Abstract:

Citrus Greening Disease is a bacterial disease from the organism *Candidatus* Liberibacter asiaticus (*C*Las). Citrus greening affects tree health as well as fruit development, ripening and quality. Secondary metabolites produced by citrus bacteria may prove to be a new source of antibiotics against *C*Las. In previous work, we showed that antibiotic natural products were produced by bacteria living inside citrus trees. For this study, I conducted a time course of bacterial strain CB729 with different media and incubation times to attempt to increase the production of amicoumacins A and B, two known compounds with antibiotic properties.

Introduction:

Citrus greening disease has infiltrated the citrus industry since its introduction to the United States. It started in Florida and has moved across the country to even Orange County, California. This disease has ravaged the citrus industry and has caused approximately 21% decrease in the fresh citrus fruit market and about a 72% decline in the production of oranges used for juice and other products. Strategies for mitigating the disease include removing infected trees or using pesticides, but a well defined method that prevents the disease has yet to be discovered.

In a typical orchard, the citrus trees are clonally propagated, yet *C*Las-infected orchards commonly contain trees with different levels of symptoms. Given that all the trees are genetically identical, the plant's genes couldn't be responsible for providing resistance to *C*Las. The Roper lab decided to explore the microbiomes of infected citrus trees, and saw differences between trees with slow progression of disease compared with trees that had severe symptoms. This

suggested that certain bacteria living inside the trees might be providing a defense against *C*Las. We hypothesized that these bacteria might be producing antibiotic natural products, which could provide a possible treatment for Citrus Greening Disease.

Over the summer, the strain CB729 became the main focus of our research as our collaborators at UCR sent us an extract that had promising activity. After separation by HPLC, we were able to isolate a single compound, and determine its structure to be that of *N*-actylamicoumacin, using LCMS and 1D- and 2D-NMR. Pure *N*-acteylamicoumacin was tested for antibiotic activity against *Liberibacter crescens*, but was found to be inactive. However, LCMS data from active fractions from CB729 showed the masses of other amicoumacins. In particular, the known antibiotics amicoumacin A and amicoumacin B were believed to be present in the active extracts.

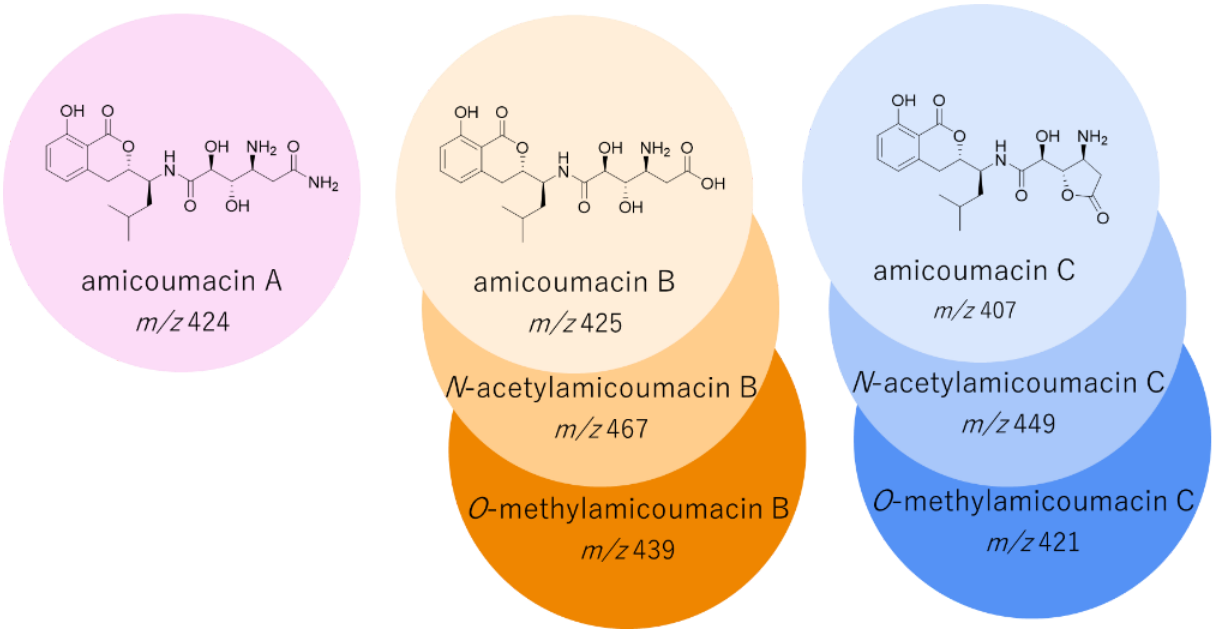

Figure 1: Amicoumacin masses observed in LCMS chromatograms of extracts of CB729

The goal of my honors project was to see if we could boost amicoumacins production, more specifically amicoumacin A and B which are active. We decided to conduct a time course to find the most ideal growing conditions. We focused on changing the media that CB729 was grown in and the amount of time that the bacteria had to incubate. Previously we had grown our citrus bacteria for a whole week but the time course focused on shorter times to see if we could get more amicoumacin A and B instead of *N*-acetylamicoumacin. The purpose of the differing times was to give the bacteria enough time to make a decent amount of amicoumacins but not enough that random *N*-acetyltransferases could come and attach a *N*-acetyl group rendering them inactive.

The two media that were used in this experiment were Tryptic Soy Broth (TSB) and A21 media. Previously we had grown CB729 in Luria Bertani Broth and Potato Dextrose Broth. TSB was tested because of its rich complex media that was predicted to have more useful nutrients for growing. A21 is a media that was used by our collaborators at UCR and was the media used to generate the CB729 extract from which *N*-acetylamicoumacin was initially isolated. A21 has more carbon and different nitrogen sources which could lead to better growing conditions.

Methods and Results:

Growing of Bacteria:

CB729 was stored at -80° C. Single colonies of CB729 grown on LBA at 30°C were used to inoculate 8 × 25 mL of LB broth in 50 mL conical tubes and incubated with shaking for 24 hours at 30 °C. 15 mL of these seed cultures were used to inoculate each of 20

×125mL cultures in 500 mL flasks (10 × TSB media and 10 × A21 media). Cultures were shaken at 30 °C until each time point (4, 8, 12, 24, and 48 hours).

Extraction of Natural Products

One flask was removed from the shaker at each time point, and the culture extracted with 3 × 75 mL portions of ethyl acetate.The ethyl acetate layers were combined and evaporated under reduced pressure, to give the crude extracts shown in Tables 1 and 2.

Name	Media	Time on Shaker (hr)	Crude Mass (mg)
A4 1	A21	4	8.3
A4 2	A21	4	9.6
A8 1	A21	8	12.6
A8 2	A21	8	14.4
A12 1	A21	12	16.8
A12 2	A21	12	20.2
A24 1	A21	24	23.7
A24 2	A21	24	16.9
A48 1	A21	48	14
A48 1	A21	48	14.4

Name	Media	Time on Shaker (hr)	Crude Mass (mg)
A4 1	A21	4	3.8
A4 2	A21	4	3.6
A8 1	A21	8	4.5
A8 2	A21	8	4.7
A12 1	A21	12	7.1
A12 2	A21	12	19.7
A24 1	A21	24	19.7
A24 2	A21	24	19.7
A48 1	A21	48	12
A48 1	A21	48	11.3

Tables 1 and 2 show the different media and time points that were grown and extracted along with the crude weights that were dried down

Analysis of Extracts:

5 mg aliquots of each extract were sent to collaborators at the University of California Riverside for filter disc assays and Liquid Chromatography Mass Spectrometry (LCMS). Filter disk assays show activity of the extracts versus *Liberibacter crescens* which is a culturable close relative to *C*Las, which is unculturable. The crude extracts are placed on filter disks applied to freshly plated lawns of *L. crescens*. If the extracts are active, rings of inhibition appear around the discs, revealing antibiotic activity against *L. crescens*. The assumption is that *C*Las and *L. crescens* are similar enough that molecules that inhibit *L. crescens likely* also inhibit *C*Las. Activity of the time course extracts is seen below in figure 2.

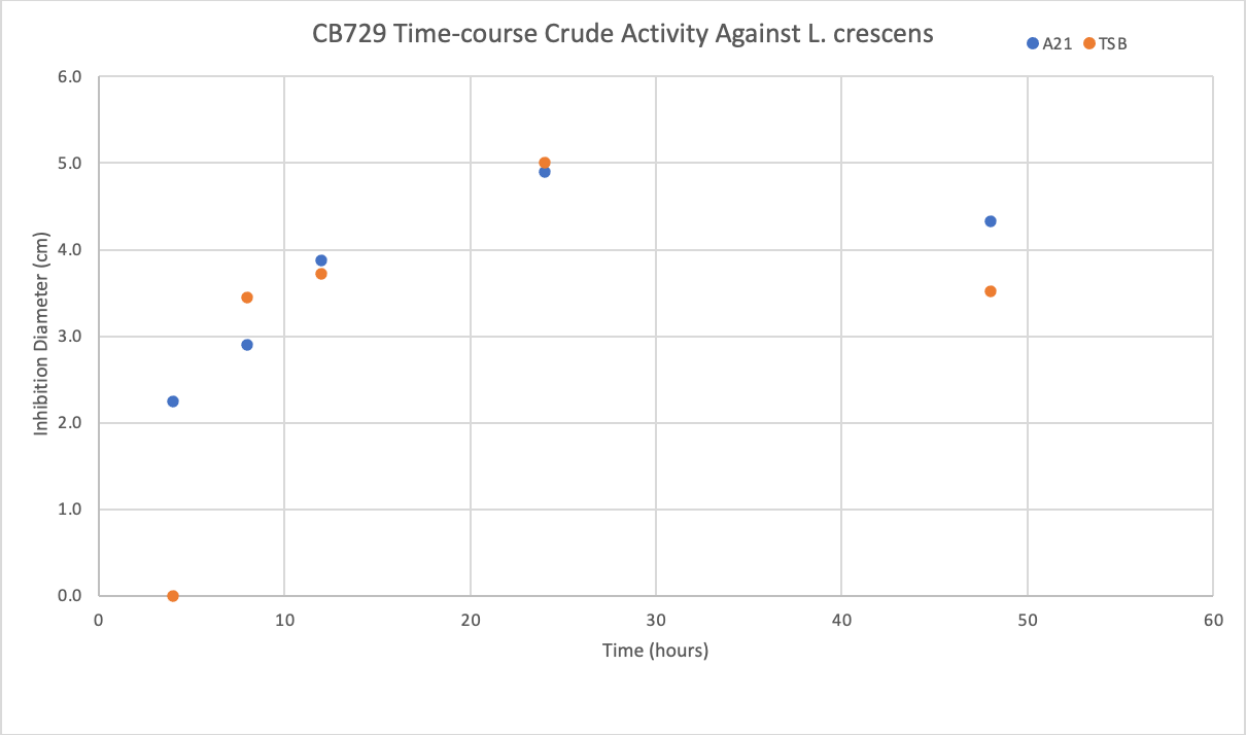

Figure 2: Activity of A21 and TSB crude extracts against L. crescens over time course.

With limited data, it is difficult to draw conclusions from the activity because we don't have data past 48 hrs. It would be helpful to run the experiment up to 72 hours or even 96 hours to see if the activity continues to decrease from the high at 24 hours, if it rebounds after the dip at 48 hours, or if it plateaus at a certain time.

At UCR they also subjected the crude extracts to high-resolution LCMS/MS. Extracted ion chromatograms (EIC) of the mass of amicoumacin A (*m/z* of 424.2084) were used to determine the abundance of what could be amicoumacin A. (By observing the MS2 spectrum of the peak at 424.2, they were also able to support the identification of amicoumacin A.) The data received is shown below in figure 3.

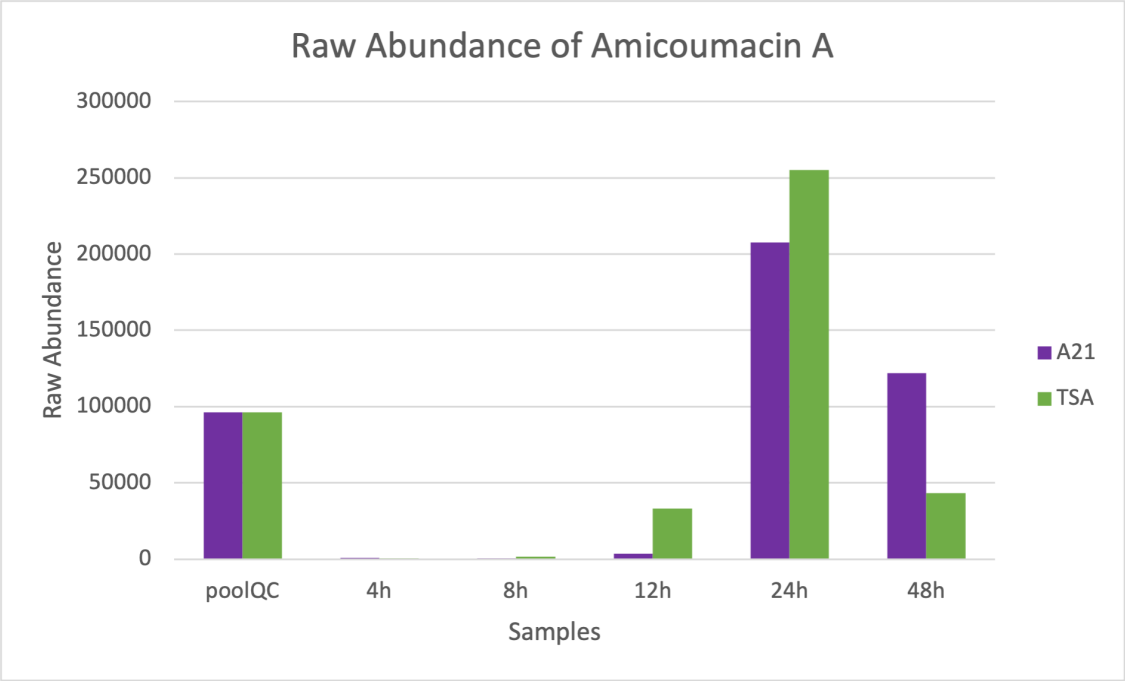

Figure 3: Abundance of amicoumacin A (m/z 424.2048) in time course extracts.

The remaining crude extracts were subjected to ^{1}H NMR specroscopy to compare the chemical composition at the different time points. Since the spectra of the different time points were very similar, the extracts from the cultures grown in each media were combined to give more mass of crude extract for ultimate compound isolation.

Appendix:

A21 Culture Medium Recipe:

Per 1 L of DI Water

- Glucose 20 g/L
- Yeast Extract 5 g/L
- K2HPO4 1 g/L
- MgSO4·7H2O 0.5 g/L
- KCl 0.5 g/L
- CuSO4 1.6mg/L
- Fe2(SO4)3 1.2mg/L
- MnSO4 0.4mg/L

Tryptic Soy Broth Medium Recipe:

Per 1 L of DI Water

- Casein 17 g/L
- Soya peptone 3 g/L
- NaCl 5 g/L
- K2HPO4 2.5 g/L
- Dextrose 2.5 g/L

References:

(1) Ferrarezi, R. S.; Qureshi, J. A.; Wright, A. L.; Ritenour, M. A.; Macan, N. P. F. Citrus production under screen as a strategy to protect grapefruit trees from Huanglongbing disease. https://www.frontiersin.org/articles/10.3389/fpls.2019.01598/full (accessed Apr 13, 2023).
(2) Ginnan, N. A.;et Al.; Department of Botany and Plant Sciences; Roper, M. C.; †Corresponding author: M. C. Roper; E-mail Address: http://orcid.org/0000-0003-4637-6225Department of Microbiology and Plant Pathology; Disease-induced microbial shifts in citrus indicate microbiome-derived responses to huanglongbing across the disease severity spectrum. https://apsjournals.apsnet.org/doi/10.1094/PBIOMES-04-20-0027-R (accessed Apr 13, 2023).
(3) Juice plant pathogen could be treated with newly identified antibacterial agent. https://www.acs.org/pressroom/newsreleases/2019/april/juice-plant-pathogen-could-be-treated-with-newly-identified-antibacterial-agent.html (accessed Apr 13, 2023).
(4) Maksimova, E. M.; et.Al Multifaceted mechanism of amicoumacin a inhibition of bacterial translation. https://www.frontiersin.org/articles/10.3389/fmicb.2021.618857/full (accessed Apr 13, 2023).
(5) Zheng, C. Citrus greening is killing the world's orange trees. Scientists are racing to help. https://cen.acs.org/biological-chemistry/biochemistry/Citrus-greening-killing-worlds-orange/97/i23 (accessed Apr 13, 2023).