Role of Exopolysaccharides in Transient Transformation and Virulence of *Agrobacterium fabrum*

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Abstract

Agrobacterium tumefaciens is a soil bacterium that can transfer DNA to plant cells. If a plant is wounded, the bacteria infect the wound and transfer a segment of DNA to host cells to induce formation of crown gall tumors. Exopolysaccharides play a role in producing a biofilm that helps protect *A. tumefaciens* from plant hosts' defense mechanisms such as hydrogen peroxide. Our research examines whether certain exopolysaccharides are required for bacterial growth in the presence of wounded plant roots, transfer of DNA, and tumor formation. Some *A. tumefaciens* mutants that fail to produce certain exopolysaccharides show reduced or no crown gall tumor formation and fail to transfer of DNA. The bacterial mutants unable to make cellulose or β-1, 2-D glucan exopolysaccharides fail to induce tumor formation and transfer DNA. These data suggest that cellulose and β-1, 2-D glucan exopolysaccharides are important for tumor formation and DNA transfer. However, not all exopolysaccharides matter for virulence and transient transformation. The bacterial mutants unable to make succinoglycan, curdlan, and the unipolar polysaccharide induce crown gall tumor formation and exhibit DNA transfer. These results suggest that succinoglycan and the unipolar polysaccharide are not important for virulence, induction of crown gall tumor formation, nor transient transformation of *A. tumefaciens*.

Introduction

Agrobacterium tumefaciens is a gram-negative bacterium found in the rhizosphere. It can transfer DNA to plant cells via horizontal gene transfer using a type IV secretion system. If a plant is wounded, the bacteria infect the wound and transfer a segment of DNA to host cells to induce formation of crown gall tumors. The constructed Ti-reporter-plasmid pGWB2nlgfp can be used to observe transient transformation in plant host cells. This tumor-inducing (Ti) plasmid contains a eukaryotic promoter and nuclear localization signal in front of a green fluorescent protein gene to allow for observation of DNA transfer to plant host cells using fluorescence microscopy. These genes are carried between the T-DNA border repeats, allowing transfer to the plant host. The Ti-plasmid also contains a gene encoding resistance to kanamycin. The kanamycin gene is not located within the T-DNA border repeats. As part of their defense against pathogens, plants produce reactive oxygen species such as hydrogen peroxide. Exopolysaccharides and short chain succinoglycan molecules are reported to play a role in resistance to hydrogen peroxide in *Sinorhizobium meliloti* (Lehman and Long, 2013). Exopolysaccharides are extracellular macromolecules that are involved in biofilm formation that may help protect bacteria from plant defenses. By providing bacterial protection, exopolysaccharides may play a role in virulence and DNA transfer. Our research examines whether exopolysaccharides are required for mutants to grow in the presence of wounded plant hosts, resistance to hydrogen peroxide, transfer DNA, and induce crown gall tumors. Virulence assays were conducted on *Bryophyllum daigremontiana* leaves and *Lycopersicum esculentum* (tomato) stems by inoculating wounds. Fluorescence microscopy was used to observe the frequency of DNA transfer by the mutant strains. Tumor formation and transient transformation were compared to the virulent C58 parent strain. These comparisons inform the discussion on the role of exopolysaccharides in the virulence and transient transformation of *A. tumefaciens* and resistance to hydrogen peroxide.

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

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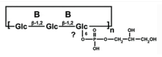
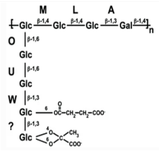
Please direct any questions or comments to Ann Matthysse, Ph.D at matthysse@bio.unc.edu, or to Caroline Chandler at cmsc@email.unc.edu. Thank you!

Known Exopolysaccharides of *Agrobacterium* and Bacterial Mutants Altered in Their Synthesis

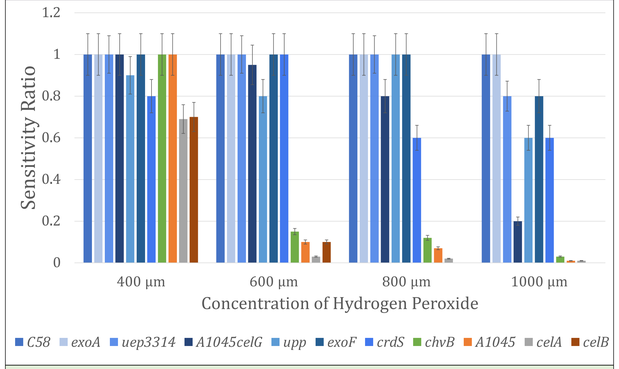
Bacterial Strain and Gene Number	Exopolysaccharide Affected	Exopolysaccharide Structure	Cellular Location
chvB (A1045)	β-1, 2-D glucan production		Periplasmic space
exoA (Atu4053)	Glycosyltransferase, required for succinoglycan synthesis		Extracellular space
exoF (Atu3326)	Polysaccharide transport, required for export of succinoglycan*		Extracellular space
celA (Atu3309)	Cellulose synthase	-(β-1, 4-D-glucose)$_n$-	Extracellular space
crdS (Atu3056)	Curdlan synthase	-(β-1, 3-D-glucose)$_n$-	Extracellular space
Δupp	Unipolar polysaccharide synthesis	Unknown	Cell surface at one pole
A1045celG (Atu8186)	Lacks β-1, 2-D glucan and overproduces cellulose	*celG* is a regulator of cellulose synthesis	
celG	Overproduces cellulose	*celG* is a regulator of cellulose synthesis	

**ExoF may be involved in the export of other unidentified polysaccharides*

Methods

Ti-Reporter-Plasmid: The plasmid pGWB2nlgfp contains a eukaryotic promoter and nuclear localization signal in front of a green fluorescent protein gene.
Germination of Plant Seeds: Tomato seeds were surface sterilized and germinated in sterile water. They were then placed into sterile 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 0.05% 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.
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." Tumor formation was scored per site inoculated.
Hydrogen Peroxide Sensitivity: Cultures were grown in Luria broth on a roller drum, diluted, and then plated on Luria agar with various concentrations of hydrogen peroxide.

Sensitivity to Hydrogen Peroxide



The sensitivity of bacterial mutants to hydrogen peroxide is shown as the percent surviving cells at various H_2O_2 concentrations. As the concentration of hydrogen peroxide increases, the mutants unable to produce the periplasmic polysaccharide β-1,2-D-glucan (*chvB* and *A1045*) or cellulose (*celA* and *celB*) decrease in survival. The *A1045celG* mutant overproduces cellulose. It survives higher concentrations of H_2O_2 better than A1045, suggesting that increased amounts of cellulose are able to partially restore the resistance of the mutant to H_2O_2. The other exopolysaccharide mutants tested are relatively unaffected in resistance to H_2O_2.

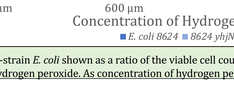
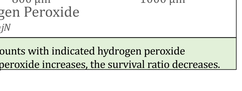
Virulence of Bacterial Strains

Bacterial strain	Virulence (percent of inoculated sites forming tumors)	
	B. daigremontiana	*L. esculentum*
C58	**88 (71/82)[A]**	**100 (39/39)**
NT1[B]	**0 (0/10)**	**0 (0/6)**
ExoA	**100 (8/8)**	**100 (8/8)**
ExoF	**100 (12/12)**	**75 (3/4)**
A1045	**0 (0/8)**	**0 (0/8)**
chvB	**0 (0/8)**	**0 (0/4)**
crdS	**100 (4/4)**	**100 (5/5)**
Δupp	**100 (8/8)**	**91 (10/11)**
celA	**12 (1/8)**	**0 (0/12)**
A1045celG	**100 (4/4)**	**100 (6/6)**
celG	**100 (4/4)**	**100 (6/6)**
3314	**100 (12/12)**	**100 (3/3)**

[A] percent inoculated sites forming tumors (number of tumors/number of inoculated sites)
[B] *NT1* is avirulent and lacks both the Ti and reporter plasmids

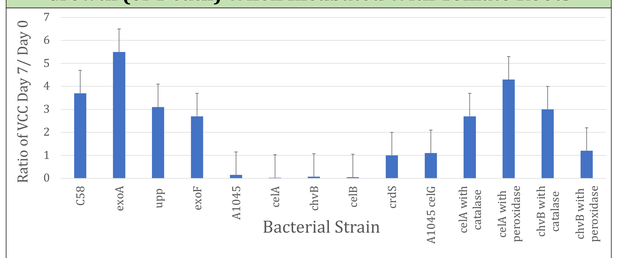
***C58, crdS, celG, exoA, exoF, 3314, and Δupp* were all virulent. The cellulose and β-1,2-D glucan mutants were avirulent. *celA* was severely attenuated on *B. daigremontiana*.**

Virulence of Bacterial Strains

Bacterial Strain	*B. daigremontiana*	*L. esculentum*
C58		
A1045		
celA		

***C58, exoA, exoF, Δupp, celG, 3314,* and *crdS* are all virulent. *A1045* is avirulent, and *celA* is severely attenuated.**

Growth (or Death) When Incubated With Tomato Roots

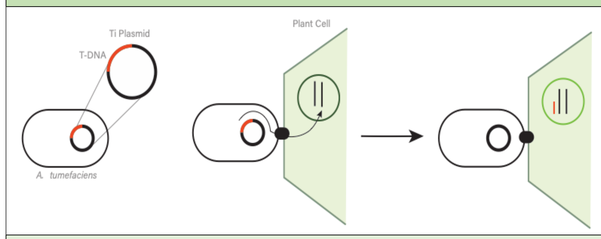


The growth (or death) of bacterial mutants, when incubated with tomato root segments, is reported as the ratio of the viable cell count at day 7 over that at day 0. A value of 1.0 indicates that the bacteria neither grew nor died. Values less than 1 indicate bacterial death. Values greater than 1 indicate bacterial growth. The bacterial mutants unable to produce cellulose (*celA* and *celB*) or produce the periplasmic polysaccharide β-1,2-D-glucan (*chvB* and A1045) died when incubated with cut tomato roots. The addition of catalase and peroxidase to growth media with these strains appears to restore some growth. The parent strain C58 and the mutants unable to produce succinoglycan (*exoA* and *exoF*) or the unipolar polysaccharide (*Δupp*) grew when incubated with tomato root segments.

Survival Ratio of *E. coli* Strains with Hydrogen Peroxide

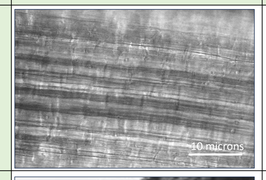
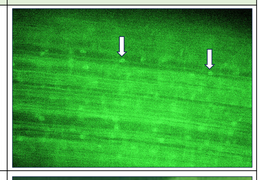
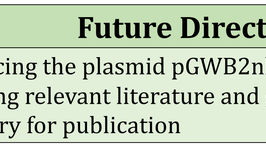
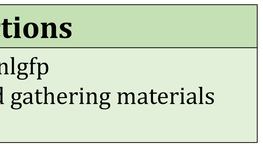
The survival of pathogenic lab-strain *E. coli* shown as a ratio of the viable cell counts with indicated hydrogen peroxide concentrations and without hydrogen peroxide. As concentration of hydrogen peroxide increases, the survival ratio decreases.

Transient Transformation



Transient transformation is a method of introducing foreign DNA into a plant cell. Here, the Ti-reporter-plasmid pGWB2nlgfp is introduced to plant host cells. Fluorescing nuclei indicate successful transfer of T-DNA to the plant host nucleus by the bacterial strains introduced.

Transient Transformation on Tomato Roots

Bacterial Strain	Nomarski Optics	Fluorescence Microscopy
C58		
celA		
celB		
chvB		

Fluorescing nuclei indicate successful transfer of T-DNA to the plant host nucleus by the *C58* parent strain. Virulent mutants are known to form tumors and transfer their DNA to the plant host genome. Avirulent mutants were examined for transient transformation based on the unpublished observation that some mutants cannot form tumors yet show transient transformation (Matthysse). The *celA*, *celB*, and *chvB* mutants do not form tumors nor transfer DNA to the plant host.

Conclusions

- Only a few exopolysaccharides play a role in resistance to hydrogen peroxide in *A. tumefaciens*
- Cellulose and β-1,2-D-glucan both are required for maximum resistance to H_2O_2
- Exopolysaccharide mutations which decrease resistance to H_2O_2 result in lack of virulence and ability to transfer DNA to the plant host genome

Future Directions

- Sequencing the plasmid pGWB2nlgfp
- Exploring relevant literature and gathering materials necessary for publication