Deciphering regulatory mechanisms in *M. xanthus* using Isotopic Ratio Outlier Analysis (IROA) for metabolome-wide quantitation

Helmholtz-Institute for Pharmaceutical Research Saarland

HELMHOLTZ ZENTRUM FÜR INFEKTIONSFORSCHUNG

UNIVERSITÄT DES SAARLANDES

IROA TECHNOLOGIES

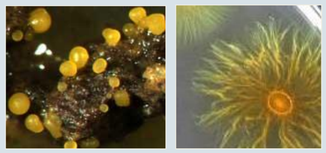
HIPS, Bruker Daltonik & IROA Technologies

Daniel Krug[1,2], Carsten Volz[1], Aiko Barsch[3], Chris Beecher[4], Felice de Jong[4], Rolf Müller[1,2]

[1] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Saarbrücken and Helmholtz Center for Infection Research (HZI), Braunschweig, Germany
[2] Saarland University, Saarbrücken, Germany
[3] Bruker Daltonik GmbH, Bremen, Germany
[4] IROA Technologies, Ann Arbor, Michigan, USA

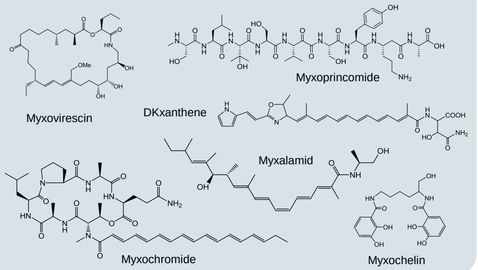
Introduction

Myxobacteria are an important source of natural products often exhibiting potent biological activity and unusual modes-of-action [1]. Their genomes - among the largest in prokaryotes - harbour intriguing numbers of secondary metabolite biosynthetic pathways, whereas the small-molecule products for many of these have yet to be discovered [2]. Myxobacteria also devote an exceptionally high proportion of their genetic capacity to regulation, as exemplified by the genome of the model strain *Myxococcus xanthus* DK1622. Deciphering complex myxobacterial regulatory networks improves our ability to manipulate natural product yields and can facilitate the discovery of novel metabolites. Here, we present a comprehensive metabolomics study using comparative LC-hrMS profiling and IROA to quantify small-molecule readouts in response to induced overexpression of a transcriptional antiterminator in DK1622.



Photography: G.J. Velicer / M. Vos

Fig. 1 Fruiting bodies of *Myxococcus xanthus* (left), swarming colony on agar surface (right), and structuresof secondary metabolite classes known from the model strain DK1622 (bottom).



Methods

- Genetically modified *M. xanthus* strains were maintained in media in which all of the carbon was isotopically defined (95% or 5% 13C), until fully transformed to the isotopic balance of the media. Cell pellets were harvested, equal aliquots of 95% and 5% 13C cells were pooled and extracted.
- Extracts were separated using a Waters BEH C18 column on an Ultimate 3000 RSLC system (Thermo Scientific). Gradient elution was at 600 µl/min (45 °C) using H2O + 0.1 % FA (A) and ACN + 0.1 % FA (B). The gradient started at 5 % B for 0.5 min, increasing to 95 % B in 19 min. MS analysis was done with a maXis UHR-Q-TOF instrument (Bruker) operated in positive ESI mode.
- The IROA ClusterFinder software was used to perform a scan-by-scan analysis of the complete dataset and identify all IROA peaks based on their extended isotopic envelopes.

Results

Quantitation of changes in myxobacterial secondary metabolomes

The IROA approach [3] was used to determine the biochemical response of complex *M. xanthus* metabolomes to overexpression of the transcriptional antiterminator gene *taA* found in the myxovirescin biosynthetic gene cluster (C. Volz, *unpublished*). A mutant of *M. xanthus* DK1622, engineered for inducible overexpression of *taA,* was grown in media in which all carbon sources were labeled with 5% ^{13}C (Fig. 2). Cells were treated with inducer (vanillic acid), and following the IROA protocol, compared with the same strain labeled at 95% ^{13}C, grown without induction. Pooling control and experimental samples mitigates several commonly encountered sources of variance, including: sample-to-sample, prep-related, and ion suppression. The approach enables the removal of false data such as noise or artifactual peaks, identified by the absence of isotopic signature. The number of carbons in each IROA peak may be calculated, and with accurate mass, used to determine the molecular formula of each metabolite with high confidence.

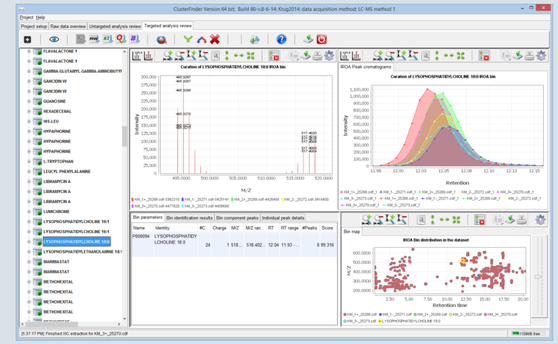


Fig. 3 A) IROA Technologies ClusterFinder: Detecting changes in the metabolome of *M. xanthus* DK1622 triggered by induction of the transcriptional antiterminator *taA* (5% ^{13}C), compared to a non-induced control cultivation (95% ^{13}C).

B) In *M. xanthus*, unusual fatty acids and lipids have been shown to be important for fruiting body formation and sporulation. Here lysophosphatidylcholine-18:0 was clearly seen to be upregulated in induced experimental vs. untreated samples based on the IROA pattern by the ClusterFinder software.

C) Molecular formulae generation by Bruker's SmartFormula: In the PeriodicTable Editor the non-natural IROA carbon isotopic ratio is defined (95% ^{12}C, 5% ^{13}C). Using this new IROA C12 element and exactly 24 C atoms as input for molecular formula generation one hit is returned: $C_{24}H_{49}NO_7P$. A public database query reveals this compound as lysophosphatidylcholine-18:0.

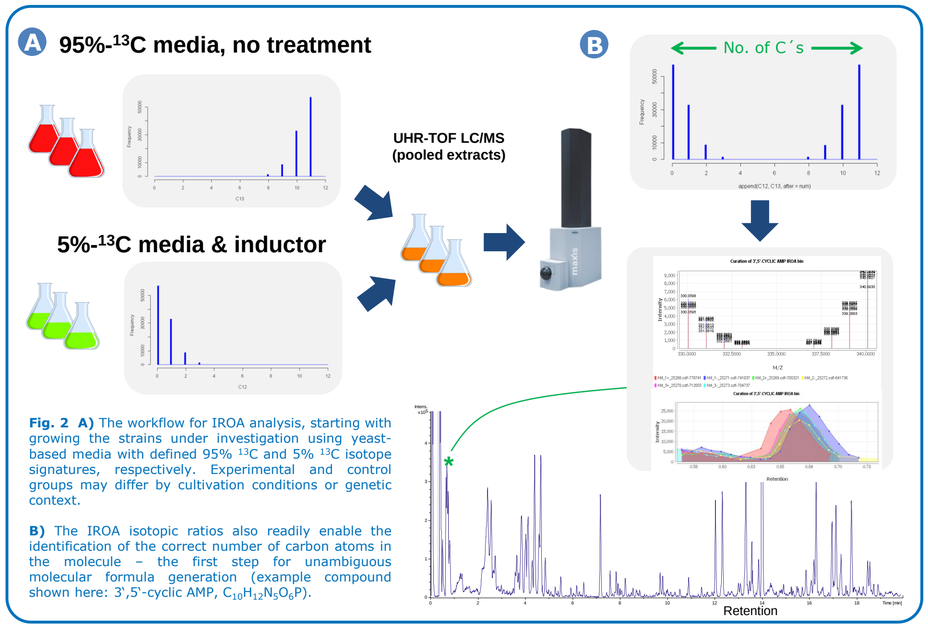


Fig. 2 A) The workflow for IROA analysis, starting with growing the strains under investigation using yeast-based media with defined 95% ^{13}C and 5% ^{13}C isotope signatures, respectively. Experimental and control groups may differ by cultivation conditions or genetic context.

B) The IROA isotopic ratios also readily enable the identification of the correct number of carbon atoms in the molecule – the first step for unambiguous molecular formula generation (example compound shown here: 3',5'-cyclic AMP, $C_{10}H_{12}N_5O_6P$).

Metabolite identification through generation of reliable molecular formulae

Peaks of biological origin are perfectly paired: each IROA envelope is half control and half experimental. The ratio of these halves quantitates the response relative to the control. As an example, the IROA envelope for lysophosphatidylcholine 18:0 has two halves separated by a 24 carbon mass difference (Fig. 3). The sum of the base peaks and their associated isotopic peaks are used to calculate their respective areas accurately and their ratio is evaluated. In this study a total number of 345 metabolites affected by the transcriptional antiterminator were highlighted using the IROA protocol and database searches. 46 metabolites were upregulated more than 5-fold and molecular formulae were calculated for these to provide tentative identification. A significant portion of these compounds could be assigned as constituents of unusual lipids. Importantly, the IROA approach has the potential to reveal also previously undiscovered secondary metabolites by generating molecular formulae with increased confidence, thus allowing to judge the novelty of candidate compounds through their absence from specialized databases such as *Myxobase* for myxobacterial natural products.

References

[1] Müller R, Wink J, "Future potential for anti-infectives from bacteria - How to exploit biodiversity and genomic potential", *Int. J. Med. Microbiol.* 2014, 304, 3-13

[2] Krug D, Müller R, "Secondary metabolomics: the impact of mass spectrometry-based approaches on the discovery and characterization of microbial natural products", *Nat. Prod. Rep.* 2014, 31 (6), 768-783

[3] de Jong F, Beecher C, "Addressing the current bottlenecks of metabolomics: Isotopic Ratio Outlier Analysis (IROA®), an isotopic-labeling technique for accurate biochemical profiling", *Bioanalysis*, 2012, 4(18), p. 2303-14

Summary

- Comprehensive study of regulation in myxobacteria with an untargeted metabolomics setup using Isotopic Ratio Outlier Analysis (IROA) and UHR-Q-TOF
- Reliable relative quantitation of known and unknown metabolites from a myxobacterial mutant strain in response to induction of the transcriptional antirepressor *taA*
- Compound identification facilitated by the use of ultra-high resolution MS and the knowledge of the number of carbons in each molecule due to IROA

Metabolomics