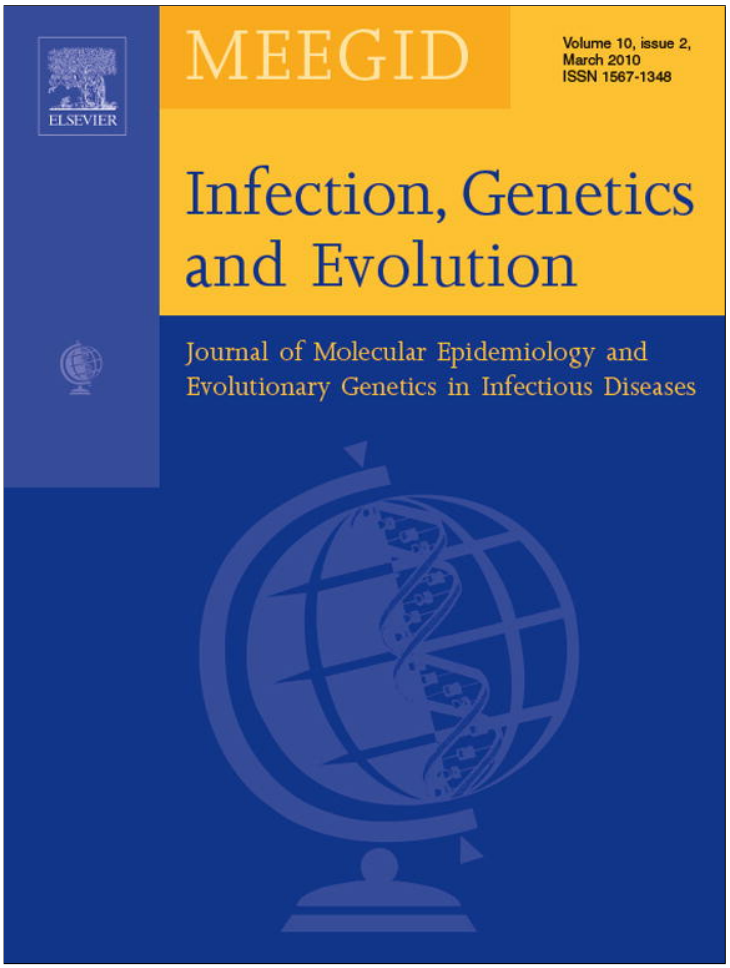

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Identification of subspecies specific genes differentially expressed in procyclic forms of *Trypanosoma brucei* subspecies

Gustave Simo [a,b,*], Stephane Herder [c], Gerard Cuny [c], Jörg Hoheisel [a]

[a] Deutsches Krebsforschungszentrum, Division of Functional Genome Analysis (B070), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany
[b] Medical Research Centre, Institute of Medical Research and Medicinal Plant Studies (IMPM), Yaoundé, Cameroon
[c] Laboratoire de Recherche et de Coordination sur les Trypanosomoses, UMR 177, IRD/CIRAD, Montpellier, France

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ABSTRACT

Trypanosoma brucei subspecies undergo establishment and maturation in tsetse flies mid-gut and salivary glands, respectively. Successful establishment of trypanosomes in tsetse mid-gut as well as their migration to saliva gland depends on the ability of these parasites to adapt rapidly to new environmental conditions and to negotiate the physical barriers. To identify subspecies specific genes which are differentially regulated during the establishment of *T. brucei* subspecies in tsetse flies mid-gut, a comparative genomic analysis between different *T. brucei* subspecies was performed using microarrays containing about 23 040 *T. brucei* shotgun fragments. The whole genome analyses of RNA expression profiles revealed about 274 significantly differentially expressed genes between *T. brucei* subspecies. About 7% of the differentially regulated clones did not match to any *T. brucei* predicted genes. Most of the differentially regulated transcripts are involved in transport across cell membrane and also in the purines metabolism. The genes selectively up regulated in *T. brucei gambiense* and *T. brucei rhodesiense* (human infective *T. brucei*) like snoRNA and HSP70 are expressed in response to stress. The high failure rate in the process of establishment and maturation of *T. brucei gambiense* during cyclical transmission in tsetse flies may result from the incapacity of this parasite to regulate its growth due to the expression of a variety of chaperones or heat shock proteins. Genes selectively up regulated in *T. brucei brucei* like NT8.1 nucleoside/nucleobase transporters and S-adenosylmethionine synthetase may favour the establishment of this subspecies in tsetse mid-gut. These genes appear as potential targets for investigations on the development of vaccine blocking the transmission of trypanosomes in tsetse flies.

1. Introduction

Salivarian trypanosomes causing Human African Trypanosomiasis (HAT) in humans and "nagana" in livestock are cyclically transmitted by tsetse flies of the genus *Glossina*. For successful transmission, *Trypanosoma brucei* subspecies undergoes two stages of differentiation in tsetse flies: first, establishment in the mid-gut and then maturation in the salivary glands (Hao et al., 2001). In the mid-gut, the mammalian bloodstream trypanosomes rapidly differentiate to procyclic forms and begin to replicate (establishment). Once established in the mid-gut, procyclic forms of *T. brucei brucei* (*T. b. brucei*) and the causative agents of HAT, *T. brucei rhodesiense* (*T. b. rhodesiense*) and *T. brucei gambiense* (*T. b. gambiense*) must mature by migrating forwards to the proventriculus of the fly where they begin to differentiate into epimastigotes and finally metacyclics which eventually colonize the salivary glands (Abbeele et al., 1999; Hao et al., 2001).

Tsetse flies are in generally refractory to trypanosome infections with typically less than half the fly population becoming infected, even under ideal conditions in the laboratory (Lehane et al., 2003). This is reflected in the field infection rates which often fail to exceed 15% of the fly population (Masiga et al., 1996; Morlais et al., 1998; Njiru et al., 2004). In experiments, many flies that become infected, fail to produce mature infections and therefore never become infective and thus are incapable of transmitting parasites (Lehane et al., 2003). Improvement of our knowledge on the cyclical transmission of trypanosomes depends largely on our knowledge of the physiology of the parasite and its arthropod host, and particularly of the interdependence of their physiologies. To understand the refractoriness or the susceptibility of tsetse fly to trypanosome infections, studies have been undertaken to identify tsetse flies related factors that may play a role during establishment and maturation of trypanosomes in tsetse flies. Factors

* Corresponding author at: Medical Research Centre, Institute of Medical Research and Medicinal Plant Studies (IMPM), P.O. Box 6163, Yaoundé, Cameroon.
E-mail addresses: gsimoca@yahoo.fr, g.simo@dkfz-heidelberg.de (G. Simo).

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including lectin levels in the gut at the time of parasite uptake, fly species, sex, age, symbiotic associations in tsetse fly and fly immunity factors have been reported to play a part in determining the success or failure of trypanosome infections (Dale et al., 1995; Milligan et al., 1995; Maudlin and Welburn, 1994; Welburn and Maudlin, 1999; Hao et al., 2001). Tsetse flies have been shown to possess mid-gut lectin levels that are capable of killing trypanosomes *in vivo* by a process resembling programmed cell death (Welburn and Maudlin, 1999). Recently, antimicrobial peptide genes like attacin, defensin and diptericin from tsetse fat body tissue were identified and their differential regulation shows that the tsetse immune system can discriminate bacteria and trypanosome infections and also different life stages of trypanosomes (Hao et al., 2001). Moreover, MacLeod et al. (2007) have shown that antioxidant can inhibit death of *T. b. brucei* in the mid-gut of tsetse flies, suggesting that reactive oxygen species play a role in killing incoming trypanosomes. Despite these considerable advances, the mechanism of refractoriness or susceptibility of tsetse flies to trypanosome infections is not fully understood but parasite related factors may play also an important role.

In tsetse fly mid-gut characterized by physico-chemical properties considerably different from the vertebrate blood, trypanosomes must adapt rapidly (e.g. *T. congolense* for example required only 3 days of lectin signal to stimulate maturation; Welburn and Maudlin, 1989) by undergoing substantial changes in their morphology, their internal structures and their metabolism. These modifications are accompanied by changes in the expression of an unknown number of genes or proteins. Successful establishment of trypanosomes in tsetse fly depends on the ability of each trypanosome subspecies to adapt, transform, growth and survive rapidly in the new micro-environment. From experimental and field studies, it has been observed that the rate of establishment and maturation of trypanosomes differ according to parasite subspecies or genotypes (Maudlin and Welburn, 1988, 1994; Dale et al., 1995; Ravel et al., 2006). Experimentally, *T. b. rhodesiense* produced significantly lower establishment and maturation (in *Glossina morsitans morsitans*) than *T. b. brucei* while the maturation of *T. b. gambiense* in any species of tsetse flies is rare (Maudlin and Welburn, 1994; Milligan et al., 1995; Ravel et al., 2006). With the exception of *T. b. gambiense*, *T. brucei* isolates routinely produce metacyclics within 12 days of establishment in tsetse flies (Maudlin and Welburn, 1994). A great variation in the establishment and maturation of *T. congolense* and *T. brucei* s.l. has been also reported (Maudlin and Welburn, 1987, 1988; Ravel et al., 2006; Hu and Aksoy, 2006). Until now, the mechanism by which some trypanosomes die or survive in tsetse fly by evading the natural immune system of tsetse flies still not yet fully understood.

Maudlin and Welburn (1994) suggested that the signal of maturation of trypanosomes is received soon after establishment in tsetse mid-gut. The installation of *T. b. gambiense* in *Glossina* took place at least 11 days after infection and the maturation occurred after 29 days (Ravel et al., 2003). Recently, MacLeod et al. (2007) reported that trypanosomes require, in tsetse fly mid-gut, a Nitric oxide (NO) signal and or the presence of L-cysteine to promote migration to salivary glands and maturation into mammalian infective forms. Establishment of trypanosome in tsetse mid-gut appears, therefore, as one of the key points determining the success or the failure of the cyclical transmission of trypanosomes. Better understanding the mechanism by which trypanosomes establish in the mid-gut can provide opportunities to identify genes that may enable the development of vaccines blocking transmission. In this study, a comparative genomic analysis between procyclic forms of different *T. brucei* subspecies (*T. b. gambiense*, *T. b. rhodesiense* and *T. b. brucei*) was performed to identify subspecies specific genes which are differentially expressed during establishment of these trypanosomes in tsetse fly mid-gut.

2. Materials and methods

2.1. Construction of genomic T. brucei microarray

The construction of microarray was performed following the protocol described by Diehl et al. (2002) and Brems et al. (2005). Briefly, independent random shotgun genomic clones from *T. brucei* strain TREU927/4 (*T. b. brucei* subspecies) were used to create PCR products which were spotted onto "Nexterion® Slide A+" (PeqLab Biotechnologie GmbH, Germany) following the spotting and post-processing protocol described by the manufacturer.

2.2. Culture of parasites and RNA extraction

Four isolates of different procyclic forms of *T. brucei* subspecies were used in this study. These trypanosome stocks were isolated between 1966 and 1980, and the stabilats were conserved in liquid nitrogen. These trypanosomes include:

- One isolate of *T. b. brucei* subspecies. This trypanosome subspecies infects a variety of animals in sub-Saharan Africa. The isolate EATRO 1125, used here, was isolated in 1966 from zebu in Uganda and it has been already characterized as *T. b. brucei* by several studies (Hide et al., 1990; Simo et al., 2005).
- One isolate of *T. b. rhodesiense* subspecies which express the SRA gene and causes the acute form of HAT in East Africa. The isolate used here, TRPZ 166, was isolated from goat in Zambia in 1982. It has been characterized as human infective by several markers (Mathieu-Daudé et al., 1995; Koffi et al., 2007).
- One isolate of group 1 *T. b. gambiense*. This subspecies is responsible of the chronic form of HAT in west and central Africa. The group 1 *T. b. gambiense* isolate (Peya) used here was isolated in 1980 from human in Congo, and has been characterized by Stevens et al. (1992).
- One isolate of group 2 *T. b. gambiense* subspecies. The parasites belonging to this group are responsible of the acute form of HAT in West Africa. The group 2 *T. b. gambiense* (Biyamina) used in this study was isolated in 1982 from human and has been previously characterized by several authors (Godfrey et al., 1990; Mathieu-Daudé et al., 1995).

These procyclic trypanosomes were growth in Cunningham medium (Cunningham, 1977) supplemented with 10% foetal calf serum. In the Cunningham medium which has a similar composition as the tsetse fly mid-gut, *T. brucei* s.l. grow as procyclics, the same stage found in the tsetse fly mid-gut. When the parasites were adapted in the culture medium, they were transferred into larger culture flasks and the culture medium was added progressively to obtain 100 ml. After 2 or 3 days, the parasites were harvested in the logarithmically growing phase of cells, when the parasite density was close to 6.4×10^7 trypanosomes per millilitre of culture medium (12.5×10^7 for *T. b. brucei* and 6.4×10^7 for the others: *T. b. rhodesiense*, *T. b. gambiense* groups 1 and 2).

Total RNA were extracted from the trypanosome pellets using RNAeasy Midi Kit (Qiagen) according to the protocol described by the manufacturer. The RNA samples were stored at −80 °C.

2.3. Synthesis of labelled cDNA and hybridization of the slides

For each hybridization reaction, fluorescent cDNA were generated from 10 μg of total RNA (*T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense* groups 1 and 2 total RNA) by direct incorporation of fluorophor-labelled dCTP during the first strand cDNA synthesis. The reaction mix contained 1 μl of random hexamer primers (0.5 μg/μl), 2 μl of either Cy3 or Cy5 dCTP (25 mmol), 10 mM each

of dATP, dGTP, dTTP and 1 mM dCTP, 0.1 M dithiothreitol, 400 units of Superscript III reverse transcriptase (Invitrogen) and 2 μl of 2× first strand buffer. The reverse transcription reactions were performed at 50 °C for 4 h. At the end of the reverse transcription, the reaction was incubated at 70 °C for 10 min followed by an addition of 1 μl of RNase H. An additional incubation was performed at 37 °C for 20 min. The labeled cDNA were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The rate of incorporation of fluorophores was then determined by photometric measurement at 550 nm for Cy3 and 650 nm for Cy5. For each hybridization reaction, labeled Cy3 and Cy5 cDNA were mixed, homogenized and dried. The pellets were re-suspended in 10 μl of 10 mM EDTA, heated at 95° C for 5 min to denature DNA and kept at room temperature for 5 min. Then 40 μl of slide hybridization buffer (SlideHyb #1, SlideHyb Survey; Ambion) were added to the labeled cDNA before the hybridization reactions.

Six hybridizations including two biological replicates were performed for each comparison (e.g.: 6 slides to compare the differential expression between *T. b. brucei* and *T. b. rhodesiense*). To prevent bias occurring during incorporation of different dyes, Cy3 and Cy5 were swapped between the two RNA preparations. Hybridization reactions were carried out under a glass cover slip (24 mm × 60 mm). The slides were kept in waterproof humidified chamber in a water-bath at 55 °C for 16–18 h. After hybridization, the slides were washed two time at 55 °C for 5 min in 2× SSC, 0.2% SDS, followed by a second wash at 37 °C for 10 min in 0.1× SSC, 0.2% SDS. An additional wash to rinse slides was performed at 37 °C for 10 min in 0.1× SSC. The slides were dried and subjected to florescence detection.

2.4. Image acquisition and data analysis

The signal intensities of the spots were determined with laser scanner according to the protocol described by Diehl et al. (2002). The images were acquired by performing subsequent scans with the Cy3 and Cy5 channel at a resolution of 10 μm. The resulting images were analyzed using Genepix software (Axon Instruments, Union City, USA), which generates numerical values for the signal intensities at each spot. In subsequent data analysis, only spots in which more than 70% of the pixels had a signal above background of at least twice the standard deviation of the local background were considered.

Data quality assessment, normalization and analysis were performed with the Multi-Conditional Hybridization Intensity Processing System (MCHIPS) software package (Fellenberg et al., 2001, 2002) (http://www.m-chips.org/). Normalization was carried out as described by Kramer et al. (2008). In all cases, the overall original signal intensities of all spots were normalized by logarithmic regression. The significance of the observed variations in the transcript levels was analyzed by statistical procedure as described by Beissbarth et al. (2000). Each individual signal was filtered for quality and reproducibility. All spots with a filtered intensity of at least twice the standard deviation above the background, a minimum/maximum separation value of 0.1, and a p value (to evaluate the reproducibility between hybridizations and different biological replicates) <0.05 were selected.

2.5. Sequencing of differentially expressed genes

Five microlitres of glycerol stocks of each clone for which a differential expression were found were picked up and cultured overnight in 5 ml LB medium containing Ampicillin (100 μg/ml). After centrifugation of the culture medium at 4000 rpm for 10 min, the plasmid DNA was purified from bacteria pellets using Qiaprep Miniprep Kit (Qiagen) following manufacturers' instructions. The presence of *T. brucei* s.l. DNA fragment was checked by PCR using M13 primers. All plasmids containing an insert of ≈2.25 kb (average) corresponding to *T. brucei* s.l. DNA fragment were sent for sequencing. Sequences were obtained by long reads from one or both ends. The sequencing reactions were performed by a commercial company (GATC Biotech AG, Germany). The identification of proteins encoding differentially regulated genes was performed by blasting the sequences obtained with *T. brucei* GeneDB predicted genes at Wellcome Trust Sanger Institute (Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA, UK) using the BLAST algorithm (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/t_brucei/).

2.6. Confirmation of the microarrays data

Quantitative real-time PCR (qRT-PCR) was performed on some randomly selected differential transcribed genes for validating microarray analysis. The same total RNA used for microarray studies was subjected to qRT-PCR. One microgram of total RNA of each *T. brucei* subspecies were treated with RNase free Dnase I (Fermentas, Life Sciences) at 37 °C for 30 min to remove the contaminant genomic DNA. Then, the treated RNA were reversed transcribed at 50 °C for 1 h using the "SuperScript Vilo cDNA synthesis Kit" (Invitrogen) according to the manufacturer's instructions. The resulting cDNA were stored at −20 °C until use.

Quantitative real-time PCR reactions were carried out using gene-specific primers. These primers were designed using the "Primer-BLAST tool for finding specific primers" and "TrypanoFAN RNAit target selection script" from the http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and http://trypanofan.path.cam.ac.uk/software/RNAit.html websites, respectively. Each primer used for qRT-PCR was checked against *T. b. brucei* and *T. b. gambiense* genome database to search for perfect match. For qRT-PCR, equal amounts of cDNA were run in triplicate and the amplification reactions were carried out in a 10 μl reaction containing 2 μl of each cDNA diluted 1000-fold, 1 μl (5 pmol) of each gene-specific primer, 5 μl of "Express SYBR Green^{TM} qPCR Super Mix universal Kit" (Invitrogen) and 1 μl of RNase free water. The amplification efficiencies were evaluated and gene expression levels were normalized to constitutive expressed mRNA encoding two reference genes. Quantification of the relative changes in the target gene expression was evaluated using a standard curve.

3. Results

3.1. Overall similarity in gene expression profiles between T. brucei subspecies

The arrays used in these studies contained about 23 040 spots of *T. b. brucei* genomic fragments. This corresponds to about 1.3× coverage of the whole genome of *T. brucei* if we assume that all fragments were present at equal probability. During the analyses of microarray data with MCHIPS software, the background was subtracted and several filters were applied to choose regulated clones. About 2% (460 clones) of clones satisfying the selection criteria (fitted intensities at least 1000, min/max separation 0.1, and p value <0.05) were found significantly differentially regulated between procyclic forms of *T. brucei* subspecies.

Of the 460 differentially regulated clones, only those with an absolute Log_2 ratio (results of microarray) greater than 0.6 between control and condition were selected for sequencing and further, for qRT-PCR. A total of 274 differentially expressed clones were selected. The most similar gene expression profiles were found between *T. b. brucei* and *T. b. rhodeisense* with only 58 differentially regulated clones. Between *T. b. brucei* and *T. b.*

Table 1
Genes differentially expressed between *T. b. brucei* and *T. b. rhodesiense*.

Gene name	Gene ID	[a]Log_2(ratio)			
		Array	qRT-PCR	Biological process	Molecular function
Genes up regulated in *T. b. rhodesiense*					
Hypothetical protein	Tb927.2.1470	1.3		Unknown	Unknown
Hypothetical protein, conserved	Tb927.6.2840	1.1		Nucleic acid metabolism	Nucleotide binding
Hypothetical protein	Tb927.6.3220	1.2		Protein biosynthesis	Translation release factor
U3 small nuclear ribonucleoprotein (snRNP)	Tb927.6.2780	1.1	0.7	Unknown	Unknown
L-Threonine 3-dehydrogenase, putative	Tb927.6.2790	1.1		Cellular metabolism	Catalytic activity
Glycerol-3-phosphate dehydrogenase	Tb927.8.3530	0.7	1.1	Carbohydrate metabolism	Catalytic activity
THT2A glucose transporter	Tb10.6k15.2020 (3)	1.4	1.9	Transport	Transmembrane transporter
Heat shock protein 70	Tb11.01.3110 (2)	1.1	1.2	Response to stress	ATP binding, folding
C/D snoRNA clusters	Tb09_snoRNA_0037[b]	0.9	1.5	rRNA processing	Direct methylation
H/ACA snoRNA clusters	Tb09_snoRNA_0040[b]	0.9	1.7	rRNA processing	Direct pseudouridinylation
ABC transporter	Tb927.3.3730	0.9		Transport	Nucleotide binding
Amino acid transporter, putative	Tb927.4.4730	0.8	1.4	Transport	Transmembrane transporter
60S ribosomal subunit protein L31, putative	Tb09.211.3280	0.7		Translation	Structural constituent of ribosome
Pteridine transporter	Tb10.6k15.1350	1.1	0.9	Biopterin transport	Transporter
Glycerol kinase, glycosomal	Tb09.211.3550	0.9		Carbohydrate metabolism	Transferase activity
Fructose bisphosphate aldolase glycosomal	Tb10.70.1370	0.9		Glycolysis	Catalytic activity
Genes up regulated in *T. b. brucei*					
Hypothetical protein	Tb927.1.4630	−0.9		Unknown	Unknown
Hypothetical protein	Tb927.7.1460	−1.1	−0.15	Protein import into nucleus	Transporter
Hypothetical protein	Tb10.70.6570	−1.3		Protein modification	Catalytic activity, binding
Hypothetical protein	Tb927.2.5020	−1.4		Metabolic process	Acyl-CoA oxidase activity
Cysteine peptidase precursor	Tb927.6.970	−0.8		Proteolysis	Endopeptidase activity
Transcription initiation protein	Tb927.2.5030	−1.4	−0.15	Nucleic acid metabolism	Structural constituent of ribosome
S-adenosylmethionine synthetase	Tb927.6.4870 (3)	−1.3	−0.8	Metabolic process	Nucleotide binding
NT8.1|nucleobase/nucleoside transporter 8.1	Tb11.02.1105 (8)	−2	−2.5	Transport	Transmembrane transporter
Nucleobase transporter	Tb11.02.1106 (2)	−2.1		Transport	Transmembrane transporter

In parenthesis number of spots with identical gene found up regulated during microarray analysis.
[a] Log_2(ratio) represent expression levels between *T. brucei* subspecies. The gene ID, biological process and molecular function are from NCBI in January 2009. Some functions and biological processes remain unconfirmed.
[b] Example of Gene ID of snoRNA gene cluster identified (many clusters were identified).

gambiense group 2 or *T. b. brucei* and *T. b. gambiense* group 1, 106 and 110 respective clones were differentially regulated. About 27% of the selected clones have been already described in previous studies (Diehl et al., 2002; Brems et al., 2005). The remainder was sequenced and the results are included in this works. However, about 5% of the remainder was not subjected to sequencing because two DNA fragments were observed during the verification of the insert by PCR. The two fragments may result from the presence of different clones in the same well (possible cross-contamination of neighbour wells). In total 256 sequences of differentially expressed clones were obtained by combining the new sequences with those described in previous studies. The blast of these sequences with *T. brucei* GeneDB predicted genes revealed 7.8% of the clones encoding hypothetical proteins or proteins of unknown function. About 32.8% of the 256 clones have shown some identical Gene ID or identical DNA sequences (e.g.: 8 clones with Gene ID Tb11.02.1105 encode for NT8.1 nucleobase/nucleoside transporters 8.1; Table 1); thus, encoding identical proteins. One hundred and thirty four clones (52.3%) revealed different DNA sequences (differentially regulated genes found only in one spot of the microarray slides). Each of these sequences encodes for a specific protein. Moreover, about 7% of sequences did not match to any *T. brucei* predicted genes (sequences not found in the GeneDB). These sequences may originate from the telomeres or mini-chromosomes (Brems et al., 2005). More details about these sequences could be obtained with the ongoing telomere, intermediate and mini-chromosomes (representing about 10% of *T. brucei* nuclear DNA; Wickstead et al., 2004) sequencing projects.

By grouping all differentially expressed genes into different functional categories, we found that the majority of these genes are involved in nucleic acids binding and transport of molecules. Some differentially regulated genes encoded proteins of unknown function (hypothetical proteins). Genes up regulated in *T. b. brucei* encode proteins of a less diverse functional profile. Most of these genes are involved in the transport of nucleoside/nucleobase and in the transformation of methionine to S-adenosylmethionine.

3.2. Comparison of global gene expression profiles between human and non-human infective *T. brucei*

Genes encoding proteins with diverse functions were up regulated in human infective parasites compared to *T. b. brucei* (Tables 1–3). Blast of 44 DNA sequences corresponding to 44 clones which have shown differential expression profiles between human (*T. b. rhodesiense* and *T. b. gambiense*) and non-human (*T. b. brucei*) infective parasites enabled to identified 10 different transcripts (Table 4). Four of them are up regulated in *T. b. brucei* (Table 4). One of these four genes encodes a hypothetical protein while the others encode proteins involved in ATP binding and the transport of nucleosides and nucleobases. The genes up regulated in all human infective parasites include genes encoding HSP70, glucose transporter and small nucleolar RNA (snoRNA). These proteins are involved in ATP binding, transport of glucose, methylation and pseudouridinylation of RNAs.

3.3. Subspecies specific genes differentially regulated between *T. brucei* subspecies

In addition to genes found differentially regulated between human and non-human infective *T. brucei*, several specific transcripts of each *T. brucei* subspecies were identified. The comparison of global gene expression profiles between *T. b. brucei* and *T. b. rhodesience* enabled the identification of genes encoding hypothetical proteins, transcription initiation protein and cysteine peptidase

Table 2
Genes differentially expressed between procyclic forms of *T. b. brucei* and *T. b. gambiense* group 2.

Gene name	Gene ID	[a]Log_2(ratio)			
		Array	qRT-PCR	Biological process	Molecular function
Genes up regulated in *T. b. gambiense* group 2					
Hypothetical protein	Tb09.211.0380	0.8		Unknown	Unknown
Hypothetical protein	Tb09.160.4460	1.2		Unknown	Unknown
Hypothetical protein	Tb09.160.4480	1.1		Transport	Unknown
Hypothetical protein, conserved	Tb11.02.4450	0.9		Transport	Unknown
Hypothetical protein, conserved	Tb927.6.3220	0.7		Protein biosynthesis	Translation release factor
RHS protein,	Tb927.2.340	0.7	2.3	Unknown	Unknown
Calpain-like protein fragment	Tb927.1.2260	0.7		Unknown	Unknown
THT2A||glucose transporter	Tb10.6k15.2020	0.8	1.6	Transport	Transmembrane transporter
Heat shock protein 70	Tb11.01.3110	0.8	0.7	Response to stress	ATP binding, folding
C/D snoRNA clusters	Tb09_snoRNA_0037[b]	0.8	1	rRNA processing	Direct methylation
H/ACA snoRNA clusters	Tb09_snoRNA_0040[b]	0.8	0.9	rRNA processing	Direct pseudouridinylation
Major surface protease gp63, putative	Tb927.8.1630	0.6		Cell adhesion, proteolysis	Hydrolase activity, ion binding
Major surface protease gp63	Tb927.8.1640 (2)	0.8		Cell adhesion, proteolysis	Hydrolase activity, ion binding
Zinc finger protein	Tb927.6.3490	0.8		Cellular metabolism	Nucleic acid binding
60S ribosomal protein L13a, putative	Tb927.4.3550	0.6		Translation	Structural constituent of ribosome
Pumilio RNA-binding protein PUF1	Tb10.70.2800	0.9		mRNA metabolism, cell growth	RNA-binding
Histone H2B	Tb10.406.0390	0.9		Nucleic acid metabolism	Nucleotide, DNA binding
Hexokinase	Tb10.70.5800	0.9		Glycolysis	Nucleotide binding, transferase activity
Hexokinase	Tb10.70.5820	1.2		Glycolysis	Nucleotide binding, transferase activity
Genes up regulated in *T. b. brucei*					
Hypothetical protein, conserved	Tb927.1.4310	−0.6		Unknown	Protein binding
Hypothetical protein, conserved	Tb927.5.1850	−0.7		Transcription, protein biosynthesis	Ribosomal protein
Procyclin-associated gene 1 polypeptide	Tb10.6k15.0040	−1.4		rRNA metabolism, DNA repair	Unknown
Hypothetical protein	Tb11.01.8270 (2)	−0.7		Proteolysis	Nucleic acid binding
Hypothetical protein	Tb927.7.1460	−1.1	−0.6	Protein import into nucleus	Transporter
Glucose-6-phosphate isomerase, glycosomal	Tb927.1.3830	−0.6		Glycolysis, gluconeogenesis	Catalytic activity
Elongation factor 1 gamma, putative	Tb11.01.4750	−0.6		Translation, biosynthesis	Translation elongation factor activity
Mitochondrial carrier protein, putative	Tb10.61.1820	−0.7		Transport	Transporter activity and binding
Phosphate repressible phosphate permease	Tb927.1.580	−0.7		Transport	Transmembrane transporter
Receptor-type adenylate cyclase GRESAG 4	Tb11.27.0001 (3)	−1.3	−0.6	Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Receptor-type adenylate cyclase GRESAG 4	Tb927.6.180	−0.8		Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Receptor-type adenylate cyclase GRESAG 4	Tb927.6.300	−0.7		Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Receptor-type adenylate cyclase GRESAG 4,	Tb927.6.790	−0.8		Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Receptor-type adenylate cyclase GRESAG 4	Tb927.6.200	−1		Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Receptor-type adenylate cyclase GRESAG 4	Tb10.389.0430	−0.6		Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Amino acid transporter	Tb927.4.4000 (3)	−1.5	−1.8	Transport	Transmembrane transporter
S-adenosylmethionine synthetase	Tb927.6.4870 (2)	−1.3	−0.8	Metabolic process	Nucleotide binding
S-adenosylmethionine synthetase	Tb927.6.4910 (5)	−1.3		Metabolic process	Nucleotide binding
S-adenosylmethionine synthetase	Tb927.6.4850 (3)	−1.3		Metabolic process	Nucleotide binding
S-adenosylmethionine synthetase	Tb927.6.4880	−1.5		Metabolic process	Nucleotide binding
NT8.1|nucleobase/nucleoside transporter 8.1	Tb11.02.1105 (7)	−1.6	−1.9	Transport	Transmembrane transporter
Nucleobase transporter	Tb11.02.1106 (3)	−1.3		Transport	Transmembrane transporter

In parenthesis number of spots with identical gene found up regulated during microarray analysis.
[a] Log_2(ratio) represent expression levels between *T. brucei* subspecies; RHS: Retrotransposon hot spot. The gene ID, biological process and molecular function are from NCBI in January 2009. Some functions and biological processes remain unconfirmed.
[b] Example of Gene ID of snoRNA gene cluster identified (many clusters were identified).

precursor which were up regulated in the first subspecies while genes encoding proteins involved in several biological functions (biopterin and ABC transporter; kinase, aldolase and dehydrogenase activities) were up regulated in *T. b. rhodesience* (Table 1).

Between *T. b. brucei* and *T. b. gambiense* (groups 1 and 2), genes encoding hypothetical proteins, elongation factor 1 gamma and receptor-type adenylate cyclase (GRESAG 4) were up regulated in animal infective parasite (Tables 2 and 3). *T. b. gambiense* groups 1 and 2 show several transcripts encoding proteins with a large spectrum of biological functions. Genes encoding HSP84, mitochondrial HSP70 and HSP60, synaptojanin, elongation factor 2 and other proteins were up regulated in *T. b. gambiense* group 1 (Table 2). In *T. b. gambiense* group 2, genes encoding proteins involved in nucleic acids binding like zinc finger protein, pumilio RNA-binding protein PUF1 and histone 2B were up regulated. Tables 1–3 report detailed results of subspecies specific transcripts between different *T. brucei* subspecies.

3.4. Verification of T. brucei subspecies microarray expression by qRT-PCR

For the verification of the microarray results, level of specific mRNAs from different *T. brucei* subspecies were evaluated by qRT-PCR. The assay was carried out using specific primers of 15 *T. brucei* specific genes which were found differentially expressed by microarray analysis. Levels of transcripts determined by qRT-PCR were normalized to two *T. brucei* genes found unregulated in all *T. brucei* subspecies by microarray experiments. These two genes (Tb927.2.2410; Tb927.2.5610) encode for hypothetical proteins. Results obtained by qRT-PCR were consistent with microarray

Table 3
Genes differentially expressed between procyclic forms of *T. b. brucei* and *T. b. gambiense* group 1.

Gene name	Gene ID	[a]Log$_2$(ratio)			
		Array	qRT-PCR	Biological process	Molecular function
Genes up regulated in *T. b. gambiense* group 1					
Calpain-like protein fragment	Tb927.1.2260	0.9		Unknown	Unknown
Hypothetical protein	Tb11.02.4450	0.6		Transport	Unknown
Hypothetical protein	Tb11.01.6380	0.8		Nucleic acid metabolism	Unknown
Hypothetical protein	Tb927.5.2170	0.8		Transport	Unknown
RHS protein (pseudogene), putative	Tb927.2.340	0.6	1.8	Proteolysis	Protein kinase
RNA-binding protein, putative	Tb09.160.3150	1		RNA processing	Nucleotide, nucleic acid binding
THT2A glucose transporter	Tb10.6k15.2020 (2)	0.9	1.3	Transport	Transmembrane transporter
Heat shock protein 70	Tb11.01.3110 (6)	0.8	1.2	Response to stress	ATP binding, folding
Heat shock 70 protein, mitochondrial precursor	Tb927.6.3750	0.9		Response to stress	ATP binding, folding
Heat shock 70 protein, mitochondrial precursor	Tb927.6.3740	1		Response to stress	ATP binding, folding
C/D snoRNA clusters	Tb09_snoRNA_0037[b]	1.1	2.4	rRNA processing	Direct methylation
H/ACA snoRNA clusters	Tb09_snoRNA_0022[b]	1.3	1.1	rRNA processing	Direct pseudouridinylation
Elongation factor 2	Tb10.70.2660	0.6	0.7	Translation	Nucleotide binding
Elongation factor 2	Tb10.70.2650 (3)	0.7		Translation	Nucleotide binding
Universal minicircle sequence binding protein	Tb10.70.0820	0.7		Nucleic acid metabolism	Nucleic acid binding
Universal minicircle sequence binding protein	Tb10.70.0800	0.6		Nucleic acid metabolism	Nucleic acid binding
Heat shock protein, mitochondrial precursor	Tb11.02.0250	0.7		Response to stress	Nucleotide and protein binding
Chaperonin Hsp60, mitochondrial precursor	Tb10.70.0430 (2)	0.7		Protein folding	Nucleotide and protein binding
Synaptojanin	Tb09.211.2020	0.7		Endocytosis	Hydrolase activity
Eukaryotic initiation factor 4a, putative	Tb09.160.3270	0.7		Translation	Nucleotide, nucleic acid binding
Trypanothione synthetase	Tb927.2.4370	0.9		Biosynthetic process	Oxidoreductase activity
Transketolase, putative	Tb927.8.6170	0.6		Pentose-phosphate shunt	Transferase activity
Glycosomal phosphoenolpyruvate carboxykinase	Tb927.2.4210	0.8		Gluconeogenesis	ATP, microtubule binding
Pteridine transporter	Tb927.1.2850	1	1.9	Biopterin transport	Transporter
Genes up regulated in *T. b. brucei*					
Hypothetical protein	Tb927.7.1460	−2	−2.1	Protein import into nucleus	Transporter
73 kDa paraflagellar rod protein	Tb927.3.4290 (2)	−0.6		Cell motility	Protein binding
Elongation factor 1 gamma, putative	Tb11.01.4750	−0.8		Translation, biosynthesis	Translation elongation factor activity
Receptor-type adenylate cyclase GRESAG 4	Tb927.6.790 (2)	−0.8		Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Receptor-type adenylate cyclase GRESAG 4	Tb927.6.310	−0.8		Cyclic nucleotide biosynthetic	Adenylate cyclase activity
Receptor-type adenylate cyclase GRESAG 4	Tb927.6.800	−0.9	−1	Cyclic nucleotide biosynthetic	Adenylate cyclase activity
S-adenosylmethionine synthetase	Tb927.6.4870	−1.9	−1.1	Metabolic process	Nucleotide binding
S-adenosylmethionine synthetase	Tb927.6.4850	−2		Metabolic process	Nucleotide binding
S-adenosylmethionine synthetase	Tb927.6.4910 (6)	−1.7		Metabolic process	Nucleotide binding
NT8.1|nucleobase/nucleoside transporter 8.1	Tb11.02.1105 (8)	−1.8	−2	Transport	Transmembrane transporter
Nucleobase transporter	Tb11.02.1106 (5)	−2		Transport	Transmembrane transporter

In parenthesis number of spots with identical gene found up regulated during microarray analysis.
[a] Log$_2$(ratio) represent expression levels between *T. brucei* subspecies; RHS: Retrotransposon hot spot. The gene ID, biological process and molecular function are from NCBI in January 2009. Some functions and biological processes remain unconfirmed.
[b] Example of Gene ID of snoRNA gene cluster identified (many clusters were identified).

Table 4
Genes differentially expressed between human and non-human infective *T. brucei* s.l.

Gene name	Gene ID	qRT-PCR	Biological function	Molecular function
Genes simultaneously up regulated in *T. b. rhodesiense, T. b. gambiense* group 1 and 2				
Heat shock protein 70	Tb11.01.3110	C (1.1)	Response to stress	ATP binding and folding
THT2A||glucose transporter	Tb10.6k15.2020	C (1.3)	Transport	Transmembrane transporter
C/D snoRNA clusters	Tb09_snoRNA_0037[a]	C (1.6)	rRNA processing	Direct methylation
H/ACA snoRNA clusters	Tb09_snoRNA_0022[a]	C (1.2)	rRNA processing	Direct pseudouridinylation
Genes up regulated in *T. b. brucei*				
Hypothetical protein, conserved	Tb927.7.1460	C (0.9)	Protein import into nucleus	Transporter
S-adenosylmethionine synthetase	Tb927.6.4870	C (0.9)	Metabolic process	Nucleotide binding
NT8.1| nucleobase/nucleoside transporter	Tb11.02.1105	C (2.1)	Transport	Transmembrane transporter

[a] Example of Gene ID of snoRNA gene cluster identified (many clusters were identified); C: confirmation of microarray results by qRT-PCR; In parenthesis: Log$_2$(ratio).

observations. All genes found up or down regulated in *T. b. brucei* by microarray were confirmed by qRT-PCR (Tables 1–4). *T. b. gambiense* specific genes like Elongation factor 2 found up regulated only in this subspecies by microarray was confirmed by qRT-PCR. Similar results were obtained for other genes like amino acid transporter, glycerol 3-phosphate dehydrogenase, and transcription initiation protein.

4. Discussion

The overall similarity in the gene expression profiles between *T. brucei* subspecies can be linked to their taxonomic relationships since few clones (58) were found differentially regulated between *T. b. brucei* and *T. b. rhodesiense* (*T. b. rhodesiense* is considered as a

variant of *T. b. brucei* that was adapted to human due to the expression of SRA gene) while more than 100 clones (106 clones between *T. b. brucei* and *T. b. gambiense* group 1 and 110 between *T. b. brucei* and *T. b. gambiense* group 2) were differentially expressed between *T. b. brucei* and *T. b. gambiense* (*T. b. gambiense* differs from the other subspecies by several biological, biochemical and genetic differences).

The differences in the gene expression profiles between *T. brucei* subspecies result from the specific response of each subspecies to new environmental conditions and insect related factors which prevent their establishment. This indicates that the genes expressed by each *T. brucei* subspecies occur to favour the death or the growth of trypanosomes. Indeed, the signal of maturation of trypanosomes is received soon after establishment and the greatest attrition in trypanosomes survival appears in tsetse mid-gut early in the infection process around day 3 (Gibson and Bailey, 2003).

Some genes found differentially regulated in this study are involved in stress response; confirming the stress encounters by trypanosomes during their establishment in insect mid-gut. Remarkably, the genes involved in stress were up regulated, especially, in *T. b. rhodesiense* and *T. b. gambiense*; indicating that human infective parasites are more sensitive to environmental stress during cyclical transmission of *T. brucei* subspecies. For example, heat shock proteins (HSP) 70 which is one of the most prominent HSP exhibiting markedly increased expression when the cell is subjected to stress is up regulated in *T. b. rhodesiense* and *T. b. gambiense* (Lindquist and Craig, 1988; Folgueira and Requena, 2007). This highlights the difficulty of these parasites to growth in tsetse mid-gut because cells that lost their ability to regulate cells growth often express high levels of HSP (Jäättelä, 1999). In such conditions, genes associated to the growth of *T. b. rhodesiense* and *T. b. gambiense* are probably down regulated. Between the two human infective parasites, *T. b. gambiense* expressed other HSPs like HSP84 and HSP60 which are able to interact with HSP70 in order to coordinate their actions for specific tasks like conformation regulation of cellular signalling proteins. Through such interactions, these chaperons are involved in various cellular processes including signal transduction and programmed cell death (Nollen and Morimoto, 2002). The high failure rate in the process of establishment and maturation of *T. b. gambiense* in tsetse flies may result from its inability to regulate its growth due to the expression of a variety of HSP.

Until now, little is known about the regulation of snoRNA levels in trypanosomes (Barth et al., 2008). Nevertheless, possible induction of high expression of snoRNA following stress activation of extragenic elements which are susceptible to regulate the transcription of snoRNAs has been reported (Barth et al., 2008). In *T. brucei*, several C/D snoRNAs and H/ACA which respective potentials to direct methylations and pseudouridinylations have been identified (Liang et al., 2001, 2005). Although the function of the modified nucleotides is currently unknown, the clustering of these nucleotides around functionally important regions in the RNAs suggests their importance (preservation of ribosomal functions under adverse environmental conditions due to the high number of modifications induce by snoRNA).

EF-2 like EF-1α of *T. brucei* is involved in GTP binding and the translation elongation factor activity. These two molecules are essential for protein synthesis in all organisms and they are useful proteins for tracing the early evolution of life (Creti et al., 1994; Nakamura et al., 1996). The expression level of EF-1α is regulated in many situations such as growth arrest, transformation and aging (Duttaroy et al., 1998). The abundance of EF-1α has been reported to characterize pro-apoptotic cells while low levels are indicative of anti-apoptosis state (Ouaissi, 2003). If we assume that EF-2 may have biological functions similar to those of EF-1α, we tempt to speculate that EF-2 might be part of the system transmitting signals towards the proteins synthetic machinery providing cells death. In the contrary, the up regulation of EF-1γ in *T. b. brucei* may be of great importance in the survival and the growth of these parasites (by providing cells significant protection from apoptotic death) because over-expression of EF-1γ was reported in *T. cruzi* showing reduced responsiveness to apoptosis stimuli (Ouaissi, 2003).

The up regulation of pteridine transporter gene in *T. b. rhodesiense* and *T. b. gambiense* indicates high rate of biopterin uptake since a direct correlation was found between the expression levels of biopterin gene product and active transport of biopterin in *Leishmania* (Lemley et al., 1999). Though the exact role of pteridines in the metabolism of trypanosomes is unknown, the up regulation of pteridine transporter gene could be essential for the survival of *T. b. rhodesiense* and *T. b. gambiense* because the uptake of biopterin was appeared critical for the survival of *Leishmania* (Lemley et al., 1999).

Most genes found differentially regulated in our study are involved in the transport across cell membrane, and the binding of nucleic acid and unfolded protein; indicating the important role of transmembrane transporters during the establishment of trypanosomes. The high number of genes involved in the binding of purines as well as the strong regulation of nucleoside/nucleobase transporter genes indicates that the metabolism of purines is an important step in the process leading to mid-gut infections. Indeed, *T. brucei* subspecies are unable to synthesize purines *de novo* and are absolutely reliant upon purine salvage from their hosts (Hammond and Gutteridge, 1984; Henriques et al., 2003). Purines are essential for the growth, multiplication and survival of these organisms. In experimental studies, Macleod et al. (2008) found that cyclic guanosine monophosphate (cGMP) could induce significantly higher rates of mid-gut infections. In addition, cGMP has been shown to have a major effect on the susceptibility of tsetse flies to trypanosome infections and to induce RNA and proteins changes in cultured procyclic trypanosomes (Macleod et al., 2008). Since the first step in purines salvage pathways is the transport of preformed purines through nucleoside/nucleobase transporters, it is obvious that the variations in the transcripts number of nucleoside/nucleobase transporters may have a direct effect on the purines metabolism and further, on the establishment of *T. brucei* subspecies. The up regulation of NT8.1 nucleoside/nucleobase transporters genes in *T. b. brucei* subspecies indicates high level of purines and their analogs consumption which leads further to high rate of mid-gut infections.

The S-adenosylmethionine synthetase found up regulated in *T. b. brucei* is an important enzyme converting methionine to S-adenosylmethionine. As a source of aminopropyl groups for polyamine synthesis, and as a methyl group donor for protein and lipid trans-methylation (Goldberg et al., 2000), S-adenosylmethionine is an important metabolite for African trypanosomes. The up regulation of S-adenosylmethionine synthetase in *T. b. brucei* suggests high rate of synthesized S-adenosylmethionine, and therefore high level of polamine synthesis or protein and lipid methylation in this trypanosome subspecies. This is in line with results of Goldberg et al. (1999) who found that the rate of methylation in *T. b. brucei* is 3–4-fold that of *T. b. rhodesiense*.

5. Conclusion

Whole genome analyses of RNA expression profiles between procyclic forms of *T. brucei* subspecies highlighted that only a small

percentage of *T. brucei* transcripts are modulated during establishment. The differences in the gene expression profiles between *T. brucei* subspecies may explain in part the differences observed during establishment of human and non-human infective parasites. The subspecies specific genes found differentially expressed are involved in the specific adaptation or establishment of trypanosomes in tsetse fly mid-gut. In this new environment, *T. b. gambiense* and *T. b. rhodesiense* expressed genes associated to reactions against stress while *T. b. brucei* adapts more easily by expressing genes like nucleoside/nucleobase transports which favour its establishment in tsetse fly mid-gut. For investigations on the development of vaccine blocking the transmission of trypanosomes, NT8.1 nucleoside/nucleobase transporters and S-adenosylmethionine synthetase genes appear as potential targets.

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