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Suitability of silica hydride stationary phase, aqueous normal phase chromatography for untargeted metabolomic profiling of *Enterococcus faecium* and *Staphylococcus aureus*

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Abstract

We report the robustness of silica hydride stationary phase, aqueous normal phase (ANP) chromatography to the chemical complexity of the intracellular metabolomes of *Staphylococcus aureus* and *Enterococcus faecium*. We specifically demonstrate that the chromatographic behavior of known metabolites is unaffected by the intracellular chemical matrix of these microbes and that this method enable untargeted profiling of their intracellular metabolites using accurate mass-retention time (AMRT) identifiers. We further demonstrate the ability of AMRT-based metabolite profiling to differentiate bacteria along genetic and phenotypic lines. Overall, these data commend the utility of ANP-based chromatography for untargeted metabolomics-based studies of microbial physiology and antibiotic resistance.

INTRODUCTION

Metabolites are the integrated output of the genome, proteome and environment and currency of most cellular activities. Metabolomics is the global study of all metabolites in a biological system under a given set of conditions [1]. Metabolomics-based approaches thus represent a phenotypically direct, but unexplored, window to cellular physiology.

Analytical studies of metabolism have traditionally been limited to one or a small set of predefined metabolites [2]. Recent advances in chromatographic separation and mass spectrometry however have now made it possible to pursue untargeted, quantitative studies of many diverse metabolites in parallel. Toward this end, Pesek *et al.* described a novel silica hydride-based stationary phase, aqueous normal phase (ANP) chromatographic method capable of achieving highly robust, class-specific separations of polar metabolites [3]. Because many of the biologically most active metabolites are polar compounds, this method has opened the door to a physiologically essential, but previously inaccessible, compartment of the metabolome. This method has thus enabled the development of untargeted, LC-MS-based metabolite profiling approaches that leverage the use of accurate mass-retention time (AMRT) pairs as metabolite identifiers [4].

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Here, we demonstrate the robustness of silica hydride stationary phase, aqueous normal phase (ANP) chromatography to the complex chemical matrix of the metabolome of 2 major clinical pathogens, *Staphylococcus aureus* and *Enterococcus faecium*. We specifically demonstrate that the chromatographic behavior of known metabolites is unaffected by the intracellular chemical matrix of these microbes and that ANP-based chromatography of these organisms yields highly reproducible AMRT features suitable for untargeted metabolomic studies. Toward this end, we demonstrate the ability of ANP-derived AMRT profiling to differentiate bacteria according to their phylogeny and antibiotic susceptibilities. Given the functional relevance of metabolites, these studies provide a conceptually novel lens on microbial physiology and the unchecked problem of antibiotic resistance.

MATERIALS & METHODS

Bacterial strains and cultivation

De-identified *S. aureus* and *E. faecium* isolates were obtained from the clinical microbiology laboratory of the Weill Cornell Medical Center. Bacteria were cultivated in liquid BHI media and grown for metabolomic profiling on SBA agar using the filter culture methodology of Brauer *et al.* [5]. Bacteria were harvested during the midlogarithmic phase of growth and metabolically quenched by immersion into a solvent mixture of acetonitrile:methanol:H₂O (40:40:20) pre-cooled to -40°C. Metabolites were extracted by mechanical lysis of bacteria in a MiniBeadBeater (BioSpec) followed by clarification of insoluble debris by centrifugation for 10 minutes at 14,000 rpm at 4°C. Bacterial biomass of individual samples was determined by measuring residual protein content using the method of Bradford [6].

Bacterial strain typing

Select bacterial isolates were typed by pulsed field gel electrophoresis as described by McDougal *et al.* [7].

Liquid chromatography-mass spectrometry

The mass spectrometer used for the studies was an Agilent Accurate Mass 6220 TOF coupled to an Agilent 1200 LC system equipped with a solvent degasser, binary pump, temperature-controlled auto-sampler and temperature-controlled column compartment containing a Cogent Diamond Hydride Type C silica column (150 mm × 2.1 mm) (MicroSolv Technologies). The flow rate was 0.4 ml/min with a gradient as follows: Solvent B (acetonitrile with 0.2% acetic acid) was held constant at 85% and solvent A (water with 0.2% acetic acid) was held constant at 15% for 2 minutes and decreased to 20% B over the next 12 minutes as detailed by Pesek *et al.* (Gradient 3) [3]. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump connected to a dual sprayer electrospray ionization source, operated in the positive-ion mode. ESI capillary voltage was set at 3500 V. The nebulizer pressure was set to 40 psig and the nitrogen drying gas was set to a flow rate of 10 L/min. The drying gas temperature was maintained at 250°C. The acquisition rate was 1.5 spectra/ sec and a stored mass range of *m/z* 50–1200. Data were collected in centroid mode in 2 GHz (extended dynamic range) mode. Detected masses were deemed identified metabolites on the basis of unique accurate

mass-retention time identifiers for masses exhibiting the expected distribution of accompanying isotopomers [4].

Data processing and statistical analysis

Mass spectra were deconvoluted by the Molecular Feature Extraction algorithm of Agilent MassHunter Qualitative Analysis software which identified individual metabolite features on the basis of a chromatographically covariant ion families conforming to definable empirical formulae [4]. Statistics and graphs were generated using GeneSpring MS and analyzed using principal components analysis and hierarchical clustering analysis [8].

RESULTS & DISCUSSION

To obtain physiologically faithful profiles of microbial metabolomes and avoid metabolite leakage, we adapted the filter culture system described by Brauer *et al.* [5]. To obtain a chemically diverse cross-section of hydrophobic and hydrophilic metabolites, metabolites were extracted into an organic solvent mixture of acetonitrile, methanol and water and chromatographically separated by the ANP method described by Pesek *et al.* [3] Using this method, we studied the intracellular metabolome of 11 clinical *E. faecium* and 13 *S. aureus* isolates. Samples were prepared under identical conditions and analyzed by positive-mode electrospray ionization, time-of-flight mass spectrometry to generate exemplary metabolomes. Using 5 µl of a metabolite extract corresponding to 1 µg of protein lysate, these studies yielded 314 and 360 “core” AMRT metabolite features that eluted beyond the chromatographic void volume and were observed in all *E. faecium* and *S. aureus* isolates with retention time variations of less than 0.05 min (FIG. 1a). These features corresponded to an ion count signal range spanning more than 3 orders of magnitude. Five replicate injections of each of 3 samples demonstrated an analytical variation in metabolite feature number and ion count signal of less than 1 and 10%, respectively. Retention time analysis of 6 known metabolites present within all samples and spanning the chromatographic space demonstrated no significant deviation from those of their chemical standards in either aqueous solution or the chemical matrix of microbial metabolomes (FIG. 1b). Taken together, these results validate the robustness of ANP-based chromatography to the chemical complexity of the intracellular matrix of 2 clinical pathogens.

Principal components (PCA) and hierarchical clustering analyses (HCT) demonstrated that the resultant untargeted metabolite profiles differentiated these bacteria along conventional phylogenetic lines (FIG. 2). Surprisingly, analyses of these data also revealed co-segregation of metabolite profiles with antibiotic susceptibility phenotypes of both organisms (FIG. 3). It was further observed that the metabolite profiles of genetically related strains clustered more closely to one another than to genetically unrelated strains of the same species (data not shown). Pulsed field gel electrophoresis (PFGE) analysis, in contrast, failed to differentiate either species of bacteria on the basis of their antibiotic susceptibility. These results thus demonstrate the potential of untargeted metabolomics approaches to inform on the physiology of antibiotic resistance in biochemically global terms.

CONCLUSIONS

These studies commend silica hydride stationary phase, aqueous normal phase chromatography for untargeted metabolite profiling studies of *E. faecium* and *S. aureus*, 2 major clinical pathogens. We specifically demonstrate that the chromatographic retention behavior of polar metabolites within the chemical matrix of the bacterial metabolome is no different than that of authentic chemical standards and that this method enables highly robust untargeted AMRT metabolite profiling of *E. faecium* and *S. aureus*. With this method, we further demonstrate that this approach can differentiate bacteria according to phylogeny and antibiotic susceptibility. Given the central role of metabolites in most cellular functions, these results commend ANP chromatography as a robust tool for pursuing untargeted metabolomics-based studies of *E. faecium* and *S. aureus* through the phenotypically relevant lens of small molecule metabolites.

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A **B**

Compound name	Formula	[M + H]	RT (STD) (min)	RT (EF) (min)	RT (SA) (min)
Hypoxanthine	C ₅ H ₄ N ₄ O	137.0458	2.08	2.04 ± 0.01	2.06 ± 0.01
Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	268.104	2.6	2.54 ± 0.01	2.38 ± 0.06
Nicotinic acid	C ₆ H ₅ NO ₂	124.0393	1.55	1.51 ± 0.01	1.51 ± 0.01
Glutamic acid	C ₅ H ₉ NO ₄	148.0604	5.45	5.29 ± 0.01	5.27 ± 0.01
Citrulline	C ₆ H ₁₃ N ₃ O ₃	176.103	8.48	8.36 ± 0.01	8.40 ± 0.02
Lysine	C ₆ H ₁₄ N ₂ O ₂	147.1128	12.25	12.28 ± 0.01	12.18 ± 0.02

Figure 1.

(a) Graphical depiction of an exemplary *E. faecium* metabolome generated by ANP chromatography coupled to positive mode electrospray ionization, time of flight mass spectrometry (taken from a screen grab of Agilent Mass Profiler software). Each metabolite (depicted by black dots) corresponds to a unique accurate mass-retention time pair observed in all biological and analytical replicates with a retention time variation of less than 0.05 minutes. (b) Chromatographic retention times (RTs) of 6 pre-selected metabolites spanning the chromatographic gradient, in pure chemical form and within the chemical matrix of the *E. faecium* and *S. aureus* intracellular metabolome. RTs were obtained from 5 separate injections of each standard or bacterial samples and reported as average values $\pm$ standard deviations (SD). For chemical standards, RT SDs were uniformly less than 0.005 minutes.

Figure 2.
(a) Principal components analysis and (b) Hierarchical clustering tree analysis of the intracellular metabolome of 11 *E. faecium* (red) and 13 *S. aureus* (yellow) isolates demonstrating segregation along canonical species classification lines.

Figure 3. (a, c) Principal components analysis and (b, d) Hierarchical clustering tree analysis of the intracellular metabolome of vancomycin-susceptible (red) and –resistant (yellow) *E. faecium* (a, b) and methicillin-susceptible (red) and –resistant (yellow) *S. aureus* (c, d) discriminate bacterial species according to antibiotic susceptibility phenotypes.