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Metabolomic analysis of *Campylobacter jejuni* by direct injection electrospray ionisation mass spectrometry

Robert M. Howlett, Matthew P. Davey and David J. Kelly

Summary

Direct injection mass spectrometry (DIMS) is a means of rapidly obtaining metabolomic phenotype data in both prokaryotes and eukaryotes. Given our generally poor understanding of *Campylobacter* metabolism, the high- throughput and relatively simple sample preparation of DIMS has made this an attractive technique for metabolism-related studies and hypothesis generation, especially when attempting to analyse metabolic mutants with no clear phenotype. Here we describe a metabolomic fingerprinting approach with sampling and extraction methodologies optimised for direct-injection electrospray-ionisation mass spectrometry (ESI-MS), which we have used as a means of comparing wild-type and isogenic mutant strains of *C. jejuni* with various metabolic blocks.

Key Words

Electrospray ionisation mass spectrometry, Metabolome, Metabolomics, Direct injection, DIMS, Metabolite extraction, Fingerprinting.

1. Introduction

Metabolomics is an approach to understand cellular metabolic pathways, either at the level of individual pathways or a specific set of metabolites (targeted approach) or through an overall assessment of a cell's global metabolite content (non-targeted approach). While methods aimed at identifying specific metabolites and analysing their cellular concentrations are not new, it has only recently been possible to analyse and process large numbers of metabolites simultaneously using high resolution nuclear magnetic resonance (NMR) or mass spectrometry (MS) based techniques. This has enabled methods to be developed for the preliminary analysis of 'silent mutations' in genes involved in central and non-central metabolic pathways, by comparing the global metabolite profiles of wild type versus mutated cells *(1)*.

Dunn *et al*. *(2)* noted the many techniques utilised for sampling the metabolome and at present no single technology dominates; each has its own biased metabolite detection and strengths for certain applications. Electrospray ionisation time of flight mass spectrometry (ESI-TOF-MS) is a technique that has been used for high throughput fingerprinting in both plants and bacteria *(1, 2)*. Although ESI-TOF-MS based metabolomics can suffer from a lack of mass accuracy its high sensitivity and high throughput make it an ideal candidate for establishing a strategy for the initial analysis of a large number of cellular metabolites. Similar methodologies were originally utilised for high-throughput clinical screening, however there have now been several microbial applications of this technique. Initially used in bacterial strain discrimination *(3, 4)*, Kaderbhai *et al*. *(5)* showed that direct injection ESI-TOF-MS techniques were capable of discriminating between tryptophan metabolism mutants in *E. coli.* Our recent work has shown that

putative metabolite allocation to ESI-TOF-MS outputs can enable visualisation of metabolic blocks in *C. jejuni* amino-acid metabolism mutants *(1)*. ESI-TOF-MS techniques are advantageous over other techniques such as NMR when attempting a non- targeted analysis, due to their far higher sensitivity; however the absence of a chromatographic step makes unambiguous metabolite identification impossible. Li *et al. (6)*, has since built on the DIMS fingerprinting techniques in *(1)* by separating and identifying the key metabolites of antibiotic resistant mutants of *C. jejuni* using UHPLC-MS. For this reason it is important that DIMS techniques are used as a 'first step' for hypothesis formation towards the function of putative metabolic genes before rigorous metabolite identification or phenotypic analysis is performed. The potential for analysing altered metabolic fluxes as organisms experience different conditions, such as changes to temperature or growth phase, also exists *(7)*.

Quenching of metabolism and metabolite extraction are areas of intense debate and concern in metabolomics, as many metabolite pools turn-over on a time scale of the same order (or shorter) as the time taken to quench metabolic reactions. When using the adenylate energy charge parameter (ECP) as an indicator of the maintenance of adenine nucleotide levels during quenching, some techniques have been shown to be too slow, as well as resulting in leakage of many cellular metabolites *(8)*. ESI-TOF-MS direct injection methods enable a quenching methodology to be employed that results in no metabolite loss, through the freezing of whole cell pellets in liquid nitrogen (-196°C). However in the case of bacterial cells like *C. jejuni* this does require a short centrifugation step *(1)*. Although some metabolic changes will inevitably occur during such processing, for the particular approach we describe here, where wild-type and mutant strains are

subjected to the same quenching and extraction conditions, valid comparisons are possible. The efficacy of metabolite extraction techniques have been shown to be organism specific *(8)*, but many comparative studies have shown cold methanol based methodologies to be the most efficient for extracting the broadest range of metabolites *(8, 9, 10)*. For this reason a simple cold methanol based extraction protocol was adopted in our work, enabling high reproducibility and extraction of a broad range of metabolites *(1, 11)*. Below, we provide details of the methods we have used to obtain metabolite profiles from *C. jejuni* strains using DIMS, based on our recent publication *(1)*.

2. Materials

2.1 Growth media

1. Columbia agar containing 5 % (v/v) lysed horse blood and 10 μg ml^{-1} amphotericin B and vancomycin (CA-AV) for sub-culturing of strains.
2. For liquid growth of isogenic mutants use Brain Heart Infusion with 5 % (v/v) foetal calf serum (BHI-FCS). No selective antibiotics are used so as not to affect metabolism **(***see* **NOTE 1).**

2.2 Metabolite Extraction

1. 2 mL polypropylene tubes or other solvent resistant type
2. Liquid nitrogen
3. HPLC grade methanol:chloroform 1:1 (v/v). **(***see* **NOTE 2)**
4. Stainless steel ball bearings (5 mm diameter). **(***see* **NOTE 3)**
5. Ultra-high purity (UHP) water.

2.3 Equipment

1. A MACS cabinet (Don Whitley Scientific, Shipley, UK) or other micro- aerobic incubator, to maintain micro-aerobic growth conditions [we routinely use 10 % (v/v) O_2, 5 % (v/v) CO_2 and 85 % (v/v) N_2].
2. A temperature controlled microcentrifuge.
3. A cell disruptor/homogenizer.
4. A Liquid chromatography (LC) electrospray ionization (EI) time-of-flight (TOF) spectrometer (such as a LCT Spectrometer, Waters Ltd, Manchester, UK) with an automated Waters 2695 Separations Module combining a high performance liquid chromatography (HPLC) pump and autosampler (Waters, Hemel Hempstead, UK) with a Lockspray™ interface.

3. Methods

Throughout all stages it is important to record as much metadata as possible regarding differences in sample preparation (*i.e* different days of extraction, different media batches) as this will be used in the final multifactorial analysis to look for the influence of methodological variables. The effects of this can be minimised by ensuring as much randomising of processing where possible.

3.1 Preparation and sampling of wild-type parent and isogenic mutant strain cultures.

Assume all growth conditions are at 37°C and under micro-aerobic conditions unless otherwise stated. Good aseptic technique must be maintained while working with cultures.

1. Maintain a culture streaked to single colonies on CA-AV plus selective

antibiotics. Use a colony to inoculate a 5 mL BHI-FCS starter culture in 25 mL conical flasks and grow for 16 hours (until stationary phase), shaking at 180 rpm. Ensure a minimum of 5 replicates for each strain to be analysed.

2. Spin down starter cultures (6,000 x *g*, room temperature ~22°C) and re-suspend in 1 mL BHI-FCS. Use to inoculate 25 mL cultures of BHI-FCS in 250 mL conical flasks to a starting OD_{600} of 0.1.
3. Grow cultures under micro-aerobic conditions with adequate shaking until they are at the required growth phase for sampling (*eg*, mid log phase or stationary). This can be checked by taking the OD_{600} of the culture and comparing to a known growth curve for the strain being sampled **(***see* **NOTE 4).**
4. Using the OD_{600}, calculate the volume required to take a standardised cell density for sampling. For example, sample the equivalent of 1 mL at OD_{600} 1.00 for all samples (in this instance a sample measuring OD_{600} 1.12 would require 893 µL for sampling).
5. Take the calculated volume and quickly place in a 2 mL polypropylene microfuge tube. Spin down the sample in a bench microfuge (14,000 x *g*, 1 minute, room temperature) and quickly aspirate off all of the liquid media before flash freezing the pellet in liquid nitrogen. During this stage it is important to be set up so processing time is minimised as much as possible **(***see* **NOTE 5)**.
6. Pellets can and must be stored at -80 °C prior to metabolite extraction.

3.2 Metabolite extraction

Unless otherwise stated samples, tubes and solvents are kept on ice at all times.

1. Remove samples from the -80°C freezer and place the samples in an ice-

bucket, open lids and quickly add one pre-chilled 5 mm stainless steel ball-bearing **(***see* **NOTE 3)**.

2. Add 1 mL of ice-cold HPLC Grade methanol:chloroform 1:1 (v/v) (stored at -20°C overnight prior to extraction) to each tube.
3. Briefly vortex each tube to homogenise the pellet.
4. Place tubes at -80°C for 1 hour.
5. Remove the samples from the -80°C freezer and place into a cell disruptor at 4°C for 1 minute.
6. Return samples to -80 °C for 1 hour.
7. Remove the samples from -80°C and briefly vortex.
8. Transfer the sample, minus the steel ball bearing, to a new pre-cooled 2 mL polypropylene tube.
9. Add 400 μL of ice-cold UHP water to the sample and briefly vortex before centrifugation (4,000 x *g*, 1 minute, 4°C).
10. Transfer the upper aqueous phase to a new 1.5 mL polypropylene tube.
11. Repeat step 9 before removing the upper aqueous phase and combining with the previous extraction.
12. Store both the lower non-polar (chloroform) phase and upper polar (methanol/water aqueous) phase at -80°C for the subsequent analysis below. The analysis method below can be used on either phases.

3.3 Mass-spectrometry based quantification of metabolites

We previously performed ESI-TOF-MS on an LCT spectrometer operating with 3.6 GHz time to digital conversion under the control of a MassLynx data system (version 4) running on Windows NT on an IBM compatible PC. The mass

spectrometer was operated at a resolution of 4000 (FWHM) at mass 200 *m/z* in positive and negative ion modes at a capillary voltage of 2800 V (positive) and 2500 V (negative), extraction cone at 3 V and sample cone at 20 V with a rangefinder lens voltage of 75 V chosen for detection of masses from 50–800 Da. Source temperature was 110°C and desolvation temperature was 120°C. Flow rates were 100 L/h for nebulisation and 400 L/h for desolvation **(***see* **NOTE 6** and *1, 11, 12* for further details**).**

1. Take the solvent phase from the -80°C and thaw on ice.
2. Samples may be loaded by one of two methods: Either using a syringe pump at a flow rate of 20 µL/min, or with an automated Waters 2695 Separations Module combining a HPLC pump and an autosampler with an inject volume of 100 µL at a flow rate of 50 µL /min. **(***see* **NOTE 7).**
3. A Lockspray™ interface should be used to give an external standard and allow automated correction of mass measurements (5 ng/µL sulphadimethoxine gives a lockmass of 309.0653 or 311.0814 for negative and positive ionisation modes respectively). Samples can be run in positive or negative ionisation mode as described above **(***see* **NOTE 8)**. Run three technical replicates for each sample. Spectra should be collected in centroid mode at a rate of one spectrum s^{-1} (0.95 s scan time, 0.05 s interscan delay) with 180 spectra summed over a 3-minute period before being exported from the MassLynx data system as text file peak lists (accurate mass to 4 decimal places vs. ion count) without background subtraction or smoothing.
4. Import text files into Microsoft Excel where the accurate masses of three replicate analyses of each sample can be compared.

5. The accepted range proposed to give the maximum number of peaks with minimum false positives was proposed by Overy *et al. (11)* to be defined best by plotting the acceptable mass variance as a linear function of the *m/z* values. The same equations were used in our work: positive mode, $y < 0.00003x + 0.0033$; for negative mode $y < 0.00003x + 0.044$ where y is the standard deviation of the three masses and x is the mean of the three masses. Microsoft Excel spreadsheets should be arranged to automate this process and those masses found to have a y value within acceptable limits can then have their mean accurate mass and mean response (as % total ion count in order to minimize sample-sample variation and normalize data sets) exported to a separate table. **(***see* **NOTE 9).**

3.4 Data Analysis

At this stage each sample should have a list of mean accurate mass and mean response as % total ion count. This can undergo various forms of analysis and the bioinformatics tools available are in constant development. Here is a brief description of two forms of analysis performed by Howlett *et al*. *(1).*

1. Principal Component Analysis (PCA)

PCA is a multivariate data analysis technique that can be used to simplify the complexity of the dataset, enabling visualisation of patterns between samples. In the case of analysing isogenic mutants it is used to visualise if the metabolome is altered such that it can be differentiated from the parent or wild type strain. It is important to also include metadata in any analysis to ensure strains are not separating due to processing variables, such as the day of extraction or the media batch used. Detected masses are used as observations across the strains,

treated as variables. Various commercial software packages are available to perform PCA (SPSS, IBM Corp® or SIMCA-P, Umetrics®) but an increasing number of excellent free online packages can be used such as Metaboanalyst http://www.metaboanalyst.ca/ *(13)*. These software packages will accept data produced in a similar format and enable the complexity of the data to be broken down into a series of orthogonal variables termed principal components. As well as analysing strain separation and the effects of methodological variation, these techniques can also be used to detect and remove outliers from future analysis. Scatter plots produced through PCA are also useful as a means of detecting specific mass/molecular weight bins that may be the cause of the separating samples. A large literature is available that gives an in depth description of PCA, for example *(14, 15)*.

2. Assigning of putative metabolites

Mean accurate masses can be assigned putative metabolite identities. This has previously been performed through the comparison of monoisotopic masses likely to be present in extracts (such as $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ for positive and negative ionisation modes respectively) against a list of over 1900 metabolites from an in house database compiled from KEGG (http://www.genome.jp/kegg/), Biocyc (http://biocyc.org/), Humancyc (http://humancyc.org/) to an accuracy of 0.2 Da *(1)* **(***see* **NOTE 10).** This was performed automatically using an in house produced macro in the Microsoft Excel software package (Prof Mike Burrell, The University of Sheffield, UK), however other online resources such as METLIN (https://metlin.scripps.edu/) and XCMS (https://xcmsonline.scripps.edu/) can also perform similar functions. More information on this process can be found in Davey

2011 *(16)*. Following the allocation of putative metabolites, relevant statistical analysis can be performed to highlight changes of interest. In order to reduce the number of false positive results, Benjamini-Hockberg or Bonferroni corrected significance levels should be used to isolate the most significant metabolite differences between strains and account for possible type 1 errors *(17).* Tools such as the biocyc (www.biocyc.org) omics viewer can then be used to visualise fold changes in the metabolites detected across the metabolic pathways of *C. jejuni*, where information on genetic and proteomic information for the pathways are also presented *(16)*.

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NOTES

1. Rich growth medium is used when global analysis of metabolism is performed. In these instances it is hoped that as many metabolic pathways are active as possible. Also where isogenic mutants may suffer from growth deficiencies it is important to optimize growth rate and phase of harvesting to be as close to the isogenic parent as possible. In different circumstances, such as in the analysis of specific growth conditions or when analysing specific metabolic

pathways, a defined growth medium would be preferable.

2. Mix methanol:chloroform 1:1 (v/v) within a fume hood and cool in a -20 °C spark-free laboratory freezer prior to use. ***WARNING***: methanol and chloroform are highly hazardous chemicals, please read all MSDS and wear appropriate personal protective equipment when handling these chemicals throughout the procedure.
3. Ball-bearings must be washed in methanol:chloroform 1:1, autoclaved and oven dried. Before use they should be placed in a -20 °C freezer overnight.
4. Where strains may have differing growth rates/dynamics it is important to ensure that samples are taken at matched points in their growth cycle. To do this you should perform a growth curve prior to analysis, or take the OD_{600} throughout to monitor growth.
5. Our method employs a 1 minute centrifugation before quenching in liquid nitrogen completely halts metabolism, enabling retention of the maximum number of metabolites. However it is important to note that changes in metabolite levels during centrifugation will undoubtedly occur. For this reason practice is required to ensure rapid manipulation for maximum reproducibility between samples and minimum time spent processing.
6. Other Direct Injection Mass Spectrometers are also suitable for this technique, though MS conditions would have to be slightly altered accordingly. Furthermore, similar analyses have been performed using nuclear magnetic resonance methods *(18)* and MALDI-TOF *(19)*, however sample processing would have to be altered.
7. Samples should be analysed in a randomised order to minimise effects of day-to-day machine variation. Other autosamplers would also be suitable for this

step.

8. Previous analyses have shown that both positive and negative ionisation modes are capable of distinguishing between bacterial strains *(8)*. However the presence of multiple positive ionisation species compared to the relatively simple negative ionisation mode data sets makes negative ionisation mode preferable for putative metabolite allocation *(1)*.
9. This methodology negates the need for a noise threshold and enables true low intensity metabolite peaks to be kept.
10. Lists of predicted *C. jejuni* specific metabolites can be compiled from genome sequence information and exist within e.g the KEGG database, however we have found these to be too restrictive, leaving many mass bins with no metabolite allocated *(1)*. The use of metabolites lists from multiple databases results in some mass bins receiving multiple metabolite identities. In these instances knowledge of the likely metabolome is required to decipher the most likely identity. Independent confirmation of metabolite identities is required to confirm any hypothesis made based on observed changes in putatively identified metabolite pools.

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