Differential Protein Expression Analysis of *Leishmania major* Reveals Novel Roles for Methionine Adenosyltransferase and S-Adenosylmethionine in Methotrexate Resistance*1

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Leishmania* is a trypanosomatid parasite causing serious disease and displaying resistance to various drugs. Here, we present comparative proteomic analyses of *Leishmania major* parasites that have been either shocked with or selected *in vitro* for high level resistance to the model antifolate drug methotrexate. Numerous differentially expressed proteins were identified by these experiments. Some were associated with the stress response, whereas others were found to be overexpressed due to genetic linkage to primary resistance mediators present on DNA amplicons. Several proteins not previously associated with resistance were also identified. The role of one of these, methionine adenosyltransferase, was confirmed by gene transfection and metabolite analysis. After a single exposure to low levels of methotrexate, *L. major* methionine adenosyltransferase transfectants could grow at high concentrations of the drug. Methotrexate resistance was also correlated to increased cellular S-adenosylmethionine levels. The folate and S-adenosylmethionine regeneration pathways are intimately connected, which may provide a basis for this novel resistance phenotype. This thorough comparative proteomic analysis highlights the variety of responses required for drug resistance to be achieved.

Leishmania, a protozoan parasite transmitted by the bite of a sandfly, can cause a species-dependent spectrum of disease ranging from cutaneous lesions (*Leishmania major*) to potentially fatal visceral infections (*Leishmania donovani*). With an estimated 12 million cases of leishmaniasis worldwide and 1.5–2 million new cases reported each year (1), this parasite has a significant impact on human populations. *Leishmania* is transmitted to the host as a motile, elongated promastigote, whereupon it is engulfed by a macrophage and transforms into a round, non-motile amastigote able to spread within the host. The parasite is primarily found in South America, Asia, southern Europe, and Africa, but recent reports have confirmed the visceral form of the disease in dogs in 21 U.S. states and in Canada (2).

Antimony-containing compounds are by far the most com-
monly prescribed antileishmanial therapies, but in some regions up to 80% of cases exhibit clinical resistance to these drugs (3). New targets for the design of novel therapeutics are vital. The folate biosynthetic and reduction pathways in *Leishmania* contain several elements not found in mammalian cells and are under intense scrutiny for drug development. Antifolates, such as the folate analogue methotrexate (MTX),1 have been successful in the treatment of several diseases as well as some cancers (for review, see Ref. 4) but have not yet been successful against *Leishmania*. One of the reasons for this is the ease with which the parasite is able to manipulate expression of its genes. *Leishmania* strains insensitive to MTX display a variety of primary resistance mechanisms. These include amplification of the dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) gene (5) encoding the primary target of the drug, amplification of the gene encoding PTr1 (6, 7), a pteridine reductase able to partially replace the function of *DHFR-TS* (8, 9), deletion of the folate transporters that allow MTX to enter the cell (10, 11), or overexpression of the gene coding for the biotin transporter BT1 (12).

The 34-megabase genome of the human pathogen *L. major* Friedlin has been sequenced by a consortium of laboratories (www.genedb.org). Many of its genes have no homologues in other organisms, and these may yield *Leishmania*-specific drug targets. Because of the number of unique open reading frames found in this organism as well as the complexity of the mechanisms of resistance exhibited by the parasite, a global analysis of the phenomenon of drug resistance is warranted. In a previous preliminary study we used a comparative proteomic approach to show increased levels of PTr1 in a *L. major* strain selected for MTX resistance *in vitro* (13). This was the first identification of a primary resistance mechanism using a proteomic approach and as such validated the use of two-dimensional gel electrophoresis in further studies of resistance.

Here we describe the results of detailed proteomic analyses of MTX exposure and resistance in *L. major*. Through these studies we have found altered expression of proteins involved in the stress response and in primary resistance mechanisms but also of proteins with less easily predicted roles in resistance. One of these corresponds to the methionine adenosyltransferase (MAT) protein, which is overexpressed both in sensitive cells shocked with MTX as well as in mutants resistant to the drug. We present evidence that the MAT protein and its

**References**

1. The abbreviations used are: MTX, methotrexate; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS/MS, tandem mass spectrometry; *DHFR-TS*, dihydrofolate (DHF) reductase-thymidylate synthase; MAT, methionine adenosyltransferase; SAM, S-adenosylmethionine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DIG, digoxigenin; HPLC, high performance liquid chromatography; PGFS, Prostaglandin F synthase.

This paper is available on line at http://www.jbc.org
product, S-adenosylmethionine (SAM), play previously unappreciated roles in MTX resistance.

EXPERIMENTAL PROCEDURES

Cell Culture—L. major LV39, mutants MTX60.2 (13), and MTX60.4 (14), and transfectants were grown in Medium 199 (Invitrogen) supplemented with 10% fetal calf serum (Wisent) and 5 μg/ml hemin (ICN). Where appropriate, MTX, paromomycin (Sigma), SbIII, and/or zeocin (Invitrogen) were added. Cultures were incubated at 25 °C.

Two Dimensional Sample Preparation and Electrophoresis—Culture samples were grown to late log phase as determined by optical density at 600 nm. Cells were harvested by centrifugation at 2500 × g for 5 min before being resuspended into 100 mm HEPES-NaCl buffer (7 M urea, 2 M thiourea, 40 mm Tris, 4% CHAPS, 0.1 mg/ml H11003 equilibration buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% TEMED) as recommended by the manufacturer. Strips were equilibrated in standard Tris-glycine electrophoresis buffer. Second dimension SDS-PAGE was run in a Hoefer DALT apparatus (Amersham Biosciences) at 40 mA/gel and 15 °C until the tracking dye was run off the gel.

Methotrexate Resistance in Leishmania cultures were harvested in late log phase and washed in HEPES-NaCl. Three independent cultures were grown for each condition. Cell pellets were weighed, resuspended in 2 volumes of 0.4 m HClO4, and incubated on ice for 20 min. Supernatants were passed through a 0.45-μm filter, separated into aliquots, and stored at −80 °C.

In the first dimension samples were run on 18-cm Immobiline Dry-Strips (Amersham Biosciences) on an IPGPhor isoelectric focusing system as recommended by the manufacturer. Strips were equilibrated in equilibration buffer (50 mm Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% TEMED (brilliant blue) containing 10 mg/ml diithothreitol for 15 min and then in equilibration buffer containing 25 mg/ml iodoacetamide for 15 min and sealed to 12% acrylamide gels using 0.5% agarose. Two-dimensional gels of four independent samples per strain or mutation were excised manually and sent for peptide mass fingerprinting (East-ern Quebec Proteomics Centre, Centre Hospitalier de l’Université Laval, Québec). Samples were prepared as described previously (13). Mass spectra were acquired on a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems) operating in the positive-ion reflector delayed-extraction mode. Protein identifications were obtained using MASCOT (MatrixScience) and searching for matching peptide mass deviations smaller than 60 ppm. A score of 257, 96, and/or zeocin (Invitrogen) were added. Cultures were incubated at 25 °C.

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overexpression as opposed to down-regulation. Only spot 1173 decreased in the mutants (Table I, Fig. 1A). Also, several of the proteins identified, such as chaperonins, antioxidants, and heat-shock proteins may have been involved in response to stress induced by drug selection. Finally, some proteins, such as PTR1 or the argininosuccinate synthase ARGG, are exclusively found in the mutant MTX60.2, whereas acyl-CoA dehydrogenase is expressed only in MTX60.4. However, most of the

![Figure 1](http://www.jbc.org/)

**Proteins differentially expressed in LV39 mutants resistant to MTX versus the sensitive parent as identified by MALDI-TOF or MS/MS**

<table>
<thead>
<tr>
<th>MS ID</th>
<th>M&lt;sub&gt;exp&lt;/sub&gt;/M&lt;sub&gt;pred&lt;/sub&gt;</th>
<th>pI (exp/pred)</th>
<th>ID</th>
<th>Accession no.</th>
<th>No. peaks % coverage</th>
<th>MASCOT score</th>
<th>Fold change</th>
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<tr>
<td>1160</td>
<td>66/60</td>
<td>5.2/5.3</td>
<td>Cpn60</td>
<td>LmjF36.2030</td>
<td>12/23</td>
<td>76</td>
<td>6.3 (p &lt; 0.003)</td>
</tr>
<tr>
<td>1161</td>
<td>50/81</td>
<td>5.0/5.1</td>
<td>Hsp83–1, N-terminal fragment</td>
<td>LmjF33.0360</td>
<td>10/15</td>
<td>42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5 (p &lt; 0.012)</td>
</tr>
<tr>
<td>1162</td>
<td>35/7</td>
<td>5.1/7</td>
<td>No ID</td>
<td>LmjF15.1140</td>
<td>4/24</td>
<td>62</td>
<td>2.2 (p &lt; 0.268)</td>
</tr>
<tr>
<td>1163</td>
<td>35/21</td>
<td>5.1/6.4</td>
<td>Thiol-specific antioxidant</td>
<td>LmjF13.0280</td>
<td>3/19</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 (p &lt; 0.037)</td>
</tr>
<tr>
<td>1164</td>
<td>17/50</td>
<td>4.7/4.7</td>
<td>α-Tubulin fragment</td>
<td>LmjF13.0280</td>
<td>3/19</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 (p &lt; 0.043)</td>
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<tr>
<td>1165</td>
<td>66/72</td>
<td>5.5/5.7</td>
<td>Mitochondrial HSP70</td>
<td>LmjF30.2470</td>
<td>19/28</td>
<td>803&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 (p &lt; 0.017)</td>
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<tr>
<td>1166</td>
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<td>5.3/5.4</td>
<td>Furaflagellar rod protein</td>
<td>LmjF16.1450</td>
<td>10/19</td>
<td>459&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1167</td>
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<td>5.6/5.6</td>
<td>MAT (AdoMetS)</td>
<td>LmjF30.3520</td>
<td>12/35</td>
<td>106</td>
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<td>1168</td>
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<td>Enolase</td>
<td>LmjF14.1160</td>
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<td>117</td>
<td>3.8 (p &lt; 0.010)</td>
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<td>5.6/5.6</td>
<td>ARG G</td>
<td>LmjF23.0280</td>
<td>16/53</td>
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<td>16.2 (p &lt; 0.006)</td>
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<td>5.1/5.3</td>
<td>MORN</td>
<td>LmjF30.3310</td>
<td>5/15</td>
<td>167&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 (p &lt; 0.016)</td>
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<td>1171</td>
<td>34/32</td>
<td>5.9/5.9</td>
<td>Prostaglandin F2α-synthase</td>
<td>LmjF31.2150c</td>
<td>8/27</td>
<td>81</td>
<td>3.7 (p &lt; 0.001)</td>
</tr>
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<td>5.1/5.3</td>
<td>HSP70 fragment</td>
<td>LmjF28.2770</td>
<td>10/15</td>
<td>78</td>
<td>3.4 (p &lt; 0.002)</td>
</tr>
<tr>
<td>1173</td>
<td>26/7</td>
<td>5.4/7</td>
<td>No ID</td>
<td>LmjF21.1860</td>
<td>13/29</td>
<td>134</td>
<td>2.9 (p &lt; 0.034)</td>
</tr>
<tr>
<td>1174</td>
<td>45/47</td>
<td>5.5/5.6</td>
<td>Argininosuccinate synthase</td>
<td>LmjF23.0260</td>
<td>10/28</td>
<td>90</td>
<td>Not present</td>
</tr>
<tr>
<td>1985</td>
<td>45/48</td>
<td>7.2/8.0</td>
<td>Acyl-CoA dehydrogenase</td>
<td>LmjF06.0880</td>
<td>12/18</td>
<td>64</td>
<td>Not present</td>
</tr>
<tr>
<td>894&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31/31</td>
<td>6.4/6.7</td>
<td>Pteridine reductase</td>
<td>LmjF23.0270</td>
<td>8/33</td>
<td>87</td>
<td>Not present</td>
</tr>
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</table>

<sup>a</sup> Although these scores are below the cut-off, this can be accounted for by modification and/or in vivo proteolytic fragmentation (note the differences between the experimental and predicted M<sub>r</sub>).

<sup>b</sup> These identifications were obtained by MS/MS.

<sup>c</sup> u, unique spot.

<sup>d</sup> Identified in Drummelsmith et al. (13).
in the presence of 1

the second proteins were extracted from wild type cells grown

attempted.

expression (Table II) were excised, and MALDI-TOF identification was

Spot labels are as in Table II. Spots with significant differences in

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/H11003

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the differences noted could in fact be due to exposure to MTX as

MTX-resistant mutants raised the possibility that several of

Two-dimensional Gel Electrophoresis

—

resistance mechanisms have evolved, other responses to MTX

differences noted such as MAT and enolase are found in both

strains, indicating that even in cases where distinct primary resistance mechanisms have evolved, other responses to MTX

are conserved.

Analysis of the Initial Responses of L. major to MTX by

Two-dimensional Gel Electrophoresis—The above analysis of

MTX-resistant mutants raised the possibility that several of

the differences noted could in fact be due to exposure to MTX or

opposed to resistance per se. Such changes in protein expression
could also represent initial steps in the acquisition of

resistance or perhaps an early protective response that allows time for the parasite to express an effective resistance

mechanism. To visualize these changes two analyses were carried out.

In the first, wild type cultures were grown to mid-log phase and

then exposed to 1000× the EC50 of MTX (100 μM) for 24 h. In

the second proteins were extracted from wild type cells grown

in the presence of 1× the EC50 of MTX (100 nM MTX). In both

cases samples were compared with proteins extracted from

wild type cells grown in the absence of drug exposure. Because

this exposure to drug causes a decrease in growth rate, care

was taken to attempt to harvest cells from the same phase of
growth. Representative gels (spanning pH 4–6.7) from this

analysis are shown in Fig. 2. Several differences between spots

that had been identified in previous analyses (Hsp70, Cpn60)

were noted (Fig. 2). Spots whose identity was unknown or

whose positions made matches uncertain were excised from the
gels and subjected to mass spectrometric identification and are

shown in Table II. No trace of PTR1 was seen in these gels,
suggesting that although this is a primary resistance mechanism,
it is not involved in the initial response to MTX. Expression

levels for most of the spots altered in these analyses showed the same trends in both treatments, although this was

not always the case. Interestingly, several of the protein changes noted in this analysis were the same as those seen in

the analysis of resistant mutants (Table I). This was expected in the case of stress response proteins such as heat-shock

proteins, antioxidants, and chaperonins but was somewhat unexpected in the case of MAT.

Overexpression by Gene Amplification and Linkage to Resist-

ance Mediators—ARGG (spots 1168 and 1174) catalyzes the

penultimate step in arginine biosynthesis. This protein is

highly overexpressed in mutant MTX60.2 but not in mutant

MTX60.4 (Table I). Leishmania often uses gene amplification to

respond to drug pressure (17, 18). Southern hybridization

revealed that the ARGG gene was greatly amplified in strain

MTX60.2 compared with the wild type (Fig. 3A). The PTR1

protein overexpression observed in MTX60.2 is also correlated
to amplification of the gene (Fig. 3B). This raised the possibility

that there are multiple amplicons in strain 60.2. However, it

has been known for a long time that ARGG and PTR1 are

physically linked in Leishmania tarentolae (19). Sequencing of

the L. major genome indicated that ARGG and PTR1 genes are

also closely linked on chromosome 23 in this species and are in

fact separated by a segment of only 4.4 kilobases. Therefore,

ARGG is most likely overexpressed due to the proximity of its

structural gene to that of PTR1 and, thus, its inclusion on the

same DNA amplicon. Acel-CoA dehydrogenase overexpression

is seen solely in mutant MTX60.4 (Table I). This overexpres-
sion is also correlated to gene amplification (Fig. 3C). Interest-

ingly, in L. major this gene is 4.6 kilobases downstream of

DHFR-TS on chromosome 6 (www.genedb.org). The DHFR-TS

gene, coding for the primary target of MTX, was also found to

be amplified in this mutant (Fig. 3D).

Prostaglandin F Synthase, MAT, MORN, and Enolase Are

Overexpressed in Both Resistant Mutants—Several proteins

found to be overexpressed in both independent MTX muta-

tants studied. One of these, enolase, is part of the glycolytic

pathway. Overexpression of several glycolytic enzymes, includ-
ing enolase, has been observed to result from various stresses

such as heat shock or osmotic stress (20–22), and thus, its

presence is probably not related specifically to MTX resistance.

There were also several proteins whose presence could not be

accounted for by stress responses. Prostaglandin F synthase

(PGFS) activity has only recently been described in Leishmania

(23), and its biological relevance is unclear. The PGFS protein

was found to be overexpressed 4.0- and 3.7-fold in mutants

MTX60.2 and MTX60.4, respectively, whereas a protein of un-

known function but with several MORN repeat motifs encoded

by the open reading frame LmjF30.3310 but which we will

herein call MORN was overexpressed by 10.4- and 6.6-fold in

the two mutants. This is a protein of unknown function but

which has homologues in several organisms including other

parasites such as Cryptosporidium parvum (www.genedb.org)

and Plasmodium falciparum (24) as well as bacteria, plants,

and mammals. The L. major MORN protein contains 15 puta-
tive MORN repeat motifs. These motifs are found in junctophilins, which are present in junctional complexes between the

plasma membrane and the endoplasmic reticulum (25). They

are also found in phosphatidylinositol-4-phosphate 5-kinases,

which are involved in many cellular processes including cy-

toskeleton assembly, vesicular trafficking, signal transduction,

and DNA synthesis (reviewed in Loijens et al. (26)). Finally,

MAT was overexpressed in both resistant mutants (Table I)

and, interestingly, was also increased in sensitive cells exposed
to MTX (Table II). This suggested to us that it may have an

important role in initial cellular responses to MTX in Leishma-

nia. MAT catalyzes the synthesis of SAM (for review, see Refs.

27 and 28). This metabolite is involved in many essential cel-

lular pathways, such as one-carbon metabolism, sulfuration,

and the biosynthesis of polyamines. In L. major, two copies of

this gene are present within a 7-kilobase region of chromosome

30 and are separated by a hypothetical open reading frame

(www.genedb.org). A similar organization is seen in Leisha-

nia infantum (29).

Southern hybridization indicated that unlike in the cases of

PTR1, DHFR, ARGG, and acyl-CoA dehydrogenase, overexpres-
sion is not mediated by DNA amplification of their respec-
tive genes (Fig. 4A). Therefore, we explored other mechanisms

that could have led to increased levels of these proteins. Al-
though occurring less frequently than gene amplification, RNA

overexpression without gene amplification has been observed

in drug-resistant Leishmania (30). Previously, DNA microar-
rray data and northern hybridization experiments had indicated

LV39 wt  LV39 wt + 100nM MTX

Fig. 2. Representative two-dimensional gels comparing MTX-
sensitive L. major LV39 in the absence and presence of MTX.
Spot labels are as in Table II. Spots with significant differences in
expression (Table II) were excised, and MALDI-TOF identification was
attempted. wt, wild type.
...that the MAT transcript was overexpressed in the strain MTX60.2 (14). Fig. 4B shows that this is also the case in mutant MTX60.4. Using a probe specific for the MORN gene, a similar trend was seen (Fig. 4B). Unexpectedly, however, northern blotting with a PGFS probe revealed that mRNA levels of this gene were unchanged in both mutants and the parent strain (Fig. 4B).

To evaluate the potential role, if any, of the PGFS and MORN proteins in MTX resistance, their respective genes were amplified by PCR and independently cloned into expression vectors, producing plasmids pSPoZEOaPGFS and pSPoZEOaMORN, respectively. These plasmids as well as the vector pSPoZEOa were independently transfected into L. major LV39. The abilities of the vector, PGFS, and MORN transfectants to grow in increasing levels of MTX were assessed. Growth curves showed no differences in MTX resistance levels between these strains (results not shown).

The MAT Protein and MTX Resistance—The MAT gene was similarly amplified by PCR and used to generate pSPoNEo0MAT. Both this plasmid and the vector pSPoNEo0 were independently transfected into L. major LV39. Initially, the ability of vector and MAT transfectants to grow in the presence of increasing levels of MTX was assessed. No difference in MTX EC50 was seen between these strains (Table I), indicating that MAT is not directly responsible for resistance. However, we postulated that MAT overexpression might aid in the acquisition of resistance, since it was found to be overexpressed during initial growth and shock with MTX (Table II) as well as in mutants (Table I). To investigate this possibility we attempted to judge the ease with which the MAT transfectant was able to become resistant to MTX. Because resistance is due to independent genetic events whose effects cannot be averaged, this type of analysis was not done in a quantitative manner. However, common trends might be expected to emerge. After an initial period of growth in relatively low levels of MTX (100 nM), no differences in growth were noted, which made it clear that cells overexpressing MAT were able to grow at much higher levels of MTX that those carrying the vector alone (Fig. 5A). This test was replicated a total of six times. Cells carrying vector alone showed modest increases in EC50 of 5–10-fold. However, 4 of 6 strains overexpressing MAT showed highly elevated EC50 for the drug (80–200×), one did not show elevated resistance, and one was even able to grow well beyond
Whereas triangles—results not shown). The differences in growth characteristics observed among these strains suggest that distinct changes may be at play, and as such, they have been presented independently. We also tested whether MORN or PGFS could be implicated in a similar phenotype, but this did not appear to be the case (results not shown).

These results raised the question as to whether the protective effect conferred on sensitive *Leishmania* cells by MAT was specific for MTX treatment or more general in nature. To investigate this possibility, growth curves for the pSP<sub>NEO</sub><sub>a</sub> and pSP<sub>NEO</sub><sub>a</sub>MAT transfectants were carried out in the presence of increasing concentrations of trivalent antimony. Although in this case, cells were passaged four times, no difference in growth between the two strains was apparent (results not shown).

The effects of MTX treatment and resistance on SAM levels—The implication of MAT in the MTX resistance phenotype prompted us to test whether the observed differences in levels of the MAT protein were reflected in terms of its product, SAM. HPLC analysis of cellular extracts of *L. major* LV39 wild type and MTX60.4 revealed that levels of SAM were approximately doubled in the mutant strain (Fig. 6A, p = 0.034). Overexpression of the MAT protein alone lead to a modest but statistically insignificant increase in SAM levels even though the gene was present in multicopy (Fig. 6B, *vector versus MAT*). However, analysis of a MAT transfectant that had acquired MTX resistance after growth in 0.1 μM MTX and subsequently 10 μM MTX (from Fig. 5B) showed a statistically significant increase in SAM levels (Fig. 6B, *vector versus MAT plus MTX; p = 0.025*).

Thus, an increase in SAM is correlated to the selection and emergence of MTX resistance in *Leishmania*.

**DISCUSSION**

The ability of our proteomic analyses to identify primary resistance mechanisms has already been proven. Indeed, we had identified a primary MTX resistance mechanism, namely overexpression of the PTR1 protein, in an *L. major* LV39 MTX-resistant mutant using a proteomic approach (13). Previously, DNA microarray analyses of mutant MTX60.4 revealed the overexpression of DHFR-TS (14), which is a known primary resistance mechanism to MTX in *Leishmania* (5, 31). Our proteomic analysis of mutant MTX60.4 failed to detect this primary resistance mechanism. However, we identified acyl-CoA dehydrogenase as a unique spot in this mutant. The structural gene for this protein is closely linked (4.6 kilobases) to that of DHFR-TS. Thus, our proteomic approach can lead to the localization of a primary resistance protein even when it is not detected directly. A similar situation was seen in mutant MTX60.2 for ARGG (Fig. 3), the gene for which is adjacent to the *PTR1* gene. *Leishmania* are well known for their ability to overexpress a particular protein by amplifying relatively large sections of chromosomal DNA, and this has been found to be involved in resistance of this organism to several classes of drug (19). This phenomenon could prove to be very useful in the search for resistance mechanisms. The discovery of an overexpressed spot could suggest the presence of an amplicon that could, in turn, point to the presence of a primary resistance mediator in that region. Gene amplification is a frequent event implicated in drug resistance and tumor progression in cancer cells (32, 33), and a proteomic approach as described here could point to proteins that, by virtue of gene proximity, might be implicated in the phenotype being studied.

During the course of the analysis presented here, many differences in protein expression were found, and among those identified were proteins involved in the stress response (chaperons, heat-shock proteins, possibly enolase; Tables I and II). These resistant mutants display slightly longer doubling times that the wild type, suggesting that although they have been passaged for more than a year in high levels of MTX they have not yet fully adapted to these conditions. This observation could reflect either an inability of the resistance mechanisms being expressed to completely abrogate the effects of the drug or a significant energy cost associated with the expression of resistance for which the cells have not yet compensated. Gels of proteins extracted from these mutants grown in the absence of MTX for several rounds show that some of these spots are...
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Although our proteomic efforts to this point had led to primary resistance mechanisms and an appreciation for the role of the stress response in resistance, our goal for these studies is to identify novel elements involved in resistance, its acquisition, or its maintenance. Thus, we turned our attention to overexpressed proteins for which we could not easily provide a rationale. Among these, we chose to concentrate on the PGFS and MORN proteins, which are both overexpressed to a relatively high degree in the two independent MTX mutant strains (Table I) as well as MAT, which is overexpressed in both mutants and was also identified in our analysis of the initial responses to MTX (Tables I and II).

Although gene amplification is a frequent mode of gene and indirectly of protein overexpression, we found that the structural genes for these proteins were not amplified at the DNA level but that MAT and MORN mRNA levels increased in the mutants (Fig. 4). The levels of PGFS transcript appeared to be unchanged in MTX-resistant mutants (Fig. 4). It would therefore appear that a translational mechanism of regulation is at play in the case of PGFS overexpression. Very little is known concerning transcriptional and translational control in Leishmania and related parasites. In several cases, mRNA amounts are often affected by differential mRNA stability conferred by untranslated regions (35–37). In other cases, mRNA levels are unable to account for protein amount (38, 39), but as yet no mechanism for translational regulation has been described. Further study of PGFS expression and also the mechanisms by which MAT and MORN mRNAs are increased might provide new insight into poorly understood post-transcriptional regulatory mechanisms in this important class of pathogen.

No role in MTX resistance for MORN or PGFS was found by overexpressing their cognate genes in sensitive L. major cells. Neither of these proteins was induced upon exposure to MTX, but both were overexpressed in resistant mutants. It is possible that these proteins either do not contribute to a small degree to MTX resistance or are required for partial compensation of growth defects caused by such resistance. The growth curves used to assess the impact of overexpression of these genes on MTX resistance levels might not be sensitive enough to detect small changes. Equally possible is that alone, these proteins do not cause a measurable resistance phenotype. Further experiments, for example by co-transfecting these genes into strains carrying multicopy PTR1 or DHFR-TS and assessing any synergistic effects, might help to elucidate any role they may have in resistance.

Unlike MORN and PGFS, the MAT protein was found overexpressed not only in resistant mutants but upon exposure to MTX. This raised the possibility that MAT overexpression might be required for acquisition of MTX resistance or might have a protective effect that would give the cells time to express more effective resistance mechanisms. The growth curve experiments presented here support this hypothesis. In general, MTX resistance selection takes numerous passages in slowly increasing concentrations of drug. Here, however, cells overexpressing MAT were able to withstand initial drug treatment and quickly became capable of growing in fairly high levels of MTX (Fig. 5).

Although this was a novel and unexpected finding, there is evidence that MTX can affect SAM levels. The folate and SAM pathways intersect through the enzyme methionine synthase, which regenerates methionine from homocysteine, a metabolite produced via S-adenosylhomocysteine through the use of SAM in various transmethylation reactions (for review, see Refs. 40 and 41; Fig. 7). Methionine synthase requires 5-methyltetrahydrofolate (5-MeTHF) as a methyl donor. In mammalian tissues, the presence of MTX inhibits DHFR-TS, which leads to increases in dihydrofolate (DHF) pools (42). This phenomenon is also true in L. major. MTX reduces levels of 5,10-methylenetetrahydrofolate (5,10-MeTHF) (43), and high levels of DHF reduce the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (44). Thus, the availability of this cofactor to methionine synthase is diminished, which should result in an imbalance of SAM/S-adenosylhomocysteine in the cell. This balance is critical to several metabolic functions in the cell. Depletion of the SAM pool has been shown to be a cause of MTX-associated hepatotoxicity during cancer treatment (42). MTX treatment in cancer and psoriasis patients has in fact been shown to increase levels of homocysteine (45) and decrease cellular methylation (46).

Our proteomic analyses suggest that MAT levels are increased early during exposure to MTX (Fig. 2, Table II) and together with metabolite analyses (Fig. 6) suggest that increased MAT and SAM facilitate the emergence of MTX resistance. SAM levels are significantly increased in a mutant strain highly resistant to MTX (Fig. 6A) even when compared with MAT transfectants (Fig. 6B), implying that further changes occur during resistance acquisition and adaptation that act with MAT overexpression to increase SAM levels. There are numerous candidates for these changes. For example, mammalian MAT activity is regulated by phosphorylation (47), which could lead to quick changes in MAT activity. Post-translational modification of MAT may also exist in Leishmania. Furthermore, increased levels of methionine synthase activity might be required to provide substrate for the MAT enzyme (Fig. 7). The predicted methionine synthase enzyme in L. major is quite large (138.5 kDa); thus, it is unlikely that it would have been visualized by the two-dimensional gel method employed here. However, transcriptomic analysis of strain MTX60.4 did not see an increase in methionine synthase mRNA (14). Another potential mechanism to explain increased intracellular SAM levels is increased transport. In the related parasite Trypanosoma brucei, SAM uptake was found to account for 50% of the intracellular pool (48), suggesting that exogenous SAM is the primary source of this metabolite. Leishmania has SAM transport activity (49), but the identity of the transporter is unknown.

2 J. Drummelsmith and M. Ouellette, unpublished information.

3 J. Drummelsmith, I. Girard, and M. Ouellette, unpublished information.
An aid to dissecting the role of SAM in MTX resistance would be to greatly perturb SAM levels. Unfortunately, given that MAT overexpression had minimal effects on SAM levels (Fig. 6), it is not likely that disrupting some or all of the MAT genes would provide the desired background. The complex nutritional requirements for *in vitro* culture of *Leishmania* preclude growth in a SAM-depleted medium, and since the transporter is unknown we cannot perturb this activity. We are, however, in the process of attempting to identify this transporter, which may allow us to further characterize the effect of SAM levels on MTX resistance.

MAT overexpression may contribute to MTX resistance by compensating for some of the deleterious effects of MTX itself or might have a more direct role in resistance. Either way, the implication of MAT and SAM in MTX resistance could prove to have a profound effect on drug design. MTX, for instance, has thus far proven ineffective in the treatment of leishmaniasis. The high level of drug required to affect the parasite *in vivo* is toxic for the host, and on top of this the pathogen quite easily acquires resistance. A combination of inhibitors of key enzymes of the folate and SAM pathways could prove to have a synergistic effect, thereby decreasing the levels of MTX or other antifolates required for treatment. In addition, inhibiting a process required for the acquisition of resistance, such as increased MAT activity or SAM uptake, might significantly reduce or even eliminate its development. These results could also be applicable to mechanisms of MTX resistance in cancer cells.

There have been comparatively few global analyses of the effects of antimicrobial treatment and resistance. In this analysis we have shown that a proteomic approach can directly or indirectly reveal primary resistance mechanisms. It also shows the potential of such approaches to discover subtle, yet potentially important protein and metabolic changes involved in drug resistance.

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Differential Protein Expression Analysis of \textit{Leishmania major} Reveals Novel Roles for Methionine Adenosyltransferase and S-Adenosylmethionine in Methotrexate Resistance

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