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Exploring Bacterial Metabolome Alterations Mediated by Genetic Mutations

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ABSTRACT

Purpose: Demonstrating how high-resolution mass spectrometry performed on a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer can provide insight into how genetic mutations perturb cells' metabolome and lipidome.

Methods: Small molecules and lipids were extracted from seven different mutant strains of *E. coli* that altered enzyme expression related to the TCA cycle.Data were analyzed using Thermo Scientific Compound Discover 3.1 and Lipid Search 4.2.

Results: AcnB and acnA mutant strains showed the most unique metabolic profiles of all mutants tested, with no indications of oxidative stress. Lipid profiles showed a doubling in amounts of total PG for the acnB mutants relative to WT, possibly resulting from membrane imbalance

Figure 1. The Orbitrap Exploris 480 high resolution mass spectrometer used to acquire the metabolite and lipid data for this study.

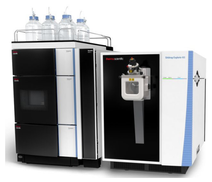
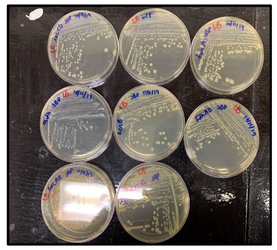


Figure 2. Culture plates of E. Coli



INTRODUCTION

Escherichia Coli (*E. coli*) is a well-studied model organism, which can easily be mutated at the gene level to create robust and reproducible biological variants. In this study, seven single gene mutations were made within the central carbon metabolism. Each mutant strain must alter its metabolic processes in a unique manner to survive and replicate. Since the mutations occur in the same biochemical pathway, many of the same compounds will be present but at amounts that span multiple orders of magnitudes, in addition to the presence or absence of other distantly related compounds.

MATERIALS AND METHODS

Sample Preparation Details

E. Coli MG1655 was used as wild type (WT) for the experiment and mutants were generated via mini λ recombination with a kanamycin cassette to generate the 7 mutant strains, each one knocking out a specific enzyme in the tricarboxylic acid (TCA) cycle. Cells were collected during exponential growth phase (OD ~0.4) for metabolite and lipid analyses. All strains were collected in replicates of five onto 0.4 µm polycarbonate filter disks, along with media, extraction and solvent blanks. Metabolite extraction used an acidic acetonitrile extraction solvent (40% acetonitrile, 40% methanol, and 20% water with 0.1M formic acid.[1] Supernatant was dried under nitrogen and resuspended in 300 µL of 90:10 water: methanol spiked with phenylalanine-d8 as internal standard at a final concentration of 100 nM.

Lipid extraction was optimized for the recovery of phospholipids using a mixture of ethanol, water, diethyl ether, pyridine and ammonium hydroxide.[2] Supernatant was dried under nitrogen, then desalted using water-saturated butanol and dried again. Final suspension was made using 300µL of 50:50 IPA: ACN spiked with Avanti SPLASH® LIPIDOMIX® as an internal standard at 1:1000 dilution from the purchased standard.

Liquid Chromatography Details

A Thermo Scientific™ Vanquish™ Horizon UHPLC coupled to a Thermo Scientific™ Exploris 480™ mass spectrometer was used for all sample analyses. For metabolomics analysis, two microliters of sample were loaded onto a Thermo Scientific™ Hypersil GOLD™ column (150 mm x 2.1 mm 1.9µm). Chromatographic separation used 0.1% formic acid in water (solvent A), and 0.1% formic acid in methanol (solvent B) at a flow rate of 450 µL/min and a column temperature of 55 ºC. Elution profile was a follows: 0 min 0.5%B, 5.5 min 50%B, 6.0 min 98%B, 12 min 98%B, 12 min 0.5%B, 15 min 0.5%B.

For lipidomics analysis, two microliters of sample were loaded onto a Thermo Scientific™ Accucore™ C18+ column (150 mm x2.1 mm 1.5 µm). Chromatographic separation used 60:40 ACN: water + 10 mM ammonium formate (solvent A) and 90:10 IPA: ACN + 10 mM ammonium formate (solvent B) at a flow rate of 260 µL/min and a column temperature of 45 ºC. Elution profile was as follows: 0 min 10%B, 1 min 10%B, 2 min 30%B, 3.5 min 50%B, 7 min 60%B, 17 min 70%B, 18 min 80%B, 20 min 95%B, 22 min 100%B, 28 min 100%B, 28 min 10%B, 32 min 10%B

Mass Spectrometry Details

Metabolomic samples were run once per ionization mode in full scan, with fragmentation data obtained using data-dependent MS2 mode on pooled samples for each mutant strain. Global pooled samples were injected every 10 samples for QC based area normalization. Lipidomic analyses were run in full scan data-dependent MS2 once per ionization mode for all samples.

Data Analysis

Metabolomic data files were analyzed using Thermo Scientific™ Compound Discoverer™ 3.1 using the untargeted metabolomics workflow, and lipidomic data files were analyzed using LipidSearch 4.2™ software.

RESULTS

Metabolomics

The calculated CV for the internal standard was 3.05%.Normalization within Compound Discoverer was performed by two distinct processes: first, all compound areas were normalized using a pooled QC sample injected every 10 injections to reduce the influence of compound degradation and fluctuation across the sequence; secondly, areas of compounds were scaled to optical densities to minimize biological variation due to cell quantities.

Detected compounds were filtered based intensity (<1E6), above background levels (>5x), and having usable QC values. This resulted in 1479 compounds- 639 in positive mode, and 840 in negative mode being considered in analyses. Metabolites present in the TCA pathway were identified by matching data to the mzCloud™ spectral library.

Figure 3. Trend line chart comparing glutathione and glutathione disulfide across mutant strains

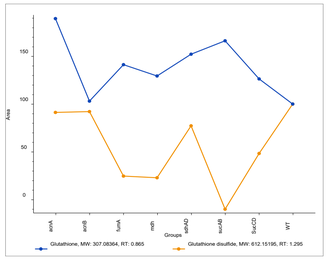


Figure 4. Principle Component Analysis (PCA) for compounds in negative mode

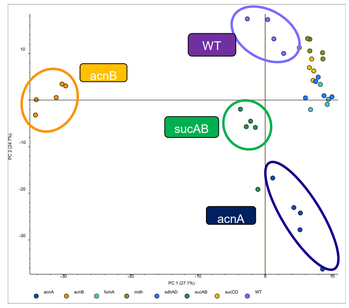
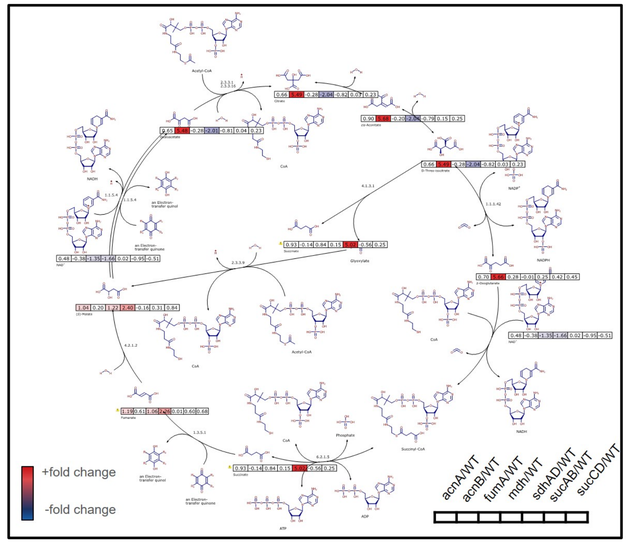


Figure 5. Metabolika pathway of TCA cycle with overlaid log2 fold change for the mutant strains vs. WT (8 of 9 major compounds in the TCA cycle were detected in the study)



Lipidomics

Avanti SPLASH® LIPIDOMIX® was spiked into all samples for quantitation and quality control purposes. By plotting the area for the PG internal standard across each sample group, the reproducibility of the analysis can be measured by the CV. After performing peak detection and alignment in LipidSearch 4.2™, the major lipid classes present in the *E.coli* mutants were found to be phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). This is consistent with the known membrane composition of this bacterium. While there were several triglycerides and phosphatidylcholine lipids identified in the samples (Table 1), their origin could easily be determined to not culture dependent with the use of media blanks used as controls.

Quantitative lipid data was generated from the internal standard, which contains one labeled lipid per common compound class. Using a 1:1000 dilution and a one-point calibration curve, a response factor was calculated in LipidSearch 4.2 and automatically applied to all lipids within a class to estimate individual lipid concentrations. PG lipids are hypothesized to change most in accordance to carbon metabolism disturbances and representative species are reported in Table 2, with **increases** and decreases highlighted

Table 1. Summary of lipids detected in all strains of E.coli

Lipid Class	ChE	DG	LPC	LPE	LPG	PA	PC	PE	PG	PS	TG	Total
# detected before filtering	35	23	26	33	7	23	482	287	82	81	291	1370
# detected after filtering	1	2	9	2	7	6	1	70	35	6	95	234

Table 2. Estimated concentrations (µg/mL) of select PG lipids for all E. coli strains based on measurement of PG(15:0-18:1(d7)) internal standard and normalized to optical density.

Lipid	WT	acnA	acnB	sucAB	sucCD	sdhAD	Mdh	fumA	Media
PG(16:0_16:1)	12.19	9.39	**15.60**	9.21	6.11	9.30	8.58	8.21	*0.00*
PG(16:0_18:1)	6.17	4.24	**8.05**	4.50	3.33	4.26	4.59	3.84	*0.00*
PG(17:1_16:0)	3.57	2.28	**9.36**	2.28	1.28	2.53	1.98	1.78	*0.00*
PG(16:1_18:1)	2.81	1.61	2.52	2.21	1.83	1.90	1.72	1.39	*0.00*
PG(18:1_18:1)	1.63	0.83	1.53	1.09	0.81	0.97	1.06	0.78	*0.00*
PG(16:0_16:0)	0.90	0.70	**1.61**	0.41	0.43	0.59	0.55	0.60	*0.00*
PG(16:0_16:1)	0.62	0.09	**4.13**	**6.32**	**4.09**	**2.92**	**2.74**	**2.67**	*0.00*
PG(16:1_16:1)	0.48	0.25	0.48	0.54	0.34	0.43	0.37	0.31	*0.00*
PG(16:0_14:0)	0.48	0.38	**0.76**	0.31	0.25	0.33	0.27	0.30	*0.00*
PG(16:1_14:0)	0.38	0.24	0.39	0.28	0.23	0.29	0.22	0.22	*0.00*
Total PG	29.23	20.00	**44.44**	27.13	18.70	23.52	22.07	20.10	*0.01*

CONCLUSIONS

The Orbitrap Exploris 480 collects high resolution full scan spectra with demonstrated low intragroup variation and fast, high quality MS2 spectra at scan speeds up to 40 Hz, allowing for confident metabolite identification. By setting the cycle time to 0.7 seconds, it was possible to gather up to 12 MS2 scans per cycle at a resolution of 15k (data not shown).

The collected data show that the metabolic profiles of acnA, acnB, and sucAB mutant strains are highly unique from each other and other strains tested (Figure 4). Even though all studied mutations target the TCA cycle, only acnB mutants had a significant impact on the abundance of the metabolites in this cycle.

Lipids from six unique phospholipid groups were identified in the samples and species within each group could be quantified using a new feature of LipidSearch 4.2™ together with a lipid internal standard mixture to give estimated concentrations. It was seen that the top 10 most common PG in bacterial cells are more abundant in the acnB mutant stain, suggesting substantial metabolic diversion to lipid biosynthesis and potential cell membrane anomalies.

REFERENCES

1. Rabinowitz, J. D.; Kimball, E., Acidic Acetonitrile for Cellular Metabolome Extraction from Escherichia coli. Analytical Chemistry 2007, 79 (16), 6167-6173.

2. Guan, X. L.; Riezman, I.; Wenk, M. R.; Riezman, H., Chapter 15 - Yeast Lipid Analysis and Quantification by Mass Spectrometry. In Methods in Enzymology, Academic Press: 2010; Vol. Volume 470, pp 369-391

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TRADEMARKS/LICENSING

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