IROA approach enabling detection of metabolites whose production is initiated or ceased in response to treatment

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ABSTRACT

In previous IROA metabolomics protocols, treatment and control organisms were grown in medias containing uniformly-labeled carbon sources with different 12C:13C ratios (95:5 vs. 5:95), pooled prior to chemical extraction and LC/MS. Analytes below the limit of detection for treatment or control group evaded recognition, since they did not form a pair of corresponding 12C- and 13C-enriched MS peaks. This limits the utility of established IROA protocols for natural product studies, where molecules and their biosynthetic pathways are commonly identified by comparing metabolite profiles between natural product producing and non-producing organisms (e.g. wild type vs. gene elimination mutant organisms). Here we devised an IROA protocol enabling the detection of metabolites whose production is initiated or halted in response to experimental conditions.

INTRODUCTION

Over 65% of known microbial secondary metabolites are produced by actinomycetes. *Nocardiopsis* sp. prevail in a variety of environments and produce a vast variety of bioactive compounds with proven clinical use for treatment of microbial infections, cancer, and other maladies, including many antibiotics that may aid as signaling molecules to protect their existence under different conditions, stressors and against competing microorganisms[1].

Modified metabolomics-guided approach to investigate metabolites produced by *Nocardiopsis*. The majority of Actinomycete genomes are not expressed under typical laboratory fermentation conditions, however experimental approaches such as the heterologous expression of biosynthetic genes and fermentation environment manipulation have shown promise for increasing expression of biosynthetic genes. The success of these approaches is reliant upon accurate methods for the evaluation of metabolic changes in response to experimental manipulations. Typically MS-based metabolomics methods have relied on isotopically labeled standards to ensure accurate identification as they behave physically and chemically identical to the analytes under measurement[2].

As such, in previous studies we implemented Isotope Ratio Outlier Analysis (IROA)[3,4] global LC/MS metabolomics to evaluate changes in the metabolome of a model *Nocardiopsis* species resulting from treatment with an iron chelating reagent, bipyridyl. Although our previous IROA LC/MS approach revealed several metabolites unique to the *Nocardiopsis* sp. it did not enable the detection of metabolites whose production is initiate or halted in response to experimental conditions, namely the introduction of bipyridyl. **Hence we modified our approach by developing a set of standards that included metabolites produced in response to the iron chelator 2,2'-bipyridyl (Fig. 1).**

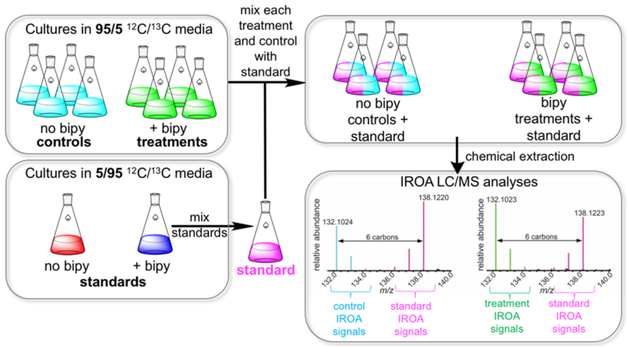


Figure 1. Overview of IROA experimental approach for detecting metabolites whose production is up- or downregulated or is exclusive to treatments or controls. This approach was used to evaluate impacts of 2,2'-bipyridyl (bipy) on the Nocardiopsis sp. metabolome. Bipyridyl treatment (n = 4) and no-bipyridyl control (n = 4) bacteria were fermented in uniformly 95:5 ^{12}C:^{13}C-labeled media alongside equivalent standard cultures in 5:95 ^{12}C:^{13}C-labeled media. Standard cultures were then pooled to provide a mixture containing distinctly labeled isotopes of every metabolite from treatments or controls. Each treatment and control culture was supplemented with this internal standard mixture prior to chemical extraction and LC/MS. The ratio of control (blue) or treatment (green) MS signal integrals relative to corresponding differentially isotope-labeled standard signals (magenta) was determined. Statistical comparison of these treatment:standard and control:standard isotope ratios enabled determination of metabolites whose production was impacted by bipyridyl treatment. Evaluation of the m/z difference between fully ^{12}C-labeled metabolites and fully ^{13}C-labeled isotopes indicated the number of carbon atoms in each molecule, as illustrated by the IROA peak pair for leucine from *Nocardiopsis* sp.

THEORY AND DISCUSSION

Bacteria utilize siderophores, low molecular weight binding iron-binding complexes, to acquire and transport iron for important cellular processes. The effect of iron limitation on siderophore secondary metabolite production was examined using a modified IROA Basic Protocol (Fig. 1). The approach enables the measurement of metabolic changes, the removal of false data such as noise or artifactual peaks identified by the absence of ^{12}C/^{13}C isotopic peak pair observed for each metabolite, and also the calculation of the number of carbon atoms which together with accurate mass for either of the monoisotopic peaks makes it possible to unambiguously determine the empirical formula for masses below 400. Of the 1,332 metabolites revealed by IROA peak pair analyses (Fig. 2), identities of 107 molecules were supported by correspondence between number of carbon atoms, exact masses of molecular ions, and retention times with a library of standard compounds. Identified metabolites encompassed common classes of biomolecules including amino acids, dipeptides, carbohydrates, and metabolites from nucleic acid pathways. These data support the suitability of IROA approaches for evaluating actinomycete metabolomes and detecting a broad range of metabolites from Nocardiopsis sp.

RESULTS

Nocardiopsis sp. metabolome revealed by untargeted IROA UHPLC/MS

High-resolution ESI+ and ESI- UHPLC/MS data for chemical extracts of bipyridyl treatment and no bipyridyl control cultures, each supplemented with internal standard, revealed 1,332 pairs of corresponding 95:5 and 5:95 ^{12}C:^{13}C IROA MS peaks. Computation of the difference in molecular ion m/z's between fully ^{12}C- and ^{13}C-labeled peak pair signals supported that the number of carbon atoms in detected metabolites ranged from 2 to 36 (Fig. 2).

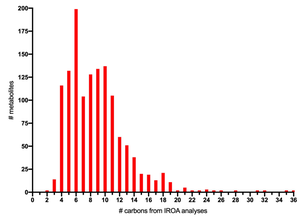


Figure 2. Histogram of the number of carbon atoms in each of the 1,332 Nocardiopsis sp. metabolites detected by IROA LC/MS. The number of carbon atoms in each molecule was proposed by finding the *m/z* difference between the fully ^{12}C- and ^{13}C-labeled molecular ion peak pair for each analyte.

IROA LC/MS differentiates treatment and control Nocardiopsis sp. metabolomes and suggests the production of novel metabolites: univariate and supervised/unsupervised multivariate analyses

For each IROA MS peak pair detected from Nocardiopsis sp., the ratios of 95:5 ^{12}C:^{13}C-labeled signals (from control or treatment, n = 4) and isotopic 5:95 ^{12}C:^{13}C-labeled signals (from standard) were determined. Control:standard and treatment:standard isotope ratio t-tests revealed that abundances of 253 metabolites were altered in response to bipyridyl treatment with $p < 0.001$ (not shown). PCA distinguished samples between control and treated group (Fig. 3a) and the PCA scree plot (Fig. 3b) showed that the first two PCs account for most of the variation between groups. RF differentiated all treatments from controls (Fig. 3c), and the mean decrease accuracy (MDA) was determined for individual metabolites (Fig. 3d). The 40 metabolites with highest MDA scores included molecules up- and downregulated in response to bipyridyl treatment spanning multiple classes of primary metabolites. Molecules from amino acid pathways (i.e. L-phenylalanine, 4-guanidinobutanoic acid, 2-aminobenzoic acid, L-isoleucine) were prominent among metabolites with largest MDA values.

Figure 3. Multivariate analyses of bipyridyl treatment and no-bipyridyl control Nocardiopsis sp. metabolomes. (a) PCA score plot revealed the impact of bipyridyl. Data points corresponding to controls are shown in red (S001-S004); clearly differentiated from light blue data points corresponding to treatments (S005-S008). (b) Scree plot of PCA component variances. (c) RF analysis clearly segregated samples between treatment and control groups. Data points are colored as in (a). (d) RF variable importance plot for the 40 metabolites contributing most toward RF separation of treatment and control groups. Annotated metabolites associated with amino acid metabolism, based on the KEGG database, are listed in green. Previously reported secondary metabolites are named in red, and novel predicted MF with >10C are in purple. Blue data points indicate metabolites that decreased in response to bipyridyl treatment and orange indicates metabolites whose abundance increased.

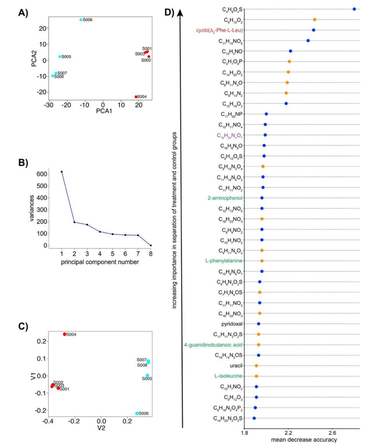
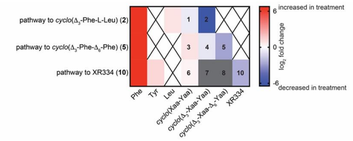


Figure 4. Application of IROA metabolomics for evaluating impacts of small molecules on targeted natural product biosynthetic pathways. Impacts of bipyridyl on Nocardiopsis sp. production of DKPs 1-10 were evaluated. (a) Previously reported biosynthetic pathway for 1-10 includes aminoacyl tRNA synthetase (AARS)-catalyzed ligation of amino acids with tRNAs, followed by cyclodipeptide synthase (CDPS)-catalyzed formation of DKPs 1, 3, and 6 from different combinations of aminoacyl-tRNAs [38]. Tailoring these DKP intermediates by a cyclic dipeptide oxidase (CDO) and/or methyltransferase (MT) leads to final products 2, 5, and 10. (b) LC/MS2 supported identification of each DKP from Nocardiopsis sp. extracts. Comparison of MS2 fragments for 1, 3, and 6 from extracts with synthetic standards supported their ID, dehydrogenated and/or methylated derivatives confirmed by correspondence between observed fragments shown in color with literature values. (c) Heatmap (see 3rd column) of mean (n = 4) log2 fold changes in abundance of DKP pathway metabolites in response to bipyridyl treatment. From left-to-right, the heatmap is arranged according to the order of precursors leading to final DKPs 2, 5, and 10. Map fields marked with "X" indicate metabolites not required for production of the final DKP indicated in that row, fields in gray indicate previously reported pathway intermediates not detected in our experiments, and numbers superimposed within the heatmap correspond to compound numbers from (a). Xaa and Yaa indicate Phe, Leu, or Tyr residues of DKPs.

IROA enables rapid assessment of the perturbation of targeted metabolic pathways

To establish IROA as a tool for evaluating mediators of targeted pathways, we assessed the effects of bipyridyl on a previously reported nocazine/XR334 diketopiperazine (DKP) biosynthetic pathway from Nocardiopsis sp.[5] (Fig. 4a). Production of nocazine/XR334 pathway final products (i.e. 2, 5, 10) is catalyzed by a suite of promiscuous enzymes, with these DKP products assembled from different amino acid precursors and differing in methylation and/or dehydrogenation tailoring (Fig. 4a). The IROA dataset revealed MS exact masses and carbon numbers consistent with DKPs 1-6 and 10 and amino acid precursors leucine, phenylalanine, and tyrosine; DKPs 7-9 were not found within the IROA dataset nor upon manual inspection of the raw LC/MS data. The identity of the amino acid precursors was confirmed against LC/MS RT and exact mass of authentic standards and structures of DKPs 1-6 and 10 confirmed by tandem mass spectra (Fig. 4b) of Nocardiopsis sp. metabolites and commercial standards. The abundance of each nocazine/XR334 pathway precursor, intermediate, and product (Fig. 4a) was compared between bipyridyl treatment (n = 4) and no bipyridyl control (n = 4) cultures. A similar trend was observed along the pathways leading to final products 2, 5, and 10. For each, amino acid precursors were upregulated in treatments, while downregulation of late stage intermediates and products was observed (Fig. 4c below). This finding supports that bipyridyl perturbs the nocazine/XR334 pathway, and suggests a particularly negative impact on later stages of the pathway.

Figure 4c



METHODS

Experiment design: *Nocardiopsis* sp. were maintained in media that promoted homogenous growth. Cells were washed, and resuspended with inorganic buffer. 20uL aliquots of washed cell suspension were added to 3 mL of isotopically defined (95% or 5% U-^{13}C), until fully transformed to the isotopic balance of the media. Bipyridyl treatment (n = 4) and no-bipyridyl control (n = 4) bacteria were fermented in uniformly 95:5 ^{12}C:^{13}C-labeled media alongside equivalent standard cultures in 5:95 ^{12}C:^{13}C-labeled media. Standard cultures were pooled to provide a mixture containing distinctly labeled isotopes of every metabolite from treatments or controls. Each treatment and control culture was supplemented with this internal standard mixture prior to chemical extraction with ethyl acetate. Samples were stored at -20 °C .

LC-MS: Pooled (95%/5%) sample extracts were analyzed by reverse phase UHPLC-HRMS using an Ace Excel PFP-C 18 2.1x100 mm, 2.0 µm column. The UHPLC pump was a Dionex Ultimate 3000, and the mass spectrometer was a Thermo Fisher Q-Exactive Orbitrap. Solvent A was 0.1% formic acid in water, and solvent B was acetonitrile. The collected data were initially processed by Xcalibur Workstation software (version 3.0).

Data processing and analysis: The Thermo RAW datafiles were converted to mzXML data files which were imported directly into the IROA ClusterFinder Software Program for peak picking and compound identification and the IROA portal for statistical analysis.

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SUMMARY

The modified IROA analytical and bioinformatic metabolomics approach was used to interrogate samples treated with and without iron-chelation to look for potential *Nocardiopsis* sp. natural products suggest broad-reaching metabolic consequences of bipyridyl (Fig. 3). Our study illuminates the impacts of bipyridyl on the metabolome of a model actinomycete and provides the first implementation of IROA global metabolomics for study of actinomycetes. Future studies are anticipated to promote dereplication of known metabolites and facilitate rapid prioritization of promising novel metabolites.

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