**Leishmania major** Expresses a Single Dihydroxyacetone Phosphate Ayltransferase Localized in the Glycosome, Important for Rapid Growth and Survival at High Cell Density and Essential for Virulence*

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Despite major advances in the understanding of pathogenesis of the human protozoan parasite *Leishmania major*, little is known about the enzymes and the primary precursors involved in the initial steps of synthesis of its major glycerolipids including those involved in virulence. We have previously demonstrated that the initial step of acylation of the precursor glycerol 3-phosphate is not essential for the synthesis of ester and ether phospholipids in this parasite. Here we show that *Leishmania* expresses a single acyltransferase with high specificity for the precursor dihydroxyacetone phosphate and shows the best activity in the presence of palmitoyl-CoA. We have identified and characterized the *Lm* DAT gene encoding this activity. *Lm* DAT complements the lethality resulting from the loss of both dihydroxyacetone phosphate and glycerol-3-phosphate acyltransferase activities in yeast. Recombinant *Lm* DAT exhibits biochemical properties similar to those of the native enzyme of the promastigote stage parasites. We show that *Lm* DAT is a glycosomal enzyme and its loss in a Δ*lmdat*Δ*lmdat* null mutant results in complete abrogation of the parasite dihydroxyacetone phosphate acyltransferase activity. Furthermore, lack of *Lm* DAT causes a major alteration in parasite division during the logarithmic phase of growth, an accelerated cell death during stationary phase, and loss of virulence. Together, our results demonstrate that *Lm* DAT is the only dihydroxyacetone phosphate acyltransferase of the *L. major* localized in the peroxisome, important for growth and survival and essential for virulence.

Worldwide, protozoan parasites of the genus *Leishmania* cause a large spectrum of important human diseases collectively named leishmaniasis. These parasites develop within the digestive tract of the sand fly vector as flagellated, mobile promastigotes and differentiate into and multiply as non-motile amastigotes within the phagolysosomal compartment of vertebrate host macrophages.

Glycerolipids constitute 70% of total lipids in the protozoan parasite *Leishmania* (1–3). They are classified into ester and ether lipids depending on the substitution at position 1 of the glycerol backbone. Ester lipids harbor an acyl group, whereas ether lipids carry a fatty alcohol moiety. Lipids of *Leishmania* parasites have been a focus of extensive studies because some of their derivatives, such as lipopolysaccharide and glycosylphosphatidylinositol anchored protease gp63, were shown to be important for parasite virulence and development (for review, see Refs. 4–8). Lipids are also essential cell constituents and, therefore, must be constantly synthesized to allow multiplication of the parasite. This suggests that the pathways leading to their synthesis are essential for parasite proliferation and pathogenesis and, thus, offer a reasonable target for rational design of new anti-leishmanial drugs. In fact, a lipid-based drug, miltefosine, is a potent antileishmanial compound that inhibits parasite growth in vitro and in vivo and is currently used for treatment of visceral and mucocutaneous forms of leishmaniasis (9–12). The acylation of dihydroxyacetone phosphate (DHAP) 3 by a DHAP acyltransferase (DHAPAT) represents the initial and obligatory step in the biosynthesis of ether lipids in most organisms that synthesize alklyglycerolipids (13). The product of this first acylation reaction, 1-acyl-DHAP, is then converted to 1-alkyl-DHAP by a FAD-dependent alky DHAP synthase (14), which is further reduced to 1-alkyl-glycerol-3-phosphate (1-alkyl-G3P) by an NADPH-dependent 1-acyl/1-alkyl-DHAP reductase. 1-Alkyl-G3P serves as the obligate precursor for all ether phospholipids. Alternatively, 1-acyl-DHAP can be reduced to 1-acyl-G3P by an NADPH-dependent 1-alkyl/1-acyl-DHAP reductase, which is then used for the biosynthesis of ester phospholipids. The relative contribution of the DHAP acylation step in the biosynthesis of ester phospholipids has not yet been firmly established (15, 16).

DHAPAT activity has been characterized biochemically in different organisms (17–19). In most animal tissues, DHAPAT is found in a membrane-bound fraction (15, 20) and localized in the luminal side of peroxisomes (15, 21). This enzyme was also found to be part of a heterotrimeric complex that includes the 1-alkyl-DHAP synthase (22, 23). Alterations in DHAPAT function have been associated with various human diseases such as neonatal adrenoleukodystrophy, infantile Refsum, disease, hyperpippecolic academia, and rhizomelic chondrodysplasia punctata (24–26).

We have previously reported the characterization of the first acyltransferase *Lm* DAT specific for the lipid precursor G3P. We showed that this activity was encoded by a single gene, *Lm* DAT, whose deletion resulted in a major defect in the synthesis of triacylglycerols but had little or no effect on the biosynthesis of ester and ether phospholipids, suggesting that G3P is not the primary lipid precursor for membrane biogenesis in *Leishmania* promastigotes (27). In an attempt to evaluate the importance of dihydroxyacetone phosphate in parasite lipid metab-

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3 The abbreviations used are: DHAP, dihydroxyacetone phosphate; DHAPAT, DHAP acyltransferase; G3P, glycerol 3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; PrI, propidium iodide; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; PrI, propidium iodide.

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Characterization of Leishmania DHAPAT

**TABLE 1**

Yeast strains used in this work

| Strains | Genotype | Source |
|---------|----------|--------|
| cmy228 (Sc21) | MATα his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1Δ::TRP1 gat2Δ::HIS3 ura3-1 [pGAL1::GAT1 URA3] | Ref. 32 |
| gat1Δgat2Δ + [GAL1::GAT1] + [ADH1::LmDAT] (Sc9) | MATα his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1Δ::TRP1 gat2Δ::HIS3 ura3-1 [pGAL1::GAT1 URA3] | This work |
| gat1Δgat2Δ + [GAL1::GAT1] + [ADH1] (Sc4) | MATα his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1Δ::TRP1 gat2Δ::HIS3 ura3-1 [pGAL1::GAT1 URA3] | This work |
| gat1Δgat2Δ + [ADH1::LmDAT] (Sc12) | MATα his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1Δ::TRP1 gat2Δ::HIS3 ura3-1 [pBEVY-L.LmDAT] | This work |

olism and development, we have isolated and characterized the gene _LmDAT_ encoding the DHAPAT enzyme involved in the acylation of this precursor. We provide evidence that _LmDAT_ is the only dihydroxyacetone phosphate acetyltransferase of the parasite, residing in peroxisome-like organelles termed glycosomes in _Leishmania_ and related parasites, important for optimal growth during the exponential phase and for survival at high cell density and essential for virulence. These data suggest that acylation of DHAP may represent the major pathway for the synthesis of glycerolipids in _Leishmania_.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—Promastigotes of _Leishmania major_ Friedlin V1 strain (MHOM/IL/80/Friedlin) were maintained in liquid and semi-solid M199-derived medium (28). Transfection was performed according to Ngo et al. (29), and selection was applied as appropriate in the presence of G418, nourseothricin, blasticidin, or puromycin (10, 150, 5, and 50 μg/ml, respectively). To examine parasite proliferation, parasites were collected at mid-log phase, diluted to 5 × 10^5 parasites/ml, and incubated at 26 °C. Growth was monitored by collecting samples over time and counting them using a hemacytometer. Limiting dilution assays were carried out by diluting parasite cultures with fresh media to 5 parasites/ml. 200-μl samples of this diluted culture were transferred into a 96-well plate. The plates were incubated for 2 weeks at 26 °C, and the presence of parasites was monitored by light microscopy.

_Saccharomyces cerevisiae_ strains used in this study are listed in Table 1. Yeast was cultivated at 30 °C in YPD (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose), or synthetic minimal media supplemented as required to maintain cell growth. Limiting dilutions on solid YPD and YPGal plates were performed as described previously (27).  

**Plasmids—** _LmDAT_ was PCR-amplified from _L. major_ genomic DNA using the primer pair BN-LmDHAPAT-5′ (5′-GGGATCCCACTATAGAGCCTTCCACCCACTGCGG-3′) and LmDHAPAT-B-3′ (5′-GGGATCCCACTCTTGGATGCTGGTGT-3′). The resulting PCR product was digested with BamHI and ligated into the BamHI site of pBluescript II KS (+) (Invitrogen), resulting in pCR-3′- _LmDAT_. The 3′-untranslated region was excised from _LmDAT_ by PCR using the primers LmDAT 5′-GTCCGCTGGTGTAGTCTTGTTAT-3′ and LmDAT 5′-ACCGTCACTATTCGCTGCTGCT-3′. The resulting PCR product was digested with BamHI and ligated into the corresponding sites of pBluescript II KS (+) (Invitrogen), resulting in pCR-3′- _LmDAT_.

**Contra-selection to discard the** _ADH1_ **cassette**

| Yeast strains used in this work |
|--------------------------------|
| MATα his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1Δ::TRP1 gat2Δ::HIS3 ura3-1 [pGAL1::GAT1 URA3] | This work |

**DHAPAT activity** was measured as previously described (27).  

**Activity Assays**—The yeast strain _gat1Δgat2Δ + [ADH1::LmDAT]_ was grown in YPD to an _OD_ of 0.8–1.0. Cells were washed twice in ice-cold water, and cell pellets were frozen at −80 °C until use. Yeast and _Leishmania_ protein extracts were prepared as described previously (27, 32). Protein concentration was determined by the bicinchoninic acid assay using bovine serum albumin as a standard. Glycerol-3-phosphate acyltransferase (GPA) activity was measured by previously described (27) using 100–200 μg of protein. DHAPAT activity was measured.
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assessed by measuring the acylation rate of DHAP. This substrate was synthesized from fructose-1,6-bisphosphate as described by Bates and Saggerson (34). The assay was performed in a 0.5-ml final volume in a buffer containing 120 mM KCl, 50 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 8 mM NaF, 4 mg/ml fatty acid poor albumin, 100 μM acyl-CoA, 0.25 unit of aldolase, 7 units of triose phosphate isomerase, 0.1% CHAPS, and 0.5–1 mg of protein. Assays were performed at 30 °C unless otherwise stated. For the pH-dependent DHAPAT assay, a 100 mM sodium phosphate buffer set up at different pH values was used. The specificity of L-[U-¹⁴C]glycerol 3-phosphate (Amersham Biosciences) and L-[fructose-U-¹⁴C] 1,6-bisphosphate (MP Biomedicals) were 149 and 295 mCi/mmol, respectively. DHAPAT and GPAT reaction products were verified by analytical thin layer chromatography using Silica Gel 60A plates (Whatman) and a solvent made of chloroform, methanol, acetic acid, 5% aqueous sodium metabisulfite (75:30:9:3, v/v/v/v). The plates were sprayed with EN³HANCE (PerkinElmer Life Sciences) before autoradiography. The Rf values for 1-acyl-G3P and 1-acyl-DHAP were 2.1 and 3.5, respectively.

Malate dehydrogenase assay was performed in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.21 mM oxaloacetate and 0.26 mM NADH. The assay was initiated by the addition of 20 μg of Leishmania protein extracts, and the decrease in absorbance was monitored spectrophotometrically at a wavelength of 340 nm.

Microscopy—Differential interference contrast (DIC) analysis was performed using promastigotes fixed with 4% paraformaldehyde in phosphate-buffered saline and mounted on polylysine-coated slides. Immunofluorescence assay was performed with wild-type parasites alone or transformed with pL-BSD.HV-LmDAT (Ec247) as described previously (28). The recombinant His-5,5'-tagged HV-LmDAT was revealed with V5 monoclonal antibodies (Invitrogen) and hypoxanthine-guanine phosphoribosyltransferase with specific rabbit polyclonal immunoglobulins (generous gifts of A. Jardim). Both antibodies were used at a 1:500 dilution. Images were taken with a Nikon fluorescence microscope.

Analysis of Cell Death—Parasites were washed once in phosphate-buffered saline and stained in the presence of 1 μg/ml propidium iodide (PI) in phosphate-buffered saline for 10 min before being analyzed by flow cytometry.

Virulence Assays in Mice—Virulence assays were performed as described in Zufferere et al. (28). Briefly, parasites derived from a 3-day stationary culture were washed once in cold saline buffer. 1 × 10⁵ or 1 × 10⁶ cells were injected subcutaneously in the right rear footpad of 6–8-week-old female BALB/c mice (Charles River Laboratory). Infections were monitored by comparing the thickness of the footpad over time using a Vernier caliper.

RESULTS

Characterization of the Dihydroxyacetone Phosphate Acyltransferase Activity in L. major Promastigotes—Our genetic and biochemical characterization of the L. major GPAT enzyme LmGAT suggested that the lipid precursor G3P does not play an essential function in parasite development or biosynthesis of its major acyl- and alkylglycerolipids. To examine the importance of the second lipid precursor DHAP in parasite membrane biogenesis and development, we characterized its first acylation step (DHAPAT activity) in whole cell extracts. DHAPAT activity was linear during ~8 min of incubation and reached a plateau thereafter when measured at 30 °C (Fig. 1A). When the assay was performed at 0 °C, only marginal activity could be detected, indicating that this reaction is enzymatic. Kinetic studies were conducted by varying the concentration of fructose 1,6-bisphosphate to obtain a substrate saturation curve. DHAPAT activity reached its maximum when the substrate concentration was ~2 mM (Fig. 1B). From these data, a Vₘₐₓ of 208.8 ± 33.6 pmol/min/mg and a Kₘ of 0.47 ± 0.1 mM could be calculated, indicating that DHAPAT activity in Leishmania involves a relatively low affinity DHAPAT enzyme(s). The specificity of DHAPAT was assessed by testing different fatty acyl-CoA donors that differ in length and/or saturation. The best activities were obtained when palmitoyl-CoA was present in the assay, whereas myristoyl-CoA, stearoyl-CoA, and linoleoyl-CoA all resulted in lower activities. The least effective fatty acyl-CoA donors were dodecyl-, palmitoleoyl-, oleoyl-, and arachidoyl-CoA (Fig. 1C).

Identification of LmDAT—To isolate the gene encoding the L. major DHAPAT activity, we searched the L. major genome for proteins harboring the four signature motifs important for acyltransferase activity (35). In addition to the LmGAT gene, we identified an open reading frame of ~4.3 kilobases encoding a protein of 1436 amino acids, which we termed LmDAT (for dihydroxyacetone phosphate acyltransferase) that exhibits significant similarity (~35% identity and ~55% similarity) to other eukaryotic DHAPATs (Fig. 2B). The most conserved region in the enzyme encompasses amino acids 680–975 and harbors the four canonical domains involved in substrate recognition and catalysis (Fig. 2B (35, 36)). Interestingly, unlike human and other higher eukaryotic
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DHAPTs, LmDAT contains an unusual N-terminal domain of ~650 amino acids. Homologs of LmDAT in the Trypanosomatidae family such as Trypanosoma cruzi and Trypanosoma brucei also harbor this N-terminal domain and share a high degree of identity and similarity with LmDAT over the whole protein length. A specific homology search of available protein databases with this N-terminal domain failed to identify any similarity to known proteins. The C-terminal domain of LmDAT over the whole protein length. A specific homology search of available protein databases with this N-terminal domain failed to identify any similarity to known proteins. The C-terminal domain of LmDAT contains a signature tripeptide SKM for localization to the glycosomes (37).

Subcellular Localization of LmDAT—To determine the cellular localization of LmDAT, a hexahistidine-V5 tag was added to the N-terminal portion of the enzyme, and the resulting recombinant protein (HV-LmDAT) was expressed in wild-type Leishmania. Immunofluorescence analysis using a V5-specific monoclonal antibody revealed a signal diagnostic of glycosomal proteins. Indeed colocalization studies revealed a perfect colocalization between HV-LmDAT and the glycosomal marker, hypoxanthine-guanine phosphoribosyltransferase (38) (Fig. 3). No signal could be detected in non-transfected cells using anti-V5 antibody (Fig. 3). These data suggest that LmDAT is localized to the glycosomes (21).

Functional Analysis of LmDAT in Yeast—We have previously shown that the yeast S. cerevisiae can be used as a surrogate system to genetically and biochemically analyze the function of heterologous GPAT enzymes (27, 33). Yeast harbors two acyltransferases, GAT1 and GAT2, which catalyze the acylation of both G3P and DHAP (32, 39). GAT1 “gat2Δ” and gat1ΔGAT2” single deletion mutants are viable, whereas a double mutant, gat1Δgat2Δ, is lethal. To genetically assess the function of LmDAT, we examined whether LmDAT can rescue the lethal phenotype of the yeast double mutant gat1Δgat2Δ lacking the GAT1 and GAT2 genes (32, 39). To this end, we generated a strain gat1Δgat2Δ+[GAL1::GAT1]+[ADH1::LmDAT] that expresses LmDAT under the constitutive ADH1 promoter in the strain cmy228 (gat1Δgat2Δ+[pGAL1::GAT1 URA3]) (32). This strain bears chromosomal deletions of GAT1 and GAT2 but expresses episomally the endogenous GAT1 under the control of the inducible GAL1 promoter. A control strain, gat1Δgat2Δ+[GAL1::GAT1]+[ADH1], which carries the vector alone, was also generated. Both strains were serially diluted on complete media containing either glucose or galactose as a sole carbon source. As expected, both strains grew on galactose-containing media, which promotes expression of the GAT1 transgene (Fig. 4A). However, whereas the gat1Δgat2Δ+[GAL1::GAT1]+[ADH1::LmDAT] strain was capable of growing on glucose-containing media that represses GAT1 expression, the gat1Δgat2Δ+[GAL1::GAT1]+[ADH1] failed to give colonies on this medium, suggesting that LmDAT complements the loss of the endogenous GAT1 and GAT2 genes. We next selected 5-fluoroorotate-resistant derivatives of the gat1Δgat2Δ+[GAL1::GAT1]+[ADH1::LmDAT] strain that lost pGAL1::GAT1 URA3 plasmid. Consequently, a strain gat1Δgat2Δ+[ADH1::LmDAT], which lacks yeast GAT1 and GAT2 genes and relies for survival solely on LmDAT, was successfully obtained, thus eliminating the
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possibility of complementation due to expression of the endogenous GAT1 gene. The presence of LmDAT or the absence of the episome expressing the GAT1 gene in this strain was verified by PCR analyses using primers specific to GAT1 and LmDAT (Fig. 4B). As a positive control, PCR analyses using primers specific for the choline transporter gene HNM1 showed the presence of this gene in all strains (Fig. 4B). Together, these data demonstrate that LmDAT can functionally complement the loss of the yeast dual DHAPAT/GPAT acyltransferase activity.

We next characterized the activity of recombinant LmDAT in yeast. LmDAT DHAPAT activity measured in the complemented cell extracts at 30 °C was linear for ~6 min before reaching a plateau. In contrast, only minimal activity could be detected when the assay was carried out at 0 °C (Fig. 5A). The DHAP concentration in the reaction mixture was varied to obtain a substrate saturation curve. DHAPAT activity was optimal at a substrate concentration of ~2 mM, and from the Michaelis-Menten representation, a $K_m$ of 0.69 ± 0.06 mM and a $V_{max}$ of 2.39 ± 0.36 nmol/min × mg were obtained (Fig. 5B). The specificity of the fatty acyl-CoA donor was then determined by testing different chain lengths and saturated/non-saturated fatty acyl-CoA donors. LmDAT showed the best activity with palmitoyl-CoA. Lower activity was obtained with lauroyl-, myristoyl-, palmitoleoyl-, stearoyl-, linoleoyl-, oleoyl-, and arachidoyl-CoA (Fig. 5C). These results are consistent with the fatty acid composition of Leishmania membranes where C16, C18 fatty acids prevail (3, 28) and with the activity of the endogenous enzyme. To assess whether LmDAT can also acylate G3P, GPAT assays were performed. Although GPAT activity could be detected in LmDAT-expressing cells, this activity represented only ~8.5% of its DHAPAT activity (Fig. 5D).

![FIGURE 4. LmDAT complements the lethal phenotype of a yeast double mutant lacking GPAT and DHAPAT activity. A: Growth assay of $gat1$Δ $gat2A$ [GAL1::GAT1] + [ADH1::LmDAT] (1) and $gat1$Δ $gat2A$ [GAL1::GAT1] + [ADH1::LmDAT] (2) serially 10-fold diluted on complete medium containing either glucose or galactose as sole carbon source. The highest dilution contains $3 \times 10^5$ cells. Plates were incubated at 30 °C for 4 days. B, agarose gel electrophoresis of PCR products obtained by amplifying genomic DNA from $gat1$Δ $gat2A$ [GAL1::GAT1] (1), $gat1$Δ $gat2A$ [GAL1::GAT1] + [ADH1::LmDAT], (2), and $gat1$Δ $gat2A$ + [ADH1::LmDAT](3) with GAT1-, LmDAT-, and HNM1-specific (positive control) primers as described under “Experimental Procedures.”](image-url)

![FIGURE 5. GPAT and DHAPAT activities of LmDAT expressed in S. cerevisiae. The acylation rate of DHAP produced by catabolism of fructose-1,6-bisphosphate by an aldolase and triose phosphate isomerase measured as described under “Experimental Procedures.” A, time-dependent DHAPAT activity from cell extracts of $gat1$Δ $gat2A$ + [GAL1::LmDAT] grown in YPD. The assay was performed as described under “Experimental Procedures.” B, kinetics of DHAPAT activity of LmDAT. The substrate saturation curve was fitted to the Michaelis-Menten equation, and the double reciprocal plot is depicted as an inset. $K_m$ and $V_{max}$ were calculated to be 0.69 ± 0.06 mM and 2.39 ± 0.36 nmol/min × mg. C, fatty acyl-CoA donor specificity of LmDAT. Samples were incubated at 30 °C for 5 min in the presence of 100 mM fatty acyl-CoA. D, DHAPAT activity of LmDAT represents only 8.5 ± % of GPAT activity. GPAT activity of LmDAT performed in the presence of 100 mM palmitoyl-CoA during 5 min. E, LmDAT activity is pH-dependent. All assays were performed at least twice, and only one representative experiment is depicted. The S.D. is shown.](image-url)

![FIGURE 6. Generation of an LmDAT null mutant of L. major. A, genomic organization of the LmDAT locus in the wild-type and mutant versions of the allele. The probe (thick black line) and scale are depicted. B, Southern blot analysis of genomic DNA digested with BamHI and SalI. Lane 1, wild type; lane 2, the heterozygote mutant (LmDAT: PAC/LmDAT) with PAC-replaced allele; lane 3, homozygote mutant (LmDAT:PAC/LmDAT:SAT) with both LmDAT alleles replaced with PAC and SAT cassettes, respectively. The size and identity of the bands are shown. The probe used is depicted in A.](image-url)
LmDAT DHAPAT activity was optimal at a neutral pH, decreased by 30% at pH 9, and was almost absent at pH 5.5 (Fig. 5E).

L. major Lacking LmDAT Gene Are Deficient in DHAPAT Activity—To assess the role of LmDAT in parasite physiology, a null mutant was generated. Two rounds of transformation and selection were performed to obtain the homozygous knock-out mutant. The first and second alleles were replaced by a puromycin (PAC) and a nourseothricin (SAT) cassette, respectively. A line, \( {\Delta lmdat} / {\Delta lmdat} \), carrying both mutations was obtained, and the loss of the endogenous LmDAT alleles was verified by Southern blot analysis (Fig. 6). This result demonstrates that LmDAT is not essential for parasite viability. The DHAPAT activity of the \( {\Delta lmdat} / {\Delta lmdat} \) null mutant was compared with its isogenic parental strain (wild type) and to a \( {\Delta lmdat} / {\Delta lmdat} + [LmDAT BSD] \) complemented strain, carrying a wild-type LmDAT gene on an episome in the \( {\Delta lmdat} / {\Delta lmdat} \) knock-out background. Although the wild-type and complemented lines exhibited comparable DHAPAT activity levels, the mutant completely failed to acylate DHAP (Fig. 7A). This result suggests that LmDAT is the sole DHAPAT enzyme in L. major promastigotes.

To assess whether the loss of DHAPAT activity in the \( {\Delta lmdat} / {\Delta lmdat} \) mutant could be compensated by an increase in the cellular GPAT activity catalyzed by the LmGAT enzyme, GPAT activity of the \( {\Delta lmdat} / {\Delta lmdat} \) was compared with that of the wild-type and complemented strains. No differences in GPAT activity could be detected between the three strains (Fig. 7B). As a control, similar levels of the malate dehydrogenase activity that is not related to lipid metabolism were found in the three strains (Fig. 7C).

L. major Lacking LmDAT Gene Have Altered Growth during Exponential Phase and Increased Cell Death during Stationary Phase—Growth assays revealed that the null mutant \( {\Delta lmdat} / {\Delta lmdat} \), although viable, grew at a ~45–50% reduced growth rate when compared with wild-type cells (Fig. 8A; Table 2). This growth defect could be compensated by the addition of the LmDAT gene (Fig. 7A). The doubling time of biomass accumulation was 14.2 ± 2.5 h for \( {\Delta lmdat} / {\Delta lmdat} \) mutant cells versus 7.6 ± 0.5 h for wild-type and 7.9 ± 0.7 h for complemented cells (Fig. 8A; Table 2). Furthermore, the \( {\Delta lmdat} / {\Delta lmdat} \) mutant reached only half the maximal cell density compared with that of the wild type and the complemented strains (\( \sim 3 \times 10^7 \) versus \( \sim 6 \times 10^7 \) cells/ml; Fig. 8A). These results demonstrate that LmDAT plays an important role in parasite development during the linear phase of cellular division. Interestingly, whereas the cell number of wild-type and complemented strains remained the same several days after they reached their maximum cellular density, the cell number of the \( {\Delta lmdat} / {\Delta lmdat} \) mutant dramatically decreased soon after reaching its maximum cellular density (Fig. 8A). Microscopic analysis revealed a major alteration in the morphology of the \( {\Delta lmdat} / {\Delta lmdat} \) cells with an increase in the number of unflagellated, rounded, and lysed cells in the culture (Fig. 8C). This defect was not observed during the linear phase of growth of the \( {\Delta lmdat} / {\Delta lmdat} \) mutant (data not shown). In contrast, the morphology of wild-type and complemented cells was unaltered during exponential and stationary phases of growth (Fig. 8C).

We further analyzed cell survival during the stationary phase by plating wild type, knock-out, and complemented strains on fresh media and counting the number of resulting colonies. The null mutant \( {\Delta lmdat} / {\Delta lmdat} \) gave ~70% decreased colony number when compared with wild-type and complemented cells (Fig. 8B). These results demonstrate that LmDAT is essential for cell viability during the stationary phase. Parasite death after maximum growth was further demonstrated using PrI to stain necrotic cells followed by quantification of PrI-positive cells by flow cytometry. Wild-type, knock-out, and complemented cells did not take up PrI during the logarithmic phase of development and replication (Fig. 9A). Upon entry into stationary phase, however, 14% of \( {\Delta lmdat} / {\Delta lmdat} \) cells were permeable to this dye, whereas only ~1–2% of wild-type and complemented cells were PrI-positive (Fig. 9B).

L. major Lacking LmDAT Gene Are Avirulent—To examine the role of LmDAT in L. major virulence, wild-type, null mutant, and complemented strains were examined for their ability to form a footpad lesion using a mouse model of cutaneous leishmaniasis. Whereas wild-type
and complemented strains formed a lesion within 4 weeks after injection with $10^6$ parasites, no lesions could be detected in mice injected with the null mutant 11 weeks post-injection with $10^6$ or $10^7$ parasites (Fig. 10).

**DISCUSSION**

The characterization of the enzymatic machinery involved in the initiation of glycerolipid biosynthesis in *Leishmania* is essential for understanding how the parasite synthesizes its virulence factors and develops and multiplies to cause the pathological symptoms associated with human leishmaniasis. Two reactions initiate the glycerolipid biosynthetic pathway, acylation of G3P by a GPAT enzyme or of DHAP by a DHAPAT. We have previously shown that in *Leishmania*, the GPAT enzyme *LmGAT* is not essential for promastigote development and survival and that mutants lacking this gene were affected in the synthesis of triglycerides but had unaltered synthesis of the major ester and ether phospholipids and were as virulent as wild-type cells in a mouse model of cutaneous leishmaniasis (27). To understand the importance of the alternative pathway (acylation of DHAP) in parasite physiology, we have isolated the gene *LmDAT* encoding the DHAPAT protein, characterized its enzymatic properties, and generated a null mutant.

*LmDAT* and its orthologs from *T. brucei* and *T. cruzi* are unusually large enzymes harboring, in addition to their C-terminal DHAPAT domain, a large N-terminal domain of 650 amino acid residues. This domain does not share homology with any known proteins in the available databases. The presence of an unusual polypeptide extension has also been found in the subunit II of RNA polymerase I of *T. brucei* and *L. major*, which harbor an N-terminal 250 amino acid extension (40, 41), and the 3-mercaptopyruvate sulfur-transferase enzymes of *L. major* and *Leishmania mexicana*, which contain 70 additional amino acids at their C terminus (42). So far no function could be assigned to these domains.

Our results showed that *LmDAT* complements the lethal phenotype of the yeast double mutant *gat1Δgat2Δ*. Although yeast cells lack ether phospholipids, their GAT1 and GAT2 enzymes are capable of acylating both G3P and DHAP (32, 39). The 1-acyl-DHAP formed from DHAP is reduced by an NADPH-dependent reductase to 1-acyl-G3P, which is subsequently used for the synthesis of ester phospholipids (43, 44).

***FIGURE 10.*** The *Δlmdat/Δlmdat* mutant is avirulent. Groups of four mice were injected with $10^6$ wild type (black circles), $10^6$ complemented line *Δlmdat/Δlmdat + [LmDAT BSD]* (gray circles) or either $10^6$ (open circles) or $10^7$ (open triangles) *Δlmdat/Δlmdat* mutant parasites as described under “Experimental Procedures,” and lesion formation was followed. The thickness of the footpad was measured with a vernier caliper.
synthesis of ester phospholipids and showed normal growth in logarithmic and stationary phases (27). Thus, the slow growth phenotype of the Δimdat/Δimdat mutant and its accelerated death in stationary phase are unlikely to be due to loss of synthesis of ether lipids or reduced synthesis of triglycerides. Therefore, we propose that the acylation of DHAP by LmDAT may represent the primary route for the synthesis of ester phospholipids in this parasite and that abrogation of this metabolic pathway is responsible for the slow growth phenotype. Alternatively, because glycolysis in Leishmania and related protozoa occurs within the glycosomes (52), it is possible that deletion of LmDAT may lead to increased levels of DHAP within these organelles. Accumulation of this precursor has previously been shown to be deleterious to Leishmania (53). Studies to evaluate these two hypotheses are warranted.

The slow growth phenotype and accelerated death after entry into the stationary cell stage of the Δimdat/Δimdat strain are reminiscent of a Leishmania mutant devoid of sphingolipids (54). Similar to fungi, Leishmania contains mainly inositol phosphoceram ide instead of sphingomyelin or glycosphingolipid in its membranes. Deletion of LmDAT may impair inositol phosphoceramide biosynthesis indirectly because of a lack of the precursor phosphatidylinositol.

A significant finding reported in this paper is the attenuated virulence phenotype of L. major resulting from the deficiency of LmDAT. Although the exact mechanism by which LmDAT promotes virulence remains to be elucidated, the Δimdat/Δimdat mutant might be considered as a vaccine candidate.

The phenotype of the Δimdat/Δimdat mutant is more severe than that of the Δads/Δads mutant, which lacks ether lipids, or that of the Δlpg1/Δlpg1 mutant, which lacks lipophosphogycan (28, 55). This difference is not surprising because LmDAT-deficient parasites die rapidly upon entry into the stationary cell stage. Cessation of growth at high cell density has been shown to trigger differentiation to metacyclics (56).

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