Modulation of *Leishmania* ABC Protein Gene Expression through Life Stages and among Drug-Resistant Parasites†

Philippe Leprohon, Danielle Légaré, Isabelle Girard, Barbara Papadopoulou, and Marc Ouellette*

Centre de Recherche en Infectiologie et Division de Microbiologie, Université Laval, Québec City, Québec, Canada

Received 26 May 2006/Accepted 21 July 2006

The ATP-binding cassette (ABC) protein superfamily is one of the largest evolutionarily conserved families and is found in all kingdoms of life. The recent completion of the *Leishmania* genome sequence allowed us to analyze and classify its encoded ABC proteins. The complete sequence predicts a data set of 42 open reading frames (ORFs) coding for proteins belonging to the ABC superfamily, with representative members of every major subfamily (from ABCA to ABCH) commonly found in eukaryotes. Comparative analysis showed that the same ABC data set is found between *Leishmania major* and *Leishmania infantum* and that some orthologues are found in the genome of the related parasites *Trypanosoma brucei* and *Trypanosoma cruzi*. Customized DNA microarrays were made to assess ABC gene expression profiling throughout the two main *Leishmania* life stages. Two ABC genes (*ABCA3* and *ABCG3*) are preferentially expressed in the amastigote stage, whereas one ABC gene (*ABCF3*) is more abundantly expressed in promastigotes. Microarray-based expression profiling experiments also revealed that three ABC genes (*ABCA3*, *ABCC3*, and *ABCH1*) are overexpressed in two independent antimony-resistant strains compared to the parental sensitive strain. All microarray results were confirmed by real-time reverse transcription-PCR assays. The present study provides a thorough phylogenetic classification of the *Leishmania* ABC proteins and sets the basis for further functional studies on this important class of proteins.

ATP-binding cassette (ABC) proteins form the largest family of transmembrane proteins and are found in all living organisms (31). Most of these proteins are involved in the ATP-dependent transport of a variety of molecules across biological membranes, including amino acids, sugars, peptides, lipids, ions, and chemotherapeutic drugs (reviewed in reference 31). The functional significance of this family of proteins is reflected by the diversity of Mendelian and complex disorders associated with human genetic diseases involving ABC genes (reviewed in references 7 and 12). Proteins of the ABC superfamily contain in their sequences a strongly conserved nucleotide-binding domain (NBD) with two major motifs (13, 31). Along with the Walker A and B motifs found in many ATPase families, the NBD is composed of a characteristic ABC signature, the “C” motif, which is located between the two Walker motifs, just upstream of the Walker B site (33). Furthermore, in addition to the nucleotide-binding domains, ABC proteins involved in transport also contain transmembrane domains (TMD) composed of multiple transmembrane α-helices. A functional transporter appears to require a minimum of at least two NBDs coupled with two TMDs. Eukaryotic ABC transporter genes are organized either as full transporters containing two TMDs and two NBDs or as half transporters containing only one of each domain encoded on the same molecule (33). The half transporter molecules need to assemble as homodimers or heterodimers in the membrane to create a functional transporter. Some ABC proteins apparently not involved in transport activities but implicated in other conserved cellular processes do not have any TMDs and are composed of two NBDs fused on the same molecule. On the basis of gene structure similarity and homology in the NBD sequence, eukaryotic ABC proteins can be divided into eight different subfamilies (ABCA to ABCH), seven of which (ABCA to ABCG) are found in the human genome (2). The ABCH subfamily has been identified for the first time in *Drosophila melanogaster* (14).

In the protozoan parasite *Leishmania*, several ABC transporters have already been characterized. At least 20 different *Leishmania* species are responsible for a variety of clinical manifestations ranging from self-healing skin ulcers (e.g., *Leishmania major*) to life-threatening visceral diseases (e.g., *L. donovani* and *L. infantum*) (30). *Human leishmaniasis* has a prevalence of 12 million cases, an estimated population of 350 million at risk and an incidence of 2 million new cases annually (43). *Leishmania* has a relatively simple life cycle, with two main stages: the flagellated promastigotes, which are found in the gut of the insect vector, and the intracellular amastigotes, which live inside macrophages of the mammalian host. No effective vaccine is yet available against this parasite, and its control relies primarily on chemotherapy. Antimony containing compounds such as sodium stibogluconate (Pentostam) and *N*-methylglucamine (Glucantime) remain the mainstay against all forms of *Leishmania* infections (43), despite the emergence of antimony-resistant parasites now described on a frequent basis in several regions where this organism is endemic (27, 41, 48).

The first ABC protein identified in *Leishmania* is MRPA (PGPA) (45), a member of the ABCC subfamily able to confer antimony resistance by sequestering thiol-metal conjugates in an intracellular vesicle (40). MRPA was shown to be part of a large gene family with at least four other members (39). More recently, another ABCC member named PRP1 was shown to confer pentamidine resistance and antimony cross-resistance.
Comparisons of the tide-binding domains of ABC proteins were extracted by performing Pfam genetic analyses. The ABC sequences of correction distance method of the MEGA package version 3.1 (38). The robustness resulting multiple sequence alignments were subjected to analyses using the maxi-
 searches with the PF00005 motif as a query. ORFs were retrieved using the Pfam browse option at GeneDB and using BLAST
 presence of flanking Walker A and Walker B conserved sequences. Furthermore, microarray-based expression analyses were conducted to evaluate the ABC gene expression throughout parasite life stages and in parasites resistant to antimonal drugs.

**MATERIALS AND METHODS**

**Database searches and sequence analyses.** The putative L. major ABC genes were retrieved from the GeneDB database (www.genedb.org) using the Motif search tool with the ABC signature (“C”) motif as query sequence. ORFs identified as encoding proteins containing such motif were then screened for the presence of flanking Walker A and Walker B conserved sequences. Furthermore, a series of BLAST searches were conducted on L. major predicted proteins in GeneDB by using the model ABC domain ABC_tran (accession PF000005) as a query. A series of BLAST searches were done on the assembled contig sequences of L. major Friedlin until no more new ABC coding genes could be identified. Finally, the complete L. major ABC gene data set was used as a query for multiple BLAST searches in the GeneDB database to reveal the complete sequence of the L. infantum orthologues. The amino acid sequence of nucleo-
side-binding domains of ABC proteins were extracted by performing Pfam searches at the Sanger Institute Web site (www.sanger.ac.uk/Software/Pfam/).

Comparisons of the Leishmania ABC gene families to homologues present in other sequenced eukaryotic genomes were made using BlastP at the NCBI Web site. Sequences were assigned as orthologues if they showed the highest score in BLAST search analyses and if they clustered on the same tree branch in phylogen-
etic analyses. The ABC sequences of Trypanosoma brucei and Trypanosoma cruzi were retrieved using the Pfam browse option at GeneDB and using BLAST searches with the PF000005 motif as a query.

**Phylogenetic analyses.** Multiple sequence alignments were performed on the complete protein sequences or on the amino acid sequences of the ATP-binding domains by using CLUSTAL W (56) with the default settings. The two NBDs of full-length ABC proteins were treated independently for alignments. If needed, gaps were removed from the alignment by using the BioEdit software (28). The resulting multiple sequence alignments were subjected to analyses using the maxi-
mum-parsimony algorithm and the neighbor-joining algorithm (49) with the Poisson correction distance method of the MEGA package version 5.1 (38). The robustness of the neighbor-joining tree was assessed by 1,000 bootstrap resamplings.

**Cell lines.** L. infantum amastigotes were kept in culture as axenic amastigotes by culturing at 25°C in

**Gene expression throughout parasite life stages and in parasites resistant to antimonal drugs.**

**RAHANA TSA labeling and detection kit (Perkin-Elmer) according to the supplier’s recommen-
dations. For each reaction, fluorescein-labeled and biotin-labeled CDNs were synthesized from 2 μg of purified total RNA previously mixed with two exogenous mRNAs (CAI1 at 2 pg/μl and NAC1 at 2 pg/μl from Anabidopsis thaliana; Strategene) added as external standards to adjust for variations in the incorporation efficiency of the modified nucleotides and for differences in first-
strand cDNA synthesis reaction. The labeled CDNs were precipitated with isopropanol, washed with 70% ethanol, dried, and resuspended each in 15 μl of hybridization buffer (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), 0.1% sodium dodecyl sulfate, 25 μg of salmon sperm DNA/ml, 460 μg of yeast tRNA/ml. Two differentially labeled target CDNs were pooled for hybridization overnight at 42°C under a coverslip (ERIE Scientific) in a hybridization chamber (Corning). After hybridization, the microarrays were washed once with 2× SSC-0.1% sodium dodecyl sulfate preheated at 42°C and then with 1× SSC, 0.2× SSC, and 0.05× SSC successively at room temperature. Hybrid-
ized targets from fluorescein-labeled and biotin-labeled CDNs were detected on the microarray using Cy3-tyramide and Cy5-tyramide reagents, resectively. Each hybridization experiment was performed in four replicates using four independent RNA extractions and two dye-swapping procedures.

**Microarray data processing and analysis.** Detection of the Cy5 and Cy3 signal was sequentially performed on a ScanArray 4000XL scanner (Perkin-

**Reverse transcription-PCR (RT-PCR).** Primers were designed based on the ABC sequences of L. infantum using GeneRunner software. cDNA was synthesized from 2 μg of total RNA using Superscript II RNase H reverse transcriptase (Invitrogen) and Oligo(dT)12-18 primers (Invitrogen) according to manufacturer’s instructions. Each PCR was performed with enzyme PCR was performed with the following program (21). The relative amount of PCR products generated from each primer set was determined based on the threshold cycle (Ct) value and ampli-
cation efficiencies and was normalized by dividing the values by the relative amount of the GAPDH gene used as a control.

---

**Figure 1.** Diagram illustrating the approach used in this study. A. The ABC gene data set in GeneDB was used to identify ABC genes in L. major and L. infantum. B. Low-density DNA microarrays were designed to express this data set as a resource for future functional genomic investigations of the ABC gene expression throughout parasite life stages and in parasites resistant to antimonal drugs. C. Transcriptional analysis of these family members could provide further insights on the role of these related proteins in the biology of the parasite and in drug resistance. Low-density DNA microarrays are well suited for that purpose given their relative simplicity and reproducibility. As a prelude to and resource for future functional genomic investigations of this relevant group of proteins, we present here a complete inventory and phylogenetic classification of the ABC proteins found in the protozoan parasite Leishmania. Moreover, microarray-based expression analyses were conducted to evaluate the ABC gene expression through-out parasite life stages and in parasites resistant to antimonal drugs.
### TABLE 1. Leishmania ABC proteins

<table>
<thead>
<tr>
<th>Gene nomenclature</th>
<th>L. major</th>
<th>L. infantum</th>
<th>Leishmania alias</th>
<th>Leishmania gene topology</th>
<th>T. brucei</th>
<th>T. cruzi</th>
<th>Orthologous sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>LmjF2.0300</td>
<td>LinJ0.0220</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCA7</td>
<td>LmjF15.0760</td>
<td>LinJ15.0790</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCA8</td>
<td>LmjF27.0970</td>
<td>LinJ27.0550</td>
<td>LtrABC2.1</td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCA9</td>
<td>LmjF27.0990</td>
<td>LinJ27.0560</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCA10</td>
<td>LmjF29.0620</td>
<td>LinJ29.0770</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB2</td>
<td>LmjF32.3080</td>
<td>LinJ32.3640</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB3</td>
<td>LmjF34.0990</td>
<td>LinJ34.0950</td>
<td>MDR1</td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB4</td>
<td>LmjF33.1430</td>
<td>LinJ31.1810</td>
<td>PRP1</td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB6</td>
<td>LmjF33.1860</td>
<td>LinJ33.1860</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB8</td>
<td>LmjF34.0670</td>
<td>LinJ34.0690</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCB9</td>
<td>LmjF34.0670</td>
<td>LinJ34.0690</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCD1</td>
<td>LmjF27.0470</td>
<td>LinJ27.0380</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCD3</td>
<td>LmjF33.1860</td>
<td>LinJ33.1860</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCF1</td>
<td>LmjF33.0160</td>
<td>LinJ33.0160</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCF3</td>
<td>LmjF33.0310</td>
<td>LinJ33.0340</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG1</td>
<td>LmjF06.0080</td>
<td>LinJ06.0080</td>
<td>NBD-TM</td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG2</td>
<td>LmjF06.0090</td>
<td>LinJ06.0090</td>
<td>NBD-TM</td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG3</td>
<td>LmjF06.0100</td>
<td>LinJ06.0100</td>
<td>NBD-TM</td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG4</td>
<td>LmjF15.0890</td>
<td>LinJ15.0940</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG5</td>
<td>LmjF23.0380</td>
<td>LinJ23.0420</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG6</td>
<td>LmjF36.2890</td>
<td>LinJ36.3580</td>
<td>NBD-TM</td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG7</td>
<td>LmjF11.0040</td>
<td>LinJ11.0040</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG8</td>
<td>LmjF29.1640</td>
<td>LinJ29.1640</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCG9</td>
<td>LmjF30.1330</td>
<td>LinJ30.1700</td>
<td></td>
<td>[TM-NBD]₂</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued on following page
RESULTS

The Leishmania ABC protein family. The combined use of systematic BLAST searches against the L. major genome (www.genedb.org) using the ABC signature sequence as a query and of the analysis of the current annotated database allowed the identification of 42 ORFs that could be assigned to the ABC superfamily. This represents 0.5% of the total number of genes (approximately 8,300) in L. major. The retrieved sequences were designated as ABC proteins based on the presence of the characteristic ABC signature motif flanked by the Walker A and B motifs. Of the 42 ORFs, the L. major genome was found to encode 32 intrinsic membrane ABC proteins and 10 ABC proteins without any TMD. Among the intrinsic membrane proteins, full transporters were more frequent than half transporters (20:12). The number of predicted transmembrane spans varies from 10 to 14 for the full molecules, whereas in half molecules the number of putative transmembrane spans varies from 2 to 6. A detailed inventory of these ORFs, including names, GeneDB database accession numbers for the L. major and L. infantum orthologues, overall structural organization, and most similar ORFs found in the genome of the related parasites T. brucei and T. cruzi is presented in Table 1.

Classification of the Leishmania ABC proteins. An alignment of the ATP-binding domains was generated and used for phylogenetic analysis to classify the 42 ABC proteins encoded by the Leishmania genome. Figure 1 displays a neighbor-joining tree clustering the 42 ABC proteins into eight subfamilies, seven of which are found in the human genome. As shown in Fig. 1, Leishmania has 10 members of the ABCA family (ABC1), 4 members of the ABCB family (MDR/TAP), 8 members of the ABCC family (MRP/CFTR), 3 members of the ABCD family (ALD), 1 member of the ABCE family (OABP), 3 members of the ABCF family (GNC20), and 6 members of the ABCG family (WHITE). We also found three members belonging to the ABCH family and four proteins not clustering to any of the above-mentioned subfamilies. The same clusters were obtained when a maximum-parsimony phylogenetic analysis was used (data not shown). The Leishmania ABC genes are largely dispersed in the genome and are found on 19 different chromosomes (Fig. 2). Most of the genes are found alone on chromosomes, but some are grouped in a head-to-tail fashion as part of two- or three-gene clusters (Fig. 2). All of these clusters contain members of the same subfamily: ABCA genes (chromosomes 11 and 27), ABCC genes (chromosomes 23 and 31), or ABCG genes (chromosome 6). Interestingly, the noncontiguous ABCA2 and ABCC4 genes of chromosome 11 share a 100% identity at the nucleotide level.

Orthology of the ABC proteins in trypanosomatids. We compared the ABC data set of L. major to the L. infantum contig sequences and to the related parasite T. brucei (6) and T. cruzi (19) genomes. Table 1 shows that an L. infantum orthologue was found for every L. major ABC gene with a conserved synteny. Furthermore, the L. infantum contig sequences did not reveal any additional ABC protein coding ORF that has not been previously found in the L. major genome. With its 42 ABC genes, Leishmania is the parasite with the largest ABC data set in the Trypanosomatidae family, with the T. cruzi and T. brucei genomes encoding 28 and 22 ABC proteins, respectively (Table 1). This variation in the number of ABC genes between the three organisms seems to be the result of an expansion of the ABCA, ABCC, and ABCG subfamilies that occurred in Leishmania after the split with the Trypanosoma lineage and of the loss of some ABC genes in Trypanosoma. Indeed, only one cluster of orthologous genes was found between Leishmania, T. cruzi, and T. brucei compared to Leishmania, but only some of these could be assigned as orthologous sequences by phylogenetic analyses. These clusters of orthologous genes, where homologues found in the three genomes are grouped on the same cluster in a phylogenetic tree, are observed in the most evolutionary stable subfamilies (Table 1 and Fig. 3). Indeed, only one cluster of orthologous genes was found between Leishmania, T. cruzi, and T. brucei in the ABCA subfamily (Table 1 and Fig. 3A), whereas two have been identified in the ABCC subfamily (Table 1 and Fig. 3B). No ABCB orthologue could be found between Trypanosomas and Leishmania. The ABCD, ABCE, and ABCF subfamilies show the highest level of orthology, with all of the Leishmania genes forming unambiguous pairs with their T. brucei and T. cruzi homologues. Within the ABCG subfamily, three of the six Leishmania genes paired with an orthologue in the Trypanosoma genomes (Table 1 and Fig. 3G). Several Leishmania ABC proteins either included in the ABCB class or still unclassified had orthologues
in usually both *T. brucei* and *T. cruzi*. One exception is LmjF32.2060, which has no orthologue in the sequenced *Trypanosoma* genes (Fig. 3H).

The *Leishmania* ABC proteins paralogous relationship. One way to address the putative function of a gene is to identify orthologues of known function. One such approach based on phylogenetic analysis was applied here in order to compare the ABC proteins of *Leishmania* to those of other eukaryotic genomes whose ABC gene inventory and classification have already been done (*Homo sapiens*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Dictyostelium discoideum*). As exemplified by the ABCA subfamily in Fig. 4A, the *Leishmania* proteins cluster in phylogenetic branches along with their trypanosomatid homologues but apart from proteins encoded by the other eukaryotic genomes. This is also the case for the proteins of the ABCC and ABCG subfamilies (results not shown). Among the other subfamilies, very few clear orthologues have been identified by phylogenetic analyses. Among these are the *L. major* ABCB3 protein, which is found on a strong cluster with the yeast ATM1 protein, and the *L. major* ABCE1 protein, which is an orthologue of the yeast ABCE1-like YDR091c protein. The three *L. major* ABCF proteins were also successfully paired; ABCF1 is clustering with the human ABCF3 and the yeast GCN20 proteins, ABCF2 is clustering with the human ABCF2 protein among others, and ABCF3 is found on a cluster along with the human ABCF1 protein (Fig. 4B). In some instances (ABCB1, ABCD3, and ABCG5 proteins), *Leishmania* ABC proteins are clustering with more than one homologue of another organism (data not shown), rendering the assignment of a putative function more ambiguous.

The *Leishmania* genome encodes seven ABC proteins not
clustering with any of the mammalian ABC subfamilies. These proteins were referred to as members of the ABCH subfamily or as unclassified ABC proteins (“others” in Table 1), whether they clustered or not with representatives of the *Drosophila* ABCH proteins (Table 1). Most of them do not possess any transmembrane domain, with LmjF32.2060 being the only exception with six predicted amino-terminal membrane-spanning α-helices. Furthermore, with the exception of LmjF30.1330,
FIG. 4. Phylogenetic tree of selected ABC proteins with other eukaryotic ABC proteins. Phylogeny was derived and is displayed according to the procedure outlined in Fig. 1, except that complete protein sequences were used instead of just the NBD. (A) Phylogenetic tree of ABCA proteins in seven eukaryotic genomes. (B) Phylogenetic tree of the ABCF proteins in Leishmania, with human and yeast proteins. Im, L. major; Te, T. cruzi; Tb, T. brucei; Hs, H. sapiens; Dm, D. melanogaster; Ce, C. elegans; Dd, D. discoideum; Sc, S. cerevisiae.
which possesses a carboxy-terminal NBD and a degenerated amino-terminal NBD, all unclassified proteins possess a single NBD usually located at the carboxy termini of the molecule (Table 1). Phylogenetic analyses revealed that the LmjF11.0040 and LmjF29.1640 proteins are orthologues of the D. discoideum ABCh.2 and ABCh.1 proteins, respectively, and that they are found along with LmjF30.1330 on a cluster containing the D. melanogaster CG9990, CG6162, and CG11147 ABCH proteins (data not shown). All of the other unclassified proteins of Leishmania seem to be more related to bacterial ABC proteins by BLASTp searches.

Generation of DNA microarrays to study ABC gene expression in the two main life stages of L. infantum. L. major and L. infantum have the same complement of ABC proteins (Table 1), and oligonucleotides were designed to detect the genes of both species. Changes in mRNA abundance were examined by customized DNA microarrays comprising 70-mer oligonucleotides representing the entire ABC data set of Leishmania in multiple replicates in addition to PCR fragments of several genes known to be involved in the trypomastigote biosynthetic pathway or in antimony resistance (26). In order to estimate the accuracy of the protocol, arrays were first hybridized to Cy3- and Cy5-labeled cDNA generated from the same RNA preparation, and hybridization was found to be uniform (data not shown). Once the spotting, hybridization, and washing conditions were optimized and hybridization signals could be consistently reproduced, the arrays were used for the parallel analysis of ABC gene expression throughout the life cycle of L. infantum and in antimony-resistant mutants of L. infantum.

The ABC gene expression profiling between promastigotes and axenic amastigotes of L. infantum is shown in Fig. 5A. From the selected genes printed on the array, one ABC gene, ABCF3, appeared to be consistently upregulated in the promastigote stage (2.5-fold, \( P < 0.01 \)). The expression of another gene, S-adenosylhomocysteine hydrolase (SAHH), is also increased in promastigotes (2.8-fold, \( P < 0.01 \)). When we applied a cutoff of at least twofold differential expression, three ABC genes showed a consistent upregulation in the amastigote stage. One corresponds to the ABC transporter ABCA3 (2.5-fold, \( P < 0.01 \)), the second one corresponds to the ABC transporter ABCG3 (2.0-fold, \( P < 0.01 \)), and the last one corresponds to ABCF2 (3.0-fold, \( P < 0.02 \)). The expression of the aquaglyceroporin AQP1 also seems to be increased in axenic amastigotes (2.0-fold, \( P < 0.02 \)). No other ABC gene showed a significant differential expression in any of the two life stages when using a cutoff between 1.5 and 2. The putative differential expression of ABC genes was further confirmed by real-time RT-PCR, and the increased mRNA levels of ABCF3 in promastigotes and of ABCA3 (Fig. 5B) and ABCG3 in amastigotes were confirmed by this technique (Fig. 5C).
ABCC4, a gene that was not found to be differentially regulated by microarrays, was also found to be similarly expressed between the two stages of *Leishmania* in real-time RT-PCR experiments (Fig. 5C). Although *ABCF2* was found to be significantly overexpressed in the amastigote stage by microarrays, this could not be confirmed by real-time RT-PCR (Fig. 5C).

**ABC gene expression in antimony-resistant mutants.** The role of the ABC transporter MRPA (ABCC3) in antimonial resistance is well established (20, 40). Since antimony resistance is multifactorial, it is possible that other ABC proteins could also be implicated (44). To test whether antimony resistance is correlated with the differential expression of other ABC genes, we performed microarray analyses using an antimony-sensitive *L. infantum* promastigote wild-type (WT) strain compared to an antimony-resistant *L. infantum* promastigote mutant strain (Sb2000.1). The ABC gene expression between the *L. infantum*-sensitive (WT) and -resistant (Sb2000.1) strains is shown in Fig. 6A. As expected, the majority of the spots aligned along the regression curve, suggesting that the genes represented by these spots are equally expressed in both samples. When applying a cutoff of at least twofold differential expression, the only gene found to be differentially expressed between the sensitive and the resistant strains is the one coding for the ABC transporter MRPA (ABCC3) (3.8-fold, *P* < 0.01). However, when applying a cutoff of between a 1.5- and a 2-fold difference in mRNA abundance, two more ABC genes, *ABCA3* (1.8-fold, *P* < 0.02) and *ABCH1* (1.8-fold, *P* < 0.01), showed a statistically significant higher expression in the antimony-resistant strain. Interestingly, the same mutant was recently studied with a *Leishmania* full genome array and, while the expression of several genes was modulated, the expression of the same three ABC protein genes was found to be upregulated (unpublished observations). The differential expression of these three genes in the antimony-resistant mutant was confirmed by real-time RT-PCR experiments (Fig. 6B and C). The overexpression of *ABCA3* and *ABCH1* is described for the first time in an antimony-resistant *Leishmania* strain. To test whether this is also the case in other antimony-resistant mu-
tants, we analyzed by real-time RT-PCR the expression of these genes in the *L. infantum* Sb4000.4 promastigote line selected for resistance to Sb(III). Interestingly, MRPA was grossly overexpressed, but *ABCA3* and *ABCH1* were also found to be increased in this independent mutant (Fig. 6D). As observed in the Sb2000.1 mutant, the *ABCE1* gene was not found to be differentially expressed in the Sb4000.4 line (Fig. 6D). Transfection of MRPA in *L. infantum* conferred a three-fold increase in Sb(III) resistance. Despite the overexpression of *ABCA3* and *ABCH1* in two independent mutants, attempts to overexpress *ABCA3* or *ABCH1* in a *L. infantum* WT background did not result in antimony resistance, however (result not shown). The coexpression of either *ABCA3* or *ABCH1* with *ABCC3* in a WT background did not confer higher resistance than *ABCC3* overexpression alone (result not shown).

**DISCUSSION**

The completion of the *Leishmania* genome sequencing project (34) has allowed the characterization of 42 members of the *Leishmania* ABC gene family, a number considerably higher in comparison to related trypanosomatids (Table 1) or to the Apicomplexa parasites *Plasmodium* (fewer than 20 members) (22) and *Toxoplasma gondii* (20 members) (50). Phylogenetic analysis has allowed the classification of the *Leishmania* ABC proteins in different subfamilies using a nomenclature ABCA to ABCH adopted by the community of investigators working with eukaryotic ABC proteins (14). Another classification of the *L. major* ABC genes has also been done using the TCDB system (www.tcdb.org), and 45 *Leishmania* ABC proteins were classified in 22 different groups belonging to the 3.A.1 TCDB class (6). The discrepancy between the 42 proteins highlighted in the present study and the 45 pinpointed by a preliminary analysis of the *L. major* genome is explained by the inclusion of four proteins in the latter analysis (6) (LmjF21.0880, LmjF27.1700, LmjF29.0930, and LmjF34.2070) that are, according to all of the search criteria used here, unlikely to be ABC proteins, and by the omission of LmjF33.3040 (see Fig. 3H), which is clearly an ABC protein. For the remainder of this discussion, we shall use the ABCA to ABCH family nomenclature.

*Leishmania* has representative members of each subfamily, and the proportion of proteins in each of them seems relatively well conserved compared to other eukaryotes, especially for the ABCD, ABCE, and ABCF subfamilies (13). It is interesting that the *Leishmania* genome encodes for three proteins of the ABCH subfamily originally discovered in *D. melanogaster* (14). Interestingly, three of the four unclassified ABC proteins are well conserved in trypanosomatids (see Table 1). Homologues of this heterologous group of unclassified proteins have already been identified in other genomes (13, 15, 54) and appear to be more related to bacterial ABC proteins. A comparison of the ABC proteins of *Leishmania* to those of five other eukaryotic genomes revealed few unambiguous orthologous sequences. This is in agreement with previous studies that evaluated the frequency of orthologous ABC transporter pairs between different eukaryotic genomes (14, 53). Therefore, no detailed predictions of function in *Leishmania* ABC proteins can be drawn on the basis of phylogeny alone. As previously observed (14, 53), the frequency of orthologous sequences was found to be lower in the transporter subfamilies A, B, C, D, and G compared to those not involved in transport activities (subfamilies E and F).

The difference in ABC gene number in *Leishmania* compared to other eukaryotic microorganisms is mostly due to an expansion of the *ABCA, ABCC,* and *ABCG* genes, several of which have likely resulted from gene duplication events occurring after the split between the *Leishmania* and *Trypanosoma* lineages (Fig. 3). Several genes coding for members of those subfamilies have been found grouped in a head-to-tail fashion in the genome of *Leishmania* (Fig. 2), a finding consistent with gene duplication. The duplication events seem to have occurred recently given the high similarity between the paralogues (Fig. 3A, C, and G). This difference in the ABC complement between *Leishmania* and *Trypanosoma* could result from the different environments encountered by the parasitic organisms which translates into distinct needs and maintains a selective pressure on the ABC gene data set. The known function of members of the ABCA, ABCB, and ABCG subfamily in other eukaryotes allows tentative explanations as to why the expansion of these ABC proteins occurred in *Leishmania* compared to other eukaryotic microorganisms. The lifestyle and life cycle of *Leishmania* differs considerably from other eukaryotic pathogens. Indeed, it migrates and differentiates in the digestive tract of the sand fly vector and, in contrast to most eukaryotic pathogens, it remains within the phagolysosome of the host macrophages. These inhospitable habitats are likely to produce a number of toxic molecules for which the parasite has to defend itself. The ABCs are best known to provide protection against a vast repertoire of xenobiotics and endobiotics and their glutathione, glucuronide, and sulfate conjugates (reviewed in reference 16). Thus, it is possible that the expansion of the ABC subfamily in *Leishmania* (Table 1 and Fig. 3) is necessary to protect the cells in both life stages against toxic molecules. Interestingly, two ABCB proteins of *Leishmania*, MRPA (ABCC3) and PRP1 (ABCC7), have already been shown to have a role in dealing with xenobiotics (11, 40). Some eukaryotic members of the ABCG, most notably ABCG2, are involved in the detoxification of drugs and an expansion of the ABCG proteins in *Leishmania* (Fig. 3) (the parasitism ABCG1, -2, and -3 are distantly related to the human ABCG2) may also help the parasites to cope with the variety of adverse conditions they are encountering.

*Leishmania* has 10 ABCA proteins, but these are absent in yeast (15) and in Apicomplexa parasites (50), and their number is considerably less in *Trypanosoma*. The ABCA and the ABCG proteins are best known for the transport of a variety of lipids, including cholesterol, plant sterols, sphingolipids, and phospholipids (37; reviewed in reference 55). At least two *Leishmania* ABCA transporters have demonstrated an ability to transport phospholipids (5, 46). There is considerable evidence that lipid transport and salvage are implicated in several aspects of *Leishmania* biology, and this provides a plausible explanation for the expansion of the ABCA and ABCG subfamilies. Indeed, the major sphingolipid of *Leishmania*, inositol phosphorylceramide, is absolutely required for metacyclogenesis (infectious promastigotes) in *L. major* (17, 60). Sphingolipid metabolites have been shown to modulate a wide variety of cellular events in a number of cells including *Leishmania* (18). Surprisingly, *Leishmania* amastigotes cannot make...
these sphingolipids and need to salvage them from the host (17, 59, 60) and ABC proteins could possibly be implicated in this phenomenon as well. Interestingly, and consistent with the proposal described above, our targeted DNA microarrays indicated that the expression of ABCA3 and ABCG3 was consistently upregulated in axenic amastigotes (Fig. 5). A homologue of the L. major ABCA3 in T. cruzi (TcABC1) was shown to be overexpressed in the epimastigote and amastigote stages (57), and the preferential expression in amastigotes of a gene coding for an ABCA protein has recently been reported in Leishmania using random genomic DNA microarrays (1). Glycosylinsitol phospholipids are highly abundant and important in host-parasite interactions and were shown to be translocated across membranes (47), and this could also involve ABC proteins. Leishmania has specialized sterols including ergosterol (24) and, if ABCGs were required for their transport, then there may also explain partly the expansion of ABCGs in Leishmania.

DNA microarrays are useful in the field of parasitology, as exemplified by numerous studies on stage specific expression and on drug resistance in Leishmania (1, 3, 20, 26, 32, 51). Furthermore, custom-made ABC transporter-targeted microarrays have already been used to study multidrug resistance in cancer cells (4, 23). The only ABC gene found to be preferentially expressed in the promastigote stage of Leishmania is ABCF3, an orthologue of the human ABCF1. The human ABCF1 protein copurifies with the eukaryotic initiation factor 2 and associates with the ribosomes in an ATP-dependent manner (58). Given the orthologous relationship with L. major ABCF3, one can expect a similar role in translation initiation for the Leishmania protein. Stage-specific regulation of gene expression in Leishmania is often controlled at the translation level (8, 42), and thus it is possible that genes expressed in a stage-specific manner such as ABCF3 are involved in stage-specific gene regulation.

The customized DNA microarrays were also used for the analysis of ABC gene expression in antimony-resistant mutants. The gene MRPA was found overexpressed in the Sb2000.1 resistant mutant, in agreement with previously reported results (20). ABCA3 and ABCCH1 were also overexpressed in the Sb2000.1 mutant (Fig. 6C). Interestingly, the same three genes—MRPA, ABCA3, and ABCCH1—were found to be overexpressed in an independent novel SbIII L. infantum resistant mutant (Fig. 6D). Furthermore, given the involvement in vesicular trafficking and exocytosis pathway of an ABCA3 homologue in T. cruzi (57), an attractive scheme to the antimony resistance pathway in the Sb2000.1 or Sb4000.4 mutants would be an increased sequestration of the thiol-SbIII complexes in intracellular vesicles by the overexpression of MRPA, followed by an increased exocytosis of those vesicles resulting from ABCA3 overexpression. Preliminary analysis of an antimony-resistant L. donovani field isolate also suggests that ABCA3 expression may be increased in these resistant parasites (unpublished observations). Overexpression of ABCA3 or ABCCH1 in a WT background was not sufficient to observe an antimony resistance phenotype (result not shown), so the role, if any, in antimony resistance requires further experimental work. The ABCA3 and/or ABCCH1 genes may contribute to resistance, however, in other contexts such as when other mutations are present as in the mutants Sb2000.1 or Sb4000.4.

Our work has highlighted, in contrast to other protozoan parasites, the magnitude of the ABC protein family of Leishmania. Given the multiple proteins found in the transporter subfamilies, Leishmania seems equipped to export a wide variety of compounds. The present study has also illustrated the usefulness of small targeted microarrays of 70-mer oligonucleotides. These ABC arrays will be useful tools for studying the physiological function of ABC proteins and to detect modulation in gene expression in Leishmania parasites resistant to various chemotherapeutic drugs.

ACKNOWLEDGMENTS
We thank the Sanger Center, D. Smith and J. Mottram for the availability of the L. infantum sequences (www.sanger.ac.uk). We thank Eric Leblanc for help in initial phylogenetic analyses and Jean Morisette for using the GeneSpring 7.2 software.

This study was funded in part by the CIHR group GR14501 and operating grants to M.O., through a FQRNT group grant and through a Wellcome Trust-Burroughs Wellcome Fund new initiative in infectious diseases program grant to M.O., P.L. is a recipient of a CIHR studentship. B.P. and M.O. are Burroughs Wellcome Fund New Investigators and Scholar in Molecular Parasitology, and M.O. holds a Canada Research Chair in Antimicrobial Resistance.

REFERENCES
2. Allikmets, R., B. Gerrard, A. Hutchinson, and M. Dean. 1996. Characterization of the human ABCG1 protein copurifies with the eukaryotic initiation factor 2 and associates with the ribosomes in an ATP-dependent manner (58). Given the orthologous relationship with L. major ABCF3, one can expect a similar role in translation initiation for the Leishmania protein. Stage-specific regulation of gene expression in Leishmania is often controlled at the translation level (8, 42), and thus it is possible that genes expressed in a stage-specific manner such as ABCF3 are involved in stage-specific gene regulation.

The customized DNA microarrays were also used for the analysis of ABC gene expression in antimony-resistant mutants. The gene MRPA was found overexpressed in the Sb2000.1 resistant mutant, in agreement with previously reported results (20). ABCA3 and ABCCH1 were also overexpressed in the Sb2000.1 mutant (Fig. 6C). Interestingly, the same three genes—MRPA, ABCA3, and ABCCH1—were found to be overexpressed in an independent novel SbIII L. infantum resistant mutant (Fig. 6D). Furthermore, given the involvement in vesicular trafficking and exocytosis pathway of an ABCA3 homologue in T. cruzi (57), an attractive scheme to the antimony resistance pathway in the Sb2000.1 or Sb4000.4 mutants would be an increased sequestration of the thiol-SbIII complexes in intracellular vesicles by the overexpression of MRPA, followed by an increased exocytosis of those vesicles resulting from ABCA3 overexpression. Preliminary analysis of an antimony-resistant L. donovani field isolate also suggests that ABCA3 expression may be increased in these resistant parasites (unpublished observations). Overexpression of ABCA3 or ABCCH1 in a WT background was not sufficient to observe an antimony resistance phenotype (result not shown), so the role, if any, in antimony resistance requires further experimental work. The ABCA3 and/or ABCCH1 genes may contribute to resistance, however, in other contexts such as when other mutations are present as in the mutants Sb2000.1 or Sb4000.4.

Our work has highlighted, in contrast to other protozoan parasites, the magnitude of the ABC protein family of Leishmania. Given the multiple proteins found in the transporter subfamilies, Leishmania seems equipped to export a wide variety of compounds. The present study has also illustrated the usefulness of small targeted microarrays of 70-mer oligonucleotides. These ABC arrays will be useful tools for studying the physiological function of ABC proteins and to detect modulation in gene expression in Leishmania parasites resistant to various chemotherapeutic drugs.

ACKNOWLEDGMENTS
We thank the Sanger Center, D. Smith and J. Mottram for the availability of the L. infantum sequences (www.sanger.ac.uk). We thank Eric Leblanc for help in initial phylogenetic analyses and Jean Morisette for using the GeneSpring 7.2 software.

This study was funded in part by the CIHR group GR14501 and operating grants to M.O., through a FQRNT group grant and through a Wellcome Trust-Burroughs Wellcome Fund new initiative in infectious diseases program grant to M.O., P.L. is a recipient of a CIHR studentship. B.P. and M.O. are Burroughs Wellcome Fund New Investigators and Scholar in Molecular Parasitology, and M.O. holds a Canada Research Chair in Antimicrobial Resistance.

REFERENCES
2. Allikmets, R., B. Gerrard, A. Hutchinson, and M. Dean. 1996. Characterization of the human ABCG1 protein copurifies with the eukaryotic initiation factor 2 and associates with the ribosomes in an ATP-dependent manner (58). Given the orthologous relationship with L. major ABCF3, one can expect a similar role in translation initiation for the Leishmania protein. Stage-specific regulation of gene expression in Leishmania is often controlled at the translation level (8, 42), and thus it is possible that genes expressed in a stage-specific manner such as ABCF3 are involved in stage-specific gene regulation.

The customized DNA microarrays were also used for the analysis of ABC gene expression in antimony-resistant mutants. The gene MRPA was found overexpressed in the Sb2000.1 resistant mutant, in agreement with previously reported results (20). ABCA3 and ABCCH1 were also overexpressed in the Sb2000.1 mutant (Fig. 6C). Interestingly, the same three genes—MRPA, ABCA3, and ABCCH1—were found to be overexpressed in an independent novel SbIII L. infantum resistant mutant (Fig. 6D). Furthermore, given the involvement in vesicular trafficking and exocytosis pathway of an ABCA3 homologue in T. cruzi (57), an attractive scheme to the antimony resistance pathway in the Sb2000.1 or Sb4000.4 mutants would be an increased sequestration of the thiol-SbIII complexes in intracellular vesicles by the overexpression of MRPA, followed by an increased exocytosis of those vesicles resulting from ABCA3 overexpression. Preliminary analysis of an antimony-resistant L. donovani field isolate also suggests that ABCA3 expression may be increased in these resistant parasites (unpublished observations). Overexpression of ABCA3 or ABCCH1 in a WT background was not sufficient to observe an antimony resistance phenotype (result not shown), so the role, if any, in antimony resistance requires further experimental work. The ABCA3 and/or ABCCH1 genes may contribute to resistance, however, in other contexts such as when other mutations are present as in the mutants Sb2000.1 or Sb4000.4.


32. El-Fadili, K., N. Messier, P. Leprohon, G. Roy, C. Guimond, N. Trudel, N. G.
Gagnon, D. Richard, N. Messier, B. Papadopoulou, J. Corbeil, M. G.


41. Ivens, A. C., C. C. Peacock, E. A. Worthy, L. Murphy, G. Aggarwal, M.
Berriman, E. Sik, M. A. Rajandream, E. Adlem, R. Aert, A. Anupama, Z.
Apostolou, P. Attipoe, N. Bason, C. Bauer, A. Beck, S. M. Beverley, G.
Blaisseutrin, K. Borzym, G. Bothe, C. V. Breschi, M. Collins, E. Cadag, L.
Ciarloni, C. Clayton, R. M. Coulson, A. Cronin, A. K. Cruz, R. M. Davies,
Frasch, A. Fraser, M. Fuchs, C. Gabel, A. Goble, A. Godfau, D. Harris, C.
Herriger, F. Woll, H. Hilbert, D. Horn, Y. Huang, S. Kielgas, A. Knights, M.
Kube, N. Larke, L. Lvitin, A. Lord, T. Louie, M. Marra, D. Masny, K.
Matthews, S. Michaela, J. C. Mottam, S. Muller-Auer, H. Munden, S.
Nelson, H. Norbertekz, K. Oliver, S. O’Neill, M. Pentony, T. M. Pohl,
C. Price, J. Purnelle, E. Quail, A. Rallbonwitowis, R. Reinhardt, M. Rieger,
J. Rinta, J. Robben, L. Robertson, J. C. Ruiz, S. Rutter, D. Saunders, M.
Schafer, J. Schein, D. C. Schwartz, K. Seeger, A. Seyler, S. Sharp, H. Shin,


44. Kobayashi, A., Y. Takezawa, T. Hirata, Y. Shimizu, K. Misasa, N. Kioka,


47. Légarié, D., D. Richard, R. Mukhopadhyay, Y. D. Steriehoft, B. P. Rosen, A.

48. Lira, R. S., S. Sundar, A. Makharia, R. Kenney, A. Gan, E. Saraiya, and D.
Sacks. 1999. Evidence that the high incidence of treatment failures in Indian Kala-Azar is due to the emergence of antimony-resistant strains of Leishmania donovani. Infect. Dis. 130:564–567.

49. McNeill, F., M. Muller, S. Cloutier, N. Boilard, A. Rochette, M. Dube, and


55. Rojas, R., L. Valderrama, M. Valderrama, M. X. Varona, M. Ouellette, and


