Integrated Metabolic Flux and Omics Analysis of *Leishmania major* metabolism

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Abstract

Leishmaniasis is a virulent parasitic infection that causes a significant threat to human health worldwide. The existing drugs are becoming less effective due to the ability of *Leishmania spp.* to alter its metabolism to adapt to harsh environments. Understanding how this parasite manipulates its metabolism inside the host (e.g., sandfly and human) might underpin new ways to prevent the disease and develop effective treatment strategies.

Despite significant advances in omics technologies, biochemistry of parasites still lacks the understanding of molecular components that determine the metabolic behavior under varying conditions. Metabolic network modeling might be of interest to identify physiologically relevant nodes in a metabolic network.

The present work proposes a metabolic model iSK570 (an extension of the iAC560 model) with additional reactions for the metabolism of lipids, long chain fatty acids and carbohydrates to study the metabolic behavior of this parasite. Gene Inactivity Moderated by Metabolism and Expression (GIMME) algorithm was used to verify the consistency between model flux predictions and gene expression data. Improved flux distributions were obtained, allowing a more accurate understanding of stage-specific metabolism in of promastigotes and amastigotes.

1. Introduction

Protozoan parasites from the genus *Leishmania* belong to the family Trypanosomatidae, and cause a spectrum of human diseases affecting around 12 million people worldwide (www.who.int). Existing treatment therapies involving drugs such as e.g. sodium stibogluconate and meglumine antimoniate, amphotericin B and miltefosine are limited by lack of efficacy [1,2]. Considering endemic severity of the disease, there is an urgent need for understanding *Leishmania* metabolism which can subsequently help in developing novel anti-leishmanial therapies.

Significant alterations have been observed in the metabolism exhibited by *Leishmania* at different stages of its life cycle, where it faces different nutritional environments [3]. For example, the promastigote form (inside sandfly) of *Leishmania* preferably uses glucose and L-proline via glycolysis pathways and TCA cycle; while amastigote uses glucosamine (GlcN) and its derivative N-acetylglucosamine (GlcNAc) along with some lipids and amino acids [4,5]. Availability of various sugars, such as hexoses (e.g. glucose, mannose, and galactose) and amino sugars (e.g. GlcN and GlcNAc) are determining factors for parasitic metabolic phenotype, especially for synthesizing essential glycans and glycoconjugates [6].

Unfortunately, no previous studies have explained the metabolic basis leading to the biosynthesis of glycans and glycoconjugates in the presence of different environments. In fact, it is still unknown if observed metabolic changes are resulting from, or arising out of the different parasitic stages. For example, under promastigote stage, only a few enzymes from the TCA cycle are active, while in amastigote stage, glycolytic enzymes are less functional.

Metabolic network modeling is an effective and sophisticated approach for systematically study the metabolic behaviour of an organism, as well as to understand the relationship between its genotype and phenotype. Previously, these methods have been used to understand the cellular metabolism as well as to identify essential genes in many medically important organisms, such as *Mycobacterium tuberculosis* [7], *Acinetobacter baumannii* [8], *Francisella tularensis* [9] including human parasites like *Leishmania major* [10] and *Plasmodium falciparum* [11]; though with the low prediction accuracy. One of the most probable and obvious reasons for the low prediction accuracy might be associated with the lack of use of experimental data (e.g. transcriptomics, proteomics, and metabolomics etc.) to constrain the model and unavailability of the suitable strategies to use omics data in the metabolic network analyses.

Integrating omics data with metabolic network analysis can improve our understanding on various aspects, such as metabolic alterations associated with the environmental conditions, essential genes and metabolic flux variability of the essential reactions [12]. The relevant data can be integrated into the metabolic model to provide an extra layer of metabolic flux constraints to improve its overall prediction efficiency. Various methods like GIMME [13], iMAT [14], MADE [15], E-Flux [15] and PROM [16] have been made available for the integration of transcriptomics and genomics, fluxomics [17], and metabolomics [18] data into metabolic models. Successful examples include the integration of RNAseq data into the *Leishmania infantum* model [19], proteomics data into a metabolic model of *Enterococcus faecalis* [20], and multi-omics data into metabolic models of *Escherichia coli* [21] to understand the metabolism and associated phenotypes. The strategy has also improved drug target predictions in many medically important organisms such as *Aspergillus fumigatus* [22], *Plasmodium falciparum* [23] and *L. major* [24].

In spite of the availability of abundant omics data and various methodologies, only a few studies have employed these strategies to understand the metabolism of *Leishmania* [10,19,25]. Here, we applied omics data with metabolic modeling approaches to understand the metabolic profile of *L.
major under different environmental conditions. The workflow mainly includes the integration of gene expression data from promastigote and amastigote stages into our metabolic model using Gene Inactivity Moderated by Metabolism and Expression (GIMME) method [13].

2. Methodology

2.1. Model extension and refinement

The existing metabolic model iAC560 [10] was extended to include sugar nucleotides biosynthetic pathways, which reactions and enzyme-coding genes were collected from databases like KEGG [26] and LeishCyc [27]. As some of the reaction steps were not associated with a specific gene, homology search tools like BLAST [1], were applied to find the highest scoring gene sequences (% identity ≥ 40%, alignment length ≥ 70% and E-value 1.0e-30), as described in [19] and associate those to the corresponding reactions. Additionally, based on experimental evidence, several metabolic reactions were altered in terms of reversibility and/or compartments, while new transport reactions for sugar nucleotides, lipids, and fatty acids were also included. Refer to Supplementary material S1 for added, deleted or altered reactions.

2.2. Biomass composition

The macromolecular composition of L. major cells was also corrected. Protein, DNA and RNA contents were estimated from L. donovani studies [19], while carbohydrates, lipids, and polyamine contents were calculated using experimental data from protozoan Tetrahymena [28,29] and L. mexicana [30]. Individual carbohydrates, such as mannan, lipophosphoglycan (LPG), glycoinositol phospholipid (GIPL), and N-glycans, were estimated as follows: mannan contents were assumed to represent 80% and 90% of all carbohydrates in promastigote and amastigote stage, respectively [30], while LPG, GIPL, and N-glycans would represent 20% and 10% in total, respectively. The relative mass fractions (w/w) of LPG, GIPL, and N-glycans were estimated based on previous studies [31–34]. Further details on biomass calculations can be found in Supplementary material S2.

2.3. In-silico media formulation

2.3.1. Modified Media for Promastigote (MMP)

MMP was formulated for L. major growth under promastigote stage, which includes 16 nutrient sources: L-arginine, L-cysteine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tyrosine, L-valine, hypoxanthine, phosphate, oxygen, proline, and glucose. The nutrients, in particular, glucose and proline were considered based on the previous studies [35,36], explaining that both the compounds are major carbon source for Leishmania promastigote, while remaining ones were included considering the experimental studies [37,38] and computational predictions in [10], which concluded that Leishmania can grow in these nutrients.

2.3.2. Modified Media for Amastigote (MMA)

MMA includes all 16 nutrients from MMP with additional amino sugars, amino acids, lipids and fatty acids, making a total of 21 nutrients. GlcN and GlcNAc sugars were added considering findings from Naderer et al. (2010) studies [4] that showed the degradation of glycosaminoglycans inside macrophages to provide GlcN and GlcNAc as carbon sources during the amastigote stage. Fatty acids like stearyl acid and lipids, e.g. phosphatidylethanolamine were also considered, based on different studies that show that Leishmania utilizes lipids from host cells and transports them into the cytosol [39,40]. The consumption of the lipids and fatty acids during amastigote stages were also supported by other experimental studies discussing the possibility of growth of Leishmania axenic amastigote in lipid and fatty acid-rich medium [41–43]. The amino acids aspartate and alanine were also added to MMA, based on higher consumption measurements of these amino acids as carbon sources by amastigotes [44].

2.4. Reaction flux constraints in FBA-based simulations

Model simulations under amastigote and promastigote stages were estimated using different reaction constraints. For example, the uptake flux for proline was reduced by 90% in amastigote compared to promastigote simulations, based on the previous study showing a decrease in the consumption of this particular amino acid in L. mexicana amastigotes [35]. Also, glucose uptake flux was constrained to 90% less than that in the promastigote stage, considering previous findings [45,46], which concluded that parasitophorous vacuole is a compartment poor in glucose. Furthermore, the oxygen uptake in amastigote stage was significantly reduced as compared to that in the promastigote stage, considering the fact that Leishmania-infected macrophage is an oxygen-deficient entity [47,48]. The upper and lower limits for uptake fluxes for all other nutrients were set unconstrained (See Supplementary material S2).

2.5. Metabolic network analysis

The gene expression data (FPKM\(^1\) values) of 10275 genes from Leishmania spp. [49] was integrated with the extended metabolic model (termed as iSK570) by applying GIMME approach. OptFlux modules [50] were used to run GIMME algorithm and to perform FBA-based analyses under different environmental conditions. Briefly, GIMME implementation considers genes (and associated reactions) with an expression level below the threshold as inactive, and thus removes those from the simulation. The algorithm may reconsider few of these

\(^1\) FPKM (Fragments Per Kilobase Million) is method for estimating relative abundance of transcripts in terms of fragments observed in RNA-Seq experiment.
inactive reactions, especially the essential ones and so-called metabolically important reactions (MIRs), back in the simulation to achieve an optimal solution. The remaining reactions are blocked and termed as metabolically unwanted reactions (MURs) in that particular metabolic state. Inconsistencies between the metabolic model and gene expression data are estimated based on MIRs that are re-inserted in the model; however, GIMME solves a linear programming (LP) on reconsidered reactions to minimize this inconsistency. As such, inconsistency scores (IS) are calculated and associated with each metabolic reaction. Accordingly, metabolic reactions can be categorized as follow:

1. inactive (expression levels below the threshold and metabolic flux equal to zero);
2. potentially inactive (expression levels below the threshold and metabolic flux is non-zero);
3. potentially active (expression levels above the threshold and metabolic flux equal to zero);
4. active (expression levels above the threshold and metabolic flux is non-zero).

Different threshold values were tested, and inconsistency scores (IS) were recalculated as described in [13] (Figure 1). Furthermore, flux spans based on Flux Variability Analysis (FVA) and PFBA flux distributions were compared. The predicted changes in metabolic operability of reactions after GIMME implementation were also compared with proteomic data from Pawar et al. (2014) [51].

3. Results and Discussion

3.1. Consistency between metabolic model iSK570 and gene expression data in promastigote conditions

Based on different tests, where the gene expression threshold values were changed, it was observed that IS values increase with the threshold values (Figure 2A), particularly above threshold values of 11 (Figure 2B). Below this threshold, IS values are close to zero, indicating that there are only a few inconsistencies between predicted fluxes and gene expression levels associated to the corresponding reactions. As such, while increasing the threshold value more reactions with predicted fluxes different from zero, but with low expression levels, i.e. reactions that should be active, are included, which increases the level of inconsistency between expression data and flux predictions. Although the number of potentially inactive reactions, i.e. reactions with expression levels below the threshold and predicted zero flux, increases with the threshold value, agreeing with metabolic predictions; the fact is that increasing the threshold value tends to exclude reactions that should be active as predicted by FBA-based simulations.

\[ IS = \frac{\text{flux}_{R_2} \times (\text{threshold} - \text{g}_1) + \text{flux}_{R_3} \times (\text{threshold} - \text{g}_2)}{\text{flux}_{R_2} \times (3.5 - 3) + \text{flux}_{R_3} \times (3.5 - 1.0)} \]

For threshold = 3.5,

\[ IS = \frac{4.08 \times (3.5 - 3) + 1.0 \times (3.5 - 1.0)}{4.08 \times (3.5 - 3) + 1.0 \times (3.5 - 1.0)} \]

\[ = 1.0 \]

Figure 1: A) Workflow for integrating gene expression data into the metabolic. B) An exemplifying scheme for calculating inconsistency score (IS) using gene expression and flux values.
3.2. FVA and PFBA analyses

FVA analyses were performed based on GIMME results using a threshold value of 12. Briefly, the idea was to evaluate changes in metabolic predictions imposed by GIMME constraints (especially blocked reactions or MURs) and estimate the impact in the predicted metabolic flexibility under the defined conditions. Therefore, FVA analyses with and without GIMME constraints were compared. Reactions were categorized as such: type1, minimum and maximum FVA fluxes equal to zero; type2, minimum and maximum FVA fluxes different from zero (either positive or negative); and type3, minimum and maximum FVA fluxes equal to upper and lower bounds of reactions (Table 1).

Results show that the number of reactions type3 decreased, while reactions type1 and type2 increased, which suggests that GIMME-based constraints reduced metabolic flexibility associated with large FVA spans as defined by FVA fluxes of type 3 reactions. Also, reactions type 1 with FVA spans of zero (i.e. blocked reactions) contribute to decrease this metabolic flexibility, as the number of possible alternatives for carbon distribution within the network also decreases. Minimum and maximum flux values from FVA analyses with and without GIMME constraints for each reaction are presented in Supplementary material S1.

As shown in Figure 2A, the number of potentially inactive reactions (i.e. with gene expression less than the threshold and predicted flux equal to zero) increases to a maximum of 400 at the highest expression threshold value (368). In general, GIMME considers these reactions as MURs (or metabolically unwanted reactions) and, ultimately they do not have an impact on the flux distribution. Similarly, potentially active reactions (i.e. with gene expression less than threshold and flux equal to zero) can be associated with MIRs and GIMME might need to reconsider some of these reactions during the simulation process. These are almost 250 at a maximum threshold value of 368. Although the number of MIRs are lower than the number of MURs at a particular threshold value, these contribute far more to increase IS values. Therefore a threshold value should be carefully selected. In the following analysis, a threshold value of 12 (equivalent IS = 5.9x10^2) was chosen to perform GIMME simulations, which predicted 30 genes (out of 570) with expression levels below the threshold value, corresponding to 23 reactions from which 16 were considered MURs and 7 MIRs.

### Table 1: Number of reactions classified as type1, type2 and type3 from FVA results considering simulations with and without GIMME-based constraints (i.e. deleting MURs).

<table>
<thead>
<tr>
<th>Reaction Category</th>
<th>Minimum (min) and maximum (max) FVA values</th>
<th>Number of reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without GIMME-based constraints</td>
<td>With GIMME-based constraints</td>
</tr>
<tr>
<td>type1</td>
<td>min = 0 and max = 0</td>
<td>472</td>
</tr>
<tr>
<td>type2</td>
<td>min/max &lt; 0 or min/max &gt; 0</td>
<td>239</td>
</tr>
<tr>
<td>type3</td>
<td>min = lower bound and max = upper bound</td>
<td>466</td>
</tr>
</tbody>
</table>

Additionally, PFBA and GIMME flux distributions were compared. In general, flux distributions did not change significantly, most likely because of small differences in the number of active and non-active reactions (Figure 3A); however, few reactions changed their flux values from zero to non-zero and vice-versa. The reactions with these binary changes are mostly transport reactions, but reactions associated with metabolic pathways like “Glycerolipid metabolism” (30.3 %) and “Pyrimidine metabolism” (9.09%) (Figure 3B) were also found. Changes in flux operability of these reactions can be supported by proteomic data for L. major from Pawar et al., 2014 [51], which showed that genes associated with eight reactions (out of ten) that changed their fluxes from zero to non-zero, are expressed at the protein level (Table 2). This indicates that GIMME-based flux analyses improve model predictions.
Table 2: List of reactions which showed binary changes (zero to non-zero) in their fluxes after GIMME implementation (threshold value of 12), and which associated enzymes have positive expression at protein level.

<table>
<thead>
<tr>
<th>Reaction ID</th>
<th>PFBA flux value</th>
<th>Associated genes</th>
<th>Protein expression [51]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without GIMME</td>
<td>With GIMME</td>
<td></td>
</tr>
<tr>
<td>R_AGPAT1_LM</td>
<td>0</td>
<td>4.80</td>
<td>LmjF32.1960 yes</td>
</tr>
<tr>
<td>R_CDPDSPm__LM</td>
<td>0</td>
<td>3.26</td>
<td>LmjF14.1200 yes</td>
</tr>
<tr>
<td>R_GPAM_LM</td>
<td>0</td>
<td>4.80</td>
<td>LmjF34.1090 yes</td>
</tr>
<tr>
<td>R_HEXg</td>
<td>0</td>
<td>99.30</td>
<td>(LmjF21.0250 or LmjF36.2320 or LmjF21.0240) yes</td>
</tr>
<tr>
<td>R_ME1x</td>
<td>0</td>
<td>162.49</td>
<td>LmjF24.0970 yes</td>
</tr>
<tr>
<td>R_PAPam_LM</td>
<td>0</td>
<td>1.04</td>
<td>(LmjF18.0440 or LmjF19.1350) yes</td>
</tr>
<tr>
<td>R_PNS1</td>
<td>0</td>
<td>10000</td>
<td>LmjF29.2800 yes</td>
</tr>
<tr>
<td>R_UPPRTr</td>
<td>0</td>
<td>-10000</td>
<td>LmjF34.1040 yes</td>
</tr>
</tbody>
</table>

4. Conclusion

The work described the application of GIMME algorithm in combination with flux-based analysis to integrate gene expression data into genome-scale models to determine consistency between data and metabolic model ISK570. The strategy has been used to put an extra layer of stoichiometric constraints on reactions to predict more accurate fluxes across various pathways of L. major. The predicted activation/inactivation of the metabolic reactions in a particular environment was supported by expression of the associated enzymes at the protein level. Improved flux distribution further used to describe stage-specific metabolism and drug target predictions in Leishmania (not described here due to page limitations). All supplementary data mentioned in this manuscript can be provided on demand.

5. Funding and Acknowledgement

This work was supported by the Initial Training Network, GlycoPar, funded by the FP7 Marie Curie Actions of the European Commission (FP7-PEOPLE-2013-ITN-608295). The authors gratefully express appreciation to SilicoLife Lda for providing required infrastructural facilities related to this work. We also thank Bruno Pereira (systems biologist at SilicoLife) and Hugo Giesteira (programmer at SilicoLife) for scientific and technical assistance during various phases of the project.

6. References

10. Arvind K. Chavali, Jeffrey D. Whittemore, James A. Eddy, Kyle T.