A defined medium for *Leishmania* culture allows definition of essential amino acids

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Abstract

Axenic culture of *Leishmania* is generally performed in rich, serum-supplemented media which sustain robust growth over multiple passages. The use of such undefined media, however, obscures proteomic analyses and confounds the study of metabolism. We have established a simple, defined culture medium that supports the sustained growth of promastigotes over multiple passages and which yields parasites that have similar infectivity to macrophages to parasites grown in a conventional semi-defined medium. We have exploited this medium to investigate the amino acid requirements of promastigotes in culture and have found that phenylalanine and tryptophan are essential for viability in culture, while the absence of arginine, leucine, lysine, and valine results in significantly impaired, almost negligible growth. Most of the 20 proteogenic amino acids promote growth of *Leishmania* promastigotes, with the exception of alanine, asparagine, and glycine. This defined medium will be useful for further studies of promastigote substrate requirements, and will facilitate future proteomic and metabolomic analyses.

Introduction

Leishmania parasites cause a spectrum of important diseases in animals, including humans, which are collectively termed leishmaniasis. *Leishmania* have a digenetic life-cycle that involves an alternation between two distinct forms, promastigotes and amastigotes, and a transmission between two hosts, an invertebrate and a vertebrate host. While it is possible to study *Leishmania* biology in insect and mammalian host models, there are significant advantages to studying parasites in axenic culture, in particular the possibility to generate large quantities of uncontaminated parasite material under controlled conditions. However, the nutrient media that are routinely employed for axenic cell culture contain a variety of carbon sources and are typically supplemented with undefined animal sera. Such rich, serum-supplemented media may elicit phenotypes that differ from those which prevail in the nutritionally-sparse environments that the parasites encounter in the sand fly gut and the macrophage phagolysosome. Furthermore, the presence of undefined components in the culture media complicates the interpretation of biochemical data, and is a particular problem for proteomic and metabolomic studies, since heterologous proteins may obscure the characterisation of the parasite proteins of interest and the provenance of metabolites is unknown. The addition of undefined sera to culture media has specific safety implications for the production of tools such as vaccines.

A variety of media for growing *Leishmania* promastigotes have previously been reported (Table 1) [1 - 32], but the addition of either serum or protein has been necessary to support continuous growth over multiple passages. Many of the earlier media, such as the Novy-MacNeal-Nicolle (NNN) [9] and LIT-R9 [18], contain complex, undefined additions such as blood components or infusion from brain or liver [11, 12]. Such components can be substituted with peptone and yeast extracts, in media such as PY A-FBS 10 [17], Y-P [21], AJM-1 [22], and CML [27] (Table 1), or by the addition of animal serum. Work by Trager [10], Steiger and Steiger [13, 14], and Berens [15] established that animal serum could be replaced by bovine albumin for *in vitro* culture of *Leishmania*, resulting in the development of simplified yet still incompletely defined culture media.

Alternative medium formulations have also included components such as urine [19, 23], casein hydrolysate [22], and lemon extract [29]. These additions are less costly than animal sera but, like serum, are undefined and potentially variable. Trager [10] and Steiger and Black [33] were the first to report chemically defined, serum- and protein-free media that were able to support growth of *Leishmania* species (Table 1). Subsequent publications have expanded the number of media with a more completely defined composition (Table 1) [34 - 37], including the more recent work of Fritsche *et al.* [28] and Merlen *et al.* [38]. SFP medium [28] is supplemented with protein and RNA and CDM/LP medium [38] has an elaborate composition, including cholesterol.

We thus set out to develop a completely defined, synthetic medium able to support continuous growth of *Leishmania* promastigotes in axenic culture. As a foundation, we used the most simplified medium developed by Steiger and Black [33], then omitted components that were not chemically defined and systematically modified the composition to determine the relative importance of amino acids, co-factors, vitamins, and metals. This led to the development of a defined medium, termed Nayak Medium (NM) which supports continuous growth of *L. mexicana* promastigotes. We observed that the kinetics of *L. mexicana* promastigote growth in NM medium approach those seen in a conventional rich, serum-supplemented medium, and that the promastigote morphology and infectivity to macrophages are similar to cells grown in a conventional media.

Table 1. Media proposed for culture of *Leishmania*.

	Media	Composition	Reference
Serum- and/or protein-supplemented media	**M199**	Supplemented with 5% serum	Morgan *et al.*, 1950
	DMEM	Supplemented with 10% serum	Dulbecco and Freeman,1959
	RPMI 1640	Supplemented with 10% serum	Moore *et al*., 1967
	Grace's insect tissue-culture medium	Supplemented with 20% serum	Grace, 1962; Childs *et al*., 1978
	Mitsuhashi-Maramorsch medium	Supplemented with 20% serum	Mitsuhashi and Maramorsch, 1964; Childs *et al*., 1978
	HOMEM	Supplemented with 20% serum	Berens *et al*., 1976
	Schneider's *Drosophila* medium	Supplemented with (30%) serum	Schneider, 1972
	NNN	Contains blood agar and 0.6% NaCl	Nicolle, 1908
	Medium S	Supplemented with bovine plasma fraction V	Trager, 1957
	GLSH	Contains glucose, lactalbumin, heamoglobin, supplemented with 10% serum	Jardin and Le Ray, 1969
	--	Contains rabbit blood lysate, beef and liver infusion, salts, and glucose	Dwyer, 1972
	RE I	Contains minimal number of components, including bovine albumin	Steiger and Steiger, 1976
	RE III	Simplified RE I	Steiger and Steiger, 1977
	HOSMEM-II	MEM-based, supplemented with bovine albumin fraction IV	Berens and Marr, 1978; Schuster and Sullivan, 2002
	PY A-FBS 10	Contains peptone, yeast autolysate, salts, supplemented with 10% serum	Palomino, 1982
	LIT-R9	Contains liver infusion and tryptose	Sadigursky and Brodskyn, 1986
	--	M199 supplemented with urine	Armstrong and Patterson, 1994
	--	Contains bovine hemoglobin powder	Agarwal and Jain, 1996
	P-Y	Contains peptone, yeast extract, salts, supplemented with 10% serum	Limoncu *et al*., 1997
	AJM-1	Contains peptone, casein hydrolysate, beef and yeast extracts	Ali *et al.*, 1998
	--	M199 supplemented with 10% human urine	Singh *et al.*, 2000
	--	Contains Brain Heart Infusion (BHI), supplemented with hemin	Meehan *et al.*, 2000
	cRPMI	RPMI 1640 supplemented with filtered and dialyzed, protein- and low molecular weight component-free serum fractions	Santarem *et al.*, 2003
	--	M199 supplemented with cow, buffalo, or goat milk	Muniaraj *et al.*, 2007
	CML	Supplemented with peptone and yeast extract	Sharief *et al.*, 2008
	SFP	Supplemented with BSA (SFP(I), BSA and hemin (SFP(II)), PEG-hemin (SFP(III))	Fritsche *et al.*, 2008
	GALF-1	Contains minimal number of components, lemon extract, 2% urine, and 20% serum	Tasew *et al.*, 2009
	PBHIL	Contains glucose, salts, peptone, BHI, liver infusion broth, and hemin	Rodrigues *et al.*, 2010
	SNB-9	Contains blood agar, neopeptone, and 0.6% NaCl	Grekov *et al.*, 2011
	--	Contains nutrient agar, nutrient broth, and 10% serum	Aksoy Gokmen *et al.*, 2015
Serum- and/or protein-free media	**Medium C**	Contains minimal number of components	Trager, 1957
	RE IX	Simplified RE III, lacks bovine albumin	Steiger and Black, 1980
	RE X	RE IX without glucose	
	MD29	Contains a variety of purines and pyrimidines	Melo *et al.*, 1985
	--	Rich in nutrients	O'Daly and Rodriguez, 1988
	S-α-MEM	α-MEM and RPMI 1640 supplemented with hemin, HEPES, glutamine, glucose, folic acid, biotin, and adenine	Kar *et al*., 1990
	S-RPMI		
	--	M199 supplemented with HEPES, co-factors, and vitamins	McCarthy-Burke *et al.*, 1991
	CDM/LP	Contains complex fatty acids and amino acid intermediates	Merlen *et al.*, 1999

Experimental procedures

Cell culture

Leishmania mexicana MNYC/BZ/62/M379, *L. donovani* MHOM/SD/63/Khartoum, and *L. major* MHOM/IL/80/Friedlin were passaged at an initial density of 5.0 x 10^5 cells/mL in hemoflagellate-modified minimum essential medium (HOMEM) [7] (Invitrogen, Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Biosera), and cultured at 27^{o}C. Viability and integrity of the cells was assessed using the trypan blue dye (Sigma-Aldrich, Merck) exclusion test [39]. Parasite growth was followed by diluting a 0.01 mL aliquot of the cell culture with 2% (w/v) formaldehyde-phosphate buffered saline (PBS) (Sigma-Aldrich, Merck) and counting the cells on an improved Neubauer haemocytometer.

Medium preparation

Based on Steiger and Black's RE IX medium [33] and the nutrient requirements of *Leishmania* promastigotes determined in this study, a base medium (BM) and an optimised defined medium (NM) were developed. Compound stock solutions were prepared by dissolving individual components (Sigma-Aldrich, Merck) in Milli-Q water, except adenosine, biopterin, folic acid, hemin, and lipoic acid which were dissolved in 1N NaOH. The stock solutions were then filter sterilized through 0.22 μm Express PLUS membrane filters (Millipore, Merck) and stored in aliquots at -20^{o}C. The pH of all media was adjusted to 7.4 and the media were also filter sterilized.

Estimation of nutrient requirements

To assess amino acid requirements, 1.0 x 10^6 cells/mL log phase promastigotes grown in HOMEM were harvested by centrifugation at 1000 x *g* for 10 min at room temperature (RT), then the pellets were washed with NM lacking amino acids (NM-AA) and re-suspended in Nayak single amino acid "knock out" media, containing all amino acids bar one.

To assess metal and cofactor requirements, 1.0 x 10^6 cells/mL log-phase promastigotes grown in HOMEM were harvested by centrifugation at 1000 x *g* for 10 min at RT, then the pellets were washed twice with BM and re-suspended in BM supplemented with cocktails of co-factors and metals (Sigma-Aldrich, Merck). The co-factor cocktail contained 10μM of biopterin, lipoic acid, and the vitamins biotin, folic acid, and riboflavin. Hemin was not included in the tested cocktail. The metal cocktail included magnesium, calcium, zinc, iron, cobalt, copper, and manganese.

The growth data presented comprise representative information from 5 independent assays, each carried out in triplicate (n=15). All data were analysed using one-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test and a significance threshold of $p<0.0001$ (Graph-pad Prism software). Images of cells cultured in the single amino acid "knock out" medium were prepared by smearing 200μL of culture medium from each condition onto glass slides (Marienfeld). The slides were then air-dried and the cells fixed by

dipping the slides into ice cold methanol for 2 min. The slides were air-dried again and stained with Giemsa (Sigma-Aldrich, Merck) (diluted in 10mM phosphate buffer, pH 7.2) for 20 min. Dried slides were visualised using light microscopy (Axiovision,) and the images analysed by Fiji-Image J software, with a standard scale bar of 10µm assigned for a constant number of pixels. 50 cells from each slide were measured and cell body length plotted as their frequency distribution. For statistical significance, one-way ANOVA was performed with Dunnett's multiple comparison test.

Estimation of protein concentration

1.0 x 10^7cells in total, grown in single amino acid "knock out" media, were harvested by centrifugation at 1000 x *g* for 10 min at RT, re-suspended in PBS containing 0.05% Triton X100, 5% glycerol, 2mM 1,10-phenanthrolin, 10µM leupeptin, 7µM pepstatin, and 10µM E64 (Sigma-Aldrich, Merck) and lysed by sonication. Protein concentration was determined by Bradford assay (Bio-Rad). Data were derived from three independent experiments, each performed in triplicate (n=9).

Estimation of infectivity

THP1 cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific) supplemented with 10% FCS at 37ºC and 5% CO_2. Cultures were initiated at a density of 5.0 x 10^5 cells/mL and sub-passaged every five days. THP1 cells were differentiated using 100nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Merck) and plated on Nunc Lab-Tek Chamber slides (Sigma-Aldrich, Merck) 24 h prior to infection. After a four-hour incubation, *L. mexicana* promastigotes, cultured in either HOMEM or NM to stationary phase, and heat-killed parasites were collected by centrifugation at 1000 x *g* for 10 min at RT, then resuspended in RPMI 1640 medium, and added to THP1 cells at a 1:10 ratio. The chamber slides were incubated for 4 h at 32ºC and 5% CO_2, then the medium replaced to removed extracellular promastigotes. At intervals of 4, 24, 48, 96 and 144 h post-infection, THP1 cells were washed three times with PBS, fixed by immersion in ice cold methanol and stained with Giemsa for 20 min as described above. Dried slides were visualised using light microscopy (AxioVision, Zeiss) and the percentage of infectivity and number of amastigotes per macrophage calculated.

Estimation of drug sensitivity

Alamar blue assay was used to assess the IC_{50s} of three leishmanicidal compounds as described by Raz *et al.* [40]. Briefly, 1.0 x 10^6 cells/mL were diluted in either HOMEM or NM media and 5.0 x 10^5 cells/mL were inoculated into 96-well flat-bottomed microtiter plates (Sigma-Aldrich, Merck) and incubated for 72 h at 27ºC with amphotericin B, pentamidine, and methotrexate (Sigma-Aldrich, Merck). The latter were diluted in either HOMEM or NM media, in a 8-fold dilution series that covered a range from 200µM to 0.2µM. Then 0.49mM of the fluorescent dye resazurin (Sigma-Aldrich, Merck) was added to each cell suspension and the incubation was continued for further 48 h. Fluorescence was measured on a PHERAstar *FS* spectrometer (BMG Labtech) at excitation and emission wavelength of 544 nm and 590 nm, respectively. Regression analysis was used to determine

the IC_{50} of the compounds. The data were log transformed and sigmoidal dose-inhibition curves plotted using GraphPad Prism. Data were derived from three independent experiments, each performed in duplicate (n=6).

Results

Development of defined media for axenic culture of *Leishmania* promastigotes

We have developed a serum- and protein-free medium with a simple, defined composition. As a reference point, we supplemented a previously reported growth medium, RE IX [33], to produce an amino acid-rich basal medium (BM) (Table 2), in which we performed nutrient requirement analyses. These experiments led to the development of Nayak medium (NM) (Table 2), which is able to support continuous culture of *L. mexicana* promastigotes at a growth rate comparable to that of cells grown in conventional rich, serum-supplemented media such as HOMEM [7]. By systematically modifying NM, we investigated the importance of individual amino acids for sustaining *L. mexicana* promastigote growth.

Table 2. Composition of base medium (BM) and Nayak medium (NM) for axenic culture of *Leishmania* promastigotes. *, present in the media but not in the co-factor cocktail.

Components	BM (concentration, mM)	NM (concentration, mM)
Salts		
KCl	5.36	5.36
NaCl	136.8	136.8
NaH_2PO_4	0.33	0.33
$NaHCO_3$	3.57	3.57
Vitamins		
Biotin	-	0.01
D-Calcium pantothenate	0.002	0.002
Folic acid	0.002	0.012
Nicotinamide	0.008	0.008
Pyridoxal HCl	0.004	0.004
Riboflavin	0.002	0.012
Thiamine HCl	0.002	0.002
Co-factors		
p-Aminobenzoic acid	-	0.01
Biopterin	-	0.01
Hemin*	0.0077	0.0077
Lipoic acid	-	0.01
Metals		
$CaCl_2$	-	0.72
$CoCl_2$	-	0.000032
$CuSO_4$	-	0.00003
$Fe(NO_3)_3$ $9H_2O$	-	0.009
$MnCl_2$	-	0.000045
$MgSO_4$ $7H_2O$	-	0.81
$ZnCl_2$	-	0.03
Amino Acids		
L-Arginine HCl	0.5	0.5
L-Leucine	0.5	0.5
L-Lysine HCl	0.5	0.5
L-Phenylalanine	0.5	0.5
L-Tryptophan	0.5	0.5
L-Valine	0.5	0.5
L-Alanine	0.5	0.5
L-Asparagine	0.5	0.5
L-Aspartic acid	0.5	0.5
L-Cysteine	0.5	0.5
L-Glutamic acid	0.5	0.5
L-Glutamine	0.5	0.5
Glycine	0.5	0.5
L-Histidine HCl H_2O	0.5	0.5
L-Isoleucine	0.5	0.5
L-Methionine	0.5	0.5
L-Proline	0.5	0.5
L-Serine	0.5	0.5
L-Threonine	0.5	0.5
L-Tyrosine	0.5	0.5
Other		
Adenosine	0.074	0.074
Choline chloride	0.007	0.007
D-Glucose	16.6	16.6
HEPES	25.01	25.01
myo-Inositol	0.01	0.01
Sodium pyruvate	0.99	0.99

Assessment of amino acid requirements

RE IX medium included the 12 amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, and glutamine. Arginine, for which *Leishmania* appear to be auxotrophic [13, 14, 41, 42], was not included. To investigate the influence of a complex amino acid content over promastigote growth, we modified the composition of RE IX to include equimolar concentrations (0.5 mM) of all 20 proteogenic amino acids and found that this medium, henceforth referred to as basal medium (BM) (Table 2), significantly enhanced growth of *L. mexicana* promastigotes compared with RE IX (Figure 1A).

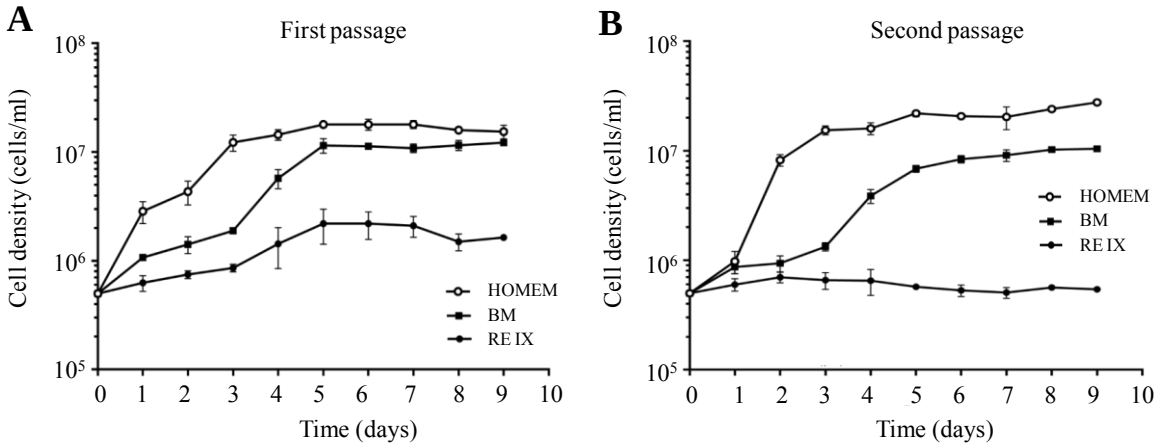

Figure 1. Comparison of growth of *L. mexicana* promastigotes in semi-defined serum-supplemented and defined media. A. Growth of *L. mexicana* promastigotes in RE IX medium (black circles), basal medium (BM) (squares), and HOMEM medium (white circles) shown over a 9 day period. B. Growth of *L. mexicana* promastigotes sub-passaged on day 4 in fresh RE IX, BM, and HOMEM media shown over a 9 day period. Abbreviations: BM, basal medium; HOMEM, hemoflagellate-modified minimum essential medium. Error bars = mean ± standard deviation (SD); n=15.

Assessment of metals and cofactor requirements

As shown by the growth analysis, the BM medium supported robust growth of *L. mexicana* promastigotes (Figure 1A). Nevertheless, there was a pronounced lag phase in the replication rate of freshly passaged cultures, and growth was reduced when cultures were sub-passaged in BM (Figure 1B). We therefore tested the importance of a range of co-factors, vitamins, and metals for promastigote growth. Initial growth analyses (data not included) defined which compounds supported *L. mexicana* promastigote growth and the optimal concentration, and those were consequently used in the requirement analyses. The latter revealed that addition of 10µM of biopterin alone or 10µM of biopterin, folic acid, lipoic acid, and riboflavin to BM led to a decrease in the time needed by the promastigotes to double in number (Figure 2A). Supplementing BM with magnesium, calcium, zinc, iron, cobalt, copper, and manganese also promoted a significant reduction in the doubling time (Figure 2B). Addition altogether of co-factors, vitamins, and metals to BM further reduced the doubling time of the promastigotes (Figure 2B). Higher concentrations of the seven metals, however, inhibited promastigote growth (data not included).

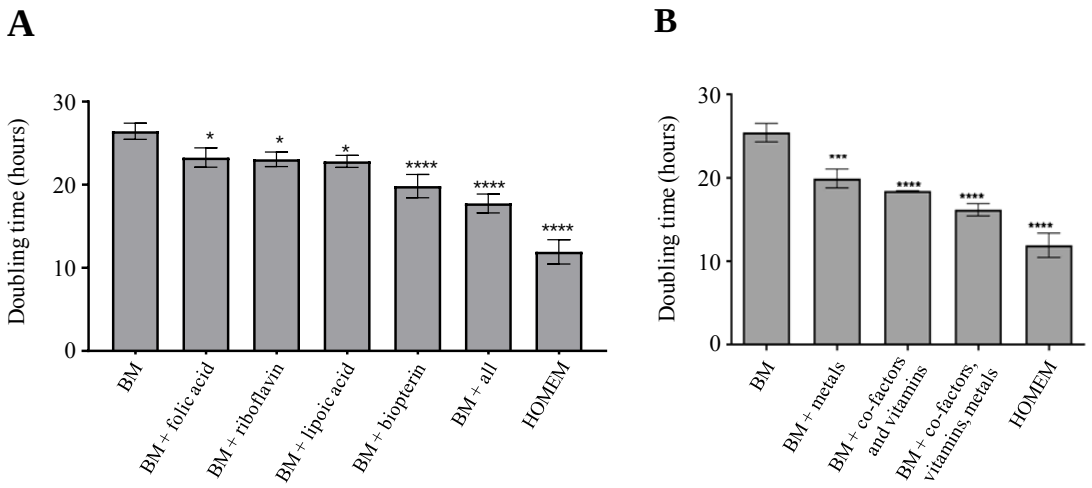

Figure 2. Effect of co-factors, vitamins, and metals on growth of *L. mexicana* promastigotes. Abbreviations: BM, base medium; HOMEM, hemoflagellate-modified minimum essential medium. Error bars = mean ± SD; n=15; ****, $p<0.0001$; ***, $p<0.0005$, *, $p<0.05$.

Assessment of promastigote growth in defined Nayak medium

Addition of metals and cofactors to BM resulted in a decrease in the doubling time of *L. mexicana* promastigotes from 30 to 16 h, compared with a doubling time of 12 h in HOMEM. We then tested the ability of the supplemented BM, henceforth referred to as Nayak medium (NM), to support continuous growth of *L. mexicana* promastigotes in sequential passages. We established stable cultures which we were able to maintain for more than 15 sub-passages (Figure 3A). We followed the growth of the promastigotes cultured in NM and HOMEM and observed comparable rates (Figure 3A), although the density of stationary phase promastigotes grown in NM was somewhat lower (data not included). Growth comparison between *L. mexicana*, *L. donovani*, and *L. major* promastigotes additionally revealed that all three species had analogous growth patterns (Figure 3B). Phenotypic comparison between *L. mexicana* promastigotes cultured in NM and HOMEM furthermore showed that log-phase promastigotes grown in NM had shorter body length (Figure 3, C and D) but higher cell volume (data not included) compared with log-phase promastigotes grown in HOMEM, while the opposite, longer body length (Figure 3, C and D) and lower cell volume (data not included), was observed for stationary phase promastigotes grown in NM compared with log-phase promastigotes grown in HOMEM.

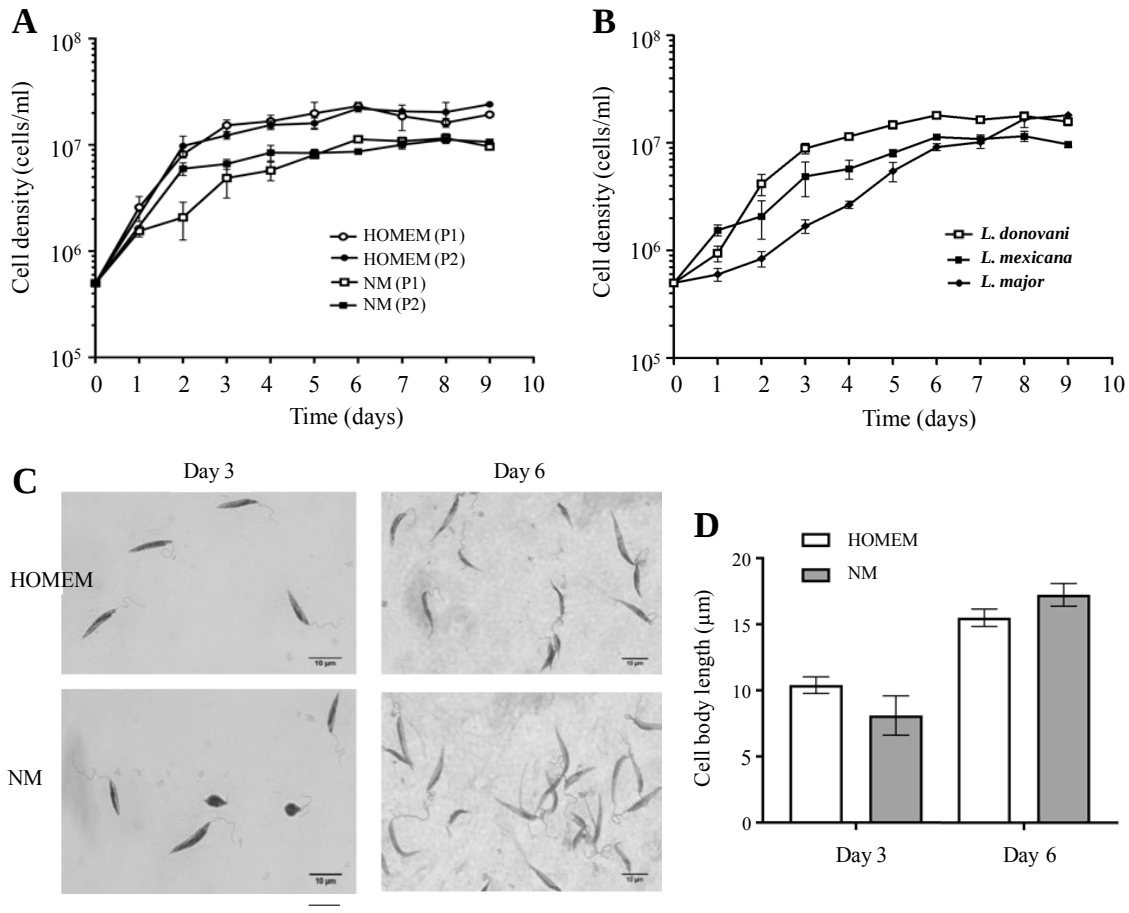

Figure 3. Growth and morphometric properties of *Leishmania* promastigotes grown in defined Nayak medium. A. Growth kinetics of *L. mexicana* promastigotes cultured in NM and HOMEM. B. Growth kinetics of *L. donovani* (white squares), *L. mexicana* (black squares), and *L. major* (diamonds) promastigotes cultured in NM. C. Representative images of log phase (day 3) and stationary phase (day 6) *L. mexicana* promastigotes grown in NM and HOMEM. The standard scale bar is 10µm. D. Morphometric comparison of body length of *L. mexicana* promastigotes grown in NM and HOMEM. Abbreviations: NM, Nayak medium; HOMEM, hemoflagellate-modified minimum essential medium; P1, first passage; P2, second passage. Error bars = mean ± SD; n=15.

Assessment of relative importance of specific amino acids

All amino acids in the NM medium were supplemented in free form, allowing to study the importance of each amino acid by individually omitting them from the medium. To assess the relative contribution of each amino acid to the growth of *L. mexicana* promastigotes, we created 20 single amino acid "knock out" media. Our growth assessment showed that omission of phenylalanine and tryptophan halted cell division within 4 h while the absence of arginine, lysine, and leucine resulted in considerably impaired, almost negligible, promastigote growth (Figure 4A). For each of these conditions, no viable cells were observed after 6 days in the respective cultures (data not included). Omission of valine led to very slow growth; absence of aspartate, cysteine, glutamate, glutamine, histidine, isoleucine, methionine, serine, threonine, and tyrosine resulted in an increase in the doubling time; and finally, absence of alanine, asparagine, glycine, and proline imposed no effect over promastigote growth when compared to promastigote growth in NM (Figure 4A).

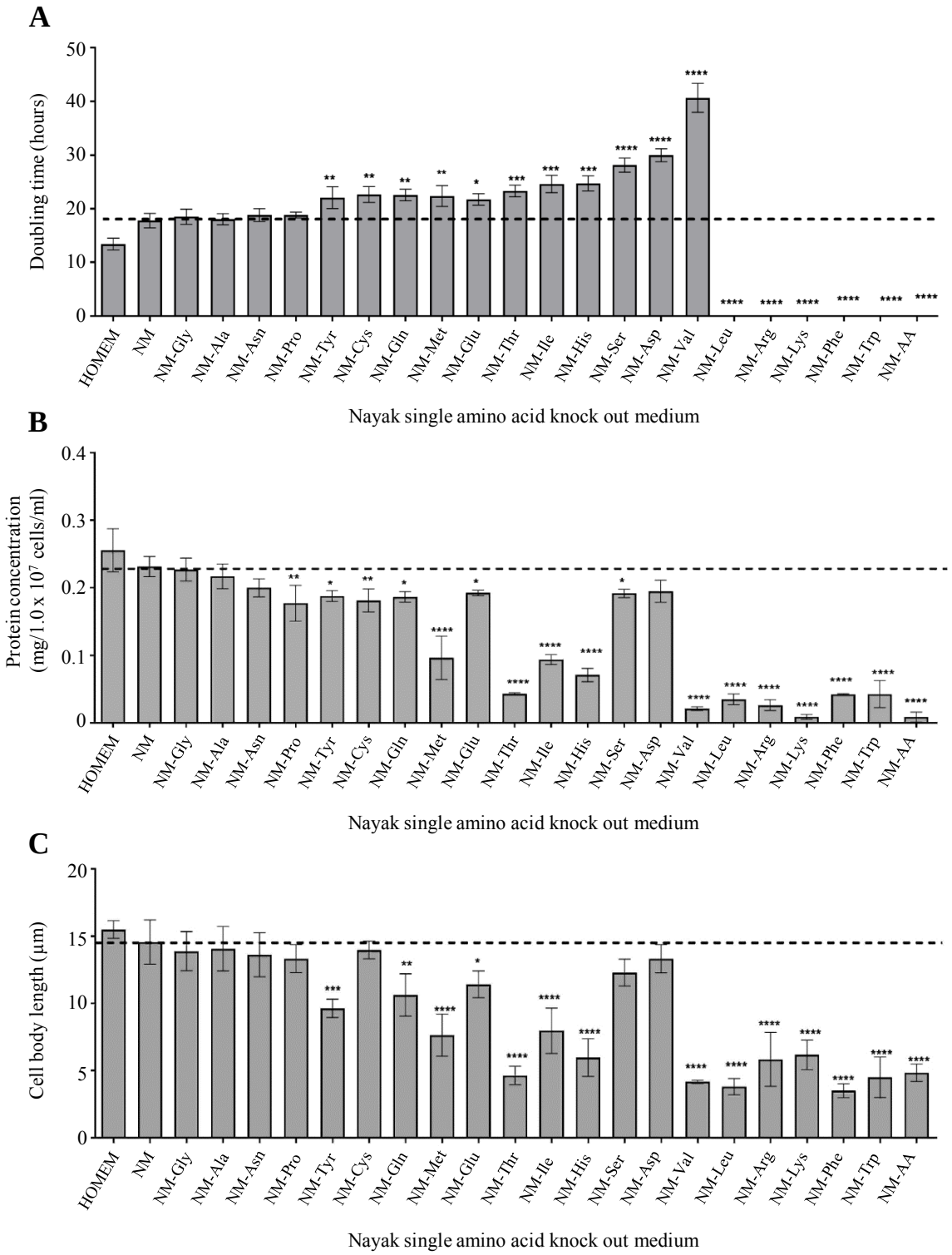

Figure 4. Single amino acid "knock out" media analysis. A. Growth of *L. mexicana* promastigotes in NM single amino acid "knock out" media, expressed as doubling time. B. Protein content of *L. mexicana* promastigotes grown in single amino acid "knock out" media. C. Comparison of cell size of *L. mexicana* promastigotes grown in single amino acid "knock out" media. Abbreviations: HOMEM, hemoflagellate-modified minimum essential medium; NM, Nayak medium; Gly, glycine; Ala, alanine; Asn, asparagine; Pro, proline; Tyr, tyrosine; Cys, cysteine; Gln, glutamine; Met, methionine; Glu, glutamate; Thr, threonine; Ile, isoleucine; His, histidine; Ser, serine; Asp, aspartate; Val, valine; Leu, leucine; Arg, arginine; Lys, lysine; Phe, phenylalanine; Trp, tryptophan; NM-Trp, Nayak medium without tryptophan; NM-AA, Nayak medium without amino acids. Error bars = mean ± SD; n=9; ****, $p<0.0001$; ***, $p<0.0005$, **, $p<0.001$, *, $p<0.05$.

Absence of several amino acids is associated with reduced protein synthesis in *Leishmania* [43, 44]. To evaluate the importance of each amino acid for protein synthesis, we maintained *L. mexicana* promastigotes for 6 days in NM single amino acid "knock out" media. As expected, the protein content of promastigotes grown in the same media that did not support cell growth, namely the media without arginine, leucine, lysine, phenylalanine, and tryptophan (Figure 4A), was also dramatically decreased (Figure 4B). More surprising, however, was the observation that the omission of histidine, isoleucine, methionine, and threonine, which only moderately reduced promastigote growth, led to an equally pronounced decrease in protein content (Figure 4B). Reduced protein content suggested that cell size might also be decreased, so we undertook a morphometric analysis of the cells grown in the amino acid "knock out" media. We found a direct correlation between protein content and cell size, namely, that the cells that had lower protein content (Figure 4B) had also dramatically reduced cell size (Figure 4C).

Assessment of promastigote infective competence in defined Nayak medium

The infectivity of *L. mexicana* promastigotes grown in HOMEM and NM was compared *in vitro* by infecting differentiated THP1 macrophages. Intensity and prevalence of infection was similar for both (Figure 5).

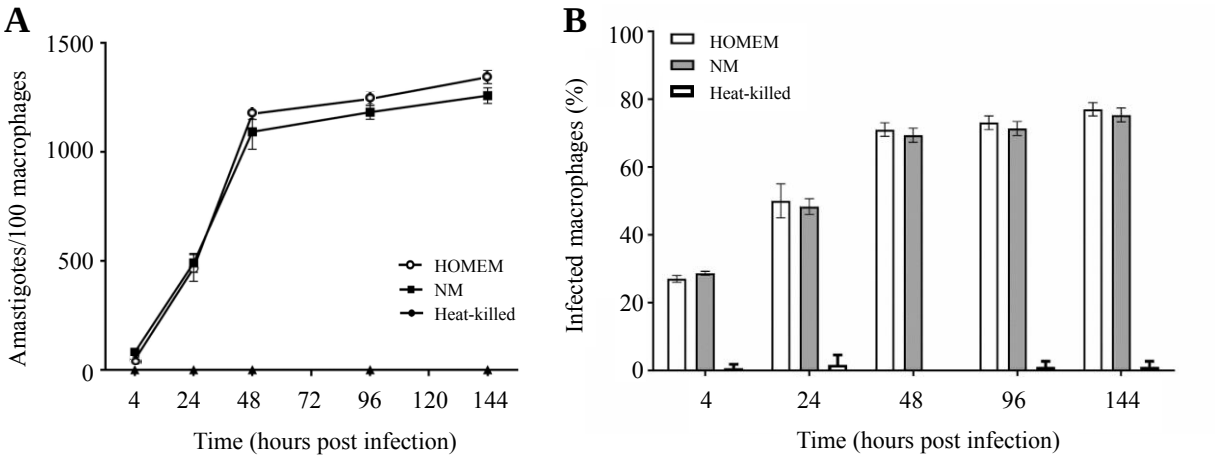

Figure 5. Evaluation of infectivity of *L. mexicana* promastigotes in defined Nayak medium. A. Number of amastigotes per 100 macrophages infected with *L. mexicana* promastigotes grown in HOMEM (circles), NM (squares), and heat-killed parasites (triangles). The latter was used as a negative control. B. Percentage of infected macrophages with *L. mexicana* promastigotes grown in HOMEM (white bars) and NM (grey bars), and heat-killed parasites (thick-lined white bars). Abbreviations: NM, Nayak medium; HOMEM, hemoflagellate-modified minimum essential medium. Error bars = mean ± SD; n=9.

Assessment of promastigote sensitivity to anti-leishmanial drugs

Drug sensitivity of promastigotes grown in HOMEM and NM was compared using the Alamar blue assay [40]. The IC_{50} measured for methotrexate and pentamidine were significantly lower when cells were grown in NM compared with HOMEM, while the IC_{50} for amphotericin B was similar under both conditions (Figure 6).

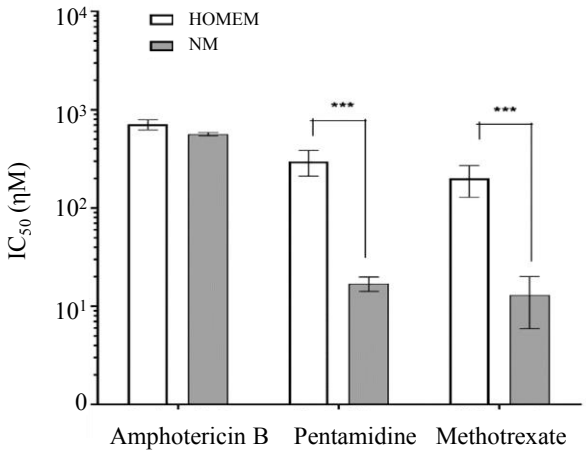

Figure 6. Drug sensitivity of *L. mexicana* promastigotes grown in defined Nayak medium. Toxicity of amphotericin B, pentamidine, and methotrexate on promastigotes grown in HOMEM (white bars) and NM (grey bars) was tested by an Alamar blue assay. Abbreviations: NM, Nayak medium; HOMEM, hemoflagellate-modified minimum essential medium. Error bars = mean ± SD; n=6; ***, $p<0.001$.

Discussion

Genomic analysis of *Leishmania* has yielded extensive information about their evolution, distribution, life-cycle, metabolism, and pathogenesis, and has suggested that different species have distinct strategies to cope with the environmental challenges they encounter during development [42, 45 - 49]. However, a recent genomic comparison of several species of *Leishmania* revealed minimal variation in the genome of these organisms [50 - 55]. That suggested that species-specific features are the result of post-transcriptional regulatory mechanisms. The transcriptome, and particularly the proteome and metabolome of *Leishmania*, are highly dynamic and detection of subtle differences in the abundance of components of these systems requires the use of tightly controlled experimental conditions and cutting-edge instrumentation. The use of undefined culture media can confound the interpretation of proteomic and metabolomics datasets, because the proteome or metabolome that is analysed is of mixed origin. The development of defined media could remedy this issue, as well as potentially reduce the cost and the risk of contamination with infectious agents. To facilitate proteomics and metabolomics analysis of *Leishmania*, we have developed a simple, chemically defined growth medium, termed Nayak medium (NM), that supports continuous parasite growth, allows determination of nutrient requirements, and enables proteomics and metabolomics analysis of *Leishmania* to be performed on samples that comprise proteins and metabolites of solely parasite origin.

Leishmania growth in Nayak medium

Comparative growth analyses in NM revealed that *L. mexicana* promastigotes grow at a marginally slower rate and to a lower stationary-phase density but show similar morphology and macrophage infectivity to cells grown in our standard rich, serum-supplemented culture

media (HOMEM). Interestingly, we found that the IC_{50} for pentamidine and methotrexate was significantly lower in promastigotes grown in NM, compared with HOMEM. We hypothesize that this may be result from interactions between these compounds and serum proteins that are present in HOMEM, and we are currently testing this possibility. Importantly, promastigote growth can be maintained indefinitely in this defined medium, confirming that the latter includes all nutrients required by the organisms, and will permit the generation of large quantities of biological material. Among the medium components in NM, we chose to further investigate amino acid, co-factor, vitamin, and metal requirements.

Amino acids essential for *Leishmania* viability

Arginine, leucine, lysine, phenylalanine, tryptophan, and valine

Amino acids are primary nutrients and as such are acquired or synthesized by organisms. Bioinformatic analysis of *Leishmania* genome sequence datasets suggests they are able to synthesize alanine, aspartate, asparagine, cysteine, glutamate, glutamine, proline, serine, methionine and threonine but must salvage arginine, histidine, isoleucine, leucine, lysine, phenylalanine, tryptophan, tyrosine, and valine from the environment [42]. We tested the importance of each of these substrates for *Leishmania* viability in NM, a background where the only appreciable alternative carbon source was glucose, and were able to confirm that a subset of essential amino acids, namely arginine, leucine, lysine, phenylalanine, tryptophan, and valine (Table 3) are required by *L. mexicana* promastigotes to survive in culture for even a single passage (corresponding to fewer than 5 population doublings). Of these 6 amino acids, transporters only for arginine and lysine have been characterised in *Leishmania* and the respective permeases have been designated AAP3 [41] and AAP7 [56].

Internalised arginine is directed to intermediary metabolism for the synthesis of important biomolecules such as proteins, polyamines, and nitric oxide (Figure 7) [41]. Additionally, a recent metabolomic analysis has revealed that arginine is also converted to citrulline, argininosuccinate (the linking intermediate in the aspartate-to-fumarate interconversion), and argininic acid, and excreted as such by *Leishmania* (Figure 7) [57]. The same study further showed that *L. mexicana* utilizes more arginine compared with *L. major* and *L. donovani* which suggested that the amino acid may have discrete fate and function in the different *Leishmania* sub-species.

Lysine is involved in protein synthesis and is a key site for post-translational modifications (PTMs) of histone and non-histone proteins. Genomic analysis has confirmed that *Leishmania* contain genes for methyltransferases, demethylases, acetyltransferases, and deacetylases [50] and following characterisation of a number of histone acetyltransferase (HAT) genes has suggested that PTMs of lysine play a role in *Leishmania* survival [58 - 61]. No further metabolic function of lysine, however, has been described in *Leishmania*.

Leucine, through 4-methyl-2-oxopentanoate (4mop) and hydroxymethylglutaryl-CoA (HMG-CoA), and valine, through propionyl-CoA and succinyl-CoA, can be converted to acetyl-CoA and then metabolised through the tricarboxylic acid cycle (TCA cycle) (Figure 7) [42]. The products of leucine and valine degradation, namely 4mop and 3-hydroxyisovalerate (HMB),

respectively, are excreted by *Leishmania* [57] while the generated HMG-CoA is used in isoprenoid synthesis [62].

Phenylalanine can be converted to tyrosine, phenyllactate, and phenylpyruvate while tryptophan can be converted to indole 3-lactate, indole 3-pyruvate, indole 3-acetate, and kynurenate, which are all excreted by *Leishmania* (Figure 7) [42, 57]. No intracellular metabolic function has been assigned to these metabolites. It has been proposed, however, that when excreted, the products of aromatic amino acid breakdown are involved in parasite-host interactions and pathogenesis [63, 64].

Table 3. Relative importance of individual amino acids in Nayak defined medium. Amino acid colours correspond to those in Figure 7 and reflect their importance.

Critical for viability	Support growth	Support protein synthesis	Non-essential (in the presence o f glucose)
L-Arginine	L-Aspartate	L-Histidine	L-Alanine
L-Leucine	L-Glutamate	L-Isoleucine	L-Asparagine
L-Lysine	L-Glutamine	L-Methionine	L-Cyteine
L-Phenylalanine	L-Serine	L-Threonine	Glycine
L-Tryptophan			L-Proline
L-Valine			L-Tyrosine

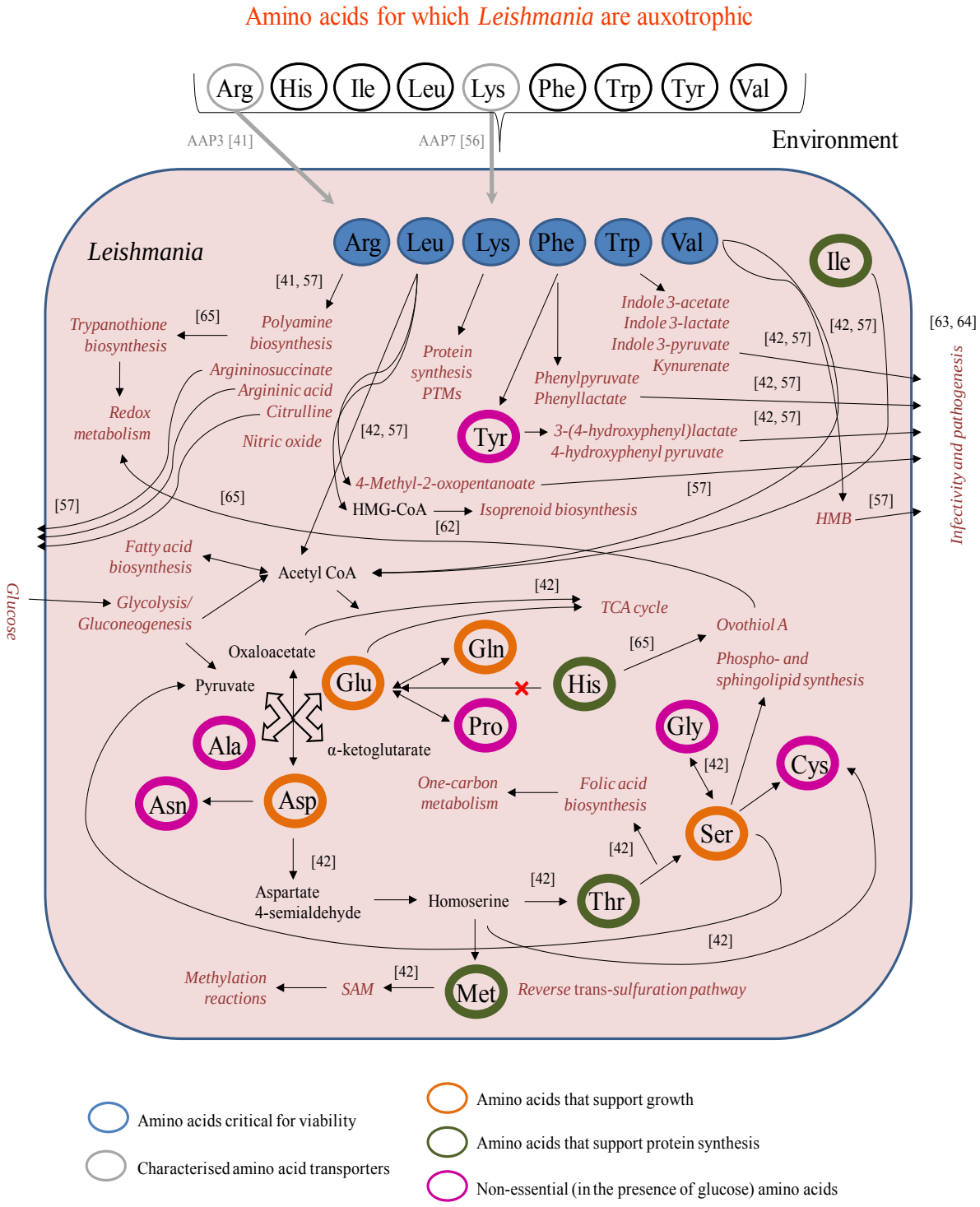

Figure 7. Amino acids shown to be crucial for *Leishmania mexicana* viability in defined Nayak medium. Abbreviations: AAP, amino acid permease; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Cys, cysteine; Glu, glutamate; Gln, glutamine; Gly, glycine; His, histidine; HMB, 3-hydroxyisovalerate; HMG-CoA, hydroxymethylglutaryl-CoA; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; SAM, S-adenosyl-L-methionine; Ser, serine; TCA, tricarboxylic acid cycle; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

Amino acids required for growth and protein synthesis in *Leishmania*

The observation that arginine, leucine, lysine, phenylalanine, tryptophan, and valine were crucial for promastigote survival was not surprising, as bioinformatics analyses suggest that *Leishmania* are auxotrophic for these amino acids [42]. They are also predicted to be auxotrophic for isoleucine and histidine, but we observed moderate growth in media lacking these amino acids. However, the protein content and size of these cells was dramatically reduced, suggesting that isoleucine and histidine limitation does impact the cells. It is likely that growth would not be sustained in a subsequent passage into histidine or isoleucine knockout media. The histidine-glutamate pathway does not operate in *Leishmania* and the amino acid thus cannot be used as an energy source [42]. Histidine, instead, serves as a precursor for ovothiol A and as such is proposed to be involved in defence against oxidative stress [65]; a property that may be dispensable in axenic culture but critical in the host. Isoleucine is converted to acetyl-CoA and used in lipid synthesis [66].

Tyrosine can be synthesized from phenylalanine and excreted as 3-(4-hydroxyphenyl)lactate and 4-hydroxyphenil pyruvate [42, 57]. In 2014, Moreno and colleagues elucidated the crystal structure of *L. infantum* tyrosine aminotransferase (TAT) and showed that TAT is a cytoplasmic enzyme of the Iγ subfamily of aminotransferases that is expressed at a higher rate in amastigotes [63, 64]. The latter suggests that tyrosine, similar to other aromatic amino acids whose oxidation end-products have been related to *Leishmania* infectivity in macrophages [63, 64], could be more important, and even vital, for *Leishmania* amastigotes.

Culture of promastigotes in NM that was deficient in lysine resulted in a profound decrease in protein synthesis that was equal to that observed in promastigotes grown without any amino acid. Similarly, omission of methionine and threonine significantly inhibited protein synthesis while that of aspartate and serine slowed, to a great extent, promastigote growth. Aspartate serves as a carbon and energy source and supports asparagine synthesis (Figure 7), TCA cycle anaplerosis, and carbohydrate synthesis via gluconeogenesis [42, 67 - 69]. Serine and methionine are precursors for cysteine (Figure 7) [70] while serine, glycine, and threonine can reversibly be converted into each other by the serine hydroxymethyltransferase (SHMT) and tetrahydrofolate (THF)-dependent glycine cleavage system (GCS) [42]. SHMT and GCS, additionally, are involved in the folic acid biosynthesis pathway whose operation leads to the formation of one-carbon units that are used for the synthesis of thymidylate, purines, and methionine [71, 72]. The latter is a precursor for S-adenosyl-L-methionine (SAM) (Figure 7) which is used in polyamine synthesis [73] and as a methyl donor in various methylation reactions. Cysteine, glycine, and glutamate, in turn, are precursors for glutathione whose conjugation with spermidine leads to the generation of trypanothione, the central antioxidant of trypasomatids [65]. Serine, additionally, is an important building block in phospho- and sphingolipid biosynthesis while threonine is used for production of acetyl-CoA and acetate (unpublished data). Interestingly, aspartate, glutamate, glutamine and serine, which markedly promote promastigote growth are among the most abundant amino acids in the honeydew on which the insect vectors of *Leishmania* feed [74 - 76].

Of the 20 proteogenic amino acids, only alanine, asparagine, glycine, and proline could be

omitted from NM without significant consequences to promastigote growth. These amino acids can directly be synthesized from pyruvate, aspartate, serine, and glutamate, respectively, and may not be of benefit when the latter substrates are in sufficient quantities.

Alanine plays a key role in osmoregulation [77, 78] and proline, which is a primary carbon and energy source for *L. mexicana* promastigotes when glucose cannot be acquired [67], is not as important under amino acid- and glucose-replete conditions. Metabolism of alanine, asparagine, glycine, and proline might be of greater importance in the gut of the insect vector where energy substrates are variable and osmotic challenges more extreme. In the defined NM medium, however, the four amino acids are non-essential.

Co-factors, vitamins, and metals required for *Leishmania* viability

Previous studies on medium formulation and nutrient requirements and transport have established that *Leishmania* must salvage biotin, pterins, folic acid, pantothenate, pyridoxine, riboflavin, nicotinate, and heme from exogenous sources [10, 33, 42, 72, 79 - 81]. In this study, we showed that biopterin has the highest impact on *in vitro* growth of promastigotes, followed by lipoic acid, folic acid, riboflavin, and *p*-aminobenzoic acid (pABA). *Leishmania* encode multiple genes for folate/biopterin transporters [81 – 84]. When internalised, folic acid is conjugated to pteridine moieties and pABA to form folates which are reduced by pteridine reductase (PTR1) and dihydrofolate reductase–thymidylate synthase (DHFR-TS) and used as co-factors in one-carbon metabolism (Figure 8) [42, 72]. The latter is interweaved with many essential processes, such as the synthesis of thymidylate, methionine, and 10-formyltetrahydrofolate (required for mitochondrial protein synthesis), in *Leishmania* [71, 72, 81]. Folate transport and metabolism, furthermore, have been associated with drug resistance in *Leishmania* [72, 81, 82] and are validated chemotheraputic targets.

Lipoic acid and its reduced form, dihydrolipoic acids, are highly reactive antioxidants which scavenge reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, singlet oxygen, chromanoxyl radicals of vitamin E, and ascorbyl radicals of vitamin C [85, 86]. Lipoic acid, additionally, is an essential co-factor in several multienzyme complexes, such as pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH), and branched-chain 2-oxo acid dehydrogenase (BCDH) complexes [87]. Similarly, riboflavin, in the form of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), is a co-factor in many flavoproteins which participate in reduction-oxidation reactions, including such leading to energy generation (Figure 8).

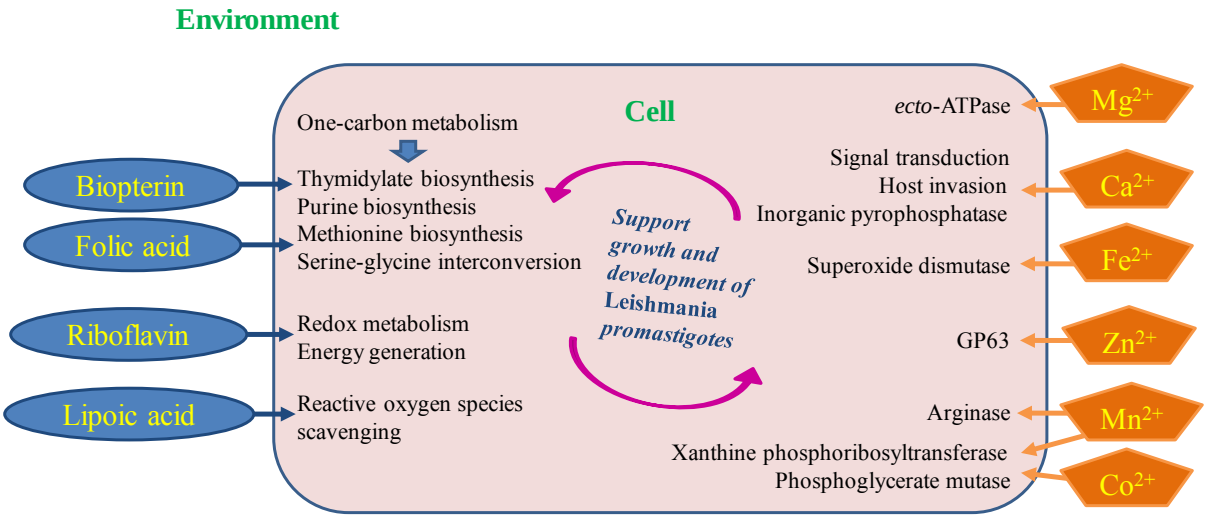

Figure 8. Co-factors, vitamins, and metals shown to be important for *Leishmania* growth in defined Nayak medium. Abbreviations: Mg^{2+}, magnesium ion; Ca^{2+}, calcium ion; Fe^{2+}, ferrous ion; Zn^{2+}, zinc ion; Mn^{2+}, manganese ion; Co^{2+}, cobalt ion.

Although we observed growth without specific addition of metals, it is likely that some metal ions are required for growth, and we cannot exclude the possibility that trace amounts of essential metals are added as impurities in other medium components. Nevertheless, we observed that addition of calcium (Ca^{2+}), cobalt (Co^{2+}), copper (Cu^{2+}), iron (Fe^{2+}), manganese (Mn^{2+}), magnesium (Mg^{2+}), and zinc (Zn^{2+}) were important for *Leishmania* growth (Figure 8). Co^{2+}, Cu^{2+}, and Mn^{2+} were effective at much lower concentrations than the other 4 metals. Accordingly, it has been determined that Mg^{2+}, Zn^{2+}, and Fe^{2+} are the most abundant divalent cations in the cytosol of the related organism, *Trypanosoma brucei,* whereas Co^{2+} is present in trace amounts [88]. Two potential CorA-like Mg^{2+} transporters, designated MGT1 and MGT2, have recently been identified in *L. major* and were shown to be important for parasite propagation and infectivity in mice [89]. Mg^{2+}, similar to the other cations discussed here, is an important metal biocatalyst and as such, it has been linked to activation of enzymes such as the *ecto*-ATPase of *L. tropica* which has been implicated in virulence (Figure 8) [90]. Ca^{2+} is stored in acidic compartments termed acidocalcisomes and plays a critical role in signal transduction and host invasion in *Leishmania* [91]. A number of genes encoding putative Ca^{2+}-binding proteins have been identified in the genome of trypanosomatids, and some of these proteins, including protein kinases, peptidases, peroxiredoxins, and ion channels, have been characterised in *Leishmania* [91 - 93]. Metabolically, Ca^{2+} is required for the proper operation of enzymes such as the inorganic pyrophosphatase of *L. major* (Figure 8) [94].

Acquisition of external iron has recently been elucidated and involves the conversion of Fe^{3+} into Fe^{2+} and import of the latter in the *Leishmania* milieu [95]. Fe^{2+}, Zn^{2+}, and Mn^{2+} are components of three well-characterised metalloenzymes of *Leishmania*, namely the antioxidant and virulence factor superoxide dismutase (SOD) [96], the major surface glycoprotein and virulence factor GP63 [97, 98], and arginase [87] (Figure 8). All three enzymes have been associated with host invasion, evasion of host immune response, and establishment of infection [96 - 99]. Mn^{2+}, additionally, is a co-factor for xanthine

phosphoribosyltransferase [100] while Co^{2+} is required by phosphoglycerate mutase [101] (Figure 8).

Although NM was developed as a simple defined medium, our study suggests that many components are not essential for *L. mexicana* growth, as observed for most amino acids. We have tested still more simple media compositions but we observed that growth rates and sustainable growth are reduced as components are removed (data not shown), limiting the use of such minimal media to studies where there is a specific advantage to its use

The development of a defined medium for the axenic culture of amastigotes would be of great use, though this may be more challenging as many of the factors present in animal sera will be relevant to the parasite persistence in the vertebrate host. Accumulating data suggests that amino acids are key substrates throughout the *Leishmania* life-cycle, and our data underscore the relative order of importance of the 20 proteogenic amino acids for parasite growth. Although their phenotypes are very different, exogenous amino acids that are essential to promastigotes are likely essential also in amastigotes, and the routes by which these are acquired and metabolised represent potential drug targets.

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Author Contributions

AN and SA contributed equally to this work. AN contributed to experimental work, data analysis and writing. SA contributed to data analysis and writing. MB contributed to experimental design and writing. RB conceived and directed the study and contributed to data analysis and writing.

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Notes

The authors declare no competing financial interest.

Abbreviations

4mop, 4-methyl-2-oxopentanoate; AAP, amino acid permease; Ala, alanine; ANOVA, Analysis of Variance; Arg, arginine; Asn, asparagine; Asp, aspartate; BCDH, branched-chain 2-oxo acid dehydrogenase; BM, base medium; Ca, calcium; Co, cobalt; Cu, copper; Cys, cysteine; DHFR-TS, dihydrofolate reductase–thymidylate synthase; FAD, flavin adenine dinucleotide; FBT, folate biopterin transporters; FCS, fetal calf serum; Fe, iron; FMN, flavin mononucleotide; GCS, glycine cleavage system; Glu, glutamate; Gln, glutamine; Gly, glycine; HAT, histone acetyltransferase; His, histidine; HMB, 3-hydroxyisovalerate; HMG-CoA, hydroxymethylglutaryl-CoA; HOMEM, hemoflagellate-modified minimum essential medium; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Mg, magnesium; Mn, manganese; NM, Nayak medium; NM-AA, Nayak medium lacking amino acids; OGDH, 2-oxoglutarate dehydrogenase; pABA, *p*-aminobenzoic acid; PBS, phosphate buffered saline; PDH, pyruvate dehydrogenase; PEG, Polyethylene glycol; Phe, phenylalanine; PMA, phorbol 12-myristate 13-acetate; Pro, proline; PTM, post-translational modification; PTR1, pteridine reductase; RT, room temperature; SAM, S-adenosyl-L-methionine; SD, standard deviation; Ser, serine; SOD, superoxide dismutase; TAT, tyrosine aminotransferase; TCA, tricarboxylic acid cycle; THF, tetrahydrofolate; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; Zn, zinc.

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