

Genome sequencing of the lizard parasite *Leishmania tarentolae* reveals loss of genes associated to the intracellular stage of human pathogenic species

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ABSTRACT

The *Leishmania tarentolae* Parrot-Tarll strain genome sequence was resolved to an average 16-fold mean coverage by next-generation DNA sequencing technologies. This is the first non-pathogenic to humans kinetoplastid protozoan genome to be described thus providing an opportunity for comparison with the completed genomes of pathogenic *Leishmania* species. A high synteny was observed between all sequenced *Leishmania* species. A limited number of chromosomal regions diverged between *L. tarentolae* and *L. infantum*, while remaining syntenic to *L. major*. Globally, >90% of the *L. tarentolae* gene content was shared with the other *Leishmania* species. We identified 95 predicted coding sequences unique to *L. tarentolae* and 250 genes that were absent from *L. tarentolae*. Interestingly, many of the latter genes were expressed in the intracellular amastigote stage of pathogenic species. In addition, genes coding for products involved in antioxidant defence or participating in vesicular-mediated protein transport were underrepresented in *L. tarentolae*. In contrast to other *Leishmania* genomes, two gene families were expanded in *L. tarentolae*, namely the zinc metallo-peptidase surface glycoprotein GP63 and

the promastigote surface antigen PSA31C. Overall, *L. tarentolae*'s gene content appears better adapted to the promastigote insect stage rather than the amastigote mammalian stage.

INTRODUCTION

Leishmania is an early-branching unicellular eukaryote that belongs to the Kinetoplastida order and the family Trypanosomatidae. *Leishmania* species are transmitted by the bite of female phlebotomine sand flies as extracellular flagellated metacyclic promastigotes and replicate in mammalian macrophages as intracellular aflagellated amastigotes. *Leishmania* infections represent a global health problem with 350 million people at risk, an annual incidence of 2 million and an overall prevalence estimated at 12 million people worldwide (1). At least 20 *Leishmania* species cause a large spectrum of clinical manifestations ranging from self-resolving skin lesions (*L. major*) to mucocutaneous manifestations (*L. braziliensis*) reaching to life-threatening visceral diseases (*L. donovani*/*L. infantum*).

Through phylogenetic analyses, *Leishmania* was divided in three distinct subgenera: the *Leishmania*, the *Viannia* and the *Sauroleishmania* (2). The classification of lizard *Leishmania* as a distinct genus was once debated (3,4) but the molecular evidence did not support this assumption. *Leishmania* (*Sauroleishmania*) *tarentolae* was first

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isolated from the lizard *Tarentola mauritanica* in 1921 (5) and is probably the most widely studied *Leishmania* (*Sauroleishmania*) species. In lizards, the parasites live predominantly as promastigotes in the lumen of the cloacae and intestine or in the bloodstream (6). Amastigotes, either free or inside monocytes, are rarely observed in lizards (4,6), although both free promastigotes and amastigotes from the blood were reported (7). The ability of *L. tarentolae* to develop into amastigote forms in the lizard is still debated but, as for several *Leishmania* species infecting lizards (6), *L. tarentolae* is able to enter human phagocytic cells and differentiate into amastigote-like forms. However, there is no clear evidence for their efficient replication within macrophages (8–10). Because of its rapid growth in defined media and lack of pathogenicity to humans, *L. tarentolae* has been used widely as a model organism for studies on gene amplification (11–13) or RNA editing (14). Furthermore, *L. tarentolae* has been used as a platform for the production of recombinant proteins (15) and as a potential vaccine candidate (9,10).

The genomes of two *Leishmania* (*Leishmania*) species, *L. major* and *L. infantum*, and one *Leishmania* (*Viannia*) species, *L. braziliensis*, have already been completed and annotated (16,17), and more are being sequenced (www.tritrypdb.org). The 32.8 Mb genome of *L. major* clone Friedlin spreads over 36 chromosomes and is presently the best annotated *Leishmania* genome (16). A 5-fold shotgun sequencing was provided for *L. braziliensis* clone M2904 and *L. infantum* clone JPCM5. The genomes of the various *Leishmania* species contain a similar number of genes, estimated at 8200 (Table 1). Despite the 20–100 million years of divergence within the *Leishmania* genus, a recent sequence comparison of the genomes of *L. major*, *L. infantum* and *L. braziliensis* has revealed a strong conservation of gene content and synteny across the genus (17). Comparative genomics of *L. major*, *L. infantum* and *L. braziliensis* have shown that approximately 200 genes were differentially distributed between the species and that only 78 genes were unique to one species (17). Notable differences were observed in *L. braziliensis*, which contains a putative RNA interference pathway and two types of transposons (TATES) and retrotransposons (SLACS) absent in the two other species.

Here, we sequenced and assembled *de novo* the *L. tarentolae* strain Parrot-TarII using next-generation

high-throughput DNA sequencing technologies. The comparison of the *L. tarentolae* genome with the published genomes of pathogenic *Leishmania* species revealed a high degree of synteny. We identified 95 predicted coding sequences unique to *L. tarentolae* and 250 genes present in the pathogenic species but absent in *L. tarentolae*. Interestingly, several of the genes lacking from *L. tarentolae* are expressed preferentially in the intracellular amastigote stage of pathogenic species. This may explain in part why *L. tarentolae* is less well adapted to infect human macrophages and why these parasites are mostly reported as free organisms in the lizards.

MATERIALS AND METHODS

Sample preparation and sequencing

Promastigotes of *L. tarentolae* (strain Parrot-TarII) were grown up to the late log phase in SDM-79 (Schneider's *Drosophila* medium) supplemented with 5 µg/ml hemin and 5% heat-inactivated fetal calf serum (FCS) (Multicell, Wisent Inc.). High molecular weight genomic DNA was extracted from parasites after chemical lysis with SDS (1%) followed by two sequential phenol extractions, proteinase K and RNase A treatments. Chromosomal DNA was further purified on cesium chloride gradients to limit the extent of kinetoplastid DNA inclusion (18). Purified *L. tarentolae* genomic DNA was sequenced with next-generation sequencing technologies using paired (2.5 kb inserts) and unpaired GS-FLX or Titanium sequencing procedures (Roche 454). GS-FLX and Titanium sequencing were performed at the McGill University and Génome Québec Innovation Centre, Montréal, Canada. Genomic sequences using unpaired Solexa/Illumina sequencing were performed at the Netherlands Cancer Institute. Sequencing runs are described in Supplementary Table S1.

Assembly

Sequences produced on the GS-FLX and Titanium sequencers were assembled using Newbler 2.0 (Roche). The N₅₀ scaffold length of the *L. tarentolae* assembly was 61 619 bp. Sequences produced by Solexa/Illumina were assembled with the Velvet version 0.7.55 software (19). Additional validation of the assembly was performed

Table 1. Summary of sequenced *Leishmania* spp. genomes

	<i>Leishmania tarentolae</i>	<i>Leishmania major</i> (V5.2)	<i>Leishmania infantum</i> (V3a)	<i>Leishmania braziliensis</i> (V2)
Strain	Parrott-TarII	Friedlin	JPCM5	WHOM/BR/75/M2904
Number of chromosomes	36	36	36	35
Genome size (bp)	30 440 719	32 816 678	32 134 935	32 005 207
Overall G+C content (%)	57.2	59.7	59.3	57.8
Coding G+C content (%)	58.4	62.5	62.5	60.4
Number of coding genes in database (time of study)	8201	8304	8216	8133
Number of OGs	7449	7530	7506	7353
Mean nucleotide identity with <i>L. tarentolae</i> (%)	–	84.9	85.0	79.2
Mean amino acid identity with <i>L. tarentolae</i> (%)	–	81.9	82.4	74.8
Reference	Current study	(16)	(17)	(17)

using our own *de novo* assembler Ray (20). The assemblies were merged using the minimus2 scaffolder found in the AMOS package version 2.0.8 (21). Chromosome-like scaffolds were created by comparing gene order in the *de novo* assembled scaffolds and contigs to the gene order in the version 5.2 of the reference *L. major* genome using BLAST and custom python scripts.

Annotation

Gene identification was performed by comparing the assembled *L. tarentolae* genome sequence to the putative genes of *L. infantum*, *L. major* and *L. braziliensis* using BLAST 2.2.21 (22). Sequences from the assembly were compared to the coding sequences (CDS) of the three other species and all *L. tarentolae* open reading frames (ORFs) larger than 100 nucleotides were translated and compared to the gene sequences of the three other species. Similar analysis was also performed on *T. cruzi* and *T. brucei*. Another set of genes was predicted with Augustus using evidence from protein homology to *Trypanosomatidae* and an *ab initio* model trained for *L. tarentolae* (23–25). A consensus set of genes was compiled by pooling the two annotation sets, which were filtered in order to identify the most probable genes in the *L. tarentolae* sequence. All putative genes were mined for domain structures and motifs using HMMER 2.0 and the pfam database (26,27). Further gene ontology (GO) analyses were performed using Blast2GO (28). Additional phylogenetic analyses were conducted in MEGA4 (29). Synteny maps were drawn using the R software (<http://www.r-project.org/>) based on chromosome comparisons with BLAST (blastn). The genome sequence and annotation of *L. tarentolae* Parrot-TarII is available on TriTrypDB (www.tritrypdb.org).

Orthologous group identification

Putative *L. tarentolae* genes were compared to genes from other sequenced *Leishmania* species using the OrthoMCL 2.0 web tool (30,31). Genes from *L. major*, *L. infantum* and *L. braziliensis* have already been clustered in groups of orthologs, which are publicly available in the OrthoMCL database (30). Each group of orthologous genes contains orthologs (related genes found in different species) and paralogs (related genes found within a species). Orthologous groups (OGs) may include whole gene families, subfamilies or single genes, depending on the extent and variability of the gene family. Genes qualified as absent from *L. tarentolae* were validated manually by searching the sequence of this gene in the original reads and by assembling the matching reads using CAP3 (32).

Validation of copy number variation

Reads were mapped onto the *L. tarentolae* scaffolds using the BWA software (33). The number of reads corresponding to each nucleotide position was calculated and the mean read coverage for each gene was estimated. For each OG, the read coverages of all genes were summed to estimate the total coverage for each OG. The total read coverage of OG were compared to the number of genes in the OG. Orthologous groups for

which copy number variation were observed between *L. tarentolae* and the other species were manually inspected to validate that total coverage of OG confirmed the number of genes.

Southern blot hybridizations

Promastigotes of *L. tarentolae* strain Parrot-TarII, *L. tarentolae* strain S125, *L. major* strain Friedlin, *L. infantum* strain JPCM5 and *L. braziliensis* strain WHOM/BR/75/M2904 were grown at 25°C in SDM-79 medium supplemented with 10% heat-inactivated FCS and 5 µg/ml of hemin. Total DNA from each culture was prepared with DNazole (Invitrogen), digested with XhoI restriction enzyme (New England Biolabs, Pickering, ON, Canada) and run on standard agarose gels. Southern blot analyses with [α -³²P]-dCTP labelled DNA probes were performed according to standard protocols (18). All probes were generated by polymerase chain reaction using primer sets listed in Supplementary Table S2. For each target, we generated a specific probe for each *Leishmania* species, which were co-hybridized on a blot of total digested genomic DNAs. Equal amount of DNA was layered for each strain and monitored by hybridization with the single copy *PTR1* gene.

Comparative genomic hybridization

Leishmania whole-genome DNA microarrays used in CGH experiments, which included 8100 60-mer probes that were designed to hybridize all genes of *L. major* 5.2 and *L. infantum* 3.0a, were obtained from Agilent Technologies (Mississauga, ON, Canada). Microarray platform details and probe sequences were deposited in the GEO database under the accession GPL11330. Sample preparation, pre-hybridization and hybridization steps were performed as previously described (34). Normalization and data analysis were done in R with LIMMA 2.7.3. (35). Multiple testing correction was done using the false discovery rate (FDR) method and probes were considered significant when $P < 0.05$ and \log_2 ratio > 2 . The entire data set was deposited in GEO under the reference number series GSE27184.

H₂O₂ IC50 assay

Promastigotes of *L. major* LV39, *L. infantum* MHOMMA#67#ITMAP-263 and *L. tarentolae* S125 were grown at pH 7.0 and 25°C in SDM-79 medium supplemented with 10% fetal bovine serum, 5 µg/ml hemin and 5 µM biopterin (Sigma-Aldrich, St-Louis, MO). *Leishmania* promastigotes (5×10^6) were inoculated in 5 ml medium and H₂O₂ (Rougie Pharma, Mirabel, QC, Canada) was added at various concentration (100 µM, 250 µM, 500 µM, 1000 µM, 2500 µM and 5000 µM). OD^{600nm} values were taken after 72 h. Each curve was performed in triplicate. IC50 were calculated for each curve and the mean and 95% confidence intervals were calculated.

RESULTS

Leishmania tarentolae genome sequencing

The genome of *L. tarentolae* strain Parrot-TarII was resolved using high-throughput sequencing technologies (Supplementary Table S1) to a 16-fold mean coverage and 23-fold peak coverage. The assembled *L. tarentolae* genome contains a total of 30 440 719 bases with 95.1% of the GS-FLX and Titanium reads found in 773 scaffolds and the remaining 4.9% distributed in 2499 contigs. Reads obtained by Illumina sequencing were also incorporated in the final sequence to assist in the assembly. After *de novo* assembly, sequences of specific chromosomes were built using contigs and scaffolds based on their homology to *L. major*. Directed assembly on *L. major* allowed the mapping of 29 862 062 bases (98.1%) leaving 578 657 bases (1.9%) in 1315 small contigs. A summary of the sequencing statistics of the *L. tarentolae* genome and the other published *Leishmania* spp. genomes are presented in Table 1. CHEF analysis of the *L. tarentolae* genome showed 24–28 chromosomal bands (36). Similar analysis of the *L. infantum* genome showed a comparable number of band, which were further resolved in 36 chromosomes using hybridization of genome fragments (37).

Leishmania tarentolae is syntenic with other *Leishmania* species

De novo assembled contigs and scaffolds were generally syntenic with both *L. major* and *L. infantum*. Most differences between *L. tarentolae* and the other species consisted in gene insertions or deletions distributed randomly or in tandem arrays throughout the genome. Full synteny maps comparing *L. tarentolae* to *L. infantum* and *L. major* are provided in Supplementary Figures S1–S36. Although *L. tarentolae* possesses similar percent identity with *L. infantum* and *L. major* (Table 1), its synteny is closer to the latter. For example on chromosome 28, a stretch of 90 kb is syntenic between *L. tarentolae* and *L. major* but the gene ordering is different in *L. infantum* (Figure 1A). *De novo* assembled scaffolds are, in this case, long enough to confirm that *L. tarentolae* is more syntenic to *L. major*. Similar differences were found at the proximal region of chromosome 7 (Figure 1B) and at the distal end of chromosome 35 (Figure 1C), both suggesting greater synteny to *L. major* than to *L. infantum*. Other loci where *L. tarentolae de novo* assembled scaffolds were syntenic to *L. major* but not to *L. infantum* can be found in Supplementary Figures S7, S9, S11–13, S32 and S35. Overall, the synteny of *L. tarentolae* is closer to the *L. (Leishmania)* species than to the *L. (Viannia)* subgenus. Indeed, several loci are in common between *L. tarentolae* and *L. major*, although they are absent from *L. braziliensis*. Breaks in synteny are also more frequent with *L. braziliensis* than *L. major* (data not shown).

Leishmania tarentolae gene content

Genome annotation of *L. tarentolae* indicated a total of 8201 putative protein-coding genes, a number similar to the other sequenced *Leishmania* species (Table 1).

Annotation was performed by comparing the *L. tarentolae* genome to other *Leishmania* and *Trypanosoma* species along with *ab initio* annotation using the Augustus software trained for *Leishmania* gene detection (23–25). Given that assembly of repeated gene clusters is more difficult, the count of genes found in *L. tarentolae* may be biased, especially for genes present in high copy number.

Using the OrthoMCL web tool, the set of *L. tarentolae* putative genes was compared to the OrthoMCL database (www.orthomcl.org) in order to assign each gene to a group of orthologs (30). This allowed us to readily compare the gene content of the four sequenced *Leishmania* species, determine which genes are unique to a given species and calculate which OG of genes vary in copy number between the different species. These results were further confirmed by interspecies comparative genomics hybridization (CGH) microarrays, read depth analysis and, in selected cases, by Southern blots analyses. The gene content of *L. tarentolae* is highly similar to the three pathogenic *Leishmania* species sequenced, which contain a similar number of OG (Table 1).

Figure 2 compares the gene content of *L. tarentolae* to other *Leishmania* species with emphasis on *L. major*. Overall, 7331 OG are shared by *L. tarentolae* and *L. major*. Of these, 7225 OG (7662 genes in *L. tarentolae* and 7845 genes in *L. major*) have a similar copy number in *L. tarentolae* and at least another *Leishmania* species, 20 OG (32 genes in *L. tarentolae* and 131 genes in *L. major*) have a lower copy number in *L. tarentolae* than the three other species (Supplementary Table S3) and 86 OG (363 genes in *L. tarentolae* and 133 in *L. major*) have a higher copy number in *L. tarentolae* than the three other species (Supplementary Table S4). More than a third of the OGs with varying copy numbers have a putative function (see Figure 2 and Supplementary Tables S3 and S4).

A total of 250 *L. major* genes distributed between 188 OGs were found to be absent from *L. tarentolae* (Figure 2 and Supplementary Table S5). Of these, 83 OG were shared by the three pathogenic species, 74 OG by *L. major* and *L. infantum*, 5 OG by *L. major* and *L. braziliensis* and 26 OG were unique to *L. major* (Figure 2).

A total of 73 OG (95 genes) were unique to *L. tarentolae* (Supplementary Table S6). From these, 31 OG had orthologs in other non-*Leishmania* species, including 29 in *Trypanosoma* spp. We also found 42 OG (65 genes) that were sequence orphans with no similarity to sequences found in databases.

Genes absent from *L. tarentolae* or present in lower copy number compared to the pathogenic *Leishmania* spp

Leishmania tarentolae lacks several genes coding for proteins implicated in trafficking. Indeed, the $\beta 1/\beta 2$ -adaptins (LmjF11.0990; LmjF36.5595), μ -adaptin (LmjF31.3035) and the epsilon-adaptin (LmjF30.1545) (Figure 2 and Supplementary Table S5) were absent from *L. tarentolae*. Adaptins are involved in the formation of clathrin-associated adaptor protein (AP) complexes, which play a key role in the transport of proteins by regulating the

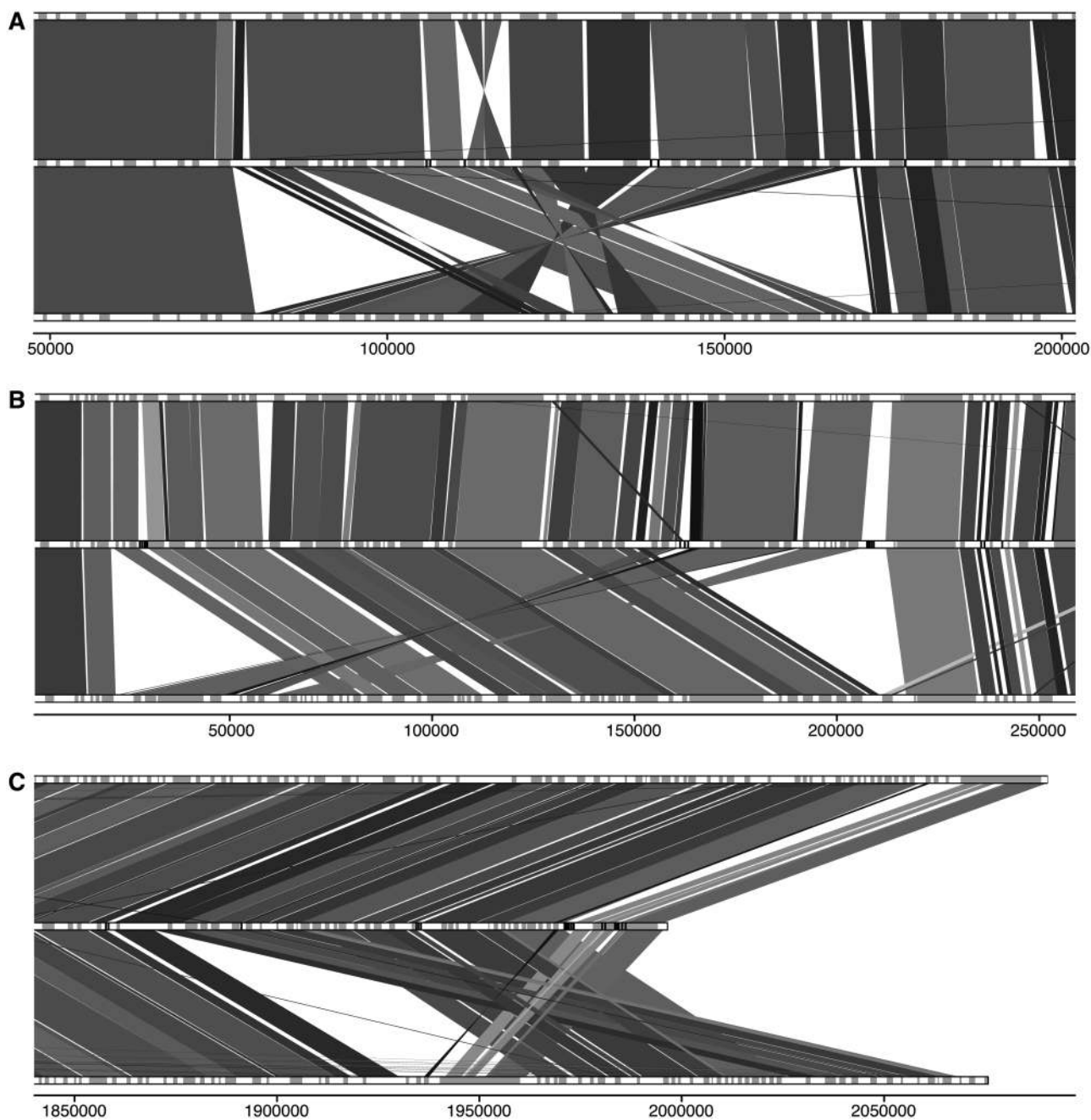


Figure 1. Synteny map of *L. tarentolae* (middle) compared to *L. major* (top) and *L. infantum* (bottom). Genes on chromosome tracks are grey. *Leishmania tarentolae* contig delimitation is indicated in black in the middle lane. Shade of synteny blocks is proportional to sequence identity, the darker the more similar are the sequences. The scale represents nucleotide position on the chromosome. (A) 5' region of chromosome 28. (B) 5' region of chromosome 7. (C) 3'-end of chromosome 35.

formation of transport vesicles as well as cargo selection between the trans-Golgi network, endosomes, lysosomes and the plasma membrane (38,39). The calcium-dependent membrane binding proteins copines (LmjF28.1190) and Ras-like small GTP-binding proteins (LmjF36.1820), both involved in cell signalling and/or membrane trafficking/transport pathways and exocytosis, were also absent from *L. tarentolae*. The endosomal/lysosomal membrane-bound acid phosphatase (LmjF28.2650), potentially involved in intracellular trafficking (40), is also

missing from *L. tarentolae*. *Leishmania tarentolae* also lacks the phosphatidylinositol 3-kinase 2 gene (LmjF14.0020) and the phosphatidylinositol-4-phosphate 5-kinase gene (LmjF26.2495) (Figure 2 and Supplementary Table S5) whose activities were linked to a diverse set of key cellular functions, including intracellular trafficking (41). The Tubby protein 1, that has been reported to bind phosphatidylinositol 4,5-bisphosphate on the plasma membrane and facilitate macrophage phagocytosis (42), is not present in *L. tarentolae*.

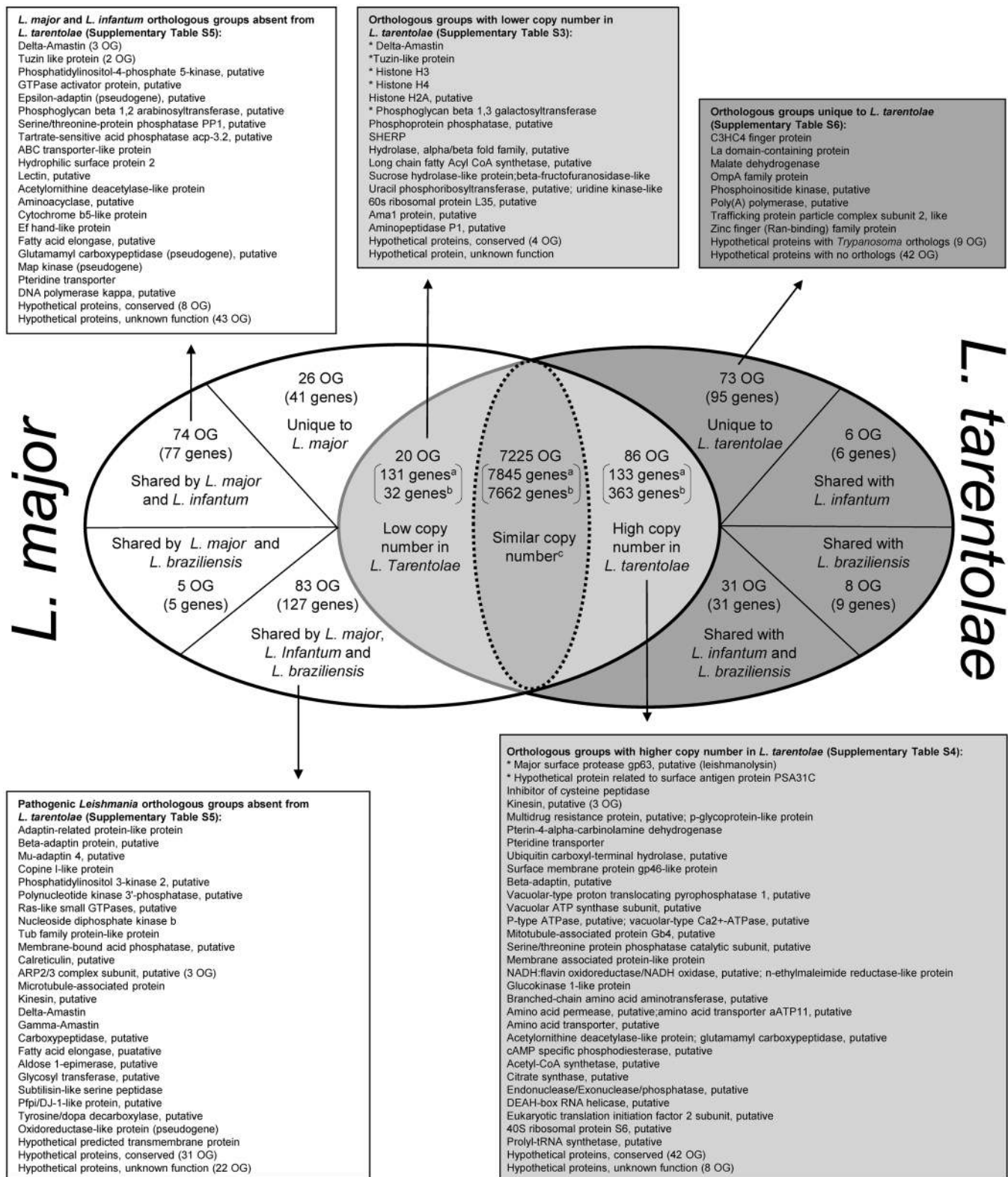


Figure 2. Differential distribution of genes and OGs of genes between *L. tarentolae* and *L. major*. ^aGene counts referring to *L. major*. ^bGene counts referring to *L. tarentolae*. Lists include the description of OGs of genes that have differential distribution between *L. tarentolae* and the three sequenced *Leishmania* pathogenic species. Counts of genes within the different OG are in parenthesis. The complete list of genes and OGs for selected categories is shown in Supplementary Tables S3–S6. Asterisk indicates genes with the highest copy number variability in *L. tarentolae*.

Leishmania tarentolae lacks three of the seven subunits of the Arp2/3 complex, notably the p40/ARPC1 (LmjF18.0920) p20/ARPC4 (LmjF02.0600) and p15/ARPC5 (LmjF05.0285) (Figure 2 and Supplementary Table S5). Only one Arp2/3 complex subunit coding gene was found and annotated in the *L. tarentolae* genome (not shown). The Arp2/3 complex is required for actin polymerization and reported to associate with several other cytoskeletal components, including microtubules (43,44). Interestingly, a number of microtubule-associated proteins (9 on chromosome 19) and a kinesin microtubule-associated protein (LmjF16.1580) are also missing from the *L. tarentolae* genome (Figure 2 and Supplementary Table S5).

Genes reported to play a role in the resistance to oxidative stress are absent from *L. tarentolae*. These include a subtilisin (LmjF28.2380) belonging to the S8A subfamily of proteases, shown recently to process the terminal peroxidases of the trypanothione reductase system in *Leishmania* (45); a Pfp/DJ-1-like protein (LmjF35.3910) that defends cells against reactive oxygen species and mitochondrial damage (46); and a tyrosine/dopa decarboxylase (LmjF30.2500), a precursor of catecholamines, which may act as scavengers of free radicals (47) (Figure 2 and Supplementary Table S5). Collectively, these data suggest that *L. tarentolae* may deal less efficiently with oxidants than *L. major*. Interestingly, *L. tarentolae* strains were found to be >3.5-fold more sensitive to hydrogen peroxide than *L. major* and *L. infantum*. Indeed, the IC₅₀ of *L. tarentolae* to H₂O₂ was 152 ± 7 μM but 514 ± 115 μM for *L. major* and 574 ± 121 μM for *L. infantum*.

Gene content analysis also revealed differences in the glycoprotein content between *L. tarentolae* and the other pathogenic species. Genes involved in lipophosphoglycan (LPG) modifications were found to be either in lower copy number or absent from *L. tarentolae*. Chromosome 2 displayed important differences between *L. tarentolae* and

the two other sequenced Old World species in terms of phosphoglycan transferases (Figure 3). This region extends on two *de novo* assembled scaffolds in *L. tarentolae*, providing reliable information on this locus. We found that phosphoglycan β 1,3 galactosyltransferase OG of genes necessary for the attachment of the side chain Gal residues to the LPG phosphoglycan (PG) repeats (48) are present in 6–8 copies in other *Leishmania* species but only in 2 copies in *L. tarentolae* (Supplementary Table S3). Within the same locus, phosphoglycan β 1,2 arabinosyltransferase (LmjF02.0180; LmjF02.0220), involved in the terminal capping oligosaccharide of LPG in *L. major* (49), is found in *L. major* and *L. infantum* but not in *L. tarentolae* (Supplementary Table S5). *Leishmania tarentolae* also lacks two glycosyltransferase genes (LmjF35.5250, LmjF29.2110), enzymes that catalyze the transfer of a monosaccharide unit to a glycosyl acceptor molecule. Moreover, *L. tarentolae* lacks calreticulin (LmjF31.2600), an endoplasmic reticulum (ER) chaperone ensuring the proper folding and quality control of newly synthesized glycoproteins destined for secretion or cell surface expression (50) (Supplementary Table S5).

Amastins, one of the largest family of surface proteins in *Leishmania* (17) shown to be expressed primarily in the intracellular stage of the parasite (51,52), are under-represented in *L. tarentolae* (Supplementary Table S3). Amastins, whose function has yet to be determined, are divided into four subfamilies based on their phylogeny and genomic positioning (53). We show that *L. tarentolae* contains the amastins of the α, β and γ subfamilies that are also shared with *Trypanosoma* or *Crithidia*, but lacks all but two of the amastins of the delta subfamily (Figure 4A). This subfamily is expressed preferentially in the intracellular stage of the parasite (51). No new amastins were discovered in *L. tarentolae*. Interestingly, tuzins, which are often associated on the same chromosomal locus with amastin genes in the pathogenic

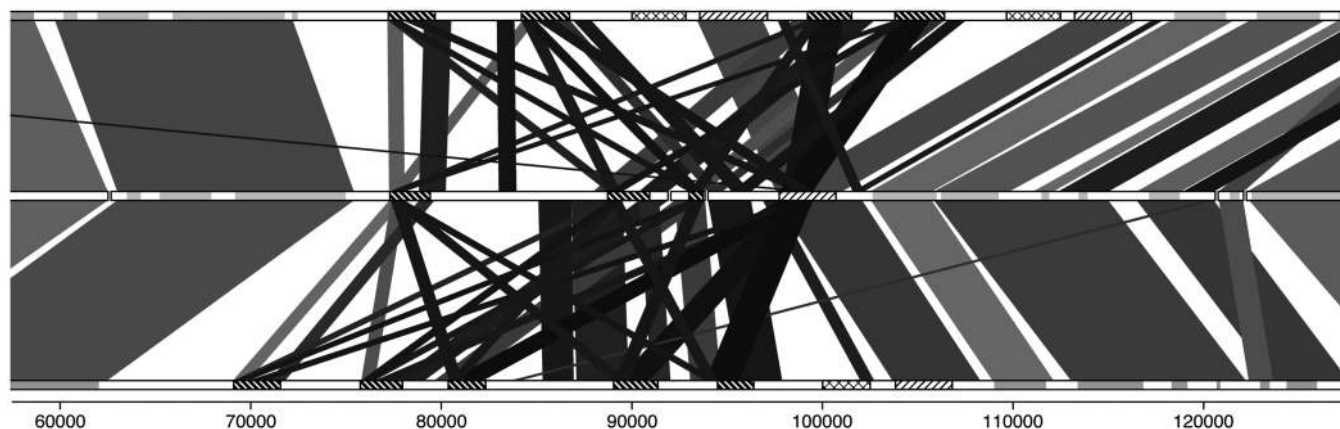


Figure 3. Differential distribution of genes involved in lipophosphoglycan and phosphoglycan modification in *L. tarentolae* (middle) as compared to *L. major* (top) and *L. infantum* (bottom). Phosphoglycan β 1,2 arabinosyltransferase is shaded with crosses, a first group of phosphoglycan β 1,3 galactosyltransferase is shaded with thin lines and a second group with bold lines. The other genes on chromosome tracks are grey. *Leishmania tarentolae* contig delimitation is in black in the middle lane. Shade of synteny blocks is proportional to sequence identity, the darker the more similar are the sequences. The scale represents nucleotide position on chromosome 2.

Leishmania species, were less diverse in *L. tarentolae* with only 4 tuzin genes compared to 6 and 28 genes in *L. infantum* and *L. major*, respectively. The amastin- and tuzin-rich region within chromosome 8 contains only two copies of the tuzin gene in *L. tarentolae* (Figure 4B). On *L. tarentolae* chromosome 34, amastin and tuzin genes are present in single copy as opposed to the situation in *L. major* where they are organized in tandem, suggesting co-expression (Figure 4C).

Gene family expansion in *Leishmania tarentolae*

Genome comparative analysis between the *L. tarentolae* and the other sequenced *Leishmania* pathogenic species revealed that two surface-associated protein gene families were particularly enriched in the non-pathogenic *L. tarentolae*. The first family is leishmanolysin, a major surface zinc metalloprotease, also known as GP63; which is mostly, but not exclusively, expressed in the promastigote stage of several *Leishmania* species (54–56). The GP63 OG in *L. tarentolae* is highly expanded with 49 putative GP63 genes as compared to 29 in *L. braziliensis*, 7 in *L. infantum* and 5 in *L. major* (Supplementary Table S4). Due to the high copy number and sequence variability, assembly of the GP63 gene family was limited and resulted in several partial gene sequences. The density of read coverage for GP63 indicated portions of the gene that were well conserved in *L. tarentolae* while other regions were less represented, supporting a sequence diversity of GP63 in *L. tarentolae* (Figure 5A). Sequence diversity in the *L. tarentolae* GP63 gene may affect its function. Indeed, a gelatin zymography assay showed no protease activity in *L. tarentolae*, in contrast to the pathogenic *Leishmania* species (Figure 6).

The second surface protein gene family that was expanded in *L. tarentolae* is distantly related to the promastigote surface antigen proteins (PSA) located on chromosome 31. PSA-2 was originally described in 1989 (57). The phylogenetic history of this gene family has recently been studied and members have been assigned to subfamilies based on their sequences and chromosomal locations (58). We observed that the *L. tarentolae* PSA genes are orthologous to *PSA31C*. There are 63 paralogs in *L. tarentolae*, while only a single copy is present in *L. major* and *L. infantum* and two copies in *L. braziliensis* (Supplementary Table S4). *Leishmania tarentolae* PSA proteins seem to contain only the leucine-rich repeated motif (LRR) (Figure 5B), a domain often implicated in the binding to other proteins or glycolipids. This gene is proposed to be involved in host–pathogen interactions (58).

Smaller copy number variations were observed for 84 additional OGs, which are shown in Supplementary Table S4.

Genes unique to *Leishmania tarentolae*

We found 73 OG (95 genes) unique to *L. tarentolae* (Figure 2 and Supplementary Table S6). Of these groups, 31 (42%) had OrthoMCL orthologs in species other than *L. infantum*, *L. major* and *L. braziliensis*,

including *Trypanosoma* spp. (26 groups; 36%). Putative functions could be ascribed to 10 OG (14%). These include a C3HC4 finger protein, a trafficking protein particle complex subunit 2-like protein, a La domain-containing protein, a malate dehydrogenase, an OmpA family protein, a phosphoinositide kinase, two surface protein GP63 OGs, a zinc finger (Ran-binding) family protein and a poly(A) polymerase, which is different from the poly(A) polymerases found in the pathogenic *Leishmania*. The remaining OG are sequence orphans with no orthologs found in the orthoMCL database.

Experimental validation for gene content and copy number variations

We validated experimentally some of the changes highlighted from the genome sequence comparisons using comparative genomic hybridizations (CGH), Southern blot and read depth analysis. Full *Leishmania* genome microarrays designed for *L. major* and *L. infantum* were used to co-hybridize *L. tarentolae* with either the *L. major* or the *L. infantum* DNA to test whether we could identify genes specific to pathogenic species or genes with altered copy number. We found that 31–35% of the genes absent in *L. tarentolae* showed significantly higher signals with the DNA derived from the pathogenic species, which is statistically significant when compared to the overall ratio of 8% due to better hybridization of the DNAs derived from the pathogens ($P < 0.001$, one sample test for binomial proportion). Similarly, 20% of the *L. tarentolae* genes with lower copy number were validated by the CGH experiment ($P < 0.001$) confirming in part the sequencing data. These correlations are explained by the design of the microarray that contains several probes (45%) that have more than 10 mismatches with *L. tarentolae*, and by the fact that this microarray did not allow testing for the presence of *L. tarentolae*-specific genes. Considering these limitations, the CGH results allowed the validation of many differentially distributed genes between the *Leishmania* species. However, genes that are not validated by CGH may still be differentially distributed between the species. Genes confirmed by CGH are highlighted in Supplementary Tables S3 and S5.

The correlation between sequence data and gene copy number was validated by Southern blot hybridizations for a few chosen genes (Figure 7). The delta-amastin subfamily is present in high copy number in *L. major* and *L. infantum* but not in *L. tarentolae* (Supplementary Table S3). The low copy number of delta-amastins in the TarII-Parrot *L. tarentolae* strain predicted from the sequencing data and their high copy number in the pathogenic species were confirmed by Southern blot hybridization (Figure 7A, lanes 3–5). Indeed the intense hybridization signals in the pathogenic species were due to the highly repetitive nature of a cluster of amastins giving rise to single restriction fragments (see Figure 7A). Interestingly, the copy number of delta-amastins was low not only in the TarII-Parrot strain (Figure 7A, lane 1) cultured for decades in the laboratory, but also in a more recent isolate from lizard with a limited history of

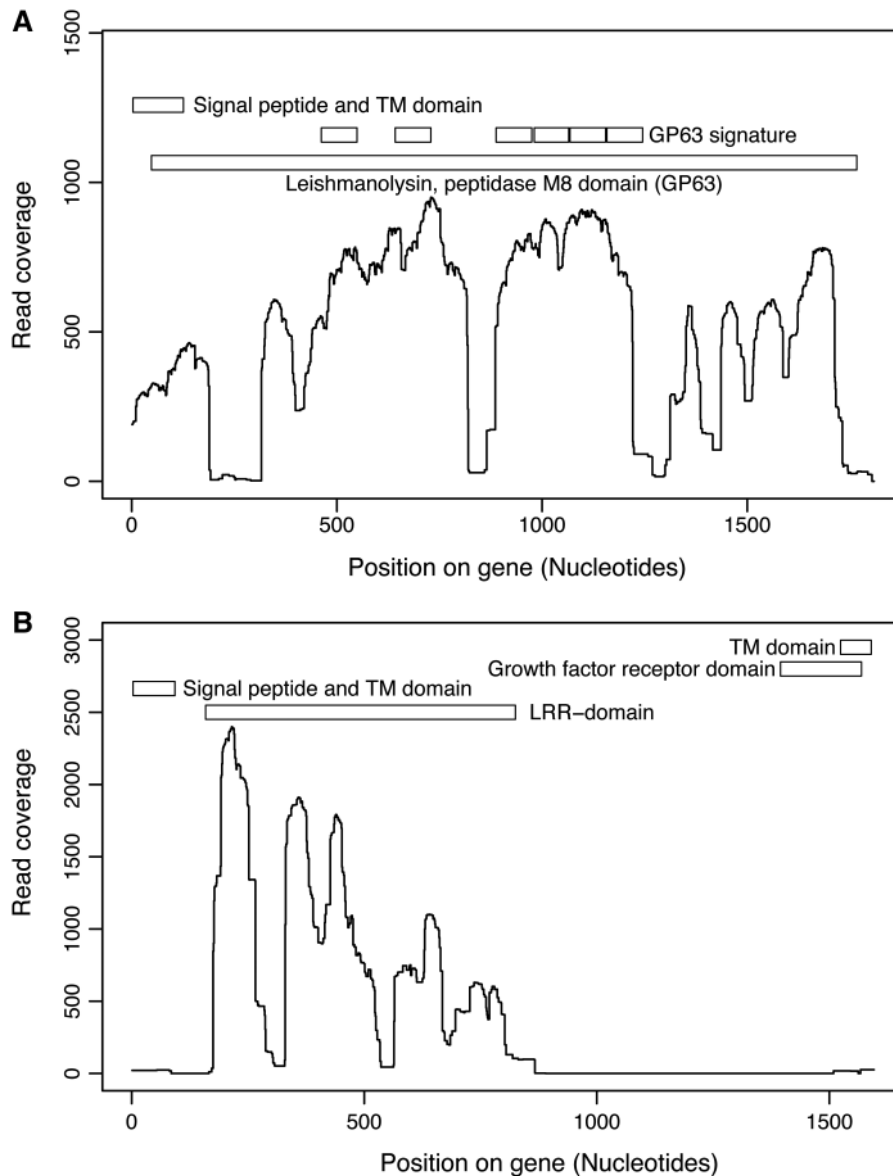


Figure 5. Density of read coverage for genes present in high copy number in *L. tarentolae*. For each position of the reference *L. major* genes, the number of corresponding reads were counted and plotted on the graph. Protein domains are indicated on the upper portion of each graph. (A) Leishmanolysin (*GP63*) gene; LmjF10.0480 is used as a reference. (B) Promastigote surface antigen *PSA31C* gene; LmjF31.1440 is used as a reference.

in vitro culture passages (Figure 7A, lane 2). As a control, we used a probe recognizing the proto-delta amastin gene, which according to the sequence data is present in similar copy numbers in all species. Southern blots have indeed validated this assumption (Figure 7B).

In an independent set of experiments, Southern blot hybridizations corroborated sequencing data for the two genes involved in LPG side chain addition and LPG modification, indicating that phosphoglycan β 1,3 galactosyltransferase was present in lower copy number in *L. tarentolae* (Figure 7C) whereas phosphoglycan β 1,2 arabinosyltransferase was absent from this species (Figure 7D). Also, Southern blot analyses confirmed the expansion of the *GP63* and *PSA31C* protein gene families

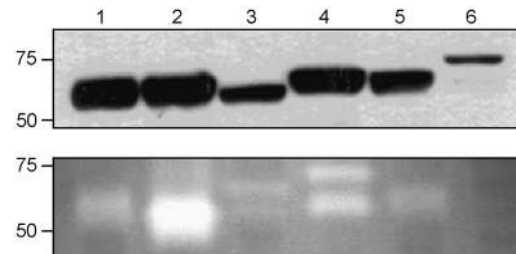


Figure 6. Protease activity of *GP63* in six *Leishmania* species. (A) Western blot using monoclonal antibody targeting *GP63* shows the quantity of this protease in each sample. (B) Gelatin zymography assay determining the protease activity of *GP63*. No signal was observed for *L. tarentolae*, suggesting the absence of *GP63* activity in this species. Lane 1, *L. mexicana*; lane 2, *L. major*; lane 3, *L. donovani*; lane 4, *L. infantum*; lane 5, *L. amazonensis*; and lane 6, *L. tarentolae*.

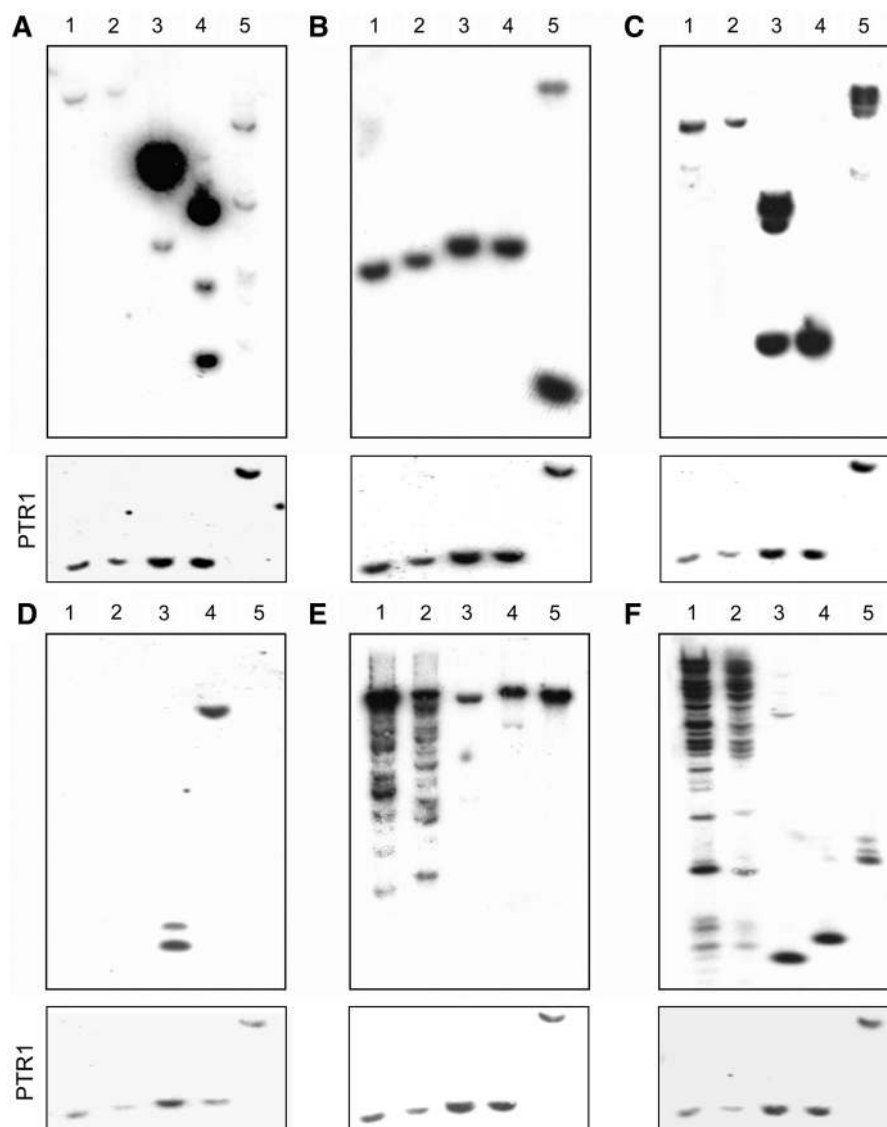


Figure 7. Southern blot hybridization of genes whose copy number varies between *L. tarentolae* and the other *Leishmania* pathogenic species. Total genomic DNA of *Leishmania* isolates was digested with XhoI, run on agarose gels, blotted and hybridized with a combination of PCR-specific probes derived from each species (see Supplementary Table S2 for primer sequences and probe details). (A) Delta-amastin; (B) Proto-delta-amastin (shared by the four species) as a control; (C) Phosphoglycan β 1,3 galactosyltransferase; (D) Phosphoglycan β 1,2 arabinosyltransferase; (E) *GP63*; and (F) Surface antigen protein *PSA31C*. DNA loading was estimated by hybridizing the same blot with the single copy gene *PTR1* (shown in the lower portion of each panel). Lanes 1, *L. tarentolae* Parrott-TarII; lane 2, *L. tarentolae* S125; lane 3, *L. major* Friedlin; lane 4, *L. infantum* JPCM5; lane 5, *L. braziliensis* WHOM/BR/75/M2904.

in *L. tarentolae* as deduced from the sequencing data (Figure 7E and 7F). The heterogeneity in the hybridizing fragments gives some credence to the size diversity of *GP63* (Figure 5A) and *PSA31C* (Figure 5B) genes, as suggested by the sequencing data. Moreover, both gene families have a high read coverage, 3413 reads per nucleotide for *GP63* and 7280 reads per nucleotide for *PSA31C*, which supports the idea that these genes are in high copy number (Supplementary Table S4). Read depth analysis also supports the higher copy number of genes listed in Supplementary Table S4 and the lower copy number of genes listed in Supplementary Table S3. The mean read coverage for single-copy genes was 22.4 read/nucleotide.

Orthologous groups of genes varying in copy number or missing from *L. tarentolae* are developmentally regulated in pathogenic *Leishmania* species

We show here that some genes preferentially expressed in the amastigote stage, such as the amastins, are present in lower copy number or are absent from *L. tarentolae*, while genes reputed to have a higher expression in the insect stage (e.g. *GP63* or *PSA31C*) are represented in higher copy number in *L. tarentolae* (Figure 7E and 7F). We performed additional bioinformatics analyses to test whether OGs of genes with a variable copy number between *L. tarentolae* and the pathogenic species were stage-regulated in the later. Specific expression of these

groups to *Leishmania*'s life cycle stages was based on published transcriptomics and proteomics studies (59–71). Overall, 23% of the 7694 OG were associated to one or the other developmental stage of the parasite with 890 OG associated to the promastigote stage and 1013 OG associated to the amastigote stage (Table 2). Association to either developmental stage is indicated in Supplementary Tables S3–S5. Comparison of each category to overall distribution was performed using the Fisher's exact test. Interestingly, 70% (16/23) of the OG with a lower copy number and ~34% (29/86) of the OG with a higher copy number in *L. tarentolae* were differentially expressed in either life stage of the pathogenic *Leishmania* species (Table 2). Remarkably, OG absent from *L. tarentolae* were mainly associated to the amastigote life stage in other *Leishmania* ($P < 0.001$). Indeed, of the 66 OGs present in pathogenic *Leishmania* spp. but absent from *L. tarentolae* that were associated to one or the other developmental stage of the parasite, 18 genes were linked to the promastigote life stage, 52 to the amastigote stage and 4 to both life stages (Table 2).

Examples of genes that were absent from *L. tarentolae* but preferentially expressed in the amastigote stage of other *Leishmania* species are the delta-amastins and the hydrophilic surface protein gene family HASPA1, HASPA2, HASPB1, HASPB2 (Supplementary Table S5), known to be preferentially expressed in *L. major*, *L. infantum* and *L. donovani* amastigotes as determined by DNA microarray studies (68,69). This list also includes a 3,2-trans-enoyl-CoA isomerase, an ABC transporter-like protein, a class I nuclease-like protein, a D-isomer-specific 2-hydroxyacid dehydrogenase-protein, an Ef hand-like protein, a GTPase activator protein, a pteridine transporter and a tyrosine/dopa decarboxylase. The remaining amastigote OGs absent from *L. tarentolae* encode hypothetical proteins (Supplementary Table S5).

DISCUSSION

We have used next-generation DNA sequencing technologies to obtain a high-quality draft (72) of the genome sequence of *L. tarentolae*, the first non-human trypanosomatid pathogen sequenced. Genome sequence analysis revealed that *L. tarentolae* is syntenic to the three-sequenced pathogenic *Leishmania* species and that >90% of the approximately 8200 genes are shared by all the species. However, a number of genes shown to be either important for pathogenesis or preferentially expressed in the intracellular parasitic stage in the pathogenic species

or more relevant to the insect stage were either absent from *L. tarentolae* or present at variable copy number, supporting the hypothesis that some of these genes may be responsible for the reduced capacity of *L. tarentolae* to live as an intracellular parasite and its diminished pathogenic potential to humans.

Among the genes absent from *L. tarentolae*, there was a significant bias for those expressed specifically in the amastigote intracellular stage in the pathogenic species (Table 2). Most of these genes correspond to hypothetical conserved or hypothetical proteins of unknown function (Supplementary Table S5) further highlighting the gaps in our understanding of the biology of intracellular parasites. Still, well-studied genes, such as the amastin gene family, especially the delta-amastins, are in low copy number in *L. tarentolae* (only 2 found, see Figure 4A) whereas high numbers of these genes (12–25 members) are found in the pathogenic species. The delta subfamily contains a diverse clade that is exclusive to *Leishmania* with one locus (proto-delta-amastins) found in other trypanosomatids (53). Although the function of amastins is still unknown, most of the delta-amastins undergo a stage-specific regulation and are preferentially expressed in amastigotes (51,52,68,73,74) and few in infective metacyclics (59). Given their specific expression in amastigotes, it has been argued that their expansion should be viewed as the adaptation of the parasite to a novel life stage after the acquisition of vertebrate parasitism (53). *Leishmania tarentolae* also lacks tuzins, conserved transmembrane proteins with unknown function, which are often contiguous with delta-amastins. Moreover, the majority of *L. tarentolae* genes displaying high variation in their copy number (e.g. *GP63*, *PSA31C*, phosphoglycan β 1,3 galactosyltransferase, *ama1*, etc) are developmentally regulated in the pathogenic *Leishmania*.

Two of the most studied abundant surface constituents of *Leishmania* promastigotes are the GPI-anchored molecules lipophosphoglycan (LPG) (75) and GP63 zinc metallo-protease (76). In contrast to the mammalian parasites, lizard *Leishmania* such as *L. tarentolae* and *L. adleri* seem to lack LPG (77). Sequencing data did not reveal any specific gene, absent or mutated, that could explain the lack of LPG. LPG has been implicated in many steps required for the establishment of initial macrophage infection, notably in the inhibition of phagolysosome biogenesis and in resistance to oxidants (75), but also in the development of innate immunity (78,79). Indeed, LPG-deficient *L. major* mutants showed attenuated virulence (80,81) although this was not the case for *L. mexicana*

Table 2. Association of OGs of genes in *L. tarentolae* with differential expression in promastigote and/or amastigote life stages in pathogenic *Leishmania* species

	<i>n</i>	Promastigote	Amastigote	<i>P</i> -value*
All OGs	7694	890	1013	NA
Higher copy number in <i>L. tarentolae</i>	86	20	20	0.75
Lower copy number in <i>L. tarentolae</i>	23	11	11	0.83
OGs absent from <i>L. tarentolae</i>	278	18	52	<0.001

*Promastigote versus amastigote comparison using two-tailed Fisher's exact test.

NA: Not Applicable.

LPG-deficient mutants (82). In addition to LPG, *L. tarentolae* lacks enzymes that are involved in LPG modification like the phosphoglycan β 1,2 arabinosyltransferase, which is involved in the terminal capping of LPG in *L. major* (49), and has less copies of the β 1,3 galactosyltransferase gene, which adds the β 1,3 galactoside to the repeating PG of LPG (48) (Figure 3). These modifications play a role in *Leishmania*–sand fly interactions (48,49,83).

The major surface protease GP63, a well known virulence factor implicated in phagocytosis, parasite evasion of complement-mediated lysis, and induction of the host immune response (76,84), is highly expanded in *L. tarentolae*, as confirmed by Southern blot (Figure 7E) and read coverage analyses (Figure 5A). In the sand fly, GP63 is thought to be implicated in nutrient acquisition and attachment of promastigotes to the gut wall (76,85). Expansion of *GP63* genes in *L. tarentolae* may thus facilitate nutrient acquisition, and in the absence of LPG, GP63 may be important for the parasite to maintain its attachment to the midgut. More recently, it was shown that during *Leishmania*–macrophage interaction, GP63 is internalized by macrophages, where it interacts and cleaves several intracellular macrophage proteins, including actin cytoskeleton regulators, protein tyrosine phosphatases, and transcription factors (86–89). Collectively, these cleavage-dependent activation events lead to the down-regulation of IFN- γ signalling and downstream macrophage activation, including NO production (90). Interestingly, *L. tarentolae* was shown to lack protease activity (91), as also validated here by gelatin zymography (Figure 6). While *GP63* genes were difficult to assemble, read analysis showed that some portions of the gene, especially the N-terminal and C-terminal regions, were underrepresented or had increased sequence variability while other portions were conserved (Figure 5A). It has been reported previously that the C-terminus of GP63 contains a GPI membrane anchor (92). Moreover, it has been shown recently that deletion of 180–211 amino acids from the C-terminal domain of GP63 resulted in 50% loss of its catalytic activity (93). Our data (Figures 5A and 6) and published reports (86,91) support the possibility that the high sequence variability of the *L. tarentolae* *GP63* gene may affect GP63 protease's activity.

In addition to *GP63* genes, *L. tarentolae* has expanded the promastigote surface antigen PSA31C for which only a single copy is present in the pathogenic *Leishmania* (58). The PSA proteins are part of eight subfamilies that have usually a signal peptide, a cysteine-rich region, leucine-rich repeats, a threonine/serine-rich region and a domain possibly acting as a GPI anchor (58). PSA were found to be either membrane-bound, secreted or soluble but little is known about their function (58). The expression of some *PSA* genes was found to be increased in metacyclic parasites (94). In *L. tarentolae*, the only recognizable feature in the *PSA31C* subfamily is the leucine-rich repeats domain (Figure 5B). The expansion of this subfamily in *L. tarentolae* (Figure 7F) is striking and warrants further analysis. One hypothesis is that the *PSA31C* gene has expanded in lizard parasites to facilitate promastigote survival either in the insect vector or in the lizard.

Another interesting feature of the *L. tarentolae* genome is the lack of a number of genes encoding key functions of vesicular protein trafficking. In fact, *L. tarentolae* lacks the large adaptin subunits (β 1/ β 2) and the medium-sized- μ -adaptins that are part of the heterotetrameric AP-1 complex, which is involved in the formation of clathrin-coated vesicles (CCVs) mediating protein transport between the *trans*-Golgi network and endosomes (38). *Leishmania* mutants lacking AP-1 subunits showed significant defects in Golgi structure, endocytosis or exocytic transport and displayed reduced rates of endosome-to-lysosome transport (95). Moreover, it was shown that Sigma 1- and μ 1-adaptin homologues of *L. mexicana* are required for parasite survival in the infected host (96). In addition, the absence of membrane-bound acid phosphatase localizing to endosomal/lysosomal compartments (40) and of copines, soluble, calcium-dependent membrane-binding proteins with C2 domains known to be involved in cell signalling and/or membrane trafficking pathways and exocytosis (97) further suggests that *L. tarentolae* may have a deficient vesicular protein transport. In line with this, *L. tarentolae* lacks members of the Ras-like small GTP-binding proteins that have emerged as master regulators of cellular membrane transport by interacting with C2 protein domains (98). Also, *L. tarentolae* lacks three of the seven subunits of the Arp2/3 complex, which has been shown to function in cellular processes ranging from cell motility, cytokinesis and endocytosis to trafficking and cell–cell communication (44). Arp2/3 complex is a key player in initiating actin polymerization at sites of endocytosis (99). Since in the absence of ARPC1, ARPC4 and ARPC5, the Arp2/3 complex cannot be formed (44,100), it is possible that *L. tarentolae* shows defects in endocytosis, which may impact the capacity of the parasite to communicate efficiently with the intracellular environment in the macrophage phagolysosome.

Some other genes known to play a role in virulence were also missing from *L. tarentolae*. Indeed, *L. tarentolae* lacks one of the two subtilisin (SUB) proteases found to process the terminal peroxidases of the trypanothione reductase system (45). Subtilisin promotes survival of *Leishmania* amastigotes by serving as a maturase for the trypanothione reductase system, which is essential to maintain redox homeostasis in the host macrophage and to protect the parasite against oxidative damage (101). Interestingly, *SUB*-deficient *Leishmania* showed increased sensitivity to hydroperoxides and reduced viability in mouse infection models (45). In addition, *L. tarentolae* lacks DJ-1, a multifunctional oxidative stress response protein that defends cells against reactive oxygen species and mitochondrial damage (46). This is consistent with the observed decrease of *L. tarentolae* survival in the presence of H₂O₂.

Here, we provide a high-quality draft of the genome sequence of the lizard *L. tarentolae* using next-generation sequencing (NGS) technologies. Limitations of NGS technologies are well known (102) and similar problems were observed with the *L. tarentolae* genome assembly. The size of the *L. tarentolae* genome is somewhat smaller (~5%) than that of the other sequenced *Leishmania* spp. due to collapse of regions with identical nucleotide

sequence. Also, repeated genes such as *GP63* or *PSA31C* had a tendency to be fragmented in the assembly. However, these problems were attenuated by read number analysis. Homopolymers are an important cause of sequencing errors when using the 454 platform and can lead to frame shifts that make it difficult to differentiate actual sequencing errors from pseudogenes. In most cases, comparison of these specific regions to reads obtained by Illumina sequencing validated the presence or absence of genes. Most importantly, the current sequence draft provides additional research avenues to investigate *Leishmania* pathogenesis. The absence of LPG, acid phosphatase and delta-amastin glycoproteins on the surface of *L. tarentolae* combined to GP63 lacking most likely its protease activity could lead to more vulnerable parasites, which may be more sensitive to complement-mediated lysis with a diminished capacity of survival within the host macrophage. In addition, a possible defect in the vesicular trafficking of glycoproteins, plasma-membrane proteins and secreted proteins may have important consequences on the ability of *L. tarentolae* to survive as an intracellular parasite. Furthermore, *L. tarentolae* lacks several proteins of unknown functions (including the delta-amastins) shown to be expressed preferentially in the amastigote stage. The absence of these proteins may explain why *L. tarentolae* cannot replicate efficiently in mammalian macrophages. In summary, this study provides insights into the reconstruction of the steps leading to increase adaptation for intracellular parasitism and suggests a number of hypothesis that can be experimentally tested.

ACCESSION NUMBER

The genome sequence of *L. tarentolae* has been deposited in tritrypdb.org. Upon inclusion, the sequence will be transferred to Genbank.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–6, Supplementary Figures 1–36.

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