The genetic toolbox for *Leishmania* parasites

Sigrid C. Roberts

Pacific University School of Pharmacy; Hillsboro, OR USA

Keywords: *Leishmania*, genetic manipulation, targeted gene deletion, episomes, overproducers

Abbreviations: AGO, argonaute; BSD, blasticidin S deaminase; CPA, cysteine peptidase A; DCL, dicer; HYG, hygromycin phosphotransferase; NEO, neomycin phosphotransferase; ODC, ornithine decarboxylase; PAC, puromycin acetyltransferase; PCR, polymerase chain reaction; PHLEO, bleomycin/phleomycin binding protein; SAT, streptothricin acetyltransferase; spp, species; RNAi, RNA interference

Leishmania parasites cause a variety of devastating diseases in tropical areas around the world. Due to the lack of vaccines and limited availability of drugs, new therapeutic targets are urgently needed. A variety of genetic tools have been developed to investigate the complex biology of this parasite and its interactions with the host. One of the main techniques is the generation of knockout parasites via targeted gene replacement, a process that takes advantage of the parasites ability to undergo homologous recombination. Studying the effect of gene deletions in vitro and in infectivity models in vivo allows understanding the function of a target gene and its potential as a therapeutic target. Other genetic manipulations available include episomal and chromosomal complementation and the generation of overproducer strains. However, there are also limitations, such as the lack of RNA interference machinery in most *Leishmania* species and limited options for inducible expression systems. The genomes of several *Leishmania* species have now been sequenced and will provide powerful resources in combination with the genetic tools that are available. The increasing knowledge of parasite biology and host parasite interactions derived from these studies will raise the number of potential therapeutic targets, which are sorely needed to combat leishmaniasis.

Introduction

Protozoan parasites of the genus *Leishmania* are the etiologic agents of leishmaniasis, a spectrum of devastating and potentially deadly diseases. The ailment affects an estimated 12 million people worldwide and no vaccine is available. Unfortunately, the current small inventory of available drugs to treat leishmaniasis is far from ideal, mainly because of toxicity and therapeutic unresponsiveness. Thus, a better understanding of parasite biology and host interactions is urgently needed in order to identify, characterize, and validate novel therapeutic targets. An impressive array of genetic tools has been developed for *Leishmania* over the years making this parasite a model organism for elucidating pathways, cellular processes and host interactions. These

tools have facilitated the use of reverse genetic approaches (from gene to phenotype) as a more targeted approach to study gene function than the classical or forward genetic method (from phenotype to gene). This minireview will describe the current methods that are available for molecular manipulations of *Leishmania*, with a focus on targeted gene replacement. Cited references are representatives only and not all inclusive of the available literature.

Cultivation of *Leishmania*

Leishmania species are digenetic parasites that exist as both insect vector and mammalian forms. Promastigotes corresponding to the parasite found in the gut of the sand fly vector can easily be cultured in vitro in large quantities and apparently indefinitely. In the mammalian host, *Leishmania* promastigotes are taken up by macrophages and delivered to the phagolysosome,¹⁻³ an intracellular organelle that is usually well equipped to combat foreign microorganisms. *Leishmania* parasites, however, change into the smaller non-motile amastigote form and thrive in this hostile environment.¹⁻³ Axenic or extracellular amastigote model systems have been developed that mimic the phagolysosome by reducing pH and increasing temperature.^{4,5} In vitro infectivity studies in primary macrophages (mainly derived from murine bone marrow or peritoneal cavity) or immortalized macrophage cell lines and in vivo infectivity models in mice or hamsters have been developed to study the medically more relevant amastigote form of this parasite.

Leishmania Genetics

Leishmania parasites have unusual genetic properties, foremost a surprising lack of transcriptional control. Genes are transcribed into polycistronic pre-mRNAs that are subsequently processed into mature mRNAs by trans splicing, which involves the addition of a spliced leader or miniexon, and polyadenylation.⁶ Control of gene expression does not occur at the transcriptional level but rather at the level of RNA stability, translation and protein turnover.⁷ Typical promoter elements for protein expressing genes have not been identified, and transcription occurs processively over long stretches in a single direction.^{8,9} However, strand switch regions appear to function similarly to promoters and

Correspondence to: Sigrid C. Roberts; Email: sroberts@pacificu.edu
Submitted: 07/25/11; Revised: 09/22/11; Accepted: 09/23/11
<http://dx.doi.org/10.4161/bbug.2.6.18205>

to define the direction of transcription.¹⁰ Although *Leishmania* parasites have generally been considered to be diploid organisms, aneuploidy may be a more accurate term. In *L. major*, individual chromosomes have been found in different ploidy states giving the genome a mosaic structure.¹¹ Genetic exchange has only recently been discovered but sexual crosses occur infrequently and apparently only in the insect vector¹² limiting the usefulness of crossing for genetic studies. Gene amplification via the creation of extrachromosomal elements that mediate drug resistance takes place frequently in *Leishmania* and the occurrence of aneuploidy or polyploidy further demonstrates the plasticity of the genome. Another unique feature of *Leishmania* and related parasites is the kinetoplast, an unusual organelle where RNA editing, a templated modification of pre-mRNA sequences, often leads to substantial sequence differences between the pre-mRNA and the mature mRNA.

Leishmania Genome Databases

The first leishmanial genome to be sequenced completely was that of *L. major* (Friedlin strain).¹³ More recently, the genomes of *L. infantum* (clone JPCM5), *L. braziliensis* (clone MHOM/BR/75M2904), and *L. mexicana* (MHOM/GT/2001/U1103) have been sequenced and currently underway is the sequencing of the *L. donovani* genome (<http://www.sanger.ac.uk/resources/downloads/protozoa/>). Genomic information is available from sources such as GeneDB (<http://www.genedb.org>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). In addition, TriTrypDB, an integrated database designed to ease data mining and interpretation, has been developed (<http://tritrypdb.org>).¹⁴ These genome databases are invaluable resources for identifying putative genes of interest, in silico modeling of pathways and networks, genome wide evolutionary analysis and the identification of putative therapeutic targets. Despite the tremendous usefulness of the genome sequences, it should also be noted that many putative gene functions are still unknown or hypothetical. Remarkably, efforts to sequence whole genomes of individual *Leishmania* isolates from patients have become more feasible and will undoubtedly provide useful information, for example on drug resistance mechanisms.

Targeted Gene Replacement

The technique of targeted gene replacement to generate knockout or gene deletion parasites was pioneered in the early 1990s¹⁵⁻¹⁸ and is now routinely used to investigate the importance or function of genes of interest. *Leishmania* parasites effectively undergo homologous recombination, which is used to exchange target genes with drug resistance markers where the introduced drug resistance markers provide a selection mechanism (Fig. 1A). Targeting constructs are designed in which upstream and downstream regions corresponding to the flanking sequences of the target gene are joined to a drug resistance cassette. Previously, time-consuming cloning steps were involved in the generation of targeting DNAs. However, more recently several

techniques have been developed that simultaneously assemble multiple DNA fragments and considerably simplify the assembly of targeting constructs. Examples include the use of a PCR fusion-based strategy¹⁹ and a one step multi-fragment ligation technique.²⁰ The linearized and purified constructs are transfected into parasites via electroporation. Subsequently, transfected parasites are typically plated on semi-solid agar plates and drug resistant colonies are selected to isolate isogenic clonal cell lines. DNA gel blots and polymerase chain reactions (PCR) are performed to verify that the expected homologous recombination event has occurred. Because *Leishmania* spp are diploid organisms, two rounds of targeted gene replacement have to be performed. One round of targeted gene replacement followed by negative selection for loss-of-heterozygosity can also be achieved but depends on the availability of a suitable selection scheme.^{21,22} It should be noted that several drug resistance markers are currently available (Table 1).

Targeted deletion of non-vital or conditionally essential genes facilitates studies of promastigotes and axenic amastigotes in defined growth media to address the function of target proteins and to elucidate metabolic pathways and cellular function.²³⁻³⁰ Macrophages and in vivo animal infectivity studies in mouse and hamster models using gene deletion strains address whether the corresponding proteins are involved in virulence or survival of intracellular parasites.³¹⁻³⁵ Together, the results can identify putative targets for the development of therapeutic strategies. One example for the value of this approach is the elucidation of the polyamine biosynthetic pathway in *Leishmania* and the validation of ornithine decarboxylase (ODC) as a potential therapeutic target. Gene deletion mutants were generated for each of the polyamine biosynthetic enzymes and revealed that each enzyme is essential for polyamine biosynthesis, i.e., no alternative pathway exists.^{24,27-29} Furthermore, substantial differences compared with the mammalian pathway were discovered, for example the lack of a spermine synthase and a backconversion pathway in the parasite.²⁴ Finally, in vivo infectivity studies in mice revealed that arginase gene deletion mutants were able to infect animals whereas ODC gene deletion mutants exhibited a profoundly reduced infectivity phenotype.^{32,36}

It is impossible to generate knockouts of essential genes if gene function cannot be rescued by nutrient supplementation or other rescue manipulations. However, the ability to generate chromosomal null mutants in the presence, but not absence, of episomes that express the targeted gene is generally accepted as evidence for the essentiality of a specific gene.^{19,37} It should also be noted that attempts to replace essential genes often reveals chromosomal amplification or aneuploidy, a testament to the unusual plasticity of the *Leishmania* genome.¹⁷

Complementation

Complementation or add-back experiments are considered necessary to verify that the observed phenotype can indeed be ascribed to the loss of the target gene and not to other spontaneous, unrelated mutations. Target genes are usually cloned into an episome containing a third drug resistance (selective)

Figure 1. For figure legend, see page 323.

Table 1. Available drug resistance markers

Drug resistance gene	Abbreviation	Drug	Reference
<i>Neomycin phosphotransferase</i>	NEO	Geneticin	Cruz and Beverley 1990
<i>Hygromycin phosphotransferase</i>	HYG	Hygromycin	Cruz et al. 1991
<i>Puromycin acetyltransferase</i>	PAC	Puromycin	Freedman and Beverley 1993
<i>Bleomycin/Phleomycin binding protein</i>	PHLEO	Phleomycin	Freedman and Beverley 1993
<i>Streptothricin acetyltransferase</i>	SAT	Nourseothricin	Joshi et al. 1995
<i>Blasticidin S deaminase</i>	BSD	Blasticidin S	Goyard 2000

marker (Fig. 1B). However, the vectors currently available for *Leishmania* transfections have several limitations, including the fact that they are not always stably maintained in the absence of selective pressure. The instability of episomal vectors rarely impacts studies with parasites in cultures containing the selective drug. However, this drawback poses serious concerns for long-term infectivity studies in animals in which *Leishmania* are obligatorily passaged for several weeks or months in the absence of the selective drug. Because the episomal vectors lack active segregation mechanisms, equal distribution between daughter cells is not guaranteed. Furthermore, the level of gene expression from an episome cannot be controlled and will fluctuate among individual parasites depending upon the episomal copy number. Thus, expression of the target protein may be low and insufficient for rescue or too high and have deleterious effects on parasite growth. Overall, the need for improved complementation systems to validate targets in *Leishmania* is considerable. Sequence elements that confer mitotic stability and segregation have been identified in artificial chromosomal elements^{38–41} and will hopefully aid in the development of stable episomal constructs. Other strategies have targeted the add-back gene copy to chromosomal locations (Fig. 1C). In one case, the *L. donovani* *ODC* gene has been re-introduced into its original locus into a Δ *Ldodc* strain.⁴² The selective pressure of restoring putrescine prototrophy was sufficient to isolate complemented cell lines.⁴² The advantage of such a knock-in strategy is that no additional drug resistance marker—and thus no exogenous sequence—is required, that the gene is expressed at wild type levels and that it will be maintained stably.⁴² However, this strategy is only possible if a selection scheme, such as restoring prototrophy, is available. In a similar strategy, the gene encoding the *L. infantum* cysteine peptidase (*LiCPA*) gene was re-introduced into the *CPA* chromosomal locus of two Δ *Licpa* strains.³³ However, this construct contained the blasticidin resistance gene to provide a selective marker and the presence of such exogenous sequences may interfere with gene expression levels.³³ It should be noted that integration of DNA into chromosomal loci is not always stable, for example repetitive clusters such as the 18S RNA and the tubulin gene cluster appear to have low genetic stability.⁴³

Negative Selection

Negative selection techniques have been applied to *Leishmania* in order to minimize the number of drug resistance markers or to remove exogenous DNA sequences. Introduction of genes encoding enzymes like thymidine kinase and cytosine deaminase confer sensitivity to ganciclovir and 5-fluorocytosine, respectively.^{44,45} One example is a hit and run strategy that has been applied to delete one allele of the CPA gene array: the thymidine kinase gene was integrated into the target area followed by its removal using a null targeting fragment containing no exogenous DNA.⁴⁶ Furthermore, transgenic, drug sensitive *Leishmania* strains may be a potential tool for the development of safe life vaccine strains.^{44,47}

RNA Interference

RNA interference (RNAi) targets the degradation of specific mRNAs with corresponding short double stranded RNA sequences. This pathway is present in higher eukaryotes, presumably to regulate gene expression and as a defense mechanism against viruses and transposons. RNAi has become a useful tool for molecular geneticists in *Trypanosoma brucei*, a parasite closely related to *Leishmania*, and is now commonly being used to study the effect of silencing or knock down of target genes.⁴⁸ However, most *Leishmania* species lack the RNAi machinery^{49,50} with the exception of *L. braziliensis* and other *Leishmania* species within the subgenus Viannia.^{49,50} A comparison of genome data for genes encoding proteins of the RNAi machinery indicates that RNAi was lost after the separation of the *Leishmania* subgenus Viannia from other *Leishmania* species.⁴⁹ It is unclear at this time what evolutionary pressure could have caused retention of the RNAi machinery in one *Leishmania* genus and loss in others but viral invasion, genome plasticity and gene amplification may all have played a role.⁴⁹ Genes encoding orthologs of argonaute (AGO1) and dicer (DCL1 and DCL2) are present in the *L. braziliensis* genome, and RNAi knockdown of *AGO1* in *L. braziliensis* confirmed both that RNAi functions in this *Leishmania* species and the function of the putative AGO1 for RNAi.⁴⁹ The usefulness of RNAi as a tool to inducibly knockdown genes in *L. braziliensis*

Figure 1 (See opposite page). Targeted gene replacement and complementation strategies. (A) Generating knockout parasites by two rounds of targeted gene replacement. (B) Complementation of knockout parasites by re-introducing the target gene on an episomal vector. (C) Complementation of a knockout strain by re-integrating the target gene into the original chromosomal locus. The following drug resistance markers are indicated as examples: NEO: neomycin phosphotransferase, HYG: hygromycin phosphotransferase, BSD: blasticidin S deaminase. Crosses indicate the event of homologous recombination.

was further confirmed by targeting the mRNA for the paraflagellar rod protein, a vital component of the flagellum.⁴⁹

The advantage of RNAi over traditional knockout approaches is that a knockdown can be induced, thus it allows the study of essential genes. Furthermore, a traditional knockout approach requires at least two rounds of targeted gene replacement and sometimes more if multiple copy numbers or chromosome amplification is present. RNAi, however, targets the mRNA, thus the gene copy number is irrelevant. On the other hand, a major disadvantage of RNAi is that sometimes only partial reduction of gene expression is achieved whereas targeted gene replacement provides a clean knockout.

Expression Systems

Leishmania parasites can be transfected with episomes in order to express endogenous or heterologous proteins. The episomes contain drug resistance markers to facilitate selection and raising the drug concentration increases the copy number of the episomes thus yielding higher protein expression.⁵¹ The overproducer strains can be used as tools to investigate the mode of action of cytotoxic analogs in Leishmania because they will be more resistant to a drug specifically targeting the overproduced protein.^{51,52} Overexpression of certain genes can also help to understand their biological function as the phenotypic effects caused by the investigated protein will be amplified.^{53,54} A novel system utilizing artificial linear episome-based structures shows promise for expressing large quantities of target proteins in *L. tarentolae* for the purpose of protein purification.^{55,56}

A unique challenge to the development of regulated expression systems is posed by the unusual lack of transcriptional control in Leishmania parasites. Attempts to develop systems where gene expression can be induced have been disappointing thus far but are being explored further.⁵⁶⁻⁵⁸ As mentioned above, RNAi for knock-down of gene expression is not possible in most Leishmania species. However, a technique that regulates the stability of specific proteins has recently been modified for the use in Leishmania.^{59,60} Here, synthetic molecules target

degradation domains that can be fused to a specific target protein while the addition of a specific ligand can shield the protein from degradation. In Leishmania, the expression of lipophosphoglycans has been inducibly downregulated using this destabilizing method.⁶⁰

Insertional Mutagenesis

Because homologous recombination occurs more frequently than random integration, insertional mutagenesis has been limited in Leishmania. However, the heterologous *Drosophila mariner* transposon system has been modified for use in Leishmania and allows in vivo mutagenesis or shuttle mutagenesis, as well as tagging or trapping of genes by fusion to reporter proteins.^{8,61-64} This system can potentially be used to study the regulation of gene expression and the localization of proteins, and may facilitate whole proteogenomic approaches.^{8,65,66}

Summary

A substantial number of genetic tools are available to manipulate Leishmania, making this parasite an ideal model organism. Foremost, the ability to generate gene deletion mutants has facilitated an understanding of the function and importance of a number of targeted genes. The combination of genetic advances and available genome sequences will be a powerful resource for in depth analyses. With these advanced tools, the function and importance of genes, metabolic pathways and cellular processes can be explored and will yield a better understanding of the parasite and especially its interaction with the host during infections. This increased knowledge will undoubtedly enhance the number of potential therapeutic targets, which are sorely needed to combat the devastating diseases caused by Leishmania.

Acknowledgments

The author's research that is described in this article was supported in part by Grant AI041622 from the National Institute of Allergy and Infectious Disease.

References

- Chakraborty P, Basu MK. Leishmania phagolysosome: drug trafficking and protein sorting across the compartment. *Crit Rev Microbiol* 1997; 23:253-68; PMID:9347223; DOI:10.3109/10408419709115139.
- Handman E. Cell biology of Leishmania. *Adv Parasitol* 1999; 44:1-39; PMID:10563394; DOI:10.1016/S0065-308X(08)60229-8.
- Lodge R, Descoteaux A. Leishmania invasion and phagosome biogenesis. *Subcell Biochem* 2008; 47:174-81; PMID:18512351; DOI:10.1007/978-0-387-78267-6_14.
- Debrabant A, Joshi MB, Pimenta PF, Dwyer DM. Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. *Int J Parasitol* 2004; 34:205-17; PMID:15037106; DOI:10.1016/j.ijpara.2003.10.011.
- Doyle PS, Engel JC, Pimenta PF, da Silva PP, Dwyer DM. *Leishmania donovani*: long-term culture of axenic amastigotes at 37°C. *Exp Parasitol* 1991; 73:326-34; PMID:1915747; DOI:10.1016/0014-4894(91)90104-5.
- LeBowitz JH, Smith HQ, Rusche L, Beverley SM. Coupling of poly(A) site selection and trans-splicing in Leishmania. *Genes Dev* 1993; 7:996-1007; PMID:8504937; DOI:10.1101/gad.7.6.996.
- Cohen-Freue G, Holzer TR, Forney JD, McMaster WR. Global gene expression in Leishmania. *Int J Parasitol* 2007; 37:1077-86; PMID:17574557; DOI:10.1016/j.ijpara.2007.04.011.
- Beverley SM, Akopyants NS, Goyard S, Matlib RS, Gordon JL, Brownstein BH, et al. Putting the Leishmania genome to work: functional genomics by transposon trapping and expression profiling. *Philos Trans R Soc Lond B Biol Sci* 2002; 357:47-53; PMID:11839181; DOI:10.1098/rstb.2001.1048.
- Clayton CE. Life without transcriptional control? From fly to man and back again. *EMBO J* 2002; 21:1881-8; PMID:11953307; DOI:10.1093/emboj/21.8.1881.
- Martínez-Calvillo S, Yan S, Nguyen D, Fox M, Stuart K, Myler PJ. Transcription of *Leishmania major* Friedlin chromosome 1 initiates in both directions within a single region. *Mol Cell* 2003; 11:1291-9; PMID:12769852; DOI:10.1016/S1097-2765(03)00143-6.
- Sterkers Y, Lachaud L, Crobu L, Bastien P, Pages M. FISH analysis reveals aneuploidy and continual generation of chromosomal mosaicism in *Leishmania major*. *Cell Microbiol* 2011; 13:274-83; PMID:20964798; DOI:10.1111/j.1462-5822.2010.01534.x.
- Akopyants NS, Kimblin N, Secundino N, Patrick R, Peters N, Lawyer P, et al. Demonstration of genetic exchange during cyclical development of Leishmania in the sand fly vector. *Science* 2009; 324:265-8; PMID:19359589; DOI:10.1126/science.1169464.
- Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, et al. The genome of the kinetoplastid parasite, *Leishmania major*. *Science* 2005; 309:436-42; PMID:16020728; DOI:10.1126/science.1112680.

14. Aslett M, Aurrecochea C, Berriman M, Brestelli J, Brunk BP, Carrington M, et al. TriTrypDB: a functional genomic resource for the Trypanosomatidae. *Nucleic Acids Res* 2010; 38:457-62; PMID:19843604; DOI:10.1093/nar/gkp851.
15. Cruz A, Beverley SM. Gene replacement in parasitic protozoa. *Nature* 1990; 348:171-3; PMID:2234081; DOI:10.1038/348171a0.
16. Cruz A, Coburn CM, Beverley SM. Double targeted gene replacement for creating null mutants. *Proc Natl Acad Sci USA* 1991; 88:1710-4; PMID:1651496; DOI:10.1073/pnas.88.16.1710.
17. Cruz AK, Titus R, Beverley SM. Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting. *Proc Natl Acad Sci USA* 1993; 90:1599-603; PMID:8381972; DOI:10.1073/pnas.90.4.1599.
18. Curotto de Lafaille MA, Wirth DE. Creation of Null/+ mutants of the alpha-tubulin gene in *Leishmania enriettii* by gene cluster deletion. *J Biol Chem* 1992; 267:23839-46; PMID:1429722.
19. Mukherjee A, Roy G, Guimond C, Ouellette M. The gamma-glutamylcysteine synthetase gene of *Leishmania* is essential and involved in response to oxidants. *Mol Microbiol* 2009; 74:914-27; PMID:19818018; DOI:10.1111/j.1365-2958.2009.06907.x.
20. Fulwiler AL, Soysa DR, Ullman B, Yates PA. A rapid, efficient and economical method for generating leishmanial gene targeting constructs. *Mol Biochem Parasitol* 2011; 175:209-12; PMID:21055426; DOI:10.1016/j.molbiopara.2010.10.008.
21. Gueiros-Filho FJ, Beverley SM. Selection against the dihydrofolate reductase-thymidylate synthase (DHFR-TS) locus as a probe of genetic alterations in *Leishmania major*. *Mol Cell Biol* 1996; 16:5655-63; PMID:8816478.
22. Hwang HY, Ullman B. Genetic analysis of purine metabolism in *Leishmania donovani*. *J Biol Chem* 1997; 272:19488-96; PMID:9235951; DOI:10.1074/jbc.272.31.19488.
23. Beverley SM, Turco SJ. Identification of genes mediating lipophosphoglycan biosynthesis by functional complementation of *Leishmania donovani* mutants. *Ann Trop Med Parasitol* 1995; 89:11-7; PMID:8745922.
24. Jiang Y, Roberts SC, Jardim A, Carter NS, Shih S, Ariyanayagam M, et al. Ornithine decarboxylase gene deletion mutants of *Leishmania donovani*. *J Biol Chem* 1999; 274:3781-8; PMID:9920931; DOI:10.1074/jbc.274.6.3781.
25. Joshi PB, Sacks DL, Modi G, McMaster WR. Targeted gene deletion of *Leishmania major* genes encoding developmental stage-specific leishmanolysin (GP63). *Mol Microbiol* 1998; 27:519-30; PMID:9489664; DOI:10.1046/j.1365-2958.1998.00689.x.
26. Murray AS, Lynn MA, McMaster WR. The *Leishmania mexicana* A600 genes are functionally required for amastigote replication. *Mol Biochem Parasitol* 2010; 172:80-9; PMID:20307588; DOI:10.1016/j.molbiopara.2010.03.008.
27. Roberts SC, Jiang Y, Jardim A, Carter NS, Heby O, Ullman B. Genetic analysis of spermidine synthase from *Leishmania donovani*. *Mol Biochem Parasitol* 2001; 115:217-26; PMID:11420108; DOI:10.1016/S0166-6851(01)00293-6.
28. Roberts SC, Scott J, Gasteier JE, Jiang Y, Brooks B, Jardim A, et al. S-adenosylmethionine decarboxylase from *Leishmania donovani*. Molecular, genetic and biochemical characterization of null mutants and overproducers. *J Biol Chem* 2002; 277:5902-9; PMID:11734561; DOI:10.1074/jbc.M110118200.
29. Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. Arginase plays a pivotal role in polyamine precursor metabolism in *Leishmania*. Characterization of gene deletion mutants. *J Biol Chem* 2004; 279:23668-78; PMID:15023992; DOI:10.1074/jbc.M402042200.
30. Rodriguez-Contreras D, Feng X, Keeney KM, Bouwer HG, Landfear SM. Phenotypic characterization of a glucose transporter null mutant in *Leishmania mexicana*. *Mol Biochem Parasitol* 2007; 153:9-18; PMID:17306380; DOI:10.1016/j.molbiopara.2007.01.010.
31. Beverley SM, Turco SJ. Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*. *Trends Microbiol* 1998; 6:35-40; PMID:9481823; DOI:10.1016/S0966-842X(97)01180-3.
32. Boitz JM, Yates PA, Kline C, Gaur U, Wilson ME, Ullman B, et al. *Leishmania donovani* ornithine decarboxylase is indispensable for parasite survival in the mammalian host. *Infect Immun* 2009; 77:756-63; PMID:19064633; DOI:10.1128/IAI.01236-08.
33. Denise H, Poot J, Jimenez M, Ambit A, Herrmann DC, Vermeulen AN, et al. Studies on the CPA cysteine peptidase in the *Leishmania infantum* genome strain JPCM5. *BMC Mol Biol* 2006; 7:42; PMID:17101050; DOI:10.1186/1471-2199-7-42.
34. Gilroy C, Olenyik T, Roberts SC, Ullman B. Spermidine Synthase Is Required for Virulence of *Leishmania donovani*. *Infect Immun* 2011; 79:2764-9; PMID:21536795; DOI:10.1128/IAI.00073-11.
35. Turco SJ, Spath GF, Beverley SM. Is lipophosphoglycan a virulence factor? A surprising diversity between *Leishmania* species. *Trends Parasitol* 2001; 17:223-6; PMID:11323305; DOI:10.1016/S1471-4922(01)01895-5.
36. Gaur U, Roberts SC, Dalvi RP, Corraliza I, Ullman B, Wilson ME. An effect of parasite-encoded arginase on the outcome of murine cutaneous leishmaniasis. *J Immunol* 2007; 179:8446-53; PMID:18056391.
37. Chawla B, Jhingran A, Singh S, Tyagi N, Park MH, Srinivasan N, et al. Identification and characterization of a novel deoxyhypusine synthase in *Leishmania donovani*. *J Biol Chem* 2010; 285:453-63; PMID:19880510; DOI:10.1074/jbc.M109.048850.
38. Casagrande L, Ruiz JC, Beverley SM, Cruz AK. Identification of a DNA fragment that increases mitotic stability of episomal linear DNAs in *Leishmania major*. *Int J Parasitol* 2005; 35:973-80; PMID:15996670; DOI:10.1016/j.ijpara.2005.04.004.
39. Dubessay P, Ravel C, Bastien P, Crobu L, Dedet JP, Pages M, et al. The switch region on *Leishmania major* chromosome 1 is not required for mitotic stability or gene expression, but appears to be essential. *Nucleic Acids Res* 2002; 30:3692-7; PMID:12202753; DOI:10.1093/nar/gkf510.
40. Tamar S, Papadopolou B. A telomere-mediated chromosome fragmentation approach to assess mitotic stability and ploidy alterations of *Leishmania* chromosomes. *J Biol Chem* 2001; 276:11662-73; PMID:11152684; DOI:10.1074/jbc.M009006200.
41. Boucher N, McNicoll E, Laverdiere M, Rochette A, Chou MN, Papadopolou B. The ribosomal RNA gene promoter and adjacent cis-acting DNA sequences govern plasmid DNA partitioning and stable inheritance in the parasitic protozoan *Leishmania*. *Nucleic Acids Res* 2004; 32:2925-36; PMID:15161957; DOI:10.1093/nar/gkh617.
42. Roberts SC, Kline C, Liu W, Ullman B. Generating knock-in parasites: integration of an ornithine decarboxylase transgene into its chromosomal locus in *Leishmania donovani*. *Exp Parasitol* 2011; 128:166-9; PMID:21354142; DOI:10.1016/j.exppara.2011.02.020.
43. Mureev S, Kushnir S, Kolesnikov AA, Breitling R, Alexandrov K. Construction and analysis of *Leishmania tarentolae* transgenic strains free of selection markers. *Mol Biochem Parasitol* 2007; 155:71-83; PMID:17658188; DOI:10.1016/j.molbiopara.2007.05.007.
44. Davoudi N, Tate CA, Warburton C, Murray A, Mahboudi F, McMaster WR. Development of a recombinant *Leishmania* strain sensitive to ganciclovir and 5-fluorocytosine for use as a live vaccine challenge in clinical trials. *Vaccine* 2005; 23:1170-7; PMID:15629360; DOI:10.1016/j.vaccine.2004.08.032.
45. LeBowitz JH, Cruz A, Beverley SM. Thymidine kinase as a negative selectable marker in *Leishmania major*. *Mol Biochem Parasitol* 1992; 51:321-5; PMID:1315415; DOI:10.1016/0166-6851(92)90082-U.
46. Denise H, Coombs GH, Mottram JC. Generation of *Leishmania* mutants lacking antibiotic resistance genes using a versatile hit-and-run targeting strategy. *FEMS Microbiol Lett* 2004; 235:89-94; PMID:15158266; DOI:10.1111/j.1574-6968.2004.tb09571.x.
47. Muyombwe A, Olivier M, Harvie P, Bergeron MG, Ouellette M, Papadopolou B. Protection against *Leishmania major* challenge infection in mice vaccinated with live recombinant parasites expressing a cytotoxic gene. *J Infect Dis* 1998; 177:188-95; PMID:9419187; DOI:10.1086/513821.
48. Ullu E, Tschudi C, Chakraborty T. RNA interference in protozoan parasites. *Cell Microbiol* 2004; 6:509-19; PMID:15104593; DOI:10.1111/j.1462-5822.2004.00399.x.
49. Lye LF, Owens K, Shi H, Murta SM, Vieira AC, Turco SJ, et al. Retention and loss of RNA interference pathways in trypanosomatid protozoans. *PLoS Pathog* 2010; 6:1001161; PMID:21060810; DOI:10.1371/journal.ppat.1001161.
50. Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, Quail MA, et al. Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nat Genet* 2007; 39:839-47; PMID:17572675; DOI:10.1038/ng2053.
51. Roberts SC, Jiang Y, Gasteier J, Frydman B, Marton LJ, Heby O, et al. *Leishmania donovani* polyamine synthetic enzyme overproducers as tools to investigate the mode of action of cytotoxic polyamine analogs. *Antimicrob Agents Chemother* 2007; 51:438-45; PMID:17116678; DOI:10.1128/AAC.01193-06.
52. Nunes VS, Damasceno JD, Freire R, Tosi LR. The Hus1 homologue of *Leishmania major* encodes a nuclear protein that participates in DNA damage response. *Mol Biochem Parasitol* 2011; 177:65-9; PMID:21291918; DOI:10.1016/j.molbiopara.2011.01.011.
53. Bhargava P, Kumar K, Chaudhary SS, Saxena AK, Roy U. Cloning, overexpression and characterization of *Leishmania donovani* squalene synthase. *FEMS Microbiol Lett* 2010; 311:82-92; PMID:20722739; DOI:10.1111/j.1574-6968.2010.02071.x.
54. Uliana SR, Goyal N, Freymuller E, Smith DE. *Leishmania*: overexpression and comparative structural analysis of the stage-regulated meta 1 gene. *Exp Parasitol* 1999; 92:183-91; PMID:10403759; DOI:10.1006/expr.1999.4410.
55. Kushnir S, Cirstea IC, Basiliya L, Lupilova N, Breitling R, Alexandrov K. Artificial linear episome-based protein expression system for protozoan *Leishmania tarentolae*. *Mol Biochem Parasitol* 2011; 176:69-79; PMID:21167214; DOI:10.1016/j.molbiopara.2010.12.002.
56. Kushnir S, Gase K, Breitling R, Alexandrov K. Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolae*. *Protein Expr Purif* 2005; 42:37-46; PMID:15939291; DOI:10.1016/j.pep.2005.03.004.
57. Yan S, Myler PJ, Stuart K. Tetracycline regulated gene expression in *Leishmania donovani*. *Mol Biochem Parasitol* 2001; 112:61-9; PMID:11166387; DOI:10.1016/S0166-6851(00)00345-5.
58. Yao C, Luo J, Hsiao CH, Donelson JE, Wilson ME. *Leishmania chagasi*: a tetracycline-inducible cell line driven by T7 RNA polymerase. *Exp Parasitol* 2007; 116:205-13; PMID:17320870; DOI:10.1016/j.exppara.2007.01.001.

59. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* 2006; 126:995-1004; PMID:16959577; DOI:10.1016/j.cell.2006.07.025.
60. Madeira da Silva L, Owens KL, Murta SM, Beverley SM. Regulated expression of the *Leishmania major* surface virulence factor lipophosphoglycan using conditionally destabilized fusion proteins. *Proc Natl Acad Sci USA* 2009; 106:7583-8; PMID:19383793; DOI:10.1073/pnas.0901698106.
61. Augusto MJ, Squina FM, Marchini JF, Dias FC, Tosi LR. Specificity of modified *Drosophila* mariner transposons in the identification of *Leishmania* genes. *Exp Parasitol* 2004; 108:109-13; PMID:15582507; DOI:10.1016/j.exppara.2004.08.002.
62. Damasceno JD, Beverley SM, Tosi LR. A transposon toolkit for gene transfer and mutagenesis in protozoan parasites. *Genetica* 2010; 138:301-11; PMID:19763844; DOI:10.1007/s10709-009-9406-7.
63. Gueiros-Filho FJ, Beverley SM. Trans-kingdom transposition of the *Drosophila* element mariner within the protozoan *Leishmania*. *Science* 1997; 276:1716-9; PMID:9180085; DOI:10.1126/science.276.5319.1716.
64. Robinson KA, Goyard S, Beverley SM. In vitro shuttle mutagenesis using engineered mariner transposons. *Methods Mol Biol* 2004; 270:299-318; PMID:15153636.
65. Coelho AC, Tosi LR, Cotrim PC. Mapping of a *Leishmania major* gene/locus that confers pentamidine resistance by deletion and insertion of transposable element. *Rev Inst Med Trop Sao Paulo* 2004; 46:109-12; PMID:15141283; DOI:10.1590/S0036-46652004000200010.
66. Goyard S, Tosi LR, Gouzova J, Majors J, Beverley SM. New Mos1 mariner transposons suitable for the recovery of gene fusions in vivo and in vitro. *Gene* 2001; 280:97-105; PMID:11738822; DOI:10.1016/S0378-1119(01)00779-X.