

THE ROLE OF TOXINS IN PATHOGENESIS OF THE ANTHRAX-LIKE

BACILLUS CEREUS STRAIN G9241

by

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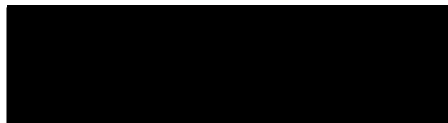
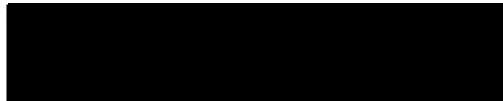
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DEDICATION

For my parents,

Спасибо за Вашу поддержку и любовь,

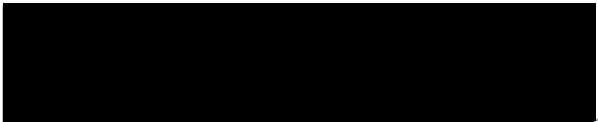
и за Вашу веру в меня.

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February 6, 2018

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ABSTRACT

Title of Dissertation:

The Role of Toxins in Pathogenesis of the Anthrax-Like *Bacillus cereus* Strain G9241

Yuliya Seldina, Doctor of Philosophy, 2017

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Bacillus cereus G9241 was the etiologic agent of a severe pulmonary infection in which the disease presentation resembled that of inhalational anthrax, an illness that is usually caused by *B. anthracis*. Like *B. anthracis*, G9241 carries two large virulence plasmids, designated pBCXO1 and pBC210. The pBCXO1 plasmid is nearly identical to the pXO1 virulence plasmid of *B. anthracis* and carries genes for production of the anthrax toxin components. These genes, *pagA* (*pagA1*), *lef*, and *cya* encode protective antigen (PA), lethal factor (LF) and edema factor (EF), respectively. The pBC210 plasmid carries a homolog of PA, called PA2 (*pagA2*) and a gene (*cer*) for a novel ADP-ribosyltransferase toxin, Certhrax. Recent studies showed that Certhrax post-translationally modifies human vinculin in HeLa cells and, similar to LF and EF, requires PA for entry into cells. Here, experiments were carried out to determine the role that the anthrax toxins play in pathogenesis of G9241 and whether PA2 and Certhrax contribute to virulence as well.

For this purpose, a series of isogenic markerless deletion mutants of the toxin gene components were constructed through allelic exchange and then were assessed for virulence in subcutaneous (s.c.) and inhalational (i.n.) models of infection in A/J and C57BL/6 mice.

Our results indicate that PA and LF are essential for full virulence of *B. cereus* G9241. Additionally, we observed a slight attenuation in virulence in the $\Delta pagA2$ mutant following s.c. challenge of mice, a finding that suggests that PA2 contributes, albeit minimally, to pathogenesis of G9241. We also found that PA2 cannot functionally replace PA1 as the primary binding subunit of anthrax toxin. Surprisingly, we showed that Certhrax is an anti-virulence factor of G9241; *i.e.* a $\Delta cer\Delta lef$ mutant was more virulent than a Δlef mutant following s.c. inoculation of A/J mice. Moreover, the enzymatic activity of Certhrax was responsible for this phenotype. Restoration of *cer* in the $\Delta cer\Delta lef$ mutant attenuated the virulence to Δlef levels, while an enzymatically inactive Certhrax did not. Furthermore, we showed that Certhrax elicits its effects early in infection.

Collectively, we show that toxins can both negatively and positively affect virulence of *B. cereus* G9241.

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CHAPTER 1: Introduction

PUBLIC HEALTH SIGNIFICANCE OF *B. CEREUS SENSU LATO*

The *Bacillus cereus sensu lato* is a group of Gram positive, soil-dwelling bacteria that are ubiquitous in the environment throughout the world. These rod-shaped, facultative anaerobes are endospore formers, a characteristic that permits them to persist in the soil for long periods of time. In the presence of a favorable environment, such as increased temperature and improved nutrient availability within a host, the enzymatically inactive endospore germinates into a vegetative form that can replicate and produce virulence factors to cause disease.

The best known members of this genus are *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringensis*. *Bacillus thuringensis* is an insect pathogen that is often used as a pesticide in agriculture. In fact, it is one of the most widely applied biopesticides worldwide, as it is safe for humans and does not persist in the environment for extended periods of time. Additionally, the pesticidal toxins of *B. thuringensis* have been used to engineer insect-resistant transgenic crops for several decades (91).

Bacillus anthracis is an animal and human pathogen and is the etiologic agent of anthrax. There are four forms of disease based on the route of infection: cutaneous, gastrointestinal, inhalational, and injectional. Anthrax most commonly affects wild and domestic animals that become sick when they ingest or inhale spores from the contaminated environment. Animal cases have been reported in agricultural regions worldwide, in particular in developing nations with limited public health infrastructure and no animal vaccination programs (18). In the United States, outbreaks occur sporadically in wild grazing animal populations, such as bison and deer. Human

infections are typically associated with handling of infected animals or animal products and can become lethal if not treated immediately. Indeed, *B. anthracis* is considered a Tier 1 agent by the Centers for Disease Control and Prevention (CDC) because of its lethal potential and historic use as a biologic weapon. In 1979, more than 60 people died of inhalational anthrax in the Soviet city of Sverdlovsk following accidental release of spores from a biological weapons manufacturing facility in the city (71). In 2001, 5 people died and 17 others became sick following the intentional release of anthrax spores in the U.S. postal system (46).

B. cereus, on the other hand, is most commonly associated with an acute but mild foodborne illness caused by ingestion of contaminated food, such as improperly handled meats and vegetables. According to the CDC, *B. cereus* is responsible for an average of 63,400 annual cases of foodborne illness, although the number of cases is predicted to be underreported (95). When isolated from patients in the hospital, *B. cereus* is often considered a contaminant. However, it can also be an opportunistic pathogen. In fact, it has been a source of more severe infections such as endocarditis, osteomyelitis and wound infections in immunocompromised individuals (50). Certain groups of individuals, such as neonates and those undergoing surgical procedures, are also considered to be at higher risk for infection.

Emergence of more virulent *B. cereus* strains

In 1994, a patient presented to a hospital in Louisiana with fever, chills, hemoptysis (coughing up blood), nausea, vomiting, and had an abnormal chest X-ray (42). He sought care two days after onset of symptoms. The patient was a welder by

occupation, had no underlying health issues, and was a non-smoker. Despite severe pulmonary illness, as reflected by the need for mechanical ventilation for 44 days, treatment with numerous antibiotics, and a partial lobectomy, the patient survived. The clinical features of this illness were consistent with those described in patients who contracted inhalational anthrax in the Amerithrax (US Postal Service) attack of 2001. Surprisingly, the strain isolated from the blood and sputum of the welder was a *B. cereus* strain, subsequently named G9241. Further genetic typing showed that G9241 shared many virulence traits with *B. anthracis* (42). These features include the production of anthrax toxins and a capsule produced as a result of the presence of large virulence plasmids.

Several other *B. cereus* strains have been identified as causes of severe anthrax-like infections in immunocompetent individuals since the isolation of G9241 (Table 1). The majority of these strains caused fatal respiratory infections in metal workers in the southern regions of the United States. Most of the strains appear to be distinct in their molecular makeup, although *B. cereus* strain 03BB87, which was isolated from a miller operator in Texas in 2003, seems to be indistinguishable by Polymerase Chain Reaction (PCR) analyses from G9241 (43). *B. cereus* strain BcFL2013 was the first known *B. cereus* isolate from a cutaneous anthrax case in Florida. That infected individual worked with horses (70). Additionally, strains *B. cereus* CI and CA, collectively referred to as *B. cereus* biovar *anthracis*, were isolated from great apes in Africa. These strains have now been placed on the HHS Select Agent list, as they express the poly- γ -D-glutamic acid capsule (PDGA) of *B. anthracis* (14; 48).

Table 1. *B. cereus* isolates from anthrax-like infections.

<i>B. cereus</i> strain	Source	Year isolated	Genetic composition ^a	Reference
<i>B. cereus</i> G9241	Welder (not fatal) Louisiana, USA	1994	Tox+, PDGA ^c Cap- pBCXO1+, pBC210+	Hoffmaster <i>et al.</i> (2004)
<i>B. cereus</i> G9898	2 welders (fatal) Louisiana, USA	1996	Cap+ ^b	Miller <i>et al.</i> (1997)
<i>B. cereus</i> 03BB87	Muller operator (fatal) Texas, USA	2003 (Oct.)	Tox+, PDGA Cap-, pBCXO1+, pBC210+	Hoffmaster <i>et al.</i> (2006)
<i>B. cereus</i> 03BB102	Welder (fatal) Texas, USA	2003 (Nov.)	Tox+, PDGA Cap+, pBC210-	Hoffmaster <i>et al.</i> (2006)
<i>B. cereus</i> 03BB108	Environmental Texas, USA	2003	Tox-, PDGA Cap+, pBC210-	Hoffmaster <i>et al.</i> (2006)
<i>B. cereus</i> Ecl2	Welder (fatal) Texas, USA	unspecified	Tox+, pXO2-, Cap+ ^b	Wright <i>et al.</i> (2011)
<i>B. cereus</i> BcFL2013	Cutaneous eschar Florida	2013	Tox+, PDGA Cap- pXO1+, partial pBC210	Marston <i>et al.</i> (2016)
<i>B. cereus</i> biovar <i>anthracis</i>	Great apes (fatal) Cote d'Ivoire, Cameroon Africa	2001-2004	Tox+, PDGA Cap+ HA Cap+	Klee <i>et al.</i> (2006)

^a Genetic composition as confirmed via PCR with target specific genes

^b Capsule positive via stain

^c Poly- γ -D-glutamic acid capsule (PDGA)

The emergence of more virulent *B. cereus* strains raises certain questions. These questions include the following: are *Bacillus* strains recombining in the environment to exchange genes for virulence factors? Does the emergence of these strains challenge the known principles of pathogenesis in *B. anthracis*? Should new diagnostic tests be developed to more rapidly identify and type the *Bacillus* isolates to ensure proper treatment for the patient? Additionally, many of the isolates to date came from metal workers in the southern U.S. Is infection with virulent *B. cereus* strains a work-related exposure, and, if so, what steps need to be taken to prevent more incidents? A clearer understanding of the biology of these *B. cereus* strains and their potential to cause severe human illness is of importance to public health.

Molecular biology of the *B. cereus sensu lato*

Despite significant differences in the pathogenic potential between *B. anthracis* and typical *B. cereus* strains, there are many similarities between the organisms on a molecular level. Indeed, some studies suggest that the two are chromosomally indistinguishable (40). In fact, the two strains exhibit greater than 99% sequence similarity in their 16S ribosomal RNA gene, a locus that is often used in phylogenetic typing of bacterial strains (6). Historically, *B. anthracis* was genetically separated from other *Bacillus* strains primarily due to the presence of two large virulence plasmids that together are required for full pathogenicity of the bacterium. The two plasmids, pXO1 and pXO2, are 182kb and 96kb, respectively. The pXO1 plasmid carries three genes involved in the production of the anthrax toxins (Fig. 1A): *pagA* for protective antigen (PA), *lef* for lethal factor (LF), and *cya* for edema factor (EF). The toxins that result from

expression of these genes are lethal toxin (LT) that is comprised of LF and PA and edema toxin (ET), comprised of EF and PA. On the other hand, pXO2 carries the operon involved in the synthesis of the PDGA capsule, *capBCADE*. The capsule is an essential factor involved in pathogenesis because it interferes with opsonization of the bacterium by phagocytes (27; 68). Lethal toxin and edema toxin contribute to bacterial dissemination and are responsible for many of the symptoms of anthrax. Early antibiotic treatment of patients is imperative for their survival during infection. Treatment with antimicrobials becomes less effective when bacteria replicate to high levels in the body and produce large quantities of toxins that are secreted in the bloodstream to cause severe tissue damage (102).

The expression of virulence factors is a tightly regulated process by most bacterial pathogens, and *B. anthracis* is no exception. Indeed, the *atxA* gene for Anthrax toxin activator (AtxA) that is the master transcriptional regulator of virulence in *B. anthracis* is located in close proximity (on the pXO1 plasmid) to the anthrax toxin gene components. AtxA is involved in the regulation of numerous genes on both virulence plasmids as well as the chromosome (11). AtxA positively, and directly, regulates expression of the anthrax toxin components and indirectly affects expression of *capBCADE* through the regulation of *acpA*, which encodes a capsule activator (26; 49; 109). Thus, an *atxA*-null mutant strain is highly attenuated *in vivo* (24).

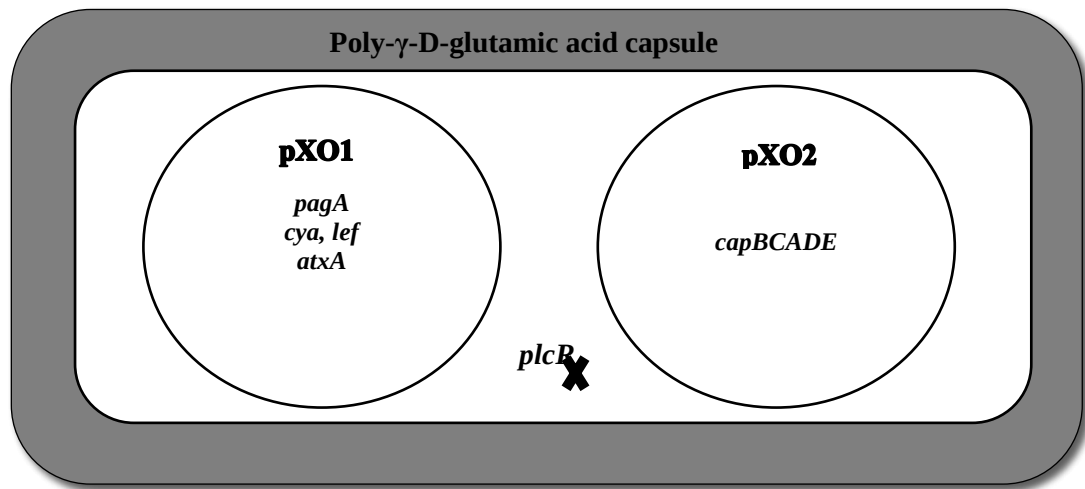
The mechanism behind AtxA-mediated regulation has not yet been established, as AtxA does not appear to bind to any consensus sequence on DNA despite the fact that

Fig. 1. Virulence plasmids of *B. anthracis* and *B. cereus* G9241.

A. *B. anthracis* contains two virulence megaplasms, pXO1 and pXO2. The pXO1 plasmid contains genes for the anthrax toxin components: *pagA* for protective antigen (PA), *cya* for edema factor (EF), and *lef* for lethal factor (LF). Additionally, the plasmid carries *atxA* that encodes AtxA, a master virulence regulator. The pXO2 plasmid carries the *capBCADE* operon that is involved in the synthesis of the poly- γ -D-glutamic acid capsule. The *plcR* gene is located on the chromosome. PlcR is a regulator of many secretory proteins in *B. cereus* and *B. thuringiensis*. However, no PlcR is made in *B. anthracis* due to the presence of a mutation in the gene, designated by an “x” in the figure.

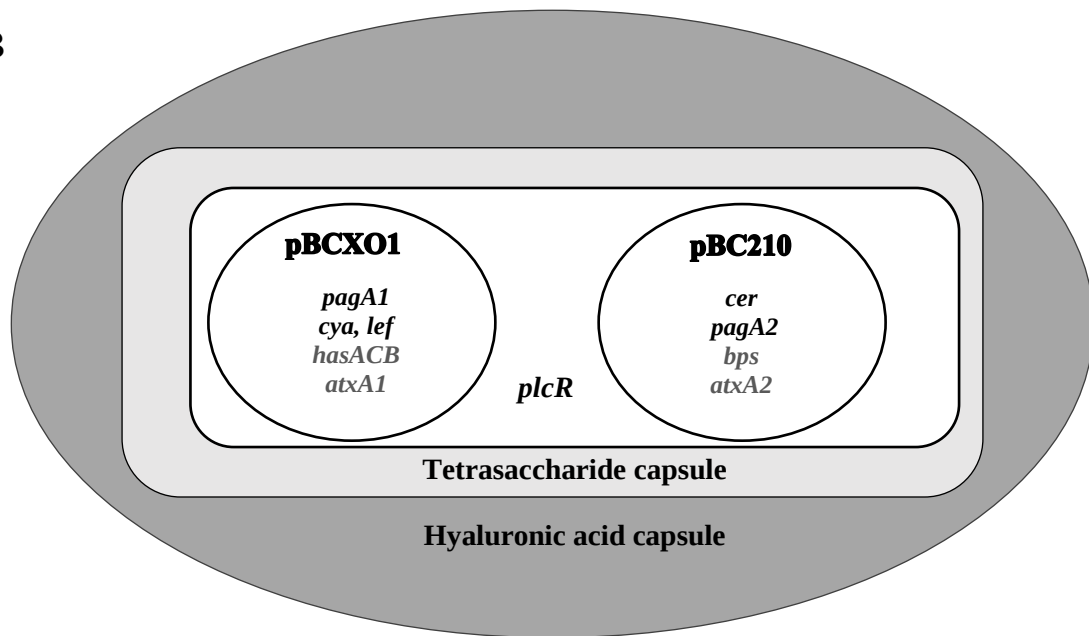
B. *B. cereus* G9241 also carries two large virulence plasmids, pBCXO1 and pBC210. The pBCXO1 plasmid is homologous to pXO1 and carries genes for the anthrax toxin components, *pagA*, *cya*, *lef*, as well as *atxA1* that encodes a homolog of AtxA called AtxA1. The pBCXO1 plasmid also carries an operon, *hasACB*, involved in the synthesis of the hyaluronic acid capsule. The pBC210 plasmid carries *atxA2* that encodes a second homolog of AtxA, AtxA2, and a second capsule synthesis operon, *bps* that is involved in the expression of the tetrassacharide capsule. Additionally, pBC210 contains *cer* and *pagA2*, genes that express Certhrax and Protective Antigen 2 (PA2), respectively. G9241 has a genetically intact *plcR* gene on the chromosome and therefore expresses PlcR.

A



Bacillus anthracis

B



Bacillus cereus G9241

structural analysis of the protein suggests it should have DNA-binding activity (107). However, the upstream region of AtxA-regulated genes appears to have a higher adenine/thymine (AT) percentage as compared to the overall genome content of the bacterium, and A- and T- tracts are associated with the formation of kinks in the DNA. Therefore, curvature of the DNA in the promoter regions is suggested to play a role in the way that AtxA acts on its targets (37). Several studies show that AtxA-regulated genes are expressed to higher levels in the presence of CO₂ and sodium bicarbonate and that AtxA is more active under such conditions (39; 49). Of note, CO₂/bicarbonate levels influence numerous cellular and physiological processes in humans and other mammals (17). Thus, various pathogens appear to have adopted ways to detect and respond to changes in environmental CO₂ levels. For example, pathogens such as Group A *Streptococcus* and *Vibrio cholerae* show enhanced virulence factor production in the presence of CO₂ *in vitro* (16; 97).

Besides the presence of the two virulence plasmids, another notable difference between *B. anthracis* and *B. cereus* lies in the *plcR* gene that is located on the chromosome (54). PlcR is a transcriptional activator of several extracellular virulence factor genes in *B. cereus* and *B. thuringensis*. The products of these genes include degradative enzymes, enterotoxins, and other secretory proteins (33; 34). Unlike AtxA, PlcR binds to a specific consensus sequence, referred to as the PlcR-box, that is located upstream of the regulated gene (1). Due to a single nucleotide insertion in the *plcR* of *B. anthracis*, the gene product is a truncated PlcR polypeptide that has no activity as a transcriptional activator (1). Therefore, *B. anthracis* is non-hemolytic and does not express *B. cereus*-specific virulence factors such as phospholipase C. *B. cereus*, on the

other hand, is beta-hemolytic. Additionally, *B. anthracis* is sensitive to penicillin and gamma phage lysis, while *B. cereus* is resistant to both (41). These distinctions, in conjunction with the fact that *B. anthracis* is non-motile due to mutations in the flagellar genes, are used in clinical settings to differentiate *B. anthracis* from typically non-pathogenic *B. cereus* isolates.

***B. CEREUS* G9241**

As described in Hoffmaster *et al.* (42), G9241 was identified through retrospective analyses of various *Bacillus* isolates from cases of severe disease. Further genomic analysis showed that the isolate carries two large virulence plasmids, called pBCXO1 and pBC210, that contain homologs of various *B. anthracis* virulence factors and a third plasmid, pBClin29 that encodes putative phage proteins. The pBCXO1 and pBC210 plasmids are required for complete virulence of G9241 in mice (117). The pBCXO1 plasmid is almost identical to the pXO1 plasmid of *B. anthracis*, with 99.6% amino acid identity, and also contains the structural genes of the anthrax toxins as well as the gene for the regulator AtxA (AtxA1) (Fig. 1B). Additionally, the plasmid carries a functional three-gene capsule synthesis operon, *hasACB*, that is involved in the production of a hyaluronic acid capsule (81). There is a single nucleotide insertion in the *hasA* gene in *B. anthracis* and, therefore, the hyaluronic acid capsule is not expressed in that pathogen (83). The other plasmid in G9241, called pBC210 (previously pBC218), is unlike pXO2 and was never before described in *Bacillus* until the paper by Hoffmaster *et al.* (42). A recent epidemiologic study showed that pBC210 is not common in *Bacillus* isolates, as it was not present in 103 strains tested. However it was subsequently found in

several soil samples obtained from Florida (66). The pBC210 plasmid contains a second functional capsule biosynthesis operon and a second homolog of the gene for AtxA, *atxA2*. AtxA2 exhibits 78% amino acid identity to AtxA of *B. anthracis* (42; 81). A recent study showed that AtxA1 is the primary regulator of virulence in G9241 and is involved in positively regulating expression of toxins and both capsules (96).

Nevertheless, AtxA2 appears to primarily regulate the expression of the tetrasaccharide capsule found on pBC210. Moreover, both regulators are required for full virulence of G9241 *in vivo* (96). Additionally, pBC210 carries a homolog of the gene for protective antigen, called *pagA2* (PA2 has 60% amino acid identity to PA), and a gene called *cer*. Certhrax exhibits 36% amino acid identity to LF but has been identified as a novel ADP-ribosyltransferase toxin (111).

In keeping with the general biology of *B. cereus*, G9241 is beta-hemolytic and motile (42). In addition to the presence of two virulence plasmids that contain the regulators AtxA1 and AtxA2, the *plcR* gene is genetically intact, and the protein is functional as a regulator. This latter finding is particularly interesting as it was originally believed that *plcR* and *atxA* loci were not compatible within a single organism (74).

The contributions of genes located on the pXO1 and pXO2 virulence plasmids to pathogenesis of *B. anthracis* have been studied for several decades. Conversely, the role in pathogenesis of the *B. cereus* G9241 capsule and toxin genes, encoded on the megaplasmids pBCXO1 and pBC210, is only now a subject of investigation. The fact that G9241 appears to have an additional set of regulatory proteins compared to *B. anthracis* adds another layer of complexity to the study of the virulence mechanisms of G9241.

***In vivo* virulence of G9241**

There are several animal infection models used to assess virulence of *Bacillus* strains. These models involve the infection of various animal species by different routes of spore inoculation. A/J mice, for example, are highly susceptible to infection even with the attenuated *B. anthracis* Sterne strain (pXO1+/pXO2-) and are often used to evaluate the role of toxins in pathogenesis (113). The A/J mice are complement C5a deficient and, therefore, are unable to recruit neutrophils and phagocytes to the site of infection as efficiently as other mice (116). C57BL/6 mice, on the other hand, are less susceptible to infection with *Bacillus* and are often referred to as the immune-competent mouse model (114).

Hoffmaster *et al.* first assessed virulence of G9241 by inoculating A/J mice via the intraperitoneal (i.p.) route with either 10^4 or 10^6 spores, and observed 100% lethality in both groups. The pathogen disseminated throughout the host and was recovered from the spleen, liver, heart and lungs of the mice. G9241 that was cured of either plasmid was attenuated in A/J and C57BL/6 strains following i.p., subcutaneous (s.c.), or intranasal (i.n.) inoculation of spores (81; 117), findings that substantiate the requirement for both large plasmids in full virulence of the pathogen. Additionally, as each plasmid carries a distinct capsule synthesis operon, Oh *et al.* assessed the contribution of each capsule to virulence of G9241 *in vivo*. Single and double capsule deletion mutants were engineered and assessed for virulence in C57BL/6 mice inoculated i.p. with 1×10^5 of spores. Forty percent of mice survived inoculation with the hyaluronic acid capsule deletion mutant, and 50% survived inoculation with the tetrasaccharide capsule mutant. Unencapsulated *B. cereus* G9241 was avirulent when inoculated at that dose of spores (81). These data suggest that both capsules are necessary for full virulence of G9241.

Despite the presence of a similar repertoire of virulence factors to *B. anthracis*, Wilson *et al.* showed that G9241 was avirulent in New Zealand White rabbits following s.c. inoculation of spores and was attenuated approximately 100-fold as compared to published data associated with fully-virulent *B. anthracis* Ames following aerosol administration of spores to rabbits (117; 122). Oh *et al.* showed that G9241 also had a higher LD₅₀ following aerosol challenge of C57BL/6 mice, as compared to published data for Ames (82). Of note, Scarff *et al.* (96) showed that wild-type G9241 behaved similarly to Ames following s.c. and i.n. challenge of A/J and C57BL/6 mice when spores were prepared in liquid Difco sporulation medium. The spores used in the rabbit challenge experiments by Wilson *et al.* were prepared in liquid medium as well. Could the difference in pathogenesis between G9241 and Ames in the rabbit model be attributed solely to the lack of the PDGA capsule in G9241, or are there also variabilities in toxin expression in G9241 that contribute to this virulence phenotype?

Toxins expressed by G9241

As previously mentioned, G9241 contains genes involved in the production of the anthrax toxins, LT and ET. These toxins belong to a family of toxins referred to as binary toxins (7). The pBCXO1 plasmid carries genes for protective antigen, edema factor and lethal factor, as noted above. The proteins share 99.7%, 96%, and 99% amino acid identity with PA, EF and LF of *B. anthracis*, respectively. Additionally, the amount of PA expressed by G9241 is comparable to that seen in *B. anthracis* Ames and Sterne (42). In *B. anthracis*, protective antigen is expressed and secreted as an 83kDa protein (PA₈₃) that acts as the binding subunit of the anthrax toxins. As previously mentioned, EF bound

to PA forms ET, whereas LF bound to PA forms LT. Each component is non-toxic on its own (9). PA₈₃ binds to the anthrax toxin receptors on the eukaryotic cell surface (Fig. 2). The two receptors are tumor endothelial marker 8 (TEM8, or ATXR1) and capillary morphogenesis protein 2 (CMG2, or ATXR2). Both are abundantly expressed in various tissues, although recent studies suggest that CMG2 is the more important receptor in anthrax toxin-mediated lethality *in vivo* in a mouse model (58). PA₈₃ protein gets cleaved by furin, a cell-surface protease, into a 63kDa monomer (PA₆₃) that then oligomerizes into a heptamer or octomer (47; 75). This oligomerization exposes PA binding sites for EF and LF. A maximum of three molecules of EF, LF or a mixture can bind at a time (23). The complex is then endocytosed through a clathrin-mediated mechanism and trafficked to the early endosome where, in the presence of an acidic environment, the PA oligomer forms a pore in the membrane of the endosome and LF and EF destabilize and unfold (51). This unfolding permits the translocation of the two toxin components into the cytosol of the cell. ET is a calmodulin-dependent adenylyl cyclase that induces production of cyclic adenosine monophosphate (cAMP). This molecule disrupts a wide variety of cell-signaling pathways and leads to the production of edema (53). Lethal toxin is a zinc-metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKKs) and disrupts numerous signaling pathways, such as those involved in cell cycle regulation and immune modulation; this disruption leads to cell death (3).

A transcriptional analyses study showed that in the presence of 14% CO₂ and bicarbonate, conditions that induce expression of virulence factors in *B. anthracis*, the G9241 genes for the anthrax toxin components were transcribed to higher levels as compared to an aerobic environment (87). Capsule synthesis genes were also upregulated

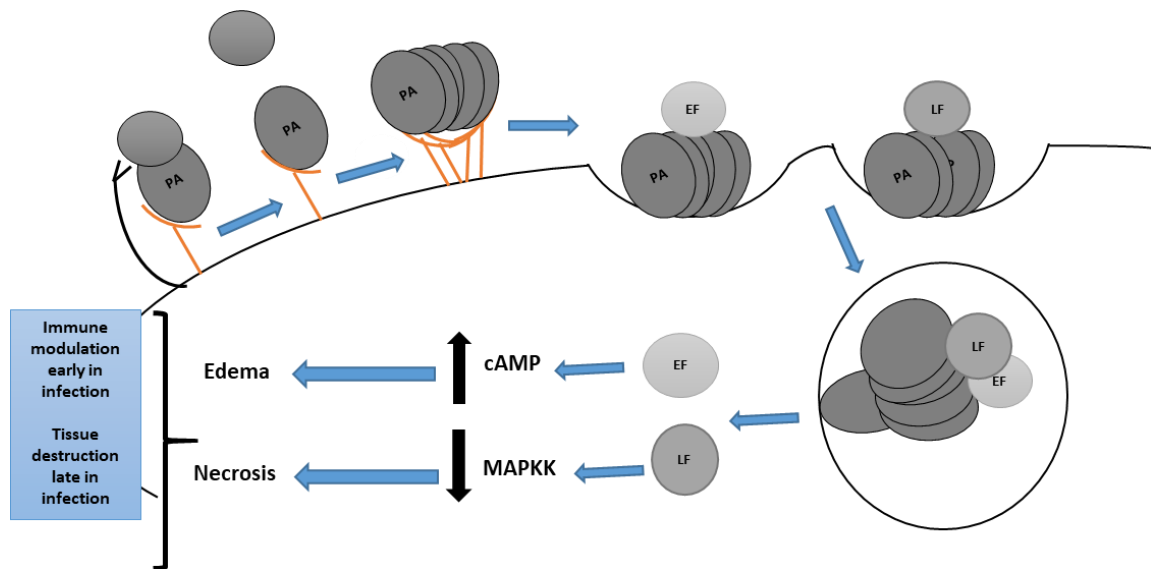


Fig. 2. Anthrax toxin translocation into the host cell.

The 83 kDa protective antigen (PA₈₃) binds to the cell-surface receptor of a target cell and gets cleaved by a surface protease, furin, into a 63 kDa monomeric protein. The monomer oligomerizes to expose binding sites that permit edema factor (EF) and lethal factor (LF) to bind. The bound toxin complex gets endocytosed and trafficked to an early endosome, where, in the presence of an acidic pH, the PA oligomer forms a pore in the endosomal membrane. This step allows edema toxin (ET), a calmodulin-dependent adenylyl cyclase, and lethal toxin (LT), a zinc-metalloprotease, to enter the cell. ET induces expression of large quantities of cAMP that causes edema. LT cleaves mitogen-activated protein kinase kinases (MAPKKs), an event that disrupts various cell signaling pathways and ultimately causes necrosis. Collectively, ET and LT are involved in immune modulation early in infection and tissue destruction late in infection. Modified from: Prince. 2003. *J. Clin. Invest.* 112:656–658.

under these conditions, as well as various other genes located on the two virulence plasmids. Up-regulation of the gene expression also extended to *pagA2* and *cer*, loci found on the pBC210 plasmid. These transcriptome analyses revealed that pathogenic strains of *Bacillus*, both G9241 and *B. anthracis* Sterne, were more sensitive to overall atmospheric changes as compared to the non-pathogenic strain *B. cereus* strain 10987 (87). Since *cer* and *pagA2* are expressed under conditions necessary for the expression of known virulence factors of *B. anthracis*, these data suggest that PA2 and Certhrax could have potential roles in pathogenesis.

Protective antigen

The role of protective antigen in pathogenesis of *B. anthracis* was first described in the early 1950s, when researchers observed that filtered serum, obtained from *B. anthracis*-infected guinea pigs, was lethal when injected into other animals intravenously (101). Subsequent studies showed that protective antigen was one of three components involved and that it was a combination of PA and one of the other two components that induced lethality in animals (9). As noted previously, protective antigen is the binding component of the anthrax toxins, and its expression is essential for ET and LT toxicity; EF and LF are not toxic to cells without it. PA is strongly immunogenic and is the primary component of the human anthrax vaccine used in the U.S., called Anthrax Vaccine Adsorbed (AVA) or BioThrax. As mentioned previously, PA is expressed from a gene located on the pXO1 plasmid in *B. anthracis*, and a homolog is located on the pBCXO1 plasmid in G9241. The gene is 2319 base pairs (bp), where the first 29 codons

encode a signal peptide (115). Expression of *pagA* appears to be more complex than what is seen with the other toxin components as the *pagA* gene contains two transcriptional start sites, P1 and P2, and PA is expressed from two promoters (49). The transcriptional initiation at the P1 site, located 58bp upstream of the translational start, is increased in the presence of elevated CO₂ levels. This CO₂-mediated effect has been attributed to transcriptional activation by AtxA (24). Conversely, AtxA does not have any effect on transcript levels of mRNA that map to the P2 start site, located 26bp upstream of the translational start. Additionally, *pagA* is the first gene in a bicistronic operon with a 300bp gene, *pagR*. PagR is a weak autorepressor of the *pagAR* operon and is an intermediate effector in the AtxA regulatory cascade (44; 73).

PA is a 735 amino acid protein that is comprised of four domains (88) (Fig. 3). Domain 1, residues 1-258, contains the furin proteolytic cleavage site, RKKR, required to generate the nicked PA₆₃ form of the protein. Following oligomerization, domain 1 forms the binding sites for EF and LF. Domain 2, residues 259-487, is involved in the formation of the transmembrane pore that allows ET and LT to enter the cytosol of the cell. Domain 3, residues 488-595, is involved in oligomerization of PA₆₃, where five specific residues (Phe552, Phe554, Ile562, Leu566, and Ile574) appear to be essential for proper oligomerization (4). Domain 4, residues 596-735, binds to the cellular receptors, TEM8 and CMG2. Certain genetically constructed PA variants can selectively bind to either TEM8 or CMG2 (20; 21). For example, a PA mutant with amino acid substitutions R659S and M662R shows increased specificity for TEM8, whereas PA I656Q and I656V show reduced specificity for TEM8 but unchanged specificity for CMG2 (20; 21). Such studies were done with the idea that the alteration of PA binding tropism could create

important therapeutic opportunities, such as the development of a targeted cancer treatment.

In 1991, Pezard *et al.* (89) demonstrated that a PA deletion mutant (PA-) of *B. anthracis* Sterne was avirulent following s.c. inoculation of Swiss mice. This avirulence of a *B. anthracis* Sterne PA mutant was also observed in an aerosol challenge experiment of A/J mice, as well as in aerosol challenges of rabbits with a PA- *B. anthracis* Ames (63; 89). However, some virulence results are mouse strain specific. For example- a PA- mutant of *B. anthracis* UT500 (pXO1+/pXO2+) was attenuated following s.c. challenge of BALB/C mice, while a PA- mutant of Ames was still fully virulent (19). Despite some variability in *in vivo* data, it is still apparent that protective antigen is essential for complete virulence of the bacterium.

The protective antigen homolog located on the pBCXO1 plasmid of G9241, termed PA1, is essentially identical to PA of *B. anthracis*. To date, two studies have explored the contribution of PA1 to virulence of G9241, and each showed that a deletion mutant strain of *pagA1*, $\Delta pagA1$, was attenuated following i.p. inoculation of C57BL/6 mice with 1×10^5 spores (81; 82). In one study 40% of mice survived that dose while in a second report 90% survived the same dose. Additionally, there was a decrease in bacterial counts in tissue of moribund animals infected with the deletion strain as compared to wild-type G9241. The $\Delta pagA1$ mutant was also attenuated following aerosol challenge with 1×10^6 spores (82). These data suggest that, like PA of *B. anthracis*, PA1 of G9241 is important for virulence of G9241.

The enzymatic components of anthrax toxin: lethal factor and edema factor

Lethal factor and edema factor are the enzymatic moieties of anthrax toxin. They are 90kDa and 93kDa in size, respectively, and share approximately 35% sequence identity in their N-terminal region (residues 1-254). This region is involved in binding to the protective antigen oligomer (5). In particular, there are seven conserved amino acids that are essential for the interaction of LF and EF with PA: D182, D187, L188, Y223, H229, L235, Y236 (52). Mutations in any of these residues, particularly H229, significantly decrease translocation of toxin into the cell. Early studies showed that injection of LF into Fischer rats, in conjunction with PA, resulted in rapid lethality, while injection of EF and PA into guinea pigs resulted in the production of edema (9).

Lethal factor is expressed as an 809-residue immature protein that is encoded by a 2427 bp gene. The first 33 amino acids constitute a signal sequence that gets cleaved to produce a mature protein of 776 amino acids (13). Beyond the N-terminal PA-binding region, often referred to as LF_N, lethal factor consists of three other domains (Fig. 3) (84). Domain 1 only weakly interacts with domain 2 and is stable on its own. LF_N can therefore be used in the construction of fusion proteins for therapeutic purposes, where the generated protein can use PA to enter target cells. Such a fusion protein, for example, was constructed with the gp120 component of the Human Immunodeficiency Virus (HIV)-1 cell envelope to deliver gp120 to antigen-presenting cells in an attempt to develop a new T-cell vaccine against HIV (35). Domain 2 of LF shares structural similarity to a *Bacillus cereus* VIP2 toxin (ADP-ribosyltransferase). However, this domain is enzymatically inactive due to mutations in conserved catalytic residues and an insertion in domain 3. Domain 4 is the enzymatic zinc-dependent metalloprotease domain and is essential for the activity of the toxin. The toxin targets and cleaves MAPKKs near

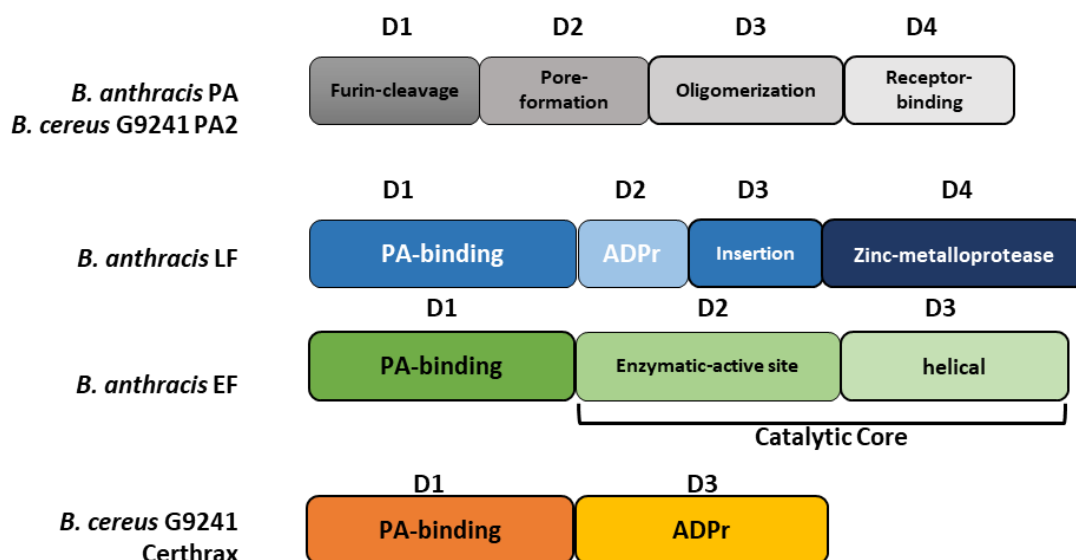


Fig. 3. Domain structures of PA, LF, and EF of *B. anthracis* and PA2 and Certhrax of *B. cereus* G9241.

The protective antigen (PA) of *B. anthracis* and PA2 of *B. cereus* G9241 both consist of four domains. Domain 1 (D1) contains the proteolytic cleavage site required to generate PA₆₃; D2 forms the transmembrane pore required for ET and LT translocation into the cytosol; D3 is involved in oligomerization; D4 is involved in binding to the CMG2 and TEM8 receptors on the cell surface. Lethal factor (LF) contains an N-terminal PA-binding domain (D1), an enzymatically inactive ADP-ribosyltransferase domain (D2) due the presence of an insertion sequence (D3) and an enzymatic zinc-metalloprotease domain (D4). Edema factor (EF) consists of an N-terminal PA-binding domain (D1) and a catalytic core made up of two domains that undergo a conformational change once bound by calmodulin: one that contains the enzymatic active-site of the adenylyl cyclase (D2) and a second helical domain (D3). Certhrax consists of a PA-binding domain (D1) and an enzymatic ADP-ribosyltransferase domain (D2). The domains are not drawn to scale. The PA and PA2 figure was modified from Oh *et al.* (82), while the LF and Certhrax domain structure was modified from Visschedyk *et al.* (111).

their amino terminal regions and prevents phosphorylation of downstream components of the MAPK pathway, such as the p38, Janus kinase (JAK) and extracellular signal-regulated kinase (ERK) pathways. These downstream pathways are all involved in regulation of cell stress, proliferation, migration and apoptosis, and other vital cell functions (28; 105; 112). The effect of LF-dependent MAPKK cleavage leads to multisystem damage of the host (106).

Edema factor, although originally characterized for the high levels of edema it causes in animals, also affects a multitude of cell signaling pathways via the production of very high levels of cAMP (53). EF does not share structural homology with other known adenylyl cyclases. The protein is expressed in an inactive state and undergoes significant conformational changes when it is bound and activated by calmodulin. Besides the N-terminal PA-binding domain, EF consists of a catalytic core domain that contains the enzymatic active site and a third helical domain (Fig. 3). Calmodulin binds to and inserts itself between the second and third domain of EF. This insertion induces a conformational change that then stabilizes the ATP substrate-binding site of EF (25). Overexpression of cAMP ensues and leads to increased activation of various cell signaling pathways, such as activation of protein kinase A and the subsequent phosphorylation of the cAMP response element-binding protein (PKA/CREB). The activation of these pathways results in transcriptional changes as well as cytoskeletal morphological modifications (22; 53). Although ET was originally only associated with production of edema, the toxin clearly has more far-reaching effects that impact cell function.

The specific effects reported for each toxin during infection conflict among some publications. These contradictory findings are likely due to the use of multiple *in vivo* models and different *Bacillus* strains in the various research studies. Nevertheless, the consensus among anthrax researchers is that edema and lethal toxins both play important roles throughout the course of the infection and affect a breadth of cell types and pathways (30; 60; 77). Early in infection the toxins are thought to modulate the host immune response in such a way as to interfere with bacterial killing. Specifically, many studies explored toxin effects on macrophages *in vitro* because mice that do not express the CMG2 receptor on the surface of myeloid cells are resistant to anthrax intoxication (59). Most of this toxin-dependent pathogenesis has been attributed to lethal toxin, as MAPKK cleavage can induce apoptosis in macrophages (85). However, only certain animal strains produce macrophages that are sensitive to LT toxin lysis. Furthermore, some studies show that LT-mediated lethality does not depend on macrophage sensitivity (76). This contradiction could be partially attributed to the fact that certain mouse strains contain polymorphisms in the *Nalp1b/Nlrp1b* inflammasome that renders their macrophages susceptible to cleavage by LT (12). However, this susceptibility also appears to be protective, as cleavage of *Nalp1b/Nlrp1b* activates caspase-1 that leads to induction of pro-inflammatory cytokines and a more robust immune response (104). LT not only can affect macrophages but also disrupts dendritic cell responses, as well as B and T cell immunity (3). Since *Bacillus* can infect phagocytic cells and yet use them to disseminate within the host, there must be a “checks and balances” system in place to slow down the rate of immune cell death. ET has been implicated in such a role. The toxin may aid in the delay of cell death since the production of high levels of cAMP

induces transcription of the plasminogen activator inhibitor type-2 (PAI-2) through the PKA/CREB pathway and thereby protects cells from apoptosis (86). Levy *et al.* also showed that ET plays a role in the suppression of neutrophil function (80).

The dampening of the innate immune response by the toxins allows for bacteria to replicate and eventually spread throughout the host. Systemic anthrax largely affects the cardiovascular system and can lead to shock-induced death. However, early studies suggested that the shock and late stage disease were a result of immunopathology following sepsis (64). Recent work shows that the toxins are themselves responsible for the observed tissue damage. LT can induce lethality through hypoxia-mediated toxicity and preferentially targets cardiomyocytes and vascular smooth muscle cells (61; 76). ET, on the other hand, targets hepatocytes and leads to significant liver damage through production of edema (61). Overall, anthrax toxins exert effects on a multitude of physiologic systems and can lead to irreversible cell and organ damage and subsequent death of the host.

Many of the originally documented *in vivo* anthrax toxin effects were based on the administration of purified toxins. This approach did not lead to a complete understanding of the role of toxins in virulence. It wasn't until the early 1990s that researchers were able to generate deletion mutants of *B. anthracis* for pathogenesis studies. Multiple *in vivo* studies done with toxin deletion mutants of unencapsulated *B. anthracis* strains suggest that LT is the more important toxin in virulence (63; 65; 89). Lowe *et al.* (65) generated Sterne strains that produced varying amounts of LF, in a Δlef background, and showed that strains that produced 100% and 40% of LF were as virulent as the wild-type Sterne in A/J mice, while the 10% LF producers were able to colonize

the site of infection but did not disseminate as efficiently. The 0.3% LF producers were cleared from the mice. Together these findings highlighted the probable role of LF in early establishment of infection and subsequent dissemination. However, the production of LT is not necessarily sufficient for full virulence of *B. anthracis*, because ET also contributes to pathogenesis in some animal models. Thus, during infection with encapsulated *B. anthracis* Ames, the presence of capsule and at least one of the toxins is sufficient to cause lethality in rabbits following inhalational administration of spores (62). Additionally, at least one of the toxins is necessary for dissemination of the bacterium (62). A similar observation was made in the guinea pig model following s.c. and i.n. inoculation with the Vollum strain (pXO1+, pXO2+), where the deletion of just one toxin gene does not affect lethality (56; 57). Of note, analyses that measured *in vivo* toxin levels found that LT concentrations during infection exceed those of ET by as much as a 5:1 ratio (78). One might speculate that LT appears to be more critical in pathogenesis because ET concentrations do not reach high enough systemic levels to illicit a greater pathologic effect. However, it is clear that *B. anthracis* pathogenesis is complex and variables such as the mouse strain used in studies can affect the conclusion derived from the experiment.

The novel toxin components of G9241: PA2 and Certhrax

While the pBCXO1 plasmid of G9241 appears to play a more important role in virulence of the pathogen than pBC210, the roles of gene products of the latter plasmid are not yet understood. The pBC210 plasmid is predicted to contain 188 putative open reading frames, approximately 47% of which are upregulated in the presence of CO₂ and

sodium bicarbonate (87). The genes for protective antigen 2 and Certhrax, *pagA2* and *cer*, are upregulated 40- and 30-fold, respectively, as compared to growth under aerobic conditions (87). As previously mentioned, PA2 shares 60% overall amino acid homology with PA of *B. anthracis*, and Certhrax shares 36% identity with LF, primarily in the PA-binding domain of LF. This latter observation suggests that Certhrax may also bind PA. Since *pagA2* and *cer* are located just 600 bases apart, the possibility exists that PA2 could be the binding component involved in translocation of Certhrax into the cell (82). It is common for the enzymatic and binding subunit genes of binary toxins to be located in close proximity to each other, as seen in some *Clostridium* strains (103).

PA2 is predicted to have a similar structure to PA of *B. anthracis* and PA1 of G9241, but it exhibits only 61%, 70%, 59% and 45% amino acid identity in domains 1 through 4, respectively (82). Additionally, PA2 is secreted and can be found in culture supernatants as with the other anthrax toxin components, (96). Oh *et al.* (82) generated a markerless deletion mutant strain of *pagA2* and assessed its contribution to virulence following i.p. inoculation of C57BL/6 mice. Mice were challenged with a single dose of 1×10^5 of spores with either wild-type G9241 or the mutant strain. Although an actual LD₅₀ was not determined, the mutant strain appeared as lethal as the wild-type G9241 and showed a similar bacterial load in tissue of euthanized moribund animals. These findings suggest that PA2 may not be important in virulence of G9241; however, it is unclear if similar results would be observed in a different infection model.

Certhrax, on the other hand, was identified as a new ADP-ribosyltransferase toxin (ADPRT) in an *in silico* analysis done by Fieldhouse *et al.* (29). ADPRTs belong to a group of toxins that bind nicotinamide adenine dinucleotide (NAD), cleave it into ADP-

ribose and nicotinamide components, and then facilitate the transfer of the ADP-ribose to a specific amino acid of a target protein that is then inactivated. There are many well-known ADPRTs that cause diseases in humans, such as diphtheria toxin produced by *Corynebacterium diphtheriae*, pertussis toxin produced by *Bordetella pertussis*, and cholera toxin produced by *Vibrio cholerae* (45; 98). Some known targets of ADPRTs are actin, Rho GTPases, and eukaryotic elongation factor 2. ADP-ribosylation of these proteins leads to actin depolymerization, disruption of the actin cytoskeleton and cessation of protein synthesis, respectively. The ADPR toxins generally do not share sequence homology but instead share structural similarities and conserved catalytic residues. In particular, there are three conserved amino acid motifs of importance for ADPRTs: a catalytic arginine in region 1 that is important in binding NAD; a serine-threonine-serine (STS) motif in region 2 that forms the frame for the active site; and a catalytic glutamine-x- glutamic acid (QXE) motif in region 3 that is essential for the enzymatic activity of the toxin. Certhrax has all of the conserved motifs (29; 111). The protein, comprised of 476 amino acids, is predicted to be 55kDa and consist of two domains: an N-terminal PA-binding domain, residues 1-241, and a C-terminal enzymatic domain, residues 242-476 (Fig 3). The structure of Certhrax was modeled after LF, as LF was the closest fold recognition match. No signal sequence was identified for Certhrax, an observation that suggests that the toxin is exported differently than the anthrax toxin components.

Visschedyk *et al.* (111) showed that Certhrax requires PA for cell entry. RAW264.7 macrophages were intoxicated with Certhrax alone, or with PA from *B. anthracis*, and cell viability was measured. There was a loss in percent cell viability only

when Certhrax was administered in conjunction with PA. This effect was PA-binding dependent, as macrophage cells that lack the PA receptor, R3D cells, were resistant to Certhrax plus PA toxicity. Additionally, Certhrax was made enzymatically inactive through the mutation of the catalytic QXE residues, a finding that further confirms that Certhrax behaves as an ADPRT. Vinculin was subsequently identified as the target of Certhrax in HeLa cells (99). Vinculin is an important component of focal adhesion complexes as it coordinates actin polymerization and promotes the interaction between the actin cytoskeleton and the extracellular matrix. Vinculin is also involved in cell migration (8). HeLa cells transfected with the enzymatic domain of Certhrax (CerADPr), show disruptions in the actin cytoskeleton as early as 4 hours post transfection. The cells later become rounded, lose adhesion, and eventually die (100). These morphological phenotypes can be rescued with the addition of exogenous vinculin (99). The publication by Simon and Barbieri was the first report of vinculin as a target for ADPR toxins. Those authors proposed that, in a G9241 infection, Certhrax targets and ADP-ribosylates vinculin that then disrupts the focal adhesion complexes to cause actin depolymerization. Actin depolymerization leads to disruption of the epithelial barrier which, in turn, promotes G9241 dissemination. Additionally, decreased association of actin with the anthrax toxin receptors increases the affinity of PA for the receptor and thereby enhances G9241 toxin involvement (99). Therefore, Certhrax could potentially have an important role in pathogenesis of G9241.

PURPOSE OF THE RESEARCH AND THE PROPOSED SPECIFIC AIMS

As described above, *Bacillus cereus* G9241 is a complex pathogen that produces multiple toxins and two distinct capsules. Few studies have explored how the pathogen causes disease, and many questions still remain. Despite the presence of two large virulence plasmids in G9241 and the fact that the organism caused a severe illness in a person, G9241 does not behave as does the fully virulent *B. anthracis* Ames strain in certain animal models. This apparent reduced virulence of G9241 could presumably be attributed to the lack of the PDGA capsule. However, the pathogen produces both the anthrax toxins and two novel putative toxin components, but the effect of these on pathogenesis has not yet been fully delineated. The work conducted here aims to fill some of these gaps. We hypothesize that the anthrax toxins play an essential role in the pathogenesis of *B. cereus* G9241, and that Certhrax and PA2 are also required for full virulence of the bacterium. The following aims were proposed to test the hypotheses:

Specific Aim 1: Evaluate what role, if any, Protective Antigen 2 plays in the virulence of G9241 *in vivo*.

Subaim 1.1: Construct a *pagA2* deletion mutant strain, as well as a double *pagA1/pagA2* mutant strain, and test each for virulence in s.c. and i.n. inoculation models of A/J and C57BL/6 mice.

Subaim 1.2: Generate a *pagA2* knock-in mutant strain in *B. cereus* G9241 $\Delta pagA1 \Delta pagA2$ to assess if PA2 can functionally replace PA1 in virulence.

Specific Aim 2: Assess the role that Certhrax plays in pathogenesis of G9241 *in vivo*.

Subaim 2.1: Identify *in vitro* conditions that promote Certhrax gene and protein expression.

Subaim 2.2: Construct a *cer* deletion mutant strain to be used for s.c. and i.n challenge of A/J and C57BL/6 mice.

Subaim 2.3: Assess whether the production of lethal toxin affects the role of Certhrax *in vivo* via construction of a single *lef* deletion mutant strain and a double *cer/lef* mutant strain.

CHAPTER 2: Materials and Methods

BACTERIAL GROWTH CONDITIONS AND SPORE PREPARATION

The bacterial strains and plasmids used in this study are listed in Table 2. The *Bacillus cereus* G9241 and *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) broth (BD Biosciences, East Rutherford, NJ) overnight at 37°C with shaking at 225 rpm. Antibiotics were added as needed at the following concentrations: kanamycin (Kan) at 100 µg/ml, spectinomycin (Sp) at 200 µg/ml and erythromycin (Erm) at 5 µg/ml for *B. cereus*; ampicillin (Amp) at 100 µg/ml and Kan at 50 µg/ml for *E. coli*. To promote toxin expression, an overnight culture of G9241 was grown in heart infusion (HI) broth (BD Biosciences) and diluted 1:1,000 in HI broth supplemented with 0.8% sodium bicarbonate. That culture was then incubated for 4 hours at 37°C in 5% CO₂ with shaking (called toxin-inducing conditions). To promote capsule expression, an overnight culture of G9241 grown in HI broth was sub-cultured 1:1,000 in HI broth supplemented with 0.8% sodium bicarbonate and 50% heat-inactivated horse serum (Life technologies, Carlsbad, CA), then grown overnight at 37°C in 14% CO₂ and with no shaking (called capsule-inducing conditions).

G9241 spores were prepared as described by Scarff *et al.* (96). Briefly, G9241 was grown overnight in 50 ml of brain heart infusion (BHI) broth (BD Biosciences) at 37°C with shaking at 225 rpm. The whole culture was added to 1L of Difco sporulation medium (DSM) that consisted of 0.8% nutrient broth, 0.012% MgSO₄, 0.1% KCl, 1mM Ca(NO₃)₂, 6mM NaOH, 1µM FeSO₄, and 0.01 mM MnCl₂. The culture was grown for 5 days at 30°C, with shaking at 225 rpm. The culture was pelleted by centrifugation at

Table 2. Strain list

Name	Description	Reference
Strain		
<i>E. coli</i>		
Mach1TM- T1^R	Chemically competent <i>E. coli</i>	Life Technologies
<i>dam</i>-/<i>dcm</i>-	Chemically competent <i>E. coli</i> to generate unmethylated DNA	New England Biolabs
<i>B. cereus</i> G9241		
G9241	Wild-type strain	Hoffmaster <i>et al.</i> (2004)
Δcer	G9241 Δcer	This work
Δlef	G9241 Δlef	This work
$\Delta cer\Delta lef$	<i>lef</i> deleted in G9241 Δcer	This work
<i>cer.rest</i>Δlef	<i>cer</i> restored in G9241 $\Delta cer\Delta lef$	This work
<i>inact.cer.rest</i>Δlef	<i>cer</i> (Q429A,E431A) restored in G9241 $\Delta cer\Delta lef$	This work
$\Delta pagA2$	G9241 $\Delta pagA2$	This work
$\Delta pagA1$	G9241 $\Delta pagA1$	This work
$\Delta pagA1\Delta pagA2$	<i>pagA2</i> deleted in G9241 $\Delta pagA1$	This work
<i>pagA2in</i>$\Delta pagA1$	<i>pagA2</i> cloned into <i>pagA1</i> site of G9241 $\Delta pagA1\Delta pagA2$	This work
pBCXO1-/pBC210-	Plasmid-cured strain of G9241	Wilson <i>et al.</i> (2011)
Plasmids		
pJMS1	Temperature-sensitive vector derived from pLM4 (Oh <i>et al.</i> , 2011); Kan ^R	Scarff <i>et al.</i> (2016)
pJMS1::<i>cerU/D</i>	1kb <i>cer</i> flanking genes cloned into pJMS1 to generate Δcer	This work
pJMS1::<i>lefU/D</i>	1kb <i>lef</i> flanking genes cloned into pJMS1 to generate Δlef	This work
pJMS1::<i>uCerd</i>	<i>cer</i> ligated into pJMS1:: <i>cerU/D</i> to restore <i>cer</i> in $\Delta cer\Delta lef$	This work
pJMS1::<i>inact.cer</i>	pJMS1:: <i>uCerd</i> with mutations introduced in <i>cer</i> (Q429A, E431A)	This work
pJMS1::<i>pagA2U/D</i>	1kb <i>pagA2</i> flanking genes cloned into pJMS1 to generate $\Delta pagA2$	This work
pJMS1::<i>pagA1U/D</i>	1kb <i>pagA1</i> flanking genes cloned into pJMS1 to generate $\Delta pagA1$	This work
pJMS1::<i>pagA2in</i>	<i>pagA2</i> ligated into pJMS1:: <i>pagA1U/D</i> to generate a <i>pagA2</i> knock-in at the <i>pagA1</i> site in $\Delta pagA1\Delta pagA2$	This work
pUTE657	IPTG-inducible expression vector derived from pBC16 and pDR111 with P _{hyper-spank} ; Amp ^R in <i>E. coli</i> , Sp ^R in <i>Bacillus</i>	Pflughoeft <i>et al.</i> (2011)
pCer	pUTE657-derived expression vector for Certhrax; putative Certhrax ribosomal binding site and coding region (position 84 to 1530) under the control of P _{hyper-spank}	This work
pHisCer	pUTE657-derived expression vector for His-Certhrax (N-terminal His ₆ tag); putative Certhrax ribosomal binding site and coding region (position 84 to 1530) under the control of P _{hyper-spank}	This work

pHT304-18z	Promotorless <i>lacZ</i> expression vector; Amp ^R in <i>E. coli</i> , Erm ^R in <i>Bacillus</i>	Agaisee <i>et al.</i> (1994)
p18z::<i>cerUp1</i>	pHT304-18z-derived <i>cer</i> promoter- <i>lacZ</i> fusion construct; contains sequence 109bp upstream from translational start	This work
p18z::<i>cerUp2</i>	pHT304-18z-derived <i>cer</i> promoter- <i>lacZ</i> fusion construct; contains sequence 267bp upstream from translational start	This work
p18z::<i>cerUp3</i>	pHT304-18z-derived <i>cer</i> promoter- <i>lacZ</i> fusion construct; contains sequence 540bp upstream from translational start	This work
p18z::<i>pagA2up1</i>	pHT304-18z-derived <i>pagA2</i> promoter- <i>lacZ</i> fusion construct; contains sequence 155bp upstream from translational start	This work
p18z::<i>pagA2up2</i>	pHT304-18z-derived <i>pagA2</i> promoter- <i>lacZ</i> fusion construct; contains sequence 273bp upstream from translational start	This work
p18z::<i>pagA2up3</i>	pHT304-18z-derived <i>pagA2</i> promoter- <i>lacZ</i> fusion construct; contains sequence 549bp upstream from translational start	This work
pET15b+	Expression vector for N-terminal His-tagged proteins; Amp ^R	Novagen
pET15b::<i>cer</i>	pET15-derived expression vector for His-Certhrax; <i>cer</i> cloned	This work
pET42b+	Expression vector for N-terminal GST-His-S-tagged proteins with a C-terminal His tag; Kan ^R	Novagen
pET42::<i>pagA2</i>	pET42-derived expression vector for GST-S-PA2-His; <i>pagA2</i> cloned without the signal sequence, N-terminal His tag was removed via PCR	This work

8,000 x *g* for 15 min, washed three times with sterile water, re-suspended in 10 ml sterile water, and heat treated at 65°C for 1hr to kill vegetative cells. The heat-resistant spores were stored at 4°C.

All work with *B. cereus* G9241 vegetative cells and spores was done in a Biosafety Cabinet, in accordance with BSL2+ safety standards. The Personal Protective Equipment (PPE) for researchers who worked with spores included an N95 respirator, extended cuff disposable gloves, safety goggles, and a disposable lab coat.

CONSTRUCTION OF *B. CEREUS* MUTANT STRAINS AND RECOMBINANT PLASMIDS

B. cereus deletion mutations were generated through homologous recombination with a temperature sensitive plasmid as described by Scarff *et al.* The plasmid, pJMS1, was derived from pLM4 (81). For each mutant strain, 1kb regions flanking the gene of interest were amplified via Polymerase Chain Reaction (PCR) with gene-specific primers listed in Table 3. The PCR products were first separately cloned into pGem®-T vectors (Promega, Madison, WI). Each vector was then digested with restriction endonucleases. The 3' flanking region was excised from pGem-T and was ligated into the construct that carried the 5' 1kb region such that the 3' region immediately followed the 5' flanking region. A restriction enzyme site marked the spot of the absent gene. The 2kb fragment was then sub-cloned into pJMS, and the recombinant plasmid was then transformed into One Shot® Mach1™- T1^R chemically competent *E. coli* cells (Invitrogen by Life Technologies). The plasmid was isolated from the Mach1 cells and subsequently transformed into *dam-/dcm-* *E. coli* (New England Biolabs, Ipswich, MA) to obtain unmethylated DNA. One microgram of the purified unmethylated plasmid DNA was

electroporated into *B. cereus* G9241 as described by Vergis *et al.* (110). Half of the transformants were recovered on LB agar plates supplemented with kanamycin after 4 hrs of growth at 30°C, while the rest were recovered after overnight growth at 30°C.

Transformant *B. cereus* G9241 colonies were re-streaked, and overnight cultures were passaged at a 1:100 dilution 2-3 times (once in the morning and once in the afternoon) at 37°C in the presence of kanamycin to force plasmid integration into the chromosome within the homologous region of DNA. The integrants, so obtained, were passaged 8-9x at 30°C in the absence of antibiotic to promote secondary recombination and loss of the pJMS1 vector. Colonies were screened for kanamycin sensitivity through patching on plain LB agar plates and on plates supplemented with Kan. At each point of the mutagenesis process, clones were tested for their capacity to sporulate. Specifically, clones were grown overnight in 1 ml of DSM broth at 37°C, visualized microscopically for formation of the forespore, then heat killed at 65°C for 30min. Only clones that survived the spore test were used in the subsequent steps. Deletion mutations were confirmed through Southern blot analyses and a PCR plasmid screen. A series of PCR reactions (Table 3) were done with 24 primer sets that anneal to areas spanning the pBCXO1 and pBC210 plasmids, as well as the *bclA* gene of the chromosome, to ensure that the plasmids remained intact in all mutant strains (96; 117).

The Δcer mutant strain was generated with primers P1-P4; P1/P2 were used to amplify the upstream region of *cer*, P3/P4 the downstream region. Primer sets P5/P6 and P7/P8 were used to amplify the flanking 3' and 5' 1kb region of *pagA2*, respectively, to generate $\Delta pagA2$. The Δlef mutant strain was obtained with primer sets P9/P10 and P11/P12, for amplification of the upstream and downstream *lef* region, respectively. The

$\Delta cer\Delta lef$ double deletion mutant strain was generated by deleting *lef* in the Δcer background. Primers P13/P14, P15/P16 were used to amplify the upstream and downstream regions to generate the $\Delta pagA1$ background. The $\Delta pagA1\Delta pagA2$ double deletion mutant strain was generated via deletion of *pagA2* in $\Delta pagA1$. To generate the *cer* restorant, *cer.rest* Δlef , *cer* was PCR-amplified with the P23 and P24 primers and ligated into the pJMS vector used to make Δcer following vector and fragment digest with *Bam*HI. Primers P25/P26 were used in a mutagenesis reaction to remove the 5' *Bam*HI site left from the ligation. The construct (pJMS1::*uCerd*) was electroporated into $\Delta cer\Delta lef$ and the restorant was generated through allelic exchange as outlined above. To generate the *inact.cer* Δlef restorant, primers P27 and P28 were used to mutate the two catalytic residues of *cer* in pJMS1::*uCerd*. The clone was then electroporated into $\Delta cer\Delta lef$ G9241. The *pagA2-knockin* strain was constructed through a similar approach. Primer set P17/P18 was used to amplify *pagA2* that was then ligated into the construct used to generate the $\Delta pagA1$ mutant strain. Primers P19/P20 and P21/P22 were used to remove the 5' and 3' *Eco*RI restriction sites within the clone, which was subsequently electroporated into $\Delta pagA1\Delta pagA2$ and the transformant sub-cultured as previously described.

Vector pUTE657 (90) was used to generate two distinct IPTG-inducible *cer* expression constructs through application of In-Fusion®HD cloning technology (Clontech, Mountain View, CA). Primers P35 and P37 were used to amplify *cer* (84-1530bp) in order to construct plasmid pCer, whereas primers P36 and P37 were used to amplify *cer* with an N-terminal Histidine tag (sequence CATCATCACCATCACCAC) to generate plasmid pHisCer. The In-Fusion cloning reaction mixture contained the proprietary In-

Fusion HD enzyme, pUTE657 digested with *Sall* and *SphI*, the amplified *cer* PCR product and water, per the manufacturer's guidelines. The reaction was incubated at 50°C for 15 min, and 2µl were transformed into *E. coli* Mach 1 competent cells (Ap^R). The transformants were confirmed through PCR and sequencing. The pCer and pHisCer vectors, as well as the pUTE657 empty vector, were transformed into *dam-/dcm-* cells and subsequently electroporated into Δcer G9241.

The *lacZ* promoter fusion constructs of *cer* and *pagA2* were generated with plasmid pHT304-18z (2). Three different sized upstream regions were amplified by PCR for each gene and cloned into the pCR®- Blunt II- TOPO® vector (Invitrogen by Life Technologies) as per the manufacturer's guidelines. The following primers were used to generate the fragments for *cer*: primers P38 and P41 amplified *cerup1* (109bp upstream from ATG), primers P39/P41 amplified *cerup2* (267bp upstream from ATG), primers P40 and P41 amplified *cerup3* (540bp upstream of ATG). For the *pagA2* promoter fusion, the following primers were used: primers P42 and P45 for *pagA2up1* (155bp upstream of ATG), primers P43 and P45 for *pagA2up2* (273bp upstream of ATG), primers P44/P45 for *pagA2up3* (549bp upstream of ATG). The fragments were liberated from TOPO® with enzymes *XbaI/BamHI* for the *cer* constructs and enzymes *HindIII/BamHI* for *pagA2*, ligated into pHT304-18z, and confirmed by PCR with the same primers that were used to generate the fragments. Each clone, as well as the empty vector, was electroporated into wild-type G9241 and recovered on Erm LB agar plates.

Table 3. Primers used in the study.

Primer Name	Primer Sequence	Primer Purpose
P1	GGATCCAAATATAGGAGTACTAACAGTAATAGTAGTACTAAACATGAAACAC	<i>cer</i> upstream 1kb region to generate Δcer
P2	GTCGACCTCAATCTATTACAGAATTAAGTAGTCAATTAAAGAACTCTAAAATAAATC	<i>cert</i> upstream 1kb region to generate Δcer
P3	GCGGCCGCTGTAGATCAGATGTCCTATTCATAACTTCTTGATCGTTAACCG	<i>cer</i> downstream 1kb region to generate Δcer
P4	GGATCCAGTCTATTAAGGAATCAAAAAAGGATATATATCTTAAGTAGAGACATG	<i>cer</i> downstream 1kb region to generate Δcer
P5	GCGGCCGCATAATACCTTTTATCAATTAATCTAGAGTGATAGC	<i>pagA2</i> downstream 1kb region to generate $\Delta pagA2$
P6	GGATCCCTTAATTATCCTCATAAAAAAGTTGTAATAAAATTCTGATGCTAAGG	<i>pagA2</i> downstream 1kb region to generate $\Delta pagA2$
P7	GGATCCATACTGGCTCCTTTTATATTTATTTTCATATTAATATAAATG	<i>pagA2</i> upstream 1kb region to generate $\Delta pagA2$
P8	GTCGACCAGAGCTACAATTTGGTAAAAAATATGAGGATTATAATC	<i>pagA2</i> upstream 1kb region to generate $\Delta pagA2$
P9	GTCGACATTAATTTTGGTTCCTGTGTCTAATGTAGCAGATACATCTAG	<i>lef</i> upstream 1kb region to generate Δlef
P10	GGATCCATTTAATCTCTCCTTTTTTATAAGTGAACATTTCAAC	<i>lef</i> upstream 1kb region to generate Δlef
P11	GGATCCGTAATGTATTAAAAATTTCAAATGGATTAAATAATAATAAACGGG	<i>lef</i> downstream 1kb region to generate Δlef
P12	GCGGCCGCAAAATATTTGCTATGAGTTTCTTAGCAATGAGGG	<i>lef</i> downstream 1kb region to generate Δlef
P13	GTCGACGAGATGAAAATGGTAATATAGCGAATAAATTAAAAAAC	<i>pagA1</i> upstream 1kb region to generate $\Delta pagA1$
P14	GAATTCATACGTTCTCCTTTTGTATAAAATTAATTTATATTATATTG	<i>pagA1</i> upstream 1kb region to generate $\Delta pagA1$
P15	GAATTCGGTAATTCTAGGTGATTTTAAATTATCTAAAAACAG	<i>pagA1</i> downstream 1kb region to generate $\Delta pagA1$
P16	GCGGCCGCATGTGCCATTGTTTTTAAAAG	<i>pagA1</i> downstream 1kb region to generate $\Delta pagA1$
P17	GAATTCATGAAAAAAGAAAAGTATTCATCCCATTATTAGC	Amplify <i>pagA2</i> to generate <i>PA2knock-in</i>
P18	GAATTCCTTATTCTTTAGCGAAAGGACTAAAACC	
P19	CAAAAAGGAGAACGTATATGAAAAAAGAAAAGTATTCATCC	Remove the 5' <i>EcoRI</i> site of the pJMS1::PA2in construct
P20	GGATGAATACTTTTCTTTTTTTCATATACGTTCTCCTTTTGTG	
P21	GTCCTTTCGCTAAAGAAATAAGGTAATTCTAGGTG	Remove the 3' <i>EcoRI</i> site of the pJMS1::PA2in construct
P22	CACCTAGAATTACCTTATTCTTTAGCGAAAGGAC	
P23	GGATCCATGACCACTGTACAAGGTGCGAGTAGTC	Amplify <i>cer</i> to generate <i>cer</i> restorant
P24	GGATCCCTATTTTCCCTATTCAAGTTCCTAAATAGATTGAAAG	
P25	GTTAGTACTCCTATATTTATGACCACTGTACAAGGTGCGAGTAG	Remove the 5' <i>BamHI</i> site of the pJMS1::Up-Cer-Down construct
P26	CTACTCGCACCTTGACAGTGGTCATAAATATAGGAGTACTAAC	
P27	GATATGAGTGAATATCCAGGGGCATATGCAATGTTAATAAATAGAGG	Generate Q429A and E431A mutation in <i>cer</i>
P28	CCTCTATTTATTAACATTGCATATGCCCTGGATATCTACTCATATC	
P29	GCAATTATCCGTGATACCTTTCCTTTAAATCAG	<i>cer</i> -specific probe for Southern blot analysis
P30	CGCCTCCTATATTGGGATAATTCTCCG	
P31	GCTGCATCTGCCGTATTACACCTAATGTC	

P32	GGACCACTCCAGATAATACAACAGAAACAATTCCG	<i>pagA2</i> specific probe for Southern blot analysis
P33	GTGAGAATGGGGATACTAGTACCAACGGGATC	<i>pagA1</i> probe for Southern blot analysis
P34	GTAATTAGTTCATTGGCGCTCGTTTCGTCTAATCTAC	
P35	GATAACAATTAAGCTTAGTCGACGAATAAAGAAGAACGAATGAAAGAAATAATACGC	In-fusion cloning of <i>cer</i> into pUTE657
P36	GATAACAATTAAGCTTAGTCGACGAATAAAGAAGAACGAATGCATCATCACCATCACCACAAAGAAATAATACGC	In-fusion cloning of <i>his-cer</i> into pUTE657
P37	CACCGAATTAGCTTGCATGCCTATTTTCCCTATTCAAGTTCCTAAATAG	In-fusion cloning of <i>cer</i> / <i>his-cer</i> into pUTE657; reverse primer
P38	GTAAAGCATGGAAGGATGTAGATAACATGATAAAG	Amplify <i>cerup1</i> for promoter fusion construction
P39	CCCTAAAGCGAAAGTATTATAATTGGTTAGGCTAAG	Amplify <i>cerup2</i> for promoter fusion construction
P40	CCGGATTCCCTCCTATATCTGTCAC	Amplify <i>cerup3</i> for promoter fusion construction
P41	GGATCCTAAATATAGGAGTACTAACAGTAATAGTAGTTACTAAACATGAAAC	Reverse primer for <i>cer</i> promoter fusion constructs
P42	AAGCTTGAAATTTGTTCAATCGTATTGCTCAAATTAATAATTTAATTCC	Amplify <i>pagA2up1</i> for promoter fusion construction
P43	AAGCTTCCACATCTATTATTTCCCTATCACATTGATTGAATGC	Amplify <i>pagA2up2</i> for promoter fusion construction
P44	AAGCTTGGAGGTATGACTTATTTAATAATGGACACTTTACTACTAAC	Amplify <i>pagA2up3</i> for promoter fusion construction
P45	GGATCCATACTGGCTCCTTTTATATTTATTTTCATATTAATATAAATGAAATAGAAAAG	Reverse primer for <i>pagA2</i> promoter fusion constructs
P46	ATGAGATTTAAGGAAATAAGTGATGATGTAAAAGAAAAGAACAAAAATG	Clone the ADPr domain of Certhrax into pet42b
P47	ATGGACCTCGAGCTATTTTCCCTATTCAAGTTCCCTAAATAGATTG	
P48	GGTATTGAGGGACGCAATACAACGCAAGAGGACAGTATATTACAAGG	Clone <i>pagA2</i> into pet42b to express GST-PA2
P49	GGTGGTGGTGCTCGAGTTTCTTTAGCGAAAGGACTAAAACCCCTTTTTC	
P50	CGCGCGGCAGCCATATGACCACTGTACAAGGTGCGAGTAGTC	Clone <i>cer</i> into pet15b to express His-Certhrax
P51	GTTAGCAGCCGGATCCCTATTTTCCCTATTCAAGTTCCCTAAATAGATTG	
P52	CGTTCGGATCTACTGCTGCTACTCTTC	RT-PCR of <i>pagA2</i>
P53	GATCAATACTTACACCGTGGGTACCTACTGTAC	
P54	CTCCCGTTTCTAAAGTTGTGATTACCGTTGGATTTC	RT-PCR for <i>cer/pagA2</i> co-transcript
P55	GCAGATATGAGTGAATATCCAGGGCAATATGAGATG	

Table 4. PCR plasmid screen primer list.

Plasmid	Primer name	Primer Sequence	Reference
pBCXO1	XO1.1s	GGAGGTCCATAGAATAATATAGAACGGGATGC	(117)
	XO1.1a	CAGCACTATCAACACTGGAGCGATTC	
	XO1.2s	TATCAAACAGATGTCAACCGTATCG	
	XO1.2a	CAGCATCTTTAACCCTAGACCTAAC	
	XO1.3s	CTACATTGCTGACATTCAAAGGTAG	
	XO1.3a	GTACGGTGATACAACACAAATTGAC	
	XO1.4s	GTTTGCCAGCTCCATTCCCAATAAG	
	XO1.4a	GGCTGAAAGCGTTAAGGCATATTCC	
	XO1.5s	CCTTCTGCGGTGTACTTGTTAATGG	
	XO1.5a	AATGCCTGACGGCACAACAATGTTC	
	XO1.6s	TACGTGATTGAGCAGCACATAGTAG	
	XO1.6a	TACAGCGATACCAGATACTCCTGTC	
	XO1.8s	AAGGTTTCATCTTCAGGCACAGATTC	
	XO1.8a	CAGCTCTTGATCCACTATAGGATTC	
	XO1.9s	CTTCTACAGTCACTTGGTCTCTAGTGAGTGGAC	
	XO1.9a	CATCCATTGAATTAGGTTTCATCTTGGCATATGGATC	
	XO1.10s	GTACAAGAGGAAGAGCAAGTAAATC	
	XO1.10a	CTCATTCCCATTTCATTCTCCTTATC	
	XO1.11s	GTGAAGATTAGAAAAGCGATTATCCCAGCAG	
	XO1.11a	TTATTTATTGATAAGTTTATCTCGAGTGATATTTCTCAAATAA CTC	
pBC210	218.1s	GTTACGCAGCATCTCGAATATTAAC	(96)
	218.1a	AAATCTTGCCCATGTCCAATAAGG	
	218.2s	CACTCATATCTGCTATATAAGCCCCATGTA	
	218.2a	GTTGGAAGTGGATCAGATGAGATTTAAGG	
	218.3s	CGACTAAATTGCTGAAGGCAGTAAC	
	218.3a	TTAGGAGGAAGGTAAATGAGGAAAG	
	218.4s	CGCTTATATGCCAATATCCCTAATG	
	218.4a	AAATCTCTTTTCGGTAGGGAAATCTG	
	218.5s	ACCCACTATATTCGGACTAAATACC	
	218.5a	ATGACCTTTGGGCACAGTATGTAAC	
	218.6s	ATTCTCCTCGTCTCCCTCCTGATTC	
	218.6a	GCACGGGACTTTCCGATAGACATGC	
	218.7s	ATACATACCGTGACTTCGTGTTCTC	
	218.7a	TAGGTGTTTATGGGACTACGGTTAC	
	218.8s	ACCGATCAACTCCTCAAGAACTGATCC	
	218.8a	TCCGGCTTCAACCTATTCCATCTTCAGTC	
	218.9s	CAGTATTCGTGCTAAAGGTTATAGG	
	218.9a	ACCGTAATATCCAATCATTAGGTG	
	218.10s	AGCGTGCTGTAAAGGTGTTAGAATC	
	218.10a	CATTTGCTTCATCAGGTCAATAGG	
	218.11s	ATTATCAACCACGAGTGGTACGTAG	
	218.11a	AGGATTTCCGGTGTTAATAGCTGAC	
	218.12s	GTATTAGCGAAATGAGACGGAGAAG	
	218.12a	CTTTGTAATTTCTCCACCGACAGAC	
Chromosome	<i>bclA</i>	CCA CCG TTT ACC CTT CCT ACC GGA CCA AC	(96)

		CTT CAA CTA ATG ATG GAG TTG TCG TAA TTT GCG	
pBCXO1	<i>atxA1</i>	CTCAGTTTCATGTAATGTAACGCCGAAAGAG	
		CTGATTATCTACAGGTCGCAGATAAAACAGTCC	

SOUTHERN BLOT ANALYSES TO CONFIRM *B. CEREUS* G9241 MUTATIONS

Southern blot analysis was carried out to confirm each deletion mutant strain of *B. cereus* G9241. The approach used digoxigenin (DIG)-labeled probes prepared through PCR amplification as per the manufacturer's guidelines (Roche, Pleasanton, CA). Briefly, each probe was generated in a PCR reaction with approximately 40ng of wild-type G9241 DNA and the gene-specific primers listed in Table 2. Total *Bacillus* DNA was isolated with the Wizard® Genomic DNA Purification kit (Promega). For each Southern blot, 2-5 µg of wild-type and mutant strain G9241 DNA were digested for 7-8 hrs at 37°C with specific restriction endonucleases (New England Biolabs). Each 20 µl sample, as well as a DIG-labeled DNA molecular weight marker VII (Millipore Sigma, St. Louis, MO), were loaded onto a 0.8% agarose gel and subjected to gel electrophoresis overnight at 30V. The DNA was transferred onto a positively-charged nylon membrane (GE Healthcare) for 3-4 hrs as outlined in the Roche protocol. The membrane was cross-linked by ultra violet light (UV) and sealed in a plastic bag with DIG Easy Hyb (Promega) in a pre-hybridization step for 30 min at 42°C. The labeled probe was denatured at 92°C for 12 min, added to DIG Easy Hyb, and then hybridized to the nylon membrane overnight at 42°C. The following day the membrane was exposed to low stringency (2x SSC, 0.1% SDS) and high stringency (0.5x SSC, 0.1% SDS) washes, blocking solution and antibody solution (1:10,000 Anti-Digoxigenin-AP), then washed with wash buffer and detection buffer (Roche). The CDP-*Star* substrate (Roche) was added, and the blots were exposed with the use of an ImageQuant LAS4000 imager (GE Life Sciences, Piscataway, NJ).

The *cer* deletion was confirmed with a *pagA2*- specific probe synthesized with primer set P31/P32. The DNA was digested with *Bam*HI and *Bmg*BI restriction endonucleases. The Δ *pagA2* mutant strain was confirmed with a *cer*-specific probe that was generated with primer set P29/P30 and hybridized to DNA digested with *Bam*HI and *Hind*III enzymes. The Δ *pagA1* and Δ *lef* mutant strains were both confirmed with the *pagA1* probe that was generated with primers P33/P34. To confirm the Δ *pagA1* mutant strain, DNA was digested with *Eco*RI and *Hind*III; *Eco*RV was used in the digestion of Δ *lef*. The *cer.rest* Δ *lef* and *inact.cer.rest* Δ *lef* strains were both confirmed with the *cer* probe, following digest with *Bam*HI alone. The *pagA2*in Δ *pagA1* mutant strain was confirmed with the *pagA2*-specific probe following DNA digestion with *Bam*HI.

WESTERN BLOT ANALYSES TO DETECT TOXIN EXPRESSION

Western blot analyses were carried out to detect PA, LF, EF and PA2 in culture supernatants and Certhrax in cell lysates of *B. cereus* G9241 strains grown under toxin- or capsule-inducing conditions. Twenty-five ml cultures were grown to late-exponential phase, and their absorbance recorded at a wavelength of 600nm (OD₆₀₀). The cultures were centrifuged at 10,000 x *g* for 15 min. The supernatants were collected and filtered through a 0.22 μ m filter (Corning, Corning, NY) then standardized to the lowest OD₆₀₀. Proteins were precipitated with the addition of ice-cold ethanol at a 1:1 ratio and stored at -20°C overnight. The samples were centrifuged at 10,000 x *g* and the supernatants discarded. The resultant protein pellets were washed once in PBS then re-suspended in 250 μ l of 4x SDS-PAGE loading buffer [141 mM Tris Base, 106 mM Tris HCl, 2% SDS, 10% glycerol, 0.51 M EDTA].

G9241 cell lysates were prepared based on the protocol described by Hammerstorm *et al.* (39). The cell pellets were washed once with 1 ml KTE-PIC buffer [10mM Tris-HCl pH 8, 100mM KCl, 10% ethylene glycol, and 1x Complete EDTA-free proteinase inhibitor (Roche by Millipore Sigma)]. The pellet was re-suspended in 550 μ l of KTE-PIC and transferred to a tube that contained approximately 400 of μ l 0.1 mm Zirconia/Silica Beads (BioSpec Products, Bartlesville, OK). The pellet was beaten in the presence of the beads for 2 min with a Turbo Mixer vortex attachment, and the sample was then centrifuged at 10,000 x g at 4°C. The supernatant was collected, and 4x SDS-PAGE buffer was added to the sample.

The culture supernatants and cell lysates were denatured at 92°C for 12 min and separated with a 4-12% Bis-Tris protein gel (Life Technologies) in MOPS buffer (Teknova, Hollister, CA) at 150V. An Oriole stain was applied to the gel to detect total protein (Bio-Rad, Hercules, CA). For Western blot analyses, the total protein was transferred to a nitrocellulose membrane with the iBLOT Dry Blotting System apparatus (Life Technologies). The membrane was blocked at room temperature for at least an hour in phosphate buffered saline (PBS) that contained 0.1% Tween-20 (PBS-T) and 5% skim milk. Primary antibody was added to the membrane in fresh blocking solution, and the blots were then rocked overnight at 4°C. The blots were washed three times with PBS-T and incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody (Bio-Rad) at a 1:10,000 dilution for at least 1 hr at room temperature; goat anti-mouse Ig was used for PA and LF Western blot while goat anti-rabbit Ig was used for EF, PA2 and Certhrax detection. The blots were washed once with PBS-T and twice with PBS, the

HRP-antibodies bound to the protein were mixed with ECL reagent (GE Healthcare), and the blots viewed with the ImageQuant LAS4000.

The following primary antibodies were used to detect the anthrax toxin components: mouse monoclonal anti-PA and anti-LF antibodies at 1:10,000 dilutions (GenWay, San Diego CA); rabbit polyclonal anti-EF sera at 1:1,000 dilutions (generously provided by Dr. Stephen Leppla). PA2 was detected with a 1:100 dilution of a rabbit peptide antibody generated by GenScript (Piscataway, NJ) as described by Scarff *et al.* (96). Certhrax was detected with anti-ADPr antibody purified from sera of a rabbit immunized with the purified His-ADPr portion of Certhrax (Pocono Rabbit Farm & Laboratory Inc., Canadensis, PA 18325) that was generated through a collaboration with Joseph Barbieri and Nathan Simon as described in Simon *et al.* (100). The rabbit ADPr anti-sera was concentrated via affinity purification with a GST-ADPr column. The GST-ADPr was first expressed from a pET-42b(+) expression vector (Novagen by Millipore Sigma) that was generated via PCR amplification of the *cer* ADPr region with primers P46/P47. The amplified product and pET-42b were digested with *PshAI* and *XhoHI* endonucleases and the ligated. The construct was transformed into BL21 (DE3) pLysS *E.coli* cells (ThermoFisher Scientific, Waltham, MA) for expression. The culture was grown and induced with 100 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). The cells were pelleted and re-suspended in binding buffer [10mM Imidazole, 50mM Sodium Phosphate, 200mM NaCl, pH 8] and subsequently lysed via sonication, and then centrifuged to collect the culture supernatant. The supernatant was filtered with a 0.4 μ m membrane. The supernatant that contained the GST-ADPr was then bound to a glutathione affinity column (GE Healthcare) for purification, as described in the

manufacturer's manual. The purified GST-ADPr was subsequently covalently bound to a N-hydroxysuccinimide (NHS)-activated Fast protein liquid chromatography (FPLC) column from GE Healthcare as described in the manufacturer's user manual. The rabbit anti-serum sample was loaded onto the column and was eluted with 0.1M glycine, pH 2.8. The eluent was desalted into PBS then incubated with plasmid-cured G9241 lysates to remove non-specific antibodies to *Bacillus*. The non-specific antibody-bacteria mixture was pelleted by centrifugation, and the supernatant was filtered through 0.22µM membrane. The anti-ADPr antibody was used at a 1:500 dilution for Western blot analyses.

PURIFICATION OF RECOMBINANT PROTEINS

Recombinant PA, PA2 and Certhrax were used to detect antibodies in mouse serum or as positive controls in Western blot analyses. The rPA was purchased from the Critical Reagents Program (Aberdeen, MD). GST-tagged PA2 was purified in collaboration with Joseph Barbieri and Amanda Przedpelski. The *pagA2* gene was amplified with primers P48/P49, ligated into the pET-42b+ expression vector and then transformed into BL21 (DE3) pLysS *E. coli*. The protein was purified by sequential Nickel-affinity and then GST- chromatography in the Barbieri laboratory, and eluted in 10mM Tris pH8, 200 mM NaCl, and 40% Glycerol.

The His-tagged recombinant Certhrax was purified from a pET15b::*cer* expression vector through nickel-affinity chromatography. The construct was generated with primers P50/P51 and cloned into the pET15b vector (Novagen). The resultant plasmid was transformed into the BL21 (DE3) pLysS cells. The *E. coli* strain expressing

recombinant His-Cer was grown to early log-phase and induced with IPTG. Four hours post induction, cells were pelleted; suspended in Binding Buffer [10mM Imidazole, 50mM Sodium Phosphate, 200mM NaCl, pH 8], and sonicated to lyse the bacteria. Insoluble material was removed by centrifugation at 12K rpm for 20min. Cell lysate supernatants were collected and filtered through a 0.4µm membrane. Recombinant His-Cer was purified via nickel-affinity chromatography over His-Trap prepack columns (GE Healthcare) run with a 0.5 ml/min flow rate. Specifically bound His-tagged protein was eluted with a step gradient of 500mM imidazole, 50mM sodium phosphate, 200mM NaCl, pH 8. The column fractions with the highest 280nm absorbance were retained and assayed for His-Cer by SDS-PAGE separation. Those fractions with the highest concentration of the 55-60 kDa protein were pooled and used in subsequent experiments.

***IN VITRO* EXPRESSION OF CERTHRAX IN *E. COLI* AND *B. CEREUS* G9241 CELL LYSATES**

The IPTG-inducible constructs, pCer and pHisCer, transformed into either *E. coli* Mach 1 cells or electroporated into the Δcer mutant strain of *B. cereus* G9241, were used to detect Certhrax expression via Western blot analyses with the anti-ADPr antibody. The *E. coli* strains that contained the pCer and pHisCer constructs were grown overnight in LB broth supplemented with Amp. The overnight cultures were subcultured at a 1:50 dilution into two tubes that contained fresh LB-Amp and grown to an OD₆₀₀ of 0.4-0.8. One of the cultures was induced with 100 µM IPTG and further grown overnight at 37°C with shaking, while the other culture was left un-induced and grown overnight as a control. The samples were centrifuged and the cell pellets were re-suspended in 200 µl of

SDS buffer. The samples were denatured at 92°C for 12 min and subjected to SDS-PAGE electrophoresis.

WT G9241, Δcer , Δcer pCer, Δcer pHisCer, and Δcer pEmpty (empty vector control) G9241 strains were grown under toxin-inducing conditions, except the cultures were grown for 24 hours without shaking instead of 4 hours with shaking. During the 24 hour incubation period, the cultures were induced with 100 μ M IPTG at 0 and 22 hours. Following incubation, the cultures were centrifuged, and the cell lysates were collected as previously described. The cell lysates were subjected to SDS-PAGE electrophoresis.

The *E. coli* and *B. cereus* G9241 cell lysates were examined via Western blot analyses with the anti-ADPr antibody at a 1:500 dilution in PBS-T-SM, and an anti-His antibody at a 1:1000 dilution (Novagen).

VISUALIZATION OF THE TETRASACCHARIDE AND HYALURONIC ACID CAPSULES

B. cereus G9241 capsules were visualized microscopically as described by Scarff *et al.* (96) after samples were exposed to the Maneval stain (69). Briefly, G9241 strains were grown under capsule-inducing conditions for 24 hrs. A 900 μ l sample of cells was aliquoted, fixed with 100 μ l of formaldehyde (Fisher Scientific), and stored at 4°C until used. To remove excess formaldehyde, 100 μ l of cells were washed 3x with PBS with two 5 min centrifugal spins at 16,100 x g (maximum speed) and one 10 min spin at the maximum speed. The pellet was re-suspended in 100 μ l of PBS and then split into two samples, where half was treated with 200 units of hyaluronidase (Millipore-Sigma) to visualize the pBC210 tetrasaccharide capsule, and the other half of the sample received 10 μ l of H₂O to allow visualization of the hyaluronic acid capsule. Samples were

incubated for 2hrs at 37°C, washed once with PBS, and then were re-suspended in PBS. The cells were visualized microscopically following staining with the Maneval stain (Carolina Biological Supply Co., Burlington, NC). Eight microliters of each sample were added to a few drops of 1% Congo red on a microscope slide, the materials were mixed, smeared on the slide, and then the samples were allowed to air dry. The smear was flooded with Maneval solution, which was allowed to sit for 5 min. The slide was then gently washed with H₂O. The slide was air-dried and examined on an Olympus BX60 microscope under 1,000X magnification. Images were taken with a SPOT Insight Gigabit Color Camera (SPOT Imaging Solutions, Sterling Heights, MI).

GROWTH CURVES OF G9241 MUTANT STRAINS

Overnight cultures of the G9241 strains were grown in LB broth and sub-cultured at a dilution of 1:100 into 25 ml of LB. The strains were then grown for 6 hrs at 37°C with shaking at 250 rpm. Samples were obtained every hour to measure absorbance reads (OD₆₀₀), and for enumeration of colony forming-units (CFU/ml) on LB agar. Data were plotted with GraphPad Prism Software. Three biological replicates were obtained at each time point and the mean OD₆₀₀ and geometric mean CFU/ml determined.

SPORE GERMINATION ASSAY

G9241 mutant strain spores were assessed for their capacity to germinate *in vitro* in the presence of L-alanine and inosine (Millipore-Sigma). A germinant solution was prepared with 100mM L-alanine, 25mM inosine and 1M Tris, pH 7.4. Ten µl of 10⁸-10⁹ G9241 spores were added to 190 µl of germinant in a 96-well plate that was immediately

placed onto a BioTek EL800 microplate reader (BioTek, Winooski, VT) to measure changes in relative fluorescent units (RFU) over a period of 30min at a wavelength of 562nm. The experiment was carried out in triplicate for each strain. Data were recorded by application of Gen5 1.11 Software (BioTek).

RNA EXTRACTION AND REVERSE-TRANSCRIPTASE PCR

Bacillus RNA was extracted via protocols modified from Lessard (55) and Gilbreath *et al.* (31). *Bacillus* cultures were grown under toxin-inducing conditions to mid-exponential phase for 4 hrs. Half of the culture (12.5ml) was pelleted via centrifugation at 10,000 x g for 15min at 4°C. The pellet was re-suspended in 1 ml lysozyme (20mg/ml in TE buffer) (Millipore Sigma) and incubated for two hours at 37°C, with shaking at 250 rpm. Two hundred microliters of 20% SDS (Millipore Sigma) were added to lyse the cells, followed by 10 µl of 20mg/ml proteinase K (Millipore Sigma). The samples were then incubated for one hour at 37°C with shaking. Three milliliters of TRIzol (Thermo Fisher Scientific) were added, and the tubes were shaken in a vortex 3x for 5 seconds each and then allowed to sit at room temperature for 3-5min. Chloroform was added at 0.2 ml per 1ml of TRIzol, and the samples were vortexed for 15sec. The samples were centrifuged at 3,000 x g for 15 min at 4°C, and the top aqueous phase was transferred to a fresh 15ml conical tube (Corning). An equal volume of 100% isopropanol was added, and the samples were shaken in a vortex at low speed. The RNA was subsequently isolated via a vacuum manifold procedure (31), where the samples were loaded onto RNeasy columns (QIAGEN, Germantown, MD) placed onto the vacuum manifold followed by the addition of RW1 buffer (QIAGEN). The columns were

then placed into collection spin tubes, DNase was added with RDD buffer (QIAGEN), and the samples were incubated for 60 min at room temperature. The samples were washed with RW1 buffer, and the flow through discarded following a spin at 12,000 x g for 30 sec. The samples were then washed twice with RPE buffer (QIAGEN) with a 30 sec spin at 12,000 x g, and another spin for 2 min. The flow-through was discarded after each spin. The columns were centrifuged one more time (12,000 x g for 2 min) and then placed into a new collection tube. The RNA was eluted with RNase-free H₂O two times. The RNA was subjected to gel electrophoresis on a 1% agarose and 0.5X TBE gel to assess the quality of the RNA.

The RNA was converted to cDNA with the Quantitect Reverse Transcription kit (QIAGEN). Reverse transcriptase PCR was done to assess whether *cer* and *pagA2* were co-transcribed. The PCR was carried out with the following primer pairs: primers P29 and P30 to detect *cer* transcript, primers P52/P53 for the *pagA2* transcript, and primers P54/P55 for the co-transcript.

DETECTION OF B-GALACTOSIDASE ACTIVITY IN *LACZ* PROMOTER FUSION CONSTRUCTS

As described above, three different promotor fusion constructs were generated for *cer* and *pagA2* with the pHT304-18z vector, where different-sized DNA fragments were inserted in front of a promotorless *lacZ* gene. To detect β -galactosidase activity, the *B. cereus* G9241 strains carrying the promoter constructs or an empty vector as a control were grown under toxin-inducing conditions for 4 hrs with shaking. The strains were further incubated overnight at 37°C in 5% CO₂ without shaking. At each time point, the samples were streaked on HI agar plates that were overlaid with 40 μ l of 20 mg/ml 5-

Bromo-4-Chloro-3-Indolyl β -D-Galactopyranoside (X-gal) substrate (Thermo Fisher Scientific). The colonies were observed for the presence of blue pigmentation indicative of β -galactosidase activity.

MOUSE INFECTION AND DISSEMINATION STUDIES

All mouse infections were done under ABSL-2 conditions and in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Uniformed Services University. Six-week-old female C57BL/6 and A/J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed in filter-top cages with standard food and water. Five mice per group were inoculated with a range of *B. cereus* G9241 spore doses via the s.c. or i.n. routes as described by Wilson *et al.* For s.c. inoculation, 100 μ l of spores were injected into the right flank of the mouse. For the i.n. inoculation, mice were lightly anesthetized via isoflurane inhalation, and 50 μ l of spore inoculum was pipetted into each nare to be inhaled. A/J and C57BL/6 mice were inoculated via both routes with the following strains: Δ *cer*, Δ *lef*, Δ *cer* Δ *lef*, Δ *pagA2*, Δ *pagA1*, and Δ *pagA1* Δ *pagA2*. Additionally, A/J mice were challenged s.c. only with the *cer.rest* Δ *lef*, *inact.cer.rest* Δ *lef*, and the *pagA2in* Δ *pagA1* G9241 mutant strains. At least two independent experiments were done with each strain and for each inoculation route. Following inoculation, mice were observed for morbidity and mortality, and moribund animals were humanely euthanized. The data were collected and the LD₅₀ values calculated via probit analyses with SPSS software (IBM, Armonk, NY). The Reed-Muench method (93) was used to calculate the LD₅₀ when the requirements for the probit analysis were not met. Median time to death was plotted with GraphPad Prism software

and assessed for statistical significance by the Kruskal-Wallis post-test with Dunn's multiple comparisons. The percent survival of mice inoculated s.c. with the $\Delta pagA2$ or Δcer mutant strains was compared to the WT G9241 strain value by Fischer's exact test.

A/J mice that survived the s.c. challenge with either $\Delta pagA1$ or $\Delta pagA1\Delta pagA2$ spores were challenged with equivalent doses of the same strain at day 14 and were monitored for an additional two weeks. Cardiac punctures were used to collect blood for detection of anti-PA2 antibodies. The blood was collected in 1.5ml microcentrifuge tubes (USA Scientific, Ocala, FL) and allowed to sit at room temperature for two hours. The tubes were held at 4°C overnight and subsequently centrifuged for 10min at 1700 x g. The serum was collected and pooled from mice given the same dose. The pooled sera were used as primary antibodies (1:100 dilution) in Western blot analyses against 1 µg of purified rPA or GST-PA2 proteins. Additionally, Western blot analyses were done with GST-PA2 protein that was cleaved with Factor Xa (New England Biolabs) to remove the GST tag. Briefly, 20 µg of GST-PA2 were digested overnight at room temperature with 1 µl of Factor Xa or 1 µl of H₂O as a control. The samples were re-suspended in 4x SDS buffer, denatured, and then subjected to SDS-PAGE electrophoresis. The protein was transferred to a nitrocellulose membrane for Western blot analysis with the pooled mouse sera as a probe.

Forty A/J mice were inoculated via the s.c. route in a dissemination experiment. Twenty mice were inoculated with 10⁶ of *cer.restΔlef* and the rest with 10⁶ *inact.cer.restΔlef*. For each group, 5 mice per cage were humanely euthanized at 6hr, 24hr, or 72hr post inoculation, while mortality in one cage of mice was followed through day five of infection. Any surviving mice in that cage were humanely euthanized at day 5

post inoculation. For each time point, mouse abscesses and spleens were collected for enumeration of colony forming units. The mouse abscesses were excised from the site of injection and placed into 1 ml of PBS in 2 ml microcentrifuge tubes (USA Scientific). The abscesses were weighed and then mashed with a plunger from a 10 ml syringe (BD, Franklin Lakes, NJ). The solid tissue was removed and discarded. The spleens were collected in 15 ml tubes with 1 ml PBS. The organs were weighed and homogenized. For both tissues, half of each sample was heat killed at 65°C for 30 min, then both halves were serially diluted and plated on LB agar plates for enumeration. The data were plotted and analyzed with GraphPad Prism software. Data were compared with a two-way ANOVA and the Bonferroni adjustment for multiple comparisons.

CHAPTER 3: Results

CONTRIBUTIONS

We thank Dr. Courtney Petro for her help in construction of pJMS1::*uCerd*, pJMS1::*inact.cer*, and pJMS1::*pagA2in*. These constructs were used to generate several mutant strains of *B. cereus* G9241. We thank Dr. James Sinclair for his aid in His-Certhrax protein purification, as well as purification of the anti-ADPr antibody. We thank Dr. James Vergis for generating the pET15b::*cer* and pET15b::*pagA2* constructs that were used to purify recombinant proteins, as well as the insertion of the upstream and downstream *cer* gene-flanking regions into pGem-T. We thank Dr. Roberto Cieza for his help with mouse cardiac punctures. We thank the laboratory of Dr. Joseph Barbieri for their purification of the GST-PA2 protein. Finally, we thank Dr. Stephanie Servetas for her help in RNA isolation.

SPECIFIC AIM 1: EVALUATE WHAT ROLE, IF ANY, PROTECTIVE ANTIGEN 2 PLAYS IN THE VIRULENCE OF G9241 *IN VIVO*.

Subaim 1.1: Construct a *pagA2* deletion mutant strain, as well as a double *pagA1/pagA2* mutant strain, and test each for virulence in s.c. and i.n. inoculation models of A/J and C57BL/6 mice.

Characterization of a markerless *pagA2* deletion mutant strain of *B. cereus* G9241, Δ *pagA2*

To assess the role of protective antigen 2 in pathogenesis of G9241, we generated an isogenic deletion mutant strain that we refer to hereafter as $\Delta pagA2$. The construction of $\Delta pagA2$ was described in the methods. We verified the loss of *pagA2* through Southern blot analysis (Fig. 4A). In this and all other Southern blot analyses, digoxigenin (DIG)-labeled probes were created through PCR amplification with gene-specific primers. To confirm the *pagA2* deletion, the probe was made with primers designed to anneal within the *cer* gene located approximately 600bp upstream of *pagA2*. For wild-type (WT) G9241 DNA that was digested with *Bam*HI and *Hind*III restriction endonucleases, the *cer*-specific probe was expected to hybridize to 2187 and 1514bp DNA fragments. When *pagA2* was deleted, a *Bam*HI site was introduced at the site of mutagenesis. Therefore, in the $\Delta pagA2$ mutant strain, the *cer*-specific probe was expected to hybridize to DNA fragments 1514 and 1413bp in size (Fig. 4A). DNA isolated from a plasmid cured strain of G9241 (pBCXO1-/pBC210-) was used as a negative control for the experiment.

Because the mutagenesis process involved several passaging steps, a PCR plasmid screen was used to ensure that the two virulence plasmids, pBCXO1 and pBC210, remained intact in the $\Delta pagA2$ mutant strain. The plasmid screen involved a set of 24 PCR reactions with primer pairs that anneal throughout the pBCXO1 and pBC210 plasmids, including a pair that annealed within the *bclA* gene on the chromosome of G9241. As seen in Fig. 5, the $\Delta pagA2$ mutant strain showed no loss of DNA from either of its plasmids.

Subsequently, $\Delta pagA2$ was assessed for any changes in growth rate, toxin and capsule expression, and spore germination as compared to WT G9241. The growth rate

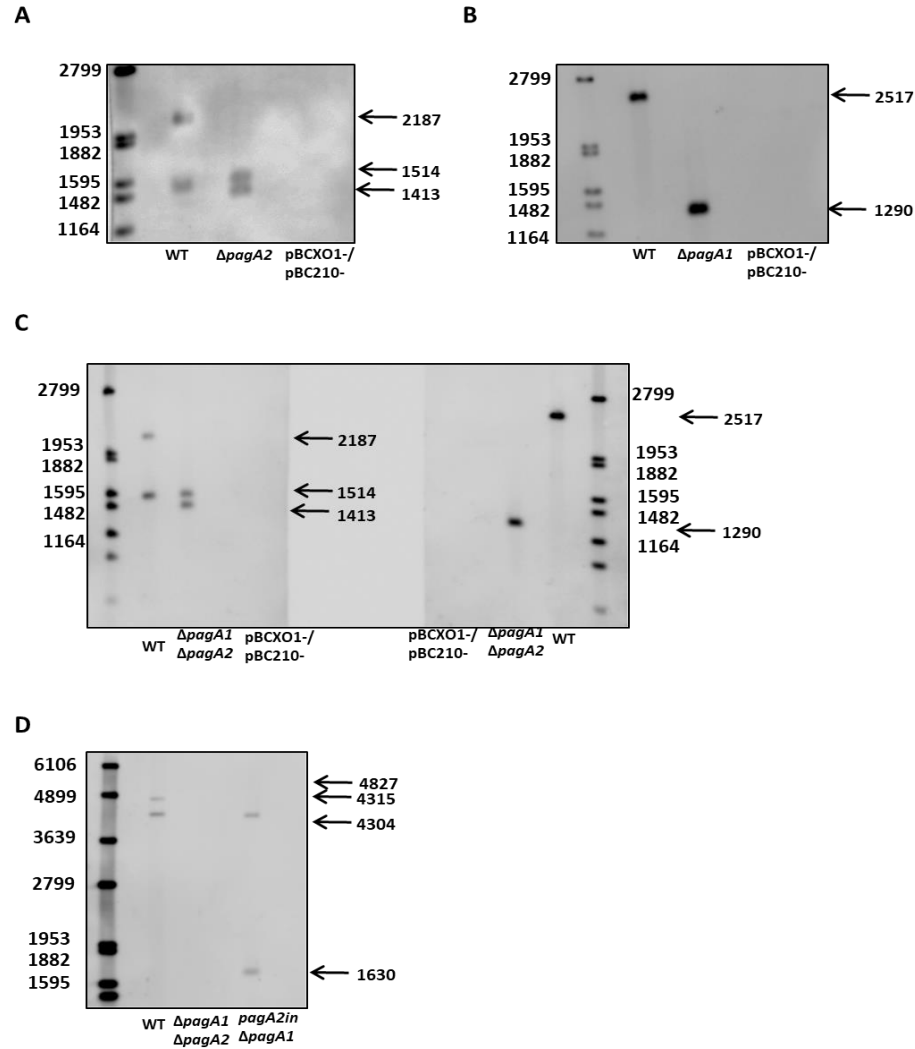


Fig. 4. Southern blot confirmation of $\Delta pagA2$, $\Delta pagA1$, $\Delta pagA1\Delta pagA2$, and $pagA2in\Delta pagA1$ mutant strains.

Southern blot analyses were done with DIG-labeled gene-specific probes hybridized to strain DNA digested with restriction endonucleases, as described in the methods. DNA from wild-type (WT) and plasmid-cured (pBCXO1-/pBC210-) G9241 strains were used as positive and negative controls, respectively. The $\Delta pagA2$ mutation (**A**) was confirmed with a *cer*-specific gene that was expected to hybridize to 2187bp and 1514bp bands in WT DNA, and 1514 and 1413bp bands in the mutant strain. The $\Delta pagA1$ mutation (**B**) was confirmed with a *pagA1*-specific probe that was expected to hybridize to a 2517bp band in WT DNA and a 1290bp band in the mutant strain. The same two probes were used to confirm the $\Delta pagA1\Delta pagA2$ mutant strain (**C**), such that the deletion of the *pagA2* gene is depicted on the left of the figure, and the *pagA1* deletion is depicted on the right. The $pagA2in\Delta pagA1$ knock-in mutant strain (**D**) was confirmed with a *pagA2*-specific probe that was expected to hybridize to a 4827 and 4315bp band in WT DNA, and a 4304 and 1630bp band in the mutant strain. The arrows depict the actual size of the observed bands.

experiment was carried out in LB broth over a period of six hours, where samples of *ΔpagA2* or WT were collected every hour for measurement of culture turbidity (OD₆₀₀) and for enumeration of colony-forming units. The experiment was done with three biological replicates for each strain. As shown in Fig. 6A, the *ΔpagA2* mutant strain had no growth defects as compared to WT G9241 in the rich medium.

Western blot analyses were carried out to show that the *ΔpagA2* deletion mutant strain did not, in fact, express PA2 following growth under toxin-inducing conditions and also to ensure that the strain still produced PA, LF and EF. Protein samples from supernatants of cultures of wild-type G9241, *ΔpagA2*, and the plasmid cured strain of G9241 were concentrated by precipitation with ice-cold ethanol as described in the methods. Oriole stain was used to observe total protein of each sample on SDS-PAGE gels to ensure similar protein concentrations were loaded on the gel (Fig 7A). The Western blot analyses were done with anti-PA (Fig. 7B), anti-LF (Fig. 7C), anti-EF (Fig. 7D) and anti-PA2 (Fig. 7E) primary antibodies as probes. As shown in the figure, the *ΔpagA2* mutant strain expressed PA, LF and EF toxin components in culture supernatants but not PA2.

To assess capsule expression, the mutant was grown under capsule-inducing conditions, as described in the methods, and a portion of the culture was fixed with formaldehyde for capsule staining. Half of the sample was treated with hyaluronidase to digest the hyaluronic acid (HA) capsule so the tetrassacharide capsule could be observed. The other half of the sample was treated with water as a control to view the HA capsule. The samples were stained with the Maneval stain and observed under the microscope at

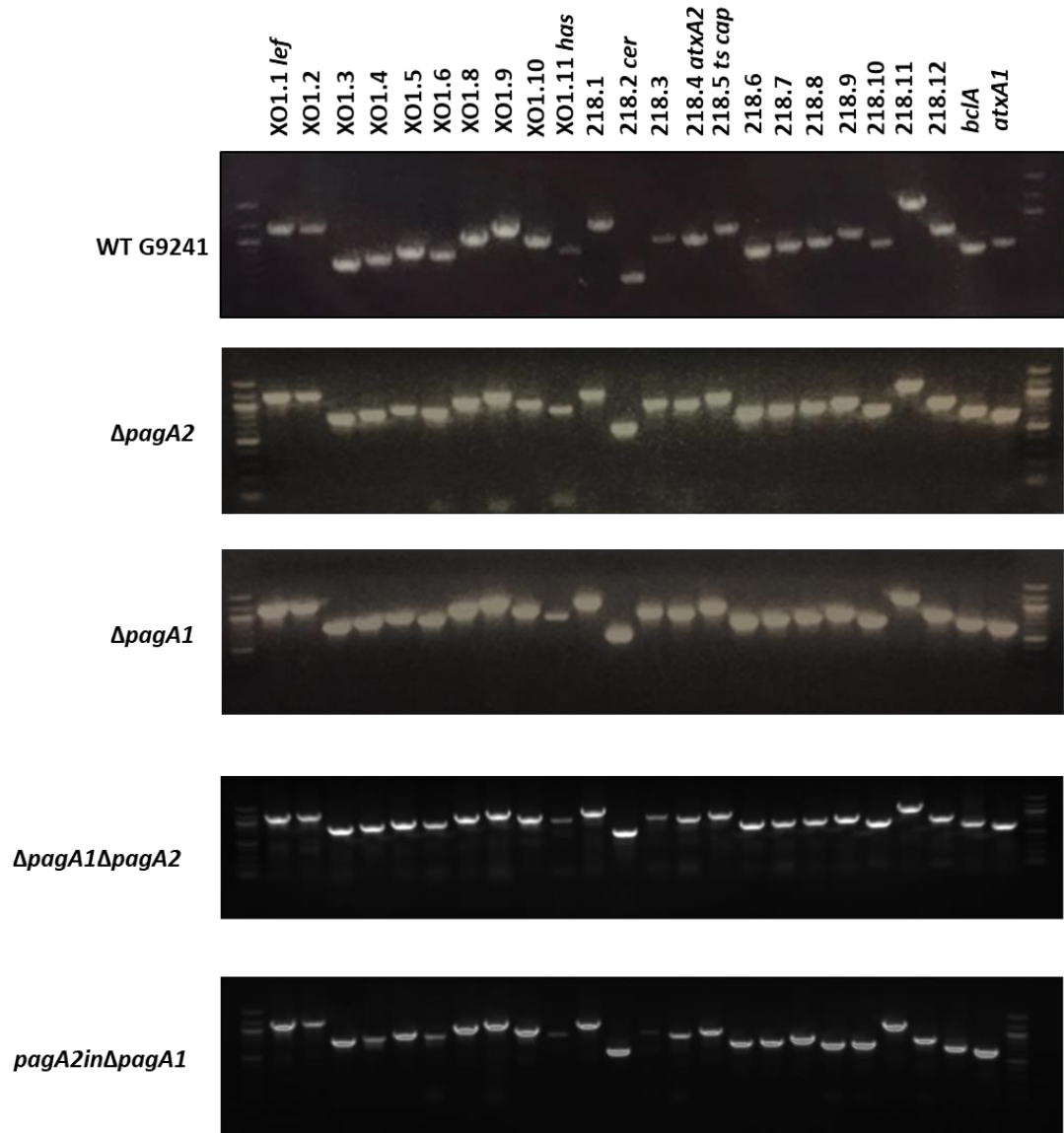


Fig. 5. PCR plasmid screen of $\Delta pagA2$, $\Delta pagA2$, $\Delta pagA1\Delta pagA2$, and $pagA2in\Delta pagA1$ mutant strains as compared to wild-type G9241.

A set of 24 PCR reactions were done for each mutant strain with primers that anneal to regions along the pBCX01 and pBC210 plasmids, as well as to a chromosomal gene, *bclA*.

1000X. Like WT G9241 (Fig. 8A, B), the $\Delta pagA2$ mutant strain expressed both the HA capsule (Fig. 8C) and the tetrassacharide capsule (Fig. 8D). On the other hand, the plasmid-cured strain of G9241 that was used as a negative control in the experiment was unencapsulated (Fig. 8K,L).

Once the molecular integrity of the $\Delta pagA2$ mutant strain was established, we generated $\Delta pagA2$ spores, as detailed in the methods. The spores were then assessed for their capacity to germinate *in vitro* in the presence of two germinants, L-alanine and inosine. The mutant spores were added to the germinant solution in a 96-well plate, and germination was assessed via measurement of changes in optical density over a period of 30 minutes. As compared to WT G9241 (Fig. 9A), the $\Delta pagA2$ spores (Fig. 9B) had no germination defect.

In vivo pathogenesis of $\Delta pagA2$ G9241

To determine whether PA2 contributes to virulence of G9241, we inoculated six week-old female A/J and C57BL/6 mice with $\Delta pagA2$ spores through the subcutaneous (s.c.) and intranasal (i.n.) routes. For the s.c. inoculation, mice were injected with 100 μ l of 10^5 - 10^6 spores into the right flank. For the i.n. experiments, mice were first anesthetized with isoflurane and 50 μ l of spores, ranging in dose from 10^2 to 10^7 , were then pipetted onto each of the nares to be inhaled. Mice were subsequently monitored for 14 days for morbidity and mortality, and the 50% lethal dose (LD₅₀) was calculated using SPSS probit analysis. As shown in Table 4, there was no significant difference in lethality in A/J mice inoculated through either route with the $\Delta pagA2$ spores as compared to WT

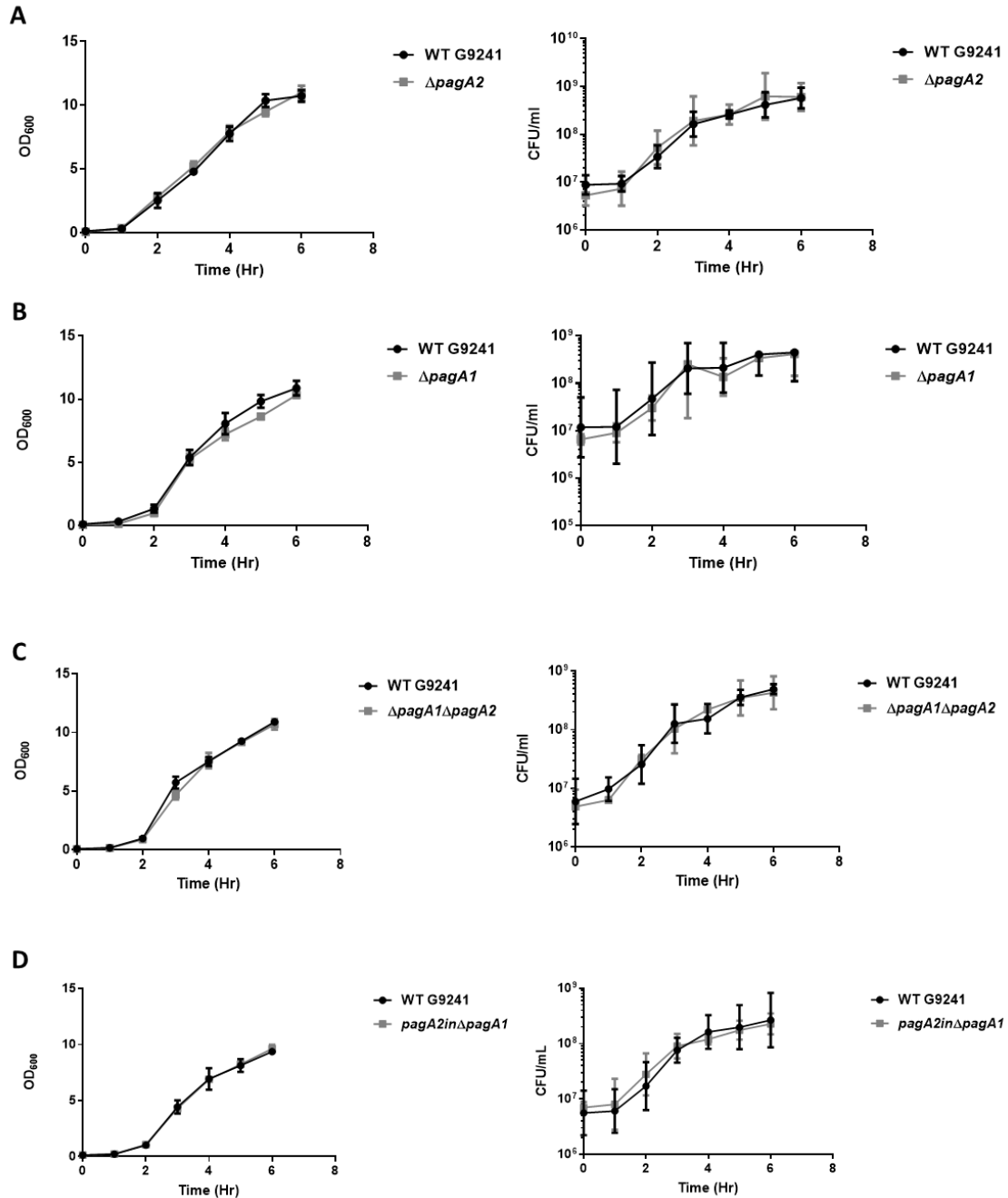


Fig. 6. Growth curves of $\Delta pagA2$, $\Delta pagA1$, $\Delta pagA1\Delta pagA2$, and $pagA2in\Delta pagA1$ mutant strains as compared to wild-type G9241.

Cultures of wild-type (WT) G9241 and $\Delta pagA2$ (A), $\Delta pagA1$ (B), $\Delta pagA1\Delta pagA2$ (C), or $pagA2in\Delta pagA1$ (D) were grown in LB broth for six hours with aeration. Samples were collected hourly to measure optical density (OD₆₀₀) and were serially diluted for enumeration of colony-forming units (CFU/ml). The graphs represent an average (OD₆₀₀) or geometric mean (CFU/ml) of three biological replicates with standard error depicted with the bars.

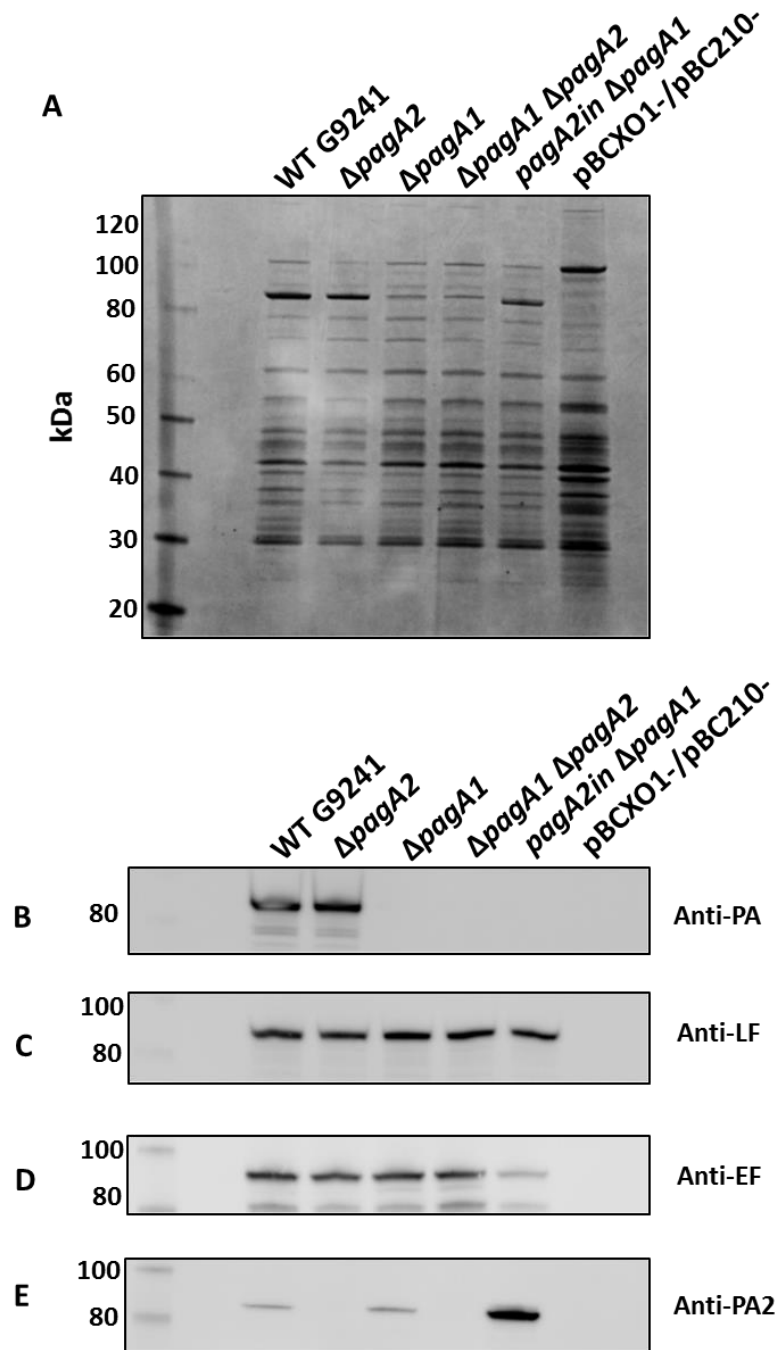


Fig. 7. Western blot analyses of toxin component expression of $\Delta pagA2$, $\Delta pagA1$, $\Delta pagA1 \Delta pagA2$, and $pagA2in \Delta pagA1$ mutant strains. Cultures of the *B. cereus* G9241 mutant strains were grown under toxin-inducing conditions. Supernatant proteins were precipitated with ice cold ethanol and subject to SDS-PAGE electrophoresis. Oriole stain (A) was used to view total protein. Proteins were transferred to nitrocellulose membranes and probed with anti-PA (antibody against PA of *B. anthracis*) (B), anti-LF (C), anti-EF (D), and anti-PA2 (E) antibodies for Western blot analyses.

G9241. The LD₅₀ values were calculated to be 20 and 1x10⁴ spores for the s.c. and i.n. experiments, respectively. Additionally, the median time to death was assessed for each mouse inoculated with the lower doses of the *ΔpagA2* mutant strain, and compared to mice challenged with WT G9241 (Fig. 10A). For the A/J mice that actually succumbed to the infection following challenge with the mutant spores, there was no difference in the time to death as compared to WT. But, there was a statistically significant difference in the average median time to death between WT and the mutant strain for the lowest inoculum group because there were fewer mice that died following challenge with *ΔpagA2* spores ($p=0.0065$ via Mann-Whitney rank sum test). The calculated percent survival in A/J mice inoculated with the 10¹ dose of *ΔpagA2* spores was 60%, which is statistically significantly higher than the 13.3% survival rate of mice inoculated with WT ($p= 0.0280$ via Fischer's exact test). In the C57BL/6 mouse model, there was no difference in LD₅₀ values in mice inoculated via the intranasal route, where the LD₅₀ was 6x10⁵ for the *ΔpagA2* mutant strain and 4x10⁵ for WT, but there was a statistically significant attenuation in virulence in mice subcutaneously inoculated with the *ΔpagA2* spores [LD₅₀ of 200 spores for *ΔpagA2* as compared to 40 for WT (Table 4)]. Moreover, we observed a similar pattern in the median time to death in the s.c. model of C57BL/6 mice to what was seen in A/J mice, such that there was no difference in the time to death in mice that did succumb to the infection with the lowest tested dose of the *ΔpagA2* mutant strain (Fig. 10B), as compared to WT G9241. There was, however, a statistical increase in the average median time to death for that inoculum dose in mice inoculated with the mutant strain due to differences in survival rates ($p= 0.0131$ via Mann-Whitney rank sum test). Indeed, 80% of C57BL/6 mice survived inoculation with 10¹ of *ΔpagA2*

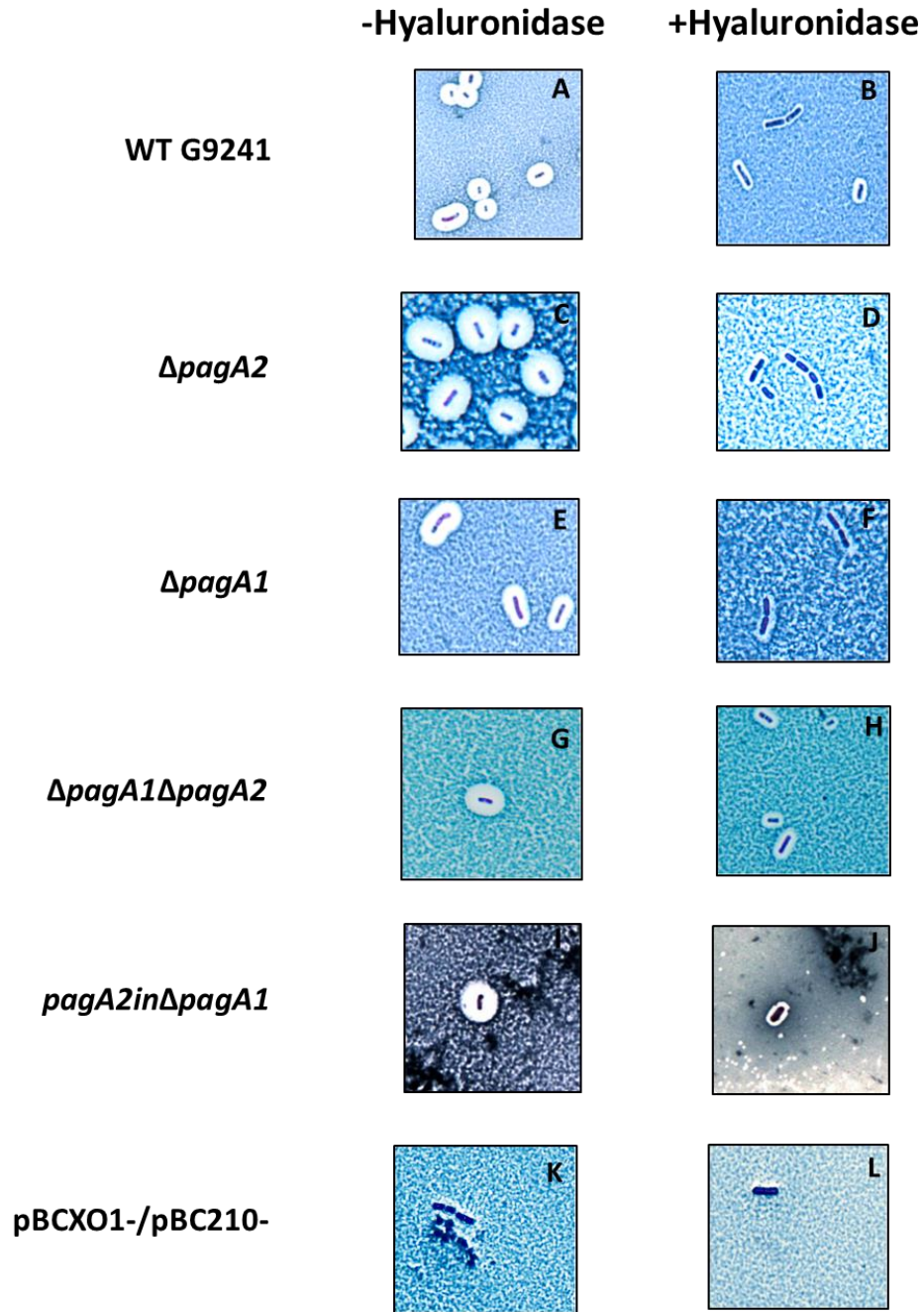


Fig. 8. Capsule production of the *pagA* mutants of G9241. Cultures of wild-type (WT) G9241 (A,B), $\Delta pagA2$ (C,D), $\Delta pagA1$ (E,F), $\Delta pagA1\Delta pagA2$ (G,H), $pagA2in\Delta pagA1$ (I,J) and pBCXO1-/pBC210- G9241 were grown under capsule-inducing conditions for 24 hours, as described in the methods. The samples were treated water (A, C, E, G, I, K) or hyaluronidase (B, D, F, H, J, L) for two hours at 37°C and subsequently stained with the Maneval stain to visualize the hyaluronidase and tetrassacharide capsules, respectively. Images were captured at 1000X magnification.

spores and only 27% survived WT G9241 for the same dose ($p=0.0154$). These results suggest that PA2 does contribute to virulence of *B. cereus* G9241 but in a limited capacity as the effect can only be seen at the lowest inoculum doses. The data also implicated PA1 as the more essential toxin binding subunit in G9241.

Characterization of $\Delta pagA1$ and $\Delta pagA1\Delta pagA2$ mutant strains of G9241

Since PA1 appears to be the more essential binding subunit involved in anthrax toxin translocation in G9241, we sought to determine whether expression of PA1 masked more substantial *in vivo* effects of PA2 in the two mouse models used in this study. To do so, we generated an isogenic deletion mutant of *pagA1*, $\Delta pagA1$, and a double *pagA1/pagA2* deletion mutant, called $\Delta pagA1\Delta pagA2$. The $\Delta pagA1$ mutant strain was generated via a similar approach that was used for $\Delta pagA2$. Instead of a *Bam*HI site, an *Eco*RI restriction site was left to mark the *pagA1* deletion following homologous recombination. The successful deletion of *pagA1* was confirmed via Southern blot analysis but with a *pagA1*-specific probe that hybridized to the 3' end of the gene and to a portion of the downstream DNA. For the analysis, WT G9241, $\Delta pagA1$ and pBCXO1-/pBC210- DNA samples were digested with *Eco*RI and *Hind*III restriction endonucleases. The probe was expected to hybridize to a 2517bp band in the WT G9241 and a 1290bp band in the $\Delta pagA1$ mutant strain. As seen in Fig. 4B, the full *pagA1* gene was indeed deleted in the $\Delta pagA1$ mutant strain. To generate the $\Delta pagA1\Delta pagA2$ mutant strain, we deleted *pagA2* in the $\Delta pagA1$ mutant strain background, as previously described. Both the *cer*- and *pagA1*-specific DIG probes were used to confirm the double deletion

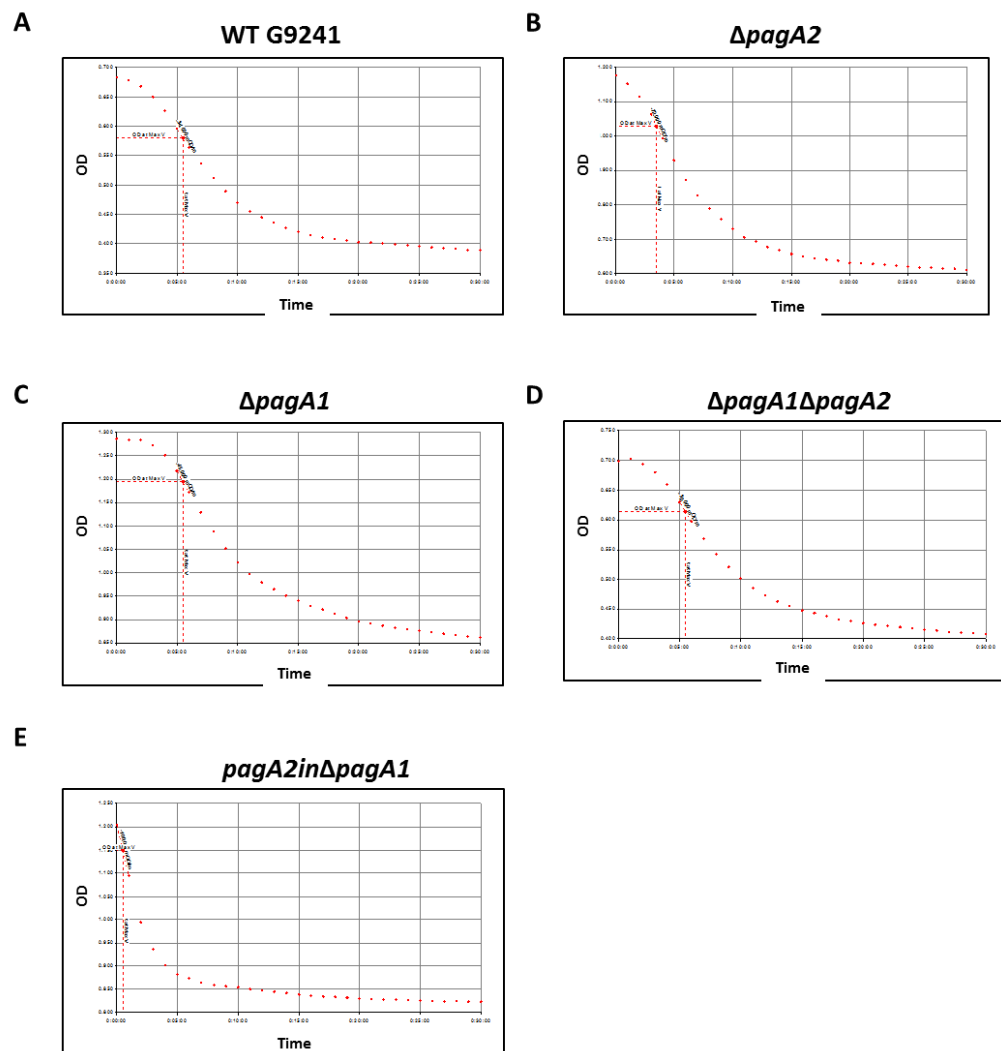


Fig. 9. *In vitro* germination of $\Delta pagA2$, $\Delta pagA1$, $\Delta pagA1\Delta pagA2$, and $pagA2in\Delta pagA1$ mutant spores.

Spores of wild-type (WT) G9241 (A), $\Delta pagA2$ (B), $\Delta pagA1$ (C), $\Delta pagA1\Delta pagA2$ (D), or $pagA2in\Delta pagA1$ (E) were incubated with germinants L-alanine and inosine for 30min. The optical density was recorded at a wavelength of 562nm every minute with a plate-reader. Each graph is a representative of three separate experiments for each strain.

mutant strain (Fig. 4C). The *cer* probe hybridized to 2187 and 1514bp bands of WT DNA, and 1514 and 1413bp bands in the mutant strain to confirm the absence of the *pagA2* gene. The *pagA1* probe, on the other hand, hybridized to a 2517bp DNA fragment of WT G9241, and a 1290bp fragment in the mutant, due to the deletion of the *pagA1* gene (Fig. 4C).

The PCR plasmid screen showed that the pBCXO1 and pBC210 plasmids were intact in both new mutant strains (Fig. 5). Additionally, neither $\Delta pagA1$ nor $\Delta pagA1\Delta pagA2$ had a growth defect as compared to WT G9241 (Fig. 6B, C) when grown in rich medium.

To further characterize the $\Delta pagA1$ and $\Delta pagA1\Delta pagA2$ mutant strains, Western blot analyses were carried out to assess toxin expression while Maneval staining was used to observe capsule expression. As seen in Fig. 7B, $\Delta pagA1$ and $\Delta pagA1\Delta pagA2$ did not express PA1 in culture supernatants. This observation further confirmed the successful deletion of the *pagA1* gene. Additionally, $\Delta pagA1\Delta pagA2$ did not express PA2 *in vitro* (Fig. 7E). However, both mutants still produced LF and EF, as well as PA2 in the case of $\Delta pagA1$. Following growth of cultures under capsule-inducing conditions, it was apparent that both strains produced the HA and tetrassacharide capsules (Fig. 8G-J).

To assess virulence of $\Delta pagA1$ and $\Delta pagA1\Delta pagA2$ *in vivo*, we generated spores of both mutant strains. The spores had no *in vitro* germination defects, as seen in Fig. 9C and 9D. We first inoculated A/J and C57BL/6 mice with the $\Delta pagA1$ mutant spores. The mutant strain was avirulent in the s.c. inoculation model of C57BL/6 mice, and just a few mice died in the 10^7 dose following i.n. inoculation. There was a significant attenuation in virulence of $\Delta pagA1$ in the A/J mice as well when compared to WT G9241. We

Table 4. LD₅₀ spore values of G9241 and *pagA* mutant strains following inoculation of A/J or C57BL/6 mice by the subcutaneous (s.c.) or inhalational (i.n.) route.

	A/J		C57BL/6	
	s.c.	i.n.	s.c.	i.n.
WT G9241	10 ^a	3x10 ^{3a}	40 ^a (16-100)	4x10 ^{5a} (2x10 ⁵ -9x10 ⁵)
$\Delta pagA2$	20 ^a	1x10 ^{4a}	200 ^{a,c} (70-500)	6x10 ^{5a} (2x10 ⁵ -3x10 ⁶)
$\Delta pagA1$	2x10 ^{4b}	2x10 ^{5a,c} (6x10 ⁴ -5x10 ⁵)	>3x10 ⁷	2x10 ^{6b}
$\Delta pagA1\Delta pagA2$	6x10 ^{4b}	3x10 ^{5a,c} (1x10 ⁵ -7x10 ⁵)	-----	-----
<i>pagA2in</i> $\Delta pagA1$	3x10 ^{5b}	-----	-----	-----

- LD₅₀ values calculated via probit analyses with 95% confidence intervals provided in parentheses.
- LD₅₀ values calculated by the Reed and Muench method, when the criteria for probit analysis were not met.
- Values are significantly different from WT G9241 as determined by probit analyses ($p < 0.05$).

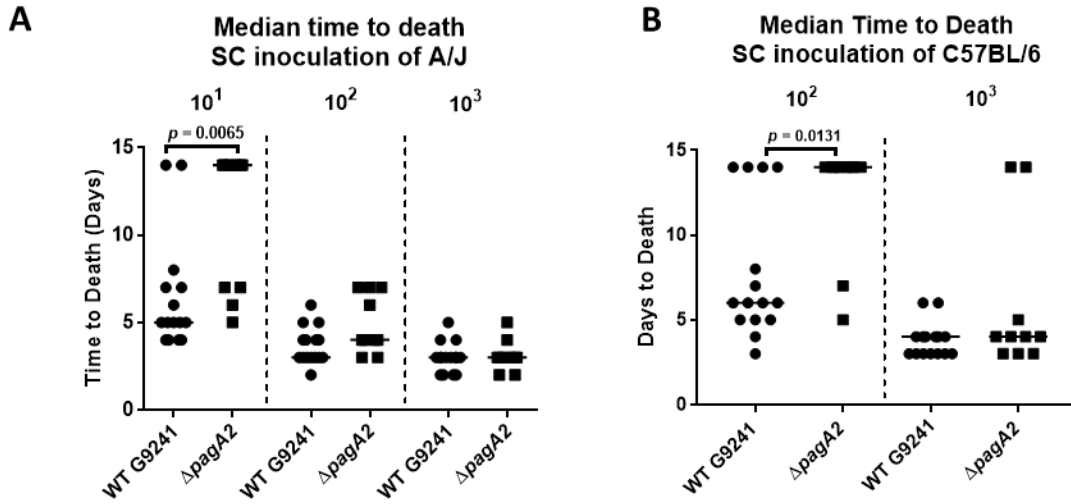


Fig. 10. Median time to death of mice inoculated subcutaneously with 10^1 - 10^3 of *B. cereus* G9241 spores.

A/J (**A**) and C57BL/6 (**B**) six-week old female mice were inoculated with either WT G9241 (●) or $\Delta pagA2$ (◻) spores. Each point represents the time to death of a single mouse and the dashed line represents the group median. For this calculation, time to death of animals that did not die in the 14-day observation period was recorded as 14 days. The medians were compared with the Mann-Whitney rank sum test, and significant values depicted with their appropriate p values.

observed approximately a two log attenuation in mice inoculated via the i.n. route in the A/J mice. The LD₅₀ value for the s.c. route was calculated to be 2x10⁴ via the Reed and Muench method (Table 4). That procedure was used because there wasn't a single inoculum group where 100% of the mice died, and, therefore, the data did not satisfy the criteria necessary for probit analysis. The results were consistent in two different experiments with 5 mice per group inoculated per dose.

Since the $\Delta pagA1$ mutant strain was almost avirulent by both inoculation routes in the C57BL/6 mice, only the A/J mice were inoculated with the $\Delta pagA1\Delta pagA2$ mutant spores (Table 4). The double knock-out was as attenuated as the $\Delta pagA1$ mutant strain in mice inoculated via the i.n. route, with a calculated LD₅₀ of 3x10⁵. This value was statistically different when compared to WT G9241. The double knock-out was highly attenuated when given to A/J mice s.c. with an LD₅₀ value calculated to be 6x10⁴ via the Reed and Muench method. Thus, there was no difference in virulence between the $\Delta pagA1$ and $\Delta pagA1\Delta pagA2$ mutant strains. Therefore, the data further support the dominant role of PA1 in the pathogenesis of G9241 and the minimal involvement of PA2.

In vivo expression of PA2

The construction of the $\Delta pagA1$ and $\Delta pagA1\Delta pagA2$ mutant strains additionally provided the means to explore PA2 expression *in vivo*. As described in the methods, A/J mice that survived the s.c. inoculation with either mutant strain were subsequently inoculated with an equal dose of spores and monitored for an additional 14 days. Cardiac punctures were done to collect blood so as to isolate serum from each mouse. Serum

samples collected from the same inoculum group were pooled and used in Western blot analyses to probe against purified recombinant PA (rPA) or a purified GST-tagged PA2 (GST-PA2). The proteins are approximately 83kDA and 120kDA, respectively (Fig. 11D). We observed strong reactivity on Western blots of pooled sera from mice inoculated with $10^7/10^6$ dose of $\Delta pagA1$ with GST-PA2 (Fig. 11A). A very faint band was observed on the blot that used serum pooled from mice inoculated with 10^5 of $\Delta pagA1\Delta pagA2$ (Fig. 11B). We suspected that the faint band was due to cross-reactivity with the GST tag. Indeed, when the tag was cleaved with Factor Xa, there was no reactivity of the serum with 20 μ g of cleaved protein (Fig. 11C). These data suggest that PA2 is indeed expressed *in vivo* in mice following inoculation with G9241 and that there is an elicited host response against the protein.

Subaim 1.2: Generate a *pagA2* knock-in mutant strain in *B. cereus* G9241 $\Delta pagA1\Delta pagA2$ to assess if PA2 can functionally replace PA1 in virulence.

Characterization of a *pagA2* knock-in mutant strain, where *pagA2* is cloned into the site of *pagA1*

Our experiments with the *pagA2* and *pagA1* deletion mutant strains suggest that PA2 has a minimal role in pathogenesis, as compared to PA1. Beyond just amino acid differences between PA1 and PA2, there are also differences in the upstream promoter regions of the two genes. Indeed, these regions are not homologous. Additionally *pagA1* is part of an operon with the auto-regulator *pagR*, while there is no *pagR* homolog downstream of *pagA2*. Therefore, we sought to investigate whether PA2 could

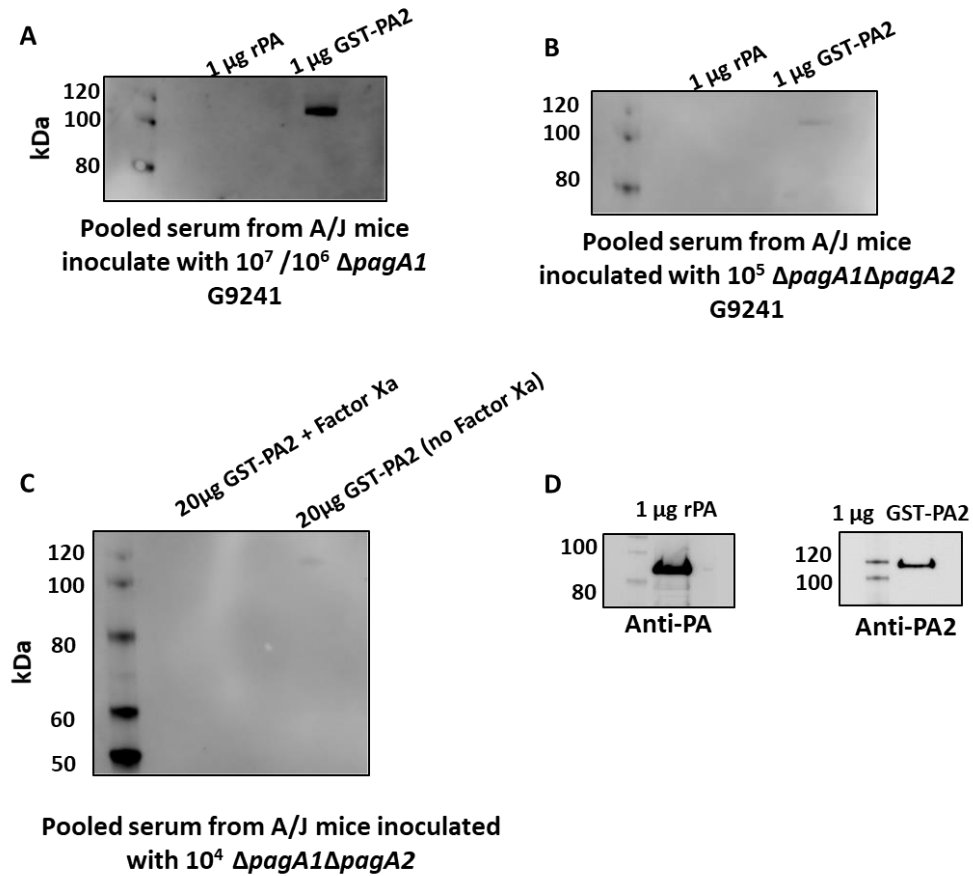


Fig. 11. Western blot analyses to detect anti-PA2 antibodies in sera of mice inoculated subcutaneously with $\Delta pagA1$ or $\Delta pagA1\Delta pagA2$.

Cardiac punctures were done to obtain serum from A/J mice that survived s.c. inoculation with either of the two mutant strains. One microgram of rPA or GST-PA2 were probed with pooled sera from mice inoculated with $10^7/10^6$ dose of $\Delta pagA1$ G9241 (A) or pooled sera from mice inoculated with 10^5 of $\Delta pagA1\Delta pagA2$ spores (B). Sera were diluted at a 1:100 concentration in PBS-T-SM. The GST-PA2 protein (20 μg) was digested with Factor Xa to test if the GST tag reacted non-specifically with serum (C). Undigested GST-PA2 protein was used as a control. Blots probed with anti-PA (PA from *B. anthracis*) or anti-PA2 antibodies were used to test the integrity of the rPA and GST-PA2 proteins, respectively (D).

functionally replace PA1 if all other expression conditions were the same between the two. Therefore, we generated a *pagA2* knock-in mutant strain, where *pagA2* was cloned into the site of *pagA1* within the $\Delta pagA1 \Delta pagA2$ mutant strain background, as described in the methods. A *pagA2*-specific DIG- labeled probe was used to confirm the knock-in mutant strain. WT, $\Delta pagA1 \Delta pagA2$ and *pagA2* $\Delta pagA1$ DNA were digested with the *Bam*HI endonuclease. In the case of WT DNA, the *pagA2* probe hybridized to the expected size bands of 4827 and 4315bp (Fig. 4D) and did not hybridize to the DNA of the $\Delta pagA1 \Delta pagA2$ deletion mutant strain. When *pagA2* was inserted into the *pagA1* site in the knock-in mutant strain, the probe hybridized to a 4304bp and 1630bp DNA fragment, as anticipated (Fig. 4D). Additionally, we confirmed that there was no loss of DNA across the pBCXO1 and pBC210 plasmids (Fig. 5) and that the *pagA2in* $\Delta pagA1$ mutant strain had no growth defects as compared to WT G9241 (Fig. 6D)

The successful construction of the *pagA2in* $\Delta pagA1$ mutant strain was further confirmed through Western blot analysis. Whereas the parent strain, $\Delta pagA1 \Delta pagA2$, did not express PA1 or PA2, the knock-in mutant did express PA2 (Fig. 7E). The banding pattern seen on the Western blot, as well as the Oriole stain (Fig. 7A) also suggested that there may be more PA2 made in the knock-in mutant strain than in WT G9241. The band intensity of PA2 was assessed on the blot by measuring the integrated density (i.d.) of the band with the Photoshop software, and the value was adjusted to reflect differences in the total protein density measured on the Oriole stain. The calculated integrated density of the PA2 band in the knock-in mutant was four times higher than the intensity of the protein in the WT G9241. Additionally, the mutant expressed LF and EF (Fig. 7C, D), as

well as both of the capsules (Fig. 8I, J). Finally, the *pagA2inΔpagA1* spores had no *in vitro* germination defects (Fig. 9E).

Since the PA2 effect was observed only in the s.c. inoculation model, A/J mice were inoculated with a range of doses of the *pagA2inΔpagA1* spores and monitored for 14 days. The morbidity and mortality pattern of the knock-in mutant was similar to that of *ΔpagA1* and *ΔpagA1ΔpagA2* G9241, with the LD₅₀ value calculated to be 3x10⁵ via the Reed and Muench method. These data suggest that PA2 cannot functionally replace PA1 to effectively translocate anthrax toxins to the host cells.

SPECIFIC AIM 2: ASSESS THE ROLE THAT CETHRAX PLAYS IN PATHOGENESIS OF G9241 *IN VIVO*.

Subaim 2.1: Identify *in vitro* conditions that promote Certhrax gene and protein expression.

When we began our studies, the expression of *cer* in *B. cereus* G9241 had only been shown in the transcriptome analyses carried out by Passalacqua *et al.* (87). The growth conditions used in the study are different from the toxin-inducing conditions we use in the laboratory. Therefore, we sought to establish that *cer* was indeed expressed when G9241 was grown in HI broth supplemented with 8% sodium bicarbonate and 5% CO₂ at 37°C. RNA was isolated from three different sets of WT G9241 cultures as described in the methods. One microgram of RNA was used to make cDNA for each replicate, and another microgram was used as a negative control (no RT) to ensure that the RNA was free of DNA contamination. PCR reactions were set up with *cer*- specific primers to test for the presence of *cer* transcript (primer pair 1 in Fig. 12). Additionally,

pagA2- specific primers (primer pair 2 in Fig. 12) were used as a positive control to confirm that the growth conditions were conducive to toxin component expression. WT DNA and sterile water were further used as positive and negative controls in the experiment, respectively. As seen in Fig. 12, both *cer* and *pagA2* were expressed in all three samples of WT cDNA. No product was observed in any of the ‘no RT’ samples, an observation that indicated that there was no DNA contamination in the isolated RNA. Additionally, with the use of primers that amplified the intergenic region between *cer* and *pagA2* (primer pair 3 in Fig. 12), we observed a transcript. Thus, we concluded that the two genes are, in fact, co-transcribed as an operon.

To better understand how *cer* and *pagA2* are transcribed, we constructed several promoter fusions for each. The goal was to identify where the promoter for *cer* is located and if *pagA2* has its own promoter despite being the downstream gene in the operon. As depicted in Fig. 13A, three different-sized upstream regions were amplified for each gene and cloned into the pHT304-18z vector, such that the fragments were inserted in front of a promoterless *lacZ* gene. For *cer*, the primers CerUp1, CerUp2, and Cerup3 amplified 109, 267, and 540bp fragments, respectively, where the reverse primer annealed to DNA immediately upstream of the ATG of the protein. For *pagA2*, the fragments generated via PCR were 155, 273, and 549bp upstream of the start codon of the open reading frame. Once the fragments were cloned into the pHT304-18z vector each construct, as well as an empty vector control, was electroporated into wild-type G9241. The strains were grown under toxin-inducing conditions for 4 hours, as previously described, and were further incubated overnight at 37°C, 5% CO₂ without shaking. At each time point, cultures were streaked out on HI agar plates, supplemented

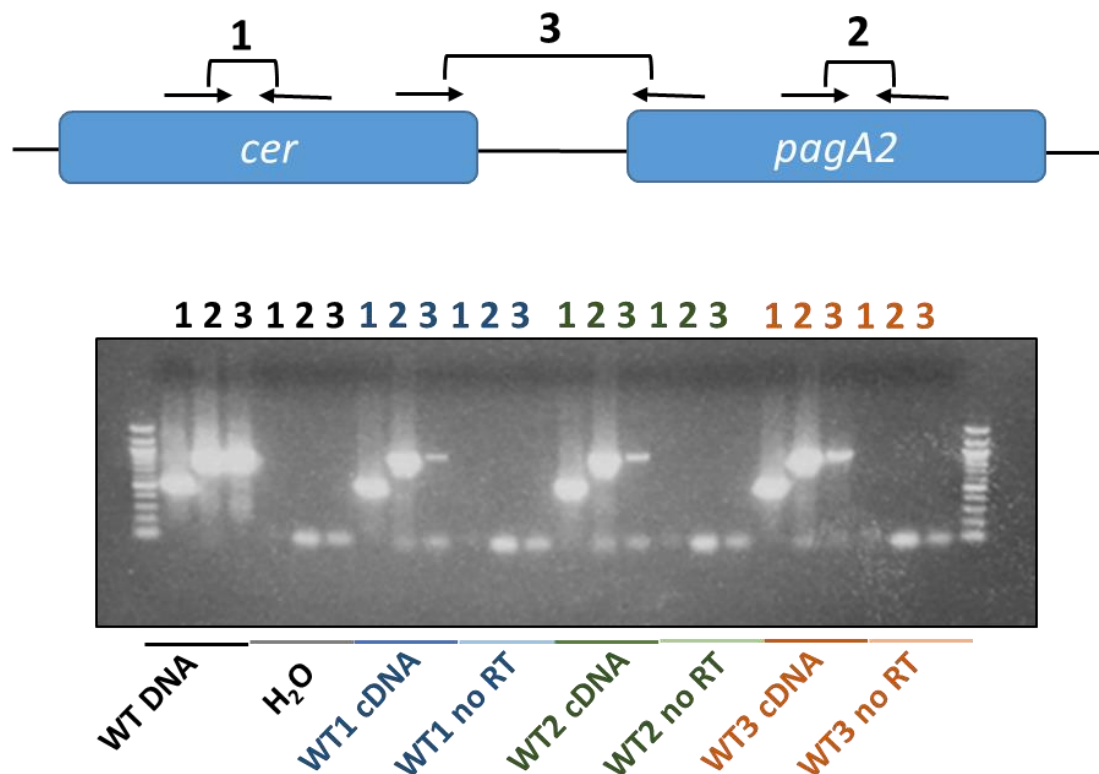


Fig. 12. PCR analyses to detect *cer*, *pagA2* and co-transcript expression in WT G9241 cDNA.

RNA was isolated from three sets of cultures of WT G9241 grown under toxin-inducing conditions. One microgram of RNA was used to make cDNA and a no RT control, where no reverse-transcriptase was added. PCR reactions were done with *cer*- specific primers (1), *pagA2*- specific primers (2), and primers that amplified the *cer-pagA2* intergenic region (3) as shown in the top diagram. WT DNA and water were used as positive and negative controls, respectively.

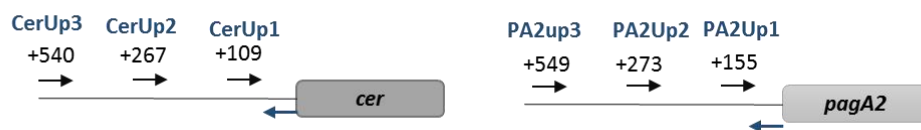
with erythromycin and overlaid with the X-gal substrate. The colonies on the plates were evaluated for β -galactosidase (β -gal) activity (blue color) after overnight incubation at 37°C. As pictured in the captured images for the *cer* constructs (Fig. 13B) the expression of *lacZ* was observed in the pHT304-18z::*cerup2* and pHT304-18z::*cerup3* G9241, but not in pHT304-18z::*cerup1*. The data were consistent for both time points and across several replicate experiments. No β -gal activity was observed in any of the empty vector controls. These results suggest that the *cer* promoter may be located within 267 bases upstream of the *cer* ATG, and that the first 109bp are insufficient to promote β -gal expression. Furthermore, the data shown in Fig. 13C suggest that *pagA2* does in fact have its own promoter. The β -gal activity was noted for all *pagA2* promoter constructs but not in the empty vector control. Therefore, we surmised that the *pagA2* promoter may lie within 155bp upstream of the *pagA2* ATG.

Beyond the above mentioned transcription analyses, experiments were carried out to show expression of Certhrax protein *in vitro*. Based on the amino acid composition, the predicted size for Certhrax was 55kDa. To generate an antibody against the protein, serum was collected from a rabbit that was immunized with a purified histidine-tagged ADPr portion of Certhrax. The anti-ADPr antibody was subsequently purified, as described in the methods, and used for Western blot analyses at a 1:500 dilution in PBS-T-SM to detect Certhrax in G9241 supernatants or cell lysates. However, no protein was detected on the blots from cultures grown under toxin- or capsule-inducing conditions (data not shown). To determine whether that was due to low Certhrax concentrations in the samples, *cer* was cloned into an IPTG-inducible vector, pUTE657. Thus, expression of the gene was placed under the control of an inducible promoter,

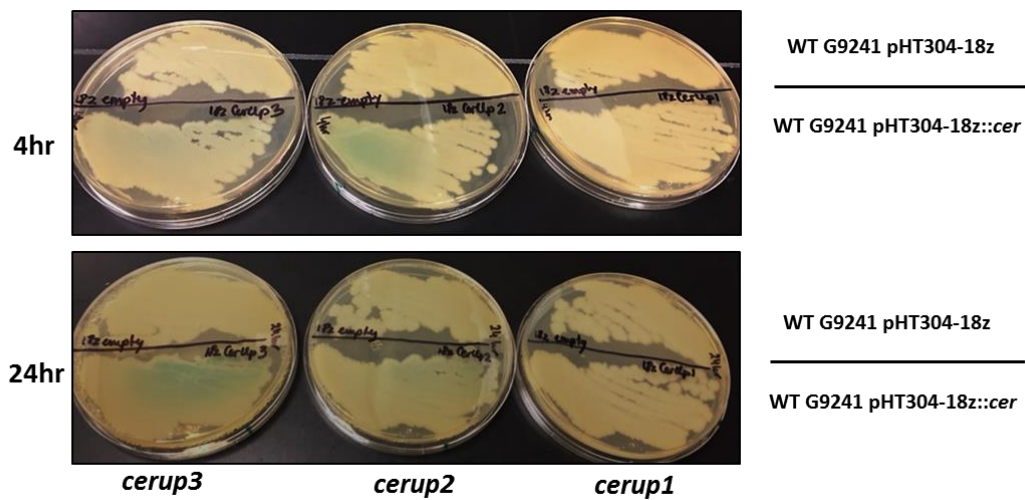
Fig. 13. β -galactosidase activity of *cer* and *pagA2* promoter fusion constructs.

Three sets of primers were used to amplify by PCR different-sized regions upstream of *cer* and *pagA2* (A). The amplified products were inserted in front of a promotorless *lacZ* gene in the pHT304-18z vector. The generated constructs were: pHT304-18z::*cerup1*, pHT304-18z::*cerup2*, and pHT304-18z::*cerup3* for *cer*; pHT304-18z::*pagA2up1*, pHT304-18z::*pagA2up2*, pHT304-18z::*pagA2up3* for *pagA2*. The constructs and an empty vector control were electroporated into WT G9241. The strains were grown under toxin-inducing conditions for 4 and 24 hours and were subsequently streaked out on Heart Infusion (HI) agar plates that were supplemented with erythromycin and overlaid with X-gal substrate. Production of blue pigment was indicative of *lacZ* expression and β -galactosidase activity. Each agar plate had WT G9241 pHT304-18z (empty vector) streaked out on the top and the *cer* (B) or *pagA2* (C) promoter fusions on the bottom.

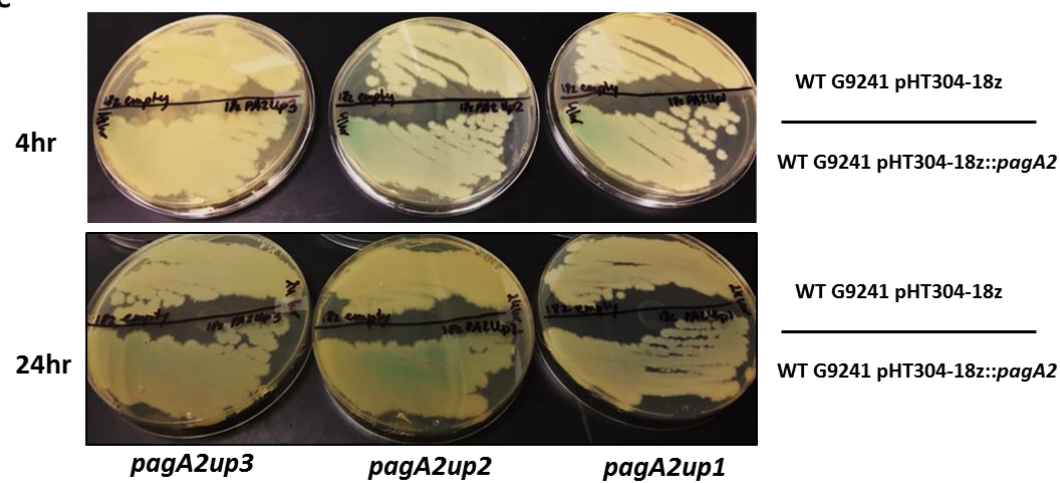
A



B



C



Phyper-spank. Additionally, *cer* was cloned into the vector with an N-terminal His-tag so that an anti-His antibody could be used to detect the protein in conjunction with the anti-ADPr. The two constructs, pCer and pHisCer, respectively, were transformed into *E. coli* Mach 1 competent cells (Fig. 14A) and were also electroporated into a *cer* deletion mutant strain of G9241, Δcer (Fig. 14B). The deletion mutant strain will be further discussed below. The *E. coli* transformants were induced with 100 μ M IPTG, and cell lysates prepared as in the methods. The induced and un-induced cell lysates were probed with anti-ADPr or anti-His antibodies (Fig. 14A) by Western blot. In *E. coli*, Certhrax and His-Certhrax were expressed at approximately 55kDa and were detected in induced and un-induced cell lysates. The expression was especially clear when probed with the anti-His antibody for the pHisCer construct. Of note, the purified recombinant His-Certhrax protein used as a control was no longer detected with the anti-His antibody but was detected with anti-ADPr (Fig. 14A). To detect expression in G9241, the Δcer pCer strain, in conjunction with WT G9241, Δcer , and Δcer carrying the empty pUTE657 vector (pEmpty), were grown under toxin-inducing conditions as previously described, except the strains were grown for 24hrs without shaking instead of four hours. The cultures were induced with 100 μ M IPTG at 0 and 22hrs and cell lysates and supernatants were collected. As shown in Fig. 14B, there was a detectable band in the Δcer pCer cell lysates at approximately 55kDa and a stronger band at approximately 50-52kDa (Fig. 14B). No product was observed in the WT or Δcer cell lysates or in the empty vector control. Additionally, no protein was detected in the culture supernatants for any of the strains when probed with anti-ADPr. Of note, the Δcer pHisCer cell lysates did not react with the anti-His antibody as was seen with the *E. coli* lysates (data not shown).

Collectively, these data suggest that Certhrax is a cell-associated protein, approximately 55kDa in size, which may undergo N-terminal processing in *B. cereus* G9241 to a protein approximately 50-52kDa in size. Additionally, the protein may be expressed at low levels in G9241 *in vitro* and can therefore be detected only when over-expressed.

Subaim 2.2: Construct a *cer* deletion mutant strain to be used for s.c. and i.n challenge of A/J and C57BL/6 mice.

Characterization of the Δcer mutant strain of *B. cereus* G9241

To determine whether Certhrax plays a role in pathogenesis of G9241, a markerless deletion mutant strain, Δcer , was constructed through a similar approach as described for the *pagA* mutants. The Δcer mutant strain was confirmed through Southern blot analysis with a DIG probe that hybridizes within the downstream *pagA2* gene. DNA, isolated from WT G9241, Δcer , and the plasmid cured G9241 were digested with *Bam*HI and *Bmg*BI enzymes. The probe hybridized to two fragments in WT DNA that were 3745 and 1790bp, as expected (Fig. 15A). Since a *Bam*HI site was left at the site of the deleted *cer* gene, the probe hybridized to the 3745bp band in Δcer and a smaller 1383bp fragment. There was no reactivity with the DNA of the plasmid-cured strain (Fig. 15A).

The Δcer was further assessed for integrity of the pBCXO1 and pBC210 plasmids via the PCR plasmid screen, as previously described. One of the primer pairs used in the screen, 218.2, anneals within the *cer* gene. Therefore, as shown in Fig. 16, there was no product detected with that PCR reaction with Δcer DNA. There was no other loss of

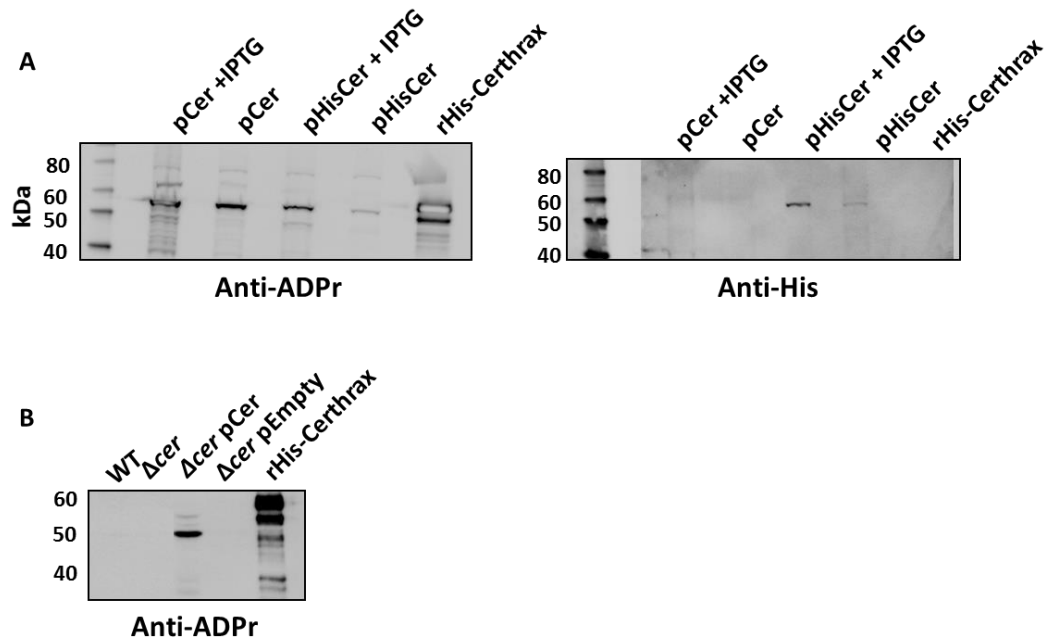


Fig. 14. *In vitro* Certhrax expression in IPTG-inducible expression constructs.

The pUTE657 vector was used to generate constructs pCer and pHisCer, where promotorless *cer* or an N-terminally His-tagged *cer* were placed under control of an IPTG-inducible promoter, respectively. **(A)** Constructs were induced in *E. coli* Mach1 cells with 100 μ M IPTG. Cell lysates of induced and un-induced *E. coli* transformed with pCer or pHisCer were subjected to SDS-PAGE and the proteins were transferred to nitrocellulose membranes for Western blot analyses with anti-ADPr or anti-His antibodies. **(B)** The pCer clone, or an empty vector, were electroporated into WT G9241 Δ cer to generate Δ cer pCer and Δ cer pEmpty, respectively. The strains, in conjunction with WT G9241 and the Δ cer mutant strain, were grown under toxin-inducing condition for 24 hours without shaking and were induced with 100 μ M IPTG at 0 and 22 hours. The cell lysates were subjected to SDS-PAGE and the proteins were transferred to a membrane for Western blot analysis with anti-ADPr. Recombinant His-tagged Certhrax protein was used as a control.

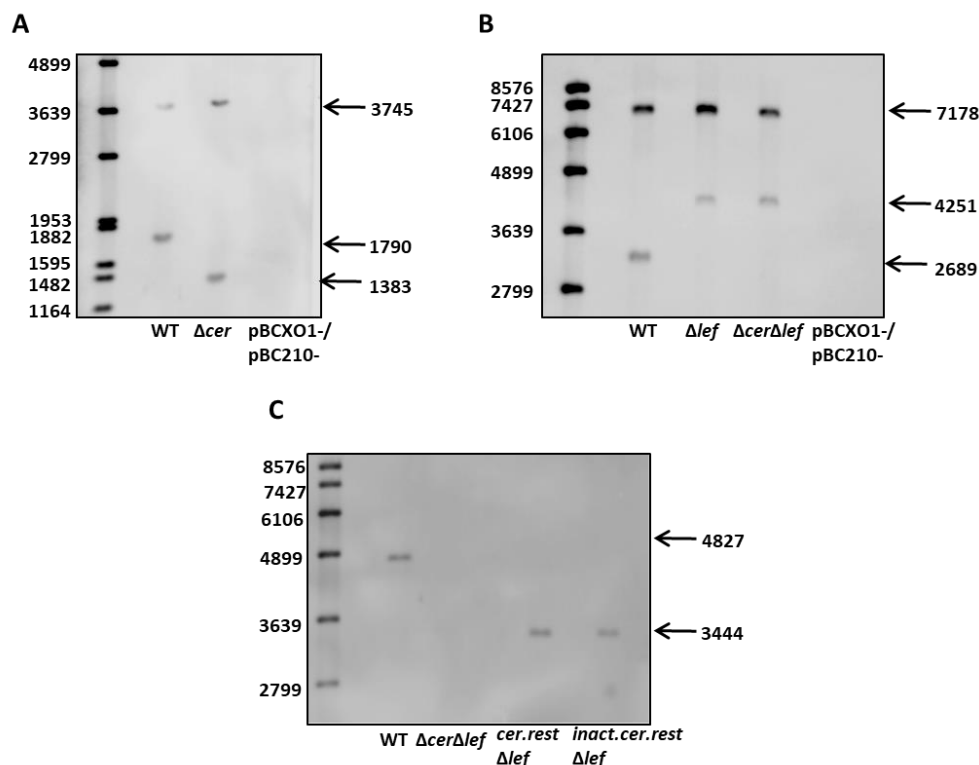


Fig. 15. Southern blot confirmation of Δcer , $\Delta cer\Delta lef$, Δlef , *cer.rest* Δlef , and *inact.cer.rest* Δlef mutant strains of G9241.

DIG-labeled gene-specific probes were hybridized to DNA of the different strains that was digested with restriction endonucleases, as described in the methods. The Δcer mutation (**A**) was confirmed with a *pagA2*-specific probe that was expected to hybridize to 3735 and 1790bp bands of WT DNA, and 3745 and 1383bp bands of mutant strain DNA. The *lef* deletion in the Δlef and $\Delta cer\Delta lef$ mutant strains (**B**) was confirmed with the *pagA1* probe that was expected to hybridize to 7178 and 2689bp bands of WT DNA, and 7178 and 4251bp bands in the two mutant strains. The *cer.rest* Δlef and *inact.cer.rest* Δlef mutant strains (**C**) were confirmed with a *cer*- specific probe that was expected to hybridize to a 4826bp band of the WT DNA, and a 3444bp band of each mutant strain DNA. The DNA isolated from the plasmid-cured (pBCXO1-/pBC210-) G9241 was used as a negative control. The arrows depict the actual size of the observed bands.

plasmid DNA in the mutant strain. Additionally, the mutant strain showed no growth defect in rich medium when compared to WT G9241 (Fig. 17A).

We subsequently examined toxin and capsule expression capabilities of Δcer . The mutant strain expressed all of the anthrax toxin components and PA2 in culture supernatants following growth under toxin-inducing conditions (Fig. 18A-E). When grown under capsule-inducing conditions, we observed the presence of the HA capsule (Fig. 19C) and the tetrassacharide capsule post hyaluronidase treatment (Fig. 19D).

Virulence of Δcer G9241 following s.c. and i.n. inoculation of A/J and C57BL/6 mice

The Δcer spores were made as described in the methods section and were first assessed for germination *in vitro*. The mutant spores showed no germination defects as compared to WT G9241 (Fig. 20B) and were, therefore, ready for the mouse inoculation experiments. A/J and C57BL/6 mice were inoculated with Δcer spores through s.c. and i.n. routes and monitored for morbidity and mortality for 14 days. The LD₅₀ values for each inoculation model were calculated through probit analyses (Table 5). The mutant was as virulent as WT G9241 in all of the *in vivo* inoculation models. In fact, we saw 100% lethality even at the lowest 10¹ inoculum dose following s.c. inoculation of A/J mice, compared to 13% survival in the WT G9241 group. The LD₅₀ value of the mutant strain for the s.c. route in A/J mice was therefore <20 spores, while in the C57BL/6 mice it was calculated to be 70 spores. For the i.n. route, the LD₅₀ values were 5x10² and 3x10⁵ in A/J and C57BL/6 mice, respectively. Additionally, there was no statistical difference in the median time to death during s.c. inoculation of either mouse strain (Fig. 21) even at the lowest inoculum dose. However, it was noted that A/J mice inoculated with the

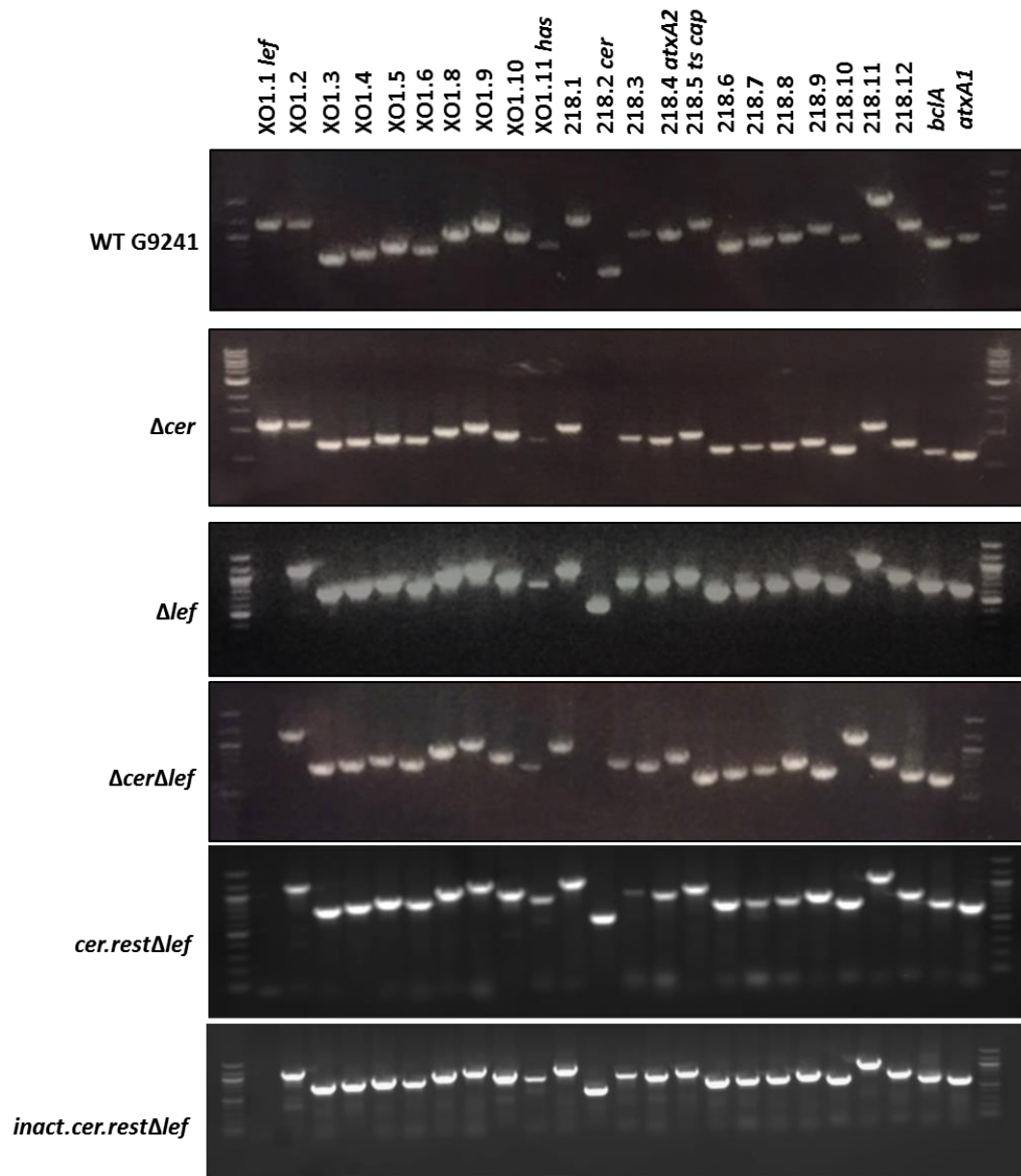


Fig. 16. PCR plasmid screen of Δcer , $\Delta cer\Delta lef$, Δlef , $cer.rest\Delta lef$, and $inact.cer.rest.\Delta lef$ mutant strain DNA.

Twenty four PCR reactions were set up with primers that anneal throughout the pBCX01 and pBC210 plasmids, as well as a pair that anneals within the chromosomal *bclA* gene. PCR reactions were done to check plasmid integrity post mutagenesis with DNA from each mutant strain.

mutant strain through the s.c. route appeared to form a larger abscess at the site of inoculation (a large bump at the right flank of the mouse) as compared to WT G9241. Collectively, these data suggest that Certhrax does not play a prominent role as a virulence factor in pathogenesis of G9241.

Subaim 2.3: Assess whether the production of lethal toxin affects the role of Certhrax *in vivo* via construction of a single *lef* deletion mutant strain and a double *cer/lef* mutant strain.

Characterization of the Δlef and $\Delta cer\Delta lef$ mutant strains of *B. cereus* G9241

Since lethal toxin is an important virulence factor in *B. anthracis*, studies were carried out to characterize the role of LT in pathogenesis of G9241 and to determine if its *in vivo* activity could mask any observable effects of Certhrax. For these experiments, we generated a markerless deletion mutant strain of *lef*, Δlef , as well as a double *cer/lef* knock-out, $\Delta cer\Delta lef$. The $\Delta cer\Delta lef$ mutant strain was constructed via deletion of *lef* in the Δcer mutant strain background. The successful deletion of *lef* in both strains was confirmed via Southern blot analysis with the same DIG-labeled probe that was used to confirm the *pagA1* deletion. However, in this case, DNA was digested with *EcoRV* to generate 7178 and 2689bp fragments in WT G9241 (Fig. 15B). Due to the deletion of the *lef* gene that contained an intergenic *EcoRV* cut site, the probe hybridized to the 7178bp fragment and a larger 4251bp DNA fragments in the two mutant strains (Fig. 15B). The successful deletion of *lef* was further noted in the PCR plasmid screen, since the XO1.1 primer pair anneals within the gene. Therefore, in Δlef , there was no product in the XO1.1 PCR reaction, while the rest of the pBCXO1 and pBC210 plasmid DNA was

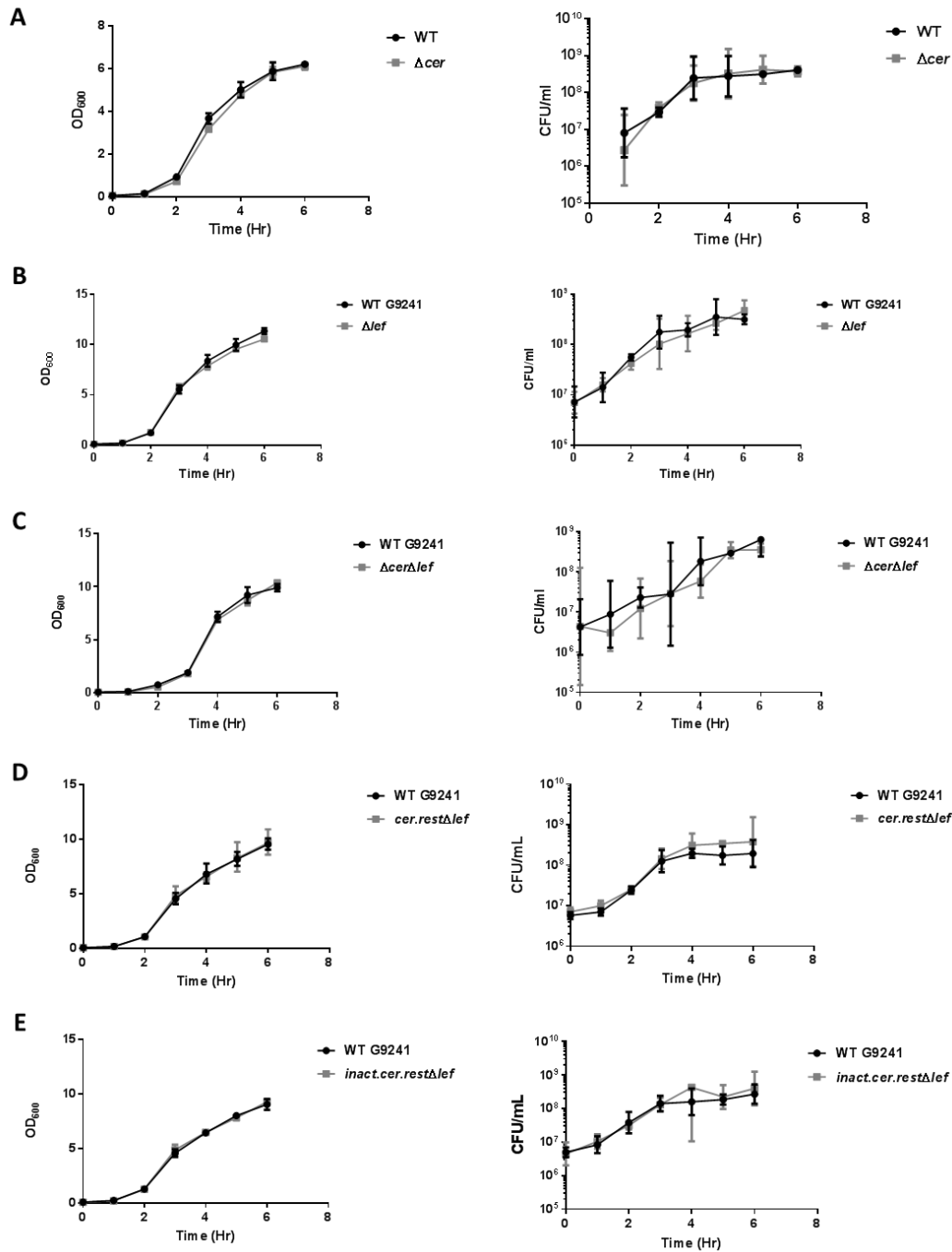


Fig. 17. Growth curves of Δcer , $\Delta cer \Delta lef$, Δlef , $cer.rest \Delta lef$, and $inact.cer.rest \Delta lef$ mutant strains as compared to WT G9241.

Three biological replicates of WT G9241 and Δcer (A), Δlef (B), $\Delta cer \Delta lef$ (C), $cer.rest \Delta lef$ (D), $inact.cer.rest \Delta lef$ (E) were grown in LB broth for six hours at 37°C with shaking. Samples were collected hourly to measure culture turbidity (OD₆₀₀) and for enumeration of colony-forming units (CFU/ml). The graphs represent an average (OD₆₀₀) or geometric mean (CFU/ml) of the three biological replicates with standard error depicted as bars.

intact (Fig. 16). In the case of the $\Delta cer\Delta lef$ mutant strain, no bands were observed with the XO1.1 or 218.2 primer pairs, as the strain is missing both *lef* and *cer* (Fig. 16). The mutant strains were also confirmed via Western blot analyses as neither expressed LF in culture supernatants but both expressed the rest of the tested toxin components (Fig. 18). Additionally, we showed that the mutagenesis process did not affect general *B. cereus* G9241 biology as both Δlef and $\Delta cer\Delta lef$ mutant strains had no growth defects as compared to WT G9241 (Fig. 17B, C) and expressed both G9241 capsules (Fig. 19E-H). As the Δlef and $\Delta cer\Delta lef$ spores had no *in vitro* germination defects (Fig. 20C, D) they were subsequently used to assess virulence of the mutant strains. A/J and C57BL/6 mice were inoculated s.c. and i.n. with the Δlef mutant strain of G9241. As shown in Table 5, the deletion of *lef* significantly attenuated virulence in both mouse strain. In fact, the mutant was avirulent during s.c. inoculation of C57BL/6 mice and just a few mice died following intranasal inoculation with the highest 10^7 dose of mutant spores. There was also a significant attenuation in virulence of the mutant strain compared to WT G9241 in A/J mice. The calculated spore LD₅₀ values were 7×10^5 and 2×10^5 for the s.c. and i.n. routes, respectively (Table 5). These data show that LT is essential for full pathogenesis of *B. cereus* G9241 in these mouse models.

To determine whether expression of LF could mask any *in vivo* effects of Certhrax, mice were inoculated with $\Delta cer\Delta lef$ G9241 spores. As shown in Table 5,

the $\Delta cer\Delta lef$ mutant strain had comparable LD₅₀ values to those of Δlef following i.n. inoculation of A/J mice and for both administration routes in C57BL/6 mice. In each case, the double knock-out was significantly attenuated when compared to WT G9241,

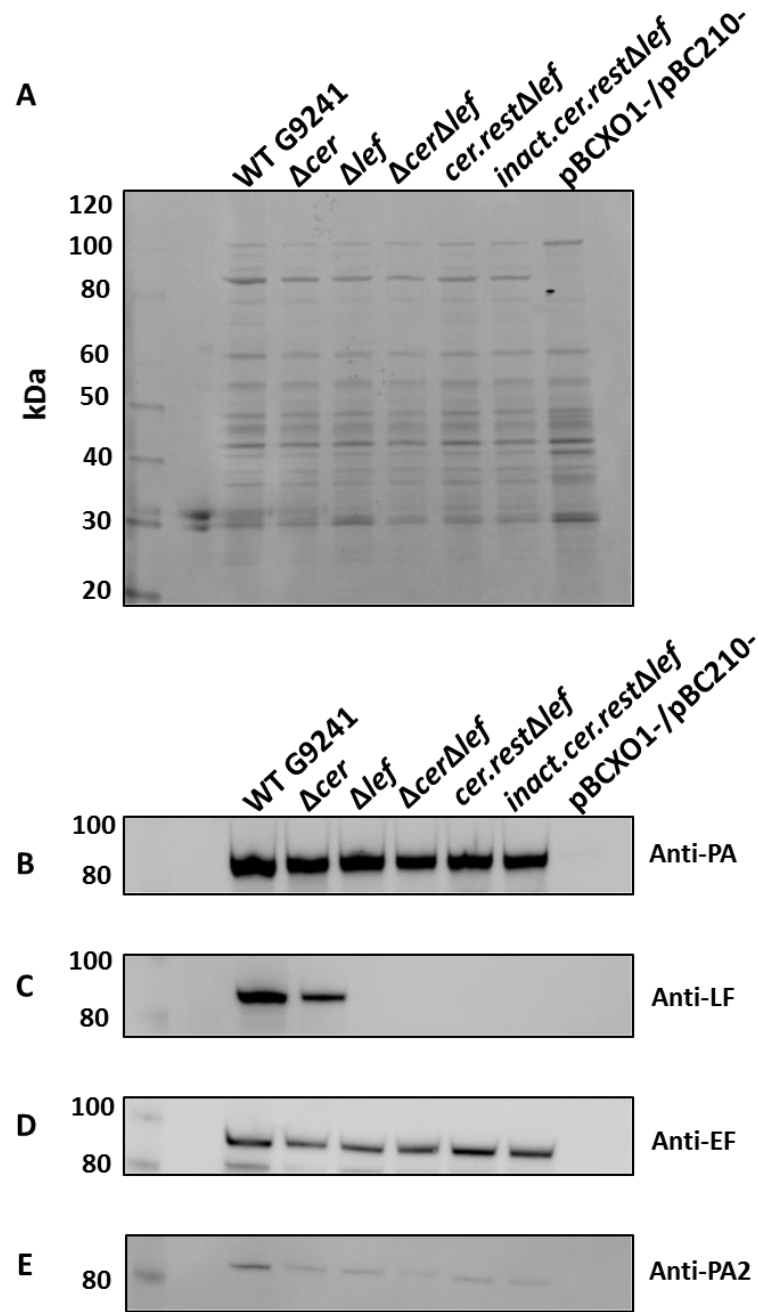


Fig. 18. Western blot analyses of toxin production in Δ cer, Δ lef, Δ cer Δ lef, cer.rest Δ lef, and inact.cer.rest Δ lef mutant strains of *B. cereus* G9241.

Cultures were grown under toxin-inducing conditions and the isolated supernatants were concentrated through ethanol precipitation. Samples were subject to SDS-PAGE electrophoresis. Total protein was visualized with Oriole stain (A) and gels were transferred to nitrocellulose membranes for Western blot analyses with anti-PA (B), anti-LF (C), anti-EF (D), and anti-PA2 (E) antibodies. Supernatants from WT G9241 and a plasmid cured G9241 (pBCXO1-/pBC210-) were used as positive and negative controls, respectively.

but it was not different from Δlef . However, an exception was noted following the s.c. inoculation of A/J mice. The calculated LD₅₀ value for $\Delta cer\Delta lef$ in the s.c. model was 1×10^3 spores, which is statistically significantly more virulent than what was observed with the Δlef mutant. These results were consistent in three separate infection experiments and suggested that *cer* behaves as an anti-virulence factor during subcutaneous inoculation of A/J mice.

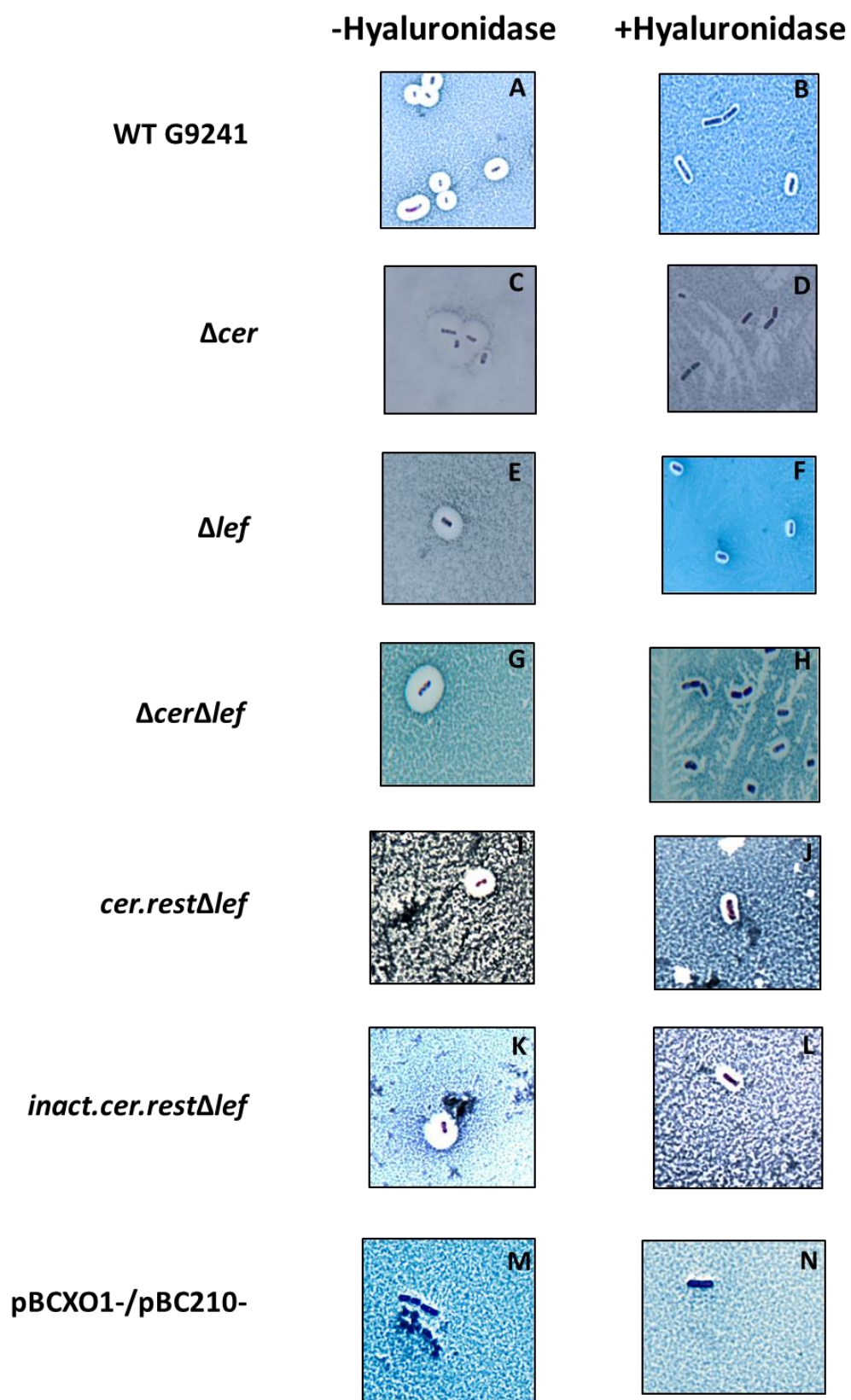
To assess whether Certhrax was expressed *in vivo*, a similar approach was taken to that described for PA2. Specifically, A/J mice that survived the s.c. inoculation with Δlef and $\Delta cer\Delta lef$ (from LD₅₀ studies above) were subsequently inoculated with the same dose of the mutant strains again and were monitored for an additional 14 days. At four weeks, cardiac punctures were used to collect serum to look for a host antibody response against Certhrax. The sera were collected and used as the primary antibody in Western blot analyses to probe for recombinant purified Certhrax. However, there was no reactivity between rCer and any of the serum samples (data not shown). Nevertheless, the difference in virulence between Δlef and $\Delta cer\Delta lef$ provides indirect evidence that Certhrax is indeed expressed *in vivo* during *B. cereus* G9241 infection.

Characterization of $\Delta cer\Delta lef$ restorants with enzymatically active vs. inactive *cer*

To determine whether the increased virulence phenotype of $\Delta cer\Delta lef$, when compared to Δlef , is due to Certhrax competing for binding with LF and EF or due to its enzymatic activity, two *cer* restorants were generated within the $\Delta cer\Delta lef$ mutant strain

Fig. 19. Capsule expression by Δcer , Δlef , $\Delta cer\Delta lef$, $cer.rest\Delta lef$, and $inact.cer.rest.\Delta lef$ mutant strains of *B. cereus* G9241.

G9241 cultures of wild-type (WT) G9241 (**A,B**), Δcer (**C,D**), Δlef (**E,F**), $\Delta cer\Delta lef$ (**G,H**), $cer.rest\Delta lef$ (**I,J**), $inact.cer.rest\Delta lef$ (**K,L**) and pBCXO1-/pBC210- G9241 (**M,N**) were grown under capsule-inducing conditions for 24 hours. The samples were treated water (**A, C, E, G, I, K,M**) or hyaluronidase (**B, D, F, H, J, L,N**) for two hours at 37°C and subsequently stained with the Maneval stain to visualize the hyaluronidase and tetrassacharide capsules, respectively. Images were captured at 1000X magnification.



background. One restorant, called *cer.restΔlef*, was generated by cloning wild-type *cer* into its native location in *ΔcerΔlef* G9241 while the other mutant, *inact.cer.restΔlef*, was made with an enzymatically inactive *cer* as described in the methods. The successful restoration of the gene in both mutant strains was confirmed through Southern blot analysis with the use of a *cer*- specific DIG-labeled probe. Following restriction digestion with *Bam*HI, the probe hybridized to a 4827bp DNA fragment in WT G9241 and did not hybridize to *ΔcerΔlef* DNA (Fig. 15C). A 3444bp DNA fragment was detected in both restorant mutant strains, as expected (Fig. 15C).

Additionally, restoration of the gene in the *cer.restΔlef* and *inact.cer.restΔlef* mutant strains was confirmed during the PCR plasmid screen, since a DNA product was detected with the 218.2 primer pair reaction, while it was absent in the *ΔcerΔlef* parent strain DNA (Fig. 16). Furthermore, both restorant strains were still missing *lef* as shown in the PCR reaction with the XO1.1 primers (Fig. 16) and the Western blot probed with anti-LF antibody (Fig. 18C). The *cer.restΔlef* and *inact.cer.restΔlef* mutant strains still produced PA, EF and PA2 proteins in the supernatants of cultures grown under toxin-inducing conditions (Fig. 18B, D, E) and expressed both capsules when grown under capsule-inducing conditions (Fig. 19I-L). The two strains had no growth defects as compared to WT cultures (Fig. 17D, E) nor any spore germination defects *in vitro* (Fig. 20E, F).

The virulence phenotype of each restorant strain was assessed in the s.c. inoculation model of A/J mice. As shown in Table 5, the *cer.restΔlef* mutant strain of G9241 was highly attenuated as compared to WT G9241 with a calculated LD₅₀ of 5x10⁶. Furthermore, this restorant was significantly more attenuated than the parent *ΔcerΔlef* strain, with a similar phenotype to *Δlef*. The *inact.cer.restΔlef* mutant strain, on the other

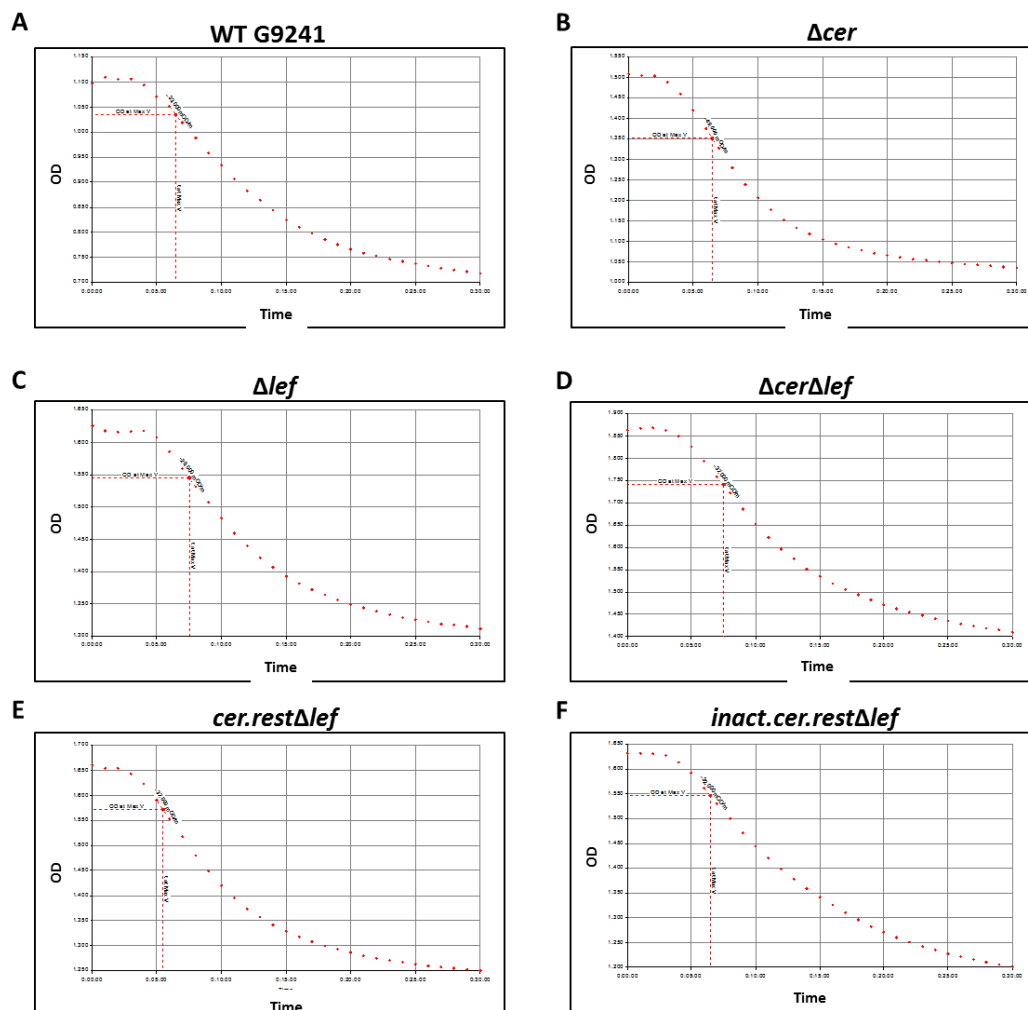


Fig. 20. Germination of wild-type (WT) G9241, Δcer , Δlef , $\Delta cer\Delta lef$, *cer.rest* Δlef , and *inact.cer.rest* Δlef mutant spores in the presence of germinants L-alanine and inosine.

Spores of wild-type (WT) G9241 (A), Δcer (B), Δlef (C), $\Delta cer\Delta lef$ (D), *cer.rest* Δlef (E), or *inact.cer.rest* Δlef (F) were incubated with germinants L-alanine and inosine for 30min. A plate reader was used to record changes in the optical density at a wavelength of 562nm over a 30-minute period. Each graph is a representative of three separate experiments for each strain.

Table 5. LD₅₀ spore values of G9241 and *cer/lef* mutant strains following inoculation of A/J or C57BL/6 mice via the subcutaneous (s.c.) or inhalational (i.n.) route.

	A/J		C57BL/6	
	s.c.	i.n.	s.c.	i.n.
WT G9241	10^a	3x10^{3a}	40^a (16-100)	4x10^{5a} (2x10⁵-9x10⁵)
<i>Δcer</i>	<20	5x10^{2a} (4x10¹-4x10³)	70^a (20-200)	3x10^{5a} (1x10⁵-8x10⁵)
<i>Δlef</i>	7x10^{5a,c} (2x10⁵-3x10⁶)	2x10^{5a,c}	>2x10⁷	5x10^{6b}
<i>ΔcerΔlef</i>	1x10^{3a,c,d} (2x10²-5x10³)	2x10^{5a,c} (5x10⁴-5x10⁵)	>2x10⁷	4x10^{6b}
<i>cer.restΔlef</i>	5x10^{6b}	-----	-----	-----
<i>inact.cer.restΔlef</i>	2x10^{4a,c} (4x10³-10⁵)	-----	-----	-----

- LD₅₀ values calculated via probit analyses with 95% confidence intervals provided in parenthesis.
- LD₅₀ values calculated by the Reed and Muench method, when the criteria for probit analysis were not met.
- Values are significantly different from WT G9241 as determined by probit analysis ($p < 0.05$).
- Value is significantly different from *Δlef* G9241 as determined by probit analysis ($p < 0.05$).

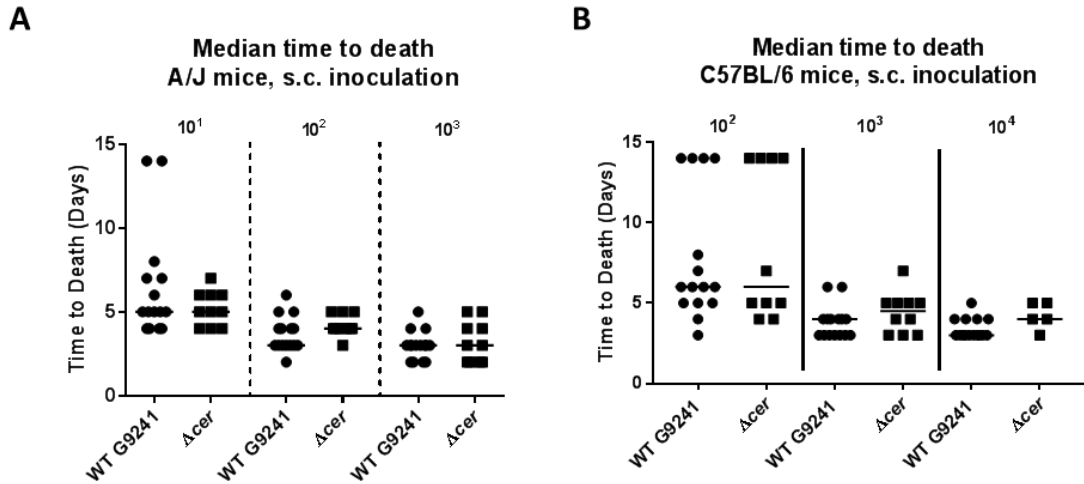


Fig. 21. Median time to death of mice inoculated subcutaneously with 10^1 - 10^4 of *B. cereus* G9241 spores. A/J (A) and C57BL/6 (B) mice were inoculated with either WT G9241 (●) or Δcer (▫) spores. Each point represents the time to death of a single mouse and the dashed line represents the group median. For this calculation, time to death of animals that did not die in the 14-day observation period was recorded as 14 days. The medians were compared statistically with the Mann-Whitney rank sum test.

hand, had a similar LD₅₀ value to the $\Delta cer\Delta lef$ mutant strain of G9241. In fact, the LD₅₀ values of the two strains were not statistically different. These results show that the enzymatically inactive *cer* did not complement the virulence phenotype of the $\Delta cer\Delta lef$ mutant to Δlef levels, while the enzymatically active *cer* did complement. Therefore, these data suggest that the enzymatic activity of *cer* is responsible for the observed virulence difference between Δlef and $\Delta cer\Delta lef$.

We sought to assess if there was a difference in *in vivo* dissemination between the two restorant strains of G9241. To do so, 20 A/J mice were subcutaneously inoculated with a dose of 10⁶ spores of the *inact.cer.restΔlef* while another group of 20 mice were inoculated with the same dose of *cer.restΔlef* spores. The intent was to measure differences in bacterial burden over time in the abscess formed at the site of inoculation and in the spleen of the mice inoculated with the two strains. Five mice per strain were euthanized at 6, 24 and 72 hours post inoculation. Mice in the last group were allowed to succumb to disease naturally, and any survivors were euthanized five days post inoculation. For that group, mice inoculated with the *inact.cer.restΔlef* all died of the infection: one mouse died on day 2, two mice died on day 3, one on day 4 and the last on day 5 after inoculation. For the 5 mice inoculated with *cer.restΔlef*, one mouse died on day 3 and the rest were euthanized at the end of the experiment on day 5. At each time point, the abscess was removed from the site of inoculation for each mouse, weighed and then mashed in 1ml of PBS. The spleens were also collected at each time point, weighed, and then homogenized in PBS. Half of each sample was heat-killed for 30min at 65°C to allow for spore enumeration, while the other half of the sample was used to count the total bacterial burden. Figure 22 depicts weights of the mouse abscesses (Fig. 22A)

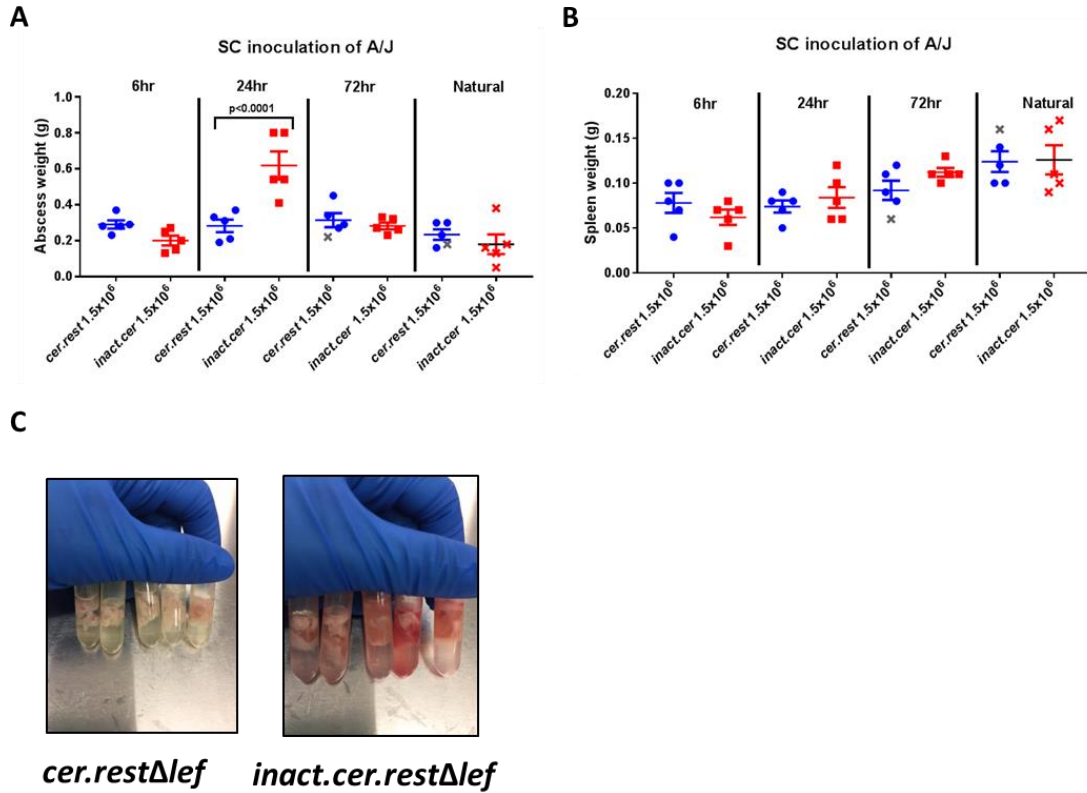


Fig. 22. Weights of tissue collected from A/J mice following s.c. inoculation with *cer.rest Δlef* or *inact.cer.rest Δlef* mutant spores. Mice were inoculated with a 10^6 dose of *cer.rest Δlef* (blue) or *inact.cer.rest Δlef* (red) mutant spores. At 6, 24, and 72 hours post inoculation, five mice per group were euthanized and their spleens and abscesses, formed at the site of inoculation, were collected. The last group of five mice for each strain succumbed to the infection and the tissues were collected upon death. The abscesses (**A**) and spleens (**B**) were weighed when collected. X's identify mice that were dead upon tissue isolation. The dash represents average weights for each group and the error bars represent the standard error of the mean. Abscesses collected at the 24hr time point are pictured in the figure (**C**), where the abscesses collected from *cer.rest Δlef* -infected mice are in yellow tubes and ones collected from *inact.cer.rest Δlef* -infected mice are in orange tubes. Statistical differences were assessed with a two-way ANOVA; $p < 0.05$ is considered significant.

and spleens (Fig. 22B) collected at each time point. There was no difference in spleen weights between mice inoculated with the *cer.restΔlef* or *inact.cer.restΔlef* spores, but there was a gradual increase in the average spleen weights over time. However, a significant difference in abscess weights was noted at 24 hours post inoculation between the two inoculum strains ($p < 0.0001$ as calculated via a two-way ANOVA with a Bonferroni adjustment for multiple comparisons) (Fig. 22A). Mice inoculated with the *inact.cer.restΔlef* restorant formed significantly larger abscesses at the site of inoculation than mice inoculated with the other mutant strain. The abscesses were photographed and are shown in Fig. 22C. The abscess weights were comparable at all other time points between the two strains. Furthermore, despite the difference noted at 24 hours, there was no difference in the overall bacterial burden (CFU/g) in the collected abscesses between the two mutant strains at any time point (Fig. 23A). However, the average spore count (per gram of tissue) was higher in the abscesses of mice inoculated with *inact.cer.restΔlef* at the 6 hour time point (Fig. 23B). The numbers of spores per gram were comparable at the later time points. Conversely, there was a larger overall bacterial burden in the spleen of mice inoculated with the *cer.restΔlef* mutant of G9241 at 6 and 24 hours post inoculation. At 72 hours the CFU/g were comparable between the two strains. We also noted that mice that died of the infection (marked with an x in Fig. 23), or were moribund, had a bacterial burden greater than 10^7 in the spleen. Mice that were inoculated with *cer.restΔlef* and did not die of the infection had a lower bacterial count in the spleen as compared to the mouse that succumbed to the illness in the same group. The mice inoculated with *inact.cer.restΔlef* and died all had a high bacterial burden in the

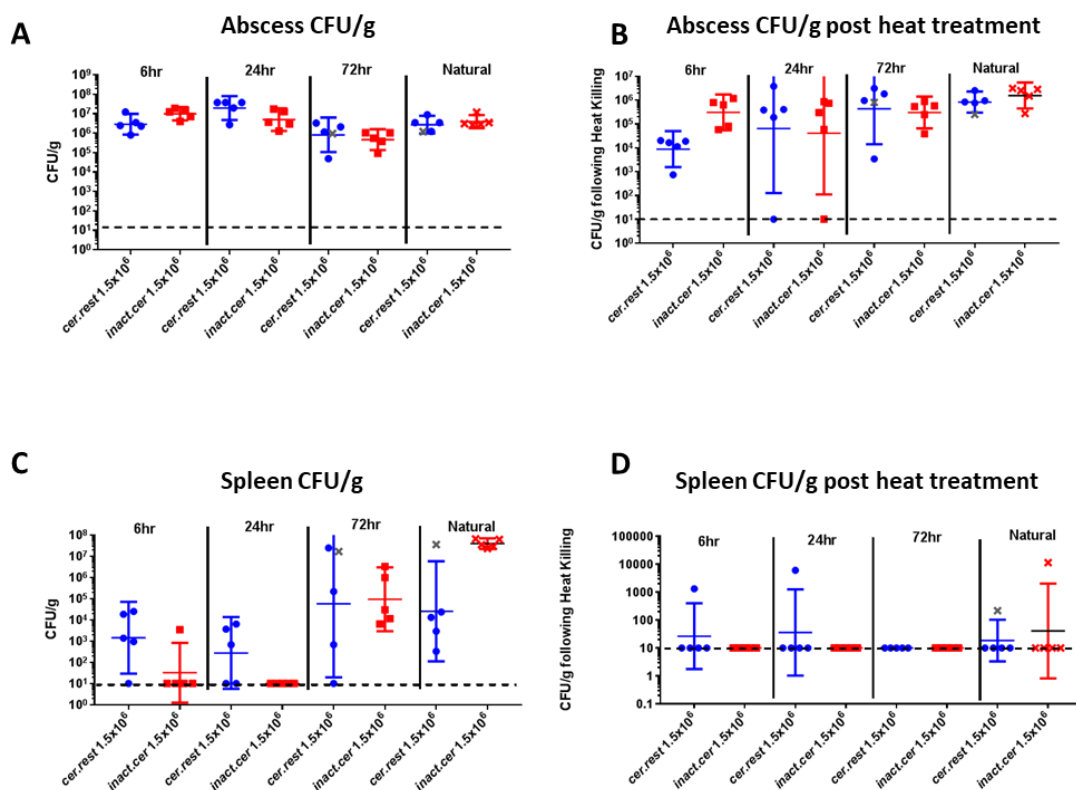


Fig. 23. Bacterial burden in abscesses and spleens of A/J mice inoculated with spores of the *cer.rest*Δ Δ *lef* or *inact.cer.rest*Δ Δ *lef* mutant strains of *B. cereus* G9241. Mice were inoculated with a 10^6 dose of *cer.rest*Δ Δ *lef* (blue) or *inact.cer.rest*Δ Δ *lef* (red) mutant spores. Abscesses, formed at the site of inoculation, and spleens were collected from five mice per group at 6, 24, and 72 hours post inoculation. The last group of five mice for each strain succumbed to the infection (natural) and the tissue were collected upon death. The tissue were first weighed, and then the abscesses were mashed in PBS, while the spleens were homogenized. Half of each sample were heat-treated to kill vegetative cells (B,D) while the other half were not (A,C). All samples were serially diluted and plated for enumeration of colony forming units (CFU). The calculated abscess (A,B) and spleen (C,D) CFU/g are represented for each mouse. The line represents the geometric mean for each group and the error bars represent the 95% confidence interval. The X's represent mice that were dead upon tissue isolation. The dashed black line represents the limit of detection.

spleen. Additionally, with the exception of a few mice, most of the bacteria in the spleen were vegetative cells and not spores (Fig. 23D). Collectively, the data suggest that the *inact.cer.restΔlef* mutant strain persists locally at the site of inoculation longer than the *cer.restΔlef* mutant strain that starts to disseminate within the first 6 hours post inoculation. Despite the earlier dissemination, however, mice inoculated with *cer.restΔlef* G9241 appear to control the infection better than what is observed with *inact.cer.restΔlef*-infected mice, as the bacterial counts remain lower in the spleen of those mice. Therefore, Certhrax may play a role as an attenuating virulence factor of *B. cereus* G9241 early in infection.

CHAPTER 4: Discussion

The over-arching goal of the work described here was to determine the role that toxins play in pathogenesis of the anthrax-like *B. cereus* strain G9241. In particular, we sought to understand the contribution to virulence of two novel toxin components, PA2 and Certhrax. The large number of putative virulence factors encoded by G9241 complicates analysis of the *in vivo* role of a single protein in pathogenesis. Nevertheless, we showed that the anthrax toxins, PA and LF specifically, are essential for full virulence of *B. cereus* G9241 whether A/J or C57BL/6 mice are infected by the subcutaneous or intranasal routes. Additionally, while the actual function of PA2 is still not clear, our findings suggest that the protein does contribute to G9241 pathogenesis, albeit in a more limited capacity than PA and LF. Our studies also revealed that there are differences in the functional capabilities between PA and PA2. Furthermore, and more unexpectedly, we showed that Certhrax, a novel ADP-ribosyltransferase, behaves as an anti-virulence factor following subcutaneous challenge of A/J mice with *B. cereus* G9241 spores.

The approach taken in this study was to generate a series of single and double toxin component deletion mutant strains, and, subsequently, to characterize their virulence phenotype. The mutagenesis process involved the use of a temperature-sensitive (ts) plasmid that carried upstream and downstream regions flanking the gene of interest. Each mutation was generated by homologous recombination, wherein the plasmid was electroporated into G9241 and the transformant subsequently exposed to several passages at plasmid-permissive and non-permissive temperatures, with and without antibiotic pressure, respectively. The successful mutagenesis process occurred at

a low frequency. Due to the pressures placed on the bacterium during these subculturing events, it was important to ensure that there were no unintended mutations introduced in the organism that might affect the biology of the bug. These mutations could manifest as sporulation defects, random loss of virulence plasmid DNA, growth defects, etc. Each mutant strain was therefore examined for such unintended effects through a series of procedures, such as the PCR plasmid screen, Southern and Western blot analyses, spore germination assays, *in vitro* growth rate assessments, and other assays. Only when all these tests indicated that the *in vitro* phenotype was as expected for that strain did we assess *in vivo* pathogenesis.

The virulence of each G9241 mutant strain was explored in two different mouse strains. In general, the use of mice as a surrogate to study pathogenesis of a bacterium that causes disease in humans comes with certain advantages and limitations. There are differences in structural physiology and anatomy, as well as in the immune system between mice and humans. For example, mice have fewer airway branches than do humans, and mice have no cough reflex (72). Additionally, mice have bronchus-associated lymphoid tissue (BALT), while such tissue is mostly absent in healthy humans. There are also differences in the balance of lymphocytes and neutrophils in the blood, such that mouse blood is rich in lymphocytes, while human blood has a higher concentration of neutrophils. Despite such variances, mice have historically been used in *B. anthracis* research because they are affordable, small and thus easy to handle, and there are many genetically distinct strains that can be used to answer specific scientific questions. Indeed, mice serve as good models to study the early stages of disease following *B. anthracis* spore administration, as well as toxin involvement later in

infection (36). The subcutaneous administration of *Bacillus* spores results in an initial localized infection, where bacilli replicate at the site of inoculation and release toxins to cause toxemia. From the subcutaneous site, the bacteria later disseminate to other tissue, such as the spleen and the lungs (32). During intranasal inoculation, the spores germinate and bacilli disseminate throughout the body to cause bacteremia (94; 108).

The two mouse strains used in the study, A/J and C57BL/6 mice, are commonly employed to evaluate *B. anthracis* virulence. A/J mice are often so tested because of their susceptibility to anthrax infection, even with the attenuated un-encapsulated *B. anthracis* Sterne strain or nontoxigenic (Tox-) but encapsulated strains of the organism. This susceptibility makes them a good tool to study the role of toxins in virulence of the bacterium and in evaluation of toxin-based therapeutics (113). The increased sensitivity of the A/J mice, even to the attenuated *B. anthracis* strains, is due to their deficiency in the expression of the C5 component of complement that leads to a weakened host immune response to infection. C5 is a key serum chemoattractant. Therefore, A/J mice are unable to recruit neutrophils and phagocytes to the site of infection as quickly as typical C5+ mouse strains. C57BL/6 mice, which are C5+, are considered to be of intermediate susceptibility to infection with *B. anthracis* wild-type strains (67; 116). Thus, following s.c. inoculation with wild-type *B. anthracis*, C57BL/6 mice display a prolonged time to death and have higher LD₅₀ values to those observed in A/J mice (116). Therefore, C57BL/6 mice may provide a longer period of time for researchers to study the effects of a toxin of interest or to evaluate the impact of countermeasures. Moreover, the combined use of these two mouse strains (A/J and C57BL/6), occasionally reveals

roles for virulence factors of infecting *B. anthracis* strains that may be otherwise masked in anthrax infections of other inbred murine strains.

We observed that, as with *B. anthracis*, A/J mice appear to be more susceptible to inoculation with G9241 spores as compared to C57BL/6. This susceptibility difference is particularly apparent in the intranasal inoculation model, where there is a two log increase in LD₅₀ values for WT G9241 following challenge of C57BL/6 mice compared to infection of A/J mice by the same route. Overall, wild-type G9241 behaves similarly to the fully virulent *B. anthracis* Ames strain in these mouse models when comparing LD₅₀ values for G9241 to those published for *B. anthracis* Ames. Since toxins play a role in *B. anthracis* pathogenesis, it was, therefore, not a surprise to observe the involvement of anthrax toxins in pathogenesis of G9241 in this study.

A $\Delta pagA1$ mutant strain of G9241 was previously assessed for lethality after intraperitoneal challenge of C57BL/6 mice with 10⁵ of spores, and that mutant strain caused fewer deaths at that dose than WT (81; 82). Our data show that the $\Delta pagA1$ mutant strain is essentially avirulent following s.c. challenge of C57BL/6 mice. The strain is also highly attenuated in the A/J mouse model by the same inoculation route, but we noted more variability in the dose response in these mice. For example, a similar number of mice died following s.c. inoculation with the 10⁶ and 10⁵ dose of spores. Since none of the doses inoculated led to 100% lethality in a single group of mice, not even the 10⁷ inoculum, we had to use the Reed and Muench method to estimate the LD₅₀. Furthermore, some of the A/J mice displayed symptoms of neurologic distress, such as head tilting and spinning when lifted by the tail (15) and were humanely euthanized during the experiment. These data suggest that perhaps when PA1 is not expressed, there

is a difference in the type of disease caused by the mutant strain, or a difference in the progression of the infection in A/J mice. During intranasal inoculation of both A/J and C57BL/6 mice, we observed approximately a two log and a one log attenuation in virulence, respectively, when *pagA1* was deleted. The finding of a reduction but not loss of virulence when the Δ *pagA1* mutant strain was inoculated intranasally suggests that the G9241 capsules may play a greater role in dissemination of bacteria during inhalational anthrax than do toxins. Such a conclusion is consistent with the role the PDGA capsule plays in *B. anthracis* pathogenesis. Drysdale *et al.* showed that a toxigenic but unencapsulated mutant strain of *B. anthracis* UT500 is unable to disseminate to the spleen of mice following intratracheal inoculation of spores (27).

In addition to confirming the critical role of PA1 in G9241 pathogenesis, we have for the first time described the contribution of LT to pathogenesis of this bacterium. We observed significant attenuation in virulence of a *lef* deletion mutant strain in both mouse strains following s.c. inoculation. In fact, the Δ *lef* mutant spores were essentially avirulent in C57BL/6 mice, *i.e.* 100% of mice survived even at the highest (10^7 spores) dose during s.c. challenge. Following i.n. inoculation, the Δ *lef* mutant strain behaved similarly to the Δ *pagA1* mutant strain of G9241. The contribution of lethal toxin to *B. anthracis* pathogenesis has been documented in several different animal models, although the relative importance of this toxin varies depending on the model used. For example, an LF- mutant strain of the unencapsulated *B. anthracis* Sterne was avirulent following aerosol challenge of A/J mice (63) and s.c. challenge of Swiss mice (89). LT was also required for dissemination in A/J mice challenged s.c. with Sterne spores (65). However, an LF- mutant strain of the fully virulent *B. anthracis* Ames was as virulent as WT

following s.c. challenge of BALB/c mice (19). Conversely, pulmonary challenge of rabbits with *B. anthracis* Ames or Vollum spores revealed that LT plays a role in virulence in that model (56; 62). Our results for the Δlef mutant strain appear to be more consistent with the virulence phenotype of the LT- mutant strains of *B. anthracis* Sterne.

Further research is needed to determine the precise role that lethal toxin plays in the two murine inoculation models described here. For example, dissemination experiments could be undertaken to determine whether the *lef* mutant strain can spread from the site of inoculation as effectively as WT G9241, or whether the mutant strain can establish an infection at the site of inoculation as well as WT. Moreover, a complete assessment of anthrax toxin involvement in the virulence of G9241 would require the creation and *in vivo* evaluation of a Δcya mutant strain, as well as a $\Delta lef \Delta cya$ double deletion mutant strain. While LT is more consistently linked to virulence of *B. anthracis* than is ET, several studies showed that ET can contribute to *B. anthracis* pathogenicity, particularly in certain murine challenge models with Sterne (89) and in rabbits with the fully virulent *B. anthracis* strains (57).

While anthrax toxin involvement in pathogenesis of G9241 was one focus of the work described here, the major focus of the investigation was on the two less well characterized toxin components, PA2 and Certhrax. PA2, as a homolog of protective antigen, was considered to play a role in toxin translocation into host cells. However, we were uncertain how Certhrax might contribute to virulence of G9241 but hypothesized a role as follows. Certhrax is a novel ADP-ribosyltransferase toxin that targets vinculin *in vitro*; a target that prior to the study by Simon and Barbieri had not been described for ADPRTs (99). These investigators theorized that Certhrax could contribute to bacterial

spread within the host following infection with G9241 because the toxin, when transfected into cells, disrupts actin polymerization and subsequently changes cell morphology. We subsequently speculated that, since *cer* and *pagA2* are located in close proximity on the pBC210 plasmid, PA2 may be the preferred binding component for Certhrax translocation into the cell.

Another reason to propose that PA2 may be the binding component for Certhrax is that there are several *Clostridial* strains that express ADP-ribosyltransferase toxins, where the genes for the enzymatic and binding subunit are located in close proximity to each other on the chromosome or plasmid (103). Some of these toxins also target and disrupt the actin cytoskeleton but via inactivation of G-actin protein. These toxins include *C. botulinum* C2 toxin, *C. difficile* iota toxin, *C. perfringens* iota toxin and binary enterotoxin (CBE), as well as *C. spiroforme* CST toxin. Additionally, the *B. cereus* vegetative insecticidal protein (VIP) also affects the actin cytoskeleton and is structurally similar to the *Clostridial* toxins. These binary enterotoxins are expressed as separate A and B components and, like PA, the binding subunits are inactive until they are cleaved by a serine-type protease. Most of the A and B components contain signal peptides and are therefore secreted into culture supernatants (103). By contrast, the C2 and BEC enzymatic components do not contain a signal sequence and their expression is linked to sporulation of the bacterium (79; 121). These latter toxin subunits are released from the bacterial cell following lysis during the sporulation process.

Protective antigen 2, similar to PA of *B. anthracis*, does have a signal sequence, and is secreted into culture supernatants. We showed that the protein is also expressed *in vivo* as we detected PA2-specific antibodies in sera of A/J mice inoculated s.c. with the

ΔpagA1 mutant strain of G9241. Conversely, Certhrax does not appear to have a signal sequence and cannot be detected even in cell lysates of the WT G9241 culture grown *in vitro*. In fact, Certhrax seems to be expressed at such low levels that we were only able to detect protein when it was over-expressed in an IPTG-inducible vector transformed into the *Δcer* mutant strain of G9241. Our studies suggest that the protein undergoes N-terminal processing in G9241; we detected multiple protein bands in the transformant cell lysates probed with an anti-ADPr antibody that were approximately 55 and 52 kDa in size. Furthermore, Certhrax that was expressed with an N-terminal histidine tag was not detected with an anti-His antibody in *Bacillus* cell lysates but was detected with an anti-ADPr antibody. The low levels of Certhrax expression *in vitro* may explain why we were unable to detect anti-Certhrax antibodies in mouse sera following inoculation with the *Δlef* mutant strain of G9241. The difference in lethality between the *Δlef* and *ΔcerΔlef* mutant strains in A/J mice, however, does provide indirect evidence that Certhrax is expressed during G9241 infection. Since Certhrax is not secreted in culture supernatants like PA2 and the anthrax toxin components, preliminary studies were carried out to determine whether Certhrax expression was linked to sporulation of the bacterium as is the case with C2 and BEC toxins of *Clostridium* (see above). Therefore, WT G9241 cultures were grown in sporulation medium and supernatants were isolated at 24, 48 and 72 hours for western blot analyses with the anti-ADPr antibody as the probe. Despite the detection of a protein band of approximately 50kDa (data not shown), the findings were inconclusive. Further analyses must be undertaken to determine whether the 50kDa protein band detected is in fact Certhrax.

Beyond characterization of Certhrax and PA2 expression, we conducted several experiments to evaluate transcription of the *cer* and *pagA2* genes. Passalacqua *et al.* (87) showed that the expression of the two genes was upregulated in the presence of sodium bicarbonate and 14% CO₂, as was the case for the anthrax toxin component and capsule-synthesizing genes. Therefore, we anticipated the detection of *pagA2* and *cer* transcripts when cultures were grown under the toxin-inducing conditions used in our laboratory. We did not, however, anticipate that the two genes would be co-transcribed, as was evident from our data. Specifically, there are approximately 600bp that separate the two genes, but a co-transcript was detected in three different PCR reactions with wild-type G9241 cDNA. These data imply that PA2 may, indeed, play a role in Certhrax translocation into host cells. However, each gene appears to also be expressed alone as *pagA2* has its own promoter. Through the use of promoter fusion constructs, we identified the approximate location of the *cer* and *pagA2* promoters to be within 267 and 155 bp upstream of the translational start for each, respectively (Fig. 13). Primer extension was attempted with γ -³²P -labeled primers to identify the transcriptional start site for *cer* and *pagA2*, but the experiments were technically unsuccessful. Nevertheless, the finding of a co-transcript and promoter for each gene suggests that some form of regulation is involved in transcription. Indeed, the anthrax toxin component expression is regulated by AtxA in *B. anthracis* (49), and AtxA1 has been implicated in regulation of toxin expression in G9241 (96). The presence of kinks in the secondary structure of DNA has been suggested to play a role in AtxA regulation of *B. anthracis*, and these kinks are more prevalent in A- and T- rich regions of DNA (37). Of note, the upstream regions of the anthrax toxin component genes, *pagA2* and *cer* have A- and T- rich upstream regions

as compared to the *Bacillus* chromosome. Indeed, adenine and thymine constitute 81% of the 155 upstream bases of *pagA2* and 78.7% of the 267 upstream bases of *cer*. The chromosome of *B. cereus* G9241, in comparison, is 64.6% AT-rich. Therefore, it is possible that AtxA1 also regulates expression of these two genes. This hypothesis can be more directly tested by qRT-PCR to measure *pagA2* and *cer* transcript levels in the $\Delta atxA1$ mutant strain of G9241.

The role of PA2 in G9241 pathogenesis was previously assessed in an IP inoculation model of C57BL/6 mice (82), and at a 10^5 spore dose the $\Delta pagA2$ mutant strain was as virulent as WT G9241. Tissue recovered from moribund or dead mice had similar G9241 loads to those described in mice exposed to WT spores. Oh *et al.* therefore concluded that PA2 is not involved in pathogenesis (82). In the s.c. and i.n. inoculation models described in this study, the $\Delta pagA2$ mutant strain was also as virulent as WT G9241 at the higher spore inoculum doses. However, we observed a slight but significant decrease in LD₅₀ values in C57BL/6 mice injected subcutaneously with the $\Delta pagA2$ mutant spores as compared to WT G9241 spores. Furthermore, there was a decrease in the percent of deaths when both A/J and C57BL/6 mice were inoculated with the lowest dose of the mutant strain, as compared to the mice exposed to WT G9241 spores. The mice that did die in those inocula groups had a comparable median time to death to the mice that succumbed to the disease in the WT group (Fig. 10).

To further dissect the contribution of PA2 to pathogenesis, we explored the virulence of a $\Delta pagA1\Delta pagA2$ double deletion knock-out in the A/J mice. The double deletion mutant strain had comparable LD₅₀ values to the $\Delta pagA1$ mutant strain. As with the $\Delta pagA1$ mutant strain, A/J mice inoculated with the double knock-out strain had a

variable response to individual doses. Therefore, lethality was assessed by the Reed and Muench method. Collectively, these data suggest that PA2 contributes only minimally to the pathogenesis of *B. cereus* G9241. In other words, when *pagA2* is deleted, expression of PA1 is sufficient for the translocation of the anthrax toxins into host cells to cause lethality following s.c. inoculation with G9241 spores.

The question that arose was could PA2 functionally replace PA1 if it was expressed under the same conditions as the latter protein? Besides the amino acid differences between the two proteins, and the fact that they are expressed from two different virulence plasmids, there were other variables that could be at play. First, the promoter regions between the two genes are not homologous. And second, *pagA1* is the first gene in an operon with the auto-repressor, *pagR*. While we now know that *pagA2* is part of an operon, there is no *pagR*- like gene immediately downstream of *pagA2*. Therefore, we generated a *pagA2inΔpagA1* knock-in mutant strain in the background of $\Delta pagA1\Delta pagA2$, where *pagA2* was inserted into the location where *pagA1* had been. We confirmed that the knock-in indeed expressed PA2 *in vitro*, and it appeared that PA2 was expressed to higher levels in this strain as compared to WT G9241. Furthermore, we confirmed that the *pagR* transcript was expressed in the knock-in strain through PCR with *pagR*- specific primers (data not shown). However, the *pagA2inΔpagA1* knock-in was as attenuated as the $\Delta pagA1$ and $\Delta pagA1\Delta pagA2$ mutant strains of G9241 following s.c. challenge of A/J mice. Therefore, PA2 cannot functionally replace PA1 in anthrax toxin translocation in *B. cereus* G9241.

There are several different possibilities that could explain why PA2 cannot substitute for PA1 as an anthrax toxin binding component. These alternatives are

summarized as follows: A) PA2 does not bind to the CMG2 or TEM8 receptors; B) PA2 does not oligomerize, or does not form a stable oligomer; C) EF and LF cannot bind to the PA2 oligomer; and, D) PA2 cannot form a pore to translocate EF and LF into the cytosol of the host cell. Each of these possibilities is graphically depicted in Figure 24.

PA of *B. anthracis* binds to two receptors on the cell surface, CMG2 and TEM8. These receptors are located on the surface of many eukaryotic cells, including HeLa cells and RAW264.7 macrophages. In fact, HeLa cells predominantly express the TEM8 receptor on their surface, while RAW264.7 cells express CMG2 (92). Therefore, these cell lines can be used to determine whether PA2 can bind to either of the receptors and translocate purified LF or EF into the cytosol to cause toxicity. Based on the amino acid composition, there is just 45% amino acid identity between the receptor-binding domain of PA2 and PA of *B. anthracis* (82). Therefore, it is possible that PA2 uses different receptors for cell entry.

If, on the other hand, PA2 can bind to one of these receptors, the 83kDa monomer would then have to be cleaved by a cell-surface protease to an active 63kDa monomer and then oligomerize so as to bind LF and EF. According to its amino acid sequence, PA2 does appear to have the furin cleavage site in the N-terminal region of the protein that would allow for its activation. Additionally, PA2 contains the five conserved amino acids that are involved in oligomerization of PA, and should therefore be able to form an oligomer (4). However, the capacity for PA2 to oligomerize has not been experimentally tested, and it is therefore possible that the protein cannot so assemble (Fig. 24B). One way to test for oligomerization of PA2 would be to follow the protocol of Milne *et al.*

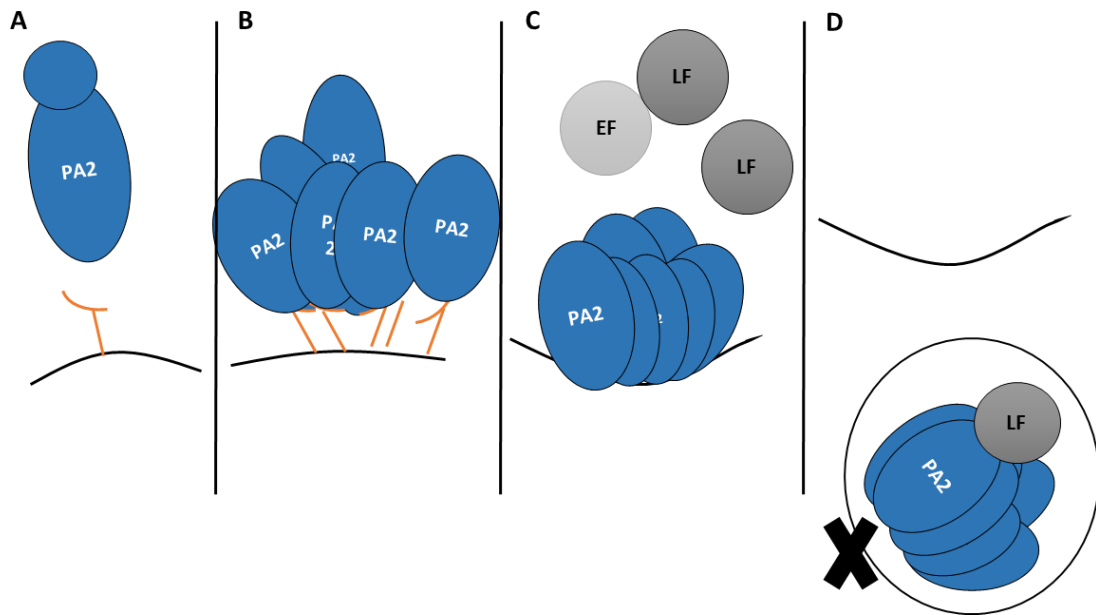


Fig. 24. Model to explain why PA2 cannot functionally replace PA1 in pathogenesis of *B. cereus* G9241.

(A) PA2 does not bind to the CMG2 or TEM8 receptors to which PA binds. **(B)** PA2 does not oligomerize or does not form a stable oligomer. **(C)** LF and EF are unable to bind to the PA2 oligomer. **(D)** The PA2 oligomer cannot form a pore in the endosomal membrane to translocate LF or EF into the cytosol of the cell.

(75). By that procedure, the first step would be to treat purified PA2 with trypsin to generate a nicked PA₂₆₃, a size that could be observed via SDS-PAGE or Western blot analyses. Next, the nicked monomer would be incubated with LF or EF to promote oligomer formation in solution that could be visualized on a native gradient gel as a high molecular weight band. The formed complex could subsequently be subjected to an acidic pH and assessed for resistance to boiling in SDS buffer. The SDS-resistant form is said to correspond to the pore conformation of the oligomer of PA (75; 120).

If PA2 can, in fact, oligomerize the way PA does in *B. anthracis*, it is conceivable that the formed oligomer may not be capable of binding LF and EF (Fig. 24C).

According to Cunningham *et al.* (23) there are seven residues in domain 1 of PA₆₃ that comprise the binding site for LF and EF in the oligomer. If any of the residues are mutated, there is essentially a complete loss of ligand binding capability. These residues are R178, K197, R200, P205, I207, I210, and K214. Based on an amino acid alignment of PA2 to PA of *B. anthracis*, it appears that all of the residues are conserved in PA2 except for R178. Instead of an arginine, a positively charged residue, PA2 contains glutamic acid, a negatively charged amino acid at that location. R178, together with K197 and R200, are located in a basic interface between two bound PA subunits. When any of these residues are mutated to alanine, there is over 90% loss of LF_N binding (23).

Therefore, it is likely that the glutamic acid substitution in PA2 could affect LF and EF binding to the oligomer. An approach to test this hypothesis would be to introduce an E178R mutation in PA2 of the *pagA2inΔpagA1* knock-in strain and determine whether virulence could be restored to wild-type levels following s.c. inoculation of A/J mice.

Additionally, *in vitro* cytotoxicity assays with HeLa or RAW264.7 macrophages could be

used to assess cytotoxicity following administration of nicked PA2 and LF proteins, as compared with intoxication after treatment of macrophages with PA and LF.

After PA oligomerizes and binds EF and LF, the protein complex gets endocytosed and trafficked to an endosome. In the presence of an acidic pH within the endosome, the PA oligomer undergoes conformational changes to form a pore that allows for EF and LF translocation into the cytosol. If PA2 is unable to form a stable oligomer, it may not be capable of forming the pore in the endosomal membrane (Fig. 24D). Lipid bilayer membranes have been used to assess PA₆₃ channel formation and ion current translocation (10; 119). Such a technique can also be used to assess the ability of PA2 to form a membrane channel for successful translocation of LF or EF.

Since our studies showed that *cer* and *pagA2* are co-transcribed, PA2 may be the preferred binding subunit involved in Certhrax translocation into host cells, and it may not be involved in translocation of the anthrax toxin components at all. Further studies need to be done to explore such a hypothesis. As for the role that Certhrax plays in virulence, the data yielded some unexpected results. When *cer* was deleted in *B. cereus* G9241, the Δ *cer* mutant strain was as virulent as WT G9241 in A/J and C57BL/6 mice, following s.c. and i.n. inoculation. Therefore, like PA2, Certhrax is not a major contributor to pathogenesis in G9241, such that when Certhrax is not expressed G9241 is still a highly virulent bacterium. There was also no difference in the median time to death at any of the doses between WT and the mutant strain. However, 100% of A/J mice inoculated with the 10¹ dose of Δ *cer* spores died as compared to 87% lethality in mice challenged with the same dose of WT G9241 spores (Fig. 21). Additionally, A/J mice challenged with Δ *cer* spores formed larger abscesses at the site of inoculation than when

mice were inoculated with the WT strain. This finding suggests that there may be more pathology at the site of inoculation with the mutant spores, and that the mutant strain may in fact be more virulent than WT G9241. The calculated LD₅₀ values for Δcer did not reflect an increased virulence phenotype when compared to WT G9241, but the LD₅₀ for WT G9241 in this model is already so low that it approaches the limit of detection. Thus, it would be difficult to calculate an LD₅₀ value lower than 10 spores.

The construction of the $\Delta cer\Delta lef$ double deletion mutant strain and its subsequent evaluation in mice lent further credence to the idea derived from the data described above that Certhrax may be an anti-virulence factor of G9241 (Table 5). Indeed, the double mutant strain had a lower LD₅₀ in A/J mice following s.c. challenge than the single *lef* deletion mutant strain. This phenotype was only observed in A/J mice, while in the C57BL/6 mouse model $\Delta cer\Delta lef$ was avirulent. As the host immune response accounts for the biggest difference between the two mouse strains, we speculated that Certhrax may be involved in immune modulation. This possibility would be in keeping with some of the effects observed from the anthrax toxins as well.

Certhrax has an N-terminal PA-binding domain and a C-terminal ADP-ribosyltransferase domain. Therefore, we developed two hypotheses to explain the increased virulence phenotype of the $\Delta cer\Delta lef$ mutant strain. The first theory was that Certhrax competes with EF and LF for binding to the PA oligomer. EF and LF have seven conserved amino acids that are necessary for each of them to bind to the PA subunit (52). Based on amino acid alignments to EF and LF, it appears that the seven amino acids are also conserved in the N-terminal region of Certhrax. Furthermore, Visschedyk *et al.* determined that Certhrax does require PA for entry into host cells to

cause cytotoxicity (111). Thus, if Certhrax competes for binding with EF and LF, more of the anthrax toxin components could bind PA in the Δcer mutant strain and more anthrax toxin would be translocated into the cell to cause pathology. More edema toxin could subsequently result in the production of more edematous fluid at the site of inoculation to form a larger abscess, as seen in the A/J mice.

The second hypothesis was that the ADP-ribosylating activity of Certhrax was responsible for the virulence phenotype of the $\Delta cer\Delta lef$ mutant strain of G9241. Vinculin has been identified as the target for Certhrax in HeLa cells (99). Vinculin is a critical protein in cell-cell and cell-matrix adhesion complexes, as it binds actin to promote its polymerization and recruits remodeling proteins to the complexes. When vinculin is absent, actin polymerization is compromised, which, in turn, causes a detrimental effect on cell morphology (8). Vinculin inactivation also results in defects in cell migration (118). Therefore, the inactivation of vinculin in G9241-infected cells perhaps disrupts their migration and prevents G9241 from rapidly spreading within the host. Therefore, when *cer* is deleted and vinculin is unaffected, G9241 spreads more rapidly.

To test the latter hypothesis directly, we generated enzymatically active and enzymatically inactive *cer* restorants in the $\Delta cer\Delta lef$ mutant strain background. Certhrax was enzymatically inactivated by mutating two catalytic residues in the ADPr domain, as it has been previously described in literature (100; 111). These mutants were examined for virulence in the s.c. challenge model of A/J mice. The *cer.rest* Δlef mutant strain showed an attenuated virulence phenotype as compared to the parent $\Delta cer\Delta lef$ strain, and the LD₅₀ was comparable to the Δlef mutant strain of G9241. The enzymatically inactive mutant strain, on the other hand, remained as virulent as the $\Delta cer\Delta lef$ mutant strain.

Therefore, these data suggest that the ADP-ribosylating activity of Certhrax is responsible for the difference in virulence between $\Delta cer\Delta lef$ and Δlef , not the fact that the protein competes for PA binding.

We further sought to determine whether the *inact.cer.rest Δlef* mutant strain was more pathogenic because it was able to disseminate more rapidly within the host than *cer.rest Δlef* . A dose of 10^6 spores of each mutant strain was administered subcutaneously to A/J mice and a group of five mice was euthanized at 6, 24, and 72 hours post inoculation with each strain. An additional five mice were inoculated per mutant strain and were left to succumb to the infection, or were euthanized at the conclusion of the experiment on day five. The bacterial burden was assessed within the abscess formed at the site of inoculation and within the spleen. The bacterial load in the spleen was used as a marker for dissemination. Several overall observations were made at the end of the experiment. First, vegetative cells were isolated from the site of inoculation in all mice as early as six hours post inoculation (Fig. 23A). These data support the hypothesis that *Bacillus* spores indeed germinate at the site of inoculation and not after being trafficked by macrophages to the draining lymph nodes. Despite the multitude of studies done in the *B. anthracis* field, the site of spore germination is still a controversial topic. Second, mice that succumbed to the infection, or were moribund upon euthanasia, had high bacterial counts in the spleen. Independent of which strain of *B. cereus* G9241 was administered, these mice had over 10^7 CFU/ml of bacteria in the spleen and, with just a few exceptions, all of the bacteria were vegetative cells and not spores (Fig. 23C-D). The spleens that did carry G9241 spores were mostly collected from mice that died overnight and therefore

the carcasses were not collected immediately. Any vegetative cells in the organs could have started to sporulate over time.

There were several differences observed between the two restorant strains during the experiment. Many of the differences occurred at the first time point evaluated. These kinetics suggest that Certhrax elicits its effect early in infection. At six hours post inoculation, no bacteria were isolated from spleens of most mice inoculated with *inact.cer.restΔlef* (Fig. 23C). Spleens collected from mice inoculated with the *cer.restΔlef* mutant strain, on the other hand, had an average of 10^3 CFU/ml of bacteria (Fig. 23C). This observation may indicate that early in infection the *inact.cer.restΔlef* does not disseminate from the site of inoculation as rapidly as does the *cer.restΔlef* mutant strain. This finding is the opposite outcome from what was initially hypothesized. At that same time point, abscesses collected from mice inoculated with the *inact.cer.restΔlef* restorant had more spores in the tissue as compared to the *cer.restΔlef* strain (Fig. 23B). This difference in counts was also true for the vegetative bacteria at that time, but the difference was not as pronounced (Fig. 23A). These results imply that the enzymatically inactive restorant remains at the site of infection longer but appears to disseminate more effectively and at larger numbers later in infection. The latter supposition is supported by the fact that all five mice inoculated with the *inact.cer.restΔlef* mutant strain, which were part of the challenge group permitted to succumb to the infection, died by the fifth day. Only one mouse inoculated with the *cer.restΔlef* died before the termination of the experiment.

The most striking difference between the two inocula strains, however, was the difference in abscess size at 24 hours post-inoculation (Fig. 22A, C). The abscesses

collected from mice challenged with the *inact.cer.restΔlef* mutant strain were much larger at that time point. Furthermore, there was no difference in bacterial counts in the abscesses at the 24 hour time point, an observation that raises the question of what is the actual composition of the abscesses? Later in infection, however, the abscess sizes were comparable between the two mouse groups. This once again suggests that Certhrax appears to act early in infection, and the effect may be more localized to the site of inoculation. Additionally, as mentioned above, since this effect is only observed in A/J mice, Certhrax may have a greater effect on mice whose immune response is compromised.

These results paint a complex picture for Certhrax involvement in pathogenesis as some of the data is counter-intuitive. As depicted in Figure 25, perhaps when Certhrax is expressed by *B. cereus* G9241, as is the case in WT G9241 and the *Δlef* and *cer.restΔlef* mutant strains, Certhrax is produced by germinated bacilli inside phagocytes and is able to target and ADP-ribosylate vinculin in those phagocytic cells (Fig. 25, panel A). Inactivation of vinculin subsequently leads to actin rearrangement and loss of cell integrity, and these events allow the bacteria to escape the cell to disseminate in the blood. This dissemination occurs soon after inoculation, and, therefore, low levels of bacteria are detected in the spleen at the six-hour time point. Additionally, if vinculin is inactivated there may be a disruption in cell migration of the immune cells. Therefore, as the infection progresses, the phagocytes cannot be used effectively by G9241 as a vehicle to disseminate throughout the host.

Conversely, when Certhrax is enzymatically inactive or the gene is deleted, G9241 spores get taken up by the phagocytic cells. Vinculin is not targeted by

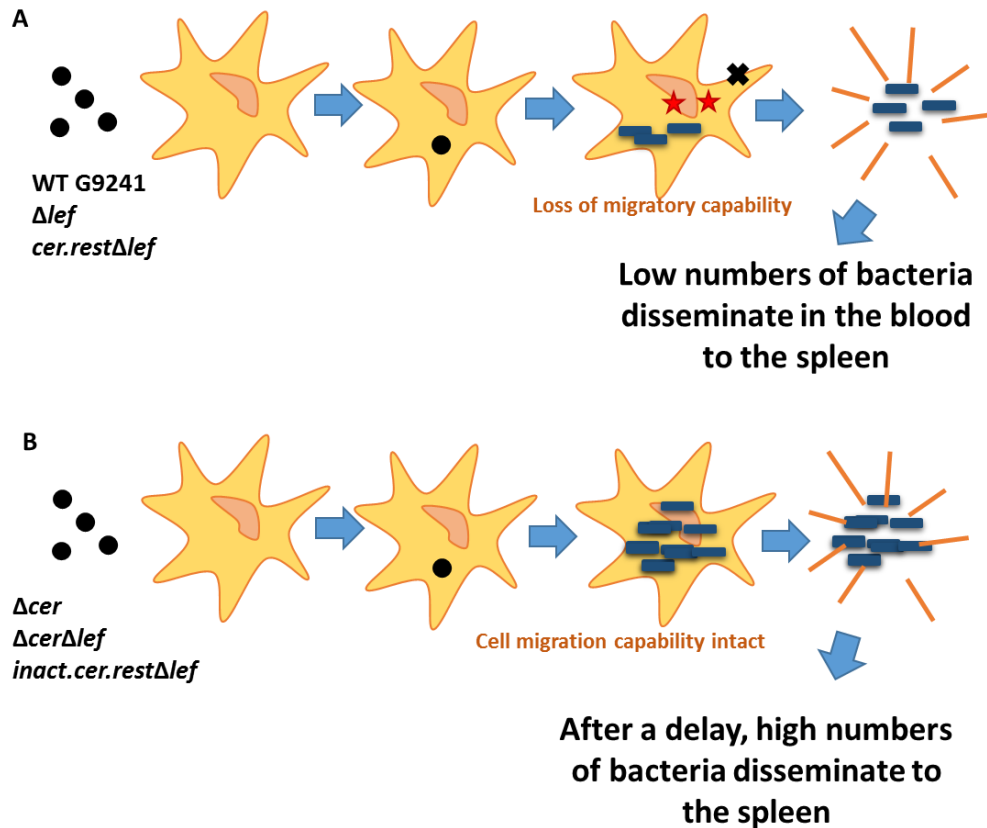


Fig. 25. Model to describe Certhrax involvement in pathogenesis of *B. cereus* G9241. The bacterial spore is represented by the black circle, a phagocytic cell is the yellow star-like shape, vegetative bacterial cells are the dark blue rods, and the active Certhrax ADPRT is represented by the red star. **(A)** Spores of *B. cereus* G9241 that can express Certhrax (WT G9241, Δlef , and *cer.rest* Δlef) get engulfed by phagocytic cells at the site of the inoculation. The spores germinate into vegetative bacteria in the phagocytic cell, where some bacteria are killed by the phagocyte, while others survive and begin to replicate. The vegetative cells secrete various virulence factors and Certhrax. Certhrax ADP-ribosylates vinculin, which, in turn, leads to actin depolymerization and loss of cell integrity. The phagocytic cells lyse and bacteria disseminate into the blood. Furthermore, a vinculin inactivation disrupts cell migration so that the phagocytic cells do not efficiently support bacterial dissemination. Thus, low numbers of bacteria reach the spleen early in infection. **(B)** Spores of *B. cereus* G9241 strains that do not express an enzymatically active Certhrax (Δcer , $\Delta cer\Delta lef$, *inact.cer.rest* Δlef) are engulfed by phagocytic cells, where the spores begin to germinate. The vegetative cells that survive killing replicate but do not express Certhrax. The phagocytic cell remains intact until the bacteria replicate to high enough levels to lyse the cell with a subsequent delay in dissemination. Since vinculin remains unaffected, some phagocytic cells with intracellular bacteria are able to migrate throughout the host and promote bacterial spread. Upon lysis of the phagocyte, high numbers of bacteria are released into the blood stream and seed the spleen in larger quantities than in panel A.

the inactive Certhrax, produced by bacilli after spore germination, and the phagocytic cells remain intact (Fig. 25, panel B). Phagocytes are able to kill some of the germinated bacteria but not all. The surviving organisms replicate within the immune cells until the sheer number of bacteria lyse the cell. This delay in bacterial dissemination at the early time points amplifies the infection and allows for more bacteria to disseminate to the spleen later in infection. Additionally, since vinculin is active, the immune cells are able to migrate from the site of infection more efficiently to distribute G9241 within the host.

Another possibility is that enzymatically active vinculin actually enhances the *B. cereus* G9241 infection. This has been observed in *Staphylococcus aureus* pathogenesis (38). In the case of *S. aureus*, researchers found that vinculin binds Rab5 and cooperatively enhances *S. aureus* uptake in non-phagocytic cells. Rab5 is a membrane-trafficking protein located in membranes of early endosomes and the plasma membrane. This protein plays a role in the internalization of many molecules, including bacteria. Over-expression of vinculin enhances *S. aureus* phagocytosis, while knock-down of vinculin with siRNA technology results in a reduced infection (38). Therefore, it is possible that vinculin also enhances *B. cereus* G9241 phagocytosis by non-phagocytic or phagocytic cells at the site of inoculation.

More data are needed to fully understand the involvement of Certhrax in pathogenesis of *B. cereus* G9241. First, it is important to analyze the composition of the abscesses. This can be achieved through microscopy of the isolated abscesses material (after a cytospin perhaps followed by a Giemsa stain), to determine what immune cells are predominantly present. The presence of pus suggests that neutrophils may be involved. Additionally, it is possible that Certhrax targets a protein other than vinculin.

Vinculin was identified as the target in HeLa cells (99; 100), but it is conceivable that there could be a different target in phagocytic cells, like RAW264.7 macrophages. Macrophage membranes and cytosol could be incubated with the ADPr domain of Certhrax, as was done by Simon *et al.* (100), to determine whether Certhrax binds to any protein besides the 120kDa vinculin. Furthermore, a dissemination study should be done with WT G9241 and the Δcer mutant strain to determine whether the deletion of *lef* has any effect on the outcome of the experiment. This study would need to be carried out with a much lower inoculum dose than used with the restorant strains, as these two strains have LT and are highly pathogenic in A/J mice.

In conclusion, this body of work shows that the anthrax toxins play an essential role in pathogenesis of *B. cereus* G9241. The homolog of protective antigen, PA2, minimally contributes to virulence of this pathogen, as we observed some attenuation at the lower inoculum doses in the absence of the *pagA2* gene. However, PA2 cannot functionally replace PA in translocation of the LF and EF components into the cell. Defining the reason for this outcome can advance our understanding of how PA functions in *B. anthracis*. We also identified Certhrax, a novel ADP-ribosyltransferase, as an anti-virulence factor of G9241. In the absence of Certhrax, G9241 becomes more virulent during subcutaneous inoculation of A/J mice. This may be one of the first studies to identify a toxin as an anti-virulence factor. Additional studies are required to understand how Certhrax elicits this effect, and if it is in fact involved in immune modulation during infection. Collectively, the results described here expand the information on pathogenicity of members of the *Bacillus cereus sensu lato* and raise novel questions for future research endeavors. With the emergence of more virulent *B. cereus* strains that

cause human and primate disease and carry anthrax-like virulence factors, it is important to define how these pathogens cause illness, how different they are from each other, and what information can be extrapolated to better define the pathogenesis of *B. anthracis* or anthrax-like organisms.

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