Isotopic Ratio Outlier Analysis (IROA) of Myxobacteria using ultra high resolution mass spectrometry

Helmholtz-Institute for Pharmaceutical Research Saarland
HELMHOLTZ ZENTRUM FÜR INFEKTIONSFORSCHUNG
UNIVERSITÄT DES SAARLANDES
IROA Technologies

ASMS 2013, MP 079

HIPS, Bruker Daltonik & IROA Technologies

Daniel Krug[1,2]; Ullrich Scheid[1]; Aiko Barsch[3]; Gabriela Zurek[3], Rolf Müller[1,2]; Chris Beecher[4], Felice de Jong[4]

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

Introduction

Myxobacteria represent an important source of novel natural products exhibiting a wide range of biological activities [1]. Some of these so-called secondary metabolites are investigated as potential leads for novel drugs. Traditional approaches to discovering natural products mainly employ bioassays and activity-guided isolation, but genomics-based strategies and "metabolome-mining" approaches become increasingly successful to reveal additional compounds. These newer methods hold great promise for uncovering novel secondary metabolites from myxobacterial strains, as the number of known compounds identified to date is often significantly lower than expected from genome sequence information [1,2]. Analytical challenges for comprehensive MS-based profiling of myxobacteria include the need to reliably detect the significant differences between secondary metabolomes, e.g. as a consequence of gene knock-outs or regulatory effects, as well as the robust quantitation of known and unknown target compounds and their identification.

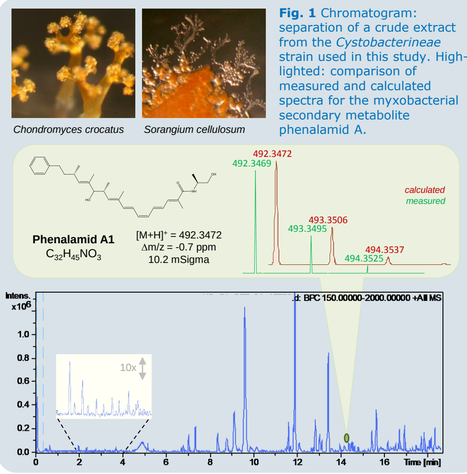

Fig. 1 Chromatogram: separation of a crude extract from the *Cystobacterineae* strain used in this study. Highlighted: comparison of measured and calculated spectra for the myxobacterial secondary metabolite phenalamid A.

Methods

- Myxobacteria were maintained in yeast-based VY/2 media in which all of the carbon was isotopically defined (95% or 5% ^{13}C, respectively, Cambridge Isotope Laboratories, Inc.). Two successive rounds of cultivation were performed (96 hours each) in order to transform bacterial cell mass to the isotopic balance of the media. Cell pellets and adsorber resin were harvested, equal aliquots of 95%- and 5%-^{13}C material were pooled and extracted.
- Extracts were separated using a Waters BEH C18 column on an UltiMate 3000™ RSLC system (Dionex). Gradient elution was at 0.6 ml/min (45 °C) using H_2O + 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 another 19 min. MS analysis was done with a UHR-Q-TOF instrument (Bruker Daltonik maXis 4G).
- 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

The IROA protocol [3] has been applied to the analysis of myxobacterial secondary metabolomes. In this IROA experiment, myxobacteria were grown in media in which all carbon sources were labeled with 95% ^{13}C and compared with a control cultivation labeled at 5% ^{13}C. Biological compounds from samples associated with 95% ^{13}C and 5% ^{13}C media are differentiatable and therefore control and experimental samples can be pooled and prepared simultaneously, removing sample-to-sample variance and ion suppression. Artifactual information identified by their absence of isotopic signature is removed and the identification of each compound enabled by the use of ultra-high resolution mass measurement and the knowledge of the number of carbons in each molecule.

Quantitation of changes in myxobacterial secondary metabolomes

In this study we used differential iron supply as a test case for IROA with secondary metabolite-producing myxobacteria (Figure 2).

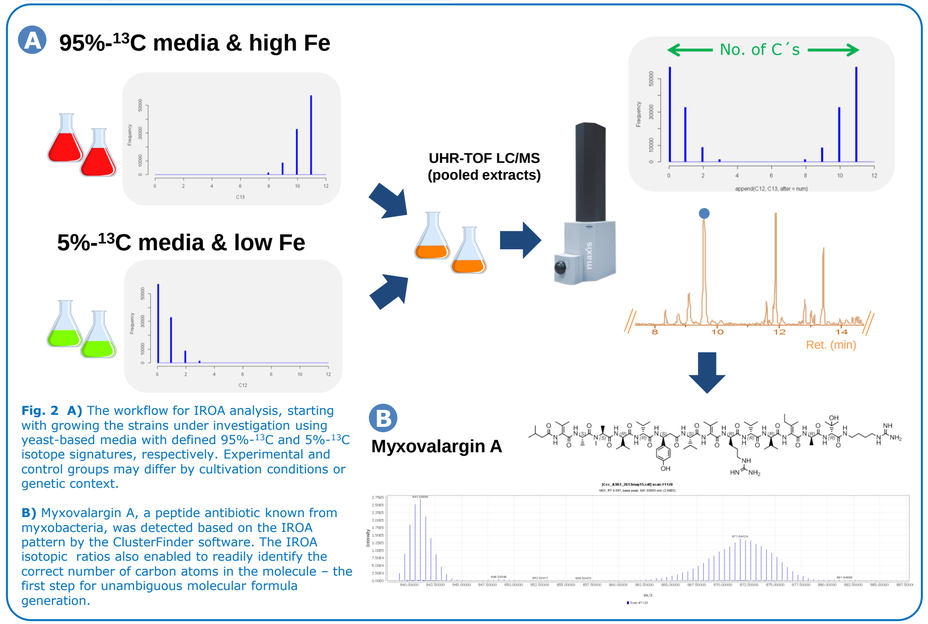

Fig. 2 A) The workflow for IROA analysis, starting with growing the strains under investigation using yeast-based media with defined 95%-^{13}C and 5%-^{13}C isotope signatures, respectively. Experimental and control groups may differ by cultivation conditions or genetic context.

B) Myxovalargin A, a peptide antibiotic known from myxobacteria, was detected based on the IROA pattern by the ClusterFinder software. The IROA isotopic ratios also enabled to readily identify the correct number of carbon atoms in the molecule – the first step for unambiguous molecular formula generation.

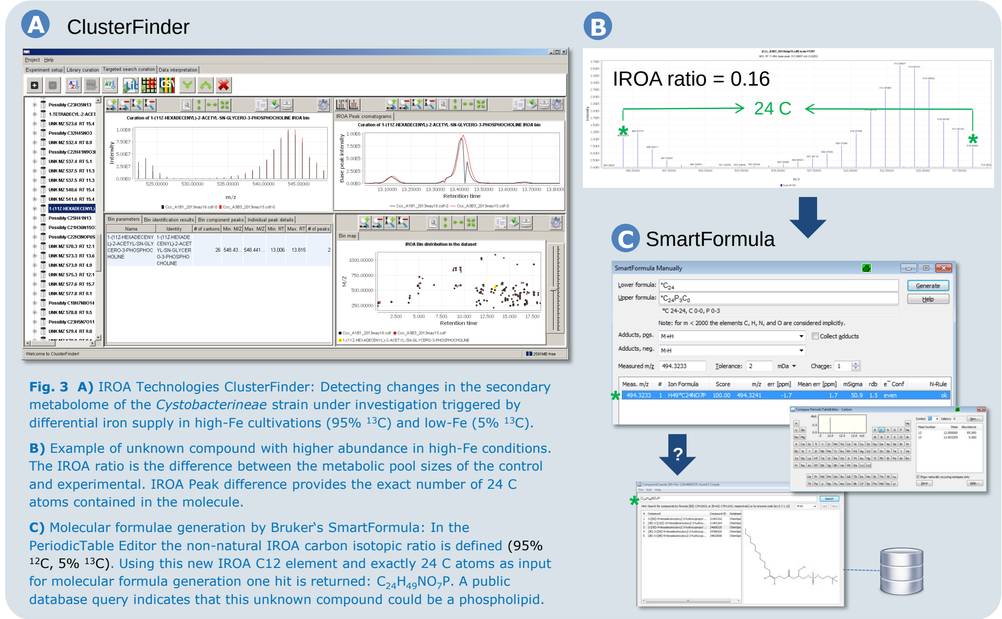

Fig. 3 A) IROA Technologies ClusterFinder: Detecting changes in the secondary metabolome of the *Cystobacterineae* strain under investigation triggered by differential iron supply in high-Fe cultivations (95% ^{13}C) and low-Fe (5% ^{13}C).

B) Example of unknown compound with higher abundance in high-Fe conditions. The IROA ratio is the difference between the metabolic pool sizes of the control and experimental. IROA Peak difference provides the exact number of 24 C atoms contained in the molecule.

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 indicates that this unknown compound could be a phospholipid.

Identification of novel metabolites through generation of reliable molecular formulae

The IROA protocol [3] enforces that the number of carbons in any mass spectral object is always known. When this is combined with high resolution mass spectrometry the formula for small molecules may be unambiguously determined. Since only compounds of biological origin can develop IROA patterns, all IROA peaks are of biological origin, and all artifacts are correctly identified as artifacts. The IROA ClusterFinder ™ software identified 160 IROA peaks, at the most stringent level (an additional 200+ were found for manual curation that was not done). The iron limitation in the media (5%-^{13}C culture) significantly changed the levels of many compounds.

Complimentary to the IROA ClusterFinder, the analysis tool SmartFormula™ allowed for the verification of molecular formula suggestions by combining accurate mass and isotopic pattern information.

In this experiment all of the expected compounds were identified, and a number of novel molecules were identified by examination of their IROA patterns (Figure 3). The formulae of these peaks have been tentatively determined and the identity of these molecules is currently being examined.

References

[1] "The biosynthetic potential of myxobacteria and their impact on drug discovery", Curr. Opin Drug Disc Dev., 2009, 12(2), p. 220-230

[2] "Myxoprincomide; a natural product from *Myxococcus xanthus* discovered by comprehensive analysis of the secondary metabolome", Angew. Chem. Int. Ed., 2012, 51(3), p. 811-816

[3] "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 secondary metabolome analysis of myxobacteria using Isotopic Ratio Outlier Analysis (IROA) and UHR-Q-TOF
- Reliable relative quantitation of known and unknown myxobacterial metabolites in response to differential iron supply
- 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