Secondary metabolites

The focus of biodiscovery and perhaps the key to unlocking new depths in taxonomy

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Introduction

Drug discovery is driven, either directly or indirectly, by biodiversity. Over half of the new chemical entities approved by regulatory authorities over the past decade are from natural sources [1-4]. Australia's natural resources contain unique, diverse and geographically distinct pools of biodiversity that have been only superficially utilised.

Microbial Screening has been harnessing Australian microorganisms as a source of novel natural products since 1994. These discovery efforts require a balanced approach to biological and chemical screening. Biological screening focuses attention on finding interesting pharmacological activity in bioassays. Chemical screening focuses on the characterisation of the secondary metabolites produced by the microorganisms.

In the present article we examine the 'management' of secondary metabolites, the key products of microbial biodiversity. These studies shed light on the fundamental unit of biodiversity, the species, and complementary approaches to taxonomy of microorganisms.

Metabolites, co-metabolites and COMET

Under appropriate fermentation conditions microorganisms produce secondary metabolites. These metabolites are neither essential to the organism's viability nor end-products of metabolism.

Secondary metabolites constitute a vast array of chemical substances that can be extracted in varying quantities. Most, but not all, metabolites appear to be specific to a species. Metabolites are not produced in isolation, rather a culture will produce 1 to 10 major co-metabolites detectable at the µg/ml level with literally hundreds of minor metabolites. While there is much debate about the role of secondary metabolites, it is perhaps not unreasonable to hypothesise that they play an integral role in mediating the dynamic interactions between microorganisms.

Historically, the discovery of novel metabolites within these mixtures could be achieved by a simple bioassay and chromatography to isolate the active compound. However, with the discovery of over 100,000 natural products and over 25,000 from microbial sources, these days are long gone. Today, high throughput bioassays require the recognition of known active metabolites early in the discovery process. This recognition and dereplication is typically achieved by the separation and analysis of the individual metabolites in the extract.

High performance liquid chromatography (HPLC) coupled to diode array detection (DAD) and/or mass spectrometric (MS) detection has emerged as the dominant technology for metabolite analysis. At Microbial Screening our focus has been on the UV spectra of metabolites using DAD as the most cost effective, robust and rapid throughput technology.

Despite the complexity of a microbial extract, metabolite patterns are not random. For virtually all microorganisms (and macroorganisms), patterns are highly diagnostic for any given species. Using this principle, we have developed metabolite recognition software, COMET that compiles and analyses *co-met*abolite patterns of natural product extracts. COMET takes the data generated by DAD-HPLC and creates compact databases of the chromatograms and UV spectra. Typically, an extract (Figure 1) is represented by a chromatogram with resolved metabolite peaks and the UV spectra of these peaks. The UV spectra provide an inherent classification of metabolite relatedness. For MST-MF2825, the screen shot shows the presence of nine different UV spectra belonging to six differing classes.

COMET provides flexible analytical tools to interrogate the database to determine whether a culture or any of its metabolites are known. With over 1 million UV spectra, COMET provides the infrastructure for chemical screening of biodiversity.

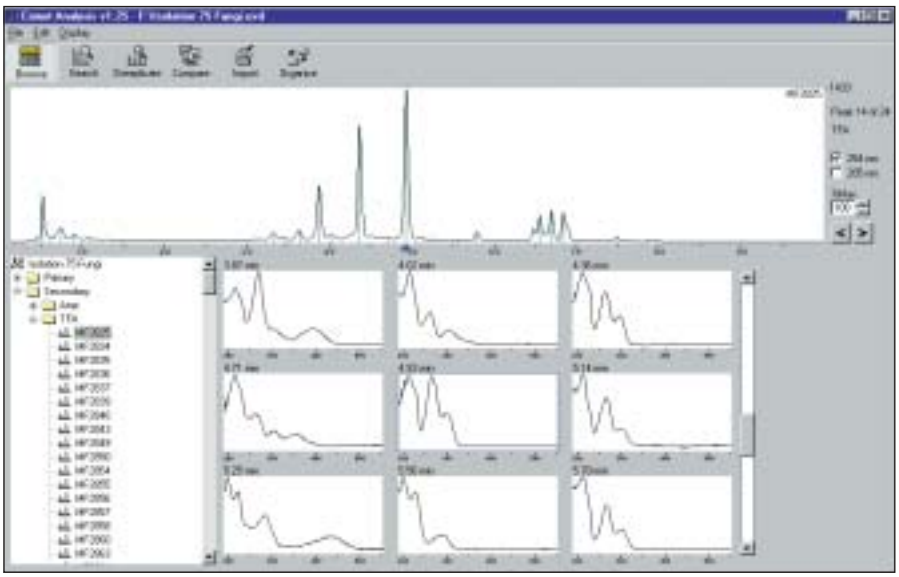
Metabolite patterns as essential aids in phenotypic based taxonomy

Molecular approaches have revolutionised taxonomy over the past 20 years. This transition has filled a need in microbiology as its rapid and almost universal uptake would demonstrate. The more labour intensive and costly phenotypic characterisation of cultures is used more selectively and is often secondary to molecular approaches. Analysis of metabolites represents a phenotypic approach that can achieve a rapid throughput comparable to molecular approaches.

Within Microbial Screening we are attempting to integrate a traditional phenotypic view of taxonomy with the genomic view using metabolite diversity. The concept of a species may seem, from a practical point of view, somewhat esoteric in drug discovery but it is central to any notion of a fundamental unit of biodiversity. Understanding these relationships has led to considerable advances in the taxonomy of Penicillium and other fungal genera [5].

A recent investigation of the species *Aspergillus carneus* serves to illustrate the role of metabolite diversity as a tool in understanding taxonomy. As part of a

Figure 1. A screen shot of COMET in Browse mode demonstrates the co-metabolite pattern of MST-MF2825, the number of peaks, production level and the UV spectra of each of the peaks in detail.



collaboration with Professor Rob Capon (Institute of Molecular Biosciences) and Dr Tom Friedel (Novartis Animal Health), a culture producing a novel family of cyclic peptides, the aspergillicins, was discovered[6]. The producing culture, MST-MF156, was identified by Ailsa Hocking (Food Sciences Australia) as *A. carneus*.

A comparative study using MST-MF156, the *A. carneus* type strain and five other independent strains of the species was undertaken. Extracts of agar plate cultures of the seven cultures were compared by HPLC and analysed in COMET. A total of 28 major metabolites with clearly resolved UV spectra were identified by their retention times in the chromatogram (Note: the aspergillicins were not sufficiently diagnostic to include their spectra in this analysis).

The distribution of the metabolites in each of the strains is presented in Table 1. The blue bands indicate the 'consensus metabolites' found in more than one of the strains, while the yellow bands indicate metabolites unique to a single strain. Most strains contain a composite of common and unique metabolites. The seven 'consensus' metabolites provide a notional concept of *A. carneus*.

In strains A, B, C and D these 'consensus' metabolites dominate the HPLC profile and the strains are therefore considered variants. A search of the COMET database reveals these profiles are unique and thus likely to constitute a species. Strains E and MST-MF156 display only a single 'consensus' metabolite and their profiles are dominated by unique co-metabolites. Strain F shares no consensus metabolites.

Overall strains E, F and MST-MF156 are more remarkable for their unique metabolite diversity rather than their fit to a 'consensus' *A. carneus*. Do the strains all belong to a single species or are they a mixture of unrelated species? Our research suggests the cultures belong to four different species. Irrespective of the answer, this study illustrates the heterogeneity implicit in conventional phenotypic approaches.

Examination of metabolite distribution in over 50 genera and species has shown that the traditional taxonomic view of a species leads to a very conservative measure of biodiversity. In many cases it is evident that the species structures of most genera are insufficient to accommodate the metabolite diversity. Have phenotypic approaches simply run out of phenotypes to key? Despite the large size of genera like Streptomyces and Penicillium, it is highly probable that they are both grossly under-speciated.

Conclusion

Microbial metabolites are the driving force behind biodiversity in drug discovery. In an era where metabolite analysis is complementary to high-throughput bioassays, rapid methods are essential. Discovery of novelty is not simply a matter of dereplicating known actives, rather it involves understanding the pattern of co-metabolite production and the correlation of these patterns to bioassay activity. Innovations in metabolite analysis offer enormous windfalls in taxonomy. Furthermore, analysis of metabolite production provides a complementary technology to molecular approaches.

References

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Table 1. Metabolite distribution within a selection of *A.carneus* strains. This indicates the presence (+) or absence of a metabolite's UV spectrum in a gradient HPLC trace of the culture. The 'consensus' metabolites cultures are coloured blue.

	2.12	3.03	3.36	3.87	4.26	4.27	4.34	4.48	4.52	4.54	4.58	4.65	4.76	4.92	5.09	5.22	5.34	5.38	5.53	5.63	5.91	5.94	6.01	6.17	6.25	6.37	6.67	6.84
Strain A	+		+		+							+				+			+							+		
Strain B					+							+				+			+							+		
Strain C		+	+		+	+						+										+		+		+		
Strain D					+						+								+				+					
Strain E							+			+				+				+			+	+			+		+	
Strain F									+						+		+				+							
MST-MF156				+				+					+			+												+

Retention Time (min)