Quantitative Metabolomic Analysis of *Acinetobacter baumannii* Biofilms

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ABSTRACT

With the advent of powerful new molecular profiling tools and ever growing rates of microbial infection, there is intense interest in understanding biofilm metabolism. Biofilms are incredibly robust microbial communities that rival some multicellular organisms in complexity, resiliency, and adaptation. Limited antibiotic penetration, the presence of persister cells, and increased horizontal gene transfer contribute to the near 1000-fold increase in antibiotic resistance seen in biofilms. The metabolic changes contributing to biofilm resilience have yet to be fully understood. An ongoing project here at MSU seeks to resensitize biofilms to antibiotics by characterizing and comparing the transcriptomes and metabolomes of three bacterial species in three modes of living; planktonic, biofilm, and biofilm treated with antibiotic. **This work focuses specifically on the uptake of amino acids, phosphate, and central carbon metabolites from the growth media via gas chromatography-mass spectrometry (GC-MS).** One notable aspect of the project seeks to provide data for the Department of Defense's biofilm computer models. However, this project's true scope is much broader in that it will serve to elucidate the dramatic differences in metabolic regulation seen in biofilms and bring about novel methods to resensitize biofilms to antibiotics.

INTRODUCTION

Acinetobacter baumannii is a coccobacillus Gram negative bacteria that became a major concern in the US when soldiers returned from the Iraq War carrying the microbe. Playing a prominent role in nosocomial infections in both military and civilian hospitals, multidrug resistant *A. baumannii* is a major concern for health care providers.

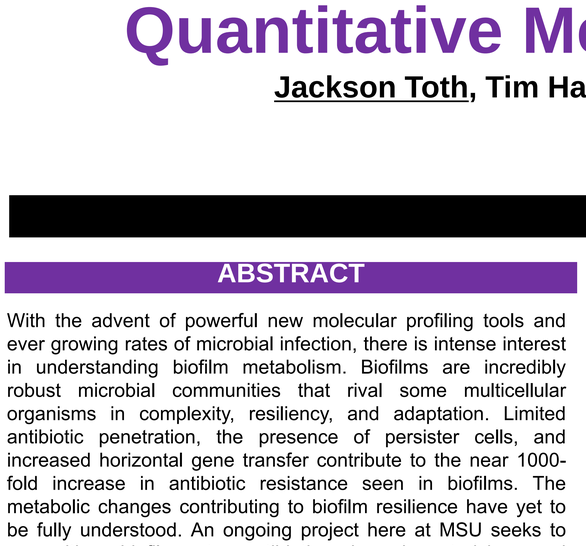

Figure 1. a) Glass slide with *A. baumannii* biofilm growing on it. b) Scanning electron micrograph of the coccobacillus *A. baumannii* on a filter. c) Diagram of drip flow reactor used to grow the biofilms. Media was dripped on the top of the slide at 1 mL/hour and allowed to travel down the slide. The effluent was collected, filtered, and then subjected to GC-MS analysis.

A. baumannii is a potent biofilm former. *A. baumannii* biofilms were cultured in a drip flow reactor (**Figure 1c**). A media was developed to mimic the exudate from a chronic wound. This media was run over the biofilms and then collected. Media doped with tigecycline was also used.

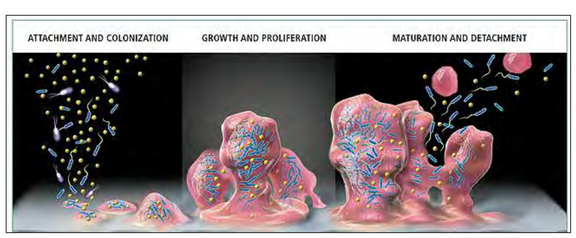

Figure 2. Biofilm formation is a complex process.

METHODS

Samples must be derivatized prior to GC-MS analysis

- 100 µL of effluent was dried down under N_2
- Excess ACN and silylating reagent N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) was added to the neat samples
- Vials were heated at 50˚C for 30 minutes

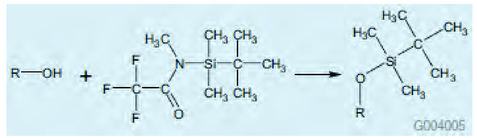

Figure 3. Reaction diagram of MTBSTFA. Derivatization caps polar moieties (mostly hydroxyls and amines), thus decreasing intermolecular interactions and increasing volatility for GC analysis.

- GCMS Method parameters
  - Columm- Zebron 5MS 30m x 250 µm x 0.25 µm
  - Flow- 1.5 mL/min
  - Carrier gas- He, constant flow
  - Inlet temp- 325 ˚C
  - Detector temp- 325 ˚C
  - Oven- 60 ˚C for 2 min, 20 ˚C/min to 120 ˚C. Then 6 ˚C/min to155 ˚C. Then 14.5 ˚C/min to 300 ˚C and hold for 10 min (30.83 min total run time)
  - Injection volume- 1 µL

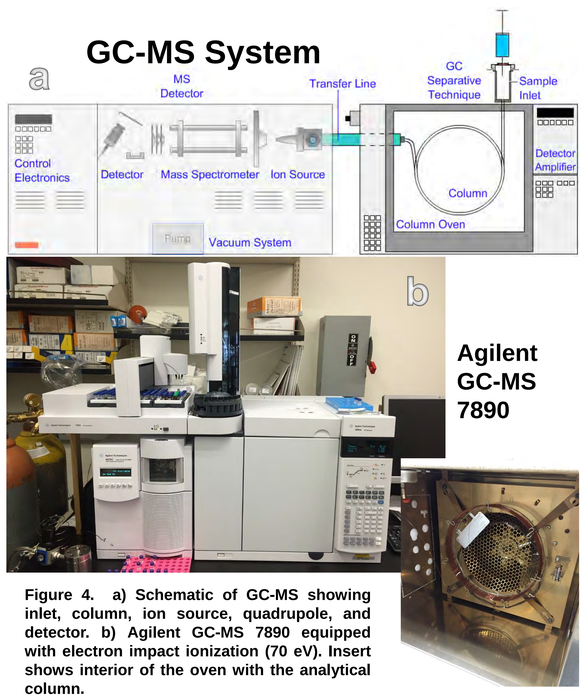

Figure 4. a) Schematic of GC-MS showing inlet, column, ion source, quadrupole, and detector. b) Agilent GC-MS 7890 equipped with electron impact ionization (70 eV). Insert shows interior of the oven with the analytical column.

- After the total ion chromatogram (TIC) was acquired, peaks were identified using the NIST database and retention times were recorded
- Unique m/z's were determined for each metabolite
- Extracted ion chromatograms (EIC) were then performed for each metabolite and peaks were manually integrated with Qualitative Analysis software
- Dilutions (0.25x, 0.5x, and 1x) of the media were used to create standard curves and lines of best fit (**Figure 5)**
- Using the lines of best fit, the final concentration of each metabolite in the effluent was calculated

DISCUSSION/RESULTS

The metabolic differences between planktonic, biofilm, and biofilm treated with antibiotic are studied here. The results show specific metabolites have statistically significant fold changes. Because the media contained relatively small amounts of carbon energy sources, it follows logically that no positive fold changes were observed for the amino acids, phosphate, and lactate. Comparison of the three experimental groups shows promising differences in metabolite uptake between the life stages and possible areas for targeting.

Seventeen amino acids, lactate, and phosphate were detected in the effluent after derivatization. Succinate, acetic acid, and formic acid were also detected in the effluent but have yet to be quantified. The retention time (RT) is listed for each metabolite along with the unique m/z that was used to generate the EIC that was used for quantification. The original concentration of each metabolite in the media is listed as 1x. Biofilm and planktonic groups were run with five biological replicates, Tigecycline treated biofilms were run with four biological replicates. Averages of these replicates are reported below **(Table 1**).

Table 1. Final concentrations of the observed metabolites.

ID	RT	Unique m/z	[1x] (mg/L)	Biofilm Conc (mg/L)	Planktonic Conc (mg/L)	Treated Conc (mg/L)
Lactic Acid	9.44	261	1500	542.9766	543.0749	543.0749
Ala	9.76	158	70	19.65495	19.65944	19.65944
Gly	9.91	218	48.1	13.11718	13.13075	13.13077
Val	10.45	186	58.8	17.51222	17.51422	17.51422
Leu	10.67	200	73.6	20.27551	20.2774	20.2774
Ile	10.85	200	36.9	10.44148	10.44263	10.44263
Pro	11.12	184	167	50.72829	50.74007	50.74007
PO4	11.89	383	180	28.5754	28.67459	28.67493
Met	12.12	292	11.5	3.348533	3.350063	3.350064
Ser	12.17	362	33.3	3.323337	3.326277	3.326279
Thr	12.33	303	34.7	7.362325	7.394025	7.394162
Phe	12.71	336	46.3	12.75484	12.77209	12.77211
Asp	12.94	418	60	22.30298	29.00399	31.01733
Glu	13.46	432	67.7	10.73872	10.73877	10.73877
Lys	13.97	300	92.3	29.35971	29.47141	29.47183
Gln	14.19	431	86.1	14.14572	14.16634	14.16637
His	15.11	196	25.9	5.844021	5.847224	5.847225
Tyr	15.36	466	56.4	1.874223	1.875864	1.875866
Trp	15.8	375	13.1	4.301588	4.310359	4.310376

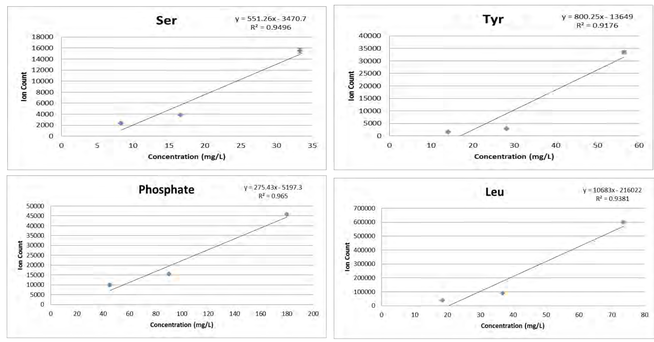

Figure 5. Select standard curves for serine, tyrosine, phosphate, and leucine. Dilutions of 0.25x, 0.5x, and 1x of the original media concentration were used. Linear lines of best fit were generated and used to calculate final concentration of each metabolite.

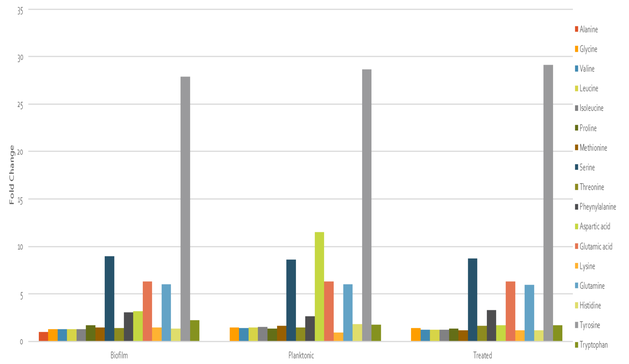

Figure 6. Bar graph of the fold change for each amino acid between the three experimental groups. Most metabolites exhibited fold changes below 2. However, serine, aspartic acid, glutamic acid, glutamine, and tyrosine exhibit significant fold changes (>5). Aspartic acid in the planktonic state shows a much higher fold change than the two biofilm states, suggesting a possible target for antibiotic resensitization.

CONCLUSIONS

- Several amino acids exhibited statistically significant fold changes and possible targets for resensitization to antibiotics
- A reliable method for the detection of 23 metabolites was developed
- Data was collected for the DOD biofilm flux models

FUTURE WORK

- Perform the same metabolomics analysis with *Staphylococcus aureus* biofilms
- Quantify central carbon metabolites
- Expand the scope of analysis to include fatty acids

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