Modulation of gene expression in *Leishmania* drug resistant mutants as determined by targeted DNA microarrays

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ABSTRACT

In the protozoan parasite *Leishmania*, drug resistance can be a complex phenomenon. Several metabolic pathways and membrane transporters are implicated in the resistance phenotype. To monitor the expression of these genes, we generated custom DNA microarrays with PCR fragments corresponding to 44 genes involved with drug resistance. Transcript profiling of arsenite and antimony resistant mutants with these arrays pinpointed a number of genes overexpressed in mutants, including the ABC transporter *PGPA*, the glutathione biosynthesis genes γ-glutamylcysteine synthetase (*GSH1*) and the glutathione synthetase (*GSH2*). Competitive hybridisations with total RNA derived from sensitive and methotrexate resistant cells revealed the overexpression of genes coding for dihydrofolate reductase (*DHFR-TS*), pteridine reductase (*PTR1*) and *S*-adenosylmethionine synthase (*MAT2*) and a down regulation of one gene of the folate transporter (*FT*) family. By labelling the DNA of sensitive and resistant parasites we could also detect several gene amplification events using DNA microarrays including the amplification of the *S*-adenosyl homocysteine hydrolase gene (*SAHH*). Alteration in gene expression detected by microarrays was validated by northern blot analysis, while Southern blots indicated that most genes overexpressed were also amplified, although other mechanisms were also present. The microarrays were useful in the study of resistant parasites to pinpoint several genes linked to drug resistance.

INTRODUCTION

Leishmania is a protozoan parasite distributed worldwide for which we have limited means to control its spread. No vaccines are available and chemotherapy relies mainly on pentavalent antimony containing drugs, although in endemic regions with high antimony resistance the palliative miltefosine appears very useful (1,2). Unresponsiveness of parasites to antimonials is a serious health problem and resistance has now reached epidemic proportions in parts of India (3). Numerous resistance mechanisms have been uncovered from *in vitro* studies that were related to either transport defect, amplification of genes coding for drug targets, or altered metabolism (reviewed in 4). Antimony is given as pentavalent antimony (SbV) sugar conjugates but the metal is likely to be reduced *in vivo* to the trivalent form (SbIII). Therefore, a loss of this reduction activity could lead to resistance (5). The route of entry of SbV is not known, but reduced uptake could also lead to resistance and cells selected for SbIII resistance show a reduction in the steady-state accumulation of the metal (6). All antimonite and arsenite resistant *Leishmania* mutants share a metabolic signature: a significant increase in the intracellular trypanothione (TSH) level (7,8). TSH is a glutathione–spermidine conjugate (9), which is formed following several enzymatic steps (Fig. 1B). Overexpression of some of the genes in this pathway can lead to an increase in the intracellular level of TSH (10,11). It is proposed that an Sb–TSH conjugate is formed inside the parasite cell and the complex is either extruded outside the cell by an ATP-dependent efflux system (Fig. 1D) or sequestered within a vacuole by the intracellular ABC transporter PGPA (12). Other genes not related to the metabolic pathways shown in Figure 1D can also contribute to Sb resistance (13,14).

Similarly, resistance to the model antifolate drug methotrexate (MTX) in *Leishmania* can be explained by several mechanisms (Fig. 1A) including reduced uptake mediated by the modulation of the expression of a series of folate

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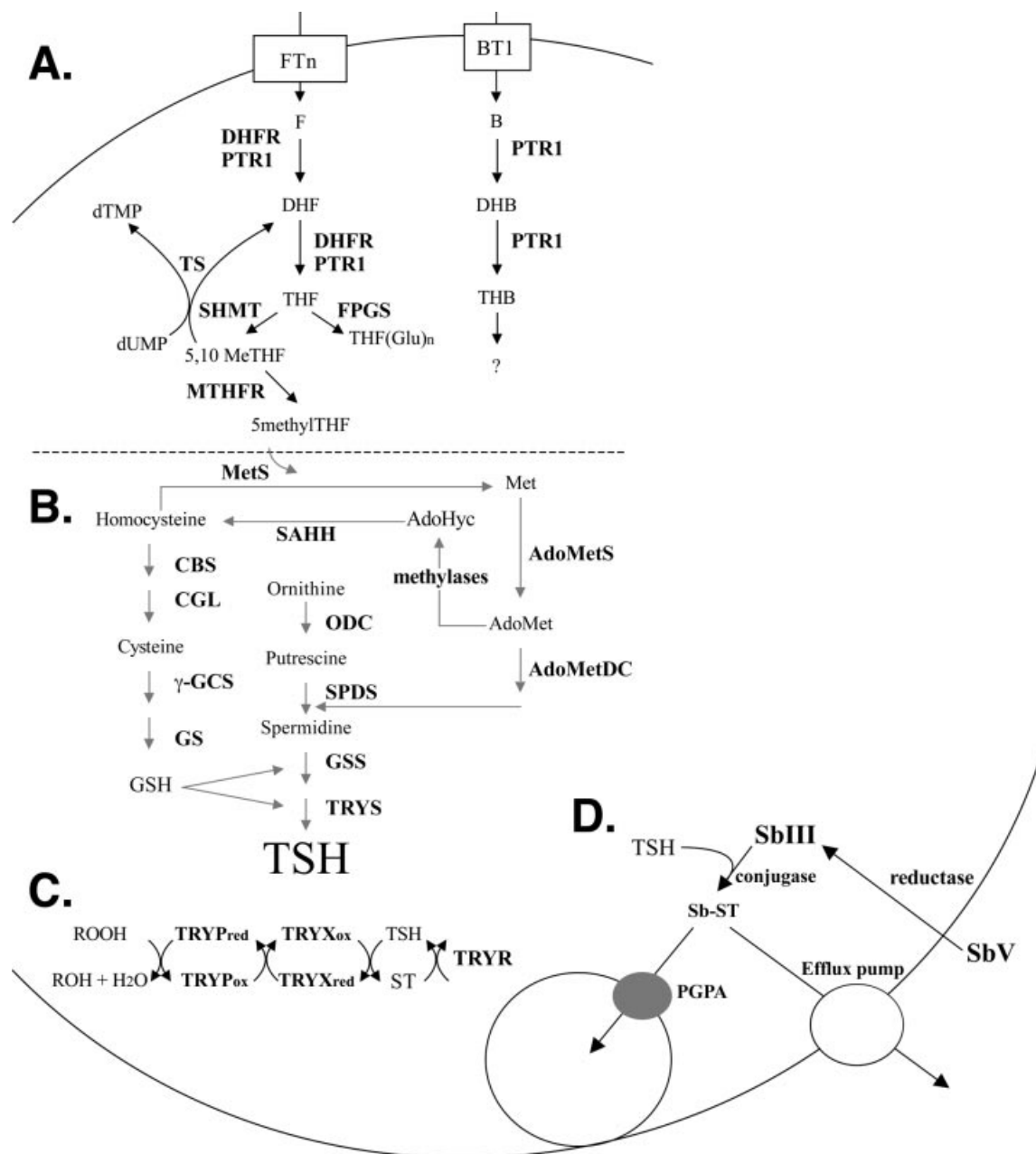Régis Peytavi, Centre de Recherche en Infectiologie du CHUL

Figure 1. Biochemical pathways of TSH and folate metabolism in *Leishmania*. (**A**) Folate and pterin metabolic pathways. (**B**) TSH biosynthetic pathway including selected aspects of cysteine and methionine pathways. (**C**) TSH-dependent peroxide reduction pathway. (**D**) Model for antimony resistance in *Leishmania*. All annotated genes involved in these pathways and their abbreviations are listed in Table 1 and were arrayed on DNA microarrays. F, DHF, THF, B, DHB and THB correspond to Folate and Biopterin and their Dihydro and Tetrahydro forms.

transporters (FT) (15,16). This is often compensated for by an overexpression of the biopterin transporter BT1 (17,18). Amplification of the target gene dihydrofolate-reductase-thymidylate synthase *DHFR-TS* (19) or of the pterin reductase *PTR1* that has folate reductase activity (20,21) are two other frequent MTX resistance mechanisms. Modulation in the level of polyglutamylation of folates and MTX was also shown to contribute to MTX resistance in this parasite (22).

Several different mechanisms are responsible for resistance to a drug and multiple resistance mechanisms may co-exist in the same cell and act either additively or synergistically. The

multiplicity of resistance mechanisms to a certain class of drug justifies an approach allowing the simultaneous analysis of the expression of several genes. DNA microarrays are well suited for that purpose. The utility of this technology has been demonstrated for drug responses and resistance mechanisms in microorganisms and cancer cells (23–29), and could allow the identification of novel pathways potentially involved in resistance. DNA microarrays have recently been used to investigate stage-specific gene expression in *Leishmania* (30–32). Since drug resistance in this parasite is often linked to alterations in gene expression due to either gene amplification

(33,34), gene deletion (15) or other mechanisms (11), we chose DNA microarrays since it is well suited for analysing drug resistant mutants. The *Leishmania* genome-sequencing project is near completion (www.genedb.org) and most of the genes involved in the TSH biosynthetic pathway or in the folate metabolism pathway shown in Figure 1, are now part of the public domain. We have generated custom microarrays with DNA fragments corresponding to genes related to these two metabolic pathways as well as the linked pathways of cysteine and methionine metabolism (Fig. 1). We present a study of the expression of these genes in *Leishmania* mutants selected for metal or MTX resistance.

MATERIALS AND METHODS

Cell lines and cultures

The *Leishmania tarentolae* wild-type cell line TarIIWT, the arsenite resistant mutant TarIIAs50.1 and the antimonite resistant mutant TarIISbIII400.1 have been described previously (8,35). The *Leishmania major* LV39 strain was selected step by step for MTX resistance in M199 medium. This process generated two independent clones named MTX60.2 and MTX60.4, both resistant to 60 μM of MTX (EC_{50} of wild-type cell is 100 nM) (36). The growth properties of the different wild-type species as well as of the drug resistant mutants were carefully monitored to ensure that cells were collected in the same growth phase.

Total RNA preparation and labelling

RNA was isolated from 10^8 *Leishmania* cells during the mid-log growth phase using Trizol reagent (Invitrogen) as described by the manufacturer. The RNA preparation was treated with DNase I (Ambion) to avoid any genomic contamination and purified further using the RNeasy kit (Qiagen). Purified total RNA was quantified spectrophotometrically at 260 and 280 nm, and ratios$_{A260 nm/A280 nm}$ between 1.9 and 2.1 were considered acceptable. RNA integrity was assessed by visualising the ribosomal bands on a 1% TAE agarose gel. RNA was converted to fluorescent cDNA probes by direct incorporation of Cy3/Cy5-linked dUTP (Amersham Pharmacia) mediated by the Superscript II reverse transcriptase (RT) (Invitrogen) according to the supplier recommendations. For each labelling reaction, 15 μg of purified RNA were spiked with two exogenous RNAs (*CAB1* at 2 pg/μl and *NAC1* at 5 pg/μl from *Arabidopsis thaliana*; Stratagene) added as references to adjust for variations in the incorporation efficiency of the two dyes and for differences in first strand cDNA synthesis reactions. Briefly, total RNA was first denatured by heating at 95°C for 5 min and the reverse transcription was performed at 42°C for 3 h with the RT enzyme, random nonamers and anchored oligo(dT)$_{25}$. The RNA was then degraded by an RNase treatment and the cDNA was purified using the Qiaquick PCR purification kit (Qiagen). The synthesised cDNA was quantified at 260 nm while the Cy3 and Cy5 fluorophor incorporations were monitored at 550 and 650 nm, respectively. Aliquots of cDNA containing 20 pmol of incorporated dye were lyophylised and stored at –80°C until use.

DNA labelling

Total DNA was isolated using the DNAzol technique (Invitrogen). Thirty micrograms were digested with Sau3AI, purified using Microcon 30 filter columns (Millipore) then quantified at 260 nm. Two micrograms of digested DNA were labelled by direct incorporation of Cy3/Cy5-linked dUTP using a random priming method with a highly concentrated Klenow enzyme (NEB). In addition to the genomic template DNA, each labelling reaction contained random hexamers, 120 μM of each of dATP, dGTP and dCTP, 60 μM dTTP, 60 μM Cy3 or Cy5-dUTP in a Tris-MgCl$_2$ buffer. Reactions were performed at 37°C for 2 h. The DNA preparation was then purified with Microcon 30 filter columns and quantified using procedures similar than those described above for labelled cDNAs. Aliquots containing 20 pmol of incorporated dye were lyophylised and stored at –80°C until use. Hybridisations were performed with the same protocol used for labelled cDNA.

Generation of customised DNA microarrays

When this work was initiated, not all genes found in Table 1 were known and some were obtained by PCR homology cloning, but with the *Leishmania* genome-sequencing project advancing steadily (and now almost completed) it has been possible to obtain most genes from *in silico* screening. The remaining exception is the cystathionine γ-synthase (Table 1). Prior to genome data, several genes were cloned using degenerated oligonucleotides derived from the conserved sequences of aligned homologues of these proteins. For all 44 genes, depending on the size and sequence, one to three pairs of primers were synthesised and used to amplify 500 bp fragments from *Leishmania* genomic DNA. These PCR fragments (one to three fragments per gene, see Table 1) were cloned in the pGEMt-easy vector system (Promega), which contains the sequences necessary for amplification using T7 and SP6 primers. The identity of all clones was confirmed by sequencing. For layering on DNA arrays, each cloned fragment was amplified by PCR using T7 and SP6 primers, purified on a Qiaquick PCR column (Qiagen) and quantified using the Picogreen reagent (Molecular Probes). Aliquots containing 2 μg of each PCR fragment were transferred to a 384 well plate, dried and subsequently resuspended in 5 μl of a 50% DMSO solution. The DNA was denatured by heating at 95°C for 15 min and printed onto CMT-GAPS II slides (Corning) using an SDDC-2 Arrayer (Virtek) in a 70% humidity constant atmosphere. Slides were UV cross-linked at 200 mJ then baked at 80°C for 2 h. Printing quality controls of each slide batch include staining with ToTo-3 iodine (Molecular Probes) and hybridisation with Cy3-labelled random oligonucleotides (Spotcheck, Genetix).

Prehybridisation/hybridisation and statistical analysis

Slides were prehybridised at 42°C for 60 min in 5× SSC, 0.1% SDS, 1% BSA, 100 μg/ml ssDNA. For hybridisation, Cy3 and Cy5 labelled cDNAs were first resuspended and mixed together in the hybridisation solution (50% formamide, 5× SSC, 0.1% SDS, 25 μg/ml ssDNA, 460 μg/ml tRNA) preheated to 42°C. The mixture was heated at 95°C for 5 min, cooled slowly to 42°C and applied under a coverslip on the array. Hybridisation was performed at 42°C for at least 12 h

Table 1. Genes represented in custom DNA microarrays

Genes	Protein	Access. #	Species used	# PCR Fragment
ABCTP1	ABC transporter	AC005766	*L.major*	2
Actin	Actin[a]	L16961	*L.major*	1
MAT2	*S*-Adenosylmethionine synthase (AdoMetS)	AF179714	*L.donovani*	2
AdoMetDC	*S*-Adenosylmethionine decarboxylase	U20091	*L.donovani*	2
α-*tubulin*	Alpha-tubulin[a]	U09612	*L.donovani*	2
Amastine	Amastigote-specific gene	AF195531	*L.donovani*	1
β-*tubulin*	Beta-tubulin[a]	X93566	*L.major*	3
BT1	Biopterin transporter 1	AF244919	*L.major*	3
CBL	Cystathionine β-lyase	AL359773	*L.major*	3
CBS	Cystathionine β-synthase	AF256080	*L.tarentolae/L.major*	2
CGS	Cystathionine γ-synthase	PCR homology	*L.major*	1
DHFR-TS	Dihydrofolate reductase thymidylate synthase	X51733	*L.major*	3
FPGS	Folylpolyglutamate synthase	AF284554	*L.tarentolae*	2
FTn	Folate transporters	AF084469	*L.donovani*	3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase[a]	AF047497	*L.major*	1
GSH2	Glutathione synthetase (GS)	AL356246	*L.major*	2
GSH1	γ-Glutamylcysteine synthetase (γ-GCS)	Y10049	*L.tarentolae/L.major*	3
GSS	Glutathione spermidine synthase	AQ849191	*L.major*	1
HSP70	Heat shock protein 70	X13441	*L.major*	2
L4468.01	Transporter, pot. ATP-dep. permease precursor	AL121864	*L.major*	3
L673.01	Transporter, MDR copy 1, Pgp	AL135898	*L.major*	3
L673.02	Transporter, MDR copy 2, Pgp	AL135898	*L.major*	1
L8329.03	Transporter, bacterial type ABC transporter	AL446004	*L.major*	3
MDR	Transporter, multiple drug resistance related	U63320	*L.donovani*	2
METS	Methionine synthase	AQ848249	*L.major*	1
METH	Methionine synthase cobalamine dependant	Chr7-11_tmp.570[b]	*L.major*	1
METE	Methionine synthase cobalamine independent	AQ845725	*L.major*	1
ODC	Ornithine decarboxylase	AF159564	*L.tarentolae/L.major*	3
ORF SbV	Na stibogluconate resistance protein	AF047351	*L.tarentolae*	2
ORF19	Cysteine-leucine rich protein gene	AF262948	*L.tarentolae*	2
PAH	Phenylalanine hydroxylase	CHR28_tmp.72[b]	*L.tarentolae*	2
PFK1	Phosphofructokinase I[a]	AY029213	*L.major*	2
PGPA	p-Glycoprotein A (ABC transporter)	X17154	*L.tarentolae/L.major*	1
PGPE	p-Glycoprotein E, (ABC transporter)	L29485	*L.tarentolae*	2
PTR1	Pterine reductase 1	L01699	*L.major*	1
SAHH	*S*-Adenosylhomocysteine hydrolase	AQ902547	*L.major*	2
SHMT	Serine hydroxymethyltransferase	AL138973	*L.major*	2
SOD A	Fe-dep. superoxide dismutase A	AF003964	*L.major*	2
SOD B	Fe-dep. superoxide dismutase B	AF003963	*L.major*	1
SPDS	Spermidine synthase	AF298195	*L.donovani*	1
TRYR	Trypanothione reductase	Z23135	*L.donovani*	3
TRYP	Tryparedoxin peroxidase	AF069386	*L.major*	1
TRYX	Tryparedoxin	AQ849312	*L.major*	1
TRYS	Trypanothione synthetase	AL133443	*L.major*	1

[a]Housekeeping and structural genes used as controls.
[b]Temporary systematic name in *Leishmania* Gene databank.

under immersion (Corning chambers). Slides were first washed at 55°C for 5 min in 1× SSC, 0.03% SDS. Subsequent washes were at room temperature in 0.2× SSC and 0.05× SSC, each for 5 min under agitation. Slides were dipped in water and 99% ethanol then dried by centrifugation. Scanning was performed using a confocal 4000XL scanner (GSI Lumonics). Spots were manually examined and those of low intensity or presenting unusual shape were ignored. Data were analysed using QuantArray software. Four different RNA preparations of each mutant and their corresponding wild-type cell line were analysed, corresponding to two dye-swapping experiments. Data were corrected for local background, and ratios were adjusted using the two spiked RNAs as references. Statistical significance was calculated by the Student's test using StatView software, and the two spiked RNAs as controls. A cut-off of two for significant differences was chosen although we do agree that smaller differences may

also be of interest when statistically significant. Genes with consistent altered expression (at least 2-fold differences) were further studied by Southern and northern blot analysis, while following standard protocols (37) to confirm results obtained by DNA microarrays. Blots were hybridised to the same PCR DNA fragments layered on the arrays, and the hybridisation intensity was derived by densitometry analysis using an alphaImager 2000 (Alpha Innotech) with the software Alpha Ease version 4, and also a BioImage Visage 100S and compared with the signal intensity of blots hybridised with an α-tubulin probe.

RESULTS

Generation of DNA microarrays

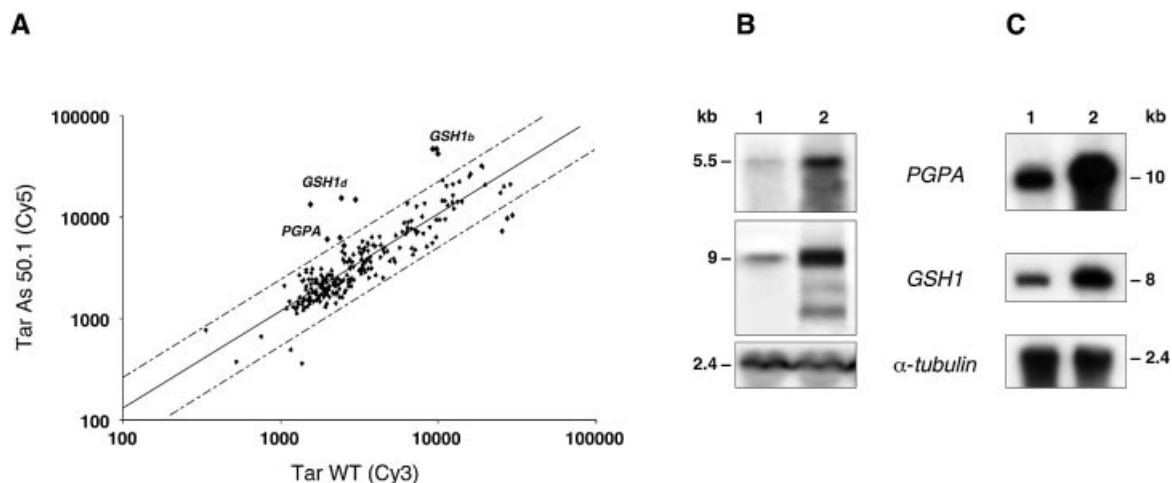The strategy to generate the DNA microarrays is laid out under Materials and Methods. Our goal was to build DNA

Figure 2. Gene expression analysis of the *L.tarentolae* As50.1 mutant as determined by DNA microarrays and northern blot analysis. (**A**) Scatter plot of hybridisation intensities between TarAs50.1 (Cy5) and wild-type *L.tarentolae* cells (Cy3). The expression of genes represented by dots within the dashed lines are considered as similar in the two tested strains. Dashed lines indicate 2-fold differences and genes whose expression differ significantly are indicated. (**B**) Confirmation of DNA microarray results by northern blot analysis showing that *PGPA* and *GSH1* were overexpressed in the same mutant. Presumably, for genes grossly overexpressed, microarray results can only be qualitative as the signals are rapidly saturated. An α-tubulin hybridisation was performed to monitor RNA and DNA loading. For each figure we show one representative of several control gels used for the various genes. For quantification see Table 2. (**C**) Southern blot analysis to test whether increased RNA expression is mediated, at least in part, by gene amplification. The DNA of the parasites was digested with HindIII. 1, *L.tarentolae* wild-type cell; 2, TarII As50.1. Sizes were determined using the 1 kb Plus DNA ladder and the 0.24–9.5 kb RNA ladder from Invitrogen.

microarrays containing most of the genes known to be implicated in antimony and MTX resistance and to complete these arrays with genes known to be involved in TSH and folate metabolic pathways including some involved in cysteine and methionine metabolic pathways (Fig. 1) as well as a number of 'housekeeping' genes. The 44 genes investigated, with their corresponding accession numbers, are listed in Table 1. Each PCR fragment was spotted in triplicate on the array. Arrays were hybridised to Cy3 and Cy5 labelled cDNA from the same sample preparation and hybridisation was found to be uniform (not shown). Once the spotting, hybridisation and washing conditions were optimised, and hybridisation signals could be consistently reproduced, the arrays were used for the parallel analysis of gene expression in *Leishmania* drug resistant mutants.

Analysis of metal resistant mutants by DNA microarrays

Our previous work on metal resistance in *Leishmania* has highlighted the importance of TSH biosynthesis and the increased expression of the ABC transporter PGPA (38). All the known genes of the TSH metabolic pathway were thus included on the array. To validate the use of our custom DNA microarrays, we first studied the arsenite resistant mutant TarIIAs50.1, a mutant in which *PGPA* is known to be overexpressed (35) and in which the level of TSH is known to be increased (7). Most genes were equally expressed between wild-type and TarIIAs50.1, except for γ-glutamylcysteine synthetase (*GSH1*) ($P < 0.0001$), the gene product of which catalyses the rate limiting step of glutathione biosynthesis, and *PGPA* ($P < 0.0177$), which according to this (Fig. 2) and other experiments were found to be overexpressed in the mutant. The same hybridisation profile was obtained whether the mutants were grown in the presence of drugs or in the absence of drugs for one passage (result not shown). It is also worth noting that arrays containing mostly genes derived from

L.major (Table 1) are suitable for analysing the expression of genes of *L.tarentolae*. DNA microarray expression results were confirmed by northern blot analysis (Fig. 2B). While microarrays suggested a 2–3-fold overexpression, quantification of northern blots indicated that *GSH1* was overexpressed by 11-fold and *PGPA* by 15-fold (Table 2). However, when the microarrays were scanned with a lower laser power, the fold difference values were higher and closer to northern blot values but weaker signals were lost. Thus, in some experiments, our custom microarrays became rapidly saturated for genes highly overexpressed.

The antimonite resistant mutant *L.tarentolae* TarIISbIII400.1 has been described previously. It is already known that *GSH1* and *PGPA* are overexpressed (8) in this strain, but revertants of these cells having lost the *GSH1* containing amplicon nonetheless had higher TSH levels than wild-type cells (8), suggesting that another gene of the TSH biosynthetic pathway might be increased. Analysis of the competitive hybridisation between labelled wild-type and TarIISbIII400.1 cDNAs on our custom DNA microarrays indeed confirmed that *PGPA* and *GSH1* ($P < 0.0079$ and $P < 0.0001$) were overexpressed, but we also found that the glutathione synthase (*GSH2*) gene, the second step in glutathione biosynthesis (Fig. 1), was also overexpressed ($P < 0.0001$) (Fig. 3). The *Leishmania GSH2* gene has not yet been described but was revealed by the ongoing *Leishmania* genome project (accession number AL356246). The gene product shows 35 and 38% identity with the GSH synthase proteins of yeast and human (accession numbers Y138804 and BC007927), respectively. These array results were confirmed by northern blot analysis (Fig. 3B). In this mutant a better correlation was observed between microarrays and northern blots to estimate the fold increase in gene expression, although saturation was evident for the highly overexpressed genes (Table 2).

Table 2. Correlation between microarray results, RNA expression and DNA copy number

| Strains	Genes	Average fold increase compared to wild-type cells		
		Microarrays	Northern	Southern
TarAs50.1	*GSH1*	3.0 ± 0.2*[a]	10.9	12.9
	PGPA	2.0 ± 0.3**[b]	15	3.2
TarIISbIII400.1	*GSH1*	11.5 ± 0.9*	44.7	$3.9 (1.9 \pm 0.1$*$)$[c]
	PGPA	2.2 ± 0.3**	2.9	$12.7 (6.2 \pm 0.5$*$)$[c]
	GSH2	2.8 ± 0.3*	3.6	$0.8 (1.0)$[c]
	SAHH	0.7 ± 0.1**	1.1	$2.2(2.8 \pm 0.1$*$)$[c]
LV39 MTX60.2	*PTR1*	25.3 ± 4.0*	20	17.2
	MAT2	4.1 ± 0.2*	4	1.1
LV39 MTX60.4	*FT*	0.6*	0.12[d]	0[e]
	DHFR	2.8 ± 0.1*	11.5	7.1

[a]*P value <0.0001.
[b]**P value <0.02.
[c]Values within parenthesis correspond to gene amplification as determined using DNA microarrays.
[d]This signal is probably coming from one of the several other FT members.
[e]Some of the copy number of the FT family remains unchanged but other genes, indicated by asterisks, were deleted.
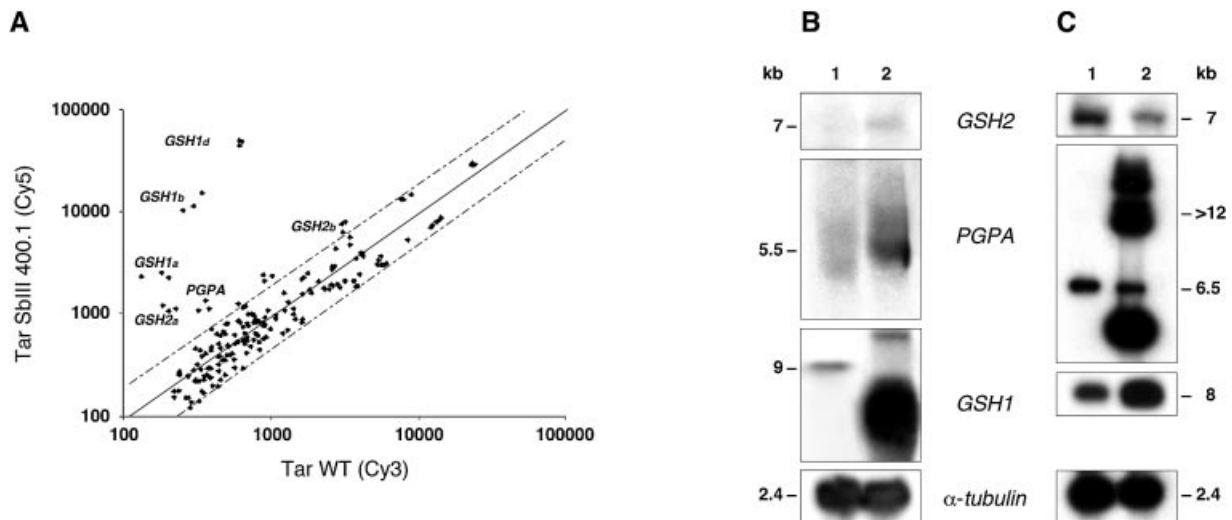
Figure 3. Gene expression analysis of the *L.tarentolae* TarIISbIII400.1 mutant as determined by DNA microarrays and northern blot analysis. (**A**) Scatter plot of hybridisation intensities between Tar SbIII400.1 (Cy5) and wild-type *L.tarentolae* cells (Cy3). Dashed lines indicate 2-fold differences. (**B**) Confirmation of DNA microarray results by northern blot analysis showing that *PGPA*, *GSH1* and *GSH2* are overexpressed. An α-tubulin hybridisation was performed to monitor RNA and DNA loading. For each figure we show one representative of several control gels used for the various genes. For quantification see Table 2. (**C**) Southern blot analysis to test whether increased RNA expression is mediated by gene amplification. The DNA of the parasites was digested with HindIII (blots hybridised to *GSH2* and tubulin probes); EcoRI (blot hybridised to a *GSH1* probe); and BamHI (blot hybridised to a *PGPA* probe). 1, *Leishmania tarentolae* wild-type cell; 2, TarIISbIII400.1. Sizes were determined using the 1 kb Plus DNA ladder and the 0.24–9.5 kb RNA ladder from Invitrogen.

Analysis of *L.major* mutants selected for MTX resistance by DNA microarrays

Our success in analysing metal resistance using DNA microarrays has prompted us to investigate MTX resistant mutants of *L.major* that had not been systematically characterised previously. The first mutant analysed was *L.major* MTX60.2 and DNA microarrays have revealed that *PTR1* ($P < 0.0001$) as well as *PGPA* ($P < 0.0001$) (Fig. 4A) were overexpressed. *PTR1* is a known MTX resistance gene (39,40) and its overexpression was confirmed by northern blots (Fig. 4B). In addition to *PTR1*, the microarrays suggested that the *S*-adenosylmethionine synthase gene (*MAT2*), recently described in *L.infantum* (41) and involved in a

critical step in methionine metabolism (Fig. 1), was also overexpressed ($P < 0.0001$). This microarray result was validated by northern blot analysis (Fig. 4B). As for mutant TarIISbIII400.1 there was a good correlation between the fold expression determined by DNA microarrays and northern blots (Table 2).

The analysis of the mutant *L.major* MTX60.4 using DNA microarrays has revealed a different set of genes whose expression was altered compared with the *L.major* MTX60.2 mutant. Indeed, *DHFR-TS* was overexpressed ($P < 0.0001$) while the expression of one of the FT genes was down regulated ($P < 0.0001$) (Fig. 5A). These expression results were confirmed by northern hybridisations where *DHFR-TS* RNA was increased (Fig. 5B). Several messengers hybridised
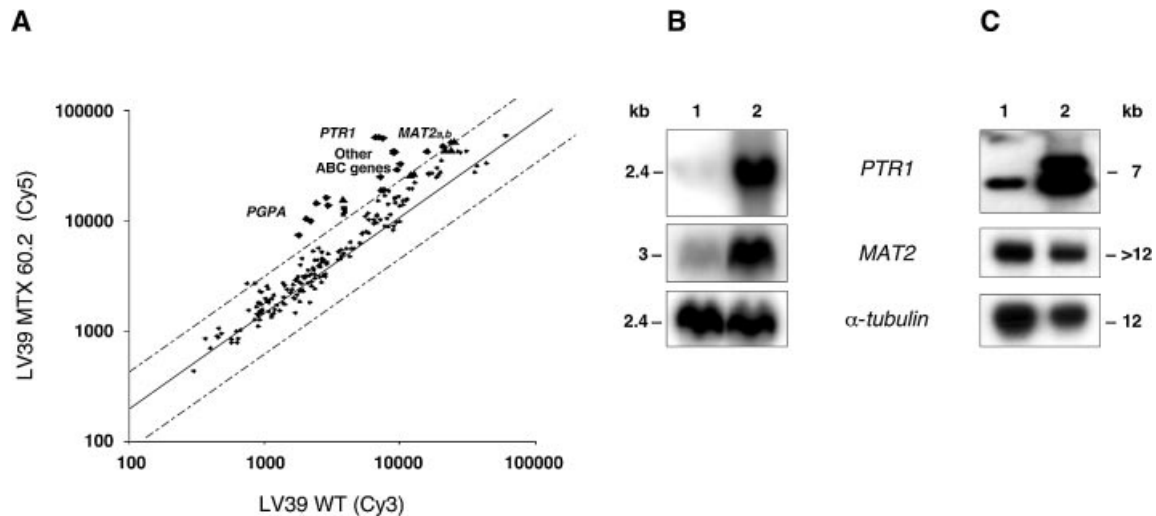
Figure 4. Gene expression analysis of the *L.major* LV39 MTX60.2 mutant as determined by DNA microarrays and northern blot analysis. (**A**) Scatter plot of hybridisation intensities between LV39 MTX60.2 (Cy5) and wild-type *L.major* LV39 cells (Cy3). Dashed lines indicate 2-fold differences. A number of fragments spanning ABC transporter genes cross-hybridised to the overexpressed PGPA. (**B**) Confirmation of DNA microarray results by northern blot analysis showing overexpression of *PTR1* and *MAT2*. An α-tubulin hybridisation was performed to monitor RNA and DNA loading. For quantification see Table 2. (**C**) Southern blot analysis to test whether increased RNA expression is mediated by gene amplification. The DNA of the parasites was digested with HindIII except for the blot hybridised to the *PTR1* probe where the DNA was digested with SacI. 1, *Leishmania major* wild-type cell; 2, *L.major* LV39 MTX60.2. Sizes were determined using the 1 kb Plus DNA ladder and the 0.24–9.5 kb RNA ladder from Invitrogen.

to an FT probe, but the expression of at least one message was significantly down-regulated (Fig. 5B, marked with an asterisk). The down-regulation of FT expression is translated into a marked decrease of folate/MTX transport in mutant cells (Fig. 5D). There was a lack of correlation in the estimation of expression levels of *DHFR-TS* between microarrays and northern blots (Table 2).

Gene overexpression is mediated by gene amplification and other mechanisms

Gene overexpression in *Leishmania* is often due to gene amplification (33,34). We have tested whether DNA microarrays could be useful to detect gene amplification events by carrying out competitive hybridisations with digested total DNAs derived from sensitive cells and resistant mutants labelled with Cy3 and Cy5, respectively. Results are presented for the mutant TarIISbIII400.1 in which three genes, *PGPA*, *GSH1* and *GSH2* were found to be overexpressed (Fig. 3). While *PGPA* was amplified, and the copy number of *GSH1* was also slightly increased, *GSH2* was not amplified (Fig. 6). These array results were confirmed by Southern blot analysis (Fig. 3C) (Table 2). While *GSH1* is amplified, the increase in copy number is not commensurate with the increase in RNA overexpression, suggesting that another mechanism than gene amplification also contributes to *GSH1* overexpression. Moreover, since *GSH2* is overexpressed without gene amplification (Fig. 3), we tested whether all the other genes found to be overexpressed in the other resistant mutant studied were amplified or not using Southern blots. In mutant TarIIAs50.1, overexpression of both *GSH1* and *PGPA* is correlated to amplification of their respective genes (Fig. 2C). In the MTX resistant mutant *L.major* MTX60.2, the overexpression of *PTR1* is due to gene amplification (Fig. 4C). *PGPA* is closely linked to *PTR1* on the same locus (42,43) and

thus the overexpression of *PGPA* observed by microarray (Fig. 4) is likely due to the co-amplification of its gene. In contrast to *PTR1*, the *MAT2* gene in *L.major* MTX60.2 is overexpressed (Fig. 4B) but its gene is not amplified (Fig. 4C). In mutant *L.major* MTX60.4, the overexpression of *DHFR-TS* is due to the amplification of its gene (Fig. 5C). In the same mutant, one of the FT was down-regulated. We probed the DNA with a FT probe recognising several gene members (15) and observed a deletion event in the *FT* locus (Fig. 5C, marked with an asterisk). In general, a higher copy number of a gene will lead to more RNA but the correlation is only qualitative (Table 2). Copy number and RNA expression correlated best for *PTR1* in LV39MTX60.2 and for *GSH1* in TarIIAs50.1 (Table 2).

In our analysis of DNA amplification events using microarrays, we found in the mutant TarIISbIII400.1 (Fig. 6A), a new gene corresponding to *S*-adenosyl homocysteine hydrolase (*SAHH*) (Fig. 1), a putative drug target in *Leishmania* (44). This array result was validated by Southern blot analysis (Fig. 6C). This result was in contrast to the microarray results, however, which did not show that the *SAHH* gene was overexpressed. A northern blot using a *SAHH* probe revealed that in this mutant there were two RNA species, as opposed to one in the wild-type cell (Fig. 6B). The fold increase in expression was low, however, and explains why this was missed by the microarray analysis. Further detailed analysis has confirmed that there is no statistical difference in the expression array data for this gene between wild-type and TarIISbIII400.1 (Table 2).

DISCUSSION

DNA microarrays are powerful tools now being employed in the field of parasitology. They have already showed their
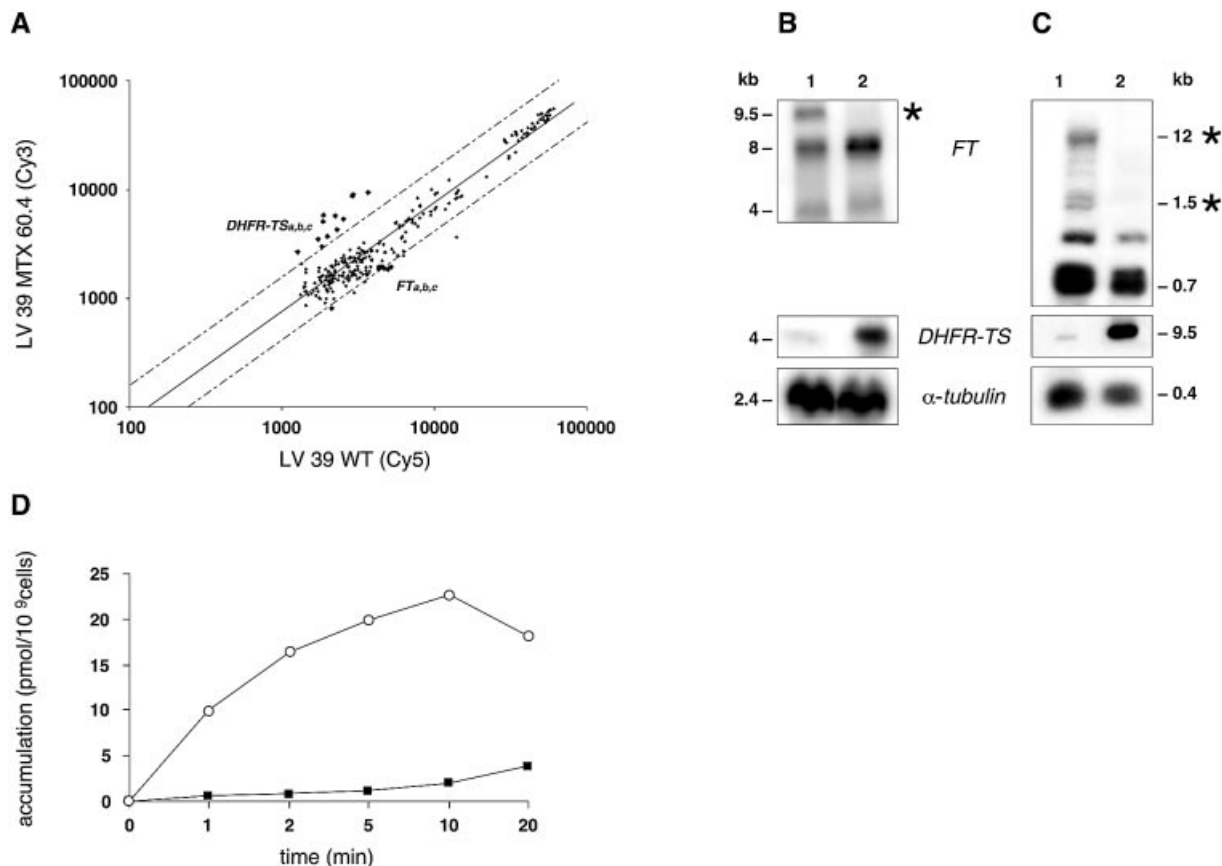
Figure 5. Gene expression analysis of the *L.major* LV39 MTX60.4 mutant as determined by DNA microarrays and northern blot analysis. (**A**) Scatter plot of hybridisation intensities between LV39 MTX 60.4 (Cy3) and wild-type *L.major* LV39 cells (Cy5). Dashed lines indicate 2-fold limits. (**B**) Confirmation of DNA microarray results by northern blot analysis showing overexpression of *DHFR-TS*, while the expression of one member of the FT family labelled with an asterisk is repressed. An α-tubulin hybridisation was performed for the determination of equal RNA and DNA loading. For quantification see Table 2. (**C**) Southern blot analysis to test whether variation in RNA expression is mediated by variation in gene's copy number. The DNA of the parasites was digested with SalI for the FT and α-tubulin blots and with SacI for the *DHFR-TS* blot. 1, *Leishmania major* wild-type cell; 2, *L.major* LV39 MTX60.4. Sizes were determined using the 1 kb Plus DNA ladder and the 0.24–9.5 kb RNA ladder from Invitrogen. (**D**) Transport experiment showing a decrease in MTX accumulation in the mutant LV39 MTX60.4 cells (filled squares) compared with wild-type *L.major* LV39 cells (open circles).
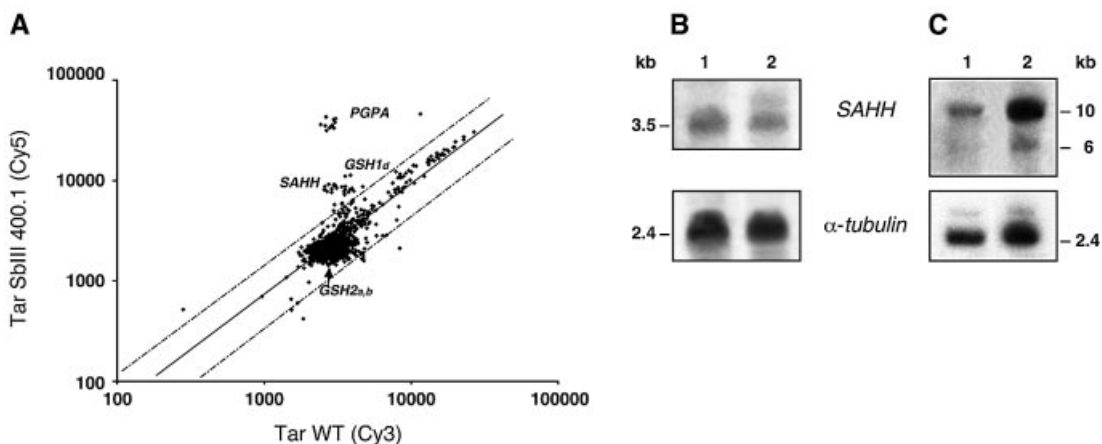

Figure 6. Gene amplification events in *L.tarentolae* TarIISbIII400.1 as determined by DNA microarrays. (**A**) Scatter plot of hybridisation intensities between TarIISbIII400.1 and wild-type digested total DNA and labelled, respectively, with Cy5 and Cy3. This corresponds to a second generation of array with 50 genes each spotted 12 times. Dots outside the dotted lines representing 2-fold differences suggest changes in copy number in the mutant. (**B**) Northern blot analysis of the *SAHH* gene. 1, *Leishmania tarentolae* wild-type cell; 2, TarIISbIII400.1. Sizes were determined using the 1 kb Plus DNA ladder and the 0.24–9.5 kb RNA ladder from Invitrogen. (**C**) The amplification of the *SAHH* gene in this mutant was confirmed by Southern blot of DNA digested with HindIII, while the other amplification events are shown in Figure 3.

usefulness in malaria research (45–48) and also for looking at gene expression in *Trypanosoma brucei* (49). Both cDNAs and random 1 kb genomic DNAs of *L.major* spotted on microarrays appear to have provided useful results to study differential gene expression between *L.major* promastigotes and metacyclics (30–32). We took advantage of the ongoing *Leishmania* genome-sequencing project and analysed four diverse resistant mutants. These studies have validated the use of microarrays in studying drug resistance in *Leishmania* and have pinpointed new genes overexpressed either by gene amplification or by other mechanisms.

A number of studies in bacterial or mammalian cells have dealt with changes of expression in the presence of drugs (24–28) but few have dealt with the analysis of resistance *per se*. One example is the use of microarrays for studying the genesis of resistance to fluconazole in *Candida albicans* (29). While it was known that TSH levels were increased in TarIIAs50.1 (7), the molecular mutation responsible for this increase was unknown. In other mutants, we have shown that overexpression of *GSH1* and/or of the ornithine decarboxylase gene *ODC* were associated with increased TSH biosynthesis (11). This is due to *GSH1* amplification in TarIIAs50.1. The work presented here has also pinpointed a novel gene corresponding to *GSH2*, whose overexpression is possibly linked to increased TSH in metal resistant mutants. Although *GSH1* has been shown to be overexpressed in a number of circumstances in several organisms including *Leishmania* (10), this appears to be the first report of *GSH2* overexpression in any drug resistant cells. While GS is not rate limiting under standard conditions, this situation could change following the amplification of *GSH1* in *Leishmania*, as observed in TarIISbIII400.1. In rats, treatment known to increase the expression of *GSH1* also increase the expression of *GSH2* (50). While overexpression of *GSH2* in yeast does not appear to increase GSH levels (51), induction of GS increased glutathione levels in a human liver cell line (50).

The custom DNA microarrays have also been used for the analysis of MTX resistant mutants where several genes known to be implicated in resistance, such as *PTR1*, *DHFR-TS* and *FT* were pinpointed. The analysis of LV39 MTX60.2 has highlighted the overexpression of *MAT2*. Overexpression of this gene is novel and has not been described in any other drug resistant organism. Ongoing proteomic studies have shown that overexpression of the MAT2 protein is a frequent event in MTX resistant mutants or in cells put in contact with MTX, but transfection of *MAT2* alone does not seem to directly confer MTX resistance (J.Drummelsmith and M.Ouellette, unpublished). Since the *MAT2* gene is not amplified, its overexpression cannot be explained by co-amplification of another nearby resistance gene. Genes in *Leishmania* are transcribed as large polycistronic RNAs and only one region on the entire chromosome 1 appears to correspond to a pol II-like promoter (52). Thus, we think it is unlikely that the overexpression of *MAT2* is due to a mutation in a promoter-like element of an upstream gene. Mutations in either the 5′ or 3′ untranslated regions of *MAT2* or mutations in a *trans* acting factor may explain the increase in RNA level of this gene or of the other genes revealed here that are overexpressed without an increase in copy number. Because *MAT2* is often overexpressed following MTX selection, we believe that it is linked to MTX selection. Since transfection excludes a

direct role (unpublished observation), we must assume a more indirect one. AdoMet is not only an intermediate metabolite in methionine catabolism but also a key intracellular molecule involved in several processes (53). One of these may be perturbed upon alteration of folate metabolism. Since folate and methionine pathways are linked (Fig. 1), a modulation in folate metabolism may indeed have consequences on the expression of genes involved in downstream pathways.

Upon drug selection, *Leishmania* often uses DNA amplification to overexpress its genes (33,34). In the mutants studied, we found that the *GSH1*, *PGPA*, *PTR1* and *DHFR-TS* genes were amplified (Figs 2–5) and that some members of the FT family were deleted. The ability of DNA microarrays to detect gene amplification events is less exploited than for expression profiling, but it was used, for example, in the study of human breast tumours (54). We have demonstrated the suitability of DNA microarrays to detect gene amplification events in *Leishmania* (Fig. 6 and results not shown). While simple techniques such as comparison of digested DNA on ethidium bromide stained gels can lead to the detection of gene amplification events (19,55), this does not identify the number of unique amplicons nor their identity. These arrays have allowed the detection of the amplification of the *SAHH* gene in a SbIII resistant mutant (Fig. 6). The amplification of this gene does not seem to lead to an increase in RNA levels, as this was not detected using microarray. Northern blot analysis (Fig. 6) indicated that while the main message was at an estimated size of 3.5 kb, another band at 3.8 kb seems to be unique to the mutant. It is possible that this second *SAHH* RNA species is more easily transported or translated and thus could lead to increase gene product expression. It is intriguing that the expression of another gene of the methionine pathway is changed in drug resistant mutants, although in this case a more simple explanation can be put forward. An increase in SAHH will lead to increase homocysteine, the precursor of *de novo* cysteine biosynthesis, and cysteine is the main building block of TSH which is increased in mutants.

The overexpression of most genes detected in our custom arrays were linked to gene amplification events. Nonetheless, two genes *GSH2* and *MAT2* were overexpressed without gene amplification (Figs 3 and 4) and often, for other genes, the fold increase in expression is not commensurate with the level of gene amplification (Table 2). Increased expression without amplification was found previously for *ODC* overexpression in arsenite resistant mutants (11) and for overexpression of *BT1* or *PTR1* in cells grown in biopterin poor medium for prolonged periods (56). As there seems to be no control at the level of transcription initiation in *Leishmania*, it is possible that an increased in RNA stability is the reason for the augmented levels of RNA, as uncovered using microarrays and confirmed by northern blot analysis. This could either be due to changes in untranslated regions or to mutations in *trans* acting factors.

Overall, we have shown the utility of DNA microarrays to study drug resistance in *Leishmania*. Indeed, we could find several genes in the various mutants that were overexpressed that correspond to known resistance gene, hence validating the technique. Our small targeted arrays have not only allowed the efficient analysis of several resistant mutants but have also permitted the discovery of three new genes *GSH2*, *SAHH* and

MAT2, whose expression was modulated in drug resistant mutants. Once the whole genome of *Leishmania* become available and is put on arrays, many more genes could be found overexpressed (or repressed) and/or amplified (or deleted) in the same mutants. These arrays will be a valuable tool to study the mechanisms of resistance in field isolates.

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