Opening moves: early events in anthrax pathogenesis and their consequences for dissemination.

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Abstract

Bacillus anthracis is a Gram positive sporulating bacterium that is the causative agent of anthrax. Early steps in dissemination are understudied due to the asymptomatic and asynchronous nature of *B. anthracis* infections. The goals of these studies are to establish a model of the early steps of *B. anthracis* infections and examine how the initial site of infection and exotoxin production affect bacterial dissemination. In these studies, a mouse model was used in combination with a bioluminescent signature tagged library to determine how *B. anthracis* disseminates through the host and the roles of an exotoxin component, lethal factor, in colonization and dissemination. This library allowed for real time observation of dissemination and analysis of bacterial population dynamics. In the first set of experiments, mice were infected via inhalational or subcutaneous routes with the tagged library to determine how the bacterial population changed during dissemination. In inhalational and subcutaneous infections, the disseminated population was comprised of a small subset of the original library. This indicated the presence of a population bottleneck, a random decrease in genetic diversity during the infection. Surprisingly, the diminishment and location of the bottleneck varied depending on the initial site of infection. This suggested *B. anthracis* exploits multiple host environments and some sites may be more permissive for dissemination than others. In the second set of experiments, mice were infected with a signature tagged library where a portion of clones lacked lethal factor. Lethal factor is known to be an important virulence factor as deletion results in attenuated or complete loss of virulence in most animal models. Host survival increased when $\leq$ 10% of the library produced lethal factor, but few other differences were observed. Furthermore, decreases in the proportion of lethal factor producing clones led to fewer clones disseminating. These findings suggest that a threshold of lethal factor must be

produced for colonization and dissemination to occur *in vivo*. In total, this work provides an understanding of how *B. anthracis* is able to rapidly disseminate from a several tissues and gives insights in where future therapeutics should be focused to reduce disease incidence.

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Chapter 1: Introduction

Bacillus anthracis and anthrax

Bacillus anthracis is a Gram positive sporulating bacterium in the *Bacillus* genus, more specifically in the *Bacillus cereus sensu lato* group, that is the cause of the disease anthrax [1]. Unlike the other members of the *sensu lato* group, the vast majority of which are non-pathogenic, *B. anthracis* has key differences that highlight its niche as a pathogen. Comparative analysis of the genome to several other closely related species suggested that *B. anthracis* is capable of surviving in soil, but adapted to protein-rich environments of animals [2]. These include unique regulators of virulence factors, two mega plasmids that encode the genes for a tripartite exotoxin and the biosynthetic machinery for a poly-gamma-D-glutamate capsule and specific germination and sporulation systems [1, 3-8]. Before exploring how these adaptations lead to disease, however, a brief discussion of anthrax and its disease incidence should be discussed.

Anthrax

Bacillus anthracis is the causative agent for anthrax and is enzootic in areas of several continents, including Europe, Asia, Africa, and both North and South America [9]. Anthrax can occur in at least 4 forms: cutaneous, injectional, gastrointestinal, and inhalational. Although primarily considered a bioweapon in the developed world, anthrax has traditionally been considered a disease of livestock and people who work with animal products. Precise numbers of the animal and human disease burden is difficult to measure as many of the infections occur in developing rural areas where medical and veterinary care is less common; however, large outbreaks are still reported [10-15]. As such, anthrax remains a disease of economic and public health concern that requires attention and management.

By far the most common reported form of human anthrax is cutaneous anthrax, which is commonly reported in enzootic regions [9, 16]. Cutaneous anthrax typically leads to the formation of black eschars on the infected tissues. These eschars also are responsible for the name of the disease (from Greek '*anthrax*' meaning coal). The eschars develop from small painless, itchy lesions that occur about 3 to 5 days after infection [16, 17]. These lesions will then develop edema and later develop a black necrotic center, known as an eschar [17, 18]. After the appearance of the eschar, the host will present with fever, malaise, and lymphadenitis [18]. While cutaneous anthrax is the most commonly reported type of anthrax, it spontaneously resolves in about 80% of cases even in the absence of antibiotics and approaches 100% with appropriate antibiotic administration [16, 19].

More recently a new form of anthrax deemed "injectional" anthrax has been reported in heroin users in Northern Europe [14, 20, 21]. These infections have typically involved patients who injected heroin in to the skin and not directly into the bloodstream. Unlike cutaneous anthrax, with its characteristic eschar and low mortality, injectional anthrax had only slight changes to the skin that resembled pathologies common to intravenous injection drug users and had higher mortality [20]. Given the relatively few cases of injectional anthrax it is unclear why injection into the skin leads to higher rates of mortality. It is possible that injection is delivering a higher concentration of spores or that some areas of the skin have unique factors that limit the infection that is not present in other areas.

Gastrointestinal anthrax has been reported to have a higher mortality than cutaneous with reports of ~25-60% of patients succumbing to the disease [22, 23]. Some have subdivided gastrointestinal anthrax in to two forms: oropharyngeal and intestinal [16, 19, 22, 23]. Oropharyngeal anthrax develops from an ulcer in either the mouth or upper gastrointestinal

tract and progresses with edema, lymphadenitis, and bacteremia [19, 23]. Infections that arise from the lower gastrointestinal tract leads to intestinal anthrax and symptoms include fever, diarrhea, nausea, and vomiting [19, 23, 24]. Traditionally, gastrointestinal anthrax has been thought to occur by the ingestion of contaminated food products; however a recent case in New England involved a woman developing gastrointestinal anthrax through playing a contaminated African drum [16, 19, 23, 25]. The patient was found to have a pinworm infection as indicated by pinworm larvae in her stool, but it is not known if this had a role in the development in the gastrointestinal anthrax [25]. As such, gastrointestinal anthrax may not just be a threat to people eating contaminated food, but also those with pre-existing or chronic damage to the intestines.

The most dangerous form of anthrax is the inhalational form, but it is also the least reported. Inhalational anthrax has a mortality rate approaching 100% and only reduces to ~50% with aggressive antibiotic therapy [19, 23]. Although rare today, inhalational anthrax was more common in the 19th century due to the exposure of wool sorters in textile mills to wool contaminated with *B. anthracis* spores [26, 27]. These cases decreased as vaccination procedures for livestock and better working conditions developed in Western Europe [26, 27]. However, the development of *B. anthracis* as a bioweapon has led to both accidental and purposeful exposures in the 20th century. Post mortem analysis from an accidental release in Sverdlovsk, USSR and the intentional release in the 2001 United States Postal Service attacks led to greater understanding of the inhalational form of the disease [28-30]. In particular, it was noted that inhalational anthrax was a fulminant disease that had a variable latency period [16, 19]. This had previously been reported in primate models, where culturable spores could be detected in lung tissues 100 days after exposure [31]. Further, autopsies from the Sverdlovsk incident found

evidence of a focal lesion in the lung and meningitis in a large proportion of patients, which may have been the primary site of dissemination [29].

The difficulty in managing inhalational anthrax lies with the variable period of dormancy and the asymptomatic nature of the disease until late in infection. Patients first present an influenza-like illness with an unproductive cough, fever, and malaise, yet this is actually late in infection when many patients have both bacteremia and toxemia [16, 19, 23]. The disease rapidly progresses to cause hypotension, shortness of breath, and death. An additional challenge with treatment of inhalational anthrax is the associated toxemia in addition to the high bacterial burden [23, 26]. As such, even if antibiotics are given and the bacteria are killed, the exotoxin levels can be at a level where death or tissue damage still occurs [26, 32]. Given the fulminate nature and high mortality, it is clear that a fundamental understanding of the early events in pathogenesis is critical in development of prophylactics that target *B. anthracis*. Such advances in combination with antibiotic therapy would lead to a substantial decrease in mortality or morbidity.

Disease incidence and potential threats

Though high mortality can occur with *B. anthracis*, the advent of antibiotics and an effective livestock vaccine have greatly reduced its reported incidence [9]. Indeed, reports from the 19th century demonstrate that the anthrax disease burden was problematic in both urban and agricultural settings [33, 34]. The incidence of anthrax infection amongst wool sorters was particularly high, leading to workers drawing lots to determine who would work with particular batches of animal wool [35]. These issues continued until factories improved ventilation and legislation in 1919 required certain animal hair to be disinfected [26, 27]. Similarly, anthrax outbreaks were so severe before widespread vaccine and antibiotics became

available that an estimated 20-30 percent of cattle and sheep were killed by anthrax in France [33]. In one reported example, between 30,000 and 60,000 animals were thought to have died in South Africa in 1923 [34, 36].

While such continuous outbreaks are not seen today, anthrax is still enzootic in several areas of North America, South America, Asia, Africa, and Mediterranean Europe. In several areas anthrax is reported in domesticated animals, such as cattle, and can reemerge after long periods of inactivity. Within the United States, there were at least 10 states with at least one epizootic outbreak between 1996 and 2001 [37]. Similarly, wild animals have also been observed to have periodic outbreaks, such as with American bison in sections of Canada [38]. Interestingly, recent attempts to maintain large hunting ranches with white tail deer in Texas have also been reported to have anthrax outbreaks [9, 10].

Beyond animal infections, human infections continue to occur in agricultural areas of less-developed nations, such as Turkey, Bangladesh, and India [11, 15, 39]. Some areas with higher incidences of human anthrax have surprisingly low incidences of animal anthrax; however, when animal anthrax does occur there are peaks in human incidences [9]. Other incidental human exposures include contaminated heroin or use of contaminated animal hides, as described previously, could help explain some this disparity between the rates of human and animal anthrax infections [14, 25]. However, it is clear that there are large gaps in understanding the epidemiology of anthrax.

Another concern for human anthrax is its long history as a biological warfare agent. After the use of chemical and biological weapons in the First World War, a number of countries were concerned about their use in the World War II [40]. The first clearly documented use of anthrax as a bioweapon on humans was during the Japanese occupation of Manchuria [16, 41].

This increased development of biological weapons did not abate after 1945 due to the Cold War [40]. The United States and several other countries formally ended offensive research in anthrax in the 1972 by signing the Bioweapon and Toxic Weapons Convention. The Soviet Union, however, continued with a clandestine bioweapons program that produced tons of anthrax spores [40]. These activities would have largely gone unnoticed except for the accidental release of anthrax spores in Sverdlovsk, USSR [28]. The news of this outbreak was tightly suppressed by the Soviet government and it was not until the fall of the Soviet Union that a clear picture arose of the outbreak [28, 29]. Since the end of the Cold War, intentional anthrax outbreaks have been due to bioterrorism. Compared to the Sverdlovsk incident, these attacks have had a greater psychological impact than actual mortality. The Japanese cult Aum Shinrikyo released anthrax in Tokyo; however, they released a highly attenuated vaccine strain that resulted in no infections [40]. In the United States the 2001 US Postal System attacks highlighted the potential threats and consequences of an anthrax attack [42, 43]. Such anthrax attacks also led to large costs in site remediation [44]. Therefore, while anthrax does not affect livestock or humans to the extent that was seen in previous centuries, there is considerable need to understand the organism as it still could be exploited to cause disease. A fundamental understanding of the pathogenesis in animal models is necessary to develop potential therapeutics to mitigate such threats.

The infection cycle: insights in to bacteriology and pathogenesis of *B. anthracis*

B. anthracis has an unusual infection cycle that moves between long periods of dormancy as a spore and rapid growth of bacilli in the host [17]. The infection cycle can be divided in to 5 steps: the spore stage, uptake in the host and germination, replication of the vegetative bacilli, death of the host, and sporulation (Figure 1).

Each part of the infection cycle has areas of intense research and represent stages for therapeutic development. Further, each stage of infection reveals how *B. anthracis* has adapted to use this cycle to become a successful pathogen. In the section below, each stage will be examined to highlight the bacteriology and pathogenesis of *B. anthracis*; however, key aspects such as the effects of the exotoxin and dissemination will be further described in other sections.

The Spore Stage

B. anthracis has two forms in nature, the spore and the vegetative bacilli, that have distinct roles in the infection process [17]. The spore is the infectious particle for *B. anthracis* [17, 45]. *B. anthracis* spores are typical to other spores from the *Bacillus* genera in terms of composition and ability to survive for long periods of time in a dormant state. As an extreme example of the latter, spores from *Bacillus* species have been isolated from salt crystal formed 250 million years ago and from 25-40 million year old insects suspended in amber [46, 47]. More specific studies of *B. anthracis* spore hardiness and the issues of remediation can be observed through the remediation efforts of a Scottish island used by the British to develop anthrax as a bioweapon [48]. After 40 years, the island was decontaminated by delivering 25-50 liters of 5% formalin per square meter. The mechanisms for resistance can be illustrated by its structural anatomy. *B. anthracis* shares a large number of homologues and structural similarities to the well-studied model organism, *B. subtilis* [49]. Structurally, a spore is composed of the core, surrounded by the cortex, and covered with a proteinaceous coat. Some *Bacillus* species, including those in the *B. cereus* group containing *B. anthracis*, also have an outermost layer known as the exosporium (Figure 2A) [50].

The core contains the DNA and all proteins necessary to initiate germination (described below) [50, 51]. The core is dehydrated and mineralized with dipicolinic acid and calcium ions

[51]. On the outside of the core is the cortex, which has a thick layer of peptidoglycan which aids in heat resistance and helps the core keep its dormancy through maintaining its dehydrated state [50]. Surrounding the cortex is the spore coat, a complex organization of proteins that aid in chemical and physical resistance [52]. This resistance occurs during spore development and includes cross-linking lysines and tyrosines on the surface of the coat [52-54].

The outermost structure of the *B. anthracis* spore is the exosporium. The exosporium has a paracrystalline basal layer with thin hair like appendages and is separated ~10-20 nm from the rest of the spore [50]. The dominant protein on the exosporium is BclA, a collagen-like protein that covers the exosporium with a hair-like nap [55]. However, other important proteins are present on the exosporium, such as the superoxide dismutase and an alanine racimase, which have important roles in pathogenesis and germination [56-58].

Despite the presence of proteins known to be involved with pathogenesis being present on the exosporium, the role of the exosporium in anthrax pathogenesis is debated. The most abundant protein, BclA, is considered to have a role in facilitating macrophage uptake [59]. Spores isolated from strains that lack BclA were found to have greater adhesion to epithelial cells, but had no difference in virulence compared to wild type spores [60]. This suggests that BclA is an anti-adhesin that guides spores to the professional phagocytes [59, 60]. In contrast, other groups have found infection with Δ*bclA* spores led to quicker germination and a shorter median time to death than the wildtype [61]. BclA may also play a role in epithelial uptake of spores, as the complement protein C1q acts as a bridging molecule between BclA and the α2β1 integrins on epithelial cells [62]. While the exosporium may not be necessary for infection in animal models, it may guide the spore to certain cell types and promote infection [60]. As such, the spore is able to play two roles in infection: first, it allows the spore to remain in the

environment for long stretches of time and secondly, it acts as the infectious particle of the disease.

Spore Germination

After uptake into the host, the spores are able to sense the correct host environment and transition from the spore form to the vegetative bacilli by a process known as germination. As described in the previous section, spores have a number of attributes that allow them to survive for long periods in the environment. Equally important is the spores' ability to sense the correct host environment and initiate germination. When spores are in a favorable environment for growth, they can sense key molecules called germinants [63]. For *B. anthracis* these include the L-amino acids, such as L-alanine, L-histidine, and L-serine, as well as sugars such as inosine [64]. While L-amino acids are important in triggering germination, D-amino acids inhibit germination [65, 66]. The alanine racemase enzyme on the exosporium takes advantage of this activity to prevent germination at inopportune times in the host [58]. Alanine racemase converts L-alanine to D-alanine when there is a low concentration in the environment, thereby acting like a germination buffer. However, when there is a large concentration of L-alanine in the environment, the racemase cannot convert enough alanine to prevent germination. These germinants and anti-germinants act either alone or in combinations and signal through five functional germination receptors, *gerH, gerL, gerK, gerS*, and *gerX*, on the spore coat [63, 64, 67, 68]. The need for several germination receptors may not only reflect redundant mechanisms for sensing germinants, but also may be a mechanism for *B. anthracis* to sense different sites in the host for germination. While most germination receptors were sufficient for germination in an intratracheal instillation model, spores delivered subcutaneously could only germinate if they

had *gerH* [69]. However, whether germination in different sites leads to differences in dissemination or population structures is a poorly understood area.

Spore germination is an irreversible and rapid process [64]. After the germinants have bound the germination receptors, a germination cascade occurs that leads to rehydration of the core, release of cations, and a loss of bacteriocidal resistance [63]. The first phase of germination after receptor signaling is the release of calcium and dipicolinic acid from the core, letting the core partially hydrate [70]. Partial hydration then allows for Stage II, where the core continues to hydrate, expand, and the cortex begins to degrade [63, 70]. In the final stage, termed outgrowth, the core continues to expand and begins metabolism [63].

An important aspect of *B. anthracis* pathogenesis is the site the spores germinate. A widely considered model of dissemination involves the uptake of spores into macrophages where they germinate en route to the draining lymphatics (discussed at greater length below) [71, 72]. Spores are highly resistant to macrophage mediated killing, but this resistance is lost during germination [73]. Yet, several studies have indicated that the bacteria can and do germinate in macrophages *in vivo* [74-77]. The ability of the spores to germinate and escape from the macrophages is due to their ability to express the exotoxin and dependent on the amount of spores within the macrophage [75-77]. Similarly, germination within a mucosal associated lymphoid tissue, such as the NALT or Peyer's patch, would require rapid expression of virulence factors in order to survive in a phagocyte dense environment [78]. Other sites of germination, such as the ear dermis in subcutaneous infections or epithelial cells, may not have such immediate threats from host innate immune cell [79, 80]. The germinated bacilli would have to quickly act, however, to prevent the surrounding epithelial or endothelial cells from detecting their presence and producing cytokines or chemokines . As such, it seems that the

germinating spores need to rapidly respond to the host environment after germination in order for the bacteria to survive.

The Vegetative Bacilli

The vegetative bacillus is a rod shaped, capsulated bacterium, whose exotoxin and dissemination causes the disease anthrax. The bacilli forms long chains of rods and the length of the bacilli may have a role in dissemination [81]. The surface of the bacilli is composed of the membrane, peptidoglycan, an S-layer, and the capsule [82]. The membrane of *B. anthracis* is relatively understudied compared to other parts of the surface, but is considered to have several similarities to the model organism *B. subtilis* [82]. The peptidoglycan layer was previously thought to lack lipoteichoic acid, but recent studies have determined the presence and gene products necessary for its formation [83]. In general, the peptidoglycan has a structure similar to the closely related *B. cereus* [82]. Similar to other Gram positive organisms, proteins can be both covalently and non-covalently attached to the peptidoglycan [84-86]. Covalently attached proteins have an LPXTG, NPKTG or an LPNTA motif at the C-terminus which is recognized by sortase proteins [87]. These sortases are able to cleave the protein at the threonine position in the conserved motif and attach the protein to peptidoglycan. Non-covalently attached proteins are attached to the peptidoglycan by an S-layer homology (SLH) domain. Closely apposed to the peptidoglycan is the surface layer, or S-layer [17]. The S-layer is a paracrystalline layer surrounding the entire bacteria that is composed of two proteins, Sap and EA1 [88-90]. While the S-layer is not necessary for virulence, some proteins with SLH domains have roles in virulence [91, 92]. The outermost layer of the surface is composed of the capsule. The capsule is composed of a poly-gamma-D-glutamatic acid polymer [17]. This peptide-based capsule is atypical from other bacterial capsules, including many of those in *Bacillaceae* [17, 82]. The

capsule is considered a major virulence factor, as infection with a capsule deficient strain has drastically reduced mortality in most hosts, yet it remains poorly studied compared to the exotoxin [93-96]. The capsule is thought to act to prevent phagocytosis, but more recent studies have suggested that the capsule also interacts with the exotoxin to increase virulence [97, 98].

While the chromosome of *B. anthracis* is quite similar to those in the *B. cereus* group, it is the presence of pX01 and pX02 that allow *B. anthracis* to act as a pathogen. The genes that encode for the biosynthetic machinery of the capsule and the exotoxin components are found on two mega plasmids, pX01 and pX02 [3, 6, 8]. The exotoxin subunits, as wells as the germination receptor *gerX* and the master virulence regulator *atxA*, are located on the pX01 plasmid [6, 8, 99]. The other plasmid, pX02, contains the *cap* operon, which encodes *capBCADE*. The proteins from the *cap* operon form a complex at the membrane, produce glutamic acid polymer, and regulate its attachment to the peptidoglycan [100]. Additionally, two transcriptional regulators of the *cap* operon are encoded on the surface of pX02, which are under the control of AtxA [101]. These two plasmids act in a coordinated manner with chromosome when in the host to allow the bacteria to rapidly disseminate and reach a high bacterial burden in the host without eliciting an immune response until very late in infection [17, 102].

The major virulence factors of *B. anthracis* are the exotoxin and the capsule [95, 96, 103, 104]. The exotoxin will be further discussed in the next section due to the wealth of research on its role in infection. However, there are several other proteins that play minor roles in infection of the host. These include proteases, phosphatases, phospholipases, and a cholesterol-dependent cytolysin. *B. anthracis* has sixteen different proteases that are secreted *in vitro* [105-107]. The two proteases that have been the most studied, Npr599 and InhA, are metalloproteases that degrade extracellular matrix proteins, syndecan-1, and several proteins

involved with fibrinolysis [107]. Interestingly, the secreted proteases InhA and HtrA also appear to regulate secreted *B. anthracis* proteins including the adhesin BslA [108-110]. *B. anthracis* also expresses three phospholipases, PlcA, PlcB, and SmcA. These target phosphatidylinositol, phosphatidylcholine, and sphingomyelin, respectively [2]. Purified phosopholipases were able to cause hemolysis and cleavage of GPI linked proteins from dendritic cells [111, 112]. *In vitro* and *in vivo* studies suggest that these phospholipases are functionally redundant as only the Δ*plcA* Δ*plcB*, Δ*smcA* triple mutant has defects in macrophage and mouse models of infection [113, 114]. In the closely related *B. cereus* strain, these phospholipases are controlled by the PlcR regulator [115]. However, it is not well understood how the *B. anthracis* phospholipases are regulated since its PlcR homologue has a nonsense mutation [116]. Another virulence factor implicated with phospholipases is anthrolysin O (ALO), a cholesterol-dependent cytolysin (CDC). CDCs are found in several Gram-positive organisms and are often necessary for virulence [117]. The precise role of ALO *in vivo* is poorly understood. The Δ*alo* mutant is fully virulent, but a Δ*alo* Δ*plcA* Δ*plcB* Δ*smcA* mutant is attenuated *in vivo* [113]. Further, purified ALO is capable of lysing several immune cells and disrupting barrier function of *in vitro* when added to epithelilal monolayers [118, 119]. Interestingly, *B. anthracis* releases vesicles that contain ALO and components of the exotoxin [120]. The role of these vesicles in not understood, but it may play a role in disseminating exotoxin components through the host.

In addition to the proteases and cytolysins, there are also minor virulence factors that directly down regulate the immune system. On the extracellular surface of *B. anthracis* is adenosine synthase A (AdsA), a 5'-ectonucleotidase that dephosphorylates AMP to produce adenosine [121]. The production of adenosine is then able to signal to host immune cells and

reduce inflammation [122]. *In vitro* survival assays in whole blood have demonstrated that *B. anthracis* has increased survival in blood when compared to the Δ*adsA* mutant. More recently, a novel virulence factor was identified that repressed NF-κB by methylating human histone H1 [123]. SET domains, or suppressor of variegation, enhancer of zeste, trithorax domains are typically found in eukaryotic organisms and used to regulate transcription through epigenetic modification [124]. More recently, homologous SET domains were found in a variety of pathogens, including *B. anthracis* [125]. BaSET was found to be secreted by *B. anthracis* and translocated to the nucleus of macrophages [123]. Once in the nucleus, BaSET was found to methylate histone H1, leading to inhibition of NF-κB activation. Further, the ΔBaSET was found to be avirulent in mouse models [123]. The production of virulence factors allow the bacilli to replicate and disseminate through the host in a short period of time. Yet, it is unknown if and how these virulence factors are regulated over time. It is possible that the expression of one set of virulence factors is required or helpful at one point of the infection but dispensable or detrimental at another point. Understanding which virulence factors are expressed and their relative amounts throughout the infection would yield a deeper understanding of anthrax pathogenesis.

Death of the host

The production of the exotoxin, capsule, and other virulence factors allow the bacilli to reach high levels in the host in a relatively short time. While figures vary depending on the animal model, the terminal blood colony forming units (CFU) counts tend to vary between 10^4-10^9 CFU/mL [9]. However, there is debate as to whether toxemia or sepsis due to bacteremia is the cause of death in the animal [126-129]. *B. anthracis* infections lead to high levels of the exotoxin being produced in the host, in one case 100μg/mL of protective antigen, a component

of the exotoxin, have been detected after death [130]. Further, antibiotic therapy of late stage anthrax still has a high mortality despite effectively killing the bacteria due to the effects of toxemia [26]. In support of the previous finding, genetic deletion of the exotoxin receptors on immune cells, cardiac cells and smooth muscle cells protect mice from toxemia [128]. In contrast to the toxemia mediated death model, others have argued that the exotoxin merely enables the bacilli to reach a high bacterial burden, which in turn triggers sepsis [95, 126]. Pathological examination of animals that died from injected exotoxin did not show evidence of fibrin deposits, vasculitis, inflammation, or pleural effusions that are often seen at end stages of anthrax [129, 131]. Given the number of mammals that *B. anthracis* infects and the variable resistance to intoxication within a species, *B. anthracis* may use the exotoxins primarily as a mechanism to increase bacterial burden and dissemination [132]. However, in order to complete their infection cycle and infect new hosts, the bacteria must kill the host and sporulate [17]. Therefore, in exotoxin resistant animals the exotoxin may allow the bacteria to reach the bloodstream and reach a sufficient bacterial burden to elicit sepsis in order to kill the host and continue the infection cycle.

Sporulation

B. anthracis cannot be spread via host-to-host transmission [16]. As such, in order to complete its infection cycle and infect the next host, the bacilli must transition back to the spore, a process known as sporulation [4]. *B. anthracis* sporulation occurs after the death of the host and is not thought to occur during the dissemination stage [17, 81, 133]. The molecular mechanisms behind *B. anthracis* sporulation ensure that the bacilli only sporulate at the appropriate moment. This is done through two approaches: the first to prevent the bacilli from sporulating too soon and secondly to induce sporulation after death. Sporulation occurs when

five sensor kinases detect decreasing nutrients and increased oxygen tension [134, 135]. The sensor kinases phosphorylate the response regulator Spo0F, which eventually phosphorylates Spo0A through Spo0B. Spo0A then upregulates a number of genes involved in sporulation [136]. To prevent early sporulation, *B. anthracis* has at least two regulatory networks that prevent early sporulation [5]. Both systems target the Spo0F response regulator that initiates the sporulation cascade [134, 137, 138]. Two proteins on the pX01 and pX02 plasmid have areas of homology to the signaling region of a sporulation sensor kinase BA2291. It is hypothesized that the two plasmid proteins, pX01-118 and pX02-61 bind to the same signal as BA2291 and thus compete for the signal. Further, BA2291 is thought to be a kinase for Spo0F when activated, but acts as a phosphatase when inactive [134]. Thus, pX01-118 and pX02-61 not only compete for the same signal, they cause BA2291 to further repress sporulation by preventing activation of the Spo0F protein [137]. A second method to inhibit premature sporulation is the Phr-Rap system encoded on the pX01 plasmid [138]. Rap is similarly able to dephosphorylate Spo0F, but is inhibited by the protein Phr. Phr, however, must undergo maturation through an export/import pathway [139]. As such, when the bacilli are at low concentration in the blood stream, Phr is exported and diffuses away. Whereas at later stages of infection where the bacilli are in high numbers the Phr concentration is higher and able to be imported where it inhibits Rap's phosphatase activity [138]. Thus *B. anthracis* sporulation not only uses nutrient and environmental signals to sporulate, it also has genes encoded on the virulence plasmids to prevent premature sporulation. These actions allow the bacilli to reach higher numbers in the host, which will develop to a greater number of spores and increase the likelihood of infecting a new host.

Evolution and global phylogenetic analysis of strains

B. anthracis is considered a monomorphic or clonal organism due to the high degree of conservation within its genome [2, 140]. This is a fundamental issue for *B. anthracis* in terms of evolution as it limits how the organism can adapt to a changing environment. Further, it is also complicates analyses to determine where an outbreak originated [141, 142]. *B. anthracis* is considered an evolutionarily recent pathogen, despite being recorded through most of human history. This is due to the bacteria maintaining much of its life as a dormant spore [141]. Further, when it is the metabolically active vegetative bacilli it typically has less than a week for growth before host death and sporulation occurs [141]. Thus, the bacteria have a very narrow time to mutate, develop genetic insertions/deletions, or undergo horizontal gene transfer. In spite of the monomorphic genome, whole genome sequencing has identified several SNPs and unique areas between strains termed, Variable Number Tandem Repeat (VTNR) regions, which can differentiate these strains [143, 144]. From this data, three groups, named A, B, and C, were identified [143]. Interestingly, the A group has the greatest distribution, followed by the B group [145]. It is not understood why the A group has such greater geographic distribution relative to the other strains. Areas in which evolution could happen is also an area of interest. Some groups have proposed that *B. anthracis* can germinate in soil or outside of the mammalian host environment. Studies have shown that *B. anthracis* can form biofilms in soil and infect earthworms when they interact with phage [146]. Others have found the bacilli in the rhizosphere of plants or infect and kill amoeba [147, 148]. However, very little is known about the evolutionary implications that occur when infecting the host. Several bacterial pathogens are known to undergo a population bottleneck during infection [149-151]. Population bottlenecks occur when there is a non-selective decrease in an organism's population that leads to a decrease in the genetic diversity of a population. A consequence of these drastic reductions is higher rates of genetic drift for the population [152]. Genetic drift can be quite harmful to the

evolution of an organism as it increases the likelihood of deleterious mutations and decreases the likelihood of a beneficial mutation of an organism [153]. Since *B. anthracis* is primarily in its metabolically active form in mammalians hosts, a greater emphasis should be put on examining the evolutionary changes that occur during infection the bacteria.

The cellular and physiological role of the anthrax exotoxin

The following is an excerpt from the previously published review, "The cellular and physiological effects of the anthrax exotoxin and its relevance to disease"[154].

B. anthracis is known to have two main virulence factors, a secreted exotoxin and an anti-phagocytic capsule. The exotoxin is composed of a host-cell receptor binding protein named protective antigen (PA) and two enzymatic proteins, lethal factor (LF) and edema factor (EF) [155, 156]. LF and EF have no known activity on the host until they bind and then are subsequently translocated to the target-cell cytosol by PA. Once LF or EF is bound to PA, they are referred to as lethal toxin (LT) or edema toxin (ET), respectively. Although there are a multitude of studies that focus on the cellular and *in vivo* effects of LT and ET individually, bacterial infections in most animal models suggest that the two exotoxin components work cooperatively. For example, mouse and rabbit models have a higher LD_{50} when either EF or LF is genetically deleted. Therefore, the exotoxin should be considered as a single complex (PA, LF, and EF), particularly since the relative amounts of LF and EF within individual cells is not well understood. Additionally, the holotoxin can contain all three components. For most hosts, bacteria must have both the capsule and the exotoxin in order to be fully virulent. The enzymatic subunits target key signaling cascades that are present in several types of cells across

many physiological systems. Similarly, the exotoxin receptors are highly expressed in a wide variety of tissues, allowing for the exotoxin to act on several systems simultaneously. This has complicated the understanding of the exotoxins' effects on the host since these pathways have several targets in a cell, which can lead to unique effects for different cell types and organs. Since the 1950's, research has been aimed to define the molecular mechanism of cellular intoxication and its relation to the disease. Of particular interest to this body of work are the effects of the exotoxin on the host immune system and cardiovascular effects in terms of initial survival in the host and dissemination which leads to host death.

Components of the exotoxin and mechanism of internalization

Host cell intoxication begins when PA binds to either tumor endothelial marker 8 (TEM8, also known as anthrax toxin receptor 1 (ANTXR1)) or capillary morphogenesis gene 2 (CMG2, also known also anthrax toxin receptor 2 (ANTXR2)) [42, 157, 158]. PA can also bind to β1 integrins, which potentiate the uptake of PA by macrophages [159]. The physiological role of TEM8 in mammals has been implicated in binding the extracellular matrix, directing endothelial migration and adhesion [160, 161]. Less is known about CMG2 but evidence suggests a role in endothelial proliferation [162]. As their names and functions suggest, TEM8 and CMG2 are highly expressed in vasculature, but several studies have also noted their presence in epithelial cells (particularly in respiratory epithelium, intestines, and keratinocytes) as well as in immune cells [42, 157, 163]. Genetic and biochemical studies have recently demonstrated that PA has higher affinity for CMG2 and plays a larger role in susceptibility than TEM8 in mice [164]. After PA binds to CMG2 or TEM8, it is cleaved by Furin or a Furin-like protease to a 63 kDa isoform (PA_{63}) that heptamerizes [165-167]. Alternatively, PA can be cleaved by serum proteases independently from cell binding [168, 169]. Reports differ as to

whether this phenomenon is a species specific effect in mice or occurs in several animal models. While the exact protease has not been discovered, it is sensitive to the protease inhibitor leupeptin, heat sensitive, and dependent on calcium. Once PA_{63} has heptamerized, it can bind both LF and EF with up to three potential binding sites per heptamer [170]. A more recent study also provides evidence that PA can oligomerize into either a heptamer or an octomer. In comparison to the heptamer, the octomer is more stable when unbound to the host exotoxin receptor, perhaps enabling it to oligomerize and pre-form the exotoxin complex in serum [171].

PA oligomerization is necessary for its association with lipid rafts and clathrin-mediated endocytosis [172]. After endocytosis, the exotoxin is sorted into intraluminal vesicles in the early endosome [173]. The decreasing pH in the early endosome causes PA/TEM8 to undergo a critical conformational change to insert into the membrane and form a translocation pore [167]. PA bound to CMG2, however, needs a lower pH than TEM8 (Rainey et al., 2005). In either case, this leads to LF/EF translocation into the intraluminal vesicle, which then reaches the cytosol when the intraluminal vesicles fuse back into the late endosome membrane or the autophagosome [173, 174].

Once in the cytosol, LF and EF can exert their effects on the cell. LF is a zinc metalloprotease that cleaves the N-terminus from several MEKs and prevents the activation of Erk1/2, p38, and JNK-pathways [175-177]. Edema factor is a calmodulin dependent adenylyl cyclase toxin that raises cAMP levels to supra-physiological levels [178]. In contrast to other cAMP elevating toxins, such as *Bortadella pertussis* adenylate cyclase toxin and cholera toxin, ET produces high amounts of cAMP perinuclearly rather than at the cell membrane [179]. The increased cAMP can then activate protein kinase A (PKA), exchange protein activated by cAMP (Epac), and the guanine exchange factor Rap1 [180].

While considerable research has led to fundamental understandings in how the exotoxin is internalized and acts on their respective targets, the fact that LF and EF target proteins that are central in so many cellular functions complicates the analysis of how it alters the cellular and physiological function. What is clear is that some actions are essential for both the bacteria's initial survival and subsequent dissemination. Historically, these systems were thought to be the immune system and more recently the cardiovascular system.

Immunological effects

The ability of *B. anthracis* to subvert the immune system is of the utmost importance for the pathogen to complete its lifecycle [17]. This fact has not escaped the attention of researchers as there are a multitude of studies and reviews that investigate how the anthrax exotoxin affect the immune system in order to establish and exploit a niche in the host [181, 182]. Further, when mice with the CMG2 exotoxin receptor genetically deleted from myeloid cells were infected with *B. anthracis*, there was a complete loss of mortality [183]. How the exotoxin exploits the immune system is harder to pinpoint as it is able to affect the immune system in several ways. In particular, the exotoxin is able to reduce the immune response by altering three main functions: chemotaxis, bacteriocidal activity, and exotoxin induced pyroptosis/apoptosis of immune cells.

Neutrophils are among the first immune cells to respond to infections and are essential for clearance of many bacterial and fungal pathogens. Therefore, it stands to reason that a swift influx of neutrophils is necessary to contain an infection by *B. anthracis*. Neutrophils play a key role in clearing *B. anthracis* infection and the exotoxin is capable of abrogating their protective effects [183-185]. Further, early production of pro-inflammatory cytokines, such as IL-1β, can increase host survival mediated by neutrophils [186]. Both purified LT and ET inhibit

neutrophil chemotaxis by reducing F-actin formation *in vitro* [187, 188]. This leads to reductions in random velocity, directed velocity toward formyl-Met-Leu-Phe (a well characterized chemoattractant), and reduces polarization of the leading and lagging edges of the cell. F-actin is further reduced when the cells are treated with both LT and ET simultaneously, demonstrating an additive role for both exotoxins [188]. ET also reduces the expression of CD11b/CD18 (Mac-1 or CR3), a β2 integrin that is important in neutrophil adhesion and extravasation. This presumably would reduce diapedesis through capillaries or epithelial layers to the site of infection; however, this has not been tested [188]. These studies are in contrast to earlier work from Wade *et al*. which found ET increases PMN chemotaxis and an *in vivo* observation that greater amounts of neutrophils were found near injection sites of ET [189, 190]. The latter observation may reflect the indirect effects of ET increasing production of inflammatory lipid mediators which recruit neutrophils. Other *in vivo* studies have indicated that LT is capable of reducing chemotaxis to PMA mediated inflammation in the ear in mice models [184].

Macrophage chemotaxis is altered by both LT and ET similarly to neutrophils. *In vivo* clodronate depletion of macrophages sensitizes mice to infection during inhalational anthrax, demonstrating a net protective function of macrophages [191, 192]. *In vitro* treatment of macrophages and peripheral blood lymphocytes with either purified LT or ET reduces phosphorylation of Erk, presumably by independent mechanisms, which lessens chemotaxis toward the chemoattractants SDF-1α and MIP-1α [193]. The reduction is specific for the exotoxins since the MEK inhibitor PD98059 is able to phenocopy the decrease in chemotaxis, supporting a role for Erk1/2 in chemotaxis, and the ET inhibitor adefovir is able to partially rescue chemotaxis. Interestingly, the F-actin decrease found in During's research [187] was not observed in neutrophils treated with PD98059, suggesting that chemotaxis was not inhibited in

their experimental system. This could be due to MEK1 having unique effects in macrophages and neutrophils or the F-actin reduction occurs via another MAPK pathway. Similarly, purified ET increases the cell migration of bone marrow derived macrophages (BMDM) [194]. This migration is due to protein kinase A (PKA) stimulation activating CREB. Several genes are then induced via CREB, including *Sdc1*, which is necessary for the increased migration and actin redistribution that occurs in ET treated BMDMs. It is important to note that there was no indication that the macrophages were moving in the direction of a chemoattractant. This is a salient point in *B. anthracis* exotoxin-mediated chemotaxis assays since an increase in random migration is distinct from chemotaxis. As such, differentiation between random migration versus chemotaxis should be noted when interpreting these observations. Recombinant ET administration, however, leads to an increase in total circulating neutrophils and monocytes [195]. This may be the result of an increase in chemotaxis out of the bone marrow, as the bone marrow is depleted of pluripotent stem cells. Indeed, G-CSF, a potent maturation factor for neutrophil progenitors, has a cAMP response element in its promoter, which may explain both the marked increase of neutrophils and G-CSF in the serum.

Lastly, dendritic cells, which represent a crucial link between innate and adaptive immunity, have an increased ability to chemotax and invade matrigel when treated with purified ET [196]. LT has been shown to inhibit chemotaxis in PBMC's; however, addition of purified LT with ET also leads to an increase in chemotaxis compared to untreated DCs [193, 196]. Additionally, recombinant ET is capable of activating glycogen synthase kinase (GSK) in DCs, which in turn is able to fully activate CREB-mediated transcriptional changes shown by Kim *et al*. to be involved with chemotaxis [194, 197]. It is unknown if GSK is involved with CREB activation in either neutrophils or macrophages.

In addition to their potent actions on chemotaxis, the *B. anthracis* exotoxins are also able to reduce the bactericidal activity and activation of the innate immune system. Intoxicating human neutrophils with purified ET reduces phagocytosis, which is not surprising given the potent effect ET has on neutrophil actin formation [198]. Additionally, purified ET reduces the neutrophil's oxidative burst. Recombinant LT's role in the neutrophil oxidative burst has also been examined with two discordant studies showing it either increases or decreases the oxidative burst in human neutrophils [199, 200]. Purified LT also reduces the amount of pro-inflammatory cytokines and chemokines in NB-4 cells, a neutrophil-like immortal cell line [201, 202]. The effects of LT on human neutrophils may be less pronounced due to the ability of human α-defensins to neutralize LT, though it is unclear in what environments *in vivo* α-defensin concentrations would be at sufficiently high levels to mediate this inhibition [203].

While it is tempting to assume that an exotoxin's effect on the bactericidal activity of circulating phagocytes will be similar to that of a resident phagocyte, research has indicated there are important differences. Resident phagocytes have previously been implicated as acting as a Trojan horse for *B. anthracis* spores [71, 74, 76]. That is, the spore is phagocytosed by alveolar macrophages (AMs) or lung dendritic cells (DCs), which then migrate with the spore to the draining mediastinal lymph node. During migration the spore germinates, replicates, and escapes from the phagocyte into the lymph node where it can later spread to the blood. However, human AMs are more resistant to MEK1 cleavage and LT-mediated cytokine inhibition than peritoneal macrophages or the murine RAW264.7 macrophage-like cell line [204]. Further, PA does not bind AMs well, despite similar transcription levels of CMG2 compared to RAW264.7 cells. This might be due to single nucleotide polymorphisms (SNPs) in human macrophages. Genetic susceptibility to exotoxin uptake was explored in an *in vitro* system where lymphoblastoid cells derived from 234 cohorts in the HapMap Project [132]. Cells

were treated with PA and FP59, a heterologous cargo protein for the PA oligomer that rapidly kills lymphocytes in a manner independent from either LT or ET, to assess their relative capacity for intoxication. Susceptibility to infection varies 30,000-fold, with three cell lines showing a much higher level of resistance. Excluding these three outliers, there is still a 250-fold range of resistance between individuals. This suggests that human cells have varying ability for exotoxin uptake. Further, a SNP that causes a P357A substitution in the human CMG2 region involved with PA internalization reduces the amount of PA uptake in transgenic RAW264.7 cells [132]. It is likely, however, that AMs are uniquely resistant to LT-mediated death as the AMs in Wu's studies were likely collected from several individuals [204]. AMs from Cynomolgus macaques, however, do have reductions in cytokine production when intoxicated with purified LT *in vitro*, likely due to the effects of MEK1 cleavage [205]. Therefore, in addition to differential responses between human and mouse AMs, important species difference in the intoxication of immune cells exist even in very closely related non-human primates and humans. Despite the difference in cytokine production, both studies demonstrate stark differences in the viability of AMs when compared to circulating monocytes/macrophages and macrophage-like cell lines [175, 204, 206]. ET intoxication of Guinea pig AMs with purified ET reduces their secretion of Type-IIA phospholipase A2 [207]. This enzyme not only activates innate immunity through the production of arachadonic acid, but also is able to exert a direct bactericidal activity. Accordingly, infection of Guinea pigs with an EF deficient *B. anthracis* strain does not reduce phospholipase A2 activity. It would be interesting to determine if ET can induce increased cAMP and a decrease in Type-IIA phospholipase A2 in human AMs given the report of poor PA binding to these cells.

There is also conflicting data as to whether circulating monocytes/macrophages produce more or less proinflammatory cytokines after exposure to purified LT. This is in part

due to the ability of macrophages in some rodent inbred strains to undergo pyroptosis in response to LT intoxication (described in further detail below) [127, 208, 209]. Addition of LT to monocytes prevents differentiation into macrophages *in vitro* and reduces the amount of TNFα, IL-1β, and antimicrobial peptides produced by macrophages in response to stimuli [202, 210, 211]. Further, both low doses of recombinant LT and ET reduce the ability of macrophages to phagocytose [212, 213]. In agreement with the protective roles of macrophages, spore-infected mice supplemented with mutant macrophages that are resistant to LT-mediated lysis (see more details below) had higher survival rates than those supplemented with exotoxin-sensitive RAW264.7 cells. The mice with the mutant LT-resistant macrophages had lower bacterial burdens 18 hours post infection than controls. There is no difference in mouse survival between mutant LT-resistant and parental macrophages when infected with vegetative bacteria, potentially indicating an exotoxin-independent mechanism, such as capsule for phagocyte evasion [72].

NK cells were first identified as being involved in anthrax immunity when it was noted that formaldehyde fixed spores (FIS) induce a large increase in IFN-γ and IL12p40 in murine splenocytes [214]. Incubating splenocytes with anti-IL-12 antibodies abrogates the increase in IFN-γ production, strongly suggesting that IL-12 production is necessary for the increase in IFN-γ. Further, this increase is dependent on MyD88 signaling as splenocytes from MyD88$^{-/-}$ mice do not respond with an increase of either of these cytokines. Within the spleen, the CD11b^{+} population (macrophages and some DCs) are responsible for the IL-12 production, whereas the CD49b^{+} population (the majority of NK and NKT cells) produces the IFN-γ. Addition of purified LT to splenocytes disrupts a paracrine feedback loop that is known to increase the bacteriocidal activity of macrophages and monocytes as well as increase inflammation in the host.

A recent study further delineated the mechanism of the macrophage-NK interactions and its role *in vivo* [215]. BMDM induce IFN-γ production in NK cells after exposure to *B. anthracis* spores through direct contact of macrophage RAE-1 ligand to their activating NKG2D receptor. Neutralization of either RAE-1 or NKG2D by monoclonal antibodies leads to a decrease in IFN-γ and this IFN-γ decrease is additive if both antibodies are included. Furthermore, addition of spores to BMDM led to an increase in RAE-1 expression. RAE-1 is expressed due to TLR signaling through MyD88, in agreement with the previous publication noting that MyD88 is necessary for this macrophage-NK paracrine function [216]. Furthermore, IL-18 and IL-15 are necessary for the IL-12 mediated IFN-γ production. Purified LT decreases IFN-γ release from NK cells by preventing IL-12/IL-18 production via reduced phosphorylation of p38, JNK, and ERK. LT treatment does not lead to increases in cell death as determined by cell permeability, but it does reduce metabolism when analyzed using mitochondrial reductase assay. While purified LT acts on NK cytokine secretion, purified ET leads to only a slight reduction in IFN-γ production at high concentrations when splenocytes are exposed to FIS. However, purified ET reduces IL-12 released by macrophages/DCs to basal levels, thereby reducing IFN-γ production indirectly. ET also reduces IL-18 production when exposed to FIS, as addition of IL-18 can restore IFN-γ levels in splenocytes treated with purified ET. Further, both purified LT and ET reduce the ability of NK cells to lyse MHC class I-deficient cells *in vitro* and *in vivo*. Lastly, infection of mice with either ET only or LT only-expressing strains leads to a decrease in migration of NK and $F4/80^{+}$ cells to draining lymph nodes.

Gonzales *et al*. similarly found a decrease in IFN-γ, but no changes in apoptosis when incubating human NKs and monocytes with purified LT [217]. Conversely to mouse NK cells, human NK cells treated with purified LT or ET were still capable of lysing MHC class I deficient K562 cells. Both studies demonstrated that NK's delayed dissemination of *B. anthracis* in both

cutaneous and inhalational models of infection; however, Gonzales *et al*. noted that NK deficiency did not alter the pathology or the survival kinetics. In agreement with this, NK cells greatly enhance the bactericidal activity of infected monocytes and surprisingly can cause a ~2 $\log_{10}$ decrease in extracellular vegetative bacilli. While NK-macrophage/monocyte enhancement was well described in Klezovich-Bénard's work, the mechanism by which NK cells are able to kill bacilli is unknown. The authors suggest that natural cytotoxicity receptors, which have been shown to have an effect on other bacteria, may bind a yet to be discovered ligand on *B. anthracis*. It is unknown if these cytotoxic receptors are altered by exotoxin.

LT can also induce anergy in Type I CD1d-restricted natural killer T (NKT) cells [218]. These cells are a class restricted T cell that express a semi-invariant T cell receptor, CD1d lipid specific antigen presentation molecule, and the NK1.1 molecular marker. These cells occupy a unique niche in the host between the innate and adaptive immune systems. NKT cells also express CMG2 and TEM8 at greater quantities and bind PA to a greater extent than other splenocytes. Intoxication with purified LT leads to a large decrease in NKG2D expression by NKTs, yet this is not seen in NK cells. Further, addition of purified LT to NKT cells leads to decreases in CD69 as well as increases in Ly49 inhibitory receptors. Similarly to NK cells, there is no decrease in cell viability. NKT cells are also noted for their ability to be stimulated with the glycolipid alpha-galactosylceramide (α-GC) to produce IFN-γ and IL-4. *Ex vivo* stimulation of splenocytes with α-GC from a mouse treated with purified LT led to a decrease in both IL-4 and IFN-γ. Co-culture assays using splenocytes harvested from an NKT deficient mouse treated with purified LT and LT treated NKTs expanded *ex vivo* demonstrate that the IFN-γ production comes from the NKT cell and that splenocytes and NKT cells are synergistically involved in IFN-γ production.

DCs, T, and B cells are also affected by exotoxins, though as mentioned in a previous review, it is not certain what role this plays given that anthrax is an acute disease and there is evidence that survivors have long lasting immunity [39, 219]. It is possible that DCs, T, and B cells increase the activation and bacteriocidal activity of the innate immune system indirectly through cytokine and chemokine production. In support of this, *in vitro* addition of purified exotoxin reduces the amount of pro-inflammatory cytokines produced by human and murine dendritic cells [220-223]. Splenic DCs that are intoxicated with purified LT are deficient in stimulating T cell proliferation [220]. Further, there is evidence that purified LT can induce cell death in immature, but not mature, DCs [224, 225]. This is complicated by the fact that recombinant ET can induce maturation of immature DCs, a feature that has been demonstrated with other cAMP increasing agents [196, 226]. It is worth noting that DCs are a very diverse class of cells that originate from separate precursors and have different roles [227]. Thus, anthrax exotoxins may have unique effects on different subtypes of DCs. In support of this, bone marrow derived DCs infected with a *B. anthracis* strain expressing both exotoxins do not have defects in maturation [223]. Yet, infecting lung DCs (LDCs) with spores from a strain that produces LT with or without ET leads to a decrease in LDC activation markers that are not expressed when LDCs are infected with Sterne strain (LT^+ and ET^+) [222]. Therefore, it is possible that in addition to alveolar macrophages, resident lung DC's have unique responses to LT and ET.

Beyond the previously mentioned decrease in DC-mediated stimulation of T and B cells, both LT and ET are capable of directly suppressing immune cell proliferation, similar to the effect of ET on the murine J774.A1 macrophage-like cell line [228-231]. Purified exotoxins are also sufficient to reduce cytokine and IgM production [229]. As discussed above, T and B cells have powerful roles in regulating the innate immune system and may have an important role in

directing the innate immune response. This is underscored by purified ET inducing the development of T helper cells to a T_H2 and T_H17 phenotype, depending on the amount of ET added [232, 233]. The latter development is surprising given that T_H17 invokes a potent antimicrobial effect. At present, there is little data on the effect of LT on T helper polarization, so it is unknown if these effects would be mitigated during co-intoxication. It is tempting to speculate that the reduction of IFN-γ from NK cells plays a role in this polarization toward T_H2/T_H17.

Finally, host immune cells are particularly susceptible to multiple forms of cell death when intoxicated by *B. anthracis*' exotoxins. Macrophages are vulnerable to LT mediated death [209, 234], but a large body of work has focused on the ability of some mouse strain macrophages to generate a potently pro-inflammatory and rapid cell death called pyroptosis (further discussed below) [127, 208]. For the purposes of this dissertation, macrophages that undergo pyroptosis are referred to as LT-sensitive and macrophages that experience a slow apoptotic death are referred to as LT-resistant. Interestingly, mice that have LT-sensitive macrophages are generally resistant to anthrax spore infections and those strains that succumb to anthrax tend to have LT-resistant macrophages [103]. This sensitivity is due to several genetic factors, but the majority of research has focused the allelic variants of Nalp1b/Nlrp1b [235]. Yet, there are exceptions to this correlation between Nlrp1b-sensitive mice and rapid death due to purified LT injection; e.g. C3H mice macrophages rapidly lyse when exposed to high concentrations of purified LT, but the mice have greater survival after LT injection than the LT-sensitive Balb/c strain [236]. These exceptions suggest that there may be several loci that confer lethality to the host, but the Nalp1b-sensitive allele is sufficient for macrophage pyroptosis [236, 237]. Indeed, there is some data that pyroptosis by itself may have host protective effects. Transgenic mice that have an LT-sensitive Nalp1b allele in an otherwise resistant background

have a greater resistance to spore challenge than the parental LT-resistant Nalp1b mouse strain and greater recruitment of neutrophils into the peritoneal cavity during a peritoneal infection [238]. Therefore, mice with the LT-sensitive Nalp1b allele have a greater early pro-inflammatory response due to LT intoxication, suggesting that an increased pro-inflammatory response is protective against *B. anthracis* infection. Similar findings support that this Nalp1b-sensitive protection is linked to neutrophil recruitment in a subcutaneous infection model [186]. Finally, pyroptosis in response to purified LT exposure has only been reported in macrophages and DCs of mouse and rat strains [224, 239]; therefore, the role of Nlrp1b in human pathogenesis is unclear. At lower doses (or higher doses for non-sensitive strains/organisms) purified LT induces apoptosis in macrophages and DCs [206, 224, 225, 240]. Only Voth *et al*. have reported ET to be directly cytotoxic to both zebrafish embryos and RAW264.7 macrophage-like cells [241]. However, ET inhibits cell cycle progression in the J774.1A and RAW264.7 macrophage-like cell line [197, 230]. It is unknown if this delay leads to apoptosis or whether it is unique to macrophages.

The exotoxin's impact on the immune system is contrarily simple and complex. It is obvious to see that the exotoxin works rapidly in the host to inhibit early inflammation and sustain this anti-inflammatory state despite high bacterial burdens in the blood, spleen, and other organs. Yet, the exotoxin works on a systemic and cellular level to reduce the immune response in a multifaceted manner by coordinating decreases in the ability of immune cell chemotaxis, bactericidal activities, and increases cell death through various mechanisms. What remains to be understood are how the exotoxin coordinates these events in real time and what the relevant concentrations of the exotoxin components are during infection.

Cardiovascular and endothelial effects

While intoxication of the immune system allows for survival of the bacteria in the host, its effect on the cardiac tissue and vasculature are potentially necessary dissemination through the host and the final stages of infection in many animal models and human case studies [242-245]. The ability to delete the exotoxin receptor in specific tissue with the Cre/lox system in mice indicated that cardiac and smooth muscle cells are crucial for exotoxin mediated death [128]. Additionally, several lines of evidence indicate that the exotoxin leads to increases in vascular permeability in the host. Such changes are necessary for the bacteria to disseminate beyond the initial site of colonization and lead to the death of the host.

Early anthrax research suggested that the exotoxin induced immunopathology due to sepsis and this led to the vascular damage and shock at terminal stages. In the past 10 years, however, there has been growing credence to the hypothesis that the shock-like phenotype of anthrax is due to cardiovascular damage directly from exotoxins rather than immunopathology from intoxicated host cells. Injection of purified LT in mice leads to host tissue damage more consistent with hypoxia, rather than endotoxin/macrophage induced septic shock [129]. Post-mortem histology from LT-injected BALB/cJ mice demonstrates a lack of indicators of cytokine-induced shock, e.g. fibrin clots and capillary thrombosis in the liver, kidney dysfunction, or extended increased production of pro-inflammatory cytokines. Rather, hypoxic response proteins rapidly increase after LT injection. This contrasted earlier work from Hanna and Collier which concluded that the rapid death from LT injection was due to increased cytokine production from macrophages [127]. Further, depletion of macrophages and antibody mediated neutralization of IL-1 protects mice from LT-mediated death. Adoptive transfer of RAW264.7 cells, which are sensitive to LT-mediated pyroptosis and secrete IL-1β, into macrophage-depleted mice re-establishes the rapid death phenotype [127]. Subsequent studies, however, have shown evidence that LT predominantly suppresses pro-inflammatory cytokine production

even when LPS is used as the stimulus [175, 243, 246]. Similarly, slow infusion of LT into rats reduces cytokines in serum when compared to rats that received LPS only. Delivery of purified ET can also exert pro-inflammatory effects with some cytokines, but since ET does not induce pyroptosis, it has never been implicated as causing pro-inflammatory septic shock [195].

Infusions or boluses of purified exotoxin do not completely replicate the pathology of anthrax and toxemia should not be thought of as recapitulating the bacterial infection. In contrast to the Moayeri hypoxia study, necropsies from *B. anthracis* infections show fibrin deposits and vasculitis in several animal models [96]. What is striking is that the bacilli can reach such high numbers in hosts before there are any overt signs of morbidity. Since the majority of data support an anti-inflammatory role of LT and ET, it is possible that anthrax exotoxins are temporarily reducing immunopathology by reducing the amount of pro-inflammatory cytokines produced by the host. Similar to the effect of anthrax exotoxin reducing the amount of pro-inflammatory cytokines in immune cells, several reports have shown that LT reduces pro-inflammatory cytokines in endothelial cells. IL-8 transcripts are destabilized by LT via dephosphorylation of the RNA regulation protein tristetraprolin [22, 247]. In addition, purified LT suppresses tissue factor production and NF-κB up regulation when endothelial cells are stimulated by LPS [248]. Perhaps the reduction of the inflammatory response is critical early in infection to protect the bacteria from innate defenses as they initially colonize and then progress towards dissemination to the blood stream. Although few studies have looked at the effect of *B. anthracis* peptidoglycan on host cells, the pro-inflammatory responses induced by peptidoglycan require the p38 MAP kinase pathway that are in turn inhibited by purified LT [202, 249, 250]. *In vitro* addition of LT to purified human white blood cells inhibits antimicrobial peptide production [202]. Additionally, sub-lethal doses of LT can result in slight reductions of pro-inflammatory cytokines in rats injected with *E. coli* or its purified LPS [246]. In contrast to

the anti-inflammatory role of LT to the endothelium, LT has an additive effect with TNF-α to up regulate VCAM expression in human endothelial cells [251]. Further studies are necessary to understand the mechanism and limitations of exotoxin to prevent immunopathology via peptidoglycan.

Although the exotoxins decrease host immunopathology at some stages of infection, there is evidence that LT and ET induce damage to the vasculature and increase permeability. Increased leakage or hemorrhage of the vasculature is commonly associated with anthrax in non-human primate models [96]. Treatment of primary human endothelial monolayers with purified LT, but not its individual components, increases the permeability in a concentration and time dependent manner [252]. Microscopic analysis further shows cell elongation, interepithelial gaps, and a reduction of surface vascular endothelial cadherins. While there is a small decrease in cell metabolism and low levels of apoptosis at 48 and 72 hours post addition, this is not sufficient to cause the increased permeability seen with LT treatment. In agreement with this, treatment with a cocktail of MAP kinase inhibitors rescues the permeability defect. Another study found that addition of purified LT to human umbilical endothelial cells (HUVEC) induced significant amounts of apoptosis [253].

A unique method of investigating the effect of injected purified LT on vasculature *in vivo* utilized transparent zebrafish embryos [254]. This model allows visualization of the progressive endothelial permeability and cardiovascular dysfunction in real time. Enlargement of the cardiac chambers first occurs, followed by a rapid increase in vascular permeability and intersegmented vessel collapse. Later a narrowing of the outflow tract from the heart and pericardial edema results in a cessation of blood flow. Vascular collapse occurs with no signs of decreased cell proliferation and with little cell death. Endothelial growth, proliferation, and permeability are in part regulated by vascular endothelial growth factor (VEGF) and VEGF

receptors (VEGFR). VEGFR inhibitors can partially abrogate the effects of LT on vascular permeability [254]. Whether LT's effect on VEGF pathways is independent from MAPK pathways is unknown. Vascular leakage in mouse models shows similar results using a Miles assay, which uses intravenously-delivered Evan's blue dye to quantify macromolecular vascular leakage. Subcutaneous injection of purified LT results in a rapid (15-25 minutes) and dose dependent response in mice [255]. This rapid response to LT suggests that vascular leakage is due to a transcription/translation independent event. The fact that ketotifen, a histamine inhibitor, can greatly reduce the amount of leakage lends credence to this idea, though it is uncertain via which mechanism or on which cell ketotifen is functioning. The ability and degree of leakage varies between inbred mice strains and has no correlation to strain susceptibility to LT-mediated death [255]. Therefore, LT mediated death may be due both to vascular leakage and the host's response to the leakage. Moreover it is unknown how MEK cleavage by LT could lead to such a rapid response.

LT and ET can also affect endosomal recycling pathways, which can result in vasculature leakage. LF and EF reduces both Notch receptor and surface protein levels of the Notch ligand Delta when endogenously expressed by a GAL4 promoter in *Drosophila melanogaster* [256]. Notch and Delta are members of the Notch signaling pathway, which have crucial roles in embryogenesis and cell to cell communication. EF also down regulates the production of Serrate, another Notch ligand. Both of these exotoxins target the recycling of the Delta ligand that is necessary for proper DE-cadherin expression at cell junctions. The decreased cadherin expression leads to reduced cell to cell contact and therefore increases permeability. EF interferes with the Rab11 and Sec15 complex, whereas LF targets only Sec15 vesicles. Sec15/Rab11 are also inhibited by ET and LT in human brain microendothelial cell lines

(hBMEC), leading to a decrease in membrane cadherin expression. Transwell assays also demonstrated an increase in hBMEC permeability.

There is conflicting data on ET's role in inducing vascular leakage. Addition of purified ET induces transendothelial cell macroapertures (TEM) in HUVEC monolayers and their formation correlates with the increase in permeability. These TEMs are resealed by the host via Missing in Metastasis protein through actin polymeration, suggesting a complicated balance in the maintenance of endothelial integrity [257]. Conversely, Tessier *et al*. found that administration of purified ET to HUVECs increased transepithelial electrical resistance (TEER) and suggested that ET induced edema indirectly by increasing inflammatory lipid mediators *in vivo* [189]. Furthermore, pharmacological inhibition of prostanoids, histamines, and neurokinins, which were postulated to induce histamine production, reduced edema formation via vascular leakage. The source of neurokinins is unknown, but it could come from immune cells, fibroblasts, endothelial, or quite intriguingly, sensory neurons.

Finally, host control of endothelial permeability has a very important role in regards to the blood brain barrier (BBB), which is composed of a single layer of brain microendothelial cells (BMEC). Anthrax is not commonly thought of as a disease that results in meningitis; yet, data has shown that ~50% of patients with systemic anthrax developed meningitis, far greater than many other bacterial causes [29]. *B. anthracis* is able to adhere to and be internalized by the BMEC and this invasion is potentiated by the presence of the exotoxin encoding genes. Additionally, infection in a meningitis model leads to inflamed and thickened meninges in 63% of infected mice [258]. Further, adherence and invasion is aided by both bacterial exotoxins and the adhesin BslA. ZO-1^{+} tight junctions in BMEC monolayers exposed to non-encapsulated Sterne strains are disrupted to a greater extent than either the Δ*bslA* mutant or the ΔET/LT mutant [259]. Infections with exotoxin deficient bacterial strains demonstrate that ET is

necessary to disrupt the barrier function in BMEC monolayers, but LT is necessary for invasion. In vivo meningitis models of CD-1 mice show that LT is necessary for bacteria to reach the brain; however, it could be that the LT deficient strain is attenuated and cannot establish a productive infection in these mice.

While exotoxin mediated immunomodulation is required for initial survival of the host, there is the potential for other cell types to play a role in dissemination and death. Thus, the exotoxin must not only avoid clearance by the host, it must disseminate and induce the death of the host in order to continue its lifecycle. The increased permeability of the endothelial and blood brain barrier and the ability to cause the death of the host are two likely ways that the bacteria induce dissemination and aid in the death of the host, yet their molecular mechanisms are only recently being understood.

Dissemination of *Bacillus anthracis* in the host

Given the high mortality of inhalational anthrax and its potential as a biological weapon, the majority of dissemination studies have focused on inhalation routes; however, some studies have investigated dissemination in subcutaneous and gastrointestinal *B. anthracis* infections [79, 81, 260-263]. Regardless of the route of infection, *B. anthracis* has a common method of dissemination (Figure 3) [264, 265]. Spores germinate in the initial site of the infection and the vegetative bacilli produce the exotoxin and capsule (Weiner et al., 2014). Within this initial site, also known as a portal of entry, the bacilli replicate and increase the exotoxin concentration in the local environment. From this portal of entry, bacilli disseminate to the draining lymphatics and repeat the process of replication and increase exotoxin concentration in the host [184] . Finally, the bacilli disseminate from the draining lymph node to the bloodstream via efferent lymphatics [74, 81, 266]. From there, the bacteria could reach other organs, such as the kidneys,

spleen, and liver [81, 95, 260, 266]. Despite the conservation of dissemination between these routes, differences in the initial site or dissemination to the lymphatics must occur as some routes of infection higher mortality than others.

Since the late 19th century, there has been controversy as to how and from where *B. anthracis* disseminates [267-269]. In part these differences are due to the difficulty of studying *B. anthracis* in the early stages of pathogenesis [270]. These are further compounded by the speed with which *B. anthracis* is able to disseminate [266, 270]. As such, there remain several areas of dissemination that are not understood well. In particular, researchers have used several animal models and post mortem analysis of humans to understand if the lungs act as the initial site of infection or if they are an organ that the spores must pass through to cause infection [74, 81, 266-268, 271, 272]. These differences can be viewed through two different models of dissemination, a phagocyte mediated dissemination and a non-phagocyte mediated dissemination method.

Phagocyte mediated dissemination to lymphatic tissue

Early research with inhalation anthrax in animal models suggested that *B. anthracis* did not infect the lungs [273]. Observations from several studies noted that bacilli were rarely found within the alveolar spaces of the lungs, but spores and germinated bacilli were often found in macrophages and the lung associated lymph nodes [74, 95, 266, 267, 272, 274]. Additionally, inhalational anthrax does not cause broncheopneumonia like several other lung pathogens, but an interstitial pneumonia with pathological evidence that it is secondary to dissemination [96, 267, 272]. Histological analysis of guinea pigs infected intratracheally with *B. anthracis* spores suggested that phagocytes could act as a vehicle to deliver spores or vegetative bacilli to the draining mediastinal lymph nodes [74]. Similarly, dendritic cells are a capable of spore uptake and germination [221, 222]. Dendritic cells with spores are able to migrate from the alveolar

spaces to the draining lymph nodes in as few as 45 minutes post infection [222]. Despite these findings, there are several caveats to these studies. A crucial piece of data supporting phagocyte mediated dissemination is a histology section containing a macrophage with germinated spores within a lymphatic vessel [74]. However, histological analysis does not clearly indicate the direction of migration of bacterial laden macrophages. Indeed it is possible those spores are being transported away from the mediastinal lymph nodes. Secondly, the presence of spore or bacillus within an immune cell in a lymph node does not conclusively demonstrate that the bacteria are viable or continue on to infection. As such, the germinated spores in Ross' histology image could have been destroyed by the macrophage en route to the lymph node. In support of the latter, mice where macrophages were depleted with clodronate liposomes were more susceptible to inhalational anthrax [191]. Therefore, there may be phagocyte independent methods of dissemination.

Phagocyte independent dissemination in the host

While the phagocyte dependent methods of dissemination have been well researched over the past 50 years, there is relatively little research on how phagocyte independent dissemination occurs. A recent review from our laboratory examining these differences posed a Jailbreak model of dissemination that synthesized many of the features of phagocyte independent dissemination [264]. This model proposes that *B. anthracis* spores not only sense the host, but they sense favorable environments in the host for germination. After germination the combination of exotoxin, proteases and other factors allow the bacilli to replicate locally, disrupt the epithelial or endothelial barrier, disseminate to the draining lymph node and ultimately the throughout the host.

Investigations of *B. anthracis* infections with bioluminescent strains in mice have demonstrated that infections can initiate outside the lymph nodes [81, 260, 270, 275]. Inhalational infections and gastrointestinal infections were found to initiate first in the nasal associated lymphoid tissue (NALT) or Peyer's patch, respectively [81]. From these mucosal associated lymphoid tissues, the bacilli disseminate to the draining region lymph nodes and the access the blood stream from the efferent lymphatics [81, 270]. Subcutaneous infections do not initiate in a mucosal associated lymphoid tissue, but appear to germinate and disseminate from the initial site of injection in the ear dermis [79, 81, 261]. Spores are capable of germination without prior engulfment by phagocytes and germinated within 15 minutes in a guinea pig model [79]. These infections appear to spread to lymphatics in a phagocyte independent manner, as debridement of the tissue prevents the infection from disseminating, despite the presence of spores in the draining cervical lymph nodes [261].

Infections are also observed to occasionally germinate and initiate in the lumen of the lungs [133, 275, 276]. This is a surprising finding as the lungs are generally refractory to germination [74, 214, 275-277]. Compared to the subcutaneous and NALT based infections, lung based infections are detected later and it is unclear how these infections disseminated from the lung lumen. Moreover, how these lung-based infections initiate are not well understood. Some have suggested that lung based infections occur at higher doses and initiate from macrophages engulfing too many spores [75, 275, 276]. The spores would then be able to germinate and kill and escape from the macrophage before it has time to migrate to the mediastinal lymph nodes [275].

An alternative model for phagocyte independent dissemination is that the spores and vegetative bacilli can be internalized by epithelial cells directly [62, 271, 278-281]. These studies

are supported by *in vitro* and *in vivo* studies that demonstrate that the spores are able to adhere, invade, and transcytose through the epithelial layer [271, 279, 280]. Additionally, *B. anthracis* spores are unique from related *Bacillus* spores in that they can reside in the lung for long periods of time [278]. Whether these bacilli contribute to the infection or germinate within the epithelial cell *in vivo* is unknown.

Clonal analysis as a tool to understand population dynamics *in vivo*

Beyond the controversies in how dissemination occurs, there is very little known about how dissemination affects the bacterial population dynamics *in vivo*. Though tempting to consider, bacterial infections do not necessarily take advantage of one site and disseminate unidirectionally from that portal of entry [149-151, 282]. Pathogens can disseminate through multiple routes, leading to several smaller populations colonizing unique areas of the host or a small population arising from an initial site that goes on to disseminate throughout the host [150, 282]. The use of clonal analysis allows for these *in vivo* population dynamics to be understood. Clonal analysis uses a series of equally virulent clones that differ only by a unique tag, such as antibiotic markers, fluorescent tags, or recombinant DNA tags. Animal models are infected with this library and the infection is allowed to disseminate. After death or at key time points, the animal is dissected and the clonal proportions within organs are analyzed. The changes in proportion can then be used to detect population bottlenecks, dissemination pathways, or to analyze the effects of virulence factors on the ability to disseminate [133]. *B. anthracis* is known to disseminate to the blood stream frequently but can use several different routes. While the use of bioluminescent *B. anthracis* strains have been useful in understanding where the bacilli disseminate, they tell little of what percentage of the inoculum go on to disseminate and kill the host. Nor do such studies illustrate how virulence factors, such as lethal

factor, work in concert with dissemination to cause disease. As such, clonal analysis could be used to understand how early events in pathogenesis and exotoxin expression affect dissemination.

Dissertation goals and significance

The goal of this dissertation is to understand the early events in anthrax and how it influences dissemination. This is divided into two chapters: Chapter 2 investigates how the initial sites of infection impact dissemination and Chapter 3 investigates how the amount of lethal factor affects early stages in colonization and dissemination. While *B. anthracis* can disseminate through several routes, the frequency of dissemination varies between these routes [16]. As such, a comparison of these routes may provide an understanding of the challenges to dissemination the bacteria face in each route. For example, a dissemination route that allows fewer clones to disseminate suggests that the host is more capable of clearing a bacterial infection when compared to an infection where several clones disseminate. The knowledge that some routes are more permissive for infection than others will allow for future research that can identify why these sites are more permissive. Furthermore, little is known about why these infections initiate in the mucosal associated lymphoid tissue, dermis, or the lung lumen. These tissues environments are different from each other and may force *B. anthracis* to disseminate in unique ways.

The second goal is to understand the role lethal factor has for dissemination in a mouse model. Lethal factor is known to be important in pathogenesis, as infection with a strain that lacks lethal factor (LF) has either reduced virulence or is completely avirulent [95, 270]. What is poorly understood is the relative amount of lethal factor that is needed for distinct points in the infection and whether LF produced from one bacillus in a population can benefit a bacillus that

cannot produce LF. Such studies would demonstrate how potent LF is at inhibiting the immune system and allowing dissemination. If ΔLF strains could disseminate when only a small amount of LF is produced, it would suggest that the evolutionary pressure on LF is relatively low and that a mutant bacillus that produce less LF would not face heavy selection pressure in a population. Further, it would allow researchers to understand how much LF is needed for colonization and subsequent dissemination. Such data would be useful in understanding pathogenesis of *B. anthracis* in a host model and would provide a clear understanding of the host immune response to the bacteria.

Figures for Chapter 1

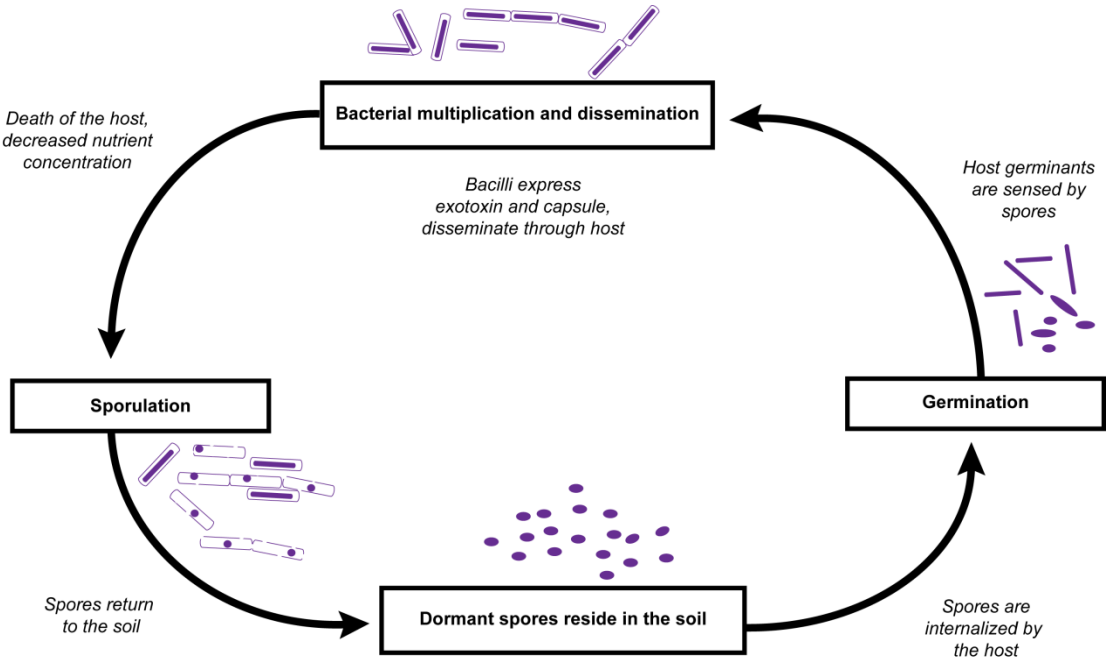


Figure 1. Diagram of the *B. anthracis* infection cycle.

Dormant spores reside in the soil until they are taken up by the host. Once in the host, the spores germinate to form the vegetative bacilli. The bacilli produce both a capsule and an exotoxin which allow the bacilli to multiply to a high number and disseminate through the host. The combination of bacteremia and toxemia lead to the death of the animal. The decreased nutrients and increased oxygen tension in the dead animal cause the bacilli to sporulate, which leads to the spores return to the soil. Diagram from [17] and used with permission.

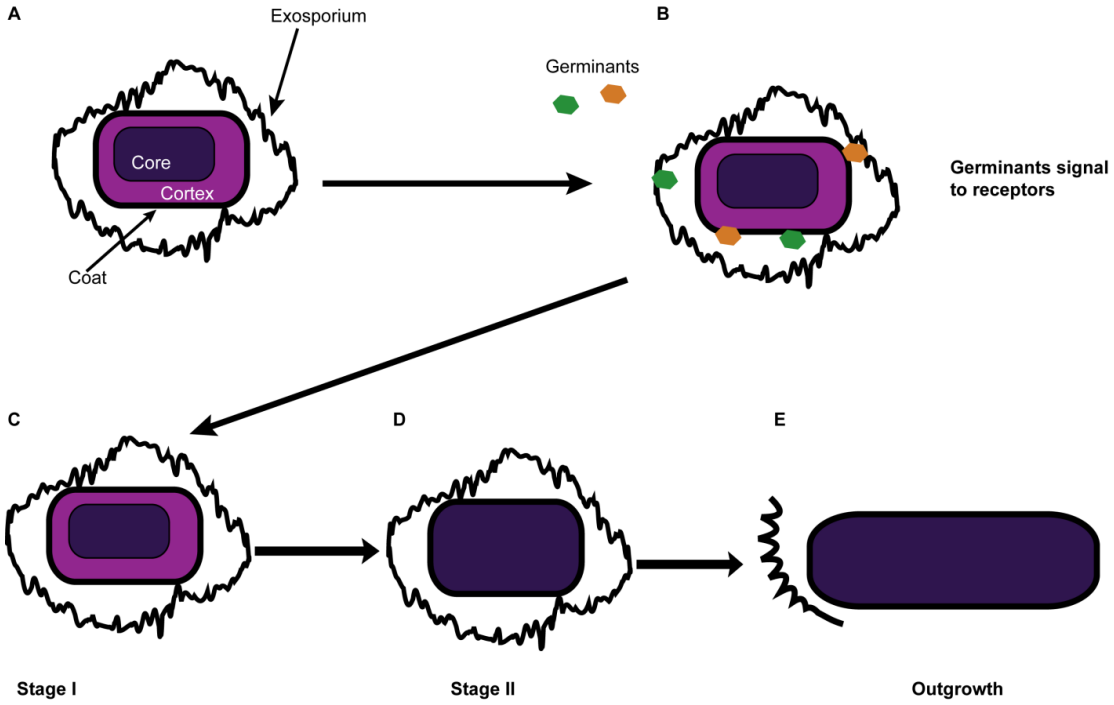


Figure 2. Diagram of spore structure and germination.

A) The core, cortex, coat, exosporium are labeled. **B)** Germination initiates when host germinants bind to germination receptors on the spore coat. Receptors signal and lead to Stage I **C)** Stage I leads to partial hydration and cation release from the core. **D)** Stage II leads to cortex degradation, core expansion, and further hydration. Stage II causes the spore to become more susceptible to heat and other forms of killing. **E)** The final stage is outgrowth where the exosporium is released from the bacteria and the bacilli begins metabolism, exotoxin production, and capsule biosynthesis.

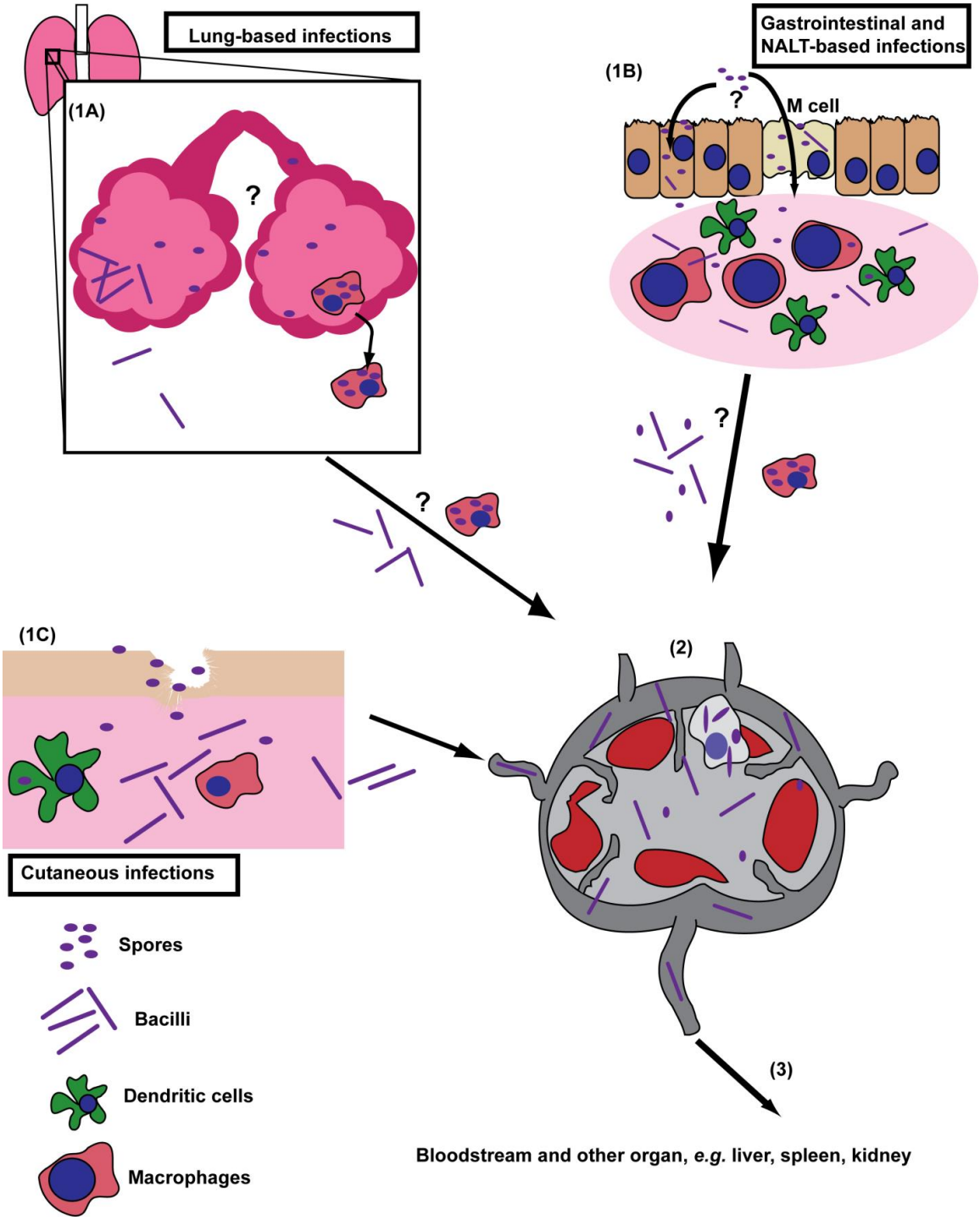


Figure 3. Diagram of potential dissemination pathways for *B. anthracis*.

B. anthracis can disseminate from several routes, but has similarities between different routes. Infections can initiate from two possible routes in the inhalation routes, the lungs (1A) or the

NALT (1B). Similarly, gastrointestinal routes begin in the Peyer's patches (1B). Skin infections have spores germinate in the local tissue (1C) and then bacilli disseminate to the draining lymph node (2). In contrast, mucosal associated lymphoid tissue and alveolar spaces could have either local germination in the portal of entry and then disseminate to the lymph node, or could have phagocytes deliver spores to the lymph nodes (2). From the lymph nodes, the bacilli are able to access the bloodstream and reach other organs (3).

Chapter 2: Early events in *Bacillus anthracis* infections affect dissemination and are dependent on the portal of entry

This chapter is a modified version of the previously published article, "*Bacillus anthracis* Has Two Independent Bottlenecks That Are Dependent on the Portal of Entry in an Intranasal Model of Inhalational Infection" and was used with permission. Copyright © American Society for Microbiology, [*Infect. Immun*., 81(12), 2013, 4408-4420. doi:10.1128/IAI.00484-13]

Introduction

The high mortality rate associated with inhalational anthrax is in part due to the bacteria's success in dissemination through the host and a lack of diagnostic clinical signs of an infection until late in infection. Early research with *B. anthracis* suggested that inhalational infections disseminate via resident phagocytes acting as a "Trojan horse" [74, 283]. In the Trojan horse model, inhaled spores are deposited in the alveoli and phagocytosed by resident lung phagocytes. Either in transit or within the mediastinal lymph nodes (mLN), the spore germinates, escapes from the phagocyte, and replicates in the mLN; the bacteria eventually disseminate to the blood stream via efferent lymphatics [71, 74, 266]. However, experimentally testing this model for early events in pathogenesis is challenging in animal models due to the fulminant nature of the infection and its asynchronous dissemination [270, 284].

Bioluminescent imaging (BLI) of *B. anthracis* strains with luminescent reporters enabled researchers to dissect early events in pathogenesis that previously were difficult to study. These BLI studies identified two novel sites of establishment and dissemination of infection [81, 261, 275, 276]. In the upper respiratory passage, bacteria were first observed to replicate and disseminate from the nasal associated lymphoid tissue (NALT) [81, 260, 270, 275]. A second

portal of entry was identified in the lower respiratory passage in mice where germination and outgrowth occurred directly in the alveolar lumen [275, 276]. Germination and dissemination from the lung was a surprising result, since *B. anthracis* does not generally provoke a classic bronchopneumonia and there is evidence that the lung is refractory to germination [74, 81, 275, 276, 284, 285]. However, inducing germination in the lungs or pre-existing damage to the lungs has been previously shown to permit spore germination and outgrowth in the lungs [45, 81, 286]. While several studies have sought to understand the Trojan horse model of dissemination, fewer studies have investigated the dissemination from NALT and lung-based portals of entry.

A current challenge in understanding bacterial dissemination is rooted in the fact that the activities and behavior of bacteria within the host is poorly understood. Bacterial growth, migration, and metabolism within the host are in constant flux due to the changing host environment over time. These changes in growth and behavior are known as population dynamics. Most host-pathogen studies have focused on dissemination and disease through the amount and location of bacterial colony forming units (CFU) in the host at defined time points. The drawback to this approach is that measuring the amount of CFU in an organ at a particular point in time is limited in temporal scope and only represents the net growth of the bacteria at that particular time (Figure 4A). Thus, the researcher can only investigate a narrow spectrum of *in vivo* population dynamics. Even the information that is gained from the CFU counts present at a time point is limited. For example, CFU counts arise from the difference between the bacterial replication/immigration from other sites and the death/emigration to other sites. Even when techniques are used that allow for real time observation, such as bioluminescent imaging, the population structure and growth rates remain difficult to establish. Such changes in the population structure could lend important insights into the dissemination process of *B. anthracis*

which would be useful for developing future therapeutics or identifying host barriers that are effective at preventing dissemination.

The dissemination patterns of several bacterial pathogens have been analyzed through clonal analysis to clarify portals of entry and understand how the host alters the pathogen's population dynamics [149-151, 287, 288]. Clonal analysis consists of creating a library of isogenic clones that vary only by a signature tag, *e.g.* an antibiotic marker or recombinant DNA sequence. The host is then infected with an equally proportioned mixture of clones that are isolated from tissues of interest at particular stages of the infection to determine if and how the proportions of clones vary during dissemination. Such an approach allows for analysis of the changes in population structure, migration, and replication that occurred within the host during the infection (Figure 4B). These analyses have been used to determine the sequence of dissemination as well as identify population bottlenecks for several pathogens [149-151, 287]. A bottleneck within an infected host occurs when there is a non-selective decrease in the proportion of bacteria relative to a previous infected site. Such bottlenecks increase the rate of genetic drift and allelic fixation, leading to drastic reduction of genomic diversity at the population level, which can be measured by effective population [152]. Effective population differs from population size because it measures the number of individuals within a population that will pass their alleles on to the next generation, rather than the number of individuals. As such, the effective population can measure the intensity or rate of genetic drift on a population. Additionally, the knowledge that only a portion of the inoculum goes on to be the disseminated population brings novel insights into the connection between the dose required to initiate an infection, *e.g.* an infectious dose that would cause an infection for 50% of animals (ID_{50}), and the amount required to kill the host. While previous research has demonstrated that NALT-based

bottlenecks occur as *B. anthracis* bacilli disseminate, it is unknown whether a bottleneck occurs in lung-based infections [288]. If a bottleneck were to occur in both sites, population dynamics during the infection could be compared to inform researchers about how the site of establishment influences the ensuing infection. This could then be used to understand how to reduce the likelihood of dissemination from the sites.

The research described here compared the dissemination of *B. anthracis* from the lungs and dissemination from the NALT using a library of luminescent DNA-tagged clones of *B. anthracis* Sterne strain. The simultaneous use of BLI with clonal analysis was essential to dissect how spatial and temporal events alter the bacterial population dynamics *in vivo*. Lung-based infections initiate when a small number of spores germinate in a focal region and spread rapidly through the host. Since lung infections initiate from a small, distinct focal area and rapidly disseminate through the host, it is difficult to envision such an event being captured by classic pathological or diagnostic methods with significant frequency. When compared to NALT-based infections, lung-based infections undergo bottlenecks at the site of germination and have a significantly higher effective population. This suggests that *B. anthracis* is able to establish infections in the unique micro-environments of the NALT or lung lumen within the host respiratory system and that these locations impart distinct characteristics on the resultant disseminated bacterial population that reflect how the infection unfolds within the host.

Materials and Methods

Bacterial strains, culture procedures and media.

Bacillus anthracis Sterne strain 7702 (pX01^{+}, pX02^{-}) was used for construction of clonal mutants (see below) and was obtained from BEI (Manassas, VA). A luminescent *B. anthracis* 7702 strain, BIG23, was constructed by integrating the plasmid pIG6-19 into pX01, a kind gift

from Michele Mock [261]. This strain contains the *luxABCDE* operon from *Photorhabdus luminescens* under the control of the PA promoter, leading to light expression *in vivo*. BIG23 was used as the base strain for all mouse infections. *B. anthracis* was grown in BHI broth (Becton Dickson, MD) supplemented with 0.5% glycerol at 37°C unless otherwise noted. Spores were produced by plating on NBY media with antibiotics when necessary and incubated at 30°C for 10 days, then harvested and purified on an Omnipaq gradient (350 milligrams of iodine/mL) (GE Healthcare, NJ), similar to previously described methods [81, 261, 289]. *Escherichia coli* strains were either α-select (Bioline, MA) or *dcm, dam*-deficient GM119 (*dcm-6, dam-3)* [290] and grown in LB broth with appropriate antibiotics when necessary (Becton Dickson, MD). All strains and plasmids are described in Table 1.

Construction of *B. anthracis* clonal mutants.

Unique DNA-tagged clones were constructed by inserting recombinant DNA from a progressive 100bp DNA step ladder (Promega, WI) into the chromosomal *B. anthracis eag* gene. DNA tags were inserted into *B. anthracis* by allelic exchange with pSE02 and pCZ03, which are modified from the pHY304 temperature sensitive allelic exchange vector, kindly provided by S. Leppla (Table 1) [291]. To facilitate allelic exchange in *B. anthracis*, a 2kb section of the *eag* gene was constructed by splice by overlap PCR (SOE-PCR) using primers FIG4, RIG4, FIG5, and RIG5 which also introduced a BamHI site (Table 2). The modified *eag* fragment was then inserted into pHY304 to generate pIG13. The pIG13 then had two different multiple cloning site (MCS) inserted into the recombinant BamHI site in *eag* to allow for greater flexibility for cloning in DNA- tags. The pSE02 vector contains a short DNA linker with an EcoRV and SalI site flanked by BamHI sites, whereas pCZ03 has the linker flanked by EcoRI, SpeI, and NotI sites. The pSE02 vector was constructed by SOE PCR where the overlap region contained an EcoRV,

SpeI, SalI and BamHI site within the *eag* gene. The two SOE regions were amplified from pIG13 using RDL22 and F SOE linker and FIG8 and R SOE linker and spliced together by PCR using FIG8 and RDL22 primers. The pCZ03 vector was generated by inserting the linker into the MCS of pGEM-T Easy (Promega, WI), then isolating the MCS containing pGEM-T Easy plasmid by EagI digestion and blunted using the NEB Quick Blunting Kit (NEB, MA). Next, pIG13 was digested with BamHI blunted using the NEB Quick Blunting Kit and the MCS was inserted into pIG13. The fragments from the DNA ladder were blunted with the NEB Quick Blunt kit and ligated into the EcoRV site in the *eag* region of homology of either pCZ03 or pSE02's MCS using T4 ligase (NEB, MA).

After pSE02 and pCZ03 were ligated with fragments from a progressive 100 bp Step-ladder, they were transformed into α-select chemically competent *E. coli* (Bioline, MA). Transformants were then screened with M13 primers to ensure the fragments ligated into the plasmid. The verified plasmid was then passaged through GM119 to remove DNA methylation and purified before electroporation into 7702 as previously described [292]. Mutants were passaged at 42°C twice to select for merodiploid insertion of the temperature-sensitive plasmid carrying erythromycin (Erm) resistance into *eag*, then repeatedly passaged at 37°C until bacteria became Erm-sensitive, indicating that allelic exchange had occurred. PCR was used to identify bacteria with the DNA ladder insert and confirm the presence of pX01. Lastly, to make the tagged strains luminescent, pIG6-19 was conjugated into tagged 7702 strains and selected on BHI plates supplemented with 5 μg/ml Erm and 60 μg/mL polymyxin B [293]. Strains were sporulated and purified as described above.

Determination of growth rate of *B. anthracis* clones.

Strains were grown overnight in BHI media at 37°C and back-diluted to an optical density at 600 nm of ~0.005 in 50 mL of fresh BHI media. The optical density was then measured every hour for 8 hours, then at 12 hours and 24 hours. This was independently repeated 3 times.

Infection and dissection of mice.

Mice manipulations and husbandry were performed in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All techniques and animal husbandry was approved by the University of Virginia Animal Care and Use committee (protocol #3671) and were designed to minimize distress and pain for the animals. All mice were 6-12 week old female A/J mice (Jackson Labs, ME) and were housed and/or bred using specific pathogen-free rearing procedures at the University of Virginia. Infections were performed by anesthetizing mice with methoxyflurane (metafane, Matrix Scientific, SC) in a nose cone and then spores were delivered by intranasal instillation as previously described [81]. The infections were monitored by anesthetizing mice with a 2.5% (%v/v) isoflurane-oxygen mix with an XGI-8 gas anesthesia system (Perkin Elmer, MA) and light production was followed in the host with either the IVIS100 or the IVIS Spectrum (Perkin Elmer, MA) until light was detected in the NALT or ventral thoracic cavity after 1 minute exposure with large binning. Light production was measured and quantified using LivingImage Software (version 3.2, Perkin Elmer, MA). Any mouse with light in the kidneys was considered to have a disseminated infection and was euthanized.

Mice were euthanized and the NALT, cervical lymph nodes, mediastinal lymph nodes, lungs, and kidney were removed and homogenized on ice using Dounce homogenizers and ice-cold 1xPBS as previously described [192]. The homogenates were divided in half with one half

heated to 65°C for 20 minutes to kill vegetative bacilli such that the remaining CFU represent spores. Both fractions were serially diluted and spread on LB plates with 5 μg/mL Erm to enumerate colony forming units (CFU). A portion of each sample was also used to inoculate 3 mL BHI broth with 5 μg/mL Erm and grown overnight at 37°C. If any bacterial growth occurred in overnight cultures an aliquot was brought to 10% glycerol (%v/v) and stored as frozen samples, in order to confirm clonal proportions or increase the number of colonies analyzed at a later date, if necessary.

Determination of clonal identity in organs.

Clonal proportions were established for each organ that contained at least 30 CFU. Organs that contained less than 30 CFU were precluded from further study as small numbers of bacteria are subject to greater skewing of clonal proportions due to chance. For each organ, 48 colonies were individually lysed using the HotSHoT technique to extract DNA [294]. Using 5 μL of the lysed colony DNA sample, clonal identity was established by PCR amplification of the *eag* region flanking the DNA tag using Agilent Paq5000 polymerase (Agilent, CA) and primers FDL26 and RDL 26 to yield unique fragment sizes for each clone.

Probability modeling to determine the size of the clonal library.

The probability of two exact clones passing through a bottleneck was determined by using the hypergeometric formula: $h(x; N, n, k) = [\ {}_kC_x\ ]\ [\ {}_{N-k}C_{n-x}\ ] / [\ {}_NC_n\ ]$, where N is the inoculum delivered in CFU, x are the number of identical clones found in the kidney, k is the number of CFUs from a particular clone in the inoculum, and n is the number of clones that pass through the bottleneck (bottleneck size). The probability was then calculated for a given bottleneck size for a given number of clones in the library.

Statistical analysis.

Student's T tests, one-way and two-way ANOVAs, and Tukey multiple comparisons post test were performed using Graphpad Prism (version 6.03, Graphpad Software, CA). Growth rates were statistically compared by non-linear regression and comparing F-values from each strain. Fisher's exact tests were performed using the R project for statistical programming version 2.15.0 (The R Foundation for Statistical Programming, Vienna, Austria)

Results

Bacterial burdens are similar when *B. anthracis* disseminate from NALT and lung portals of entry.

BLI analysis of infections identified the NALT and the lung lumen as sites of initial germination and growth for *B. anthracis* [81, 275, 276]. Because BLI analysis indicated the bacteria had different dissemination patterns between NALT and lung-based infections, it was hypothesized that these routes of dissemination may have different levels of bacterial burden in similar lymph nodes. To test this hypothesis, A/J mice were infected intranasally with BIG23, a luminescent derivative of 7702. BIG23 has the genes necessary for light production under the control of the protective antigen promoter and produces light when vegetative bacteria reside in the toxin-inducing conditions found throughout the host [261]. Intranasally infected mice developed NALT, lung or lymphatic-based infections (Figure 5). Concurrent dual-portal infections were rarely observed and mice were only analyzed if there was luminescence exclusively in the NALT, lungs, or when seen in the lymphatics. After either a NALT, lung, or lymph-based infection was detected, the cervical lymph nodes (cLN), lungs, mLN, and kidneys were homogenized and the total bacteria and spore populations were analyzed. Since spores are more resistant to heat than the bacilli, any colony-forming unit obtained from the heated homogenate is considered to have been derived from an ungerminated spore. Thus, the

proportion of the bacterial population consisting of spores can be isolated by heating an aliquot of the tissue homogenate and comparing it to an unheated aliquot.

A NALT infection was indicated when the mouse had measurable luminescence above background solely in the nasal passages (Figure 5A). In agreement with previous publications, these mice had luminescence detected in the NALT, cLN, and if the infection were allowed to progress, in the kidney (data not shown). The kidneys were chosen as a proxy to indicate systemically disseminated bacilli since the kidneys reach a high bacterial burden, lack spores, and are the first major organs to become luminescent after dissemination. Mice that had infections disseminate from the NALT did not show luminescent foci in their lungs when dissected (data not shown) [275, 276]. Bacteria in the lymph nodes and kidneys were almost exclusively heat-sensitive, as all CFUs from heat-resistant spores were below the limit of detection (Figure 5B). In contrast, the lungs in these NALT-based infections had 10-fold more vegetative bacteria than heat-resistant spores in disseminated infections.

A lung infection was defined as when light was detected solely through the ventral thorax as previously described (Figure 5C) [81, 276]. Upon closer examination after dissection, the luminescence in lung infections were confined to a single focal region in the lungs and would regularly be detected in the draining mLNs. If the lung infection was allowed to progress to later stages, the kidneys would also display measurable luminescence, in agreement with previous publications (data not shown) [81, 275, 276]. Similarly, in lung-based infections the mLNs and the kidneys contained high amounts of heat-sensitive CFU and all heat-resistant CFU were below the limit of detection (Figure 5D). Additionally, the lungs in lung-based infections contained 12-fold more heat-sensitive bacteria than the heat-resistant bacteria. The

cLNs, however, rarely had CFU present and, when present, they were always below the limit of detection (<20 CFU).

Lymph based dissemination was also observed in mice that were intranasally infected (Figure 5E). These infections differed from NALT and lung-based infections due to their rapid appearance of luminescence in the kidneys and lymph nodes, as well as the number of lymph nodes that were infected. With NALT and lung-based infections, the first site of luminescence was either the NALT or the lung lumen. Then the luminescence would be detected in the draining lymphatics or kidneys. Lymph-based infections would show no luminescence the 24 hours prior to detection. When luminescence was detected, it was evident that dissemination occurred rapidly as the kidneys and several lymph nodes were be luminescent, most commonly the cervical, mediastinal, inguinal, and sciatic lymph nodes. In contrast to lung-based infections, the cLNs contained a high proportion of vegetative bacilli (Figure 5F).

The bacteria pass through distinct bottlenecks based on their route of dissemination.

B. anthracis undergoes a bottleneck as it disseminates from the NALT to the draining cLN [288]. A population bottleneck occurs when there is a dramatic population decrease that leads to a non-biased reduction in genetic variability relative to the original population. BLI analysis has revealed that *B. anthracis* has two portals of entry for inhalational anthrax [275]. Given that the respiratory mucosa in the alveoli is distinct from the NALT and there were differences in the bacterial burden in the cLN, we tested to see if lung-based infections also passed through a bottleneck. Probability modeling determined that 5 clones was the optimum number of DNA-tagged clones to allow for the greatest resolution of the bottleneck without causing unnecessary experimental burden (Figure 6). DNA tagged strains were then tested for growth rate defects and *in vivo* virulence to ensure that *B. anthracis* is undergoing a bottleneck

and not selection against a less virulent strain. A comparison of the *in vitro* growth rates showed no significant growth defects between any of the tagged strains and the parent BIG23 strain (Figure 7A, Non-linear regression and comparison of F-values, *P*-value= 0.9991). Additionally, there were no statistically significant differences between the frequencies of clones in the kidneys compared to those delivered to the mouse, supporting the conclusion that no single clone had a competitive advantage over the others and that passage through a bottleneck is random (Figure 7B, Fisher's Exact Test *P*-value = 0.521).

NALT-based infections were found to undergo a significant reduction in the number of clones between the NALT and cLN, as all organs other than the NALT contained an average of 1 clone (Figure 8A, One way ANOVA with Tukey multiple comparisons post test, ****P-value <0.0001) [288]. Mice with lung-based infections had a less pronounced decrease in the number of clones represented as the bacilli disseminated from the lung lumen (Figure 8B). Organs were dissected and clonal identities were determined when light was detected in the ventral thoracic cavity, but before luminescence was detected in the kidneys. This allowed for the determination of the average number of clones in each organ before the bacteria returned to the lungs in large numbers from the circulatory system, which would confound analysis. The number of clones represented in the whole lungs was not significantly different from the number of clones delivered in the initial inoculum (Figure 8B, One way ANOVA with Tukey multiple comparison post test, *P*-value > 0.05). The average number of clones in the mLNs, which drain bacteria from the lungs, ranged from 1 to 4 clones with a significantly lower average of 2 clones represented compared to the initial inoculum's 5 clones (Figure 8B, One way ANOVA with Tukey multiple comparison post test, ****P-value < 0.0001). In areas where heat-resistant spores were never found, *i.e.* the kidneys, there was an average of 2 different clones, significantly lower

than the inoculum (Figure 8B, One way ANOVA with a Tukey multiple comparison post test, ****P-value < 0.0001). Lymph-based infections had clonal proportions that were unique from NALT and lung-based infections. There were fewer clones in the organs in all organs analyzed, with an average of 2 clones in the draining mLNs and kidney (Figure 8C, One way ANOVA with Tukey's multiple comparison test, **P-value <0.01). The lungs were slightly higher with an average of 3 clones present, lower than the number found in lung-based infections, but higher than in NALT-based infection (Figure 8C, One way ANOVA with Tukey's multiple comparison test, *P-value <0.05). Two mice did not have enough CFU present in the cLN to permit clonal analysis; however, the other two were found to have only 1 clone present in the cLN.

To test if there were differences between the bottleneck in NALT-based, lymph-based, and lung-based infections, the number of different clones found in the organs was compared. Mice with lung infections did not have bacteria present in their cLN until they were moribund and were thus not compared. Mice with a lung-based infection had a significantly higher number of clones represented in the lungs than those with NALT or lymph-based infections (Figure 8D, Two way ANOVA with Tukey's multiple comparison test, **P-value <0.01, ****P-value <0.0001).

Lung-based infections have disseminated clonal populations that do not match populations in the mLN and the whole lung, whereas NALT-based infections have disseminated populations that resemble the cLN.

In order to determine whether the clone or clones that pass through the bottleneck are the founders of the disseminated population in the host, the clonal proportions in each organ were analyzed. BLI was used to synchronize the infections to when light was first detected in the kidneys. Organs were then removed from the infected mice and the proportions and

identities of clones were compared between the organs to determine dissemination patterns. In agreement with previous publications, NALT-based infections were found to have no difference in the clonal numbers or proportions between the NALT and the initial inoculum early in infection (Figure 9). NALT-based infections were found to be almost completely comprised of 1 clone in all organs and the clone found in the cLN had the same identity as the kidneys (Figure 10A, Fisher's exact test, *P*-value >0.05). The lungs in mouse 1 had all clones present; however, the lung homogenates were further tested to determine if this population represented ungerminated spores in the lungs. The percent germination was calculated by comparing heated and unheated lung tissue homogenates. There was a small difference in CFU between the heated (spore only) and unheated lung (bacilli and spores) homogenates ($1.2x10^5$ spore CFU/lung and $1.8x10^5$ total bacteria CFU/lung) implying that most bacteria in the lungs remained as spores. Additionally, there was no statistical difference between the clonal proportions from the heat-resistant bacteria in the lung homogenates and total bacteria lung homogenates for mouse 1, further suggesting that the lungs contained only spores (Figure 10A, Fisher's exact test, *P*-value >0.05). This suggests that the increased number of different clones in the lungs in mouse 1 is due to being dissected before the disseminated bacteria returned to the lungs after dissemination from the NALT and cLN.

In agreement with the data in Figure 8, the clonal proportions in lungs and mLN present in lung-based infections were more diverse than in NALT-based infections. Out of six mice, only mouse E and F had a single clone constitute the majority of the whole lung, 82% and 52% respectively (Figure 10B, Fisher's exact test, *P*-value >0.05). Importantly, mice E and F were dissected later than the other four mice, meaning that the bacilli had a longer time to replicate and disseminate through the mouse, which could allow recolonization of the lung from the

periphery. The other 4 mice had no more than 35% of the clonal proportions containing a single clone. Therefore, if a bottleneck occurred, clonal analysis was not sufficient to detect lung bottlenecks when the whole lungs were analyzed in a single homogenate. Similarly, only one mouse out of six had no statistical difference between the kidneys and mLNs, potentially suggesting the bacilli disseminated without using the lung lymphatics (Figure 10C, Fisher's exact test, ****P-value <0.0001).

The focal regions of bacterial growth arise from a few clones that replicate in a small area and these founders go on to disseminate through the host.

The population structure of the disseminated bacteria from lung-based infections suggested a bottleneck occurred by the time the bacteria were systemically disseminated, but whole lung analysis was potentially hindered by the high spore burden in the lungs (Figure 5D and Figure 8B). Since previous publications have noted that lung germination and outgrowth occurs in distinct focal regions in the lung, it was investigated if these regions contained a higher proportion of bacilli than spores [275, 276]. Moreover, the high degree of luminescence within this region suggests a higher bacilli proportion, which would reduce the likelihood of sampling spores and allow for greater ability to determine if a bottleneck occurred in the lungs. After luminescence was detected in the ventral thoracic cavity, the lungs were removed and separated into luminescent and non-luminescent regions (Figure 11A). The focal luminescent region had greater percentage of bacilli than the non-luminescent regions as determined by heat-sensitivity (Figure 11B, Student's T test ****P-value <0.0001). Germination in the non-luminescent region averaged 39% (S.D. 15.9%) indicating that the majority of the bacteria were still in spore form. However, an average of 86% (S.D. 18.9%) of the bacteria in the luminescent

focal region were heat-sensitive in addition to expressing a luminescent reporter that only produces light in metabolically active vegetative bacilli.

If a bottleneck occurred in the focal region, it could suggest that a few spores in the focal region germinated, replicated, and eventually disseminated. Alternatively, it is possible that all 5 clones in the library germinated in a small area, but only the first few clones that reached the draining lymph node or blood stream were the founders of the disseminated population. To differentiate between these two scenarios, the clonal diversity of the focal regions were analyzed separately from the rest of the lungs and compared to the clonal diversity of a similarly sized non-luminescent area of the lung. Mice were first dissected when there was clearly luminescence present in the thoracic cavity, which corresponded to > $3.5x10^6$ CFU/g in the focal region. In these mice, all 5 mice had significant differences in clonal diversity between the initial inoculum and the focal region (Figure 12A, Fisher's exact test ****P-value <0.0001). Two mice, mouse 4 and mouse 5, had the same clonal population comprise a majority in the focal region as well as the non-luminescent region. These mice had among the greatest amount luminescence and bacterial burden (>$9.5x10^6$ CFU/g) of the group. A second group of mice was dissected at the earliest sign of luminescence, which corresponded to <$3.5x10^6$ CFU/g, to determine how early in infection the bottleneck could be detected. In this group, there were no significant differences between the initial inoculum and the focal region in 3 out of 4 mice (Figure 12B). Most non-luminescent regions were significantly different from the focal region, but no single subpopulation was greater than 42%. This suggests slight shifts in clonal proportions occur as spores transit from the nasal passage to the lungs. Germination varied in the focal regions of these mice, with mouse 6 having only 38% germination, mouse 9 having 91% germination, and mouse 7 and mouse 8 being >95% germinated. To ensure that the

clonality in the focal region is not an artifact due to the intranasal delivery of the spores to the lungs, the heated homogenates from the focal regions were analyzed. All clonal populations were present in the focal regions as spores, but most mice showed statistically significant shifts from the focal regions (Figure 12C). However, averaging the clonal proportions in all heated homogenates of the focal regions showed no difference between the initial inoculum.

The dominant clone in the lung focal region is the founder of the disseminated population.

To test whether the dominant clone in the focal region goes on to disseminate through the host, the clones in the kidneys were compared to the clones in the focal region. A comparison between the subpopulations in the focal regions and kidneys in five out of eight mice did not show a significant difference, suggesting that the dominant population in the lung went on to disseminate through the mouse (Figure 13A, Fisher's exact test P-value>0.05). Focal regions in mice 6 and 7 had a significant difference compared to the kidneys, but were dissected early after luminescence was detected in the lungs. A bottleneck, however, occurred between the focal region and the kidneys. Mouse 3 had two populations comprise 76% of the focal region and the same two populations comprise 97.9% of the kidney, but the two populations were found to be significantly different from each other (Figure 13A). The mLNs, however, had unique clonal proportions from the kidneys and focal regions of five out of six mice, suggesting that these populations did not contribute to the disseminated population (Figure 13B).

Infection can initiate and disseminate from the cervical and mediastinal lymph nodes.

The clonal proportions of lymph-based infections were analyzed in 4 mice when luminescence was detected in several lymph nodes and there was no previous indication of infection the previous day. Half of the analyzed mice did not have enough CFU in the cLN to permit clonal analysis. The other half, however, had highly similar populations between the

disseminated population in the kidney and the cLN (Figure 14). Similarly, the lungs and mLN clonal proportions were very similar to the disseminated population (Figure 14). The similarity between the mLN and the disseminate population illustrates the difference between the clonal proportions found in organs with lung-based dissemination and lymph-base dissemination.

The lung-based bottleneck allows more clones to pass through and has a lower effective population.

In order to determine if the lung-based bottleneck allowed more clones to pass through the bottleneck, additional mice were infected to increase the number of lung infections analyzed. The average number of clones represented in the disseminated population was measured by analyzing the average number of different clones in the kidneys from NALT-based and lung-based infections. The average number of different clones in the kidneys was significantly greater in lung-based infections than in NALT-based infections (Figure 15, Student's T-test *P-value = 0.014). The effective population (N_e) could then be calculated from the number of clones in the disseminated population and used to compare the size of the bottlenecks the bacilli pass through from different portals of entries. Effective population (N_e) size is an idealized population that has the same rate of genetic drift as the actual population size and allows for a quantitative comparison of bottlenecks between portals of entry. One method of determining N_e is to analyze the number of offspring between non-overlapping generations. If there is a decrease in population due to a bottleneck then the effective population will decrease in proportion to the stringency of the bottleneck. In a *B. anthracis* infection, the generations occur between the inoculation with spores and the death of the host. As such, one can calculate the effective population by determining the number of clones in the disseminated population, *i.e.* the kidney clonal population. The effective populations were significantly

higher in lung-based infections than in NALT-based infections (Table 3, Student's T-test *P-value = 0.014).

Discussion

Our work provides insight on the ability of *B. anthracis* to colonize and disseminate from unique microenvironments in the host respiratory tract by combining BLI technology with clonal analysis. All mice had *B. anthracis* pass through a bottleneck regardless of where the infection initiated. Clonal analysis of lung-based infections suggested that a bottleneck occurred in the lumen of the lung, possibly as a few bacteria germinated in the lungs. These bacteria then rapidly disseminate from the lungs to the bloodstream and other organs. Further, the clone population in most mLNs had a statistically different population from their respective kidneys and focal growth in the lung. NALT-based infections, however, passed through a bottleneck between the NALT and the cLNs. In contrast to the lung-based infections, the cLNs always had the same tagged clone as their NALT and kidneys. Analysis of the kidneys from mice with lung-based infections had a greater mean number of clones and a higher effective population than mice with NALT-based infections. Overall, these data suggest that *B. anthracis* disseminate differently from the two inhalational portals of entry and that some routes of inhalational dissemination allow greater amounts of bacilli to disseminate; thus the portal of entry defines the characteristics of the subsequent disseminated infection.

The experiments performed in this study were done using the BSL-2 Sterne strain of *B. anthracis* in the A/J mouse model, which has its advantages and disadvantages. Since the Sterne strain lacks the pX02 plasmid encoding the anti-phagocytic capsule, the experiments can be performed without the difficulties of BSL-3 facilities, providing a more economical model of study. Additionally, several studies have noted key similarities between the A/J mouse model

of anthrax with larger animal models [103, 104, 270, 275, 295]. These include a similar dissemination pattern and a high level of bacteremia within the host [270, 275, 295]. As such, mice are useful models to analyze colonization and early steps in pathogenesis [96, 295]. A disadvantage, however, is the lack of capsule, which is thought to account for pathological differences found during the bacteremic stages of infection. Acapsular strains have limited splenic colonization and do not cause vasculitis and mediastinitis [96]. Therefore, comparisons between the Sterne-A/J model and human anthrax must be considered carefully. It is important to note that the focus of this study was to investigate the dissemination of *B. anthracis* in inhalational infections, not to model the effects of late stage anthrax.

In our studies, A/J mice were given spores by intranasal instillation to cause inhalational infections. A concern with the intranasal model is effective delivery of spores to the lungs [104]. In our hands, mice lungs would contain about 5% of the inoculum at the time of dissection. However, when the clonal proportions were analyzed both within the focal region and in the non-luminescent regions, all clonal populations were present and when averaged were not significantly different from the inoculum. This suggests that while our mice had fewer spores reach the lungs compared to previously published amounts in aerosol models, all clonal identities were capable of reaching the focal regions [295]. As such, the bottlenecks detected in the focal region are unlikely to occur due to uneven delivery of the spores to the lungs.

The high spore burden relative to vegetative bacteria within the lung from the initial inoculum was likely the factor that obscured the detection of a bottleneck at early time points. If an organ has a large and random population of spores, there is a greater chance of sampling ungerminated spores of a dissimilar clonal subpopulation. While heat treatment of homogenates can differentiate spores from total bacteria, there is no current methodology for

isolating only the vegetative bacilli for clonal analysis which, if possible, would permit earlier analysis of the bottleneck.

To circumvent the high spore burden in the lungs, luminescent focal regions and non-luminescent regions were separated to 1) attempt to isolate a region with a higher proportion of bacilli to spores and 2) to determine the extent to which the bacilli can spread through lung tissue without hematogenous dissemination. The luminescent focal areas were found to mainly comprise vegetative bacilli, whereas the rest of the lung tissue had the majority of bacteria in spore form (Figure 11). While previous studies found germination and outgrowth occurs in the lungs, it is interesting to note that the bacilli do not migrate far from the focal region without prior systemic dissemination through the host. This suggests that the site of lung germination as previously reported in Sanz *et al*. may represent the earliest moments of the focal infection formation in the lung [276].

The luminescent focal regions were examined at various levels of bacterial burdens to determine when the bottlenecks could be detected. When focal regions were analyzed with $\geq$ $3.5x10^6$ CFU/g, there were large increases in only 1 or 2 clones. However, a bottleneck could not be detected when mice were dissected at the earliest observation of luminescence. This suggests that detection of the lung bottleneck is dependent on both germination and bacterial replication and that the disseminated population arises from a small number of spores. Interestingly, bacilli could be found in the kidneys before the bottleneck could be detected in the lungs; this suggests rapid dissemination from the lungs after germination. Mice were also dissected when there was a high level of luminescence ($>9.5x10^6$ CFU/g in the focal regions) and were found to have increased amounts of the disseminated population in the non-luminescent regions. As such, it

suggests that the non-luminescent areas of the lung have the disseminated population return secondarily, rather than primarly spreading through the lung.

In lung-based infections, bacterial dissemination from the portal of entry to the draining lymph nodes is sufficient, but not necessary for dissemination. A comparison between the mLNs and the kidneys found that six out of twelve mice had the greatest clonal proportion in the mLN in common with the kidney. As such, it may be that the bacilli are disseminating without directly utilizing a lymphatic route. Interestingly, other routes of dissemination in murine anthrax, such as subcutaneous and gastrointestinal infections, have also shown dissemination through the lymph node to be required [81, 261]. We speculate that such a route could be possible given that both exotoxin components, edema toxin and lethal toxin, are capable of increasing vascular and alveolar epithelial permeability [252, 296]. However, future studies will need to be performed in order to determine if and to what degree the mouse lung epithelium becomes permeabilized. Epithelial layers have also been identified as being capable of spore uptake and to allow transcytosis of spores [271]. Therefore, another possibility is that spores are directly internalized by the lung epithelium to then transit directly to the circulatory system and thereby bypass the lymph nodes.

Lymph based infection disseminate suddenly and are characterized by luminescence being present in several lymph nodes. Typically, the infections would not have prior luminescence in the NALT or lung lumen, suggesting that the infections initiated from the lymph nodes. While previous experimental data has suggested that infections begin in the lymph nodes based on exclusionary evidence, *i.e.* an absence of vegetative bacteria in the lung, or histological evidence, our data strongly suggests that the infection originated from the lymphatics. These experiments, however, have not conclusively demonstrated how the bacilli

reach the lymphatics. Further, it why these infections occur in the lymphatics rather than the NALT or lung remains elusive, particularly since these mice were infected intranasally with similar doses. It is interesting to note the speed at which the dissemination occurs compared to NALT or lung based infections. Unlike NALT or lung-based infections, lymph-based infections were in part characterized by the absence of infection ~24 hours prior to the detection of luminescence in lymph nodes and kidneys. NALT, lung, and subcutaneous infections, which likely disseminate via the Jailbreak model, typically have a period of growth prior to disseminating from the portal of entry that allows for greater concentrations of the exotoxin to accumulate in the host. As such, it remains unresolved how the bacilli can disseminate so quickly when there is less time for exotoxin concentrations to accumulate.

The dramatic clonal reductions that occur in inhalational anthrax may also play a role in the paucity in chromosomal diversity in *B. anthracis*. It has previously been speculated that *B. anthracis*' lack of genetic diversity occurs partly because of its long dormancy in spore form (Reviewed in [141]). Population bottlenecks occurring *in vivo* might also play a role in the lack of genomic diversity between strains. In our experiments, NALT-based infections almost always led to allelic fixation, *i.e.* there was only a single clone disseminated in almost every instance (Figure 10A). Lung-based infections permitted a significantly more diverse population of bacteria to expand than those that arose from NALT-based infections, as indicated by the greater average number of clones and effective population in the kidneys (Figure 8, Table 3). When one examines the diversity of isolates in many countries, it is apparent that there are regions within countries and continents where specific strains are far more prevalent, which would be expected if a bottleneck is occurring between hosts [145, 297]. Conversely, multiple bottlenecks occurring simultaneously throughout a continent can increase diversity over time if

there is relatively little exchange between each geographic region. As such, *in vivo* bottlenecks may provide a mechanism to drive diversity on a global level for bacteria that spend a large amount of time in spore form.

Germination and outgrowth from the lungs is not without controversy. The potential of lungs to act as primary sites of infection was first suggested by preliminary work which found bacterial replication and damage in the lung and bronchi [268, 269]. It is worth noting that several of these studies used experimental animals in which the lungs were compromised by an unrelated previous infection or experimental manipulation [286, 298, 299]. Similarly, a majority of male victims in the Sverdlovsk anthrax outbreak were moderate to heavy smokers and the most common occupation was welder; factors which increase the risk of lung damage and disease [28]. Therefore, previous damage to the lungs may skew infections to germinate and disseminate from the lungs, rather than disseminate from the NALT or lymphatics. Conversely, there is support for lymphatic dissemination from the lungs since large scale germination does not occur in the lungs and there is no sign of bronchiopneumonia [74, 274, 283-285, 295]. A central caveat to previous pathological analyses and determination of bacterial burden is that the infection was analyzed either at defined time points or after death and in particular organs. Mice in this current study had a similar bacterial burden in most organs at the time of dissection regardless of where the infection initiated. This suggests that determining dissemination patterns by quantifying bacterial loads in organs may not be conclusive by itself. In a number of animal models, lung lesions have been observed upon post-mortem analysis, but had no real-time assays to track the infection in the host [29, 272, 286, 298-302]. Therefore, caution should be used when concluding where an infection began given that there is still little known about the early events in pathogenesis *in vivo* or how differences in delivery and environment could alter

the portal of entry. Further development of *in vivo* analysis of inhalation *B. anthracis* infections can assist in identifying the early immune interactions which determine host survival and identify factors that lead to germination and colonization of a portal of entry, and point to areas of further research in larger animal models.

Our results provide a possible reconciliation between conflicting reports on *B. anthracis* infections that initiate and later disseminate from the lung and previous observations of initiation and dissemination from the mLN. This is not to say that mLN based infections do not occur; rather it is to argue that lung-based infections can occur and may not be easily detected. Clonal analysis suggests that a small number of spores germinate in a small, discrete location in the lung. The lungs of these mice contained bacteria in spore form throughout most of the lungs, except for the focal area where a small number of spores germinated and replicated. Rather than spreading primarily through the lungs, the bacilli rapidly spread either through the lymphatics or directly to the blood stream. Therefore, even in our mice with a lung infection, the lungs on the whole were mostly refractory to germination and overt lung damage was limited to a portion of a lobe that could have easily gone undetected in past studies that did not utilize BLI. Thereby when the lungs were studied as a complete unit, rather than focusing on an important sub region that contained the growth as we have achieved, it would have been difficult to detect vegetative growth from this small area above the background of CFU from ungerminated spores within the rest of the lung tissues. Moreover, a post-mortem dissection of the animal without the BLI data would have greater difficulty discerning if the lung lesions occurred prior to dissemination.

Tables and Figures for Chapter 2

Table 1. Strains and plasmids used in Chapter 2

Strain or plasmid	Relevant characteristics	Reference
Strain		
7702 Sterne	pX01^{+}, pX02^{-} BSL-2 strain of *B. anthracis*.	BEI Resources, VA
BIG23	Luminescent derivative of 7702. Contains the *luxABCDE* operon from *Photorhabdus luminescens* under the control of the PA promoter, which produces light in vegetative bacilli when under toxin producing conditions. Erm^{R}.	[261]
α-select	Chemically competent *E. coli* strain which allows for transformation and blue/white screening.	Bioline, MA
GM119	*dcm-6, dam-3*; prevents DNA methylation of cytosines and adenines.	[290]
Ec138	GM119 containing pRK2. pRK2 contains an *oriT* which enables conjugation from *E. coli* to *B. anthracis*.	Kind gift from Richard Calendar
Plasmid		
pHY304	Multicopy shuttle vector; Erm^{R} for both Gram-positive and Gram-negative, contains MCS	[291]
pIG13	2 kb *eag* fragment with a recombinant BamHI site inserted in the pHY304 vector, used to facilitate allelic exchange of recombinant tags.	This study
pSE02	Shuttle plasmid that allowed for blunt insertion of DNA ladder fragments to create recombinant tagged clones; a variation of pIG13 which inserted a SalI, SpeI, and EcoRV site between BamHI sites in the *eag* fragment.	This study
pGEM-T Easy	Linear cloning vector with ampicillin resistance and 3' thymidine overhangs to facilitate ligation of PCR products.	Promega, WI
pCZ03	Shuttle plasmid that allowed for blunt insertion of DNA ladder to create recombinant tagged clones; variation of pIG13 which inserted an EcoRI, SpeI and NotI site from the pGEM-T easy vector into the BamHI site in the *eag* fragment.	This study
pIG6-19	Confers luminescence to *B. anthracis.* The *luxABCDE* operon from *P. luminescens* under control of the PA promoter in the pAT113 suicide vector.	[81]

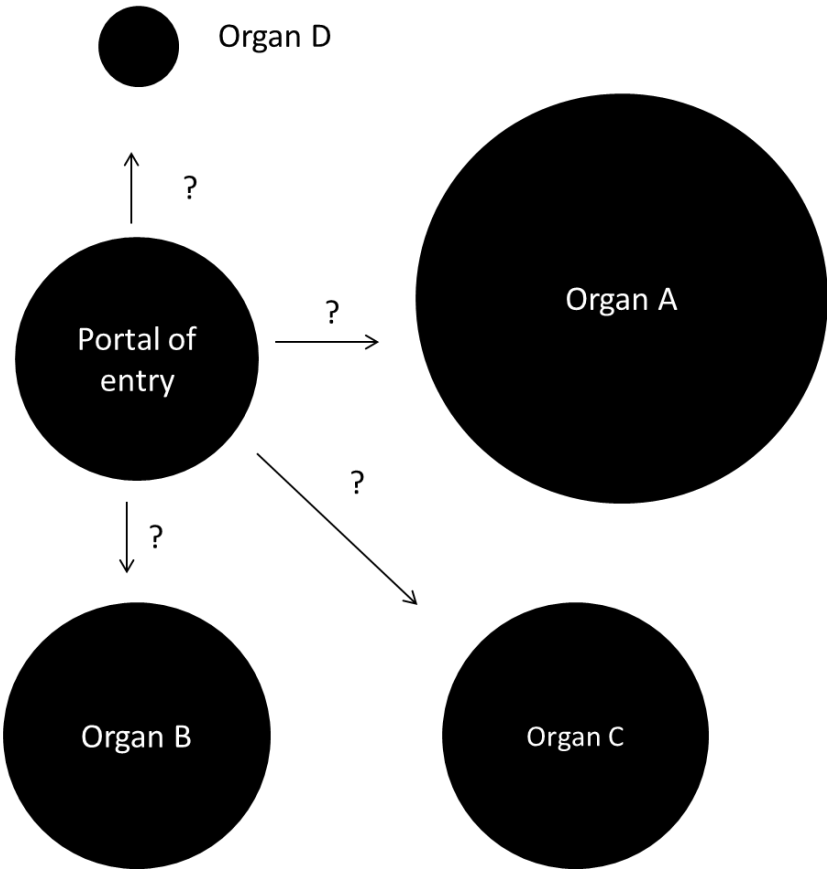
Table 2. Primers used in Chapter 2

PCR primers	Sequence	Reference
FIG4	5′-AAAGAATTCGCTCAGCCTTCTTTCAAAGATGC-3′	This study
RIG4	5′-AAAAAAGTCGACATATCATCCAGGTTACTCTTCTCAAGC-3′	This study
FIG5	5′-GTAAACTATGGCAGGATCCTTTCTGGTAAAG-3′	This study
RIG5	5′-CTTTACCAGAAAGGATCCTGCCATAGTTTAC-3′	This study
FIG8	5′-AAAGCGGCCGCTCAGCCTTCTTTCAAAGATGC-3′	This study
FDL5	5′-CCGATATCCATCGAAAAGGA-3′	This study
RDL5	5′-CGTTTTTCAAGCAGTGCAGA-3′	This study
F SOE linker	5′-GGTGTTGTAAACTATGGCAGGATCCACTAGTGATATCGTCGACGGATCCATAGCCAAAACTTAGTTGTTGGTGAAAAAGC-3′	This study
R SOE linker	5′-GCTTTTTCACCAACAACTAAGTTTTGGCTATGGATCCGTCGACGATATCACTAGTGGATCCTGCCATAGTTTACAACACC-3′	This study
RDL22	5′-CGTCCCCTCGAGCATTCAGGCTGCATCAACTT-3′	This study
FDL26	5′-CTTAGACGGTTCACCAGCAA-3′	This study
RDL26	5′-CGCCATATTGGTCAGTTGTT-3′	This study
M13F	5′-GTAAAACGACGGCCAG-3′	This study
M13R	5′-CAGGAAACAGCTATGAC -3′	This study

Table 3. Effective population of disseminated bacteria from different portals of entry

Site of bottleneck	N_e
No bottleneck	5
NALT-based	0.72
Lung-based	1.65

A



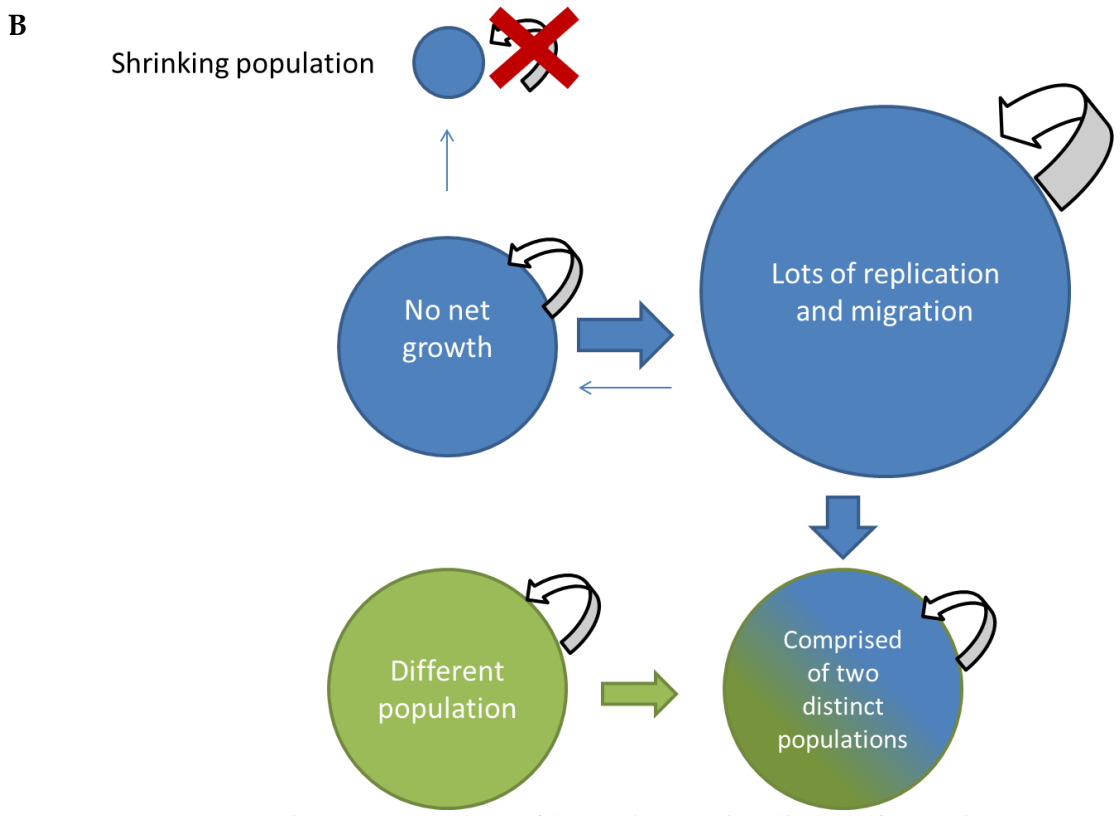


Figure 4. CFU enumeration and location of bacteria provide little information regarding population dynamics within a host.

A) Bacterial burdens in tissues of the host can only provide limited insight on the population dynamics within a host. Organs are represented by circles, with the area of the circle representing the relative amount of CFU in the tissue. Bacteria potentially migrate from the portal of entry to other organs and the migration is represented by arrows. The amount of bacteria which migrate and the order by which they migrate cannot be easily assessed. **B)** In reality, infection has several factors that lead to growth and dissemination to and from organs. These factors include the amount of migration to and from the organ, the amount of replication, and the amount of bacterial death. These factors work together over time to lead to the bacterial burden within an organ. Organs are represented by circles, with the area of the circle representing the relative amount of

CFU in the tissue. Each color represents a unique population of bacteria. Arrows represent migration from one organ to another and the size of the arrow is relative to the amount of bacteria that migrate. White curved arrows represent bacterial replication within the organ, with the size of the arrow representing the relative amount of bacterial replication.

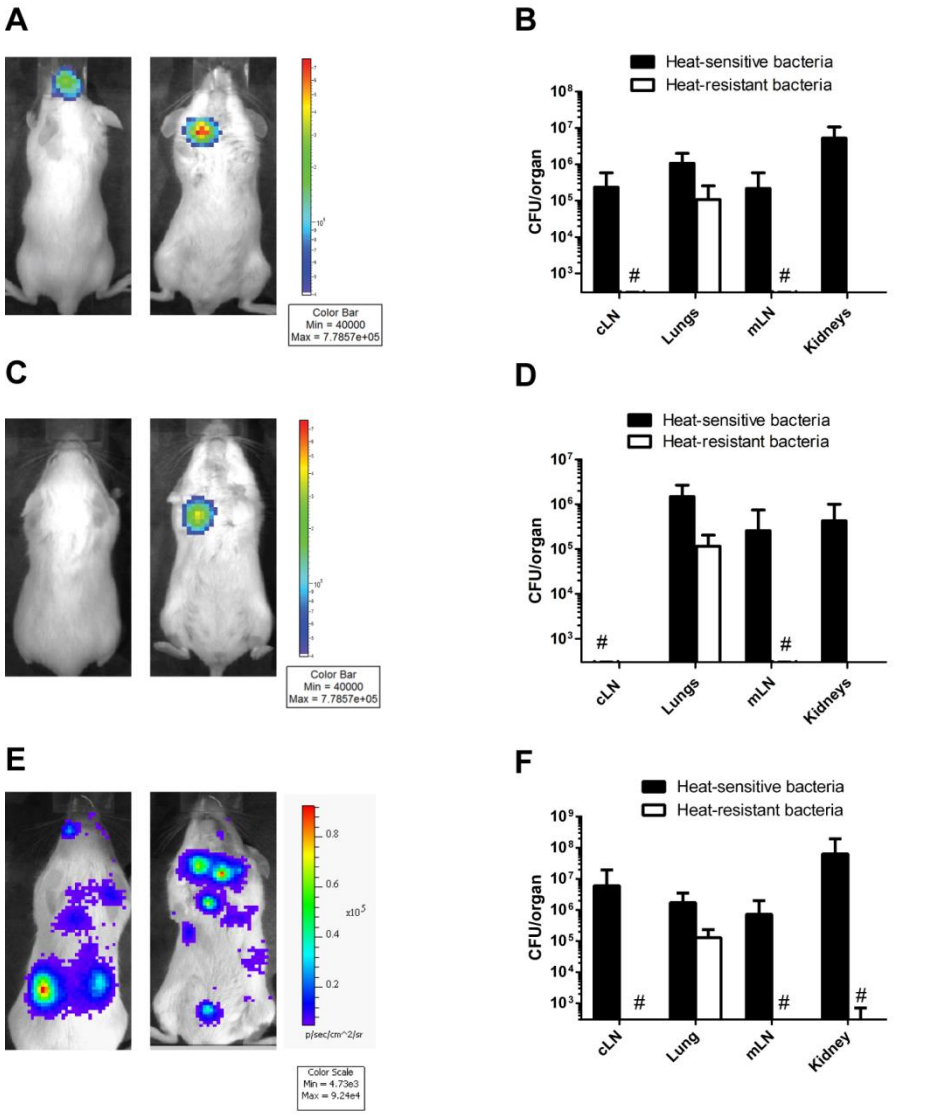


Figure 5. Inhalational *B. anthracis* infections have similar bacterial burdens in most organs. **(A)** Dorsal and ventral black and white photos of A/J mouse with pseudocolor overlay of luminescence intensity. The mouse was infected with 4.6×10^6 spores intranasally. NALT-based infections were indicated when luminescence was only detected in the nasal passages. **(B)** CFU per organ of the cLN, lungs, mLN, and kidneys in mice with NALT-based infections (n=6). Black columns are the average heat-sensitive CFU and white columns are the average heat-

resistant CFU. Bars represent the 95% confidence intervals. # represents CFU that were too few to enumerate. **(C)** Dorsal and ventral black and white photo of A/J mouse with pseudocolor overlay of luminescence intensity. The mouse was infected with $2x10^7$ spores intranasally. Lung-based infections were indicated when luminescence was detected from the ventral thorax. **(D)** CFU per organ of the cLN, lungs, mLN, and kidneys in mice with Lung-based infections (n=6). Black columns are the average heat-sensitive CFU and white columns are the average heat-resistant CFU. Bars represent the 95% confidence intervals. # represents CFU that were too few to enumerate. Lung-based infections were detected when luminescence was detected in the ventral thoracic cavity. **(E)** Dorsal and ventral black and white photo of A/J mouse with pseudocolor overlay of luminescence intensity. The mouse was infected with $2x10^7$ spores intranasally. Lymph-based infections were indicated when luminescence was detected when several lymph nodes are luminescent. **(F)** CFU per organ of the cLN, lungs, mLN, and kidneys in mice with lymph-based infections (n=4). Black columns are the average heat-sensitive CFU and white columns are the average heat-resistant CFU. Bars represent the standard deviation. # represents CFU that were too few to enumerate. Lymph-based infections were detected when luminescence was detected in several lymph nodes. Figure 1A, C, and E are representative of all mice (n=12 for NALT infection, n=16 for lung infections, n=8 for lymph infections) and the pseudocolor scale next to A, C and E represent luminescence in photons sec^{-1} cm^{-1} $steradian^{-1}$.

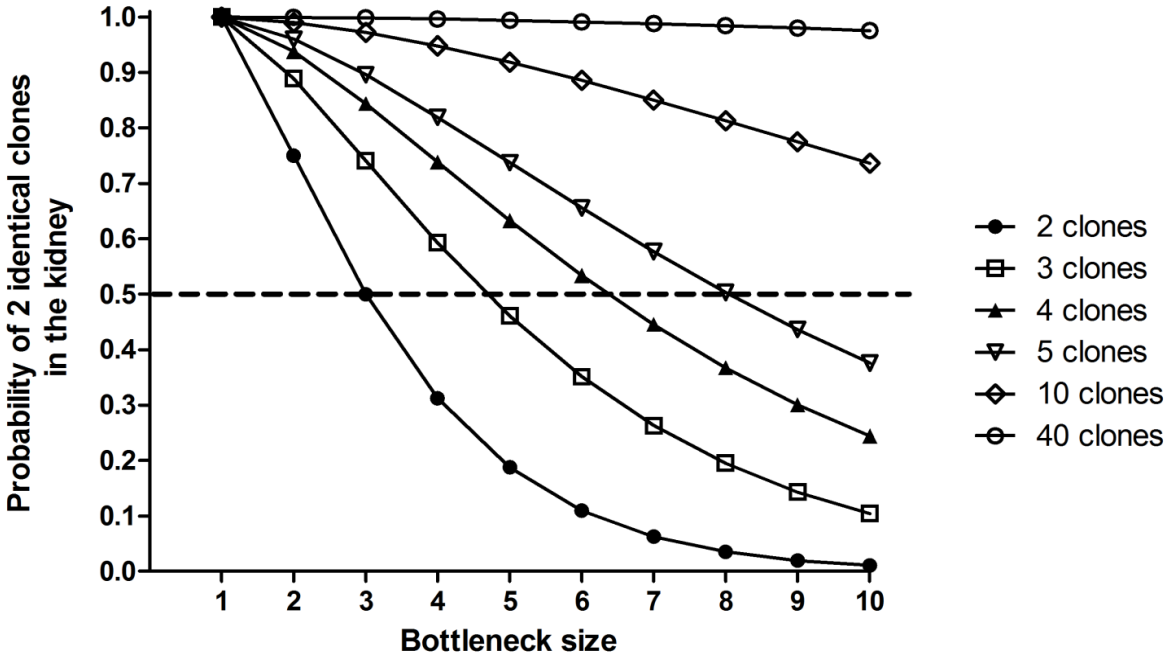


Figure 6. Probability modeling suggests that a library of 5 clones is sufficient for clonal analysis.

The probability of finding two identical clones in the kidney after passing through a bottleneck is dependent on the size of the bottleneck and the number of clones in the library. The probability of finding two exact clones in the kidney was calculated using hypergeometric distribution for differently sized clonal libraries, using the same size inoculum and assuming the clones are equally proportioned. The values were then plotted for differently sized bottlenecks; *i.e.* the number of clones that made it from the portal of origin to the kidney. Although using libraries with more clones would enable greater resolution of larger bottlenecks, our preliminary experiments indicated that it was uncommon for more than 3 *B. anthracis* clones to pass through the NALT and lung-based bottleneck. This allowed us to conclude that a library of 5 clones would be necessary to achieve a 90% likelihood that two clones with the same tag passing through the bottleneck would not be mistakenly scored as a

single clone. If fewer than 5 clones were used, confidence would drop to 74% for three clones and 50% for two clones. Moreover, ensuring evenly mixed inoculums and subsequent analysis of 10 or 40 clones presents technical difficulties that outweigh their benefit; *i.e.* there is a diminishing return for each clone added, such that increasing clones in the theoretical library from 10 to 40 would only gain ~2% in confidence for a bottleneck allowing 3 clones to pass through.

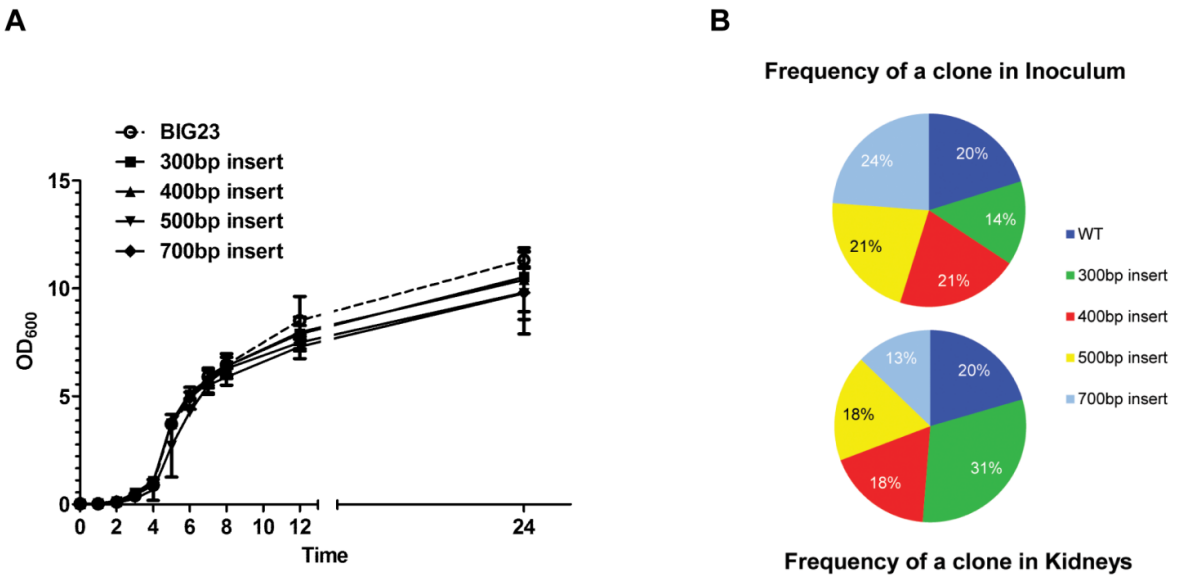


Figure 7. All clones in the clonal library are equally capable of growth and pathogenesis. **(A)** The *in vitro* growth rates of each clonal strain was compared for 24 hours (Non-linear regression and F-value comparison, *P*-value = 0.9991). **(B)** The frequency of the clones in the initial inoculum was compared to the frequency of the clone found in the kidney from lung-based infections. The frequency was compared using a Fisher's exact test to determine if there was a statistical difference between the two frequencies (Fisher's Exact test, *P*-value = 0.521).

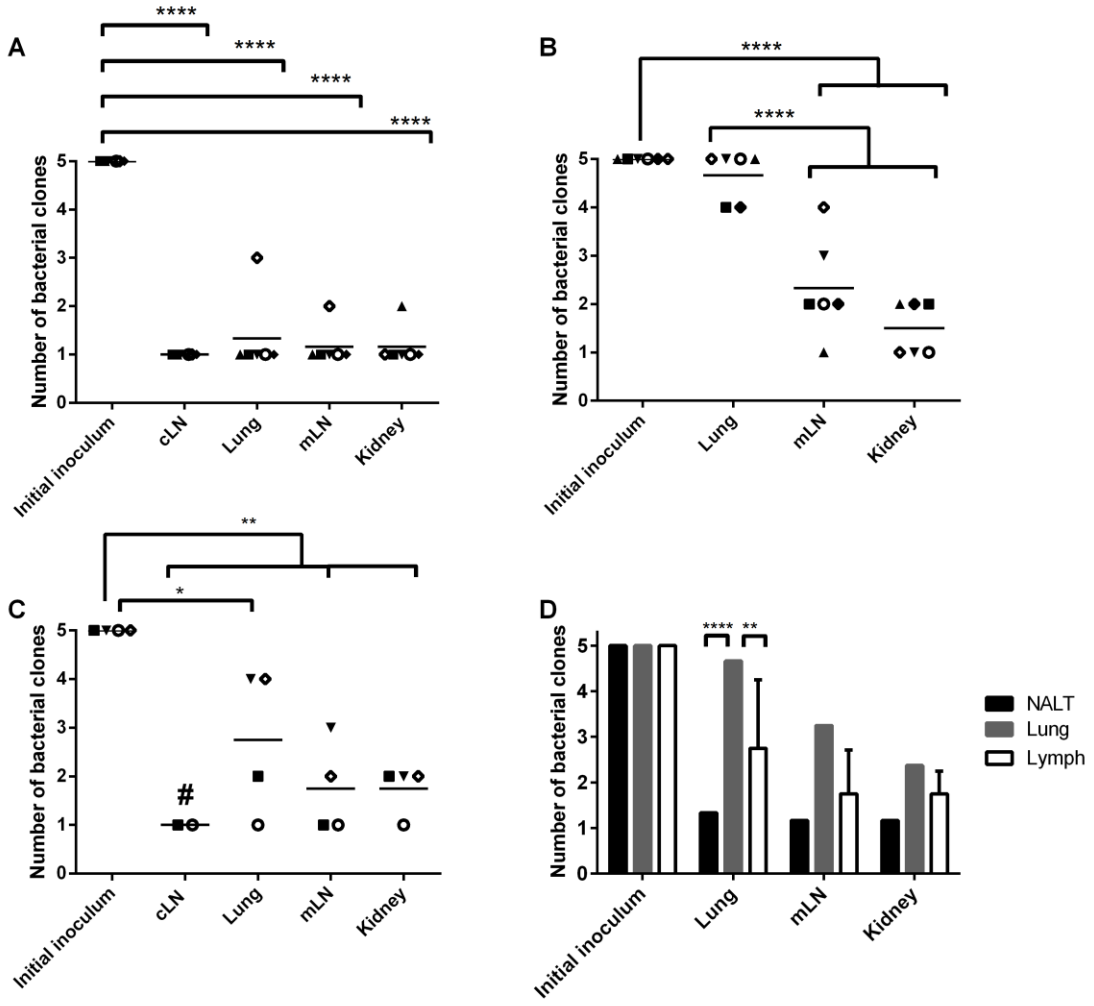


Figure 8. **Lung-based bottleneck have a higher number of clones in the lungs and mLN.** **(A)** The number of different clones represented in each organ of mice dissected after a NALT-based infection had progressed to the draining cLN or kidneys. Each symbol represents a unique mouse and the bars represent the mean (n=6). Asterisks indicate a statistical difference from the initial inoculum (One way ANOVA test with Tukey's multiple comparisons, ****P-value <0.001). **(B)** The number of different clones represented in the organs of mice dissected

when luminescence was detected in the thoracic cavity. Each symbol represents a unique mouse (n=6). Bars represent the grand mean and asterisks indicate a statistical difference from the initial inoculum (One way ANOVA test with Tukey's multiple comparisons, ****P-value <0.001). **(C)** The number of different clones represented in the organs of mice dissected when luminescence was detected in the lymph-based infections. Each symbol represents a unique mouse (n=4), # represents that 2 mice had too few clones for clonal analysis to be performed. Bars represent the grand mean and asterisks indicate a statistical difference from the initial inoculum (One way ANOVA test with Tukey's multiple comparisons, ***P-value <0.001, *P-value <0.05). **(D)** The average number of different clones represented in the lungs, mLN, and kidneys in both NALT (closed circles, n=6) and lung-based (open squares, n=6) infections. Bars represent the means of different clones represented in each organs. Asterisks indicate a statistical difference between the NALT and lung-based infections (Two way ANOVA with Tukey's multiple comparison test, **P-value <0.01, ****P-value <0.0001).

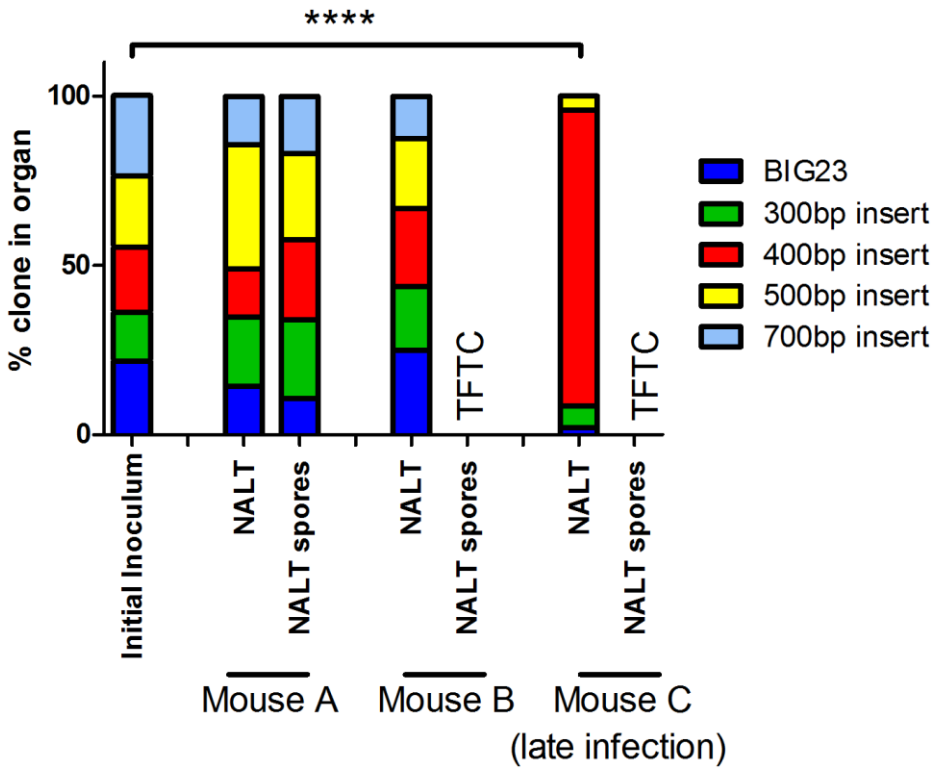
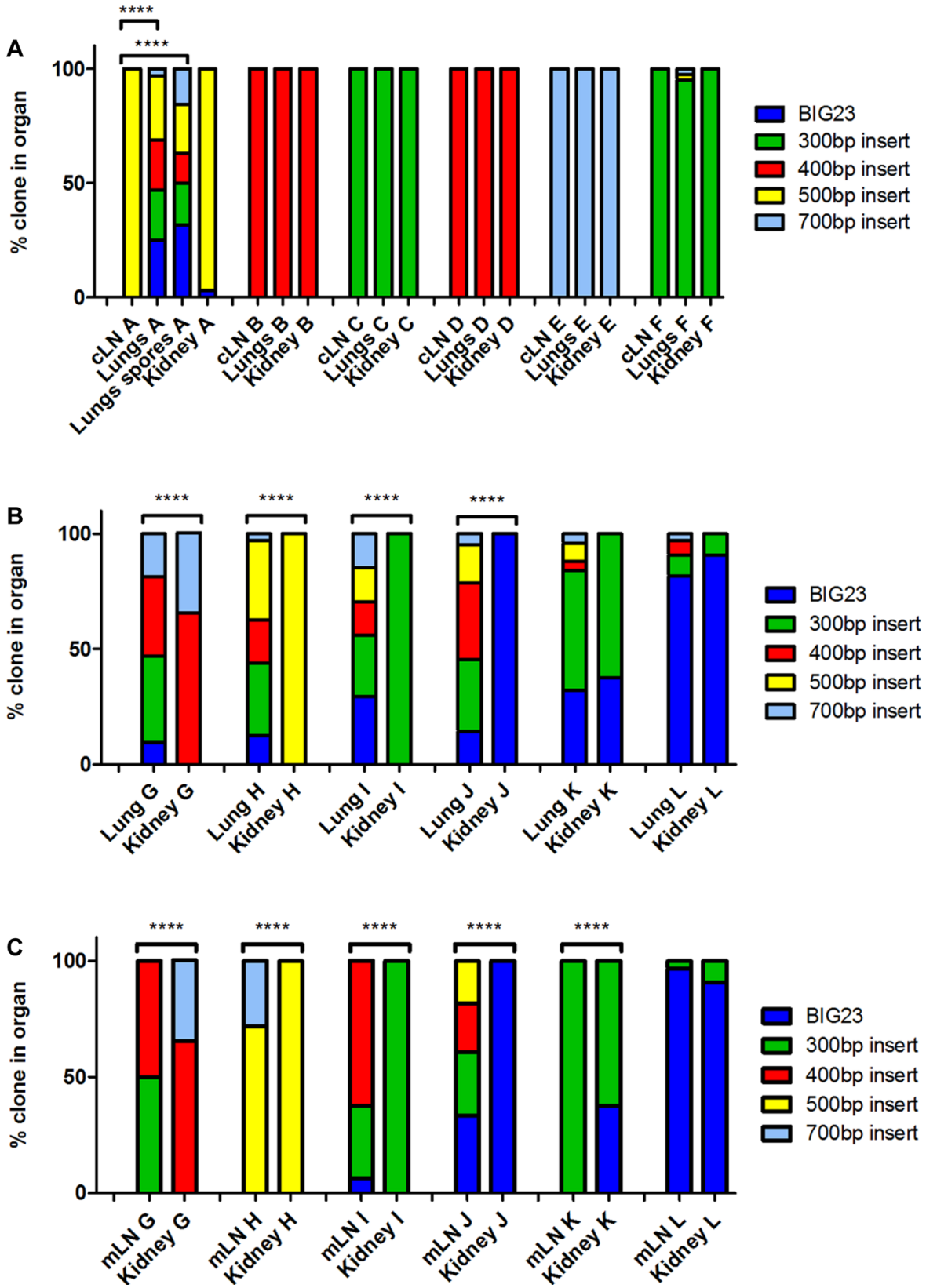


Figure 9. NALT-based infections have all spores germinate in the NALT.

The percentages of clones from unheated homogenates of the NALT (bacilli and spores) were compared to the heated homogenates of the NALT (spores only) from 3 separate mice. Mouse A and B were dissected when luminescence was detected in the NALT and cLN. Mouse C was dissected after dissemination had occurred as evidenced by luminescence in the kidney (data not shown). Each column represents the indicated organ for a single mouse, with stacked percentages for each clone. Each group of columns represents a single mouse. Mice with fewer than 20 CFU in the NALT were labeled as too few to count (TFTC) and were excluded from analysis. Asterisks indicate statistically different populations when comparing the number of colonies of each clone (Fisher's Exact test, ****P-value <0.0001).


A
% clone in organ
100
50
0
****
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cLN A
Lungs A
Lungs spores A
Kidney A
cLN B
Lungs B
Kidney B
cLN C
Lungs C
Kidney C
cLN D
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Kidney D
cLN E
Lungs E
Kidney E
cLN F
Lungs F
Kidney F
BIG23
300bp insert
400bp insert
500bp insert
700bp insert
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% clone in organ
100
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Lung G
Kidney G
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Kidney H
Lung I
Kidney I
Lung J
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Lung K
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Kidney K
mLN L
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BIG23
300bp insert
400bp insert
500bp insert
700bp insert

Figure 10. Clonal proportions in NALT-based infections were similar between organs, whereas lung-based infections showed greater diversity.

(A) The clonal proportions in the cLNs, lungs, and kidneys of mice that had a NALT-infection that disseminated at least to the draining cLN (n=6). Asterisks indicates a significant difference of clonal proportions between the initial inoculum, the lungs, and heated lung homogenates (for Mouse 1 only) (Fisher's exact test, ****P-value <0.0001). Each column represents the indicated organ for a single mouse identified from 1 to 6, with stacked percentages for each clone. **(B)** The clonal proportions found in the lungs and kidneys of mice with disseminated lung infections (n=6). Each column represents the indicated organ for a single mouse, with stacked percentages for each clone. Asterisks signify a statistical difference between the lungs and kidney for a single mouse identified A through F (Fisher's Exact test, ****P-value <0.0001). **(C)** The clonal proportions found in the mLNs and kidneys of mice with a disseminated lung infection (n=6). Each column represents the labeled organ for a single mouse identified as A through F, with stacked percentages for each clone. Asterisks signify a statistical difference between the mLN and kidney for a single mouse (Fisher's Exact test, ****P-value <0.0001).

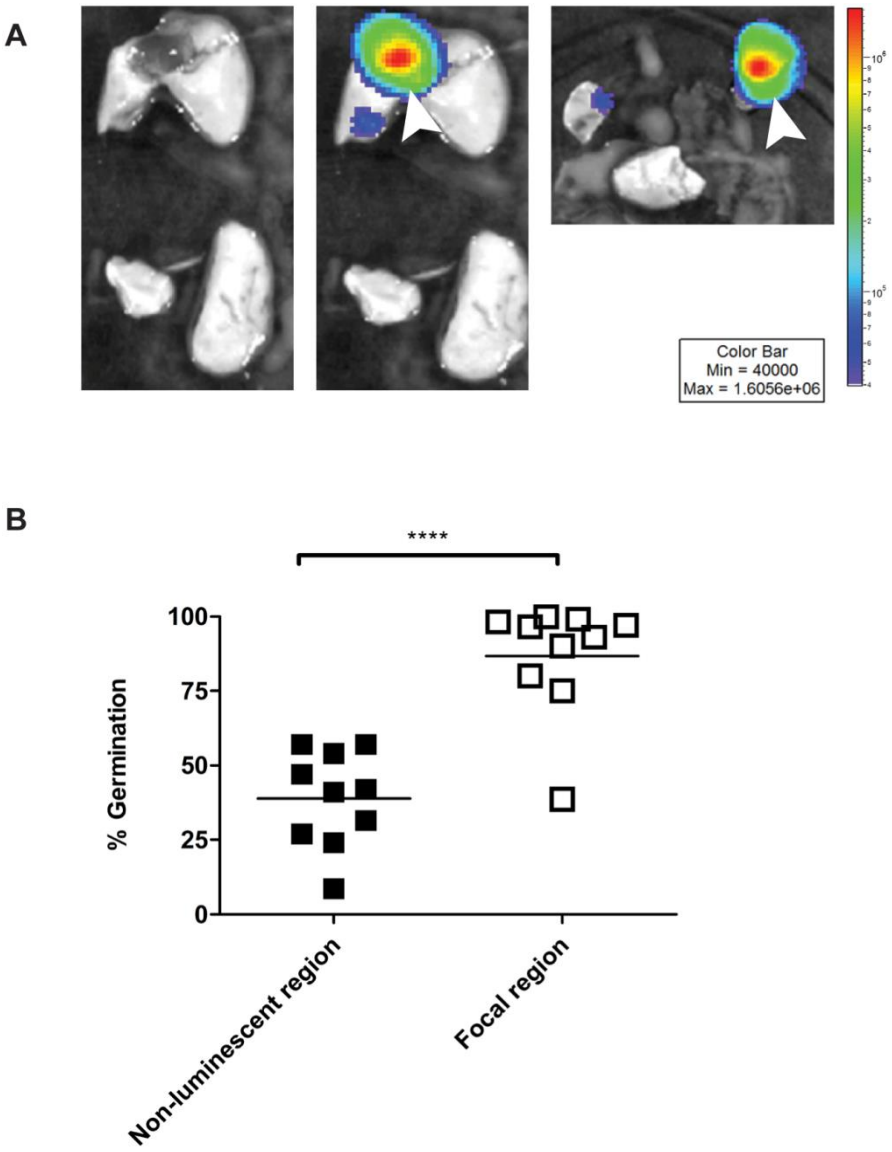
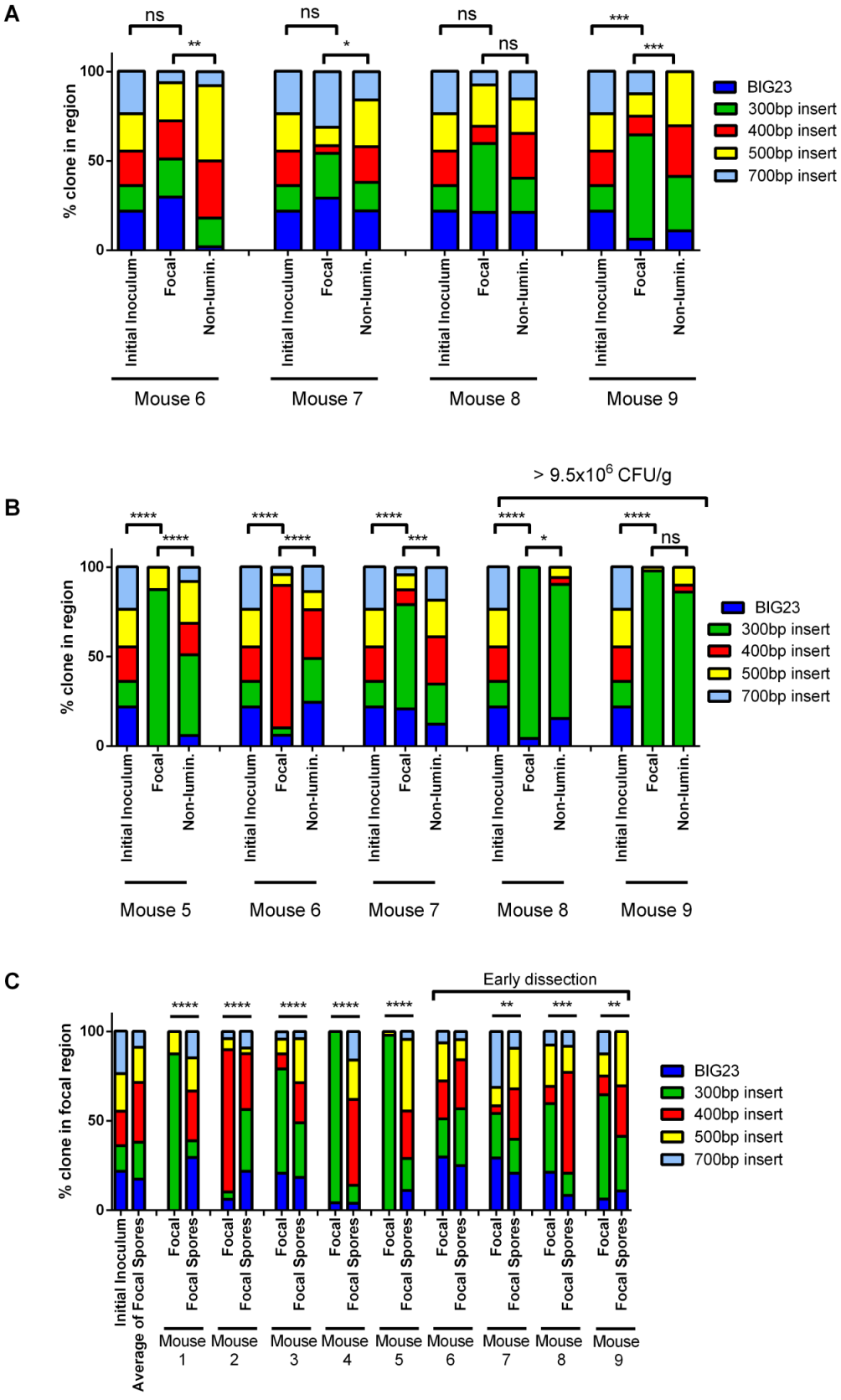


Figure 11. Lung-based infections occur in distinct focal regions that are mainly composed of vegetative bacilli.

(A) Photograph of a dissected mouse lung with luminescent focal region with a pseudocolor overlay representing luminescence intensity as indicated on the scale to the right. The lung was further dissected to isolate the luminescent focal region (white arrowhead) and homogenized to determine the clonal identities in the focal region. **(B)** The percentage of vegetative bacilli was compared between a non-luminescent area of the lungs (closed squares) and the focal region

(open squares). Bars represent the mean. Asterisks indicate statistically different means (N=10 for both focal regions and non-luminescent regions, Student's T test, ****P-value <0.0001).


A
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ns
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*
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% clone in region
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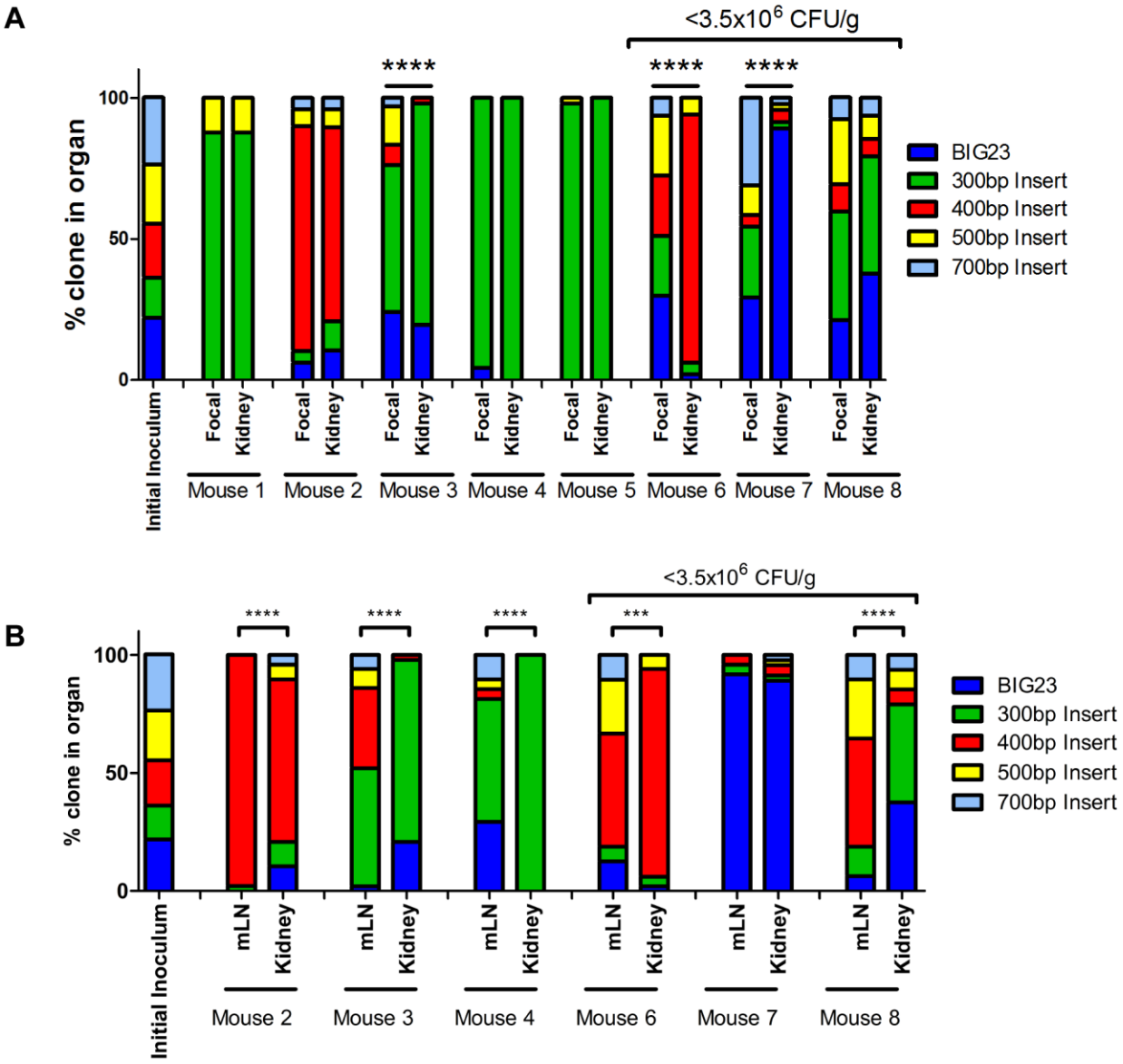
50
0
BIG23
300bp insert
400bp insert
500bp insert
700bp insert
Initial Inoculum
Focal
Non-lumin.
Mouse 6
Mouse 7
Mouse 8
Mouse 9
B
> 9.5x10^6 CFU/g
****
****
****
****
****
***
****
*
****
ns
% clone in region
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9
C
Early dissection
****
**
***
**
% clone in focal region
Initial Inoculum
Average of Focal Spores
Focal
Focal Spores
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9

Figure 12. The luminescent focal region is the site of the bottleneck in lung-based *B. anthracis* infections.

The percentages of clones in the focal region were compared to the initial inoculum and a non-luminescent region outside of the focal region when there was either **(A)** >$3.5x10^6$ CFU/g present in the lungs or **(B)** <$3.5x10^6$ CFU/g present in the lungs. **(C)** The percentages of clones in the focal region were compared to the spores found in the focal region regardless of the amount of CFU present. Each column represents the labeled organ for a single mouse, with stacked percentages for each clone. Each group of columns represents a single mouse. Asterisks indicate statistically different populations when comparing the number of colonies of each clone (Fisher's Exact test, * *P*-value <0.05, ***P*-value <0.01, ****P*-value <0.001, *****P*-value <0.0001).


A
<3.5x10^6 CFU/g
****
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% clone in organ
100
50
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BIG23
300bp Insert
400bp Insert
500bp Insert
700bp Insert
Initial Inoculum
Focal
Kidney
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
B
<3.5x10^6 CFU/g
****
****
****
***
****
% clone in organ
100
50
0
BIG23
300bp Insert
400bp Insert
500bp Insert
700bp Insert
Initial Inoculum
mLN
Kidney
Mouse 2
Mouse 3
Mouse 4
Mouse 6
Mouse 7
Mouse 8

Figure 13. Clonal proportions in the lung focal regions resemble those in the kidney, but not mLNs.

(A) The percentages of clones in the focal region or **(B)** mLNs were compared to the percentage clones in the kidneys. Each column represents the indicated organ for a single mouse, with stacked percentages for each clone. Each group of columns represents a single mouse. Asterisks indicate statistically different populations when comparing the number of colonies of each clone (Fisher's Exact test, **P*-value <0.05, ***P*-value <0.01, ****P*-value <0.001, *****P*-value <0.0001).

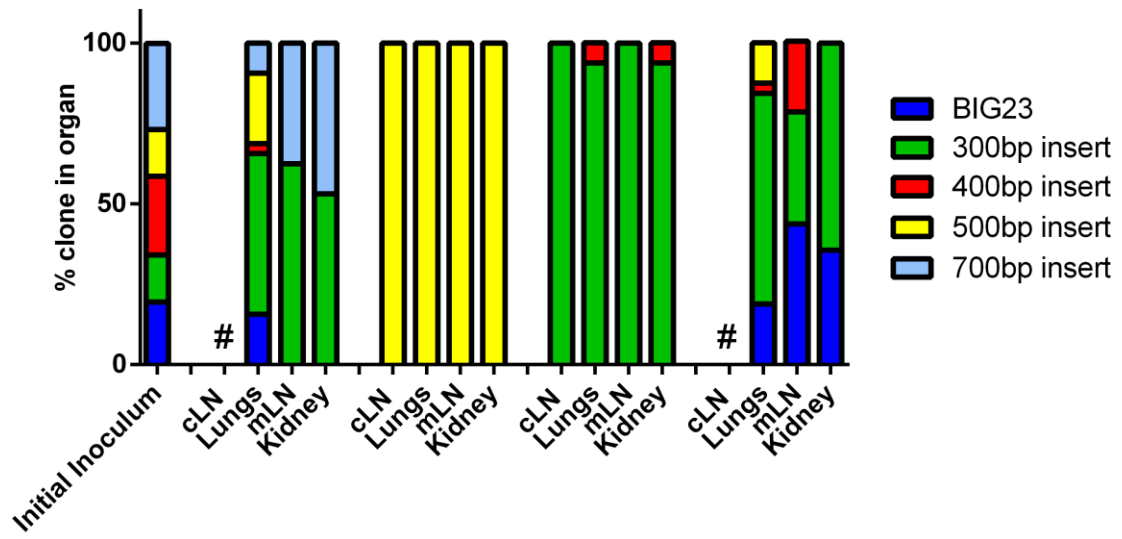


Figure 14. Clonal proportions from lymph-based infections are similar between the mLN and kidneys.

The percentages of clones in the lungs, cLNs, mLNs were compared to the percentage clones in the kidneys. Each column represents the indicated organ for a single mouse, with stacked percentages for each clone. Each group of columns represents a single mouse. Asterisks indicate statistically different populations when comparing the number of colonies of each clone (Fisher's Exact test, **P*-value <0.05, ***P*-value <0.01, ****P*-value <0.001, *****P*-value <0.0001).

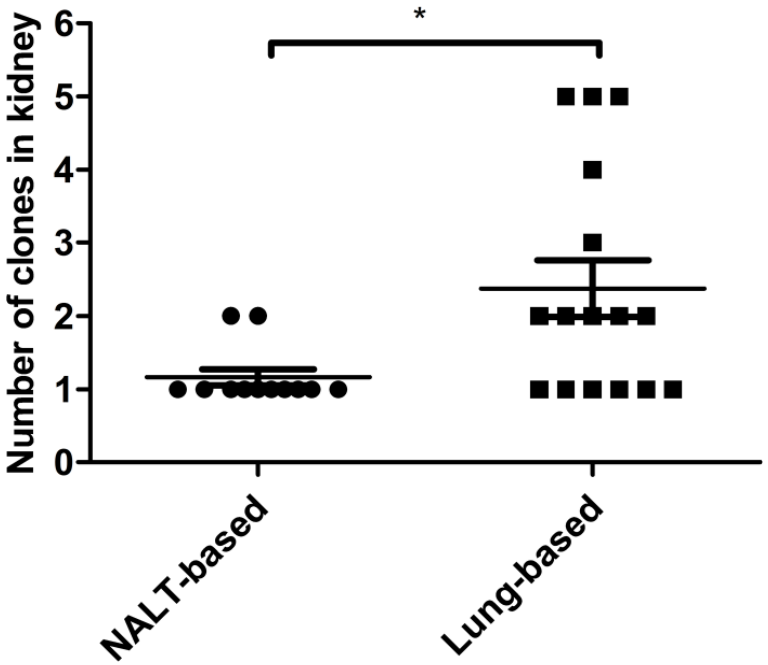


Figure 15. Lung-based bottleneck have a higher number of clones in the kidneys.

The mean number of different clones represented in the kidneys of mice with NALT-based (closed circles, n=12) and lung-based (closed squares, n=16) were compared. Each symbol represents a single mouse; bars represent the mean and error bars represent the 95% confidence interval. Statistical difference is indicated by an asterisk (Student's T-test, **P*-value = 0.013).

Chapter 3: Lethal factor is important for both colonization and dissemination in the early stages of infection.

Introduction

While our research supports the finding that *B. anthracis* encounters barriers to dissemination in the host model, it is unknown which bacterial factors are needed in order to bypass these barriers. Within the host the vegetative bacillus expresses lethal factor, edema factor, and the receptor-binding subunit protective antigen (see Chapter 1), which assemble in a complex known as the exotoxin [154]. The exotoxin is rapidly expressed in the host after germination and the exotoxin production is critical for both the establishment of the infection and death in most animal models [81, 95, 270, 303]. Both LF and EF are capable of increasing endothelial permeability, reducing chemotaxis, limiting the bactericidal activity of immune cells, and inducing cell death [154, 304]. Deletion of CMG2/ANTXR2 in myeloid cells led to host resistance to infection with an uncapsulated strain in mice [183]. LF in particular is important for disabling the immune response early in infection [184]. In addition to targeting local immune cells at the initial site of infection, the exotoxin affects key tissues which contribute to the death of the animal [128]. As such, LF makes an attractive candidate for allowing *B. anthracis* to pass through host barriers and reach the bloodstream. Yet, the amount of LF necessary for either establishment of infection or dissemination is not well understood. Further, it is not known if decreased amounts of LF lead to reductions in bacterial burden or delays in dissemination.

The individual roles of LF and EF *in vivo* have been examined using genetic deletions to delineate their roles in pathogenesis. Most animal models require PA to be secreted for infection

to occur, underscoring the importance of the exotoxin in disease [95, 270, 305]. In some animal models, such as rabbits, the presence of either LF or EF in addition to PA is necessary for disease [95, 305]. Rabbit infections with either the LF deficient mutant (ΔLF) or the edema factor deficient mutant (ΔEF) strains led to higher 50% lethal doses and increased host survival compared to infection with wild type *B. anthracis* [95, 303, 305]. Interestingly, the secreted exotoxin could not be complemented *in trans* when rabbits were infected with a mixed inoculum of capsulated fully toxigenic *B. anthracis* and a capsulated PA knockout mutant (ΔPA) [95]. This suggested that in this scenario the protective effect of the exotoxin is limited to an area near the bacilli (*i.e.* each bacterium must secrete its own exotoxin in order for the exotoxin to successfully protect that single bacterium). In mouse models, the effect of the individual exotoxin components using bioluminescent strains has differing results that are dependent on the presence of capsule. Unlike other animal models, mice can be infected with non-toxigenic, capsulated strains and will have the disease progress to death [306]. In capsulated *B. anthracis* strains, the absence of LF led to an altered dissemination pattern compared to ΔEF or the parental strains in inhalational infections [260]. In mice infections with the uncapsulated *B. anthracis* strain, ΔLF, ΔPA, and ΔLF/ΔEF/ΔPA strains could not disseminate while ΔEF strains had decreased virulence [270]. While mouse models have differences from other animal models, they allow for real time analysis of the early stages of anthrax infection that are otherwise difficult to study. Therefore, the mouse model of infection was used in these studies to further understand the role of LF in early pathogenesis.

To better understand the roles of LF in the early stages of infection, mice were challenged with *B. anthracis* libraries that produced lower amounts of LF at the population level. Signature tag libraries were made with varying ratios of LF expressing (LF^+) and ΔLF mutants.

The libraries had 100%, 40%, 10% or 0.3% of the population comprised of LF producing strains; the remainder of the population was comprised of ΔLF mutants. The bacteria in these libraries had unique DNA tags inserted into the chromosome to further define the role of LF on the disseminating population structure. When the bacteria were induced *in vitro* to produce exotoxin with R media, there was no difference in the ability of the 40% LF^+ inoculum to lyse macrophages. However, the 10% and 0.3% LF^+ inoculums were not able to induce significantly more killing than the media control. Similarly, *in vivo* infections of mice found no difference in survival between mice infected with the 100% LF^+ and 40% LF^+ libraries. Mice infected with the 40% LF^+ library also had no difference in dissemination kinetics and or bacterial burden when compared to the 100% LF^+ inoculum. However, mice infected with the 10% and 0.3% LF^+ library had increased survival compared to the 100% LF^+ library. Infection with a 0.3% LF^+ inoculum failed to colonize, disseminate, or cause mortality; yet, the 10% LF^+ library could colonize the ear for several days but was less capable of dissemination. Since it was previously shown that the protective role of the exotoxin cannot be complemented *in trans*, it was hypothesized that the ΔLF clones would not survive at the initial site of infection and the population would not be able to disseminate. When the mice were infected with the 40% and 10% LF^+ inoculum, the ΔLF bacteria were still capable of survival and dissemination. Infections with 10% LF^+ inoculum, however, showed ΔLF clones were less capable of dissemination and were less competitive than their LF^+ counterparts. These data suggest that discrete amounts of LF are necessary for colonization and dissemination in a mouse model. Furthermore, secreted LF can act *in trans* to promote the growth of ΔLF bacteria when at near equal numbers of wild-type and mutant bacteria in a subcutaneous mouse model.

Materials and Methods

Bacterial strains and culturing

Bacillus anthracis 7702 Sterne strain (pX01$^+$, pX02$^-$) was kindly provided by Molly Hughes, Division of Infectious Diseases, University of Virginia (Charlottesville, VA). ΔLF 7702 was a kind gift from Scott Stibitz [293]. Construction of LF$^+$ strains with recombinant DNA tags has been previously described in Chapter 2 and the same cloning and allelic exchange strategy was used to insert recombinant tags into ΔLF strains [133]. Previous studies have demonstrated that these clones had no defect in *in vitro* growth rates or ability to disseminate *in vivo* (Figure 7). Strains were made luminescent by transforming the plasmid pIG6-19 into either the 7702 or ΔLF 7702 strain and selecting for erythromycin (Erm) resistance. Briefly, pIG6-19 is a suicide vector with homology to the upstream region of Protective Antigen and allows the plasmid to insert into the pX01 plasmid through a single crossover event. This recombination did not interrupt the gene encoding Protective Antigen or its native promoter. The pIG6-19 plasmid contains the *luxABCDE* operon under the control of the Protective Antigen promoter allowing for luminescence *in vivo* and was a kind gift from Michèle Mock [81]. The strain BIG23L is a luminescent derivative of ΔLF 7702 and does not contain any insertion in the *eag* locus. DNA tagged ΔLF and LF$^+$ strains were also transformed with pIG6-19 to allow for bioluminescent analysis. DNA tag sizes were confirmed by PCR analysis and bioluminescence was confirmed by incubating bacteria overnight on Cap media using the IVIS100 (Perkin Elmer, MA) [307]. Clones were then induced to sporulate by plating bacteria on NBY media with 5μg/mL Erm at 30°C for 10 days. The spores were then collected from plates and purified on an Omnipaq gradient (350 mg of iodine/mL, GE Healthcare, NJ) as described previously [81, 264, 289]. Spores of DNA tagged clones were CFU enumerated and mixed to make libraries with various ratios of LF$^+$ and ΔLF bacilli. These libraries were kept at 4°C or kept on ice until used for experiments.

Cell culture and cytotoxicity measurements

The libraries were induced to produce exotoxin *in vitro* by incubating in R media [308]. Libraries were grown from spores in LB supplemented with 5μg/mL Erm and shaken overnight at 37°C. The overnight cultures were then diluted 1:10 in 100 μL LB in a 96 well plate in a humid chamber and incubated overnight at 37°C overnight without shaking. Finally, fresh R media was prepared and supplemented with 0.8% sodium bicarbonate and the overnight bacterial cultures were diluted 1:10 into the R media in a 96 well plate. Samples were incubated in a 37°C incubator with 5% CO_2 without shaking for 6 hours. The supernatant was collected and spun down to remove any bacteria. Supernatants were kept at -80°C until needed for cytotoxic experiments.

RAW264.7 cells (ATCC, VA) were grown in high glucose DMEM (Invitrogen-Gibco, CA) supplemented with 10% fetal bovine serum (Invitrogen-Gibco, CA) and 1000 units/mL penicillin-streptomycin (Invitrogen-Gibco, CA) and incubated at 37°C with 5% CO_2. For cytotoxicity assays, 50,000 cells in 200μL were seeded into a 96 well plate and allowed to adhere for at least 2 hours. The supernatants or R media were added at $1/10^{th}$ the final volume and allowed to incubate for 4 hours. In some wells recombinant LF was added at a final concentration of 2.5 μg/mL in 200μL. Cytotoxicity was measured by % LDH released into the media using the Promega CytoTox96 Non-radioactive Cytotoxicity (Promega). The %LDH release was normalized to the amount of LDH released by the supernatant from the 100% LF^+ inoculum.

Mouse infection and bioluminescent monitoring of dissemination

All mice were 6-12 week A/J mice (Jackson Labs) and were housed in pathogen-free caging at the University of Virginia. Mice were infected by subcutaneous injection in the ears as

previously described [81, 264]. Briefly, mice were anesthetized with a 3% Isoflurane-oxygen (v/v%) mix using a Isotec 5 vaporizer (Absolute Anesthesia) and 10 µL of spores in PBS were injected into the ear using a 0.5cc syringe. The inoculum varied between $2x10^5$ and $5x10^5$ spore CFUs per mouse. Mice were observed once a day for 7 days after infection and bioluminescence was monitored using an IVIS100 (Perkin Elmer). The bioluminescent radiance was measured using LivingImage Software (version 3.2, Perkin Elmer). For fold difference of luminescent radiance over background, a region of interest (ROI) was measured over the luminescent ear and an ROI of the same size was measured over the uninfected ear. Fold difference was calculated as the radiance of the infected ear divided by the radiance of the non-infected ear. Any mouse with luminescence detected in the kidneys was considered to have a fatal infection and was euthanized to reduce pain or distress.

Clonal analysis to determine proportions of signature tagged mutants in organs

The proportion of clones in the organs were determined by selecting 48 clones from each indicated organ of a mouse that had a disseminated infection and using PCR to determine the size of the signature tag. Since small numbers of clones could skew the clonal analysis, organs were only analyzed if there were >300 CFU per organ. Colonies were chosen at random and lysed using the HotSHoT method to extract DNA [294]. Each clone had a tag inserted into the *eag* locus and PCR amplification used primers which flanked the insertion region and conditions as previously described in Chapter 2 [133].

Statistical analysis

Statistical analysis for Student's T Test, one way ANOVAs, two way ANOVAs, and post-hoc tests were calculated using Graphpad Prism (version 6.03, Graphpad Software). Fisher exact tests were calculated using R: A language and environment for statistical computing [309].

Results

Reductions in LF producing bacilli lead to increases in host survival.

The elimination of LF production decreases virulence in most animal models and is required for inhalational infections to occur in the Sterne-A/J model [95, 270, 305]. Few studies, however, have analyzed the relative amount of LF that is required for colonization or dissemination from the initial site of the infection. In order to assess the role of LF for dissemination from the portal of entry, four signature tag libraries were designed with decreasing ratios of 7702 (LF^+) to Δ*lef* 7702 (ΔLF). The amount of LF that could be produced by a population was controlled by altering the proportion of the inoculum that encodes for LF. Importantly, the amount of PA and EF produced by the library would remain the same as solely LF decreased. A previously developed bioluminescent signature tag library, which differed only by the presence of uniquely sized pieces of recombinant DNA, was constructed in the ΔLF and LF^+ 7702 strains [133]. The ΔLF clones either had no DNA tag inserted, designated BIG23L, or either a 300bp or 400bp DNA tag inserted in the S-layer *eag* locus in the chromosome. The LF^+ clones had either a 500bp or a 700bp DNA tag inserted into the *eag* locus. Previous work established that insertion of the tag in these clones did not alter the clones' growth *in vitro* or ability to disseminate *in vivo* [133]. The proportions in each inoculum library were constructed to have all LF^+ clones (100% LF^+), 3 ΔLF clone to 2 LF^+ clones (40% LF^+), 10 ΔLF to 1 LF^+ clone (10% LF^+), or 300 ΔLF to 1 LF^+ clone (0.3% LF^+).

To test if reducing the proportion of LF^+ bacilli in a population reduced LF production, the four signature tag libraries were incubated in R media for 6 hours to induce exotoxin production and their ability to lyse RAW264.7 cells was analyzed. RAW264.7 cells are highly sensitive to a rapid, pro-inflammatory death when treated with LT [208]. Therefore, if less LF is being produced by the population when there are fewer LF^+ bacilli, the ability to lyse macrophages should decrease. LDH release was highest when cells were treated with the 100% LF^+ library and slightly lower for the 40% LF^+ library. The 10% and 0.3% LF^+ library caused very little LDH release, suggesting that these populations produced a lower level of LF than the 100% LF^+ and 40% LF^+ populations (Figure 16A). To confirm that this decrease in LDH release is not due to poor exotoxin induction, recombinant LF was added back at a concentration of 2.5 μg/mL per well and the LDH release was measured. When LF was added back to the 10 and 0.3% LF^+ supernatants, but not R media alone, there was an increase in LDH release (Figure 16A). This suggests that the bacterial population still produces sufficient levels of PA but have reduction in LF.

Mice were infected subcutaneously to determine if a decrease in LF producing bacteria increased survival. These mice were then compared to mice infected with a similar sized inoculum of the 100% LF^+ library. In agreement with previous publications the infections initiated in the pinna of the ear disseminated to the draining cervical lymph node (cLN), then disseminated throughout the mouse (data not shown) [81, 261]. After dissemination from the cLN, the kidneys were the first organ to luminesce and were used to indicate a disseminated infection. Mice were considered to have a fatal infection when the bacteria had disseminated from the cLN to the kidney. When mice were challenged with the 100% LF^+ library, approximately 70% of mice had a fatal infection by day 7. When mice were challenged with the

40% LF+ library, there was not a significant change in survival (Figure 16B). Mice that were infected with either the 10% LF+ or the 0.3% LF+ library, however, had statistically significant increased survival relative to the 100% LF+ library (Log rank test, *P*-value = 0.02). The 10% LF+ library was still capable of disseminating in the mouse; however, the 0.3% LF+ library was unable to cause any death in mice at a similar sized inoculum. This suggests that decreasing the proportion of LF+ bacilli in a population decreases the virulence of the library.

The 0.3% LF+ library fails to colonize the host, but dissemination kinetics and bacterial burdens do not differ between the other libraries.

The 10% and 0.3% LF+ library had increased host survival, but it is not known whether this increased survival is due to a defect in colonization, dissemination, and/or bacterial burden. For these experiments, colonization was considered to be the ability to replicate within the ear, regardless of whether dissemination from the ear occurs. Dissemination was considered to be the ability to replicate in the ear and then disseminate to other organs, such as the cLN or kidney. The 100% LF+, 40% LF+ and the 10% LF+ libraries were capable of replication in the ear, as the bioluminescent radiance in the infected ear would be >10-fold over background for an average of 3.5, 2.75 and 4.4 days, respectively, during the infection period (Figure 17A). In contrast, the 0.3% LF+ library never exceeded > 2-fold over background. This suggests that although the 10% LF+ library cannot disseminate as often as the 40% LF+ or the 100% LF+ library, the 10% LF+ library can still replicate within the ear for several days. The 0.3% ΔLF library, however, was not able to colonize the ear to the same extent.

Since LF is known to be important for colonization and survival in the Sterne-A/J mouse model, the dissemination timing was analyzed in mice with the 40% LF+ and 10% LF+ libraries and compared to the 100% LF+ library. Mice were compared by the day luminescence was first

detected in the draining lymph nodes and kidneys. The average time to luminescence was detected in the draining lymph nodes for LF^+ library was 2.2 days (95% CI = 1.3-3.1 days) (Figure 17B). The 40% and 10% LF^+ libraries were not significantly different from the 100% LF^+ library; the average day luminescence was detected in the cLN for the 40% LF^+ library was 3.0 days (95% CI = 1.8-4.2 days) and for the 10% LF^+ library was 2.7 days(95% CI = 1.8-3.6 days) (Figure 17B). Similarly, there was no difference between the day luminescence was detected in the kidneys of mice. The average day that luminescence was detected in the kidney in mice infected with the 100% LF^+ library was 4.1 days (95% CI = 2.8-5.5 days), which was not significantly different from the 40% LF^+ library average, 3.8 days (95% CI = 1.8-5.8 days), or the 10% LF^+ library average, 3.0 days (95% CI = 2.3-3.7 days). The 0.3% LF^+ library had no detectable luminescence in the ear, or any other part of the host, during the 7 days the mice were observed.

Lastly, the bacterial burdens in organs were compared when the 100% LF^+, 40% LF^+, and 10% LF^+ libraries disseminated in mice. Since mice with the 0.3% LF^+ did not disseminate they were excluded from these analyses. *B. anthracis* infections are asynchronous and therefore mice were compared only if the mice were at the same stage of infection; *i.e.* when luminescence was detected in the kidneys, but before mice were moribund. Though there was variability in the bacterial burdens in tissue, no significant difference could be detected in disseminated organs between the libraries (Figure 17C). Therefore, we conclude that if *B. anthracis* can disseminate systemically that no significant differences occur in the bacterial burden when the initial inoculum consists of at least 10% LF^+ bacteria.

ΔLF clones survive and disseminate throughout the host when there is a 40% reduction of LF producing bacteria.

Since there were increases in host survival with the 10% LF$^+$ library, but no defects in dissemination kinetics or bacterial burden, it was tested whether this observation reflected the failure of ΔLF clones to replicate and/or disseminate in the host. Clonal analysis was used to determine if the ΔLF clones could survive *in vivo* and determine how well LF can be complemented *in trans* by the LF producing bacilli for the 40% LF$^+$ and 10% LF$^+$ libraries. Clonal analysis uses a library of clones that are differentiated only by a single tag or marker. Animal models can be infected with these libraries and then dissected to determine the signature tag identities present in the host. The changes of the proportions of tagged bacteria during dissemination through the host can then be used to determine how the bacterial population structure was altered during infection. Previous research, however, has demonstrated that *in vivo* bottlenecks occur in *B. anthracis* infections in mice [133, 288]. Since stringent bottlenecks could complicate analysis of *in trans* complementation in our model, clonal analysis was first performed with the 100% LF$^+$ library to determine if a stringent bottleneck occurred in the subcutaneous model, which has not been addressed in previous research. Mice were infected with between $2.0x10^5$ and $5.0x10^5$ spore CFU in the pinna of the ear. The infection was allowed to disseminate until light was detected in the kidney, indicating that the host is bacteremic.

Signature tag proportions were analyzed in the ear infected with the 100% LF$^+$ library to determine whether the bacteria experienced a bottleneck in the initial site. In four of seven mice at least two clones comprised >70% of the population, suggesting a bottleneck occurred in the ears of these mice (Figure 18A). Mouse #7's bacterial population did not subsequently disseminate, but a bottleneck still occurred. The other three mice did not indicate a bottleneck took place in the ear since no clone comprised more than 45% of the population. This suggests that a bottleneck can occur in the ear of mice, but does not necessarily occur in every infection.

As a control for *in vivo* selection against the DNA tag interrupting the *eag* locus, the signature tag proportions found in the ears were compared to the initial inoculum. If there is no *in vivo* selection in an organ, the signature tag proportions should be statistically equal to the inoculum. When these proportions were averaged for all 7 mice there was not a significant difference between the average proportions and the initial inoculum (Figure 18A, Fisher exact test *P*-value = 0.132). This suggests that an *in vivo* growth defect does not occur from a tag interrupting the *eag* locus when all clones produce LF. When the cLNs were analyzed, four out of six mice had a bottleneck given the above criteria (70% of the population comprised of $\leq$ 2 clones). The three mice with bottlenecks in the ear corresponded with three of the cLNs with bottlenecks. The averaged proportion of the clones did not significantly vary from the inoculum, implying that all clones were equally capable of reaching the cLN (Figure 18B, Fisher's exact test *P*-value = 0.063). Therefore, a bottleneck can occur in either the ear or as the bacteria migrate from the ear to the cLN. When the disseminated populations in the kidneys were examined, there were pronounced bottlenecks in 7 of 8 mice with 1 or 2 clones comprising >85% of the population (Figure 18B). Similar to the bacterial populations in the cLN, the averaged kidney populations did not have a significant difference from the initial inoculum, suggesting that the bottlenecks were not due to host selection against specific tagged clones (Fisher's exact test *P*-value = 0.14). Those mice that did not have a bottleneck in the lymph node passed through a bottleneck en route to the kidney. Thus, the subcutaneous route has multiple bottlenecks that occur, with the most stringent typically occurring between the cLN and the kidney. Yet, the subcutaneous route demonstrated that several clones are present in the ears, cLNs, and at times the kidney. Disseminated populations of bacteria from intranasal routes, however, encountered a bottleneck either at the initial site of infection or as the bacteria disseminate to the lymphatics [133, 288]. This early bottleneck would lessen or preclude the

ability of the LF+ clones to complement the ΔLF clones during dissemination. This would greatly increase the number of animals needed to determine if the ΔLF clones could still disseminate. Therefore, the subcutaneous model of infection is the most appropriate model for testing the role of LF by *in trans* complementation.

When the signature tag proportions were analyzed in the ears of mice that received the 40% LF+ library, the signature tag proportions were not statistically different from the initial inoculum (Figure 19A), Fisher's Exact Test *P*-value = 0.81). Similar to the 100% LF+ library, half of the mice had >70% of the population be comprised of ≤ 2 clones. The draining cLNs from mice infected with the 40% LF+ library only had a bottleneck in 1 of the 8 mice; the other mice had no sign of a bottleneck in the cLN (Figure 19B). When averaged, the signature tag proportions in the cLNs did not significantly differ from the initial inoculum, suggesting that all clones were equally capable of reaching and replicating within the draining lymphatics (Figure 19B, Fisher's Exact Test *P*-value = 0.89). Lastly, the kidneys were analyzed to determine if the ΔLF clones could disseminate throughout the host. Similar to the 100% LF+ library, the signature tag proportions suggested a stringent bottleneck occurred between the cLN and the kidneys. Five of the eight mice (62.5%) had a majority of the population composed of ΔLF clones and there was no statistical difference between the averaged signature tag proportions in the kidneys and the initial inoculum (Figure 19B, Fisher's Exact Test *P*-value = 0.29). These data suggest that the LF+ clones are capable of complementing *in trans* for the ΔLF clones to such an extent as to permit the dissemination of the normally avirulent ΔLF throughout the mouse.

The ΔLF clones have decreased proportions and fewer clones disseminate when only 10% of the population contains LF producing bacteria.

Mice infected with the 10% LF$^+$ clonal library were analyzed to determine if the ΔLF clones could survive and replicate as they disseminate through the host in the presence of limited LF production. In mice where the infection disseminated through the host, the ears had an increased proportion of BIG23L relative to the initial inoculum in all eight mice (Figure 20A). This increase in BIG23L came at the expense of the other ΔLF strains as on average 93% of the population was ΔLF and resulted in a significant difference from the initial inoculum delivered (Figure 20A, Fisher's exact test *P*-value = 0.00017). This result was surprising, as there was no indication that the 300bp or 400bp insert clones having any *in vivo* survival defects when the mice had a 40% LF$^+$ or 100% LF$^+$ in the ear (Figure 18A and Figure 19A). Moreover, there were no growth or *in vivo* dissemination defects for the 300bp or 400bp insert in inhalational infections [133]. After disseminating from the ear to the cLN, LF$^+$ clones comprised a significantly greater percentage of the population, including 2 mice with a majority of the population comprised by a LF$^+$ clone (Figure 20B). The average proportions of clones in the cLN demonstrate that there is a decrease in ΔLF clones and in particular the BIG23L clone (Figure 20B). The decrease in ΔLF clones relative to the ear continued when the kidneys were analyzed. In half of the kidneys analyzed by clonal analysis, the LF$^+$ clones grew to represent the majority of the population. Further, 7 of the 8 mice had the percentage of LF$^+$ clones at least double from their proportion in the ear (Figure 20B). This suggests that the LF$^+$ clones are more likely than the ΔLF clones to disseminate when 90% of the population cannot produce LF.

Lastly, the number of clones that were able to disseminate to the kidney were compared between the 100% LF$^+$, 40% LF$^+$ and 10% LF$^+$ library. There was a significant decrease in the amount of clones that were able to disseminate to the kidneys in the 10% LF$^+$ library compared

to either the 100% LF$^+$ or 40% LF$^+$ library (Figure 21). This suggests that LF influences the ability of clones to pass through the bottlenecks in a subcutaneous infection.

Discussion

The production of LF *in vivo* is an important virulence factor that promotes bacterial survival and contributes to host mortality. While genetic deletions of LF have highlighted its importance for disease, the relative amounts of LF needed in the early phases of pathogenesis are poorly understood. A principle finding of this study was that decreasing amounts of LF$^+$ bacilli in the inoculum had discrete effects on the local site of colonization. When the population was 40% LF$^+$ bacteria, there were no differences in survival, colonization, or bacterial burden in the analyzed tissues. A further reduction of LF$^+$ bacilli to 10% of the population led to an increase in host survival, but not in dissemination kinetics, bacterial burden, and colonization of the ears. This suggests that the amount of LF produced by the 10% LF$^+$ library is sufficient for colonization, but resulted in less frequent dissemination. The library with the lowest amount of LF$^+$ bacilli, the 0.3% LF$^+$ library, never had luminescence that was detectable over background ($\geq$ 2-fold) when injected into the ear, implying the bacteria colonized poorly. As such, it appears that when the LF$^+$ clones are reduced by 90%, the population is less likely to disseminate from the ear. However, the library must have at least 10% of the population producing LF in order to colonize the ear. Thus, the amount of LF producing bacteria in a site can reveal the multifaceted roles LF plays early in infection.

Anthrax is an acute disease in which the bacteria rapidly disseminate through the host [81, 95, 133, 270]. Given this acute nature, a promising area for therapeutic development would be the early stages when the bacteria begin to overwhelm the host defenses in order to disseminate. When the amount of LF producing strains was reduced to 10% of the population,

host survival increased. Yet, there were no significant differences between the bacterial burden, dissemination kinetics, or ability to colonize the host. This suggests that in a murine subcutaneous infection, the initial site of infection is a crucial point that determines whether the host will survive the infection. Similar studies have found that removing the infected portion of the ear or genetically deleting the CMG2 receptor from myeloid cells leads to increases in mouse survival [183, 264]. Our findings, however, imply that LF is critical for this survival and dissemination from the initial site.

Reducing the number of LF producing bacilli illustrates the protective effect of the exotoxin on a population of bacteria. Our current and previous findings found no difference in ability to disseminate between strains with a DNA tag interrupted the *eag* gene and its parental counterpart when there was at least 40% of the population produced LF. Furthermore, when *eag* was interrupted with the DNA tags, there was no difference in the *in vitro* growth rates. Yet, in populations where 90% of the population was ΔLF, there was an increase in the *eag*$^+$ BIG23L clone. This change predominantly came due to a decrease in the 300bp insert and 400bp insert ΔLF clones, since average BIG23L proportion of the population doubled while the LF$^+$ population only decreased from 10% to 7% of the population. While EA1, the protein encoded by *eag*, does not seem to have a direct role in pathogenesis, it is a major component of the S-layer. The S-layer has several proteins that are attached to it which have been shown to be important for adhesion, pathogenesis, and iron uptake [92, 310, 311]. It is possible that in this animal model the decrease in LF producing strains reveals that the S-layer may play a role in pathogenesis, but the exotoxin obscures the defect in most circumstances. We speculate that the exotoxin can mask minor survival defects *in vivo* by effectively inhibiting the host response.

Since bacterial burdens are the same after dissemination despite increases in host survival, clonal analysis may be a more sensitive method to measure decreased virulence.

In the inhalational rabbit model of anthrax, ΔPA strains cannot be trans-complemented when co-infected with the fully toxigenic strain [95]. As such, it was surprising to find that both the 40% LF^+ and 10% LF^+ populations had the LF^+ clones trans-complement the ΔLF clones. Since this study uses different animal models, bacterial strains, and infection routes, direct comparisons between these data are difficult. Further, since capsulated non-toxigenic bacilli disseminate in mice, the strains used in Lovchik *et al*.'s rabbit model would be inappropriate for the analysis of the role of LF early in infection. Nonetheless, mouse models have several advantages over the rabbit model which can be used to answer questions regarding early *B. anthracis* pathogenesis. Notably, mice permit the use of bioluminescent imaging, which allowed for the observation that the increased host survival was due to the decreased dissemination in the 10% LF^+ library and the inability of the 0.3% LF^+ library to colonize the ear. Further, the use of mice infected with bioluminescent *B. anthracis* indicated that the average time from dissemination from the ear to the draining lymph node and from the lymph node to the kidney are statistically indistinguishable despite increased host survival. This was crucial for determining that early events in *B. anthracis* infections either lead to clearance and survival or dissemination and death.

Our research indicates that LF, and perhaps the exotoxin in general, influences the number of signature-tagged clones that disseminate from the initial site of infection. Previous research has noted that the host tissue environment where the infection establishes impacts the population structure as it disseminates [133]. In this study clonal analysis indicated that when LF is reduced to the level produced by the 10% LF^+ library fewer clones comprise the

disseminated population. Given that different routes allow distinct numbers of clones to disseminate, this may also explain the discrepancy between our results and Lovchik *et al.* It is quite possible LF acts in close proximity to the secreting bacilli when being transported to the lymph nodes intracellularly, but is less influential in other routes of infection. This suggests that LF, and the exotoxin as a whole, plays a role in defining the stringency of the population bottleneck.

B. anthracis dissemination has two models widely recognized of dissemination through the host: the Trojan horse model and the Jailbreak model [71, 264]. The Trojan horse model involves spores being internalized by professional phagocytes which deliver the spores to the lymph nodes. Once in the lymph nodes, the spores germinate, escape the phagocyte, and begin the infection in the draining lymph node [71]. In contrast to the Trojan horse model of infection, the Jailbreak model argues that germination occurs extracellularly and the exotoxin and/or secreted proteases lead to the destruction of the tissue barriers and disrupt the host immune response [264]. The subcutaneous mouse model appears to support the Jailbreak model as spores can germinate extracellularly in the ear and debridement of infected ear tissue increases host survival [79, 261]. This could explain how population-based decreases in LF led to an overall decrease in dissemination for the ear as decreased levels of LF producing bacteria would be less likely to suppress the immune response or breakdown tissue barrier function. Yet, a bottleneck occurred during the disseminated infections described here and only a few bacteria were capable of causing an infection which led to the host death. This argues that exotoxin production from all bacteria is acting synergistically to increase the chance of dissemination and host death while at the same time any single bacterium can disseminate and cause the death of the animal. This resembles the partial synergistic action hypothesis in infection [312]. It would

be interesting to see if this infection action model is applicable to other pathogens or routes of *B. anthracis* infection.

The route of infection or which specific exotoxin component that is being complemented may also play a role in the ability of *in trans* complementation to occur. Some animal models were found to have a high bacterial burden in the lung associated lymph nodes but not in the lungs, suggesting that the spores were trafficked to the lung associated lymph nodes by phagocytes acting as a Trojan horse [95]. In contrast to these inhalational infections, subcutaneous ear infections are known to spread from the colonized tissue in the ear, rather than from spores that migrated to the lymph node [264]. This raises the interesting possibility that the amount of LF needed in a site is dependent on the environment that is colonized. Another possibility for the differences in the data reported here from others is that different exotoxin components were being complemented. It is possible that while LF is able to be complemented, PA cannot; perhaps reflecting unappreciated differences between these two exotoxin component's biochemical functions, such as the timing of formation of biologically active holotoxin relative to the proximity to the bacterium. Further research is necessary to understand how exotoxin components interact *in vivo* as well as whether these interactions are similar in all tissues.

While much has been gained in previous analysis of the anthrax exotoxin in disease and dissemination there are still areas in further need of study. Our research indicates that a certain amount of LF^+ strains in a population are necessary for dissemination in a subcutaneous model of infection, but a lower level is needed for the initial colonization. Thus, it follows that a greater focus should be placed on understanding the relative concentrations of LF that exist in the host

in particular time points in the infection. Such advances in basic science would likely hold great promise in development and validation of future therapeutics in animal models.

Figures from Chapter 3

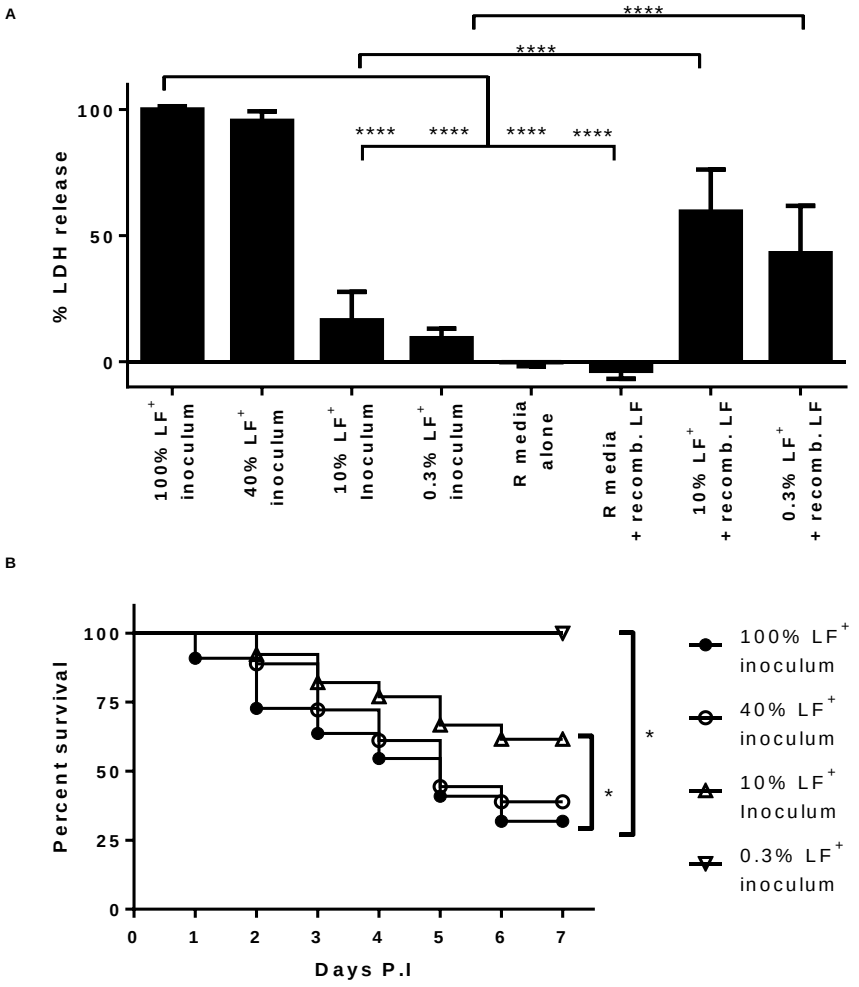
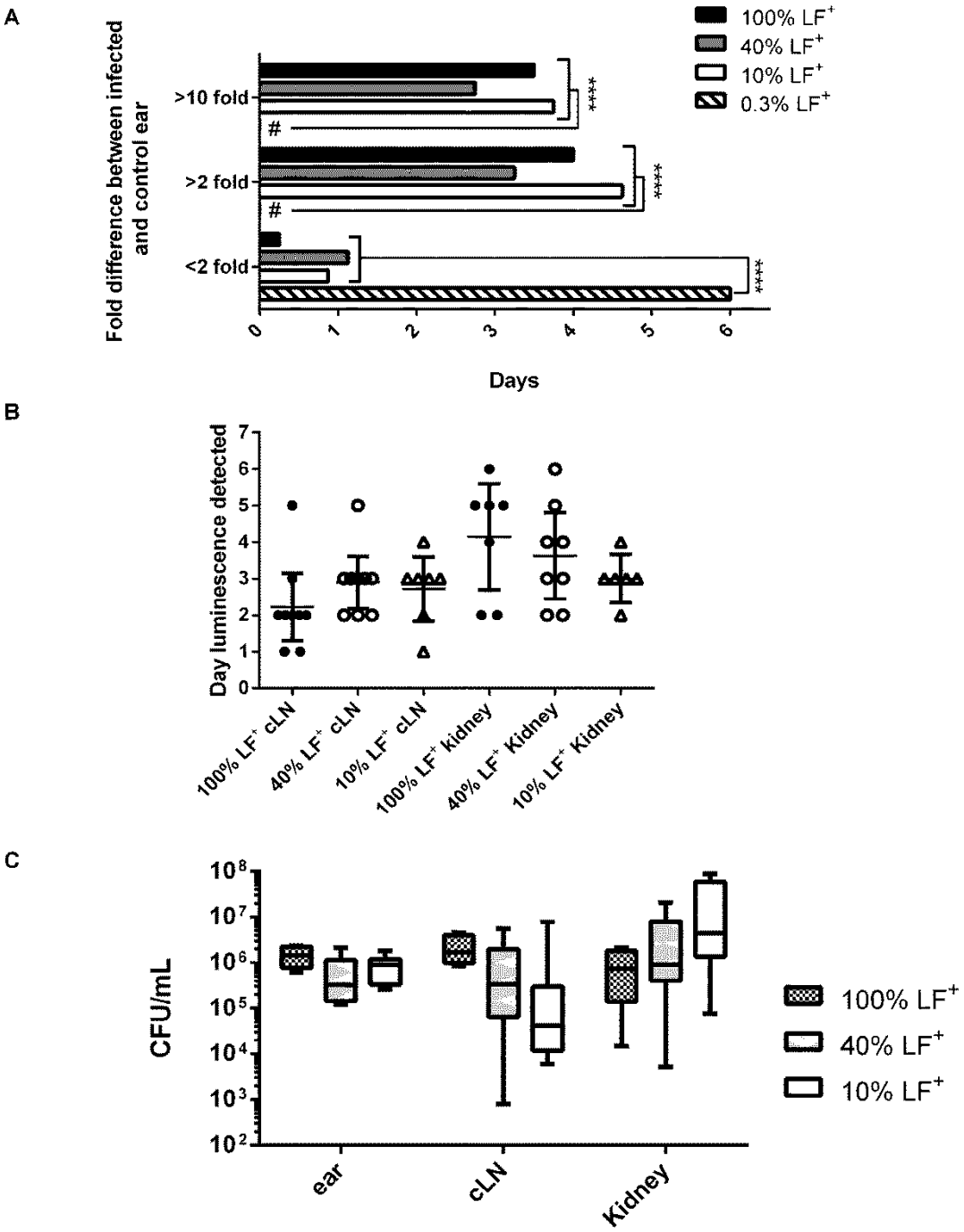


Figure 16. Decreasing LF producing clones in a signature tag library led to reductions in virulence.

(A) Decreasing the amount of LF producing clones in a library led to reductions in macrophage cytotoxicity. RAW264.7 cells were plated in 96 well plates and treated with bacterial supernatants from exotoxin inducing R media. LDH release was measured and the % LDH release was calculated relative to the 100% LF^+ supernatant. Experiments were repeated at least three independent times with at least 3 replicates per run. Asterisks represent a significant difference (One-way ANOVA with Tukey's multiple comparisons test. ****P-value <0.0001) Error bars represent the 95% confidence interval. **(B)** Kaplan-Meier curve of mice infected with 2x 10^5 CFU spores subcutaneously in the ear pinna. Mice were observed for 7 days. Solid circle represent mice infected with a library where all clones are LF^+ (n=22), open circles represent 40% LF^+ library (n=14), empty upward triangle represent 10% LF^+ library (n=39), empty downward triangle represent 0.3% LF^+ clones library (n=8). Asterisk represents a significant difference from the 100% LF^+ library (Log rank test, *P-value <0.05).


A
100% LF+
40% LF+
10% LF+
0.3% LF+
Fold difference between infected and control ear
>10 fold
>2 fold
<2 fold
#
#
****
****
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0
1
2
3
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5
6
Days
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Day luminescence detected
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4
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6
7
100% LF+ cLN
40% LF+ cLN
10% LF+ cLN
100% LF+ kidney
40% LF+ Kidney
10% LF+ Kidney
C
CFU/mL
10^8
10^7
10^6
10^5
10^4
10^3
10^2
ear
cLN
Kidney
100% LF+
40% LF+
10% LF+

Figure 17. Increased survival was due to defects in colonization or dissemination.

(A) Number of days luminescence was detected at a particular fold difference over background for each signature tag library. Fold difference represents the increase of the infected ear over the non-infected ear. Solid black bars represent mice infected with the 100% LF^+ (n=4), grey bars represent 40% LF^+ library (n=8), diagonal hashed bars represent 10% LF^+ library (n=8), white bars represent 0.3% LF^+ library (n= 8). Number signs signify that library never had luminescence detected over background at the given fold difference. Asterisks represent a statistical difference from the 0.3% LF^+ library (Two way ANOVA with Dunnett's Multiple comparison test; **** *P*-value <0.0001). **(B)** There is no temporal difference in dissemination between the signature tag libraries that are capable of dissemination. Columns indicate the average day luminescence was detected in the draining cervical lymph node and kidney with the 100% LF^+ library (solid circles, n=9 for cLN, n=7 for kidney), 40% LF^+ library (open circles, n=9 for cLN, n=8 for kidney), 10% LF^+ library (upward facing open triangles, n=7 for cLN, n=6 for kidney). Mice were observed at least once per day. Error bars represent the 95% confidence interval. No significant difference when comparing the days for either the cLNs or kidneys (One way ANOVA, *P*-value < 0.05) **(C)** CFU enumeration in organs with infected with differing levels of LF. CFU were enumerated from mice infected with $2x10^5$ CFU spores of either the 100% LF^+ library (checkered boxes, n=4), 40% LF^+ signature tag library (gray boxes, n=9), or 10% LF^+ signature tag library (white box n=8). Mice were dissected after luminescence was detected in the kidney, but before mice were moribund. Error bars represent the 10-90 percentiles.

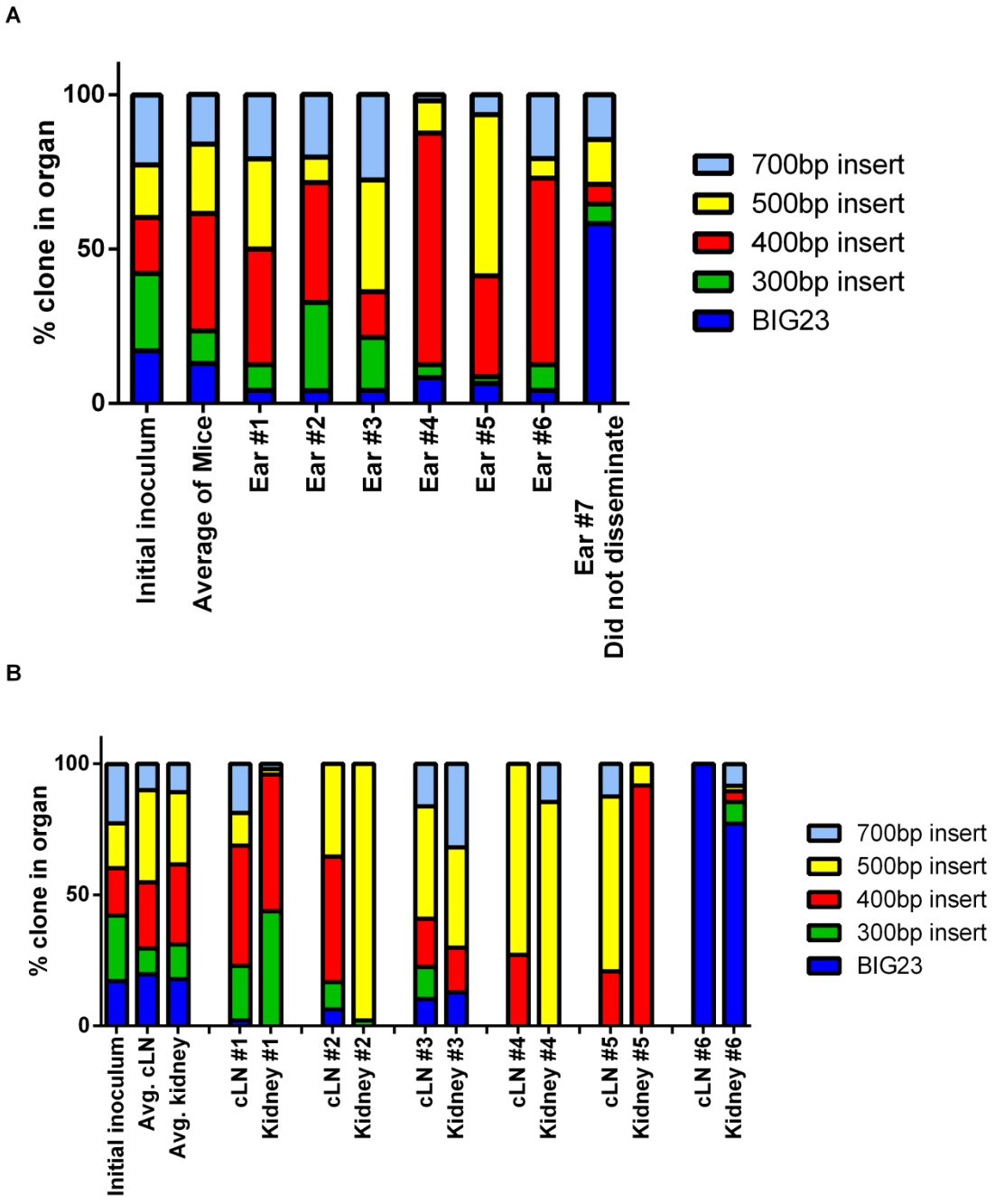

Figure 18. Multiple bottlenecks occur in the subcutaneous routes, but several clones are capable of dissemination.

(A) Graphs indicate the signature tag proportions found from the bacterial population that resided in the ear of mice infected with the 100% LF^+ library. Each column is a stacked percentage bar where each clone is represented by a different color. **(B)** Bottlenecks often occur in the draining cLN and are always present by the time the bacteria reach the kidneys. Graphs indicate the signature tag proportions found of the bacterial population that resided in the cLN or kidney of mice infected with the 100% LF^+ library. Each column is a stacked percentage bar where each clone is represented by a different color.

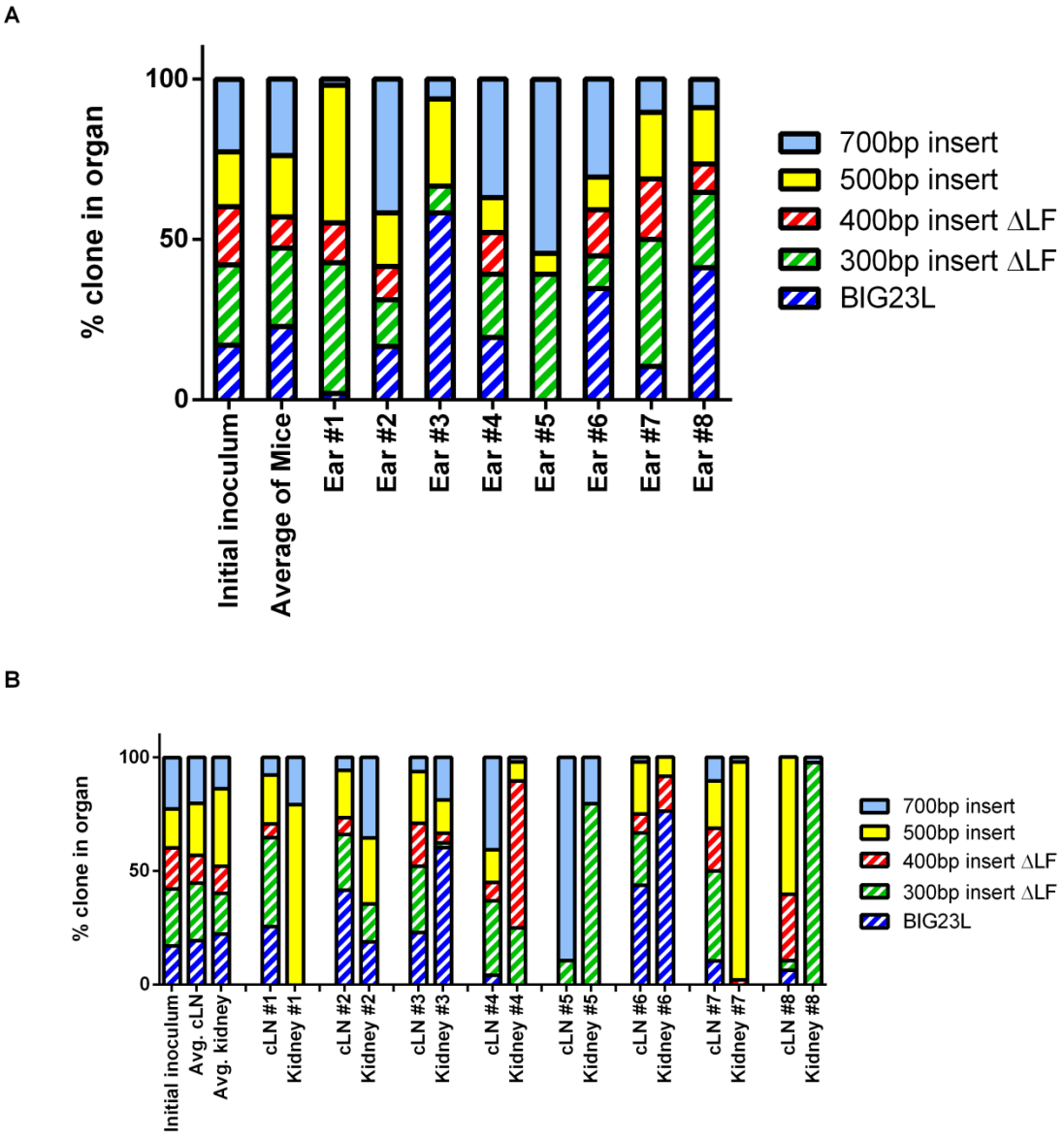

Figure 19. ΔLF clones can be trans complemented when 40% of the library produces LF.

(A) ΔLF clones are capable of colonization and replication when LF is reduced by 60%. The signature tag proportions found of the bacterial population that resided in the ear of mice infected with the 40% LF^+ library. Each column is a stacked percentage bar where each clone is represented by a different color. **(B)** ΔLF clones disseminate to the cLN and kidneys when there is a 60% reduction in LF producing bacteria. The signature tag proportions found of the bacterial population that resided in the cLN and kidney of mice infected with the 40% LF^+ library. Each column is a stacked percentage bar where each clone is represented by a different color.

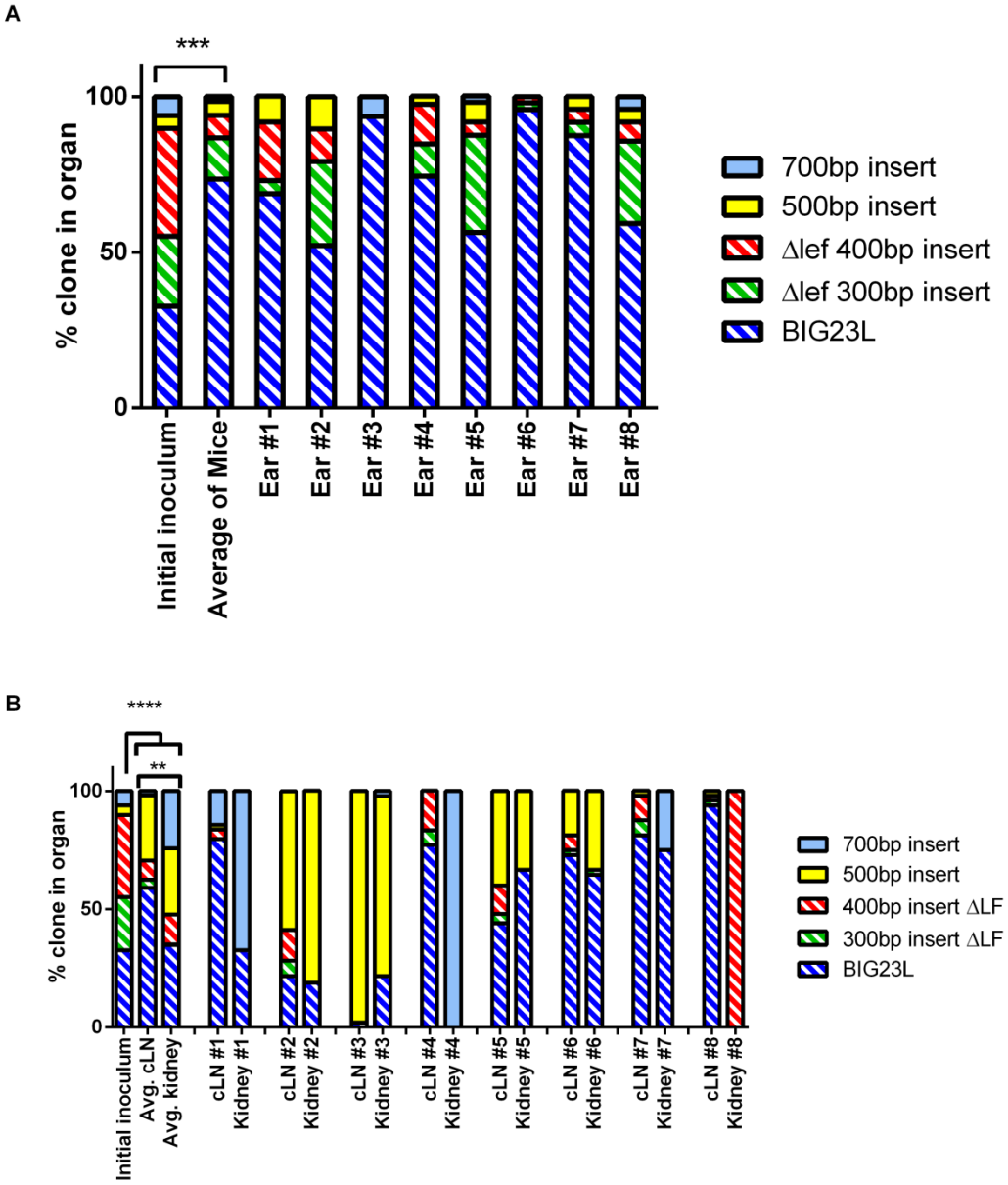
A
***
100
50
0
% clone in organ
Initial inoculum
Average of Mice
Ear #1
Ear #2
Ear #3
Ear #4
Ear #5
Ear #6
Ear #7
Ear #8
700bp insert
500bp insert
Δlef 400bp insert
Δlef 300bp insert
BIG23L
B
****
**
100
50
0
% clone in organ
Initial inoculum
Avg. cLN
Avg. kidney
cLN #1
Kidney #1
cLN #2
Kidney #2
cLN #3
Kidney #3
cLN #4
Kidney #4
cLN #5
Kidney #5
cLN #6
Kidney #6
cLN #7
Kidney #7
cLN #8
Kidney #8
700bp insert
500bp insert
400bp insert ΔLF
300bp insert ΔLF
BIG23L

Figure 20. ΔLF clones are less capable of survival and dissemination when 10% of the library produces LF.

(A) BIG23L is the dominant clone in the ear of mice when there is a 90% reduction of LF producing bacteria in the inoculum. The signature tag proportions found of the bacterial population that resided in the ear of mice infected with the 10% LF^+ library. Each column is a stacked percentage bar where each clone is represented by a different color. Asterisk represents a significant difference from the initial inoculum (Fisher's Exact test, *** *P*-value <0.001) **(B)** ΔLF clones are less abundant in the disseminated population when LF is reduced by 90%. The signature tag proportions found of the bacterial population that resided in the cLN and kidneys of mice infected with the 10% LF^+ library. Each column is a stacked percentage bar where each clone is represented by a different color. Asterisk represents a significant difference from the initial inoculum (Fisher's Exact test, ** *P*-value <0.01; **** *P*-value <0.0001).

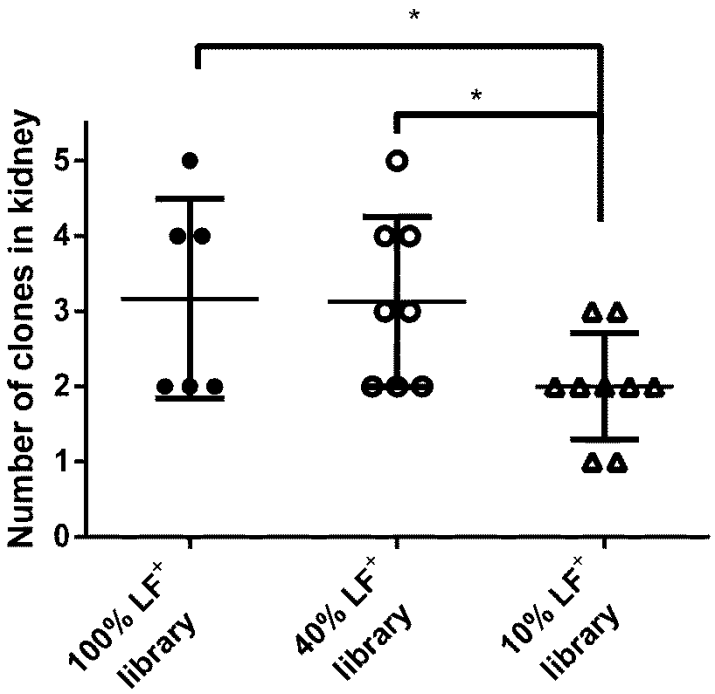

Figure 21. Fewer clones disseminate when the library only has 10% of the population produce LF.

There are fewer clones that disseminate when LF producing bacteria are reduced by 90% in the inoculum. The number of clones that disseminated to the kidneys were enumerated and compared between the libraries. Each symbol represents a mouse. Bars represent the mean and error bars represent the standard deviation. Asterisks represent a statistical difference between the means (Student's T Test, *P-value = 0.044).

Chapter 4: Summary and Future Directions

Until recently the theories of *B. anthracis* dissemination have focused on the role of phagocytes that delivery spores or vegetative bacilli to the regional lymph nodes of the lungs [71, 74, 285]. Alternative models of dissemination have been recently suggested after the observation that germination and outgrowth can occur in mucosal associated lymphoid tissues or in the lumen of the lungs [81, 270, 275, 276]. By using bioluminescent strains of *B. anthracis*, these studies indicated where the infection initiated and the later organs that were infected, but how the bacteria disseminate from the portals of entry was not investigated. *B. anthracis* infection models have differing amounts of virulence in terms of LD_{50}'s, which may be a reflection of some routes having greater barriers for dissemination than others [103]. As such, an understanding of how the bacilli disseminate from unique environments could allow for identification of host factors that limit dissemination. One factor that we explored and may play a role in dissemination from the portals of entry is the secreted exotoxin [95, 270, 303, 305]. In particular, lethal factor is known to play a role in the disease as infection with a ΔLF strain leads to decreased virulence [95, 270, 305]. However, it is not well understood how decreasing LF early in the infection would affect an inoculum of *B. anthracis*. These studies described in this thesis investigated the early stages of *B. anthracis* pathogenesis, concentrating on actions that occur at the portal of entry and how they affect disseminated population.

The effect of the portal of entry on *B. anthracis* dissemination

Our initial studies were focused on how the population dynamics changeas *B. anthracis* disseminates through the host, focusing on bottlenecks as they may represent crucial moments where the host presents barriers to dissemination. Several pathogens have had their

dissemination routes investigated through clonal analysis of signature tagged mutants [133, 150, 282, 313, 314]. These studies have redefined the understanding of how pathogens disseminate through the host and clarified the portal of entry for infection. For example, *Yersinia pseudotuberculosis* was thought to invade the Peyer's patch through M cells and then disseminate through the host via host lymphatics [315-317]. Clonal analysis using an inoculum of signature tagged mutants, however, suggested that bacteria invaded and replicated in the intestines. This allowed the bacteria to continuously disseminate by exploiting multiple niches over a period of time, rather than have one chance of invading the M cells and colonizing the Peyer's patches [150]. Similarly, *L. monocytogenes* infections of Guinea pigs indicate that the bacteria first infect the small intestinal villi [313]. *L. monocytogenes* primarily invades villus enterocytes, causing their death and leading to release of infected cells. The bacteria can then spread to other intestinal cells, such as other enterocytes, invade the Peyer's patches, and macrophages in the lamina propria. [313]. After invading the Peyer's patch or infecting macrophages in the lamina propria, the bacteria can go on to disseminate to the spleen and liver [313]. As such, it seems a common theme in pathogenesis is the establishment of population of bacteria within the host and then subsequent dissemination from the replicating pool.

Several of these pathogens encountered a population bottleneck within the host. A bottleneck is a decrease in population size that leads to a reduction in the genetic diversity. Population decreases could occur from environmental changes, such as movement between hosts or from one host site to another, or from a selective pressure for an allele that confers greater fitness. Both a bottleneck and a selective event lead to reduced genetic diversity; therefore, it should be emphasized that a bottleneck leads to a reduction of genetic diversity by random sampling of a population. This key aspect, random sampling, means that the alleles

which pass through the bottleneck do not necessarily lead to increased fitness for the population. In fact, encountering a bottleneck often leads to reduced fitness since it cannot select for beneficial mutations or against detrimental mutations. Since passage through a bottleneck reduces the number of alleles available for future progeny, the population over time becomes more homogenous and less able to adapt to new environments or conditions. From a pathogenesis stand point, bottlenecks can represent a moment in infection where the host imposed a barrier on the pathogen's dissemination. Therefore, identifying the sites and results of these bottlenecks would inform researchers on how *B. anthracis* exploits the host for dissemination. In particular, it was unknown if *B. anthracis* uses a common mechanism of dissemination between portals of entry.

At the time these studies were initiated, it was known that *B. anthracis* can disseminate from several portals of entry in inhalational routes, but the lung epithelium and the NALT are quite different structures that have different roles in preventing infection [78, 80]. NALT, lung, and subcutaneous *B. anthracis* infections appeared to support the Jailbreak model of dissemination [264]. That is, the inoculum germinates and colonizes outside of a lymph node, then migrates to the lymph node secondarily without transportation via a phagocyte. To test how *B. anthracis* disseminated from these different environments, we used clonal analysis to compare the dissemination from the NALT and the lung epithelium [150, 313]. Our data from clonal analysis suggested that *B. anthracis* has independent ways of reaching the bloodstream [133]. In inhalational infections NALT-based infections were found to disseminate through the lymphatics as suggested by the bioluminescent analysis previously published [81, 275]. However, during migration from the NALT to the cervical lymph nodes (cLNs) a pronounced bottleneck occurred [133, 288]. This would suggest that the bottleneck in NALT-based infections

is migration mediated and the first bacterium to reach the draining lymph node will become the founder of the population. In contrast, the lung-based infections had a bottleneck occur at the portal of entry and the dominant population at the portal of entry was also the dominant population at the dissemination sites. Further, the disseminated population did not necessarily need to disseminate through the lymph nodes, which is different from subcutaneous, gastrointestinal, and NALT-based infections [133]. Such differences may be due to the NALT being a germination permissive environment and the lung lumen typically acting as a germination-restrictive site [74, 95, 133, 267, 285]. Therefore, it seems that the first spore to germinate and replicate goes on to be the founder of the disseminated population.

Subcutaneous infections were distinct from both forms of inhalational infection. The infection initiated outside of lymphoid tissue, like the lungs, but always disseminated to the cLN before reaching the bloodstream, like the NALT. Further, the subcutaneous infection had multiple bottlenecks yet allowed the most clones to pass through, whereas the other infections only had a single stringent bottleneck. This may suggest that several bottlenecks do not necessitate or imply stringency. Further, it may imply that certain host barriers are a more effective at restricting dissemination than others. It is difficult to determine the causes of the bottlenecks in the subcutaneous infections. Cutaneous injection of spores into the ears of mice leads to rapid germination of 76% of the inoculum, with roughly half germinating extracellularly and half germinating within phagocytes [79]. Further, CFU counts remained stable in the ear 6 hours after injection despite widespread germination. This suggests that there is a robust immune response to the nascent bacilli and perhaps this response is strong enough at times to lead to a bottleneck. Bottlenecks that occur in the cLN or after the bacteria disseminate to other organs most likely occur due to migration, similar to the NALT-based infection. Therefore, while *B. anthracis* is capable of disseminating from many sites, the disseminated population has unique

features based on the initial site it enters the host. Lymph -based infections had clear bottlenecks in the lymphatic tissues that matched the disseminated population. Most likely, the bottleneck occurred because of the phagocyte acting as a Trojan horse. The phagocyte caused the bottleneck by bringing a random sample of the inoculum to the lymph node. Therefore, a bottleneck is a common feature of anthrax infections regardless of how the bacteria disseminate to the lymph node.

Interestingly, the subcutaneous route had the most clones disseminate, but required the smallest inoculum to cause disease. In our lab, the LD_{50} of subcutaneous infections were approximately 50-fold lower than inhalational infection. Yet, subcutaneous infection had significantly more clones in the disseminated population than mice with NALT-based infections. It is tempting to conclude that bottlenecks are due to host factors that limit the spread of bacteria; however, the subcutaneous infection has the greatest amount of clones and the most bottlenecks. This seems to imply the stringency of a bottleneck has a greater effect on reducing population than several weak bottlenecks. This could also be due to the amount of bacteria that are present in the respective portal of entry. A subcutaneous infection is able to have 10-fold more spores delivered to the portal of entry an intranasally delivered infection. Alternatively, there may be factors about the bottleneck that are not well understood which contribute to the resistance of dissemination. Therefore, future studies should identify what barriers exist in the cutaneous model and how *B. anthracis* overcomes them.

Future studies should investigate how bacteria interact with the host in these portals of entry to determine what leads to resolution of the infection versus colonization and dissemination. While mice were only analyzed when the bacteria disseminated from the portal of entry, there were some mice that developed NALT and subcutaneous infections that resolved

in the mouse without dissemination. Thus, it appears that some host factors are capable of controlling infection when they reduce bacterial replication early. Given the high percentage of infections that occur with subcutaneous injections in the ear, a low dose of spores (~1 LD_{50}) could be delivered to mice and candidates for the barrier could be removed or otherwise antagonized to see if they led to an increase in mortality or persistence in the ear. In contrast, lung-based infections were invariably fatal if luminescence was detected in the thoracic cavity. These factors could be due to particular cells being present or from these cells effectively recruiting cells from other locations. Given the depth of tissue, however, there may have been lung infections that were resolved by the host and were thus never detected. *B. anthracis* is capable of germination in the lung, but it is unclear how long the nascent bacilli survive in the lung. It is clear from clonal analysis that only one germination event leads to colonization and dissemination, but it could be possible that other spores germinate and are cleared by the host. It is unknown if such unsuccessful germination events contribute to the lung-based infection. Recent advances in measuring exotoxin concentration could be used on intranasally infected mice to see if lungs contain detectable levels of LF or EF in the absence of luminescence in the lungs or NALT. If exotoxins are present, follow up studies could be performed to see if the lungs are more permeable or if immune cells isolated by bronchial lavage are less bactericidal.

Another interesting area to explore would use clonal analysis to investigate why non-toxigenic, encapsulated strains are capable of dissemination through the mouse, as we described in Chapter 3. The studies described here were conducted with the uncapsulated toxigenic 7702 strain derivatives. It remains to be determined how capsule affects dissemination and if the presence of capsule and exotoxin alter the *in vivo* population dynamics. Currently, it is not know why mice can be infected with capsulated non-toxigenic bacteria and still have a

fatal infection and this aspect has been used as a critique to the murine anthrax model [95, 96]. A further understanding of this difference could either alleviate or further define the mechanism of this model.

Lastly, a future direction should examine whether the bottleneck is beneficial or detrimental to the bacteria. A number of pathogens are known to pass through a bottleneck as they disseminate through the host, but it still remains unclear how this phenomenon affect the bacteria in the long run. Given that *B. anthracis* sporulates after the death of the host and remains in a metabolically and evolutionarily dormant state, the infectious period in the host represents the only moments when most evolutionary processes can occur. This may be beneficial for *B. anthracis,* as most bacterial mutations are biased toward deletions and new genes often arrive by horizontal gene transfer [153]. Interestingly, there is little evidence of gene loss or horizontal gene transfer between *B. anthracis* strains since its evolutionary origin [318, 319]. Therefore, *B. anthracis* may utilize these bottlenecks to maintain genes that are necessary for survival in the host. This could be tested by passaging one population of *B. anthracis* through a mouse, letting the bacilli sporulate, and then infecting a new mouse. Simultaneously, another population of *B. anthracis* would be passaged in rich media *in vitro*, allowed to sporulate and then allowed to germinate and grow in fresh media. These cycles would be repeated several times and then the two populations would be tested to see if there was any decrease in virulence between the populations.

The role of lethal factor in colonization and dissemination in a subcutaneous model of infection.

After it was found that the location and extent of the dissemination bottlenecks varied depending on the portal of entry, we sought to identify virulence factors that were necessary for

passage through the bottleneck. As there are several immune cells present in the ear dermis and the immune cells may not be the direct cause of the bottleneck, it was decided to focus on a bacterially produced factor that might have a role in passing through the bottleneck. Concurrent studies in our lab indicated that a component of the exotoxin, lethal factor, reduced neutrophil chemotaxis and their bacteriocidal activity [184]. Yet, it remained undetermined how much lethal factor was necessary for the bacteria to colonize or disseminate in the host. If the bacilli were able to colonize and/or disseminate with a large reduction of lethal factor, it would suggest that local intoxication occurred quickly and there is a short time for the host to clear the infection. Conversely, if the bacilli were not able to colonize and/or disseminate with a small reduction of lethal factor, it suggest that the host response is almost able to clear the response and that lethal factor disables the immune response at higher concentrations.

Our studies found that lethal factor deficient strains (ΔLF) were able to disseminate through the host when at least 40% of the inoculum contained LF^+ strains. Decreasing the amount of LF^+ strains to 10% of the population, however, resulted in increased host survival and decreased bacterial dissemination. Furthermore, the 10% LF^+ population led to the ΔLF clones being less competitive than the wild type and fewer clones disseminating. This suggests that at least 10% of the population needed to produce LF in order for dissemination to occur. Inocula in which only 0.3% of the population was LF^+ were not capable of colonizing the ear, whereas the 10% LF^+ population was able to replicate for several days in the ear but had a defect in dissemination. As such, it appears that discrete amounts of LF must be present for colonization and higher levels for dissemination, but the targets for LF remain unknown. Further, by decreasing the amount of LF in a population the entire population becomes less likely to disseminate.

A possibility that remains to be addressed is whether the bacilli are able to survive in the kidney without LF production due to some unique attributes of the kidney. That is, it may be that the ΔLF bacilli are able to survive in the kidney because LF is not needed for survival in that particular location. The kidneys are known to have resident macrophages and dendritic cells and previous models of glomerulonephritis have indicated that lymphocytes and several innate immune cells are capable of infiltrating the kidneys and responding to bacterial infections [320, 321]. Still, experiments should be performed that demonstrate that the presence of LF *in trans* must be present in order for ΔLF strains to survive in the kidneys. One approach to test this is to inject bioluminescent ΔLF vegetative bacilli intravenously into mice and determine if vegetative bacilli colonize and survive in the kidney and, if so, how long the ΔLF bacilli can survive. If the bacteria can survive in the kidneys without LF, it would suggest that some parts of the body are more susceptible to infection than others or that particular exotoxin components are needed in some organs, but not others. When analyzing the role of exotoxin component in contributing to mortality, LF was found to cause toxemia by targeting cardiocytes, while EF toxemia caused death through targeted hepatocytes [128]. Therefore, it is possible that different exotoxin components would target different organs. Alternatively, if the bacilli cannot colonize the kidney, it would suggest that LF intoxication is necessary for survival and growth in the kidney. To test this, one could attempt to intoxicate the mouse with purified LT (LF + PA) prior to injection of ΔLF bacilli intravenously. This assumes that one could reach similar levels of system exotoxin intoxication *in vivo* by injected purified exotoxin. If the bacteria are able to colonize the kidney after LT intoxication, it would suggest that LF intoxication of the kidney is necessary for survival of ΔLF in the kidney. However, if the ΔLF bacteria cannot survive in the kidney after LF intoxication, it would suggest either that the other factors are necessary for ΔLF survival in the kidneys or that injection of purified exotoxin components do

not adequately recapitulate a natural infection. Another approach would be to analyze several other organs in the host, such as the liver, spleen and heart. While one organ may have unique attributes that permit growth without LF, it is unlikely that several organs would also have such attributes. Thus, if these other organ had ΔLF clones present in similar proportions as the kidney, it would suggest that these bacteria can colonize and replicate in the host without LF.

In the mouse model, at least, it seems that the subcutaneous, NALT-based, and lung-based models all disseminate through the Jailbreak model of dissemination [264]. The subcutaneous route has the most evidence for the dissemination with the Jailbreak model. The bacilli are able to germinate without phagocytosis by immune cells, precluding a hallmark of the Trojan horse dissemination models [79]. Spores are found in the draining lymph node early after infection, but debriding the infected portion of the ear prevents infection from disseminating in mice [261]. This suggests that the delivery of spores to the lymph node is not sufficient for a disseminated infection in the subcutaneous route. Lastly, the production of LT in the ear of the mouse led to decreased neutrophil accumulation in the ear [184, 193]. Given the conserved targets in immune cells, it is likely that macrophages are also less able to chemotax toward the infection to the ear when the bacteria are producing LT. As such, there would be fewer phagocytes to deliver the bacilli to the draining lymph node in a subcutaneous infection. Therefore, the subcutaneous route of infection has several lines of evidence that suggest the bacteria do not use the Trojan horse model of dissemination to reach the bloodstream. There are some facets of the model that still need to be investigated. The Jailbreak model also proposes that the bacteria disseminate to the lymphatics due to increasing the permeability of either the epithelial or endothelial layers by the exotoxin or secreted proteases. These studies, however,

have not been performed *in vivo* and it is not known if permeabilization precedes dissemination nor which factors are responsible for increasing permeability.

The NALT and lung-based infections also have several similarities to the Jailbreak model. These infections first occurred outside of the regional lymph node and, in the lung-based infection, did not necessarily need to disseminate through the lymph nodes. It is likely that the exotoxin and proteases also increase permeability in the tissues surrounding the portal of entry [296]. Further work, however, is necessary to confirm that the surrounding epithelial or endothelial tissue is intoxicated or otherwise impaired by bacterial factors. The use of *in trans* complementation of LF^+ produced by a subset of the inoculum was attempted for inhalational infections, however, luminescence was not detected in any mice (data not shown). This may suggest that inhalational infections require higher amounts of LF than subcutaneous infections. Such a hypothesis could be tested by infecting mice intranasally with an inoculum where the ΔLF proportion comprises the minority of the population. Germination is rare in the lung based infection and arises from only a few spores. As such, *in trans* complementation may not occur in lung-based infections. Therefore, any attempt to characterize the Jailbreak model in lung-based infections would have to look at whether the lung epithelial is permeabilized before dissemination and which bacterial factors are required to do so.

A detailed understanding of dissemination from the ear should be the first area of future studies from this work. Inocula where only 10% of the population produced LF survived in the ear several days longer than the 0.3% LF^+ inoculum. The 10% LF^+ inoculum, however, does not disseminate as well as the 40% LF^+ inoculum, which is equally virulent to the wild-type strain. Therefore, the 40% LF^+, 10% LF^+, and 0.3% LF^+ inocula could be used to further understand how the bacilli disseminate from the ear dermis and which host barriers are effective at preventing

dissemination. LF is known to prevent immune cell accumulation at the ear and increases the permeability of endothelial layers. Thus it should be investigated whether the LF produced by these inocula increase permeability and prevent immune cell accumulation. Histological and immunofluorescent analysis of the ear dermis could be used to determine if there are differences in the number and composition of immune cells in the dermis. If mice infected with the 10% LF^+ inoculum have the same proportion of immune cells as mice infected with the 0.3% LF^+ inoculum, it would suggest that the immune cells are able to accumulate, but have a defect in bactericidal activity. Alternatively, it is possible that there would be fewer immune cells in the dermis of mice infected with the 10% LF^+ inoculum. It has been suggested that local concentrations of lethal toxin (LF + PA) are more important to prevent neutrophil accumulation than systemic LT levels [184]. Therefore, a decreased amount or altered proportions of immune cells in the dermis would suggest the 10% LF^+ inoculum is able to prevent immune cell accumulation and likely interferes with the bactericidal activities of the resident immune cells.

Precisely how the bacilli disseminate from the dermis to the cervical lymph node is still not understood well. One possibility is that the bacteria are able to increase the permeability of the endothelium [252, 254, 256]. This would possibly allow the bacilli to more easily access the lymphatic capillaries and disseminate to the draining lymph node. Several fluorescent dyes are available for the measurement of endothelial permeability, such as TRITC-labeled dextran and Evan's Blue. If permeability is increased, as measured by the amount of dye accumulated in the tissue, it would suggest that LF is also increasing endothelial permeability. Therefore, one could compare the amount of permeability between the 40% LF^+ inoculum, the 10% LF^+ inoculum, and the 0.3% LF^+ inoculum. If the 0.3% LF^+ and 10% LF^+ has decreased permeability compared to the 40% LF^+ inoculum, it would suggest that the defect in dissemination is due to the

decreased permeability. Alternatively, if the permeability is similar between the 40% LF^+ inoculum and the 10% LF^+ inoculum, it may suggest that the amount of systemic exotoxin is important for survival in the lymph node early in dissemination. As such, these experiments would lead to novel insights into how *B. anthracis* disseminates from the dermis to the lymphatics and the role of LF in these steps.

While the majority of the experiments described have focused on a phagocyte-independent route of dissemination, phagocyte mediated dissemination could play a role and should be investigated. An *in vitro* infection of macrophages with a library of LF^+ and ΔLF clones could provide insight on phagocyte mediated dissemination. Bacterial clearance by the macrophage is dependent in part to the number of spores phagocytosed by the macrophage [75]. While *in trans* complementation of LF is possible in the ear, it is not known if a population of bacilli can complement LF within a macrophage or the efficiency of intracellular *in trans* complementation. It is quite possible that *in trans* complementation of LF is not as effective at preventing bacterial killing in a macrophage compared to subcutaneous infections and therefore there would be a greater amount of bacterial killing, reducing the chance of dissemination. Rabbits that are infected by bronchoscopy with a fully virulent strain are thought to have dissemination occur by the Trojan horse model. Necropsy of infected rabbits found vegetative bacilli in the lung-associated lymph nodes, but not in the lumen of the lungs [95]. When rabbits were infected with a mixed inoculum of fully virulent and ΔPA spores, there was a 3 $\log_{10}$ decrease in the bacterial burden of fully virulent clones in the lung associated lymph nodes [95]. In contrast, our mice models showed that infections initiated outside of lymph nodes in most infections and likely did not migrate to the lymph nodes using a phagocyte. This may account

for differences between our data and previous publications and would suggest that the Trojan horse model occurs in certain animal models.

What still remain unclear are the roles of the other exotoxin components. Studies using mixed infections in an inhalational rabbit model found that ΔPA mutants were less capable of dissemination to the regional lymph nodes compared to the fully toxigenic wild type strain. This could be due to differences in the host model, infection route, or exotoxin component. As for the latter, there could be distinct requirements for an exotoxin component production by an individual bacterium. That is, it may be absolutely necessary for the bacilli to produce PA, but less so for LF or EF. Why this may occur is currently unknown as few studies have been able to analyze exotoxin levels at the early stages when their concentrations are low [184]. Further examination of these differences may lead to a greater understanding of the role intoxication plays in the development of the infection.

Decreasing LF in a population also revealed that a genetic deletion of the EA1 protein in ΔLF strains made them less competitive relative to its $EA1^+$ ΔLF strain. This was surprising since this was not observed when mice were infected with the 100% or 40% LF^+ library. As such, the exotoxin may mask slight defects in survival. Several proteins on the spore, such as BclA, or on the vegetative bacilli, such as ALO or phospholipases, have been indicated as having some role in virulence, but clear phenotypes have been difficult to obtain. Although these targets might not be developed independently in to therapeutic targets, it could identify gene products that are necessary for metabolism, germination, or dissemination.

Overall conclusions

Recent use of bioluminescent strains identified novel portals of entry for *B. anthracis*, suggesting the Trojan horse model may not adequately describe dissemination. Therefore,

research was necessary to delineate how *B. anthracis* disseminates from these sites and more closely understand whether there are host restrictions to dissemination. A restriction to dissemination would be indicated by a severe decrease in the bacterial population during colonization or migrating to another site. A library of equally virulent signature tagged strains was used to identify bottlenecks, since a dramatic decrease in the population would result a decreased number of clones in a tissue. A bottleneck was detected for each route analyzed but was present in unique places of the host. Moreover, the amount of LF produced by an inoculum can determine the amount of clones that are able to disseminate throughout the host. This suggests that each route has different barriers for dissemination and that components of the exotoxin are important for allowing dissemination to occur. Therefore, the work described here demonstrates novel findings regarding *B. anthracis* pathogenesis by investigating population dynamics inside of the host.

Appendix A: Spore adhesion and internalization by epithelial cells.

Introduction

B. anthracis has several proposed mechanisms to disseminate from the lungs. Perhaps the best studied is the Trojan horse model, where spores are internalized by professional phagocytes and delivered to the mediastinal lymph nodes [71, 72, 222]. After engulfment, spores germinate and rapidly produce the exotoxin within the phagocyte [285]. This allows the bacilli to kill the phagocyte and escape in to the lymph node, where the bacilli continue to replicate and disseminate through the host [95, 272]. However, dissemination by subverting phagocytes appears to be a difficult event as macrophages are capable of killing germinated spores and vegetative bacilli [73, 75, 322]. Furthermore, if phagocytes were solely responsible for delivering viable spores to the lymph node, depletion of macrophages should increase host survival. Yet, depletion of macrophages by clodronate liposomes or supplementation of toxin resistant macrophages increases host survival [72, 191, 192]. Other proposed routes of dissemination suggest that spores are taken into mucosal associated lymphoid tissue, where they germinate and disseminate to the regional lymph nodes [81]. These findings occurred in a murine model of anthrax and due to limitations of bioluminescent technology cannot be performed in other animal models. Therefore, it is unknown if similar routes of dissemination also occur in other animal models. Further, *B. anthracis* is capable of germination and dissemination when injected subcutaneously in the ear or in the lung lumen, where there is no clear mucosal associated lymphoid tissue [81, 275]. Therefore, *B. anthracis* may use other routes of dissemination that are less well characterized.

A more recent model of dissemination has been suggested whereby the spores are internalize or transcytose through lung epithelial cells [271, 280]. *In vitro* assays with lung and colonic epithelial cell lines found that spores were capable of adherence and internalization into epithelial cells, as well as transcytosis through epithelial layers [271, 280]. Spores were found to bind to epithelial cells in a concentration dependent manner, suggesting a receptor mediated adherence [60].Furthermore, *in vivo* analysis of mice lungs after intranasal instillation of spores found that within 2 hours of infection spores were both adhered to and internalized into lung epithelial cells. Yet, at the time these studies were commenced, the bacterial and host proteins that mediated adherence and internalization were unknown.

In order to identify gene products that are necessary for adherence and internalization of spores to the epithelial layer, transposon site hybridization (TRaSH) was planned to be used in combination with *in vitro* cell adherence and internalization assays. TRaSH is a negative selection screen that combines transposon mutagenesis with microarray analysis (Figure 22) [323, 324]. Animals are infected with a transposon library and the infection is allowed to progress to either a defined time point or death. Transposon insertions where interrupted genes necessary for survival in the host would have been selected against and thus would no longer be present in the library. The transposon library would then have the genomic regions surrounding the transposon amplified and labeled with a fluorescent dye. A duplicate library that had not passed through an animal would also have the genomic regions amplified and labeled with a different fluorescent dye. This library would represent all clones that were present when the animal was infected. The two labeled cDNA would be combined and hybridized on a microarray chip for analysis. A spot where both fluorescent dyes hybridized

would suggest that the gene was not necessary for the screen, whereas a spot with only the unselected library would suggest the gene is necessary to pass through the screen.

While the development of a transposon library was successful and spores were found to bind to the epithelial cells, the number of cells that adhered and internalized were too low to use transposon site hybridization. As such, the project was terminated and focus was moved to investigate other areas of *B. anthracis* pathogenesis.

Materials and Methods

Bacterial strains and cell cultures

Bacillus anthracis 7702 was kindly provided by Dr. Molly Hughes, Division of Infectious Diseases, University of Virginia (Charlottesville, VA). *Bacillus subtilis* subsp. *subtilis* str. 168 was from the Bacillus Genetic Stock Center (Columbus, OH). *Salmonella typhimurium* SL1344 was a kind gift from Dr. James Casanova, Department of Cell Biology, University of Virginia. *Listeria monocytogenes* and *Listeria innocua* were a kind gift from Dan Portnoy, Department of Molecular and Cellular Biology, University of California, Berkeley. *E. coli* DH5α strains were from Bioline Reagents (Bioline, MA). All strains were cultured in LB broth at 37°C unless otherwise stated. To produce spores, vegetative bacilli were plated on NBY media, supplemented with antibiotics if necessary, and incubated at 30°C for 10 days. Spores were then harvested and separated from vegetative bacilli on an Omnipaq gradient (350 milligrams of iodine/mL) (GE Healthcare, NJ), similar to previously described methods [81, 261, 289]. MDCK and Caco2 cell lines were grown in DMEM + 10% FBS and incubated at 37°C with 10% CO_2. A549 cell lines were grown in F12K media + 10% FBS and incubated at 37°C with 10% CO_2.

Association and internalization of bacteria with cell lines

Cells were harvested when they were 60-70% confluent and seeded into 24 well plates with ~$2x10^5$ cells/well and allowed to incubate overnight. The next day the cells were washed with 1x PBS and fresh media was added. *Salmonella* and *Listeria* were prepared for infection as previously described [325, 326]. *B. anthracis* spores were added with 2.5 mM D-alanine to prevent germination in the media. For the association assay, the cells were lysed with 0.1% saponin after 1 hour incubation at 37°C and the CFU enumerated by serial dilution. For the internalization assay, bacteria were incubated with the cells for 1 hour at 37°C, washed 3 times with 1xPBS, and then fresh media with 2.5 mM L-alanine and 100μg/mL gentamicin was added. The antibiotic containing media was allowed to incubate with the cells for 1 hour at 37°C and then cells were washed 3x with 1x PBS. Finally, cells were lysed with 0.1% saponin and the CFU were enumerated by serial dilution.

Results

B. anthracis has higher percent adherence to epithelial cells than *B. subtilis*, but little internalization.

To determine if *B. anthracis* spores are more capable of adherence to epithelial cells than a non-pathogenic *B. subtilis* strain, spores were incubated with MDCK cells and Caco2 cells and their association were quantified. Association refers to cells that are both adhered to the cell and those that were internalized into the cell. *B. anthracis* had a significantly higher association with both Caco2 cells and MDCK cells compared to *B. subtilis* (Figure 23A). Since the inoculum of bacteria added to the cells was ~10^6 CFU of spores, this resulted in ~10^4 spores adhered and internalized to epithelial cells. While this could be enough for TRaSH, the percent association was approximately a tenth of what was previously reported for epithelial cells [280].

Next the percentage internalization was analyzed by treating cells with gentamicin and L-alanine for one hour. The L-alanine would allow for germination of spores and the gentamicin would only kill extracellular bacteria as it is cell impermeable. As such, only the internalized bacteria could be harvested from these cells. Since *B. subtilis* spores were unable to adhere to epithelial cells, it was hypothesized that the internalization would also be minimal. Therefore, known intracellular bacteria, such as *Listeria monocytogenes* and *Salmonella typhimurium*, were used as positive controls and *E. coli* DH5α and *Listeria innocua* were used as non-invasive controls. All strains were found to be capable of association with MDCK and A549 cells, in particular *S. typhimurium* (Figure 23B). Compared to *S. typhimurium*, *B. anthracis* invaded epithelial cells poorly (Figure 23C). However, there was no significant difference between *E. coli* and *B. anthracis* internalization. Invasion of A549 cells with either *B. anthracis* or *L. monocytogenes* had similar amounts of internalization, resulting in ~10^4 cells internalized (Figure 23D). However, these amounts of bacteria were too low to use a negative selection screen, such as TRaSH and orders of magnitude lower than previous reports [280]. This suggested that in our lab *B. anthracis* is less capable of internalization than pathogens that are known to internalize epithelial cells.

Since the internalized CFU from *B. anthracis* were so low, it was tested if these CFU were from bacteria that were internalized by the cells or if they represented extracellular spores that did not germinate in response to the L-alanine/gentamicin treatment. MDCK cells were treated with either with cytochalasin D, fixed with paraformaldehyde, or left untreated and spores were allowed to adhere and internalize. Cytochalasin D and paraformaldehyde should prevent internalization by inhibiting F-actin polymerization or by fixing the cells, respectively. While *S. typhimurium* had significant decreases when MDCK cells were treated with either cytochalasin

D or paraformaldehyde, no differences were seen between the *B. anthracis* conditions (Figure 24). This suggests that the CFUs recovered after gentamicin treatment were either ungerminated spores or a small amount of bacteria that resisted antibiotic killing.

Discussion

B. anthracis has previously been indicated as being capable of internalization and possibly dissemination from lung epithelial cells [271, 279, 280]. To identify gene products that facilitate adherence and internalization to epithelial cells, we had planned to use TRaSH but first had to confirm the adherence and internalization phenotype. In contrast to previously published data, we could not see similar levels of adherence to epithelial cells or any significant amount of internalization. Moreover, the amounts of spores present on the epithelial cells were too low for TRaSH analysis or other genetic screens. This precluded us from identifying gene products necessary for epithelial uptake.

It is unknown why spores had lower adherence and a near absence of internalization in our lab. While it is possible that differences between handling cell lines contributed to this decrease, *S. typhimurium* was capable of being internalized into our cells. Further, non-invasive bacteria such as *E. coli* and *L. innocua* were not able to be internalized. This suggested that our cells were capable of internalizing intracellular bacteria. Furthermore, while our internalization differed with finding from Russell *et al*., they were similar to Bozue *et al*.'s findings with the fully virulent Ames strain [60, 280]. As such, it is unknown why our findings differ from Russell *et al.*, but they have been replicated by an independent laboratory.

While adherence was decreased from previously published accounts, *B. anthracis* spores were found to be more adherent than non-pathogenic *B. subtilis*. An interesting cause for the increased adherence could be due to the presence of the exosporium. *B. subtilis* is not thought to

have an exosporium, whereas the exosporium on *B. anthracis* has been implicated by some to have a role in virulence [50, 59, 62, 322]. The exosporium, and in particular the protein BclA, has mostly been implicated as preventing adhesion to epithelial cells and signaling for phagocytosis in macrophages [59, 60]. However, BclA was found to adhere to epithelial cells through a complex composed of the C1q complement protein and integrins [62]. Therefore, more research is needed to examine the factors that would enable or prevent spore uptake into the epithelial cells.

Genetic screens, including TRaSH, require a large number of bacteria in order for the screen to identify candidates. If one were to consider that the library contained 1 transposon insertion for every gene in the *B. anthracis* chromosome, it would require at least $5x10^6$ cells being internalized. However, we frequently found internalized bacteria in the 100-1000 order of magnitude. This would have required at least 2000 24 well plates in order to have 1-fold coverage of the genome. As such, it was not feasible to identify gene products that lead to adhesion or internalization by this method.

Figures for Appendix A

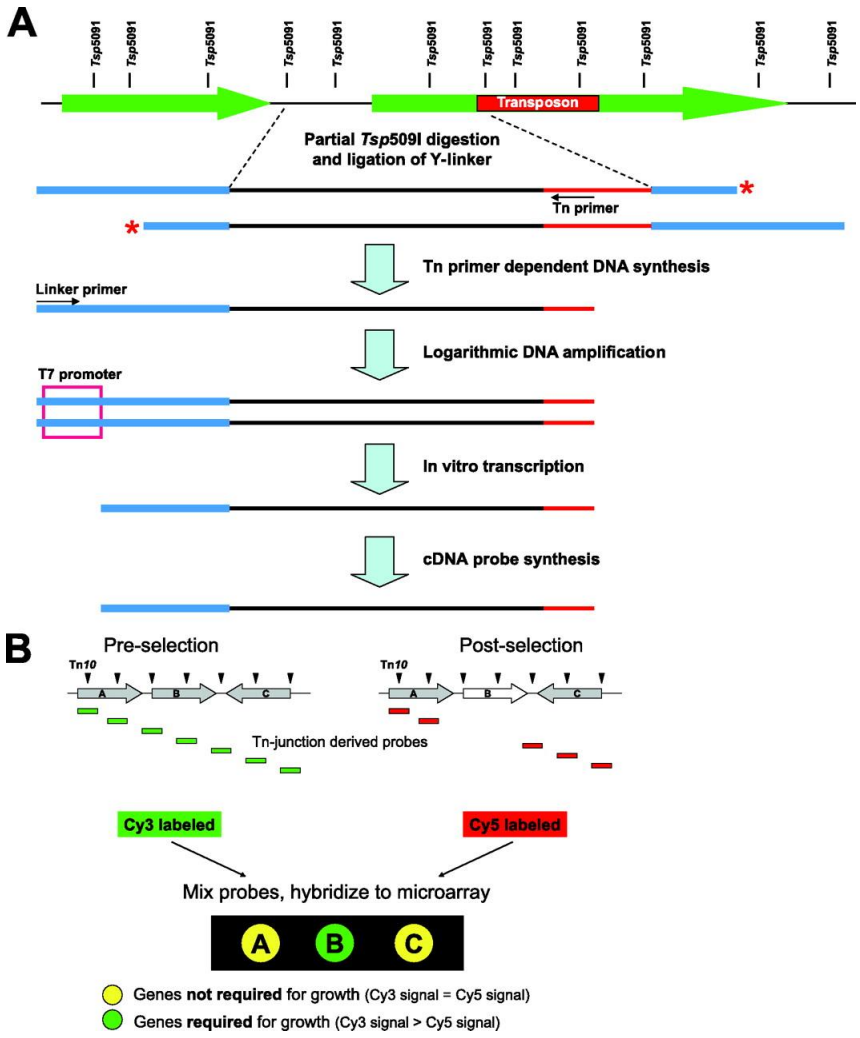

Figure 22. Diagram of transposon site hybridization (TRaSH)

Transposon site hybridization identifies gene products that are required to pass through a selection, such as survival in a host or resistance to an antimicrobial peptide. **(A)**Transposon interruptions are detected by partially digesting the genomes of a library that passed through a condition and one that has not passed through a condition. The regions next to the transposon are then amplified by linker ligation, *in vitro* transcribed, and then converted to cDNA. The cDNA is labeled with fluorescent dyes. **(B)** The fluorescent cDNA is then hybridized to a microarray chip and analyzed. Spots where both fluorescent labels are present suggest that the transposon interruption was not necessary for passing through the selection, whereas the

presence of only the pre-selection labeled dye suggest the gene is required for survival. Diagram from [327] and used with permission.

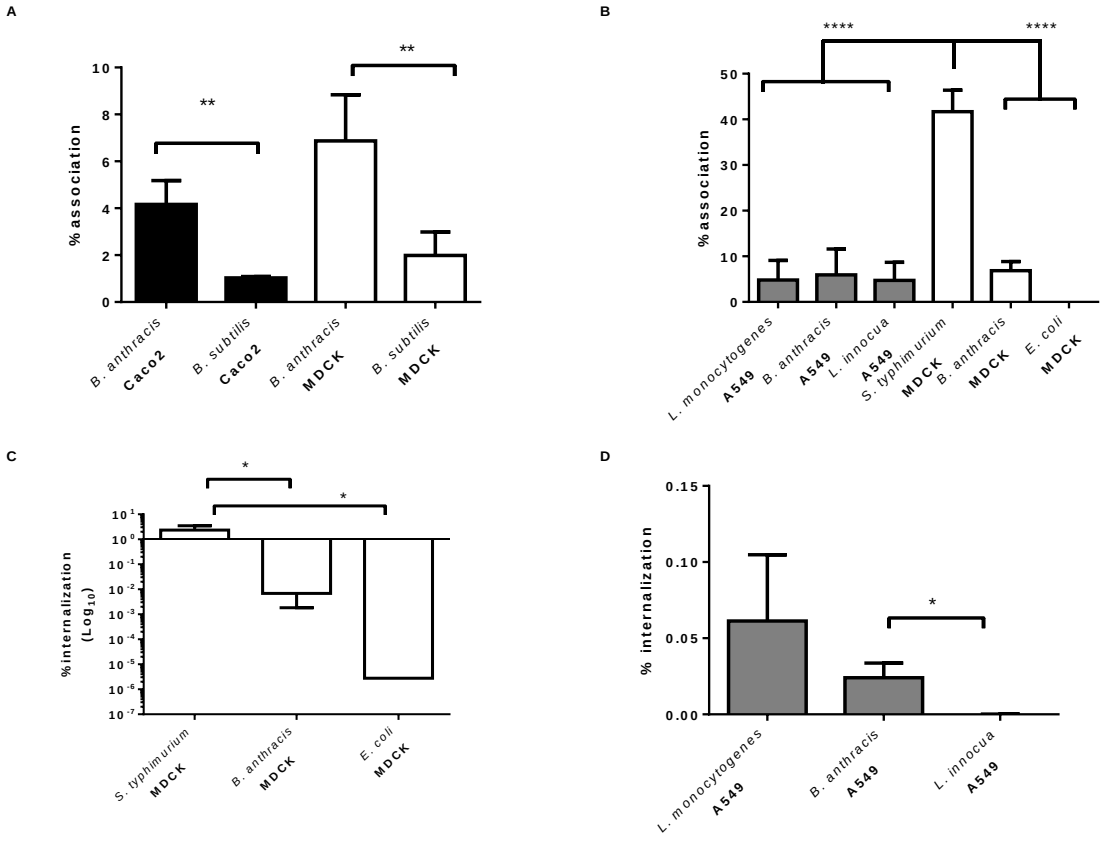

Figure 23. Association and internalization of *B. anthracis* spores on epithelial cell lines.

(A) The percent association of *B. anthracis* or *B. subtilis* spores when incubated with Caco2 or MDCK cells for 1 hour. Experiments were performed in triplicate and repeated twice. Bars represent the standard deviation. Asterisks represent a significant difference (Student's T-test, ** *P*-value <0.01). **(B)** The percent association of *B. anthracis*, *L. monocytogenes,* or *L. innocua* with A549 lung epithelial cells and *S. typhimurium, B. anthracis*, and *E. coli* DH5α with MDCK epithelial cells. Experiments were performed in triplicate and repeated twice. Bars represent the standard deviation. Asterisks represent a significant difference (One way ANOVA, ****P-value <0.0001). **(C)** The percent internalization of *S. typhimurium, B. anthracis*, and *E. coli* DH5α with MDCK epithelial cells. Experiments were performed in triplicate and repeated twice. Bars represent the standard deviation. Asterisks represent a significant difference (Student's T-test, * *P*-value <0.05). **(D)** The percent internalization of *B. anthracis*, *L. monocytogenes,* or *L. innocua* with A549 lung epithelial cells. Experiments were performed in triplicate and repeated twice. Bars represent the standard deviation. Asterisks represent a significant difference (Student's T-test, * *P*-value <0.05).

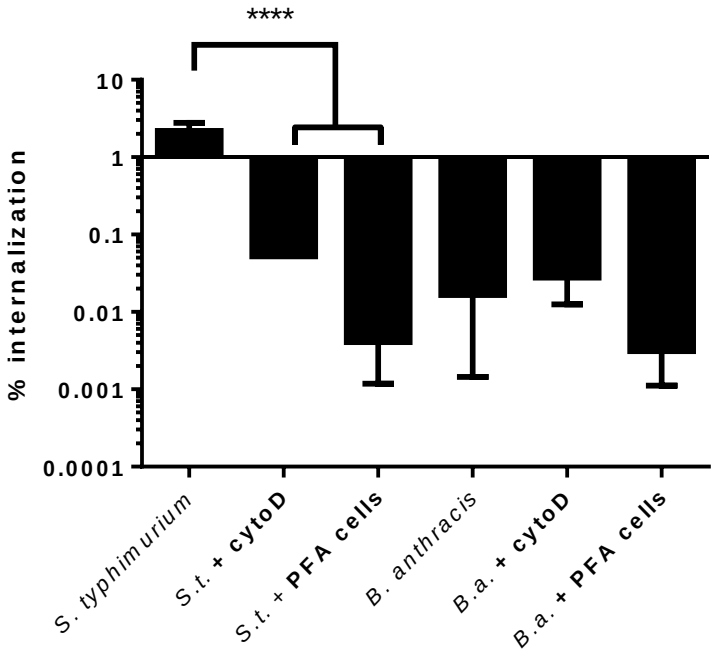

Figure 24. The percent internalization does not decrease for *B. anthracis* when cells are treated with cytochalasin D or 4% paraformaldehyde.

MDCK cells were either treated with 5 µg/ml cytochalasin D (cytoD) or 4% paraformaldehyde (PFA) for 30 minutes and *S. typhimurium* or *B. anthracis* spores were added. Experiments were performed two independent times in triplicate. Bars represent the standard deviation. Asterisks represent a significant difference (One-way ANOVA with Sidak's Multiple comparison test, ****P-value <0.0001).

Appendix B: The role of an extracellular adenosine monophosphatase in *B. anthracis* pathogenesis.

Introduction

B. anthracis has several minor virulence factors that are thought to contribute to infection in addition to the exotoxin and capsule. These include proteases and cholesterol dependent cytolysins, which degrade host proteins or lyse immune cells, as well as other factors that reduce the inflammatory response to the bacteria [105-114, 118-120]. One such immune modulating protein is the extracellular adenosine synthase A (AdsA) [121]. AdsA acts as an ecto-nucleotidase which dephosphorylate 5'-AMP to produce adenosine [121]. Extracellular adenosine is used to regulate several physiological roles in the host, including both inflammatory and anti-inflammatory processes [328]. This gives the host several mechanisms to strictly regulate the amount of extracellular adenosine [328]. Adenosine modulates the inflammatory response by signaling through four adenosine receptors, which are G-coupled protein receptors on the host membrane, that either inhibit or stimulate cyclic AMP production in the cell (Figure 25) [328]. Two of the receptors, A_1 and A_3, are inhibitory receptors that prevent the production of cAMP and thereby increase inflammation [328, 329]. The other two receptors, A_{2A} and A_{2B}, are stimulatory receptors that increase cAMP and reduce inflammation [328, 329]. The signaling is further regulated due to the differences in affinity for the adenosine receptors. Half of the receptors, A_1 and A_{2A}, are high affinity and can signal with very low concentrations of adenosine and the other receptors, A_{2B} and A_3, have low affinity and require higher levels of adenosine. This complicated mechanism of adenosine signaling allows the host to rapidly respond to the changing environment in order to initiate or resolve inflammation.

Several Gram-positive pathogens have a homolog of AdsA which is attached to peptidoglycan by Sortase A [121]. The majority of research has investigated how *Staphylococcus aureus* uses AdsA to reduce the host immune response and cause disease. *S. aureus* strains that lack AdsA did not survive as well in whole blood or produce as severe abscesses in mouse kidneys compared to its wild-type counterpart [121]. Further, while *S. aureus* was phagocytosed by neutrophils, the presence of AdsA allowed the bacteria to survive within the neutrophil [121]. In addition to the experiments with *S. aureus,* the AdsA homolog in *B. anthracis* was also found to have decreased survival in whole blood compared to its parental. While AdsA confers a survival advantage for *B. anthracis* in *ex vivo* experiments, it is not known if this also occurs *in vivo*. Further, adenosine signaling to stimulating receptors leads to an increase in cAMP at the membrane, whereas edema toxin is known to increase cAMP concentrations perinuclearly [197, 328]. Since cAMP production can have distinct effects based on its cellular location, adenosine stimulated cAMP may have different effects than edema toxin stimulated cAMP [197, 330].

To investigate the role of AdsA in *B. anthracis* pathogenesis and dissemination, an AdsA mutant was constructed in the 7702 and BIG23 *B. anthracis* background. Infection of mice led to conflicting results depending on the route of infection. Subcutaneous infections had a 5-fold higher LD_{50} suggesting that the Δ*adsA* mutant was less able to survive compared to wild type. In contrast, intranasal infections had a 6-fold lower LD_{50} suggesting the Δ*adsA* mutant was more virulent than the parental. There were no differences between the bacterial burden of the parental or mutant strains at death with the luminescent BIG23 strain. This suggests that the effect of AdsA occurs at the early moments of infection. Due to the inability to find a consistent phenotype, it was decided that the project should not be continued.

Materials and Methods

Bacterial strains and Animals

Bacillus anthracis 7702 (pX01^{+}, pX02^{-}) was a kind gift from Dr. Molly Hughes, Division of Infectious Diseases, University of Virginia (Charlottesville, VA). BIG23, a luminescent derivative of 7702, was a kind gift from Michele Mock and was constructed by integrating the pIG6-19 plasmid in to the pX01 plasmid. Spores were produced by plating an aliquot of vegetative bacilli on NBY agar and left to incubate for 10 days at 30°C. After 10 days, the bacteria were removed from the plate and the spores were purified by passing through an Omnipaq gradient (350 milligrams of iodine/mL), similar to previously described methods [81, 261, 289]. The *E.coli* strains used were either α-select (Bioline, MA) or *dcm, dam*-deficient GM119 (*dcm-6, dam-3)* [290]. All strains were grown in either BHI or LB broth with appropriate antibiotics when necessary. All strains and plasmids, except for Δ*adsA* 7702 and Δ*adsA* BIG23 are outlined in Table 1.

Mice manipulations and husbandry were performed under the precedents set forth in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments, housing, and husbandry was approved by the University of Virginia Animal Care and Use committee (protocol #3671) and were designed to minimize distress for the animals. BALB/c and C57BL/6J mice were obtained from Jackson Labs (Jackson Lab, ME).

Construction of the Δ*adsA B. anthracis* strains.

The Δ*adsA* strain was designed by using allelic exchange to introduce a markerless deletion of *adsA* into the *B. anthracis* chromosome. A markerless deletion allele was constructed by splice by overlap PCR (SOE-PCR) of two homologous regions which deleted 98% of the coding region of *adsA* (BAS4031). The upstream region or homology was amplified by FDL21 (5'- TAAACTAGTACCCGCAGTTCCAACATTAC-3') and AdsA SOE Rev (5'-

AAAATGCTGGCTGGAGCAATTGTCGACAAAGCAAATAGATAATCGAGA-3'). FDL21 has a SpeI restriction site that is underlined and the AdsA SOE Rev primer has a SalI site underlined. The downstream region of homology was amplified using RDL20 (5'-TTTGAGCTCTCCGCTTCAAAATGAGCTTT-3') and AdsA SOE Fwd (5'-TCTCGATTATCTATTTGCTTTGTCGACAATTGCTCCAGCCAGCATTTT -3'). RDL21 has a SacI site that is underlined and AdsA SOE Fwd has a SalI site that is underlined. Due to the difficulties of splicing together the two SOE homology regions via PCR, the homology regions were independently subcloned into the pGEM-T easy vector. The upstream homology region and the allelic shuttle vector pHY304 were digested with SpeI and SalI and ligated with T4 DNA ligase (NEB, MA). The vector was then transformed into α-select *E. coli* cells and colonies with successful transformants were identified by PCR. The vector was the isolated by miniprep and, along with the downstream homology region, digested with SalI and SacI and ligated using T4 DNA ligase. Transformants were then analyzed by PCR to confirm that the region contained the Δ*adsA* allele and then further confirmed by DNA sequencing.

The Δ*adsA*/pHY304 vector was then transformed in GM119 to produce an unmethylated vector and purified by miniprep. The unmethylated vector was then transformed into *B. anthracis* 7702 and incubated overnight at 30°C on BHI plates supplemented with 5 μg/mL erythromycin (Erm). Transformants were screened by PCR to confirm the Δ*adsA* allele and then grown at 41°C overnight twice to select for bacteria where Δ*adsA*/pHY304 had crossed into the chromosome by homologous recombination. The bacteria were passaged at 37°C until bacteria loss Erm resistance indicating that the allelic exchange occurred. Erm sensitive mutants were then analyzed by PCR to determine if they had the Δ*adsA* allele. Finally, pIG6-19 was conjugated into Δ*adsA* 7702 to contruct a luminescent strain, as previously described [293].

Blood survival assays with *B. anthracis* strains

Mice were anesthetized with 3% (v/v%) Isoflurane-oxygen mix using a Isotec 5 vaporizer (Absolute Anesthesia). Unconscious mice were exsanguinated using a 1-mL syringe and 26.5G needle that contained heparin sulfate. Blood from multiple mice was pooled and 100µL of *B. anthracis* was added to 900µL of blood. The blood samples were incubated at 37°C with slow shaking for one hour. At defined time points, 100µL of the bacteria-blood mixture would be withdrawn and serially diluted on to BHI plates to enumerate CFU.

Determination of LD_{50} for *B. anthracis* and Δ*adsA B. anthracis*.

To determine the LD_{50} for subcutaneous and inhalational infections, 4 groups of 4 mice were infected Δ*adsA* 7702, Δ*adsA* BIG23, 7702, or BIG23 spores. Intranasal infections were performed by anesthetizing mice with methoxyflurane (metafane, Matrix Scientific, SC) nose and then spores were delivered by intranasal instillation as previously described [81]. Subcutaneous infections were performed by subcutaneous injection in the ears as previously described [81, 264]. Mice were anesthetized with a 3% (v/v%) Isoflurane-oxygen mix using a Isotec 5 vaporizer (Absolute Anesthesia) and 10 µL of spores in PBS were injected into the ear pinna via a 0.5cc syringe. Mice were then observed and any survivors were euthanized on the tenth day. LD_{50} were calculated as previously described [331].

Statistical analysis

Student T-Test and Log Rank test were performed by Graphpad Prism version 6.03 (Graphpad Software, CA).

Results

Δ*adsA* mutants do not have a clear defect in whole blood survival with either C57BL/6J or BALB/c mouse blood.

Previous research indicated that Δ*adsA* 7702 strains have a survival defect when incubated in whole rat blood for one hour [121]. After the Δ*adsA* strain was constructed in our lab, it was tested to see if incubation in mouse blood gave similar results. Both C57BL/6J and BALB/c blood was isolated and either the Δ*adsA* strain or 7702 strain were incubated in whole blood for one hour. There were no significant differences between the Δ*adsA* and 7702 strain for either blood (Figure 26). Since there was no difference in survival between strains when incubated in whole blood, it was tested if there were defects *in vivo*.

B. anthracis strains that lack AdsA are less virulent in a subcutaneous route, but more lethal in an inhalational route.

AdsA was found to have decreased virulence in *ex vivo* whole blood survival assays. To test if AdsA also has less virulence *in vivo,* mice were infected with Δ*adsA* 7702 and the parental 7702 intranasally. Mice infected intranasally with the Δ*adsA* 7702 strain had a 6-fold lower LD_{50} compared to the wildtype 7702 strain (LD_{50} for Δ*adsA* = $5.4x10^5$ CFU, LD_{50} for 7702 = $3.2x10^6$; Figure 27A and Figure 27B). Of these doses, the Δ*adsA* strain had a significantly higher survival than the wildtype only at the 10^7 CFU spore dose (Figure 27C). This suggests that in an inhalational infection, the lack of AdsA made the bacteria more virulent than wildtype. Additionally mice were infected subcutaneously with Δ*adsA* BIG23 and parental BIG23 strain. In contrast to inhalational infections, a subcutaneous infection using the luminescent BIG23 strain found that Δ*adsA* BIG23 had a 5-fold higher LD_{50} than the wildtype BIG23 strain (LD_{50} for

Δ*adsA* = 3.5x10^5 CFU, LD_{50} for 7702 = 7x10^4; Figure 28). Thus, while the inhalational infection showed Δ*adsA* strains were more virulent, subcutaneous infections appeared to be less virulent. Finally, to see if the presence AdsA lead to higher bacterial burdens compared to the Δ*adsA* mutant at the time of death in mice, bacterial burdens in the spleen and kidneys were compared. There was no difference between the wild type and Δ*adsA* mutant in either the spleen or the kidney (Figure 29).

Discussion

The Δ*adsA* knockout strain in our hands had a complex phenotype that was difficult to analyze. In contrast to previous studies, the Δ*adsA* strain did not have decreased survival in whole blood compared to the parental strain. Survival curves from intranasal and subcutaneous infections were contradictory, with intranasal infections finding that the Δ*adsA* mutant was more virulent than the 7702 strain and subcutaneous infections finding that Δ*adsA* was less virulent than wildtype. Since there was difficulty determining if Δ*adsA* was more or less virulent than its parental counterpart and an *in vitro* assay that showed decreased survival could not be developed, the project was discontinued.

Previous studies with AdsA in the 7702 strain found a decrease in survival when bacteria were incubated in Sprague-Dawley rat blood for one hour [121]. *S. aureus* strains that lacked AdsA also had a survival defect when incubated in BALB/c blood, but defect was not detected with *B. anthracis* strains [121]. Further, the survival defect could not be detected in C57BL/6J strains either. Differences between our findings and previous publications may be due to the amount of bacteria added to the blood as the CFU dose was not listed in the previously published materials and methods [121]. Although the deletion of *adsA* removed the catalytic domain from the chromosome, the amount of adenosine produced by either the

wildtype or Δ*adsA* strain was not measured. As such, it is possible that our strain of *B. anthracis* was not properly induced to produce adenosine in blood.

When the mice were infected with the Δ*adsA* mutant, the virulence either increased or decreased depending on if it was an inhalation or subcutaneous infection. Subcutaneous infections had a 5-fold lower LD_{50} compared to the wildtype BIG23 strain. Inhalational infections, however, had a 6-fold increased LD_{50} compared to the 7702 strain. The latter finding is troublesome as it could be viewed as having dual use potential. Dual use data is considered data that has the potential for both the treatment and detection of select agents as well as the potential for misuse by enhancing the virulence of the pathogen [332]. The differential virulence defect, however, does raise interesting questions about why AdsA has a virulence defect in subcutaneous infections, but not in inhalational infections. One could speculate that the difference is due to the concentration of adenosine that AdsA can produce in these sites. Clonal analysis has shown subcutaneous injection of spores in the ear leads to a high level of germination in the ear, which would lead to a great number of bacilli that can cleave 5'-AMP. This would theoretically lead to a high level of adenosine with in the area around the injection site, which would decrease the inflammatory response. In contrast, intranasal infections would have a smaller amount of bacilli in the NALT (as discussed in section 4.1) or have only a few spores germinant in the lungs. This would theoretically result in lower concentrations of adenosine that could lead to increased inflammatory responses with the $AdsA^+$ strains. Thus, the Δ*adsA* strain could multiply with less inflammation than the wildtype. However, one must first have a firm understanding of which immune cells respond to the germinated bacilli in these sites and be able to analyze relative adenosine concentrations in these sites before such a hypothesis could be tested. Given the challenges that answering this hypothesis would have

required, it was decided to forgo this project in order to understand how *B. anthracis* disseminates from different portals of entry.

Figures from Appendix B

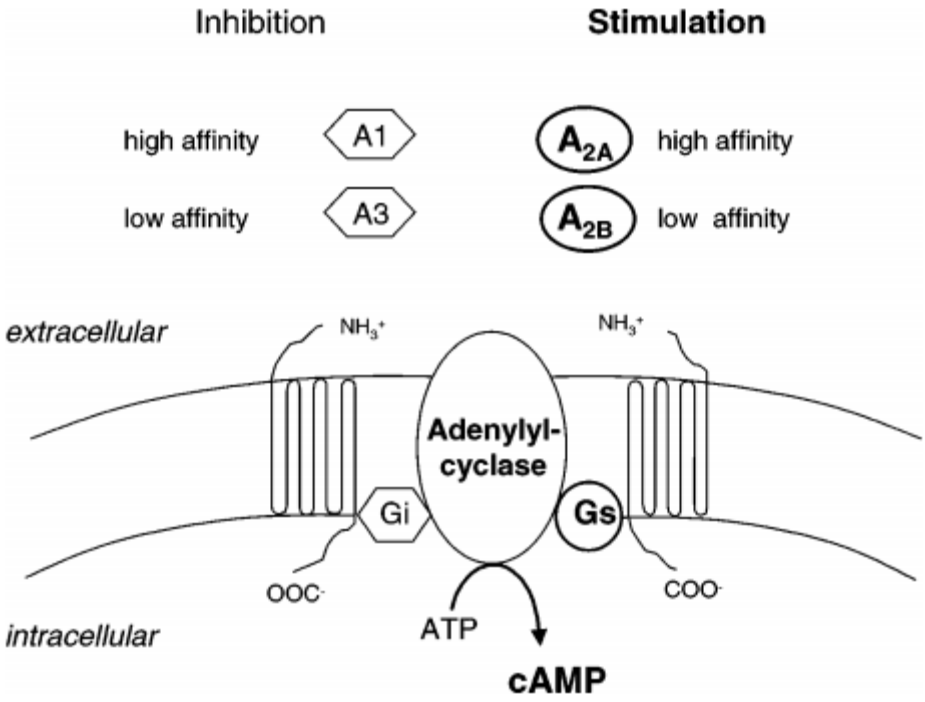

Figure 25. Adenosine signaling occurs through four receptors regulate inflammation through cAMP production.

Extracellular adenosine can signal through four membrane bound G coupled protein receptors. These membrane receptors either stimulate cAMP production and reduce inflammation or inhibit cAMP and promote inflammation. Additionally, these receptors have differential affinities which signal based on the amount of extracellular adenosine in the local environment. Image from [328] and used with permission.

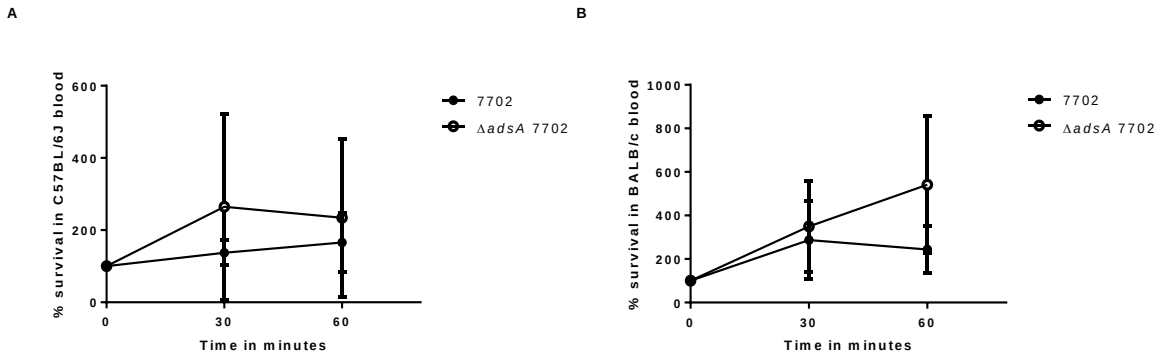

Figure 26. Δ*adsA* 7702 has no significant difference from the parental strain for survival in blood.

(A) Percent survival of 7702 and Δ*adsA* in whole C57BL/6J mouse blood for one hour. C57BL6/J blood had either ~2x10^6 CFU 7702 added or between either 5x10^5 or 2.8x10^6 CFU Δ*adsA* 7702 added. Closed circles represent the mean percent survival of 7702 bacteria and open circles represent the mean percent survival of the Δ*adsA* 7702 strain. Bars represent the standard deviation. Experiments were repeated two independent times in triplicate. (B) Percent survival of 7702 and Δ*adsA* in whole BALB/c mouse blood for one hour. BALB/c blood had 2x10^6 to 5x10^6 CFU of either 7702 or Δ*adsA* 7702 added. Closed circles represent the mean percent survival of 7702 bacteria and open circles represent the mean percent survival of the Δ*adsA* 7702 strain. Bars represent the standard deviation. Experiments were repeated three independent times in triplicate.

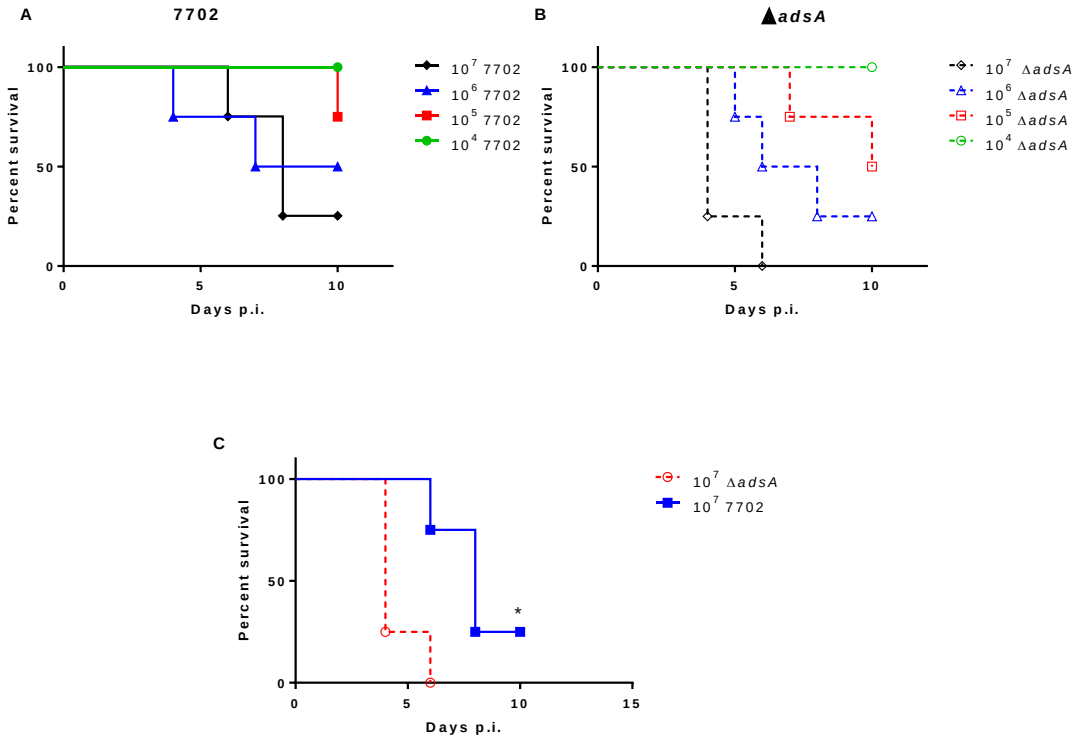

Figure 27. Δ*adsA* is more virulent in intranasal models than 7702.

(A) Kaplan Meier survival curve of an intranasal 7702 infection comparing the percent survival over 10 days. Mice (n=4 for each dose, N=16) were infected with Log_{10} dilutions via intranasal instillation. (B) Kaplan Meier survival curve of an intranasal Δ*adsA* 7702 infection comparing the percent survival over 10 days. Mice (n=4 for each dose, N=16) were infected with Log_{10} dilutions via intranasal instillation. (C) Comparison of the 10^7 dose between 7702 and Δ*adsA* strains. Asterisk represents a significant difference (Log-rank test; *P*-value <0.05).

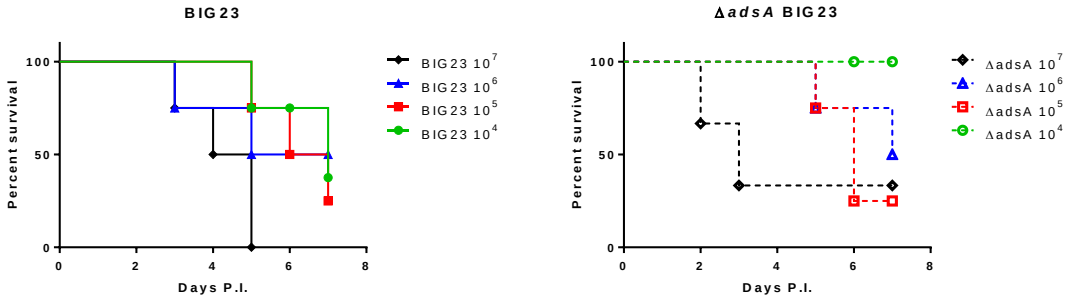

Figure 28. Survival curve of mice infected subcutaneously with Δ*adsA* BIG23 and BIG23. (A) Kaplan Meier survival curve of a subcutaneous BIG23 infection comparing the percent survival over 10 days. Mice (n=4 for each dose, N=16) were infected with Log_{10} dilutions via subcutaneous injection in the ear pinna. (B) Kaplan Meier survival curve of a subcutaneous Δ*adsA* BIG23 infection comparing the percent survival over 10 days. Mice (n=4 for each dose, N=16) were infected with Log_{10} dilutions via subcutaneous injection in the ear pinna.

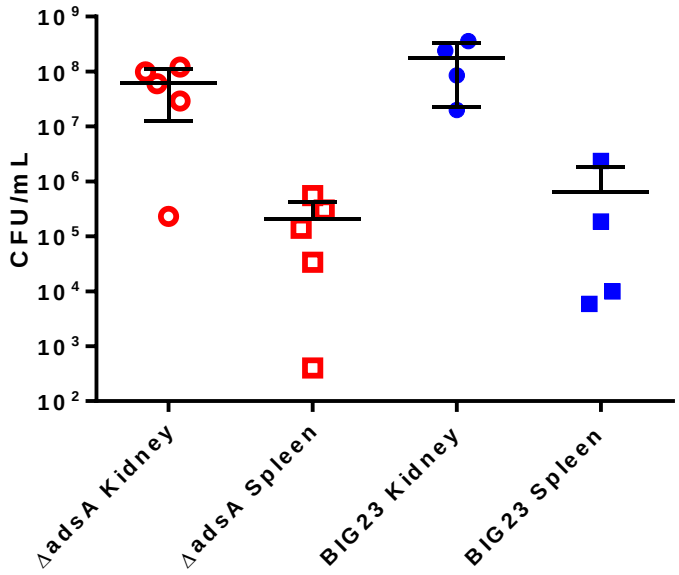

Figure 29. Bacterial burdens do not differ between Δ*adsA* and BIG23 strains.

Bacterial burdens were compared between the Δ*adsA* BIG23 and wildtype BIG23 strains in the spleens and kidneys of mice after death. Δ*adsA* BIG23 strains are represented by red open circles and squares (n = 5). Wildtype BIG23 strains are represented by blue closed circles and squares (n = 4). Bars represent the mean and error bars represent the standard deviation.

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