Developing an assay for tryptophan metabolites in order to determine their impact on the pathogenicity of Chlamydia trachomatis

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Introduction

The aromatic compound indole is produced and secreted by numerous bacterial species. Secreted indole acts as a pleiotropic signaling molecule that alters gene expression, affects growth behavior (including biofilm formation), stress response pathways (including antibiotic tolerance) and virulence traits. Given its prominent role as an extracellular signaling molecule, it is not surprising that indole has emerged as an important regulator of complex microbial communities as found in the human gut and genital tract (GT). Indeed, a number of GT and/or gut-resident bacteria, including Peptostreptococcus species, Fusobacterium species, Bacteriodes species and Escherichia coli, are known indole producers.

In addition to producing and sensing indole, some bacteria are also known to consume exogenous indole. One prominent example is Chlamydia trachomati. C. trachomatis is a tryptophan auxotroph that normally acquires tryptophan from its infected host cell. During an immune response, human cells can deplete their intracellular tryptophan stores and effectively starve C. trachomatis of this essential nutrient. In order to survive the immune response, C. trachomatis has evolved to convert exogenous, micobiota-derived indole into tryptophan.

Additionally, indole may trigger the production of metabolites by members of the genital microbiota.

The goal of this study was to develop an assay for the detection of tryptophan metabolites by applying a non-targeted GC/MS approach. To achieve this we began to analyze cultures of the bacterium E. coli, a known indole producer and indole sensor. In the long run we hope to use the data gathered as part of this study to develop an additional method to detect and quantify indole and other tryptophan metabolites in clinical GT samples and to determine whether or not the relative concentration of any of these compounds correlates with disease outcomes of C. trachomatis infections.

We showed that gas chromatography-mass spectrometry (GC/MS) is a robust platform for rapid profiling of multiple classes of small metabolites in diverse biological matrices. We used a well established oximation-silylation protocol that renders small, polar metabolites sufficiently volatile for GC/MS instrumentation.

Experimental

Sample Preparation Procedure

Fresh cultures of Luria-Bertani were inoculated with 1/200 aliquots of E. coli stock cultures TM1061 and MC1061. These strains are isogenic except that TM1061 is unable to convert tryptophan into indole. The cultures were grown at 37°C with aerolisation in the presence or absence of 100 µg ampicillin/ mL. Aliquots were taken at five and nine hours which correlates to late log and mid stationary timepoints, respectively. The aliquots were diluted in water and sonicated for 20 seconds to lyse the cells. The proteins were precipitated with 10 parts methanol. The samples were then centrifuged twice at 15K g for 10 min at 4°C. The supernatants were dried on a SpeedVac concentrator. The carbonyl groups were first protected by methoximation. The compounds were then derivatized by MSTFA + 1 % TMCS in accordance with the protocol developed by Palazoglu M. and Fiehn O., (Agilent Application Note 5990-3638en),

Analytical Methodology

An Agilent 6890 / 5975 GC/MS was equipped with a large-volume, independently heated ProSep inlet. This inlet was used because of its ability to selectively vent the derivatizing agent out the vent port. Two 15-m DB-5ms (0.25mm id x0.25um) columns joined by a Purged Ultimate Union were used for the separation. The purged union permitted back flushing of the first column to eliminate the very close eluting high boilers while the analytes of interest continued on the second analytical column.

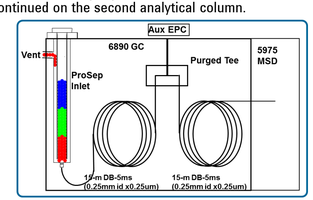

Figure 1: GC/MS System Configuration.

The ProSep program events:

At injection, the split flow is 25-mL/min while the ProSep is at 80°C. This is done to vent most of the derivatizing agents. At 0.25-min the split vent is closed and analytes are directed to the analytical column while the ProSep is rapidly ramped (100°C/min) to 280°C and held for 5 min. During this time, at 4 minutes, the split vent is again opened and additional flow is added (250-mL/min total in ProSep split mode) to flush out the inlet. At the end of the run during backflushing the temperature is raised to 325°C to flush matrix out the split vent.

Experimental and Results

GC temperature program:

At injection hold the oven at 60° for 1 min. Then heat it to 325°C at 10°C/min and hold at that temperature for 3.5 min. There is a 6.5 min backflush at 325°C to bring the total run time to 31 minutes.

GC flow program:

A constant flow of 1.2mL/min of helium is used through both columns until the end of the run at 31 minutes when the first 15m-column pressure is dropped so that the flow is reversed. During Post-Run backflushing the front column flow is slightly under -4.3 mL/ min and the rear column flow is +4.3 mL/min.

The samples were injected with a 1:10 split.

Metabolite identification is aided by the use of a retention time locked (RTL) metabolite library (Kind T et al., Anal Chem 81(2009):10038), to which we have added many analytes of interest to our group.

Chromatographic Peak Deconvolution

A GC/MS scan method can benefit from chromatographic deconvolution to identify unique compounds in complex matrices. The software goes through four steps:

1. **Noise Analysis** calculates a noise factor for each ion chromatogram.
2. **Perceive Compounds** uses the noise analysis results along with retention time to determine a model peak shape for each chromatographic component.
3. **Spectral Deconvolution** takes a least-squares approach to creating a spectrum for each component.
4. **Compound Identification** compares each of these components against an EI spectral library.

One of the benefits of chromatographic deconvolution is that it is an unbiased approach that does not take targeted ions into consideration. The spectral de-skewing and the peak shape information determined during compound perception allows for the identification of components that have similar peak shapes and apexes in adjacent scans.

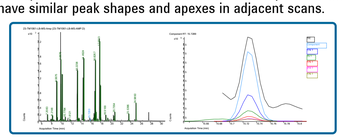

Figure 2: Peak deconvolution helps identify compounds near their detection limit in complex matrices. In this case we see the Total Ion Chromatogram with a small peak found at 15.723 min on the left and to the right we see the de-convoluted ions. It is putrescine with a match factor of 89.

Mass Profiler Professional Statistical Evaluation

Mass Profiler Professional (MPP) was used for statistical evaluation of known metabolites. In this interpretation we are comparing Ampicillin treated wild type to knock-out E. coli strains. The steps are as follows:

1. Define the experiment type, workflow, and organism.
2. Select the data source as MassHunter Qual.
3. Import CEF files from MassHunter Qual.
4. Set abundance and model ion filters.
5. Define retention and match factor alignment parameters. (Total: 89 identified of 296)
6. Select internal, external, or no calibration.
7. Choose baseline correction.
8. Set condition filter flags. (Total Compounds: 62)
9. Filter based on fold change with a threshold of 2 and significance p < 0.05. (Total Compounds: 5)

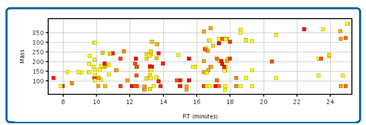

Figure 3: Mass vs. retention time plot after peak alignment. Entities designated with a red dot are low frequency and the yellow ones show up in all samples. Typically most of the entities are low frequency and are easily removed by setting appropriate condition filters. In this case we are only evaluating known metabolites and have a high percentage of high-frequency metabolites.

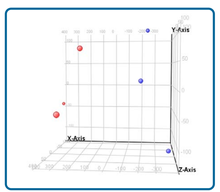

Figure 4: Principal Component Analysis (PCA) after peak alignment. Blue dots represent Ampicillin treated wild type E. coli strains and blue dots represent knock-out E. coli strains. This exploratory step reveals trends and outliers.

Results and Discussion

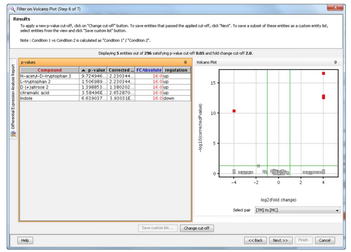

Figure 5: The Volcano Plot shows fold-change for each entity on the x-axis and significance on the y-axis. In this case the null hypothesis is that treatment with Ampicillin has no effect on E. coli metabolism. We can reject the null hypothesis since the p value is less than 0.05 (or 5 %). The four metabolites in the upper right hand corner are up regulated in the Ampicillin treated wild type E. coli strains. Indole, as expected, is down regulated.

E. coli samples were extracted and profiled by GC-MS. Two E. coli strains were employed. Strain TM1061 is isogenic to MC1061 except that it carries a loss-of-function mutation (a transposon insertion) in the gene tnaA which encodes tryptophanase, an enzyme that converts tryptophan into indole. We evaluated the metabolomic differences between MC1061 and TM1061 samples by multi-variant software. Unsupervised PCA (Principal Component Analysis) of the transformed data shows differences in their metabolic profiles with complete separation of the tnaA-deficient and wild type E. coli strains on the score map. T-test analysis reveals that tryptophan metabolites are significantly increased ($p<0.05$) in the wild type bacteria compared to the tnaA-deficient bacteria.

- Tryptophan has been consumed in all the MC1061 strain samples, but not the TM1061 strain samples. Although both strains make tryptophan and incorporate it into proteins, the import of exogenous tryptophan is dependent on tnaB - a tryptophan transporter that is in the same operon as the tnaA, the gene encoding tryptophanase. The TM1061 strain is deficient for the entire tna operon, so it lacks both tnaA and tnaB and therefore can't import tryptophan. As a result exogenous tryptophan will not be consumed by the TM1061 strain.
- The induction of indole production is only seen in the Ampicillin-treated MC1061 strain but not the Ampicillin treated TM1061 strain (tnaA/B negative) - as expected. This is expected, because Amphicillin is known to substantially increase the low steady state expression of tnaA expression and because TM1061 lacks a functional tnaA gene.
- Indole can enter both MC1061 and TM1061 cells. The expression of tna operon is positively regulated by indole. In both wild type bacteria (MC1061) and tna-deficient bacteria (TM1061) exogenous indole enters the bacterial cell and is consumed by the microbe. In MC1061 but not in TM1061 strains, indole (like Ampicillin) induces tna expression. The induction becomes detectable in a bioassay at around 0.25mM and is really robust at 1mM. Therefore 1mM exogenous indole in the wild type strain induces robust tna expression and these bacteria begin to make their own indole. 50 µM is not enough indole to induce tna expression, and therefore the MC1061 strain simply acquires indole and consumes it under these conditions. In the TM1061 strain, of course, neither low nor high indole concentration will induce expression of the tna operon. So all the exogenous indole will be consumed and none produced.

Conclusions

We were able to develop an assay for the detection of tryptophan metabolites by applying a non-targeted GC/MS approach using E. coli bacterium as a Chlamydia trachomatis analog. We will use this data to develop a method to quantify tryptophan metabolites in clinical GT samples.

We also demonstrated that software tools such as chromatographic peak deconvolution and MPP help simplify statistical analysis of metabolomic data sets.

Acknowledgements

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