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Characterizing the virulence factor YapE in Yersinia pestis.

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CHARACTERIZING THE VIRULENCE FACTOR YAPE IN *YERSINIA PESTIS*

by

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B.S., Hampton University, 1998

M.S., Johns Hopkins University, 2004

M.S., University of Louisville, 2012

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A Dissertation Approved on

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DEDICATION

I dedicate this to my family and all of those who have helped me in achieving my goal. I hope that this serves as an inspiration to my children (MJ, DK, SS, DA) that no matter how hard things may get to never, ever give up and see it through. This has been one of the most challenging and rewarding endeavors that I have pursued (outside of parenting). Thank you DWT, I love and miss you much.

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Through everyone's endless patience, support and the occasional push, I have accomplished what began as merely a thought fifteen years ago.

ABSTRACT

CHARACTERIZING THE VIRULENCE FACTOR YAPE IN *YERSINIA PESTIS*

Tiva T. VanCleave

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Yersinia pestis is the causative agent of bubonic plague and is primarily transmitted by fleas. Upon infection, the bacteria rapidly travel to the regional lymph nodes causing inflammation and cellulitis in these tissues (referred to as buboes). Two outer membrane proteins, YapE and Pla, have been implicated to have roles in dissemination to the lymph nodes. Their adhesive properties have shown that they are able to interact with host macrophages thereby increasing their ability to disseminate to regional lymph nodes. More recently, we have shown that YapE is cleaved by another virulence factor important for lymph node colonization, Pla, to become an active adhesin. To further understand the biology of YapE, I used a transposon mutagenesis screen to identify possible regulators. While the current approach did not identify novel regulators, I discuss potential modifications to screening conditions that may allow for increased success in future attempts. I also investigated the role that Pla may play in the activation or degradation of other outer membrane proteins. 2-DIGE analysis identified 76 *Y. pestis* proteins that may be processed by Pla and I confirmed Pla-dependent

degradation on HmsF at 37°C. To further investigate the role outer membrane proteins may play in intracellular survival, I infected macrophages with *Y. pestis* strains lacking *pla*, *yapE*, *ail* or *ompA*. Before performing these intracellular assays, I developed an optimized gentamicin protection assay to monitor intracellular growth of *Y. pestis*. To this end, I determined that the concentration of gentamicin used for *Y. pestis* should not exceed 8 µg/ml for 1 hour. Using this assay, I verified that OmpA was required for *Y. pestis* intracellular survival in macrophages. While Y. pestis lacking Pla or YapE had minor defects in intracellular growth, an *ail* mutant showed no phenotype difference compared to WT. Future studies with mutants lacking multiple adhesins will help to define the potential contributions of these proteins to intracellular survival.

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CHAPTER ONE

YERSINIA PESTIS AND PLAGUE

Plague is an enzootic disease that has caused three pandemics worldwide [1]. These pandemics consisted of the Justinian plague (541 AD), The Black death (1334 AD) and the current, Modern plague (1900 AD). The current pandemic, Modern plague, came by way of infected rats that travelled on steamships from Asia and spread among port cities around the world. This pandemic came to the western shores of the United States, and can still be found in western states such as New Mexico, Arizona, Colorado, California, Oregon, and Nevada (https://www.cdc.gov/plague/maps/index.html). In the United States animals such as rock squirrels, wood rats, ground squirrels, prairie dogs, chipmunks, mice, voles, and rabbits can be affected by plague (CDC and https://emedicine.medscape.com/article/235627-overview#a6). Plague still remains a significant concern for public health because of its ability to be transmitted person to person through respiratory droplets and possible use in bioterrorism and biological warfare [2, 3]. Currently, *Y. pestis* is listed as a Tier 1 Category A Select Agent due to its potential to be used as a weapon [3, 4].

From 1970-2016 there have been between 1-17 cases per year reported in the United States (https://www.cdc.gov/plague/maps/index.html). Outside of the United States, plague is much more prevalent, especially in Africa and Asia. Currently, the Center for Disease Control (CDC) and the World Health Organization report that as recent as August 1 – November 22, 2017 that in Madagascar alone there were a total of 2348 confirmed cases of plague with a fatality rate 8.6% (http://www.who.int/csr/don/27-november-2017-plague-madagascar/en/).

Plague is caused by the Gram negative bacterial pathogen *Yersinia pestis* [5]. When transmitted by fleas, the bacteria initially colonizes the intradermal space before migrating to the draining lymph node, where it causes inflammation and cellulitis resulting in a bubo (bubonic plague) [6]. Left untreated, the bacteria can escape from the lymph node and disseminate systemically through the blood to various organs causing a fatal sepsis known as septicemic plague. Secondary pneumonic plague occurs during the septicemic infection when the blood circulating with the bacteria goes to the lungs. A primary pneumonic infection can also occur by direct inhalation of infectious droplets or aerosols and develops into acute pneumonia, intra-alveolar hemorrhage and edema, profound lobular exudation, fibrin deposition, and bacillary aggregation [2, 7]. Due to acute and systemic infections, the mortality rate of plague reaches 70-100% without treatment depending on routes of infection [8].

*Y. pestis vir*ulence determinants

Y. pestis belongs to the family *Enterobacteriaceae* where *Yersiniae* contains 14 species, with three being pathogenic to humans [9-11]. Two are enteric pathogens, *Y. entercolitica* and *Y. pseudotuberculosis* and the third, *Y. pestis*, is the agent of plague. *Y. pestis* diverged from *Y. pseudotuberculosis* approximately 1,500-20,000 years ago [12, 13]. During this divergence, *Y. pestis* acquired two additional plasmids (pPCP1 and pMT1) that are necessary for transmission of the bacteria from the flea vector to the mammalian host [5, 12, 13]. Furthermore, *Y. pestis* has also acquired new virulence factors and lost genes that are required for enteric infection but not for plague [5].

1. Evasion of the immune response by the Ysc Type III Secretion System (T3SS)

The T3SS is found on the pCD1 plasmid, which is present in all three pathogenic species of *Yersinia* [5]. The assembly of the T3SS allows for transport of effector proteins across the inner and outer bacterial membranes of the bacterium in addition to across eukaryotic membranes [14]. These effector proteins released through the T3SS are referred to as Yops (Yersinia outer membrane proteins). The Yops allow for disruption of the cytoskeleton, mediate cell adhesion, and facilitate the adherence of the T3SS for Yop injections [14, 15]. *In vivo*, *Y. pestis* injects their effector Yops targeting primarily neutrophils but also phagocytes such as macrophages and dendritic cells although in vitro, they are able of injecting effector Yops into essentially any cell [16]. The T3SS is expressed when inside a mammalian host (37°C) and has no role in the colonization of the flea (26°C) [5].

There are seven Yops found in all three *Yersinia* spp.; YpkA/YopO, YopH, YopE, YopJ/YopP, YopK, YopM, in addition to YopT that is only found in Y. entercolitica. YpkA/YopO, YopE, YopT and YopH play a role in the disruption of the actin cytoskeleton and target eukaryotic cell signaling components of the RhoA family of small GTPases that facilitates the inhibition of phagocytosis with the exception of YopH for the later [17, 18]. Additionally, YopH disrupts both the innate and adaptive immunity pathways that are important for the virulence of all three pathogenic species in mice [17, 19], in addition to inhibiting autophagy and blocking phagocytosis in macrophages [17, 20, 21].

YopP/J inhibit the ability to activate and release NF-Kβ, keeping the cell in an anti-inflammatory state [22-26]. YopJ also has been shown to inhibit caspase-1 in activated macrophages [27]. YopM is essential for *Yersinia* virulence because of its capacity to downregulate proinflammatory responses while upregulating IL-10, an anti-inflammatory cytokine [28-31]. Comparable to YopJ, YopM has been shown to block mature caspase-1 activity inhibiting the recruitment of pro-caspase-1 to the inflammasome complex [32-34]. Loss of the T3SS or the pCD1 plasmid results in complete attenuation of all three pathogenic species in mice [18]. This further validates the importance that the T3SS is vital for the virulence of the bacteria.

2. Modulation of LPS

One way that *Y. pestis* is able to avoid immediate clearance from the host once infection has begun is to be able to sense the change in environment, and change the conformation of its LPS when going from the flea to the mammalian host. At 26°C (flea), the LPS is hexa-acylated which is recognized by TLR-4, inducing proinflammatory cytokines such as IL-8, IL-1 and TNF. During a temperature shift, that happens during infection into a mammalian host (37°C), the LPS changes to the tetra-acylated form that does not stimulate TLR-4 and is an antagonist for hexa-acylated LPS [35, 36]. To further validate the role the change in LPS has on virulence, Montminy and co-workers altered the LPS of *Y. pestis* to be a more powerful TLR-4 stimulant. The change to the stimulatory form of LPS caused *Y.pestis* to be rendered completely avirulent after subcutaneous infection [36].

3. Iron uptake

Siderophore-based iron acquisition has been seen as a hallmark for virulence in successful pathogenic bacteria [37]. This is true for the pathogenicity of *Yersiniae* and its ability to acquire iron. All three pathogenic species of *Yersinia* use the siderophore Yersiniabactin (Ybt) [37]. The iron accumulated from the host allows for metabolic stability for the purpose of multiplying to cause systemic infections [38]. Yersiniabactin synthesis in *Y.pestis* consists of *irp1* and *2* and *ybtU,T,E* and *S,* and are clustered on a mobile genetic element known as the High Pathogenicity Island that makes it possible to be widely disseminated in *Enterobacteriacea* [37]. This siderophore plays an important role in the acquisition of iron and murine virulence [37, 39, 40]. Inactivation of Ybt has been shown to significantly attenuate *Yersinia* [37, 39, 41-45].

4. Plasminogen activation by Pla

Pla is encoded on the plasmid pPCP1. *pla* was identified as one of the most exceedingly expressed genes in *Y. pestis* isolated from bubos of infected rats [46, 47]. Pla is a member of the omptin family of proteases that has evolved specifically for bubonic and pneumonic forms of plague [48]. Pla is able to convert plasminogen to plasmin allowing for the degradation of extracellular matrices permitting *Y. pestis* to quickly invade the host and migrate to draining lymph nodes [17, 49-51]. During subdermal or subcutaneous infection, *Y. pestis* expressing Pla had wide-spread bacterial foci with minimal recruitment of inflammatory cells [48, 52]. Conversely, when Pla was not expressed, it resulted in an accumulation of bacteria at site of infection, causing an inability to

disseminate and thereby increasing the recruitment of immune cells [48]. Unlike the bubonic (subcutaneous) model, Pla is not necessary for the dissemination from the initial site of infection for primary pneumonic plague. Pla is pivotal for the progression of primary pneumonic plague by assisting in the growth or survival and proliferation of *Y. pestis* in the lungs [46, 53]. Pla is important for the fitness of the bacteria for both bubonic and pneumonic plague although through differing mechanisms. This shows the multifaceted nature of Pla, that while it is dispensable for the spread of *Y. pestis* in pneumonic plague, its protease activity is important during bubonic plague.

5. The Capsular antigen fraction 1 (Caf1) Capsule

The Caf1 capsule is made up of the Fraction 1 (F1) antigen, a fimbrial protein that accrues on the bacterial surface to form a capsule unique to *Y. pestis* [54]. Caf1 capsule is strongly influenced by temperature, where essentially no capsule is identified in the flea vector or at temperatures <35°C [54]. The *caf1* gene is the most highly expressed genes during infection of the mammal, indicating that it is a virulence factor [54]. Studies have shown that the capsule works in tandem with the T3SS to make *Y. pestis* resistant to uptake by macrophages and PMNs [55-58]. Although the *caf1* gene is highly expressed, there has been conflicting data on its requirement for virulence [56, 59]. Some studies with bubonic plague in mice and guinea pigs, and pneumonic plague in primates, have shown that when the F1 capsule is not present, there is no effect on virulence [56, 60, 61]. Conversely, Gonzalez and co-workers were able to show that in C57BL/6J mice a Δ*caf1*Δ*psaA* strain had a significant attenuation in

dissemination or colonization into deeper tissues from the lymph node compared to the WT *Y. pestis* [59]. However, the same Δ*caf1*Δ*psaA* strain was not attenuated in BALB/cJ mice [62]. The previous findings by Weening and co-workers showed that differences in virulence with *caf1* was predicated on the mouse background [62]. Although there appears to be evidence that the capsule plays a minor role in virulence, depending upon host; it has been shown that minute amounts of the F1 subunits and the outer membrane Caf1A protein have an affinity to the IL-1 receptor on macrophages and to IL-1β expression by epithelial cells [63-65]. This may play a role in early infection by conferring adhesion or anti-immunity potential [54].

6. *Y. pestis* Adhesins

The ability to target cells for T3SS has been directly linked to bacterial adhesins [66]. In the enteropathogenic *Yersiniae*, two important adhesins, YadA and Invasin, facilitate bacterial attachment and Yop injection [14, 15]. Although these two adhesins are important for the pathogenicity of *Y. entercolitica* and *Y. pseudotuberculosis*, *yadA* and *inv* are pseudogenes and not expressed in *Y. pestis* [17]. Therefore, other adhesins, have taken on the roles of YadA and Inv.

Ail

Ail functions as a multi-factorial adhesin in *Yersiniae* [17]. During its divergence from the two enteric pathogens, it gained additional roles in the virulence of *Y. pestis* that include; 1) facilitating the injection of Yops into host cells [14, 67], 2) inhibition of the inflammatory response [68], 3) inhibition of the bactericidal

properties of complement [68-70]and 4) prevention of neutrophil recruitment to the lymph nodes [69, 70]. Ail has been shown to be the primary adhesin in the attachment of the T3SS to host cells in *Y. pestis* where its deletion exhibits significant reduction in binding to epithelial and monocytic cells [17, 70]. Interestingly, the transcription of Ail can also be altered when other outer membrane components are mutated [70]. In addition when comparative proteome strategies were used to identify new virulence targets found that Ail clustered together with virulence associated proteins on the plasmid pCD1.

Pla

In addition to being a protease, Pla also has adhesive properties [71]. It has been linked to invasion of host immune cells and dissemination by its interaction with CD205 on macrophages [72]. A *pla* mutant is attenuated in a mammalian host due to both its inability to disseminate from the site of infection and through its uptake by macrophages. In addition to attachment to phagocytes, Pla is also necessary for the positioning of the T3SS for the delivery of Yops [71].

YadB/C

YadB and YadC have the ability to form oligomers that lead to aggregation and mediate invasion to epithelial cells, although they are not necessary for host cell adherence [72, 73]. Studies have shown that when both proteins are mutated, there is a substantial decrease in the ability for the bacteria to invade HeLa cells and type I pneumocytes, but does not affect overall adherence to cells [73].

Autotransporters

Autotransporters are found in Gram-negative bacteria and are comprised of a N-terminal signal sequence, passenger domain, and C-terminal β-domain. Within the Yersiniae species there are nine Yersinia autotransporter proteins (Yaps) that produce functional proteins [74]. Autotransporters can contribute to pathogenesis through adherence to eukaryotic cells, proteolysis, complement resistance and cytotoxicity [75]. One particular autotransporter that has been conserved in all three pathogenic species is YapE.

YapE

YapE has been maintained in all the pathogenic species of *Yersiniae* [76]. The conservation of *yapE* in *Yersinia* suggests that YapE may be a virulence factor. YapE is capable of facilitating contact between bacteria and eukaryotic cells through its adhesive properties [76]. YapE also promotes adherence to eukaryotic cells. This was demonstrated by Lawrenz and co-workers with the evaluation of the adherence of bacteria to RAW264.7 macrophage cells. They found that the expression of native YapE in *Y. pestis* had a significant increase of adherent bacteria (3.5 fold increase, $P<0.0001$).

YapE also allows for of aggregation in *Y. pestis* and *Y. pseudotuberculosis* but not in its distant relative*, Y. entercolitica* when expressed in *E.coli* [76]. A study by Lawrenz and co-workers showed that the processing of YapE by the omptin, Pla, is specific to *Y.pestis* but not its enteric predecessors [77]. The post-translational modification of YapE appears to have been acquired after *Y. pestis* diverged

from *Y.pseudotuberculosis* and *Y. entercolitica* [77]. As well as allowing for bacterial aggregation, this processing was also shown to be necessary for the adhesion to eukaryotic cells [76]. Their data showed that processing by Pla is necessary for YapE to play a role in bacterial aggregation and eukaryotic cell adhesion.

7. Role of intracellular survival

Y. pestis is considered a facultative intracellular pathogen due to its ability to replicate in macrophages and neutrophils [5, 78, 79]. During a subcutaneous infection (bubonic), there is a robust innate immune response with an immediate arrival of both neutrophils and macrophages. However, initial neutrophil killing of the bacteria is greater because the bacteria has not had an opportunity to employ many of its anti-phagocytic properties during the transitional phase from the flea to a mammalian host. Following infection with *Y. pestis*, neutrophils are rapidly employed to the site and phagocytose the bacteria. Macrophages also engulf the bacteria, although to a lesser degree, and appear to move away from the site of infection [80]. It has been shown that although neutrophils have a high success rate of eliminating *Y. pestis*, a small number of *Y. pestis* is still able to replicate inside the hostile environment [78]. Due to the attenuated lifespan of neutrophils, dying neutrophils are cleaned up by resident macrophages. Once the bacteria is taken up by the macrophage, it is housed in a Yersinia containing vacuole (YCV) where it is able to subvert the phagolysosomal degradation pathway [81]. The intracellular lifecycle of *Y.pestis* has been shown to be important for virulence. This has been exhibited by lower bacterial burden when animals were depleted

for dendritic/macrophage populations during plague infection and survival defect, in macrophages, by a *phoPQ* mutant [30, 80, 82-85].

Central hypothesis and Aims

YapE mediates interactions with macrophages and is required for disseminated disease. Therefore, my central hypothesis is that YapE-dependent interactions with macrophages contribute to overall virulence of *Y. pestis*. The following aims were designed to further understand the biology associated with YapE.

Aim 1: Identify genetic regulators of *yapE* in *Y. pestis*.

Aim 2: Determine if other outer membrane proteins are processed by Pla.

Aim 3: Determine if YapE and/or Pla contribute to intracellular infection.

CHAPTER TWO

IDENTIFICATION OF REGULATORS OF YAPE

Introduction

Bacteria regulate the expression of genes in response to the environment in order to survive in specific niches. Virulence determinants are tightly and coordinately regulated during infection. Virulence-related regulators can sense host signals (e.g., changes in temperature) and then differentially regulate large sets of genes required for adaptation to the host niche [86]. In the case of pathogens, virulence genes are regulated in a coordinated fashion to allow the bacterium to infect its host. *Y. pestis* is maintained in an enzootic cycle between mammalian and insect hosts [87] and several studies have shown tight regulation of virulence factors in *Y. pestis* dependent on host [74, 88, 89].

yapE transcription levels are very low during *in vitro* growth [74]. To evaluate *in vivo* transcription, Lenz and co-workers used both a bubonic and pneumonic plague model, and compared transcription levels of *yapE*, *yapG* and *yapC* grown at 26° in broth cultures [74]. They observed a significant increase in *yapE* expression *in vivo* compared to *in vitro*. Following subcutaneous infection, *yapE* transcript increased 15-fold increase in expression of *yapE* in cervical lymph nodes by 72 h and an approximate 5-fold in the lungs during pneumonic plague [74]. This increase of expression *in vivo*, for both the pneumonic and bubonic models, suggest that YapE may be important for mammalian infection [6]. In addition to observing the differing levels of transcription in both models. Differences in levels of *yapE* expression between the lymph nodes and lungs may suggest that *yapE* has a more substantial role in bubonic infection than pneumonic infection. This was confirmed when using a bubonic mouse model (subcutaneous) and

challenging with a *yapE* mutant and parental WT (CO92) strain. Colonization of proximal lymph nodes had a noticeable delay in colonization compared to WT by 36 hours post infection. When the same challenge was performed on a pneumonic plague model (intranasal), the mutated *yapE* strain did not significantly alter the ability for the lungs to be colonized [76].

To further understand what stimuli is responsible for the change of expression between the *in vivo* and *in vitro* testing, the bacteria were subjected to varying stimuli such as temperature, iron, calcium, and pH *in vitro* [74]. None of these environmental ques changed the expression of *yapE in vitro* [74]. To this end, it was surmised that there must be other signals in vivo, that allows the bacteria to streamline gene expression.

These findings support the hypothesis that *yapE* transcription is regulated in *Y. pestis*. Here I attempted to elucidate the regulatory mechanisms for *yapE* expression. Defining these mechanisms will help us to better understand how *Y. pestis* regulates gene expression in response to host colonization.

Materials and methods

Construction of the yapE reporter in Y. pestis CO92 pCD1-ΔlacZΔyapE background

The bacterial strains and plasmids utilized in this study are listed in Table X. *Y. pestis* was grown in Brain Heart Infusion (BHI) broth at 26°C. Antibiotics were used at the following concentration: chloramphenicol, 12.5 µg/ml (*Yersinia*).

The promoter regions upstream from the *yapE* gene were amplified by PCR with primers DNA278 (5' – GG GGT ACC C TGG CTA TCA TTG TTT TAC GAA AGC – 3') and DNA368 (5' – G GGA TCC CTT ATG GCT TGC TGA TAA TAG CAG AGT – 3'). The resulting product was gel purified and digested with KpnI and BamHI. The resulting fragment was ligated onto pFU99 and transformed into YPA109 to generate YPA111.

Transposon Mutagenesis

YPA111 was grown in BHI broth at 26°C in chloramphenicol 12.5 µg/ml and *E. coli* (S17) containing the transposon pAJD428 (LOU117) was grown in Luria-Bertani (LB) broth at 37°C in Kanamycin 50 µg/ml. In a separate tube, a 3:1 (LOU117:YPA111) was added to sterile 10 mM $MgSO_4$. The mixture was then placed into the 3 cc syringe attached to a Swiznet filter containing a membrane filter (0.45 um HV) allowing the solution to be pushed through the membrane filter. Using flame sterilized tweezers, the membrane was removed from the filter and placed on a BHI agar plate that has no antibiotic and allowed the bacteria to grow overnight at 26°C. The following day the membrane was removed from the agar plate and placed into a 1.5 ml tube with 1X sterile PBS and vortexed for 45 seconds. The bacteria was then plated the rehydrated bacteria (straight) onto BHI agar plates containing; Kanamycin 100 µg/ml, Chloramphenicol 12.5 µg/ml, Polymyxin 25 µg/ml, X-gal80 µg/ml and IPTG 1mM and allowed to grow for 2 days at 26°C. Colonies that were blue were then grown for further evaluation.

DNA Isolation

Blue colonies that developed on the mating plate were isolated and grown in BHI broth overnight. The following day 1.5 mls of the bacteria were pelleted by centrifugation and re-suspended in a buffer of 50mM Tris and 50 mM EDTA. 10%SDS and Pronase were added to the re-suspended bacteria and allowed to incubate for 1 hour at 42°C until the solution was clear. Phenol was added and the mixture was shaken for one hour. To the mixture, chloroform was added and centrifuged for 15 minutes at 7,000 x g. The top phase was transferred into a clean 1.5 ml Eppendorf tube and 3M of sodium acetate (NaOAc) and 95% ethanol was added and inverted allowing the DNA to precipitate out of solution and the DNA was rinsed with cold ethanol. To the rinsed DNA, TE and RNase was added and allowed to incubate at 37^{O}C for 1 hr allowing the DNA to unwind into solution. After incubation, sodium acetate and cold isopropyl alcohol is added to the solution containing the DNA and centrifuged for 10' @ 13,000 RPM. The remaining pellet was washed 2 x with 70% EtOH and the DNA was allowed to dry for 30 minutes at room temperature. The dried pellet was then re-suspend DNA 200 in µl H_2O for further evaluation.

Arbitrary PCR

To identify the insertion site of the transposon from the isolated DNA above, we performed two rounds of PCR with the first round amplifying the junction between the TnMod-RKm'-laclq-tacp (pAJD428) and the *Y. pestis* chromosome using 5'-GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN ACG CC-3' (DNA457) and

5'-GAC AAT TAA TCA TCG GCT CG-3' (DNA458). The PCR was ran on an 0.8% agarose gel and then the PCR product was then purified using the Promega PCR clean up kit and eluted in ddH2O. The purified PCR product was then amplified for the transposon using primers 5'-GGC CAC GCG TCG ACT AGT AC-3' (DNA459) and 5'-TCG GCT CGT ATA ATG TGT GG-3' (DNA460). Again, the PCR was ran on a 0.8% agarose gel for verification. The resulting PCR was then then purified using the Promega PCR clean up kit and eluted in ddH2O and sent for sequencing using M13 reverse primer 5'-AGC GGA TAAC AAT TTC ACA CAG GA-3' (DNA461). When the results were returned, the inverted repeats were identified (TGAACACATATTCTCAGTC) and the sequence upstream of the inverted repeats were placed in NCBI BLAST to identify the region of insertion in the *Y.pestis* chromosome.

Construction of the inducible phage construct

The genetic regions YPO2277-2281 were amplified using 5'- CGC ACT GAC CCA ATT CGA GCA GAA CAG AAG CGC C -3' (DNA567) and 5'- TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC TCA CCA ATC AGG CCT -3'(DNA581) and the inducible plasmid, pMWO-034 was amplified using 5'- GGC GCT TCT GTT CTG CTC GAA TTG GGT CAG TGC G -3' (DNA570) and 5'- AGG CCT GAT TGG TGA GTC GAC GGT ATC GAT AAG CTT GAT ATC GAA -3' (DNA580). The PCRed vector, pMWO-034, was digested with DpnI and incubated at 37 for 1 hour. Gibson cloning was performed by adding the amplified phage region (YPO2277-2281) to the vector and incubated at 50° for 1 hour and placed on ice. The assembled

reaction was dialyzed for 20 minutes and electroporated into DH5α cells and recovered in room temperature LB broth. The electroporated DH5α cells were incubated on the roller drum for 1 hour at 37°C. The reaction was then plated onto LB/Kan50 plates and placed in the 37°C overnight.

Confirmation of phage insertion into pMWO-034 plasmid

Clones from the assembled Gibson cloning of the phage region (YPO2277-2281) were grown in LB broth overnight at 37°C and the plasmids were extracted using the Wizard® Plus SV Minipreps DNA Purification System. The plasmids were then subjected to restriction digest with BamHI for 3 hours and then ran on an 0.8% agarose gel to confirm insertion.

Miller Assay for induction of phage in high salinity and high osmolarity

Bacterial strain YPA111 was grown in BHI at 26°C overnight and diluted 1:12.5 into 5 mls of M9 media and allowed to grow for 1 hour at 26°C. After the first hour, 1 mM IPTG was added to the media containing the phage containing strain YR076 and placed on the roller drum to incubate for 2 hours at 26°C (T=3h). To the respective sample, to test for stress induced by salinity, 5M NaCl was added to the media for a final concentration of 0.5M NaCl for high salinity; the bacteria experiencing high osmolarity sorbitol was added to the culture for a final concentration of 0.5M sorbitol for 20 minutes. OD_{600} readings were taken after 20 minutes of growth for all samples NaCl, sorbitol and both controls. Assay tubes were set up to include the Z buffer containing 60 mM disodium phosphate, 40 mM monosodium phosphate, 10 mM potassium chloride and 10 mM magnesium sulfate and β-mercaptoethanol. To the buffer, chloroform and 0.1% SDS was

added. Cells were added to working buffer and lysed immediately by vortexing for 10 seconds. The samples were allowed to incubate at room temperature for 5 minutes.

The β-galactisidase reaction began by adding ONPG as well as the timer to begin the reaction and quickly vortexed. After sufficient color change, the reaction was completed by adding Na_2CO_3 and the stop time was recorded. The sample was quickly vortexed and allowed to sit for 5-10 minutes allowing the particulates settle. Once the reactions were completed, the top layer was transferred into a cuvette, and the absorbance was measure at OD_{420} and OD_{550}.

1. Determine Miller Units using the following formula:

Miller Units = $1000 \times [(OD_{420} - 1.75 \times OD_{550})] / (T \times V \times OD_{600})$

- OD_{420} and OD_{550} are read from the reaction mixture.
- OD_{600} reflects cell density when harvested.
- T = time of the reaction in minutes.
- V = volume of culture used in the assay in mLs.

Results:

Transposon mutagenesis screen

To identify gene(s) that are involved in the regulation of *yapE*, we designed a reporter system where we fused the promoter region of *yapE* to a *lacZ* gene that gives a colormetric readout when the transposon inserts itself into gene(s) that may influence *yapE* regulation (Fig. 2-1). Using this reporter strain, a transposon library was generated, using the Tn5 pAJD428 transposon. The pAJD428 transposon is different from the traditional Tn5 transposon that it also contains an

outward facing *lac* promoter. This allows for the observation of both positive and negative regulators. The limitation of the Tn5 is that the only regulator that can be screened is a negative regulator. The pAJD428 transposon, because of the outward facing *lac* promoter, is able to also screen positive regulators. This can be observed when plated in the presence of IPTG that will drive the *lac* promoter allowing continued transcription of the gene in which the transposon inserted. Over 54,000 colonies were screened using the pAJD428 with 100 containing the blue phenotype indicating increased expression of *yapE*.

To determine if the transposon inserted itself into a positive or negative regulator of *yapE*, I grew each blue colony in the presence or absence of 1 M IPTG. Colonies that turned white in the absence of IPTG, were designated IPTG dependent, suggesting transposon insertion upstream of a positive regulator. Those that remained blue in the absence of IPTG were designated IPTG independent, suggesting insertions into negative regulators (Table 2-2). Of the 100 original colonies 36 were IPTG dependent.

Identification of transposon insertions

Genomic DNA was isolated from each clone and arbitrary PCR was performed to identify the genomic area where the transposon inserted. 22 were found in sugar transport and metabolizing genes, 56 were inserted into the reporter plasmid, 1 into an *E.coli* DNA polymerase II, and 1 into a phage-related membrane protein. I was not able to identify insertion sites for 20. Due to the nature of the screen, the sugar transport and metabolizing genes were not further investigated because they fell into an operon containing *lacI* and a putative galactosidase gene (Table 2-1).

Since the readout is from cleavage of X-gal by β-galactasidase it was determined that these may be false positive.

Identification of Phage insertion

From the 100 colonies that were isolated, one insertion, that was IPTG independent, was in a phage related membrane protein (Table 2-1). During the screen there were instances where there was a secondary insertion one in the reporter plasmid and one in the secondary region. To ensure there was no secondary insertion causing the change in *yapE* expression, plasmid isolated from the clone containing the phage insertion (YR076), the vector plasmid, and a sample that had a known insertion into the plasmid (YR100) were cut by restriction digest to linearize the plasmid and compare the sizes (Figure 2-3). An increase in size would denote an insertion of the transposon in the reporter plasmid. To this end, it was verified that there were no additional insertions in the reporter plasmid.

Determining if insertion in the Phage-like protein influences yapE expression.

To determine if the transcription of the phage shock protein is regulating *yapE*, an inducible phage was designed consisting of the phage-like and secretion proteins sequence and the two hypothetical proteins flanking that region. The transposon inserted itself between YPO2278 and YPO2279 and I wanted to include the genes upstream and downstream of the insertion site (Fig.2-5).

The genomic region used for the inducible plasmid codes for the filamentous phage, YpfΦ. We first wanted to determine if induction of this phage would alter the growth of the bacteria. To determine if there was a growth defect, OD_{600}

readings were taken every 2 hours for 8 hours of YR076 (induced with IPTG and uninduced), vector containing strain (YPA111) and WT (YPA035). Induction of YR076 was performed after 1 hour of initial growth. Over the first 6 hours there was no difference between ODs of each of the samples. By hour 8, there was a slight decrease in OD in both the induced and uninduced YR076 although it was shown to be statistically insignificant (Fig. 2-4). Since there was no change in growth when the phage protein was expressed by the YR076 clone, I decided to perform a β-galactosidase assays placing the YapE reporter strain (YPA111) in stress conditions to activate the native phage.

Phages are activated under stressful conditions and produce a protein termed phage shock protein (psp). The phage shock protein response is well characterized in *Y. pestis* predecessor, *Y. entercolitica*. These proteins are required for regulation and stress tolerance [90]. Lenz and co-workers have previously shown that when *Y. pestis* is placed under environmental stressors, there was an influence in the expression of several Yaps (Table 2-2). Although, there was no change in vitro when placed under these stressors, there was an increase in vivo of *yapE* at 37°C. Three stressors that were not evaluated in the Lenz screen were, low Mg^{2+} concentration, osmolality and salinity. To determine if one of the aforementioned stressors may have some influence on the phage transcription, I decided to perform a Miller assay (β-galactosidase assay) using the *yapE* reporter strain (YPA111). Compared to the control, YR076, there was no change in expression of the *yapE* promoter region for any of the three stressors (Fig 2-7 and 2-8).

Because I was unable to activate the native phage, the inducible phage protein plasmid that was constructed was tested next. By Western blot analysis, the inducible plasmid containing strain (YPA131) was compared to an inducible strain of CO92 containing a *yapE* overexpression plasmid (YPA024). Because endogenous YapE is so low *in vitro*, the use of an overexpressing strain for *yapE* allows for verification of YapE in addition to having the ability to quantify the overall amount of protein produced. The both samples (YPA131 and YPA024) were induced with anhydrous tetracycline (aTc) or no anhydrous tetracycline were added to the samples (negative control). Samples were acquired every 2 hours for 6 hours and the Western blot membrane was probed with 1:1000 α-*yapE*. Compared to the overexpression YapE strain, there was no visible detection of YapE at any of the time points from the samples containing the inducible phage (Fig. 2-6). As stated earlier, previous studies by Lenz and Lawrenz, YapE expression is low in vitro and an overexpression plasmid was required to observe YapE by Western blot. Another possibility for a negative blot is that the amount of YapE expressed may be below the limit of detection by Western blot analysis.

Discussion

Initially, I previously screened over 15,000 clones using a Tn5 transposon and acquired 75 clones containing the blue phenotype. The initial use of the Tn5 limited the scope of identifying regulators because the only regulators that could be identified were negative regulators. To increase the scope of the screen, I changed to the pAJD428 transposon. Much like the Tn5, pAJD428 consists of inverted repeats and a kanamycin cassette but it also contained an outward

facing inducible *lac* promoter. If the transposon inserted itself into a negative regulator, thereby inactivating the repressor, would allow for increased activity of *yapE* promoter. This allowed for the observation of both negative and positive regulators by way of dependence or independence of IPTG. To this end, I was able to perform a >10-fold coverage screen of the *Y. pestis* genome, screening 54,931 colonies for changes in *yapE* expression using the pAJD428 transposon.

YapE is a highly conserved autotransporter and has been shown to be a virulence factor due to its increased upregulation in vivo at 37°C, ability to adheres to RAW macrophages by 3.5-fold, and displays a delay in dissemination and colonization of the lymph nodes [76]. Because bacteria coordinate expression of virulence factors, we sought to investigate the mechanism of how *yapE* was being regulated. To this end, we used a transposon mutagenesis screen to try to identify gene(s) responsible for *yapE* regulation. We performed a 10-fold coverage of the *Y. pestis* genome and were able to isolate 100 clones that had the blue phenotype representing an insertion into a possible regulator of *yapE* either a positive regulator or a negative regulator. What we found is ~85% of the transposons inserted themselves into our reporter or sugar transport and metabolizing genes. The remaining clones were either not verifiable due to not being able to locate the inverted repeats or we were unable to obtain verifiable data from sequencing. We did find one insertion a filamentous phage, YpfΦ that has been shown to confer some selective properties to *Y. pestis* during the infection process [91].

Filamentous phage infections produce the protein, phage shock protein, when the bacteria are in stressful conditions [92]. When infection occurs from the flea vector to the human host, *Y. pestis* goes through copious changes due to its ability to infect two different host the flea at 26°C and the mammalian host at 37°C. Once inside the mammalian host, the bacteria are subjected to temperature shift, pH change, in addition to the influx of neutrophils and macrophages that phagocytize the bacteria placing them under highly stressful conditions. Lenz showed that using environmental cues in vitro had no effect on *yapE* transcription levels but in vivo (37°C) they showed that *yapE* transcript increased 15-fold by 72 hours [74]. This indicates that there may be other stress inducers or environmental cues in vivo that may influence transcription levels of *yapE*.

Although we were not able to detect any *yapE* transcription by way of our reporter, this may be due to the length of time the bacteria was placed in high stress environment (up to 12 hours). Lenz showed the increase of transcript of *yapE,* in vivo, was between 48 and 72 hours [74]. The time points analyzed here may not have been long enough to allow for the regulator to be induced.

In addition to the genes of the phage, we also cannot rule out the possibility that this was another case of false positive due to a *lacI* transcriptional regulatory protein downstream of the phage related membrane proteins (Fig. 2-2) and the insertion in the phage operon may have induced expression of this gene and production of a cryptic β-galactosidase.

Another caveat to the transposon screen is that *yapE* expression is already low in vitro. When designing the reporter, there is a chance there was YapE expression but the limit of detection by Western blot was not able to detect the protein. Alternately, YapE expression in a potential *yapE* regulatory mutant may have been toxic. To try to avoid this, I used a Δ*yapE* strain.

When identifying gene regulators, there is the possibility that regulation may be multifactorial. The screens limitation is that it did not taking into consideration the impact of temperature, pH, oxygen or other environmental cues may have on *yapE* regulation. In the future, screens for *yapE* regulators should be performed in the presence of factors that mimic the host would be advantageous. This would include growing the cultures containing the transposon insertion at both 26° and 37° and in the presence of 5% CO_2. Because *yapE* is upregulated in the host, another possibility is to use to perform the screen in macrophages. After infection, macrophages could be lysed and bacterial RNA purified and perform qRT-PCR performed on *yapE* to determine if insertion influences transcriptional change in *yapE*.

An alternative possibility is that the regulator may be a sRNA. Small RNAs, are small (50-250 nucleotide) non-coding RNA molecules produced by bacteria [93]. These small molecules have been shown to have regulatory function that include, gene expression that affects protein synthesis at the post-transcriptional level [94]. We may have missed sRNAs in our transposon mutagenesis screen due to their small nature.

Although we were unable to identify a regulator of *yapE*, we believe that this should be followed up using alternative methods and to possibly investigate sRNAs as possible regulators that would not be identified by this method.

Table 2-1 Transposon mutagenesis screen.

ID Number	Transposon Type	IPTG-	IPTG+	Sequencing data
YR01A)1212A	pAJD428		Blue	hit reporter (cc) (IR located)
YR02A)1212C	pAJD428		Blue	hit reporter
YR03A	pAJD428		Blue	hit reporter (cc) (IR located)
YR04A	pAJD428		Blue	hit reporter (cc) (IR not located)
YR05A	pAJD428		Blue	hit reporter (cc) (IR not located)
YR06A	pAJD428		Blue	hit reporter (cc) (IR not located)
YR07A	pAJD428		Blue	putatitive galactosidase/maltodextrin permease protein (IR not located)
YR08A	pAJD428		Blue	putative sugar binding protein/sugar transport ATP binding protein(cc)(IR located)
YR09A	pAJD428		Blue	hit reporter (cc) (IR not located)
YR10A	pAJD428		Blue	hit reporter (cc) (IR located)
YR11A	pAJD428		Blue	putative maltodextrin transport permease (cc)(IR located)
YR12A	pAJD428	Blue	Blue	hit reporter (cc) (IR located)
YR13A	pAJD428	Blue	Blue	no sequence obained
YR14A	pAJD428	Blue	Blue	hit reporter (cc) (IR not located)
YR15A	pAJD428	White	Blue	hit reporter (cc) (IR located)
YR16A	pAJD428	Blue	Blue	putative betagalactosidase/putative galactosidase (IR)
YR17A	pAJD428	Blue	Blue	hit reporter
YR18A	pAJD428	Blue	Blue	putative galactosidase/maltodextrin permease (IR)
YR19A	pAJD428	Blue	Blue	hit reporter (IR)
YR20A	pAJD428	Blue	Blue	putatitive galactosidase
YR21A	pAJD428	Blue	Blue	lactose operon repressor
YR22A	pAJD428	Blue	Blue	hit reporter
YR23A	pAJD428	White	Blue	hit reporter
YR24A	pAJD428	Blue	Blue	hit reporter
YR25A	pAJD428	White	Blue	putatitive galactosidase
YR26A	pAJD428	Blue	Blue	hit reporter
YR27A	pAJD428	Blue	Blue	hit transposon?
YR28A	pAJD428	White	Blue	hit reporter
YR29A	pAJD428	White	Blue	hit reporter
YR30A	pAJD428	Blue	Blue	hit transposon?
YR31A	pAJD428	White	Blue	hit reporter
YR32A	pAJD428		Blue	no sequence obained
YR33A	pAJD428	White	Blue	hit reporter
YR34A	pAJD428		Blue	no sequence obained
YR35A	pAJD428		Blue	no sequence obained
YR36A	pAJD428		Blue	hit reporter
YR37A	pAJD428	White	Blue	hit reporter
YR38A	pAJD428		Blue	no sequence obained
YR39A	pAJD428		Blue	no sequence obained
YR40A	pAJD428	Blue	Blue	hit reporter
YR41A	pAJD428	White	Blue	putatitive galactosidase/maltodextrin permease protein
YR42A	pAJD428	White	Blue	hit reporter (DC)
YR43A	pAJD428	Blue	Blue	putatitive galactosidase/maltodextrin permease protein
YR44A	pAJD428	Blue	Blue	hit reporter (DC)
YR45A	pAJD428	Blue	Blue	hit reporter (DC)
YR46A	pAJD428	White	Blue	hit reporter (DC)
YR47A	pAJD428	White	Blue	hit reporter (DC)
YR48A	pAJD428	White	Blue	putative autotransporter (hit reporter)
YR49A	pAJD428	White	Blue	hit reporter (DC)
YR50A	pAJD428	White	Blue	359 bp at 5' side YPO3984 and 186 bp at 3' side YPO3985
YR51A	pAJD428	White	Blue	hit transposon?

YR52A	pAJD428	White	Blue	359 bp at 5' side YPO3984 and 69 bp at 3' side YPO3985
YR53A	pAJD428	Blue	Blue	no sequence obained
YR54A	pAJD428	Blue	Blue	hit reporter (pFU99)
YR55A	pAJD428	Blue	Blue	putatitive galactosidase/maltodextrin permease protein
YR56A	pAJD428	White	Blue	putatitive galactosidase/maltodextrin permease protein
YR57A	pAJD428	Blue	Blue	E. coli DNA polymerase II (?)
YR58A	pAJD428	Blue	Blue	hit reporter
YR59A	pAJD428	Blue	Blue	hit reporter
YR60A	pAJD428	Blue	Blue	hit reporter (cc) (IR located)
YR61A	pAJD428	Blue	Blue	hit reporter (cc) (IR located)
YR62A	pAJD428	White	Blue	putative sugar binding protein (IR located)
YR63A	pAJD428	White	Blue	hit transposon?
YR64A	pAJD428	White	Blue	putative galactosidase/maltodextrin permease
YR65A	pAJD428	Blue	Blue	putative galactosidase/maltodextrin permease (IR located)
YR66A	pAJD428	White	Blue	hit reporter (cc) (IR located)
YR67A	pAJD428	Blue	Blue	putative galactosidase
YR68A	pAJD428	Blue	Blue	putative galactosidase/maltodextrin permease
YR69A	pAJD428	White	Blue	putative galactosidase (IR located)
YR70A	pAJD428	White	Blue	hit reporter (cc) (IR not located)
YR71A	pAJD428	Blue	Blue	hit reporter (cc) (IR located)
YR72A	pAJD428	White	Blue	hit reporter (cc) (IR located)
YR73A	pAJD428	Blue	Blue	hit reporter
YR74A	pAJD428	Blue	Blue	hit reporter
YR75A	pAJD428	White	Blue	putative sugar binding protein
YR76A	pAJD428	Blue	Blue	phage hypothetical protein/phage-related membrane protein
YR77A	pAJD428	White	Blue	hit reporter (cc) (IR located)
YR78A	pAJD428	Blue	Blue	hit transposon?
YR79A	pAJD428	Blue	Blue	hit transposon?
YR80A	pAJD428	White	Blue	hit reporter (cc) (IR located)
YR81A	pAJD428	White	Blue	putative galactosidase/maltodextrin permease (IR located)
YR082A	pAJD428	White	Blue	putative galactosidase/maltodextrin permease
YR083A	pAJD428	White	Blue	hit reporter (cc) (IR located)
YR084A	pAJD428	White	Blue	putative betagalactosidase/putative galactosidase
YR085A	pAJD428	Blue	Blue	hit reporter
YR086A	pAJD428	White	Blue	hit reporter (cc) (IR located)
YR087A	pAJD428	White	Blue	hit reporter
YR088A	pAJD428	Blue	Blue	hit reporter
YR089A	pAJD428	Blue	Blue	hit reporter
YR090A	pAJD428	Blue	Blue	hit transposon?
YR091A	pAJD428	Blue	Blue	hit transposon?
YR092A	pAJD428	Blue	Blue	hit reporter
YR093A	pAJD428	White	Blue	hit transposon?
YR094A	pAJD428	Blue	Blue	no sequence obained
YR095A	pAJD428	Blue	Blue	no sequence obained
YR096A	pAJD428	White	Blue	hit reporter
YR097A	pAJD428	Blue	Blue	hit reporter
YR098A	pAJD428	Blue	Blue	no sequence obained
YR099A	pAJD428	Blue	Blue	no sequence obained
YR100A	pAJD428	White	Blue	hit reporter

Table 2-2 Growth and stress conditions of *Yersinia* autotransporter proteins

Bacteria	Type of stress	Results
Y.pestis	Temperature (not extreme temp)	• ≥2-fold @37°-*yapF, yapJ, yapK, yapL and yapM* • 15-fold increase in *yapE* (in lymph nodes)
	Calcium	• Only 2 genes responded to CA levels, *yapK* and *yapM*. • *yapM* was induced @26° and • *yapK* was repressed in the presence of calcium at 37° • other yap genes showed no change in expression levels
	Iron	*yapM* was the only gene found to be responsive to low-iron conditions where it was repressed at both 26° and 37°
	pH	• Under acidic conditions (pH 6.0) no *yap* genes were induced • @ pH of 6.0 *yapG* was repressed >2-fold • @pH of 8.0 *yapG* was repressed 2.5 fold @ 26° • *yapH* was induced 3.5-fold at pH 8.0 and 26°

Table adapted from Lenz, JD et al., 2011 [74]

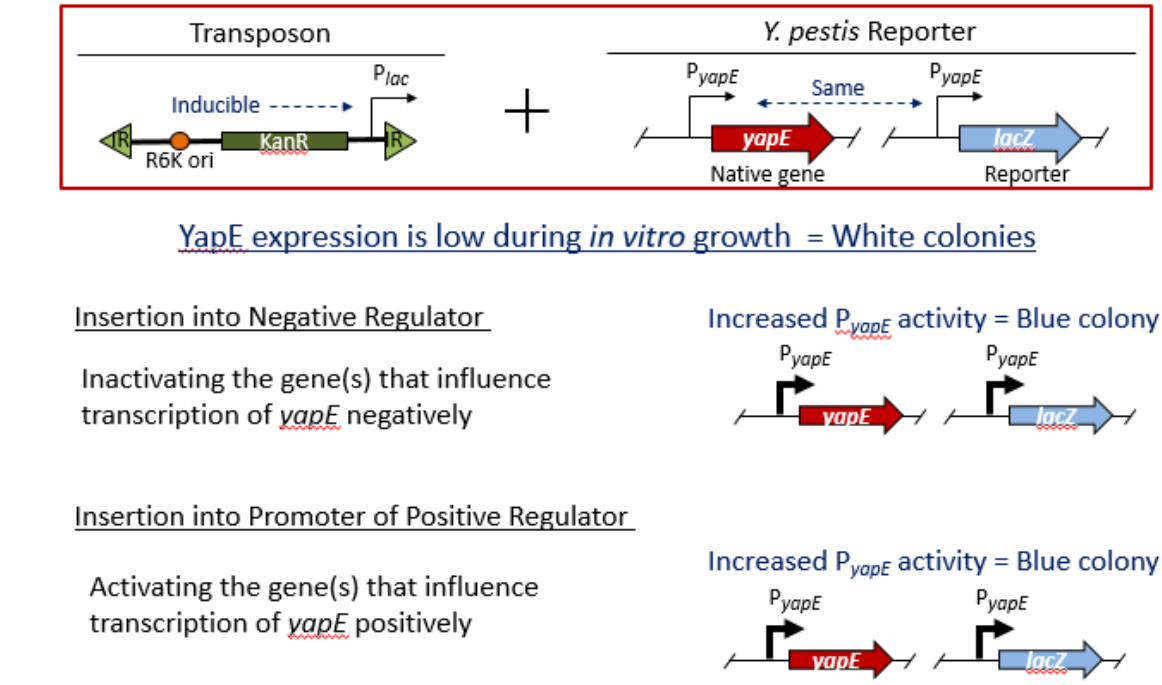


Figure 2-1. Schematic of Transposon Mutagenesis Screen

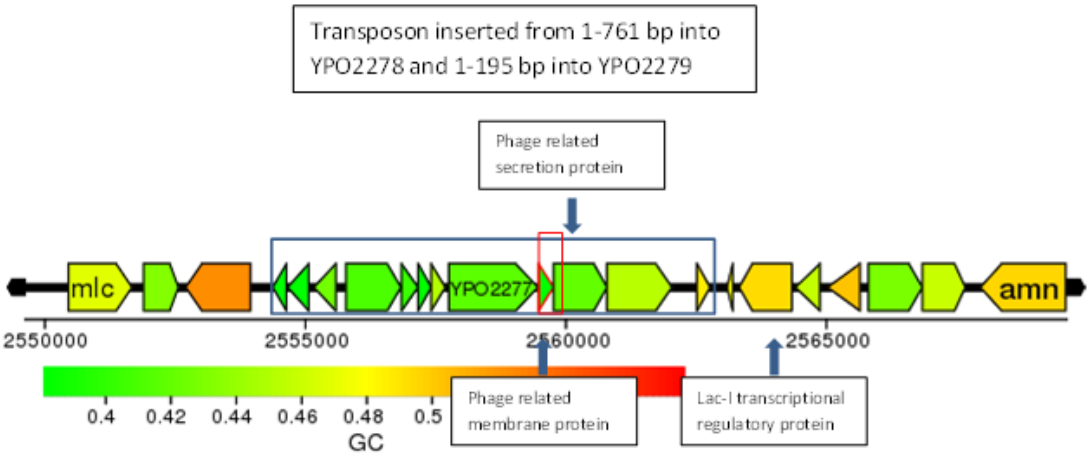


Figure 2-2. Transposon insertion site into Phage related membrane protein

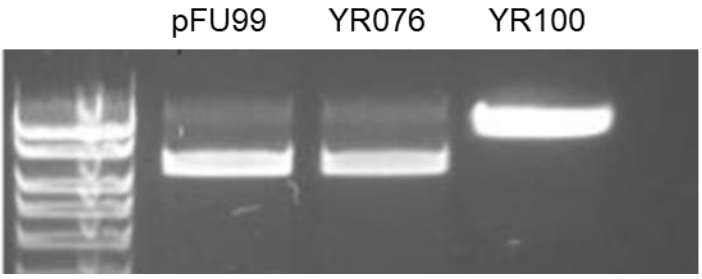


Figure 2-3. There is no secondary insertion in clone YR076. Plasmid DNA from the reporter (pFU99), YR076, and a clone containing an inserted transposon in the reporter (YR100) was linearized using BamHI and ran on a 0.8% agarose gel. The reporter and clone YR076 both migrated to approximately 6.8 kb showing that there is no other insertion found in YR076.

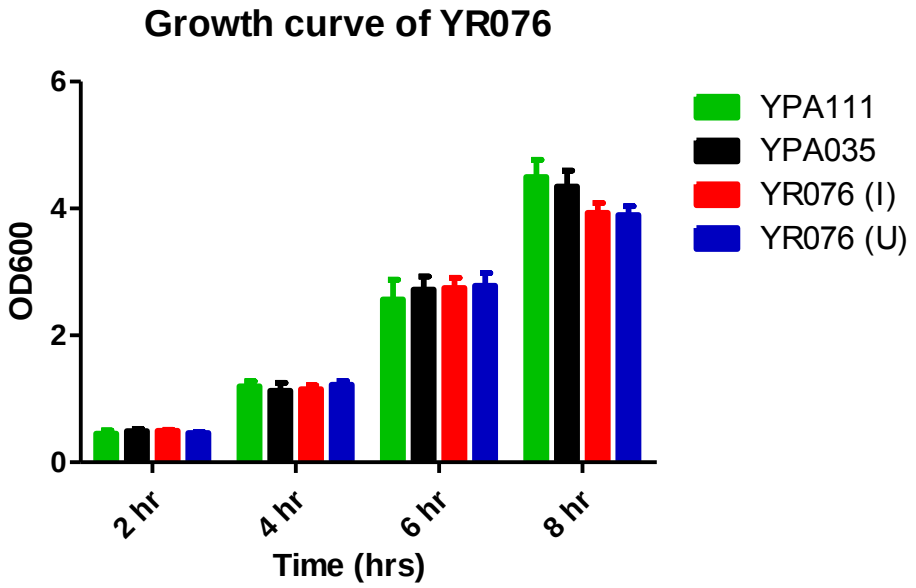


Figure 2-4. Growth curve of YR076 clone. Growth curve showing OD_{600} readings over 8 hours of growth when clone YR076 is induced with IPTG and not induced compared with the vector strain YPA111 (pFU99::yapE) and the wild-type strain YPA035 (CO92 pCD-). 2-way ANOVA showed no significance at the 8 hour time point.

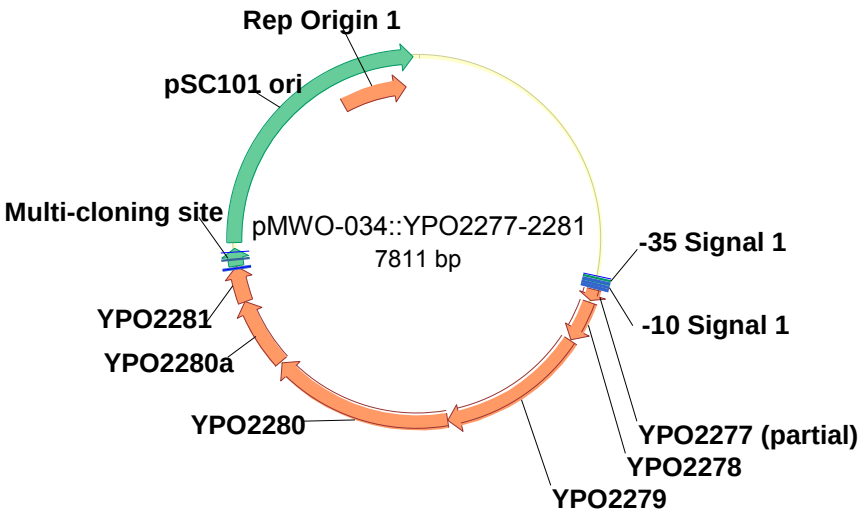


Figure 2-5. Inducible plasmid for the phage-like membrane protein

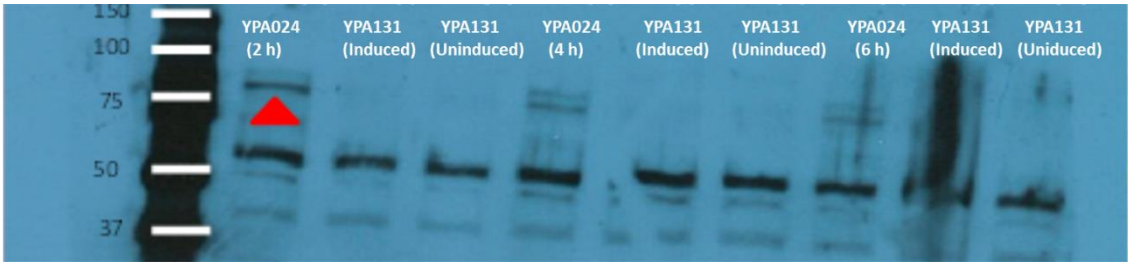


Figure 2-6. Western blot analysis of inducible phage plasmid. YPA001 w/ $YapE_{Ypestis}$ tetO expresion plasmid (YPA024) and the phage-inducible plasmid (YPA131) induced and uninduced were grown at 26°. Whole cell lysate was acquired at T=2h, 4h, and 6h. The red arrow shows YapE.

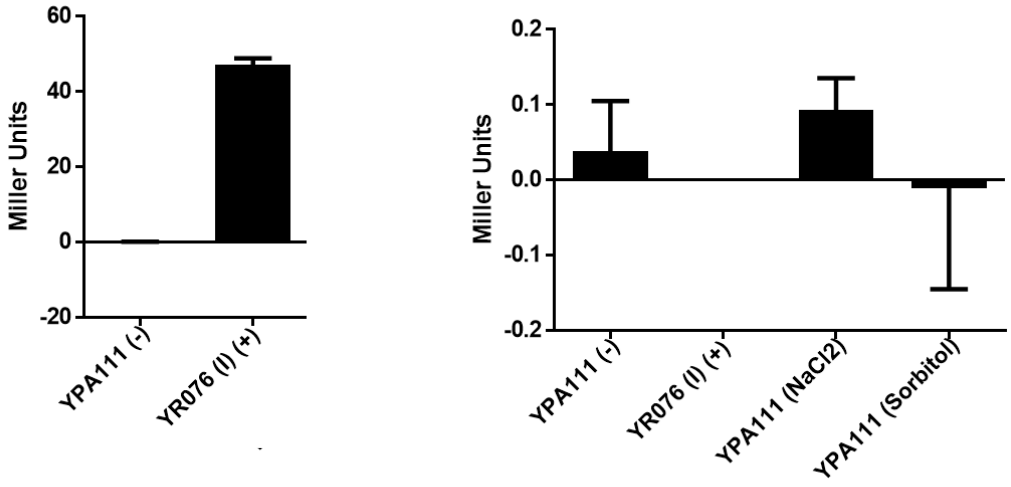


Figure 2-7. The native phage, YpfΦ, was not induced by salinity or osmolarity. The reporter strain (YPA111) was grown at 26° in the presence of 5M $NaCl_2$ or 5M sorbitol for 20 minutes and yapE::lac expression was measured by Miller Assay. The clone containing the transposon insertion into the phage, YR076, was induced with IPTG and used as a positive control. YPA111, grown in media with no added stressors for a negative control.

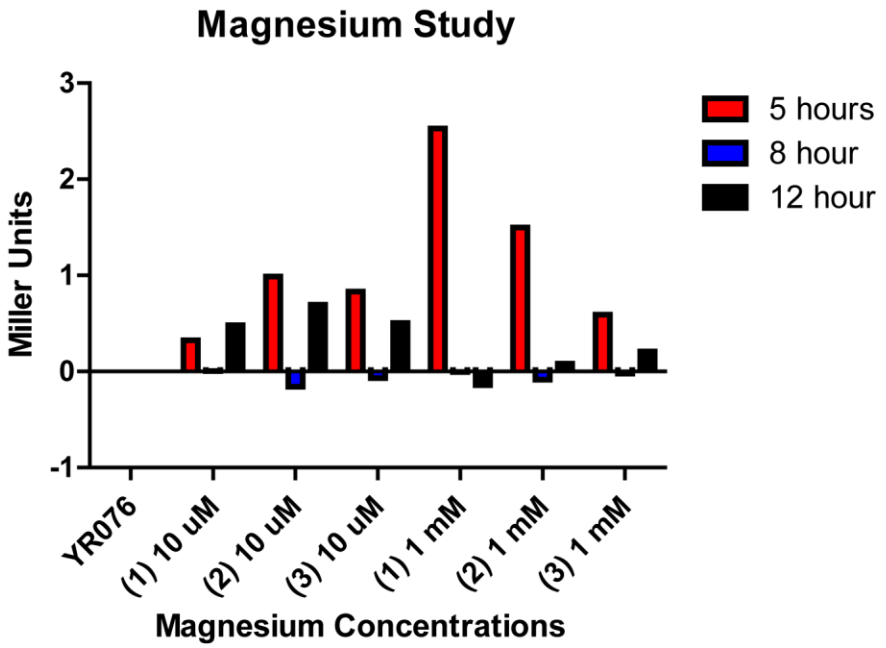


Figure 2-8. Low Magnesium is not an environmental cue for YapE.

The reporter strain (YPA111) was grown at 26° in the presence of 10 μM $MgCl_2$ or 1mM $MgCl_2$ and a Miller Assay was performed to measure *yapE* promoter activity at 5, 8, and 12 h. The clone containing the transposon insertion into the phage, YR076, was induced and used as a positive control. The reporter strain, YPA111, was just grown in media with no added stressors for the negative control. Due to the extreme elevation of Miller units for the YR076 (I), this data was not shown. Data shows three biological replicates for each concentration.

CHAPTER THREE

SURFACE PROTEINS CLEAVED BY THE PLA PROTEASE

Introduction:

Pla a 9.5 kd protein located on the pPCP plasmid (also known as pPst) of *Yersinia pestis*, and has been shown to be responsible for the invasive property of plague in the mammalian host [95]. Pla, a member of the omptin family of enterobacterial surface protein, is a multifunctional protein that can activate the mammalian plasma proenzyme plasminogen into plasmin [49]. In addition to being able to activate plasminogen into plasmin, Pla also cleaves the complement C3 component [52], modifies bacterium-produced Yops (*Yersinia* outer proteins) [96, 97], has the ability to activate adhesins (YapE) and proteolytically inactivates α_2-antiplasmin [98, 99].

YapE facilitates contact between bacteria and eukaryotic cells through its adhesive properties [76]. Lawrenz and co-workers showed that YapE adhesive properties is predicated upon its cleavage by Pla. This was the first indication that Pla may cleave other bacterial outer membrane proteins.

Therefore I sought to determine if other *Y. pestis* outer membrane proteins are cleaved by Pla.

Material and Methods:

Membrane Fractionation

Y. pestis CO92 pCD1$^{(-)}$ (with and without pla) were grown, overnight, at 26°C in Brain Heart Infusion (BHI) broth (Difco). The overnight culture was then sub-diluted into BBL BHI, where $CaCl_2$ was added to the media at a final concentration of 2.5 mM and allowed to agitate on a floor shaker for approximately 3 hours at

26°C and 37°C. The bacteria was grown to an optical density of ~2 OD_{600}/ml and approximately 300 ODs were harvested. The harvested bacteria was then centrifuged for 10 minutes at 4,000 xg at 4°C. The supernatant was removed and the bacteria were washed 3X with 25 mls of 10 mM HEPES, pH 7.5, 1x protease inhibitor. Bacteria was re-suspended into 10 mM HEPES, pH 7.5, 1x protease inhibitor and transfer to 50 ml conical for sonication. The bacteria was then sonicated in 3 cycles at 30% amplitude for one minute on ice. The sonicated fractions were then centrifuged for 2 minutes at 1,000 x g at 4°C. The supernatant was transferred into ultracentrifuge tubes for ultracentrifugation at 100,000 xg for 30 minutes at 4°C. Supernatant was removed and the pellet was re-suspended in 0.3% Sarkosyl, 10 mM HEPES, pH 7.5, 1x Protease inhibitor and incubated at room temperature for 30 minutes. The outer membranes were pelleted by ultra-centrifugation at 100,000 x g for 30 min. at 20°C. The inner membrane proteins in the supernatant were removed and the pellet was dissolved in molecular grade water.

Chloroform-methanol extraction

To the outer membrane fraction methanol was added and vortexed thoroughly. Chloroform and water were added, vortexed, then centrifuged for 1 min at 14,000 x g. The top aqueous layer was removed and placed in a separate Eppendorf tube where methanol was added and vortexed. The fraction was then centrifuged for 5 min at 20,000 x g. The methanol was removed leaving a pellet that contains the outer membrane proteins. The pellet was then lyophilized by speed vac. The pellet was then sent to ABI for 2-D gel analysis.

2-D gel analysis

2-D DIGE gel analysis was performed on the lyophilized protein sample (~500 ug) from the outer membrane prep stated previously. The sample was sent to AppliedBiomics where they performed 2-D DIGE analysis on the 4 samples consisting of WT CO92 *Y. pestis* at 26° and 37° and CO92 Δpla at 26° and 37°. 2-D DIGE analysis consist of cross-gel analysis containing an internal control, comprised of an equal mix of all 4 samples, loaded on both of the analytical gels. The first analytical gel compared the protein profile of WT CO92 *Y. pestis* at 26° and CO92 *Δpla* at 26°and the second gel consisting of WT CO92 *Y. pestis* at 37° and CO92 *Δpla* at 37°. By having an internal standard being loaded on both gels allowed for the comparison of spots across the two gels.

The protein expression profiles acquired by AppliedBiomics consisted of: i) the raw data of both gels (WT26°/*Δpla*26° and WT37°/ *Δpla*37°),ii) a protein ID number, iii) master number, iv) appearance, and v) ratio treatment/control.

Gel image report. The overlay of both gel images were compared where the red and green spots represent up- and down- regulated proteins, respectively. DeCyder analysis software was employed to analyze the quantitative changes in protein between the samples. The software was able to calculate the log value of the standardized abundance of the selected spot for each sample. The samples were then grouped into control and treatment and the average was taken of each group. It also allowed for the evaluation of the fold change (increase of decrease) of each spot was indicated.

Results:

2DIGE and MS/MS determination of outer membrane proteins processed by Pla

To ascertain outer membrane proteins whose expression changes when Pla is absent or present, I employed the methods of 2-DIGE and mass spectrometry. WT and Δ*pla* strains were grown at 26° and 37°, outmembrane proteins isolated and labeled with a fluorescent dye. Proteins were separated by 2-DIGE and compared (WT/Δ*pla* at 26° and 37°) Differences between the proteins were analyzed. Applied Biosystems (ABI) was able to identify 76 distinct protein IDs that were assigned by their DeCyder software. Of the 76 proteins identified, we chose 10 to further investigate by way of MS/MS. Our criteria for choosing the protein was that there was a ≥ 2-fold increase or decrease when grown at both 26° and 37° between WT and Δ*pla*. To this end, we chose the proteins identified in Fig.1. Of these spots, serine endoprotease (spot 16), SodC (spot 61), Glutamine sythetase (spot 63) and Inosine-5'-monophosphate (spot 72) are not outer membrane proteins and removed from further consideration. The remaining six peptides were two forms of Pla (spot 37 & 48), HmsF (spot 5), OmpA (spot 40), OmpX/Ail (spot 51) and Omp85/YaeT (spot 9).

Western blot analysis for Ail and HmsF:

To confirm the results from 2DIGE, I chose two proteins, Ail and HmsF. Total outer membrane proteins were isolated from WT or a Δ*pla* strain of *Y. pestis* grown at 26°C to 37°C and separated by SDS-PAGE. Proteins were probed with α-Ail or α-HmsF antibodies. When probing with α-Ail, there was no change in Ail expression from 26°C and 37°C or between WT and Δ*pla* (Fig. 3-2). When

evaluating the difference in protein expression of HmsF, at 26°C there was no difference in protein amounts between the HmsF over expressing strain, the *pla* mutant and WT (Fig. 3-3). Conversely, a substantial reduction of HmsF was observed at 37°C in WT *Y. pestis* versus the ∆*pla* mutant, indicating that Pla degrades HmsF.

Discussion:

Using a *pla* mutant we were not surprised that the first and highest on the list was Pla itself. Pla is able to autoprocess itself into three forms, alpha, beta, and gamma. These forms of Pla consist of α-Pla (43%), β-Pla (30%) and γ-Pla (16%) [99]. α-Pla corresponds to the full-size mature Pla where β and γ-Pla are the subsequent processing of α-Pla [100] [97, 101]. The autoprocessing of Pla was further shown by Kukkonen and co-workers using mutations in Pla that compromised its proteolytic activity, resulting in loss of the beta form of Pla. During our screen we found it interesting that HmsF was found to be significantly increased when Pla was removed from *Y. pestis*. HmsF [102] is one of two outer membrane proteins that are in the *hmsHFRS* operon. The *hmsF* operon is located in the pgm locus and is required for hemin storage. This operon has been showed to play an important role in its ability to block the proventricular valve in the flea allowing for the transmission of the bacteria from the flea to mammalian hosts [102]. Although Perry and co-workers showed that the expression of the *hmsHFRS* operon was not predicated by temperature we found that there was a difference in protein when Pla was absent at 37° [102]. The reason for the possible reduction of HmsF at 37°C is that Pla activity is optimal at 37°C where

Pla activity is reduced at 26°C. Since the genes expressed in *hmsHFRS* operon play a role in biofilm formation, this phenotype would not be advantageous to the bacteria when moving into a mammalian host. This leads us to conclude that the reason that posttranslational modification to HmsF allows for the inhibition of the biofilm that is needed for transmission from the flea but would hinder its mobility inside of a mammalian host.

OmpA has been a highly characterized outer membrane protein that has been shown to facilitate adhesion to and/or invasion of eukaryotic cells, in addition to conferring serum resistance [103]. OmpA has also been shown to mediate adhesion and/or invasion of eukaryotic cells and serum resistance [104].

The attachment invasion locus (Ail) is an outer membrane protein that is expressed in all three pathogenic species of *Yersinia* and is thermally regulated and is expressed fully at 37°C [105, 106]. Although Ail is expressed in all three species to aid in the adherence of host cells, its role in the virulence of *Y. pestis* is more pronounced. This is due to *Y. pestis* no longer having functional YadA and invasin due to a frame shift mutation or transposon insertion [107]. One of Ail's primary role is to facilitate the attachment to host cells for the delivery of Yop effector proteins from the T3SS. In addition to attachment, Ail is also important in the evading of host immune responses allowing for rapid progression of infection [108].

It is also important to note that the regulation of these genes are varied with respect to *pla* expression (Table 3-3). To this end, we have a better understanding of the dual nature of Pla and its interplay among various outer

membrane proteins and possible explanations on its attenuation when mutated outside of a reduction in dissemination, but also the attenuation it has intracellularly. Although we only evaluated the top 10 proteins, other proteins identified in the 2-D gel analysis may give us further insight into novel proteins that are either activated or degraded by Pla, and whether Pla process contributes to virulence.

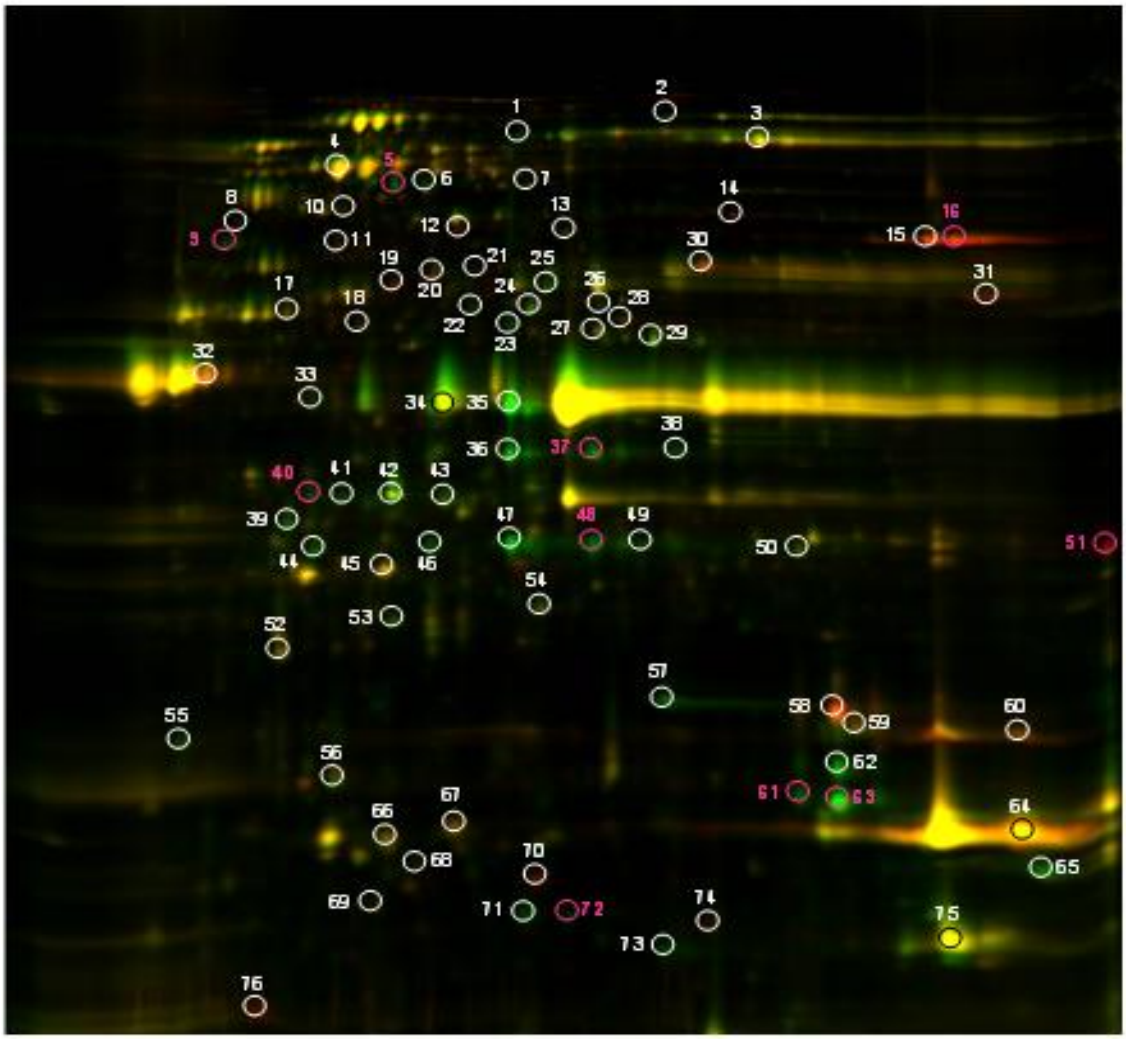


Figure 3-1. 2-DIGE outer membrane protein spot identification. Outer membranes from WT (green) and ∆pla (red) grown at 37°. The first analytical gel compared the protein profile of WT CO92 *Y. pestis* at 26° and CO92 Δpla at 26°and the second gel consisting of WT CO92 *Y. pestis* at 37° and CO92 Δpla at 37°. The overlay of both gel images were compared where the red and green spots represent up- and down- regulated proteins, respectively. The white circles indicate proteins where the ratios were ≥2-fold difference between temperature and strain. The pink circles indicated the proteins chosen for MS/MS analysis.

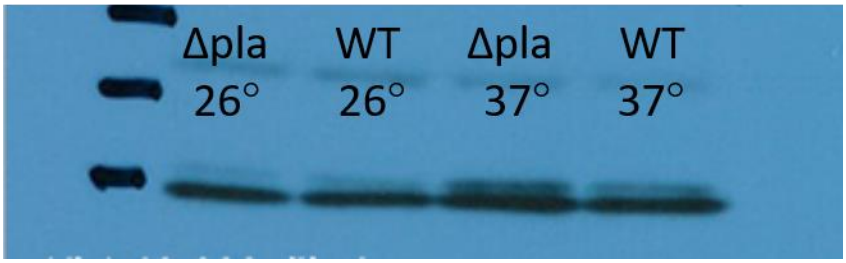


Figure 3-2. Western blot analysis of Ail at 26°C and 37°C in the presence or absence of pla . WT and Δ*pla* strains were grown to mid-exponential phase at 26°C and 37°C and collected by centrifugation. Outer membrane proteins were extracted, and 1.5 OD_{600} of each sample was analyzed by Western blot and probed with 1:40,000 α-Ail.

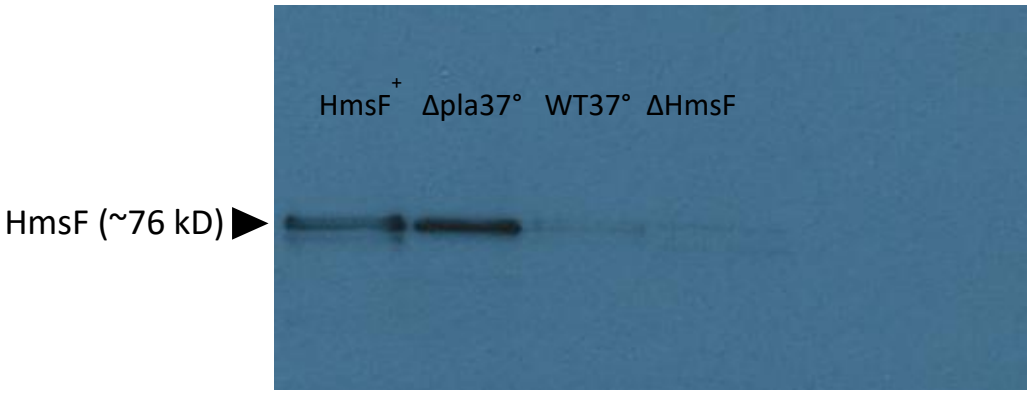


Figure 3-3. Western blot analysis of HmsF in the presence and absence of pla at 37°C. WT and Δ*pla* strains were grown to mid-exponential phase at 26°C and 37°C and collected by centrifugation. Outer membrane proteins were extracted, and 1.5 OD_{600} of each sample was analyzed by Western blot and probed α-HmsF (1:40,000). $HmsF^{+}$(over expression strain: positive control), ΔHmsF (negative control).

Table 3-1. 2-DIGE Analysis

Protein ID	Master No.	Appearance	Δpla26/ WT26	Δpla37/WT37	WT37/WT26	Δpla37/ Δpla26	Δpla37/ WT26	Δpla26/ WT37
1	429	6 (6)	-15.75	-3.77	-1.17	3.58	-4.41	-13.49
2	371	6 (6)	-3.12	-1.83	-1.82	-1.06	-3.32	-1.72
3	447	6 (6)	1.20	1.06	7.76	6.90	8.25	-6.48
4	537	6 (6)	-1.94	1.72	1.41	4.69	2.42	-2.73
5	590	6 (6)	-5.99	-10.84	1.42	-1.27	-7.62	-8.52
6	592	6 (6)	-6.67	-3.22	1.39	2.88	-2.31	-9.28
7	610	6 (6)	1.83	1.05	-2.75	-4.80	-2.62	5.03
8	731	6 (6)	1.23	-1.04	-5.14	-6.56	-5.35	6.31
9	797	6 (6)	-15.01	-9.15	-3.17	-1.94	-29.04	-4.73
10	677	6 (6)	2.19	1.34	1.28	-1.28	1.72	1.72
11	793	6 (6)	1.86	2.06	-2.14	-1.93	-1.04	3.97
12	759	6 (6)	2.39	6.46	-1.42	1.90	4.54	3.41
13	765	6 (6)	1.39	1.23	-4.15	-4.68	-3.37	5.77
14	700	6 (6)	1.23	4.41	-6.56	-1.82	-1.49	8.03
15	799	6 (6)	5.72	7.51	4.80	6.29	36.03	1.19
16	803	6 (6)	7.22	8.53	5.25	6.21	44.81	1.38
17	961	6 (6)	2.50	1.14	-1.35	-2.95	-1.18	3.37
18	998	6 (6)	1.38	1.30	-3.97	-4.22	-3.06	5.49
19	878	6 (6)	2.32	4.99	-2.44	-1.14	2.04	5.66
20	854	6 (6)	1.25	1.93	-3.95	-2.54	-2.04	4.92
21	855	6 (6)	1.51	1.48	-2.41	-2.46	-1.63	3.64
22	952	6 (6)	-1.11	-3.17	5.15	1.80	1.62	-5.70
23	1011	6 (6)	-2.42	-5.12	3.44	1.63	-1.49	-8.32
24	963	6 (6)	-1.38	-2.64	10.37	5.42	3.93	-14.31
25	904	6 (6)	-5.80	-3.38	1.23	2.12	-2.74	-7.16
26	957	6 (6)	1.39	-1.79	6.44	2.60	3.61	-4.64
27	1027	6 (6)	1.24	-1.02	3.61	2.85	3.53	-2.92
28	997	6 (6)	1.63	1.96	3.65	4.37	7.15	-2.23
29	1053	6 (6)	-1.07	-2.29	2.49	1.16	1.09	-2.65
30	844	6 (6)	1.49	2.86	1.62	3.10	4.63	-1.08
31	950	6 (6)	1.78	3.34	2.44	4.57	8.15	-1.37
32	1127	6 (6)	2.99	2.12	3.88	2.75	8.23	-1.30
33	1164	6 (6)	-2.92	-1.29	-2.27	-1.00	-2.92	-1.29
34	1151	6 (6)	-2.68	-1.90	-2.32	-1.64	-4.41	-1.16
35	1190	6 (6)	-7.38	-25.07	2.14	-1.59	-11.72	-15.78
36	1312	6 (6)	-37.36	-36.65	1.92	1.96	-19.09	-71.71
37	1307	6 (6)	-10.87	-27.63	2.83	1.11	-9.78	-30.71
38	1305	6 (6)	-2.15	-6.74	2.84	-1.11	-2.37	-6.09
39	1486	6 (6)	-2.44	-7.89	3.54	1.09	-2.23	-8.63
40	1404	6 (6)	-14.11	-6.14	-2.62	-1.14	-16.10	-5.38
41	1413	6 (6)	-2.64	-4.97	3.20	1.70	-1.55	-8.44
42	1400	6 (6)	-5.24	-2.35	-2.58	-1.15	-6.05	-2.04
43	1419	6 (6)	-3.20	-2.10	-2.24	-1.47	-4.72	-1.43
44	1551	6 (6)	-2.49	-8.11	3.33	1.02	-2.43	-8.31
45	1604	6 (6)	1.28	1.56	2.03	2.47	3.16	-1.59
46	1534	6 (6)	-6.88	-5.85	1.87	2.19	-3.13	-12.84
47	1541	6 (6)	-99.61	-68.33	2.37	3.45	-28.88	-235.64
48	1546	6 (6)	-11.55	-23.18	2.39	1.19	-9.69	-27.63
49	1552	6 (6)	1.09	-3.00	-1.08	-3.52	-3.22	1.17
50	1560	6 (6)	-1.19	-2.34	7.08	3.61	3.03	-8.45
51	1554	6 (6)	3.38	12.15	1.65	5.94	20.07	2.04
52	1841	6 (6)	1.15	1.23	6.78	7.22	8.32	-5.87
53	1755	6 (6)	-3.87	-2.42	-2.36	-1.48	-5.71	-1.64
54	1723	6 (6)	-1.07	-1.37	-3.09	-3.94	-4.23	2.88
55	2074	6 (6)	1.17	-5.25	6.99	1.14	1.33	-5.99
56	2115	6 (6)	1.28	-1.30	-2.99	-4.96	-3.88	3.82
57	1981	6 (6)	-1.51	-5.90	9.35	2.40	1.58	-14.15
58	2001	6 (6)	1.57	4.43	-1.90	1.48	2.33	2.99
59	2047	6 (6)	-1.45	1.42	2.92	5.99	4.14	-4.22
60	2064	6 (6)	1.37	2.14	1.28	2.00	2.74	1.07
61	2162	6 (6)	-2.21	-9.91	8.59	1.92	-1.15	-19.03
62	2107	6 (6)	-2.54	-38.63	4.45	-3.42	-8.68	-11.29
63	2171	6 (6)	-3.96	-31.02	11.41	1.46	-2.72	-45.22
64	2237	6 (6)	1.37	2.64	1.19	2.28	3.13	1.16

Table 3-2. MS/MS Analysis of peptides chosen from the 2-DIGE Analysis.

Spot number	MALDI well number	Top Ranked Protein Name [Species]	Accession No.	Protein MW	Protein PI	Pep Count	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%	Comments or Other Possibilities
5	E1	HmsF [Yersinia pestis]	gi|4321107	76,637	5.5	22	606	100	463	100	see hit #2
9	E2	outer membrane protein assembly factor YaeT [Yersinia pestis KIM10+]	gi|22127003	87,784	5.4	22	1170	100	1036	100	degradation product?
16	E3	serine endoprotease [Yersinia pseudotuberculosis IP 31758]	gi|153950722	49,835	9.0	19	1110	100	970	100	different strain
37	E4	protease 7 (Protease VII) (Omptin) (Outermembrane protein 3B) (Protease A) [Yersinia pestis biovar	gi|165940405	31,546	5.9	15	754	100	631	100	see hit #3
40	E5	outer membrane protein OmpA [Yersinia pestis KIM10+]	gi|22126613	39,249	8.6	15	944	100	793	100	degradation product?
48	E6	outer membrane protease [Yersinia pestis biovar Microtus str. 91001]	gi|45478719	34,602	5.9	16	763	100	632	100	see hit #2
51	E7	attachment invasion locus protein [Yersinia pestis KIM10+]	gi|22125223	21,555	8.5	10	154	100	69	100	post-translational modification?
61	E8	copper/zinc superoxide dismutase [Yersinia pseudotuberculosis IP 31758]	gi|153948541	20,044	6.3	3	129	100	100	100	different strain
72	E10	inosine 5'-monophosphate dehydrogenase [Yersinia pestis Antiqua]	gi|108808306	51,792	6.3	8	206	100	176	100	degradation product?

Table 3-3. *Yersinia pestis* outer membrane regulators.

Gene	Regulator	Upregulated at 26°	Upregulated at 37°
pla	cyclic AMP receptor protein (Crp)		√
hmsF	*fur*	√	
ompA	*micA*		√
ail		√	√

CHAPTER FOUR

IMPACT OF GENTAMICIN CONCENTRATION AND EXPOSURE TIME ON INTRACELLULAR *YERSINIA PESTIS*[1]

[1] VanCleave TT, Pulsifer AR, Connor MG, Lawrenz MB. Impact of Gentamicin Concentration and Exposure time on Intracellular *Yersinia pestis*. Front Cell Infect Microbiol. 2017 Dec 11;7:505. doi:10.3389/fcimb.2017.00505. PubMed PMID: 29312891.

Introduction

Many bacterial pathogens have evolved mechanisms to infect and survive within host cells. Therefore, in order to study and understand the pathogenesis of intracellular pathogens, cell culture-based *in vitro* assays have been developed. For obligate intracellular pathogens, like *Chlamydia* species, which cannot grow outside of a host cell, these assays are relatively straight forward. Bacteria are added directly to cells, allowed to adhere and invade for a certain period of time, and the remaining extracellular bacteria are removed by washing the cells [109, 110]. However, for facultative intracellular bacteria that can also replicate in tissue culture medium, additional steps are needed to inhibit the growth of extracellular bacteria not removed by washing. The most common approach to limit extracellular growth is to include an antibiotic to the medium after bacteria have had time to invade cells. Importantly, the antibiotic chosen should have a limited ability to cross the plasma membrane of the cell. Thus, intracellular bacteria are protected from the antibiotic while extracellular bacteria are inhibited by the antibiotic (commonly referred to as an antibiotic protection assay) [111, 112]. Application of the antibiotic protection assay has allowed the study of intracellular virulence mechanisms across multiple facultative intracellular bacteria [113].

While the antibiotic protection assay is commonly used to study intracellular pathogens, strong evidence indicates that antibiotics originally thought to be completely excluded by the host cell can slowly enter and accumulate within cells. For example, aminoglycosides such as streptomycin and gentamicin are

often employed in the antibiotic protection assay because they are not lipid soluble and original studies indicating poor cell permeability [114-116]. However, subsequent studies have demonstrated that despite poor membrane permeability, aminoglycosides can still accumulate within cells to levels that inhibit bacterial growth, especially for organisms that remain in vacuolar compartments. Such phenomenon has been reported for antibiotic protection assays involving *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and *E. coli* [117-120]. The increased sensitivity of organisms within phagosomal compartments to aminoglycosides, as opposed to those that escape into the cytoplasm, has been attributed to the believed primary route of antibiotic uptake – pinocytosis of the extracellular milieu [117]. Pinocytosed vesicles containing the antibiotic in turn readily fuse with phagosomes/endosomes, delivering the aminoglycoside to the bacterial containing compartment. However, it should be noted that aminoglycosides have also been shown to accumulate in the cytosol depending on the cell type used and length of incubation time with the antibiotic, and thus, addition of aminoglycosides may also artificially influence intracellular growth of cytoplasmic pathogens [117, 121]. Therefore, these data should be taken into consideration when designing antibiotic protection assays to study intracellular growth.

Yersinia pestis, the causative agent of plague, is a facultative intracellular pathogen that is able to invade and replicate in host cells [58, 78, 81, 82, 122-124]. Of particular interest has been *Y. pestis* intracellular survival in macrophages [81, 125]. However, *Y. pestis* has also been shown to survive in

epithelial cells [107] and more recently, within neutrophils [122, 126-128]. As *Y. pestis* does not express many extracellular virulence factors in the flea vector, it is believed that intracellular survival is important during the early stages of infection after transmission from the flea, and provides a protective niche for the bacterium to initiate expression of the factors needed to exist extracellularly [81, 82, 88, 124, 129]. Once *Y. pestis* invades a cell, it is able to generate a replicative niche within a phagosomal compartment (called the *Yersinia* containing vacuole or YCV) by subverting the normal maturation of the phagosome [83, 130-132]. The YCV appears to be maintained throughout the intracellular infection until the host cell eventually lyses, releasing the bacteria into the extracellular environment [131].

As *Y. pestis* is able to grow in most cell culture media, in order to study intracellular interactions with host cells *in vitro*, the antibiotic protection assay is needed to inhibit extracellular growth. In most cases gentamicin is the primary antibiotic employed for *Y. pestis* intracellular studies because the bacterium is sensitive to the antibiotic (MIC ~2 μg/ml) [133]. Surprisingly, in light of the potential influence gentamicin has on the intracellular growth of other bacteria, a large variety of gentamicin concentrations (ranging from 0.016-256 μg/ml) and incubation times (from 15 min to 2 h) have been reported in the *Y. pestis* literature [78, 122, 134-138]. Not surprisingly, variation in *Y. pestis* intracellular survival has also been observed. Importantly, characterization of potential *Y. pestis* pathogenesis factors involved in intracellular infection, or attempts to repeat published data, could be influenced simply by the concentration of

gentamicin used in the antibiotic protection assay. Therefore, we felt it was imperative to understand the potential influence of gentamicin concentrations on *Y. pestis* intracellular growth in order to eliminate any potential for unintended influence of the gentamicin protection assay on the interpretation of intracellular studies with *Y. pestis*.

MATERIAL AND METHODS

Cell culture and bacterial strains

Primary peritoneal macrophages were harvested from 8-12 week old C57/Bl6 mice (University of Louisville IACUC Protocols 16651 and 16723) injected with 3 mls of sterile Brewer's thioglycolate medium. Four days after injection, mice were humanely euthanized and peritoneal macrophages were recovered in 20 mls of Hank's Buffered Salt Solution (HBSS). Cells were isolated by centrifugation and resuspended in 10 mls of DMEM (DMEM, 100 mM glucose, sodium pyruvate; Hyclone) + 10% FBS (Biowest) as previously described [139]. Cells were quantified, transferred to microtiter plates or dishes, and allowed to adhere for 3 h. Non-adherent cells were then removed from adherent macrophages by washing with HBSS three times. RAW264.7 macrophages were originally obtained from ATCC and cultured in DMEM + 10% FBS. RAW264.7 cells were propagated for only up to 15 passages for these studies. *Y. pestis* strains used in these studies are listed in Table 1. Bacteria were propagated in Difco Brain Heart Infusion (BHI) (BD, Co.). We have previously shown that the bioluminescence generated by the $Lux_{P\mathit{tolC}}$ bioreporter directly correlates with bacterial numbers

and can be used to kinetically monitor *Y. pestis* intracellular survival in gentamicin protection assays [130, 140].

Live Cell Microscopy

5 x 10^5 peritoneal macrophages in FluoroBrite DMEM (ThermoFisher), 4 mM glutamine + 10% FBS were added to a 35 mm glass bottom FluoroDish (World Precision Instruments). Macrophages were allowed to adhere to the dish for 3 h prior to infection. *Y. pestis* was grown overnight in BHI broth at 26°C and then diluted 1:25 in fresh BHI broth and grown for an additional three hours until the culture reached an absorbance at 600 nm of ~1.0. The bacteria were diluted into FluoroBrite DMEM, 4 mM glutamine + 10% FBS and added to macrophages at a multiplicity of infection (MOI) of 3 bacteria per macrophage. To facilitate cell-bacteria interactions, FluoroDishes were centrifuged at 200 x g for 5 minutes and returned to the CO_2 incubator. Fifteen min later, the medium was removed, the cells were washed three times with sterile 1X PBS to remove extracellular bacteria, and fresh FluoroBrite DMEM, 4 mM glutamine + 10% FBS was added. Live imaging was performed using a Nikon A1 confocal microscope (Nikon Instruments, Inc.) with a live cell chamber equilibrated to 5% CO_2 and 37°C prior to imaging. Either a 488 nm or 561 nm laser with filter sets 525±50 nm or 595±50 nm were used to visualize EGFP or mCherry, respectively. Images were taken using a 40x Plan Fluro Oil objective every 20 min beginning 1 h post-infection and continuing for 24 h. The Nikon Perfect Focus System and field tiling were used to collect six fields (287 x 287 μM per field) at each time point. Bacterial

numbers were estimated by calculating the area of the fluorescent signal for each field using FIJI [141].

Monitoring bacteria using a bioluminescence

1.5 x 10^5 macrophages were aliquoted into the wells of a white 96 well microtiter plate (Greiner Bio One) and allowed to adhere for 3 or 15 h (peritoneal or RAW264.7 cells, respectively). *Y. pestis* was grown overnight in BHI broth at 26^{o}C and then diluted 1:25 in fresh BHI broth and grown for an additional three hours until the culture reached an absorbance at 600 nm of ~1.0. The bacteria were diluted into 37^{o}C DMEM +10% FBS and added to macrophages at a MOI of 10 bacteria per macrophage. To facilitate cell-bacteria interactions, microtiter plates were centrifuged at 200 x g for 5 minutes and returned to the CO_2 incubator for an additional 15 minutes, at which point gentamicin was added directly to the wells without washing to achieve the final desired concentrations. Concentrations of gentamicin used were not cytotoxic to macrophages. Bacterial numbers as a function of bioluminescence produced by the Lux_{PtolC} bioreporter [130, 140] was monitored kinetically to limit manipulation of the macrophages using a Synergy HT plate reader (0.5 sec read, sensitivity of 135) (BioTek) or IVIS Spectrum camera system (15 min read, 500 nm emission filter) (Caliper).

Determination of gentamicin minimal inhibitory concentration

Y. pestis was grown overnight in BHI broth at 26^{o}C and then diluted 1:25 in fresh BHI broth and grown for an additional 3 h until the culture reached an absorbance at 600 nm of ~1.0. The bacteria were diluted into 37^{o}C DMEM + 10% FBS and aliquoted at 1.5 x 10^6 CFU per well in a 96 well plate. Gentamicin was

diluted in 37^{o}C DMEM + 10% FBS and added to the bacteria to achieve the final desired concentration (0 – 128 µg/ml). One h after treatment, bacterial numbers were determined by conventional enumeration using serial dilution and plating on BHI agar [140].

Enumerating intracellular bacteria by conventional enumeration

For short incubations with the antibiotic, bacterial numbers were directly enumerated by serial dilution to allow for separate calculations of extracellular and intracellular bacterial numbers after gentamicin treatment. Macrophages were infected with *Y. pestis* as described above and treated with gentamicin for 1 h. After treatment with gentamicin, the culture medium was collected into a separate tube, the cells were washed three times with sterile 1X PBS, which was also collected and combined with the collected medium, the combined medium + 1x PBS washes was serial diluted and plated on BHI agar plates to enumerate the extracellular bacteria. For the intracellular bacteria, washed macrophages were lysed with 0.1% Triton X100 and serial dilutions were plated on BHI agar plates for enumeration [140].

Statistical Analysis

All studies were repeated three times to ensure reproducibility. When needed, mean values from individual treatment groups were compared to the 0 µg/ml gentamicin group using the ANOVA with the Dunnett's post test or across all samples with the Tukey post test. A *p*-value less than 0.5 was consider to be statistically significant.

RESULTS

Macrophage - *Y. pestis* interactions in the absence of gentamicin. To establish the fate of *Y. pestis* during infection of macrophages in the absence of gentamicin treatment, primary peritoneal macrophages were infected with a *Y. pestis* strain expressing the mCherry fluorescent protein (YPA165). 20 min post-infection, the macrophages were washed to remove extracellular bacteria and imaged every 20 min by laser confocal microscopy for 24 h (data not shown). Changes in bacterial number, as a function of the total area of mCherry signal, were calculated at each time point (Fig. 4-1). Over the first ~12 h, most bacteria appeared to be intracellular and we observed a steady increase in the area of mCherry over time, indicating intracellular survival and growth (Fig. 4-1A). However, a small number of infected macrophages began to lyse ~12 h post-infection and release *Y. pestis* into the culture medium, though cell death was not synchronous and most infected cells were still intact through the first 18 h (Fig 4-1C). We continued to observe increases in the area of mCherry through 24 h of observation (Fig. 4-1B), and while bacteria appeared to continue to grow intracellularly, extracellular bacteria released from dead macrophages were also replicating in the medium and represented a large portion of the mCherry signal at later time points (Fig. 4-1B and C). By 24 h post-infection, almost all of the infected macrophages had lysed and the majority of the mCherry signal was from extracellular bacteria (Fig. 4-1C).

To determine if the *Y. pestis* Ysc type 3 secretion system (T3SS) influenced the intracellular fate of the bacterium, macrophages were infected with a *Y. pestis*

strain lacking the pCD1 virulence plasmid encoding the Ysc T3SS and expressing EGFP (YPA081) and imaged by laser confocal microscopy as described above. As observed for the T3SS positive strain, total area of EGFP increased over the entire course of the experiment (Fig. 4-1A and B). However, unlike the *Y. pestis* pCD1$^{(+)}$ strain, we did not observe macrophage lysis, bacterial release, or extracellular proliferation over the 24 h time frame of the experiment, indicating that the increased area of EGFP was a function of only intracellular growth. Together these data: 1) confirm data from previous *in vitro* assays that *Y. pestis* survives intracellularly in primary macrophages [123, 132]; 2) confirm that *Y. pestis* lacking the T3SS can survive in macrophages [78, 123, 132]; 3) demonstrate that macrophages infected with *Y. pestis* with a functional T3SS lyse more rapidly than macrophages infected with *Y. pestis* lacking the T3SS; 4) macrophage cell death releases intracellular bacteria into the environment; and 5) bacteria released from macrophages are viable and can replicate in the cell culture medium. While intracellular vs. extracellular bacteria can be differentiated during live imaging, *in vitro* assays that do not use microscopy are not able to differentiate between the two, thus requiring the use of an antibiotic protection assay. However, as a wide variety of concentrations of gentamicin have been reported in the *Yersinia* literature [78, 122, 134-138], we next wanted to determine if extended incubation with gentamicin could influence intracellular proliferation of *Y. pestis*.

Extended incubation with gentamicin impacts intracellular survival of *Y. pestis*. The simplest antibiotic protection assay would be to add gentamicin to

the infected cells and maintain that concentration in the medium throughout the entire course of the study. However, extended incubations have been shown to influence the intracellular proliferation of other bacteria [111, 117, 118]. To determine if extended incubations with gentamicin impacted survival of intracellular *Y. pestis*, peritoneal macrophages were infected with a *Y. pestis* strain containing the $Lux_{P\mathit{tolC}}$ bioreporter (YPA050), which we have previously shown can be used to accurately monitor intracellular *Y. pestis* as a function of bioluminescence [130, 140]. Twenty min post-infection, serial dilutions of gentamicin were added to each well to achieve final concentrations of 128 to 1 μg/ml and beginning 2 h after infection, and bacterial numbers, as a function of bioluminescence, were determined every 3 h for 20 h using a plate reader. We observed that at the lowest concentrations tested, 1 and 2 μg/ml, that bacterial bioluminescence increased over time, although at 2 μg/ml, bioluminescence was approximately 2-3 fold lower than at 1 μg/ml (Figure 4-2A and 4-2B). At concentrations greater than 2 μg/ml, bioluminescence no longer increased, indicating that bacteria were no longer able to proliferate. At 4 μg/ml, we observed an initial decrease in bioluminescence between 2 and 5 h, at which time bioluminescence remained constant over the remainder of the assay. Gentamicin concentrations greater than 4 μg/ml resulted in a steady decrease in bacterial bioluminescence over the entire course of the assay (Fig. 4-2D-H). Compared to our live imaging experiments without gentamicin, these data demonstrate that extended incubation with even low concentrations of

gentamicin can have a dramatic influence on *Y. pestis* intracellular survival, and can lead to intracellular killing of the bacterium.

Sensitivity to gentamicin is cell type dependent. As pinocytosis is suggested to be the main mechanisms for gentamicin uptake by macrophages, sensitivity of intracellular bacteria to gentamicin could vary depending on cell type used. RAW264.7 cells are an immortal cell line originally derived from mouse macrophages and are commonly used as a macrophage model [142]. To determine if the sensitivity of intracellular *Y. pestis* to gentamicin could vary depending on cell type, RAW264.7 cells were infected with *Y. pestis* YPA050. 20 min later, serial dilutions of gentamicin were added to each well to achieve final concentrations of 128 to 1 μg/ml and cells were incubated for 20 h. Bacterial numbers were monitored as a function of bioluminescence, normalized to T=2 h, and compared to similarly treated primary peritoneal macrophages (Fig. 4-3). Similar to peritoneal cells, bacterial bioluminescence increased in RAW264.7 macrophages when treated with 1 μg/ml, indicating intracellular proliferation. However, unlike bacteria in peritoneal macrophages, *Y. pestis* bioluminescence continued to increase in RAW264.7 cells until gentamicin concentrations reached 16 μg/ml. After 16 μg/ml, bioluminescence began to decrease overtime, indicating bacterial killing, and approached levels similar to that seen for peritoneal macrophages at 128 μg/ml. These data highlight that gentamicin inhibition of intracellular *Y. pestis* can vary between cell types.

Short incubation with high concentrations of gentamicin inhibits intracellular growth. Since extended incubations with gentamicin dramatically inhibited the proliferation of intracellular *Y. pestis*, we next sought to identify the minimal concentration of gentamicin needed to inhibit *Y. pestis* growth in tissue culture medium in a 1 h period. Towards this end, *Y. pestis* YPA050 was diluted in DMEM, 10% FBS with increasing concentrations of gentamicin. One h after addition of gentamicin, bacteria were serially diluted and plated on BHI agar to determine remaining viable bacteria (Fig. 4-4A). All doses of gentamicin tested resulted in a significant decrease in the number of recovered bacteria compared to untreated ($p \leq 0.0001$) and the degree of inhibition was dependent on the concentration of the antibiotic. While bacteria were reduced ~1,000-fold within one h with 4 μg/ml gentamicin, no detectable viable bacteria were recovered after incubation with ≥8 μg/ml. Similar inhibition was observed for the Medivalis biovar KIM D19 (Fig. 4-7).

Next we sought to determine whether the presence of macrophages impacted the minimal concentration of gentamicin required to inhibit *Y. pestis* growth in cell culture medium. Peritoneal macrophages were infected with *Y. pestis* YPA050 for 20 min and serial dilutions of gentamicin were added to each well to achieve final concentrations of 128 to 1 μg/ml. One h after addition, the gentamicin containing medium was removed and combined with washes and viable extracellular bacteria were determined by serial dilution and conventional enumeration (Fig. 4-4B). Extracellular bacterial numbers were significantly lower in all the samples that received greater than 1 μg/ml of gentamicin as compared

to absence of gentamicin ($p \leq 0.0001$). However, no significant differences were observed in bacterial numbers between concentrations of 4 to 32 μg/ml. Furthermore, while we observed no significant difference in bacterial numbers between the 64 and 128 μg/ml concentrations, we observed ~2.5-fold lower numbers of extracellular bacteria at 64 and 128 μg/ml samples (average 180 CFU) compared to the 4 to 32 μg/ml samples (average 458 CFU) that was statistically significant ($p \leq 0.05$).

Finally, we enumerated the number of intracellular bacteria in macrophages after 1 h treatment with gentamicin at both 1 and 24 h post-treatment to determine if gentamicin concentration during short incubations could impact intracellular survival of *Y. pestis* (Fig. 4-5). At 1 h post-treatment, statistically significant differences in the number of intracellular bacteria recovered were not observed until gentamicin concentrations were greater than 8 μg/ml (Fig. 4-5A). In concentrations greater than 8 μg/ml, a dose dependent decrease in viable intracellular was observed. However, even at the highest doses, intracellular numbers only varied by ~1.3-fold as compared to macrophages receiving lower doses of antibiotic. To determine if long term intracellular survival of *Y. pestis* was affected by short exposures to gentamicin, a separate group of infected cells were washed 1 h after gentamicin treatment to remove residual gentamicin and fresh medium without gentamicin was added. 23 h later, the medium was removed, the cells were washed, lysed, and intracellular bacterial numbers were enumerated (Fig. 4-5B). We observed bacterial proliferation in all samples containing less than 64 μg/ml gentamicin, and there were no significant

differences in intracellular numbers in samples containing 0 to 32 μg/ml gentamicin. However, bacteria did not proliferate in macrophages treated with 64 μg/ml of the antibiotic, and at the highest dose of 128 μg/ml, intracellular bacterial numbers decreased over time. Together these data indicate that at least 4 μg/ml of gentamicin is required to achieve optimal killing of extracellular *Y. pestis* in 1 h during macrophage infection assays. However, as gentamicin concentrations increase, even short incubations with the antibiotic can impact proliferation of intracellular *Y. pestis*, especially at higher doses of the antibiotic.

Optimized gentamicin protection assay for *Y. pestis*

Together these data suggest that an optimized gentamicin assay for *Y. pestis* should include two doses of gentamicin. The first should be an initial dose to efficiently kill the majority of the extracellular bacteria in a short incubation period (*e.g*., one hour) without inhibiting intracellular survival. The data from short term incubations with gentamicin (Fig. 4-5) suggest that this concentration should not exceed 8 μg/ml. After the one hour incubation, the medium should be removed as continued incubation in 8 μg/ml results in inhibition of intracellular bacteria (Fig. 4-2). The medium should then be replaced with a lower dose that inhibits extracellular bacterial growth but not intracellular survival of the bacteria during extended incubations. The data from extended incubations with gentamicin (Fig. 4-2) suggest that this maintenance dose should not exceed 2 μg/ml. To test these empirically derived gentamicin concentrations, peritoneal or RAW264.7 macrophages were infected with YP043, a fully virulent strain of *Y. pestis* CO-92

expressing the Lux$_{P_{tolC}}$ bioluminescent bioreporter [140]. Twenty min later, gentamicin was added at a final concentration of 8 μg/ml. Cells were incubated for 1 h, washed with 1X PBS, and the medium was replaced with medium containing 2 μg/ml gentamicin. Intracellular bacterial survival was monitored by bacterial bioluminescence every 2 h for 20 h using an IVIS optical imager (Fig. 4-6). In peritoneal macrophages, bioluminescence slightly decreased over the first ~8 h of infection, indicating an initial inhibition of bacterial survival by the macrophages, but then at ~12 h, bacterial bioluminescence began to increase, reaching a steady state level at 16 h that was maintained for the remainder of the infection (Fig. 4-6A). Based on our live imaging experiments, this steady state level at later time points likely represents a balance between intracellular replication and gentamicin killing of extracellular bacteria from lysed macrophages (Fig. 4-1). In RAW264.7 macrophages we did not observe an initial decrease in bacterial bioluminescence, supporting that this cell type is more permissive to intracellular bacterial infection (Fig. 4-6B). Instead, we observed a ~2-fold increase in bioluminescence over the first 4 h, which plateaued at this level for the first 10 h of the infection. Then, similar to the peritoneal macrophages, at ~12 h post-infection, bacterial bioluminescence began to increase, indicating further intracellular bacterial proliferation.

Discussion

The antibiotic protection assay has been a key technique for the study of bacterial intracellular pathogenesis. The assay was originally developed on the

premise that certain classes of antibiotics are excluded from eukaryotic cells, resulting in antibiotic-mediated killing of extracellular bacteria but not intracellular bacteria. While researchers have more recently recognized that antibiotics originally thought to be excluded can inhibit the growth of several bacterial pathogens, only a few systematic studies have been performed to characterize the impact of antibiotic treatment on specific pathogens [116, 117]. We took particular notice to this in the *Yersinia* field, where large variations in antibiotic concentrations and exposure times have been reported in the literature [78, 122, 134-138, 143]. We were concerned that such variations in the antibiotic protection assay between laboratories had the potential to decrease reproducibility of experiments and potentially produce artificial phenotypes due to unanticipated influence by the antibiotic. As such, our goals here were to specifically demonstrate that variations in the gentamicin protection assay could alter *Y. pestis* intracellular growth and suggest that researchers carefully consider antibiotic concentrations and exposure times when designing future intracellular experiments with *Y. pestis* (or any bacteria).

Through the use of live-cell microscopy we were able to observe *Y. pestis* interactions with macrophages in the absence of gentamicin. These observations suggest that washing of infected cells removes the majority of bacteria not phagocytosed by the macrophages, resulting in the absence of extracellular growth during the first several hours of the experiment. However, infected macrophages eventually lyse and release viable *Y. pestis* into the medium. These bacteria then replicate extracellularly. Importantly, our experience with live

cell microscopy suggests that macrophage lysis is not synchronized and the timing of when macrophages lyse and initiation of extracellular growth can vary from experiment to experiment. Therefore, more conventional assays to study intracellular proliferation without microscopy will require addition of an antibiotic in the medium to ensure that extracellular growth is not mistaken for intracellular growth. However, the data reported here also suggest that researchers should carefully optimize the gentamicin protection assay to minimize potential artificial influence on the system. Using an empirical approach, we defined the maximum concentrations of gentamicin that should be used in a two-step gentamicin protection assay as 8 μg/ml and 2 μg/ml (Fig. 4-6). These concentrations represent the maximum concentrations of gentamicin that inhibit *Y. pestis* growth without demonstrating a significant impact on intracellular numbers (as compared to live microscopy in the absence of antibiotic). However, important considerations in optimizing intracellular assays are not only antibiotic concentration and exposure time, but also how *Y. pestis* is handled prior to interactions with host cells and the cell type being used. For example, while our data indicates that one hour incubation with 8 μg/ml of gentamicin is the minimum bactericidal concentration for *Y. pestis*, this was determined for early logarithmically growing bacteria. We have observed that for stationary phase cultures, 16 μg/ml of gentamicin is required to eliminate all extracellular bacteria in one hour [130, 140]. Furthermore, our data comparing peritoneal and RAW264.7 macrophages support that researchers should not assume that empirically determined gentamicin concentrations that do not influence

intracellular growth in one cell type will not alter growth in another. Wendte et al. also recognized differences in intracellular *Y. pestis* sensitivity to gentamicin between THP-1 and RAW264.7 macrophages [144]. Therefore, different macrophages may require concentrations different from those used here. In summary, we hope these studies help researchers reconsider the potential impact of gentamicin on *Y. pestis* intracellular growth when designing future experiments using the antibiotic protection assay.

Table 4-1. *Yersinia pestis* strains used in this study.

Strain	Relevant Characteristics	Bioreporter	Source
YPA165	CO92pgm$^{(-)}$ pCD1$^{(+)}$	pGEN222::mCherry	*This study*
YPA081	CO92 pgm$^{(+)}$ pCD1$^{(-)}$	pGEN222::EGFP	[130]
YPA050	CO92 pgm$^{(+)}$ pCD1$^{(-)}$	Lux$_{PtolC}$	[140]
YP043	CO92 pgm$^{(+)}$ pCD1$^{(+)}$	Lux$_{PtolC}$	[140]

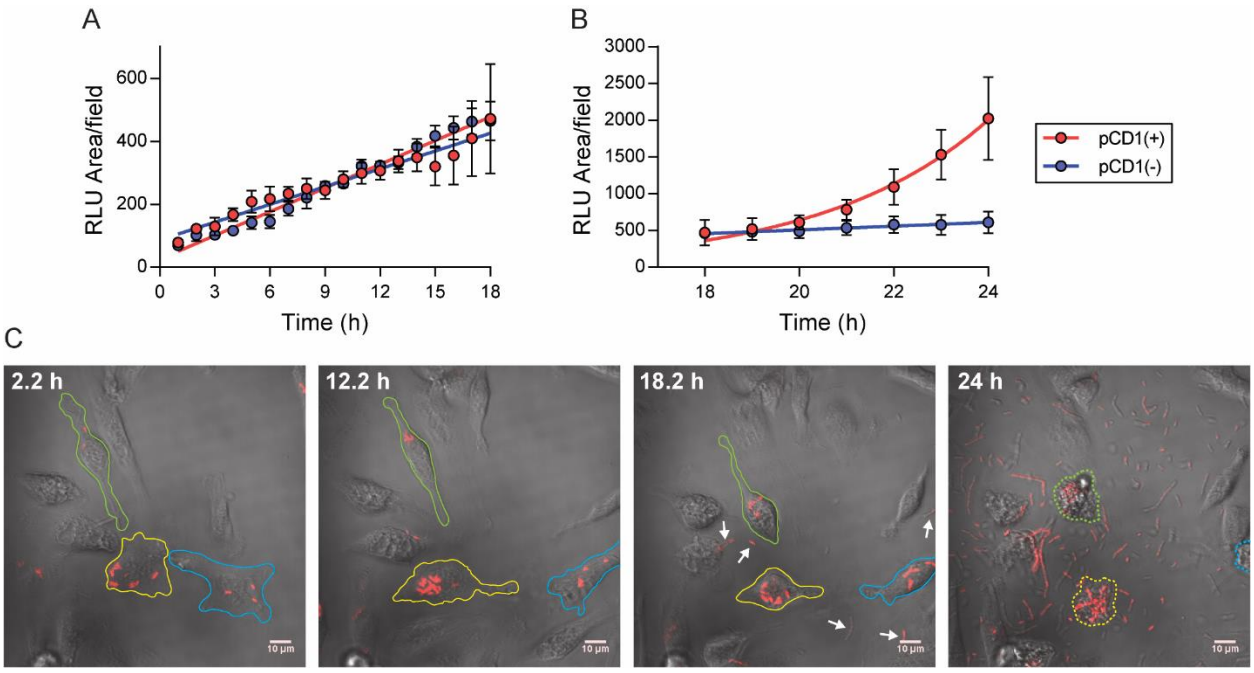


Figure 4-1. Live imaging of *Y. pestis* in the absence of gentamicin. (A and B) Primary peritoneal macrophages were infected with *Y. pestis* YPA165 expressing mCherry (red symbols; pCD(+)) or *Y. pestis* YPA081expressing EGFP (blue symbols; pCD(-)) at a MOI=3. One h after infection, cells were washed to remove extracellular bacteria and macrophages were imaged by live confocal microscopy. Bacterial numbers as a function of total fluorescent (RLU) area per field were determined every 1 h for 24 h. Each point represents the mean ±S.D. calculated from four imaged fields at each time point (an average of 16 infected cells per field). (C) Representative images of *Y. pestis* YPA165 infected macrophages at indicated time points during the infection. Individual macrophages tracked through the entire course of the experiment are outlined in different colors. Lysed macrophages at 24 h are highlighted by dotted lines. Presence of extracellular bacteria at 18.2 h are highlighted by arrows.

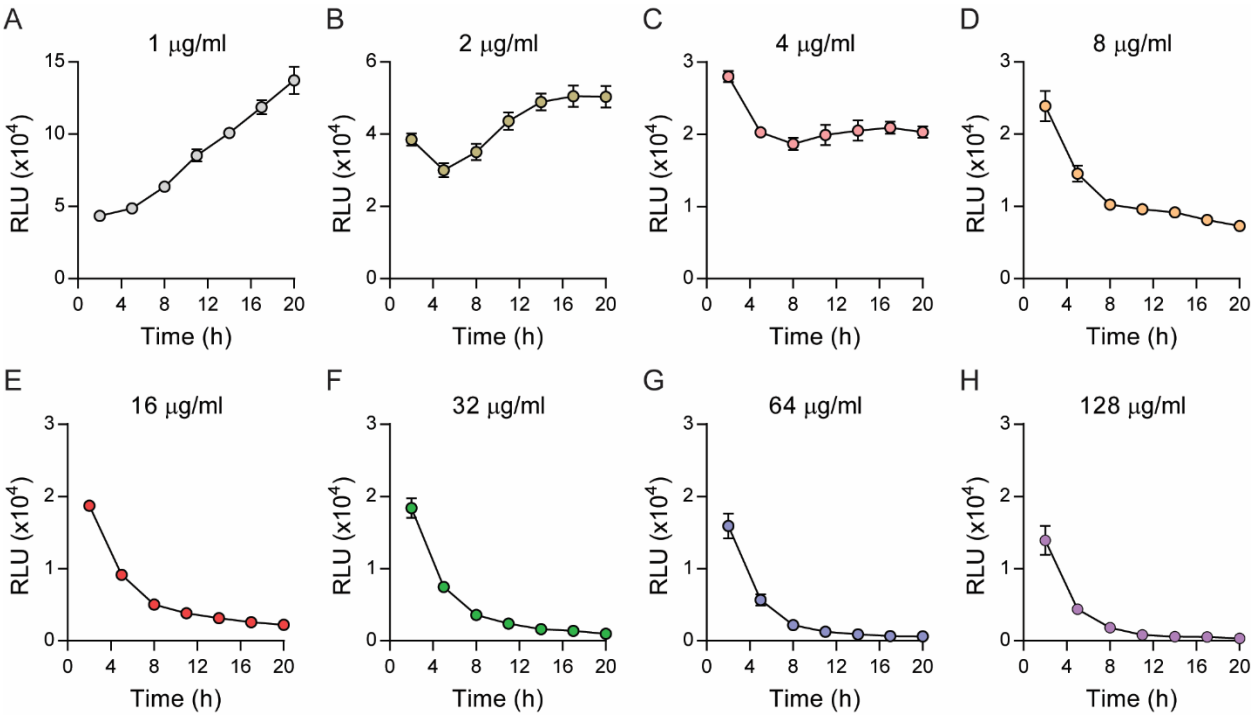


Figure 4-2. Extended incubations with gentamicin inhibit *Y. pestis* intracellular survival. Primary peritoneal macrophages were infected with *Y. pestis* YPA050 at an MOI of 10 (n=6). 20 min post-infection, macrophages were washed and the medium was replaced with medium containing gentamicin at indicated concentrations. Bacterial numbers as a function of bioluminescence were determined every 3 h for 20 h using a plate reader and displayed as the mean ±S.D. (in some cases the S.D. is smaller than the symbol size). Data is shown from one representative experiment of three independent experiments.

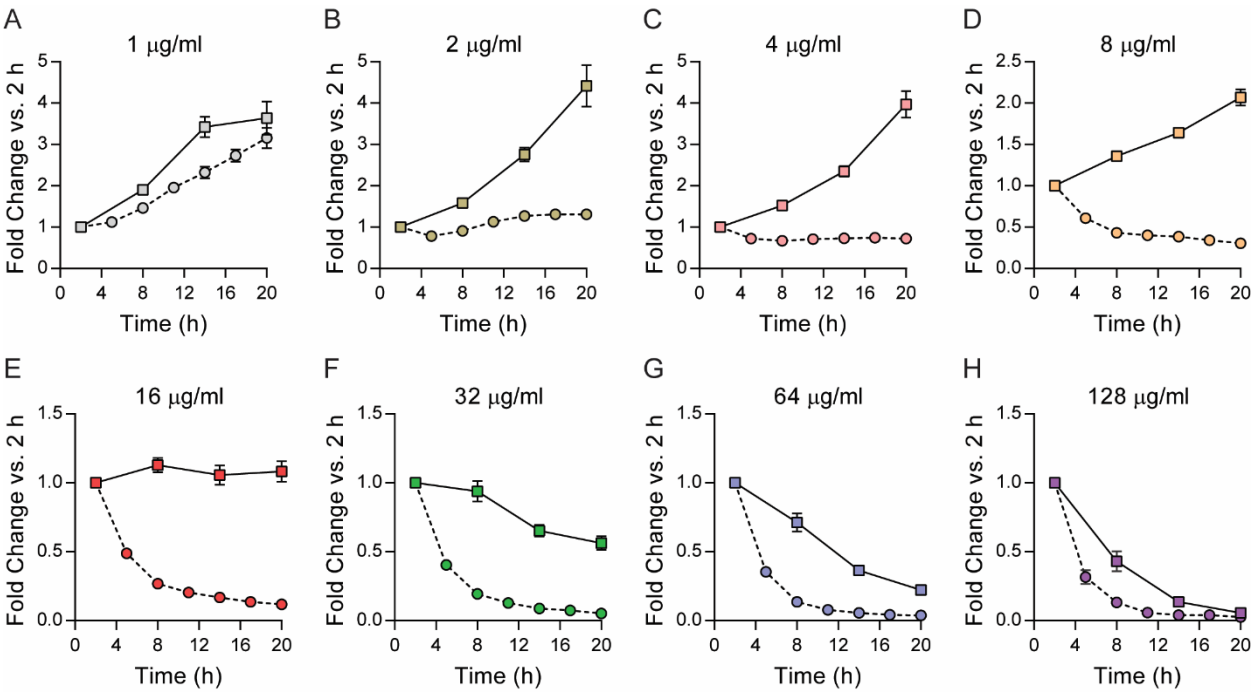


Figure 4-3. Cell type can influence intracellular sensitivity of *Y. pestis* to gentamicin. Primary peritoneal macrophages (circles, dotted lines) or RAW264.7 macrophages (squares, solid lines) were infected with *Y. pestis* YPA050 at an MOI of 10 (n=6). 20 min post-infection, macrophages were washed and the medium was replaced with medium containing gentamicin at indicated concentrations. Bacterial numbers as a function of bioluminescence were determined every 3-6 h for 20 h using a plate reader. Bioluminescent data from each time point was normalized to fold change compared to the 2 h time point by dividing each time point by the 2 h time point and is displayed as mean fold change ±S.D. Data is shown from one representative experiment of three independent experiments.

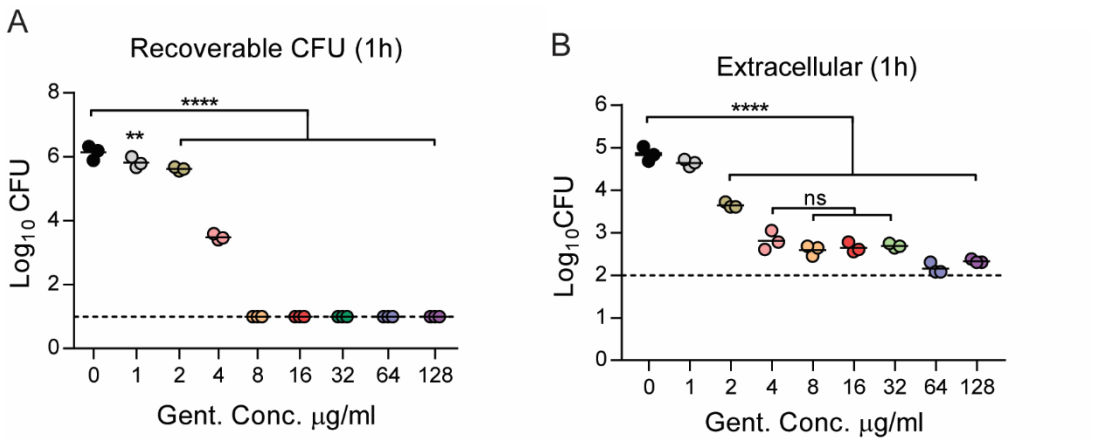


Figure 4-4. Minimum inhibitory concentration of gentamicin for *Y. pestis*. Logarithmically growing *Y. pestis* YPA050 was diluted to 1.5 x10^6 CFU per well in a 96 well plate containing gentamicin at the indicated concentrations. (A) One h after inoculation into gentamicin, bacteria were removed, serially diluted in 1x PBS, and enumerated on agar to determine bacterial viability after exposure to gentamicin (n=3). (B) Primary peritoneal macrophages were infected with *Y. pestis* YPA050 at an MOI of 10 (n=3). 20 min post-infection, gentamicin was added at indicated concentrations. One h later, the medium and washes were collected, combined, and serially diluted to enumerate viable bacteria by plating dilutions on BHI agar. Each point represents one sample and the bars represent the mean CFU. The dotted lines indicate the limit of detection. Data is shown from one representative experiment of three independent experiments. (A and B) ANOVA with Dunnett's post hoc analysis was used when comparing samples to 0 μg/ml gentamicin. (B) ANOVA with Tukey's post hoc analysis was used when comparing between the 4 – 32 μg/ml samples. **= p≤0.01; ****= p≤0.0001; ns= not significant.

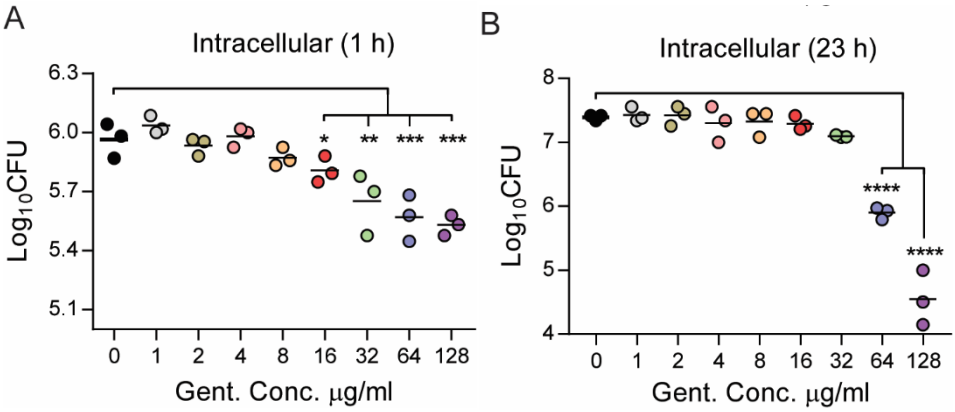


Figure 4-5. Short incubations with gentamicin impact *Y. pestis* intracellular survival in a dose dependent manner. Primary peritoneal macrophages were infected with *Y. pestis* YPA050 at an MOI of 10 (n=3). 20 min post-infection, the medium was replaced with medium containing gentamicin at indicated concentrations. One h later, gentamicin was removed, and (A) cells were washed three times, lysed with 0.1% Triton, and intracellular bacterial were enumerated by serial dilution and plating on BHI agar plates, or (B) fresh medium without gentamicin was added and cells incubated for an additional 23 h. At 23 h post-gentamicin treatment, the medium was removed, cells were washed three times, lysed with 0.1% Triton, and intracellular bacterial were enumerated by serial dilution and plating on BHI agar plates. Each point represents one sample and the bars represent the mean CFU. Data is shown from one representative experiment of three independent experiments. ANOVA with Dunnett's post hoc analysis compared to 0 μg/ml gentamicin: *= p≤0.05; **= p≤0.01; ***= p≤0.001; ****= p≤0.0001.

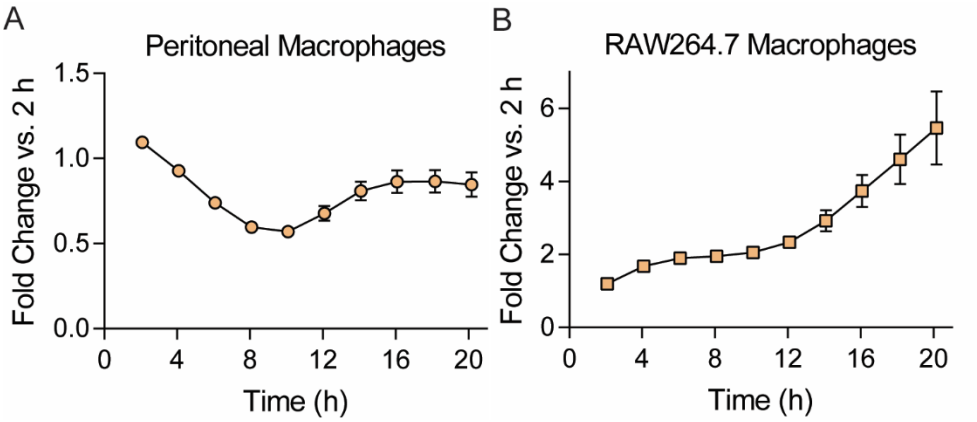


Figure 4-6. Optimized gentamicin protection assay. Primary peritoneal macrophages were infected with *Y. pestis* YP043 at an MOI of 10 (n=6). 20 min post-infection, macrophages were washed and the medium was replaced with medium containing 8 µg/ml gentamicin. One h later, culture medium was removed, cell were washed with 1X PBS, and fresh medium containing 2 µg/ml gentamicin was added. Bacterial numbers as a function of bioluminescence were determined every 2 h for 20 h using an IVIS Spectrum optical imager. Bioluminescent data from each time point was normalized to fold change compared to the 2 h time point by dividing each time point by the 2 h time point and is displayed as mean fold change ±S.D. (in some cases the S.D. is smaller than the symbol size). Data is shown from one representative experiment of two independent experiments.

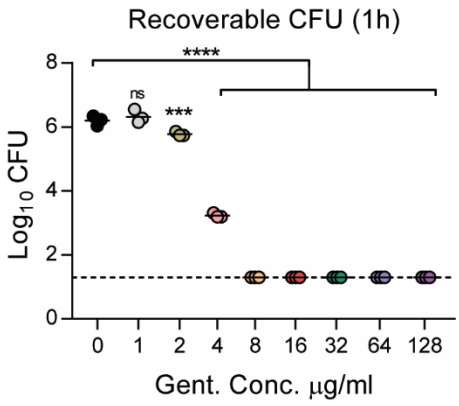


Figure 4-7. Minimum inhibitory concentration of gentamicin for KIM D19 *Y. pestis*. Logarithmically growing KIM D19 *Y. pestis* pCD1$^{(-)}$ Lux$_{PtolC}$ was diluted to 1.5 x10^{6} CFU per well in a 96 well plate containing gentamicin at the indicated concentrations. (A) One h after inoculation into gentamicin, bacteria were removed, serially diluted in 1x PBS, and enumerated on agar to determine bacterial viability after exposure to gentamicin (n=3). Each point represents one sample and the bars represent the mean CFU. The dotted lines indicate the limit of detection. Data is shown from one representative experiment of three independent experiments. ANOVA with Dunnett's post hoc analysis compared to 0 µg/ml gentamicin: **= p≤0.01; ****= p≤0.0001; ns= not significant.

CHAPTER FIVE

ROLE OF YAPE AND PLA IN INTRACELLULAR SURVIVAL OF *Y. PESTIS*

Introduction:

Recent studies have shown that the uptake of *Y. pestis* by host cells such as macrophages, and to a lesser degree, neutrophils, is important for survival and replication [122] [132]. During initial infection, outer membrane proteins are expressed that aid in the uptake of the bacteria into the phagocytes. Many of these outer membrane proteins are adhesins that help mediate adherence to eukaryotic cells.

One adhesin, Ail, has been shown to target neutrophils in vivo, although there does not appear to be a specific cell type that it directly mediates binding [145]. Although the characteristics of Ail in relation to the T3SS have been extensively studied, to date, there is little to no information on the role Ail may play in intracellular survival. The adhesin, OmpA, has been shown to contribute to intracellular survival [104]. Bartra and co-workers infected RAW264.7 macrophages using KIM5-3001 WT strain or an Δ*ompA* strain, and showed that by 24 hours there was a statistical difference in bacterial survival ($P<0.05$) [104].

Two adhesins that have played an integral role in the virulence of *Y. pestis* are YapE and Pla. YapE and Pla have both demonstrated that they mediate adherence to macrophages [77]. Moreover, strains deficient in either of these adhesins are attenuated in dissemination and colonization of lymph nodes and organs [53, 76]. It has been observed that at the site of subcutaneous infection, macrophages migrate away from the site and travel to a draining lymph node. The absence of either adhesin may cause a reduction of bacteria taken up by the macrophages. This reduction of bacteria may have downstream effects that can

influence the bacterial colonization. Another possibility for the reduction in dissemination and colonization is that these adhesins play a role in intracellular survival.

To confirm the role these known adhesins may play in intracellular survival, strains lacking these genes (Δ*ail*, Δ*ompA*, Δ*yapE,* and Δ*pla*) were used to infect macrophages and their survival was monitored by bioluminescence.

Results:

Absence of outer membrane adhesins can influence intracellular survival

To investigate intracellular survival of the above mentioned adhesins, I used a bioluminescent reporter strain containing individual mutations in *ail*, *ompA*, *yapE* or *pla* and compared survival against WT *Y. pestis* (Table 5-1) in RAW264.7 macrophages. Macrophages were infected with an MOI of 10 monitored over 22 h. YPA147 (Δ*ail*) showed no change in intracellular survival compared to WT over the course of the experiment (T=22h) (Fig 5-1). Conversely, there was a significant reduction of viable bacteria seen in the YPA148 (Δ*ompA*) strain compared to WT by the 11h time point and continued for the duration of the experiment (T=22 h) ($P<0.0001$) (Fig 5-2). YPA145 (Δ*pla*) showed a reduction in bacterial numbers starting at 12 h post-infection ($P<0.01$) and showed higher statistical significance at 24 h ($P<0.0001$) (Fig. 5-3). YPA087 (Δ*yapE*) showed statistical differences at both 10 h ($P<0.001$) and 24 h ($P<0.01$) (Fig.5-4).

Discussion:

Adhesins are important to the spread of disease due to their ability to adhere to eukaryotic cells, avoid complement and other subversions of the immune system. These systems are in place to help with the fitness of the bacteria and allow for increased virulence. I have shown that Ail does not impact intracellular survival, while an *ompA* mutant does have a survival defect intracellularly [104]. Although I did observe a survival defect with the *ompA* mutant, it is not due to reduced adherence or invasion. Bartra and co-workers examined the initial uptake, by cultured macrophages, of an *ompA* mutant and an isogenic *ompA*+ strain and found the uptake comparable [104]. It was only at the later time point they saw a reduction in bacterial numbers. This is because OmpA does not have the same function of promoting adherence and invasion in *Y. pestis* like other bacterial pathogens; to this end, their data identified *ompA* as a pro-survival gene that promotes intracellular survival in *Y. pestis* [104]. The two other adhesins that have played an integral role in the virulence are YapE and Pla. The intracellular attenuation of Δ*yapE*, although statistically significant, may not be biologically significant. There was a more significant attenuation at the 10 h time point than seen at 24 h. This may be in part because other adhesins are compensating for loss of YapE.

For Pla, vanLier and co-workers showed that the single *pla* mutant was not able to survive in RAW264.7 macrophages [143]. I too was able to show an attenuated phenotype for a *pla* mutant intracellularly but not complete clearance. To this end, I tried to recapitulate their findings only to show that the clearance

they reported may be a byproduct of antibiotic killing by way of the gentamicin protection assay. While other studies have shown that there is an attenuation in intracellular survival, they showed complete clearance by 12 hours where we saw less than a log difference when using 8 μg/ml of gentamicin (Fig. 5-3A). To confirm that the concentration of gentamicin was impacting intracellular growth, I used 128 μg/ml to kill for 1 hour and saw virtually the same reduction in bacterial numbers (Fig. 5-3B). One possible reason for the difference in bacterial clearance demonstrated by the van Lier group is that the strain of bacteria they used contained the pgm locus where our study did not use a strain containing the pgm locus. This is confounding because the *rip* (*required for intracellular proliferation*) operon is located within the pgm locus that allows for survival and replication intracellularly. It would be assumed that if the pgm locus is present the bacteria would have an advantage to survive intracellularly where the removal should result in the reduction of bacterial numbers. One reason I did not see the reduction in bacterial numbers with the ∆pgm strain is that the *rip* operon is important for *Y. pestis* replication in activated macrophages [146, 147]. When performing the gentamicin protection assay, the macrophages were not stimulated prior to infection. This is why the absence of the pgm locus did not appear to effect intracellular survival in this case.

Adherence and invasion assays were performed with the *yapE* and *pla* mutants and for both there was no difference in adherence compared to WT (Fig 5-5 and Fig. 5-6 respectively). Although there was a statistical significance see in invasion with the *pla* mutant because the percentage is so minute, I do not

believe that this finding is biologically significant. This shows that the adherence and uptake by the macrophages is not impacted by the mutations of *yapE* and *pla*. Further studies are needed to understand the mechanisms that these adhesins use that may mediate intracellular survival of *Y. pestis*. The survival defect observed with a *pla* mutant may also be predicated on other adhesins that *pla* may either activate or degrade.

Table 5-1. *Yersinia pestis* strains used in this study.

Strain	Mutation	Relevant Characteristics	Bioreporter	Source
YPA143		CO92pgm$^{(-)}$ pCD1$^{(+)}$	LuxP_{tolC}	This study
YPA145	Δ*pla*	CO92 pgm$^{(-)}$ pCD1$^{(+)}$	LuxP_{tolC}	This study
YPA147	Δ*ail*	CO92 pgm$^{(-)}$ pCD1$^{(+)}$	LuxP_{tolC}	This study
YPA148	Δ*ompA*	CO92 pgm$^{(-)}$ pCD1$^{(+)}$	LuxP_{tolC}	This study
YPA087	Δ*yapE*	CO92 pgm$^{(+)}$ pCD1$^{(-)}$	LuxP_{tolC}	This study
YPA050		CO92 pgm$^{(+)}$ pCD1$^{(-)}$	LuxP_{tolC}	[140]

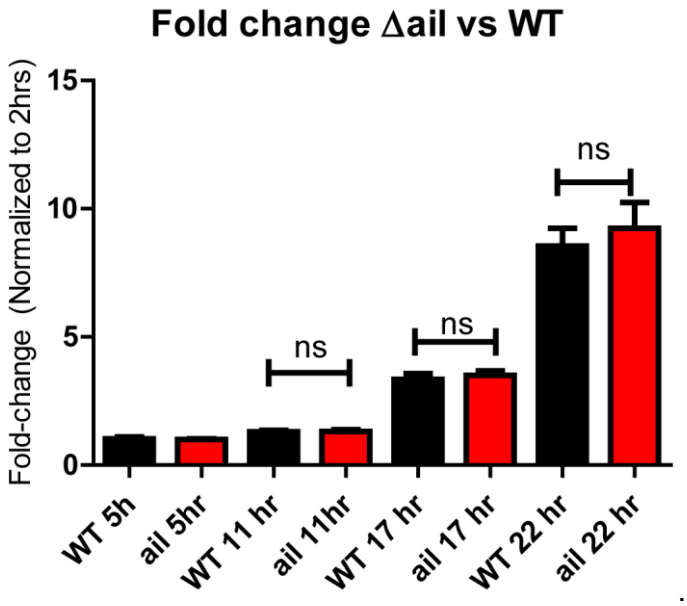


Figure 5-1 Δail does not exhibit an intracellular survival defect. . RAW264.7 macrophages were infected with *Y. pestis* YPA147 (n=12) or YPA143 (n=12) at an MOI of 10. Bacterial numbers were determined at 5, 11, 17 and, 22h using a plate reader measuring bioluminescence. Each time point was normalized to fold change compared to the 2 h time point by dividing each time point by the 2 h time point and is displayed as mean fold change ±S.D. (ns = no significance) Data shown is from one representative experiment of three independent experiments.

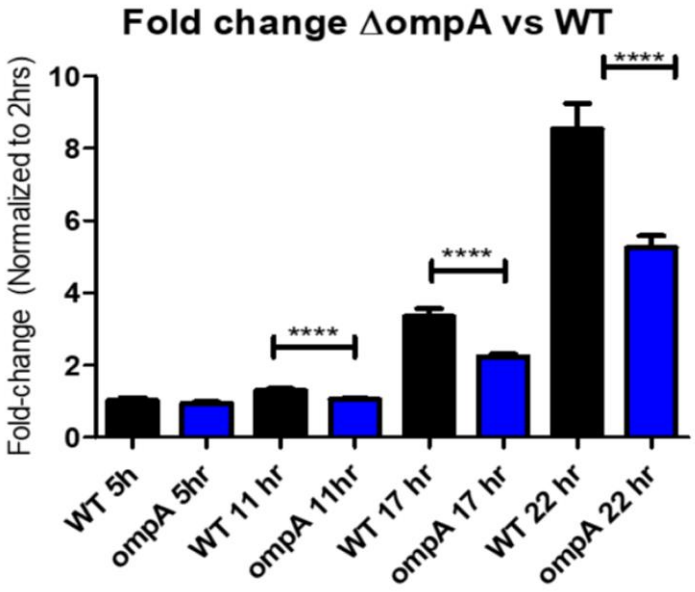
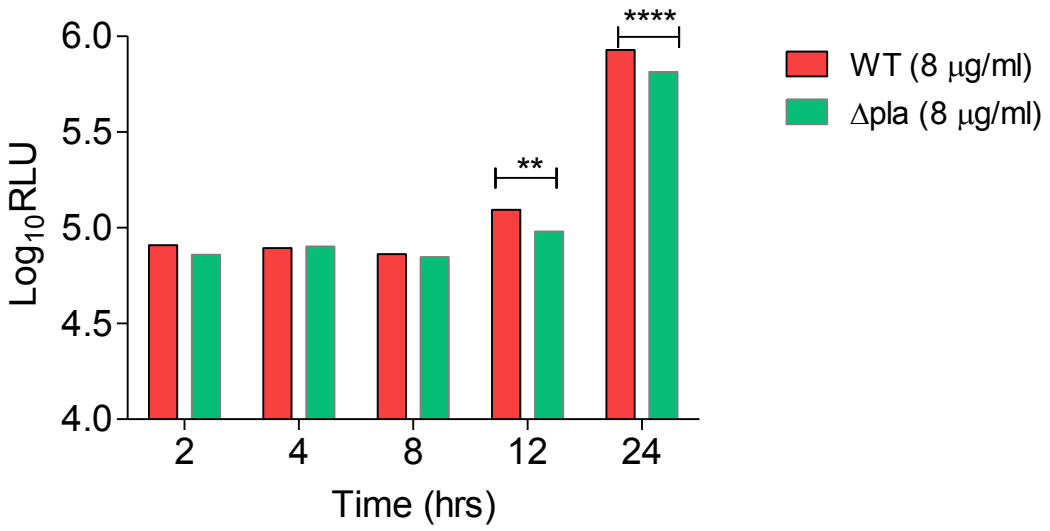


Figure 5-2 Δ*ompA* is attenuated intracellularly. RAW264.7 macrophages were infected with *Y. pestis* YPA148 (n=12) or YPA143 (n=12) at an MOI of 10. Bacterial numbers were determined at 5, 11, 17 and, 22h using a plate reader measuring bioluminescence. Each time point was normalized to fold change compared to the 2 h time point by dividing each time point by the 2 h time point and is displayed as mean fold change ±S.D. (**** = P<0.0001)

A.



B.

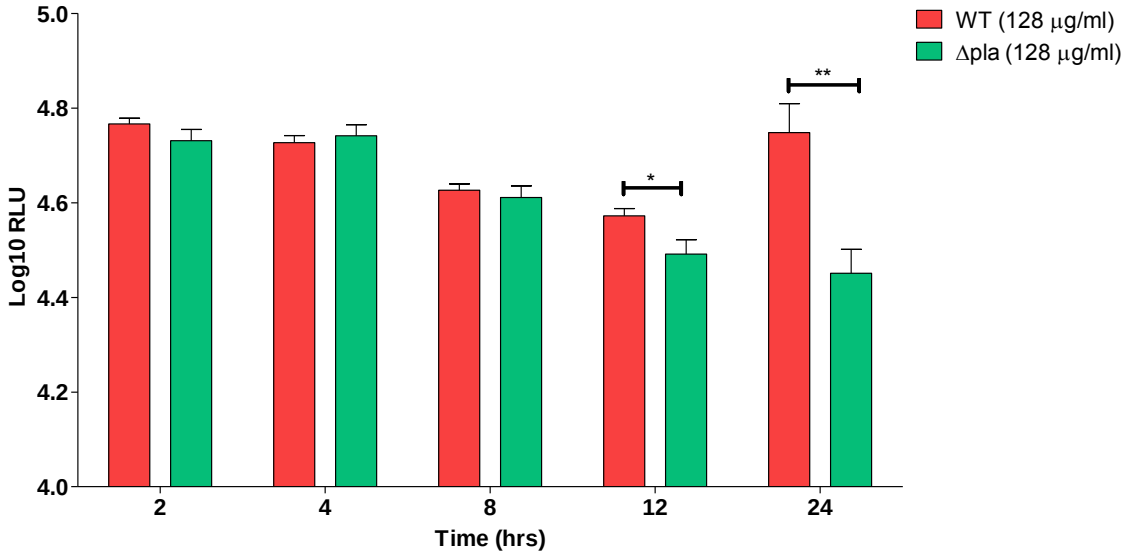


Figure 5-3 Δpla is attenuated intracellularly. (A and B) RAW264.7 macrophages were infected with Y. pestis YPA148 (n=12) or YPA143 (n=12) at an MOI of 10. Gentamicin protection assay was used to determine intracellular bacteria viability using killing concentrations of 8 µg/ml (A) and 128 µg/m (B). Bacterial numbers for each experiment were determined at 2, 4, 8, 12 and 24h using a plate reader measuring bioluminescence. Each time point is reflective of the bioluminescence emitted as a reflection of bacterial numbers. Student t-test was used (**** = P<0.0001).

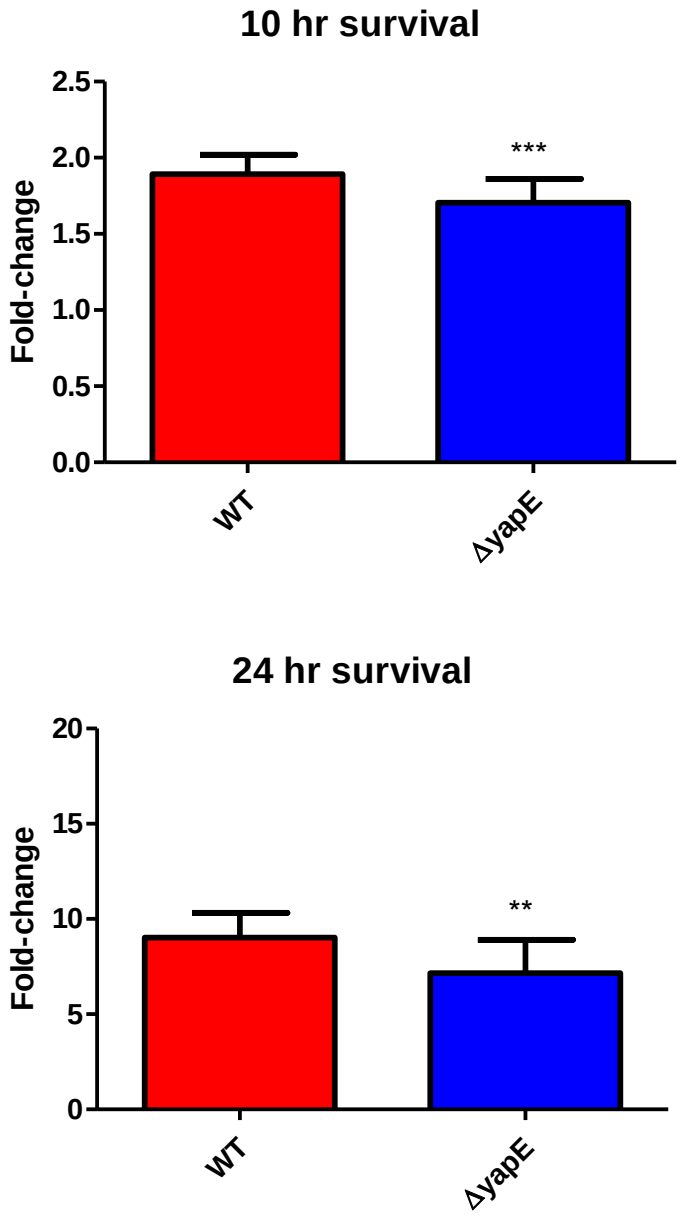


Figure 5-4. ΔyapE exhibits an intracellular survival defect. . RAW264.7 macrophages were infected with *Y. pestis* YPA087 (n=18) or YPA050 (n=18) at an MOI of 10. Bacterial numbers were determined at 10 and 24 h using a plate reader measuring bioluminescence. Time points were normalized to fold change compared to the 2 h time point by dividing each time point by the 2 h time point and is displayed as mean fold change ±S.D. (*** = P<0.001, ** = P<0.01)

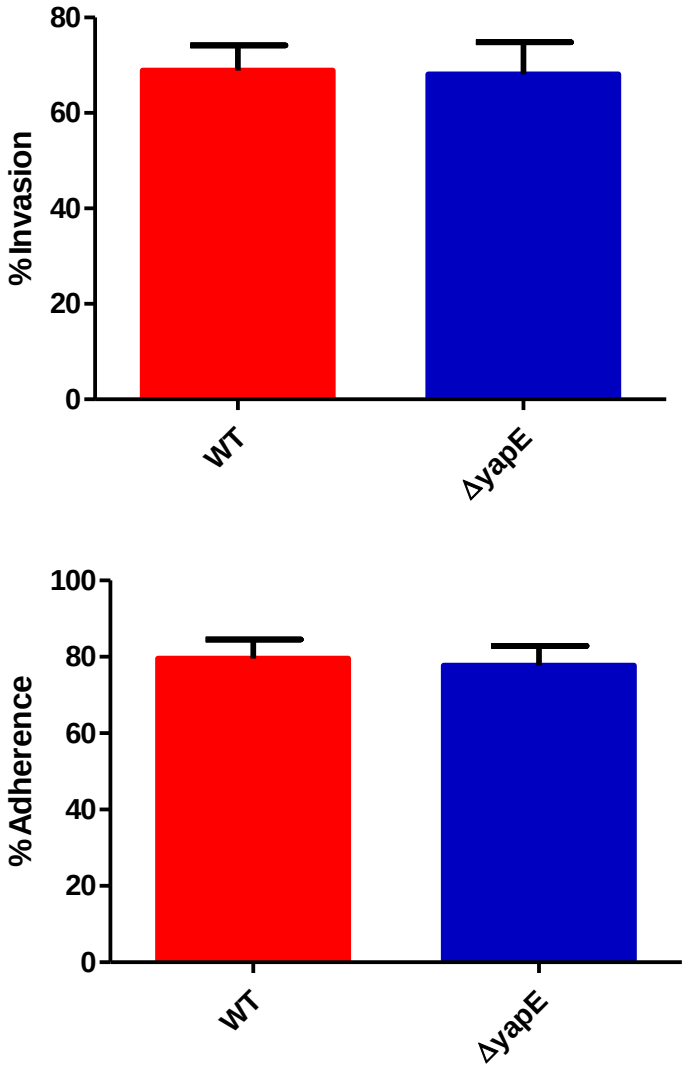


Figure 5-5 Δ*yapE* does not influence invasion or adherence. RAW264.7 macrophages were infected with *Y. pestis* YPA087 (n=18) or YPA050 (n=18) at an MOI of 10. Bacterial numbers for invasion was determined by taking bioluminescent readings after the bacteria was added to the macrophages (T=5 min) and dividing by the 2 h time point after the gentamicin protection assay to get a percentage of intracellular bacteria compared to initial bacterial numbers. Adherence was determined by dividing bacteria added to the macrophages (T=5 min) and dividing by the 20 minute time point after the cells had been washed twice with sterile 1X PBS. Invasion and adherence data was acquired using RLU readout as a function of bacterial numbers.

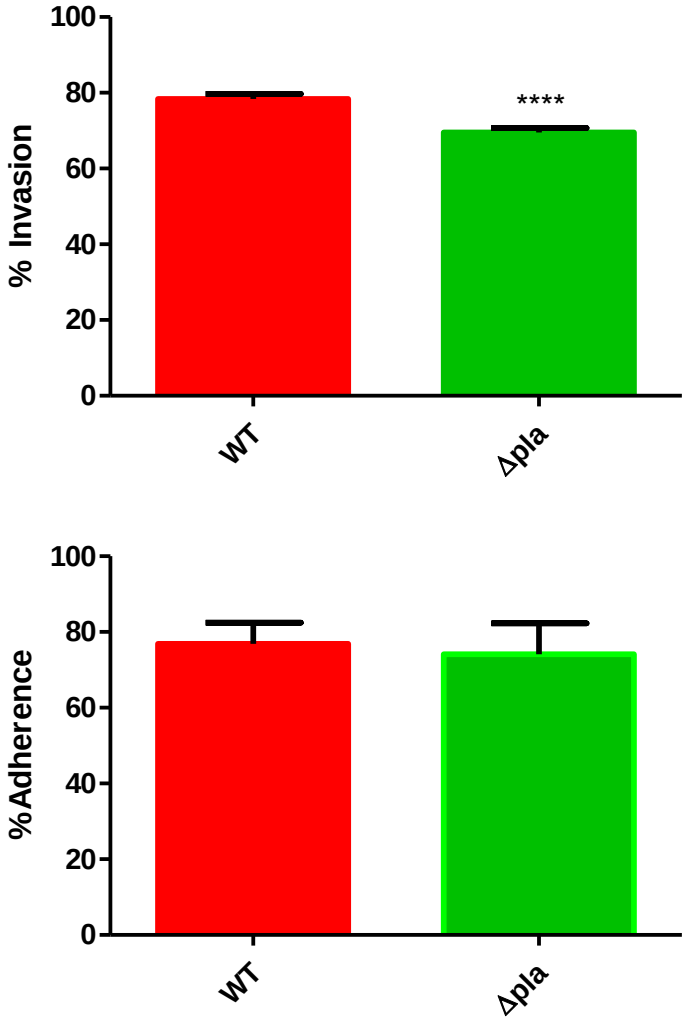


Figure 5-6 Δ*pla* does influence invasion but not adherence. RAW264.7 macrophages were infected with *Y. pestis* YPA138 (n=18) or YPA050 (n=18) at an MOI of 10. Bacterial numbers for invasion was determined by taking bioluminescent readings after the bacteria was added to the macrophages (T=5 min) and dividing by the 2 h time point after the gentamicin protection assay to get a percentage of intracellular bacteria compared to initial bacterial numbers. Adherence was determined by dividing bacteria added to the macrophages (T=5 min) and dividing by the 20 minute time point after the cells had been washed twice with sterile 1X PBS. Invasion and adherence data was acquired using RLU readout as a function of bacterial numbers. (*** = P<0.0001)

CHAPTER SIX

DISCUSSION AND FUTURE DIRECTIONS

Research Summary

The evaluation of the role adhesins play in the virulence of *Y. pestis* has been widely studied in how they are able to resist complement, aggregate for wider dissemination and their contribution to overall fitness of the bacteria. In the studies that have been previously outline, I explore the regulation and intracellular survival of the virulence factors yapE and Pla.

While investigating how *yapE* is regulated, we found that many of the transposon insertion sites were found in sugar processing and metabolizing genes. There was one insertion into a putative phage protein, where I was unable to induce the expression of *yapE*. I believe that this is because the true regulator is either 1) an essential gene or 2) the regulator is a sRNA that has not been annotated within the bacterial sequence. After doing and extensive search of the YapE sequence against the published sRNAs, I was unable to find a match between the annotated sRNA with the promoter region of *yapE*. I believe that further investigation of sRNAs should be continued because data has shown that sRNAs have regulatory control over stress response such as iron depletion and sugar stress. This could be another reason why I was able to get a positive response when sugar transport and metabolism genes had transposon insertion. sRNAs are also implicated in the regulation of outer membrane proteins including porins and adhesins [94]. To this end, I recommend the following future aims to evaluate sRNAs for regulation of *yapE*: To determine how *yapE* is being regulated I would focus on 3 sRNAs that have been shown to regulate crp, hfq and Ysr170 By modulating the expression

levels of target genes, sRNAs can enable rapid adaptation of cellular physiology in response to environmental change. [148].

Outer membrane proteins processes by pla:

Pla has been shown to process and/or degrade outer membrane proteins and analysis performed by 2-DIGE and mass spectrometry showed at least 76 proteins. Because I only looked at the top ten proteins under the parameters of >2-fold increase or decrease of expression, there is a strong chance that I missed out on some that may have worked in concert with other proteins that may have influence other factors of cleavage or degradation. Future directions is to further investigate the proteins that were not evaluated and explore the role those proteins may play in the virulence of *Y.pestis.*

Intracellular survival of mutations ∆ail, ∆ompA, ∆yapE and ∆pla

When evaluating intracellular survival by the *yapE* mutant, there was only a slight attenuation observed that was statistically significant but not believed to be biologically relevant. This may be in part to the redundancy of other autotransporters and adhesins that may compensate for the lack of one of their expression. As stated previously, the attenuation observed in the ∆*ompA* strain may be more attributed to its inability to resist complement and targeted by PMNs early in infection and thereby decreasing the amount of bacteria that is being taken up by the macrophages. As for the *pla* mutation, this may also be a two-fold reason for the reduction of bacteria due to its primary role of being a protease. The inability to activate plasminogen to allow for the dissemination of the bacteria allows for fewer bacteria to migrate to draining lymph nodes. Moreover, as shown in previous

studies, Pla also degrade Yops. Intracellularly, if Yops are identified early in the macrophage they are able to illicit a response from the inflammasome that can cause the cell to enter pyroptosis effectively killing the bacteria.

Future direction:

Is ∆pla attenuated intracellularly due to the inability to degrade Yops?

To evaluate if the degradation of Yops are a fundamental cause for the survival defect observed in a *pla* mutant, I would infect macrophages with a T3SS/*pla* double mutation and compare the viability against the single *pla* mutant. In addition to an infection study to observe the intracellular survival defect, after infecting with a ∆*pla* strain of *Y.pestis*, test for ruptured plasma membrane, and/or DNA fragmentation. Both signal that the cell is going under pyroptosis and a hallmark of inflammasome activation.

Does double elimination of yapE and pla increase attenuation?

To investigate attenuation intracellularly when *pla* and *yapE* are mutated, I would look at performing an infection using a strain that was ∆*pla* and ∆*yapE* to see if the attenuation is more severe than they are individually. Previous studies have shown when 2 or more outer membrane proteins (Ail and Pla) were mutated that there was significant attenuation [149]. Ail being one of the outer membrane proteins that we found in our 2-D/mass spec screen showed that there was a decreased in a pla mutant. This may show that Pla's involvement in intracellular survival may be in tandem with other outer membrane proteins. When using a ∆*pla* and ∆*yapE* dual mutant, I would look at the reduction of bacterial attachment and uptake with macrophages. This may show that the processed form of YapE that is key to

activation for adhesion but its full form may contribute to being noticed by the cell that would increase its elimination by the cell. To follow up on the processing event of YapE, I would also investigate mutating the site Pla recognizes to cleave YapE and if that may play a role in intracellular survival.

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APPENDIX

Acknowledgements

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Komolafe, O., **Templeton, T.**, Srivastava, D., Thacker, B., Lavender, N., Doll, M., Hein, D., Brock, G., Kidd, L.R. Multifaceted Analytical Approach for Predicting Prostate Cancer Susceptibility Among African-American Men. ***School of Public Health and Information Sciences***, University of Louisville, Louisville, KY, April 24, 2007.

Templeton, T.M., Thacker, B., Srivastava, D.S., Komolafe, O.O., Pihur, V., Brock, G., Doll, M.A., Hein, D., Kidd, L.R. Interaction Among Carcinogen-Biotransformation Genes (*N*-Acetyltransferase 1 and 2) and Prostate Cancer Risk Using a Comprehensive Analytical Approach. ***5th Annual James Graham Brown Cancer Center Retreat Research Louisville***, University of Louisville, Louisville, KY, November 29, 2006.

Komolafe, O.O., Srivastava, D.S., Thacker, B., **Templeton, T.M.**, Doll, M.A., Hein, D.W., Pihur, V., Brock, G. and Kidd, L.R. Combined Effects of Variant N-Acetyltransferase 1 (NAT1) and Multiple Drug Resistance 1 Genes (*NAT1* and *MDR1*) in Prostate Cancer Risk. ***5th Annual James Graham Brown Cancer Center Retreat Research Louisville***, University of Louisville, Louisville, KY, November 29, 2006.

Srivastava, D.S., Komolafe, O.O., **Templeton, T.M.**, States, J.C., Brock, G., Kidd, L.R. Interaction Among Variant Base Excision Repair Genes (hOGG1, APE-1, and XRCC1) in Relation to Prostate Cancer Risk in African-American Men. ***5th Annual James Graham Brown Cancer Center Retreat Research Louisville***, University of Louisville, Louisville, KY, November 29, 2006.

Komolafe, O.O., Srivastava, D.S., Thacker, B., **Templeton, T.M.**, Doll, M.A., Hein, D.W., Pihur, V., Brock, G. and Kidd, L.R. Combined Effects of Variant N-Acetyltransferase 1 (NAT1) and Multiple Drug Resistance 1 Genes (*NAT1* and *MDR1*) in Prostate Cancer Risk. ***11th Annual Research Louisville***, University of Louisville, Louisville, KY, October 10, 2006.

Thacker, B., **Templeton, T.M.**, Srivastava, D.S., Komolafe, O.O., Pihur, V., Brock, G., Doll, M.A., Hein, D., Kidd, L.R. Interaction Among Carcinogen-Biotransformation Genes (*N*-

Acetyltransferase 1 and 2) and Prostate Cancer Risk Using a Comprehensive Analytical Approach. ***11th Annual Research Louisville***, University of Louisville, Louisville, KY, October 10, 2006 **(Received Honorable Mention During Poster Presentation Competition)**.

Srivastava, D.S., Komolafe, O.O., **Templeton, T.M.**, States, J.C., Brock, G., Kidd, L.R. Variant Oxidative Stress Gene (hOGG1) and Prostate Cancer Risk Among African-American Men. ***11th Annual Research Louisville*****,** University of Louisville, Louisville, KY, October 14, 2006.

Kidd, L.R., **Templeton, T.**, Kittles, R. Germ-line *BCL-2* sequence variants and inherited predisposition to prostate cancer. ***4th Annual Brown Cancer Center Retreat***, University of Louisville, School of Medicine, Louisville, KY, September 2005.

Fellowships

2010-2012 University of Louisville IPIBS Graduate Fellowship
2011-2014 Southern Regional Education Board Doctorial (SREB) Fellow

Awards

2015 Graduate School Travel Award: Midwest Microbial Pathogenesis Conference (MMPC)
2015 CODRE/School of Interdisciplinary and Graduate Studies Diversity Research Grant
2018 School of Medicine Diversity Award

Service and memberships

2010-Present	Microbiology and Immunology Graduate Student Organization (MISO)
2010-2014	Black Biomedical Graduate Student Organization
2009	American Society of Human Genetics (ASHG)
2009	American Society of Clinical Oncology (ASCO)
2011-2014	American Society for Microbiology (ASM)
2015-2016	Science Policy and Outreach Group (SPOG) (Secretary)

Employment

Ph.D Candidate 2010–Present

Microbiology & Immunology Ph.D Thesis Research
Dr. Lawrenz Lab, Center for Predictive Medicine; Louisville, KY

Title: Characterizing the Virulence Factor YapE in *Yersinia pestis*.

Goal: To characterize the role YapE and Pla play in intracellular survival

Research Coordinator (2007–2010)

James Graham Brown Cancer Center, (U of L) Louisville, KY
Brown Cancer Center conducts both intensive clinical research and basic-science cancer research.

- Conduct, organize, manage and supervise data collection, clean-up, and analysis activities; coordinate and schedule research activities

- Develop administrative policies and procedures and establish standard operating procedures
- Organize and conduct statistical analysis of data generated from study results
- Collect, edits, and presents research outcomes and recommend new ideas for ongoing lines of research
- Conduct research on past literature, monitor current research literature for relevant information, summarize information gathered in the form of table and figures
- Participated in grant proposal, preparation and development, interpret and advise faculty of the extramural funding source guideline requirements. Assisted with the administrative portion of grant applications as needed
- Manage the laboratory including coordinating/supervising, ordering lab supplies/equipment, ensure operation of equipment, organizing paper/binders/references, maintaining files, contacting vendors/IT to repair computers and other major laboratory equipment

Researcher Technologist Senior, Acting Lab Manager (2006-2007)

James Graham Brown Cancer Center, (U of L) Louisville, KY
Brown Cancer Center conducts both intensive clinical research and basic-science cancer research.

- Design/introduce/evaluate/test/apply genotyping assays
- Train/supervise post-doc, students and summer interns and lab technicians in lab techniques and computer programs/databases
- Assist with maintenance and management of lab to ensure timely and effective completion of all planned research activities and projects
- Order lab supplies/equipment and prepare biological reagents
- Assist in writing, editing, organizing final drafts and laboratory records for exhibition, lectures, research proposals and grants
- Synthesize research information and prepare tables, figures and descriptive statistics
- Use complex databases to demonstrate that selected genetic changes alter protein structure/function or alter gene regulation
- Participate in the planning and development of objectives and project management (i.e., laboratory and/or field work).
- Establish standard operating procedures for research projects and activities

Research Technologist II (2004-2006)

James Graham Brown Cancer Center, (U of L) Louisville, KY

- Performed genotyping assays for xenobiotic metabolism genes
- Developed assays and protocols for primer design utilizing Primer3 and DS Gene Software
- Perform critical analyses of literature relevant to research undertaken.
- Conduct and coordinate experimental tests and plan the processing and analysis of research data.
- Developed and tested experimental designs and analytical methods

- Organize data search and analysis procedures.
- Participated in the planning and development of general goals for the research project and laboratory and/or fieldwork.
- Assisted in the preparation and editing of research papers and manuscripts for publication and presentation at conferences and workshops
- Developed and/or revise existing research methodologies
- Perform related duties as assigned
- Molecular Genetic skills
    - ABI 7700, 7900
    - UV Spectrophotometer
    - Polymerase Chain Reaction
    - Pyrosequencing PSQ HS96

Senior Genetic Technician II (1999-2004)

Center for Inherited Disease Research, (CIDR) Baltimore, MD

The Center for Inherited Disease Research (CIDR) is a centralized facility established to provide genotyping and statistical genetics services for investigators seeking to identify genes that contribute to human disease.

- Performed various assays to assist in developing multiplex reactions of mouse oligos
- Set up polymerase chain reactions; trouble shoot such reactions
- Prepare, load and perform electrophoresis of samples on ABI 377, ABI 3700 and ABI 3730 Fluorescent Sequencers
- Analyze data with ABI Genescan and Genotyping software
- Performed SNP assays utilizing Illumina BeadArray chemistry, SAMs, Tecan Genesis Workstation 150 and LIMS
- Promotion from Senior Genetic Technician I to Senior Genetic Technician II after six months of employment
- Assisted statistical analysis and bioinformatics group with data analysis