Role of ClpX and ClpP in antibiotic resistance in *Bacillus anthracis*

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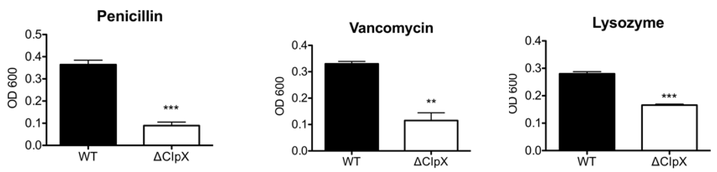
ABSTRACT

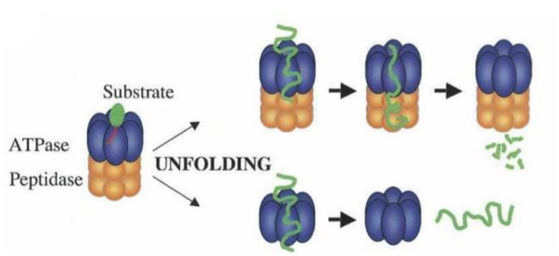
Bacillus anthracis is a Gram-positive bacterium that causes anthrax in humans. It is a significant microorganism in that many proteins important to virulence or pathogenesis are highly conserved in many other pathogenic bacteria. Our lab has previously identified the protein ClpX in *Bacillus anthracis* as metabolically significant in antibiotic resistance. Specifically, *B.* anthracis lacking the *clpX* gene (ΔClpX) are significantly more susceptible to antibiotics that target the bacterial cell wall such as penicillin than the wild type. ClpX has multiple functions; primarily it interacts with ClpP to form a proteolytic complex that degrades dysfunctional or obsolete proteins. ClpX also has an independent chaperone function, moving proteins around the cell. This project has focused on determining if the pathway of decreased antibiotic resistance in mutant *B. anthracis* is dependent on ClpX interactions with ClpP, or if ClpX can function independently. To test this, a point mutation (I265E) was made in the *ClpX* gene at the site that has been previously identified as the site of interaction between ClpX and ClpP in *Staphylococcus aureus.* The ClpX genes in *B. anthracis* and *S. aureus* exhibit a high degree of conservation particularly in this region, and it is expected that this site will also be critical for ClpX and ClpP interaction in *B. anthracis*. The mutated *ClpX* gene (I265E) has been confirmed with sequencing and has been transformed as an inducible expression plasmid into the ΔClpX *B. anthracis* strain. Preliminary assays to determine the antibiotic resistance of the mutant strain have shown marked decrease in resistance to penicillin as compared to the wild-type or the complemented strain.

BACKGROUND

ClpX is a regulatory ATPase that functions along with ClpP as a subunit of the ClpXP protease, which is essential for regulating the degradation of proteins in *Bacillus anthracis.* Previous studies show that deletion of *clpX* (Δ*clpX*) results in an increased susceptibility to antimicrobial agents that target or interact with the cell wall. ClpX also has an independent chaperone function which is being explored in this project.



Question: Is ClpX-mediated antibiotic resistance dependent on ClpP through the ClpXP protease, or is it through independent chaperone functions of ClpX alone?



This figure shows the two ClpX pathways; association with ClpP to form a proteolytic complex or independent protein processing

CONSTRUCTION OF CLPXI265E MUTATION

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Sa ClpX:   MFKFNEDEENLKCSFCGK   DQDQVKKLVAGSGVYICNEC IELCSEIVEEELAQNTSEA
Ba ClpX:   MFKFNDEKGQLKCSFCGK   TQTQVRKLVAGPGVYICDEC IELCTEIVQEELAKDEEVE
           *****::: :********    * **:*****.*****:** ****:***:****:: .
Sa ClpX:   ITELPTPKEIMDHLNEYVIG QEKAKKSLAVAVYNHYKRIQ QLGPKEDDVELQKSNIALIG
Ba ClpX:   FKDVPKPVEIREILDEYVIG QDNAKKALAVAVYNEYKRIN SN-SKIDDVELAKSNIALIG
           :.::*.* ** : *:***** *:.***:************: .  .* **************
Sa ClpX:   PTGSGKTLLAQTLAKTLNVP FAIADATSLTEAGYVGDDVE NILLRLIQAADFDIDKAEKG
Ba ClpX:   PTGSGKTLLAQTLARILNVP FAIADATSLTEAGYVGEDVE NILLKLIQAADYDVEKAEKG
           **************: **** ******************:*** ****:******:*::*****
Sa ClpX:   IIYVDEIDKIARKSENTSIT RDVSGEGVQQALLKILEGTT ASVPPQGGRKHPNQEMIQID
Ba ClpX:   IIYIDEIDKVARKSENPSIT RDVSGEGVQQALLKILEGTV ASVPPQGGRKHPHQEFIQID
           ***:*****:******.*** ******************. ************:**:****
Sa ClpX:   TTNILFILGGAFDGIEEVIK RRLGEKVIGFSS-NEAD    KYDEQALLAQIRPEDLQAYG
Ba ClpX:   TTNILFICGGAFDGIEPIIK RRLGEKVIGFGSEKKNA    DVNEKHVLSHVLPEDLLRFG
           ******* ******** :** **********.* ::      . :*: :*::: ****  :*
Sa ClpX:   LIPEFIGRVPIVANLETLDV TALKNILTQPKNALVKQYTK MLELDDVDLEFTEEALSAIS
Ba ClpX:   LIPEFIGRLPVIANLEPLDE DALVDILTKPKNALVKQFQK LLELDDVELEFEEGALIEIA
           ********:*::*****.**   ** :***:********: * :******:*** * **  *:
Sa ClpX:   EKAIERKTGARGLRSIIEES LIDIMFDVPSNENVTKVVIT AQTINEETEPELYDAEGNLI NNSKTSA
Ba ClpX:   KKAIERKTGARGLRSIIEGL MLEVMFELPSRKDIEKCILT KETVADNAAPKLVLQDGTVL D-TKTSA
           :******************   :::::**::**.::: * ::*  :*: ::: *:*   :*.:: : :****|
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The alignment of the *clpX* gene in *s. aureus* and *B. anthracis*. Amino acids that are identical are marked with an asterisk, those that are "interchangeable" are marked with a semicolon, and those that are chemically similar to a lesser extent are marked with a period. The bolded amino acids are part of a highly conserved region that is important for ClpP association, and the amino acid marked in red is the target of site-directed mutagenesis.

SITE DIRECTED MUTAGENESIS

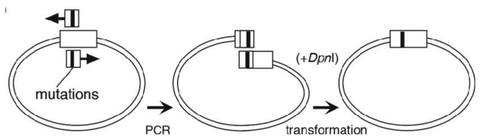

Image provided by IntechOpen.com

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I265E Mutant   CCAATTATTAAACGCCGTCTTGGTGAAAAGGTAGAGGGATTTGGTTCTGAGAAGAAAAAT
               ||||||||||||||||||||||||||||||||||||||    ||||||||||||||||||||||
Sterne Strain  CCAATTATTAAACGCCGTCTTGGTGAAAAGGTAATTGGATTTGGTTCTGAGAAGAAAAAT
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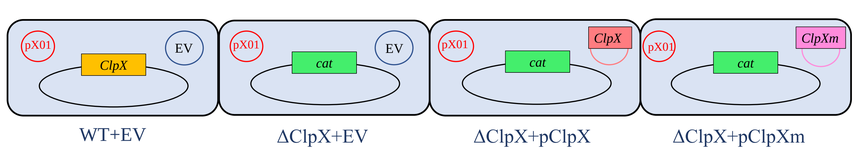
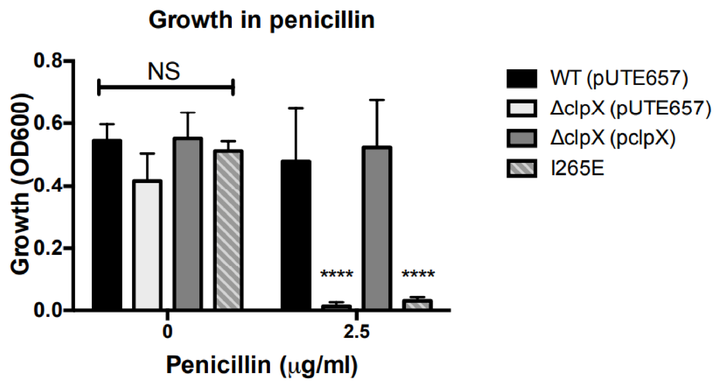


The four strains of *B. anthracis* that were used in the Minimum Inhibitory Concentration Assays. Site-directed mutagenesis was used to create the I265E mutant plasmid (pClpXm) which was then transformed into the ΔClpX strain.

RESULTS

MIC Assays were performed with the four previously described strains of *B. anthracis* in increasing concentrations of penicillin. The wild type and complement strain showed phenotypical levels of antibiotic resistance, whereas the knockout and I265E mutant complement had no growth even in the lowest concentration of penicillin. This is consistent with the ClpP-dependent pathway



NS indicates no significance, **** indicates a P value of <0.0001. Statistical analysis performed with a 2-way ANOVA and Tukey analysis.

CONCLUSIONS

Preliminary MIC Assay results strongly suggest that this pathway is ClpP dependent in nature. The mutant strain (I265E) did not show restored phenotype of resistance, suggesting that the process of resistance requires association of ClpX and ClpP into a proteolytic complex. Additional MIC assays will be performed with other antimicrobials such as vancomycin and lysozyme. Additionally, *Bacillus anthracis* is unique in that it has two forms of the ClpP protein, ClpP1 and ClpP2. The next step would be to determine if these two different isotypes have different functions within the cell, and if the differences effect the ClpXP proteolytic pathway of antibiotic resistance.

REFERENCES

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