Adenylosuccinate Synthetase and Adenylosuccinate Lyase Deficiencies Trigger Growth and Infectivity Deficits in Leishmania donovani

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Background: Purine salvage in Leishmania is an essential nutritional function.

Results: Null mutants deficient in either adenylosuccinate synthetase or adenylosuccinate lyase impact growth and infectivity phenotypes of Leishmania donovani.

Conclusion: Adenylosuccinate synthetase and adenylosuccinate lyase are central enzymes in purine salvage by L. donovani.

Significance: Adenylosuccinate lyase has been validated as a potential drug target in L. donovani.

Leishmania are auxotrophic for purines, and consequently purine acquisition from the host is a requisite nutritional function for the parasite. Both adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ASL) have been identified as vital components of purine salvage in Leishmania donovani, and therefore Δadss and Δasl null mutants were constructed to test this hypothesis. Unlike wild type L. donovani, Δadss and Δasl parasites in culture exhibited a profoundly restricted growth phenotype in which the only permissive growth conditions were a 6-aminopurine source in the presence of 2′-deoxycoformycin, an inhibitor of adenine aminohydrolase activity. Although both knock-outs showed a diminished capacity to infect murine peritoneal macrophages, only the Δasl null mutant was profoundly incapacitated in its ability to infect mice. The enormous discrepancy in parasite loads observed in livers and spleens from mice infected with either Δadss or Δasl parasites can be explained by selective accumulation of adenylosuccinate in the Δasl knock-out and consequent starvation for guanylate nucleotides. Genetic complementation of a Δasl lesion in Escherichia coli implied that the L. donovani ASL could also recognize 5-aminomidazole-N-succinylcarboxamide ribotide as a substrate, and purified recombinant ASL displayed an apparent Km of ~24 μM for adenylosuccinate. Unlike many components of the purine salvage pathway of L. donovani, both ASL and ADSS are cytosolic enzymes. Overall, these data underscore the paramount importance of ASL to purine salvage by both life cycle stages of L. donovani and authenticate ASL as a potential drug target in Leishmania.

Protozoan parasites that infect humans constitute a phylogenetically diverse assortment of organisms that cause a variety of devastating and often fatal diseases in humans and their domestic animals. Indeed, afflictions of parasitic etiology represent some of the most consequential diseases worldwide in terms of human suffering and economic spoliation. Leishmania donovani is the causal agent of visceral leishmaniasis, a disease that is invariably lethal if untreated. L. donovani, like all Leishmania species, is a digenetic protozoan parasite that is present as the flagellated, extracellular promastigote in the phlebotomine sandfly vector and as the immotile, intracellular amastigote within phagolysosomes of macrophages of the infected mammalian host. There is no effective vaccine against leishmaniasis, and the standard antileishmanial drugs are far from ideal. These drugs are toxic, expensive, and necessitate prolonged and invasive administrations, and furthermore drug resistance has rendered the chemotherapies too often ineffective (1–4). Thus, the need for new drugs, as well as new drug targets, is acute.

The implementation of rational, selective, and effective antiparasitic drug therapies relies upon the exploitation of underlying biochemical and/or metabolic disparities between the parasite and mammals. Among the most conspicuous of the metabolic discrepancies between Leishmania and its mammalian hosts is the pathway by which purine nucleotides are synthesized. Whereas mammals are prototrophic for purines and synthesize purine nucleotides from amino acids and one-carbon fragments, Leishmania, like all protozoan parasites, cannot generate the purine ring de novo (5–7). Accordingly, each parasite genus expresses a distinctive complement of nutritionally indispensable purine salvage and interconversion enzymes that allow the parasite to scavenge host purines.

The purine pathway of Leishmania is complex, intertwined, and capable of assimilating effectively every purine nucleobase or nucleoside from the host or culture medium into the parasite nucleotide pool (5–8). Leishmania express four enzymes that are capable of converting host or extracellular purines into nucleotides: 1) hypoxanthine-guanine phosphoribosyltransferase (HGPRT)2; 2) hypoxanthine-guanine phosphoribosyltransferase; XPR; xanthine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; AHH, adenine aminohydrolase; dCF, 2′-deoxycoformycin; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; IMPDH, inosine 5′-monophosphate dehydrogenase; SAICAR, 5-aminimidazole-4-succinylcarboxamide ribotide; N9-9′-NTA, nickel-nitroacetic acid; NEO, neomycin resistance; HYG, hygromycin phosphotransferase; BSD, blastidicin deaminase; EGFP, enhanced GFP; G418, Geneticin; EHNA, erythron-9-(2-hydroxy-3-nonyl)idenone; LPI, low phosphate induction medium.

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‡ This article contains supplemental Fig. S1 and Tables S1 and S2.

The abbreviations used are: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPR, xanthine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; AHH, adenine aminohydrolase; dCF, 2′-deoxycoformycin; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; IMPDH, inosine 5′-monophosphate dehydrogenase; SAICAR, 5-aminimidazole-4-succinylcarboxamide ribotide; Ni9-9′-NTA, nickel-nitroacetic acid; NEO, neomycin resistance; HYG, hygromycin phosphotransferase; BSD, blastidicin deaminase; EGFP, enhanced GFP; G418, Geneticin; EHNA, erythron-9-(2-hydroxy-3-nonyl)idenone; LPI, low phosphate induction medium.

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xanthine phosphoribosyltransferase (XPRT); 3) adenine phosphoribosyltransferase (APRT); 4) and adenosine kinase (2, 5, 9, 10). HGPRT and XPRT are confined within the glycosome (11, 12), a membrane-bound microbody organelle that is found exclusively among trypanosomatid parasites (13–15), whereas APRT has been definitively localized to the cytosol (12). Genetic studies in L. donovani reveal that none of the four enzymes by itself is essential for parasite survival because promastigotes deficient in the activity of any one of the four purine salvage enzymes are viable and do not exhibit a fitness deficit (16–20). However, the construction and phenotypic characterization of a conditionally lethal Δhgprt/Δxprt double mutant offers robust genetic verification for the conjecture that essentially all purine salvage by L. donovani is mediated through HGPRT or XPRT and that APRT and adenosine kinase are functionally superfluous. Whereas wild type L. donovani can grow in virtually any purine nucleobase or nucleoside as the sole purine source, the Δhgprt/Δxprt double knock-out can grow only in adenine or adenosine and only when adenine amidohydrolase (AAH) is pharmacologically obstructed with 2′-deoxycoformycin (dCF) (21). Furthermore, the Δhgprt/Δxprt null mutant is highly compromised in its ability to establish a visceral infection in mice (21), implying a central role for HGPRT and XPRT in purine salvage by amastigotes as well. These findings demonstrate, therefore, that virtually all host or extracellular purines are funneled into substrates of HGPRT or XPRT and strongly intimate the functional importance of the downstream nucleotide interconversion enzymes that distribute the enzymatic products of HGPRT and XPRT into adenylate and guanylate nucleotides. These nucleotide interconversion enzymes include adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ASL), which convert IMP to AMP, IMP dehydrogenase (IMPDH) and GMP synthase, which generate GMP from IMP, and AMP deaminase and GMP reductase, which back-convert AMP and GMP into IMP, respectively (5, 7, 8, 22) (see Fig. 1). A battery of nucleotide kinases then converts the nucleoside monophosphates to diphosphates and triphosphates (6).

ADSS catalyzes the GTP-dependent formation of adenylosuccinate from IMP and aspartic acid, whereas ASL cleaves a fumarate molecule from the adenylosuccinate product of the ADSS reaction. The native L. donovani ADSS and ASL enzymes have been partially purified from intact parasites and characterized kinetically with respect to their unusual capacities to metabolize nucleotide products of the noted antileishmanial pyrazolopyrimidine nucleobase and nucleoside analogs, e.g. allopurinol, 4-thiopurinol, and formycin B (23–25). The functional importance of either enzyme, however, to either the promastigote or the amastigote was heretofore unknown. To investigate the function of ADSS and ASL in L. donovani, Δadss and Δasl knock-outs were created by double targeted gene replacement protocols, and the growth and infectivity phenotypes of the knock-out lines were assessed. Both Δadss and Δasl parasites exhibited the same constrained growth phenotype as the previously characterized Δhgprt/Δxprt double mutant (21), i.e. the only permissive growth conditions required the presence of dCF and either adenine or adenosine as the purine source. The infectivity phenotypes between the Δadss and Δasl null lines in mice were diametric opposites, however, with the former causing a robust visceral infection, whereas the parasite burdens in mice infected with the Δasl knock-out were greatly reduced. These infectivity data validate ASL as a promising therapeutic target for the treatment of leishmaniasis. Recombinant ASL was purified and shown to be catalytically active, and the gene complemented asl-deficient Escherichia coli in a manner that suggested that it was a bifunctional enzyme, like its human counterpart, which also could catalyze the cleavage of 5-aminoimidazole-(N-succinylcarboxamid) ribotide (SAICAR) to 5-aminoimidazole-4-carboxamide ribotide and fumarate (26).

Both ADSS and ASL were localized to the parasite cytosol using immunocytochemistry and/or cell fractionation, implying a potentially intriguing portioning of the adenylate and guanylate branches of the L. donovani purine salvage pathway. This investigation is the first to validate a single component of the purine salvage pathway of Leishmania as a prospective antileishmanial drug target.

EXPERIMENTAL PROCEDURES

Materials, Chemicals, and Reagents—[8-14C]Hypoxanthine (51 mCi/mmol) was bought from Moravek Biochemicals (Brea, CA), and [α-32P]dCTP was acquired from MP Biomedicals (Irvine, CA). Unlabeled purine bases, nucleosides, and nucleotides were procured from Sigma-Aldrich® or Fisher Scientific, adenylosuccinate was purchased from Sigma-Aldrich®, and dCF was obtained from Tocris Bioscience (Ellisville, MO). Restriction enzymes were purchased from New England Biolabs (Beverley, MA). The TOPO® TA Cloning™ kit, pCR®2.1-TOPO® vector, Champion™ pET200/D-TOPO® expression vector, and BL21 Star™ (DE3) One Shot™ competent cells, as well as the goat anti-rabbit Oregon Green-, goat anti-mouse Oregon Green-, goat anti-mouse Alexa Fluor® 568-, and goat anti-guinea pig rhodamine red-conjugated secondary fluorescent antibodies, were bought from Invitrogen. IMPDH antisera was produced in guinea pigs as reported (27), and mouse monoclonal anti-α-tubulin (DM1A1) antibody was obtained from EMD Millipore (Billerica, MA). Mouse monoclonal anti-human influenza hemagglutinin (HA) and anti-γ-glutamyl transpeptidase (GTP) primary antibodies were bought from Sigma-Aldrich® and BD Biosciences, respectively. Goat anti-rabbit HRP-conjugated and goat anti-mouse HRP-conjugated sec-
ondary antibodies were purchased from Thermo Fisher Scientific. Complete Mini EDTA-free protease inhibitor tablets were procured from Roche Diagnostics, the nickel-nitroloacetic acid (Ni\(^{2+}\)-NTA)-agarose beads were from Qiagen (Valencia, CA), and the BioSafe™ Coomasie and Bio-Rad protein assay kits were from Bio-Rad. The pX63-NEO, pX63-HYG, and pXG-BSD leishmanial expression vectors harboring the neomycin resistance (NEO), hygromycin phosphotransferase (HYG), and blasticidin deaminase (BSD) markers were used to confirm their identities. The PCR fragments were then used to amplify gene fragments from wild type parasites by double targeted gene replacement using the transfection parameters and plating methods reported previously (34,35). The linearized drug resistance cassettes enclosing the ADSS flanks were excised from pX63-NEO-Δadss and pX63-HYG-Δadss with HindIII-BglII, and the X63-NEO-Δadss and X63-HYG-Δadss targeting constructs were used to sequentially create ADSS/Δadss heterozygotes followed by Δadss null mutants using electroporation and plating methods as detailed (34,35). The ADSS/Δadss heterozygotes were isolated on semi-solid growth plates containing 20 μg/ml Geneticin (G418) or 50 μg/ml hygromycin supplemented with 100 μM adenine, whereas the Δadss knock-outs were selected on plates containing 20 μg/ml G418, 50 μg/ml hygromycin, 100 μM adenine, and 20 μM dCF. The Δaah/Δadss double knock-out was generated within the Δaah parental strain (42) using linearized X63-NEO-Δadss and X63-HYG-Δadss targeting constructs. The Δaah/ADSS/Δadss heterozygotes and Δaah/Δadss knock-outs were isolated on semi-solid medium containing selective concentrations of G418 and/or hygromycin, as appropriate for the selective marker, and supplemented with 100 μM adenine in the absence of dCF.

Similarly, the X63-NEO-Δasl and X63-HYG-Δasl gene replacement constructs were employed to successively generate the Δasl heterozygotes followed by the construction of the Δasl knock-outs from their corresponding heterozygotes.

Identification of the ADSS mRNA Spliced Leader Addition Site Using Rapid Amplification of cDNA Ends PCR—First strand ADSS cDNA was generated from 2 μg of L. donovani total RNA via reverse transcription with Superscript® III (Invitrogen) polymerase at 50 °C according to the manufacturer’s protocol using the ADSS gene-specific primer listed in supplemental Table S1. A portion of the cDNA reaction was then subjected to PCR using a sense primer corresponding to the spliced leader sequence, which is trans-spliced onto the 5′-termini of all Leishmania mRNAs (41), and the nested antisense primer listed in supplemental Table S1. The resulting PCR products were gel-purified and sequenced to identify the position of the spliced leader addition site on the ADSS mRNA relative to the predicted translation start site.

Generation of Targeting Constructs—To generate the pX63-NEO-Δadss and pX63-HYG-Δadss gene targeting vectors, ~850 and 950 bases of ADSS 5′- and 3′-UTRs were amplified from a purified ADSS cosmid by PCR, subcloned into the PCR®2.1-TOPO® vector (Invitrogen), sequenced to ensure fidelity, and inserted into the appropriate restriction sites of the pX63-NEO and pX63-HYG vectors (28). The primers used to amplify the ADSS UTRs are shown in supplemental Table S1. Similarly, the pX63-NEO-Δasl and pX63-HYG-Δasl targeting vectors were generated after ~900 and 1200 bases of the Δadss knock-outs were PCR-amplified from a purified ASL cosmid, subcloned into the PCR®2.1-TOPO® vector, sequenced, and inserted into the appropriate restriction sites of the pX63-NEO or pX63-HYG vectors (29). The primers used to amplify the ASL UTRs are listed in supplemental Table S2.

Creation of Null Mutants—The Δadss and Δasl knock-outs were generated from wild type parasites by double targeted gene replacement using the transfection parameters and plating techniques reported previously (34,35). The linearized drug resistance cassettes enclosing the ADSS flanks were excised from pX63-NEO-Δadss and pX63-HYG-Δadss with HindIII-BglII, and the X63-NEO-Δadss and X63-HYG-Δadss targeting constructs were used to sequentially create ADSS/Δadss heterozygotes followed by Δadss null mutants using electroporation and plating methods as detailed (34,35). The ADSS/Δadss heterozygotes were isolated on semi-solid growth plates containing 20 μg/ml Geneticin (G418) or 50 μg/ml hygromycin supplemented with 100 μM adenine, whereas the Δadss knock-outs were selected on plates containing 20 μg/ml G418, 50 μg/ml hygromycin, 100 μM adenine, and 20 μM dCF. The Δaah/Δadss double knock-out was generated within the Δaah parental strain (42) using linearized X63-NEO-Δadss and X63-HYG-Δadss targeting constructs. The Δaah/ADSS/Δadss heterozygotes and Δaah/Δadss knock-outs were isolated on semi-solid medium containing selective concentrations of G418 and/or hygromycin, as appropriate for the selective marker, and supplemented with 100 μM adenine in the absence of dCF.

Similarly, the X63-NEO-Δasl and X63-HYG-Δasl gene replacement constructs were employed to successively generate the Δasl heterozygotes followed by the construction of the Δasl knock-outs from their corresponding heterozygotes.

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The heterozygotes were selected on plates containing suitable concentrations of G418 and/or hygromycin supplemented with 100 μM adenine and 20 μM dCF, and the Δasl knock-outs were selected on plates containing 20 μg/ml G418, 50 μg/ml hygromycin, 100 μM adenine, 100 μM guanine, and 20 μM dCF. The ASL/asl heterozygote was created within the Δaah background to generate Δaah/ASL/asl parasites, which were then transfected with the second replacement construct in an attempt to generate a Δaah/Δasl double knock-out. The putative Δaah/Δasl parasites were selected in 20 μg/ml G418, 50 μg/ml hygromycin and supplemented with either 100 μM adenine or a purine mixture consisting of 200 μM adenine, 200 μM guanine, and 200 μM xanthine. The genotypes of all heterozygotes and knock-outs were verified using standard genomic DNA isolation and Southern blotting protocols (43). The hybridization probes harboring either the ADSS or ASL ORFs or the 5′- or 3′-UTR were PCR-amplified from the ADSS and ASL cosmids, respectively, and gel-purified using a Wizard SV gel and PCR clean-up kit (Promega, Madison, WI).

Generation of Episomally Complemented Lines—The pXG-BSD vector (29) was used for episomal complementation of Δadss and Δasl parasites. The primers used to PCR-amplify the ADSS and ASL ORFs are shown in supplemental Tables S1 and S2, respectively. The coding sequence of each gene was subcloned independently into the pCR®2.1-TOPO® vector (Invitrogen), sequenced to ensure fidelity, excised from pCR®2.1-TOPO® with Smal-BamHI, and inserted into the Smal-BamHI sites of the pXG-BSD expression plasmid (29). The pXG-BSD-ADSS and pXG-BSD-ASL episomes were then used to generate Δadss[pADSS] and Δasl[pASL] “add-back” cell lines from the Δadss and Δasl knock-outs. The complemented cell lines were selected in 20 μM blastidin and supplemented with 100 μM adenine. Southern blotting was performed to confirm the genotypes of the “add-backs” as described above.

Growth Phenotypes—To evaluate the capacity of wild type, Δadss, Δasl[pADSS], Δaah, Δaah/Δadss, Δasl, and Δasl[pASL] promastigotes to grow in different purine sources, exponentially growing parasites were washed several times with PBS, resuspended at a density of 5 × 10⁶ cells/ml in 1.0-ml aliquots of modified Dulbecco’s modified Eagle medium—Leishmania (16) containing 100 μM purine and 5% dialyzed FBS, and dispensed into individual wells of 24-well tissue culture plates (Sarstedt Inc., Newton, NC). The ability of these lines to grow in either adenine or adenosine was also determined in the presence of dCF. In addition, the adenosine growth profile of the Δaah/Δadss double knock-outs was also assessed in the presence of 20 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (44) to inhibit the adenosine deaminase activity found in FBS (45), thereby preserving the nucleoside. After 7–10 days, parasites were enumerated visually by hemocytometer.

ADSS and ASL Activity Measurements in Intact Parasites—The ability of wild type, Δadss, and Δasl promastigotes to convert [8,14C]hypoxanthine into various metabolites was assessed using TLC. 5.0 × 10⁷ parasites were washed and resuspended in 20 μl of PBS; [8,14C]hypoxanthine (51 mCi/mmole) was added to a final concentration of 100 μM, and the cells were allowed to incubate at 26°C. At 30 min and 2 h, 10 μl of the parasite mixture was centrifuged at 13,000 rpm in an Eppendorf Centrifuge 5415D, the supernatant was removed, and the parasite pellet was resuspended in 5 μl of glacial acetic acid to lyse the cells and terminate the reaction (46). The lysate was spotted onto a PE-SIL-G TLC plate with fluorescent indicator (GE Healthcare), so that nonradioactive standards could be detected, and developed in dioxane/ammonium hydroxide/water at a ratio of 6:1.5 (v/v/v) (47). The TLC plate was exposed to X-ray film at −80°C and developed in a standard X-ray film developer.

Macrophage Infections—Macrophage infectivity experiments with wild type, Δadss, Δadss[pADSS], Δasl, and Δasl[pASL] promastigotes were performed using either peritoneal macrophages harvested from BALB/c mice or cultured J774 murine macrophages (ATCC, Manassas, VA) (DMEM supplemented with 4 mM l-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, and 10% FBS but without added purine) and incubated at 37°C in a humidified 5% CO₂ incubator as described (16, 21, 48, 49). 72 h post-infection, macrophages were washed, stained using the Diff-Quik kit (International Medical Equipment Inc., San Marcos, CA), and amastigotes were enumerated (16, 21, 49).

Mouse Infections—Prior to injection, all parasites were cycled back and forth several times between the promastigote and axenic amastigote forms (33, 34). Groups of five 7-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were inoculated by tail vein injection with 5 × 10⁶ late log or stationary phase wild type, Δadss, Δadss[pADSS], Δaah/Δadss, Δasl, or Δasl[pASL] promastigotes (48, 49). Four weeks post-infection, mice were sacrificed and their livers and spleens harvested as reported (48, 49). Single-cell suspensions from mouse organs were prepared by passage through a 70-μm cell strainer (BD Falcon), and parasite burdens were then determined in 96-well microtiter plates using a standardized limiting dilution assay (50). The organ-derived wild type, Δadss[pADSS], Δaah/Δadss, or Δasl[pASL], parasites were titered in modified Dulbecco’s modified Eagle medium—Leishmania (16) supplemented with 5% FBS and 100 μM adenine, whereas the Δadss and Δasl mutants were grown in medium containing 100 μM adenine and 20 μM dCF.

Bacterial Complementation—The ASL ORF was amplified by PCR from the ASL cosmid using primers that attached 5′-NdeI and 3′-EcoRI restriction sites (supplemental Table S2), subcloned into the pCR®2.1-TOPO® vector, excised via NdeI-EcoRV digest, gel-purified, and ligated into the pBACE bacterial expression vector (51). The pBace and pBACE-ASL plasmids were then electroporated independently into SØ200 (purB68, Δ[fumC-nth]84, relA1, rpsL254(strR), metB1) asl-deficient bacteria (26), and the transformed bacteria were tested for their ability to grow on low phosphate induction medium (LPI) agar alone or LPI agar supplemented with 200 μM adenine, 200 μM hypoxanthine, or 200 μM adenine plus 200 μM hypoxanthine (26).

Similarly the ΔADSS ORF was also PCR-amplified from the ADSS cosmid, subcloned, excised from pCR®2.1-TOPO®, and ligated into the pBACE vector after an NdeI-EcoRV restriction digest (supplemental Table S1), and the pBACE and pBACE-ΔADSS plasmids were electroporated independently into several strains of adss-deficient E. coli (52), including ES4 (fhuA2, lacY1, or...
lacZ4, tsx-1, or tsx-70, glv44(AS), galE6, mtlA2, purA4S), PC1523 (thr-25, hisA49, argF58, relA1, serA27, spoT1, purA54, argE61), JW4135-2 (Δ(ara-D-araB)567, ΔlacZ4787::rrnB-3), λ−, rph-1, Δ(hadR-hadB)568, ΔpurA727::kan, hsdR514), TX595 (araBAD-0, Δlac-8888, purA206 (stable, Temp,ApR):Mud), and H1238 (thr-25, hisA49, argF58, relA1, spoT1, purA54, argE61), obtained from the E. coli Genetic Stock Center at Yale (New Haven, CT). The transformed Es4, PC1523, JW4135-2, or TX595 bacteria were then grown on LPI agar alone or on LPI agar supplemented with 200 μM adenine (52). The H1238 cells were grown on LPI agar alone, on LPI agar supplemented with 200 μM adenine, or on M9 agar to which 1 mM MgSO4, 0.1 mM CaCl2, 1 mM threonine, 1 mM arginine, 0.2% glucose, and 0.2 mM isopropyl-β-D-thiogalactopyranoside and 100 μg/ml ampicillin were added as detailed (53).

Expression and Purification—The ASL and ADSS ORFs were amplified by PCR from the ASL and ADSS cosmids, respectively, and inserted separately into the pET200/D-TOPO® E. coli expression plasmid, which automatically attaches a His6 tag to the NH2 terminus of the inserted gene product, and the constructs were sequenced to confirm the gene sequences. The pET200/D-TOPO®-ASL and the pET200/D-TOPO®-ADSS constructs were then independently transformed into BL21 Star™ (DE3) One Shot® E. coli (Invitrogen). After induction in 0.5 mM IPTG, the recombinant L. donovani ASL protein was purified to virtual homogeneity over a Ni2+-NTA-agarose column from E. coli extracts that had been prepared using a French press as described (54). Recombinant ASL was eluted from the Ni2+-NTA-agarose with 250 mM imidazole as detailed (54). Separation of the purified recombinant ASL fractions on a 10% SDS-polyacrylamide gel and subsequent staining with Bio-safe™ Coomassie (Bio-Rad) confirmed the purity of the recombinant protein. A Thermo Labsystems Multiscan Ascent plate reader (Thermo Scientific) was employed at 600 nm to determine the protein concentration and yield of the purified ASL after the addition of Bio-Rad protein assay reagent (54). Yields of purified recombinant ASL protein were ~25–30 mg/liter of bacterial culture. Efforts to purify recombinant ADSS under both native and denaturing conditions were also made.

Antibodies and Immunoblotting—Polyclonal ASL antiserum was generated in rabbits by Open Biosystems (Huntsville, AL) using purified recombinant ASL as an immunogen and standard injection protocols. Western blotting protocols were performed as detailed (43) using rabbit anti-ASL and mouse anti-α-tubulin primary antibodies followed by goat anti-rabbit HRP-conjugated and goat anti-mouse HRP-conjugated secondary antibodies, respectively.

ASL Assay—The ability of purified, recombinant ASL to utilize adenylosuccinate as a substrate was assessed by a spectrophotometric assay in which the cleavage of adenylosuccinate to AMP and fumarate (24) was measured by a decrease in absorbance at 282 nm (24, 55) using a Beckman DU 640 spectrophotometer (Beckman Coulter). All kinetic studies with ASL were carried out under experimental conditions that were linear with time and enzyme concentration. Briefly, ~1.25 μg of recombinant ASL was added to a quartz cuvette containing 1.0 ml of 20 mM HEPES-KOH, pH 7.0, and adenylosuccinate varying in concentration from 1 to 125 μM. The reaction was carried out at 25 °C and pH 7.0 over a 90-s time course, and absorbance at 282 nm was measured. The amount of adenylosuccinate consumed was calculated from the A282 reading at each time point. The molar extinction coefficient (ε) of adenylosuccinate was calculated experimentally to be 16,430 M⁻¹·cm⁻¹ under these reaction conditions. Michaelis-Menten analysis (56) (GraphPad Prism 4.0) was used to determine the Km of ASL for adenylosuccinate. The pH optimum was determined by plotting the rate of consumption of 25 μM adenylosuccinate versus pH. The pH of the HEPEs-KOH buffer was adjusted by the addition of the following solutions: 0.5 M 2-[bis(2-hydroxyethyl)amino]l-2-(hydroxymethyl)propane-1,3-diol (pH 5.8, 6.0, 6.5, and 7.0), 0.5 M N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (pH 7.5, 8.0, and 8.5), and 0.5 M N-cyclohexyl-2-aminothiogusulanic acid (pH 9.0, 9.5, and 10.0).

ADSS Localization—PCR was employed to attach an HA tag onto either the 5′- or 3′-end of the ADSS ORF. Smal and BamHI restriction sites (supplemental Table S1) were included on the HA-ADSS constructs, and the DNA fragments were ligated into the pXG-BSD leishmanial expression vector (29) to generate pXG-BSD-5′-HA-ADSS and pXG-BSD-3′-HA-ADSS plasmids, allowing the parasites to produce ADSS protein that was HA-tagged at either the NH2 or COOH terminus. The plasmids were transfected separately into wild type L. donovani, and immunofluorescence assays were performed using polyclonal mouse anti-HA primary antibody followed by goat-anti mouse Oregon Green- (1:10,000) or goat anti-mouse Alexa Fluor 568-conjugated (1:10,000) secondary antibody to visualize the HA-tagged ADSS. Parasites were co-stained with guinea pig anti-IMPDH antibodies (1:500) and goat anti-guinea pig rhodamine red-conjugated (1:10,000) secondary antibody to differentiate glycosomal IMPDH. Cells were visualized on a Zeiss Axiowert 200 inverted microscope (Carl Zeiss Microimaging, Thornwood, NY) with a ×63 oil immersion lens. Photos were taken with a Zeiss AxioCam MR camera using Axioscience 4.2 software and compiled using Adobe Photoshop Creative Suite 4.

ASL Localization by Subcellular Fractionation—The separation of glycosomal and cytosolic fractions from 3–4 × 10⁹ L. donovani promastigotes was performed as detailed (42). A linear 20–70% sucrose gradient was prepared as described (43) and allowed to equilibrate overnight at 4 °C. Post-nuclear whole cell lysates were overlaid on the sucrose gradient and centrifuged for 6 h at 36,000 rpm in a Beckman SW41 rotor. The gradient was fractionated, and the proteins were precipitated with trichloroacetic acid and analyzed by standard Western blotting procedures (43) using polyclonal ASL and PEX14 (57) antisera.

ASL Localization by Microscopy—Purified ASL protein was bound to AminoLink coupling resin (Thermo Scientific), and crude ASL antiserum was purified against ASL protein using an AminoLink Immobilization kit (Thermo Scientific) according to the manufacturer’s instructions. The immunofluorescence assay was performed on L. donovani promastigotes as described (11, 12, 27, 48) using a 1:1000 dilution of recombinant anti-ASL antibody and a 1:10,000 dilution of secondary goat anti-rabbit Oregon Green-conjugated antibody (Invitrogen).
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Parasites were co-stained with guinea pig anti-IMPDH antibodies (1:500) and goat anti-guinea pig rhodamine red-conjugated (1:10,000) secondary antibody to detect glycosomal IMPDH. Cells were photographed as detailed above.

The Adsl ORF was amplified by PCR from the ASL cosmid using primers that attached appropriate 5′- and 3′-restriction sites (supplemental Table S2), subcloned into the pCR®2.1-TOPO® vector, excised via an NdeI-EcoRV restriction digest, and ligated into the pLCNEO-EGFPCO vector to create pLCNEO-EGFPCO-ASL. pLCNEO-EGFPCO-ASL was then transfected into wild type L. donovani promastigotes, the transfectants were selected in 20 μM G418, and EFGP-tagged ASL protein was visualized as described above.

RESULTS

Primary Structures of L. donovani ADSS and ASL—The L. donovani ADSS (ABS11225) and ASL (ABS11226) genes and their flanking sequences were isolated from purified cosmids clones that hybridized to the putative ADSS (LmjF.13.1190) and ASL (LmjF.04.0460) ORFs, respectively, from L. major (38). The L. donovani ADSS ORF predicted a 710-amino acid protein with a molecular mass of ~78.2 kDa that was 93–99% identical to ADSSs from L. major, Leishmania mexicana, and Leishmania infantum and 58, 61, 16, 17, 16, and 17% identical to the ASL sequences from Trypanosoma brucei, Trypanosoma cruzi, Plasmodium falciparum, Homo sapiens, Saccharomyces cerevisiae, and E. coli, respectively. A multiple sequence alignment among phylogenetically diverse ADSS proteins revealed that the Leishmania ADSS ORF s encompass a sizeable NH2-terminal extension (38) as well as two internal insertions of 20 and 55 residues, which render the leishmanial ADSSs much longer than ADSSs from other organisms (supplemental Fig. S1A). A portion of the NH2-terminal extension, as well as the entirety of both internal insertions, is also found in the putative ADSSs from T. brucei and T. cruzi, two protozoan pathogens that are evolutionarily related to Leishmania, but not in ADSSs from other organisms (supplemental Fig. S1A). Because the length of the predicted Leishmania spp. ADSS ORF created some uncertainty about the translation initiation site, a modified rapid amplification of cDNA ends technique was employed to determine whether the mRNA start site of ADSS was 5′ to the predicted ATG start site. Two spliced leader addition sites were identified (data not shown) upstream of the predicted ATG start (203 and 365 bp), with no downstream sites detected. This is consistent with the predominant spliced leader addition sites subsequently reported on TriTrypDB and indicates that the predicted initiating ATG is, in fact, present on the L. donovani ADSS mRNA. These data corroborate the observation by Specter et al. (24) that the partially purified L. donovani ADSS protein appeared to have a larger particle weight than other sources of ADSS. Additional analysis of the L. donovani ADSS amino acid sequence revealed that this protein possesses many residues that are found within the active site of both the P. falciparum and E. coli ADSS crystal structures (59, 60).

The L. donovani ASL encodes a protein of 479 amino acids with a molecular mass of ~53.7 kDa. Pairwise alignments of the L. donovani ASL with ASLs from other organisms (supplemental Fig. S1B) demonstrated 93–100% identity to ASLs from other Leishmania species (38) and 67, 71, 39, 16, 18, and 49% amino acid identity to the predicted ASL sequences from T. brucei, T. cruzi, P. falciparum, H. sapiens, S. cerevisiae, and E. coli, respectively. A multiple sequence alignment of L. donovani ASL with ASLs from other organisms revealed several residues that are conserved among all of the aligned ASL proteins (supplemental Fig. S1B).

Confirmation of Δadss, Δaah/Δadss, and Δasl Genotypes—To test the functionality of ADSS and ASL in intact parasites, Δadss and Δasl null mutants were created by targeted gene replacement from wild type L. donovani as described under “Experimental Procedures.” It was necessary to include dCF in the second round of transfection for both knock-outs to prevent deamination of adenine and compel its incorporation into the parasite nucleotide pool through APRT. A Δaah/Δadss double knock-out was also created within the Δaah background in order to eliminate the pharmacological requirement for dCF and to demonstrate that AAH activity is critical to the conditionally lethal Δadss mutation (42). Because dCF is a specific inhibitor of AAH (42, 61), dCF was not included in the selection of Δaah/Δadss parasites from the Δaah/ΔADSS/adss progenitor. Efforts to create a Δaah/Δasl double knock-out from a Δaah/ASL/asl heterozygote were unsuccessful, even after supplementation of the culture medium with a purine mixture consisting of adenine, guanine, and xanthine that should theoretically have supplied precursors for both adenylylate and guanylate nucleotides.

Southern blots of genomic DNA from wild type, ADSS/adss, Δadss, Δadss[pADSS], Δaah/ΔADSS/adss, and Δaah/ΔADSS/adss parasites using either the ADSS ORF or 5′-UTR as a hybridization probe verified the allelic replacements in the heterozygotes and null mutants (Fig. 2). The hybridization signals from the genomic DNA samples prepared from wild type and genetically manipulated parasites corresponded to the expected restriction fragments for each cell line and confirmed the specific gene rearrangements at the ADSS locus in ADSS/adss and Δadss parasites (Fig. 2). Western blotting was not carried out for the transgenic strains harboring rearrangements at the ADSS locus because of the lack of specific anti-ADSS antisera.

The Δasl genotype was also confirmed by Southern analysis. Southern blots of genomic DNA from wild type, ASL/asl, Δasl, and Δasl[pASL] parasites using either the ASL ORF or 3′-UTR as a hybridization probe confirmed the allelic substitutions (Fig. 3, A and B). The hybridization signals from the genomic DNA samples were the expected size of restriction fragments predicted from mapping of the wild type ASL locus. Additionally, the specific allelic rearrangements observed in the genetically manipulated transgenic parasites confirmed the null genotype of the Δasl knock-out clone (Fig. 3, A and B). Western blotting with polyclonal anti-ASL antibodies verified the specificity of the ASL antisera, the absence of ASL protein in the Δasl mutant, and the presence of ASL in wild type and Δasl[pASL] parasites (Fig. 3C).

Growth Phenotypes—The impact of Δadss and Δasl lesions on the nutritional phenotype of L. donovani promastigotes was then evaluated by ascertaining the capabilities of wild type, null mutant, and add-back parasites to grow in a variety of different purines (Fig. 4A). Whereas wild type and Δadss[pADSS] pro-
mastigotes could proliferate in medium supplemented with any of the purine nucleobases or nucleosides tested, the Δadss null mutant could not grow on guanine, guanosine, hypoxanthine, inosine, or xanthine as the sole purine source (Fig. 4). Because the Δaah/Δadss double knock-out could not deaminate adenine to hypoxanthine, this strain was able, as expected, to grow in adenine in the absence of 20 μM dCF (Fig. 4A). The absence of a hybridization signal in genomic DNA from Δadss and Δaah/Δadss parasites (lanes 4 and 9) confirmed the null genotype of these cell lines. B, the 1650-bp hybridization signal produced from genomic DNA that had been cut with HindIII/BglII/EcoRI and probed with the AAH ORF (lanes 1–5) encompasses 765 bp of the AAH ORF and 885 bp of the AAH 5’-UTR. The absence of an AAH hybridization signal in DNA from parasites containing the Δaah lesion (lanes 6–9) verified the genotype of these cell lines.

FIGURE 2. Southern blot analysis of Δadss knock-outs. A, total genomic DNA from wild type (lane 1), ADSS/adss heterozygotes (lanes 2 and 3), Δadss (lane 4), Δaah/Δadss parasites (lanes 5 and 6), Δaah/ADSS/adss (lanes 7 and 8), and Δaah/Δadss parasites (lane 9) was digested with HindIII/BglII/EcoRI (lanes 1–4 and 6–9) or SmaI/BamHI (lane 5), fractionated on a 0.8% agarose gel, transferred to nylon membranes, and hybridized under stringent conditions with a probe containing the full-length ADSS ORF. The ~3500-bp genomic DNA fragment (lanes 1–3 and 6–8) encompasses 849 bases of the ADSS ORF and ~2700 bp of the 5’-UTR, and the ~3800-bp band (lanes 1–3 and 6–8) includes the remaining 1284 bp of the ADSS ORF and ~2500 bp of the 3’-UTR. The coding sequence of ADSS was excised from the pXG-BSD-ADSS episome by digestion with SmaI/BamHI, and presents as a 2133-bp fragment (lane 5). The absence of a hybridization signal in genomic DNA from Δadss and Δaah/Δadss parasites (lanes 4 and 9) confirmed the null genotype of these cell lines. B, the 1650-bp hybridization signal produced from genomic DNA that had been cut with HindIII/BglII/EcoRI and probed with the AAH ORF (lanes 1–5) encompasses 765 bp of the AAH ORF and 885 bp of the AAH 5’-UTR. The absence of an AAH hybridization signal in DNA from parasites containing the Δaah lesion (lanes 6–9) verified the genotype of these cell lines.

Δadss cells, grown in adenine plus EHNA (Fig. 4A), demonstrating both that extracellular adenine is deaminated to inosine by serum adenine deaminase and that EHNA is not an inhibitor of the leishmanial AAH.

The growth phenotypes of Δasl and Δasl[pASL] parasites were similarly assessed. Restrictive and permissive growth conditions for the Δasl knock-out were comparable to those of the Δadss parasites. Predictably, wild type and Δasl[pASL] promastigotes could proliferate in medium supplemented with any of the purine nucleobases or nucleosides appraised, whereas the

FIGURE 3. Southern and Western blot analysis of Δasl knock-outs. A, total genomic DNA from wild type (lane 1), ASL/adsl heterozygotes (lanes 2 and 3), Δasl (lane 4), and Δasl[pASL] parasites was digested with EcoRI/ClaI (lanes 1–4) or SmaI/BamHI (lane 5), fractionated on a 0.8% agarose gel, and blotted onto nylon membrane, and the blot was hybridized under stringent conditions with a probe containing the full-length ASL ORF. The ~9100-bp wild type ASL EcoRI/ClaI genomic DNA fragment (lanes 1–3) encompasses the entire ASL ORF as well as 4258 and 3419 bp of the 5’- and 3’-UTRs, respectively. The absence of a hybridization signal in genomic DNA from Δasl parasites (lane 4) confirmed that both copies of ASL had been deleted from this cell line. The full-length coding sequence of ASL was excised from the pXG-PHILO-ASL episome by digestion with SmaI/BamHI and is present as a 1440-bp fragment (lane 5). B, the fragments produced when digested genomic DNA was probed with the ASL 3’-UTR were as predicted from the ASL cosmids isolated from an L. donovani cosmids library. C, lysates of exponentially growing wild type (lane 1), Δasl (lane 2), and Δasl[pASL] (lane 3) parasites were analyzed by immunoblotting with monospecific polyclonal antisera to ASL as shown. The amount of protein loaded onto each lane was normalized using commercially available mouse anti-α-tubulin monoclonal antibody.
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Δasl null mutant could not grow with guanine, guanosine, hypoxanthine, inosine, or xanthine as the purine in the medium (Fig. 4B). The only permissive growth conditions for the Δasl line were a 6-aminopurine source, either adenine or adenosine, in medium to which 20 μM dCF was added.

**ADSS and ASL Activity Measurements in Vivo**—To demonstrate that ADSS and ASL are functional in intact parasites, the capacities of wild type and null mutant *L. donovani* promastigotes to metabolize [14C]hypoxanthine into various products was determined. Wild type parasites converted [14C]hypoxanthine into numerous detectable products, including di- and triphosphorylated nucleotides, adenylosuccinate, IMP, AMP, and nucleosides (Fig. 5). In contrast, Δasl parasites amassed high levels of adenylosuccinate, synthesized no AMP, and produced diminished amounts of total nucleotides (Fig. 5 and Table 1). Interestingly, a band that migrates above the nucleotides but below hypoxanthine (indicated by an arrow in Fig. 5; see also Table 1) appeared after TLC separation of the Δasl cellular metabolites. The nature of this band is unclear, but its location in the chromatogram suggests that it is a nonphosphorylated product, perhaps dephosphorylated adenylosuccinate. As expected, Δadss parasites did not metabolize [14C]hypoxanthine into any detectable amounts of adenylosuccinate or AMP, and the majority of the radiolabeled substrate was converted to IMP as well as other nucleotides (Fig. 5). The extra spot that accumulates in Δasl cells is absent in the Δadss lanes of the chromatogram. These data confirm the biochemical activities of ADSS and ASL in *L. donovani* promastigotes and the metabolic deficits in metabolism incurred by the Δadss and Δasl genetic lesions.

**Macrophage Infections**—To ascertain whether a genetic deficiency in either Δadss or Δasl compromised the ability of *L. donovani* to infect host cells, infectivity assays with stationary phase wild type, knock-out, and add-back promastigotes were performed in murine peritoneal macrophages. Whereas wild type, Δadss[pADSS], and Δasl[pASL] parasites were capable of infecting and sustaining robust infections in the macrophages, the parasite loads in macrophages infected with Δadss and Δasl parasites were 4.2 and 19.8%, respectively, relative to what was obtained for the wild type parasite infections (Fig. 6A).

**TABLE 1**

Percentage of products produced by each cell line when given [14C]hypoxanthine

| Products | Wild type | Δasl | Δadss |
|----------|-----------|-----|-------|
|          | 30 min | 120 min | 30 min | 120 min | 30 min | 120 min |
| Hyp      | 3.2    | 2.4    | 3.4    | 2.4    | 36.1   | 20.1   |
| Unknown  | 0.0    | 0.0    | 6.8    | 10.0   | 0.0    | 0.0    |
| Nucleosides | 0.0  | 3.8    | 3.8    | 2.9    | 4.6    | 14.0   |
| AMP      | 5.0    | 9.7    | 0.0    | 0.0    | 0.0    | 0.0    |
| IMP      | 0.0    | 2.9    | 7.1    | 7.1    | 39.0   | 29.0   |
| AMPs     | 6.0    | 6.6    | 64.6   | 53.9   | 0.0    | 0.0    |
| NDPS     | 86.2   | 74.7   | 14.3   | 23.7   | 20.3   | 36.9   |

Values are expressed as a percentage of the total amount of products produced by each cell line at 30 and 120 min. The products are listed in the left-most column. Hyp, hypoxanthine; AMP, adenosine; AMPS, adenylosuccinate.
Mouse Infections—Because both Δadss and Δasl parasites exhibited markedly reduced parasite loads in macrophages, the ability of the null mutants and their respective complements to infect BALB/c mice, a well characterized rodent model for L. donovani infections (48, 49, 62–64), was evaluated. Unexpectedly, despite the reduced parasite numbers observed in peritoneal macrophages infected with Δadss parasites (Fig. 6A), the inoculation of BALB/c mice with stationary phase Δadss promastigotes resulted in a robust infection in which parasite loads were equivalent to those obtained from mice injected with wild type parasites (Fig. 6B). The parasite burdens in livers and spleens from mice infected with the Δadss[pADSS] add-back were predictably equivalent to those obtained from mice inoculated with wild type parasites (Fig. 6B). The Δaah/Δadss double knock-out was also evaluated for its ability to establish an infection in mice. Parasite burdens were reduced by 1 to 2 orders of magnitude in livers but were equivalent to wild type levels in the spleens (Fig. 6B). Parasite loads in the livers and spleens of mice infected with the Δasl knock-out were 4 and 3.5 orders of magnitude lower, respectively, relative to the parasite loads obtained from mice inoculated with wild type L. donovani (Fig. 6C). Complementation of the genetic defect in the Δasl[pASL] add-back cell line essentially restored parasite burdens in livers and spleens to wild type levels (Fig. 6C).

Bacterial Complementation—To confirm that the L. donovani ASL encodes a functional ASL protein, the leishmanial ASL was used to complement the asl deficiency of SΦ200 E. coli (26). The SΦ200 bacteria are incapable of de novo purine synthesis because they lack the bifunctional E. coli enzyme that catalyzes both the adenylosuccinate and SAICAR lyase activities. SAICAR lyase is an early step in the purine biosynthetic pathway that occurs prior to the synthesis of IMP in purine prototrophic organisms (26). SΦ200 cells are, therefore, auxotrophic for purines but can be propagated in minimal medium containing both adenine and hypoxanthine, with the former necessary to bypass ASL to synthesize adenylate nucleotides and the latter to circumvent SAICAR lyase to make guanylate nucleotides. SΦ200 cells, however, cannot grow in either adenine or hypoxanthine alone (26). SΦ200 E. coli transformed with pBAce-ASL were capable of growing on minimal medium plates lacking purine or supplemented with adenine or hypoxanthine, whereas cells transformed with pBAce alone were only capable of growth on minimal medium when provided with both adenine and hypoxanthine (Fig. 7). In contrast, transformation of the ADSS chimeric construct pBAce-ADSS into a variety of adss-deficient E. coli strains harboring a purA mutation, including ES4, PC1523, JW4135-2, TX595, and H1238, did not complement the purine auxotrophy conferred by the adss lesion.
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Protein Expression, Purification, and Kinetic Analysis—Recombinant His₆-tagged L. donovani ADSS was purified to essential homogeneity over a Ni²⁺-NTA column and its catalytic activity assessed spectrophotometrically by monitoring the conversion of adenylosuccinate to AMP. ASL activity was first tested over a range of pH values and was found to be optimal at pH 7.0 (data not shown). ASL kinetics were then determined by varying the adenylosuccinate concentration at pH 7.0, the pH at which ASL displayed optimal activity. Michaelis-Menten analysis (56) (Fig. 8) revealed that the apparent Kₘ of ASL for adenylosuccinate was ~24 μM with a Vₘₐₓ of 2.1 μmol·min⁻¹·mg protein⁻¹. A kₗ₅ value of 1680 min⁻¹ was then calculated from the kinetic data (Fig. 8).

Attempts to purify sufficient, recombinant, His₆-tagged L. donovani ADSS using a Ni²⁺-NTA column were unproductive. Although ADSS production from pET200/D-TOPO was robust, virtually all of the recombinant ADSS protein was insoluble and not amenable to kinetic analysis, despite a number of induction and purification schemes that were implemented. The presence of ADSS as inclusion bodies could account for the failure of the pBAce-ADSS vector to complement multiple adss-deficient E. coli strains.

ADSS and ASL Localization—ADSS was localized to the cytosol by immunofluorescence of L. donovani parasites that expressed either NH₂- or COOH-terminal HA-tagged ADSS using anti-HA primary and Oregon Green- or Alexa Fluor®-568-conjugated secondary antibodies (Fig. 9A). The cytosolic location of ADSS was also supported by the lack of colocalization with IMPDH, a known glycosomal marker (27) (data not shown). Western blot analysis of whole cell lysates from 5′-HA-ADSS- and 3′-HA-ADSS-expressing parasites ensured that the HA tag was not cleaved from ADSS and that an HA-ADSS product of the correct molecular weight was produced in these parasites (data not shown).

ASL was localized to the cytosol using a variety of techniques including subcellular fractionation, immunofluorescence assay, and direct fluorescence. Separation of L. donovani lysates into organellar and cytosolic components by differential centrifugation and subsequent immunoblotting revealed that ASL was localized almost entirely to the cytosolic compartment (Fig. 9B). The differential centrifugation protocol that was employed has been shown previously to yield intact glycosomes (65, 66). Further fractionation of the post-nuclear cell lysates on a sucrose gradient revealed that PEX14, a known glycosomal marker, cosedimented with the glycosomes (lower portion of the gradient, fractions 15–28) (57), whereas ASL could be detected exclusively at the top of the sucrose gradient (fractions 1–14) with other cytosolic enzymes (Fig. 9C and data not shown). An immunofluorescence assay validated the cytosolic context of ASL in intact wild type and Δasl[Δsl] promastigotes and confirmed the absence of ASL in Δasl parasites (Fig. 9D). The cytosolic location of ASL was also corroborated by direct fluorescence in wild type L. donovani that had been transfected with pLCNEO-EGFPCO-ASL (Fig. 9D). The cytosolic milieu for ASL was further reinforced by the lack of colocalization with IMPDH, a glycosomal enzyme (27) (data not shown).

DISCUSSION

The striking non-infectious phenotype of the Δhgp/Δxprt double knock-out implicated the downstream nucleotide interconversion enzymes ADSS, ASL, IMPDH, and GMP synthase as crucial bottlenecks for purine metabolism by L. donovani and, therefore, potential therapeutic targets. To test this hypothesis, Δadss and Δasl mutants were generated by double targeted gene replacement and their growth and infectivity phenotypes determined. As predicted, both null strains exhibited a strikingly restricted growth phenotype that was virtually identical to that of the previously characterized Δhgp/Δxprt cell line. The only permissive growth conditions for Δadss and Δasl promastigotes were a 6-aminopurine, either adenine or adenosine, as the purine source, and dCF, the AAH inhibitor (Fig. 4). These growth studies clearly establish that both ADSS and ASL are essential enzymes for the conversion of IMP to AMP. Episomal complementation of the null lines restored the wild type growth spectrum, authenticating that the nonpermissive growth conditions could be ascribed to the specific genetic lesions in the mutant lines and not to secondary genetic alteration(s) that may have been introduced through the genetic manipulations required for strain construction.

To assess the impacts of the Δadss and Δasl lesions on the viability of the amastigote stage of L. donovani, the abilities of
**FIGURE 9. Localization of ADSS and ASL.** A, L. donovani promastigotes harboring ether the pXG-5’-HA-ADSS (I–III) or pXG-ADSS-3’-HA (IV–VI) episome were subjected to immunofluorescence analysis using mouse anti-HA antibodies, and goat anti-mouse Oregon Green-conjugated secondary antibody was used to detect the anti-HA antibodies. Parasites were also stained with DAPI (II and V) and phase contrast images are shown in panels III and VI. B, L. donovani promastigote cell lysates were fractionated by sedimentation at 45,000 × g for 1 h at 4°C, and the total cytosolic (lane 1 (C) and crude organellar (lane 2 (O)) fractions were subjected to Western blot analysis using anti-ASL antibody. C, post-nuclear whole cell lysate pellets were resolved by centrifugation on a linear sucrose density gradient. All 28 fractions collected from the sucrose gradient were then subjected to Western blot analysis using anti-ASL and anti-PEX14 antibodies. The gradient fractions are indicated on the top of the panels. D, fixed and permeabilized exponentially growing wild type (I–III), Δasl (IV–VI), or Δadss [ΔPEX14] (VII–IX) L. donovani promastigotes were incubated with rabbit anti-ASL antisera, and the primary antibodies were visualized with goat anti-rabbit Oregon Green-conjugated secondary antibody (X–XII). EGFP-tagged ASL was also visualized by direct fluorescence (X). DAPI staining is shown in panels II, V, VIII, and XI, and phase contrast images are illustrated in panels III, VI, IX, and XII.

the null strains to establish infection were assessed in murine macrophages and in BALB/c mice. Intracellular parasite numbers were substantially reduced in peritoneal macrophages infected with either Δadss or Δasl parasites, and these infectivity deficits were eliminated in macrophages infected with the episomally complemented add-back lines (Fig. 6A). Interestingly, Δadss parasites retained their capacity to establish a robust infection in mouse livers and spleens with parasite loads that were equivalent to those obtained from mice infected with either wild type or Δadss[ΔADSS] parasites. The disparate capacity of Δadss parasites to infect macrophages in vitro and mice in vivo could be explained by discrepant purine availability in the two experimental models. The capability of Δadss L. donovani to establish visceral infection levels equivalent to those of wild type parasites (Fig. 6B) intimates that L. donovani amastigotes are able to scavenge purines from the mouse that fulfill both their adenylate and guanylate nucleotide requirements using ADSS-independent routes. Presumably, the guanylate nucleotide requirements of the Δadss parasites could be fulfilled by guanine or guanosine derived directly from host breakdown of guanylate nucleotides. Alternatively, hypoxanthine, a breakdown product of host nucleotide metabolism, can be converted to GMP by the successive actions of HGPRT or XPR, IMPDH, and GMP synthase (see Fig. 1). To fulfill its adenylate nucleotide requirement, however, alternative metabolic routes using 6-aminopurine sources derived from the host must be utilized, because IMP to AMP conversion is blocked in the Δadss knock-out. These alternative routes include adenosine kinase and/or APRT (see Fig. 1). In contrast, mice inoculated with Δasl parasites displayed parasite loads in livers and spleens that were 4 and 3 orders of magnitude lower, respectively, than those in mice infected with wild type parasites. These reduced parasite burdens in mice infected with Δasl parasites were effectively restored to wild type levels by episomal complementation with ASL (Fig. 6C). Thus, the infectivity data in mice are analogous to those obtained in vitro with the peritoneal macrophages (Fig. 6, A and C) and bolster ASL as a possible target for therapeutic manipulation.

The discrepant parasite loads obtained from mice infected with L. donovani harboring either a Δadss or Δasl lesion was surprising but can be explained by their respective metabolic functions. When exposed to [14C]hypoxanthine as the extracellular purine, Δadss promastigotes primarily accumulate [14C]IMP, nucleosides, and other (presumably guanylate) nucleotides (Fig. 5). Conversely, the Δasl promastigotes accrete, as expected, adenylosuccinate and exhibit decreased di- and
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triphosphorylated nucleotide synthesis as compared with wild type parasites (Fig. 5). One can speculate, therefore, that Δasl L. donovani amastigotes also build up adenylosuccinate at the expense of nucleotide production; thus the adenylosuccinate serves as a metabolic “dead end” sink for Δasl parasites. Conversely, because adenylosuccinate synthesis is blocked in Δadss parasites, IMP is converted to guanylate nucleotides in both life cycle stages, whereas the amastigote adenylate pool is replenished from adenosine and/or adenine supplied by the host through the parasite adenosine kinase and/or APRT (Fig. 1). Thus, the Δadss knock-out can generate adenylate and guanyl rate nucleotides from host purines, whereas the Δasl amastigotes funnel host purines into adenylosuccinate via ADSS, thereby precluding sufficient nucleotide production in the parasite. Consistent with this scenario is the fact that we were unable to generate a Δasl/Δasl double knock-out in promastigotes under any experimental condition, presumably because exogenous purines were shunted to adenylosuccinate, a metabolic dead end for L. donovani harboring a Δasl mutation.

Because the infectivity data bolstered ASL as a promising therapeutic target, the ASL enzyme was characterized further. Recombinant ASL was purified to effective homogeneity, kinetically and functionally characterized, and localized. The recombinant enzyme catalyzed the conversion of adenylosuccinate to AMP and fumarate with high affinity (Fig. 8), and the L. donovani ASL gene was capable of complementing an asl-deficient (purB) strain of E. coli (Fig. 7). Because the bacterial ASL catalyzes two discrete steps in the purine biosynthesis pathway of E. coli, the SAICAR lyase activity that precedes IMP formation as well as adenylosuccinate lyase (26), the ability of the L. donovani ASL to complement the bacterial purB lesion demonstrates that the parasite enzyme catalyzes both activities. The fact that the L. donovani ASL enzyme can cleave SAICAR to 5-aminoimidazole-4-carboxamide ribotide and fumarate, the antepenultimate step in IMP biosynthesis in purine prototrophs, is a bit of a curiosity, because SAICAR lyase would be effectively unproductive in an organism such as Leishmania that lacks the remainder of the purine biosynthetic machinery.

ADSS was localized to the cytosol via indirect immunofluorescence (Fig. 9A). ASL was also shown to be cytosolic by subcellular fractionation, indirect immunofluorescence, and direct fluorescence (Fig. 9B–D). Many other vital components of the purine salvage pathway, however, including HGPRT, XPRT, and IMPDH (11, 12, 27), are sequestered within the glycosome. The targeting of HGPRT, XPRT, and IMPDH to the glycosome is mediated by an archetypical COOH-terminal triad, peroxisomal targeting signal 1 (11, 12, 27), which is lacking in ADSS and ASL. ADSS and ASL also lack the well characterized NH2-terminal peroxisomal targeting signal 2 (67). Thus, the purine salvage pathway is distributed between the cytosol and glycosomal compartments. The location of ADSS and ASL within the cytosolic milieu proves that the glycosomal membrane is permeable to nucleotides, as all salvaged purines are phosphoribosylated to the nucleotide level by glycosomal HGPRT and/or XPRT (2, 11, 12, 21). To generate adenylate nucleotides, therefore, the IMP product of HGPRT and XPRT must exit the glycosome intact in order to be converted in the cytosol to AMP by the sequential actions of ADSS and ASL.

This genetic dissection of the adenylate pathway advances ASL as a critical bottleneck in purine salvage by L. donovani and intimates that this last enzyme in AMP synthesis could serve as a possible drug target for visceral leishmaniasis and perhaps other forms of leishmaniasis triggered by other Leishmania species. Whether ASL is also a putative target for other genera of protozoan parasites remains to be explored, although ASL is not expressed by all protozoan parasites that infect humans (58). The purine salvage pathways of T. brucei and T. cruzi, protozoan pathogens derived from a genus closely related to Leishmania, are similar but not identical to that of Leishmania, suggesting that ASL could be essential for these pathogens, but this has not been tested. Despite the complexity of the purine pathway of Leishmania, this is, at least to date, the only purine-metabolizing enzyme that, by itself, is vital to both life cycle stages of the parasite. Previous studies have established that HGPRT and XPRT together are indispensable to the parasite, but neither is essential on its own (16, 21). Further therapeutic validation of ASL is therefore warranted.

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