sequence (Nafissi, Chau et al. 2012). Intriguingly, this work determined that a putative Shine-Dalgarno sequence located 9 nucleotides upstream from the Fis start codon is not important for translation of this protein. Instead, the 3' end of *dusB* seems to play an important role in promoting the translation of Fis. This region contains an AU-rich segment 35 nucleotides upstream of the *fis* translation start site that is critical for translation of Fis (Nafissi, Chau et al. 2012) (Fig 1-5). AU-rich sequences are often found in 5' untranslated regions and are believed to enhance translation by facilitating ribosome accessibility and serving as binding sites for the ribosomal S1 protein, which is essential for translation of proteins lacking Shine-Delgarno sequences (Sorensen, Fricke et al.

1998). Additionally, in *E.coli,* a stem-loop formed at the 3' end of the *dusB* transcript is important for enhancing translation of Fis (Nafissi, Chau et al. 2012) (Fig 1-5). Notably, both the AU segment and stem loop regions at the 3' end of *dusB* are conserved in a number of *Gammaproteobacteria,* including *Yersinia* (Nafissi, Chau et al. 2012). Altogether, these results support a model in which *dusB* largely functions at the RNA level by enhancing translation of Fis.

Figure 1-5. The conserved *dusB-fis* operon

The *dusB-fis* operon is conserved in in *Enterobacteriaceae* family members of the *Gammaproteobacteria*. In *E. coli,* these two genes are co-regulated and transcribed from a single promoter located upstream of *dusB.* **(A)** The 55-nt sequence immediately upstream of the *fis* start codon, which includes the intergenic region and the 3' end of the *dusB* mRNA, is important for translation of Fis. The underlined sequence marks the lower right arm of the putative stem loop structure shown in B. The bolded sequence represents an AU-rich region at the -35 site that is believed to serve as the ribosome binding site for translation of the *fis* mRNA. The boxed sequence marks the *dusB* stop codon. **(B)** Schematic depicting the 44-nt putative RNA stem-loop structure upstream of the *fis* start codon, which enhances translation of Fis through an unknown mechanism.



Adapted from (Nafissi, Chau et al. 2012).

1.3.2 Functions of Fis in other organisms

Fis, or factor for inversion stimulation, belongs to a class of proteins known as nucleoid associated proteins, or NAPs. NAPs are small, abundant proteins that affect chromosomal compaction by bending, wrapping, and bridging DNA (Dorman 2014). Additionally, many NAPs positively and negatively influence transcription of a broad range of genes (Dorman 2014). Examples of conserved NAPs in gram-negative bacteria include Fis, H-NS, HU, and IHF. In general NAPs are believed to exert their functions at the DNA topological level by coordinating the architecture of the bacterial chromosome, which can indirectly impose regulatory constraints on a variety of genes (Dorman 2013, Dorman 2013). Additionally, these proteins can also interact with RNA polymerase and bind promoter regions to directly regulate transcription (Dillon and Dorman 2010).

While Fis has not been previously characterized in *Yersinia*, it is highly conserved in the Gammaproteobacteria, where a large number of studies, particularly in *E. coli* and *Salmonella*, have been performed to characterize its functions and roles in bacterial physiology. Fis was originally identified as an accessory protein for the site-specific recombinases Hin of Salmonella and Gin of bacteriophage Mu (Johnson, Bruist et al. 1986, Koch and Kahmann 1986). However, this protein is now recognized as playing broad roles in a number of cellular processes. Fis assists in the lifecycles of bacteriophages lambda and Mu; in lambda it contributes to integration and excision, while in Mu, it cooperates with the Mu repressor to maintain lysogeny (Ball and Johnson 1991, Ball and Johnson 1991, Betermier, Poquet et al. 1993, van Drunen, van Zuylen et al. 1993). Fis also plays a role in the transposition of Tn*5* and IS*50*, where it serves as an

accessory factor (Weinreich and Reznikoff 1992). In *E. coli*, Fis is involved in initiating chromosomal replication at the oriC (Gille, Egan et al. 1991).

Notably, Fis serves as a key genome-structuring element, by contributing to the formation and stability of the looped domain architecture of the nucleoid, as well as by regulating both gyrase and topoisomerase at the transcriptional level (Schneider, Travers et al. 1999, Ohniwa, Morikawa et al. 2006, Skoko, Yoo et al. 2006). It can also affect chromosome topology directly by binding to DNA. Specifically, Fis can preserve intermediate supercoiled forms of DNA, thereby acting as a "topological buffer," and protecting them from extreme shifts toward more relaxed or more negatively supercoiled ends of the topological spectrum (Travers and Muskhelishvili 2005). However, this control of DNA topology also involves other NAPs. Finally, like other NAPs, Fis regulates the expression of a large number of bacterial genes, both as a transcriptional activator and repressor. In some cases, Fis acts as a traditional transcription activator, where it makes contact with RNA polymerase at promoter regions (Bokal, Ross et al. 1997) (McLeod, Aiyar et al. 2002). In other cases, its effects on transcription are indirect and involve the modulation of DNA supercoiling at promoters in ways that enhance transcription initiation (Auner, Buckle et al. 2003).

A number of studies have been performed to determine the direct and indirect transcriptional targets of Fis at different stages of bacterial growth. Fis-regulated genes contribute to several critical bacterial processes, including the organization of genetic material and the production of a variety of components of the translational machinery (Bosch, Nilsson et al. 1990, Nilsson, Vanet et al. 1990, Ross, Thompson et al. 1990, Schneider, Travers et al. 1999, Ohniwa, Morikawa et al. 2006, Skoko, Yoo et al. 2006).

Interestingly, despite these the numerous genes whose expression is altered in the absence of fis, Δ*fis* mutants are viable; indicating that there is likely redundancy in the systems Fis regulates. However, *fis* mutants do have defects under some conditions. *E. coli* and *Salmonella* strains containing deletions of fis have an altered morphology, display an extended lag phase, and are outcompeted following extended co-culture with in isogenic WT strain (Nilsson, Vanet et al. 1990, Filutowicz, Ross et al. 1992, Osuna, Lienau et al. 1995, Schneider, Travers et al. 1997, Skoko, Yoo et al. 2006). Interestingly, *E. coli* strains overexpressing Fis protein also display reduced growth rates (Richins and Chen 2001), indicating that the expression this protein must be carefully regulated to keep it within certain limits.

The expression of *dusB-fis* is largely growth stage and nutrient-state dependent. Studies performed in *E. coli* and *Salmonella* have found that Fis is highly expressed during early exponential phase growth, where it is among the most abundant proteins in the cell (Ball, Osuna et al. 1992, Osuna, Lienau et al. 1995). Conversely, by the onset of stationary phase, levels of Fis decline rapidly (Bosch, Nilsson et al. 1990, Ball, Osuna et al. 1992, Keane and Dorman 2003). Work performed to characterize the mechanisms of this expression pattern have determined that Fis is not subject to rapid turnover by proteolysis; rather, its decline in concentration appears to be primarily due to dilution as cells divide without synthesizing new Fis protein. Fis negatively regulates its own promoter, and thus, high protein levels would feed back onto its own transcription (Mallik, Pratt et al. 2004). The *dusB-fis* promoter is also negatively regulated by the stringent response, which is consistent with its low expression during stationary phase or nutrient-limited growth (Mallik, Pratt et al. 2004). Additionally, the *fis* promoter is

sensitive to DNA supercoiling; it operates optimally when DNA is more negatively supercoiled than average, which is consistent with the state of the bacterial nucleoid during rapid exponential phase growth (Schneider, Travers et al. 2000).

In addition to regulating factors that contribute to bacterial growth, Fis also serves as a transcriptional regulator of virulence factors in many pathogens, including the mammalian pathogens *Vibrio cholerae*, *Shigella flexneri*, *Pasteurella multocida*, *Salmonella typhimurium*, and pathogenic *Escherichia coli*, as well as the plant pathogen *Dickeya dadantii* (Falconi, Prosseda et al. 2001, Wilson, Libby et al. 2001, Schechter, Jain et al. 2003, Kelly, Goldberg et al. 2004, Lautier and Nasser 2007, Lenz and Bassler 2007, Saldana, Xicohtencatl-Cortes et al. 2009, Steen, Steen et al. 2010, Rossiter, Browning et al. 2011) (Duprey, Reverchon et al. 2014). Accordingly, *fis* mutants are attenuated for infection in a number of these pathogens (Duprey, Reverchon et al. 2014).

Interestingly, though the sequence of *fis* is highly conserved between these different species, the virulence programs regulated by this protein are very diverse, ranging from quorum sensing, to capsule production, to adhesion, to Type 3 Secretion (Kelly, Goldberg et al. 2004, Lenz and Bassler 2007, Saldana, Xicohtencatl-Cortes et al. 2009, Steen, Steen et al. 2010). The mechanism for this diversity is not clearly understood, though it is believed that pathogens may utilize Fis's sensitivity to fluctuations in nutrient states and DNA topology to coordinate transcriptional responses to different host environments (Duprey, Reverchon et al. 2014). For example, *Salmonella* utilizes Fis to sense changes in DNA topology that reflect bacterial location during different stages of infection. More specifically, during growth of *Salmonella* in the mammalian GI tract, anaerobic conditions lead to compaction of the nucleoid; this

change in topology is detected and stabilized by Fis and leads to transcriptional induction of the SPI-1 pathogenicity island, which is important for extracellular growth of this pathogen (Wilson, Libby et al. 2001, Schechter, Jain et al. 2003, O Cróinín, Carroll et al. 2006, Duprey, Reverchon et al. 2014). By contrast, during intracellular growth of *Salmonella* within macrophages, exposure to oxidative stress leads to nucleoid relaxation. During early stages of macrophage infection, Fis enhances this relaxation, leading to induction of SPI-2 gene expression, which is critical for intracellular growth (O Cróinín, Carroll et al. 2006).

Based on these observations, we hypothesize that *Yptb* utilizes Fis to sense and adapt to different environmental niches encountered during infection; this hypothesis will be explored more in Chapter 4 of this work.

CHAPTER 2: MATERIALS AND METHODS

2.1 Strain Construction

Strains utilized in this work are listed in Table 2-1 and primers are listed in Table 2-2. *Yptb* knockouts were generated in pIB1^{-} YPIII and pIB1^{+} IP2666, as indicated in Table 2-1. Knockouts containing in-frame scar sequences were created using allelic exchange as follows: Primers were designed to amplify 800bp regions flanking each targeted gene (Table 2-1), where primers p1 and p2 amplified upstream regions and p3 and p4 amplified downstream regions. Primers p2 and p3 also contained overlapping sequences necessary to create a ~60bp scar sequence after gene deletion. Overlapping products were combined using splicing by overlap extension (SOE) PCR and ligated into the *sacB*-based vector pCVD442 following restriction digestion. The resulting plasmids were introduced into *E.coli* DH5αλpir or SY327λpir and integrated into the *Yptb* chromosome by mating, with the help of a tri-parental mating strain containing pRK600. Deletions were confirmed by PCR, utilizing primers p1 and p4 to screen for a truncated product. To complement *dusB-fis,* the entire operon, as well as 800bp of sequence both up and downstream, were amplified with the p1 and p4 primers utilized to generate the original deletion. The resulting PCR product was cloned into pCVD442 by restriction digestion and ligation. This plasmid, pCVD442- *dusB-fis,* was introduced into *E. coli* DH5αλpir and mated into *Yptb* as described above. Successful complementation was confirmed by PCR as described above. Strains containing YopE-ETEM fusions were generated as follows: *Yptb* strains were mated overnight with an SM10λpir strain containing the plasmid pSR47-YopETEM, and plated on media containing kanamycin and irgasan to select for crossover of the chimeric YopE-TEM gene into the *yopE* locus. Successful crossover was confirmed by PCR.

2.2 Media and Growth Conditions

All *Yptb* cultures were grown in L or 2XYT broth, with the exception of nitric oxide and H_2O_2 sensitivity assays (described below). Following mouse infections, tissue homogenates were plated onto L agar containing 0.5 μg/mL irgasan, or with a combination of irgasan and 50 μg/mL kanamycin to select for marked bacterial strains. During strain construction, carbenicillin and irgasan were used to select for strains containing integrated plasmids following matings, and 10% sucrose was utilized to select for strains that had resolved the integrated plasmid. With the exception of the beta-lactamase assays (described below), all cultures were incubated at 26 °C with shaking. For animal infections, strains were inoculated into L broth 48-hours prior to infection. Following overnight growth, these strains were diluted 1:40 and incubated for ~8 hours, after which they were diluted 1:100 and incubated overnight.

2.3 Animal work

2.3.1 Mouse infections

All infections were performed by intravenous injection in 8-10 week C57Bl/6 or C67Bl/6 gp91$^{phox-/-}$ mice. For infections with strains constructed in pIB1^{-} YPIII, mice were inoculated with 1 x 10^4 bacteria. For infections with strains constructed in pIB1^{+} IP2666, mice were inoculated with 1 x 10^3 bacteria. Competition experiments were performed using a 1:1 mixture of an unmarked strain and a strain harboring an insertion of miniTn5 Kan^R in a neutral locus (Logsdon and Mecsas 2003). Following infections, spleens and livers were isolated, weighed, homogenized, and plated on L agar containing 0.5 μg/mL irgasan. The quantity of CFU/gram was determined by dividing the number of recovered CFU by the weight of the tissue sample extracted. For competition

experiments, tissue homogenates were plated onto non-selective media as well as onto media containing 50 μg/mL kanamycin and the CFU count for each strain was determined by subtracting the number of Kan^R colonies from the total number of colonies recovered on non-selective plates. The proportion of each strain in the inoculum was confirmed using the same methods. C.I values were determined by the following equation: C.I. =(mutant/WT output ratio)/(mutant/WT input ratio).

2.3.2. Immune cell depletions

For Ly6G and Gr1 cell depletions, mice were intraperitoneally injected with 50 μg of 1A8 (Fisher) or RB6-8C5 (eBioscience) antibody 24 hours prior to and 24 hours post-infection. For inflammatory monocyte depletions, mice were intraperitoneally injected with 20 μg of MC21 antibody (gift from Matthias Mack (Maus, Wellmann et al. 2005)) 1 day prior to infection and each day after until completion of the experiment. To confirm successful neutrophil and inflammatory monocyte depletion, a portion of infected spleens was used to generate single cell suspensions and treated with collagenase D (Roche). 100 μl of cells were incubated with 50 μl of FACS buffer (PBS+1% HIS) containing a 1：200 dilution of Mouse BD Fc Block (BD) for 10 minutes at 4°C. 50 μl of FACS buffer containing the fluorescent antibodies CD11b PE-Cy7 (eBioscience) and Gr1 PE Cy-5 (eBioscience) was then applied to each sample, such that final dilution of stains was 1:75. Following a 30 minute incubation at 4°C, samples were analyzed by flow cytometry. Gates were established to define populations as indicated in Fig 2-10.

2.4 TnSeq and mini-TnSeq Screens

2.4.1. TnSeq to identify genes required for growth of pIB1⁻ in livers

2.4.1.1 Generation of transposon library

Two transposon library pools were constructed by Greg Crimmins as follows: The vector pSC189 containing Himar1 was mutated on one end of the transposon recognition sequence to produce an *Mme*I restriction site. To perform transpositions in a *Yptb* strain, the Himar1(*Mme*I) transposon was introduced into pIB1⁻ YPIII by mating with SM10λ*pir*. Briefly, 25 mL of pIB1⁻ YPIII was grown overnight in 2XYT broth at 26°C, and 75 mL of SM10λ*pir* (pSC189Himar1(*Mme*I) was grown O/N at 37°C in L broth containing 30 μg/mL Kan and 100 μg/mL Amp. The SM10λpir cultures were washed 3X with 1X PBS, pelleted, and resuspended in the pIB1⁻ YPIII culture. Matings were incubated for 16–24 hours at 37°C in the spent *Yptb* culture, standing. Bacteria were then pelleted, resuspended in 5 ml 2XYT, and spread on 10 L agar plates containing 30 ug/ml kanamycin and 2 μg/ml irgasin. Libraries of approximately 10,000 colonies were scraped off plates, pelleted, resuspended in 50% glycerol and stored at −80°C.

2.4.1.2 Mouse infection, tissue processing, and preparation of libraries for sequencing

Each library pool of ~10,000 insertion mutants was adjusted to yield a total of 200,000 colonies on L agar, then scraped and resuspended in 2XYT. Small aliquots were used to start overnight cultures in 2XYT at 26°C, with shaking, and alsoa volume equivalent to ~$2x10^9$ CFU was used to generate “input” genomic DNA using the Qiagen DNeasy kit. Overnight cultures of libraries were then diluted in 1X PBS and used as the inoculum for tail vein injections, with a dose of 1×10^5 bacteria/mouse. Each library was

injected on two separate days, into 5 mice, for a total of 10 mice/library and 20 mice between both libraries. At 3 days post-infection, livers were isolated and homogenized, and bacteria were recovered on L agar containing 30 ug/ml kanamycin and 1 ug/ml irgasan. Colonies were scraped off plates and "output" genomic DNA was isolated from a volume equivalent to ~$2x10^9$ CFU using the Qiagen DNeasy kit. Input and output samples were prepared for Illumina sequencing, as previously described (van Opijnen, Bodi et al. 2009). Briefly, genomic DNA was digested with the restriction enzyme MmeI, which cuts ~15-17 bp downstream of its recognition site, allowing for inclusion of *Yptb* sequence flanking the transposon insertion in the digested product. Digested DNA was treated with calf intestinal phosphatase and purified by phenol: chloroform extraction. Purified DNA was precipitated and an adapter molecule containing a 4bp index was ligated onto the 2bp overhang left after MmeI digestion. A PCR was performed using the adapter-ligated DNA as a template, with primers complementary to the transposon and adapter sequences. A ~180bp PCR product was gel- purified after <30 cycles of amplification, in order to avoid introducing a bias toward amplifying a random transposon insertion clone. PCR products were then sequenced using the Illumina Genome Analyzer.

For the 37°C temperature sensitivity screen, both libraries were plated out to yield 200,000 colonies, scraped, combined, and small volumes of the libraries were diluted into 2XYT. These cultures were incubated overnight at 26°C. The following day, stationary phase cultures were diluted into 2XYT and grown overnight again this time in duplicate, so that one sample was incubated at 26°C and the other at 37°C. Genomic DNA was then extracted and prepared for sequencing as described above.

2.4.1.2 Data analysis

After Illumina sequencing, reads were mapped and insertions in the first 5% or last 10% of a gene, as well as all intergenic insertions, were discarded. The total number of reads for all insertion mutants in each gene were summed. The abundance all insertions in a gene in a given input or output pool was then determined by dividing the number of reads for a gene by the total number of reads in the pool. The ratio of output/input for each gene was then calculated by dividing the abundance of each gene in an output sample by the abundance of that gene its corresponding input sample. The output/input ratio of each gene in each organ was averaged all liver samples infected with a given library pool (a total of 10 mice). Additionally, another analysis was performed where the output/input ratio of the 1977 genes mutated in both libraries was averaged across all 20 samples. The Log_2 values of output/input ratios were used for further statistical analysis, including determining the average ratio and standard deviation in single and pooled library analyses.

2.4.2. Mini-TnSeq assay

2.4.2.1 Mouse infections and preparations of libraries for sequencing

Individual overnight cultures of pIB1$^-$ YPIII and pIB1$^+$ IP2666 in-frame mutants containing scar sequences were grown at 26°C. The next day, cultures of individual strains were mixed so that each putatively attenuated mutant would represent ~3% of the inoculum, and each of the 2 neutral mutants would each represent ~25% of the inoculum. Libraries were intravenously injected into 10 C57Bl/6 mice and 7-8 C57Bl/6 mice treated with either RB6-8C5 or with 1A8, at a dose of $1x10^4$ for pIB1$^-$ YPIII libraries and $1x10^3$ for pIB1$^+$ IP2666 libraries. Each condition was split into two separate infections. At 3

days post-infection, tissues were isolated and plated for CFUs on 150mm agar plates so that each plate would contain ~1x10^4 CFUs. Bacteria were scraped off plates, mixed, and genomic DNA was extracted from a volume equivalent to ~2x10^9 CFUs, using the Qiagen DNeasy Blood and Tissue kit. DNA libraries were prepared for sequencing using the homopolymer tail-mediated ligation PCR technique as previously described (Lazinski and Camilli 2013). Briefly, genomic DNA was sheared by sonication, and treated with terminal deoxytransferase in order to generate a 3' poly C-tail sequence. Two rounds of nested PCR were then employed to amplify regions immediately downstream of deleted genes. These products were multiplexed using 6bp indexing primers and sequenced on the Illumina Hi-Seq 2500.

2.4.2.2 Data analysis

Following sequencing, reads were mapped to the region immediately downstream of the deleted genes and the total number of reads for each mutant in a given organ or input pool was determined. The abundance of each mutant in an input or output pool was calculated by dividing the number of reads for that mutant by the total number of reads for a given organ or input sample. Fitness values were obtained by dividing the abundance of a mutant in a given organ by its proportion in the input pool. To determine statistical differences between "attenuated" mutants and neutral mutants, fitness values were $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis with the Dunnett's multiple comparison post-test, when comparing the fitness scores of each mutant to those of both of the neutral mutants, pooled.

2.5 BETA-LACTAMASE ASSAYS

2.5.1 Type 3 Secretion assay

Strains containing chimeric YopE-TEM fusions were grown overnight at 26°C with shaking, then diluted 1:40 into L broth containing 20mM sodium oxalate + 20mM $MgCl_2$. Cultures were grown for 2 hours at 26°C with shaking and then shifted to 37°C for 2 hours. After growth, cultures were centrifuged and a fraction of the culture supernatant was applied to an equal volume of nitrocefin (at 500 μg/mL). After 5 minutes of incubation, the A_{490} of samples was measured using a BioTek Synergy HT plate reader.

2.5.2 CCF4 effector translocation assay

Strains containing chimeric YopE-TEM fusions were grown overnight at 26°C with shaking, then diluted 1:40 into L broth containing 20mM sodium oxalate + 20mM $MgCl_2$. Cultures were grown for 2 hours at 26°C with shaking and then shifted to 37°C for 2 hours. After growth, cultures were used to infect HEp-2 cells at multiplicities of infection of 10 and 40. After 1 hour, cells were treated with gentamicin to stop the infection. Cells were lifted from plates using trypsin and then loaded with 1 μg/ml CCF4 (Invitrogen) and 1.5 mM probenecid (Sigma). Following a 20 minute incubation, cells were analyzed by flow cytometry to quantify fluorescence following excitation at 388 (blue) and 440 (green) nm wavelengths, where blue fluorescence indicated presence of translocated effectors inside of the cell. Gates were established to define populations as indicated in Fig 4-4.

2.6 In-vitro stress Tolerance assays

2.6.1 Growth in low pH and low iron

For low pH growth assays, WT and Δ*dusB-fis Yptb* were grown overnight at 26°C with shaking, then diluted 1:100 into either L broth or L broth at pH 5.5. Cultures were grown at 26°C with shaking, and the OD_{600} of cultures was measured at 1-hour intervals for 12 hours. For low-iron growth assays, cultures were grown overnight as described above and diluted 1:100 into a well of a 96-well plate containing L broth or L broth + 31 μM 2,2'- Bipyridyl (Sigma). Plates were incubated for 20 hours in a BioTek Synergy HT plate reader at 26°C with shaking, and OD_{600} measurements were recorded for each well at 15-minute intervals.

2.6.2 Nitric oxide and H_2O_2 sensitivity assays

Stationary phase cultures were diluted 1:40 into L broth for 4 hours at 26°C with shaking. Cultures were then washed 3 times with 1X PBS and diluted 1:50 into M9 glucose medium or into M9 glucose medium containing either 1.5mM H_2O_2 or 2.5mM of the nitric oxide donor DETA NONOate (Cayman Chemical). Samples were incubated at 26°C with shaking for 1 hour, and plated onto L agar in order to quantify surviving bacteria.

2.7 qRT-PCR

Bacteria were exposed to H_2O_2 treatment as described above. Treated and control samples were pelleted and resuspended in buffer RLT (Qiagen) + ß-mercaptoethanol, and RNA was isolated using the Qiagen RNeasy kit. DNA contamination was eliminated using the DNA-free kit (Ambion), and RNA was reverse transcribed into cDNA using M-

MLV reverse transcriptase (Invitrogen), in the presence of RNase-OUT (Invitrogen). Additionally, control samples were prepared that lacked M-MLV treatment, to confirm elimination of DNA. cDNA was utilized as a template in qPCR reactions with 0.5μM F and R primers (Table 2-2) and SYBR Green (Applied Biosystems), using the BioRad CFX Real-Time PCR detection system. Samples were normalized to an endogenous 16s RNA control and relative expression was determined using the ΔΔCT method (Applied Biosystems), when comparing H_2O_2-treated to untreated samples.

Table 2-1. Strains and plasmids used in this study.

Strains	Description	Source or Reference
E.coli		
ERG5	DH5α pRK600	Logsdon et al (2003)
ERG73	DH5αλpir pCVD442-Δ*aroA*	This study
ERG60	DH5αλpir pCVD442-Δ*aroE*	This study
ERG63	DH5αλpir pCVD442-Δ*purM*	This study
ERG99	SY327λpir pCVD442-Δ*YPK_3179*	This study
ERG86	DH5αλpir pCVD442-Δ*YPK_3184*	This study
ERG181	SY327λpir pCVD442-Δ*YPK_3185*	This study
ERG78	DH5αλpir pCVD442-Δ*rfaH*	This study
ERG79	DH5αλpir pCVD442-Δ*wecC*	This study
ERG69	DH5αλpir pCVD442-Δ*arnDT*	This study
ERG49	DH5αλpir pCVD442-Δ*dusB-fis*	This study
ERG71	DH5αλpir pCVD442-Δ*YPK_1920*	This study
ERG72	SY327λpir pCVD442-Δ*oppD*	This study
ERG75	DH5αλpir pCVD442-Δ*flgD*	This study
ERG76	DH5αλpir pCVD442-Δ*YPK_2594*	This study
ERG41	SY327λpir pCVD442-Δ*psaABCEF*	This study
ERG100	SY327λpir pCVD442-Δ*YPK_3600*	This study
ERG87	SY327λpir pCVD442-Δ*YPK_3656*	This study
ERG77	DH5αλpir pCVD442-Δ*YPK_3765*	This study
ERG64	DH5αλpir pCVD442-Δ*YPK_1604*	This study
ERG40	SY327λpir pCVD442-Δ*YPK_2061*	This study
ERG261	DH5αλpir pCVD442-*dusB-fis*	This study
FM036	SM10λpir pSR47-YopETEM	Maldonado-Arocho et al (2013)
Yersinia pseudotuberculosis		
ERG97	IP2666	Simonet and Falkow (1992)
ERG224	IP2666 *yopH*-NdeI Kan^R	Logsdon et al (2003)
ERG318	IP2666 *yopE::mcherry*	Crimmins et al (2012)
ERG169	YPIII pIB1$^-$ Δ*aroA*	This study
ERG115	YPIII pIB1$^-$ Δ*aroE*	This study
ERG113	YPIII pIB1$^-$ Δ*purM*	This study
ERG194	YPIII pIB1$^-$ Δ*YPK_3179*	This study
ERG96	YPIII pIB1$^-$ Δ*YPK_3184*	This study
ERG199	YPIII pIB1$^-$ Δ*YPK_3185*	This study
ERG110	YPIII pIB1$^-$ Δ*rfaH*	This study
ERG140	YPIII pIB1$^-$ Δ*wecC*	This study
ERG95	YPIII pIB1$^-$ Δ*arnDT*	This study
ERG121	YPIII pIB1$^-$ Δ*dusB-fis*	This study
ERG149	YPIII pIB1$^-$ Δ*YPK_1920*	This study
ERG174	YPIII pIB1$^-$ Δ*oppD*	This study
ERG114	YPIII pIB1$^-$ Δ*flgD*	This study
ERG193	YPIII pIB1$^-$ Δ*YPK_2594*	This study
ERG51	YPIII pIB1$^-$ Δ*psaABCEF*	This study
ERG205	YPIII pIB1$^-$ Δ*YPK_3600*	This study
ERG221	YPIII pIB1$^-$ Δ*YPK_3656*	This study
ERG147	YPIII pIB1$^-$ Δ*YPK_3765*	This study
ERG116	YPIII pIB1$^-$ Δ*YPK_1604*	This study
ERG56	YPIII pIB1$^-$ Δ*YPK_2061*	This study
ERG166	IP2666 Δ*aroA*	This study
ERG124	IP2666 Δ*aroE*	This study
ERG185	IP2666 Δ*purM*	This study
ERG198	IP2666 Δ*YPK_3179*	This study
ERG120	IP2666 Δ*YPK_3184*	This study
ERG201	IP2666 Δ*YPK_3185*	This study
ERG127	IP2666 Δ*rfaH*	This study
ERG158	IP2666 Δ*wecC*	This study
ERG186	IP2666 Δ*arnDT*	This study
ERG146	IP2666 Δ*dusB-fis*	This study
ERG187	IP2666 Δ*YPK_1920*	This study
ERG164	IP2666 Δ*oppD*	This study
ERG125	IP2666 Δ*flgD*	This study
ERG162	IP2666 Δ*YPK_2594*	This study
ERG148	IP2666 Δ*psaABCEF*	This study
ERG207	IP2666 Δ*YPK_3600*	This study
ERG211	IP2666 Δ*YPK_3656*	This study
ERG163	IP2666 Δ*YPK_3765*	This study
ERG179	IP2666 Δ*YPK_1604*	This study
ERG222	IP2666 Δ*YPK_2061*	This study
ERG316	IP2666 *yopH*-NdeI Kan^R Δ*dusB-fis*	This study
ERG301	IP2666 Δ*dusB-fis::dusB-fis*	This study
ERG291	IP2666 ETEM	Harmon et al (2010)
ERG292	IP2666 Δ*yscF*-ETEM	This study
FM034	IP2666 Δ*yopB*-ETEM	Maldonado-Arocho et al (2013)
ERG293	IP2666 Δ*dusB-fis*-ETEM	This study
ERG259	IP2666 Δ*hmp*	Davis et al (2015)

Table 2-2. Primers used in this study.

Primer name	Primer sequence
aroA p1	5'-ATTATCTAGACATTGACGGCGTTGCGATTAATGAACAAC-3'
aroA p2	5'ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATGGGAAAAACATTCTCCAGTCTTGTATAG-3'
aroA p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTGATAGCACCTGAATTGAATGGGTTCTATCACA-3'
aroA p4	5'-ATTA GAGCTCTGCAACTGTAGCATACGTCTGTGTGC-3'
aroE p1	5'-ATTATCTAGACAAGGGAAACTGTTACAACGTAAGTCTC-3'
aroE p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATACAATATCCTACCCTTGGCGAAAC-3'
aroE p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACGGTAAATAGAAGCACACACCAATGAATGC-3'
aroE p4	5'-ATTAGCATGCGATGCGCATTATAGGGAGTCGATTTTTTTG-3'
purM p1	5'-ATTATCTAGATGGTCATATGGGCCGAACCGGA-3'
purM p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATAATCTAAGTTTCTCTCGTCTTCGAAGC-3'
purM p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTTAGGTTAATAAGTTAAAGTGAGAAGGGTATTGAAG-3'
purM p4	5'-ATTAGCATGCCTGCAGAACATGGAATGATCACATTGAATC-3'
YPK_3179 p1	5'-ATTATCTAGAATTGCAAAAGTATTTTTCGATAATACTGTGGATGCTTG-3'
YPK_3179 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATATATATTCTCTTTATGACTTTAGAGTAAATCAGTAGTGAC-3'
YPK_3179 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTAATGTTAATATAATAACTTGGGAGGATAATATTTAATTTTAAAGTTGG-3'
YPK_3179 p4	5'-ATTAGCATGCGGATTAATATAGTTAATTAAGTGTTCAACATAGTCATTAATAATAGATATTTTT-3'
YPK_3184 p1	5'-ATTATCTAGAGTAGTTCTATATTCAATATTGCAAATATCCAAAGGC-3'
YPK_3184 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATTTCTCACCAACCAACTATTTTATTTAGCATTAGAA-3'
YPK_3184 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACAACAAATAAAATGGATTACAATATATGACTAAGATTGCGCT-3'
YPK_3184 p4	5'-ATTAGCATGCAACGGTAATTTGCTTGCCTGTTGCAATAAC-3'
YPK_3185 p1	5'-ATTATCTAGATTAGGGCCAGCGGCTATAATTCCATTTG-3'
YPK_3185 p2	5'ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCAAAGTTAACTCCAATTAAAATAAAACATCACATTATTCTTATACCG-3'
YPK_3185 p3	5'TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTAAAATAGTTGGTTGGTGAGAAATGAAGATAATTTACGA-3'
YPK_3185 p4	5'ATTAGCATGCTCGCTTCTAATATTGGAATACCAAAACCTTCATAG-3'
rfaH p1	5'-ATTATCTAGACCAATACCTGCTGGCGATAGATGC-3'
rfaH p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATAGTTTCACATACCTTTGGGTACTATTTAGCA-3'
rfaH p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTAATAACACGCTGACTTACAATAATCTGGATGA-3'
rfaH p4	5'-ATTACCCGGGTGCGTTGAAAGATGCAGTTCCTGAGCTA-3'
wecC p1	5'-ATTATCTAGACATACCTGTCGGCCATGTCGAG-3'
wecC p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTACTCATAGCGTCACCTGATGATTCTTTAAAG-3'
wecC p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTGGCGTTGAGGCGAATTCTCGTCA-3'
wecC p4	5'-ATTAGCATGCCGCAAATGGTTTCAACTACTTCAATATTCTTGC-3'
arnDT p1	5'-ATTAATCTAGACTATTGCGACACCCATTGAATACACCC-3'
arnDT p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATGCGTTCAACTCATCTTTAGGGGCT-3'
arnDT p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTGAACAGCTACCTACTCTTGCCGATG-3'
arnDT p4	5'-ATTAGCATGCGCTGGCGTGGTACATCGTTACGG-3'
dusB-fis p1	5'-ATTAGCATGCCCAATATTCTGGCAGGCCCATTACG-3'
dusB-fis p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATAGACAAAGAATGACCACACTGTGTCC-3'
dusB-fis p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTGATACTAGTCAGTTAACTTGTTGTTTAAAAAGGCAC-3'
dusB-fis p4	5'- ATTAGAGCTCAAAGCTACGATGATATCGTTGGCTTGTTGAT-3'
YPK_1920 p1	5'-ATTATCTAGATGTCATGTCGTATCGTTTACAGAACCATC-3'
YPK_1920 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATTCCTTCGCCCTCGTCGGTATT-3'
YPK_1920 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACCTTTTCTAAGGAACCATCATGAAAAAGCATACTTTA-3'
YPK_1920 p4	5'-ATTAGAGCTCTCTTCGTATTTATGGAAAGTTGGCACCAAG-3'
oppD p1	5'-ATTATCTAGAGGGCTGTTGGCCAGTAATGGTCG-3'
oppD p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATACTAATTCCCCTACCGGCTTATAACAG-3'
oppD p3	5'-TTCCTATTCTCAAGGTCGACCTGTATTTAAGTGGTCCAACTAACTTGGTGATTTTCTGTGATTACGTCTGCAT-3'
oppD p4	5'-ATTAGCATGCCTGGGAACATATTTTTAGTTTCCGCAACCATC-3'
flgD p1	5'-ATTATCTAGAAGCGCTTAATTTGCGTGCACAACGG-3'
flgD p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATTATGCTGGCTCCTGTTATTGACCC-3'
flgD p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTTATAAGTCTGTTGTTAGGCTTTCATAGAACTGTTA-3'
flgD p4	5'-ATTAGCATGCTTGATTTCATGGCGGTTACCCAAGCTAT-3'
YPK_2594 p1	5'-ATTATCTAGACACTGCTGTCAGTATATGCAACATAAACC-3'
YPK_2594 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCAAGCTGTCCTCATGCCACACCA-3'
YPK_2594 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACGAGAAATAAGGGAATATCATTGTGAATAAGAAAAACAG-3'
YPK_2594 p4	5'-ATTAGCATGCTATCTTCAGGCC AGTGAATAGTATTGATCG-3'
psaABCEF p1	5'-GGTTAAAAAGGATCGATCCTCTAGAATGGGCGGCCCAGTGAATAAAGCG-3'
psaABCEF p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATTTGCCCTCACCTCCCCTGAT-3'
psaABCEF p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTAAAGGCATCCGCGATGACACAATTCC-3'
psaABCEF p4	5'-CCGGGAGAGCTCGATATCGCATGCTGAGAAATTTAAACTCGCGTGTGAGAAGTACG-3'
YPK_3600 p1	5'-ATTATCTAGAATCAGTTTCAGAATTTCCAAACCAATATTCACTGC-3'
YPK_3600 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATCATGACATCCTATTTATACCGTCATACTTC-3'
YPK_3600 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACCAGCACTAAAATATGTCTGTAAGTGACACCA-3'
YPK_3600 p4	5'-ATTAGCATGCTATTTTTAGCCTCGTGAGTGAAGGGTTGA-3'
YPK_3656 p1	5'-ATTATCTAGAGGGGCGCAATATTTTTCAGTCAGATGCGC-3'
YPK_3656 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATAGAAACTCCTGTTTTATATGGTGGTCACA-3'
YPK_3656 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTTGAAGTAATCCGATAAGGGTCACTGG-3'
YPK_3656 p4	5'-ATTACCCGGGGTCCCCGGCACTCCTACGC-3'
YPK_3765 p1	5'-ATTATCTAGACTTCGTTGATGGAATACATGCAACCGG-3'
YPK_3765 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATAAATCTTCCGTATTAATAAGCTTAAAATTTGTAGCC-3'
YPK_3765 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTTGGATTAGGCTGGATAAAATATCCCCTG-3'
YPK_3765 p4	5'-ATTAGAGCTCTTCGCACGTTTAGTCAAAATCACTTATTCAATTG-3'
YPK_1604 p1	5'-ATTATCTAGAACTGGTCGGTGCACAAGGTATTACCT-3'
YPK_1604 p2	5'ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATTGATGGCTCCTCCTATCAGGTGA- 3'
YPK_1604 p3	5'TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTAACCCGAGCCATCGGAGGCCAT-3'
YPK_1604 p4	5'-ATTAGCATGCGCAATATAAGGGGTTATCTTTGAGCATGGG-3'
*YPK_2061*p1	5'-ATTATCAGATTTACTCCTCTGCCTATCCTCACCGTT-3'
YPK_2061 p2	5'-ACAGCGTCGACCTTGAGAATAGGAACTTCGGAATAGGAACTCATAATGATTCCATTTATTAAAAGTTAATTTTTGATAAGGCAGGA-3'
YPK_2061 p3	5'-TTCCTATTCTCAAGGTCGACGCTGTATTTAAGTGGTCCAACTAATATTCTGTTGATTAACGCAGATAACCAGAATGAC-3'
YPK_2061 p4	5'-ATTAGCATGCACGCAAAAGTATTGAGATAGACTATTGTCCAAATTG-3'
mini-TnSeq F1	5'-AGTTCCTATTCCGAAGTTCCTATTCTCAAG-3'
mini-TnSeq R1	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGG-3'
mini-TnSeq F2	5'-CAAGGTCGACGCTGTATTTAAGTGGTCCAACT-3'
mini-TnSeq R2	5'-CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGG3'-
mini-TnSeq Illumina sequencing primer	5'-CAAGGTCGACGCTGTATTTAAGTGGTCCAACT-3'
16s RNA qPCR F	5'-CAGCCACACTGGAACTGAGA-3'
16s RNA qPCR R	5'-GTTAGCCGGTGCTTCTTCTG-3'
sodA qPCR F	5'-TGCAAATACGGTGTTGGAGA-3'
sodA qPCR R	5'-GATAGCGGCTTTCAGGTCAC-3'
ahpC qPCR F	5'-CGTGGTGTTGAAGTTGTTGG-3'
ahpC qPCR R	5'-TGTCGATCAGGAATGAACCA-3'
katG qPCR F	5'-GAAATGCAGCCATTGAGGAT-3'
katG qPCR R	5'-TAGGCGAAAGCGTGTTTCTT-3'
grxA qPCR F	5'-TTACTGTGTCCGTGCCAAAGAG-3'
grxA qPCR R	5'-CGTAGGCTTCGAAATCAGT-3'
rpoC qPCR F	5'-ACGGGTAGCGGTAAAGACCT-3'
rpoC qPCR R	5'-AATACGCGCCAAGGTATCAC-3'

CHAPTER 3: DISCOVERY AND CHARACTERIZATION OF CHROMOSOMAL FACTORS CONTRIBUTING TO *YERSINIA PSEUDOTUBERCULOSIS* PATHOGENESIS

Experiments summarized in Figure 2-1 and Table 2-1 were performed in collaboration with Gregory Crimmins and are included in the following manuscript:

Crimmins, G. T., S. Mohammadi, E. R. Green, M. A. Bergman, R. R. Isberg and J. Mecsas (2012). "Identification of MrtAB, an ABC transporter specifically required for *Yersinia pseudotuberculosis* to colonize the mesenteric lymph nodes." PLoS Pathog **8**(8): e1002828.

3.1 BACKGROUND

Previous studies have demonstrated that strains of *Yptb* lacking the T3SS-encoding pIB1 virulence plasmid retain the ability to colonize and grow within lymphoid tissue sites, indicating that chromosomal factors contribute to infection within these organs (Simonet, Mazigh et al. 1984, Une and Brubaker 1984, Balada-Llasat and Mecsas 2006). Though several chromosomal virulence factors have been previously identified and studied in *Yersinia* infection models, much work in the field has focused on characterizing the functions and targets of T3SS effector proteins during mammalian infection (Viboud and Bliska 2005, Aepfelbacher, Trasak et al. 2007, Matsumoto and Young 2009). Several transposon-based screens have been performed to identify *Yersinia* virulence factors; however, prior to the publication of this study(Crimmins, Mohammadi et al. 2012), no screens utilizing transposon libraries with a complexity of more than 2,000 insertion mutants had been reported (Darwin and Miller 1999, Karlyshev, Oyston et al. 2001, Mecsas, Bilis et al. 2001, Flashner, Mamroud et al. 2003, Flashner, Mamroud et al. 2004). Additionally, no screens had been performed using libraries generated in the absence of the virulence plasmid, which could mask the contributions of chromosomal virulence factors. Therefore, we hypothesized that the *Yptb* chromosome encodes many virulence factors that have not yet been identified and carried out a high-throughput screen to identify such factors.

3.2 TnSeq screen to identify chromosomal factors required for T3SS-independent infection

To identify chromosomal factors that contribute to colonization and/or growth of plasmid-deficient *Yptb* in deep tissue sites, we performed a transposon-based, TnSeq screen. TnSeq utilizes massively parallel sequencing of transposon junctions to quantify insertion mutants, which allows for the screening of complex libraries containing tens of thousands of unique clones (van Opijnen, Bodi et al. 2009). Therefore, two transposon library pools, each containing ~10,000 insertion mutants, were generated in the pIB1$^-$ YPIII strain. The number of *Yptb* clones that colonize the small intestine and organ sites following oral inoculation is extremely small (Mecsas, Bilis et al. 2001, Barnes, Bergman et al. 2006); therefore, we chose to infect mice by the systemic route and analyze colonization and growth of transposon mutants within the liver, where roughly 50% of clones present in the inoculum were recovered at 3 days post-infection (Crimmins, Mohammadi et al. 2012).

Each library pool was infected into 10 C57Bl/6 mice by intravenous injection, at a dose of $1x10^5$, which ensured 10-fold coverage of each insertion mutant in the inoculum. Collectively, these two pools contained insertions in 3088 genes out of 4250 annotated genes in the genome, with 1977 genes containing transposon insertions in both libraries (Fig 3-1A). After 3 days, bacteria were recovered from the livers of infected mice and DNA "output" libraries were prepared for Illumina sequencing. Input libraries were also generated from the inoculum. Additionally, in order to identify any mutants that were generally defective for growth at high temperatures, a second screen was performed to determine genes essential for growth at 37°C.

The proportion of reads for a given gene within each output sample was determined and divided by that gene's proportion in its corresponding input pool. Output/input ratios were Log_2 transformed and statistical significance was defined as having a log_2 output/input ratio of $\geq$2.5 standard deviations from the mean of all genes (Fig 3-1). This analysis was performed with 3 separate groups of genes: 1) all genes mutated in both libraries, 2) all genes mutated in Library #1, and 3) all genes mutated in Library #2. Additionally, all genes with a log_2 output/input ratio of $\geq$2.5 standard deviations from the mean in the 37°C screen were eliminated from the analysis.

Using the cutoff of $\geq$2.5 standard deviations from the mean of all genes, we identified 33 chromosomal factors that contributed to growth of pIB1$^-$ YPIII in livers, including many genes that had been previously uncharacterized in *Yersinia* infection models (Table 2-1). A large subset of these factors was related to regulation and biosynthesis of LPS, which is known to modulate the virulence of *Yersinia* (Bengoechea, Zhang et al. 2002, Montminy, Khan et al. 2006, Perez-Gutierrez, Llobet et al. 2010). Another subset included factors involved in the biosynthesis of amino acids and purines, indicating that these molecules may be rare or depleted in the animal host. We also identified a number of genes encoding known *Yptb* virulence factors, including Invasin, pH6 antigen, and SufI, which suggested that this screen identified factors important for infection of both plasmid-deficient and WT *Yptb.* Additionally, we identified several genes with unknown or previously uncharacterized functions, including genes involved in response to envelope stress, flagellin biogenesis, and multiple genes encoding membrane transporters.

Figure 3-1. Characterization of TnSeq libraries and histogram of results from screen for chromosomal virulence factors

(A) Number of genes containing mutations in each transposon mutant pool (Dashed line = Library #1. Solid line = Library #2) **(B)** Histogram of the 1977 genes mutated in both transposon libraries. X-axis measures the Log_2 transformed Output/Input ratios for each gene following infection of the liver. Y-axis measures the number of genes corresponding to each X-interval value.

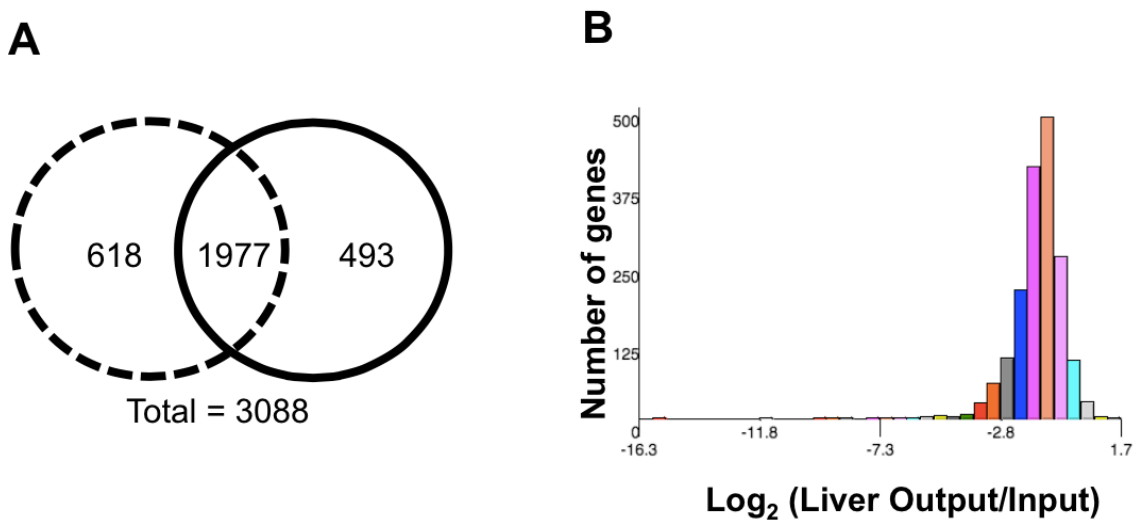


Table 3-1. Identification of genes required for growth of pIB1$^-$ *Yptb* within the liver

Shown are genes that contained transposon insertions that displayed Log_2 Output/Input values that were ≥2.5 standard deviations from the mean Log_2 Output/Input of the libraries analyzed during liver infection. Output/Input values were determined by dividing the fraction of sequencing reads for a given gene in an output sample by its fraction in its respective input pool. All genes with Log_2 Output/Input ratios that were ≥2.5 standard deviations from the mean following growth at 37° C were eliminated from our analysis.

	Library	Annotation	Output/Input (Std dev. from mean)
Known virulence factors			
YPK_2757	#1 and #2	*psaC*	1.07E-02 (-4.72)
YPK_0665	#1 and #2	*sufI*	1.86E-02 (-3.99)
YPK_2429	#1 and #2	*invasin*	1.98E-02 (-3.91)
YPK_2759	#1 and #2	*psaA*	3.08E-02 (-3.32)
Metabolism			
YPK_0321	#1 and #2	*aroE*	3.28E-04 (-9.27)
YPK_1253	#1 and #2	*purM*	5.28E-03 (-5.67)
YPK_0357	#1 and #2	*purH*	1.80E-02 (-4.04)
YPK_2670	#1 and #2	*aroA*	3.06E-02 (-3.33)
YPK_2047	#1 and #2	*trpA*	3.33E-02 (-3.22)
YPK_2528	#1 and #2	*hisB*	3.42E-02 (-3.18)
YPK_0356	#1	*purD*	9.53E-03 (-2.58)
YPK_1364	#2	*purC*	0 (NA)
LPS			
YPK_3937	#1 and #2	*rfaH*	2.36E-05 (-12.87)
YPK_3184	#1 and #2	O-Ag	1.52E-03 (-7.32)
YPK_3179	#1 and #2	O-Ag	2.32E-03 (-6.77)
YPK_3183	#1 and #2	O-Ag	5.38E-03 (-5.64)
YPK_4030	#1 and #2	*wecC*	2.61E-02 (-3.55)
YPK_1834	#1 and #2	*arnD*	4.77E-02 (-2.74)
Other			
YPK_3221	#1 and #2	*mrtB*	0 (NA)
YPK_3222	#1 and #2	*mrtA*	2.19E-03 (-6.84)
YPK_1234	#1 and #2	phage protein	2.69E-03 (-6.57)
YPK_2423	#1 and #2	*flgD*	3.03E-02 (-3.34)
YPK_1292	#1 and #2	*rodZ*	4.07E-02 (-2.95)
YPK_2066	#1 and #2	*oppD*	4.40E-02 (-2.85)
YPK_1713	#1 and #2	Hypothetical	5.20E-02 (-2.63)
YPK_2406	#1	Hypothetical	0 (NA)
YPK_3656	#1	Hypothetical	0 (NA)
YPK_0453	#1	*dusB*	1.73E-04 (-5.98)
YPK_0688	#1	Hypothetical	4.46E-04 (-5.17)
YPK_2424	#1	*flgC*	8.56E-03 (-2.67)
YPK_3600	#1	Hypothetical	9.96E-03 (-2.54)
YPK_2199	#2	Hypothetical	0 (NA)
YPK_0208	#2	Hypothetical	8.67E-03 (-2.75)

3.3 Design and implementation of mini-TnSeq screen to confirm findings of transposon-based screen

Using large pools of transposon mutants in animal infection models can sometimes result in "false negatives," as libraries are subject to bottleneck constraints and transposon insertions can have polar effects on nearby loci. Therefore, to further evaluate the loci identified in our TnSeq screen, we devised and implemented a high-throughput, sequencing-based approach (a "mini" TnSeq) to simultaneously compare the survival of multiple in-frame deletion mutants in small infection pools (Fig. 3-2). Eighteen mutants containing an in-frame scar sequence (Fig 3-3) were generated in both the plasmid deficient (pIB1$^-$) YPIII strain, as well as in a plasmid-containing (pIB1$^+$) IP2666 strain, to determine whether the *in vivo* contributions of some of these genes may be masked by the T3SS. We chose the IP2666 pIB1$^+$ strain because it encodes the known virulence factor, *phoP*, which is mutated in YPIII (Grabenstein, Marceau et al. 2004).

The 18 operons and genes represented the broad functional classes identified in the original screen, which included biosynthesis of metabolic compounds, LPS synthesis and modification, as well as several factors that have been previously uncharacterized in *Yersinia* infection models (Table 3-2). In order to test our statistical cutoff of 2.5 standard deviations from the mean, we also chose to evaluate 4 genes, *YPK_3185, YPK_1920, YPK_2594,* and *YPK_3765*, that fell slightly below this cutoff, but were either components of operons and pathways containing multiple attenuated mutants, or appeared interesting because of their potential to encode functions critical within tissue environments. Additionally, because 4 genes chosen for this analysis, *dusB, arnD,* and *psaA,* and *psaC,* were predicted to be in operons with genes that did not contain

insertions in either transposon library, entire operons of those loci were deleted to control for possible polar effects that transposon insertions may have caused in the TnSeq screen.

In order to ensure that the bacterial pools used to infect mice contained an equal mixture of attenuated mutants and WT bacteria, we also constructed deletions of two "neutral genes," *YPK_1604* and *YPK_2061*, selected because transposon disruptions in these genes had no deleterious effects in the original TnSeq screen. Mice were infected intravenously with 10^4 CFU of the pIB1$^-$ library or 10^3 of the pIB1$^+$ library. In each pool, the two neutral strains comprised roughly 50% of the inoculum, while the remaining 18 mutants each comprised ~3% of the population (Fig 3-2). Because use of smaller mutant pools eliminated bottleneck constrictions, the phenotypes of these mutants were evaluated in both livers and spleens. Following recovery of bacteria from infected livers and spleens at 3 days post-infection, DNA was processed for Illumina sequencing and fitness values were calculated for each mutant by dividing the proportion of reads for a mutant in an "output" liver or spleen by its proportion in the inoculum. Statistical significance was determined by comparing the mean fitness values of mutants to that of the neutral mutants pooled together.

Figure 3-2. Depiction of mini-TnSeq experiment

Infection inoculums were prepared so that each putatively attenuated mutant represented ~3% of the pool, while each of the two neutral strains represented 25% of the inoculum. Mice were infected with pIB1^{+} and pIB1^{-} libraries, and after 3 days, spleens and livers were isolated, homogenized, and plated to retrieve surviving bacteria. DNA was prepared for sequencing as indicated in Fig 2-3, and fitness values were calculated by determining the percentage of reads for a mutant in an organ divided by the percentage of that mutant in its respective input pool.

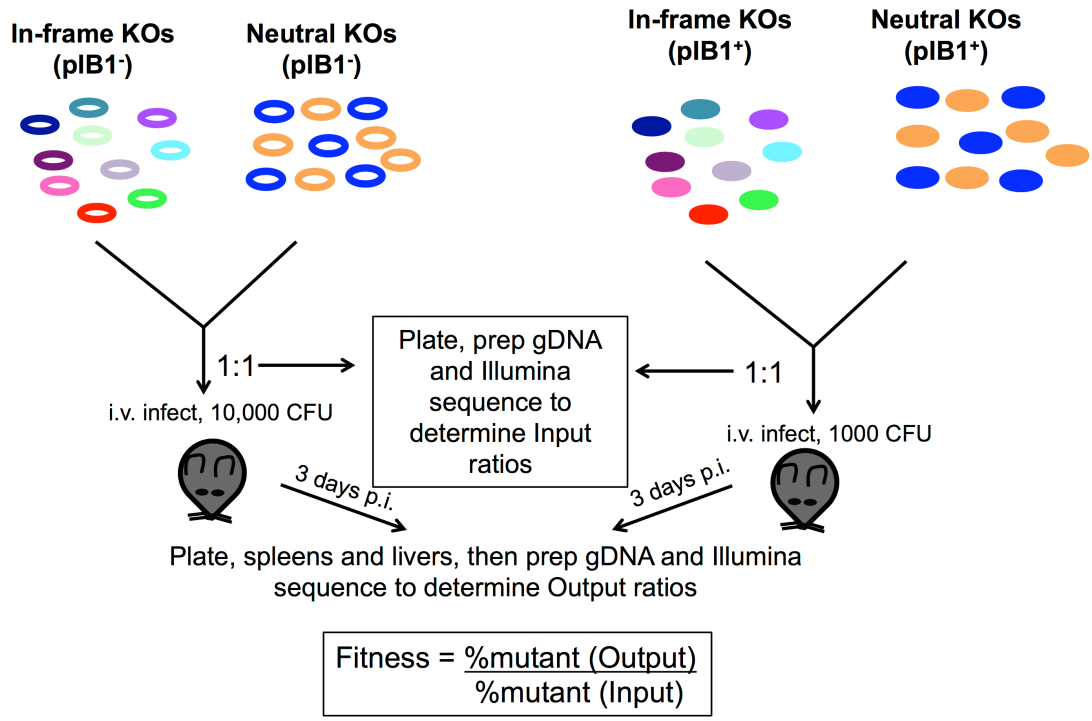
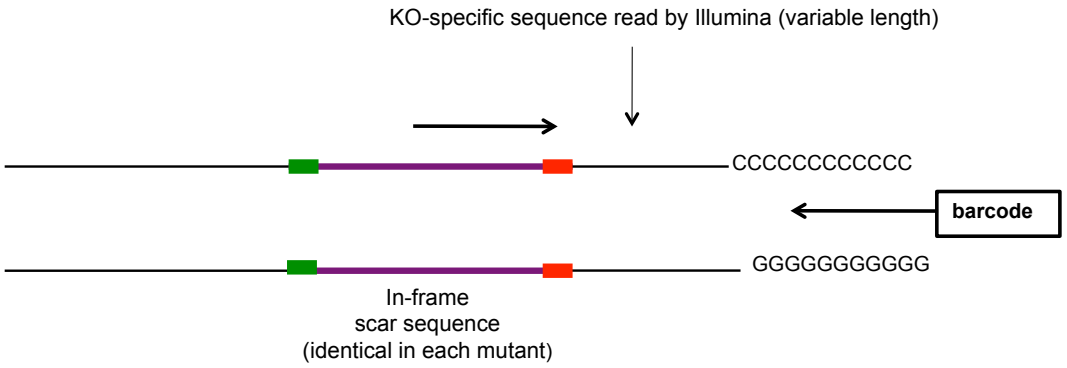


Figure 3-3. Preparation of mini-TnSeq libraries for Illumina sequencing

Mutants containing in-frame scar sequences were prepared for Illumina sequencing as indicated below. Briefly, genomic DNA was isolated from bacterial input and output pools, sheared by sonication, and treated with terminal deoxytransferase in order to generate a 3' poly C-tail sequence. Two rounds of nested PCR were then employed to amplify regions immediately downstream of deleted genes. These products were multiplexed using 6bp indexing primers and sequenced on the Illumina Hi-Seq 2500.



Using this approach, we found that 14/18 mutants generated in the pIB1$^-$ YPIII background had statistically significant virulence defects in infected liver tissues. Strikingly, 13 of those 14 genes were also critical for growth of pIB1$^+$ IP2666 within the liver (Fig 3-4, Table 3-2). These findings suggested that our TnSeq screen succeeded in identifying factors important for *Yptb* infection, and also indicated that nearly all genes evaluated were required for virulence, even in the presence of the T3SS. Additionally, of the 4 genes we evaluated that fell below our statistical cutoff in the TnSeq screen, only one, *YPK_3185*, was dispensable for virulence in this assay. This indicates that our earlier statistical cutoff may have been conservative and that even more genes identified in the TnSeq screen may be worthy of further study.

Mutants that were attenuated for growth within the liver in the mini-TnSeq assay included the auxotrophic strains Δ*aroA* and Δ*aroE*, which are unable to produce aromatic amino acids, and Δ*purM,* which lacks a component of the purine biosynthesis pathway. With the exception of one strain (Δ*YPK_3185*), all of the mutants containing deletions of LPS synthesis and modification genes were attenuated for virulence in at least one tissue site. Importantly, several factors that had not been previously characterized in *Yersinia* infection models, including *YPK_2594*, which has no predicted function, *YPK_1920*, which is predicted to encode a lipoprotein, *YPK_3765*, predicted to encode a murein peptide ligase, and the *dusB-fis* operon, which encodes the nucleoid associated protein Fis, were critical for infection. Additionally, we identified six mutants, Δ*aroA,* Δ*YPK_3184,* Δ*arnDT,* Δ*YPK_1920,* Δ*YPK_2594,* and Δ*psaABCEF,* which were defective for growth of the pIB1$^+$ strain in the liver (Fig 3-4), but not the spleen (Fig 3-5) (Table 3-2).

Four mutants evaluated in this assay, Δ*YPK_3185, ΔYPK_3185, ΔYPK_3600,* and Δ*YPK_3656* did not have virulence defects in pIB1$^-$ YPII. As mentioned, one of these genes, *YPK_3185*, fell below our initial statistical cutoff. Additionally, it is in an operon with other O-antigen biosynthesis genes important for virulence; the lack of a phenotype in this in-frame deletion indicates that the insertion was likely polar on downstream genes in the original TnSeq screen. Two genes, *YPK_3600* and *YPK_3656*, were predicted to encode hypothetical proteins and were only present in one of the two libraries tested in the original TnSeq screen. Thus we hypothesize that bottleneck effects might have influenced their significance in that assay. The last mutant to not repeat was a deletion of *flgD*, which is predicted to contribute to flagellin synthesis. Because *Yersinia* does not express flagellin genes at 37°C (Badger and Miller 1998), it is not surprising that this gene was not essential for virulence. Potentially, in the original screen the insertion in this gene may have had polar effects on the nearby *inv* locus, which also contained attenuated insertion mutants in our TnSeq analysis. Alternatively, the virulence of insertion mutants in this gene may have been influenced by their low frequency in the transposon mutant pool, as small defects in growth or colonization could be easily overcome by increased representation in the inoculum.

Table 3-2. Phenotypes of mutants in mini-TnSeq assay

Shown are p-values representing comparisons between the fitness scores of individual mutants evaluated in mini-TnSeq assay and the fitness score of the two neutral mutants (pooled together), as described in Fig. 2-4, Fig 2-5 and Experimental Procedures. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$.

	pIB1$^-$		pIB1$^+$	
	Liver	**Spleen**	**Liver**	**Spleen**
Metabolism				
ΔaroA	****	ns	****	ns
ΔaroE	****	****	****	****
ΔpurM	****	****	****	****
LPS				
ΔYPK_3179	****	****	***	***
ΔYPK_3184	****	**	**	ns
ΔYPK_3185	ns	ns	ns	ns
ΔrfaH	****	**	*	**
ΔwecC	****	ns	**	****
ΔarnDT	***	ns	****	ns
Other				
ΔdusB-fis	****	***	**	****
ΔYPK_1920	****	ns	****	ns
ΔYPK_2066	*	ns	ns	ns
ΔflgD	ns	ns	ns	ns
ΔYPK_2594	**	ns	****	ns
ΔpsaABCEF	****	****	*	ns
ΔYPK_3600	ns	ns	ns	ns
ΔYPK_3656	ns	ns	ns	ns
ΔYPK_3765	****	****	**	****
Neutral				
ΔYPK_1604	N/A	N/A	N/A	N/A
ΔYPK_2061	N/A	N/A	N/A	N/A

Figure 3-4. Fitness of mutants in mini-TnSeq assay in livers

Fitness of knockouts generated in YPIII/pIB1$^-$ **(A)** or 10^3 IP2666/pIB1$^+$ **(B)** at 3 days post-infection of mini-TnSeq libraries. Fitness values were obtained by dividing the proportion of sequencing reads for a mutant in the liver by its proportion of reads in the inoculum. Each data point for a mutant represents the fitness value in an individual mouse. The experiment was repeated twice with 3-5 mice per experiment for a total of 7-10 mice. Fitness values were $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis with Dunnett's multiple comparison post-test. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$. P-values represent comparisons between the fitness scores of individual mutants with the combined fitness scores of the neutral mutants.

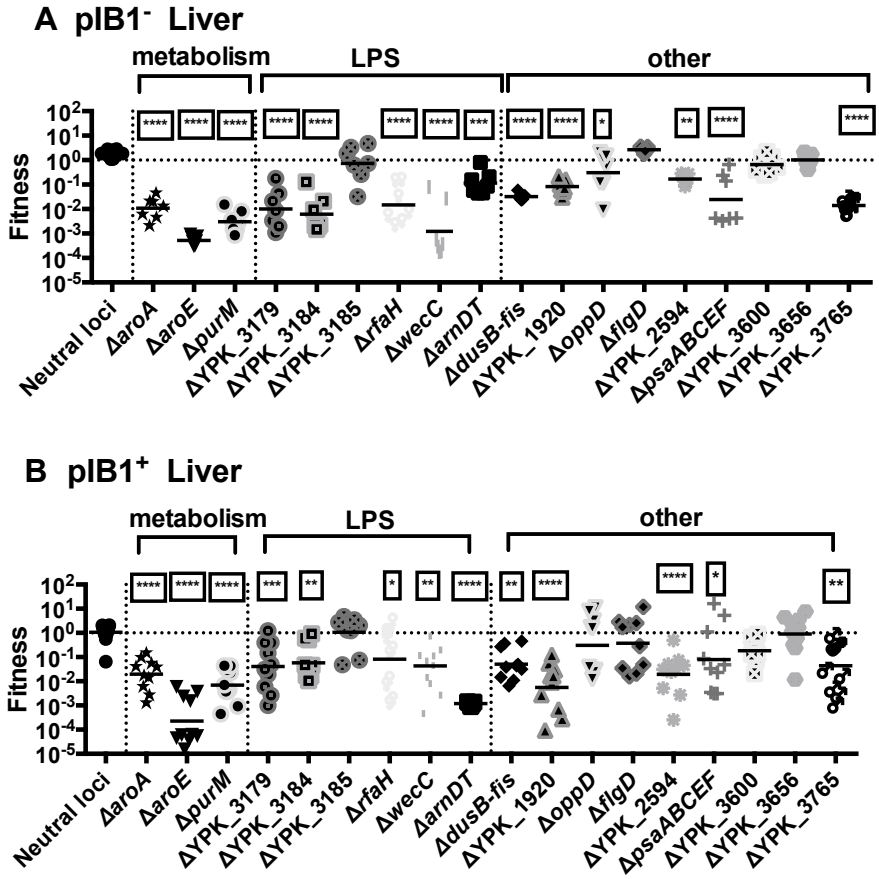
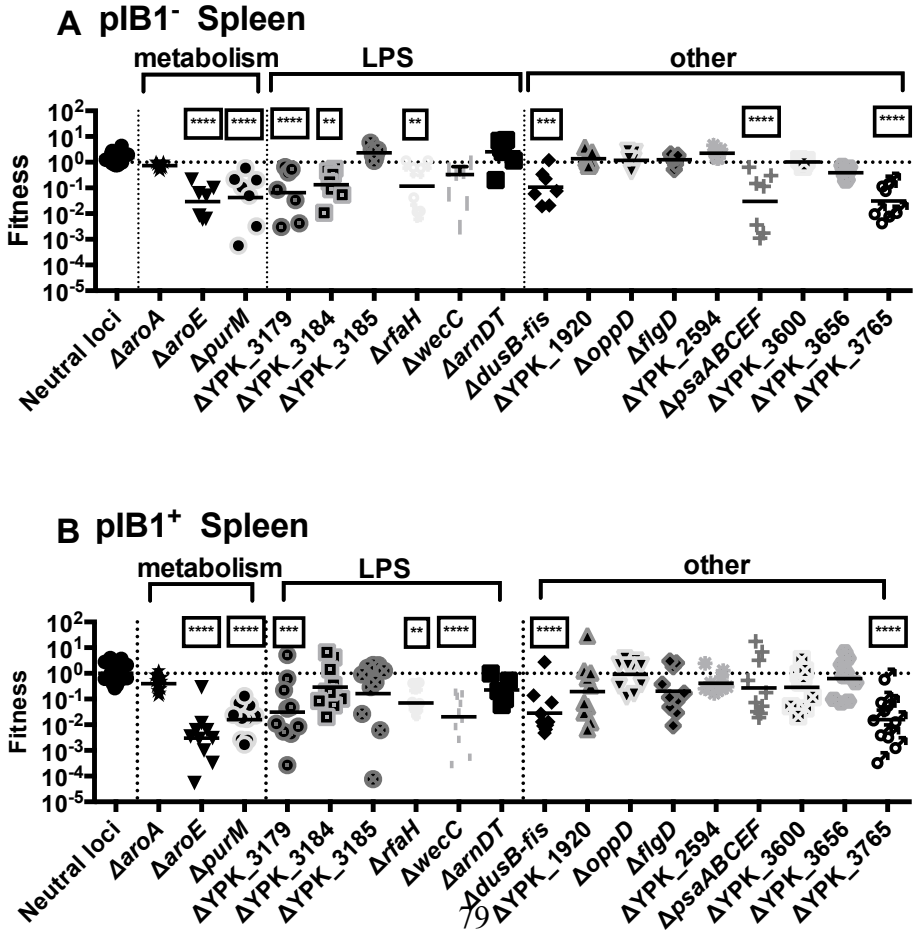


Figure 3-5. Fitness of mutants in mini-TnSeq assay in spleens

Fitness of knockouts generated in YPIII/pIB1$^-$ **(A)** or 10^3 IP2666/pIB1$^+$ **(B)** at 3 days post-infection of mini-TnSeq libraries. Fitness values were obtained by dividing the proportion of sequencing reads for a mutant in the spleen by its proportion of reads in the inoculum. Each data point for a mutant represents the fitness value in an individual mouse. The experiment was repeated twice with 3-5 mice per experiment for a total of 7-10 mice. Fitness values were $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis with Dunnett's multiple comparison post-test. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$. P-values represent comparisons between the fitness scores of individual mutants with the combined fitness scores of the neutral mutants.

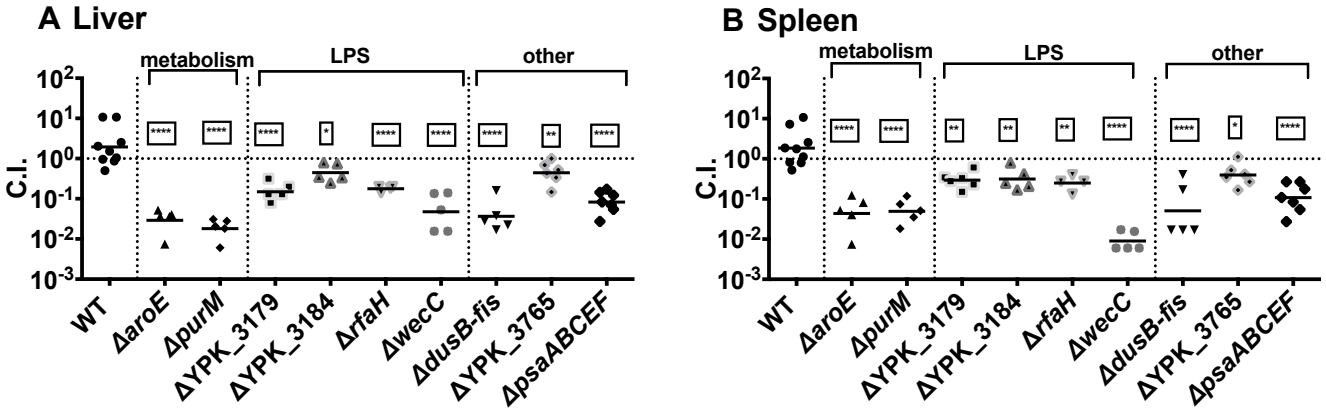


3.4 MINI-TNSEQ ACCURATELY PREDICTED OUTCOMES OF 1:1 INFECTIONS

To evaluate whether mutants were attenuated when they were not in a small minority of the input pool, traditional competition experiments were performed using bacterial mutants generated in the pIB1^{+} background. Mutants were mixed at a 1:1 ratio with a drug resistant WT strain, and C.I. values were obtained (Fig 3-6). Importantly, all of the mutants evaluated were attenuated in this assay. In conclusion, using our efficient and highly sensitive mini-TnSeq assay, we found that many bacterial mutants were attenuated in both WT and plasmid-deficient *Yptb*, indicating that most of these loci do not have redundant roles with the T3SS.

Figure 3-6. Mini-TnSeq accurately predicted outcomes of 1:1 co-infections

1:1 competition experiments with selected mutants from mini-TnSeq library recapitulate screen results. Mice were inoculated intravenously with a pool of 10^3 bacteria, containing a 1:1 mixture of WT-Kan^R and a drug-sensitive mutant. During infection, a drug-sensitive WT strain was also competed against WT-Kan^R. Livers **(A)** and spleens **(B)** were dissected at 3 days post-infection, homogenized and plated onto selective and non-selective plates to determine the competitive index (C.I.). Each data point represents an individual mouse. A total of 4-10 mice were infected per strain and each strain was tested 2-3 times. Each C.I. data value was $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis with Dunnett's multiple comparison post-test, when comparing the C.I. values of mutant strains with those of the sensitive WT strain. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$.



3.5 MINI-TNSEQ SCREEN TO DETERMINE INTERACTION OF MUTANTS WITH INNATE IMMUNE CELLS

Infection with *Yptb* produces a pronounced inflammatory response, where bacteria grow in tissue sites surrounded by phagocytic cells (Balada-Llasat and Mecsas 2006, Crimmins, Mohammadi et al. 2012, Davis, Mohammadi et al. 2015). Therefore, we hypothesized that some of the genes evaluated in the mini-TnSeq assay may encode proteins that directly interface with phagocytic cells, or are important for surviving in the face of anti-microbial responses generated by these cells. To test this, mice pre-treated with the RB6-8C5 antibody, which depletes $Gr1^{pos}$ cells ($Ly6G^{pos}$ neutrophils and $Ly6C^{pos}$ inflammatory monocytes, dendritic cells, and lymphocytes) or the 1A8 antibody, which depletes $Ly6G^{pos}$ cells (neutrophils only) were infected with the $pIB1^{+}$ mini-TnSeq library. Surprisingly, very few significant changes in fitness scores were detected following infection of $Gr1^{pos}$ cell-depleted (Fig 3-7) and $Ly6G^{pos}$ cell-depleted (Fig 3-8) mice, suggesting that most of the genes in our mini-library are important for bacterial colonization and growth in animal tissues, regardless of the presence of phagocytes. However, 4 mutants displayed significantly altered fitness scores upon infection of immunocompromised mice (Fig 3-7 and Fig 3-8).

Growth of Δ*YPK_3765* was restored to WT levels in the livers and spleens of both RB6-8C5- and 1A8-treated mice (Fig 3-7 and Fig 3-8), indicating that this gene is required for resisting growth restriction by phagocytic cells. Strikingly, depletion with the RB6-8C5 and 1A8 antibodies resulted in decreased growth of the Δ*psaABCEF* mutant in spleens (Fig 3-7B and Fig 3-8B), while depletion with 1A8 decreased the growth of Δ*rfaH* in livers (Fig. 3-8A), demonstrating that neutrophils may actually protect these

mutants from further growth restriction in those tissue sites. Because treatment with 1A8 was sufficient to alter growth of Δ*YPK_3765*, Δ*rfaH,* and Δ*psaABCEF*, the fitness of these mutants *in vivo* appears to be specifically modulated by neutrophils (Fig 3-8).

By contrast, growth of Δ*dusB-fis* was restored in the livers and spleens of mice treated with the RB6-8C5 antibody (Fig. 3-7), but not in mice treated with the 1A8 antibody (Fig 3-8). This result suggested one of three possibilities: that Δ*dusB-fis* is sensitive to all $Gr1^{pos}$ cells, to one or more $Ly6C^{pos}$ cell types (inflammatory monocytes, dendritic cells, and lymphocytes), or to $Ly6G^{pos}$ neutrophils and a subset of $Ly6C^{pos}$ cells. To distinguish among these possibilities, we performed 1:1 co-infection experiments with Δ*dusB-fis* in mice treated with an antibody (MC21) that blocks the chemokine receptor CCR2 and prevents recruitment of $Ly6C^{pos}$ inflammatory monocytes to tissue sites from the bone marrow (Mack, Cihak et al. 2001, Bruhl, Cihak et al. 2007, Mildner, Djukic et al. 2008, Reich, Schmidbauer et al. 2013). Additional cohorts of mice were treated with 1A8, with RB6-8C5, or with a combination of the 1A8 and MC21 antibodies prior to infection. Depletion of cell subsets was confirmed by flow cytometry, using Gr1 and Cd11b markers to distinguish between neutrophil and inflammatory monocyte populations (Fig 3-9). Treatment with either 1A8 or MC21 alone did not restore the virulence of the Δ*dusB-fis* mutant (Fig. 3-10), suggesting that the presence of either $Ly6G^{pos}$ or $Ly6C^{pos}$ cell type(s) at the site of infection was sufficient to restrict the growth of this mutant. By contrast, depletion with a combination of the MC21 and 1A8 antibodies restored growth of Δ*dusB-fis* in livers and spleens, demonstrating that Δ*dusB-fis* is specifically sensitive to neutrophils and CCR2-recruited inflammatory monocytes during tissue infection. However, it is also possible that during infection, Δ*dusB-fis*

primarily interfaces with neutrophils and is only sensitive to inflammatory monocytes when neutrophils have been depleted. The experiments performed in this study cannot rule out this possibility.

Importantly, growth of Δ*dusB-fis* was also restored when we complemented this mutant by re-introducing the *dusB-fis* genes into the deleted strain (Fig. 3-10). In summary, these results demonstrate that *dusB-fis* promotes resistance to or evasion of killing by both neutrophils and inflammatory monocytes during mouse infection.

Figure 3-7. Fitness of mini-TnSeq library in mice depleted of $Gr1^{pos}$ cells

Mice were intraperitoneally injected with RB6-8C5 24 hours prior to and 24 hours post-infection. Mice were inoculated intravenously with libraries of knockouts generated in IP2666/pIB1^{+} at a dose of 10^3 CFU. Fitness values were obtained by dividing the proportion of sequencing reads for a mutant in the depleted liver **(A)** or spleen **(B)** by its proportion of reads in the inoculum. Each data point for a mutant represents the fitness value for a mutant an in individual mouse. Each depletion experiment was performed twice with a total of 4-8 mice. Non-depleted fitness values are the same as shown in Fig 2-4 and Fig 2-5. Fitness scores values were $\log_{10}$ transformed and an unpaired t-test, followed by a Holm-Sidak post-test to correct for multiple comparisons, was performed to calculate statistical differences between the fitness scores of specific bacterial mutants in depleted and non-depleted mice. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$.

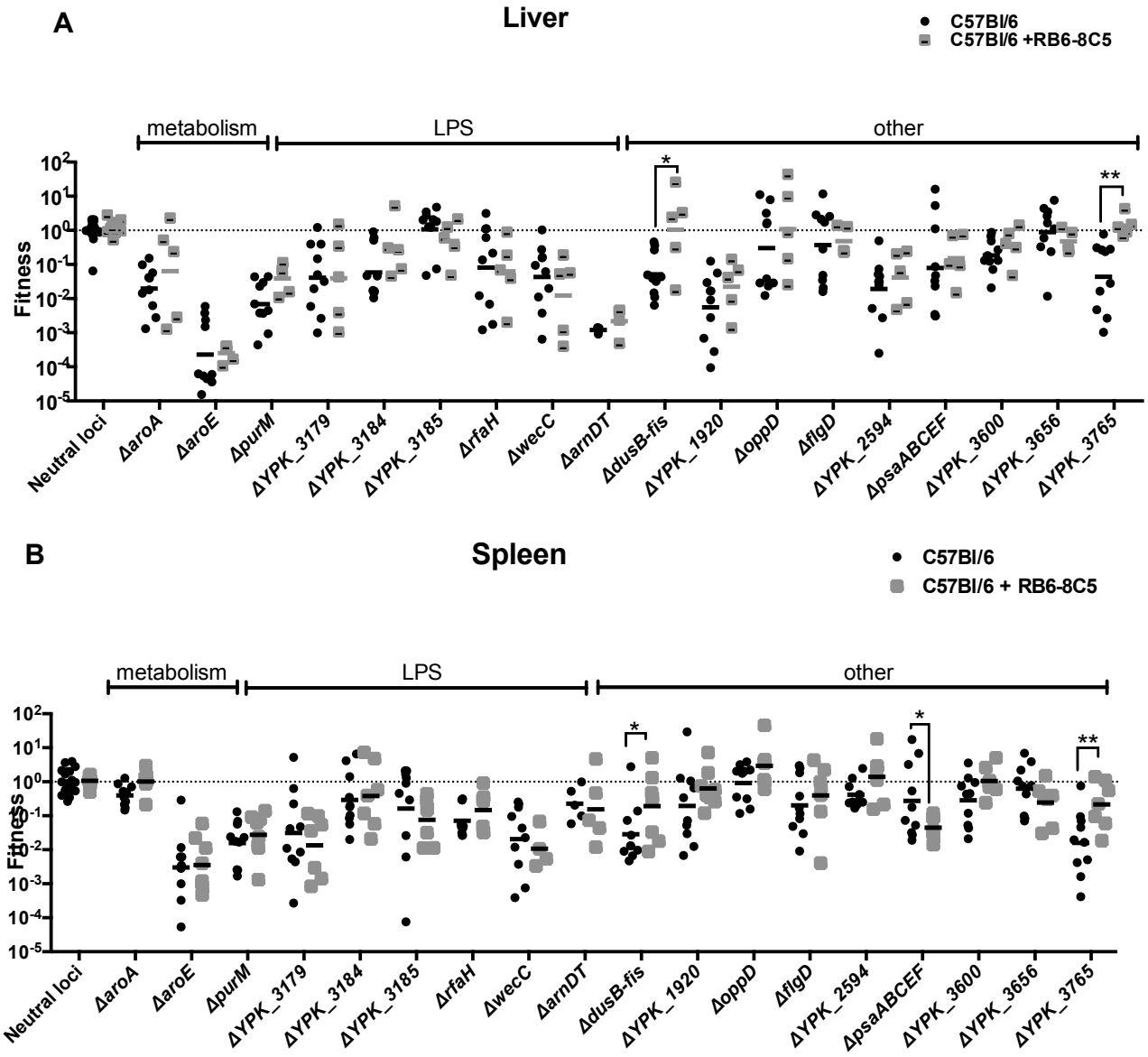

A
Liver
C57Bl/6
C57Bl/6 +RB6-8C5
metabolism
LPS
other
Fitness
Neutral loci
ΔaroA
ΔaroE
ΔpurM
ΔYPK_3179
ΔYPK_3184
ΔYPK_3185
ΔrfaH
ΔwecC
ΔarnDT
ΔdusB-fis
ΔYPK_1920
ΔoppD
ΔflgD
ΔYPK_2594
ΔpsaABCEF
ΔYPK_3600
ΔYPK_3656
ΔYPK_3765
B
Spleen
C57Bl/6
C57Bl/6 + RB6-8C5
metabolism
LPS
other
Fitness
Neutral loci
ΔaroA
ΔaroE
ΔpurM
ΔYPK_3179
ΔYPK_3184
ΔYPK_3185
ΔrfaH
ΔwecC
ΔarnDT
ΔdusB-fis
ΔYPK_1920
ΔoppD
ΔflgD
ΔYPK_2594
ΔpsaABCEF
ΔYPK_3600
ΔYPK_3656
ΔYPK_3765

Figure 3-8. Fitness of mini-TnSeq library in mice depleted of Ly6G^{pos} cells

Mice were intraperitoneally injected with the 1A8 antibody 24 hours prior to and 24 hours post-infection. Mice were inoculated intravenously with libraries of knockouts generated in IP2666/pIB1^{+} at a dose of 10^3 CFU. Fitness values were obtained by dividing the proportion of sequencing reads for a mutant in the depleted liver **(A)** or spleen **(B)** by its proportion of reads in the inoculum. Each data point for a mutant represents the fitness value for a mutant an in individual mouse. Each depletion experiment was performed twice with a total of 4-8 mice. Non-depleted fitness values are the same as shown in Fig 2-4 and Fig 2-5. Fitness scores values were $\log_{10}$ transformed and an unpaired t-test, followed by a Holm-Sidak post-test to correct for multiple comparisons, was performed to calculate statistical differences between the fitness scores of specific bacterial mutants in depleted and non-depleted mice. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$.

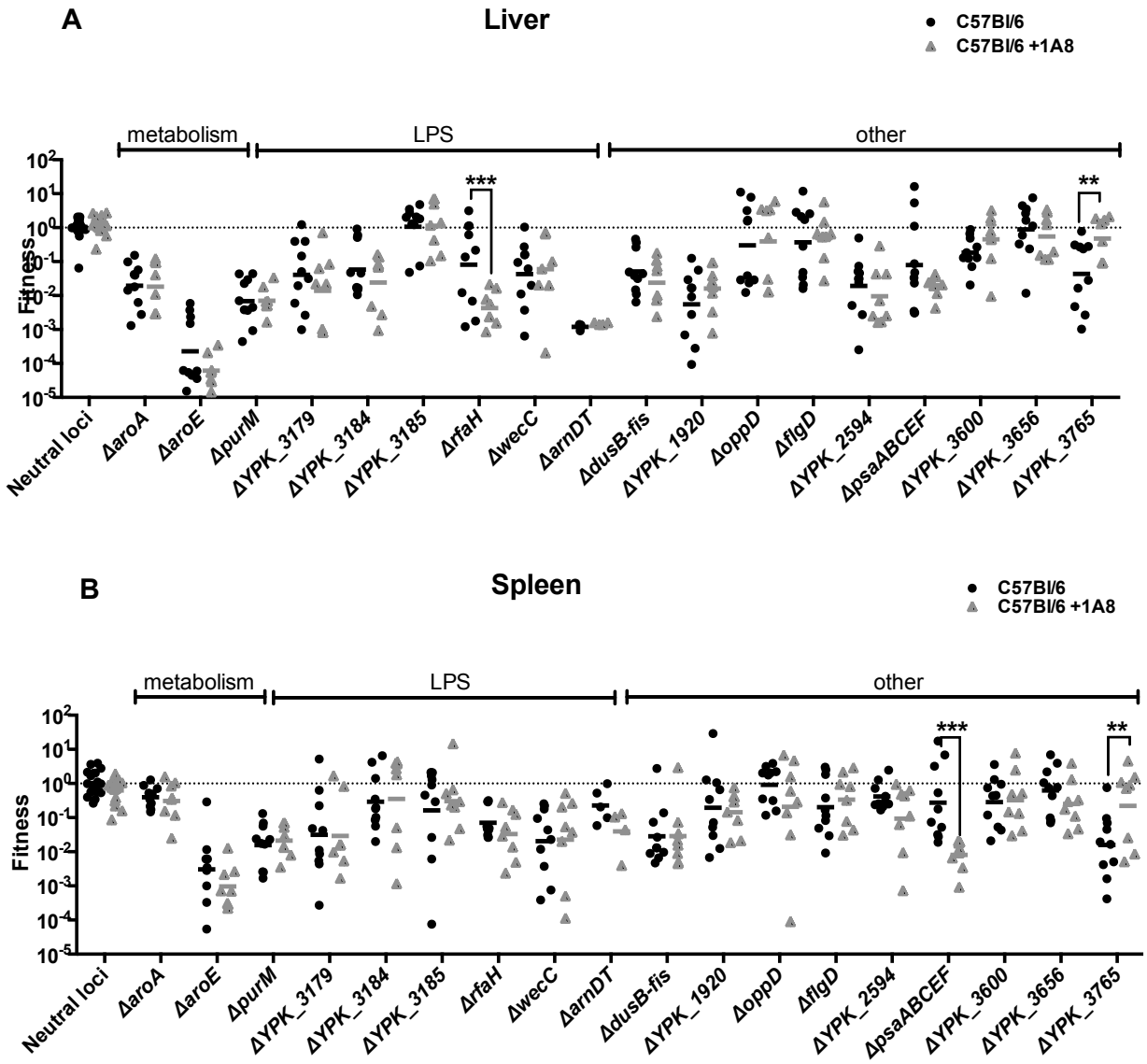

A
Liver
C57Bl/6
C57Bl/6 +1A8
metabolism
LPS
other
Fitness
***
**
Neutral loci
ΔaroA
ΔaroE
ΔpurM
ΔYPK_3179
ΔYPK_3184
ΔYPK_3185
ΔrfaH
ΔwecC
ΔarnDT
ΔdusB-fis
ΔYPK_1920
ΔoppD
ΔflgD
ΔYPK_2594
ΔpsaABCEF
ΔYPK_3600
ΔYPK_3656
ΔYPK_3765
B
Spleen
C57Bl/6
C57Bl/6 +1A8
metabolism
LPS
other
Fitness
***
**
Neutral loci
ΔaroA
ΔaroE
ΔpurM
ΔYPK_3179
ΔYPK_3184
ΔYPK_3185
ΔrfaH
ΔwecC
ΔarnDT
ΔdusB-fis
ΔYPK_1920
ΔoppD
ΔflgD
ΔYPK_2594
ΔpsaABCEF
ΔYPK_3600
ΔYPK_3656
ΔYPK_3765

Figure 3-9. Confirmation of neutrophil and inflammatory monocyte depletions

(A-B) Shown are representative FACs plots from spleens isolated from infections of non-depleted mice, or mice treated with either 1A8 or MC21 antibodies. Tissues were extracted 3 days post-infection and single cell suspensions were prepared for FACs analysis by staining with Gr1 and Cd11b antibodies. Gating was performed **(A)** selecting the live cell population and **(B)** within that population, designating neutrophils as those cells expressing high levels of both Gr1 and Cd11b, and inflammatory monocytes as those cells expressing intermediate levels of Gr1 and high levels of Cd11b. (**C)** Quantitation of FACs analysis described above. Statistical significance was determined using One Way ANOVA analysis with Dunnett's multiple comparison post-test by comparing the % total cell values of each depletion condition with that of non-depleted mice. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$.

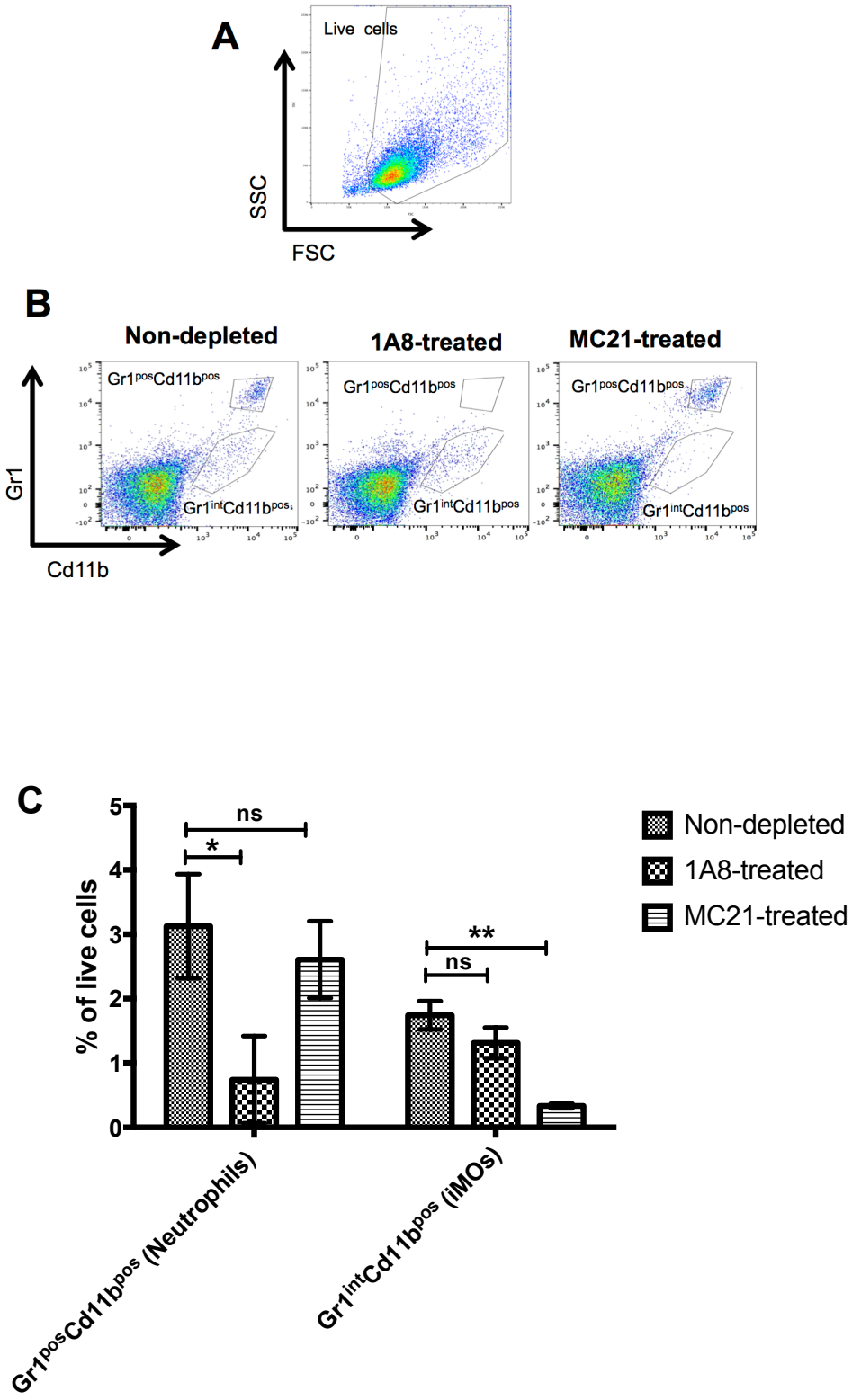
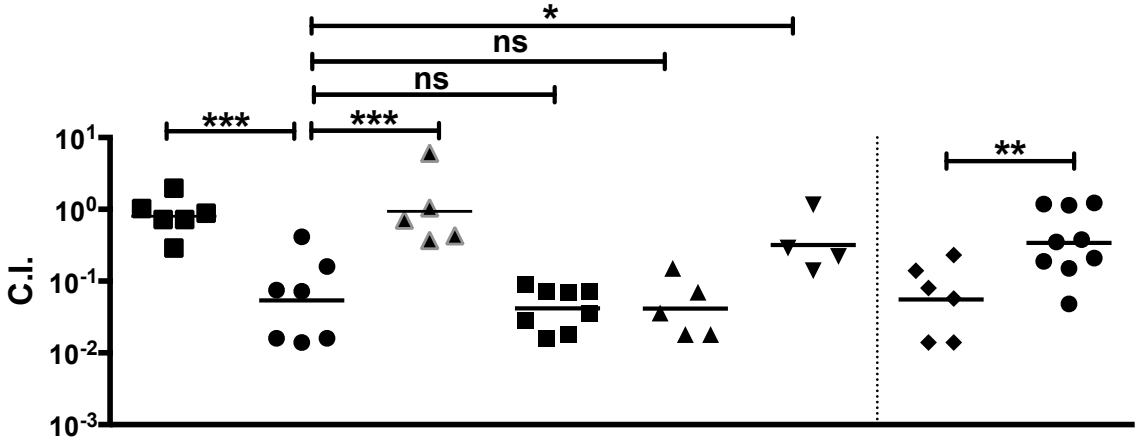

A
Live cells
SSC
FSC
B
Non-depleted
1A8-treated
MC21-treated
Gr1posCd11bpos
Gr1intCd11bpos
Gr1
Cd11b
C
% of live cells
ns
*
**
ns
Non-depleted
1A8-treated
MC21-treated
Gr1posCd11bpos (Neutrophils)
Gr1intCd11bpos (iMOs)

Figure 3-10. *ΔdusB-fis* is sensitive to neutrophils and inflammatory monocytes *in vivo*

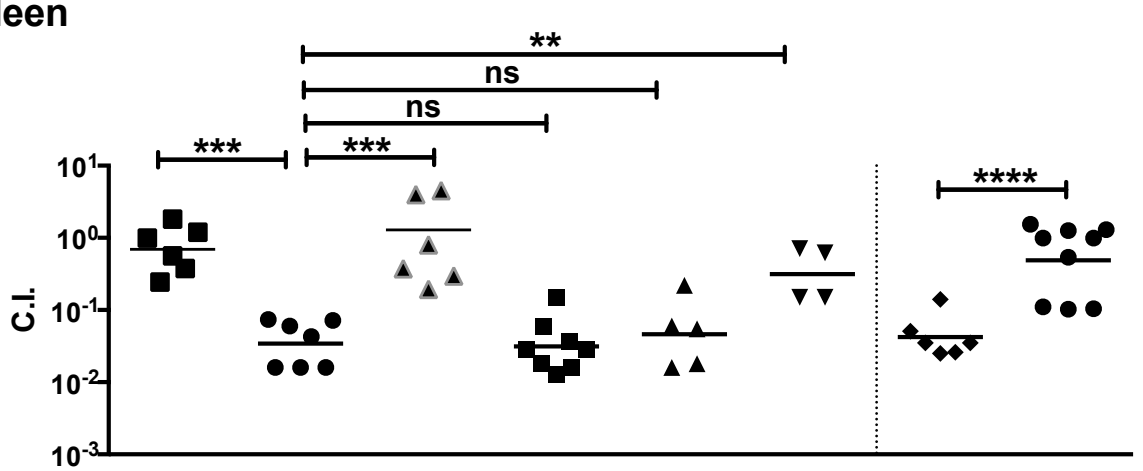
C57BL6/J mice were inoculated intravenously with a pool of 10^3 bacteria, containing an equal mixture of *ΔdusB-fis*-Kan^R and WT. Twenty-four hours prior to and post-infection, some mice were depleted with either the 1A8 or RB6-8C5 antibody. Other mice were intraperitoneally depleted with the MC21 antibody 1 day prior to infection and each day after until completion of experiment. At 2 days (RB6-8C5 treated mice) or 3 days post-infection, mice were euthanized and livers **(A)** and spleens **(B)** were dissected. Dilutions of bacteria recovered from tissues were plated onto selective and non-selective media to determine the competitive index (C.I.). Each C.I. value was $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis the Dunnett's multiple comparison post-test by comparing the C.I. values of mutant strains in non-depleted and depleted mice. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$.

A **Liver**



Strain competed against WT-Kan^{R}	WT	*ΔdusB-fis*	*ΔdusB-fis::dusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*
Mouse condition	non-depleted	non-depleted	non-depleted	1A8 Ab	MC21 Ab	1A8 +MC21 Ab	non-depleted	RB68C5 Ab
Days post-infection	3	3	3	3	3	3	2	2

B **Spleen**



Strain competed against WT-Kan^{R}	WT	*ΔdusB-fis*	*ΔdusB-fis::dusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*	*ΔdusB-fis*
Mouse condition	non-depleted	non-depleted	non-depleted	1A8 Ab	MC21 Ab	1A8 +MC21 Ab	non-depleted	RB68C5 Ab
Days post-infection	3	3	3	3	3	3	2	2

3.6 PERSPECTIVES ON IDENTIFICATION OF CHROMOSOME-ENCODED *YERSINIA* VIRULENCE FACTORS

In these studies, we used TnSeq to identify at least 33 factors that contribute to liver colonization and growth by a plasmid-deficient *Yptb* strain. By utilizing two highly complex libraries with over 10,000 unique insertion mutants each, this study represents the most expansive screen of *Yptb* transposon mutants in an infection model performed to date. Notably, several of the genes identified in our screen have been previously characterized as virulence factors in *Yersinia*; this result validated our experimental design and also indicated that using the plasmid-deficient strain did not prevent identification of factors required for WT infection. Importantly, in addition to the known virulence factors, our screen also identified many factors that had been previously uncharacterized in *Yersinia* infection models, including genes contributing to metabolism, LPS synthesis, stress response, as well as several genes encoding hypothetical proteins.

After designing and implementing a mini-TnSeq assay, we further evaluated the role of 18 of these genes in a systemic infection model, by constructing in-frame gene or total operon deletions containing scar sequences, in both plasmid-deficient and WT *Yptb*. Interestingly, we found that the vast majority of mutants with defects in the absence of the virulence plasmid were also attenuated in pIB1^{+} *Yptb*, indicating that the majority of genes identified in the screen are essential for virulence, regardless of the presence of the T3SS. In fact, only one mutant analyzed, Δ*oppD*, was only attenuated for growth in livers in the absence of the virulence plasmid, but not in its presence. Additionally, two

mutants, Δ*YPK_3184* and Δ*psaABCEF*, were attenuated in the spleen only in the pIB1^{-} background, but not in the WT background.

Interestingly, six mutants, Δ*aroA,* Δ*YPK_3184,* Δ*arnDT,* Δ*YPK_1920,* Δ*YPK_2594,* and Δ*psaABCEF,* were defective for growth of the WT strain in the liver, but not the spleen. This result both reflects the fact that our original screen was performed in the liver and indicates that different tissue sites can influence the repertoire of bacterial virulence factors required during infection. Indeed, it has previously been established that mammalian organs differ in their mechanisms of sensing and responding to microbial infections (Raz 2007, Hu and Pasare 2013) and that, consequently, bacteria may utilize genes to survive in some tissues that are dispensable in others. Therefore, *Yptb* may require certain factors, such as *aroA*, to survive in the liver because their products are limiting in this organ. It is interesting to speculate that certain mutants, such as Δ*YPK_3184* and Δ*arnDT,* may be more readily detected by pattern recognition receptors in the liver and would therefore fail to colonize or sustain growth in this organ. Alternatively, these mutants may be more susceptible to bactericidal factors that are produced in higher levels in the livers than in the spleen.

By infecting the mini-TnSeq libraries into mice depleted of Ly6G^{pos} and Gr1^{pos} cells, we identified two mutants, Δ*dusB-fis* and Δ*YPK_3765,* which were sensitive to these cells during tissue infection. Interestingly, while *YPK_3765* was important for specifically preventing neutrophil-mediated clearance, growth of the Δ*dusB-fis* mutant was only restored when mice were depleted of both neutrophils and inflammatory monocytes, indicating that both of these cell types are capable of restricting this mutant *in vivo*. Another surprise from this assay was the finding that the virulence defects of the

ΔpsaABCEF and *ΔrfaH* mutants were exacerbated following neutrophil depletion during *Yptb* infections of the spleen, and liver, respectively, indicating that these loci may promote survival in a non-inflammatory niche or in the presence of a host cell subset "unmasked" by neutrophil depletion.

Altogether, our findings reinforce the argument that *Yptb* relies on a number of chromosome-encoded factors to grow within tissue sites and withstand restriction by immune cells. In particular, 4 loci, *dusB-fis*, *YPK_3765*, *psaABCEF*, and *rfaH*, encode factors that play critical roles in interfacing with phagocytic cells during tissue infection.

CHAPTER 4: CHARACTERIZING THE ROLE OF THE *DUSB-FIS* OPERON IN PROMOTING *YERSINIA PSEUDOTUBERCULOSIS* PATHOGENESIS

4.1 BACKGROUND

Our previous study identified at least 33 genes or operons required for growth of *Yptb* following systemic infection. One of these loci, *dusB-fis,* was specifically required for resistance to clearance by neutrophils and inflammatory monocytes, as depletion of both cell types prior to infection restored growth of the Δ*dusB-fis* mutant strain. The *dusB-fis* operon is conserved in *Enterobacteriaceae* family members of the *Gammaproteobacteria* (Nafissi, Chau et al. 2012) and encodes the NAP Fis. In *E. coli*, the two genes are co-regulated and transcribed from a single promoter upstream of *dusB* (Ball, Osuna et al. 1992, Beach and Osuna 1998), where the *dusB* mRNA transcript is believed to play a regulatory role in promoting translation of Fis (Nafissi, Chau et al. 2012).

While no published work has characterized a function for Fis in *Yersinia*, Fis and other NAPs have been well studied in *E. coli* and other organisms, where these small, histone-like proteins play important roles in modulating DNA architecture, as well as in directly and indirectly regulating transcription at a global level (Duprey, Reverchon et al. 2014). Interestingly, Fis serves as a transcriptional regulator of virulence factors in several mammalian pathogens, including *Vibrio cholerae, Shigella flexneri, Pasteurella multocida, Salmonella typhimurium*, and pathogenic *Escherichia coli* (Falconi, Prosseda et al. 2001, Wilson, Libby et al. 2001, Schechter, Jain et al. 2003, Kelly, Goldberg et al. 2004, Lenz and Bassler 2007, Saldana, Xicohtencatl-Cortes et al. 2009, Steen, Steen et al. 2010, Rossiter, Browning et al. 2011) (Duprey, Reverchon et al. 2014). In these organisms, it activates a diverse range of virulence functions, from quorum sensing, to

capsule production, to adhesion, to Type 3 Secretion (Kelly, Goldberg et al. 2004, Lenz and Bassler 2007, Saldana, Xicohtencatl-Cortes et al. 2009, Steen, Steen et al. 2010).

Therefore, in this chapter, we investigate how, in *Yptb,* Fis promotes resistance to clearance by both neutrophils and inflammatory macrophages.

4.2 Δ*dusB-fis* CAN COLONIZE, BUT IS UNABLE TO SUSTAIN GROWTH IN SYSTEMIC TISSUE SITES

Because the virulence of the Δ*dusB-fis* mutant was restored in the absence of neutrophils and inflammatory monocytes, it is possible that these immune cells restrict survival of this mutant in the bloodstream after intravenous infection, thereby preventing high levels of tissue colonization. Alternatively (or in addition), neutrophils and inflammatory monocytes may restrict the growth of Δ*dusB-fis* in tissue sites once these cells surrounded the bacteria. To distinguish between these two possibilities, the growth kinetics of the Δ*dusB-fis* mutant were determined during systemic mouse infection at 4, 24, 48, and 72-hour time-points, using both co-infection and single-strain infection models. In both co-infections with a WT strain (Fig 4-1) and in single-strain infections (Fig 4-2), the Δ*dusB-fis* mutant colonized tissue sites and grew for the first 24 hours post-infection with similar kinetics to WT *Yptb*. However, by 48 hours post-infection, the level of Δ*dusB-fis* failed to increase as rapidly as WT, indicating that the growth of this strain was not restricted until after initial seeding and expansion with tissue sites. Combined with our findings from the depletion experiments, these results suggest that Δ*dusB-fis* cannot adapt to a change in the tissue environment, likely due to the influx and/or activities of neutrophils and inflammatory monocytes.

Figure 4-1. *ΔdusB-fis* can colonize, but is unable to sustain growth in systemic tissue sites following co-infection with WT *Yptb*

C57BL6/J mice were inoculated intravenously with a pool of 10^3 bacteria, containing an equal mixture of WT *yopE::mcherry* and *ΔdusB-fis*-Kan^R. Cohorts of mice were euthanized at 4-hour, 24-hour, 48-hour, and 72-hour time-points post-infection, and their spleens and livers were dissected. Dilutions of bacteria recovered from livers **(A)** and spleens **(B)** were plated onto non-selective media and onto kanamycin selective plates. The number of bacteria recovered from selective and non-selective plates was used to determine quantity of WT *yopE::mcherry* and *ΔdusB-fis*-Kan^R bacteria in each organ sample. CFU/g values were $\log_{10}$ transformed and statistical significance was calculated using an unpaired t-test with the Holm-Sidak post-test to correct for multiple comparisons, for comparing the CFU/g values of *ΔdusB-fis*-Kan^R to WT *yopE::mcherry* bacteria in each tissue at each time point. ns = not significant, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, and **** indicates $p \leq 0.0001$.

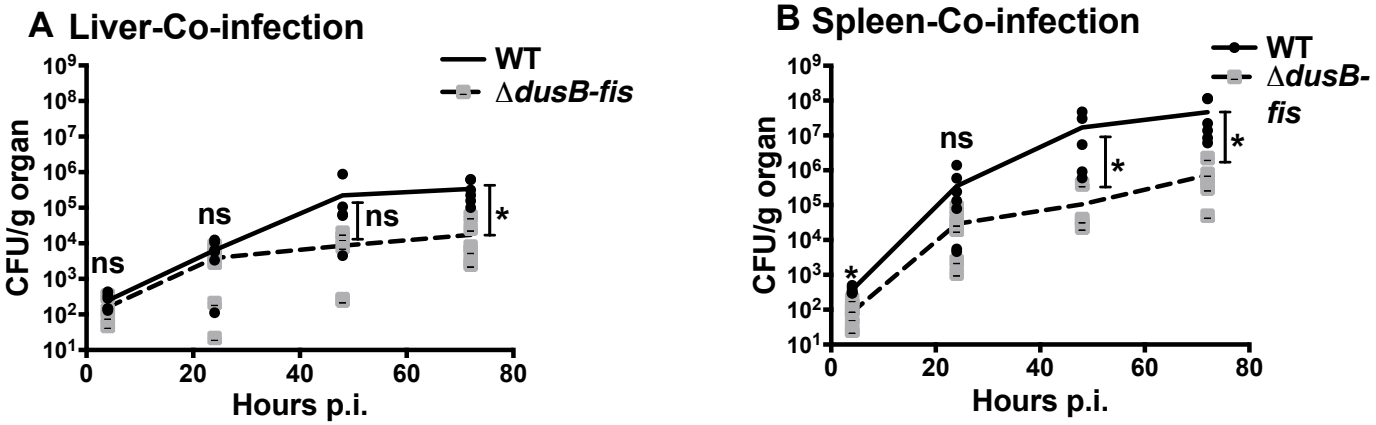
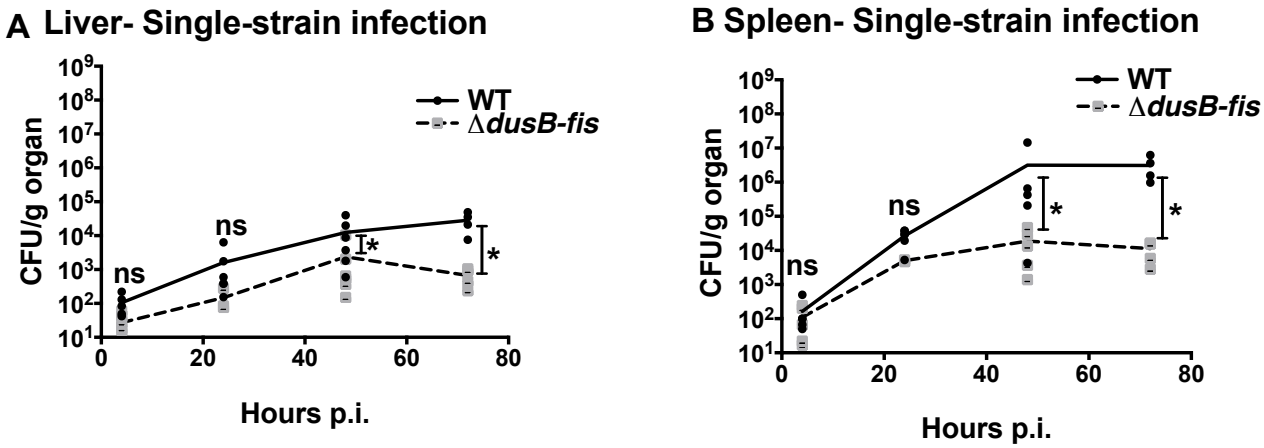


Figure 4-2. *ΔdusB-fis* can colonize, but is unable to sustain growth in systemic tissue sites following single-strain infection

C57BL6/J mice were inoculated intravenously with either WT *yopE::mcherry* or *ΔdusB-fis*-Kan^R at a dose of 10^3 CFU. At 4-hour, 24-hour, 48-hour, and 72-hour time-points post-infection, mice were euthanized and spleens and livers were dissected. Dilutions of bacteria recovered from livers **(A)** and spleens **(B)** were plated onto non-selective media and the quantity of CFU/gram was determined by dividing recovered CFU by the weight of the tissue sample extracted. CFU/g values were $\log_{10}$ transformed and statistical significance was calculated using an unpaired t-test with the Holm-Sidak post-test to correct for multiple comparisons, for comparing the CFU/g values of *ΔdusB-fis*-Kan^R to WT *yopE::mcherry* bacteria in each tissue at each time point. ns= not significant, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, and **** indicates $p \leq 0.0001$.



4.3 *ΔdusB-fis* IS NOT DEFECTIVE FOR TYPE III SECRETION OR EFFECTOR TRANSLOCATION

Because Fis serves as a transcriptional regulator of virulence factors in several pathogens (Duprey, Reverchon et al. 2014), we speculated that the virulence defect of Δ*dusB-fis* observed in our studies was due to an inability to mount a transcriptional response to protect against the bactericidal actions of neutrophils and inflammatory monocytes in systemic tissue sites. Neutrophils and inflammatory monocytes use a variety of mechanisms to restrict bacterial growth upon recruitment to tissue sites, including the phagocytosis of bacteria, release of toxic granules, release of diffusible reactive gases (ROS and reactive nitrogen species), and chelation of metals (Serbina, Jia et al. 2008, Kruger, Saffarzadeh et al. 2015). Because T3SS effectors interfere with many of these processes (Matsumoto and Young 2009), and because Fis regulates expression of the SPI-1 and SPI-2 pathogenicity islands in *Salmonella* (Wang, Liu et al. 2013), we tested whether Fis positively regulated expression of the T3SS or the translocation of T3SS effectors. Engineered strains of WT and Δ*dusB-fis Yptb* containing the beta-lactamase, TEM, fused to the first 100 amino acids of the T3SS effector YopE, were used to measure effector secretion and translocation (Durand, Maldonado-Arocho et al. 2010). Under conditions that induce expression and secretion of T3SS effectors, Δ*dusB-fis* secreted effectors into culture supernatants at equivalent levels to WT *Yptb* (Fig 4-3). Because Fis could regulate other factors, such as adhesins, which also contribute to efficient effector translocation into host cells (Maldonado-Arocho, Green et al. 2013), the ability of Δ*dusB-fis* to translocate T3SS effectors into cultured epithelial cells was measured using the CCF4-FRET based translocation assay. The Δ*dusB-fis* mutant had no

defect in translocating T3SS effectors into cultured cells (Fig 4-4), suggesting that Fis plays no role in regulating the expression of the *Yptb* T3SS machinery or in regulating the expression of other factors that promote efficient effector translocation through this system.

Figure 4-3. *ΔdusB-fis* is not defective for Type 3 Secretion

Stationary phase cultures of WT-YopE-TEM, *ΔyscF*-YopE-TEM, and Δ*dusB-fis*-YopE-TEM strains were diluted 1:40 into L broth depleted of calcium. Cultures were grown for 2 hours at 26°C and then shifted to 37°C for 2 hours. After growth, cultures were centrifuged and a fraction of the culture supernatant was applied to an equal volume of nitrocefin (at 500 μg/mL). After 5 minutes of incubation, the A_{490} of samples was measured using a BioTek Synergy HT plate reader. Statistical significance was calculated using One Way ANOVA analysis with Dunnett's multiple comparison test post-test to compare each strain to WT. ns= not significant, and **** indicates $p \leq 0.0001$. Each bar represents the mean and SEM of 3 biological replicates.

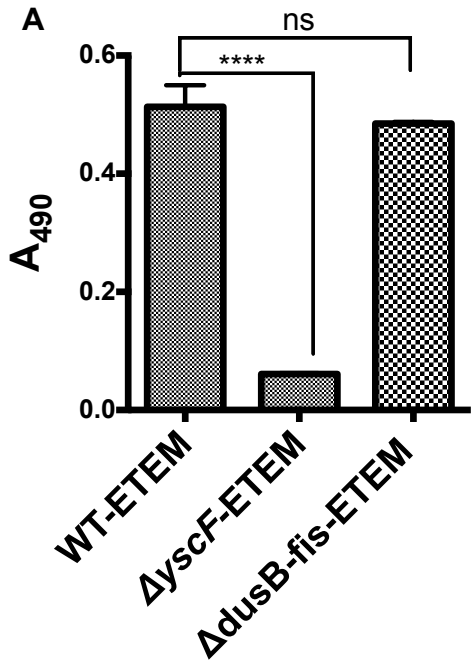
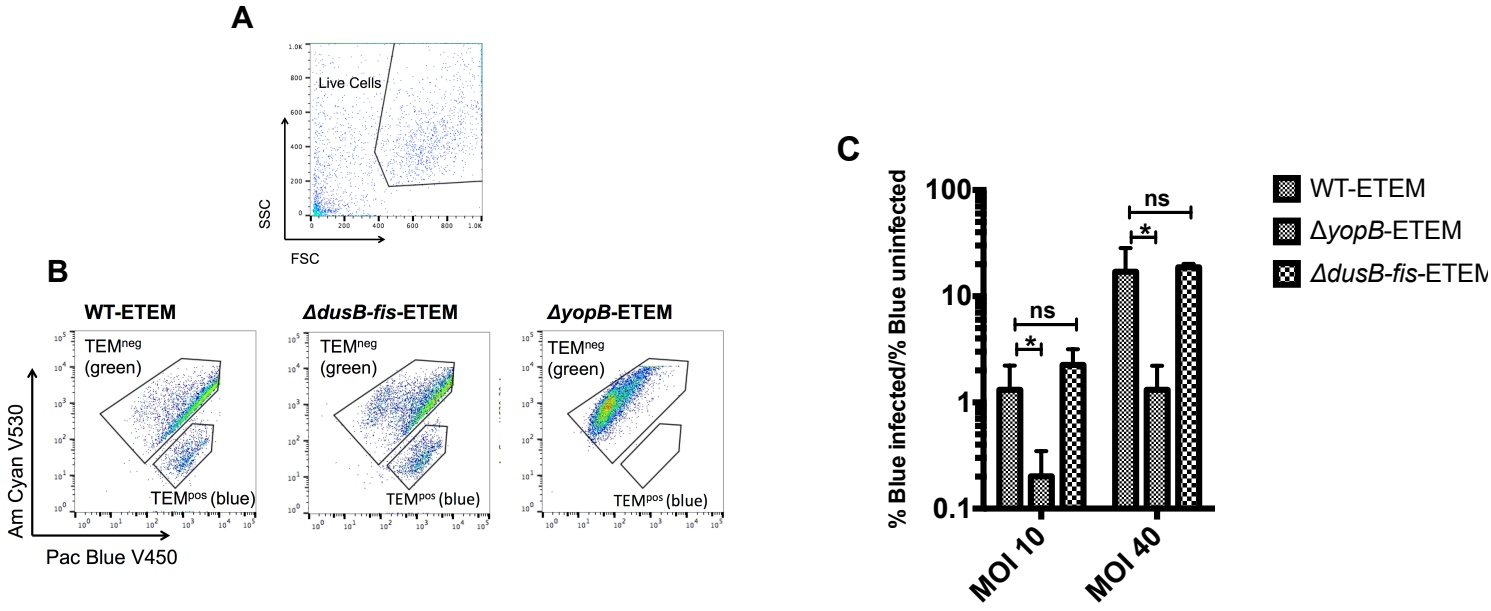


Figure 4-4. *ΔdusB-fis* is not defective for T3SS effector translocation

WT-ETEM *ΔyopB*-ETEM, and Δ*dusB-fis*-ETEM strains were grown overnight and diluted into low-calcium broth as in Fig 4-3. After growth, bacteria were used to infect HEp-2 cells at the indicated multiplicities of infection for 1 hour and then cells were exposed to CCF4 and the percentage of cells containing translocated effectors (% blue) was determined by FACs. **(A-B)** Shown are representative FACs plots, where live cells were selected as indicated in **(A)** and that population was separated into non-translocated ETEM^{-} "green" and translocated ETEM^{+} "blue" gates **(B). (C)** The %blue value for each sample was determined by dividing the percentage of blue cells by the total number of cells counted. This value was then normalized to the mean %blue value measured in uninfected samples in order to control for background fluorescence. Data was $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis with Dunnett's multiple comparison test to compare each strain to WT. * indicates $p \leq 0.05$. Each bar represents the mean and SEM of 3-6 biological replicates.



4.4 *DUSB-FIS* PROMOTES RESISTANCE TO ROS *IN VITRO*

To evaluate whether Fis coordinates a transcriptional response to one or more of the bactericidal stresses imposed by neutrophils and inflammatory monocytes, the Δ*dusB-fis* mutant was exposed to several conditions that simulate the actions of these cells. These conditions included exposure to low pH (Fig 4-5), low iron concentrations (Fig 4-5), nitric oxide (Fig 4-6), and ROS (Fig 4-6). While Δ*dusB-fis* was often delayed in emerging into exponential growth compared to WT, its growth rate in broth with a low pH or low iron was not more impaired relative to its growth rate under non-stress conditions (Fig 4-5). Additionally, exposure to the nitric oxide donor DETA NONOate did not affect survival of Δ*dusB-fis*, but did result in killing of a mutant lacking *hmp*, which is known to play a role in nitric oxide detoxification in *Yptb* (Davis, Mohammadi et al. 2015) (Fig 4-6). By contrast, the survival of Δ*dusB-fis* was significantly impaired after exposure to H_2O_2 (Fig 4-6), suggesting that Fis promotes resistance to ROS.

To determine whether Fis protects against ROS by regulating the expression of one or more ROS-detoxifying genes, we performed qRT-PCR on transcripts isolated from WT and Δ*dusB-fis* following exposure to H_2O_2. Four genes predicted to contribute to resistance to and/or detoxification of ROS, *sodA, katG, ahpC,* and *grxA,* were expressed at higher levels in WT than in the Δ*dusB-fis* mutant following exposure to H_2O_2 (Fig 4-7). By contrast, there was no difference between WT and Δ*dusB-fis* in the expression of a non-ROS inducible gene, *rpoC.* Notably, while the transcription of *katG, ahpC,* and *grxA* was induced in a WT strain following exposure to H_2O_2, in this strain, levels of *sodA* transcript were only marginally higher than in an untreated sample. However, this expression was still markedly higher than in a Δ*dusB-fis* mutant, where

sodA transcription appeared to be down-regulated following exposure to H_2O_2. This result suggests that *dusB-fis* may provide some intrinsic protection against ROS by preventing repression of *sodA* during conditions of oxidative stress. Combined, these results indicate that Fis provides intrinsic protection against killing by ROS by either directly or indirectly regulating the transcription of genes required for resistance to oxidative stress.

Figure 4-5. *ΔdusB-fis* is not defective for growth at a low pH or in iron limiting conditions

(A) Stationary phase cultures of WT and *ΔdusB-fis* were diluted 1:100 into L broth and L broth adjusted to pH 5.5 and the OD_{600} of cultures was measured at 1-hour-intervals for 12 hours. Each symbol represents the mean of 2-4 biological replicates. (**B)** Stationary phase cultures were diluted 1:100 into a well of a 96-well plate containing L broth or L broth + 31 μM 2,2'- Bipyridyl and OD_{600} measurements were recorded at 15-minute intervals. Lines represent the mean of 3 biological replicates.

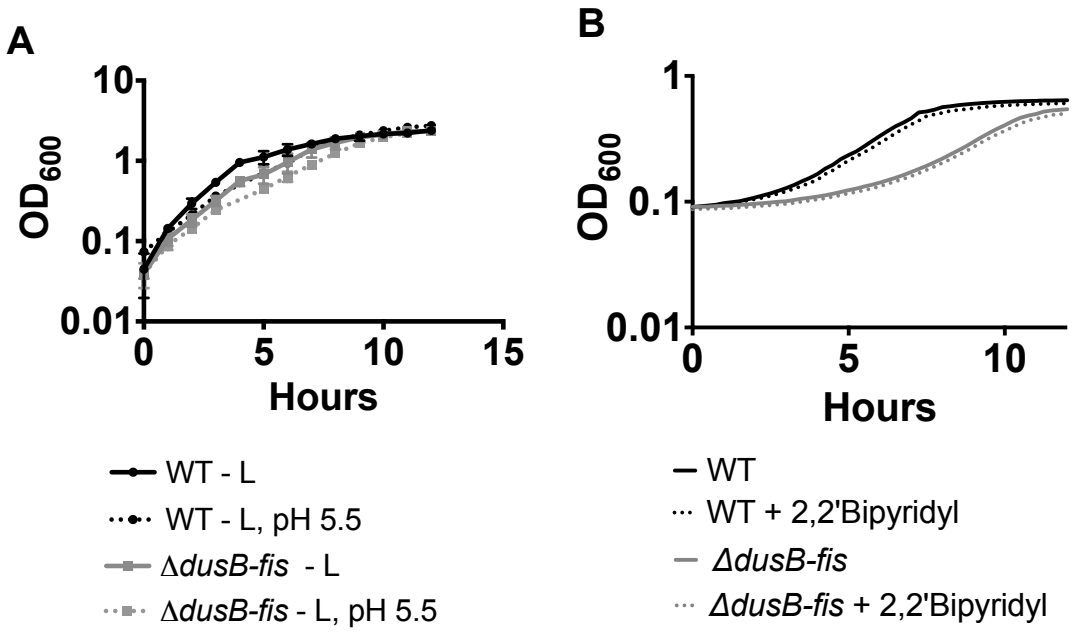


Figure 4-6. *dusB-fis* is required for resistance to H_2O_2, but not nitric oxide

(**A)** Exponential phase cultures were washed and back diluted into M9 glucose medium or M9 glucose containing 2.5mM DETA NONOate for 1 hour. Survival indicates the number of CFUs recovered following NO treatment divided by the number of CFUs recovered from untreated cultures. Bars represent the mean and SEM of 3 biological replicates. Survival values were $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis with Dunnett's multiple comparison post-test to compare each strain to WT. (**B)** Exponential phase cultures were washed and back diluted into M9 glucose medium or M9 glucose containing 1.5mM H_2O_2. Survival indicates the number of CFUs recovered following H_2O_2 treatment divided by the number of CFUs recovered from untreated cultures. Bars represent the mean and SEM of 11 biological replicates. Survival values were $\log_{10}$ transformed and statistical significance was calculated using a Mann-Whitney t-test. ns=not significant, ** indicates $p \leq 0.001$, and *** indicates $p \leq 0.001$.

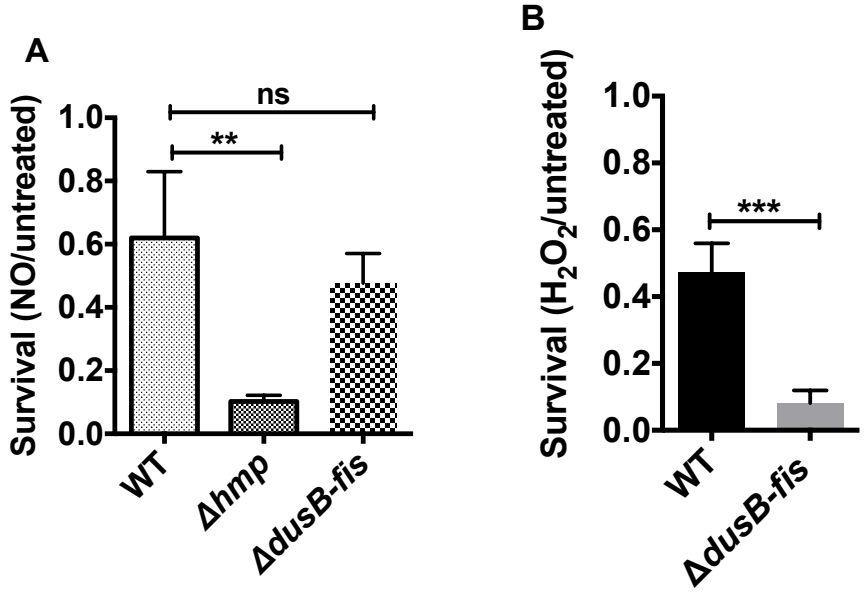
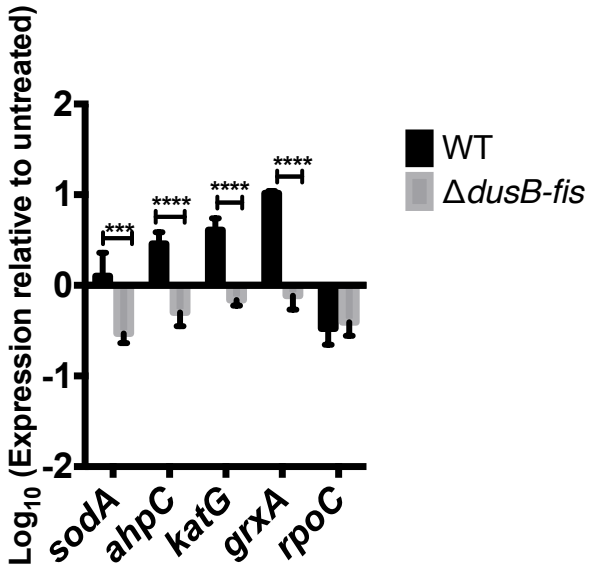


Figure 4-7. *ΔdusB-fis* fails to up-regulate ROS-responsive genes after exposure to H_2O_2.

Cultures were treated with H_2O_2 as in (Fig 4-6B). RNA isolated from treated and untreated samples was used to generate cDNA, and qPCR reactions were performed using the BioRad CFX Real-Time PCR detection system. Relative expression was determined by normalizing to 16S RNA as well as to expression in untreated samples using the ΔΔCT method (Applied Biosystems). Bars represent the mean and SEM off 4 biological replicates. Relative expression values were $\log_{10}$ transformed and an unpaired t-test with the Holm-Sidak correction for multiple comparisons was performed to calculate statistical differences between expression in WT and *ΔdusB-fis*. ** indicates p≤0.001*** indicates p≤0.001, and **** indicates p≤0.0001.



4.5 *DUSB-FIS* PROMOTES RESISTANCE TO ROS PRODUCED BY THE NADPH OXIDASE COMPLEX DURING TISSUE INFECTION

To test the possibility that Fis protects *Yptb* from ROS in tissue sites, gp91$^{phox-/-}$ mice, which cannot assemble a productive NADPH oxidase complex (Pollock, Williams et al. 1995), were infected with a mixture of WT and Δ*dusB-fis*. Strikingly, Δ*dusB-fis* had no growth defect in these mice (Fig 4-8), indicating that this mutant is restricted by ROS during tissue infection. Interestingly, gp91$^{phox-/-}$ mice were only slightly more susceptible to *Yptb* infection, as total bacterial loads in spleens and livers were only ~3.5x higher than those in tissues recovered from C57Bl/6 mice (Fig 4-9). Furthermore, most of this increase was attributed to the relief in Δ*dusB-fis* growth restriction in the gp91$^{phox-/-}$ mice, as analysis of the CFU burden of each individual bacterial strain recovered from co-infected mice showed little difference in WT CFU levels between gp91$^{phox-/-}$ and C57Bl/6 mice (Fig 3-10). This result, coupled with our earlier observations, indicates that the primary role of *dusB-fis* during *Yptb* infection within deep tissue sites is to protect against ROS produced by neutrophils and inflammatory monocytes, by regulating a transcriptional response that enables *Yptb* to detoxify ROS that have entered the bacterial cell.

Figure 4-8. The competitive index of *ΔdusB-fis* is restored in gp91$^{phox-/-}$ mice

C57Bl/6 or C57Bl/6 gp91$^{phox-/-}$ mice were inoculated intravenously with a 1:1 mixture of WT and *ΔdusB-fis*-Kan^{R} at a dose of 1x10^{3} CFU. Livers **(A)** and spleens **(B)** were dissected, weighed and plated for CFUs on selective and non-selective agar at 3 days post-infection. The number of bacteria recovered from selective and non-selective plates was used to determine the C.I. of *ΔdusB-fis*-Kan^{R}. Each symbol represents an individual mouse, and open circles indicate organs that contained no *ΔdusB-fis*-Kan^{R} at our limit of detection. C.I. values were $\log_{10}$ transformed and statistical significance was calculated using the Mann-Whitney t-test. ** indicates $p \leq 0.001$, and *** indicates $p \leq 0.001$

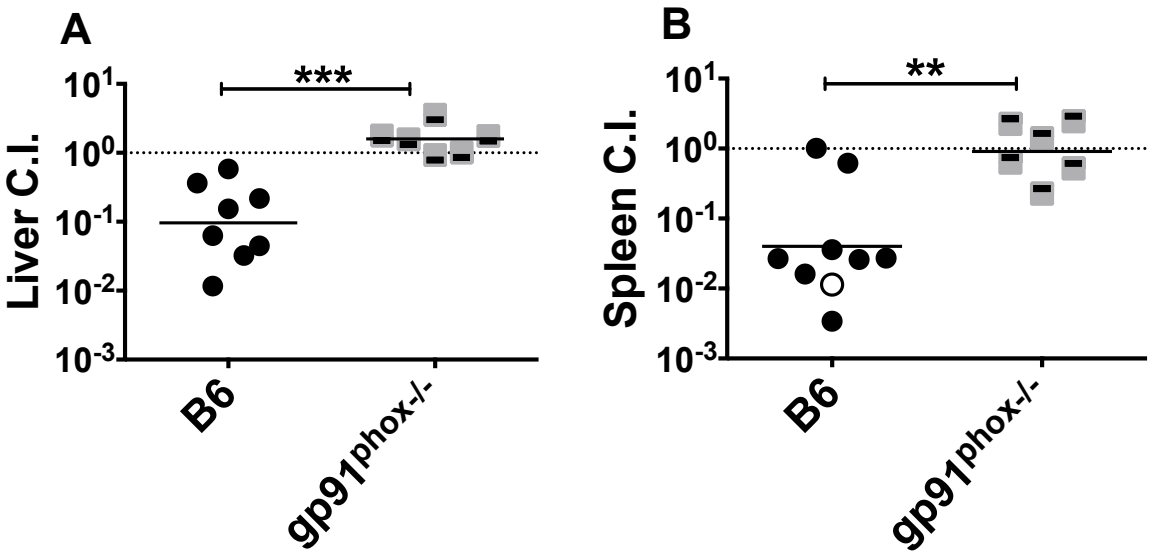


Figure 4-9. Mice unable to produce ROS are only slightly more susceptible to colonization by *Yptb*

C57Bl/6 or C57Bl/6 gp91$^{phox-/-}$ mice were inoculated intravenously with a 1:1 mixture of WT and *ΔdusB-fis*-Kan^{R} at a dose of 1x10^{3} CFU. Livers **(A)** and spleens **(B)** were dissected, weighed and plated for CFUs on selective and non-selective agar at 3 days post-infection. The quantity of bacteria/gram was determined by dividing the total number of recovered CFU (on non-selective plates) by the weight of the tissue sample extracted. CFU/g values were $\log_{10}$ transformed and statistical significance was calculated using the Mann-Whitney t-test. ns= not significant, and * indicates $p \leq 0.001$.

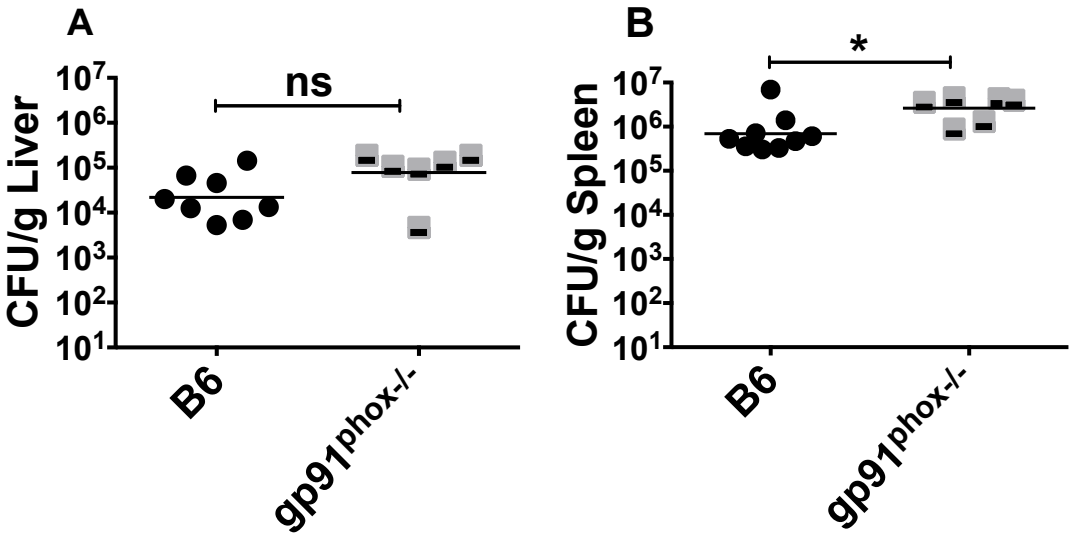
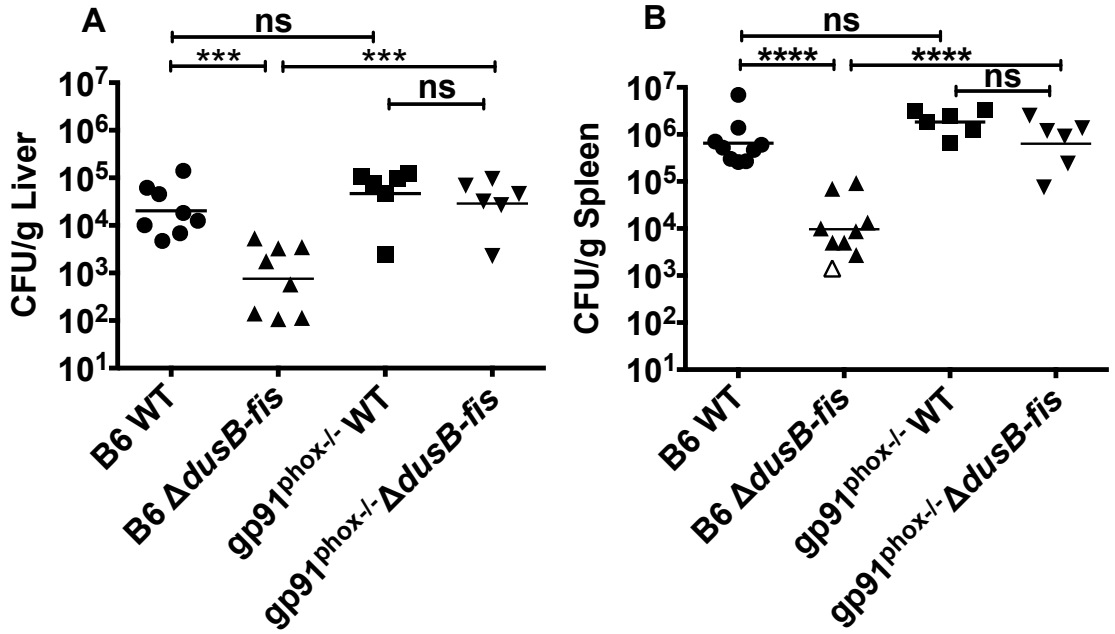


Figure 4-10. Growth of *ΔdusB-fis,* but not WT, *Yptb* is altered in mice unable to produce ROS

C57Bl/6 or C57Bl/6 gp91$^{phox-/-}$ mice were inoculated intravenously with a 1:1 mixture of WT and *ΔdusB-fis*-Kan^R at a dose of 1x10^3 CFU. Livers **(A)** and spleens **(B)** were dissected, weighed and plated for CFUs on selective and non-selective agar at 3 days post-infection. The number of bacteria recovered from selective and non-selective plates was used to determine the quantity of *ΔdusB-fis*-Kan^R and WT bacteria in each organ sample. The quantity of bacteria/gram was determined by dividing the number of recovered CFU for each bacterial strain by the weight of the tissue sample extracted. Each symbol represents an individual mouse, and open triangles indicate organs that contained no *ΔdusB-fis*-Kan^R at our limit of detection. CFU/g data was $\log_{10}$ transformed and statistical significance was calculated using One Way ANOVA analysis with Dunnett's multiple comparison post-test to compare the CFU/g values of each bacterial strain in both WT and knockout mice. ns = not significant, *** indicates $p \leq 0.001$, and **** indicates $p \leq 0.0001$.



4.6 PERSPECTIVES ON THE ROLE OF *DUSB-FIS* IN PROMOTING *YERSINIA* VIRULENCE

Our results, in aggregate, support a model whereby *Yptb* uses the well-conserved *dusB-fis* operon to detoxify ROS that are generated by the NADPH oxidase complex in neutrophils and inflammatory monocytes surrounding bacteria in the spleen and liver. Specifically, (1) *dusB-fis* was important for defense against both neutrophils and inflammatory monocytes, as the growth of this mutant was only restored when both immune cell populations were depleted (Fig 2-10); (2) Δ*dusB-fis* initially colonized spleens and livers, but was unable to sustain growth in these tissue sites by 48 post-infection (Fig 3-1, Fig 3-2); (3) *dusB-fis* was required for protection against oxidative stress (Fig 3-6) and regulated the transcription of at least 4 genes, *sodA, katG, ahpC,* and *grxA*, that are predicted to contribute to response and/or resistance to ROS in *Yersinia* (Fig 3-7); and (4) growth of Δ*dusB-fis* was restored in gp91$^{phox-/-}$ mice, whose immune cells lack a functional NADPH oxidase complex and thus cannot undergo oxidative burst (Fig 3-8). Remarkably, these mice contained equal bacterial loads of WT and Δ*dusB-fis*, which supports the idea that the primary contribution of this operon to *Yptb* infection is to prevent against restriction by ROS.

These findings were unexpected for several reasons. First, previous studies of *Yersinia* gene expression in animal models have observed little induction of ROS-detoxifying genes during infection of lymphoid tissue sites, suggesting that *Yersinia* may not experience oxidative stress during growth in these organs (Sebbane, Lemaitre et al. 2006, Davis, Mohammadi et al. 2015). By contrast, a prior study analyzing the phenotype of a Δ*sodA* mutant determined that this gene was critical for growth of *Y. enterocolitica*

within livers and spleens, suggesting that *Yersinia* do encounter ROS during tissue infection (Roggenkamp, Bittner et al. 1997). Second, it has been well established that two T3SS effectors, YopE and YopH, prevent oxidative burst in Yop-intoxicated immune cells (Bliska and Black 1995, Songsungthong, Higgins et al. 2010). However, YopE and YopH can only function within the cells to which they have been delivered. As only a small fraction of immune cells are intoxicated with Yops during infection of systemic tissue sites (Durand, Maldonado-Arocho et al. 2010), our results indicate that *Yptb* may encounter ROS produced by non-injected cells and requires mechanisms to detoxify these species. Because Fis is dispensable for T3SS effector translocation, but is required for protection against ROS both *in vivo* and *in vitro*, our work suggests that these species must be coming from neighboring immune cells not intoxicated with Yops. Furthermore, the observation that $gp91^{phox-/-}$ mice were no more sensitive to WT *Yptb* than C57Bl/6 mice suggests that WT *Yptb* is completely resistant to ROS produced by the immune response during infection.

In summary, we propose that *Yptb* utilizes both offensive and defensive measures to counteract ROS produced by phagocytic cells during mammalian infection. *Yptb* directly prevents oxidative burst in T3SS-intoxicated host cells, and also detoxifies ROS produced by non-intoxicated phagocytic cells through a Fis-dependent transcriptional response.

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

5.1 Significance of This work

5.1.1 Identification of novel chromosomal factors contributing to the virulence of Yersinia pseudotuberculosis

The initial goal of this work was to identify novel chromosomal factors that contribute to the virulence of *Yptb.* We hypothesized that there was large potential to uncover novel chromosome-encoded virulence factors for two reasons: 1) *Yptb* lacking its ~70kb virulence plasmid pIB1 displays a remarkable ability to colonize and persist within mammalian tissue sites (Simonet, Mazigh et al. 1984, Une and Brubaker 1984, Balada-Llasat and Mecsas 2006), and therefore chromosomal factors must be sufficient for this phenotype; 2) Prior to our study, no genetic screen in *Yersinia* infection models had previously been performed with more than 2,000 transposon insertion mutants; therefore we hypothesized that many chromosomal virulence factors had not yet been uncovered. Additionally, the development of genetic techniques that allow for massively parallel sequencing of transposon libraries (van Opijnen, Bodi et al. 2009) gave us the ability to screen a highly complex library of >20,000 unique insertion mutants representing insertions in 3088 out of 4250 total genes during murine infection.

Using TnSeq, we determined the phenotype of these 20,000 insertion mutants generated in the plasmid-deficient *Yptb* background during infection of the liver. At the time of publication of this work, this was the most complex *Yersinia* transposon library that has been analyzed in a mouse infection model, and is this most expansive screen performed with *Yptb* to-date (Crimmins, Mohammadi et al. 2012). Using this assay, we

determined that 33 genes were critical for growth of plasmid-deficient *Yptb* within the liver, but were otherwise dispensable for *in vitro* growth at both 26°C and 37°C.

These putative virulence factors belonged to several distinct functional classes, including LPS synthesis and modification, purine synthesis, amino acid synthesis, as well as several previously uncharacterized genes and genes encoding hypothetical proteins. Additionally, mutants containing insertions in several genes that have been previously established as *Yersinia* virulence factors, including *psaA, psaC,* and *sufI,* were attenuated in our screen. Because these genes had been identified as virulence factors in previous screens and/or studies of the plasmid-containing *Yersinia,* this result served as a good positive control for our assay and also implied that the genes we identified might be important for growth of both plasmid-deficient and WT strains.

Despite identifying previously recognized virulence factors, using transposon insertion libraries to assess the virulence of bacterial mutants in animal models can often suffer from several drawbacks: one being the extreme bottlenecks present in tissue infection models, where only a fraction of transposon mutants in a library are capable of simultaneously infecting a given organ. Another drawback is that transposon disruptions within genes can often have polar effects on nearby loci. Therefore, we designed and implemented a mini-TnSeq assay that allowed us to assess the virulence of multiple in-frame deletion mutants in one infection pool using Illumina sequencing as a read-out. Using this assay, we further characterized 18 mutants that were attenuated in our TnSeq screen. The 18 loci represented several broad functional classes, including metabolism, LPS synthesis and modification, as well as several previously uncharacterized virulence factors. Notably, 4 of the 18 genes we evaluated in this assay did not pass our statistical

cutoff in the original TnSeq screen; however these genes were chosen because they were either components of operons and pathways that contained multiple attenuated mutants in the original TnSeq screen, or appeared interesting because of their potential to encode functions critical within tissue environments.

Strikingly, we found that 14 of the 18 mutants we evaluated were significantly attenuated for growth within the liver during infection of plasmid-deficient (pIB1$^-$) YPIII. As this was the primary strain background and tissue site examined in our original TnSeq screen, these results further validated the results of that screen. Importantly, of the 4 mutants we evaluated that did not pass our statistical cutoff in the original TnSeq screen, only one, Δ*YPK_3185,* did not exhibit a virulence defect in this assay. This suggests that even more genes characterized in the original screen could be reconsidered as virulence factors. Furthermore, *YPK_3185* resides in a multi-cistronic operon that contained several attenuated insertion mutants in the original screen. Therefore, the insertion in *YPK_3185* was likely polar on downstream genes.

Additionally, we utilized the mini-TnSeq assay to evaluate the contribution of each of these 18 loci to infection in the presence of the T3SS, by infecting mice with pools of mutants constructed in the WT background. Surprisingly, we determined that the majority of mutants with defects in the absence of the virulence plasmid were also attenuated in pIB1$^+$ *Yptb*. In fact, only one mutant, Δ*oppD*, was only attenuated for growth in livers in the absence of the virulence plasmid (Table 3.2); additionally, two other mutants, Δ*psaEFABC* and Δ*YPK_3184*, were only attenuated for growth in the spleen in the pIB1$^-$ background. Because plasmid-deficient *Yptb* recruits fewer inflammatory host cells to the site of infection (Balada-Llasat and Mecsas 2006,

Crimmins, Mohammadi et al. 2012), it is possible that these genes may be important for survival when bacteria are growing in a non-inflammatory niche. Alternatively, they may share functional redundancy with the T3SS or another pIB1-encoded factor.

5.1.2 Identification of mutants whose survival is modulated by the presence of phagocytic cells during tissue infection

The second objective of this project was to determine whether any genes identified in the original TnSeq screen interacted with phagocytic cells or prevented phagocytic cell-mediated clearance, using our mini-TnSeq assay. We hypothesized that we would identify genes that interacted with phagocytic cells for two reasons: 1) Although plasmid-deficient *Yptb* induces a weaker immune response than WT, phagocytic cells infiltrate tissues and can surround pIB1$^-$ *Yptb* microcolonies during lymphoid tissue infection (Balada-Llasat and Mecsas 2006). Because this strain is able to persist under these conditions for several days, it is likely that the chromosome encodes factors that suppress killing by these cells, and that these factors may have been identified in our original TnSeq screen. 2) Many of the mutants characterized in our mini-TnSeq assay were highly attenuated in the pIB1$^+$ background, where phagocytic cells are almost always found surrounding the bacteria (Balada-Llasat and Mecsas 2006, Crimmins, Mohammadi et al. 2012, Davis, Mohammadi et al. 2015). Therefore it is possible that some of the genes evaluated may play a role in preventing phagocytic cell clearance under those conditions.

Using our mini-TnSeq assay (with libraries generated in pIB1$^+$ *Yptb*), we identified 4 mutants that displayed altered virulence following infection of mice depleted

of neutrophils or of all Gr1^{pos} cells. Intriguingly, 2 of these mutants, Δ*psaABCEF* and Δ*rfaH,* were even less virulent in neutropenic mice, suggesting that these genes may be important for growth of *Yptb* within a non-inflammatory niche or in the presence of a immune cell type that is "unmasked" following neutrophil depletion because this cell type now comes into direct contact with bacteria, for example. These phenotypes may be consistent with our findings that these mutants were highly attenuated in a pIB1^{-} background, where a weaker immune response is recruited and neutrophils are not detected at bacterial infection sites (Balada-Llasat and Mecsas 2006, Crimmins, Mohammadi et al. 2012).

Additionally, we identified two mutants, Δ*YPK_3765* and Δ*dusB-fis*, which exhibited restoration in virulence following immune cell depletion. Because neutrophil depletion was sufficient to rescue the virulence of Δ*YPK_3765,* we hypothesize that this gene, encoding a putative murein peptide ligase, plays a role in preventing neutrophil-mediated killing. Murein peptide ligases are important for peptidoglycan synthesis and recycling, therefore, aberrant peptidoglycan production or release by this mutant may result in enhanced neutrophil recruitment or killing. In contrast to Δ*YPK_3765,* the virulence of Δ*dusB-fis* was restored after Gr1^{pos} cell depletion, but not after neutrophil depletion, suggesting that this mutant was either susceptible to killing by a Ly6C^{pos} subset, or that it is sensitive to all Gr1^{pos} cells. We distinguished among these possibilities by performing infections of this mutant in CCR2-depleted mice and determined that Δ*dusB-fis* is specifically sensitive to neutrophils and inflammatory monocytes.

Altogether, this screen resulted in several novel findings about the roles of chromosomal factors in preventing phagocytic cell restriction of *Yptb* during tissue

infection. In particular, this is the first report of the requirement for the conserved *dusB-fis* operon, which is known to contribute to virulence in other pathogens, during *Yersinia* infection.

5.1.3 Characterization of the role of the dusB-fis operon in protecting Yptb from phagocytic restriction

Using a variety of *in vivo* and *in vitro* analyses, we investigated the mechanism by which *dusB-fis* prevents against neutrophil- and inflammatory monocyte- mediated clearance. First, we determined that the Δ*dusB-fis* mutant is dispensable for spleen and liver colonization, but is required for sustaining growth in these tissue sites at later time-points. This result suggested that this mutant was not being cleared in the blood and that it may be sensitive to phagocytic cell subsets that are recruited to sites of bacterial infection in the spleen and liver. Additionally, we verified that *dusB-fis* is dispensable for Type 3 Secretion and effector translocation. While it was unlikely that Fis would regulate the T3SS, given the importance of *dusB-fis* in infection of plasmid-deficient *Yptb*, this finding confirms that, during tissue infection, *dusB-fis* likely protects bacteria from an antibacterial response that is either not inhibited or not fully inhibited by T3SS effectors.

Because Fis serves as an environmental sensor in other pathogens (Duprey, Reverchon et al. 2014), we hypothesized that properties of phagocytic cells may induce a Fis-mediated transcriptional response that protects *Yptb* from growth restriction at later stages of infection. To test this, we evaluated the sensitivity of Δ*dusB-fis* to a variety of *in vitro* stresses that simulated the antibacterial actions of neutrophils and inflammatory monocytes in tissue sites, and determined that this mutant was specifically sensitive to

killing by H_2O_2. Using qRT-PCR, we confirmed that *dusB-fis* controlled the transcription of several ROS-responsive genes, including *katG, ahpC, sodA,* and *grxA.* This result suggests that, in *Yptb*, Fis somehow senses ROS in the environment and induces the transcription of factors required for detoxification of and resistance to these species. This is consistent with a previous study of *E. coli,* which found that Fis was required for growth in the presence of oxidative stress (Weinstein-Fischer, Elgrably-Weiss et al. 2000). This study found that, under conditions of oxidative stress, Fis promoted relaxation of DNA; this unwinding is believed to make certain promoter sites more available to transcriptional regulators, which could enhance expression of the OxyR and SoxR regulons. Additionally, *Salmonella typhimurium* also utilizes Fis to detect changes in DNA topology. During growth of *S. typhimurium* in macrophages, Fis senses and maintains DNA relaxation, potentially brought on by exposure to ROS; this DNA relaxation results in transcriptional induction of the SPI-2 pathogenicity island, which is critical for intracellular survival (O Cróinín, Carroll et al. 2006). Therefore, it is possible that *Yptb* may utilize Fis in a similar manner, to detect and "buffer" DNA topology changes in response to ROS exposure, thereby allowing for the transcription of certain factors. Alternatively or additionally, it is possibly that in *Yptb,* Fis may directly regulate the transcription of oxidative response genes or their regulators. This latter hypothesis could be explored by further qRT-PCR and gel-shift experiments.

5.1.3 Clarification of the role of oxidative burst in controlling Yersinia infection

To determine whether Δ*dusB-fis* is sensitive to ROS *in vivo*, we evaluated the virulence of Δ*dusB-fis* in gp91$^{phox-/-}$ mice. Because gp91^{phox} is an essential component of the NOX2 complex, the phagocytic cells of these mice are unable to undergo oxidative burst. Strikingly, we found that the virulence of Δ*dusB-fis w*as completely restored in these mice. This finding confirmed our hypothesis that the primary role of *dusB-fis* during animal infection is to protect against ROS produced by the NOX2 complex of neutrophils and inflammatory monocytes. Because Fis promotes the transcriptional induction of several ROS-responsive genes following *in vitro* exposure to H_2O_2, we hypothesize that Fis-dependent upregulation of these genes may be critical for tissue infection; however, we have not yet tested this hypothesis.

The observation that growth of Δ*dusB-fis* was restored in gp91$^{phox-/-}$ mice was critical in clarifying the role that phagocyte-meditated oxidative bursts play in controlling *Yersinia* infection. As mentioned in Chapter 1, the extent to which *Yersinia* experiences oxidative stress during tissue infection has been unclear. While *Yersinia* utilizes the effectors YopE and YopH to suppress oxidative burst in phagocytic cells (Bliska and Black 1995, Songsungthong, Higgins et al. 2010), a Δ*sodA* mutant in *Y. enterocolitica* is still attenuated for the splenic growth (Roggenkamp, Bittner et al. 1997), suggesting that translocation of Yops may not be sufficient to prevent any exposure to ROS *in vivo.* In possible contrast to this finding, a recent study found that *Yptb* does not up-regulate *sodA* or *katG* during splenic infection, and that a *katG* transcriptional reporter is not highly induced in this tissue site (Davis, Mohammadi et al. 2015). However, *sodA* RNA levels observed during *Yptb* tissue infection were similar to those reported in stationary phase growth, where bacteria can experience some endogenous oxidative stress. In regard to the

katG reporter result, it is possible that the levels of ROS present in *Yptb* microcolonies in the spleen are not uniformly high; therefore transcriptional induction of this gene might only occur in several bacteria per colony. This is consistent with reports that *katG* requires high concentrations of H_2O_2 to be induced (Hillar, Peters et al. 2000).

By contrast, a microarray analysis analyzing *Y. pestis* gene expression during bubo infection suggests that this bacterium may not encounter large sources of oxidative stress in this tissue site (Sebbane, Lemaitre et al. 2006). However, this could be reflective of the different mechanisms of virulence between the pathogenic *Yersinia* and/or the impact that infection stage and tissue sites have on the role of ROS in controlling bacterial infection. Indeed, late-stage bubo infection by *Y. pestis* is often characterized by necrosis and extremely high bacterial burdens, with as many as 10^7-10^9 bacteria growing in extensive masses (Sebbane, Lemaitre et al. 2006). By contrast, splenic infection of *Yptb* is characterized by bacterial growing in clonal microcolonies, usually consisting of small clusters of bacteria surrounded closely by a shell of phagocytes (Davis, Mohammadi et al. 2015). At the time-points we characterized in this work, the total *Yptb* burden in spleens and livers was no more than 10^6 CFU.

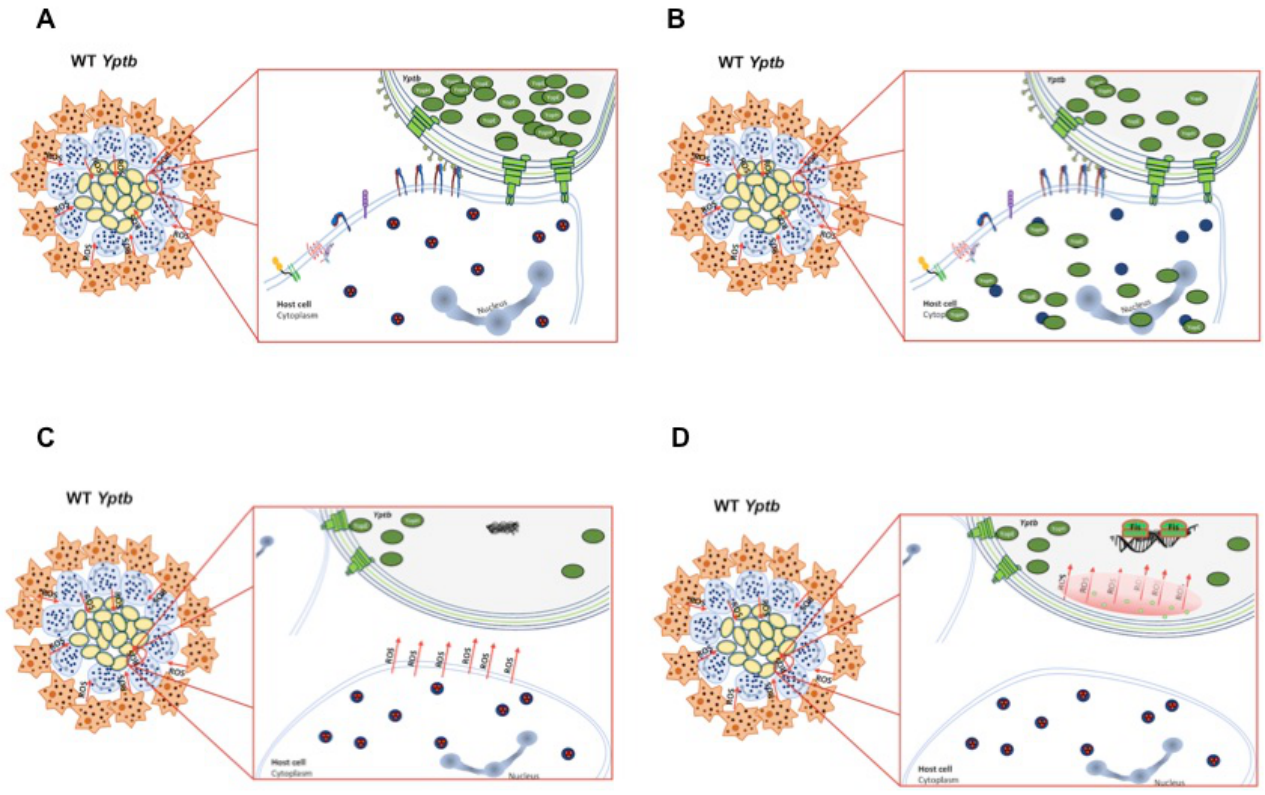
Furthermore, the observation that the virulence of Δ*dusB-fis* is restored in gp91$^{phox-/-}$ mice indicates that *Yptb* must encounter ROS at some point in infection of spleens and livers, and that it is likely coming from neutrophils and inflammatory monocytes, as depletion of these cell types also restores growth of the mutant. Additionally, because *dusB-fis* was dispensable for Yop secretion and translocation, NADPH oxidase inhibition by YopE and YopH must not be sufficient to eliminate all ROS from the environment. Potentially, ROS are being produced by neighboring

phagocytic cells that are not intoxicated with Yops; alternatively, some phagocytes trigger oxidative burst after sensing bacterial PAMPs but prior to Yop translocation. Interestingly, we observed no statistically significant differences in the WT bacterial burdens between C57Bl/6 and gp91$^{phox-/-}$ mice, suggesting that *Yptb* is almost completely resistant to ROS during infection. However, because the virulence of Δ*dusB-fis* is restored in gp91$^{phox-/-}$ mice, this resistance must occur through both Yop-mediated suppression of oxidative burst, as well as through a Fis-dependent mechanism.

Altogether, our results support a model (Fig 5-1) in which, during infection of *Yptb* in systemic tissue sites, 1) some neutrophils and inflammatory monocytes recruited to bacterial microcolonies undergo oxidative burst at these sites, while others do not, due to the presence of YopE and YopH within those cells; 2) bacteria within the microcolonies encounter (potentially low levels of) ROS released by those cells; and 3) those ROS are detoxified in a Fis-dependent manner, likely through the Fis-mediated transcriptional induction of ROS-responsive genes.

Figure 5-1. Proposed model of *Yptb* resistance to ROS during growth within systemic tissue sites

During growth within livers and spleens, *Yptb* forms extracellular aggregates or microcolonies. Following stimulation of the immune response by these bacteria, neutrophils, macrophages, and inflammatory monocytes are recruited to sites of infection. **(A-B)** Some cells in close contact with bacteria on the periphery of the microcolony become translocated with T3SS effectors, resulting in inhibition of NOX2 activation and oxidative burst. **(C)** Other cells, which are not translocated with Yops, undergo oxidative burst in response to bacterial contact and/or PRR activation. **(D)** In WT bacteria, ROS released by these cells diffuse into the bacterial microcolony and are detoxified in a Fis-dependent manner, potentially through Fis-mediated transcriptional induction of ROS-responsive genes. However, in Δ*dusB-fis* infections, mutants are unable to detoxify ROS and are killed and/or restricted for growth.


A
WT Yptb
Host cell
Cytoplasm
Nucleus
B
WT Yptb
C
WT Yptb
D
WT Yptb

5.2 FUTURE DIRECTIONS

5.2.1 Determine the impact of Fis on DNA supercoiling in response to ROS

In this work, we determined that, following *in vitro* exposure to H_2O_2, Fis mediates upregulation of a variety of ROS-responsive genes. However, the mechanism by which this occurs is still unknown. It is possible that, in a similar manner to its actions in *E. coli* and *Salmonella,* Fis in *Yersinia* responds to changes in DNA topology to facilitate transcriptional responses that enable the bacteria to adapt to this environmental stress. In particular, a study of Fis in *E. coli* determined that in response to H_2O_2 in the environment, Fis was critical for DNA unwinding and for positive regulation of *topA1*, which encodes topoisomerase I (Weinstein-Fischer, Elgrably-Weiss et al. 2000). It was postulated that Fis-mediated DNA unwinding might make certain promoter sites, potentially those of oxidative stress response genes, more available for binding of RNA polymerase. To determine whether a similar phenotype occurs in *Yptb*, we could analyze the level of DNA relaxation in WT and Δ*dusB-fis* strains following exposure to exogenous H_2O_2, using the pUC plasmid as a reporter and the DNA intercalating agent chloroquine, which enhances separation of different topoisomers of DNA on agarose gels. If we observe more highly supercoiled DNA species in the Δ*dusB-fis* mutant than in WT, it would suggest that Fis plays a role in promoting or "preserving" a relaxed DNA topology in response to oxidative stress.

5.2.2 Evaluate the role of Fis in mediating regulation of ROS-responsive genes during tissue infection.

In this work, we determined that *dusB-fis* is required for controlling the transcription of several ROS-resistance genes, including *sodA, ahpC, katG,* and *grxA* in response to exogenous H_2O_2 *in vitro*. However, we have not determined whether these genes are regulated in a Fis-dependent manner during tissue infection. To test this, we could construct fluorescent transcriptional reporters for a number of oxidative stress response genes and use microscopy to monitor their expression in WT and Δ*dusB-fis Yptb* during tissue infection. Importantly, because Δ*dusB-fis* is severely attenuated for growth at later time-points, this experiment would only work if infecting this strain at a higher dose allowed the bacteria to at least partially overcome this defect so that microcolonies could be visualized by microscopy. Alternatively, we could extract bacterial RNA from WT and Δ*dusB-fis*-infected tissues and use qRT-PCR to measure expression of several of these genes. Again, lower levels of Δ*dusB-fis* would require that we use much higher does of this mutant in initial infections in order to recover sufficient levels of bacteria to evaluate the RNA.

5.2.3 Assess the survival of ΔdusB-fis in GI tissue sites following oral infection

Two studies suggest that *Yersinia* may encounter fewer ROS during infection of GI tissue sites than in systemic tissues: one reported that the full ROS-inhibitory activity of YopE was not essential for growth in Peyer's patches (Songsungthong, Higgins et al. 2010), while another paper reported that *Y. enterocolitica* lacking *sodA* was attenuated for

growth in the spleen and liver following systemic infection, but not in the GI tissues following oral infection (Roggenkamp, Bittner et al. 1997). Therefore, it is possible that *Yersinia* encounters fewer ROS in the gut than in the systemic tissue sites. To test this hypothesis, we could determine the phenotype of a Δ*dusB-fis* mutant in GI tissues following oral infection of WT mice, $Gr1^{pos}$ cell-depleted mice, and $gp91^{phox-/-}$ mice. If Δ*dusB-fis* has a defect in these tissue sites that is restored by $Gr1^{pos}$ cell-depletion or in $gp91^{phox-/-}$ mice, it would suggest that *Yptb* does encounter ROS in the gut, and that Fis performs a similar function in the GI tissues as in the systemic sites. If Δ*dusB-fis* has a no defect in the GI tissues of WT or immunocompromised mice, it would imply that ROS are likely not produced by phagocytic cells in these tissues, or that *Yptb* are not in sufficient proximity to ROS that are being produced to be restricted by these species. Finally, if Δ*dusB-fis* has a defect in the GI tissues, but that defect is not ablated by $Gr1^{pos}$ cell-depletion or in $gp91^{phox-/-}$ mice, it would imply that Fis promotes growth in these sites through a mechanism distinct from that in the systemic tissue sites.

5.2.4 Use a global, un-biased approach to determine direct and/or indirect regulatory targets of Fis following exposure to oxidative stress

While we determined 4 genes, *sodA, ahpC, katG,* and *grxA,* that were regulated in a Fis-dependent manner following exposure to H_2O_2, there are likely many more genes that could be either directly or indirectly activated by Fis during this condition, including genes they may contribute to other critical oxidative stress defense processes, such as DNA damage repair and/or iron-sulfur cluster repair. Therefore, we could use an unbiased approach to determine which genes exhibit Fis- dependent transcriptional

changes in response to oxidative stress, using RNA-Seq. Alternatively, or additionally, we could use genetic interaction mapping with saturating transposon libraries generated in WT and Δ*dusB-fis Yptb* to determine genes that interact with Fis to permit survival during conditions of oxidative stress. In contrast to an RNA-Seq screen, genetic interaction mapping would allow us to determine which Fis-transcriptional targets are essential for growth under conditions of oxidative stress. These approaches would also provide insight into how *Yptb* in general responds to oxidative stresses, which could be applied to our *in vivo* studies.

Following identification of Fis-regulatory targets, we could construct bacterial mutants to determine whether these targets are essential for resistance to ROS and for mouse infection. We could also generate transcriptional reporters or perform qRT-PCR to evaluate their expression during tissue infection.

FINAL CONCLUSIONS

In summary, this work has uncovered a key mechanism by which *Yptb* ensures its survival within host tissues and demonstrated that *Yptb* senses ROS and uses both chromosomal factors and the T3SS to prevent killing by these species in tissue infection. Fis is undoubtedly critical for other biological functions and resolving its role in the physiology of pathogenic *Yersinia* could lead to insights into how gram-negative bacteria survive and persist in tissues.

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