A NOVEL ROLE FOR TELLURIUM RESISTANCE GENES IN THE PATHOGENESIS OF *BACILLUS ANTHRACIS*

By

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Introduction

Bacillus anthracis is a gram positive, spore-forming bacterium and the causative agent of the deadly disease anthrax. This disease has plagued humans since antiquity, having been implicated in the 5th and 6th plagues of Egypt (Cieslak and Eitzen Jr, 1999; Hart and Beeching, 2002). Anthrax, primarily a disease of herbivores, is historically known as 'Woolsorter's' disease, afflicting those who had direct and frequent contact with infected animals (Hart and Beeching, 2002). Diseased animals are highly infectious upon death. Once the animal is moribund, the vegetative bacteria sporulate almost immediately upon contact with air. Spores are highly resistant to drastic environmental factors such as low nutrient supply and harsh weather, allowing them to survive in this dormant state for decades (Kolstø et al., 2009).

Spores are the infectious agent and disease presentation depends on mode of entry. In cutaneous anthrax, spores enter the body through a lesion or break in the skin. This is the least severe of the three modes of infection, resulting in a 30% mortality rate if left untreated. Gastrointestinal anthrax occurs by ingestion of infected animal products with a mortality rate of 35-60%. Finally, inhalation anthrax, the most deadly, results in 90-95% mortality. Treatment initiated more than 48 hours post onset of flu-like symptoms, still result in ~95% fatality (Cieslak and Eitzen Jr, 1999). Due to the high virulence, relative ease of spore dispersal, and deadly nature, anthrax is feared as a possible bioterrorist threat, as occurred in the September 11, 2001, attacks (Hudson et al., 2008).

During infection, *B. anthracis* spores are phagocytosed by alveolar macrophages and dendritic cells. En route to the nearest lymph node, bacteria germinate, survive the phagolysosome and break out of the cell. Subsequent extracellular replication in the capillaries leads to widespread septicemia (Dixon, 1999). In most pathogens there is an evolutionary trade-

off in regards to virulence in the sense that if the microbe kills its host too quickly, then the pathogen will also die. However, for the lifecycle of *B. anthracis* to be completed, the bacterium must replicate expeditiously and kill the host quickly to ensure a high bacillary load (10^9 cfu/ml in terminal animals) is released into the surrounding environment (Cote et al., 2011). It has been hypothesized that a combination of toxins, plasmid and chromosomally encoded, contribute to toxemia and bacteremia resulting in the death of the host.

Three major virulence factors exist on two extra-chromosomal plasmids, pXO1 and pXO2, coding for the anthrax toxins, lethal and edema toxin, and the capsule, respectively (Kolstø et al., 2009). Edema toxin is a calmodulin-dependent adenylate cyclase that acts to increase cyclic-AMP levels leading to an offset of the water homeostasis in the cell (Dixon et al., 2000). Edema toxin inhibits neutrophil function and priming, decreases NADPH oxidase activity, decreases migratory dendritic cell function, decreases T-cell function and increases the T-helper response.

Lethal toxin disrupts mitogen-activated protein kinase kinases leading to a decrease in both dependent and independent products of this cascade (Nalp1b inflammasome dependent death, the pro-inflammatory cytokines IL-8, IL-10, and TNFα secretion). Lethal toxin decreases monocyte differentiation, macrophage function, bactericidal activity and chemotaxis of macrophages, neutrophil migration and priming, NADPH oxidase activity, B-cell proliferation, IgM production, function and maturation of migratory DCs, and T cell function and chemotaxis. Lethal toxin also increases apoptosis of alveolar macrophages and migratory dendritic cells as well as up-regulating the Th2 response of T cells (Tournier et al., 2009). These toxins successfully disrupt both the innate and adaptive arms of the immune system in the host.

Located on the pXO2 plasmid is the negatively charged poly-glutamyl capsule, which allows vegetative *B. anthracis* to resist phagocytosis by the host (Baillie and Read, 2001) due to its low immunogenicity (Candela and Fouet, 2005). Capsule synthesis is reported to begin immediately after phagocytosis of the spores but is not visible until the bacteria are vegetative. The capsule can be sloughed off and act as an immune decoy (Ezzell and Welkos, 1999; Cote et al., 2011). Both plasmids are required for full virulence (Little and Ivins, 1999). The Sterne strain, a widely accepted model strain of *B. anthracis*, lacks the pXO2 plasmid and is highly attenuated for virulence.

While the virulence factors located on the two plasmids have been extensively studied, the ~5,000 chromosomal genes have not been thoroughly investigated. There is experimental evidence suggesting that these genes play a role in pathogenesis (Baillie and Read, 2001). For example, the transition from spore to vegetative bacteria, which is essential for pathogenesis, is initiated by germinants. Seven have been discovered to date, six of which are chromosomally encoded on the *ger* operon (*gerA, gerH, gerK, gerS*, and *gerT*) with only one on the pXO1 plasmid (*gerX*) (Cote et al., 2011). Sequencing of the genome has yielded numerous other examples including haemolysins (homologues that contribute to pathogenesis of *B. cereus* and *B. thuringensis*), phospholipases (homologous to *L. monocytogenes*) and iron acquisition functions (Read et al., 2003). While many of the chromosomal genes remain unclassified or putative/hypothetical, it is likely many more genes contribute to pathogenesis.

A transposon based mutagenesis system was employed to randomly disrupt chromosomal genes in *B. anthracis* Sterne (McGillivray et al., 2009). 5,000 different mutants were generated, each representing an independent gene disruption. The invertebrate model *C. elegans*, which has been used previously to model infections with pathogens (Glavis-Bloom et al., 2012), was used

to screen transposon mutants for their ability to infect the worms. A large number of human, plant, animal and insect pathogens have been shown to infect *C. elegans* by replacing its food source with the desired pathogen and observing health and development of the nematode (Sifri et al., 2005). Transposon-based screens have successfully been used to identify attenuated mutants in *C. elegans*, which were later verified in mammalian systems (Sifri et al., 2005). Many biochemical, developmental and cell biological pathways have been highly conserved evolutionarily between the nematode and mammals (Aballay and Ausubel, 2002). The MAPK, transforming growth factor-β and an insulin/insulin-like growth factor pathway, which are found in *C. elegans*, induce programmed cell death, production of reactive oxygen species (ROS) (Chávez et al., 2007) secretion of antimicrobial peptides (Kato et al., 2002) and lysozymes in mammals (Sifri et al., 2005).

Using this screening system, 11 mutants were identified as unable to establish infection within the worm. The most highly attenuated mutant had a transposon insertion in an operon containing six tellurium resistance genes (McGillivray, unpublished data). Tellurium is an elemental metal found in the earth's crust and exists as water-soluble tellurite (TeO_3^{-2}). Tellurium compounds possess antimicrobial properties and have been used historically to treat diseases such as leprosy, tuberculosis, dermatitis, cystitis and eye infections (Taylor, 1999). Tellurite is toxic to bacteria and the ability to reduce it to tellurium, a less toxic form, is essential for bacterial survival. Little is known about the specific mechanisms that contribute to tellurium resistance (Te^R) although there are at least 5 different genetic Te^R determinants (Taylor, 1999). Three determinants are found on the bacterial chromosome of *Pseudomonas syringae* (*tmp*), *Escherichia coli* K12 (*tehA/B*), and *Rhodobacter sphaeroides* (*trgA/B,cysK*). Two Te^R prototypes are plasmid mediated and have been extensively studied on the R478 plasmid (*terZABCDEF*) and IncPα (*kilA, telA/B*) (Taylor, 1999).

However, this does not explain why bacteria, such as human pathogens, which are unlikely to come into contact with the soil-based tellurite, would possess tellurium resistance operons within their genome. Plasmid-mediated Te^R has been shown to confer resistance to infection by bacteriophage and pore-forming colicins as well as resistance to tellurite, suggesting an alternate role of the genes in R478 (Taylor, 1999). Although the actual cause of tellurite toxicity is unknown, the current hypothesis suggests associated oxidative stress, either because tellurite is a strong oxidant or because it results in the formation of ROS (Summers and Jacoby, 1977). These last two statements taken together suggest another role for the tellurium resistance operon found in *B. anthracis*, such as resistance to ROS, an important innate immune defense.

Methods

Strains and Growth Conditions

The parent strain used was *Bacillus anthracis* Sterne, which lacks the pXO2 plasmid encoding the capsule. Two mutants were used that have disruptions in the *yceG* gene of the tellurium resistance operon. Transposon mutant 1 (TN1) has a 7kb transposon inserted in the *yceG* region and was created using a previously described transposon mutagenesis system (McGillivray et al., 2009). Insertional mutant 1 (IM1) has the 5kb pHY304 plasmid inserted in the *yceG* region and was constructed previously (McGillivray unpublished data). To create a complemented strain, the *yceG* and *yceH* genes were amplified as one large amplicon, cloned into the pdcERM expression plasmid and transformed into the TN1 mutant (TN1+pEF). Overnight cultures were grown in Brain Heart Infusion (BHI) growth medium at 37°C with agitation. The following antibiotic concentrations were used: 50 μg/ml kanamycin (TN1), 5 μg/ml erythromycin (IM1) and 50 μg/ml kanamycin + 5 μg/ml erythromycin (TN1+pEF) when indicated.

Bacterial Growth Curves

Equal amounts of WT, TN1 and IM1 overnight cultures were added to 3 ml BHI medium (Hardy Diagnostics). Growth of each strain was monitored by optical density (OD) every hour for eight hours. Absorbance was recorded at 600nm (ThermoScientifc Spectronic 20D+). Equal amounts of WT, TN1 and IM1 overnight cultures were added to 3 ml BHI + 30 μg/ml potassium tellurite. Cultures grew for 8 hours before OD readings were recorded. Readings were taken every hour from hour 8 to hour 24. Growth curves were repeated a minimum of three times. Representative graphs are shown.

Transmission Electron Microscopy:

Bacterial cultures were grown overnight in the presence and absence of increasing amounts of potassium tellurite. The bacteria were pelleted and washed in phosphate buffer (1 X PBS), fixed in 2.5% glutaraldehyde and stained using 1% osmium tetroxide. A series of alcohol washes were carried out and samples embedded in EPON/Araldite embedding resin mixture (20g EMbed 812, 16g DDSA, 8G NMA, and 1.1-1.3g DMP 30). The fixed samples were baked at 60°C for 3-5 days in a Precision Scientific, Co. heated vacuum desiccator. Sectioning of samples was performed using Reichert-Jung Ultracut E. Sections were placed on carbon-coated grids and visualized under the TEM (JEOL 2100) at 200kV. Figures are representative of 3 independent sample preps.

Oxidant Assays

10 ml of Brain-Heart Infusion (BHI) media was inoculated with 500 μL of overnight WT and mutant cultures. The 10 ml cultures were grown to early logarithmic stage (OD 0.4). Cells were washed and resuspended in BHI (H_2O_2 and hypochlorite) or RPMI + 5% Lysogeny broth (LB- Hardy Diagnostics) (paraquat) (Hardy Diagnostics and Cellgro, respectively). *B. anthracis* parental and mutant strains were diluted 1:2 (H_2O_2), 1:10 (hypochlorite) or 1:100 (paraquat) (all Sigma) in the absence or presence of indicated amounts of antimicrobial agents and added to 96-well plates. The plates were incubated overnight at 37°C under static conditions. At 24 hours each well was mixed using multichannel pipettes and optical density was measured (Floustar Omega) at 600 nm. All assays were repeated at least 3 times in triplicate, combined and presented as mean +/- SEM.

Antimicrobial Assays

3 ml of BHI medium was inoculated with 100 ul of overnight WT and mutant cultures and grown to early log phase (OD 0.4). Cells were washed and resuspended in RPMI + 5% LB. WT and mutant strains were diluted 1:20 (Nisin (Sigma), bacitracin (Sigma) and CRAMP (Anaspec)), 1:100 (HNP-2; Anaspec) and 1:10 (LL-37; Anaspec) and added to 96-well plates in the absence and presence of antimicrobial agents. Plates were incubated overnight at 37°C under static conditions. At 24 hours wells were mixed with multichannel pipettes and optical density was measured (Floustar Omega) at 600 nm. Nisin and bacitracin assays were repeated at least 3 times in triplicate, combined and presented as mean +/- SEM. LL-37 assays were repeated at least three times in singles, combined and presented as mean +/- SEM

Survival in Macrophages:

WT and TN1 survival in macrophages was assessed using J774 cells, an immortalized macrophage-like cell line. Cells were grown at 5% CO2 at 37°C in RPMI+ 10% FBS and maintained between 3-9 x 10^5 cells/ml. 24-well plates were seeded with 4 x 10^5 cells 12 hours prior to exposure with bacteria. Bacteria were grown to early log phase and incubated with cells for 30 minutes to allow for phagocytosis. 50 μg/ml gentomycin was added to wells after phagocytosis and incubated for 30 minutes to kill extracellular bacteria. Cells were washed twice in PBS and allowed to incubate for 3.5 additional hours at 37 °C plus 5% CO_2. 0.25% triton-X was added to macrophages and triturated at 0 and 4 hours after initial addition of bacteria. Surviving colony forming units (cfu) were enumerated by dilution plating.

Survival in Human Whole Blood

Blood collected from healthy donors (use and procedures approved by the University of California, San Diego, Human Research Protections Program) was incubated with 10^5 cfu *B. anthracis* in a total volume of 500 μl and agitated at 37 °C. At 15 minutes, aliquots were removed, blood was lysed in water and surviving bacteria were enumerated by serial dilution plating. Experiments were performed using blood from three individual donors and results are combined and presented as mean +/- SEM.

Gene Expression Profiling:

WT bacterial cultures were grown overnight in BHI and appropriate antibiotic media. RNA was extracted using RNeasy Mini Kit (Qiagen) including second RNA elution step, treated with DNase (Ambian), and reverse transcribed to cDNA (Applied Biosystems) per

manufacturer's protocol. PCR primers designed for three genes in the operon (*yceC*, *yceG* and *yceH*) were used to amplify the cDNA via semi-quantitative PCR (S1000 Thermal Cycler).

RT-PCR		PCR	
1 cycle	25°C for 10 min	1 cycle	95°C for 3 min
1 cycle	37°C for 2 hrs	30 cycles	95°C for 30 sec
1 cycle	85°C for 5 min		54°C for 30 sec
1 cycle	4°C forever		72°C for 1 min
		1 cycle	12°C forever

Gel electrophoresis was performed on the PCR products and visualized using Gel Logic 200 Imaging System under UV light for the presence of bands representing the corresponding cDNA. The band intensity of each gene was analyzed using Kodak 1D software.

Name of Primer		Sequence (5'->3')
FusA Fwd	------	AAG CTG GTG GTG CTG AAG CAC
FusA Rev	------	TTC CCA ATC AGC TTC TCC TTG AAG
YceC IM Fwd	------	AGT CAA GCT TCA ATC AGG TGG GTT TCT GT
YceC Confirm Rev	------	TGA TGC CAT ATA CAA TTC CTC CTC TGT
YceG IM Fwd #2	------	CGT CCA CAA TAT GAC CTG ACA G
YceG Rev #4	------	GAG CTA CAG GTG TAT AGG AAC G
YceH IM Fwd	------	AGT CAA GCT TTG GAC CGA TTC GAT AGC AA
YceH IM Confirm Rev	------	CGA CTT TCT TCA CGC TCA CGC

RNA extraction, cDNA synthesis and second strand cDNA synthesis were repeated at least 3 times and a representative figure is shown.

QPCR

cDNA from WT, TN1 and IM1 was analyzed via an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). SYBR Green PCR mix (Life Technologies) was used as the

indicator. Amplification of genes was conducted in 40 cycles, and relative expression calculated by the Livak method.

Name of Primer		Sequence (5'->3')
FusA Fwd	------	AAG CTG GTG GTG CTG AAG CAC
FusA Rev Short	------	CCA TTT GAG CAG CAC CAG TGA
YceC Fwd Short	------	GTA TCG CCA CGG AAG TGA ATG
YceC IM Confirm Rev	------	TGA TGC CAT ATA CAA TTC CTC CTC TGT
YceG Fwd Short #2	------	CGT CCA CAA TAT GAC CTG ACA G
YceG Rev #4	------	GAG CTA CAG GTG TAT AGG AAC G
YceH IM Fwd	------	AGT CAA GCT TTG GAC CGA TTC GAT AGC AA
YceH IM Rev Short	------	CAC GGC CCA TCG TTT GAT AC

Antimicrobial Disk Diffusion

WT, TN1 and IM1 were streaked on BHI agar plates containing 0 or 30 μg/ml potassium tellurite as indicated and then challenged with 6mm sterile paper disks (Becton Dickinson) inoculated with 30 μg chloramphenicol, 10 μg streptomycin, 10 U penicillin or 30 μg tetracycline. Plates were incubated overnight at 37°C and zone of clearing was measured and area of clearing calculated. Results from 3 independent experiments were combined and presented as mean +/- SEM.

Potassium Tellurite Disk Diffusion

WT, TN1 and IM1 were streaked on BHI agar plates and challenged with 6 mm sterile paper disks (Becton Dickinson) inoculated with a disk soaked in 10 mg/ml potassium tellurite. Plates were incubated overnight at 37°C and zone of clearing was measured and area of clearing calculated. Results from 3 independent experiments were combined and presented as mean +/- SEM.

Mouse Infection Studies

Bacteria were grown to OD 0.4 at wavelength 600 nm, washed in PBS and resuspended at a 1:30 dilution in PBS (approximately $2x10^7$ cfu/ml). Parental (WT) and mutant (TN1) *B. anthracis* Sterne were mixed at a 1:1 ratio and 0.1 ml was injected intravenously via the tail vein into 8-week old female CD1 mice. The initial starting numbers of bacteria were determined by serially diluting the 1:1 mixture on BHI plates (total cfu) and BHI Kan plates (TN1 cfu). WT cfu was determined by subtracting the Kan cfu from the total cfu. After 2 days, animals were euthanized and the kidneys were isolated and weighed. 1 ml of PBS was added per gram dry weight and kidneys were homogenized using a bead beater. Surviving bacteria were enumerated by serial dilution on BHI and BHI Kan plates and cfu/gram kidney was determined for WT and TN1 as described above.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. Disk diffusion, H_2O_2, Hypochlorite, paraquat, nisin, polymyxin, bacitracin, LL-37, and survival assays were evaluated with a one-way ANOVA followed by a Tukey's *post hoc* analysis using GraphPad Prism software. *In vivo* competition mouse infection data was log transformed and analyzed by a paired student's t-test.

Results

Identification of attenuated transposon mutants.

C. elegans was used as a model system to identify transposon mutants unable to successfully establish an infection. Of the ~5,000 mutants screened, 11 presented with attenuated virulence in *C. elegans* (less than 40% in comparison to an 80% infection rate with wild-type)

(**UNRESOLVED**). The most reliably attenuated mutant, TN1, infected and killed at a rate of less than 20% (Figure 1A). Infected worms are dark, rigid and immotile whereas worms that were able to clear the infection are motile, transparent and curved, allowing for easy phenotypic recognition of attenuated mutants (Figure 1B).

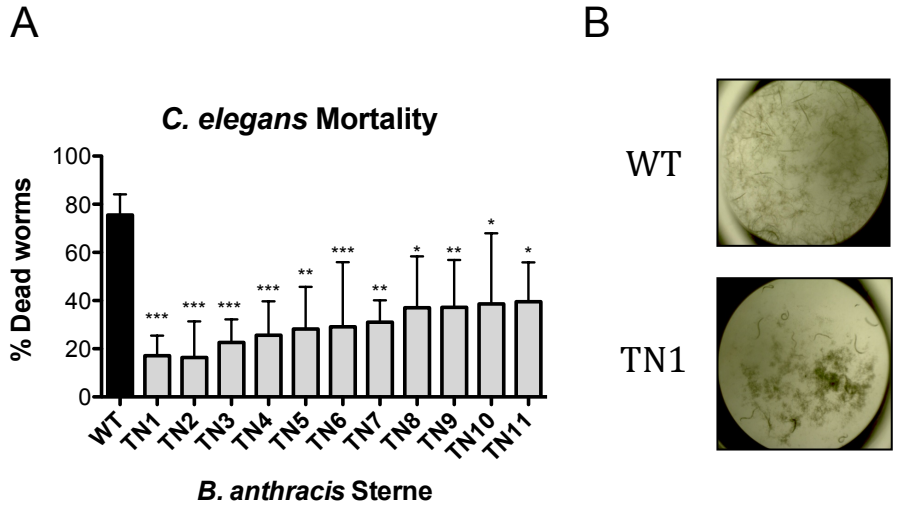

Figure 1. Identification of transposon mutants with attenuated virulence using *C. elegans*. **A**) Worm death was assessed at 72 hours. 11 mutants were found to have attenuated virulence. Data evaluated by one-way ANOVA followed by Tukey's *post hoc* analysis. *,p-value<0.05; **,p-value<0.01; ***,p-value<0.001. **B**) Worms were infected with wild-type (WT) or the transposon mutant (TN1). Infected worms are dark, rigid and immobile worms (WT) while uninfected worms are curvilinear (TN1).

Tellurium Resistance in *B. anthracis*

The TN1 transposon was inserted in the 5th gene of a six-gene operon containing tellurium resistance genes (Figure 2, black arrow). Tellurium resistance genes were originally identified in the R478 plasmid in *Escherichia coli* but have been found in other bacterial species including *Bacillus subtilis*, *Pseudomonas syringae*, *Rhodobacter sphaeroides* and *Yersinia pestis* (Taylor, 1999). Because of the homology of *B. anthracis* to *B. subtilis*, we have kept the gene

names originally assigned *yceC, yceD, yceE, yceF, yceG,* and *yceH* (Kumano et al., 1997). Homology also exists between the *yce* genes of *B. anthracis* and the *ter* genes in the R478 plasmid with *yceC* and *yceD* having homology to both *terD* (37.0% and 54.2%) and *terE* (40.1% and 53.6%) (percent identity). Many bacterial species possess multiple *terD* paralogs arranged in tandem with *terC* genes. This is the case for the *Bacillus yce* operon where, in terms of homology, the operon is arranged *terD-terD-terD-terC-yceG-telA*, with the addition of *terC* and *telA* contributing to a higher complexity (Anantharaman et al., 2012).

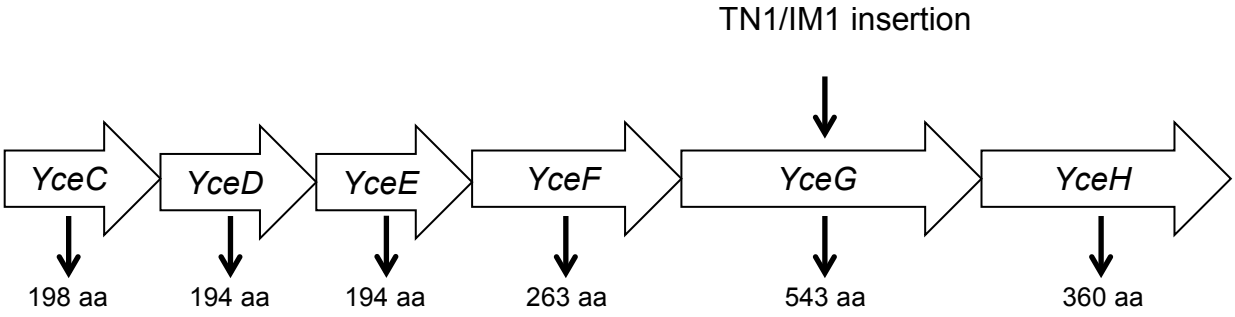

Figure 2. Tellurium Resistance Gene Operon found in *B. anthracis*. Location of the transposon insertion or plasmid insertion for TN1 and IM1, respectively, is indicated (arrow). Protein size (aa) indicated underneath.

An independent mutation (IM1) was generated to confirm the phenotype seen with the TN1 was due to a disruption in the *yceG* gene. The transposon mutant completely knocked down the expression of *yceG* and *yceH*, whereas the insertional mutant completely knocked down the expression of *yceG* and but had little to no effect on the expression of *yceH* (Figure 3A and Figure 3B).

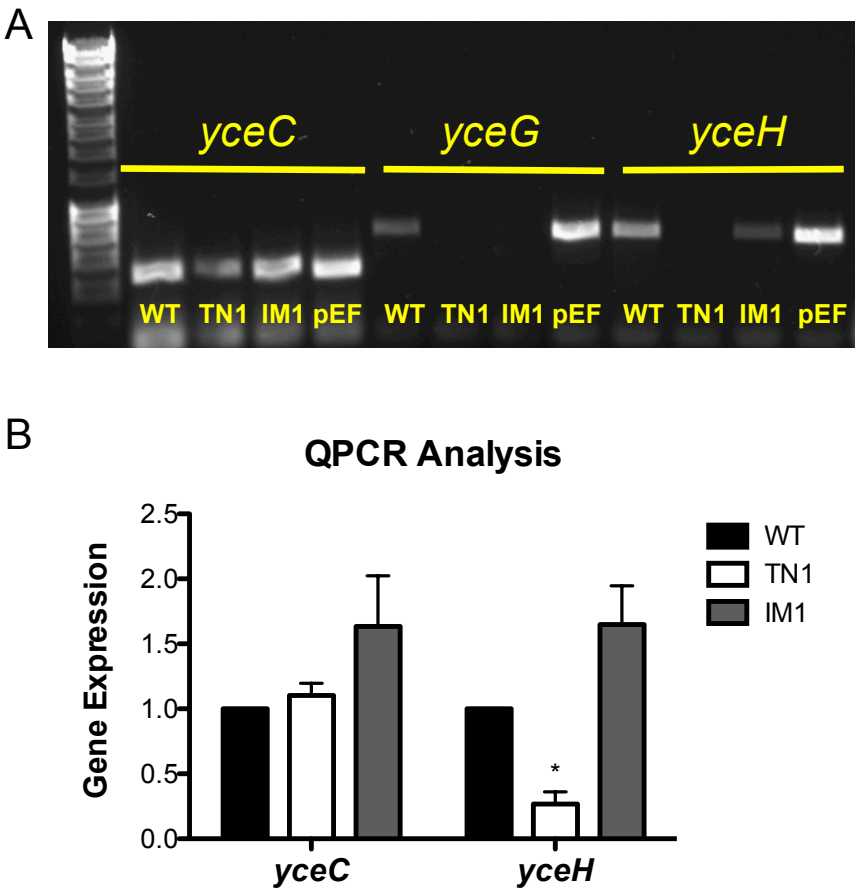

Figure 3. Tellurium resistance gene expression. **A)** Semi-quantitative PCR was performed on cDNA from WT, TN1, IM1 and the complemented strain, TN1+pEF (abbreviated pEF) showing relative gene expression (*yceC, yceG* and *yceH*). **B)** Quantitative-PCR (QPCR) analysis of *yceC* and *yceH* in WT, TN1 and IM1. *,p-value<0.05; evaluated by one-way ANOVA, followed by Tukey's *post hoc* analysis.

Tellurium exists in nature as tellurite, TeO_3^{2-}, which is toxic to most bacteria. If bacteria are challenged with TeO_3^{2-}, the organism can reduce the compound to the less toxic form, Te^0. This reduction of tellurite causes a black precipitant to form producing black colonies (Figure 4A). We found that both WT and mutant bacteria were able to reduce tellurite as evidenced by black metallic tellurium deposits in the bacterial membrane via transmission electron microscopy (Figure 4B, red circles).

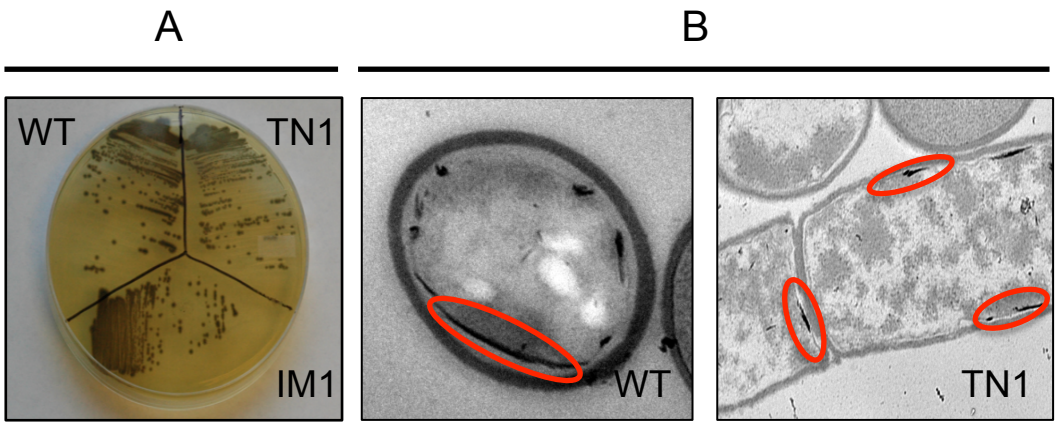

Figure 4. Reduction of potassium tellurite by *B. anthracis*. A) Colony morphology of WT, TN1 and IM1 post incubation on plate containing 30 μg/ml potassium tellurite. **B)** Tellurium deposits (red circles) seen in WT and TN1 via transmission electron microscopy.

However, when exposed to potassium tellurite in liquid culture there was a delay in growth between the WT and mutant bacteria (Figure 5B). This delay in growth was due strictly to the challenge with potassium tellurite, as no difference in growth rate was seen in BHI alone (Figure 5A).

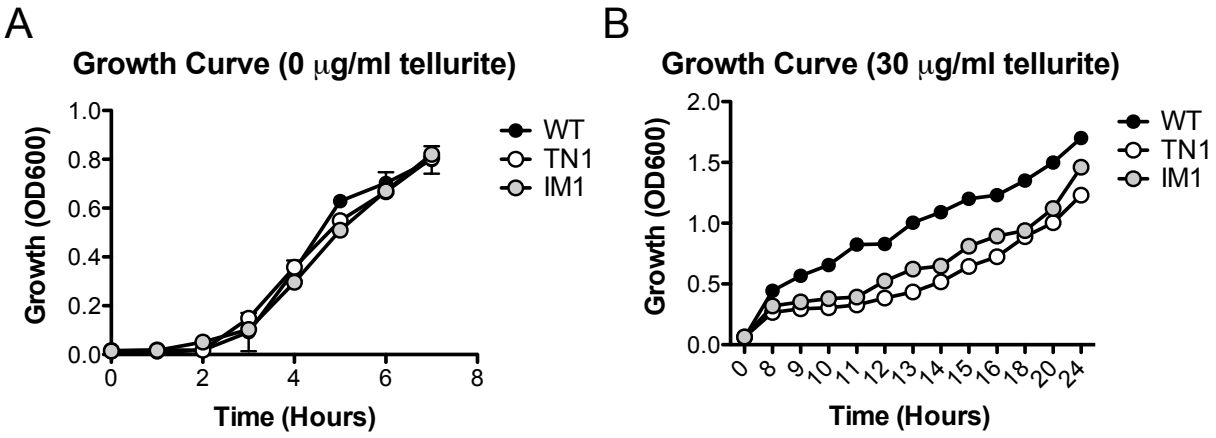

Figure 5. WT and mutant bacteria growth analysis. Bacterial growth of WT (black circles), TN1 (white circles) and IM1 (gray circles) in the absence **A**) and presence **B**) or 30 μg/ml potassium tellurite.

In addition, growth on agar plates with potassium tellurite impregnated disks yielded significant increases in the zone of growth inhibition in both the IM1 and TN1 mutants in comparison to

WT (Figure 6A). We conclude that loss of *yceG* and *yceH* tellurium resistance genes decreases, although does not completely ablate, tellurium resistance in *B. anthracis* Sterne.

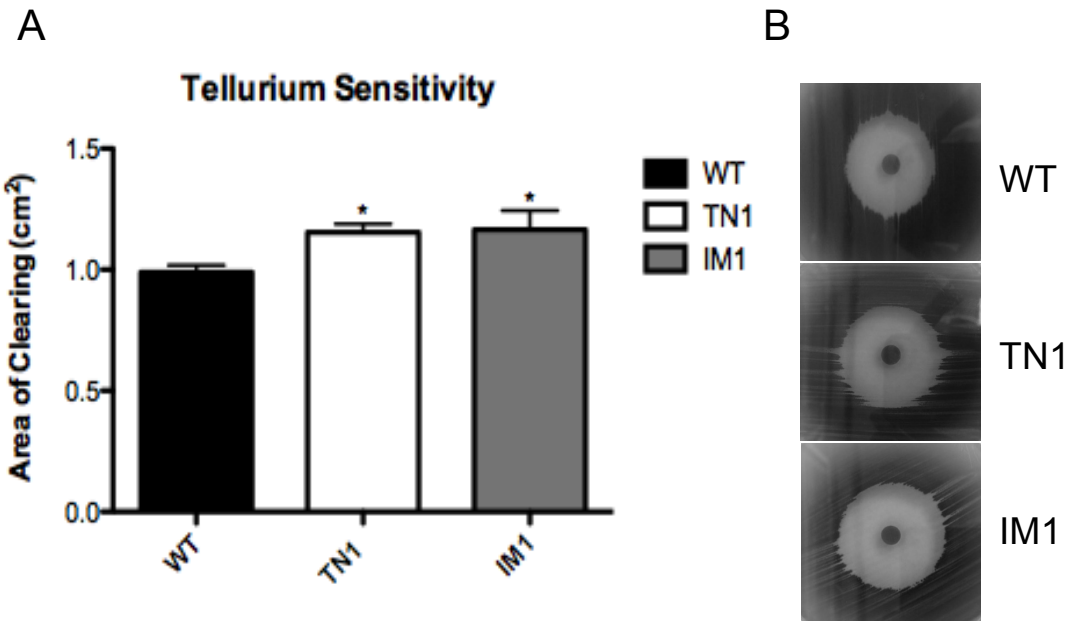

Figure 6. Susceptibility to potassium tellurite (K_2TeO_3). A) Growth inhibition surrounding tellurite impregnated disks for WT (black bar), TN1 (white bar) and IM1 (gray bar). *,p-value<0.05 from K_2TeO_3 treated WT by one-way ANOVA, followed by Tukey's *post hoc* analysis. **B)** Photographic representation of growth inhibition when challenged with K_2TeO_3 disks.

Susceptibility to Reactive Oxygen Species (ROS)

Although there was a slight decrease in growth rate in our mutants when exposed to tellurium, this does not explain why the loss of these two specific genes led to decreased virulence in the *C. elegans* infection model since tellurite toxicity is not a host defense strategy. The speculated toxicity of tellurite is hypothesized to be due to its strong oxidizing effect or by initiating production of ROS. *C. elegans* use an oxidative burst similar to that of mammalian phagocytic cells in response to infection by pathogens (Chávez et al., 2007). Due to the overlap of ROS production by *C. elegans* and the strong oxidant nature of TeO_3^2, we tested bacterial

susceptibility to several different ROS. When TN1 and IM1 were grown in the presence of 0% and 0.02% H_2O_2, a ROS commonly found in the phagolysosome, there was a statistically significant decrease in growth in comparison to WT, while there was no difference in BHI alone (Figure 7A). The next ROS we investigated was hypochlorite, the active ingredient in bleach. When neutrophils are activated, a reaction takes place converting chloride and hydrogen peroxide into hypochlorous acid (Klebanoff, 1968; Klebanoff, 1974; Klebanoff, 2005). When mutant bacteria were grown in the absence and presence of 0.03% hypochlorite, there was a statistically significant decrease in TN1 and IM1 resistance in comparison to WT (Figure 7B). Superoxides are short-lived anions that are generated post-phagocytosis. These dismutate quickly to form secondary ROS such as H_2O_2 and hypochlorite (Kobayashi et al., 2005). We found no significant difference in sensitivity to superoxides between the two mutants and WT (Figure 7C) indicating that TN1 and IM1 are more sensitive to some, but not all, ROS.

An important method used in molecular biology to ascertain if a phenotype is due solely to the loss of a specific gene is to complement the genes by reintroducing the gene into the genome, often on a plasmid. If complementation is achieved, the phenotype should resemble wild-type. We complemented our TN1 mutant by transforming it with a plasmid containing the genes *yceG* and *yceH*. Although we restored gene expression (Figure 3A, pEF lanes), we failed to restore phenotype when challenged with H_2O_2 (Figure 7D). This may be due to the fact that the proteins must be expressed at the proper levels and overexpression, as occurs on a plasmid, is as detrimental as the lack of expression seen in our mutants.

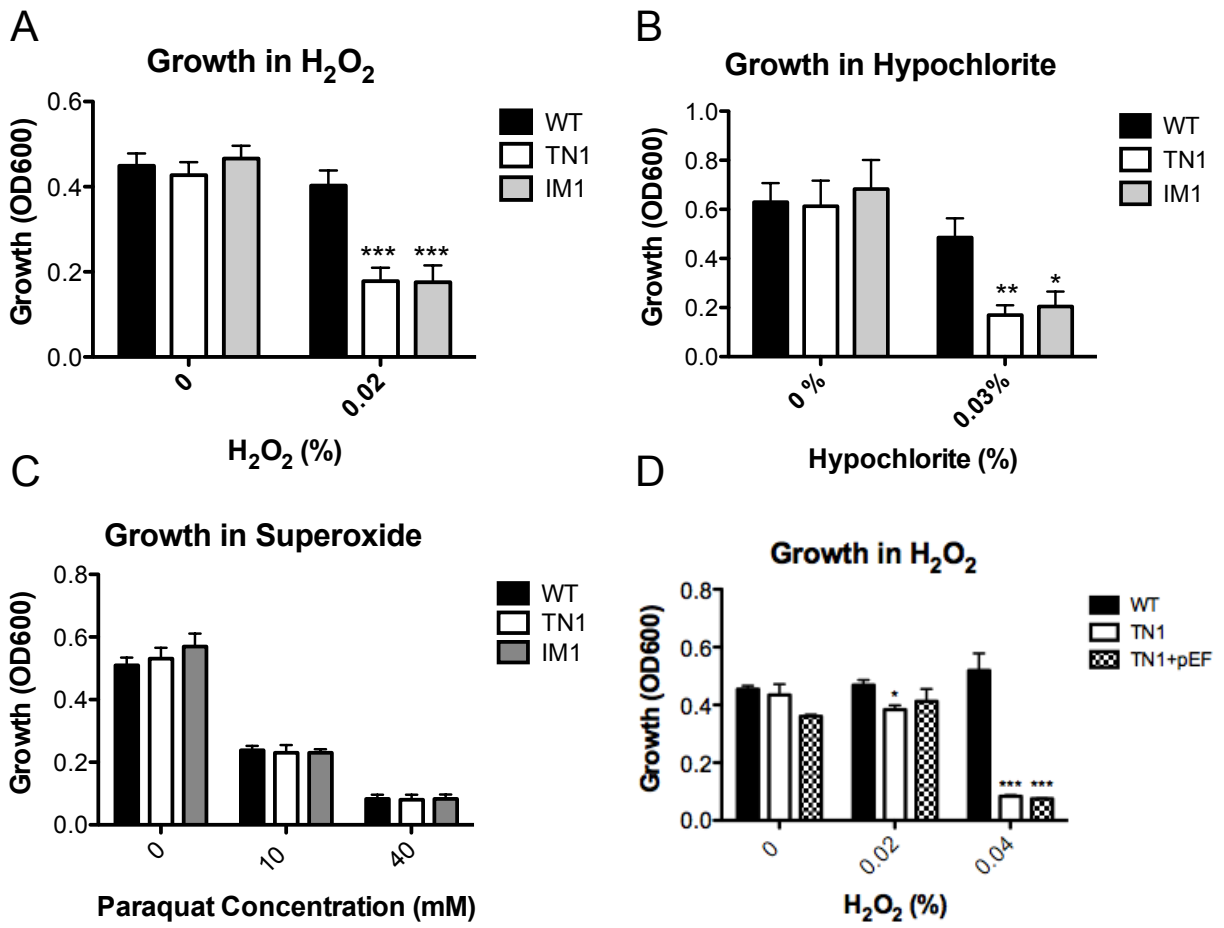

Figure 7. Bacterial Growth in Reactive Oxygen Species. Bacterial growth assessed at 24 hours in the presence of **A**) H_2O_2 **B**) hypochlorite or **C**) superoxide. **D**) Complementation of *yceG* and *yceH* genes. Bacterial growth of wild type (WT; black bars), transposon mutant 1 (TN1; white bars), and the complemented strain (TN1+pEF; checkered bars) in presence of indicated concentrations of H_2O_2. All data evaluated by one-way ANOVA, followed by Tukey's *post hoc* analysis. *,p-value<0.05; **,p-value<0.01; ***,p-value<0.0001.

Tellurium Resistance and Antibiotic Challenge

In 1932, Fleming first reported the antibacterial effect tellurite had on coliform bacteria. This is of interest due to the increasing emergence of multi-drug resistant bacteria. It was recently found that the use of sub-lethal doses of TeO_3^{2-} can increase the efficacy of certain antibiotics in *E. coli* (Molina-Quiroz et al., 2012) (Figure 8A). There is a significant increase in

the zone of growth inhibition measured for WT in the presence (checked bars) of 30 µg/ml potassium tellurite impregnated BHI plates when challenged with chloramphenicol, tetracycline and penicillin in comparison to when tellurite was absent (black bars). In order to test if the increase in susceptibility to antibiotics was due to the action of tellurite resistance genes, we compared WT and TN1 inhibition to various antibiotics in the presence of tellurite and saw no difference (Figure 8B). This suggests the increase in antibiotic susceptibility is not due to loss of the *yceG* and *yceH* genes.

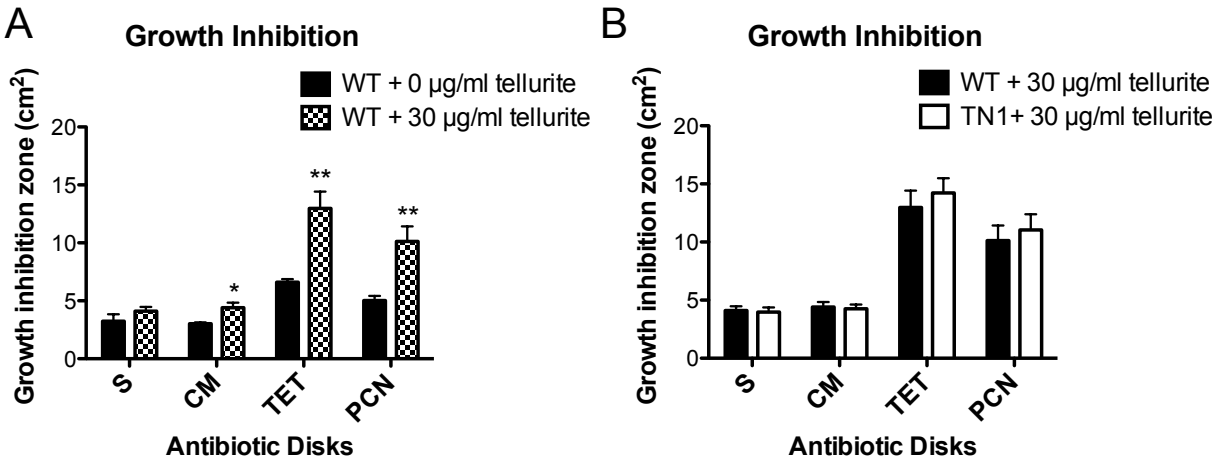

Figure 8. Potassium tellurite decreases resistance to some antibiotics. **A)** Susceptibility of *B. anthracis* WT to streptomycin (S), chloramphenicol (CM), tetracycline (TET) and penicillin (PCN) in the absence (black bars) and presence of 30 µg/ml potassium tellurite (checkered bars) . Data analyzed by one-way ANOVA, followed by Tukey's *post hoc* analysis. *,p-value<0.05; **,p-value<0.01. **B)** Comparison of WT (black bars) and TN1 (white bars) growth inhibition zones to S, CM, TET and PCN in the presence of 30 µg/ml potassium tellurite.

Antimicrobial Peptide (AMP) Resistance

C. elegans live in a relatively hostile environment, surrounded by bacteria. In order to compensate for their lack of adaptive immunity, the nematode must rely solely on their innate immune response, which includes various antimicrobial effector proteins such as neuropeptide

like proteins, caenacins, antibacterial factor peptides and caenopores (Bogaerts et al., 2010). Because our mutants were pulled out of a screen using *C. elegans*, we wanted to look into different facets of the nematode immune response, including antimicrobial peptides. In addition, Collins et al. demonstrated *telA*, a tellurium resistance gene found in *Listeria monocytogenes*, is required for resistance to several antimicrobials including the antimicrobial peptides nisin, gallidermin, bacitracin and the beta-lactam antibiotics cefuroxime and cefotaxime (Collins et al., 2010). The *telA* gene in *L. monocytogenes* has significant homology to *yceH*, which is knocked down in our TN1 mutant. Therefore we chose to look at whether our tellurium mutants had increased susceptibility to AMPs, as well as the cell wall acting antibiotic penicillin.

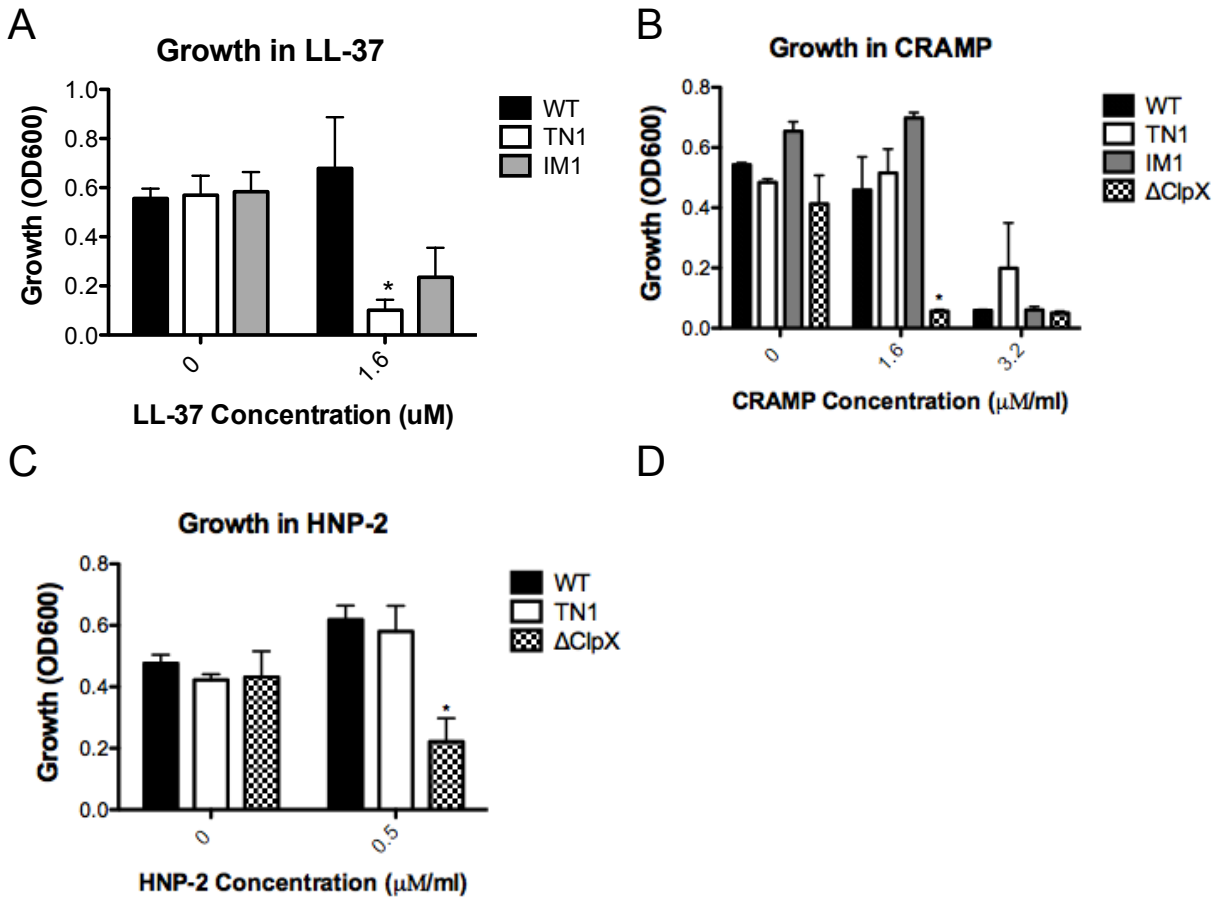

Figure 9. Bacterial growth in antimicrobial peptides. Growth of *B. anthracis* WT (black bars), TN1 (white bars), IM1 (gray bars) and ΔClpX (checkered bars) in **A**) LL-37, **B**) CRAMP

and **C**) HNP-2 at the indicated concentrations. Data evaluated by one-way ANOVA, followed by Tukey's *post hoc* analysis. *,p-value<0.05.

We first investigated the effect of two classes of mammalian AMPs, the cathelicidins, LL-37 and CRAMP and the defensin, HNP-2. AMPs bind bacterial membranes, forms pores and disrupts homeostasis of the bacterial cell. In the presence of LL-37, the human cathelicidin, we observed a statistically significant reduction in growth of TN1 in comparison to WT, but not in IM1 (Figure 9A). However, we saw no difference between WT, TN1 and IM1 susceptibility when grown in the presence of the murine cathelicidin, CRAMP (Figure 9B) or the defensin, HNP-2. In contrast, we did see a significant decrease with *B. anthracis* Sterne lacking the *clpX* gene (*ΔclpX*), which has previously shown to be sensitive to several classes of AMPs including HNP-2 and cathelicidins (McGillivray et al., 2009).

We next tested three bacterial produced AMPs, nisin and bacitracin, which were linked to *telA* in *L. monocytogenes* (Collins et al., 2010). When grown in the indicated amounts of nisin, we saw no difference in susceptibility amongst WT, TN1 or IM1 when assays were performed in BHI (Figure 10A) or the cell-culture media RPMI (Figure 10B) even though a significant difference was seen in the *ΔclpX* mutant (Figure 10B). When grown in the presence of bacitracin in RPMI, we again saw no difference between WT and mutant bacteria (Figure 10C). We also tested susceptibility of our mutants to polymyxin, an antimicrobial peptide that disrupts the cell membrane by interfering with phospholipids. When grown in the absence or presence of the indicated amounts of polymyxin, we observed no difference in susceptibility between WT and our mutants (Figure 11A). Collins et al. found the loss of *telA* in *L. monocytogenes* leads to increased susceptibility to the beta-lactam antibiotics, cefuroxime and cefotaxime (Collins et al., 2010). We tested susceptibilities of WT and our mutants to the beta-lactam antibiotic, penicillin, and observed no difference in penicillin susceptibility of the TN1 and IM1 mutants (Figure 11B).

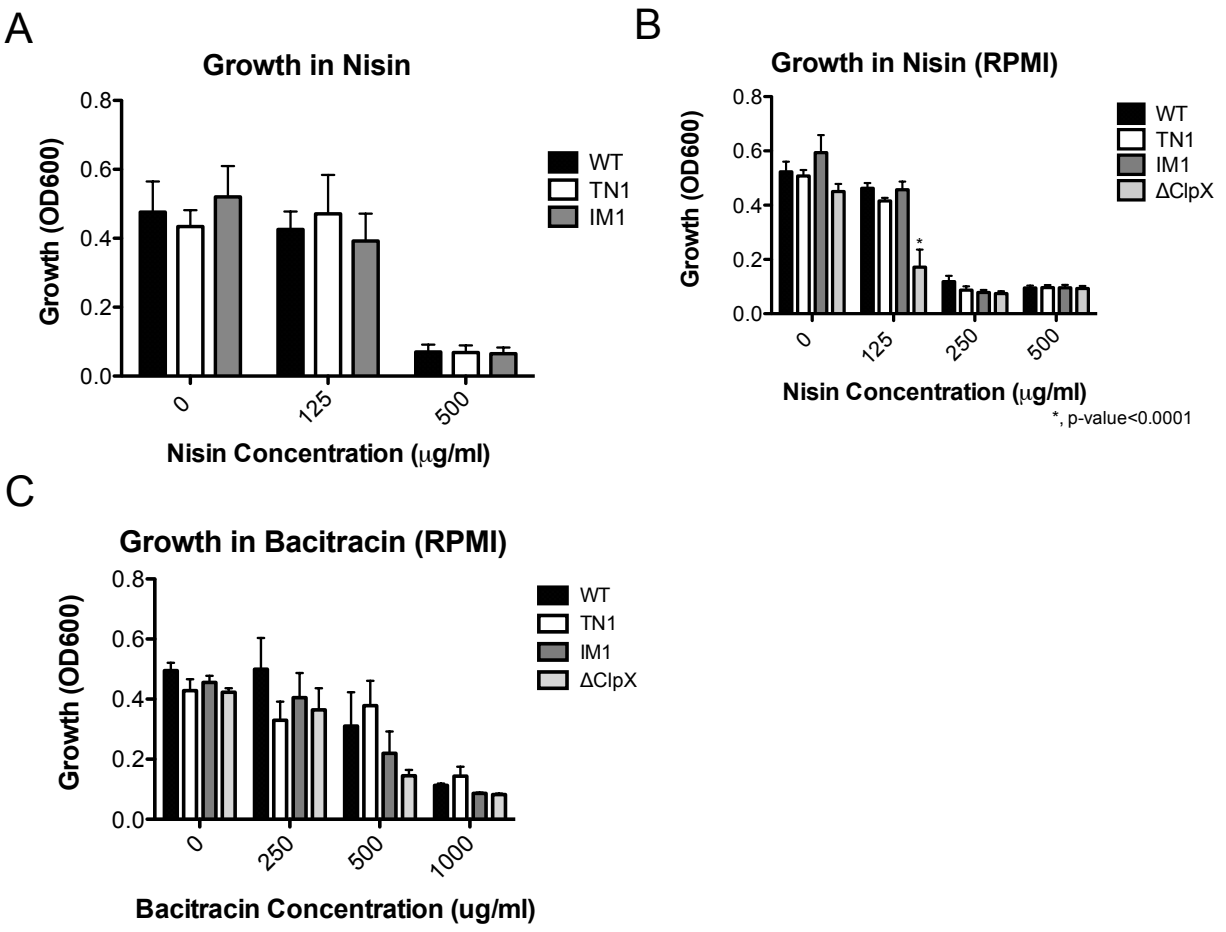

Figure 10. Growth in the presence of antibacterial agents in BHI and RPMI media. *B. anthracis* WT (black bars), TN1 (white bars), IM1 (dark gray bars) and *ΔClpX* (light gray bars) growth in the absence and presence of **A**) nisin in BHI, **B**) nisin in RPMI and **C**) bacitracin in RPMI at indicated amounts.

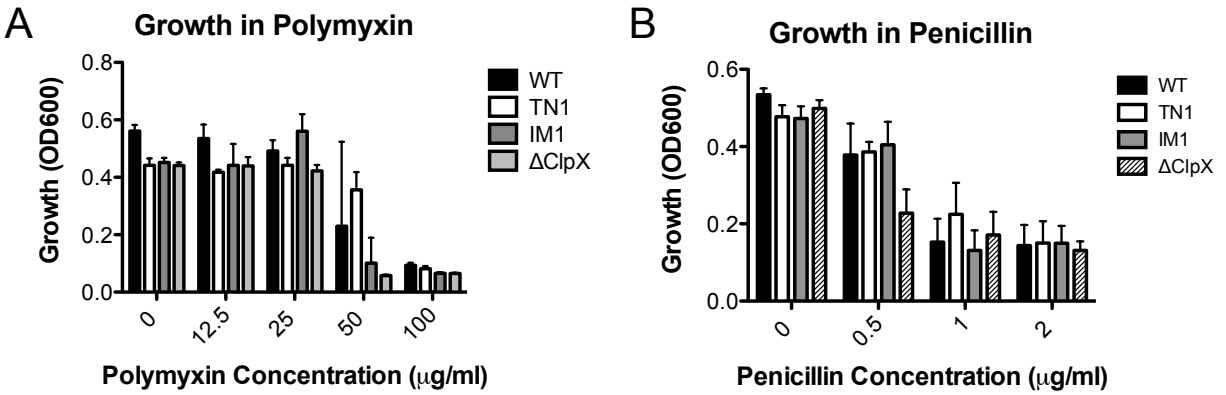

Figure 11. Growth in polymyxin and pencillin is not hindered by the loss of tellurium resistance genes. Bacterial growth of WT (black bars), TN1 (white bars), IM1 (dark gray bars) and *ΔClpX* (light gray bars) in **A**) polymyxin and **B**) penicillin at the indicated amounts.

Role of *yceG* in Mammalian Host Defense

When *B. anthracis* spores are inhaled, an immune response is mounted in the lungs where phagocytic cells, such as macrophages, are recruited to the area of infection and begin clearing the pathogen. *B. anthracis* germinates post phagocytosis of the spores, and the vegetative bacteria survive exposure to the phagolysosome. Instead of killing *B. anthracis*, the phagocytic cells carry it to the lymph nodes. Eventually *B. anthracis* lyses the host cell, replicates extracellularly and eventually spreads throughout the circulatory system (Guidi-Rontani et al., 1999). Since production of ROS is a key method of killing in the phagolysosome of macrophages, we looked at bacterial survival in the murine macrophage-like cell line, J774. WT and TN1 were incubated with J774 cells allowing phagocytosis to occur. Cells were then lysed at 4 hours and plated overnight on BHI. Surviving cfu were enumerated to determine percent survival. TN1 had a substantial decrease in survival in comparison to WT (Figure 12A).

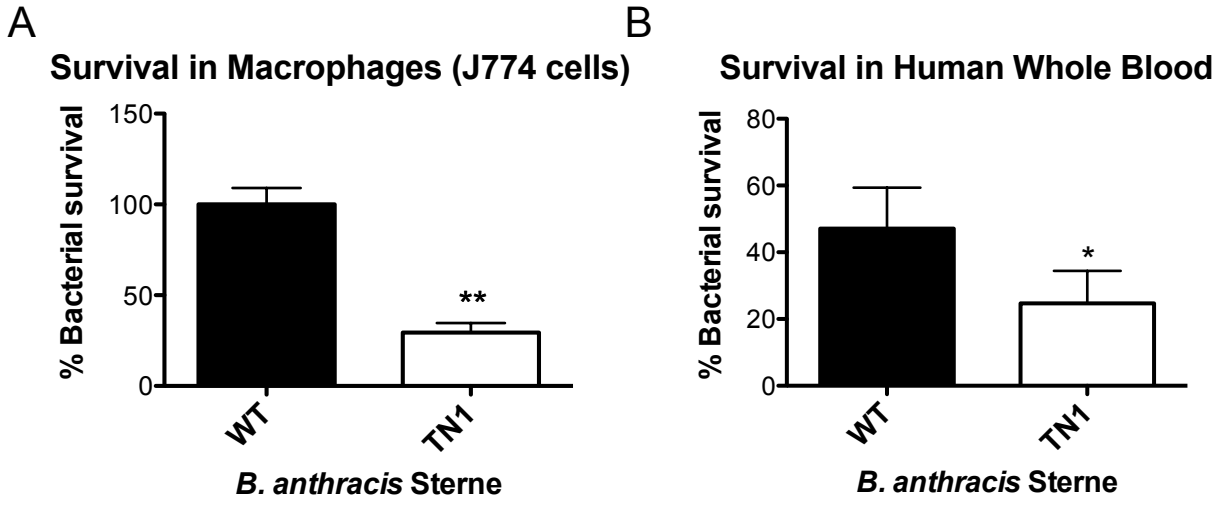

Figure 12. Bacterial survival against mammalian cellular defenses. 12A Survival of WT (black bar) and TN1 (white bar) in the murine macrophage-like cell line, J774. **12B** Survival of WT and TN1 in human whole blood. Data analyzed by student's paired t-test. *,p-value<0.05; **,p-value<0.01.

Neutrophils and dendritic cells have also been implicated in phagocytosis of spores, although not much is known about their interaction (Cote et al., 2011). Dendritic cells have proven critical for the transport of spores from the alveolar space to draining lymph nodes (Brittingham et al., 2005). Both cell types are found in human whole blood. We compared survival of the WT and TN1 mutant after 15 minutes exposure to human whole blood to determine if our mutants had an increased susceptibility to immune defenses found in the blood. We observed a significant decrease in survival of TN1 in comparison to WT when exposed to human whole blood (Figure 12B).

Mouse models are imperative in studying a multitude of human afflictions, ranging from identifying drug targets to curing acute promyelocytic leukemia. Mice share 99% of the same genes with humans, making them a valuable model organism. To determine whether TN1 has a decreased ability to survive in a whole-animal model, we performed a competition assay where WT and TN1 bacteria were mixed in a 1:1 ratio and injected intravenously into 8-week old mice. Once mice were moribund, the kidneys were removed and a homogenate was plated to enumerate surviving cfu. We found a 1-log decrease in surviving TN1 bacteria in comparison to WT (Figure 13). This suggests *yceG* and *yceH* are important for establishing an infection *in vivo* and this is likely linked to the increased susceptibility seen to host innate immune defenses such as ROS and/or LL-37.

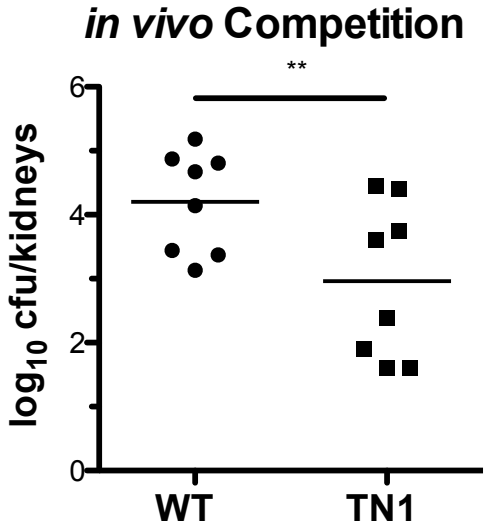

Figure 13. CD1 mice were injected with a 1:1 ratio of wild type (WT) and transposon mutant 1 (TN1). Enumeration of surviving cfu from kidneys 2 days after challenge. **,p-value<0.001 by t-test.

Discussion

Results presented in this study indicate that tellurium resistance genes found in *Bacillus anthracis* Sterne contribute to reactive oxygen species resistance and potentially other host defenses, such as the human antimicrobial peptide LL-37. We have shown that *C. elegans* is a useful and sufficient model of infection for identifying chromosomal genes essential for pathogenesis (Figure 1). The transposon mutant we focused on, TN1, had a transposon insert in the fifth gene of a six-gene operon containing tellurium resistance genes (Figure 2). This operon possesses substantial homology to the *yceCDEFGH* operon found in *Bacillus subtilis* and some homology to the *terZABCDEF* operon (Figure 2). Another independent mutation was created, insertional mutant 1, IM1, with a disruption in the fifth gene, *yceG*, of the operon. The two mutants presented with similar phenotypes although TN1 was more susceptible in most assays.

This could be attributed to the way the disruptions interfere with normal expression of the operon. TN1 has a complete knockdown of *yceG* and *yceH*, while IM1 has a complete knockdown of *yceG* but only reduced expression of *yceH* (Figure 3). This leads us to believe that the loss of both genes contributes to a more susceptible phenotype. We have demonstrated complementation of the *yceG* and *yceH* genes, but could not restore the WT-like phenotype, suggesting expression of the genes on the plasmid may be at higher levels than desired for the bacterium (Figure 3A and Figure 7D).

Using electron microscopy, we demonstrated TN1 is capable of reducing TeO_3^{2-} to Te^0 and storing the black metallic tellurium crystals in the inner membrane as does WT (Figure 4B, red circles). We see a lag in growth of TN1 and IM1 in comparison to WT, but not a complete inhibition. (Figure 5, Figure 6). Because the bacteria can continue to grow and survive with the loss of the genes in the presence of potassium tellurite, and *C. elegans* are not using tellurite as a defense mechanism, we have proposed an alternative role for the *yce* operon.

We first tested sensitivities to various ROS because C. elegans utilize an oxidative burst in response to pathogens (Chávez et al., 2009) and because tellurite itself has been linked to ROS production {Summers 1977; Taylor 1999). The decrease in resistance to H2O2 and hypochlorite of TN1 and IM1 suggest the yceG and yceH genes contribute to the organism's ability to neutralize reactive oxygen species (Figure 7A, Figure 7B). In contrast, we see no difference in resistance to another ROS, superoxide (Figure 7C). This suggests specificity in the action of yceG/H. Pohl et al. observed that genes of the ter operon are upregulated in the presence of H2O2 but there is no change in regulation in the presence of paraquat. They believe this is due to the more damaging nature of H2O2 and its ability to generate highly reactive hydroxyl radicals (Pohl et al., 2011). There is undoubtedly a difference in the stress caused by

superoxide and hydrogen peroxide leading to upregulation of different classes of genes. Activated genes in the presence of superoxide include genes involved in uptake of iron compounds, iron compound transporters and genes involved in the synthesis of siderophores. The main genes upregulated in response to H_2O_2 are involved in DNA metabolism and repair, protein degradation, damage protection and detoxification (Pohl et al., 2011).

We see an increase in susceptibility to antibiotics upon co-incubation with sub-lethal doses of potassium tellurite, although this is not related to the loss of tellurium genes (Figure 8). The increase zone of clearing upon tellurite incubation could be due to a combination of antibiotic targeting as well as the induction of oxidative stress caused by potassium tellurite. Therapeutically, this could be useful in preventing antibiotic resistance because the bacteria would have to develop resistance to two targets: tellurite and the antibiotic. Tellurite also has low reactivity with eukaryotic cells (Molina-Quiroz et al., 2012).

Collins et al. determined that loss of *telA*, a tellurite resistance gene, made *Listeria monocytogenes* more susceptible to the AMPs, bacitracin and nisin, and the beta-lactam antibiotics, cefuroxime and cefotaxime (Collins et al., 2010). However, although *yceH* has significant homology to *telA* and expression of *yceH* is knocked down in TN1 (Figure 3), we see no difference in resistance to nisin or bacitracin (Figure 10). We also observe no difference in growth between our mutant and WT bacteria in the presence of polymyxin or penicillin (Figure 11A and Figure 11B). We wanted to include a wide range of AMPs and also investigated the human cathelicidin, LL-37, the mouse cathelicidin CRAMP, and the human defensin, HNP-2. Although we saw no difference in susceptibility to HNP-2 or CRAMP with the TN1 or IM1 mutant, we did observe a significant difference with TN1 in comparison to WT in the presence of LL-37 (Figure 9C, Figure 9B and Figure 9A). LL-37 and CRAMP, both of which are

cathelicidins, are important in the first line of defense to invading pathogens at barriers including respiratory epithelium. It is surprising we found no difference with CRAMP considering the strong homology it has to LL-37 at both the sequence and structure level (Pestonjamasp et al., 2001). Interestingly, we find that TN1 is more susceptible to LL-37 but the IM1 is not. Because *yceG* is knocked out in both TN1 and IM1 and *yceH* is knocked out only in TN1 and not IM1, we conclude *yceH*, but not *yceG*, is important in the neutralization of this peptide. YceG has characteristics that are believed to interact with the bacterial membrane and is usually in close linkage with TelA in other bacterial species (Anantharaman et al., 2012), therefore these proteins may somehow alter the interaction of AMPs with the bacterial membrane. At the protein level, *B. anthracis* YceH and *L. monocytogenes* TelA share 24% identity and therefore they may function in a similar manner, although YceH appears to be more specific in its resistance mechanisms to AMPs than TelA.

A potential role of Te^R genes in host defense has long been speculated (Taylor, 1999) but to our knowledge, this is the first report directly linking them to increased susceptibility to mammalian infection models. We find that the *yceG* and *yceH* genes are important against mammalian host defenses including macrophages and human whole blood (Figure 12A and Figure 12B) as well as virulence *in vivo* (Figure 13). Macrophages, dendritic cells and neutrophils all use an oxidative burst in their microbicidal repertoire which adversely affect membrane synthesis and nucleic acids as well as other vital cellular processes. Since we have shown that resistance to oxidative stress is mediated in part by the *yceG* and *yceH* genes, it is likely this contributes to the decreased virulence.

Tellurium resistance genes are interesting, although not much is known about their roles. As mentioned previously, the tellurium resistance operon of *B. anthracis* consists of a *terD-terD-*

terD-terC-yceG-telA gene arrangement. *TerD* paralogs are hypothesized to form a multi-subunit complex with potential to bind cAMP. *TerC* is often found in conjunction with multiple repeats of *terD*, suggesting a strong association between the transmembrane unit formed by *terC* and the multi-subunit complex formed by *terD* (Anantharaman et al., 2012). A recent comparative genomics study indicates the *Ter* and *telA* families may have a diverse array of functions including signal transduction, soluble metabolite biosynthesis, DNA repair and RNA-based regulation (Anantharaman et al., 2012). Therefore, loss of these genes could render bacteria more susceptible to a variety of potential environmental stresses including, but not limited to, exposure to strong oxidants. This would help explain their widespread and ubiquitous nature in a wide variety of bacterial species including several human pathogens. Further study will hopefully help clarify the roles and mechanism of action of this intriguing and mysterious family of genes.

References

Aballay, A., and Ausubel, F.M. (2002). Caenorhabditis elegans as a host for the study of host pathogen interactions. Curr Opin Microbiol *5*, 97-101.

Anantharaman, V., Iyer, L.M., and Aravind, L. (2012). Ter-dependent stress response systems: novel pathways related to metal sensing, production of a nucleoside-like metabolite, and DNA-processing. Mol Biosyst

Baillie, L., and Read, T.D. (2001). Bacillus anthracis, a bug with attitude!. Curr Opin Microbiol *4*, 78-81.

Bogaerts, A., Beets, I., Schoofs, L., and Verleyen, P. (2010). Antimicrobial peptides in Caenorhabditis elegans. Invertebrate Survival Journal *7*, 45-52.

Brittingham, K.C., Ruthel, G., Panchal, R.G., Fuller, C.L., Ribot, W.J., Hoover, T.A., Young, H.A., Anderson, A.O., and Bavari, S. (2005). Dendritic cells endocytose Bacillus anthracis spores: implications for anthrax pathogenesis. The Journal of Immunology *174*, 5545-552.

Candela, T., and Fouet, A. (2005). Bacillus anthracis CapD, belonging to the gamma glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. Mol Microbiol *57*, 717-726.

Chávez, V., Mohri-Shiomi, A., and Garsin, D.A. (2009). Ce-Duox1/BLI-3 generates reactive

oxygen species as a protective innate immune mechanism in Caenorhabditis elegans. Infect Immun *77*, 4983-89.

Chávez, V., Mohri-Shiomi, A., Maadani, A., Vega, L.A., and Garsin, D.A. (2007). Oxidative stress enzymes are required for DAF-16-mediated immunity due to generation of reactive oxygen species by Caenorhabditis elegans. Genetics *176*, 1567-577.

Cieslak, T.J., and Eitzen Jr, E.M. (1999). Clinical and epidemiologic principles of anthrax. Emerging Infectious Diseases *5*, 552.

Collins, B., Joyce, S., Hill, C., Cotter, P.D., and Ross, R.P. (2010). TelA contributes to the innate resistance of Listeria monocytogenes to nisin and other cell wall-acting antibiotics. Antimicrob Agents Chemother *54*, 4658-663.

Cote, C.K., Welkos, S.L., and Bozue, J. (2011). Key aspects of the molecular and cellular basis of inhalational anthrax. Microbes Infect *13*, 1146-155.

Dixon, T.C. (1999). Anthrax. NEJM

Dixon, T.C., Fadl, A.A., Koehler, T.M., Swanson, J.A., and Hanna, P.C. (2000). Early Bacillus anthracis-macrophage interactions: intracellular survival survival and escape. Cell Microbiol *2*, 453-463.

Ezzell, J.W., and Welkos, S.L. (1999). The capsule of bacillus anthracis, a review. J Appl Microbiol *87*, 250.

Glavis-Bloom, J., Muhammed, M., and Mylonakis, E. (2012). Of model hosts and man: using

Caenorhabditis elegans, Drosophila melanogaster and Galleria mellonella as model hosts for infectious disease research. Adv Exp Med Biol *710*, 11-17.

Guidi-Rontani, C., Weber-Levy, M., Labruyère, E., and Mock, M. (1999). Germination of Bacillus anthracis spores within alveolar macrophages. Molecular microbiology *31*, 9-17.

Hart, C.A., and Beeching, N.J. (2002). A spotlight on anthrax. Clin Dermatol *20*, 365-375.

Hudson, M.J., Beyer, W., Böhm, R., Fasanella, A., Garofolo, G., Golinski, R., Goossens, P.L., Hahn, U., Hallis, B., et al. (2008). Bacillus anthracis: balancing innocent research with dual-use potential. Int J Med Microbiol *298*, 345-364.

Kato, Y., Aizawa, T., Hoshino, H., Kawano, K., Nitta, K., and Zhang, H. (2002). abf-1 and abf 2, ASABF-type antimicrobial peptide genes in Caenorhabditis elegans. Biochem J *361*, 221-230.

Klebanoff, S.J. (1968). Myeloperoxidase-halide-hydrogen peroxide antibacterial system. J Bacteriol *95*, 2131-38.

Klebanoff, S.J. (1974). Role of the superoxide anion in the myeloperoxidase-mediated antimicrobial system. J Biol Chem *249*, 3724-28.

Klebanoff, S.J. (2005). Myeloperoxidase: friend and foe. J Leukoc Biol *77*, 598-625.

Kobayashi, S.D., Voyich, J.M., Burlak, C., and DeLeo, F.R. (2005). Neutrophils in the innate immune response. ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS-ENGLISH EDITION- *53*, 505.

Kolstø, A.B., Tourasse, N.J., and Okstad, O.A. (2009). What Sets Bacillus anthracis Apart from Other Bacillus Species? Annu Rev Microbiol

Kumano, M., Tamakoshi, A., and Yamane, K. (1997). A 32 kb nucleotide sequence from the region of the lincomycin-resistance gene (22 degrees-25 degrees) of the Bacillus subtilis chromosome and identification of the site of the lin-2 mutation. Microbiology *143 (Pt 8)*, 2775-782.

Little, S.F., and Ivins, B.E. (1999). Molecular pathogenesis of Bacillus anthracis infection. Microbes Infect *1*, 131-39.

McGillivray, S.M., Ebrahimi, C.M., Fisher, N., Sabet, M., Zhang, D.X., Chen, Y., Haste, N.M., Aroian, R.V., Gallo, R.L., et al. (2009). ClpX contributes to innate defense peptide resistance and virulence phenotypes of Bacillus anthracis. J Innate Immun *1*, 494-506.

Molina-Quiroz, R.C., Muñoz-Villagrán, C.M., de la Torre, E., Tantaleán, J.C., Vásquez, C.C., and Pérez-Donoso, J.M. (2012). Enhancing the Antibiotic Antibacterial Effect by Sub Lethal Tellurite Concentrations: Tellurite and Cefotaxime Act Synergistically in Escherichia coli. PLoS One *7*, e35452.

Pestonjamasp, V.K., Huttner, K.H., and Gallo, R.L. (2001). Processing site and gene structure for the murine antimicrobial peptide CRAMP. Peptides *22*, 1643-650.

Pohl, S., Tu, W.Y., Aldridge, P.D., Gillespie, C., Hahne, H., Mäder, U., Read, T.D., and

Harwood, C.R. (2011). Combined proteomic and transcriptomic analysis of the response of Bacillus anthracis to oxidative stress. Proteomics *11*, 3036-055.

Read, T.D., Peterson, S.N., Tourasse, N., Baillie, L.W., Paulsen, I.T., Nelson, K.E., Tettelin, H., Fouts, D.E., Eisen, J.A., et al. (2003). The genome sequence of Bacillus anthracis Ames and comparison to closely related bacteria. Nature *423*, 81-86.

Sifri, C.D., Begun, J., and Ausubel, F.M. (2005). The worm has turned--microbial virulence modeled in Caenorhabditis elegans. Trends Microbiol *13*, 119-127.

Summers, A.O., and Jacoby, G.A. (1977). Plasmid-determined resistance to tellurium compounds. J Bacteriol *129*, 276-281.

Taylor, D.E. (1999). Bacterial tellurite resistance. Trends in Microbiology *7*, 111-15.

Tournier, J.N., Paccani, S.R., Quesnel-Hellmann, A., and Baldari, C.T. (2009). Anthax toxins: a weapon to systematically dismantle the host immune defenses. Mol Aspects Med

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Education

Texas Christian University
M.S. Biology, May 2013
Advisor: Shauna M. McGillivray, Ph.D.

Texas Christian University
B.S. Biology, May 2011

Research Experience

Master's Thesis
I have shown tellurium resistance genes play an active role in the neutralization of reactive oxygen species using in vitro minimal inhibitory concentration assays. We have shown a direct correlation between tellurium resistance genes and virulence of this pathogen.

Research Techniques

Microbiology
Bacterial growth and culture
Minimal inhibitory concentration assays
Genetic manipulation

Microscopy
Confocal microscopy
Fluorescent microscopy
Scanning electron microscope
Transmission electron microscope

Molecular biology
PCR, RT-PCR, Q-PCR
RNA Extraction
Gel electrophoresis
Western Blot
ELISA
Tissue culture
Cloning

Teaching & Outreach

Graduate Teaching Assistant
Texas Christian University, Fall 2011- Spring 2013
Majors Introductory Biology I and II Laboratory (10504 and 10514)

Graduate Student Liaison
"Science Major for a Day" Half-day outreach activity for 4th grade students from Sycamore Elementary, a Title 1 Crowley ISD school

Awards & Honors

S.E. Sulkin Award
First place award in medical microbiology for outstanding scientific achievement for an oral presentation give at a state meeting. Texas Branch American Society for Microbiology 2012 Fall Meeting. Waco, TX. Oral Presentation. (2012)

2nd Place Poster Presentation
North Texas Life Science Research Symposium. Fort Worth, TX. Graduate Division. (2012)

Dean's List
Texas Christian University, 2011

National Society of Collegiate Scholars
Texas Christian University, 2009-present

Phi Theta Kappa
Northlake College. 2008- present

Funding

Teaching Assistantship Award
Amount covers cost of tuition, student fees, and stipend

Adkins Summer Research Grant
Texas Christian University, Biology Department, Summer 2012
$3000 Awarded

Professional Affiliations

American Society for Microbiology
Fall 2011- present

Texas Society for Microscopy
Spring 2012- present

Manuscripts

Tellurium resistance genes in *Bacillus anthracis.*
Franks SE, Ebrahimi C, Hollands A, Okumura C, Aroian R, Nizet V and McGillivray SM.

Manuscript in progress; planned submission to the Journal of Bacteriology

Presentations

Franks SE, Ebrahimi C, Hollands A, Okumura C, Aroian R, Nizet V and McGillivray SM. The Role of Tellurium Resistance Genes in *Bacillus anthracis*. (2012). Texas Branch American Society for Microbiology 2012 Fall Meeting. Waco, TX. ***Oral Presentation.***

Franks SE, Ebrahimi C, Hollands A, Okumura C, Aroian R, Nizet V and McGillivray SM. The Role of Tellurium Resistance Genes in *Bacillus anthracis*. (2012). North Texas Life Science Research Symposium. Fort Worth, TX. ***Poster Presentation.***

Franks SE, Ebrahimi C, Aroian R, Nizet V and McGillivray SM. Investigation of Novel Virulence Factors in *Bacillus anthracis*. (2012). 112th General Meeting of the American Society for Microbiology. San Francisco, CA. ***Poster Presentation.***

Franks SE, Ebrahimi C, Ernest Couch, Aroian R, Nizet V and McGillivray SM. Discovery of Novel Virulence Factors in *Bacillus anthracis*. (April 2012). Texas Society of Microscopy, Fort Worth, TX. ***Oral Presentation.***

Franks SE, Ebrahimi C, Aroian R, Nizet V and McGillivray SM. Investigation of Novel Virulence Factors in *Bacillus anthracis*. (2012). Student Research Symposium, Texas Christian University. Fort Worth, TX. ***Poster Presentation.***

Franks SE, Ebrahimi C, Aroian R, Nizet V and McGillivray SM. Investigation of Novel Virulence Factors in *Bacillus anthracis*. (2011). Texas Branch American Society for Microbiology 2011 Fall Meeting. Arlington, TX. ***Poster Presentation.***

Franks SE and McGillivray SM. Construction of a genetic knockout of the ClpC ATPase in *Bacillus anthracis*. (April 2011). Student Research Symposium, Texas Christian University. Fort Worth, TX. ***Poster Presentation.***

References

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ABSTRACT

A NOVEL ROLE FOR TELLURIUM RESISTANCE GENES IN THE PATHOGENESIS OF *BACILLUS ANTHRACIS*

by Sarah Elizabeth Franks, M.S. 2013

Department of Biology

Texas Christian University

Thesis Advisor: Dr. Shauna M. McGillivray, Assistant Professor of Biology

Bacillus anthracis must avoid an array of antibacterial defenses by the host during infection. Although anthrax toxin and capsule, located on extra-chromosomal plasmids, play important roles in the pathogenesis of this disease, evidence indicates chromosomal genes also contribute. A random chromosomal mutant library of *B. anthracis* Sterne was employed to identify novel chromosomal virulence factors. Mutants were screened for loss of virulence in *Caenorhabditis elegans*. Those unable to infect *C. elegans* were selected for and the site of transposon insertion was identified. The most highly attenuated mutant had a disruption in an operon containing multiple tellurium resistance genes, leading to an increased susceptibility to potassium tellurite as well as host defenses (ROS and AMPs). In addition, the mutant displays attenuated virulence in whole blood and macrophage survival in comparison to WT. We conclude the tellurium resistance operon of *B. anthracis* has a novel role in resistance to critical host defenses.