Metabolomic analysis of protozoan parasites





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Protozoan parasites cause a number of important diseases in humans, including malaria, African trypanosomiasis, Chagas disease and the leishmaniases. Current therapeutics for these diseases are limited and their effectiveness is being further undermined by the emergence of drug-resistant parasite strains. Parasite genome sequencing projects have provided new insights into the metabolic capacity of these pathogens and have highlighted potential drug targets. However, these genome-based reconstructions of metabolic networks are incomplete and we still have only a limited understanding of the metabolic requirements of these pathogens during infection. Metabolomics has emerged as a powerful new tool for investigating parasite metabolism and host responses, complementing more established omics technologies as well as being useful as a stand-alone technique.

Protozoan parasites are an evolutionarily diverse group of eukaryotes, colonising a wide range of extracellular and intracellular niches within their mammalian hosts (Figure 1). Analysis of parasite metabolism is complicated by the fact that, with few exceptions, little is known about the nutrient composition of these host niches, and only a minority (typically <40%) of genes in these pathogens have a predicted or experimentally verified function. Furthermore, it is often difficult to identify clear differences in the metabolic capacity of different parasite stages from changes in gene transcription or protein expression, reflecting the importance of post-translational regulatory mechanisms in these organisms[1,2]. Finally, all of these pathogens scavenge a variety of complex metabolites from their hosts, complicating assessment of the importance of *de novo* biosynthetic and catabolic pathways[3,4]. Metabolomics refers to the quantitative analysis of all low molecular weight (<1500 Dalton) metabolites in a biological extract and is increasingly being used to probe the metabolism of other microbial pathogens. While the metabolomic analysis of parasitic protozoa is still in its infancy, recent studies have highlighted the utility of these approaches in assessing the physiological state and metabolic flexibility of different parasite developmental stages, in identifying new or unanticipated metabolic pathways, and in dissecting the mode of action of anti-protozoal drugs. Metabolomic approaches can also be used to analyse host metabolic responses to infection, potentially highlighting new therapeutic strategies and diagnostic biomarkers.

Metabolomics – the methodology

Metabolomic analysis can be undertaken on cultured parasites, infected host cells and relevant biofluids and tissues from infected hosts (Figure 2). In all cases, it is critical that metabolic processes are rapidly and effectively quenched prior to metabolite extraction. A rapid chilling protocol has been developed for Leishmania parasites that appears to be readily applicable to other parasite and mammalian cell systems[5,6]. Metabolites are commonly extracted from the metabolically quenched cell pellets or culture supernatants using organic solvent-aqueous mixtures[6]. In some cases, these crude metabolite extracts are further fractionated by solvent partitioning to obtain fractions that are enriched for either polar or lipidic metabolites.

Metabolite extracts can be subsequently analysed using multiple analytical platforms (Figure 1, Table 1)[7]. Hyphenated mass spectrometry approaches provide the greatest coverage and sensitivity – a key factor when isolating pathogens from host cells or tissues. Gas chromatography-mass spectrometry (GC-MS) has been used extensively to identify several hundred metabolites in crude cellular extracts. GC-MS provides robust metabolite identification (based on mass spectrum and retention time), high reproducibility and sensitivity and is amenable to automation[5,6]. Liquid chromatography-mass spectrometry (LC-MS) approaches

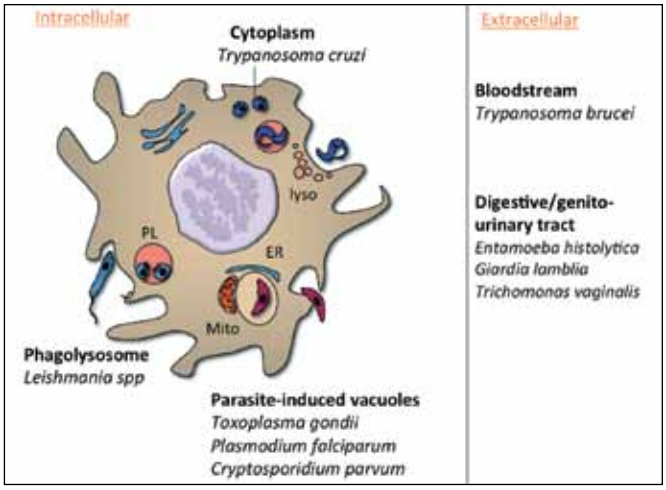


Figure 1. Parasitic protozoa are responsible for a number of important disease in humans and domestic livestock. These pathogens occupy a range of intracellular and extracellular niches in their mammalian host(s), many of which are poorly defined in terms of nutrient availability. Significant diversity exists in the vacuoles induced by apicomplexan parasites, particularly in their association with host Cell orghanelles such as the mitochondria (Mito) and endoplasmic reticulum (ER). T. cruzi transiently occupies mature lysosomes (Lyso) prior to escaping into the cytoplasm.

provide complementary metabolite class coverage[8]. Major advantages of LC-MS approaches include the ability to analyse samples without derivatisation, greater sensitivity for many metabolites and a larger number of mass spectrometer detection options. In particular, the use of LC in conjunction with ultra-high resolution mass spectrometers greatly facilitates metabolite identification. Other approaches, such as nuclear magnetic resonance spectroscopy (NMR) are also commonly used in metabolomic studies. While NMR is generally less sensitive than mass spectrometric approaches, it provides a highly quantitative and reproducible measure of some metabolite classes that are difficult to detect by other methods (for example, ATP)[9]. These platforms, particularly when used in parallel, are capable of

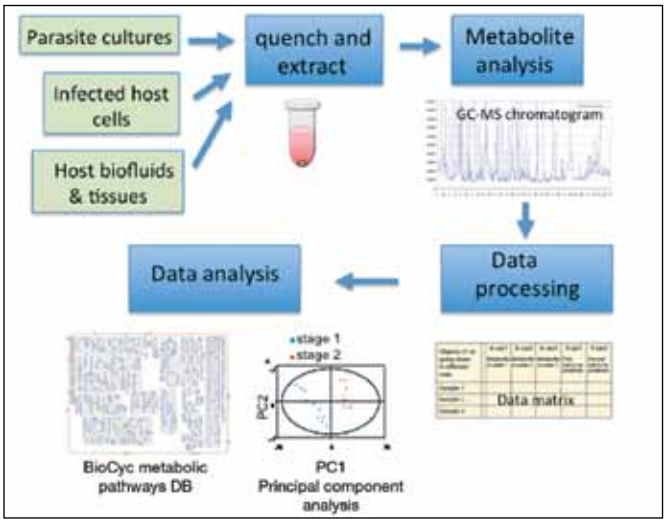


Figure 2. A metabolomics pipeline. Cultured parasite stages of infected host cell/tissues are metabolically quenched prior to metabolite extraction and analysis by high resolution mass spectrometry or NMR. Raw analytical =data is processed (peak deconvolution, alighnment and nomalization) prior to univariate and multivariate statistical analysis and pathway visualisation.

detecting more than a 1000 metabolites in cellular extracts. To put this in context, it has been estimated that the number of non-lipidic metabolite species in a cell may vary from ~200 (such as *Mycoplasma pneumonia*), to ~1000 (for single-celled eukaryotic pathogens such as pathogenic fungi and protists), to 5000 in metazoan organisms, such as humans[10], suggesting that current technologies are already capable of covering a significant fraction of the parasite and host cell metabolomes.

Detecting new metabolic pathways in parasitic protozoa

Metabolomic technologies have made a number of significant contributions to our understanding of central carbon metabolism in several human parasites, including *Plasmodium falciparum*, *Trypanosoma brucei* and *Leishmania* spp. (Figure 1). The major disease-causing stages of the malarial parasite, *P. falciparum,* are the asexual stages that develop in mammalian red blood cells. These stages are thought to be primarily dependent on the uptake and catabolism of glucose to lactate for energy and growth, resulting in hypoglycaemia and lactic acidosis in clinical cases of malaria. However, recent LC-MS and NMR-based metabolomic analysis of infected red blood cells showed that levels of TCA cycle intermediates were elevated in *P. falciparum*-infected, as compared to uninfected red blood cells, suggesting that intracellular parasite stages have an active mitochondrion metabolism[8,9]. Subsequent labelling experiments with ^{13}C-glucose and ^{13}C-glutamine, followed by measurement of ^{13}C-incorporation into intracellular metabolite pools by LC-MS, demonstrated

Table 1. Applications of metabolomics to study host-parasite interactions.

Sample	Analytical methods	Applications
Cultured parasite life cycle stages	GC-MS/LC-MS ^{1}H/^{13}C-NMR	• Complete inventory of all parasite metabolites • Identification of new/unanticipated metabolic pathways • Analysis iof parasite mutants (gene function) • Mode of drug action • Intracellular metabolic fluxes • Systems biology analysis (integration with other Omics datasets
Culture medium	GC-MS/LC-MS ^{13}C-NMR	• Nutrient and carbon source utilization
Parasite lysates	LC-MS[1]	• Identification and analysis of enzyme activity
Host biofluids & tissues	GC-MS/LC-MS ^{13}C-NMR	• Biomarkers for diagnosis/ • Host responses to parasite infections • Physiological state of parasites in situ • Responses of specific host immune cells to infection

1 LC-MS has been used to monitor changes in crude cell extracts or synthetic metabolite mixtures fllowing addition of recombinant enzymes. However, other profiling methods could be used instead.

that intracellular *P. falciparum* stages catabolised glutamine via a bifurcated TCA cycle (operating in both an oxidative and reductive mode) providing a source of reducing equivalents for the respiratory chain, as well as precursors for biosynthetic reactions[11]. While the expression of TCA cycle enzymes in these parasite stages had been suggested from transcriptomic and proteomic analyses, this example demonstrates the utility of metabolomics profiling and stable isotope labelling approaches for dissecting how these parasites have exploited canonical metabolic pathways in novel ways. Interestingly, little correlation was observed between the metabolite levels in the *P. falciparum* red blood cell stages and corresponding enzymes involved in their synthesis or catabolism, further highlighting the importance of post-translational mechanisms in regulating parasite metabolism.

Recent metabolomic analyses of trypanosomatid parasites, *T. brucei* and *Leishmania* spp. have further highlighted the extraordinary metabolic diversity of these parasites[12]. In one of the most comprehensive analyses of its type, the uptake and catabolism of six different ^{13}C-carbon sources by *Leishmania* parasites was assessed using GC-MS and ^{13}C-NMR[6]. These analyses revealed a direct link between hexose catabolism and amino acid biosynthesis that was not anticipated from genomic models of metabolism[6]. These findings have helped to explain the loss of virulence in *Leishmania* mutants lacking hexose transporters or the enzymes involved in carbohydrate metabolism[13]. Ultra-high resolution mass spectrometry has also been used to identify key intermediates in a novel pathway of carbohydrate reserve material in these parasites[14]. These examples highlight the utility of metabolomics approaches, in both identifying new metabolic pathways and dissecting novel variations in core pathways of central carbon metabolism.

Using metabolomics to identify new diagnostic biomarkers and understand host responses

Many of these parasites induce non-specific symptoms that can complicate accurate and timely diagnosis. High-resolution ^{1}H-NMR metabolomic analyses of serum and urine samples from *P. berghei* and *T. brucei*-infected mice have revealed marked changes in many metabolites providing a highly sensitive measure of disease progression[15,16]. While some of these changes reflect contributions from parasite metabolism, others were attributed to changes in both host metabolism and the host gut microflora, presumably reflecting changes in nutrient availability and/or host immunity. Metabolomic analyses have also revealed unanticipated aspects of host-parasite interactions. For example, *P. falciparum* was found to utilise arginine at a rate far greater than is required for normal growth, suggesting a parasite-driven mechanism for reducing arginine levels in the surrounding host milieu[8]. As arginine is required for key host microbicidal processes, such as nitric oxide production, this is evidence that *P. falciparum*, like some other parasites, may up-regulate arginine catabolism in order to generate cytoprotective polyamines and to prevent host nitric oxide synthesis[13].

Other applications for metabolomics to study host-parasite interactions

A high priority of future metabolomics research is to obtain a complete inventory of all metabolites (scavenged or synthesised) in mammalian-infective parasite stages (Table 1). This goal will require a major effort in characterising unknown metabolite peaks in mass spectrometric analyses, and will greatly aid ongoing genome annotation efforts. A second area of interest is to develop a truly systems biology approach to understanding parasite metabolism, incorporating metabolomics and other profiling data. Considerable progress has been made in developing mathematical models of metabolism for *T. brucei* bloodstream stages utilising enzyme kinetic data[17], and these models will be considerably advanced by measures of intracellular metabolic flux calculated from ^{13}C-labelling experiments. While current ^{13}C-flux measurements in bacteria typically require that cells are labelled to steady-state[18], new approaches have recently been developed that allow quantitative fluxes to be measured using dynamic measurements over much shorter periods[19,20]. Metabolomic ^{13}C-flux analyses will allow a more accurate identification of potential drug targets, such as metabolic choke points, the further characterisation of parasite mutants and the analysis of a drug's mode of action. Another innovative use of metabolomics technology is to test for enzyme activities in proteins of unknown function, by incubating the recombinant protein with crude parasite extracts[21]. Finally, metabolomic approaches are increasingly being used to assess the physiological state of pathogens *in vivo*[22]. This is particularly relevant for pathogens such as *Leishmania* and *T. cruzi* that may enter into a long latent state that is intrinsically resistant to current drug treatments[13]. The detection and analysis of physiologically distinct parasite populations *in vivo* will be facilitated by the development of imaging mass spectrometers that are capable of detecting spatial changes in metabolite levels across infected tissue sections[23].

Acknowledgements

We thank members of the McConville laboratory and the Bio21 Institute node of Metabolomics Australia laboratory for discussions and contributions to the development of some of the approaches described here. Work from the authors' laboratory was supported by the NHMRC. MJM is a NHMRC Principal Research Fellow.

References

1. Foth, B.J. *et al.* (2008) Quantitative protein expression profiling reveals extensive post-transcriptional regulation and post-translational modifications in schizont-stage malaria parasites. *Genome Biol.* 9, R177.
2. Leifso, K. *et al.* (2007) Genomic and proteomic expression analysis of *Leishmania* promastigote and amastigote life stages: The *Leishmania* genome is constitutively expressed. *Mol. Biochem. Parasitol.* 152, 35–46.
3. Tarun, A.S. *et al.* (2009) Redefining the role of *de novo* fatty acid synthesis in *Plasmodium* parasites. *Trends Parasitol.* 25, 545–550.
4. Kirk, K. *et al.* (2009) Purine uptake in *Plasmodium*: transport versus metabolism. *Trends Parasitol.* 25, 246–249.
5. De Souza, D.P. *et al.* (2006) Progressive peak clustering in GC-MS Metabolomic experiments applied to *Leishmania* parasites. *Bioinformatics* 22, 1391–1396.
6. Saunders, E.C. *et al.* (2011) Isoptopomer profiling of *Leishmania mexicana* promastigotes reveals important roles for succinate fermentation and aspartate uptake in TCA cycle anaplerosis, glutamate synthesis and growth. *J. Biol. Chem.*
7. van der Werf, M.J. *et al.* (2007) Microbial metabolomics: toward a platform with full metabolome coverage. *Anal. Biochem.* 370, 17–25.
8. Olszewski, K.L. *et al.* (2009) Host-parasite interactions revealed by *Plasmodium falciparum* metabolomics. *Cell Host Microbe* 5, 191–199.
9. Teng, R. *et al.* (2009) Metabolite profiling of the intraerythrocytic malaria parasite *Plasmodium falciparum* by ^{1}H NMR spectroscopy. *NMR Biomed.* 22,

292–302.
10. Psychogios, N. *et al.* (2011) The human serum metabolome. *PLoS One* 6, e16957.
11. Olszewski, K.L. *et al.* (2010) Branched tricarboxylic acid metabolism in *Plasmodium falciparum*. *Nature* 466, 774–778.
12. Tielens, A.G. and van Hellemond, J.J. (2009) Surprising variety in energy metabolism within Trypanosomatidae. *Trends Parasitol.* 25, 482–490.
13. McConville, M.J. and Naderer, T. (2011) Metabolic pathways required for the intracellular survival of *Leishmania*. *Annu. Rev. Microbiol.* 6, 543–561.
14. Sernee, M.F. *et al.* (2006) *Leishmania* β-1,2-mannan is assembled on a mannose-cyclic phosphate primer. *Proc. Natl. Acad. Sci. USA* 103, 9458–9463.
15. Basant, A. *et al.* (2010) Alterations in urine, serum and brain metabolomic profiles exhibit sexual dimorphism during malaria disease progression. *Malar. J.* 9, 110.
16. Wang, Y. *et al.* (2008) Global metabolic responses of mice to *Trypanosoma brucei* infection. *Proc. Natl. Acad. Sci. USA* 105, 6127–6132.
17. Bakker, B.M. *et al.* (2010) The silicon trypanosome. *Parasitol.* 137, 1333–1341.
18. Zamboni, N. and Sauer, U. (2009) Novel biological insights through metabolomics and ^{13}C-flux analysis. *Curr. Opin. Microbiol.* 12, 553–558.
19. Amador-Noguez, D. *et al.* (2010) Systems-level metabolic flux profiling elucidates a complete, bifurcated tricarboxylic acid cycle in *Clostridium acetobutylicum*. *J. Bacteriol.* 192, 4452–4461.
20. Yuan, J. *et al.* (2008) Kinetic flux profiling for quantitation of cellular metabolic fluxes. *Nat. Protoc.* 3, 1328–1340.
21. Baran, R. *et al*. (2009) Mass spectrometry based metabolomics and enzymatic assays for functional genomics. *Curr. Opin. Microbiol.* 12, 547–552.
22. Eisenreich, W. *et al.* (2010) Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nat. Rev. Microbiol.* 8, 401–412.
23. Liu, W.T. *et al.* (2010) Imaging mass spectrometry of intraspecies metabolic exchange revealed the cannibalistic factors of *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* 107, 16286–16290.

Biographies

Eleanor Saunders became interested in metabolomics and its application to *Leishmania* parasite during her PhD studies at the University of Melbourne. She is currently doing a postdoctorate and developing new approaches for measuring metabolic fluxes in the protozoan parasites.

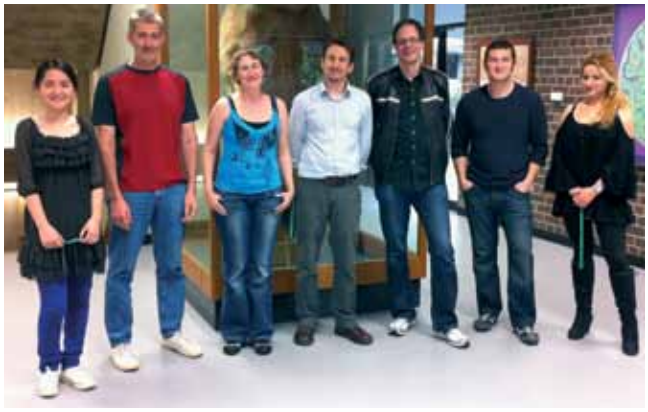
David de Souza has utilised metabolomics technologies to study a wide variety of microbial, animal and plant systems. He is completing his PhD studies at the University of Melbourne on parasite metabolomics as well as working as a research officer in the Bio21 node of Metabolomics Australia.

James MacRae completed his PhD studies at the University of Dundee, Scotland, studying the surface glycoproteins of the *Trypanosoma cruzi* parasite. He is currently utilising metabolomic approaches to identify potential drug targets in the apicomplexan parasites at the University of Melbourne.

Vladimir Likić is a bioinformatician and computational biologist with a focus on parasitic protozoa. He is a senior research fellow at the Bio21 Institute and head of Bioinformatics at Metabolomics Australia.

Malcolm McConville has had a long-term interest in parasite metabolism. He is currently a NHMRC Principal Research Fellow and head of the Metabolomics Australia node at the Bio21 Institute, Melbourne University.

From omics to systems biology: Exploring the mystery box of microbial life



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Microbial molecular biology has traditionally used very reductionist approaches; for example, find a gene of interest, clone it or knock it out and see if you can detect a phenotype. The genomics era has opened up the possibility of analysing microbes and communities at a systems level by combining high-throughput experimental data from genomic, transcriptomic, proteomic and phenomic techniques. This parallels earlier reductionist approaches by going from DNA to RNA to protein to phenotype, albeit on a global rather than individual gene scale.

In our group we are applying systems approaches at two levels; firstly we are looking at reconstructing the metabolism and physiology of pathogenic and environmental microbes. For example, we have been identifying the key drug resistance factors and elucidating the role of multidrug efflux pumps in the opportunistic pathogen *Acinetobacter baumannii*[1,2] and the regulation and biosynthesis of secondary metabolites in plant-associated pseudomonads[2]. Secondly, we are exploring the identity and function of populations within microbial communities to clarify their activity in global nutrient cycles. Following on from whole-community metagenomics projects, such as the Global Ocean Survey[3], we have been digging deeper into the functional diversity of marine cyanobacteria by combining