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Review

Eating at the Table of Another: Metabolomics of Host-Parasite Interactions

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The application of metabolomics, the global analysis of metabolite levels, to the study of protozoan parasites has become an important tool for understanding the host-parasite relationship and holds promise for the development of direly needed therapeutics and improved diagnostics. Research advances over the past decade have opened the door for a systems biology approach to protozoan parasites with metabolomics, providing a crucial readout of metabolic activity. In this review, we highlight recent metabolomic approaches to protozoan parasites, including metabolite profiling, integration with genomics, transcription, and proteomic analysis, and the use of metabolic fingerprints for the diagnosis of parasitic infections.

Introduction

Enzymes are central to every aspect of biology, and metabolite levels (as substrates, products, and cofactors) are the crucial readout of the combined enzyme and transporter activity within cells. Technological advances during the past decade now allow for the rapid and accurate measurement of the abundance levels of hundreds to thousands of metabolites simultaneously from complex mixtures. These techniques, collectively referred to as metabolomics, fill a crucial gap alongside data from other global approaches characterizing genes, transcripts, and proteins in the push toward a systems-level characterization of cells. Metabolomic analysis has been widely applied to study the systems biology of numerous model organisms across the tree of life, including Archeae (Trauger et al., 2008), Eubacteria, (Rabinowitz, 2007), fungi, (Brauer et al., 2006), plants (Cho et al., 2008; Lisec et al., 2008), animals (Pedersen et al., 2008; Sun et al., 2007), and human cell tissue culture (Khoo and Al-Rubeai, 2007). Application of metabolomics to the study of protozoan parasites, while still in its infancy, is rapidly emerging as a fertile approach to better understand the host-parasite interaction.

Protozoan parasites cause serious infections in humans and animals, including life-threatening diseases such as malaria (*Plasmodium spp.*), African sleeping sickness (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and leishmaniasis (*Leishmania spp.*). Though these diseases affect few in the developed world, they continue to threaten the health and livelihood of a large portion of humanity. To make matters worse, once efficacious antimalarial drugs, such as the antifolates and 4-aminoquinolines, have been rendered useless due to widespread parasite resistance (Guerin et al., 2009).

From a systems biology perspective, studying parasitic disease is particularly enticing, as it inherently involves the complex interplay of two interconnected biological systems with a net flow of energy and nutrients between the host and the parasite. In the process of adapting to their host niche, these organisms have evolved reduced metabolic capacity, often drastically so, while expanding mechanisms for avoiding host defense and for utilizing metabolites from their hosts. Some, like the African trypanosome, are so adept at avoiding the host defense mechanisms that they grow freely in the blood stream, where they are bathed in nutrients. Others, such as the malaria parasite, have adapted to life within host cells, which offers certain protections but can place additional barriers to nutrient acquisition. Many parasites must also infect multiple host species in order to complete their lifecycle, which can require a drastic remodeling of parasite metabolism to adapt to an entirely different metabolic milieu, as in the mammal/arthropod transitions of vector-borne parasites (Aly et al., 2009).

The current paucity of effective preventative or therapeutic options is another strong motivation for the study of parasite metabolism. No effective vaccine exists for the prevention of protozoan parasitic disease in humans, and the number of effective and affordable drugs, small to begin with, is progressively shrinking with rising incidence of resistance, making the development of novel antiparasitic drugs imperative. Most drugs against infectious disease exploit differences in metabolism by targeting pathogen enzymes that are absent or highly divergent from those of the host (Agüero et al., 2008). This strategy has been highly successful for evolutionarily distant bacterial pathogens, but protozoan parasites, as fellow eukaryotes, present fewer divergent drug targets due to a shared eukaryotic ancestry and their reduced metabolic capacity. Understanding parasite metabolic networks, including the role of novel organelles such as the apicomplexan apicoplast (the nonphotosynthetic remnant of an algal symbiont) (Seeber et al., 2008) or the mitosomes/hydrogenosomes of low branching protozoa (Hackstein et al., 2006), will be crucial for developing effective new drugs.

The current state of research on protozoan parasites is primed for systems biology approaches, including global metabolite analysis. With few exceptions, one or more stages of the important protozoan pathogens of humans can be grown in culture to densities that allow isolation of significant amounts of DNA, RNA, proteins, and metabolites. Annotated draft sequences exist of genomes from all major groups of protozoan pathogens that infect humans (many accessible via http://www.eupathdb.org). The available genomes and expressed sequence tags have

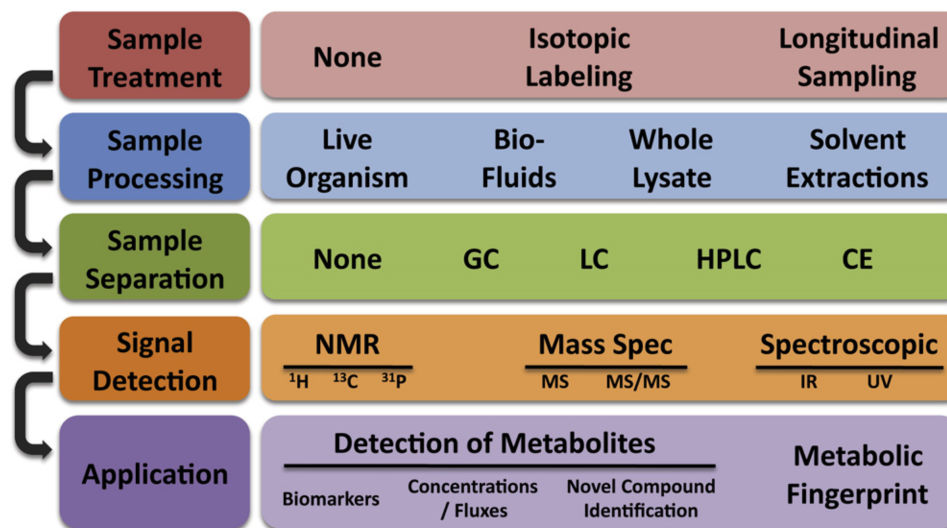

Figure 1. Commonly Used Methodologies in Metabolomics

The diagram above outlines the major steps of metabolomic experiments, along with some commonly used options available for each step. Prior to sample processing, biological materials of interest may be isotopically enriched to follow the metabolism of labeled compounds over time. Some technologies, such as nuclear magnetic resonance (NMR) spectroscopy, allow for the monitoring of metabolites within live organisms or assaying whole lysate directly, but often, sample complexity is reduced by either limiting the material sampled to specific biofluids (e.g., urine or plasma) or performing solvent extractions (e.g., methanol/water, methanol/chloroform, or perchlorate). Furthermore, mass spectrometry (MS) and spectroscopic (infrared [IR] or ultraviolet [UV]) approaches often utilize chromatography (gas [GC], liquid [LC], or high-performance liquid [HPLC]) or capillary electrophoresis (CE) to limit the sample complexity entering the detector at any one time and to determine a characteristic retention time. Mass spectrometry utilizes the retention time for a given metabolite, as well as the mass/charge ratio (MS), often in conjunction with the mass/charge ratio of fragmentation daughter ions in tandem MS (MS/MS), for identification. NMR exploits structurally dependent changes in the magnetic resonance of suitable nuclei (e.g., ^{1}H, ^{13}C, or ^{31}P) for metabolite identification. Light spectroscopic approaches do not perform as well for the individual identification of metabolites but represent a more cost-effective approach to determining changes in the overall metabolic profile or fingerprint of a sample. Both NMR and MS approaches offer the possibility for the detection of dozens to hundreds of individual metabolites, but recent advances also permit more accurate measurements of metabolite concentrations, which are critical for determining metabolite fluxes within a metabolic network.

opened the door for proteomics by allowing peptide mapping and for the design of DNA microarrays that have already been used extensively for transcriptome analysis of numerous parasites, including *Plasmodium spp.*, *T. gondii*, and the kinetoplastids.

Integrating metabolomics with the analysis of the genomic, transcriptomic, and proteomic data already in place opens the door for systematic study of the host-parasite interaction that will lead to a deeper biological understanding and is likely to unveil additional targets for prevention and therapy. In this review, we aim to outline the current status of using metabolomics in the study of protozoan parasites and highlight numerous promising applications.

Definitions and Methodology

Broadly defined, the "metabolome" is the collection of all small molecules (<1 kDa) of a given system, and "metabolomics" is the characterization of this set by one or more techniques. The more narrowly defined term "metabonomics" refers to the quantitative measurement of metabolic responses over time within a system in response to an event (infection, drug treatment, starvation, etc.) (Holmes et al., 2008). In the case of a host-parasite system, metabolomics often involves the characterization and interplay of each organism's metabolome or a characterization of the host cell's metabolome in the absence or presence of the parasite, known as the cometabolome (Holmes et al., 2008).

Historically, enzyme-based assays and tracing of radiolabeled nutrients were the major tools for assaying parasite metabolism (Sherman, 1998). Today, nuclear magnetic resonance (NMR) spectroscopy and separation-linked single (MS) or tandem (MS/MS) mass spectrometry are the major tools in use for metabolomics. Their use in metabolomics has been expertly reviewed elsewhere (Bollard et al., 2005; Lenz and Wilson, 2007; Want et al., 2007). Two recent reviews by Besteiro et al. and Scheltma et al. also provide a helpful overview of available metabolomic approaches in parasitology as applied to the analysis of phospholipid biosynthesis in *Plasmodium spp.* (Besteiro et al., 2010) and *Leishmania* (Scheltema et al., 2010), respectively. We provide a cursory overview of various methodological options in Figure 1, but a more detailed description and comparison of these technologies is outside its scope.

Laying the Groundwork: Profiling Parasite Metabolomes

A number of pioneering studies published in the 1980s first applied NMR spectroscopy to parasite metabolism (expertly reviewed by S.N. Thompson [Thompson, 1991]). Many of these early studies focus on the profiling of major parasite metabolites. To avoid significant overlap with this comprehensive review, our review will focus only on subsequently published work.

Since 1990, ^{31}P-NMR has continued to be used for profiling various aspects of parasite phosphate metabolism in *Plasmodium spp.*, *Toxoplasma gondii*, and *Cryptosporidium parvum* (Moreno et al., 2001), as well as in the kinetoplastids (Mendoza et al., 2002). Similarly, the use of ^{13}C-NMR for metabolite mapping has also continued, including the characterization of abundant metabolites in *Entamoeba histolytica*- (Bakker-Grunwald et al., 1995) and *P. falciparum*-infected erythrocytes (Lian

et al., 2009), measuring glucose flux in *P. falciparum*- and *P. yoelii*-infected erythrocytes (Mehta et al., 2006), and measuring the suppression of glycolytic activity in uninfected erythrocytes by malaria-conditioned medium (Mehta et al., 2006).

With increasing magnet strength and improved signal filtering, the use of ^{1}H-NMR for metabolite mapping has steadily grown and yielded several studies mapping the metabolome of kinetoplastid parasites, including *L. donovani* (Gupta et al., 1999) and *T. cruzi* (Penin et al., 1998), as well as the lipidome of *L. donovani* (Adosraku et al., 1993). A recent metabolic analysis of *P. falciparum* trophozoite extracts compared several extraction conditions and identified more than 50 metabolites with measured concentrations in the high nM/low μM range for 40 separate metabolites (Teng et al., 2009). A surprising finding of this study included the accumulation of HEPES within the parasite, a buffering agent used in culture medium, further highlighting the considerable differences between in vitro and in vivo growth conditions (reviewed in LeRoux et al., 2009). In a complementary study using an LC-MS/MS approach, Olszewski et al. sampled synchronous *P. falciparum* blood-stage cultures at 8 hr intervals and assayed 89 known metabolites across the 48 hr intraerythrocytic cycle (Olszewski et al., 2009).

Kamleh and colleagues compared the metabolome of *T. brucei* procyclic forms grown on glucose, the preferred carbon source, or proline, which is abundantly available in the Tsetse fly vector. Using hydrophilic interaction chromatography (HILIC) and electrospray ionization (ESI) mass spectrometry, more than 1000 distinct peaks were resolved, including many phospholipids and lipophilic amino acids, which can be difficult to resolve using standard reverse-phase HPLC. Numerous metabolites changed significantly in abundance following a switch in carbon source from glucose to proline, including several glutamine derivatives and phospholipids (Kamleh et al., 2008). Finally, an earlier study determined the uptake and catalysis of polyamines and thiols by *T. cruzi* trypomastigotes and amastigotes by HPLC/MALDI-TOF MS (Ariyanayagam et al., 2003).

Toward Parasite Systems Biology: Integrating Global Approaches

Whereas small molecule profiling provides an important baseline for the metabolic characterization of parasites, studying their metabolic response (and underlying regulation) due to environmental or genetic changes has the potential to yield greater insight into parasite biology by investigating how the interplay of genetics and environment affects changes in the metabolome and how changing metabolites feed back into the regulation of gene expression and enzymatic activity.

Inferring Metabolic Networks: Pitfalls and Promises

The sequencing of parasite genomes has yielded unprecedented insights into how parasites have adapted to their various niches and have become increasingly dependent on their hosts by sacrificing the ability to synthesize nutrients de novo for less-energy-intensive scavenging mechanisms. Identification of conserved metabolic enzyme pathways (Pinney et al., 2005) and nutrient transporters (Ginsburg and Stein, 2005; Martin et al., 2005) by comparative genomics has allowed the construction of metabolic networks for these organisms in silico (Chavali et al., 2008; Ma and Zeng, 2003). These networks have been a powerful tool in guiding parasite research by laying out parasites' anabolic and catabolic potential (Pinney et al., 2005), highlighting potential drug targets (Cornish-Bowden and Cárdenas, 2003; Pinney et al., 2007), and attempting to identify metabolic gaps and novel pathways (Huthmacher et al., 2008; Zamboni and Sauer, 2009).

A variety of interfaces and databases are available to browse the annotated genomes of protozoan parasites, with http://www.eupathdb.org being the most comprehensive. The annotation depth and quality of parasite genomes varies considerably between species according to funding and human capital invested to date. Protein coding genes are generally automatically annotated by a variety of homology methods (Interproscan, OrthoMCL-DB) and assigned Gene Ontology function terms and Enzyme Commission numbers when possible. A number of automated and hand annotated approaches have been used to build metabolic networks of protozoan parasites from functionally annotated genes, including the metaTiger KEGG interface, the ApiCyc and LeishCyc databases, and the Malaria Parasite Metabolic Pathways (MPMP) database. In a recent review, Hagai Ginsburg outlines advantages and shortcomings of these approaches for building metabolic networks of the malaria parasite—lessons that are likely to apply generally to attempts of building metabolic pathways of other protozoa (Ginsburg, 2009). An inherent shortcoming of homology-based approaches for predicting metabolic networks is that they cannot predict the function of novel or highly divergent metabolic genes. Such ‘‘hypothetical’’ genes of unknown function represent a sizable fraction of predicted genes in the available parasite genomes (Carlton et al., 2008). Depending on their activity, unidentified metabolic genes among them could alter the structure of metabolic networks considerably.

Finding Missing and Essential Links: Using Metabolomics to Improve Metabolic Networks

In silico metabolomic networks are particularly valuable for identifying metabolic gaps, ‘‘missing’’ enzymes based on otherwise complete pathways or orphan biochemical activities, and for highlighting differences in host and parasite metabolism that may be exploited for drug therapy. Apart from enzymatic evidence, various sources of experimental data have been used to improve the accuracy of metabolic networks based on genomic sequence, including phylogenetic conservation of pathways (Chen and Vitkup, 2006), patterns of coexpression at the transcript (Brauer et al., 2005) or protein level (May et al., 2008), and, increasingly, metabolite presence and abundance (Brauer et al., 2006; Breitling et al., 2008; May et al., 2008). Recently, Mohanty et al. used a homology-based search for missing enzymes to assign putative function to several ‘‘hypothetical’’ genes and fill 14 of their 69 predicted metabolic gaps in *P. falciparum* (Mohanty and Srinivasan, 2009). Comprehensive profiling of parasite metabolomes is certain to reveal additional gaps through the identification of ‘‘orphan’’ metabolites or activities. Ultimately, the use of metabolomics for the identification of previously uncharacterized metabolites will also set off the search for the enzymes involved in their synthesis (Kalisiak et al., 2009).

Recent studies have used metabolic network analysis to identify essential activities in protozoan parasites that may be promising targets for drug design. In an excellent example of this approach, Chavali et al. mapped a metabolic network of

Leishmania infantum that incorporated 560 genes, 1112 reactions, 1101 metabolites, and 8 subcellular localizations across both the amastigote and trypomastigote life stages. Using this network, the authors were able to predict minimal parasite nutritional requirements, as well as metabolic reactions that are essential to parasite survival for both single- and double-knockout mutations (Chavali et al., 2008). In a similar approach, Roberts et al. used a combination of genomic and proteomic data to generate a compartmental model of *T. cruzi* epimastigote metabolism covering 215 genes, 162 reactions, and 158 metabolites. In addition to using this approach for predicting essential enzymatic activities, they identified several enzymes that are critical for epimastigote survival that were previously thought to be nonessential, and also found reactions that were previously thought essential, which were dispensable. Taking their research a step further, these predictions were validated using RNA interference (Roberts et al., 2009). Using an in silico approach, Yeh et al. (Yeh et al., 2004) and Fatumo et al. (Fatumo et al., 2009) used metabolic network analysis to identify essential enzyme activities in *Plasmodium falciparum*. Analyses such as these are currently being combined with other considerations to prioritize parasite drug targets by the WHO in pathogens causing neglected diseases (Agüero et al., 2008).

Many properties of metabolic networks cannot be fully determined based on interconnectivity and coexpression alone but depend critically on the reaction rates of each step (Sauer, 2006). Metabolite flux through the network can be probed by feeding isotopically labeled nutrients and following label incorporation into downstream metabolites by successive metabolomic analyses (Zamboni et al., 2009). Isotopic labeling can reveal nonintuitive network behavior and network redundancy (Blank et al., 2005), highlighting potential drug targets (Munger et al., 2006) and mechanisms of action (Kwon et al., 2008) that are not apparent based on interconnectivity alone. With the exception of work on the *E. histolytica* glycolysis pathway (Moreno-Sánchez et al., 2008), very little work has been done on metabolic flux analysis in protozoan parasites. The application to host-parasite systems is likely to be an important tool for rational drug design of novel antiparasitic compounds and understanding the mechanisms of action of existing drugs, such as artemisinin, which remain contentious (Eastman and Fidock, 2009).

These studies illustrate how interpreting parasite metabolism in light of the genome alone can be misleading, and it is important to remember that in silico metabolic networks only reveal the metabolic potential of these organisms. The actualization of this potential is restricted by numerous levels of regulation, including lifecycle-specific gene expression, subcellular protein localization, enzyme concentration and posttranslational regulation, metabolite transport, and substrate, product, and cofactor abundance. Global transcriptional and proteomic analyses have helped narrow these questions by revealing which parts of the networks are coexpressed but unfortunately cannot directly describe the cellular metabolic status. Hence, integrating mechanisms of regulation with the actual readout of metabolic activity, metabolite levels, and fluxes is crucial for a systematic understanding of parasite metabolism and rational identification of drug targets. Of course, obligate intracellular parasites pose a unique challenge to flux analysis because two interconnected metabolic networks are at work: that from the host and that of the parasite. Therefore, defining the source of the labeled metabolites is more difficult. Furthermore, nutrients that are solely obtained from the host cell will be difficult to label for the study of parasite metabolism, making flux analysis virtually impossible for these nutrients.

Break Something and Measure What Happens: Applications to Reverse Genetics

Combining metabolomics with reverse genetics (genetic or chemical knockouts/knockdowns) is very appealing, particularly for targets with potential enzymatic activity, as it allows for the rapid characterization of hundreds of metabolic phenotypes simultaneously. For this reason, it has been one of the areas in the study of parasites in which metabolomics has taken a solid foothold. In the Apicomplexa, due to their haploid genomes, knockout parasite lines are particularly well suited for metabolomic studies. The susceptibility of *T. brucei* to RNA interference (Wang et al., 2000) enables a powerful combination of reverse genetics and metabolomics among the kinetoplasts.

Van Weelden et al. used a combination of HPLC-UV/conductivity detection, ^{14}C-radiolabeled glucose, and ^{13}C-NMR to measure glycolysis and TCA cycle intermediates, as well as ATP/ADP/AMP/P_i levels, in a *T. brucei* wild-type strain and two independent aconitase knockout strains to validate the dispensability of the TCA cycle for energy generation in *T. brucei* procyclic forms (van Weelden et al., 2003). In a second metabolomic study of *T. brucei*, Coustou et al. used ^{13}C-NMR to compare the utilization of D-[1-^{13}C]-glucose (preferred) or L-[4-^{13}C]-proline (highly available in the insect host) as the primary carbon source for wild-type procyclic forms. Combining this approach with RNAi knockdown mutants, the authors were able to develop a detailed metabolic model of proline utilization in the presence and absence of glucose (Coustou et al., 2008).

Studies using 13C-NMR in *Leishmania* have taken a metabolomics approach to investigate the critical role of fructose-1,6-bisphosphatase in gluconeogenesis for *L. major* survival in macrophages by illustrating the parasite's inability to utilize labeled fatty acids as gluconeogenic substrates and demonstrating the loss of virulence in fructose-1,6-bisphosphatase knockout parasites (Naderer et al., 2006). De Souza et al. used the effect of two gene deletions (a glucose transporter and phosphomannose isomerase) on the *L. mexicana* metabolome to validate improved noise filtering using a new peak clustering algorithm, comparing metabolite extracts from wild-type and knockout strains (De Souza et al., 2006).

In an apicomplexan example, Olszweski et al. used LC-MS of growth medium from cultures of synchronized malaria parasites to link arginine depletion and ornithine enrichment to the parasite arginase by demonstrating stable arginine and ornithine levels in ex vivo cultures of arginase knockout *P. berghei* parasites (Olszewski et al., 2009).

Currently, there are numerous genetically altered parasite lines available (http://www.mr4.org, http://www.atcc.org, and http://www.pberghei.eu), which could be used to assay metabolic perturbations due to genetic lesion. Recent studies have generated type II fatty acid synthesis (FAS II) knockout mutants in *Plasmodium spp.* (Vaughan et al., 2009; Yu et al., 2008) and *Toxoplasma gondii* (Mazumdar et al., 2006) and demonstrate genetically that FAS II is essential only during late-stage liver development in Plasmodium but necessary for tachyzoite

growth in *Toxoplasma*. Such mutants are ideal for the characterization of the metabolic phenotypes downstream of these mutations under both lethal and nonlethal conditions using metabolomic approaches for both parasites.

From Metabolites to Genes: Applications to Forward Genetics

The application of quantitative trait locus analysis to quantitative measurements of global metabolite levels (mQTL) has emerged as a powerful tool for dissecting the genetic basis of metabolism in plants (Keurentjes et al., 2006). Assaying 84 metabolites (50 of known chemical structure) by GC-MS in shoots of 429 genotyped inbred *A. thaliana* lines, Lisec et al. were able to identify 157 metabolite-specific QTLs, mapping to enzymes in the appropriate metabolic pathway for 67% of QTLs. The number of contributing loci varied from just 1 for each of 42 metabolites to 6 QTLs contributing to tyrosine metabolism. Furthermore, the study revealed the presence of mQTL hot spots, loci that affect the levels of many different metabolites (Lisec et al., 2008).

Several sets of genotyped F1 lines are available from crosses in both *P. falciparum* (Hayton et al., 2008; Walliker et al., 1987; Wellems et al., 1990) and *T. gondii* (Sibley and Boothroyd, 1992; Su et al., 2002). Using QTL analysis, these crosses have been used successfully to map the genetic basis of a number of phenotypes, including resistance to a variety of drugs (Ferdig et al., 2004; Fidock et al., 2000; Mu et al., 2003; Nair et al., 2008; Yuan et al., 2009), host cell binding (Hayton et al., 2008), and virulence (Saeij et al., 2006; Taylor et al., 2006). Given a sufficient difference in metabolic phenotypes of the parental strains, metabolomic analyses of daughter lines from these crosses could be used to map the genetic basis of a variety of phenotypes, including starvation responses, compensatory mechanism in response to manipulation of metabolic genes, and metabolic effects of drug treatment, potentially identifying unintended, secondary drug targets.

Controlling the Machinery: Metabolomics and Gene Regulation

Understanding the interplay of an organism's environment and regulation of gene expression were among the first problems of molecular genetics (Jacob and Monod, 1961). What started out as a simple system of less than a dozen components controlling lactose utilization by *E. coli* has grown into the study of systems biology, monitoring gene expression across an organism's entire genome and measuring hundreds of metabolites within the organism and the environment.

Correlating transcriptional responses to environmental stimuli has yielded a deeper, systematic understanding of the biology of a great variety of organisms, such as environmental adaptation by extremophiles (Trauger et al., 2008), optimizing bacterial biofuel production (Yang et al., 2009), understanding yeast metabolic cycle changes in response to environmental disturbances (Gasch et al., 2000), and temperature stress in fruit flies (Pedersen et al., 2008). But combining the study of gene expression at either the transcript or protein level with metabolomic approaches has been rare in the study of host-parasite interaction.

In order to compare the response of mice either resistant or susceptible to cerebral malaria, Rae et al. measured gene transcript levels of 21 metabolic and immune response genes expressed in the brain and determined brain metabolite levels using ^{13}C- and ^{31}P-NMR but were unable to correlate changes in gene expression and metabolite levels in mice susceptible to cerebral malaria (Rae et al., 2004). This could possibly be due to the fact that correlations of gene expression and metabolite changes occur in a more localized fashion and are lost when integrated over a whole organ as diverse as the brain.

Two *P. falciparum* studies have correlated changes in malaria gene expression with metabolite measurements over the intraerythrocytic developmental cycle using global transcription microarray and LC-MS/MS analysis. Olszewski et al. monitored the levels of 119 metabolites in synchronized *P. falciparum* culture at 8 hr intervals, sampling both the media and infected red blood cells while simultaneously extracting total RNA for microarray analysis. Several metabolites correlated strongly with transcript levels of enzymes involved in their synthesis and/or catabolism, including 5-methylthioinosine and α-ketoglutarate (Olszewski et al., 2009).

Taking a broad systems approach, van Brummelen et al. characterized the global effect of inhibiting the bifunctional enzyme PfAdoMetDC/ODC on the *Plasmodium falciparum* intraerythrocytic developmental cycle by following global transcript, protein, and metabolite abundance, using DNA microarrays, 2D PAGE-MALDI-MS/MS, and LC-MS/MS, respectively (van Brummelen et al., 2009). PfAdoMetDC/ODC inhibition resulted in growth arrest and compensatory changes at the transcript and protein level, including downregulation of several enzymes in the polyamine synthesis pathway downstream of PfAdoMetDC/ODC, while ornithine decarboxylase, which generates the PfAdoMetDC/ODC substrate ornithine, increased along with lysine decarboxylase, which produces the polyamine cadaverine independently of PfAdoMetDC/ODC. As expected, metabolite analysis revealed a significant drop in parasite polyamine levels, specifically spermidine and putrescine, with a concomitant rise in the upstream metabolite S-adenosylmethionine.

These studies mark the first steps toward parasite systems biology and illustrate how parasite metabolite levels fluctuate in response to changes in parasite gene expression at the transcriptional and protein level and how metabolite concentrations can feed back to regulate expression of metabolic genes.

It Takes Two to Tango: Metabolomics of Host-Parasite Systems

By its very nature, parasite metabolism cannot be understood without integrating it with that of the host. Metabolomic profiling of host fluids and tissues and changes in response to infection with protozoan parasites are critical for understanding parasite pathogenesis and show promise for use in diagnostics.

Biomarkers of Infection

A major goal of the application of metabolomics to the study of parasites is the identification of diagnostic biomarkers. Standard diagnostic methods for parasitic infections rely heavily on microscopy, which is time intensive and requires highly trained personnel, as well as specialized equipment. Metabolomic identification of infection biomarkers is an important step toward the development of rapid and cost-effective diagnostics (Holmes et al., 2008). Identification of infection biomarkers has been successful in a variety of experimental systems, including in the plasma of lymphocytic choriomeningitis virus-infected mice (Wikoff et al., 2009b), in the cerebral spinal fluid of simian immunodeficiency virus-infected rhesus macaques (Wikoff et al.,

2008), and in sera of Hepatitis B virus-infected humans (Yang et al., 2006).

Sample acquisition for diagnostic purposes can be intrusive and requires sterile equipment not always available, making the use of biomarkers from nonintrusive samples (saliva/urine) particularly appealing. This approach has already been applied successfully in the diagnosis of a number of nonparasite disease states (reviewed in Nordstrom and Lewensohn, 2009), including early diagnosis of various cancers, markers of renal failure, and response to a variety of drugs. The development of metabolic biomarkers of parasite infection for diagnostic purposes remains in its infancy, but promising work has emerged during the past few years.

Recently, several studies have used both NMR (Li et al., 2009; Nishina et al., 2004; Saric et al., 2009; Wang et al., 2004) and UV spectroscopy (Angulo et al., 2009) for metabolomic analysis of host samples (plasma, urine, or stool) to identify biomarkers of infection with a variety of parasitic helminths. These approaches were able to differentiate infected from uninfected animals, often early in the course of infection, using either abundance changes in the metabolomic profile or metabolites unique to infected animals. Similarly, metabolite analysis of histological samples was able to demonstrate tissue-specific responses to infection. A shared thread of these studies was significant changes in metabolites produced by the intestinal flora following infection with intestinal and extraintestinal helminths alike, indicating that changes in the composition of the intestinal flora can have significant effects on the metabolite profile of the host (Wikoff et al., 2009a; Yap et al., 2008) and are a common outcome of infection.

Markers of Protozoan Infection

Fewer metabolomic studies have attempted to develop biomarkers of protozoan infections. Using ^{1}H-NMR and working in the *P. berghei* mouse model, Li et al. found drastic decreases in plasma glucose levels and a concomitant rise of lactate and pyruvate as the result of anaerobic glycolysis by the parasite, as well as a drop in glycerophosphoryl choline, which acts as a source of choline for the parasite. Analysis of urine from infected mice again found evidence of changes in the intestinal flora and, of note, excretion of pipecolic acid and two unknown metabolites absent from the urine of uninfected mice, though many of these changes only became apparent when parasitemia exceeded 15% of circulating erythrocytes (Li et al., 2008).

Using ^{13}C- or ^{31}P-NMR and *P. berghei*, Rae et al. compared brain glucose metabolism and bioenergetics in response to infection with the ANKA strain, which causes cerebral malaria, to infection with the K173 strain, which does not. In mice with cerebral malaria, there was a notable reduction in brain metabolic activity along with a shift in D-[1-^{13}C]-Glucose utilization from the TCA cycle to (1) anaerobic metabolism, (2) the glutamate/glutamine cycle, and (3) the GABA shunt. The increased utilization of the glutamate/glutamine cycle preceded increases in lactate and alanine, suggesting that increased gluteminergic metabolism is not the result of brain hypoxia but is due to other metabolic disturbances. Mice infected with the K173 strain, on the other hand, displayed evidence of increased ($\sim$2-fold) cerebral metabolic rate with elevated glucose utilization and relatively uniform incorporation into metabolites. Energy metabolism as monitored by ^{31}P-NMR revealed a significant increase in inorganic phosphate and phosphocreatine, consistent with increased anaerobic metabolism and cerebral hypoxia in mice with cerebral malaria (Rae et al., 2004).

A third study compared the effects of *P. berghei* ANKA infection in mice susceptible (CBA/J) or resistant (BALB/c) to cerebral malaria via in vivo NMR and ^{1}H/^{31}P-NMR metabolite profiling of blood and brain extracts. Cerebral malaria-susceptible mice showed brain anomalies absent from resistant mice at similar parasitemia levels, including an unexpected elevation of cerebral blood flow. Measuring brain metabolite levels revealed a notable drop in choline-derived compound levels, important for membrane and myelin homeostasis (Penet et al., 2007). Furthermore, this study found increases in brain glutamine and alanine levels in susceptible mice, consistent with increased blood ammonia levels, as well as the decreased levels of brain *myo*-inositol, which are thought to compensate for increased intracellular osmolarity due to glutamine accumulation. These changes are compatible with hepatic encephalopathy and were supported by significantly increased blood bilirubin levels, significant changes in liver enzyme function, and an overall increase of hepatic tissue pathology in susceptible mice (Penet et al., 2007).

Tracking the urine and plasma metabolome for 33 days by ^{1}H-NMR allowed for the differentiation of mice infected by African trypanosomes from uninfected animals, starting at 1 day postinfection, with differences becoming more pronounced as the infection progressed (Wang et al., 2008). Notable changes in urine were seen for several markers, including 3-carboxy-2-methyl-3-oxopropanamine as a unique marker of infection. Analysis of plasma metabolite levels was able to correlate increases in lactate, oxaloacetate, and glucose, as well as decreases in glutamine and lysine with infection. Starting on day 7, there were also notable changes in plasma markers. The identification of several unique urinary metabolites present early in disease progression is promising, as treatment options for African trypanosomiasis are considerably better while the parasite remains confined to the bloodstream. Once the parasite crosses the blood-brain barrier, prospects of survival dim considerably. Identification of urine or plasma biomarkers of central nervous system invasion by the parasite would mark a major advance beyond the diagnostic standard of microscopic inspection of cerebral spinal fluid.

So far, metabolomic biomarker studies of parasite infections have performed well in differentiating infected from uninfected animals and in highlighting the effect of infection on the intestinal flora. However, these approaches remain far from being useful in a diagnostic context, as many of these biomarkers appear to simply be indicators of infection rather than being pathogen specific (Koulman et al., 2009). Blinded studies using several pathogens and the same host will be needed to validate the use of metabolomics in producing correct differential diagnoses. For instance, changes in the composition of the host's intestinal microflora can result in major changes of blood and urine metabolites (Wikoff et al., 2009a; Yap et al., 2008) and appear to be a common side effect in parasitic infections. The recurrence of cometabolites (e.g., trimethylamine or hippurate) produced by the intestinal flora as prominent biomarkers in these model systems using controlled nutrition and isogenic hosts foreshadows the large potential for variability when the diversity of

host genetics, intestinal flora composition, and nutritional status is considerably higher.

One current application of metabolomics to medical parasitology has been in the diagnosis and monitoring of large hydatid cysts in the brains and peritoneal cavity that are caused by cestodes in the genus *Echinococcus*. These cysts can be diagnosed and monitored by MRI and display characteristically high end product metabolites of the parasite's anaerobic glycolysis, which decrease with successful treatment (Seckin et al., 2008). Such metabolite-based diagnostics hold great promise for the future and will ultimately enable rapid assessment of patients in the field.

Future Directions

Global metabolite analysis is a powerful tool for understanding the biology of protozoan parasites and host-parasite interactions. In this review, we have presented a wide variety of applications for metabolomics to protozoan parasites, including work toward integrating global metabolite levels with genomic, transcriptional, and proteomic analysis in search of a systems-level understanding of parasite metabolism and gene regulation. Given the dire need for better antiparasitic drugs and diagnostics, metabolomics is becoming a valuable tool in identifying potential drug targets and defining markers of infection. In addition, characterizing the effect of novel drugs on host and parasite metabolite levels promises to identify their mechanisms of action. A number of fascinating questions in parasite biology are likely to benefit tremendously through metabolomic analysis. Of great interest is the dissection of potentially noncanonical biochemical pathways and of parasite-specific metabolism. One such example is deciphering the actual function of the tricarboxylic acid (TCA) cycle of blood-stage malaria, as it is known not to be a major source of energy or reducing power in these parasites (Mogi and Kita, 2009; Seeber et al., 2008). Probing the TCA cycle using metabolite flux with ^{13}C-enriched nutrients is likely to yield novel insights into how these parasites utilize TCA cycle enzymes and their contribution to central carbon metabolism.

Recent work by Blume et al. showed that TgGT1, the major glucose transporter at the parasite plasma membrane of *T. gondii*, is nonessential for parasite growth in vitro but results in growth and motility defects that can be partially ameliorated by increasing media glutamine levels (Blume et al., 2009). Metabolomic characterization of this substrate switch could provide insight into the robustness of *T. gondii* metabolism and its ability to adapt to changing nutritional environments.

Other promising applications include understanding how parasite metabolic requirements vary upon differentiating from one life stage to the next or following transmission to new host species. For example, as *Toxoplasma* parasites differentiate into slow-growing bradyzoites, they become increasingly resistant to many drugs effective against the fast-growing tachyzoite stage, yet the underlying metabolic changes responsible for this resistance are still poorly understood. Similarly, when malaria parasites differentiate into sexual-stage gametocytes, they become increasingly resistant to many drugs that are effective against the other blood stages. A further area of interest would be the use of metabolomics to explore the host response to parasite infection both in terms of how parasites siphon energy and metabolites from the host and how the host can modify its metabolism to combat the infection, as in the case of tryptophan restriction in mice to control growth of *Toxoplasma* (Däubener et al., 2001). With the recent advances in methodologies for measuring complex mixtures of cellular biomolecules, metabolomic studies of parasites hold great promise for the improved understanding and control of protozoan parasites.

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