Succinoglycan and β-lactamase Production Confers Resistance to External Stresses in *Agrobacterium tumefaciens*

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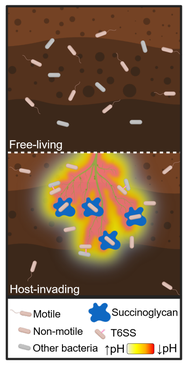
What is succinoglycan?



Model of plant invasion

Succinoglycan is a negatively charged exopolysaccharide that is required for *Agrobacterium tumefaciens* to invade a plant host. This invasion is the initial step of Crown Gall disease, where the plant grows large tumor-like growths. The mechanistic role of succinoglycan in this process remains largely unknown.

Here, we find that succinoglycan may play a broad role in response to stressful environments, including acid stress, outer-membrane stress, and antibiotic stress.

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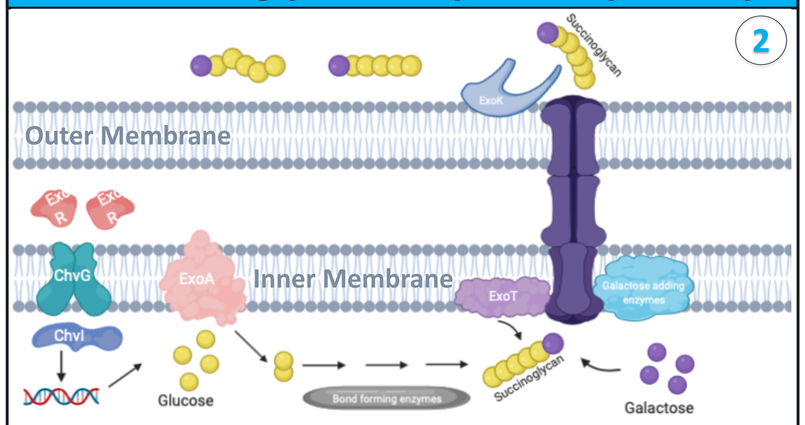
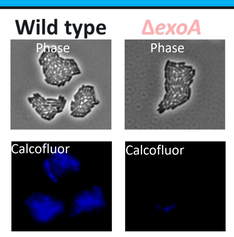
The succinoglycan biosynthesis pathway



2

The succinoglycan biosynthesis pathway is activated by the **ChvG-ChvI** two-component system. **ExoR** is know to be degraded in the presence of acid, leading to phosphorylation of **ChvI** and succinoglycan biosynthesis. Succinoglycan biosynthesis requires ExoA, a glycosyltransferase.

Succinoglycan production and export is dependent on ExoA



3

Calcofluor white binds succinoglycan allowing for visualization of succinoglycan using fluorescence microscopy. Here we show that ExoA is required for succinoglycan production.

Objective and Hypothesis

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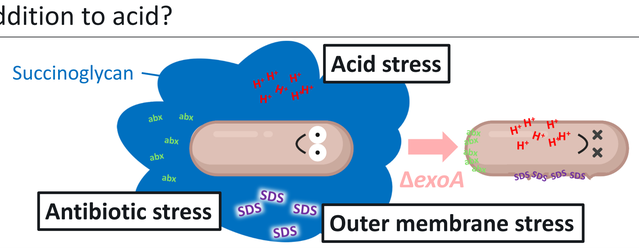
Research Question:

Does succinoglycan protect against environmental stressors in addition to acid?



Hypothesis: *Agrobacterium tumefaciens* secretes succinoglycan to protect itself from acid, antibiotic, and outer membrane stresses.

Succinoglycan production provides protection in acidic environments

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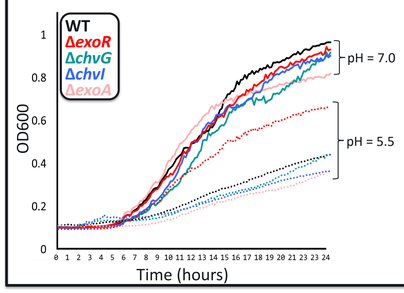
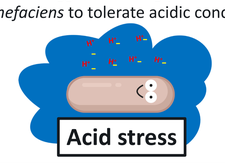
Growth of ***ΔexoR*** (succinoglycan overproducing), ΔexoA (does not produce succinoglycan), **ΔchvG**, **ΔchvI**, and **WT** was monitored over 24 hours in liquid media at neutral (7.0) or low (5.5) pH.



ΔexoR grows better than all other mutant strains tested in acidic media

ΔexoA grows worse than all other mutant strains tested in acidic media

These data suggest succinoglycan secretion allows *A. tumefaciens* to tolerate acidic conditions.



Succinoglycan provides protection against an anionic detergent

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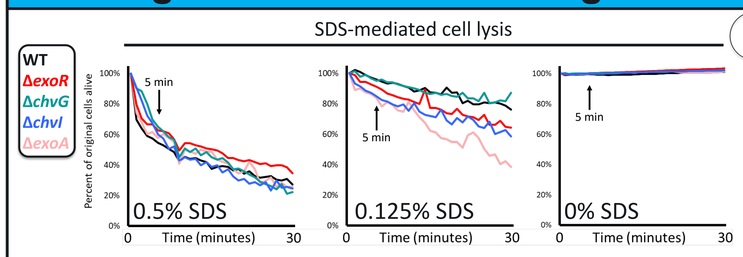


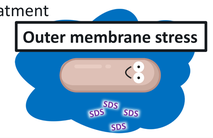
Sodium dodecyl sulfate (SDS) is a detergent that disrupts outer membranes. Mixing the cells with low concentrations of SDS can cause cell lysis as indicated by the decreased cell abundance.

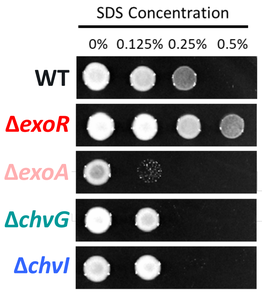
After 5 minutes of SDS treatment, cells are spotting on growth media to determine how well the cell can recover from this stress.

exoR recovers better than all strains following SDS treatment

ΔexoA is the most sensitive to SDS treatment

These data suggest succinoglycan protects the outer membrane of *A. tumefaciens*.





Succinoglycan production confers resistance to cell wall-targeting antibiotics

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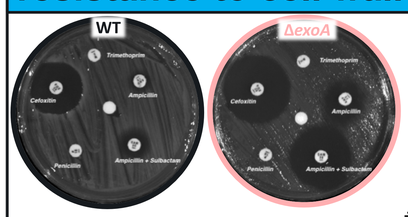
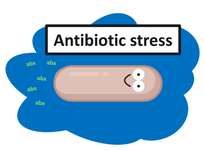
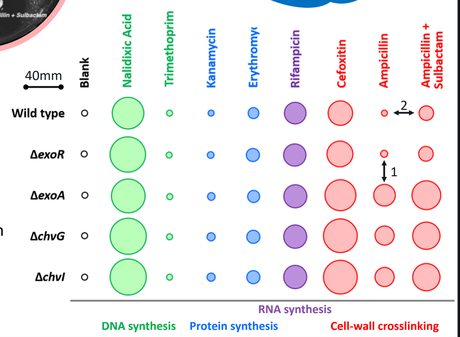




Using the antibiotic disc diffusion assay we find that ΔexoA, **ΔchvG**, and **ΔchvI**, show increased sensitivity to β-lactam antibiotics. We also see increased size in zones of inhibition when adding sulbactam, a β-lactamase inhibitor. From these data we can conclude 2 things:

1) Succinoglycan contributes to β-lactam resistance.
2) There are β-lactamases contributing to β-lactam resistance.



Summary of Results

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- ***ΔexoR*** constitutively produces succinoglycan, providing resistance to each of the external environmental stressors.
- ΔexoA does not produce succinoglycan and is the most sensitive to each of the external environmental stressors tested suggesting succinoglycan plays a direct role in the protecting the cells from these agents
- **ΔchvG** and **ΔchvI** are also sensitive to the external environmental stressors suggesting that this regulatory pathway likely plays an indirect role in protection from stress
- The disparity in the zones of inhibition between amp and amp + sulbactam across all strains suggest the presence of beta-lactamase activity

The genome of *A. tumefaciens* encodes two β-lactamases that are differentially regulated

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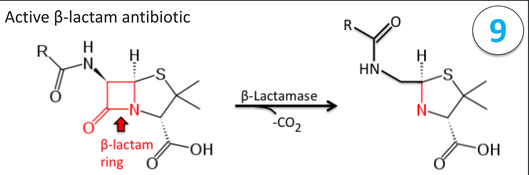
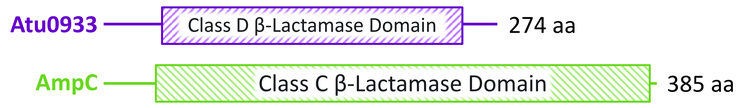
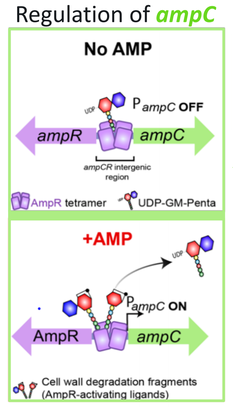
Function of β-lactamases:





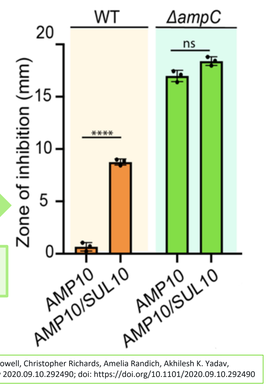
Bioinformatic analysis suggests that Atu0933 is a class D β-lactamase, an understudied group of β-lactamases. AmpC belongs to a well-studied group of β-lactamases that can be inhibited by sulbactam. Our lab has shown that AmpC is likely the culprit for the differences in zones of inhibition upon addition of sulbactam.

Regulation of *ampC*



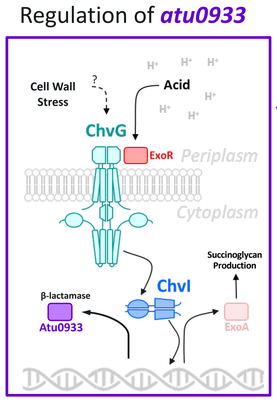
Transcription of *ampC* is regulated by AmpR. When treated with ampicillin, transcription is turned on.

Deletion of *ampC* increases sensitivity to ampicillin.

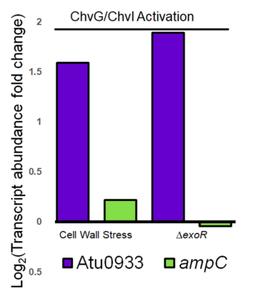


Wanda M. Figueroa-Cuilan, Matthew Howell, Christopher Richards, Amelia Randich, Akhilesh K. Yadav, Felipe Cava, Pamela J.B. Brown. bioRxiv 2020.09.10.292490; doi: https://doi.org/10.1101/2020.09.10.292490

Regulation of *atu0933*



Transcriptional data indicate that transcription of *atu0933* is upregulated under cell wall stress conditions and upon deletion of *exoR*, encoding the ChvG/ChvI regulator.



Transcription of *atu0933* is regulated by ChvG/ChvI. Transcription is turned on ExoR-mediated acid sensing or under cell wall stress.

Future Directions

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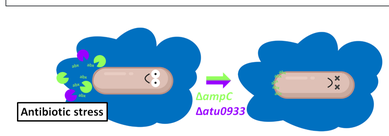
Ongoing Research Question:

How does *A. tumefaciens* tolerate β-lactam antibiotics?

Hypothesis: *A. tumefaciens* secretes β-lactamases which contribute to survival during cell wall stress. Succinoglycan production alone may not be sufficient.



Research Plan:

1. Construct mutants
   - *atu0933* deletion
   - *atu0933/ampC* double mutant
2. β-lactam treatment
   - Disc diffusion assays to determine sensitivity to β-lactam antibiotics
   - Characterize AmpC and Atu0933 substrate specificities
3. Inhibitor studies
   - Determine impact of β-lactamase inhibitors such as sulbactam, clavulanic acid, and EDTA to explore differences in AmpC and Atu0933 activities
4. Biochemical characterization
   - Nitrocefin assays to quantify the amount of secreted β-lactamase in WT, *ampC*, and *atu0933* strains

Acknowledgements

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