

Identifying Difficidin in the Supernatant of *Bacillus velezensis* using High Resolution Mass Spectrometry

Ben Nasisi, Conor Jenkins, Madison Jermain, Wade Winkler

Background/Introduction

Difficidin is a naturally occurring extracellular antibiotic produced by *Bacillus velezensis*. The *dfn* operon encodes for the difficidin biosynthesis proteins. This operon (*dfn*) is transcriptionally regulated by intrinsic transcription termination sites that are spread throughout the 70 kilobase operon and that limit *dfn* expression. LoaP is a specialized transcription elongation protein that promotes readthrough of these terminators. We hypothesize that LoaP associates with RNA polymerase to promote readthrough of the termination sites; however, the LoaP regulatory mechanism is still unknown. To better study the LoaP Mechanism of action, a direct readout of difficidin production is needed. We have developed a mass spectrometry based assay to quantify levels of difficidin.



Figure 1 - Schematic of the *dfn* operon

Methods

Three strains - wild-type, Δ *loaP*, and Δ *dfn* - were utilized to test native levels, low levels, and absence of difficidin, respectively. Strains were grown overnight to an OD₆₀₀ of ~2.0 in 150 mL of rich medium (LB broth). Cells were pelleted and extraction of difficidin was performed by incubation of the supernatant at 4°C overnight with Amberlite XAD-16 resin. Metabolites were then eluted from the resin using 8 mL of methanol. Samples were dried down and run on a reverse phase chromatography system in tandem with high resolution mass spectrometry. Data was analyzed by Compound Discoverer and Skyline.

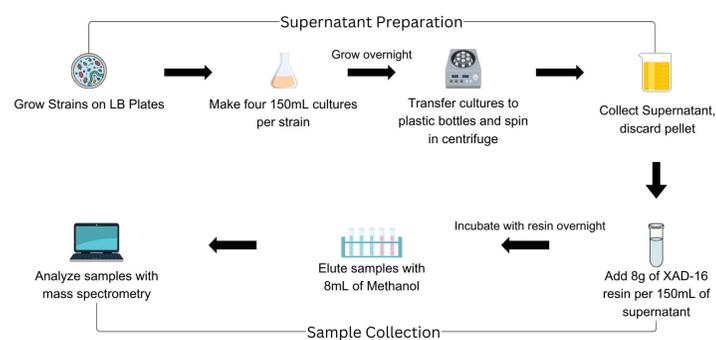


Figure 2 - Workflow of the extraction protocol

Difficidin Identification Utilizing Untargeted Mass Spectrometry

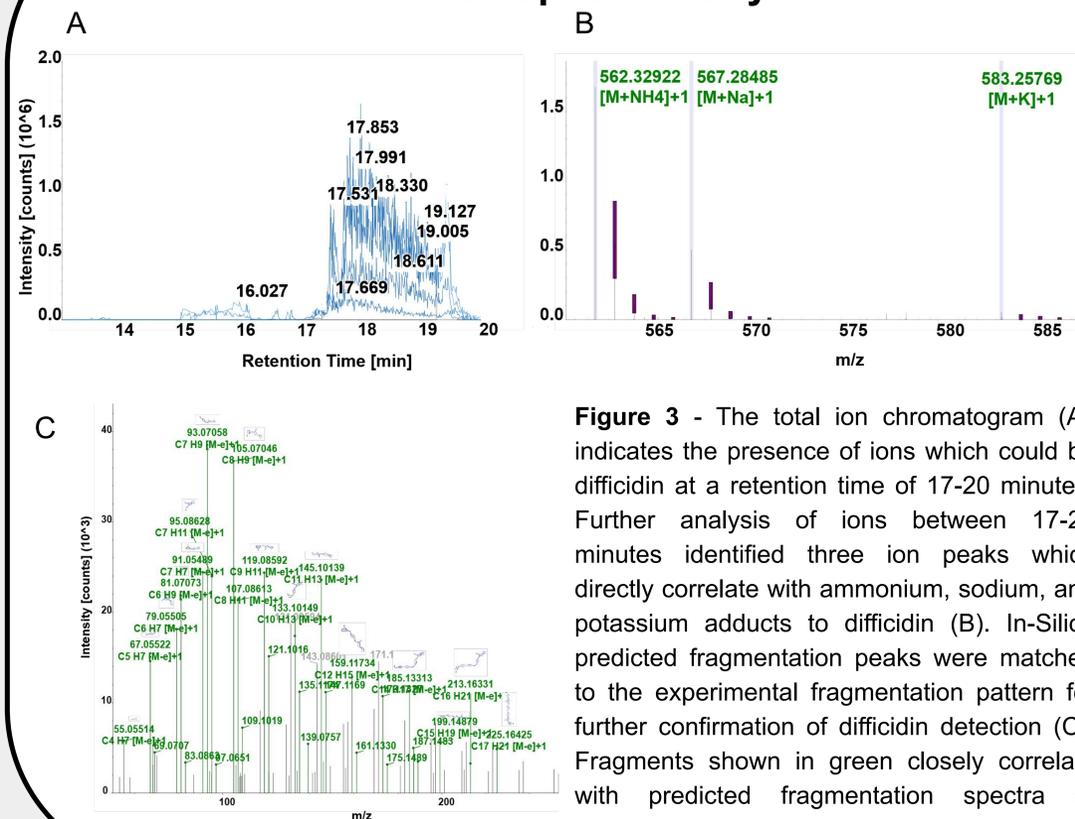


Figure 3 - The total ion chromatogram (A) indicates the presence of ions which could be difficidin at a retention time of 17-20 minutes. Further analysis of ions between 17-20 minutes identified three ion peaks which directly correlate with ammonium, sodium, and potassium adducts to difficidin (B). In-Silico predicted fragmentation peaks were matched to the experimental fragmentation pattern for further confirmation of difficidin detection (C). Fragments shown in green closely correlate with predicted fragmentation spectra of difficidin.

Conclusions & Future Directions

This mass spectrometry based assay has provided the framework to continue studying difficidin and, by association, the LoaP mechanism of action. By using the methods described in this experiment, we present the ability to study point mutations on LoaP to identify residues important to its activity and potential interaction partners utilizing difficidin as a readout. Future testing involves stains containing specific point mutations *LoaP* knockout strains and *LoaP* complementation strains.

References

- Goodson JR, Klupt S, Zhang C, Straight P, Winkler WC. LoaP is a broadly conserved antiterminator protein that regulates antibiotic gene clusters in *Bacillus amyloliquefaciens*
- Adams KJ, Pratt B, Bose N, et al. Skyline for Small Molecules: A Unifying Software Package for Quantitative Metabolomics.
- Wu L, Wu H, Chen L, Yu X, Borriss R, Gao X. Difficidin and bacilysin from *Bacillus amyloliquefaciens* FZB42 have antibacterial activity against *Xanthomonas oryzae* rice pathogens.

Method Optimization and Quantification in Wild-Type and *dfn* knockout

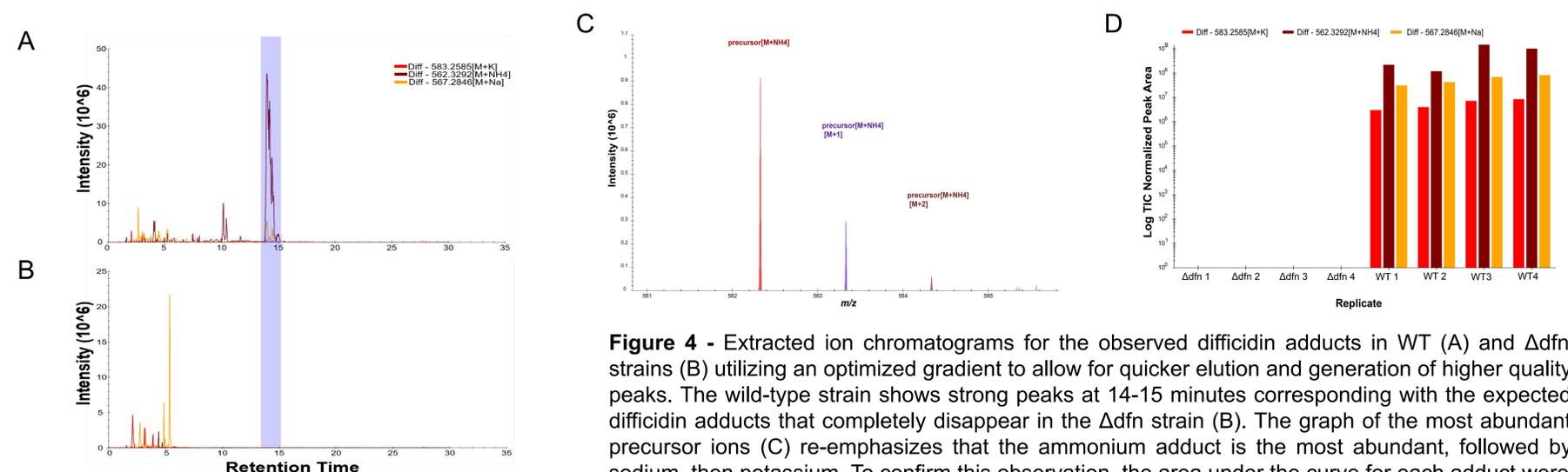


Figure 4 - Extracted ion chromatograms for the observed difficidin adducts in WT (A) and Δ *dfn* strains (B) utilizing an optimized gradient to allow for quicker elution and generation of higher quality peaks. The wild-type strain shows strong peaks at 14-15 minutes corresponding with the expected difficidin adducts that completely disappear in the Δ *dfn* strain (B). The graph of the most abundant precursor ions (C) re-emphasizes that the ammonium adduct is the most abundant, followed by sodium, then potassium. To confirm this observation, the area under the curve for each adduct were calculated (D) showing the quantification of these peaks utilizing biological replicates