Examining the Effect of Quorum Sensing Molecules on the Type-Three Secretion System in *Yersinia pestis*

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PhD Candidacy Exam
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Aims:

**Aim 1:** To determine if C8 and oxo-C8 affect the ability of *Y. pestis* to modify host cell immune responses. Studies have shown that the TTSS injects *Yersinia* outer protein (Yop) effectors into host cells and these effectors are able to block the production of proinflammatory cytokines and disrupt phagocytosis, increasing the chances of survival for the invading bacterium [15]. I propose to determine if down-regulation of TTSS genes by exposure to C8 and oxo-C8 prevents *Y. pestis* from making any of these host cell modifications by measuring phagocytosis of *Y. pestis* bacteria, production of host cell cytokines, and apoptosis. I will achieve this by performing double immunofluorescence, sandwich ELISA, and Annexin V/PI staining, respectively. I will also measure the amount of pro-apoptotic proteins to further characterize the level of apoptosis of host cells.

**Aim 2:** To determine if C8 and oxo-C8 affect TTSS apparatus assembly and/or secretion. YscC is an outer membrane protein that belongs to the family of secretins. This protein forms a stable multimeric complex which comprises the outer membrane ring of the injectisome apparatus. Down-regulation or defective expression of YscC prevents the formation of the injectisome and therefore prevents secretion of the Yop effectors [16-17]. I propose to determine if the injectisome is being assembled by performing immunoblot assays using antibodies against the YscC protein. Not only does exposure of *Y. pestis* to C8 or oxo-C8 prevent *Y. pestis* from making any of these host cell modifications by measuring phagocytosis of *Y. pestis* bacteria, production of host cell cytokines, and apoptosis. I will achieve this by performing double immunofluorescence, sandwich ELISA, and Annexin V/PI staining, respectively. I will also measure the amount of pro-apoptotic proteins to further characterize the level of apoptosis of host cells.

**Aim 3:** To determine if the down-regulation of the TTSS and Yop effectors is a result of the inability of ClpXP and Lon proteases to degrade YmoA. YmoA is a small, histone-like protein that regulates the transcriptional activation of VirF. When YmoA is present, DNA remains bent and/or supercoiled and transcription cannot occur, so this protein is readily degraded when cultures of *Y. pestis* are shifted to TTSS-activating conditions [11-14]. ClpXP and Lon are proteases that are essential for the temperature-dependent degradation of YmoA, thus relieving the repression of TTSS-specific transcription [12]. I propose to determine if C8 and oxo-C8 causes down-regulation of TTSS genes through ClpXP and Lon disruption. I will test this by measuring protein levels and degradation of YmoA by immunoblot assays in TTSS-inducing conditions. I will also test ΔymoA and ΔClpXPΔLon mutants for expression of *yopE* or *yscC* in the presence of C8 or oxo-C8 by *lacZYA* expression assays.
B. BACKGROUND AND SIGNIFICANCE

B.1. *Yersinia pestis*, an obligate parasite

The genus *Yersinia* is composed of at least 15 species, three of which (*Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Yersinia pestis*) are infectious to mammals. Bacteria within this genus are Gram-negative, rod-shaped, facultative anaerobes (Fig. 1). *Y. enterocolitica* and *Y. pseudotuberculosis* are food- and water-borne pathogens that cause often non-fatal gastrointestinal disease in mammalian hosts. *Y. pestis*, which is thought to have diverged from *Y. pseudotuberculosis* within the last 1,500 to 20,000 years, is the causative agent of bubonic, septicemic, and pneumatic plague [13-15, 19-24]. Multiple pandemics of plague have been recorded throughout history, including the infamous “Black Death” which claimed an estimated 17 million to 28 million Europeans (roughly 30%-40% of the European population) in the mid 14th century. Still today, thousands of cases of plague are reported each year worldwide. This deadly pathogen has also been used as a weapon of bioterrorism. In the 14th century, the corpses of plague victims were catapulted over city walls. Ceramic bombs containing plague infected fleas were dropped on Chinese villages in the early part of the 20th century. Later in the 20th century, a more sophisticated means of aerosolizing the organism was investigated by several countries [13, 23-26]. The fact that *Y. pestis* is classified as a re-emerging pathogen by the World Health Organization (WHO) [27] and the potential to use this organism as a deadly weapon only strengthens the need for a better understanding of this bacterium’s strengths and weaknesses. Understanding the mechanism of quorum sensing molecules and their effects on different virulence factors in *Y. pestis* will pave the way for novel therapies against this bacterium.

Unlike *Y. pseudotuberculosis*, *Y. pestis* is a flea-borne pathogen that is most commonly associated with small rodents, although this bacterium can infect most mammals [2-3, 13, 19-20, 23-25, 28-29]. *Y. pestis* is thought to persist in maintenance cycles, in which there is an enzootic or latent/persistent phase and an epizootic or active phase. During the epizootic phase, significant amplification of infections occurs within highly susceptible populations, resulting in epidemics and/or pandemics [20, 28]. Many speculations have been made as to how *Y. pestis* can persist in between epizootic cycles. Proposed explanations include heterogeneous infection within highly resistant populations, persistence in fleas, and survival in soil [20, 28]. Although some studies have claimed that *Y. pestis* cannot live outside a vector or host, several groups have demonstrated the viability and virulence of *Y. pestis* after prolonged growth in sterilized soil [28]. The dominant paradigm for flea-borne transmission of plague has been dissemination by “blocked fleas”. In 1914, Eisen and colleagues showed that plague bacilli form a blockage in the proventriculus of oriental rat fleas, *Xenopsylla cheopis* [20]. The proventriculus, a valve that connects the esophagus to the midgut in fleas (Fig 2A), is lined on its interior wall with spines composed of the same material as the insect’s exoskeleton (Fig 2B). *Y. pestis* is able to adhere to these spines and form a biofilm that eventually blocks blood meal from reaching the midgut, leaving the flea in a state of perpetual hunger (Fig 2C&D). As a result, the flea will...
continually feed in an attempt to satisfy this hunger, as well as regurgitate blood meal that is overloaded with plague bacilli [2-3, 20]. Interestingly, *Oropsylla montana*, which is considered the North American primary flea vector of *Y. pestis* to humans, rarely experiences blockage of the proventriculus by plague biofilms. The fact that this flea can still infect highly susceptible California ground squirrels (*Spermophilus beechyii*) and rock squirrels (*Spermophilus variegatus*) during epizootic periods suggests that a mode of transmission alternative to the “blocked flea” paradigm is present in *Oropsylla-Spermophilus* systems [20].

Plague can be transmitted to humans by (1) infected flea bites or coming in contact with infected rodents; (2) consuming infected animals that have contracted the disease; or (3) handling cats that were infected by eating rodents contaminated with the bacterium [23]. Human-to-human transmission is also possible through the inhalation of respiratory droplets from an infected individual, although this mode of infection is specific to the pneumonic form of plague [21, 23, 26]. Bubonic plague is the most common form affecting humans. Following an incubation period of 3-7 days, patients experience fever, malaise, nausea, vomiting, diarrhea, and subsequently the formation of buboes (tender, swollen lymph nodes), due to the replication of bacteria in the draining lymph nodes. Contraction of bubonic plague can also lead to septicemic or secondary pneumonic plague. Primary pneumonic plague, which is much more severe than bubonic plague, rapidly progresses from flu-like symptoms to bloody sputum after a short incubation period of 1-3 days. The mortality rate of this pathogen is on average around 15%, with proper antibiotic treatment. If, however, proper treatment is not taken or the disease is diagnosed too late, mortality can climb to 70% to 100%, depending on the route of infection [21, 23-24, 30].

In laboratory animals, bacterial concentrations in the blood will climb to $10^8 - 10^9$ cfu/ml. Since fleas consume such small amounts of blood (0.1 - 0.3 µl) per meal, high concentrations of plague bacilli in a host system are needed to efficiently infect these insect vectors [29]. Contrary to ticks and mosquitoes which seek out a new host for each feeding, fleas tend to feed repeatedly on the same host until such events as death or the appearance of a newly born host. Once bacterial concentrations in a host blood stream reach the high level needed to infect feeding fleas, death of the host is likely, which will force the newly infected fleas to seek out new hosts, thus increasing the likelihood of transmission [29]. Once inside a human host, *Y. pestis* causes a biphasic infection. The early stage of infection is distinguished by an immeasurable host immune response and replication of bacilli intracellularly and extracellularly. During the later stage of infection, plague bacilli will continue to replicate, although mostly extracellularly, and an influx of phagocytic cells will result in inflammation, cytokine production, and tissue necrosis in infected areas [25]. *Y. pestis* is able to either resist phagocytosis or avoid destruction within phagolysosomes due to the induction of a number of virulence factors including a Type Three Secretion System (TTSS), the formation of a capsule composed of F1 antigen, and several other secreted and expressed effector proteins [1, 13-14, 22, 24, 30-34].

The plague genome contains three plasmids, pCD1, pPCP1, and pMT1, the latter two being unique to *Y. pestis*. Within these three plasmids are encoded a multitude of genes that play significant roles in the pathogenesis of *Y. pestis* [13, 19, 24]. Within the flea (28°C), plague bacilli do not express many host-specific virulence factors; only genes needed for colonization or basic survival within the vector, but at host body temperature (37°C), a wide variety of virulence factors are up-regulated for colonization and dissemination of disease [11, 13, 24]. Several very important virulence factors are components of the TTSS of *Y. pestis*. Encoded on the pCD1 plasmid, TTSS-specific genes can be divided into three general groups: (1) the secretion apparatus (Ysc), (2) secreted effector proteins (Yops), (3) regulators of the TTSS, such as chaperones (Sycs) and transcriptional regulators (VirF/LcrF) [16, 35]. The secretion apparatus consists of a basal body topped by a needle-like apparatus (Fig. 3A). The basal body, which

![Figure 3: The Ysc injectisome. (a) Schematic representation of the Ysc injectisome spanning the outer membrane (OM), the peptidoglycan layer (PG) and the cytoplasmic membrane (CM) of the bacterium. (b) An electron micrograph of injectisome needles protruding from *Yersinia enterocolitica* E40. Image courtesy of L. Journet, P. Broz and G.R.C., University of Basel (Biozentrum), Switzerland [1].]
contains the protein pump, spans the two bacterial membranes and the peptidoglycan layer and the needle-like apparatus protrudes from the cell (Fig. 3B) [1, 15, 36-38]. YscC polymerizes and forms the distal part of the basal body [1, 16]. The needle itself is formed by the polymerization of YscF, while YscP regulates the length of the needle [1, 15, 17]. To date, six Yop effector proteins have been characterized in Y. pestis, YopH, YopO/YpkA, YopE, YopJ/YopP, YopT, and YopM (summarized in Fig. 4) [1, 13-15, 19, 30, 33, 37-38].

B.2. Interspecies and Intraspecies Communication by means of Quorum Sensing

Quorum sensing can be defined as the regulation of gene expression in response to variations in cell-population density. Organisms create and release specific signal molecules referred to as autoinducers. As cell density increases, the concentration of these autoinducers increases. Once a minimal threshold concentration is detected by an organism, alterations in gene expression are made. This phenomenon was first identified over 30 years ago in the luminescent bacteria Vibrio fischeri and Vibrio harveyi. Researchers observed that light emission only occurred at high cell-population densities. There are many different mechanisms by which organisms secrete autoinducers into their medium. Passive diffusion, efflux pumps, and specific transporters have been shown to be involved in autoinducer export [39-41].

Scientists have discovered a diverse array of physiological activities, such as symbiosis, sporulation, motility, biofilm formation, competence, conjugation, and antibiotic production, which are regulated by quorum sensing. Surface-associated biofilms have been shown to be partially regulated by quorum sensing in environmental and medically relevant bacteria [39-41]. To increase the chances of receiving DNA from closely related strains, some organisms use quorum sensing to regulate the uptake of extracellular DNA. Quorum sensing has also been implicated in host-microbe interactions for both pathogens and symbionts that live in association with plant or animal hosts [42].

Not only does quorum sensing occur within a species, but it can also occur between species. In response to indole produced by Escherichia coli, Pseudomonas aeruginosa, which does not produce indole, will increase biofilm production. Alternatively, E. coli will decrease biofilm production in the presence of acyl-HSL produced by P. aeruginosa (E. coli does not produce acyl-HSL) [39].

Several human pathogens use quorum sensing to alter virulence factors and ensure successful colonization and/or dissemination. For example, Staphylococcus aureus normally exists as a benign commensal organism in humans, but if this bacterium penetrates host tissues, it can become very harmful if not deadly. At low cell density, protein factors specific for attachment and colonization are expressed, but when cell density crosses a certain threshold, these protein factors are down-regulated and replaced by secreted toxins and proteases used in dissemination. The quorum sensing system of S. aureus is responsible for this density-dependent change [41]. Contrary to this pattern, researchers recently reported that a buildup of AHLs leads to the down-regulation of the TTSS in Pseudomonas aeruginosa [43]. Interestingly, a second study found that essential components of the TTSS of Y. pestis were also down-regulated by AHLs (C8 and o xo-C8) exogenously added to buffered culture medium [9].
B.3. Quorum Sensing and *Yersinia pestis*

In order to produce autoinducers and respond to their presence, Gram-negative bacteria need a synthase (LuxI) as well as a signal transduction network, which usually involves a response regulator (LuxR) and/or a sensor kinase protein. When levels of autoinducers reach a threshold, they bind to the “LuxR” response regulator and induce expression of the “LuxI” synthase, which produces more autoinducers, thereby cycling in a positive feedback mechanism [40-41]. In *Y. pestis*, two LuxR-LuxI systems have been identified, YspR-YspI and YpeR-YpeI, which produce AHLs as autoinducers. [10]. High-performance liquid chromatography (HPLC) has revealed that four distinct AHLs are produced in large quantities by plague bacilli (*Y. pestis*), N-hexanoyl-HSL (C6), N-(3-oxohexanoyl)-HSL (oxo-C6), N-octanoyl-HSL (C8), and N-(3-oxooctanoyl)-HSL (oxo-C8) [8-10]. In *Y. pseudotuberculosis*, a close relative to *Y. pestis*, the production of AHLs is regulated by temperature, as well as acidity of the medium [44]. The possibility of exogenously controlling the TTSS of *Y. pestis* could prove invaluable for the treatment of plague infections, especially the more severe pneumonic form.

C. RESEARCH DESIGN AND METHODS

C.1. Aim 1: To determine if C8 and oxo-C8 affect the ability of *Y. pestis* to modify host cell immune responses.

C.1.1. Rationale: The goal of this aim is to determine if *Y. pestis* can suppress the immune responses of phagocytic cells in the presence of C8 or oxo-C8. The TTSS of *Y. pestis* is an essential component of pathogenesis, as mutants unable to express a variety of TTSS-specific proteins are either attenuated in virulence or completely avirulent in murine models. Therefore, I hypothesize that the down-regulation of multiple TTSS-specific components by exogenous C8 and oxo-C8 will inhibit the ability of plague bacilli to silence immune cell activation and recruitment.

Macrophages and Neutrophils are cells of the innate immune system that serve as the first line of defense against invading pathogens. One feature of innate immune cells is the presence of pattern recognition receptors (PRRs), which become activated upon contact with generalized features of different invading organisms. These pathogen-associated molecular patterns (PAMPs) include ssRNA from viruses, β-glucan from fungi, and lipopolysaccharide (LPS) from Gram-negative bacteria [45-46]. Hexa-acylated lipid A is a potent activator of TLR4 in conjunction with MD-2. Interestingly, *Y. pestis* expresses hexa-acylated lipid A at 21°C to 27°C, but switches to tetra-acylated lipid A at 37°C, which has a much lower stimulatory effect on TLR4 and MD-2 [47]. Once activated by bacterial components, macrophages and neutrophils undergo a series of events to facilitate phagocytosis, cytokine and chemokine production, and in some cases apoptosis to kill intracellular pathogens [45-46]. Phagocytosis is a process, by which the eukaryotic cell must rearrange its filamentous actin (F-actin) through activation of Rho GTPases (Rho, Rac, and Cdc42) (Fig. 5). Rho is responsible for the formation of stress fibers, Rac1 induces production of lamellipodia, and Cdc42 activates the assembly of filopodia. These GTPases can be found cycling between their inactive GDP-bound state and their active GTP-bound state. Guanine-nucleotide exchange factors (GEFs) activate GTPases through the exchange of a GDP for a GTP and GTPase-activating proteins (GAPs) inactivate them by hydrolysis of GTP. They can be maintained in their inactive GDP-bound state by guanine nucleotide dissociation inhibitors (GDIs) [48-50]. YopH, YpkA, YopE, and YopT all function to prevent phagocytosis by disruption of the actin cytoskeleton in macrophages [1, 15, 33, 37-38]. In addition to phagocytosis, macrophages and neutrophils have signaling pathways that lead to changes in gene expression. Activating these mitogen-activated protein kinase (MAPK) and IκB kinase (IKK) signaling pathways leads to the induction of proinflammatory cytokines and chemokines, such as tumor necrosis factor α (TNF-α) and interleukin-8 (IL-8). Additionally, activation of NFκB through the IKK pathway results in the induction of anti-apoptotic genes and the down-regulation of pro-apoptotic genes.
YopJ prevents the induction of inflammatory cytokines and chemokines, as well as promotes apoptosis of macrophages [37]. Although the effector function of YopM has not been fully elucidated, one study observed that the secretion of this effector protein correlated with a decrease in the expression of IL-15 on macrophages and the α chain of IL-15 receptor on natural killer (NK) cells. IL-15 is important in the activation and maintenance of circulating NK cells and YopM is postulated to inhibit the recruitment of activated NK cells to sites of plague infection (summarized in Fig. 4) [53].

These *Y. pestis* effector proteins are extremely important in preventing the host innate immune system from mounting a strong response against plague infection, as five of the six proteins have been shown to be essential for full virulence of the pathogen [33, 37]. I predict that down-regulation of Yops by exogenous C8 and o xo-C8 prevents these effectors from inhibiting phagocytosis and the secretion of cytokines, as well as inducing apoptosis within macrophages.

### C.1.2. Experimental Design:

The experimental approach will be to use various techniques to test the ability of macrophages to internalize plague bacilli, secrete cytokines and chemokines, and to avoid apoptosis in the presence of *Y. pestis*. To achieve this, I will expose two strains, *Y. pestis* KIM5 and *Y. pestis* KIM5/ΔyscC, to different concentrations (100µM or 1mM) of C8 or o xo-C8 in dimethyl sulfoxide (DMSO) or DMSO alone. *Y. pestis* KIM5 will serve as the wild-type strain with unaffected expression and secretion of Yops and *Y. pestis* KIM5/ΔyscC will serve as a control strain, as deletion of yscC, one of the core components of the injectisome, will prevent secretion of Yops independent of AHLs. I will obtain the *Y. pestis* KIM5/ΔyscC strain from Dr. Susan Straley [18].

The THP-1 human monocyte cell line, which is routinely used to mimic *in vitro* activity of activated macrophages, will be utilized in this study. Plague bacilli will be grown in TMH-LC (low calcium medium) [54], unless otherwise specified. THP-1 cells will be grown in complete Dulbecco’s Modified Eagle Medium (DMEM) (DMEM + 4.5 mg/ml glucose, 10% Fetal Bovine Serum [FBS], 2mM L-glutamine, 1mM sodium pyruvate, 50mM 3-(N-morpholino)propanesulfonic acid [MOPS], pH 7.0) unless otherwise specified. *Y. pestis* will be grown in TMH-LC at 28°C with shaking to an OD₆₀₀ of 0.1, at which time C8, o xo-C8, or DMSO will be added to cultures and tubes will be incubated for an additional hour at 28°C with shaking. THP-1 cells, maintained in complete DMEM at 37°C with 5% CO₂, will be collected, counted, and diluted to 2 x 10⁷ cells/ml in complete DMEM. 1 x 10⁶ cells of plague bacilli (MOI 5) will be added to appropriate wells and plates will be centrifuged briefly to facilitate adherence. Wells with THP-1 cells alone will serve as controls (Table 1). This experimental setup will be used for all assays in this section. For measurement of cytokine/chemokine production and apoptosis, the same medium will be collected for measuring cytokine/chemokine production and the cells will be used for the measurement of pro-apoptotic protein levels.

<table>
<thead>
<tr>
<th>THP-1 Alone</th>
<th><em>Y. pestis</em> KIM5</th>
<th><em>Y. pestis</em> KIM5/ΔyscC</th>
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<tr>
<td>+DMSO</td>
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<tr>
<td>+100µM C8</td>
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</table>

Table 1: Growth conditions for quorum sensing experiments.

C.1.2.1. Measurement of phagocytosis

Sterile glass coverslips will be placed at the bottom of each well before the addition of any medium or cells for adherence. Once cells are added to all wells, plates will be incubated at 37°C with 5% CO₂ for 30 minutes. Coverslips will be removed, washed three times in phosphate-buffered saline (PBS), and maintained at 4°C. Double immunofluorescence will be employed to calculate extracellular and intracellular bacteria. For extracellular bacteria, coverslips will be incubated with rabbit anti-*Yersinia* antiserum (Ake Forsberg [31]) for 15-20 minutes at -20°C to inhibit phagocytosis. Coverslips will be washed three times in PBS, fixed in ice-cold methanol for 90 seconds, and air dried. TRITC (tetramethyl rhodamine isothiocyanate)-conjugated anti-rabbit secondary antibodies (Thermo Scientific) will be exposed to coverslips for 30 minutes at 37°C. After three
washes in PBS, coverslips will be again exposed to rabbit anti-Yersinia antiserum for 1 hour at 37°C to stain all intracellular and extracellular bacteria. After another three washes, I will incubate coverslips with FITC (fluorescein isothiocyanate)-conjugated anti-rabbit secondary antibodies (Thermo Scientific) for 1 hour at 37°C. Coverslips will be washed in PBS to remove excess antibodies and mounted on glass slides with mounting solution (20% Airvol [Air Products] and 4% Citifluor [Citifluor, Ltd.] in 20mM Tris-HCl, pH 8.5). A fluorescence microscope equipped with a Plan-apochromat 63X/1.40 oil immersion objective will used to examine samples. The extracellular bacteria will be detected by excitation at 530 to 585 nm and total bacteria will be examined by excitation at 450 to 490 nm. The percentage of intracellular bacteria will be calculated.

C.1.2.2. Measurement of cytokine and chemokine production

All plates will be incubated at 37°C with 5% CO₂ for 6 hours. The culture medium from each well will be collected at 0, 1, 3, and 6 hours. These supernatants will be stored at -20°C until all time points have been collected, upon which time a sandwich enzyme-linked immunosorbent assay (ELISA) assay will be performed. Antibodies to Tumor Necrosis Factor (TNF-α) and Interleukin (IL-8) (2µg/ml) in 5% nonfat milk will be used to coat enhanced protein-binding ELISA 96 well plates (BD Biosciences) overnight at 4°C. Once unbound antibodies are washed away with PBS/Tween, 5% nonfat milk will be added to block non-specific binding. Samples will be added to the wells and allowed to bind to antibodies overnight at 4°C. Wells will be thoroughly washed with PBS/Tween and biotinylated detection antibodies to TNF-α and IL-8 (1µg/ml) in 5% nonfat milk will be added to each well and incubated for 1 hour at room temperature. After extensive washes in PBS/Tween, Avidin-horseradish peroxidase will be added to wells for 30 minutes at room temperature, after which ABTS (2,2’-Azipinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate solution will be used to facilitate a color change. Once sufficient color has been created, reactions will be stopped with a stop solution (20% sodium dodecyl sulfate [SDS]/50% dimethylformamide [DMF]) solution and reactions will be read at OD₄₀₅. Exogenous TNF-α and IL-8 will be added to control wells for positive control of color change. All needed antibodies or reagents will be obtained from BD Biosciences. The level of cytokine and chemokine production will be calculated relative to negative and positive controls.

C.1.2.3. Measurement of phagocyte apoptosis

All plates will be incubated at 37°C with 5% CO₂ for 6 hours. To assess apoptosis of the THP-1 cells, I will use two approaches (1) measure the level of proteins known to be involved with apoptosis (caspase 8, caspase 3, BclXL, and RIP) and (2) stain cells with Annexin V-FITC and propidium iodide (PI) for Flow Cytometry (FACS) analysis. (1) Cells will be collected by scraping and transferred to microfuge tubes at 0, 1, 3, and 6 hours. After washing the cells once in 1x PBS, I will resuspend the pellet in SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) sample buffer. I will run the samples on an SDS-PAGE 12.5% polyacrylamide gel and transfer proteins to a Polyvinylidene Fluoride (PVDF) membrane. After blocking the membrane in 5% nonfat milk, I will incubate the membrane with primary monoclonal antibody (BD Biosciences) diluted in 5% nonfat milk for 2 hours at room temperature, after which I will thoroughly wash the membrane with 1x Tris-buffered saline/Tween (TBST). HRP-conjugated secondary antibody (Amersham Biosciences) diluted in 5% nonfat milk will then be incubated with the membrane at room temperature for 1 hour, after which subsequent thorough washes will occur. I will activate chemiluminescence by using Enhanced Chemiluminescence (ECL) reagents made fresh in lab (in 10 ml 100mM Tris-HCl, pH 8.5, 50 µl luminal, 22 µl cumaric acid, 3 µl H₂O₂). I will be able to visualize protein bands after removal of ECL reagents from the membrane by laying a film down with the membrane (wrapped in saran wrap) for 1 min to 5 min, depending on the strength of the signal. Each antibody will be used separately, followed by stripping of the membrane for the next reaction. I will strip the membrane by incubating it in a Tris/SDS/2-mercaptoethanol solution for 30 min at 50 °C with shaking. I will then wash the membrane thoroughly with 1x TBST and proceed with the next antibody. As a control, I will probe for tubulin (Calbiochem). Protein concentrations will be determined by using a Typhoon 8600 and ImagQuant 5.1 software. I will run an identical gel and stain with coomassie blue as a loading control. (2) At 0, 1, 3, and 6 hours, I will add Annexin V-FITC (Millipore) directly to culture medium to a final concentration of 3 µg/ml (away from light). After a 3 minute incubation, cells will be collected by scraping and washed several times in complete DMEM to remove excess Annexin V-FITC and resuspended to a final concentration of 1 x 10⁶ cells/ml. 500 µl samples will be separated from cultures and PI (Millipore) will be added to samples to a final concentration of 1 µg/ml for 15 minutes at room temperature in the dark. After staining, I will wash cells
and fix them with 4% paraformaldehyde overnight to be analyzed by FACS the next day. The percentage of apoptotic cells relative to total cells will be calculated.

C.1.3. Expected Outcomes:

C.1.3.1. Measurement of phagocytosis

<table>
<thead>
<tr>
<th>THP-1 Alone</th>
<th>Expected Results</th>
<th>KIM5 + THP-1</th>
<th>Expected Results</th>
<th>KIM5/ΔyscC + THP-1</th>
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<td>+ o xo-C8</td>
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Table 2: Expected results for phagocytosis experiment

I expect that the KIM5 strain treated with either C8 or o xo-C8 will show an increase in phagocytosis compared to the KIM5 strain not treated with the AHLs (Table 2). The Yop genes in the KIM5/ΔyscC strain are not secreted due to the loss of YscC, an essential component of the injectisome. This strain serves as a positive control and I expect these bacilli to be readily phagocytosed by the THP-1 cells, regardless of treatment with C8 or o xo-C8. I also expect that the increase in phagocytosis will correlate to the amount of C8 or o xo-C8 added to the medium, since more AHLs lead to a more pronounced down-regulation of TTSS components [9].

C.1.3.2. Measurement of cytokine and chemokine production

<table>
<thead>
<tr>
<th>THP-1 Alone</th>
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<td>+ o xo-C8</td>
<td>TNF-α/IL-8</td>
<td>+ o xo-C8</td>
<td>TNF-α/IL-8</td>
<td>+ o xo-C8</td>
<td>TNF-α/IL-8</td>
</tr>
</tbody>
</table>

Table 3: Expected results for cytokine/chemokine experiment

I expect that treatment of the KIM5 strain with C8 or o xo-C8 will prevent the inhibitory effects on proinflammatory molecules (Table 3). I expect to measure significant amounts of TNF-α and IL-8 in the medium surrounding cells, which will correlate with the concentration of C8 or o xo-C8 added to the medium. I also expect to see this response for the KIM5/ΔyscC strain, regardless of exposure to C8 or o xo-C8. For the untreated KIM5 strain, I expect to see a significantly lower amount of TNF-α and IL-8 in the medium, as this strain expresses all TTSS genes.

C.1.3.3. Measurement of phagocyte apoptosis

<table>
<thead>
<tr>
<th>THP-1 Alone</th>
<th>Expected Results</th>
<th>KIM5 + THP-1</th>
<th>Expected Results</th>
<th>KIM5/ΔyscC + THP-1</th>
<th>Expected Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>+DMSO</td>
<td>Low Apoptosis</td>
<td>+DMSO</td>
<td>Increased Apoptosis</td>
<td>+DMSO</td>
<td>Decreased Apoptosis</td>
</tr>
<tr>
<td>+ C8</td>
<td>Low Apoptosis</td>
<td>+ C8</td>
<td>Decreased Apoptosis</td>
<td>+ C8</td>
<td>Decreased Apoptosis</td>
</tr>
<tr>
<td>+ o xo-C8</td>
<td>Low Apoptosis</td>
<td>+ o xo-C8</td>
<td>Decreased Apoptosis</td>
<td>+ o xo-C8</td>
<td>Decreased Apoptosis</td>
</tr>
</tbody>
</table>

Table 4: Expected results for apoptosis experiment

I expect to see a significantly lower number of apoptotic THP-1 cells when exposed to KIM5 with either C8 or o xo-C8 (Table 4). Since the inhibition of NFkB activation by YopJ leads to the induction of apoptosis in macrophages, taking away the expression of this protein should prevent the cell-mediated death of THP-1 cells. Therefore, I expect to see fewer cells stained with Annexin V and PI when compared to the KIM5 untreated strain. I also expect to see an increase in pro-apoptotic protein expression for the untreated KIM5 strain, contrary to the KIM5 strain treated with C8 and o xo-C8. As stated before, I do expect to see a correlation between the number of cells undergoing apoptosis or the expression levels of pro-apoptotic proteins and the amount of AHL added to the culture for the KIM5 strain. I also expect to see fewer apoptotic THP-1 cells when exposed to KIM5/ΔyscC, regardless of C8 and o xo-C8 treatment.

C.1.4. Anticipated Problems and Alternative Strategies: Certain acyl-homoserine lactones from Pseudomonas aeruginosa have exhibited immunomodulatory activity towards macrophages in vitro. Researchers discovered the N-(3-oxododecanoyl)-L-homoserine lactone (oxo-C12) inhibits IL-12 and TNF-α production in LPS-
stimulated peritoneal macrophages. They also tested N-(3-oxohexanoyl)-L-homoserine lactone (oxo-C6), which was not active against macrophages [55]. This raises the possibility that C8 and oxo-C8 from Y. pestis also independently affect macrophages, although the study done with P. aeruginosa showed that the larger AHL (oxo-C12) was active and the smaller AHL (oxo-C6) was not. I propose to measure TNF-α and IL-8 production of LPS-stimulated (Sigma) THP-1 cells with and without the addition of C8 and oxo-C8 at the above mentioned concentrations before beginning the experiments mentioned above. This will tell me if these AHLs are causing defects in immune function. I do not expect this to be the case, since C8 and oxo-C8 are smaller AHLs similar to oxo-C6. If this is the case, I will attempt to adjust the concentrations of C8 and oxo-C8 to lower amounts to try and avoid an overpowering immunomodulatory effect.

C.2. Aim 2: To determine if C8 and oxo-C8 affect TTSS apparatus assembly and/or secretion.

C.2.1. Rationale: The goal of this aim is to determine if exogenous C8 or oxo-C8 directly down-regulates the transcription of essential components of the TTSS injectisome and all Yop proteins or if these proteins are still translated and assembled, but cannot function properly, thereby blocking further transcription of the genes. I hypothesize that there will be a significant reduction in the expression of YscC complexes, as well as a decrease in the number of Yops within the cytosol of the bacteria. I also hypothesize that no Yops will be secreted into the growth medium in TTSS-inducing conditions and that Y. pestis will fail to experience a growth restriction in TTSS-inducing conditions when exposed to AHLs.

The TTSS is an essential virulence factor encoded on the pCD1 plasmid of Y. pestis. Genes related to the TTSS are down-regulated at 28°C and in high calcium medium, suggesting the TTSS is only transcribed at set times. Also, when plague bacilli are shifted to low calcium medium or 37°C, researchers noticed a cessation of growth. Scientists have postulated that this is a result of energy expenditure on the assembly and secretion of TTSS components [1, 13-15, 18]. TTSS-specific genes can be divided into three general groups: (1) the secretion apparatus, (2) secreted effector proteins, (3) regulators of the TTSS, such as chaperones and transcriptional regulators [16, 35]. The basal body of the injectisome, which contains the protein pump, spans the peptidoglycan layer and two bacterial membranes (inner and outer) and a needle-like apparatus protruding from the cell (Fig. 2A&B) [1, 15, 36-38]. Inside the cell, YscN, the ATPase that is responsible for substrate recognition and generation of energy for Type III Secretion reactions, is speculated to polymerize to form the lower part of the cylinder [1, 15, 56]. YscQ is one of the components of the C ring, an antechamber to the protein channel located in the bacterial cytoplasm. YscV, YscU, YscR, YscT, and YscS are all components of the basal body that are in contact with the cytoplasmic membrane [1, 17]. YscC polymerizes and forms the distal part of the basal body [1, 15-16, 18]. YscJ and YscW are lipoproteins that help localize YscC and the other components of the basal body to the cytoplasmic and outer membranes. YscF, YscO, YscP, and YscX are all components of the external parts of the injectisome [1, 35]. The needle itself is formed by the polymerization of YscF, while YscP regulates the length of the needle [1, 15]. Many researchers have postulated that effector proteins are secreted in different stages. If the early proteins are not secreted, then the late stage proteins are blocked from secretion [17, 57-58]. Among early stage secretion proteins are LcrV, YopD, and YopB. YopD and YopB are hydrophobic and are therefore proposed to aid in forming pores in the eukaryotic cell membrane into which the needle is inserted [1, 15, 38, 57]. LcrV is deposited at the tip of the needle and functions in late stage Yop secretion specificity [1, 9, 14-15, 36]. Autoproteolytic cleavage of YscU, a component of the basal body, sends a signal to the TTSS that early effectors have been released and late effectors can now be secreted [1, 17]. Functions of the six late stage Yop effector proteins, YopH, YopO/YpkA, YopE, YopJ/YopP, YopT, and YopM are summarized in Figure 4.

Studies have shown that many TTSS genes are under negative feedback regulation. When there is a defect in the injectisome preventing secretion of Yops, or a negative regulation of secretion that cannot be lifted, synthesis of Yops significantly lowers [1, 15]. Also, the TTSS is proposed to expend a major amount of energy and researchers have eluded this to being the reason why bacteria have significantly slowed growth at 37°C in low calcium medium [1, 14-15, 18]. Therefore, it is possible that Y. pestis down-regulates expression of the TTSS later in infection to facilitate exponential growth and dissemination. Theoretically, as the number of bacteria increases, so does the amount of AHLs that are present in the environment. I predict that the artificial presence of large concentrations of AHLs, such as C8 and oxo-C8, trick plague bacilli into thinking they have reached a population level in which late stage virulence factors are needed, instead of the TTSS.
These virulence factors, such as the F1 capsule, still protect bacilli from phagocytosis and complement-dependent destruction, while also allowing exponential growth to achieve dissemination of the host.

C.2.2. Experimental Design: The experimental approach will be to use various techniques to measure the abundance of YscC complexes, as well as Yops both inside and outside the bacterial cell. I will also test the growth rate of plague bacilli. To achieve this, Y. pestis KIM5 and Y. pestis KIM5/ΔyscC (to measure complex formation) or Y. pestis KIM5/ΔpPCP1 and Y. pestis KIM5/ΔyscC (to measure individual Yop presence inside and outside the cell) will be grown in TMH-LC (described in section C.1.2) or TMH-HC (TMH-LC + 2.5mM CaCl₂) where indicated. I will expose strains to either different concentrations (100µM or 1mM) of C8 or oxo-C8 in DMSO or DMSO alone. As previously stated (section C.1.2), KIM5/ΔyscC is devoid of YscC, which is a major component of the injectisome. The KIM5/ΔpPCP1 does not contain the pPCP1 plasmid which encodes the plasminogen activator (Pla). Using a strain lacking the Pla protease will ensure that Yops secreted into the medium are not degraded. I will obtain this strain from Dr. Susan Straley [58].

For a number of experiments, I will require the use of primary antibodies specific to each Yop, as well as one for YscC. To acquire these, I will tag each Yop and YscC separately and use the protein collected to immunize rabbits. I will use PCR to purify each gene from Y. pestis genomic DNA. Each primer will have specific attB recombination sequences to facilitate cloning by the Gateway system (Invitrogen). I will clone each gene into pDEST 17, which encodes a 6x His tag that is fused to the N-terminal region of any insert and is under the control of the T7 promoter. Each plasmid will be transformed into BL21 (DE3) cells (Invitrogen). From here, I will grow bacteria in 0.1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce expression of each gene of interest and collect the resulting protein using Nickel-Nitrilotriacetic Acid (Ni-NTA) agarose beads. Protein will be eluted from beads and Trichloroacetic Acid (TCA) precipitated for further experiments.

To create antibodies, I will inject a rabbit with each purified protein in Freund’s complete adjuvant subcutaneously. At 4 and 8 weeks, I will boost rabbits with the purified protein in Freund’s incomplete adjuvant. Once I collect the antiserum, I will purify the antibodies using affinity chromatography on a CNBr-activated sepharose column (Amersham Pharmica) loaded with the corresponding protein I originally collected from BL21 (DE3).

C.2.2.1. Measurement of the YscC Complex
I will grow KIM5 and KIM5/ΔyscC in TMH-LC at 28°C with shaking to an OD₆₀₀ of 0.1. C8, oxo-C8, or DMSO will be added to both strains and cultures will be allowed to grow further at 28°C with shaking for 4 hours before being shifted to 37°C for 4 hours. After sufficient growth, bacterial density at OD₆₀₀ of all cultures will be measured to calculate cell count and a total of 5 x 10⁶ cells will be transferred to microfuge tubes. Pelleted bacteria will be washed once with 1x PBS and resuspended in SDS-PAGE sample buffer. I will also add 5 x 10⁶ cells of the untreated KIM5 strain to a separate tube for visualization of YscC as a monomer. In the untreated KIM5 culture, YscC should exist as a complex, since the formation of the injectisome will not be disturbed. To dissociate the complex, I will sonicate the bacteria to disrupt the cells, precipitate the protein in acetone, and treat with phenol at 70°C for 10 minutes. Protein will be run on an 8% polyacrylamide gel, transferred, and probed with antibodies as previously described in section C.1.2.3. I will use an anti-YscC primary antibody with an HRP-conjugated secondary antibody (Sigma) for detection of protein bands with ECL reagents. Protein concentrations will be determined by using a Typhoon 8600 and ImagQuant 5.1 software. I will run an identical gel and stain with coomassie blue as a loading control.

C.2.2.2. Measurement of Yop secretion
I will grow KIM5/ΔpPCP1 and KIM5/ΔyscC in TMH-LC or TMH-HC at 28°C with shaking to an OD₆₀₀ of 0.1. C8, oxo-C8, or DMSO will be added to both strains and cultures will be allowed to grow further at 28°C with shaking for 1 hour before being shifted to 37°C with shaking for 4 hours. I will measure the bacterial density at OD₆₀₀ of all cultures to calculate cell count and transfer a total of 5 x 10⁶ cells to microfuge tubes. Pelleted bacteria will be washed once with 1x PBS and resuspended in SDS-PAGE sample buffer for visualization of proteins within the bacterial cell. Proteins in the culture medium will be collected by TCA precipitation (5% final concentration) at 4°C for 18 hours. After TCA precipitation, protein will be pelleted and resuspended in solubilization buffer (500mM Tris-HCl, pH 8.0, 0.5x PBS, 0.1% SDS) to a standard final concentration based on the optical density reading before protein collection. Samples will be gently warmed to
encourage solubilization and protein equivalent to $5 \times 10^6$ cells will be run by SDS-PAGE on a 12% polyacrylamide gel, which will be transferred to a PVDF membrane. The membrane will be probed with antibodies as previously described. I will use a primary antibody with an HRP-conjugated secondary antibody (Sigma) for detection of protein bands with ECL reagents. Protein concentrations will be determined by using a Typhoon 8600 and ImagQuant 5.1 software. I will run an identical gel and stain with coomassie blue as a loading control. The membrane will be stripped and reprobed as described in section C.1.2.3. Antibodies to be used are anti-YopE, anti-YopH, anti-YopT, anti-YpkA, anti-YopJ, and anti-YopM.

C.2.2.3. Growth Time Course
I will grow KIM5 and KIM5/ΔyscC for nine generations in TMH-LC and TMH-HC at 28°C with shaking, after which cultures will be diluted to an OD$_{600}$ of 0.1. C8, oxo-C8, or DMSO will be added to tubes and cultures will continue to grow at 28°C with shaking for another hour. At this time, I will shift cultures to 37°C with shaking and grow for an additional 6 hours, taking samples and measuring OD$_{600}$ every hour.

C.2.3. Expected Outcomes:

C.2.3.1. Measurement of the YscC Complex

<table>
<thead>
<tr>
<th>KIM5</th>
<th>Expected Results</th>
<th>KIM5/ΔyscC</th>
<th>Expected Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>+DMSO</td>
<td>Full YscC complex</td>
<td>+DMSO</td>
<td>YscC complex</td>
</tr>
<tr>
<td>+ C8</td>
<td>Decreased YscC complex</td>
<td>+ C8</td>
<td>No YscC complex</td>
</tr>
<tr>
<td>+ oxo-C8</td>
<td>Decreased YscC complex</td>
<td>+ oxo-C8</td>
<td>No YscC complex</td>
</tr>
</tbody>
</table>

Table 5: Expected results for YscC complex formation experiment

I expect to see decreased amounts of YscC complex present with the KIM5 strain treated with the AHLs, since I predict that the down-regulation of Ysc genes is happening at a transcriptional level (Table 5). As discussed before, YscC is an essential component of the TTSS injectisome that polymerizes to create the distal portion of the basal body. This complex can be dissociated with phenol treatment to visualize the monomer. If Ysc genes are being down-regulated due to an inability of the complex to properly form, then I would still expect to see YscC complex formation or I would expect to see expression of YscC in the monomer form, instead of the polymer form. I would not expect to see any YscC complexes in the KIM5/ΔyscC control, as this strain does not produce any YscC proteins to form a complex. I would expect to see a “wild-type” level of YscC complex for the KIM5 untreated strain, which would also serve as a control.

C.2.3.2. Measurement of Yop secretion

<table>
<thead>
<tr>
<th>KIM5/ΔpPCP1</th>
<th>Expected Results</th>
<th>KIM5/ΔyscC</th>
<th>Expected Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>+DMSO</td>
<td>Normal Yop secretion</td>
<td>+DMSO</td>
<td>No Yop secretion</td>
</tr>
<tr>
<td>+ C8</td>
<td>Decreased Yop secretion</td>
<td>+ C8</td>
<td>No Yop secretion</td>
</tr>
<tr>
<td>+ oxo-C8</td>
<td>Decreased Yop secretion</td>
<td>+ oxo-C8</td>
<td>No Yop secretion</td>
</tr>
</tbody>
</table>

Table 6: Expected results for Yop secretion experiment

Several Yop effectors are down-regulated by C8 and oxo-C8, therefore I expect to see a significant decrease if not a complete absence in Yop secretion from the KIM5/ΔpPCP1 strain treated with C8 and oxo-C8 (Table 6). I expect a complete lack of Yop secretion for the KIM5/ΔyscC control strain in all conditions. For the untreated KIM5/ΔpPCP1 strain, I expect to see secreted Yops in the medium. Also, I predict that the down-regulation of Yop genes is happening at a transcriptional level, so I do not expect to see a significant amount of Yops in the cell fraction of the KIM5/ΔpPCP1 strain treated with C8 and oxo-C8, compared to the untreated KIM5/ΔpPCP1 strain. This is what I also expect for the KIM5/ΔyscC control strain treated with C8 or oxo-C8, but I do expect to see levels of intracellular Yops comparable to the untreated KIM5/ΔpPCP1 strain in the untreated KIM5/ΔyscC control strain. If, however, the down-regulation of Yops occurred as a result of improper Ysc complex formation, I would expect to see a significant amount of Yops in the cell fraction of the KIM5/ΔpPCP1 strain treated with C8 and oxo-C8, which would mean that the Yops are being produced, but cannot be secreted and are subsequently experiencing a halt in further transcription.
C.2.3.3. Growth Time Course

<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition</th>
<th>Expected Results</th>
<th>Strain</th>
<th>Condition</th>
<th>Expected Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM5</td>
<td>+DMSO</td>
<td>Growth arrest in TMH-LC</td>
<td>KIM5/ΔyscC</td>
<td>+DMSO</td>
<td>No Growth arrest in TMH-LC</td>
</tr>
<tr>
<td>+ C8</td>
<td>No Growth arrest in TMH-LC</td>
<td>+ C8</td>
<td>No Growth arrest in TMH-LC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ oxo-C8</td>
<td>No Growth arrest in TMH-LC</td>
<td>+ oxo-C8</td>
<td>No Growth arrest in TMH-LC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Expected results for Y. pestis growth experiment

I expect to see an absence of growth arrest for all strains in all conditions, except for the KIM5 untreated strain in TMH-LC, which should show the “wild-type” phenotype of growth restriction (Table 7).

C.2.4. Anticipated Problems and Alternative Strategies: Since all of these experiments have been successfully completed before, without the addition of C8 or oxo-C8, I do not anticipate any problems. If, however some experiments prove to be irreproducible or outcomes are ambiguous, alternative strategies can be employed. If I cannot measure protein concentrations of YscC or any of the Yops, I could instead measure gene expression using a lacZYA construct. This way, I would be able to see if yscC and Yops are being transcribed at lower levels or normal levels. If expression levels of these genes are at normal levels, then I would expect the effect of C8 and oxo-C8 to be on TTSS assembly and/or Yop secretion and not at the transcriptional level. Although the TTSS does exhibit negative feedback regulation, I would expect initial levels of gene expression to be normal and then to drop dramatically if this feedback regulation is taking place. Alternatively, if C8 and oxo-C8 are directly acting on gene expression, then I would expect expression levels to be lower than normal from the beginning of induction. For visualization of the Ysc complex, Electron Microscopy (EM) could be used in conjunction with quantum dot nanocrystals (QDs) to possibly see areas of concentration of YscC at the cell membrane, indicating the formation of a TTSS injectisome. I would use an anti-YscC antibody, a biotin-conjugated secondary antibody, and a Streptavidin conjugate of QD525 [59]. For the growth time course, I could alternatively plate out samples of cultures at different times and measure colony-forming units (CFU).

C.3. Aim 3: To determine if the down-regulation of the TTSS and Yop effectors is a result of the inability of ClpXP and Lon proteases to degrade YmoA.

Rationale: The goal is to determine if the down-regulation of these TTSS proteins as a result of the presence of exogenous C8 and oxo-C8 is due to the decrease in ClpXP and Lon expression and subsequent buildup of stable YmoA. I hypothesize that C8 and oxo-C8 affect the temperature-induced degradation of YmoA by down-regulating transcription of proteases clpXP and lon, thereby causing a negative regulation of TTSS transcription. Therefore, YmoA proteins will be more stable at 37°C. Also, I hypothesize that the down-regulation of clpXP and lon is a result of the direct binding of either YspR or YpeR to the promoter region of clpXP and lon.

The TTSS of Y. pestis is regulated in both positive and negative ways by a multitude of environmental conditions and regulatory elements. Calcium starvation and temperature shift (28°C to 37°C) are among several in vitro environmental cues used to activate the TTSS. When plague bacilli are grown in rich medium low in calcium at 37°C, one noticeable phenotype is growth arrest [1-6]. This growth response is postulated to be connected to the up-regulation of genes specific to the TTSS and to the secretion of Yop effectors, as the formation and activity of this complex expends a large amount of energy. The TTSS is also highly up-regulated when bacteria come in contact with eukaryotic cells, which is not surprising since the TTSS is a virulence determinant [1-6]. The fact that the TTSS is only induced and active under certain conditions suggests that there are negative regulators controlling expression of genes on the pCD1 plasmid and secretion of TTSS substrates.

VirF (LcrF), a trans-acting regulatory element, positively regulates numerous genes on the pCD1 plasmid. When plague bacilli are shifted to 37°C, VirF is transcribed and subsequently induces the expression of the TTSS. Interestingly, over-expression of VirF does not induce the expression the TTSS genes at 28°C, which suggests another element is involved in VirF-mediated activation. Studies show that VirF can still bind to promoter elements on the pCD1 plasmid at 28°C, so this protein does not require any conformational changes to activate transcription at 37°C [3-5, 7]. This led scientists to speculate that the promoter elements
themselves must undergo a conformational change to favor transcriptional activation at 37°C. Indeed, it was shown that there are many regions of intrinsic bending within the pCD1 plasmid at temperatures below 28°C [3, 7]. YmoA, a small histone-like protein, is involved in the compaction of DNA at lower temperatures [3-5, 7]. When temperature is raised to 37°C, YmoA is degraded and the DNA is free to be transcribed by VirF. Further experiments implicated two proteases, ClpXP and Lon, in the temperature-induced degradation of YmoA. Indeed, when both proteases are deleted from Y. pestis, YmoA builds up in the cell and transcription of the TTSS is repressed (Fig. 1a) [7]. Interestingly, in Burkholderia pseudomallei, the PmlI-PmlR quorum sensing system has been shown to modulate production of MprA, a metalloprotease, at high cell density. Inactivation of PmlI led to overproduction of MprA at high cell density [8]. Furthermore, LasR of Pseudomonas aeruginosa directly binds as a dimer to the promoter region of several genes regulated by quorum sensing [9].

I hypothesize that C8 and oxo-C8 affect the temperature-induced degradation of YmoA by down-regulating transcription of proteases clpXP and lon, thereby causing a negative regulation of TTSS transcription (Fig. 1b). Therefore, transcription of TTSS-specific genes is down-regulated, even in inducing conditions. I also hypothesize that the down-regulation of clpXP and lon is a result of the direct binding of either YspR or YpeR to the promoter region of clpXP and lon.

Experimental Design:

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM5.01</td>
<td>KIM5ΔysplΔypeIΔyscC ΔlsyI ΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), Expresses lacZYA (yscC) &amp; lacYFF (yopI)</td>
</tr>
<tr>
<td>KIM5.02</td>
<td>KIM5ΔysplΔypeIΔyosMΔyscC ΔlsyI ΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), histone-like protein (ymoA)</td>
</tr>
<tr>
<td>KIM5.03</td>
<td>KIM5ΔysplΔypeIΔclpXPΔlonΔyscC ΔlsyI ΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), proteases (clpXP &amp; lon)</td>
</tr>
<tr>
<td>KIM5.04</td>
<td>KIM5ΔysplΔypeIΔyopM ΔyosM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), Expresses lacZYA (ymoA)</td>
</tr>
<tr>
<td>KIM5.05</td>
<td>KIM5ΔysplΔypeIΔyopM ΔyopRΔyosMΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), proteases (clpXP &amp; lon)</td>
</tr>
<tr>
<td>KIM5.06</td>
<td>KIM5ΔysplΔypeIΔyopRΔyosMΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), AHL regulator (yopR)</td>
</tr>
<tr>
<td>KIM5.07</td>
<td>KIM5ΔysplΔypeIΔyopRΔyosMΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), AHL regulator (yopR)</td>
</tr>
<tr>
<td>KIM5.08</td>
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<td>Lacks AHL synthase (yopI &amp; yopE), AHL regulator (yopR)</td>
</tr>
<tr>
<td>KIM5.09</td>
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<td>Lacks AHL synthase (yopI &amp; yopE), AHL regulator (yopR)</td>
</tr>
<tr>
<td>KIM5.10</td>
<td>KIM5ΔysplΔypeIΔyopRΔyopMΔyopR ΔlacZYA ΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), AHL regulator (yopR)</td>
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<tr>
<td>KIM5.11</td>
<td>KIM5ΔysplΔypeIΔyopRΔyopMΔyopR ΔlacZYA ΔyopM ΔlacZYA</td>
<td>Lacks AHL synthase (yopI &amp; yopE), AHL regulator (yopR)</td>
</tr>
</tbody>
</table>

The experimental approach will be to test the effects of C8 and oxo-C8 on multiple steps leading to the transcription of the TTSS (Fig. 1a) in different mutant backgrounds (Table 1). I will measure yscC and yopE transcription (step 3), ymoA transcription and protein stability (step 2), and clpXP and lon transcription (step 1). Furthermore, I will test for an interaction between either YspR or YpeR and the promoter regions of clpXP and lon. All experiments will be conducted in a ΔysplΔypeI background to ensure that the amount of C8 or oxo-C8 in culture medium is controlled. To create deletion mutant strains, I will generate plasmids carrying an antibiotic resistance cassette surrounded by flanking regions for each gene to be deleted. These deletion plasmids will be electroporated into KIM5ΔysplΔypeI and allelic exchange will be
verified by PCR. All strains will be grown in TMH-LC and exposed to either different concentrations (100µM or 1mM) of C8 or o xo-C8 in DMSO or DMSO alone.

Expression of yopE and yscC

To determine if C8 and o xo-C8 are affecting expression of the TTSS through ClpXP, Lon, and YmoA, I will measure the transcription of yopE and yscC using lucFF and lacZYA reporter constructs, respectively, in a variety of mutant backgrounds. To make strains KIM5.01, KIM5.02, and KIM5.03 that contain the lucFF and lacZYA cassette under the control of the yopE and yscC promoters, respectively, I will create a pCD1 plasmid containing a lacZ YA cassette in place of yscC and a lucFF cassette in place of yopE. The altered pCD1 plasmid will be electroporated into all three Y. pestis strains (KIM5/ΔyspIΔypeI, KIM5/ΔyspIΔypeI/ΔymoA, and KIM5/ΔyspIΔypeI/ΔclpXPΔlon) and positive transformants (replacement of pCD1 with pCD1-lacZYA/lucFF) will be verified by PCR. KIM5.01 will serve as a “wild-type” strain for AHL treatment. KIM5.02 will serve as a negative control for AHL treatment, since this strain lacks the YmoA histone-like protein and therefore has constitutive expression of yscC and yopE. KIM5.03 will serve as a positive control for AHL treatment, since this strain lacks the ClpXP and Lon proteases and therefore has stable expression of YmoA and repression of yscC and yopE transcription. For the analysis of yopE and yscC expression, I will grow KIM5.01, KIM5.02, and KIM5.03, in TMH-LC medium at 28°C. I will add C8, o xo-C8, or DMSO and continue to grow cultures at 28°C. After preliminary growth, I will shift all cultures to 37°C and continue growth for an additional 3 hours. Firefly luciferase activity and β-galactosidase activity will be measured using a NovaBright β-galactosidase and firefly luciferase dual enzyme reporter gene detection system (Invitrogen). Luciferase activity will be detected immediately and β-galactosidase activity will be detected 60 minutes later. Therefore, luciferase measurements will not have background β-galactosidase activity (this needs to build up over time) and β-galactosidase measurements will not have background luciferase activity (this breaks down quickly). Chemiluminescence will be quantified on a luminometer.

YmoA expression and protein degradation

To determine if exposure of Y. pestis to C8 and o xo-C8 is altering TTSS expression through the presence of YmoA, I will measure the protein stability of YmoA in conditions favoring degradation. I will also measure ymoA transcription using a lacZYA reporter. To achieve this, I will electroporate a plasmid carrying a lacZYA cassette under the control of the ymoA promoter into KIM5/ΔyspIΔypeI, KIM5/ΔyspIΔypeI/ΔclpXPΔlon, KIM5/ΔyspIΔypeI/ΔyspR, KIM5/ΔyspIΔypeI/ΔypeR, and KIM5/ΔyspIΔypeI/ΔyspRΔypeR. Positive transformants will be verified by PCR. KIM 5.04 will serve as the “wild-type” for AHL treatment. KIM 5.05 will serve as a positive control for AHL treatment, since this strain does not contain ClpXP and Lon proteases and therefore does not exhibit YmoA degradation. KIM5.06, KIM5.07, and KIM5.08 lack one or both of the quorum sensing transcriptional regulators. These strains have the potential to serve as negative controls for AHL treatment, since loss of the regulator responsible for repressing clpXP and lon expression will result in proper degradation of YmoA. I will grow KIM5.05, KIM5.06, KIM5.07, KIM5.08, and KIM5.09 in TMH-LC medium at 28°C. I will then add C8, o xo-C8, or DMSO and continue to grow cultures at 28°C. After preliminary growth, I will shift cultures to 37°C and grow for 3 hours. I will then add chloramphenicol (40 µg/ml) to block further protein synthesis and grow cultures for an additional 3 hours. Samples will be collected at 0, 15, 30, 60, 120, and 180 minutes from all cultures. For the YmoA degradation experiment, all protein will be electrophoresed, transferred, and probed with antibodies to be detected by Chemiluminescence. I will use anti-YmoA and anti-YscQ antibodies. YscQ will serve as a protein control for YmoA stability experiments. Protein concentrations will be determined by using a Typhoon and ImagQuant software. I will run an identical gel and stain with coomassie blue as a loading control. For ymoA transcription, LacZYA expression will be measured using a NovaBright β-galactosidase reporter gene assay (Invitrogen). Chemiluminescence will be quantified on a luminometer.

Expression of clpXP and lon

To determine if C8 and o xo-C8 are affecting the TTSS through the loss of clpXP and lon expression, leading to stability of YmoA, I will measure the transcription of both proteases using lacZYA and lucFF reporter constructs in a variety of mutant strains. To make strains of KIM5.09, KIM5.10, KIM5.11, and KIM5.12 that contain the lucFF and lacZYA cassettes under the control of the lon and clpXP promoters, respectively, I will
create a plasmid containing a lacZYA cassette in place of clpXP and a lucFF cassette in place of lon. The plasmid will be electroporated into four Y. pestis strains (KIM5/ΔyspIΔypeI, KIM5/ΔyspIΔypeIΔyspR, KIM5/ΔyspIΔypeIΔypeR and KIM5/ΔyspIΔypeIΔyspRΔypeR) and positive transformants will be verified by PCR. KIM5.09 will serve as a "wild-type" for AHL treatment. KIM5.10, KIM5.11, and KIM5.12 lack one or both of the quorum sensing transcriptional regulators. These strains have the potential to serve as negative controls for AHL treatment, since loss of the regulator will prevent the AHL-mediated down-regulation of clpXP and lon. Using these three strains will also help determine which regulator (YspR or YpeR) is responsible for the AHL-mediated down-regulation of clpXP and lon. For the analysis of clpXP and lon expression, I will grow KIM5.09, KIM5.10, KIM5.11, and KIM5.12, in TMH-LC medium at 28°C. I will add C8, o xo-C8, or DMSO and continue to grow cultures at 28°C. After preliminary growth, I will shift all cultures to 37°C and continue growth for an additional 3 hours. Firefly luciferase activity and β-galactosidase activity will be measured using a NovaBright β-galactosidase and firefly luciferase dual enzyme reporter gene detection system (Invitrogen) as described above. Chemiluminescence will be quantified on a luminometer.

"LuxR" binding to clpXP and lon

To determine if either YspR, YpeR, or both directly bind to the promoter regions of clpXP and lon to alter RNA transcription of these proteases, I will perform both 1.) an Electrophoretic Mobility Shift Assay (EMSA) and 2.) a DNase I protection assay. 1.) For the EMSA, I will first acquire YopE, YpeR, and YspR by fusing an N-terminal 6x-His tag to each gene under the control of the T7 promoter and over-express each protein in E. coli using IPTG (.1mM) and C8 or o xo-C8 (for YpeR and YspR only). I will use AHLs to collect soluble YspR and YpeR because it has been shown in P. aeruginosa that LasR, the quorum sensing transcriptional regulator, must form a complex with 3-oxo C12 to reside in the soluble fraction of protein. Otherwise, LasR will form insoluble aggregates [9]. Protein will be collected using (Ni-NTA) agarose beads, eluted from beads, and Trichloroacetic Acid (TCA) precipitated. The regulatory regions of clpXP and lon will be obtained by PCR and end-labeled on both sides with [γ-32P] ATP and T4 nucleotide kinase (Invitrogen). EMSA will be performed by mixing either YspR, YpeR, or both with either the clpXP or lon end-labeled regulatory region, either C8 or o xo-C8, Herring sperm DNA (Abcam) (to eliminate non-specific binding), and DNA binding buffer. A separate reaction using YopE instead of YspR or YpeR will be performed to control for specificity of band shifts. All reactions will be electrophoresed on a native polyacrylamide gel and band shifts will be visualized using PhosphorImager technology. Once shifts have been verified, unlabeled clpXP and lon regulatory regions will be used to show competition and specificity. 2.) For the DNase I protection assay, I will use the regulatory regions obtained for EMSA, but only have one end labeled with [γ-32P] ATP, rather than both as was used for the EMSA. I will make mixtures as I did for the EMSA and after proper incubation, reactions will be exposed to DNase I. All reactions will be stopped with footprint stop solution and DNA will be ethanol precipitated. Digestion products will be separated on a denaturing polyacrylamide gel and visualized using PhosphorImager technology.

Expected Outcomes: YmoA, a histone-like protein, regulates expression of TTSS-specific genes on the pCD1 plasmid via the VirF transcription factor. When Y. pestis is grown below 28°C, YmoA is stable and DNA cannot be transcribed due to intrinsic bending. If, however, Y. pestis is grown at 37°C, YmoA is readily degraded and DNA undergoes a conformational change to promote transcription. The degradation of YmoA is mediated by two proteases, ClpXP and Lon. Researchers have reported that a loss in ClpXP and Lon results in the stable expression of YmoA at 37°C and subsequent down-regulation of the TTSS [7]. In Burkholderia pseudomallei, scientists discovered that quorum sensing molecules mediate the down-regulation of MprA protease [8]. Therefore, I hypothesize that C8 and o xo-C8 are down-regulating transcription of clpXP and lon to prevent the degradation of YmoA. Furthermore, I hypothesize that either YspR, YpeR, or both are directly binding to the promoter regions of clpXP and lon to cause this down-regulation. In P. aeruginosa, LasR directly binds as a dimer to the promoter region of several genes regulated by quorum sensing [9].
Expression of yopE and yscC

I expect that *lucFF* (*yopE*) and *lacZYA* (*yscC*) will either not be expressed or be expressed at significantly lower levels in the KIM5.01 strain treated with C8 or oxo-C8, as well as the KIM5.03 control strain (both treated and untreated) (Table 2). I also expect that while the untreated KIM5.01 strain will show normal expression levels of *lucFF* (*yopE*) and *lacZYA* (*yscC*) in the KIM5.02 strain treated with AHLs to be similar to the treated KIM5.01 strain.

YmoA expression and protein degradation

I expect to see no degradation of YmoA in the KIM5.04 strain treated with AHLs, which is also what I expect to see with the KIM5.05 control strain in all conditions (Table 3). I expect to see proper YmoA degradation in the KIM5.06, KIM5.07, or KIM5.08 strains, depending on which regulator is responsible for *clpXP* and *lon* down-regulation. I also expect to see proper degradation in the untreated KIM5.04 strain.

Expression of clpXP and lon

I expect that *lacZYA* (*clpXP*) and *lucFF* (*lon*) will either not be expressed or be expressed at significantly lower levels in the KIM5.09 strain treated with C8 or oxo-C8, (Table 4). I expect to see normal expression of *lacZYA* (*clpXP*) and *lucFF* (*lon*) in the KIM5.10, KIM5.11, or KIM5.12 strains, depending on which regulator is responsible for the down-regulation of *clpXP* and *lon*. I also expect to see normal expression of *lacZYA* (*clpXP*) and *lucFF* (*lon*) in the untreated KIM5.09 strain.

“LuxR” binding to clpXP and lon

I hypothesize that either YspR, YpeR, or both are directly interacting with the regulatory regions of both *clpXP* and *lon* to cause down-regulation of these proteases, subsequently preventing YmoA degradation and down-regulating TTSS transcription. If this is the case, I expect to see a band shift for both promoter regions when exposed to the proper “LuxR” protein (YspR or YpeR) and either C8 or oxo-C8. It is possible that both regulatory quorum sensing proteins interact with *clpXP* and *lon*, in which case I would expect to see a band shift for all reactions except the control reaction with YopE. I also expect to see a reduction in the amount of DNA that shifts when unlabeled regions are added to reactions, demonstrating specificity of binding and competition between the labeled and unlabeled DNA regions. For the DNase I protection assay, I expect to see an area on both regulatory regions where the DNA is protected from DNase treatment, indicating that protein (either YspR, YpeR, or both) is binding to the promoter region. I do not expect to see this “protected area” when using YopE.

Anticipated Problems and Alternative Strategies: Since all of these experiments have been successfully completed before, without the addition of C8 or oxo-C8, I do not anticipate any problems. If assays turn out to be ambiguous, alternative strategies can be taken. I would expose a strain of *Y. pestis* that overproduces...
ClpXP and Lon to C8 or oxo-C8 to see if down-regulation of the TTSS-specific genes is reversed. I could measure this by either lacZYA expression or Real-Time PCR. If C8 and oxo-C8 do act through ClpXP and Lon to prevent degradation of YmoA, then I would expect to see up-regulation of the TTSS in the presence of C8 or oxo-C8. If C8 or oxo-C8 does not directly bind to the promoter regions of clpXP and lon to cause down-regulation of transcription, I could measure protease activity of the two proteases to determine if perhaps their activity is affected and not their transcription. I could also test the stability of clpXP and lon mRNA. It is possible that transcription itself is not affected by treatment with C8 or oxo-C8, but that mRNA is readily degraded, preventing translation to occur.
References


