Phenotypic Characterisation of Glucose Transporter Knockout *Leishmania mexicana*

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Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy

College of Medical, Veterinary & Life Sciences

Institute of Infection, Immunity & Inflammation

University of Glasgow

September 2012

Abstract

The aim of this project was to investigate nutrient acquisition by glucose transporter null mutant *Leishmania mexicana*, to understand how genetic ablation of glucose transporter genes in these protozoan parasites affects their phenotype. It has previously been demonstrated that glucose transporter null mutant *Leishmania* (ΔLmGT) promastigotes have lost the capacity to transport glucose as well other hexoses. Glucose is a potential carbon source for promastigotes, which are typically maintained in glucose-rich media and which encounter high levels of glucose in the sandfly midgut. Despite inability to transport glucose, the null mutant parasite is able to survive and grow, although at a reduced rate in media that contains non-carbohydrate potential carbon sources. This finding suggesting that the null mutant may utilise other carbon sources. In this project, a variety of approaches have been applied to investigate the nature of these alternative carbon source and the mechanisms by which they are acquired and metabolised.

Amino acid uptake assays revealed that glucose transporter null mutant promastigotes take up several key amino acids at a significantly enhanced rate, compared with wild type promastigotes. A comparative metabolomic analysis was applied for more comprehensive comparison between the wild type and glucose transporter null mutant. Though the uptake assay and metabolomic analysis showed distinct changes in nutrient uptake and metabolisms between the two cell lines, these results does not tell whether the changes are due to changes in transporter activity or expression, nor does it inform on the enzymes involved in these processess. Thus, a proteomic approach was applied. Since membrane transporter were a focus of interest, but are known to be highly hydrophobic and of relatively low abundance, it was necessary to develop protocols for membrane protein enrichment and fractionation in *Leishmania mexicana*.

A previous comparative proteomic analysis, which did not represent membrane proteins, had highlighted changes in the abundance of a major component of the paraflagellar rod. This unexpected result was further investigated, by Western blotting, electron microscopy and taxis assay. The results point to changes in flagellar structure of function in glucose transporter null mutant *Leishmania*.

It is no doubt the experiments carried out in this project have shown interesting changes in glucose transporter null mutant *Leishmania*, in terms of nutrient acquisition and phenotypic characteristics. The data provide a new set of ideas as to how *Leishmania* thrive in a condition where glucose is scarce. These data may have relevance to leishmaniasis in mammals, as it is likely that glucose is constitutively scarce in the intracellular environment inhabited by the amastigote. However, due to time constraints, it was not possible to extend these investigations to the amastigote stage of *Leishmania*.

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Acknowledgements

I am heartily thankful to my supervisor, Dr Richard Burchmore, whose encouragement, supervision and support from the beginning to the concluding level enabled me to develop an understanding of the subject. This thesis will not be complete without your patience in teaching and guiding throughout the period of my PhD.

I also would like to thank my assessors, Professor Michael Barrett and Professor Lawrence Tetley for the advice and contribution of ideas to the project. To Dr Karl Burgess and Dr Darren Creek, thank you for your generous advice and invaluable help in the metabolomic analyses. To Dr Christina Naula, thank you for making the start of my PhD with less anxiety and to Gordon Cambell, thank you for assisting me in the lab. Finally, a big thank to all my labmates in level 5 GBRC for helping me directly or indirectly in labwork or in the completion of my thesis writing, for without their help I don't think I could have made it this far.

I would like to particularly thank my parents for their devotion, sacrifices, love and care to me and my two brothers; we are truly blessed to have you as our parents. To my dad, I want you to know that since I was a little girl, your faith in me is what has made me to never stop pursuing my dream.

And finally, to the 3 pillars of my life, my husband and my lovely daughters, Raja Khairul Adli, Raja Ayesha Nazneen and Raja Hawa Sufia, there are no words to describe how thankful I am for having all of you in my life. You give me the courage and confidence to take whatever challenges I face to have a better future for us. Thank you for all your sacrifices, love, understanding, patience in supporting me to pursue my dream.

Author's Declaration

I declare that the results presented in this thesis are my own work, except when stated otherwise.

Dhilia Udie Lamasudin

September 2012

Miscellaneous

Some of the work within this thesis has been previously presented at the following international and local meetings.

2009- British Society for Proteome Research (BSPR) Meeting 2009, Hinxton, Cambridge,UK

2009- 8th Annual World Congress, Human Proteome Organisation (HuPO), Toronto, Canada

Abbreviations

ΔLmGT	Glucose transporter null mutant *Leishmania mexicana*
1-SDS PAGE	1-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
2-DE	2-dimensional gel electrophoresis
ACN	Acetonitrile
Amp	Ampere
ATP	Adenosine triphosphate
AmBic	Ammmonium bicarbonate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
Ci	Curie
CL	Cutaneous leishmaniasis
DDT	Dichlorodipenyltrichloroethane
DTT	1,4-Dithio-DL-threitol
DiGE	Differential gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
GT1	Glucose transporter null mutant promastigotes expressing LmGT1
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing

IMP	Integral membrane proteins
iNOS	Inducible nitric oxide synthase
IPG	Immobilised pH gradient
kDa	Kilo Dalton
LmGT1/2/3	Glucose transporter proteins isoform 1/2/3 *Leishmania mexicana*
mA	Miliampere
ML	Mucocutenous leishmaniasis
mM	Milimolar
M_r	Mobility
MOWSE	Molecular Weight Search
MS	Mass spectrometry
MVB	Multi-vescular bodies
MVT	Multi-vescular tubule
Myc-GT2	myc-tagged glucose transporter 2 *Leishmania mexicana*
LCMS	Liquid chromatography mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
ORF	Open reading frame
PA	Polyamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
pmoles	Picomoles

RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RT	Retention time
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
TEMED	N, N, N',N'-tetramethyl ethylenediaminide
TMD	Transmembrane domain
V	Volt
VL	Visceral leishmaniasis
WT	Wild type

Chapter 1

General Introduction

1.1 The Kinetoplastida

Kinetoplastida are a group of eukaryotes that have a unique structure called the kinetoplast, which is the mitochondrial DNA of trypanosomatid protozoa (Shapiro & Englund 1995) and it is the structure located near the basal body of flagellum and is the genome of the mitochondrion.

Another unique feature of this genus is the flagella structure and, depending on the groups, kinetoplastids may exist as uniflagelatte (Trypanosomatids) or biflagellate (Bodonids). The flagellum facilitates the motility and may play a role in biosensing (Ginger et al. 2008). Kinetoplastids can be divided into two sub-groups; free living and parasitic. However, the most important group in the order is the trypanosomatidae, particularly *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp. (Simpson et al. 2006), all of which cause serious infectious diseases in humans and domestic animals. Genetic sequencing of some of the species showed that trypanosomes and *Leishmania* share many similarities in their genome, although each different species has different manifestations upon infection of animals or humans (Stuart et al. 2008).

Other parasitic protozoans in the group are *Phytomonas* spp.(plant parasite) and *Crithidia fasciculata* (insect parasite) (Shapiro & Englund 1995).

1.2 Leishmania

1.2.1 Life Cycle

Leishmania spp. exists in two stages; the aflagellate amastigote mammalian stage and flagellated promastigote sandfly stage. The different developmental forms can be differentiated by their different habitat

requirement and morphology (Besteiro et al. 2007). The transmission of the parasite occurs when the sandfly, injects promastigotes into the skin of a mammal during a bloodmeal. These promastigotes will be taken up by macrophages and transform into amastigotes before start to multiply. The multiplication continues until the macrophage bursts and releases the amastigotes which then will infect neighbouring cells.

When a sandfly takes a bloodmeal from an infected mammal, the infected macrophages may be ingested into the sandlfy midgut and transform into promastigotes and start to divide. The promastigotes will move to the proboscis area of the midgut, ready to be transmitted to another mammalian host when the sandfly takes another bloodmeal.

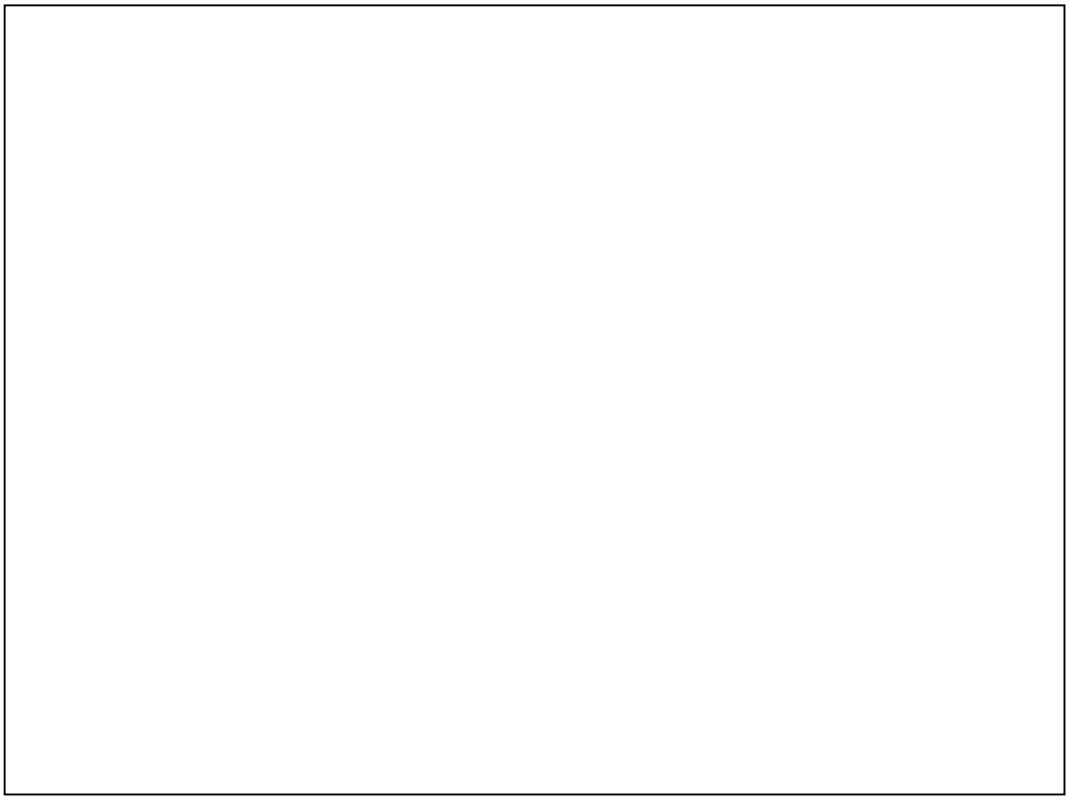


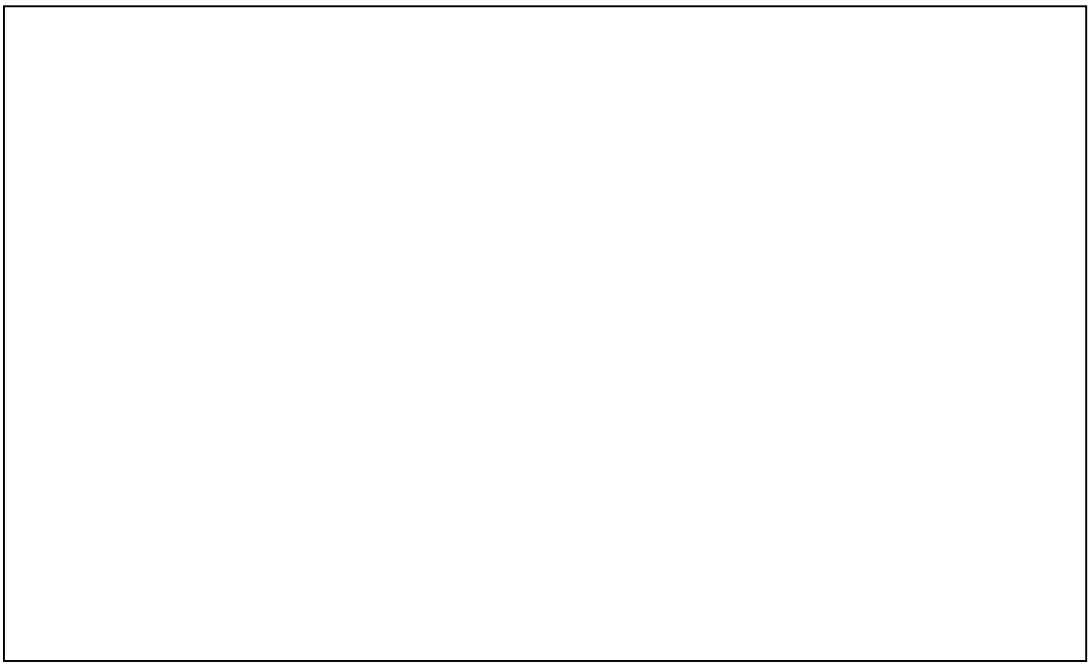
Figure 1-1 Digenetic life cycle of Leishmania. Diagram has been removed due to copyright restrictions.

1.2.2 Life cycle forms

Leishmania exists in two forms depending on the environment they are residing. The cycle alternates between two main morphological forms i.e. intracellular amastigotes which reside in mammalian hosts and motile promastigotes that reside in the sandfly vector.

There are several forms of the sandfly phase of promastigotes that have been described and the metacyclic form which is the mammalian-infective stage that are best known for the promastigotes form (Gossage et al., 2003). A study on *Leishmania (Leishmania) mexicana* and *Leishmania (Leishmania) infantum* (syn. *chagasi*) demonstrated that there are two distinct, sequential growth cycles during development in the sandfly vector, *Lutzomyia longipalpis* involving four separate life cycle stages (Gossage et. al. 2003). The growth cycles begun by procyclic promastigote (short and ovoid promastigotes), which reside in the abdominal midgut that contains the bloodmeal. Subsequently, the cells transform to non-dividing forms called the nectomonad promastigotes (long and slender promastigotes) and this is the form of the cells when the anterior migration occurs. Later, the transformation from the nectomonad into leptomonad forms (short and broad promastigotes) occurs, indicating the start of the second growth cycle in the anterior midgut. Subsequently the differentiation of leptomonad promastigotes into non-dividing metacyclic promastigotes arises, preparing the to be transmitted into a mammalian host (Gossage et al. 2003).

However there are two morphological forms that are known to exist but regarded to be 'unresolved issues' i.e. haptomonad promastigotes and paramastigotes (Gossage et al. 2003). As described by Killick Kendrick, haptomonad has two populations where both have the same small, broad electron-lucid forms but can be found either as free living in the gut lumen or attached to the cuticular surfaces of the gut (Killick-Kendrick et al. 1974). On the other hand, paramastigotes which are both free-living and attached, have been described as infective stages. The form arising from haptomonad promastigotes or degenerate forms are found in the foregut in later stages of infection (Gossage et al. 2003). However, both haptomonad and paramastigotes are comparatively a minor subpopulation and their role in the life cycle is uncertain (Gossage et al. 2003).



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1.2.3 Intracellular Organelles

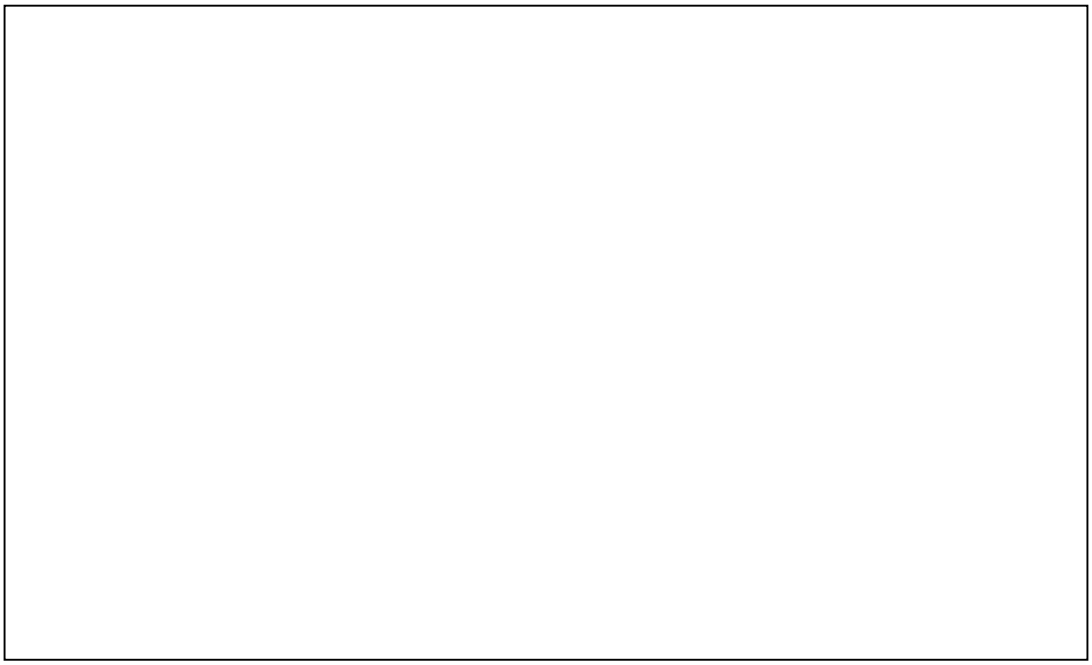


Figure 1-3 The image has been removed due to copyright restrictions.

One of the unique features of the Trypanosomatidae organism is the existance of an organelle called kinetoplast DNA. The kinetoplast DNA represents a complex associate composed of a network of thousands of minicircles and a few dozen maxicircles. While maxicircles molecules occupy 5% of total DNA, minicircles exist in 5-50 thousands molecules per cell (reviewed in (Yurchenko & Kolesnikov 2000)). Maxicircles are analoug to other mitochondrial DNAs as they encode for mitochondrial rRNA and mitochondrial protein (Shapiro 1993). In minicircles, there is a variable region on the structure that carries information or so-called "guide RNAs" (gRNA) which participate in the posttranscriptional modification of mRNA known as "uridylate editing" (reviewed in (Yurchenko & Kolesnikov 2000).

Whereas glycolisis in most all organisms occurs in cytosol, in tyrpanosomatids, the major part of the pathway, occur in compartmentalised organelle called glycosome; a microbody related to a higher eukaryote organelle, called peroxisome (reviewed in (Moyersoen et al. 2004)). Just like other peroxisome-like organelles in other oraganisms, glycosomes are bounded by a single phospholipid bilayer membrane, have an electron-dense proteinaceous matrix and do not have DNA (reviewed in (Moyersoen et al.

2004)). Other metabolisms may occur in glycosome (or at least in part). A study on *Leishmania mexicana* have shown that the enzymes from the first three steps in ether lipid biosynthesis are glycosomal (reviewed in Parsons et. al 2001). Ether lipids in trypanosomatids have vital roles in the association of major surface molecules with the plasma membrane through glycosylphosphatadylinositol (GPI) anchors. In addition, the GPI anchor of *Leishmania* promastigotes lipophosphoglycan (LPG) also has a unique ether lipid i.e. 1-*0*-alkyl-phosphatidylinositol or lyso-1-*0*-alkyl-phosphotidylinositol suggesting that it is likely that ether lipids have an important function in *Leishmania* (reviewed in (Parsons et al. 2001). Trypanosomatids are unable to synthesise purine, thus they rely on purine salvage from the host. Unlike several purine salvage enzymes in eukaryotes that are cytosolic, those enzymes i.e. hypoxanthine:guanine phosphoribosyl transferase, adenine phosphoribosyl transferase and xanthine phosphoribosyl transferase are exclusively localised in glycosome (reviewed in (Parsons et al. 2001). A study has showed that that compartmentalisation of glycolysis in glycosome is vital in trypanosomatid protozoans. Expression of the glycosomal enzyme phosphoglycerate kinase (PGK), which convert 1,3-bis phosphoglygerate to 3-phosphoglycerate, in the cytosol proven to be toxic to the protozoan(reviewed in (Parsons et al. 2001)). Other functions of the glycosomal compartmentalisation in the trypanosomatid include avoiding osmotic effects when the glucose is high, recovery from starvation i.e. the closed glycosomal system avoid the usage of ATP, thus allow the glycolysis to restart only when substrates are available (reviewed in (Parsons et al. 2001)).

As they cycle between the vertebrate and invertebrate hosts vector, the protozoan parasite *Leishmania* undergo various developmental changes in their growth rates andnutritional requirements which include vivid changes in their morphology and function in organells involved in the secretory and endocytic pathway (Waller & McConville 2002). Flagellar pocket has been suggested to be the sole exocytosis and encytosis site as several organelles involved in secretory pathway and endocytosis pathway, residie around this area in the cell (Waller & McConville 2002). The secretory function in the parasite is played by the endoplasmic reticulum (ER) and Golgi apparatus and these two major organelles in the secretory pathway are morphologically well developed in dividing

promastigotes(Waller & McConville 2002). However, endocytic and lysosomal compartments in the promastigotes stage are still relatively poorly characterised (Waller & McConville 2002). In *Leishmania*, it has been identified that endocytic pathway involve three compartments that are morphologically different i.e. 1)endosomes localised near the flagella pocket, 2)multi-vescular bodies (MVB) found at the anterior end of the protozoan promastigotes and 3)multi-vescular tubule-lysosome that runs along the anterior-posterior axis of the cell body(Waller & McConville 2002).

MVT-lysosome is the terminal compartment in the promastigote endocytic pathway and was first recognised in *Leishmania mexicana* promastigote (reviewed in (Waller & Mcconville 2002)). Despite being the terminal lysosome compartment in promastigotes, this structure has a low pH level and low lytic capacity (reviewed in (Waller & Mcconville 2002)). Interestingly, promastigotes that are incubated in acidic media showed an increasing rate of degradation of chimera. This observation demonstrates that MVT-lysosome's lytic capacity may be regulated by the acidity of the environment rather than the level of proteases expression (reviewed in (Waller & Mcconville 2002)). The MVT-lysosome's structure and morphology has been suggested to be maintained by microtubules and this is supported by the finding that the MVT-lysosome rapidly depolymerised into one or two large vesicles if promastigotes are treated with microtubule disrupting agents like thioridazine (reviewed in (Waller & Mcconville 2002)).

MVBs that are located near the flagellar pocket have been suggested to be candidate intermediates that mediate transport from the Golgi apparatus or endosomes to the MVT-lysosomes (reviewed in (Waller & Mcconville 2002)).These MVBs could diffuse through the cytosol compartment or be transportered along the cytoplasmic microtubules by motor proteins as they have also been found in other trypanosomatids (reviewed in (Waller & Mcconville 2002)).

Endocytic orgenalles mentioned above i.e. MVT-lysosome (multi-vescular tubule), endosomes and multi-vescular bodies(MVB) are different from acidocalcisomes (reviewed in (Waller & Mcconville 2002)). Acidocalcalcisomes are organelles that can be found in a diverse range of organisms and were first

defined in trypanosomes (Docampo et al. 1995; Vercesi, Moreno, and Docompo 1994). They are known as acidic organelles with a high concentration of phosphorus and exist in forms of pyrophosphate (PPi) and polyphosphate (poly P), and these phosphorus are complexed with calcium and other cations. The membrane of acidocalcisomes contain a number of pumps (Ca^{2+}-ATPase, V-H^+-ATPase, H^+-PPase), exchangers (Na^+/H^+, Ca^{2+}/H^+) and channels (aquaporins) (reviewed in (Moreno & Docompo, 2009)).

In protists, acidocalcisomes are the compartments used for the major storage of phosphorus e.g. Pi, PPi and poly P. PP_i is a byproduct of the biosynthesis of nucleic acids, coenzymes and proteins, activation of fatty acids and biosynthesis of isoprenoids. But the information on how PPi is transported across acidocalcisomes and why it is stored is not really known. Unlike PPi, the functions of poly P in trypanosomatids are recognised and the changing concentrations of long and short chains of poly P in *Trypanosoma cruzi* during life cycle and when encountered with hyposmotic and hyperosmotic environments suggest that acidocalcisomes are involved in stress response to environmental changes (reviewed in (Moreno & Docompo, 2009)). As acidocalcisomes are the main storage compartments for poly P and calciums and other cations, this suggest that acidocalcisomes could be involved in energy sources and signaling processes respectively (Moreno & Docompo 2009, Lopez-Martin et al 2008). In addition, acidocalcisomes may also be involved in pH homeostasis as poly P could be involved in intracellular pH regulation through the H^+ produced from its hydrolysis. Acidocalcisomes are also suggested to be involved in osmoregulation as acidocalcisomes react to osmotic stress by altering the content of sodium and chloride (Moreno & Docompo 2009, Lopez-Martin et al 2008,LeFurgey et al. 2001).

Transition from the promastigote to amastigote stage is accompanied by changes in the intracellular organelles morphology. In lesion-derived amastigotes, the ER and Golgi apparatus appeard to be discreet and it has been reported that the latter has been shown in a majority of cases to be disseminated and hard to find (reviewed in (Waller & Mcconville 2002)). In amastigotes, the lysysome system is expanded and it is most obvious for members in the *Leishmania mexicana* complex, where the large lysosomal

vacuoles termed as megasomes exist (reviewed in (Waller & Mcconville 2002)). The term megasome reflect the large lysosomal organelles found in *Leishmania mexicana* complex amastigotes and the term was introduced by Alexander and Vickerman in 1975 (reviewed in (de Souza 2010)). Megasomes, which take up to 15% of the cell volume in *Leishmania mexicana* complex amastigotes correlates with a great increase of cysteine proteases (reviewed in (de Souza 2010,Waller & Mcconville 2002)). However the size of megasomes is different between species (the size of megasomes in *Leishmania chagasi* amastigotes are only 5% of the cell volume) and it is also suggested that megasomes are a characteristic of the *Leishmania mexicana* complex (reviewed in (de Souza 2010)). It has been suggested that megasomes and their constituents may be important in the infectivity and virulence of *Leishmania* species (reviewed in (de Souza 2010).

Autophagosome is a structure that is formed by a membranous structure that engulfs the cytoplasm/organelles that are to be degraded (reviewed in (Besteiro et al. 2006)). Autophagosome transports the internalised materials to the lysosomal compartment to be degraded and it is suggested that autophagosome needs to have strong interactions with the endosomal compartments to achieve its full degradative potential (reviewed in (Besteiro et al. 2006). Autophagosome also believed to be involved in the architectural modifications that occur during the development and differentiation processes (reviewed in (Besteiro et al. 2006)).

1.2.4 Cytoskeleton of Leishmania

Trypanosomatids like *Trypanosoma* and *Leishmania*'s cell shapes are defined by their internal cytoskeletons (Gull 1999). The plasma membranes in most eukaryotic cells are well-known to be intimately connected with the cytoskeleton (Dagger et al. 1989, De Souza 1984). Cytoskeletons are made up of parallel rows of microtubules that run under the pellicle, and this assembly pattern by microtubules form a unique and complex structure i.e. subpellicular microtubule and like any other organisms, α/ß tubulin heterodimers, are the major proteins constituents of subpellicular microtubules (De Souza 2010). These subpellicular microtubules in Trypanosomatids are cross-linked to each other and to the plasma membrane (Gull 1999).

Subpellicular microtubules exist throughout the cell body but not at the flagella pocket area. Instead, at one point, the microtubule corset is substituted by a filamentous structure called the Flagellum Attachment Zone (FAZ) (De Souza 2010). FAZ is linked with microtubules that are closely associated with the cisternae of endoplasmic reticulum and is derived near to the basal bodies (De Souza 2010).

The primary function of subpellicular microtubules is maintaining the cell shape and it is clear that experiments involving treatments that hamper the microtubules organisation, led to the changes in cell asymmetry (De Souza 2010). In addition to cell shape maintainance, the microtubules's function also involves the maintainance of the plasma membrane's rigidity. Therefore, the assembly of endocytic vesicles and fusion of cytoplasmic vesicles with the membrane could be avoided (De Souza 2010). A few studies have demonstrated that endocytic and exocytic activities occur in the area that is not associated with the subpellicular membrane i.e. flagellar pocket (De Souza 2010, Field & Carrington 2004, Overath & Englster 2004).

Glycosomes are the microbodies of the kinetoplastida and are related to peroxisomes and there is no evidence indicating that they have a nucleus. Studies on biogenesis of glycosomes in trypanosomatids are extensive and regarded as important as it is a potential candidate for drug targets (reviewed in (de Souza 2010)).

1.3 Leishmaniasis

Leishmania spp. (order, Kinetoplastidae; family, Trypanosomatidae), a protozoan parasite of mammals, is the causative agent of the infectious disease named leishmaniasis. Depending on the species of the protozoan, the disease is characterised by the manifestations of either one of the 3 major categories of the clinical symptoms i.e. self-healing cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) (Lynn & McMaster 2008). While cutaneous leishmaniasis can be debilitating and disfiguring, and can lead to serious secondary bacterial infection, visceral leishmaniasis is always fatal without treatment.

Apart from the clinical symptoms, *Leishmania* spp. can be categorised according to the geographical distribution. The term "Old world" refers to species endemic to Asia, Middle East and Africa, e.g. *Leishmania major* , *Leishmania infantum* and *Leishmania tropica* whereas the term "New World" refers to species endemic to Southern and Central America,e.g. *Leishmania mexicana*, *Leishmania (Vianna) braziliensis* and *Leishmania panamensis* (David & Craft 2009)

It is estimated 1.5-2 million people acquire leishmaniasis every year, with 350 million population are at risk (David & Craft 2009). It is possible that the figures will increase because of global warming and changes in human ecology(Bailey & Lockwood 2007;Desjeux 2001).

Factors that are believed to facilitate the spread of leishmaniasis are urbanization, immigration and deforestation. It also has been reported that soldiers who have served in Iraq have contracted the disease, as have tourists who have visited regions where leishmaniasis is endemic (Bailey & Lockwood 2007;Harms et al. 2003;Weina et al. 2004).

Aas the disease is affecting mostly the poorest countries, efforts to combat leishmaniasis seem to be never-ending and the resources for diagnostics, treatment and control are costly. Many infected individuals reside in remote areas from where it is hard to access medical assistance.

Despite extensive research over several decades, the aim to find the most accurate, high-sensitivity, affordable, less invasive, low toxicity drugs, treatments and diagnostic of the disease has yet been successful. Hence, research on various aspects of the parasite must be not neglected as it provides better insight into mechanisms of infection and pathology and will speed up our mission to contain the spread of the disease.

1.4 Diagnosis and Treatment

Clinical features of fatal visceral leishmaniasis are fever, often associated with rigor and chills, splenomegaly, pancytopenia, weight loss and if leave untreated will lead to fatality (Sundar et al 2011). Some of the symptoms of CL are similar to other diseases (e.g. leprosy, skin cancers, tuberculosis, cutaneous mycoses, bacterial or fungal infections), which make the diagnosis difficult and misleading. In addition, delayed diagnosis could lead to mucosal infection (Blum & Hatz 2009a;Reithinger et al. 2007) . While cutaneous leishmaniasis is non-fatal and self-healing, the infection can cause multiple lesions leading to mutilating scars, which in turn will cause aesthetic stigma to the patient (Desjeux 2004). The aim in the treatment of cutanueous leishmaniasis is to speed up the healing process and so to lessen the scarring especially in cosmetic sites and to prevent spreading of the parasite (i.e. mucosal leishmaniasis) or relapse (Herwaldt 1999;Reithinger et al. 2007)

1.4.1 Diagnosis

Diagnosis of the disease is important to detect and confirm the organisms causing the infection. A rapid, accurate and non-risks methods are ideal criterias in diagnosing the disease.

Traditionally, for VL , the method of testing on serologic specimen and as well by demonstration of the parasite through microscopic examination and in vitro isolation have been accepted as the diagnosis procedure (Sundar & Rai 2002, Antinori et al. 2007). Sera obtained from patients are tested with Indirect Flourescent Antibody Test (IFAT) and Enzyme-Linked- ImmunoSorbent Assay (ELISA), culture and Geimsa-stained paper (Sakru et al. 2011). For CL, the parasite presence can be tested by direct microscopy, a typical histology, positive culture and some indirect skin test like Montenegro skin test, positive serology or the presence of leishmanial DNA (Vega-Lopez 2003). However, molecular diagnosis by the polymerase chain reaction (PCR) has been accepted for leishmaniasis diagnosis (Vega-Lopez 2003, Antinori et al. 2007).

However, serologic specimens has a few undesireable criteria that make it less favourable as it has less sensitivity i.e. in patients with severe immunosuppression, unable to discriminate between active or past-infections and unable to monitor the response of the parasite to specific theraphy (Sundar & Rai 2002, Antinori et al. 2007). Antinori (Antinori et al. 2007) has demonstrated that the PCR performed on whole blood samples produced more sensitive results as compared to PCR performed on serologic testing, in vitro culture and direct bone marrow specimen.

Detection of *Leishmania* amastigotes in CL and ML mostly been performed using invasively obtained clinical specimens such as lesions aspirates, scrapings or biopsies (Veland et al 2011).However these techniques causing discomfort to patients as they are invasive procudures which posing risks of causing bleeding and infection (Veland et al. 2011). The procedures are difficult to perform especially to paediatric patients, in remote areas and areas with intercurrent bacterial or fungal infection (Veland et al. 20011).

1.4.2 Treatment

For decades, pentavalent antimonials (meglumine antimoniate, sodium stibogluconate) have remained the first line drugs to treat CL from the new world (Blum & Hatz 2009, Seifert 2011). Sodium stibogluconate (Pentostam) is where the pentavalent antimony is reacted with gluconic acid to generates a number of compounds where the structures are unknown and whereas meglumine antimoniate (Glucantime) is in which the pentavalent antimony is reacted with the sugar meglumine to form the same unknown compounds (Berman 1996). Response rate are over 95% in previously untreated VL patient but it has been reported of acquired resistance to pentavalent antimony in some areas that have high-prevalance and high-epidemic transmission. Not only that, the situation is aggravated by the fact that in some cases the drug has caused adverse side effect like cardiotoxixity (Seifert 2011). The drug also requires a long treatment duration which can take between 20-28 days and followed for one year (Markle & Makhoul 2004).

A polyene antibiotic or Amphotericin B which was extracted from *Streptomyces nodosus* is used as first line treatment in areas of high-rate of unresposiveness to pentavalent antimonials and second line elsewhere (reviewed in Seifert 2011). Treatment using amphotericin B deoxycholate has it drawbacks such as it need hospitalisation, regular supervision of patients, prolonged period of treatment and infusion-related side effects like fever, chills and trombophlebitis (reviewed in Seifert 2011). Athough liposomal amphoterecin B is much safer and effective than amphoterecin B deoxycholate, the high cost for this drugs limits it comprehensive use in many endemic regions (reviewed in Seifert 2011).

Miltefosine, which originally is a drug cancer, was the first oral anti-Leishmanial treatment available to the market. It is a potential tool in elimination programme in some countries like India and Bangladesh and Nepal whereas in Colombia and Bolivia it is use as a second line treatment. However the drug has been reported to cause side effect i.e. gastrointestinal tract and also causing an elevation of hepatic enzymes (Seifert 2011).

Other anti-Leishmanial drugs are paromomycin (injection for VL and topical treatment for CL), pentamidine (first line drugs for certain forms of CL and second line treatment for VL) and sitamaquine (Seifert 2011).

However, mounting occurrence of resistance to these drugs and side effectes has becoming a problem for the current anti-leishmaniasis treatments, hence a development for a new drugs has been a focus for researchers.

1.5 Environment Adaptation

As they move through their life cycle, *Leishmania* parasites adapt to survive in 2 different environments i.e. the parasitophorous vacuole in a macrophage that has low pH, with a temperature of 37°C and elevated CO_2 (Besteiro et al. 2007;Burchmore & Barrett 2001) whereas the sandfly midgut has neutral pH with a temperature that fluctuates with the environment of the insect.

Both promastigotes and amastigotes have the ability to avoid the killing mechanisms of the macrophage by altering the macrophage signalling and functions (Abu-Dayyeh et al. 2010). For example, several studies have shown that both forms are capable of blocking the nitric oxide production that is fatal to many invading pathogens (Forget et al. 2006;Proudfoot, O'Donnell, & Liew 1995). The ability of the amastigote to thrive in these hostile conditions shows that it is an opportunistic organism which has evolved means for survival, has strategies to evade microbial activity and their components on their plasma membrane are compatible with the environment (Antoine et al. 1990).

1.6 Metabolism in the life cycle of *Leishmania*

As the different stages of the parasite reside in two distinct environments, the parasite must adapt nutrient requirements and energy metabolism according to the surroundings. Proteins in the plasma membrane of the parasite are in the front line of this adaptation, both sensing changes in the environment and mediating the selective transfer of material from host to parasite.

Nutrient availability changes as *Leishmania* move through their life cycle. Between bloodmeals, sandflies feed on plant sugar, hence *Leishmania* promastigotes in this habitat encounter high sugar levels in the midgut and use this as source of energy and general nutrition (Schlein 1986). Unlike promastigotes that constantly bathe in a sugar-rich environment, amastigotes reside in a macrophage parasitophorous vacuole (PV), which known to have a low level of free sugars (Burchmore & Barrett 2001;McConville et al. 2007;Naderer & McConville 2008)

In a previous study, it was shown, in *L.amazonensis*-infected macrophages, that material was delivered to the parasitophorous vacuole (Rabinovitch et al. 1985), suggesting that amastigotes depend on the endocytosed materials from outside vacuole to gain nutrients. Russell and colleagues have described the findings of endocytosed material in the flagellar pocket and inside amastigotes, further confirming that parasite uptake of intra-vacuolar substance (Russell et al. 1992). The expression of various plasma

membrane transporters in both amastigotes and promastigotes enable the parasites to exploit a range of molecules such as sugars, amino acids, polyamines and nucleoside metabolites available in sandfly midgut and macrophage phagolysosome (Burchmore & Barrett 2001). These endocytosis and lysosomal degradation provide extra means for the parasite to gain important nutrients (Waller & McConville 2002) essential for metabolism and survival of the parasites

1.6.1 Energy Metabolism

Glucose is a primary source for energy metabolism for promastigotes and as promastigotes live in a high-sugar environment in the sandfly gut, glucose transporters play a pivotal role in maintaining the glucose supply. Glucose mostly metabolise via glycolysis and pentose phosphate pathway (Barrett 1997). Like any other trypanosomatids, the end products of glucose aerobic metabolism in *Leishmania* are mostly incompletely oxidised i.e succinate, L-alanine and acetate with traces of glycerol which 75% of the carbon comes from glucose (Maugeri et al. 2003).The remaining of the glucose may be oxidised in tricarboxilic acid cyle which generate CO_2 (Cazzulo et al. 1988).

It is reported that the *Leishmania* amastigote has higher activities for enzymes that catalyse the ß-oxidation of fatty acids but low activities of glycolitic pathway enzymes (Coombs et al. 1982), suggesting that the amastigotes uses other carbon sources than glucose to support energy metabolism, probably because is glucose is scare in the parasitophorous vacuole (Burchmore & Barrett 2001).

Many metabolic pathways are essential for survival i.e. defence against oxidative stress, generation of molecules for nucleotides and nucleic acid synthesis, or generation of molecules essential for organism building block. To survive, the *Leishmania* must ensure that critical pathways remain provided, despite changes in the availability of key nutrients. The existence of essential catabolic, anabolic and anaplerotic pathways in the glycosomes of *Leishmania* provide them main targets for chemotheraphy (Hart & Opperdoes 1984).

1.6.1.1 Glycolysis

Glycolysis is a pathway that converts one molecule of glucose to two molecules of pyruvate which follow by the generation of two molecules of ATP. The breakdown of polysaccharides and molecules i.e glucose, related hexose sugars and sugar phosphates, generated from metabolism of oligosaccharide, make their way to the glycolysis pathway. In eukaryotic cells glycolysis take place in the cytosol and yielded pyruvate will continue further oxidation in mitochondria. However, trypanosomatidae parasites like *Leishmania* spp. carry the first seven reactions of glycolysis in an cytplasmic organelle called glycosome (Mathews et al. 2000).

Data for the occurrence of usual glycosomal enzyme distributions in promastigote life cycle of *Leishmania major*, *Leishmania mexicana*, *Leishmania b. braziliensis* and *Leishmania donovani* was published although little discrepancy has been noticed in enzymes composition between *Leishmania* spp. or with other members of the Trypanosomatidae (Hart & Opperdoes 1984).

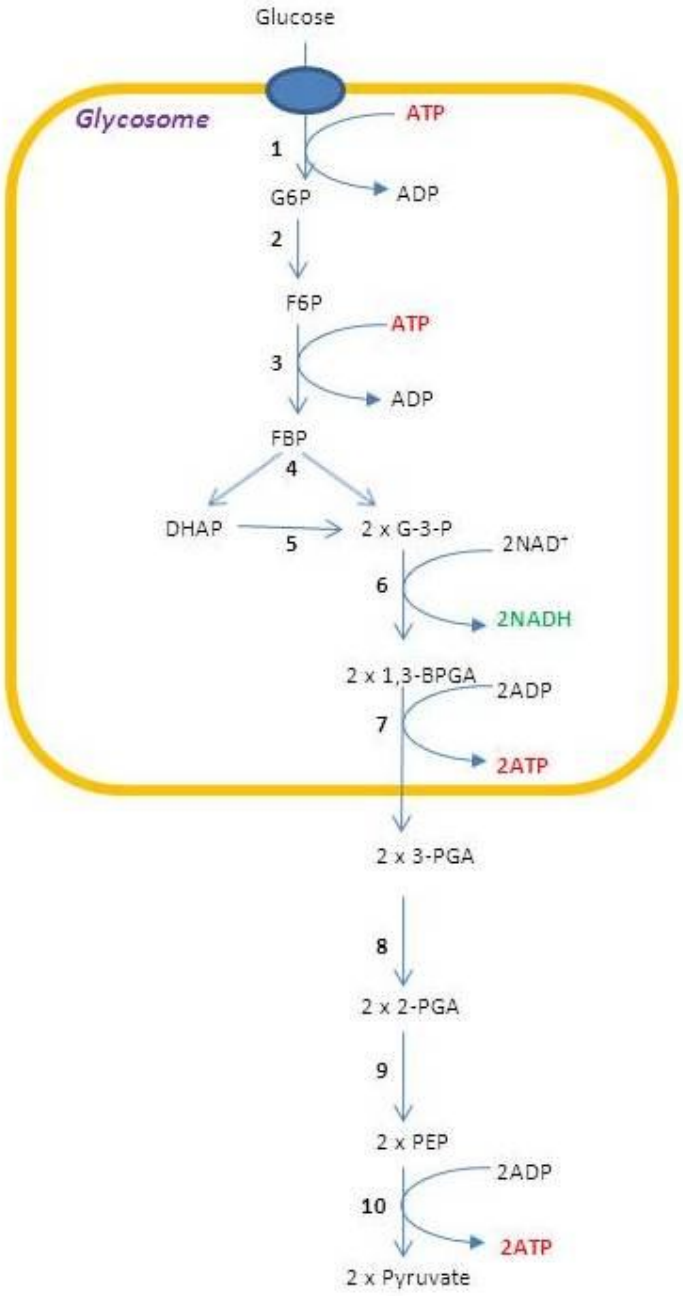


Figure 1-4: Glycolysis pathway in *Leishmania* (The diagram was adapted from Saunders et al. 2010 and Hannaert et al. 2003). In contrast to the other organisms where the glycolytic enzymes are cytosolic, in *Leishmania* and other Trypanosomatids, the seven glycolytic enzymes converting glucose to 3-phosphoglycerate are restricted in peroxisome-like oganelles called glycosomes (Verlinde et al. 2001). Enzymes (1) Hexokinase : (2) glucose-6-phoshate dehydrogenase: (3) phosphofructokinase : (4) aldolase : (5) triosephosphate isomerase : (6) glyceraldehyde-3-phosphate dehydrogenase : (7) posphoglycerate kinase : (8) phosphoglycerate mutase : (9) enolase : (10) pyruvate kinase. Abbreviations: G6P, glucose 6-phosphate ; F6P, fructose 6-phosphate ; FBP, fructose 1,6-bisphosphate ; DHAP, dihydroxyacetone ; G-3-P, glyceraldehyde 3-phosphate ; 1,3-BPGA, 1,3-bisphosphoglycerate ; 3-PGA, 3-phosphoglycerate ; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate (Mottram& Coombs, 1985, Verlinde et al. 2001, Pabon et al. 2006, Quinones et al. 2007, Norwicki et al. 2009, Saunders et al. 2010)

1.6.1.2 Pentose Phosphate Pathway

Pentose phosphate pathway (PPP) is an anabolic pathway in cytosol. The primary function of PPP is to supply NADPH for reductive biosynthesis and ribose-5-phosphate for nucleotide and nucleic acid biosynthesis (Mathews et al. 2000).

PPP has been less studied compared to glycolysis despite its importance in metabolism for many parasitic protozoa and in host-parasite relationship (Barrett 1997).

PPP supplies NADPH to organisms, which involves in oxidative stress. It was highlighted in previous published paper (Rodriguez-Contreras et al. 2007) that glucose transporter null mutant is sensitive to oxidative stress.

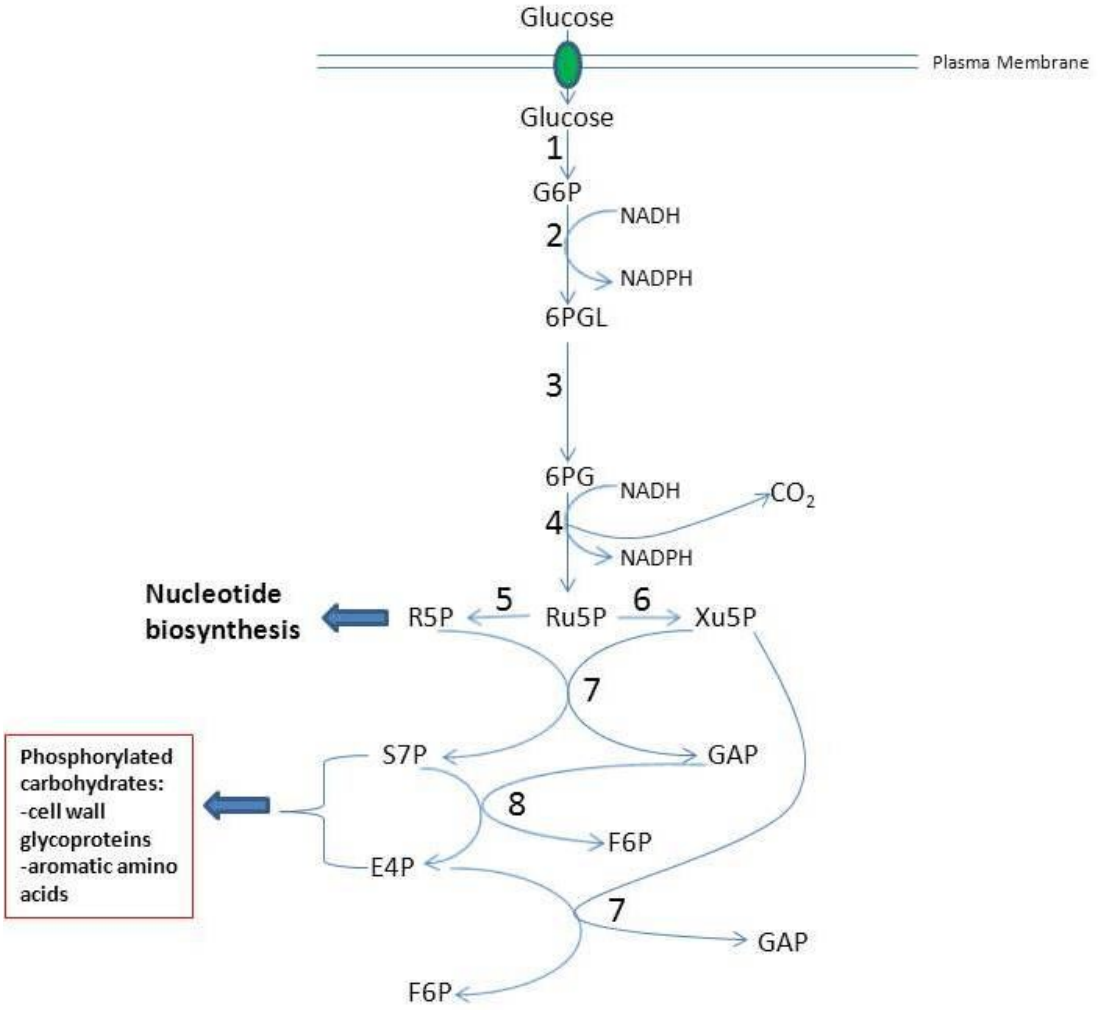


Figure 1-5: Glucose metabolism in pentose phosphate pathway. Glucose from the extracellular transported by the glucose transporter (green-coloured oval) converted to glucose-6-phosphate (G6P) which metabolise by the pentose phosphate pathway (PPP). PPP generates NADPH which is important for cellular redox (reduction of glutathione, trypanothione and thioredoxin; detoxification (cytochrome P450, glutathione-S-transferase); and reductive biosynthesis. Apart from NADPH generation, PPP generates important ribose-5-phosphate (for nucleotide biosynthesis) and intermediates for phosphorylated carbohydrates production. Abbreviations: G6P, glucose 6-phosphate: 6PGL, 6-phosphogluconolactone: 6PG, 6-phosphogluconate: Ru5P, ribulose 5-phosphate: R5P, ribose 5-phosphate: Xu5P, xylulose 5-phosphate: S7P, sedoheptulose 7-phosphate: E4P, erythrose 4-phosphate: F6P, fructose-6-phosphate: GAP, glyceraldehyde-3-phosphate. Enzymes indicated by numbers: 1, hexokinase: 2, glucose-6-phosphate dehydrogenase: 3, 6-phosphogluconolactonase: 4, 6-phosphogluconate dehydrogenase: 5, pentose phosphate isomerase: 6, ribulose-5-phosphate-3'-epimerase: 7, transketolase: 8, transaldolase. (Description and pathway image was adapted and amended from Barrett, 1997)

1.7 Transporters

Nutrients and essential molecules for the survival of *Leishmania* need to be transported into the cell. Membrane transporters are critical in transporting molecules into the cell compartment where various metabolisms operate.

1.7.1 Glucose transporters

As glucose is the primary source for energy metabolism in organism, the glucose transporter has been the major focus of researchers (Manolescu et al. 2007). In *Leishmania* spp., the central role of glucose uptake has make it important to better understand the process at it molecular, cellular and genetic level to enhance our biochemistry and physiology knowledge of the parasite, therefore gives more information for researches to develop a novel therapeutics target that selectively impede the function of vital permeases of the parasites without affecting the host's glucose transporter (Landfear 2010).

It is unclear of whether *Leishmania* glucose transporter is a facilitative transporter or concentrative proton symporters although it is reported that glucose uptake in parasite is inhibited by protonophores and sodium azide (respiratory inhibitors) (Burchmore & Hart 1995;Zilberstein & Dwyer 1984;Zilberstein & Dwyer 1985), indicating the existence of proton symport (Landfear 2010).

The big number(40%) of metacyclic-like cultures in stationary phase culture and increasing expression of glucose transporter in *Leishmania mexicana* cultured under surrounding that induced metacyclogenesis suggesting metacyclogenesis process is coupled with increased glucose uptake and utilisation (Bates & Tetley 1993;Burchmore & Hart 1995).

The genome of *Leishmania mexicana* contains a cluster of 3 genes named *LmGT1, LmGT2 and LmGT3* (Burchmore & Landfear 1998) which are link to each other. These gene encode glucose tansporter proteins and these proteins are related in sequence and predicted secondary structure to mammalian facilitative glucose transporters, contain 12 transmembrane domains although only approximately 20% of the sequence reported to be similar to the closest

homologue, human glucose transporter GLUT1 (reviewed in (Landfear 2010)). These 3 *L.mexicana's* glucose transporter proteins are isoforms that similar but differ to each other over the region containing transmembrane domains and connecting loops. However, these exhibited to be significantly diverge in sequence in the hydrophilic domains at the NH_2 and COOH termini (Burchmore & Landfera 1998).

Each isoform has been expressed in *xenopus* oocytes and glucose transporter *Leishmania mexicana* and from these experiments it showed that these proteins transported hexose sugars i.e. glucose, fructose, mannose and galactose (Burchmore & Landfear 1998, Rodriguez-Contreras et al. 2007).

Pro-1 protein, which is a glucose transporter in *Leishmania enriettii* exists in two isoforms i.e. isoform 1 and isoform 2 (Piper et al. 1995) and the protein is encoded by a sigle family of tandemly clustered genes which contain 8 copies of 3.6 kilobaserepeat unit (Stein et al. 1990). Isoform 2 has been reported to be closely related to LmGT2 of the *Leishmania mexicana* (Burchmore & Landfear 1998). Glucose transporters in parasitic parasites i.e. Leishmania,Trypanosoma and Plasmodium are related in sequence and structure to mammalian facilitative glucose transporters of the SLC family althoughthey are quite divergent in sequence (reviewed in (Landfear 2010)).

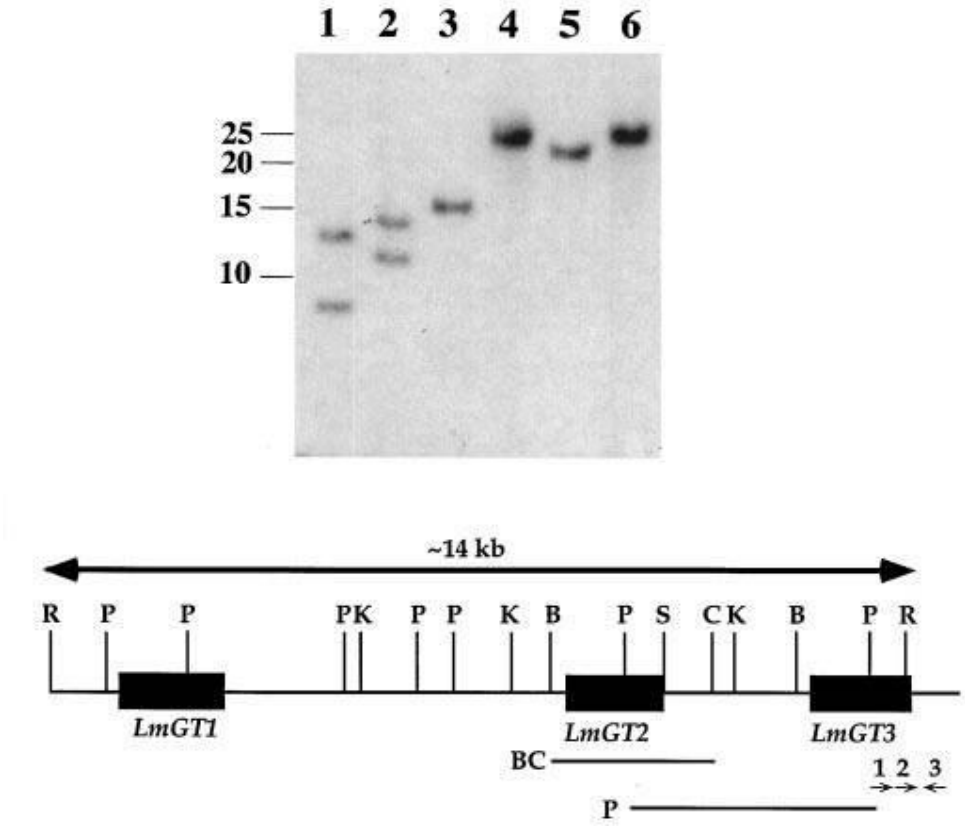


Figure 1-6 Structure of the *LmGT* gene locus in *L.mexicana*. (Upper) A Southern blot analysis showing genomic DNA restriction fragments separation in the electrophoresis , transferred to a nylon membrane and hybridised with a protein-coding region of *L.enrietti* Pro-1 glucose transporter gene. Lane 1, *Bgl*II; lane 2, *Not*I; lane 3, *Eco*RI; lane 4, *Hind*III; lane 5, *Bam*HI; lane 6, *Eco*RV. The analysis revealed the single hybridising bands in *Eco*RI, *Hind*III, EcoRV and BamHI digests, suggesting that most or all of the *LmGT* locus was contained within the ~14 kb *Eco*RI. (Lower) A picture showing the restriction map of ~14 kb *Eco*RI genomic clone. Southern blot analysis showed that glucose transporter homologs mapped to three fragments in a *Kpn*I-*Eco*RI digest of this genomic clone. The three fragments that were subcloned and partially sequenced are an *Eco*RI-*Kpn*I (~5.2 kb), a *Kpn*I (~3.8kb) and a *Kpn*I-*Eco*RI (~2.7 kb). The solid boxes in the picture indicates protein coding regions and were deduced and revealed that the single *L.mexicana* glucose transporter locus comprises 3 clustered genes that encode 3 glucose transporter isoforms i.e. LmGT1, LmGT2 and LmGT3. Coding and 3'-UTR sequences downstream of the ~14 kb EcoRI fragment were acquired from cDNA using primers at locations designated by the small arrows marked 1, 2 and 3. R, *Eco*RI; K, *Kpn*I; S, *Sca*I; B, *Bgl*II; P, *Pst*I; C, *Cla*I. Restriction fragments for *Bgl*II/*Cla*I (BC) and *Pst*I (P) are indicate by the solid lines below the map. Image from Burchmore & Landfear 1998).

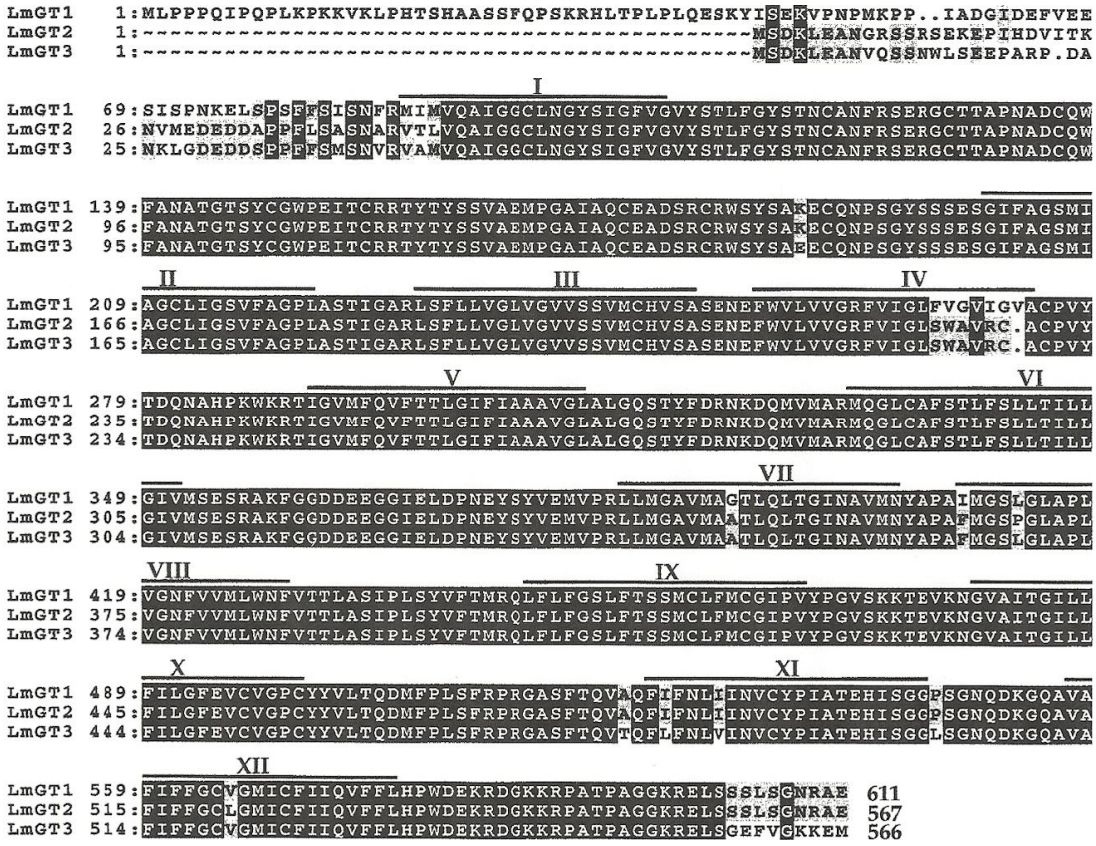
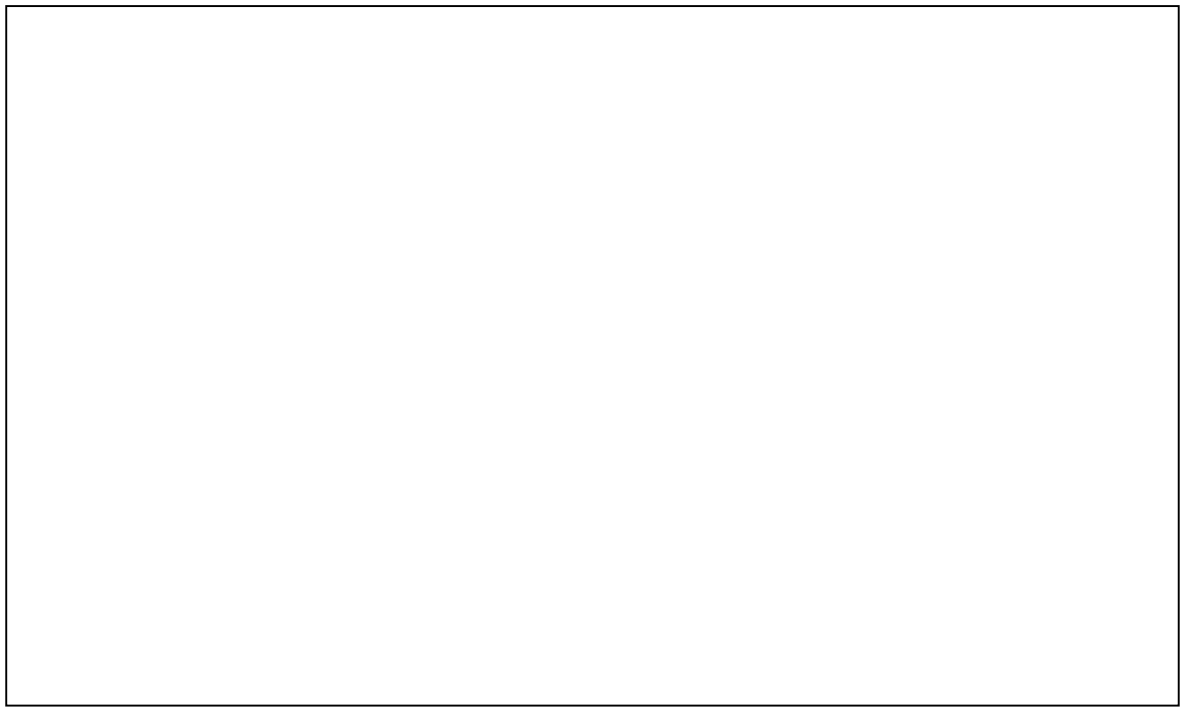


Figure 1-7 The predicted amino acids sequences alignment for *L.mexicana*'s glucose transporters LmGT1, LmGT2 and LmGT3. The white backgrounds indicate the non-identical regions in all three proteins, while regions that are identical in two out 3 sequences are showed by a gray background. The positions for amino acids in each sequence show by the the numbers on the left, whereas the total number of constituent amino acids indicated by the numbers at the end of each sequence. Spaces presented to optimize the alignment are demonstrated by a period. The roman numbered bars above the aligned sequences indicate the predicted transmembrane domains. Image from Burchmore & Landfear 1998.



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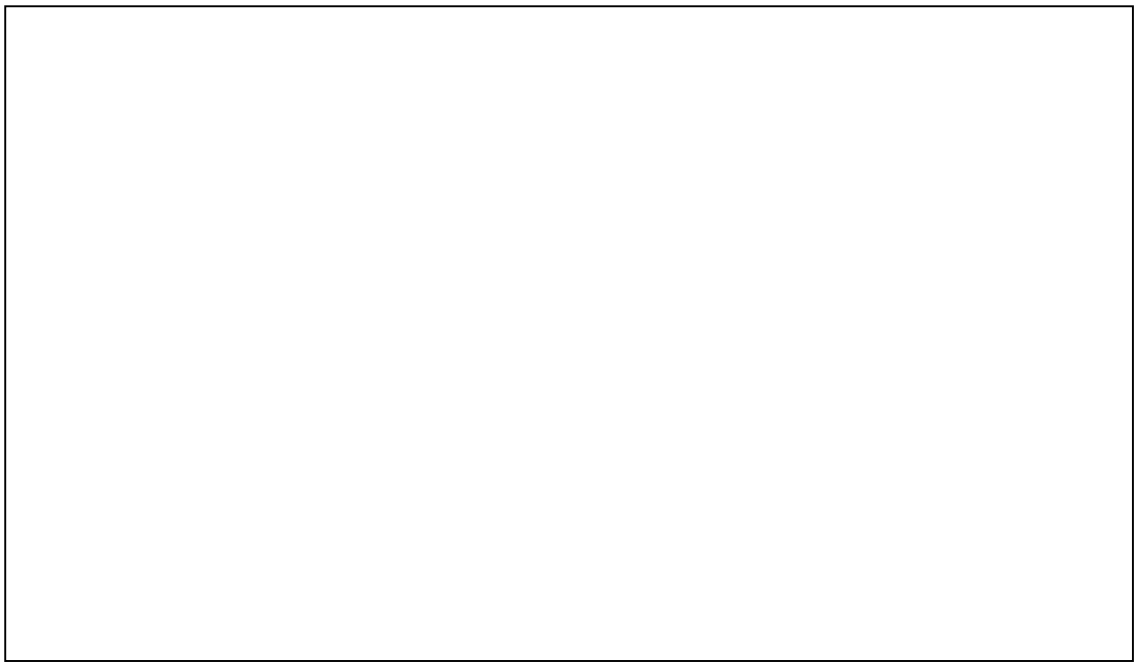
1.7.2 Amino acid transporter

Refer to Chapter 2.

1.7.3 Myo-inositol transporter

The plasma membrane of *Leishmania* generally composed of inositol containing phospholipids or glycoproteins which attach to the membrane by an anchor called glycosylphosphotidyinositol anchors (McConville & Ferguson 1993). Lipophosphoglycan , which is the main glycolipid found in promastigotes(Turco & Descoteaux 1992), is structurally modified of inositol-phospholipid. Both lipophosphoglycan and glycoprotein of the parasites are thought to facilitate the attachment of the parasite to the epithelium of the sandfly midgut and as well as serving as a protection to the parasites as the parasite able to survive and replicate in a known hostile condition. From the biosyenthetic pathway of lipophosphoglycan (Turco & Descoteaux 1992) and glycosylphosphotidylinositol anchors (McConville & Ferguson 1993), it has been found to be essential as their building block. Drew et al. have shown that myo-inositol, i.e. the biosynthetic precursor for abundant glycolipids, is transported across the plasma membrane of *Leishmania donovani* promastigotes by a specific transporter and this transporter exhibits a structural associate to mammalian facilitative glucose transporters (Drew et al. 1995). The study using tow-electrode voltage clamp experiments have demonstrated that the transporter is a symporter that utilises a proton gradient to accumulate myo-inositol within the cell. In addition, immunolocalisation experiments from the same paper (Drew et al. 1995) revealed the presence of this protein in the *Leishmania donovani*'s plasma membrane. The gene that encodes for this transporter was named D1 (or also known as MIT (myo-inositol/proton co-transporter)) and it was cloned and sequenced from *L.donovani* (Langford et. al 1992). This gene sequence is shown to be related to ITR1 and ITR2 genes i.e.genes encode for inositol transporters from the yeast, *Sachharomyces cerevisiae*(Drew et al. 1995) and it is reported to have a number of conserved residues and motifs from the sugar transporter superfamily and these residues are important for the function of inositol uptake (Seyfang et al. 1997 & Seyfang & Landfear 2000).



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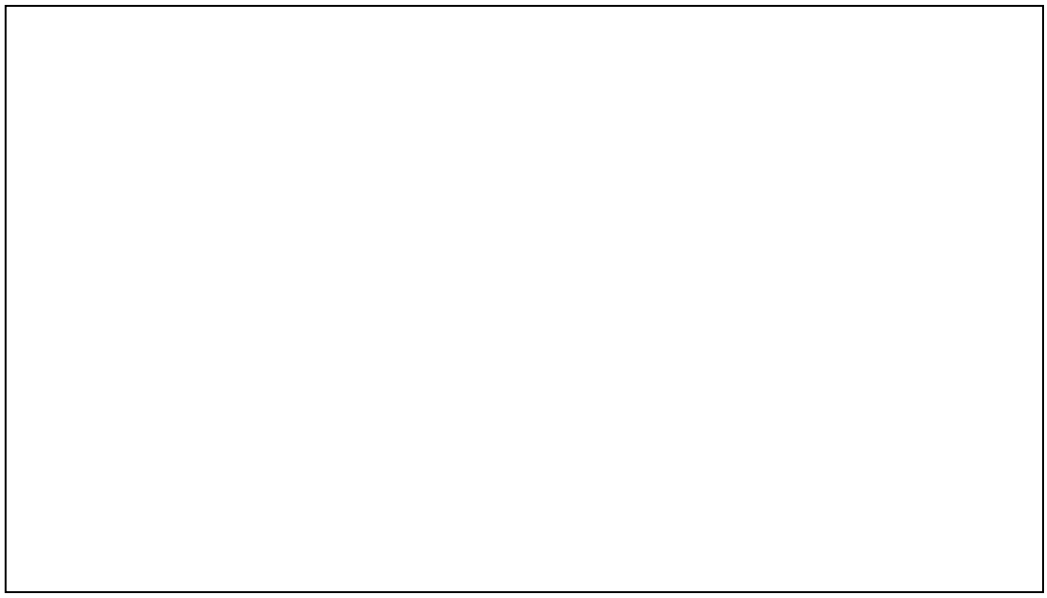
1.7.4 Nucleoside transporter

Unlike the mammalian cells that possess the ability to synthesised purines de novo, *Leishmania* spp. and other protozoan parasites depend on the various kinds of nucleoside transporters to salvage these compounds from their hosts. This lack of de novo purine synthesis is in concurrence with the parasite surroundings that plentiful with pre-formed purine (Lafon et al. 1982). However, contrary to purine metabolism, *L.mexicana* has the capacity for pyrimidine biosynthesis (Galbraith 1991;Marr 1983). The reason of huge interest of scientist to study these transporters in *Leishmania* is also because that they mediate the uptake of a variety of cytotoxic drugs (Landfear et al. 2004).

Genetic and biochemical studies done by Iovannisci et al. showed that *Leishmania donovani* promastigotes have 2 nucleoside transport activities with different substrate specificities (Iovannisci et al. 1984).The first is a transport activity of adenosine and pyrimidine nucleoside and as well as a cytotoxic analog of adenosine i.e. tubercidin, while the other is a transport activity for guanosine, inosine and the cytotoxic inosine isomer, formycin B (Aronow et al. 1987). Mutant cell lines from *Leishmania donovani* promastigotes i.e. TUBA5 (tubercidin-resistant) and FBD5 (formycin B resistant) have been used to clone the respective nucleoside transporter genes by functional complementation (Vasudevan et al. 1998 & Carter et al. 2000). The genetic locus of *LdNT1* encompasses 2 closely related genes i.e. *LdNT1.1* and *LdNT1.2* (Vasudevan et al. 1998). It has been shown that these nucleoside transporter proteins, LdNT1.1 and LdNT1.2 are related to mammalian equilibrative transporters but have higher substrate specificity for adenosine and pyrimidine nucleosides (Aronow et al. 1987) compared to mammalian equilibrative transporters which have broad substrate specificities which transport all of the nucleosides (Yao et al. 1997)). LdNT1.1 and LdNT1.2 proteins are predicted to have 11 transmembrane domains (Vasudevan et al. 1998). When TUBA5 *Leishmanias* were transfected with a cosmix library containing the *LdNT1.1* and *LdNT1.2* genes, the cells showed restoration of sensitivity to the cytotoxic adenosine analog tubercidin and ability to transport [^{3}H] adenosine and [^{3}H]uridine. In addition, expression of LdNT1.2 ORF in Xenopus oocytes enhanced the uptake of [^{3}H]adenosine, hence these findings confirm that the LdNT1.1 and LdNT1.2 proteins are functional nucleoside transporters (Vasudevan et al. 1998).

Gene *LdNT2* encodes a polypeptide comprising 499 amino acids which also exhibit significance homology with other members of the equilibrative nucleoside transporters family (Carter et al. 2000). Molecular analysis done on *Leishmania donovani* genome has demostrated that *LdNT2* gene exists as a single copy gene which encodes a single transcript of 3 kilobase pairs (Carter et al. 2000). Expression of *LdNT2* in nucleoside transport null mutant *L.donovani* and in *Xenopus laevis* oocytes had proved that LdNT2 is an inosine-guanosine transporter with high affinity with a singular predicted membrane topology (Carter et al. 2000).



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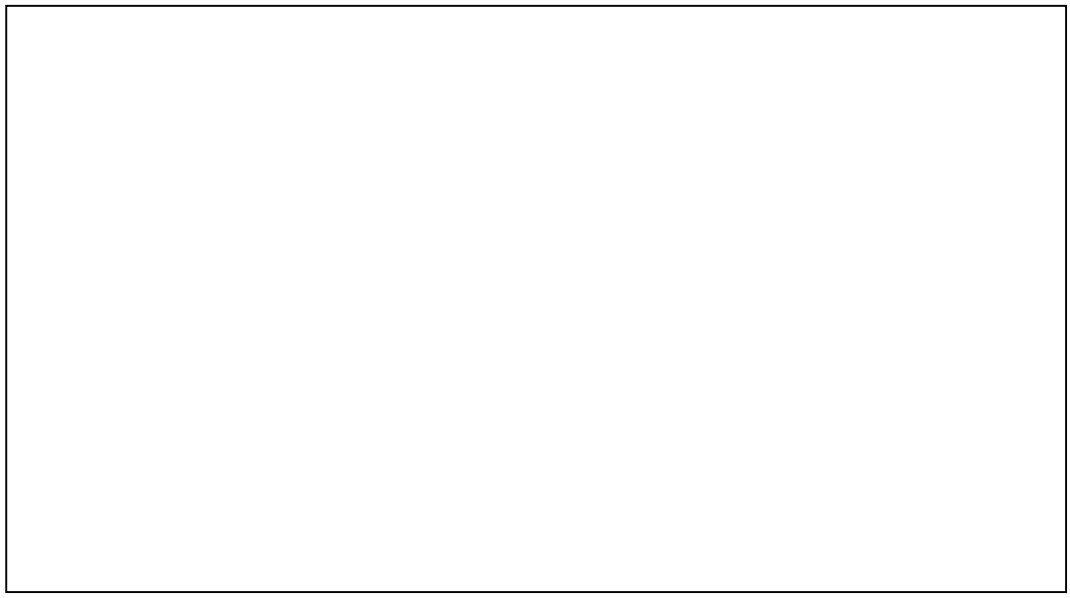

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1.8 Glucose transporter null *Leishmania mexicana*

Leishmania mexicana has a cluster of family of 3 related single copy glucose transporter genes and they encode for proteins LmGT1, LmGT2 and LmGT3 (Burchmore & Landfear 1998) . The proteins are similar isoforms but different from each other at the transmembrane domains and connecting loops and significantly diverge at the amino and carboxy-hydrophylic domains(Burchmore & Landfear 1998) . Although LmGT2 mRNA is reported to be more highly expressed in promastigotes compared to amastigotes, the LmGT1 and LmGT3 mRNAs expression in both development stages are the same (Landfear 2000).The phenotype of these transporters genes were analysed by reverse genetics. Targeted gene replacement technique was employed by knockout *LmGT1*, *LmGT2* and *LmGT3* genes residing on both homologs chromosomes and replace them with drug resistance marker and this glucose transporters genes deletion was generated at promastigote stage (Burchmore et al. 2003). The study showed that glucose transporter null mutant *Leishmania* (ΔLmGT) lost the ability to uptake hexoses sugar i.e. glucose, fructose, mannose and galactose and the uptake of all 4 sugars is restored by the complementation of the null mutant with each open reading frame on an episomal expression vector (Rodriguez-Contreras et al. 2007). Apart from the lost capacity of the hexoses uptake, the growth of ΔLmGT promastigotes is slower and to a lower density compared to the wild type. However by complementation of all 3 *LmGT* open reading frame the growth rate are restored to almost to the same level of the wild type (Landfear 2010).

In the studies involving murine primary macrophages, ΔLmGT promastigotes able to infect macrophages (Burchmore et al. 2003). While the wild type *Leishmania mexicana* amastigote replicate, ΔLmGT was unable to replicate and died over the course 6 days later(Landfear 2010). Nevertheless, the growth of ΔLmGT amastigotes in macrophages is partially restored by complementation with *LmGT2* or *LmGT3*. Furthermore, ΔLmGT promastigotes were unable to transform into axenic culture form amastigotes , and wild type axenic amastigotes were unable to survive with withdrawal of glucose from growth medium (Burchmore et al. 2003). These findings show that, although down-regulation of glucose transport(Burchmore & Hart 1995) and metabolism (Hart & Coombs 1982;Rainey & MacKenzie 1991) are observed in *Leishmania*

amastigotes, glucose transporter expression is important for amastigotes survival (Landfear 2010).A study on *Leishmania mexicana* showed that glucose transporter LmGT2 and LmGT3, can mediate ribose uptake(Naula et al. 2010). LmGT2 has the higher affinity for the pentose sugar D-ribose (Naula et al. 2010). *Leishmania* has the capacity to transport (Maugeri et al. 2003;Pastakia & Dwyer 1987) and metabolise ribose (Berens, Deutsch-King, & Marr 1980). Amastigote may use ribose as an energy source (Burchmore & Barrett 2001). These observations support the suggestion that glucose transport is essential for the survival of both *Leishmania* life cycle stages as it is has high affinity for other molecules than glucose thus *Leishmania* can thrive in a condition with scarce glucose supply.

However, Feng has demonstrated that these null mutants have suppressed the avirulent phenotype and found the amplification of the gene called *LmGT4*, a gene that encodes an alternative hexose transpoter, LmGT4. The suppressors null mutant display an increased expression of *LmGT4* mRNA and protein (Feng et al. 2009). Other phenotypes exhibited by the suppressor null mutants recover the capability of transporting hexose sugar, boost its resistance to stress; oxidative stress, heat shock and starvation for nutrients, and enhance their carbohydrate ß-mannan storage, as well as increasing the cell size compared to the unsuppressed null mutants.In addition, the growth as a promastigote stage is increased compared to the unsuppressed null mutants (Feng et al. 2009).

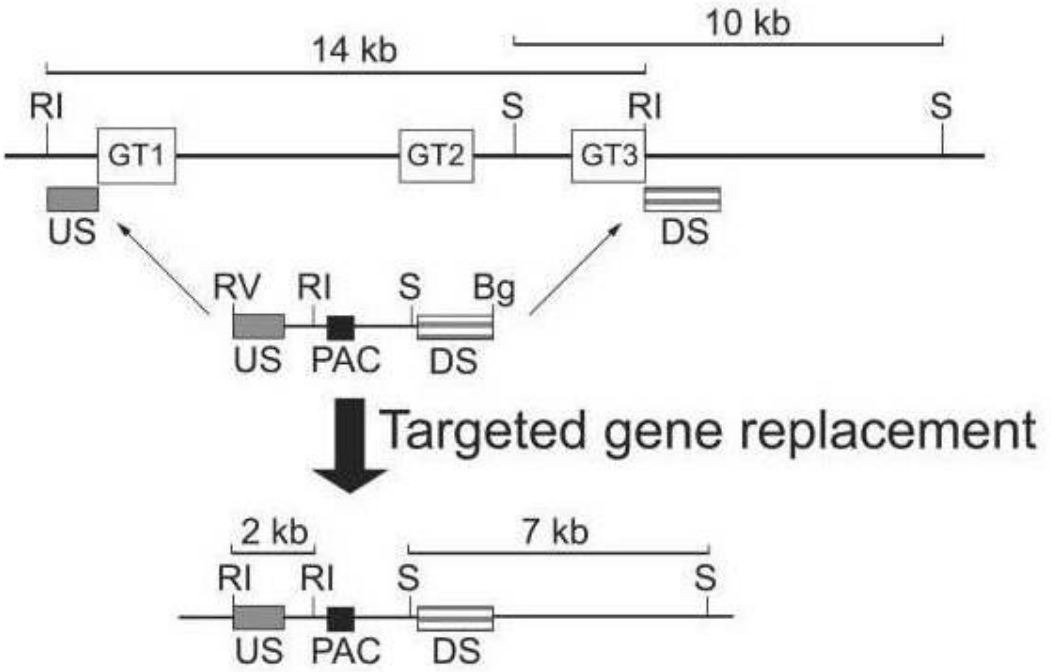


Figure 1-12 Construction of glucose transporter null mutant. The mutant was generated by targeted gene replacement strategy. (Upper) To target homologous recombination of the gene disruption constructs, the *LmGT*gene locus including the three ORFs (open reading rectangles marked GT1, GT2 and GT3), the 10kb *Sma*I and 14 kb *Eco*RI restcition fragments, and the location of the upstream (US) and downstream (DS) segments were used in the strategy. The example of one of the disruption constructs structure is showed immediately below the LmGT locus. The construct includes the US and DS segments, the ORF for the PAC selectable marker , the *Eco*RV and *Bgl*II terminal polylinker restriction sites, and the internal *Eco*RI and *Sma*I restriction sites. The sites for the homologous integration indicated by the thin arrows. (Lower) Below the thick arrow shows the event of the targeted gene replacement, showing the structure of the resulting chromosomal locus and the predicted 2 kb *Eco*RI and 7 kb *Sma*I restriction fragments that are diagnostic of the correct homologous integration event. The restriction fragments are indicated by the symbols : RI, *Eco*RI; S, *Sma*I; RV, *Eco*RV; Bg, *Bgl*II. As required, a null mutant need a second targeted gene replacement using similar gene disruption cassette containing a SAT marker. (Burchmore et al, 2003).

1.9 Proteomics

Why is there still a need to measure protein level when there are many other methods e.g. DNA chip arrays, can accurately measure mRNA levels?

The truth is, the level of mRNA does not reflect the actual amount of protein present in the cell (Conn 2003). Several studies have showed poor correlation between transcriptome and proteome in yeast (Gygi et al. 1999) and human liver (Anderson & Seilhamer 1997). Furthermore, the phenotype expressed somehow is influenced by the surroundings, thus, study on mRNA or protein profile expressed must be done under strictly controlled conditions (Burchmore et al. 2001).

Unlike genome which the content is static, proteome may change as it goes through post-translational modification(PTM) like proteolysis, glycosylation, acetylation, deamination, palmitoylation, sulfation,phosphorylation and so on, or formation of large protein complexes (Cho 2007;Conn 2003). Ultimately, a single gene may encode as many as 50 different proteins type. Thus, genome content does not predict the active of protein and RNA quantitation does not represent the protein levels (Cho 2007).

Proteomic studies have reported higher percentage in predicting the proteome compared to transcriptomic studies as stage-specific protein are specifically regulated at translational and post-translational level (Bente et al. 2003;Cohen-Freue et al. 2007;Leifso et al. 2007;McNicoll et al. 2006;Rosenzweig et al. 2008).

However, proteomics studies can be less conclusive in elucidating phenotype or diversity of pathology. For example in a drug resistance study (Drummelsmith et al. 2003;Drummelsmith et al. 2004;El Fadili et al. 2009;Sharma et al. 2009;Singh et al. 2008;Vergnes et al. 2007) and species diversity study(Brobey et al. 2006), a number of differently expressed proteins lacking annotated functions were listed and this may make the process of identifying of how different proteins in the biological systems of organisms relate to each other and hence, may raise many other new questions(Scheltema et al. 2010).

1.9.1 Proteomics & challenges for membrane protein

The central proteomic technology is mass spectrometry but in order to achieve significant invention and findings other technologies, resources and expertise are needed (Brewis & Brennan 2010). Prior to the identification of proteins (or peptides) by MS, there are certain procedures that a sample protein needs to go through. A choice of separation, fractionation and identification techniques needs to be considered carefully in order to get the most valuable results.

There are major challenges imposed upon the study of membrane proteomes. Firstly, membrane proteins are mostly present at low levels in biological membranes and it is thus difficult to isolate proteins of interest in sufficient quantities for further analysis or identification. This can be solved by enriching membrane proteins using biochemical approaches. Secondly, the high hydrophobicity characteristic of membrane proteins makes them less soluble in an aqueous environment. Alternative methods for membrane protein solubilisation are thus required (Seddon et al. 2004).

Challenges are also present at the downstream protocol of the experiment. As membrane protein is highly hydrophobic, it needs to be solubilised in a suitable detergent. Unfortunately this detergent may interfere with separation particularly those with LC and produce noise in analytical analysis i.e in ESI-MS, so detergents need to be carefully removed before being further analysis(Blonder et al. 2002) analysis steps.

1.9.2 Protein Enrichment

Enrichment is the first key steps in my proteomic experiment and this step, which defines the plasma membrane starting material, is undoubtedly the most important (Sprenger & Jensen 2010). Unlike DNA, protein unable to be amplified within minutes using PCR. Depends on the working hypotheses, nature or target of the experiment, the commonly used enrichment strategies are selective precipitation and/or subcellular fractionantion of proteins or organelles by density gradient centrifugation and two-phase partitioning, cationic colloidal silica fractionation, immunoisolation and affinity enrichment plasma membrane

proteins by using cell-surface biotinylation chemistries or by using lectins (Cordwell & Thingholm 2010;Josic & Clifton 2007;Sprenger & Jensen 2010).

1.9.3 Protein Separation

Enriched protein samples contain numerous of complex protein and peptides. In order to optimise the characterisation of each protein/peptide, one has to choose the most suitable separation technique hence easier to identify each molecule.

The most used separation techniques in proteomic experiments are liquid chromatography or gel electrophoresis, either one-dimensional (1-DE) or two-dimensional (2-DE). 2DE, which was first described in 1975 (O'Farrell, 1975) relies on separating proteins based on 1) pI, isoelectric focusing, (first dimension) and 2) molecular weight (second dimension) and stained for visualisation using Coomassie blue and silver staining. Liquid chromatography although offering a wide choice of matrices for protein separation and the potential for automation and direct feed into MS, also has disadvantage that made this less attractive. The direct feeding of samples into MS limit the time to analyse each fraction individually as each fraction is a moving target (Minden 2007).

As 2-DE separate proteins in gel, this permits more time to spend on analysing each fraction individually i.e. predict the molecular weight by looking at the position of the fraction in the gel and more than one samples can be run simultaneously unlike column chromatography which is limited to one sample per apparatus which may take longer time for analysis and is not suitable for comparative proteomics.

However, 2-DE has been proven be incompatible for membrane proteins separation as it poorly resolve basic and hydrophobic protein (McDonough & Marban 2005) and has low sensitivity in detecting protein with low abundance (Ning et al. 2008).

To overcome these problems, one approach is combination of gel based fractionation, particularly 1-dimensional polyacrylamide sodium dodecyle

sulphate gel electrophoresis (1D-SDS PAGE) with isoelectric focusing (IEF) fractionation in solution.

1.9.4 Comparative proteomic challenges

Comparative study in biological science can be interpreted as experimentation where 2 or more samples are being compared against their biological states i.e. the biochemistry or physiological processes, molecular content/level etc of their cells, tissues or organs depending on the hypotheses and aims of the experiment.

It is widely used in biomedical studies and has shed some light on understanding the pathology of diseases. Proteomic approaches have been incorporated in comparative studies as it is considered to be a highly powerful technology to investigate the differences of proteome in cells, tissues or organs which facilitates addressing the issues in biomedical research.

Comparative studies in *Leishmania* have been widely used in understanding several important issues i.e. how one of the stages of the life cycle can be pathogenic to humans whereas the other stage does not, investigating the mechanism of survival of the intracellular form and simultaneously pinpointing therapeutic targets (Walker et al. 2006) and, how one stage of the life cycles can strive in harsh conditions and be more infective to mammals compared to the another (Bente et al. 2003;Nugent et al. 2004).

However, before one wants to start a comparative proteomics study, some challenges need to be considered as if these challenges are not taken seriously, it could influence the experimental outcome. Such challenges are:

1. How to resolve complex proteins and identify the few proteins that differs between the samples being compared (Minden 2007).

The chemical complexity of the proteome is far greater than is encountered in genomic and transcriptomic profiling. These complexities are

contributed by the protein mass, protein isoelectric points (pI) and protein abundance where the mass can range from 1000-1,000,000 Da, the pI ranges between 3-10 and abundance ranges from 1000-10,000,000 copies per cell. To find proteins with small amounts that are different between samples and sifted through these highly complex proteins, one will need to design a fractionation method that reduces the complexity without modifying the chemical nature of the proteins although the detection method nowadays has advanced in identifying proteins rapidly.

2. Dynamic range

The Dynamic range of proteome poses another challenge to comparative study. One can have hypotheses to search for proteins that might be as high $1x10^6$ molecules per cell to as low as 50 molecules per cell (Ghaemmaghami et al. 2003). Tubulin is known to have high-abundance in kinetoplastida and due through their extensive modification and processing together with the existence of numerous isoforms, detection of low abundance proteins is difficult and could lead to underrepresentation of low copy number proteins (Foucher et al. 2006).

1.9.5 Membrane Protein

The plasma membrane serves as the cell boundary and contains a numerous number of transporters and other proteins that are important for the survival, growth and virulence of parasites. Membrane protein which exist as a lipid bilayer, play various important roles in cellular processes i.e. signal transduction, cell adhesion, metabolite and ion transport, endocytosis etc. Comparative proteomic analyses, which have demonstrable value in understanding parasite phenotypes, tend to be biased against the plasma membrane proteome because these proteins are often of low abundance and has poor solubility. The importance of membrane protein to organisms is further supported by the fact that the membrane protein represents around 30% of total proteins (Wallin & von Heijne 1998).

Extensive researches on membrane proteins have been ongoing for several decades and yet it remains as difficult tasks for membrane protein experiments because of its contents/building blocks, which requires a balanced hydrophilic and lipophilic surrounding, which optimises the differences among different proteins (Sprenger & Jensen 2010).

Membrane consists primarily of protein and lipid. Protein can comprise greater than 50% by mass of membrane and carries out most of the tasks of the membrane. Depending on the type of the cell, the ratio of the weight of membrane-associated proteins to lipids may range from 1.0 for blood cells to 3.0 for mitochondrial membranes except for myelin where it is only 0.23 as it function as an insulator unlike other cell types that serve as 'conductors' (Singer 2004).

1.10 Metabolomics

Metabolomics aim to understand biology by studying the metabolic profiling of subject under study. As none of the post genomic technologies i.e genomics, transcriptomics, proteomics can stand alone in studying the phenotypic characterisation of organisms, metabolomic investigations may needed in order to complement more conventional –omic studies. By using the *L.major* genome sequence, two different studies (Chavali et al. 2008;Doyle et al. 2009;Scheltema et al. 2010) have predicted around 1000 metabolites that comprise central metabolism and related with enzyme-catalysed reactions. However this number may reduce to 600 as cell-based metabolomics covers varies cellular sections, so the same metabolites may exist in more than one cellular compartment (Scheltema et al. 2010). Transcription and protein events, which are intermediate processes in the information flow from genotype to phenotype, are much more complex than the metabolome, but the chemical diversity of the metabolome is far greater than for the nucleic acid or protein. While proteins are the primary effectors molecules in cells, the metabolites are the substrates and products that are processed to drive living processes.

Nevertheless, it is impossible to predict metabolite levels by looking at the protein and transcript levels as metabolic levels or metabolome profile may

influenced by non-local control structure of metabolic network i.e enzyme inhibitors operating at the remote points in metabolic system as showed by metabolic control investigation (Kell & Westerhoff 1986).

Metabolomic analysis has potential to reveal important processes that may be specific to *Leishmania* and that may consequently present excellent drug targets. Indeed most drugs act as metabolite mimics. Metabolomic approaches were exploited in this project to complement proteomic analyses, with the assumption that cells unable to acquire a key metabolite like glucose would display significant changes at the metabolomic level.

1.11 Mass spectrometry

Over the years scientists have paid their attention to the sequential isolation, structural characterisation and functional assay of proteins. These studies are no doubt have been giving invaluable insights to understand the functional biochemisty of proteins. However these approaches do not suitable for studying 'global' biological studies (Ferguson & Smith 2003) where a complex mixture of proteins are studied at once rather than targeting a particular protein of interest. Mass spectrometry has been used widely in proteomic studies as it has enabled rapid protein identification and characterisation, owing to its speed, specificity and sensitivity (Reid & McLuckey 2002).

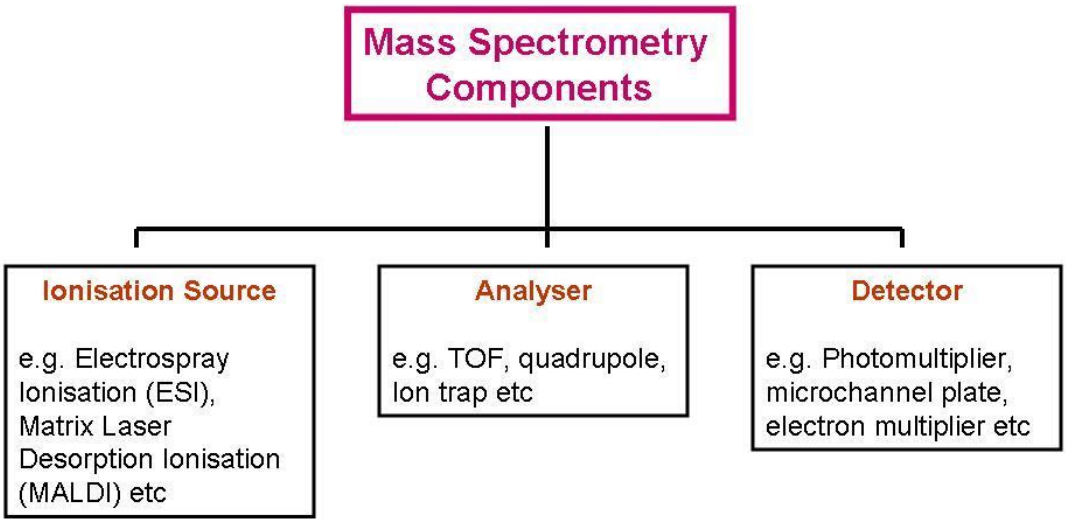


Figure 1-13 Mass spectrometry consists of three components. As a sample of study subjected to mass spectrometry , it is first ionised into a gas phase by the ionisation technique e.g. electrospray ionisation (ESI) and matrix laser desorption ionisation (MALDI) etc. The gas phase comprises ionised molecules and ions are pass through an anlyser which can be only one kind e.g. time-of-flight (TOF), quadrupole and ion trap etc or a combination of 2 more of the mentioned analyser. The analyser will sort out the ions and separate them according to their mass and charge ratio (m/z). Finally, the separated ions are detected and measured.

Basically the samples, either mixtures of proteins or peptides originated from whole cell lysate or tissue homogenate, are initially separated in microcapillary format liquid chromatography or electrophoresis before being transformed to gas phase by electron ionisation followed by mass analysis by the mass analyser.

In the current project, ESI-QTOF MS (Electrospray ionisation-quadrupole time-of-flight mass spectrometry) was used. It has an ESI source, hybrid mass analysers (quadrupole and time of flight) and a multichannel plate detector. In the studies, experiments were operated in both MS and tandem MS modes. MS mode uses the TOF to measure peptide ion mass, then MS/MS mode uses the quadrupole to isolate and fragments specific peptie ions and the TOF to measure their mass.

1.11.1 Ionisation

Ionisation techniques and instruments to be used depends on the samples and what kind of analysis is to be conducted. The most common ionisation techniques used in biological studies are electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI).

1.11.1.1 Electrospray Ionisation (ESI)

The history of ESI began with the work of Malcolm Dole, who described it in the studies of ions from syntheticpolymers in 1968 (Zhou & Veenstra 2008). However, only in the late 1980's ESI has made an impact in biochemical studies when John Bennett Fenn showed that it is possible to analyse high molecular weight proteins (Zhou & Veenstra 2008). ESI generates intact ions in vacuum from large and complex species and makes it applicable for analysis that involve fragile polar molecules which may have important roles in biological systems (Fenn et al. 1989).

Figure 1-14 This image has been removed due to copyright restrictions.

1.11.2 Analyser

An alternative approach is using the tandem mass spectrometry (MS/MS) (Ferguson & Smith 2003). Usually, this is done using triple quadrupole (TQ), quadrupole ion trap (QIT), Fourier transform ion-cyclotron resonance (FTICR) or the hybrid that was used in this project (Ferguson & Smith 2003) i.e. quadrupole time-of-flight (QTOF) mass spectrometers.

1.11.2.1 Quadrupole

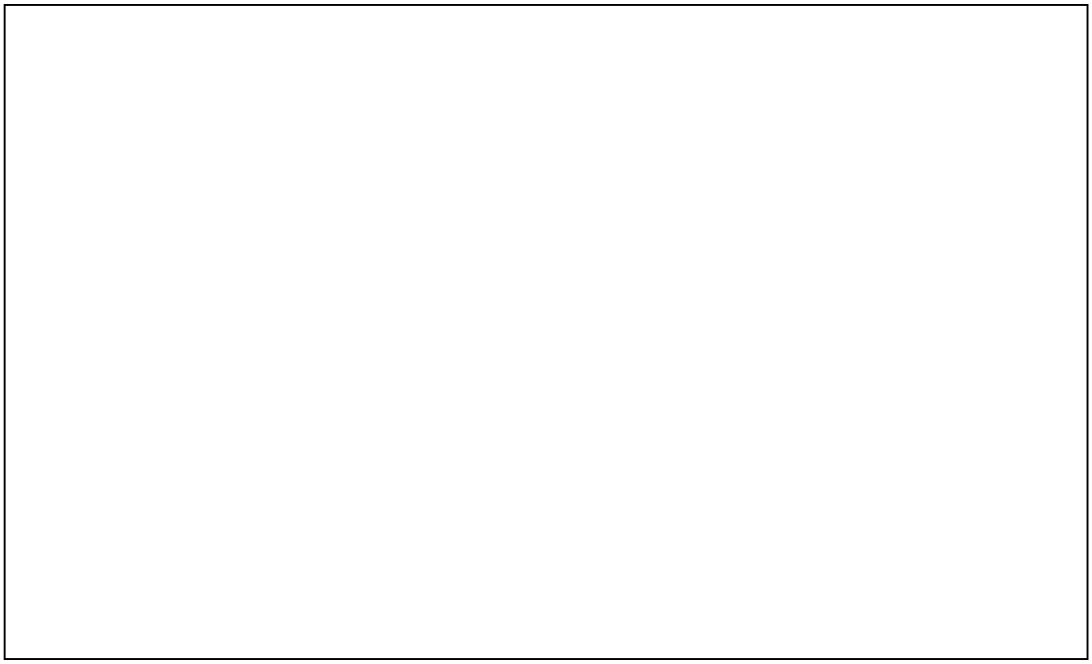


Figure 1-15 This image has been removed due copyright restrictions.

1.11.2.2 Time-of-Flight (TOF)

Time-of-flight is based on the simple principle of mass separation. The velocites of ions are related to their mass to charge ratio (m/z). If ionised species were at the same initial position, when they were accelerated by a constant homogenous electric field, the ions will move and the time taken to reach reach to the destination directly indicate their masses.

1.12 Project Aims

1. Look for changes in nutrient acquisition by glucose transpoter null *Leishmania*.

2. Further investigate proteomic changes seen in a previously performed whole proteome comparative analysis.

3. Develop a plasma membrane protein enrichment strategy for *Leishmania.*

4. Apply this to look for differences in the plasma membrane proteome between wild type and glucose transporter null *Leishmania*.

5. Comparative metabolomic study.

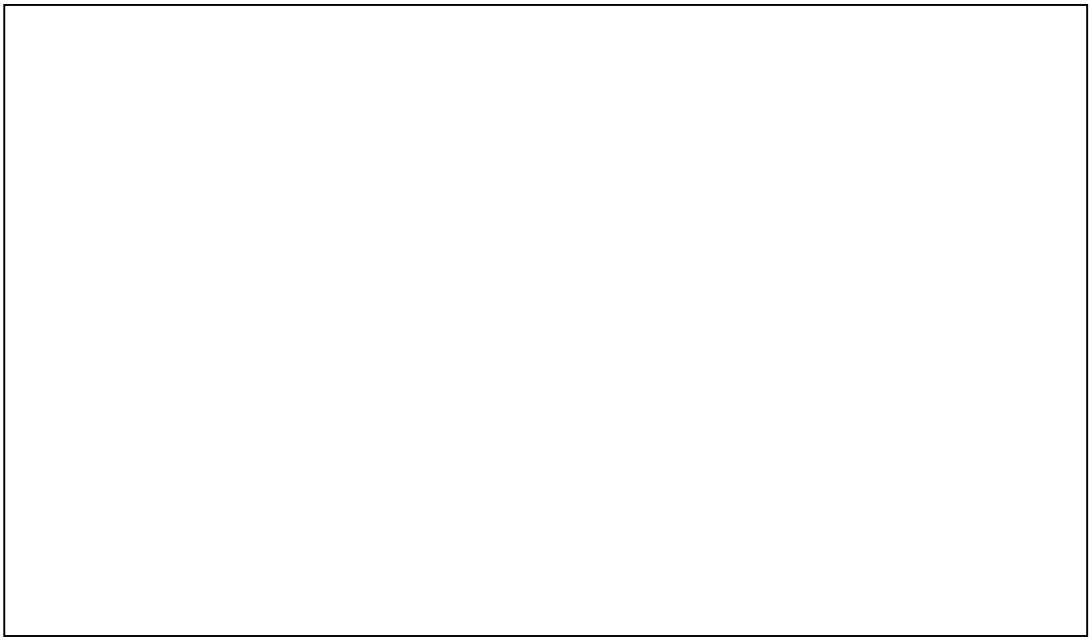
Chapter 2

Nutrient Uptake in Glucose transporter null mutant *Leishmania*

2.1 Introduction

The sand fly vector of *Leishmania* feeds primarily on plant material, which is rich in carbohydrates but relatively poor in other carbon sources (Burchmore et al. 2003;Young et al. 1980). Sugars are thus the major energy source available to the promastigote stage as it multiplies in the sand fly gut. Consistent with this, genetic ablation of glucose transporters in *Leishmania* gives rise to cells that cannot acquire glucose and that are poorly able to survive in the sand fly host (Burchmore et al. 2003). However, glucose-transport-null *Leishmania* promastigotes are viable in rich media, indicating that this stage has the ability to acquire other carbon sources from the environment. The major carbon sources that are presented to *Leishmania* in standard promastigote growth media (Appendix) are glucose and a variety of amino acids. To investigate the amino acid uptake capacity of *Leishmania* promastigotes, and to compare amino acid transport in wild type and glucose transport-null *Leishmania*, a series of transport assays were performed, together with a comparative metabolomic analysis of these lines.

Table 2-1 The table has been removed due copyright restrictions.



(Refer to Table 2-1) The cells were left to starve up to 3 hours to measure the amino acid in starvation experiments. Some amino acids were found to be enhanced during the experiment including isoleucine, phenylalanine and arginine and this accumulation maybe because of continued protein breakdown, as slowly metabolised compounds or as catabolic products of other cellular components (Simon et al. 1983). However, other amino acids that were found to be decreased during starvation are proline, valine, methionine, aspartic acid , threonine and serine and this fall in concentration may be due to continued catabolism or the inability of the cells to synthesise during starvation (Simon et al. 1983). As proline has been shown to have special physiological importance in *Leishmania sp.*, (Krassner & Flory 1969 and Krassner & Flory 1972) Simon et al. decided to look for effects in the amino acid pool (Simon et al 1983). Following the 3 hours of incubation of the cells with 5 mM proline, the amino acids that have been elevated are glutamic acid, serine, glucine, arginine, tyrosine and lysine. The observation of theeffect of proline on the amino acids pool may be associated with the proline's suitability as a precursor of other amino acids (Simon et al. 1983). Furthermore, the chromatography of cell extracts after incubation with [^{14}C]proline generates a number of label amino acids, hence, signifying that proline is a precursor of other amino acids (Simon et al. 1983). The use of sodium fluoride (metabolic inhibitor) in the study (Simon et al 1983) is in view that metabolic inhibitors are demonstrated to increase proteolysis in

L.tropica (Simon & Mukkada 1983). Following prolonged intoxication with sodium fluoride, several of the amino acids were reduced in concentration i.e. threonine, serine, glutamic acid, methionine, aspartic acid, histidine, proline and alanine. Nevertheless, the majority of the amino acids have increased in concentration during the first 30 minutes of incubation (data not shown in the paper Simon et al 1983), hence this finding is well-matched with the previous observation (Simon & Mukkada 1983) of increased proteolysis which is caused by metabolic inhibitor i.e. in this experiment, sodium fluoride (Simon et al. 1983).

The experiments in Table 2-1, show changes in the concentrations of amino acid pool and these changes may be a sign that these components are on demand in *Leishmania* to support their physiological metabolism (Simon et al. 1983). Serine , threonine, glycine, alanine were identified to be amongst the major components in the amino acid pool in *Leishmania tropica* promastigotes and these can be converted to intermediates in glycolysis or Krebs cycle. It is proposed that amino acids that came from proteolysis could be oxidised through Krebs cycle (Simon et. 1983). The identification of several enzymes that are involved in amino acids-carbohydrate transformations further strenghten the possibility of the usage of amino acids as readily feasible sources for carbon and energy metabolisms (Simon et al. 1983). The important links between proline and carbohydrate metabolism were provided by the reversible transamination for the formation of aspartic and glutamic acids from corresponding keto acids (Simon et al. 1983).

The amino acids that were shown to be reduced during both starvation and treatment with the metabolism inhibitor (Table 2-1) i.e. proline, serine, aspartic acid and methionine, highlight their importance as this observation signify the usage of these amino acids to support carbon and energy sources for survival during critical conditions. (Further explaination of serine and proline (including arginine and glutamine) are available in the discussion section in this chapter).

2.1.1 Amino acid Transport in Leishmania

Several amino acid transporters have been identified in *Leishmania*. In eukaryotes amino acids play essential biological functions such as osmolytes, precursor for protein biosynthesis or other biosynthetic pathways. *Leishmania* and other eukaryotes have the ability to synthesise a subset of amino acids whereas others need to be taken up from the host environment. However, there may be permeases (transporters) for amino acids that are unable to be synthesised by a cell in order to supplement the supply provided by biosynthesis (Landfear 2011). Several amino acid transporters have been characterised in *Leishmania*. Although the transport of glutamate and methionine are found to exist in *Leishmania*, the transporters for these amino acids have not been cloned in *Leishmania* spp.

Arginine transport: An arginine transporter, LdAAP3, was the first *Leishmania* amino acid permease to be cloned and functionally characterized, in *Leishmania donovani* (Shaked-Mishan et al. 2006). The transpoter is make up by 480 amino acids and predicted to have 11 membrane spanning domain (Shaked-Mishan et al 2006). *Saccharomyces cerevisiae* mutant transfected with the gene *LdAAP3* showed that the encode for high-affinity arginine transporter with K_m recorded to be at 14μM (Shaked-Mishan et al. 2006). *LdAAP3* gene exists in two copies which located next to the other in chromosome 31 and these copies are only different in their 3'UTR (Shaked-Mishan et al. 2006). Arginine transports in trypanosomatidae and mammals are probably to be fundamentally different as they are belong to two different family (LdAAP3 closely related family of AAP which is exclusive to members in the family trypanosomatidae and arginine transpoters of the mammals like CAT1-3 belong to amino acid-polyamine-organocation (APC) family) (Reviewed in (Shaked-Mishan et al. 2006).

Arginine is an important amino acid in *Leishmania* and must be obtained from the host(Opperdoes & Coombs 2007). The amino acid is used for protein synthesis and serves as precursor in polyamine synthesis (Roberts et al. 2004). The uptake of arginine is regulated by the internal arginine levels as starvation of this amino acid for 4 hours leads to a ~5 fold increase of the arginine uptake and increased expression of LdAAP3 protein and mRNA (Darlyuk et al. 2009).

Lysine transport: Unlike mammals, in which the same permease transports both arginine and lysine, trypanosomatid protozoa appear to have separate transport systems for both (Inbar et al. 2010). Trypanosomatids do not have the key enzyme essential for lysine synthesis that can be found in plants, thus they depend on the import of lysine from outside of the cell (Opperdoes & Coombs 2007). A transporter for lysine has been identified and characterised in *Leishmania donovani* (Inbar et al. 2010). In *L. donovani*, the gene *LdAAP7* encodes a lysine transporter. Attempts made to delete *LdAAP7* in *Leishmania donovani* have failed because of gene duplication (Inbar et al 2010). Analysis on the gene's sequence revealed that L.donovani ISR chromosome 32 that contains LdAAP7 gene is present at only 2 alleles (Reviewed in (Inbar et al. 2010)). Therefore, it has been concluded that the LdAAP7 duplication in the knockout experiments may suggest that the gene is important for the survival of the parasite and the only lysine transporter in the promastigote stage (Inbar et al. 2010).

The LdAAP7 protein belongs to the large amino acid/auxin permease (Inbar et al, 2010). While overexpression of this transporter in *T.cruzi* increased lysine uptake, this was not the case in *L.donovani*. This maybe because *Leishmania* regulate the intracellular lysine accumulation strictly (Inbar et al,2010) in accordance with the observation that the concentration remain stable during starvation (Darlyuk et al, 2009). Lysine transport decreases during growth in culture and stationary phase cultures, which also transport less arginine and proline than cultures in logarithmic growth, transport almost no lysine (Inbar et al. 2010). Lysine is used solely for protein biosynthesis, unlike proline and arginine (Darlyuk et al, 2009) which are utilised in other anabolic and catabolic processes. Given the toxic effect of lysine to many organisms (Inbar et al, 2010) , reduced transport in non-dividing stationary phase parasites may be an adaptive mechanism to avoid the accumulation of lysine (Inbar et al, 2010).

Glutamate Transport: Enzymes responsible for glutamate metabolism, such as glutamate dehydrogenase, have been shown to exist in several species of *Leishmania* (Martin et al. 1976). The transport of glutamate has been reported in *Leishmania (Leishmania) amazonensis* (Paes et al. 2008). The transport of the amino acid has been characterised by Paes et al as an active transport, shown to

be partially inhibited by analogues such as glutamine, aspartate, α-ketoglutarate and oxaloacetate, methionine and alanine.

Methionine transport : Methionine uptake in *Leishmania tropica* promastigotes was characterised by Mukkada et al (Mukkada & Simon 1977) and shown to occur against a concentration gradient and via a saturable system which requires metabolic energy and was sensitive to temperature. Influx of methionine is inhibited by increased levels of methionine in the intracellular pools (feedback inhibition) and increased levels of other amino acids, including some that have no affinity for the methionine carrier (i.e. lysine and tyrosine) can also inhibit methionine uptake, through an incompletely understood phenomenon described as transinhibition (Simon & Mukkada 1977).

Proline transport: Geraldo et al have shown that the putative proline transporter La-PAT1 gene in *Leishmania amazonensis* is up-regulated in amastigotes and, it was suggested that two distinct transporters that are developmentally regulated exist for proline uptake in amastigotes and promastigotes (Geraldo et al. 2005). The single copy gene is upregulated in amastigote stage and it encodes a putative amino acid transporter that belongs to the amino acid/auxin permease family, a group of H(+)/amino acid symporters (Geraldo et al. 2005).

Leishmania promastigotes and amastigotes exhibit different kinetic properties and pH sensitivity for proline transport(Glaser & Mukkada 1992;Krassner & Flory 1972). Uptake of proline in both life stages is by active transport, driven by proton motive force, and this is might be important to maintain the acidic intravacuolar environment (Geraldo et al. 2005).

Serine transport: Serine uptake has been characterised in both promastigote and amastigote stages of *Leishmania (Leishmania) amazonensis* (dos Santos et al. 2009). The serine uptake is characterised to be a saturable transport system and the uptake increased linearly with temperature in the range of 20°C to 45°C. The uptake is pH dependent and optimum transport of the amino acid is at pH 7.5.

2.2 Materials and Methods

Homem (Invitrogen), Foetal bovine serum (PAA), L-[3,4-3H (N)]- glutamine, L-[2,3,4-3H]-monohydrochloride arginine, L-[2,3-3H]-proline, L-[3H(G)]-serine (all radioactive amino acids are from Perkin Elmer), chloroform (Fishcer Scientific), methanol (Sigma), L-serine, L-proline, L-arginine, L-glutamine (all from Sigma), PBS (Sigma), mineral oil (Sigma), formaldehyde (Sigma), dibutyl phthalate (Sigma)

2.2.1 Culturing Leishmania mexicana

L. mexicana WT MNYC/BZ/62/M379 promastigotes, and a glucose transporter null line derived from this parent (Burchmore et al, 2003) were grown in modified Eagle's minimal essential medium, HOMEM, with 10% (v/v) heat-inactivated foetal calf serum (FBS) and maintained at 25°C.

2.2.2 Amino Acid Uptake Assay

Cells were grown up to the density of 1 X 10^7 cells/ml, counted and washed twice in cold PBS at 1200 x g for 10 minutes at 4°C. The cells were resuspended at a density of 5 x 10^8/ml in PBS and were preincubated in a 25°C water bath for 5-10 minutes. Uptake assays were performed in triplicate. 1.7 ml Eppendorf tubes, with caps removed, were prepared with 200µl dibutyl phthalate/mineral oil. Then, 100µl of labelled substrate, prepared in PBS at 2x the desired final concentration, was added and the tubes were centrifuged at 13000 x g for 1 minute.

The substrates were labelled with either of these 4 amino acids i.e. L-[3,4-^{3}H (N)]- glutamine, L-[2,3,4-^{3}H]-monohydrochloride arginine, L-[2,3-^{3}H]-proline, L-[^{3}H(G)]-serine (Perkin Elmer)

To initiate the assay, 100µl of cell suspension was added into the aqueous layer of the substrate and timed from the instant of addition (5X10^8/1ml(concentration) X 100µl = 5X10^7cells). The uptake was stopped by

centrifugation at 13,000 x g for 1 minute. Tubes containing pelleted cells were immediately transferred into liquid nitrogen to snap freeze the cell pellets. The base of each frozen tube was then clipped off, with the cell pellet intact, into scintillation vials. To estimate the specific activity of the labelled substrate stock, 10µl aliquots of the 2x substrate were transferred to scintillation vials. 200µl of 2% SDS was added to each scintillation vial and these were incubated for ~30minutes with occasional agitation before addition of 3 ml of scintillation fluid. Vials were sealed, agitated, and incubated o/n in the dark at room temperature before scintillation counting (Perkin Elmer, 1450 Microbeta Wallac Trilux Liquid Scintillation & Luminescence counter).

An aliquot of each cell suspension was fixed by 100-fold dilution into 1% formaldehyde and accurate cell count was performed to determine the number of cells in each assay. As a control for non-specific binding, extracellular substrate trapping etc, identical assays were performed with cells that had been pre-incubated for 10minutes with 1% formaldehyde.

2.2.3 Metabolomics

2.2.3.1 Cell Preparation

Promastigote cultures of *Leishmania mexicana* were grown as described in 2.2.1 but merely grown up to $2x10^4$ cells/ml and only used 20ml per sample.

2.2.3.2 Metabolomic sample preparation

Cells were quenched by submersion of tube in dry ice/ethanol bath until culture temperature reached 4°C. This may take approximately 1 minute. For subsequent steps, all were performed at 0-4°C. Cells were centrifuged for 10minutes at 100g and supernatant was removed to approximately 500µl. For analysis of spent medium, 5µl of supernatant was kept. Pellets were resuspended and transferred to 1.5ml eppendorf tube. Centrifuged for 10minutes at 1000g and supernatant was removed completely.

The pellet was suspended in 200 µl of chloroform/methanol/water (1:3:1) and vigorously mixed on a cooled shaker for 1 hour. Cells were centrifuged for 3

minutes at 13,000g. Supernatant was taken and stored at -80°C until analysis by LCMS.

Medium sample (5 µl) was extracted with solvent according to the same protocol for samples extraction, prior to LCMS analysis. This is to allow removal of medium contaminants at the data-analysis stage.

2.2.3.3 Mass Spectrometry

The analysis of samples using mass spectrometry were done by Dr Karl Burgess and Dr Darren Creek (Creek et al. 2012). The experiments were only repetead technically hence the results are preliminary data.

The protocol begins with the samples being analysed on an Exactive Orbitrap mass spectrometer (Thermo Fischer) in both positive and negative modes (rapid switching), coupled to a U3000 RSLC HPLC (Dionex) with a ZIC-HILIC column (Sequant) (Vincent et al. 2010 and Vincent et al. 2012). All samples from each experiment were analysed in the same analytical batch (Vincent et al. 2012. Analysis of quality control samples, internal standards and total ion chromatograms were done to test the quality of the chromatography and signal reproducibility. Samples that showed undesirable analytical variation (retention time drift) were taken out from the analysis (Vincent et al. 2012). To facilitate metabolite identification, a standard mix comprising approximately 200 metabolites runs from the start of the experiment (Vincent et al. 2012.

Raw files generated by the mass spectrometer were converted into mzXML files by ReAdW converter. These mzXML files were split into positive and negative ions and subsequently processed in with XCMS (http://www.scripps.edu) to detect peaks. MzMatch (http://mzmatch.sourceforge.net/) was used to convert the files into peakML files, combine data from all samples and annotate related peaks. Further processing was conducted using in-house Excel VBA scripts to identify metabolites by looking at the combination of mass and retention times. A filtering process was then applied to remove related and duplicated peaks.

All putatively identified metabolites abundance were compared between wild type, glucose transporter null mutant, HOMEM media and spent HOMEM media from wild type and glucose transporter null mutant culture.

2.3 Results

2.3.1 Amino Acid Uptake Assays

To determine relative levels of amino acid uptake in wild type and glucose transporter null *Leishmania*, uptake of radiolabelled [^{3}H]- serine, [^{3}H]-arginine, [^{3}H]-proline and [^{3}H]-glutamine was determined at 4 time points i.e 30 seconds, 60 seconds, 90 seconds and 120seconds. These four amino acids were chosen as they are amongst the most abundant in the HOMEM media (Appendix) that was used to maintain the cells in culture. The activity of the labelled amino acids are 1mCi (37MBq)/1ml for ^{3H}L-proline and 250µCi (9.25MBq)/250µl for ^{3H}L-arginine, ^{3H}L-glutamine and ^{3H}L-serine.

Table 2-2: Uptake of amino acids in wild type (WT) and glucose transporter null mutant (ΔGT). Timetable summarised experiments that were done 2 times for each amino acid (each experiment was run in triplicate). Graphs for uptake for individual amino acid are shown in the next page. Mean±SEM, n=6.

Time (Seconds)	Amino Acid Uptake (pmoles/10^7 cells)							
	Proline		Serine		Arginine		Glutamine	
	WT	ΔGT	WT	ΔGT	WT	ΔGT	WT	ΔGT
30	5.47 ±2.60	13.76 ±1.86	3.91 ±0.99	222.98 ±30.54	26.55 ±3.481	17.39 ±3.73	1.77 ±0.28	28.01 ±6.01
60	6.49 ±2.25	17.69 ±3.10	9.79 ±3.36	222.09 ±17.20	55.66 ±6.50	29.32 ±4.47	3.44 ±0.47	27.62 ±2.78
90	10.14 ±4.27	44.34 ±6.26	18.11 ±6.12	340.54 ±44.26	97.37 ±8.84	61.88 ±13.82	5.47 ±0.97	53.26 ±4.99
120	29.66 ±11.47	55.81 ±6.33	30.51 ±10.14	198.28 ±24.86	109.86 ±19.79	68.74 ±12.05	9.14 ±0.56	54.43 ±6.50

2.3.1.1 Serine Uptake Assay

Serine uptake was assayed at 30μM in wild type and glucose transporter null mutant promastigotes. We found that uptake of serine was significantly higher in glucose transporter null *Leishmania*. The initial rate of serine uptake (i.e. the first 30 seconds of the assay) in the glucose transporter null mutant was 222.98 pmoles per $1X10^7$ cells whereas the initial rate of uptake in wild type cells, assayed in parallel over the same period, was 3.91 pmoles per $1X10^7$ cells. The initial rate of serine uptake in glucose transporter null *Leishmania* was 57 times higher than in wild type *Leishmania*. However, over the uptake assay time course (120 sec), glucose transporter null *Leishmania* did not continue to accumulate labelled serine while wild type *Leishmania* accumulated serine at a linear rate.

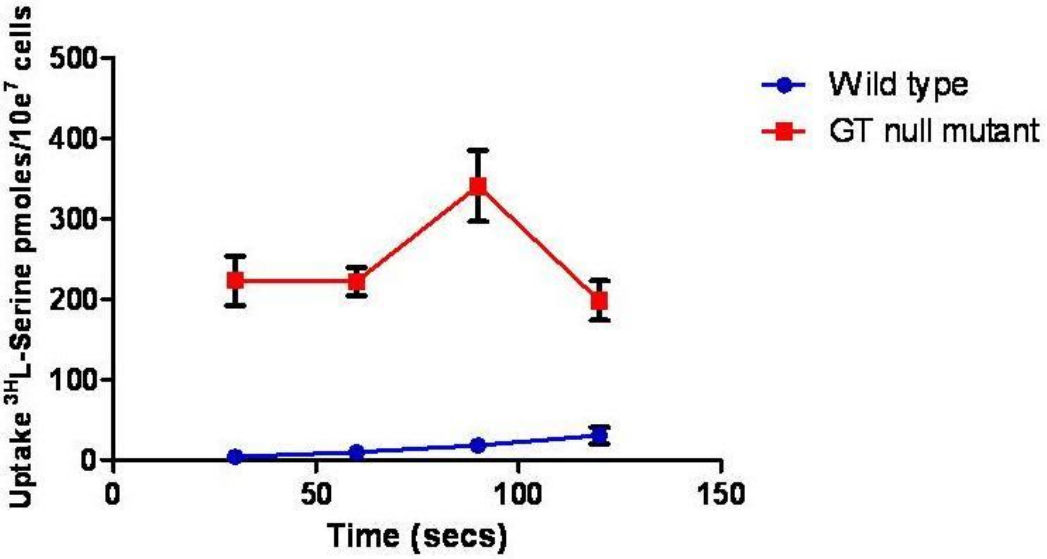


Figure 2-1: Serine uptake assay by glucose transporter null mutant and wild type *Leishmania* promastigotes. Experiments were done twice in triplicate with all triplicates were run in parallel. Each point represents the mean of 6 replicates. GT null mutant: glucose transporter null mutant *Leishmania*.Cell numbers used, ($5X10^8$cells/1ml(concentration) X 100μl = $5X10^7$cells).

2.3.1.2 Arginine Uptake Assay

Uptake of arginine by glucose transporter null mutant and *Leishmani*a WT was investigated over 120 seconds with exposure to 30 µM. The uptake showed that wild type has a slightly higher uptake compared to the null mutant. At 30 seconds, the wild type accumulates arginine at 26.55 pmoles per $1x10^7$ cells whereas the null mutant accumulates arginine 1.5 times lower than the wild type i.e. 17.39 pmoles per $1x10^7$ cells. At 60 seconds, wild type accumulates 1.9x more arginine compared to the null mutant i.e. 55.66 pmoles per $1x10^7$ cells and 29.32 pmoles per $1x10^7$ cells respectively. At 90 seconds, the wild type and null mutant continue to accumulate arginine at 97.37pmoles per $1X10^7$ cells and 61.88 pmoles per $1X10^7$ cells respectively. The uptake of the amino acids continues at 120 seconds with wild type accumulate 109.86 pmoles per $1X10^7$ cells and 68.73 pmoles per $1X10^7$ cells for null mutant.

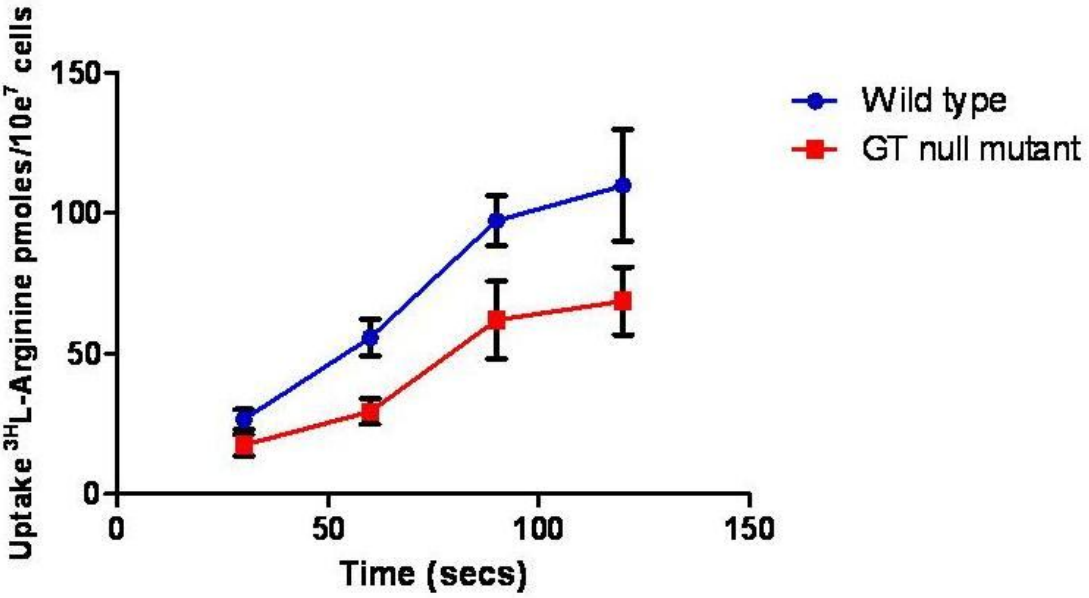


Figure 2-2: Arginine uptake assay by glucose transporter null mutant and wild type *Leishmania* promastigotes. Experiments were done twice in triplicate with all triplicates were run in parallel. Each point represents the mean of 6replicates. GT null mutant: glucose transporter null mutant *Leishmania*. Cell numbers used, ($5X10^8$cells/1ml(concentration) X 100µl = $5X10^7$cells).

2.3.1.3 Proline Uptake Assay

Measurement of proline transport along a time course was done with proline concentration of 30 µM. The accumulation of proline at the first time point is significantly higher in the null mutant compared to the wild type i.e. 2.4X higher in the null mutant compared to the wild type which is 5.47 pmoles per $1x10^7$ cells in wild type and 13.76 pmoles per $1x10^7$ cells in null mutant. At 60 seconds, the accumulation continues with the uptake in wild was recorded at 6.49 pmoles per $1X10^7$ cells and 17.69 pmoles per $1X10^7$ cells in null mutant. The uptake of proline in the null mutant at 90 seconds was 44.34 pmoles per $1x10^7$ cells whereas the wild type uptake for proline at this point was 10.14 pmoles per $1x10^7$ cells i.e 4.4X difference between the cell lines. At 120 seconds, null mutant accumulates 55.81 pmoles per $1X10^7$ cells of proline whereas the wild type accumulates 29.66 pmoles per $1X10^7$ cells of proline.

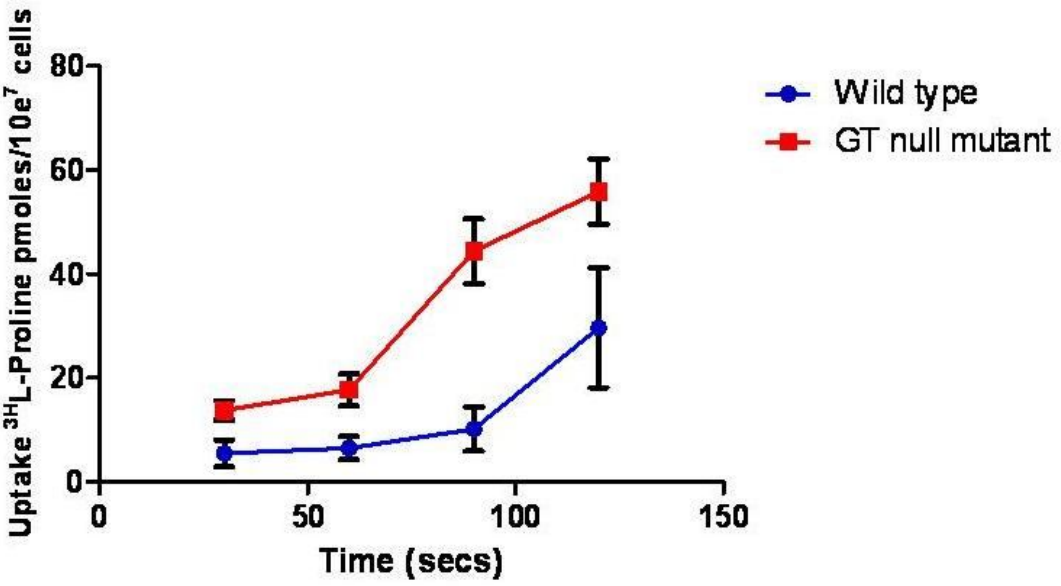


Figure 2-3: Proline uptake assay by glucose transporter null mutant and wild type *Leishmania* promastigotes. Experiments were done twice in triplicate with all triplicates were run in parallel. Each point represents the mean of 6 replicates. GT null mutant: glucose transporter null mutant *Leishmania*. Cell numbers used, ($5X10^8$cells/1ml(concentration) X 100µl = $5X10^7$cells).

2.3.1.4 Glutamine Uptake Assay

Glutamine uptake for both wild type and null mutant was analysed and the concentration of ^{3H}-glutamine is set at 30 µM and the uptake is observed over 120seconds. At the first time point, null mutant accumulate 28.01 pmoles of glutamine per $1x10^7$ per cells, 15.8X higher compared to wild type i.e 1.77 pmoles per $1x10^7$ cells. Both cell lines continue the uptake for glutamine after 120seconds and null mutant remained to transport glutamine higher compared to wild type with the final time's record for the uptake was 9.14 pmoles per $1x10^7$ cells for the wild type and 54.43 pmoles per $1x10^7$ cells for null mutant.

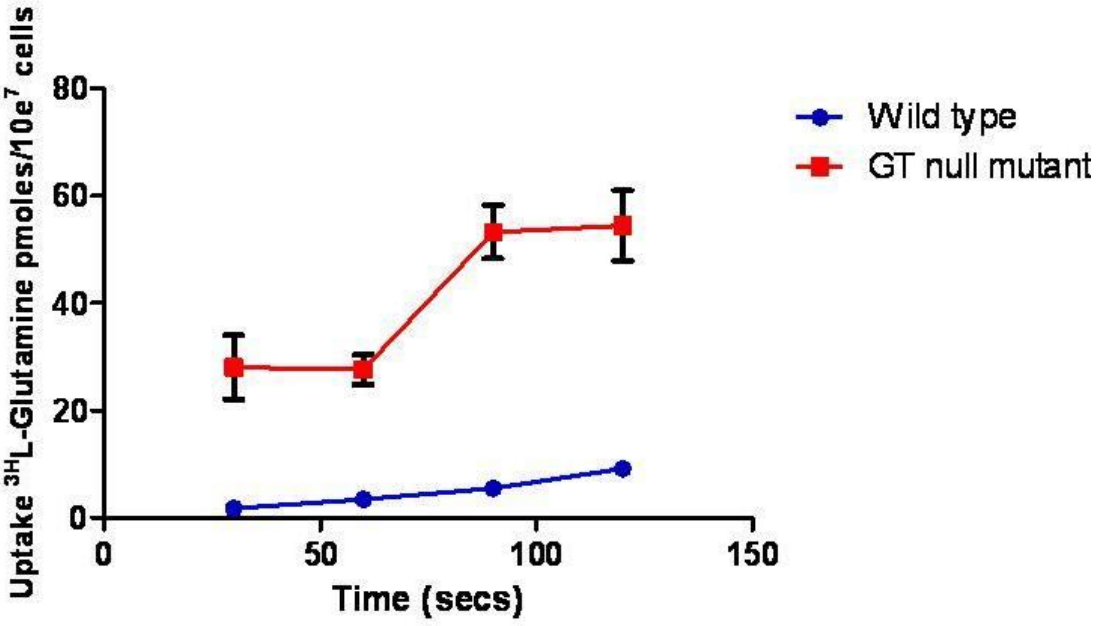


Figure 2-4: Glutamine uptake assay by glucose transporter null mutant and wild type *Leishmania* promastigotes. Experiments were done twice in triplicate with all triplicates were run in parallel. Each point represents the mean of 6 replicates. GT null mutant: glucose transporter null mutant *Leishmania*. Cell numbers used, ($5X10^8$cells/1ml(concentration) X 100µl = $5X10^7$cells).

2.3.2 Metabolomics

The analysis of samples using mass spectrometry was done by Dr Karl Burgess and Dr Darren Creek (Creek et al. 2012). The experiments were only repetead technically hence the results are preliminary data.

The Identification Macro programme (http://mzmatch.sourceforge.net/) has identified 803 metabolites in all samples analysed based on putative identification. The metabolite levels are expressed as mean peak heights, relative to the wild type cells. However, only 71 metabolites (refer to Table 2-2 to Table 2-7) have the confidence level of 10 which is the highest confidence level in the analysis process. The metabolites that have confidence levels below 5 were excluded from the analysis. The confidence level of the metabolites is based on several factors including:

1) Retention times (RT) from the HPLC chromatography. The software has a record of RT for a number of known metabolites and this is called standard RT. The metabolites that match this standard RT scored the confidence level of 10, while metabolites that do not have a standard RT in the database are assigned a confidence level of 8 if the predicted retention time is within a predicted RT window.

2) The existence of the metabolites in *Leishmania* metabolism. This is done by referring to the database called LeishCyc (http://leishcyc.bio21.unimelb.edu.au/), which is a database of metabolic pathways for *Leishmania*. Confidence levels for putative identifications not present in LeishCyc were reduced by 1.

3) Related peaks were assigned by the mzMatch software to indicate possible MS artefacts. Putative metabolites assigned as related peaks were given lower confidence levels (-2).

The mass spectrometry procedure was done by Dr Daren Creek and Dr Karl Burgess. This metabolomic study is a preliminary data as it the the experiment was only technically repeat instead of biologically repeat.

Amino Acid Metabolism

Name	Formula	Isomers	Homem	WT-C	WT-H	ΔGT-C	ΔGT-H
L-Aspartate	**C4H7NO4**	**4**	**0.36**	**1**	**0.08**	**0.6**	**0.09**
L-Cysteine	C3H7NO2S	2	0	1	0.003	0.42	0
L-Glutamate	**C5H9NO4**	**14**	**0.26**	**1**	**0.12**	**0.74**	**0.09**
L-Valine	C5H11NO2	16	0.22	1	0.27	1.46	0.25
L-Isoleucine	**C6H13NO2**	**11**	**0.3**	**1**	**0.32**	**0.94**	**0.22**
L-Leucine	C6H13NO2	11	0.42	1	0.34	1.17	0.32
5'-Methylthioadenosine	**C11H15N5O3S**	**2**	**0**	**1**	**0**	**1.96**	**0.22**
S-Adenosyl-L-Methionine	C15H23N6O5S	1	0	1	0	0.83	0
Putrescine	**C4H12N2**	**1**	**0.03**	**1**	**0.02**	**1.5**	**0.04**
4-Aminobutanoate	C4H9NO2	14	0.27	1	0.29	0.9	0.32
Spermidine	**C7H19N3**	**1**	**0.08**	**1**	**0.12**	**0.85**	**0.11**
L-Ornithine	C5H12N2O2	6	0.12	1	0.11	0.4	0.11
L-Arginine	**C6H14N4O2**	**3**	**0.34**	**1**	**0.25**	**0.5**	**0.29**
L-Tyrosine	C9H11NO3	11	0.35	1	0.37	0.9	0.37
L-Tryptophan	**C11H12N2O2**	**6**	**0.43**	**1**	**0.39**	**0.77**	**0.39**
L-Kynurenine	C10H12N2O3	2	0.38	1	0.43	0.57	0.44
3-(2-Aminoethyl)-1H-indol-5-ol	**C10H12N2O**	**4**	**0.17**	**1**	**0.28**	**0.55**	**0.26**
L-Glutamine	C5H10N2O3	6	0.2	1	0.14	1.13	0.1
L-Phenylalanine	**C9H11NO2**	**7**	**0.32**	**1**	**0.34**	**0.93**	**0.35**
Phenylacetylglycine	C10H11NO3	8	0.33	1	0.38	0.57	0.43
L-Methionine	**C5H11NO2S**	**5**	**0.42**	**1**	**0.33**	**1.56**	**0.32**
N6,N6,N6-Trimethyl-L-lysine	C9H20N2O2	2	0.91	1	0.38	2.23	0.65
L-Carnithine	**C7H15NO3**	**2**	**1.71**	**1**	**0.7**	**1.62**	**1.4**
Trimethylammoniobutanoate	C7H15NO2	6	0.63	1	0.49	0.75	0.6
L-Lysine	**C6H14N2O2**	**7**	**0.29**	**1**	**0.33**	**0.61**	**0.3**
L-Histidine	C6H9N3O2	4	0.34	1	0.32	0.88	0.32
L-Threonine	**C4H9NO3**	**11**	**0.24**	**1**	**0.25**	**0.43**	**0.13**
L-Serine	C3H7NO3	3	0.57	1	0.24	0.16	0.06
L-Cystathionine	**C7H14N2O4S**	**4**	**0**	**1**	**0**	**0.56**	**0.03**
Choline	C5H14NO	1	0.4	1	0.35	0.82	0.4
Trypanothione disulfide	**C27H47N9O10S2**	**1**	**0**	**1**	**0**	**1.26**	**0**
Glutathione	C10H17N3O6S	3	0	1	0	0.38	0
L-Cystine	**C6H12N2O4S2**	**2**	**0.66**	**1**	**0.7**	**0.22**	**0.39**
S-glutathionyl-L-cysteine	C13H22N4O8S2	2	0	1	0	0.13	0
Glycine	**C2H5NO2**	**3**	**0.28**	**1**	**0.2**	**2.02**	**0.3**
Pantothenate	C9H17NO5	1	0.27	1	0.25	0.44	0.31
L-Proline	**C5H9NO2**	**4**	**0.02**	**1**	**0.03**	**0.04**	**0**
L-Asparagine	C4H8N2O3	6	0.26	1	0.21	0.21	0.04
L-Alanine	**C3H7NO2**	**9**	**0.03**	**1**	**0.06**	**0.1**	**0.01**
O-Acetylcarnitine	C9H18NO4	1	1.89	1	0.59	1.26	1.69

Table 2-3: Metabolites from amino acid metabolism. The metabolite levels are expressed as mean peak heights, relative to the wild type cells (WT-C). Homem is mean peaks from neat Homem, WT-C is mean peaks from wild type cells, WT-H is the mean peak for spent Homem in wild type culture, ΔGT-C is mean peak from glucose transporter null mutant cells and ΔGT-H is mean peaks from Homem used for glucose tranporter null mutant culture. Isomers refer to the number of alternative isomers that exist within the Scotmet metabolite database.

Carbohydrate Metabolism

Name	Formula	Isomers	Homem	WT-C	WT-H	ΔGT-C	ΔGT-H
Succinate	**C4H6O4**	**7**	**0.02**	**1**	**0.29**	**0.07**	**0.02**
2-Oxoglutarate	C5H6O5	7	0	1	0.16	0.21	0.14
Fumarate	**C4H4O4**	**3**	**0.1**	**1**	**0.1**	**0.45**	**0.2**
Pyruvate	C3H4O3	3	0.47	1	0.43	0.6	0.45
D-Glucose 6-phosphate	**C6H13O9P**	**46**	**0**	**1**	**0**	**0.29**	**0**
D-Glucose	C6H12O6	57	0.33	1	0.41	0.71	0.45
Sucrose	**C12H22O11**	**42**	**0.78**	**1**	**0.64**	**0.11**	**0.08**
Glycerol	C3H8O3	1	2.72	1	1.21	2.18	2.64
(s)-Malate	**C4H6O5**	**4**	**0.03**	**1**	**0.08**	**0.26**	**0.07**

Table 2-4: Metabolites from carbohydrate metabolism. The metabolite levels are expressed as mean peak heights, relative to the wild type cells (WT-C). Homem is mean peaks from neat Homem, WT-C is mean peaks from wild type cells, WT-H is the mean peak for spent Homem in wild type culture, ΔGT-C is mean peak from glucose transporter null mutant cells and ΔGT-H is mean peaks from Homem used for glucose tranporter null mutant culture. Isomers refer to the number of alternative isomers that exist within the Scotmet metabolite database.

Metabolism of Cofactors and Vitamins

Name	Formula	Isomers	Homem	WT-C	WT-H	ΔGT-C	ΔGT-H
Pyridoxal	**C8H9NO3**	**9**	**0.89**	**1**	**0.77**	**1.35**	**0.89**
Thiamin	C12H17N4OS	1	0.37	1	0.43	0.77	0.39
Riboflavin	**C17H20N4O6**	**2**	**0.44**	**1**	**0.44**	**0.68**	**0.42**
Folate	C19H19N7O6	1	0.37	1	0.41	0.59	0.36
Nicotinamide	**C6H6N2O**	**4**	**1.33**	**1**	**0.63**	**0.77**	**1.07**

Table 2-5: Metabolites from metabolism of cofactors and vitamins. The metabolite levels are expressed as mean peak heights, relative to the wild type cells (WT-C). Homem is mean peaks from neat Homem, WT-C is mean peaks from wild type cells, WT-H is the mean peak for spent Homem in wild type culture, ΔGT-C is mean peak from glucose transporter null mutant cells and ΔGT-H is mean peaks from Homem used for glucose tranporter null mutant culture. Isomers refer to the number of alternative isomers that exist within the Scotmet metabolite database.

Nucleotide Metabolism

Name	Formula	Isomers	Homem	WT-C	WT-H	ΔGT-C	ΔGT-H
UMP	**C9H13N2O9P**	**4**	**0.01**	**1**	**0**	**1.48**	**0.01**
AMP	C10H14N5O7P	7	0.08	1	0.03	1.57	0.09
Xanthine	**C5H4N4O2**	**3**	**0.35**	**1**	**0.03**	**0.59**	**0.07**
Hypoxanthine	C5H4N4O	3	0.16	1	0.06	0.31	0.08
Guanine	**C5H5N5O**	**3**	**0.09**	**1**	**0.05**	**0.35**	**0.09**
Inosine	C10H12N4O5	3	4.09	1	0	0.48	0
IMP	**C10H13N4O8P**	**3**	**0**	**1**	**0**	**0.91**	**0**
Adenosine	C10H13N5O4	3	0.31	1	0.13	1.07	0.26
Allantoin	**C4H6N4O3**	**3**	**0.59**	**1**	**0.43**	**0.75**	**0.59**

Table 2-6: Metabolites from nucleotide metabolism. The metabolite levels are expressed as mean peak heights, relative to the wild type cells (WT-C). Homem is mean peaks from neat Homem, WT-C is mean peaks from wild type cells, WT-H is the mean peak for spent Homem in wild type culture, ΔGT-C is mean peak from glucose transporter null mutant cells and ΔGT-H is mean peaks from Homem used for glucose tranporter null mutant culture. Isomers refer to the number of alternative isomers that exist within the Scotmet metabolite database.

Energy Metabolism

Name	Formula	Isomers	Homem	WT-C	WT-H	ΔGT-C	ΔGT-H
Orthophosphate	**H3O4P**	**1**	**0.51**	**1**	**0.57**	**0.89**	**0.56**
NAD+	C21H28N7O14P2	1	0.84	1	0	1.77	0.74

Table 2-7: Metabolites from energy metabolism. The metabolite levels are expressed as mean peak heights, relative to the wild type cells (WT-C). Homem is mean peaks from neat Homem, WT-C is mean peaks from wild type cells, WT-H is the mean peak for spent Homem in wild type culture, ΔGT-C is mean peak from glucose transporter null mutant cells and ΔGT-H is mean peaks from Homem used for glucose tranporter null mutant culture. Isomers refer to the number of alternative isomers that exist within the Scotmet metabolite database.

Lipid Metabolism

Name	Formula	Isomers	Homem	WT-C	WT-H	ΔGT-C	ΔGT-H
Choline phosphate	**C5H15NO4P**	**1**	**0**	**1**	**0**	**3.1**	**0**
sn-glycero-3-Phosphocoline	C8H21NO6P	1	0.41	1	0.05	1.04	0.13
sn-Glycerol 3-phosphate	**C3H9O6P**	**3**	**0.01**	**1**	**0.01**	**0.15**	**0.02**

Table 2-8: Metabolites from lipid metabolism. The metabolite levels are expressed as mean peak heights, relative to the wild type cells (WT-C). Homem is mean peaks from neat Homem, WT-C is mean peaks from wild type cells, WT-H is the mean peak for spent Homem in wild type culture, ΔGT-C is mean peak from glucose transporter null mutant cells and ΔGT-H is mean peaks from Homem used for glucose tranporter null mutant culture. Isomers refer to the number of alternative isomers that exist within the Scotmet metabolite database.

Generally, the metabolomic data shows that there are differences in the accumulation of amino acids between the glucose transport null mutant *Leishmania* and wild type *Leishmania*. The observation on serine accumulation in neat Homem and cells (refer to Table 2-3 which compares amino acid contents in neat Homem and cells labelled as C-WT and C-ΔGT) are showing that there is a probability of increased reliance on this amino acid metabolism as a carbon and energy source as the serine's content was recorded to be very low in null mutants (metabolomic data, Table 2-3) but high in uptake(Figure 2-1), and the serine's concentration in neat HOMEM is high (Table 2-3). These observations indicate that serine may be have been utilised heavily in the null mutant.

The levels of metabolites associated with carbohydrate metabolism are uniformly lower in glucose transport null mutant *Leishmania*, as would be expected for cells that cannot acquire exogenous glucose. Interestingly, the level of glycerol is higher in glucose transport null mutant *Leishmania*. Glycerol, which can be directed to carbohydrate production or for energy generation, may derive from increased lipid metabolism.

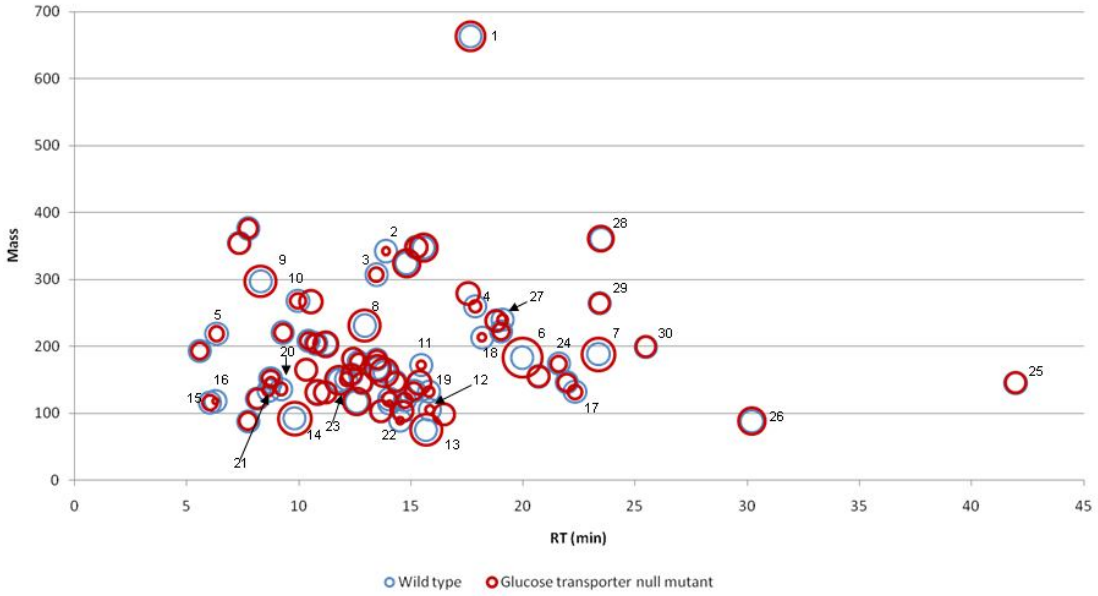


Figure 2-5: Bubble plot of metabolites of wild type and glucose transporter null mutant's cells from metabolomic analysis. Wild type is represented by the blue circles and glucose transporter null mutant is represented by the red circles. The radius of each circle is proportionate to the mean peak of the respective metabolite. This plot includes displays of the 71 metabolites that have a confidence score of 10 found in both cell lines.

Bubble Number	Metabolites
1	NAD+
2	Sucrose
3	Glutathione
4	D-Glucose-D-phosphate
5	Pantothenate
6	Choline Phosphate
7	N6,N6,N6-Trimethyl-L-Lysine
8	MOPS
9	5'-Methylthioadenosine
10	Inosine
11	sn-Glycerol-3-phosphate
12	L-Serine
13	Glycine
14	Glycerol
15	Fumarate
16	Succinate
17	L-Ornithine
18	S-Glutathione
19	L-Asparagine
20	Hypoxanthine
21	(S)-Malate
22	L-Alanine
23	L-Methionine
24	L-Arginine
25	Putrescine
26	Spermidine
27	L-Cystine
28	Trypanothione disulfide
29	Thiamine
30	S-Adenosyl-L-Methionine

Table 2-9: The table shows the name of metabolites of the numbered bubbles from the bubble plot (Figure 2-5).

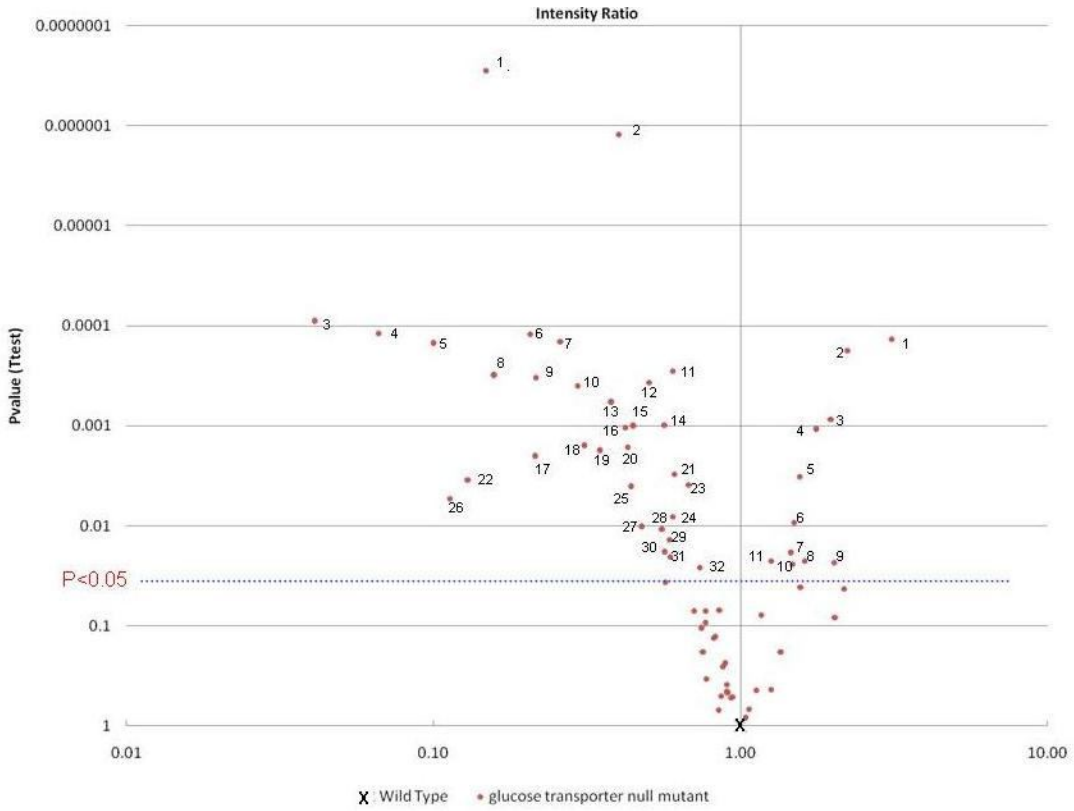


Figure 2-6: Volcano plot of wild type and glucose transporter null mutant from metabolomic analysis. It plots significance or T-test or p-value (y-axis) versus magnitude of change (x-axis). Metabolites that have low P-value (P<0.05) are regarded as highly significant and appear towards the top of the plot. Values that appear on the left denotes the downregulation of the metabolites in null mutant compared to wild type and values on the right denotes the upregulation of the metabolites in null mutant compared to wild type. The dotted blue line denotes the border of P<0.05. The metabolites names of the numbered red dots are listed in Table 2- 10(refer to next page).

Numbering	Metabolites down-regulated in Glucose transporter null mutant	Numbering	Metabolites up-regulated in Glucose transporter null mutant
1	Sn-glycerol 3 phosphate	1	Choline phosphate
2	L-Ornithine	2	N6,N6,N6-Trimethyl-L-lysine
3	L-Proline	3	5'-methylthioadenosine
4	Succinate	4	NAD+
5	L-alanine	5	L-Methionine
6	L-Asparagine	6	Putrescine
7	(S)-malate	7	L-Valine
8	L-Serine	8	L-Carnitine
9	L-Cystine	9	Glycine
10	D-glucose-6-phosphate	10	UMP
11	L-Aspartate	11	Trypanothione disulfide
12	L-Arginine		
13	Glutathione		
14	L-Cystathionine		
15	Fumarate		
16	L-Cysteine		
17	2-Oxoglutarate		
18	Hypoxanthine		
19	Guanine		
20	L-Threonine		
21	L-Lysine		
22	S-gluthathionyl-L-lysine		
23	Riboflavin		
24	Pyruvate		
25	Pantothenate		
26	Sucrose		
27	Inosine		
28	3-(2-aminoethyl)-1H-indol-5-ol		
29	Xanthine		
30	L-Kynurenine		
31	Folate		
32	L-Glutamate		

Table 2-10: Table listing labelled metabolites from volcano plot (Figure 2-6).

The bubble plot (Figure 2-5) helps in identifying metabolites that differ in expression level between wild type and null mutant. The difference of metabolite expression in each cell type is easy to see as the diameter of the bubble is proportionate to the mean level of the metabolite. Although the bubble plot displays all 71 metabolites that have a confidence level of 10, Table 2-9 list down all the 'most' easiest bubbles to be characterised in the plot. Some of them were not listed as the bubbles from different metabolites were piling on top of each other hence were difficult to be identified individually.

The volcano plot (Figure 2-6) displays the metabolites from null mutant in comparison to wild type. The comparison was based in terms of the metabolites

significance (p-value,t-Test) in it occurrence in the results (y-axis) and level of the metabolites abundance in the null mutant in comparison to the wild type (x-axis). The red dots (represent metabolites from the null mutant) in the plot, which appear at the left side ($x<1$) of the plot are metabolites found to be down-regulated in the null mutant but higher in the wild type cells. While the red dots that appear on the right side ($x>1$) of the plot are metabolites that have been found to be up-regulated in the null mutant but lower in the wild type cells. Metabolites that have P-value lower than 0.05 ($P<0.05$) are regarded to be highly significant and these metabolites appear towards the top of the plot. The t-test assesses whether the mean peaks of metabolites derived from null mutant cells and wild type cells are statistically different from each other.

The volcano plot shows that amongst metabolites that were down-regulated in the null mutant are intermediates from citric acid cycle and glycolysis i.e. succinate, (S)-malate, fumarate, D-glucose-6-phosphate and pyruvate. However, NAD+, the 'electron acceptor' that highly used in glycolysis and citric acid cycle was upregulated in the null mutant. Other than those metabolites, amino acids were among metabolites being down-regulated (L-proline, L-alanine, L-serine, L-aspartate, L-arginine, L-cysteine, L-threonine, L-lysine and L-glutamate) and upregulated (L-methionine and L-valine) in the null mutant cells. Other metabolites of interest that appeared on the volcano plot are sucrose, L-ornithine,L-cystine and L-kynurenine (all down-regulated in null mutant) and putrescine (upregulated in null mutant).

2.4 Discussion

2.4.1 Amino Acid in Organisms

Amino acid intake generally exceeds the basic requirement for essential protein synthesis and other biosynthesis. The nitrogen from protein usually degraded whereas the carbon skeleton will be metabolised in the citric acid cycle. Therefore, protein can contribute significantly as a source of energy. The processes of amino acid degradation start with the removal of the α-amino group to generate the corresponding α-keto acid. Subsequently, the carbon skeleton will proceed either to oxidation in the citric acid cycle or for biosynthesis of carbohydrate, depending on the physiological state of the organism. Pyruvate and oxaloacetate are generated by carbon skeletons coming from alanine and aspartate and subsequently converted to carbohydrate via gluconeogenesis whereas amino acids are metabolised in ketogenesis leading to acetyl-CoA and acetoacetyl-CoA (Mathews et al. 2000).

My experiments to investigate nutrient uptake by glucose transporter null mutant *Leishmania* show that this mutant takes up significantly more amino acids than the wild type. These mutants grow at a slower rate than wild type *Leishmania* promastigotes. This observation and facts about organism dependence on amino acids or proteins help us to understand the physiological importance of amino acids whenever the glucose availability is scarce i.e in the parasitophorous vacuole.

It has been suggested that an intracellular amino acid pool is one way that *Leishmania* may use to keep a storage of metabolic intermediates that are capable of providing energy and facilitate the maintenance of ionic balance (Simon et al.1983). From our metabolomic data, it appeared that the wild type contains significant amount of alanine and serine and this supports the observation of Simon et al in their experiment with *Leishmania tropica* promastigotes, which showed that serine and alanine were amongst the amino acids in the protozoan's intracellular amino acid pool.

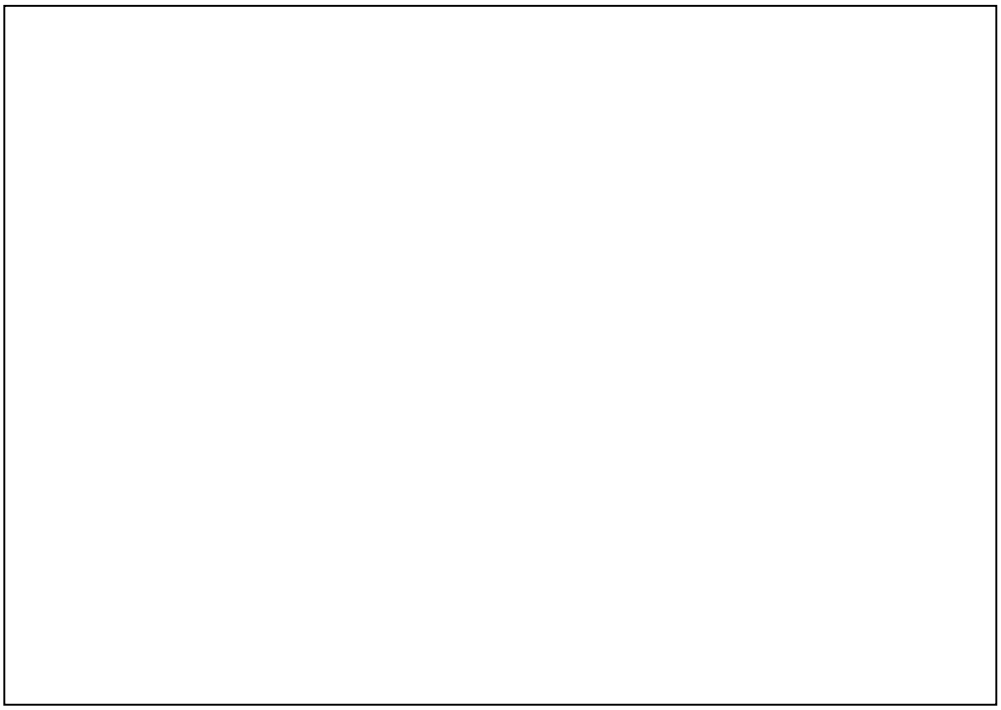


Figure 2-7: The diagram has been removed due to copyright restrictions.

2.4.2 Serine

In the serine uptake assay, the null mutant showed a very high uptake of serine. This high initial uptake of serine was also demonstrated by promastigotes and amastigotes of *Leishmania amazonensis* (dos Santos et al. 2009), which the amastigotes had higher uptake compared to promastigotes.However the uptake in null mutant is decreasing at 90seconds. This may be because that the null mutant cells are saturated with serine or (more interestingly) one could speculate that the null cells are utilising serine as an energy source, and are thus releasing radiolabelled products into the media. The metabolomic data (Table 2-3) support the serine uptake result. The level of L-serine in null mutant cell is significantly lower compared to the level in the wild type cell i.e serine was 6.25X (refer to Table 2-3)lower in null mutant than in wild type and clearly the spent media from the null mutant indicated to have 4X lower serine compared to the wild type's spent media. This demonstrated that not only the uptake of serine was higher in null mutant but the usage of serine was also higher in the null mutant compared to the wild type. These findings were not surprising as previous published papers have indicated the importance of serine in *Leishmania* and the extra increases of serine uptake and usage indicates that in an environment where glucose is scarce and where the capability of glucose uptake is lacking in the parasite, serine is probably one of the amino acids used as an energy source. Serine is one of the amino acids that is essential for *Leishmania* and may be captured from the surroundings (Hanada 2003). Serine is a substrate for the synthesis of sphingolipids and phosphatidylserine (dos Santos et al. 2009). Phosphatidylserine can be found ubiquitously in eukaryotic and prokaryotic cells as a membrane phospholipid(Vance & Steenbergen 2005). In *Leishmania* promastigotes, serine is required for de novo synthesis of ethanolamine which is essential for the metacyclogenesis of *Leishmania*. A mutant deficient in ethanolamine synthesis shows enhances cell deaths as it enters stationary phase, despite normal growth during the log phase(Zhang et al. 2007). Evidence shows that serine is essential for the differentiation and metacyclogenesis processes of the parasites. The condensation of L-serine and palmitoyl-CoA into 3-ketosphingosine (3-ketodihydrosphingosine or 3-KDS) leads to

the de novo synthesis of sphingolipids and this process is catalysed by the enzyme serine palmitoyltransferase(Zhang et al. 2003). Parasites that lack the catalytic subunit of this enzyme showed high frequencies of cell shape abnormality and, upon entry into the stationary phase, showed poor infectivity in macrophage and animal infections, and reduced viability as amastigotes(Zhang et al. 2003).

Simon et al have demonstrated that serine and ethanolamine ,in addition to alanine, glycine, threonine, α-aminobutyric acids, were the principal constituents of the free amino acids in *Leishmania tropica* promastigotes (Simon et al. 1983).

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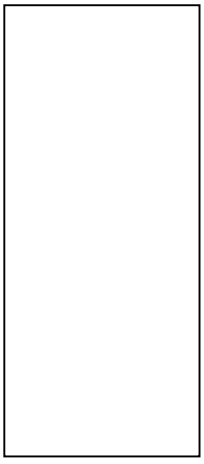

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2.4.3 Arginine

In this study, arginine uptake (Figure 2-2) was demonstrated to be slightly higher in the wild type. This observation was supported by results from the metabolomic comparison of wild type and glucose transporter null mutant lines. Unlike serine uptake, both the wild type and null mutant showed that the arginine uptake still continue even after 90seconds.

Arginine is essential for *Leishmania* as it has been demonstrated that *Leishmania* promastigotes are unable to survive in a L-arginine-free medium (Krassner & Flory 1971). This suggests that mechanisms for transport and utilisation of arginine exist in *Leishmania* promastigotes (Wanasen & Soong 2008). L-arginine is involved in the activation of macrophage function and, depending on the extracellular stimuli, L-arginine can be catabolised by an enzyme called inducible nitric oxide synthase (iNOS) to produce nitric oxide or an enzyme called arginase for polyamine synthesis. The upregulation of iNOS in macrophages after expose to stimuli such as Th1 cytokines leads to nitric oxide production which promotes parasite killing. On the contrary, stimulation by Th2 cytokines induce L-arginine that leads to the production of polyamines (Iniesta et al. 2002). In addition, stimulation of Th2 triggers the expression and activity of arginase that is involved in conversion of arginine to L-ornithine, which is subsequently converted to putrescine by ornithine decarboxylase (ODC) (Raina & Janne 1968). Activities of iNOS and arginase are suggesting to be influenced by L-arginine (Wanasen & Soong 2008) as it has been shown that counter-regulatory mechanisms exist between iNOS and arginase. It has been documented that an intermediate product of the iNOS pathway, N-omega-hydroxy-L-arginine (LOHA) inhibits arginase function (Iniesta et al. 2001) and by competing for L-arginine, an arginase can decrease the iNOS activity (Gotoh & Mori 1999).

LdAAP3 has been identified as an arginine transporter *in Leishmania donovani* (Shaked-Mishan et al. 2006). It is an amino acid permease that comprises 480 amino acids with 11 predicted *trans*-membrane domains. LdAAP3 is highly specific for arginine, as it was not inhibited by other amino acids or arginine-related compounds.

As previous papers have shown that arginine is important in *Leishmania* (Wanasen & Soong 2008), the small reduction in arginine uptake in the glucose transporter null mutant in the current experiment could be due to impairment of the arginine uptake system. Another explanation is that arginine may not be needed in high amount in promastigotes. Arginine can be used to generate nitric oxide and polyamines, as well as urea and ammonia. Urea and ammonia were found to be abundant in late log and stationary phase cultures of *L. mexicana* (Hart & Coombs 1982), which may suggest that arginine is important for the metacyclogenesis process. The experiments reported here were conducted in promastigotes, where arginine utilisation is likely less critical to the parasite. Moreover, as arginine is important as a defence mechanisms, the slightly reduced rate of arginine uptake in the null mutant may be the reason for null mutant amastigotes are unable to survive in macrophages (Burchmore et al. 2003;Rodriguez-Contreras et al. 2007).

2.4.4 Proline

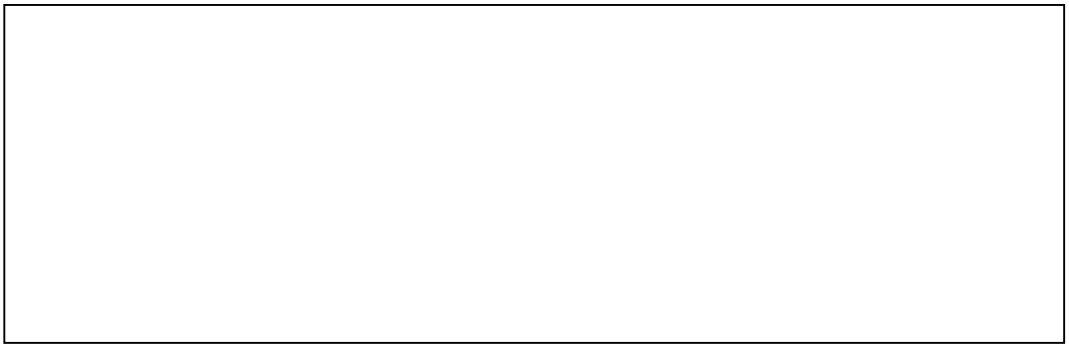
The result from proline uptake (Figure 2-3) showed that the null mutant transports higher level of proline compared to wild type. The metabolomic result showed that in the null mutant the level of proline is 25X lower compared to the wild type. This may be explained by rapid incorporation of proline, such that the radiolabel is accumulated but the proline is only transiently present within cells. Previous reports suggest that proline has an important role in amino acid metabolism (Krassner & Flory 1972). Thus, in conditions where glucose maybe scarce, proline plays an important part as an energy source for *Leishmania*.

In *Leishmania donovani* promastigotes , it was demonstrated that oxygen uptake was stimulated by L-proline and to a lesser extent by L-glutamate and L-arginine (Krassner & Flory 1972). Moreover, L-proline is an energy source for *L. tarentolae* and as such may be replaced by D-glucose(Krassner 1969) and it may play an important role in amino acid metabolism as its presence supports growth in the absence of any one of 5 amino acids i.e. L-methionine, L-alanine, L-glutamic acid, L-isoleucine and L-aspartic acid. All these 5 amino acids are essential in the absence of L-proline. Based on a study in *L.donovani* promastigotes, proline has been found to be oxidised at a high rate and to be

present in a huge amount in the hemolymph. Promastigote culture media is presumed to approximate the habitat ,encountered in the insect host and proline is thought to be a major substrate in insect metabolism (reviewed in (Krassner & Flory 1972)). Through that observation it may be reasonable to suggest that the insect form of hemoflagellates have access to an abundant energy source in the form of proline, a habitat that supports the development of a proline oxidase system (Krassner & Flory 1972).

2.4.5 Glutamine

The uptake assay of glutamine showed very little transport of this amino acid in both glucose transporter null mutant and wild type, although uptake was slightly higher in the null mutant compared to the wild type. Consistent with this, in the metabolomics result, the accumulation of L-glutamine in the null mutant was slightly higher in null mutant. Unlike uptake of serine, arginine and proline, uptake of glutamine in both cell lines shows a linear uptake even after 90seconds. This may be because glutamine (together with asparagine) is formed directly from glutamate and aspartate (Mathews et al. 2000) and transport of glutamine from outside cells may not necessary.



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Glutamine can supply its amide nitrogen to be used in several reactions generating other amino sugars, purine and pyrimidine nucleotides, nicotinamide nucleotides and glycoproteins (Mathews et al. 2000). Carbamoyl phosphate synthetase is an enzyme that is involved in the pyrimidine biosynthesis pathway. The enzyme that uses glutamine as substrate is named carbamoyl phosphate synthetase II (CPSaseII) whereas CPSaseI, which localises in mitochondria uses ammonia as the substrate (Mathews et al. 2000;Tatibana & Shigesada 1972).

2.4.6 Metabolomic Data

In general, the metabolomic results do support the results from the amino acid uptake assay. From the metabolomic data, L-arginine levels were lower in the null mutant, and, in uptake assays, arginine uptake in the null mutant was lower compared to the wild type. All 3 other amino acids tested i.e. serine, proline and glutamine, were shown to be transported into the null mutant at higher levels compared to the wild type. Metabolomic analysis for L-alanine showed that this amino acid is present in a significant amount in wild type cells. The level of L-alanine was significantly high in wild type and very low in null mutant. Alanine is one of the end products of glucose catabolism (Bringaud et al. 2006;Hart & Coombs 1982;Saunders et al. 2011;Tielens & van Hellemond 2009) which supports the lack of glucose transport in null mutant hence reduced the secretion of alanine. Previous observations reported that alanine and serine (which is also significantly high in wild type promastigotes) are among the most abundant amino acids, that can be found in the pool of intracellular amino acids in *Leishmania tropica* promastigotes (Simon et al. 1983). It was postulated that aminotransferases were essential enzymes for the conversion of pyruvate to alanine (reviewed by (Krassner & Flory 1972)) thus contribute to the abundance of alanine in *Leishmania*. Thus, the high content of pyruvate and alanine in wild type compared to glucose transporter null mutant shown by metabolomic analysis further supports the incapability of the null mutant to use glucose as a carbon source for energy metabolism. Aminotransferases are enzymes that catalyse transamination reactions, a process whereby amino acids can replenish citric acid cycle intermediates (Mathews et al. 2000).

NAD+ is consumed during glycolysis when two glyceraldehyde-3-phosphates are converted by glyceraldehyde-3-phosphate dehydrogenase to two 1,3-bisphosphoglycerates (Mathews et al. 2000). In a volcano plot (Fig 2-6), NAD+ was high in null mutant cell. This is not surprising as NAD+ consumption is exist in metabolism like glycolysis that uses glucose as the carbon source.

In *Leishmania mexicana* promastigotes it has been demonstrated that alanine and acetate are highly utilised under glucose replete -conditions (Saunders et al. 2011). In the current experiment, metabolomic results revealed that L-alanine was down-regulated in glucose transporter null mutant. Given the finding by Saunders, this is not surprising as wild type cells still utilised significant amount of alanine. Thus promastigotes that lack glucose transporter may use more alanine as they depend solely on carbon sources other than glucose to support energy metabolism and other important physiological and biochemical processes.

Saunders et al (Saunders et al. 2011) also suggest that the replenishment of citric acid cycle intermediates is essential to maintain the cataplerotic synthesis of glutamate and biosynthetically related amino acids like proline and glutamine. Both proline (96% lower in null mutant) and glutamate (26% lower in null mutant) in glucose transporter null mutant were down regulated in the current metabolomic experiment. This may show that the heavy utilisation on these amino acids by the null mutant to support various biochemical and physiological processes has caused the constant low level of the amino acids in the cell.

As the glucose transporter null mutant cannot acquire glucose as an energy source, glycolysis may be running at a reduced rate. The low uptake of glucose and low rate of glycolysis also reflects on the level of D-glucose-6-phosphate which is one of the intermediates in glycolysis. In glycolysis, the phosphorylation of glucose by hexokinase generates D-glucose-6-phosphate, which in turn is converted to D-fructose-6-phosphate by the enzyme phosphoglucoisomerase (Mathews et al. 2000).

Gluthathione was also down regulated by 62% in the null mutant while trypanothione disulfide level in the null mutant were just 21% higher than wild type. Trypanothione disulfide($T[S]_2$) plays a vital function in the defence against oxidative stress and is an unusual form of glutathione found in parasitic protozoa (Castro-Pinto et al. 2008). In mammals and other eukaryotes, the thiol cofactor glutathione is the substrate for glyoxalase enzymes but in *Leishmania*, trypanothione is used for their activity(Padmanabhan et al. 2005;Vickers et al. 2004). Glutathione is important as a defence mechanism against nitric oxide cytotoxicity of macrophages (Romao et al. 1999). Romao et al demonstrated that macrophages and *L.major* depleted of glutathione were more susceptible to cytotoxic effects of nitric oxide donors, S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). Therefore, the decrease of glutathione in the metabolomic results may explain why glucose transporter null mutant *Leishmania mexicana* promastigotes could not survive in macrophages (Rodriguez-Contreras et al. 2007).

Polyamines (PA) also appeared to give interesting results in the metabolomic analysis. Polyamines are important metabolites in eukaryotes as they participate in a variety of proliferative processes and in *Leishmania* and trypanosomes, PAs take part in additional functions in the synthesis of the thiol trypanothione (Colotti & Ilari 2011). PA metabolites that only showed increases in the null mutant were putrescine (33%higher in null mutant) and the rest PAs were down-regulated in the null mutant i.e L-arginine (50% lower), L-ornithine (60% lower), spermidine (15% lower) and s-Adenosyl-L-methionine or AdoMet (17% lower).

Comparative metabolomic analysis reveals significant differences between wild type and glucose transporter null mutant promastigotes. In addition to increased amino acid transport the result generated from metabolomic results provides a proof of principle that metabolomic analysis can be utilised to investigate the mechanisms behind phenotypic changes in *Leishmania*.

Nevertheless, the complex data generated by metabolimic analysis are challenging to analyse and the changes in the light of the specific lesion in glucose transport are difficult to interpret with the knowledge and current understanding of intermediary metabolism in *Leishmania*. Furthermore, there is

no obvious correlation between metabolomic and proteomic datasets, suggesting that the current resolution of "global" omic analyses leaves much to be desired.

2.5 Conclusion

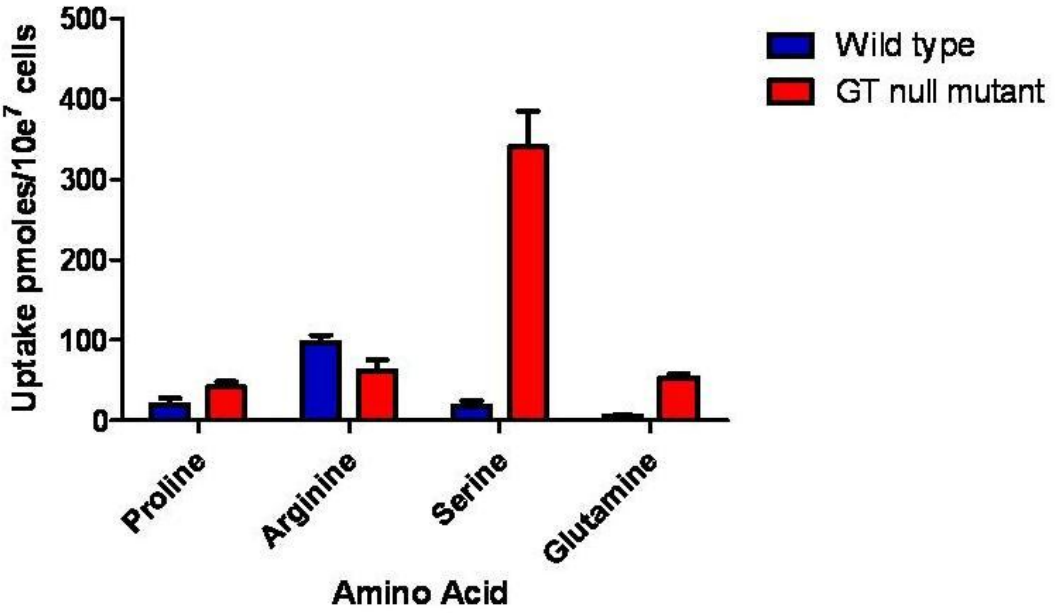


Figure 2-11: Amino acid uptake at 90secs by wild type and glucose transporter null mutant promastigotes (GT null mutant).

The uptake of 3 out of 4 amino acids tested in the assay were significantly higher in glucose transporter null *Leishmania*. This higher rate is consistent with utilisation of these amino acids as an energy source in glucose transporter null parasites, while the same substrates may have primarily anabolic fates in wild type cells, that are able to use glucose as a primary energy source. The metabolomic results indicate that levels of several metabolites were down regulated in the glucose transporter null mutant. This may be because the utilisation of other metabolites in the null mutant is significantly high as 'dependence' on glucose as the main energy source inevitably has to be shifted to other metabolites that are available to the null mutant. Hence, although the null mutant transports significantly high amounts of several amino acids (uptake assay) i.e. serine and proline, these amino acid were found to be low in the null mutant cell according to the metabolomic result. The metabolomic data also shows components of lipid metabolism are up regulated in the null mutant thus it could be that the null mutant relies on lipids as a source of energy beside

amino acids. In general it appears, perhaps not surprisingly, that glucose transporter null mutant *Leishmania* exhibit a different metabolic economy, as an adaptation to the loss of glucose as an exogenous carbon and energy source.

Chapter 3

Characterisation of the plasma membrane subproteome of *Leishmania mexicana* promastigotes

3.1 Introduction

3.1.1 Plasma membrane proteomics

The surface of *Leishmania* parasites is the interface with the environment and with the host. Surface proteins are tethered to the plasma membrane, either directly or indirectly by interaction with membrane proteins. The plasma membrane proteome is a defining feature of all eukaryotes but is of particular interest in *Leishmania* in the context of host: parasite interactions.

Recently, the *Trypanosoma brucei* bloodstream form plasma membrane proteome was characterised (Bridges et al. 2008). Although *Leishmania* and trypanosomes are phylogenetically related, methods that were developed to enrich *T. brucei* bloodstream form plasma membranes were not easily adapted to *Leishmania* promastigotes (R. Burchmore PhD thesis, 1993). A major goal of this PhD project was thus to develop efficient methods for the enrichment of a *Leishmania* promastigote plasma membrane fraction, and to exploit this to characterise the proteome of this fraction.

Integral membrane proteins (IMPs) have important functions in cell-cell interactions, molecular transport and signal transduction. However, analysing IMPs is a challenging task because of the relatively hydrophobic nature of proteins that are soluble in the phospholipid bilayer which comprises the plasma membrane. Hydrophobic proteins are difficult to solubilise in aqueous solvents, but this is a prerequisite for conventional proteomic analyses. Furthermore, most integral membrane proteins are of relatively low abundance, as they are constrained to the 2-dimensional space of the lipid bilayer (Speers & Wu 2007).

As a consequence of these features, membrane proteins are typically under-represented in proteomic analyses (Lu et al. 2008).

3.1.2 Enrichment of the membrane proteins, subcellular fractionation and removal of membrane-associated proteins

The generally low abundance of membrane proteins has been a major issue in large scale identification of integral membrane proteins (IMPs). Thus, the strategy of enriching IMPs plays a major part in sample preparation. A common strategy for membrane protein enrichment is differential ultracentrifugation to achieve subcellular fractionation. Cells are lysed and subcellular components are separated by a series of centrifugations at increasing speeds to recover fractions that have different densities. Following each centrifugation, the organelles that have sedimented to the bottom of the tube are recovered in the pellet. The supernatant is then recentrifuged at higher speed to sediment organelles or subcellular fractions with progressively lower densities.

We applied a salt-extraction protocol(Piper et al. 1995) to remove membrane associated proteins (Pedersen et al. 2003;Schluesener et al. 2005) with the aim of further enriching IMPs. As a result of their high hydrophobicity, IMPs and membrane associated proteins are poorly soluble in aqueous solvents and are mainly soluble in detergents or organic solvents. Alkaline sodium carbonate was used to remove the membrane-anchored protein by disrupting the electrostatic interactions between proteins. In principle, this strategy should result in the release of hydrophilic peripheral membrane proteins into the aqueous buffer, leaving hydrophobic IMPs in the lipid bilayer. Removal of the peripheral membrane-associated protein should reduce sample complexity and facilitate a focus on IMPs.

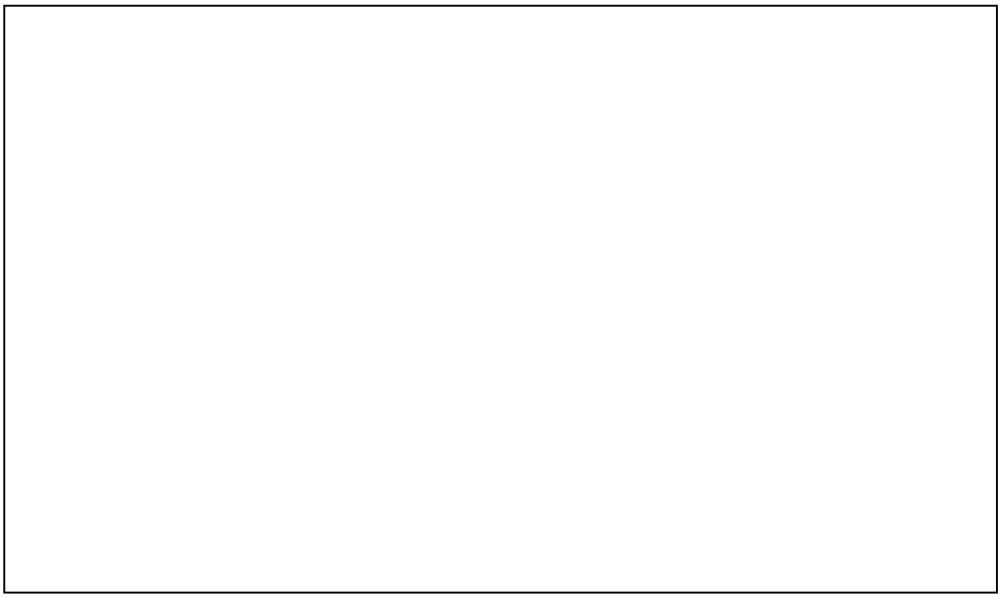
Extraction with sodium carbonate for IMP enrichment has been widely exploited in a variety of systems (Pedersen et al. 2003;Rahbar & Fenselau 2005;Zhang et al. 2006), suggesting that a similar approach may be useful in the isolation and identification of IMPs from *Leishmania mexicana*.

We also tried an extraction protocol using Triton X-114. The principle of this protocol is to exploit the solubility of the membrane proteins in detergent to preferentially solubilise IMPs (Bordier 1981).

3.1.3 Protein Separation

We applied both 1 and 2 dimensional strategies for protein separation. Proteins were separated in one dimension using conventional SDS PAGE based on apparent molecular weight. To increase the resolution of the protein separation, we used solution-phase isoelectric focussing, to generate fractions of proteins separated by isoelectric point. These fractions were then separated by SDS-PAGE.

Solution-phase isoelectric focussing was achieved using the Agilent 3100 OFF-GEL fractionator, a new system for protein and peptide separation. This instrument generates a voltage potential and a pH gradient across a chamber that is divided into multiple (12 or 24) wells. This enables isoelectric fractionation of proteins in solution, to generate a fraction that can be further resolved by electrophoresis.



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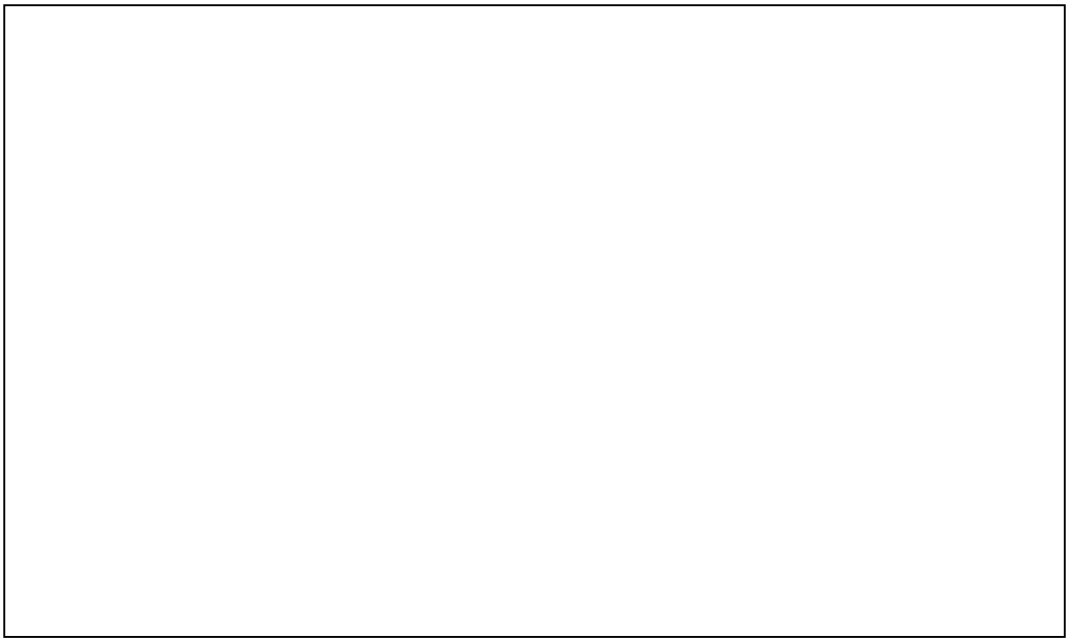

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3.1.4 Monitoring plasma membrane enrichment

Subcellular fractionation can result in enrichment of specific subcellular compartments and organelles. It is important to identify markers that can be exploited to monitor recovery of subcellular components. Although some enzymatic markers have been identified for the *Leishmania* plasma membrane (Gottlieb & Dwyer 1981;Zilberstein & Dwyer 1988), I wished to use an integral membrane protein as a marker. Despite previous efforts in the Burchmore lab, it was not possible to obtain an antibody against native integral membrane proteins. Therefore, an epitope-tagged glucose transporter was generated for this purpose.

A myc-tagged glucose transporter as a marker for plasma membrane enrichment, to provide quality control for membrane fractionation. It is an epitope tag of the sequence 408-420 AEEQKLISEEDL of human c-myc (http://www.biocompare.com/). Dr Christina Naula had designed and synthesised a plasmid construct (px63NEO) (Figure 3-2) containing this myc-epitope within the coding sequence of the LmGT2 glucose transporter (LeBowitz et al 1990, Cruz et al 1991, Goyard & Beverley 2000). The heterologous epitope tag was inserted into a hydrophilic loop region of LmGT2 (Figure 3-1), at a location that has previously been shown to tolerate sequence change without affecting function (Snapp & Landfear 1997). This construct was transfected into glucose transporter knockout *Leishmania* (episome) and maintained in 50µg/ml of neomycin. Functional expression in the plasma membrane of the parasites was confirmed by glucose transport assay (Figure 3-3). Myc-glucose transporter was expressed from pX63 neo vector. Expression is driven by flanking regions from DHFR-TS (dihydrofolate reductase-thymidilate synthase).

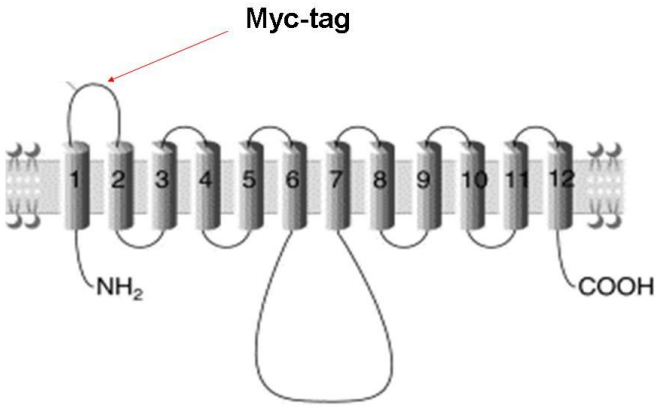

Figure 3-1: myc-tagged glucose transporter. The glucose transporter has 12 integral membrane spanning helices. The myc tag is located in the first loop of the amino-terminus of the transporter. Picture from Dr Richard Burchmore, University of Glasgow.

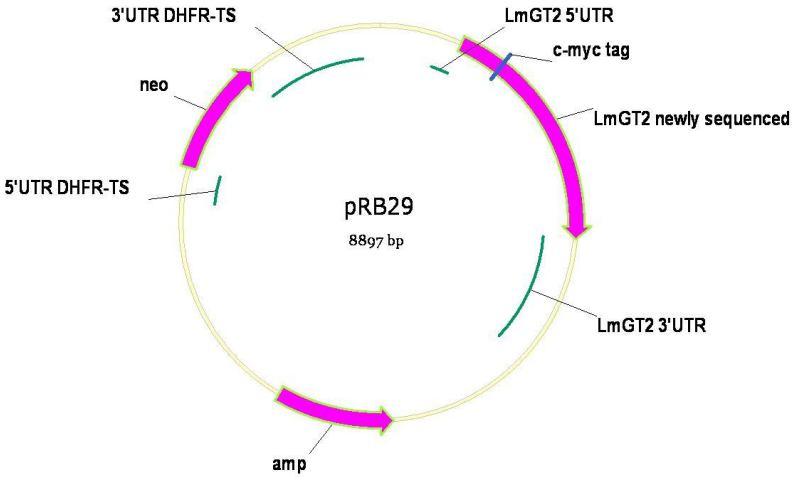

Figure 3-2: Plasmid construct encoding a myc-tagged glucose transporter. The construct was designed and synthesised by Dr Christina Naula. The glucose transporter is an integral component of the *Leishmania* plasma membrane. I performed western Blot analysis of *Leishmania* cells that express this construct, in order to monitor the recovery efficiency for plasma membrane proteins in differential subcellular fractionation. Picture from Dr. Richard Burchmore and Dr. Christina Naula.

3.2 Materials and Methods

3.2.1 Materials

G418 (Calbiochem), HOMEM (Invitrogen),Foetal Bovine Serum(PAA , The Cell Culture Company), G418 sulfate (Calbiochem),Tris-CL (Fischer Scientific), EDTA (SIGMA), Sarkosyl(PROMEGA), NaCl (Fishcer Scientific), ethanol (Fischer Scientific), Tween-20(Sigma), Phosphate buffer saline (PBS) (Sigma), leupeptin (Sigma), pefabloc(Sigma), pepstatin (Sigma), phenanthroline (Sigma),sucrose (Fischer Scientific), ^{3H}Glucose (GE Healthcare),Glucose (Sigma), HEPES (Fischer Scientific), $NaHCO_3$ (Fischer Scientific), sodium dodecyl sulphate (SDS)(Sigma), the *E.coli* positive control (Abcam), Tween (Sigma), powder milk (Marvel), CyDyes (GE Healthcare), Lysine (Sigma), acetonitrile (Sigma), ammonium carbonate (Sigma), formic acid (Fishcer), sequencing grade modified trypsin(Promega), DTT (Agilent), c-Myc antibody (Cell Signalling Technology), iodoacetamide (Sigma) ,anti-mouse secondary antibody HRP (Calbiochem), Prestained protein marker (New England Biolabs), SuperSignal®Substrate (Novagen), bromophenol blue (Sigma), glycerol (Sigma)

3.2.2 Cell culture

Promastigotes from 3 cell lines (wild type *Leishmania mexicana*, glucose transporter null mutant *Leishmania mexicana*(ΔLmGT), myc-tagged glucose transporter *Leishmania mexicana*(myc-GT2) (drug selection of transfected cells – G418 50μg/ml)) were used in the study. The cell lines were grown in modified Eagle's minimal essential medium HOMEM with 10% (v/v) heat inactivated foetal bovine serum (FBS) and maintained at 25°C.

3.2.3 Glucose uptake

The protocol is similar as for the amino acid uptake in Chapter 2. However, we used wild type and myc-tagged glucose transporter cell line.

3.2.4 Protein from whole cell lysate preparation

Promastigotes with the density of $1x10^7$ cells/ml were pelleted at 1200xg for 10minutes at 4°C and pelleted again 2 times with cold 1x PBS using the original volume. The cells were resuspended to a density of $1x10^7$cells/10µl or $6x10^7$cells/15µl of PBS containing protease inhibitors (the final concentration for each inhibitor, for example in 10ml PBS, must be as follows: leupeptin 100µg/ml, pefabloc sc 50µg/ml, pepstatin 5µg/ml, phenanthroline 50µg/ml and 1mM EDTA).

An equal amount of 2x Leammli buffer were added. The cells then were heated up at 65°C for 5 minutes, quick spin for 1 minute and stored in a freezer until needed. Prior to loading of samples on gel, the proteins were heated again at 65°C for 5 minutes.

3.2.5 Salt-extracted membrane proteins

The protocol was amended from Piper et al (Piper et al. 1995). Cells with a concentration of $1x10^7$cells/ml (with each cell line has a volume of 380ml) were pelleted at 1200xg for 10minutes at 4°C and rinsed twice in HES buffer containing 20mM Hepes, pH 7.5, 1mM EDTA, 255mM sucrose. Cells were resuspended in 5mls HES buffer on ice, and the cells were lysed using a sonicator (15 pulses with 3 seconds each pulse, in a cold room). The lysates were diluted with 15ml of $NaHCO_3$, pH 10.6 to remove peripheral membrane proteins. Divided into 2 fractions i.e. whole cell lysate (add the Leamli buffer and kept in -20°C freezer immediately) and the remainder was spun in an ultracentrifuge (rotor ,70Ti: Beckman Instruments, Inc., Fullerton, CA) for 1½ hour at 40,000 rpm at 4°C.

Both supernatant and membrane preparation (the pellet) were added to Leamli buffer to make $3x10^{10}$ cells/ml and kept in -20°C freezer until further analysis. Before the samples were used for a western blot, 2µl of 2-ß-mercaptoethanol was added into 18µl ($5.4x10^8$cells) of each samples. I tried various cell counts and $5x10^7$ cells showed the expression of myc-tagged glucose transporter.

3.2.6 Triton X-114 extraction

Protocol from Bordier et al (Bordier 1981) and Burchmore et al (PhD Thesis, 1993). Cells harvested from 200 ml at 5 X10^7 cells/ml by spinning at 2.4 k x g for 10 minutes at 4°C. Cells resuspended in 50ml of ice cold PBS and harvested by repeating the spinning. Pellet resuspended in 5ml of ice-cold 1% Triton X-114 in PBS with a protease inhibitor cocktail (the final concentration for each inhibitor, for example in 10ml PBS, must be as follows: leupeptin 100µg/ml, pefabloc sc 50µg/ml,pepstatin 5µg/ml, phenanthraline 50µg/ml and 1mM EDTA). The pellet mix was stirred vigorously on ice for 90 minutes, then a 500 µl aliquot was removed and frozen at -20°C (total lysis). The remainder was spun at 40,000 xg for 30 minutes at 4°C, supernatant removed and repeated again. The pellets were combined and frozen at -20°C (detergent insoluble fraction). The supernatant was incubated at 30°C for 10minutes, then spun at 5000 xg for 20 minutes at 30°C without using the brake when the machine is decelerating. The upper (aqueous) phase is separated and both were placed on ice. Both portions were returned to a volume of 5 ml by adding ice-cold PBS to detergent phase and ice-cold 10% Triton X-114 in PBS to the aqueous phase. Both stirred vigorously on ice for 60 minutes. Both samples were incubated at 30°C for 10 minutes, then spun at 5000 xg for 20 minutes at 30°C. The aqueous wash was separated from the original detergent phase and the detergent phase from the original aqueous phase and discarded. Samples were stored at -20°C until analysis by SDS-PAGE.

3.2.7 1-SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on Novex 4-20% Tris-glycine pre-cast gels (Invitrogen). Pre-stained protein marker, Broad Range (6-175kDa) (New England BioLabs) was used as molecular weight (Mw) marker. The proteins were separated by standard method. The *E.coli* positive control whole lysate expressing 11 different epitope tags (Abcam) was used. Gels that served as loading control were stained with Coomassie blue and gels that need for protein screening were proceed to Western Blot procedure. Prior to loading, samples were incubated at 60°C for 5 minutes.

3.2.8 Western Blot

Three different transfer buffers were tested, to identify the best conditions for blotting of integral membrane proteins:

1. 25mM Tris-Base, 192mM Glycine, 20% Methanol pH 8.3(standard buffer)

2. 0.1% SDS in buffer 1. (Small et al. 1988)

3. 0.7M Glycine, 25mM Tris-Base pH 7.7 (Small et al. 1988)

Different time for blotting have been used for each buffer, between 40minutes to 4hrs 30minutes. 2 different transfer voltages were also tested i.e. 200volt and 0.10-0.15Ampere.

The proteins were electroblotted from the gel onto Hybond-ECL nitrocellulose membrane (Amersham Hyperfilm, GE Healthcare) using a mini transblot apparatus (Bio Rad Laboratories) according to the manufacturer's instruction.

The blots were blocked with milk 5% (W/V) in PBS-Tween 0.1% for 1 hour at room temperature, washed 3 times with PBS-Tween 0.1% for 15 minutes for each wash, incubated 1 hour to overnight with anti-c-Myc mouse monoclonal antibody (9B11) with the concentrations used for each western blot were mentioned in Figure 3-6, Figure 3-7, Figure 3-8 and Figure 3-9 (Cell signaling Technology), repeat the same washing procedure and incubated with goat anti-mouse IgG peroxidase conjugate (Calbiochem)(concentrations of secondary antibody used are mentioned in Figure 3-6, Figure 3-7, Figure 3-8 and Figure 3-9).

The blot(s) was developed using SuperSignal ®Substrate (Novagen). Developed blots were exposed to high performance chemiluminescene film (Amersham Hyperfilm™ ECL, GE Healthcare Ltd. UK).

3.2.9 Labelling and separation of membrane proteins

In the protocol that used the incorporation of CyDye, CyDye labelling was performed prior to IEF fractionation, samples from the wild type and glucose transporter null mutant was suspended in 10 µl Lysis buffer (pH 9) prior to labelling. Then, protein samples were labelled with CyDyes (400 pmol of dye for each sample) named Cy3 and Cy5 respectively and incubated in the dark for 30 minutes. The reaction was stopped by the addition of 1µl of 10mM lysine into each sample. CyDye labelled samples were mixed together prior to the IEF fractionation. The mixture volume was 140µl.

The fractionation was conducted using an AGILENT 3100 Off-Gel fractionator according to the manufacturer's instructions. First, OFFGEL stock solutions with a concentration of 1.25X, comprises thiourea (entire tube, AGILENT), DTT (entire tube, AGILENT), 6ml glycerol solution (AGILENT) and 600µl OFFgel Buffer (AGILENT) were prepared by mixing all the solutions mentioned, and bring the total volume to the 50ml with dH2O.This mixture is for 24 wells frame. As the sample (i.e. the mixture of dye-labelled WT and ΔLmGT) is only 140µl and it is in an OFFGEL compatible solution and the volume is less than 0.72ml, 0.58ml (0.72ml minus sample volume in ml) of dH2O was mixed in 2.88ml 1.25X OFFGEL stock solution and mix. Then, the 140µl CyDye-labelled sample was added. Meanwhile, an IPG strip rehydration solution was prepared by mixing 0.48ml OFFGEL Stock Solution(1.25X). Following the manufacturer's instruction, an IPG strip (pH 3-10) was placed in one of the lanes in the tray. After placing the frame onto the IPG strip, IPG strip was rehydrated by pipetting 40µl of IPG Strip Rehydration Solution into each well. Following the rest of the instruction in the manual, after adding the sample (CyDye-labelled samples mixture in the OFFGEL solution mix) with equal amounts into each well,all protocols were followed. The current was applied on the fractionator, and the voltage ranged from as low as 200V to as high as 1500V.

The fractionation took approximately for 4 days (96hours) to complete. At the end of the fractionation, all samples in 24 wells were collected and kept at -20°C until further analysis.

Prior to SDS-PAGE analysis, the samples were heated at 65°C for 5 minutes. 5µl of loading buffer was added to each samples and subsequently loaded into wells in precast gel, Novex ®4-12% Tris –Glycine mini gel (Invitrogen).

3.2.10 Bands Picking and Cutting

The SDS gels that contained protein samples that have been fractionated in AGILENT 3100, Scanned with Typhoon 9400 Variable Mode Imager (GE Healthcare) according to the manufacturer's protocol.

Depending on the purpose of the experiment, each sample in their respective lane was sectioned into 30 equal-sized bands, or bands of interest. The cutting is done manually. Lanes that showed strong differences in expression were chosen and cut for analysis. Spots of interest were cut from the gel using a scalpel and kept in a fridge until in-gel trypsin digestion.

3.2.11 In-gel Trypsin Digest for Coomassie-stained proteins

Gel pieces were washed in 500µl of 100mM ammonium bicarbonate for 1 hour. The gel sections were then washed in 50% acetonitrile, 100mM ammonium bicarbonate for another 1 hour before being reduced by the addition of 150µl of 100mM ammonium bicarbonate and 10µl of 45mM DTT and the pieces were then incubated for 30 minutes at 60°C. To alkalyte, the pieces were let to cool to room temperature and 10 µl of 100mM iodoacetamide and incubated in the dark for 30 minutes. The solvent was then discarded and the gel pieces were washed again in 500 µl of 50% acetonitrile in 100 mM ammonium bicarbonate with shaking for 1 hour. Then 50 µl of acetonitrile was added to shrink the gel pieces and the solution was removed after 10 minutes and the gel pieces were dried in a vacuum centrifuge. Afterwards, a 0.2µg/µl sequencing grade modified Porcine Trypsin (Promega) in 25 mM ammonium bicarbonate was sufficiently added to rehydrate the shrank gel plugs. Add more the trypsin solution until gel bands were fully rehydrated. Approximately 20 µl of 25mM ammonium bicarbonate was added and enough to cover the gel pieces and the gels were leave to digest overnight at 37°C. Acetonitrile was added to the digest to make approximately

50% acetonitrile in the digest and subsequently incubated for 20 minutes. The tubes containing the samples were briefly centrifuge to pellet the gel bands. All liquid was transferred to a clean tubes and care must be taken to make sure no gel was transferred together. Next, 1 % formic acid was added and sufficient to cover the gel pieces and centrifuge briefly to pellet the gel. All liquid was transferred to the same tubes used for the first extract and care was taken to avoid any transfer of the gel and this step was repeated for one more time. A sufficient amount of acetonitrile was added to cover the gel pieces and incubated for 10 minutes and transferred all liquid into the same tube for the first extract and avoid to transferred the gel pieces. This extract step is repeated again for one more time and subsequently, the combined extracts were completely dried in the Speedvac. The samples are now ready for mass spectrometry analysis.

3.2.12 Mass Spectrometry

Tryptic peptides were solubilized in 0.5 % formic acid and fractionated on a nanoflow HPLC system (Famos / Switchos / Ultimate, LC Packings) before being analysed by electrospray ionisation (ESI) mass spectrometry on a Q-STAR® Pulsar i hybrid MS/MS System. Peptide separaration was performed on a Pepmap C18 reversed phase column (LC Packings), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min. The flow rate was maintained at 0.2 μl / min. Mass spectrometric analysis was performed using a 3 second survey MS scan followed by up to four MS/MS analyses of the most abundant peptides (3 second per peak) in Information Dependent Acquisition (IDA) mode, choosing 2+ to 4+ ions above threshold of 30 counts, with dynamic exclusion for 120s.

Data generated from the Q-STAR® Pulsar i hybrid mass spectrometer was analysed using Applied Biosystems Analyst QS (v1.1) software and the automated Matrix Science Mascot Daemon server (v2.1.06). Protein identifications were assigned using the Mascot search engine, which gives each protein a probability based MOWSE score. In all cases variable methionine oxidation was allowed in searches and carbamidomethylation of cysteines was selected as a fixed

modification. An MS tolerance of 1.2 Da for MS and 0.4 Da for MS/MS analysis was used.

3.2.13 Screening for integral membrane protein

The results from MS analysis that were analysed by MASCOT were further examined to search for integral membrane proteins (IMPs). The accession numbers of each protein identified in the samples were entered in the TriTrypDB website (tritrypdb.org/) to search the transmembrane domain (TMD) in the protein. The protein that showed the existence of TMD was concluded to be the membrane protein in IMPs group. Because IMPs may have a substantial fragment of their sequence embedded in the membrane, analysis of TMD peptides magnifies the chance of categorised IMPs (Speers & Wu 2007).

3.3 Results

3.3.1 Expression of an epitope-tagged glucose transporter

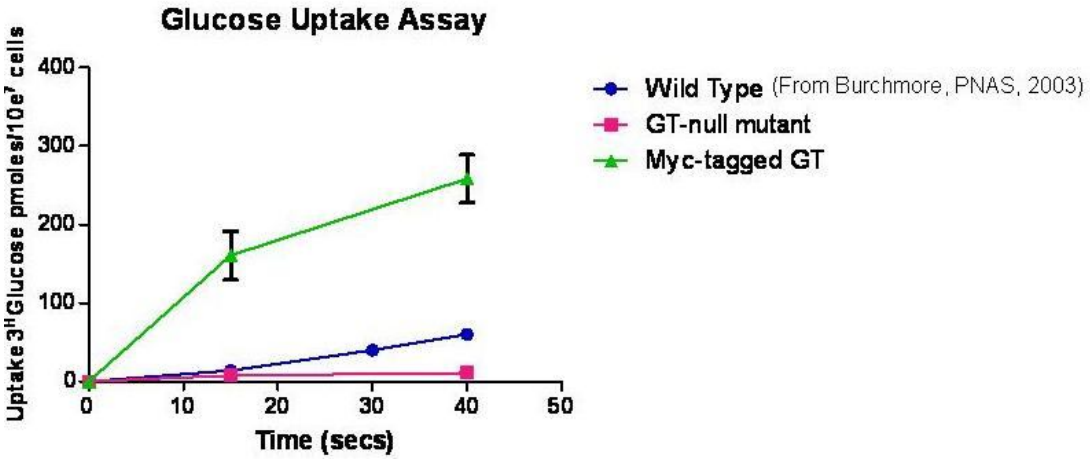

Figure 3-3: Glucose uptake of wild type, glucose transporter null mutant (GT-null mutant) and myc-tagged glucose transporter (Myc-tagged GT). The graph shows that the recovery of glucose uptake ability with the introduction of plasmid containing Myc-tagged glucose transporter.

An uptake assay was performed to see the functionality of the restored glucose transporter through transformation of plasmid into glucose transporter null mutant *Leishmania*. A graph line for wild type taken from a published paper from Burchmore et al (Burchmore et al. 2003) was extrapolated in the graph. Two clones of the myc-tagged glucose transporter *Leishmania*, Myc-tagged glucose transporter showed a very high uptake of glucose compared to the wild type whereas the null mutant line shows negligible glucose uptake.

3.3.2 Fractionation efficiency by western blot tracking for glucose transporter and proteomic analysis

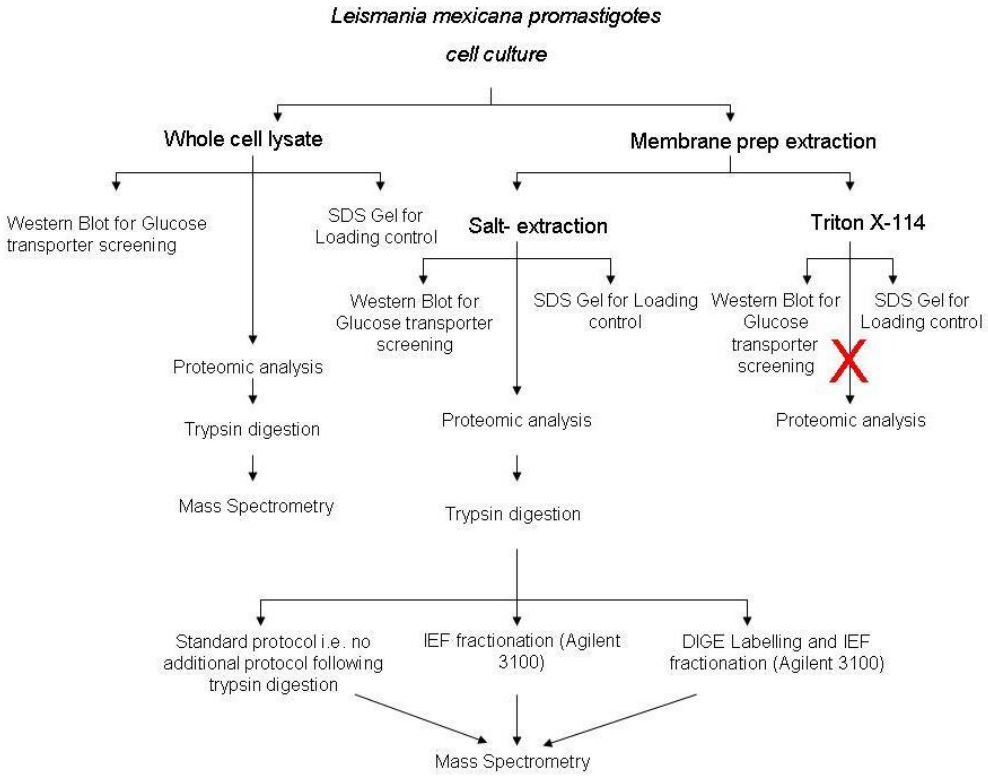

Figure 3-4: Flowchart shows the workflow of whole cell lysate and membrane fractionation. Both whole cell lysate and membrane prep samples were analyse for glucose transporter recovery. This is done to investigate the expression of myc tagged glucose transporter. The 'quality' of the myc-tagged glucose transporter's expression in western blot is an indicator for fractionation efficiency. Samples from membrane preparation were tested in 2 extraction protocols i.e. Salt-extraction and Triton X-114 membrane extraction. Based on the Western blot result, only whole cell lysate and membrane extraction from salt-extraction protocol were analyse for proteomic analysis. Samples from Triton X-114 were not giving satisfying result in western blot analysis and performing proteomic analysis on these samples thought to be not necessary. SDS gel loaded with samples were used as a loading control.

3.3.3 Detection of myc-tagged LmGT2 in *Leishmania mexicana*

In order to test the efficiency of membrane fractionation, 3 samples should be collected i.e. the whole cell lysate, the supernatant and membrane preparation. The efficiency of the preparation of the samples and the extraction are based on whether the myc-tagged glucose transporter is seen in 2 fractions of the cell i.e. the whole cell lysate and the membrane fraction. And this myc-tagged glucose transporter should not be seen in the supernatant.

Thus, in the experiment three fractions i.e. whole cell lysate, supernatant, membrane preparation from the sample preparation were collected from both the wild type and null mutant cells and immunoblotted to screen for the myc-tagged glucose transporter recovery. All three fractions must be collected from the same source i.e. from the same flask. It was necessary to test different cell counts of cells to see the expression of myc-tagged glucose transporter i.e $2.7x10^8$ cells to $2.7x10^7$ cells from the cell cultures that have been grown up to the cell density of $1X10^7$cells/ml. It appeared that the lower the cell counts that I used, the better was the expression. Immunoblotting for integral membrane proteins can be difficult to achieve because these proteins are typically of low relative abundance. Furthermore, hydrophobic proteins blot less efficiently than more hydrophilic proteins (Small et al. 1988).

In addition, the higher level of 2-β-mercaptoethanol has shown to give a better expression of the band. 1-SDS gel (Figure 3-5) loaded with proteins from whole cell lysate, membrane protein fraction and supernatant from all 3 cell lines tested (wild type, glucose transporter null mutant and myc-tagged glucose transporter), serves as a loading control(Welinder & Ekblad 2011). Western blots were done for whole cell lysate (Figure 3-6), supernatant (Figure 3-7) and membrane fraction (Figure 3-8) to monitor the myc-tagged glucose transporter recovery and the approximate band size for myc-tagged glucose transporter is between 46-58kda. A band's expression was seen in whole cell lysate (Figure 3-6) and lane loaded with myc-tagged glucose transporter cell line in membrane fraction (lane 4, in Figure 3.8). But in Figure 3-8, i.e. membrane protein fractionation, although lane 4 and lane 5 loaded with the same myc-tagged-glucose transporter cell lines, a band's expression was stronger in lane (5) and mildly in lane (4) (lane 4 has higher protein content than lane 5). No expression was seen in supernatant fraction from all cell lines (Figure 3-7). However the molecular weight of these bands expressed in whole cell lysate and membrane fraction was slightly higher (in whole cell lysate, Figure 3-6) and slightly lower (in membrane fractionation, Figure 3-8) compared to glucose transporter molecular weight which is around 52kDa. This may caused by various reasons such as post-translatonal modification (for Figure 3-6 and Figure 3-8) or the protein may have been aggregated due to the chemical used in extraction thus the protein moved slower in the gel(in Figure 3-8).

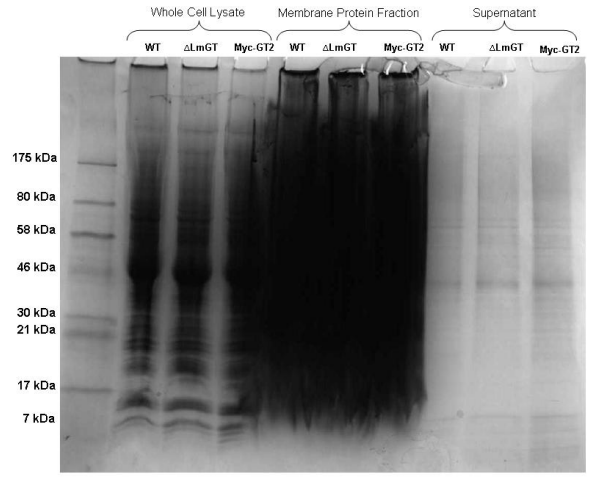

Figure 3-5: 1-SDS gel stained with Coomassie blue for analysis of the salt-extraction protocol to monitor the recovery of membrane proteins where myc expression serves as the indicator. All 3 fractions i.e. whole cell lysate, membrane protein and supernatant were collected to screen for the myc expression. This gel serves as quality control to show that all proteins in all lanes were loaded into the respective well at equal amounts (Welinder & Ekblad 2011). (20µl samples were loaded for all lanes which in the membrane protein fraction's lanes, may contain $6x10^8$ cells per lane and this cell count is likely be less in the other lanes i.e. whole cell lysate and supernatant).

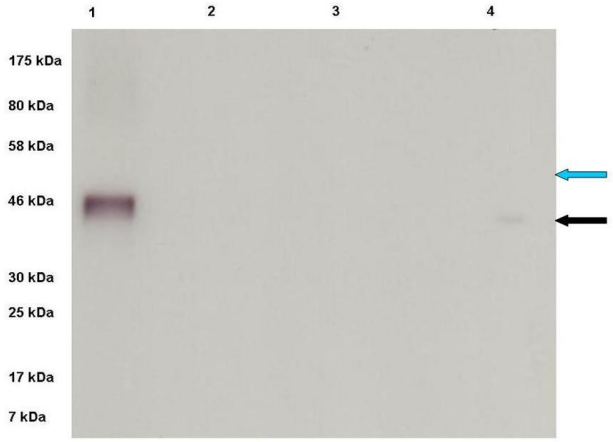

Figure 3-6: Western blot of whole cell lysate from salt-extraction. Screening for glucose transporter expression probed with c-Myc Mouse mAB (1:200) and anti-mouse secondary antibody (1:50,000). 1: c-Myc positive control, 2: Wild type, 3: glucose transporter null mutant, 4: myc-tagged glucose transporter expressing cells. Blue arrow shows the approximate molecular weight for myc-tagged glucose transporter (52kDa) and the black arrow shows a band.

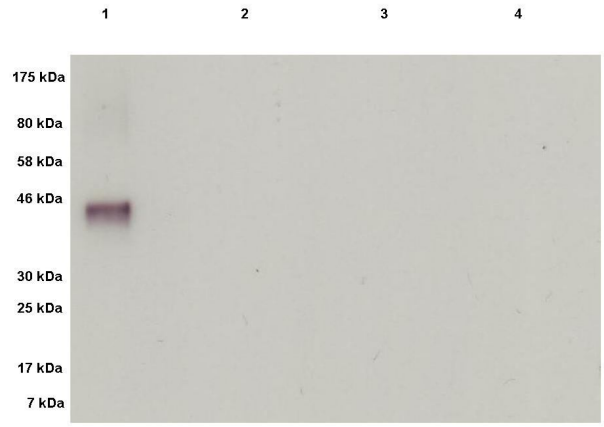

Figure 3-7: Western blot of supernatant fraction from salt-extraction. Screening for glucose transporter expression probed with c-Myc mouse mAB (1:200) and anti-mouse secondary antibody (1:50,000). 1: c-Myc positive control, 2: Wild type, 3: glucose transporter null mutant, 4: myc-tagged glucose transporter.

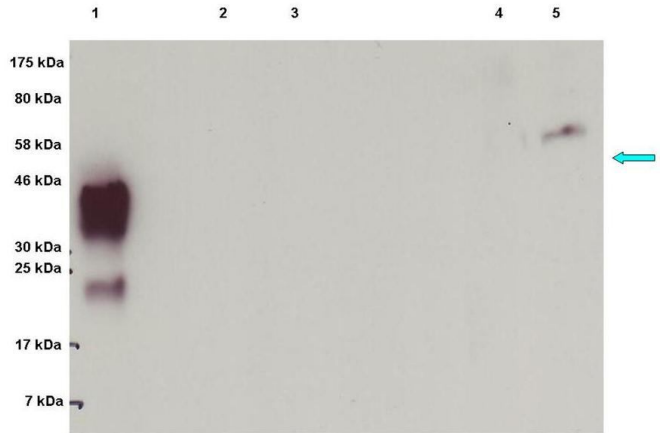

Figure 3-8: Western blot of membrane preparation fraction from salt-extraction. Screening for glucose transporter expression probed with c-Myc Mouse mAB (1:200) and anti-mouse secondary antibody (1:10,000). 1: c-Myc positive control, 2: Wild-type, 3: glucose transporter null mutant, 4: myc-tagged glucose transporter ($5.4x10^7$ cells), 5: myc-tagged glucose transporter ($2.7x10^7$ cells). The arrow on the right-handside shows the approximate molecular weight for myc-tagged glucose transporter i.e. approximately 52kDa.

3.3.4 Western blot for samples from Triton X-114 extraction. Recovery for myc-tagged glucose transporter Leishmania mexicana

Another membrane protein extraction protocol using Triton X-114 was tested. 20µg proteins were loaded in each well. Two fractions of the extraction i.e. detergent phase and aqueous phase from each cell line i.e. wild type, glucose transporter null mutant and myc-tagged glucose transporter, were collected and the myc-tagged expression was monitored on a western blot (Figure 3-9). A band was detected in lane 7 which was loaded with myc-tagged glucose transporter cell line from detergent phase. However, the expressed band was not resolved by molecular weight but rather smeared. This is likely due to the formation of mixed micelles between Triton X-114 and SDS in the sample (Burchmore, R.J., PhD thesis, 1993). A strong band is seen between 31kDa-46kDa in all cell lines from the aqueous phase (lane 2, 3, 4). This could be some cross reaction with an unrelated protein as the wild type and glucose transporter null parasites were not transfected with a myc-tagged construct. The positive control (lane 1) showed a very strong expression but the lower band is considered to be the positive control as the molecular weight for the positive control is 41kDa (from manufacturer's instruction sheet).

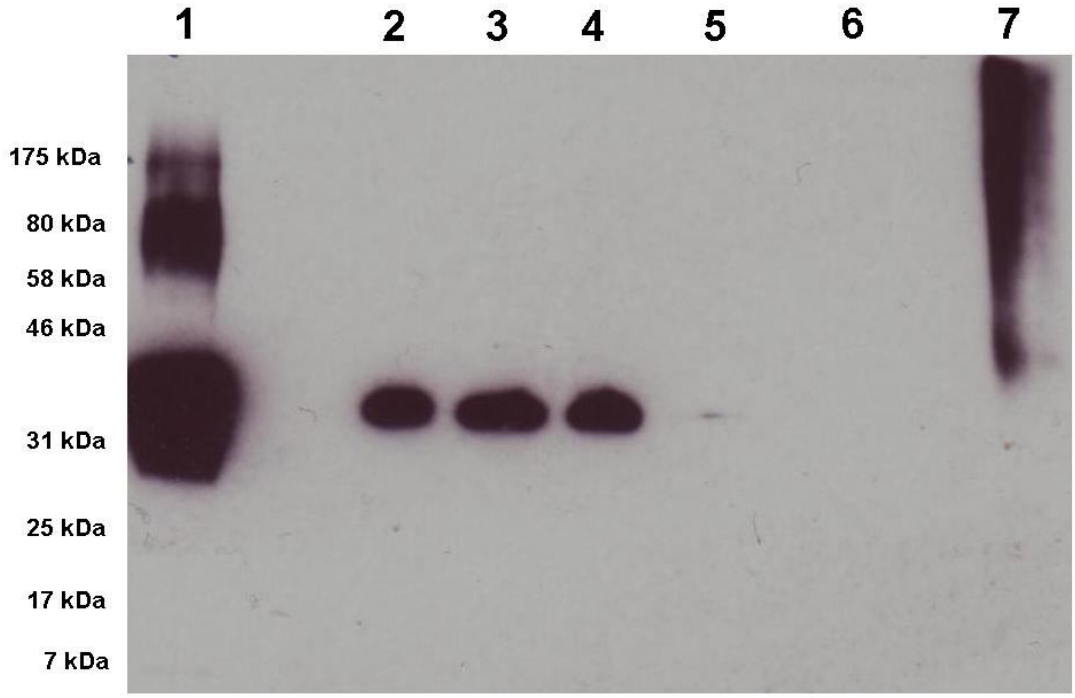

Figure 3-9: Western blot of protein samples from Triton X-114 extraction. 1: myc positive control, lane 2,3 and 4 are aqueous phase and lane 5,6 and 7 are detergent phase . Lane 2&5: Wild type, lane 3&6: glucose transporter null mutant, lane 4&7: myc-tagged glucose transporter. Positive control is a *E.coli* positive control (whole lysates) containing a 41kd recombinant protein expressing 11 tags, including C-Myc (Abcam).

3.3.5 Proteomics for Whole Cell Lysate

A comparative analysis of whole proteome was carried out between wild type (WT) and glucose transporter null mutant (ΔLmGT) to detect whole protein content expression differences. Figure 3-10 shows the 1-SDS gel used in the proteomic analysis. 35 equal-sized bands were cut out from both lanes i.e WT and ΔLmGT and bands were analysed by mass spectrometry. Results from the mass spectrometry analysis are summarised in Table 3-1 (Band 1-Band 35).

Based of the observation of hydrophobic predicted Transmembrane domains (TMD), several IMPs were identified in whole cell proteome in both cells. Some IMPs were exclusively identified in wild type i.e. MDR1-glycoprotein(LmjF34.0990), ATP-dependent zinc metallopeptidase (LmjF18.0610), E1F3-interacting protein-like protein (LmjF36.0250) and protein transport protein sec 61 gamma subunit (LmjF25.1015). IMPs that were exclusively found in the null mutant whole cell proteome were calcium motive p-type ATPase (LmjF35.2080), fatty acid elongase (LmjF14.0650), hypothetical protein (LmjF23.0640) and Gim5A protein (LmjF35.3700).

Integral protein that was found in both whole cell proteome from both cells analysed was only glucose regulated protein 78 (LmjF28.1200).

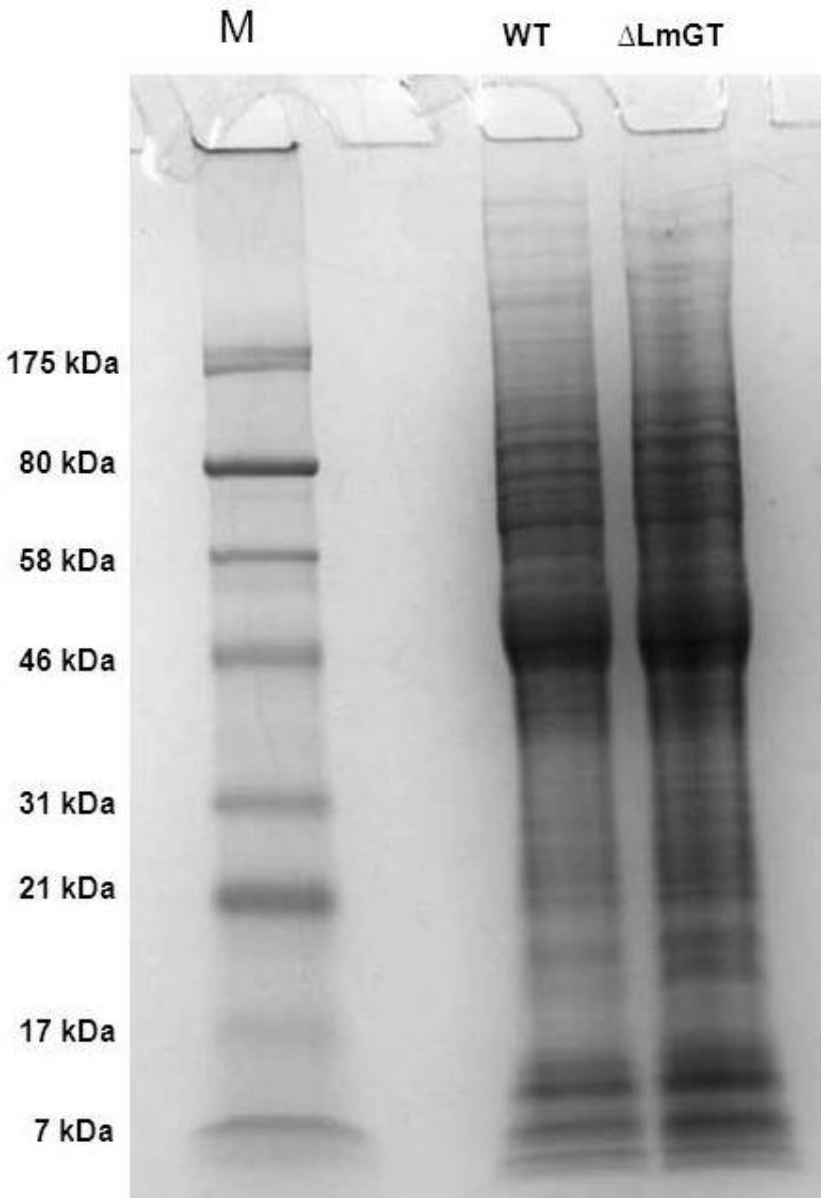

Figure 3-10: 1-SDS gel stained with Coomassie blue. This gel serves as loading control for whole cell lysate proteomic analysis. Each lane was loaded with $2x10^7$ cells (Welinder & Ekblad 2011).

3.3.5.1 Whole Proteome Comparison

Table 3-1: Results from mass spectrometry analysis for whole cell protein comparison between wild type (WT) and glucose transporter null mutant (∆LmGT) were summarised in the table below (Band 1-Band 35). 35 bands from each cell line were excised from the gel. TMD (transmembrane domain) was search in each found protein to confirm whether the protein was a membrane protein. (Mwt=molecular weight in kDa). Blue column= proteins (with TMD) found only in wild type, red columns= proteins (with TMD) found only in glucose transporter null mutant, yellow columns= proteins (with TMD) found both in wild type and glucose transporter null mutant.

Band 1

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF19.1070	Hypothetical protein, conserved	103031	35	0	0	1
	LmjF34.3330	Cytochrome p450-like protein	60567	30	1	0	1
∆GT	LmjF21.1860	Beta tubulin	50395	162	16	0	6
	LmjF16.1460	Kinesin, putative	247359	60	0	0	1
	LmjF17.0080	Elongation factor 1-alpha	49485	44	0	0	1
	LmjF20.0160	Hypothetical protein, conserved	88683	39	0	0	1
	LmjF25.2155	Hypothetical protein, conserved	27588	32	4	0	1

Band 2

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF15.0440	Hypothetical protein	1527956	101	0	0	1
	LmjF13.0280	Alpha tubulin	50526	74	3	0	1
∆GT	**No significant hits to report**						

Band 3

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF08.1230	Beta tubulin	50302	28	2	0	1
∆GT	LmjF15.0440	Hypothetical protein, unknown function	1527956	92	0	0	1
	LmjF27.0500	Calpain-like cysteine peptidase	702676	31	0	0	1

Band 4

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF27.0500	Calpain-like cysteine peptidase, putative	702676	28	0	0	1
∆GT	LmjF13.1650	Dynein heavy chain, putative	532336	42	0	0	1
	LmjF25.0980	Dynein heavy chain, putative	538395	40	0	0	3

Band 5

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF25.0980	Dynein heavy chain, putative	538395	88	0	0	2
	LmjF08.1230	Beta tubulin	50302	57	3	0	1
	LmjF14.1440	Hypothetical protein, conserved	104705	56	1	0	1
	LmjF13.0280	Alpha tubulin	50526	50	3	0	1
	LmjF13.1650	Dynein heavy chain, putative	532336	42	0	0	2
	LmjF27.0500	Calpain-like cysteine peptidase	702676	30	0	0	1
∆GT	LmjF13.1650	Dynein heavy chain, putative	532336	99	1	0	4
	LmjF05.0380	Microtubule-associated protein, putative	206884	49	0	0	1

Band 6

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF03.0690	Hypothetical protein, conserved	249125	83	1	0	2
	LmjF13.0280	Alpha-tubulin	50526	60	3	0	1
	LmjF21.0800	Hypothetical protein, conserved	250810	50	0	0	1
	LmjF14.1440	Hypothetical protein, conserved	104705	28	0	0	1
∆GT	LmjF03.0690	Hypothetical protein, conserved	249125	55	0	0	1
	LmjF13.0280	Alpha-tubulin	50526	50	3	0	1
	LmjF21.0800	Hypothetical protein, conserved	250810	39	0	0	1

Band 7

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF16.0590	Carbomoyl-phosphate synthase, putative	208165	90	2	0	2
	LmjF29.2110	Hypothetical protein, conserved	193313	46	0	0	1
	LmjF03.0690	Hypothetical protein, conserved	249125	43	0	0	1
∆GT	LmjF16.0590	Carbamoyl-phosphate synthase, putative	208165	48	0	0	1

Band 8

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF36.1630	Clathrin heavy chain, putative	192062	140	1	0	2
	LmjF13.0280	alpha-tubulin	50526	52	3	0	1
	LmjF29.2110	Hypothetical protein, conserved	193313	51	0	0	1

	LmjF09.1510	Hypothetical protein, conserved	175536	40	0	0	1
ΔGT	LmjF36.1630	Clathrin heavy chain, putative	192062	80	2	0	3
	LmjF13.0280	Alpha-tubulin	50526	56	6	0	2
	LmjF11.0370	Hypothetical protein, conserved	67774	28	1	0	1

Band 9

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF34.0990	MDR1, p-glycoprotein	148015	258	3	10	3
	LmjF20.1180	Calpain-like cysteine peptidase, putative	109171	63	1	0	1
	LmjF13.0280	Alpha-tubulin	50526	58	3	0	1
	LmjF29.2630	Hypothetical protein, conserved	132406	46	1	0	1
	LmjF22.1500	ATP-dependant DEAD/H RNA helicase, putative	120849	42	1	0	1
	LmjF31.1500	Hypothetical protein, conserved	120776	42	1	0	1
	LmjF36.1630	Clathrin heavy chain, putative	192062	28	0	0	1
ΔGT	LmjF35.2080	Calcium motive p-type ATPase, putative	123373	66	1	8	1
	LmjF35.3100	ATP-dependant RNA helicase, putative	100525	44	1	0	1
	LmjF22.1500	ATP-dependant DEAD/H RNA helicase, putative	120849	28	1	0	1

Band 10

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF34.0990	MDR1, p-glycoprotein	148015	100	1	10	1
	LmjF35.3100	ATP-dependant RNA helicase, putative	100525	14	3	0	2
	LmjF13.0280	Alpha-tubulin	50526	52	3	0	1
	LmjF31.1900	Ubiquitin-fusion protein	14958	38	12	0	1
ΔGT	LmjF35.1180	NADH-dependant fumarate reductase, putative	124204	93	1	0	1

Band 11

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF15.1010	Glutamate dehydrogenase	115588	119	1	0	1
	LmjF36.0180	EF2-1, elongation factor 2	94928	94	1	0	1
	LmjF32.3010	Hypothetical protein, conserved	97475	94	1	0	1

	LmjF13.0280	Alpha-tubulin	50526	57	3	0	1
	LmjF22.1540	Alanyl-tRNA synthetase, putative	106882	50	1	0	1
	LmjF15.1480	cAMP specific phosphodiesterase, putative	105161	25	1	0	1
ΔGT	LmjF36.0180	EF2-1, elongation factor 2	94928	162	8	0	4
	LmjF32.3010	Hypothetical protein, conserved	97475	100	1	0	1
	lmjF15.1010	Glutamate dehydrogenase	115588	87	1	0	1
	LmjF13.0280	Alpha-tubulin	50526	66	3	0	1
	LmjF18.0510	Aconitase, putative	98229	53	1	0	1
	LmjF15.1480	cAMP specific phosphodiesterase, putative	105161	52	1	0	1
	LmjF30.3420	Hypothetical protein, conserved	97869	40	1	0	1

Band 12

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF36.0180	EF2-1, elongation factor 2	94928	378	14	0	8
	LmjF13.0280	Alpha-tubulin	50526	116	7	0	2
	LmjF18.0510	Aconitase, putative	98229	106	3	0	2
	LmjF36.6980	Eukaryotic translation initiator factor 3 subunit 8, putative	82437	80	1	0	1
	LmjF28.2620	Coatomer gamma subunit, putative	96256	55	1	0	1
	LmjF36.1370	Transitional endoplasmic reticulum ATPase, putative	87582	45	3	0	2
	LmjF12.0250	Cysteinyl-tRNA synthetase, putative	88887	34	1	0	1
	LmjF15.1480	cAMP specific phosphodiesterase	105161	33	1	0	1
ΔGT	LmjF36.0180	EF2-1, elongation factor 2	94928	327	13	0	6
	LmjF13.0280	Alpha-tubulin	50526	114	6	0	2
	LmjF36.6980	Eukaryotic translation initiator factor 3 subunit 8, putative	82437	79	1	0	1
	LmjF29.1750	Paraflagellar rod protein 1D, putative	69390	74	2	0	1
	LmjF27.0870	Hypothetical protein, conserved	85382	68	2	0	1
	LmjF31.0390	Calpain-like cysteine peptidase, putative	97703	68	3	0	1
	LmjF11.1000	Pyruvate phosphate dikinase, putative	101609	47	2	0	1
	LmjF36.1370	Transitional endoplasmic reticulum ATPase, putative	87582	32	2	0	1

Band 13

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF29.1750	Paraflagellar rod protein 1D, putative	69390	130	4	0	2
	LmjF31.0010	5-methyltetrahydropteroyltriglutamate--homocyst ei nemethyltransferase, putative	86627	115	3	0	2
	LmjF36.0180	EF2-1, elongation factor 2	94928	78	2	0	2
	LmjF13.0280	Alpha-tubulin	50526	62	3	0	1
	LmjF34.0230	Hypothetical protein, conserved	81994	58	1	0	1
	LmjF28.2770	Heat-shock protein hsp70, putative	71893	53	3	0	2
	LmjF31.0440	Cytoskeleton-associated protein CAP5.5, putative	81042	51	2	0	1
	LmjF01.0520	Long chain fatty acid CoA ligase, putative	78255	27	3	0	1
	LmjF32.2960	Minichromosome maintenance (MCM) complex subunit, putative	81251	25	1	0	1
ΔGT	LmjF31.0010	5-methyltetrahydropteroyltriglutamate--homocyst ei nemethyltransferase, putative	86627	145	5	0	3
	LmjF16.1425	Paraflagellar rod protein 2c	69257	118	2	0	2
	LmjF13.0280	Alpha-tubulin	50526	97	6	0	2
	LmjF30.2460	Heat-shock 70-related protein 1, mitochondrial precursor, putative	69532	87	3	0	1
	LmjF29.1750	Paraflagellar rod protein 1D, putative	69390	81	4	0	2
	LmjF28.1200	Glucose-regulated protein 78, putative	72067	74	3	1	1
	LmjF28.2770	Heat-shock protein hsp70, putative	71893	68	3	0	2
	LmjF32.0400	ATP-dependant RNA helicase, putative	67276	46	1	0	1

Band 14

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF28.2770	Heat-shock protein hsp70, putative	71893	181	7	0	4
	LmjF16.1425	Paraflagellar rod protein 2C	69257	149	2	0	2
	LmjF30.2460	Heat shock 70-related protein 1	69532	109	3	0	1
	LmjF29.1750	Paraflagellar rod protein 1D, putative	69390	106	4	0	2

	LmjF34.3670	Vacuolar ATP synthase catalytic subunit a, putative	68206	90	3	0	1
	LmjF28.1200	Glucose-regulated protein 78, putative	72067	90	3	1	1
	LmjF13.0280	Alpha-tubulin	50526	88	6	0	2
	LmjF32.0400	ATP-dependant RNA helicase, putative	67276	80	8	0	3
	LmjF26.1570	Thimet oligopeptidase, putative	77775	78	1	0	1
	LmjF23.0540	Acetyl-CoA synthetase, putative	79156	53	2	0	1
	LmjF24.1560	Hypothetical protein, conserved	67813	50	3	0	1
ΔGT	LmjF28.2770	Heat-shock protein hsp70, putative	71893	312	13	0	6
	LmjF16.1425	Paraflagellar rod protein 2C	69257	268	8	0	5
	LmjF28.1200	Glucose-regulated protein 78, putative	72067	211	13	1	4
	LmjF30.2460	Heat shock 70-related protein 1, mitochondrial precursor, putative	69532	155	3	0	1
	LmjF26.1570	Thimet oligopeptidase, putative	77775	138	3	0	2
	LmjF29.1750	Paraflagellar rod protein 1D, putative	69390	102	4	0	2
	LmjF34.3670	Vacuolar ATP synthase catalytic subunit a, putative	68206	91	3	0	1
	LmjF24.1560	Hypothetical protein, conserved	67813	85	3	0	1
	LmjF13.0280	Alpha-tubulin	50526	78	6	0	2
	LmjF23.0640	Hypothetical protein, conserved	63257	71	2	1	1
	LmjF26.1240	HSP70.4, heat shock protein 70-related protein	71001	70	1	0	1
	LmjF05.0960	Dipeptidylpeptidase III, putative	76262	66	2	0	1
	LmjF32.0400	ATP-dependant RNA helicase, putative	67276	56	5	0	2
	LmjF23.0540	Acetyl-CoA synthetase, putative	79156	46	2	0	1

Band 15

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF28.2770	Heat shock protein hsp70, putative	71893	323	14	0	6
	LmjF13.0280	Alpha-tubulin	50526	114	14	0	4
	LmjF30.2460	Heat shock 70-related protein 1, mitochondrial precursor, putative	69532	104	3	0	1

	LmjF29.1310	Carnitine/choline acetyltransferase, putative	68959	99	3	0	2
	LmjF24.1630	Succinate dehydrogenase flavoprotein, putative	67605	98	2	0	1
	LmjF29.1750	Paraflagellar rod protein 1D, putative	69390	96	4	0	2
	LmjF32.1000	Chaperonin containing t-complex protein, putative	59978	80	4	0	1
	LmjF28.1200	Glucose-regulated protein 78, putative	72067	70	3	1	1
	LmjF16.1425	Paraflagellar rod protein 2C	69257	59	2	0	1
	LmjF08.1230	Beta-tubulin	50302	53	3	0	1
	LmjF08.1110	Stress-induced protein sti1	62638	49	2	0	1
	LmjF36.6650	PGAM, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	60903	45	7	0	2
	LmjF26.1570	Thimet oligopeptidase, putative	77775	39	1	0	1
	LmjF16.1180	Coatomer delta subunit-like protein	58116	38	2	0	1
	LmjF32.2960	Minichromosome maintenance (MCM) complex subunit, putative	81251	27	1	0	1
ΔGT	LmjF28.2770	Heat-shock protein hsp70, putative	71893	312	11	0	6
	LmjF13.0280	Alpha-tubulin	50526	236	17	0	5
	lmjF36.2030	Chaperonin Hsp60, mitochondrial precursor	59623	140	7	0	2
	LmjF08.1230	Beta-tubulin	50302	120	6	0	2
	LmjF32.1000	Chaperonin containing t-complex protein, putative	59978	118	6	0	2
	LmjF24.1630	Succinate dehydrogenase flavoprotein, putative	67605	96	2	0	1
	LmjF28.1200	Glucose-regulated protein 78, putative	72067	67	3	1	1
	LmjF35.3860	t-complex protein 1, eta subunit, putative	62507	59	3	0	1
	LmjF29.1310	Carnitine/choline acetyltransferase, putative	68959	53	2	0	1
	LmjF25.1170	ATPase beta subunit, putative	56541	29	2	0	1
	LmjF02.0370	Proteasome regulatory non-ATPase subunit 6, putative	59200	6	3	0	1

Band 16

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF13.0280	Alpha-tubulin	50526	218	17	0	5

	LmjF28.2770	Heat-shock protein hsp70	71893	207	11	0	5
	LmjF24.1630	Succinate dehydrogenase flavoprotein, putative	67605	90	2	0	1
	LmjF08.1230	Beta tubulin	50302	68	3	0	1
	LmjF25.1170	ATPase beta subunit, putative	56541	63	13	0	3
	LmjF18.0610	ATP-depndant zinc metallopeptidase, putative	65040	53	3	1	1
	LmjF32.3270	Chaperonin alpha subunit, putative	59497	53	3	0	1
	LmjF32.0400	ATP-dependant RNA helicase, putative	67276	48	1	0	1
	LmjF27.1805	Glycosomal phosphoenolpyruvate carboxylase, putative	58670	46	2	0	1
	LmjF36.0250	EIF3-interacting protein-like protein	732223	46	2	1	1
	LmjF26.1570	Thimet oligopeptidase, putative	77775	33	1	0	1
	LmjF29.1310	Carnitine/choline acetyltransferase, putative	68959	31	2	0	1
∆GT	LmjF08.1230	Beta tubulin	50302	273	16	0	6
	LmjF25.1170	ATPase beta subunit, putative	56541	157	5	0	2
	LmjF13.0280	Alpha tubulin	50526	66	3	0	1
	LmjF27.1805	Glycosomal phosphoenolpyruvate carboxylase, putative	58670	40	2	0	1

Band 17

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF33.0792	Beta tubulin	50335	701	46	0	17
	LmjF13.0280	Alpha tubulin	50526	322	23	0	7
	LmjF27.1805	Glycosomal phosphoenol pyruvate carboxykinase, putative	58670	111	7	0	3
	LmjF36.2030	Chaperonin Hsp60, mitochondrial precursor	59623	91	5	0	2
	LmjF25.1170	ATPase beta subunit, putative	56541	62	7	0	2
	LmjF36.1520	Hypothetical protein, conserved	57081	55	2	0	1
	LmjF11.0100	Seryl-tRNA synthetase, putative	53850	51	2	0	1
	LmjF35.0020	Pyruvate kinase, putative	57833	36	4	0	1
∆GT	LmjF33.0792	Beta tubulin	50335	368	27	0	10
	LmjF13.0280	Alpha tubulin	50526	186	21	0	6
	LmjF17.0080	Elongation factor 1-alpha	49485	58	5	0	1
	LmjF32.3130	Ribosomal protein 13, putative	47743	49	3	0	1

	LmjF36.2020	Chaperonin Hsp60, mitochondrial precursor	60484	29	3	0	1

Band 18

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF33.0792	Beta tubulin	50335	629	41	0	18
	LmjF13.0280	Alpha tubulin	50526	217	20	0	6
	LmjF32.3310	Dihydrolipoamide dehydrogenase, putative	51173	58	2	0	1
	LmjF17.0080	Elongation factor 1-alpha	49485	40	8	0	2
	LmjF24.2110	Hypothetical protein, conserved	55619	27	2	0	1
	LmjF27.1805	Glycosomal phosphoenolpyruvate carboxykinase, putative	58670	26	2	0	1
∆GT	LmjF33.0792	Beta tubulin	50335	512	40	0	17
	LmjF13.0280	Alpha tubulin	50526	373	28	0	8
	LmjF17.0080	Elongation factor 1-alpha	49485	48	2	0	1
	LmjF19.0160	Aminopeptidase, putative	43006	47	6	0	2
	LmjF21.0240	Hexokinase, putative	52200	41	2	0	1

Band 19

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF08.1230	Beta tubulin	50302	438	21	0	1
	LmjF13.0280	Alpha tubulin	50526	388	32	0	10
	LmjF17.0080	Elongation factor 1-alpha	49485	105	18	0	6
	LmjF14.1160	ENOL,enolase	46689	56	14	0	4
	LmjF36.3910	S-adenosylhomocysteine hydrolase	48425	33	2	0	1
∆GT	LmjF08.1230	Beta tubulin	50302	115	6	0	2
	LmjF16.0550	Orotidine-5-phosphate decarboxylase/orotate phosphoribosyltransferase, putative	49811	43	10	0	2

Band 20

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF35.3230	Cystathione gamma lyase, putative	45074	140	9	0	2
	LmjF21.1552	RNA helicase,putative	49741	65	3	0	1
∆GT	LmjF35.3230	Cystathione gamma lyase, putative	45074	162	9	0	2

	LmjF30.2970	Glyceraldehyde 3-phosphate dehydrogenase, glycosomal	39370	116	4	0	1
	LmjF36.2950	Succinyl-CoA ligase [GDP-forming] beta-chain, putative	44616	54	2	0	1

Band 21

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF30.2970	Glyceraldehyde 3-phosphate dehydrogenase, glycosomal	39370	112	4	0	1
	LmjF29.1070	Ribosomal protein L1a, putative	41109	80	5	0	1
	LmjF35.3230	Cystathione gamma lyase, putative	45074	29	5	0	1
∆GT	LmjF30.2970	Glyceraldehyde 3-phosphate dehydrogenase, glycosomal	39370	119	4	0	1

Band 22

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF34.0140	Malate dehydrogenase	33622	85	12	0	2
	LmjF30.0880	Adenosine kinase, putative	37703	63	7	0	1
	LmjF13.0280	Alpha tubulin	50526	42	3	0	1
	LmjF21.1250	Adenylate kinase, putative	30212	41	5	0	1
∆GT	LmjF34.0140	Malate dehydrogenase	33622	142	5	0	1

Band 23

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF34.0140	Malate dehydrogenase	33622	109	5	0	1
∆GT	LmjF34.0140	Malate dehydrogenase	33622	136	5	0	1
	LmjF32.0450	40S ribosomal protein S2	28811	89	5	0	1
	LmjF28.2740	LACK1,activated protein kinase C receptor (LACK)	34891	89	5	0	1
	LmjF13.0280	Alpha tubulin	50526	62	3	0	1

Band 24

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF34.0140	Malate dehydrogenase	33622	83	5	0	1
	LmjF32.0450	40S ribosomal protein S2	28811	72	5	0	1
	LmjF13.0280	Alpha tubulin	50526	68	3	0	1

ΔGT	LmjF32.0450	40S ribosomal protein S2	28811	84	5	0	1
	LmjF13.0280	Alpha tubulin	50526	70	3	0	1

Band 25

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	**No significant hits to report**						
ΔGT	LmjF34.2580	Hypothetical protein, conserved	22634	29	9	0	1

Band 26

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF18.0620	60S ribosomal protein L10a	24970	31	7	0	1
ΔGT	LmjF18.0620	60S ribosomal protein L10a	24970	65	7	0	1

Band 27

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF18.0620	60S ribosomal protein L10a, putative	24970	78	7	0	1
	LmjF14.0190	Hypothetical protein, conserved	22485	61	6	0	1
ΔGT	LmjF18.0620	60S ribosomal protein L10a, putative	24970	57	7	0	1
	LmjF14.0190	Hypothetical protein, conserved	22485	55	6	0	1

Band 28

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF27.0760	Small GTP-binding protein Rab1, putative	22424	87	8	0	1
	LmjF04.0760	Nascent polypeptide associated complex subunit-like protein, copy 2	26675	83	11	0	1
	LmjF21.1700	Proteosome alpha2 subunit, putative	25309	42	8	0	1
ΔGT	LmjF27.0760	Small GTP-binding protein Rab1, putative	22424	79	8	0	1
	LmjF04.0760	Nascent polypeptide associated complex subunit-like protein, copy 2	26675	63	11	0	1
	LmjF28.0110	Proteosome beta 3 subunit , putative	22790	62	7	0	1
	LmjF18.0620	60S ribosomal protein L10a, putative	24970	57	7	0	1

	LmjF14.0190	Hypothetical protein, conserved	22485	57	6	0	1
	LmjF14.0650	Fatty acid elongase, putative	32476	34	4	7	1

Band 29

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	**No significant hits to report**						
ΔGT	LmjF27.0760	Small GTP-binding protein Rab1, putative	22424	74	8	0	1
	LmjF35.3700	Gim5A protein, putative	25339	56	5	1	1

Band 30

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF31.2790	adp-ribosylation factor, putative	20290	39	6	0	1
ΔGT	LmjF11.0960	40S ribosomal protein S5	21398	93	7	0	1
	LmjF30.3600	ATP synthase, epsilon chain, putative	20429	83	6	0	1
	LmjF31.2790	adp-ribosylation factor, putative	20290	58	11	0	1
	LmjF35.3700	Gim5A protein, putative	25339	42	5	1	1

Band 31

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF24.2210	60S ribosomal protein L12, putative	17698	98	9	0	1
	LmjF30.3600	ATP synthase, epsilon chain, putative	20429	57	6	0	1
ΔGT	LmjF36.0980	40S ribosomal protein S10, putative	18752	57	6	0	1

Band 32

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF36.2860	S24E-1, 40S ribosomal protein S24e	15874	121	21	0	3
	LmjF22.0420	40S ribosomal protein S15, putative	17424	121	10	0	1
ΔGT	LmjF22.0420	40S ribosomal protein S15, putative	17424	107	10	0	1
	LmjF36.2860	S24E-1, 40S ribosomal protein S24e	15874	88	11	0	2

Band 33

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF22.0420	40S ribosomal protein S15, putative	17424	127	10	0	1
	LmjF36.2860	S24E-1, 40 ribosomal protein S24e	15874	77	10	0	1
	LmjF36.3270	60S ribosomal protein L22, putative	15050	57	22	0	1
	LmjF16.0600	Histone h3, putative	14826	48	24	0	1
	LmjF09.0910	Calmodulin, putative	16814	38	24	0	1
∆GT	LmjF22.0420	40S ribosomal protein S15, putative	17424	131	10	0	1
	LmjF21.0730	60S ribosomal protein 136, putative	11992	73	13	0	1
	LmjF25.2090	Hypothetical protein, conserved	17898	67	17	0	1
	LmjF13.0570	40S ribosomal protein S12, putative	15826	45	17	0	1
	LmjF19.1100	Hypothetical protein, conserved	139733	41	1	0	1
	LmjF35.2050	60S ribosomal protein L32	15367	36	13	0	1

Band 34

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF15.1470	Ribosomal protein S6, putative	13564	107	12	0	1
	LmjF12.0520	Vacuolar ATP synthase subunit, putative	15656	74	16	0	1
	LmjF05.0500	ATPase alpha subunit	62794	47	2	0	1
	LmjF27.1530	Hypothetical protein, conserved	11488	40	15	0	1
∆GT	LmjF05.0500	ATPase alpha subunit	62794	54	2	0	1
	LmjF21.0730	60S ribosomal protein 136	11992	51	13	0	1

Band 35

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF26.0620	10kDa heat shock protein, putative	10651	70	14	0	1
	LmjF05.0500	ATPase alpha-subunit	62794	47	2	0	1
	LmjF33.0610	Hypothetical protein, conserved	21883	40	6	0	1
	LmjF25.1015	Protein transport protein sec 61 gamma subunit, putative	7884	38	18	1	1

ΔGT	LmjF36.3750	40S ribosomal protein S27-1, putative	10135	66	15	0	1
	LmjF05.0500	ATPase alpha-subunit	62794	65	2	0	1
	LmjF15.1470	Ribosomal protein S6, putative	13564	52	12	0	1

3.3.6 Membrane proteome from salt-extraction protocol

3.3.6.1 Membrane proteome comparison from standard protocol (without IEF and DIGE)

Proteomic analysis was carried out to compare the membrane proteomes of wild type (WT) and glucose transporter null mutant (ΔLmGT) *Leishmania*. A salt-extraction protocol was chosen over the Triton-X 114 protocol for sample preparation for mass spectrometry analysis as detergent may cause interference to the mass spectrometry and in electrophoretic separation. 1-SDS gel loaded with the cell lines tested served as loading control (Figure 3-11) and subsequently 30 pieces of equal-sized bands were excised each from the samples lane. However, due the MS availability, only 9 bands (Band 2-Band 10) from each sample were analysed. The results from the mass spectrometry analysis are summarised in Table 3-2.

A number of membrane proteins were identified from both samples, and some were exclusively found in either of the samples. Proteins that were exclusively found in wild type were 1) LPG1R, beta-galactofuranosyltransferase-like protein (LmjF33.0300), 2) LPG3, lipophosphoglycan biosynthetic protein, putative (LmjF29.0760), 3) Biopterin transporter, putative (LmjF35.5150), 4) 3'-nucleoside/nuclease, putative (LmjF12.0400), 5) Pretranslocation protein, alpha subunit, putative (LmjF11.1050), 6) Fatty acid desaturase, putative (LmjF10.0010), 7) Fatty acid desaturase, putative (LmjF33.3270).

Membrane proteins that were identified exclusively in the null mutant were as follows: 1) ABC transporter, putative (LmjF29.0620), 2) Glucose-regulated proetein 78, putative (LmjF28.1200), 3) Vacuolar-type protein translocating pyrophosphate 1, putative (LmjF31.1220), 4) GP63-1, GP63, leishmolysin (LmjF10.0470), 5) Hypothetical protein conserved, (LmjF28.0930), 6) Nucleoside transporter 1, putative (LmjF15.1230), 7) NAD(p)-dependant steroid dehydrogenase-like protein (LmjF06.0350) and 8) Cytochrome C, oxidase VII, putative (LmjF25.1130).

Among other membrane proteins that were found in both cell lines are: 1) H1A-1, p-ATPase, putative (LmjF18.1510), 2) NT2, nucleoside transporter 1,

putative (LmjFF36.1940), 3) Hypothetical protein, conserved (LmjF30.1760), 4) Multidrug resistance protein, putative (LmjF23.0220), 5) Nucleobase/nucleoside transporter 8.1, putative (LmjF13.1210), 6) GP63-1, GP63, leishmolysin (LmjF10.0460), 7) Hypothetical protein, conserved (LmjF21.1555) and 8) Membrane-bound acid phosphatise 2, putative (LmjF36.2590).

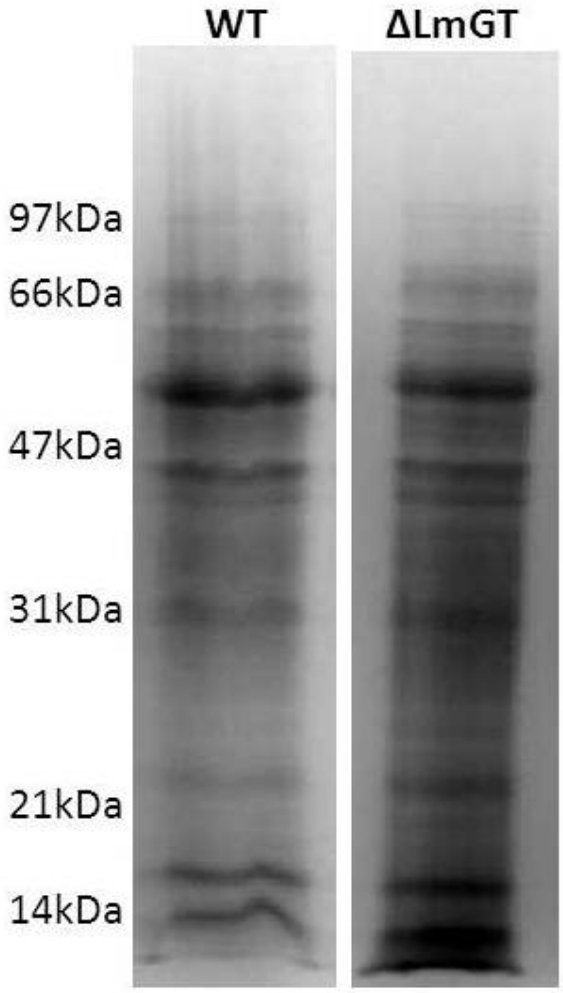

Figure 3-11: SDS gel of membrane extraction from wild type (WT) and glucose transporter null mutant (ΔLmGT), stained with Coomassie blue. Each well were loaded with 2µl=2x10^{7}cells.

Table 3-2: Results from mass spectrometry analysis comparing membrane proteomic between wild type and glucose transporter null mutant from salt-extraction protocol were summarised in this table. Blue column= proteins (with TMD) found only in wild type, red columns= proteins (with TMD) found only in glucose transporter null mutant, yellow columns= proteins (with TMD) found both in wild type and glucose transporter null mutant.

Band 2

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF33.0300	LPG1R,beta-galactofuronosyltransferase-like protein	52125	33	1	1	3
	LmjF26.1190	Hypothetical protein, conserved	184507	31	1	0	3
ΔGT	LmjF05.0380	Microtubule-associated protein, putative	206884	46	0	0	1
	LmjF27.0240	Kinetoplast-associated protein-like protein	78813	38	1	0	1
	LmjF35.2450	Hypothetical protein, conserved	254600	35	1	0	3
	LmjF33.2550	Isocitrate dehydrogenase, putative	46591	30	2	0	1

Band 3

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
wild type	LmjF27.0240	Kinetoplast-associated protein-like protein	78813	51	1	0	1
	LmjF05.0380	Microtubule-associated protein, putative	206884	41	1	0	2
	LmjF18.1510	H1A-1, P-ATPase, putative	108036	38	5	8	6
	LmjF14.0800	Hypothetical protein, conserved	42371	30	2	0	1
	LmjF23.1540	Hypothetical protein, conserved	106312	29	2	0	2
ΔGT	LmjF27.0240	Kinetoplast-associated protein-like protein	78813	70	1	0	1
	LmjF36.4800	Hypothetical protein, conserved	57011	40	5	0	6
	LmjF14.0800	Hypothetical protein, conserved	42371	35	2	0	1
	LmjF29.0620	ABC transporter, putative	209023	31	1	16	5
	LmjF23.0220	Multidrug resistance protein, putative	175138	31	0	6	2
	LmjF12.0110	Hypothetical protein, unknown function	91587	30	7	0	5

Band 4

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF23.1540	Hypothetical protein, conserved	106312	34	2	0	5
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	32	2	10	1
ΔGT	LmjF18.1510	H1A-1, P-type H1-ATPase, putative	108036	69	3	8	5
	LmjF05.0800	Microtubule-associated protein, putative	206884	35	1	0	2

	LmjF28.1880	Hypothetical protein, conserved	140389	33	1	0	4
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	30	2	10	1

Band 5

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF15.1010	Glutamate dehydrogenase	115588	71	4	0	6
	LmjF29.0760	LPG3, lipophosphoglycan biosynthetic protein, putative	86561	46	9	1	7
	LmjF11.1000	Pyruvate phosphate dikinase, putative	101609	40	6	0	5
	LmjF23.1540	Hypothetical protein, conserved	106312	37	4	0	4
ΔGT	LmjF15.1010	Glutamate dehydrogenase	115588	104	12	0	14
	LmjF30.1760	Hypothetical protein, conserved	210216	32	1	1	5
	LmjF13.1210	Nucleobase/nucleoside transporter 8.1, putative	55252	31	1	11	1
	LmjF23.1540	Hypoyhetical protein, conserved	106312	31	1	0	2
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	29	3	10	2

Band 6

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF33.0312	HSP83, heat shock protein 83-1	80997	49	11	0	7
	LmjF08.1230	Beta tubulin	50302	46	16	0	7
	LmjF15.1010	Glutamate dehydrogenase	115588	45	7	0	8
	LmjF23.1540	Hypothetical protein, conserved	106312	38	1	0	2
	LmjF35.5150	Biopterin transporter, putative	70130	36	1	14	3
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	31	2	10	1
	LmjF20.1090	Hypothetical protein, conserved	207668	30	2	0	4
ΔGT	LmjF15.1010	Glutamate dehydrogenase	115588	35	2	0	2
	LmjF31.1210	Hypothetical protein, conserved	552349	34	1	0	5
	LmjF30.2830	Hypothetical protein, conserved	92082	34	0	0	1
	LmjF23.1540	Hypothetical protein, conserved	106312	34	3	0	7
	LmjF25.1350	Hypothetical protein, conserved	280794	32	1	0	3
	LmjF32.3150	Hypothetical protein, conserved	273868	30	1	0	3

Band 7

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF25.2420	Hypothetical protein, conserved	30623	40	5	0	3
	LmjF30.1760	Hypothetical protein, conserved	210216	31	3	1	5

	LmjF23.0220	Multidrug resistance protein, putative	175138	30	1	6	3
ΔGT	LmjF28.1200	glucose-regulated protein 78, putative	72067	283	28	1	19
	LmjF16.1425	Paraflagellar rod protein 2C	69257	230	30	0	17
	LmjF30.2470	Heat shock 70-related protein 1, mitochondrial precursor, putative	72346	195	23	0	15
	LmjF08.1230	Beta tubulin	50302	175	27	0	13
	LmjF30.2460	Heat shock 70-related protein 1, mitochondrial precursor, putative	69532	143	19	0	14
	LmjF29.1750	Paraflagellar rod protein 1D	69390	121	25	0	16
	LmjF15.1010	Glutamate dehydrogenase	115588	92	10	0	10
	LmjF28.2770	Heat-shock protein hsp70, putative	71893	78	15	0	9
	LmjF36.2590	Membrane bound acid phosphatase 2, putative	63722	77	6	1	4
	LmjF13.0280	Alpha tubulin	50526	77	20	0	8
	LmjF31.1220	Vacuolar-type protein translocating pyrophosphatase 1, putative	84195	47	8	15	6
	LmjF33.0312	HSP83, heat shock protein 8.1, putative	55252	39	1	0	1
	LmjF24.2060	Tranketolase, putative	72363	33	4	0	2
	LmjF30.1160	Hypothetical protein, conserved	177511	30	2	0	5
	LmjF22.1110	Dynein heavy chain, cytosolic, putative	624243	30	1	0	11
	LmjF23.1540	Hypothetical protein, conserved	106312	29	2	0	4

Band 8

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF33.0792	Beta tubulin	50335	565	53	0	33
	LmjF13.0280	Alpha tubulin	50526	551	51	0	27
	LmjF21.1860	Beta tubulin	50395	503	47	0	32
	LmjF25.1180	ATPase beta subunit, putative	56513	147	24	0	10
	LmjF30.2470	Heat shock 70-related protein 1, mitochondrial precursor, putative	72346	109	15	0	10
	LmjF17.0080	Elongation factor 1-alpha	49485	71	6	0	3
	LmjF29.1750	Paraflagellar rod protein 1D, putative	69390	48	5	0	4
	LmjF25.1120	ALDH2, aldehyde dehydrogenase, mitochondrial precursor	54712	43	3	0	2
	LmjF29.0530	Hypothetical protein,conserved	315663	40	1	0	5
	LmjF16.1425	Paraflagellar rod protein 2C	69257	38	10	0	6
	LmjF35.2320	Protein kinase, putative	123069	35	6	0	5
	LmjF13.1210	Nucleobase/nucleoside transporter 8.1, putative	55252	35	1	11	1
	LmjF24.2110	Hypothetical protein	55619	34	2	0	1
	LmjF28.1710	Hypothetical protein	230686	33	3	0	6
	LmjF10.0460	GP63-1, GP63, leishmolysin	65039	33	2	1	2

	LmjF34.0820	eEF1B beta 1, elongation factor 1-beta	25630	33	10	0	2
	LmjF25.2420	Hypothetical protein	30623	31	6	0	9
	LmjF23.1540	Hypothetical protein, conserved	106312	31	2	0	3
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	30	4	10	2
ΔGT	LmjF08.1230	Beta tubulin	50302	280	41	0	19
	LmjF25.1170	ATPase beta subunit, putative	56541	211	31	0	12
	LmjF13.0280	Alpha tubulin	50526	163	37	0	13
	LmjF36.2030	Chaperonin Hsp60, mitochondrial precursor	59623	112	18	0	8
	LmjF36.2020	Chaperonin Hsp60, mitochondrial precursor	60484	112	12	0	5
	LmjF27.1805	Glycosomal phosphoenolpyruvate carboxykinase,putative	58670	96	13	0	8
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	55	6	10	3
	LmjF03.0200	delta1-pyrroline-5-carboxylate dehydrogenase, putative	62658	52	7	0	5
	LmjF10.0470	GP63-1, GP63, leishmolysin	70545	40	2	2	2
	LmjF27.1880	Hypothetical protein, conserved	57214	36	1	0	1
	LmjF24.0770	Malic enzyme, putative	64003	34	1	0	1
	LmjF16.1425	Paraflagellar rod protein 2C	69257	34	14	0	9
	LmjF25.1130	Cytochrome C oxidase VII, putative	19058	33	8	1	1
	LmjF31.1220	Vacuolar-type proton translocating pyrophasphatase 1, putative	84195	32	5	15	4
	LmjF18.1510	H1A, P-type H+-ATPase, putative	108036	29	1	8	1

Band 9

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF05.0500	ATPase alpha subunit	62794	241	28	0	14
	LmjF08.1230	Beta tubulin	50302	234	30	0	16
	LmjF21.0240	Hexokinase, putative	52200	112	28	0	11
	LmjF36.2950	Succinyl-CoA ligase [GDP-forming] beta chain, putative	44616	90	22	0	7
	LmjF13.1210	Nucleobase/nucleoside transporter 8.1, putative	55252	69	1	11	1
	LmjF10.0290	Isocitrate dehydrogenase [NADP], mitochondrial precursor, putative	48802	64	22	0	8
	LmjF36.6940	PDI-2, protein disulfide isomerase	52799	59	14	0	6
	LmjF21.1555	Hypothetical protein, conserved	46704	55	16	1	6
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	52	4	10	4

	LmjF28.2770	Heat-shock protein hsp70, putative	71893	44	5	0	4
	LmjF31.1640	Thiolase protein-like protein	47260	41	5	0	2
	LmjF13.0280	Alpha tubulin	50526	37	11	0	5
	LmjF19.1160	Hypothetical protein, conserved	41275	35	2	0	1
	LmjF23.1540	Hypothetical protein, conserved	106312	33	6	0	6
	LmjF28.2385	DNA replication licensing factor, putative	97755	33	4	0	4
	LmjF12.0400	3'-nucleoside/nuclease, putative	41184	33	12	2	4
	LmjF35.4130	Poly(a) binding protein, putative	65536	31	2	0	2
	LmjF35.1620	Hypothetical protein, conserved	214361	30	1	0	5
ΔGT	LmjF08.1230	Beta tubulin	50302	297	39	0	17
	LmjF16.0550	Orotidine-5-phosphate decarboxylase, orotate phosphoribosyltransferase, putative	49811	158	24	0	11
	LmjF13.0280	Alpha tubulin	50526	130	27	0	10
	LmjF32.3130	Ribosomal protein 13, putative	47743	72	20	0	8
	LmjF17.0080	Elongation factor 1-alpha	49485	69	14	0	5
	LmjF28.0930	Hypothetical protein, conserved	45661	53	13	1	6
	LmjF30.2470	Heat shock 70-related protein 1, mitochondrial precursor, putative	72346	46	10	0	6
	LmjF10.0460	GP63-1,GP63, leishmolysin	65039	45	2	1	2
	LmjF28.2420	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyl transferase, putative	41934	38	6	0	3
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	37	3	10	2
	LmjF36.6940	PDI-2, protein disulfide isomerase	52799	35	12	0	5
	LmjF15.1230	Nucleoside transporter 1, putative	72867	30	2	10	2

Band 10

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild type	LmjF08.1230	Beta tubulin	50302	398	37	0	27
	LmjF21.1860	Beta tubulin	50395	383	37	0	27
	LmjF13.0280	Alpha tubulin	50526	338	41	0	16
	LmjF08.1100	Hypothetical protein	42247	162	28	0	14
	LmjF30.2970	Glyceraldehyde 3-phosphate dehydrogenase, glycosomal	39370	65	28	0	11
	LmjF36.2380	Sterol 24-c-methyltransferase, putative	40484	61	3	0	1
	LmjF17.0080	Elongation factor 1-alpha	49485	59	4	0	2
	LmjF36.2590	Membrane-bound acid phosphatase 2, putative	63722	46	3	2	2

	LmjF11.1050	Pretranslocation protein, alpha subunit, putative	54275	42	8	9	5
	LmjF25.1180	ATPase beta subunit, putative	56513	42	17	0	8
	LmjF27.1805	Glycosomal phosphoenolpyruvate carboxykinase, putative	58670	35	3	0	2
	LmjF23.1680	Hypothetical protein, conserved	176704	34	2	0	4
	LmjF34.0820	eEF1B beta 1, elongation factor 1-beta	25630	34	21	0	4
	LmjF21.0825	Hypothetical protein, conserved	349096	34	1	0	6
	LmjF10.0010	Fatty acid desaturase, putative	45438	33	4	6	2
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	33	2	10	1
	LmjF21.0780	Hypothetical protein, conserved	86910	32	3	0	2
	LmjF23.1540	Hypothetical protein, conserved	106312	32	1	0	2
	LmjF15.1010	Glutamate dehydrogenase	115588	32	0	0	1
	LmjF33.3270	Fatty acid desaturase, putative	45949	31	8	5	4
	LmjF27.0420	Ribokinase, putative	35719	31	9	0	3
	LmjF16.0550	Orotidine-5-phosphate decarboxylase/orotate phosphoribosyltransferase, putative	49811	30	3	0	2
	LmjF35.1620	Hypothetical protein, conserved	214361	29	1	0	5
ΔGT	LmjF08.1230	Beta tubulin	50302	243	29	0	16
	LmjF30.2970	Glyceraldehyde 3-phosphate dehydrogenase, glycosomal	39370	220	32	0	1
	LmjF13.0280	Alpha tubulin	50526	194	33	0	11
	LmjF05.0500	ATPase alpha subunit	62794	137	17	0	8
	LmjF29.1070	Ribosomal protein L1a, putative	41109	126	20	0	7
	LmjF08.1100	Hypothetical protein, conserved	42247	64	11	0	5
	LmjF13.1210	Nucleobase/nucleoside transporter 8.1, putative	55252	63	1	11	1
	LmjF36.1260	ALD, fructose-1,6-bisphosphate aldolase	41201	62	17	0	8
	LmjF19.1160	Hypothetical protein, conserved	41275	54	4	0	2
	LmjF27.0930	Isovaleryl-coA dehydrogenase, putative	44973	54	8	0	3
	LmjF17.0080	Elongation factor 1-alpha	49485	49	8	0	4
	LmjF36.1940	NT2, nucleoside transporter 1, putative	54305	48	3	10	2
	LmjF12.0670	Cytochrome C oxidase subunit IV	39581	48	15	0	6
	LmjF06.0350	NAD(p)-dependant steroid dehydrogenase-like protein	43231	43	3	1	2
	LmjF14.0990	ADP/ATP mitochondrial carrier-like protein	40715	34	6	0	3
	LmjF36.2030	Chaperonin Hsp60, mitochondrial precursor	59623	34	9	0	4

	LmjF30.2460	Heat shock 70-related protein 1, mitochondrial precursor, putative	69532	33	5	0	4
	LmjF21.1555	Hypothetical protein, conserved	46704	33	4	1	2
	LmjF23.1540	Hypothetical protein, conserved	106312	31	2	0	5
	LmjF31.0760	Hypothetical protein, unknown function	33719	30	7	0	2
	LmjF18.1380	Pyruvate dehydrogenase E1 component alpha subunit, putative	43359	29	8	0	3

3.3.7 IEF Fractionation

Solution-phase isoelectric focusing (IEF) was tested as a means to further fractionate membrane protein samples, to generate a less complex protein mixture for mass spectrometry analysis. The membrane protein extraction protocol alone generated a mixture of proteins that was too complex to resolve fully by SDS-PAGE. Direct MS analysis of such SDS-PAGE fraction led to the identification of large numbers of non-integral membrane proteins, particularly tubulins, hence limiting the identification of low abundant membrane proteins, IEF was exploited to decrease the unrelated membrane proteins in the samples.

Unlike the standard protocol where the whole lane will be cut into 30-35 equal-sized bands, here we only chose bands that show an apparent discrepancy in their expression on the gel. After the salt-extraction, the protein samples were subjected to IEF fractionation using an AGILENT 3100 Off-Gel fractionator. In the machine, after the samples were prepared according to the manufacturer manual, each sample was loaded into 24 well in the AGILENT 3100's tray with each well receiving the same volume of sample. The fractionation started as the current was applied (first dimension fractionation). At the end of the fractionation, samples were loaded onto a SDS gel for second dimension fractionation of the protein. 12 bands from each sample (Figure 3-12) were excised and subjected to trypsin digest and mass spectrometry analysis. The results from mass spectrometry are summarised in Table 3-3.

The samples fractionated on the SDS gel showed a better resolution compared to samples that were not fractionated by IEF (Figure 3-12). However, subsequent MS analysis showed only non-membrane related proteins and tubulins were found to be more abundant compared to the previous membrane proteomic result. Most of the proteins found are beta and alpha tubulins and hypothetical proteins with no TMD (transmembrane domain). Other proteins found were kinesin (only in ΔLmGT), heat shock protein (hsp70) and ATP synthase epsilon chain.

3.3.7.1 IEF Fractionation without DiGE labelling

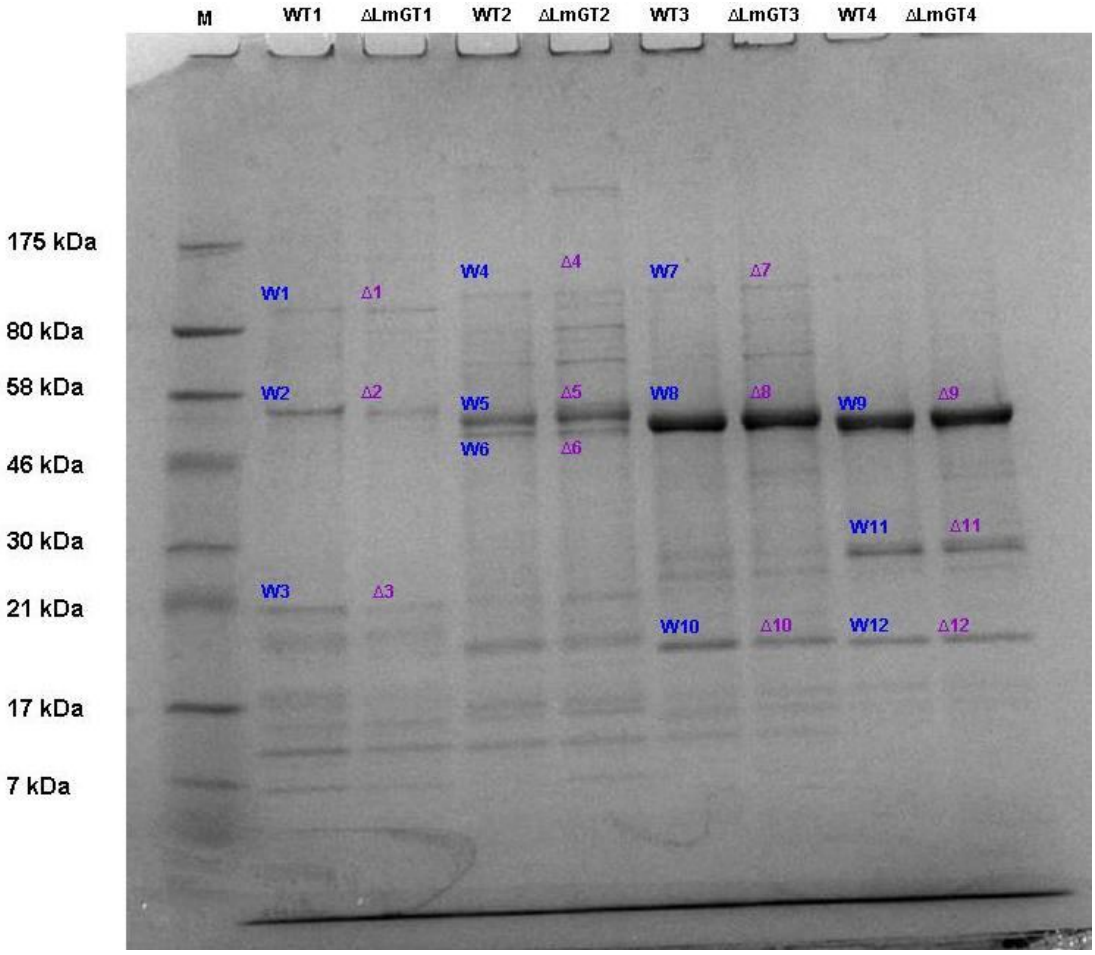

Figure 3-12: 1-SDS gel stained with Coomasie blue, loaded with membrane protein samples of WT (wild type) and ΔLmGT (glucose transporter null mutant) from salt extraction after IEF fractionation. Lanes WT1-WT4 loaded with samples from tray number 1-4 respectively which fractionates WT samples. Lane ΔLmGT 1-ΔLmGT 4 loaded with samples from tray number 1-4 respectively which fractionates samples ΔLmGT. The blue coloured marks (W1-W12) indicate bands excised from WT and purple coloured marks (Δ1-Δ12) indicate bands excised from ΔLmGT. These bands that are labelled with coloured marks were chosen to be excised and further analysed as their expression on the gel were different. Hence the protein levels between bands from 2 adjacent lanes could be different from each other.

3.3.7.2 Membrane proteomic comparison from IEF fractionation

Table 3-3: Membrane proteomic result from membrane fractions fractionated with isoelectric (IEF) focusing and 1-SDS gel.

Band 1

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF10.1040	Hypothetical protein, conserved	125995	44	1	0	2
	LmjF31.2655	Hypothetical protein, unknown function	60979	30	1	0	2
ΔLmGT	**No significant hits to report**						

Band 2

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF08.1230	Beta tubulin	50302	32	11	n/a	5
	LmjF03.0720	Hypothetical protein, conserved	94247	31	1	0	1
ΔLmGT	**No significant hits to report**						

Band 3

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF33.0792	Beta tubulin	50335	58	26	0	7
ΔLmGT	LmjF08.1230	Beta tubulin	50302	86	11	0	5

Band 4

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	**No significant hits to report**						
ΔLmGT	LmjF19.0580	Hypothetical protein, conserved	295765	31	0	0	2

Band 5

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF28.2770	Heat shock protein hsp70, putative	71893	32	5	0	4
ΔLmGT	LmjF28.2770	Heat shock protein, hsp 70, putative	71893	83	23	0	14

Band 6

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF08.1230	Beta tubulin	50302	275	34	0	19
	LmjF21.1860	Beta tubulin	50395	257	34	0	19
	LmjF03.0280	Hypothetical protein	103798	30	3	0	2

ΔLmGT	LmjF08.1230	Beta tubulin	50302	532	48	0	29
	LmjF21.1860	Beta tubulin	50395	507	48	0	29

Band 7

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF08.1230	Beta tubulin	50302	80	17	0	7
	LmjF29.0380	Hypothetical protein, conserved	104683	36	2	0	2
ΔLmGT	LmjF28.2770	Heat shock protein, hsp 70, putative	71893	85	21	0	11
	LmjF08.1230	Beta tubulin	50302	69	11	0	4
	LmjF23.1280	Hypothetical protein, conserved	125443	33	0	0	1
	LmjF19.0680	Kinesin, putative	119533	32	2	0	3

Band 8

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF33.0792	Beta tubulin	50335	569	63	0	35
	LmjF21.1860	Beta tubulin	50395	489	57	0	34
	LmjF13.0280	Alpha tubulin	50526	34	24	0	8
ΔLmGT	LmjF33.0792	Beta tubulin	50335	535	62	0	32
	LmjF13.0280	Alpha tubulin	50526	155	30	0	13

Band 9

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF13.0280	Alpha tubulin	50526	337	62	0	27
	LmjF33.0792	Beta tubulin	50335	267	47	0	19
	LmjF21.1860	Beta tubulin	50395	216	41	0	18
ΔLmGT	LmjF33.0792	Beta tubulin	50335	408	57	0	25
	LmjF13.0280	Alpha tubulin	50526	274	69	0	25
	LmjF06.0810	Hypothetical protein, unknown function	348424	33	0	0	1

Band 10

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF13.0280	Alpha tubulin	50526	201	36	0	17
	LmjF08.1230	Beta tubulin	50302	124	17	0	9
	LmjF30.3600	ATP Synthase, episilon chain, putative	20429	33	6	0	1
ΔLmGT	LmjF13.0280	Alpha tubulin	50526	162	39	0	18
	LmjF33.0792	Beta tubulin	50335	148	27	0	13
	LmjF34.3690	Hypothetical protein, conserved	143897	33	4	0	4
	LmjF30.3600	ATP synthase, epsilon	20429	32	6	0	1

		chain, putative					
	LmjF31.1470	Hypothetical protein, unknown function	110948	30	1	0	1
	LmjF13.0520	Hypothetical protein, unknown function	31830	30	5	0	2
	LmjF23.1280	Hypothetical protein, conserved	125443	30	0	0	1

Band 11

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF08.1230	Beta tubulin	50302	262	25	0	12
	LmjF21.1860	Beta tubulin	50395	232	25	0	12
∆LmGT	LmjF33.0792	Beta tubulin	50335	187	32	0	12

Band 12

	Accession Number	Gene Annotation	MWt	Protein Score	Sequence Coverage (%)	Number of predicted TMD	Queries Matched
Wild Type	LmjF08.1230	Beta tubulin	50302	107	16	0	8
	LmjF13.0280	Alpha tubulin	50526	63	21	0	8
∆LmGT	LmjF08.1230	Beta tubulin	50302	137	13	0	8

3.3.7.3 DiGE

We incorporated a DiGE protocol in an attempt to quantitate the proteome of the membrane (For method, refer to '3.2.9 Labelling and separation of membrane proteins'). First, membrane preparation samples from the wild type and glucose transporter null mutant were tagged with Cy3 and Cy5 respectively and mixed prior to the IEF fractionation according to the manufacturer instruction. Subsequently, the mixture was subjected to isoelectric focusing fractionation (AGILENT 3100) according to the manufacturer's protocol in the manual. Following the IEF fractionation, all samples in all wells were loaded onto 1-SDS for a second dimension fractionation. These gels (Figure 3-13), were then visualised immediately using 'Image Quant 5.2', and stained with Coomassie blue. We decided, based on what we have seen under the 'Image Quant 5.2', to choose lane number 6 to cut into 30 equal-sized bands for the further mass spectrometry analysis. The fluorescent image was meant to help tp detect the level of proteins different between WT and ΔLmGT.

Due to time constraint, the bands were not analysed further in mass spectrometry.

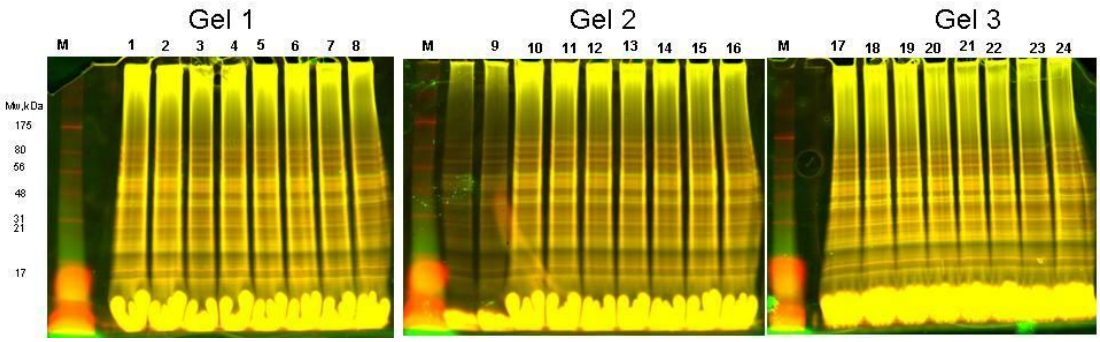

Figure 3-13: Membrane preparation samples from salt-extraction in SDS gel after IEF and SDS page fractionation. The membrane extraction in the IEF compatible solution were labelled with Cydyes (Cy5 (WT) and Cy3 (ΔLmGT)) and both samples were mixed together prior to fractionation by IEF 24-well tray in AGILENT 3100. After the IEF fractionation was completed, each individual well containing the sample was fractionated again in 1-SDS gel. The gels were viewed under 'Typhoon 9400 Variable Mode Imager' software as soon as the electrophoresis wascompleted. Numbers on top of each gel indicate the number of the well in tray that each of samples came from after the fractionation. Three gels were needed as 24 samples were retrieved in the Agilent 3100. The imager uses 2 filters with different UV wavelengths i.e. filter with 532nm UV's wavelength fluoresces green colour on null mutant and filter with 633nm UV's wavelength fluoresces red colour on the wild type. The red colour on the gel indicates the upregulation of the protein in the wild type and green colour indicates the upregulation of the protein in null mutant. The yellow colour indicates the proteins that have similar amounts in both cells. Due to time constraint, the bands were not analysed further in mass spectrometry.

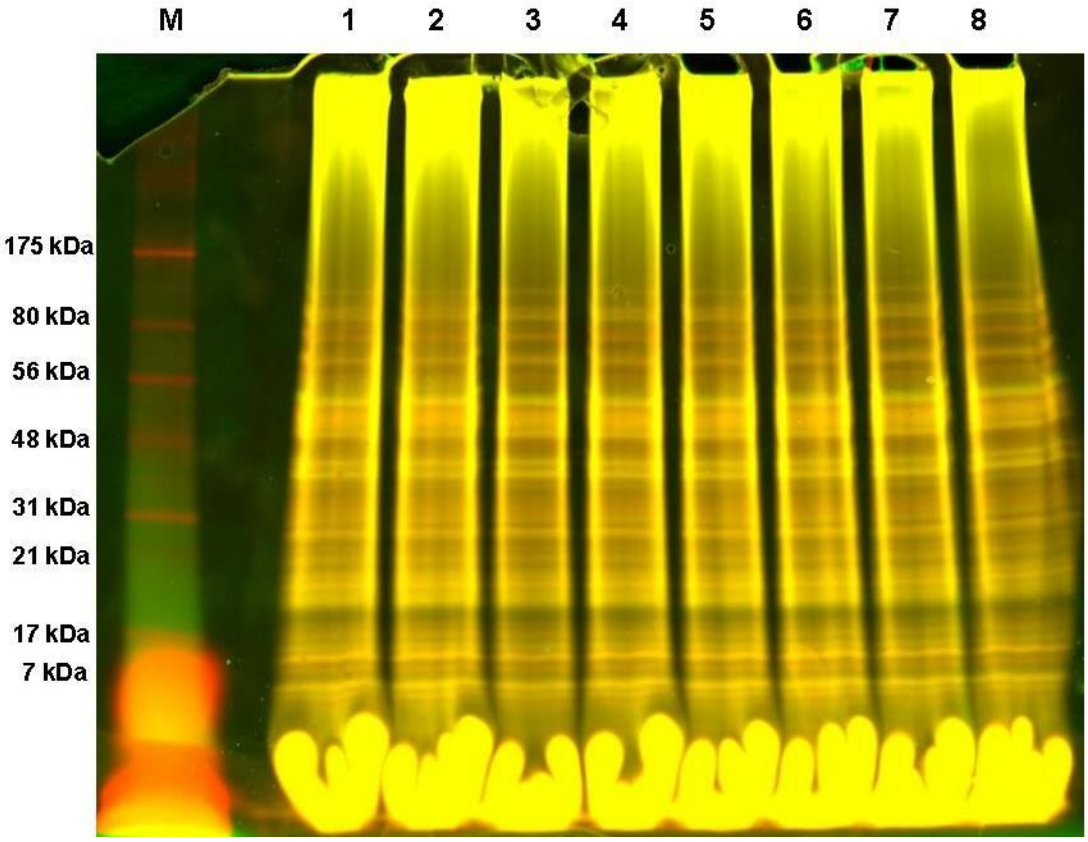

Figure 3-14: The same gel 1 (loaded with membrane protein) from Figure 3-13.Unfortunately, the differences in the bands expression couldn't be detected. This could be due to cell loss or protein loss during the experiment. This method should be improvised first before any conclusion can be made regarding its performance and efficiency in membrane protein analysis. However, due to time constraints, the bands were not analysed further in mass spectrometry.

3.4 Discussion

In this chapter, the goal is to analyse the efficiency of the enrichment, fractionation and identification protocol for integral membrane proteins of *Leishmania mexicana*. The efficiency of the techniques used is reflected in how many integral membrane proteins were identified after the MS analysis.

The plasma membrane is a phospholipid bilayer with fatty acids facing inwards and hydrophilic polar heads located on the cell's external and the cytosolic environment. Within the bilayer are a number of significantly hydrophobic proteins with different properties and functions. These proteins include (Cordwell & Thingholm 2010):

1) Integral membrane proteins, spanning the bilayer structure with 1 or more hydrophobic transmembrane domains (TMD),

2) Integral membrane proteins (IMPs), that are clustered to form a channel or pore to transport nutrients and/or for the elimination of toxic substances

3) Integral membrane proteins that are embedded in the inner or outer surface of the bilayer but do not span the entire bilayer structure.

4) Proteins that are located peripherally to the lipid bilyayer, and are indirectly associated with the bilayer through protein:protein interaction, as opposed to protein:lipid interaction.

As the protein mixtures recovered by subcellular fractionation can represent, at best, enriched populations of these proteins, MS analysis of these fractions will not only result in the identification of proteins that are associated with the membrane under physiological conditions, but also of proteins that have become artifactually associated with membrane fractions during subcellular fractionation. The later proteins are contaminants, but it is not possible to distinguish them from *bona fide* peripheral membrane proteins, without the application of targeted approaches such as subcellular localisation by immunofluorescene analysis.

Transmembrane spanning integral membrane proteins (class 1 and 2 above) are characterised by the possession of multiple hydrophobic transmembrane domains, which can be revealed bioinformatically by hydropathy analysis (http://tritrypdb.org/tritrypdb/). This approach can be used to predict proteins that have a likely integral membrane localisation.

To monitor the efficiency of the extraction protocols, a myc-tagged glucose transporter (myc-GT2) was chosen to be the marker for plasma membrane recovery. The observation that the myc-tagged glucose transporter was able to mediate robust glucose uptake confirms that the tagged protein is expressed in a functional conformation in the plasma membrane.

Two techniques of membrane fractionation were applied i.e. salt-extraction and Triton X-114. The salt extraction protocol was used by Piper et al (Piper et al. 1995) to determine the subcellular location of Pro-1 and Pro-2 glucose transporters in *Leishmania enrietti*. They used epitope tagging to differentiate and identify the location of the 2 individual glucose transporter isoforms. In the current experiment, an epitope tag named myc was used to monitor the membrane fractionation recovery by screening myc expression in western blot. The salt extraction protocol exploits alkaline sodium carbonate to disrupt the electrostatic interactions between proteins, liberating hydrophilic proteins that are tethered to the membrane by protein:protein interactions, but retaining proteins that are associated with the membrane by solubility in the lipid bilayer. By removing the membrane associated proteins, it was hoped to reduce interference from peripheral (non-integral) membrane proteins(Speers & Wu 2007).

Figure 3-6, Figure 3-7 and Figure 3-8 show the western blot screening for myc expression in all 3 fractions i.e. the whole cell lysate, supernatant and membrane extraction that came from wild type, glucose transporter null mutant and myc-tagged glucose transporter cells lines. All 3 fractions were collected from the same sample preparation i.e. the whole cell lysate, supernatant and membrane extractions were prepared from the same preparation. Figure 3-6, is a western blot of a whole cell lysates from wild type, glucose transporter null mutant and myc-tagged glucose transporter cell lines using myc antibody and anti-mouse secondary antibody. The western blot (Figure 3-6) shows a band

expressed in a lane containing the myc-tagged glucose transporter cell line. As expected, no expression was seen in the supernatant fraction (Figure 3-7) from all three cell lines tested. In western blot loaded with membrane extraction (Figure 3-8), a strong expression was seen in the lane loaded with lower cell amounts of myc-tagged- glucose transporter compared to the lane that was loaded with same cell amount but with higher cell counts. This could be explained by the fact that with more cells loaded in a lane, the faster the substrate was consumed by the peroxidase.

However, the molecular weights of the bands expressed in the myc-tagged glucose transporter lanes from whole cell lysate (Figure 3-6) and membrane preparation (Figure 3-8) are different. The bands may represent the same molecule e.g. myc-tagged glucose transporter, but due to stringent extraction technique, the molecule may have undergone some changes i.e. phosphorylation. Nevertheless, this can only be confirmed if the gels were originally where the blots were pressed against, are analysed further by cutting the gel area where the the bands were located and sent for mass spectrometry analysis.

Triton X-114 is reported to preferentially solubilise hydrophobic integral membrane proteins (Bordier 1981;Maclay 1956;Tanford & Reynolds 1976). Expression of the myc-tagged glucose transporter was screened in aqueous and detergent phase fractions from Triton X-114 extracted *Leishmania*. As in integral membrane protein is highly hydrophobic the myc –tagged glucose transporter is expected to be found only in the detergent phase. Anti-myc antisera reacted only with material in the lane loaded with myc-tagged-glucose transporter, but no clear band was visible. One possible explanation for this could be the formation of mixed micelles of SDS and Triton in the gel, resulting in poor separation in SDS-PAGE (Piper et al. 1995). It was noticeable that electrophoresis proceeded much more slowly than normal with these samples, suggesting that the presence of Triton X-114 may interfere with SDS-PAGE. Bands of the same molecular weight were also revealed in aqueous phase, suggesting that Triton X-114 extraction is of limited efficiency for the isolation of integral membrane proteins such LmGT2.

Although both extraction techniques showed some potential for the enrichment of integral membrane proteins, salt-extraction of membrane-enriched subcellular fractions appeared to be more reliable than detergent extraction. However further investigation need to be done to explain on expression of bands on the myc-tagged glucose transporter lane from both whole cell lysate and membrane fraction which have different molecular weightsb. Furthermore, the interference produced by the presence of Triton in detergent extracted samples hampered subsequent gel-based separation. In this light, only salt-extracted membrane fractions were subjected to membrane proteomic analysis.

Proteomic Analysis: This study was focused on the identification of membrane proteins from *Leishmania*. Membrane proteins are of relatively low abundance and are detected rather infrequently in whole organism proteomic studies. Nevertheless, to establish a baseline for the detection of membrane proteins by proteomic approaches, a preliminary proteomic analysis was performed on unfractionated *Leishmania* lysates. Several integral membrane proteins were identified in this whole cell proteome. Some IMPs were exclusively identified in wild type and some were exclusively found in the null mutant. The only protein found in both whole cell proteome was glucose regulated protein 78 (LmjF28.1200). In David Henderson's (Henderson, D., MRes Project, University of Glasgow 2007)proteomic analysis, paraflagellar rod protein 2C (LmjF16.1425) was found to be upregulated in glucose transporter null mutant *Leishmania*. This protein was found in both cell lines but, as it was not a quantitative proteomic analysis, the results can only reveal the presence of the proteins and it is not possible with the current data to make any inference as to the relative abundance of the paraflagellar rod protein, nor any other proteins.Three techniques were used to facilitate membrane proteomic analysis:

1) Samples where directly sent from SDS gel to trypsin digestion prior to MS analysis

2) Samples were separated by IEF separation prior to SDS-PAGE, followed by trypsin digestion

3) Samples where Cy-Dye labelled, fractionated by IEF followed by SDS- page prior to MS analysis.

Due to constraints on mass spectrometry access, only samples generated from protocols 1 and 2 were analysed by MS. The results from protocol 3 are nevertheless of interest because they represent an innovative approach to relative quantitation of membrane subproteomes.

Separation of enriched membrane proteins by SDS-PAGE, followed by tryptic digest of apparent molecular weight fractions, led to the identification of a greater number of putative IMPs than were observed in a similar analysis of whole cell lysate. This suggests that the subcellular fractionation and salt-extraction method does enrich membrane proteins from the *Leishmania mexicana* promastigotes.

Fujiki et al (Fujiki et al. 1982) used 100mM $Na_2 CO_3$ treatment for isolation of intracellular membranes and obtained membranes that, by several criteria, were intact and relatively pure :

1) They preserved integral membrane proteins (cytochrome b5 , cytochrome P-450, ribophorins I and II)

2) They maintained the active of several integral membrane enzymes (NADPH-cytochrome c reductase and NADH-ferricyanide reductase).

In the current experiment, membrane enrichment followed by salt extraction did lead to the identification of quite a number of IMPs (Sauvage et al. 2009).

However, the complexity of the sub-proteomic fractions that were analysed was still very significant and the stained gel lanes revealed many hundred protein bands that were poorly resolved in this 1 dimensional separation. It was not possible to see any clear differences in banding pattern between wild type and glucose transporter null mutant lines.

Although it is tempting to suggest that nucleobase and nucleoside transporter might be upregulated in glucose transporter null *Leishmania*, perhaps reflecting a change in nutrient acquisition, these proteomic data are not quantitative and comparisons of protein identifications from these two parallel analyses are not justified.

To address the issue of relative quantitation, we wished to enhance the resolution of our proteomic separation by adding a second dimension. We chose to employ solution-phase isoelectric focusing, as an alternative to gel-based IEF that might be better suited to separation of hydrophobic membrane proteins. The second membrane proteomic analysis protocol thus employed in-solution IEF fractionation prior to SDS-PAGE. This approach gave rise to a number of fractions that, when resolved on SDS-PAGE as a second dimension separation, did indeed appear to be less complex than samples separated by SDS-PAGE in a single dimension. Furthermore, a number of significant differences were visible between adjacent, comparable lanes loaded with samples from wild type and glucose transporter null mutant fractions. Several of these gel regions were excised for analysis, but most of the identified proteins were tubulins. Tubulins are amongst the most abundant proteins in *Leishmania*, and are indeed associated with membrane as they are the major constituent of the cytoskeleton, which lies beneath, and in intimate contact with, the plasma membrane. The presence of tubulin would likely hamper the identification of low abundance IMPs, and no IMPs were identified by this approach. It is not clear why tubulin was present in all of the samples analysed, but one possibility is that tubulins did not separate well by solution-phase IEF and were thus present in every sample that was loaded on SDS-PAGE. Alternatively, it is possible that the salt extraction procedure was inefficient during the fractionation of these particular samples, giving rise to fractions that were unusually heavily contaminated with tubulin.

Some significant differences were expected to be detected between adjacent lanes which compared lanes loaded with samples from wild type and glucose transporter null mutant fractions (Figure 3-12). However none significant differences were seen. Samples were prepared by the same same salt-extrcation protocol but samples extracted were labelled with spectrally resolvable CyDyes prior to 2 dimensional separation by solution-phase IEF followed by SDS-PAGE.

The Cy Dyes used are mass and charge matched, so that identical proteins in each sample will migrate together but are independently detectable by fluorescent scanning at specific wavelengths. This protocol represents a novel adaptation of the Difference Gel Electrophoresis (DiGE) protocol, for which these CyDye tags were developed. Labelled samples were mixed and separated by IEF and SDS-PAGE and the resulting gel was scanned at 2 discrete wavelengths, to generate images that could be overlaid. This approach revealed several protein bands that showed differential expression between wild type and glucose transporter-null mutant samples. The enhanced resolution of the 2 dimensional separation should enable excision and MS analysis of discrete protein bands, but it was not possible to complete this during the present project.

Isolation, separation, identification and quantitation of integral membrane proteins are all challenges in proteomics. The data presented in Chapter 2 clearly demonstrate functional differences between wild type and glucose transporter null mutant *Leishmania*. These differences, such as changes in amino acid transport activity, would likely be apparent at the proteomic level, if appropriate tools were available to look. Furthermore, there may be other changes in membrane protein expression that are not revealed by targeted analyses of the type reported in Chapter 2, and that could only be revealed by comparative membrane proteomic analysis. These results presented here underscore the challenges involved, but some progress has been made with each of these steps and the data may contribute to future efforts to investigate changes in membrane protein expression by proteomic approaches.

3.5 Conclusion

Despite the challenges to the study of integral membrane proteins, the results reported in this chapter show that, by improving the enrichment and fractionation strategy, comparison of membrane protein expression between wild type and glucose transporter null mutant is possible. Although there are many issues that still need to be addressed, by integrating methods that have high sensitivity for detecting low abundance and highly hydrophobic proteins, progress has been made towards comparative membrane proteomic analysis.

Chapter 4

Phenotypic Changes in Glucose Transporter null *Leishmania mexicana*

4.1 Introduction

Leishmania mexicana have three genes that encode putative glucose transporters. These genes, named *LmGT1*, *LmGT2* and *LmGT3* are present at the same genomic locus and are very similar to each other, and to functionally characterised glucose transporters in other trypanosomatid species. In previous studies, both heterologous and homologous expression has confirmed that the LmGT genes encode *bona fide* glucose transporters. The individual isoforms display unique expression patterns, substrate specificity and kinetic properties (Burchmore et al. 2003;Burchmore & Landfear 1998). Genetic ablation of the complete LmGT locus gives rise to mutant parasites that grow well when cultured in vitro in a complex media but which are unable to transport glucose. Several studies have shown that glucose transporter null mutant (ΔLmGT) *Leishmania* promastigotes have phenotypic differences compared to the wild type (WT) promastigotes. Some of the changes reported are that they are smaller in volume, unable to replicate as amastigotes thus explaining the non-viability of this mutant inside macrophage (Burchmore et al. 2003;Rodriguez-Contreras et al. 2007).

Significant phenotypic changes are to be expected in cells that can no longer utilise exogenous glucose as an energy source, and we set out to undertake a comparative proteomic characterisation of WT and ΔLmGT, to reveal some of the molecular changes behind observed phenotypic changes and to highlight changes that might give rise to more subtle phenotypic consequences. At the outset of this project, a comparative 2D gel-based analysis was performed by David Henderson, as part of a Masters project (Henderson, D., MRes Project, University of Glasgow 2007). This study highlighted several proteomic changes that I have investigated, using a variety of approaches, during my PhD studies. From David Henderson's study, he found that there are distinct changes in the proteome of *Leishmania mexicana* due to abnormality of genes in charge for the transport of glucose from the external medium. The

removal of genes *LmGT1*, *LmGT2* and *LmGT3* causing the differential regulation of proteins involved in glucose metabolism and carbohydrate formation, oxidative stress and cellular structure. He demonstrated that there as down-regulation of enzymes that were involved in carbohydrate metabolism i.e. mannose-1-phosphate guanylyltransferase and myo-inositol-1-phosphate synthase in the null mutant, down-regulation of tryparedoxin peroxidase in the null mutant which in return increased sensitivity of the null mutant toward oxidative stress and up-regulation of paraflagellar rod protein 2C (protein PFR 2C) in null mutant which is one of the structural in flagellar formation.

Thus, following observations from David Henderson's Masters Project in 2007, I was interested in investigating further on proteins such as tryparedoxin peroxidase (TP) and paraflagellar rod protein 2C (PFR). In this chapter we will investigate these differences by several methods.

4.1.1 Flagellar Structure in Leishmania mexicana promastigotes

One of the distinguishing organelles possessed by all of the trypanosomatid protozoa is the flagellum. The motility and sensory abilities are among the functions currently recognised for trypanosomatid's flagella. The surface membrane of *Leishmania* species and other kinetoplastid protozoa can be divided into 3 morphologically divergent subdomains i.e. the flagellar membrane, the flagellar pocket and the pellicular plasma membrane (Balber 1990) where each domain is known to embody a highly specialised membrane with characteristic functions with unique protein and, possibly, lipid composition(Landfear & Ignatushchenko 2001). It has been reported that the flagellum has increased sterol content and reduced integral protein content (Vickerman & Tetley 1990). One possible reason for increased sterol content in the flagellar membrane might be that it makes the flagellar membrane more rigid, therefore lowering the risks of distortion during movement. The reduced integral membrane protein might be possible because of the elimination of transport sites for molecules which are not essential for flagellar activity but only vital for bodily function (Vickerman & Tetley 1990).

The flagellum of *Leishmania* and of related organisms contains a structure called the paraflagellar rod (PFR; also known as paraxial rod). The PFR is a massive network of woven cytoskeletal filaments which resides along the axoneme in the flagella. The function of the axoneme is believed to control the beating in all eukaryotic flagella(Portman & Gull 2010). Two PFR structure is a unique feature that can be found in all kinetoplastids studied to date, with the exception of the amastigote, where the condensed flagellum does not arise from the flagellar pocket (Portman & Gull 2010). The PFR comprises 2 major subunits, PFR1 and PFR2, depending on the organism. PFR1 and PFR2 are relatively large proteins (mobility (M_r) can range from 70,000 to 80,000 for PFR1 and from 68,000 to 72,000 for PFR2). *PFR1* and *PFR2* genes are highly conserved in*T.brucei*, *T.cruzi* and *Leishmania* species, with over 80% amino acid identity (Maga & LeBowitz 1999) . PFR 1 and PFR2 assemble into a complex structure, and null mutants in *L. mexicana* of either one of the subunits or both of the subunits assemble abnormal PFR structures (Maga & LeBowitz 1999). It has been reported that protozoans lacking native PFR structure demonstrate a distinct reduction in cell motility Santrich et al. 1997, Maga & LeBowitz 1999). It also has been shown that *Leishmania mexicana* PFR null mutants swim 4-5 times slower than the wild type in aqueous media (Maga et al. 1999;Santrich et al. 1997). In the experiment conducted by Santrich et al, they described and characterised *L.mexicana* as lacking the *PFR-2* genes which was constructed by targeted gene replacement (Santrich et al. 1997). In their report, it reveals that PFR-2 null mutants have a residual inner substructure of PFR which contains PFR-1 protein. These null mutants however exhibit significant changes in the flagellar beat waveform and forward swimming velocity and reduced the internal elastic bending resistance. These results signify an important role of PFR and flagellum for *Leishmania*'s motility (Santrich et al. 1997).

No experiment has been done to look at specific changes in the expression of adjacent genes to GT locus. However, the proteins that were found to be differentially expressed by Feng (Feng et al. 2011) and David Henderson (Master Project) were not adjacent to LmGT locus. The microarray experiment in Feng (Feng et al. 2011) did not reveal modulation of adjacent genes. In fact, there was no significant change in any other genes on the relevant chromosome. Also,

through our observation, the phenotype doesn't seem to change whether or not antibiotic selection is maintained.

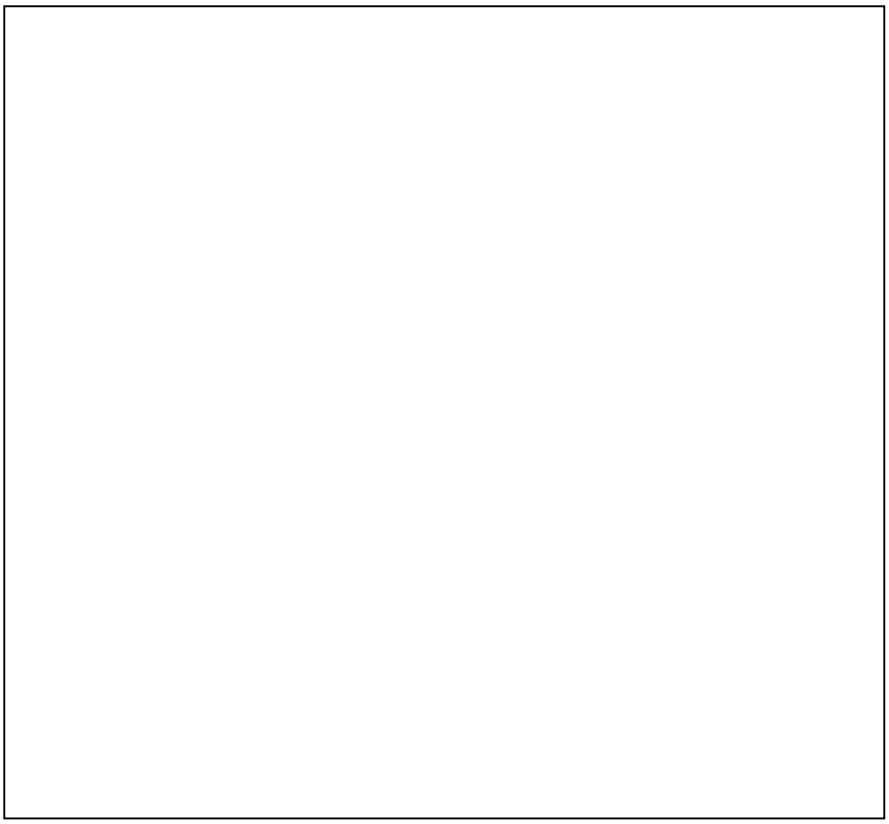

Figure 4-1: The images have been removed due to copyright restrictions.

4.1.2 Oxidative stress response in Leishmania mexicana

Tryparedoxin peroxidase is one component of the thioredoxin system, which is important for guarding the cells against oxidative challenge by reducing harmful peroxides such as H_2O_2. The down-regulation of this protein in ΔLmGT, suggested by observations in a previous comparative proteomic analysis (Henderson, D., MRes Project, University of Glasgow 2007), provides a possible explanation for increased susceptibility to oxidative stress.

As digenetic organisms, response to oxidative stress is an important factor in the survival of trypanosomatids such as *Leishmania* spp. Upon entry to the mammalian host, the protozoan encounters a great amount of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), which are produced by the host as a means to form a hostile living condition for intruding pathogenic microorganisms, hence facilitating their elimination from the host (Iyer et al. 2008). All living cells generate the intermediates of the partial reduction of oxygen, O_2 i.e. O_2^-. and hydrogen peroxide (H_2O_2) through side reactions of physiological electron transfer which is run by multienzyme redox systems (Chance et al. 1979). A cascade of enzymes in trypanosomatids that are involved in the defence mechanisms of toxic ROI and RNI generated by the host or vector has attracted the attention of researchers in finding a drug target in combating the disease. Trypanosomatids lack the enzymes catalase and selenium-dependent glutathione peroxidase which exist in higher eukaryotes. The enzymes that are involved in combating toxic peroxides in these protozoa are trypanothione reductase, tryparedoxin and tryparedoxin peroxidase and the mediator that supplies reducing equivalents is called trypanothione (N^1,N^8-bis(glutathionyl)-spermidine) (Fairlamb & Cerami 1992;Iyer et al. 2008;Jaeger & Flohe 2006;Nogoceke et al. 1997). It also has been reported that overexpression of tryparedoxin peroxidase is linked to the resistance to arsenite (Lin et al. 2005) and antimony (Wyllie et al. 2008).

Daneshvar et al have demonstrated that a *Leishmania infantum* attenuated by selection with the aminoglycoside antibiotic gentamicin (H-Line) was unable to survive within bone-marrow-derived macrophages of BALB/c mice while unselected *L.infantum* wild type survived and multiplied in the macrophages (Daneshvar et al. 2010). Proteomic studies indicated that several proteins

involved in thiol redox control were differentially regulated in *L.infantum* H-line (Daneshvar et al, 2011). Redox control is important for *Leishmania* as they are exposed to an oxidative burst when they encounter their host cell's macrophages (Goyal et al. 1996;Miller et al. 2000;Pearson et al. 1982;Wilson et al. 1994). The H-Line *L.infantum* has a different pattern in the expression of tryparedoxin peroxidise, peroxidoxin and tryparedoxin (Daneshvar et al. 2011). These enzymes are the main machinery of trypanothione-mediated hydroperoxide metabolism in *Leishmania*(Castro et al. 2008).

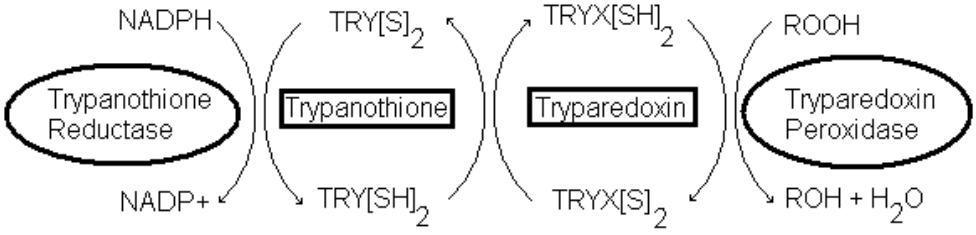

Figure 4-2: Cascade of Trypanothione peroxidase. (Picture by Dr Richard Burchmore, University of Glasgow)

In the light of the published observation that ΔLmGT promastigotes show increased sensitivity to oxidative stress (Rodriguez-Contreras et al. 2007), we have further explored the expression of tryparedoxin peroxidase in ΔLmGT.

4.1.3 Taxis Response in Leishmania mexicana

Leishmania promatigotes are highly motile organisms. Movement is facilitated by the flagellum, located at the anterior end of the protozoan. During development of promastigotes in the sand fly vector stage, transformation to the infectious metacyclic stage is accompanied by migration from the multiplication site in the midgut to the pharynx and proboscis (mouth area) of the sand fly vector, ready to be transmitted to the mammalian host vector (Bray 1983;Leslie et al. 2002). Motility may be important for this migration, which may also involve environmental sensing to enable chemotaxis. Motility may also be important in the initial stages of mammalian infection. A study by Bray (Bray

1983), found that promastigotes of *Leishmania* gradually move towards a concentration gradient of various sugars. Sugars have been postulated to play an important function as chemotaxins that attract the protozoan to move from the midgut to the oesophagus of the sand fly before being transmitted to the mammalian host as an infectious metacyclic form.

Oliveira et al (Oliveira et al. 2000) have tested the movement of promastigotes of *Leishmania chagasi* and *Leishmania amazonensis* towards concentrations of a variety of sugars i.e glucose, fructose, sucrose, raffinose, mannose, galactose, maltose, melibiose. The results produced the proof of sugar chemotaxis (Leslie et al. 2002;Oliveira et al. 2000).

Leslie et al performed a similar assay to that conducted by Oliveira et al. In this experiment, the assay measured the accumulation of promastigote cells that move from a buffered cell suspension into the open end of a capillary tube that contained a solidified agarose gel containing a putative chemoattractant, over a period of 1 hour. A concentration gradient was generated within and diffused from the open end of the capillary tube (Leslie et al. 2002;Oliveira et al. 2000). In the experiment, the promastigote's movement has been tested in two conditions i.e. in the presence and absence of solute gradients. It has been demonstrated that promastigotes were not passively carried up concentration gradients but moved actively up an osmotic gradient (Leslie et al. 2002).

When sand flies feed on plant material (usually sap or aphid nectar), ingested material is diverted to a blind-ending sugar crop, where water is extracted before sugars are released to enter the midgut for digestion and absorption. This anatomical feature may lead to the establishment of a sugar concentration gradient between oesophagus and midgut. Hence osmotaxis or chemotaxis may explain how promastigotes move from the midgut to the mouthparts. Additionally, or alternatively, osmotaxis may represent an adaptation for promastigotes to avoid osmotic stress (Leslie et al. 2002).

Leishmania mexicana has 3 isoforms of glucose transporters encoded by LmGT glucose transporter gene superfamily namely glucose transporter 1 (LmGT1), glucose transporter 2 (LmGT2) and glucose transporter 3 (LmGT3) (Burchmore & Landfear 1998). Subcellular localisation of all three isoforms has

been investigated by Burchmore et al (Burchmore et al. 2003) and it has been demonstrated that LmGT2 and LmGT3 are localised to the cell body whereas LmGT1 was localised specifically to the flagellum structure. However, a functional characterisation study revealed that LmGT1 has lower affinity for glucose compared to LmGT2 and LmGT3, hence cells transfected with plasmid containing only LmGT1, transport less glucose compared to the other 2 isoforms. Axoneme -based structures, such as flagella, are known to be involved in environmental sensing (Landfear & Ignatushchenko 2001) , and there are several examples of glucose transporter proteins that function as glucose sensors (Ozcan et al. 1996;Ozcan et al. 1998)I wished to test the hypothesis that a glucose transporter, localised to the anteriorly-oriented flagellum, might sense sugars and mediate chemotaxis (Burchmore et al. 2003).

In this project, to further characterise the LmGT1 in *Leishmania mexicana*, a taxis assay was performed to look at the response of towards glucose LmGT1 transfected *Leishmania* compared with a glucose transporter null mutant (ΔLmGT). The method used was based on the modification of methods from Oliveira et al (Oliveira et al. 2000) and Leslie et al (Leslie et al. 2002).

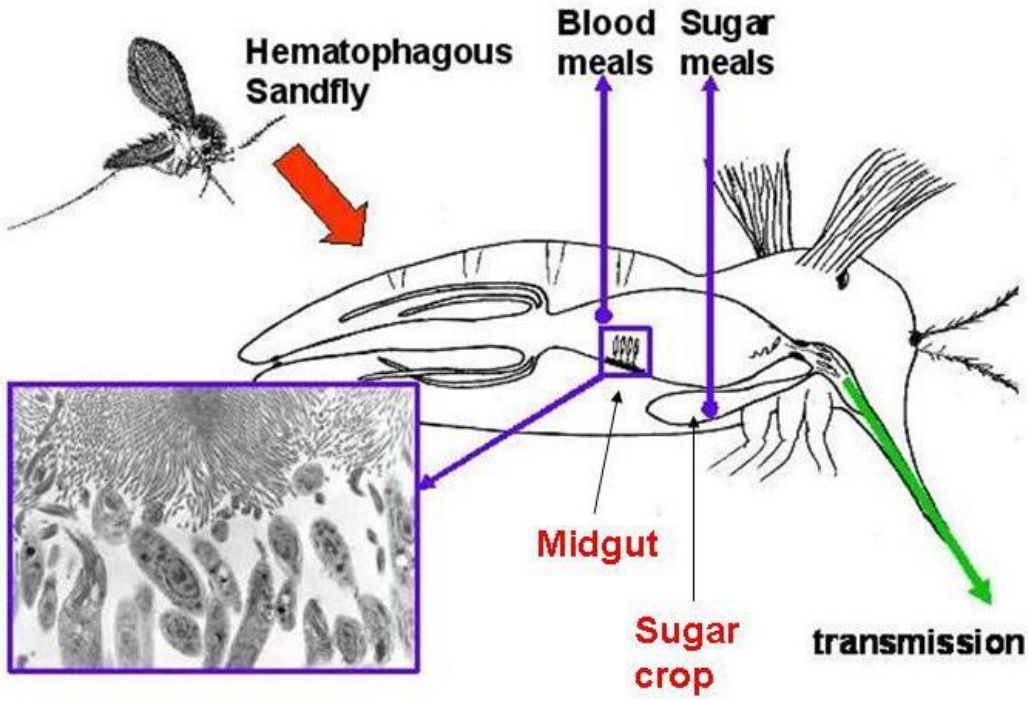

Figure 4-3: Sand fly feeds on bloodmeal and plant sugar (sugar meals) alternately. When the sand fly feeds on plant, the sugar meal will be moved to the sugar crop and stored here and become concentrated by removing excess water and release slowly into the midgut under the control of stamodial valve (purple-lined box) (Tang & Ward 1998). Whereas when the sand fly takes a bloodmeal, it will be transfered directly into the midgut (Killick-Kendrick 1978;Killick-Kendrick 1999;Schlein 1986). Killick-Kendrick suggested that a sugar gradient exists in the alimentary tract of the sand fly thus stimulating the migration of promastigotes during development (Killick-Kendrick 1978). (Picture by Dr Richard Burchmore, University of Glasgow).

4.2 Materials and Methods

4.2.1 Materials

Homem (Invitrogen), PBS (Sigma), Sodium ß-glycerophosphate (Fluka analytical), NaCl , KCl, $MgCl_2$ (Fischer Scientific), BSA (Sigma) and agarose (Invitrogen), glutaraldehyde solution (Sigma), osmium tetroxide solution (Sigma) , cacodylate (Sigma), ethanol(Fischer), propylene oxide (icis), borax buffer (Fishcer), uranyl acetate (Sigma), sucrose (Sigma), toluidine blue (Sigma), Chicken polyclonal antirat IgG peroxidase conjugate(Abcam), Goat Anti-Mouse IgG peroxidase conjugate (Calbiochem), Goat Anti-Rabbit IgG Peroxidase Conjugate (Merck), FBS Gold(PAA), G418 sulfate (Calbiochem), Prestained protein marker (New England Biolabs), peroxidase SuperSignal® substrate (Novagen), Tryparedoxin peroxidase antisera(A gift from Dr Susan Wyllie, University of Dundee), L8C4 and L13D6 antibodies (Both were gifts from Professor Keith Gull, University of Oxford) .

4.2.2 Western blot for PFR1&2, PFR2 and Tryparedoxin peroxidase

Wild type and glucose transporter null mutant *Leishmania* promastigote cultures were grown, as described in Chapter 2, to the concentration of up $1X10^7$ cells/ml and the Western Blot protocol, as in Chapter 3, was followed. Monoclonal antibodies used in this study were L8C4 which recognises PFR2 in *Leishmania mexicana* by Western blot and L13D6 which recognises PFR1 and PFR2 in *Trypanosoma brucei*. Both antisera were raised against *Trypanosoma brucei* and were gifts from Professor Keith Gull, University of Oxford. Tryparedoxin peroxidase antisera raised in adult male Wister rat (Wyllie et al. 2008) was a gift from Dr Susan Wyllie, University of Dundee. For the PFR1&2 immunoblots the primary antibody used was 1:1000 and 1:50,000 for the anti mouse secondary antibody. The PFR2 immunoblots, primary antibody was diluted to 1:500 and 1:50,000 for the anti mouse secondary antibody, respectively. For tryparedoxin peroxidase immunoblot, 1:2000 and 1:50,000 of tryparedoxin peroxidase antisera and anti rat secondary antisera, respectively.

4.2.3 Processing Cells for Transmission Electron Microscopy and Scanning Electron Microscopy

4.2.3.1 Transmission Electron Microscopy

Glucose transporter knock-out and wild type cell line were harvested at $1x10^7$ cells /ml in 10mls each. The cultures were centrifuged at 1000rcf for 10 minutes. The pellets were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 30 minutes. Then the pellets were rinsed a few times with 0.1M buffer with 2% sucrose followed by fixing in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour followed by a wash with distilled water to remove the osmium solution. The cells were dehydrated from 70% alcohol through 90%, and 3 changes of absolute alcohol at 10 minutes each. The final change is absolute ethanol dried over molecular sieve, decanted carefully to avoid sieve residue carryover. The processing of the specimens was continued by replacing the alcohol with propylene oxide twice at 5 minutes each. Add resin (araldite/epoxy) to prop oxide in 1:1 and mix. Replace final change of pure prop oxide with resin/PO and leave on rotator in the fume hood for 3 hrs or overnight. Replace with resin mix plus accelerator and rotate, capped for 6 hours.

Flat embed pellet pieces in fresh resin/accelerator in silicon moulds or in BEEM capsules and polymerize at 60^oC for 48hours. Finally, semi-thin sections were obtained from trimmed blocks and stained in borax buffered 1% toluidine blue on a glass slide for 30secs on a hotplate, rinsing in water to carefully wash off excess stain. Cell preservation and position in the block was confirmed by LM. Re-trimming was performed if needed and ultrathin sectioning for TEM performed.

This experiment and the results were done with help from Professor Laurence Tetley and Margaret Mullin from FBLS integrated Microscopy Facility, University of Glasgow.

4.2.3.2 Scanning Electron Microscopy

Cells were pre-washed from culture medium in 0.1M phosphate buffer, rinse and concentrated by spinning at 1000g , 2 x 5 mins and fixed by resuspending the pellet in 2.5% glutaraldehyde /0.1M PO_4 buffer for 30 - 40mins.

Terminate fixation by replacing fix with 0.1M PO_4 / 2% sucrose rinse, spinning 3 x 5mins. Resuspend between washes. In all subsequent steps care must be taken to avoid drying the specimens until the final stage.

Apply droplets of concentrated suspension in rinse buffer to 10mm diameter PLL-coated glass coverslips and allow 15 mins for cells to attach. Remove unattached cells by washing off with rinse buffer before adding 2% OsO_4 in distilled water in equal volume to rinse buffer. Leave to fix for 30 mins. Remove OsO_4 with 3 changes of distilled water and add 0.5% aqueous. Uranyl Acetate for 30 mins in dark. Wash in distilled water twice and dehydrate cells using an alcohol series

70% Alcohol	- 5mins
90% Alcohol	- 5mins
Absolute Alcohol	- 2x5mins
Dried Abs Alcohol	- 5mins

Transfer coverslips cell-side up into a covering volume of hexamethyldisilazane (HMDS).

Parasites were dried from HMDS by overnight evaporation of the solvent in the fume hood. The specimens were then mounted on stubs using conductive tape and coated with gold/palladium in a Polaron SC515 sputter coater prior to examination in a JEOL 6400 SEM operating at 6kV and secondary electron images recorded using an ADDA3 digital capture system (Olympus/SIS, Germany).

This experiment and the results analysis were done with the help from Professor Laurence Tetley and Margaret Mullin from FBLS integrated Microscopy Facility, University of Glasgow.

4.2.4 Taxis assay

Cell cultures of GT1 and ΔLmGT were grown, as described in Chapter 2, to the concentration of up $1X10^7$ cells/ml and these are the cell suspensions to be used in for the taxis assay. The method was modified from a previous paper(Oliveira, Melo, & Gontijo 2000). Washing and incubation solution (WIS) was prepared, composing of 30mM sodium ß-glycerophosphate (Fluka analytical), 87mM NaCl , 27 mM KCl, 2mM $MgCl_2$ (pH 7.1) (Fischer Scientific), 0.004% enriched BSA (Sigma) and 0.2% agarose (Invitrogen). Before the gel solidified, 6 capillaries were filled with WIS containing 100mM glucose by capillary action, where one of capillary ends was submerged in WIS buffer for a few minutes until the WIS buffer filled up approximately ¾ of the capillary length. After the WIS in all capillaries had solidified, one end of the capillary was submerged into a cell suspension of $5X10^6$ cells/ml in PBS (with 3 capillaries in each GT1 and ΔLmGT cell suspensions, respectively) and left for 1 hour at room temperature.

After 1 hour, cell suspension from each capillaries were drawn and counted under the microscope (the cell suspension was diluted 3X for easier counting i.e 8µl cell suspension + 16µl counting buffer).

4.3 Results

4.3.1 Western blot loading control

Pre-cast gradient SDS gel, Novex 4-20% Tris Glycine (Invitrogen) loaded with whole cell lysate from wild type and glucose transporter null mutant was used as a loading control for western blot for the PFR analysis and tryparedoxin peroxidase (Figure 4-4) analysis. Transketolase was one of the loading controls that were initially chosen for the analysis. However, after several trials, TKT had to be excluded as a loading control as it gave an inconsistent result throughout the experiments i.e. it expressed in only a few trials. Furthermore, transketolase expression in glucose transporter null mutant has not been tested, therefore the result is questionable.

Hence, in this project Coomassie staining was chosen as loading control (Welinder & Ekblad 2011).A whole cell lysate from wild type and glucose transporter null mutant grown to equal cell density i.e. $3x10^{10}$cells/ml, were loaded into several wells in the gel using various volumes (or cell numbers) and after electrophoresis, the gel was stained with Coomassie blue (Figure 4-4). Proteins in all lanes were compared for their expression intensities, and the volume (or cell number) that showed similar intensity of expression for wild type and glucose transporter null mutant was chosen for western blot analysis of PFR and tryparedoxin peroxidase (Daneshvar et al. 2011)).

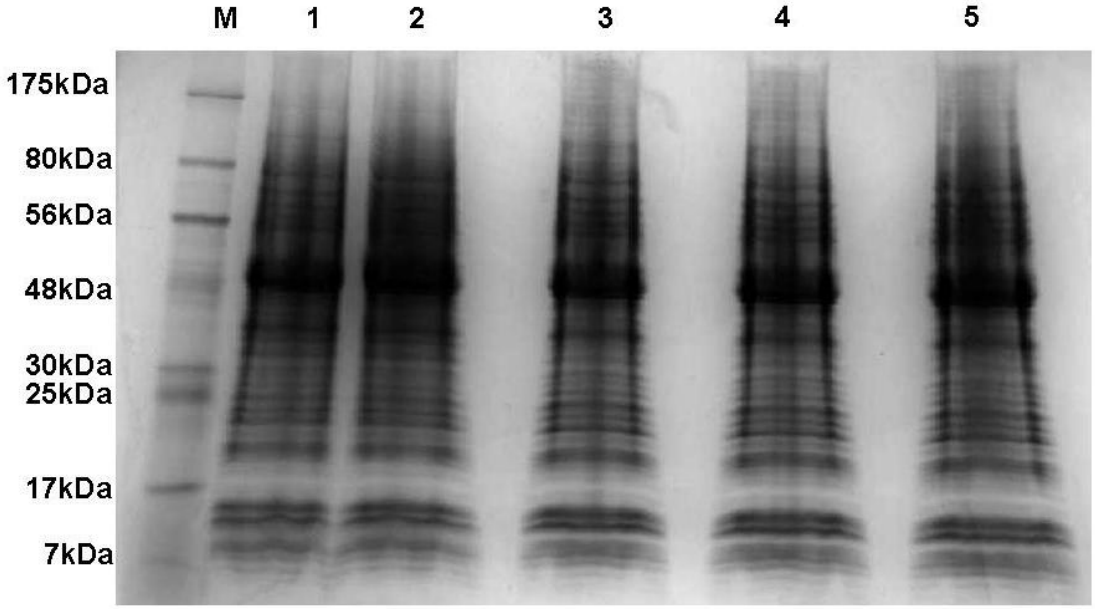

Figure 4-4: Gel from SDS-PAGE stained with Coomassie blue that served as a loading control. Gel was loaded with whole cell lysate of wild type and glucose transporter null mutant cells of *Leishmania mexicana*. M is the protein ladder. At the end of the whole cell lysate (WCL) preparation, both WT and ΔLmGT cells were resuspended at the concentration of $3x10^{10}$cells/ml. Each lane was then loaded with different volumes (or number of cells). Volume (number of cells) from WT and ΔLmGT lanes that show the same intensity of the protein expression after staining will then be chosen to be used in the following western blot. Lane 1 and 2 are loaded with WT with lane 1 loaded with 20µl WCL (cell numbers, 6×10^8 cells=20µl x $3x10^{10}$cells/ml) and lane 2 loaded with 25µl WCL (cell numbers, 7.5×10^8 cells=25µl x $3x10^{10}$cells/ml). Lane 3, 4 and 5 are loaded with ΔLmGT. Lane 3 loaded with 25µl WCL (cell numbers, 7.5×10^8 cells=25µl x $3x10^{10}$cells/ml), lane 4 loaded with 30µl WCL (cell numbers, 9×10^8 cells=30µl x $3x10^{10}$cells/ml), lane 5 loaded with 35 µl WCL(cell numbers, 1.05×10^9 cells=35µl x $3x10^{10}$cells/ml). Although the chosen volume and cell numbers are not the same between cell lines, they may have the same protein content. It has been reported that the null mutants are smaller in size and volume but null mutants may need more cells to have the same amount of protein in WT with lower cell numbers.

4.3.2 Paraflagellar Rod Protein

Monoclonal antibodies raised against conserved epitopes of *Trypanosome brucei* PFR1 and PFR 2 were obtained from Professor Keith Gull. One antibody recognises both PFR1 and PFR2 (L13D6; anti-PFR1/2), another recognised PFR2, but not PFR1 (L8C4; anti-PFR2), in *T. brucei*. These antibodies have previously been observed to recognise PFR in *Leishmania mexicana*, but it is not clear that they have the same capacity to distinguish between PFR1 and PFR2. Studies in the Gull lab suggest that L8C4; anti-PFR2 recognises *L. mexicana* PFR2 by Western blot, but may also recognise PFR1. L13D6; anti-PFR1/2 has not previously been tested with *L. mexicana*. These antibodies were used to probe lysates of WT and ΔLmGT promastigotes on Western blots.

Figure 4-5 shows a Western blot probed with anti-PFR1/2. A single band is observed that is much more intense in wild type than in ΔLmGT. Although this band is at approximately the expected size for *L. mexicana* PFR, we cannot conclude that the band represents either (or both) of the *L. mexicana* PFR isoforms.

Figure 4-6 shows a Western blot probed with L8C4; anti-PFR2. A single band is observed in wild type, but a clear doublet is seen in ΔLmGT. Each lane presents a band of similar size and intensity, but the ΔLmGT lane presents an additional, slightly larger band that is not observed in WT. This suggests that an additional PFR isoform is expressed in ΔLmGT. Since this additional band has a larger apparent molecular weight, it is likely that it represents a post-translational modification of PFR that is induced in ΔLmGT.

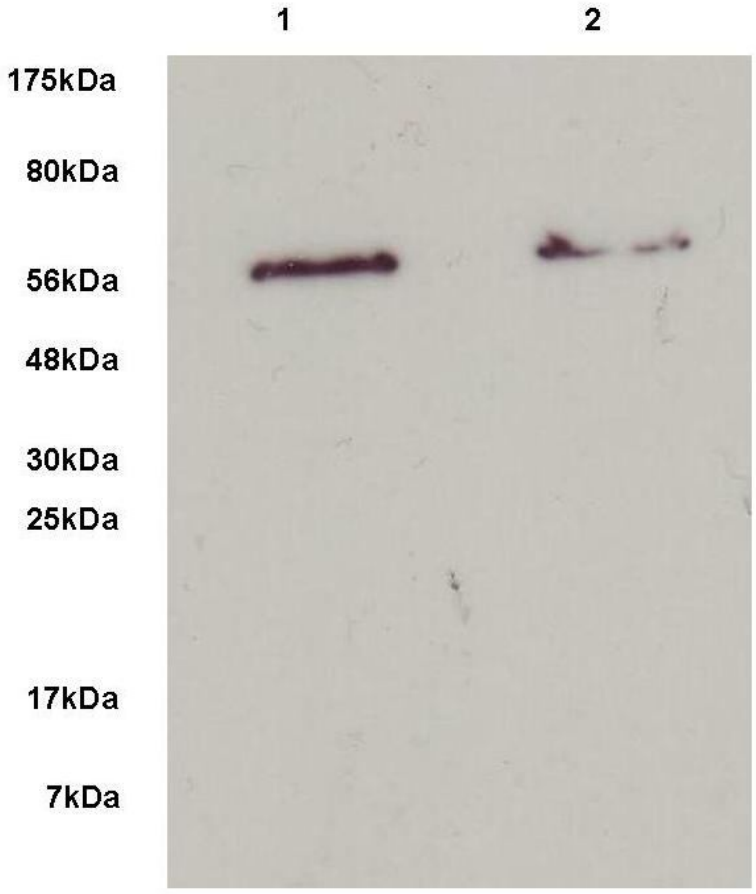

Figure 4-5: Western blot analysis of PFR1&2. Whole cell lysate prepared from wild type and glucose transporter null mutant *Leishmania mexicana* and PFR1&2 monoclonal antibody (L3D16) was used to probe the protein contained in the whole cell lysate in the western blot. Lane 1 is whole cell lysate from wild type and lane 2 is whole cell lysate from glucose transporter null mutant. The proteins were probed with PFR1&2 monoclonal antibody at 1:1000 dilution and anti-mouse secondary antibody at 1:50,000 dilution. The volume for WT and null mutant used were 20µl (cell numbers, 6 x 10^8 cells=20µl x 3x10^{10}cells/ml) and 30µl (cell numbers, 9 x 10^8 cells=30µl x 3x10^{10}cells/ml) respectively.

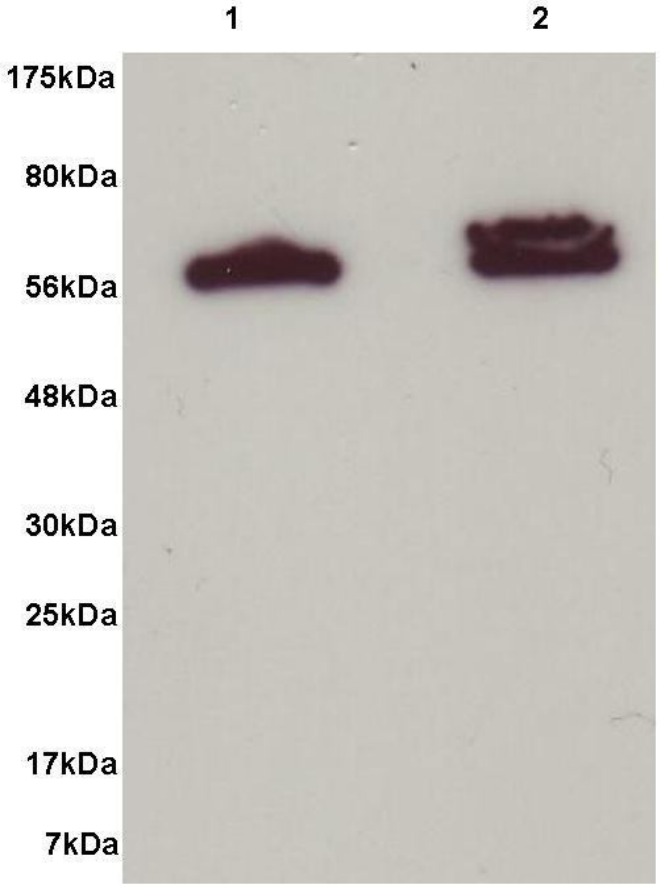

Figure 4-6: Western blotting analysis of PFR2. Whole cell lysate prepared from wild type and glucose transporter null mutant *Leishmania mexicana* and PFR2 monoclonal antibody (L8C4) was used to probe the protein contained in the whole cell lysate in the western blot. Lane 1 is whole cell lysate from wild type and lane 2 is whole cell lysate from glucose transporter null mutant. The proteins were probed with PFR2 monoclonal antibody at 1:500 dilution and anti-mouse secondary antibody at 1:50,000 dilution. The volume for WT and null mutant used were 20µl (cell numbers, 6 x 10^8 cells=20µl x 3x10^{10}cells/ml) and 30µl (cell numbers, 9 x 10^8 cells=30µl x 3x10^{10}cells/ml) respectively.

4.3.3 Tryparedoxin Peroxidase

Tryparedoxin peroxidase in *Leishmania mexicana* wild type and glucose transporter null mutant was analysed by Western blot (Figure 4-7). Equal numbers of WT and ΔLmGT promatigotes were lysed in sample buffer and separated by SDS-PAGE. The gel was blotted and probed with antisera raised against tryparedoxin peroxidase from *Leishmania donovani*. The antisera detected a single band at the anticipated molecular weight for tryparedoxin peroxidase. The band appeared to have similar intensity in both WT and ΔLmGT lanes.

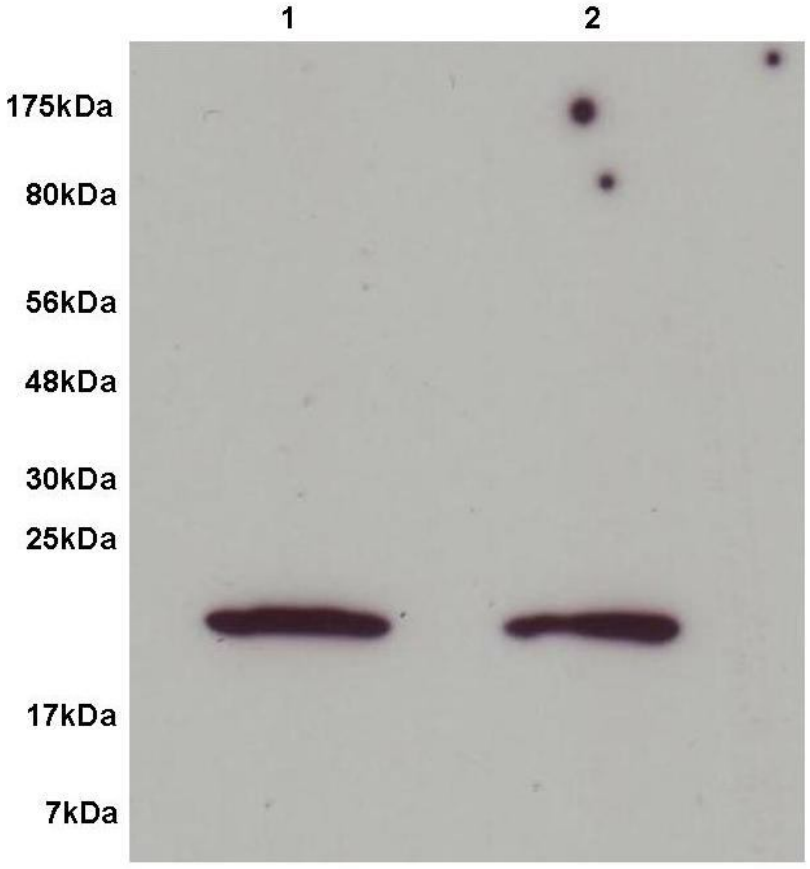

Figure 4-7: Western blotting analysis of tryparedoxin peroxidase. Whole cell lysate prepared from wild type and glucose transporter null mutant *Leishmania mexicana* and tryparedoxin peroxidise antisera were used to probe the protein contained in the whole cell lysate in the western blot. Lane 1 is whole cell lysate from wild type and lane 2 is whole cell lysate from glucose transporter null mutant. The proteins were probed with tyrparedoxin peroxidase antisera at 1:2000 dilution and anti-rat secondary antibody at 1:50,000 dilution. The volume for WT and null mutant used were 20µl (cell numbers, 6 x 10^8 cells=20µl x 3x10^{10}cells/ml) and 30µl (cell numbers, 9 x 10^8 cells=30µl x 3x10^{10}cells/ml) respectively.

4.3.4 Flagellar structure analysis

The ultrastructure of the flagellum in wild type and ΔLmGT promastigotes was compared by both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). TEM images were taken to analyse the structure of PFR in WT and ΔLmGT.

The structure of the flagellum of wild type seems to be intact after the treatment for TEM (Figure 4-8). The cross-sections of the several flagella were also seen and both the axoneme and microtubule tubule seem to be intact.

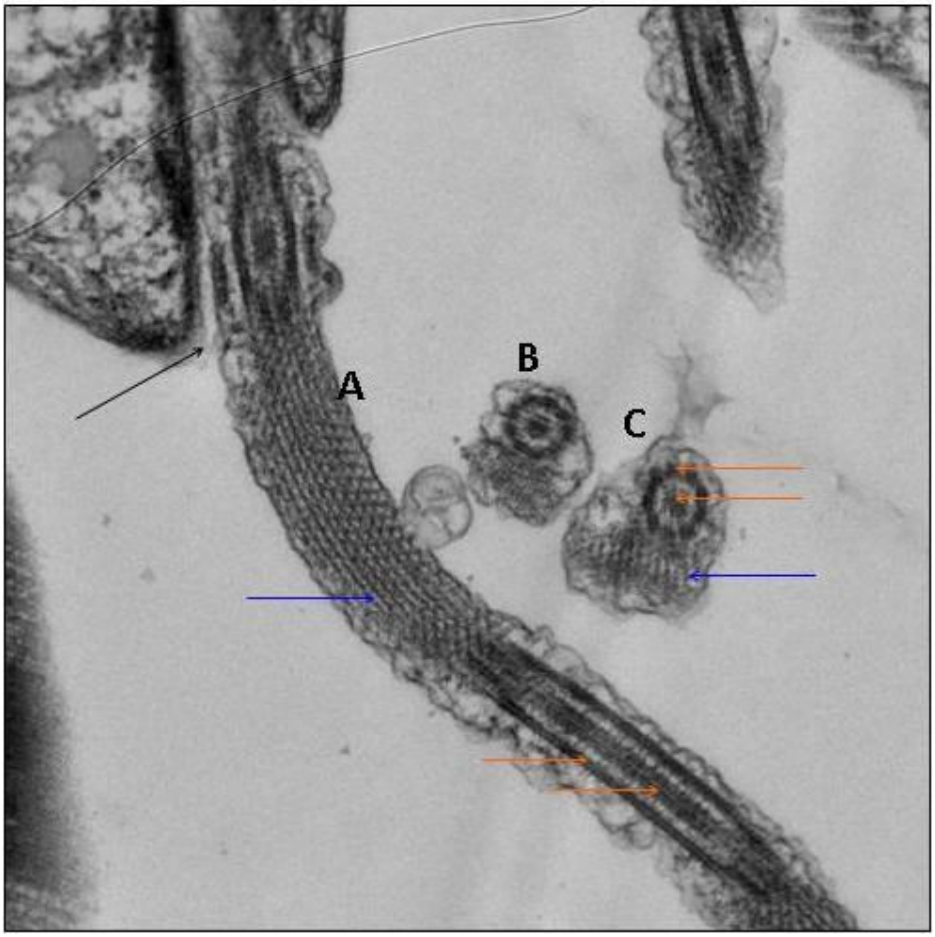

Figure 4-8: TEM image of wild type *L.mexicana*. The Black (⟵) arrow shows the opening of the flagellar pocket. The blue (⟵) arrows show the PFR of the flagellum and the orange (⟵) arrows show the axoneme structure. 'A' shows a longitudal section. 'B' and 'C' are the cross sections of flagellum. This was a preliminary experiment.

Figure 4-9(1) shows that the flagellum was truncated at the opening of the flagellar pocket (indicated by the pink arrow). In Figure 4-9(2), the flagellum started to 'degrade' (C) at the opening of the flagellar pocket. The cross-section of the flagellum (D), which is from another cell, indicates that the axoneme and microtubule were present in both WT and ΔLmGT. Intact flagella were rarely seen in TEM of the glucose transporter null mutant, indicating that their flagella were probably degraded during the TEM treatment.

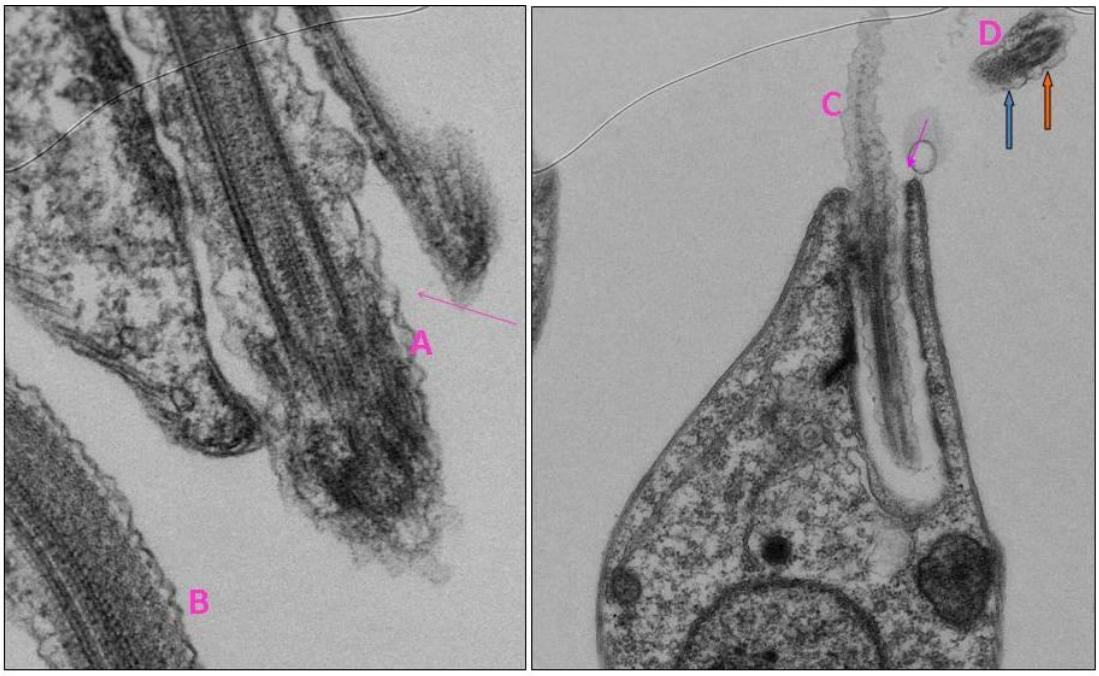

Figure 4-9: TEM images of glucose transporter null mutant. (1) A and B show the structure of the flagellum. The pink arrows (←) in the GT knockout mutants show the opening of the flagellar pocket, the flagellum A, seems to be truncated at the opening of the flagellar pocket. (2) C is the flagellum remain that seem to be degraded at the opening of the flagellar pocket (arrow). D is a cross section of a flagellum. The cross section, although not clearly showing the details of the structure, does show that the axoneme (orange arrow →) and the PFR (blue arrow →) structure are intact. This was a preliminary experiment.

4.3.5 Analysis of the cell shapes and flagellar length

The TEM images did not show the intact flagella of the glucose transporter null mutant. Scanning Electron Microscopy (SEM) was done to measure the body of the promastigote and its flagellum. The measurement is taken using software called 'iTEM' (The TEM Imaging Platfrom, Olympus) and then the results were compared between the promastigotes of wild type and glucose transporter null mutant. 268 cells from each cell line were randomly selected from the SEM images produced (Figure 4-10 (WT) and Figure 4-11 (ΔLmGT)) and the measurements were taken by iTEM software. From the images, we can see a reasonable number of cells that are from different physiological stages i.e. procyclic amd metacyclic promastigotes. Metacyclic promastigotes were found to be represented fairly equally in both the wild type and ΔLmGT samples. Generally, the shape of the promastigotes between the WT and ΔLmGT do not really show significant differences.

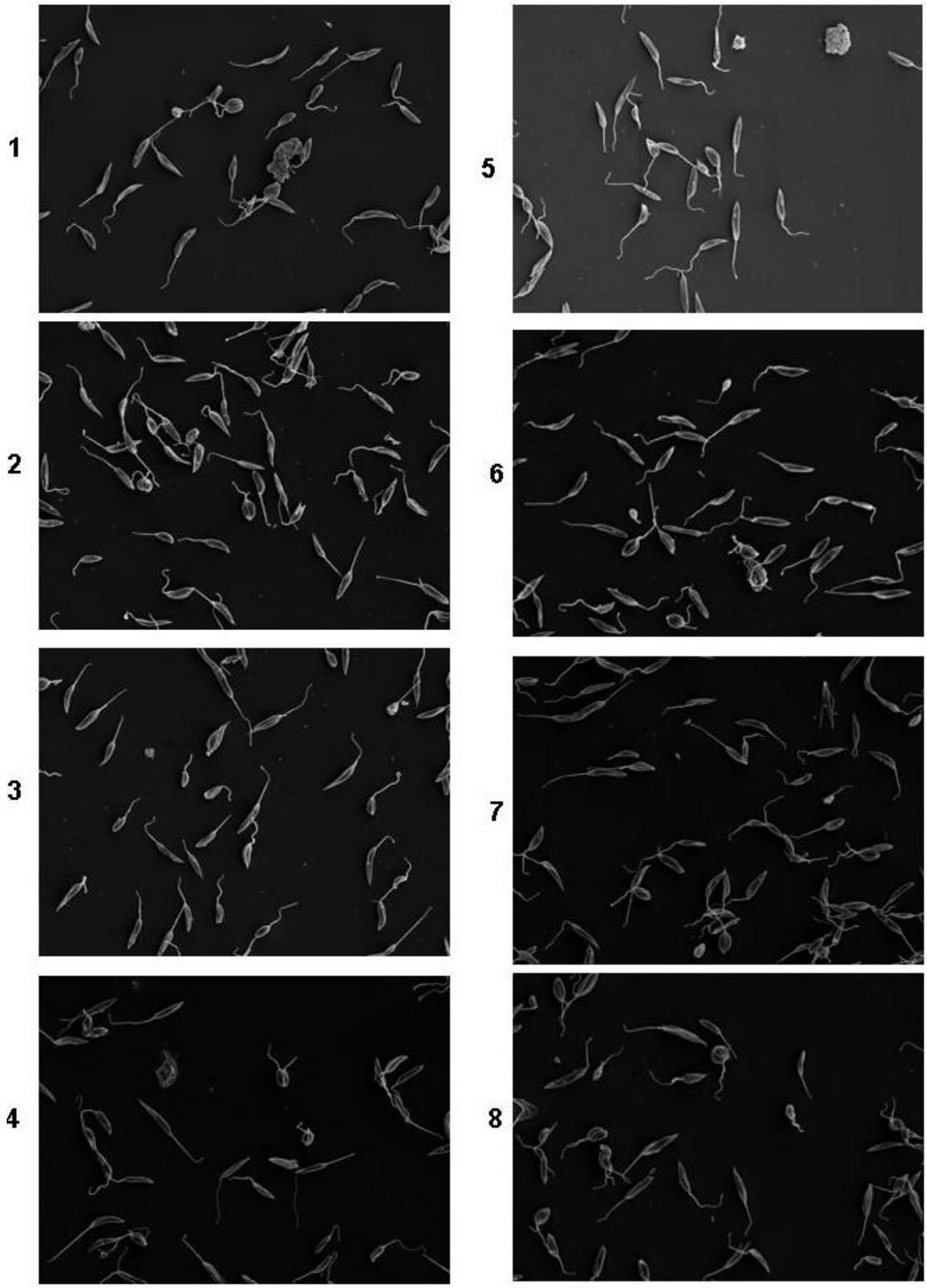

Figure 4-10: SEM images of wild type (WT). 268 cells were randomly chosen and the body length and the flagella were measured by iTEM software.

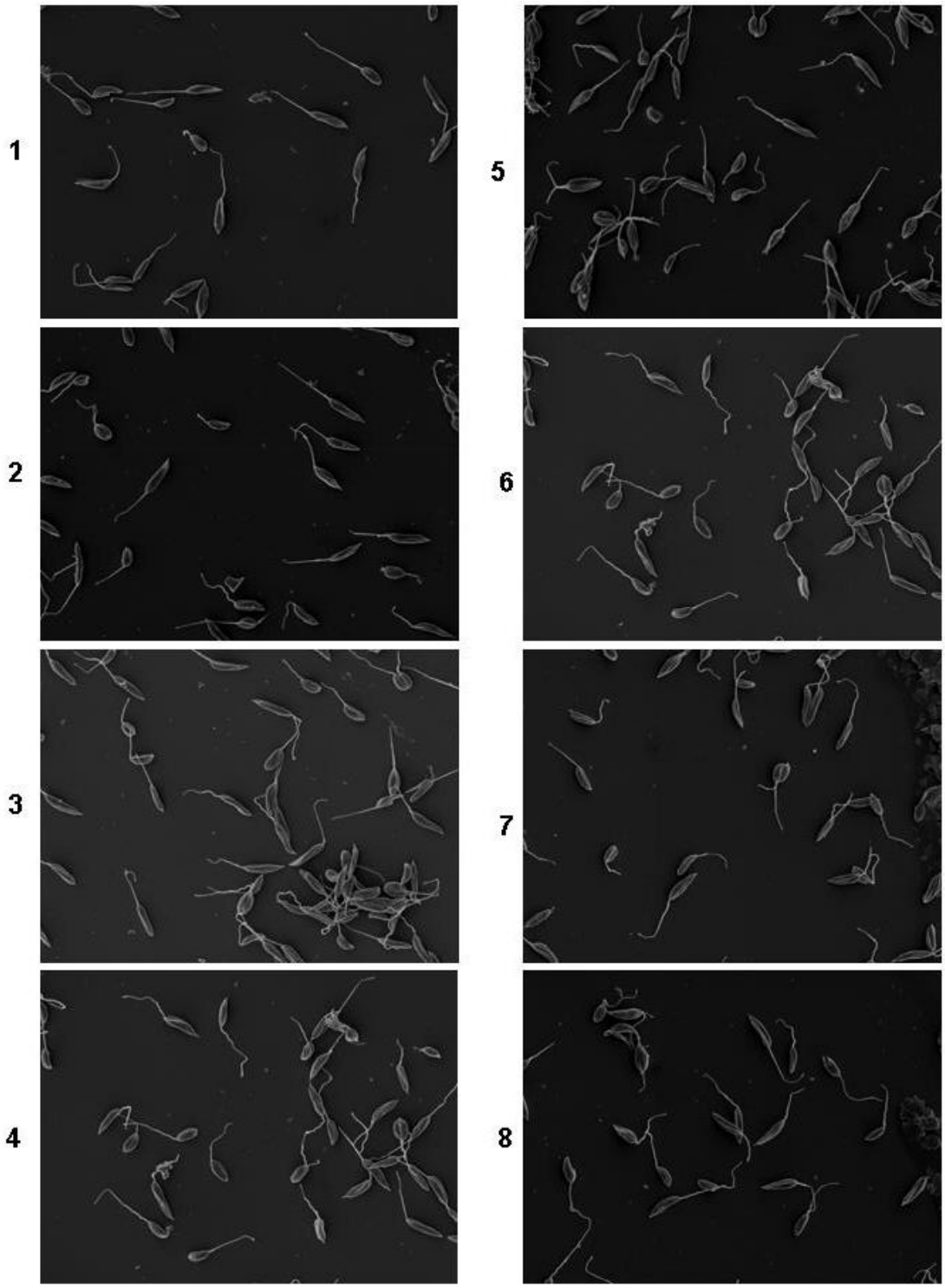

Figure 4-11: SEM images for glucose transporter null mutant (ΔLmGT). 268 cells were randomly chosen and the body length and the flagella were measured by iTEM software.

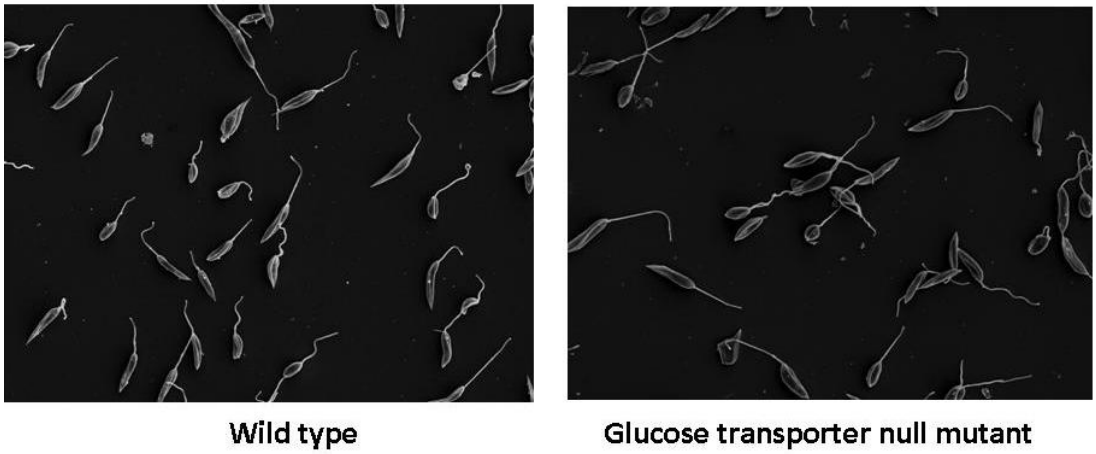

Figure 4-12: SEM images from wild type and glucose transporter null mutant promastigotes *Leishmania mexicana*.

The measurements of the body length and the flagellae of promastigotes were collected, the mean of each measurement was calculated and plotted on a histogram (Figure 4-13), showing mean body and flagellum length for wild type and glucose transporter null mutant *Leishmania*. A t-test was performed to test the significance of the data. 268 cells from each cell line were chosen randomly and each cell's body length and flagellum length were measured by iTEM software, and means were calculated. There was no significant difference in body length between wild type and glucose transporter null mutant (P=0.474). However, the mean flagellum length of the glucose transporter null mutant promastigotes was significantly greater than the mean flagellum length of wild type promastigotes (P<0.001).

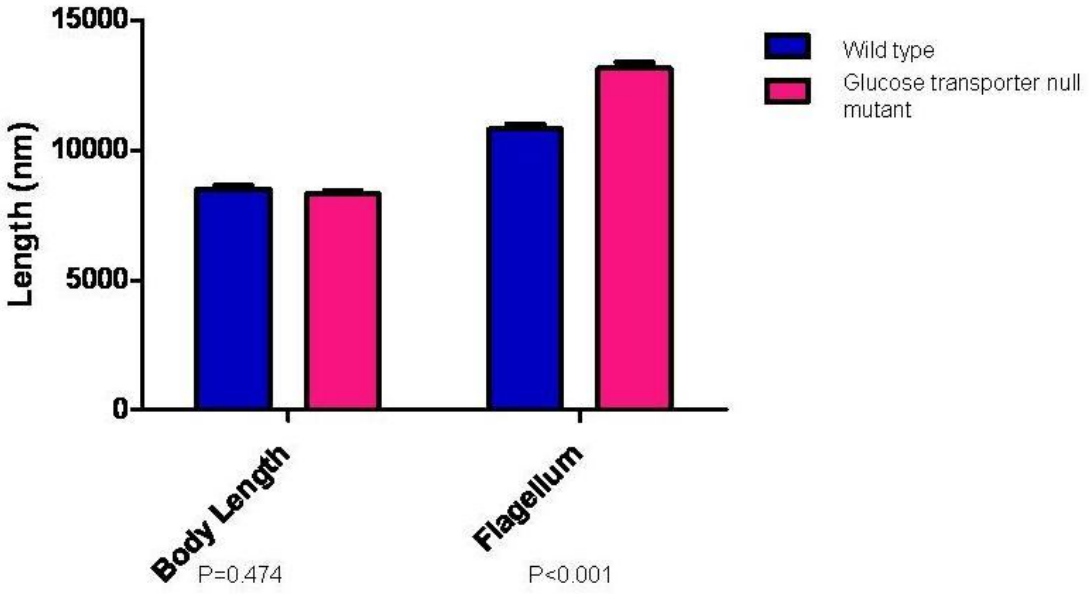

Figure 4-13: Comparison of the body length and flagellum length of wild type and glucose transporter null mutant. Body length and flagellum was measured for representative populations of the two cells line and means were compared. The body length of the two cell lines has similar measurement whereas the glucose transporter null mutants have significantly longer flagellae than the wild type promastigotes.

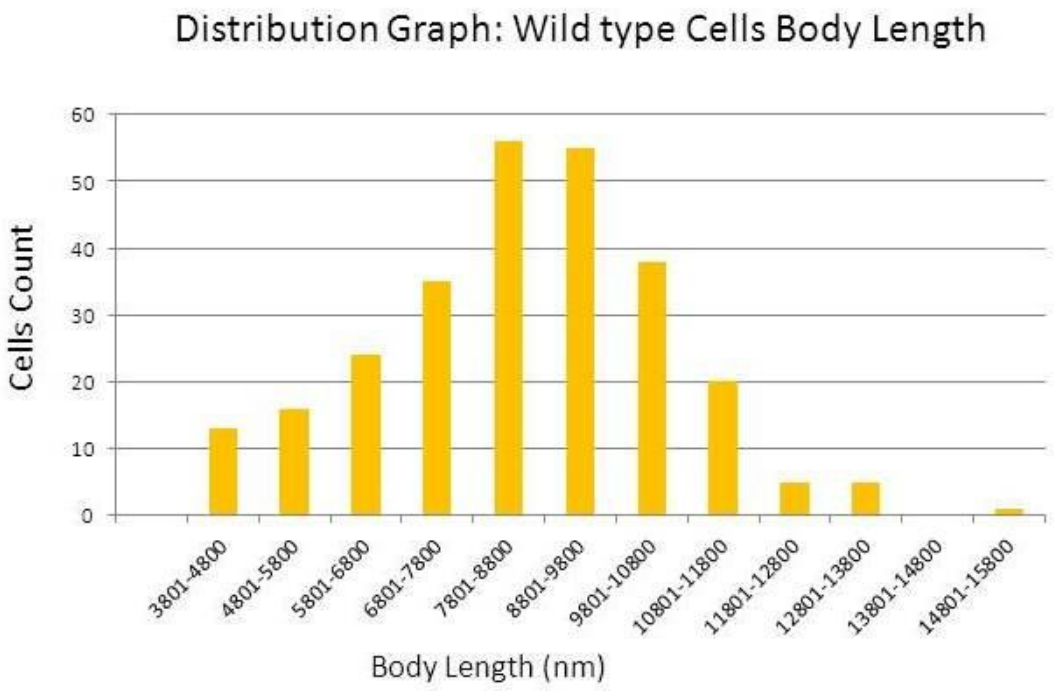

Figure 4-14 Distribution graph of wild type cells body length.

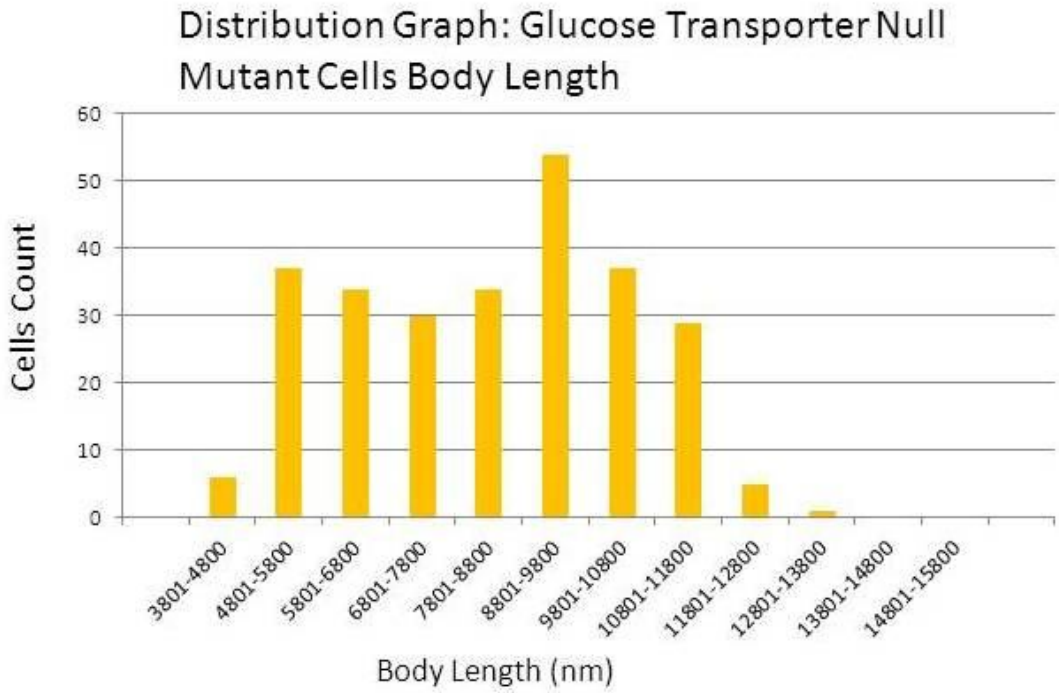

Figure 4-15 Distribution graph of glucose transporter null mutant cells body length.

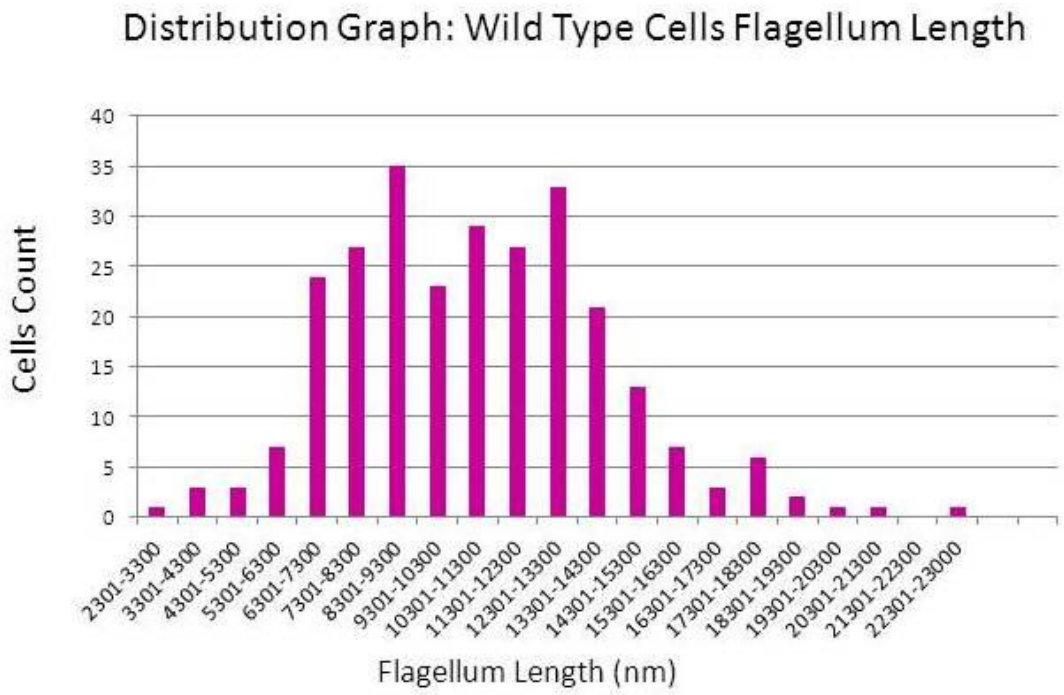

Figure 4-16 Distribution graph of wild type cells flagellum length.

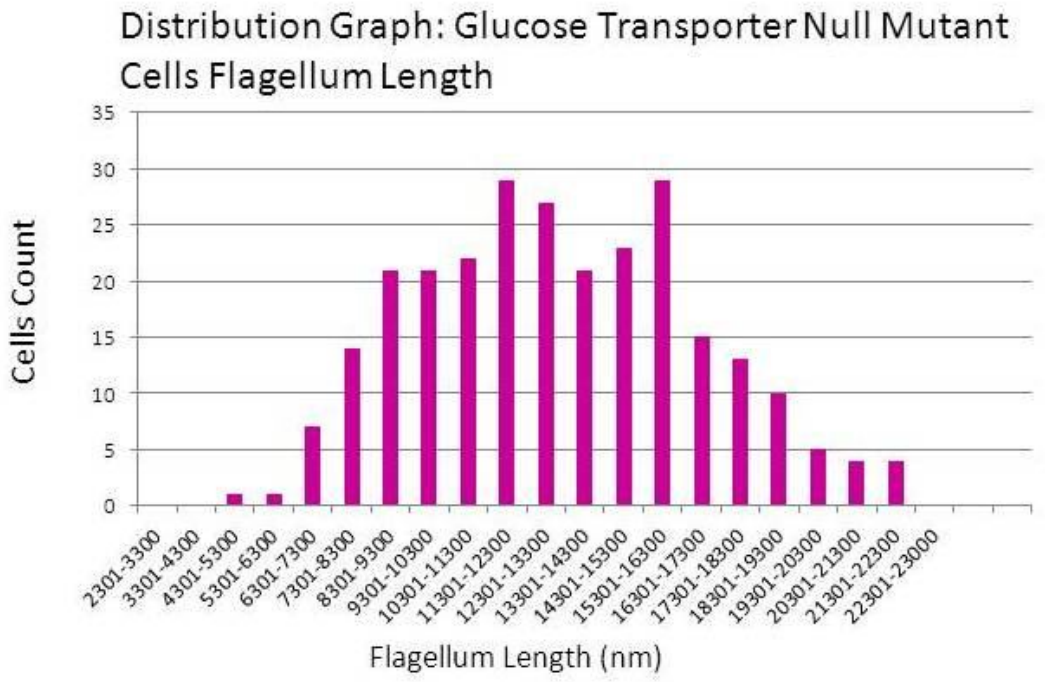

Figure 4-17 Distribution graph of glucose transporter null mutant cells flagellum length.

4.3.6 Characterisation of Taxis response in LmGT1 and ∆LmGT

To investigate the possibility that differences in flagellar length and composition might result in changes in motility or taxis, chemotaxis assays were performed. The response of promastigotes to exogenous glucose tested using a previously developed chemostaxis assay (Leslie et al. 2002). The chemotaxis response of glucose transporter null mutant promastigotes (ΔLmGT) was compared with glucose transporter null mutant promastigotes expressing LmGT1 (GT1), a glucose transporter isoform that has previously been found to localise to the flagellum. LmGT1 expression was maintained via an episome which is vector pX63 NEO.This is selected with 50µg/ml G418 (neomycin). Expression was validated by functional assay i.e. glucose transport was measured in LmGT1 expressors (Naula et al. 2010). The concentration on glucose that was used in the experiment was 100mM.

It was found that LmGT1 promastigotes were more attracted to glucose compared to ΔLmGT promastigotes. The mean of the LmGT1 calculated in the capillary tube after 1 hour incubation of the capillary tube containing WIS and glucose in cell suspension was $1.2X10^6$ and mean reading for ΔLmGT cells drawn from the capillary tube was $4.1\ X10^5$ cells, where the difference of means was $7.95X10^5$.

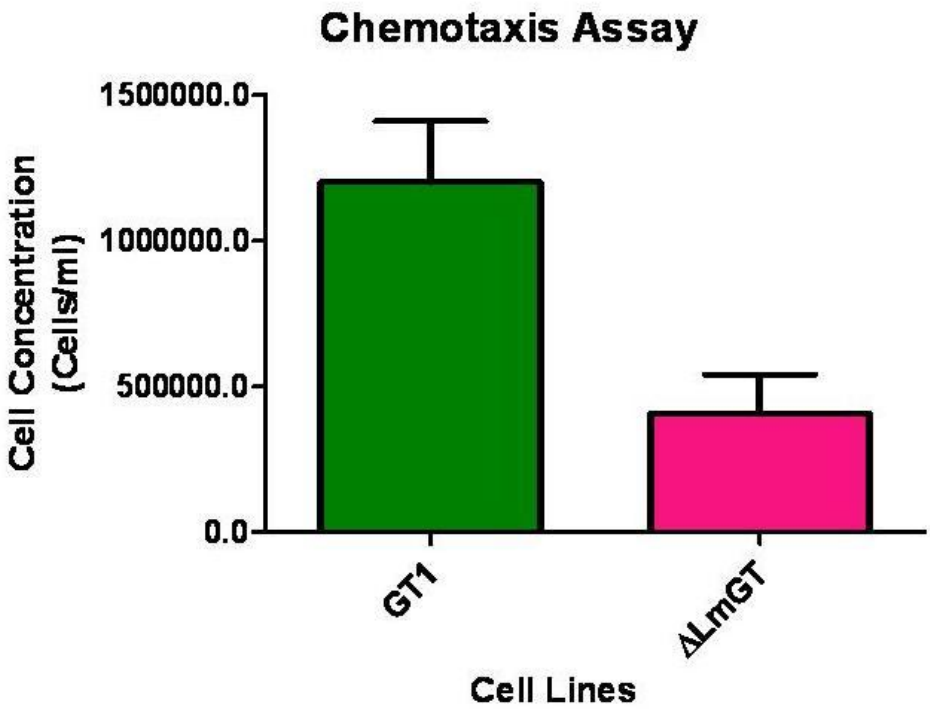

Figure 4-18: Chemotaxis assay of GT1 and ΔLmGT. The experiments were done in triplicate. The plotted number of each cells in the graph represents the mean number of promastigotes cells in the capillary tube containing WIS buffer mixed with 0.1M glucose. GT1 mean±SEM,n=2 was $1.2X10^6$ ± 2.1 X 10^5 and for ΔLmGT mean±SEM,n=2 was $4.1X10^5$ ±1.35 x 10^5.(SEM:Standard error mean). GT1- glucose transporter null mutant promastigotes expressing LmGT1, ΔLmGT-glucose transporter null mutant.

4.4 Discussion

Paraflagellar Rod Protein:

A whole cell proteomic investigation on wild type and glucose transporter null mutants, performed as part of a Masters project by David Henderson has revealed that a paraflagellar rod protein, PFR2, is expressed at a significantly higher level in glucose transporter null mutant promastigotes than in wild type promastigotes.

Western blotting was performed to investigate the expression of the 2 most abundant PFR isoforms in *Leishmania mexicana* i.e. PFR 1 and 2, to validate the previous proteomic experiment done by David Henderson. L13D6, a mouse monoclonal antibody (raised against *Trypanosoma brucei brucei* Ca^{2+} flagellar prep which recognised both PFR1 and PFR2 in *T.brucei* (Gadelha et al. 2004;Kohl et al. 1999), recognised a band in both wild type and glucose transporter null mutant cell lysates, though the band was less intense in the null mutant lane. Although this single clear band appears at an apparent molecular weight that is consistent with detection of PFR 1 and/or PFR2, this antibody has not previously been tested against *Leishmania* and may not efficiently recognise PFR 1 or PFR2 homologues in *L. mexicana*.

However, a second monoclonal antibody, L8C4, which recognises *T. brucei* PFR2 but not PFR1, has previously been shown also to recognise PFR2 in *L. mexicana*. This antisera recognised a single intense band at the expected molecular weight in wild type *L. mexicana* lysates, but gave 2 bands, of similar intensity, in glucose transporter null mutant lysates. The additional band detected in the glucose transporter null mutant may represent expression of a post-translationally modified isoform of PFR2, a result that is consistent with the original proteomic study that suggested upregulation of PFR2 in glucose transporter null mutant promastigotes.

One possibility is that the additional band detected in the glucose transporter null mutant, which is of a slightly higher apparent molecular weight, might represent a phosphorylated form. The observed differences in expression of PFR2 between wild type and glucose transporter null mutant promastigotes

may contribute to some of the structural and functional changes observed in this mutant

Other experiments should be carried out to further confirm the results as there are many factors. Hence, a transmission electron microscopy analysis on the PFR structure was performed. The transmission electron microscopy images were intended for structural investigation particularly on the axoneme and paraflagellar rod protein of wild type and glucose transporter null mutant.

In figure 4-8, TEM images from wild type cross-section clearly show axoneme structure with PFR structure next to it. These PFR structure seem to be intact in the images. However, the discrepancies that were expected in PFR structure between wild type and glucose transporter null mutant were unable to be analysed as the flagellar structure in the glucose transporter null mutant (Figure 4-9) cells seem to be degraded following the TEM samples preparation. The degradation seems to exhibit the 'fragility' of the flagellum to the chemicals used in the treatment. This 'fragility' must be further investigated in order to give a better insight of the causes that result in the failure of the flagellum to remain intact after the TEM treatment. As it is well understood that removing the glucose transporter in *Leishmania mexicana* promastigotes changes certain phenotypes of the protozoan, the strength of the flagellum structure could be one of the phenotype changes. However, this can only be confirmed if such investigation was carried out where one of the methods of studying this is by performing the optical tweezer method (Pozzo et al. 2009). TEM analysis in the project was done once hence it was a preliminary result.

In view of the fact that TEM could not give a satisfactory finding, the Scanning Electron Microscopy (SEM) images were generated to investigate the difference in the body and flagellum length between the wild type and glucose transporter null mutant. From the SEM images generated for wild type (Figure 4-10) and glucose transporter null mutant (Figure 4-11), the cell shapes between the cell lines did not show a clear difference. Both populations seem to have a similar proportion of metacyclic and procyclic promastigotes. There were numbers of dividing cells found in the wild type and glucose transporter null mutant. Randomly, approximately 268 cells were chosen from each cell line to measure the length of the cell body and flagella. The average length of the cell

body and flagellum were then calculated and a bar graph was generated (Figure 4-13). The cell body length of the two cell lines were almost the same with P=0.474. The flagellum length showed a significant difference with the average length for wild type and glucose transporter null mutant at 10267.26µm and 13009.17µm respectively. In addition, the wild type's flagellum is 21% shorter than the mutant's flagellum and P<0.001. Rodriguez-contreras et al. have showed that glucose transporter null mutant decreased in cell volume (Rodriguez-Contreras et al. 2007) and with the current experiment, it can be concluded that the exclusion of *LmGT* genes superfamily in *Leishmania mexicana* promastigotes affected the cell volume of the null mutant to decrease but it did not change the body length of the cell.

Tryparedoxin peroxidase: Figure 4-7 shows Western blot expressing tyrparedoxin peroxidase in whole cell lysate from wild type and glucose transporter null mutant exposed to tryparedoxin peroxidase antisera. Tryparedoxin peroxidase is involved in oxidative stress defense mechanism in *Leishmania*. It has been reported that glucose transporter null mutants are more prone to oxidative stress hence they are unable to survive as amastigotes in macrophages (Rodriguez-Contreras et al. 2007). However, in the current experiment, no significant discrepancies were noticed on the band expression between wild type and null mutant after being probed with tryparedoxin peroxidase antisera. Nevertheless, antisera is prone to have a cross reaction with other proteins. Thus, whether or not the tryparedoxin peroxidase in null mutant has similar regulation as in wild type, the question remains to be answered.

Taxis assay: Taxis can be defined as a response by free motile organisms or cells that move towards or away from certain environment stimuli. There are a number of different kinds of taxis characterised by organisms depending on the type of the stimuli. For example, chemotaxis is a taxis response to certain chemical or substances like glucose, NaCl or even toxic molecules. When an osmotic gradient is applied to the environment, cells will change its adaptation to the condition by moving towards an environment that is favourable to their survival and this response to osmotic gradient is defined as osmotaxis.

The result from the taxis assay showed a different response between the glucose transporter null mutant, and a line expressing only one glucose transporter isoform. The stronger taxis response observed in the line expressing LmGT1 (GT1 cell lines), which has a flagellar localisation, suggests that this glucose transporter might be involved in mediating a response to glucose in the environment of the cell. Further experiments are required to determine whether expression of other glucose transporter isoforms can also have this affect, and to test whether expression of LmGT1 leads to a change in expression of proteins such as PFR2.

However, this response may be a result of the osmotaxis of the cells. To determine if this response is either chemotaxis or osmotaxis, another experiment could give a better insight as performed by (Barros et al. 2006). Oliveira et al (Oliveira et al. 2000) performed an experiment to test the chemotaxis capability of *Leishmania amazonensis* and *Leishmania chagasi* towards a number of chemoattractants that were mixed in WIS buffer which partially filled the capillary tube that was submerged in a cell suspension. Among the chemoattractants that were tested were glucose, fructose, sucrose, raffinose, manose, glalactose, maltose and melibiose. The number of promastigotes that moved into the capillary was compared to the control (WIS buffer with no added chemoattractant) and counted and the mean average was calculated. The result from the experiment was concluded as chemotaxis of the cell. However, Leslie et al. (Leslie et al. 2002) carried out an experiment where the protocol was based on the modification of the experiment conducted by Oliveira et al. (Oliveira et al. 2000). The result generated from the experiment challenged the result from Oliveira et al's by stating that the result from Oliveira may be a resultant of an osmotaxis. In 2006, Barros et al (Barros et al. 2006) performed an experiment that could determine the taxis response of a cell to be either chemotaxis or osmotaxis.

From the experiment, it showed that even at very low concentration, sucrose, lactose, mannitol and glycine promastigotes showed a taxis response towards these chemoattractant but when the same concentration of NaCl, Hepes and guanosine were submitted to the environment, no taxis responses were recorded.

Hence, from this previous work done by Barros et al, the result from the current experiment can be concluded to be chemotaxis, and not osmotaxis as the concentration of glucose used was 100mM (Barros et al. 2006). As the current experiment was intended to investigate the LmGT1 protein function in the flagellum, it can be concluded that the sensor capability of the flagellum was therefore facilitated by LmGT1 as the number of promastigotes lacking this protein attracted towards the glucose in the capillary tube was lower than the LmGT1's. As LmGT1 was demonstrated to promote a very small increase in growth over the level of ΔLmGT and is low-affinity glucose transporter in addition to its subcellular location in the flagellum (Burchmore et al. 2003), the possibility that LmGT1 functions as glucose sensor is very likely. Apart from the sensory capability, the result from the graph also suggested that LmGT1 expressing cells may have moved faster towards the glucose-containing capillary compared to ΔLmGT.

4.5 Conclusion

The experiments in this chapter support the hypothesis that ablation of glucose transport in *Leishmania* promastigotes gives rise to cells that have altered flagellar structure (length and robustness) and function (motility and/or sensing). It is not known how these changes are related to loss of glucose transport capacity. One interesting possibility is that the imposed shift in energy source, from glucose to alternatives such as amino acids, results in compensatory changes in the flagellum. These changes may have little consequence to cells in culture, but may have more profound effects in the insect host.

Chapter 5

General discussion

Like all parasites, *Leishmania* acquire their nutrients only through their hosts and this requires an efficient transport system within the parasites. As the nutrient transport systems are crucial for survival to all living organisms, *Leishmania* nutrients acquisition systems are potential drug targets and worth to be researched and investigated in order to gives more insight on how does the mechanisms work. For this reason, the current project's interest is focuses on the mechanisms of nutrient acquisition in *Leishmania*.

5.1 The Problem

It is known that the promastigote stage of *Leishmania* spp.use glucose as their main energy source for their survival (Burchmore & Hart 1995;Hart & Coombs 1982) as they living in a vector's midgut that feed on plants sugar (Schlein 1986;Tang & Ward 1998) hence they are bathed in sugar-rich environment in the sandfly host. In culture, promastigotes acquire glucose from media via membrane transporters (Burchmore & Hart 1995;Burchmore & Landfear 1998) and metabolise glucose to generate energy and for anabolic processes (Hart & Coombs 1982;Langford et al. 1994). However, it has been demonstrated that promastigotes can also grow in glucose-free media although they grew slowly compared to the when glucose is available (Burchmore et al. 2003).

A glucose transport null mutant *Leishmania* mutant has been generated by targeted gene knockout (Burchmore et al. 2003). The three genes i.e. *LmGT1*, *LmGT2* and *LmGT3*, are single copy genes that are clustered together at a single locus, encodes three distinct glucose transporter isoforms i.e. LmGT1, LmGT2 and LmGT3 (Burchmore & Landfear 1998). Although this line does not have the capacity to transport glucose, nevertheless, it is able to survive in culture. Interestingly, it has been shown that glucose transporter null mutant promastigotes are significantly compromised in ability to survive in the sandfly host. However they were demonstrated to be unable to multiply and survive in macrophage host cells (Burchmore et al. 2003;Rodriguez-Contreras et al. 2007).

These observations raised a suggestion on the existence of possible alternative mechanisms for nutrient acquisition in glucose transport null mutant *Leishmania* for energy requirement and carbon supply important for their growth and survival in glucose free environment although these alternative mechanisms may not sufficient to permit the protozoan parasites to grow and survive as the clinically-relevant stage, the amastigotes. Hence, together with the observations and suggestions that have been raised, the potential that glucose transport system may have as antileishmanial drug targets is highlighted and it is worthwhile to study and research further the system to shed light on how these system works in *Leishmania*.

5.2 The Approach

The central aim of this project was to investigate the molecular mechanism behind the phenotypic changes observed in glucose transporter null mutant *Leishmania.* This aim was approached by incorporation of different fields of research methods i.e. combination of targeted analyses, using conventional and analytical approaches such as uptake assays and western blotting, and through more global proteomic and metabolomic analyses. The results generated demonstrate that glucose transport null *Leishmania* have undergone changes in metabolism and some phenotypic changes.

At the beginning of the current project, a master student (Henderson., D. Master Student, University of Glasgow, 2007) had undertaken a comparative proteomic analysis of wild type and glucose transporter null promastigotes of *Leishmania mexicana*. The most pronounced proteomic change observed in this study was in the expression of a major structural component of the flagellum i.e. the paraflagellar rod protein. Paraflagellar rod proteins are essential for full motility and provides support for metabolic regulators that may control flagellar beating(Gadelha et al. 2005) and deletion genes encode for these proteins results in impairment to flagellum movement (Santrich et al. 1997). Rodriguez-Contreras et al. showed that glucose transporter null mutant *Leishmania mexicana* decreased in cell volume compared to the wild type (Rodriguez-Contreras et al. 2007) but none has mentioned on flagellum structure change in glucose transporter null mutant. Henderson's study revealed that, despite the decreasing in volume size, the paraflagellar rod protein 2C (PFR2, accession:

LmjF16.1425) was significantly upregulated within the null mutant *Leishmania*. In order to validate this finding and to investigate the flagellar structure and function, Western blotting, microscopic analysis and taxis assay were carried out.

Lysates of wild type and glucose transporter null promastigotes were probed with 2 antisera raised against flagellar components of *Trypanosoma brucei*. Both antibodies detected bands at the expected apparent molecular weight for PFR proteins but L8C4 detected a clear doublet in lysates of the glucose transporter null mutant, but not in wild type. This result is consistent with expression of an additional isoform of PFR2 in the glucose transport null line. This isoform might be post-translationally modified, and it would be of interest to investigate this possibility in future work. These data validate the observation from a proteomic screen which suggested that PFR2 expression is upregulated in the glucose transporter null mutant.

Transmisson electron microscopy (TEM) analysis revealed interesting images for glucose transporter null mutant's flagellum. Initially , TEM was applied to look at PFR and axoneme structures as these structure were linked to the abnormality phenotype found in PFR null mutant *Leishmania mexicana* (Maga & LeBowitz 1999). Western blotting supported the hypothesis that expression of paraflagellar rod components is altered in the glucose transporter null mutant, which might result in some structural changes in the flagellum of glucose transporter null mutant *Leishmania*. However, unlike the wild type's TEM images which were intact and the structure of flagellum was clearly shown, the null mutant's flagellum seems to either 'degrading' or 'truncated' at the flagellar pocket of the protozoan parasite. Hence, the axoneme and PFR structures and composition were unable to be analysed in the null mutant. There is no evidence that the flagellum is truncated in vivo, so it seems likely that the flagellum of the glucose transporter null mutant is more fragile under the fixation conditions used to prepare samples for TEM. Though this "fragility" meant that it was not possible to obtain images of glucose transporter null mutant with intact flagellae, it does indicate a significant structure difference between the flagellae of wild type and null mutant promastigotes.

Scanning electron microscopy (SEM) analysis was done to look at the length of the cell body and flagellum of the *Leishmania* cells. Rodriguez-Contreras et al. has reported that cell volume in the glucose transporter-null mutant has decreased compared to the wild type's. However none published paper has reported on the flagellum's length. From the SEM analysis, the body length of both cell lines did not show a large difference. The flagellum length on the other hand, has showed that the null mutant has significantly longer flagellum compared to the wild type.

Taxis assay was performed to look at the *Leishmania* taxis response towards glucose. LmGT1 is one of 3 glucose transporter isoforms and has been shown to be located at the flagellum (Burchmore et al. 2003). As the flagellum is also hypothesised to be involved in environmental sensing (Landfear & Ignatushchenko 2001), LmGT1 was suggested to be involved in glucose sensing based on its location on the flagellum (Burchmore et al. 2003). In the taxis assay that performed in the current project, restoration of LmGT1 in glucose transporter null mutant *Leishmania* seems to have different phenotype of GT1 line compared to glucose transporter null mutant. The number of GT1 cells drawn from the capillary tube containing the glucose-contaning WIS gel was significantly higher compared to the null mutant's. This result show that the hypotheses that LmGT1 in flagellum may involves in glucose sensing (Burchmore et al. 2003) may be is true. Given the position of flagellum to be in the anterior of the cell, this may further support the involvement of flagellum and LmGT1 in environmental sensing and not just in motility. These results, though preliminary, are of considerable interest and future work may focus on the chemotaxis phenotype of wild type and of other relevant mutants.

Changes in flagellar structure and function analyses through Western blot, electron microscopy and taxis assay in the current project have confirmed that phenotypic differences between the flagellae of wild type and glucose transporter null promastigotes but it was not possible to relate these changes to the lesion in glucose transport capacity. It would be of interest in the future to further investigate these changes in glucose transporter null mutant *Leishmania* line that express individual glucose transporter isoforms(one of which has a flagellar localisation) but these experiments need to be well planned and longer

duration to be completed hence the experiments were beyond the timescale available for this project.

Glucose transporter null mutant *Leishmania mexicana* under study has a lesion in membrane transport. Hence, one of the aims of the current project was to look at these membrane transporters expressions in the null mutant. However, it is well-known that the challenges of investigating membrane transporters can be cumbersome because membrane proteins have relatively low abundance and are typically under-represented in proteomic analyses (Speers & Wu 2007).

Comprehensive analysis of cell proteomes is very challenging due to its complexity and dynamic range of protein concentrations. Hence the success of the proteome analysis is greatly dependent on the quality of the fractionation employed prior to MS analysis(Horth et al. 2006). For this reason, comparative analysis of similar fractions derived from wild type and glucose transporter null mutant promastigotes required further resolution of the complex mixture of proteins that comprised this plasma membrane-enriched fraction. An alternative approach which combine the use of solution-phase isoelectric focusing (IEF), 1-SDS page and differential labelling of proteins (DiGE) were applied. Solution-phase isoelectric focusing gives rise to unique fractions of reduced complexity, nevertheless the MS analysis revealed that there were great amount of tubulins and this may be the reason the low abundant proteins and IMPs could not be identified (Table 3-3) although previously in the same project where samples that had not been fractionated by OFFGEL has identified some IMPs both in the wild type and glucose transport null mutant (Table 3-2). The reason for not being able to identify IMPs in OFFGEL-fractionated samples could be due loss of samples during the fractionation due to leakage or drying of samples due to the long fractionation period which took 96 hours to complete. In addition to that, prior to fractionation, the membrane preparation samples had to be diluted into OFFGEL compatible solution hence this will decreasing the concentration of the IMPs which are already of relatively low abundance. This could be improved by using 2-5 higher concentration of proteins.

DiGE labelling involves incorporating fluorescent dyes , Cy3-NHS and Cy5-NHS, which are cyanine based, molecular-weight matched, amine reactive and

positively charged (Viswanathan et al. 2006). The combination of OFF-Gel with DiGE to analyse the membrane preparation samples did give an encouraging outcome. Differential labelling of proteins isolated from wild type and glucose transport null mutant promastigotes *Leishmania* enabled comparison of protein expression between these samples and some apparent differences were detected. However, due to time constraint and limited resources, further analysis i.e. mass spectrometry analysis, could not be carried out to complete proteomic analysis of these sub-fractions.

The *Leishmania* genome project reveals many genes that encode putative nutrients transporters (Aslett et al. 2010;Myler et al. 2001;Myler & Stuart 2000;Peacock et al. 2007). It has been suggested that high concentration of amino acids in *Leishmania* are accumulated by plasma membrane permeases (Zilberstein & Gepstein 1993). HOMEM, the growth media used in this study for *Leishmania* culture has a high concentration of numerous amino acids (Appendix). Amino acid in the culture media are the most likely used by the parasite as a carbon source (Krassner & Flory 1971). In culture, *Leishmania* can use amino acids as a carbon source when glucose is limiting for growth(Saunders et al. 2010). In addition, catabolic metabolism of numerous amino acids that generate intermediates for Krebs cycle have also been revealed or predicted (glutamine/glutamic acid, proline, asparagine/aspartic acid, alanine, serine, glycine, threonine, isoleucine, methionine, valine, cysteine) in *Leishmania* (Opperdoes & Coombs 2007). Ablation of glucose transport in glucose transporter null mutant promastigotes may have been possible because the cells were able to utilise amino acids as an alternative carbon and energy source to glucose. To test this possibility, the capacity of wild type and glucose transporter null mutant promastigotes to acquire various amino acids was compared.

The results of uptake assays for these four particular amino acid are clear and encouraging. But to assay for the uptake of individual amino acids, or of nutrients in general is time consuming and costly. Furthermore, targeted assays can only report on the metabolites that are under study and for which tracer compounds exist. Hence a global approach is an attractive alternative that could facilitate our understanding of uptake or requirements of other compounds than glucose that may be essential to *Leishmania* when glucose is limiting for growth.

To make a more global approach, I chose to perform an experiment which incorporate's the used of LC-MS-based comparative metabolomic analysis in order to screen for changes on metabolic pathways between wild type and glucose tranpporter null mutant *Leishmania* promastigotes. These analyses supported the results obtained through amino acid upatake assays. On top of that, it has also revealed a fascinating diversity of metabolic changes in glucose transport null mutant promastigotes.

The results generated from the current project have showed clearly that there are considerable changes in nutrient acquisition, phenotype and metabolism in glucose transporter null mutant *Leishmania mexicana*. These changes reflect the ability of the null mutant to adapt to a genetic lesion in the glucose transport capacity and the upregulation of the amino acid uptake activity is the most obvious adaptation metabolism seen in these null mutants where glucose is unable to be transported into the cells for energy metabolism.

5.3 Future Directions

Many previous publications have showed that *Leishmania* comprise genes that encodes a battery of putative amino acid transporters in promastigotes stage. However, there is little evidence that these promastigotes encounter abundant amino acids in the sandfly host and it is well known that the sandfly host feeds primarily on plant sap. The current project presented herein show that uptake of amino acids in glucose transporter null mutant promastigotes but it can not specify if this upregulation was due to augmented expression of the transporters or to changes in transport activity. In that light, genomic study of *Leishmania* could offer details of the regulation of gene expression and it would be interesting to investigate whether some amino acid transporter genes are amplified in glucose transport null mutant *Leishmania*.

The molecular analysis involving western blot, taxis assay and transmission electron microscopy and scanning electron microscopy has provide proof that lesion in membrane transport in *Leishmania* not only caused changes in nutrient acquisition adaptation and sensitivity to environment stimuli but as well as structural changes

Both uptake assay and metabolomic results have proven that certain amino acids are important to the glucose transporter null mutant *Leishmania* and probably are equally important in growth conditions where glucose is unavailable or scarce. It would be of interest in future studies to look at systems that may be involved in the regulation of transporter expression or activity. This could give insights on how important are those amino acids and could the lesion in those amino acid transport impose lethal effect on the cells.

Despite the inability to prove whether the bands expressed in membrane fractionation was a myc-tagged glucose transporter (Figure 3-6 and Figure 3-8), the data from proteomic results from whole cell lysate (Table 3-1) and salt-extraction method (Table 3-2), demonstrate that salt-extraction needs to be improvised before the method can be declared as 'not suitable' for the *Leishmania's* membrane protein extraction. The combination of subcellular fractionation and solution phase isoelectric focussing will present a high resolution method for plasma membrane proteomics and these approaches might successfully be combined with differential CyDye labelling to facilitate comparative plasma membrane proteomics. Hence they should be tested again in the future.

Nevertheless, although each aspects of study in this project has proved that all disciplines applied were challenging in terms of its reproducibility or technicality, the results demonstrate that the genetic lesion in glucose transport in *Leishmania* has caused major changes to the structural, phenotype and nutrient acquisition in the extracellular promastigotes of protozoan *Leishmania*. However, the study has also raised interesting new sets of questions that could be addressed in future research. Since serine accumulation and uptakes seem to be significantly different between wild type and glucose transporter null mutant, it would probably be best to generate a null mutant cell line that lack important proteins, where they can either be enzymes or transporters, that are responsible in transporting or converting serine *Leishmania*.

5.4 Summary

This project has highlighted some of the challenges that surround attempts at comparative proteomic analyses, particularly where membrane

proteins are in the spotlight. In addition, the uptake assays and metablomic analyses have further confirmed the importance of amino acids for the survival and growth of *Leishmania* in an environment where glucose is scarce. Despite the complexity of the metabolomic results generated from the project, it has also revealed the potential for comparative metabolomic analysis as an alternative unbiased approach to give a better insight into the molecular changes behind a complex phenotype. The work described in the project has no doubt provided a new set of knowledge that could be used for improving related research in the future.

Appendices

Appendix 1

HOMEM Recipe (Invitrogen-GIBCO)

Sodium Dihydrophosphate 2H2O	1.58 gram
Magnesium Sulphate 7H2O EP	0.2 gram
Sodium Chloride EP	6.8 gram
Potassium Chloride E	0.4 gram
Sodium Pyruvate	0.11 gram
Dextrous Anhydrous E	3 gram
L-Glutamine	0.292 gram
D-Biotin EP+	0.0001gram
P-Aminobenzoic acid	0.001gram
Phenol RED NA Salt	0.0gram
HEPES	5.9575gram
Sodium bicarbonate	0.3gram

MEM Non-Essential AM (100x liquid) 0.01 liter

L-Alanine	890 mg/L
L-Asparagine	1320mg/L
L-Aspartic Acid	1330mg/L
L-Glutamic Acid	1470mg/L
Glycine	750mg/L
L-Proline	1150mg/L
L-Serine	1050mg/L

MEM Amino Acid Solution (50x liquid)	0.03 liter
L-Arginine HCL	6320mg/L
L-Cysteine	1201.00mg/L
L-Histidine.HCL.H_2O	2096.00mg/L
L-Isoleucine	2623.00mg/L
L-Leucine	2623.00mg/L
L-Lysine HCL	3653.00mg/L
L-Methionine	746.00mg/L
L-Phenylalanine	1651.00mg/L
L-Threonine	2382.00mg/L
L-Tryptophan	510.00mg/L
L-Tyrosine	1811.00mg/L
L-Valine	2343.00mg/L

MEM Vitamin Solution (100x liquid)	0.01 liter
NaCl	8500.00mg/L
D-Ca Pantothenate	100.00mg/L
Choline Chloride	100.00mg/L
Folic Acid	100.00mg/L
i-Inositol	200.00mg/L
Nicotinamide	100.00mg/L
Pyridoxal HCl	100.00mg/L
Riboflavin	10.00mg/L
Thiamine HCl	100.00mg/L

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