Role of Exopolysaccharides in Resistance to Hydrogen Peroxide and Virulence of *Agrobacterium Tumefaciens*

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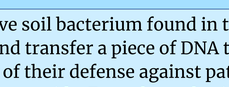
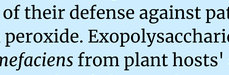
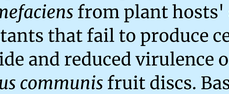
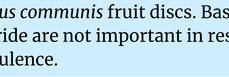
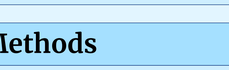
Abstract

Agrobacterium tumefaciens is a gram-negative soil bacterium found in the rhizosphere. If a plant is wounded, the bacteria infect the wound and transfer a piece of DNA to the host cells to induce the formation of crown gall tumors. As part of their defense against pathogens, plants produce reactive oxygen species including hydrogen peroxide. Exopolysaccharides play a role in producing a biofilm that helps protect *A. tumefaciens* from plant hosts' defense mechanisms like hydrogen peroxide. Some *A. tumefaciens* mutants that fail to produce certain exopolysaccharides show reduced resistance to hydrogen peroxide and reduced virulence on tomato stems, *Bryophyllum daigremontiana* leaves, and *Pyrus communis* fruit discs. Based on these data, succinoglycan and the unipolar polysaccharide are not important in resistance to hydrogen peroxide, transient transformation, and virulence.

Methods

Ti-Reporter-Plasmid: The plasmid pGWB2nlgfp contains a eukaryotic promoter and nuclear localization signal in front of a green fluorescent protein gene. It was obtained from and constructed by the Alan Jones laboratory at the University of North Carolina at Chapel Hill. The plasmid was introduced into the C58 parent strain by Dr. Matthysse, Lauren Burke, and Janine Corley. Caroline Chandler introduced the plasmid to the *ExoA, ExoF, A1045, CelA, CrdS,* and *Δupp* mutant bacterial strains. The strains were then streaked on Luria agar plates that contained kanamycin to select for strains containing the kanamycin resistant plasmid.
Germination of Plant Seeds: Tomato seeds were surface sterilized and germinated in sterile water. They were then placed into sterile petri dishes with sterile water and germinated in a dark drawer at room temperature.
Preparation of Bacterial Cultures: The cultures containing the Ti-Reporter plasmid were grown overnight in 2 mL of Luria broth with kanamycin on a shaker.
Inoculation of Plant Roots: In a containment hood, the tomato roots were cut into thin 0.5 cm sections. The roots were inoculated with 0.3 mL bacterial culture and placed in petri dishes with ¼ MS salts + 0.1% glucose.
Inoculation of *P. Communis Fruit* Discs: In a containment hood, pears were surface sterilized and cut using sterile instruments. Pears were then sliced into thin and thick discs. The thin discs were inoculated with 0.3 mL bacterial culture and placed in petri dishes with ¼ MS salts + 0.1% glucose. The thick discs were placed on water agar with 0.1 mL of bacterial culture and incubated for up to 14 days.
Virulence Assays: Tumor assays were performed on *B. daigremontiana* leaves and tomato stems using techniques described in "Glycoside Hydrolase Genes Are Required for Virulence of *Agrobacterium tumefaciens* on *Bryophyllum daigremontiana* and Tomato." Bacterial virulence was determined on *P. communis* fruit discs by inoculating thick discs on water agar and incubating the discs for up to six weeks. Tumor formation was scored per site inoculated forming tumors.
Preparation of Bacterial Cultures for Hydrogen Peroxide experiments: 2 mL of Luria broth was added to a 5.75 inch screw cap tube. An inoculation loop was used to obtain a bacteria colony from an agar plate by dragging the loop across the surface of the plate. The loop was then placed in the broth and spun to transfer the colonies to the tube. This process was repeated for the desired amount of colonies.
Preparation of Luria Agar Plates with Hydrogen Peroxide: 50 mL of Luria agar was liquified using a microwave and placed into a 55ºC water bath until flasks were cool enough to touch. A hydrogen peroxide dilution was made with 1 mL of distilled H_2O and 0.1 mL of H_2O_2. 0.05 of cycloheximide was added to added to the flasks along with 0.02 mL of the H_2O/H_2O_2 solution to one flask and 0.03 to another to make a 40 mM and 60 mM concentration respectively. The flasks were poured into plates and sat for 24 hours.
Hydrogen Peroxide Resistance Procedure: The procedure followed the work done by W. Eiamphungporn et al.[1], with the following modifications: 40 mM and 60 mM concentrations of hydrogen peroxide were used in order to determine resistance levels. The H_2O_2 was added to agar plates instead of being directly added to the cell cultures. A spectrophotometer was used to determine concentration bacterial concentration in 0.9% NaCl to then perform a dilution. The strains that were used were the wild-type C58 and the mutants *ExoF, ExoA, Δupp, CelA,* and *CrdS.*

List of Exopolysaccharides

Bacterial Strain and Gene Number	Exopolysaccharide Affected	Exopolysaccharide Structure	Cellular Location
***chvB* (A1045)**	B-1, 2-D glucan production		Periplasmic Space
***CelA* (*Atu3309*)**	Cellulose synthase		Extracellular Space
***CrdS* (*Atu3056*)**	Curdlan synthase		Extracellular Space
***ExoA* (*Atu4053*)**	Glycosyltransferase required for succinoglycan synthesis		Extracellular Space
***ExoF* (*Atu3326*)**	Polysaccharide transport required for export of succinoglycan*		Extracellular Space
Δupp	Unipolar polysaccharide synthesis	Unknown	Cell surface at one pole

*** ExoF may be involved in other unknown roles**

References

Mathews, S. L., Hannah, H., Samagaio, H., Martin, C., Rodriguez-Rassi, E., & Matthysse, A. G. (2019). Glycoside Hydrolase Genes Are Required for Virulence of Agrobacterium tumefaciens on *Bryophyllum daigremontiana* and Tomato. *Applied and environmental microbiology*, *85*(15), e00603-19. https://doi.org/10.1128/AEM.00603-19

Matthysse, A.G. (2018). Exopolysaccharides of *Agrobacterium tumefaciens*. In: Gelvin, S. (eds) Agrobacterium Biology. Current Topics in Microbiology and Immunology, vol 418. Springer, Cham. https://doi.org/10.1007/82_2018_100

Benjaphorn Prapagdee, Warawan Eiamphungporn, Panatda Saenkham, Skorn Mongkolsuk, Paiboon Vattanaviboon, Analysis of growth phase regulated KatA and CatE and their physiological roles in determining hydrogen peroxide resistance in *Agrobacterium tumefaciens*, *FEMS Microbiology Letters*, Volume 237, Issue 2, August 2004, Pages 219–226, https://doi.org/10.1111/j.1574-6968.2004.tb09699.x

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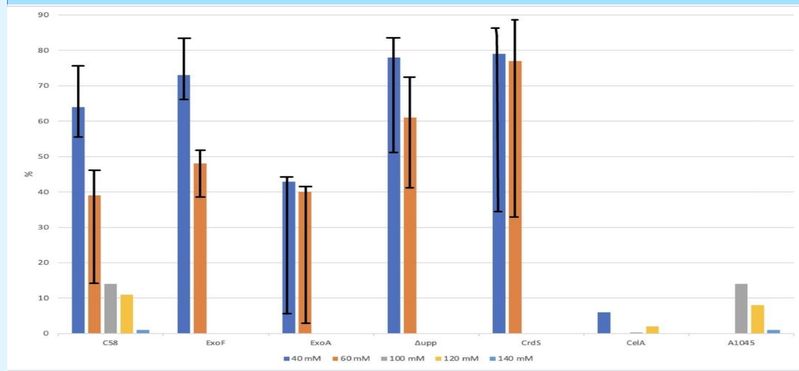
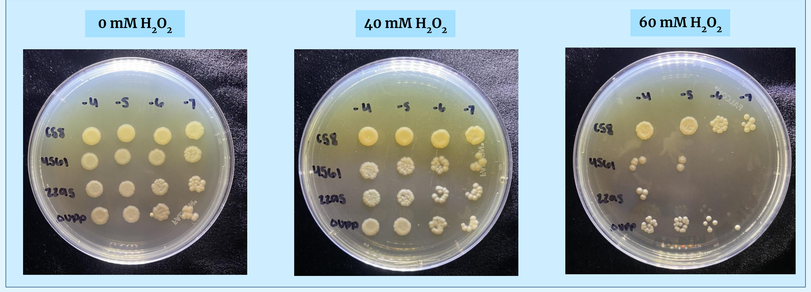
% Survival of C58 and Mutant Strains



*Data for CelA and A1045 was collected by Kayla Crouch in a previous semester. For bars without error bars, there was only one piece of data.

C58 and Δupp Dilutions at Three Different Concentrations of H_2O_2

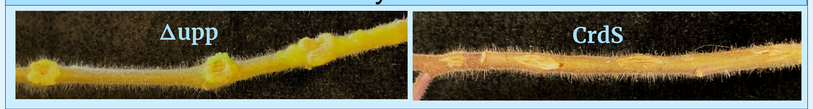


Virulence and Transient Transformation of Exopolysaccharides

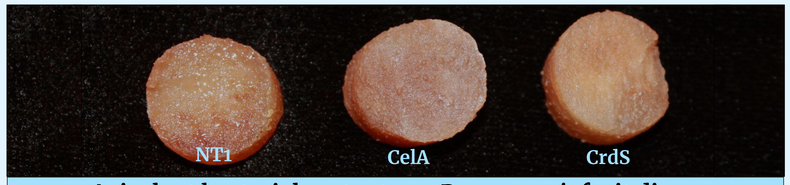
Bacterial Strain	Virulence [B]			DNA Transfer [C]	
	B. daigremontiana	*P. communis*	*Tomato*	*P. communis*	*Tomato*
C58	88	100	100	1.0	1.0
NT1 [A]	0	0	0	0.0	0.0
A1045	0	IP [D]	IP	IP	IP
CelA	0	0	0	0.0	0.0
CrdS	0	0	0	0.0	0.0
CrdSCelA	0	0	0	0.0	0.0
ExoA	IP	93	IP	1.0	0.5
ExoF	100	100	50	1.0	1.0
Δupp	100	100	50	1.0	1.0

[A] NT1 is avirulent and lacks both the Ti and reporter plasmids
[B] Percent of inoculated sites forming tumors
[C] Scored by rate of transformation (1.0 = transformation present, 0.5 = spotty transformation, and 0.0 = little to no transformation present)
[D] Trials in progress

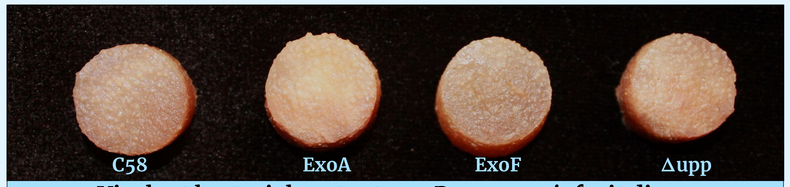
Virulence Assays on Tomato Stems



Virulence Assays on *B. daigremontiana*

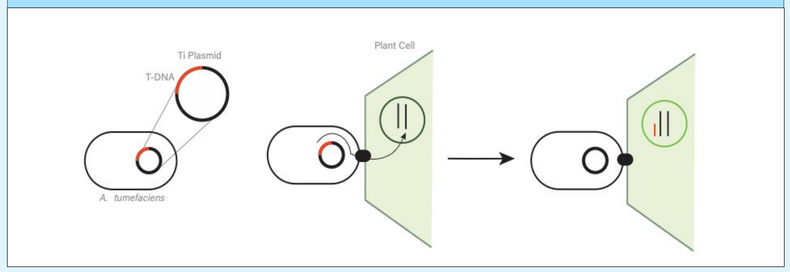


Avirulent bacterial mutants on *P. communis* fruit discs.

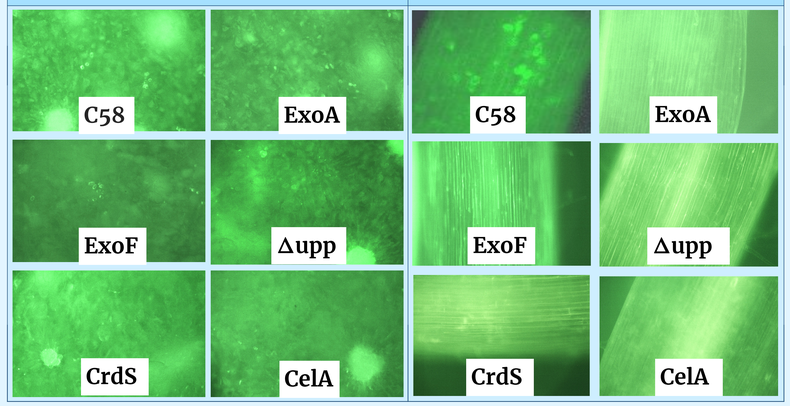


Virulent bacterial mutants on *P. communis* fruit discs.

Transient Transformation



Transient Transformation of Exopolysaccharides



Conclusion

- C58, *ExoA*, and *ExoF* have higher levels of resistance to hydrogen peroxide than *Δupp* and *CelA*.
- *CrdS* was resistant to H_2O_2 but did not induce tumor growth.
- C58, *ExoA*, *ExoF*, and *Δupp* were virulent and induced tumor formation.
- Based on these data, succinoglycan and the unipolar polysaccharide are not important in resistance to hydrogen peroxide, transient transformation, and virulence.

Future Directions

- Continue trials assessing the role of various exopolysaccharides in *A. tumefaciens* virulence and resistance to hydrogen peroxide.
- Repeat trials with *CrdS* and *CrdSCelA* to verify results.