Development of a Antibacterial Disc Diffusion Assay to Differentiate Fusarin C from Non-Fusarin C Activity in Early Screening of Mutant *Fusarium graminearum* Extracts

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Abstract

The discovery of novel compounds with antibacterial properties continues to be critically important.[3] One potential source of such compounds is the cryptic genome of fungi known to produce biologically active molecules. A *kmt6* mutant of the cereal pathogen *Fusarium graminearum* was previously developed through a histone H3 lysine 27 methyltransferase gene knockout, and has been shown to express hundreds of additional genes compared to wild type under the same growth conditions.[1] Both the *kmt6* mutant and the wild type *F. graminearum* produce the biologically active metabolite fusarin C, shown in Figure 1, and its analogs. Activity due to fusarin C can mask any activity of non-fusarin C compounds during early screening of extracts.

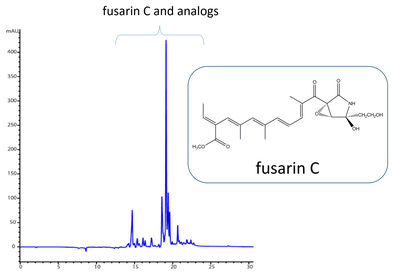

Figure 1. HPLC profile of "fusarin C isolate" sample, with fusarin C at RT 19 minutes.

Using a broad panel of bacteria, two samples were tested for differentiating activity profiles. These included an ethyl acetate extract of *kmt6* growth media (i.e., the "**160001E**" fraction), and a more purified flash column fraction containing fusarin C and its analogs (i.e., the "**fusarin C isolate**"). Intriguingly, both *Bacillus cereus* and *Staphylococcus aureus* were inhibited by the 160001E extract, but were not inhibited by the fusarin C isolate. This indicates that activity from the 160001E fraction is not due to fusarin C or its analogs, but rather due to non-fusarin C compounds. With these results, a panel of 5 bacteria, including both gram negative and gram positive strains, has been developed as an early screening tool to differentiate activity between fusarin C isolates and non-fusarin C compounds from *kmt6* mutant *F. graminearum*. This will aid in the bioassay-guided fractionation of extracts exhibiting antibacterial activity due to non-fusarin C compounds and toward the important aim of identifying novel compounds with antibacterial activity.

Background and Introduction

The Loesgen laboratory supplied the fusarin C isolate sample from *F. graminearum*, and the Freitag laboratory at OSU supplied the *kmt6* mutant of *F. graminearum*. Initial investigations of the antibacterial range of the fusarin C isolate were performed using an agar disc diffusion assay with the fifteen bacteria listed in Table 1. In the disc diffusion assay approach to antibiotic testing, filter discs loaded with known concentrations of the compound to be assessed are applied to agar previously inoculated with bacteria. On plates where the bacteria is inhibited by the compound being analyzed, a zone of inhibition develops around the disc.[4] Based on susceptibility to fusarin C isolates, the length of optimum growth, and the clinical relevance, the five bacterial strains found in Table 2 were selected for further disc diffusion assay investigation with the *kmt6* 160001E extract. Figure 2 shows the overall flowchart for the experimental design of this research.

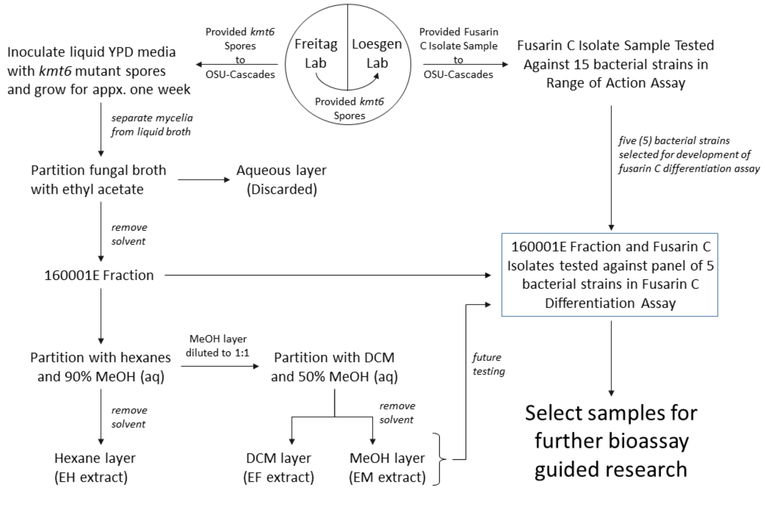

Figure 2. Overview of sample sources, experimental design, and flow processes.

Materials and Methods

F. graminearum Growth and Fractionation

Petri plates with YPD solid media were inoculated with spores and grown for 7 days at 30°C in the dark as seen in Figure 3. Liquid YPD media was inoculated using tissue from YPD plates and grown for 7 days in the dark. Mycelia were strained and discarded, and aqueous broth extracted with ethyl acetate and dried of residual solvent.

Sample Preparation for Range of Action Assay

Fusarin C isolate was diluted in ethyl acetate to 3.2 mg/mL, 0.32 mg/mL and 0.032 mg/mL. Ampicillin was diluted in sterile water to 0.02 mg/mL and trimethoprim (TMP) was diluted in LB broth to 1 mg/mL.

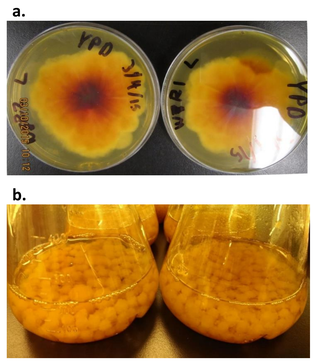

Figure 3. *F. graminearum* grown (a) on petri plates with solid YPD media; and (b) in YPD liquid media.

Sample Preparation for Fusarin C Differentiating Disc Diffusion Assay

Fusarin C isolate was diluted in ethyl acetate to 3.2 mg/mL. Ampicillin was diluted in sterile water to 0.02 mg/mL and 0.002 mg/mL. TMP was diluted in LB broth to 1.0 mg/mL and 0.01 mg/mL. The *kmt6* E fraction (Cascades code: 160001E) was diluted in ethyl acetate to 3.2 mg/mL.

Disc Diffusion Assay

Overnight bacterial cultures were diluted 1:100 in sterile phosphate buffer (pH 7.0) and 150 uL was spread onto LB agar plates. 25 µL of sample or control antibiotic were added to a disc and placed onto bacterial lawn. The plates were incubated for 24 hours. Zone of inhibition was recorded using the mean distance from edge of disc to edge of inhibition zone.

Results & Discussion

Fusarin C Isolate Dose Response

Initial dose response studies were conducted. As shown in Figure 4, there was no discernable activity below a fusarin C isolate concentration of 3.2 mg/mL in dose response studies. Therefore, this concentration was used for the remainder of the studies.

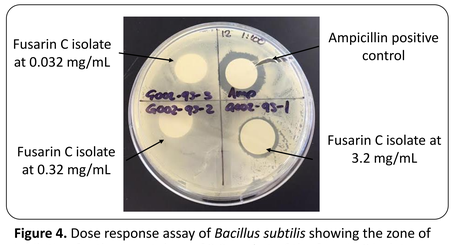

Figure 4. Dose response assay of *Bacillus subtilis* showing the zone of inhibition for the target dose of 3.2 mg/mL of the fusarin C isolate.

Fusarin C Isolate Initial Activity

As shown in Table 1, the fusarin C isolate inhibited bacterial growth of 4 of the original 15 bacteria tested in the initial disc diffusion assay. These four strains, as well as one other, were selected for further analyses.

Table 1. Panel of 15 bacteria initially used for testing the fusarin C isolate.

Bacterial Strains Used in Initial Range of Action Assay*				
Gram negative	**Acinetobacter calcoaceticus**	*Enterobacter aerogenes*	***Enterobacter cloacae***	*Escherichia coli (B)*
	Klebsiella pneumoniae	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Serratia marcescens*
	Pseudomonas aeruginosa	*Serratia marcescens*		
Gram positive	*Bacillus cereus*	***Bacillus subtilis***	***Bacillus megaterium***	*Enterococcus faecalis*
	Lactococcus lactis	*Sarcina aurantiaca*	*Staphylococcus aureus*	

* Bacteria names in bold were inhibited by the fusarin C isolate

160001E Fraction Activity

Table 2 shows the five (5) bacterial strains selected for further testing, where four were inhibited by the 160001E fraction. Two of the strains, namely *Staphylococcus aureus* and *Bacillus cereus*, were inhibited by the 160001E fraction but were not susceptible to inhibition by the fusarin C isolate.

Results & Discussion (continued)

Table 2. Zone of inhibition results of fusarin C isolate and 160001E samples in the differentiating disc diffusion assay.

Bacteria	Significance of Activity	Ampicillin or TMP Zone of Inhibition* (mm)	Fusarin C isolate Zone of Inhibition (mm)	160001E Zone of Inhibition (mm)
Bacillus cereus (+)	Differentiator	1.5	0	0.75
Bacillus subtilis (+)	Positive Control	5.0	2.5	2 .0
Enterobacter cloacae (-)	Positive Control	1.0	2.0	1.0
Escherichia coli B. (-)	Negative Control	5 .0	0	0
Staphylococcus aureus (+)	Differentiator	1.0	0	1.3

* Measurements represent an average of the zone of inhibition from two disc diffusion assays.

Figure 5 shows the results of the differentiating assay, and suggests that the activity from the 160001E sample against *B. cereus* and *S. aureus* is due to compounds that are not present in the fusarin C isolate sample.

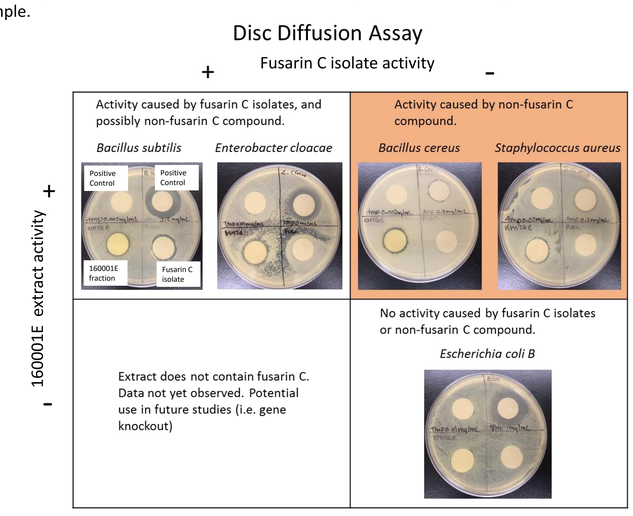

Figure 5. Disc diffusion assay results of fusarin C isolate and 160001E (*kmt6* E fraction) and controls.

Conclusion

A fusarin c isolate was initially screened in a disc diffusion assay against a broad panel of fifteen (15) bacteria. The fusarin C isolate showed activitiy against both gram positive and gram negative bacteria, supporting previous findings of wide-range bioactivity.[2] Results from this initial assay provided rationale for choosing a smaller panel of five (5) bacteria, including both gram positive and gram negative bacterial strains, to be developed in efforts to differentiate activity between fusarin C isolates and non-fusarin C compounds. Importantly, *B. cereus* and *S. aureus* were not inhibited by the fusarin C isolate, but were inhibited by the 160001E fraction. This indicates that activity from the 160001E fraction is not due to fusarin C or its analogs, but rather it is due to non-fusarin C compounds.

Literature Citations

1. Connolly, L.R.; Smith, K.S.; and Freitag, M. The Fusarium graminearum Histone H3 K27 Methyltransferase KMT6 Regulates Development and Expression of Secondary Metabolite Gene Clusters. *PLOS Genetics* **2013**, *9*, e1003916.
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Acknowledgements

- OSU-Cascades (CBURG): Gaskin's Fund and Circle of Excellence Grant
- OSU (Freitag): NIH (GM097637) and NSF (MCB1515998)
- Conner Burck for previous work on *F. graminearum*
- Dr. Pat Ball and Stephanie Johnson for bioassay consultation